Kinetic Analyses of Calcium Movements in HeLa Cell Cultures

I. Calcium influx

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ABSTRACT Calcium influx was studied in monolayers of HeLa cells to determine the number of exchangeable and nonexchangeable pools and the rate constant of the different fluxes. Of the two exchangeable pools, one has a very fast rate of exchange with a half-time of 1.54 min, a compartment size of 1.06 mM moles/mg cell protein, and an exchange rate of 474 mM moles/(mg protein·min). This compartment is likely to be extracellular and could represent calcium exchange between the extracellular fluids and surface binding sites of the cell membrane. The second exchangeable pool has a half-time of exchange of 31 min, a compartment size of 2.69 mM moles/mg cell protein (0.224 millimole calcium/kg cell water), and a flux rate of 0.0546 μmole cm⁻² sec⁻¹. This compartment can be considered to be the intracellular pool of exchangeable calcium. An unexchangeable intracellular pool of calcium of 3.05 mM moles/mg cell protein was detected implying that only 45% of the intracellular calcium is exchangeable. In addition, a large extracellular pool of calcium has been found to be unexchangeable, probably a part of the cell glycocalix. Finally, dinitrophenol 10⁻⁸ M does not affect the slow component of the calcium uptake curve which brings new evidence that calcium entry into the cell is not a metabolically dependent process.

The study of cellular calcium metabolism presents several difficulties. A cell, for instance, cannot be considered an homogeneous pool of calcium. There are at least two calcium compartments of different magnitude showing markedly different properties (1, 2). One of these compartments is even not part of the cell itself but lies outside the plasma membrane and has been called “cell coat” or “glycocalix” (1–3). The second, the cellular calcium compartment, is undoubtedly divided into several pools, but for the time being we shall consider intracellular calcium as one compartment. Until now we have attempted to dissect these two main compartments by chemically stripping the cells from their coat with trypsin-EDTA in order to identify the properties of
each pool separately. To study calcium fluxes in cell cultures with radiotracer techniques would obviate some of the limitations imposed by the previous method and provide more information with regard to the number and the magnitude of calcium pools present in the system without destroying it. A more precise estimate of the intracellular pool of calcium and of the fraction which is exchangeable would help determine the magnitude of the concentration difference between the extracellular and the cellular compartments and thus bring more information about the thermodynamic conditions of the system.

Another problem has to do with the active or passive nature of the calcium fluxes in and out of the cell. There is a chemical potential difference between the extracellular fluids (ECF) and the cell, but the magnitude of the gradient is uncertain (1, 2, 4). There is also an electrical potential difference of \(-17.5\) mV in HeLa cells, the interior of the cell being negative with regard to the medium (5). Such an electrochemical potential difference could permit an energy-independent influx of calcium and would require an active process for the efflux of calcium. The measurement of calcium fluxes and the influence upon them of some metabolic inhibitors could provide more evidence to support this hypothesis.

The experiments reported here confirm the existence of two exchangeable calcium compartments, and two unexchangeable. These studies also support the idea that calcium influx is a passive phenomenon.

**METHODS**

HeLa cells were grown for 4 days in milk bottles and in Minimum Essential Medium (MEM) with Earle's salt solution containing 10% calf serum and antibiotics. The day of the experiment, the medium was decanted and the cells were preincubated for 1 hr in 15 ml of Krebs-Henseleit bicarbonate buffer containing 10 mm glucose and 1.3 mm Ca. At 0 time, 20 \(\mu\)C of \(^{45}\)Ca was added to the flasks and the cells were incubated on a rocking platform in a 37°C incubator room. The cells were harvested at various time intervals, from 1 min to 4 hr, by decanting the radioactive medium and washing the monolayers six times with cold buffer. 4 ml of deionized water was then added to the flask, the cells scraped from the glass with a plastic policeman, transferred into a plastic centrifuge tube, and homogenized with a high intensity ultrasonic probe. Aliquots of the homogenate were analyzed for \(^{46}\)Ca (6), \(^{45}\)Ca, and protein (7). The medium was also analyzed for \(^{46}\)Ca and \(^{45}\)Ca. The isotope was counted on a Packard Tri-Carb scintillation spectrometer in Bray's mixture.

**CALCULATIONS**

We have shown that a cell monolayer comprises at least two calcium compartments (1, 2): one is the calcium pool of the cell coat or glyocalix (3) and the other is the cellular calcium compartment. Ours can thus be considered a three compartment closed system. In our mathematical model, we first con-
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considered the parallel case of a three compartment system, represented schematically as follows:

\[ S_1 \xrightarrow{k_{12}} S_2 \xrightarrow{k_{23}} S_3 \]

Coat  Medium  Cell

The symbols used in our equations were taken from Robertson et al. (8) and are defined as follows.

<table>
<thead>
<tr>
<th>Symbols</th>
<th>Definitions</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S_i )</td>
<td>Amount of Ca in compartment ( i )</td>
<td>( \mu )moles</td>
</tr>
<tr>
<td>( R_i )</td>
<td>Amount of tracer ( ^{45})Ca in compartment ( i )</td>
<td>cpm</td>
</tr>
<tr>
<td>( X_i )</td>
<td>Specific activity of tracer in ( S_i )</td>
<td>cpm/( \mu )moles</td>
</tr>
<tr>
<td>( X_{i0} )</td>
<td>Specific activity of tracer in ( S_i ) at time zero</td>
<td>cpm/( \mu )moles</td>
</tr>
<tr>
<td>( E )</td>
<td>Equilibrium (infinite time) specific activity</td>
<td>cpm/( \mu )moles</td>
</tr>
<tr>
<td>( \phi_{ij} )</td>
<td>Flux of calcium from compartment ( i ) to compartment ( j )</td>
<td>( \mu )moles/min</td>
</tr>
<tr>
<td>( k_{ij} )</td>
<td>Fraction of ( S_i ) transferred to compartment ( j ) per unit time, ( k_{ij} = \frac{\phi_{ij}}{S_i} )</td>
<td>( \text{min}^{-1} )</td>
</tr>
</tbody>
</table>

**Assumptions**

1. Since in each culture flask containing 15 ml of medium and an average of 5.3 mg of cell protein the compartment size of \( S_2 \) (medium = 19,500 \( \mu \)moles) is 85 times greater than \( S_1 + S_3 \) (cell + coat = 230 \( \mu \)moles) and since the fluxes in and out of the medium are so small as to be negligible, \( S_2 \) and \( X_2 \) are constants. This assumption is supported by actual measurements of the medium specific activity.

2. From assumption 1, it follows that

\[ X_{20} = X_2 = E \]

3. The system is in a steady state, therefore we can write

\[ \phi_{12} = -\phi_{21} \text{ and } \phi_{23} = -\phi_{32} \]

and all fluxes are constant.

4. The change in specific activity in each compartment can be described by the following exponential function

\[ 1 - \frac{X_i}{E} = e^{-\lambda \tau} \]  \( (1) \)

5. After the tracer is added in compartment [2], the increase in tracer activity
of the monolayer which comprises compartments [1] and [3] is equal to its disappearance from compartment [2].

Therefore

\[ -\frac{dR_3}{dt} = \frac{dR_1}{dt} + \frac{dR_3}{dt} \]  

(2)

or

\[ -\frac{dR_3}{dt} = -\frac{dR_{1+3}}{dt} \]  

(3)

We can state

\[ \frac{dR_{1+3}}{dt} = (\phi_{11} + \phi_{33})X_2 - \phi_{12}X_1 - \phi_{32}X_3 \]  

(4)

Since from equation (1)

\[ X_1 = E(1 - e^{-\lambda_1 t}) \]  

(5)

we can write

\[ \frac{dR_{1+3}}{dt} = (\phi_{11} + \phi_{33})X_2 - \phi_{12}[E(1 - e^{-\lambda_1 t})] - \phi_{32}[E(1 - e^{-\lambda_3 t})] \]  

(6)

Since \( X_2 = E = \) constant and since all \( \phi ' \)s are constants

\[ \phi_{12}E + \phi_{32}E - (\phi_{11} + \phi_{33})X_2 = \text{constant} \]  

(7)

or

\[ E(\phi_{12} + \phi_{32} - \phi_{11} - \phi_{33}) = C \]  

(8)

Since at steady state \( C = 0 \), therefore

\[ \frac{dR_{1+3}}{dt} = -\phi_{12}e^{-\lambda_1 t} - \phi_{32}e^{-\lambda_3 t} \]  

(9)

From the experimental points expressed as cell \(^{46}\)Ca activity/medium specific activity or \( \frac{dR_{1+3}}{dt} \), the uptake curve was drawn by hand. \( \frac{dR_{1+3}}{t} \) was determined from the hand drawn curve and plotted on semilog paper, and the parameters of the different components were obtained by graphical analysis. The slopes and intercepts of both components were obtained by computer.
RESULTS

Fig. 1 illustrates the calcium uptake obtained by incubating the cells with $^{45}$Ca from 1 min to 4 hr in eight separate experiments. The hand drawn curve represents at least two exponential functions. The two components obtained by plotting $-\frac{dR}{dt}$ on semilog paper are shown in Fig. 2. These two exponential functions identified by graphical analysis follow the following general formulation

$$\frac{dR_{1+2}}{dt} = -Ae^{-\lambda_1 t} - Be^{-\lambda_2 t}$$

(10)

If we assume that the compartments are in parallel, we can write that $A = \phi_1$, $B = \phi_2$, $\lambda_1$, and $\lambda_2$, and solve for the fluxes, rate constant, and compartment size of the two phases. If the three compartments were placed in series, this oversimplification of the solution of the equations could
overestimate the pool size of the slow component, as pointed out by Huxley (13). However, when the rate constants differ widely, the error becomes negligible. In our case, since there is a 20-fold difference between $\lambda_1$ and $\lambda_3$ the distinction between the parallel and the series case becomes somewhat academic and will be overlooked. The half-times of the fast and slow components are 1.54 and 31 min, respectively. The intercepts are 474 and 60 $\mu$moles/(mg protein·min) for the fast and slow components (Table I). To verify the derived constants, a curve was reconstructed according to the following equation

$$\frac{dR_{1+3}}{dt} = \frac{1}{E} (474 \cdot e^{-0.448t} - 60 \cdot e^{-0.0223t})$$  (11)

**TABLE I**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fast phase</th>
<th>Slow phase</th>
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<tbody>
<tr>
<td>Half-time, min</td>
<td>1.54</td>
<td>31.0</td>
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<tr>
<td>Rate constants</td>
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<tr>
<td>$k_{12}$, min$^{-1}$</td>
<td>0.448</td>
<td>0.0223</td>
</tr>
<tr>
<td>$k_{23}$, min$^{-1}$</td>
<td></td>
<td></td>
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<tr>
<td>Fluxes</td>
<td></td>
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</tr>
<tr>
<td>$\phi_{12}$, $\mu$moles/(mg protein·min)</td>
<td>474</td>
<td></td>
</tr>
<tr>
<td>$\phi_{23}$, $\mu$moles/(mg protein·min)</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Compartment size*</td>
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<td></td>
</tr>
<tr>
<td>$S_1$, $\mu$moles/mg protein</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>$S_3$, $\mu$moles/mg protein</td>
<td>2.69</td>
<td></td>
</tr>
</tbody>
</table>

*Compartment size $S_i = \frac{\phi_{ij}}{k_{ij}}$
The reconstructed curve was compared to the actual data and appears on Fig. 1, as the dotted line. All points fall within the standard error of the mean of the eight individual experiments. When the data were expressed as the relative specific activity (cell specific activity/medium specific activity), the steady-state value obtained between 3 and 4 hr was 0.074 indicating that only 7.4% of the total calcium of the monolayer had exchanged. Since the total calcium pool of a HeLa cell monolayer contains 43.52 ± 2.60 μmoles/mg protein (1), compartments $S_1 + S_3$ should be 3.27 μmoles/mg protein, which compares well with the value of 3.75 μmoles/mg protein ($S_1 + S_8 = 1.06 + 2.69 = 3.75 \text{ μmoles/mg protein}$) obtained by compartmental analysis. When different compartments are kinetically defined by radiotracer technique it is difficult to assign to each phase a morphological or physiological label. It is likely, however, that the fast phase is extracellular and that the slow phase represents some cellular compartment. First, we can compare the values obtained by chemical analysis (1) with those in the present study which are shown in Table II. The value of the cell coat obtained previously (1) is much greater than our fast phase $S_1$ (37.78 vs. 1.06 μmoles/mg protein respectively). On the other hand, the “naked” cell Ca, 5.74 μmoles/mg protein (1), is not very different from the value of 2.69 μmoles/mg protein obtained by tracer kinetics. In this case the probable explanation for the difference is the presence in the cell of one or several unexchangeable pools of calcium. We know that in addition to binding to organic and inorganic anions, calcium can be accumulated and sequestered in mitochondria and endoplasmic reticulum (4). On the basis of the measurements in HeLa cells presented in Table II it would appear that only 45% of the intracellular calcium would be exchangeable. In support of these calculations, studies of calcium exchange in muscle and nerve also reveal that 40% only of the intracellular calcium pool is exchangeable (9–12). When the cellular calcium concentration is expressed on the basis of the water content of the cells, it is found to be 0.224 mmole/kg cell water. Since only a fraction of the exchangeable cell calcium is ionized (4),

<table>
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<th>Chemical analysis (1)</th>
<th>Kinetic analysis</th>
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<tbody>
<tr>
<td>Extracellular Ca, μmoles/mg protein</td>
<td>37.78</td>
<td>1.06</td>
</tr>
<tr>
<td>Cell Ca, μmoles/mg protein</td>
<td>5.74</td>
<td>2.69</td>
</tr>
<tr>
<td>Cell Ca, mmoles/kg cell water*</td>
<td>0.47</td>
<td>0.224</td>
</tr>
</tbody>
</table>

* Calculated on the basis of the water content of HeLa cells which is 12.29 ± 0.95 mg water/mg cell protein (1).
the activity gradient between the ECF with a calcium concentration of 1.3 mM and the intracellular compartment is considerable.

There is a puzzling discrepancy between the cell coat as determined by chemical dissection with trypsin-EDTA (37.78 μmoles/mg protein), and the fast phase $S_1$ obtained by our compartmental analysis (1.06 μmoles/mg protein). We have also to reconcile the fact that although 87% of the monolayer calcium is in the cell coat, a pool which can be removed with trypsin-EDTA and should be readily exchangeable (1), only 7% of total calcium of the monolayer had exchanged in 4 hr, according to our kinetic analyses. The only possible interpretation is that the large extracellular pool of calcium which is part of the cell coat or glyocalix is unexchangeable. This is not impossible since the calcium in this compartment is part of the coacervation of proteins, mucopolysaccharides, and prosthetic groups from the lipid plasma membrane which might involve very strong chemical bonds. The fast exchanging compartment $S_1$ could be a small exchangeable fraction of this glyocalix pool or more likely could represent binding to the plasma membrane. This calcium binding to the membrane could be a preliminary step for the translocation of calcium into the cell or it could be independent of it. In any case, we can visualize the different pools of calcium and their magnitude (expressed as μmoles/mg protein) as follows:

**Calcium Fluxes** In order to compare the calcium fluxes obtained in HeLa cells with those reported in the literature for other cell systems, we had to estimate the surface area of our cells. This was done as follows: Protein analyses, viable cell counts, and measurements of the cell diameter were performed on HeLa cells in suspension. The cell counts were made with a hemocytometer after staining the cells with erythrosin B, and the cell diameter was measured on a Reichert inverted microscope with an ocular micrometer and using a stage micrometer as reference. As shown in Table III, the mean diameter was found to be 17.1 μm. Accepting that a cell in suspension is a nearly perfect sphere, its surface area can be calculated to be 916 μm² per cell, 9.16 cm² per 10⁶ cells, or 36.5 cm²/mg cell protein. Since cells grown in monolayers flatten to attach to the glass of the culture flask, one-half of their
surface area is in close contact with the glass and thus unavailable for exchange. In our calculations of calcium fluxes the surface area was therefore halved to 18.3 cm²/mg cell protein. The validity of these calculations is supported by the fact that a complete monolayer covering the whole surface of a T-60 culture flask contains an average of 16 × 10⁶ cells or 4 mg of cell protein. According to our calculations, the surface area of this monolayer is 73 cm² as compared with the actual measurement of 72 cm² for the floor surface of a T-60 culture flask.

With this information, it is now possible to calculate the calcium fluxes in HeLa cells and to compare them with those reported in other tissues. Table IV presents these calculations for the slow and fast phases of our cell system. The exchange rate between the extracellular pool and the medium is fast, 0.430 μmole cm⁻² sec⁻¹. This is about one order of magnitude faster than those reported for transmembrane calcium fluxes (14-16). This fact supports our assumption that the fast phase of exchange represents a calcium compartment located outside the plasma membrane. On the other hand, the exchange rate of the slow phase, 0.0546 μmole cm⁻² sec⁻¹, is in excellent agreement with the transmembrane calcium fluxes obtained in nerve and muscle. Hodgkin and Keynes report a calcium influx of 0.076 μmole cm⁻² sec⁻¹ in squid axon (14); Bianchi and Shanes, an influx of 0.094 μmole cm⁻² sec⁻¹ in sartorius muscle (15); and Winegrad and Shanes, an influx in heart muscle varying from 0.016 to 0.048 μmole cm⁻² sec⁻¹ depending upon the extracellular calcium concentration (16). The close similarity between our results and those obtained in nerve and muscle strongly supports our assumption that the slow component of exchange in our system does represent calcium exchange between the intracellular pool and the ECF. It also suggests that the basal or resting exchange rate of calcium is not very different from cell to cell. But it does not follow, however, that, under different conditions, the basal rates of exchange will be similarly affected. The distinction between excitable and unexcitable mem-

### Table III

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of measurements</th>
</tr>
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<tbody>
<tr>
<td>Cell proteins, μg/10⁶ cells</td>
<td>28</td>
</tr>
<tr>
<td>Cell diameter, μm</td>
<td>100</td>
</tr>
<tr>
<td>Cell surface, μm²/cell</td>
<td>916</td>
</tr>
<tr>
<td>Cell surface, cm²/10⁶ cells</td>
<td>9.16</td>
</tr>
<tr>
<td>Cell surface, cm²/mg protein</td>
<td>36.7</td>
</tr>
<tr>
<td>Surface available for exchange in a</td>
<td>18.3</td>
</tr>
<tr>
<td>monolayer, cm²/mg protein</td>
<td></td>
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</tbody>
</table>
branes is a case in point. In addition, it remains to be shown whether agents affecting calcium transport, such as parathyroid hormone, thyrocalcitonin, and vitamin D, will uniformly influence calcium exchange in every cell system.

**Influence of DNP on Calcium Influx** We have stated earlier that the thermodynamic conditions prevailing in our system could permit a passive influx of calcium from the ECF to the cell. Actually we have already shown that in HeLa cells calcium uptake is totally unaffected by anaerobic conditions, by $10^{-4}$ M dinitrophenol, and by $10^{-3}$ M iodoacetic acid (1). DNP is also without effect on the calcium uptake of another cell strain, monkey kidney cells (17). Thus, if our reasoning is correct, calcium influx should not be affected by metabolic inhibitors. To test this hypothesis, HeLa cells were incubated as described above in the presence of 20 μC of $^{44}$Ca and $10^{-3}$ M DNP. The inhibitor was added 30 min before the isotope. The cells were harvested after various incubation times, from 1 min to 4 hr. The results from three experiments are shown in Fig. 3. The time points do not deviate significantly from the solid line representing the control curve of Fig. 1, drawn on the graph as reference. However, since the fast component of influx represents a large percentage of the total uptake and since we have no reason to believe that it would be affected by DNP if we assume it to represent calcium binding to the membrane, the effects of DNP on the slow phase might not be detectable. We performed, therefore, a mathematical dissection of the two compartments by subtracting from the original data the fast phase which we recalculated from its derived parameters. The results of this dissection are shown in Fig. 4. It is clear that no significant effect of DNP can be detected on the slow component of influx. No more information can be derived from these results, because we do not know whether the system is still in steady state in the presence of DNP and whether our earlier assumptions are still valid. But we can conclude that DNP does not interfere with calcium influx in HeLa cells. This would support
the suggestion already advanced by several investigators in their study of calcium transport in nerve and muscle that calcium influx is a passive phenomenon in the sense that it is not dependent upon the expenditure of metabolic energy (9, 14, 18).

Figure 3. Calcium uptake in HeLa cells in the presence of $10^{-4}$ M DNP. Each point represents the mean ± SEM of three experiments. The solid line is the hand drawn curve of the control calcium uptake of Fig. 1 included here as reference.

Figure 4. Calcium uptake by the slow compartment with and without DNP after mathematical dissection of the fast phase. From each experimental point, the corresponding value for the fast phase calculated from the derived constants was subtracted. Each point represents eight experiments for the controls and three experiments for DNP.
DISCUSSION

It is difficult to have a precise idea of the calcium concentration of a cell because of the ubiquity of calcium in every tissue. Calcium is an integral part of the plasma membrane, it is bound to the glycoproteins of the cell coat, to the collagen and mucopolysaccharides of the ground substance, and to many other anions and structural constituents of the cell. We have previously suggested that the major part of the calcium could very well be extracellular in showing that even in cell cultures 90% of the calcium can be removed by trypsin-EDTA (1). It is certain now that all cells are coated with a layer of glycoproteins which binds a significant amount of calcium (3). Since trypsin removes the cell coat without affecting the plasma membrane, the calcium which was removed with the cell coat was, therefore, considered extracellular (1, 3). It was debatable, however, whether some of the cellular calcium could also be removed in the process of harvesting the cells with trypsin-EDTA. We have shown now with techniques which do not involve the use of trypsin-EDTA that this is very unlikely. The experiments presented in this paper confirm the existence of several compartments of calcium in a cell monolayer. There are at least two exchangeable pools and two unexchangeable ones. The magnitude of the fast exchanging pool and its rate of exchange with the medium strongly support our assumption that it is extracellular. It represents presumably calcium binding to the surface of the cell membrane. The slow exchanging component has a rate of exchange and a magnitude which are compatible with an intracellular compartment. The pool size and the influx rate are practically the same as those reported for muscle and nerve (4, 14, 19–21). Furthermore, this exchangeable pool is roughly 40% of the total cellular calcium measured in cells deprived of their coat by trypsin-EDTA (1) and is identical to the exchangeable calcium reported in muscle and nerve (4, 9–12). We conclude, therefore, that the slow component measured in our system represents the exchangeable intracellular calcium compartment. In addition, there are two unexchangeable calcium pools: one intracellular and the other extracellular. The magnitude of the latter might be characteristic of a cell monolayer although it is likely that slices and tissues also contain large amounts of extracellular bound calcium (9, 16, 22). It is our contention that the intracellular calcium concentration is not very different from cell to cell. The close fit of the values in nerve, muscle, kidney cells, and HeLa cells supports our argument (1, 14, 19–21). It is likely, therefore, that any value of intracellular calcium exceeding $0.5 \times 10^{-8}$ M is a reflection of some contamination from extracellular calcium in the determinations.

It is evident, however, that even when different compartments are kinetically defined, it is difficult to assign to each phase a morphological or physiological entity. The choice has to be made between the most likely possibilities.
on the basis of the most reasonable assumptions. In our particular case, the slow phase could possibly be assigned to other extracellular pools, distinct from the fast exchanging compartment: a slowly exchanging calcium-binding pool in the cell coat, for instance, or another class of binding sites on the plasma membrane. It would be unusual, however, for calcium binding of either of these extracellular pools to be affected by metabolic inhibitors. Since we have identified by calcium efflux studies a slowly exchanging pool with a time constant practically identical to that of the slow component of influx, it is reasonable to assume that these are one and the same compartment (23). And since calcium efflux from this slow compartment is markedly inhibited by metabolic inhibitors (23), it is very unlikely that the slow phase represents binding to an unidentified extracellular pool.

It is clear now that an activity and an electrical potential difference exists between the ECF and the intracellular compartment. The fact that DNP does not affect the uptake curve of calcium in our system brings new evidence that calcium entry into the cell is not metabolically dependent (1, 9, 14, 18). Our hypothesis is that calcium influx is a facilitated diffusion process. This would imply that any experimental conditions affecting calcium influx do so by increasing some parameter of the membrane "permeability," e.g. calcium binding to a carrier or the diffusion coefficient of a calcium-carrier complex and not by stimulating an active or metabolically dependent process.

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