Ligands of Surface Ig Raise Cytoplasmic Free Ca^{++} in Human B Cells

H. C. CLEVERS, A. C. BLOEM, F. GMELIG-MEYLING & R. E. BALLIEUX

Department of Clinical Immunology, University Hospital, Utrecht, The Netherlands

Clevers, H.C., Bloem, C.A., Gmelig-Meyling, F. & Ballieux, R.E. Ligands of Surface Ig Raise Cytoplasmic Free Ca^{++} in Human B Cells. *Scand. J. Immunol.* 22, 557–562, 1985.

With the use of the fluorescent Ca^{++} indicator Quin-2, we have measured changes in intracellular calcium levels in human B cells in response to anti-Ig antibodies, to *Staphylococcus aureus* (Staph) or to protein A. Cells of an Epstein-Barr virus-transformed $\mu\lambda$ -carrying B-cell line, AZU-1, increased free cytosolic calcium after addition of anti- μ or anti- λ antibodies; F(ab')₂ fragments with anti- μ specificity were equally effective. Fab fragments of sheep anti-Ig antibodies only induced a rise in calcium levels after addition of a second anti-sheep Ig antiserum. Cross-linking of non-Ig surface determinants did not influence calcium homeostasis. The calcium channel blockers verapamil (100 μM), nifedipine (20 μM), and LaCl_3 (200 μM) inhibited the anti- μ -induced calcium influx. Peripheral blood B cells reacted in essentially the same way in response to anti- μ antibodies. The B cell mitogens protein A and Staph also induced a rise in intracellular calcium. These observations indicate that Ca^{++} may play a role as a messenger in the activation of human B cells via surface Ig.

H. C. Clevers, Department of Clinical Immunology, University Hospital, Catharijnesingel 101 3511 GV Utrecht, The Netherlands

It is generally accepted nowadays that cross-linking of antigen-receptors in the membrane of B lymphocytes is an essential step in B-cell activation. In vitro this can be achieved by incubation with anti-IgM antibodies or with *Staphylococcus aureus* (Staph) preparations that interact with surface Ig via protein A. It is assumed that these ligands mimic the action of multivalent antigens [8, 9]. Recently it has been shown with the use of Quin-2, an intracellular fluorescent Ca^{++} indicator [18], that T cells respond with a rapid Ca^{++} influx upon binding of monoclonal antibodies to the antigen receptor-associated T3 molecule [19]. A rise in cytosolic free calcium ($(\text{Ca}^{++})_i$) has also been reported in murine splenocytes in response to anti-Ig antisera [4, 15]. In addition, voltage-gated Ca^{++} channels have been demonstrated in the cell membrane of mouse myeloma cells [7]. These findings prompted us to investigate a possible rise in $(\text{Ca}^{++})_i$ induced by ligands binding to cell surface components in human cells of B lineage. The data presented here indicate that Ca^{++} ions play a

crucial role in the early phase of B cell activation, induced by cross-linking of surface Ig.

MATERIALS AND METHODS

Isolation of cells. The AZU-1 cell line, an Epstein-Barr virus (EBV)-transformed surface $\mu^+\lambda^+$ human B cell line, was kept in continuous culture in RPMI 1640 (Gibco Europe, Hoofddorp, The Netherlands) supplemented with 10% fetal calf serum, 2×10^{-5} M β -mercaptoethanol, L-glutamine, and antibiotics.

Peripheral blood B cells. Mononuclear cells were isolated from buffy coats of healthy volunteers by centrifugation on Ficoll-Isopaque (1.077 g/cm³). The cell preparation was depleted of T cells by two rounds of rosette formation with the use of S-(2-aminoethyl) isothiuronium bromide hydrobromide-treated sheep erythrocytes. Non-T cells were separated in a monocyte- and a B-cell-enriched fraction by centrifugation on a discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden), with densities ranging from 1.053 to 1.126 g/ml. The upper layer (M ϕ) contained approximately 97% monocytes and 3% lymphocytes; the lower layer, enriched for B cells (B), was contaminated with approximately 13%

monocytes and 3% T lymphocytes, as detected by immunofluorescence with the appropriate monoclonal antibodies.

Quin-2 assay [17]. Quin-2 acetoxymethylester ((Sigma, St. Louis, Mo., USA); 10 mM in DMSO) was added to a cell suspension containing 10^8 cells per ml of RPMI 1640 to a final concentration of $50 \mu\text{M}$; the mixture was incubated for 20 min at 37°C . The suspension was then diluted 10-fold and incubated for a further 30 min. Cells were centrifuged for 10 min at 200g, resuspended in RPMI at $10^7/\text{ml}$, and kept at room temperature. Immediately before the assay $250 \mu\text{l}$ of the suspension was centrifuged for 5 sec at $14,000g$; the pellet was resuspended in $250 \mu\text{l}$ of assay buffer (NaCl 145, KCl 5, Na_2HPO_4 1, CaCl_2 1, MgSO_4 0.5, glucose 5, Na HEPES 10 mM, pH 7.40). Quin-2 fluorescence was recorded with a Perkin-Elmer MPF-3 spectrofluorimeter thermostated to 37°C . Excitation and emission wavelengths were 339 and 492 nm, with 4 and 10 nm bandwidths.

At selected times, $5 \mu\text{l}$ of a given ligand was added. Antisera were used at stock concentrations unless otherwise indicated:

goat anti-human IgM (Tago, Burlingame, USA): αIgM ,

goat anti-human IgE (Tago): αIgE ,

goat anti-human IgG (Tago): αIgG ,

goat anti-mouse IgM + IgG (Tago): GaMIg ,

rabbit anti-human κ light chain (Central Laboratory of the Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands): $\alpha\kappa$,

rabbit anti-human λ light chain (CLB): $\alpha\lambda$,

F(ab')₂ sheep anti-human IgM (prepared in our laboratory, 1 mg/ml): (Fab')₂ αIgM ,

Fab sheep anti-human total Ig (CLB): (Fab) αIg ,

rabbit anti-sheep Ig (Nordic, Tilburg, The Netherlands): RashIg ,

protein A (1 mg/ml; Pharmacia, Uppsala, Sweden),

Staphylococcus aureus, formalinized, 1×10^8 organisms/ml, kindly supplied by Dr R. K. B. Schuurman, University of Leyden, The Netherlands [17].

anti HLA-Dr (Becton-Dickinson, Mountain View, USA),

5D2 monoclonal antibody (Dr B. van Camp, University of Brussels, Belgium; 1:100 ascites), recognizing a B cell differentiation antigen present on AZU-1.

For calibration, fluorescence was measured after release of the indicator from the cells with NP40 (Fluka, Buchs, Switzerland) 0.02% v/v at Ca^{++} concentrations of $<1 \text{ nM}$ ($F_{\min}$) and 1 mM ($F_{\max}$). $(\text{Ca}^{++})_i$ is calculated by the equation $(\text{Ca}^{++})_i = (115 \text{ nM}) (F - F_{\min}) / (F_{\max} - F)$.

Blocking experiments. Quin-2-loaded AZU-1 cells were incubated for 5 min at room temperature with the Ca^{++} channel blocker verapamil (Sigma, St Louis, Mo., USA) in end concentrations of 0, 25, and $100 \mu\text{M}$ or with nifedipine (Sigma) at 0, 5, and $20 \mu\text{M}$; each sample then contained 0.5% ethanol. At time points indicated, $5 \mu\text{l}$ of αIgM was added. Alternatively, cells were preincubated with LaCl_3 (Merck, Darmstadt, FRG) at 0, 50, and $200 \mu\text{M}$ in assay buffer.

RESULTS

Cells of the AZU-1 line or purified peripheral blood B cells were loaded with Quin-2 and stimulated in suspension with various ligands of surface Ig or with antibodies to cell-surface structures of a non-Ig nature.

As shown in Fig. 1, the concentration of free Ca^{++} in the cytosol of resting AZU-1 cells was

FIG. 1. AZU-1 cells were loaded with Quin-2 and at the times indicated $5 \mu\text{l}$ of different antisera was added. The time course of fluorescence intensity was recorded and calibrated in terms of $(\text{Ca}^{++})_i$.

about 95 nM ; upon addition of αIgM or $\alpha\lambda$, $(\text{Ca}^{++})_i$ increased about 5-fold. The effects of αIgM and $\alpha\lambda$ were not additive. The divalent F(ab')_2 fragments of αIgM were equally effective, indicating that Ig-Fc receptor interactions are not involved in the induction of the increase. Monovalent $(\text{Fab})\alpha\text{Ig}$ did not exert a measurable effect on Ca^{++} homeostasis. However, cross-linking of these fragments on the cell membrane by RashIgG did induce Ca^{++} mobilization (Fig. 2); this established the necessity of aggregation of surface-bound Ig in this process.

No Ca^{++} mobilization was observed after incubation with αIgG , αIgE (results not shown), or $\alpha\kappa$; these 3 antisera are directed

Fig. 2. Crosslinking of Fab fragments of sheep anti-human total Ig by rabbit anti-sheep antiserum induced calcium mobilization in AZU-1 cells. In a control experiment phosphate buffered saline (PBS) was added instead of (Fab) α Ig.

against antigens not detectable on AZU-1 cells. In addition, incubation of the AZU-1 line cells with monoclonal antibodies directed towards HLA-DR framework determinants or the B-cell differentiation marker 5D2, did not result in an increase of (Ca⁺⁺)_i, even after subsequent crosslinking of surface-bound monoclonal antibodies by G α Mlg.

Addition of verapamil, an agent which blocks cation flow through Ca⁺⁺ channels, inhibited the α IgM induced raise in (Ca⁺⁺)_i almost completely at 100 μ M, as shown in Fig. 3. In addition, nifedipine, another organic Ca⁺⁺ channel blocker, partially inhibited transmembrane Ca⁺⁺ flow at 5 μ M; at 20 μ M the calcium flux was virtually abolished. Also, LaCl₃ at 200 μ M blocked Ca⁺⁺ mobilization induced by α IgM. In control experiments it was noticed that the addition of each of these agents resulted in a gradual decrease of measured (Ca⁺⁺)_i at a rate of 4 nM/min at maximum, probably due to a disturbance of the balance between transmembrane calcium fluxes. These changes were considered to be relatively insignificant, as compared to those induced by α IgM.

Fig. 3. AZU-1 cells were loaded with Quin-2 and incubated with 0, 25, or 100 μ M verapamil in assay buffer for 5 min, each sample containing 0.5% ethanol; at the time indicated 5 μ l of α IgM was added.

Essentially identical observations were made using human blood B cells. These B lymphocytes can also be induced to raise (Ca⁺⁺)_i upon incubation with α IgM (Fig. 4). The addition of antibodies to IgE (α IgE), which was not detectable on the cells by immunofluorescence, did not induce a measurable increase in (Ca⁺⁺)_i. Calcium mobilization was also induced by protein A and, at a slower rate, by Staph.

The measured calcium influences were not due to an increase of (Ca⁺⁺)_i in monocytes contaminating the B cell preparations, as shown by the lack of response in control experiments using an almost pure monocyte preparation (Fig. 4).

DISCUSSION

A novel mechanism functioning as second messenger has been recognized recently. Within seconds after receptor-ligand binding two biologically active split products of the membrane component phosphatidyl inositol [4, 5] bisphosphate are found: diacylglycerol, an activator of protein kinase C, and inositol trisphosphate (ITP), which activates calcium ion channels. Since ITP is water-soluble, it can traverse the cytosol; consequently, calcium channels in the cell membrane as well as in the membrane of cell organelles are probably opened up, resulting in a transient rise in (Ca⁺⁺)_i (reviewed by Berridge & Irvine [1]).

The role of calcium in lymphocyte activation has been extensively documented using different experimental approaches [5]. The recent development of the Quin-2 assay enables direct measurement of changes in cytosolic calcium levels. With the use of this assay it has been shown that T-cell mitogens such as phyto-

FIG. 4. Peripheral blood B cells (B) and monocytes (M) were isolated and loaded with Quin-2. At times indicated 5 μ l of a ligand of surface immunoglobulin was added.

haemagglutinin [18] or monoclonal antibodies binding to the antigen receptor-associated T3 molecule [19] induce a rapid rise in $(Ca^{++})_i$. Furthermore, murine splenocytes were shown to respond with a calcium influx upon addition of anti-Ig antisera [15].

Here we report that cells of human B lineage, too, increase $(Ca^{++})_i$ in response to crosslinking of sIg. Cells of the $\mu\lambda$ carrying, EBV-transformed AZU-1 line increased $(Ca^{++})_i$ upon addition of antisera directed towards μ or λ only. Crosslinking of sIg appeared to be essential, since $F(ab')_2$ fragments of sheep anti-Ig antibodies did not exert any measurable effect, whereas addition of rabbit anti-sheep antiserum restored the original capacity of inducing calcium mobilization. These results cannot be explained by aspecific membrane perturbation: binding and subsequent crosslinking of monoclonal antibodies directed towards non-Ig surface determinants, i.e., anti-HLA-DR and ζ D2, did not influence $(Ca^{++})_i$.

Calcium flow could be inhibited by the organic calcium channel blockers verapamil and nifedipine at concentrations reported to inhibit lymphocyte mitogenesis in *in vitro* cultures [2, 3, 6] and by $LaCl_3$. However, calcium fluxes in excitable tissues are blocked at 10 to 100-fold lower concentrations [11]. Verapamil has recently been shown to inhibit K^+ efflux currents in T lymphocytes at concentrations comparable to those used in this study [6]. Thus involvement of K^+ channels in the α IgM-induced rise in $(Ca^{++})_i$ cannot be excluded by these results. However, La^{3+} at 200 μ M is considered to be a specific calcium-channel blocker [5], suggesting that Ca^{++} ion channels play a crucial role in the phenomena reported in this paper. Recently, La^{3+} has been reported to be an effective blocker of Ca^{++} shifts in human T cells stimulated by anti-T3 antibodies [14].

Blood B lymphocytes were also shown to raise $(Ca^{++})_i$ upon addition of α IgM. The response, however, was less dramatic than in

AZU-1 cells; this is probably due to the heterogeneity of the cell preparation used, of which only the IgM-carrying B cells will increase their (Ca⁺⁺)_i upon addition of α IgM. As alternative anti-Ig reagents, Staph or its protein A were assayed; protein A present on the Staph particles is able to bind to the Fab as well as the Fc portion of human Ig. By interactions with the F(ab')₂ part of the surface-bound Ig, crosslinking is established, resulting in an activation signal [16]. The signal provided by Staph leads to a transition of B cells from a resting state into the S phase [13]. Soluble protein A can act as a B-cell mitogen as well [12]. Both protein A and Staph were found to induce calcium mobilization in blood B lymphocytes and not in the monocytes contaminating the B-cell preparation (Fig. 4). The particulate nature of Staph probably results in slower recombination with sIg, explaining the different kinetics found. Human T cells increase (Ca⁺⁺)_i in response to protein A in the presence of monocytes only [10]. In the present study we cannot exclude that the B cell response to protein A is monocyte-dependent.

Most information on early events in the activation of human lymphocytes has been derived from studies on the effect of mitogens, such as PHA and Con A, on T cells. The data presented in this communication show that multivalent ligands, binding to surface IgM or its light chain on human B cells, induce a rapid calcium mobilization, thus establishing the central role of calcium ions early in the process of activation induced by crosslinking of B-cell antigen receptors.

ACKNOWLEDGMENT

This work was supported partly by the Foundation 'De Drie Lichten'.

REFERENCES

- Berridge, M.J. & Irvine, R.F. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315, 1984.
- Birz, D.L., Berger, M. & Fleisher, T. The interference of T cell activation by calcium channel blocking agents. *J. Immunol.* **133**, 2904, 1984.
- Blitstein-Willinger, E. & Diamantstein, T. Inhibition by isoptin (a calcium antagonist) of the mitogenic stimulation of lymphocytes prior to the S phase. *Immunology* **34**, 303, 1978.
- Braun, J., Sha'afi, R.I. & Unanue, E.R. Cross-linking by ligands to surface immunoglobulin triggers mobilization of intracellular ⁴⁵Ca²⁺ in B lymphocytes. *J. cell. Biology* **82**, 755, 1979.
- Campbell, A.K. Intracellular calcium. *Its Universal Role as Regulator*. John Wiley & Sons Limited, Chichester, 1983.
- Chandy, K.G., DeCoursey, T.E., Cahalan, M.D., McLaughlin, C. & Gupta, S. Voltage-gated potassium channels are required for human T lymphocyte activation. *J. exp. Med.* **160**, 369, 1984.
- Fukushima, Y. & Hagiwara, S. Voltage-gated Ca²⁺ channel in mouse myeloma cells. *Proc. natn. Acad. Sci. USA* **80**, 2240, 1983.
- Kehrl, J.H., Muraguchi, A., Butler, J.L., Falkoff, R.J.M. & Fauci, A.S. Human B cell activation, proliferation and differentiation. *Immunol. Rev.* **78**, 75, 1984.
- Kishimoto, T., Yoshizaki, K., Kimoto, M., Okada, M., Kuritani, T., Kikutani, H., Shimizu, K., Nakagawa, T., Nakagawa, N., Miki, Y., Kishi, H., Fukunaga, K., Yoshikubo, T. & Taga, T. B cell growth and differentiation factors and mechanism of B cell activation. *Immunol. Rev.* **78**, 97, 1984.
- Lederman, H.M., Lee, J.W.W., Cheung, R.K., Grinstein, S. & Gelfand, E.W. Monocytes are required to trigger Ca⁺⁺ uptake in the proliferative response of human T lymphocytes to *Staphylococcus aureus* protein A. *Proc. natn. Acad. Sci. USA* **81**, 6827, 1984.
- Lee, K.S. & Tsien, R.W. Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature, Lond.* **302**, 790, 1983.
- Lipsky, P.E. Staphylococcal protein A, a T cell-regulated polyclonal activator of human B cells. *J. Immunol.* **125**, 155, 1980.
- Muraguchi, A. & Fauci, A.S. Proliferative responses of normal human B lymphocytes. Development of an assay system for human B cell growth factor (BCGF). *J. Immunol.* **129**, 1104, 1982.
- Oettgen, H.C., Terhorst, C., Cantley, L.C. & Rosoff, P.M. Stimulation of the T3-T cell receptor complex induces a membrane potential-sensitive calcium influx. *Cell* **40**, 583, 1985.
- Pozzan, T., Arslan, P., Tsien, R.Y. & Rink, T.J. Anti-immunoglobulin, cytoplasmic free calcium and capping in B lymphocytes. *J. cell. Biology* **94**, 335, 1982.
- Romagnani, S., Giudizi, M.G., Biagiotti, R., Almerigogna, F., Maggi, E., del Prete, G. & Ricci, M. Surface immunoglobulins are involved in the interaction of protein A with human B cells and in the triggering of B cell proliferation induced by protein A-containing *Staphylococcus aureus*. *J. Immunol.* **127**, 1307, 1981.
- Schuurman, R.K.B., Gelfand, E.W. & Dosch, H.-M. Polyclonal activation of human lymphocytes in vitro. 1. Characterization of the lympho-

- cyte response to a T-cell-independent B cell mitogen. *J. Immunol.* **125**, 820, 1980.
- 18 Tsien, R.Y., Pozzan, T. & Rink, T.J. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature, Lond.* **295**, 68, 1982.

- 19 Weiss, A., Imboden, J., Wiskocil, R. & Stobo, J. The role of T3 in the activation of human T cells. *J. Clin. Immunol.* **4**, 165, 1984.

Received 12 April 1985