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Factors Influencing Calcium Influx in Endotoxin-Challenged Fibroblasts[1] (41983)

A. SUBRAMONIAM, HARISH PADH, AND J. J. ALEO

Department of Pathology, Temple University School of Dentistry, 3223 North Broad Street, Philadelphia, Pennsylvania 19140

Abstract. The role of cell density and pH on calcium influx was studied in normal and endotoxin-challenged cultured 3T6 fibroblasts. In normal fibroblasts, at low cell densities, there was no marked difference in calcium influx at pH 6.6, 7.4, and 7.8, whereas at high cell densities, the calcium influx was markedly higher at pH 6.6 as compared to that at pH 7.8. Endotoxin treatment for 4 hr at low cell density and in alkaline pH (7.4–7.8) increased calcium influx in a dose-dependent manner. In contrast, at high cell density and low pH (6.6), endotoxin treatment markedly decreased calcium influx in a dose- and time-dependent manner. These endotoxin-induced changes in calcium influx were not fully compensated by altered calcium efflux because total calcium content of the cells was found to be altered. The efficacy of the endotoxin varied depending on the bacterial source of the endotoxin and the method of purification. There was a relationship between the effect of different endotoxins on the increase in calcium influx and the inhibition of cell proliferation. Endotoxin did not decrease, but slightly increased cell proliferation when added to high cell density cultures even at a concentration of 200 μg/ml.

Endotoxin, a lipopolysaccharide of gram-negative bacterial cell walls, is toxic to most mammalian cell types, and endotoxemia is a clinical problem of considerable importance (1–3). Lethal infections with gram-negative bacteria are thought to be due in large part to the toxicity of cell-wall lipopolysaccharides.

Several studies suggest that the toxic effect of lipopolysaccharides could be partly due to an increased level of calcium and calcium–calmodulin complex. Administration of lipopolysaccharide to mice in a sublethal quantity elevated liver ^{45}Ca uptake by 40% within 4 hr (4). Lipopolysaccharide induces lipolysis in isolated human adipocytes, which is also induced by a calcium ionophore, A23187 (5). Furthermore, physiological studies suggest that endotoxin induces alterations in calcium flux across excitable membranes (6, 7). Experiments with certain preparations of *Escherichia coli* endotoxins show a preliminary increase in transmitter release which is followed by a profound depression of spontaneous transmitter release at neuromuscular junctions in the frog. Therefore, endotoxin may act as a partial calcium ionophore during the early stage of exposure but subsequently, it blocks calcium access to the membrane (8).

Fibroblasts are one of the cell types most susceptible to the action of endotoxin (9). Pulp fibroblasts are able to respond to low levels of endotoxin under *in vitro* conditions by increasing cell division and synthesis of connective tissue matrix (10). Moderate concentrations of endotoxin depress the proliferation of cultured fibroblasts without compromising cell viability; but high concentrations of endotoxin depress both cellular proliferation and viability (11, 12).

The concentration of calcium in the culture medium is known to affect the growth of several cell types, including fibroblasts (13–15). Besides, several lines of studies suggest that a marked increase in the intracellular calcium concentration could lead to cell death (16–23). On the other hand, a very slight increase in calcium could stimulate cell proliferation (13, 15, 24).

Cell density is known to remarkably influence calcium uptake by microsomes of cultured fibroblasts (25). The pH of the medium decreases at high cell density in cultured cells as compared to the pH at low cell density. Therefore, the effect of pH and cell density on calcium influx was studied in normal and endotoxin-challenged fibroblasts to determine whether or not the interaction of endotoxin with the cultured cells influences calcium influx at different pHs and cell densities.

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Materials and Methods. *Cell culture.* 3T6 mouse fibroblasts (ATCC) were grown in plastic tissue culture flasks (25 cm^2 growth surface) in the Dulbecco–Vogt modification of Eagle's medium (GIBCO) supplemented with 10% of heat inactivated calf serum (GIBCO) and 100 μg/ml of gentamicin (GIBCO). The cultures were incubated for 1 to 6 days (depending on the experiment to be performed) at 37°C in an atmosphere of 7.5% CO_2.

Endotoxins were purchased from Sigma and used without further purification. Fresh endotoxin solutions were prepared in sterile normal saline or in tissue culture medium. To study the effect of endotoxin on multiplication of cells, cultures were seeded with (experimental) or without (control) endotoxin with identical amounts of trypsinized cell suspension (typically 80,000–100,000 cells/ml) in each flask. The cultures were refed after 48 hr, unless otherwise stated. Whenever refeeding was done, endotoxin was included in the experimental culture. Cells were trypsinized and the number of viable cells per culture was determined by trypan blue exclusion. Triplicate experiments were performed in each case.

^{45}Ca-influx measurements. The last change of the growth medium was done 12–16 hr prior to the experiment. After decanting the growth medium without disturbing the attached cell sheet, cells were incubated in 4.8 ml Hepes medium (unless otherwise specified). The Hepes medium was composed of 145 m*M* NaCl, 7 m*M* KCl, 0.8 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, 20 m*M* glucose, and 25 m*M* Hepes. The desired pH was obtained by adjusting with NaOH. After 1 hr of preincubation in Hepes medium, endotoxin (in 0.2 ml saline) was added to the experimental flasks (0.2 ml saline was added to the control flasks) and incubated for 4 hr, unless otherwise specified, at 37°C in air. After the incubation, the medium was changed with Hepes medium containing $^{45}CaCl_2$ (0.7–0.9 μCi/ml, New England Nuclear) and incubated for 2.5 min. Incubation was stopped by decanting the medium and washing the cell sheet four times with 5 ml of ice-cold Hepes washing buffer which was composed of the Hepes medium described above in which glucose was omitted and the NaCl concentration was increased to 155 m*M*. The pH of the washing buffer was 7 at 4–6°C. The washed cell sheet was digested overnight in 2.5 ml of 0.4 *N* NaOH. An aliquot (2 ml) of the dissolved cells was mixed with 10 ml of Aquasol (NEN) in a glass scintillation vial. Radioactivity was counted in a Beckman 150 scintillation counter (^{14}C channel).

A time course study of ^{45}Ca uptake showed that the uptake was increasing with time for at least 20 min. To minimize the effect of efflux, cells were exposed to ^{45}Ca for 2.5 min in the influx studies. The ^{45}Ca present in the pericellular area was also included in the influx value. However, the value obtained at 0 time incubation (a quick wash with radioactive medium) was subtracted from each value.

Determination of calcium content in fibroblasts. Cultures were seeded with fewer cells in growth medium containing $^{45}CaCl_2$ (approximately 0.8 μCi/ml) and were allowed to proliferate. Refeeding was also done with the radioactive medium. $^{45}CaCl_2$ (0.8 μCi/ml) was included in the buffered Hepes medium used for toxin treatment. Total calcium content was expressed per milligram protein (1 nmole Ca^{2+} = 600 to 700 cpm).

Protein determination. Total cellular protein was measured by the method of Bradford (26) using bovine serum albumin as a standard.

Results. *Effect of pH and cell density on calcium influx.* The effect of pH on calcium influx at different cell densities is given in Fig. 1. At low cell densities (0.3×10^6 or 0.6×10^6 cells/ml), there were no marked differences in calcium influx at 3 different pHs tested (pH 6.6, 7.4, or 7.8), whereas at high cell density (2.8×10^6 cells/ml), the calcium influx was markedly higher in acidic pH (6.6) as compared to that in alkaline pH (7.8). ^{45}Ca-influx rate was in the increasing order when the pH was in the decreasing order. This pH effect, at high cell density, on calcium influx was reversible since the increased influx observed in cells treated at pH 6.6 was reversed to a lower level upon transfer to alkaline pH (7.8) and vice versa (data not given).

At high cell density, the calcium content of the cells was also changed by the pH of the medium. Cells treated in alkaline pH

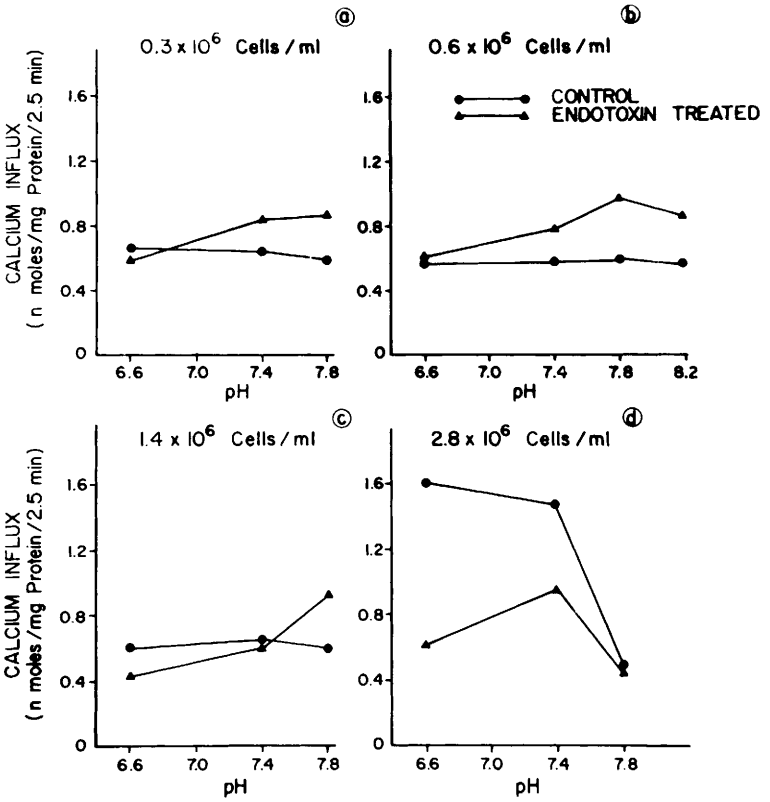

FIG. 1. Effect of pH and cell density on Ca influx in control and endotoxin-treated fibroblasts. Cultures were seeded with approximately 8×10^4, 16×10^4, and 32×10^4 cells/ml and incubated for 48 hr at 37°C in 7.5% CO_2 atmosphere to obtain 0.3×10^6, 0.6×10^6, and 1.4×10^6 cells/ml respectively. 2.8×10^6 Cells/ml were obtained by incubating for 72 hr with a seeding density of 32×10^4 cells/ml. *E. coli* endotoxin (serotype 0127:B8, butanol extract) was used at a concentration of 200 μg/ml. (As given under Materials and Methods, the culture was treated with the endotoxin for 4 hr at the appropriate pH in Hepes medium.)

(7.8) for 5 hr were found to have less total calcium content as compared to those treated for the same period in acidic pH (6.6). This effect was not observed at low cell density (Table I).

When the same high cell density (approximately 2.8×10^6 cells/ml) was obtained by seeding with fewer numbers of trypsinized cells and allowing to proliferate for 120 hr, or by seeding trypsinized cells at high cell density and allowing to proliferate for 24 hr, there were marked differences in calcium influx between the two groups at pH 6.6. The calcium influx was found to be remarkably higher in the latter case as compared to that in the former case. The calcium content of the cells was also found to be more with the increase of calcium influx (Table I). The increased calcium influx and calcium content could be due to better cell–cell contact resulting from seeding trypsinized cells at high density.

The entire cellular calcium was exchanged for radioactive calcium within 4 hr. The ^{45}Ca content was found to be almost the same in cultures treated with ^{45}Ca-containing medium for 4 hr and those cells grown in ^{45}Ca medium for 6 days starting from a density of 10,000 cells/ml (data not given). It is interesting to note that nearly 15 to 20% of total cellular calcium was exchanged for radioactive calcium within 2.5 min (Table I).

Effect of endotoxin on ^{45}Ca influx at various pHs and cell densities. At low cell density

TABLE I. CALCIUM INFLUX AND TOTAL CALCIUM CONTENT IN CULTURED FIBROBLASTS UNDER DIFFERENT EXPERIMENTAL CONDITIONS

No. of cells/ml:	0.6×10^6		2.8×10^6		
Age of culture (hr):	48		24	120	
pH:	6.6	7.8	6.6	6.6	7.8
Calcium influx (nmole/mg protein/2.5 min)					
Control	0.564 ± 0.04	0.549 ± 0.06	4.42 ± 0.11	1.58 ± 0.16	0.468 ± 0.03
Endotoxin treated	0.587 ± 0.05	0.985 ± 0.07*	1.99 ± 0.15*	0.501 ± 0.07*	0.456 ± 0.05
Total calcium content (nmole/mg protein)					
Control	3.80 ± 0.18	3.38 ± 0.26	15.66 ± 0.57	8.26 ± 0.29	4.24 ± 0.25
Endotoxin treated	3.62 ± 0.13	4.26 ± 0.12*	9.51 ± 0.27*	5.42 ± 0.21*	4.19 ± 0.20

Note. Cultures were seeded with varying numbers of cells and incubated for specified periods of time to obtain a final cell density of 0.6×10^6 or 2.8×10^6 cells/ml. *E. coli* 0127:B8 (butanol extract) endotoxin was used at a concentration of 200 μg/ml and treated for 4 hr as given under Materials and Methods. Total calcium content was also determined as given in methods. Calcium influx values at a cell density of 0.6×10^6 cells were taken from Fig. 1 for comparison. Values are means ± SE of triplicate experiments.

* Significantly different from untreated control, $P < 0.01$.

($0.3–0.6 \times 10^6$ cells/ml), endotoxin treatment for 4 hr increased ^{45}Ca influx in fibroblasts at pH 7.4 and 7.8 but not at 6.6. The increase was greater at pH 7.8 than that at pH 7.4. However, further increase of pH to 8.2 lowered the effect. At a medium cell density (1.4×10^6 cells/ml), the increase in calcium influx was evident only at pH 7.8 and not at 7.4. In contrast, at high cell density, endotoxin treatment markedly decreased calcium influx at pH 6.6 and 7.4 but it had no effect at pH 7.8. The decrease was more at pH 6.6 than that at pH 7.4 (Fig. 1).

The increase in calcium influx induced by endotoxin in fibroblasts at low cell density and at pH 7.8, as well as the endotoxin induced decrease in calcium influx at high density and at pH 6.6 were dependent on the endotoxin dose used. These effects increased with the increase of endotoxin concentration from 50 to 200 μg/ml (Figs. 2, 3).

An increase in the calcium influx rate occurred after 10 min of endotoxin treatment. This effect slightly decreased after 4 hr of treatment (Fig. 4). But before endotoxin treatment, the cells must be kept at an alkaline pH (7.4–7.8) for considerable time (5 hr) for this effect to occur after 10 min; 1 hr pretreatment in alkaline pH was found to be not sufficient. In contrast to this, the decrease in calcium influx induced by endotoxin, at a high cell density and at pH 6.6, was a delayed action (Fig. 4). Endotoxin treatment for 10 min was not sufficient for the reduction to occur, and the degree of the reduction in calcium influx was increasing with the period of treatment up to the 4 hr studied.

Endotoxin-induced increase in calcium influx (at low cell density and pH 7.8) was not fully compensated by increased extrusion of calcium from the cells because total cellular calcium content was increased by 20–28% in 4 hr of treatment. Similarly, the decrease in calcium influx induced by endotoxin at high cell density and low pH (6.6) was also not compensated by a decrease in calcium efflux. Under these conditions, endotoxin treatment for 4 hr resulted in a marked reduction in calcium content (Table I).

In 5 hr, the pH of the Hepes medium was found to decrease due to the metabolic products of the cells approximately from 6.6, 7.4, and 7.8 to 6.59, 7.32, and 7.64, respectively, in the low density culture (0.7×10^6 cells/ml), and to 6.59, 7.20, and 7.5, respectively, in the high density culture (2.8×10^6 cells/ml). When compared to control cultures, there was a slight but consistent decrease in pH (0.015 to 0.02) of the culture medium after toxin treatment for 4 hr.

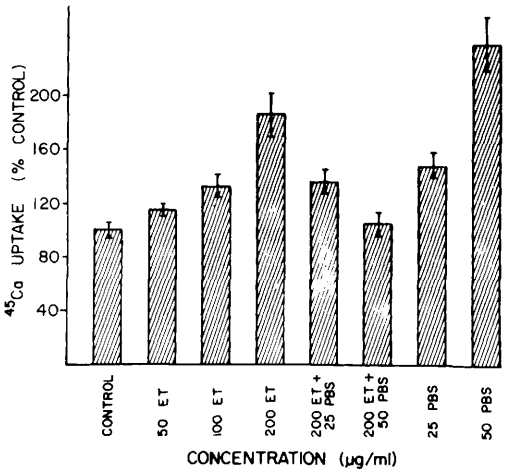

FIG. 2. Effect of polymyxin B sulfate-bound endotoxin or different concentrations of free endotoxin on calcium influx at low cell density and alkaline pH. Cells were grown to a density of approximately 0.7×10^6 cells/ml. Growth medium was removed and the cultures incubated in Hepes medium (pH 7.8). After 1 hr of incubation different concentrations of endotoxin (ET:*E. coli* 0127:B8, butanol extract) or polymyxin B sulfate (PBS) in 0.2 ml saline were added in each flask to give the indicated final concentrations. Polymyxin B sulfate and endotoxin were mixed to a ratio of 1:4 (1.25 mg polymyxin B sulfate:5 mg endotoxin in 1 ml) or 1:8 (1:25 mg polymyxin B sulfate:10 mg endotoxin in 2 ml) in saline and incubated for 15 min at 37°C before addition to the culture. The mixture was added to the culture to give the indicated concentrations of polymyxin B sulfate and endotoxin. After 4 hr of treatment with polymyxin B sulfate and/or endotoxin, ^{45}Ca influx was measured as given under Materials and Methods at pH 7.8. Data are means ± SD of triplicate experiments.

Effect of polymyxin B sulfate-bound endotoxin or polymyxin B sulfate on calcium influx. Polymyxin B prevents many of the toxic effects of endotoxin (27, 28, 29), probably by virtue of its ability to bind with the lipid-A moiety of endotoxin (30). Treatment of endotoxin solution in saline with polymyxin B sulfate to a ratio of 4:1 (w/w of endotoxin:polymyxin B sulfate) for 15 min at 37°C, before addition to the culture, prevented the effect of endotoxin on calcium influx. But polymyxin B sulfate induced a remarkable increase in calcium influx when added to the culture alone, but not with endotoxin, in the same alkaline medium at low cell density (Fig. 2). These results suggest that lipid-A is involved in endotoxin-induced increase in calcium influx and that the lipid-A-bound polymyxin B sulfate is no longer able to induce an increase in calcium influx. In contrast, the decrease in calcium influx induced by endotoxin at pH 6.6 and at high cell density was not influenced by treatment with polymyxin B sulfate. At this pH and cell density, in the absence of endotoxin, polymyxin B sulfate did not alter calcium influx significantly even at a concentration of 200 μg/ml.

Efficacy of diverse endotoxins on calcium influx. The efficacy of various endotoxins in increasing calcium influx at low cell density and at pH 7.8 was dependent on the bacterial source and the method of purification of the endotoxin used (Fig. 5). Endotoxin obtained by TCA extraction from *E. coli* 055:B5 (Sigma, Lot No. 91F-4016) was found to be effective while the phenol extract from the same bacteria (Sigma, Lot No. 89C-052A) was almost ineffective. But TCA extract from *Salmonella minnesota* (Sigma, Lot No. 22F-0152) was not as effective as the TCA extract

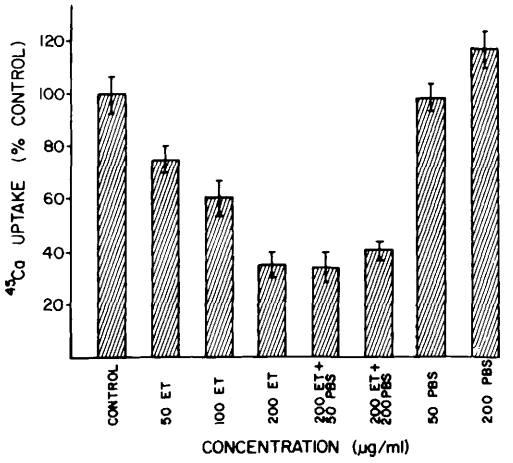

FIG. 3. Effect of polymyxin B sulfate-bound endotoxin or different concentrations of free endotoxin on calcium influx at high cell density and pH 6.6. Cells were grown to a density of approximately 2.8×10^6 cells/ml by 72 hr of incubation. ^{45}Ca influx was determined as given under Materials and Methods. Hepes buffer at pH 6.6 was used for toxin treatment and ^{45}Ca-influx measurement. Polymyxin B sulfate (PBS) and endotoxin (ET) were mixed to a ratio of 1:2 or 1:4 (w/w) in saline and proceeded as given in legend of Fig. 2. Data are means ± SD of triplicate experiments.

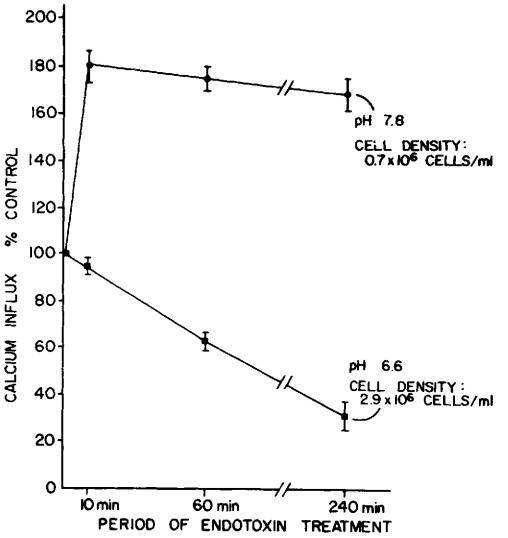

FIG. 4. Time course of endotoxin treatment on calcium influx at different pHs and cell densities in fibroblasts. Cells were grown to a density of 0.7×10^6 or 2.9×10^6 cells/ml. Cells were incubated with Hepes medium, pH 7.8 (0.7×10^6 cells/ml) or 6.6 (2.9×10^6 cells/ml) for 290 min (for 10-min toxin treatment) or 240 min (for 60-min toxin treatment) or 60 min (for 240-min toxin treatment). Then, endotoxin (*E. coli* 0127:B8, butanol extract; 200 μg/ml final concentration) in 0.2 ml saline was added in the experimental flasks and incubated for 10, 60, or 120 min. (In control 0.2 ml saline was added). Data are means ± SD of triplicate experiments.

from *E. coli* (055:B5). Of the four endotoxins tested, butanol extract from *E. coli* 0127:B8 (Sigma, Lot No. 101F-6829) was found to be the most effective. Unlike the increase in calcium influx induced by endotoxin, the decrease in calcium influx (at high cell density and pH 6.6) was induced by all endotoxins tested, but the degree of the inhibition of influx differed among different endotoxins. Endotoxin prepared by butanol extraction from *E. coli* 0127:B8 was found to be more efficient in inducing both of these opposite effects on calcium influx.

Effect of endotoxins on cell proliferation. When cells were grown for 48 hr in medium containing endotoxin at a concentration of 200 μg/ml, there was a decrease in the number of viable cells depending on the bacterial source and the method of purification of the endotoxins tested. Butanol extract from *E. coli* 0127:B8 and TCA extract from *E. coli* 055:B5 showed significant inhibition in cell proliferation while the TCA extract from *S. minnesota* and the phenol extract from *E. coli* 055:B5 did not inhibit cell proliferation (Fig. 6). Thus, there was a relationship between the ability of diverse endotoxins to increase calcium influx and to decrease cell multiplication.

When cells were grown with the most potent endotoxin tested (*E. coli* 0127:B8, butanol extract) at a concentration of 200 μg/ml, a decrease in viable cell counts appeared after 24 hr. This decrease slightly progressed up to 72 hr of growth (Fig. 7). After 72 hr of multiplication (approximately 1.8×10^6 cells/ml), there was a catch up growth in experimental cultures resulting in no significant difference in the number of viable cells, as compared to control cultures, after 120 hr of multiplication. Interestingly, when endotoxin was added to the control culture after 72 hr of incubation (after reaching a cell density of $1.6–1.8 \times 10^6$ cells/ml) there was no reduction in the number of viable cells, but after 144 hr of incubation (72 hr after the addition of endotoxin) there

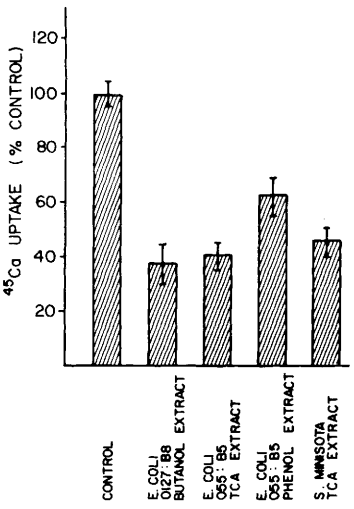

FIG. 5. Effects of various endotoxins on calcium influx in high cell density fibroblasts at pH 6.6. Fibroblasts were grown to a density of 2.8×10^6 cells/ml by 72 hr of incubation. Cultures were treated with various endotoxins to a final concentration of 200 μg/ml for 4 hr in Hepes medium (pH 6.6) and calcium influx was determined at pH 6.6 as described under Materials and Methods. Values are means ± SD of triplicate or duplicate experiments.

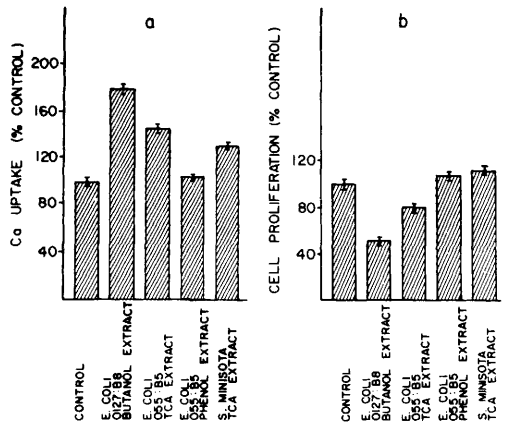

FIG. 6. Effects of various endotoxins on (a) calcium influx and (b) cell proliferation. (a) Fibroblasts were grown to a density of 0.7×10^6 cells/ml and calcium influx was determined at pH 7.8 as given under Materials and Methods. Cultures were treated with various endotoxins for 4 hr at a concentration of 200 μg/ml in Hepes medium (pH 7.8). (b) Fibroblasts were seeded at a density of 8×10^4 cells/ml in normal growth medium with various endotoxins at a concentration of 200 μg/ml. After 48 hr of incubation, cell sheet was trypsinized and counted. Values are means ± SE of triplicate experiments.

was a trend to increase the number of cells as compared to control (Fig. 7). These results may suggest that the increase in calcium influx observed at low cell density may be directly or indirectly inducing a decrease in cell multiplication.

Discussion. The dynamism of calcium metabolism was evident by the fact that 15–20% of cellular calcium (including pericellular) could be exchanged to ^{45}Ca in 2.5 min, and almost the entire calcium could be exchanged to ^{45}Ca within 4 hr. This rapid exchange rate is not unique to fibroblasts. Kurzinger *et al.* (31) have shown that in neural cells in culture all the cellular calcium could be exchanged for ^{45}Ca in 60 to 120 min.

The present study reveals the importance of pH at high cell density on calcium influx and cellular calcium content. A shift from acid to alkaline pH for 4 hr markedly decreased cellular calcium content. This effect was reversed when the pH was changed to acidic. These observations have great significance not only in understanding calcium metabolism but also in determining cellular calcium content or calcium influx by different methods under different experimental conditions.

Moore and Pastan showed that energy-dependent calcium-uptake activity by microsomes from high cell density cultures of mouse fibroblasts is markedly higher as compared to calcium uptake by microsomes from low cell density cultures (25). The studies of Moore and Pastan at pH 6.8 suggest that cell–cell contact could increase calcium uptake by microsomes (32). In the present study, the cell-density-dependent increase in calcium influx rate or cellular calcium content was marked at pH 6.6 (and to a smaller degree at pH 7.4) and not at pH 7.8. Therefore, it is conceivable that at a higher pH (7.8) the possible receptor mediated cell–cell contact may not occur.

Seeding the trypsinized cells at high cell density resulted in a significant increase in calcium influx when compared to cultures seeded with fewer cells and grown to the same cell density; seeding trypsinized cells at high cell density appears to have increased cell–cell contact. Moore and Pastan (25, 32) have also noted an elevation of calcium uptake by microsomes from trypsinized cells, at high density.

It is well known that specific binding between macromolecules are dependent on pH to a large extent. Some investigators suggest that endotoxin triggers its toxic actions from a specific receptor site (33). If this is true, the increase in calcium influx induced by endotoxin could be due to toxin receptor binding. In that case, the toxin binding at a specific receptor may occur in slightly alkaline pH (7.4–7.8) under minimal cell–cell contact. Probably, increased cell–cell contact may render the specific receptor(s) for endotoxin unavailable or unsuitable for binding. Another possibility is the specific receptor(s) may be produced in favorable configuration and orientation in low-density cells treated in slightly alkaline pH (7.4–7.8) for a considerable time.

Endotoxin-induced increase in calcium influx was only partially compensated by an increase in calcium efflux. When there was 55–65% increase in cellular ^{45}Ca in 2.5 min, there was only 20–25% increase in cellular

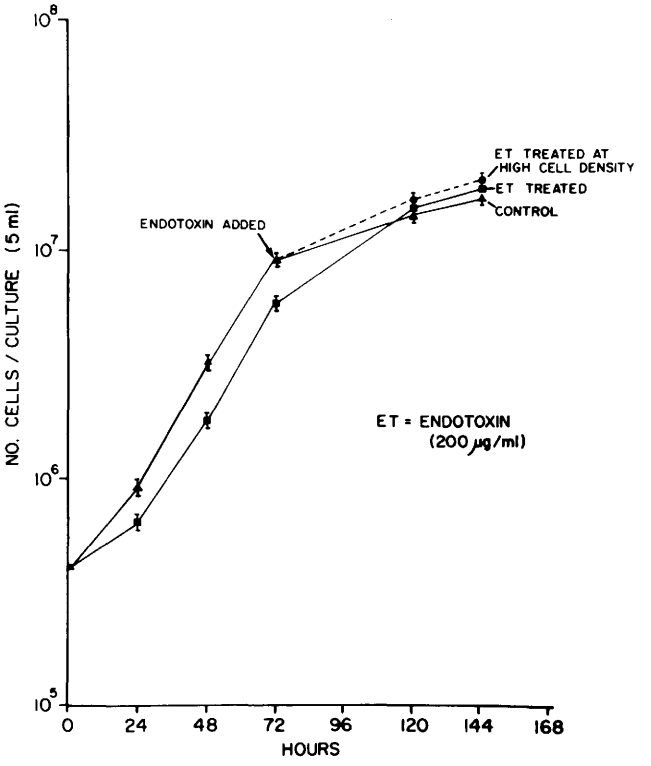

FIG. 7. Effect of *E. coli* endotoxin on fibroblast proliferation. Mouse 3T6 fibroblasts were cultured in normal growth medium with (experimental) or without (control) 200 μg/ml endotoxin (*E. coli*, 0127:B8, butanol extract). The culture medium was changed every 48 hr of culture. In one set of control flasks endotoxin was included after 72 hr of culture. After the indicated periods of incubation, cell counts were made. (In each case triplicate experiments were made.)

^{45}Ca in 4 hr. Total cellular calcium content was exchanged to ^{45}Ca within 4 hr.

Some believe that endotoxin initiates its biological responses through alterations in membrane lipids (34, 35). An activation of phospholipase A induced by endotoxin administration has been reported in liver (36) and in heart (37). Phospholipase A activation changes membrane lipid fluidity (38). An increase in cytoplasmic-free calcium could increase the activity of phospholipase A (39). Therefore, it is reasonable to assume that the increase in calcium influx observed in fibroblasts in the present study could alter membrane lipids.

Unlike the increase in calcium influx which occurred as early as 10 min of treatment, endotoxin-induced decrease in calcium influx did not occur significantly when treated with the toxin for 10 min. Endotoxin from *S. abortus equii* inhibited the ATP-dependent calcium uptake by canine myocardial sarcoplasmic reticulum under *in vitro* conditions; it required 15 min of preincubation for this effect to occur significantly (40). Hess and Briggs (38) feel that the large size of the molecule prevents its ready penetration into the membrane and the lag phase is related to this problem. Furthermore, endotoxin decreases the leakage of sucrose from phosphatidylcholine liposomes from 27 to 4% in 5 hr. These findings suggest that endotoxin increases the molecular packing of phosphatidylcholine bilayers (41). In view of these facts, it is reasonable to assume that at high cell density and in acidic pH (6.6), lipopolysaccharides may penetrate the lipid bilayer and decrease the permeability to calcium ions. Cell–cell contact may help for the insertion of endotoxin into the plasma membrane lipids.

Free and bound lipid-A exhibits a high affinity for cell membranes, for other lipids and proteins. The lipid-A moiety of endotoxin from different gram-negative bacteria are distinctive (42). Therefore, it is conceivable that the effect of endotoxin on cellular calcium influx could differ depending upon the bacterial source, as observed in the present study. It is also possible that the method of extraction of the endotoxin is important for the preservation and expression of endotoxin's efficacy (42).

The relationship observed in this study between the increase in calcium influx and decrease in cell proliferation induced by various endotoxins suggests that the decrease in cell proliferation could be due to an increase in calcium influx. Furthermore, at high cell density, addition of endotoxin to the culture did not decrease cell proliferation. At this density, endotoxin treatment did not increase, but decreased calcium influx at pH 6.6 (and at 7.4). When pH decreases in culture at high cell density, the control cells have high levels of calcium influx already. Under these conditions, when endotoxin decreases calcium influx to some extent, this may not affect the cell adversely. Endotoxin could have a beneficial effect at this cell density and acidic pH.

However, all the biological effects of endotoxin are not attributable to changes in calcium influx at least in all cell types. The effect of endotoxin may be cell specific. *E. coli* lipopolysaccharide (phenol extract) induced time- and dose-dependent bovine endothelial cell injury which is manifested initially by cell detachment from culture substratum. This effect was not prevented by chlorpromazine or trifluoperazine (43).

In any case, the present study indicates clearly that endotoxin alters calcium influx and cellular calcium content in cultured fibroblasts depending on cell–cell contact and culture pH. Since calcium–calmodulin plays a pivotal role in cellular metabolism by regulating the activities of many enzymes (44, 45), an alteration in the cellular flux of calcium induced by endotoxin could affect the normal metabolism and function of the cell.

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