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THE EFFECTS OF CAFFEINE AND RYANODINE ON THE ELECTRICAL ACTIVITY OF THE CANINE CORONARY SINUS

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SUMMARY

1. Cells of the coronary sinus of the canine heart can exhibit triggered activity in which each action potential arises from a depolarizing after-potential that follows the previous action potential; an early after-hyperpolarization commonly precedes the delayed after-depolarization and both are increased in amplitude by the addition of noradrenaline. The delayed after-depolarization is thought to be caused by an inward current activated by a rise in intracellular Ca^{2+} that is, in turn, caused by Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmic reticulum (s.r.). The effects of caffeine and of ryanodine on the electrical activity of the coronary sinus were investigated because each of those agents is thought to affect the handling of intracellular Ca^{2+} by the s.r.

2. The steady-state effect of exposure to 5 mM-caffeine is to cause the delayed after-depolarization to move much earlier in the cycle, and become too small to give rise to an action potential so that preparations cannot show triggered activity; moreover, if a burst of activity is in progress it is terminated by exposure to 5 mM-caffeine.

3. Exposure to 0·5 mM-caffeine causes the delayed after-depolarization to move earlier in the cycle but to become larger so that triggered activity is more easily induced and longer lasting than in the absence of caffeine.

4. Shortly after the addition (or wash-out) of 5 mM-caffeine the after-depolarization transiently resembles that seen in the presence of 0·5 mM-caffeine so that bursts of triggered activity may occur just after the addition or removal of 5 mM-caffeine.

5. Exposure to 5 mM-caffeine abolishes early rapid repolarization (phase 1), shifts the plateau to a more positive level and retards the completion of repolarization. The effect on phase 1 is mimicked by exposure to solutions low in Cl^-; the effect on the plateau is mimicked by exposure to 20 mM-tetraethylammonium (TEA); fibres exposed to solutions containing 20 mM-TEA and 21 mM-Cl^- show action potentials very like those of fibres exposed to 5 mM-caffeine.

6. If a fibre already exposed to a low Cl^-, TEA-containing solution is then exposed to 5 mM-caffeine, no further change occurs in the action potential but the characteristic effects of caffeine on the after-depolarization appear.

7. Exposure to ryanodine prevents the appearance of the delayed after-

depolarization but leads to the appearance of an exceptionally long depolarizing after-potential that begins very early in diastole and, though waning, persists almost throughout diastole.

8. Many of our findings can be interpreted in terms of generally accepted effects of caffeine on the handling of intracellular Ca^{2+} by the s.r. and by the effects of intracellular Ca^{2+} on the 'cation channel'; however, several additional assumptions are required to interpret all of the effects, which are numerous, complex and concentration dependent.

INTRODUCTION

It has been suggested (Kass, Tsien & Weingart, 1978; Colquhoun, Neher, Reuter & Stevens, 1981; Kass & Tsien, 1982; Orchard, Eisner & Allen, 1983) that the membranes of many kinds of cardiac cells have in them 'cation channels' that open when the level of intracellular Ca^{2+} is high and that, when open, admit both Na^+ and K^+, thereby giving rise to a current that has a reversal potential near zero. That current would move the membrane potential towards zero whether the potential is at a positive level (as it is during the end of the upstroke and the beginning of the plateau) or at a negative level (as it is during the later part of phase 2, during phase 3, and during diastole). (See Fig. 1, Niedergerke & Page, 1981.)

A rise in myoplasmic Ca^{2+} can induce a release of Ca^{2+} from the sarcoplasmic reticulum (s.r.) and cause myoplasmic Ca^{2+} to rise still further (Fabiato & Fabiato, 1975; Fabiato 1983). If that occurs during diastole the resulting intracellular Ca^{2+}-induced opening of the cation channel could cause the delayed after-depolarizations (Kass *et al.* 1978; Matsuda, Noma, Kurachi & Irisawa, 1982) that can give rise to one or more non-driven action potentials and to triggered arrhythmias (Cranefield, 1975; 1977).

Since caffeine has effects on the ability of the s.r. to take up Ca^{2+} and on Ca^{2+}-induced Ca^{2+} release (Weber & Herz, 1968; Endo, 1977; Fabiato, 1983; Hess & Wier, 1984), as well as on the electrical and mechanical activity of cardiac tissue (deGubareff & Sleator, 1965; Blinks, Olson, Jewell & Braveny, 1972; Clark & Olson, 1973; Henderson, Brutsaert, Forman & Sonnenblick, 1971; Ohba, 1973; Kimoto, 1972; Vasalle & Lin, 1979; Niedergerke & Page, 1981; Clusin, 1983; Paspa & Vassalle, 1984; Satoh & Vassalle, 1984; Vassalle & DiGennaro, 1984), we studied its effects on the action potentials, after-potentials, and triggered activity of the canine coronary sinus. These effects proved to be concentration dependent and complex. Caffeine affects the shape and duration of the action potential, has transient effects on the maximum diastolic potential and resting tonus, changes the amplitude and time of occurrence of the delayed after-depolarization, can induce an early after-depolarization, and can either facilitate or inhibit triggered activity.

Many of our findings can be explained by generally accepted properties of the intracellular Ca^{2+}-opened cation channel and by generally accepted effects of caffeine on the s.r., but caffeine has effects not easily explained by a single action on the s.r. We also describe an interaction between caffeine and ryanodine that is difficult to explain in terms of previously published interpretations of the action of ryanodine. A preliminary report of some of these results has appeared in an abstract (Wit, Aronson & Cranefield, 1984).

METHODS

Mongrel dogs weighing 10–25 kg were anaesthetized with sodium pentobarbitone (30 mg/kg I.V.). The hearts were removed rapidly through a thoracotomy and rinsed in cool modified Tyrode solution with the following composition in mM: NaCl, 137; KCl, 4; $NaHCO_3$, 24; dextrose, 5·5; NaH_2PO_4, 1·8; $MgCl_2$, 0·5; $CaCl_2$, 2·7. The entire coronary sinus was removed from the heart, cut open along its length and pinned out flat (Wit & Cranefield, 1977). Then, under a dissecting microscope, small strips of tissue 2–4 mm long and less than 1·5 mm wide were carefully dissected from the internal surface of the opened coronary sinus (Boyden, Cranefield, Gadsby & Wit, 1983). The coronary sinus strips were suspended between 100 μm wide insect pins in the narrow channel of a fast-flow system (Gadsby & Cranefield, 1977) and superfused with Tyrode solution at a rate of 6 ml/min. The fast flow system allowed us to change the solution in the perfusion chamber with a half-time of less than 1 s. Equilibration of the extracellular space in the small atrial strips with the superfusing solutions occurs within several seconds as has been shown in previous reports from this laboratory (Gadsby & Cranefield, 1977; Boyden *et al.* 1983). The superfusing solution was gassed with 95 % O_2–5 % CO_2 and pre-heated before entering the chamber. The pH of the solution was 7·4. The temperature was monitored with a small thermistor bead positioned close to the preparation. The experiments were carried out at 35–37 °C but during any single experiment the variation in temperature was less than $\pm$ 0·2 °C.

In five experiments, one end of the small coronary sinus strip was ligated with 5·0 surgical silk which was used to attach it to a force displacement transducer (Cambridge Instruments). The other end of the strip was fixed to an insect pin 100 μm in width. The strip was suspended between the transducer and the pin in the fast flow system.

The preparations were initially superfused for 30–60 min with Tyrode solution. After that time resting potentials were around −60 mV (Wit & Cranefield, 1977; Boyden *et al.* 1983). Then, in all experiments the preparations were perfused with Tyrode solution containing noradrenaline (Breon Laboratories), $1·5–3 \times 10^{-6}$ M. The disodium salt of ethylenediaminetetraacetic acid (EDTA, 10 μM) was added to prevent oxidation of the catecholamine. The concentration of noradrenaline used in each experiment was sufficient to produce hyperpolarization to between −70 and −90 mV (Wit & Cranefield, 1977).

The preparations were stimulated with external bipolar electrodes (Teflon-coated silver wire). Stimuli were rectangular pulses 1·5 to 2 times threshold and 1–2 ms in duration. Transmembrane potentials were recorded with conventional micro-electrodes filled with 3 M-KCl. Membrane potentials were recorded on an Ampex or Racal tape recorder and Gilson or Gould chart recorder while being simultaneously displayed on a Tektronix 511 D storage oscilloscope. The Gould recorder was used to record tension.

The appropriate amount of caffeine or ryanodine was weighed in the powdered form and added directly to the Tyrode solution superfusing the preparation. In addition to using the Tyrode solution described above, some experiments were also done in a low Cl^- solution containing in mM: Na isethionate, 146; K^+ methylsulphate, 4; HEPES, 5; $MgCl_2$, 0·5; Ca^{2+}, methanesulphonate, 2·7; and dextrose, 5·5. In some experiments 20 mM-tetraethylammonium chloride (TEA) was added to the low Cl^- solution.

Terminology

Throughout this article we use the term *early after-depolarization* to describe a depolarizing after-potential that begins before the completion of repolarization. We use the term *early after-hyperpolarization* to describe an after-potential that is continuous with terminal repolarization but carries the membrane potential to a level negative to that prevailing before the upstroke of the action potential. We use the term *delayed after-depolarization* to describe either a depolarizing after-potential that arises after the return of the membrane potential to the diastolic level or one that arises during an early after-depolarization (see Cranefield, 1975, 1977; Wit & Cranefield, 1977).

RESULTS

After-depolarizations and triggered activity

Fig. 1 shows the effects of 5 mM-caffeine on the electrical activity of a strip of canine coronary sinus exposed to noradrenaline. Fig. 1*A–D* shows recordings at high amplification; the after-depolarizations can be seen but the action potentials are largely off the screen of the oscilloscope. The first two action potentials of Fig. 1*A*

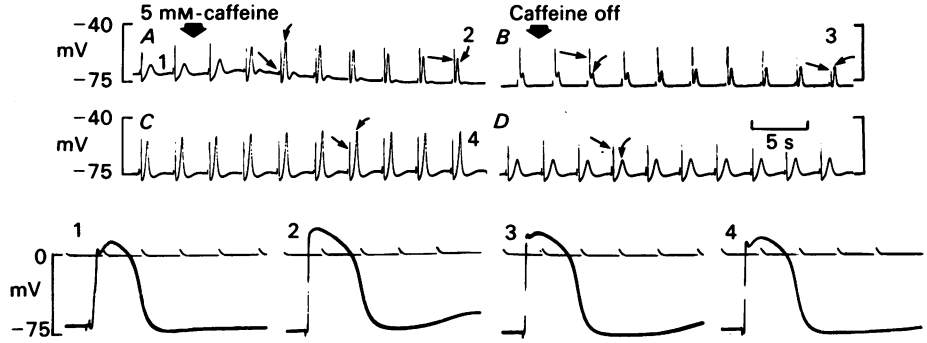

Fig. 1. Effects of caffeine and removal of caffeine on after-depolarizations and action potentials. Noradrenaline (3×10^{-6} M) was present throught the experiment. The narrow straight arrows indicate the terminal phase of repolarization of the action potential and the curved arrows indicate the peaks of the delayed after-depolarizations. The numbers indicate when the corresponding action potentials in the lower part of the Figure were recorded; time marks, 100 ms. *A*, effects of the exposure to 5 mM-caffeine (broad arrow). *B*, initial effects of removal of caffeine (broad arrow) after a steady-state effect had been achieved (about 5 min of exposure). *C*, later effects of removal of caffeine. *D*, return to steady state after caffeine had been washed out for 30 min.

are followed by an early after-hyperpolarization. Within 3 s after the solution was changed to one containing 5 mM-caffeine (large arrow, Fig. 1*A*) changes are seen in the electrical activity of the preparation. (a) During the first few beats that follow the addition of caffeine the resting potential shifts in the positive direction and then in the negative direction, ending up a few mV negative to the level it had before exposure to caffeine. (b) The early after-hyperpolarization increases in amplitude during the first and second beats after the addition of caffeine, reaching a maximum when the positive swing of the resting potential is the greatest. Thereafter the early after-hyperpolarization becomes progressively smaller until it not only vanishes but becomes replaced by an early after-depolarization. (c) The delayed after-depolarization becomes larger, appears earlier and is often followed by a further oscillatory after-potential of low amplitude. The delayed after-depolarization sometimes became large enough to induce a burst of triggered activity; if it did not, it continued to move earlier but became progressively smaller. (d) In the steady state, reached within 5 min, the delayed after-depolarization is early and small and almost merges with the caffeine-induced early after-depolarization from which it arises, to constitute a marked and prolonged delay of terminal repolarization (first action potential of Fig. 1*B*).

If the preparation then was exposed to a caffeine-free perfusate (Fig. 1*B*, large arrow) the changes described above occurred in reverse order except that there was no transient change in the resting potential. During wash-out of the caffeine the after-depolarization transiently increased in amplitude more than it did during the early stage of exposure to caffeine (compare Fig. 1*C* with Fig. 1*A*); thus a burst of

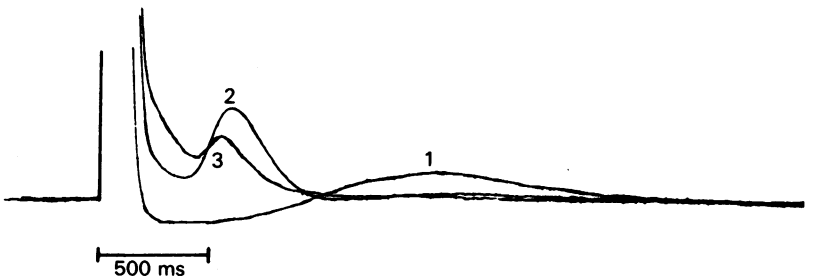

Fig. 2. Three action potentials recorded at high gain and paper speed superimposed to highlight the effects of caffeine on after-depolarizations. Only the lower portion of the action potentials were recorded at this high gain. Noradrenaline (3×10^{-6} M) was present throughout the experiment. Trace 1, noradrenaline alone. Trace 2, early effects of exposure to 5 mM-caffeine. Trace 3, steady-state effects of exposure to 5 mM-caffeine.

triggered activity may appear early during the wash-out of caffeine. The after-depolarizations eventually return to their previous amplitude and configuration (Fig. 1*D*). Similar events were seen in eight experiments.

The superimposed records in Fig. 2 were taken on a time and voltage scale designed to emphasize the caffeine-induced changes in the after-potentials; the amplifications are such that little of the action potential proper is seen. Trace 1 shows the electrical activity of a fibre exposed to noradrenaline but not to caffeine. The action potential, from upstroke to the return of the membrane potential to the previous diastolic level, is only 200 ms long. It is followed by an early after-hyperpolarization which lasts 800 ms and gives way to a delayed after-depolarization which lasts a further 1400 ms. The peak of the delayed after-depolarization thus occurs about 1600 ms after the upstroke of the action potential. Traces 2 and 3 were obtained from the same preparation (still exposed to noradrenaline) after exposing it to 5 mM-caffeine. Trace 2 shows the early effect of caffeine, during the period when the amplitude of the delayed after-depolarization is enhanced. The action potential is slightly prolonged; the early after-hyperpolarization is abolished and is replaced by an early after-depolarization, i.e. repolarization is arrested a few mV short of the diastolic membrane potential. The delayed after-depolarization is about 600 ms long and its peak occurs about 600 ms after the upstroke of the action potential. Trace 3 shows the steady-state effect of 5 mM-caffeine. The action potential is further prolonged and the early after-depolarization begins at a more positive level and is longer than it is in trace 2, whereas the subsequent after-depolarization is smaller and shorter and reaches its peak sooner than in trace 2. The peak of the delayed after-depolarization in trace 3 occurs some 1200 ms earlier than the peak of the after-depolarization seen in trace 1. The prolongation of the action potential and the early and delayed

after-depolarizations (traces 2 and 3) occur within the interval taken up by the early after-hyperpolarization in the absence of caffeine.

The changes seen when 5 mM-caffeine is added or removed may reflect the change in the concentration of caffeine as it rises to its steady-state level and then falls to zero, since the steady-state effects of lower concentrations of caffeine (0·5–2·5 mM)

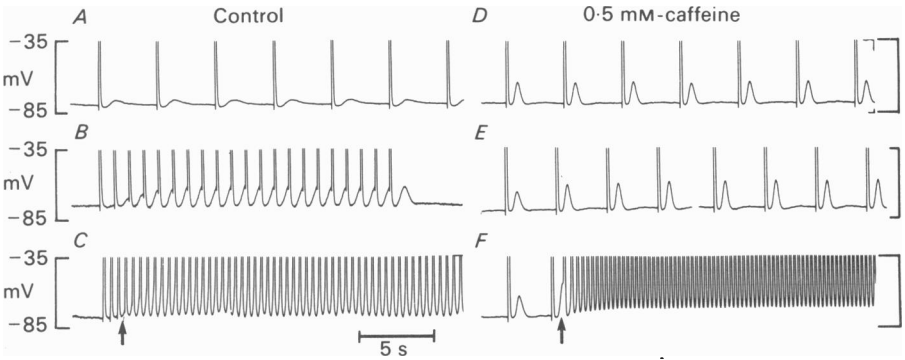

Fig. 3. Effects of a low concentration of caffeine on triggered activity. Noradrenaline (3×10^{-6} M) was present throughout the experiment. *A–C* caffeine-free solution. *D–F*, caffeine-containing solution. The arrows indicate the onset of triggered activity which occurred when the preceding cycle length of stimulation was decreased to 400 ms in *C* and to 3000 ms in *F*.

are similar to those shown in the middle of Fig. 1*A*. The steady-state effects of exposure to 0·5 mM-caffeine are shown in Fig. 3, the preparation being exposed to noradrenaline throughout the experiment. Fig. 3*A–C* is taken in caffeine-free solution and Fig. 3*D–F* in the presence of 0·5 mM-caffeine. The cycle length in Fig. 3*A* is 4000 ms. The delayed after-depolarization increased in amplitude when the cycle length was reduced to 1000 ms (Fig. 3*B*) but not until the stimulus cycle length was decreased to 400 ms (Fig. 3*C*) did triggered activity occur (at the arrow). Exposure to 0·5 mM-caffeine caused the delayed after-depolarization to increase in amplitude and to move earlier (Fig. 3*D*). The delayed after-depolarization increased in amplitude when the stimulus cycle length was decreased only slightly to 3500 ms (Fig. 3*E*). Triggered activity was evoked (Fig. 3*F*) at a much longer cycle (3000 ms) than under control conditions. Bursts of triggered activity lasted longer in the presence of 0·5 mM-caffeine than in its absence. The rate during the burst in the absence of caffeine is about 120/min, whereas it is about 240/min during the burst in the presence of caffeine, presumably because, as seen by comparing Fig. 3*A* and *D*, the time from the upstroke of one action potential to the peak of the after-potential that initiates the next action potential is much shortened in the presence of caffeine.

Exposure to higher concentrations of caffeine (10 mM) almost immediately caused after-depolarizations like those shown at the beginning of Fig. 1*B* or in trace 3 of Fig. 2. The transient increase in the amplitude of the delayed after-depolarization shown in the middle of Fig. 1*A* was not seen in preparations exposed to 10 mM-caffeine. Triggered activity could not be induced by decreasing the stimulus cycle length during steady-state exposure to either 5 mM- or 10 mM-caffeine.

Preparations in which bursts of triggered activity regularly lasted 5–10 min were exposed to caffeine soon after a burst of activity began. Exposure to low (0·5 mM) concentrations of caffeine increased the rate of triggered activity and prolonged the duration of the bursts (not shown). Exposure to higher concentrations (5 mM) terminated bursts of triggered activity. Fig. 4 shows records from a preparation in

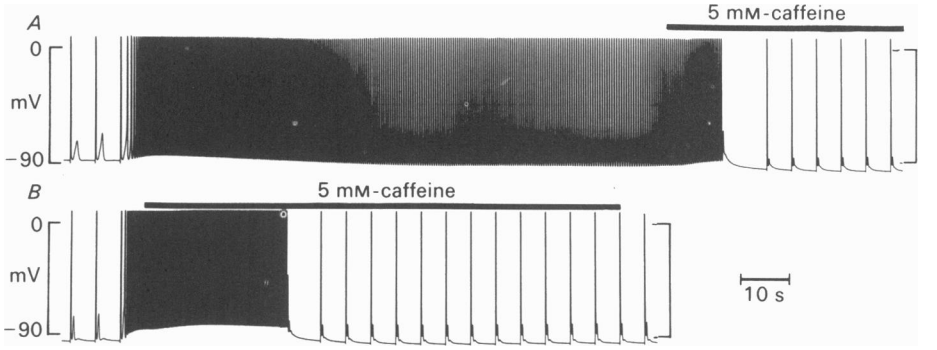

Fig. 4. Inhibition of triggered activity by caffeine. The horizontal bar indicates the period of exposure to caffeine. Noradrenaline (3×10^{-6} M) was present throughout the experiment. *A*, inhibition of triggered activity induced after the third driven action potential by exposure to caffeine. *B*, inhibition of triggered activity that developed after wash-out of caffeine by re-exposure to caffeine.

which bursts of triggered activity always lasted more than 5 min. In Fig. 4*A* a burst of triggered activity began after the third driven action potential. After about 100 s the preparation was exposed to 5 mM-caffeine (Fig. 4*A*, horizontal bar). A positive shift in the maximum diastolic potential was soon followed by cessation of triggered activity. The caffeine was washed out and, after 5 min, another burst of triggered activity was initiated (Fig. 4*B*). The solution was changed to one containing 5 mM-caffeine 5 s after the triggered activity began (Fig. 4*B*, horizontal bar) and activity ended within 30 s.

Mechanical activity

Since after-depolarizations are accompanied by after-contractions, we measured tension during exposure to and wash-out of 5 mM-caffeine. Fig. 5 shows that exposure to caffeine caused changes in the after-contractions that paralleled those of the after-depolarizations. The amplitude of the after-contraction increased and the after-contraction occurred earlier after the preceding contraction. Twitch tension also increased during the first few stimulated beats. Thereafter, twitch tension decreased, even as the after-contraction was still increasing. During the initial phase of enhancement of twitch tension, after-depolarizations and after-contractions, there were changes in diastolic membrane potential and tonic tension. The very small transient positive shift of the resting potential was accompanied by an increase in tonic tension that had a similar time course. The subsequent shift of the diastolic potential to a level more negative than that recorded prior to exposure to caffeine was accompanied by a gradual fall in tonic tension. The amplitude of the

after-contraction usually remained somewhat elevated even after the after-depolarization had decreased in amplitude.

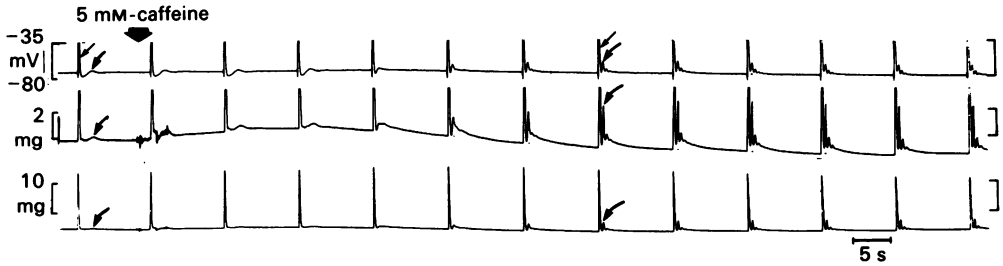

Fig. 5. Effects of caffeine on after-depolarizations and after-contractions in a canine coronary sinus preparation exposed to noradrenaline (3×10^{-6} M). The upper trace shows after-depolarizations; the middle and lower traces show simultaneously recorded mechanical activity at high and low gain. The narrow arrows indicate the terminal part of repolarization of the action potential; the curved arrows indicate the peaks of the after-depolarizations and the peaks of after-contractions; the broad arrow indicates the moment of exposure to caffeine.

Action potentials

The action potentials labelled 1 and 2 in Fig. 1 were recorded prior to and during exposure to 5 mM-caffeine; action potential 3 was recorded soon after caffeine was washed out. In the presence of caffeine rapid initial repolarization (phase 1) was abolished, the plateau (phase 2) began at and remained at a more positive level and was prolonged, and phase 3 slowed. Further prolongation of the terminal part of phase 3 repolarization results from the appearance of an early after-depolarization (see Fig. 2). These effects of caffeine were present but less prominent at lower concentrations.

Certain studies have suggested that outward currents carried by Cl^- and/or K^+, currents possibly activated by intracellular Ca^{2+}, contribute to the shape of the action potential (Dudel, Peper, Rudel & Trautwein, 1967; Fozzard & Hiraoka, 1973; Kenyon & Gibbons, 1979; Siegelbaum & Tsien, 1980; Goto & Colatsky, 1982; Hess & Wier, 1984) and that at least one of those currents may be blocked by caffeine (Corabouef & Carmeliet, 1982). Therefore, we did experiments designed to determine whether the blockade of outward currents carried by Cl^- or K^+ can mimick the effects of caffeine on the action potential.

Within 30 s after exposure to a virtually Cl^--free solution, the phase of rapid initial repolarization disappeared (not shown) so that the plateau shifted to a more positive voltage but the duration of the action potential did not increase as it does in fibres exposed to caffeine. Exposure to 20 mM-TEA caused a gradual increase in the duration of the plateau but had little effect on the phase of early rapid repolarization and thus caused little shift in the level of the plateau (not shown). The delayed after-depolarization became larger in fibres exposed to either virtually Cl^--free solution or to TEA.

The records in Fig. 6 show that by exposing the preparation to 20 mM-TEA and

low (21 mM) Cl^- the effects of caffeine on the action potential could be fully mimicked: early rapid repolarization was abolished, the plateau shifted to a more positive level and the duration of the action potential increased (Fig. 6*B*). Although this action potential resembles that of a fibre exposed to 5 mM-caffeine, the further addition of caffeine produces other typical caffeine-induced effects, namely interruption of

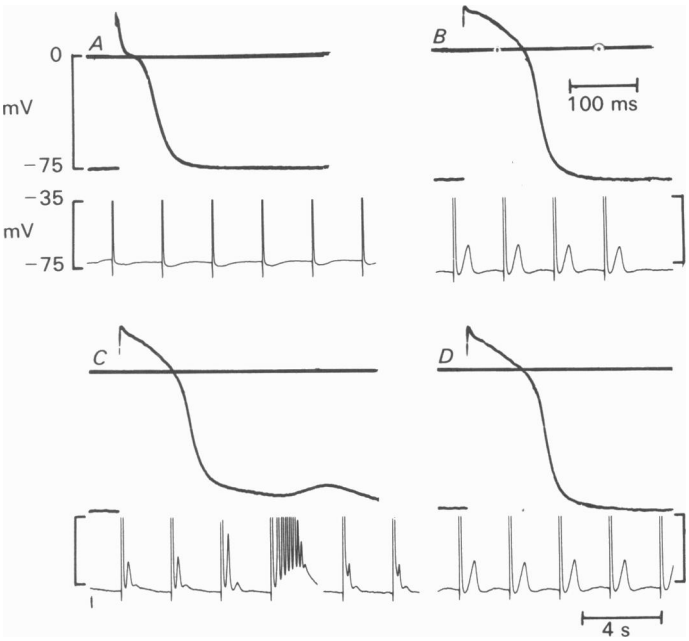

Fig. 6. Effects of low Cl^-, TEA-containing Tyrode solution and caffeine on action potentials. Noradrenaline (3×10^{-6} M) was present throughout the experiment. *A*, normal Tyrode solution. *B*, Tyrode solution with low Cl^- (21 mM) and 20 mM-TEA-Cl. *C*, Tyrode solution with low Cl^- (21 mM), 20 mM-TEA-Cl, and 5 mM-caffeine. *D*, Tyrode solution with low Cl^- (21 mM) and 20 mM-TEA-Cl after removal of caffeine.

repolarization by an early after-depolarization and the evoking of a burst of triggered activity (Fig. 6*C*). These effects, which vanish after the removal of caffeine (Fig. 6*D*), thus may be independent of the effects of caffeine on the amplitude, duration and shape of the action potential.

To determine whether the effects of caffeine are caused by an effect on the intracellular handling of Ca^{2+} or by an effect on Ca^{2+} influx we relied on the assumption that reduction in extracellular [Ca^{2+}] presumably causes no immediate significant change in the internal Ca^{2+} stores (Chapman & Leoty, 1976), whereas exposing a regularly stimulated fibre to low extracellular [Ca^{2+}] for several minutes probably does deplete intracellular [Ca^{2+}].

If caffeine was added and extracellular [Ca^{2+}] was maintained at 2·7 mM, an early and a delayed after-depolarization began to appear within 10 s and increased in amplitude during the next 60 s (Fig. 7*A*). The amplitude of the delayed after-depolarization is plotted below as a function of time ($\times$). If caffeine was added and

extracellular [Ca^{2+}] was simultaneously lowered to 0·25 mM, the after-depolarizations characteristic of fibres exposed to caffeine were still seen (Fig. 7*B*). During the first 30 s the after-depolarizations had the same amplitude as they did when the fibre remained exposed to 2·7 mM-extracellular-Ca^{2+} (filled circles in graph). After 45 s of exposure to a solution containing low extracellular [Ca^{2+}] and caffeine, the after-depolarizations did decline in amplitude, presumably secondary to an eventual

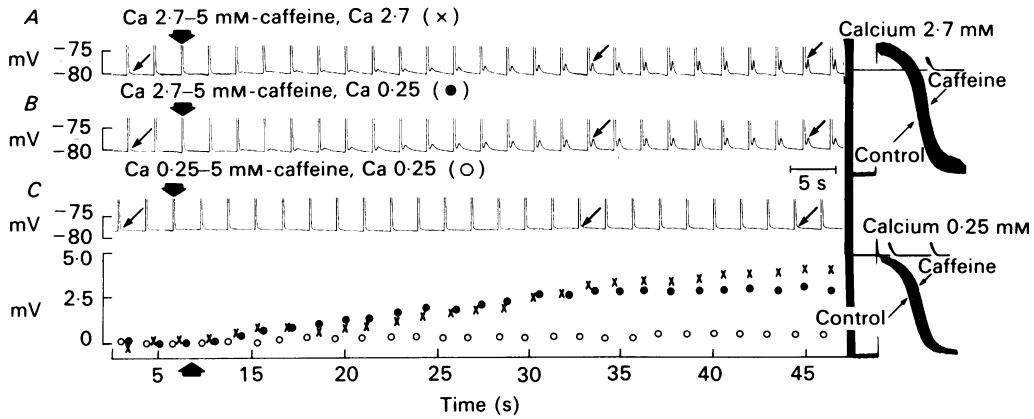

Fig. 7. Effects of extracellular [Ca^{2+}] on the actions of caffeine. Noradrenaline (3×10^{-6} M) was present throughout the experiment. The graph at the bottom of the Figure is a plot of the peak amplitude of the delayed after-depolarizations as a function of time under the experimental conditions indicated by the symbols above the records in *A–C*. The superimposed action potentials at the right of the Figure show the effects of exposure to caffeine (5 mM) on the shape of the action potential with normal extracellular [Ca^{2+}] (2·7 mM) and with low extracellular [Ca^{2+}] (0·25 mM). The broad arrows indicate when the solution was changed and the narrow arrows indicate the peaks of the delayed after-depolarizations. *A*, exposure to caffeine (5 mM) and normal extracellular [Ca^{2+}] (2·7 mM) after prior perfusion with normal extracellular [Ca^{2+}] (2·7 mM). *B*, exposure to caffeine (5 mM) and low extracellular [Ca^{2+}] (0·25 mM) after prior perfusion with normal extracellular [Ca^{2+}] (2·7 mM). *C*, exposure to caffeine (5 mM) and low extracellular [Ca^{2+}] (0·25 mM) after prior perfusion with low extracellular [Ca^{2+}] (0·25 mM).

fall in intracellular [Ca^{2+}]. The preparation was again exposed to caffeine-free solution containing 2·7 mM-Ca^{2+}. Subsequent exposure for 3 min to a caffeine-free solution containing 0·25 mM-Ca^{2+} led to a decrease in mechanical activity which suggests depletion of internal Ca^{2+} stores. Exposure to 5 mM-caffeine (Fig. 7*C*, broad arrow) induced only a small early after-depolarization, seen as a prolongation of the final phase of repolarization. If the exposure to 0·25 mM-extracellular Ca^{2+} for 3 min prior to caffeine administration in Fig. 7*C* reduced intracellular [Ca^{2+}], then the records in Fig. 7*B* and *C* suggest that intracellular [Ca^{2+}] is an important determinant of the magnitude of the initial effect of caffeine.

During wash-out of caffeine the delayed after-depolarization may become much larger than it is under control conditions (Fig. 1*C*), but that increase in amplitude does not occur in preparations exposed to 0·25 mM-extracellular Ca^{2+}, prior to the withdrawal of caffeine. Fig. 8*A* shows the reduction in the amplitude of the delayed after-depolarization that results when extracellular [Ca^{2+}] is reduced from 2·7 to

0·25 mM. After the preparation had become mechanically quiescent, caffeine (5 mM) was added (Fig. 8*B*) and a small early after-depolarization followed by a small delayed after-depolarization resulted. The delayed after-depolarization was much smaller than that seen in the presence of 2·7 mM-extracellular Ca^{2+} and no caffeine. Finally, the preparation was exposed to a caffeine-free solution containing 2·7 mM-

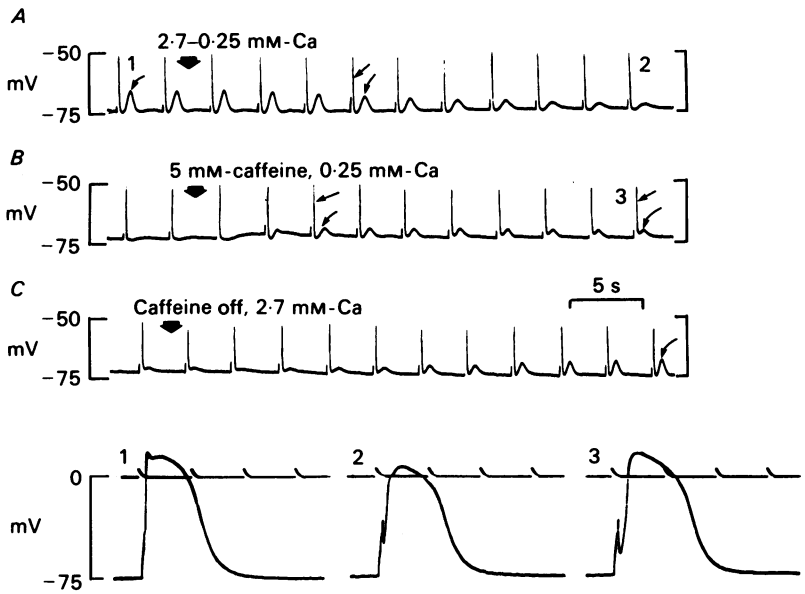

Fig. 8. Effects of extracellular [Ca^{2+}] and caffeine on after-depolarizations and action potentials. Noradrenaline (3×10^{-6} M) was present throughout the experiment. The broad arrows indicate when the external solution was changed; the narrow straight arrows indicate the terminal phase of repolarization and the curved arrows indicate the peaks of delayed after-depolarizations. The numbers indicate when the corresponding action potentials in the lower part of the Figure were recorded; time marks, 100 ms. *A*, exposure to low extracellular [Ca^{2+}] (0·25 mM) after prior perfusion with normal extracellular [Ca^{2+}] (2·7 mM). *B*, exposure to caffeine (5 mM) and low extracellular [Ca^{2+}] (0·25 mM) after prior perfusion with low extracellular [Ca^{2+}] (0·25 mM) long enough to cause mechanical activity to disappear. *C*, exposure to caffeine-free solution and normal extracellular [Ca^{2+}] (2·7 mM) after prior exposure to caffeine (5 mM) and low extracellular [Ca^{2+}] (0·25 mM).

extracellular Ca^{2+} (Fig. 8*C*). The delayed after-depolarization returns to the amplitude seen in a fibre exposed to a caffeine-free solution containing 2·7 mM-Ca^{2+} but there is no sign of the great increase in amplitude seen when caffeine is withdrawn from a preparation in which internal Ca^{2+} stores are normal (see e.g. Fig. 1*C*).

Effects of ryanodine

Ryanodine is thought to block release of Ca^{2+} from the s.r. (Sutko & Kenyon, 1983). If after-depolarizations in coronary sinus fibres are driven by a cyclic uptake and release of Ca^{2+} from the s.r. then ryanodine should inhibit their development in the absence of caffeine and block their enhancement by caffeine.

We studied the effects of ryanodine on fibres exposed to noradrenaline that did not have after-depolarizations and on fibres that had large after-depolarizations. Ryanodine did not cause delayed after-depolarizations in fibres that did not initially have them. In preparations that showed delayed after-depolarizations, adding ryanodine (1 μM) decreased the amplitude of the after-depolarizations until they almost disappeared (Fig. 9). The coupling interval between the upstroke of the action

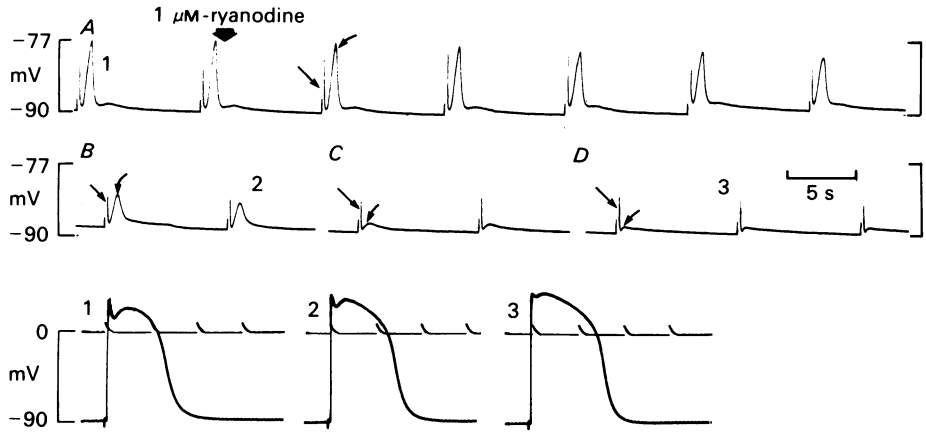

Fig. 9. Effects of ryanodine on after-depolarizations and action potentials. Noradrenaline (3×10^{-6} M) was present throughout the experiment. The broad arrow indicates the moment of exposure to ryanodine (1 μM); the narrow straight arrows indicate the terminal part of repolarization of the action potential and the curved arrows indicate the peaks of delayed after-depolarizations. The numbers indicate when the corresponding action potentials at the bottom of the Figure were recorded; time marks, 100 ms. *A*, immediately after exposure to ryanodine. *B*, after exposure to ryanodine for 95 s. *C*, after exposure to ryanodine for 160 s. *D*, after exposure to ryanodine for 250 s.

potential and the after-depolarizations did not decrease as it did after exposure to caffeine. However, a small early after-depolarization persists and often, in the presence of ryanodine, becomes exaggerated both in amplitude and duration, sometimes lasting throughout diastole. For example, in Fig. 9*D* the action potential is followed by a small early after-depolarization that decays almost linearly throughout diastole.

Fig. 10 shows the electrical activity of a fibre that developed only very small delayed after-depolarizations when exposed to noradrenaline. Some 30 s after exposure to 5 mM-caffeine (Fig. 10*A*) large after-depolarizations and a burst of triggered activity are seen. The preparation was re-exposed to a caffeine-free solution until a new steady state was reached. Then the preparation was exposed to ryanodine (Fig. 10*B*) and, after two action potentials, to 5 mM-caffeine. This resulted in a slow but prolonged and sustained increase in maximum diastolic potential and the appearance of a very prolonged early after-depolarization. After fifteen action potentials the maximum diastolic potential increased by more than 10 mV and the early after-depolarization attained about the same amplitude (10 mV) and was about

2 s long. The preparation was then exposed to a caffeine-free solution; 25 min later the early after-depolarization had increased slightly in amplitude and greatly in duration, the amplitude being about 12 mV and the duration nearly 3 s (last five action potentials of Fig. 10*B*, and action potential 4 of the bottom traces). Ryanodine also increased the amplitude of the action potential, abolished early rapid repolarization, and moved the plateau from + 20 to + 50 mV (see Fig. 9). These effects are similar to those of caffeine.

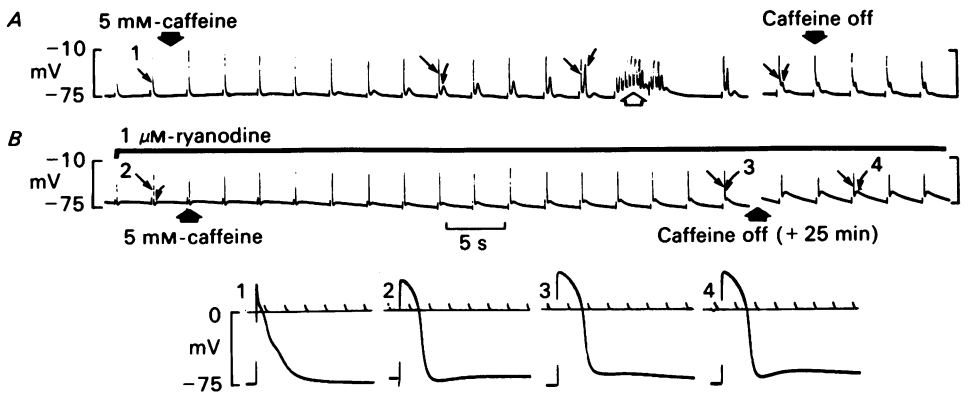

Fig. 10. Effects of caffeine and caffeine after treatment with ryanodine on after-depolarizations and action potentials. Noradrenaline (3×10^{-6} M) was present throughout the experiment. Filled broad arrows indicate when caffeine-containing solution was started and stopped; filled small straight arrows indicate the terminal phase of repolarization of the action potential; filled curved arrows indicate the peaks of the delayed after-depolarizations; the unfilled broad arrow indicates triggered activity. The numbers indicate when the corresponding action potentials at the bottom of the Figure were recorded; time marks, 100 ms. *A*, exposure to and removal of caffeine. *B*, exposure to caffeine shortly after treatment with ryanodine (horizontal bar).

DISCUSSION

Caffeine, triggered activity and after-potentials

The effects of caffeine on the rate of triggered activity and on the changes in rate when its action terminates triggered activity are consistent with its effects on the delayed after-depolarization. Exposure to 0·5 mM-caffeine causes the delayed after-depolarization to move earlier and to increase in size, which explains why bursts of activity evoked in the presence of low concentrations of caffeine show a higher rate than those evoked in its absence and why, if 0·5 mM-caffeine is added during a burst of activity, the rate increases. The steady-state effect of 5 mM-caffeine is to cause the after-depolarization to be sub-threshold, and not to increase in size in response to increases in drive rate. Under such conditions the preparation cannot be triggered nor presumably, could it sustain an existing burst of after-potential-dependent rhythmic activity. If an existing burst of activity is terminated by adding 5 mM-caffeine the rate increases towards the end of the burst, i.e. at a time when the early transient effects of 5 mM-caffeine on the delayed after-depolarization would be expected to cause it to appear earlier and be larger.

The effects of caffeine on the resting potential and the shape of the action potential

The early loss of resting or maximum diastolic potential might result from an early release of Ca^{2+} from the s.r. opening the cation channel. That such a release occurs is suggested by the loss of resting potential that accompanies the brief increase in tonus observed after exposure to caffeine (Fig. 5). Although this transient depolarization can be explained by a transient inward current induced by caffeine (Clusin, 1983), we cannot explain the hyperpolarization and the decrease in diastolic tonus that occur during prolonged exposure to caffeine.

Caffeine-induced changes in the shape of the action potential similar to those we observed have been reported in other types of cardiac cells (deGubareff & Sleator, 1965; Kimoto, 1972; Clark & Olson, 1973; Kimoto, Sito & Goto, 1974; Niedergerke & Page, 1981). An increase in the density of the slow inward current could cause such changes in the shape of the action potential (Goto, Yatani & Ehara, 1979) but Hess & Wier (1984) report that caffeine reduces the density of the slow inward current in cardiac Purkinje fibres. On the other hand, the action potential of a cell exposed to 5 mM-caffeine is virtually identical to that of a cell exposed to a solution containing low (21 mM) Cl^- and 20 mM-TEA, i.e. a cell that may be assumed to have lost some of the outward currents normally carried by Cl^- and K^+. Caffeine might block the channels that carry such currents or it might prevent a release of intracellular Ca^{2+} that normally activates outward currents during the plateau. That such intracellular Ca^{2+}-activated currents may exist has been suggested by several authors (Dudel *et al.* 1967; Fozzard & Hiraoka, 1973; Kenyon & Gibbons, 1979; Siegelbaum & Tsien, 1980; Goto & Colatsky, 1982; Hess & Wier, 1984). In Purkinje fibres caffeine may markedly reduce a very early intracellular Ca^{2+} release that follows the upstroke of the action potential, and another release that occurs during the first 50–100 ms of the plateau (Hess & Wier, 1984). Such reductions might reduce outward currents normally activated by intracellular Ca^{2+}.

A similar reduction in intracellular Ca^{2+}-activated outward currents might explain the fact that caffeine slows terminal repolarization and abolishes the early after-hyperpolarization, but, as we discuss below, in fibres exposed to caffeine intracellular $[Ca^{2+}]$ may actually be abnormally high towards the *end* of the action potential. It is thus at least possible that caffeine exerts a direct TEA-like effect on the potassium conductance responsible for delayed rectification.

The delayed after-depolarization

Fluctuations in intracellular Ca^{2+} occur in cardiac fibres at all times and increase in size under conditions of 'intracellular Ca^{2+} overload' (Matsuda *et al.* 1982; Kort & Lakatta, 1984). Those fluctuations are intracellular Ca^{2+}-dependent but they are time independent and no persuasive explanation of how the passage of an action potential can synchronize them has been advanced. It is more often assumed that the Ca^{2+} that enters during the action potential causes the intracellular Ca^{2+}-dependent *and* time-dependent process of Ca^{2+}-induced Ca^{2+} release not only to occur but to occur after a long delay and in a synchronized fashion. Since we do not know why delayed after-depolarizations arise in the first place, we cannot be sure why they may be induced or enhanced by noradrenaline. We can thus be even less sure of any

explanation of the effects of adding caffeine to fibres already exposed to noradrenaline.

Delayed after-depolarizations can occur very late in diastole, as late as 1·6 s after the upstroke. Noradrenaline might induce such late after-depolarizations by enhancing uptake of Ca^{2+} by the s.r. After a sufficient period of s.r. loading in the presence of noradrenaline, Ca^{2+}-induced Ca^{2+} release might occur. The occurrence of after-contractions and after-depolarizations rather late in diastole would reflect the time required for the overload induced by noradrenaline to develop. In support of this view are the observations of Fabiato & Fabiato (1973) on the effects of catecholamines on tension development in disrupted cardiac cells that retain parts of the sarcolemma. They found that exposure to isoprenaline (10^{-6} M) increased the length and amplitude of the contraction, an effect tentatively attributed to an increase in the capacity of Ca^{2+} storage leading to a longer time being required to reach the level of Ca^{2+} needed to induce Ca^{2+} release.

Our results suggest that the effects of caffeine on after-depolarizations are due to changes in myoplasmic Ca^{2+} rather than to changes in Ca^{2+} influx (see Figs. 7 and 8). Support for this interpretation is provided by studies showing that in skinned rat ventricular cells low doses of caffeine (1–2 mM) increase the frequency of contractions while slowing the rate of tension development and relaxation (Fabiato & Fabiato, 1975) and that caffeine releases Ca^{2+} from the s.r. and inhibits Ca^{2+} uptake by the s.r. in cardiac and skeletal muscle (Weber & Herz, 1968; Blinks *et al.* 1972; Endo, 1977).

There is a basic difference between events attributable to a diminished increase in potassium conductance, g_K (such as the prolongation of the action potential and the appearance of an early after-depolarization) and a renewed positive swing of the membrane potential (i.e. a delayed after-depolarization). The latter event requires a phasic *increase* in *net* inward current and it seems more plausible to explain such an increase by a rise and fall in actual inward current than by a fall and rise in outward current.

A phasic rise in inward current during the first few hundred ms after repolarization might always be present, its effects being not only masked but overwhelmed by a simultaneous and larger increase in outward current. On the other hand, if early sequestration of Ca^{2+} is impaired, then intracellular [Ca^{2+}] might reach the level needed to induce Ca^{2+} release earlier in the cycle, thus causing the delayed after-depolarization to move progressively earlier. The eventual decrease in the amplitude of that delayed after-depolarization could be attributed to depletion of the Ca^{2+} stock of the s.r. The more or less sustained enhancement of the delayed after-depolarization seen during exposure to 0·5 mM-caffeine would then be explained by assuming that low concentrations of caffeine reduce the threshold of Ca^{2+}-induced Ca^{2+} release, causing it to occur earlier and in increased amount. We cannot, however, rule out the possibility that low concentrations of caffeine cause the cation channel to open in response to lesser increases in intracellular [Ca^{2+}].

We began the present investigation in the belief that the previously described effects of caffeine would make it a simple tool for a straightforward elucidation of the relationship between Ca^{2+}-induced Ca^{2+} release and the delayed after-depolarizations seen when fibres of the coronary sinus are exposed to noradrenaline.

Its effects on the electrical activity of the coronary sinus proved to be too various, too complex and too concentration dependent to make it useful for that purpose.

Ryanodine is said either to block release of Ca^{2+} from the s.r. (Sutko & Kenyon, 1983) or to induce a leak of Ca^{2+} from the s.r. (Hilgemann, Delay & Langer, 1983; Hunter, Haworth & Berkoff, 1983). Blockade of Ca^{2+}-induced Ca^{2+} release could explain our finding that ryanodine abolishes both the delayed after-depolarization seen in the presence of noradrenaline (Fig. 9) and that seen after caffeine is added. Ryanodine also causes a change in the shape of the action potential (Fig. 9) that could be explained if ryanodine prevents Ca^{2+}-induced Ca^{2+} release from occurring during the action potential, thus preventing the appearance of intracellular Ca^{2+}-activated outward currents carried by Cl^- and K^+. Sutko & Kenyon (1983) have reported that ryanodine can block a possible intracellular Ca^{2+}-dependent outward current in Purkinje fibres of the calf heart.

Fibres exposed to ryanodine develop a small but very long early after-depolarization (see Fig. 9*B*). The addition of caffeine to a preparation already exposed to ryanodine (Fig. 10*B*) has two striking effects, an increase in maximum diastolic potential and an increase in both the amplitude and the duration of the after-depolarization. Exposure to ryanodine or to ryanodine plus caffeine can thus create rather dramatic changes in the electrical activity of fibres of the coronary sinus, changes which might well alter the mechanical activity of the cell.

We suggest that caffeine, ryanodine and ryanodine plus caffeine may exert effects not previously allowed for in studies in which those compounds have been used to analyse the role of the s.r. in the electrical and mechanical activity of cardiac cells.

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