Changes in intracellular calcium free and calcium stored balance in children granulocytes after stimulation. Preliminary results

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Abstract

In human granulocytes calcium fluxes are crucial for effective production of oxygen intermediates in signal transduction pathways originating from chemoattractant receptors. The evidence rise that factors stimulating oxidative burst independently from calcium signaling pathways may exert modulatory effects on intracellular calcium free level. The aim of our study was to examine the impact of well known receptor (fMLP) and non-receptor (PMA) related oxidative burst inducers on calcium kinetics in children suffering from recurrent infections. Granulocytes were withdrawn from group of healthy children and children suffering from recurrent infections. Simultaneous calcium free and calcium stored measurement was performed by flow cytometer. PMA-induced, deep and long lasting (over 420 sec.), decline in calcium free level was more evident in children suffering from recurrent infections during first 240 sec. Also in this group, prolongation of recovery was visible after stimulation with fMLP. In conclusion we suggest that: 1. calcium kinetics is shifted in children suffering from recurrent infections, 2. PMA interfere with intracellular calcium free level.

Key words: *calcium intracellular, Fluo-3, Fura Red, fMLP (N-Formyl-L-methionyl-L-leucyl-L-phenylalanine), PMA (Phorbol 12-myristate 13-acetate), recurrent infections*

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Introduction

Cytosolic free calcium (Ca^{2+}) is believed to play a key role in the regulation of cellular functioning [1]. In vascular smooth muscle cells Ca^{2+} control excitation-contraction coupling [2]. In non-excitable cells like platelets and fibroblasts Ca^{2+} may influence tyrosine phosphorylation [3] or gate the signal for collagen synthesis and deposition [4]. Several authors have reported that leukocytes signal transduction pathways originating from chemoattractant receptors culminate in intracellular calcium fluxes [5, 6].

Since granulocytes constitute an important line of human immunological system, its proper functioning is crucial for effective host defense. Although it is widely known that, following stimulation, human granulocytes produce reactive oxygen intermediates in a process termed oxidative burst [7, 8]. The cellular mechanisms underlying this process are still poorly understood. Particularly, the role of intracellular calcium free and calcium stored balance (calcium ratio) in granulocytes from subjects with impaired immunological response has yet to be investigated.

Flow cytometry may be a valuable tool for calcium intracellular evaluation. Fluo-3 and Fura Red calcium indicators used simultaneously allows calcium ratio measurement by flow cytometer equipped with argon laser [9]. Fluo-3 fluorescence increases in the green region when is bound to calcium while Fura Red exhibits inverse behavior, fluorescing most intensely in the red region when calcium is not bound (Fig. 1).

The aim of this study was to assess the effects of receptor (fMLP) and non-receptor (PMA) related oxidative burst inducers on calcium free and calcium stored balance in granulocytes obtained from children suffering from recurrent infections.

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Material and Methods

Patients

Venous blood was collected from two groups of children: healthy volunteers (n=7) and those suffering from recurrent upper respiratory tract infections (n=5). These patients had at least eight episodes of sore throat and fever per year. Healthy volunteers had negative history of recurent upper respiratpry infections. During the study, all of the children had no signs of infection and the results of carried out basic laboratory tests (blood count and smear, erythrocyte sedimentation rate, urinalysis) were all within the normal range. None of the children recieved any medications during the study. Children with recurrent infections were studied 7-10 days after the end of treatment.

Cells and reagents

Briefly, granulocytes were isolated from heparinized blood by Ficoll-Hypaque (Sigma Chemicals, St. Luis, MO, USA) gradient centrifugation. Cells were divided into aliquots and then pelleted and resuspended in 1-2 x 10^7/ml in RPMI-1640 medium (Sigma Chemicals, St. Luis, MO, USA). The cells were incubated in the dark for 45 min. at 37°C media containing 5 µM concentrations of Fluo-3 and Fura Red (all acquired from Molecular Probes, Eugene, OR, USA and prepaRed as 10 mg/ml DMSO solutions). The cells were then washed once with RPMI and resuspended in fresh medium at 2.0 x 10^6/ml. Cells were kept at room temperature until warmed to 37°C 5 min. prior to analysis at 37°C.

The research and ethical commitee of Medical Univesity of Warsaw approved the investigation.

Flow cytometry

Analyses were performed on a Coulter Epics XL flow cytometer (Coulter, Hialeh, FL, USA) equipped with argon laser. Granulocytes were discriminated by flow cytometric measurements of cellular forward angle and right angle scatter. Cytoplasmic calcium changes were monitored by using simultaneously the calcium indicators Fluo-3 and Fura Red. Fluo-3 and Fura Red were excited at 488 nm with Fluo-3 emission detected at 515-535 nm and Fura Red emission detected at 665-685 nm. After 40 sec. the analysis was interrupted, the stimuli added: fMLP or PMA (Sigma Chemicals, St. Luis, MO, USA), and the measurement continued. Data were collected in histograms displaying: Fluo-3 fluorescence vs. time and Fura Red fluorescence vs. time. Later on Fluo-3/Fura Red ratio vs. time was calculated. Changes in calcium intracellular after stimulation are expressed as a percent of initial, resting value (100%).

Results

Effects of fMLP on children granulocytes are shown in Tab. 1. Stimulation with fMLP evoked rapid and transient

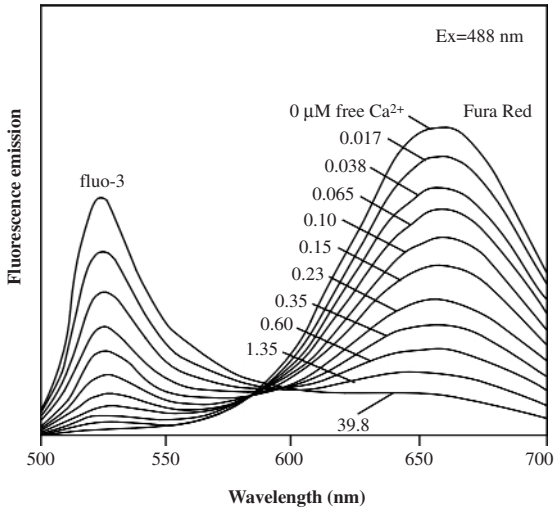


Fig. 1. Fluorescence emission spectra of 1 1:10 mole : mole mixture of fluo-3 and Fura Red indicators, simultaneously excited at 488 nm, in solutions containing 0-39,8 µM free Ca^{2+}. Courtesy of Molecular Probes Inc.

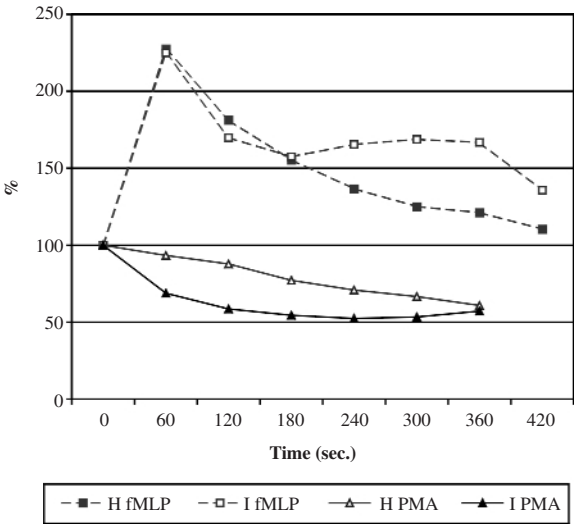


Fig. 2. Effects of fMLP and PMA on children granulocytes (H – healthy subjects, I – suffering from recurrent infections, sec. – second); changes after stimulation are expressed as a percent of initial, resting value (100%)

increase of calcium free associated with calcium bound decline both in cells obtained from healthy volunteers and those with recurrent infection. None significant differences between these two groups have been observed. Calcium ratio reached its peak after 60 sec. in both groups and remained significantly above initial level during 180 sec. in control group and 360 sec. in group of children with recurrent infections (Fig. 2).

Table 1. Effect of fMLP on children granulocytes (H - healthy subjects, I - suffering from recurrent infections, *p<0.01 - resting calcium ratio vs. calcium ratio after stimulation); changes after stimulation are expressed as a percent of initial, resting value (100%)

Groups/Time (sec.)	60 SD	120 SD	180 SD	240 SD	300 SD	360 SD	420 SD
H Fluo-3, n = 7	162.2 ±43.0	144.8 ±36.6	130.4 ±31.8	121.0 ±29.1	114.7 ±26.0	108.2 ±16.2	99.5 ±15.6
H Fura Red, n = 7	77.0 ±6.8	84.4 ±14.5	86.7 ±8.9	90.9 ±13.8	94.3 ±6.7	93.7 ±4.9	96.3 ±6.0
H Calcium ratio, n = 7	227.5* ±78.2	181.2* ±63.7	155.4* ±48.5	136.7 ±43.3	125.1 ±35.3	121.1 ±21.0	110.4 ±19.8
I Fluo-3, n = 5	169.4 ±67.5	143.5 ±58.5	137.4 ±55.5	140.7 ±56.9	143.2 ±53.4	135.4 ±59.5	126.1 ±54.8
I Fura Red, n = 5	77.4 ±12.0	84.7 ±9.5	88.2 ±9.6	86.9 ±10.3	86.6 ±11.8	85.0 ±14.6	93.8 ±9.6
I Calcium ratio, n = 5	224.9* ±126.7	169.8* ±101.5	157.4* ±90.6	165.5* ±92.4	168.7* ±93.2	166.8* ±113.6	135.7 ±79.6

Table 2. Effect of PMA on children granulocytes (H - healthy subjects, I - suffering from recurrent infections, sec. - second, *p<0.01 resting calcium ratio vs. calcium ratio after stimulation, **p<0.01 between groups); changes after stimulation are expressed as a percent of initial, resting value (100%)

Groups/Time (sec.)	60 SD	120 SD	180 SD	240 SD	300 SD	360 SD
H Fluo-3, n = 7	89.8 ±7.0	84.4 ±11.8	76.8 ±11.0	72.3 ±10.5	70.4 ±10.7	63.8 ±15.1
H Fura Red, n = 7	104.7 ±5.2	106.3 ±6.6	108.9 ±8.3	110.4 ±8.9	113.0 ±11.4	114.6 ±15.2
H Calcium ratio, n = 7	93.3* ±8.7	87.8* ±12.7	77.1* ±11.6	70.8* ±10.7	66.6* ±11.3	60.8* ±17.0
I Fluo-3, n = 5	71.3 ±15.2	63.7 ±17.6	60.6 ±16.3	58.9 ±14.3	58.7 ±13.3	60.0 ±11.6
I Fura Red, n = 5	107.3 ±9.8	111.8 ±12.7	114.3 ±13.4	115.7 ±14.4	113.2 ±15.7	110.5 ±11.1
I Calcium ratio, n = 5	68.8** ±17.5	58.6** ±20.4	54.4** ±18.4	52.4* ±16.7	53.3* ±16.0	57.2* ±13.3

Effect of PMA on children granulocytes are shown in Tab. 2. Administration of PMA provoked deep and long lasting decline of calcium free associated with moderate rise of calcium bound in both groups. Calcium ratio decrease was significantly deeper in the granulocytes from subjects with recurrent infections during first 180 sec. in comparison with control group (Fig. 2).

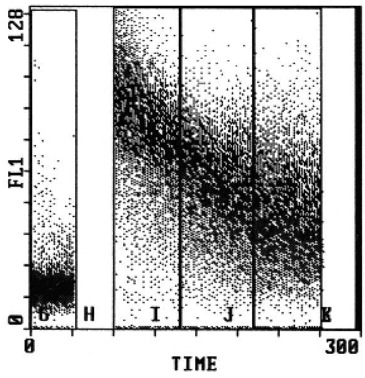
Discussion

In this paper we have assessed the acute effects of fMLP and PMA on Ca^{2+} in granulocytes obtained from children suffering from recurrent infections. Using the two indicators Fluo-3 and Fura Red we were able to monitor simultaneously two pools of calcium intracellular: calcium free and calcium stored. Fig. 3 show representative tracings of response to added stimuli.

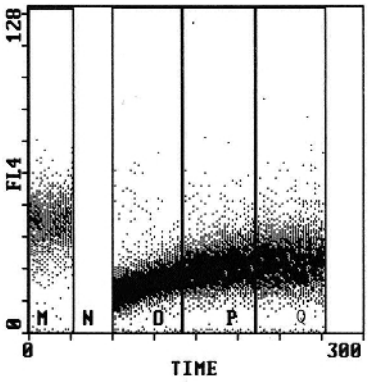
More specifically, we found that in granulocytes from healthy children fMLP calcium ratio elevation is combined with deep decrease of calcium bound level what may suggest that mobilization of Ca^{2+} is generated mostly from internal stores. Recently, aberrations in chemoattractant-induced signaling in neonatal neutrophils have been reported [10]. Our results play in concert with earlier data acquired from adult subjects [5, 7].

Moreover, we demonstrated that the fMLP-induced Ca^{2+} mobilization mechanism is not impaired in children

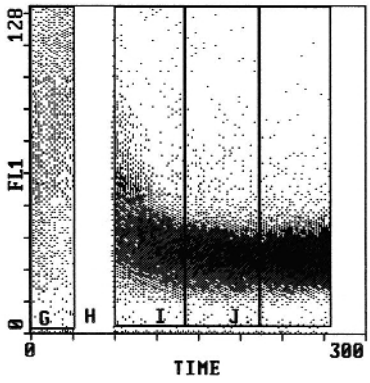
A. fMLP, Fluo-3



B. fMLP, Fura Red



C. PMA, Fluo-3



D. PMA, Fura Red

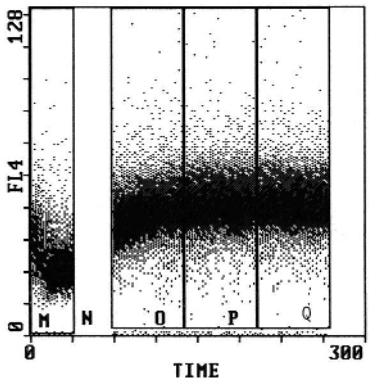


Fig. 3. A-D. Flow cytometric determination of fluctuations of granulocytes cytoplasmatic calcium concentration by measurement of cellular Flou-3 and Fura Red before and after fMLP and PMA stimulation. Gates: G (Fig 3.A and C), M (Fig 3.B and D) show Ca^{2+} level before stimulation (resting level). Gates: H (Fig 3.A and C), N (Fig 3.B and D) show the time of stimuli addition. Gates I, J, K (Fig 3.A and C) and gates O, P,Q (Fig 3.B and D) show response on the stimulants

suffering from recurrent infection. However, the decay of calcium ratio to resting level was prolonged in cells from those children. It was mostly due to sustained higher calcium free level. Some authors indicate that pro-inflammatory factors like soluble E-selectin may prolong the elevation of Ca^{2+} without effecting the peak response in neutrophils treated with platelet activating factor but not fMLP [11]. Consequences of such enhanced exposition to elevated Ca^{2+} remain obscure. Nevertheless, clinical relevance of altered Ca^{2+} kinetics shall not be neglected [12]. Due to the last report Ca^{2+} after peak decline may be exaggerated by anti-inflammatory agents acting via cyclic AMP [13].

Wide consensus has been reached that PMA may produce an oxidative burst in human granulocytes acting downstream and independently from Ca^{2+} – related signaling pathways [5, 14, 15]. According to some authors PMA does not exert any sudden effects on Ca^{2+} in adult human leukocytes [5]. Other suggests that PMA promotes calcium influx in Red blood cells via protein kinase C dependent mechanism [16].

We demonstrated that PMA diminishes calcium ratio in granulocytes obtained from healthy children. Decrease of calcium free combined with very moderate increase of calcium bound might indicate that Ca^{2+} efflux is stimulated. Furthermore, our results show that in the group of children suffering from recurrent infections decrease of calcium ratio is significantly deeper in the first 180 sec. and associated with even more visible calcium free decline. As far as we know, we are the first to report that PMA exerts such effect on children granulocytes. Detailed investigation is needed to clarify the mechanisms underlying the observed results. More precisely, the role of protein kinase C, phosphatidylinositol 3-kinase and Akt signaling pathway shall be explored.

Generally, our observation that long term pathological condition like recurrent infections shifts calcium kinetics in children granulocytes, is consistent with rising evidence that "blocks of Ca^{2+} puffs" are crucial for signal gating and, albeit, for regulation of cellular responses in diverse cell types [1, 4, 12, 17]. Specifically, we found that in granulocytes withdrawn form children suffering from recurrent infections Ca^{2+} kinetics is significantly changed. Furthermore we demonstrated that PMA in children neutrophils may induce calcium ratio decline.

References

1. Berridge MJ, Lipp P, Bootman MD (2000): The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1 (1): 11-21.
2. Baro I, Eisner DA (1995): Factors controlling changes in intracellular Ca^{2+} concentration produced by noradrenaline in rat mesenteric artery smooth muscle cells. J Physiol 482 (2): 247-258.
3. Vostal JG, Jackson WL, Shulman NR (1991): Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. J Biol Chem 266 (25): 16911-16916.
4. Ceolotto G, Pessina AC, Iori E, et al. (1998): Modulatory effect of insulin on release of calcium from human fibroblasts by angiotensin II. J Hypertension 16 (4): 487-493.
5. Lund-Johansen F, Olweus J (1992): Signal transduction in monocytes and granulocytes measured by multiparameter flow cytometry. Cytometry 13: 693-702.
6. Wymann MP, Sozzani S, Altruda F, et al. (2000): Lipids on the move: phosphoinosittide 3-kinases in leukocyte function. Immunology Today 21: 260-264.
7. Babior BM (1978): Oxygen-dependent microbial killing by phagocytes. N Engl J Med 298: 721-725.
8. Zeman K, Kantorski J, Paleolog EM, et al. (1996): The role of receptors for tumour necrosis factor-alpha in the induction of human polymorphonuclear neutrophil chemiluminescence. Immunol Lett 53 (1): 45-50.
9. Nowak EJ, Rabinowitch PS (1994): Improved sensitivity in flow cytometry intracellular ionized calcium measurement using Fluo-3/Fura Red fluorescence ratios. Cytometry 17: 135-141.
10. B, Laskin DL, Mariano TM, et al. (2001): Mechanisms underlying reduced responsiveness of neonatal neutrophils to distinct chemoattractants. J Leukoc Biol 70 (6): 969-76.
11. Rauchaud-Sparagano MH, Walker TR, Rossi AG, et al. (2000) Soluble E-selectin acts in synergy with platelet-activating factor to activate neutrophil β2-integrins. J Biol Chem 275 (21): 15758-15764.
12. Tovey SC, de Smet P, Lipp P, et al. (2001): Calcium puffs are generic InsP(3)-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. J Cell Sci 114 (22): 3979-3989.
13. Tintinger GR, Theron AJ, Anderson R, Ker JA (2001): The anti-inflammatory interactions of epinephrine with human neutrophils in vitro are achieved by cyclic AMP-mediated accelerated resequestration of cytosolic calcium. Biochem Pharmacol 61 (10): 1319-1328.
14. Pozzan T, Lew DP, Wollheim CB, Tsien RY (1983): Is cytosolic ionized calcium regulating neutrophil activation. Science 221: 1413-1415.
15. Rossi F, Della BV, Grześkowiak M, Bazzoni F (1989): Studies on molecular regulation of phagocytosis in neutrophils. J Immunol 142: 1652-1660.
16. Andrews DA, Yang L, Low PS (2002): Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human Red blood cells. Blood 100 (9): 3392-3399.
17. Podesta M, Zocchi E, Pitto A, et al. (2000): Extracellular cyclic ADP-ribose increases intracellular free calcium concentration and stimulates proliferation in human progenitors. FASEB J 14: 680-690.