CROSSLINKING OF SURFACE IMMUNOGLOBULIN AND Fc RECEPTORS ON B LYMPHOCYTES INHIBITS STIMULATION OF INOSITOL PHOSPHOLIPID BREAKDOWN VIA THE ANTIGEN RECEPTORS

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B lymphocytes carry clonally distributed immunoglobulin (Ig) molecules on their surface, which are receptors for antigen. Ligation of Ig receptors on mouse B cells by $F(ab')_2$ fragments of rabbit anti-mouse Ig antibodies (anti-Ig) results in polyclonal proliferation of a substantial proportion of these cells, and it is likely that this affords a polyclonal model for antigen-induced B cell activation (reviewed in 1). It is also well established that intact (IgG) rabbit anti-Ig does not induce murine B cells to proliferate (1), except those from relatively old mice (2). Instead, intact anti-Ig inhibits B cell proliferation induced by the $F(ab')_2$ fragments and by lipopolysaccharide (LPS),[1] as well as LPS-stimulated antibody secretion (3–5). Recent evidence (6) has, however, shown that intact anti-Ig does stimulate B cells, but only drives them into a transitional activation state between deep quiescence and G_1 proper.

All in all, it thus appears that crosslinking of surface Ig and Fc receptors on a B cell by intact anti-Ig produces a dominant inhibitory signal (4, 5), that allows the cells to just leave G_0, but prevents further progress into the cell cycle (6). It is an attractive possibility that this system therefore provides a model for studying the regulation of antigen-specific B cell responses by the binding of antigen-antibody complexes to their Fc receptors (7). The biochemical basis of these inhibitor phenomena has not been elucidated.

In many cell types, binding of ligands to their receptors provokes an early increase in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and rapid degradation of phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and 1,2-diacylglycerol (1,2-DG) (reviewed in 8). A substantial body of evidence (8, 9) now indicates that both Ca^{2+} mobilization and the formation of IP_3 and 1,2-DG are important signals for the induction of a number of cellular responses, including cell growth. In support of this latter concept, it has been found that $F(ab')_2$ anti-Ig induces PIP_2 degradation as well as Ca^{2+} mobilization in mouse B cells (10, 11). We therefore wished to study the effects of intact anti-Ig on these

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[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; Con A, concanavalin A; 1,2-DG, 1,2-diacylglycerol; IP, inositol (poly)phosphate (with subscript); LPS, lipopolysaccharide; PIP_2, phosphatidylinositol bisphosphate; PMA, phorbol myristate acetate; SPA, staphylococcal protein A.

two early biochemical responses in B lymphocytes. The results presented here indicate that PIP_2 degradation, but not Ca^{2+} mobilization is abrogated by crosslinking of surface Ig and Fc receptors. These findings might provide a framework for explaining the mode of action of Fc receptors in regulating the activation of B cells via their Ig receptors.

Materials and Methods

Animals. 3–6-mo-old male (CBA × C57BL/6)F_1 mice, bred under specific pathogen-free conditions at the National Institute for Medical Research, were used.

Reagents. 2-[^{3}H]inositol (15 Ci/mmol), 5-[^{3}H]uridine (2–5 Ci/mmol), and quin2 acetoxymethyl ester were from Amersham International, Amersham, United Kingdom. Protein A was from Pharmacia Fine Chemicals, Uppsala, Sweden. Affinity-purified rabbit anti-mouse Fab antibodies and F(ab′)$_2$ fragments of these antibodies were prepared as described previously (6). The monoclonal anti-mouse Fc receptor antibody 2.4G2 (a rat/mouse hybrid molecule) (12) was purified from tissue culture supernatant by affinity chromatography, using a column of Sepharose 4 B conjugated with goat anti-mouse Ig antibodies, which also bind rat Ig. All other chemicals were analytical grade.

Cell Preparations. B lymphocytes were prepared from spleens of mice by depleting T cells and adherent cells as described earlier (11). The resulting cell preparations contained >90% surface Ig-positive cells by immunofluorescent analysis.

Assay of [^{3}H]Uridine Incorporation. Aliquots of 10^6 B cells were cultured in flat-bottomed microtiter wells in 0.2 ml RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 1 mM pyruvate, nonessential amino acids, penicillin, streptomycin, and 5% (vol/vol) fetal calf serum (complete medium). After 16 h of incubation, 1 μCi of [^{3}H]uridine was added, and incorporation of label was determined 4 h later by harvesting on glass fiber discs followed by liquid scintillation counting.

Assay of Inositol Phosphates. B cells, suspended at 8×10^7 cells/ml in Hanks' balanced salt solution containing 0.5% (wt/vol) gelatin and buffered to pH 7.2 with 20 mM Hepes, were labeled with [^{3}H]inositol (0.5–2.5 μCi/10^6 cells) for 4 h at 37°C in a 6.5% CO_2 atmosphere. After labelling, the cells (>95% viable) were washed and resuspended to 5.6×10^6 cells/ml in complete medium. Aliquots of 0.54 ml were preincubated for 30 min at 37°C in a humidified 6.5% CO_2 atmosphere. Then, the cells were stimulated by adding anti-Ig, in 60 μl of medium. The incubations were terminated by successively adding 0.12 ml of 0.22 N HCl and 2.7 ml of chloroform/methanol (1:2, vol/vol). Phases were separated by adding 0.9 ml of chloroform and 0.9 ml of water. Aliquots of 2–3 ml of the upper phase were diluted eightfold with water and applied to columns containing ~0.5 ml of Dowex-1 in the formate form. First, [^{3}H]inositol was washed through with 10 ml of water. For detailed analyses, radioactive phosphate esters were then eluted sequentially with 10-ml volumes of 5 mM disodium tetraborate and 30 mM sodium formate solution (glycerophosphoinositol), 0.1 M formic acid and 0.2 M ammonium formate solution (inositol monophosphate), 0.1 M formic acid and 0.5 M ammonium formate solution (inositol bisphosphate), and 0.1 M formic acid and 1.0 M ammonium formate solution (inositol trisphosphate). However, in most experiments, glycerophosphoinositol was eluted first, then the three inositol phosphate species were eluted together with 10 ml of 0.1 M formic acid and 1.0 M ammonium formate solution. Validation of the method has been discussed previously (11).

Determination of $[Ca^{2+}]_i$. The procedures are described in detail elsewhere (11). In short, B cells were loaded with the fluorescent Ca^{2+} indicator, quin2, to intracellular concentrations of 1.1–2.2 mM, by incubating the cells with its membrane-permeable acetoxymethyl ester. The fluorescence of the quin2-loaded cells (10^7 cells/ml in Hanks' containing 10 mM Hepes, pH 7.4) was monitored with a spectrofluorometer in a thermostatically controlled cell, kept at 37°C. The signals were calibrated by lysing the cells with 0.05% (vol/vol) Triton X-100 in the presence of 10 μM diethylenetriaminepentaacetic acid, followed by quenching the fluorescence with 0.2 mM $MnCl_2$.

Statistical Analyses. Differences were tested for significance by Wilcoxon's two-sample test (13).

Results

Intact Anti-Ig Induces Abortive Release of Inositol Trisphosphate in B Cells. We initially compared the capacity of the mitogenic [$F(ab')_2$] and nonmitogenic (intact) forms of anti-Ig to induce inositol phospholipid breakdown in B cells. The degradation of PIP_2 was assayed by measuring the release of [^{3}H]inositol trisphosphate (IP_3) from prelabelled inositol phospholipids. IP_3 is rapidly sequentially dephosphorylated to IP_2, IP, and finally, free inositol.

Fig. 1 shows the levels of [^{3}H]IP_3 (Fig. 1 *A*) and of total [^{3}H]inositol phosphates (IP + IP_2 + IP_3) (*B*) in B cells at various times after addition of a (potentially) mitogenic dose of $F(ab')_2$ anti-Ig or an equimolar amount of the intact antibody. After 30 s, the two forms of anti-Ig had induced substantial and indistinguishable increases in the levels of IP_3 and total inositol phosphates. However, at later time points, inositol phosphates levels in cells incubated with $F(ab')_2$ anti-Ig were higher than in those incubated with intact antibodies, and after 4 min the differences had become highly significant ($p < 0.02$).

The results of further experiments (Fig. 2) clearly confirm that intact anti-Ig induces a much smaller release of inositol phosphates in B cells than the $F(ab')_2$ form, even after 1 h of incubation. These experiments were done in the presence of Li^+, which inhibits the final step in the dephosphorylation of inositol phosphates (14). This therefore causes accumulation of inositol phosphates, thus considerably amplifying the response (11).

$F(ab')_2$ and Intact Anti-Ig Induce Similar Changes in Cytoplasmic Ca^{2+}. Since breakdown of PIP_2 is typically associated with Ca^{2+} mobilization (8), we subsequently studied the effects of the two forms of anti-Ig on $[Ca^{2+}]_i$ in B cells. Quin2-loaded B cells were incubated with equimolar amounts of $F(ab')_2$ or intact anti-Ig, either in the presence of 1.3 mM Ca^{2+} (Fig. 3 *A*), or immediately after reduction of extracellular Ca^{2+} to <250 nM by EGTA (Fig. 3 *B*). In the presence of extracellular Ca^{2+}, $F(ab')_2$ anti-Ig induced a rapid, three- to fourfold rise in

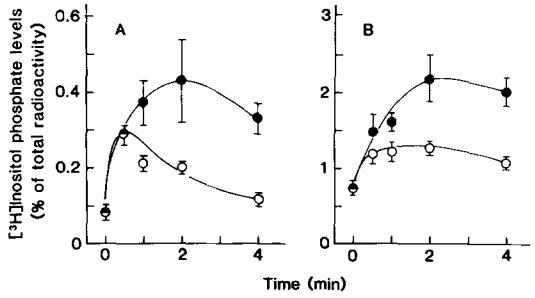

FIGURE 1. Effects of intact and $F(ab')_2$ anti-Ig on the levels of inositol trisphosphate (*A*) and total inositol phosphates (*B*) in B lymphocytes. $F(ab')_2$ (●) or intact (○) anti-Ig were added to aliquots of [^{3}H]inositol-labelled cells, to final concentrations of 50 μg/ml and 75 μg/ml, respectively. At the times indicated, [^{3}H]inositol trisphosphate levels (*A*) and total [^{3}H]inositol phosphate levels (inositol mono-, bis-, and trisphosphate; *B*) were determined. The results are given as percentages of the total cellular radioactivity (100,000 dpm, average). Levels in cells incubated with medium alone did not change significantly. The points are means ± SEM of four replicates from two separate experiments.

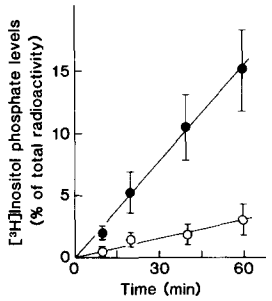

FIGURE 2. Effects of intact and F(ab')$_2$ anti-Ig on inositol phosphate levels of mouse B cells in the presence of 5 mM LiCl. Aliquots of [^{3}H]inositol-labelled cells, in medium containing 5 mM LiCl, received F(ab')$_2$ (●) or IgG (○) anti-Ig to final concentrations of 50 μg/ml and 75 μg/ml, respectively. At the times indicated, total [^{3}H]inositol phosphate levels were determined. The results are expressed as percentages of the total cellular radioactivity (15,000 dpm, average), and are corrected for the levels present at starting time (1.4 ± 0.3%). Levels in cells incubated with medium alone did not change significantly. Points are means ± SEM of four replicates from two separate experiments.

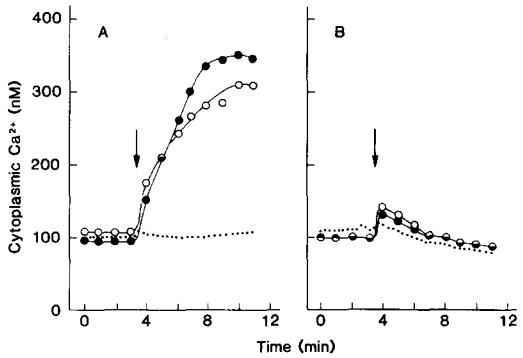

FIGURE 3. Effects of F(ab')$_2$ and intact anti-Ig on $[Ca^{2+}]_i$ in B lymphocytes in the presence of normal (*A*) or low (*B*) extracellular Ca^{2+} levels. Quin2-loaded B cells were incubated at 37°C in a medium containing 1.3 mM Ca^{2+} and 0.9 mM Mg^{2+}, and their fluorescence was recorded. At the time indicated by the arrows, F(ab')$_2$ anti-Ig (25 μg/ml; ●), intact anti-Ig (37.5 μg/ml; ○), or medium alone (····) were added. ~15 sec before these additions, the cells received either EGTA to 1.8 mM (*B*), or an equal volume of medium (*A*). Addition of EGTA immediately reduced extracellular Ca^{2+} to <250 nM. The observed fluorescence was corrected for the dilution due to additions made. The traces shown are means of four replicates obtained in two separate experiments. SEM were <10%.

$[Ca^{2+}]_i$ (see also 11 and 15), and the intact antibodies elicited a very similar response. When extracellular Ca^{2+} was depleted, the increases were much smaller, but again, both forms of anti-Ig induced very similar responses. Ca^{2+} depletion with EGTA, however, did not affect inositol phosphate release (measured after 1 h of incubation in the presence of 5 mM LiCl; results not shown).

Thus, it appears that only a small proportion of the increase in $[Ca^{2+}]_i$ in B cells is due to release from intracellular stores, with the majority coming from the exterior. By using quin2, the contribution of the internal stores might be underestimated, because quin2 buffers changes in Ca^{2+} concentrations. The newly developed Ca^{2+} indicator indo-1 can be used at much lower intracellular concentrations than quin2 (16). Preliminary experiments with indo-1 yielded

essentially the same results, although the initial transient $[Ca^{2+}]_i$ increases were higher, which presumably reflects the lower level of Ca^{2+} buffering with this indicator.

Fig. 4 shows that $[Ca^{2+}]_i$ increases induced by $F(ab')_2$ and intact anti-Ig were virtually the same over a wide concentration range. However, at every concentration tested, IgG anti-Ig induced much less inositol (poly)phosphate release than the $F(ab')_2$ antibodies.

Intact Anti-Ig Inhibits Inositol Phosphate Accumulation Induced by $F(ab')_2$ Antibodies. Since intact anti-Ig is known (3–6) to inhibit DNA synthesis induced by $F(ab')_2$ fragments, we were interested in determining the effects of the intact antibody on inositol phosphate release induced by the mitogenic form of the ligand. [^{3}H]inositol-labelled B cells were stimulated with $F(ab')_2$ anti-Ig (50 μg/ml) in the presence of varying doses of intact antibodies (Fig. 5). Even small amounts of intact anti-Ig effectively suppressed the release of inositol phosphates provoked by $F(ab')_2$ fragments. [^{3}H]inositol phosphate levels in cells incubated with equimolar concentrations of $F(ab')_2$ and IgG anti-Ig were virtually the same as those in cells incubated with intact antibody alone (13 ± 5% and 12 ± 4%, respectively).

Thus, intact anti-Ig not only provokes minimal PIP_2 breakdown in B cells, but also supresses the response induced by $F(ab')_2$ fragments.

Role of Fc Receptors in Inhibition of Inositol Phospholipid Degradation by Intact Anti-Ig. If Fc receptors on B cells are implicated in the abrogation of inositol

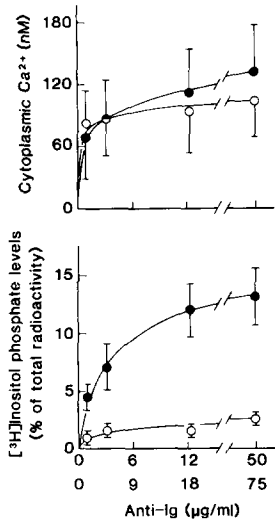

FIGURE 4. Dose-dependence of inositol phosphate formation and increase in $[Ca^{2+}]_i$ induced by $F(ab')_2$ and intact anti-Ig. The upper panel shows the increases $[Ca^{2+}]_i$ following addition of varying concentrations of $F(ab')_2$ (●) or IgG (○) anti-Ig to quin2-loaded B cells in medium with 1.3 mM Ca^{2+}. The changes in $[Ca^{2+}]_i$, determined as in Fig. 3, are given as maximal increases over control values (medium added: 94–97 nM). The lower panel gives inositol phosphate levels in [^{3}H]inositol-labelled cells incubated for 1 h with the indicated concentrations of $F(ab')_2$ anti-Ig (●) or IgG anti-Ig (○), in the presence of 5 mM LiCl. The results are expressed as percentages of the total cellular radioactivities (39,000 dpm, average), and are corrected for levels in unstimulated controls (1.4 ± 0.2%). All points are means ± SEM of four or five replicates from four separate experiments.

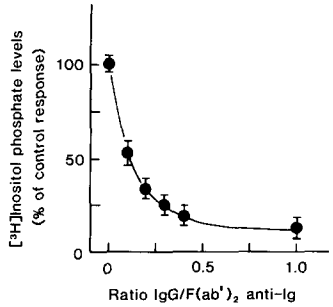

FIGURE 5. Effect of intact anti-Ig on F(ab')$_2$ anti-Ig–induced inositol phosphate formation. Aliquots of [^{3}H]inositol-labelled B cells (in medium with 5 mM LiCl) were incubated with F(ab')$_2$ anti-Ig (50 μg/ml), mixed with increasing amounts of intact anti-Ig up to a molar ratio of 1:1. The amounts of radiolabel in the total inositol phosphate fraction were determined after 1 h. The results, corrected for levels in unstimulated cells, are expressed as percentages of the control response in cells incubated with F(ab')$_2$ anti-Ig alone. [^{3}H]inositol phosphate levels in these controls were 26.8 ± 0.8% of the total cellular radioactivity (23,000 dpm, average). Points are means ± SEM of four replicates from two separate experiments.

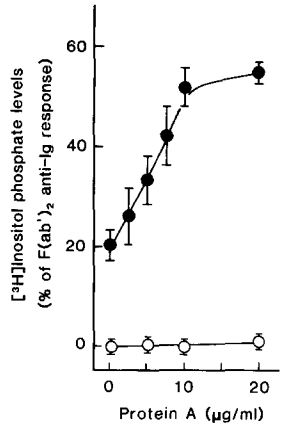

FIGURE 6. Effect of SPA on the capacity of IgG anti-Ig to induce inositol phosphate formation. Aliquots of [^{3}H]inositol-labelled B cells, in medium with 5 mM LiCl, received 30 μg/ml IgG anti-Ig plus 0–20 μg/ml SPA (●), or 0–20 μg/ml SPA alone (○). The mixtures of SPA and antibodies had been preincubated for 1 h at 37°C before addition to the cells. After 1 h of incubation, cellular [^{3}H]inositol phosphate levels were determined. The results, corrected for levels present in unstimulated cells, are expressed as percentages of the response to an equimolar concentration (20 μg/ml) of F(ab')$_2$ anti-Ig. [^{3}H]Inositol levels in the latter were 16.8 ± 0.2% of the total cellular radioactivity (35,000 dpm, average). Points are means ± SEM of four replicates from two separate experiments. F(ab')$_2$ anti-Ig–induced inositol phosphate formation was not affected by SPA (data not shown).

phosphate release by intact anti-Ig, reagents that bind to the Fc portion of anti-Ig, or to the Fc receptor should have a protective effect on the response. We therefore studied the effect of staphylococcal protein A (SPA), which binds tightly to the Fc portion of rabbit IgG, forming a soluble complex (17). It has been shown (18) that SpA markedly increases the proliferative response of mouse B cells to intact rabbit anti-Ig.

In the experiments summarized in Fig. 6, [^{3}H]inositol-labelled B cells were incubated for 1 h with 30 μg/ml intact anti-Ig plus 0–20 μg/ml SPA, or with 0–

20 μg/ml SPA alone. The release of inositol phosphates induced by intact anti-Ig alone was only 20% of the control response to the $F(ab')_2$ fragments. However, increasing amounts of SPA significantly enhanced the response to a maximum of 55% of the control. SPA alone did not induce any inositol phosphate formation. To monitor B cell activation, [^{3}H]uridine uptake was measured after 16 h of culture with 30 μg/ml intact anti-Ig, with or without 20 μg/ml SPA. SPA increased uptake from 26 ± 3% to 65 ± 5% of the response to the $F(ab')_2$ antibodies (not shown).

We next studied the effects of the anti-Fc receptor monoclonal antibody 2.4G2 (12) on the release of [^{3}H]inositol phosphates in B cells incubated with intact anti-Ig (Fig. 7). In the absence of 2.4G2, intact anti-Ig induced a barely detectable response. In striking contrast, it induced a considerable increase in inositol phosphate levels in 2.4G2-treated cells. The monoclonal antibody itself did not elicit an appreciable response. Inositol phosphate release provoked by $F(ab')_2$ anti-Ig was not increased by 2.4G2, but in fact slightly (~40%) decreased (results not shown). Intact anti-Ig alone (20 μg/ml) did not increase incorporation of [^{3}H]uridine in B cells. However, in combination with 2.4G2 (100 μg/ml), it induced a large increase in [^{3}H]uridine uptake, which was twice that induced by $F(ab')_2$ anti-Ig. This increase is probably partly due to protection of signalling via surface Ig, and partly to a direct action of 2.4G2 on the cells, since the latter induced 60–70% of the response to $F(ab')_2$ anti-Ig. Recently, Lamers et al. (19) reported that the 2.4G2 hybridoma produces, in addition to anti-Fc receptor antibodies, a mitogen that copurifies with the antibody. It is therefore possible that stimulation of [^{3}H]uridine uptake by our 2.4G2 preparation was due to this contaminant, rather than to the anti-Fc receptor antibody.

Taken together, the results in this section support the hypothesis that interaction of the Fc portion of intact anti-Ig with its receptor on B cells inhibits release of inositol phosphates (and B cell proliferation) induced by ligation of surface Ig.

Fc Receptors and Cytoplasmic Ca^{2+}. Young et al. (20) reported recently that

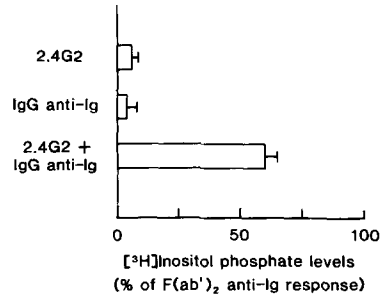

FIGURE 7. Effect of the anti-Fc receptor antibody 2.4G2 on inositol phosphate formation in IgG anti-Ig-stimulated B cells. Aliquots of [^{3}H]inositol-labelled B cells, in medium with 5 mM LiCl, were preincubated for 30 min with or without 2.4G2 (100 μg/ml). Then, the cells received intact anti-Ig to 20 μg/ml, or medium alone. Total [^{3}H]inositol phosphate levels were determined 1 h later. The results, corrected for levels in unstimulated cells, are expressed as percentages of the response to an equimolar concentration (13.3 μg/ml) of $F(ab')_2$ anti-Ig. [^{3}H]Inositol phosphate levels in the latter were 11.7 ± 0.9% of the total cellular radioactivity (19,000 dpm, average). Results shown are means ± SEM of four replicates from two separate experiments.

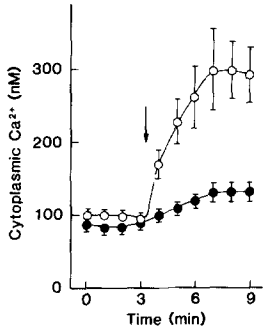

FIGURE 8. Effects of monoclonal antibody 2.4G2 and intact anti-Ig on cytoplasmic Ca^{2+} levels in B cells. Quin2-loaded B cells (in medium with 1.3 mM Ca^{2+}) received (at the time indicated by the arrow) IgG anti-Ig (75 μg/ml; ○), or 2.4G2 (100 μg/ml; ●). Changes in $[Ca^{2+}]_i$ were determined as in Fig. 3. The points are means ± SEM of four replicates from two separate experiments. Addition of medium alone to the cells did not significantly alter their $[Ca^{2+}]_i$.

crosslinking of Fc receptors on mouse macrophages with 2.4G2 induced an increase in $[Ca^{2+}]_i$ in these cells. We therefore studied the effect of 2.4G2 on $[Ca^{2+}]_i$ in mouse B cells. Fig. 8 shows that 2.4G2 increased $[Ca^{2+}]_i$ in B cells from a resting level of 84 ± 7 nM to a maximum of 134 ± 13 nM. This increase was, however, much lower than that induced by intact anti-Ig, which caused a rise from 98 ± 5 nM to 297 ± 57 nM.

We cannot exclude the possibility that the small increase in $[Ca^{2+}]_i$ induced by our 2.4G2 preparation is in fact provoked by the possible contaminant discussed above. However, if it is a genuine effect of the anti-Fc receptor antibody, our results suggest that the increase in $[Ca^{2+}]_i$ induced by intact anti-Ig may not be solely due to ligation of surface Ig, but that crosslinking of Fc receptors may contribute to the increase as well.

Discussion

The precise function of Fc receptors on B cells is still undefined, although there is considerable evidence that they play an important role in immunoregulation via antigen-antibody complexes, by inhibiting B cell proliferation and/or maturation to antibody secretion (reviewed in 7 and 21). It appears likely that intact rabbit anti-Ig antibodies mimic the effects of antigen-antibody complexes by crosslinking surface Ig and Fc receptors (4, 5). This system therefore provides an attractive polyclonal model to study the consequences of these ligand-receptor interactions on B cells. We recently reported (11) that the mitogenic $F(ab')_2$ form of anti-Ig induces the rapid and prolonged release of inositol (poly)-phosphates in murine B cells. We show here that, in marked contrast, the nonmitogenic intact antibodies induce abortive release of inositol phosphates, in particular of IP_3. The response was initially comparable to that induced by $F(ab')_2$ anti-Ig, but was abrogated after some 30 s (Fig. 1). In more prolonged experiments, we found that the amounts of inositol phosphates released by the intact antibody were only 10–20% of those released by $F(ab')_2$ anti-Ig. Furthermore, small amounts of the intact antibody dramatically reduced inositol phosphate release provoked by $F(ab')_2$ fragments (Fig. 5).

On the other hand, both forms of anti-Ig induced comparable increases in $[Ca^{2+}]_i$ (Fig. 3*A*). This was an unexpected finding, since recent evidence (22) has indicated that IP_3 releases Ca^{2+} from intracellular stores. It is of course possible that the small amount of IP_3 released by intact anti-Ig is sufficient to generate a normal $[Ca^{2+}]_i$ increase. Our Ca^{2+}-depletion data (Fig. 3*B*), however, suggest that in B cells the increase in $[Ca^{2+}]_i$ induced by both forms of the antibody is mainly due to influx, and thus may not be mediated by IP_3. The anti-Fc receptor antibody 2.4G2 induced a modest increase in $[Ca^{2+}]_i$ (Fig. 8). We cannot, therefore, exclude the possibility that intact anti-Ig also induces a smaller increase in $[Ca^{2+}]_i$ than the $F(ab')_2$ antibody, but that this is masked by the Ca^{2+}-mobilizing activity of the Fc receptors. However, attempts to induce Ca^{2+} flux in B cells by ligating Fc receptors with heat-aggregated human Ig were unsuccessful (our unpublished data).

It has been shown earlier (23, 24) that intact anti-Ig induces resting B cells to enter a transitional activated state between G_0 and classical G_1 (6), while $F(ab')_2$ anti-Ig induces (dose-dependent) progress of B cells through G_1 towards eventual DNA synthesis. The $F(ab')_2$ fragments induce PIP_2 degradation by phospholipase C, which yields 1,2-DG, in addition to IP_3 (10, 11). 1,2-DG is believed to act as second messenger by activating protein kinase C, an enzyme that appears to play a key role in the transduction of extracellular signals into intracellular responses (25). Our data show that inositol phosphate release, and thus 1,2-DG formation, is suppressed in B cells cultured with intact anti-Ig. It is an attractive possibility that the abrogation of 1,2-DG production explains the nonmitogenicity of IgG anti-Ig. We have attempted to test this hypothesis by coculturing B cells with the intact antibody, together with phorbol myristic acetate (PMA), which substitutes for 1,2-DG by activating protein kinase C (26). The results were negative, perhaps because PMA is a very potent inhibitor of B cell proliferation induced by conventional mitogens (27). Further experiments using synthetic 1,2-DG are in progress.

Previous studies (27–30) have shown that agents like PMA, concanavalin A (Con A), and calcium ionophores also induce quiescent B cells to enter a transitional activation state between G_0 and classical G_1. PMA induces neither Ca^{2+} mobilization, nor PIP_2 degradation (11). Calcium ionophores and Con A, on the other hand, resemble intact anti-Ig in that they do not induce appreciable inositol phosphate release in B cells, but do cause increases in $[Ca^{2+}]_i$ (29, and M. K. Bijsterbosch and G. G. B. Klaus, manuscript in preparation). We speculate that intact anti-Ig, Con A, and Ca^{2+} ionophores induce resting B cells to leave G_0 by activating a common Ca^{2+}-dependent mechanism. This concept is supported by the finding that the immunosuppressive drug cyclosporine inhibits activation of B cells by these Ca^{2+}-elevating agents, but not by agents that do not alter $[Ca^{2+}]_i$ (29, 31).

The biochemical basis of our findings is as yet unknown. Inhibition of mitogenesis by intact anti-Ig is apparently a consequence of crosslinkage of the surface Ig and Fc receptors on the same B cell, since the effect cannot be reproduced by ligating Ig and Fc receptors separately with $F(ab')_2$ anti-Ig and aggregated Ig or antigen-antibody complexes, respectively (3–5, and our unpublished results). This is predictable if the effects of intact anti-Ig do indeed mimic those of

antigen-antibody complexes, to ensure antigen specificity in the regulation of appropriate B cell clones by endogenously produced immune complexes. Our results are in line with this, since the inhibition of PIP_2 degradation by the intact antibody could be substantially reversed by either blocking Fc receptors, or by blocking the Fc portion of the anti-Ig (Figs. 6 and 7). It is highly unlikely that the phenomenon involves binding of the ligand to Fc receptors on macrophages, for two reasons: (*a*) we used macrophage-depleted B lymphocytes; (*b*) it is improbable that formation of macrophage–B cell clusters and subsequent inhibitory events could account for such an extremely rapid effect.

In conclusion, we can envisage several hypotheses to explain our observations. It has been found (32) that Fc receptors on human B leukemia cells are associated with a phospholipase A_2, and that binding of aggregated IgG to them activates the enzyme. If this is also true for mouse B cells, then this could have at least two possible consequences: (*a*) the resulting release of arachidonic acid metabolites, in particular prostaglandins, could cause elevation of cyclic AMP levels in the cells, which is known (25) to inhibit inositol phospholipid degradation. We think that this explanation is unlikely, because we have not found elevated cyclic AMP levels in B cells incubated with intact anti-Ig (our unpublished results). Moreover, indomethacin, which blocks the cyclooxygenase pathway, failed to affect inositol phosphate release induced by intact anti-Ig (data not shown). A second possibility is that stimulation of the putative phospholipase A_2 interferes more directly with phospholipase C–mediated PIP_2 degradation by affecting the levels of inositol phospholipids available for the enzyme. Experiments to test this hypothesis are in progress.

Summary

$F(ab')_2$ fragments of rabbit anti–mouse Ig induce proliferation of murine B lymphocytes, whereas the intact antibodies are not mitogenic. $F(ab')_2$ anti-Ig stimulates the rapid breakdown of inositol phospholipids in B cells, resulting in the prolonged release of inositol (poly)phosphates and diacylglycerol. In marked contrast, intact anti-Ig initially induces a comparable response, which is abrogated after some 30 s. Blocking either the Fc receptors on the B cells or the Fc portion of the antibodies significantly reversed the inhibitory effect. On the other hand, both forms of anti-Ig elicited comparable increases in free cytoplasmic Ca^{2+} levels in B cells.

These results therefore indicate that crosslinkage of Fc and surface Ig receptors on B cells inhibits inositol phospholipid breakdown (but not Ca^{2+} flux) resulting from ligation of the antigen receptors. Since there is evidence implicating inositol phospholipid breakdown in the induction of cell growth, this effect could provide a biochemical explanation for the known capacity of antigen-antibody complexes to inhibit B cell activation.

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