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Plasma membrane potential interferes with the respiratory burst of peripheral granulocytes

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Abstract

Membrane potential is involved in the regulation of several immune functions developed by granulocytes. The Na^+/K^+ gradient across the plasma membrane, mainly generated by the Na^+/K^+ pump, plays a key role in the maintenance of membrane potential. This study is focused on the correlation between plasma membrane potential and the *in vitro* receptor – triggered respiratory burst of normal human peripheral granulocytes. The respiratory burst was measured as superoxide anion release by the cytochrome *c* reduction test and plasma membrane potential was modulated by experimental changes of the extracellular potassium concentration. Results show a differentiated cellular response, depending on the *in vivo* activation state and on the signals received *in vitro* by granulocytes *via* CR3 or FcγR. Alteration of the membrane potassium gradient modulates the respiratory burst of unstimulated and CR3-activated cells, whilst it does not seem to significantly interfere with the signals delivered by FcγR.

Keywords: granulocytes - respiratory burst - superoxide anion - plasma membrane potential

Introduction

At the inflammatory situs various stimuli that act on granulocytes (PMNs) produce post–receptor events at the level of the plasma membrane potential, intracellular calcium concentration, pH and finally trigger oxygen-dependent and oxygen-independent cytotoxic functions [1, 2]. The Na^+/K^+ pump in the plasma membrane plays a key role in the production and maintenance of membrane potential, generating a Na^+, K^+ gradient across the plasmalema. The Na^+/K^+ pump is an ATP - ase that functions as an antiportal system, transporting Na^+ and K^+ against their concentration gradients [3].

Changes of the membrane potential following ligand-receptor interactions have been described in both excitable cells (neurons), but also in non-excitable cells, such as cells of the immune sys-

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tem. Plasma membrane potential is involved in the regulation of several functions developed by immune cells [4, 5]. For instance, a persistent raise of the Na^+/K^+ ratio inhibits macromolecules synthesis and cell proliferation in lymphocytes [6]. In PMNs and lymphocytes membrane potential acts as an important modulator in the receptor–dependent signaling pathways [7]. Experimental data show a strong bond between membrane potential and intracellular calcium ions level in immune cells [8,9].

In PMNs, small transient plasma membrane depolarization triggered by N-formylated peptides receptors or the PKC activator phorbol myristate acetate (PMA) appears to be a physiological feedback mechanism, which diminishes the changes of intracellular calcium concentration [10,11]. Experimental plasma membrane depolarization lowers the calcium mobilization from internal stores and calcium influx into neutrophil cell lines [7], thus limiting the respiratory burst induced by chemoattractants (fMLP). PMA depolarizes the plasma membrane in human PMNs by a mechanism involving both intra- and extracellular calcium ions [12,13].

There is strong evidence regarding the depolarizing action of reactive oxygen species (ROS) showing that oxygen radicals modulate the activity of the Na^+/K^+ pump and the membrane potential [14]. The electrogenic activity of the NADPH oxidase, the enzymatic complex that initiates ROS chain by forming superoxide anion, is associated with depolarization of the plasma membrane in activated PMNs, exerting a negative feedback on its own activity [14,15]. However, in spite of numerous experimental evidence, the physiological relevance of plasma membrane potential changes is still to be explored.

The aim of this paper was to investigate the influence exerted by plasma membrane potential on the respiratory burst of human normal peripheral blood PMNs stimulated *in vitro via* physiologically relevant receptors. Membrane depolarization was induced by altering transiently or in a persistent manner the extracellular K^+ concentration. Our study shows that the plasma membrane depolarization interferes with superoxide anion release by PMNs, the response being dependent on the cellular activation state and on the receptor that triggers cellular activation.

Material and methods

Reagents and stimuli

Cytochrome *c* from horse heart, Hank's balanced salt solution (HBSS), eosin, potassium chloride (KCl) and human IgG were purchased from Sigma. Zymosan particles (Z) were prepared in the Immunobiology Laboratory, "Victor Babes" Institute, Bucharest according to the method described by Lachman [16]. Heat aggregated IgG (aggIgG) was prepared by incubation of IgG at 60°C for 20 minutes [17].

Healthy volunteers

We investigated a group of 19 healthy volunteers presenting no clinical signs of disease, 10 males, 9 females, with ages between 35 - 55 years.

Cells

Blood was collected by venipuncture and PMNs were isolated within two hours from blood collection using the method described by Boyum [18]. Briefly, PMNs were isolated by density gradient centrifugation on Hystopaque and further red blood cells lysis (lysing solution: 0.83% NH_4Cl and 0.084% $NaHCO_3$). PMNs were resuspended in HBSS supplemented with 1mM calcium chloride and 1mM magnesium chloride (complete HBSS). Cellular viability, estimated by the eosin exclusion test, exceeded 95%.

Superoxide anion release

The extracellular release of superoxide anion was evaluated by the cytochrome c reduction test [19]. Test samples containing 0.98 mg/ml cytochrome *c*, 1 x 10^6 cells/ml in the presence of stimuli (Z or aggIgG) and/or KCl were adjusted to a final volume of 1 ml with complete HBSS. Samples containing complete HBSS and cytochrome c were used as controls. In order to induce a persistent membrane depolarization, meant to affect the activity of the plasma membrane Na^+/K^+ - ATPase, PMNs were pre-incubated for 20 minutes at 37°C with 50 mM KCl, then introduced in the activation and detection system (10mM final KCl concentration). Controls and test samples were incubated for 30 minutes at 37°C

with continuous gentle stirring. The reaction was stopped on ice and supernatants were collected. Superoxide anion release was calculated as the optical densities difference at 550 nm and 535 nm [ODD = $(OD)_{550} - (OD)_{535}$]. In order to induce a persistent membrane depolarization, meant to affect the activity of the plasma membrane Na^+/K^+ - ATPase, PMNs were preincubated with 50 mM KCl, prior to experimental activation *via* CR3 or FcγR. The effect exerted by KCl was expressed as ODD in the absence of KCl/ODD in presence of KCl.

Statistics

Experimental results were expressed as mean value ± standard error of the mean (SEM). Results were statistically compared using the Student two-tailed t test, paired two samples.

Results

PMNs play a major role in host defense against invading microorganisms. They are rapidly recruited from the blood stream to the site of infection upon activation by chemoattractants and develop non-specific microbicidal mechanisms that include generation of ROS [20]. It is known that ROS exert a depolarizing effect on the plasma membrane, acting as modulators of the Na^+/K^+ pump activity [21]. In this context, our aim was to indirectly investigate the influence of transmembrane potential changes on the superoxide anion released by PMNs in various conditions of cellular activation (with Z or aggIgG, targeting CR3 and FcγR, respectively).

We indirectly induced plasma membrane depolarization by raising the extracellular K^+ concentration in two ways; a slight transient alteration of the K^+ ions homeostasis was produced by concomitantly treating isolated PMNs with 10mM KCl and stimuli. PMNs isolated from peripheral blood of healthy volunteers release superoxide anion *in vitro* without experimental priming, both in their basal state (ODD = 25.68 ± 5.65) and when activated *via* CR3 (ODD = 37.63 ± 6.33) or FcγR (ODD = 38.63 ± 4.81).

Individual data analysis points out that unstimulated PMNs show an important individual variability of their basal oxidative responses (Fig.1). In this respect, we identified groups of individuals with high (H), medium (M) and low (L) basal respiratory burst (Fig. 1A). We noticed that most of the healthy volunteers belong to the M group (9/19), followed by L (7/19) and H (3/19) groups.

Superoxide release is significantly stimulated upon CR3 activation for the total group of 19 individuals ($p<0.006$). The L group ($p<0.004$) inflicts this pattern of CR3-triggered respiratory burst while response to Z in the other groups is not significant (Fig. 1B). The same pattern of activation was registered for superoxide release induced *via* FcγR, the stimulatory effect noted for the total group ($p<0.01$) being mainly sustained by the L group ($p<0.02$). aggIg also tends to stimulate the oxidative burst of PMNs isolated from the M group (Fig. 1C).

In this view, a functional analysis on groups of differently responsive individuals provides more accurate data with respect to cellular responses developed *in vitro* by PMNs in various condition of cellular activation and modulation.

Transient change of the extracellular potassium concentration

Transient perturbation of the membrane potential due to small doses of KCl (10 mM) significantly enhances the basal superoxide anion release in L group samples ($p<0.02$) (Fig. 2).

Transient membrane depolarization significantly stimulates the oxidative burst of PMNs, triggered by Z *via* CR3 ($p<0.08$) only in the M group (Fig. 3).

No functional changes were recorded when PMNs were concomitantly treated with 10mM KCl and aggIgG.

Persistent change of the extracellular potassium concentration

A persistent change of the transmembrane K^+ equilibrium significantly enhances the basal superoxide anion release in L group samples ($p<0.06$) (Fig. 2). When cells were activated *via* CR3, KCl preincubation inhibited superoxide anion release ($p<0.06$) in the M group (Fig. 3).

Persistent changes of extracellular K^+ concentration reduce respiratory burst induced *via* FcγR by aggIgG in group H ($p<0.05$) (Fig. 4).

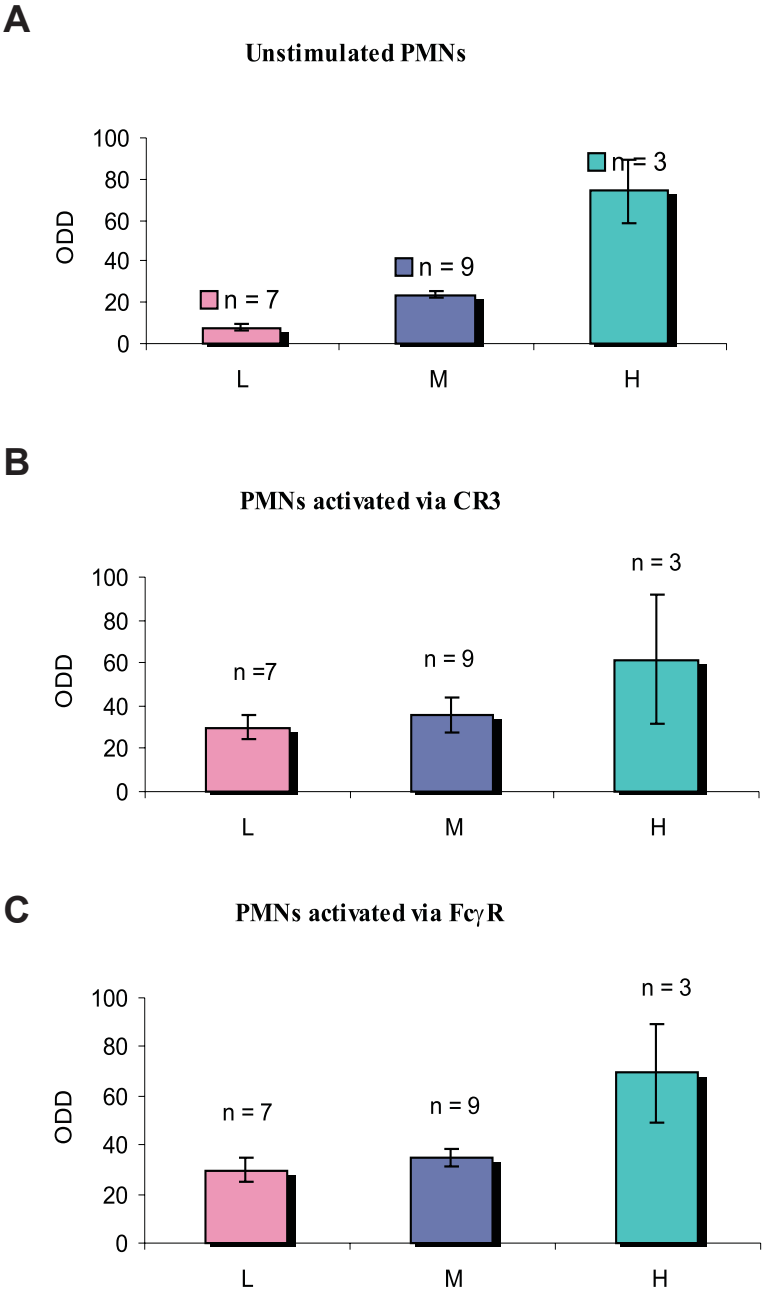

Fig. 1. Superoxide anion release by PMNs: **A.** basal cellular response; **B.** CR3-triggered cellular response; **C.** FcγR -triggered cellular response. Healthy volunteers were grouped according to the intensity of the basal cellular response: high (H), medium (M) and low (L) responses. n = number of healthy volunteers.

Discussion

We identified three distinct groups of healthy volunteers characterized by PMNs with low, medium and high basal respiratory burst. This individual variability is probably due to the *in* vivo cellular activation status or to distinct reactivities of cells to isolation procedures. We noticed that the mentioned groups also exhibit distinct functional patterns when cells are activated *via* CR3 or FcγR. Thus, only PMNs from the low responsive group develop a significant respiratory burst when challenged *in vitro*, suggesting that ROS generation is restrained by intracellular mechanisms that control the balance between the bactericidal and host tissue-damaging effects of ROS, which can trigger cancer [22].

Both transient and persistent membrane depolarization stimulate the low intensity basal superoxide anion release, suggesting that, in certain cases, membrane potential might limit the basal respiratory burst. It is also

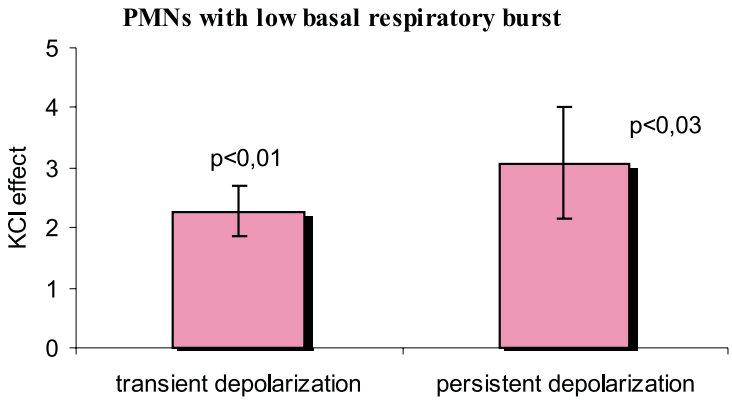

Fig. 2. The effect exerted by membrane depolarization on superoxide anion release by PMNs in individuals of the group with low intensity basal respiratory burst (n = 7).

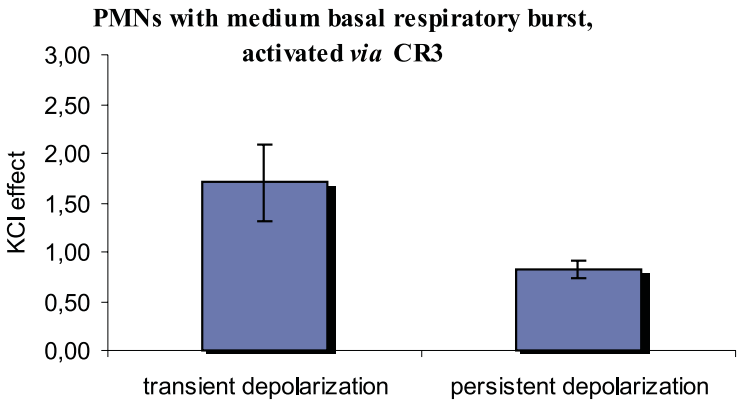

Fig. 3. The effect exerted by membrane depolarization on superoxide anion release by PMNs in individuals of the group with medium basal respiratory burst (n = 9) activated *via* CR3.

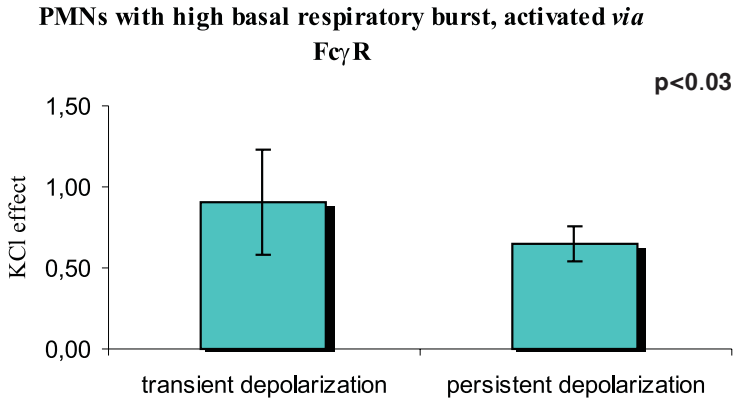

Fig. 4. The effect of membrane depolarization on the superoxide release elicited by PMNs in individuals of the group with high basal respiratory burst (n = 3) activated *via* FcγR.

worth noticing that only PMNs showing a weak basal activity are sensitive to changes of the membrane potential and, as mentioned before, to activation signals delivered by receptors with physiological relevance.

When PMNs develop significant oxidative *in vitro* responses triggered *via* CR3 or FcγR, profound membrane depolarization inhibits superoxide anion release, indicating that membrane potential sustains the oxidative activity of activated cells.

Signals delivered by CR3, but not by FcγR, are sensitive to a small raise in extracellular potassium concentration. Possibly, membrane potential controls the CR3-mediated oxidative burst of PMNs by two distinct mechanisms that either hinder, or potentiate cell function.

PMNs activated *via* CR3 or FcγR, that respond to changes of the transmembrane K^+ gradient, correspond to the groups of individuals with medium and high basal oxidative burst, respectively.

Therefore, the effect induced by membrane depolarization might be correlated to the degree of priming acquired by PMNs *in vivo*, prior to isolation and *in vitro* activation.

In conclusion, the involvement of the membrane potential in the superoxide release by PMNs is dependent on the magnitude of experimental membrane depolarization, on the receptor that triggers cellular activation and on the respiratory burst intensity. However, our experimental model does not clarify whether membrane depolarization affects superoxide anion generation or radical exocytosis.

This study is also relevant for evaluating the respiratory burst of PMNs in patients with K^+ disturbances associated to various pathologies or therapies. Persistent exposure of PMNs to K^+ hinders the tissue-damaging oxidative stress induced *via* CR3 and FcγR, but might also impair the host defense relying on the oxygen-dependent microbicidal functions.

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