8. G. I. Grebler, J. Biol. Chem., **234**, No. 8, 3112 (1961).
9. P. K. Tublus and P. V. Gauland, Methods Enzymol., **12**, 535 (1969).
10. I. P. Williamson and B. E. Corney, Methods Enzymol., **13**, 434 (1969).

MEASUREMENT OF THE INTRACELLULAR Ca^{2+} CONCENTRATION IN MACROPHAGES AND THE EFFECT OF PLATELET ACTIVATION FACTOR

V. G. Pinelis, D. V. Zagulova, A. A. Galkin, M. B. Baskakov, and Kh. M. Markov

UDC 616.155.33-008.924.1-074

KEY WORDS: Ca^{2+}, platelet activation factor, macrophages, fluorescent indicator

The basic functions of macrophages, namely chemotaxis, degranulation, phagocytosis, and the "oxygen burst" [1, 11, 14] determine their key role in inflammatory reactions. Stimulated macrophages, in turn, secrete mediators of inflammation, namely prostaglandins, leukotrienes, platelet activation factor (PAF), superoxide anions, and interleukin-1 [12, 13]. An important role in these processes is played by Ca^{2+}, namely by changes in the intracellular concentration of this cation. For instance, an increase in $[Ca^{2+}]_i$ in macrophages, produced by means of ionophore A_{23187}, causes the appearance of tumoricidal activity and the secretion of tumor necrotic factor (TNF), expression of various antigens on the membrane surface, and an "oxygen burst," whereas antagonists of calcium ions and calmodulin inhibit activity of the macrophages [7, 9, 15]. There is some evidence in the literature [3, 8, 10] of a change, through the influence of $[Ca^{2+}]_i$ agonists in macrophages stimulated by sodium thioglycollate, although definite functional differences are known in the responses of resident and stimulated macrophages to the action of biologically active substances [1, 3]. It must also be pointed out that in [3], $[Ca^{2+}]_i$ was determined with the aid of the fluorescent probe quin-2, which has definite shortcomings (toxicity, low quantum yield, lower dissociation constant).

In the present investigation a new Ca^{2+}-sensitive fluorescent indicator fura-2 was used to study changes in $[Ca^{2+}]_i$ in resident and activated macrophages under the influence of PAF. PAF is a natural mediator of inflammatory reactions, of phospholipid nature, synthesized in many cells including macrophages (see above). In turn, mechanisms whereby the released PAF stimulates macrophages have not been finally established, and data in the literature are contradictory [3, 8].

EXPERIMENTAL METHOD

Resident and stimulated macrophages obtained from mice killed by cervical dislocation were used. In the latter case, 4 days before isolation of the cells the animals were given an intraperitoneal injection of 1.5 ml of a 2% solution of sodium thioglycolate; cells from peritoneal washings, obtained by injection of 5-7 ml of Hanks' solution (pH 7.35) into the peritoneal cavity, were sedimented on coverslips measuring 7 × 15 mm in order to obtain a monolayer, which was recorded under the microscope, or cell suspensions were prepared.

Research Institute of Pediatrics, Academy of Medical Sciences of the USSR. A. V. Vishnevskii Institute of Surgery, Moscow. Tomsk Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR M. Ya. Studenikin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 111, No. 6, pp. 579-581, June, 1991. Original article submitted October 2, 1989.

 0007-4888/91/0006-0752$12.50

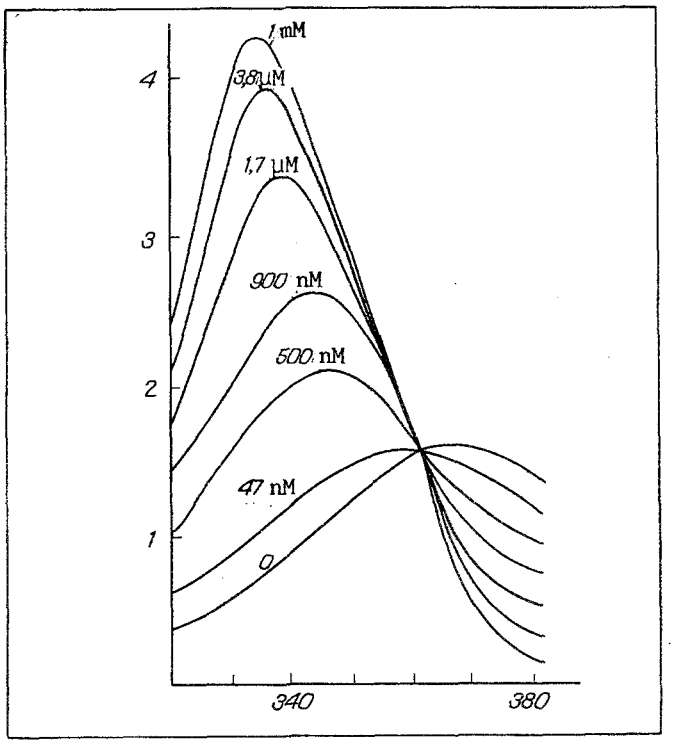

Fig. 1. Excitation spectra of 1 μM fura-2 in Ca^{2+} buffer solutions (0-1 mM Ca^{2+}; 100 mM KCl; 10 mM EGTA; 20 mM NaCl; 1 μM fura-2; pH 7.0; 37°C). Abscissa, wavelengths of excitation (in nm); ordinate, intensity of fluorescence with emission at 505 nm (in relative units).

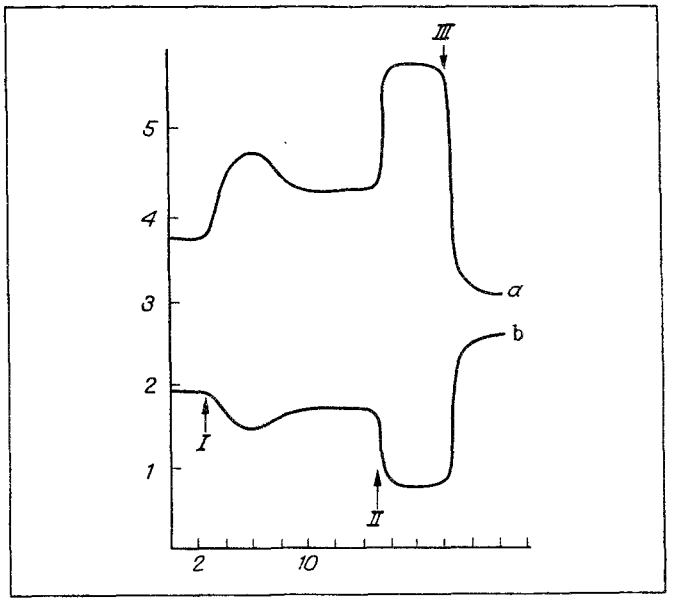

Fig. 2. Changes in intensity of fluorescence of fura-2-loaded macrophages at 340 nm (a) and 380 nm (b) excitation and 505 nm emission. Numbers next to arrows: I) PAF (10^{-7} M), II) digitonin, III) EGTA. Absicssa, time (in min); ordinate, intensity of fluorescence (in relative units).

The intracellular Ca^{2+} concentration was determined by means of the Ca-sensitive dual-wave fluorescent probe fura-2, which penetrates into the cell in the form of a hydrophobic ester, is hydrolyzed by intracellular esterases, and binds with Ca^{2+}; during binding the maximum of fluorescence is shifted and its intensity changes. Coverslips with a monolayer or the cell suspension was transferred into Hanks' solution (pH 7.0) containing 2.5 μM fura-2/AM, and incubated at 16-17°C (preliminary experiments showed that at this temperature the maximum of penetration of the probe into the cell is obtained, and exo- and endocytosis are reduced) for 60 min on a shaker. The plates were then washed twice for 10 min each time in Hanks' solution (pH 7.35) containing 1% BSA, and in the suspension case, centrifugation was used for washing (800*g*, 10 min). Until the investigations the cells were kept in a refrigerator at 8°C. Fluorescence was measured on a "Hitachi 650-60" spectrofluorometer (Japan) at 37°C. By means of a microprocessor it was possible to allow for background fluorescence and to record at two excitation wavelengths (340 and 380 nm) with emission at 505 nm. During measurement of fluorescence of the monolayer the coverslip was fixed in the quartz cuvette of the spectrofluorometer diagonally at an angle of 45° to the excitation and emission slits, whose width was 5 nm. The concentration of free intracellular Ca^{2+} was calculated by the method of Grynkiewicz and Tsien [5] for dual-wave probes:

$$[Ca^{2+}\,I_i = K_d\,(R - L_{min})\,/\,(R_{max} - R),$$

where R denotes the ratio of values of fluorescence of the probe at 340 and 380 nm, K_d the dissociation constant, and I_i a coefficient of proportionality, which was calculated by using the pure form of fura-2.

EXPERIMENTAL RESULTS

Data on changes in fluorescence of accurately prepared solutions with different concentrations of Ca and 1 mM $MgCl_2$ are given in Fig. 1. On the basis of the fluorescence curves thus obtained values of K_d and I_i were calculated, and amounted to 210 and 6.84 nM respectively, roughly the same as values obtained by Grynkiewicz and Tsien [5]. These coefficients in solutions not containing magnesium were significantly smaller, and this must be taken into account when working with this particular probe.

Measurements of the Ca^{2+}-signal were made both in suspension and in a monolayer of macrophages. The use of the monolayer, according to data in the literature, has certain advantages compared with the cell suspension: 1) up to 95% of macrophages are usually found in the monolayer, whereas their proportion in suspension is much less; 2) leakage of the probe from the cells into the monolayer is less marked; 3) the adherent cells are washed more easily and rapidly; 4) superfusion of the monolayer is possible.

Our investigations showed that the Ca^{2+}-signal in the suspension was unstable and the basal level was difficult to monitor (not given). Meanwhile in the monolayer, fluorescence of the probe-loaded macrophages was stronger and more stable (Fig. 2).

The investigations showed definite differences in $[Ca^{2+}]$ in resident and sodium thioglycolate stimulated macrophages (Fig. 3). Second, the basal level of $[Ca^{2+}]_i$ was much higher in the stimulated cells (100 + 2 nM compared with 64 + 10 nM in residents, $n = 8$, $p < 0.05$). Elevation of the basal level in stimulated macrophages is evidence of changes in rates of Ca^{2+} exchange and may be associated, as data obtained by several workers have shown [1, 6, 7], with the higher functional activity of these cells (more intensive activity of acid hydrolases, lactate dehydrogenase, aminopeptidase). Second, in resident macrophages PAF (10^{-7} M) induced a rapid but small (not more than 150 nM) transient increase in free cytosol calcium. By contrast, in macrophages treated with sodium thioglycollate, PAF in the same concentration caused a persistent and greater (up to 400 nM) increase in $[Ca^{2+}]_i$. The prolonged calcium response to the action of PAF in the stimulated macrophages is probably associated, on the one hand, with reduction of calcium ATPase activity (a similar action of PAF has been described in smooth muscle cells [2]), and on the other hand, with the so-called additive effect (priming effect) of PAF of preactivated cells [2].

The considerable increase in the intracellular Ca^{2+} concentration discovered in the present investigation by the use of fura-2, and also in studies by Rink and co-workers [3] on quin-2-loaded macrophages under the influence of PAF is connected with the increased input of Ca^{2+} into the cells through receptor-mediated channels, for this effect was reduced in calcium-free solution (EGTA buffer) (the lower curves in Fig. 3). However, as these curves show, about 40% of the increase of $[Ca^{2+}]$ in response to PAF was due also to release of this cation from the intracellular depots. It is interesting to note that, unlike in the present investigation, in which the new fluorescent probe fura-2 was used as Ca^{2+} indicator, in

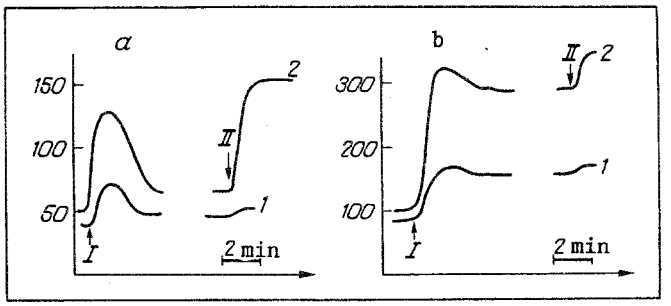

Fig. 3. Effect of PAF (10^{-7} M) on $[Ca^{2+}]_i$ in resistant (a) and sodium thioglycollate stimulated peritoneal macrophages (b) (2 μM ionomycin was added to obtain R_{max}, R_{min} after addition of Mn^{2+}). Ordinate, $[Ca^{2+}]_i$ (in nM); numbers next to arrows: I) PAF, II) ionomycin. 1) $[Ca^{2+}]_i$ in macrophages at $[Ca^{2+}]_0$ = 0, 1-2 mM EGTA added to buffer. 2) $[Ca^{2+}]_i$ in macrophages at $[Ca^{2+}]_0$ = 1 mM.

the investigations of Rink and co-workers [3], who used quin-2, the release of Ca^{2+} from the intracellular depots was considerably less (about 10%). Gelfand and co-workers [4] found that fura-2 is most suitable for the detection of Ca^{2+} release from intracellular depots by the fluorescent probe.

Thus the results of the present investigation showed that PAF, by interaction with specific plasma membrane receptors of macrophages, leads to an increase in $[Ca^{2+}]$, which plays an important role in the triggering of most; intracellular reactions, evidently including hyperpolarization of the cells through activation of calcium-dependent K^+-channels, as has been shown for endothelial cells [2].

LITERATURE CITED

1. D. O. Adams and T. A. Hamilton, Ann. Rev. Immunol., **2**, 283 (1984).
2. P. Braquet, M. Paubert-Braquet, and R. H. Bourgain, J. Lipid Med., **1**, 75 (1989).
3. G. W. Conrad and T. J. Rink, J. Cell Biol., **103**, 439 (1986).
4. E. W. Gelfand, B. G. Mills, R. K. Cheung, and S. Grinstein, Immunol. Rev., **95**, 59 (1987).
5. G. Grynkiewicz, M. Poenie, and R. Y. Tsien, J. Biol. Chem., **260**, 3440 (1985).
6. H. P. Hartung, M. G. Parnham, and J. Winkelman, Int. J. Immunopharmacol., **8**, 118 (1983).
7. A. Holian and R. P. Daniel, J. Cell Physiol., **113**, 87 (1982).
8. A. Holian and H. D. Stickle, J. Cell Physiol., **123**, 39 (1985).
9. J. M. Mencia-Huerta, R. Roubin, J. L. Morget, and J. Benveniste, J. Immunol., **129**, 804 (1982).
10. T. Nakamura, Y. Nishisawa, and C. Yamato, Eur. J. Pharmacol., **148**, 35 (1988).
11. D. S. Nelson, Clin. Exp. Immunol., **45**, 225 (1981).
12. J. A. Rankin, M. Hitchcock, and W. W. Merril, J. Immunol., **132**, 1993 (1984).
13. R. Snyderman and M. C. Pike, Ann. Rev. Immunol., **2**, 257 (1984).
14. E. R. Unanue, Ann. Rev. Immunol., **2**, 395 (1984).
15. J. E. Weiel, D. O. Adams, and T. A. Hamilton, J. Immunol., **134**, 293 (1985).