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THE EFFECTS OF STIMULATION RATE ON CALCIUM-DEPENDENT ACTION POTENTIALS RECORDED FROM CHICK EMBRYO HEART CELL AGGREGATES

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SUMMARY

1. Action potentials were recorded from aggregates of heart cells prepared from 3- or 7-day chick embryos. At 3 days the maximum rate of rise ($+\dot{V}_{max}$) was insensitive to TTX; at 7 days it was considerably reduced by TTX.

2. In the presence of TTX the action potential overshoot was dependent on $[Ca]_o$; the results may be fitted using constant field theory and assuming that the membrane is over a hundred times more permeable to Ca than to Na or K.

3. An increase in stimulation rate in the range 0·2–2 Hz led to an increase in both overshoot and $+\dot{V}_{max}$. This effect was not seen after addition of 20 mM-tetraethylammonium ions, nor when Sr was substituted for Ca in the external medium. We suggest that these rate-dependent changes may result from partial inactivation of an outward K current.

INTRODUCTION

In adult cardiac muscle, the shape of the action potential is influenced by the rate of firing (reviewed by Cranefield & Hoffman, 1958; Carmeliet, 1977; Boyett & Jewell, 1980). The firing rate appears to exert its effects both by affecting the degree of recovery of the ionic currents from one action potential to the next, and by altering the ionic concentrations close to the membrane.

Embryonic heart tissue has somewhat different electrophysiological properties from those of adult cardiac muscle (Shigenobu & Sperelakis, 1971, 1972; Sperelakis, Shigenobu & McLean, 1973). We have used aggregates of heart cells isolated from 7-day chick embryos (Moscona, 1961) in order to study the effects of rate on the action potential of such tissue. Such aggregates are relatively easy to record from and retain similar properties to those of intact tissue of a corresponding age (McDonald & Sachs, 1975). In particular the fast Na channels in this tissue may be blocked by tetrodotoxin, leaving a 'calcium' action potential, which appears to result from inward current flowing through a channel with very similar properties to that underlying the slow inward current, I_{si}, in adult tissue. (In adult Purkinje fibres Grabowski, Lüttgau & Schulze (1978) have shown that action potential plateau height is a good measure of I_{si}.) In this paper we characterize the ionic dependence of the

action potential in the presence of TTX and describe the effects of rate on this simplified action potential.

METHODS

Tissue culture

Fertilized eggs from White Leghorn hens were incubated at 37 °C for the required number of days. The hearts were aseptically removed from the embryos and were cut into pieces of about 1 mm diameter. The tissue was placed into a solution of collagenase 0·5 mg/ml. (Worthington) in Ca/Mg-free Tyrode solution at 37 °C for 4–8 min. The composition of this Tyrode solution was as follows (mM): NaCl, 137; KCl, 2·7; NaH_2PO_4, 0·3; NaH_2CO_3, 11·9; glucose, 5·5. The tissue was gently sucked through a 21 g and then a 25 g syringe needle to produce a suspension of single cells. The cells were washed in culture medium and finally placed in a 30 ml. silicone rubber stoppered bottle at a density of 10^5–10^6 cells per 3 ml. culture medium. The culture medium was a modification of that used by Nathan, Pooler & De Haan (1976) and was as follows: 25 % 199 medium (Gibco), 2 % horse serum (Flow Laboratories), 4 % fetal calf serum (Flow Laboratories), 0·5 % gentamycin (Flow Laboratories), 68·5 % Earl's balanced salt solution containing the following components (mM): NaCl, 116·0; $MgSO_4$, 0·8; NaH_2PO_4, 0·9; $NaHCO_3$, 26·2; $CaCl_2$, 1·8 and glucose 5·5, gassed with 5 % CO_2, 20 % O_2, 75 % N_2. The bottles were placed on a gyratory shaker at 60–70 rev/min at 37 °C and the cells were allowed to aggregate for 1–4 days. The aggregates were transferred to 30 mm sterile tissue culture dishes (Sterilin) and were left for several hours to allow attachment to the bottom of the dish.

Electrophysiological recording

Culture dishes containing the aggregates were transferred to a heated platform and maintained at 30 ± 1 °C. At least one hour before recording commenced, the culture medium was exchanged for the recording medium which was either L-15 medium (Flow Laboratories) with 2 % fetal calf serum and 2 % horse serum, or a modified Hanks salt solution which consisted of the following (mM): NaCl, 120; KCl, 4·8; KH_2PO_4, 0·6; $MgSO_4$, 0·6; $CaCl_2$, 1·2; $NaHCO_3$, 11·9; glucose 5·6; HEPES, 20. The solution was bubbled with 95 % O_2, 5 % CO_2 and the pH was maintained at 7·2. Recordings were made from either spontaneously beating aggregates or aggregates stimulated by means of square wave pulses of 5 msec duration and intensities slightly above threshold delivered through Pt wire electrodes placed on either side of the aggregate. Action potentials were recorded intracellularly using 20–35 MΩ KCl micro-electrodes and standard electrophysiological techniques. Maximum rate of rise was measured using a differentiating circuit or from a fast sweep speed oscilloscope trace. Results are given as mean ± S.E. of the mean throughout.

RESULTS

Aggregates of 100–200 μm diameter produced from 7-day embryos and maintained *in vitro* for 2–4 days were used for most of the experiments. The Ca-dependence of the action potential was also investigated in aggregates from 3-day embryos, maintained *in vitro* for 1–2 days. Most aggregates beat spontaneously, though around 20 % would beat only if electrically stimulated. Table 1 shows the maximum diastolic potential (m.d.p.), overshoot and maximum rate of rise ($+\dot{V}_{max}$) of spontaneously beating 7-day and 3-day aggregates. All three parameters, and in particular $+\dot{V}_{max}$, were lower in 3-day aggregates. Our value for $+\dot{V}_{max}$ in 7-day aggregates is considerably lower than that reported by McDonald & Sachs (1975); this may be explained in part by the lower temperature which we used (30 °C rather than 38 °C). Addition of tetrodotoxin (TTX, 10^{-6} g/ml.) to the recording medium greatly reduced $+\dot{V}_{max}$ in 7-day aggregates, but did not affect this parameter in 3-day aggregates. M.d.p. and overshoot were not affected by TTX. Since we wished to study the Ca-

and rate-dependence of the action potential in the absence of fast Na inward current, TTX was added to the recording medium of all subsequent experiments.

Effects of Ca on overshoot and maximum rate of rise

In order to avoid changes in overshoot resulting from changes in firing rate these experiments were performed using aggregates which were paced at 0·5 Hz. Increasing $[Ca]_o$ increased both $+\dot{V}_{max}$ and overshoot, the responses being complete within 2 min. $+\dot{V}_{max}$ increased from 2·9±0·4 V/sec ($n = 8$) at 0·6 mM-Ca to 8·1±0·5 V/sec

TABLE 1. Action potential parameters of embryonic heart cell aggregates

Age	Maximum diastolic potential (mV)	Action potential overshoot (mV)	Maximum rate of rise (V/s)	Maximum rate of rise in presence of TTX 10^{-6} g/ml. (V/s)
3-day ($n = 5$)	−54·0±4·1	+20·2±1·7	7·7±1·0	7·4±0·8
7-day ($n = 10$)	−68·8±1·6	+26·5±1·6	31·8±6·6	5·1±0·8

at 5·6 mM-Ca. Fig. 1 shows the effect of varying $[Ca]_o$ from 0·6 mM to 5·6 mM on the overshoot. The overshoot increased by about 23 mV for a 10-fold increase in $[Ca]_o$ (Fig. 1*B*). These results could be fitted using an appropriate form of the constant field equation (Goldman, 1943; Fatt & Ginsborg, 1958).

$$V = \frac{RT}{F} \ln \frac{4P'_{Ca}\alpha[Ca]_o + P_{Na}\alpha[Na]_o + P_K\alpha[K]_o}{P_{Na}\alpha[Na]_i + P_K\alpha[K]_i}, \quad (1)$$

where

$$P'_{Ca} = P_{Ca}\left\{\frac{1}{\exp(VF/RT)+1}\right\}$$

which can be solved by a quadratic method. V, R, T and F have their usual meanings; $\alpha[K]$, $\alpha[Na]$ and $\alpha[Ca]$ are K, Na and Ca ion activities. For internal ionic activities we took the values measured by Fozzard & Sheu (1980) in 7-day chick embryonic heart: $\alpha[K]_i = 82{\cdot}6$ mM, $\alpha[Na]_i = 8{\cdot}7$ mM. The activity co-efficients (α) of ions in the external solution were calculated according to Blaustein (1974) giving α for Na and K a value of 0·75 and for Ca of 0·31. $\alpha[Ca]_i$ was assumed to be effectively zero.

The best fit was obtained using the ratio of permeabilities $P_{Ca}:P_{Na}:P_K = 1:0{\cdot}006:0{\cdot}006$ which indicates that, at the peak of the action potential, the membrane is over 100 × more permeable to Ca than to Na or K.

We also fitted our results making the asumption of fixed negative charges at the inner membrane surface giving a surface potential, V', (Frankenhauser, 1960; Meves & Vogel, 1973; Reuter & Scholtz, 1977).

Under these conditions P'_{Ca} in eqn. (1) is given by

$$P'_{Ca} = P_{Ca}\left\{\frac{1}{\exp[(V-V')F/RT]+1}\right\}$$

and the equation may then be solved by successive approximation. Taking $V' = +50$ mV (cf. Meves & Vogel, 1973) the ratio of P_K and P_{Na} to P_{Ca} was increased 2·5-fold but is still very low. A good fit in the presence of surface charge was obtained

with $P_{Ca}:P_{Na}:P_K = 1:0{\cdot}015:0{\cdot}015$. These ratios are rather similar to those used by Reuter & Scholtz (1977) to fit their results for slow inward current in adult ventricular muscle.

In addition, experiments were carried out in which the Na concentration was roughly halved from 131·9 to 65 mM (replaced by Tris-HCl). The overshoot of the

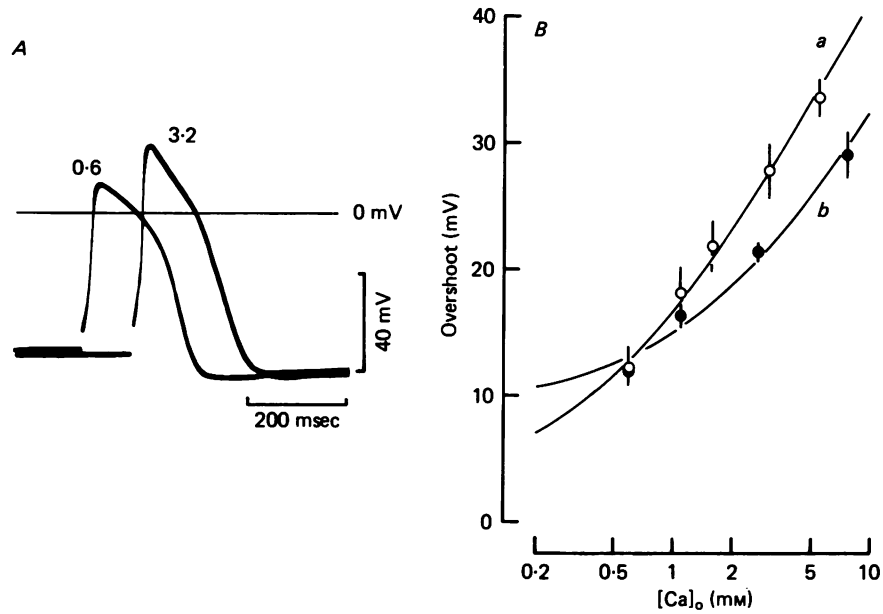

Fig. 1. Calcium dependence of heart cell aggregate action potentials. *A*, action potentials recorded from aggregates produced from 7-day chick embryo heart cells at 0·6 and 3·2 mM external Ca concentration. The rapidly rising phase of the action potential has been traced in this and subsequent records. *B*, means and standard errors of action potential overshoots plotted against log of external Ca concentration in the presence of TTX. 10^{-6} g/ml. ○, overshoots recorded from aggregates produced from 7-day chick embryo hearts ($n = 7$–14). ●, overshoots recorded from aggregates produced from 3-day chick embryo heart ($n = 6$). The lines are drawn to eqn. (1) taking (*a*) $P_{Ca}:P_{Na}:P_K = 1:0{\cdot}006:0{\cdot}006$; (*b*) $P_{Ca}:P_{Na}:P_K = 1:0{\cdot}02:0{\cdot}016$.

action potential was reduced by $6{\cdot}4 \pm 1{\cdot}4$ mV ($n = 4$) which is quite close to the predicted reduction of 5·7 mV obtained using equation (1) and $P_{Ca}:P_{Na}:P_K = 1:0{\cdot}006:0{\cdot}006$.

Although the membrane appears from these results to be much more permeable to Ca than to Na and K, about half the inward current will be carried by Na ions since $\alpha[Na]_0$ is so much greater than $\alpha[Ca]_0$. The constant field equations for I_{Na} and I_{Ca} with the above permeability ratios predict that in 1·2 mM-Ca saline 50·8 % of the inward current will be carried by Ca and 49·2 % by Na.

We also examined the relation between overshoot, $+\dot{V}_{max}$ and $[Ca]_0$ in 3-day aggregates. $+\dot{V}_{max}$ increased from $5{\cdot}2 \pm 0{\cdot}6$ V/sec ($n = 6$) at 0·6 mM-Ca to $11{\cdot}4 \pm 1{\cdot}2$ V/sec at 7·7 mM-Ca. The overshoot increased by approximately 16 mV for a 10-fold increase in $[Ca]_0$ (Fig. 1*B*). The curve was fitted using Fozzard & Sheu's

(1980) values for 4-day chick embryos of $\alpha[Na]_i$ = 12·5 mM and $\alpha[K]_i$ = 71·3 mM and taking $P_{Ca}:P_{Na}:P_K = 1:0{\cdot}02:0{\cdot}016$.

Effects of stimulation rate on the action potential

In 7-day aggregates in the presence of TTX an increase in stimulation rate from 0·2 to 2 Hz led to an increase in overshoot of 3–4 mV and in $+\dot{V}_{max}$ of 1–2 V/sec. At frequencies greater than 2 Hz most aggregates displayed a decrease in both overshoot and $+\dot{V}_{max}$.

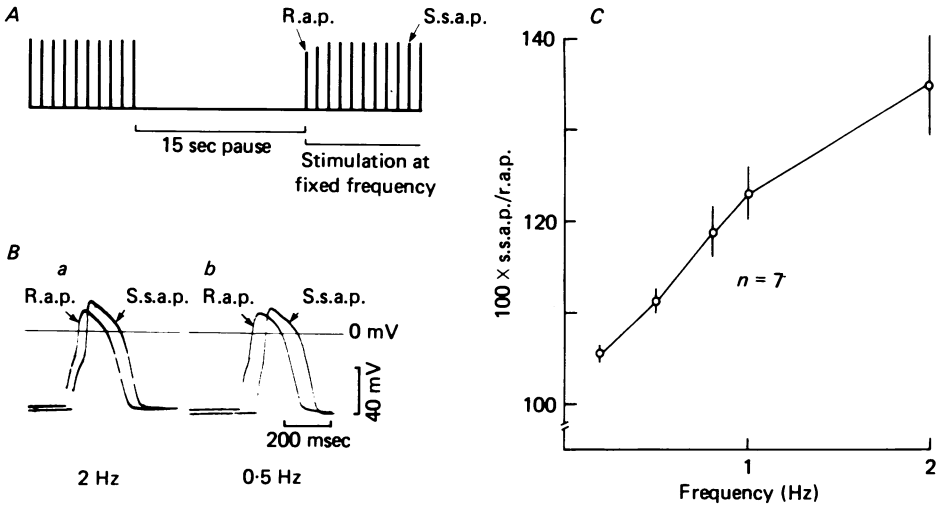

Fig. 2. *A*, diagram to show the experimental procedure used to determine the effects of rate on action potential shape (see text for details). *B*, action potentials recorded from aggregated 7-day chick embryo heart cells: (*a*), the rested action potential and the steady state action potential at a frequency of 2 Hz; (*b*), the rested action potential and the steady state action potential at a frequency of 0·5 Hz. *C*, relationship between steady state action potential overshoots as a percentage of the rested state action potential overshoot and frequency of stimulation. Action potentials were recorded from heart cell aggregates produced from 7-day chick embryos in L-15 medium containing TTX. 10^{-6} g/ml.

In order to examine the action potential changes at frequencies up to 2 Hz we used an interrupted stimulation protocol (Fig. 2*A*). Aggregates were driven at a given frequency in the range 0·2–2 Hz and we allowed at least 2 min for the action potentials to reach a steady state. Such action potentials are referred to as steady-state action potentials (s.s.a.p.). Stimulation was stopped for 15 sec and the first action potential recorded after this pause is referred to as the rested action potential (r.a.p.). The r.a.p. had a lower overshoot and $+\dot{V}_{max}$ and was of shorter duration than the s.s.a.p., and these differences were more marked when the s.s.a.p. was driven at a higher frequency (Fig. 2*B*). In Fig. 2*C* the overshoot of the s.s.a.p. expressed as a percentage of the overshoot of the r.a.p. is plotted against stimulation frequency. Both overshoot and $+\dot{V}_{max}$ recovered to the steady-state level over the first two to three action potentials after the pause.

In addition to these changes in overshoot and $+\dot{V}_{max}$ the resting membrane potential became more negative (by up to 8 mV) during continuous stimulation. This did not increase with frequency in the range studied however, being already maximal at 0·2 Hz. The change was abolished in TEA saline (Fig. 3*A*). Such increases in resting potential on stimulation have been explained in terms of an increased stimulation of a Na–K pump which hyperpolarizes the membrane either because it lowers $[K]_o$ below the normal value or because it is electrogenic (Vasalle, 1970; Glitsch, 1973).

A number of mechanisms could explain the rate-dependent changes in overshoot and $+\dot{V}_{max}$. They do not seem to be simply a consequence of the change in resting potential, since this was maximal at 0·2 Hz, whereas overshoot and $+\dot{V}_{max}$ increase progressively in the range 0·2–2 Hz. It is also unlikely that an increase in $[K]_o$ plays an important role; this would depolarize the membrane whereas a hyperpolarization is observed.

It seems likely, therefore, that the observed changes in overshoot and $+\dot{V}_{max}$ result from a change in membrane conductance. Possible mechanisms are a facilitation of the slow inward current system, or a reduction in an outward K current. In order to affect overshoot and $+\dot{V}_{max}$, such a K current would have to be either an early K current or to persist from the previous action potential. This current would be fully activated after the 15 sec gap, but would become partially inactivated when stimulation is recommended, thus giving rise to an increase in $+\dot{V}_{max}$ and overshoot.

To examine possible involvement of I_{si} facilitation we looked at the effect of increased external Ca concentration on the rate dependent changes in overshoot and $+\dot{V}_{max}$. When external Ca concentration was increased from 0·6 to 3·1 mM in four experiments, the difference in overshoot and V_{max} between the r.a.p. and s.s.a.p. were similar, suggesting that external Ca concentration had no effect on the rate-dependent process. Such an experiment does not absolutely rule out Ca facilitation, however. We used eqn. (1) to predict the change in P_{Ca} needed to give the observed change in overshoot at a $[Ca]_o$ of 3·1 mM and then calculated the change in overshoot which would result from such a P_{Ca} change at 0·6 mM-$[Ca]_o$. The predicted difference in the overshoot changes between the r.a.p. and the s.s.a.p. was only 2·3 mV which may not have been easily detectable.

Effect of TEA

If the rate-dependent changes in the action potential do depend on changes in K current then it might be expected that they would be affected by K current blockers. We examined the effect of 20 mM-tetraethylammonium chloride (TEA) added to the bathing saline. Fig. 3*A* shows that TEA increased both the height and duration of the s.s.a.p. and that, in the presence of TEA, the r.a.p. was almost identical to the s.s.a.p., so that the rate dependent changes were abolished (Fig. 3*B*). Since 20 mM-TEA increased the tonicity of the medium, we also increased the tonicity by the same amount using sucrose. 40 mM-sucrose had no effect on either the r.a.p. or s.s.a.p.

Effect of Sr

In a second set of experiments the divalent cation strontium was substituted for the external Ca. In the absence of Ca, Sr readily permeates the slow inward current channel (Vereecke & Carmeliet, 1971; Kohlhardt, Haastert & Krause, 1973) but under these conditions the transient outward current is strongly suppressed in calf cardiac

Purkinje fibres (Siegelbaum & Tsien, 1980). When 3 mM-Ca was replaced by 3 mM-Sr a slight depolarization of 2–3 mV was usually observed and a decrease in height and an increase in the duration of the action potential also occurred (Fig. 4). In five experiments the rate-dependent changes in overshoot and $+\dot{V}_{max}$ between r.a.p. and s.s.a.p. did not occur in the Sr solution.

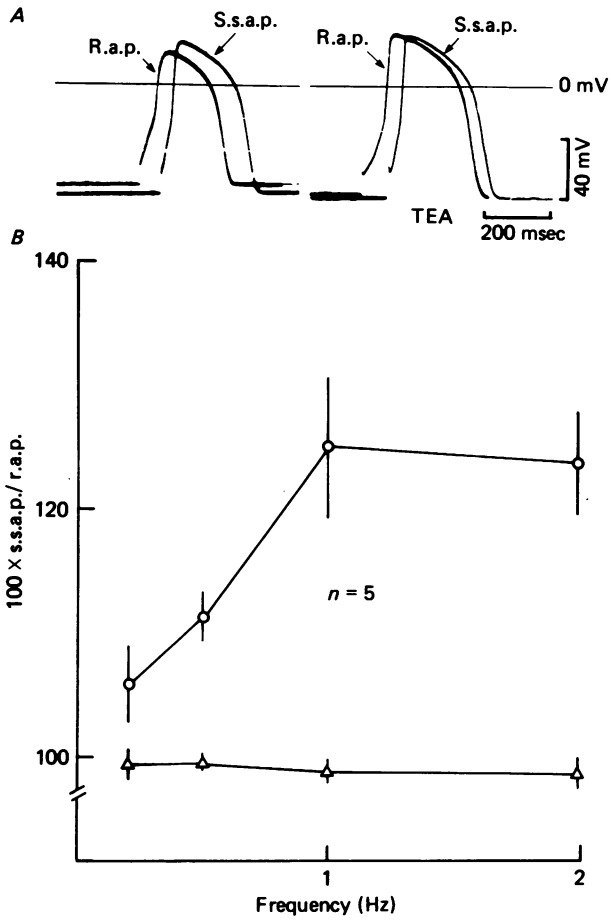

Fig. 3. *A*, the rested action potential and the steady state action potential recorded at a frequency of 1 Hz before and after the addition of 20 mM-TEA. *B*, steady state action potential overshoot as a percentage of rested action potential overshoot plotted against frequency of stimulation both before (○) and after (△) the addition of 20 mM-TEA. Action potentials were recorded from heart cell aggregates produced from 7-day chick embryos in L-15 medium containing TTX 10^{-6} g/ml.

DISCUSSION

Our results show that 3-day aggregates had a low rate of rise which was unchanged by TTX. 7-day aggregates had faster rates of rise which were TTX sensitive; in the presence of TTX the $+\dot{V}_{max}$ was reduced to the 3-day value. These findings are in agreement with those reported for intact embryonic tissue at a similar age (Pappano,

1977). Constant-field fits to the relation between action potential peak and $[Ca]_o$ in the presence of TTX suggest that the membrane is much more permeable to Ca than Na or K at this time, the ratios being similar to those previously reported for I_{si} in adult tissue (Reuter & Scholtz, 1977).

In common with other cardiac tissue (Boyett & Jewell, 1980) the embryonic heart cell aggregates used in the present study show rate-dependent action potential

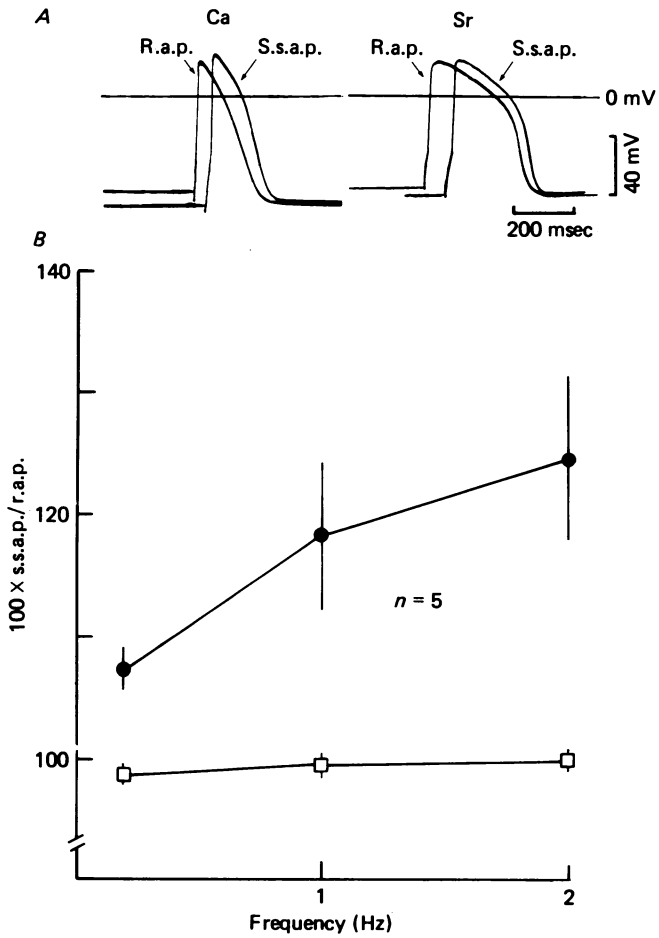

Fig. 4. *A*, the rested action potential and steady state action potential recorded at a stimulation frequency of 1 Hz in the presence of 3 mM-Ca or 3 mM-Sr. *B*, steady state action potential overshoot as a percentage of resting action potential overshoot plotted against frequency of stimulation, (●) 3 mM-Ca solution, (□) 3 mM-Sr solution.

changes. Overshoot and $+\dot{V}_{max}$ increased with increasing frequency up to 2 Hz. Increases in action potential plateau height with moderate increases in frequency have been reported in adult ventricular tissue of dogs (Hoffman & Suckling, 1954; Moore, Preston & Moe, 1965; Greenspan, Edmands & Fisch, 1967), rabbits (Gibbs & Johnson, 1961) and cats (Bass, 1975).

Although the resting potential increased on stimulation in our experiments this seems unlikely to be the basis for changes in overshoot and $+\dot{V}_{max}$ for reasons given

earlier (p. 6). Also, Sr saline completely abolished changes in overshoot and $+\dot{V}_{max}$ (Fig. 4*A*), whereas a change in resting potential, though rather smaller than in normal saline, was still seen. The most likely mechanisms for these changes in overshoot and $+\dot{V}_{max}$, then, are inward current facilitation or outward current depression.

In adult tissue, facilitation of the slow inward current, I_{si}, has been reported in ventricular fibres (Hiraoka & Sano, 1976), Purkinje fibres treated with cardiac glycoside (Weingart, Kass & Tsien, 1978) and frog atrium (Noble & Shimoni, 1981). While such a mechanism could explain our observations the lack of effect of changing $[Ca]_o$ and the action of TEA in removing the rate-dependent changes argues for the alternative possibility that an outward K current is depressed during repeated stimulation. It is possible that the K current involved is an early transient outward current. Such a current in Purkinje fibres is inhibited both by TEA (Kenyon & Gibbons, 1979) and by substitution of Sr for Ca in the bathing saline (Siegelbaum & Tsien, 1980). In addition Siegelbaum & Tsien, (1980) have reported a beat-dependent decline in transient outward current during trains of pulses of 0·5–1·67 Hz. Such a transient outward current was not reported in chick embryo heart cell aggregates by Nathan & De Haan (1979), but it is possible that it could be masked by inward currents, in particular I_{si}, since these two currents overlap in both time course and voltage dependence in Purkinje fibres (Vitek & Trautwein, 1971; Gibbons & Fozzard, 1975). An early outward K current could cause the rate-dependent effects which we observe if it recovered fully from inactivation during the 15 sec gap, but became partially inactivated when stimulation was recommenced, due to the briefer period between successive action potentials. Thus, this current would be larger in the r.a.p. than the s.s.a.p. and increasing frequency would increase the degree of inactivation of this current by shortening the time for its recovery. Since $\alpha[K]_i$ is so much larger than $\alpha[Ca]_o$ the K permeability underlying any such current need only be very small compared to P_{Ca}. In view of the effect of TEA and strontium we feel that such a K current mechanism provides the most probable explanation of the rate-dependent increases in overshoot and $+\dot{V}_{max}$ which we observed.

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