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EFFECT OF POTASSIUM DEPOLARIZATION ON SODIUM-DEPENDENT CALCIUM EFFLUX FROM GOLDFISH HEART VENTRICLES AND GUINEA-PIG ATRIA

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SUMMARY

1. ^{45}Ca fluxes were studied in normal and potassium-depolarized goldfish ventricles as a function of the external Na concentration. Some of the experiments were also performed on guinea-pig auricles.

2. When the external K concentration was increased from 5·4 to 142 mM, keeping osmolarity constant by adding 137 mM-Li or choline (hyperosmotically) to the low K solution, the ^{45}Ca efflux was reversibly inhibited, whereas the [^{3}H]sucrose efflux was unaffected.

3. Goldfish ventricles, which have been depolarized with 142 mM-K for 100 min, repolarized within 20 min, from *ca.* −15 mV to *ca.* −70 mV, following the application of 5·4 mM-K. This repolarization was independent of the presence of external Na. During the repolarization the ^{45}Ca efflux was reactivated. This reactivation, however, depended on the external Na concentration. Comparable results were obtained in guinea-pig atria.

4. A similar repolarization and Na-dependent reactivation of ^{45}Ca efflux was obtained in goldfish ventricles superfused with 10^{-6} M-Ca^{2+} (4·5 mM-Ca, 5 mM-EGTA, pH 7·1), provided that the ^{45}Ca washout was started in high K.

5. In 10^{-6} M-Ca^{2+}, 137 mM-Na, 5·4 mM-K and 137 mM-choline goldfish ventricles depolarized to about −25 mV within 80 min. If the choline was now replaced by 137 mM-K, the membrane potential moved to *ca.* −15 mV, and under these conditions the ^{45}Ca efflux was slightly *increased*.

6. Following Na-free perfusion for 100 min, and at normal external Ca concentrations, the ^{45}Ca efflux from goldfish ventricles was stimulated by the addition of Na. The curve relating this stimulation to the external Na concentration had a sigmoidal shape and was shifted to the right by K-depolarization. In guinea-pig atria the inhibition of the Na-stimulated Ca efflux by depolarization was of a non-competitive type.

7. Following a Na-free incubation of 100 min and a subsequent period of 20 min in 137 mM-Na, the intracellular Na content of goldfish ventricular cells was some 20% lower in K-depolarized cells than in cells at the resting potential.

8. ^{45}Ca influx in goldfish ventricles in the presence of 137 or 68·5 mM-Na was not significantly changed by K-depolarization.

9. The results show that the Na-dependent fraction of Ca efflux is inhibited by high external K. The effect is probably due to depolarization, which may be an argument in favour of electrogenic n $Na^+ - 1$ Ca^{2+} exchange, with $n \geqslant 3$.

INTRODUCTION

In cardiac muscle a coupling of opposite movements of Ca and Na across the sarcolemma may be the basic mechanism for the maintenance of a low intracellular Ca ion concentration (Reuter & Seitz, 1968). In this hypothesis net outward transport of Ca is energetically driven by net influx of Na down its electrochemical gradient. It can be calculated that, if Ca–Na exchange is the only system which reduces sarcoplasmic Ca, an exchange ratio of three or more Na ions for one Ca ion is required to reduce the sarcoplasmic Ca to below 10^{-7} M, the estimated concentration at rest (Fabiato & Fabiato, 1975).

Such a stoichiometry, however, would mean that the Na–Ca transport will depend on the membrane potential, as in each exchange cycle a net transport of electric charge occurs. Recent results from measurements of contracture tension (Chapman & Tunstall, 1981) and membrane potential (Coraboeuf, Gautier & Guiraudou, 1981) are in keeping with this view. The most convincing evidence of an electrogenic Na–Ca exchange, however, comes from studies of Ca movements in isolated preparations of heart sarcolemma where a three Na^+–one Ca^{2+} exchange stoichiometry and effects of membrane potential on Ca fluxes were observed (Pitts, 1979; Bers, Philipson & Nishimoto, 1980; Lamers & Stinis, 1981). It is therefore surprising that Ca flux studies in intact heart muscle have so far failed to demonstrate unequivocal effects of membrane potential (compare Niedergerke, 1963; Wollert, 1966; Jundt, Porzig, Reuter & Stucki, 1975). However an effect of membrane potential on Ca efflux in frog heart has been reported recently (Chapman, Tunstall & Yates, 1981). In the present paper I have re-investigated the effects of K-depolarization on Ca efflux and especially on the fraction of Ca efflux which depends on extracellular Na.

A preliminary account of part of this work has been published elsewhere (Busselen, 1981).

METHODS

Preparations. For the efflux experiments performed on goldfish (*Carassius auratus*) the ventricles were dissected and cut in half as described previously (Busselen & Van Kerkhove, 1978). In mammalian experiments left or right auricles from adult guinea-pigs were opened and divided into two halves taking care to minimize damage to the trabeculae. For measurements of contractile force and membrane potential, strips with a diameter of about 0·5 mm and a length of about 5 mm were excised from the heart of either species, following as closely as possible the orientation of the muscle fibres.

The composition of the various solutions used is given in Table 1. When in the text the name of a solution is followed by a number in parentheses, this number refers to the solution in Table 1. The experiments were carried out at 20 °C for goldfish ventricles and at 37 °C for guinea-pig atria. The solutions were saturated with 100 % O_2.

Determination of tissue Na and [^3H]sucrose space. Preparations were loaded with [^{3}H]sucrose in a solution containing 1 mM-carrier sucrose. [^{3}H]sucrose was chosen as a marker of the extracellular space because its uptake reached a plateau, amounting to about 60 % of the Na space, within 1 hr. After the loading period the preparations were gently blotted between filter paper (Whatman no. 42),

weighed (= wet wt.) and extracted in 2 ml. of 0·15 N-HNO_3 overnight. The Na and [^{3}H]sucrose concentrations of these solutions were then measured using atomic absorption spectrophotometry (Varian AAS) or liquid scintillation counting (Packard Tricarb-2425). These procedures yielded values which were not different from those determined, in separate experiments, after ashing with H_2O_2 for Na assay and after digestion with Soluene (Packard) for [^{3}H]sucrose determination.

^{45}Ca and [^{3}H]sucrose efflux. For effluxes which started in Na-containing or Na-free solutions, the preparations were loaded with ^{45}Ca for 60 min in normal Na-saline or in Na-free saline respectively (Li or choline was used to replace Na). The preparations were then washed out into the first three

TABLE 1. Composition of experimental solutions

	NaCl (mM)	LiCl (mM)	Choline Cl (mM)	KCl (mM)	$MgCl_2$ (mM)	$CaCl_2$ (mM)	EGTA (mM)	Glucose (mM)	Tris (mM)	pH
(1)	137	—	—	5·4	0·5	1·8	—	5·5	10	7·3
(2)	137	137	—	5·4	0·5	1·8	—	5·5	10	7·3
(3)	137	—	137	5·4	0·5	1·8	—	5·5	10	7·3
(4)	137	—	—	142	0·5	1·8	—	5·5	10	7·3
(5)	—	274	—	5·4	0·5	1·8	—	5·5	10	7·3
(6)	—	—	274	5·4	0·5	1·8	—	5·5	10	7·3
(7)	—	137	—	142	0·5	1·8	—	5·5	10	7·3
(8)	—	—	137	142	0·5	1·8	—	5·5	10	7·3
(9)	137	137	—	5·4	0·5	4·5	5	5·5	10	7·1
(10)	137	—	137	5·4	0·5	4·5	5	5·5	10	7·1
(11)	137	—	—	142	0·5	4·5	5	5·5	10	7·1
(12)	—	274	—	5·4	0·5	4·5	5	5·5	10	7·1
(13)	—	—	274	5·4	0·5	4·5	5	5·5	10	7·1
(14)	—	137	—	142	0·5	4·5	5	5·5	10	7·1
(15)	—	—	137	142	0·5	4·5	5	5·5	10	7·1

Reference names: (1) normal Na-saline; (2)–(15) hypertonic saline; (4) (7) (8) and (11) (14) (15) high K solutions; (9)–(15) low Ca^{2+} solutions (Ca^{2+} was *ca.* 10^{-6} M, assuming an apparent binding constant for Ca to EGTA of $7·6 \times 10^6$ M^{-1} at pH 7·1, according to Portzehl, Caldwell & Rüegg, 1964); (5)–(8) and (12)–(15) Na-free solution. Intermediate concentrations of Na were prepared by mixing corresponding Na-containing and Na-free solutions.

efflux tubes for 20 min; thereafter the tubes were changed at 10 min intervals. ^{45}Ca was measured by scintillation counting. At the end of the experiment the tissues were blotted, weighed and extracted as described above. Extraction with 0·15 N-HNO_3 gave similar results to those obtained after digestion with H_2O_2. In some experiments preparations were simultaneously loaded with ^{45}Ca and [^{3}H]sucrose. The settings for both channels of the liquid scintillation counter were adjusted to obtain optimal separation of the two tracers. The effluxes of both isotopes were calculated making the appropriate corrections.

When effluxes were performed at low external Ca concentration, the Ca^{2+} concentration was buffered at 10^{-6} M using 5 mM-EGTA and 4·5 mM-Ca, pH 7·1 (at this pH, the Ca EGTA binding constant was $7·6 \times 10^6$ M^{-1} according to Portzehl, Caldwell & Rüegg, 1964). The presence of EGTA and 4·5 mM-Ca would reduce an artifactual decrease in the rate of ^{45}Ca efflux due to extracellular Ca binding or backflux (Busselen & Van Kerckhove, 1978). The viability of the preparation was confirmed from separate experiments recording normal membrane potential and contractions in tissues which had been superfused with 10^{-6} M-Ca^{2+} and afterwards transferred again to normal Ca-contaning Na-saline. (Stronger reduction of Ca^{2+} by applying 0 Ca, 5 mM-EGTA, caused irreversible depolarization and loss of contractility, after returning to normal Na-saline).

^{45}Ca influx. Following the immersion of a tissue for a short period of time in saline containing ^{45}Ca, only a small proportion of the isotope is taken up into the cells whereas a larger proportion remains in the extracellular space and is bound to the cell surfaces. In order to measure the true Ca influx, the preparations, after an initial ^{45}Ca loading period of 10 min, were washed for 20 min in a Na- and Ca-free solution containing 10 mM-$LaCl_3$ and 137 mM-Li. It is expected that La^{3+} ions

would displace Ca^{2+} from the extracellular binding sites (van Breemen & McNaughton, 1970) and that, in the absence of external Na and Ca, cellular Ca efflux (Na–Ca and Ca–Ca exchange) would be retarded. The ^{45}Ca remaining in the tissue after this treatment gives a measure of intracellular Ca. The tissues were desiccated in an oven at 90 °C and weighed (dry weight), and following extraction with 0·15 N-HNO_3 the ^{45}Ca was measured by scintillation counting.

Contractile force and membrane potential. Muscle strips were mounted horizontally in an open superfusion bath with one end fixed to a Sanborn FTA-10-1 isometric transducer. Membrane potentials of the cells were recorded with conventional intracellular micro-electrodes, filled with 3 mM-KCl. For each preparation, the membrane potential was determined by at least five impalements (usually between ten and twenty impalements). Values reported in the text refer to mean membrane potential ±S.E. of the mean for n preparations.

Statistics. Values in the text are given as means ±S.E. of mean. Statistical analysis of the difference between the experimental and control halves of the same hearts was performed using a Student's t test for paired data. $P < 0.05$ was considered significant.

RESULTS

Effects of K-depolarization on ^{45}Ca efflux as a function of the external Na concentration

The Na-activated Ca efflux across membranes depolarized by K^+ is difficult to evaluate because of possible changes in specific activity of ^{45}Ca at the inside of the cells. The apparent inhibition of Ca efflux when the external K^+ concentration is raised or the external Na^+ concentration is reduced, is not necessarily an indication of impaired outward Ca transport, as a coincident increase of cold Ca influx (see Niedergerke, 1963) might dilute the intracellular tracer concentration, so that the measured ^{45}Ca efflux underestimates the real efflux (but not the rate coefficient). Further if the outward transport is mediated by a carrier, the rate coefficient of the ^{45}Ca efflux may be depressed by an increasing competition between ^{45}Ca and inactive Ca for the intracellular binding sites (provided that the K_m value for binding is not much larger than the actual intracellular ^{40}Ca concentration). These problems may be overcome by studying the reactivation of Ca efflux upon the *reduction* of K or *increase* of Na (for if the apparent inhibition of Ca efflux were due to a decrease in specific activity in high K^+ or low Na, returning to normal K or Na should not reactivate Ca efflux as a spontaneous increase in specific activity is unlikely). Alternatively the experiments may be carried out at a very low external Ca^{2+} concentration (which would prevent changes in intracellular ^{40}Ca concentration caused by a varying influx); or by measuring Ca influx in normal and depolarized preparations (which would give information about the magnitude of the possible tracer dilution).

In goldfish ventricles the resting potential in normal Na-saline (1) is $-68{\cdot}6 \pm 0{\cdot}1$ mV ($n = 4$). When the external K is raised to 142 mM (4) it decreases reversibly to $-16{\cdot}1 \pm 2{\cdot}4$ mV ($n = 4$). After re-application of low K, peak tension for isometric contractions, elicited by field stimulation (frequency 1/min) is restored within 3 min, even when the superfusion with high K lasted for 40 min. Fig. 1 shows the effect of increasing K from 5·4 mM (2) to 142 mM (4) on the rate coefficient of ^{45}Ca efflux into a solution containing 137 mM-Na and 1·8 mM-Ca. It shows a relatively small decrease in rate coefficient which is rapidly reversed upon returning to the normal low K concentration. As the Ca efflux is composed of a Na-dependent, a Ca-dependent and residual Ca efflux (Reuter & Seitz, 1968), it is not possible to say which fraction of Ca efflux is inhibited by high K. In an attempt to answer this question similar experiments were carried out in different external Na concentrations. As it is easier to measure an increase in the rate coefficient than a decrease, in these experiments the efflux was started in 142 mM-K (4, 7); after 100 min, K was reduced to 5·4 mM. K was substituted isosmotically with either Li (2, 5) (Fig. 2*A*), choline, Tris or sucrose. In some experiments, K was lowered without osmotic compensation. The

results are qualitatively similar and are discussed together. In the same preparations [^{3}H]sucrose efflux was measured (Fig. 2*B*). As K is not seen to affect the washout of [^{3}H]sucrose, the inhibition and reactivation of ^{45}Ca efflux cannot be caused by changes in the external diffusion pathways.

The effects of repolarization on ^{45}Ca efflux in the different extracellular Na concentrations are summarized in Fig. 3. The re-activation of Ca efflux upon K

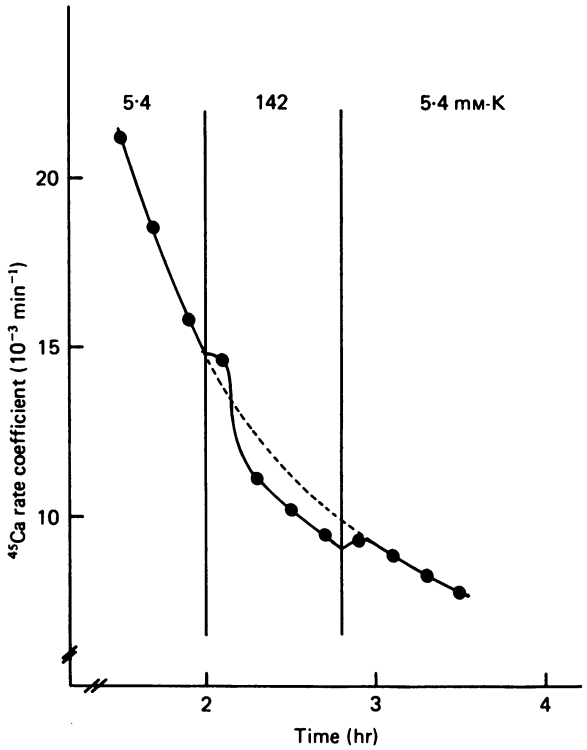

Fig. 1. Effect of high (142 mM) K on ^{45}Ca efflux in the presence of 137 mM-Na and 1·8 mM-Ca. 137 mM-LiCl was added to the normal K solution in order to keep constant osmotic pressure and ionic strength throughout the experiment. The dashed line is calculated from the rate coefficients obtained in normal (5·4 mM) K. Mean of eleven preparations.

reduction is estimated by subtracting extrapolated curves in high K from the values obtained in low K. The extrapolation (Fig. 3, inset), is produced by mathematically fitting (least squares method) an exponential curve, $k = ax^b$, to the last four points obtained in high K, where k is the rate coefficient, x is the efflux time, and a and b are constants determined separately for each efflux. The figure shows that the difference in rate coefficient (Δk) between the extrapolated line and that observed following the repolarization-induced reactivation of Ca efflux, clearly depends on the external Na concentration. Between external Na concentrations of 68·5 mM and 137 mM Δk is maximum, but is reduced as the external Na concentration is reduced. In the virtual absence of Na a small negative value may be observed, possibly due to some stimulating action of high K on Ca efflux (see later). Similar efflux

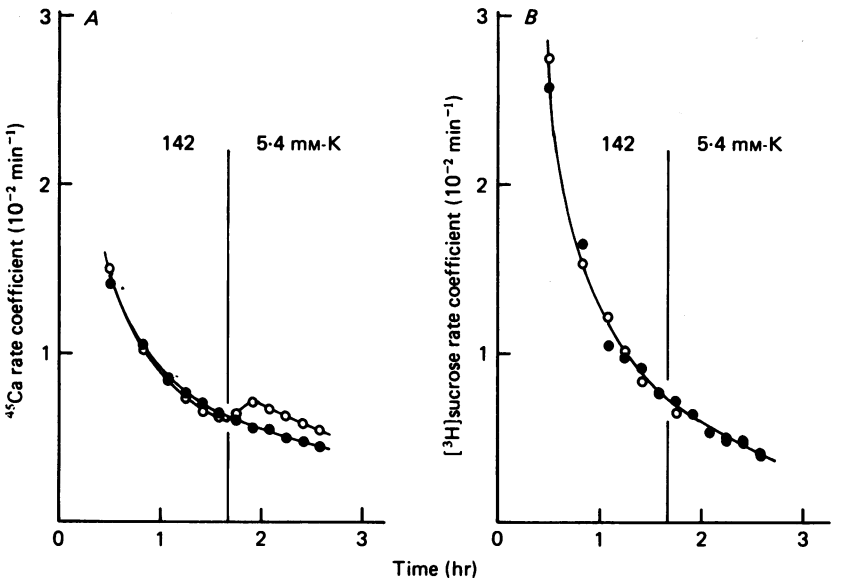

Fig. 2. Effect of lowering K from 142 mM to 5·4 mM on a simultaneous ^{45}Ca efflux (*A*) and [^{3}H]sucrose efflux (*B*) from the same half ventricles in the presence of 137 mM-Na and 1·8 mM-Ca (○). Control halves of the same hearts were washed out in 5·4 mM-K throughout (●). Osmolarity and ionic strength were kept constant by adding LiCl to the low K solutions. Each curve mean of six preparations.

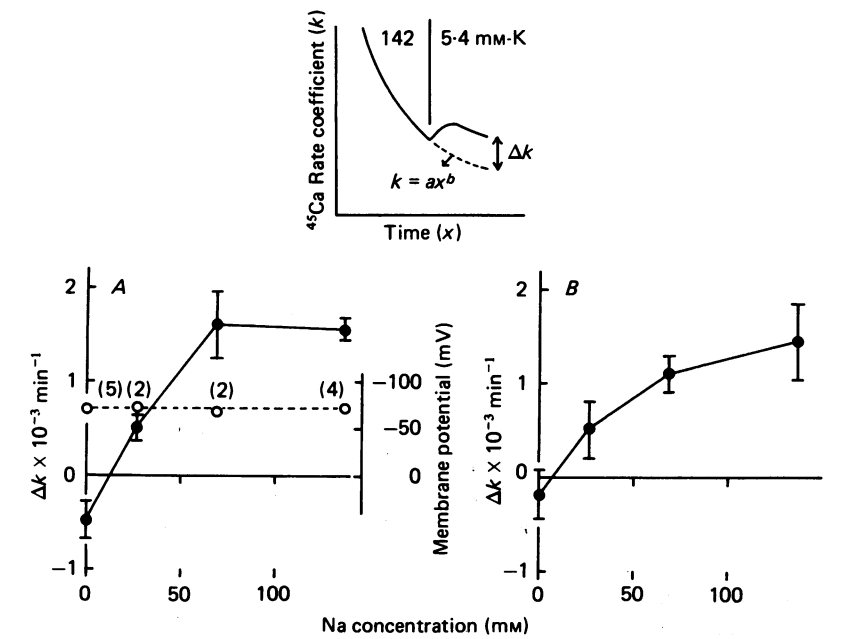

Fig. 3. Changes in rate coefficients of ^{45}Ca efflux (Δ*k*) induced by lowering the K concentration from 142 mM to 5·4 mM, as a function of the extracellular Na concentration. Δ*k* calculated as shown in the inset. For full description see text. Osmolarity was kept constant by substituting Li for both K and Na. Each value mean ± S.E. of six preparations. The resting membrane potential (○) was measured in separate experiments *ca.* 20 min after reducing K from 142 mM to 5·4 mM. Number of preparations in brackets. S.E. of the mean was smaller than the size of the symbols. *A*, goldfish ventricles; *B*, guinea-pig atria.

experiments on guinea-pig atria gave strikingly comparable results (Fig. 3*B*). This effect of Na was not due to incomplete repolarization at lower Na concentrations, as values of the membrane potential, measured at *ca.* 20 min after lowering K in the efflux solution from 142 mM to 5·4 mM are independent of Na (Fig. 3*A*).

When goldfish ventricles are superfused with low Ca, high K solutions (11, 15) for 100 min, and K is thereafter reduced to 5·4 mM (10, 13), the membrane potential recovers within 20 min from *ca.* −10 mV to −71 ± 1 mV (three preparations), in the

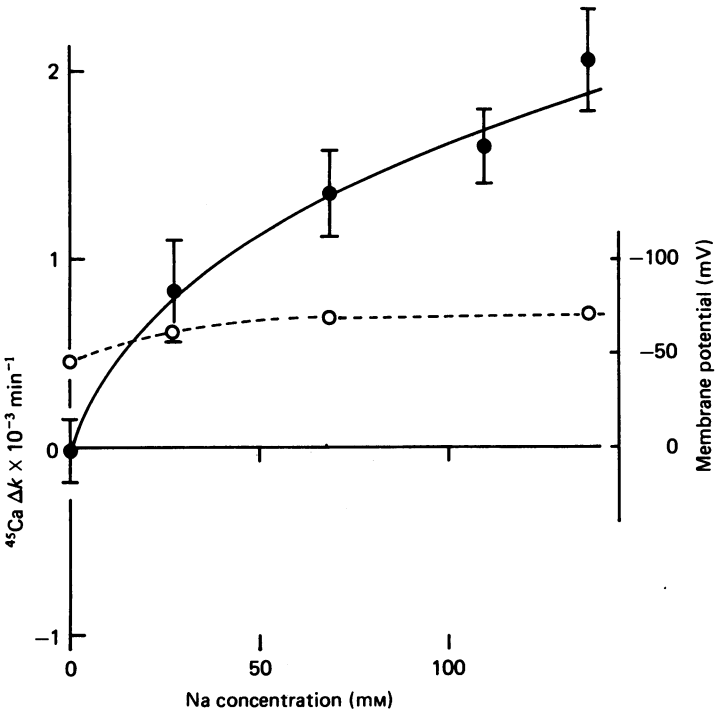

Fig. 4. Changes in rate coefficients of ^{45}Ca efflux (Δ*k*) from goldfish ventricles during repolarization plotted against the external Na concentration (●; means ± S.E.M., $n = 6$). Choline was used for osmotic compensation of changes in the Na and K concentration. Δ*k* was calculated as shown in Fig. 3. The solutions contained 4·5 mM-Ca and 5 mM-EGTA, pH 7·1. Assuming an apparent binding constant for the Ca EGTA complex of $7{\cdot}6 \times 10^6$ M^{-1} at pH 7·1 (Portzehl *et al.* 1964), the free Ca^{2+} concentration should be *ca.* 10^{-6} M. Resting membrane potential (○) was determined about 20 min after the re-application of normal K. Each value mean of three preparations. S.E. was smaller than size of symbols.

presence of normal external Na (137 mM). When Na is reduced the repolarization is less complete (Fig. 4), a feature which may influence the shape of the reactivation curve. The lack of reactivation in the absence of Na is unlikely to be due to the relatively small repolarization (to −46 mV) as in a separate experiment in 0 Ca, 5 mM-EGTA, in the presence of Na (137 mM), a substantial increase in rate coefficient was still found for a repolarization to only −40 mV. The reactivation of Ca efflux associated with the repolarization however is still clearly dependent on the external Na concentration (Fig. 4).

Is the effect of high K on Ca efflux due to depolarization?

Using the techniques described here it is not possible to depolarize the membranes by means other than by increasing K. When the external Ca concentration is reduced, however, a reversible depolarization develops in goldfish ventricles, superfused with Na-saline containing 5·4 mM-K, 4·5 mM-Ca, 5 mM-EGTA and 137 mM-hypertonic choline-Cl (10), which become spontaneously active. Action potentials of increasing

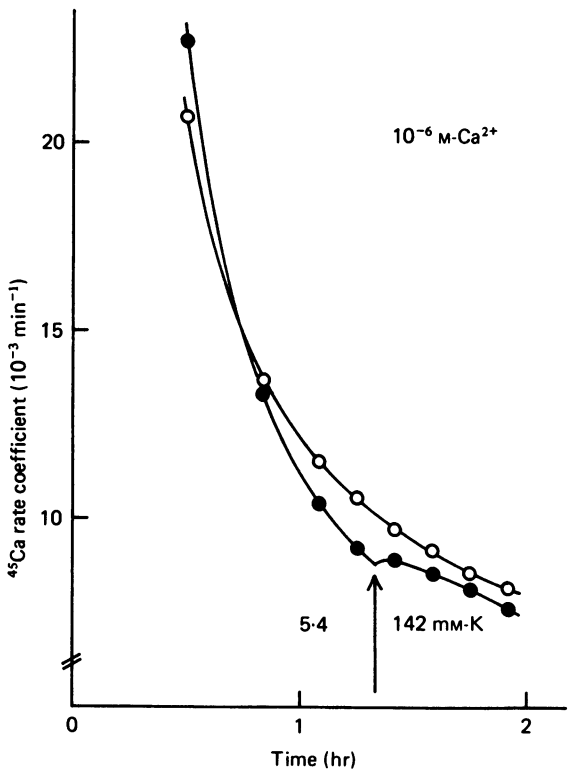

Fig. 5. The effect of raising (arrow) external K from 5·4 mM to 142 mM on ^{45}Ca efflux (●) from ventricles washed out in Na-saline containing 10^{-6} M-Ca^{2+} (4·5 mM-Ca, 5 mM-EGTA, pH 7·1). Control halves of the same hearts (○) were continuously washed out in the presence of 142 mM-K. Each curve mean of six preparations. Choline was used to replace 137 mM-K at normal K concentration.

duration (from *ca.* 0·5 sec to above 1 min) can be recorded for about 1 hr, and the apparent resting membrane potential gradually declines from −70 mV to −25 mV within 100 min. Perhaps the membrane potential at that stage represents the prolonged plateau of an action potential (see Miller & Mörchen, 1978). When choline is then replaced by K (11), the membrane depolarizes further to −15 mV. If the inhibition of Ca efflux were due to high K *per se* and not to depolarization, one could expect that the Ca efflux would still be reduced by raising K. Fig. 5 shows that this is not the case. On the contrary, the rate coefficient of Ca efflux is slightly increased.

The Na-activated fraction of Ca efflux in normal and depolarized heart muscle

To determine the effects of membrane potential upon the Na-activated fraction of the Ca efflux, the preparations were loaded with ^{45}Ca in Na-free solution for 60 min. The efflux was then started in Na-free medium (5, 6, 7, 8), and after 100 min Na was re-admitted (2, 3, 4). The increase in rate coefficient due to Na re-application (the Na-activated fraction of Ca efflux) is estimated by subtraction of an extrapolation

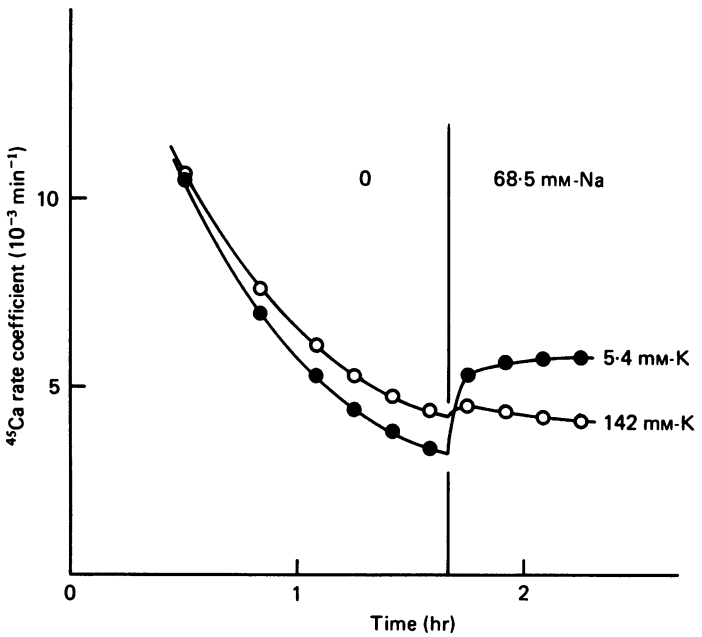

Fig. 6. The effect of K-depolarization on the Na-activated fraction of Ca efflux (increase of rate coefficient after re-admission of Na). Goldfish ventricles were loaded with ^{45}Ca in Na-free solution and Na (in this case 68·5 mM) was re-admitted after 100 min (vertical bar). [K] was 142 mM during the whole efflux for one half ventricle (○), whereas the other half of the same heart was washed out in normal K (●). Osmolarity was kept constant (hyperosmotically) using choline to replace K and Na. Each curve mean of six half ventricles.

of the initial (Na-free) part of the efflux curve beyond the onset of Na re-application from the observed rate coefficient (Fig. 7 inset). The mean difference between the last three rate coefficients in the presence of Na and the corresponding but extrapolated Na-free rate coefficients is taken as the measure of Na-dependent Ca efflux. The extrapolation was a mathematical fitting of the curve, as described in the first paragraph. An example of such an experiment (Fig. 6) shows that the reactivation of Ca efflux by Na is strongly inhibited in K-depolarized ventricles, whereas in Na-free medium high K causes an increase in the rate coefficient of Ca efflux. In Fig. 7, the Na-activated fraction of Ca efflux (Δk) is plotted against the external Na concentration. Fig. 7*A* and *B* were obtained when either choline of Li were used for osmotic compensation respectively. The curves relating Δk to external Na are sigmoidal (see

also Busselen & Van Kerkhove, 1978) and were fitted (continuous line) by the expression $\Delta k = \Delta k_{max}/1+(K_{Na}/Na)^n$, with $n = 4$ (see Mullins, 1979). It should be noted, however, that with $n = 3$, the curves fitted the experimental points also reasonably well. The empirical value of n therefore does not provide a good basis for making decisions concerning the degree of co-operativity. In the presence of high K, the curves are shifted to the right, to show a relative inhibition of Δk which is stronger

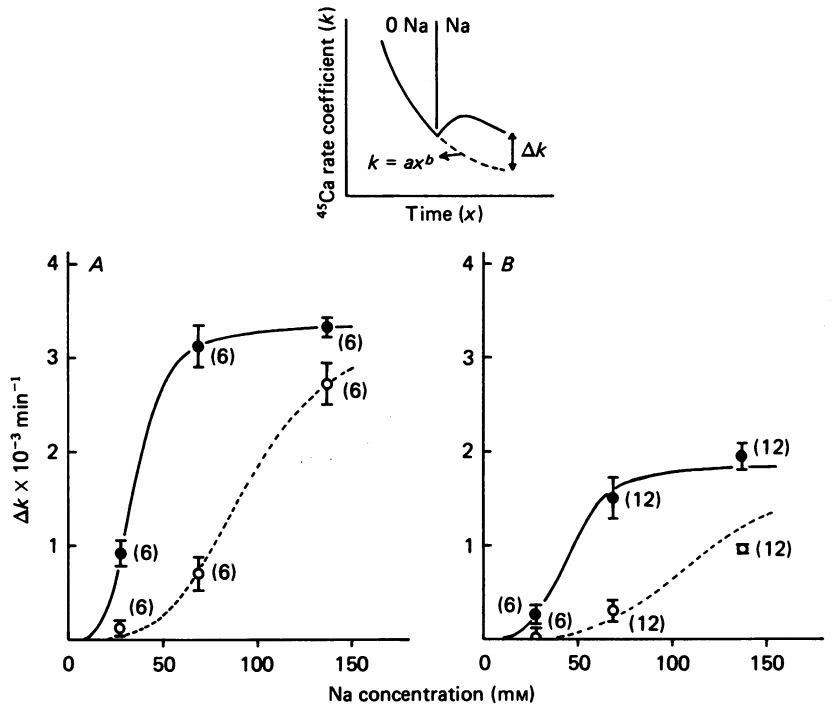

Fig. 7. The Na-activated fraction of ^{45}Ca efflux (Δk) in normal (●) and K-depolarized (○) goldfish ventricles as a function of the external Na concentration. In order to maintain a constant osmotic pressure either choline (*A*) or Li (*B*) were used to replace both Na and K. Results in normal and high K were obtained in paired experiments on halves of the same hearts. S.E. of the mean indicated by vertical bars. Number of ventricles in parentheses. Δk was calculated as shown in the inset, and explained in the text. The continuous lines are drawn to the expression: $\Delta k = \Delta k_{max}/1+(K_{Na}/Na)^4$, with $\Delta k_{max} = 3{\cdot}3 \times 10^{-3}$ min^{-1} and $K_{Na} = 35$ mM (*A*) and $\Delta k_{max} = 1{\cdot}8 \times 10^{-3}$ min^{-1} and $K_{Na} = 43$ mM (*B*) respectively. The dashed lines are drawn to the same expression using the same values of Δk_{max}, but with $K_{Na} = 95$ mM (*A*) and $K_{Na} = 117$ mM (*B*).

in low Na than in high Na. The dashed curves (Fig. 7*A* and *B*) were fitted by the same expression as above, using the value of Δk_{max} obtained for the ventricles immersed in low K. The shift of the curves in depolarized preparations might indicate a decreased affinity of Na (or a higher affinity of Ca) for the Na–Ca exchange carrier. The curves in the presence of either choline or Li are quantitatively different (compare Figs. 7*A* and *B*), suggesting that these cations may interfere with the Na–Ca or Ca–Ca exchange system or with its dependence on K.

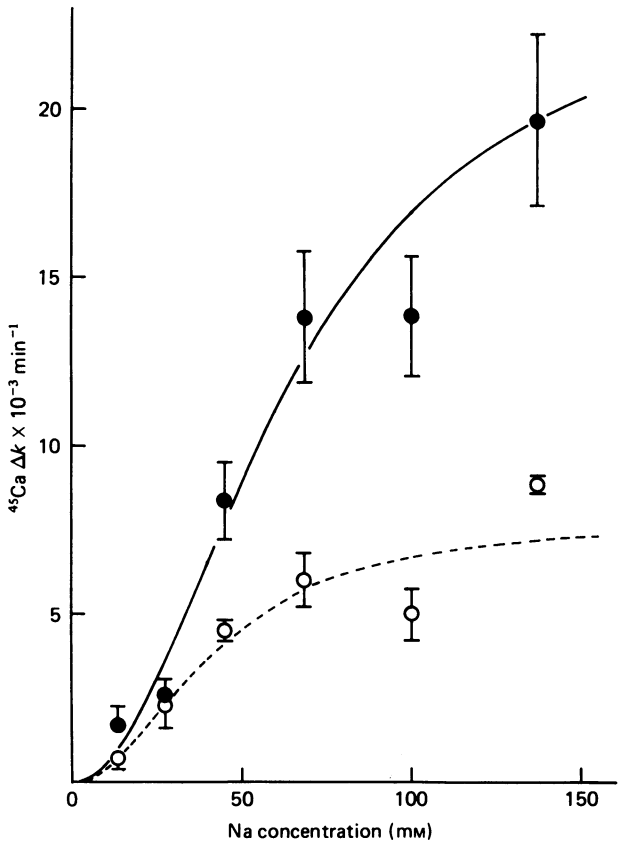

Fig. 8. The effect of K-depolarization on the Na-dependent fraction of ^{45}Ca efflux (Δk) in guinea-pig atria as a function of the external Na concentration. Results in normal (●) and K-depolarized (○) tissues from parallel experiments on halves of the same atria. Each symbol represents the mean Δk of three preparations, S.E. of mean indicated by vertical bars. Δk was determined as explained in Fig. 7. Choline has been used to adjust osmotic pressure. The lines are drawn to the equation $\Delta k = \Delta k_{max}/1+(K_{Na}/Na)^2$ with $\Delta k_{max} = 24{\cdot}1 \times 10^{-3}\ min^{-1}$, $K_{Na} = 65$ mM (●) and $\Delta k_{max} = 7{\cdot}8 \times 10^{-3}\ min^{-1}$, $K_{Na} = 42$ mM (○).

Similar experiments were performed on halves of guinea-pig atria (Fig. 8). In this case the curves were better fitted by the foregoing expression, when n was 2. The relative effect of high K was nearly constant in the various external Na concentrations.

Effluxes in 10^{-6} M-Ca^{2+} (4·5 mM-Ca, 5 mM-EGTA) appeared unsuccessful because the preparations depolarized in Na-free solutions containing 5·4 mM-K (12, 13).

Ca influx

In order to evaluate an eventual contribution of changes in Ca influx due to depolarization, Ca influx was measured under experimental conditions where Na-dependent Ca efflux was clearly inhibited by high K. Any significant difference was

not observed between the 10 min Ca uptake in low and high K, in either 137 mM-Na (hypertonic Li) (2, 4) or in 68·5 mM-Na (hypertonic choline) (3, 6 and 4, 8).

Measurement of intracellular Na

The intracellular Na content was measured under the same experimental conditions as those used for the determination of the Na-dependent fraction of Ca efflux, viz. after Na-free superfusion and re-admission of Na in either low or high K. [^{3}H]sucrose

TABLE 2. Effect of K-depolarization on 10 min ^{45}Ca influx

	Concentration of main cations in solution (mM)					10 min ^{45}Ca influx (mM-Ca/kg dry wt.) mean ± S.E.
	Na	Li	Choline	K	Ca	
(*a*)	137	137	—	5·4	1·8	1·70 ± 0·10 (n = 18)
(*b*)	137	—	—	142	1·8	1·68 ± 0·08 (n = 18)
(*a*)	68·5	—	205·5	5·4	1·8	1·85 ± 0·07 (n = 12)
(*b*)	68·5	—	68·5	142	1·8	1·95 ± 0·10 (n = 12)

Goldfish ventricles were adapted to the given solutions for 1 hr. Halves of the same hearts were compared, one half (*a*) being immersed in low K, the other half (*b*) in high K. Then ^{45}Ca was added for 10 min. After the uptake of ^{45}Ca, extracellular Ca was removed by washing the preparations for 20 min in Na- and Ca-free solution containing (mM): $LaCl_3$, 10; LiCl, 137; KCl, 5·4; $MgCl_2$, 0·5; glucose, 5·5; Tris, 10 (pH 7·3). The influxes in low and high K were not significantly different (Student's t test, paired case).

TABLE 3. Cellular Na after Na-free immersion for 1 hr and re-admission of Na for 20 min, in low and high K

	Main cations in final solution (mM)			Na concentration (m-moles/kg wet wt.) mean ± S.E.M, n = 11		
	Na	K	Li	Tissue Na (1)	Extracellular Na (2)	Cellular Na (1)–(2)
(*a*)	137	5·4	137	60·04 ± 5·25	35·84 ± 3·10	24·19 ± 3·11
(*b*)	137	142	—	55·28 ± 4·03	35·72 ± 1·72	19·56 ± 2·69

Cellular Na was determined by subtracting extracellular Na (2), calculated from the [^{3}H]sucrose space, from the total Na (1) measured by atomic absorption spectrophotometry. (*a*) and (*b*) are paired results on halves of the same ventricles. Preparations were immersed successively in the following solution: Na-free saline (137 Li, 1·8 Ca) for 60 min, Na-free saline containing [^{3}H]sucrose for 40 min and Na-saline containing [^{3}H]sucrose for 20 min (the specific activity of [^{3}H]sucrose was the same for Na-free and Na-containing solutions). The difference in cellular Na content between preparations (*a*) and (*b*) was significant ($P < 0·05$, Student's t test for the paired case).

was used for measuring the extracellular space. Table 3 shows that the intracellular Na content was lower by *ca.* 20% in K-depolarized preparations, suggesting a decreased rate of Na influx (which is expected if the hypothesis of an electrogenic Na–Ca exchange holds true). The different Na content should be taken into account for the interpretation of the Ca efflux data (competition of Na and Ca at the internal site of the membrane).

DISCUSSION

The present results show that, in heart preparations of such unrelated vertebrates as the goldfish (ventricular muscle) and the guinea-pig (atrial muscle), Ca efflux is inhibited during K-depolarization. These direct results on intact cardiac muscle therefore reinforce recent findings, indicating electrogenic exchange of one Ca ion for at least three Na ions, from studies on subcellular vesicles of heart sarcolemma (Pitts, 1979; Bers *et al.* 1980; Lamers & Stinis, 1981), from voltage-clamp experiments (Horackova & Vassort, 1979), and from recordings of contractile tension and membrane potential (Chapman & Tunstall, 1980, 1981; Coraboeuf *et al.* 1981). In squid axon it is well established that Na–Ca exchange is sensitive to membrane potential (Mullins & Brinley, 1975; Baker & McNaughton, 1976*a*, *b*) due to a coupling ratio of at least three Na^+ for one Ca^+ (Baker, Blaustein, Hodgkin & Steinhardt, 1969; Blaustein & Russell, 1975). Reports of Ca efflux from heart preparations, however, have failed to demonstrate clearly any effect of depolarization on Ca efflux, probably due to the experimental procedures used. Niedergerke (1963) described a stimulation of Ca efflux by K-depolarization during very short efflux times: after the initial 10–11 min of perfusion, high K was applicated for only 5 min. As other contracture fluids (with reduced Na) gave a similar effect, this result may be due to a mechanical 'squeezing' of the tissue due to the coincident contracture, as a similar stimulation of [^{3}H]sucrose efflux can be recorded under the same experimental conditions (P. Busselen, unpublished results on goldfish ventricles). A squeezing effect may also explain the phasic stimulation of Ca efflux from goldfish ventricles at the onset of high K application (see for example Fig. 1). Wollert (1966) in his experiments increased external K by replacing NaCl isotonically by KCl, and noted a decrease in Ca efflux. This is presumably explained by the reduction of external Na and not by the depolarization *per se*. The experiments by Jundt *et al.* (1975) on guinea-pig auricles, although carried out under almost identical conditions to those in this study (hypertonic K for choline substitution and re-application of Na after Na-free superfusion), did not demonstrate any appreciable K effect. This result is not easily explained, but could again be related to the experimental technique, the first hour of their ^{45}Ca efflux being in Ca-free Tyrode solution and the second hour in normal Tyrode solution. In this study Ca-free perfusion is omitted to avoid the risk of the so called 'Ca-paradox', in consequence of tissue damage when Ca is re-admitted after Ca-free superfusion (Zimmerman & Hulsmann, 1966).

The best indication that high K does inhibit Na-dependent Ca efflux is that this phase of the efflux is increased when the K concentration in the bathing solution is reduced from 142 to 5·4 mM (Figs. 2–4). As this effect is of similar magnitude in both 10^{-3} M and 10^{-6} M external Ca^{2+}, the effects of any change in intracellular specific activity of ^{45}Ca upon the rate coefficient are ruled out. This notion is supported as the ^{45}Ca uptake is not significantly different in normal and depolarized preparations (Table 2). However, an unaltered Ca uptake is not easily reconciled with an electrogenic Na–Ca exchange nor with the observations of Niedergerke (1963). Niedergerke (1963) reported a substantial increase in ^{45}Ca accumulation during the K-depolarization of frog ventricle, which is in line with the increased Ca influx inferred from contractile and voltage-clamp experiments (for review see Chapman,

1979). The experiments reported here, however differ from Niedergerke's in two main respects: (1) a 20 min wash in Na- and Ca-free solution containing La is included to remove extracellular Ca, and (2) the influx is measured *after* exposure of the preparations to the depolarizing solution for 1 hr. As depolarization is known to decrease the intracellular Na^+ concentration (Ellis, 1977; P. Busselen, unpublished results on trout hearts) and as Ca influx depends on internal Na (Glitsch, Reuter & Scholz, 1970), any stimulation of the Ca influx by depolarization might have been abolished under the present experimental conditions. Further the reduced internal Na concentration might have stimulated Ca efflux by reducing the competition between Ca^{2+} and Na^+ at the inside of the membrane. In consequence the inhibition of Ca efflux during depolarization is probably underestimated.

An electrogenic Na–Ca exchange is also suggested by the observations that reactivation of ^{45}Ca efflux when Na is re-admitted to the Na-free efflux solutions (Fig. 6–8) is reduced in depolarized ventricles, and that the cellular reuptake of Na is inhibited in depolarized preparations (Table 3).

It is still not clearly established, however, whether the effect of high K is due to depolarization or to another action of K. Evidence that depolarization actually decreases Ca–Na exchange was obtained only under rather unphysiological conditions (Fig. 5). In *Myxicola* giant axons Na efflux which is dependent on external Ca (the reverse of external Na-dependent Ca efflux) is increased by raising external K an effect not due to the membrane potential *per se* (Sjodin & Abercrombie, 1978). Other unspecified effects of K^+ on the Ca transport mechanism have been suggested (Morad & Klitzner, 1978; Morad, Reeck & Rao, 1981), and K may enhance Ca–Ca exchange (Blaustein & Russell, 1975; Philipson & Nishimoto, 1981) which could account for the high K stimulation of Ca efflux in Na-free solution (Fig. 6).

In the final series of experiments the Na-dependent fraction of Ca efflux in various Na concentrations is measured for normal and depolarized preparations. The curves obtained in goldfish ventricles (Fig. 7) are shifted to the right in high K, indicating an altered affinity of the transport mechanism for external Na or Ca. However, as other factors (such as intracellular Na concentration, other actions of K) may determine the shape of the curves and their shift, it is difficult to draw conclusions from this observation. In guinea-pig atria, however, the inhibition is of a non-competitive type (Fig. 8).

The evidence for a Ca–Na exchange is quite compelling; however its stoichiometry is still open to question. If two Na ions exchange for each Ca ion, as proposed by Reuter & Seitz (1968), the energy drawn from the Na gradient would be insufficient to build up the large Ca gradient across the sarcolemma. Mullins (1979) from a theoretical point of view suggests that a four Na^+–one Ca^{2+} coupling mode would be necessary to establish a physiological external Ca–internal Ca concentration ratio of around 10^5. This exchange stoichiometry accounts for a probably partial uncoupling of the Ca for Na exchange. A residual Ca efflux (Ca leak and/or Ca pump?) in the absence of external Na and Ca is known to occur (Reuter & Seitz, 1968; Busselen & Van Kerkhove, 1978), and for this reason the curves in Fig. 7 were fitted assuming a 4 Na–1 Ca^{2+} ratio. However the data is not accurate enough to discriminate between a three or four power function, and the curves for guinea-pig auricles are better fitted with a two power function (Fig. 8). Furthermore the sigmoid shape for

the curve might be exaggerated by a higher Ca uptake in low Na than in the virtual absence of Na (Busselen & Van Kerkhove, 1975; see also Baker *et al.* 1969). In view of the membrane potential dependence, however, three or more Na ions must exchange for one Ca ion unless the mechanism whereby opposite movements of Ca and Na are coupled is more complicated (for example see Benninger, Einwächter, Haas & Kern, 1976).

Although the hypothesis, that the energy for Ca extrusion is exclusively provided for by the Na gradient, is attractive, it is not unlikely that ATP-dependent Ca pumping may contribute to the Ca homeostasis. ATP-mediated Ca transport and Ca-ATP ase have indeed been described in isolated cardiac sarcolemma (Trumble, Sutko & Reeves, 1980; Caroni & Carafoli, 1980; Morcos, 1981; Lamers & Stinis, 1981). ATP-driven transport of Na (Na–K transport) and of Ca probably cooperate to establish the steady internal Ca concentration at rest, whereas during contraction and relaxation changes in membrane potential may affect the internal Ca concentrations by changing the equilibrium conditions for the electrogenic three Na^+–one Ca^{2+} exchange.

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