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THE EFFECT OF THE INTERNAL SODIUM CONCENTRATION ON CALCIUM FLUXES IN ISOLATED GUINEA-PIG AURICLES

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SUMMARY

1. Calcium efflux from guinea-pig auricles followed saturation kinetics when $[Ca]_o$ and $[Na]_o$ were changed while the ratio $[Ca]_o/[Na]_o^2$ was kept constant. The Michaelis constant, $K_m^{Ca+Na} = 40$ mM, suggests that a hypothetical carrier system, responsible for sodium–calcium exchange, is far from saturation with the inside concentrations of these ions.

2. $[Na]_i$ was altered in the auricles between 12·5 and 60 mM/kg fibre water while total cellular calcium concentration ($[Ca]_t$) at the beginning of the influx period was not significantly different in the various groups of preparations.

3. ^{45}Ca influx increased appreciably with increasing $[Na]_i$. ^{45}Ca influx from sodium-poor solution corresponded to an almost equal increase in $[Ca]_t$, while $[Ca]_t$ did not change much in preparations loaded with ^{45}Ca in Tyrode solution. When the sodium-activated fraction of calcium influx was plotted against $[Na]_i^2$ the resulting curve indicated saturation with $K_m^{Na} = 3500$ (mM $[Na]_i)^2$ and maximal influx rate, $J_{i,max}^{Ca'} = 1{\cdot}35$ mM/kg wet weight × 10 min.

4. When the preparations were re-equilibrated for various times in normal Tyrode solution after $[Na]_i$ had been increased, both the sodium-activated component of calcium influx and $[Na]_i^2$ decreased with approximately the same rate constants.

5. Calcium efflux from auricles with high $[Na]_i$ was increased when it was measured in Tyrode solution while the efflux in sodium-poor solution was inhibited.

6. Auricles with increased $[Na]_i$ showed a positive inotropic contractile response.

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7. The main conclusion reached by these experiments is that calcium influx is affected by $[Na]_i$ in a way which is compatible with a carrier-mediated sodium–calcium exchange system.

INTRODUCTION

In a previous study it has been shown by Reuter & Seitz (1968) that calcium efflux from isolated cardiac preparations is dependent on the calcium and sodium concentrations in the bathing fluid. The results were interpreted in terms of a facilitated diffusion system involving a carrier which is occupied by $[Ca]_o$ and $[Na]_o$ in a competitive way. In such a system the energy required for extruding calcium ions out of the cell against a large electrochemical gradient could be provided by the downhill movement of calcium and sodium into the cell. Net outward transport of calcium has been shown to result from the exchange of internal calcium against external sodium. Independently Blaustein & Hodgkin (1968, 1969) have demonstrated that a similar calcium transport system operates in squid axon. Furthermore, results obtained by Baker, Blaustein, Hodgkin & Steinhardt (1969) indicate that internal sodium in squid axon can promote calcium influx.

The present paper deals with the influence of the internal sodium concentration on calcium influx and efflux in isolated guinea-pig auricles. The interpretation of the results is given in terms of a carrier-mediated calcium transport system of the type which has recently been proposed by Reuter & Seitz (1968) for cardiac muscle and by Baker *et al.* (1969) for squid axon. Some of the results have already been published in preliminary notes (Glitsch, Reuter & Scholz, 1969; Reuter, 1970).

METHODS

Material. The experiments were performed on left auricles isolated from guinea-pig hearts.

Alteration of $[Na]_i$. After the dissection all preparations were first equilibrated for 30 min in normal Tyrode solution at 35° C. Thereafter the auricles were either left in this solution for another 30–90 min or the external conditions were changed in order to alter the intracellular sodium concentration, $[Na]_i$, of the preparations. $[Na]_i$ was reduced by soaking the preparations for 45 min in Tyrode solution containing 32·4 mM-KCl, 27 mM-NaCl of the solution being replaced by an equimolar amount of KCl. $[Na]_i$ was increased either by rapid stimulation (300/min) for 30 min in KCl-poor (0·65 mM), $CaCl_2$-poor (0·2 mM) Tyrode solution or by cooling the preparations in the same solution at 5° C for 90 min. The sodium and potassium contents of the auricles achieved under these conditions were determined.

Calcium influx measurements. The uptake of ^{45}Ca in resting guinea-pig auricles with different $[Na]_i$ was measured by immersing the preparations for 10 min at 35° C either in radioactive Tyrode solution with 0·65 mM-KCl or in a corresponding sodium-poor solution (solution 2 in Table 1). In order to avoid spontaneous electrical

and contractile activity of the preparations in the K-poor Tyrode solution acetylcholine (10^{-7}–10^{-6} g/ml.) was added which has been shown to have no effect on ^{45}Ca influx in resting guinea-pig auricles (Hoditz & Lüllmann, 1964). At the end of this time great care was taken to wash the extracellular space as free from calcium as possible. For this purpose the preparations were transferred during five successive periods of 5 min each through large volumes of inactive solution at room temperature. This rinsing solution (solution 3 in Table 1) was completely free of sodium and calcium since the loss of cellular calcium has been shown to be largely inhibited under this condition (Reuter & Seitz, 1968; Blaustein & Hodgkin, 1969; this paper). The time of 25 min in well stirred large volumes (about 1 l. each) seemed to be sufficient for extracellular tracer-washout since it has been shown that additional 15 min of washout had no further effect on radioactivity in comparable groups of preparations. Afterwards the auricles were gently blotted on filter paper under constant pressure, cut edges were removed and the preparations were weighed and ashed for radioactive and chemical analysis.

TABLE 1. Composition of solutions (mM)

Components	Solution 1 Tyrode	Solution 2 Na-poor	Solution 3 Na-, Ca-free
NaCl	137	—	—
Choline-Cl	—	137	137
KCl	5·4	0·65	5·4
$CaCl_2$	1·8	1·8	—
$MgCl_2$	1·05	1·05	1·05
$NaHCO_3$	11·9	11·9	—
NaH_2PO_4	0·42	0·42	—
Tris-buffer + HCl to pH 7·4	—	—	5
Glucose	5	5	5

KCl and $CaCl_2$ in Tyrode solution were sometimes varied as indicated in the text. Choline-Cl solutions contained atropine sulphate (10^{-5} g/ml.). Solutions 1 and 2 were equilibrated with 95 % O_2 + 5 % CO_2, solution 3 with 100 % O_2.

Calcium efflux measurements. The method of measuring calcium efflux from the resting auricles by means of ^{45}Ca was essentially the same as the one described by Reuter & Seitz (1968).

Analytical procedures. The methods used for determinations of ^{45}Ca and calcium content in the ash of the auricles and in the uptake solutions have been described extensively by Reuter & Seitz (1968). The sodium and potassium contents of the preparations were measured by flame photometry (Eppendorf Flame Photometer). The water content of the tissue $(H_2O)_T$ was derived from the difference between wet weight and dry weight of each preparation. The extracellular space, ECS, was assumed to be 300 ml./kg wet weight (Bauer, Lüllmann & Richter, 1963). Thus the apparent intracellular ion concentrations, C_i, could be estimated by

$$C_i = \frac{C_T - C_o \times \mathrm{ECS}}{(H_2O)_T - \mathrm{ECS}},$$

where C_T and C_o are the total tissue concentration of the ion and its concentration in the bathing fluid, respectively.

Solutions. The composition of the bathing solutions is given in Table 1.

Statistical methods. Whenever possible, values are presented as means ± S.E. of means. Significance of differences of means was checked by Student's *t* test.

RESULTS

Saturation of calcium efflux by $[Ca]_o + [Na]_o$. Reuter & Seitz (1968) have demonstrated that calcium efflux from cardiac muscle is dependent on $[Ca]_o$ and $[Na]_o$. Both ions compete for binding sites presumably at the outer surface of the cell membrane. The respective magnitudes of the calcium- and sodium-activated components of total calcium efflux are

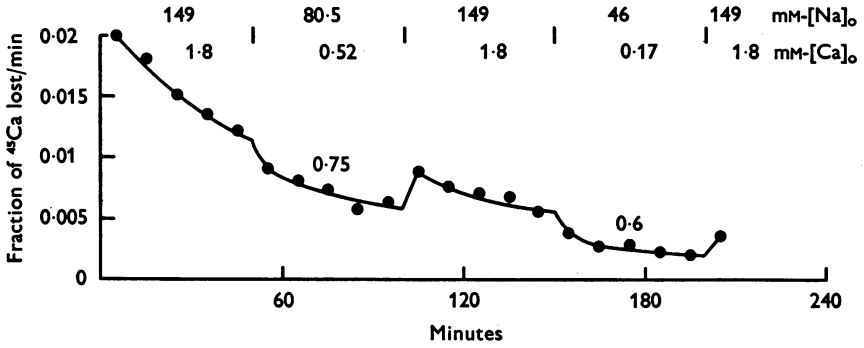

Fig. 1. Calcium efflux from a guinea-pig auricle during reduction of $[Ca]_o$ and $[Na]_o$ at constant ratio $[Ca]_o/[Na]_o^2$ ($0{\cdot}81 \times 10^{-4}$ mM^{-1}). $[Ca]_o$ and $[Na]_o$ were reduced twice between periods in normal Tyrode solution by isosmotic replacement of $CaCl_2$ and NaCl by choline-Cl in the solution. The numbers within the curve give the reduction of calcium efflux during the periods with decreased $[Ca]_o + [Na]_o$ relative to the preceding period in normal Tyrode solution. The mean values of several similar figures obtained in different experiments are plotted in Fig. 2 as relative rate of calcium efflux (J_e^{Ca}). Ordinate: fraction of ^{45}Ca lost/min (rate coefficient); abscissa: time in min.

determined by the ratio $[Ca]_o/[Na]^2$. In the present study the total concentration of both ions in the bathing fluid was changed systematically while this ion ratio was kept constant. Therefore, the fractions of total efflux governed by each of these ions were constant and the efflux became dependent only on the variation of the sum $[Ca]_o + [Na]_o$. NaCl in the Tyrode solution was replaced by either LiCl, choline-Cl or sucrose, since it has been shown that none of these substances has a stimulatory effect on calcium efflux (Reuter & Seitz, 1968; Glitsch & Reuter, 1968). The efflux measured in resting guinea-pig auricles as fraction of ^{45}Ca lost per minute (rate coefficient) decreased when the sum of both ions in the Tyrode solution was reduced (Fig. 1). Furthermore, when the relative rate of calcium efflux, J_e^{Ca}, was plotted as function of the sum $[Ca]_o + [Na]_o$ a hyperbolic saturation curve was obtained. The curve could be fitted by the equation

$$J_e^{Ca} = \frac{J_{e,max}^{Ca}([Ca]_o + [Na]_o)}{K_m^{Ca+Na} + [Ca]_o + [Na]_o}. \quad (1)$$

The maximal relative rate of calcium efflux, $J^{Ca}_{e,max}$, and the apparent Michaelis constant, K_m^{Ca+Na}, were derived from the Lineweaver–Burk plot of the results in Fig. 2. Each point of this plot represents a mean of ten to fourteen measurements. The total efflux was half saturated, K_m^{Ca+Na}, by 40 mM-$[Ca]_o + [Na]_o$ when the ratio $[Ca]_o/[Na]_o^2$ was $0{\cdot}81 \times 10^{-4}$ mM^{-1}. From the previous data (Reuter & Seitz, 1968) it can be concluded that K_m^{Ca+Na} of total efflux decreases when this ion ratio increases.

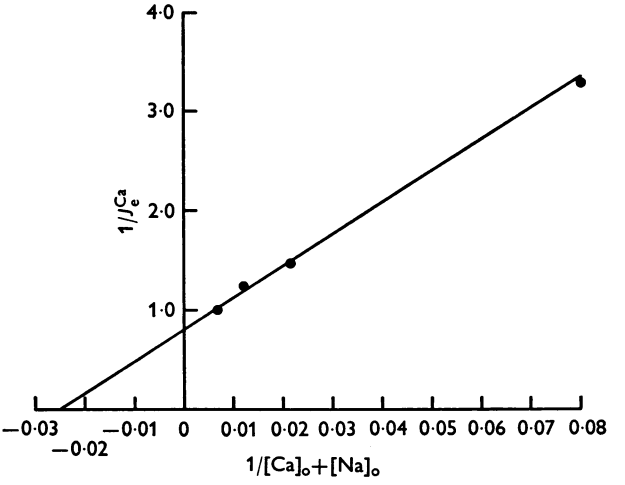

Fig. 2. Double reciprocal plot of relative rate of calcium efflux (J_e^{Ca}; normalized values of fraction of ^{45}Ca lost/min; ordinate) from guinea-pig auricles against the sum $[Ca]_o + [Na]_o$ (abscissa) in Tyrode solution with constant ratio $[Ca]_o/[Na]_o^2$ ($0{\cdot}81 \times 10^{-4}$ mM^{-1}). Each point is the average result of ten to fourteen measurements. The straight line is the reciprocal of the saturation curve calculated from eqn. (1) with $K_m^{Ca+Na} = 40$ mM and $J^{Ca}_{e,max} = 1{\cdot}25$.

These results are mainly presented to demonstrate that with the much lower internal concentrations of both ion species (Table 2) presumptive binding sites (carriers) at the inner surface of the membrane are expected to be far from saturation. Therefore, if the carrier is loaded at both sides of the membrane in the same way and if it traverses the membrane much faster when it is loaded, an increase in internal concentration of either of these ions could induce more carrier to move from inside to outside. In this case more carrier would be loaded per unit time at the outside and an increase in influx of both ions should occur. To test this prediction, in the present study calcium influx was determined as a function of internal sodium concentration.

Ion concentrations in auricles at the beginning of the influx periods. In one series of experiments sodium and calcium concentrations were determined

in guinea-pig auricles which were exposed to various conditions in order to alter $[Na]_i$. The data presented in Table 2 indicate that the intracellular sodium concentration is not different in auricles soaked in normal Tyrode solution for 1 and 2 hr respectively. $[Na]_i$, however, was reduced by increasing $[K]_o$ while it was increased by rapid stimulation and even further by cooling. $[K]_i$ (not listed in Table 2) increased or decreased inversely to $[Na]_i$. The total cellular calcium concentration in the tissue, $[Ca]_t$, was not significantly different in the various groups of auricles at the end of each incubation period. However, the groups of auricles with increased $[Na]_i$ (groups 4 and 5 in Table 2) after the equilibration period in

TABLE 2. Intracellular sodium concentration ($[Na]_i$; mM/kg fibre water), and total cellular calcium content in the tissue after subtraction of calcium in the extracellular fluid ($[Ca]_t$; mM/kg tissue) of guinea-pig auricles soaked in different bathing fluids. Mean ± S.E.; number of auricles in the different groups between 6 and 15

External condition	$[Na]_i$	$[Ca]_t$
1 norm. Tyrode, 1 hr	20·0 ± 3·4	1·6 ± 0·2
2 norm. Tyrode, 2 hr	21·0 ± 5·0	1·7 ± 0·1
3 Tyrode, 32·4-KCl	12·5 ± 1·7	1·8 ± 0·2
4 Tyrode, 0·65-KCl, 0·2-$CaCl_2$, stim. 300/min for 30 min	40·5 ± 4·8	1·6 ± 0·1
5 Tyrode, 0·65-KCl, 0·2-$CaCl_2$, 5° C for 90 min	59·7 ± 6·6	1·6 ± 0·1

normal Tyrode solution were soaked in calcium-poor solution. Therefore, it is possible that these preparations initially lost (cf. Fig. 6) and afterwards regained calcium in the course of these incubation periods. But this question was not investigated further since the experiments listed in Table 2 were performed only to present data on ion concentrations in the tissue at the beginning of the ^{45}Ca influx periods. At the end of each equilibration period the preparations contracted upon stimulation, but did not show any sign of sustained contracture.

The effect of $[Na]_i$ *on calcium influx.* The various groups of preparations used for ^{45}Ca influx measurements were first treated in exactly the same way as those listed in Table 2. Therefore, these ion concentrations are representative for the beginning of each influx period. In order to avoid appreciable changes of $[Na]_i$ during the subsequent 10 min influx periods the radioactive uptake solutions contained 0·65 mM-KCl only. The low $[K]_o$ itself had no significant effect on ^{45}Ca uptake. This has been checked by comparing calcium influx from Tyrode solutions containing 0·65 and 5·4 mM-KCl in two groups of resting auricles (six preparations each) with 'normal' $[Na]_i$. The influx was 0·42 ± 0·05 and 0·44 ± 0·05 mM-Ca/kg wet wt. during a 10 min exposure to ^{45}Ca.

Fig. 3 shows the effect of different $[Na]_i$ on calcium influx in isolated

resting guinea-pig auricles. The values of $[Na]_i$ are the same as those listed in Table 2. The results of the lower curve (filled circles) were obtained with auricles exposed to ^{45}Ca-containing Tyrode solution, those of the upper curve (circles) with preparations loaded with ^{45}Ca in sodium-poor solution (solution 2 in Table 1). The groups of auricles in both uptake solutions were treated in the same way before exposure to ^{45}Ca and hence are supposed to have identical $[Na]_i$ (cf. Table 2). There is evidently a striking influence

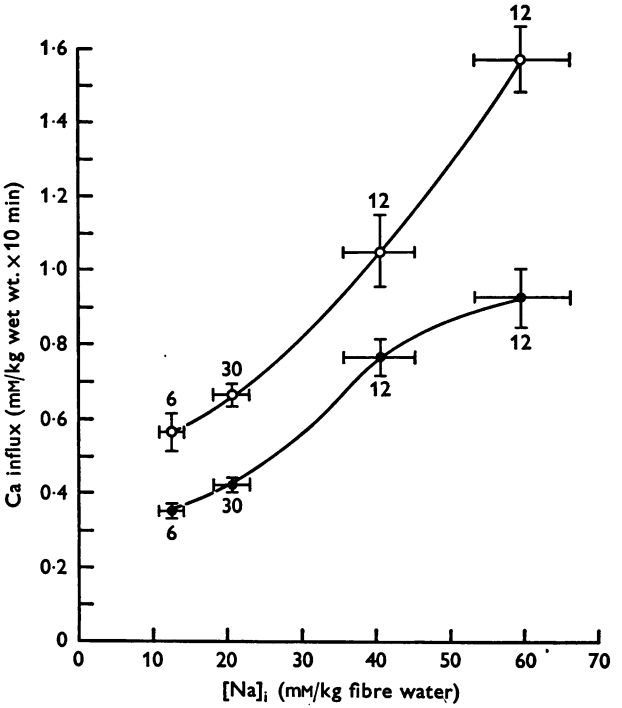

Fig. 3. Effect of internal sodium on calcium influx in guinea-pig auricles during a 10 min exposure to ^{45}Ca-containing Tyrode solution (●, lower curve) or ^{45}Ca-containing Na-poor solution (○, upper curve; NaCl was replaced by choline-Cl; solution 2 in Table 1). The average values (± S.E.) for $[Na]_i$ (mM/kg fibre water; abscissa) are taken from Table 2. The numbers refer to the auricles used for measuring ^{45}Ca influx at each condition; vertical bars are ± S.E. about the mean. Ordinate: calcium influx (mM/kg wet weight × 10 min).

of $[Na]_i$ on calcium influx irrespective of whether the influx was measured from Tyrode solution or sodium-poor solution. In both cases calcium influx increased almost threefold by increasing $[Na]_i$ from 12·5 to 60 mM/kg fibre water. With auricles exposed to ^{45}Ca-containing Tyrode solution the largest effect of $[Na]_i$ occurred between 20 and 40 mM where calcium influx was increased from $0{\cdot}43 \pm 0{\cdot}02$ to $0{\cdot}76 \pm 0{\cdot}05$ mM/kg wet wt. × 10 min.

The average calcium influx from sodium-poor solution was larger than from Tyrode solution, an effect which has been described already for various cardiac tissues (Niedergerke, 1963; Langer, 1964; Wollert, 1966). This extra uptake of calcium in sodium-poor solution is a net uptake and apparently independent of $[Na]_i$ since both curves illustrated in Fig. 3 run parallel with $[Na]_i$ between 12·5 and 40 mM. But there is another important difference between both ^{45}Ca uptake conditions. As has been demonstrated by Reuter & Seitz (1968) calcium net efflux from guinea-pig auricles is dependent on $[Na]_o$. Therefore one would expect an increase in calcium content in the auricles with high $[Na]_i$ which were immersed in sodium-poor solution. This prediction was checked by using additionally a fluorometric method (von Hattingberg, Klaus, Lüllmann & Zepf, 1966) to determine the calcium content at the end of the influx periods in all auricles used for ^{45}Ca uptake measurements. In preparations exposed to sodium-poor solution the net entry of calcium was almost as large as the increase in influx measured by ^{45}Ca. Thus, $[Ca]_t$ increased by $0·1 \pm 0·08$, $0·47 \pm 0·09$ and $0·71 \pm 0·1$ mM/kg wet wt. × 10 min after $[Na]_i$ was raised from 12·5 mM to 20, 40 and 60 mM, respectively. However, there was only a small net uptake of calcium in sodium-rich auricles during the 10 min exposure to ^{45}Ca-containing Tyrode solution. Under this condition the maximal increase in $[Ca]_t$ between groups of auricles with 12·5 and 60 mM $[Na]_i$ was $0·2 \pm 0·06$ mM/kg wet wt. × 10 min ($P < 0·05$) while calcium net uptake in the other groups was not significant ($0·02 \pm 0·06$ and $0·1 \pm 0·05$ mM/kg wet wt. × 10 min). These results support strongly the conclusion drawn by Reuter & Seitz (1968) that calcium net transport into the outward direction is governed by the sodium-activated component of calcium efflux. They are also in agreement with the hypothesis on sodium–calcium exchange across the membrane presented in the Discussion. This theory predicts an effective outward transport of calcium provided $[Na]_o$ is higher than $[Na]_i$. This is the case with all preparations loaded in Tyrode solution but not in sodium-poor solution. Thus the sodium concentration gradient will determine the net uptake of calcium during the period of increased calcium exchange when $[Na]_i$ is high.

Since the results illustrated in Fig. 3 indicate clearly that a fraction of total calcium influx is dependent on $[Na]_i$ it was of interest to investigate whether this sodium-activated component of calcium *influx* has similar kinetic features as the sodium-activated component of calcium *efflux*. This component of calcium efflux has been shown to depend on the square of $[Na]_o$ and to follow saturation kinetics (Reuter & Seitz, 1968). The sodium-activated component of calcium influx was obtained from the results shown in Fig. 3. Both curves were extrapolated to zero $[Na]_i$. The intersects with the ordinate gave the sodium-independent components

of calcium influx (0·29 and 0·50 mM/kg wet wt. × 10 min) which were subtracted from each of the influx values in Fig. 3. The difference between sodium-independent fraction and experimental values is considered to be the sodium-activated component of calcium influx. When these components were plotted against the square of $[Na]_i$ the resulting curves showed a tendency toward saturation. This was confirmed by plotting the reciprocal of the sodium-activated component of calcium influx against the reciprocal of $[Na]_i^2$. The resulting straight line indicates that this component of calcium influx, $J_i^{Ca'}$, can be described by the equation

$$J_i^{Ca'} = \frac{J_{i,max}^{Ca'} [Na]_i^2}{K_m^{Na} + [Na]_i^2}, \tag{2}$$

where $J_{i,max}^{Ca'}$ is the maximal calcium influx which depends on $[Na]_i^2$ and K_m^{Na} is the apparent Michaelis constant of this influx component. Fig. 4

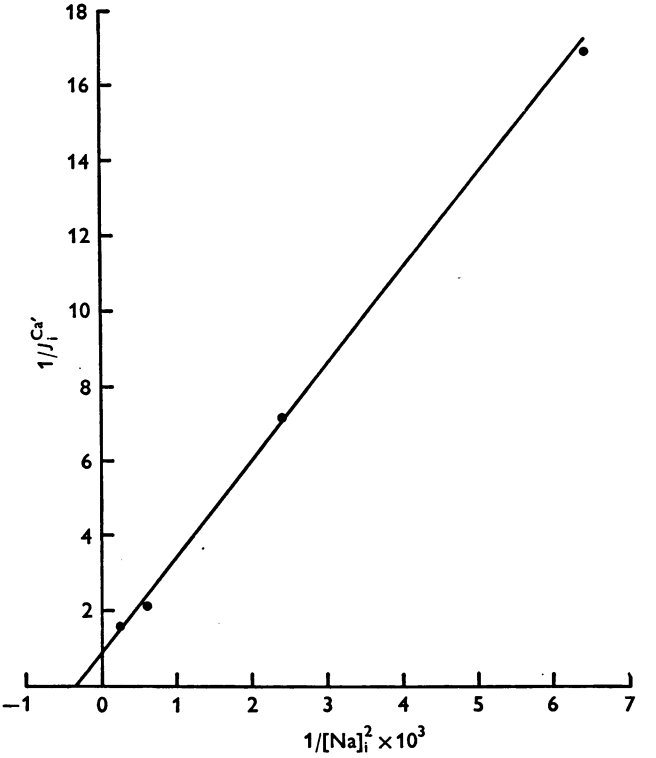

Fig. 4. Double reciprocal plot of sodium-activated calcium influx ($J_i^{Ca'}$; mM/kg wet weight × 10 min; ordinate) against $[Na]_i^2$ (mM/kg fibre water; abscissa). The values were derived from Fig. 3 (lower curve) as described in the text. The straight line is the reciprocal of the saturation curve calculated from eqn. (2) with K_m^{Na} = 3500 (mM)² and $J_{i,max}^{Ca'}$ = 1·35 mM/kg wet weight × 10 min.

shows the double reciprocal plot of the sodium-activated component of calcium influx measured in Tyrode solution. $J^{Ca'}_{i,max}$ and K^{Na}_m derived from this plot are 1·35 mM/kg wet wt. × 10 min and 3500 (mM $[Na]_i)^2$. The same plot of the influx results obtained in sodium-poor solution indicated a small tendency of $J^{Ca'}_{i,max}$ to increase under these conditions. It could be fitted better by taking $J^{Ca'}_{i,max}$ as 1·8 mM/kg × 10 min. However, the results

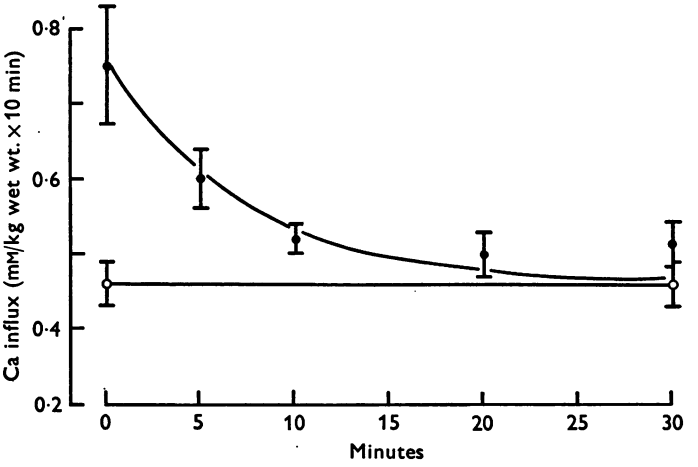

Fig. 5. Decline of calcium influx in auricles at various times after [Na] had been increased by rapid stimulation. Ordinate: calcium influx (mM/kg wet weight × 10 min); abscissa: minutes of re-incubation in normal Tyrode solution after increase of $[Na]_i$. Control preparations with 'normal' $[Na]_i$ (○) were soaked only in Tyrode solution before exposure to ^{45}Ca-containing Tyrode solution. The decline of calcium influx in the test preparations (●) various times after $[Na]_i$ had been increased could be fitted by an exponential (upper smooth curve) with a rate constant $k = 0{\cdot}14\ min^{-1}$. Each mean ± S.E. gives the results of ten preparations.

obtained in sodium-poor solution were complicated by the net uptake of calcium which could account for this slight variation of maximal influx rate.

High $[Na]_i$ declines exponentially to approximately 20 mM when auricles are re-incubated for 30 min in normal Tyrode solution (solution 1 in Table 1; cf. Glitsch, 1969). Since calcium influx is partly dependent on $[Na]_i^2$ both this fraction of calcium influx as well as $[Na]_i^2$ may decrease with the same rate constants under this condition. Fig. 5 illustrates the results of experiments in which $[Na]_i$ in guinea-pig auricles was first increased by rapid stimulation as described above. Afterwards the preparations were re-immersed in normal Tyrode solution for different times before calcium influx from sodium-containing solution was measured. Calcium influx was essentially the same in two groups of control preparations (circles) soaked

in Tyrode solution for 60 and 90 min without stimulation before exposure to ^{45}Ca. In the other groups of auricles (filled circles) in which $[Na]_i$ had been increased before incubation in normal Tyrode solution, calcium influx was much higher immediately after stimulation. It decreased exponentially when the preparations were re-incubated in normal Tyrode solution for various times after stimulation. The rate constant of the

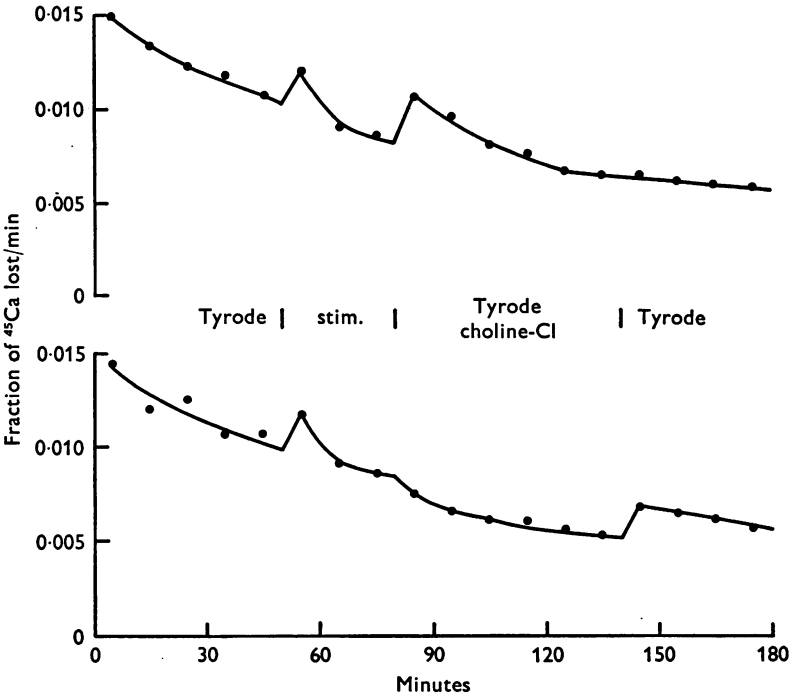

Fig. 6. Calcium efflux from resting auricles in normal Tyrode solution (upper curve) and sodium-poor solution (NaCl) replaced by choline-Cl (lower curve) after periods of rapid stimulation (stim. 300/min in potassium-, calcium-poor Tyrode solution). Ordinate: fraction of ^{45}Ca lost/min; abscissa: time.

exponential which fits the decline of calcium influx in Fig. 5 best was 0·14 min^{-1}. This rate constant is similar to the one obtained for the decrease of $[Na]_i^2$ under identical conditions (approximately 0·12 min^{-1}).

Increased $[Na]_i$ and calcium efflux. Calcium efflux was studied when $[Na]_i$ was increased by rapid stimulation similarly to the procedure used for calcium influx measurements. Results of two auricles are shown in Fig. 6. After a resting period in normal Tyrode solution the transient increase in ^{45}Ca efflux during the phase of rapid stimulation in calcium-poor, potassium-poor Tyrode solution may reflect an initial net loss of

calcium from the auricles. When the auricles were transferred afterwards to sodium-poor, choline-Cl solution (lower curve) calcium efflux decreased. Under the same condition calcium influx occurred as net influx in preparations loaded with ^{45}Ca (p. 32). The average inhibition of calcium efflux from three auricles in sodium-poor solution was $25 \pm 3\,\%$. However, net efflux of calcium was probably inhibited to a greater extent since the calcium-activated component of calcium efflux becomes larger while the sodium-activated component decreases under this condition (Reuter & Seitz, 1968).

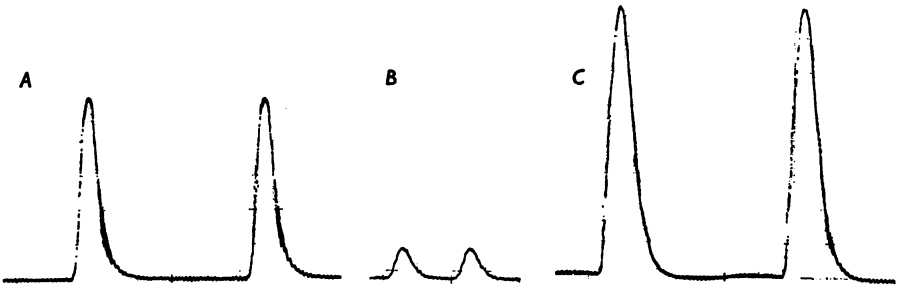

Fig. 7. Contractions of guinea-pig auricles in Tyrode solution before (*A*) and after (*C*) a 30 min period of rapid stimulation (300/min) in potassium-, calcium-poor Tyrode solution (*B*). Frequency of stimulation in *A* and *C*, 120/min.

The average increase in calcium efflux from four auricles exposed to normal Tyrode solution after the stimulation period in calcium-poor, potassium-poor Tyrode solution (Fig. 6, upper curve) was $40 \pm 2\,\%$ during the first collecting period. This result is not immediately obvious from the theory on sodium-calcium exchange presented in the Discussion. However, several factors which are in agreement with this theory could cause an increase in calcium efflux under the present condition. Thus, Reuter & Seitz (1968) have shown that the efflux increases after a rise of $[Ca]_o$ because the calcium-activated fraction of calcium efflux is smaller in calcium-poor than in normal Tyrode solution and presumably more carrier will be activated. In addition, it cannot be excluded that a small liberation of bound calcium from intracellular structures (cf. Palmer & Posey, 1967; Dransfeld, Greef, Schorn & Ting, 1969) could account for a small increase in calcium efflux in Tyrode solution when $[Na]_i$ becomes high. The increase in calcium efflux is formally in agreement with increased isotopic exchange since ^{45}Ca influx was appreciably larger than calcium net uptake under a comparable condition (Fig. 3 and p. 32). Unfortunately experimental limitations make it very difficult to separate quantitatively the

different factors which may be responsible for this increase in calcium efflux.

Effect of increased $[Na]_i$ *on contractility.* Since $[Na]_i$ had a marked influence on calcium influx in guinea-pig auricles it was of interest to investigate the contractility of the preparations when $[Na]_i$ was high. This is illustrated by Fig. 7. Record *A* shows single contractions of an auricle in normal Tyrode solution while record *B* was obtained during rapid stimulation in potassium-poor, calcium-poor Tyrode solution. Tension increased strongly in normal Tyrode solution immediately after the period of rapid stimulation (record *C*) and decreased afterwards to a lower steady-state level within about 20 min. Similar results were obtained with six auricles.

DISCUSSION

The main results which have to be discussed in greater detail are (i) the dependence of calcium influx on $[Na]_i$ in isolated guinea-pig auricles and (ii) the kinetic features of the sodium–calcium exchange system which maintains $[Ca]_i$ at a rather low level.

Baker & Blaustein (1968) and Baker *et al.* (1969) were the first to describe a calcium influx component which is dependent on $[Na]_i$ in crab nerve and squid axon. The investigation by Reuter & Seitz (1968) on cardiac tissue provided evidence for a calcium efflux component which depends on $[Na]_o$, and the interpretation of the results implied that $[Na]_i$ might also influence calcium influx. This has been confirmed by the present study. Even a slight increase in $[Na]_i$ above the 'normal' concentration, which is expected to be between 10 and 20 mM in guinea-pig auricles, increases calcium influx appreciably. An interesting finding in this respect is the increase in contractility of auricles which have been loaded with $[Na]_i$ (Fig. 7). This supports strongly the speculations about the cardiotonic action of digitalis recently proposed by Baker *et al.* (1969). A small increase in the amount of calcium which can be released during an action potential from intracellular stores could account for the positive inotropic effect of digitalis (cf. Klaus, 1963). This could be achieved by the promotion of calcium influx when the sodium pump is inhibited (Glynn, 1964; Repke, 1964). A large accumulation of $[Na]_i$ by toxic concentration of digitalis induces an appreciable net uptake of calcium (Klaus, 1963) because the carrier system becomes less effective for net transport of calcium into the outward direction (see below). This may cause contracture, especially since it has been shown that calcium uptake into sarcoplasmic reticulum (Palmer & Posey, 1967) or mitochondria (Dransfeld *et al.* 1969) of cardiac muscle is inhibited when $[Na]_i$ is high. Other hitherto unexplained results

by Niedergerke & Harris (1957) and Thomas (1960) could also be interpreted by the present findings. These authors demonstrated an increase in strontium and calcium influx in frog heart preparations soaked in potassium-free solution. Under this condition an accumulation of $[Na]_i$ did occur (Thomas, 1960) which promotes calcium influx and hence contractility.

Further speculations along these lines have been discussed by Baker *et al.* (1969) and by Reuter (1970). It should be mentioned, however, that despite the influence of the sodium–calcium exchange on cardiac contractility, twitch tension is also strongly dependent on calcium inward current during the plateau of the action potential (Reuter & Beeler, 1969; Beeler & Reuter, 1970). The rest of the discussion deals with kinetic features of the sodium–calcium exchange system.

When a carrier exists in the membrane which is loaded specifically by two sodium ions and one calcium ion at both surfaces one would expect that either of these ions at one surface can promote the unidirectional flux of the own species as well as that of the competing ion into the opposite direction. Therefore, when the concentration ratios $[Ca]_o/[Ca]_i$ and $[Na]_o^2/[Na]_i^2$ are unity in both cases, exchange of these ions without net flux should occur provided the affinity constants are the same on either surface of the membrane. In excitable tissues such as cardiac muscle or nerve, however, large concentration gradients for sodium and calcium exist across the membrane. In this case the simple ion exchange system becomes kinetically more complicated and the sodium gradient may provide the energy for extruding calcium against its electrochemical potential (Reuter & Seitz, 1968; Blaustein & Hodgkin, 1969). Furthermore, in the results obtained with guinea-pig auricles the apparent affinity (= reciprocal of the Michaelis constant) of the carrier for intracellular sodium seems to be at least two times smaller than for extracellular sodium. Since, however, the methods of determining the extracellular space may involve systematic errors (Page, 1962) there is some uncertainty about the absolute values of $[Na]_i$ and this difference in affinity cannot be considered as being entirely proved. At the outer surface of the membrane the affinity for sodium decreases when the affinity for calcium increases (Reuter & Seitz, 1968). It is not unreasonable to assume that the same happens to be the case at the inner surface. It is very satisfactory in this context that a fraction of total calcium influx is dependent on the square of $[Na]_i$ similarly to the sodium-activated component of calcium efflux which depends on $[Na]_o^2$ (Reuter & Seitz, 1968). The fraction of total carrier, F, on either side of the membrane which is occupied by sodium can thus be obtained by the application of the law of mass action (cf. Lüttgau & Niedergerke, 1958; Niedergerke, 1963; Reuter & Seitz, 1968; Baker *et al.*

1969). Since one calcium ion and two sodium ions act as competitors F is given by

$$F = \frac{[\mathrm{Na}]^2}{[\mathrm{Na}]^2 + K_m^{\mathrm{Na}}[\mathrm{Ca}]}, \tag{3}$$

where K_m^{Na} is the apparent dissociation constant of the sodium-activated calcium flux component.

K_m^{Na} increases with increasing [Ca] (Reuter & Seitz, 1968). In cardiac muscle the fraction of exchangeable $[\mathrm{Ca}]_t$ which is in equilibrium with the membrane-bound calcium competing with internal sodium is unknown. The results obtained by Blaustein & Hodgkin (1969) on squid axon indicate that it might be the free calcium ion concentration which is in equilibrium with the carrier-bound calcium. Provided this fraction of $[\mathrm{Ca}]_t$ does not change eqn. (3) shows that F becomes primarily a function of $[\mathrm{Na}]_i^2$. Therefore, when the carrier at the inner surface of the membrane is far from saturation as indicated by Fig. 2, calcium influx should increase proportionally to $[\mathrm{Na}]_i^2$. Under this condition eqn. (2) becomes applicable and describes adequately the sodium-activated component of calcium influx which has been investigated in the present study (Fig. 4).

Under steady-state conditions in Tyrode solution the flux ratio for sodium-activated calcium fluxes can be derived from eqns. (2) and (3). Provided $J_{i,\max}^{Ca'}$ and $J_{e,\max}^{Ca'}$ are equal the ratio for the unidirectional sodium-activated calcium fluxes would be given by

$$\frac{J_i^{Ca'}}{J_e^{Ca'}} = \frac{[\mathrm{Na}]_i^2}{[\mathrm{Na}]_o^2} \times \frac{[\mathrm{Na}]_o^2 + K_m^{\mathrm{Na_o}}[\mathrm{Ca}]_o}{[\mathrm{Na}]_i^2 + K_m^{\mathrm{Na_i}}[\mathrm{Ca}]_i} \tag{4}$$

(cf. Stein, 1967). Assuming that $[\mathrm{Ca}]_i$ in closest vicinity to the inner surface of the membrane is $1{\cdot}55 \times 10^{-5}$ M, a value which is derived from eqn. (5), the flux ratio would be approximately unity with $K_m^{\mathrm{Na_o}} = 1670\ (\mathrm{mM})^2$ (Reuter & Seitz, 1968) and $K_m^{\mathrm{Na_i}} = 3500\ (\mathrm{mM})^2$. This ratio indicates the ability of the system to provide net flux of calcium in the outward direction when $[\mathrm{Ca}]_i$ becomes higher than 10^{-5} M under these conditions. The calcium-activated fractions of the calcium fluxes shuttle calcium forth and back only.

The concentration gradient of sodium across the membrane is maintained by the sodium pump, while the free energy required for net transport of calcium against its concentration gradient could be derived from the free energy present in the concentration ratio $[\mathrm{Na}]_o^2/[\mathrm{Na}]_i^2$. In agreement with this assumption is the finding that calcium content increases in DNP-poisoned auricles because of a large net uptake of sodium, although total calcium efflux is enhanced under this condition (Reuter & Seitz, 1968; cf. also Blaustein & Hodgkin, 1969, and Rojas & Hidalgo, 1968, for

squid axon). In favour of this assumption is also the low activation energy for calcium efflux from cardiac preparations (5·9 kcal/mole; Reuter & Seitz, 1968; Reuter, 1970). In such a system in which sodium influx is coupled with calcium efflux the steady-state distribution ratio of calcium can be expressed by

$$\frac{[Ca]_i}{[Ca]_o} = \frac{[Na]_i^2/K_m^{Na_i}}{[Na]_o^2/K_m^{Na_o}}. \tag{5}$$

With $[Na]_i^2 = 400$ (mM)^2, $[Na]_o^2 = 22200$ (mM)^2, and $K_m^{Na_i}$ and $K_m^{Na_o}$ as in eqn. (4), $[Ca]_i$ could be reduced to $1{\cdot}55 \times 10^{-5}$ M at the inner surface of the membrane.

The conclusions one can draw from these results are fourfold: (1) the carrier which exchanges sodium and calcium across the membrane is loaded with these ions on both sides of the membrane in principally the same way according to the law of mass action; (2) the carrier is almost saturated by the normal concentrations of calcium and sodium in Tyrode solution, i.e. outside the membrane, while it is far from saturation by the inside concentrations of these ions; (3) the affinity of the carrier for sodium may be smaller at the inner surface of the membrane than at the outer surface; (4) since a smaller fraction of the carrier is loaded with sodium at the inside of the membrane compared to the outside (cf. Reuter & Seitz, 1968), a net transport of calcium in the outward direction occurs as a result of the sodium-calcium exchange. When $[Na]_o$ is reduced or $[Na]_i$ becomes very high calcium accumulates within the cell.

The precise over-all free calcium ion concentration, $[Ca^{2+}]_i$, in the myoplasm of cardiac muscle during relaxation is not known, although one can assume that it is not much higher than 10^{-7} M (Portzehl, Caldwell & Rüegg, 1964; Katz & Repke, 1966; Weber, Herz & Reiss, 1967). Thus the sodium–calcium exchange system may be responsible for calcium net transport into the outward direction only when $[Ca^{2+}]_i$ exceeds 10^{-5} M. Intracellular calcium transport systems of sarcoplasmic reticulum and mitochondria (Weber *et al.* 1967; Patriarca & Carafoli, 1968), however, may reduce $[Ca^{2+}]_i$ in the myoplasm much further.

This interpretation of the sodium–calcium exchange in terms of a carrier hypothesis does not involve the electric field across the membrane, i.e. the membrane potential. Provided the loaded carrier is charged and/or the association of the ions with the carrier is influenced by the electric field one must introduce a term for this field as an additional factor (cf. Reuter, 1970). This could be done by applying the principles of the constant field theory (Goldman, 1943; Hodgkin & Katz, 1949) to the carrier model. However, up to this stage we have no experimental evidence which would allow us definitely to include such an expression. An important

problem which has to be solved in this context is the coupling ratio between calcium influx and sodium efflux. Baker *et al.* (1969) have shown that $[Ca]_o$ has a strong influence on sodium efflux from squid axon, especially when $[Na]_i$ is high. The similar rate constants for decrease of $[Na]_i^2$ and calcium influx (Fig. 5) as well as the present kinetic considerations suggest that the same might be the case in isolated cardiac preparations.

The present interpretation of the results in terms of a carrier system is based entirely on experimental criteria as saturation, ion selectivity and ion competition. However, the kinetic characteristics of a system with a limited number of adsorption sites and those of a carrier system can be very similar and in fact there may be no molecular distinction to be made (Britton, 1966). Therefore, the kinetic behaviour described above might also be a consequence of interdiffusion of ions in a porous ion exchange membrane with adsorption sites of high selectivity for calcium and sodium ions. A recent paper by van Breemen & van Breemen (1969) on calcium exchange across artificial porous phospholipid membranes also suggests the possibility of such an alternative explanation.

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