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Correlation between Plasma Membrane Potential and Second Messenger Generation in the Promyelocytic Cell Line HL-60*

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Didier Pittet, Francesco Di Virgilio‡, Tullio Pozzan§, Antoinette Monod, and Daniel P. Lew

From the Infectious Diseases Division, University Hospital, 1211 Genève 4, Switzerland, the ‡Centro Consiglio Nazionale delle Ricerche per la studio delle Fisiologia di Mitocondri and Istituto di Patologia Generale, University of Padova, I-35131 Padova, Italy, and §the Istituto di Patologia Generale, University of Ferrara, I-44100 Ferrara, Italy

The effects of plasma membrane depolarization on cytosolic free calcium ($[Ca^{2+}]_i$) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) generation were investigated in the human promyelocytic cell line HL-60 differentiated with either dimethyl sulfoxide or retinoic acid into neutrophil-like cells. Increases in $[Ca^{2+}]_i$ and accumulation of $Ins(1,4,5)P_3$ were triggered by two chemoattractants fMet-Leu-Phe and leukotriene B_4. Plasma membrane potential was depolarized by isoosmotic substitution of NaCl with KCl, by the pore-forming ionophore gramicidin D, or by long term treatment with ouabain. Both Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx across the plasma membrane were reduced by prior depolarization of plasma membrane potential regardless of the procedure employed to collapse it. Agonist-induced generation of $Ins(1,4,5)P_3$ was also reduced in parallel in pre-depolarized HL-60 cells.

The present findings provide further evidence suggesting that plasma membrane potential can be an important modulator of agonist-activated second messenger generation in myelocytic cells.

Receptor binding on the plasma membrane of several nonexcitable cells, among which are neutrophils, causes changes in the plasma membrane potential (1-5). The physiological role of these plasma membrane potential variations during cell activation has remained dubious however. Recently, evidence has accrued that plasma membrane potential in nonexcitable cells might have a regulatory role in the homeostasis of $[Ca^{2+}]_i$,[1] as it has been shown by several laboratories that membrane depolarization reduces agonist-induced Ca^{2+} inflow across the plasma membrane whereas hyperpolarization has the opposite effect (6-8). The inhibition of Ca^{2+} influx could be due, at least in part, to a decreased Ca^{2+} electrochemical gradient, but it cannot be excluded that the membrane pathways for Ca^{2+} influx in nonexcitable cells are "voltage-modulated," although not "voltage-gated" (8).

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[1] The abbreviations used are: $[Ca^{2+}]_i$, cytosolic free calcium concentration; fMet-Leu-Phe (fMLP), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; LTB_4, leukotriene B_4; $Ins(1,4,5)P_3$, inositol (1,4,5)-trisphosphate; Me_2SO or DMSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

In a previous study, we showed that, in human neutrophils, prior depolarization of plasma membrane potential reduced not only Ca^{2+} influx but also Ca^{2+} release from intracellular stores activated by the chemotactic peptide fMet-Leu-Phe (6). Conversely, incubation of the neutrophils with the ionophore valinomycin, that hyperpolarizes the plasma membrane potential, potentiated both Ca^{2+} release and Ca^{2+} influx triggered by this same agonist (6). In the same study, we postulated that reduced mobilization of Ca^{2+} from the intracellular stores in depolarized neutrophils might depend on a decreased agonist-triggered generation of $Ins(1,4,5)P_3$.

In the present investigation, we have directly tested this hypothesis by assessing the role of plasma membrane depolarization on $[Ca^{2+}]_i$ homeostasis and agonist-dependent $Ins(1,4,5)P_3$ formation in the human promyelocytic cell line HL-60 differentiated either with DMSO or retinoic acid. Two stimuli (fMet-Leu-Phe and LTB_4), acting on different receptors, were applied, and three different procedures (incubation in high KCl buffer, gramicidin D, or ouabain) were used to depolarize the plasma membrane potential in intact cells.

MATERIALS AND METHODS

Reagents and their sources were as follows: *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), *N*-*t*-BOC-L-methionyl-L-leucyl-L-phenylalanine (Sigma No. 0511), (5*S*,12*R*)-Dihydroxy-(6*E*,8*E*,10*E*,14*Z*)-eicosatetraenoic acid, leukotriene B_4 (LTB_4), gramicidin D from *Bacillus brevis*, (4β,20(22)-Cardenolide-1β,3β,-5α,11α,14,19-hexol-3-(6-deoxy-α-L-mannopyranosyl), G-strophanthin (ouabain), monensin, nigericin, retinoic acid (Sigma), fura-2 acetoxymethylester (fura-2/AM) (Calbiochem, La Jolla, CA), fetal calf serum (GIBCO, Paisley, Scotland), inositol-free RPMI (AMIMED, Geneva, Switzerland). All other analytical chemical products were of analytical grade and were obtained from Sigma, Merck, and Calbiochem AG, Inc.

The medium, referred to as "calcium medium," contained 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM Hepes, pH 7.4; the "calcium-free medium" refers to the above medium without $CaCl_2$ and supplemented with 1 mM EGTA. "KCl medium" refers to the latter where NaCl was substituted with KCl and Na_2HPO_4 with K_2HPO_4 calculated to contain (final) 50 ("50 mM KCl medium"), 100 ("100 mM KCl medium"), or 140 mM KCl ("140 mM KCl medium") and 54, 44, and 4 mM NaCl, respectively. Isoosmotic substitution rather than addition of hypermolar KCl was necessary since hyperosmolarity *per se* partially inhibits $[Ca^{2+}]_i$ rises in myelocytic cells (9). "CsCl medium" refers to the calcium-free medium where NaCl was substituted with CsCl (cesium chloride) and Na_2HPO_4 with K_2HPO_4 calculated to contain (final) 5 mM KCl. This last medium was supplemented with 1 mM EGTA.

Culture and Labeling of HL-60 Cells--The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (5 units/ml), and streptomycin (50 μg/ml). Cells were passaged twice every week, differentiated by adding DMSO (final concentration 1.3% v/v) or retinoic acid (final concentration 0.2 μM), and used for all experiments after 7 days of differentiation (10, 11). DMSO-differentiated HL-60 cells express a high concentration of fMet-Leu-

Phe, but much less LTB_4 receptor-mediated responses, while the contrary is true for retinoic acid-differentiated HL-60 cells. Inositol phospholipids and inositol phosphates were labeled as previously described (12), the cells being incubated from day 5 to day 7 in RPMI medium containing *myo*-[2-^{3}H]inositol (1.5 μCi/ml).

Measurements of Inositol Phosphates—Cells (35×10^6/0.5 ml) were warmed for 5 min at 37 °C before stimulation, and incubations were terminated by the addition of ice-cold 10% (v/v) trichloroacetic acid. Samples were kept on ice 5–10 min and centrifuged ($800 \times g \times 10$ min); supernatant was washed three times with a 5-fold excess of diethyl ether. The ether-washed and neutralized extracts were analyzed by HPLC, essentially as previously described (10). [^{3}H] Ins(1,4,5)P_3 serving as position and recovery standard was purchased from Amersham International, United Kingdom. In some experiments, inositol phosphates were separated by stepwise elution from small Dowex (anion exchange) columns as previously described (10, 11).

Measurement of Cytosolic Free Calcium and Ca^{2+} Influx Determination—Fura-2 loading was performed at 37 °C as described previously (12), at a final fura-2/AM concentration of 2 μM. After the loading procedure, the cells were kept at room temperature. Just before use, a sample of the cell suspension was centrifuged to remove BSA and resuspended in the indicated medium. Fluorescent measurements were performed with a Perkin-Elmer fluorimeter (LS 3, Perkin-Elmer Cetus Instruments, Norwalk, CT) as described (12). Excitation and emission wavelengths for fura-2 measurements were 340 and 505 nm, respectively. The values of fura-2 fluorescence were not expressed as absolute $[Ca^{2+}]_i$ values because the exact number of differentiated (responsive) cells may vary in different cell preparations (11).

Determination of Ca^{2+} influx in fura-2-loaded cells was assessed as previously described (12, 13) using the quenching properties of Mn^{2+} on fura-2 fluorescence, assessed at 360 nm excitation wavelength (isosbestic point). Ca^{2+} influx rates were deduced from the initial slope produced upon Mn^{2+} addition (100 μM) to fMet-Leu-Phe-stimulated cells and expressed as Δ of total fura-2 signal (in %/min), as described (12).

Membrane Potential Measurements—Membrane potential changes were measured with the lipophilic fluorescent dye bisoxonol as previously described for human neutrophils (6). DMSO- or retinoic acid-differentiated HL-60 cells and dye concentration in these experiments were 4×10^6/ml and 100 nM, respectively.

Ouabain Treatment—The cells (2×10^7/ml) were preincubated for 5 min at 37 °C in Ca^{2+} medium containing BSA (0.1%), before the addition of ouabain (100 μM). The cell suspension was gently shaken every 10 to 15 min to prevent sedimentation and clumping and kept at 37 °C throughout the incubation period. Under these conditions after 8 h in the presence of ouabain, trypan blue exclusion reached 6 ± 2% (mean ± S.D., $n = 4$ in two separate experiments); *i.e.* very similar to that of controls also kept at 37 °C for the same period in the absence of ouabain. Intracellular Ca^{2+} stores (assessed by the addition of ionomycin to cells incubated in Ca^{2+}-free medium) were slightly depleted after 8 h in Ca^{2+} medium both in the presence (8.5 ± 3%) and in the absence (8 ± 2%) of ouabain (mean ± S.D., $n = 5$ in two separate experiments).

Just before use, a sample of the cell suspension was centrifuged to remove BSA and resuspended in fresh medium still containing ouabain. Fura-2 loading was performed during the last hour preceding the use of the cells and did not interfere with the ouabain treatment (as assessed by membrane potential changes). In control experiments, ouabain pretreatment did not alter the fura-2 loading, as judged by comparing the intracellular fura-2 concentrations in ouabain-treated and untreated cells (14).

Presentation of Data—Unless otherwise indicated, typical experiments are shown which are representative of at least four similar experiments.

RESULTS

Depolarization of Plasma Membrane Potential Decreases $[Ca^{2+}]_i$ Release from Intracellular Stores—Ca^{2+} release from intracellular stores induced by fMet-Leu-Phe and LTB_4 was measured in HL-60 cells differentiated with DMSO (to induce expression of fMet-Leu-Phe receptors) or retinoic acid (to induce expression of LTB_4 receptors) (11). Plasma membrane potential was depolarized according to three different procedures: (i) by isoosmotic substitution of NaCl with KCl in the incubation medium; (ii) by treatment with the pore-forming ionophore gramicidin D; or (iii) by incubation with the inhibitor of the Na^+/K^+ ATPase ouabain. In order to study the effect of depolarization on Ca^{2+} redistribution from intracellular stores without interference from Ca^{2+} influx, the experiments of Fig. 1 to Fig. 7 were performed in Ca^{2+}-free media supplemented with 1 mM EGTA.

Fig. 1, *traces a, b,* and *c,* shows that the Ca^{2+} rises triggered by a submaximal concentration of fMet-Leu-Phe were greatly inhibited in DMSO-differentiated HL-60 cells by predepolarization of plasma membrane potential. Similar results were obtained in retinoic acid-differentiated cells challenged with submaximal concentrations of LTB_4 (compare *traces e* and *f* to *d*). The small increase in fura-2 fluorescence observed upon addition of gramicidin D to resting cells (exemplified in Fig. 1, *traces b, e, h,* and *k*) was found not to reflect a true change in $[Ca^{2+}]_i$ because this rise in fluorescence was not inhibitable by preincubation of the cells in the presence of the fMet-Leu-Phe antagonist BOC-Met-Leu-Phe and was more prominent

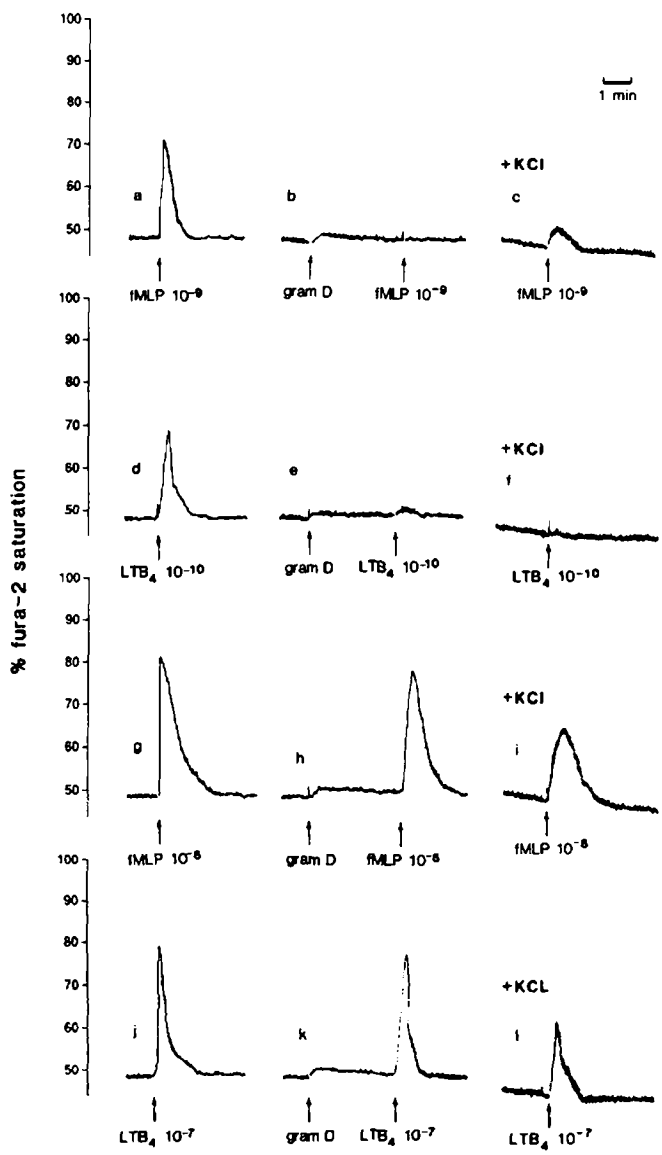

FIG. 1. **Agonist-induced $[Ca^{2+}]_i$ changes are inhibited by gramicidin D and KCl pretreatment.** Following a 5-min preincubation at 37 °C in Ca^{2+}-free, EGTA-supplemented NaCl (*traces a, b, d, e, g, h, j,* and *k*) or KCl (140 mM) medium (*traces c, f, i,* and *l*), fura-2-loaded cells were stimulated by various concentrations of fMLP or LTB_4. In *traces b, e, h,* and *k*, gramicidin D (1 μM) was added 3 min before agonist stimulation.

at the excitation wavelength of 360 nm (isosbestic point), indicating an effect on fura-2 fluorescence independent of $[Ca^{2+}]_i$ (data not shown).

At 10^{-9} M fMet-Leu-Phe, the inhibition by gramicidin D was about 100%, while that observed in 140 mM KCl ranged between 60 and 80%. However, while the inhibition by gramicidin D was relieved at high agonist concentrations (compare *trace h* with *g* and *k* with *j*), that by high KCl persisted both for fMet-Leu-Phe and LTB_4 (compare *trace i* with *g* and *l* with *j*). Fig. 2 shows however that, quite unexpectedly, Ca^{2+} mobilization by the Ca^{2+} ionophore ionomycin as well was reduced in cells incubated in high KCl media (Fig. 2, compare *b* with *c*) but unaffected by gramicidin D pretreatment (Fig. 2, compare *a* with *c*). Given that ionomycin-induced Ca^{2+} redistribution should bypass any receptor-linked step, the simplest explanation is that incubation in high KCl reduces the total Ca^{2+} content of intracellular stores. In control experiments, the ionomycin-sensitive Ca^{2+} stores were shown to be proportionally reduced by 15 ± 4, 26 ± 7, and 53 ± 5% (mean ± S.D., n = 3–5 in three separate experiments) when DMSO-differentiated HL-60 cells were preincubated for 5 min in the presence of 50, 100, and 140 mM KCl, respectively. Fig. 2 also shows that, in agreement with previous reports (15, 16), high KCl medium sensitizes the cells to low doses of fMet-Leu-Phe. Indeed, HL-60 cells depolarized with 140 mM KCl in the presence of gramicidin D still weakly responded with a rise in $[Ca^{2+}]_i$ to 10^{-9} M fMet-Leu-Phe (Fig. 2*e*), while an aliquot of the same batch of cells depolarized with 100 nM gramicidin D in Na^+ medium failed to respond to the same concentration of the chemotactic peptide (Fig. 2*d*). The residual Ca^{2+} mobilization by fMet-Leu-Phe in KCl + gramicidin D-treated cells was not due to incomplete depolarization since membrane potential was not appreciably different in the two experimental conditions (not shown). These observations indicate that incubation in high KCl media has effects on $[Ca^{2+}]_i$ homeostasis in HL-60 cells unrelated to depolarization of plasma membrane potential and make ionic substitution unsuited for the study of the effect of depolarization on receptor-triggered $[Ca^{2+}]_i$ changes.

Although gramicidin D had neither of the undesirable effects of KCl, it cannot be excluded that this ionophore too has effects on Ca^{2+} homeostasis unrelated to changes in membrane potential. Therefore, we examined the relationship

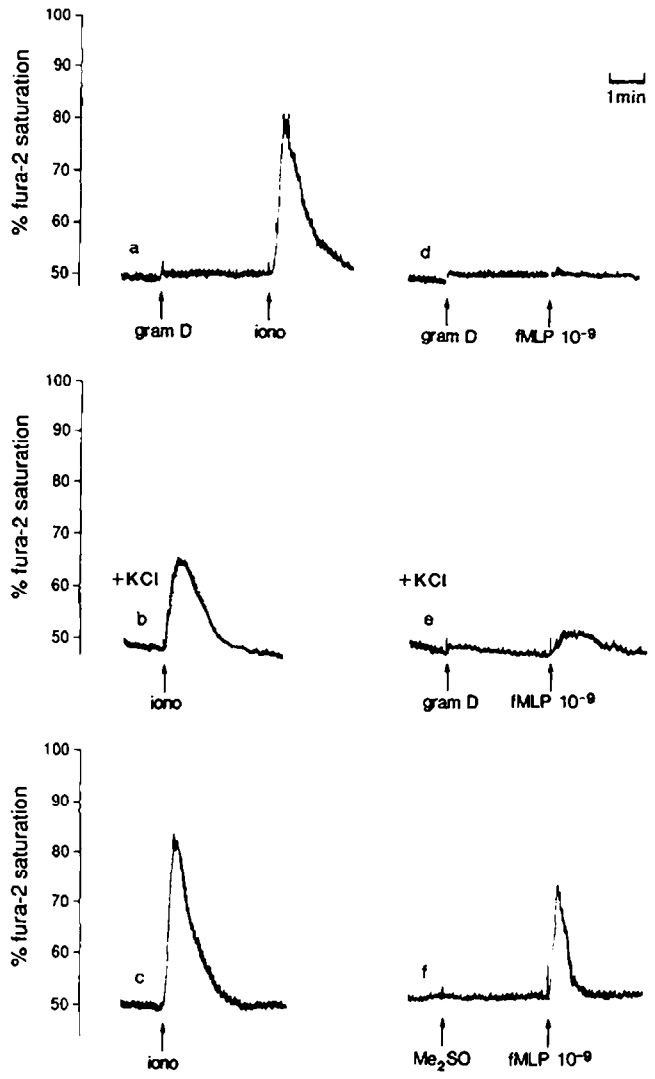

FIG. 2. **Effects of gramicidin D and KCl pretreatment on ionomycin-mobilizable Ca^{2+} stores.** DMSO-differentiated HL-60 cells loaded with fura-2 (2 μM) as described under "Materials and Methods," were incubated in Ca^{2+}-free EGTA-supplemented NaCl (*traces a, c, d,* and *f*) or KCl (140 mM) medium (*traces b* and *e*). After 5 min, ionomycin (500 nM), gramicidin D (1 μM), or Me_2SO was added. Gramicidin D concentration added in *d* was 100 nM.

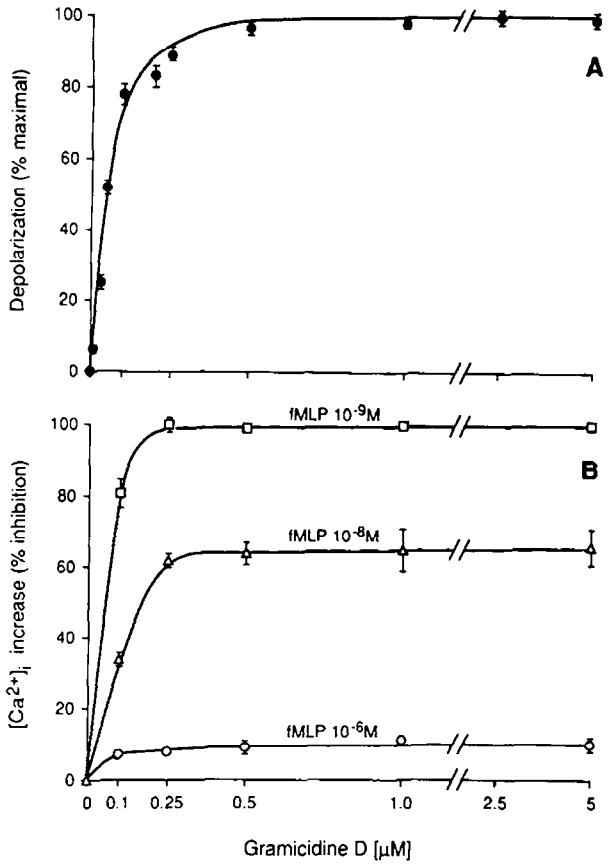

FIG. 3. **Plasma membrane depolarization and $[Ca^{2+}]_i$ rise inhibition induced by gramicidin D.** *Panel A*, plasma membrane depolarization was assessed by monitoring bisoxonol fluorescence changes induced by various concentrations of gramicidin D in DMSO-differentiated HL-60 cells (see "Materials and Methods"). Maximal change upon 5 μM gramicidin D addition was 1.6-fold the basal fluorescence of cells before the addition of gramicidin D. Values are mean ± S.D. of n = 3–10 determinations from four separate experiments. *Panel B*, fura-2-loaded cells were pretreated by various concentrations of gramicidin D for 3 min before stimulation by 10^{-6} M (○), 10^{-8} M (△), or 10^{-9} M (□) fMLP in Ca^{2+}-free medium. Values are mean ± S.D., n = two to five determinations in two separate experiments and expressed as percent inhibition of the maximal increase in $[Ca^{2+}]_i$ triggered by the addition of 10^{-6} M fMLP (84 ± 2% of fura-2 saturation).

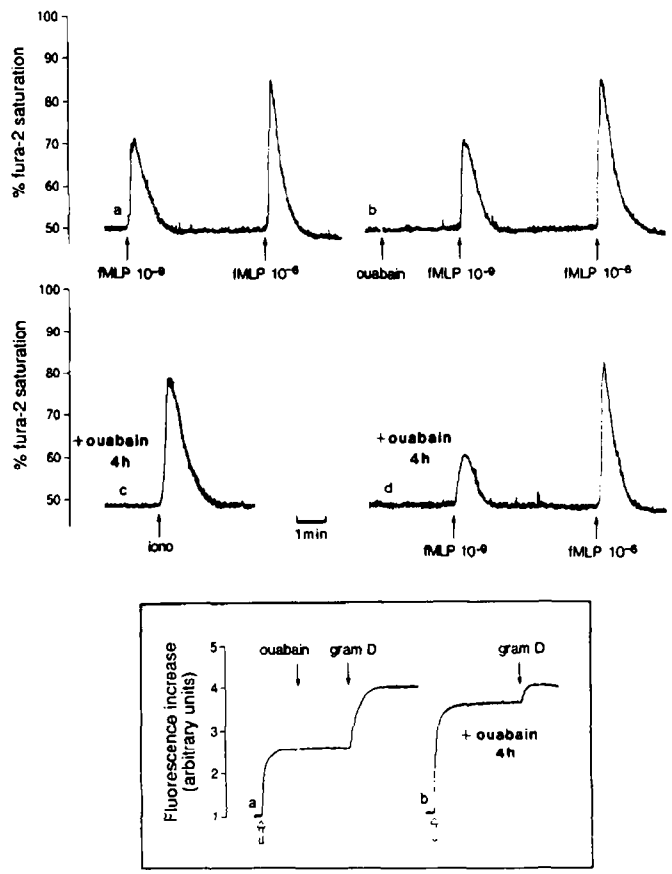

FIG. 4. **Effects of ouabain pretreatment on fMLP-induced $[Ca^{2+}]_i$ rises.** DMSO-differentiated HL-60 cells loaded with fura-2 (2 μM) were sequentially stimulated by 10^{-9} and 10^{-6} M fMLP. Prior to fMLP stimulation, cells were pretreated as follows: *a*, Me_2SO for 3 min; *b*, ouabain (100 μM) for 3 min; *c* and *d*, ouabain (100 μM) for 4 h. Ionomycin (500 nM) was added in *c*. *Inset*, bisoxonol fluorescence changes; *open arrow* refers to the addition of the cells (4×10^6/ml) to bisoxonol (100 nM) containing Ca^{2+}-free medium; cells were pretreated with ouabain (100 μM) for 3 min (*a*) or for 4 h (*b*). Gramicidin D (1 μM) was added when indicated. The *traces* shown were obtained the same day using the same batch of fura-2-loaded cells previously or acutely treated with ouabain as described under "Materials and Methods"; similar data were obtained in two additional experiments.

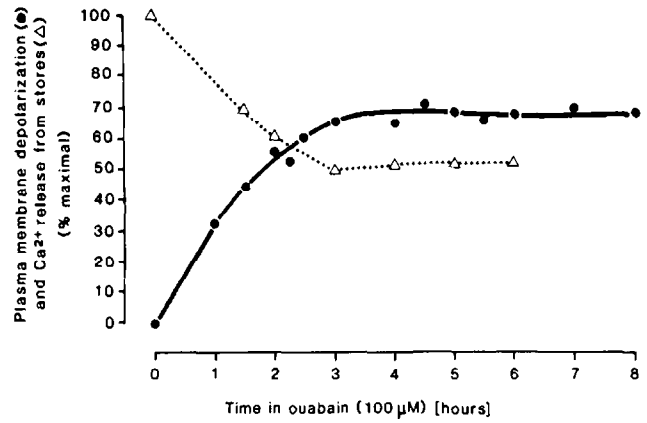

FIG. 5. **Time courses of ouabain effects on plasma membrane potential and fMLP-induced Ca^{2+} release from intracellular stores.** Plasma membrane depolarization (●) and $[Ca^{2+}]_i$ changes (△) were assessed following various times of incubation in the presence of ouabain (100 μM). Plasma membrane potential values are expressed as percentage of maximal depolarization induced by gramicidin D (1 μM) assessed in parallel in cells not previously treated with ouabain. $[Ca^{2+}]_i$ changes are expressed in percent of maximal fura-2 fluorescence saturation following the addition of 10^{-9} M fMet-Leu-Phe (72 ± 2% at time 0). Data shown were obtained the same day using the same batch of fura-2-loaded cells kept at 37 °C and continuously or not exposed to ouabain. Similar data were obtained in two different experimental days.

between the concentrations of gramicidin D needed to depolarize the plasma membrane potential and those which inhibited the $[Ca^{2+}]_i$ rise. As shown in Fig. 3*A*, gramicidin D fully depolarized HL-60 cells at a concentration of 250–500 nM. Increasing the gramicidin D concentration up to 5 μM had no further effect on membrane potential. Fig. 3*B* shows that inhibition of the $[Ca^{2+}]_i$ rise induced by fMet-Leu-Phe was also maximal at about 250 nM gramicidin D. In agreement with previous observations, the inhibition was progressively smaller at higher fMet-Leu-Phe concentrations (6). The ED_{50} for gramicidin D inhibition of the $[Ca^{2+}]_i$ rise was however independent of the fMet-Leu-Phe concentrations. Similar results were observed with LTB_4 (not shown).

HL-60 cells possess a Na^+/K^+ pump similar to that found in other cell types (17). Binding sites for ouabain, a powerful blocker of the Na^+/K^+ ATPase, have been demonstrated to be present in both DMSO- and retinoic acid-differentiated HL-60 (18, 19). Cells were thus depolarized using ouabain treatment. Ouabain had no short term effect on either plasma membrane potential (Fig. 4, *inset a*) or fMet-Leu-Phe (Fig. 4, compare *a* and *b*) and ionomycin (not shown)-induced Ca^{2+} redistribution. On the contrary, as shown in Fig. 4*d*, a 4-h incubation in the presence of 100 μM ouabain caused 56 ± 3% (mean ± S.D., $n = 5$ in three separate experiments) reduction of the $[Ca^{2+}]_i$ rise induced by 10^{-9} M fMet-Leu-Phe, whereas the $[Ca^{2+}]_i$ rise caused by ionomycin or 10^{-6} M fMet-Leu-Phe was unaffected (Fig. 4, *c* and *d*). The *inset* of Fig. 4 shows that after 4 h of ouabain treatment (*b*), membrane potential was about 68 ± 2% depolarized (mean ± S.D., $n = 8$ in four separate experiments), as compared to an optimal concentration of gramicidin D (1 μM). Fig. 5 shows a time course of the depolarizing effects of ouabain treatment (100 μM) in HL-60 cells. The ouabain-induced plasma membrane depolarization (*closed symbols*) was time-dependent, reaching its maximum after 3 to 4 h. The $[Ca^{2+}]_i$ rise inhibition, assessed in parallel (*open symbols*) on the same batch of fura-2-loaded cells, had a similar time course. Half-maximal effect for both plasma membrane depolarization and $[Ca^{2+}]_i$ rise inhibition appeared after about 1 h.

Both gramicidin D and ouabain treatment, besides depolarizing the plasma membrane potential, also affect the cytoplasmic concentration of Na^+ and K^+. It cannot be excluded therefore that the inhibitory effect on Ca^{2+} mobilization is due to perturbation of the Na^+/K^+ concentration rather than to depolarization of the plasma membrane. We mimicked the effects of gramicidin D and ouabain on the intracellular Na^+/K^+ concentration by treating HL-60 cells simultaneously with nigericin, a proton/K^+ exchanger, and monensin, a proton/Na^+ exchanger. This treatment is expected to affect the intracellular Na^+ and K^+ concentrations without major effects on membrane conductance. Nigericin (1 μM) induced a weak membrane potential depolarization, 25–30% when compared to full depolarization induced by gramicidin D (1 μM), and subsequent addition of monensin did not cause further depolarization. In nigericin + monensin-treated HL-60 cells differentiated with DMSO or retinoic acid, the $[Ca^{2+}]_i$ rise induced by 10^{-9} M fMet-Leu-Phe or LTB_4, respectively, was unaffected (data not shown).

To further rule out the possibility that inhibition of the $[Ca^{2+}]_i$ transients by gramicidin D was due to Na^+ overloading, we tested the effects of gramicidin D in HL-60 cells incubated in a medium where Na^+ was replaced by Cs^+, which is highly

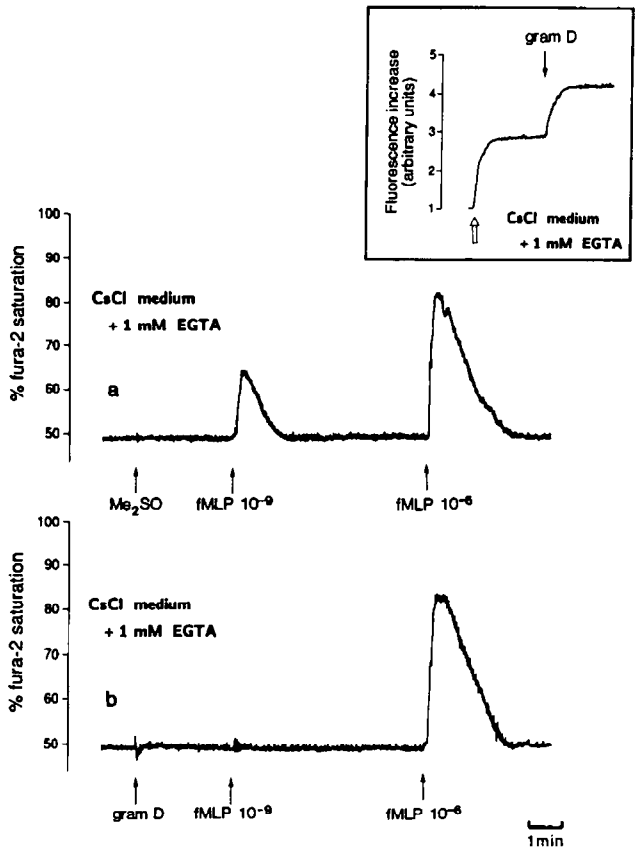

FIG. 6. **Effects of gramicidin D on plasma membrane potential and fMLP-induced Ca^{2+} release in Na^+-free, Cs^+-containing medium.** DMSO-differentiated HL-60 cells loaded with fura-2 (2 μM) were sequentially stimulated by 10^{-9} and 10^{-6} M fMLP in CsCl medium supplemented with 1 mM EGTA (and free of Na^+). Prior to fMLP stimulation, cells were pretreated for 3 min with Me_2SO (*a*) or gramicidin D (*b*) (1 μM). *Inset*, plasma membrane potential was assessed in parallel by bisoxonol fluorescence changes using the same batch of cells and in the conditions described above. Cells (4×10^6/ml) were added (*open arrow*) to CsCl medium supplemented with 1 mM EGTA containing bisoxonol (100 μM), before the addition of gramicidin D (1 μM).

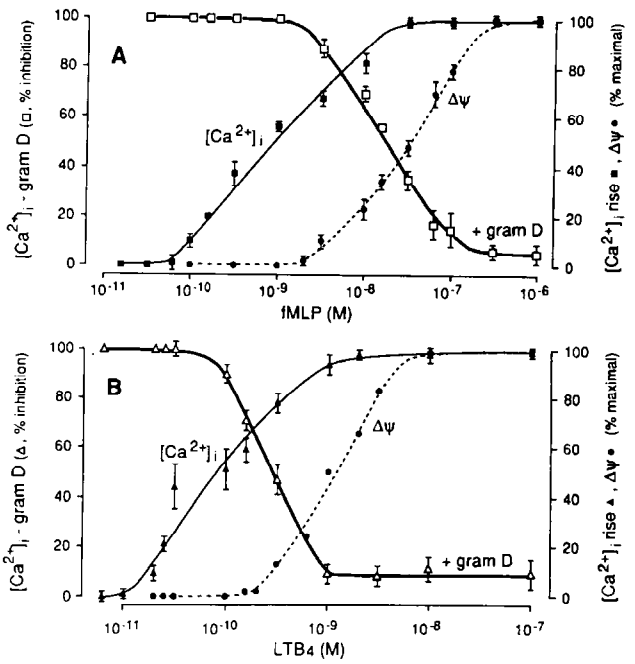

FIG. 7. **Dependence on the agonist concentration of plasma membrane depolarization, $[Ca^{2+}]_i$ rise, and gramicidin D-induced inhibition of the Ca^{2+} release from stores.** DMSO (*A*)- or retinoic acid (*B*)-differentiated HL-60 cells were loaded with fura-2 and resuspended in Ca^{2+}-free medium supplemented with 1 mM EGTA. Cells were then stimulated with increasing concentrations of fMLP (*A*) or LTB_4 (*B*). ●, plasma membrane depolarization; $[Ca^{2+}]_i$ increase upon fMLP (■, □) or LTB_4 (▲, △) addition. *Open symbols* indicate experiments in which HL-60 cells were pretreated with 1 μM gramicidin D for 5 min prior to fMLP (□——□) or LTB_4 (△——△) addition. Data are expressed as percentage of maximum response elicited by fully stimulatory fMLP (10^{-6} M) or LTB_4 (10^{-7} M) concentrations (83 ± 1 and 80 ± 2% fura-2 saturation, respectively). The effects of gramicidin D pretreatment on $[Ca^{2+}]_i$ increases (*open symbols*) are expressed as percent of inhibition of the respective maximum responses. Values are mean ± S.D., n = three to seven determinations in five separate experiments (*A*), n = two to three determinations in three separate experiments (*B*).

permeable through the gramicidin D pore (20). As shown in Fig. 6, in Cs^+-containing medium, gramicidin D induced a large depolarization and Ca^{2+} release stimulated by submaximal fMet-Leu-Phe concentrations was completely inhibited (Fig. 6, compare *traces a* and *b*).

We have previously shown that in mature human neutrophils the dose dependence for fMet-Leu-Phe-induced $[Ca^{2+}]_i$ rises was about 1 log unit shifted to the left compared to the fMet-Leu-Phe dose dependence for plasma membrane depolarization (6). This observation was confirmed in the present study; the relationship between $[Ca^{2+}]_i$ rises and plasma membrane depolarization caused by increasing concentrations of fMet-Leu-Phe or LTB_4 in HL-60 cells is shown in Fig. 7, *A* and *B*. As shown, a rise in $[Ca^{2+}]_i$ was detectable at agonist concentrations unable to depolarize the plasma membrane potential. Whereas the calculated ED_{50} for Ca^{2+} mobilization induced by fMet-Leu-Phe in DMSO- and by LTB_4 in retinoic acid-differentiated HL-60 cells were 8×10^{-10} M and 10^{-10} M, respectively, those calculated for agonist-induced plasma membrane depolarization were 5×10^{-8} M and 10^{-9} M, respectively. Fig. 7 also shows that predepolarization of plasma membrane inhibited the $[Ca^{2+}]_i$ rise completely only at the fMet-Leu-Phe concentrations that did not themselves depolarize plasma membrane potential (Fig. 7*A*). Again, a similar

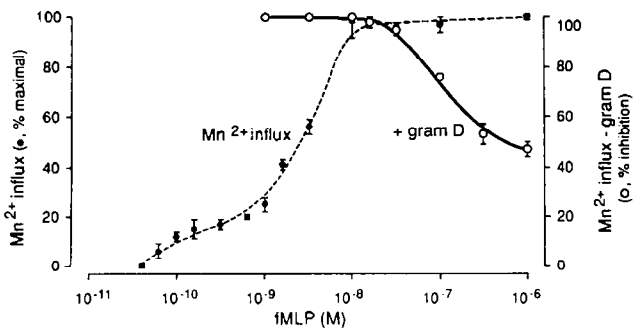

FIG. 8. **Effects of plasma membrane depolarization induced by gramicidin D on fMet-Leu-Phe-stimulated Mn^{2+} influx.** DMSO-differentiated HL-60 cells were loaded with fura-2 and resuspended in Ca^{2+} medium for 5 min prior to pretreatment with gramicidin D (1 μM, *open symbols*). Cells were then stimulated with increasing concentrations of fMLP, and Mn^{2+} influx (●) was assessed 1 min after fMLP stimulation as described under "Materials and Methods." Data are expressed as percentage of maximum response elicited by fully stimulatory fMLP (10^{-6} M) concentrations. The effects of gramicidin D pretreatment on Mn^{2+} influx (*open symbols*) are expressed as percent of inhibition of the respective maximum responses. Values are mean ± S.D., n = three to five determinations in three separate experiments.

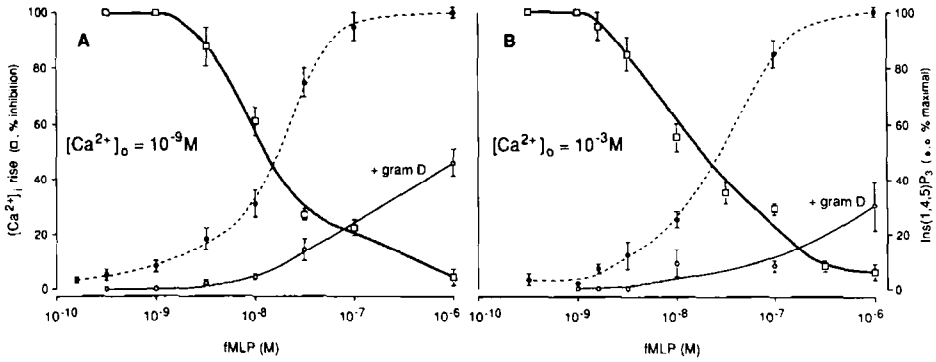

FIG. 9. **Plasma membrane depolarization by gramicidin D decreases chemotactic peptide-dependent Ins(1,4,5)P_3 production.** Fura-2-loaded cells were stimulated by increasing concentrations of fMLP in Ca^{2+} (*B*) or Ca^{2+}-free (*A*) medium; *open symbols* refer to pretreatment with gramicidin D (1 μM) for 3 min before stimulation. The trichloroacetic acid extract from *myo*-[2-^{3}H]inositol-labeled HL-60 cells (3.5×10^7/0.5 ml) stimulated by various concentrations of fMLP for 8 s was analyzed by HPLC as described under "Materials and Methods." $[Ca^{2+}]_i$ rises and inositol phosphates were assessed in parallel using the same batch of fura-2-loaded cells. Values are mean ± S.D., n = two to four duplicate determinations in three separate experiments. $[Ca^{2+}]_i$ rise is expressed as percent inhibition from maximal fura-2 saturations reached upon 1 μM fMLP (82 ± 1 and 94 ± 2% in *A* and *B*, respectively). Ins(1,4,5)P_3 values are expressed as percentage of maximum response elicited by fully stimulatory fMLP concentration (10^{-6} M): 1248 ± 25 and 1435 ± 63 dpm, in *A* and *B*, respectively. Basal values were 28 ± 1 and 110 ± 4 dpm, respectively.

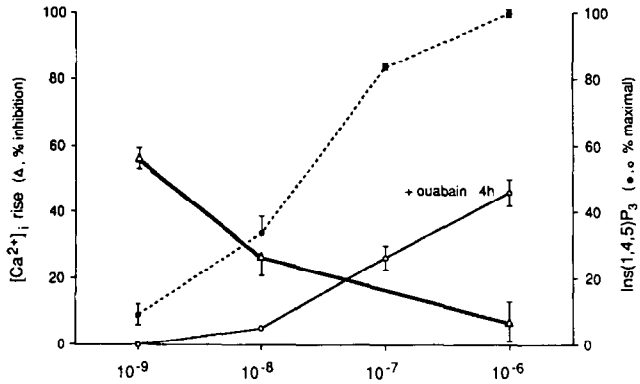

FIG. 10. **Ouabain pretreatment inhibits fMLP-stimulated $[Ca^{2+}]_i$ rises and Ins(1,4,5)P_3 production.** Fura-2-loaded cells pretreated with ouabain (100 μM) for 4 h at 37 °C (*open symbols*) were stimulated by increasing concentrations of fMLP in Ca^{2+}-free medium; control cells were also kept at 37 °C for 4 h. The trichloroacetic acid extract of 3.5×10^7 cells stimulated by various concentrations of fMLP for 30 s was separated on a Dowex column as described under "Materials and Methods." $[Ca^{2+}]_i$ rise values are mean ± S.D., n = three to four determinations in three separate experiments and expressed as percent inhibition from maximal fura-2 saturations reached upon 10^{-6} M fMLP (80 ± 2%). Ins(1,4,5)P_3 values, assessed in parallel using the same batch of cells, are expressed as percentage of maximum response elicited by fully stimulatory fMLP concentration (10^{-6} M) in the absence of ouabain (●): 846 ± 13 dpm, mean ± S.D. of duplicate determinations; substracted basal values (control unstimulated cells) were 240 ± 12 dpm. Similar data were obtained in one separate experiment.

pattern of inhibition was observed with LTB_4 in retinoic acid-differentiated cells (Fig. 7*B*).

Effect of Plasma Membrane Potential on Agonist-induced Ca^{2+} Influx—In addition to causing Ca^{2+} redistribution, agonists linked to phosphatidylinositol (4,5)-bisphosphate hydrolysis stimulate Ca^{2+} influx through the plasma membrane by an as yet poorly characterized mechanism (7, 8, 21). It has been demonstrated that Mn^{2+} can be utilized as a useful substitute for Ca^{2+} to monitor quantitatively agonist-stimulated Ca^{2+} influx (12, 13, 22). Inhibition of Mn^{2+} influx, unlike the effect on Ca^{2+} release from intracellular stores, was present all over the range of chemotactic peptide concentrations tested (Fig. 8). Inhibition of LTB_4-induced Mn^{2+} influx was more difficult to quantitate since stimulation of HL-60 cells with this agonist induced very little Mn^{2+} (or Ca^{2+}) influx.[2]

Plasma Membrane Potential Modulates Agonist-stimulated Ins(1,4,5)P_3 Production—Both fMet-Leu-Phe and LTB_4 are known to induce Ins(1,4,5)P_3 generation in differentiated HL-60 cells (10–12). Pretreatment with gramicidin D inhibited Ins(1,4,5)P_3 generation triggered by various fMet-Leu-Phe concentrations either in the absence or in the presence of extracellular Ca^{2+} (Fig. 9, *A* and *B*). Similar results were obtained with LTB_4 as a stimulus in retinoic acid-differentiated cells (not shown). Fig. 10 shows also that ouabain treatment caused a large inhibition of Ins(1,4,5)P_3 generation. However, consistent with the results on plasma membrane potential and $[Ca^{2+}]_i$ rises shown in Figs. 4 and 5, the effects of ouabain on Ins(1,4,5)P_3 generation were smaller than that of gramicidin D (compare Fig. 9*A* with 10).

Finally, no significant inhibition of Ins(1,4,5)P_3 production upon fMet-Leu-Phe stimulation could be demonstrated when DMSO-differentiated HL-60 cells were incubated in the presence of monensin (1 μM) + nigericin (1 μM) (7 ± 3%, mean ± S.D., n = 4 in two separate experiments).

DISCUSSION

It is increasingly appreciated that a single membrane surface receptor may be linked to the generation of multiple intracellular signals, often with both stimulatory and inhibitory activity on cell responses. This generates a complex interplay of intracellular messages that dictate whether a given cell response will eventually be generated or aborted. Chemotactic receptors in myelocytic cells are good examples of receptors associated to the generation of both stimulatory and inhibitory signals. In this cell type, activation of the phosphoinositide turnover, mobilization of intracellular Ca^{2+}, and increased influx of extracellular Ca^{2+} are thought to represent activatory signal, while the cyclic AMP increase has inhibitory functions (23). The physiological meaning of the changes in plasma membrane potential triggered by chemotactic substances is still puzzling (4, 9, 24); these cells are not excitable and do not possess voltage-gated Ca^{2+} channels (25, 26).

[2] D. Pittet and D. P. Lew, manuscript in preparation.

In a previous paper (6), we suggested that, in neutrophils, plasma membrane depolarization might represent a feedback mechanism devised to dampen agonist-induced increases of $[Ca^{2+}]_i$. This hypothesis was mainly based on the effects of the pore-forming ionophore gramicidin D, which inhibited both Ca^{2+} influx and Ca^{2+} mobilization. Regulation of Ca^{2+} entry into nonexcitable cells by plasma membrane potential is now a widely recognized phenomenon (6–8) and there is general agreement that depolarization decreases, while hyperpolarization increases, Ca^{2+} influx through agonist-activated Ca^{2+} channels, contrary to excitable cells where depolarization opens voltage-gated Ca^{2+} channels.

There are a number of ways to depolarize plasma membrane potential in intact cells. All of them however will also alter other cellular parameters (intracellular cation and anion distribution, pH, receptor number, and affinity, etc . . .), and, in order to make a cause-effect relationship between depolarization and $[Ca^{2+}]_i$ rises, one should exclude that the inhibitory effect is due to one of these side effects. Isoosmotic substitution of NaCl with KCl is the most classical procedure to depolarize plasma membrane potential; this treatment however proved not to be a suitable means in our cell model to test the effect of plasma membrane depolarization since high KCl (*a*) depletes intracellular Ca^{2+} stores and (*b*) increases the affinity of fMet-Leu-Phe receptor for the agonist (6, 9). Roberts *et al.* (9) have previously shown complex changes on neutrophil physiology induced by high KCl, in addition to increases in fMet-Leu-Phe receptor number and affinity (see also Ref. 6). Gramicidin D, on the other hand, appears in our model the most efficient depolarizing agent, and its inhibitory effects can most likely be attributed to membrane potential collapse. This latter conclusion is based on the following evidence: (*a*) the dose dependence inhibition of $[Ca^{2+}]_i$ rises by gramicidin D is superimposed on the dose dependence of gramicidin D-induced depolarization; (*b*) when maximal depolarization is obtained, a further increase in gramicidin D concentration has no additional effects on $[Ca^{2+}]_i$ rises, and the ED_{50} for gramicidin D inhibition is independent of fMet-Leu-Phe concentration; (*c*) the effect of gramicidin D is observed for both fMet-Leu-Phe in DMSO- and LTB_4 in retinoic acid-differentiated HL-60 cells, thus excluding a specific inhibitory effect on fMet-Leu-Phe receptor (27). An alternative to gramicidin D was long term ouabain treatment. Ouabain proved to be a convenient tool for depolarizing HL-60 cells without obvious undesirable side effects. In fact: (*a*) long term treatment with ouabain causes plasma membrane potential depolarization and inhibits $[Ca^{2+}]_i$ rises induced by both chemotactic ligands and (*b*) the extent of plasma membrane depolarization parallels its effects on Ca^{2+} release triggered by the agonist. In particular, the degree of inhibition of $[Ca^{2+}]_i$ rises by ouabain treatment was very similar to that caused by doses of gramicidin D which caused equivalent depolarization of the plasma membrane potential.

Treatment of cells with gramicidin D and ouabain alters profoundly the intracellular Na^+ and K^+ concentration by causing extensive Na^+ overloading and K^+ depletion. However, the observation that gramicidin D also inhibits the $[Ca^{2+}]_i$ rises caused by low fMet-Leu-Phe concentrations in Cs^+ medium is against a major inhibitory role of Na^+ overloading, although under these conditions K^+ depletion still occurs. Therefore, we cannot exclude that the effects of gramicidin D on $[Ca^{2+}]_i$ homeostasis are, at least in part, due to intracellular K^+ deprivation. On the other hand, treatment of HL-60 cells with nigericin plus monensin, which causes a large decrease in intracellular K^+ concentration,[3] is without significant effect on $[Ca^{2+}]_i$ transients induced by fMet-Leu-Phe or LTB_4.

The inhibition of Ca^{2+} release by plasma membrane depolarization is paralleled, and probably caused, by the inhibitory effect on Ins(1,4,5)P_3 formation. At variance with the effect on Ca^{2+} release, inhibition of Ins(1,4,5)P_3 generation was observed at all fMet-Leu-Phe concentrations tested. This apparent contradiction is however explained by the high sensitivity of the Ca^{2+} release mechanism to Ins(1,4,5)P_3. In fact, only 30–40% of the maximal amount of producible Ins(1,4,5)P_3 is sufficient to give maximal increases in $[Ca^{2+}]_i$ (compare Figs. 9 and 10). Thus, even a large inhibition of Ins(1,4,5)P_3 production by gramicidin D or ouabain (50 to 60% at 10^{-6} M fMet-Leu-Phe) still allows enough Ins(1,4,5)P_3 formation to generate maximal Ca^{2+} mobilization.

It should be stressed that the effect of membrane potential collapse on Ca^{2+} release from stores and Ins(1,4,5)P_3 formation might be specific for myelocytic cells. It remains to be seen whether the observed effect may be generalized to other agonists and whether it might also play an important regulatory role in other cellular systems.

We can only speculate about the mechanism by which depolarization inhibits Ins(1,4,5)P_3 formation and thus Ca^{2+} mobilization. Changes in plasma membrane potential might easily affect protein mobility, the lipid bilayer organization, or the activity of various transmembrane enzymes. Possible mechanisms for the observed effect might thus include changes in the accessibility of phosphatidylinositol (4,5)-bisphosphate to phospholipase C or uncoupling an important regulatory subunit, such as a G protein, from the phospholipase C.

Finally, membrane potential collapse also reduced Ca^{2+} influx triggered by fMet-Leu-Phe. Unlike the effect on Ca^{2+} mobilization, but similar to the effect on Ins(1,4,5)P_3 production, the effect on Ca^{2+} influx is observed at all concentrations of agonist tested. This inhibition can be explained by the reduction of the driving force for Ca^{2+} entry across the plasma membrane, although we cannot exclude that either the chemotactic peptide-operated Ca^{2+} channels are voltage-modulated, although not voltage-gated, or that the inhibition of Ins(1,4,5)P_3 generation might be responsible for the inhibition of Ca^{2+} influx.

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