Regulation of Capacitative Ca\textsuperscript{2+} Influx in Human Neutrophil Granulocytes

ALTERATIONS IN CHRONIC GRANULOMATOUS DISEASE\textsuperscript{*}

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Ca\textsuperscript{2+} entry through the capacitative (store-regulated) pathway was shown to be inhibited in neutrophil granulocytes by the protein kinase C activator phorbol 12-myristate 13-acetate and the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP) by a hitherto unknown mechanism. Measuring both Ca\textsuperscript{2+} and Mn\textsuperscript{2+} entry into store-depleted cells we show in the present study that inhibition of the capacitative pathway is absent in several forms of chronic granulomatous disease. To establish the possible relationship between inhibition of the capacitative pathway and ability of O\textsubscript{2} production and consequent membrane depolarization, gradual changes of the membrane potential were evoked in neutrophils of healthy individuals. This was accomplished by pharmacological manipulation of the membrane potential and by variations of the concentration and type of the stimulant. Close relationship was observed between membrane depolarization and inhibition of Mn\textsuperscript{2+} entry through the capacitative transport route. Our results provide an explanation for the inhibitory action of fMLP and phorbol 12-myristate 13-acetate on capacitative cation influx and reveal that upon physiological stimulation, Ca\textsuperscript{2+} entry into neutrophils is restricted by the depolarization accompanying O\textsubscript{2} production.

Stimulation of neutrophil granulocytes by various receptor agonists is accompanied by an increase of intracellular Ca\textsuperscript{2+} concentration. This Ca\textsuperscript{2+} transient is a crucial element in the transduction of extracellular signals, leading to the activation of different effector responses (e.g. degranulation, superoxide production) of the cells (1–3). The Ca\textsuperscript{2+} signal of neutrophils consists of two components: (i) a rapid, transient increase of [Ca\textsuperscript{2+}]\textsubscript{i}, due to Ca\textsuperscript{2+} release from the internal stores induced by inositol 1,4,5-trisphosphate and (ii) a sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, due to Ca\textsuperscript{2+} influx from the extracellular space (1).

Neutrophil granulocytes do not possess voltage-dependent Ca\textsuperscript{2+} channels, but there is growing evidence that emptying of the internal Ca\textsuperscript{2+} stores initiates Ca\textsuperscript{2+} influx from the extracellular milieu, i.e. the Ca\textsuperscript{2+} content of the stores regulates Ca\textsuperscript{2+}-conducting channels in the plasma membrane (4–7). This Ca\textsuperscript{2+} entry is often referred to as capacitative or store-operated Ca\textsuperscript{2+} influx (8).

In human neutrophil granulocytes the regulation of capacitative Ca\textsuperscript{2+} influx is more complex: agonists of several chemo-tactic receptors, N-formyl-methionyl-leucyl-phenylalanine (fMLP) and platelet-activating factor, initiate Ca\textsuperscript{2+} influx by emptying the internal stores in an inositol 1,4,5-trisphosphate-dependent manner while fMLP, but not platelet activating factor, was also shown to inhibit capacitative Ca\textsuperscript{2+} influx (9). A similar action of the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) has been reported, too (9–13). The inhibitory effect of both fMLP and PMA is absent in undifferentiated HL-60 cells and develops gradually during the differentiation induced by dimethyl sulfoxide (13). Despite the similarity in the basic feature, i.e. blocking the capacitative Ca\textsuperscript{2+} influx, several differences were also described in the characteristics of the inhibition brought about by the two compounds. (i) The fMLP-induced inhibition is transient while the effect of PMA is sustained (9). (ii) Staurosporine, an inhibitor of protein kinase C prevents the inhibitory effect of fMLP only partially but blocks the effect of PMA completely (9). (iii) The inhibitory action of fMLP is sensitive to pertussis toxin whereas the effect of PMA is not influenced by the toxin (14). (iv) Okadaic acid, an inhibitor of protein phosphatases prolongs the inhibition elicited by fMLP but it does not affect the PMA-induced response (12). On the basis of these observations a role of protein phosphorylation was suggested but the intervening kinase(s) remained unidentified (9, 12–14).

The characteristics of Ca\textsuperscript{2+} influx inhibition by fMLP and PMA are remarkably similar to the features of superoxide production, evoked by the same agents (15, 16). Generation of superoxide by the NADPH oxidase induces rapid depolarization of the cells mainly due to electron transfer from the intracellular milieu to the extracellular environment (17–21). Depolarization itself was shown to inhibit the agonist-induced Ca\textsuperscript{2+} influx in several cell types (22–24). However, the possibility of a causal relationship between inhibition of capacitative Ca\textsuperscript{2+} influx by fMLP and PMA and depolarization coupled to superoxide generation elicited by the same agents has never been raised.

In the present study two approaches were used to test this hypothesis. First the effect of fMLP and PMA on capacitative Ca\textsuperscript{2+} influx was investigated in neutrophil granulocytes of patients suffering from chronic granulomatous disease (CGD), i.e. in cells which are unable to produce superoxide, but possess...
the capacitative Ca\(^{2+}\) entry mechanism. The second approach consisted of testing the inhibitory action of FMLP and PMA on Ca\(^{2+}\) influx in healthy neutrophils, where O\(_2^-\) production or membrane potential have been modulated by pharmacological means. The obtained results reveal a close relationship between plasma membrane depolarization and inhibition of Ca\(^{2+}\) influx into neutrophil granulocytes. The suggested mechanism may play a significant autoregulatory role in healthy granulocytes, whereas in CGD cells the lack of inhibition of capacitatively regulated Ca\(^{2+}\) entry may represent an alteration of cellular Ca\(^{2+}\) metabolism. To our knowledge this is the first report showing disturbed regulation of capacitative Ca\(^{2+}\) entry in a human disease.

EXPERIMENTAL PROCEDURES

Materials—Fura-2/AM was obtained from Calbiochem; Percoll from Pharmacia; dimethyl sulfoxide, PMA, FMLP, cytochrome c, and thapsigargin were from Sigma and di-O-C\(_3\) (3) from Molecular Probes. Diphenylene iodonium (DPI) was the kind gift of Dr. Arvind Nanda (Hospital for Sick Children, Toronto, Canada). All the other reagents were of research grade.

The routinely used medium (referred to as H medium) contained in mM: NaCl 145, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 0.8, HEPES 10, glucose 5, pH 7.4. The Ca\(^{2+}\)-free medium consisted of the same constituents except for CaCl\(_2\) and was supplemented with 0.5 mM EGTA. In the KCI based H medium NaCl was replaced by KCl.

Cell Isolation—Human neutrophils were prepared from blood of healthy volunteers by dextran sedimentation followed by Percoll gradient centrifugation according to the procedure described in Ref. 25. Contaminating red cells were removed by hypotonic lysis. Cells were finally suspended in H medium and kept at room temperature. Preparations contained more than 95% neutrophils, viability as determined by erythroin B dye exclusion exceeded 97%.

Characterization of CGD Patients—The diagnosis of CGD was established on the basis of serious reduction of O\(_2^-\) production and by detection of the molecular defect using Western blot analysis. Qualitative investigation of O\(_2^-\) generation was carried out by the nitro blue tetrazolium slide test as described in Ref. 26. Quantitative measurements consisted of determination of chemiluminescence (27) and cytochrome c reduction (28) following stimulation of the cells by 300 nM PMA. The amount of cytochrome b\(_{558}\) in the separated membrane fraction was determined on the basis of the heme spectrum as detailed in Ref. 29. Expression of the different subunits of NADPH oxidase was assessed by Western blot analysis using monoclonal antibodies against gp91\(^{phox}\) and p22\(^{phox}\) and polyclonal antibodies against p47\(^{phox}\). All the applied antibodies were developed and kindly provided by Professor Dirk Roos (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands). Genetic analysis of the mutation underlying CGD was carried out by Professor Dirk Roos (CLB, Amsterdam, Netherlands) and the results were reported in Ref. 30.

Four patients (N. M., L. F., A. R., and G. B.) investigated in the present study had a mutation on the cytochrome b\(_h\) (CYBB) gene and expressed neither gp91\(^{phox}\) nor p22\(^{phox}\) in their neutrophils. One patient (L. B.) with p47\(^{phox}\) deficiency had a GT deletion on the gene neutrophil cytochrome b\(_h\) factor 1 and the cytosolic protein p47\(^{phox}\) was absent in the granulocytes of this patient. Further data on the patients investigated in the present study are detailed in Ref. 30.

Measurement of [Ca\(^{2+}\)]\(_i\)—For loading with the fluorescent dye, cells (5 \times 10\(^6\)) were incubated in the presence of 4 mM Fura-2/AM for 30 min at 37 °C. Thereafter cells were washed to remove the extracellular dye and resuspended in H medium at a density of 5 \times 10\(^6\) cells/ml. Fura-2-loaded cells were stored at room temperature.

For measurement of [Ca\(^{2+}\)]\(_i\), Fura-2-loaded cells (10\(^6\)) were suspended in 3 ml of H medium and preincubated at 37 °C for 5 min prior to addition of any agent. Changes of the fluorescence were recorded in a Deltascan dual-wavelength spectrofluorimeter (Photon Technology International, South Brunswick NJ) using wavelengths 340 and 380 nm for excitation and 505 nm for emission. Measurements were performed at 37 °C with continuous stirring. [Ca\(^{2+}\)]\(_i\) was calculated from the ratio of fluorescence excited at 340 and 380 nm, following the method detailed in Ref. 31. Data were analyzed with the Felix software (PTI).

Measurement of Mn\(^{2+}\) Influx—Mn\(^{2+}\) influx measurements were performed under the same conditions as described for [Ca\(^{2+}\)]\(_i\), except that the excitation wavelength was 360 nm. At this wavelength, fluorescence of Fura-2 is quenched by Mn\(^{2+}\) but it is not influenced by changes of [Ca\(^{2+}\)]\(_i\). Unless otherwise stated, Mn\(^{2+}\) influx was initiated by adding 300 mM MnCl\(_2\).

Measurement of Membrane Potential Changes—Changes of the membrane potential were monitored by the potential sensitive fluorescent dye 2′,7′-diphenyl-1,4-oxycarbocyanine (DiIC\(_3\)) (33) as described in Refs. 17 and 32. Cells (10\(^6\)) were suspended in 3 ml of H medium supplemented with 100 mM cytochrome c. The absorbance of the sample was followed continuously at 550 nm by a Perkin-Elmer Lambda 2 spectrophotometer. For generation of O\(_2^-\) by the xanthine/xanthine oxidase system or removal of O\(_2^-\) issued by neutrophils by superoxide dismutase had no measurable effect on the fluorescence of DiIC\(_3\) under our experimental conditions.

Measurement of O\(_2^-\) Production—The amount of O\(_2^-\) generated either by neutrophils or the xanthine/xanthine oxidase reaction was determined photometrically as the superoxide dismutase inhibitable reduction of ferricytochrome c detected at 550 nm (28). Neutrophils (10\(^6\) cells) were suspended and preincubated for 5 min at 37 °C in 1.2 ml of H medium supplemented with 100 mM cytochrome c. The absorbance of the sample was followed continuously at 550 nm by a Perkin-Elmer Lambda 2 spectrophotometer. For generation of O\(_2^-\) by the xanthine/xanthine oxidase system, 3 ml of H medium supplemented with 100 mM cytochrome c and 500 mM xanthine was incubated with 20 millimolar/ml xanthine oxidase for 5 min at 37 °C. The absorbance of the sample was read after 5 min. O\(_2^-\) production was calculated using an absorption coefficient of 21 mM\(^{-1}\) cm\(^{-1}\) for cytochrome c.

Statistical Analysis—Data are presented either as representative traces of the indicated number of experiments performed on cells of different donors or as the mean ± S.E. of the number of determinations indicated (n).

RESULTS

The Effect of FMLP and PMA on the Capacitative Ca\(^{2+}\) Influx into Neutrophils of Healthy Individuals and CGD Patients—For the selective study of the capacitative Ca\(^{2+}\) influx, neutrophil granulocytes were treated with the microsomal Ca\(^{2+}\)-ATPase inhibitor thapsigargin (TG). In this way the agonist-responsive internal Ca\(^{2+}\) stores could be depleted even in the absence of increased inositol 1,4,5-trisphosphate generation and full activation of the capacitative pathway could be achieved both in the absence and presence of extracellular Ca\(^{2+}\) (34). Comparative experiments were carried out on neutrophil granulocytes of healthy individuals which produced high amounts of O\(_2^-\) upon stimulation by FMLP and PMA, and on cells of CGD patients lacking a functional oxidase. The active O\(_2^-\) producing enzyme is a multicomponent structure and genetic defects have been described in both subunits of the membrane-bound cytochrome b\(_{558}\) (gp91\(^{phox}\) and p22\(^{phox}\)), and in the two phagocyte-specific cytosolic proteins (p47\(^{phox}\) and p67\(^{phox}\)) (30, 35, 36). We carried out measurements on granulocytes of patients with verified defects in the gene of gp91\(^{phox}\) or p47\(^{phox}\).

Fig. 1 shows the effect of FMLP and PMA on the TG-induced capacitative influx of normal (A) and two different types of CGD cells (B and C). In the absence of extracellular Ca\(^{2+}\) TG elicited a transient increase of [Ca\(^{2+}\)]\(_i\), which declined slowly, due to the extrusion of Ca\(^{2+}\) by the plasma membrane pump. The resting level of [Ca\(^{2+}\)]\(_i\), as well as the amplitude and kinetics of the TG-induced increase of [Ca\(^{2+}\)]\(_i\), were basically similar in healthy and CGD cells. The addition of 1 mM CaCl\(_2\) (raising the free Ca\(^{2+}\) to approximately 0.5 mM) after 10 min incubation in the presence of TG, induced a rapid increase of [Ca\(^{2+}\)]\(_i\), both in normal (Fig. 1A, trace 1) and in CGD (Fig. 1B, C and trace 1) cells, indicating capacitative entry of Ca\(^{2+}\) into the neutrophils. In cells not treated with TG, the addition of CaCl\(_2\) caused no detectable change of [Ca\(^{2+}\)]\(_i\), confirming the necessity of store depletion for the activation of the influx (not shown). Pretreat-
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**In Normal and CGD Neutrophil Granulocytes**—The rapid rise in [Ca\(^{2+}\)], after addition of CaCl\(_2\) to store-depleted cells reflects the sum of two opposing processes: Ca\(^{2+}\) influx through the capacitative pathway and Ca\(^{2+}\) extrusion via the plasma membrane Ca\(^{2+}\) pump. The capacitative pathway of many different cell types including human neutrophils is also permeable for Mn\(^{2+}\), whereas Mn\(^{2+}\) is not a substrate for the Ca\(^{2+}\) pump. Thus, for isolated study of the capacitative influx Mn\(^{2+}\) is a better indicator than Ca\(^{2+}\) itself. As the reaction of Fura-2 with Mn\(^{2+}\) results in quenching of the fluorescence of the dye, Mn\(^{2+}\)-entry into the cells is represented by and proportional to the detected decrease of Fura-2 fluorescence.

In the following experiments we examined the effect of fMLP and PMA on the TG-evoked Mn\(^{2+}\) entry. Cells were exposed to 100 nM TG in the presence of extracellular Ca\(^{2+}\). After a 10-min incubation, 300 μM MnCl\(_2\) was added. As shown in Fig. 2A (traces 1) the addition of MnCl\(_2\) induced a rapid quenching of fluorescence representing Mn\(^{2+}\) entry via the capacitative pathway opened by the store depletion. A rapid fall in fluorescence occurred in the first 30 s (about 80% of total decrease of fluorescence), followed by a slower phase of quenching. In control experiments, where the cells were not treated with TG, MnCl\(_2\) caused only negligible decrease in fluorescence (data not shown), proving that the plasma membrane of resting neutrophils is relatively impermeable for Mn\(^{2+}\) and the dye is localized almost exclusively in the cytosol. When normal cells were stimulated by 10 nM PMA (Fig. 2A, trace 2) or 1 μM fMLP (Fig. 2A, trace 3) 1 min before the addition of MnCl\(_2\), the quenching by Mn\(^{2+}\) was strongly reduced (69.2 ± 8.9% inhibition by PMA, n = 4 and 66 ± 3.2% by fMLP n = 4) indicating that both fMLP and PMA inhibited Mn\(^{2+}\) influx into the cells. Fig. 2, B and C (traces 2 and 3), summarizes the effect of the same agonists on the TG-induced Mn\(^{2+}\) entry in gp91\(^{phox}\)-deficient (B) and p47\(^{phox}\)-deficient (C) CGD cells. As the nearly parallel experimental recordings show, PMA and fMLP were both ineffective in reducing the TG-induced Mn\(^{2+}\) entry into the cells. In four experiments carried out on gp91\(^{phox}\)-deficient CGD patients the rate of Mn\(^{2+}\) entry in the presence of fMLP or PMA was even slightly higher (by 6.7 ± 7 and 6.2 ± 4.5% respectively) than in the absence of any stimulator. In the experiment carried out on p47\(^{phox}\)-deficient neutrophils, fMLP induced 8% and PMA affected 14% inhibition of Mn\(^{2+}\) entry.

**Control of O\(_{2}\) Production and Membrane Potential Changes in CGD Neutrophils**—Both methods, widely used for investigation of Ca\(^{2+}\) entry through the capacitative pathway provided similar results: neither fMLP nor PMA inhibits this transport route in CGD cells. We wished to establish whether the altered regulation of capacitative Ca\(^{2+}\) entry into CGD cells might be related to the characteristic functional defects observed in this disease. To this end O\(_{2}\) production and the consequent membrane potential changes had to be checked in the cells of the CGD patients investigated in this study.

When stimulated with PMA, superoxide production was not detectable in the granulocytes of three gp91\(^{phox}\)-deficient patients (N. M., A. R., and G. B.). The neutrophils of one gp91\(^{phox}\)-deficient patient (L. F.) produced less than 10%, whereas the cells of the p47\(^{phox}\)-deficient patient (I. B.) produced less than 5% of the control value.

Stimulation of intact neutrophils by fMLP or PMA was shown to induce rapid depolarization of the cells, the main component of which seems to be the electrogenic operation of the oxidase. In accordance with this suggestion, CGD cells were reported to undergo only marginal depolarization upon fMLP or PMA treatment (17–21, 37).

In our experiments addition of PMA or fMLP to neutrophils obtained from healthy individuals resulted in a sudden depo-

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Fig. 1. Effect of PMA and fMLP on the capacitative Ca\(^{2+}\) influx in healthy (A), gp91\(^{phox}\)-deficient (B), and p47\(^{phox}\)-deficient (C) CGD cells. Fura-2-loaded cells suspended in Ca\(^{2+}\)-free H medium were exposed to 100 nM TG at the time points indicated by the arrows. After 9 min, cells were stimulated by 10 nM PMA (traces 2) or 1 μM fMLP (traces 3) and 1 min later the medium was supplemented with 1 mM CaCl\(_2\) as indicated by the asterisks. To unstimulated cells (traces 1) CaCl\(_2\) was added 10 min after TG. Traces are representative of four similar experiments performed with healthy cells and two similar experiments performed with gp91\(^{phox}\)-deficient CGD cells. CGD cells with p47\(^{phox}\)-deficiency were examined in one experiment.

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The Effect of fMLP and PMA on the TG-induced Mn\(^{2+}\) Influx

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The Effect of fMLP and PMA on the TG-induced Mn\(^{2+}\) Influx in Normal and CGD Neutrophil Granulocytes—The rapid rise in [Ca\(^{2+}\)] after addition of CaCl\(_2\) to store-depleted cells reflects the sum of two opposing processes: Ca\(^{2+}\) influx through the capacitative pathway and Ca\(^{2+}\) extrusion via the plasma membrane Ca\(^{2+}\) pump. The capacitative pathway of many different cell types including human neutrophils is also permeable for Mn\(^{2+}\), whereas Mn\(^{2+}\) is not a substrate for the Ca\(^{2+}\) pump. Thus, for isolated study of the capacitative influx Mn\(^{2+}\) is a better indicator than Ca\(^{2+}\) itself. As the reaction of Fura-2 with Mn\(^{2+}\) results in quenching of the fluorescence of the dye, Mn\(^{2+}\)-entry into the cells is represented by and proportional to the detected decrease of Fura-2 fluorescence.

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In our experiments addition of PMA or fMLP to neutrophils obtained from healthy individuals resulted in a sudden depo-
lizarization of similar amplitude but with different kinetics (Fig. 3). Whereas PMA induced a stable depolarization lasting over 5 min (Fig. 3A), the fMLP-evoked depression of the membrane potential showed a partial recovery after 2 min (Fig. 3B). Both PMA and fMLP had only a marginal effect on the membrane potential of neutrophil granulocytes obtained either from gp91phox-deficient (Fig. 3) or p47phox-deficient (not shown) patients. Our measurements carried out on healthy and CGD cells are thus in full agreement with previous reports (17, 18, 37).

**Fig. 2. Effect of PMA and fMLP on the capacitative Mn2+ influx in healthy (A), gp91phox-deficient (B), and p47phox-deficient (C) CGD cells.** Fura-2-loaded cells suspended in H medium were incubated with 100 nM TG. After 9 min, cells were stimulated by 10 nM PMA (traces 2) or 1 μM fMLP (traces 3) and 300 μM MnCl₂ was added 1 min later, as indicated by the asterisks. To unstimulated cells (traces 1) MnCl₂ was added 10 min after TG. Traces are representative of four similar experiments performed with healthy cells, four experiments with gp91phox-deficient cells and show the result of one experiment carried out with p47phox-deficient CGD cells.

Correlation between Membrane Potential and Inhibition of the Capacitative Influx in Healthy Neutrophil Granulocytes—The experiments carried out on neutrophil granulocytes of CGD patients revealed that both fMLP and PMA failed to inhibit capacitative Ca²⁺ influx in these defective cells and they both failed to induce any significant depolarization. In our next experiments we wanted to test the correlation of these two parameters in healthy neutrophils under conditions where gradual changes of the membrane potential could be evoked.

As a first approach we tried to reduce the agonist induced depolarization by pharmacological means and study the effect of fMLP and PMA on the Ca²⁺ influx under these conditions. For this purpose we applied a combined pretreatment using two drugs which affect charge movements in two different ways. The NADPH-oxidase inhibitor DPI reduces the rate of electron efflux through the oxidase (38) whereas the K⁺-selective ionophore, valinomycin, allows charge compensation through outward movement of K⁺. These two agents with different sites of action were previously described to clamp efficiently the PMA-induced depolarization (33). The addition of 10 nM PMA after 10 min incubation with TG induced a rapid depolarization response (Fig. 4A, trace b) whereas pretreatment of the cells with valinomycin and DPI largely diminished the depolarizing effect of PMA (Fig. 4A, trace a). Clamping of the membrane potential by these agents was similarly effective when fMLP was used as a stimulus (Fig. 4B). Both DPI and valinomycin, when applied separately, were able to reduce only partially the membrane potential change induced by either stimulating agent (data not shown).

The effect of pretreatment with valinomycin and DPI on the PMA-induced inhibition of the capacitative influx is demonstrated in Fig. 4C. Addition of CaCl₂ to store-depleted control cells resulted in an increase of [Ca²⁺]i from 100 nM to approximately 250 nM (trace 1), whereas in PMA-stimulated cells the same treatment induced a rise of [Ca²⁺]i only to approximately 130 nM (trace 2). Pretreatment of the cells with valinomycin and DPI (trace 3) eliminated the inhibitory effect of PMA and the increase of [Ca²⁺]i was similar to the value observed without PMA treatment. The inhibitory effect of fMLP on the capacitative Ca²⁺ influx was also prevented by DPI plus valinomycin (Fig. 4D). When DPI or valinomycin was used alone to attenuate the fMLP- or PMA-induced membrane potential changes the inhibition of the influx was only partially antagonized (traces not shown). Prevention of membrane potential changes also effectively counteracted the inhibitory effect of both fMLP and PMA on Mn²⁺ entry into TG-pretreated cells (Fig. 5).

A parallel increase was observed in membrane depolarization and inhibition of Mn²⁺ entry when the concentration of the receptor agonist fMLP was varied between 1 nM and 1 μM, i.e. in the range where O₂⁻ production is augmented from just detectable to maximal intensity. Also platelet-activating factor, another chemotactic receptor agonist which evokes a similar Ca²⁺ signal as fMLP but has only slight effect on O₂⁻ production (39) induced partial depolarization and reduced the rate of Mn²⁺ entry into TG-treated cells moderately (data not shown).
The close relationship between membrane potential changes and inhibition of the capacitative Mn$^{2+}$ influx observed under these remarkably different experimental conditions suggests a causative relation.

The Effect of Superoxide Anions and Oxidase-independent Depolarization on the Capacitative Mn$^{2+}$ Influx—In the above described experiments attenuation of the agonist-induced depolarization was accompanied by a decrease in the superoxide production. To test the possible role of superoxide anions themselves in the inhibition of Ca$^{2+}$ influx via the capacitative pathway, we examined the TG-induced Mn$^{2+}$ influx under conditions where superoxide was generated by xanthine oxidase in the presence of xanthine. The concentration of the enzyme and its substrate were chosen so that a similar amount of O$_2^-$ was formed as in the case of stimulation of the cells by fMLP. Superoxide production by the xanthine/xanthine oxidase system did not affect the membrane potential of the cells (not shown) and did not inhibit the TG-induced Mn$^{2+}$ influx (Fig. 6A). Apparently O$_2^-$ anions themselves are not responsible for the inhibitory action of fMLP and PMA on capacitative Ca$^{2+}$ influx, supporting our view that the change of the membrane potential resulting from the electrogenic operation of the enzyme may be the decisive factor.

To further substantiate the role of depolarization in the agonist-induced inhibition of the store-regulated influx, we examined the effect of oxidase-independent depolarization on the capacitative Mn$^{2+}$ influx. We followed the TG-induced Mn$^{2+}$ entry into cells, suspended in a KCl-based medium supple-
Regulation of Capacitative Ca\textsuperscript{2+} Influx in Neutrophils

FIG. 6. Effect of superoxide anions (A) and NADPH oxidase independent depolarization (B) on the capacitative Mn\textsuperscript{2+} influx. A, Fura-2-loaded cells were incubated in H medium containing 500 \mu M xanthine and 100 nM TG. After 9 min, xanthine oxidase (20 milliunits/ml) and 1 min later 300 \mu M MnCl\textsubscript{2} were added as indicated by the asterisks (trace 1). To control cells (trace 2) MnCl\textsubscript{2} was added 10 min after TG. Traces are representative of three similar experiments. B, Fura-2-loaded cells were incubated in H medium (trace 2) or KCl based H medium, containing 3 \mu g/ml valinomycin (trace 1). In both cases 300 \mu M MnCl\textsubscript{2} was added 10 min after 100 nM TG. Traces are representative of two similar experiments.

Does Membrane Depolarization Interfere with Divalent Cation Entry during Physiological Stimulation of Neutrophil Granulocytes?—In all the previous experiments, Ca\textsuperscript{2+} or Mn\textsuperscript{2+} entry was investigated in TG-pretreated cells, i.e. under conditions where the capacitative pathway has been opened by extensive depletion of the agonist-responsive internal Ca\textsuperscript{2+} stores. In this experimental setup a clear correlation was revealed between membrane depolarization (induced by various mechanisms) and inhibition of the capacitative Ca\textsuperscript{2+} pathway. The question arises whether this mechanism plays any detectable role during activation of intact (non-depleted, non-predepolarized) cells. To address this problem, Mn\textsuperscript{2+} entry was followed in neutrophils incubated in H medium containing 1 mM CaCl\textsubscript{2}. In the experiment demonstrated in Fig. 7, fMLP was added after Mn\textsuperscript{2+}, so that the entry of divalent cation(s) occurring in the very early phase of cell stimulation could also be detected. As shown in trace 2, in healthy neutrophils the decrease of fluorescence indicating Mn\textsuperscript{2+} entry was delayed for almost 2 min after fMLP addition. This lag phase corresponds to the period where the majority of fMLP-induced O\textsubscript{2} production takes place and membrane depolarization reaches its maximum (17, 40). In sharp contrast to the normal cells, addition of fMLP was followed by instantaneous Mn\textsuperscript{2+} influx in the neutrophils of gp91\textsuperscript{phox}-deficient CGD patients (trace 1). The result shown in Fig. 7 clearly indicates that in healthy cells the decrease of the plasma membrane potential that accompanies O\textsubscript{2} production prevents the entry of divalent cations during the early phase of stimulation. This preventive mechanism is absent in the granulocytes of CGD patients.

The question arises whether the alteration of Ca\textsuperscript{2+} entry detected in CGD cells has any influence on the Ca\textsuperscript{2+} metabolism of these cells. In accordance with previous results obtained with Quin-2 as Ca\textsuperscript{2+}-sensitive dye (19), the Ca\textsuperscript{2+} signal induced by fMLP was basically similar in granulocytes from healthy or CGD individuals also under our experimental conditions, using Fura-2 as fluorescent probe (Fig. 8, A and C). However, investigation of the ionomycin-releasable Ca\textsuperscript{2+} pool revealed remarkable difference between the two cell populations. In the experiment showed in Fig. 8 external Ca\textsuperscript{2+} was chelated either before or 5 min after fMLP stimulation and ionomycin was added thereafter. In healthy cells the ionomycin-induced signal was only slightly different under the two conditions whereas in CGD cells the ionomycin-releasable Ca\textsuperscript{2+} pool was definitely larger when chelation of external Ca\textsuperscript{2+} occurred 5 min after fMLP addition. Apparently in healthy cells blockade of Ca\textsuperscript{2+} entry during fMLP induced O\textsubscript{2} production impairs the refilling of intracellular Ca\textsuperscript{2+} stores, whereas in CGD cells the absence of membrane depolarization allows rapid Ca\textsuperscript{2+} entry and earlier and more complete restoration of the Ca\textsuperscript{2+} pools.

DISCUSSION

Summing up the presented experimental data, we propose that the previously described inhibition of the capacitative Ca\textsuperscript{2+} pathway of neutrophil granulocytes by the stimulators fMLP and PMA is mediated via primary changes of the membrane potential. Our hypothesis is supported by the following
observations: (i) manipulation of the membrane potential by pharmacological agents or by variation of the concentration and the type of the stimulant in store-depleted cells revealed a close relationship between depolarization and inhibition of Mn$^{2+}$ entry; (ii) in CGD cells, where both O$_2$ production and membrane depolarization are absent, neither fMLP nor PMA is able to inhibit the capacitative Ca$^{2+}$ transport pathway (Figs. 1 and 2); (iii) prevention of depolarization in healthy cells abolishes the stimulus-induced inhibition of capacitative influx (Figs. 4 and 5); (iv) inhibition of capacitative Ca$^{2+}$ entry can also be achieved when the membrane is depolarized independently of O$_2$ generation or stimulation by fMLP or PMA (Fig. 6). Our suggestion that the change of the membrane potential is the common denominator provides a suitable explanation for the previously reported characteristics of inhibition of Ca$^{2+}$ entry. Differences in the kinetics, sensitivity to staurosporine and pertussis toxin described for the inhibitory action of fMLP or PMA on Ca$^{2+}$ import reflect the properties of the capacitative pathway itself or of any intermediary enzymes. Inhibition by fluorophores or by variation of the concentration of fMLP or PMA on Ca$^{2+}$ influx induced by PMA were found to be stable for more than 5 min (Figs. 2 and 3). On the other hand, addition of the K$^+$-ionophore valinomycin to neutrophil cytoplasts suspended in a Na$^+$-based medium significantly augmented the acidification following PMA stimulation (45), whereas in our experiments valinomycin partially antagonized both the inhibition of Ca$^{2+}$ and Mn$^{2+}$ entry and membrane depolarization brought about by PMA or fMLP. Apparently, changes of pH$_i$ do not occur in parallel with inhibition of Ca$^{2+}$ influx and in view of the above arguments we consider the alteration of the membrane potential as the decisive factor in determining Ca$^{2+}$ entry through the capacitative pathway.

The presented experiments allow some speculation about the possible mechanism of inhibition of capacitative Ca$^{2+}$ entry by membrane depolarization. In resting neutrophil granulocytes suspended in a medium containing 1 mM Ca$^{2+}$, the applied fluorescent dye detected an internal [Ca$^{2+}$] of about 100 nm, suggesting that a 10$^5$-fold concentration gradient (equalling 120 mV membrane potential change) could drive Ca$^{2+}$ inward. However, in view of recent reports demonstrating a significantly higher [Ca$^{2+}$] in the subplasmalemmal space than in the averaged cytosol (46, 47), the true concentration gradient is at least 1 order of magnitude smaller. The membrane potential of resting neutrophils is suggested to be around −60 mV but upon stimulation with PMA it was reported to overshoot up to positive values (20, 33). A drop of 60 mV or more in the driving force for Ca$^{2+}$ could certainly slow down the inward movement of the divalent cation considerably. This suggestion is supported by our control experiment where a decrease in the concentration of external Ca$^{2+}$ added to TG-pretreated cells (in the same experimental setting as shown in Fig. 1) from 500 to 50 μM resulted in a measurable impairment of Ca$^{2+}$ entry. Thus in the case of human neutrophil granulocytes, the decrease in the driving force for Ca$^{2+}$ ions due to the extensive depolarization following fMLP or PMA stimulation is certainly sufficient to cause detectable diminution of Ca$^{2+}$ influx by itself. Whether in addition to the drop in the driving force depolarization also affects the conductivity of the capacitative
cation pathway remains to be elucidated by electrophysiologi-

cally measurements.

Irrespective of the mechanism of action, the phenomenon that Ca$^{2+}$ entry is blocked by depolarization seems not to be restricted to neutrophil granulocytes. Various experimental means of depolarization were reported to inhibit agonist-induced Ca$^{2+}$ influx in T-lymphocytes stimulated via the antigen receptor (23), in platelets upon thrombin stimulation (22), and in parotid acinar cells upon carbachol stimulation (24). Inhibition of voltage-dependent K$^+$-channels decreased Ca$^{2+}$ import induced by anti-CD2 and anti-CD3 antibodies in T lymphocytes. This effect is probably mediated by membrane depolarization, too (48). This apparently general mechanism may have a significant biological role in phagocytic cells where physiological stimulation results in drastic drop of the membrane potential. Inhibition of Ca$^{2+}$ entry by membrane depolarization may represent an autoregulatory mechanism limiting Ca$^{2+}$ uptake following cell stimulation. According to the result shown in Fig. 7, this mechanism is operating during physiological stimulation of granulocytes: detectable influx of divalent cations begins only after the rate of O$_2$ production declines and other compensatory mechanisms (e.g. opening of the conductive H$^+$ transport pathway (49, 50)) contribute to the restoration of the membrane potential.

Investigation of neutrophil granulocytes obtained from CGD patients revealed that independently of the genetic form of the disease, neither fMLP nor PMA is able to restrict Ca$^{2+}$ influx via the capacitative pathway and stimulation of CGD neutrophils by fMLP induces intensive flow of Ca$^{2+}$ ions from the extracellular space into the cell at an earlier time point than in normal granulocytes. The difference in Ca$^{2+}$ entry is not reflected in the fluorescent signal detectable with Quin-2 (19) or fura-2 following fMLP stimulation. In evaluating this apparent contradiction it should be recalled that both Ca$^{2+}$-sensitive dyes are inefficient in detecting the correct [Ca$^{2+}$i] at specialized cell regions like the subplasmalemmal space where the [Ca$^{2+}$], can exceed the cellular average by 1 or 2 orders of magnitude (46, 47). In view of these considerations, our observation on the absence of inhibition of Ca$^{2+}$ entry and faster restoration of the internal Ca$^{2+}$ stores following fMLP stimulation may represent a biologically important alteration of Ca$^{2+}$ metabolism of these defective cells. The contribution of this alteration to the pathomechanism of CGD has to be clarified in future investigations.

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REFERENCES
1. Scharff, O., and Foder, B. (1993) Physiol. Rev. 73, 547–582
2. Pozzan, T., Lew, D. P., Wollheim, C. B., and Tsien, R. Y. (1983) Science 221, 1413–1415
3. Sengelov, H., Kjeldsen, L., and Borregaard, N. (1995) J. Immunol. 150, 1535–1543
4. Montero, M., Alvarez, J., and Garcia Sancho, J. (1993) Biochim. J. 277, 73–79
5. Demauxre, N., Monod, A., Lew, D. P., and Krause, K. H. (1994) Biochem. J. 297, 595–601
6. Demauxre, N., Schlegel, W., Várnai, P., Mayr, G., Lew, D. P., and Krause, K. H. (1992) J. Clin. Invest. 90, 830–839
7. Geiszt, M., Kaldí, K., Széberényi, J. B., and Ligeti, E. (1995) Biochem. J. 305, 525–529
8. Putney, J. W. (1990) Cell Calcium 11, 611–624
9. Montero, M., García Sancho, J., and Alvarez, J. (1993) J. Biol. Chem. 268, 13055–13061
10. McCarthy, S. A., Hallam, T. J., and Merritt, J. E. (1989) Biochem. J. 264, 357–364
11. Foder, B., Scharff, O., and Thastrup, O. (1989) Cell Calcium 10, 477–490
12. Montero, M., García Sancho, J., and Alvarez, J. (1994) J. Biol. Chem. 269, 2661–2668
13. Kvačák, T., Tordai, A., Szási, I., Sarkadi, B., and Gárdos, G. (1990) FEBS Lett. 266, 171–174
14. Sengelov, H., Kjeldsen, L., and Borregaard, N. (1993) EMBO J. 12, 3440–3450
15. Segal, A. W. (1996) Methods Enzymol. 265, 358–365
16. Roos, D., de Boer, M., Borregaard, N., Bjerum, O. W., Valerius, N. H., Segal, R. A., Muhlebach, T., Belohradsky, B. H., and Weening, R. S. (1992) J. Leukocyte Biol. 51, 164–171
17. Smith, R. M., and Curnutte, J. T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1098–1101
18. Meritt, J. E., and Rink, T. J. (1987) J. Biol. Chem. 262, 17362–17369
19. Geiszt, M., Káldi, K., Széberényi, J. B., and Ligeti, E. (1995) J. Immunol. Methods 43, 95–101
20. Johansen, K. S. (1983) Acta Pathol. Microbiol. Immunol. Scand. C 91, 349–354
21. Allen, R. C. (1986) Methods Enzymol. 133, 449–493
22. Markert, M., Andrews, P. C., and Babir, B. M. (1984) Methods Enzymol. 105, 358–365
23. Yang, S., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1999) J. Biol. Chem. 274, 10816–10820
24. Thastrup, O., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1999) J. Biol. Chem. 274, 10816–10820
25. Henderson, L. M., Chappel J. B., and Jones O. T. G. (1988) J. Lab. Clin. Med. 125, 392–401
26. Ellis, J. A., Mayer, S. J., and Jones, O. T. (1988) Biochem. J. 251, 877–891
27. Dewald, B., and Baggiolini, M. (1986) Biochim. Biophys. Acta 888, 42–48
28. Watson, F., Robinson, J., and Edwards, S. W. (1991) J. Biol. Chem. 266, 7432–7439
29. Levy, R., Rotrosen, D., Nagaoka, O., Leto, T. L., and Malech, H. L. (1990) J. Leukocyte Biol. 48, 251–257