Calcium Movement and Membrane Potential Changes in the Early Phase of Neutrophil Activation by Phorbol Myristate Acetate: A Study with Ion-selective Electrodes

CRISTINA MOTTOLA and DOMENICO ROMEO
Istituto di Chimica Biologica, Università di Trieste, 34127 Trieste, Italy

ABSTRACT To quantitate calcium movements and membrane potential changes in stimulated neutrophils, we have measured net fluxes of Ca^{2+} and of the lipophilic cation tetraphenyl phosphonium by a very sensitive ion-selective electrode system. Activation of neutrophils by 3×10^{-8} M phorbol 12-myristate, 13-acetate induces a release of ~20% of total cell calcium, with an initial lag period of <10 s. The Ca^{2+} outflux is markedly reduced in ATP-depleted cells and in the presence of a calmodulin inhibitor, thereby suggesting that it occurs by activation of the ATP-driven Ca^{2+} pump of the neutrophil plasmalemma. Activation of neutrophils also induces a transiently increased exchange of medium ^{45}Ca with cell calcium, which is measurable a few seconds after cell exposure to the stimulant and peaks at ~40 s. Stimulation of neutrophils after attainment of steady-state accumulation of tetraphenyl phosphonium (resting potential of −67 mV) results in a marked depolarization, with a lag period of ~60 s. The rate and extent of depolarization are reduced by 40 and 65%, respectively, in a low Na^+ medium but are not modified by an inhibitor of anion exchange across membranes. A high-K^+ medium depolarizes neutrophils without either modifying their resting oxidative metabolism or impairing stimulability by the phorbol ester. Phorbol 12-myristate, which also exhibits no effect on the oxidative metabolism of neutrophils, does not induce Ca^{2+} extrusion and membrane potential changes. The causal relationship between Ca^{2+} mobilization, membrane potential changes and activation of neutrophil functions is discussed.

At least with regard to the ion-dependent mechanisms of control of cell functions, the classic distinction between excitable and nonexcitable cells appears to be less categorical than in the past. In fact, evidence has been provided that several cell types different from neurons and myocytes, when subjected to some stimuli, undergo physical and biochemical changes characteristic for electrically excitable cells (1). One such cell is the neutrophil, for which a change both in the steady-state levels of cytosolic Ca^{2+} (2–7) and in transmembrane potential (8–10) has been suggested to be involved in the early triggering of activation of its response program (chemotaxis, secretion, "respiratory burst") by either particulate or soluble surface-reacting stimuli.

Experimental support for the role of Ca^{2+} is in part derived from work with the Ca^{2+}-ionophore A23187, which stimulates neutrophil oxidative metabolism (3) and secretion (4, 5), and from studies demonstrating that an intracellular Ca^{2+} antagonist can inhibit both the "respiratory burst" and secretion by neutrophils (11, 12).

Actual movements of Ca^{2+} from and/or to extracellular and membrane-associated pools have never been directly measured in neutrophils. However, several laboratories have reported that ^{45}Ca redistribution within the cell or between cells and their environment changes upon stimulation (2, 5–7, 13, 14). In addition, use of the fluorescent probe chlortetracycline has also provided some indication that mobilization of intramembranous Ca^{2+} occurs in stimulated neutrophils (15, 16). Both experimental approaches may, however, suffer from some limitations. Changes in the rate of calcium fluxes, as measured with an isotopic technique, may be artifactual and derive from $^{45}Ca/^{40}Ca$ exchange either as a primary effect or as a secondary consequence of cell stimulation (7, 13, 14). Furthermore, quantitative measurements by chlortetracycline of Ca^{2+} shifts from membranes are hampered by the lack of a suitable calibration and by the fact that chlortetracycline acts as a probe not only of membrane-bound cations but also of the physicochemical state of membranes (17).

We thought that a better approach for evaluating calcium

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pool redistribution was that of measuring *net* fluxes of Ca^{2+} into or from stimulated neutrophils by means of a very sensitive ion-selective electrode, which can monitor very small variations in the extracellular ion concentration above or below the initial background value (18, 19). Mobilization of Ca^{2+} from intracellular stores with concomitant increase in cytosolic free-ion concentration should activate the pump system, which in some cell types as well as in the neutrophil (19) is present at the cell surface. Thus, measurement of the net fluxes of Ca^{2+} into or out of stimulated cells may represent a means of both evaluating the direction of Ca^{2+} movement and precisely quantifying the amount of cation that has been removed from internal pools and expelled from the cell.

In the course of this investigation we have also applied the ion-selective electrode technique to the measurement of the movement of a lipophilic cation, tetraphenyl phosphonium (TPP^+), which can be employed as a probe of transmembrane potential (20, 21). The use of a TPP^+-selective electrode has several advantages over other methods previously adopted to detect membrane potential changes in stimulated neutrophils (8–10, 22, 23). First, it permits continuous recordings of the potential changes and calculation of $\Delta\Psi$ by the Nernst equation. Second, TPP^+ crosses membranes much more easily than the analogue probe triphenylmethyl phosphonium (21), which has been commonly used in this type of studies (8, 10, 23), thus permitting faster measurements of potential changes. Third, TPP^+ is also likely to be superior to the potential-sensitive fluorescent cyanine dyes (9, 10, 22), which by themselves can induce an increased cell membrane permeability to K^+ and membrane hyperpolarization (24), and whose fluorescence properties are modified by reactive O_2 derivatives produced by stimulated neutrophils (9).

MATERIALS AND METHODS

Cells

Neutrophils were isolated from fresh bovine blood as previously reported (25). They were maintained at room temperature in a medium containing 20 mM Tris-HCl, pH 7.4, 122 mM NaCl, 5 mM KCl, and 0.2 mM $MgCl_2$ (Na/K/Tris medium).

The plasma membrane fraction of neutrophils was purified as described elsewhere (25).

Analytical Techniques

Oxygen consumption by neutrophils was measured at 37°C by a Clark-type electrode (3).

Ca^{2+} net fluxes were followed by measuring changes in extracellular Ca^{2+} concentrations with the help of a very sensitive Ca^{2+}-selective electrode (W. Möller, Zurich)/amplifier/recorder system, as previously described (18, 19). The ion-selective membrane of the electrode contains a neutral carrier for Ca^{2+} (26) and has a contact surface with the medium of 3 mm^2. The response time of the system is <1 s. The assays were carried out in a thermostated (37°C) vessel containing 3 ml of Na/K/Tris medium with 0.1 mM $CaCl_2$ and 5 mM glucose, under continuous stirring. Upon equilibration of the electrode system, an aliquot (0.2–0.5 ml) of the medium was withdrawn and replaced by a suspension of 1×10^8 neutrophils in the same medium. Changes in the external Ca^{2+} concentration were quantified by calibration with EGTA at the end of each experiment (18, 19).

Changes in extracellular TPP^+ concentrations were measured at 37°C with the same electrode/amplifier/recorder system, in which the Ca^{2+}-selective membrane was replaced by a membrane responding preferentially to large onium ions (27) (a generous gift of W. Simon, ETH, Zürich, Switzerland). The difference between the emf measured by the TPP^+ electrode/reference electrode system in normal Na^+ medium and that in high K^+ medium, in combination with calibration values obtained with known amounts of TPP^+ at the end of each experiment, was used to calculate the uptake of TPP^+ specifically due to the membrane potential. The kinetics of response of the electrode to changes in transmembrane potential was tested with rat liver mitochondria. Addition of oxidizable substrates to mitochondria caused an immediate (<1 s) electrode response that was completed within 15–30 s.

Movements of Ca^{2+} between neutrophils and the incubation medium were also measured by a $^{45}Ca^{2+}$-labeling technique. Cell suspensions, containing 1×10^8 neutrophils in 2 ml of Na/K/Tris medium with 0.1 mM $CaCl_2$, were stirred magnetically in a thermostatically controlled (37°C) vessel. 5 μCi of $^{45}Ca^{2+}$ and the cell stimulant (see below) were added after 5 and 8 min of cell equilibration, respectively, at 37°C. 1 min before the addition of the stimulant and at 10-s intervals thereafter, 100-μl samples of the neutrophil suspension were placed in ice-chilled 0.45-ml polyethylene centrifuge tubes containing 200 μl of a low-viscosity, apolar phase (xylene + *o*-dichlorobenzene, $d = 1.050$ g/ml) layered on top of 50 μl of 1 mM lanthanum chloride in 15% (wt/wt) sucrose ($d = 1.060$ g/ml); lanthanum was included in the cell-collecting phase to displace $^{45}Ca^{2+}$ from cell surface binding sites and to inhibit calcium movement across the surface membrane (6). Each sample was immediately centrifuged at 10,000 *g* for 10 s (we used two Eppendorf centrifuges, model 3200). The whole supernatant was sucked off, the tip of the tube was severed with a lancet, and the cell pellet was solubilized in 90% formic acid. The radioactivity of these solutions was measured by liquid scintillation in 5 ml of Aqualuma (Lumac Systems, Basel, Switzerland) and converted into actual values of cell-bound ^{45}Ca by calibration with known amounts of $^{45}CaCl_2$, counted under the same conditions.

Total cell calcium was determined by atomic absorption spectrophotometry (19).

ATP was assayed fluorimetrically in K_2CO_3-neutralized $HClO_4$ extracts of $2–8 \times 10^6$ cells with glucose-6-phosphate dehydrogenase and hexokinase (28).

The activity of plasma membrane Ca^{2+}-dependent ATPase was determined as previously described (29).

Stimulants

The phorbol esters 12-myristate,13-acetate (PMA) and 12-myristate (PM) were dissolved in dimethyl sulfoxide at a concentration of 4 mg/ml. Working solutions were prepared daily from the stock solutions, kept frozen at −20°C.

RESULTS

Enhancement in Cell Respiration as an Assay of Neutrophil Activation

PMA is a lipophilic compound that induces a marked activation of oxidative metabolism and exocytosis of secondary granules in human neutrophils (9, 12, 30, 31). Further, PMA enhances the chemotactic response and exocytosis of primary granules in neutrophils exposed to either chemotaxins or secretagogues (32, 33). This drug thus provides a useful tool for investigating molecular events underlying the activation of the neutrophil functions.

From among the various functions of neutrophils that are modulated by PMA, we chose to assay cell respiration. This is a convenient bioassay, which permits a rapid and sensitive evaluation of neutrophil response to changes in the extracellular milieu. Fig. 1 shows that PMA, at a concentration of $0.6–6 \times 10^{-8}$ M, causes a rapid and marked stimulation of oxygen consumption by bovine neutrophils. The effects of the stimulant are saturable and exhibit stereospecificity. In fact, PM, an

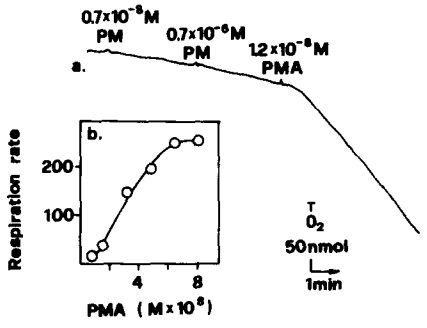

FIGURE 1 Effect of phorbol 12-myristate (*PM*) and phorbol 12-myristate, 13-acetate (*PMA*) on the respiration of neutrophils. Oxygen uptake by 6×10^7 neutrophils was measured at 37°C in Na/K/Tris medium, supplemented with 5 mM glucose and 0.1 mM $CaCl_2$ (1.8 ml). (*a*) Kinetics of respiratory activation. (*b*) Maximal rates of respiration (nmol of O_2/min) at different PMA concentrations.

analogue of PMA with an unesterified —OH in position 13, even at a concentration of 7×10^{-7} M neither stimulates neutrophil respiration nor prevents the respiratory activation promoted by PMA.

Stimulation of neutrophil oxidative metabolism by PMA is virtually independent of extracellular Ca^{2+} (Table I). Furthermore, increase of K^+ concentration from 5–100 mM, with a parallel decrease in Na^+ concentration, or substitution of 95% Na^+ by choline$^+$, has essentially no effect on either resting or stimulated respiration of neutrophils (Table I).

The latter observation is in contrast with other reports (23, 34) indicating that Na^+ is required for the activation of neutrophil oxidative metabolism. It must be emphasized, however, that the Na^+ requirement has been shown by measuring the extracellular recovery of O_2^-, the main product of O_2 reduction in stimulated neutrophils (35). The possibility thus exists that Na^+ is required either for the export of O_2^- or for the full efficiency of the redox reaction used for the extracellular detection of the radical.

Ca^{2+} Efflux from Stimulated Neutrophils

When neutrophils are exposed to 3×10^{-8} M PMA in the presence of 0.1 mM extracellular Ca^{2+}, an immediate release of Ca^{2+} from the cells is detected (Fig. 2). After an initial lag period of <10 s, the cation is transferred to the medium at a rate of 67 ± 9 pmol/s/10^8 cells, the extent of the release being 14.6 ± 2.3 nmol/10^8 cells (means of eight experiments ± SEM).

TABLE I

Activation of Neutrophil Respiration by PMA in Media with Different Ionic Compositions

	Media			
	a	*b*	*c*	*d*
Na^+, mM	122	122	27	7
K^+, mM	5	5	100	5
Ca^{2+}, mM	0.1	—	0.1	0.1
Choline, mM	—	—	—	115
Resting respiration	<4	<4	<4	<4
Activated respiration	102 ± 32	94 ± 9	110 ± 26	107 ± 25

Respiration of 6×10^7 neutrophils in Na/K/Tris medium with different ionic compositions was measured as indicated in Fig. 1. Values are maximal rates of oxygen consumption (nmol of O_2/min) before and after addition of 3×10^{-8} M PMA and are means of four determinations ± SEM.

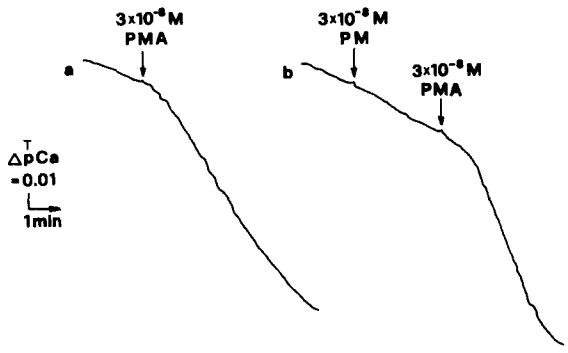

FIGURE 2 Ca^{2+} extrusion from neutrophils (1×10^8) exposed to PMA. Determinations of Ca^{2+} net fluxes were carried out at 37°C in Na/K/Tris medium (3 ml), containing 0.1 mM $CaCl_2$ and 5 mM glucose. Downward deflections in the traces indicate a rise in calcium concentration in the medium. The electrode response was calibrated by the addition of EGTA at the end of each assay.

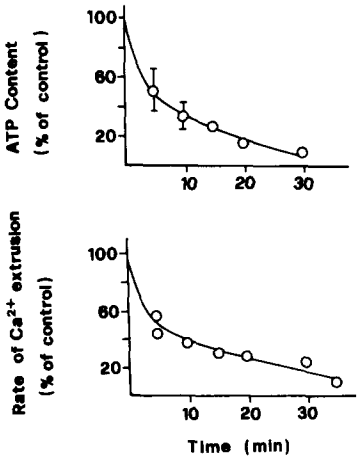

FIGURE 3 Effect of 2-deoxy-glucose on ATP content and Ca^{2+} extrusion by PMA-treated neutrophils. Cells were incubated with 20 mM 2-deoxy-glucose at 37°C and at various time intervals were assayed for ATP and capacity of extruding Ca^{2+} as a response to surface stimulation by 3×10^{-6} M PMA. The points (means of three experiments ± SEM) are % with respect to controls incubated in parallel with 20 mM glucose.

The latter value corresponds to ~20% of total cell calcium, as determined by atomic absorption spectrophotometry. Unlike PMA, PM does not induce any significant Ca^{2+} release nor does it affect the activity of PMA (Fig. 2).

Release of Ca^{2+} from neutrophils might occur through dissociation of the cation from either cell surface binding sites or exocytosed granules as well as by activation of the ATP-driven, Ca^{2+} extrusion pump that is present at the neutrophil surface (19). The second possibility can easily be ruled out. In fact, exocytosis of specific granules from neutrophils is hardly detectable in the 5 min after addition of PMA and proceeds thereafter up to 30–60 min (30, 33). Conversely, extrusion of Ca^{2+} from neutrophils is an immediate response to cell activation by PMA and is over within 4–6 min.

To select between the other two alternatives, we decreased the ATP/ADP ratio in neutrophils by means of the glycolytic inhibitor 2-deoxyglucose and studied the effect of this change of cell metabolic state on the PMA-induced Ca^{2+} release. As shown in Fig. 3, a gradual decrease in the neutrophil ATP content is accompanied by a decline in the rate of Ca^{2+} release from the cells.

Further evidence that treatment of neutrophils with PMA activates a pump derives from investigations carried out with the calmodulin inhibitor trifluoperazine (36). This drug, which is known to depress the activity of calmodulin-dependent Ca^{2+} pump systems (37), also inhibits the PMA-induced Ca^{2+} extrusion from neutrophils with an I_{50} of 35 μM.

A further step towards the elucidation of the type of interaction between the neutrophil stimulant and the Ca^{2+} pump was accomplished by studying the effect of PMA on the high affinity Ca^{2+}-activated ATPase of isolated neutrophil plasma membrane. This phosphohydrolase is very likely the enzyme manifestation of the Ca^{2+} pump, as indicated by cogent experimental evidence (19). The presence of 4×10^{-8}–1×10^{-7} M PMA in the Ca^{2+}-ATPase assay does not cause any activation of the enzyme but, on the contrary, results in a slight (5–30%) inhibition of it.

Another possibility would be that the large efflux of Ca^{2+} from the cells observed after the interaction of PMA and neutrophils is consequent to a mobilization of Ca^{2+} from intracellular stores with a concomitant activation of the pump (19). The likelihood of this mechanism, which includes an increase of the cell Ca^{2+} exchangeable pools, was tested by investigating the movement of isotopic calcium between neutrophils and the extracellular medium. As shown in Fig. 4, exposure of neutrophils to PMA, but not to either PM or the solvent dimethyl sulfoxide, causes an increased association of

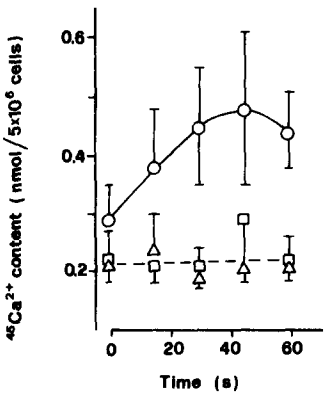

FIGURE 4 Kinetics of ^{45}Ca movement between medium and neutrophils. After 3-min equilibration at 37°C with ^{45}Ca, cells were exposed at t_0 to either 3×10^{-8} M PMA (○) or 3×10^{-8} M PM (Δ) or 0.05% (vol/vol) dimethyl sulfoxide (□). At the times corresponding to the points, samples of 5×10^6 cells were withdrawn, immediately (<10 s) separated from extracellular ^{45}Ca by centrifugation through a discontinuous density and dielectric constant gradient, solubilized, and counted. The curves represent the kinetic profile obtained in seven individual experiments (means ± SEM).

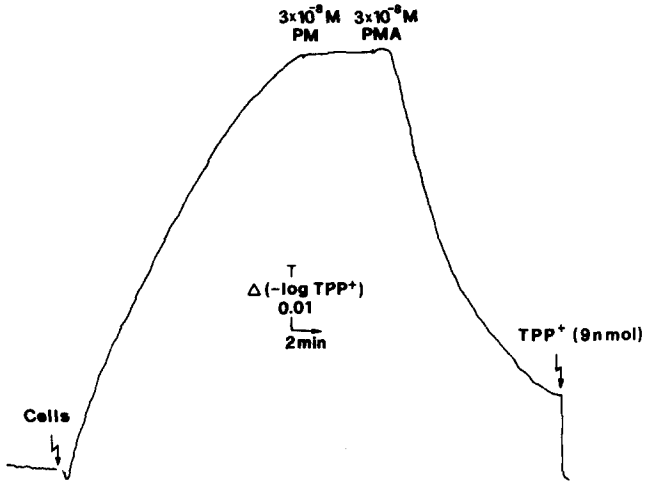

FIGURE 5 Representative experiment of TPP^+ distribution between neutrophils and medium, under either resting conditions or stimulation by PMA. 1×10^8 cells were added at 37°C to Na/K/Tris medium (3 ml), supplemented with 0.1 mM $CaCl_2$, 5 mM glucose, and 0.1 mM TPP^+. Upward deflections in the trace indicate uptake of TPP^+ by the cells (see calibration at the end of the experiment).

$^{45}Ca^{2+}$ to the cells. This shift of isotopic calcium from the medium to the neutrophils is already detectable a few seconds after addition of the stimulant to the cells, and it peaks at ~40 s.

Changes in Transmembrane Potential

TPP^+ is a lipophilic ion with the propensity for charge delocalization that allows passive equilibration with $\Delta\Psi$ (20, 21). Neutrophils suspended in a medium containing physiological concentrations of Na^+ and K^+ take up TPP^+ rapidly and achieve a steady-state level of accumulation in ~16 min (Fig. 5). Replacement of 95% Na^+ with choline$^+$ slightly decreases the time required for attainment of steady-state TPP^+ uptake but does not modify the amount of cation accumulated (Table II). Conversely, replacement of a large fraction of Na^+ with K^+ (i.e., 28 mM Na^+/100 mM K^+) depresses the steady-state level of TPP^+ accumulation by 90–100%, thereby indicating that the resting potential is almost entirely dependent on the K^+ gradient across the cell membranes.

By using an intracellular volume for neutrophils of 0.35 μl per million cells (38), it is possible to calculate the ratio TPP^+_{in}/TPP^+_{out}.

Insertion of this ratio into the Nernst equation gives −67 ± 3 mV for the apparent $\Delta\Psi$ of resting neutrophils, a value that is very close to that reported for other animal cells (1, 21).

Previous measurements of the direction of changes of resting membrane potential consequent to neutrophil stimulation has furnished contrasting results (8, 10, 22, 23), thereby precluding the possibility of identifying the ion species involved. The procedure here adopted for the continuous monitoring of membrane potential clearly shows that stimulation of neutrophils by PMA, but not PM, induces a loss of TPP^+ from the cells (Fig. 5). The time of onset of PMA-induced TPP^+ redistribution is about 60 s and the apparent depolarization is 49 mV (Table II).

In choline$^+$ medium, PMA still depolarizes the neutrophil membranes. The TPP^+ efflux, however, proceeds at a significantly slower rate and the final cation loss is ~65% lower than in normal medium (Table II). Conversely, addition to the cells of 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonate, a powerful inhibitor of the exchange of anions across the membranes of various cells, including the neutrophils (39, 40), affects neither the rate nor the extent of the PMA-induced depolarization.

DISCUSSION

An accumulating body of evidence indicates that an increase of free cytoplasmic Ca^{2+} is involved in the early triggering of the responses of neutrophils to surface stimulation by both particulate and soluble agents (2–7, 11, 12, 15, 16). This mechanism of neutrophil activation finds further support in the experiments described here. In fact, PMA, which activates a complex array of neutrophil functions, including O_2 reduction to superoxide anion, locomotion, and secretion (9, 12, 30–33), causes a very rapid mobilization of cell calcium. The effects of PMA are elicited at low concentrations and exhibit stereospecificity. Indeed, the PMA analogue phorbol 12-myristate neither activates neutrophil respiration nor induces Ca^{2+} mobilization.

Our experiments indicate that, immediately after interaction between PMA and the neutrophil surface, Ca^{2+} is released from an exchangeable pool into the cytoplasm, whence it is

TABLE II

Steady-state Distribution of TPP$^+$ between Medium and Neutrophils

	Na^+ medium	Choline$^+$ medium	*P*
Resting cells:			
Time required for steady-state TPP^+ distribution, *min*	16.7 ± 2.0	12.2 ± 0.8	<0.025
Total TPP^+ uptake, *nmol/10^8 cells*	38.3 ± 3.5	37.3 ± 3.8	NS
Apparent $\Delta\Psi$, *mV*	−67 ± 3	−66 ± 3	NS
After stimulation by PMA:			
Time of onset of TPP^+ loss, *s*	68 ± 7	58 ± 3	NS
Initial rate of TPP^+ loss, *nmol/min*	4.8 ± 0.7	2.9 ± 0.6	<0.005
Total TPP^+ loss, *nmol/10^8 cells*	31.0 ± 3.0	15.4 ± 1.7	<0.005
Apparent depolarization, *mV*	49 ± 6	17 ± 2	<0.005

Means of six experiments ± SEM. Na^+ and choline$^+$ media had the composition indicated in columns *a* and *d* of Table I, respectively. Statistical analysis was performed by the Student's *t* test (paired data); NS, not significant.

extruded by the high-affinity, ATP-dependent Ca^{2+} pump of the neutrophil plasma membrane (19). The technique used to monitor the Ca^{2+} movements permits us to answer the question of what percent of the total calcium is mobilized and subsequently expelled from the cells. From the end-point determinations of Ca^{2+} outfluxes (14.6 ± 2.3 nmol/10^8 cells), we calculate that ~20% of cell Ca^{2+} is released. It appears to be more difficult to make a precise determination of the fraction of this amount that is mobilized in the very early phase of cell activation, thereby providing the initial trigger of neutrophil activation. However, the isotopic technique employed for studying Ca^{2+} movements has shown that 15 s after PMA addition to neutrophils ~2 nmol of extracellular ^{45}Ca become associated with the cells. This transfer of ^{45}Ca from the medium to the neutrophils is not due to actual influx of the cation, because by the electrode technique only a net Ca^{2+} outflux is seen. It is thus likely that it represents an exchange of the isotope with a readily exchangeable pool of cellular Ca^{2+}.

If one assumes that the exchanged calcium is a considerable fraction of the trigger pool that is transferred from the cell Ca^{2+} stores to the cytosol, a rough estimation of the transient increase in the cytosolic free-Ca^{2+} concentration in the first 15 s of cell activation becomes possible. In fact, assuming an average volume of 0.35 μl/10^6 neutrophils (38), the cytoplasmic Ca^{2+} concentration would rise from 10^{-7}–10^{-8} M (41) to 5×10^{-5} M. This enhancement in cytosolic Ca^{2+} is sufficient to activate most Ca^{2+}-sensitive cellular processes, including the peripheral Ca^{2+} pump, and, in fact, appears excessively high. The increment is only transitory, however, and the calculations have assumed that the readily exchangeable Ca^{2+} is roughly equivalent to the mobilized cation and no equilibration with the granule and nuclear space was considered.

The concept that neutrophil stimulants induce a displacement of calcium from cell membranous compartments, as outlined here and elsewhere (3, 6, 15, 16, 19), leaves the identification of the Ca^{2+} stores still an open question. However, the rapid kinetics of medium ^{45}Ca/cell calcium exchange suggests that the trigger pool of Ca^{2+} could be localized in the plasma membrane. This conclusion is consistent with the observation that a rapid activation of neutrophil respiration can be elicited by surface-reacting, Ca^{2+}-displacing lanthanum ions (3). Furthermore, surface stimulation of neutrophils by concanavalin A bound to Sepharose beads (42) has been shown to cause a rapid Ca^{2+} displacement from the area of contact between the ligand-coated bead and the receptor-containing surface of the cell (43). Phagocytic stimuli also induce localized loss of calcium from the plasma membrane (43). Finally, a deposition of cations, probably containing calcium, has been observed on the cytoplasmic side of the plasma membrane of neutrophils stimulated by a chemoattractant and undergoing either chemokinesis or chemotaxis (44).

In this view, the plasma membrane of neutrophils would exhibit a threefold function in the regulation of the Ca^{2+}-dependent cellular processes. In fact, (*a*) it is the site of the primary interaction between the cell and the extracellular stimulatory ligands; (*b*) it probably releases the triggering pool of Ca^{2+} as a consequence of this interaction; (*c*) it affords a rapid and efficient control of cytosolic Ca^{2+} by means of the pump system, thus permitting modulation of the stimulus.

When the Ca^{2+}-dependent trigger of cell functions is discussed, much emphasis is usually placed on the transient elevation of cytosolic Ca^{2+} that results from the cation mobilization from membranous stores. However, an integrated view of the problem suggests that displacement of Ca^{2+} per se may also be of some importance for the transduction of the stimulatory signal. Removal of Ca^{2+} from cell membranes can induce a decreased constraint on either enzyme or substrate diffusion (45, 46), thereby providing a regulatory system of enzyme activity. Furthermore, asymmetric displacement of Ca^{2+} from membrane-binding sites can cause instability of the membrane (47), thus favoring a new steady-state molecular organization of the membrane and/or fusion with other membranes. It is interesting that several years ago Woodin and Wieneke (48) proposed that secretion by neutrophils is elicited by displacement of membrane-associated Ca^{2+} and ATP. In this view, the activation of the ATP-consuming, plasmalemmal Ca^{2+} extrusion system in PMA-stimulated neutrophils would also serve to prepare the cell for the secretory act.

Displacement of Ca^{2+} from the surface membrane of neutrophils could also be partially responsible for an increased permeability of the membrane to other ions and thus to a change in transmembrane diffusion potential. Both hyperpolarization (8, 22, 23) and depolarization (8–10, 22, 23) of cell membranes have actually been observed in neutrophils exposed to various stimuli. With our technique of continuous $\Delta\Psi$ monitoring, we have found that PMA elicits a depolarization of neutrophil membranes, which is measurable ~1 min after stimulation and is only partially dependent on extracellular Na^+. However, in contrast with previous suggestions (8–10), we think that a change in $\Delta\Psi$ is unlikely to be involved in the initial triggering process leading to activation of neutrophil oxidative metabolism. In fact, K^+-depolarized cells exhibit a resting respiration and are normally stimulated by PMA. Furthermore, valinomycin and nigericin, which produce hyperpolarizing effects by catalyzing a K^+ efflux, cause a very moderate metabolic stimulation of neutrophils and only at rather high concentrations (3).

The marked decrease in transmembrane potential might be involved, however, in the activation or potentiation of other neutrophil responses, such as orientation and increased frequency of locomotion. In fact, depolarization of stimulated neutrophils is in part dependent on the extracellular concentration of Na^+, and removal of Na^+ from the extracellular medium greatly decreases or even abolishes the ability of a chemotactic peptide to stimulate neutrophil movement (49). Alternatively, the collapse of membrane potential may simply be the result of a marked perturbation of the plasma membrane, without any special implication in cell activation (1). Accordingly, failure of chronic granulomatous disease neutrophils, which have a deficient "respiratory burst," to show stimulant-dependent depolarization (9, 10) could be explained by the absence of a significant membrane perturbation. It is interesting that in these genetically deficient cells the Ca^{2+}-ionophore A23187, unlike other stimulants, induces a membrane depolarization the magnitude of which is at least 50% of the response of normal cells (10). This is consistent with our suggestion that a rise in Ca^{2+} concentration in the cortical cytoplasm may be at least in part responsible for the decrease in $\Delta\Psi$.

In conclusion, we have been able to show that displacement of Ca^{2+} from intracellular stores, leading to a transient elevation in cytosolic Ca^{2+} and activation of the plasmalemmal Ca^{2+} extrusion pump, is one of the earliest responses of neutrophils to surface stimulation. Increase in free concentration of calcium in the cytosol, and also probably removal of the cation from some specific sites of cell membranes, may activate a number of cell processes, such as reduction of O_2 to superoxide anion.

Depolarization of neutrophil membranes does not appear to provide the trigger for stimulation of this metabolic pathway but may be related to the activation of other, as yet unidentified, cell processes.

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