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Effects of Calcium Ionophores on the Transport and Distribution of Calcium in Isolated Cells and in Liver and Kidney Slices

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Summary. The effects of calcium ionophores on cellular calcium metabolism were studied in cultured kidney cells, in cells freshly isolated from rat kidney, and in liver and kidney slices. In isolated cells, these ionophores decreased the total cellular Ca content and the mitochondrial Ca. ^{45}Ca efflux from prelabelled cells was also stimulated even in the absence of extracellular Ca. In slices, the ionophore A23187 increased the total slice Ca and the uptake of ^{45}Ca. However, the mitochondria isolated from these slices treated with the ionophore had a lower total Ca and a depressed relative radioactivity. These results suggest that the increased cytosolic Ca produced by Ca ionophores may be due to mobilization of intracellular Ca stores rather than to a net shift of Ca from the extracellular fluids to the cell.

The lipophilic antibiotics A23187 and X-537A are known to increase the permeability of biological membranes to divalent cations (Reed & Lardy, 1972*a*; Pressman, 1973). These ionophores are frequently used as a tool to study the role of Ca in several cellular functions. All the results published to date support the view that Ca ionophores increase the concentration of free Ca in the cell cytosol. The source of Ca responsible for the rise in cytosolic Ca is usually assumed to be extracellular. However, the contribution of any Ca sequestered in subcellular structures to the rise in cytosolic Ca is unclear. It is known that ionophores trigger the release of Ca from isolated mitochondria (Reed & Lardy, 1972*b*; Sordahl, 1974; Binet & Volfin, 1975). Furthermore, it has been observed that Ca ionophores increase cytosolic Ca in some tissues even in the absence of Ca in the extracellular fluids (Binet & Volfin, 1975; Chambers, Pressman & Rose, 1974). It is therefore possible that the Ca sequestered in subcellular structures may contribute significantly to the rise in cytosolic Ca produced by Ca ionophores.

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It has also been reported that the Ca ionophores A23187 and X-537A increase incorporation of ^{45}Ca by some cells and tissues (Reed & Lardy, 1972*a*; Prince, Rasmussen & Berridge, 1973; Massini & Luscher, 1974; Diziak & Stern, 1975). However, some investigators have demonstrated that the same ionophores decrease the cell Ca concentration in other tissues (Holland, Armstrong & Steinberg, 1975; Schudt & Pette, 1975). These ionophores could possibly increase the total cell Ca in some tissues and decrease it in others; however, the possibility of artifacts should not be excluded. Indeed one cannot exlude that an increased ^{45}Ca incorporation by a tissue may coexist with a decreased cellular Ca. This study reports the effects of Ca ionophores in cultured kidney cells, in cells freshly isolated from rat kidney, and in rat liver and kidney slices. We found that in isolated cells these ionophores decrease the cellular Ca content and deplete the mitochondria of a significant fraction of their Ca. In kidney and liver slices, on the other hand, A23187 increased ^{45}Ca uptake and the total Ca concentration of the tissue. However, the mitochondria isolated from slices which had gained Ca and had an increased ^{45}Ca were found to have a lower total Ca and a decreased relative radioactivity after the addition of A23187.

Materials and Methods

Red blood cells. Human red blood cells were obtained by venipuncture, washed twice in Krebs-Henseleit buffer and used in suspension at a concentration of 1 to 2 mg cell protein/ml of medium.

Cultured cells. Monkey kidney cells (LLC-MK_2) were grown as monolayers in minimum essential medium (MEM) and Earl's salt solutions. They were harvested after one week and incubated as a suspension in a Krebs-Henseleit buffer for the experiments.

Isolated renal cells and tubules. Renal cells and tubules were prepared by the method published by Shain (1972). The buffer used was modified slightly as follows (buffer z): 20 mM NaHEPES, pH 7.4; 120 mM NaCl; 1 mM $MgSO_4$; 3 mM K_2HPO_4; 1 mM $CaCl_2$; 1 mg/ml bovine serum albumin fraction V; 10,000 units potassium penicillin G and 10,000 μg streptomycin sulfate/100 ml (Microbiological Associates, Bethesda, Md.); 0.4 mg/ml of collagenase (Worthington Biochemical Corp., Freehold, N.J.). Sprague-Dawley rats, 100–150 g, were decapitated and bled. The abdominal cavity was opened and the aorta ligated proximal to the renal artery. A blunt needle was introduced into the abdominal aorta distal to the renal arteries and the kidneys were perfused with 40 ml of saline at 37 °C to remove the blood remaining in this vascular bed and then with 10 ml of the buffer containing collagenase. The kidneys were removed, decapsulated, minced with scissors, and incubated for 1 hr at 37 °C in buffer z. The tissue mince was dispersed by pipetting the suspension several times every 15 min. The suspension was filtered through a 110 μm sieve and the

cells thoroughly washed with buffer z. The cells were centrifuged at $150 \times g$, the suspending medium containing the collagenase was discarded, and the cells were washed once with the Krebs Heinseleit buffer to be used for the experiments. The cells were preincubated for 1 hr before the start of the experiments. Under phase contrast microscopy, the suspension consisted of isolated cells, clumps of cells, and of very small fragments of tubules.

Slices. The liver and the kidneys of 100–150 g Sprague Dawley rats were removed, after decapitation, and placed in an ice cold Krebs buffer. Slices, 0.5-mm thick, were prepared in a cold room with a Mickel microtome. The slices were incubated in Krebs Henseleit bicarbonate buffer.

Cell-fractionation. The cells were homogenized at 0° with a Bellco glass tissue grinder in 250 mM sucrose containing 0.1 mM EGTA to prevent redistribution of Ca during the fractionation process. The homogenate was centrifuged for 20 min at $800 \times g$ and the supernatant centrifuged twice at $18{,}000 \times g$. The sucrose in which the mitochondria were suspended during the second centrifugation contained no EGTA.

Slices mitochondria. Liver and kidney slices were prepared and incubated as previously described. The slices were labeled with ^{45}Ca for 2 hr as for a determination of Ca uptake. After 2 hr, A23187 was added to the incubation medium of the experimental group at a concentration of 5 μg/ml. Control groups received ethanol at a final concentration of 0.5%. Ten min after the addition of the ionophore or of ethanol, the slices were collected and washed as previously described. They were homogenized in a cold room in a 12-ml Potter-Elvehjem grinder with a Teflon pestle, in 250 mM sucrose containing 0.1 mM EGTA to prevent Ca redistribution among subcellular components. The homogenate was centrifuged for 20 min at $800 \times g$ and the supernatant centrifuged at $18{,}000 \times g$ on a Beckman L-100 ultracentrifuge. The mitochondrial pellet was washed in sucrose devoid of EGTA, resuspended and recentrifuged at $18{,}000 \times g$ in sucrose without EGTA. The final pellet was homogenized with an ultrasonic probe and its total Ca concentration, its radioactivity, and its protein concentration were determined.

Incubating medium. The medium of incubation was a Krebs Henseleit bicarbonate buffer containing (in mM): 140 Na^+, 5 K^+, 24 HCO_3^-, 121 Cl^-, 1 $MgSO_4$, 1 $CaCl_2$ and 1 Na_2HPO_4: NaH_2PO_4 at pH 7.4. The gas phase consisted of 95% air, 5% CO_2.

Ionophores. The ionophores A23187 and X-537A were dissolved in ethanol. A23187 was added to the cell suspension at concentrations from 0.01 to 5 μg/ml. The concentration of ethanol in the medium never exceeded 0.1%. A few experiments were performed with X-537A at a concentration of 10 μg/ml.

Isolated cell calcium uptake. 1.5 to 2.0 ml of cells centrifuged at $150 \times g$ were suspended in 60 ml of medium with a gas phase of 5% CO_2 in air. ^{45}Ca was added after 2 hr of preincubation and uptake determined by methods previously published (Borle, 1975).

Isolated cells calcium efflux. 0.6 ml of cells centrifuged at $150 \times g$ were first preincubated for 1 hr, then labeled with ^{45}Ca for 60 min. Isotopic desaturations were performed and the efflux rate coefficient calculated according to the method previously published (Borle, 1975).

Slices calcium uptake. After 30 min of preincubation, the incubating medium of the slices was replaced with fresh buffer containing 100 μCi of ^{45}Ca. The isotope uptake was determined for a control period of 2 hr and an experimental period of 2 hr. The slices

were removed with forceps from the incubating medium, and quickly washed in 4 different beakers containing a buffered saline solution kept at 0 °C. The slices were blotted lightly on filter paper and weighed on a Mettler balance. The uptake of isotope by the slices was determined at 5, 10 and 20 min after the addition of ^{45}Ca and every 15 min thereafter. The ionophore was added at min 126 and tissue samples were taken at min 127, 137, 145, 155 and every 15 min thereafter, until min 240. Each sample with a wet wt of 10–15 mg was placed in tared Pyrex vessel and dried for 24 hr at 95–100 °C in a vacuum oven. The dry wt was determined and the samples were then ashed overnight in a muffle furnace at 500 °C. The ash was dissolved in 0.2 ml of 2 N HCl and diluted to 2 ml with deionized water. The total Ca and the radioactivity were determined on the dissolved ash.

Definition of uptake. The uptake values of slices and cells were calculated by dividing the tissue (or cell) radioactivity by the medium specific activity. Uptake can also be called relative radioactivity and has the units of nmole/mg dry wt or nmole/mg protein: sample radioactivity (cpm/mg dry wet)/medium specific activity (cmp/nmole)=uptake (nmole/mg dry wt).

Determinations. The cells were homogenized by an ultrasonic probe, their protein concentration measured by the Lowry method (Lowry *et al.*, 1951). Ca was measured by fluorometric titration (Borle & Briggs, 1968). ^{45}Ca was assayed by liquid scintillation spectrometry using Aquasol (New England Nuclear) on a Beckman L-100 counter.

Results

Red Blood Cells

Calcium uptake by red blood cells. A23187 was added to a suspension of human erythrocytes at concentrations of 1 and 5 μg/ml. Microscopic examination of the cells with phase contrast revealed that 5 μg A23187/ml caused the cells to swell and hemolyze in less than 5 min. With an ionophore concentration of 1 μg/ml, however, there was no apparent hemolysis but the cells lost their biconcave shape. Fig. 1 shows that red blood cells do not accumulate significant amounts of tracer. ^{45}Ca uptake is extremely small, 0.013 nmole/mg protein (Table 1). When 1 μg A23187/ml of medium is added to the suspension, there is an immediate uptake of isotope. Fig. 1 shows that the cells' relative radioactivity rises from 0.013 to 3.1 nmoles/mg of cell protein, within 10 min. Thirty minutes after the addition of the ionophore, the radioactivity of the cells declines. This may be due to a progressive hemolysis, because the protein concentration of the suspension also declines in parallel fashion. Table 1 shows that after the addition of A23187 the total calcium concentration of the erythrocytes increases 62%, from 9.62 to 15.6 nmoles/mg protein. However since the cell protein concentration decreases 14%, the actual increase in Ca may be less (36%). The rise in the total cell Ca concentra-

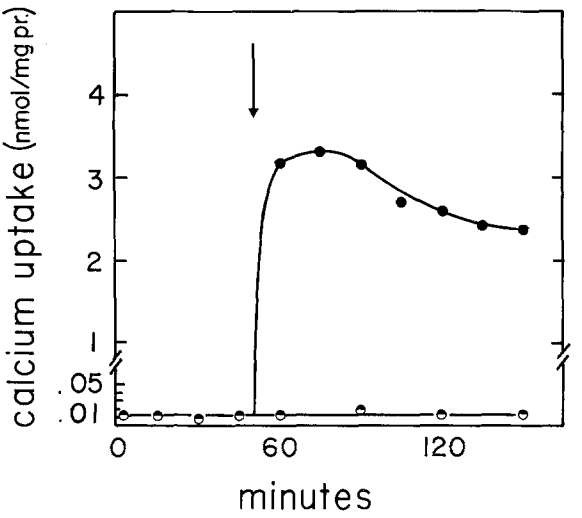

Fig. 1. Effect of A23187 on Ca uptake (relative radioactivity) in human red blood cells. A23187 (1 μg/ml) was added at min 55 (closed circles). Control cells (open circles) received ethanol (0.1%) at min 55. The graph shows the means of 2 experiments in each group

Table 1. Effect of 1 μg A23187/ml on the total Ca concentration and on Ca uptake of human red blood cells

	Control ($n=14$)	A23187 ($n=10$)	% change	P
Cell calcium (nmole/mg protein)	9.62 ±0.5	15.6 ±1.06	+62	<0.001
Calcium uptake (nmole/mg protein)	0.013±0.002	2.99±0.1	+22,900%	<0.001
Cell protein (mg/ml suspension)	1.41 ±0.03	1.21±0.05	−14%	<0.01
Cell calcium (nmole/ml suspension)	13.4 ±0.6	18.2 ±1.41	+36%	<0.01
Calcium uptake (nmole/ml suspension)	0.018±0.003	3.62±0.1	+20,000%	<0.001

Values are mean ± SE.

tion is reasonably close to the value of the Ca uptake measured with ^{45}Ca. These experiments illustrate that, in cells devoid of mitochondria, the ionophore A23187 increases the permeability of the cell membrane and allows a net shift of Ca from the extracellular fluids to the cell interior down its thermodynamic gradient.

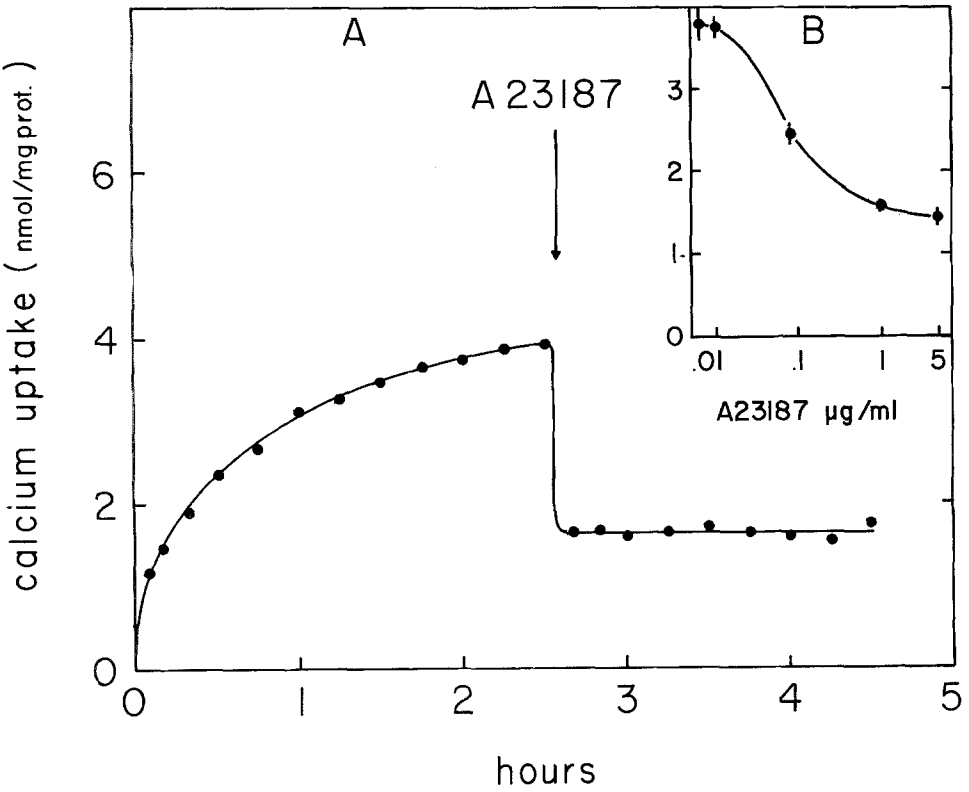

Fig. 2. (*A*): Effect of A23187 on Ca uptake (relative radioactivity) in kidney cells (LLC-MK_2). The cells were preincubated for 2 hr in a medium containing 1 mM Ca and phosphate before the addition of ^{45}Ca. A23187 was added at min 55 at a concentration of 1 µg/ml. The values are the mean of 6 experiments. The standard errors of the means were smaller than 0.15 nmole/mg protein. (*B*): Cells' relative specific activity after A23187 addition as a function of the ionophore concentration

Cultured Monkey Kidney Cells

Calcium uptake by kidney cells. The effect of ionophores on Ca uptake by kidney cells is markedly different. If the ionophore is added when the cells are near their isotopic equilibrium, the cell radioactivity drops immediately. Fig. 2 shows the results of 6 experiments obtained with 1 µg A23187/ml of medium and Fig. 3 the results of 4 experiments obtained with 10 µg X-537A/ml. Such a drop in radioactivity when the cells are close to their isotopic equilibrium can only mean a net movement of Ca out of the cells. This is confirmed by the fact that the total cell Ca drops 30% (Table 2).

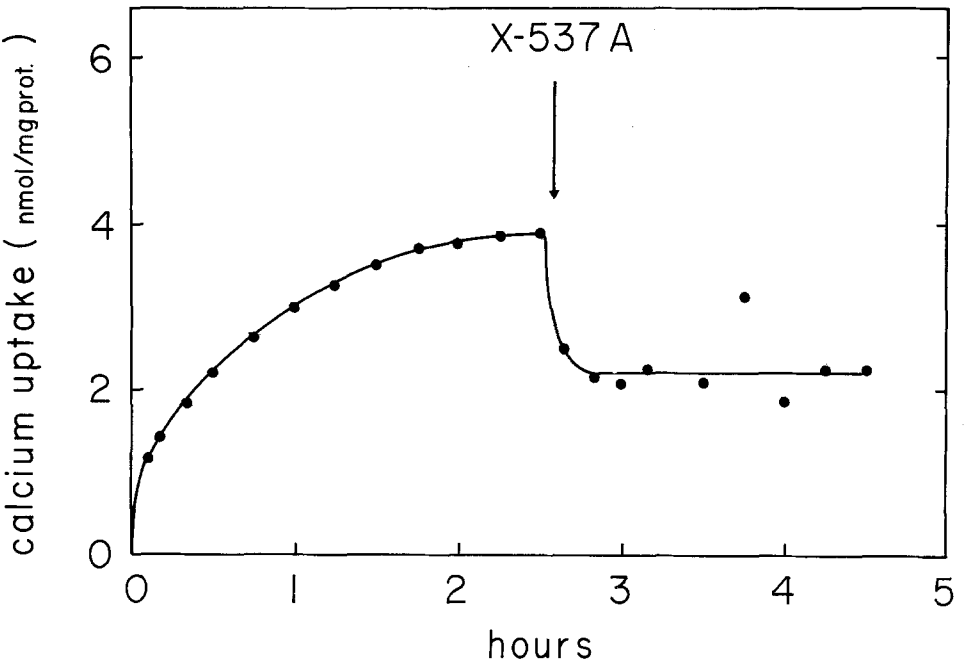

Fig. 3. Effect of X-537A on Ca uptake (relative radioactivity) in kidney cells. The conditions are the same as those described in Fig. 2. X-537A (10 μg/ml) was added at min 55. The values are the mean of 4 experiments

Table 2. Effects of A23187 and X-537A on the total calcium of kidney cells

	Control	Ionophore	% change	*P*
A23187 1 μg/ml				
Cell calcium				
(nmole/mg cell protein)	11.0 ±0.51 (15)	7.37±0.72 (16)	−33%	<0.001
(mmole/kg cell water)[a]	1.01±0.05	0.68±0.07	−33%	<0.001
X-537A 10 μg/ml				
Cell calcium				
(nmole/mg cell protein)	15.2 ±0.67 (27)	9.78±0.46 (23)	−36%	<0.001
(mmole/kg cell water)	1.39±0.06	0.90±0.04	−36%	<0.001

The values are the mean±SE. The numbers in parenthesis indicate the number of determinations.

[a] To convert nmole Ca/mg cell protein to mmole/kg cell water, the value has to be divided by 10.9 (The water content of LLC-MK_2 kidney cells is 10.9±0.39 mg/mg protein; from Borle, 1970.)

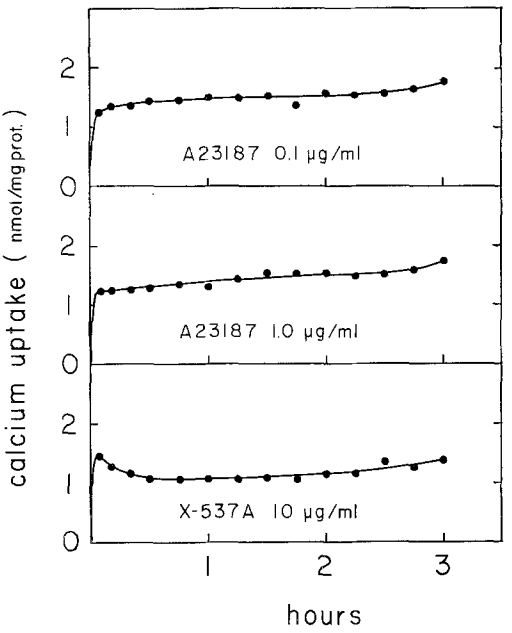

Fig. 4. Effects of A23187 and of X-537A added at time 0 of isotope uptake on the relative radioactivity of kidney cells. Each graph represents the mean of 2 experiments

When the ionophore and the tracer are added together at time 0 of the labeling period, ^{45}Ca uptake is markedly depressed as shown in Fig. 4. Under normal conditions, the curves of ^{45}Ca uptake by isolated cells comprise two kinetic phases which can be easily separated by graphical analysis (Borle, 1970, 1975) or by nonlinear least square analysis. The fast compartment of exchange has a time constant of 1 to 2 min and represents extracellular Ca binding to membrane sites or to ligands of the glycocalyx. The curves of Ca uptake shown in Fig. 4 match the first kinetic component of a normal uptake curve (Borle, 1970, 1975). This would indicate that ionophores have little or no influence on the fast component of Ca exchange in isolated cells. This view is further supported by the fact that the level of the cells relative radioactivity observed after the addition of A23187 at min 150 (Fig. 2) is close to that obtained when one adds the ionophores at time 0 (Fig. 4).

Fig. 2*A* shows that the relative radioactivity of the cells drops 60% when 1 µg A23187/ml of medium is added after 150 min of uptake. Fig. 2*b* shows that increasing the concentration of A23187 to 5 µg/ml does not enhance significantly the fall in the relative radioactivity of the cells. This also supports the view that this ionophore does not affect

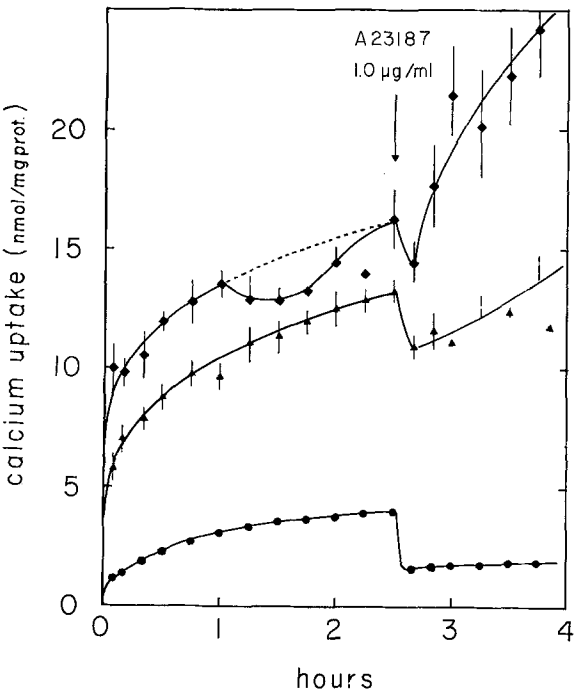

Fig. 5. Effect of A23187 (1 µg/ml) on Ca uptake by kidney cells incubated at different extracellular Ca concentrations. ● = 1 mM Ca, 1 mM phosphate. ▲ = 5 mM Ca, 0.2 mM phosphate. ◆ = 10 mM Ca, 0.2 mM phosphate. The phosphate was reduced to 0.2 mM to prevent precipitation of Ca phosphate. The ionophore was added at min 155. The values are the mean of 6 experiments at 1 mM Ca and the mean of 4 experiments at 5 and 10 mM Ca. The vertical bars represent the SEM

the first phase of Ca uptake. On the other hand, lowering the ionophore concentration decreases its effect. From 0.01 to 1.0 µg/ml, the % drop is closely related to the log of the ionophore concentration. On a weight basis, X-537A is 100 times less active than A23187. The 42% drop obtained with 10 µg X-537A ml is close to the 37% fall observed with 0.1 µg A23187/ml.

Effect of extracellular calcium on the action of A23187. Since kidney cells exposed to 1 µg A23187/ml lose 33% of their total calcium and 60% of their radioactivity when the extracellular calcium is 1.0 mM, we studied the medium Ca concentration at which the cells would actually gain Ca and accumulate more tracer. Fig. 5 and Table 3 show that with a medium Ca of 5 mM, the cells' relative radioactivity still falls immediately after the additon of the ionophore, although the cells re-accumu-

Table 3. Effect of the ionophore A23187 on the total cell Ca of cultured kidney cells

Medium Ca	Control	A23187 (1 μg/ml)	% change	*P*
	nmole/mg protein[b]			
1.3 mM	11.0±0.51 (15)	7.37±0.72 (16)	−33%	<0.001
5.0 mM[a]	22.4±1.35 (20)	24.8 ±1.2 (19)	+10%	NS
10.0 mM[a]	33.9±1.17 (24)	41.2 ±1.55 (19)	+22%	<0.001

Values are the mean±SE. Numbers in parenthesis are the number of determinations.
[a] At 5 and 10 mM Ca, the phosphate concentration was reduced to 0.2 mM to prevent the precipitation of Ca phosphate.
[b] To convert nmole Ca/mg cell protein to mmole/kg cell water the value has to be divided by 10.9. (The water content of LLC MK_2 kidney cells is 10.9±0.39 mg/mg protein; *from* Borle, 1970.)

late the tracer during the next 80 min. However the total cell Ca does not increase significantly. At a medium Ca concentration of 10 mM, A23187 first causes a small transient fall in relative radioactivity, then it significantly increases the rate of ^{45}Ca uptake; the total cell Ca rises 22%.

Effect of Calcium ionophores on calcium efflux. ^{45}Ca desaturation experiments were performed at three different Ca concentrations: 0, 1.0 and 10 mM. Fig. 6 shows the effect of 1 μg A23187/ml on the efflux rate coefficient (*ERC*) in five experiments. It is evident that the ionophore stimulates Ca efflux whether or not Ca is present in the extracellular medium and even at high medium Ca concentrations. Fig. 7 presents the effects of 10 μg X-537A/ml in the presence (1 mM) and in the absence of Ca in the medium. X-537A stimulates ^{45}Ca efflux in both conditions.

Effects of ionophores on mitochondrial calcium. We have demonstrated that with an extracellular Ca concentration of 1 mM, cultured kidney cells lose Ca when exposed to the ionophore A23187. Since a large fraction of intracellular Ca is sequestered in mitochondria, their Ca content and their radioactivity should also fall after A23187 administration. We repeated the experiments shown in Fig. 2, in which the ionophore is added 150 min after the beginning of ^{45}Ca uptake when the

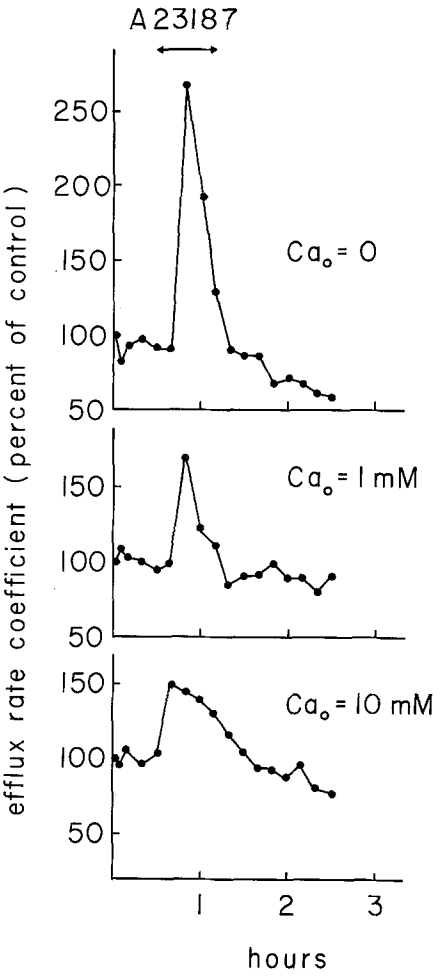

Fig. 6. Effect of A23187 (1 μg/ml) on the Ca efflux rate coefficient of kidney cells. The ionophore was added at min 40 and removed at min 90. The experiments were performed at different extracellular Ca concentrations: Ca=1 mM (n=3); Ca=0 mM (n=2); Ca=10 mM (n=1)

cells are near their isotopic equilibrium. At that time the control cells received only the solvent, ethanol without ionophore, and the experimental group received 1 μg A23187/ml of suspending medium. The cells were homogenized 10 min later according to the technique described in the method section. Table 4 presents the results of this series of experiments. Within 110 min, the ionophore depressed the total cell calcium 20% and the cell radioactivity 43%. The total Ca and the radioactivity of the mitochondria were also depressed 25% and 39%, respectively.

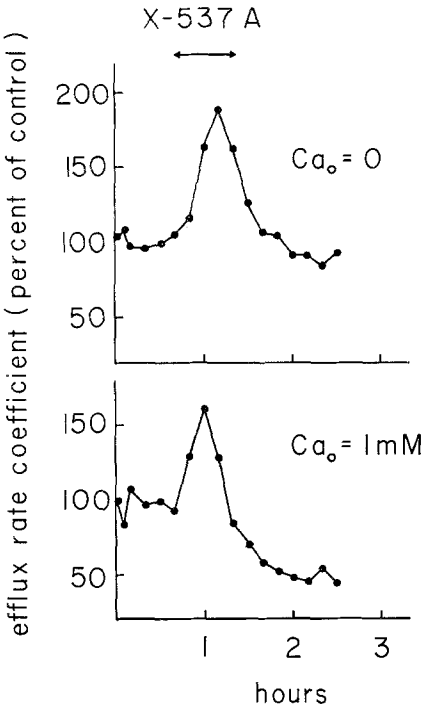

Fig. 7. Effect of X-537A (10 μg/ml) on the Ca efflux rate coefficient of kidney cells. Experimental conditions identical to those of Fig. 6

The drop in total mitochondrial Ca did not reach statistical significance probably because the determination of Ca in isolated mitochondria contains a large error, but the fall in radioactivity is significant. In control cells, the mitochondrial Ca can account for 33% of the total cell Ca. In the treated cells, 31% of the cell Ca is found in mitochondria. While the cells lost 2.6 nmoles Ca/mg cell protein after A23187 addition, the mitochondria lost 1.06 nmole Ca/mg cell protein. In other words, 41% of the total cell Ca loss is coming from the mitochondria.

Cells Freshly Isolated from Rat Kidney

To make sure that the loss in cell Ca produced by the ionophores in LLC MK_2 cells was not due to an aberrant property of cultured cells, we studied the effect of A23187 on the Ca content and the exchangeable pools of cells freshly isolated from animal tissues. The Ca uptake by freshly isolated kidney cells is shown in Fig. 8. The isotopic equilib-

Table 4. Effect of A23187 on the total Ca and on the relative radioactivity of cultured kidney cells and of their mitochondria

	Control	A23187 (1 μg/ml)	% change	*P*
Cells				
Total calcium (nmole/mg cell protein)	13.0 ±1.04 (15)	10.4 ±0.85 (16)	−20%	<0.05
(mmole/kg cell water)	1.19±0.1	0.95±0.08		
Relative radioactivity (nmole/mg cell protein)	3.85±0.19 (15)	2.21±0.16 (16)	−43%	<0.001
Mitochondria				
Total calcium (nmole/mg mito protein)	85.5 ±17.5 (7)	64.4±10.4 (7)	−25%	NS
(nmole/mg cell protein)	4.28±0.88	3.22±52		
Relative radioactivity (nmole/mg cell protein)	1.38±0.22 (7)	0.84±0.19 (7)	−39%	<0.01

Values are the mean ± SE. Numbers in parenthesis indicate the number of determinations.

rium is reached at 90 min and the relative radioactivity remains constant until 4 hr (*not shown*). After the addition of 1 μg A23187/ml, the cells relative radioactivity drops 22% (Fig. 8 and Table 5). Table 5 also shows that, after the addition of the ionophore, the total cell Ca concentration drops 17%. Thus, these results agree with those obtained in cultured kidney cells.

Kidney and Liver Slices Calcium Uptake

Figs. 9 and 10 present the Ca uptake measured in kidney and liver slices. In both cases, isotopic equilibrium is reached after 60 min and the relative radioactivity remains constant for the next three hr (*not shown*). After the addition of 5 μg A23187/ml medium, Ca uptake immediately increases. In kidney slices, the relative radioactivity reaches a new higher steady state after 60 min (Fig. 9), whereas the radioactivity constantly rises in liver slices (Fig. 10). Table 6 shows that the average rise in Ca uptake during the two hr following administration of A23187

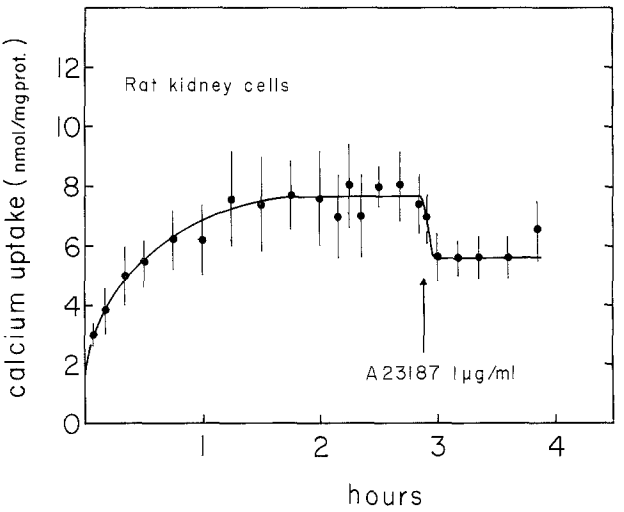

Fig. 8. Ca uptake in cells freshly isolated from rat kidneys. ^{45}Ca was added at time 0. A23187 (1 µg/ml) was added at min 175. Each point is the mean ± SE of 4 experiments

Table 5. Effect of A23187 1 µg/ml on the total Ca and ^{45}Ca uptake in cells freshly isolated from rat kidney

	Control	A23187	% change	*P*
Total cell calcium (nmole/mg protein)	15.2 ±0.9[a] (13)	12.7 ±0.3[b] (18)	−17%	<0.001
(mmole/kg cell water)	1.17±0.07	0.98±0.02	−17%	<0.001
Calcium uptake (nmole/mg protein)	7.24±0.5[c] (20)	5.66±0.3[d] (16)	−22%	<0.001

[a] Values from 140 to 170 min.
[b] Values from 180 to 230 min.
[c] Values from 130 to 170 min.
[d] Values from 180 to 220 min.

is 27% in kidney slices and 44% in liver slices. At the same time, the total Ca concentration of the slices also increases 33% in kidney and 36% in liver. A few experiments were performed in slices with 1 µg/ml of A23187. With this concentration of ionophore, no significant change was observed, and the relative radioactivity remained constant from 60 to 250 min.

Calcium content and radioactivity of mitochondria isolated from rat kidney and liver slices. We have shown above that A23187 decreases

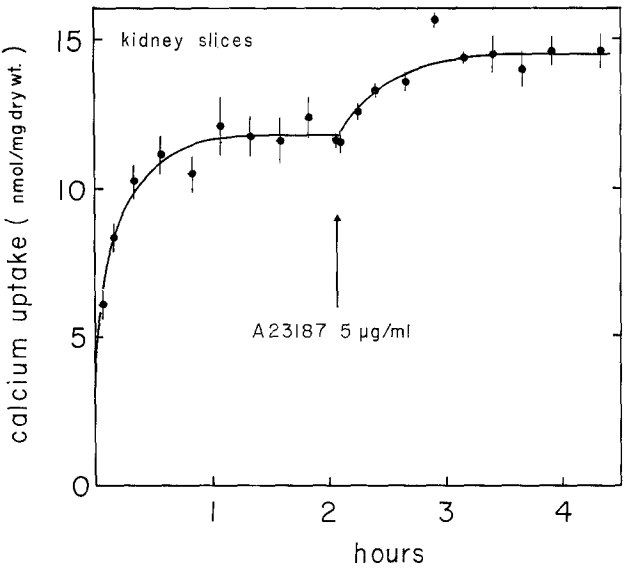

Fig. 9. Ca uptake in rat kidney slices. ^{45}Ca was added at time 0. A23187 (5 µg/ml) was added at min 126. Each point is the mean ± SE of 4 experiments

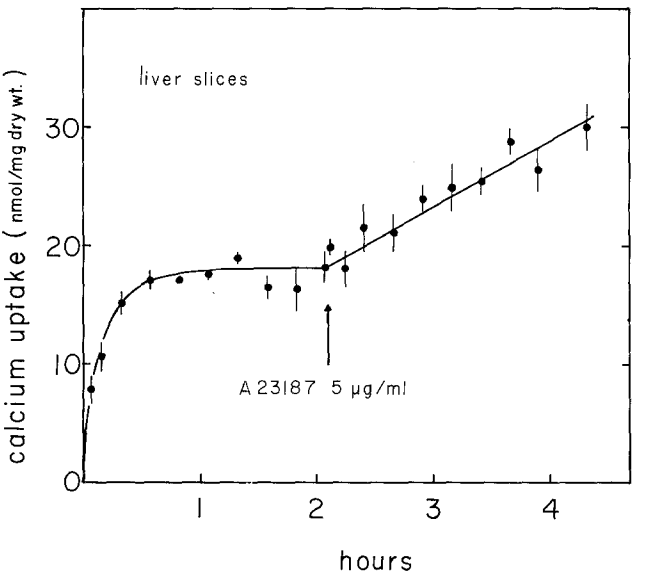

Fig. 10. Ca uptake in rat liver slices. ^{45}Ca was added at time 0. A23187 (5 µg/ml) was added at min 126. Each point is the mean ± SE of 4 experiments

Table 6. Effect of A23187 (5 μg/ml) on the total Ca and the relative ^{45}Ca radioactivity of rat kidney and liver slices

	Control	A23187	% change	*P*
Kidney slices				
Total slice calcium	13.5 ±0.4	17.9 ±0.4	+33%	<0.001
(nmole/mg dry wt)	(28)	(30)		
(mmole/kg wet wt)[e]	2.25±0.07	2.99±0.7		
(nmole/mg protein)	16.54±0.5	21.9 ±0.5		
Relative radioactivity	11.6 ±0.3[a]	14.6 ±0.2[b]	+27%	<0.001
(nmole/mg dry wt)	(28)	(30)		
Liver slices				
Total slice calcium	18.0 ±0.7	24.5 ±1.3	+36%	<0.001
(nmole/mg dry wt)	(23)	(32)		
(mmole/kg wet wt)[e]	3.26±0.13	4.44±0.24		
(nmole/mg protein)	18.0 ±0.7	24.5 ±1.33		
Relative radioactivity	17.5 ±0.5[c]	25.2 ±0.8[d]	+44%	<0.001
(nmole/mg dry wt)	(24)	(32)		

Values are the mean ± SE. Numbers in parenthesis indicate the number of determinations

[a] Mean value measured between 35 and 125 min.

[b] Mean value measured between 170 and 260 min.

[c] Mean value measured between 40 and 125 min.

[d] Mean value measured between 140 and 260 min.

[e] Kidney and liver slices contain 83.3±0.25 and 81.9±0.44% water, respectively. To convert nmole Ca/mg dry wt to nmole Ca/mg wet wt the values should be multiplied by 0.167 for kidney and by 0.181 for liver slices.

the total Ca of cultured kidney cells and that this loss of Ca was reflected in a drop in the total Ca concentration and of the radioactivity of their mitochondria. We postulated that the Ca concentration and the radioactivity of mitochondria isolated from kidney and liver slices could give some information as to whether the rise in Ca uptake of slices observed after A23187 administration is actually due to a shift of Ca into the cell. If this were so, the total Ca and the relative activity of mitochondria should also increase. Table 7 shows that this is not the case, at least in rat kidney. Indeed, despite the 33% rise in the total Ca of kidney slices and the 27% rise in their radioactivity observed on the addition of A23187 (Table 6), the mitochondria isolated from the same tissue, treated in an identical fashion, show a 36% fall in total Ca and a 32% drop in radioactivity (Table 7). These results strongly suggest that A23187 does not increase the cellular accumulation of Ca

Table 7. Total Ca and relative radioactivity of mitochondria isolated from control kidney slices and slices exposed to A23187 (5 μg/ml) for 10 min

	Control	A23187	% change	*P*
Kidney mitochondria				
Total calcium	27.2 ±7.7	17.4 ±3.5	−36%	0.05[a]
(nmole/mg mito protein)	(12)	(12)		
(nmole/mg slice protein)	9.52±2.7	6.09±1.23		
Relative activity	6.64±0.8	4.52±0.5	−32%	0.001[a]
(nmole/mg mito protein)	(16)	(16)		
Liver mitochondria				
Total calcium	24.8 ±2.5	27.6 ±2.7	+11%	NS
(nmole/mg mito protein)	(11)	(11)		
(nmole/mg slice protein)	4.98±0.5	5.55±0.54		
Relative activity	16.2 ±3.0	14.3 ±2.8	−12%	NS
(nmole/mg mito protein)	(11)	(12)		

Values are the mean ± SE. Numbers in parenthesis are the number of determinations.
[a] Correlated *t* test.

and of tracer in the cells. On the contrary, the cells seem to lose both in spite of the Ca accumulation produced by the ionophore in whole slices. The relative radioactivity of kidney slices at isotopic equilibrium is 37% larger than that of freshly isolated cells (Tables 5 and 6). Since the relative radioactivity is a measure of the exchangeable Ca pools, this suggests that 37% of the exchangeable Ca of kidney slices is extracellular. It is possible, therefore, that the net accumulation of Ca and of tracer observed in kidney slices after the addition of A23187 occurs in the extracellular interstitium and not in the intracellular compartment.

The results obtained with mitochondria isolated from liver slices are not as clear. Table 7 shows that the total mitochondrial Ca and their radioactivity are essentially unchanged. The small rise in total Ca and the small decrease in radioactivity are not statistically significant. Nevertheless, in light of the large rise in the total Ca and in the radioactivity of the liver slices (+36% and +44%, respectively, as shown in Table 6), one would have expected the ionophore to increase the mitochondrial concentration of both, if these changes had occured in the cells.

Comparison between Tables 6 and 7 also shows that, in control kidney slices, 57% of the Ca is found in mitochondria. In liver, the mitochondrial Ca accounts for 28% of the total slice Ca.

Discussion

The Ca ionophore A23187 is frequently used as a tool to study cellular functions which are believed to be regulated by cytosolic Ca. Several investigators have reported that this ionophore increases the uptake of ^{45}Ca in a variety of tissues (Reed & Lardy, 1972*a*; Prince *et al.*, 1973; Massini & Luscher, 1974; Dziak & Stern, 1975). It is also frequently observed that A23187 fails to elicit a cellular response in the absence of extracellular Ca (Prince *et al.*, 1973; Russel, Hansen & Thorn, 1974; Williams & Lee, 1974; Clyman *et al.*, 1975; Garcia, Kirpekar & Prat, 1975; Cochrane *et al.*, 1975). From such observations, many assume that A23187 produces a net shift of Ca from the extracellular milieu to the cell, increases the cytosolic Ca, and thereby triggers the process which is dependent on a rise in cytosolic Ca activity. Although such a sequence of events could very well occur, it is far from being proven by the available data. On the other hand, several reports show that A23187 can induce activation of sea urchin eggs, myogenic cell fusion, and insulin release from pancreatic islets in the absence of extracellular Ca (Schudt & Pette, 1975; Chambers *et al.*, 1974; Karl *et al.*, 1975). In these papers, the authors propose that A23187 mobilizes Ca from intracellular compartments.

The results presented in this paper strongly support the latter hypothesis. Indeed we have conclusively shown that A23187 decreases the total Ca and the radioactivity of cells and of their mitochondria. Our results also cast doubt on the validity of the argument that an increased ^{45}Ca accumulation by a tissue is evidence of a shift of Ca into the cells. It is indeed difficult to defend the postulate that an increased accumulation of Ca by the cells could coexist with a decreased Ca content and a fall in the accumulated radioactivity of their mitochondria.

Admittedly Ca ionophores increase the permeability of the cell plasma membrane and of the mitochondrial membrane to Ca (Reed & Lardy, 1972*b*; Pressman, 1973; Sordhal, 1974; Binet & Volfin, 1975). Although the concentration of cytosolic-free Ca has never been measured, some evidence supports the view that it increases after the addition of Ca ionophores. (Rose & Lowenstein, 1976; Desmedt & Hainaut, 1976). Our results obtained with red blood cells only confirm what was expected; cellular Ca uptake is dramatically increased and cell Ca concentration rises in the presence of A23187. Erythrocytes, however, may not be a good model for studying the effects of Ca ionophores because they lack mitochondria. In other cells one may wonder, first, whether these

ionophores ever reach the mitochondrial membranes and, if they do, whether the release of Ca from mitochondria significantly contributes to the rise in cytosolic Ca. Second, if Ca influx into the cell and Ca release from mitochondria both occur at the same time, what will be the net effect on the total cell Ca? If the stimulation of Ca transport across the plasma membrane predominates, the cells would presumably gain Ca. On the other hand, if the release of Ca from mitochondria is the most important effect of Ca ionophores, the cells may or may not lose Ca depending on the driving forces determining Ca movements. The results presented here suggest that Ca ionophores do penetrate the cell membrane, reach the mitochondria, and stimulate Ca release from this intracellular compartment. We cannot exclude, of course, that other unidentified compartments of kidney cells are capable of sequestering Ca and of releasing it under the influence of ionophores. An alternative explanation, yet completely unproven, could be that the high cytosolic Ca caused by an increase in Ca influx across the plasma membrane could increase the permeability of the mitochondrial membranes to Ca and release the Ca sequestered in the mitochondria. An argument against such an explanation is the fact that both A23187 and X-537A stimulate Ca efflux from the cells in the absence of extracellular Ca (Figs. 6*B* and 7*B*). In these experiments, the cytosolic Ca activity obviously could not have been raised by an increased Ca influx. Fig. 6*C* also shows that a high extracellular Ca concentration does not prevent the increased Ca efflux triggered by A23187. Whatever the mechanism, it is clear that both the mitochondria and the cells lose Ca after the addition of ionophores. The driving force causing the net shift of Ca from the cell to the extracellular fluid is unknown. The membrane potential of the kidney cells used in these experiments (LLC-MK_2) is -12.3 mV (Redmann, 1971), the inside of the cell being negative. The cytosolic Ca activity has never been measured after the addition of ionophores but there is no reason to believe that it exceeds the extracellular Ca activity. Thus, even if the ionophores depress the membrane potential to zero, we still have to explain the 30% loss of total cell Ca. One could speculate that, despite the increased plasma membrane permeability to Ca, the active transport of Ca out of the cell is maximally stimulated by the high cytosolic Ca activity and can extrude in less than 10 min 30% of the total cell Ca released from the mitochondria and possibly from other intracellular compartments. Otherwise, one has to postulate that an intracellular compartment capable of sequestering more than 30% of the cell Ca has a free Ca concentration exceeding 1 mM. In

fact, it should be significantly higher than 1 mM since, at a medium calcium concentration of 5 mM, the cells fail to gain Ca after the addition of A23187.

These results are not limited to cultured kidney cells. It has been reported that A23187 enhances the release of Ca from heart and skeletal muscle (Holland *et al.*, 1975; Schudt & Pette, 1975). The ionophores A23187 and X-537A induce parthenogenesis in sea urchin eggs, a process regulated by intracellular Ca, in the absence of external divalent cations (Chambers *et al.*, 1974). Finally, the stimulation of insulin release from pancreatic islets produced by A23187 has been shown to occur in the absence of extracellular Ca (Karl *et al.*, 1975). Our own data show that cells freshly isolated from rat kidney lose Ca after administration of A23187. Furthermore, the mitochondria isolated from kidney slices incubated with ^{45}Ca lose 20% of their Ca content and radioactivity after administration of A23187.

Presently, we have no substantiated explanation for the conflicting findings showing that A23187 increases Ca uptake by slices or by whole tissues while it decreases the Ca concentration of cells and of their mitochondria. One could postulate, however, that this ionophore increases the accumulation of Ca in the extracellular interstitium of the tissues. Hyono *et al.* (1975) have presented evidence that A23187 at low concentrations can bind two Ca ions per molecule and this ratio changes at higher ionophore concentrations to one Ca ion per two ionophore molecules. Thus, it could be theoretically possible for A23187 to attach with one binding site to the Ca bound to some ligands of the extracellular interstitium and, with the other binding site, trap in this interstititium a significant amount of Ca labelled and unlabelled from the incubating medium. Perhaps it may be relevant to note that higher concentrations of ionophore (5 μg/ml) must be used to produce an effect in tissue slices than that used in isolated cells, where 0.1 μg/ml produce significant Ca shifts. This could indicate that an important fraction of A23187 might be bound to some ligands of the interstitium.

If one postulates that ionophores increase the cytosolic Ca activity by mobilizing Ca from an intracellular compartment, the lack of action of A23187 and of other Ca ionophores in the absence of extracellular Ca remains unexplained, Again we can only speculate that a tissue incubated in Ca-free media will lose a significant fraction of its intracellular Ca. Indeed, experiments to be published (Borle, *in preparation*) show that kidney cells incubated in a Ca-free medium lose 80% of their total Ca within an hour. Since the largest fraction of the intracellular Ca

is found in mitochondria, it is evident that incubation in Ca-free media will severely deplete these internal stores of Ca. In these conditions, ionophores may not be capable of increasing cytosolic Ca by mobilizing it from depleted intracellular stores. In some cases, if the cells are not totally depleted, ionophores may still possibly increase the cytosolic Ca in the absence of extracellular Ca (Schudt & Pette, 1975; Chambers *et al.*, 1974; Karl *et al.*, 1975).

In conclusion, we believe that the increased ^{45}Ca uptake by a tissue exposed to A23187 and the lack of effect of this ionophore in Ca-free media, cannot be taken as a proof that this carboxylic antibiotic acts by producing a net shift of Ca from the extracellular fluids to the cytosol. Such results could still be consistent with the idea that ionophores increase the cytosolic Ca by mobilizing Ca from subcellular compartments (Rose & Lowenstein, 1976). Our results support the view that Ca ionophores increase the permeability of plasma and mitochondrial membranes to Ca and that they increase the cytosolic Ca concentration. However, the increased cytosolic free Ca is not due to an net shift of Ca from the extracellular fluids into the cell. On the contrary, it is clear that these divalent cation ionophores produce a net loss of cell Ca and that the source of the increased cytosolic Ca must be intracellular, presumably the mitochondria.

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