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INFLUENCE OF A CHANGE IN STIMULATION RATE ON ACTION POTENTIALS, CURRENTS AND CONTRACTIONS IN RAT VENTRICULAR CELLS

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SUMMARY

1. The effects of a change in stimulation rate on electrical activity and accompanying contraction were investigated in ventricular cells isolated from rat heart; the cells were stimulated to contract either by brief depolarization pulses which evoked action potentials, or, under voltage-clamp conditions, by step depolarizations.

2. An increase in stimulation rate from 0·3 to 3 Hz resulted in a gradual reduction in the amplitude of contraction and attenuation of the late phase of the action potential. These changes were less marked at more depolarized potentials.

3. The ventricular cells were voltage clamped at −40 mV and initially stimulated at 0·3 Hz by step depolarizations to 0 mV for 10 or 100 ms, which activated the second inward current (I_{si}) and an accompanying contraction. The amplitude and time course of contraction were similar with the two pulse durations.

4. When the duration of the depolarization was 100 ms, an increase in stimulation rate to 3 Hz caused a gradual decline in the amplitude of I_{si} and of the evoked contraction; at the same time extra contractions and small, transient inward currents appeared in addition to the evoked contractions and I_{si}s. There was a reduction in the early component of decay of I_{si} at 3 Hz.

5. With a depolarizing pulse duration of 10 ms, an increase in stimulation rate to 3 or to 4·2 Hz did not change the amplitude of the evoked I_{si} or contraction and no extra contractions or currents appeared.

6. Intracellular EGTA abolished all contractions in the cells and an increase in the rate of stimulation with 100 ms pulses did not then induce transient inward currents. There was some decrease in the I_{si} amplitude but this was not as marked as in the absence of EGTA and the time course of current decay was similar at the two rates.

7. Ryanodine prevented the appearance of extra contractions and currents when the stimulation rate was increased to 3 Hz and, as in the presence of intracellular EGTA, there was a small decrease in I_{si} amplitude while the time course of decay was similar at the two stimulation rates.

8. The time course of recovery of I_{si} from inactivation, as shown by a double-pulse

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procedure, was altered when the duration of the first pulse was reduced from 100 to 10 ms, an extra inactivation of I_{si} being seen at pulse intervals of 20–100 ms. This extra component of inactivation was not seen with intracellular EGTA or in the presence of ryanodine.

9. It is concluded that release of calcium from intracellular stores can contribute to the observed changes in electrical activity and contractions following an increase in the rate of stimulation in rat ventricular cells.

INTRODUCTION

In most mammalian ventricular tissue an increase in the rate of stimulation has a positive inotropic effect, the notable exception being the rat ventricle (cf. Koch-Weser & Blinks, 1963). In addition to changes in contraction, increasing the stimulation rate also has marked effects on action potential duration and amplitude of the second inward current (I_{si}). Generally, there is a decrease in the duration of the ventricular action potential and it has been proposed that this is brought about by an increase in intracellular calcium causing a decrease in I_{si} or an increase in an outward potassium current and hence faster repolarization (cf. Boyett & Jewell, 1980). Recently, the use of isolated cells has allowed more accurate resolution of I_{si} kinetics (see Noble, 1984) and in rat ventricular cells this current is rapidly inactivated and is not markedly contaminated by outward potassium currents during voltage-clamp steps from a holding potential of −40 mV (Mitchell, Powell, Terrar & Twist, 1983*b*). In addition, I_{si} contributes to the early plateau phase of the rat action potential (Mitchell, Powell, Terrar & Twist, 1983*a*; Schouten, 1984), the later longer phase at more negative potentials being dependent on another inward current activated by intracellular calcium and requiring extracellular sodium (Mitchell, Powell, Terrar & Twist, 1984*d*), so it is unlikely that under the conditions of our experiments the decrease in action potential duration in rat ventricle at high rates of stimulation is due to the decrease in I_{si} (Payet, Schanne & Ruiz-Ceretti, 1981).

In this paper we show that the decrease in action potential duration in isolated ventricular cells coincides with a decrease in the evoked contraction and suggest that both may result from a decrease in intracellular calcium resulting from diminution of calcium available for release from internal stores. We also demonstrate that transient inward currents associated with spontaneous contractions can be observed in single cells and that these events appear to be similar to the oscillatory effects seen in cardiac muscle under conditions where intracellular calcium is elevated, e.g. during exposure to cardiac glycosides (Lederer & Tsien, 1976; Kass, Lederer, Tsien & Weingart, 1978*a*; Kass & Tsien, 1982). Early experiments with the alkaloid ryanodine, now thought to inhibit calcium release from the sarcoplasmic reticulum (Sutko, Willerson, Templeton, Jones & Besch, 1979), indicated that rat ventricular contractions were particularly sensitive to inhibition by this compound and led to the suggestion that calcium for contraction in this tissue may be derived from a cellular source and not from influx of extracellular calcium, the accumulation of which was believed to cause the positive staircase seen in other cardiac muscle (Hajdu, 1969). Exposure of single rat ventricular cells to ryanodine produces effects which suggest that release of calcium from intracellular stores is involved in the generation of

transient inward currents and spontaneous contractions at high stimulation rates. Ryanodine was also used in double-pulse experiments monitoring the time course of I_{si} reactivation, and some inhibition of reactivation of I_{si} by stored calcium was revealed. Some of these results have been presented to the Physiological Society (Mitchell, Powell, Terrar & Twist, 1984*f*).

METHODS

Cells were isolated from rat ventricular tissue as previously described (Powell, Terrar & Twist, 1980) and mounted on a thin film of agar on a cover-slip in the centre of a Perspex chamber, through which flowed solution (8 ml min^{-1}, 36–37 °C) containing (mM): NaCl, 118·5; $NaHCO_3$, 14·5; KCl, 2·6; KH_2PO_4, 1·2; $CaCl_2$, 2·5; $MgSO_4$, 1·2; glucose, 11·1; and bovine serum albumin 5 mg ml^{-1}. Ryanodine, a gift from Merck, Sharp & Dohme, was used in some experiments at a concentration of 1 or 2 μM. Solutions were stored in water-jacketed reservoirs and flowed through water-jacketed tubing in order to minimize changes in bath temperature, which can have marked effects on electrical parameters in the cells (Mitchell *et al.* 1983*b*). The temperature was continuously monitored by a thermistor placed in solution close to the cells.

Membrane potential was recorded using 40–60 MΩ electrodes filled with 3 M-KCl and the attainment of a steady potential was aided by initially superfusing the cells with 5 mM-calcium, the potential remaining stable on reduction of the external calcium ion concentration to 2·5 mM (Powell *et al.* 1980; Mitchell, Powell, Terrar & Twist, 1984*c*). Voltage signals were fed to a pre-amplifier incorporating a bridge circuit (Dagan 8100) and the membrane potential could be changed to a different level by applying a constant current through the recording electrode. The Dagan 8100 was also employed for single-micro-electrode voltage clamping (Mitchell *et al.* 1983*b*) and the recording electrodes were of a low resistance (8–15 MΩ) to facilitate a fast response of voltage recording during switch clamping. Another switch voltage-clamping system (Axoclamp 2) was used in a few experiments, and no difference was detected between records obtained with the two systems. In some experiments the electrodes were filled with solution containing 2 M-KCl and 1 M-EGTA.

The time course of contraction was monitored by a photodiode in the microscope eyepiece, the light being restricted to the impaled cell by an adjustable rectangular slit below the condenser (Mitchell *et al.* 1983*b*). Records of current, voltage and photodiode output were stored on a Racal Store 4 FM tape recorder (3·75 or 15 in. s^{-1}) and displayed on a Gould OS4020 oscilloscope.

For analysis of current decay, traces stored on the Gould digital storage oscilloscope were transferred to a pen recorder by feeding the analogue output of the oscilloscope to the input of the pen recorder at a rate which avoided distortion of the wave form by the slow frequency response of the pen recorder. Measurement of the wave forms was usually performed by hand; to check whether any bias was introduced by this procedure, some wave forms were also digitized at 200 μs $point^{-1}$ using a VELA microprocessor-controlled interface (Educational Electronics) and transferred to a BBC microcomputer. There was good agreement between the hand and computer-measured data. When it appeared from hand-measured data that there was more than one exponential component of decay of second inward current, this could be confirmed from the computer-measured data. For example, in one experiment a regression line was fitted to computer-measured data between 6 and 22 ms following the peak of inward current (eighty time points), and 95% confidence limits within which new observations would be expected to lie were calculated; during the early part of the decay the first twenty-five points were all above the calculated line, and the first seven points were larger than the 95% confidence limits. The slope of the regression line fitted to the computer-measured points was $-0{\cdot}093 \pm 0{\cdot}013$ ms^{-1}; a regression line was also fitted to hand-measured data for the same time period (eleven points), and the calculated slope of $-0{\cdot}11 \pm 0{\cdot}01$ ms^{-1} was in reasonable agreement with that from the computer-measured data.

RESULTS

Effects of an increase in stimulation rate on action potentials and contractions

Individual rat ventricular cells were impaled initially in 5 mM-calcium, which aided the attainment of a stable membrane potential (Powell *et al.* 1980). The initial resting potential (−75 to −85 mV) remained steady when the bathing calcium ion concentration was reduced to 2·5 mM. The cells were then stimulated by applying short (0·5–1·0 ms) depolarizing pulses and the resulting action potentials were typical of the rat ventricle, having a fast initial repolarization followed, at potentials negative

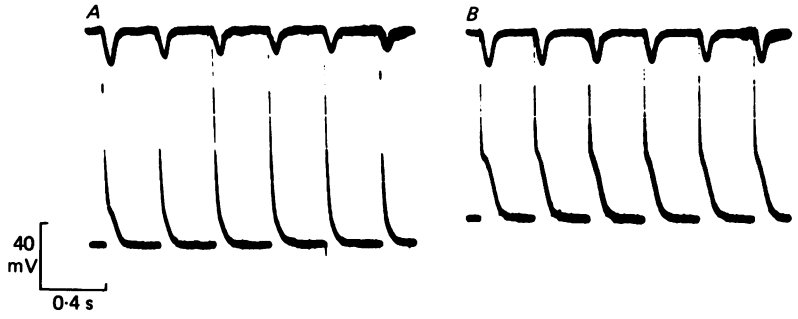

Fig. 1. Effects of an increase in stimulation rate from 0·3 to 3 Hz (at the beginning of each trace) on action potentials (lower traces) and amplitude of evoked contractions (upper traces; arbitrary units). In *A*, the resting membrane potential was −85 mV and in *B* the potential was depolarized with a constant current of 0·3 nA to −70 mV before increasing the stimulation rate. Note that the fast initial upstroke and repolarization cannot be adequately resolved at the sweep speed used here but they are not markedly changed by the increase in stimulation rate.

to −40 mV, by a long late phase of the plateau which appears to be dependent on intracellular calcium and extracellular sodium (see Mitchell *et al.* 1984*d*). The time course of the contractions was shown by the output of a photodiode (see Methods), a downward deflexion indicating a shortening of the cell. When the rate of stimulation was increased tenfold from 0·3 to 3 Hz, there was a gradual reduction in the amplitude of contraction and a decrease in the late phase of the action potential (Fig. 1*A*). The reduction in amplitude of contraction on increasing the rate of stimulation was found to be less marked at more depolarized levels as illustrated in Fig. 1*B*, where the cell was depolarized by 15 mV by applying a constant current. In addition, there was no substantial change in the resting membrane potential on increasing the rate of stimulation, which is in contrast to multicellular preparations where there is a transient depolarization followed by a hyperpolarization (Diacono, 1979).

I_{si} and time course of contraction

The rat ventricular cells were initially voltage clamped at −40 mV, at which potential the fast sodium current (Brown, Lee & Powell, 1981) and the transient outward current (Josephson, Sanchez-Chapula & Brown, 1984*b*) of these cells are inactivated. The I_{si}, initiated by a step depolarization to 0 mV, reached a peak within 3 ms and decayed with a half-time of 3 ms at 37 °C. Fig. 2*A* illustrates a typical I_{si} and the accompanying contraction seen during a depolarizing step lasting 100 ms and

while the cell was stimulated at 0·3 Hz. In these cells, contractions were initiated about 4 ms after the peak of I_{si} and had a duration of 80–100 ms, so that relaxation occurred while the cells were still depolarized at 0 mV.

In Fig. 2*B*, the duration of the depolarizing pulse was reduced to 10 ms, during which time I_{si} was fully activated but had not decayed to a steady state. On repolarization to −40 mV there appeared an inward current tail which was too slow to be due to inactivation of the I_{si} and has been found to depend on internal calcium

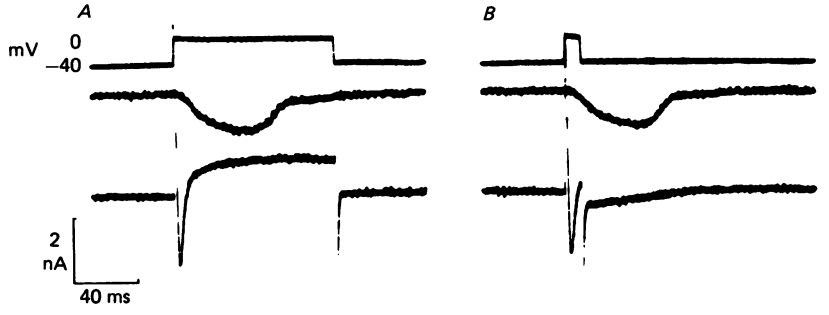

Fig. 2. Voltage-clamp records of I_{si}. The membrane potential was held initially at −40 mV and a step (upper trace) to 0 mV for 100 ms (*A*) or 10 ms (*B*) initiated the I_{si} (lower trace) and a contraction (middle trace), the time course of which was similar in both cases.

and external sodium (Mitchell, Powell, Terrar & Twist, 1984*e*). Although the duration of the depolarizing pulse was markedly reduced there was little change in the amplitude and time course of contraction. This observation is consistent with a proposed mechanism of excitation–contraction coupling in rat ventricular cells whereby calcium entering during activation of I_{si} induces release of calcium stored in the sarcoplasmic reticulum and the released calcium then initiates contraction (Fabiato & Fabiato, 1978; Mitchell, Powell, Terrar & Twist, 1984*b*). It seems possible that sufficient calcium enters even during a brief pulse to initiate the sequence of events leading to maximal contraction.

Effects of an increase in stimulation rate on currents and contractions

An increase in stimulation rate was found to induce marked changes in both currents and contractions when the duration of the depolarizing pulse was 100 ms but not when a short (10 ms) pulse was applied. Increasing the stimulation rate from 0·3 to 3 Hz with 100 ms pulses caused a decrease in the amplitude of I_{si} with each successive pulse down to a steady level of 50 ± 6 % (mean ± S.D.; range 39–59 %; $n = 9$) of the amplitude of I_{si} recorded at 0·3 Hz (Fig. 3*A*). This decrease was also seen in the presence of 1 mM-4-aminopyridine which inhibits a transient outward current probably carried by potassium (Kenyon & Gibbons, 1979) thus the reduction of the inward current was not due to a rate-dependent increase in an opposing outward current. In addition to the reduction in amplitude of I_{si} on increasing the stimulation rate, there was a change in the time course of decay of I_{si}. A semilogarithmic plot of the decay of the current recorded at 0·3 Hz showed that the time course could be fitted by at least two exponential components (see Methods) and on stimulation at

3 Hz there was a reduction or abolition of the first component of decay (Figs. 3*B* and 6*A*).

The amplitude of the evoked contraction was also reduced when the stimulation rate was increased to 3 Hz and extra contractions of increasing magnitude appeared in addition to the evoked contractions. During the development of these extra contractions, small inward currents of a transient nature also appeared between the I_{si}s (Fig. 3*A*).

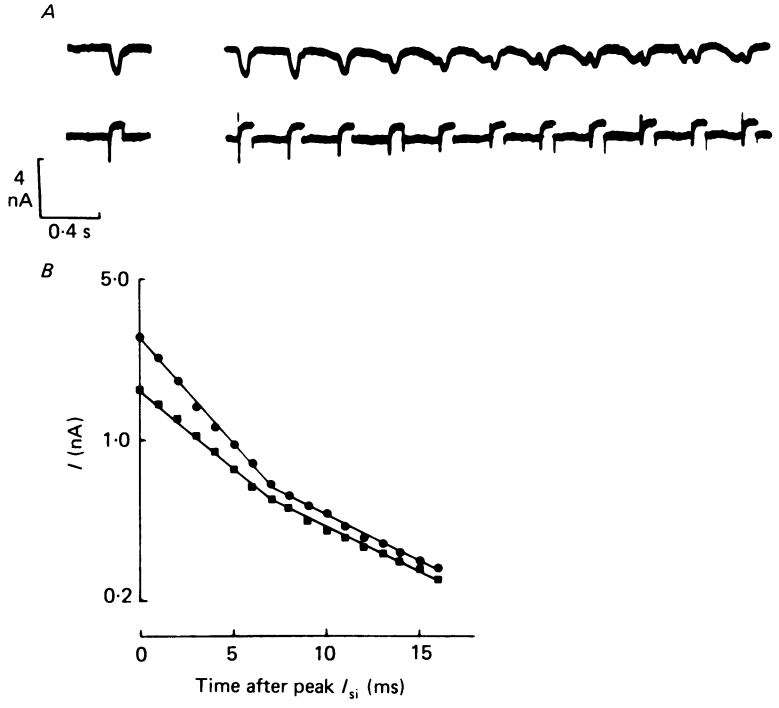

Fig. 3. Effects of an increase in stimulation rate on currents and contractions when the depolarizing pulse duration is 100 ms. *A* shows the I_{si} (lower records) and accompanying contraction (upper records) during stimulation at 0·3 Hz (left) and immediately after increasing the rate to 3 Hz (right). *B* shows a semilogarithmic plot of the decay of I_{si} before (top trace) and after (lower trace) the increase in stimulation rate.

Reduction in pulse duration and abolition of rate-dependent effects

When the pulse duration was reduced to 10 ms, the effects of an increase in stimulation rate were investigated from 0·3 to 3 Hz and also from 0·3 to 4·2 Hz (Fig. 4). The latter rate was chosen in order to keep the interval between pulses the same as that in the above experiment (Fig. 3) where the pulse duration was 100 ms. At neither of these stimulation rates was there any change in the amplitude of I_{si} or of the accompanying contractions nor was there any appearance of extra contractions and transient inward currents. The decrease in amplitude of I_{si} seen on increasing the stimulation rate with 100 ms pulses (Fig. 3) could not therefore have been due to insufficient time for reactivation of I_{si} channels. The slow inward tail of current observed on repolarization after 10 ms was unaffected by changes in stimulation rate over the range investigated.

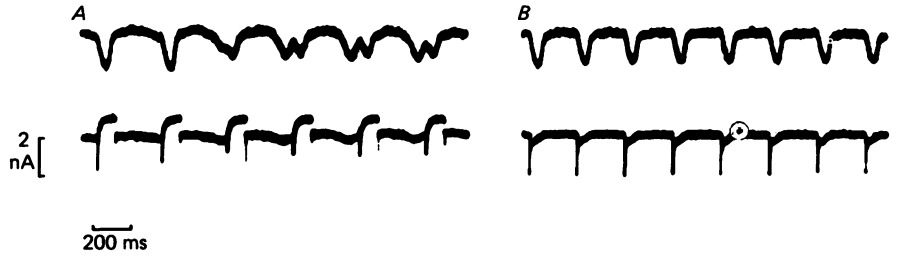

Fig. 4. Comparison of the effects of an increase in stimulation rate with two different depolarizing pulse durations. In *A* the cell was depolarized to 0 mV with a 100 ms pulse and in *B* with a 10 ms pulse. The stimulation rate was increased (at the beginning of each trace) from 0·3 to 3 Hz in *A* and from 0·3 to 4·2 Hz in *B*.

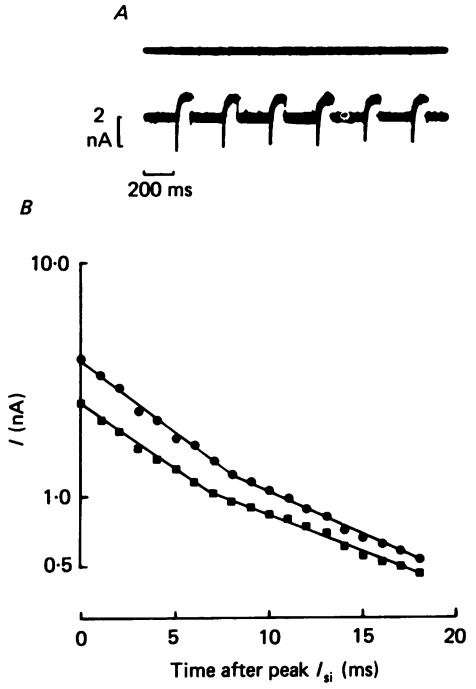

Fig. 5. Intracellular EGTA and increase in stimulation rate. The recording electrode was filled with EGTA (1 M) and KCl (2 M) and upon impalement of the cell all contraction was abolished (*A*, upper trace). The stimulation rate was increased from 0·3 to 3 Hz at the beginning of the trace in *A* and a small reduction in I_{si} occurred. *B* shows a semilogarithmic plot of the decay of I_{si} before (upper trace) and after (lower trace) the increase in stimulation rate.

Effects of intracellular EGTA and of ryanodine

Transient inward currents and associated contractions have been observed in multicellular preparations under conditions where the intracellular calcium ion concentration was raised (Kass *et al.* 1978*a*). In order to determine whether the extra currents and contractions appearing at the higher stimulation rates in single cells (cf. Fig. 3*A*) resulted from a similar mechanism, we have modified the intracellular calcium in two ways. First, cells were impaled with electrodes containing 1 M-EGTA

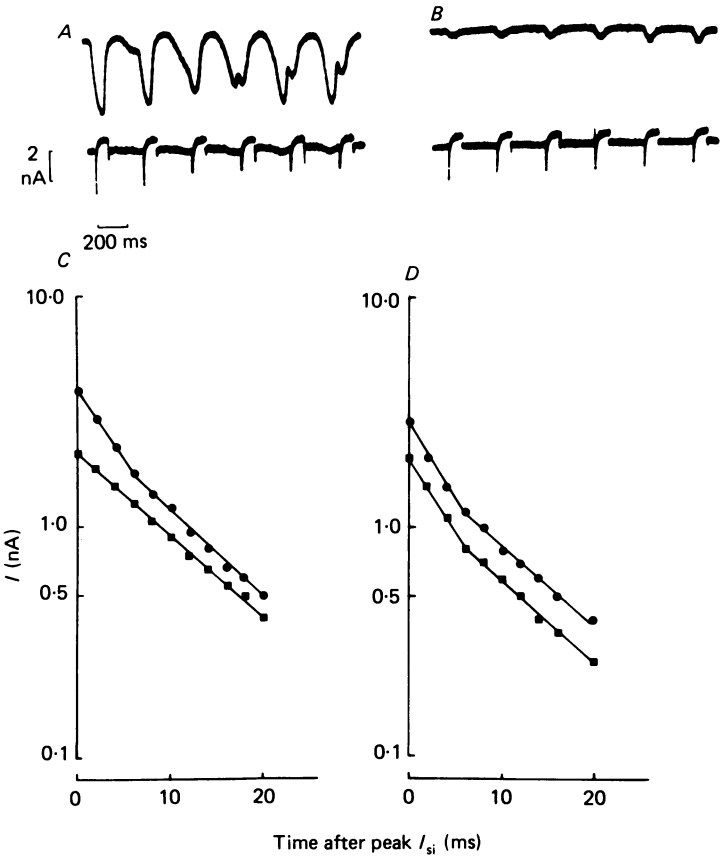

Fig. 6. Inhibition by ryanodine of the effects of an increase in stimulation rate. The stimulation rate with 100 ms pulses was increased at the beginning of the trace in both *A* and *B*, from 0·3 to 3 Hz. Ryanodine (2 μM) was added to the bathing solution after the records in *A* were taken and the cell stimulated at 0·3 Hz for 2 min, when the records in *B* were obtained. Semilogarithmic plots of current decay in the absence (*C*) and after 2 min in the presence (*D*) of ryanodine are shown at the stimulation rates of 0·3 Hz (upper traces) and 3 Hz (lower traces).

and all contractions were immediately abolished presumably as a consequence of chelation of intracellular calcium ions by EGTA which has diffused from the tip of the micropipette. In these cells, an increase in stimulation rate from 0·3 to 3 Hz (100 ms pulses) caused a gradual reduction in I_{si} amplitude (Fig. 5*A*), but only to $74 \pm 3\%$ (mean $\pm$ S.D., range 68–77 %; $n = 11$) of the amplitude at 0·3 Hz (cf. 50 % with KCl electrodes) and there was little or no change in the time course of current decay (Fig. 5*B*). The greater inactivation of I_{si} in the absence of EGTA is consistent with the presence of a calcium-induced inactivation of the current. Such a mechanism appears to reduce the first exponential component of decay in preference to the second and this inhibition is reduced in the presence of intracellular EGTA. The lack of appearance of the transient inward currents when the stimulation rate was increased during impalement with EGTA-containing electrodes also suggests that these currents were activated by intracellular calcium.

The experiments with EGTA show the importance of calcium in the effects of increasing the stimulation rate, but they give little information on the source of this calcium. One possibility is that there is an increased influx of calcium across the sarcolemma so that the cytoplasmic concentration of calcium increases and can initiate contraction and activate the transient currents. If this were the case, however, a gradual increase in tonic tension might be expected rather than fluctuating contractions. Alternatively, the concentration of calcium within the cell might be enhanced and reach a critical level for induction of calcium release from the sarcoplasmic reticulum (Fabiato, 1983); this would then initiate oscillatory contractions. In order to determine whether or not there is an involvement of stored calcium, further investigation was carried out using ryanodine, which is thought to interfere with calcium-induced calcium release from the sarcoplasmic reticulum (Sutko *et al.* 1979) and inhibits the evoked contraction in rat ventricular cells while prolonging the I_{si} (Mitchell *et al.* 1984*b*). Fig. 6*A* shows the changes in contractions and currents typical of an increase in stimulation rate (100 ms pulses), again from 0·3 to 3 Hz, at the beginning of the trace. Ryanodine (1–2 μM) was added to the superfusing solution and after 2 min exposure the evoked contraction was partially inhibited. An increase in stimulation rate induced a positive staircase but no extra contractions or currents appeared (Fig. 6*B*). In two cells where an increase in stimulation rate from 0·3 to 3 Hz, in the absence of ryanodine, induced a reduction in I_{si} amplitude to 50 and 46 %, the same increase in stimulation rate after 2 min exposure to ryanodine induced a reduction in amplitude to 70 and 65 % respectively. The decay of I_{si} at the two stimulation rates was plotted in the absence (Fig. 6*C*) and in the presence (Fig. 6*D*) of ryanodine. In the presence of ryanodine the increase in stimulation rate did not markedly affect the time course of current decay, an observation which is similar to that in the presence of intracellular EGTA (Fig. 5*B*) and is in contrast to the effects on I_{si} decay in the absence of EGTA or ryanodine, where there was a greater reduction in I_{si} amplitude and in the early exponential component of decay. The abolition or reduction by ryanodine of the effects of increasing the stimulation rate is consistent with the involvement of calcium release from intracellular stores both in the inactivation of I_{si} and in the activation of the extra contractions and transient inward currents. As in the presence of intracellular EGTA, some inactivation did occur at the higher stimulation rate in ryanodine. With more prolonged exposure to ryanodine (over 3 min) contraction was abolished while I_{si} decay was slowed and on increasing the stimulation rate from 0·3 to 3 Hz there was again little or no change in the time course of I_{si} decay.

Caffeine (1 mM), was also found to abolish the transient contractions and currents seen when the rate of stimulation with 100 ms depolarizations was increased from 0·3 to 3 Hz.

Recovery of I_{si}

The above experiments indicated that calcium release from intracellular stores could cause inactivation of I_{si}. A more detailed investigation of the recovery of I_{si} from inactivation was carried out using a double-pulse protocol in which the amplitude of I_{si} during the second pulse was compared to that during the first and where the interval between the pulses was varied between 10 and 200 ms. The

procedure was carried out twice, alternating between a first pulse duration of 10 ms and of 100 ms, while the second pulse was 100 ms in both cases (see Fig. 7). As the I_{si} did not completely decay to a steady level during the 10 ms pulse, the amplitude of this current was measured with respect to the steady level of current at the end of the depolarization during the following 100 ms pulse. The ratio of I_{si} amplitude during the second pulse to that during the first pulse was plotted against the interval

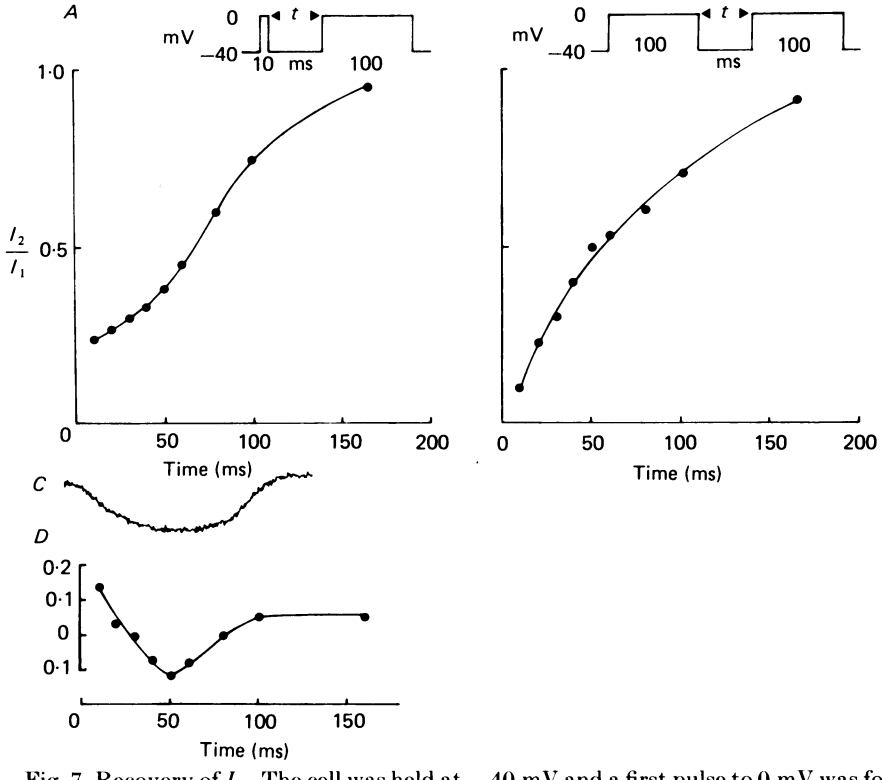

Fig. 7. Recovery of I_{si}. The cell was held at −40 mV and a first pulse to 0 mV was followed at increasing intervals by a second pulse, also to 0 mV. The ratio of the amplitude of the second evoked I_{si} (I_2) to that of the first (I_1) was plotted against the interval between pulses. For each interval, the procedure was carried out twice with a first pulse duration of 10 ms (*A*) and 100 ms (*B*), while the second pulse was constant at 100 ms (upper traces in *A* and *B*). The amplitude of I_{si} was taken as the difference between peak I_{si} and the steady-state current during the 100 ms pulses. The amplitude of I_{si} initiated by a 10 ms pulse was measured with respect to the steady-state current of the following 100 ms pulse. In *C* the time axis is the same as in *A* and illustrates the time course of contraction elicited during the procedure shown in *A*; this is activated during the 10 ms pulse so that contraction is proceeding at 0 time interval in *A*, which would correspond to the end of the 10 ms pulse. *D* shows a plot of the difference between the two curves of *A* and *B*.

between pulses. When the first pulse duration was 10 ms (Fig. 7*A*), the resulting plot showed a more complex time course than when the first pulse was 100 ms (Fig. 7*B*); there appeared to be an added inactivation of the second I_{si} at intervals between the pulses of approximately 10–100 ms (note that these times are shorter than the interval between 10 ms pulses at high stimulation rates (Fig. 4), where no inactivation of successive I_{si}s occurred). The time course of contraction initiated by the 10 ms pulse

(Fig. 7*C*) is shown in relation to the time course of recovery of I_{si} amplitude during the second pulse following a 10 ms first pulse (Fig. 7*A*). The difference between the two curves of Fig. 7*A* and *B* is plotted in Fig. 7*D* and it can be seen that there is a temporal relationship between this curve, the time course of the contraction and the extra inactivation of Fig. 7*A*. This indicates that calcium release from the sarcoplasmic reticulum may have caused a calcium-dependent inactivation of I_{si} in addition to initiating contraction. When the first pulse duration was 100 ms, however, the contraction was over by the end of this pulse and calcium released from the sarcoplasmic reticulum would have been at least partially taken up again into stores or extruded from the cell so that the added component of inactivation of the second current would not be seen. The reactivation of I_{si}, measured with a first pulse duration of 100 ms (Fig. 7*B*) followed an exponential time course for approximately 100 ms.

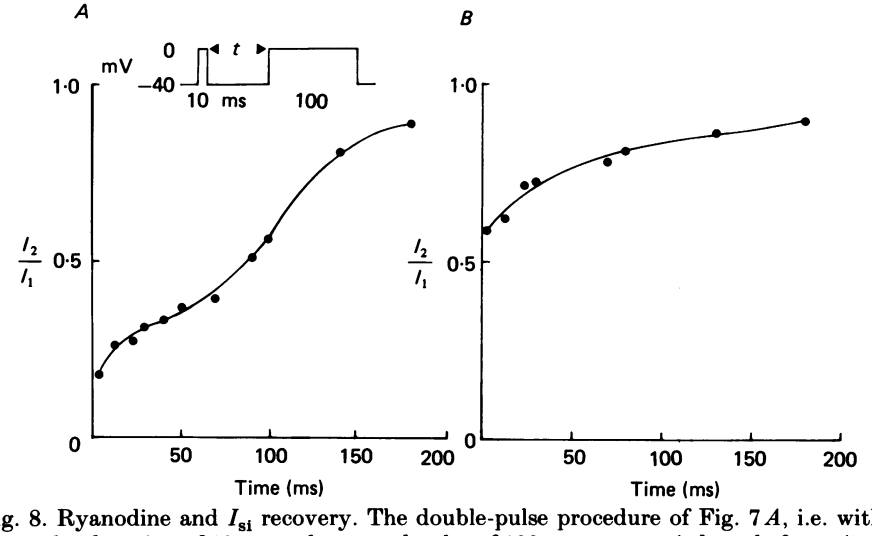

Fig. 8. Ryanodine and I_{si} recovery. The double-pulse procedure of Fig. 7*A*, i.e. with a first pulse duration of 10 ms and a second pulse of 100 ms, was carried out before (*A*) and 3 min after (*B*) exposure to 1 μM-ryanodine.

The possibility that calcium released from intracellular stores may lead to inactivation of I_{si} may be tested using ryanodine. Fig. 8*A* and *B* shows a double-pulse experiment before and after ryanodine in which the duration of the first pulse was 10 ms. Again there was an added component of inactivation between 10 and 100 ms (Fig. 8*A*), but this disappeared after 3 min exposure to the ryanodine (Fig. 8*B*). Even with very short pulse intervals there appears to be much less inactivation in the presence of ryanodine. When the recording micro-electrode contained 1 M-EGTA and contraction was suppressed, the time course of reactivation was similar to that seen in the presence of ryanodine (Fig. 8*B*) which is again consistent with the hypothesis that a decrease in intracellular calcium can relieve inhibition of the recovery of I_{si}.

DISCUSSION

Action potentials, contractions and increase in stimulation rate

In rat ventricular cells, an increase in stimulation rate caused a reduction in the evoked contraction (Fig. 1), an effect which is in contrast to that seen in other

mammalian species where an increase in stimulation rate induces a positive staircase (Morad & Goldman, 1973). A study of the effects of an increase in stimulation rate in rat ventricular muscle (Forester & Mainwood, 1974) has shown that negative inotropism occurs when the bathing calcium concentration is greater than 1·5 mM and positive inotropism occurs at lower calcium concentrations. It was suggested that the filling of an intracellular store of calcium by passive accumulation was complete at the higher external calcium concentrations, whereas at the lower concentrations the uptake increased with increasing frequency of stimulation; this would then allow a rate-dependent increase in calcium release and hence positive inotropy at the lower calcium concentrations. As calcium-dependent release of calcium from the sarcoplasmic reticulum appears to be particularly important in rat ventricular cells as a source of calcium for contraction (Fabiato & Fabiato, 1978), the reduction in amplitude of the evoked contraction on increasing the stimulation rate suggests that there has been a reduction in the amount of stored calcium available for release. The greater reduction in amplitude of contraction at more negative potentials may result from a greater proportion of calcium being extruded from the cell by sodium–calcium exchange, which would be more effective at more negative potentials (Mullins, 1979), so that less calcium is stored again in the sarcoplasmic reticulum. In other cardiac tissues where a positive staircase is normally induced on increasing the stimulation rate, it has been suggested that there is a rate-dependent accumulation of calcium in an intracellular store so that a greater evoked release of calcium would lead to an increase in contraction amplitude (Allen, Jewell & Wood, 1976; Edman & Jóhannsson, 1976). An alternative explanation is that there is a rise in the intracellular sodium concentration at high rates of stimulation and that this results in an increase in intracellular calcium (via sodium–calcium exchange) and hence an increase in contraction amplitude (Lederer & Sheu, 1983). Comparative studies of contraction in rat and guinea-pig ventricular cells suggest that there is an extra component of contraction in guinea-pig cells which is probably the result of influx of calcium at more depolarized potentials (Mitchell, Powell, Terrar & Twist, 1984*a*, *g*; see also Ochi & Trautwein, 1971); whether or not such an influx is a result of sodium–calcium exchange remains to be determined, but it could lead to differences in the effects of high rates of stimulation on contraction in different species.

The late plateau phase of the rat ventricular action potential was also reduced at the higher stimulation rate. This phase has been shown to depend on intracellular calcium and extracellular sodium (Mitchell *et al.* 1984*d*) and if there is a reduction in the amount of calcium released from intracellular stores at 3 Hz, as discussed above, this would also tend to cause a reduction in the late plateau phase. It has been suggested that a reduction in I_{si} might be involved in the decrease of the action potential duration at high stimulation rates (Payet *et al.* 1981), but this seems unlikely in view of the fact that I_{si} inactivation in rat ventricular cells is fast (Mitchell *et al.* 1983*b*; Josephson, Sanchez-Chapula & Brown, 1984*a*), and changes in this current would not therefore be expected to have a marked effect on the over-all action potential duration. It is possible, however, that a decrease in I_{si} could indirectly reduce the late phase because of a reduction in intracellular calcium.

Increasing the rate of stimulation had no obvious effects on the resting membrane potential in contrast to the transient depolarization observed in multicellular

preparations, which may be due to accumulation of potassium in the clefts between cells and subsequent activation of the electrogenic sodium–potassium exchange pump (Diacono, 1979). Accumulation would be expected to be much less in superfused single cells and so changes in resting potential arising from this would not be as marked.

I_{si} and increase in stimulation rate

There was a gradual reduction in I_{si} amplitude when the rate of stimulation with 100 ms depolarizing pulses was increased from 0·3 to 3 Hz and this could not be explained by there being too little time between pulses to allow complete recovery of the I_{si} channels, because there was no decrease in I_{si} amplitude when an increase in stimulation frequency was made with 10 ms depolarizing pulses. The reduction was not due to an increase in an opposing outward potassium current because 4-aminopyridine, which inhibits such currents, did not reduce the inactivation of I_{si} and, in any case, there is no marked contamination of I_{si} in rat ventricular cells by potassium currents (Mitchell *et al.* 1983*b*). Also, it is unlikely that large increases in the intracellular calcium ion concentration, arising from an increased influx across the sarcolemma at high stimulation rates, could reduce I_{si} to the extent seen here merely as a consequence of a reduction in the transmembrane calcium gradient. Indeed, it has been calculated in snail neurones that an increase in intracellular calcium ion concentration as high as 100 μM would not reduce the calcium current by more than 1 % (Plant, Standen & Ward, 1983).

The reduction in I_{si} amplitude was much less, though still present, in experiments where the intracellular calcium available for contraction was reduced, e.g. during impalement with EGTA-containing electrodes (Fig. 5) and in the presence of ryanodine (Fig. 6). These observations are consistent with the suggestion that a component of I_{si} may be inactivated by intracellular calcium which has been released from intracellular stores. The increase in rate also caused the reduction or disappearance of the early component of I_{si} current decay, an effect not seen with intracellular EGTA or in the presence of ryanodine. Calcium-induced inactivation of calcium currents has been proposed to be present in cardiac tissues, e.g. calf Purkinje fibres (Marban & Tsien, 1981; Kass & Sanguinetti, 1984), rabbit sino-atrial node (Brown, Kimura & Noble, 1981; Brown, Kimura, Noble, Noble & Taupignon, 1984), frog atrium (Hume & Giles, 1982; Mentrard, Vassort & Fischmeister, 1984) and rat ventricular cells (Mitchell *et al.* 1983*b*; Josephson *et al.* 1984*a*).

EGTA and ryanodine did not prevent some reduction of I_{si} after the increase in stimulation rate. It is unclear from these experiments whether there is a component of inactivation in some way caused by the prolonged (100 ms) depolarization at 0 mV or whether there is still a sufficient increase in intracellular calcium due to influx across the sarcolemma to inactivate I_{si}. If the latter were true, it would appear that EGTA was not able to buffer this calcium, possibly because of saturation of the EGTA near the membrane as proposed in *Aplysia* neurones to account for the EGTA-resistant inactivation (Eckert & Ewald, 1983) or because of the calcium being taken up into a membrane compartment near to or associated with the calcium channel, such as that proposed for insect skeletal muscle (Standen & Stanfield, 1982).

Spontaneous currents and contractions at high stimulation rates

The hypothesis that the appearance of extra contractions and transient inward currents at high stimulation rates was caused by secondary release of calcium from the sarcoplasmic reticulum is supported by the observation that these were abolished in the presence of ryanodine. Such contractions and currents did not occur when the pulse duration was only 10 ms suggesting that oscillatory release of calcium from the sarcoplasmic reticulum did not occur under these conditions. As calcium release from the sarcoplasmic reticulum is dependent on the attainment of a critical level of calcium and rate of change of calcium concentration (Fabiato, 1983), the increase in stimulation rate with the 100 ms pulse presumably caused an increase in the intracellular calcium level and turnover so that the extra release of calcium from the sarcoplasmic reticulum was initiated. This could arise from the increased influx across the sarcolemma and from the release of calcium from the myofilaments during relaxation; subsequent sequestration in the cell might be increased because of a reduced extrusion of calcium while the potential was held for 100 ms at 0 mV, where the conditions for calcium efflux via sodium–calcium exchange would be less favourable than at −40 mV (Mullins, 1979). With the 10 ms pulses, the repolarization to −40 mV would allow a more effective reduction in intracellular calcium because relaxation occurred after repolarization and hence oscillatory release of calcium would not be expected to occur.

The lack of appearance of the extra contractions and currents in the presence of caffeine, which is thought to inhibit calcium reuptake into the sarcoplasmic reticulum (Blinks, Olson, Jewell & Braveny, 1972; Hess & Wier, 1984), also provides evidence for a role of the sarcoplasmic reticulum in the observed effects of an increase in stimulation rate.

The small inward currents which appeared at the higher rate of stimulation in conjunction with the extra contractions seem to be similar to the transient inward currents observed in cardiac Purkinje fibres after exposure to cardiotonic steroids, which are thought to cause an increase in intracellular calcium as a consequence of a decrease in extrusion of calcium via sodium–calcium exchange; this arises from a reduction in the sodium gradient caused by the inhibition of the sodium–potassium pump by these drugs (Kass *et al.* 1978*a*). These transient inward currents and associated contractions were attributed to activation by oscillatory calcium release from loaded intracellular stores, the inward current being carried partly by sodium ions and proposed to be either a non-specific current or an electrogenic current reflecting calcium–sodium exchange (Kass, Tsien & Weingart, 1978*b*). Similarly, exposure to potassium-free solutions leads to the appearance of extra contractions and transient inward currents which are inhibited by ryanodine (Sutko & Kenyon, 1983). Direct measurements of intracellular calcium, using the photoprotein aequorin, show oscillatory changes in calcium which are increased when the stimulation rate is increased or after exposure to strophanthidin (Allen, Eisner & Orchard, 1984). Caffeine or ryanodine inhibited the effects of strophanthidin on the fluctuations in light and associated fluctuations in tension, again suggesting the involvement of intracellular stores.

Evoked contractions and stimulation rate

The reduction in amplitude of the evoked contraction when the stimulation rate was increased from 0·3 to 3 Hz, on stimulation by 100 ms pulses, could result from a reduction in the calcium available for release from the sarcoplasmic reticulum if extra release has recently occurred. After partial inhibition of the contractions by ryanodine, an increase in stimulation rate does induce a positive staircase. This may have resulted from an increase in cytoplasmic calcium causing a corresponding increase in release of the residual calcium still available for liberation from the stores. Alternatively, an increased cytoplasmic calcium concentration could augment the calcium released from intracellular stores and thus increase the amplitude of contraction. An additional factor is that intracellular sodium may rise during the higher rate of stimulation thus causing an increment in the intracellular calcium concentration via sodium–calcium exchange and an increase in the contraction amplitude (Lederer & Sheu, 1983).

In contrast, there is no change in the amplitude of the evoked contraction on increasing the stimulation rate when the depolarizing pulse duration is only 10 ms, suggesting that there had not been as marked a change in intracellular calcium as had occurred with the 100 ms pulses. As the duration of the depolarization at 0 mV is somewhat similar to the duration of the action potential at this potential, the reduction in the amplitude of the contractions accompanying the action potentials at 3 Hz implies that there are other factors affecting the intracellular calcium when action potentials are stimulated. As discussed in relation to Fig. 1, there may be a larger component of calcium extruded from the cell at more negative potentials and less is taken up again into the stores to maintain them at an optimum level.

Recovery of I_{si} from inactivation

The time course of recovery of I_{si} from inactivation was much slower than the time course of decay of the current. Similar findings have been reported for the I_{si} in cardiac muscle of other species, e.g. in cat ventricle (Kohlhardt, Krause, Kübler & Herdey, 1975) and frog atria (Shimoni, 1981; Mentrard *et al.* 1984), and for the calcium currents in insect skeletal muscle (Ashcroft & Stanfield, 1982) and in snail neurones (Adams & Gage, 1979). In our experiments the time course of recovery was found to be markedly different when the first pulse duration was reduced from 100 to 10 ms. With an initial pulse of 100 ms, the recovery followed an exponential time course for the first 100 ms but when the first pulse duration was only 10 ms, an added reduction in recovery of the following pulse was observed at intervals of 20–100 ms. With a 10 ms pulse, there followed a slow calcium-activated tail of inward current (Fig. 2; cf. Mitchell *et al.* 1984*e*) which might be expected to interfere with the following I_{si} and change the kinetics of recovery. This current would, however, be reduced by the following depolarization to 0 mV and it is unlikely that this could account for the observed time course of recovery of I_{si} after an initial 10 ms activating pulse since it was maximal immediately on repolarization to −40 mV, whereas the reduction in recovery was maximal at 40–50 ms. This reduction in recovery was not seen with intracellular EGTA or in the presence of ryanodine which suggests that calcium release from the sarcoplasmic reticulum had contributed to the added

inactivation. Presumably with a first pulse duration of 100 ms and relaxation occurring within this period, there would have been time for reuptake into the sarcoplasmic reticulum to prevent extra inactivation of the second I_{si}. The relationship between the time course of contraction and the added inactivation seen with a first pulse duration of 10 ms would also suggest that release of calcium from the sarcoplasmic reticulum, in addition to activating contraction, can cause some inactivation of I_{si}.

In summary, it appears that in rat ventricular cells the release of calcium from intracellular stores (particularly the sarcoplasmic reticulum) is a major factor underlying the observed effects of an increase in the rate of stimulation. The appearance of transient inward currents and contractions was consistent with an oscillatory release of calcium from intracellular stores and the decrease in I_{si} amplitude was caused, at least in part, by released calcium. The decrease in the evoked contractions accompanying the action potentials when the stimulation rate was increased is in contrast to the increase in contraction amplitude observed in other species and whether or not this difference is related to different sources of calcium for contraction remains to be investigated.

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