The Effects of Phorbol Myristate Acetate and Chemotactic Peptide on Transmembrane Potentials and Cytosolic Free Calcium in Mature Granulocytes Evolve Sequentially as the Cells Differentiate*

(Received for publication, April 7, 1986, and in revised form, September 8, 1986)

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We isolated myeloid precursors from human marrow and studied the effects of phorbol myristate acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) upon transmembrane potentials and cytosolic calcium ([Ca2+]i) as the cells matured. Using a panel of fluorescent probes, we found that membrane depolarization induced by PMA and fMLP in granulocytes, and elevation in [Ca2+]i, stimulated by fMLP, were absent in myeloblasts. When we induced differentiation with granulocyte-macrophage colony-stimulating factors, we found that both ionic responses appeared at approximately the promyelocyte stage. By using di-O-C5(3), we detected an initial phase of fMLP-induced hyperpolarization which appeared ontogenetically earlier than depolarization and which could be evoked in mature granulocytes with lower concentrations of the ligand. Hyperpolarization was partially dependent on extracellular Na+, was abrogated by increasing the external K+ concentration, and was accompanied by mild acidification of the cytoplasm. Bordetella pertussis toxin abolished both hyperpolarization and depolarization. Our findings indicate that shifts in [Ca2+]i and membrane potential changes in response to PMA and fMLP evolve as granulocytes mature. In addition, transmembrane ionic fluxes induced by fMLP appear to be more complex than previously considered, involving at least two separable phases of membrane potential change.

The tumor-promoting phorbol ester PMA† and the chemotactic peptide fMLP§ are membrane-active agonists whose ability to induce rapid changes in the transmembrane electrical potential of mature granulocytes has been well documented (1–4). In addition, fMLP stimulates a brisk rise in free cytosolic calcium ([Ca2+]i) in these cells (5–6). Because of these effects, PMA and fMLP have proven to be valuable probes for the study of the initiation of transmembrane and intracellular cation fluxes in granulocytes. The changes in transmembrane potential induced by PMA and fMLP, and the increase in [Ca2+]i, stimulated by fMLP, occur very rapidly after each of these ligands binds to the cell’s surface and, at least in the case of fMLP (7–8), appear to be initiated by the occupancy of a plasma membrane receptor(s); while such a receptor for PMA has been postulated (9), the precise mechanism for the interaction of this molecule with the cell’s surface remains unclear. In the granulocyte, considerable evidence supports the idea that membrane potential changes (3, 10) and/or a rise in [Ca2+]i (5, 11) may serve as intermediary signals that couple the surface binding of a variety of ligands to functional activation of the cell, leading to degranulation, chemotaxis, and the generation of the respiratory burst. These cytotoxic events occur primarily, if not exclusively, in mature phagocytes.

Immature myeloid cells harvested from the blood of patients with chronic myelogenous leukemia (12) and undifferentiated cells from the acute promyelocytic leukemia cell line HL-60 (13, 14) have been shown to be unable to respond to PMA or to fMLP by mounting a respiratory burst. These observations suggest either that receptors for each of these ligands develop relatively late in the ontogeny of the granulocyte, or, if the receptors are present in primitive granulocyte precursors, that they become coupled to pathways which permit signal transduction only at a later stage of granulocyte development. Since undifferentiated HL-60 cells (15, 16) and possibly also myeloid progenitor cells (17, 18) differentiate in response to PMA, and since fMLP (19) and PMA (20) receptors have been reported to be present, albeit in small numbers, on undifferentiated HL-60 cells, the lack of functional responsiveness of immature granulocyte precursors to these compounds would seem less likely to be due to a total absence of specific surface receptors than to incomplete generation of metabolic signals which serve as “second messengers” and which normally follow attachment of the ligand to its specific receptor on the cell’s surface.

In this report, we have studied the effect of PMA and fMLP on transmembrane electrical potentials and on [Ca2+]i, in normal human myeloid cells at sequential stages of matura-
tion from the myeloblast through the fully mature, segmented granulocyte. To carry out these studies, we have used a newly developed immune-rosetting technique to obtain highly en-
nriched populations of myeloid progenitor cells from normal human bone marrow, which we subsequently induced to differen-
tiate in liquid suspension cultures by the addition of the specific growth factors, granulocyte-macrophage colony-stim-
ulating factors (GM-CSF).

**MATERIALS AND METHODS**

**Isolation of Granulocytes—**50-100 ml samples of blood were collected from volunteers and anticoagulated with heparin or sodium citrate. The blood was mixed with an equal volume of 3% dextran T-500 (Pharmacia) in 0.15 M NaCl and allowed to sediment for 45 min at room temperature. The leukocyte-rich plasma was then collected and subjected to gentle centrifugation. The cell pellets were resus-
pered in 0.15 M NaCl and then centrifuged on sodium metrizoate-
Ficoll density gradients. The dense cell pellet was collected and
resuspended in cold distilled water for 30 s to lyse red blood cells, after which time an equal volume of 0.3 M NaCl was added. The cells were then resuspended in iced magnesium- and calcium-free modified Hank’s balanced salts solution (Mg2+ and Ca2+ free HBSS). Viability exceeded 99% as assessed by exclusion of the dye trypan blue (21), and the cells were >99% granulocytes as determined by 500 cell differential count performed on smears stained with Wright’s stain.

**Partial Purification of Myeloid Progenitor Cells—**Granulocyte pro-
genitor cells from the stage of the committed granulocyte-macrophage progenitor cell (colony-forming unit in culture) through the stage of the myeloblast were enriched approximately 100-fold using an im-
une-rosetting technique previously described in detail (22). In each experiment, approximately 15 ml of bone marrow were aspirated from one or two normal donors and individually subjected to fractionation by immune rosetting using the monoclonal antibodies anti-M8 (panmyeloid), anti-Mo (clone 904, panmyeloid), anti-MY4 (mono-
cyte), anti-B1 (B lymphocyte), anti-T11 (T lymphocyte), 31C6 (nat-
ural killer cell and basophil), and anti-glycophorin A (clone 39). Briefly, mononuclear cell preparations isolated from the bone marrow samples by sodium metrizoate-Ficoll centrifugation were incubated with these monoclonal antibodies and then allowed to form rosettes with sheep erythrocytes to which affinity-purified rabbit anti-mouse immunoglobulin had been covalently coupled. Rossetted cells were then removed by density gradient centrifugation. In each experiment, approximately 99% of the cells were depleted from the marrow samples by this technique, and the remaining approximately 1% of the cells were used in the experiments described below. After the separation procedure was completely carried out, marrow cells from each of two normal donors were pooled for each experiment.

**Differentiation of Myeloid Precursor Cells in Liquid Suspension Cultures—**1-2 × 106 myeloid precursor cells isolated as described above were suspended in RPMI 1640 or McCoy’s 5A medium (Gibco) containing 10% heat-inactivated fetal calf serum, 10 mM HEPES, 50 IU/ml penicillin, and 20 IU/ml streptomycin, and 50 IU/ml penicillin G at a concentration of 0.5-1.0 × 106 cells/ml. As a source of GM-CSF, 5% GCT medium (Gibco) and 5% medium conditioned by the Mo cell line (the generous gift of Dr. Steven Clark, Genetics Institute, Cambridge, MA) were added to the cell suspension, and the culture was incubated at 37 °C in a humid tissue culture incubator containing 5% CO2. At appropriate intervals, total and differential cell counts were performed, and approximately 106 cells were removed for labeling with di-O-C6(3) and indo 1 as described below.

**Measurement of Changes in Membrane Potentials Using di-S-
C3(5)—**We initially measured membrane potential changes upon stimulation of suspensions of granulocytes and of partially purified myeloid progenitor cells by fluorimetry using the cyanine dye 3,3'- dipropylthiadicarbocyanine (di-S-C5(5)) by a modification of the method described by Horne and Simons (23). When this cationic lipophilic dye is added to a cell suspension, it enters the cell membrane and equilibrates with di-S-C5(5) molecules in the extra-
cellular phase. However, its fluorescence is quenched intracellularly. In cells such as granulocytes, which maintain transmembrane elec-
trical potentials through energy-dependent cation pumps, the exter-
nal milieu of the cell is positive relative to the cell’s interior in the resting state. When these cells are loaded with di-S-C6(5), depolar-
ization of the cell causes extrusion of the probe into the extracellular phase and results in an increase in total fluorescence. Conversely, hyperpolarization of the cell results in migration of di-S-C5(5) mole-
ules into the extracellular medium into the cell, giving rise to a decrease in measurable fluorescence.

Cells prepared as described above were resuspended in Mg2+ and Ca2+ free HBSS at a concentration of 106 cells/ml. 1-ml aliquots of the cell suspension were placed into the cuvette of a Perkin-Elmer 650/10 spectrophotofluorimeter and stirred constantly at the controlled temperature of 37 °C. The sample was excited by light at a wavelength of 620 nm, and emitted fluorescence was monitored at 670 nm. Di-S-
C5(5) at a final concentration of 1.5 × 10-4 M was then added to the sample and allowed to equilibrate until a plateau in fluorescence was reached. A stimulus, or its appropriate control, was then added to the mixture in 0.005-0.02 ml volumes, and the fluorescence was moni-
tored continuously for approximately 15 min. The results were re-
ported as relative fluorescence, which represents the ratio [(Ft - F0) / F0] × 100, where F0 = fluorescence measured after addition of the stimulus, and Ft = base-line fluorescence achieved after equilibration with di-S-C5(5) but before the addition of the stimulus. As a positive control in these experiments, a change in the transmembrane electrical potential was elicited by the addition of a 5 μM concentration of the potassium ionophore valinomycin (Sigma). Neither PMMA nor FMLP, when added to cells that had not been preincubated with di-
S-C5(5), caused detectible fluorescence at the emission wavelength of the probe.

**Fluorimetric Measurement of Intracellular Calcium Using Quin 2—**

The concentration of free intracellular calcium ([Ca2+]i) was measured in initial experiments by fluorimetry using the probe quin 2 (Behring Diagnostics). Granulocytes or isolated myeloid progenitors were loaded with the acetoxymethyl ester of quin 2 (quin 2/AM) using a modification of the technique described by Tsien et al. (24). Granu-
locytes were suspended at a concentration of 5 × 106 cells/ml in HBSS, which contained a calcium concentration of 1.6 mM. Quin 2/
AM was then added to the suspension to a final concentration of 1 μM, and the mixture was then incubated at 37 °C for 20 min. The cells were then resuspended in HBSS without quin 2/AM for 30 min. Myeloid progenitor cells were loaded in a similar fashion except that the cell concentration used was 106/ml and the concentration of quin 2/AM added to the mixture was 5 μM.

Measurement of the fluorescence of intracellular quin 2 was per-
formed using the same spectrophotofluorimeter used to measure di-S-
C5(5) fluorescence. Each test sample was excited by light at a wavelength of 339 nm, and the fluorescence emitted by the sample was continu-
ously monitored at 500 nm. For each experimental point, 5 × 106 myeloid precursor cells were preincubated with quin 2/AM, washed once in a final volume of 1 ml and allowed to warm at 37 °C until an equilibrium in fluorescence was reached. An appropriate stimulus was then added to the stirring cell suspension in volumes of 0.005-0.02 ml, and changes in fluorescence were measured as a function of time after the addition. Finally, 2 mM EGTA (Sigma) containing 50 mM MgCl2 was then added to the suspension to a final concentration of 5 mM, and the mixture was then incubated at 37 °C for 2 min. The cells not preloaded with quin 2/AM and alterations in base-line fluorescence induced by the stimulus added were subtracted from the fluorescence which was experimentally measured. [Ca2+]i was then calculated from measured fluorescence as described by Tsien et al. (24), assuming the Kd for the binding of calcium to quin 2 to be 115 nM. To obtain these measurements, the maximum fluorescence for each experimental group was measured after lysing the cells with 1% Triton X-100. A minimum fluorescence signal was then obtained by adding 2 mM EGTA (Sigma) containing 0.5 mM MnCl2. Neither A23187 alone, nor A23187 added to granulocytes that had not been preincubated with quin 2/AM, gave rise to detectable fluorescence at the emission wavelength of the probe.

**Simultaneous Determination of Intracellular Fluorescence of Indo 1 and Di-O-C6(3) by Cytofluorometry—**We have described elsewhere a technique (25) which allows simultaneous observation of changes in intracellular calcium and in cellular membrane and basophil, and which is especially useful when only small numbers of cells are available. Briefly, for each experimental point in each separate experiment, 80,000-200,000 differentiating myeloid cells were removed from the culture, washed once in Krebs-Ringer phosphate buffer (pH 7.4) containing 5.5 mM glucose (KRPB), and resus-
pended in a final volume of 0.25 ml of the same buffer. The acetox-
ymethyl ester of the calcium probe indo 1 (Molecular Probes, Inc., Grand Junction, OR) was then added to the cell suspension to a final concentration of 5 μM. The mixture was incubated for 7 min at 37 °C.

**T. Hercend, J. Ritz, and J. Griffin, Dana Farber Cancer Institute, Boston, MA, unpublished observations.**

**K. Sabbath and J. Griffin, Dana Farber Cancer Institute, Boston, MA, unpublished observations.**
and then washed once with the starting buffer. The cells were stored on ice for no longer than 1 h until cytofluorimetry could be carried out. 2 min before the cells were loaded into the cytofluorimeter, a 2 × 10^{-5} M concentration of the cyanine dye di-O-C(5) was added to the mixture. This cationic lipophilic dye partitions into cell membranes in a manner similar to di-S-C3(5) and migrates in a similar fashion in response to changes in transmembrane electrical potentials. However, unlike di-S-C3(5), the fluorescence of di-O-C(5) is mainly detected when the dye is intracellular. Therefore, using this probe, depolarization of the cell membrane is recorded as a decrease in fluorescence, while hyperpolarization is measured as an increase in fluorescence.

After the cells were labeled with indo 1 and di-O-C(3), they were placed into the reservoir of a FACS 440 cytofluorimeter (Becton-Dickinson Corporation, Mountain View, CA), and cytofluorimetric analysis was begun. The cells were excited by dual argon lasers (357 and 488 nm), and fluorescence emitted at 405, 485, and 575 nm was measured simultaneously. Data were collected at the rate of approximately 200 cells/s. After a baseline reading was obtained, fMLP was injected into the sample to a final concentration of 10^{-5} M and mixed, and analysis was immediately begun. Data points, which were collected every 10 s, represented signals integrated from approximately 2000 cells. In control experiments, we determined that neither probe interfered with detection of the other and that the cells were not perturbed by either probe.

For the purposes of comparison, we isolated granulocytes from the peripheral blood as described above and labeled the cells with indo 1 and di-O-C(3) simultaneously in a manner similar to that described for myeloid precursor cells.

**Measurement of Intracellular pH**—Intracellular pH of granulocytes and isolated myeloid precursor cells was measured using the fluorescent probe 2'7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Molecular Probes, Eugene, OR). Cells were suspended in KRPB, pH 7.4, at a concentration of 10^7 cells/ml. 0.01 ml of a 1 mM solution of the tetraacetoxymethyl ester of BCECF (BCECF/AM) was then added to 2 ml of the cell suspension, and the mixture was incubated at 37°C for 5 min on a rotating mixer. The cells were then pelleted by centrifugation, resuspended in KRPB, and incubated for 15 min at room temperature. The cells were then diluted to 2 x 10^5/ml in KRPB, and 1-ml aliquots were placed in a 37°C thermostatted cuvette of a Perkin-Elmer 650/10 spectrofluorimeter. The cells were alternately excited by light at the pH-sensitive wavelength of 500 nm and the pH-invariant wavelength of 440, and emission was recorded at 530 nm. Stimuli were added after a plateau in fluorescence was reached.

**Treatment of Granulocytes with Pertussis Toxin**—Granulocytes were suspended at 10^6/ml in 0.612 M phosphate-buffered 0.15 M NaCl, pH 7.4 (phosphate-buffered saline) containing 0.1% bovine serum albumin. 500 ng of Bordetella pertussis toxin (List Biologicals, Campbell, CA) in 5 μl was then added, and the suspension was continuously agitated while incubating at 37°C for 2 h. The cells were then washed once in phosphate-buffered saline and resuspended in KRPB.

**Measurement of Membrane Potential Changes in Sodium-poor Buffers**—For these experiments, the cells were isolated and incubated with di-O-C(3) as described above in phosphate-buffered saline. 30 s before the addition of fMLP, the cells were isolated by centrifugation and resuspended in one of the following buffers. A) HBSS, B) Na+-depleted buffer, containing 125 mM choline chloride, 8 mM NaH2PO4, 2 mM NaHCO3, 5 mM glucose, 1 mM CaCl2, and 1.5 mM MgSO4, pH 7.4; or C) Ca++-enriched buffer, containing 120 mM KCl, 5 mM NaCl, 80 mM NaH2PO4, 2 mM NaHCO3, 5 mM glucose, 1 mM CaCl2, and 1.5 mM MgSO4, pH 7.4. The cells were then stimulated with fMLP, and fluorescence changes were recorded as described above.

**RESULTS**

**Measurement of Transmembrane Electrical Potentials Using di-S-C3(5)**—The effects of PMA and fMLP on the resting transmembrane electrical potentials of isolated mature granulocytes are shown in Fig. 1. For these studies, the appropriate stimulus was added at time zero to individual 1-ml aliquots of granulocytes (10^6 cells/ml in KRPB) after the cells had been equilibrated for 5 min with di-S-C3(5) at 37°C. As shown in this figure, 10^{-5} M PMA and 10^{-7} M fMLP each produced prompt membrane depolarization as assessed by this technique, reflecting a change in transmembrane cation gradients. Both stimuli induced a transient increase in total di-S-C3(5) fluorescence which was followed by a decrease in fluorescence, presumably due in part to oxidative degradation of the probe by components of the myeloperoxidase-halide-H2O2 complex produced as a result of activation of the oxidative burst (25).

When similar studies were carried out using an identical number of semipurified myeloid precursor cells (Fig. 2), comparable levels of fluorescence were achieved after equilibration of the cells with di-S-C3(5), reflecting resting transmembrane potentials which were comparable to those of mature granulocytes. However, neither fMLP nor PMA induced a change in the transmembrane electrical potentials of these cells. The potassium ionophore valinomycin, which was used as a positive control in each experiment, stimulated an immediate membrane potential change in these cells, reflecting the ex-
istence of a transmembrane $K^+$ gradient. Neither fMLP nor PMA gave rise to measurable fluorescence at the emission wavelength for di-S-C$_3$(5) when added to cells that had not been allowed to equilibrate with the dye.

**Measurement of Intracellular Free Calcium Using Quin 2**

In order to determine the concentration of cytosolic free calcium in mature granulocytes and in myeloid precursors, each of these cell populations was preincubated with quin 2/AM in HBSS which contained a calcium concentration of 1.6 mM/liter. We first examined the intracellular free calcium concentration of isolated granulocytes and found it to be $110 \pm 9 \text{ mM/liter (M} \pm S. \text{ E.}, n = 15)$. As shown in Fig. 3, when isolated granulocytes which had been preloaded with quin 2/AM were warmed to 37°C and then exposed to $10^{-7}$ fMLP, a prompt rise in $[Ca^{2+}]_i$ could be measured which reached peak levels at approximately 30 s and returned slowly thereafter to the resting concentration. The change in $[Ca^{2+}]_i$, induced by fMLP was similar to that which followed exposure of the cells to the calcium ionophore A23187, which bypasses receptors completely. We then repeated these experiments on populations of semipurified myeloid progenitor cells. As shown in Fig. 4, when these cells were treated with $10^{-7}$ M fMLP, in contrast to the effects seen in mature granulocytes, no increase in $[Ca^{2+}]_i$ occurred when these cells were treated with A23187. Neither A23187 alone nor A23187 in the presence of granulocytes that had not been preincubated with quin 2/AM gave rise to measurable fluorescence at the emission wavelength of the probe.

**Simultaneous Measurement of Intracellular Calcium and Transmembrane Electrical Potentials in Differentiating Myeloid Progenitor Cells Using Indo 1 and di-O-C$_5$(3)**

In order to gain insight into the stage of granulocyte development at which the responses to fMLP develop, we harvested semipurified myeloid progenitor cells from normal bone marrow and immediately allowed to equilibrate with quin 2/AM (1 $\mu$M) or an equivalent volume of HBSS. Fluorescence was then continuously monitored. The stimuli shown were tested five times in two separate experiments (A23187) or three times in two experiments (fMLP) with identical results.

Using GM-CSF (27). Because small numbers of these cells were available, we performed these studies by flow cytometry as described above. The total and differential cell counts obtained in a representative experiment are shown in Table 1. The total cell number decreased during the first 24 h by approximately 20%, after which time a steady increase in the total cell number occurred, reaching peak levels on day 6. Thereafter, the total cell number began to decline. As indicated by the differential counts, immediately after the cells were isolated from the bone marrow, they consisted mostly of myeloblasts and of undifferentiated, morphologically unrecognizable hemic progenitor cells which resembled medium-sized lymphocytes. By 24 h in culture, a substantial number of promyelocytes were recognizable. Thereafter, a progressive wave of granulocyte maturation took place, so that by day 6, approximately 20% of the cells consisted of fully segmented granulocytes. By day 10, most of the cells in culture were mature granulocytes.

At appropriate intervals, aliquots of the differentiating cells were removed from the cultures, labeled with di-O-C$_5$(3) and indo 1 simultaneously, and then exposed to $10^{-7}$ M fMLP. The response of differentiating myeloid precursor cells to fMLP is shown in Fig. 5. Use of the fluorescence-activated cell sorter allowed us to obtain measurements of transmembrane potentials and relative calcium concentrations simultaneously and to record them at 10-s intervals after exposure of the cells at time zero to $10^{-7}$ M fMLP. Using this technique, the gradual acquisition of responses to fMLP which developed as the cells matured was readily apparent. As shown in the left column of this figure, on day zero, at which time practically no cells beyond the stage of the myeloblast were detectable in the cell suspension, fMLP gave rise to a hyperpolarization of the cell surface which occurred within 10 s and which thereafter appeared to be sustained during the next 90 s. This response was similar to the hyperpolarization which we observed when we exposed mature granulocytes to extremely low concentrations of fMLP (Fig. 6). As the precursor cells matured in culture (Fig. 5), the initial period of hyperpolarization began to diminish by day 3. However, a later and more protracted depolarization of the cell surface began to become apparent by day 6, by which time the extent and the duration of the hyperpolarization had significantly decreased. The phase of membrane depolarization became progressively
Cation Flux in Maturing Granulocytes

**TABLE I**

Total and differential cell counts of myeloid progenitor cells induced to differentiate in vitro

| Differential cell count | Day 0  | Day 1  | Day 3  | Day 6  | Day 8  | Day 10 |
|-------------------------|--------|--------|--------|--------|--------|--------|
| (n = 1.40 × 10⁶)        |        |        |        |        |        |        |
| Undifferentiated        | 68.3   | 21.6   | 3.5    | 3.5    | 0.0    | 0.0    |
| Myeloblasts             | 17.8   | 15.7   | 10.4   | 1.7    | 0.0    | 0.9    |
| Promyelocytes           | 8.9    | 54.9   | 47.0   | 13.9   | 1.6    | 6.6    |
| Myelocytes              | 4.0    | 1.0    | 20.9   | 20.0   | 21.8   | 2.8    |
| Metamyelocytes          | 0.0    | 0.0    | 8.7    | 20.9   | 24.2   | 4.7    |
| Bands                   | 0.0    | 0.0    | 0.0    | 15.7   | 29.0   | 12.3   |
| Segmented neutrophils   | 0.0    | 0.0    | 0.0    | 21.8   | 20.2   | 64.2   |
| Macrophages             | 0.0    | 0.0    | 3.5    | 0.8    | 0.0    | 1.9    |
| Basophils               | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    | 0.9    |
| Eosinophils             | 1.0    | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    |

Approximately 2 × 10⁶ myeloid precursor cells were harvested from the marrow of a single healthy donor and incubated in vitro in the presence of GM-CSF as described. Shown are the results of one of two experiments which yielded similar results. n, total cell number.

In the center column, the fluorescence of intracellular indo 1 is shown after the cells were exposed at time zero to fMLP, and fluorescence was continuously measured. In panel A, the fluorescence of di-0-C5(3) is shown after the cells were exposed at time zero to fMLP (open circles) or 10⁻⁶ M (closed circles) fMLP. In panel B, the fluorescence of di-0-C5(3) is shown after the cells were exposed at time zero to fMLP (open circles) or 10⁻⁵ M (closed circles) fMLP. This experiment was repeated 15 times with the same results.

In the center column, the fluorescence of intracellular indo 1 is recorded in response to cellular activation by fMLP. On day zero, when the cells were stimulated by 10⁻⁶ M fMLP, no alteration in fluorescence was observed. Over the 8 days of culture, a steady progressive responsiveness to fMLP developed which was characterized by an early increase in [Ca²⁺]i, which reached peak levels in approximately 10 s. Thereafter, a steady decrease in [Ca²⁺]i took place. By day 8, the response of these cells was similar to that seen in fully mature granulocytes (Fig. 6).

**Effect of Pertussis Toxin on Membrane Potential Response**
to fMLP—In order to determine whether both the hyperpolarization and depolarization induced by fMLP are dependent upon transduction pathways that are coupled to GTP-binding proteins which can be inhibited by B. pertussis toxin, isolated granulocytes were preincubated with the toxin under the conditions described, allowed to equilibrate with di-O-C₅(3), and then stimulated with 10⁻⁷, 10⁻⁶, and 10⁻¹¹ M fMLP. As shown in Fig. 7, preincubation with B. pertussis toxin abrogated both phases of the membrane potential change induced by serial concentrations of the chemotactic peptide.

**Contribution of the External Concentration of Sodium and Potassium to fMLP-induced Hyperpolarization**—To gain insight into the transmembrane ionic movements involved in the phase of membrane hyperpolarization induced by fMLP, we investigated the individual contributions of Na⁺ and K⁺ by allowing granulocytes to equilibrate with di-O-C₅(3) in phosphate-buffered saline and then resuspending aliquots of the cells in either a Na⁺-depleted buffer or a K⁺-enriched buffer immediately before measuring the fluorescence changes induced by the addition of 10⁻¹¹ M fMLP. As shown in Fig. 8, we found that reduction of the Na⁺ concentration in the surrounding medium resulted in a moderate reduction in the degree of hyperpolarization which followed addition of the stimulus, while increase in the K⁺ concentration gave rise to a substantially greater reduction in hyperpolarization which could be measured using this probe.

**Internal pH Change Induced by fMLP**—The internal pH of isolated granulocytes and of myeloid precursor cells was monitored using the fluorescent probe BCECF. As shown in Fig. 9, 10⁻⁷ M fMLP induced a substantial decrease in intracellular pH which was followed by a secondary phase of alkalinization. While 10⁻⁶ and 10⁻¹¹ M fMLP caused quantitatively smaller changes in internal pH in these cells, both concentrations gave rise to an immediate decrease in pH. When we tested the response of myeloid precursor cells incubated for 24 h in the presence of granulocyte-macrophage colony-stimulating factors, we found that their response to 10⁻⁷ M fMLP was similar to that of granulocytes exposed to 10⁻¹¹ M fMLP. At this point in time when cellular differentiation had not proceeded significantly past the stage of the promyelocyte, stimulation of the cells with 10⁻⁷ M fMLP gave rise to hyperpolarization of the cell’s surface (see Fig. 6).

**DISCUSSION**

A major advantage of this study is our use of normal human bone marrow-derived myeloid precursor cells which can be induced to differentiate fully in vitro by the addition of the specific granulopoietic regulators, GM-CSF. Our ability to isolate sufficiently purified starting cell populations of granulocyte progenitors for these studies has resulted from the recent development of a number of monoclonal antibodies directed against specific antigens on most differentiated...
hemetic and lymphoid cells, permitting their depletion from marrow cell suspensions by techniques such as immune rosette centrifugation (22). In addition, the ability to measure fluorescence of cell-associated probes by flow cytometry has permitted experiments like those which we report herein to be carried out using extremely few cells per experimental point, making the use of normal human marrow cells feasible for such studies.

Prior to the development of this approach, a number of important experimental observations concerning the maturational development of ligand-activated membrane potential change and intracellular calcium flux were made in continuous human leukemia cell lines, such as the acute promyelocytic leukemia line HL-60. This cell line is particularly useful since, in the undifferentiated state, the cells are arrested at the level of the promyelocyte. Furthermore, they can be reliably induced to undergo maturation by a number of membrane-active agonists, giving rise to differentiation along either the monocyte-macrophage (28, 29) or the granulocyte pathway (30). Using this approach, several groups have shown that specific functional properties of mature phagocytes, such as superoxide generation (14, 31), phagocytosis (30), and chemotaxis (32), are absent at the stage of the promyelocyte and are acquired when HL-60 cells are stimulated to differentiate. Recently, Brown and co-workers (33) measured transmembrane electrical potentials in HL-60 cells as they mature in vitro, using the fluorescence probe di-O-C<sub>3</sub>(3) monitored by fluorimetry. They found that these cells in the undifferentiated state do not respond to PMA by undergoing depolarization of the cell's surface, but as the cells begin to differentiate, a progressive wave of depolarization develops in response to this ligand which increases incrementally as the cells mature. In addition, Kitagawa et al. (12) extended these findings considerably when they observed a similar evolution of membrane depolarization in cells of the HL-60 and KG-1 cell lines induced to differentiate in vitro by retinoic acid, dimethyl sulfoxide, or PMA, as well as in immature and mature granulocytes fractionated from the blood of patients with chronic myelogenous leukemia. In their study, which was carried out by fluorimetry using both di-S-C<sub>3</sub>(3) and di-O-C<sub>3</sub>(3), the acquisition of membrane potential changes in response to PMA and fMLP was found to correlates with the development of the ability of the cells to generate superoxide ions and to phagocytose latex particles. Finally, Naccache et al. (34) monitored changes in the concentration of free cytosolic calcium in quin 2-loaded HL-60 cells in response to fMLP and leukotriene B<sub>4</sub>. These investigators found that as the cells differentiate in vitro, they exhibit a transient increase in the concentration of free intracellular calcium ions. Taken together, these experimental observations, which have been made using malignant hemopoietic cells, suggest that cytotoxic activation of the mature phagocyte in response to membrane-active ligands such as PMA and fMLP is an acquired metabolic activity which slowly and progressively develops as the cells differentiate. The data which we present in this report indicate that a similar maturational sequence is involved as normal human myeloid precursor cells mature from the level of the myeloblast to that of the fully mature granulocyte and extend these observations by describing a biphasic membrane potential change induced by chemotactic peptide in cells of the granulocytic lineage.

In this report, we have examined the ontogenetic development of the membrane potential change and the transient increase in [Ca<sup>2+</sup>], stimulated by the phorbol ester PMA and the chemotactic peptide fMLP in normal human cells of the granulocytic lineage. Our results indicate that whereas PMA and fMLP induce a prompt wave of membrane depolarization in mature granulocytes, and while fMLP stimulates a rapid increase in [Ca<sup>2+</sup>], in these cells, both of these effects are absent in granulocyte precursor cells through the level of the myeloblast. Before the responses characteristic of the mature granulocyte are discernible in maturing granulocyte precursors, fMLP induces hyperpolarization of the cell's surface, a phenomenon which we found could be demonstrated in mature granulocytes stimulated by very low concentrations of the ligand (25). Our observation that both of the phases of membrane potential change were abrogated by pretreatment of the cells with B. pertussis toxin provides evidence that each separate ionic event is mediated through receptors whose transduction is coupled to a specific subset (35) of GTP-binding proteins.

In contrast to depolarization, we found that hyperpolarization could be induced by much lower concentrations of fMLP and developed earlier in the granulocyte's ontogeny. One possible explanation for these distinctions is that each of these events may be mediated by separate classes of fMLP receptors with distinctly different binding affinities. In this regard, one might, on the basis of our data postulate that hyperpolarization is governed by a high affinity receptor which develops quite early during granulocyte maturation, while depolarization is mediated by an fMLP receptor of lower affinity which appears relatively late during differentiation. In support of this hypothesis are reports that have described multiple classes of receptors on the granulocyte's surface for chemotactic peptide (8, 36-38) in which the possibility was raised that separate transduction pathways may couple individual receptors to different functions. Alternatively, these differences may not be based on separate classes of receptors, but may instead be due to separate transduction pathways which are coupled to the same fMLP receptor but which become functional at different stages of granulocyte maturation and which respond to different concentrations of the ligand or to different numbers of occupied receptors.

An additional distinction between the two phases of membrane potential change that we noted, which may prove to be of considerable value in deciphering the physiologic role of membrane potential changes in the activation of cytotoxic functions in the granulocyte, is that the extremely low concentrations of fMLP which induce only hyperpolarization of the cell's surface do not activate the oxidative burst; concentrations of fMLP which stimulate depolarization of the cell are required to induce the generation of superoxide anion (3, 25).

We attempted to gain insight into the specific transmembrane ionic fluxes which gave rise to the phase of fMLP-induced hyperpolarization that we observed by altering the concentrations of Na<sup>+</sup> and K<sup>+</sup> in the external milieu and by measuring changes in intracellular pH. That the hyperpolarization was substantially reduced by increasing the external concentration of K<sup>+</sup> while simultaneously decreasing that of Na<sup>+</sup>, but less so by depleting the concentration of Na<sup>+</sup> alone, suggests that K<sup>+</sup> efflux from the cytosol to the cell's exterior contributes significantly to the membrane hyperpolarization stimulated by fMLP. Since a mild acidification of the cell's interior accompanies the phase of hyperpolarization, we feel that it is unlikely that an efflux of protons per se could account for a substantial fraction of the cations exported, unless relatively large amounts of free acid were generated within the cell as a result of the binding of 10<sup>-11</sup> M fMLP; since there appears to be no evidence that this concentration of fMLP stimulates any significant degree of metabolic perturbation of the cell, this possibility seems unlikely.
We were interested to note that the initial phase of membrane hyperpolarization in response to fMLP, which appeared to develop early in the ontogeny of the granulocyte, was apparent when di-O-C$_3$(3) fluorescence was measured by flow cytometry but was undetectable when di-S-C$_3$(5) fluorescence was measured by fluorimetry. We feel that this dichotomy can best be explained on the basis of the difference between the sensitivities of the two techniques. Since di-S-C$_3$(5) fluoresces only in the extracellular phase, it is likely that small shifts of the dye may not be as easily detectable as with di-O-C$_3$(3), which fluoresces when it is associated with the cell. Since we measured intracellular fluorescence of di-O-C$_3$(3) by cytofluorimetry and were able to obtain measurable signals from only 2000 cells per experimental point, it is reasonable to expect a much greater degree of sensitivity from this technique than with the more frequently employed fluorimetry method.

In view of the multifaceted nature of the responses that they elicit, it is likely that when PMA and fMLP interact with the surface of the granulocyte, their attachment sets into motion a coordinated chain of events which culminates in transient elevations in cytosolic free calcium concentrations and stimulated membrane depolarization and fMLP-induced transient elevations in cytosolic free calcium concentrations evolve in a similar temporal sequence during granulocyte maturation. These observations provide further support for the concept that the ability of these two membrane-active agonists to alter the intracellular concentration of specific cations is coupled to the functional responses which they evoke.

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