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Fc-GAMMA- AND COMPLEMENT RECEPTOR MEDIATED ELEVATION IN THE CYTOSOLIC CALCIUM LEVEL IN HUMAN NEUTROPHILS

Helena Tuominen[1], Lasse Leino[2] and Karl Åkerman[1]

[1] Department of Biochemistry and Pharmacy, Åbo Academy University, Turku, Finland

[2] Department of Hematology, Turku University Central Hospital, Turku, Finland

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Summary: The effects of differently opsonized zymosan particles, acting solely at Fc-gamma or at complement receptors or at both, on the level of intracellular calcium ($[Ca^{2+}]_i$) in human neutrophils were studied. A biphasic, long-lasting increase in $[Ca^{2+}]_i$ was seen in response to IgG-, C3- and fresh serum-opsonized zymosan particles in the presence of extracellular Ca^{2+}. Unopsonized zymosan, acting mainly at CR3 failed to elevate $[Ca^{2+}]_i$. Addition of 1.4 mM EGTA reduced but did not abolish the rise in $[Ca^{2+}]_i$ triggered by opsonized zymosan, indicating that Ca^{2+} is released from intracellular stores. EGTA changed also the kinetic patterns of Ca^{2+}-responses possibly by indirectly affecting the extrusion of Ca^{2+} in neutrophils.

Introduction: The human polymorphonuclear neutrophil is known to have different types of IgG-Fc receptors (FcRI, FcRII and FcRIII) and complement receptors for opsonic fragments of C3 (CR1 and CR3) in its plasma membrane (1,2). These receptors are of importance in the immune responses mediated by the neutrophil. FcRI, expressed in significant level only after induction with interferon-gamma, is active in antibody-dependent cell-mediated cytotoxicity reactions (3). FcRII/ is essential for IgG-induced phagocytosis and superoxide production (4). The phosphatidylinositol-linked

Abbreviations: $[Ca^{2+}]_i$ intracellular free calcium concentration, FcR Fc-gamma receptors, CR complement receptors, IP_3 inositol-1,4,5-triphosphate.

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FcRIII mediates the signal for degranulation and most likely also for activation of NADPH oxidase (5,6). CR1 and CR3 participate in binding of C3b- and C3bi opsonized particles, respectively (7,8). In addition, CR3 serves as a receptor for zymosan particles and bacterial lipopolysaccharide (9,10). Both CR1 and CR3 have been shown to mediate the respiratory burst and phagocytosis (9,11).
Calcium plays an important role in many activities of the neutrophil and affects both the function and expression of opsonin receptors; especially the binding capacity and functional activity of CR3 are dependent on extracellular Ca^{2+} (12,13).
The aim of this study was to investigate the role of Ca^{2+} in signal transduction through FcR and CR by measuring the effect of opsonized particles on cytosolic Ca^{2+} using fura-2 AM.

Materials:

Fura-2 acetoxymethylester (fura-2 AM) was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A). Ethyleneglycol-bis (ß-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), N-formyl-methionyl-leucyl-phenylalanin (fMLP), and zymosan were from Sigma Chemical Co. (St. Louis, MO, U.S.A). Digitonin was purchased from Merck (Darmstadt, Germany)
Media
Na^+-based medium (BSS) was composed of (mM): NaCl, 137,; KCl, 5,; $MgCl_2$ x 6 H_2O, 1.2,; $CaCl_2$, 1,; KH_2PO_4, 0.44; $NaHCO_3$, 4.2; glucose, 10; 2-3-hydroxyl-1,1-bishydroxymethylethyl-aminoethane sulphonate, 20 (TES, Sigma, U.S.A) adjusted to pH 7.4.
The Ca^{2+}-depleted medium used during cell-isolation and for loading the cells was composed of NaCl, 137; KCl, 5.4; Na_2HPO_4, 0.34; $NaHCO_3$, 2.57; KH_2PO_4, 0.4; glucose, 5.56 adjusted to pH 7.4.

Methods:

Preparation of human neutrophils
EDTA-anticoagulated (1.5 mg EDTA/ml blood) blood samples were collected from healthy blood donors. The leucocytes were separated by sedimenting erythrocytes with 6 % dextran and granulocytes were further separated with a Ficoll-Paque density gradient (Pharmacia, Uppsala, Sweden). The erythrocyte contamination was lysed with 0.83 % ammonium chloride. Neutrophils were washed twice with Ca^{2+}- and Mg^{2+}-free BSS.
Preparation of zymosan
Unopsonized and serum opsonized zymosan preparations were made as described (14). C3-opsonized i.e. C3b/C3bi-opsonized zymosan was prepared by incubating unopsonized

zymosan with human serum from which zymosan-specific immunoglobulins had been removed (16). IgG-opsonized zymosan was prepared by incubating unopsonized zymosan (2.2 mg/ml) with an i.v. immunoglobulin preparation (Sandoglobulin R, Sandoz Pharmaceuticals Co, Switzerland) for 30 min at 37°C.

Measuring intracellular free Ca^{2+}

In order to determine intracellular free Ca^{2+}, the cells were loaded with 5 μM fura-2 AM in the Ca^{2+}-depleted medium, at 37°C for 30 min with constant agitation. Then the cells were spun down at 440 x g for 5 min, resuspended in Ca^{2+}-depleted medium and stored at room temperature. Aliquots of 300,000 cells were diluted into the Na^{+}-based medium and fluorescence was recorded at 340 nm (ex.) and 505 nm (em.) in Hitachi F-4000, Hitachi F-2000 or Perkin Elmer LS50 spectrofluorometers under constant stirring. The dye response was calibrated by sequential addition of 0.06 mg/ml digitonin and 8 mM EGTA at the end of the experiment to give maximal and minimal fluorescence, respectively. The final concentrations of fMLP and zymosan in the reaction-mixture were 76 nM and 0.14 mg/ml. Calculation of the free Ca^{2+} concentration was performed as described (15).

Results: The basal level of $[Ca^{2+}]_i$ in isolated neutrophils was 174±69 nM (mean and SD, n=250). A rise in the level of $[Ca^{2+}]_i$ was observed in normal BSS with C3-, serum- and IgG-opsonized zymosan particles, respectively (fig 1 and 2). The unopsonized zymosan particles failed to increase $[Ca^{2+}]_i$ appreciably (fig 2).

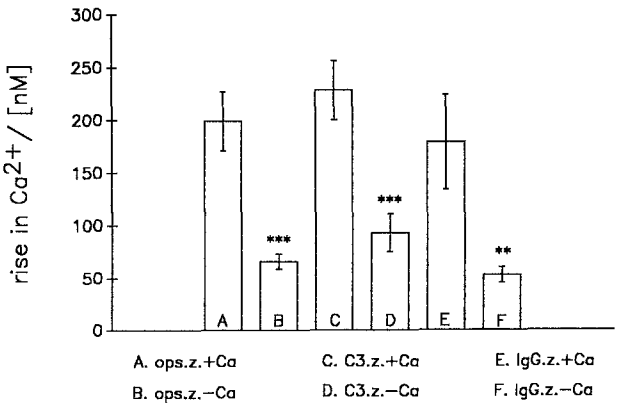

fig. 1.
Opsonized zymosan induced rise in the level of intracellular Ca^{2+} as delta Ca^{2+}. Columns A, C and E represent the rise in Ca^{2+}-containing medium with 0.14 mg/ml serum-, C3- and IgG-opsonized zymosan particles, respectively. Columns B, D and F represent the rise after addition of 1.4 mM EGTA. The values are expressed as mean and SD of at least three duplicate experiments. ** $p<0.01$; *** $p<0.001$ (Student's t test), comparison between responses in the presence and absence of EGTA.

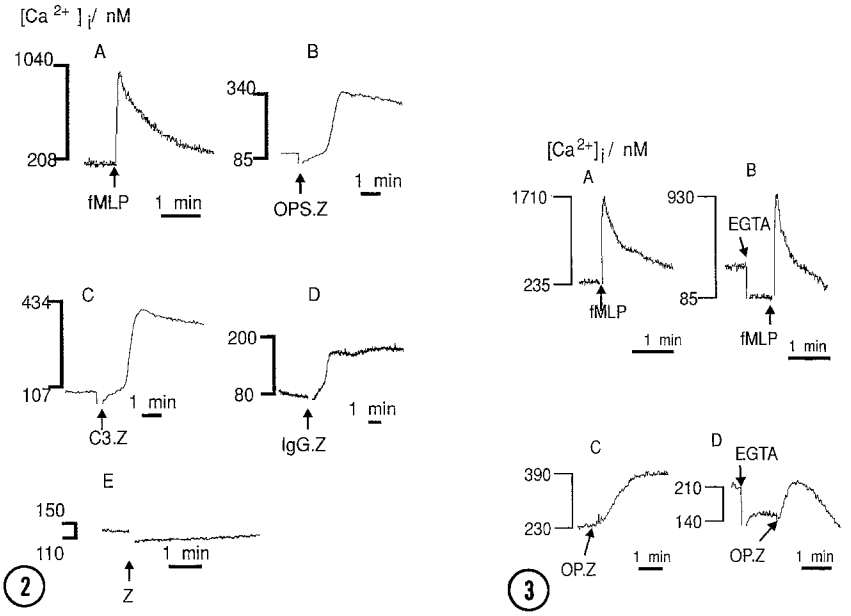

fig. 2.
Rise in the level of intracellular Ca^{2+} after stimulation with the different ligands. A fMLP, B serum-opsonized zymosan, C C3-opsonized zymosan, D IgG-opsonized zymosan and E unopsonised zymosan. Each response is representative for at least three similar experiments.

fig. 3.
The effect of EGTA on the duration of Ca^{2+}-transients. A fMLP. B 1.4 mM EGTA + fMLP. C serum-opsonized zymosan. D 1.4 mM EGTA + serum-opsonized zymosan. Each response is representative for at least three similar experiments.

The change in $[Ca^{2+}]_i$ caused by the opsonized particles was different from the rapid response of the soluble agonist fMLP (fig 2). Typically, after zymosan stimulation a two-phase rise in $[Ca^{2+}]_i$ was seen. Initially, a slow rise, lasting for 60-90 seconds occurred, followed by a rapid increase to a peak value. This two-phase phenomenon was not distinct in every experiment (fig 3C), but was a common feature. With fMLP the $[Ca^{2+}]_i$ peak started immediately to decline and reached a level near baseline in 120-180 seconds. With opsonized particles the duration of the calcium transients was markedly longer and only a slight decrease in the $[Ca^{2+}]_i$-level was observed in the first few minutes after the maximal signal.

Addition of 1.4 mM EGTA immediately before the addition of C3-, serum- or IgG-opsonized zymosan particles decreased

the Ca^{2+} signal by 60 % ($p<0.001$), 67 % ($p<0.001$) and 70 % ($p<0.01$), respectively. The $[Ca^{2+}]_i$ peak also declined rapidly to baseline level in the presence of EGTA (fig 3D). A similar change in the duration of fMLP-induced Ca^{2+}-transients was recorded (fig 3B).

Discussion: Stimulation of human neutrophils with several agonists induces an increase in the intracellular free Ca^{2+} level by releasing Ca^{2+} from internal, non-mitocondrial and IP_3-sensitive calcium pools and by increasing the plasma-membrane permeability to Ca^{2+} (17-19). Since the rise in $[Ca^{2+}]_i$ preceeds neutrophil functions, it has long been proposed that the calcium transients are part of a not yet fully characterized signal transduction pathway linking receptor occupancy to cellular responses. Recently, however, the role of Ca^{2+}-transients has been questioned, and it now seems that in neutrophils there are Ca^{2+}-transient-independent as well as Ca^{2+}-transient-dependent responses (20-22).

In the present study a clear elevation in $[Ca^{2+}]_i$ was detected in response to opsonized zymosan particles in the presence of extracellular calcium. In these conditions the reaction between cells and opsonized particles is directed by the opsonins on targets and their respective receptors on neutrophils, and presumably by zymosan sugar components acting at least at CR3 of leucocytes. Unopsonized zymosan, on the other hand, which solely interacts with CR3 and possibly also with other mannose/glucosamine receptors (23,24), could not increase $[Ca^{2+}]_i$. When the extracellular Ca^{2+} was chelated by EGTA, the opsonized particles induced $[Ca^{2+}]_i$ peaks were significantly reduced but not totally blocked. Since the removal of extracellular Ca^{2+} abolishes the CR3-dependent reactions, the functional opsonin receptors in these conditions are FcR and CR1. Thus, these results suggest that 1) CR3 (and other carbohydrate receptors), without any coupling to other opsonin receptors, cannot rapidly increase $[Ca^{2+}]_i$ in neutrophils, and 2) FcR and CR1 are able to rise $[Ca^{2+}]_i$ at least via mobilization of intracellular calcium stores. Whether the higher $[Ca^{2+}]_i$ peak in the presence of extacellular Ca^{2+} is due to the influx mediated by FcR and CR1 or the result of

co-operative action of FcR or CR1 with CR3 remains to be elucidated. Recently it was shown that the cross-linking of FcRII causes an increase in $[Ca^{2+}]_i$ by the release of calcium from intracellular stores, not by the influx (25). In addition to the Ca^{2+} peak heights, also the kinetic properities of the Ca^{2+}-transients were changed in the presence of EGTA. The typical slow, gradual rise in the $[Ca^{2+}]_i$ level after zymosan addition disappeared. Interestingly, the Ca^{2+}-transients returned also more rapidly to baseline. One explanation for this observation is that in the absence of EGTA a continuous Ca^{2+}-influx replaces the extruded calcium sustaining the long-lasting Ca^{2+} signal in cells. Alternatively, it is possible that the activation pathway in the presence of extracellular Ca^{2+} modifies the extrusion of Ca^{2+} across plasma membrane e.g. by generating a high concentration of intracellular IP_3, which has been found to inhibit both the heart sarcolemmal and human erythrocyte plasma membrane Ca^{2+}-pump activity (26,27). The latter possibility is supported by the studies, which have shown that the accumulation of IP_3 after stimulation with yeast-IgG and yeast-C3b/C3bi particles is much higher in normal neutrophils than in Ca^{2+}-depleted cells (20,21). The opsonized particles also induced a sustained production of IP_3 (21) whereas the fMLP-stimulated IP_3 formation in neutrophils is transient, returning to near basal level within 60 s (28). Clearly further work is required before the reason for different kinetic patterns is properly understood.

The data presented in this paper demonstrate that the unopsonized zymosan is unable to alter $[Ca^{2+}]_i$ in neutrophils. Yet in our previous studies unopsonized zymosan particles (and unopsonized bacteria) have mounted prominent oxidative-metabolism responses (15,29). Thus, it appears that Ca^{2+}-transients are not essential for CR3 mediated activation of NADPH-oxidase in human neutrophils.

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References:

1 Huizinga, T.W., Roos, D. & von dem Borne, A.E. (1990) Blood 75, 1211-1214

2 Ross, C.D. (1989) Curr. Opinion Immunol. 2, 50-62
3 Shen, L., Guyre, P. & Fanger, M. (1987) J. Immunol. 139, 534-538
4 Huizinga, T.W., van Kemenade, F., Koenderman, L., Dolman, K.M., Borne, A.E., Tetteroo, P.A. & Roos, D. (1989) J. Immunol. 142, 2365-2369
5 Huizinga, T.W., Dolman, K.M., van der Linden, N.J., Kleijer, M.,Nuijens, J.H., von der Borne, A.E. & Roos, D. (1990) J. Immunol. 144, 1432-1437
6 Crockett-Torabi, E. & Fantone, J. (1990) J. Immunol. 145, 3026-3032
7 Arnaout, M., Gupta, S., Pierce, M. & Tenen, D. (1988) J. Cell Biol. 106, 2153-2158
8 Ross, G. & Lambris, J. (1982) J. Exp. Med. 155, 96-110
9 Ross, G., Cain, J. & Lachmann, P. (1985) J. Immunol. 134, 3307-3315
10 Wright, S. & Jong, M. (1986) J. Exp. Med. 164, 1876-1888
11 Hoogerwerf, M., Weening, R., Hack, C. & Roos, D. (1990) Molec. Immun. 27, 159-167
12 Wright, S. & Silverstein, S. (1982) J. Exp. Med. 156, 1149-1164
13 Berger, M., Brix, D.L., Wezler, E.M., O'Shera, J.J., Brown, E.J. & Cross A.S. (1985) J. Immunol. 135, 1342-1348
14 Leino, L. & Lilius, E-M. (1990) Int. Arch. Allergy appl. Immunol. 91, 30-35
15 Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450
16 Roos, D., Bot, A., van Schaik, M., de Boer, M. & Daha M.R. (1981) J. Immunol. 126, 433-440
17 Pozzan, T., Lew, D.P., Wollheim, C.B. & Tsien R.Y. (1983) Science 221, 1413-1415
18 Korchak, H.M., Vienne, K., Rutherford, L.E., Wilkenfeld, C., Finkelstein, C. & Weissmann, G. (1984) J. Biol. Chem. 259, 4076-4082
19 Prentki, M., Wollheim, C.B. & Lew, P.D. (1984) J. Biol. Chem. 259, 13777-13782
20 Fällman, M., Lew, D., Stendahl, O. & Andersson, T. (1989) J. Clin. Invest. 84, 886-891
21 Della Bianca, V., Grzeskowiak, M. & Rossi, F. (1990) J. Immunol. 144, 1411-1417
22 O'Flatherty, J., Rossi, A., Jacobson, D. & Redman, J. (1991) Biochem. J. 278, 705-711
23 Sung, J., Nelson, R. & Silverstein, S. (1983) J. Cell Biol. 96, 160-166
24 Ross, G., Cain, J., Myones, B., Newman, S. & Lachmann, P. (1987) Complement 4, 61-74
25 Van de Winkel, J., Tax, W., Jacobs, C., Huizinga, T. & Willems, P. (1990) Scand. J. Immunol. 31, 315-325
26 Kuo, T. (1988) Biochem. Biophys. Res. Commun. 152, 1111-1116
27 Davis, F., Davis, P., Lawrence, W. & Blas, S. (1991) FASEB J. 5, 2992-2995
28 Thompson, N., Tateson, J., Randall, R., Spacey, G., Bouser, R. & Garland, L. (1990) Biochem. J. 271, 209-213
29 Leino, L., Peltonen,P. & Lilius, E-M. (1991) In Bioluminescense and Chemiluminescense: Current status, Stanley P. and Kricka L. (eds) John Wiley and Sons Ltd, Chichester, England, p 265-268