On the Effects of Divalent Cations and Ethylene Glycol-bis-(β-Aminoethyl Ether) *N,N,N′,N′*-Tetraacetate on Action Potential Duration in Frog Heart

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ABSTRACT Resting and action potentials were recorded from superfused strips of frog ventricle. Reducing the bathing calcium concentration ($[Ca^{2+}]_0$) with or without ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetate (EGTA) prolongs the action potential (AP). The change in the duration of the AP extends over many minutes, but is rapidly reversed by restoring calcium ions. Other changes (e.g., in resting potential and overshoot) are, however, only more slowly reversed. Reducing $[Ca^{2+}]_0$ with 0.2, 2, or 5 mM EGTA produces progressively greater prolongation of AP; maximum values were well in excess of 1 min. This prolongation can be reversed by other divalent cations in EGTA (Mg^{2+}, Sr^{2+}) or Ca-free (Mn^{2+}) solutions, or by acetylcholine. Barium ions increase AP duration in keeping with their known effect on potassium conductance. D600, which blocks the slow inward current in cardiac muscle, is without effect on the action potentials recorded in EGTA solutions, or on the time course and extent of the recovery to normal duration upon restoring calcium ions. It is concluded that divalent cations exert an influence on membrane potassium conductance extracellularly in frog heart. The cell membrane does not become excessively "leaky" in EGTA solutions.

INTRODUCTION

It is commonly observed that the duration of the cardiac action potential (AP) is influenced by the bathing calcium concentration. Generally, a reduction in calcium prolongs the duration of the AP, although this effect is, for example, dependent upon stimulus frequency (Bassingthwaighte et al., 1976). A number of authors have reported that calcium reduction by the use of chelating agents, e.g., ethylenediamine tetraacetic acid, and ethylene glycol-bis-(β-aminoethyl ether)*N,N,N′,N′*-tetraacetate (EDTA, EGTA) will evoke prolonged action potentials (Tritthart et al., 1973), sometimes of several seconds' duration (Hoffman and Suckling, 1956; Rougier et al., 1969). This effect is of immediate interest because both intracellular and extracellular calcium levels have been found to affect potassium conductance in a number of cell types (Meech, 1974; Meech and Standen, 1975; Isenberg, 1975; Kass and Tsien, 1975, 1976; Lew and

Ferreira, 1976), quite apart from the dependence of the "slow inward current" of cardiac muscle on the bathing calcium level (see Reuter, 1973).

The effect of extreme calcium reduction on heart muscle is also interesting since, especially in EDTA solutions, the membrane is reported to become highly permeable, allowing freer movement of ions and larger molecules into the cell (Winegrad, 1971). Until now there has been no detailed observation of the time course of the effects of EGTA on the heart or of the possible influence of divalent cations other than calcium and magnesium on the responses.

The present experiments on frog ventricle fibers were undertaken to provide information about the effects of EGTA-containing solutions on the heart under precisely defined conditions (e.g., extracellular exchange, pH, extracellular divalent cation concentration, $[X^{2+}]_0$). Special attention was paid to obtaining continuous recordings from individual cells throughout a sequence of solution changes. The results show that resting and action potentials are well maintained in divalent cation-poor media, although the duration of the AP is dramatically increased. The observations indicate that the extracellular divalent cation concentration $[X^{2+}]_0$ affects the time of onset of repolarization in frog ventricle. Changes in $[Ca^{2+}]_i$ are concluded to be of little consequence for the duration of AP under the present conditions (very low $[Ca^{2+}]_0$).

A preliminary report of some of these results has been published (Miller, 1976).

MATERIALS AND METHODS

Preparation and Mounting Procedure

A total of 20 preparations were used which comprised either a single trabeculum or a bundle of trabeculae from the ventricle of the frog *Rana esculenta*, with diameters between 200 and 700 μm and length of 2–4 mm. The muscle was mounted in a chamber similar to that of Chapman and Tunstall (1971) where one end of the muscle was tied to a fixed hook and the other to the beam of a force transducer (Endevco, 8107/20). The muscle was regularly stimulated (Devices Digitimer) generally at 4 min^{-1}, with square pulses (field stimulation, duration 2 ms and twice-threshold intensity). The superfusing solutions could be rapidly exchanged by means of a threeway tap (chamber dead space clearance <0.1 s).

Superfusing Solutions

The muscle was continuously superfused with the appropriate bathing solutions whose compositions are given in Table I. The preparation was dissected and mounted for initial superfusion in 2 mM Ca Ringer solution. A lower calcium level (0.2 mM) was used as standard to reduce contractile strength and facilitate the prolonged maintenance of the microelectrode in individual cells. The 2 mM Ca Ringer solution was occasionally reapplied for several minutes, particularly after long exposures to EGTA, to provide a check that no irreversible changes had occurred.

The free concentration of the divalent cations in the EGTA-containing solutions was calculated with a knowledge of the contamination level for calcium (c. $2 \cdot 10^{-5}$ M) and of the appropriate binding constants for the various divalent cations to EGTA (see Miller and Moisescu, 1976; Sillén and Martell, 1964). Importance is attached to the precise definition of the experimental pH in all solutions so that $[X^{2+}]_0$ may be calculated (see Table I). Although Tris is a relatively poor buffer at pH 7.0, the rapid superfusion of a

small preparation ensures sufficient buffer capacity. Experiments were made at 20.5 ± 0.5°C.

Recording and Evaluation of Data

Standard microelectrode technique was used. Intracellular potentials were measured via 3 M KCl-filled glass microelectrodes (resistance 10–18 MΩ, tip potentials 10 mV or less with capacity compensation). Potentials were recorded with either an oscilloscope camera or a UV chart recorder (galvanometer response flat to 1 kHz, maximum deflection 12 cm).

Each series of action potentials shown or evaluated was obtained during a single penetration. As far as possible, a whole series of solution changes was completed with continuous recording from one cell and the quantitative data were obtained under these

TABLE I

COMPOSITION OF THE PERFUSION SOLUTIONS

Solution	$CaCl_2$	$MgCl_2$	$SrCl_2$	EGTA	$[Ca^{2+}]_o$	$[Sr^{2+}]_o$	$[Mg^{2+}]_o$
	mM (added)				*M (calculated)*		
Normal Ringer's	2 or 0.2	–	–	–	–	–	–
Ca-free	–	–	–	–	$2 \cdot 10^{-5}$*	–	–
0.2 mM EGTA	–	–	–	0.2	$2.2 \cdot 10^{-8}$	–	–
0.2 mM EGTA + Mg	–	0.2	–	0.2	$2.25 \cdot 10^{-8}$	–	$1.98 \cdot 10^{-4}$
	–	1.0	–	0.2	$2.33 \cdot 10^{-8}$	–	$0.99 \cdot 10^{-3}$
	–	5.0	–	0.2	$2.74 \cdot 10^{-8}$	–	$4.97 \cdot 10^{-3}$
2.0 mM EGTA	–	–	–	2.0	$2.02 \cdot 10^{-9}$	–	–
2.0 mM EGTA + Sr	–	–	0.4	2.0	$2.51 \cdot 10^{-9}$	$1.58 \cdot 10^{-5}$	–
5.0 mM EGTA	–	–	–	5.0	$8.0 \cdot 10^{-10}$	–	–

All solutions contained (mM): NaCl 117, KCl 3, Tris 2.5, glucose 5, in glass-distilled water.
The divalent cations were varied as above.
The free magnesium level in the EGTA free solutions was estimated to be $\leq 10^{-6}$ M.
Acetylcholine, atropine sulfate, $BaCl_2$, and $MnCl_2$ were added as required from stock solutions, the latter two substances only to Ca-free Ringer. All solutions were adjusted to a final pH of 7.00 with HCl or NaOH. D600 (racemate) was the kind gift of Knoll AG., Ludwigshafen, Federal Republic of Germany.
* Value obtained by flame photometry and titration against EGTA (see Miller and Moisescu, 1976).

conditions. Action potential duration was measured as the time to 90% repolarization except in a few cases where an afterpotential developed in the presence of EGTA (see Fig. 3), so that fast repolarization represented only about 80% of the total. Duration was then measured to 80% or 70% repolarization, although the differences introduced were insignificant as the fast phase of repolarization remained steep under all conditions tested. The resting potential was taken as that immediately before the action potential upstroke. All the effects studied were fully reversible.

In cases where AP duration exceeded 15 s it was pointless to maintain the stimulus frequency at 4 min^{-1}, so the rate was reduced as necessary.

In several figures the alterations in duration of AP are plotted on semilogarithmic axes against time. This serves to exaggerate and clarify the small changes occurring at the beginning or end of the time courses illustrated.

RESULTS

(Normal) Ringer Solution (0.2 mM Ca)

The initial levels (± SD) for the resting potential, overshoot, and AP duration after at least 30 min in normal Ringer solution were: −74.8 ± 6.2 mV; +12.4 ± 8.6 mV; and 0.80 ± 0.19 s, respectively (47 cells). This represented a stable state. These values are in reasonable agreement with those of other authors, e.g., Niedergerke and Orkand (1966), although the resting potential is slightly lower, possibly reflecting an effect of prolonged exposure to the relatively low calcium concentration.

Calcium-Free Solutions

Mines (1913) and Daly and Clarke (1921) reported that the AP persists in calcium-free solutions even though contraction fails. This finding has frequently been confirmed and Fig. 1*a* shows comparable findings in the present study.

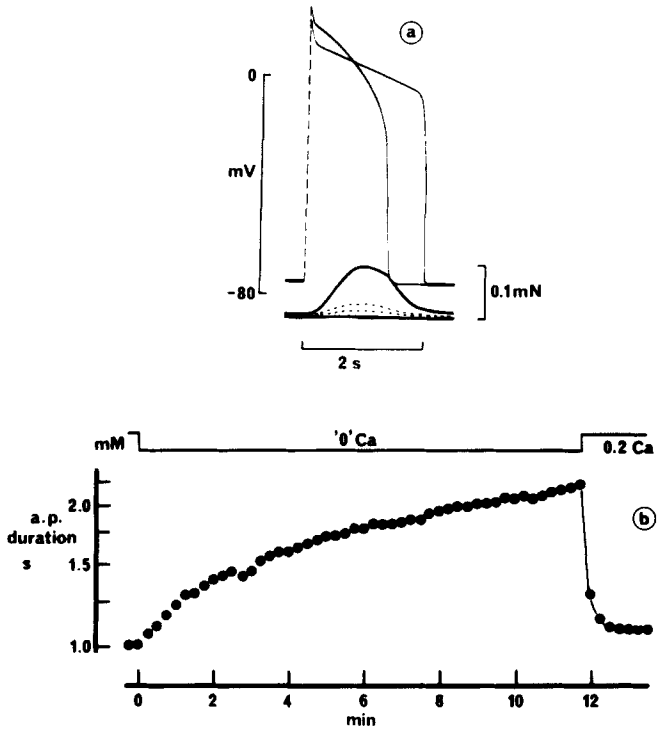

FIGURE 1. Action potentials in Ca-free Ringer's solution. (*a*) *Upper trace,* action potentials recorded in one cell immediately before, and 8 minutes after, the solution change (AP prolonged). *Lower trace,* tension output immediately before, and 15, 30 (---), and 120 s after solution change. (*b*) Action potential duration (ordinate) for successive action potentials plotted against time (abscissa) after switching from 0.2 mM Ca to Ca free Ringer solution. After 11 min 45 s 0.2 mM Ca was restored. The solid curve is an exponential with a half-time of 6 s (stimulus rate 4 min^{-1}).

The action potentials shown were recorded in the same cell immediately before and 8 min after changing from 0.2 mM Ca to Ca-"free" solution which prolonged the duration.

During the first minutes of perfusion with zero Ca Ringer's solution the AP undergoes several changes which may be summarized as follows. The early phase of the overshoot falls rapidly by several millivolts (Fig. 1*a*), the plateau in general is also reduced but assumes a more "horizontal" form as the onset of the fast repolarization is progressively delayed. AP duration increases by between 20% and 200% compared to that in normal Ringer solution. This change in duration continues over several minutes and follows a nonexponential time course (Fig. 1*b*). In some cells the first few action potentials in Ca-free (and EGTA) Ringer solution were slightly shorter than the value immediately before addition of the substance (Rougier et al., 1969). The resting potential is little affected, a slight depolarization (~5 mV) being noted in some cells.

The return to 0.2 mM Ca Ringer solution very rapidly reduces the duration of the AP ($t_{\frac{1}{2}}$ for Fig. 1*b* is 6 s) with a single exponential time course. The normal action potential form is, however, not achieved for several minutes (see later). Twitch tension falls to a very low level in these calcium-free solutions ($[Ca^{2+}]_0 = 2.10^{-5}$ M) with an approximately exponential time course (Fig. 1*a*). In the case of Fig. 1*b* (different preparation) tension was no longer detected with the first stimulus (15 s) after the solution change so that the time course could not be accurately determined (i.e. $t_{\frac{1}{2}} < 4$ s). In thicker preparations tension fell more slowly (e.g., Fig. 2). The half-time for the loss of tension thus varied considerably from one preparation to another but was always slower than the fastest phase ($t_{\frac{1}{2}}$ ~3 s) obtained with the thinner atrial trabeculae (Chapman, 1971*a*; Miller, 1975) or with the superfused half-ventricle method (Chapman and Miller, 1974). In normal Ringer solution, twitch tension recovers more slowly (see Fig. 2*b*, *d*), depending upon the duration and extent of the [Ca] reduction, as described by Chapman and Niedergerke (1970).

Effects of EGTA

The free calcium concentration can only be reduced below about 10^{-5} M by the use of calcium buffers. To be able to give the extracellular free calcium level with any certainty it is essential that the buffer capacity be adequate for the conditions near the surface of the muscle cells. Miller and Moisescu (1976) have shown that in continuously superfused thin atrial trabeculae, where extracellular exchange is rapid, a total [EGTA] of ~0.5–1 mM is necessary to hold $[Ca^{2+}]_0$ constant during the 0 Na^+ contracture (at 4.10^{-7} M Ca_0^{2+}). In the present experiments, where a greater proportion of the EGTA remains in the free form, 0.2 mM EGTA probably represents a minimum level for satisfactory buffering.

Fig. 2 shows the effect of 0.2 mM EGTA Ringer solution ($[Ca^{2+}]_0 = 2.2 \cdot 10^{-8}$ M, see Table I) on three parameters. The records in Fig. 2*a* show the form of the action potentials from a cell during, and in Fig. 2*b*, after, superfusion with 0.2 mM EGTA Ringer solution. It should be noted how slowly the prolongation of the AP occurs (Fig. 2*a*) and how quickly the effect is reversed by calcium

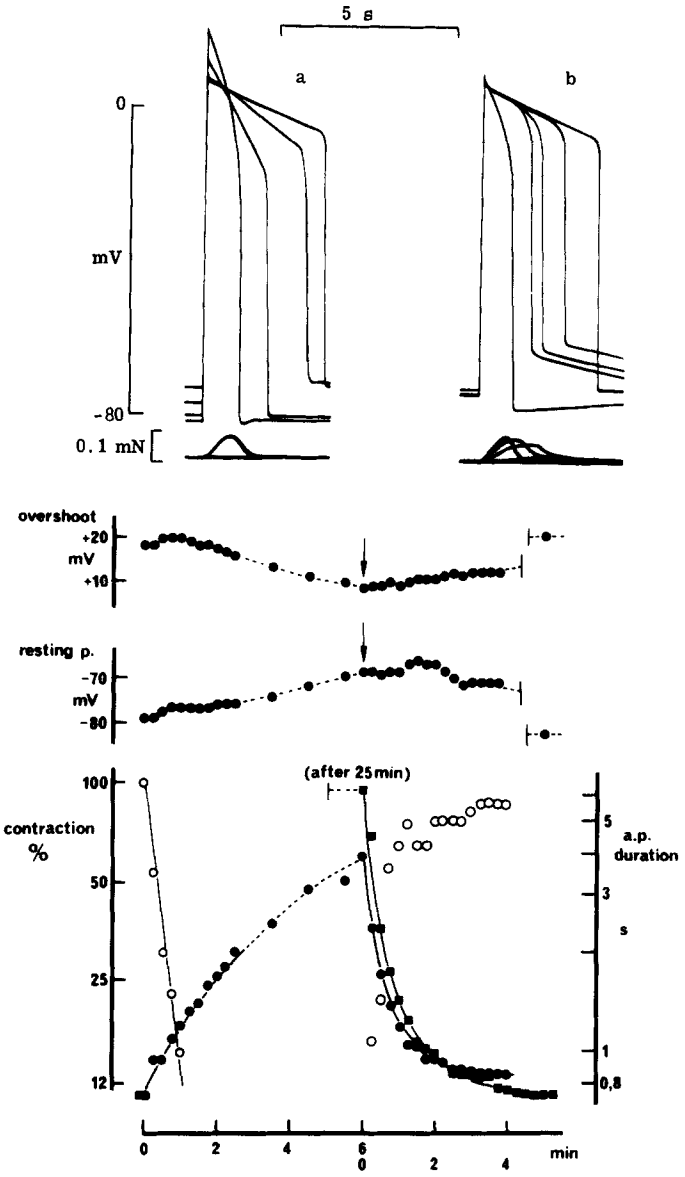

FIGURE 2. Membrane potentials in 0.2 mM EGTA Ringer's solution (see Table I). (*a*) The action potentials immediately before, and 2, 4, and 6 min after changing to Ringer + 0.2 mM EGTA (prolonged AP); and (*b*) from the same cell immediately before and 15, 30, 45, and 150 s after returning to normal Ringer solution. The lower trace in (*a*) and (*b*) shows the tension output from the muscle associated with the action potentials. (*c*, *d*) Data from one cell from another preparation (●) for action potential overshoot (upper curve, *c*) and resting potential (lower curve, *c*) and AP duration (*d*, ●, right-hand ordinate) for the first 6 min (abscissa, *d*, also applies to *c*) of perfusion with Ringer + 0.2 mM EGTA. Peak twitch tension recorded simultaneously (left-hand ordinate, *d*, ○) fell to zero.

(Fig. 2*b*). The resting potential is reduced here by about 10 mV and the fall in overshoot and the changes in the plateau can be distinguished. In almost all the cells studied, the fast repolarization phase was simply shifted along the time axis without a substantial change in its steepness. The potential at which fast repolarization occurred in this cell was more positive in EGTA. The finding is, however, not consistent as in many cases almost no change occurred (see, e.g., Fig. 3*a*). Evidently, calcium ions quickly restore the duration to normal, but the resting potential (Fig. 2*c*) and the form and height (overshoot) of the normal plateau develop somewhat later.

In this cell, a marked afterpotential developed during the recovery to normal AP duration. This may reflect a phase of potassium accumulation near the cell membrane (see Cleeman and Morad, 1976; Discussion), though it was not observed in all preparations.

The middle part of the figure (Fig. 2*c*) shows the time course of the changes in overshoot and resting potential which occurred during and after a 6-min exposure to EGTA. It can be seen that both parameters change slowly in response to the altered $[Ca^{2+}]_0$: the effects are fully reversed about 10 min after restoration of the calcium ions.

The continually recorded values of AP duration are presented in the lowest part (*d*) of Fig. 2. Although the values achieved are well in excess of those in the Ca-free solutions (Fig. 1*b*), the time course of AP prolongation is similar. In the case where normal Ringer solution was restored after 6 min (filled circles, same cell as in Fig. 2*c*), duration returned to normal with an exponential time course (Fig. 2*d*; the points fall on a solid curve which represents an exponential with $t_{\frac{1}{2}}$ = 22 s). This value is very close to the half-time for the fall in twitch tension (Fig. 2*d*, open circles, $t_{\frac{1}{2}}$ = 21 s) associated with the change to EGTA Ringer solution. Twitch tension falls to zero, the points approximating a straight line in Fig. 2*d* (semilogarithmic plot) indicating an exponential rate of fall.

(Tension normally falls with three exponential phases when $[Ca^{2+}]_0$ is reduced in frog ventricle [Chapman and Miller, 1974] but where $[Ca^{2+}]_0$ is thereby reduced to below the minimum necessary for steady-state tension production, the appearance of the two slowest phases is suppressed. This kind of behavior indicates that internal Ca stores act cooperatively with a Ca influx during the AP [see Chapman, 1971*b*; Miller, 1974].)

Later in the same experiment the action potentials were observed in another cell for 25 min in 0.2 mM EGTA (Fig. 2*d*, square symbols) after which a steady level for AP duration was achieved. The subsequent fall in duration ($t_{\frac{1}{2}}$ = 23 s) in normal Ringer solution paralleled that seen after the short exposure to

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The solid line is an exponential, $t_{1/2}$ 21 s. Normal Ringer (0.2 mM Ca) was restored after 6 min (arrows, *c*). Final values for the curves in (*c*) were obtained 9 min later. The fall in AP duration (*d*, ●) fitted by an exponential curve ($t_{1/2}$ 22 s). Twitch tension also recovers (○). Recordings made subsequently from another cell in the same preparation (■) were extended to 25 min in EGTA when a steady level for duration was reached. The fall in duration upon restoring normal Ringer solution is fitted by an exponential curve ($t_{1/2}$ 23 s). (Stimulus rate, 4 min^{-1}).

EGTA. The slow decline of both resting potential and overshoot was completed in this cell after 25 min at values of −2 mV (peak of the AP) and −60 mV (resting potential). However, such a marked depolarization was not observed in every preparation, or even in all the cells tested in any one preparation during successive exposures to EGTA. Repeated cycles of EGTA and normal Ringer solution produced very similar responses each time with continuous impalement of one cell.

Although restoring Ca_0 may be expected to alter [Ca] near the cells with the same simple time course as is seen with Ca_0 removal, the recovery of tension after exposure to EGTA is a complex phenomenon. Drastic changes in action potential duration after removing EGTA are superposed on the interaction of three "stores" for calcium normally detected in the kinetics of tension response to $[Ca^{2+}]_0$ change (Chapman and Miller, 1974). The time course of twitch recovery gives no clear information on the rate of [Ca] change near the cells as the former is subject to too many variables to permit detailed analysis. It may be noted that tension returns within 15–30 s (four beats per minute, see Fig. 2*d*) at which time the still abnormally long action potentials evoke twitches of corresponding duration which peak within 1 s and then show a slow relaxation; the onset of fast repolarization then initiates a phase of relaxation at the normal rate (see for example, Fig. 2*b*). A complete return to normal twitch strength only occurs after ~10 min stimulation in Ringer's solution.

Higher EGTA Concentrations (2 and 5 mM: Table I)

Fig. 3*a* shows an example of a cell where a marked afterpotential developed in the presence of EGTA (2 mM) although a near-normal resting potential was maintained throughout perfusion with EGTA Ringer solution. This phase of slow repolarization was often observed and prevented the resting potential from reaching a maximum when interrupted by the subsequent action potential. In this case reduction of the stimulus frequency allowed the slow repolarization to reach a steady level within about 30 s. Reducing the stimulus frequency also had the effect of prolonging the subsequent action potentials still further, as is observed in heart at more normal $[Ca^{2+}]_0$ (e.g., Bassingthwaighte et al., 1976).

The higher concentrations of EGTA produced qualitatively similar effects, although a large variation occurred from cell to cell even in the same preparation. The time course for the development of the prolonged AP in EGTA Ringer solution in two cells from the same preparation was frequently quite different although the first few AP's were nearly identical in both. This was one of several indications that some deficiences in the electrical synchronization or coordination between individual cells may develop in EGTA solutions. A possibly related phenomenon was the appearance of "humps" during the otherwise smooth prolonged plateau of the action potential.

This phenomenon was noted by Hoffman and Suckling (1956). Examples from the present experiments are given in Fig. 3*b* and *c*, which shows the development in the first six beats (Fig. 3*b*) and after 5 min (Fig. 3*c*) of the response of a cell to 2 mM EGTA Ringer solution. This odd feature in the plateau may result from the electrotonic influence of a "decoupled" cell or group of cells which repolarized earlier than that under observation.

This point could be resolved by stimultaneous recording from several cells in one preparation but, because of the rarity of this phenomenon and the technical difficulties in making multiple recordings with small preparations, this experiment was not attempted. Fig. 3*d* shows a similar hump, but in the depolarizing direction which appears to prolong the plateau. This feature was maintained for the first beat upon restoration of Ca_0^{2+} when a double contraction was evoked, reinforcing the idea that two populations of cells were

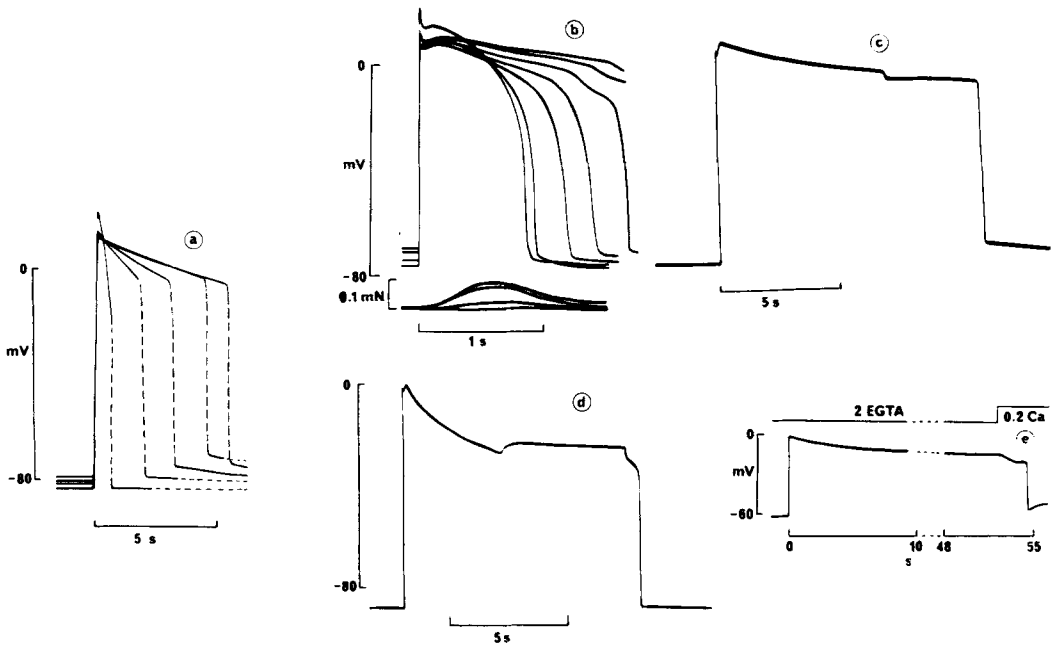

FIGURE 3. Action potentials in 2 mM EGTA (see Table 1). (*a*) Recorded immediately before, and 1, 2, 4, and 5 min after the solution change (4 min^{-1}). (*b*) *Upper trace,* action potential before and 15, 30, 45, 60, and 75 s after changing to 2 mM EGTA. *Lower trace,* twitch tension associated with the first four action potentials. (*c*) The same cell as in (*b*) (mV calibration as in *b*, note changed time base) after 5 min in EGTA. The inflection which developed at the end of the plateau in (*b*) is here a clear "jump" in the plateau (stimulus frequency reduced to 2 min^{-1}). (*d*) Depolarizing jump in the plateau which appears to delay the repolarization. AP recorded after 5 min in EGTA. (*e*) AP of indefinite duration which was terminated after 52 s by removing EGTA and adding calcium.

(temporarily) established by the EGTA solution, in one of which activity was delayed or spontaneous.

Examples of the development of AP duration with differing time courses in 2 mM EGTA are given in Figs. 4*b* and 6*a* which illustrate two different runs with one preparation. As noted earlier, this difference from run to run was not seen if the impalement of one cell was maintained throughout, again implying that action potential durations may differ from one individual cell to another in EGTA solutions.

The half-time for the increase of action potential duration therefore varied considerably. In addition, in the higher EGTA concentrations AP duration could suddenly rise to values in excess of 1 min. In these cases the normal slow development of AP duration continued until a value of about 10 s had been reached; thereafter an action potential lasting 1 min or more was recorded, followed by successive action potentials of similar length. Thus no average value could be given for the development of AP duration. In a few preparations, the AP lasted for more than 3 min and could be brought to repolarize as desired by the addition of calcium (Fig. 3*e*) and by Mg^{2+} or acetylcholine (see later sections).

These findings demonstrate the necessity for a continuous observation of the potential from a single cell since attempts to impale a new cell during such a plateau yield no reliable indication of a successful penetration and might give the false impression that the cells had permanently depolarized to near zero potential.

The occurrence of spontaneous though regular action potentials was often noted in EGTA solutions. Spontaneous activity rendered the observations invalid for quantitative treatment, but qualitative observations could be made.

Action of Other Divalent Cations

When a more or less stable action potential had been achieved in EGTA Ringer solution a number of agents were applied in an attempt to influence the AP duration. It was of interest to see whether another divalent cation could compensate for the lack of calcium ions.

Magnesium

Magnesium was tested first as it is known to have a stabilizing effect similar to that of calcium in both nerve (e.g., Frankenhaeuser and Hodgkin, 1957) and muscle (Dörrscheidt-Käfer, 1976). Magnesium also has the advantage that it binds rather weakly to EGTA so that complications of the final free magnesium and calcium concentrations are largely avoided (see Table I).

Fig. 4*a* shows that progressive increases in the concentration of magnesium in the presence of 0.2 mM EGTA reduced the AP duration stepwise to a level very near that subsequently maintained in normal Ringer solution. The rate of change of AP duration was approximately exponential in each case with half-times close to the half-time for the action of calcium. In the preparations tested in this way, 5 mM Mg^{2+} was necessary to make the duration return to normal. However, switching directly from 0.2 mM Ca^{2+} to 0.2 mM EGTA + 1 mM Mg^{2+} produced no significant change in AP duration, so that magnesium action is to some extent dependent upon the immediate history of the preparation. This behavior may reflect some changes resulting from prolonged exposure to EGTA. That Mg can compensate for Ca at a ratio of 5:1 was also found by Hoffman and Suckling (1956) for dog heart.

Strontium

Strontium ions are known to be able to replace Ca in a number of physiological processes, such as the slow inward current in the heart (Vereecke and Carmeliet,

1971). Isolated contractile proteins can be fully activated by Sr^{2+} ions (Moisescu and Thieleczek, 1975).

EGTA binds strontium somewhat less strongly than calcium so that, by suitable choice of the respective concentrations, a low $[Ca^{2+}]_0$ can be maintained

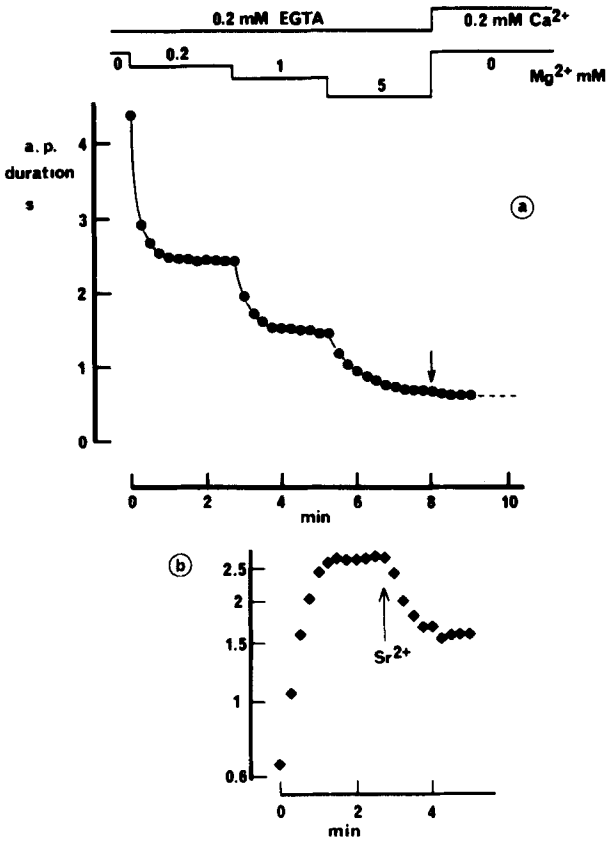

FIGURE 4. (*a*) AP duration was reduced from an initial steady level (achieved after 10 min in 0.2 mM EGTA) by successive increases in $[Mg]_0$ to 0.2, 1, and 5 mM (total) in the presence of EGTA. Associated changes in $[Ca^{2+}]_0$ are negligible ($[Ca^{2+}]_0$ = 2.2–2.7 × 10^{-8} M; see Table I). After 8 min, EGTA and Mg were removed simultaneously and Ca was raised to 0.2 mM (arrow), producing only a slight further reduction in AP duration. (*b*) Development of AP duration in 2 mM EGTA. After 2 min 45 s strontium was added (arrow, 0.4 mM $SrCl_2$; 1.6 × 10^{-5} M Sr^{2+}; see Table I) reducing AP duration with a time course similar to that of Mg^{2+} (4 min^{-1}).

in the presence of Sr^{2+}. In 2 mM EGTA, strontium (0.4 mM $SrCl_2$ = 0.016 mM Sr^{2+}, see Table I) restores the action potential duration almost to normal (Fig. 4*b*) and produces a recovery of twitch tension. Higher levels of Sr^{2+} prolong the normal AP (e.g., Vereecke and Carmeliet, 1971) probably as a result of

altered inactivation kinetics for the slow channel (Kohlhardt et al., 1973), therefore a complete restoration of the AP duration to normal is not to be expected.

Manganese

Manganese ions are well known as inhibitors of calcium current in several tissues (Narahashi, 1974) but have also been reported to carry a current in the calcium channel to mammalian heart (Ochi, 1975). Unfortunately, manganese binds more strongly to EGTA than Ca so that in general addition of Mn^{2+} to EGTA solutions results in an increase in $[Ca^{2+}]_0$ as Mn^{2+} is preferentially bound. Thus Mn^{2+} can readily be tested only with $[Ca^{2+}]_0$ above $\sim 10^{-5}$ M if Mn^{2+} concentrations above micromolar levels are of interest. 1 mM $MnCl_2$ had no detectable effect on the action potential in normal Ringer solution although contraction was blocked. However, action potentials in Ca-free Ringer solution (duration 2.1 s) were rapidly restored to normal duration (0.8 s) by 0.4 mM Mn, whose action thus resembles that of Ca, Mg and Sr.

The related divalent cations nickel and cobalt were not tested since they also bind strongly to EGTA.

Barium

Barium was tested on one preparation. Concentrations of 0.4 and 0.04 mM produced a depolarization of 10–15 mV and a prolongation of the actin potential in Ca-free solution by several seconds. The lower barium concentration, however, produced a shortening of the first AP (Fig. 5). The shortening

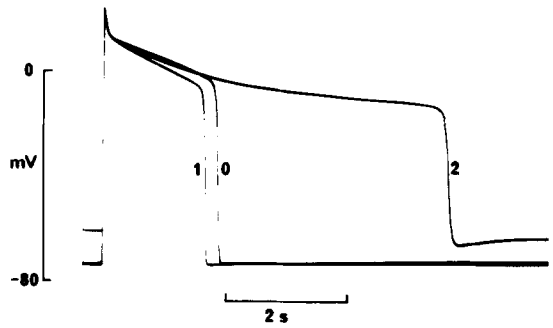

FIGURE 5. Action potentials recorded in a Ca-free Ringer solution immediately before (0), 30 s (1), and 135 s (2) after addition of 0.04 mM $BaCl_2$ (4 min^{-1}).

action may reflect an action of Ba^{2+} like that of the other divalent cations tested. The prolongation is presumed to be related to the well-known action of Ba^{2+} to reduce the potassium conductance (cf. Reuter, 1973), an action which evidently finally masks any similarity between Ba^{2+} and the other divalent cations.

D600

D600 is widely reported to block the slow inward current in heart muscle in a more or less specific manner (Kohlhardt et al., 1972; Einwächter et al., 1972).

The effects of D600 (1, 5, and 10 mg/liter = 2, 10, and 20 μM) were tested on the contraction and action potential. These levels of D600 reduce the twitch to under 5% of normal in 2 mM Ca or 0.2 mM Ca Ringer solution within 5 min. The action potential is shortened from the normal value (0.8 ± 0.19 s) to ~0.3 s (range 0.25–0.55 s, six cells in 0.2 mM Ca). This effect is reversed only after ~2 h of washout time although the contraction appears again within a few minutes of removing D600. This poor reversibility meant that D600 could reliably be tested only once on each preparation and in three muscles the drug was first added to EGTA solutions when a steady level of AP duration had already been reached. Fig. 6*a* shows the time course of the response to restoration of calcium ions (0.2 mM) after exposure to 2 mM EGTA with and without D600 (10 mg/liter). D600 had no significant effect on the prolonged action potentials in EGTA or on the time course of the recovery in Ca-containing solutions.

Stimulus Frequency

Experiments were made where the stimulus frequency was chosen in the range 1–30 min^{-1} for successive observations of the effect of calcium restoration. No significant difference was detected in either the time required for duration to reach normal, or in the time course of this recovery over the whole range of frequencies. Thus in the example shown in Fig. 6*b*, 1 minute after the solution change, the 1st (at 1 min^{-1}) or 20th (at 20 min^{-1}) action potential had the same duration. The number or frequency of the action potentials and thus the associated Ca influx are not factors which influence the time course of the membrane's response to $[Ca^{2+}]_0$ increase over this concentration range. This is consistent with the effects of D600 described above.

Acetylcholine

Acetylcholine hyperpolarizes the resting membrane and shortens the action potential in many cardiac tissues. These effects are thought to be primarily due to an increased potassium permeability (e.g., Ten Eick et al., 1976) although some effects on the slow inward current have been reported for frog atrial tissue (Giles and Noble, 1976). Acetylcholine was tested in EGTA and Ca-free Ringer solution at 10^{-6}, 5.10^{-7}, and 10^{-7} M and produced in each case a rapid reduction of the duration of AP. The time course is illustrated in Fig. 7*a*. A minimum is reached within 1 min of adding acetylcholine and is followed by a slow increase in AP duration to a slightly greater level. This biphasic time course has also been reported for guinea pig atria (Ueno, 1973). The form of the action potentials under acetylcholine is shown in Fig. 7*b* where it can be seen that its addition brings about the repolarization phase within a few seconds. Successive action potentials are progressively shortened. The dose effectiveness, although not extensively studied, was similar in both Ca-free solutions and EGTA Ringer solution, implying that extracellular calcium ions are not essential to the action of acetylcholine. These effects could be blocked by a brief (3 min) exposure to atropine (10^{-7} M) before application of the acetylcholine solution (also containing atropine). In combining observations made with acetylcholine with the results obtained with D600, one may safely conclude that the shortening of AP duration by acetylcholine reflects an increase in potassium permeability which can be achieved even in the virtual absence of

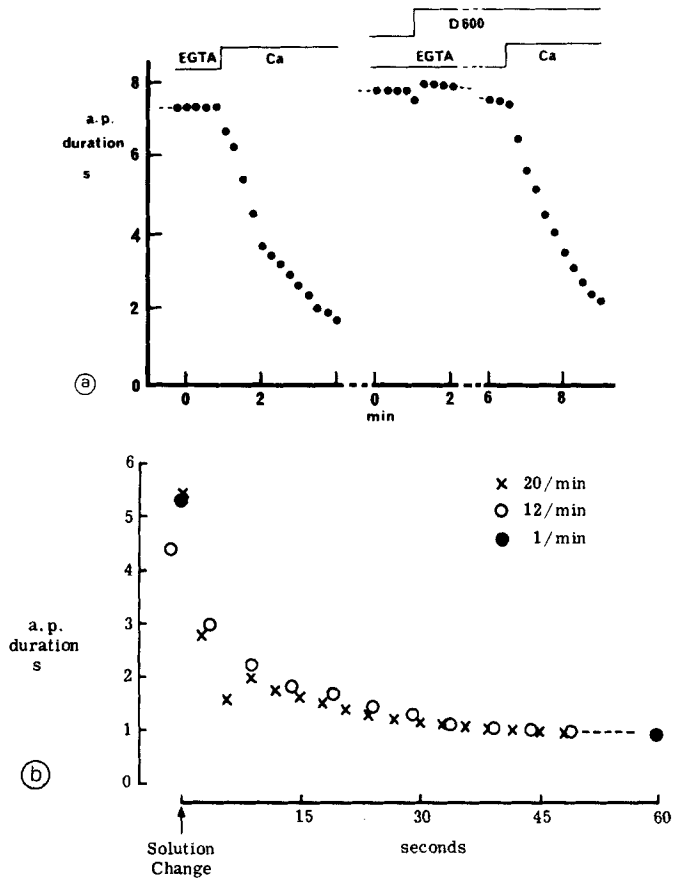

FIGURE 6. (*a*) Effect of D600 on AP duration. AP duration (ordinate) is plotted against time (abscissa). On the left a steady level has been achieved after 10 min in EGTA (2 mM). Changing to 0.2 mM Ca Ringer solution produced the normal rapid fall towards normal values (see Fig. 2). Later, in a second cell from the same preparation, the sequence was repeated (right) but first D600 (10 mg/liter) was added in the presence of EGTA (2 mM). Even after 5 min in D600, only minimal changes in AP duration had occurred. Restoring calcium (0.2 mM) produced the typical rapid fall in AP duration in the continuous presence of D600. (*b*) Effect of stimulus frequency on recovery of AP duration. A preparation was exposed to 0.2 mM EGTA until a steady AP duration was established. Stimulus frequency was then set to the desired rate for a few minutes and 0.2 Ca Ringer restored. The fall of AP duration after the solution change is plotted. All three records were obtained in the same cell after successive exposures to EGTA. (Note that at high frequencies stimuli often occurred during an AP plateau in the EGTA solution or immediately after its removal when AP duration exceeded 1/stimulus frequency.)

calcium or other extracellular divalent cations. The protracted phase of increase in AP duration after the addition of EGTA fails to occur when acetylcholine is applied. The results with acetylcholine serve to demonstrate that an increased potassium permeability will produce shortening of APD in EGTA-treated cells.

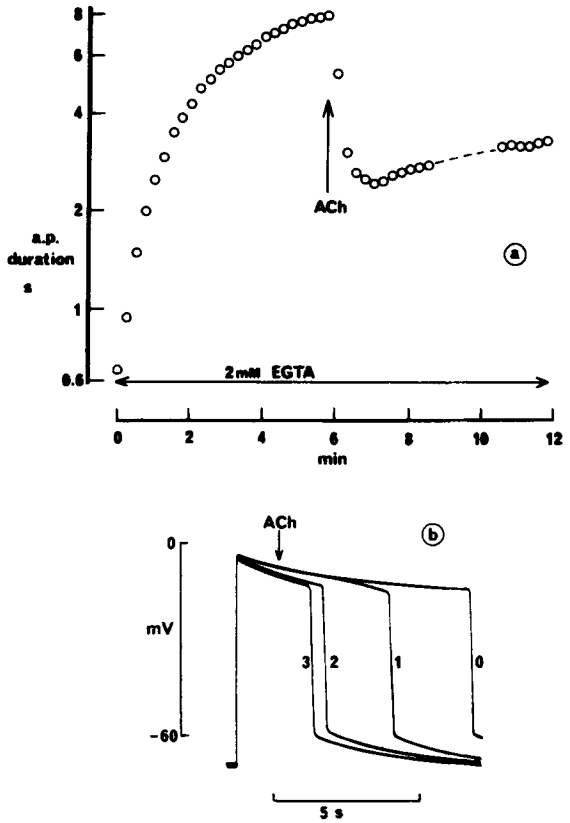

FIGURE 7. (*a*) Development of AP duration monotonically in 2 mM EGTA. After 6 min acetylcholine (5.10^{-7} M) was added (arrow) (same preparation as Fig. 4*b*). (*b*) For the same solution change, the action potentials immediately before acetylcholine addition, (0) and the first, second, and third in the presence of acetylcholine. The drug was added during the action potential labeled no. 1, along the plateau as indicated by the arrow. Repolarization was thereby accelerated, following within 3 s (4 min^{-1}).

DISCUSSION

It has previously been reported for mammalian heart (Hoffman and Suckling, 1956; Surawicz et al., 1961) and frog heart (Juncker et al., 1972; Chesnais et al., 1975) that reduced levels of calcium in the bathing medium promote prolonged action potentials. However, although the effect of extreme $[Ca^{2+}]_0$ reduction by use of Ca chelators has been reported (Chang and Schmidt, 1960; Tritthart et al., 1973), it has not been extensively investigated. The foregoing results indicate that AP prolongation is related to the $[Ca^{2+}]_0$: in 0.2 mM EGTA ($\sim 10^{-8}$ M Ca^{2+}) durations reach 2–10 s; in 2 and 5 mM EGTA ($< \sim 10^{-9}$ M Ca^{2+}, see Table I) this value often exceeds 1 min. The onset of repolarization is, however, accelerated by several divalent cations (and acetylcholine) even with $[Ca^{2+}]_0$

below 10^{-8} M. Blocking the slow inward current with D600 has no significant effect on restoration of action potential duration when calcium is readmitted after exposure to EGTA. These effects of calcium reduction develop only as long as no other divalent cations are present in significant amounts. (Fig. 4).

Divalent Cation Chelators on Frog Heart

Before an interpretation of the results in terms of the underlying ionic movements is made, some points arising from the use of EGTA need to be discussed.

It is worth noting that despite exposure to $[Ca^{2+}]_0$ of less than 10^{-9} M for periods often exceeding 0.5 h, the cell membrane does not appear to become excessively leaky: even the longest-lasting action potentials were terminated by a phase of rapid repolarization to near-normal resting potentials, although some cells exhibited marked afterpotentials (see Figs. 2, 3*a*, and 7). It seems that, at least for frog heart, experiments employing EGTA (e.g., Miller and Moisescu, 1976) can be made without risking severe disruption of the membrane. The problem of skinning of cardiac cells by EGTA/EDTA treatment is dealt with elsewhere.[1] The various binding constants of EGTA are all highly pH sensitive in the physiological range (Portzehl et al., 1964). Tris has been used as the pH buffer in the present experiments. Although it is a relatively poor buffer at the experimental pH (7.00), the effective buffer capacity is very large as a result of the rapid superfusion of the preparation (~10–20 ml/min). The problems of the adequacy of the Ca-buffer capacity of EGTA solutions were discussed in connection with Fig. 2. It should be noted that toxic effects of EGTA are difficult to distinguish from those of $[Ca^{2+}]$ reduction associated with higher [EGTA]. However, at higher free [Ca], variation of the total buffer concentration does not have marked effects (Miller and Moisescu, 1976).

Extracellular Exchange

The interpretation of the results hinges to some extent upon a knowledge of the rate of extracellular exchange after a change in the perfusing solution for this preparation of the frog ventricle. The half-time for extracellular calcium exchange under ideal conditions has been estimated to be about 3 s (Page and Niedergerke, 1972; Chapman and Miller, 1974). The fall of twitch tension in Ca-free solutions (Fig. 2) gives a rough estimate of this parameter for individual preparations (Miller, 1975). From the present experiments it seems that the extracellular calcium level, after a change to EGTA solutions, will be within a few percent of its final level within 20–60 s. (Although the EGTA buffer will diffuse somewhat more slowly than Ca^{2+} ions, the buffering action will accelerate the rate of fall of $[Ca^{2+}]_0$.) This contrasts with the development to the steady level of AP duration which extends over many minutes (Figs. 2, 4). A similar development occurs in Ca-free Ringer solution so that the cause cannot lie in either the much lower $[Ca^{2+}]_0$ of the EGTA solutions, or in the presence of EGTA itself. It seems possible that intracellular calcium is leached out of the cells in Ca-poor media and thus the amount of calcium bound in the membrane

[1] Miller, D. J. Submitted for publication.

may fall slowly even though the external change is rapid. Such a process has been suggested by Niedergerke and Orkand (1966) who also observed that in nominally calcium-free solutions, overshoot and resting potential fell only very slowly. In contrast, restoration of Ca^{2+} (Figs. 1, 2) or the introduction of other divalent cations (Fig. 4) restores AP duration with a rapid, exponential time course very close to that for the extracellular exchange in the individual preparations (see Fig. 2).

(The fall in twitch tension represents an "average" value—all cells contribute to the tension output. This is not so for the action potential duration of an individual cell unless strong electrotonic coupling ensures near-synchronous repolarization of the whole preparation. While this is likely it is by no means definite, especially in Ca-poor media [see text in connection with Fig. 3].)

In this respect the rate of action of successive $[Mg^{2+}]_0$ increases (Fig. 4*a*) or that of Sr^{2+} (Fig. 4*b*) is important since it shows that levels of the cations which are suboptimal in their effect on AP duration act as rapidly as 0.2 mM calcium; i.e., the rate of action of calcium is not accelerated by a saturation effect.

The rate of restoration of normal AP duration, like that for the fall of twitch tension in Ca-free media (Miller, 1975), is independent of stimulus frequency over a wide range (Fig. 6*b*). The similarity of this time course to that for tension fall indicates that both are strongly, even if only indirectly, dependent upon extracellular calcium—the former directly from $[Ca^{2+}]_0$, the latter from the requirement for a Ca-influx for contraction (see above) and hence a dependence on $[Ca^{2+}]_0$.

Origin of the Plateau Prolongation: External or Internal Site of Action for Divalent Cations?

The extreme duration of the action potential in media poor in divalent cations could be interpreted in several ways. Three of them are briefly considered.

The first possibility is that a Ca-influx and/or intracellular release with a subsequent binding to the inner side of the sarcolemma leads to an increase in the potassium conductance, G_K. This would be in keeping with the observations of Meech (1974) and Meech and Standen (1975) on snail neurons and of Isenberg (1975) on cardiac Purkinje fibers, that intracellularly applied Ca^{2+} (or a Ca-EGTA buffer) can increase G_K. The failure of the calcium influx in EGTA solutions would therefore limit or delay the increase in G_K necessary for repolarization.

The results demonstrate that calcium can be mimicked by several divalent cations in reducing AP duration; thus Ca and Mg acted alike (compare Figs. 2 and 4*a*). This is not in keeping with an intracellular control of G_K since it is unlikely that Mg ions can carry a current through the cell membrane (Reuter, 1973; but see Kohlhardt et al., 1973) or penetrate it to any significant extent. Any short-term entry of Mg^{2+} (and the ion acts within seconds, Fig. 4) is likely to be negligible in terms of an intracellular $[Mg^{2+}]$ change. ($[Mg^{2+}]_i \sim 1$ mM; Polimeni and Page, 1973). In any case, magnesium had the opposite effect to calcium on G_K in snail neurons (Meech, 1974). The effect of Mg^{2+} is therefore concluded to be on the outer side of the membrane.

The second interpretation is that AP duration is prolonged as a result of

slowed inactivation kinetics for the slow inward current in Ca-poor media. In the present experiments this current could not be carried by Ca^{2+} in EGTA solutions, because the driving force for calcium ions is then very small or even outwardly directed during the plateau of the action potential. Rougier et al. (1969) and Chesnais et al. (1975) have suggested, on the basis of voltage-clamp experiments with frog atrial trabeculae, that sodium ions carry the slow inward current in Ca-free solutions but that the inactivation is slowed.

The divalent cations which mimic calcium in its effect on AP duration (apart from magnesium, see above) are strontium and manganese. Both these ions can probably cross the membrane to some extent via the slow or calcium channel (Vereecke and Carmeliet, 1971; Ochi, 1975; Reuter, 1973). This was almost certainly the case for Sr^{2+} in the present experiments since contraction was restored (together with AP duration) by the cation. Manganese did not have this effect even though Mn^{2+} will activate the contractile proteins (D. G. Moisescu, personal communication).

Both the first and second interpretations for the observed changes in AP duration require that the slow inward current play a significant role in the action potentials in EGTA-containing solutions. The possibility of such a role can be excluded on the basis of the findings with D600. This substance is reported to block the slow channel fairly specifically in both mammalian (Kohlhardt et al., 1972) and frog heart (Einwächter et al., 1972), but had no significant effect on the prolonged action potentials in EGTA media or on the changes in AP duration consequent upon $[Ca^{2+}]_o$ increase (Fig. 6). The concentrations used were sufficient to block contraction in normal Ringer solution and up to 10 times higher than those used in voltage-clamp experiments to block I_{Ca}. It may be concluded that the slow inward current (in EGTA media) or calcium influx as part of the current (upon restoring $[Ca^{2+}]_o$) are not necessary for the modulation of AP duration that was observed. This is supported by the lack of a marked effect of stimulus frequency upon the time course of AP duration recovery after EGTA (Fig. 6*b*).

The third possibility to be considered is that the $[Ca^{2+}]_o$ has a direct effect upon potassium currents. This suggestion has also been made by Kaas and Tsien (1975, 1976) on the basis of voltage-clamp experiments on cardiac Purkinje fibers. These investigators report that the background potassium current, i_{K1}, is reduced when $[Ca^{2+}]_o$ is reduced. Also significant is their finding that Ca-dependent changes in i_{K1} were similar with or without activation of the slow inward current. This finding is in harmony with the present results with D600 and stimulus frequency which are also consistent with the idea that the potassium current can be influenced without involving the slow inward current. This third explanation of the results seems most satisfactory since a number of conclusions drawn earlier point to an extracellular influence of the divalent cations on the timing of AP termination.

The rapid and large effects of acetylcholine in the EGTA media serve primarily to demonstrate that increased G_K can account for altered AP duration under these conditions. The results also reveal that acetylcholine effectiveness is not markedly Ca dependent.

A most interesting feature of these long-lasting action potentials is that repolarization is so steep even after a plateau phase of many seconds' duration (e.g., Fig. 3). Afterpotentials were occasionally seen in the EGTA media (although they are more frequently seen during the recovery period after removal of EGTA, e.g., Fig. 2*a*) and are thus not strongly correlated with a long plateau. This excluded the likelihood of a significant potassium accumulation near the sarcolemma during these long action potentials of the kind described by Cleeman and Morad (1976), but this might be expected, as the outward current that they measured is low at the potential level found here for the prolonged plateau in EGTA (ca. −10 mV). Similarly, an electrogenic Ca-K exchange of the type suggested by these authors is unlikely to play a role in determining AP duration in EGTA solutions. As the foregoing sections show, APD prolongation upon exposure to EGTA is not correlated to the time course of extracellular exchange, i.e. plateau prolongation does not correlate with $[Ca]_o$ drop.

Changes in the fast sodium conductance concomitant on extreme $[Ca^{2+}]_o$ reduction have not been considered here. It is possible that extreme delay in Na inactivation could account for the findings, although a nonspecific response to extracellular divalent cations of the type found here has not been put forward in the literature. This possibility cannot, however, be excluded.

In summary, the results are consistent with the idea that membrane K conductance can be influenced by sites on the outer face of the sarcolemma which display a relatively nonspecific affinity for divalent cations. Since only relatively small changes in AP duration are observed when $[Ca^{2+}]_o$ increases from 0.2 to 2.0 mM or more (e.g., Niedergerke and Orkand, 1966), these sites are probably nearly saturated at physiological $[Ca^{2+}]_o$, although this point can be definitely resolved only by measuring the Ca_o-activated G_K changes over a wide range of $[Ca^{2+}]_o$. This system of control for G_K complements the better-documented intracellular regulation of potassium permeability which, in contrast, seems to be Ca specific.

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