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Monitoring of cytosolic free Ca^{2+} in C5a-stimulated neutrophils: Loss of receptor-modulated Ca^{2+} stores and Ca^{2+} uptake in granule-free cytoplasts

(complement/chemotaxis/secretion/O_2^- production)

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ABSTRACT **The cytosolic concentration of free Ca^{2+} in bovine neutrophils was monitored by using the intracellular Ca^{2+} indicator quin2, 2-{[2-bis(acetylamino)-5-methylphenoxy]methyl-6-methoxy-8-bis(acetylamino)}quinoline. Neutrophils at rest have a cytosolic Ca^{2+} concentration of 85 ± 5 nM, which in 2–4 min increases to 300–400 nM upon interaction with the complement fragment C5a in a concentration range of 35 pM to 1.2 μM. In the same concentration range, C5a also sequentially activates neutrophil directional migration (ED_{50} < 0.5 nM), O_2^- production (ED_{50} = 9 nM), and secretion of the contents of specific granules (ED_{50} = 39 nM). The selective Ca^{2+} ionophore ionomycin also increases cytosolic Ca^{2+} concentration above 1 μM under conditions where it stimulates neutrophil functions. Conversely, phorbol 12-myristate 13-acetate markedly activates secretion and O_2^- production without modifying the average cytosolic Ca^{2+} concentration. In the presence of EGTA ($Ca^{2+}_{out} \approx 20$ nM), with both C5a and ionomycin, cytosolic Ca^{2+} increases to less than 200 nM, and functional responses are greatly decreased. Nucleus- and granule-free neutrophil cytoplasts accumulate Ca^{2+} and produce O_2^- when exposed to ionomycin but not to C5a. These results and other considerations suggest that (*i*) activation of neutrophil functions may occur after cytosolic Ca^{2+} has exceeded the apparent threshold level of 200 nM; (*ii*) C5a receptor-mediated activation of Ca^{2+} influx may require cooperation between the neutrophil surface and some cytoplasmic organelle and/or redistribution of the C5a–receptor complexes on the cell surface; and (*iii*) the phorbol diester stimulates Ca^{2+}-dependent pathways presumably by directly activating other mechanisms such as protein phosphorylation.**

Orientation of locomotion, production of cytotoxic oxygen derivatives, and exocytosis of granule enzymes and binding proteins are characteristic components of the repertoire of behavioral changes of neutrophils, which are elicited by a variety of extracellular ligands (1–5). These changes are mimicked or modulated, or both, by Ca^{2+} ionophores and intracellular Ca^{2+} antagonists (6–10) and are associated with increased ^{45}Ca uptake (5, 11–13). Thus, the concept has emerged that a variation in the cytosolic Ca^{2+} concentration, caused by either Ca^{2+} mobilization from intracellular stores or increased inward Ca^{2+} diffusion, might promote and, perhaps, regulate the neutrophil response to surface stimulation.

Monitoring changes of cytosolic Ca^{2+} concentration in large populations of small cells such as neutrophils has been made possible recently by the introduction of a fluorescent quinoline Ca^{2+} indicator that can be trapped inside cells (14–18). We have used this technique to measure Ca^{2+} concentrations both in intact cells and in enucleated and granule-free neutrophil cytoplasts (19).

We have been able to correlate changes in cytosolic Ca^{2+} concentration with activation of neutrophil functions brought about by three different stimulants: (*i*) the complement fragment C5a, which interacts with specific receptors on the neutrophil surface (20); (*ii*) the hydrophobic ionophore ionomycin, which selectively carries Ca^{2+} across membranes (16, 18, 21); and (*iii*) phorbol 12-myristate 13-acetate (PMA), a hydrophobic neutrophil activator that binds to membranes with high affinity (13, 22).

A comparative evaluation of functional responses of intact neutrophils and cytoplasts to stimulation by these reagents has partially helped to identify the presumed mechanism of Ca^{2+}-dependent transmembrane signalling by the complement fragment C5a.

MATERIALS AND METHODS

Cells and Cytoplasts. Neutrophils were prepared from bovine blood as described (13, 23, 24) and suspended at 37°C either in 137 mM NaCl/2.7 mM KCl/0.5 mM $MgCl_2$/0.9 mM $CaCl_2$/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 7.4 (phosphate-buffered saline) or in 120 mM NaCl/5 mM KCl/0.5 mM $MgSO_4$/1 mM $CaCl_2$/1 mM Na_2HPO_4/5 mM glucose/20 mM Hepes, pH 7.4 (Hepes medium). Neutrophil cytoplasts were obtained as described by Roos *et al.* (19) after centrifugation of neutrophils at 33°C (30 min at 81,000 × *g*) through a discontinuous gradient of Ficoll containing 5 μM cytochalasin B. The material at the 12.5–16% (wt/vol) interface was collected and washed four times with Hepes medium.

Complement Fragment C5a. This was purified from yeast-activated bovine serum by a modification of the method of Fernandez and Hugli (25). The purified polypeptide migrated as a single band upon electrophoresis on a polyacrylamide gel slab (26) and reacted with an antiserum to porcine C5a (a gift of B. Damerau).

Details about the purification procedure and the structure of bovine C5a will be reported elsewhere.

Antibodies and Immunofluorescence Microscopy. A rabbit antiserum to bovine C5a was obtained after repeated immunization of rabbits with the purified C5a mixed with complete Freund's adjuvant (27). The immunoglobulins were precipitated from the antiserum with 40% sodium sulfate and further purified by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia).

For immunofluorescent detection of C5a receptors on the neutrophil or cytoplast surface, these were allowed to react for 40 min with C5a (5 μg/ml), washed three times with phosphate-buffered saline, incubated for 40 min with the pu-

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Abbreviations: $B_{12}BP$, vitamin B_{12} binding protein; quin2, 2-{[2-bis(acetylamino)-5-methylphenoxy]methyl-6-methoxy-8-bis(acetylamino)}quinoline; quin2/AM, acetoxymethyl ester of quin2; PMA, phorbol 12-myristate 13-acetate.

rified antibodies (100 μg of protein per ml), and, after three more washings with the buffered saline, fixed for 5 min with 0.5% paraformaldehyde in phosphate-buffered saline. All these operations were carried out at 0–2°C. Staining was with fluorescein isothiocyanate-labeled goat-anti-rabbit IgG (12 mg of protein per ml) at a 1:20 dilution for 30 min at room temperature. After three washings with phosphate-buffered saline, neutrophils or cytoplasts were observed by fluorescence microscopy.

Controls were run by either omitting the treatment with C5a or allowing the C5a-treated neutrophils or cytoplasts to react with bovine IgG before exposing them to the anti-C5a immunoglobulins and to the goat fluorescein isothiocyanate-labeled anti-rabbit IgG. The latter control was designed to prevent potential binding of the C5a antibodies to the neutrophil F_c fragment receptors.

Quin2 Loading and Fluorescence Measurements. Neutrophils and cytoplasts were loaded by incubation at 37°C with quin2/AM (acetoxymethyl ester of quin2, which is 2-{[2-bis(acetylamino)-5-methylphenoxy]methyl-6-methoxy-8-bis(acetylamino)}quinoline, a gift of R. Y. Tsien) in Hepes medium. The usual procedure was to add 25 μM or 10 μM quin2/AM to 5×10^7 cells per ml or 1×10^8 cytoplasts per ml, respectively, incubate for 15 min, then dilute 1:5 with Hepes medium containing 0.5% bovine serum albumin, and continue the incubation at 37°C for 45 min. From these stock suspensions, which were maintained at room temperature, an aliquot was withdrawn before each measurement, centrifuged for a few sec at 12,000 × *g*, and resuspended in 1.5 ml of Hepes medium.

Fluorescence was recorded with a Perkin–Elmer 650-40 spectrofluorimeter, equipped with a magnetic stirrer. Setting of the monochromator was 339-nm excitation (3-nm slit) and 492-nm emission (10-nm slit). Fluorescence was calibrated in terms of cytosolic Ca^{2+} concentration as described (14, 15).

Assays of Neutrophil Functions. Directional migration was assayed by a method of chemotaxis under agarose (dissolved in phosphate-buffered saline with 5 mM glucose and 0.25% gelatin) on gelatin-coated microscope slides (27). Six series of three wells, 2.5 mm in diameter and spaced 1.5 mm apart, were cut in each plate with a stainless steel punch. C5a at different concentrations and phosphate-buffered saline (10 μl) were placed in the outer wells, and the slides were incubated at 37°C for 1 hr in a humidified atmosphere. Neutrophils (3×10^5 in 10 μl) were then added to the central well, and the incubation was continued for 2 hr. Cells were fixed in methanol, stained (May–Grünwald–Giemsa), and examined by light microscopy. Directional migrations were evaluated by measuring the maximal distance migrated by three leading cells aligned in the same plane and parallel to the margin of the well (27).

Vitamin B_{12} binding protein ($B_{12}BP$) secretion was assayed as reported (24) after incubating 3×10^6 cells in 0.3 ml of Hepes medium at 37°C for 30 min with and without the appropriate stimulants.

O_2^- production was evaluated after treating (10 min at 37°C; 0.25 ml of Hepes medium) 2×10^6 neutrophils or 5×10^6 cytoplasts with the appropriate stimulants in the presence of 0.24 mM ferricytochrome *c* with or without superoxide dismutase (2, 28). The supernatants, obtained by centrifuging the reaction mixtures, were read at 550 and 468 nm, and the absorbance was converted into nmol of O_2^- produced by using an extinction coefficient (mM) of 24.5 (29).

Analytical Methods. The activities of alkaline phosphatase, myeloperoxidase, and lactate dehydrogenase were assayed as reported (23, 24). Protein and DNA were determined by the methods of Lowry *et al.* (30), with bovine serum albumin as standard, and of Labarca and Paigen (31), with salmon sperm DNA as standard, respectively.

The concentration of extracellular Ca^{2+} in equilibrium with Ca–EGTA complexes was calculated as described elsewhere (32).

RESULTS

C5a-Dependent Increase of Cytosolic Ca^{2+} Concentration in Intact Neutrophils. Intact bovine neutrophils had a cytosolic Ca^{2+} concentration of 85 ± 5 nM (mean of 20 determinations ± SEM). When exposed to C5a, there was an immediate increase in fluorescence of cell-associated quin2, which reached the top value within 2–4 min (Fig. 1). This phase was then followed by a slow decay towards resting levels of Ca^{2+}.

Fig. 2 shows that C5a elicited an increase of cytosolic Ca^{2+} to 0.3–0.4 μM over the full range of concentrations where the complement fragment exhibits its modulatory effects on neutrophil functions. Activation of oriented locomotion appeared to correlate well with the C5a-induced increase in Ca^{2+}. In fact, in the method we used to evaluate the neutrophil migration, the chemoattractant molecules diffused from wells where they were placed at suitable initial concentrations into the agarose gel and generated a chemical gradient. Thus, the actual C5a concentrations sensed by neutrophils were lower than those indicated in the abscissa of Fig. 2, with an ED_{50} for chemotaxis very likely similar to that observed for the increase in Ca^{2+}. In contrast, the ED_{50}s for the C5a-induced activation of O_2^- generation and of $B_{12}BP$ secretion were about 3 orders of magnitude higher than the C5a concentration required for half-maximal increase of cytosolic Ca^{2+}.

In the presence of ≈20 nM extracellular Ca^{2+}, C5a induced a rapid rise of cytosolic Ca^{2+}, which in general remained below 150 nM (Fig. 1). Under these conditions, which allow Ca^{2+} displacement from intracellular stores but no Ca^{2+} influx, stimulation of neutrophil functions, such as O_2^- production and especially secretion of granule content, was considerably decreased (Table 1).

Ionomycin and PMA Effects on Cytosolic Ca^{2+} Concentration and Neutrophil Functions. Ionomycin rapidly increased cytosolic Ca^{2+} concentration in neutrophils largely above 1

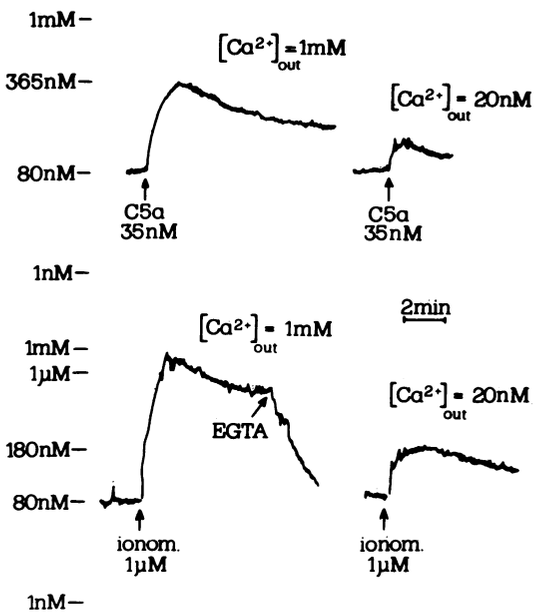

FIG. 1. Effect of C5a and of ionomycin on cytosolic Ca^{2+} concentration of neutrophils (5×10^6 per ml of Hepes medium). A decrease of Ca^{2+} concentration in the Hepes medium to ≈20 nM was achieved by adding 6.5 mM Tris-buffered EGTA. The four experiments were carried out with aliquots of the same batch of quin2-loaded neutrophils. Intracellular quin2 concentration was 0.62 mM, if one assumes a water content of 0.35 μl per 10^6 cells.

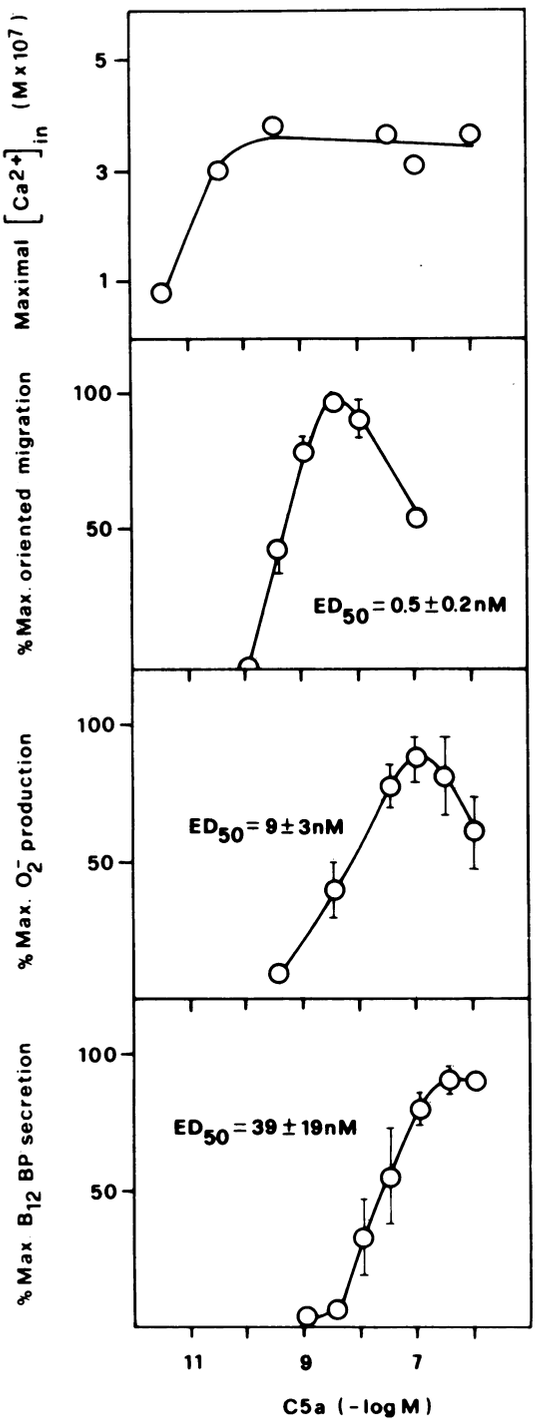

FIG. 2. Dose–response curve of C5a-dependent increase in cytosolic Ca^{2+}, orientation of locomotion, activation of O_2^- production, and of secretion of $B_{12}BP$ in neutrophils. Points related to cytosolic Ca^{2+} concentration are the average of two determinations; cytosolic Ca^{2+} concentration of untreated cells was 85 ± 5 nM (mean of 20 determinations ± SEM). Maximal (Max.) oriented migration was 563 ± 108 μm (mean of four experiments ± SEM); maximal release of $B_{12}BP$ above control was 46 ± 4% of the total cell content (mean of three experiments ± SEM); maximal O_2^- production above control was 3.8 ± 1.5 nmol/10 min per 10^6 cells (mean of six experiments ± SEM). At the concentrations of quin2 reached intracellularly (up to 0.8 mM), there was essentially no modification of the pattern of cell response to C5a.

μM (Fig. 1). Only part of this rise was due to Ca^{2+} mobilization from intracellular deposits, as indicated by the extent of fluorescence increase of cell-associated quin2 recorded in the presence of EGTA. As observed with C5a, in the latter condition, ionomycin-dependent activation of O_2^- generation and of secretion was much lower than in the presence of 1 mM extracellular Ca^{2+} (Table 1).

Conversely, with PMA as stimulant, the same increment in $B_{12}BP$ release and O_2^- production was seen either in low (≈20 nM) or high (1 mM) Ca^{2+} medium. In both conditions, the phorbol diester did not induce any change in intracellular quin2 fluorescence. Furthermore, when added to the cells before C5a or ionomycin, while not affecting the inward diffusion of the cation catalyzed by the ionophore, it completely abolished the C5a-dependent influx of Ca^{2+} (results not shown).

Increase in Cytosolic Ca^{2+} and Metabolic Responses by Neutrophil Cytoplasts. Upon centrifugation of neutrophils at 33°C through a Ficoll gradient containing cytochalasin B, a population of cytoplasts was collected that contained 31 ± 3%, 47 ± 5%, and 58 ± 7% of the original cell content of protein, alkaline phosphatase, and lactate dehydrogenase, respectively (means of six preparations ± SEM; values calculated on a per cell and per cytoplast basis). Conversely, the cytoplast population contained only 1.2 ± 0.1%, 1.3 ± 0.2%, and 3.5 ± 0.7% of the original DNA, $B_{12}BP$, and peroxidase activity, respectively. Furthermore, the cytoplasts had an internal Ca^{2+} concentration of 71 ± 10 nM (mean of 11 determinations ± SEM), as measured by the fluorescence intensity of trapped quin2.

These results indicate that (*i*) close to 100% of the isolated cytoplasts lost the nucleus and the $B_{12}BP$- and peroxidase-containing granules (24); (*ii*) cytoplasts budded from the intact cells as large vesicles (19) carrying over nearly half of the plasma membrane (alkaline phosphatase) and almost 60% of the soluble components of the cytoplasm; and (*iii*) the cytoplast-associated plasma membrane remained impermeable to Ca^{2+} and capable of maintaining the internal Ca^{2+} homeostasis (33).

Unlike intact neutrophils, quin2-loaded cytoplasts did not respond with an increase in fluorescence when exposed to ionomycin in the presence of low (≈20 nM) external Ca^{2+}. This result is unlikely to be ascribed to the Ca^{2+}-buffering activity of quin2 (14) because in the cytoplasts the cytosolic concentration of quin2 was about half of that of intact cells (compare Fig. 3 with Fig. 1). On the contrary, it suggests that the loss of nucleus and cytoplasmic granules from the neutrophils also involved loss of ionomycin-sensitive Ca^{2+} stores.

When external Ca^{2+} was 1 mM, ionomycin catalyzed the inward diffusion of Ca^{2+} into cytoplasts up to an internal concentration greater than 5 μM (Fig. 3). Under these conditions there was also stimulation of the plasmalemmal O_2^--generating enzyme (2) (Table 1).

A marked activation of O_2^- production by cytoplasts was also brought about by PMA (Table 1) without any increase in the intravesicular Ca^{2+} (not shown). Finally, C5a neither stimulated O_2 reduction by cytoplasts nor induced an inward flux of Ca^{2+} (Fig. 3).

C5a Receptors. C5a binding to both neutrophils and cytoplasts was confirmed by indirect immunofluorescence microscopy. Whereas on the intact cells the C5a–receptor complexes showed in general a polar distribution, on the cytoplast surface, these complexes were diffused as indicated by the distribution of fluorescence.

DISCUSSION

The present availability of a highly sensitive indicator of intracellular free Ca^{2+}, which can be incorporated into small cells (14–16), permits one to demonstrate the effects of extracellular ligands on cytosolic Ca^{2+} concentration, for which there was previously only indirect evidence (11–13).

By using this experimental approach, we have here inves-

Table 1. Functional responses of intact neutrophils and of neutrophil cytoplasts to various stimulants at high and low extracellular Ca^{2+}

	Intact neutrophils				Cytoplasts	
	$B_{12}BP$ secretion,* % total cell content		O_2^- production, nmol/10 min per 10^6 cells		O_2^- production, nmol/10 min per 10^6 cytoplasts	
Stimulant	Without EGTA	With EGTA	Without EGTA	With EGTA	Without EGTA	With EGTA
0.35 μM C5a	26.5 ± 1.6	2.5 ± 1.5	7.7 ± 1.6	3.2† ± 0.9	<0.5	<0.5
1 μM ionomycin	41.3 ± 6.6	5.3 ± 1.7	7.1 ± 2.0	1.0‡ ± 0.6	2.1 ± 0.8	<0.5
30 nM PMA	43.8 ± 7.9	35.8 ± 4.5	85.7 ± 8.5	88.3 ± 1.4	25.3 ± 5.0	28.4 ± 6.4

Data are means ± SEM of at least three experiments carried out with different neutrophil or cytoplast preparations and are expressed as differences with respect to values of untreated samples. Neutrophils or cytoplasts were incubated for 60 min in Hepes medium with 25 μM or 10 μM quin2/AM, respectively. Exposure to the stimulants was in 1 mM $CaCl_2$ without or with 5 mM EGTA (≈20 nM Ca^{2+}). Functional responses of quin2-loaded neutrophils or cytoplasts in 1 mM Ca^{2+} were very similar to those of untreated samples. An exception was the C5a-elicited $B_{12}BP$ release, which with quin2-loaded cells was about 1.5-fold higher than with untreated neutrophils. In the presence of EGTA, the quin2 load generated a further small depression of response to C5a and ionomycin.

*The total cell content of $B_{12}BP$ was 1.9 ± 0.1 ng per 10^6 neutrophils (mean ± SEM of eight cell preparations) and was not affected by quin2 loading. Release of cytoplasmic lactate dehydrogenase above control (<4%), either in the absence or in the presence of EGTA, was 1.7 ± 0.2% with ionomycin and 1.6 ± 0.4% with PMA; C5a did not induce any release of the cytoplasmic enzyme.

†$P < 0.01$ (EGTA present vs. EGTA absent; Student's t test for paired data).

‡$P < 0.025$ (EGTA present vs. EGTA absent; Student's t test for paired data).

tigated the correlation between cytosolic Ca^{2+} concentration and activation of neutrophil responses under conditions that mimic as closely as possible the physiological activation of neutrophils. In particular, to activate neutrophil chemotaxis, secretion, and O_2^- production, we have utilized the physiologic stimulus provided by the complement-derived polypeptide C5a (1, 3, 4, 11, 27, 28). Furthermore, secretion of granule $B_{12}BP$ was assayed in the absence of cytochalasin B, the agent commonly used to enhance neutrophil secretion of granule contents (27).

We have seen that C5a at 35 pM to 1.2 μM elevates the cytosolic Ca^{2+} concentration of neutrophils from about 80 nM to 300–400 nM. This is consistent with similar results obtained with the chemotactic peptide fMet-Leu-Phe in human and rabbit neutrophils (17, 18).

The C5a-dependent increase in intracellular free Ca^{2+} mainly relies on the stimulation of Ca^{2+} inward diffusion, although it also involves mobilization of Ca^{2+} from intracellular stores. The rapid increase in cytosolic Ca^{2+} levels is not a transient response to C5a–receptor interaction but persists for several min. In fact, upon attainment of maximal values in 2–4 min, there is a slow decrease of free calcium in the cytosol down to resting values. This could be due to internalization of C5a–receptor complex or to activation of the peripheral Ca^{2+} pump (33), or to both.

Nucleus- and cytoplasmic granule-depleted neutrophils, with a normal exposure of surface antigens and receptors (19), do not accumulate Ca^{2+} when interacting with C5a.

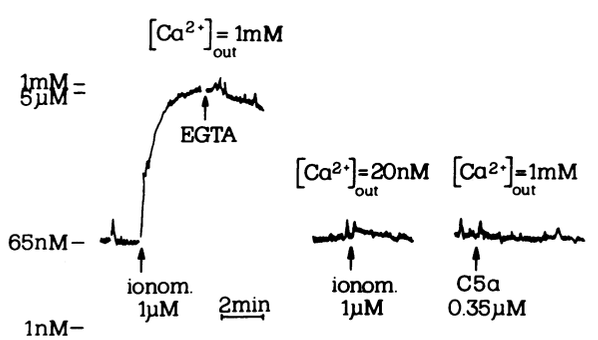

FIG. 3. Effect of ionomycin and C5a on cytosolic Ca^{2+} concentration of cytoplasts (1.3×10^7 per ml of Hepes medium). The three experiments were carried out with aliquots of the same batch of quin2-loaded cytoplasts. Internal quin2 concentration was 0.36 mM, if one assumes a water content of 0.2 μl per 10^6 cytoplasts (based on the recovery of lactate dehydrogenase activity with respect to intact neutrophils); ≈20 nM external Ca^{2+} was obtained as in Fig. 1.

This might be due to lack of formation of clusters of C5a/receptor complexes, as indicated by a comparative analysis with intact neutrophils carried out by immunofluorescence microscopy. However, alternative explanations also should be considered, such as loss from cytoplasts of a factor(s) or mechanism(s) critical for opening the Ca^{2+} channels. One of these mechanisms could simply be Ca^{2+} release from intracellular binding sites, present in intact cells but not in cytoplasts.

The results obtained with C5a and ionomycin confirm previous assumptions that chemotaxis, secretion of granule content, and activation of O_2^- production may be associated and possibly preceded by an increase in cytosolic Ca^{2+} concentration. In fact, when influx is minimized by applying the stimulants in the presence of low extracellular Ca^{2+}, the rise in cytosolic Ca^{2+} is contained (<200 nM) and activation of cell functions is barely expressed. However, when these apparent threshold levels of cytosolic Ca^{2+} are exceeded, there is no proportionality between Ca^{2+} increase and the extent of cell activation. For example, ionomycin brings cytosolic Ca^{2+} above 1 μM, yet it is a weak stimulant of O_2^- production. Furthermore, there are conditions (<1 nM C5a) in which the apparent threshold levels of cytosolic Ca^{2+} are exceeded, and yet there is stimulation of chemotaxis but not of secretion and oxidative metabolism. Thus, it appears that in neutrophils the intracellular Ca^{2+} signal requires a concomitant activation of other biochemical pathways, which might include protein phosphorylation and lipid turnover (5), to modulate the various cell functions (18).

Among the stimuli here used, PMA stands out for not inducing any change in intracellular quin2 fluorescence in either intact neutrophils or cytoplasts. This is in contrast with a previous demonstration of increased rates of cellular Ca^{2+}–medium $^{45}Ca^{2+}$ exchange and of active Ca^{2+} extrusion from PMA-treated cells (13). One possible explanation of this apparent discrepancy is that PMA induces a displacement of Ca^{2+} from binding sites localized at the inner face of the plasma membrane with an immediate and efficient removal of the cation, possibly by an activated Ca^{2+} pump (34). Ca^{2+} may diffuse rather slowly within the cytoplasm because it has many sites where it can bind. Thus, a tight coupling between Ca^{2+} mobilization, confined to the cortical cytoplasm, and extrusion of the cation, catalyzed by the surface pump, could result in no net increase of average cytosolic Ca^{2+} concentration. This mechanism is also consistent with the inhibition by PMA of the C5a-dependent Ca^{2+} influx.

The activation of Ca^{2+}-dependent pathways by phorbol esters without an increased level of average cytosolic Ca^{2+}

appears to be a general effect of these compounds on a number of cell types (15, 16). PMA has been shown to directly activate the phospholipid-dependent, Ca^{2+}-activated protein kinase C (35), an ubiquitous enzyme also present at high levels in neutrophils (36). In intact neutrophils PMA induces a marked stimulation of phosphorylation of four polypeptides (5, 37), of which at least the most heavily phosphorylated (apparent M_r, 47,000) is associated with the cytoplasts (unpublished results). Phosphorylation of specific proteins, which is also induced by the neutrophil-activating peptide fMet-Leu-Phe in a concentration-dependent manner (37), could thus provide a mechanism of sensitivity modulation of neutrophil response to a Ca^{2+} signal, permitting gradual activation of different cell functions at increasing concentrations of extracellular stimulant.

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