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THE DUAL EFFECT OF CALCIUM ON THE ACTION POTENTIAL OF THE FROG'S HEART

BY R. NIEDERGERKE AND R. K. ORKAND*
From the Department of Biophysics, University College London

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SUMMARY

1. Using ventricle strips of the frog's heart stimulated at the low rate of about 1 shock/min intracellular action potentials were recorded under conditions of varying calcium concentrations.

2. Overshoots of action potentials were increased by about 18·3 mV as a result of a 10-fold increase, within the range of 0·1–5 mM, of the calcium concentration.

3. A similar effect was obtained by strontium, but magnesium was ineffective.

4. The increase of the overshoot by high calcium was associated with an increased rate of rise of the potential during the later part of its ascending phase. The initial fast upstroke remained unaltered.

5. Another effect, a depression of the overshoot, developed during periods of repetitive stimulation, at the rate of 20/min, and this was followed by a gradual recovery during subsequent periods of rest.

6. The depression of the overshoot increased with increasing calcium concentrations reaching values of over 40 mV.

7. High concentrations of strontium and low concentrations of sodium also induced depression of the overshoot, but high magnesium was ineffective.

8. A tentative hypothesis has been proposed attributing these two effects: (*a*) to an entry of calcium through the excitable membrane thus contributing to the ionic inward current, and (*b*) to a resulting accumulation of calcium in some cellular store.

INTRODUCTION

It is known that the contraction of the frog's heart is strengthened either when the calcium concentration of the perfusion fluid is increased or when the sodium concentration is reduced (Wilbrandt & Koller, 1948) and that

* Postdoctoral fellow of the National Institute of Neurological Diseases and Blindness. Present address: Laboratory of Neurophysiology, Harvard Medical School, Boston, Massachusetts.

in both cases the calcium influx into heart cells is enhanced (Niedergerke, 1963*b*). One of the aims of the present and subsequent work has been to examine whether these changes of the contractile strength are accompanied by any corresponding changes in the size of the action potential.

Although the effects of calcium and sodium ions on the cardiac action potential have been the subject of previous investigations (Ware, 1961; Brady & Woodbury, 1960), it appeared worth while to extend the earlier results using a variety of different experimental conditions. Thus in the present work it was found that the rate at which the heart is stimulated has an important influence on the effects of calcium. For example, the overshoot of the action potential was enhanced by high calcium concentrations when the frequency of stimulation was low, but remained unchanged, or even declined, when the frequency was high. Another interesting result emerged from a comparison of the effects of calcium with those of strontium and magnesium: while strontium imitates the effect of calcium on the overshoot, magnesium does not. These changes of the 'overshoot' are to some extent parallel to those of the contractile strength produced by the same ions and may therefore help to throw some light on the process by which an action potential gives rise to a contraction.

A preliminary note of our results has already been published (Orkand & Niedergerke, 1964).

METHODS

The animals used for the present and subsequent work were either *Rana temporaria* (English or Dutch) or, on occasion, *R. pipiens*. The Ringer fluid had the composition in mM-NaCl 116, KCl 3, $NaHCO_3$ 2, and $CaCl_2$ varying between zero and 10 mM, 1 mM being the normal concentration. $CaCl_2$ was replaced, on occasion, by equivalent amounts of $SrCl_2$ or $MgCl_2$. Our calcium-free fluid contained a trace amount of about 0·01 mM of this ion as found by chemical analysis using the method of Kerr (1960).

Strips of frog ventricles were dissected as described previously (Niedergerke, 1956*a*), care being taken to remove all ventricular pacemaker tissue. After the dissection the strip was mounted in the recording chamber, held at both ends, but otherwise freely suspended in the bathing fluid, except at the region of stimulation where it was placed on a small pedestal made of methacrylate polymer (Perspex) (Fig. 1). The strip was stimulated with short negative-going square pulses from a pore electrode positioned above its upper surface, the other, earthed stimulating electrode being a narrow strip of platinum foil attached to the pedestal on which the stimulated end of the strip rested. Intracellular recording of resting and action potentials was by means of the conventional micro-electrode technique (Ling & Gerard, 1949; Nastuk & Hodgkin 1950) which was slightly modified to allow the measurement of the potential differences which frequently develop at the tip of the micro-electrodes when these are immersed in Ringer fluid (Adrian, 1956). This modification was necessary because electrodes of high resistance ($\geqslant$ 15–20 MΩ), although otherwise suitable for the recording in the small heart fibres, are prone to develop rather large 'tip potentials' and so give rise to considerable errors of the potential estimates (cf. Fig. 8B, Niedergerke & Orkand, 1966). The following electrode assembly for the measurement of the tip potentials was used (Fig. 1): Ag-AgCl/3 M-KCl (micro-electrode)/Ringer fluid/3 M-KCl (low impedance recording electrode)/Ag-AgCl. The input of the cathode follower probe was connected to a

miniature Ag-AgCl electrode enclosed in a small polythene tube whose lower end was placed inside the micro-electrode. The other Ag-AgCl electrode, at the low impedance input, was suspended in a vessel filled with 3 M-KCl, contact being made with the bathing solution through a fluid bridge of Ringer fluid. The fluid junctions at the low impedance input: Ringer/3 M-KCl and 3 M-KCl/3 M-KCl (surrounding the Ag-AgCl electrode) were through small sintered glass disks of low porosity. The 3 M-KCl fluids immediately surrounding the two Ag-AgCl electrodes were saturated with a precipitate of AgCl to prevent the chloride coating of the Ag-AgCl electrodes from dissolving. In this way slow potential drifts were kept small (Bates, 1954). At intervals the junction: Ringer/3 M-KCl at the low impedance input was renewed with fluid from the recording chamber. However, this precaution was necessary only during long recording periods (> 1 hr). The input capacitance of the system was approximately 1·5 pF.

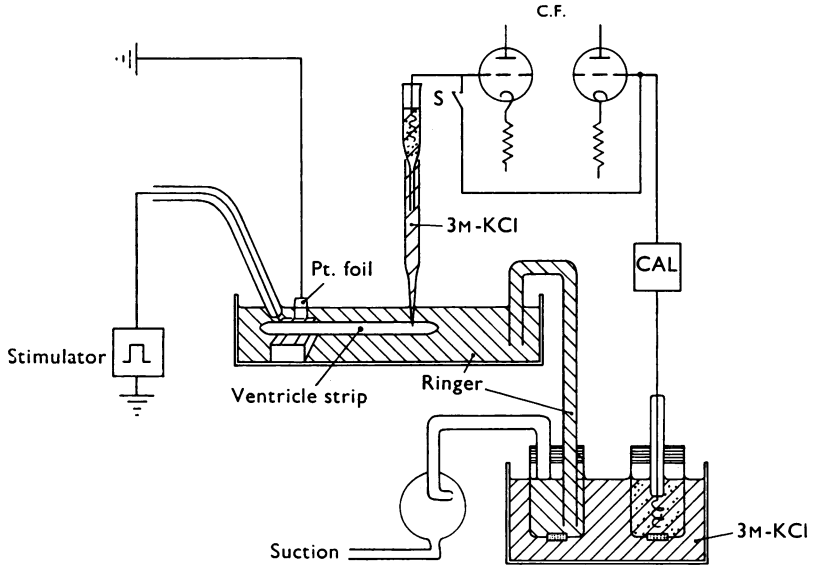

Fig. 1. Diagram of apparatus. Upper (miniature) Ag-AgCl (3 M-KCl)-electrode is inserted into micro-electrode, the indifferent Ag-AgCl (3 M-KCl)-electrode being connected with the bathing fluid through a fluid bridge filled with Ringer fluid. For stability of the electrode potentials the fluid immediately surrounding the two Ag-AgCl electrodes was saturated with a precipitate of AgCl (indicated by dots). A slight asymmetry potential, V_{As} ($\leqslant$ 2 mV) which usually existed between the two Ag-AgCl electrodes was measured, at the beginning and at the end of an experiment, by placing the upper Ag-AgCl electrode in the 3 M-KCl trough. The micro-electrode tip potential was determined by opening and closing switch S (micro-electrode being immersed in the bathing fluid) and the value V_{As} was subtracted from the V so obtained.

Potential recording was through two channels in parallel, the first being used for the direct recording of the action potential, the second, with a five times faster sweep speed, for the recording of the differentiated input to channel 1. The time constant of the differentiating network used for this purpose was 40 μsec or, occasionally, 120 μsec for the recording of slow potential changes. In some experiments made to examine in detail the rising phase of the action potential (e.g. experiments of Fig. 6) a second micro-electrode was used instead of the

bath electrode for recording. It was positioned at the surface of a fibre close to the point of insertion of the intracellular micro-electrode and served to minimize distortions of intracellular action potentials due to the electric field set up by the activity of neighbouring fibres.

The micro-electrodes used in the present work were selected to have tip potentials of less than about 3 mV. Results of insertions were rejected when there was evidence suggesting the development of a tip potential after the electrode had been inserted into a cell. Such potentials, which most frequently were negative with respect to the outside of the tip, could normally be recognized by a fairly slow apparent rise in resting potential associated with often quite considerable increases in electrode resistance. Using micro-electrodes of reasonably high resistance ($\geqslant$ 15 MΩ) the main criterion for a successful insertion was the stability of the recorded resting potential. When this declined during a period of about 30 sec after insertion the fibre under observation was abandoned. Usually six to ten insertions were made in different cells to obtain a potential estimate having a standard error of 2 mV.

All experiments were made at room temperature (18–20° C).

RESULTS

After the dissection the heart strip was kept mounted in the recording chamber and immersed in normal, 1 mM-Ca Ringer fluid for an initial period of equilibration lasting about 1 hr. Recording usually started in the presence of this fluid which was several times reapplied in the course of an experiment for further control periods of recording. Resting and action potentials were recorded in the experiments described in the first section of this paper at rather long intervals of about 1–5 min, the strip being stimulated at the rate of only 1 shock/min or less. Using these procedures resting and action potentials such as those illustrated in Fig. 2 normally remained constant over experimental periods of 12 hr or longer. The average resting potential obtained in our control fluid was 83·8 mV (40 strips; range 78–88 mV) and the average overshoot of the action potential 38·6 mV (range 28–45 mV).

Some comment is required as to the magnitude of these potentials, especially of the resting potential which appears to be smaller than that of 92·4 mV, recorded from fibres of the frog's sartorius muscle (Adrian, 1956). This difference is probably due largely to an artifact of the recording procedure using micro-electrodes whose insertion must be supposed to cause a relatively greater leak in, and subsequent shunt across, the excitable membrane of the small heart fibres (diameter of about 5 μ; Niedergerke, 1963*b*) than in the case of the larger fibres of the skeletal muscle. The effect of this leak may be estimated by assuming, as seems reasonable, that the value of the permeability ratio P_{Na}/P_K of the resting membrane of the heart is the same as that of 0·01 obtained for the skeletal muscle (Adrian, 1956). Thus, using the values for the intracellular concentration of potassium and sodium ions of frog ventricles, of 163 and 6 m-mole/kg cell water respectively (see Keenan & Niedergerke, in preparation, where

the significance of the low intracellular sodium concentration in relation to the present findings will also be discussed), the theoretically expected resting potential is

$$V_R = \frac{RT}{F} \ln \frac{P_{Na}/P_K [Na]_i + [K]_i}{P_{Na}/P_K [Na]_o + [K]_o} = \frac{RT}{F} \ln \frac{0{\cdot}01 \times 6 + 163}{0{\cdot}01 \times 116 + 3} = 92{\cdot}5 \text{ mV},$$

and the ratio of the theoretical and observed potentials (92·5/83·5 mV = 1·11) provides a correction factor which accounts for the shunt across the excitable membrane at rest. A factor of probably similar magnitude would also be required for the correction of the overshoot potentials.

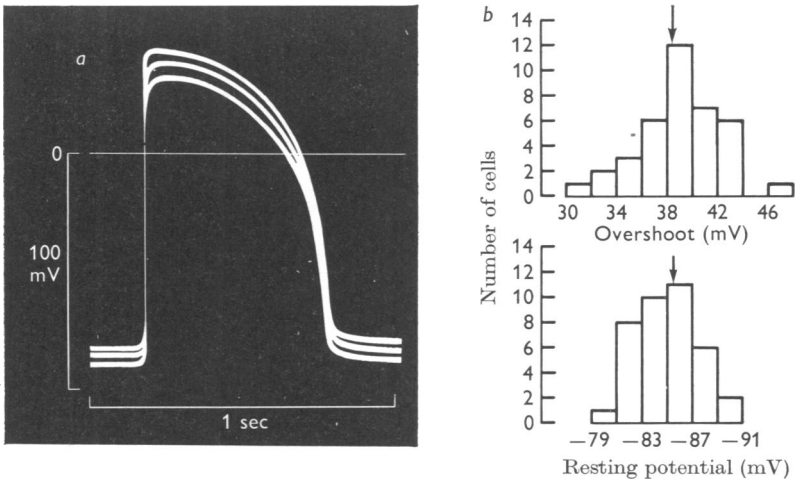

Fig. 2. Overshoot and resting potentials in the presence of 1 mM-Ca Ringer. Results from thirty-eight fibres of a single strip. 2*a* photographically superimposed records of three action potentials representative of the mean and the largest and smallest value of the potential amplitudes. Potential upstrokes have been retouched as in most other records of the present work. 2*b* Histograms of overshoots and resting potentials. Mean values of 38·5 mV and of 84·5 mV for overshoots and resting potentials, respectively, are marked by arrows.

When the calcium concentration of the bathing fluid, $[Ca]_o$, was altered resting potentials and, to a greater extent, overshoots changed. Both increased with increasing $[Ca]_o$, the effects usually being fully established only after a delay of 5–10 min, probably because of the relatively slow equilibration of the calcium in the extracellular spaces of the heart strip (cf. Niedergerke, 1957). In Fig. 3, which illustrates the combined results of fifteen ventricle strips, the difference of the resting and overshoot potentials with respect to the controls recorded in the presence of 1 mM-$[Ca]_o$ have been plotted against $\log_{10}[Ca]_o$. The points in the range between 0·1 and 5 mM-$[Ca]_o$ have been fitted by straight lines with slopes of 4·9 and 18·3 mV for a 10-fold concentration change in the case of the resting potential

and of the overshoot respectively. Outside this range the relation between the overshoot and the calcium concentration is complicated by phenomena which will be discussed in the following section.

There was little consistent change in the duration of the action potentials under these conditions. Thus although in many experiments action

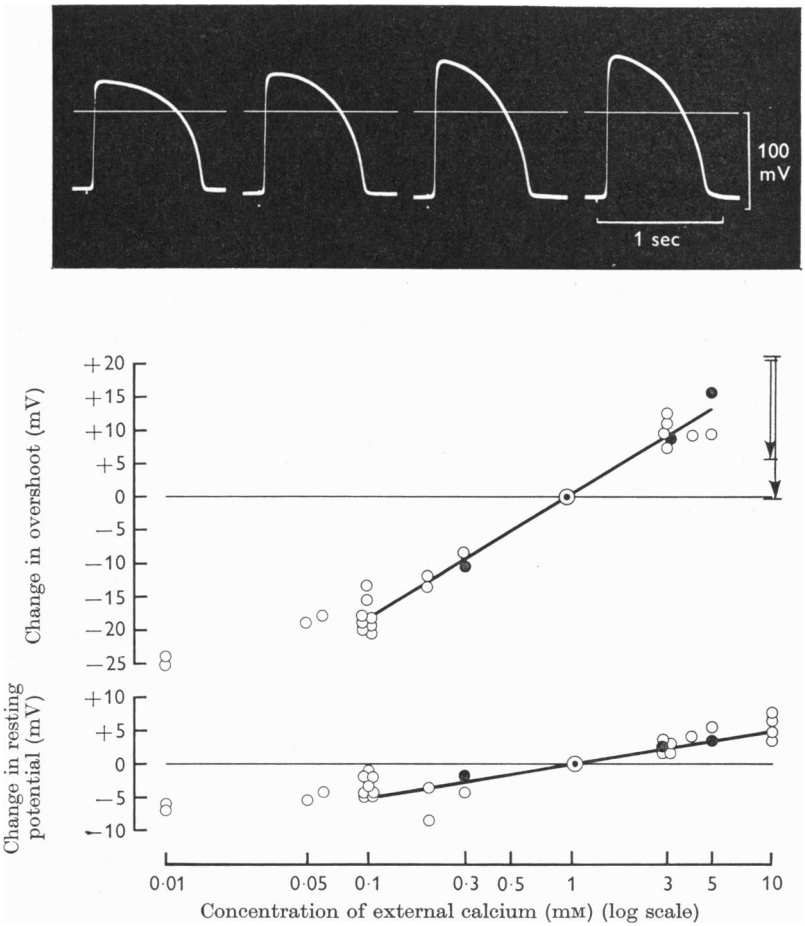

Fig. 3. Changes in resting potential and overshoot due to the variation of the external calcium concentration. Effects are plotted as the potential difference with respect to the control potentials (in 1 mM-Ca Ringer). Each point is the average estimate obtained from a series of eight or more impalements. Filled circles, results from a single strip of which records are illustrated in the upper section (from left to right: 0·3, 1, 3 and 5 mM-Ca-Ringer). The two arrows represent results of two experiments with 10 mM-Ca-Ringer, length of arrows indicates range of values, their direction the change of the overshoot with time (cf. Fig. 4*b*). Straight lines are regression lines of slope 18·3 and 4·9 mV for a 10-fold change in calcium concentration, in the case of the overshoots and resting potentials, respectively.

potentials shortened, e.g. by up to 30% of the initial duration, when $[Ca]_o$ was reduced from 1 to 0·1 mM, in others no change or even some slight lengthening of the action potential occurred as illustrated in the upper section of Fig. 3.

In view of the simultaneous changes of overshoots and resting potentials during these experiments the possibility was considered that overshoots might have been altered as a consequence of the change in resting potential. However, this is not borne out by the results of experiments during which the resting potential was increased, or reduced, by about 8–10 mV as a result of altering the external potassium concentration from the normal 3 mM concentration to either 1 or to 5 mM. Very little or no change of the overshoot was observed under these conditions (R. Niedergerke & R. K. Orkand, unpublished; cf. also discussion in connexion with Fig. 7, Niedergerke & Orkand, 1966).

It is convenient, at this stage, to propose a tentative hypothesis for the change in overshoot just discussed by making the assumption that the ionic current entering the heart cells at the time of the peak of the action potential is carried by both calcium and sodium ions. If calcium to a sufficient extent participates in depolarizing the excitable membrane, an increase of the overshoot by high concentrations of this ion would be expected. The magnitude of this effect, only 18·3 mV for a 10-fold change in $[Ca]_o$, as compared with the hypothetical maximum of 29 mV ($= RT/2F \ln 10$) for the case of a membrane which is selectively permeable to calcium (e.g. Hagiwara, Chichibu & Naka, 1964), accords with the view that other ions, such as sodium, also carry some of the depolarizing current. On the other hand the enhancement of the resting potential due to high calcium is probably related to the well-known 'stabilizing' action brought about by various divalent ions (Shanes, 1958), as is, for example, suggested by the fact that both strontium and magnesium produce very similar effects (cf. discussion in connexion with Fig. 8).

Effects of extreme changes of $[Ca]_o$. As is apparent in Fig. 3 overshoots were relatively insensitive to changes if $[Ca]_o$ in the concentration range below 0·1 mM-$[Ca]_o$ and also above 5 mM if exposure to the calcium-rich fluids was prolonged. Some information as to the nature of these effects was obtained by studying the time course with which the potentials were altered after the application of fluids containing either very low or high concentrations of calcium. In low, 0·01 mM, Ca-Ringer fluid (Fig. 4*a*) overshoots and resting potentials declined very slowly, steady state values being usually obtained only after about 1 hr of equilibration in this fluid, but the speed with which overshoots returned to their original size after application of the 1 mM-Ca fluid was much faster. An asymmetrical time course of this kind is in fact expected from the combined effects of dif-

fusion of calcium in the extracellular spaces and of the logarithmic relation between the potentials and the calcium concentration (cf. discussion of a similar phenomenon occurring during changes of the external potassium concentration; Niedergerke, 1956*b*). However, another more important factor slowing the fall of the overshoot in the presence of low $[Ca]_o$, is probably the gradual release of calcium from some cellular store (e.g. Niedergerke, 1963*a*). This would help to maintain a minimum effective calcium concentration in the cell membrane and explain (*a*) the observed flattening of the curve relating overshoots to $\log_{10}[Ca]_o$ at low calcium concentrations, and (*b*) the ability of heart strips to withstand exposure to low Ca fluids for many hours without apparent injury, as was in fact observed during the present experiments.

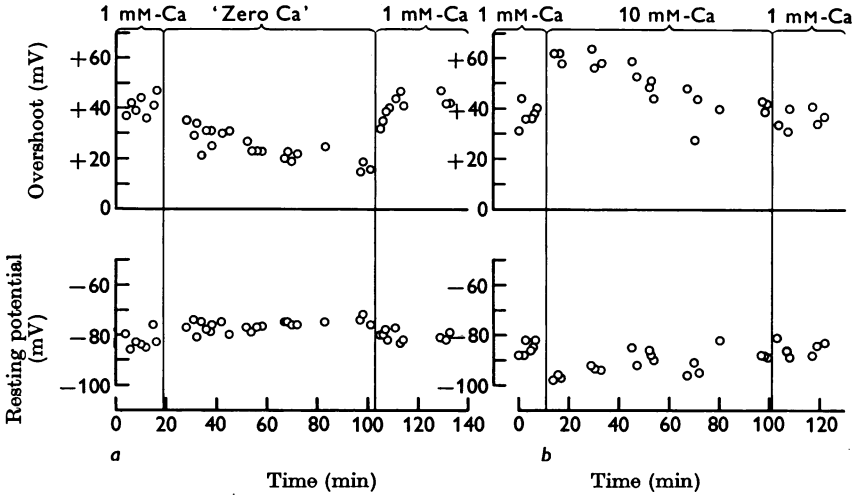

Fig. 4. Time course of the change in overshoot and resting potential on application of either 0·01 mM Ca-Ringer (4*a*) or of 10 mM Ca-Ringer 4(*b*). Results from two different strips. Each set of points (overshoot and resting potential) obtained from a record of a single cell.

As to the effects of high calcium concentrations, Fig. 4*b* shows that overshoots rapidly rose in 10 mM-Ca by about 18–20 mV following the logarithmic relationship discussed above. This effect however was transient and was followed by a slow decline of the potential to values similar to, or sometimes even lower than, those recorded in 1 mM-Ca fluids. (In Fig. 4*b* the resting potential also appeared to decline but this was not observed in other experiments of this kind.)

When studying the effects of high calcium in greater detail the size of the overshoot was found to depend on the rate of stimulation. Thus with calcium concentrations between about 4–8 mM overshoots gradually

declined by some 10–20 mV at the low stimulus rate of only 1 shock/minute but recovered during subsequent periods of rest. In the presence of calcium at concentrations of 10 mM, or above, no such recovery occurred and overshoots declined even when very occasional stimulation, at intervals of 10 min or longer, was applied.

In suggesting a possible explanation for these phenomena it may be recalled that under conditions of high concentrations of calcium and increased rates of stimulation the calcium uptake of heart cells is increased (Niedergerke, 1963*b*) and the resulting accumulation of this ion in some region of the cell may thus be a responsible factor for the depression of the overshoot. Further experiments made to examine this depressive effect of high calcium will be described in a subsequent section.

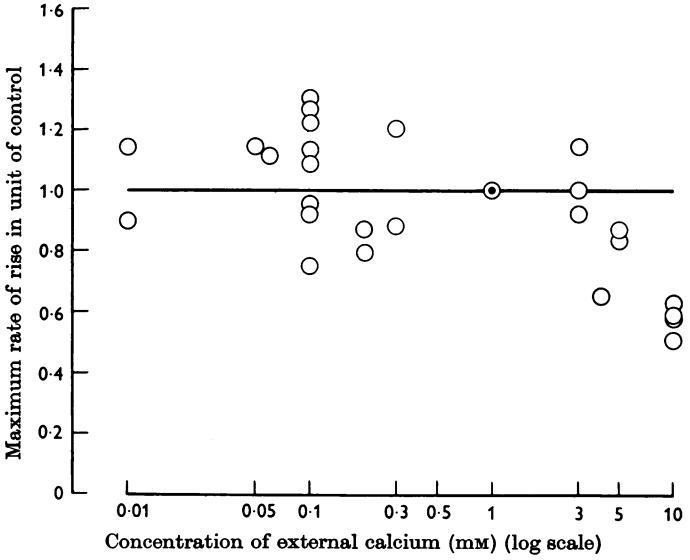

Fig. 5. Maximum rate of rise of the action potential at various calcium concentrations. Each circle is the average result from a number of different fibres, cf. Fig. 3.

Modification of the rising phase of the action potential by calcium. The rise of the action potential of the heart may be divided into two successive portions (Fig. 6; cf. also Antoni & Delius, 1965); the initial S-shaped, fast upstroke during which the membrane is depolarized to near zero potential, and the subsequent slower rise to the maximum plateau potential. As is illustrated in Figs. 5 and 6 calcium affects these two phases in different ways. Figure 5 shows that the initial rise, as measured by the maximum rate of the potential upstroke, $(dV/dt)_{max}$, was not altered in any consistent way when $[Ca]_o$ was changed, except in the range of con-

centrations above 3 mM in which enhancement of $[Ca]_o$ caused the values of $(dV/dt)_{max}$ to decline. The scatter of the values about the mean also remained unaltered under these conditions; it is therefore possible to compare action potentials with identical values of $(dV/dt)_{max}$ in different calcium concentrations. Such a comparison has been made in Fig. 6*a* in which the rising phases of two action potentials recorded in the presence of 0·2 and 3 mM $[Ca]_o$ have been photographically superimposed. It is seen that the two traces diverge from one another at a point not far from

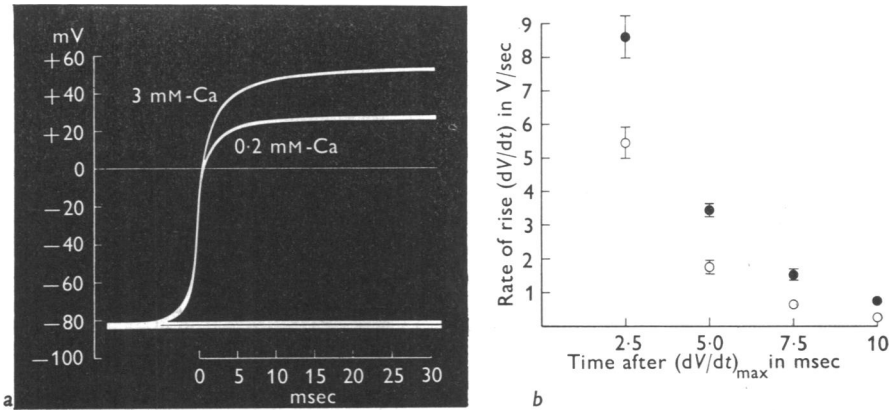

Fig. 6. Effect of increased calcium on the rising phase of the action potential. (*a*) Two photographically superimposed records obtained in the presence of 0·2 and 3 mM-Ca Ringer respectively. (*b*) Average values of (dV/dt) from the differentiated traces of nine fibres at each concentration (3 mM-$[Ca]_o$ ●; 0·2 mM-$[Ca]_o$ ○), plotted against the time after the point of $(dV/dt)_{max}$. (Vertical bars, 2 × standard error of the mean). Experiment made with an extracellular micro-electrode as the second recording electrode. Results of two fibres with greatly distorted rising phases have been omitted (cf. discussion in connexion with Fig. 7).

zero potential. In Fig. 6*b* which illustrates this in greater detail, the rate of depolarization, dV/dt, has been plotted against the time which elapsed after the point of the fastest upstroke, and it is clear that throughout this later phase the potential rises faster in the presence of the higher calcium concentration. Taking the ratio of the ordinates, in 3 and 0·2 mM $[Ca]_o$, respectively, as a measure of this effect, the values were 1·96, 2·4 and 2·8 for 5, 7·5 and 10 msec. The ratios obtained in another similar experiment were 2·2, 1·8 and 1·9, respectively.

Since the contribution of the 'local circuit' currents to the recorded changes in membrane potential progressively diminishes as the peak of the action potential is reached, the strength of the net ionic current crossing the membrane thus becoming proportional to the capacitative current, it may be concluded that the inward current during the late rising phase was

increased about twofold by high calcium under the conditions of the present experiments.

Some incidental observations on the rising phase in these experiments must be mentioned. As is well known (e.g. Hoshiko & Sperelakis, 1961) upstrokes of cardiac action potentials are frequently distorted by 'dents' or 'notches' which vary in magnitude and also in their timing within the rising phase and which cause the differentiated upstrokes to have two (or more) peaks instead of only one (cf. Fig. 7). This phenomenon, which has been interpreted in various ways (see Hoshiko & Sperelakis, 1961; Antoni & Delius, 1965), probably arises to a large extent from the potentials which are set up by the external currents of action potentials of fibres in the neighbourhood of the fibre under observation. This is suggested by the following observation: when such external potentials were directly recorded by means of a micro-electrode positioned just outside a fibre the diphasic (corresponding to the second derivative of the S-shaped upstroke) potential traces so obtained, of magnitude up to 12 mV (peak to peak), satisfactorily accounted for this type of distortion, assuming superposition of the extra and intracellular potentials (cf. also discussion in connexion with Fig. 2*M*;

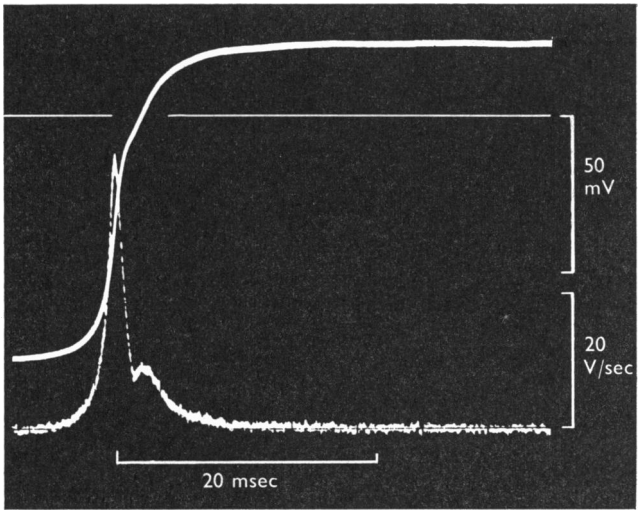

Fig. 7. Distortion of the rising phase of an action potential. Records of the rise in potential and of its differentiated form. Note dent in the upper trace and the corresponding second peak in the lower, differential trace. (External micro-electrode used as second recording electrode.)

Hoshiko & Speralakis, 1961). As a consequence, distortions of this kind were much reduced, though not altogether abolished, when the membrane action potentials were recorded using a second micro-electrode as the external reference electrode in a position close to that of the intracellular electrode (cf. Methods). This artifact is of interest since it helps to explain the large scatter of our values of $(dV/dt)_{max}$ (the standard deviation of this estimate being in some preparations as large as 70 % of the mean, cf. also the distribution of the points above and below the horizontal line in Fig. 5). Values of $(dV/dt)_{max}$ were usually markedly smaller when obtained from severely distorted upstrokes than in the absence of this distortion. Other distortions of the rising phase must be expected to occur at regions of recording where the rate of propagation of the action potential is not constant as, for example, close to a point of ramification of the cell.

Comparison of the effects of strontium and magnesium ions with those of calcium. It is known that strontium ions applied to a calcium depleted Ringer fluid strengthen the contraction of the heart in a way similar to calcium itself (e.g. Garb, 1951), while magnesium ions lack this effect (Baumecker, 1923; Garb, 1951; Antoni, Engstfeld & Fleckenstein, 1962). In the present experiments action potentials were recorded in strontium- and magnesium-rich fluids, to test whether a similar difference can be found in the effects of these ions on the overshoot of the action potential.

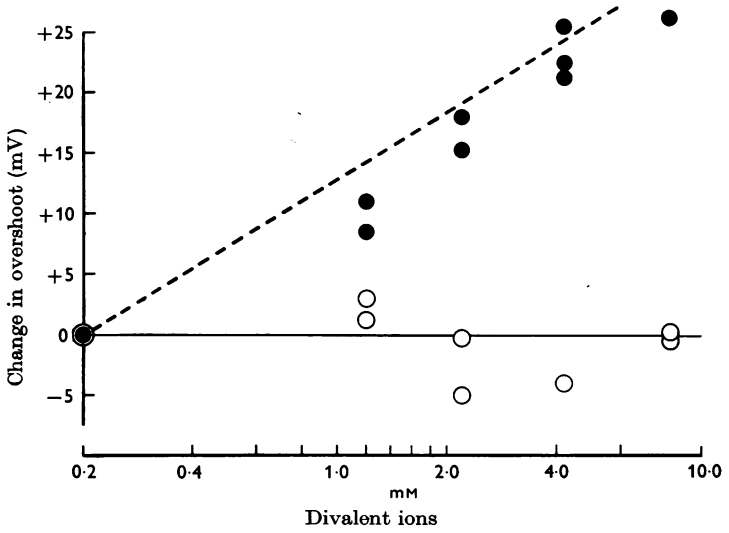

Fig. 8. Comparison of the effects of strontium and magnesium ions on the height of the overshoot. Change in overshoot, caused by the addition of $SrCl_2$, ●, or $MgCl_2$, ○, to a 0·2 mM-Ca Ringer fluid, plotted against the log (concentration of the total divalent ions). Interrupted line has the same slope (18·3 mV for a tenfold change in $[Ca]_0$) as the upper regression line of Fig. 3.

A small amount of calcium (0·2 mM), rather than a calcium-free fluid, was used because it appears that small concentrations of calcium protect the tissue against certain detrimental effects of the strontium causing a potassium loss from the cells (Thomas, 1957) and also against a conduction block which high concentrations of magnesium ($\geqslant$ 10 mM) were found to produce during preliminary experiments. Figure 8 summarizes the results from nine strips showing that magnesium ions do not significantly alter the size of the overshoot under these condition whereas high strontium is about as effective as addition of calcium in increasing the overshoot. By contrast, the effect on the resting potential of high magnesium was similar to that of strontium or calcium, e.g. resting potentials increased by about 5 mV after addition of 8 m-mole/l. $MgCl_2$ or $Sr\,Cl_2$ (cf. Fig. 3). As in the case

of increased calcium, additions of either strontium or magnesium ions in moderate concentrations (of up to 4 m-mole/l.) did not alter the maximum rate of rise of the action potential.

The effects of strontium and magnesium ions on the duration of the action potential were also different. Thus magnesium-rich fluids under present conditions caused little change of the duration (cf. also Garb, 1951) whereas the action potential was prolonged by strontium (Garb, 1951), occasionally by as much as three times or more (Fig. 9). Possibly related to this is the observation that strips immersed in strontium-rich fluids often started to beat spontaneously or responded to a single stimulus by a series of beats.

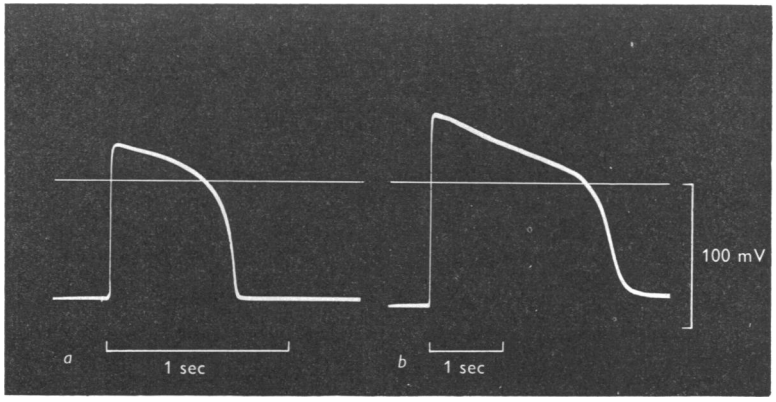

Fig. 9. Prolongation of the action potential by strontium ions. (*a*) Control record in the presence of 0·2 mM-Ca Ringer. (*b*) Record in the presence of 0·2 mM-Ca + 4 mM-Sr. Note the different time scales in *a* and *b*.

Changes produced by repeated stimulation. To test the hypothesis that progressive accumulation of calcium ions inside the cells tends to reduce the overshoot, the effects of periods of repetitive stimulation were examined using fluids of varying calcium concentrations. A constant stimulus frequency of about 20 shocks/min as previously used by Ware (1961) was chosen for most of the present experiments.

At low external calcium concentrations (between 0·1 and 0·2 mM) effects of stimulation were small. For example, in the presence of 0·2 mM-$[Ca]_o$ the overshoot in a single fibre declined by about 5 mV and the resting potential by about 3 mV, during 3 min of stimulation (Fig. 10*a*), and both potentials recovered to their original height after 2–5 min rest (Fig. 10*b*). In other experiments, with 0·1 mM-$[Ca]_o$, the effects on overshoot and resting potential tended to be still smaller, even when the heart strips were stimulated for 10 min or longer. Using calcium concentrations of 1 mM or above, it was not usually possible to record action potentials con-

tinuously from any single fibre because of the strong contractions. Records were then obtained from different cells immediately following stimulation and repeated, if possible, during several stimulation and 'recovery' runs to diminish the scatter of the values so obtained. Fig. 11 shows the results of three such recovery periods in the presence of 1 mM-Ca: overshoots were lowered by about 20 mV after stimulation and then gradually returned to

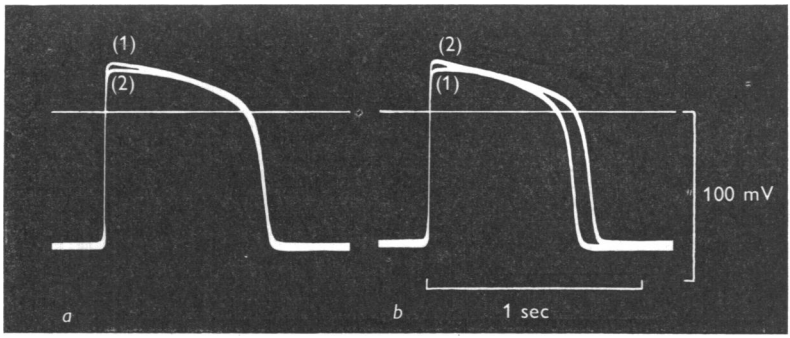

Fig. 10. Effect of stimulation in the presence of 0·2 mM-Ca Ringer. (*a*) Two superimposed records taken from a single cell before (1) and after (2) a 3 min period of stimulation at the rate of 20 shocks/min. (*b*) Two later records, from the same cell, at the beginning (1) and at the end (2) of the subsequent period of rest.

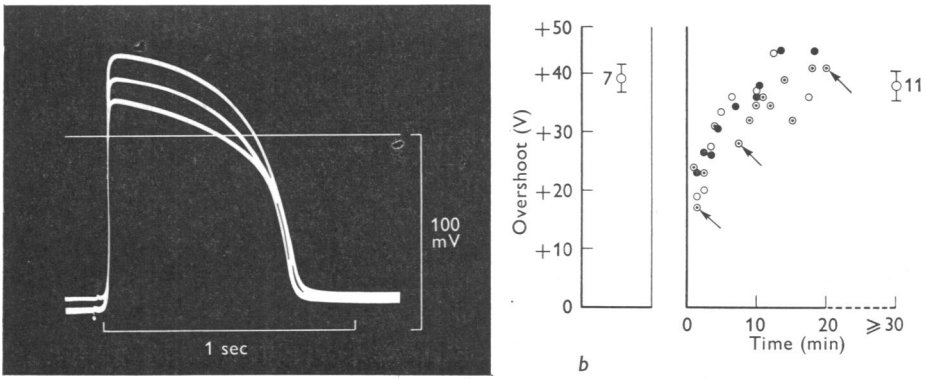

Fig. 11. Recovery of overshoots after periods of stimulation in the presence of 1 mM-Ca Ringer. Three series of action potentials (corresponding to the three different symbols) were recorded from the same strip each after 5 min of stimulation at the rate of 20 shocks/min. Sizes of overshoots (*b*) before stimulation, and at long times afterwards ($\geqslant$ 30 min) are average values, otherwise values of single records are plotted. Arrows, overshoots of the three action potentials which have been photographically superimposed in *a*.

their initial height of 38·5 mV during about 20 min of rest. Resting potentials were also reduced after stimulation, but only by some 3–5 mV and thus to a similar extent as in 0·2 mM-$[Ca]_o$. Figure 12 illustrates the

recovery of the overshoot and resting potential in two other experiments, this time in 3 mM-Ca Ringer fluid. By comparison with the result of Fig. 11 the reduction of the overshoot (by 30–40 mV), had become greater as the calcium concentration had been raised, but the decline of the resting potential was again small. It should also be noted that the rates at which the

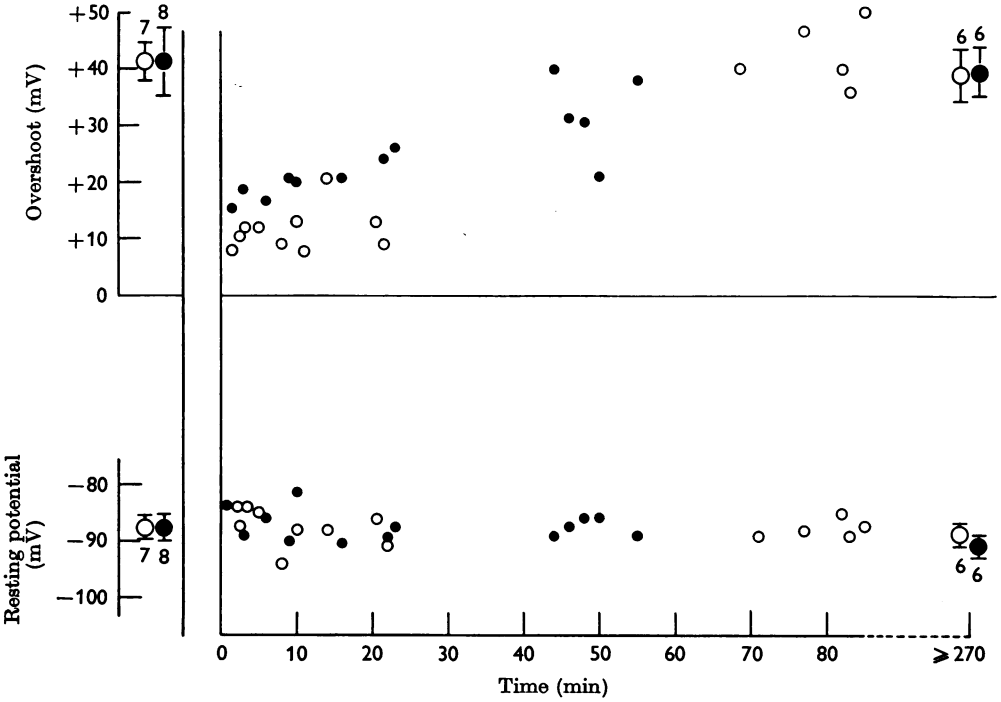

Fig. 12. Recovery of overshoots and resting potentials in the presence of 3 mM-Ca Ringer. Results of two different strips which had been subjected to a 5 min period of stimulation at 20 shocks/min. Results plotted as in Fig. 11.

potentials recovered were different, the restoration of the resting potential being completed after about 5–10 min, that of the overshoot only after 60 min of rest.

The changes in size of the action potential were accompanied by complicated, apparently also calcium-dependent, changes of its duration. Thus in the presence of low [Ca] (0·1–0·2 mM) action potentials usually shortened during stimulation and lengthened during the subsequent period of rest. At normal, 1 mM $[Ca]_o$, there was usually little effect on the duration (cf. Fig. 11), but in 3 mM-Ca fluids action potentials were frequently prolonged after cessation of stimulation and shortened in the course of the recovery (Fig. 13); for similar calcium-dependent changes of the shape of the action potential see Fig. 5, Ware (1961) and Fig. 7, Niedergerke (1956*a*).

It is interesting that the overshoots recorded immediately after stimulation at the different calcium concentrations of Figs. 10–12 were

reduced to similar heights in all these cases, between 10 and 20 mV. This result is in good agreement with that of Ware (1961) who found only small variations of the overshoot, within the range of 10–20 mV, when examining the effects of calcium in continuously beating ventricle strips.

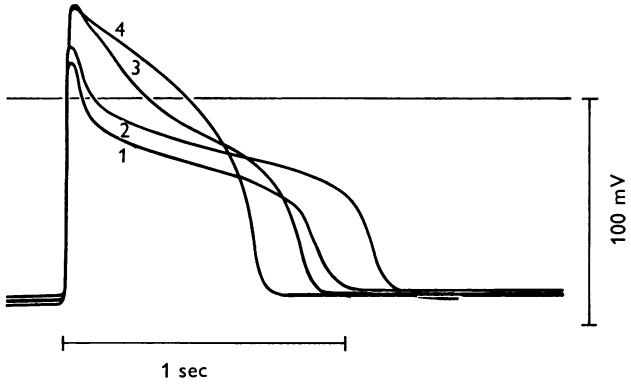

Fig. 13. Change in shape of the action potential during recovery. Similar experiment as in Fig. 12, in the presence of 3 mM-Ca Ringer. Superimposed traces of action potentials at various times of recovery after a 5 min period of stimulation (1) 5 min, (2) 10 min, (3) 70 min, (4) 100 min.

Other findings. Since a reduction of the external sodium concentration, like enhancement of the calcium concentration, facilitates calcium uptake by heart cells (Niedergerke, 1963*b*), it was of interest to know whether a depression of the overshoot is also obtained under these conditions. In a series of experiments the effects of stimulation were therefore compared using bathing fluids containing either the normal or a 25 % normal sodium concentration and in both cases a constant but reduced calcium concentration (0·15 mM). It was found that overshoots were reduced after 5 min of stimulation in the low sodium fluids by about 20 mV (average of three experiments) as compared with only 3–5 mV in the presence of the normal sodium concentration.

The question whether high strontium and high magnesium also have the effect of depressing the overshoot was tested. The fluids were similar in composition to those used during the experiments of Fig. 8 and contained 4 mM-$MgCl_2$ or $SrCl_2$ in addition to 0·2 mM-$CaCl_2$. High magnesium had no effect; the decline of the overshoot during stimulation was only by 3–5 mV as was found in the absence of magnesium. By contrast, high strontium caused a marked depression, like raised calcium: overshoots were reduced, in two experiments, from 45 to about 15 mV after 3 min of stimulation.

In some experiments strips were stimulated at frequencies of more than

70 shocks/min in fluids which contained only small amounts of calcium (0·2 mM) to make possible continuous recording of potentials from a single cell. In the experiment of Fig. 14 a strip was stimulated 25 times in 20 sec. Overshoots and resting potentials declined during this period, by 10–12 mV, and both potentials recovered rapidly afterwards, their original heights being re-established within about 30 sec of rest. By reason of the relatively large changes in resting potential and the similar time course with which resting potentials and overshoots altered, it seems likely that the depression of the overshoot in this case was to a large extent secondary to the reduction of the resting potential. It may be recalled that a similar loss of

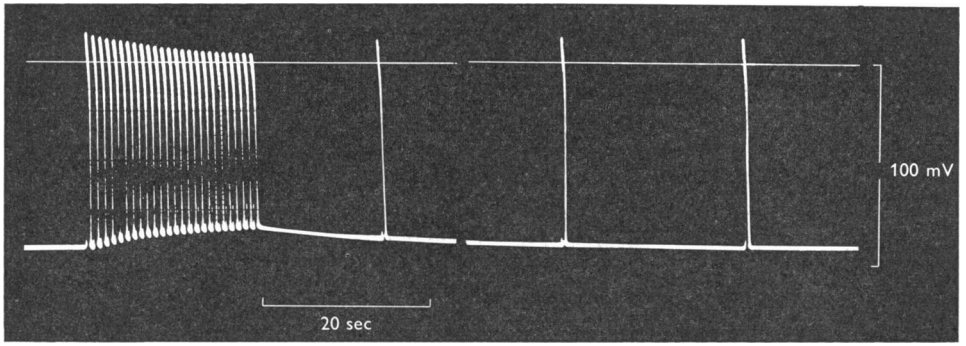

Fig. 14. Effects of stimulation at a higher rate (72 shocks/min). Single cell exposed to 0·2 mM-Ca Ringer. Break in the potential trace corresponds to gap between two successive frames of film.

overshoot associated with a fall in resting potential has been observed during periods of stimulation in the squid axon (Frankenhaeuser & Hodgkin, 1956), the probable cause being the accumulation of potassium ions in a confined space outside the cell membrane. Such an explanation may also apply to the present results.

DISCUSSION

Although some of the effects of calcium reported here resemble those obtained with other excitable cells, for instance, 'the stabilization' of the resting potential by high calcium (e.g. Shanes, 1958) there are also important differences. Thus in contrast with our findings the overshoot of Purkinje fibres was not altered significantly by varying the calcium concentration (Weidmann, 1955). However, since the experiments on Purkinje fibres had been made with high rates of stimulation (60/min) it is conceivable that a facilitation of the overshoot was concealed by a simultaneously occurring depression, similar to that shown in Figs. 10–12. One of the known effects of high calcium is the increase of the availability of

membrane sites for sodium inward transport during the action potential (Weidmann, 1955; Frankenhaeuser & Hodgkin, 1957), and it was thought at first that this might also provide an explanation for some of our results. However, the availability of these 'active' sites is critically dependent on the height of the resting potential in contrast to the facilitation of the overshoot by calcium during the present experiments. Another point of difference is in the effect of magnesium which was similar to that of calcium in the case of the squid axon (Frankenhaeuser & Hodgkin, 1957) but not so in heart muscle fibres (Fig. 8).

For an explanation of our findings it was important to know whether the intracellular sodium concentration and thus the force driving the sodium current through the excitable membrane was altered by changes of the external calcium. To this end a number of ventricles which had been exposed to fluids of different concentrations of calcium have been analysed for intracellular sodium and potassium. However, no significant differences of the intracellular sodium were obtained in these experiments (Keenan & Niedergerke, in preparation).

As already mentioned, a simple explanation which would account for the facilitation of the overshoot by high calcium is to suppose that the inward current during the action potential is carried by both sodium and calcium ions and that the observed effects are due to the changes in the fraction of the current carried by calcium. Since the calcium current is likely to be small but well maintained (cf. discussion in connexion with Fig. 9, Niedergerke, 1963*b*) effects of calcium would be expected to be negligible during the initial, fast upstroke of the action potential by comparison with the sodium current which is strong at this moment (e.g. Niedergerke & Orkand, 1966). The situation, however, is different during the latter part of the rising phase because, as in the case of the Purkinje fibres (e.g. Deck & Trautwein, 1965), both sodium and potassium permeabilities probably decline rapidly after the initial upstroke, so that the calcium current could then become a significant factor contributing to the height of the plateau potential. With these assumptions in mind the depressant effect of calcium brought out by prolonged stimulation (Figs. 10–12) might be related simply to progressive accumulation of calcium ions inside the cells as this would reduce the driving potential for this ion and thus also the part of the overshoot which depends on calcium current. This type of explanation involving calcium entry into the cells and its accumulation has much in common with a previous hypothesis made to account for the mechanical events associated with stimulation, the 'staircase phenomenon', the cumulative entry of calcium in this case being facilitory and responsible for the gradual build up of contractile strength (Niedergerke, 1956*a*).

The main difficulty in accepting this simple hypothesis becomes apparent when the current density of the inward current during the peak and plateau is calculated using the values for the calcium fluxes obtained in a previous study with ^{45}Ca-labelled fluids (Niedergerke, 1963*b*). Thus in the presence of 2 mM-Ca-Ringer fluid the extra-influx of calcium, associated with, and taken to be constant during, the plateau of an action potential, amounted to 0·1 p-mole/cm^2 sec, and so corresponds to a current density of only about 0·02 μA/cm^2. Since the effect of varying the sodium concentration on the size of the overshoot is similar to, and not greater than, that of calcium (Fig. 1; Niedergerke & Orkand, 1966) the strength of the simultaneous sodium current should also be of this order, making the total inward current to be rather less than 0·1 μA/cm^2. It appears unlikely that a current of this strength is sufficient to maintain the excitable membrane in a depolarized state, its density being, for example, more than ten times smaller than the 2–5 μA/cm^2 obtained for the smallest sodium and potassium currents measured at the plateau potential of Purkinje fibres (Deck & Trautwein, 1965). However, this argument is not necessarily decisive because (1) the current densities of the excitable membrane of frog heart fibres are not known and may be smaller than those of Purkinje fibres; (2) the magnitude of the calcium fluxes during an action potential may in fact be greater than was previously determined from tracer movements associated with series of action potentials (Niedergerke, 1963*b*). It is possible for instance that there is a large calcium influx during depolarization followed by an efflux during repolarization of similar magnitude, and such rapid changes would not have been revealed by the methods used. Some support for this idea comes from the larger movements of calcium, compared with those just described, which are observed during strong contractures of frog ventricles (Niedergerke, 1963*a*).

There remains the alternative type of explanation according to which the effects of calcium on the action potential are mediated by changes of the potassium and, or, sodium permeability, as will be discussed in the subsequent paper (Niedergerke & Orkand, 1966).

Recent findings with Purkinje fibres (Dudel & Trautwein, 1965; Reuter, 1965) have also revealed striking effects of increased external calcium concentrations on the ionic conductance of the membrane. It is again not clear whether these effects are due to a change in the calcium current through the membrane or, more indirectly, to a variation of the permeability of the membrane to other ions.

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