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Functional responses of aequorin-loaded human neutrophils. Comparison with fura-2-loaded cells

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Aequorin-loaded human neutrophils in response to chemotactic peptides and ionomycin showed a sharp rise in their intracellular Ca^{2+} concentration which decayed within 2 min. Depeletion of extracellular Ca^{2+} suppressed only the ionomycin-induced increase. Fura-2-loaded cells also showed a sharp rise in the intracellular Ca^{2+} concentration in response to each stimulator, while the decline was extremely slow in the ionomycin-induced Ca^{2+} increase. Depletion of extracellular Ca^{2+} reduced the duration of ionomycin-induced Ca^{2+} increase. Cytochalasin B almost equally potentiated the rise in the intracellular Ca^{2+} concentration induced by each stimulator. Aequorin-loaded cells showed impaired phagocytotic activity, while degranulation and oxygen radical production were not affected.

Introduction

It has been shown that the concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) plays a pivotal role in the cell activation system in a variety of cell types. However, the difficulty of directly measuring $[Ca^{2+}]_i$ in small cells, such as neutrophils, was a major obstacle in elucidating its role, until new methods for introducing either fluorescent Ca^{2+} indicators or Ca^{2+}-sensitive photoproteins into the cytoplasm of intact cells were introduced.

Quin2 and fura-2, fluorescent Ca^{2+} indicators, have been widely used to detect changes in $[Ca^{2+}]_i$. Due to its various advantages [1], fura-2 is now preferentially used, and a number of investigations using fura-2-loaded neutrophils have been carried out [2–4].

Aequorin, a jellyfish-derived photoprotein, was first used to detect $[Ca^{2+}]_i$ in smooth muscle cells by Morgan and Morgan [5]. It appears to detect localized pools of Ca^{2+}, whereas fura-2 and quin2 evenly distribute throughout the cytoplasm [6]. It has been used to detect stimulator-induced increases in $[Ca^{2+}]_i$ in various cells, including platelets and adrenal cells [7,8]. In contrast to quin 2 and fura-2, the use of aequorin in neutrophils appears to have been quite rare. To the best of our knowledge, successful aequorin loading of human neutrophils, and functional studies using it have been reported only by Klempner and Johnson, in the form of an abstract in 1984 [9]. There has been no proper investigation of agonist-induced responses of aequorin-loaded neutrophils in terms of $[Ca^{2+}]_i$ increases and phagocyte-associated functions. In the present study, we have measured changes in the $[Ca^{2+}]_i$ of aequorin-loaded human

Abbreviations: fMLP, formylmethionylleucylphenylalanine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRP, Kreb-Ringer phosphate buffer.

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neutrophils in response to fMLP, and ionomycin, a Ca^{2+} ionophore, in comparison with those loaded with fura-2. Phagocytosis, oxygen radical production, and degranulation were also measured in order to evalulate the effects of the aequorin-loading procedure on neutrophil function.

Materials and Methods

Agents. Aequorin, fura-2 A/M, and ionomycin were obtained from Baxter-Travenol (Tokyo, Japan), Funakoshi (Tokyo, Japan), and Calbiochem (Ca., U.S.A.), respectively. fMLP, cytochalasin B, cytochrome *c*, and superoxide dismutase were obtained from Sigma (MO, U.S.A.). Krebs-Ringer phosphate buffer (KRP) containing 130 mM NaCl/5 mM KCl/1 mM $CaCl_2$/1.2 mM $MgSO_4$/10 mM Na_2HPO_4/5 mM glucose/0.1% gelatin (pH 7.4), was sterilized by autoclaving and was stored at 4° C until use.

Preparation of human neutrophils. Citrate-anticoagulated blood was centrifuged at $80 \times g$ for 10 min and the platelet-rich plasma was discarded. KRP was then added to restore the blood to the original volume. Human neutrophils were purified by dextran sedimentation and Ficoll-Conray centrifugation. After contaminating red blood cells had been lyzed by hypotonic shock, purified neutrophils were washed once with KRP.

Aequorin loading and measurement of $[Ca^{2+}]_i$. Neutrophils were washed once with Hepes-Tyrode's buffer [7] containing 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and were then resuspended and incubated sequentially for 1 h at 0° C in each of the following solutions: solution A, 150 NaCl/5 mM Hepes/5 mM adenosine triphosphate (ATP)/2 mM $MgCl_2$/10 mM EGTA 0.2 mg per ml aequorin; solution B, 150 mM NaCl/5 mM Hepes/5 mM ATP/10 mM $MgCl_2$/0.1 mM EGTA.

At the end of the second incubation, the neutrophils were washed twice with KRP, and resuspended in KRP at a concentration of 10^7 cells/ml. 1 ml of this suspension was used for measurement of aequorin luminescence with a Platelet Ionized Calcium Aggregometer (Chrono-log, PA, U.S.A.). Calibration of aequorin light signals was followed essentially as described by Johnson et al. [7].

Fura-2 loading and measurement of $[Ca^{2+}]_i$. Fura-2 A/M, at a final concentration of 1 μM, was added to the purified neutrophil suspension in KRP, and incubated for 2 h at 37° C with mild shaking. After incubation, the fura-2-loaded cells were washed twice with KRP, and resuspended in KRP at a concentration of 10^7 cells/ml. Fura-2 fluorescence was measured with a Hitachi F-4000 fluorescence spectrophotometer, with the excitation wavelength alternated every 4 s from 340 nm to 380 nm, and with the emission wavelength set at 510 nm. The neutrophil suspension was kept at 37° C with constant stirring throughout the measurement. Calcium concentrations were determined from the ratio of fura-2 fluorescence intensities at excitations of 340 nm and 380 nm [1]. The data were processed with a computer fitted to the Hitachi F-4000 fluorescence spectrophotometer, and the estimated $[Ca^{2+}]_i$ levels were recorded sequentially.

Measurement of hydrogen peroxide (H_2O_2) production. H_2O_2 production by neutrophils was measured using the method of Root and Metcalf [10]. Briefly, $2.5 \cdot 10^6$ neutrophils were suspended in 2 ml of KRP containing 4 μM scopoletin and 22 nM horseradish peroxidase. After addition of 1 μM fMLP, the extinction of scopoletin fluorescence in the cell suspension was measured continuously with the excitation wavelength of 360 nm and the emission wavelength of 460 nm. The amount of H_2O_2 produced was standardized with known amounts of H_2O_2.

Phagocytosis. Emulsion of paraffin oil containing oil red O were prepared as previously described [11]. The emulsion was incubated with an equal volume of normal human serum at 37° C for 30 min for opsonization. Neutrophils ($2 \cdot 10^7$ cells/0.9 ml KRP) were incubated with 0.1 ml of the opsonized emulsion for 5 min at 37° C and then 9 ml of ice-cold KRP was added to the solution to stop the reaction. The cells were washed three times with ice-cold KRP to remove the paraffin oil droplets which had not been ingested. Paraffin oil containing oil red O was extracted with a mixture of chloroform and methanol (v/v, 1 : 2), and the absorption of the chloroform layer was determined at a wavelength of 525 nm.

Degranulation. Enzyme release from the azurophilic granules was assessed by measuring

the release of *N*-acetyl-β-D-glucosaminidase. In brief, $3 \cdot 10^6$ neutrophils in 0.5 ml of KRP were preincubated with 5 μg/ml cytochalasin B for 5 min. After the addition of 10^{-6} M fMLP, the neutrophil suspension was incubated for another 10 min. After incubation, the cell suspension was set on ice to stop the reaction. The supernatant obtained after centrifugation was assayed for enzyme content using a *N*-acetyl-β-D-glucosaminidase Test Kit Shionogi (Shionogi Pharmaceuticals, Osaka, Japan). Enzyme release was expressed as the percentage of the total enzyme present in the detergent lysate of the cell suspension.

Results

Ionomycin- and fMLP-induced $[Ca^{2+}]_i$ changes determined by aequorin luminescence

Firstly, we attempted to load human neutrophils with aequorin essentially according to the method described by Johnson et al. [7]. Although aequorin-loaded cells responded to ionomycin stimulation, fMLP induced a light peak which was only barely visible. However, when KRP instead of Hepes-Tyrode's buffer was used for suspending neutrophils, fMLP induced the cells to emit considerable luminescence. Interestingly, autoclaved KRP gave better results than did millipore-sterilized buffer. In the experiments thereafter, KRP was used for suspending neutrophils. Both

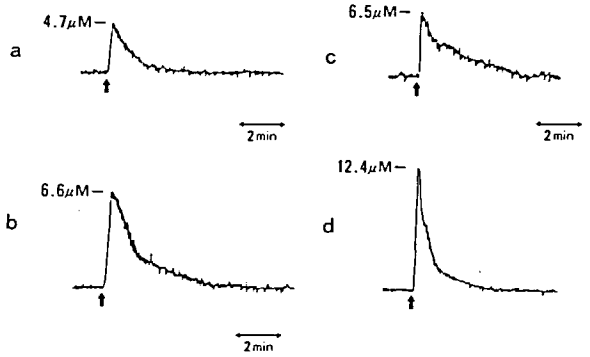

Fig. 1. fMLP- and ionomycin-induced increases in the $[Ca^{2+}]_i$ of aequorin-loaded neutrophils. The stimulator was added to the neutrophil suspension at the time indicated by the arrow, and the light peak emitted from aequorin-loaded cells was sequentially recorded. a, fMLP (10^{-7} M); b, fMLP (10^{-6} M); c, ionomycin (10^{-6} M); d, ionomycin $3.16 \cdot 10^{-6}$ M. The traces are representative of five experiments.

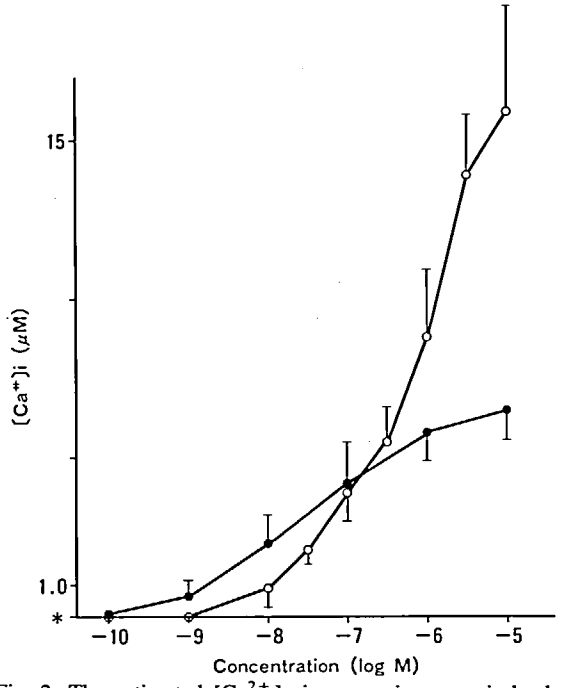

Fig. 2. The estimated $[Ca^{2+}]_i$ increases in aequorin-loaded neutrophils activated by various concentrations of fMLP (●) and ionomycin (○). The circles and bars represent the mean ± S.D. of three experiments.

ionomycin and fMLP induced increases in the $[Ca^{2+}]_i$ of aequorin-loaded neutrophils dose dependently. With fMLP stimulation, there was a lag of about 5 s before $[Ca^{2+}]_i$ started to rise, while there was almost no lag after ionomycin stimulation. After addition of either agent, the emission peak showed a sharp rise, and then declined gradually back to the starting level within 2 min (Fig. 1a and b). The duration of the light peak was not dependent upon the concentration of either agent. The estimated levels of $[Ca^{2+}]_i$ induced by various concentrations of ionomycin and fMLP are presented in Fig. 2. The optimal concentration of fMLP for induction of an increase in $[Ca^{2+}]_i$ was in the range of 10^{-6} M to 10^{-5} M, raising the $[Ca^{2+}]_i$ level to about 6 μM. With higher concentrations of ionomycin, much higher levels of $[Ca^{2+}]_i$ were attained (15.9 ± 3.3 μM).

Ionomycin- and fMLP-induced $[Ca^{2+}]_i$ changes determined by fura-2

The resting level of $[Ca^{2+}]_i$ in fura-2-loaded neutrophils was 46.3 ± 14.7 nM. fMLP induced

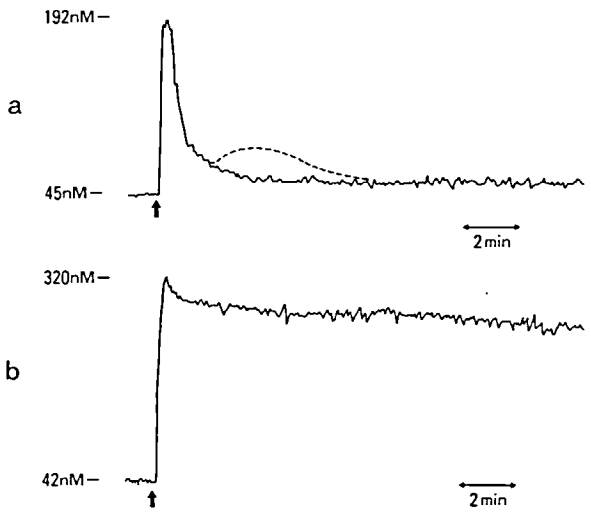

Fig. 3. fMLP- and ionomycin-induced increases in the $[Ca^{2+}]_i$ of fura-2-loaded neutrophils. The stimulator was added to the neutrophil suspension at the time indicated the arrow and changes in the $[Ca^{2+}]_i$ of fura-2-loaded cells were sequentially recorded. a, fMLP (10^{-7} M); b, ionomycin (10^{-6} M). The traces are representative of five experiments.

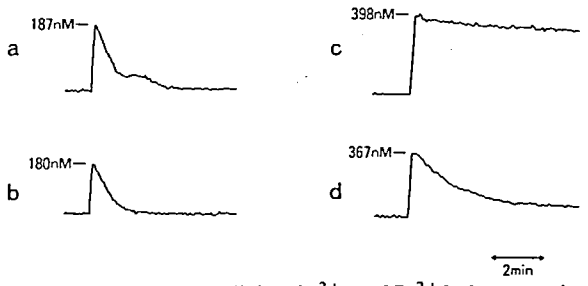

Fig. 4. Effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$ increases in fura-2-loaded cells activated by fMLP and ionomycin. a, fMLP (10^{-6} M) in the presence of extracellular Ca^{2+}; b, fMLP (10^{-6} M) in the absence of extracellular Ca^{2+}; c, ionomycin (10^{-6} M) Ca^{2+} (+); d, ionomycin (10^{-6} M) in the absence of Ca^{2+}. The traces are presentative of five experiments.

increases in $[Ca^{2+}]_i$ which declined back to almost the original level within 2 min. In some cases, a small peak lasting for 2–8 min was detected behind the main peak (Fig. 3a, dotted line). With ionomycin stimulation there was a sharp rise in $[Ca^{2+}]_i$, as with fMLP, though unlike the effect of fMLP, the induced rise declined extremely slowly (Fig. 3b). Even after 20 min the $[Ca^{2+}]_i$ remained at 60–80% of the peak $[Ca^{2+}]_i$ level.

Effects of extracellular Ca^{2+}

The effect of extracellular Ca^{2+} on the increases in $[Ca^{2+}]_i$ induced by the two stimulators were next investigated. EGTA at a concentration of 4 mM was added to the neutrophil suspension 1 min before stimulation in order to deplete the suspension of extracellular Ca^{2+}. With aequorin-loaded cells, the rise in $[Ca^{2+}]_i$ induced by 10^{-6} M fMLP, in the absence of extracellular Ca^{2+}, was found to be only slightly lower than that induced in the presence of 1 mM Ca^{2+} ($13 \pm 9\%$ reduction $n = 4$). In contrast to the results obtained with fMLP, the light peak induced by 10^{-6} M ionomycin was significantly lower in the absence of extracellular Ca^{2+} than in the presence of 1 mM Ca^{2+} ($72.5 \pm 12.2\%$ reduction $n = 4$). However, the patterns of the $[Ca^{2+}]_i$ increases were virtually the same in either the presence or absence of extracellular Ca^{2+}.

With fura-2-loaded cells, the maximum increase in $[Ca^{2+}]_i$ induced by fMLP was found to be virtually the same regardless of whether or not extracellular Ca^{2+} was present (Fig. 4a), though the small peak observed in some samples was never evident in the absence of extracellular Ca^{2+}. Although the rise in $[Ca^{2+}]_i$ induced by ionomycin did not differ significantly in the presence or absence of extracellular Ca^{2+}, there were clear differences in its subsequent decline (Fig. 4b). In the presence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ remained close to its highest level even after 5 min stimulation, whereas in the absence of extracellular Ca^{2+} it rapidly returned towards the resting level.

Effects of cytochalasin B

Cytochalasin B alone at concentrations higher than 2.0 μg/ml induced a small rise in the $[Ca^{2+}]_i$ of both aequorin- and fura-2-loaded cells. To assess the effects of cytochalasin B pretreatment on fMLP and ionomycin stimulation, the drug was added to cell suspensions 5 min before addition of the stimuli. With both aequorin- and fura-2-loaded cells, increases in the $[Ca^{2+}]_i$ induced by ionomycin were not significantly affected by cytochalasin B. However, on stimulation with fMLP, pretreated aequorin- and fura-2-loaded cells showed an increase in $[Ca^{2+}]_i$ of about 100% higher than that achieved without cytochalasin B

TABLE I

THE EFFECTS OF CYTOCHALASIN B ON THE $[Ca^{2+}]_i$ OF AEQUORIN-LOADED (A) AND FURA-2-LOADED (B) NEUTROPHILS

Increases in $[Ca^{2+}]_i$ caused by: cytochalasin B (a); 10^{-7} M fMLP added 5 min after addition of the indicated doses of cytochalasin B (b); and 10^{-6} M fMLP added 5 min after addition of the indicated doses of cytochalasin B (c). n.d., no $[Ca^{2+}]_i$ increase was detected. The data are presented as means ± S.D. of three experiments (μM).

	Cytochalasin B (μg/ml)			
	0	0.8	2.0	5.0
A. a	n.d.	n.d.	1.4 ± 1.2	2.8 ± 2.0
b	3.9 ± 1.2	4.1 ± 1.1	7.2 ± 1.9	8.3 ± 2.1
c	5.8 ± 1.8	6.2 ± 2.0	10.6 ± 2.7	12.7 ± 3.3
B. a	31 ± 7	43 ± 4	60 ± 5	74 ± 8
b	163 ± 16	208 ± 21	249 ± 36	321 ± 39
c	190 ± 30	211 ± 35	313 ± 41	406 ± 38

pretreatment. The potentiating effect of cytochalasin B on fMLP-induced increases in $[Ca^{2+}]_i$ was dose dependent with an optimal concentration of 5 μg/ml (Table I), concentrations of 10 and 15 μg/ml being no more effective.

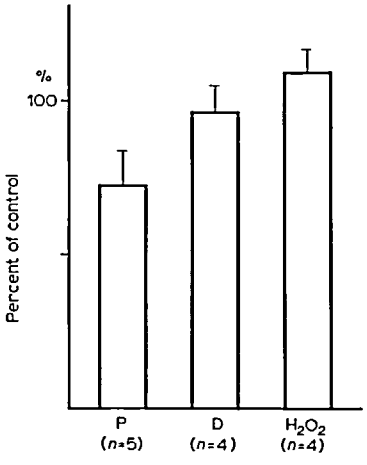

Fig. 5. Effect of aequorin loading on neutrophil function. Neutrophil function was evaluated on aequorin-loaded cells compared to non-treated cells. Non-treated cells were processed similarly to aequorin-loaded cells except for the absence of ATP, Mg^{2+} and EGTA in the buffers. The data are presented as mean ± S.D. of the number of experiments shown in parentheses. P, phagocytosis; D, degranulation; H_2O_2, H_2O_2 production.

Effect of aequorin loading on neutrophil functions

Neutrophil functions were evaluated on aequorin-loaded cells with non-treated cells as the control. Non-treated cells were processed similarly to aequorin-loaded cells except for the absence of ATP, Mg^{2+} and EGTA in solution A and solution B. Phagocytotic activity of aequorin-loaded cells was impaired, while H_2O_2 production, in response to fMLP, was only slightly elevated (Fig. 5). Degranulation (release of *N*-acetyl-D-glucosaminidase) was not significantly different from that in the control cells. The functional response of fura-2-loaded cells was not significantly different from that of the control cells (oxygen radical production and degranulation).

Discussion

In the present study, aequorin-loaded human neutrophils, when stimulated with fMLP, demonstrated a sharp rise in $[Ca^{2+}]_i$, which then declined to an undetectable level within 2 min. Our findings are in good agreement with other reports which showed that the rise in $[Ca^{2+}]_i$ detected by aequorin was in a variety of cell types which is quite transient. There has been doubt, however, as to whether this decay in aequorin luminescence truly represents a decline in $[Ca^{2+}]_i$, local consumption of aequorin being, perhaps, responsible [7]. This doubt has been based partly on findings with quin2-loaded cells that the estimated $[Ca^{2+}]_i$ induced by agonists declined gradually, returning to close to the resting level after only 10 min [7,12–14]. In the present study, we found that, as with aequorin, $[Ca^{2+}]_i$ determined by fura-2 returned to resting levels within 2 min after addition of fMLP (Fig. 1), while elevated quin2 fluorescence induced by fMLP lingered on for more than 5 min (data not shown). Since the cytoplasmic distribution and Ca^{2+} detection mechanisms of fura-2 and quin2 are the same, the differences in the $[Ca^{2+}]_i$ time courses are probably attributable to the strong buffering property of quin2; its slow release of bound Ca^{2+} enabling quin2 fluorescence to last longer than that of fura-2. Thus, since the time course for $[Ca^{2+}]_i$ changes, detected by the fluorescent Ca^{2+} indicator, fura-2, essentially coincided with that detected by the Ca^{2+}-sensitive photoprotein, aequorin, we suggest that

the $[Ca^{2+}]_i$ of fMLP-activated neutrophils does indeed return to the resting level shortly after activation.

Depletion of extracellular Ca^{2+} from the suspending buffer did not significantly affect the fMLP-induced rise in $[Ca^{2+}]_i$, as determined by both aequorin and fura-2. Thus, the fMLP-induced changes are probably due largely to mobilization of Ca^{2+} from intracellular storage sites. On the other hand, the presence of extracellular Ca^{2+} significantly modified the pattern of aequorin- and fura-2-detected ionomycin-induced $[Ca^{2+}]_i$ peaks. With aequorin-loaded cells, depletion of extracellular Ca^{2+} greatly reduced the luminescence peak, while with fura-2-loaded cells the duration of the fluorescence elevation was significantly shortened, although the peak fluorescence change was only slightly reduced. These findings suggest that the Ca^{2+} influx contributes substantially to ionomycin-induced $[Ca^{2+}]_i$ increases determined by aequorin, and that this influx helps to maintain the peak detected at a later stage by fura-2.

Cytochalasin B, which interacts with microfilaments, is known to modify neutrophil function, for instance, potentiating degranulation and oxygen radical production induced by soluble receptor-mediated stimulators, such as fMLP [15]. Although the mechanism by which cytochalasin B potentiates neutrophil responses is largely unknown, there is some evidence for its involvement in Ca^{2+} mobilization. Naccache et al. demonstrated, using ^{45}Ca, that cytochalasin B greatly potentiated Ca^{2+} mobilization induced by fMLP [16]. Korchak et al. reported that cytochalasin B elevated the fMLP-induced rise 2-fold in $[Ca^{2+}]_i$ of quin2-loaded cells [17]. On the other hand, Pozzan et al. reported, using quin2-loaded neutrophils, that cytochalasin B did not modify the rise in $[Ca^{2+}]_i$ detected by quin2, though it did greatly potentiate superoxide anion production induced by fMLP [13]. In accordance with results of Pozzan et al., we were unable to detect any effects of cytochalasin B on fMLP-induced changes in quin2 fluorescence (data not shown). However, we did observe that cytochalasin B potentiated $[Ca^{2+}]_i$ rises induced by fMLP in both aequorin- and fura-2-loaded neutrophils. Quin2, with its strong buffering property, may have minimized changes caused by cytochalasin B. Nevertheless, the possitive results obtained using two different methods for Ca^{2+} detection strongly suggest that cytochalasin B does modify Ca^{2+} mobilization induced by fMLP. Recently, Honeycutt and Niedelfound that cytochalasin B enhanced diacylglycerol production in fMLP-activated neutrophils [18]. Since inositol trisphosphate, which is able to elevate $[Ca^{2+}]_i$ and diacylglycerol are products of phospholipase C, when these findings are combined, it is suggested that cytochalasin B may act by potentiating receptor-mediated phospholipase C activation.

Aequorin loading into neutrophils utilizes ATP and EGTA for loading and Mg^{2+} for resealing. Although the precise mechanism by which aequorin molecules are set in positions in which they can detect cytoplasmic Ca^{2+} has not been elucidated, it is possible that the process for loading aequorin affects some property of the neutrophil membrane. It is also possible that small molecule metabolites and soluble protein may leak out of the cytoplasm if one assumes that aeqorin molecules pass into the cytoplasm through holes in the membranes. We, therefore, evaluated the effect of aequorin loading on various neutrophil functions. Degranulation, or the release of intracellular granule contents, in response to fMLP was not affected by aequorin loading. Knight et al., using electropermeabilized platelets, reported that the presence of ATP fully protected the impairment in degranulation caused by electropermeabilization [19]. The buffer for resealing aquorin-loaded neutrophils also contained ATP, and our findings in conjunction with those of Knight suggest that the mechanism required for the release of intracellular granule contents is well preserved after the permeabilization procedures in the presence of ATP. On the other hand, phagocytosis of endotoxin-coated particles was significantly impaired by aquorin loading. Cytochalasin B, which interferes with the cytoskeletal conformation, enhances degranulation, while it severely suppresses phagocytosis [20,21]. In the same line of reasoning, it is conceivable that some factor(s) associated with cytoskeletal integrity may be lost in aquorin-loading procedures, resulting in the impairment in phagocytosis, but not in degranulation. Oxygen radical production induced by fMLP

was not impaired after aequorin loading. Since neutrophil membrane fractions alone can produce oxygen radicals in the absence of cytosolic fractions [22], it may be understandable that the changes in cytosolic components caused by aequorin loading did not affect oxygen radical production by whole cells. Taken together, our findings suggest that the functional study of aequorin-loaded neutrophils in association $[Ca^{2+}]_i$ increases can be reliably performed in degranulation and oxygen radical production. However, results with phagocytosis should be evaluated carefully.

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