Cross-Linking of IgG Receptors Inhibits Membrane Immunoglobulin-stimulated Calcium Influx in B Lymphocytes

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Abstract. By cross-linking membrane immunoglobulins (mIg), the antigenic stimulation of B lymphocytes induces an increase in intracellular free calcium levels ($[Ca^{2+}]_i$) because of a combination of release from intracellular stores and transmembrane influx. It has been suggested that both events are linked, as in a number of other cases of receptor-induced increase in $[Ca^{2+}]_i$. Conversely, in B lymphocytes, type II receptors for the Fc fragment of IgG (FcγRII) inhibit mIg-mediated signaling. Thus, we have investigated at the level of single cells if these receptors could act on specific phases of mIg Ca^{2+} signaling.

Lipopolysaccharide-activated murine B splenocytes and B lymphoma cells transfected with intact or truncated FcγRII-cDNA were used to determine the domains of FcγRII implicated in the inhibition of the Ca^{2+} signal. $[Ca^{2+}]_i$ was measured in single fura-2-loaded cells by microfluorometry. The phases of release from intracellular stores and of transmembrane influx were discriminated by using manganese, which quenches fura-2, in the external medium as a tracer for bivalent cation entry. The role of membrane potential was studied by recording $[Ca^{2+}]_i$ in cells voltage-clamped using the perforated patch-clamp method.

Cross-linking of mIgM or mIgG with F(ab')2 fragments of anti-Ig antibodies induced a sustained rise in $[Ca^{2+}]_i$ due to an extremely fast and transitory release of Ca^{2+} from intracellular stores and a long lasting transmembrane Ca^{2+} influx. The phase of influx, but not that of release, was inhibited by membrane depolarization. The increase in $[Ca^{2+}]_i$ occurred after a delay inversely related to the dose of ligand. Co-cross-linking mIgs and FcγRII with intact anti-Ig antibodies only triggered transitory release of Ca^{2+} from intracellular stores but no Ca^{2+} influx, even when the cell was voltage-clamped at negative membrane potentials. These transitory Ca^{2+} rises had similar amplitudes and delays to those induced by cross-linking mIgs alone. Thus, our data show that FcγRII does not mediate an overall inhibition of mIg signaling but specifically affects transmembrane Ca^{2+} influx without affecting the release of Ca^{2+} from intracellular stores. Furthermore, this inhibition is not mediated by cell depolarization. Thus, FcγRII represents a tool to dissociate physiologically the phases of release and transmembrane influx of Ca^{2+} triggered through antigen receptors.

VARIATION in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[1] is one of the key events in transmembrane signaling. B lymphocytes display on their plasma membrane various receptors which can activate or inhibit Ca^{2+} signaling, and thus provide a valuable model to study this process. These cells express receptors for antigens which are the membrane-anchored forms of immunoglobulins (mIg). Stimulation by cross-linking mIg, as by a variety of receptors involved in regulation of cell growth, transduces intracellular signals which lead to the activation of phospholipase C and an increase in $[Ca^{2+}]_i$ due to both an initial release from internal stores, and a subsequent sustained Ca^{2+} entry across the plasma membrane (Bijsterbosch et al., 1986; Labaer et al., 1986; Dugas et al., 1987; for review see Rink and Meritt, 1990). The mechanism which underlies Ca^{2+} release involves, in most cases, activation by inositol 1,4,5-trisphosphate (IP_3) of Ca^{2+} channels present in the membrane of cytoplasmic Ca^{2+} storage organelles (for review see Berridge and Irvine, 1989). Information about receptor induced Ca^{2+} influx through the plasma membrane is still very limited, although it probably involves activation of a conductive pathway (Kuno et al., 1986; Lewis and Cahalan, 1989; Hoth and Penner, 1992; Lückhoff and Clapham, 1992). This is mainly due to the lack of defined pharmacological tools and to the difficulty of physiologically dissociating the phase of release from that of influx. It has been suggested in a number of cells, including lymphocytes, platelets, endothelium,

1. *Abbreviations used in this paper*: $[Ca^{2+}]_i$, intracellular free calcium concentration; FcγRII, type II receptors for the Fc fragment of IgG; mIg, membrane immunoglobulin; IP_3, inositol 1,4,5,-trisphosphate; LPS, lipopolysaccharide.

hepatocytes, and exocrine secretory cells, that stimulation of transmembrane influx of Ca^{2+} is a direct consequence of the emptying of the intracellular IP_3-sensitive Ca^{2+} stores (Scharff et al., 1988; Sage et al., 1989; Takemura, 1989; Putney, 1990; Alvarez et al., 1991; Mason et al., 1991; Meldolesi et al., 1991; Hoth and Penner, 1992). However, the mechanisms underlying the coupling between the state of filling of the store and activation of Ca^{2+} influx remain unknown and alternative pathways of activation of Ca^{2+} influx have been proposed. Particularly, IP_3 or IP_4 receptors linked to Ca^{2+} permeable channels seem to be present directly in the plasma membrane of lymphocytes (Kuno and Gardner, 1987; Khan et al., 1992*b*) and endothelial cells (Lückhoff and Clapham, 1992). Thus production of IP_3 could directly activate both Ca^{2+} release and transmembrane Ca^{2+} influx.

B lymphocytes also express the bl isoform of low affinity receptors for the Fc portion of IgG (FcγRIIbl), which inhibit cell activation triggered through mIg (for review see Fridman et al., 1992). This inhibition requires co-cross-linking of mIg and FcγRII, for example by IgG antibody-covered antigens (immune complexes), and it plays an important role in the negative feedback regulation of antibody production (Phillips and Parker, 1984; Klaus et al., 1987). We and others have shown that co-cross-linking of mIg and FcγRII markedly inhibits the mIg-triggered increase in intracellular Ca^{2+} (Wilson et al., 1987; Amigorena et al., 1992), and several reports have demonstrated that it decreases IP_3 production (Bijsterbosch and Klaus, 1985; Wilson et al., 1987; Rigley et al., 1989). However, inhibition is not complete since the early phases of both Ca^{2+} and IP_3 production seem unaffected by FcγRII (Wilson et al., 1987; Rigley et al., 1989; Amigorena et al., 1992). We thus questioned to what extent the Fc receptors could act specifically on one of the several phases of mIg triggered Ca^{2+} signaling.

To measure accurately the kinetics of Ca^{2+} signaling, $[Ca^{2+}]_i$ was recorded by microfluorometry on single, intact or voltage-clamped, fura-2-loaded cells during cross-linking of mIg alone or mIg and FcγRII. Using these methods, we analyzed the capacity of naturally occurring FcγRIIbl expressed on splenic B lymphocytes or of intact and truncated forms of FcγRIIbl transfected into a FcγRII-defective B cell lymphoma to inhibit Ca^{2+} release from internal pools or Ca^{2+} influx through the plasma membrane triggered through mIg.

Materials and Methods

Cells

Purification of resting B lymphocytes from spleens of 8–10 wk old B6D2F1 mice was performed as previously described (Amigorena et al., 1990). Lipopolysaccharide (LPS)-activated blastocytic cells were obtained by culturing these cells 48–72 h at a concentration of 5×10^5 cells/ml in RPMI 1640 (Gibco, Paisley, England) supplemented with 10% heat-inactivated FCS (J BIO, Paris, France), 1 mM glutamin, 1 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 2×10^{-5} M mercaptoethanol (Sigma Chem. Co., St. Louis, MO), 25 μg/ml LPS (Difco, Detroit, MI), and incubated at 37°C in 5% CO_2 atmosphere.

The IIA1.6 cell line (Jones et al., 1986) is a somatic variant of the A20/2J B lymphoma that has deletion of the 5′ part of the FcγRII gene (Lewis et al., 1986; Bonnerot et al., 1991) and does not express FcγRII molecules. By transfecting cDNA encoding the intact or truncated form of FcγRIIbl into this cell line, we obtained clones stably expressing $1-4 \times 10^5$ FcγRII molecules per cell (Amigorena et al., 1992). FcγRIIbl-CT79 and FcγRIIbl-CT76 are receptors deleted in the cytoplasmic portion of FcγRIIbl. They have been constructed by introducing a stop codon at positions 80 and 77 of the cytoplasmic tail as described previously (Bonnerot et al., 1992). Surface expression of FcγRIIbl-CT79 and FcγRIIbl-CT76 has been quantified by cytofluorometry using the anti-FcγR mAb 2.4G2 (Unkeless, 1979) and was found to be similar to that of the wild-type FcγRIIbl. All the experiments presented were performed on clones in which the FcγRII expression was regularly checked using this procedure. Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS (J BIO), 10 mM glutamine, 20 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and 5×10^{-5} M mercaptoethanol (Sigma Chem. Co.).

Reagents

Surface IgM and IgG were cross-linked, respectively, with affinity purified goat $F(ab')_2$ anti-mouse IgM, μ chain specific (Cappel-Organon Teknika, Fresnes, France), and affinity purified $F(ab')_2$ anti-mouse IgG, γ chain specific (Cappel-Organon Teknika). FcγRII and surface IgM or IgG were, respectively, co-cross-linked with intact rabbit anti-mouse IgM, μ chain specific (Pierce, Rockford, IL), and intact rabbit anti-mouse IgG (Cappel-Organon Teknika). The monoclonal antibody 2.4G2 was used to cross-link FcγRII alone. All chemicals were from Sigma Chem. Co.

Recording of Intracellular Ca^{2+} Levels

$[Ca^{2+}]_i$ was measured at the level of single cells using the Phocal microfluorometric system (Joyce-Loeble, Cambridge, England). 2–10 10^5 cells per ml were incubated with 1 μM fura-2-acetoxy-methyl-ester (Molecular Probes, Junction City, OR) for 15 min at 37°C in the culture medium. The loaded cells were then plated on poly-ornithine-coated (500 μg/ml, 10 min) glass coverslips. After 10–15 min at room temperature, the medium was replaced by Krebs-Ringer solution containing, in mM: NaCl, 140; KCl, 4; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 10; Glucose, 10; pH adjusted to 7.2 with NaOH. The dish was then placed on the stage of an IM-35 Zeiss inverted microscope and fluorescence levels were measured through a 40× objective at 510 nm with a photo multiplier tube (PM) (Thorn EMI, Cambridge, England). The measuring area was limited to a single cell with a diaphragm (Nikon, France) placed in the optic path before the PM. A computer controlled the rotating filter wheel which was equipped with interference filters centered at 340 and 380 nm and was placed in the light pathway of a 75 W xenon lamp that excited single cells alternatively at these two wavelengths.

The absolute intracellular Ca^{2+} concentrations were calculated on line by the Phocal software using the formula:

$$[Ca^{2+}] = K_{appt} * \frac{(R - R_{min})}{(R_{max} - R)}$$

where R is the ratio between the fluorescence levels measured using the 340 and 380 nm excitation wavelengths (the phocal system allows us to obtain one measure of $[Ca^{2+}]_i$ every 160 ms). Rmin and Rmax are the ratios obtained at 0 and saturating doses of Ca^{2+}, respectively, while K_{appt} is the apparent dissociation constant for the fura-Ca^{2+} complex, which depends on the optical set up. Rmin, Rmax, and K_{appt} equaled 0.26, 8.5, and 3 μM, respectively, and were calculated from measurements obtained from cells whole cell patch-clamped with pipette solutions buffered, at known Ca^{2+} concentrations, with EGTA or containing 10 mM Ca^{2+}. For experiments, anti-Ig antibodies were diluted in Krebs-Ringer solution at the indicated concentrations and applied by pressure ejection through a glass pipette (5–10 μm diam tip, 1.5 mm glass capillary) positioned 10–20 μm from the recorded cell. A continuous suction was applied through a second pipette (50–100 μm diam tip) positioned on the opposite side of the cell to limit diffusion of the antibody containing solution.

Perforated Patch-Clamp Recordings

Lymphocytes loaded with fura-2 as above were used. Patch electrodes (5–10 MegaΩ) were pulled from soft glass and coated with Crown sticky wax (SS white Ltd., Kingstone, UK). The bath solution was composed of the following: 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, pH 7.2 adjusted with NaOH. The pipette solution contained: 130 mM CsOH, 15 mM $CsCl_2$, 10 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.2 adjusted with Methanesulfonate. Just before the experiment, amphotericin B (Sigma Chem. Co.), in stock solution at 30 mg/ml in DMSO, was dissolved in the pipette recording solution at 30 μg/ml. For recordings, the

tip of the patch electrode was filled with a solution free of amphotericin by capillarity and backfilled with the amphotericin containing solution. After seal formation, the access resistance was monitored through compensation by an Axopatch 1-A (Axon Instrs., Foster City, CA) of the capacitative artifact produced by a 10-mV hyperpolarizing voltage pulse. Typically, an access resistance of 10–30 MegaΩ was attained after 4–8 min. The input resistance of the cell was always >5 GigaΩ. These electrical properties ensured that the cell was adequately voltage-clamped. The pipette voltage was imposed through the Axopatch 1-A, and current was monitored on a 2230 digital oscilloscope (Tektroniks, Paris, France). Simultaneous acquisition of voltage, current and fura-2 fluorescence traces was performed by the Phocal Software.

Data Analysis

All data were analyzed on a 386/25 computer (Compaq, Paris, France) using 123 spreadsheet (Lotus Corporation, Paris, France). The delay of the Ca^{2+} response was taken as the time between the onset of the addition of ligand and the point where $[Ca^{2+}]i$ had reached a value of three times that of the baseline level. The half decay time of the Ca^{2+} responses was measured as the time required for the Ca^{2+} level to decrease from its peak value to half of this maximum. All mean experimental values are given ± SD. The relative total cell fura-2 concentration was used to assess quenching of fura-2 by Mn^{2+} and was derived from measurements of fluorescence intensities measured at 340 and 380 nm excitation as follows:

If we assume a one to one binding reaction between fura-2 and Ca^{2+} with an effective dissociation constant:

$$Kd = \frac{\beta}{\alpha}, \text{ then}$$

$$Fura_{free} + Ca^{2+} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} Fura_{bound}$$

where $Fura_{free}$ is the free form of fura-2 and $Fura_{bound}$ the form bound to Ca^{2+}. According to Grynkiewicz et al. (1985), for a mixture of free and Ca^{2+} bound fura-2, at the respective concentrations [], the total fluorescence intensities at wavelengths 1 and 2 are:

$$F_1 = S_{f1} * [Fura_{free}] + S_{b1} * [Fura_{bound}] \quad (1)$$

$$F_2 = S_{f2} * [Fura_{free}] + S_{b2} * [Fura_{bound}] \quad (2)$$

if the contribution to the corresponding fluorescence is proportional to the concentration of each species with proportionality coefficients S_{f1} and S_{f2} for the free dye, measured at wavelengths 1 and 2, and S_{b1} and S_{b2} for Ca^{2+} bound dye.

Solving Eqs. 1 and 2 for $[Fura_{free}]$ and $[Fura_{bound}]$ leads to:

$$[Fura_{free}] = \frac{1}{d}(F_1 * S_{b2} - F_2 * S_{b1})$$

$$[Fura_{bound}] = \frac{1}{d}(-F_1 * S_{f2} + F_2 * S_{f1})$$

where d is the discriminant of the system of Eqs. 1 and 2 and equals

$$S_{f1} * S_{b2} - S_{f2} * S_{b1}.$$

Thus, the cell fura-2 concentration T is:

$$T = [Fura_{free}] + [Fura_{bound}] = \frac{1}{d}(F_1 * (S_{b2} - S_{f2}) + F_2 * (S_{f1} - S_{b1}))$$

$$\text{Since } R_{min} = \frac{S_{f1}}{S_{f2}}, \; R_{max} = \frac{S_{b1}}{S_{b2}}, \text{ and } K_{appt} = \frac{S_{f2}}{S_{b2}} * Kd$$

(Grynkiewicz et al., 1985)

this relationship can be written:

$$T = \frac{S_{b1}}{d * R_{max}} * (F_1 * (1 - K_{appt}) + F_2 * (K_{appt} * R_{min} - R_{max}))$$

R_{min} and R_{max} were determined experimentally as described above. K_{appt} was determined as the value for which the total cell fluorescence did not vary during a rapid change in $[Ca^{2+}]i$ (in absence of Mn^{2+}). This theoretically derived value was always very close to that determined experimentally. This equation gives T with an unknown multiplicative coefficient S_{b1}/d which was fixed arbitrarily.

Results

FcγRII-Mediated Shortening of mIg-triggered Ca^{2+} Signals

Cross-linking of mIgs by multivalent antigen was mimicked by the use of F(ab')2 fragments of anti-mIgs while co-cross-linking of mIgs and FcγRII by immune complexes was mimicked by intact IgG anti-mIgs antibodies (Phillips and Parker, 1984). Two types of lymphocytes were used which express different mIg isotypes although both express FcγRIIb1. (1) Splenic B lymphocytes activated during 48 h with LPS which express mIgM (Coutinho and Forni, 1982) and FcγRIIb1 (Amigorena et al., 1989). (2) Clonal cell lines, derived from the FcγRII-defective IIA1.6 B-cell lymphoma (Jones et al., 1986) which express functional membrane IgG2a (Justement et al., 1989; Amigorena et al., 1992), and obtained after stable transfection with the cDNA encoding intact or truncated forms of the FcγRIIb1 (see Materials and Methods).

Fig. 1 shows that cross-linking mIgM on LPS-activated spleen B lymphocytes or mIgG on IIA1.6 cells transfected with FcγRIIb1 induced a sharp rise in $[Ca^{2+}]i$ which remained elevated for hundreds of seconds. Conversely, co-cross-linking FcγRIIb1 and mIgM or mIgG only induced a transitory (15–30 s) rise in $[Ca^{2+}]i$ (Fig. 1). Both the rise time of the Ca^{2+} increase and the delay with which it occurred after addition of the ligand were similar when mIgs alone or mIg and FcRs were cross-linked. The rise occurred with a 20–40 s delay after addition of the ligand to the cell (respectively, 25 s; SD = 16 s; n = 32 for 50 μg/ml F[ab']2 anti-IgG and 30 s; SD = 11 s; n = 39 for 75 μg/ml intact anti-IgG, measured on FcγRIIb1 transfected cells) and was extremely fast since in most of the cells tested, the peak in $[Ca^{2+}]_i$ was reached in < 2–3 s. The rise speed during this explosive period was on the average of 1 μM/s and it was similar in LPS-treated and IIA1.6 cells. The peak value reached by $[Ca^{2+}]_i$ was not significantly different under both experimental conditions (respectively, 0.75 μM; SD = 0.39 μM; n = 32 for F[ab']2 anti-IgG and 0.70 μM; SD = 0.29 μM; n = 39 for intact anti-IgG, measured in FcγRIIb1 transfected cells).

When mIgs alone were cross-linked, after this short phase of sharp rise, $[Ca^{2+}]_i$ remained elevated for a length of time which varied from cell to cell and ranged between 100 s and more than 15 min (mean = 312 s; SD = 147 s; n = 32, measured on FcγRIIb1 transfected cells). By contrast, when mIgs and FcγRII were cross-linked, $[Ca^{2+}]_i$ returned rapidly to baseline levels (mean half-decay time 28.5 s; SD = 21.0 s; n = 39), even slightly lower in some cases, and remained low for the rest of the experiment. This dramatic shortening of the length of the Ca^{2+} response by FcγRII engagement in both cell types is quantified in Fig. 2.

To confirm the involvement of the Fc receptors in this inhibitory process, we performed similar experiments after preincubation of the cells with a rat monoclonal antibody, 24G.2 (Unkeless, 1979), against FcγRII. As shown in the bottom set of the traces in Fig. 1, using these conditions, the

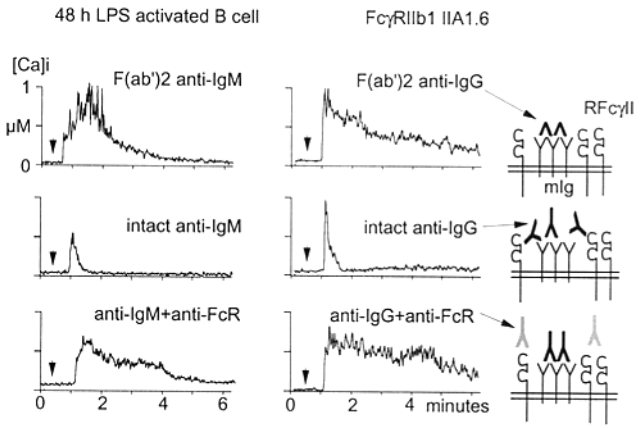

Figure 1. Inhibition of Ca^{2+} signaling by co-cross-linking of Fc receptors and surface immunoglobulins in normal and FcγRIIb1 transfected B lymphocytes. Recordings of intracellular Ca^{2+} levels in single fura-2 loaded cells during cross-linking of mIg alone (*top traces*), co-cross-linking of mIg and FcγRIIb1 in control conditions (*middle traces*), or after preincubation with an anti-FcγR antibody (*bottom traces*). The respective engagement of mIg and FcγRII in each set of experiments is represented schematically on the right side of the panels. Experiments were carried out on purified splenic B lymphocytes cultivated for 48 h with 30 μg/ml LPS (*left*) and a lymphoma cell line stably transfected with FcγIIRb1 cDNA (*right*). Stimulation of the cells (onset at *arrowhead*), respectively, with 50 μg/ml F(ab')2 anti-IgM (*top left*) or 50 μg/ml F(ab')2 anti-IgG (*top right*) induced a long lasting rise in $[Ca^{2+}]_i$, while application of 75 μg/ml intact anti-IgM (*middle left*) or 75 μg/ml intact anti-IgG (*middle right*) produced only a short peak of $[Ca^{2+}]_i$ increase. Stimulation with 75 μg/ml intact anti-IgM (*bottom left*) or 75 μg/ml intact anti-IgG (*bottom right*) after a 5-min preincubation with 8 μg/ml anti-FcγR restored a long lasting increase in $[Ca^{2+}]_i$.

intact anti-mIgM or anti-mIgG antibodies induced a long lasting rise in $[Ca^{2+}]_i$, with a length comparable to that observed when F(ab')2 fragments of the antibodies were used (Fig. 2). Addition of 24G.2 alone did not induce a rise in $[Ca^{2+}]_i$, even after the FcγRII cross-linking was augmented with a mouse anti-rat antibody (not shown). These data suggest that FcγRIIs are responsible for the inhibition of the mIgM or mIgG-mediated-Ca^{2+} responses triggered by intact anti-mIg. They also confirm that separate occupancy of

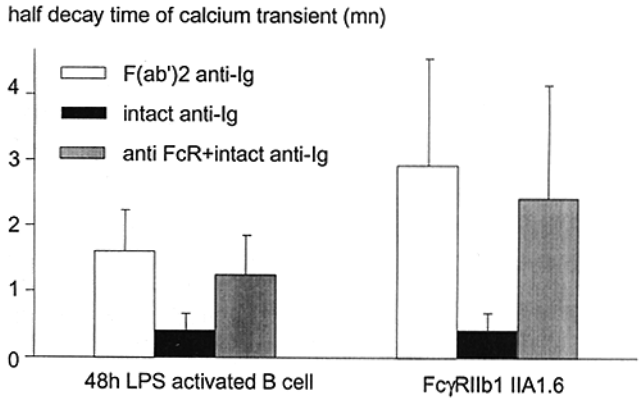

Figure 2. Mean half decay times of Ca^{2+} responses produced by cross-linking mIg with or without FcγRII. Rises in $[Ca^{2+}]_i$ were induced by F(ab')2 fragments (□) or intact anti-immunoglobulins under control conditions (■) and after 2–10 min preincubation with 8 μg/ml anti-FcγRII (▨). Data were measured on traces as in Fig. 1 and are expressed as mean ± SD of 8–39 cells.

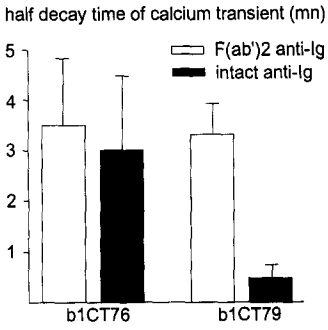

Figure 3. Disruption of FcγIIRb1 function by deletion of amino acids in its intracellular tail. Mean half decay times of Ca^{2+} responses induced by F(ab')2 fragments (□) or intact anti-immunoglobulins (■) in two cell lines transfected with the mutant FcγIIRb1 receptors b1CT79 and b1CT76, respectively. These two receptors differ respectively from the native FcγIIRb1 by a 16- and 19-amino acids deletion in the COOH terminal of the cytoplasmic tail. Only b1CT79 is capable of inhibiting Ca^{2+} signaling mediated by mIgs.

FcγRII and mIgG does not prevent signaling by mIgGs, and that co-cross-linking of both receptors is necessary for inhibition (Klaus et al., 1987).

We further analyzed the mode of operation of FcγRIIb1 by studying which structural parts of the receptor are involved in inhibition of mIg signaling. Because of alternative mRNA splicing, a second isoform of FcγRII, termed FcγRIIb2, is expressed in myeloid cells, but normally not in B lymphocytes (Lewis et al., 1986; Amigorena et al., 1989). This isoform differs from FcγRIIb1 by deletion, in the 94-amino acid cytoplasmic tail, of 47 amino acids at a site proximal to the membrane. We had previously found that the b2 isoform of FcγRII is also able to inhibit mIg-mediated activation, while a mutant receptor in which the intracellular tail is shortened to the 47-amino acid insert specific to the FcγRIIb1 sequence is not functional (Amigorena et al., 1992). These data established that this 47-amino acid insert is not necessary for inhibition. We thus questioned which of the 47 amino acids common to the cytoplasmic tail of b1 and b2 are necessary to inhibit mIg signaling by analyzing the effect of deletions in the cytoplasmic tail of the b1 isoform. Fig. 3 shows that b1CT79, a mutant receptor with 16 amino acids deleted from the COOH terminus of b1, is still fully functional. In contrast b1CT76, which differs from b1 by a 19-amino acid deletion in the COOH terminus, has lost the capacity to inhibit Ca^{2+} signaling. These results define a three amino acid region the presence of which is required for b1 receptor function and they further confirm that FcγRIIb1 is actively involved in inhibition of Ca^{2+} signaling by the intact anti-Ig.

Dose-Dependence of F(ab')2 and Intact anti-mIgG Triggered Ca^{2+} Signals

The next step toward further analyzing FcγRII-mediated inhibition of Ca^{2+} signaling was to fully characterize the rise in $[Ca^{2+}]_i$ triggered through mIgG. Fig. 4 *A* gives examples of recordings of $[Ca^{2+}]_i$ during application of different doses of F(ab')2. As the F(ab')2 concentration was decreased from 80 μg/ml to 0.1 μg/ml, the delay between the application of the ligand and the beginning of the Ca^{2+} signals increased from 32 s (SD = 13; n = 13) to 117 s (SD = 30; n = 13) although the mean peak $[Ca^{2+}]_i$ amplitude and the percentage of responding cells remained constant (Fig. 4 *B*). As the ligand concentration was further lowered, the delay to the Ca^{2+} signals did not further increase, rather, we observed a

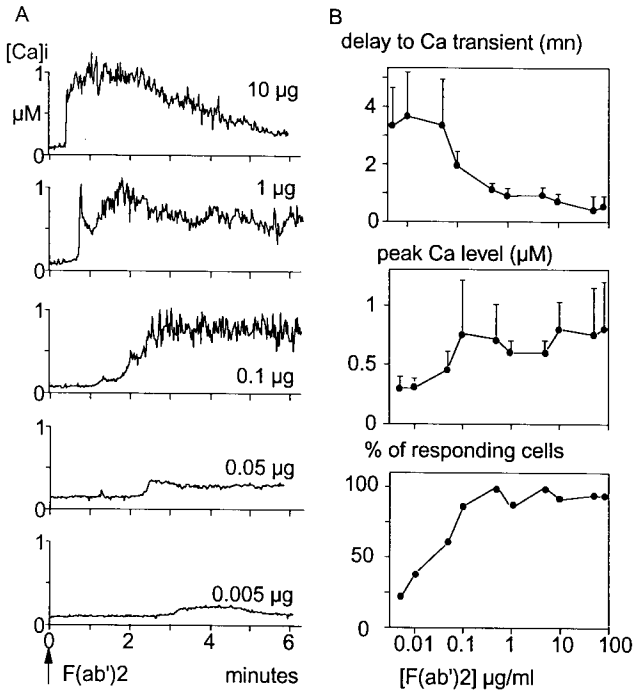

Figure 4. Dose dependence of F(ab')2 anti-IgG effects on the shape, delay, and occurrence of Ca^{2+} responses. (*A*) Recordings of intracellular Ca^{2+} levels in five different FcγRIIbl transfected B lymphoma cells during application of decreasing doses of F(ab')2 anti-IgG (as indicated at the right of each trace in μg/ml).(*B*) Variation as a function of the dose of F(ab')2 anti-IgG of the mean delay between addition of the ligand and the start of the rise in $[Ca^{2+}]_i$ (*top*), the mean peak amplitude of $[Ca^{2+}]_i$ value (*middle*) and the percentage of cells in which addition of the ligand induced a rise in $[Ca^{2+}]_i$ (*bottom*). Data are expressed as mean ± SD of 7–32 cells. The protocol used for measurements is described in Materials and Methods.

dose-dependent decrease in the mean peak $[Ca^{2+}]_i$ amplitude and in the percentage of cells which gave a response. However, at all doses $[Ca^{2+}]_i$ remained elevated for several minutes after the initial response.

Similar results were obtained when the dose of intact anti-IgG was changed (not shown). As the dose of anti-IgG was lowered, we observed an increase in the delay between the addition of the ligand and the occurrence of the response. As for F(ab')2, the amplitude of the response also decreased at concentrations of intact anti-IgG lower than 0.1 μg/ml. At all doses of anti-IgG, the duration of the transitory response remained short and usually did not exceed 30 s.

Involvement of Both Ca^{2+} Release from Intracellular Stores and of Influx through the Plasma Membrane in the Generation of F(ab')2-triggered $[Ca^{2+}]_i$-Increases

We analyzed the origin (extra-versus intracellular) of Ca^{2+} during the mIg-mediated increase in $[Ca^{2+}]_i$ by removing extracellular Ca^{2+} or by depolarizing the cell with KCl at different times with respect to the addition of F(ab')2 anti-IgG. When these manipulations were performed later than one minute after the onset of stimulation, we observed a systematic decrease of $[Ca^{2+}]_i$ back to baseline levels (Fig. 5, *A*1 and *B*1). In contrast, when the cells were stimulated in the absence of extracellular Ca^{2+} or in the presence of KCl

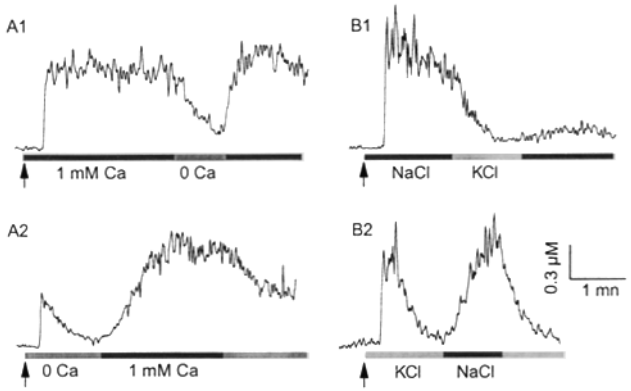

Figure 5. Sensitivity of the Ca^{2+} responses to the presence of extracellular Ca^{2+} (*A*) and membrane depolarization (*B*). (*A*1) A transitory removal of external Ca^{2+} 3 min after stimulation of FcγIIRbl transfected B lymphoma cells with 50 μg/ml F(ab')2 anti-IgG induces a reversible decrease in $[Ca^{2+}]_i$. (*A*2) The stimulation of another cell in absence of external Ca^{2+} induces a transitory increase in $[Ca^{2+}]_i$ which returns to baseline levels in less than a minute. $[Ca^{2+}]_i$ increases again when $[Ca^{2+}]_o$ is back to control levels. The recorded cells were bathed in extracellular medium containing 1 mM Ca^{2+} (■, *A*1) or 0 added Ca^{2+} and 500 μM EGTA (▩, *A*2) for no more than 3 min and stimulated at the indicated time (*arrow*) by perfusion with F(ab')2 anti-IgG diluted in the indicated medium. Switching between the different solutions was done as indicated under each recording. (*B*1) Perfusion of the recorded cell with a KCl containing extracellular medium (▩) 2 min after addition of 50 μg/ml F(ab')2 anti-IgG decreases $[Ca^{2+}]_i$. (*B*2) Stimulation of the cell in presence of extracellular KCl induces a transitory rise in $[Ca^{2+}]_i$. The perfusion was switched back to a NaCl medium at the indicated time (■).

(Fig. 5, *A*2 and *B*2), there were still sharp rises in $[Ca^{2+}]_i$, with a delay similar to those occurring in control experiments. However $[Ca^{2+}]_i$ rapidly returned to baseline levels after this initial response. The decrease in $[Ca^{2+}]_i$ induced by either removing extracellular Ca^{2+} or by adding KCl was reversed upon switching back to a Ca^{2+} and NaCl containing extracellular solution in most experiments (respectively, 26 out of 31 and 20 out of 34) thus showing that the Ca^{2+} influx pathway was still functional (Fig. 5, *A*2 and *B*2). Addition of the K^+ ionophore valinomycin (1 μM) to the solutions in order to clamp the transmembrane potential at the K^+ equilibrium potential did not modify the percentage of cells which recovered from the KCl mediated inhibition (24 out of 40 cells recovered in these conditions). This shows that the lack of reincrease of $[Ca^{2+}]_i$ in certain cells upon switching back to the NaCl containing medium after depolarization with KCl is not due to an absence of repolarization, but rather to a closure of the influx pathway.

Altogether our data show that cross-linking mIgGs induces a rise in $[Ca^{2+}]_i$ due to an early voltage-independent release of Ca^{2+} from intracellular stores and an influx of Ca^{2+} through the plasma membrane which is inhibited by membrane depolarization.

Selective Inhibition of mIg-triggered Ca^{2+} Influx by FcγRII Engagement

Co-cross-linking mIg and FcγRII triggered only a short and sharp increase in $[Ca^{2+}]_i$. This already suggested that this response is mainly the consequence of release of Ca^{2+} from

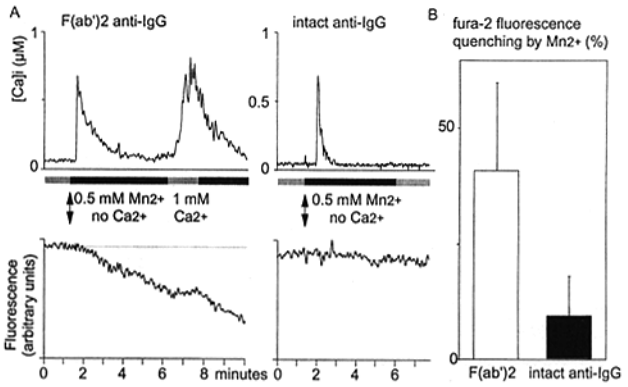

Figure 6. Absence of Mn^{2+} induced quenching of fura-2 fluorescence after co-cross-linking of FcγIIRb1 and surface-Ig. (*A*) Simultaneous displays of $[Ca^{2+}]_i$ (*top traces*) and total cell fluorescence (*bottom traces*) during perfusion of 2 FcγIIRb1 transfected B lymphoma cells with F(ab')2 anti-IgG (*left*) or intact anti-IgG (*right*) diluted in a NaCl medium containing 0.5 mM $MnCl_2$ and no added Ca^{2+} (■). The perfusion, which was switched to the control medium containing no $MnCl_2$ and 1 mM Ca^{2+} at the indicated times (▩), induced a rebound in $[Ca^{2+}]_i$ level and the cessation of fluorescence quenching in the left panels. See Materials and Methods for the equation used for calculation of total cell fluorescence. (*B*) Mean percentage of fluorescence quenching by Mn^{2+} in cells stimulated with F(ab')2 (□) or intact anti-IgG (■). The amount of total fluorescence was measured 5 min after stimulation and normalized to that recorded just before addition of the agonist. Data are expressed as mean ± SD of 10 (F(ab')2) and 11 (intact anti-IgG) cells.

intracellular stores. However other mechanisms, such as activation by FcγRII of a Ca^{2+} extrusion pump could also have accounted for the rapid return of $[Ca^{2+}]_i$ to baseline levels. To discriminate between these different hypotheses, we directly assessed the opening of a transmembrane Ca^{2+} influx pathway by using manganese (Mn^{2+}) as a tracer for divalent cation entry. Mn^{2+} binds to fura-2 and quenches its fluorescence (Grynkiewicz et al., 1985). Thus, when the extracellular medium contains Mn^{2+}, activation of a Mn^{2+} permeable Ca^{2+} influx pathway leads to Mn^{2} influx, and consequently to a decrease in total cellular fura-2 fluorescence (Hallam et al., 1989; Sage et al., 1989; Alvarez et al., 1991; Lückhoff and Clapham, 1992). It must be noted that not all the receptor-activated Ca^{2+} influx pathways allow Mn^{2+} as a permeant cation (Merritt and Hallam, 1988; Hoth and Penner, 1992). Total cellular fura-2 fluorescence must be measured using conditions where it does not depend on $[Ca^{2+}]_i$. The commonly used experimental approach consists of measuring cell fluorescence at the isosbestic wavelength for fura-2 excitation (i.e., the wavelength at which fura-2 fluorescence is independent of Ca^{2+} concentration, around 360 nm). Simultaneous recordings of $[Ca^{2+}]_i$ and cell fluorescence have been previously performed using either three wavelength (two for $[Ca^{2+}]_i$, one at the isosbestic point), or only two, one being the isosbestic wavelength. In the latter case, there is a loss in the dynamic range over which varies the fluorescence ratio used to calculate $[Ca^{2+}]_i$. These problems were avoided by calculating the theoretical fura-2 concentration from fluorescence intensity measurements at 340 nm and 380 nm excitation wavelengths (see Materials and Methods).

Experiments were performed by stimulating the cells in a medium containing no Ca^{2+} and 0.5 mM Mn^{2+}. Control experiments showed that these conditions do not lead to spontaneous decrease in cell fluorescence, indicating that these cells have a low baseline membrane permeability to Mn^{2+}. Fig. 6 *A* shows simultaneous measurement of $[Ca^{2+}]_i$ and total cell fluorescence in two FcγRIIb1-IIA1.6 cells challenged with F(ab')2 and intact anti-IgG antibodies. After a short delay both stimuli induced a transitory sharp rise in $[Ca^{2+}]_i$ due solely to release of Ca^{2+} from intracellular stores since there was no extracellular Ca^{2+}. However, while cross-linking mIgG alone led to a rapid quenching of cell fluorescence (n = 10 cells), virtually no quenching was observed when mIgG and FcγRIIb1 were co-cross-linked (n = 11 cells). The mean percentages of fluorescence quenching in both experimental conditions are given in Fig. 6 *B*. As in the experiments illustrated by Fig. 5, *A*1 and *A*2, readdition of Ca^{2+} to the extracellular medium several minutes after the onset of stimulation with F(ab')2 induced a new increase in $[Ca^{2+}]_i$, thus confirming that the Ca^{2+} influx pathway was still open. This protocol temporarily stopped fluorescence quenching since Mn^{2+} was absent during this period. In contrast, reintroduction of extracellular Ca^{2+} never led to a rise in $[Ca^{2+}]_i$ in cells stimulated with intact anti-IgG. These data indicate that the Ca^{2+} influx pathway activated through mIgG is permeable to Mn^{2+} and inhibited by engagement of FcγRIIb1.

Interestingly, in all cells tested with F(ab')2, quenching started within a few seconds after the sharp release of Ca^{2+} from intracellular stores and developed quasi-linearly during the rest of the experiment. This indicates that the transmembrane influx of Ca^{2+} is activated shortly after the cytoplasmic release. Moreover, the amount of Ca^{2+} released from intracellular stores was only slightly smaller in intact versus F(ab')2 anti-IgG stimulated cells, as indicated by measurements of the peak amplitudes and half decay times of the Ca^{2+} responses triggered in the absence of extracellular Ca^{2+} (respectively, 0.56 μM; SD = 0.41 μM and 46 s; SD = 40 s, n = 10 for F(ab')2, and 0.50 μM; SD = 0.34 μM and 38 s; SD = 25 s; n = 9 for intact anti-IgG). This shows that release of intracellular Ca^{2+} triggered through mIg is little affected by FcγRIIb1.

FcγRII Inhibition of Ca^{2+} Influx Is Not Mediated by Cell Depolarization

Activation of Ca^{2+} influx after cross-linking of mIg requires both opening of a Ca^{2+} influx pathway in the plasma membrane and a negative membrane potential to provide the electrical driving force for Ca^{2+} entry. Indeed, as shown in Fig. 5, membrane depolarization blocks Ca^{2+} influx. We thus questioned whether inhibition of Ca^{2+} influx by FcγRII was mediated by cell depolarization. To test this hypothesis, we performed experiments using the perforated patch-clamp method (Horn and Marty, 1988; Rae et al., 1991) which allows us to clamp the transmembrane voltage of cells with minimal perturbation of the cell interior. Fig. 7 shows recordings of $[Ca^{2+}]_i$ in two FcγRIIb1-IIA1.6 cells voltage-clamped at negative and positive membrane potentials. In control conditions, $[Ca^{2+}]_i$ remained low in cells voltage-clamped at −70 mV, which is close to the normal resting potential of lymphocytes (Deutsch et al., 1979; Rink et al., 1980). Cross-linking

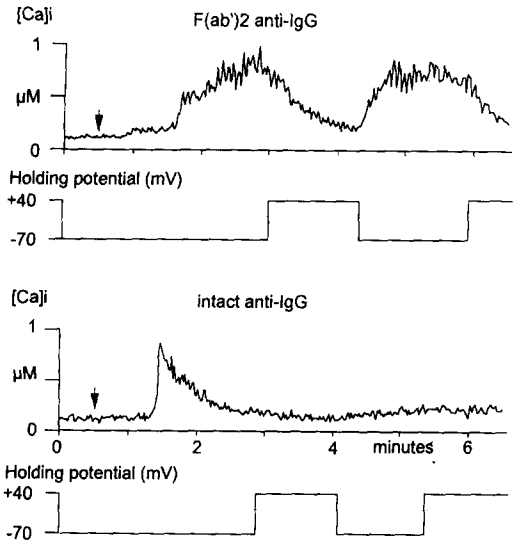

Figure 7. Inhibition of Ca^{2+} influx by FcγRII in voltage-clamped lymphocytes. Recording of $[Ca^{2+}]_i$ in two FcγRIIb1-IIA1.6 cells voltage-clamped using the perforated patch-clamp method. The transmembrane voltage was switched between −70 and +40 mV at the indicated times (traces labeled holding potential). (*Top traces*) Application (*arrow*) of 50 μg/ml F(ab')2 anti-IgG induces a sustained, depolarization-sensitive increase in $[Ca^{2+}]_i$. (*Bottom traces*) Application (*arrow*) of 75 μg/ml intact anti-IgG only induces a short transient rise in $[Ca^{2+}]_i$, despite a holding potential of −70 mV.

mIg induced a sustained rise in $[Ca^{2+}]_i$ which was reversibly inhibited by membrane depolarization (n = 5 cells). At the opposite, co-cross-linking mIg and FcγRII only produced a transient rise in $[Ca^{2+}]_i$, even though the cell was voltage-clamped at −70 mV. After this initial rise in $[Ca^{2+}]_i$, which mostly originated from release of Ca^{2+} from intracellular stores (see above), $[Ca^{2+}]_i$ remained low for the rest of the experiment. Similar results were obtained in seven cells. Thus, maintaining a negative membrane potential does not prevent inhibition of Ca^{2+} influx by FcγRII. This indicates that the mode of action of this receptor does not involve membrane depolarization.

Discussion

By measuring $[Ca^{2+}]_i$ in single cells, we have shown that (*a*) cross-linking mIgs triggers an increase in $[Ca^{2+}]_i$ due to an explosive and transitory release of Ca^{2+} from intracellular stores and a sustained influx through the membrane, and (*b*) co-cross-linking mIgs and FcγRIIb1 specifically inhibits the influx component, without affecting the release phase.

mIg Triggered Ca^{2+} Responses

Although a number of studies have analyzed the changes in $[Ca^{2+}]_i$ which occur upon mIg cross-linking (Braun et al., 1979; Pozzan et al., 1982; Bijterbosch et al., 1986; Labaer et al., 1986; Ransom et al., 1986; Dugas et al., 1987; MacDougall et al., 1988; Ransom et al., 1988; for review see DeFranco et al., 1987; Cambier et al., 1987), no details on their kinetics were available up to now since all, but one (Wilson et al., 1987) of the published investigations involved populations of a large number of cells. New information provided in this study on single cells indicates that Ca^{2+} rises to micromolar levels extremely fast (sometimes in <1 s) with a dose dependent delay of 10–200 s after addition of the ligand. This suggests that among the numerous steps which lie between mIg cross-linking and Ca^{2+} release, some initial events are rate limiting while the increase in $[Ca^{2+}]_i$ is controlled by a strongly regenerative process.

The biochemical mechanisms which underlie Ca^{2+} release have been extensively studied and several examples of strong positive cooperativity have been reported. For example, both IP_3 mediated Ca^{2+} release and phospholipase C activity, which generate IP_3, are potentiated by elevated $[Ca^{2+}]_i$ (Finch et al., 1991; Harootunian et al., 1991; Smrcka et al., 1991) and could thus participate in a regenerative mechanism. Since the delay of the responses reaches finite and nonzero asymptotic values as the doses of ligand are respectively lowered or increased (see Fig. 4 *B*), there must be some constraints upon the mechanisms by which Ca^{2+} responses are generated. One possible model is that mIgs trigger the formation of a second messenger X, likely to be IP_3 (Coggeshall and Cambier, 1984; Bijterbosch et al., 1985; Ransom et al., 1986; Fahey and DeFranco, 1987; Ransom et al., 1988) in a ligand-dose dependent manner (McConnell et al., 1992). The nonzero lower limit of the delay suggests that the intracellular concentration of X must reach a threshold value before the regenerative mechanism of Ca^{2+} release is triggered. The higher limit of the delay may be accounted for if X is permanently degraded at a fixed rate, independent of cell activation (by an IP_3-phosphatase, for example). According to these hypotheses, the doses of ligand which trigger formation of X at a lower rate than its degradation, would never produce a response, while the amplitude of the latter should be dose independent. Such a mechanism has been proposed to account for a similar behavior of PAF induced Ca^{2+} responses in macrophages (Randriamampita and Trautmann, 1989). However, this model does not fully account for our data, since we observed a decrease in the amplitude of the Ca^{2+} response at very low doses of ligand, thus showing that the process is not entirely all or none.

FcγRIIb1 Mediated Inhibition of Ca^{2+} Influx

Our data show that intact antibodies to mIgG or mIgM induce increases of intracellular Ca^{2+} much shorter in duration than those triggered by F(ab')2 fragments of anti-IgG or -IgM. Two main arguments indicate that this is due to an inhibition by FcγRII of Ca^{2+} signaling through mIgs. First, preincubation of the cells with an anti-FcγRII antibody prevented the block of signaling by intact anti-mIgs. Second, in cells transfected with a truncated and inactive form of FcγRIIb1, both intact and F(ab')2 anti-mIgs induced increases in $[Ca^{2+}]_i$ of equally long duration. Furthermore, inhibition occurs at the level of a single lymphocyte and thus does not require cooperation of receptors present on several cells. This conclusion could not be derived from studies performed on entire populations of cells (Bijsterbosch and Klaus, 1985; Wilson et al., 1987; Rigley et al., 1989).

The main outcome from our results is that FcγRII does not mediate an overall inhibition of mIg mediated signaling, but specifically affects certain of its aspects. First, by manipulating extracellular Ca^{2+} and assessing direct activation of a Ca^{2+}/Mn^{2+} influx pathway, we found that co-cross-linking FcγRII and mIg totally blocks mIg-triggered transmembrane

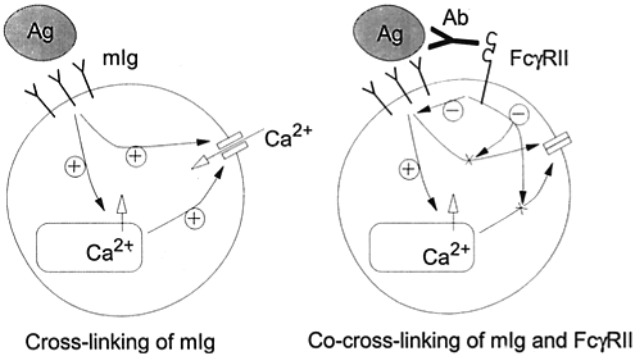

Figure 8. Schematic representation of possible sites of inhibition of mIg-triggered Ca^{2+} influx upon engagement of FcγRII. Cross-linking mIg by multivalent antigens (Ag) trigger release of Ca^{2+} from intracellular stores and transmembrane influx either independently or sequentially (*left*). Co-cross-linking mIg and FcγRII by immune complexes (Ag-Ab complex) inhibits transmembrane Ca^{2+} influx either by acting on a specific pathway of activation or by shutting off mIg signaling after the initial signals which lead to Ca^{2+} release are delivered (*right*).

Ca^{2+} influx, with little or no inhibition of mIg-induced release of Ca^{2+} from intracellular stores. This may account for the fact that co-cross-linking mIg and FcγRII does stimulate resting (G_0) B cells to increase Ia antigen expression, but only drives them to an intermediate step between G_0 and G_1, while cross-linking mIgs alone induces progression at least to the S phase of the cell cycle (Klaus et al., 1984). Second, whatever the doses of ligand used, the rises in $[Ca^{2+}]_i$ induced by F(ab')2 fragments of anti-mIgs were always long lasting, while those triggered by intact anti-mIgs were of short duration. This shows that the effect of intact anti-mIgs cannot be mimicked by lowering the dose of F(ab')2 anti-mIgs. Thus, co-cross-linking FcγRIIb1 and mIg does not solely interfere with an adequate cross-linking of mIgs and its subsequent ability to deliver activation signals to intracellular effectors. This conclusion is further supported by the observation that truncation of only three amino acids in the intracellular tail of FcγRIIb1 disrupts its function.

Thus FcγRII probably acts at a step downstream the mIg protein itself. This hypothesis is strengthened by the observation that co-cross-linking of mIgM and FcγRII cross-inhibits signaling through mIgDs present on the same cell (Klaus et al., 1985). Our voltage-clamp data show that the inhibition of Ca^{2+} influx by FcγRII is not due to a decreased transmembrane electrical driving force for this ion, but rather to the lack of opening of the influx pathway itself. Rigley et al. (1989) have shown in permeabilized cells that FcγRII inhibits the GTPγs dependent mIg signaling but not the baseline GTPγs signaling. Thus, they have suggested that FcγRII acts by uncoupling the mIg from an associated G protein. Since that FcγRII does not affect early IP_3 production (Bijsterbosch and Klaus, 1985; Wilson et al., 1987; Rigley et al., 1989) or Ca^{2+} release (this study), several biochemical pathways may be involved in mIg signaling. Along this line, recent studies have characterized tyrosine kinases associated with the mIg complex which can directly activate phospholipase C by tyrosine phosphorylation (Burkhardt et al., 1991; Carter et al., 1991; Yamanashi et al., 1991). Thus it is possible that two independent pathways lead respectively to Ca^{2+} release and transmembrane influx, only the latter being susceptible to inhibition by FcγRII. However, a current hypothesis regarding the mechanism of receptor induced Ca^{2+} influx is that it is triggered, through an unknown process, by the emptying of the intracellular Ca^{2+} pools (Sage et al., 1989; Takemura, 1989; Putney, 1990; Meldolesi et al., 1991; Hoth and Penner, 1992). In lymphocytes, a transmembrane influx of Ca^{2+} is induced after depletion of these pools, either by loading the cells with a Ca^{2+} chelator or by the Ca-ATPase inhibitor thaspsicargin, which empties Ca^{2+} stores by preventing their refilling (Scharff et al., 1988; Alvarez et al., 1991; Mason et al., 1991). Reconciliation of our data with the store-dependent-Ca^{2+}-influx hypothesis would require that FcγRII inhibit Ca^{2+} influx at a stage downstream from that of release, maybe even directly at the level of the putative Ca^{2+} channel itself. Alternatively, sustained receptor-triggered efflux of Ca^{2+} from the internal pools may be necessary for transmembrane influx to occur (Putney, 1990). This might suggest that FcγRII are initially in an inactive form and allow mIg to trigger production of an early Ca^{2+} releasing signal such as IP_3. These initial steps could then render FcγRII capable of inhibiting signaling through mIg of IP_3 production and thus prevent further release and activation of the influx pathway. It should be noted that this hypothesis holds whether influx is activated by emptying of the stores (Scharff et al., 1988; Sage et al., 1989; Takemura, 1989; Putney, 1990; Alvarez et al., 1991; Mason et al., 1991; Meldolesi et al., 1991; Hoth and Penner, 1992) or through IP_3 receptors in the plasma membrane (Kuno and Gardner, 1987; Khan et al., 1992*a*,*b*; Lückhoff and Clapham, 1992).

In summary (Fig. 8), FcγRII provides a tool which allows the physiological dissociation of the phases of Ca^{2+} release and transmembrane influx. This may help to understand how these two processes are linked and will probably involve an as yet unknown mechanism of general cellular interest.

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