EFFECT OF THE CHEMOTACTIC FACTOR FORMYL METHIONYL-LEUCYL-PHENYLALANINE AND CYTOCHALASIN B ON THE CELLULAR LEVELS OF CALCIUM IN RABBIT NEUTROPHILS

R. J. PETROSKI, P. H. NACCACHE, E. L. BECKER and R. I. SHA'AFI
Department of Pathology and Physiology, University of Connecticut Health Center, Farmington, CT 06032, USA

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1. Introduction

The essential role of divalent cations in the activation of numerous cell functions has long been recognized [1–7]. In the neutrophil, chemotactic factor-induced stimulation of locomotion, at least as measured in Boyden chemotactic chambers [8,9], lysosomal enzyme secretion [9] superoxide generation O_2^- [10] aggregation [11] all required extracellular Ca^{2+} for optimal response. In all, except the stimulation of locomotion, the response either requires or is greatly enhanced by the fungal metabolite, cytochalasin B. In addition, the divalent cation ionophore A23187, is an effective secretogogue for lysosomal enzymes and induces O_2^- production [10] and aggregation [11] in neutrophils. These activities are also greatly enhanced by cytochalasin B [10].

In attempting to understand the role of intracellular and extracellular Ca^{2+} in these functions, we found that in the presence of 250 μM extracellular calcium, the synthetic chemotactic peptide, formyl methionyl–leucyl–phenylalanine (F-Met–Leu–Phe) induced an increase in the steady-state level of cell-associated $^{45}Ca^{2+}$, whereas with 10 μM Ca^{2+}, F-Met–Leu–Phe caused a decrease in the steady-state level of cell-associated $^{45}Ca^{2+}$ [6,12–14]. We also showed that cytochalasin B greatly increased the rate and extent of uptake of $^{45}Ca^{2+}$ and the influx of $^{22}Na^+$ [6] induced by F-Met–Leu–Phe or by A23187. In addition, the degree of enzyme release caused by cytochalasin B and varying concentrations of F-Met–Leu–Phe or A23187 was a straight line function of both the increased uptake of $^{45}Ca^{2+}$ or the increase in steady-state levels of $^{45}Ca^{2+}$ also caused by these two agents.

We have systematically investigated the effect of F-Met–Leu–Phe on the levels of total and exchangeable Ca^{2+} by measuring the total cell calcium concomitantly with the $^{45}Ca^{2+}$ radioactivity. This allowed us to determine how the $^{45}Ca^{2+}$ specific activity of the cells was affected during stimulation by chemotactic factors [15]. The results of this and the aforementioned studies as well [16,17] have led us to the conclusion that the binding of chemotactic factors to their receptors leads to:

(i) A graded displacement of pre-bound Ca^{2+};
(ii) A graded increase in membrane permeability to calcium.

As mentioned above, cytochalasin B very greatly enhances the neutrophils' response to chemotactic factors both in terms of Ca^{2+} metabolism and such functions as lysosomal enzyme secretion, O_2^- generation and aggregation [9–11]. In this study we therefore have determined the effect of cytochalasin B on the total calcium and the $^{45}Ca^{2+}$ specific activity of the neutrophils before and during stimulation by F-Met–Leu–Phe.

2. Materials and methods

Polymorphonuclear leukocytes (neutrophils) were obtained as in [1,13,16]. In brief, albino rabbits (2–3 kg) were injected intraperitoneally with 400 ml sterile isotonic saline containing 0.1% glycogen and the peritoneal exudate was collected in heparinized

flasks 12–14 h later. Neutrophils were washed once with buffered ammonium chloride solution to hemolyze red blood cells [17–19]. The neutrophils were then washed once with a modified Hanks buffer solution and resuspended in this solution (5×10^6 cells/ml, 100 ml/vessel). The composition of the modified Hanks buffer is (mM): NaCl, 124; KCl, 4; Na_2HPO_4, 0.66; $MgSO_4$, 0.74; $NaHCO_3$, 15.2; Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma Chem. Corp., St Louis, MO) 10.0 and glucose, 5.56; (pH 7.2). The modified Hanks buffer solution also contained either 5 μM or 1.0 mM calcium as determined by atomic absorption spectrophotometry.

The cells were initially incubated at 37°C for 20 min. At the end of this incubation period, a known amount of $^{45}Ca^{2+}$ ($2–6 \times 10^7$ dpm contained in <5 μg $CaCl_2$) was added to the contents of each flask and the cell suspensions were allowed to equilibrate at 37°C for another 40 min. This time was sufficient for the radioactivity to reach steady-state (d(dpm)/dt = 0) where dpm are the disintegrations/min in the cells/mg protein and t is time (13). Following the 40 min incubation period, the neutrophils were challenged with either cytochalasin B (Aldrich Chem. Co. Milwaukee, WI) alone at 5 μg/ml final conc., F-Met–Leu–Phe alone at 1×10^{-8} M final conc., cytochalasin B and F-Met–Leu–Phe added simultaneously or, in the case of the control flask, in the absence of any stimuli. The cells were exposed to the stimuli for 1 min prior to centrifugation and subsequent workup. The cells were washed with La^{3+}, ashed and the total Ca^{2+} radioactivity were determined as in [15]. Protein was determined by the Lowry method [20] using bovine serum albumin (Sigma Chem. Corp., St Louis, MO) as standard. Total Ca^{2+} was determined by atomic absorption spectrophotometry and radioactivity of $^{45}Ca^{2+}$ was determined by means of liquid scintillation counting as in [15]. The specific radioactivity of the cell calcium is defined as the dpm/μg calcium.

3. Results and discussion

The effects of cytochalasin B, F-Met–Leu–Phe and cytochalasin B plus F-Met–Leu–Phe on total cell calcium were studied first in the presence of 5 μM extracellular calcium. The results are summarized in table 1. Cytochalasin B alone induces a small but statistically significant ($p < 0.01$) loss of total cell calcium as well as a slight, statistically non-significant, loss of total cell protein. These effects are possibly due to the small amount of degranulation induced by cytochalasin B in the absence of added chemotactic factor [5,21]. This suggestion is supported by the fact that the amount of calcium/mg protein does not change in the presence of cytochalasin B alone. However, in view of the short time (1 min) during which the cells were exposed to cytochalasin B in these

Table 1
Effect of cytochalasin B, F-Met–Leu–Phe and cytochalasin B plus F-Met–Leu–Phe on total cell calcium in the presence of low (5 μM) extracellular calcium

	Control	Cytochalasin B[a] (5 μg/ml)	F-Met–Leu–Phe[a] (1×10^{-8} M)	Cytochalasin B (5 μg/ml) F-Met–Leu–Phe (1×10^{-8} M)[a]
Total cell calcium (μg)	2.61 ± 0.26 (4)[b]	2.06 ± 0.23 (4)	2.32 ± 0.21 (4)	1.07 ± 0.09 (4)
Significance[c]		$p < 0.01$	n.s.	$p < 0.01$
Total cell protein (mg)	21.8 ± 1.22 (4)	18.3 ± 1.53 (4)	21.7 ± 1.15 (4)	14.4 ± 0.21 (4)
Significance		n.s.	n.s.	$p < 0.01$
Cell calcium protein (μg/mg)	0.119 ± 0.009 (4)	0.114 ± 0.011 (4)	0.107 ± 0.005 (4)	0.076 ± 0.009 (4)
Significance		n.s.	n.s.	$p < 0.01$

[a] Cytochalasin B and/or F-Met–Leu–Phe interacted with the cells (5×10^6 cells/ml, 100 ml) for 1 min
[b] The number in parenthesis refers to number of experiments; errors are standard error of the mean
[c] Significance obtained by means of paired *t*-tests. n.s., not significant

experiments, one cannot rule out the possibility that the cytochalasin B-induced changes reported in table 1 may actually reflect some of the direct effects of cytochalasin B on calcium metabolism in the neutrophils. F-Met–Leu–Phe induces a slight loss of total cell calcium that is not statistically significant and does not affect the level of total cell protein confirming the results in [15]. Cytochalasin B plus F-Met–Leu–Phe together induce significant ($p < 0.01$) losses of both total calcium (59.0%) and total protein (34%). The magnitude of the changes induced by cytochalasin B and F-Met–Leu–Phe together exceeds that induced by cytochalasin B or F-Met–Leu–Phe alone, and cannot be explained on the basis of an additive effect of the two agents.

The concentration of calcium in the lysosomes $[Ca^{2+}]_\ell$, expressed as μg calcium/mg protein, can be calculated as follows:

$$[Ca^{2+}]_\ell = \frac{(Ca^{2+})_b - (Ca^{2+})_a}{(P)_b - (P)_a}$$

where $(Ca^{2+})_b$ and $(Ca^{2+})_a$ are the amounts of calcium in the cell (in μg) measured before and after the simultaneous addition of cytochalasin B and F-Met–Leu–Phe, respectively and $(P)_b$ and $(P)_a$ represent the amount of protein in the cell (in mg) measured before and after the co-addition of the stimuli, respectively. The concentration of calcium in the lysosomes, expressed as μg calcium/mg protein, was found to be 0.254. Since this is more than double that of the untreated cell (0.119 ± 0.009) and >3-times that of the calcium remaining in the stimulated cells (0.076 μg/mg protein), it is very likely that the granule fraction is at least one site of sequestered calcium.

The effect of cytochalasin B, F-Met–Leu–Phe and cytochalasin B plus F-Met–Leu–Phe on total cell calcium was studied next in the presence of 1.0 mM extracellular calcium. The results are summarized in table 2. Cytochalasin B alone induces a small but statistically significant ($p < 0.01$) loss of total cell calcium as well as a slight, but statistically non-significant, loss of total cell protein; the amount of calcium/mg protein does not change significantly. F-Met–Leu–Phe alone does not have any significant effect on the level of total cell calcium, total cell protein or the amount of calcium/mg protein again confirming [15]. Cytochalasin B plus F-Met–Leu–Phe together do not produce any change in total cell calcium but induce a significant ($p < 0.01$) and dramatic loss (40%) of total cell protein; this is reflected by the large increase in the amount of calcium/mg protein observed after stimulation (from 0.119 ± 0.007 μg before to 0.211 ± 0.012 μg after stimulation). Presumably, the calcium in the granules that is lost during degranulation, is replaced with calcium acquired from the extracellular medium.

In order to more fully understand what takes place after stimulation of the neutrophils with cytochalasin B,

Table 2
Effect of cytochalasin B, F-Met–Leu–Phe and cytochalasin B plus F-Met–Leu–Phe on total cell calcium in the presence of 1.0 mM extracellular calcium

	Control	Cytochalasin B[a] (5 μg/ml)	F-Met–Leu–Phe[a] (1×10^{-8} M)	Cytochasalin B (5 μg/ml) F-Met–Leu–Phe (1×10^{-8} M)[a]
Total cell calcium (μg)	2.36 ± 0.19 (3)[b]	2.19 ± 0.18 (3)	2.54 ± 0.14 (3)	2.49 ± 0.10 (3)
Significance[c]		$p < 0.01$	n.s.	n.s.
Total cell protein (mg)	19.8 ± 0.99 (3)	17.1 ± 1.19 (3)	19.4 ± 1.16 (3)	11.9 ± 0.90 (3)
Significance		n.s.	n.s.	$p < 0.01$
Cell calcium protein (μg/mg)	0.119 ± 0.007 (3)	0.128 ± 0.010 (3)	0.132 ± 0.005 (3)	0.211 ± 0.012 (3)
Significance		n.s.	n.s.	$p < 0.01$

[a] Cytochalasin B and/or F-Met–Leu–Phe interacted with the cells (5×10^6 cells/ml, 100 ml) for 1 min
[b] The number in parenthesis refers to number of experiments; errors are standard error of the mean
[c] Significance obtained by means of paired t-tests. n.s., not significant

Table 3
Effect of cytochalasin B, F-Met–Leu–Phe and cytochalasin B plus F-Met–Leu–Phe on cell specific activity of radioactive calcium

Extracellular calcium concentration	Cell specific activity of radioactive calcium (relative to control)			
	Control[d]	Cytochalasin B[a] (5 μg/ml)	F-Met–Leu–Phe[a] (1×10^{-8} M)	Cytochalasin B (5 μg/ml) F-Met–Leu–Phe (1×10^{-8} M)[a]
5 μM	1.0 ± 0.10 (4)[b]	0.86 ± 0.05 (4)	0.77 ± 0.07 (4)	1.53 ± 0.16 (4)
Significance[c]		n.s.	n.s.	$p < 0.01$
1.0 mM	1.0 ± 0.10 (3)	1.13 ± 0.10 (3)	2.40 ± 0.40 (3)	6.41 ± 0.34 (3)
Significance		n.s.	$p < 0.01$	$p < 0.01$

[a] Cytochalasin B and/or F-Met–Leu–Phe interacted with the cells (5×10^6 cells/ml, 100 ml) for 1 min
[b] The number in parenthesis refers to number of experiments; errors are standard error of the mean
[c] Significance obtained by means of paired *t*-tests. n.s., not significant
[d] The specific activities of the control cells (in dpm $\times 10^4/\mu$g cell calcium) were 3.93 ± 0.55 and 0.27 ± 0.03 in the presence of 5 μM and 1.0 mM extracellular calcium, respectively

F-Met–Leu–Phe, and cytochalasin B plus F-Met–Leu–Phe in the presence of 5 μM and 1.0 mM extracellular calcium, it is necessary to examine the data concerning changes in the specific activity of the radioactive cell calcium. The results are summarized in table 3. Cytochalasin B alone had no significant effect on the cell calcium specific radioactivity at either 5 μM or 1.0 mM extracellular calcium. Confirming [15], F-Met–Leu–Phe alone induced a lowering of cell calcium specific radioactivity that was not statistically significant at an extracellular calcium concentration of 5 μM but induced a dramatic and statistically significant increase in cell calcium specific radioactivity (1.0 ± 0.1 to 2.40 ± 0.40) at 1.0 mM extracellular calcium. These and the observations in [15] are consistent with the hypothesis that the binding of the chemotactic factor to its receptor leads to:

(i) A graded displacement of pre-bound calcium;
(ii) A graded increase in membrane permeability to calcium.

Cytochalasin B and F-Met–Leu–Phe together induced a significant ($p < 0.01$) increase in cell calcium specific radioactivity from (1.0 ± 0.1 before to 1.53 ± 0.16 after stimulation at 5 μM extracellular calcium). This increase is consistent with a loss of non-exchangeable calcium during degranulation. An even larger difference in cell calcium specific radioactivity is observed between neutrophils challenged by F-Met–Leu–Phe alone and by the simultaneous addition of cytochalasin B and F-Met–Leu–Phe (from 0.77 ± 0.07 before to 1.53 ± 0.16 after stimulation).

The specific activity of $^{45}Ca^{2+}$ in the lysosomes ($(s.a.)_\ell$), defined as dpm/μg calcium, can be calculated from the data obtained in the presence of 5 μM extracellular calcium (table 1) as follows:

$$(s.a.)_\ell = \frac{(dpm)_b - (dpm)_a}{(Ca^{2+})_b - (Ca^{2+})_a}$$

where $(dpm)_b$ and $(dpm)_a$ are the disintegrations/min and $(Ca^{2+})_b$ and $(Ca^{2+})_a$ the total cell calcium (in μg) before and after stimulation, respectively. The disintegrations/min can be calculated from the product of the specific activities times the total cell calcium under each condition. The specific activity of $^{45}Ca^{2+}$ in the lysosomes so calculated comes out to be 0.533 (dpm $\times 10^4/\mu$g calcium). The specific activity of $^{45}Ca^{2+}$ in the control cells (cytochalasin B column) in table 3 is 3.376 (dpm $\times 10^4/\mu$g calcium). The specific activity of $^{45}Ca^{2+}$ in the lysosomes is thus >6-times lower than that of the control cells. This indicates that the calcium which is lost during degranulation is much less exchangeable than the rest of the calcium in the neutrophils and thus further strengthens the likelihood that the granules represent a site of sequestered calcium.

In the presence of 1.0 mM extracellular calcium,

cytochalasin B and F-Met–Leu–Phe together induce a significant, in fact, a huge increase in the specific activity of the radioactive cell calcium (from 1.0 ± 0.1 before to 6.41 ± 0.34 after stimulation). The magnitude of this change exceeds that induced by cytochalasin B or F-Met–Leu–Phe alone and cannot be explained on the basis of an additive effect of the two agents.

As already described, the results obtained in the presence of low (5 μM) extracellular calcium make it very likely that some or all of the neutrophil granules contain relatively non-exchangeable calcium and this is lost during degranulation. However, when the neutrophils undergo degranulation by F-Met–Leu–Phe and cytochalasin B in the presence of 1.0 mM extracellular calcium the total cell calcium does not change from the control under the same conditions; however, there is a 6.5-fold increase in the specific activity of the cell $^{45}Ca^{2+}$. These findings lead to the conclusion that in the presence of sufficient extracellular calcium the relatively non-exchangeable calcium of the granules that is lost during secretion is replaced by an equal amount of calcium from the medium.

Why the loss or gain of cell calcium should so well balance under these circumstances is possibly explained by the observations [22]. They observed that the empty vesicles of rabbit peritoneal polymorphonuclear leukocytes resulting from the degranulation induced by the staphylococcal toxin, leukocidin picked up Ca^{2+} from the medium. One can explain why the total cell calcium should be the same before and after degranulation if one presumes that the calcium in the granules is actively regulated to be at a fixed level and that the calcium in the granules lost during degranulation must be replaced by extracellular calcium. It must also be observed that if this interpretation is correct, the findings [13] of a linear relation between the degree of degranulation and the increase in level of $^{45}Ca^{2+}$ uptake or level of steady-state $^{45}Ca^{2+}$ becomes not a cause but a result of the degranulation.

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