Effects of Calcium Ions on Extracellular and Intracellular Processes of Production of Reactive Oxygen Species in Blood Phagocytic Cells

A. V. Bizyukin, Z. F. Kharaeva*, S. K. Soodaeva, A. G. Chuchalin

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 126, No. 9, pp. 334-337, September, 1998
Original article submitted November 22, 1996

Preincubation of phagocytes with ryosidine and EGTA increases the intracellular production of reactive oxygen species and decreases their extracellular production in cells activated with phorbol 12-myristate 13-acetate. Ryosidine and EGTA decreased intra- and extracellular production of reactive oxygen species in formyl peptide-stimulated leukocytes. The addition of the ionophore A23187 led to the appearance of reactive oxygen species in the extracellular, but not in the intracellular, medium.

Key Words: *phagocytic cells; extra- and intracellular production of reactive oxygen species; calcium ions*

Phagocytic cells play an important role in the immune response and are involved in the anti-infective mechanisms in the body. These functions of phagocytes are associated with their ability to produce reactive oxygen species (ROS) [10].

The role of Ca^{2+} in the oxidative metabolism of phagocytes is ambiguous. The stimulators of the respiratory burst: formyl peptide (N-formyl-Met-Leu-Phe, FMLP), phorbol 12-myristate 13-acetate (PMA), and calcium ionophore A23187, exert various effects on Ca^{2+} concentration in phagocytes. For example, a transient increase in cytoplasmic concentration of free Ca^{2+} precedes the production of ROS in phagocytes stimulated with FMLP [3]. PMA does not change the concentration of intracellular Ca^{2+} [13]. There was a sharp increase in the Ca^{2+} concentration in the cytoplasm after incorporation of the calcium ionophore A23187 into the phagocytic membrane [11].

A comparative study of the role of Ca^{2+} in extra- and intracellular processes of ROS generation will probably open new aspects of the mechanism of free-radical activation of phagocytes.

MATERIALS AND METHODS

Leukocytes were isolated from the blood of healthy donors [6]. The leukocyte suspension was analyzed in COBAS ARGOS 5 DIFF and COBAS HELIOS 5 DIFF automated hematological analyzers. It contained 40±6% lymphocytes, 6±2% monocytes, and 54±7% neutrophils. Lymphocytes do not produce ROS [14]. Thus, neutrophils constituted approximately 90% of leukocytes capable of producing ROS.

According to the Trypan blue. We found the suspension contained 98±2% live cells.

The production of ROS was assessed by the hydroethidium method [2]. The stock solution of the fluorochrome in dimethylformamide was stored at -18°C. The formation of ethidium from hydroethidium was monitored by measuring the intensity of ethidium fluorescence in an MPF-44A spectrofluorometer (Perkin-Elmer) in a thermostatic 1-cm cuvettes at 473 nm excitation and 610 nm emission, 37°C, and constant stirring. The intracellular and total (intracellular and extracellular) production of

Institute of Pulmonology, Ministry of Health of Russia, Moscow; *Russian State Medical University, Moscow

0007-4888/98/0009-0940$20.00

ROS was evaluated. The extracellular production of ROS was determined by calculating the difference between the obtained values.

The standard sample contained 10^6 cells/ml. Phagocytes were stimulated with PMA (2×10^{-7} M) and FMLP (2×10^{-7} M) which binds to specific receptor on the plasma membrane of human peripheral blood leukocytes. The kinetic curves representing the activation of cells with PMA and FMLP are shown in Fig. 1. PMA and FMLP did not affect the formation of ethidium from hydroethidium in the absence of cells.

The following reagents were used to study the role of Ca^{2+} in the processes of free-radical activation of phagocytic cells: 2 mM EGTA, a specific Ca^{2+}-binding agent that does not enter the cell, 2×10^{-5} M ryosidine (ryodipine, 1,4- dihydropyridine a derivative), a blocker agent of Ca^{2+} channels on the plasma and intracellular membranes (the drug used at this concentration blocks more than 50% of Ca^{2+} channels [1]), and the Ca^{2+} ionophore A23187 at a noncytotoxic concentration of 0.5 ng/ml. The reagents applied at these concentrations did not affect the viability of leukocytes (for 30-40 min), the conversion of hydroethidium into ethidium, and the luminescence of ethidium. They did not display the properties of radical traps in the Fenton's reagent.

RESULTS

Preincubation with ryosidine and EGTA affected differently the intracellular production of ROS in PMA- and FMLP-stimulated leukocytes. In PMA-stimulated leukocytes, ryosidine and EGTA increased the endogenous production of ROS by 154% and 67%, respectively (Fig. 2, *a*). In FMLP-stimulated phagocytes ryosidine and EGTA inhibited this process by 77% and 56%, respectively (Fig. 2, *b*). Ryosidine and EGTA decreased (in a similar manner) the extracellular production of ROS.

The addition of the ionophore A23187 to the leukocytes suspension stimulated the exogenous production and did not affect the intracellular production of ROS. After preincubation with A23187 phagocytes did not react to the activators.

The release of Ca^{2+} from the endoplasmic reticulum vesicles and its entry from the external medium are believed to be the essential events which precede the activation of Ca^{2+}-dependent protein kinases. Therefore, these processes are involved in the activation of NADPH oxidase and the metabolism of arachidonic acid [4].

The intracellular ROS generation may be associated with the breakdown of the mitochondrial respiratory chain [12] caused by xanthine oxidase or

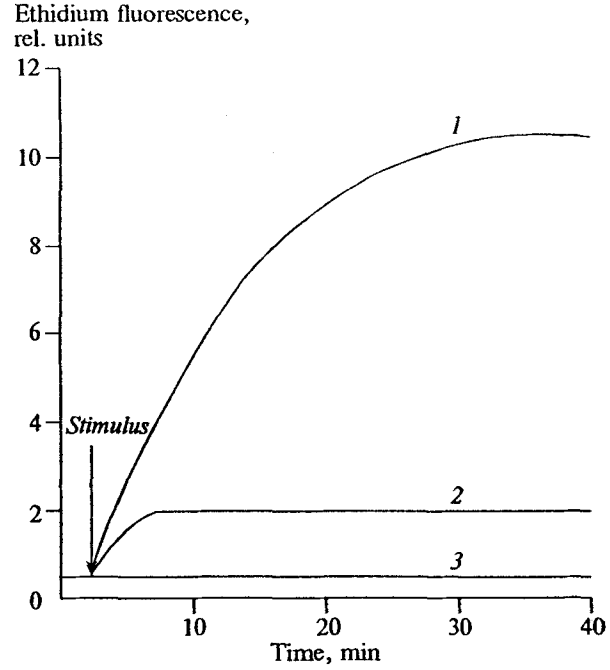

Fig. 1. Typical kinetic curves of activation of blood phagocytes with *1*) 0.2 μM phorbol 12-myristate 13-acetate, *2*) 0.2 μM formyl peptide, and *3*) hydroethidium with the stimulators mentioned above (without cells).

myeloperoxidase of neutrophils [8]. The arachidonic acid metabolites are released in or out of the plasmalemma [9]. Superoxide radicals generated by NADPH oxidase extracellularly may serve as a source of intracellular ROS. These radicals probably diffuse into the cell due to their low reactivity.

It can be suggested that if Ca^{2+} deficiency (induced by ryosidine or EGTA) similarly affects the extracellular and intracellular production of ROS, the mechanism of radical generation inside and outside phagocytes will be the same. This mechanism is probably associated with free-radical processes in the outer membrane. Similar these results were obtained after the activation of blood phagocytes with FMLP (Fig. 2, *b*). This is in agreement with the assumption of the mechanism of FMLP action [7,8].

Activation of cells with PMA produced in other effects. Ryosidine and EGTA decreased the extracellular production of ROS and increased their intracellular generation (Fig. 2, *a*). Therefore, the mechanisms of extracellular and intracellular production of ROS in PMA-stimulated phagocytes are different.

The mechanism underlying the PMA effect on the intracellular free-radical processes received little attention. PMA was shown to activate free-radical processes in mitochondria. This was confirmed by high sensitivity of this process to antimycin and cyanide [12]. There is no agreement otter the role

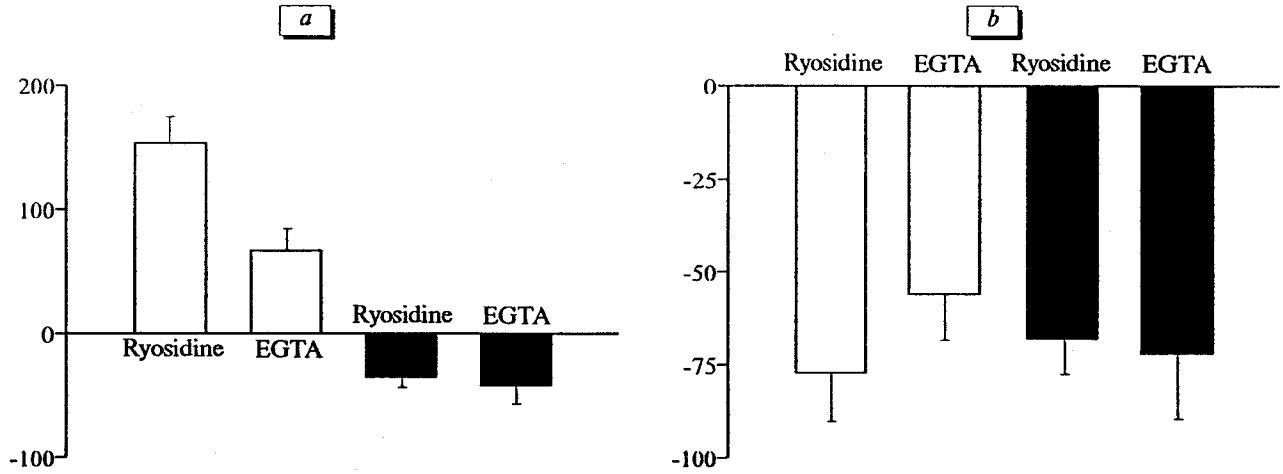

Fig. 2. Effects of ryosidine and EGTA on the intracellular (light bars) and extracellular (dark bars) production of ROS in blood phagocytes stimulated with *a*) phorbol 12-myristate 13-acetate and *b*) formyl peptide. Ordinate: $(I_i-I_c)/I_c \times 100\%$, where I_c and I_i are the intensities of ethidium luminescence in the suspension of stimulated cells in the control and after a preincubation.

of Ca^{2+} in free-radical processes in the mitochondrial respiratory chain. The interaction of PMA with mitochondria and the role of Ca^{2+} in this interaction require further investigations. Our experiments do not show what free-radical reactions in the intracellular organelles are affected by PMA. Obviously, these processes are not associated with NADPH oxidase and the metabolism of arachidonic acid.

The Ca^{2+} ionophore A23187 stimulates the entry of Ca^{2+} into phagocytes and raises by an order of magnitude the cytoplasmic concentration of free Ca^{2+}. This changes the transmembrane potential and blocks all potential-dependent Ca^{2+} channels. Calcium ionophores are believed to activate the formation of free-radical derivatives of arachidonic acid. The increase in the exogenous production of ROS in ionophore-stimulated phagocytes was observed in our experiments. Stimulatory effects of ionophores on the intracellular production of ROS (due to active metabolites of arachidonic acid produced inside of the plasma membrane) would thus be expected. However, A23187 (our data) and ionomycin [7] inhibits the intracellular production of ROS in blood phagocytes stimulated with PMA and FMLP. These data point to antimutagenic effects of calcium ionophores [5]. The presence of certain hypothetical factors that indirectly stimulate (after an increase in the extracellular ROS production) the DNA damage has been assumed. We believe that if extracellularly produced ROS are involved in the free-radical damage to DNA, the mechanism of antimutagenic effects of the ionophore A23187 is quite clear.

REFERENCES

1. G. V. Belevich, G. Ya. Dyubur, G. E. Dobretsov, *et al.*, *Biol. Membrany*, 5, No. 7, 768-776 (1988).
2. A. V. Bizyukin, L. G. Korkina, and B. T. Velichkovskii, *Byull. Eksp. Biol. Med.*, **119**, No. 4, 361-365 (1995).
3. R. A. Axtell, R. R. Sandbond, J. A. Smolen, *et al.*, *Blood*, 75, No. 6, 1324-1332 (1990).
4. P. Bellavite, *Free Radic. Biol. Med.*, **4**, 225-261 (1988).
5. H. C. Birnboim, *Biochem. Cell Biol.*, **66**, No. 5, 374-381 (1988).
6. A. Boyum, *Scand. J. Clin. Lab. Invest.*, Suppl., **21**, No. 97, 77-80 (1968).
7. C. Dahlgren, *Biochim. Biophys. Acta.*, **930**, No. 1, 33-38 (1987).
8. P. Follin, A. Johansson, and C. Dahlgren, *Cell Biochem. Funct.*, **9**, No. 1, 29-37 (1991).
9. M. F. Forgue, B. Pipy, M. Beraud, *et al.*, *Int. J. Immunopharmacol.*, **12**, No. 2, 155-163 (1990).
10. B. Halliwell and J. M. Gutteridge, in: *Free Radicals in Biology and Medicine*, Oxford (1989), pp. 58-188, 366-494.
11. T. Hoffman and E. F. Lizzio, *J. Immunol. Methods*, **112**, 9-14 (1989).
12. L. Kobzik, J. Godleski, and J. Brain, *J. Leukoc. Biol.*, **47**, No. 4, 295-303 (1990).
13. A. Perianin and R. Snyderman, *J. Biol. Chem.*, **264**, No. 2, 1005-1009 (1989).
14. J. P. Robinson, L. H. Bruner, C.-F. Bassoe, *et al.*, *J. Leukoc. Biol.*, **43**, 304-310 (1988).