Calcium Distribution in *Amoeba proteus*

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ABSTRACT A preliminary investigation of the distribution of cellular calcium in *Amoeba proteus* was undertaken. Total cellular calcium under control conditions was found to be 4.59 mmol/kg of cells. When the external Ca^{++} concentration is increased from the control level of 0.03 to 20 mM, a net Ca^{++} influx results with a new steady-state cellular calcium level being achieved in $\sim$ 3 h. At steady state the amount of calcium per unit weight of cells is higher than the amount of calcium per unit weight of external solution when the external concentration of Ca^{++} is below 10 mM. At external Ca^{++} concentrations above this level, total cellular calcium approaches the medium level of Ca^{++}. Steady-state calcium exchange in *Amoeba proteus* was determined with ^{45}Ca. There is an immediate and rapid exchange of $\sim$0.84 mmol/kg of cells or 18% of the total cellular calcium with the labelled Ca^{++}. Following this initial exchange, there was very little if any further exchange observed. Most of this exchanged calcium could be eliminated from the cell with 1 mM La^{+++}, suggesting that the exchanged calcium is associated with the surface of the cell. Increase in either the external Ca^{++} concentration or pH raise the amount of exchangeable calcium associated with the cell. Calcium may be associated with the cell surface as a co-ion in the diffuse double layer or bound to fixed negative sites on the surface of the cell. If Ca^{++}-binding sites do exist on the cell surface, there may be more than one type and they may have different dissociation constants. The cytoplasmic Ca^{++} ion activity is probably maintained at very low levels.

INTRODUCTION

Freshwater protozoans have served as model cell systems in which several basic physiological processes have been examined. For example, the freshwater amoeba has been used extensively to study amoeboid movement and pinocytosis. In addition, the distribution of inorganic ions, especially Na^+, K^+, and Cl^-, has also been investigated in freshwater amoebae (Bruce and Marshall, 1965; Prusch and Dunham, 1972). Both Na^+ and K^+ are actively transported in *Amoeba proteus*; K^+ is actively accumulated from the external medium, and Na^+ is actively extruded from the cell. This active extrusion of Na^+ from the cell may be associated with the activity of the contractile vacuole (Prusch, 1977). A considerable fraction of these ions is also maintained in part by

internal compartmentalization or binding processes, in which the cytoplasmic activities of Na^+ and K^+ are reduced. The Cl^- ion is most likely distributed passively in the amoeba.

Considerably less is known about the distribution of calcium in the amoeba, even though Ca^{++} apparently plays an important role in both amoeboid movement (Allen and Taylor, 1975) and pinocytosis (Prusch and Hannafin, 1979). Also, the Ca^{++} ion has been shown to control the overall solute permeability of the amoeba cell surface and influences the distribution of other inorganic ions in this system (Prusch and Dunham, 1972). In this regard, Tasaki and Kamiya (1964) found that the depolarization of the membrane potential in *Chaos carolinensis* by K^+ is antagonized by the presence of Ca^{++} ions, whereas Bruce and Marshall (1965) demonstrated that the Na^+ permeability of *Chaos carolinensis* surface increases in the absence of Ca^{++} ions. The membrane of *Amoeba proteus* behaves electrically as a mixed K^+-Na^+ electrode in low Ca^{++} media, but as the external Ca^{++} level is increased, the membrane potential becomes more sensitive to changes in external K^+ and less sensitive to changes in Na^+ (Prusch and Dunham, 1972). Related to this control of amoeba surface permeability by Ca^{++} is the observation that *Amoeba proteus* is capable of regulating internal Na^+ only in the presence of minimal levels of external Ca^{++}. Because it is known that the Ca^{++} ion plays an important role in the membrane processes of the amoeba, as well as in a great number of other cellular systems, the distribution of calcium in *Amoeba proteus* was investigated.

MATERIALS AND METHODS

Mass cultures of *Amoeba proteus* were maintained in Prescott-James (1955) or control medium (KCl—0.08, $CaHPO_4$—0.03, and $MgSO_4$—0.01 mM/liter) and fed on *Tetrahymena pyriformis*. The cultures were maintained at room temperature in flat, rectangular (30 × 45 cm), pyrex dishes, and were fed and washed every 48 h. In preparation for an experiment, the cells were starved for 24 h, concentrated by aspirating off most of the culture medium, and then washed three times in 500 ml of fresh control medium. The pH of the freshly washed cell suspension was approximately 6.5. In these studies, 0.4-ml aliquots of a freshly washed cell suspension were taken periodically and spun down in preweighed 0.45-ml polyethylene centrifuge tubes for 1 min in a Beckman 152 Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The supernate was then aspirated out of the tubes and the wet weight of the cell pellets was determined. The cell density of these cell suspensions was adjusted so that a 0.4-ml aliquot routinely yielded cell pellets with a wet weight of between 6 and 10 mg. In experiments involving long preincubation periods, the cells were not concentrated until just prior to the experimental or analytical procedure. During the time-course of an experiment, small samples of the cell suspension were examined with a light microscope in order to observe the condition of the cells. Extracellular space of the cell pellets was measured with [^{14}C]inulin (New England Nuclear, Boston, Mass.).

Total cellular calcium was determined by extracting the cell pellets with boiling distilled water and measuring the Ca^{++} content of the extract with an Eppendorf (Eppendorf Gerätebau, Netheler & Hinz GmbH, Hamburg, West Germany) flame photometer (Ca^{++} sensitivity 0.01 ppm with an accuracy of ±1%). Unidirectional Ca^{++} movements were studied using ^{45}Ca (New England Nuclear). Labelled Ca^{++} (2 μCi/ml) was added to a cell suspension, and aliquots of this suspension were taken at

various time intervals, spun down, and extracted as described above. The cell extract was then added to 15 ml Aquasol (New England Nuclear) in scintillation vials and counted in a liquid scintillation counter. All experiments were performed at room temperature and the results are expressed as the mean ± the standard error of the mean (number of determinations).

RESULTS

The total cellular calcium content of *Amoeba proteus* was found to be 4.59 ± 0.14 mmol/kg cells (corrected for an extracellular space of 24%), while the external Ca^{++} concentration was 0.03 mM. The rate of net Ca^{++} movement into the cell was determined by increasing the external Ca^{++} concentration from the control level of 0.03 to 20 mM with $CaCl_2$. The time-course of the observed net calcium movement is shown in Fig. 1. A steady-state cellular calcium level is established ∾ 3 h after the increase in external Ca^{++}; in this

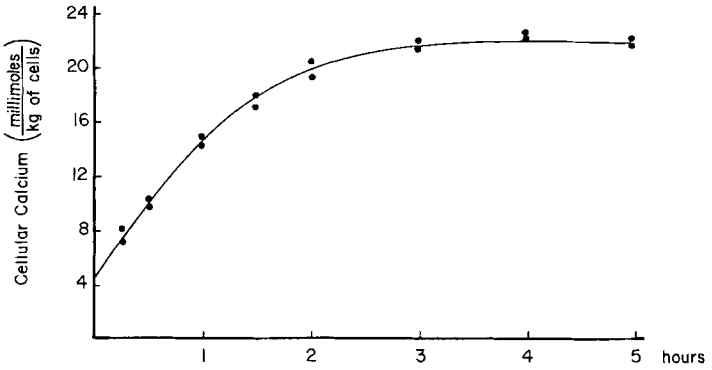

FIGURE 1. Net Ca^{++} influx. The initial external Ca^{++} concentration was increased from 0.03 to 20 mM with $CaCl_2$ and the accumulation of Ca^{++} in the cells was followed with time. Each point represents a single determination from one experiment.

case the total cellular calcium content was 22 mmol/kg cells. Steady-state cellular calcium as a function of the external Ca^{++} concentration is shown in Fig. 2. Total cell calcium was determined in cells exposed to varying external Ca^{++} concentrations for 4 h. The total amount of calcium per kilogram of cells is greater than the total amount of ionized calcium per kilogram of solution when the external concentration of Ca^{++} is low (0.03–1.0 mM). The amount of calcium per unit weight of cells increases as the external concentration of Ca^{++} increases and, above 10 mM external Ca^{++} concentration, the total cellular calcium (per kilogram cells) is approximately equal to the total amount of calcium per kilogram of external solution. These relationships suggest a large proportion of bound calcium in these cells.

Labelled calcium movements and the exchangeable fraction of the total cell calcium was determined with ^{45}Ca. A representative exchange experiment with cells in the control medium (0.03 mM Ca^{++}) is shown in Fig. 3. There is

an immediate and rapid exchange of labelled Ca^{++} (resolution time was limited to 30 s) for a fraction of the total cellular calcium after which there is an extremely slow, if any, additional exchange over the next 5 h. In a series of studies, the exchangeable cell calcium, on the basis of specific activities, was

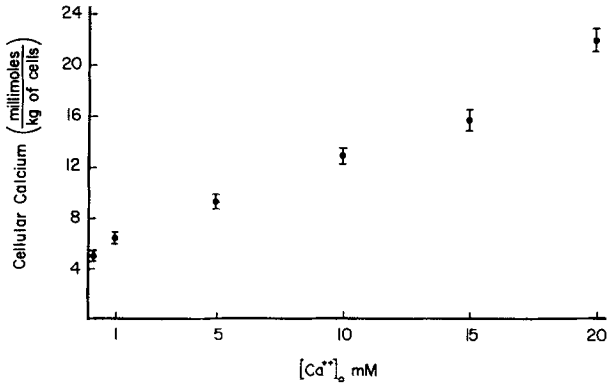

FIGURE 2. Steady-state cellular calcium as a function of the external Ca^{++} concentration. Cell suspensions were equilibrated in control medium with varying concentrations of Ca^{++} for 4 h, after which time the calcium content was measured. Each point represents the mean of eight determinations, with the vertical bar indicating ± 1 SEM.

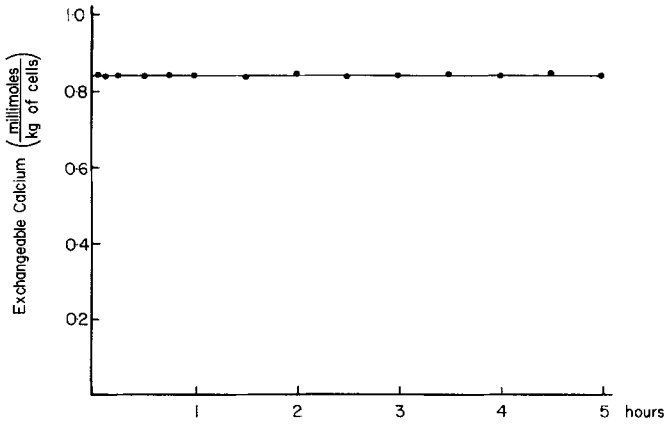

FIGURE 3. Ca^{++} exchange. ^{45}Ca (2 μCi/ml) was added to a cell suspension in the control medium (0.03 mM Ca^{++}) and the exchange monitored with time. The amount of Ca^{++} exchange was calculated on the basis of specific activities. Each point represents a single determination from one experiment.

found to be 0.84 ± 0.05 (19) mmol/kg cells. This would indicate that 18% of the total cellular calcium is exchangeable with external Ca^{++} and suggests that the rest of the total cellular calcium which may exchange very slowly may be bound to external and internal binding sites. Lanthanum, which

competes for Ca^{++}-binding sites, presumably on the basis of size and charge density (Lettvin et al., 1964), displaces most of the previously exchanged calcium (Fig. 4). That is, when $LaCl_3$ (1 mM) is added to a cell suspension which has arbitrarily been allowed to equilibrate with ^{45}Ca for 15 min, the amount of exchangeable calcium associated with the cell immediately falls to a very low and steady level of 0.03 mmol/kg cells. Addition of 1 mM $LaCl_3$ to the control medium under these conditions decreases the pH from 6.5 to 6.0. Steady-state calcium associated with cells as a function of the external La^{+++} concentration is shown in Fig. 5. Very low levels of La^{+++} (10^{-6} M) had no measurable effect on cellular calcium, but further increases in external La^{+++} progressively removed more calcium from the cell, the maximal effect of La^{+++} occurring at $\sim 10^{-3}$ M. Since most cell surfaces are impermeable to La^{+++} (Josefsson and Hanssen, 1976) and it has previously been established that La^{+++} binds to cell surfaces (Weiss, 1974), these observations indicate that La^{+++} is displacing surface calcium from *Amoeba proteus*.

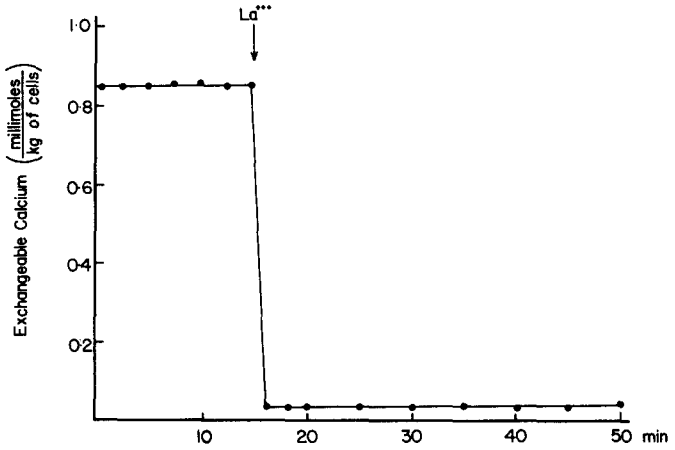

FIGURE 4. Effect of La^{+++} on Ca^{++} exchange in *Amoeba proteus*. A cell suspension in control medium (0.03 mM Ca^{++}, pH 6.5) was equilibrated with ^{45}Ca (2 μCi/ml) for 15 min and then 1 mM $LaCl_3$ was added to the suspension. Each point represents a single determination with the arrow indicating the time of La^{+++} addition.

The fraction of cell calcium measured by isotope exchange under steady-state control conditions in this system most likely represents calcium associated with the cell surface. In treating the cell as a sphere with a surface area of 5×10^{-4} cm^2 per cell with $\sim 10^6$ cells/gram wet weight (Prusch and Dunham, 1972), there would be 1.68×10^{-9} mol Ca^{++}/cm^2. Because of the highly variable shape of the amoeba, in which case the surface area is greater than that calculated for a sphere, and because surface calcium in *Amoeba proteus* may not be distributed uniformly (Shida, 1970), there may be difficulties in expressing surface calcium in this system as moles/cm^2. Consequently, cellular

calcium measured in this study by isotope exchange will be reported as millimoles/kilogram cells.

As the external Ca^{++} concentration is increased, total cellular calcium also increases (Fig. 2), although steady-state ^{45}Ca exchange kinetics remain essentially the same as under control conditions. The amount of cellular calcium exchangeable with external Ca^{++} in cells equilibrated for 4 h in media with varying Ca^{++} concentrations is shown in Fig. 6. The apparent exchangeable fraction of total cellular calcium remains relatively constant at ~1.0–1.5 mmol/kg cells over a wide range of external Ca^{++} concentrations (0.1–5 mM). Increases in the external Ca^{++} concentration above 5 mM increases the absolute amount of exchangeable calcium associated with the cell. When the external Ca^{++} level is 0.03 mM, total cell calcium is 4.59 mmol/kg cells and 18% or 0.84 mmol/kg cells of this total cell calcium is exchangeable with ^{45}Ca. In addition, 1 mM La^{+++} removes most of the exchanged calcium. On the

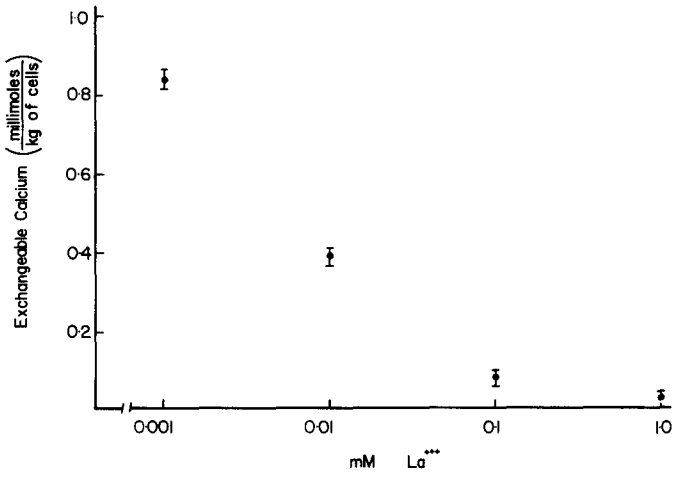

FIGURE 5. Steady-state exchangeable Ca^{++} as a function of the external La^{+++} concentration. Cell suspensions were equilibrated for 15 min with ^{45}Ca (2 μCi/ml) and varying amounts of La^{+++}, as $LaCl_3$. Each point represents the mean of eight determinations with the vertical bar indicating ± 1 SEM.

other hand, cells which have been equilibrated in control medium with 20 mM Ca^{++} have a total cell calcium content of 22 mmol/kg cells and 36% or 7.92 mmol/kg cells of this calcium is exchangeable with ^{45}Ca. In this case 1 mM La^{+++} displaces 4.2 mmol/kg cells of the previously exchanged calcium. Increasing the external La^{+++} concentration up to 10 mM did not remove additional calcium from the cell. A portion of the rapidly exchanged component of the cellular calcium (3.72 mmol/kg cells) under these conditions is not displaced by La^{+++}. This could imply that at higher external Ca^{++} concentrations, a series of previously unoccupied surface Ca^{++}-binding sites now bind Ca^{++} and because of steric considerations or dissociation energies, La^{+++} is unable to displace Ca^{++} from these sites. Alternatively, a fraction of the exchangeable calcium may have entered the cell.

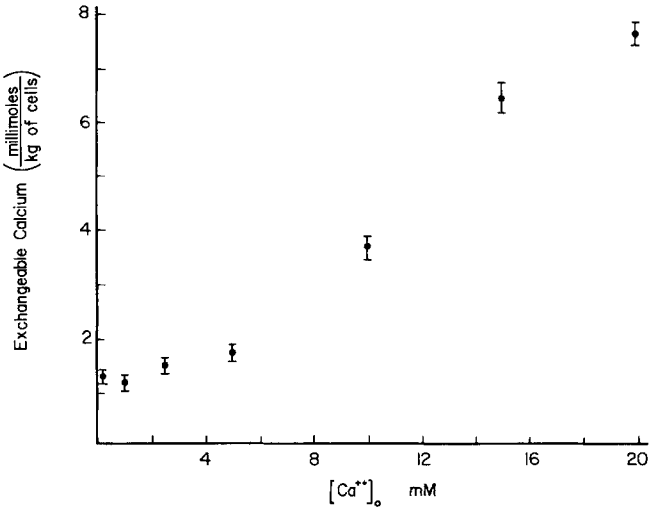

FIGURE 6. Steady-state Ca^{++} exchange as a function of the external Ca^{++} concentration. Cell suspensions were equilibrated with varying Ca^{++} concentrations for 4 h, ^{45}Ca was then added to each cell suspension (2 μCi/ml) and the amount of Ca^{++} exchange measured after another 15-min equilibration period. Each point represents the mean of 10 determinations, with the vertical bar indicating ± 1 SEM.

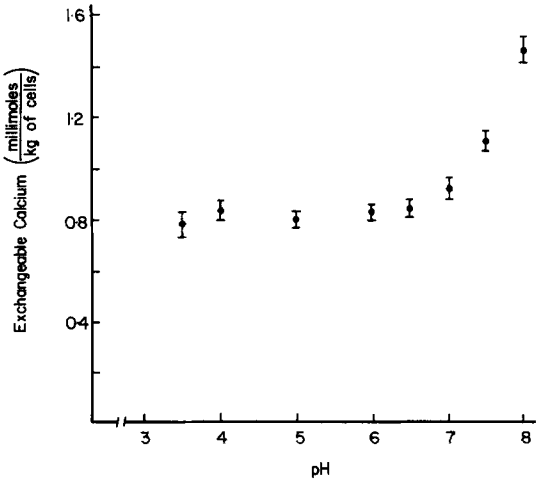

FIGURE 7. Effect of pH on Ca^{++} exchange in *Amoeba proteus*. Cell suspensions were equilibrated for 15 min in control medium (0.03 mM Ca^{++}) with ^{45}Ca (2 μCi/ml), the pH of the cell suspensions was adjusted to the indicated values with either 0.1 N HCl or NaOH. Each point represents the mean of eight determinations with the vertical bar indicating ± 1 SEM.

The effect of pH on calcium exchange in *Amoeba proteus* is shown in Fig. 7. As the pH of the medium is increased above 6.5, the amount of exchangeable calcium associated with the cell increases. Decreases in pH below the control medium pH of 6.5 do not alter the amount of exchangeable calcium associated

with the amoeba surface, indicating that the previously observed decrease in cell calcium in the presence of La^{+++} (Fig. 4) is not simply due to a decrease in pH.

DISCUSSION

The total cellular calcium content of *Amoeba proteus* under control conditions is 4.59 mmol/kg cells. On the basis of ^{45}Ca exchange kinetics and the effects of La^{+++} on exchanged calcium, a large fraction of this total cell calcium is located on the cell surface. When labelled Ca^{++} is added to a suspension of amoebae, there is a rapid initial exchange, with very little further exchange observed over the next several hours (Fig. 3). This extremely slow Ca^{++} exchange following the initial rapid uptake is not unique to amoeba; isotopic Ca^{++} exchange in the large ciliated protozoan *Spirostomum ambiguum* apparently requires a matter of days to reach steady-state distribution (Jones, 1966). Addition of 1 mM La^{+++} to an amoeba cell suspension previously equilibrated with ^{45}Ca immediately removes most of the exchanged calcium from the cells. Both the rapid initial calcium exchange and the removal of this exchanged calcium by La^{+++} indicate that this calcium is associated with the surface of the cell.

The use of labelled Ca^{++} in this study allows the approximation of surface associated calcium in *Amoeba proteus* but does not distinguish between calcium physically bound to fixed sites on the cell surface or Ca^{++} associated with the diffuse double layer (McLaughlin et al., 1971). That is, in the presence of fixed negative sites on the cell surface, a negative potential will exist at the cell surface in respect to the bulk phase of medium surrounding the cell. Brewer and Bell (1970) demonstrated previously that the surface of *Amoeba proteus* does indeed have a net negative surface charge. Because of this surface charge, divalent cations present in the bulk phase may be distributed so as to screen and reduce this surface potential resulting in a localized higher Ca^{++} concentration in the vicinity of the cell surface in respect to the bulk phase (McLaughlin, 1977). The effect of pH on surface calcium distribution in the amoeba, i.e., an apparent increase in the amount of calcium associated with the cell surface with an increase in pH (Fig. 7), could result from either a configurational change in surface Ca^{++}-binding sites, or pH could influence the negative surface charge density.

If there are at least some Ca^{++}-binding sites associated with the amoeba surface, there may be more than one type and they may have different dissociation constants. Physically, if all of the exchangeable calcium on the amoeba surface was bound by a single family of binding sites, then once these sites were occupied and therefore saturated, no additional Ca^{++} should become associated with the cell surface with further increases in external Ca^{++}. The amount of calcium apparently associated with the cell surface is relatively constant over a wide range of external Ca^{++} concentrations, from 0.1 to 5 mM (Fig. 6). An increase in the external Ca^{++} concentration above 5 mM brings about an increase in exchangeable calcium. This discontinuity in the amount of exchangeable calcium could be explained simply on the basis of more than one type of binding site for Ca^{++} (McLaughlin, 1977). Alterna-

tively, calcium maintenance on or in the vicinity of the amoeba surface may be accomplished by both a screening mechanism and by physical binding sites whose affinity for Ca^{++} may be influenced by, among other things, the membrane phospholipid composition (Hauser and Dawson, 1967).

In a number of cell systems it has now been established that a sizeable fraction of the total cell calcium is associated with the surface of the cell. In the cultured rat heart cell for example, Langer and Frank (1972) demonstrated that 15% of the total cell calcium was associated with the cell surface and this surface calcium could be displaced with La^{+++}. Apparently 87% of total cell calcium is associated with the surface of the HeLa cell (Borle, 1968), and a significant fraction of cell calcium is also associated with the membrane of the human red blood cell (Schatzmann and Vincenzi, 1969). In the giant amoeba, *Chaos chaos*, a significant amount of calcium is associated with the cell surface (Hendril, 1972), and Shida (1970) reported the cytochemical localization of calcium on the median and anterior portions of the surface of *Amoeba proteus*. In *Amoeba discoides*, La^{+++} inhibits locomotion and it was suggested that this was brought about by a La^{+++} inhibition of Ca^{++} pumps (Hawkes and Holberton, 1973). It is possible that the interference with locomotion in the amoeba with La^{+++} may be the more immediate effect of surface calcium displacement by La^{+++}.

On the basis of the Ca^{++} exchange data then, at least 18% of the total cell calcium in *Amoeba proteus* is associated with the surface of the cell. This probably represents a minimal value because there may be an unexchangeable fraction of calcium restricted to the cell surface. This still leaves a considerable amount of Ca^{++} which is probably intracellular. Borle (1968) suggests that the cytoplasmic level of Ca^{++} in a variety of cells ranges from 0.4 to 0.8 mM. In *Amoeba proteus* cytoplasmic levels of Ca^{++} may be in this range, or slightly higher, but as is the case in other cells, the cytoplasmic Ca^{++} ion activity is most likely maintained at very low levels. Several previous studies have suggested that the Ca^{++} ion activity of the amoeba cytoplasm is maintained at very low levels. For example, Taylor et al. (1973) have shown that isolated cytoplasm from the amoeba *Chaos carolinensis* contracts when the medium Ca^{++} ion activity is increased to 7×10^{-7} M, indicating that the in-vivo Ca^{++} ion activity is probably maintained at levels lower than this, and Simard-Duquesne and Couillard (1962) have demonstrated the presence of Ca^{++}-activated ATPase in the cytoplasm of *Amoeba proteus*, again suggesting the maintenance of low cytoplasmic Ca^{++} ion activities.

In this study, the maintenance of a low cytoplasmic Ca^{++} ion activity is indicated at least in part from the observed Ca^{++} exchange kinetics. The rapid Ca^{++} exchange kinetics in the amoeba (Fig. 3) can be explained if the only cellular calcium available for exchange was associated with the cell surface and cytoplasmic Ca^{++} was not available for exchange; either because of an impermeable membrane or the intracellular Ca^{++} is tightly bound or otherwise sequestered. Because there is an increase in cellular calcium when the extracellular Ca^{++} concentration is increased (Figs. 1 and 2), the amoeba surface is at least slightly permeable to Ca^{++}. Most of the Ca^{++} in the cytoplasm may then be unavailable for exchange. It is interesting to note that when the

external Ca^{++} concentration is increased, thus increasing cellular calcium, the Ca^{++} exchange kinetics do not change. That is, there is only an initial rapid surface exchange and little exchange after this, implying that the Ca^{++} which entered the cell is immediately bound. In *Spirostomum ambiguum*, where isotopic Ca^{++} exchange is very slow, Jones (1967) has suggested that most of the cytoplasmic Ca^{++} is bound in the form of apatite as it enters the cell. In amoeba, Reinold and Stochem (1972) have suggested a cytoplasmic Ca^{++} storage system, with Ca^{++} being sequestered in cytoplasmic vesicles.

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