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Pulsed electric current enhances the phorbol ester induced oxidative burst in human neutrophils

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Oxidative burst (OB) response in human neutrophils, measured with chemiluminescence (CL), has been used to determine whether pulsed electric current (PEC) might induce a functional response in these electrically nonexcitable cells, and also whether it might modify cellular response to tumor-promoting phorbol ester (PMA). Five minutes of PEC treatment caused no significant changes in neutrophil CL levels in HBSS (1.2 mM Ca^{2+} concentration) as well as in HBSS-EGTA, where the extracellular Ca^{2+} concentration was reduced to less than 30 nM. The CL level of PMA-activated neutrophils in HBSS was 52% higher than in HBSS-EGTA. In HBSS the CL level, after the combined PMA and PEC treatment, was 53% higher than in PMA-alone-treated neutrophils. Activation of the OB in HBSS-EGTA with PMA and PEC was 13% higher than in solely PMA treated neutrophils. The results suggest that in neutrophil OB response, the PEC effect is closely related with cellular calcium mobilization, since depletion of extracellular Ca^{2+} decreased the PEC effect.

Neutrophil; Oxidative burst; Pulsed electric current; PMA; Ca^{2+}

1. INTRODUCTION

The polymorphonuclear leukocytes (neutrophils) represent the front line of the internal body defense against pathogenic microorganisms. Microbial invasion stimulates neutrophils to respond more hastily. Augmented activities include chemotaxis, phagocytosis, digestion, degranulation, aggregation, and intracellular killing. The intracellular killing depends considerably on the neutrophil NADPH-oxidase system which leads to oxidative burst (OB), i.e. production of microbicidal oxidants (O_2^-, H_2O_2, $OH^\bullet$, etc.) [1].

The OB is triggered by the activation of protein kinase C (PKC), an enzyme involved in a variety of cellular responses. It is physiologically activated as the result of receptor-mediated inositol phospholipid turnover. The phorbol ester (PMA), for which PKC is a cellular target, elicits various responses from many quiescent cells similar to responses to growth factors, hormones and neurotransmitters. Treatment of neutrophils with PMA elicits OB and degranulation [2]. This biological expression of cell activation can be traced to, or at least regulated by, the movement of various ions across the plasma membrane, which results in changes in intracellular pH, membrane potential, and cytosolic free Ca^{2+} levels [3–7].

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Electrically induced nerve and muscle cell excitation has been found to be a clinically relevant procedure applied in diagnostic, therapeutic and functional electric stimulation [8,9]. Various effects of electric and electromagnetic treatment were also observed in electrically nonexcitable cells (for review see [10]) and tissues e.g. in wound- [11,12] and bone- [13] healing and in tumor growth retardation [14].

The intention of the present study was to determine whether the pulsed electric current (PEC) that elicits functional response in electrically excitable cells [8], might also affect functional response in electrically nonexcitable cells. PEC combined with the known activator PMA was used in the model of neutrophil OB measured with luminol-enhanced chemiluminescence (CL) which is an effective analytical technique for the estimation of the OB response of phagocytic cells [15]. Experiments were performed in the absence or presence of the Ca^{2+} chelator, EGTA.

2. EXPERIMENTAL

2.1. *Chemicals*

PMA, luminol (5-amino-2,3-dihydro-,4-phthalazinedione), HBSS and DMSO were obtained from Sigma (St. Louis, USA). The chemicals for modified HBSS (HBSS-EGTA), i.e. Ca^{2+}-free HBSS with 3 mM EGTA, were obtained from Merck (Darmstadt, Germany). PMA and luminol were dissolved in DMSO and added to the cell suspension to give a final concentration of 3 μM PMA, 10 μM luminol and 0.1% DMSO (v/v) in HBSS or HBSS-EGTA, respectively. The concentration of ionized calcium in HBSS-EGTA, measured by fluorescent calcium indicator fura-2 [16], was less than 30 nM.

2.2. *Blood samples*

Venous blood (5 ml) was taken from healthy adults (18–45 years old) into heparinized (50 IU) tubes. Samples were used to assess neutrophil activation without any other manipulation. Their hematological characteristics, such as the leukocyte count (Coulter Counter, USA), the differential count and erythrocyte sedimentation rate, were within normal range [17]. With regard to activation treatment, blood samples were divided into four groups in HBSS and four groups in HBSS-EGTA: control, PEC, PMA and PMA and PEC. At the beginning, 200 μl of heparinized blood was resuspended in 400 μl HBSS or HBSS-EGTA. The samples in the control groups were treated neither with PMA nor with PEC. In the PEC groups, samples were treated with electric current alone. In the PMA groups, samples were treated with PMA solution. In the PMA and PEC group, samples were treated with PMA and electric current. During treatment, samples of all groups were shaken in cuvettes with electrodes. Each experimental group consisted of 20 samples. Experiments were performed at room temperature (22°C).

2.3. *Neutrophil oxidative burst activation*

A 3 μM final concentration of PMA in HBSS and in HBSS-EGTA was used for chemical in vitro 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. PEC was applied for 5 min through Pt-Ir (90–10%) electrodes directly immersed in suspension. During treatment, signals were monitored by current probe/oscilloscope combination (Tektronix P6042/7704, USA). All hematological parameters listed above were checked after treatment with PEC. Neither 5 min nor 60 min of PEC caused significant changes. Also, PEC treatment did not cause an appreciable difference in the samples, either in pH (measured by miniature glass electrode – model MI-408; Microelectrodes Inc., USA and pH-meter model – HEK 0301; ISKRA Horjul, Slovenia) or in temperature (measured by NiCr-Ni thermosensor model TK127 and thermometer model – 2280-8; both AMR, Holzkirchen, Germany).

2.4. *The chemiluminescence assay*

CL was measured on a LKB-Wallac 1250 luminometer in polystyrene measuring cuvettes (Clinicon, Finland). After particular activation, or control treatment, 400 μl of luminol solution in HBSS or HBSS-EGTA 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. The CL level before and the CL level after 5 min treatment were chosen as the main estimation values. The significance of the results was analyzed by a paired Student's *t*-test.

3. RESULTS

3.1. *Treatment in HBSS*

Control and PEC treatment induced no significant changes in CL levels (Fig. 1). According to subjects specificity, PMA activation caused different CL response levels, but all were within the normal response range [18]. In the PMA and PEC group the CL levels were (53 ± 7)% (mean ± S.E.; n =20) higher than in the PMA group. The difference was statistically significant at a high level of confidence ($P < 0.0003$). Immediately after treatment, the PEC effect was greatest but with time it was gradually eliminated (results not presented).

3.2. *Treatment in HBSS-EGTA*

As in HBSS, control and PEC treatment induced no significant changes in CL levels (Fig. 2). Depletion of extracellular Ca^{2+} suppressed the PMA effect on OB. The CL levels of PMA-activated cells in HBSS-EGTA was (48 ± 3)% of the CL levels in the PMA group in HBSS. Activation of OB in HBSS-EGTA, with a combination of PMA and electric current (PMA and PEC group), was also lower compared to the same treatment in the HBSS. In the HBSS-EGTA the CL levels in the PMA and PEC group were only (13 ± 3)% higher than the CL levels in the PMA group which, however, the difference was still highly significant ($P < 0.0005$).

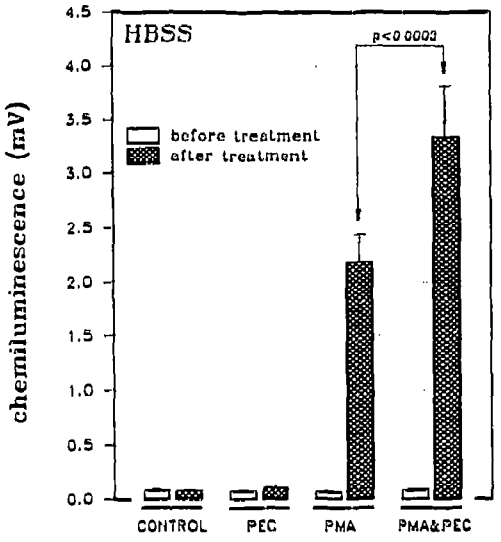

Fig. 1. The 5-min treatment effect of 20 mA amplitude PEC, 3 μM PMA and a combination of it, on neutrophil oxidative burst measured with chemiluminescence in HBSS. The results are mean ± S.E. of 20 samples. The difference between PMA and PMA and PEC treatment was shown to be statistically significant by a paired Student's *t*-test.

4. DISCUSSION

The observed increases in CL levels in the PMA and PEC groups compared to PMA groups were not due to ionization by electric current ($O_2 + e^- \rightarrow O_2^-$ and $O_2 + 2\,H_2O + 2\,e^- \rightarrow H_2O_2 + 2\,OH^-$), since no increases were noticed in the PEC groups. Since no changes were noted in pH and temperature of samples after PEC treatment (data not shown), it was possible to eliminate thermal and pH effects of applied electric current. To confirm that PEC did not affect PMA structure more than neutrophils, PMA solutions were treated, or not, with PEC for 5 min, then added to blood samples and the CL test performed. There were no appreciable differences in the CL levels between samples (data not shown). The possibility that a more potent OB response is due to electric importation of PMA into cells is not, however, completely eliminated; but the stimuli amplitude we used was at least ten times smaller than in stimuli commonly used for electroporation [19].

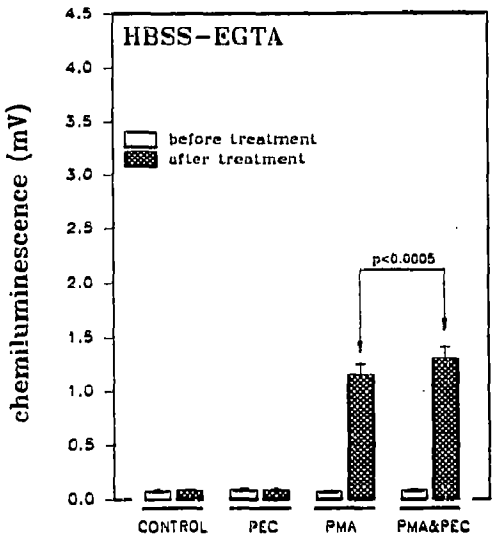

Fig. 2. The 5-min treatment effect of 20 mA amplitude PEC, 3 μM PMA and a combination of it, on neutrophil oxidative burst measured with chemiluminescence in HBSS-EGTA. The results are mean ± S.E. of 20 samples. The difference between PMA and PMA and PEC treatment was shown to be statistically significant by a paired Student's *t*-test.

The results suggest that in neutrophils, PEC does not elicit the OB response by itself, but might modify it in combination with chemical stimuli (PMA). A similar relation was also observed in some other immune relevant cells. In lymphocytes, using a combination of pulsed electromagnetic fields and phytohaemagglutinin (PHA), enhanced proliferation was observed [20]. In natural killer cells, tumoricidal activity was potentiated by a combination of direct electric current and interleukin-2 (IL-2) [21]. Evidence also exists that the sinusoidal electric field alone did not affect calvarial bone cells, but that it did diminish the neomycin suppressive action on phospholipid turnover [22].

Our results do not unequivocally indicate the mechanism by which human neutrophils respond to the PEC. However, some predictions can be made. Stimulated production of microbial oxidants in neutrophils is mainly mediated by the synergistic action of PKC activation and Ca^{2+} mobilization [23]. These membrane-mediated processes might be candidates for the PEC interactions. Although there are reports dealing with nerve cells in which high-frequency electric currents have affected phospholipids turnover [24] and translocation of PKC [25], our results suggest that in the neutrophil OB response, the PEC effect is tightly connected with Ca^{2+} mobilization. Ca^{2+} mobilization occurs by releasing Ca^{2+} from internal stores, which is mainly due to inositol trisphosphate (IP_3), the product of phospholipids turnover, and by Ca^{2+} influx from extracellular space. It is more likely that the PEC effect on OB activation was mostly due to influx from extracellular space, since depletion of extracellular Ca^{2+} highly suppressed the PEC effect.

Evidence exists that electromagnetic fields affect Ca^{2+} ion transmembrane transport (for review see [26]). Robinson theoretically described changes of Ca^{2+} transmembrane fluxes through voltage-gated and leak Ca^{2+} channels due to external direct electric current application [27]. The PEC we used may also act on other Ca^{2+} transport mechanisms. Leonard reported that direct electric current affects Ca^{2+}-ATPase activity [28], which, in addition with the Na^+/Ca^{2+} exchange system, is the part of the negative feedback control of PKC activation [7]. We cannot exclude other possible PEC involvements in signal transduction e.g. PEC might affect ligand–receptor binding interaction, surface charge, some other ion transmembrane transport and the intracellular level of cyclic AMP, since these effects were determined by different electric and electromagnetic treatments [29–34].

Finally, our results suggest that a pulsed electric current might stimulate functional response in 'electrically nonexcitable' cells. This stimulation is closely related with Ca^{2+} mobilization, but the exact regulatory pathways of the PEC remain to be investigated. The neutrophil OB model seems to be appropriate for such a study.

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