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Local Changes in Membrane Potential Intensify Neutrophil Oxidative Burst

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Abstract: The aim of this paper is to study the effects of the pulsed electrical field/current alone or combined with ionomycin, fMLP and PMA, the chemical stimuli that operate through distinctly different activation pathways, on the time course of the oxidative burst response in human neutrophils. Neither the control groups nor the neutrophils treated with electrical field alone showed any increase in oxidative burst activity measured by the luminol-enhanced chemiluminescence technique. It was found that electrical treatment potentiates chemically induced activation with either of the chemical stimulators used. The integrated oxidative burst response—which represents a cumulative amount of oxygen metabolites produced during whole response—was 87% higher in neutrophils treated with a combination of ionomycin and an electric field than in solely ionomycin treated cells, while the peak level of the response was 114% higher. In neutrophils stimulated with fMLP the electrochemical treatment caused a 32% higher integral response as well as a 22% higher peak level compared to the neutrophils treated with chemical stimulant alone. The integrated oxidative burst response in the combined PMA and electric treatment was only 4.7% higher than in the cells treated with PMA alone, and no significant difference in the peak level was found. The results suggest that electric field treatment preferentially stimulates calcium-induced activation with ionomycin rather than calcium-dependent activation with fMLP or PMA.

POLYMORPHONUCLEAR LEUKOCYTES (neutrophils) play a major role in the body's defense against harmful foreign pathogens, as evidenced by the often life-threatening infections acquired by patients with chronic granulomatous disease, neutropenia, leukemia, or congenital diseases affecting leukocyte structure and function (1). In order to perform this

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Abbreviations: CL—chemiluminescence; DMSO—dimethylsulfoxide; fMLP—N-formylmethionylleucyl-phenylalanine; HBSS—Hank's balanced salt solution; luminol—5-amino-2,3dihydro-4-phthalazinedione; O_2^-—superoxide anion; OB—oxidative burst; PEF—pulsed electric field/current; PMA—phorbol myristate acetate.

role, neutrophils migrate from the circulation to sites of tissue damage or inflammation, where they phagocytize and destroy micro-organisms and damaged tissue with an array of microbicidal oxidants, proteolytic enzymes, and antimicrobial peptides. The process of neutrophil activation includes several responses, e.g., cell shape changes, aggregation, phagocytosis, granule enzyme secretion, and stimulation of an oxidative burst (OB). The OB is a production of superoxide anion (O_2^-) and a variety of reduced oxygen metabolites that are essential for the microbial activity of neutrophils. It involves the one-electron reduction of molecular oxygen to superoxide, catalyzed by a membrane-bound NADPH oxidase complex (2).

So far, studies of the biochemical stimulus-response pathways in neutrophils have revealed the increased intracellular messengers levels, such as Ca^{2+} and protein kinases (for review see references (3–5)). Protein kinase C, an ubiquitous enzyme requiring Ca^{2+} and diacylglycerol for maximal activity, appears to play an integral role in signal transduction (6). Tumor-promoting phorbol ester, phorbol myristate acetate (PMA) may substitute for diacylglycerol and directly activates protein kinase C followed by the activation of the NADPH oxidase assembly (6). Stimulation of the neutrophil is also associated with a rise in an intracellular Ca^{2+}. The Ca^{2+} and the protein kinase C are believed to act synergistically in activating the OB response. Receptor binding, such as chemotactic peptide N-formyl-methionylleucylphenylalanine (fMLP), causes phospholipid turnover leading to the activation of the protein kinase C and the increase in intracellular Ca^{2+} (7). A rise of the cytosolic free Ca^{2+} consists of two components, a release of Ca^{2+} from intracellular stores and an influx of Ca^{2+} across the plasma membrane. Neutrophils in which intracellular Ca^{2+} concentration is driven to high levels by ionophores (A23187 or ionomycin)—the mobile ion carriers for Ca^{2+}—promptly degranulate and produce cytotoxic metabolites (8, 9).

The early events that follow the stimulation of the neutrophil also include plasma membrane potential variations and complex changes in the internal pH (10–12). There is growing evidence that the plasma membrane potential changes are an important determinant of the activity of neutrophils. Neutrophils depolarize in response to receptor agonists, but also in response to agonists that bypass the receptor mechanism, e.g. ionomycin or PMA. The physiological role of depolarization in neutrophils is different from that in excitable cells. Neither Ca^{2+} influx nor cellular activation is observed after depolarization by high extracellular K^+ or by Na^+/K^+ ionophores such as gramicidin (13). In contrast, if depolarization precedes stimulation by chemoattractants, the increase in cytosolic Ca^{2+} and cellular responses is diminished. A role of depolarization as negative feed back during neutrophil activation has therefore been proposed (13, 14). In addition, depolarization might be important for the efflux of the excessive H^+ ions generated through OB response (15). The role of plasma membrane hyperpolarization in stimulus-response coupling in neutrophils remains dubious despite suggestion that it increases the Ca^{2+} influx across membrane and potentiates the neutrophil responses (13, 16).

An external electric field/current can produce a variety of biochemical or physiological changes in biological systems. The global effect may be the result of local electric field stimulation at cellular level, in which one end of the cell is depolarized while the other end is hyperpolarized. The spatial variation of the transmembrane potential (Φ_m) induced by an external field can be calculated by solving Laplace's equation with appropriate boundary conditions. For spherical cells, like neutrophils, a simplified solution of this problem follows equation: $\Delta\Phi_m = 1.5\, E\, r \cos\theta$, where E is the electric field, r is the cell radius and θ is the

angle between the direction of the field and normal to the cell surface (17). Theoretical prediction of transmembrane potential changes ($\Delta\Phi_m$) caused by external electric field was also experimentally confirmed using potential-sensitive dyes and fluorescence microscopy imaging (18).

In our previous study (19), it was shown that an external electric field—which should theoretically change the resting membrane potential of neutrophils up to several tenths millivolts at poles faced to electrodes—amplifies the PMA-induced OB in neutrophil. The results suggested that the stimulatory effect of the external electric field was closely related to cellular Ca^{2+} mobilization from extracellular space, since depletion of extracellular Ca^{2+} highly suppressed the OB potentiation.

In the present study, the pulsed electric field/current (PEF) was used, alone or combined with ionomycin, fMLP and PMA—chemical stimuli that operate through distinctly different activation pathways—to assess a further correlation of electrically induced $\Delta\Phi_m$ changes to the OB response. The luminol-enhanced chemiluminescence (CL) technique was used for estimation of the neutrophil OB response (20).

Materials and Methods

Chemicals

PMA, fMLP, ionomycin, luminol (5-amino-2,3-dihydro-4-phthalazinedione), Hank's balanced salt solution (HBSS) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, USA). PMA, fMLP, ionomycin and luminol were dissolved in DMSO and added to the cell suspension to give a final concentration of 3 μM PMA, 1.5 μM fMLP or ionomycin, 10 μM luminol and 0.1% DMSO (v/v) in HBSS.

Neutrophil OB activation

Venous blood (5 ml) was taken from healthy adults (18–45 years old) into heparinized (50 IU) tubes. Their hematological characteristics, such as the leukocyte count (Coulter Counter, USA), the differential count and erythrocyte sedimentation rate, were within normal range (21). Blood samples were used to assess neutrophil activation without any other manipulation. At the beginning, 200 μl of heparinized blood was resuspended in 400 μl HBSS. With regard to activation treatment, blood samples were divided into four groups: control, PEF, chemical stimulation (ionomycin, fMLP or PMA) and a combination of chemical stimulant and PEF. All experimental groups consisted of seven samples. During treatment, samples of all groups were shaken in cuvettes with electrodes at room temperature.

A 1.5 μM final concentration of ionomycin or fMLP, and a 3 μM final concentration of PMA in HBSS, were used for chemical activation of OB. For electric treatment, four-second trains of biphasic, asymmetrical, charge-balanced pulses, separated by four-second pauses were used; the pulse amplitude was 20 mA, frequency 40 Hz, and the pulse duration 0.25 ms. The amplitude of the electric field in cell suspension was up to 40 V/cm. Due to the round shaped geometry of cuvettes and needle electrodes, the electric field in suspension was not uniform. Voltage drops in cuvettes were measured with Ag/Ag-Cl electrodes (Clark, UK) and a high input impedance voltage measurement device. The PEF was applied for one minute through Pt-Ir (90/10%) electrodes directly immersed in suspension. During treatment, the signals were monitored by current probe-oscilloscope combination (Tek-

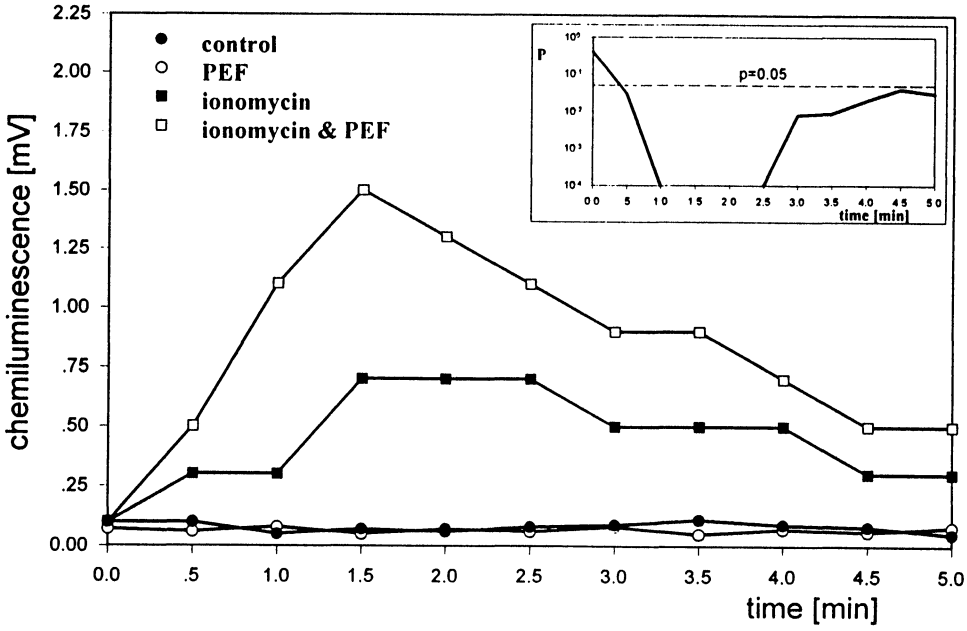

FIGURE 1. The time course of oxidative burst responses in human neutrophils stimulated with the 1.5 μM ionomycin (–■–), one minute of the pulsed electric field/current, PEF, (–○–) and their combination, ionomycin and PEF, (–□–). Filled circles, (–●–) symbolize the control. Each point represents the mean of seven samples. The statistical significance of the difference between ionomycin-stimulated oxidative burst response and the response of neutrophils treated with electric current and ionomycin was analyzed point by point by a paired Student's t-test, and is shown in the inset. The dashed line represents the statistical significance at level P=0.05.

tronix P6042/7704, USA). All hematological parameters listed above were checked after treatment with PEF. Not even 60 min of PEF caused any significant changes (data not shown).

Chemiluminescence (CL) assay

CL was measured by a LKB-Wallac 1250 luminometer in polystyrene measuring cuvettes (Clinicon, Finland). After particular activation, or control treatment, 400 μl of luminol solution in HBSS were added to each cuvette. The cuvettes were then transferred into the counting chamber, and the results, expressed in mV, are the mean values of measurements lasting for ten seconds. A time course of the OB response was chosen as the main estimation value. The statistical significance of the results was analyzed by a paired Student's t-test.

Results

The effects of the pulsed electrical field/current and three different activators, ionomycin, fMLP and PMA, on the time courses of the oxidative burst response in human neutrophils

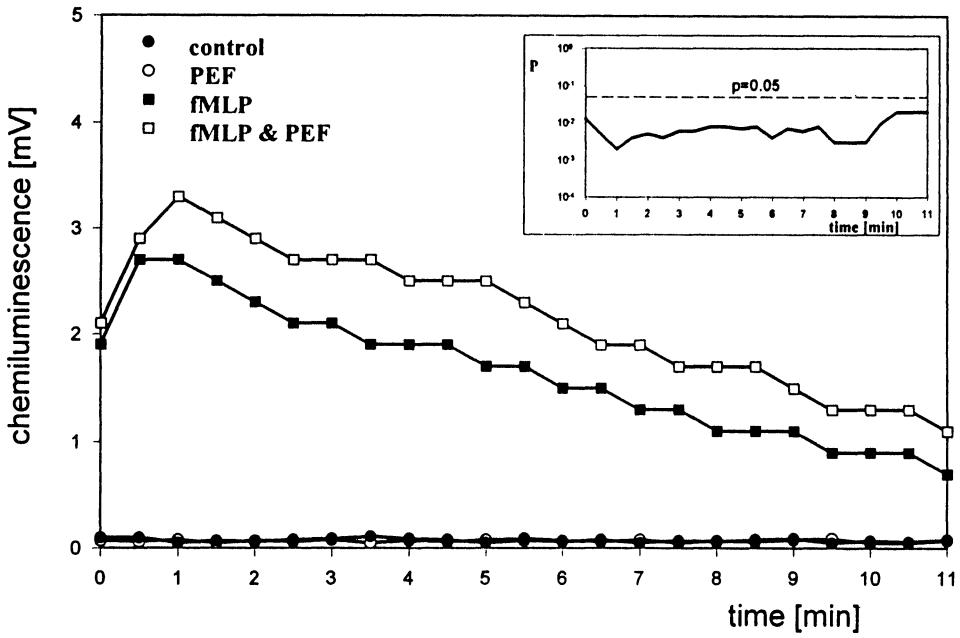

FIGURE 2. The time course of oxidative burst responses in human neutrophils stimulated with the 1.5 μM fMLP (–■–), one minute of the pulsed electric field/current, PEF, (–○–) and their combination, fMLP and PEF, (–□–). Filled circles (–●–) symbolize the control. Each point represents the mean of seven samples. The statistical significance of the difference between fMLP stimulated oxidative burst response and the response of neutrophils treated with electric current and fMLP was analyzed point by point by a paired Student's t-test, and is shown in the inset. The dashed line represents the statistical significance at level P=0.05.

were studied. In the figures, the time courses of OB responses are represented as follows: filled circles symbolizing the control; open circles, solely electric treatment; filled squares, the chemical stimulation; and open squares, the combination of chemical and electrical stimulation. Each point in the OB response curves represents the mean of seven samples.

In the control groups, the chemiluminescence of the samples after addition of luminol was measured, and no observable OB response was noticed. The OB responses of normal human neutrophils to ionomycin was characterized (Figure 1) by a gradual increase in the chemiluminescence intensity, which reached peak levels in approximately three minutes after the beginning of the stimulation. The whole OB response lasts from five to eight minutes, relative to the different blood donors.

In comparison to ionomycin the OB responses to fMLP were stronger and more rapid (Figure 2). The responses reached peak levels in the first two minutes of stimulation and were characterized by prolonged and intensive production of the oxidants for another ten to fifteen minutes.

Activation of neutrophils with PMA caused OB responses lasting almost one hour (Figure 3). There was a characteristic delay of several minutes from addition of the chemical stimulant to the onset of the oxygen metabolites production. The peak levels of OB in

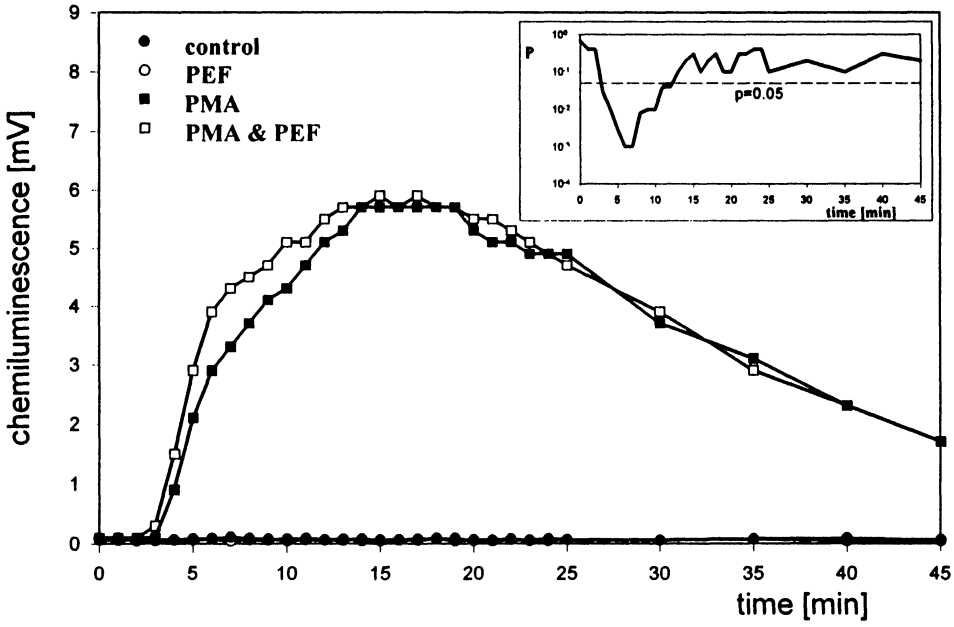

FIGURE 3. The time course of oxidative burst responses in human neutrophils stimulated with the 3 μM PMA (–■–), one minute of the pulsed electric field/current, PEF, (–○–) and their combination, PMA and PEF, (–□–). Filled circles (–●–) symbolize the control. Each point represents the mean of seven samples. The statistical significance of the difference between PMA stimulated oxidative burst response and the response of neutrophils treated with electric current and PMA was analyzed point by point by a paired Student's t-test, and is shown in the inset. The dashed line represents the statistical significance at level P=0.05.

PMA-stimulated neutrophils were relatively two times higher than in fMLP-induced responses, and four times higher than in ionomycin-induced responses.

In order to obtain a better estimation of electric field influence on the neutrophil activation by a particular chemical stimulator, three different time and chemiluminescence axes were chosen for construction of the OB response curves. Since no time shift in the peak level or change in curve shapes of the OB responses with or without electrical treatment were observed, we chose a peak level of response and an integral OB response for numerical evaluation of the electric field effects. Integral OB responses represent the cumulative amount of oxygen metabolites produced throughout the entire OB response, and these were calculated by numerical integration of the OB response curves.

Neutrophils treated by pulsed electric field without chemical stimulant did not show any increase in oxidative burst activity. The electrical treatment of neutrophils in all models of combined chemical and electrical stimulation caused statistically significant potentiation of the production of toxic oxygen metabolites, at least in the part of the OB response. The integrated OB response of neutrophils treated with the combination of ionomycin and electrical field was 87% higher than in cells treated solely by ionomycin, while the peak

level of response was 114% higher (Figure 1). In neutrophils stimulated with fMLP (Figure 2), the combination of electrical and chemical treatment resulted in a 32% higher integral response than in the neutrophils treated with chemical stimulation alone. The peak level of the fMLP response after combined electrochemical treatment was 22% higher compared to the fMLP alone activated cells. The integrated OB response in the combined PMA and electric treatment was only 4.7% higher than in the cells treated with PMA alone (Figure 3), and no significant differences in the peak levels of responses were found.

Since there are individual differences in the OB responses of the blood donors, the statistical significance of the difference between chemically induced OB response and response of neutrophils treated with a combination of electric field and chemical stimuli was analyzed point by point by a paired Student's t-test. The results of the statistical analysis are shown on the smaller graphs in the figures. The dashed lines in the statistical graphs represent the border of statistical significance at level $P=0.05$. The differences between the ionomycin and the ionomycin and electrical field, as well as the fMLP and the fMLP and electric field treated neutrophils, were statistically significant ($P<0.05$) over the whole time range except in the first 30 seconds of the ionomycin stimulated neutrophils. In the neutrophils stimulated with PMA and the combination of PMA and electric field, the difference between OB responses of these two treatments was statistically significant only between the third and the thirteenth minute of the response.

Discussion

Despite considerable progress in the understanding of the signal transduction pathways in neutrophil, the molecular mechanism and the physiological meaning underlying membrane potential changes still remain puzzling (10, 11). The fact that depolarization of the plasma membrane is among the earliest detectable event following neutrophil stimulation (13), and that the neutrophils of patients suffering from chronic granulomatous disease fail to respond with the production of microbicidal oxidants as well as with depolarization (10, 22, 23), support the hypothesis that neutrophil activation and variations in plasma membrane potential are interrelated. It is believed that membrane potential changes during neutrophil activation might have a regulatory role in the cell response through the control of intracellular Ca^{2+} homeostasis (13, 14). The lack of the Ca^{2+} voltage-gated channels in neutrophils (23) renders impossible the modulation of Ca^{2+} influx from extracellular space due to changes in membrane permeability, as is the case in electrically excitable cells. This suggests that the Ca^{2+} influx across the plasma membrane might be affected due to changes in the driving force for Ca^{2+}. In such a case, the enhanced Ca^{2+} influx should follow the hyperpolarization of the neutrophil membrane, while the depolarization should reduce the influx. The prerequisite for this influx is that Ca^{2+} channels—which are mostly closed in the resting state of the cell—have to be opened. In neutrophils, the opening of the plasma membrane channels is mainly controlled by ligand binding. Thus, the membrane permeability for Ca^{2+} might be increased by the particular chemical stimulation which opens channels, or by incorporating calcium ionophores like ionomycin or A23187 into the cell membrane. Previous presumptions could be logically correlated to experimental evidence: first, it was shown that the neutrophil depolarization with high K^+/low Na^+ extracellular solutions is not by itself sufficient to trigger the OB response (24); second, there is evidence that depolarization has the function of negative feed-back during the neutrophil activation (13); and third,

some evidence exists that the membrane hyperpolarization amplifies the neutrophil responses (16). It should be noticed, however, that the study of the influence of membrane potential changes on the neutrophil activation in extracellular Ca^{2+}-free medium has provided evidence that changes in plasma membrane potential can also modulate Ca^{2+} mobilization from intracellular stores (13). No evidence about this mechanism is provided in the paper.

When cells are exposed to external electric fields, simultaneous hyperpolarization of one hemisphere of the cell and depolarization of the other hemisphere occur. In electrically excitable cells, this leads to the activation of the voltage-gated channels on the side of depolarization and, at the end of the cascade events, to functional response of the cell, e.g. muscle contraction, hormone and neurotransmitter release. In our previous study (19), it was shown that such simultaneous hyperpolarization and depolarization of the neutrophil plasma membrane cannot elicit the OB response, but may intensify PMA-induced OB. It was also found that an intensifying effect of the external electric field was closely related to a cellular Ca^{2+} mobilization from extracellular space, since depletion of extracellular Ca^{2+} highly suppressed the OB potentiation. The predictable absence of the PMA-induced OB response and the stimulatory effect of the electric treatment in experiments with neutrophils from patients with chronic granulomatous disease was shown (25).

The focus of this study was to monitor and compare the effects of the electrical treatment on the time-courses of the PMA, fMLP and ionomycin-stimulated OB responses, chemical activators which operate through distinctly different activation pathways. We have found that the electrical treatment potentiates the OB responses induced with all chemical stimulants used. The absence of changes in the shapes of response curves suggested that the electric field amplifies chemical activation which affects the link in the normal signal transduction pathway, but does not induce parallel activation. Although, there were considerable differences in the time course of OB responses and in the absolute values of the produced microbicidal oxidants—which avoid direct comparison of responses—the results suggested that the effect of electrical potentiation was most conclusive on ionomycin induced activation, smaller in fMLP-induced and the smallest—but still statistically significant in the part of the response—in PMA-induced activation. The reason for this might be the different role of Ca^{2+} homeostasis during activation with a particular activator: i.e. ionomycin and fMLP are two potent Ca^{2+} influx agonists, while PMA is not.

Taken together, the electric field which theoretically changes the resting membrane potential of neutrophils up to several tenths of milivolts is not a trigger of OB in neutrophils but it can intensify chemically induced responses. The amplification might be in correlation with the increase of Ca^{2+} influx across the plasma membrane due to the changed driving force for Ca^{2+}. It seems that electrical treatment preferentially stimulates calcium induced activation with ionomycin rather than calcium dependent activation with fMLP and PMA, where extracellular calcium is necessary but not absolutely required for OB response. It is also evident that electrical stimulation cannot bypass general membrane failure, as is the case in the neutrophils of chronic granulomatous patients. We expect that the use of fluorescent indicators for Ca^{2+} in further experiments may yield more precise answers regarding Ca^{2+} homeostasis in electrically treated neutrophils and concerning potentiation of the neutrophils' OB with an electric field.

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Reviewer's Comment

There are more than two pathways to stimulate neutrophils. One is PKC-dependent and the other is PKC-independent. Authors should analyze the oxidative burst separately in PKC-dependent and -independent pathways.

Author's Response

The protein kinase C has been postulated to play a major role in the activation of the oxidative burst although much experimental evidence supports the existence of additional signalling pathways. In our study, we have chosen ionomycin, fMLP and PMA, three different activators of the oxidative burst. They were combined with the pulsed electric field/current to show the phenomenon of the local transmembrane potential changes being involved in intensifying the chemically induced activation of the neutrophil oxidative burst but not to clarify its mechanism of action. Regarding the PKC-dependent and -independent signalling pathways, we have not been able to analyze the initial one-minute maximum, staurosporine insensitive, the PKC-independent phase of the fMLP stimulated luminol chemiluminescence (CL) (1) because CL was first measured one minute after the particular activation.

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Reviewer's comment

Typical $[Ca^{2+}]_i$ increase in neutrophils is induced by fMLP. However, no $[Ca^{2+}]_i$ is induced by PKC-stimulating drugs, such as PMA or DG. Thus, it is necessary to measure the $[Ca^{2+}]_i$ by using a fluorescence change of Fura 2/AM.

Author's Response

Flow cytometry and Fluo-3/AM were used to measure cytoplasmic free calcium (2) after fMLP, ionomycin, PMA and PEC stimulation. Briefly, the elevation of $[Ca^{2+}]_i$ was achieved with fMLP and ionomycin. PMA and PEC did not contribute to the intracellular calcium elevations. Further data of this study are still in preparation.

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Reviewer's Comment

A more detailed explanation of pulsed electric field/current should be included.

Author's Response

The parameters of the electric field/current stimulation should be described by amplitude of an electric field or by current density in the specific medium and shape of the exposure signal. In our case, nonhomogenous conditions of the exposure system (round cuvettes with needle electrodes) dictate less precise definition of an amplitude of field and current. To ensure that cells have been exposed to the similar stimulation conditions, the exposure system was mounted on the shaker. The signal of the stimulating field was described as a biphasic train of charge-balanced pulses which are commonly used for different *in vivo*

electrotherapy including training of muscles for sport and recovery (3, 4).

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Reviewer's Comment

Several active oxygen species contribute to the chemiluminescence of the luminol. Therefore, the measurement of the superoxide generation to evaluate the effect of PEF is required.

Author's Response

Luminol chemiluminescence (LCL) is described to be an effective tool in quantitative studies and examinations of microbicidal activity of neutrophils (5, 6, 7). Because luminol freely permeates intact neutrophils it is capable of detecting reactive oxidants, both inside the cells and in the extracellular space. In contrast, cytochrome c is a large protein which cannot penetrate the cells and measurement of cytochrome c reduction only detect secreted oxidants (7). Regarding the whole blood LCL, we did not want to work with purified granulocytes because it is known that the course of preparation of purified granulocytes has many influences on their functions and furthermore, this method requires a high volume of blood.

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Reviewer's Comment

Measurement of membrane potential change of neutrophils is also required by using fluorescence change of cyanine dye or tetraphenyl phosphonium ion electrode to evaluate the PEF effect.

Author's Response

When electric-field-induced changes in transmembrane potnetial (TP) is studied, one must distinguish two different events leading to the TP change. The first event is electric-field-induced charging of the membrane capacitor, which does not involve transmembrane ion currents and second, transmembrane translocation of different ions due to changes in a driving force and in a specific membrane permeability for those ions. The first phenomenon is present each time a cell is exposed to an electric field. The second is conditional and mainly depends on the existence of voltage-dependent conductivity (voltage-dependent ion channel). In addition, the first phenomenon is a very fast event. The induction and recovery

of the field induced charging of membrane capacitance is in a range of a few tenths of nanoseconds and cannot be followed by the fluorescence dye mentioned above which is based on Nernstian distribution and has slow response time. It is also worth mentioning that the field-exposed cells are depolarized and hyperpolarized at the same time (one hemisphere versus another) which means that the global TP change is zero and if one is attempted to study such phenomenon, the local changes must be followed. All these facts bring that the only possible equipment is very fast fluorescence imaging system in combination with the fast potential-sensitive styryl probes as Di-8-ANEPS. Such a study has been performed (8) with custom-made microscopic video imaging system based on the pulsed-laser excitation of the fast voltage dyes. There are just a few laboratories in the world capable of performing such an experiment, unfortunately our lab is not among them. However, we have performed some further experiments with slow-response carbocyanine dye di-O-C_5(3) and flow cytometry. Results showed that there is no long-term global changes in transmembrane potential. Such results are interesting but do not explain events undergoing transient TP changes and changes in intracellular concentrations of specific ions relevant to neutrophil activation. So, as it does not contribute to a clarification of the underlying mechanisms of the field-intensified oxidative burst, we think it is not necessary to include in the publication.

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