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Kinetic Analysis of Calcium Movements in Cell Culture

V. Intracellular Calcium Distribution in Kidney Cells

André B. Borle

Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

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Summary. The distribution of intracellular calcium was determined in isolated kidney cells by kinetic analyses of ^{45}Ca fluxes. Isotopic desaturation curves reveal an intracellular calcium compartment with a very slow time constant. The size of this calcium compartment is markedly increased by raising the extracellular calcium, by increasing the extracellular phosphate and may contain up to 99% of the intracellular exchangeable calcium. Accumulation of calcium in this pool is completely abolished by two specific inhibitors of mitochondrial calcium uptake, Antimycin A and Warfarin®. These results suggest that this compartment represents a pool of calcium in the cell mitochondria. The sudden removal of phosphate from the medium immediately stimulates calcium efflux from the cell. Conversely, an increase in medium phosphate immediately inhibits calcium efflux. Both effects are rapidly reversible. Finally, calcium efflux from the cells is stimulated after the cells are exposed to low temperature suggesting that calcium transport out of the cell may be regulated by the cytoplasmic calcium activity. These experiments are consistent with the view that mitochondria play an important role in the control and regulation of cytoplasmic calcium activity and of calcium transport.

In previous papers, I have shown that ^{45}Ca uptake by isolated kidney cells can be adequately described by two exponential terms (Borle, 1969*a*; 1970). Calcium efflux studied in a closed system also fits a two-compartment model (Borle, 1969*b*). The fast component of calcium exchange has a half time of 1 min and can be attributed to an extracellular calcium compartment. The slow component, with a half time of 25 to 30 min, probably represents calcium transport across the plasma membrane of the cell (Borle, 1969*a*, *b*; 1970).

Desaturation experiments in an open system have been successfully used by many investigators to study calcium exchange and the effects of various agents on calcium efflux (Langer, 1964; Isaacson & Sandow, 1967). By using the same technique in isolated kidney cells, it is possible to detect at

least three exponential components, with time constants of 2, 20 and 200 min, respectively. The magnitude of the third, very slowly exchangeable compartment is markedly affected by the extracellular calcium and phosphate concentrations and by metabolic inhibitors. Indirect evidence suggests that this compartment represents a calcium pool in the cell mitochondria.

Materials and Methods

Method and Calculation

Monkey kidney cells (LLC MK_2) are grown as monolayer in 800-cm^2 roller bottles in Eagle's minimum essential medium supplemented with 10% calf serum and antibiotics. The cells are harvested from the glass with a soft rubber policeman and dispersed in a Krebs-Henseleit buffer. A cell suspension consisting of 100 to 200 mg cells (10 to 20 mg cell protein) in 6 ml of buffer is placed in a 75 × 120 mm polycarbonate test tube in a 37 °C waterbath. The cell suspension is kept dispersed with a minute teflon-coated magnet bar and an immersible magnetic stirrer. The cells are preincubated for 1 hr. After the preincubation period, 50 to 100 μC of ^{45}Ca are added to the cell suspension for exactly 60 min. At the end of the labeling period, the teflon magnet is removed from the tube and the cell suspension is centrifuged for 30 sec at 2,000 rpm on an International centrifuge kept at 37 °C. Centrifugation is stopped with the electric brake, the radioactive medium decanted from the tube, the cells are washed once and resuspended in fresh nonradioactive medium in the waterbath. The whole procedure, from the time the cells are removed from the bath until they are replaced in it with fresh medium, lasts exactly 1 min. The cell suspension is immediately divided into two fresh tubes, each containing half the original cell mass in 3 ml of medium and placed in the waterbath. Isotope desaturation is then performed simultaneously in both tubes. The medium is replaced at 1, 5, 10 and 20 min and then every 10 min for 2 to 4 hr. After each centrifugation, the 3 ml of medium is directly decanted in counting vials, 15 ml of "cocktail D" [1] are added and ^{45}Ca is measured on an ambient temperature liquid scintillation spectrometer (Beckman LS-100). At the end of the desaturation period, the cells are homogenized in 16 ml of distilled water with an ultrasonic probe. Three chemical determinations are performed on aliquots of the cell homogenate: cell proteins are measured by a modification of the Lowry method, total calcium by fluorometric titration and ^{45}Ca by liquid scintillation spectrometry.

Calculation of the Compartment Size

The sum of all the radioactivity in each wash plus the radioactivity left in the cells at t he end of the experiment is taken as the total radioactivity present in the cells at the beginning of the desaturation period. The radioactivity remaining in the cells at each time point is obtained by sequentially subtracting the radioactivity of each wash. It is plotted on semilogarithmic paper as per cent of the total initial radioactivity (Fig. 1). Three different phases of calcium efflux are obtained by graphical analysis, and the slope and intercept of each component are obtained by computer. Phase 1 has a time constant of 1 to 2 min, phase 2 of 20 to 30 min and phase 3 of about 300 min. Every

1 Cocktail D is a cocktail for ambient temperature liquid scintillation spectrometry. It will accept up to 25% of aqueous solutions. Its composition is as follows: PPO 5 g/liter, Napthalene 100 g/liter, Dioxane to 1 liter.

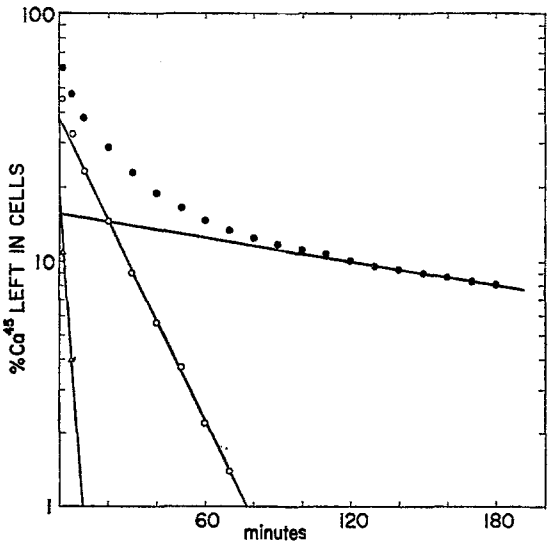

Fig. 1. The three phases of calcium efflux obtained by graphical analysis of a typical ^{45}Ca desaturation curve obtained in isolated kidney cells. Every desaturation was performed for 4 hr. The plot was consistently linear from 140 to 240 min giving clear evidence that the slowest phase is a single exponential

desaturation was performed for 4 hr. The plot was consistently linear from 140 to 240 min giving clear evidence that the slowest phase is a single exponential.

Assumptions

Assumption 1. Since the time constant of the three phases differ by at least one order of magnitude, the system can be analyzed as if it consisted of three parallel compartments, without introducing an error greater than 5 to 10% (*see below*). Since the present technique of isotope desaturation might not be fast enough to measure with an acceptable precision, the fast component of efflux having a time constant of a few minutes, only phases 2 and 3 will be considered in this paper.

Assumption 2. Since the system is at steady state, the fluxes and the rate constant of exchange are equal during the uptake of isotope and during the desaturation period. The change in specific activity X_i in each compartment i can be described by the following exponential function:

$$\frac{X_i}{E} = 1 - e^{-\lambda_i t} \tag{1}$$

where E is the specific activity of compartment i at infinite time or equilibrium specific activity.

Assumption 3. Since the fluxes in and out of the medium are so small as to be negligible (Borle, 1970) the specific activity of the medium is constant during the labeling phase and can be accepted to be equal to E. By definition

$$X_i = \frac{R_i}{S_i} \tag{2}$$

where R_i is the radioactivity and S_i the amount of calcium in compartment i. Therefore, from Eqs. (1) and (2)

$$\frac{R_i}{S_i}=E(1-e^{-\lambda_i t}) \tag{3}$$

or

$$S_i=\frac{R_i}{E(1-e^{-\lambda_i t})}. \tag{4}$$

At the end of 1 hr of ^{45}Ca uptake, $t=60$ min.

$R_{i60\,\mathrm{min}}$ and λ_i are obtained from the desaturation curve; $R_{i60\,\mathrm{min}}$ is the radioactivity of the compartment i at the beginning of the desaturation period given by the intercept of each phase at 0 time; λ_i is the slope of component i of the desaturation curve. Knowing the medium specific activity E, the amount of calcium in each compartment S_i can be calculated from Eq. (4). From the cell mass expressed in milligrams of cell protein, the surface area of the cells can be calculated (Borle, 1969*a*) and the fluxes ϕ_{ij} and the rate constant of transfer k_{ij} are obtained assuming that

$$\phi_{ij}=k_{ij}S_i. \tag{5}$$

Huxley (1960) has pointed out that when one is faced with two exponential terms describing the desaturation curve of an isotope leaving two compartments such as

$$S_2+S_3=A\,e^{-\lambda_1 t}+B\,e^{-\lambda_2 t} \tag{6}$$

it is incorrect to assume that

$$S_2=A\,e^{-\lambda_1 t} \tag{7}$$

and

$$S_3=B\,e^{-\lambda_2 t}. \tag{8}$$

This assumption often leads to a gross overestimate of the slow compartment. However, this error is often minimal when the time constants of the two phases are widely different. The error can be estimated by the equation given by Huxley:

$$S_{3_0}=\frac{AB(\lambda_1-\lambda_2)^2}{A\lambda_1^2+B\lambda_2^2} \tag{9}$$

where S_{3_0} is the compartment size of the slow compartment at time 0, and A, B, λ_1 and λ_2 are the coefficients and exponentials of the differential equation.

Table 1 gives the coefficients and exponentials of the last two phases of calcium efflux from kidney cells obtained from nine experiments.

Table 1. Coefficients and exponentials of phase 2 and phase 3 of calcium efflux[a]

Coefficients and exponentials of Eq. (9)	Experimental data
A	1.83 ± 0.22
B	2.54 ± 0.43
λ_1	0.0466 ± 0.0021
λ_2	0.00455 ± 0.00085

[a] Values are mean $\pm$ SE of nine experiments.

In our case,

$$A = 0.72\,B \tag{10}$$

and

$$\lambda_1 = 10.2\,\lambda_2. \tag{11}$$

Substituting the values of Eqs. (10) and (11) in Eq. (9) shows that

$$S_{3_0} = 0.95\,B. \tag{12}$$

Therefore, our assumption that the slow component of efflux B equals the compartment S_3 overestimates its value by 5% which is an acceptable error for these studies.

Calculation of the Efflux Rate Coefficient

To study the effects of various agents and of the external medium composition on calcium efflux, two desaturation experiments are performed simultaneously on two groups of cell suspensions which have been uniformly labeled in one single tube. At a fixed time during the desaturation, one group of cells is exposed to the test medium and its efflux rate coefficient is compared to the control group. The rate coefficient of efflux ERC is calculated according to Isaacson and Sandow (1967):

$$\mathrm{ERC}_i = \frac{\Delta C_i}{C m_i \Delta t}\,100\% \tag{13}$$

where ΔC_i is the radioactivity lost from the cells during the time interval Δt, and Cm_i is the mean radioactivity left in the cells between time t_i and $t_i + \Delta t$:

$$C m_i = \frac{C m_{t_i} + C m_{t_i} + \Delta t}{2}. \tag{14}$$

The rate coefficient of the control group is taken as 100% and the changes in the experimental group is expressed as per cent of the control group.

Results

Calcium Efflux and Intracellular Compartments

Table 2 presents the results of nine experiments performed in a Krebs-Henseleit bicarbonate buffer containing 1.3 mM Ca and 1.0 mM phosphate. Calcium efflux from the second compartment is 83.4 pmoles/(mg protein × min); calcium efflux from the third compartment is 11.1 pmoles/(mg protein × min) or approximately one order of magnitude smaller. The exchangeable calcium pools of phases 2 and 3 are, respectively, 1.83 and 2.54 nmoles/mg protein. Calcium efflux from the second phase is almost identical with the slow phase of calcium influx previously obtained by uptake experiments, 86.0 ± 8 pmoles/(mg protein × min) (Borle, 1970). Since the system is at steady state, one can assume that these fluxes represent calcium exchange with the same intracellular compartment. Calcium efflux from the second compartment can then be expressed in the classic units of

Table 2. Calcium efflux and calcium pools size determined by desaturation experiments in kidney cells[a]

Kinetic parameters of Ca efflux	Experimental data
Phase 2	
Efflux (pmoles/mg protein × min)	83.4 ±8
Derived efflux[b] (fmoles cm^{-2} sec^{-1})	41.7 ±4
Pool size (nmoles/mg protein)	1.83 ±0.22
Derived pool size (mmoles/kg cell water)[c]	0.17 ±0.02
Rate constant (min^{-1})	0.0466 ±0.0021
Phase 3	
Efflux (pmoles/mg protein × min)	11.1 ±1
Pool size (nmoles/mg protein)	2.54 ±0.43
Rate constant (min^{-1})	0.00455 ±0.00085

[a] Values are mean ± SE of nine experiments performed in a medium containing 1.3 mM calcium and 1.0 mM phosphate, at pH 7.4.
[b] Derived from the cell surface of isolated kidney cells 33.5 cm^2/mg protein (Borle, 1970).
[c] Derived from the water content of isolated kidney cells 10.9 mg/mg protein (Borle, 1970).

flux, i.e. moles cm^{-2} sec^{-1}, since it represents calcium transfer through the cell membrane. Knowing that the surface area of isolated kidney cells is 33.5 cm^2/mg protein (Borle, 1970), calcium efflux from phase 2 is 41.7 ±4 femtomoles cm^{-2} sec^{-1} (femtomoles = 10^{-3} pmoles or 10^{-15} moles). Table 3 presents the fluxes and the pool size of compartment 2 obtained by two completely different kinetic methods. The fluxes are almost identical, but the compartment size obtained by desaturation is significantly smaller. However, the second phase obtained by calcium influx represents the total intracellular exchangeable calcium since the resolution by this method cannot distinguish two separate intracellular compartments. Desaturation

Table 3. Comparison between the calcium fluxes and the compartment size of phase 2 as determined by influx and efflux experiments[a]

Experimental conditions	No. of exp.	Calcium flux (fmoles cm^{-2} sec^{-1})	Calcium pool (nmoles/mg protein)
Determined by efflux	9	41.7 ±4[b]	1.83 ±0.22
Determined by influx[c]	5	43.0 ±4	3.30 ±0.17

[a] Values are mean ± SE.
[b] Derived value obtained from the cell surface of isolated kidney cells 33.5 cm^2/mg protein (Borle, 1970).
[c] Taken from Borle (1970, Table 3).

Table 4. Influence of the medium calcium concentration on the calcium efflux and the calcium pool size[a]

Kinetic parameters	Medium calcium	
	0.6 mM	1.3 mM
Phase 2		
Efflux (pmoles/mg protein × min)	60.8 ±8.4	83.4 ±8
Derived efflux (fmoles cm^{-2} sec^{-1})	30.4 ±4.2	41.7 ±4
Pool size (nmoles/mg protein)	1.56±0.12	1.83±0.22
Phase 3		
Efflux (pmoles/mg protein × min)	8.48±0.96	11.1 ±1
Pool size (nmoles/mg protein)	2.32±0.39	2.54±0.43

[a] Values are mean ± SE of nine experiments in each group.

experiments, however, are able to detect a third phase and if one adds compartments 2 and 3 the value obtained, 4.37 nmoles/mg protein, is slightly larger than the second phase of calcium influx, 3.30 nmoles/mg protein. The second phase of calcium efflux probably represents the exchangeable calcium pool of the cell cytoplasm, whereas the third phase could represent sequestration of calcium in a separate intracellular compartment. Decreasing the medium calcium concentration from 1.3 to 0.6 mM, does not affect significantly the size of either compartment, although calcium efflux from both phases is slightly depressed (Table 4). The magnitude of the depression is similar to that obtained previously by influx experiments (Borle, 1970).

Influence of the Medium Phosphate Concentrations

Table 5 shows that raising the medium phosphate concentration from 0 to 5 mM in presence of 1.3 mM calcium produces a dramatic increase in the total cell calcium measured chemically by fluorometric titration at the end of the desaturation experiments[2]. The second and third compartments measured by isotope desaturation also increase with increasing extracellular phosphate concentration. However, there is a marked difference between the two phases: whereas the second pool rises only five times, the third pool increases 500-fold. In another series of experiments the medium calcium concentration was reduced to 0.4 mM. Fig. 2 shows the desaturation curves obtained by increasing the medium phosphate from 0 to 20 mM.

2 Phosphate was added as primary and secondary sodium phosphate in the appropriate ratio for a pH of 7.4. NaCl was reduced accordingly to obtain a final Na concentration of 142 mM.

Table 5. Influence of the medium phosphate concentration on the calcium distribution in kidney cells

Medium		Total cell calcium	Calcium pools	
Calcium (mM)	Phosphate (mM)	(nmoles/mg protein)	Phase 2	Phase 3
1.3	0	66.8	1.33	0.89
1.3	0	73.4	1.20	0.93
1.3	1	125	1.95	2.85
1.3	1	100	1.80	2.36
1.3	2	151	3.15	10.5
1.3	2	152	3.02	10.0
1.3	3	164	2.98	13.2
1.3	3	134	1.65	11.1
1.3	5	739	6.50	514
1.3	5	756	5.48	400

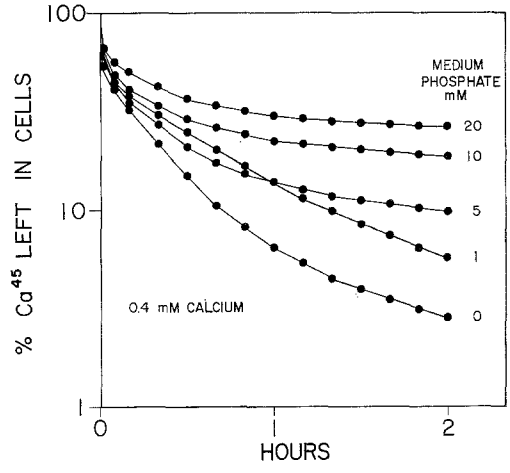

Fig. 2. Effect of increasing concentrations of extracellular phosphate on ^{45}Ca efflux from kidney cells

Fig. 3 presents the pool size of the two phases derived from 16 experiments. The pattern is identical to that obtained with 1.3 mM calcium: the pool size of the second phase rises slightly from a mean of 0.67 to 0.98 nmoles/mg protein whereas the third compartment increases from 0.35 to 25.5 nmoles/mg protein.

Influence of the Medium Calcium Concentration

The concentration of calcium in the medium also influences the calcium desaturation curves, the total cell calcium and the intracellular calcium

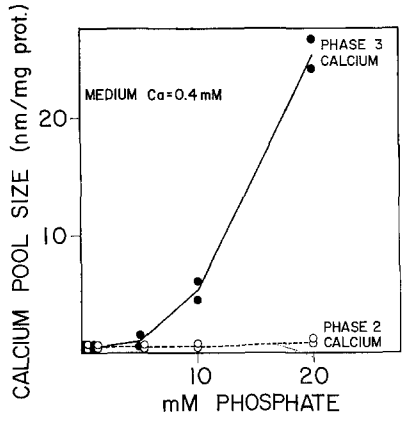

Fig. 3. Effect of increasing concentrations of extracellular phosphate on the calcium pool size of the slowest phase (phase 3) and of the second phase

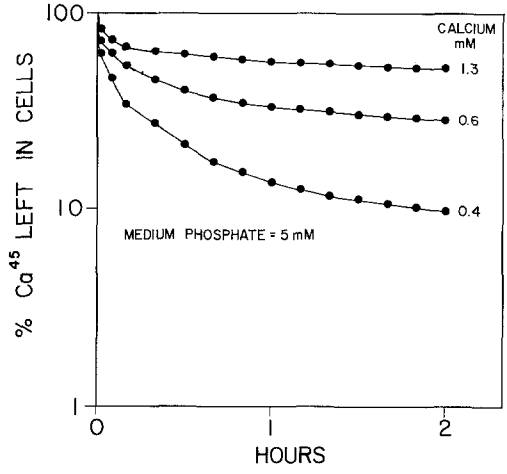

Fig. 4. Effect of increasing concentration of extracellular calcium on calcium efflux from kidney cells

distribution (Fig. 4). Table 6 shows that in a phosphate-free medium the total cell calcium is only slightly affected by the changes in the medium calcium concentration. With 1 mM phosphate, however, there is a linear relationship between the medium calcium and the total cell calcium. With 5 mM phosphate there is a very large increase in cell calcium for a relatively moderate change in extracellular calcium concentration.

Despite the rise in total cell calcium, the distribution of calcium between the two intracellular compartments does not show any significant trend in presence of 1 mM phosphate (Table 7). This would suggest that intracellular

Table 6. Influence of the medium concentration of calcium and phosphate on the total cell calcium [a]

Medium		No. of Exps.	Total cell calcium
Calcium (mM)	Phosphate (mM)		(nmoles/mg prot)
0.4	0	2	32.9 ± 6.69
0.6	0	14	38.9 ± 4.97
1.3	0	24	41.8 ± 2.68
0	1.0	2	13.6 ± 0.62
0.4	1.0	2	38.2 ± 1.1
0.6	1.0	8	59.6 ± 6.9
1.3	1.0	17	98.2 ± 7.4
2.5	1.0	2	162 ± 3.2
0.4	5.0	2	18.7 ± 0.03
0.6	5.0	2	106 ± 8.1
1.3	5.0	2	747 ± 8.5

[a] Values are mean ± SE.

Table 7. Influence of the medium calcium concentration on the calcium distribution in kidney cells (physiological medium phosphate)

Medium		Calcium pools	
Calcium (mM)	Phosphate (mM)	Phase 2 (nmoles/mg protein)	Phase 3
0.4	1.0	0.68	0.43
0.4	1.0	0.68	0.40
0.6	1.0	2.65	3.51
0.6	1.0	1.50	3.03
1.3	1.0	1.95	2.86
1.3	1.0	1.80	2.36
2.5	1.0	1.81	1.00
2.5	1.0	1.97	1.28
5.0	1.0	1.57	1.28
5.0	1.0	1.55	1.21

calcium is not affected by changes in extracellular calcium. However, Table 8 shows that in presence of 5 mM phosphate the third compartment is extremely sensitive to small changes in extracellular calcium. It increases 500-fold between 0.4 and 1.3 mM calcium in the medium. The second compartment is 50 times less sensitive, and rises only 10-fold.

In one respect, these results are similar to those obtained with increasing extracellular phosphate: the size of the second compartment increases with increasing medium concentrations of calcium and phosphate, but the rise

Table 8. Influence of the medium calcium concentration on calcium distribution in kidney cells (high medium phosphate)

Medium		Calcium pool	
Calcium (mM)	Phosphate (mM)	Phase 2 (nmoles/mg protein)	Phase 3
0.4	5.0	0.42	0.58
0.4	5.0	0.37	1.55
0.6	5.0	1.75	34.9
0.6	5.0	1.68	29.4
1.3	5.0	6.50	514
1.3	5.0	5.48	400

is only 1/50 to 1/100 of that observed in the third compartment. This suggests that the affinity of the third compartment for calcium is such that it functions as a buffer mechanism which prevents wide fluctuations in exchangeable calcium in the second compartment. Furthermore, the uptake of calcium in the third compartment depends not only on calcium but to a large extent on the phosphate concentration.

Effects of Antimycin A and Warfarin® on the Intracellular Calcium Distribution

Since the properties of the third compartment are so similar to those of isolated mitochondria (Rossi, Carafoli, Drahota & Lehninger, 1966; Leh-

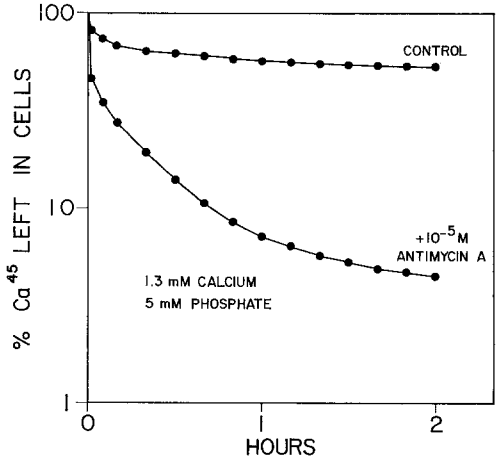

Fig. 5. Effect of 10^{-5} M Antimycin A on the ^{45}Ca desaturation curve in kidney cells

Table 9. Effect of 10^{-5} M Antimycin A and 10^{-5} M Warfarin® on the influence of phosphate on the calcium distribution in kidney cells

Medium		Inhibitor	Total cell calcium	Calcium pools	
Calcium (mM)	Phosphate (mM)	(10^{-5} M)	(nmoles/mg cell protein)	Phase 2	Phase 3
1.3	2.0	Antimycin	51.7	1.30	0.53
1.3	2.0	Antimycin	57.6	1.30	0.61
1.3	3.0	Antimycin	55.6	1.10	0.60
1.3	3.0	Antimycin	55.2	1.02	0.65
1.3	4.0	Antimycin	63.7	1.16	0.69
1.3	4.0	Antimycin	69.4	0.89	0.83
1.3	5.0	Antimycin	119	1.04	1.30
1.3	5.0	Antimycin	114	0.99	2.06
1.3	5.0	Warfarin®	87.8	1.59	1.05
1.3	5.0	Warfarin®	82.8	1.25	0.94
0.6	5.0	Antimycin	19.29	0.94	0.34
0.6	5.0	Antimycin	17.11	0.93	0.36
0.6	5.0	Warfarin®	71.3	1.04	0.87
0.6	5.0	Warfarin®	99.7	1.61	1.07

ninger, 1970), it was interesting to test the effects of mitochondrial inhibitors of calcium uptake on the intracellular calcium distribution.

Fig. 5 shows the effect of 10^{-5} M Antimycin on the desaturation curve of a typical experiment compared with its control.

Table 9 presents the results of 14 experiments obtained with 10^{-5} M Antimycin A or 10^{-5} M Warfarin® (3-(α-acetonylbenzyl)-4-hydroxycoumarin) at various concentrations of calcium and phosphate. These inhibitors completely abolish the effects previously obtained with increasing calcium and phosphate concentration on the third compartment (Table 5 and Fig. 3). Moreover, the second compartment also remains constant and does not reflect the wide concentration changes prevailing in the extracellular fluid. This suggests that the size of the calcium pool of the second phase is determined mainly by the third compartment and not by the extracellular concentration of calcium or phosphate. These results also suggest that the third compartment may represent a calcium pool sequestered in mitochondria since Antimycin A and Warfarin® are specific inhibitors of calcium uptake by mitochondria.

Effect of Extracellular Phosphate on Ca Efflux

It is possible to perturb the steady-state efflux of calcium by altering the conditions in the external medium during the desaturation experiments.

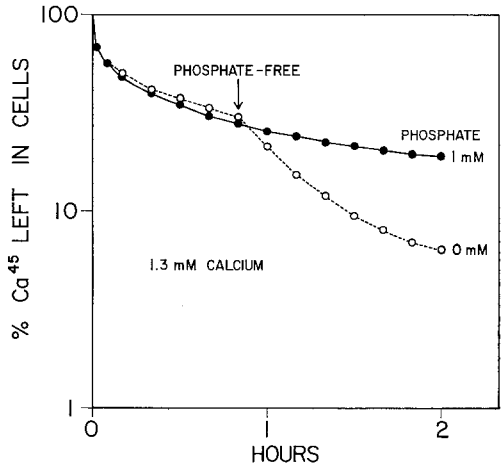

Fig. 6. Effect of the sudden removal of phosphate from the extracellular buffer on the ^{45}Ca desaturation curve in kidney cells

This method has been successfully used by Isaacson and Sandow (1967) to study the effects of quinine and caffeine on calcium efflux from muscle. The results obtained are purely qualitative and do not bring any information concerning pool sizes or rates of transport. However, they show whether a specific alteration in the external medium inhibits or stimulates calcium efflux and how rapidly the change occurs. Since the concentration of extracellular phosphate has a marked influence on the intracellular distribution of calcium, it was interesting to investigate the effects of the deletion or of the addition of phosphate in the medium on calcium efflux. Fig. 6 shows the sudden effect of a phosphate-free buffer on the desaturation curve of ^{45}Ca compared with a control experiment performed in 1 mM phosphate. Fig. 7 presents the efflux rate coefficient of six experiments in which the cells were equilibrated and labeled in a normal medium containing 1.3 mM calcium and 1.0 mM phosphate. The desaturation was performed in a medium of same composition until minute 50. At that time phosphate was omitted in the medium for six consecutive 10-min washout periods. At minute 110, the washout was continued in the initial medium containing 1.0 mM phosphate. Fig. 7 shows that immediately after the removal of phosphate from the medium, the calcium efflux rate coefficient (ERC) jumped from 100 to 210% of the control rate ($p<0.001$). In the next 10 minutes, ERC rises to 275% ($p<0.001$) and it reaches a maximum of 290%, 30 to 60 min after phosphate removal. When the medium phosphate concentration is restored to 1 mM, ERC immediately drops from 290 to 210%

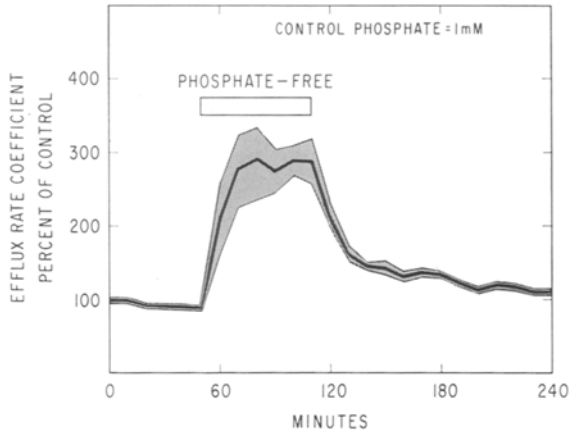

Fig. 7. Effect of the sudden removal of phosphate from the extracellular buffer on the calcium efflux rate coefficient. From 1 to 50 min the cells are desaturated in a medium containing 1 mM phosphate and 1.3 mM calcium. From 50 to 110 min. the cells are desaturated in a medium containing 1.3 mM calcium and no phosphate. After 110 min, 1 mM phosphate is again present in the medium

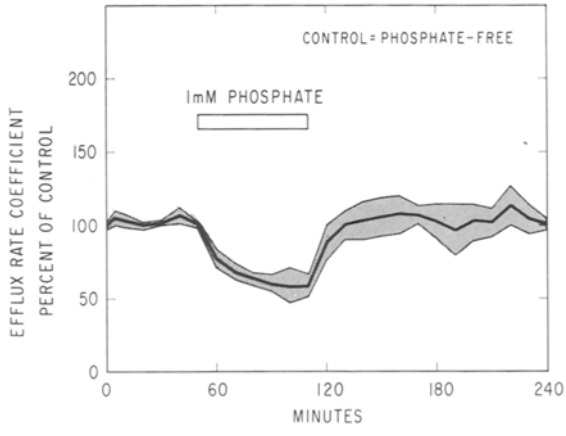

Fig. 8. Effect of extracellular phosphate on the calcium efflux rate coefficient. From 1 to 50 min the cells are desaturated in a medium containing 1.3 mM calcium and no phosphate. From 50 to 110 min, 1 mM phosphate is present in the medium. After 110 min, desaturation again takes place in a phosphate-free medium

and then slowly decreases toward the control levels. Fig. 8 shows the results of four experiments in which the reverse procedure was performed. The cells were incubated and labeled with ^{45}Ca in a phosphate-free buffer. During the first 50 minutes, desaturation was performed in the same phosphate-free medium. At minute 50, 1 mM phosphate was added in the medium

of the six consecutives washout periods. At minute 110, the washout was continued in the initial phosphate-free buffer. The results show that as soon as phosphate is added to the cell suspension, the ERC drops from 100 to 78% of the control levels to reach a minimum rate of 59%, 40 to 50 min later. When phosphate is again deleted from the washout medium, the ERC immediately returns to the control levels within 10 to 20 min.

Effects of Changes in Temperature on Calcium Efflux

Calcium efflux from the cell and calcium uptake by the mitochondria are both metabolically dependent transports. It is reasonable to assume that they will have higher Q_{10} than the presumably passive processes of calcium influx into the cytoplasm and of calcium release from the mitochondria. Consequently, lowering the temperature should cause a greater inhibition on the two processes which transport calcium out of the cytoplasm than on the two processes transferring calcium into it. The result should be an increased calcium concentration in the cytoplasm. Then, if the temperature were raised back to normal, calcium efflux should jump to levels higher than control because of the increased cytoplasmic calcium concentration. The peak of the increase should be proportional to the length of the cold exposure period. Figs. 9 and 10 illustrate two typical experiments taken from a series of 14 experiments performed to test this hypothesis. The cells were incubated, labeled and desaturated for the first 40 min in normal buffer at 37 °C. At minute 40, the cells were desaturated at 4 °C by placing them in an ice water bath for various lengths of time (20 to 80 min). Then the washout was continued at 37 °C. Fig. 9 shows that when the cells

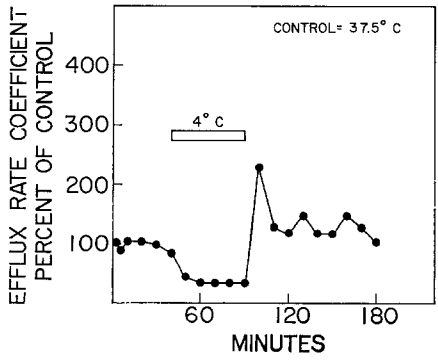

Fig. 9. Effect of a drop in the temperature of the incubating medium from 37 to 4 °C on the calcium efflux rate coefficient. The low temperature was maintained from minute 40 to 90 (total 50 min). The cells were replaced at 37 °C at time = 100 min

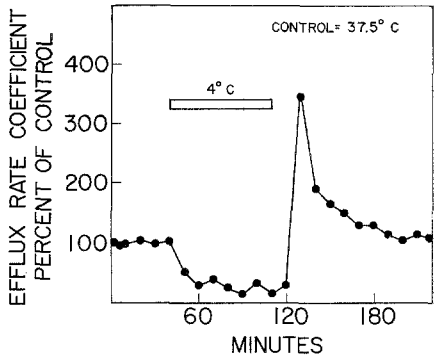

Fig. 10. Effect of a drop in the temperature of the incubating medium to 4 °C of 80-min duration (from minute 40 to 120)

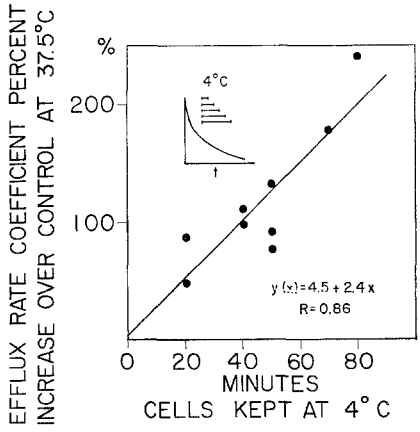

Fig. 11. Correlation between the length of the cold exposure period and the peak of the rebound in calcium efflux when the cells are replaced at 37 °C. The cells are placed at 4 °C at the same time, 40 min after the beginning of the desaturation, and replaced at 37 °C at various times, from minute 60 to 120

are placed in a 4 °C bath, the calcium ERC drops from 100 to 35% of controls. After 50 min of cold exposure, when the cells are reincubated at 37 °C, the ERC rebounds 230%. Fig. 10 illustrates a cold exposure of 80 min. At 4 °C, ERC hovers between 20 and 35% of control, when the cells are replaced at 37 °C, ERC jumps to 340% of control levels. Fig. 11 shows that there is a good correlation between the length of the cold exposure period and the peak of the rebound in calcium efflux ($R=0.86$). However, since the ERC of the control cells decreases constantly with time, this correlation may be an artifact. Indeed, the cells are placed at 4 °C at the same time, but they are replaced at 37 °C at various times (60 to 120 min

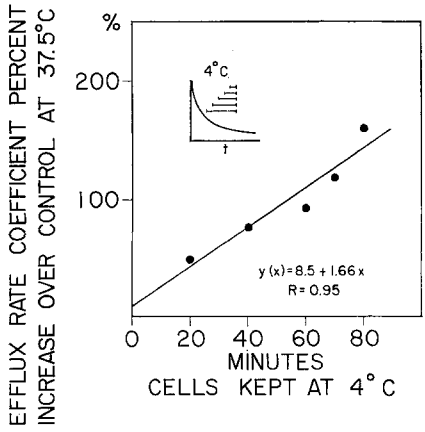

Fig. 12. Correlation between the length of time of the cold exposure and the peak of the rebound in calcium efflux when the cells are replaced at 37 °C. The cells are placed in 4 °C at various times, from minute 40 to 100 and replaced at 37 °C at the same time, 120 min after the beginning of the desaturation

after the beginning of the washout). To test the validity of the correlation, cells were placed in the cold at different times of desaturation and reincubated at 37 °C at the same time (110 min after the beginning of the washout). Fig. 12 illustrates that there is again a very good correlation between the two parameters ($R=0.95$). However, the magnitude of the effect, reflected by the slope of the regression line, is reduced 30%. These experiments suggest that cytoplasmic calcium increases when active transport processes are inhibited and that calcium efflux is dependent on the cytoplasmic calcium activity.

Discussion

^{45}Ca desaturation experiments reveal three exchangeable pools of calcium with different time constant of exchange. The half time of the fastest phase varies between 1 and 2 min. Although this cannot be measured with great accuracy by this method, it is comparable to the half time of 1.3 min of the fast kinetic component of calcium uptake which represents calcium exchange with the cell membrane or with some components of the extracellular glycocalyx (Borle, 1970). The parameters of the second phase also compare well with the slow component of calcium uptake. Its half time of exchange with the extracellular fluids is about 20 min, compared to 26 min for calcium influx. Efflux from this phase is of the same order of magnitude as the transmembrane fluxes measured in nerve and in muscle

(Hodgkin & Keynes, 1957; Winegrad & Shanes, 1962; Blaustein & Hodgkin, 1969). Thus, the second phase can be identified as the intracellular cytoplasmic pool. However, it does not represent exclusively the free calcium ion pool of the cytoplasm since calcium binding to organic molecules and to subcellular structures certainly occurs. The exchangeable pool of the second phase probably represents the free and bound calcium of the cytoplasm plus the fraction which exchanges rapidly with binding sites of subcellular components such as the nucleus, the endoplasmic reticulum and the mitochondrial surface.

The third phase has a half time of desaturation of 150 min, and a very slow rate constant of 0.00455 min^{-1}. This compartment has many characteristics of isolated mitochondria. First, when the cell accumulates calcium, most of it appears in this pool while the second phase increases only slightly. It is well known that isolated mitochondria are able to accumulate large amounts of calcium and to maintain very low calcium concentration in their external medium (Lehninger, 1970). Moreover, it has been shown that in carbon tetrachloride poisoning the liver cells accumulate large amounts of calcium and that the calcium is found mainly in mitochondria (Thiers, Reynolds & Vallee, 1960; Reynolds, Thiers & Vallee, 1962). In our system, the third slow compartment behaves as a buffer mechanism for calcium which maintains a fairly constant exchangeable calcium in the second phase, and fits well the description of mitochondrial function offered by Lehninger since it "contributes to the ionic homeostasis of the cytoplasm by serving as a temporary ion buffering system" (Lehninger, 1964). Second, accumulation of calcium in the third compartment depends on the phosphate concentration in the medium. It is well known that calcium uptake in mitochondria is stimulated by phosphate in a variety of experimental conditions (Lehninger, 1970; Borle, 1967). The sequestration and precipitation of calcium and phosphate in mitochondria has also been substantiated by electron micrographs and by microincinerations (Peachey, 1964; Scarpelli, 1965; Martin & Matthews, 1969; Sampson, Matthews, Martin & Kunin, 1970). Since liver or kidney microsomes do not possess significant calcium accumulation activity (Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965; Scarpelli, 1965), it is likely that the third phase of calcium efflux in these studies represents mitochondrial calcium. Finally, Antimycin A and Warfarin® (a derivative of dicoumarol) are specific inhibitors of calcium uptake by isolated mitochondria. These antibiotics completely abolish calcium uptake in the third calcium compartment, even at very high calcium and phosphate concentrations. This is a very strong argument indicating that the third phase represents a mitochondrial pool. It may seem puzzling

at first that calcium efflux from mitochondria has a half time of 150 min ($k = 0.00455\ \text{min}^{-1}$). Indeed calcium uptake in rat liver mitochondria is a very rapid process with a half time of a few seconds (Drahota *et al.*, 1965; Mela & Chance, 1968). Mela and Chance (1968), for instance, obtained a half time of about 8 sec which would give a rate constant of uptake of $5.2\ \text{min}^{-1}$. At steady state, the fluxes in and out of the mitochondria must be equal and proportional to the rate constant times the calcium activity of the respective compartments. Since Drahota *et al.* (1965) have shown that mitochondria are able to establish concentration gradients of calcium exceeding 1,000, it follows that the rate constant of calcium efflux by mitochondria should be at least 1,000 times smaller than the rate constant of calcium influx. And in fact, the rate constant of calcium efflux from the third compartment ($0.00455\ \text{min}^{-1}$) is roughly 1,000 times smaller than the rate of calcium uptake obtained in isolated mitochondria ($5.2\ \text{min}^{-1}$). Of course it is unlikely that the exchangeable pool size which we measured represents the calcium activity of these internal compartments since we do not know their thermodynamic activity coefficients. Nevertheless, the similarity between the measurements of the rate constant ratio and the presumed calcium activity ratio *in vivo* of the two internal pools supports our assumption that efflux from the third compartment is a reflection of calcium efflux from mitochondria. The mitochondrial pool however may not be homogenous. The free calcium inside the mitochondria is probably in equilibrium with calcium bound to the inner membrane and with an eventual solid phase of calcium phosphate precipitates. The exchangeability of these fractions with ^{45}Ca may be quite different. For instance, isotopic exchange between an aqueous and a solid phase depends more on the surface area of the solid than on its mass. Thus, an increase in the mitochondrial exchangeable pool may not necessarily mean an increased calcium mass; it could reflect an increased surface area caused by the precipitation of numerous small mitochondrial granules of calcium phosphate. The sudden appearance of such granules has been demonstrated in the mitochondria of intestinal cells after vitamin D administration (Sampson *et al.*, 1970). Nevertheless, this would reflect an increased calcium activity in the mitochondria and an increased turn-over. In these studies, however, the expansion of the third compartment is undoubtedly caused by an accumulation of calcium in mitochondria since the total cell calcium is increased significantly.

The effects of a sudden change in extracellular phosphate concentration are rapid and reversible. It is, of course, difficult to determine on which step of calcium efflux phosphate exerts its effect. Fifty minutes after the

beginning of the desaturation, the cytoplasmic pool has lost most of its radioactivity since its time constant of exchange is 22 min ($T=1/k$). Thus, the isotope appearing in the washout fluid must come from the slower compartment, presumably from the calcium sequestered in the mitochondria. Obviously, several steps are involved at this stage of the desaturation: the passage from a bound to a free form in the mitochondria, the efflux from mitochondria, the interaction with binding sites in the cytoplasm and the active transport from the cell through the plasma membrane. Phosphate could affect any of these steps. It is unlikely that a mere change in extracellular phosphate concentration has a significant influence on calcium efflux which is mainly dependent on intracellular events. Phosphate probably diffuses in or out of the cell and it is the change in intracellular phosphate concentration which affects calcium efflux. An increased intracellular phosphate resulting from the sudden addition of this anion in the medium could cause: (a) a fall in calcium activity of the cytoplasm by increasing its binding to phosphate; since the active transport of calcium out of the cell presumably depends on the free ion concentration in the cytoplasm, calcium efflux could be depressed; (b) an increased uptake of calcium by mitochondria from the cytoplasmic pool which would lower the calcium activity in the cytoplasm; (c) an increased binding or precipitation of calcium inside the mitochondria thus shifting the equilibrium from the free to the bound form and preventing the release of calcium from the very slowly exchangeable pool. Deletion of phosphate from the extracellular medium could produce the reverse effects. We presently cannot decide which of these possibilities is the most likely but these experiments underlie three important facts: (1) calcium transport can be stimulated threefold or cut in half by simply changing the external concentration of phosphate, (2) these changes occur rapidly in less than 10 min and reach their maximum within 40 min, (3) these changes are rapidly reversible.

Finally, the cold block experiments tend to support our hypothesis that the transport of calcium out of the cytoplasm either in the extracellular fluids (ECF) or into the mitochondria are energy-dependent processes whereas influx of calcium into the cytoplasm from the ECF or from the mitochondria is passive. During the cold block, the cytoplasmic calcium increases significantly. The major part of this calcium will undoubtedly be bound but the overshoot in calcium efflux occurring when the cells are replaced at 37 °C suggests that the cytoplasmic calcium activity is also increased several-fold. The cell is obviously able to correct this situation fairly rapidly since the ERC returns to normal in less than an hour. The contribution of the mitochondria in buffering this excess cytoplasmic cal-

cium is difficult to estimate. But the sharp drop in ERC immediately after the peak rebound in calcium efflux indicates that the mitochondria may participate in removing calcium from the cytoplasmic compartment.

One often wonders to which extent the results obtained in isolated subcellular systems are applicable to the whole cell with their mitochondria *in situ*. This series of experiments may not bring the proof that they are literally relevant but they are certainly consistent with the view that mitochondria play an important role in the control of intracellular calcium. They suggest that a subcellular compartment acts as a buffer for cytoplasmic calcium, that transient rise or fall in intracellular calcium activity can be rapidly corrected within the cell itself and that an increase or decreased calcium efflux from the cell may be the consequence rather than the cause of the fluctuations in calcium activity of the cell cytoplasm. If this were true, it may very well be that hormones, vitamins and metabolites such as cyclic AMP which are known to influence cellular calcium may act on the exchange of calcium between cytoplasm and mitochondria rather than affecting directly the influx or efflux of calcium in and out of the cell (Kimberg & Goldstein, 1967; Horn, Fyhn & Haugaard, 1970; Rasmussen, 1970; Ahren, Hjalmarson & Isaksson, 1971; Borle, 1971*a*, *b*; Borle, 1972).

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