Calcium Exchange and Ionized Cytoplasmic Calcium in Resting and Activated Human Monocytes

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Abstract. We have performed a comprehensive study of calcium tracer flux, distribution, content, and ionized cytoplasmic calcium during monocyte activation. A model of monocyte calcium was developed from ^{45}Ca uptake and exodus curves which indicated that cell calcium was partitioned between three compartments. The magnitude of the time constants for each pool lead us to propose cellular locations for these three compartments: a surface plasma membrane pool, a cytoplasmic pool, and an organelle pool. ^{45}Ca uptake and exodus experiments were analyzed using a nonlinear least squares fit of compartmental exchange rates and sizes. The production of superoxide was used as a reflection of the state of activation of the monocytes treated with Concanavalin A (Con A). We found that Con A-treated monocytes have an increase in the calcium exchange rate with the cytoplasmic pool from 0.04 to 0.07/min ($P < 0.05$), and an increase in the size of the cytoplasmic pool from 0.08 to 0.13 pmol/cell ($P < 0.05$). There were no significant changes in the exchange rates or sizes associated with either of the other two compartments. The cytoplasmic ionized calcium was measured with the fluorescent probe, Quin 2, which indicated a resting level of 83 nM free calcium in unadhered monocytes. Con A stimulation caused a doubling of the cytoplasmic free calcium to 163 nM within 45 s. This increment in cytoplasmic free calcium preceded the onset of superoxide following Con A treatment. These studies indicate that Con A binding to the plasma membrane increases the monocyte plasma membrane permeability to calcium. External calcium en-

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ters the cell at an increased rate and contributes to both internally bound and free calcium. The magnitude of the increase in free calcium is proportional to the concentration of Con A and stimulates calcium extrusion via the calcium transport ATPase. Moreover, there is an increased concentration of ionized cytoplasmic calcium which has the potential to interact with other cellular regulators that modulate cell activation and superoxide production.

Introduction

Alterations in cellular free calcium may induce chemical events that link external stimuli to responses in cell function. Radiolabeled calcium has been used to study the transport and content of cell calcium and to assess the effects of cell activation on calcium metabolism. Most previous studies have not considered all of the relevant parameters of calcium metabolism. Increases in the uptake of ^{45}Ca into lymphocytes treated with mitogens (1-6), granulocytes treated with chemotactic factors (7-9), platelets treated with ADP and epinephrine (10), and activated macrophages (11) have been described. Although there are reports that include both ^{45}Ca efflux and influx studies (8, 12, 13) and one report of total cell calcium and ^{45}Ca influx (7), there has not been an analysis of calcium compartments in resting and activated cells nor a description of the relationship of cellular ^{45}Ca to the changes that may occur in cytoplasmic ionized calcium. Performance of such studies in human cells has been impeded by the requirement for large numbers of highly purified populations of cells and until recently, by the lack of a practical probe for cellular free calcium. In this study we have made measurements of total monocyte calcium, calcium influx and efflux, and cytoplasmic ionized calcium, and have described the alterations in calcium exchange and content that accompany the activation of human monocytes.

Methods

Isolation of human monocytes. Human mononuclear leukocytes were isolated from plateletpheresis residues (14) using an isopaque-ficoll step gradient (15). The monocytes were separated from the lymphocytes by counterflow centrifugal elutriation by first eluting the lymphocytes from the separation chamber, leaving behind a highly enriched monocyte

population (16). The monocytes were eluted by increasing the rate of buffer flow through the separation chamber. This procedure yielded a monocyte population of >90% purity as judged by cell volume distribution, morphology on blood films treated with Wright's stain, and with a fluoride-sensitive napthyl-ASD acetate stain. Staining with propidium iodide indicated >95% viability of the monocyte population.

Measurement of superoxide. Superoxide production was quantitated by measuring the reduction of ferricytochrome C at 550 nm in a recording spectrophotometer (Beckman Model 25, Beckman Instruments, Inc., Fullerton, CA) (17). The specificity of the reducing agent was assured by the inclusion of superoxide dismutase in the reference cuvette. Cytochalasin B (5 µg/ml) was included in superoxide assays to enhance the response to Concanavalin A (Con A)[1] (17). The assay was performed in Hanks' buffered salt solution with added 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.3 (HBSS), at 37°C. Con A was added in concentrations up to 200 µg/ml, as required by individual experiments. The difference in the rate of superoxide production in the presence or absence of α-methyl mannoside (α-MM) at each Con A concentration was attributed to Con A stimulation of the monocytes.

^{45}Ca influx into monocytes. Monocytes were suspended at a concentration of 10^7 cells/ml in Medium-199 with Earle's salts containing 5 mM Ca, 5 mM K, 0.8 mM Mg, and 143 mM Na (Gibco, Grand Island, NY), 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO), 25 mM Hepes (Calbiochem-Behring, San Diego, CA), at pH 7.3, and were preincubated in siliconized plastic tubes for 30 min in a shaking water bath at 37°C. The mitochondrial inhibitors, oligomycin and potassium cyanide, were added at concentrations of 7 µM and 1 mM, respectively, at the beginning of the preincubation in appropriate experiments. Following the preincubation, $^{45}CaCl_2$ was added at an activity of 0.55 mCi/mol to initiate the experiment. In parallel duplicate tubes, ^{45}Ca was omitted and [^{3}H]sucrose, 0.5 mCi/mol, was added as an extracellular marker just before sampling. At the appropriate times, samples were removed and placed on a step gradient of F50 silicone (General Electric, Waterford, NY), specific gravity 1.050 (25°C). The samples were centrifuged at 12,800 g in a microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY). 25 µl of supernate were removed and diluted with distilled water to a volume of 0.5 ml, which was added to 10 ml Bray's solution, and the radioactivity determined in a liquid scintillation spectrophotometer (Model 2450, Packard Instruments Co., Inc., Downers Grove, IL). The microcentrifuge tubes were inverted and drained, and the tips were severed and placed in 3 ml plastic vials. 1 ml of twice distilled water was added, and the samples were dispersed and disrupted by sonication for 20 s at 40 W. 0.5 ml of each sample was added to 10 ml Bray's solution for measurement of radioactivity. The addition of an internal ^{45}Ca standard to both the cell sonicate and supernatant fractions indicated that the radioactive counting efficiencies were identical and that no correction for photon quenching was necessary. The volume of trapped space was calculated from the [^{3}H]sucrose samples and was used in the calculation of ^{45}Ca influx, ^{45}Ca efflux, and in the measurement of total monocyte calcium.

Total cell calcium measurement. Total cell calcium was measured by graphite furnace atomic absorption spectroscopy, as previously described (18). For these measurements, the vessels were washed before use in ultrapure 20% HCl, then twice in twice distilled water. 10-µl aliquots were pipetted into the graphite cuvette of a flameless atomic atomizer attached to an atomic absorption spectrophotometer (Model

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1. *Abbreviations used in this paper:* Con A, Concanavalin A; α-MM, α-methyl mannoside.

555 and 351, Instrumentation Laboratory, Inc., Lexington, MA). The time-temperature profile for the analysis was 15 s to 75°C, 45 s to 800°C, 15 s to 1,500°C, and 5 s to 2,500°C. The peak height mode of analysis was used in which the maximum recorder deflection was directly proportional to the calcium content of the sample. The successive addition of a calcium solution of known concentration to each sample was used to standardize the determination. Linear regression was used to analyze the response of peak height to added calcium. The quantity of calcium in the experimental sample was calculated from the equation:

calcium content = (peak height/slope) × (1/cell count),

where the peak height of the experimental sample is the value of y intercept of the fitted curve. The slope is the value in units of height per femtomole added calcium as determined by regression analysis. Calcium content is expressed in femtomoles per cell.

Compartmental analysis of monocyte calcium. Preliminary ^{45}Ca uptake studies were subjected to a graphical analysis plotting $^{45}Ca(\infty)$ − $^{45}Ca(t)$ (the radiolabeled calcium associated with the cells at equilibrium minus the radiolabeled calcium associated with a particular timepoint between 1 and 240 min) vs. time. In this configuration, the exponential fitting of each compartment would appear as a straight line. A peel back analysis was utilized wherein the slowest component is extrapolated back to time zero and the value of each point is removed from the value of cellular radioactivity at that point (19). This approach is continued until all the data points are accounted for by exponential components. The data were most compatible with three cellular components in the calcium exchange process. A model of cellular calcium was proposed with cellular compartments assigned to these components on the basis of the time constants for exchange, as shown in Fig. 1. The model has an external surface-bound pool of calcium, designated compartment 1, and a pool of calcium which is contained by the plasma membrane but is external to organelle membranes and is designated compartment 2. Calcium in compartment 2 is primarily chelated within the cytoplasm by proteins, nucleic acids, and membrane phospholipids, but a small portion of this pool remains ionized. The third pool includes calcium that is sequestered and presumably resides within the organelle membranes of mitochondria and endoplasmic reticulum.

Under conditions where the cell is in a steady state with respect to calcium, i.e., where net calcium flux equals zero, we can consider the following analysis. The calcium in compartment 1 is in direct exchange with the external medium so that we can describe its exchange as:

$$\frac{dR_1}{dt} = K_1T_1(S_{ext} - S_1) \tag{1}$$

where R is the radioactivity associated with the specific compartment designated by the subscript; K represents the compartmental size in femtomoles of calcium per cell at equilibrium; T represents the rate of exchange for the compartment in inverse minutes; S_1 represents the specific activity of compartment 1; and S_{ext} is the specific activity of the external medium in radioactivity per femtomole. The quantity of cell-associated calcium is sufficiently small (0.04%) when compared with the quantity of calcium in the external medium, so that this can be considered an open system.

The second calcium compartment exchanges directly with the external medium and with the third compartment so that the rate of change of its radioactivity can be represented by:

$$\frac{dR_2}{dt} = K_2T_2(S_{ext} - S_2) - K_3T_3(S_2 - S_3) \tag{2}$$
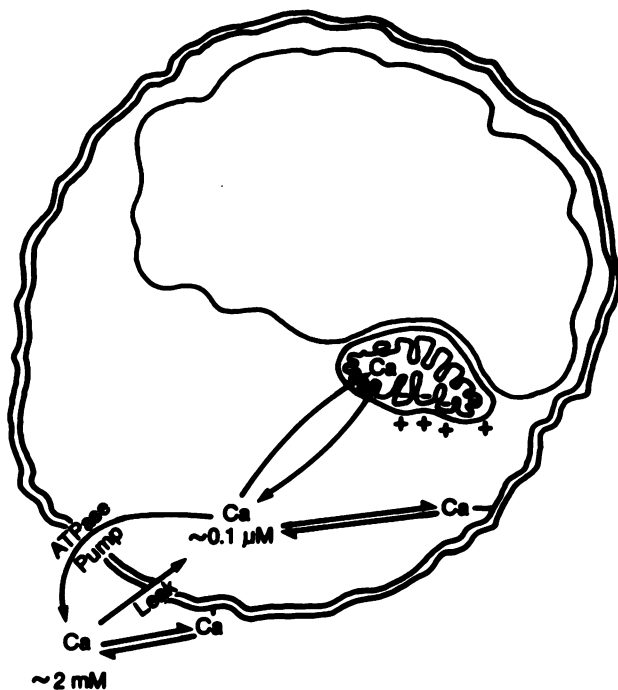
Figure 1. Calcium compartments in human monocytes. In this figure, there is an externally chelated surface pool of calcium in exchange with external calcium. The external calcium in millimolar concentration diffuses into the nanomolar ionized cytoplasmic calcium pool. This calcium leak acts to increase ionized cytoplasmic calcium, and in turn, is regulated in several ways. Cytoplasmic ionized calcium is buffered by cytoplasmic chelation to membrane phospholipids, proteins, and nucleic acids. This exchange process between cytoplasmic ionized and cytoplasmic chelated calcium is faster than the leak across the plasma membrane and is not separated by tracer exchange studies. Cytoplasmic ionized calcium can also be sequestered by organelles such as mitochondria and endoplasmic reticulum, which act to regulate the ionized calcium by sequestration. Calcium homeostasis is ultimately maintained by calcium extrusion via the plasma membrane calcium transport ATPase. This calcium pump is regulated by the concentration of ionized calcium that is found in resting and stimulated monocytes. No Na/Ca exchange process was detected in human monocytes in experiments measuring ^{45}Ca exodus in buffers containing high (140 mM) and low (15 mM) Na concentrations (unpublished results).

The third compartment exchanges only with the cytoplasmic compartment and the rate of change of the radioactivity can be described as:

$$\frac{dR_3}{dt} = K_3 T_3 (S_2 - S_3) \tag{3}$$

Substitution and integration of these equations yields the following equation that describes the total cellular radioactivity at time t:

$$R(t) = P_1(1 - e^{-\lambda_1 t}) + P_2(1 - e^{-\lambda_2 t}) + P_3(1 - e^{-\lambda_3 t}) \tag{4}$$

In this equation, $R(t)$ represents the measured radioactivity of the cells at time t. P, the size of the exchangeable pool within each compartment, has the units of radioactivity/cell which can be converted to moles per cell by multiplication with external calcium specific activity, S_{ext}. Lambda is the exponential time constant that describes the rate at which each compartment exchanges. To achieve a fit of the model to the data, an algorithm combining both Taylor series approximations and gradient method approaches was incorporated into a FORTRAN-based nonlinear least squares computer program (20). The program, run on a Prime computer system, was used to analyze the experimental data and fit the parameters P and λ of the cellular model. In the nonlinear least squares analysis, the sum of the squares of the differences between the fitted points and the observed data was <0.003 $(fmol/cell)^2$, and the maximal difference was never $>5\%$ of the observed value.

Calcium efflux experiments. Human monocytes were incubated in the presence of 0.55 mCi/mol ^{45}CaCl$_2$ at 37°C to allow exchange with radiolabeled calcium. After 4 h, the cells were diluted with fresh buffer and centrifuged at 500 g for 5 min. The labeling buffer was removed, and the labeled cells were resuspended in Medium-199, 1% BSA, and 10 mM Hepes, pH 7.3, at 37°C for measurement of calcium efflux. Samples were removed at appropriate times and centrifuged through silicone oil. The supernate was sampled, and the radioactivity was determined as described for the influx experiments. The remaining supernate was removed and the tubes were inverted to allow drainage of the silicone oil. The tube tips were then severed, placed in 3 ml tubes with 1 ml of deionized water, and sonicated for 20 s at 40 W. 0.5 ml of sonicated cell suspension was added to 10 ml Bray's solution for scintillation counting. A mathematical model of efflux was constructed that included two cellular calcium compartments. These compartments corresponded to compartments 2 and 3 of the influx model. The first compartment could not be detected in the efflux study since the radiolabeled surface calcium had exchanged during the washing and resuspension of the monocytes before the measurement of efflux. Efflux data was analyzed by use of the following equation:

$$R(t) = P_2(e^{-\lambda_2 t}) + P_3(e^{-\lambda_3 t}) + P_4$$

where $R(t)$ represents the cell-associated radioactivity at time t; P represents the size of the exchangeable compartment designated by the subscript in radioactivity per cell, and λ represents the time constant for exchange of the compartment in inverse minutes. The parameter P_4 results from the closed system used for efflux studies, where radiolabeled calcium that had left the cells, stayed in the incubation medium, and was available to reenter the cells.
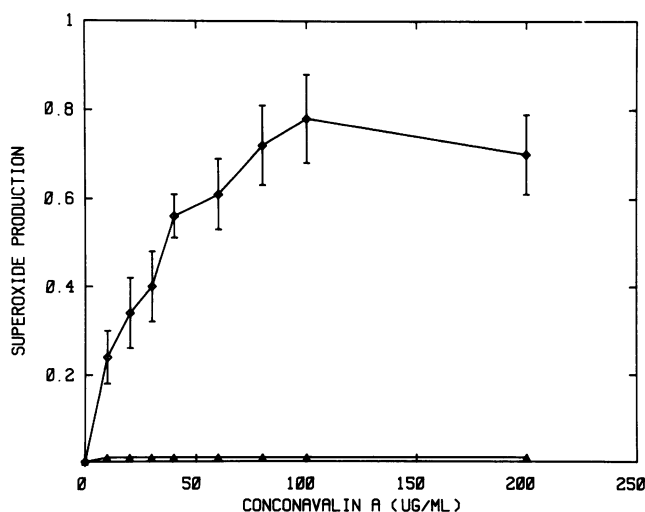
Cytoplasmic calcium in resting and stimulated monocytes. Human monocytes were labeled with the fluorescent probe, Quin 2-tetraacetomethoxyester (Quin 2-AM) (Lancaster Synthesis, England), as described by Tsien et al. (21). Briefly, the monocytes were suspended at 5×10^7–1×10^8 cells/ml in HBSS and were incubated in the presence of 5–100 μM Quin 2-AM for 30 min at 37°C in a shaking water bath. After this time, the cells were diluted with nine volumes of HBSS at 37°C and incubated for an additional hour before study. This incubation allowed Quin 2-AM to permeate the cells. Within the cells, the ester linkage was cleaved, and the dye became trapped as the free acid. The uptake of Quin 2-AM was complete in 90 min, as judged by the emission spectral shift from 435 to 490 nm. The concentration of Quin 2 within the monocytes, as determined by [^{3}H]Quin 2 uptake, was 3.1±0.08 mM in five monocyte populations. The monocytes exhibited a uniform cytoplasmic distribution of Quin 2 when viewed by fluorescence microscopy. The fluorescence intensity of the monocytes loaded with Quin 2

was monitored with a spectrofluorometer (Perkin Elmer Corp., Norwalk, CT, 650-10S) with temperature control and stirring attachments. Monocytes exhibit autofluorescence that is <10% of the total Quin 2 fluorescence at 492 nm, and the autofluorescence is unaffected by Con A stimulation. The fluorescent emission at 492 nm (slit, 6 nm) was monitored with a chart recorder. Excitation at 340 nm (slit, 6 nm) was interrupted by closing the shutter between readings to minimize bleaching of the dye.

The ionized cytoplasmic calcium $[Ca]_i$ was calculated by the use of the following formula: $[Ca]_i = K_d(F_{obs} - F_{min})/(F_{max} - F_{obs})$, where F_{obs} was the fluorescence intensity of the cytoplasmic dye within the cells. F_{max} was measured by treating the cells with 5 μg/ml lysophosphatidylcholine. This treatment increases the plasma membrane permeability to calcium and allows saturation of the intracellular dye with calcium for maximal fluorescence. The fluorescence of the calcium-Quin 2 complex was quenched by the addition of 0.5 mM MnCl$_2$ to determine the calcium independent fluorescence (F_{min}) (22). A value of 116 nM was used for the dissociation constant (K_D) of the Quin 2 calcium complex (21).

Results

Con A-stimulated superoxide production. We have used Con A-stimulated superoxide production as a reflection of monocyte activation. Superoxide production was not detected in unstimulated monocytes. The maximal rate of superoxide generation, 0.8 nmol/10^6 cells per min, occurred at a concentration of 100 μg/ml Con A (Fig. 2), and this concentration was used in the studies of monocyte calcium exchange. Con A (100 μg/ml)

caused a linear rate of superoxide production as shown in Fig. 3. The onset of superoxide generation followed Con A exposure by $\sim$60 s at all concentrations of Con A tested. 10 mM α-MM, a competitive inhibitor of Con A binding, prevented the Con A stimulation of superoxide production.

Total calcium content. To establish that the monocytes remained in a steady state with respect to unlabeled calcium during radiolabel exchange experiments, total cell calcium was determined by flameless atomic absorption spectroscopy (18). No significant alteration in total cell calcium was measured when monocytes were treated with Con A, or Con A plus α-MM (Fig. 4). The mean cellular calcium was 0.71 fmol/cell, or 1.3 mmol/liter cells in both resting and Con A-stimulated monocytes and remained constant during 4 h of observation. The results from the total calcium measurements suggest that a steady state exists with respect to calcium flux across the plasma membrane in both the resting and the stimulated monocytes. This condition is a requisite for applying the mathematical analysis which establishes the distribution of cellular calcium among physiologic pools that exchange at different rates (see parameters of calcium uptake).

^{45}Ca uptake by human monocytes. Calcium exchange in human monocytes was assessed by measuring radiolabeled calcium influx during a time course of 4 h (Fig. 5). In unstimulated monocytes, 0.287 fmol/cell of calcium had exchanged after 4 h of incubation with ^{45}Ca. Treatment with Con A (100 μg/ml) increased the quantity of ^{45}Ca that ultimately exchanged to 0.347 fmol/cell ($P < 0.01$). Pretreatment of the monocytes with α-MM blocked the increase in ^{45}Ca labeling that resulted from Con A treatment. The increase in exchangeable calcium resulting from Con A treatment, 0.060 fmol/cell, would have been detected by flameless atomic absorption spectrophotometry if the increase had resulted from a net influx of calcium. These results indicate that stimulation of monocytes with Con A causes an
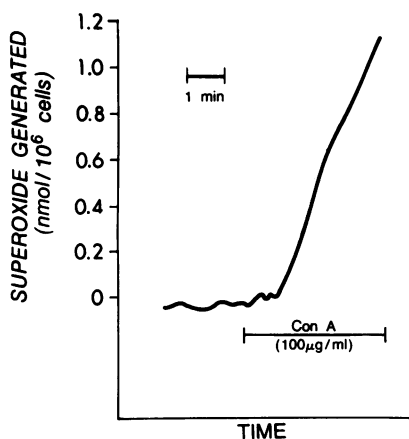
Figure 2. The effect of Con A concentration on superoxide production. The exposure of monocytes to Con A stimulated the production of superoxide within 60 s (diamonds). Superoxide was measured as the rate of superoxide dismutase-inhibitable ferricytochrome C reduction and is expressed in nanomoles of O$_2^-$ per 10^6 cells per minute. The addition of 10 mM α-MM before Con A exposure prevented the Con A-stimulated superoxide production (triangles). Unstimulated monocytes had no detectable superoxide production.

Figure 3. The response time of Con A stimulation of monocyte superoxide production. The exposure of monocytes to 100 μg/ml Con A resulted in a linear rate of superoxide production that commenced 60 s after lectin addition. Superoxide was measured as the quantity of superoxide dismutase-inhibitable ferricytochrome C reduction and is expressed as nanomoles O$_2^-$ per 10^6 cells. The addition of 10 mM α-MM before lectin exposure completely blocked superoxide production.
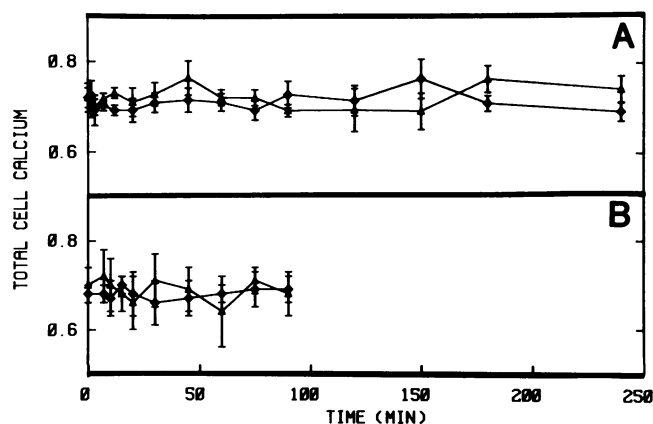
Figure 4. Total monocyte calcium content. Monocyte calcium content was determined by flameless atomic absorption spectrophotometry. Aliquots of sonicated cell suspension were pipetted into the cuvette of a graphite furnace atomizer. The time-temperature profile was optimized for sensitivity and detection limits using sonicated cell suspensions (see Methods). This technique permitted internal standardization of each sample, and was used to determine the calcium content which is expressed as femtomoles Ca per cell. (*A*) The calcium content of control (diamonds) and Con A (100 μg/ml) stimulated monocytes (triangles) during 4 h of calcium influx. Each point represents the mean±SE of five monocyte populations. (*B*) The calcium content of control (diamonds) and Con A-stimulated monocytes (triangles) during 90 min of calcium efflux. Each point represents the mean±SE of five monocyte populations.

increase in the proportion of cellular calcium that will exchange with extracellular calcium. The proportion of calcium available for exchange was 40% of total cell calcium in unstimulated cells, and 48% in Con A-treated monocytes.

Parameters of calcium uptake. To further investigate the cellular exchangeable calcium, we analyzed our data using a model which considers cell calcium distributed among three compartments. The results of a nonlinear least squares analysis of the ^{45}Ca influx data are presented in Table I. The time constants for the three calcium compartments in unstimulated monocytes were taken to represent the exchange of externally labeled calcium for unlabeled calcium in a putative extracellular surface pool, a putative internally chelated pool, and a putative organelle sequestered pool, respectively. This analysis yielded both the exponential rate at which each of the three compartments exchange calcium and the quantity of calcium contained in each pool.

When monocytes were treated with Con A at a concentration that optimally stimulates superoxide production, 100 μg/ml, a significant increase in the size and rate of calcium exchange of the second compartment, the putative internally chelated pool, was observed. The time constant for exchange between external calcium and the second compartment increased from 0.040 to 0.070/min ($P < 0.05$), and the quantity of exchangeable calcium in this compartment increased from 0.080 to 0.13 fmol/
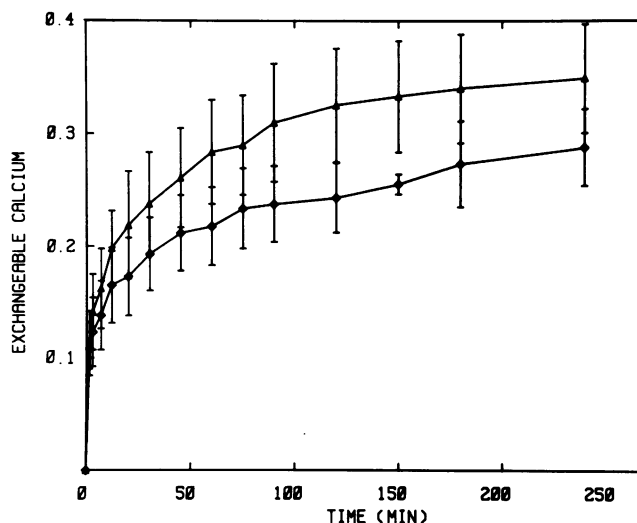
Figure 5. The uptake of ^{45}Ca into monocytes during a 4-h incubation. ^{45}Ca uptake into control monocytes (diamonds) was initiated by the addition of 0.55 mCi/mole ^{45}CaCl$_2$ to the external medium. At appropriate times, monocytes were separated from the external medium by centrifugation through a silicone oil step gradient. The ^{45}Ca uptake is expressed as exchangeable calcium in femtomoles per cell. Influx of radiolabel into the same monocytes stimulated with 100 μg/ml Con A is indicated by triangles. Each point represents the mean±SE of measurements in five populations of cells.

cell ($P < 0.05$). No significant changes were observed in the time constants or the pool sizes of the first or third compartment.

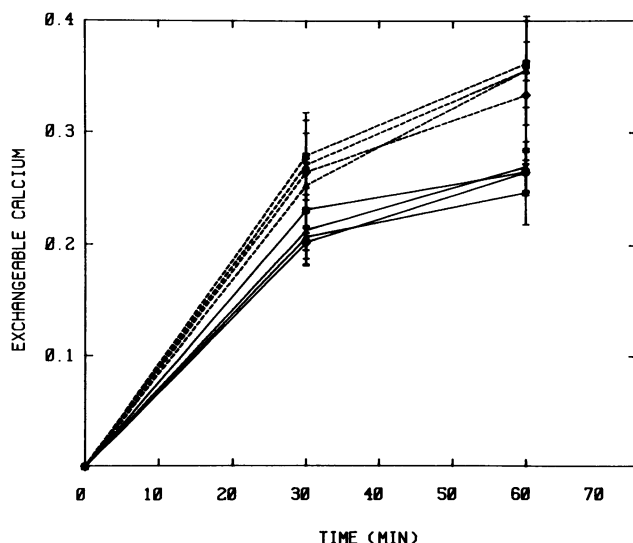*Mitochondrial inhibitors.* The results of the mathematical

Table I. Parameters of ^{45}Ca Uptake into Monocytes

Compartment	Control	Con A	Significance of difference
Time constant (min^{-1})			
1	1.3±0.2	1.3±0.1	NS
2	0.040±0.007	0.070±0.01	$P < 0.05$
3	0.0060±0.001	0.0060±0.001	NS
Pool size (*fmol/cell*)			
1	0.12±0.03	0.14±0.03	NS
2	0.080±0.01	0.13±0.01	$P < 0.05$
3	0.14±0.08	0.16±0.04	NS

Radiolabeled calcium uptake data for Con A-stimulated (100 μg/ml) and control cells from the same monocyte populations were analyzed using a computerized nonlinear least squares analysis. The analysis determined the rates at which the three compartments of calcium exchanged and the pool size of each compartment. Each parameter represents the mean±SE from five cell populations. Significance was determined by a paired Student t test analysis. NS, no significant difference.

analysis of calcium influx suggested that Con A treatment of monocytes resulted in an increase in the size of the cytoplasmic calcium pool without an increase in the surface or organelle compartments. This finding was investigated further by measuring ^{45}Ca influx in the absence and presence of mitochondrial inhibitors. The initial rate of ^{45}Ca influx in resting and stimulated monocytes is shown in Fig. 6. Neither sodium cyanide, oligomycin, or a combination of both agents altered significantly the resting or the Con A-stimulated increase in exchangeable calcium. These data indicated further that mitochondrial sequestration did not account for the Con A-stimulated increment in exchangeable calcium and supported the results of the mathematical compartment analysis.

Effect of cytoplasmic Quin 2. To investigate further the anatomical locations of the mathematically designated calcium compartments, Quin 2, an avid calcium buffer, was incorporated into monocytes and ^{45}Ca uptake was measured. Quin 2 is a fluorescent probe that is used to measure cytoplasmic free calcium (see below) and is concentrated in the cell cytoplasm (21). Three populations of monocytes were exposed to medium containing 25 μM Quin 2-AM, the concentration used in the assay for cytoplasmic ionized calcium. No difference was observed in the initial portion of the ^{45}Ca uptake when most of the tracer influx represents compartment 1 (Fig. 7 *A*). These data are consistent with a monocyte surface location for compartment 1, which was thus unaffected by the intracellular Quin 2. Al-

though Quin 2 did not alter the pool size of compartment 1, total cell-associated ^{45}Ca after 4 h of incubation was nearly twice that of control cells, suggesting a marked expansion of the intracellular calcium pools (Fig. 7 *A*). However, the influx curve was unsuitable for mathematical analysis because the ^{45}Ca exchange had not reached a steady state in 2-4 h of incubation, and the longer incubation with Quin 2 resulted in a loss of cell viability. Therefore, the sizes of pools 2 and 3 could not be measured individually in these studies.

To circumvent this problem, the amount of the intracellular calcium buffer, Quin 2, was decreased by decreasing the extracellular loading concentration of Quin 2-AM to 5 μM. In this study, the ^{45}Ca uptake curve more closely approached an isotopic steady state so that the mathematical analysis could be applied to the ^{45}Ca uptake data (Fig. 7 *B*). This analysis indicated a 74% increase in the size of pool 2 from 0.092 to 0.16 fmol calcium/cell in monocytes loaded with Quin 2, and a slight decrease in the size of pool 3. This decrease may represent a loss from pool 3 to the cytoplasmic Quin 2 or reflect the application of the computerized mathematical analysis to ^{45}Ca uptake that has not completely reached a steady state.

^{45}Ca exodus from human monocytes. To verify the calcium influx studies, the efflux of ^{45}Ca from labeled monocytes was measured. These experiments indicated that the difference in ^{45}Ca exodus after Con A treatment occurred during the initial 90 min of observation. Exposure of monocytes to Con A (100 μg/ml) caused an increase in the rate of ^{45}Ca exodus, but Con A did not affect the quantity of cell-associated radiolabel at later times (>120 min) (Fig. 8). The addition of α-MM to the incubation medium prevented the increment in efflux rate with Con A treatment.

Parameters of ^{45}Ca exodus. The results of a nonlinear least squares analysis of the efflux data are presented in Table II. The surface compartment could not be assessed in efflux studies since a rapid loss of the ^{45}Ca from the monocyte surface occurs during cell washing, and the analysis was modified accordingly. The time constants for the calcium exchange with the cytoplasmic and organelle pools in resting monocytes, 0.044 and 0.0079/min, respectively, were near those derived from influx experiments, 0.040 and 0.0060/min. Exposure of the cells to 100 μg/ml Con A significantly increased the time constant for calcium efflux from the second compartment from 0.044 to 0.073/min ($P < 0.05$). This increase was comparable with the Con A effect on the second compartment seen in influx studies, wherein the time constant increased from 0.040 to 0.070/min. The sizes of the cytoplasmic and organelle calcium pools derived from the efflux studies (Table II) were comparable with those derived from the influx data in view of the difference in experimental technique and measurements in a separate set of monocyte populations. No change was expected in the size of the calcium compartments after Con A treatment in the efflux studies since the cells were equilibrated with ^{45}Ca in the absence of Con A, and therefore, the calcium pool that is expanded by Con A was not labeled by ^{45}Ca. The monocytes were exposed
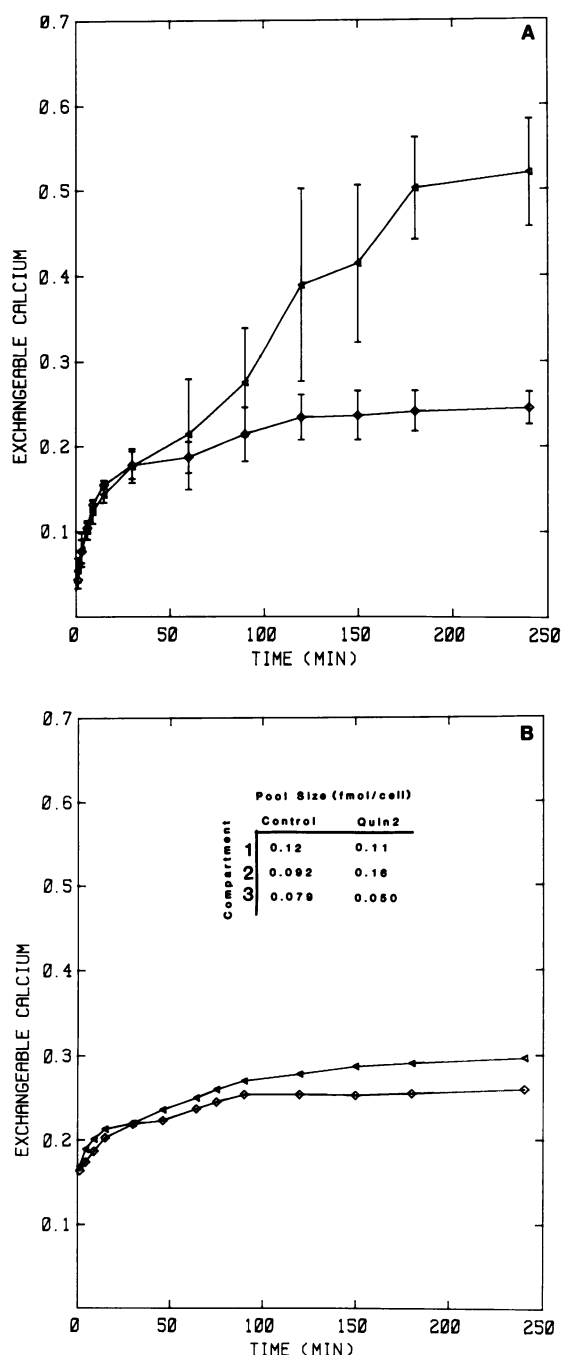

Figure 6. The effect of mitochondrial inhibitors of Con A-stimulated calcium uptake. Monocytes were preincubated in Medium-199 for 30 min at 37°C under the following conditions: no addition (diamonds), 7 μM oligomycin (crosses), 1 mM KCN (triangles), and a combination of 7 μM oligomycin and 1 mM KCN (squares). The uptake experiments were performed in untreated (solid lines) and Con A-stimulated (broken lines) cells, as described in the Methods section. Exchangeable calcium is expressed as femtomoles per cell.

Figure 7. The effect of cytoplasmic Quin 2 on ⁴⁵Ca uptake. Mono-
cytes were incubated at 37°C in Medium-199 at 10⁸ cells/ml for 60
min in the presence of 25 μM Quin 2-AM (A) and 5 μM Quin 2-AM
(B). Cells were then diluted ninefold in Medium-199 and incubated
an additional 30 min. The cells were washed twice in Medium-199
with 1% BSA and 10 mM Hepes at 37°C and resuspended in the
same medium at 10⁷ cells/ml. Calcium uptake was initiated by the
addition of 0.55 mCi/mole ⁴⁵CaCl₂, and aliquots were sampled at the
indicated time points. The time course of ⁴⁵Ca uptake is shown in
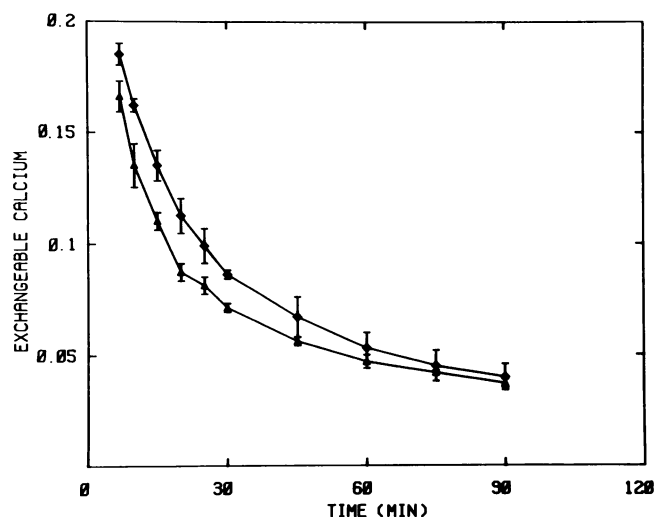
Figure 8. The time course of ⁴⁵Ca exodus from monocytes during a
90-min incubation. Monocytes were preincubated with 0.55 mCi/mol
⁴⁵CaCl₂ for 4 h. The cells were then washed by sedimentation to re-
move the labeling medium and resuspended in Medium-199. At ap-
propriate times, aliquots were taken, and the cells were sedimented
through a silicone oil step gradient. The calcium in the cell pellet in
control (diamonds) and 100 μg/ml Con A-stimulated (triangles)
monocytes is expressed as femtomoles per cell. Each data point rep-
resents the mean±SE of measurements in five populations of mono-
cytes.

to Con A only after washing by sedimentation and resuspension
prior to the measurement of calcium efflux.

Cytoplasmic free calcium. The tracer influx and efflux studies
indicated that Con A treatment resulted in an increased exchange
of calcium across the plasma membrane. To investigate the
nature of the increased exchange rate, cytoplasmic ionized cal-
cium was measured with the fluorescent probe, Quin 2. In un-
stimulated monocytes, cytoplasmic calcium was 83±18 nmol/
liter cell water (Fig. 9). Treatment of Quin 2-loaded monocytes
with 100 μg/ml Con A resulted in an increase in fluorescence
intensity reflecting an increase in cytoplasmic calcium to 163±24
nmol/liter cell water. This elevated level of cytoplasmic calcium
remained stable for >40 min indicating that the cells had reached
a new steady state following Con A exposure. The increase in
free calcium preceded monocyte superoxide production (Table
III). Monocytes showed detectable increases in Quin 2 fluores-
cence, 31±6 s after being stimulated with Con A (100 μg/ml),
while the response time for the production of superoxide was
61±9 s. This difference was highly significant (P < 0.001). The

Quin 2-loaded cells (triangles) and in similarly treated cells without
Quin 2-AM in the initial incubation (diamonds). The data in (A) rep-
resent the mean±SE of triplicate measurements in three monocyte
populations, and the data in (B) represent the mean of triplicate mea-
surements in a single monocyte population. Exchangeable calcium is
expressed as femtomoles per cell.

Table II. Parameters of ^{45}Ca Exodus from Monocytes

Compartment	Control	Con A	Significance of difference
Time constant (min^{-1})			
2	0.044±0.009	0.073±0.01	$P < 0.05$
3	0.0079±0.002	0.0082±0.002	NS
Pool size (*fmol/cell*)			
2	0.14±0.02	0.15±0.03	NS
3	0.10±0.01	0.10±0.01	NS

Radiolabeled calcium exodus data for Con A-stimulated (100 μg/ml) and control cells from the same monocyte populations were analyzed using a computerized nonlinear least squares analysis. The analysis determined the rates at which two compartments corresponding to the second and third compartments of the influx studies exchanged and established the pool size of each compartment. Each parameter represents the mean±SE from five monocyte populations. Significance was determined by a paired Student's t test analysis. NS, no significant difference.

range of the response times observed for the two cellular processes did not overlap in eight determinations. The presence of Quin 2 in the cells at concentrations of 3.1 mM did not alter either the lag time or the rate of Con A-evoked superoxide generation. The lowest concentration of Con A to cause a detectable increment in cytoplasmic calcium was 50 μg/ml. Cytoplasmic ionized calcium increased with increasing Con A concentrations up to 250 μg/ml without reaching a maximal value (Fig. 10). The continued increase in cytoplasmic calcium with concentrations of Con A greater than those that cause physiologic responses such as superoxide production may represent lectin-induced cytotoxicity and increasing permeability of the plasma membrane. Previous studies of lectin-induced potassium permeability showed toxicity at high lectin concentrations. For

Table III. Response Time in Monocyte Activation

	Lag time for increase in ionized cytoplasmic calcium concentration	Lag time for superoxide production
	s	s
Mean±SE	31.3±6	61.0±9
Range	24–40.5	45–71

The time between the introduction of Con A (100 μg/ml) and both the response of a detectable increment in ionized cytoplasmic calcium and the production of superoxide radical were determined from chart recordings. In eight individual determinations on different monocyte populations, the increment in ionized calcium preceded superoxide production ($P < 0.001$). Furthermore, in these eight cell populations, there was no overlap in response times for the two phenomena.

example, mitogenic concentrations of phytohemagglutinin increase the plasma membrane permeability to cations in human lymphocytes; higher phytohemagglutinin concentrations further increased permeability but decreased DNA synthesis (23).

The elevation of cytoplasmic calcium caused by Con A exposure could arise from the increased calcium influx across the plasma membrane, a release of chelated calcium from internal stores, or a combination of both mechanisms. To investigate the origin of the increment in cytoplasmic calcium, we measured Con A stimulation of Quin 2 fluorescence in the presence and absence of 10 mM EGTA in the medium (Fig. 11). This concentration of EGTA decreased the external ionized calcium to 28 nM. In the absence of EGTA, Con A (100 μg/ml) caused an elevation in the cytoplasmic calcium level from 80 to 160 nmol/liter cell water. In the presence of external EGTA, Con A stimulation resulted in an attenuated increase in cytoplasmic calcium to 115 nmol/liter cell water, an increase of 40% rather

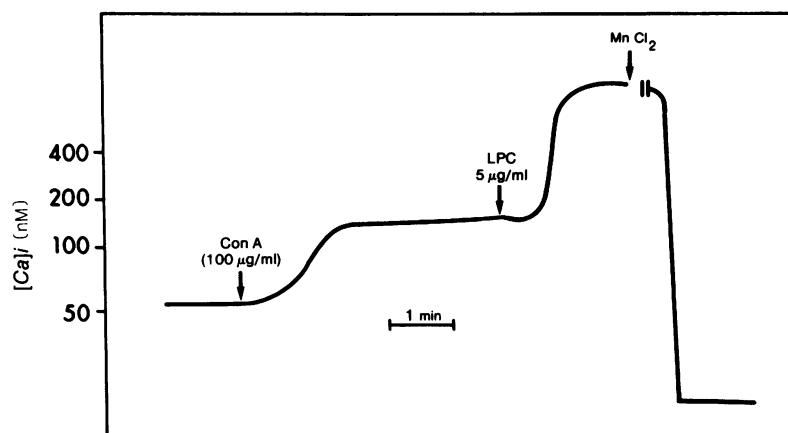*Figure 9.* Fluorescence of Quin 2-loaded monocytes. Monocytes were incubated for 90 min at 37°C in the presence of 25 μM Quin 2-AM. The cells were washed twice by centrifugation and resuspension in fresh buffer (HBSS). The excitation at 340 nm was interrupted by means of an excitation shutter to prevent photobleaching of the chromophore. The fluorescence emission at 490 nm was monitored on a chart recorder at 10-s intervals by opening the excitation shutter. The tracing was reconstructed by connecting the peaks on the chart paper. The arrow on the left side of the figure indicates the time at which the cells were exposed to 100 μg/ml Con A. The middle arrow indicates the addition of 5 μg/ml lysophosphatidylcholine to determine the maximal calcium-dependent fluorescence (F_{max}). The arrow on the right side of the figure indicates the addition of $MnCl_2$ to quench the calcium-dependent fluorescence (F_{min}).
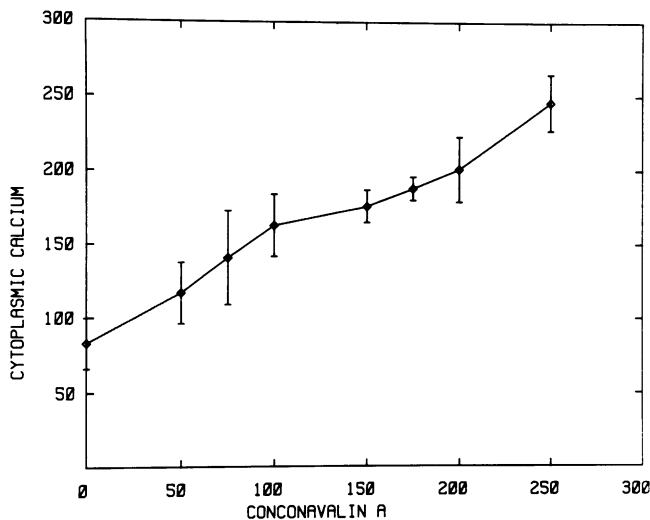
Figure 10. The effect of increasing Con A (μg/ml) concentration on the ionized cytoplasmic calcium concentration (nM). Monocytes were incubated in HBSS containing Con A concentrations from 0 to 250 μg/ml at 37°C for 30 min before fluorescence determinations. Ionized calcium was determined as described in the Methods. Each point represents the mean±SE from measurements in five monocyte populations.

than 100%, and a gradual return to the resting level after 5 min, rather than a sustained increase for >60 min. The response in EGTA-containing medium was observed with 100 μg/ml Con A, but was more pronounced with higher Con A concentrations. The presence of mitochondrial inhibitors did not affect the Con A-stimulated rise or gradual return to base line seen in the presence of EGTA. This result suggests that the gradual return to base line of ionized calcium during Con A stimulation in the absence of external calcium is not mediated by mitochondrial sequestration.

Discussion

Calcium and the calcium regulator protein, calmodulin, have been implicated in the regulation of macrophage activation (11). To characterize the alterations in calcium metabolism that are observed when monocytes are activated, we have measured and

correlated the uptake and exodus of ^{45}Ca, total calcium content, and cytoplasmic ionized calcium concentration.

The calcium in human monocytes is within the range reported for human lymphocytes (24) and several other isolated cells, such as 3T3 cells and hepatocytes (25). The exchangeable fraction of monocyte calcium after 4 h was 40%, which is similar to the exchangeable fraction in HeLa cells, 3T3 cells, and hepatocytes (25). A smaller proportion of exchangeable calcium has been reported in neutrophils, 7% (7), and lymphocytes, 15% (24), but the intracellular isotope was measured after only 30–40 min in the latter two studies, and isotope exchange may not have been complete.

The initial studies of ^{45}Ca uptake in unadhered, resting monocytes were calculated by plotting the cell-associated calcium semilogarithmically against time. Using a peel back analysis, three rate constants were required for a fit of the data. A three compartment model was suggested also by the computer-assisted mathematical analysis of the same data. This analysis allowed us to fit the data to a mathematical model with two, three, or four cellular compartments. The use of a two compartment model (26) resulted in a relatively poor fit of the data. Increasing the number of compartments in the model reduced the least squares residual as determined by computer analysis (27), and the fit was improved with a three compartment model. The use of a four compartment model reduced the residual further; however, two of the compartments had nearly identical time constants. This result could occur from an artifactual splitting of a single compartment into two similar compartments. These results lead us to analyze the calcium influx and efflux data using a model with three cellular calcium compartments. When the data from the influx studies were analyzed with a three compartment model for calcium distribution, the time constants for the exchange of calcium with each compartment were separated by nearly an order of magnitude. Such differences suggest strongly that the pools defined by our analysis represent separate physiologic compartments. Each compartment probably represents a heterogeneous group of calcium binding sites. The group with the fastest exchange rate, pool 1, is likely composed of several ligands that chelate calcium on the external monocyte surface. This location of pool 1 is also supported by the failure of the intracellular calcium buffer, Quin 2, to change the size of the pool. The second pool likely represents heterogeneous binding sites within the cytoplasm and exchanges with the ex-
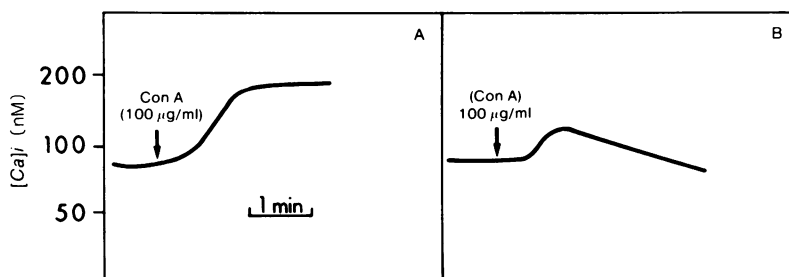
Figure 11. External calcium dependence of the Con A-stimulated increment in cytoplasmic calcium. Quin 2-loaded monocytes were suspended in either HBSS (A) or HBSS + 5 mM EGTA (B) and allowed to reach stable fluorescence. Con A (100 μg/ml) was then added to the cells at the times indicated by the arrows. The fluorescence emission at 490 nm was monitored at 10-s intervals on a chart recorder by opening the excitation shutter. The tracing was reconstructed by connecting the peaks on the chart paper.

ternal media at a rate that is substantially different from pool 1. The location of pool 2 is supported by the marked expansion of the pool by intracellular Quin 2. The third pool is most compatible with calcium sequestered in cell organelles, such as endoplasmic reticulum and mitochondria. The exchange of calcium with this pool is sufficiently slower than with pools 1 and 2, so that it can be distinguished from them mathematically.

The time constants for calcium exodus from monocyte pools 2 and 3 were nearly identical to the time constants of pools 2 and 3 measured in uptake studies. This result indicates that there was no systematic error in measuring ^{45}Ca influx and efflux. Factors such as cell death, incomplete cell recovery, or net calcium influx would have caused a disparity in the analyzed parameters of influx and efflux (28).

Con A treatment of unadhered blood monocytes was used to activate the cell. Con A binds to the plasma membrane and causes a rapid burst in the production and secretion of activated oxygen radicals such as superoxide, which comprise an integral part of the monocyte bactericidal mechanism (29). A marked change in the exchange rate of calcium and in the size of the putative cytoplasmic calcium pool occurred when monocytes were treated with Con A at a concentration that optimally stimulated superoxide production. Since there was no detectable change in the total monocyte calcium as measured by graphite furnace atomic absorption spectroscopy, these data suggest that Con A increases the plasma membrane permeability to calcium resulting in an increased rate of calcium exchange. The increase in total ^{45}Ca labeling indicates that the accessible fraction of calcium in the cytoplasmic pool has expanded so that it can exchange with external ^{45}Ca. The increase in the monocyte cytoplasmic calcium pool as measured by ^{45}Ca exchange was 0.06 fmol/cell or 180 μmol/liter cell water. Similar increases in exchangeable calcium have been reported in stimulated rabbit neutrophils (7, 9) and human lymphocytes (1). The origin of this newly exchangeable component of the cytoplasmic pool may be explained by the release of a previously unexchangeable pool from the internal surface of the plasma membrane after a stimulus binds to the external surface as described by Hoffstein (30).

The cytoplasmic ionized calcium in resting human monocytes was 0.083 μmol/liter cell water, as measured with Quin 2. This concentration is slightly lower than that reported in lymphocytes (21, 22) and rabbit alveolar macrophages (31), which were each 0.12 μmol/liter cell water. Con A activation of monocytes caused a doubling of the ionized cytoplasmic calcium from 0.083 to 0.160 μmol/liter cell water. This level of free calcium is within a range that can regulate several biological processes in mammalian cells (25). The increase in ionized calcium concentration as determined with Quin 2 was 0.080 μmol/liter cell water compared with an increase in exchangeable calcium of 180 μmol/liter cell water, suggesting that nearly all of the increase in exchangeable calcium is buffered intracellularly. This submicromolar increase in free calcium cannot be detected by direct atomic absorption spectroscopy. However, the increased exchangeable calcium of 180 μmol/liter cell water represents

~8% of the cell calcium and would have been detected by graphite furnace cell calcium content determinations if the increased ^{45}Ca labeling reflected an increase in total cell calcium.

Following Con A exposure, the cytoplasmic ionized calcium increased before superoxide generation occurred. The role of calcium as a second messenger in monocyte activation would require that the calcium response occur before physiologic responses of the cell. The chronological sequence that we have observed supports the concept that an increase in ionized calcium modulates the production of superoxide in lectin-treated monocytes (12).

The increment in ionized calcium was sustained for at least 45 min, and this suggested that perturbation by Con A results in the formation of a new steady state with an increased ionized calcium concentration. The increment in cytoplasmic calcium seen with exposure to 100 μg/ml Con A was 0.080 μmol/liter cell water. In a previous report, we have shown that the calcium extrusion pump in the monocyte plasma membrane increases approximately twofold when the cytoplasmic free calcium is increased from 0.08 to 0.16 μmol/liter cell water (16). This predicted increase in pump activity is consistent with the increase in the Ca exchange rate of 170% that we measured with radiolabeled calcium. Thus, the observed increment in cytoplasmic ionized calcium could account for an increment in calcium extrusion that is mediated by the calcium transport ATPase.

The interpretation of these data is consistent with the model illustrated in Fig. 1. Con A binds to the monocyte external plasma membrane and causes an increase in the membrane permeability to calcium and an increase in the exchangeable fraction of cell calcium. External calcium enters the cell at an increased rate along the electrochemical gradient of calcium and contributes to both the bound and free calcium in the cytoplasm. The binding of Con A increases the fraction of cell calcium that is available for exchange with the external media. The increment in free calcium plays a role in initiating superoxide production and monocyte activation and also accelerates the rate of calcium extrusion via the calcium transport ATPase (16). The calcium extrusion prevents any measurable change in total cell calcium and maintains calcium homeostasis. The increment in free calcium that we have measured is of an appropriate magnitude to interact with the calcium regulator protein, calmodulin, and with other regulatory molecules, such as cyclic nucleotides, so as to modulate the biochemical reactions that are necessary for monocyte activation.

Acknowledgments

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