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RELATIONSHIP BETWEEN INTERNAL CALCIUM AND OUTWARD CURRENT IN MAMMALIAN VENTRICULAR MUSCLE; A MECHANISM FOR THE CONTROL OF THE ACTION POTENTIAL DURATION?

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SUMMARY

1. In sheep and calf ventricular bundles, increasing the internal calcium by increasing the frequency of voltage-clamping to plateau range potentials increased the time-independent outward current. This effect was more marked with higher $[Ca]_o$, and was reduced if the Ca current blockers Verapamil or D 600 were used.

2. If the internal Ca was increased by the addition of cyanide and reduction of external sodium the outward current was also increased. The frequency-dependent increase in outward current also occurred in this Na-poor (12 mM) solution.

3. Tension measurement on the ventricular bundles showed that a Na-free solution with cyanide did not cause a contracture. On changing from Tyrode to a Na-free solution containing cyanide, and on changing back to Tyrode there was a potentiation of the twitch.

4. In Na-poor solution with cyanide, although no contracture was found, E_{Ca} was less positive, suggesting that under these circumstances Ca accumulates at the inner side of the membrane, but not around the myofibrils.

5. The prolongation of the action potential in Cl-free solution is frequency-dependent. A greater prolongation is seen at lower frequencies

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suggesting that Cl current is relatively more important for repolarization at lower frequencies of stimulation.

6. It is suggested that calcium at the inner side of the membrane sets the level of the background outward current. A feed-back mechanism on this basis is proposed for the control of the action potential duration. Various factors that could influence this basic mechanism are discussed.

INTRODUCTION

In a variety of tissues an increase in the intracellular Ca ion concentration has been shown to increase the K permeability (P_K) of the cell membrane (red cells: Whittam, 1968; Lew, 1970; Romero & Whittam, 1971; Simons, 1975; Porzig, 1975; snail neurones: Meech & Strumwasser, 1970; Meech, 1974; Meech & Standen, 1975; spinal neurones: Krnjević & Lisiewicz, 1972; squid axon: Tasaki, A. Watanabe & Lerman, 1967; but see Begenisich & Lynch, 1974). A similar association has been suggested for smooth muscle (see Bülbring, 1973; Tomita & H. Watanabe, 1973), skeletal muscle (Fink & Lüttgau, 1973), and for skate electroreceptor epithelium (Clusin, Spray & Bennett, 1974). In heart muscle a connexion between $[Ca]_i$ and P_K (manifested by an outward current) has been postulated by various authors (e.g. Morad & Greenspan, 1973; Reiter & Stickel, 1968; Prasad, 1974). Niedergerke & Orkand (1966) and McGuigan (1974) have suggested that $[Ca]_i$ sets the level of P_K and Colatsky & Hogan (1975) have suggested a similar relationship for Purkinje fibres. Evidence to support this idea has been obtained by Isenberg (1975), who injected Ca ions into short Purkinje fibre preparations and found a hyperpolarization of the membrane potential and a shortening of the action potential duration. Recently, Kass & Tsien (1976) have studied the time-independent component of the outward current in similar Purkinje fibre preparations and have found that increasing $[Ca]_o$ increases the level of the background current.

This paper reports an investigation into the effect of raising the internal Ca on such a time-independent, outward current in mammalian ventricular muscle. In our multicellular preparations (a cross-section of our bundles contained about 3000 cells) the effect of Ca injected directly into a superficial cell would be masked by electrotonic interaction with the other cells in the bundle.

Thus two indirect methods were used to load the cells with calcium. In the first, an increase in calcium was obtained by increasing the frequency at which voltage-clamp steps were applied (cf. Bassingthwaighte & Reuter, 1972) or by increasing the frequency of stimulation. In the second method an increase in cell Ca was obtained by blocking the Na/Ca

exchange mechanism, by removal of the external Na (Reuter & Seitz, 1968; Glitsch, Reuter & Scholz, 1970). It has recently been shown (Jundt, Porzig, Reuter & Stucki, 1975) that cyanide causes a release of Ca from heart mitochondria, thus cyanide was also added to the Na-poor solutions in an attempt to increase further $[Ca]_i$.

The results from ventricular bundles show that under these conditions of Ca loading there is a concomitant increase in the background outward current. In the Na-poor solution containing cyanide the reversal potential for Ca was shifted to less-positive potentials and we suggest that the increase in background outward current could be mediated through an increase in Ca at the inner side of the membrane. On the basis of these findings, a feed-back mechanism for the control of the action potential duration and contraction is presented (this is shown schematically in Fig. 8). Preliminary accounts of this work have already appeared (McGuigan & Bassingthwaighte, 1974; Fry, McGuigan & Bassingthwaighte, 1975).

METHODS

Preparation. Sheep or calf hearts were obtained from the slaughter-house and small bundles removed as described previously (McGuigan, 1974). We also obtained permission to remove such bundles from cow hearts in the slaughter-house. These were carried to the laboratory in well-oxygenated Tyrode at 4° C.

Action potential experiments. A small Perspex bath was used. The preparation was held down by threads passing over its upper surface and stimulated at one end. To pass current through the preparation during an action potential, a three-compartment chamber similar to that of Wood, Heppner & Weidmann (1969) was used.

Micro-electrodes were filled with 3 M-KCl by the fibre-glass method (K. Tasaki, Tsukahara, Ito, Wayner & Yu, 1968).

Voltage-clamp experiments. A double sucrose-gap method was used with a five-compartment chamber similar to that described by McGuigan (1974). An improved voltage-clamp circuit designed by Dr G. W. Beeler, Mayo Graduate School, Rochester, Minn. 55901 was used. This included not only a ground bath-clamp but also enabled the action potentials across both sucrose-gaps to be recorded simultaneously. Normally these action potentials differed by only a few millivolts, and were monitored continuously throughout the experiment; if a larger difference between their values occurred the experiment was discarded.

Current recording. As diagrammatically shown in Fig. 1, the current recorded (I_o) in a double sucrose-gap consists of the true membrane current, i_m, and leakage current, i_l. An increase in the measured current could thus be due either to an increase in the true membrane current or to an increase in the leakage current without an alteration of i_m. The latter situation could occur if there was an increase of the internal longitudinal resistance (r_i) of the preparation within either the test-gap or in the sucrose-gaps. This arises because an increase in r_i necessitates a larger voltage drop over the current-passing sucrose-gap to clamp the membrane to the same potential. To exclude these possibilities a number of tests were performed. A gradual increase of r_i in the sucrose-gaps will cause the current–voltage relationship I/V relationship to drift upwards during an experiment, i.e. more total current is measured at each potential. This was checked by measuring the current–voltage

relationships in Tyrode before and after application of the test solution – similar plots indicated no increase of r_i. The addition of the test solution itself, namely choline-cyanide Tyrode, could cause an increase of r_i in the test-gap. The effect of this solution on r_i in ventricular fibres was investigated separately for us by Dr Robert Weingart, Bern. In one experiment using Weidmann's (1970) method he found no change of r_i after 1 hr of application of this solution.

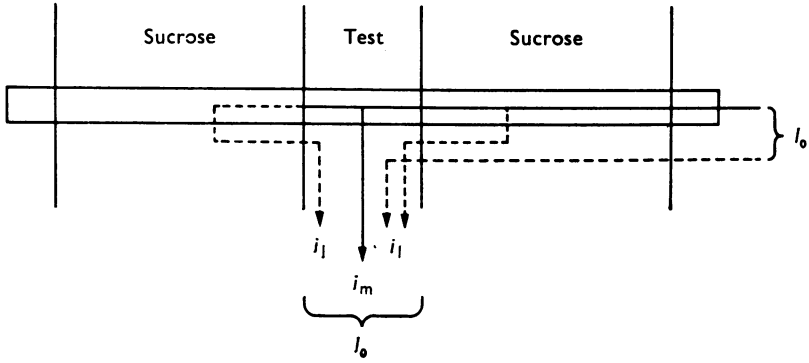

Fig. 1. Diagrammatic representation of the double sucrose gap, showing the true membrane current i_m (continuous line) and the leakage currents i_l (dashed lines). The measured current I_0 is the sum of i_m and i_l. Based on McGuigan & Tsien (1974; Appendix to McGuigan, 1974).

In the experiments in high and low $[Ca]_o$, where the clamp frequency was changed from 0·1 to 1/sec the possibility of an increase in r_i was checked by switching from voltage-clamp to current-clamp mode and recording the action potential. An increase in i_m should give a shortened action potential, whereas an increase in i_l should show no change in the action potential duration.

Definition of terms in the I/V. Only relative changes are shown, the resting potential is defined as 0 mV. All clamps were carried out from the resting potential. The resting potential in these experiments can be regarded as constant. No alteration in the resting potential was measured in the voltage-clamp experiments when changing from Tyrode to the choline solutions or to the high and low $[Ca]_o$ solutions. This was checked in two separate experiments with micro-electrodes and no measurable change was recorded.

Depolarization is a positive alteration of the potential, hyperpolarization is a negative alteration. No correction has been applied to the quoted voltage values to account for short-circuiting.

Tension recording. Tension was recorded in separate experiments using an isometric transducer (Grass XF 03). For these experiments the action potential chamber, described above, was used.

Solutions. These are given in Table 1. Isotonic sucrose solution contained 10^{-4} M-Ca ions to prevent an increase in r_i of the preparation in the sucrose-gaps (Kléber, 1973). Cl was replaced by isethionate, which does not alter the level of ionized Ca (Anderson & Foulks, 1973). NaCl was replaced by choline chloride, 20·9 g/l. replacing 136·9 mM-Na (Silvio Weidmann, Bern, personal communication). The desired Ca concentration was achieved by the addition of $CaCl_2$ from a stock solution. Ouabain (Merck, Darmstadt), Verapamil and D 600 (Knoll AG, Ludwigshafen am

TABLE 1. Concentration (mM)

Solution ...	Na	K	Ca	Mg	Cl	HCO_3	H_2PO_4	Sucrose	Choline	Isethionate	Tris	CN
Tyrode	149·2	5·4	1·8	0·5	146·9	11·9	0·4	—	—	—	—	—
Sucrose	—	—	0·1	—	0·2	—	—	292·1	—	—	—	—
Isotonic KCl	12·3	155·4	1·8	0·5	160·0	11·9	0·4	—	—	—	—	—
Choline–cyanide Tyrode	14·3	5·4	1·8	0·5	159·7	11·9	0·4	—	149·7	—	—	2
Choline Tyrode	12·3	5·4	1·8	0·5	159·7	11·9	0·4	—	149·7	—	—	—
Na-free cyanide	—	5·4	1·8	0·5	169·7	—	—	—	149·7	—	12	2
70% Na	104·4	5·4	1·8	0·5	151·1	11·9	0·4	—	49·0	—	—	—
Cl-poor	149·2	5·4	1·8	0·5	10·0	11·9	0·4	—	—	136·9	—	—

All solutions contained 5 mM glucose.

Rhein), tetrodotoxin (Sankyo, Japan) and cyanide (Merck) concentrations are given in the text.

Temperature. All experiments were carried out at 30° C.

Recording. Current and/or voltage were displayed on a four-beam oscilloscope (Tektronix 565) and photographed. Some voltage-clamp experiments were also recorded on a Watanabe pen-recorder.

RESULTS

Alteration of internal Ca by variation of stimulation frequency and $[Ca]_o$

Voltage-clamp experiments. The internal Ca was raised by increasing the frequency at which voltage-clamp steps were applied. Clamp steps of fixed duration and amplitude were employed at frequencies of 0·1 and 1/sec. The $[Ca]_o$ was either 0·6 or 5·4 mM. The steps were applied for 2–3½ min, to allow an approximate steady-state condition to be reached. The amount of outward current at the end of a clamp step was measured, either at various times during the stimulation, or at the end of the frequency run. A typical result for a clamp step of 500 msec duration and 90 mV amplitude for the two $[Ca]_o$, 0·6 and 5·4 mM is shown in Fig. 2*A*. The experiment was first carried out in 5·4 mM-Ca. The test solution was then changed to 0·6 mM-Ca and the experiment repeated. Finally, the experiment was repeated in 5·4 mM-Ca. Increasing the frequency from 0·1 to 1/sec in 5·4 mM-Ca caused a large increase in outward current in contrast to a slight increase in 0·6 mM-Ca.

At the end of each series of clamp steps the stimulation was switched from a voltage- to current-clamp mode and the action potentials recorded (Fig. 2*B*). At 0·11 sec the action potential in 5·4 mM-Ca is, if anything, slightly longer than in 0·6 mM-Ca. In contrast, at 1/sec the action potential is markedly shorter in the high Ca test solution, supporting the idea that an increase in membrane outward current has occurred.

It is convenient to mention at this stage that the results are presented as the amount of outward current at the end of a clamp pulse. However, at a high stimulation frequency the whole current trace moved up, i.e. there was not an increase in the delayed rectification; but rather an increase in the background outward current.

When the test solution contained 5·4 mM-Ca, at the high stimulation a quasi steady-state was reached after 2–3 min. A further period of clamping at this frequency produced a sudden increase in the outward current. This effect was irreversible in that on return to a stimulation rate of 0·11 sec the current never returned to its initial value. This was not investigated further, but probably resembles the membrane break-down described by Meech (1974) in some injection experiments. Decoupling was also considered, but De Mello (1975) has shown that such changes are rapidly reversible.

It is possible that the results presented above are due not to an increase in an outward current, as such, but rather to a decrease of the inward Ca

current as the clamp frequency is increased. The *I/V* shown in Fig. 2*C* shows that at a frequency of 0·1 sec there was no difference between the curves in 5·4 and 0·6 mM-Ca. The difference in outward current in Fig. 2*A*

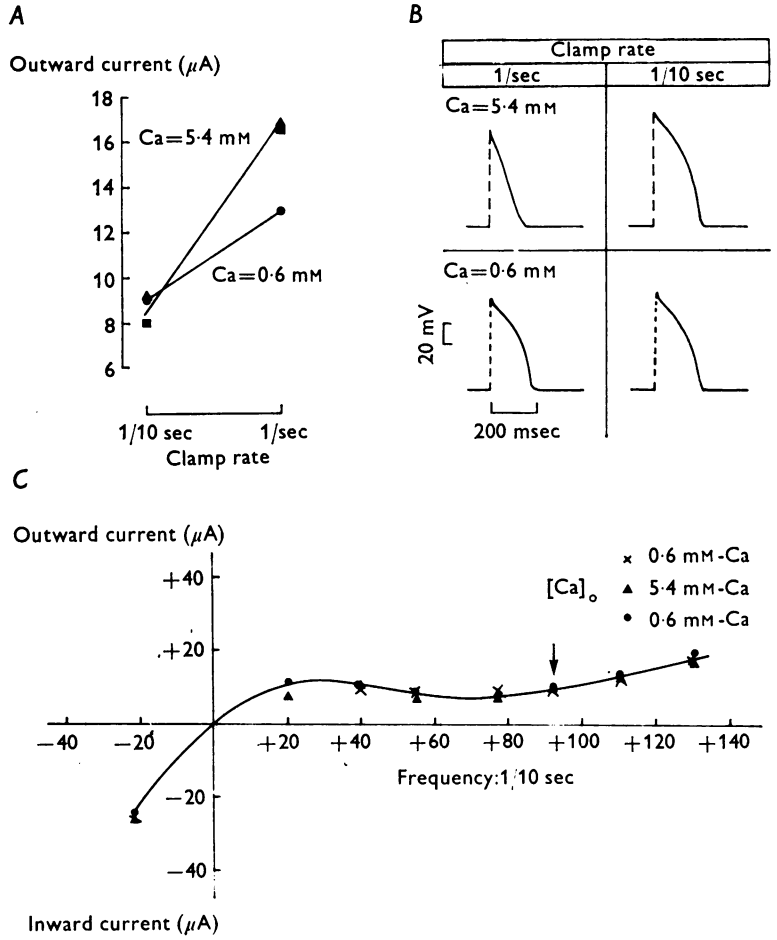

Fig. 2. Voltage-clamp rate experiment in 0·6 and 5·4 mM-Ca.

A, plot of the outward current at the end of the clamp step at two different frequencies 1/10 sec and 1/sec. ■, first experiment in 5·4 mM-Ca; ●, experiment in 0·6 mM-Ca; ▲, repeat in 5·4 mM-Ca. Clamp of 90 mV and 500 msec duration. Pulses applied for 3 min at each frequency and the outward current after 3 min of stimulation is plotted. Between the first and the second run at 1/10 sec in 5·4 mM-Ca there was a drift of 1 μA. After this initial point the currents remained constant.

B, action potentials obtained on switching from voltage clamp to stimulation at 1/10 and 1/sec in 0·6 and 5·4 mM-Ca.

C, *I/V* measured at 1/10 sec, ×, first experiment in 0·6 mM-Ca; ▲, experiment in 5·4 mM-Ca; ●, repeat in 0·6 mM-Ca.

at a stimulation frequency of 1/sec was 4 μA and such a difference should have been visible in the current–voltage relationship.

We interpret this similarity of the I/V in 0·6 and 5·4 mM-Ca to mean that at the end of the clamp step nearly all of the inward Ca has been inactivated. However, it could still be argued that in both Ca concentrations an appreciable, but similar amount of inward calcium current remains at the end of the 500 msec clamp step, i.e. in this experiment 4 μA. At the stimulation rate of 1/sec, due to Ca accumulation, the Ca current in 5·4 mM-Ca becomes less than in 0·6 mM-Ca. This seems unlikely because if Verapamil is added to block the Ca current the I/V before and after its addition are similar. Secondly, at a low frequency the inward Ca current in high Ca is larger than in low Ca, and hence the small fraction remaining after 500 msec (Beeler & Reuter, 1970; New & Trautwein, 1972). Finally, H. Reuter, Bern (personal communication), has demonstrated that at a frequency of 1/sec in 0·45 and 1·8 mM-Ca the inward Ca current in 1·8 mM-Ca is always greater than in 0·45 mM-Ca.

At a frequency of 1/sec the current–voltage relationship in 0·6 and 5·4 mM-Ca were also similar. This similarity of the relationship in the two test solutions at the high stimulation rate does not contradict the results shown in Fig. 2*A*. A complete I/V at 1/sec could be taken within 10 sec, the increase shown in Fig. 2*A* was found after 3 min. In other words, if one point is taken on the I/V relationship and clamp steps are applied at a frequency of 1/sec for 3 min there will be an increase in the outward current, so that this increase in outward current takes time to develop. Similar results were obtained in three further experiments in Tyrode and in one experiment in 70 % Na-Tyrode.

Inhibition of inward Ca current by calcium blocking agents and their effect on the outward current. If there exists a connexion between $[Ca]_i$ and the outward current, then blocking the inward Ca current during a clamp step should prevent accumulation of Ca ions at the inner side of the membrane. This should reduce the increase in outward current found when the clamp frequency is increased. Verapamil or D 600 were used to block the inward Ca current (Kohlhardt, Bauer, Krause & Fleckenstein, 1972). The result of one such experiment is shown in Fig. 3. The experiment was first carried out in Tyrode and then in Tyrode containing Verapamil (4 mg/l.) after 20 min equilibration in this solution. The clamp step was 66 mV and had a duration of 580 msec. Increasing the clamp frequency caused an increase in the outward current, which was reversible on decreasing the frequency to the original level. The addition of Verapamil reduced this increase in outward current. The I/V relationship measured in Tyrode and Tyrode-containing Verapamil were identical.

In two further experiments with D 600 (1 mg/l.) despite a slight increase in outward current at the low stimulation frequency there was a reduction in the amount of outward current at 1/sec.

Two experiments were carried out in 12 mM-Na solutions containing

D 600 (1 mg/l.) and gave similar results to Fig. 3, suggesting that this effect is not mediated through Na ions.

The interpretation we place on the results so far is that during the high-frequency stimulation there is an increase in $[Ca]_i$ mediated by the inward calcium current, which causes an increase in P_K. This is the explanation we offer for the shortening of the action potential in high $[Ca]_o$ (see Hoffman & Cranefield, 1960) at a high frequency of stimulation. An

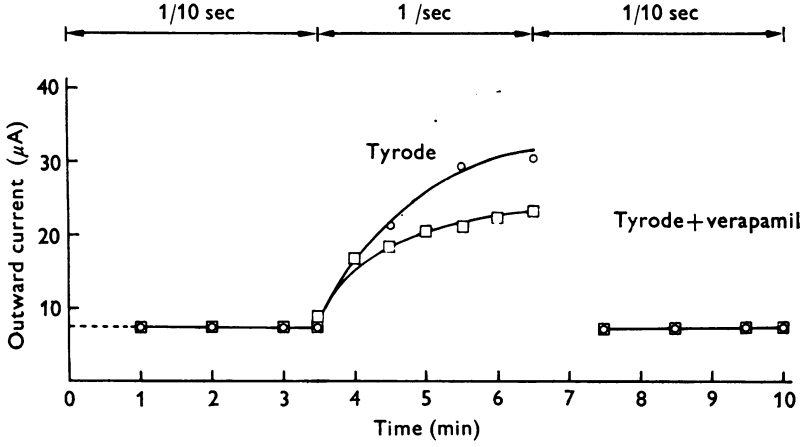

Fig. 3. Voltage-clamp rate experiment in Tyrode (○) and after 20 min in Tyrode and verapamil (□) (4 mg/l.). The outward current at the end of the clamp pulse is plotted. Clamp of 66 mV, duration 580 msec.

accumulation of K ions as a possible cause of the shortening of the action potential at a high frequency of stimulation was considered by McGuigan (1974, see discussion) but was deemed to be unlikely.

However, two criticisms can be levelled at this interpretation. Firstly, the shortening of the action potential at 1/sec stimulation in 5·4 mM-Ca is due not to an increase in the outward current but rather to a more rapid inactivation of the calcium current during the action potential (Kohlhardt, Krause, Kübler & Herdey, 1975). Secondly, the shortening of the action potential in high $[Ca]_o$ is due to the activation of more outward current, induced by the increase in the overshoot observed in this solution (see Hoffman & Cranefield, 1960). In order to answer these two specific points the following action potential experiments were undertaken.

Action potential experiments: Effect of $[Ca]_o$. While it is often stated that the action potential duration in high $[Ca]_o$ is shorter than in low $[Ca]_o$ our experiments with the voltage-clamp method have suggested that the situation could be more complicated (i.e. Fig. 2*B*). Experiments were carried out in 0·54 and 5·4 mM-Ca at frequencies ranging from 1/sec to 1/3

min. The results of one experiment are shown in Fig. 4*A*, where it is seen that the curves relating action potential duration and stimulation frequency in 0·54 and 5·4 mM-Ca cross each other, i.e. the action potential in

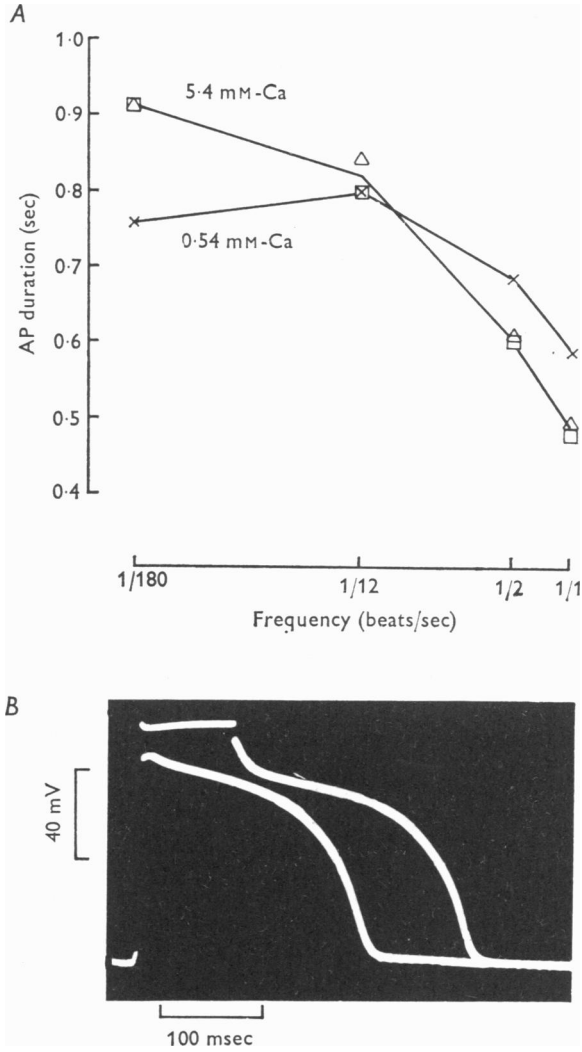

Fig. 4. *A*, action potential (AP) duration measured at four different frequencies in 5·4 and 0·54 mM-Ca. The experiment was first carried out in 5·4 mM-Ca (△), then in 0·54 mM-Ca (×) and then repeated in 5·4 mM-Ca (□). The frequency scale is logarithmic.

B, prolongation of the action potential duration by a constant current pulse applied at the same time as the upstroke. $[Ca]_o$ was 0·36 mM and the current was applied through a single sucrose gap. Temperature in both experiments 30° C.

high $[Ca]_o$ is shorter than in low $[Ca]_o$ only at frequencies greater than 1/12 sec. Similar results were obtained in two other experiments. These experiments argue against the hypothesis of a more rapid inactivation of calcium current as the cause of the action potential shortening in high $[Ca]_o$ because this hypothesis would predict that the action potential in high $[Ca]_o$ would be shorter over the entire frequency range.

The second criticism, i.e. of a higher overshoot switching on more outward current, was investigated by passing current pulses through the preparation in 0·6 mM-Ca (to increase the overshoot). The results from three fibres were identical and a typical result from a fibre stimulated at 1/2 sec is shown in Fig. 4*B*. Such current pulses prolonged the action potential: pulses as short as 50 msec induced a prolongation. Similar, but less pronounced results have been reported previously (Cranefield & Hoffman, 1958; Sakamoto, 1969; Šumbera, 1970). Thus the shortening of the action potential does not seem to be due to the increase in overshoot.

Effect of $[Cl]_o$. In the course of these experiments it was observed sometimes that at very low stimulation frequencies the action potential duration decreased, i.e. the curve of action potential duration versus stimulation frequency passed through a maximum (see also fig. 3 of Reiter & Stickel, 1968). This suggested that at such low stimulation frequencies another current could be playing a more important role in repolarization than i_K, e.g. i_{Cl}. Cl ions were partly replaced by isethionate ions, the Cl-poor solution containing 10 mM-Cl instead of 146·9 mM-Cl. The results of one experiment are given in Fig. 5. Fig. 5*A* shows a plot of the action potential duration against stimulation frequency in Tyrode and isethionate solution. It appears that at lower stimulation frequencies the prolongation of the action potential is relatively greater in the Cl-poor solution. This is seen more clearly when the percentage increase in duration induced by isethionate at each frequency is plotted as in Fig. 5*B* (100% = action potential duration in Tyrode). The plot shows that as the stimulation frequency is reduced the chloride current becomes relatively more important. One other experiment gave similar results. In four further experiments the curve relating action potential duration to frequency did not return to its original level after exposure to isethionate, but was shifted slightly upwards (three experiments) or downwards (one experiment). In these experiments the average between the two runs in Tyrode was taken, the results being similar to Fig. 5.

Alteration of internal Ca by external Na removal and the addition of cyanide

Voltage-clamp experiments. In these experiments $[Na]_o$ was reduced to 14 mM, the remainder being replaced by choline. 2 mM Cyanide was added to the test solution to release Ca ions from the mitochondria (see

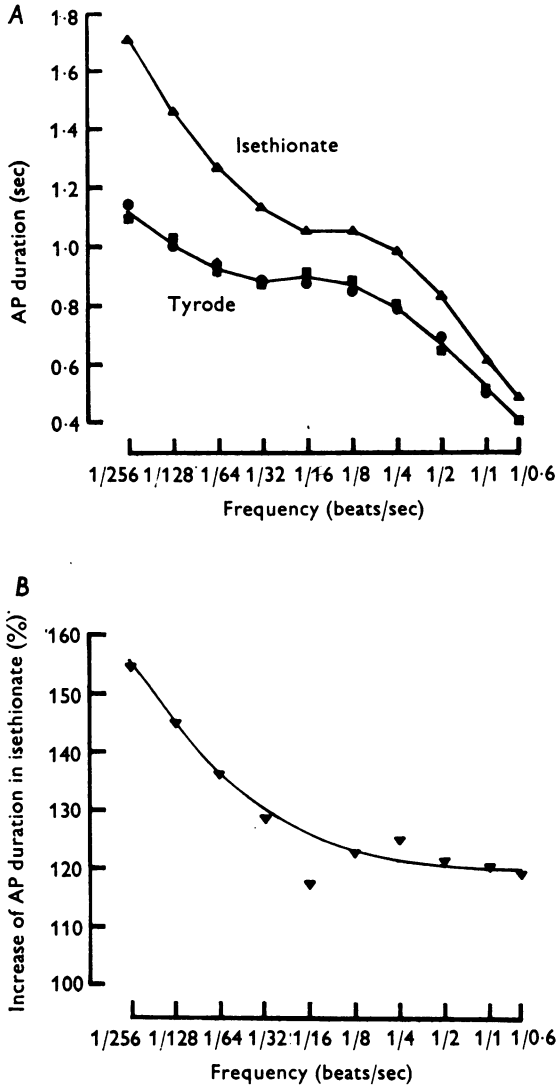

Fig. 5. *A*, plot of frequency against action potential (AP) duration in Tyrode and Cl^--poor solution (isethionate replacing Cl^-). Experiment carried out first in Tyrode (■), then in isethionate (▲) and was then repeated in Tyrode (●).

B, same experiment as shown in *A* but the increase in action potential duration has been plotted as a percentage. The action potential duration in Tyrode at each frequency has been taken as 100%. The line drawn through the points is to show the general trend. It has no special significance.

Introduction). I/V was measured first in Tyrode, then in choline-cyanide Tyrode and finally in Tyrode to check for drift of I/V (see Methods). A typical result is shown in Fig. 6, where the I/V in choline-cyanide Tyrode (measured after 25 min, action potential disappearing after 2–3 min) is clearly shifted upward when compared with Tyrode. On return to Tyrode I/V returned to very nearly its original level. Similar results were obtained in four other experiments. However, in two of these experiments on returning to Tyrode the outward current, while reduced in comparison to the

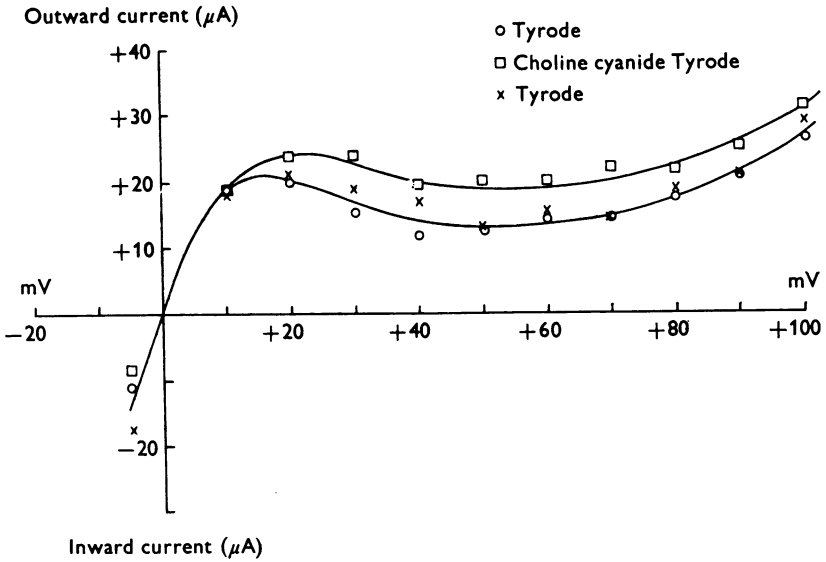

Fig. 6. I/V in Tyrode and in choline-cyanide Tyrode. ○, initial experiment in Tyrode; □, after 25 min in choline-cyanide Tyrode; ×, repeat in Tyrode.

outward current in choline-cyanide Tyrode, did not return completely to the original level, the curve being shifted slightly upwards when compared to the first run in Tyrode. This was probably due to drift during the course of the experiment (see Methods). The course of the curve in choline-cyanide Tyrode in the hyperpolarizing direction was not studied in detail.

That this effect was not due to cyanide alone was shown in two further experiments with 2 mM cyanide added to normal Tyrode. Even after 30 and 40 min exposure to this solution no change was seen in the I/V relationships when compared to Tyrode both before and after application of the cyanide-containing solution. Another possibility is that the increase in outward current in Na-poor solution is due to a removal of a background Na current. This seems unlikely for three reasons. Firstly, removal of cyanide from the low-Na solution caused a reduction of the outward current, i.e. I/V was moved downwards. Secondly, if tetrodotoxin (10^{-6} g/ml.) was

used to block the Na current an increase in outward current was found on going into Na-free + cyanide solution (one experiment). Thirdly, if the upward shift of the curve was due only to the removal of the Na background current, it would cross the curve in Tyrode at E_{Na} and not simply be moved upwards at all potentials.

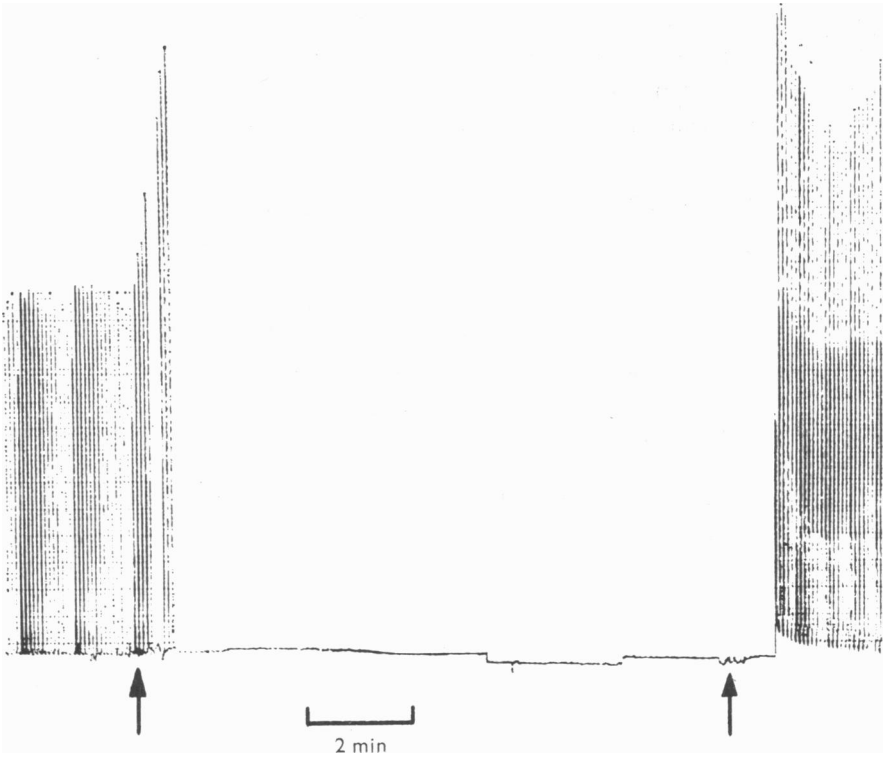

Fig. 7. Recording of the isometric twitch tension. Between arrows Na-free cyanide solution was applied. The gap in the trace after application of the Na-free solution was caused by sucking the solution out of the small bath. This is also seen as small oscillations of the base line on returning to Tyrode. The sudden drop and rise of tension during the application of the Na-free solution is an artifact.

Simple reduction of $[Na]_o$ also caused an upward shift of I/V. However, in contrast to the above results, the effect was variable. In three experiments a 10 min exposure to Na-poor solution produced an upward movement in current–voltage relationship. In two further experiments no change was seen after a 12 and 15 min exposure. To investigate the effect of time of exposure to Na-poor solutions two experiments were performed in which the I/V was monitored regularly throughout the exposure to the Na-poor solution. In each case, a definite increase in outward current was found, in one experiment after 10 min and in the second after 20 min. Thus,

while Na removal can by itself produce an increase in the measured current at a given voltage, a consistent shift of the I/V relation was observed only when cyanide was added to the test solution.

Tension experiments. An increase in the resting tension produced by ventricular bundles should be indicative of an increase in the free $[Ca]_i$. Therefore, the effect of the Na-poor solution on the resting tension was investigated. From seven experiments in Na-poor solution and two experiments in Na-free solution (both containing 2 mM cyanide) only one (in Na-poor solution) showed a small rise in resting tension (about 4 % of the twitch tension). An example of the result of an exposure to a Na-free, cyanide-containing solution is seen in Fig. 7, the Na-free solution being applied between the arrows. There was an initial period of twitch potentiation on changing to the Na-free solution whilst equilibrium was being established. This potentiation effect of Na removal on mammalian ventricle has also been described by other authors (see, for instance, Prasad, 1970). Once Na-free conditions were achieved the twitch disappeared due to failure of the propagated action potential. Often there was an increase in resting tension during the twitch potentiation which returned to the base line on cessation of the twitch. On return to Tyrode a marked twitch potentiation was also found, with a transient increase in resting tension. Scholz (1969) also found no contracture in calf ventricle in Na-free solutions. Thus there is a difference between sheep and calf ventricular preparations and guinea-pig atrial preparations where exposure to a Na-free, cyanide solution elicits a contracture (Jundt *et al.* 1975; see discussion by Scholz, 1969).

E_{Ca} measurements. The results of the tension experiments suggest that on exposure to a Na-free solution, with or without cyanide, there is little, or no, increase in the free Ca surrounding the myofibrils. However, I/V in choline-cyanide Tyrode has been observed to move upwards suggesting an increase in $[Ca]_i$, if not throughout the cell, at least at the inner surface of the membrane. To test this idea E_{Ca} was measured in Tyrode and after application of choline-cyanide Tyrode.

A double-step clamp method similar to that of Bassingthwaighte & Reuter (1972) was used in which i_{Na} was inactivated by holding the membrane potential at about -40 mV and then applying a test pulse. The double sucrose-gap method, while convenient, is not ideal for such measurements (see McGuigan, 1974) and no attempt has been made to plot the instantaneous I/V relationships, but E_{Ca} has been determined by plotting I/V for the inward Ca current and taking E_{Ca} as the point where this curve crosses the voltage axis. It is possible to use this method in sheep, calf and cow bundles because the time-dependent changes in outward current are minimal (McGuigan, 1974).

The results of four such experiments are presented in Table 2 and show that in each case the reversal potential in choline-cyanide Tyrode is less positive than in Tyrode. The average shift when correction for short-circuiting is made, using the factor of 1·1 (McGuigan, 1974) is 9 mV.

Action-potential experiments: effect of 70 % $[Na]_o$. A reduction of $[Na]_o$ to 70 % reduced both the duration and overshoot of the action potential compared to the values in Tyrode. It was originally argued (McGuigan & Bassingthwaighte, 1974) that the reduction in duration was due to an increase in $[Ca]_i$, thereby increasing P_K. However, this conclusion is probably wrong as the I/V in Tyrode and 70 % Na Tyrode showed no difference, and reduction of $[Na]_o$ by 30 % would not be expected to alter markedly the Na/Ca exchange system (H. Reuter, personal communication).

TABLE 2. E_{Ca} (mV depolarization)

Experiment	ΔV	Tyrode	Choline cyanide Tyrode	Tyrode
1C	7	97	90	100
2C	8	98	90	113
3C	8	105	97	127
6C	10	100	90	100

ΔV refers to the difference between the first experimental run in Tyrode and choline-cyanide Tyrode.

Moreover, if the overshoot of the action potential in 70 % Na Tyrode was increased by current pulses, through a single sucrose-gap, the action potential duration was increased. This result showed that the reduction in duration was at least partly due to the decrease in the overshoot. However the situation could be more complicated as small changes in the I/V relationship would not be measured. Also, if Na as well as Ca ions contribute to the slow inward current (see review by Reuter, 1973*a*) then a reduction of Na would also cause a reduction in i_{Ca}, and thus shorten the action potential.

Alteration of $[Ca]_i$ by variation of $[Na]_i$. In our original experiments (Fry *et al.* 1975) ouabain was added in order to block the Na pump and so, by increasing $[Na]_i$ increase $[Ca]_i$. However, we have now found that the actions of ouabain are more complicated. Ouabain, at a concentration of 10^{-6} M, in 0·36 mM-Ca Tyrode, causes, at a stimulation frequency of 1/2 sec, an initial prolongation of the action potential duration (see also Dudel & Trautwein, 1958; Kassebaum, 1963). While we measured an increase in outward current in voltage-clamp experiments, the interpretation of such measurements is difficult, since ouabain also increases the internal longitudinal resistance (Weingart, 1975). We now conclude that ouabain has actions other than simply increasing $[Na]_i$ (see also Isenberg & Trautwein, 1974; 1975; Cohen, Daut & Noble, 1975; Lederer & Tsien, 1975).

DISCUSSION

It has been shown in this report that the time-independent outward current can be modified in a number of ways: (1) raising the stimulation frequency increased the current and the increase was greater as $[Ca]_o$ was elevated; (2) Verapamil and D 600, agents known to block i_{Ca} in mammalian ventricular myocardium (Kohlhardt *et al.* 1972), reduced the frequency-induced increase of outward current; (3) reduction of $[Na]_o$ increased the current.

We suggest that these effects act through a change of $[Ca]_i$ at the inner side of the membrane, since there is evidence that under the above conditions Ca accumulates inside the cell. ^{45}Ca flux studies by Langer & Brady (Langer & Brady, 1963; Langer, 1965) have suggested that in dog papillary muscle an increase in the stimulation frequency increases the Ca influx into the cell, over an initial 10 min period. Electrophysiological evidence for a stimulation-induced increase in Ca at the inner side of the membrane also exists. Bassingthwaighte & Reuter (1972) observed a shift in the reversal potential of the Ca current to more negative potentials as longer test pulses were used (up to about 100 msec). Reuter (1973*b*) also showed that, after a period of rest, depolarization of ventricular muscle at a rate of 1/sec initially gave a larger inward current than the steady-state value.

There is also considerable evidence that reducing $[Na]_o$ increases internal Ca in mammalian ventricular muscle (e.g. Langer, 1968; Reuter & Seitz, 1968) and the results presented here also suggest that such a situation exists. Thus, on return to Tyrode after treatment with a Na-poor solution the twitch was greatly potentiated, suggesting that the internal stores had become loaded with Ca. Also our double-step voltage-clamp experiment indicated that E_{Ca} shifts in a direction that suggests an increase of Ca at the inner membrane surface on exposure to Na-poor solutions. This idea of a compartmentalization for Ca has been proposed by numerous authors and is supported by both flux and electrophysiological measurements (reviewed by Langer, 1973).

Another possible explanation for the above results is a coupled Ca/K carrier mechanism (Morad & Greenspan, 1973; Prasad, 1974). However, such a mechanism would have to be electrogenic and, in order to explain the cross-over of the action potential duration/frequency curves in different $[Ca]_o$, the degree of coupling would have to be variable. Further modifications of the basic scheme would have to be proposed to explain the effects of Verapamil and reduction of $[Na]_o$. While such modifications are possible there exists no experimental evidence for them. Indeed evidence against this idea has been presented by Reiter & Stickel (1968),

who showed that in the range of $[K]_o$ from 4·8 to 9·6 mM the force of contraction was not altered despite a diminution of the resting potential.

A further explanation is that the action of the increased $[Ca]_o$ and the Ca current blockers is to shift the activation curves of the slow outward current, similar to the x_1 in Purkinje fibres described by Noble & Tsien (1969). Kass & Tsien (1975) have studied such an effect and find that both Ca and D 600 move the activation curves for x_1 to more positive potentials. However, this is in the wrong direction for the effect of increased $[Ca]_o$ (see Fig. 2*A*). It could explain the effects of D 600 and verapamil, but x_1 is of but minor importance in sheep and calf ventricle as evinced by the lack of increasing outward current in the course of a 500 msec depolarizing clamp step. Also I/V in Tyrode and in Tyrode containing verapamil were identical. It is thus concluded that the major effect of the Ca blockers is on the Ca current and not on the outward K current.

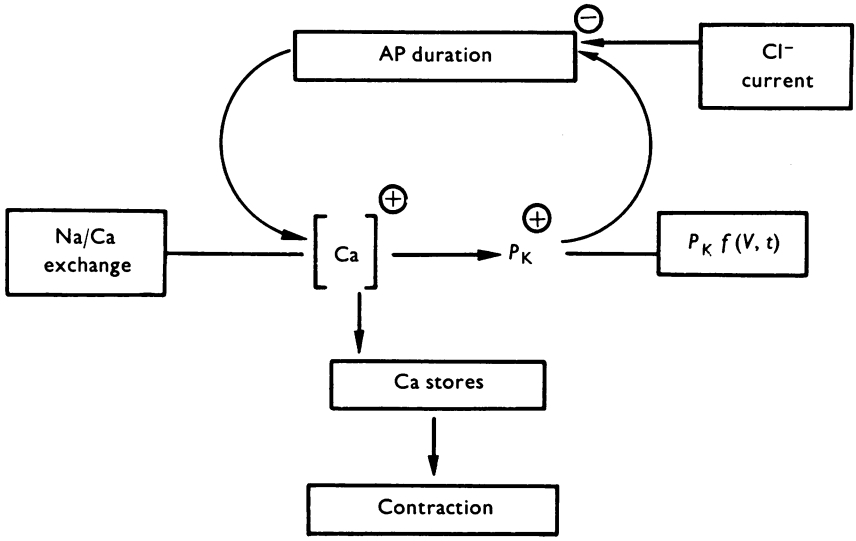

Fig. 8. Diagram of the suggested feed-back mechanism between action potential (AP) duration, contraction and internal Ca. For further details see text.

Regulation of the action potential duration. In sheep and calf ventricular muscle repolarization of the action potential is due to a decrease in the inward Ca current against a background, time-independent outward current (see Discussion, McGuigan, 1974), i.e. delayed rectification does not play an important role. Thus, increasing the background current, as manifested by an upward shift in the I/V relation, would reduce the action potential duration. The conclusions of our experiments are summarized by

Fig. 8 which shows the feed-back loop we propose for controlling the action potential duration. We would like to stress the tentative nature of our model.

If $[Ca]_o$ or the stimulation frequency is increased then Ca ions will accumulate under the membrane, causing an increase in the background outward current. This shortens the action potential duration reducing the inflow of Ca ions, thus preventing an excessive rise in $[Ca]_i$ which could cause decoupling (De Mello, 1975; Rose & Loewenstein, 1975). Kass & Tsien (1976) have shown that increasing the external Ca in Purkinje fibres also increases the background outward current. They suggested that the level of external Ca is involved in setting the level of the background outward current, and that this increase in outward current contributes to the shortening of the action potential seen in high Ca.

McGuigan (1974) has demonstrated in sheep and calf ventricle preparations a small voltage- and time-dependent outward current, similar to the x_1 current in Purkinje fibres. The existence of two mechanisms for increasing the outward current is supported by the fact that application of Verapamil or D 600 reduced, but did not abolish, the frequency-induced increase in outward current (cf. Meech & Standen, 1975).

The scheme can explain the observation that at high stimulation frequencies, i.e. 1/sec, the action potential in high $[Ca]_o$ is shorter than in low $[Ca]_o$, the opposite being true at low frequencies. With a high $[Ca]_o$ more Ca ions will enter the cell during each beat and at high frequencies will lead to an accumulation inside the cell. In opposition to this the action potential in high $[Ca]_o$ will have a greater overshoot and because the inactivation of i_{Ca} is slower at positive potentials (Beeler & Reuter, 1970; Trautwein, McDonald & Tripathi, 1975) the action potential will be lengthened. Thus, the actual duration will depend on the relationship between the transmembrane Ca ion flux per beat and the actual amount of Ca accumulated within the cells. At low stimulation frequencies the effect of the increased overshoot in high $[Ca]_o$ will then prolong the action potential relative to that in low $[Ca]_o$. The results also suggest that Cl ions play a role in determining the action potential duration. This effect is more pronounced at low than at high frequencies. The reason for this is probably twofold. At low frequencies the internal Ca-mediated increase in outward current is small so that the ratio of P_{Cl}/P_K will be greater than at high frequencies. Also at low frequencies Cl ions will have more time to distribute themselves in accordance with the level of the resting potential and thus contribute a larger amount of repolarizing current in the course of an action potential, on the assumption that Cl is passively distributed (but see Ladle & Walker 1975).

This basic scheme can also be influenced by modification of the Na/Ca

exchange system, leading to an indirect increase in the internal Ca. Blocking the Na pump will indirectly increase $[Ca]_i$ by increasing $[Na]_i$. This mechanism has been proposed for the action of K-free and K-poor solutions on heart (Reiter, Seibel & Stickel, 1971; who also give reference to earlier literature), although the authors do not interpret their results as showing a connexion between $[Ca]_i$ and P_K.

Na removal and tension. Removal of most (or all) of the external Na ions plus the addition of cyanide initially increased the twitch height. A similar potentiation was also seen on returning to Tyrode solution. Jundt *et al.* (1975) have shown that cyanide releases Ca from heart mitochondria. Since no contracture was found in our experiments it must be assumed that this Ca was taken up by the sarcoplasmic reticulum. Such a mechanism would lead to an increase in the internal stores of Ca, this conclusion being supported by the potentiation seen on going into Na-free solution and by the marked potentiation on returning to Tyrode. By this hypothesis the increase of resting tension seen initially, and on return to Tyrode would be caused by incomplete uptake of the increased Ca release. A similar conclusion that Na removal induces loading of internal Ca stores has been reached by Bass (1976) using paired pulse stimulation. These factors, i.e. Na removal or increased stimulation rate, which lead to an increased internal Ca, give rise to an increased filling of the internal Ca stores and hence to potentiated contractions. This is shown schematically in Fig. 8.

In conclusion, the feed-back mechanism suggests a connexion, via internal Ca, between the various factors that can influence action potential duration and contraction. In this context it is worth noting that Niedergerke (1956) suggested that the positive staircase in frog ventricle was due to an accumulation of Ca at the inner side of the cell membrane.

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