Role of the phosphoinositide-derived second messengers
in regulating B lymphocyte growth

by

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This thesis is dedicated to my husband,

Michael Lawrence Page,

for his patience, his loving support, and his wonderful way

of keeping things in perspective.

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Dawne M. Page

ABSTRACT

Stimulation of the antigen receptor of WEHI-231 B lymphoma cells with anti-IgM antibodies causes irreversible growth arrest. This system may be a model for antigen-induced tolerance to self components in the immune system. Anti-IgM also induces phospholipase C to hydrolyze inositol phospholipids - producing diacylglycerol, which activates protein kinase C, inositol 1,4,5-trisphosphate, which causes release of calcium from intracellular stores, and other inositol polyphosphates. The roles of two of these second messengers, diacylglycerol and calcium, in mediating growth arrest were assessed by activating protein kinase C with phorbol 12,13-dibutyrate (PdBu) and by elevating cytosolic calcium with ionomycin. Doses of these reagents were chosen that produced comparable phosphorylation of a protein kinase C substrate and elevation of cytosolic calcium as did anti-IgM. Either anti-IgM or the mimicking reagents caused growth inhibition by arresting cells in the G_1 phase of the cell cycle, not by lengthening the cell cycle. The efficiency of G_1 arrest increased with the length of time during which the cells received signalling before reaching the G_1 arrest point. Antigen-receptor stimulation, however, was twice as efficient as stimulation via PdBu and ionomycin. Correspondingly, these mimicking reagents induced only partial cell volume decreases as compared to anti-IgM. These results suggest that diacylglycerol and calcium play roles in mediating the effects of anti-IgM on WEHI-231 cells, but that other second messengers may also be involved.

A genetic approach was also used to analyze the relationship between phosphoinositide hydrolysis and growth arrest in these cells. Five mutants of WEHI-231 that were resistant to anti-IgM-induced growth arrest were found to have defects in the phosphoinositide signaling pathway. One of these appeared to be altered in a component(s) that responds to

both calcium and diacylglycerol. The other mutants exhibited decreased production of inositol phosphates, probably because of an alteration in phospholipase C. The use of ionomycin with anti-IgM corrected these latter mutants' defective growth responses to anti-IgM, suggesting that calcium is the limiting second messenger in these mutants. These results provide further evidence that calcium is an essential second messenger in mediating the anti-IgM-induced growth arrest of WEHI-231 B lymphoma cells.

Advisor: J. DeFranco

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INTRODUCTION

The immune system is composed of many different organs and cell types which interact in a complex fashion to recognize and eliminate infectious agents and tumors. Two of the major cell types involved in this process are B lymphocytes and T lymphocytes. B cells recognize and bind foreign agents, i.e. antigens, via receptors on their cell surface. B cells then internalize bound antigen, process it, and present it on their cell surface in a form that can be recognized by T cells that possess receptors specific for that antigen. This recognition activates the T cells to proliferate and to produce growth and differentiation factors. The combination of antigen binding and T cell-derived factors induces the antigen-specific B cells to proliferate and to produce secretory forms of their surface antigen receptors (antibodies), which then bind to the antigen and act to remove it from the system.

In order to respond to antigens, both B and T cells must transmit the signal that antigen has been bound to the interior of the cell. Several possible signal transduction mechanisms have been described for cell surface receptors of hormones, growth factors, and neurotransmitters. These include production of cyclic nucleotides, activation of ion channels, activation of tyrosine kinases, and hydrolysis of inositol phospholipids. The latter signal transduction pathway can generate different intracellular second messengers depending upon which inositol phospholipid is hydrolyzed. The most common reaction involves hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate, which causes release of calcium from intracellular stores. Protein kinase C can then regulate the activity of other proteins by phosphorylating them on serine and threonine residues, and calcium can regulate the activity of other proteins via calcium-binding proteins such as calmodulin. Alternatively, hydrolysis of phosphatidylinositol produces diacylglycerol and an inositol monophosphate, which does not cause calcium to be released from intracellular stores and has no known second messenger function. Thus, it is possible that different receptors use this signalling pathway in various ways to produce only one stimulus, i.e. protein kinase C

activation via diacylglycerol, or to produce two stimuli, i.e. protein kinase C activation and increases in calcium. In addition, many other inositol polyphosphates can be produced via control of the metabolism of inositol 1,4,5-trisphosphate, and these may have second messenger functions as well, although that possibility remains to be established.

Stimulation of the B cell antigen receptor causes hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in activation of protein kinase C and increases in calcium. I wished to examine the role of this signalling pathway in mediating the effects of antigen-receptor stimulation on B cells. In particular, I wished to address the relative importance of diacylglycerol and calcium as second messengers in this system. I have employed two experimental strategies, one involving the use of pharmacological agents to artificially supply the second messengers to the cells and the other involving inhibition of the action of the second messengers. Since specific inhibitors of the phosphoinositide signal transduction pathway are not available, a system was chosen in which it was possible to select for mutants that are resistant to antigen-receptor stimulation. It was hoped that the analysis of the defects in these mutants would give insight into the mechanism of antigen receptor-mediated control of B cell growth. The system used was the immature B lymphoma cell line WEHI-231. The growth of WEHI-231 cells is irreversibly inhibited by stimulation of its antigen receptor with anti-immunoglobulin antibodies (anti-Ig). Thus, mutants resistant to antigen-receptor stimulation can be easily isolated by their continued growth in the presence of anti-Ig. The system used is described in greater detail in Chapter 1. Chapters 1-3 then present experiments which compare the effects on WEHI-231 cell growth of antigen-receptor stimulation to those of reagents which can mimic the actions of calcium and diacylglycerol. Chapter 4 presents experiments which investigate the status of the phosphoinositide signal transduction pathway in mutant WEHI-231 cells that are resistant to stimulation of their antigen receptors. Together, these experiments indicated that calcium and diacylglycerol are likely playing roles in mediating the effects of antigen-

receptor stimulation on WEHI-231 cells, but that other second messengers may also be involved.

Chapter One:

Role of phosphoinositide-derived second messengers in mediating anti-IgM-induced growth arrest of WEHI-231 B lymphoma cells

ABSTRACT

Anti-IgM irreversibly inhibits the growth of WEHI-231 B lymphoma cells and induces phosphoinositide hydrolysis - producing diacylglycerol, which activates protein kinase C, inositol 1,4,5-trisphosphate, which induces the release of calcium from intracellular storage sites into the cytoplasm, and other inositol polyphosphates. The roles of two of the possible second messengers, cytoplasmic free calcium (Ca^{2+}_i) and diacylglycerol, in mediating the action of anti-IgM on WEHI-231 cells were assessed by elevating $[Ca^{2+}]_i$ with ionomycin and by activating protein kinase C with phorbol 12,13-dibutyrate (PdBu). The combination of 250 nM ionomycin and 4-7 nM PdBu was found to cause growth arrest and cell volume decrease responses in WEHI-231 cells which were similar to those caused by anti-IgM, although clearly slower. Both anti-IgM and the combination of mimicking reagents induced growth arrest of WEHI-231 cells in the G_1 phase of the cell cycle. In both cases, this growth arrest was mitigated by addition of bacterial lipopolysaccharide. Moreover, 250 nM ionomycin plus 4-7 nM PdBu did not inhibit the growth of two other murine B lymphoma cell lines, each of which did exhibit increased phosphoinositide hydrolysis but not growth arrest in response to anti-Ig. Taken together, these results suggest that ionomycin and PdBu, at the concentrations used, did not inhibit WEHI-231 growth by general toxicity, but rather by mimicking the effects of the natural second messengers generated from antigen receptor crosslinking. Thus, the phosphoinositide-derived second messengers Ca^{2+}_i and diacylglycerol are capable of playing important roles in mediating the action of anti-IgM on WEHI-231 B lymphoma cells. However, the response of WEHI-

231 cells to anti-IgM could not be fully reproduced with ionomycin and phorbol diester. These results suggest that another second messenger induced by anti-IgM may also play an important role in mediating the growth arrest of these cells.

INTRODUCTION

Crosslinkage of membrane immunoglobulin (mIg), the B cell receptor for antigen, with anti-Ig antibodies dramatically affects B cell growth. For example, anti-IgM stimulates mature, resting B cells to increase their expression of class II major histocompatibility (MHC) proteins (1), to enter the G_1 phase of the cell cycle (2), and in combination with T cell-derived lymphokines, to proliferate and to differentiate into antibody-secreting cells (3). In contrast to promoting the growth of mature B cells, anti-IgM inactivates immature B cells, a response which is believed to represent one mechanism for inducing tolerance to self antigens (4). Anti-IgM also irreversibly inhibits the growth of the murine B lymphoma cell line WEHI-231. Thus, WEHI-231 may be a tumor model for the inhibitory effects of anti-IgM on immature B cells (5-8).

Membrane IgM appears to transmit growth regulatory signals via phosphoinositide hydrolysis (9-11), a signal transduction mechanism which is also utilized by the T cell antigen receptor (12) and by receptors for many hormones and neurotransmitters (13). Incubation of mature, splenic B cells with anti-Ig (11) or antigen (14) induces hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) to produce several intracellular second messengers: inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which induces the release of Ca^{2+} from intracellular storage sites into the cytoplasm (15-17), and diacylglycerol, which activates protein kinase C (18). Anti-IgM also stimulates $PtdInsP_2$ hydrolysis in WEHI-231 cells, causing the production not only of $Ins(1,4,5)P_3$ and diacylglycerol, but also of $InsP_4$ and $Ins(1,3,4)P_3$ (19). The latter two InsP's increase in several

tissues upon hormone-stimulation (20-22), and they are believed to be derived from Ins(1,4,5)P_3 (22-24). Interestingly, $InsP_4$ synergizes with $InsP_3$ in the activation of sea urchin eggs (25) and in the activation of Ca^{2+}-dependent K^+ channels in acinar cells (26).

Recent evidence indicates that cytoplasmic free calcium (Ca^{2+}_i) and diacylglycerol play important roles as second messengers in mediating the effects of anti-IgM on mature B cells. The ability of these second messengers to mediate the biological effects of anti-IgM has been assessed by using Ca^{2+}-ionophores to increase $[Ca^{2+}]_i$ (27) and phorbol diesters to activate protein kinase C (18). Incubation of mature B cells with the combination of these reagents induces increased expression of class II MHC proteins (28, 29), entry into the G_1 phase of the cell cycle (30), and proliferation - either alone (29, 31-34) or in combination with the T cell-derived lymphokine interleukin 4 (35). Thus, the combination of phorbol diester and Ca^{2+}-ionophore mimics many of the effects of anti-IgM on mature B cells. These results suggest that Ca^{2+}_i and diacylglycerol from phosphoinositide breakdown mediate these effects of anti-IgM on mature B cells.

We wished to analyze the roles that the phosphoinositide-derived second messengers play in mediating the growth arrest of WEHI-231 cells. This analysis may aid in determining whether the same signal transduction system can lead to different biological responses in immature B cells and mature B cells, and if so, how this occurs. In this report, we show that elevation of $[Ca^{2+}]_i$ and activation of protein kinase C partially reproduce the actions of anti-IgM on WEHI-231 cells. These results suggest that Ca^{2+}_i and diacylglycerol can

play important roles as second messengers in mediating the action of anti-IgM on these cells. However, the inability to fully replace anti-IgM with Ca^{2+}-ionophore and phorbol diester raises the possibility that another second messenger also plays an important role in the growth arrest.

MATERIALS AND METHODS

Reagents

Phorbol 12,13-dibutyrate (PdBu) was obtained from Sigma Chemical Co., St Louis, MO, and was stored at -20°C as a 100 to 300 μg/ml solution in ethanol. Ionomycin (calcium salt, stored at -80°C in the dark as a 1.0-1.7 mM solution in dimethylsulfoxide), propidium iodide (stored at 4°C in the dark as a 1 mg/ml solution in 70% ethanol), and 5-bromo-2'-deoxyuridine (BrdUrd) were obtained from Calbiochem Brand Biochemicals, La Jolla, CA. The concentration of ionomycin solutions was determined by measurement of optical density in methanol at 300 nm (36). Lipopolysaccharide (LPS), lipid A, and gliding bacterial adjuvant preparations were obtained as previously described (7). LPS from Escherichia coli 0111:B4 was used in all experiments, except where noted. Goat anti-IgM and Bet 1, a rat monoclonal anti-mouse IgM, were also obtained as previously described (17) and were purified by affinity chromatography on MOPC104E (IgM)-Sepharose.

Cell culture

The mouse B lymphoma cell lines WEHI-231 (37; from Dr. N. Warner, Becton-Dickinson, Mountain View, CA), BALENLM 17 (38; from Dr. M. Davis, Stanford University, Palo Alto, CA), and 2PK3 (37; from Dr. L. Lanier, Becton-Dickinson) were cultured in RPMI 1640 (M. A. Bioproducts, Walkersville, MD, or Gibco Laboratories, Santa Clara, CA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT, or Applied Scientific, San Francisco, CA), 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol at 37°C in an atmosphere containing 5% CO_2. Some cultures also contained either 50 U/ml penicillin and 50 μg/ml streptomycin or 40-50 μg/ml gentamicin.

Cells were periodically screened for mycoplasma contamination (39) and were mycoplasma-free. Cells used in experiments were obtained from cultures in the exponential phase of growth with a density below 5 x 10^5 cells/ml, and experiments were generally done at least three times.

Cell proliferation analysis

Proliferation of WEHI-231, BALENLM 17, and 2PK3 cells was assessed by measuring the incorporation of radiolabeled thymidine. Cells (1 x 10^5/ml for WEHI-231 and 2PK3, 5 x 10^4/ml for BALENLM 17) were cultured with various reagents in a final volume of 0.2 ml in 96-well flat-bottomed plates (Costar, Van Nuys, CA). [^{3}H]-thymidine (1 μCi/well, 3.4 or 6.7 Ci/mmol; ICN, Irvine, CA) was added at the indicated times, and the cultures were harvested 3 to 4 hours later with an automated cell harvester (PHD harvester, Cambridge Technologies, Cambridge, MA). Incorporation of [^{3}H]-thymidine was determined by liquid scintillation counting. All determinations were performed at least in triplicate. In some experiments errors are expressed as 95% confidence limits; these were calculated by the method of Snedecor and Cochran (40) for the comparison of two samples of unequal sizes following logarithmic transformation of the data to approximate a normal distribution.

Cell volume analysis

WEHI-231 cells (1 x 10^5/ml) were cultured with various reagents in a final volume of 1 ml for 24 to 28 hours in 24-well flat bottomed plates (Costar, Van Nuys, CA); ten thousand to fifteen thousand cells per sample were subsequently analyzed with a Coulter Counter and Channelyzer (Coulter Electronics, Hialeah, FL) as described (41).

Cell cycle analysis

The cell cycle status of WEHI-231 cell populations treated in various ways was determined by two parameter flow-cytometric analysis of total DNA content and recently incorporated BrdUrd (42). Briefly, 15 ml of cells at 1 x 10^5/ml were cultured in 25 cm^2 tissue culture flasks (Corning Glass Works, Palo Alto, CA) for 24 hr, and 15 μM BrdUrd was added to the cells during the last half hour of culture. The cells in each sample were then pelleted by centrifugation, fixed by addition of 5 ml of cold 70% ethanol, and stored in the dark at 4°C until staining, which could be done on another day. Fixed cells were collected by centrifugation and resuspended in 2 ml of 2.5 M HCl containing 0.5% Triton X-100. Following incubation for 25 min at room temperature, 5 ml wash buffer (0.5% Tween 20 in phosphate-buffered saline) were added to each sample, which was then centrifuged and washed twice. Next, each sample was incubated for 30 min in 0.5 ml incubation buffer (wash buffer containing 1.5% Carnation Nonfat Dry Milk, w/v) containing the mouse monoclonal anti-BrdUrd antibody IU-4 at a dilution of 1:2000 or 1:4000 of the ascites fluid. (IU-4 was developed at Lawrence Livermore National Laboratories by J. W. Gray; the ascites fluid was a generous gift of Dr. F. Waldman, University of California at San Francisco, San Francisco, CA.) Following two washes, each sample was incubated with 3 μg fluorescein-conjugated goat anti-mouse IgG, (Fc fragment specific, Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in 0.3 ml incubation buffer for 20 min. The samples were subsequently washed twice, incubated in 1 ml phosphate-buffered saline containing 10 μg/ml propidium iodide for 30 to 60 min in the dark, filtered through a 37 μm mesh (Small Parts, Inc., Miami,

FL) and analyzed on a Becton Dickinson FACS-II flow cytometer. Fractions of cells in G_1 phase, S phase, or G_2+M phases were determined by integration of the relevant areas of a two-dimensional plot of fluorescein fluorescence versus propidium iodide fluorescence.

Measurement of $[Ca^{2+}]_i$

WEHI-231 cells were suspended at 2 x 10^7/ml in complete growth medium (see above) containing 3.3 - 11 μM of the acetoxymethyl ester of indo-1 (Molecular Probes, Inc., Junction City, OR). After a 20 min incubation, with occasional mixing, at 37°C in an atmosphere containing 5% CO_2, the cells were diluted 10-fold with growth medium, incubated an additional 20 min, pelleted by centrifugation, and resuspended in fluorometer medium (complete growth medium containing 20 mM HEPES, but lacking phenol red and pyroxidine HCl), which was used to approximate the tissue culture conditions used in the other assays. This was important for assessing the effect of ionomycin on $[Ca^{2+}]_i$, as serum decreased the potency of the ionophore. The intracellular concentration of indo-1 following loading was estimated to be 0.1-0.4 mM, concentrations at which buffering of $[Ca^{2+}]_i$ by the dye appeared to be minimal. The cells were kept at room temperature in the dark and were pelleted by centrifugation and resuspended in fresh fluorometer medium just before use. To measure $[Ca^{2+}]_i$, 3 ml of cells were incubated 5-10 min at 37°C in the dark to equilibrate the temperature and then were placed in a temperature-controlled (37°C) quartz fluorescence cuvette. Indo-1 fluorescence was analyzed with a Spex F2C Spectrofluorometer (excitation 334 nm, emission 400 nm); data points were collected at 1 sec intervals. The calcium concentration measured by indo-1 fluorescence was determined for each data point

RESULTS

Effect of various doses of anti-IgM on WEHI-231 proliferation, cell volume, and $[Ca^{2+}]_i$.

We wanted to assess the roles of the second messengers generated from phosphoinositide hydrolysis in mediating the growth arrest caused by anti-IgM in WEHI-231 cells. To do this, we attempted to mimic two of the second messengers, Ca^{2+}_i and diacylglycerol, by using the Ca^{2+}-ionophore ionomycin to increase $[Ca^{2+}]_i$ (27) and phorbol 12,13-dibutyrate (PdBu) to activate protein kinase C (18). Ideally, the concentrations of ionomycin and PdBu would be chosen on the basis of their ability to reproduce the anti-IgM-generated elevation of $[Ca^{2+}]_i$ and activation of protein kinase C. This goal can be readily approached for the Ca^{2+}-ionophore, since $[Ca^{2+}]_i$ can be accurately measured with intracellular fluorescent dyes.

First, it was necessary to compare the effects of various doses of anti-IgM on the biological responses of WEHI-231 cells and on the elevation of $[Ca^{2+}]_i$. Typically, the monoclonal anti-IgM Bet 1 was fully effective at inhibiting proliferation at doses of 0.1 μg/ml and higher (Fig. 1). In addition to inhibiting growth, anti-IgM causes WEHI-231 cells to decrease their cell volume by more than half within 24 hr (41), and this response showed a similar concentration dependence (Fig. 1).

Next, the effect of anti-IgM on $[Ca^{2+}]_i$ was measured at the same cell density and in essentially the same medium as that used in the biological assays. Thus, the dose response curves for anti-IgM can be directly compared between these two types of experiments. The increase in $[Ca^{2+}]_i$ induced by the monoclonal anti-IgM Bet 1 was found to vary

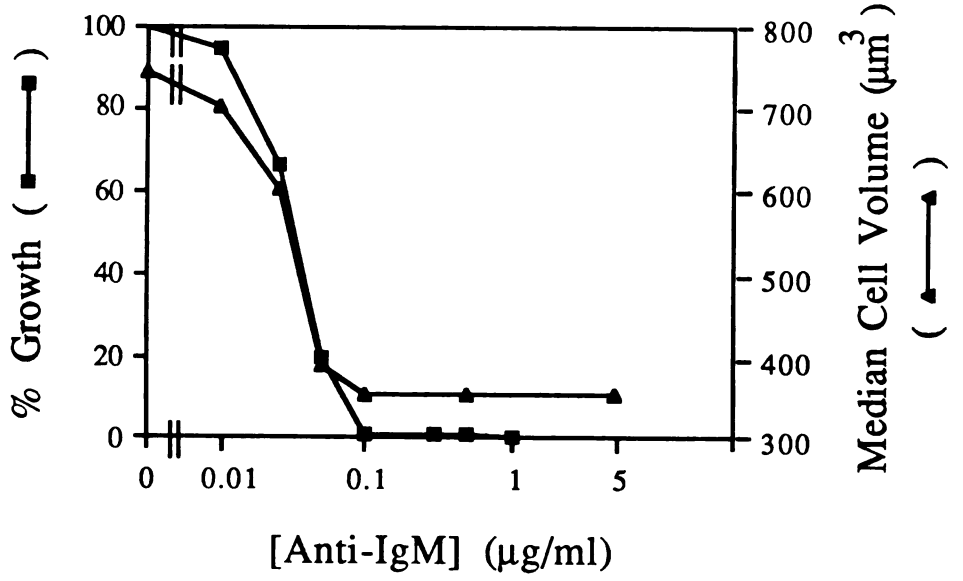


Figure 1. Effect of various doses of anti-IgM on the proliferation and cell volume of WEHI-231 cells. Cells at 10^5/ml were cultured in growth medium for 26 or 29 hr with the indicated concentrations of the monoclonal anti-IgM Bet 1. The median cell volume of each cell population from the 26 hr cultures was determined with a Coulter Counter and Channelyzer. [^{3}H]-thymidine was added for the final 3 hr to the 29 hr cultures and incorporation was determined. The incorporation of cells with no addition of Bet 1 was 125,000 $\pm$ 2,000 cpm (S.E.M.) in this experiment. Results are expressed as % growth, which is 100% x [cpm (experimental)/cpm (no addition)], and the errors for each data point were $\leq$ 5% (S.E.M.).

with the dose of anti-IgM used (Fig. 2). Addition of 5 μg/ml Bet 1 to WEHI-231 cells caused $[Ca^{2+}]_i$ to increase from a pre-stimulatory level of 135 nM to a peak of approximately 580 nM at 30-40 sec. $[Ca^{2+}]_i$ then slowly declined, reaching an elevated plateau of 230 nM after 4-6 min. In contrast, antibodies directed against cell surface structures other than mIgM (e.g., class II MHC proteins) do not cause increases in $[Ca^{2+}]_i$ in WEHI-231 cells (17). Lower concentrations of anti-IgM (0.1-1 μg/ml Bet 1) caused slower increases in $[Ca^{2+}]_i$ culminating in smaller peak values, but with similar plateau $[Ca^{2+}]_i$ levels 4-6 min after addition of anti-IgM (Fig. 2). A similar dose response behavior has also been observed with a human B cell line (44). In contrast to the small, slow increase in $[Ca^{2+}]_i$ caused by 0.1 μg/ml Bet 1, $[Ca^{2+}]_i$ was not significantly elevated upon addition of buffer alone (Table I) or of 0.01 μg/ml Bet 1 (Fig. 2), a dose which did not inhibit the proliferation or decrease the volume of WEHI-231 cells (Fig. 1). The data shown in Fig. 2 were typical of many experiments, as illustrated by the summary in Table I. Thus, the initial effect of anti-IgM on $[Ca^{2+}]_i$ in WEHI-231 cells was greatly dependent upon the dose of anti-IgM used, but 4-6 min after addition of anti-IgM, the elevation of $[Ca^{2+}]_i$ was similar with doses of anti-IgM ranging from 0.1-5 μg/ml. Since 0.1 μg/ml anti-IgM was also the minimum dose that fully inhibited proliferation and decreased cell volume, these results suggest that a small, sustained elevation in $[Ca^{2+}]_i$ is more important than a large, transient increase in $[Ca^{2+}]_i$ is for the biological response of WEHI-231 cells to anti-IgM.

Effect of ionomycin on WEHI-231 $[Ca^{2+}]_i$.

The increases in $[Ca^{2+}]_i$ induced by several doses of the Ca^{2+}-

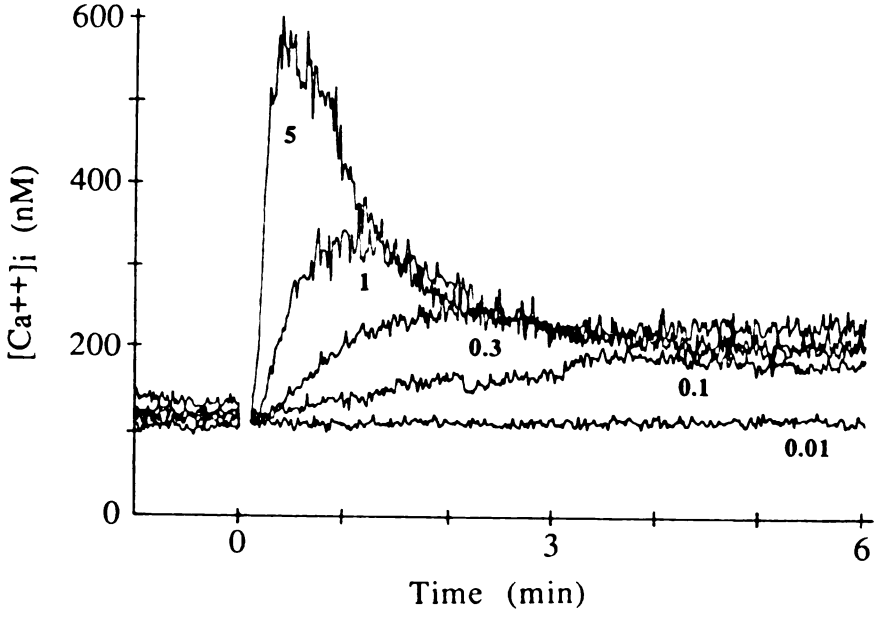


Figure 2. Increases in $[Ca^{2+}]_i$ of WEHI-231 cells induced by various doses of anti-IgM. Cells were loaded with the fluorescent Ca^{2+}-indicator indo-1, and the fluorescence of 10^5 cells/ml was monitored at 37°C with a spectrofluorometer; individual data points were converted to $[Ca^{2+}]_i$ (nM). Additions at time zero were 5.0, 1.0, 0.3, 0.1 or 0.01 μg/ml of the monoclonal anti-IgM Bet 1, as indicated.

Table I

Effect of various doses of anti-IgM or of ionomycin

on the $[Ca^{2+}]_i$ in WEHI-231 cells

Additions[a]	Peak elevation in $[Ca^{2+}]_i$ over prestimulatory level (nM)	Elevation in $[Ca^{2+}]_i$ at 4-6 min over pre-stimulatory level (nM)
Anti-IgM		
5 μg/ml (15)[b]	338 ± 85[c]	49 ± 24
1 μg/ml (3)	231 ± 19	78 ± 9
0.3 μg/ml (3)	135 ± 16	85 ± 17
0.1 μg/ml (4)	70 ± 5	64 ± 6
0.01 μg/ml (2)		9 ± 1
Buffer (3)		-4 ± 5
Ionomycin		
500 nM (11)	586 ± 231	66 ± 31
250 nM (17)	293 ± 59	58 ± 19
175 nM (4)	229 ± 50	41 ± 16
125 nM (4)	163 ± 38	50 ± 6
62.5 nM (4)	93 ± 27	26 ± 12
31.25 nM (3)	51 ± 4	18 ± 11

[a] Cells were treated as described in the legend to Figure 2 with additions at time zero as indicated.

[b] The number in parentheses indicates the number of times the experiment was done.

[c] Results are expressed as mean ± SD.

ionophore ionomycin were compared to the vigorous $[Ca^{2+}]_i$ increase induced by 5 μg/ml anti-IgM (Fig. 3). Both the peak and sustained elevation in $[Ca^{2+}]_i$ caused by 5 μg/ml Bet 1 were closely approximated by treating WEHI-231 cells with 250 nM ionomycin. In contrast, treatment of WEHI-231 cells with 500 nM ionomycin generally produced a peak change in $[Ca^{2+}]_i$ that was 1.5-3 times greater than that produced by 5 μg/ml Bet 1; however, 500 nM ionomycin did induce a plateau level of $[Ca^{2+}]_i$ that was similar to that induced by 0.1-5 μg/ml Bet 1. Ionomycin at 125-175 nM produced less of an initial increase in $[Ca^{2+}]_i$ than did 5 μg/ml Bet 1, but also produced a similar plateau level of $[Ca^{2+}]_i$ (Fig. 3 and Table I). Lower doses of ionomycin (31.25-62.5 nM) produced both a lower initial increase in $[Ca^{2+}]_i$ and a lower sustained level of $[Ca^{2+}]_i$ than that attained with 5 μg/ml Bet 1. Overall, the data indicate that 250 nM ionomycin most closely reproduced the initial and sustained increase in $[Ca^{2+}]_i$ produced by 5 μg/ml Bet 1, and 125-500 nM ionomycin generated the sustained increase in $[Ca^{2+}]_i$ produced in response to all biologically active doses of Bet 1.

In general, when receptors induce phosphoinositide hydrolysis and elevate $[Ca^{2+}]_i$, it is the phosphoinositide hydrolysis that causes the increase in $[Ca^{2+}]_i$ and not vice versa (13). This is true in WEHI-231 cells as well (19). However, unphysiologically high Ca^{2+} concentrations will activate the inositol phospholipid-specific phospholipase C; for example, concentrations of Ca^{2+} greater than 100 μM will activate this enzyme in WEHI-231 cells (45). The concentrations of ionomycin used in these experiments did not elevate $[Ca^{2+}]_i$ that high (Fig. 3) and did not cause production of any InsP's

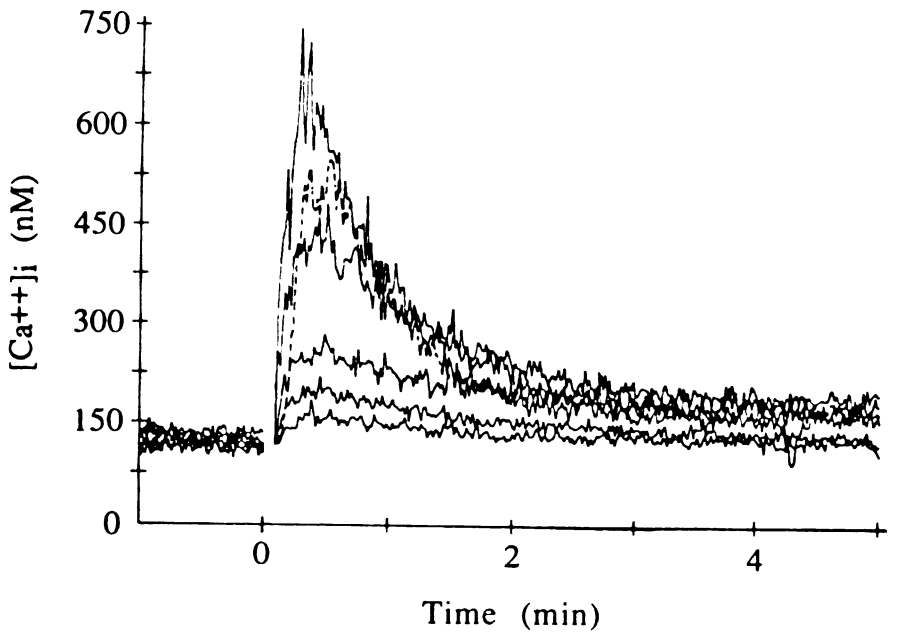


Figure 3. Increases in $[Ca^{2+}]_i$ of WEHI-231 cells induced by anti-IgM or by various doses of ionomycin. The fluorescence of indo-1 loaded cells (10^5/ml) was monitored at 37°C, and individual data points were converted to $[Ca^{2+}]_i$ (nM). Additions at time zero were 5 μg/ml of the monoclonal anti-IgM Bet 1 (dashed line), or 500 nM, 250 nM, 125 nM, 62.5 nM, or 31.25 nM ionomycin (upper solid line to lower solid line, respectively).

in WEHI-231 cells in a buffer that mimicked growth medium conditions, whereas anti-IgM did (data not shown). Thus, the doses of ionomycin used in this report produced increases in $[Ca^{2+}]_i$, but not increases in the other second messengers generated by phosphoinositide breakdown (i.e., InsP's and diacylglycerol).

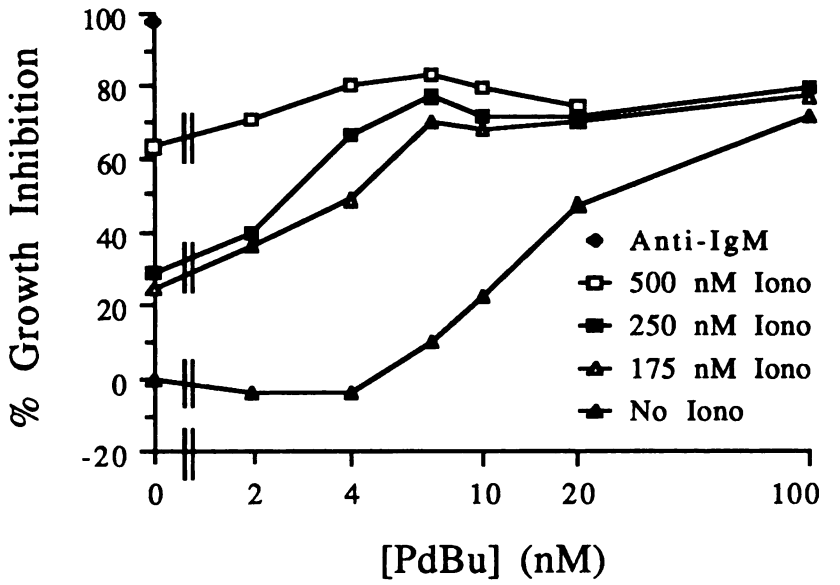
Effect of PdBu and ionomycin on WEHI-231 proliferation and cell volume.

We next examined whether elevation of $[Ca^{2+}]_i$ with ionomycin and activation of protein kinase C with PdBu could mimic the action of anti-IgM on WEHI-231 cells. In the representative experiment shown in Fig. 4A, anti-IgM inhibited WEHI-231 growth by 98% after 24-27 hr. PdBu and ionomycin also inhibited WEHI-231 growth, although always less well than did anti-IgM. For example, 100 nM PdBu inhibited growth by only 72% and 500 nM ionomycin inhibited growth by only 63%. In combination, lower concentrations of these reagents were able to induce a more complete growth arrest, although in no combination were these reagents as effective as anti-IgM. For example, in the presence of 175-500 nM ionomycin, only 7 nM PdBu was required to achieve nearly optimal inhibition of WEHI-231 proliferation (70-83%). Decreasing doses of ionomycin had decreasing ability to inhibit growth in combination with low doses of PdBu (Fig. 4B). Interestingly, the combination of low doses of the mimicking reagents (e.g 125-250 nM ionomycin plus 4-7 nM PdBu, or 62.5 nM ionomycin plus 4 nM PdBu) acted synergistically to inhibit growth. These results suggest that Ca^{2+}_i and diacylglyerol act in concert to regulate the growth of WEHI-231 cells.

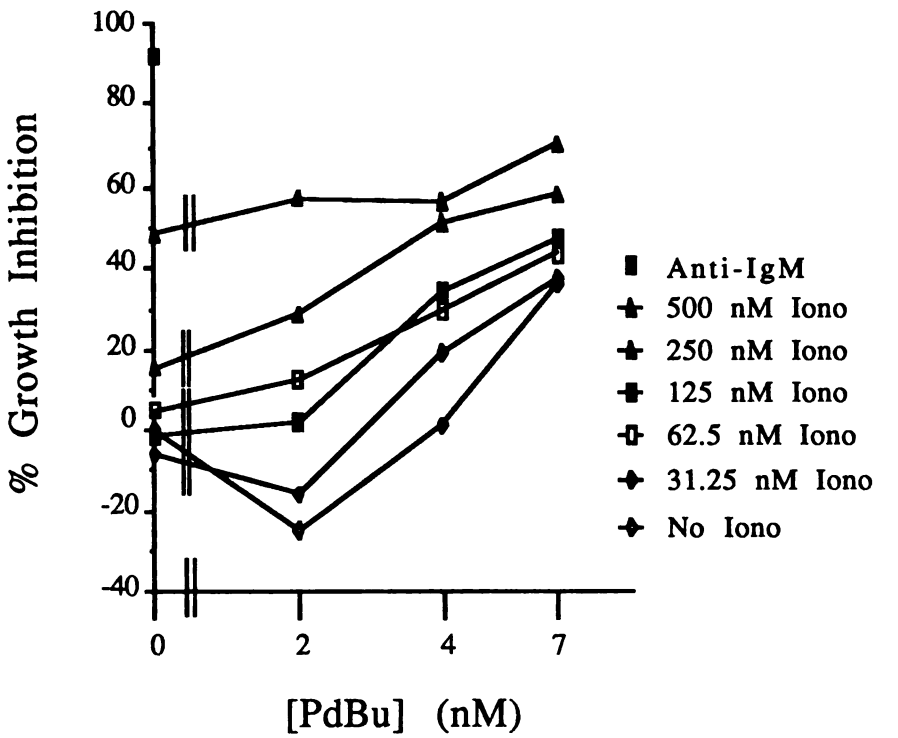
The ability of PdBu and ionomycin to decrease WEHI-231 cell

Figure 4. Effect of anti-IgM or PdBu plus ionomycin on the proliferation of WEHI-231 cells. Cells at 10^5/ml were cultured in growth medium for 25-27 hr with the monoclonal anti-IgM Bet 1 (3.75-5 μg/ml) or with the indicated concentrations of PdBu and ionomycin. [^{3}H]-thymidine was added for the final 3 hr of culture and incorporation was determined. In panel A, the incorporation of cells with no additions was 112,000 $\pm$ 2,000 cpm (S.E.M.). The results are expressed as % growth inhibition, which is 100% x {1 - [cpm (experimental)/cpm (no addition)]}, and the errors for each data point were $\leq$ 11% (S.E.M.). In panel B, the incorporation of cells with no additions was 123,000 $\pm$ 5,000 cpm, and the errors for each data point were $\leq$10% (S.E.M.).

A


% Growth Inhibition
100
80
60
40
20
0
-20
0
2
4
10
20
100
[PdBu] (nM)
Anti-IgM
500 nM Iono
250 nM Iono
175 nM Iono
No Iono

B


% Growth Inhibition
100
80
60
40
20
0
-20
-40
0
2
4
7
[PdBu] (nM)
Anti-IgM
500 nM Iono
250 nM Iono
125 nM Iono
62.5 nM Iono
31.25 nM Iono
No Iono

volume was also examined (Fig. 5). Consistent with previous observations (41), we found that WEHI-231 cells in logarithmic growth had a median volume of 803 μm^3, whereas cells treated with anti-IgM for 25.5 hr had a median volume of 368 μm^3. PdBu and ionomycin also caused WEHI-231 cells to decrease in size, although not as well as did anti-IgM. The greatest decrease obtained with these reagents occurred when cells were treated with 500 nM ionomycin plus 4 nM PdBu (median volume, 525 μm^3). Lower doses of ionomycin (down to 31.25 nM) in combination with 4-7 nM PdBu were progressively less effective at decreasing cell volume (data not shown). As was the case for the proliferation response (Fig. 4), the combination of PdBu and ionomycin was more effective than either reagent alone, yet much less effective than anti-IgM. Thus, the data in Fig. 4 and 5 suggest that PdBu and ionomycin, at the concentrations used, produce some, but not all, of the effects that anti-IgM does in WEHI-231 cells after 24-28 hr.

An interesting phenomenon was additionally apparent in the size decrease experiments. The low doses of PdBu that acted synergistically with ionomycin to inhibit growth (Fig. 4) also optimally decreased cell volume in the presence of 175-500 nM ionomycin. However, in the presence of 175-500 nM ionomycin, the size decrease began to reverse at concentrations of PdBu greater than 7 nM, and at concentrations of PdBu greater than 20 nM, the size decrease was completely abolished (Fig. 5). These results suggest that high concentrations of PdBu induce events in WEHI-231 cells that anti-IgM does not, as the anti-IgM-induced size decrease is constant from 0.1-100 $\mu g/ml$ of the monoclonal anti-IgM Bet 1 (M. R. Gold, D. M. Page, and A. L. DeFranco, unpublished results). For this reason, the remainder of the

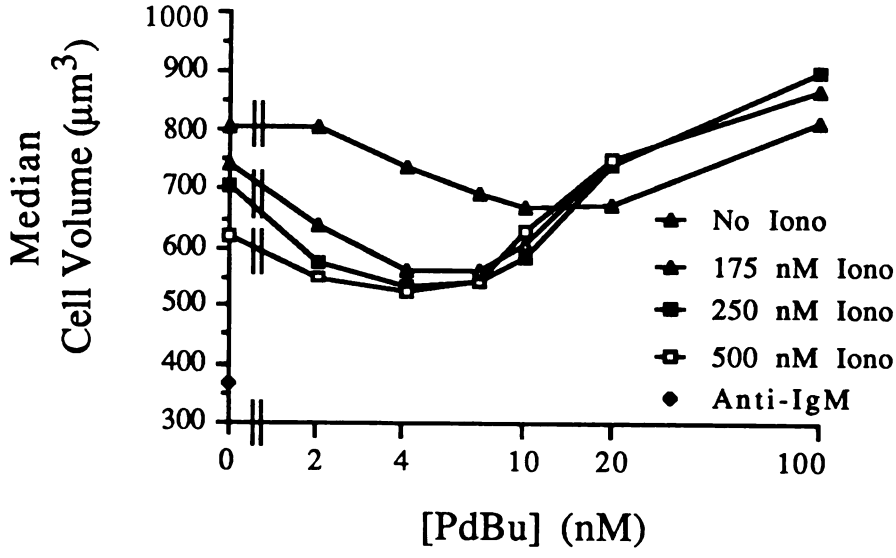


Figure 5. Effect of anti-IgM or PdBu plus ionomycin on the volume of WEHI-231 cells. Cells at 10^5/ml were cultured in growth medium for 25.5 hr with the monoclonal anti-IgM Bet 1 (5 μg/ml) or with the indicated concentrations of PdBu and ionomycin. The median cell volume was then determined for each cell population with a Coulter Counter and Channelyzer.

experiments reported here were done with 4-7 nM PdBu. The remainder of the experiments were also done with a narrow range of concentrations near 250 nM ionomycin, as this dose closely mimicked both the initial and sustained increase in $[Ca^{2+}]_i$ produced by a fully active dose (5 μg/ml) of anti-IgM. (It should be noted that 7 nM PdBu did not affect the increase in $[Ca^{2+}]_i$ caused by 250 nM ionomycin, at least over the first 5 min (data not shown).) The validity of these choices is further addressed in the discussion.

Kinetics of the growth arrest induced by anti-IgM or by PdBu plus ionomycin.

Although PdBu plus ionomycin inhibited the proliferation and decreased the volume of WEHI-231 cells, these reagents consistently did so less well than did anti-IgM after 24-28 hr (Fig. 4 and 5). To explore the nature of this difference, the kinetics of the proliferative response to anti-IgM and to PdBu plus ionomycin were examined (Fig. 6). Anti-IgM caused a rapid inhibition of WEHI-231 proliferation, whereas 4-7 nM PdBu plus 250-500 nM ionomycin caused a slower, but eventually complete, growth arrest. All concentrations of PdBu plus ionomycin tested, except for 250 nM ionomycin plus 4 nM PdBu, produced approximately the same level of inhibition by 46 hr. Thus, these reagents affected each of the WEHI-231 cells in the population; however, they exerted their effects more slowly than did anti-IgM.

Similarity in the action of anti-IgM and the combination of 250 nM ionomycin and 4-7 nM PdBu.

Since PdBu and ionomycin can simulate some of the biochemical events normally triggered by anti-IgM in WEHI-231 cells, we postulated

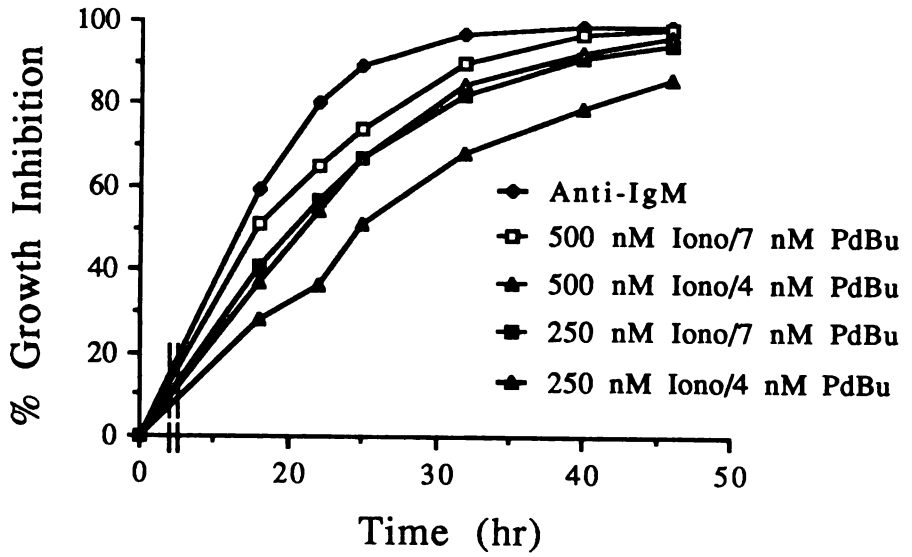


Figure 6. Kinetics of the growth arrest of WEHI-231 cells treated with anti-IgM or with PdBu plus ionomycin. Cells at 10^5/ml were cultured in growth medium for various times with the monoclonal anti-IgM Bet 1 (2 μg/ml) or with the indicated concentrations of PdBu and ionomycin. [^{3}H]-thymidine was added for the final 3-4 hr of culture and incorporation was determined. The indicated times represent the middle of the [^{3}H]-thymidine pulse. In this experiment, the incorporation of cells with no additions was 101,000 $\pm$ 2,000, 131,000 $\pm$ 2,000, 138,000 $\pm$ 1,000, 123,000 $\pm$ 2,000, 197,000 $\pm$ 3,000, and 236,000 $\pm$ 6,000 cpm (S.E.M.) for 18, 22, 25, 32, 40, and 46 hr, respectively. Results are expressed as % growth inhibition (see legend to Figure 4), and the errors for each data point were $\leq$10% (95% confidence limits).

that PdBu plus ionomycin inhibited proliferation and decreased cell volume because they were mimicking the anti-IgM-generated second messengers. However, it is possible that the effects of PdBu plus ionomycin resulted from some sort of toxicity rather than from triggering the same biological responses that anti-IgM triggers. To address this issue, we compared the nature of the growth arrests induced by anti-IgM and by PdBu plus ionomycin, and we also compared the effects of these reagents on other B cell lines.

Although the growth of WEHI-231 cells is largely inhibited after 24 hr of treatment with anti-IgM, as assessed by [^{3}H]-thymidine incorporation, the cells remain mostly viable (ca. 80%), as assessed by Trypan Blue dye exclusion. By 48 hr, however, most of the cells are no longer viable (7). Thus, anti-IgM inhibits proliferation prior to causing cell death. Indeed, WEHI-231 cells treated with anti-IgM arrest in the G_1 phase of the cell cycle (46), which is typical of regulated growth arrest in mammalian cells. Similarly, treatment of WEHI-231 cells with 250-500 nM ionomycin plus 4-7 nM PdBu inhibited proliferation prior to causing cell death (Table II) and also caused a marked increase in the number of cells in G_1 phase (see below). Thus, by these parameters, the growth inhibition induced by 4-7 nM PdBu plus 250-500 nM ionomycin was similar to that induced by anti-IgM.

Most murine B lymphomas continue to grow in the presence of anti-Ig. If PdBu plus ionomycin inhibited WEHI-231 growth by mimicking anti-IgM, then these reagents should not inhibit the growth of B cell lines that are not inhibited by anti-Ig. This issue was examined with the B lymphoma cell lines 2PK3 and BALENLM 17, which were chosen because they have the surface phenotype of mature B cells (38, 47) and

Table II

Viability of WEHI-231 cells treated with anti-IgM or with PdBu plus ionomycin

Additions[a]	[^{3}H]Thymidine Incorporation (% of untreated control)[b]	No. of Viable Cells Recovered (% of untreated control)[c]
Anti-IgM	10 (±2)	64 (±3)
[Ionomycin] (nM) + [PdBu] (nM)		
250 + 4	33 (±5)	61 (±4)
250 + 7	29 (±3)	61 (±2)
500 + 4	25 (±4)	67 (±1)
500 + 7	24 (±4)	54 (±2)

[a] WEHI-231 cells at 10^5/ml were cultured in growth medium with the monoclonal anti-IgM Bet1 (5 μg/ml) or with the indicated concentrations of PdBu and ionomycin.

[b] [^{3}H]Thymidine incorporation was measured between 24 and 27 h after additions were made. The incorporation of untreated cells (control) was 108,000 ± 3,000 cpm (SEM) in this experiment. The results are expressed as percent incorporation relative to control cultures (± 95% confidence limits).

[c] The number of viable cells, defined as cells excluding the dye trypan blue, was determined 25.5 h after additions were made. The results are expressed as the percent of viable cells recovered (± range of duplicate cultures) relative to untreated control cultures. This value represents decreases in cell number due to decreased rates of proliferation and due to cell death. The number of viable cells/ml (± range of duplicate cultures) recovered in the control cultures was 4.18 (± 0.18) x 10^5/ml.

because they have retained the anti-Ig-induced $PtdInsP_2$ hydrolysis seen in normal, mature B cells (11, 48, and unpublished observations of K. A. Fahey, D. M. Page and A. L. DeFranco). Anti-Ig did not significantly inhibit the proliferation of 2PK3 and BALENLM 17 cells (Table III). In contrast, WEHI-231 cell growth was inhibited by 97% in the same period of time in a parallel experiment. Correspondingly, the combination of 250 nM ionomycin and 4-7 nM PdBu inhibited WEHI-231 cell growth by 60-70%, but did not inhibit the growth of 2PK3 or BALENLM 17 cells (Table III). Similarly, 48 hr of treatment with 250 nM ionomycin $\pm$ 4-7 nM PdBu or with anti-Ig did not inhibit the growth of 2PK3 or BALENLM 17 cells (data not shown), demonstrating that the differences in the growth inhibition of the various cell lines were not merely kinetic ones. In some cases, 500 nM ionomycin with or without 4-7 nM PdBu modestly inhibited the growth of 2PK3 and BALENLM 17 cells -- inhibition ranging from -7 to 36% at 24 hr and from -6 to 49% at 48 hr (Table III and data not shown). These results suggest that 500 nM ionomycin may have some general toxicity associated with its growth inhibitory effects on WEHI-231 cells. On the other hand, 250 nM ionomycin with or without 4-7 nM PdBu appeared to inhibit WEHI-231 cell growth not by general toxicity, but rather by mimicking the biochemical events induced by anti-IgM.

Effect of lipopolysaccharide (LPS) on the PdBu plus ionomycin-induced growth arrest of WEHI-231 cells.

LPS and other bacterial products completely protect WEHI-231 cells from anti-IgM-induced growth inhibition for the first 36 hr of treatment (7, 8). After that time, protection wanes due to desensitization of the cells to the bacterial products (7). LPS does

Table III

Effect of anti-IgM or PdBu plus ionomycin on the proliferation of three murine B lymphoma cell lines

	[^{3}H]Thymidine Incorporation (% of untreated control)[b]		
Additions[a]	WEHI-231	2PK3	BALENLM 17
Anti-IgM	3	102	89
[Ionomycin] (nM) + [PdBu] (nM)			
0 + 4	115	130	102
0 + 7	99	128	97
250 + 0	82	97	95
250 + 4	43	114	102
250 + 7	33	102	101
500 + 0	61	64	99
500 + 4	20	91	105
500 + 7	20	93	107

[a] Cells were cultured in growth medium with 5 μg/ml goat anti-IgM or with the indicated concentrations of PdBu and ionomycin for 27 to 28 h. (It should be noted that this preparation of polyclonal goat anti-IgM likely acts on the mIgG$^+$ 2PK3 cells because κ- and λ-specific antibodies were co-purified with the μ-specific antibodies.)

[b] [^{3}H]Thymidine was added for the final 3 h of culture and incorporation was determined. In these experiments, the incorporation of cells with no additions (control) was 120,000 ± 1,000, 62,000 ± 1,000, and 135,000 ± 16,000 cpm (SEM) for WEHI-231, 2PK3, and BALENLM 17, respectively. The results are expressed as percent incorporation relative to control cultures, and all errors were ≤ 7%, ≤ 8%, and ≤ 20% (95% confidence limits) for WEHI-231, 2PK3, and BALENLM 17, respectively.

not inhibit anti-IgM-stimulated $PtdInsP_2$ hydrolysis and subsequent second messenger production (unpublished observation of J. P. Jakway, K. A. Fahey and A. L. DeFranco). Thus, LPS presumably prevents growth inhibition by interfering with the actions of the second messengers. It follows that LPS should protect WEHI-231 cells from the effects of PdBu plus ionomycin, if these reagents act by mimicking the anti-IgM-generated second messengers. Accordingly, we investigated the effects of PdBu, ionomycin, and LPS on WEHI-231 cell cycle position (Table IV). Treatment of WEHI-231 cells with anti-IgM for 24.5 hr caused the fraction of cells in the $S+G_2+M$ phases of the cell cycle to decrease dramatically from 83% to 12%. Consistent with a previous report (46), the cells that had not progressed into the $S+G_2+M$ phases were mostly arrested in the G_1 phase of the cell cycle. Some cells were found to have less DNA content than the G_1 phase; presumably these cells were in a phase intermediate between G_1 and cell death. PdBu plus ionomycin also caused a large decrease in the number of WEHI-231 cells in the $S+G_2+M$ phases of the cell cycle. In agreement with the results obtained in the growth inhibition and size decrease assays (Fig. 4 and 5), these reagents induced a similar, but less complete (presumably slower, see Fig. 6), cell cycle arrest than did anti-IgM after 24 hr.

As expected, treatment of WEHI-231 cells with anti-IgM in the presence of LPS resulted in impressive protection from growth arrest (Table IV). Similarly, the growth arrest induced by PdBu plus ionomycin was considerably abated in the presence of LPS. Curiously, LPS did not protect the cells from growth arrest induced by PdBu plus ionomycin as well as it protected them from growth arrest induced by anti-IgM (57-69% protection versus 89% protection), despite the fact

Table IV

Cell cycle status of WEHI-231 cells treated with anti-IgM,

PdBu, ionomycin, and LPS

Additions[a]	Percent Cells in S+G2+M Phases -LPS	+LPS[a]	Percent LPS Protection[b]
Medium	83	79	
Anti-IgM	12	71	89
[Ionomycin] (nM) + [PdBu] (nM)			
250 + 4	34	64	69
250 + 7	41	61	57
500 + 4	31	60	63
500 + 7	41	63	62
0 + 4	70	ND	ND
0 + 7	63	ND	ND
250 + 0	71	ND	ND
500 + 0	51	ND	ND

[a] WEHI-231 cells at 10^5/ml were cultured in growth medium for 24.5 h with the monoclonal anti-IgM Bet1 (2 μg/ml) or with the indicated concentrations of PdBu and ionomycin, either with or without 10 μg/ml LPS. The cell cycle status was then determined as described in *Materials and Methods*.

[b] Percent LPS protection is defined as 100% x {1 - [% cells in S+G2+M (experimental with LPS) - % cells in S+G2+M (LPS alone)]/[% cells in S+G2+M (experimental without LPS) - % cells in S+G2+M (medium alone)]}.

that anti-IgM caused greater cell cycle arrest in the absence of LPS. Furthermore, when growth inhibition was assessed by [^{3}H]-thymidine incorporation rather than by cell cycle analysis, LPS was less effective at protecting WEHI-231 cells treated with the higher concentrations of PdBu plus ionomycin from growth arrest. The reasons for these discrepancies are not known. However, the fact that LPS partially protected WEHI-231 cells from the PdBu plus ionomycin-induced growth arrest supports the idea that PdBu and ionomycin act, at least in part, by simulating the second messengers generated in response to anti-IgM rather than in a non-specific toxic manner.

The ability of LPS to protect WEHI-231 cells from growth arrest was probably not due to some nonspecific effect of the E. coli 0111:B4 LPS on PdBu or ionomycin, as other bacterial products (i.e., gliding bacterial adjuvant, lipid A, and LPS's isolated from other bacteria) were also able to protect WEHI-231 cells from anti-IgM-induced or PdBu plus ionomycin-induced growth arrest, as measured by [^{3}H]-thymidine incorporation (7 and data not shown). Furthermore, LPS (E. coli 0111:B4) did not inhibit the immediate actions of ionomycin and PdBu. For example, the effect of LPS on PdBu potency was examined by looking at the ability of PdBu to inhibit anti-IgM-stimulated phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ (41). We observed a partial inhibition of the anti-IgM-induced elevation of $[Ca^{2+}]_i$ with 14 nM PdBu, and this partial inhibition was not affected by LPS (data not shown). Similarly, the increase in $[Ca^{2+}]_i$ induced by 250 nM ionomycin was not affected by LPS (data not shown). Thus, LPS appears to overcome the inhibitory effects of PdBu and ionomycin by altering the response of WEHI-231 cells to the mimicking reagents.

DISCUSSION

In order to evaluate the roles of the phosphoinositide signaling pathway second messengers Ca^{2+}_i and diacylglycerol in mediating the action of anti-IgM on the growth of WEHI-231 cells, we have used ionomycin to elevate $[Ca^{2+}]_i$ and PdBu to activate protein kinase C. Like anti-IgM, the combination of PdBu and ionomycin dramatically inhibited the growth of WEHI-231 cells. The treated cells arrested in the G_1 phase of the cell cycle (Table IV), with a concomitant decrease in cell volume (Fig. 5). Either PdBu or ionomycin alone was capable of inhibiting cell growth if added at high concentrations; in combination, however, low concentrations of these reagents often had a synergistic effect on WEHI-231 growth and cell volume.

Several lines of evidence suggest that the combination of 4-7 nM PdBu and 250 nM ionomycin caused a growth arrest similar to that caused by anti-IgM. Both anti-IgM and 250 nM ionomycin plus 4-7 nM PdBu caused a growth arrest in the G_1 phase of the cell cycle, and this growth arrest preceeded cell death (Table II, IV). Increasing the doses of ionomycin plus PdBu above 250 nM and 4-7 nM, respectively, did not greatly increase the inhibition of proliferation, cell cycle arrest, or cell size decrease achieved with this combination of reagents. Furthermore, LPS, which prevents the growth arrest caused by anti-IgM (7,8), also partially prevented the growth arrest caused by 250 nM ionomycin plus 4-7 nM PdBu (Table IV). This combination of reagents was also similar to anti-Ig in its lack of effect on two other murine B lymphoma cell lines, 2PK3 and BALENLM 17, which did exhibit phosphoinositide breakdown in response to anti-Ig (Table III). In contrast to what was seen when 250 nM ionomycin $\pm$ 4-7 nM PdBu was

added to 2PK3 or BALENLM 17 cells, 500 nM ionomycin ± 4-7 nM PdBu did occasionally inhibit the growth of these cells, suggesting that 500 nM ionomycin may have some general toxicity associated with its effects on WEHI-231 cells.

These results demonstrate that Ca^{2+}_i and diacylglycerol are capable of mediating the growth inhibitory effects of anti-IgM on WEHI-231 cells. It should be noted, however, that the combinations of PdBu and ionomycin tested never completely reproduced the actions of anti-IgM. For example, these reagents never inhibited the proliferation (Fig. 4), or arrested the cells in G_1 phase (Table IV) as well as did anti-IgM by 24-28 hr. This discrepancy reflected a slower effect of the mimicking reagents on growth (Fig. 6). This slower effect on growth may explain the recent results of Scott et al (49), who found that anti-IgM, but not PdBu plus ionomycin, prevented WEHI-231 cells synchronized in the M phase of the cell cycle from entering the next S phase. Interestingly, the discrepancy between the effects of anti-IgM and those of PdBu plus ionomycin was particularly noticeable in the examination of cell size, which decreased markedly in response to anti-IgM, but less so in response to PdBu plus ionomycin (Fig. 5).

There are several possible reasons for the inability of PdBu plus ionomycin to mimic fully the biological effects of anti-IgM on WEHI-231 cells. One possibility is that Ca^{2+}_i and diacylglycerol are responsible for mediating the effects of anti-IgM on WEHI-231 size and growth, but that the actions of these second messengers cannot be reproduced with PdBu and ionomycin because ionomycin and PdBu did not elevate $[Ca^{2+}]_i$ and/or activate protein kinase C as much as did anti-

IgM. This explanation seems unlikely for several reasons. First of all, 250 nM ionomycin appeared to reproduce the initial and sustained elevation in $[Ca^{2+}]_i$ generated by a fully active dose of anti-IgM, at least over the first 5 min (Fig. 3). Correspondingly, as discussed above, increasing the dose of ionomycin above 250 nM did not greatly improve the growth inhibition or size decrease obtained in combination with 4-7 nM PdBu. It is also unlikely that 4-7 nM PdBu activated protein kinase C less well than did diacylglycerol from anti-IgM-induced phosphoinositide hydrolysis. First of all, in the presence of 175-500 nM ionomycin, doses of PdBu greater than 7 nM did not significantly increase the growth arrest (Fig. 4A), whereas they actually reversed the cell volume decrease seen at lower doses (Fig. 5). Secondly, concentrations of PdBu greater than 10 nM inhibit anti-IgM-induced phosphoinositide breakdown in WEHI-231 cells (41). Since diacylglycerol generated from phosphoinositide hydrolysis would presumably have the same effect, anti-IgM-induced signaling likely produces an equilibrium between signal generation and feedback inhibition that precludes stable, continuous activation of a large fraction of protein kinase C. According to this viewpoint, concentrations of PdBu below 10 nM, which activate one-half or less of the protein kinase C molecules (18), are appropriate for mimicking anti-IgM-induced activation of the enzyme. Our experimental observations agree with this viewpoint, as activation of protein kinase C with doses of PdBu greater than 7 nM in the presence of 175-500 nM ionomycin resulted in biological effects not seen upon activation of the enzyme with anti-IgM (e.g. increased cell size). Thus, we think it unlikely that the inability of ionomycin plus PdBu

to reproduce fully the effects of anti-IgM in WEHI-231 cells was due to insufficient elevation of $[Ca^{2+}]_i$ or to insufficient activation of protein kinase C.

Another possible explanation is that PdBu and/or ionomycin were not fully effective because they failed to reproduce some of the actions of the natural second messengers. This is unlikely in the case of ionomycin since the second messenger, Ca^{2+}_i, was actually the same. It is also unlikely that PdBu exerted its effects on WEHI-231 cells through a mechanism other than activation of protein kinase C, as another phorbol diester, 4β-phorbol didecanoate, also inhibited proliferation and decreased cell volume in combination with 250 nM ionomycin, whereas the biologically inactive stereoisomer, 4α-phorbol didecanoate, did not (data not shown). Activation of partially purified protein kinase C *in vitro* shows the same stereospecificity (50). Thus, these results suggest that the ability of PdBu or 4β-phorbol didecanoate to contribute to decreasing the volume and inhibiting the proliferation of WEHI-231 cells is due to activation of protein kinase C. It is also unlikely that PdBu did less than what diacylglycerol does, as Hornbeck and Paul (51) showed that a related compound, phorbol 12-myristate 13-acetate, induces phosphorylation of all the major substrates that anti-IgM does in WEHI-231 cells. On the other hand, PdBu may have done more than the natural second messenger diacylglycerol did, if the amounts of PdBu used activated protein kinase C more than did anti-IgM-generated diacylglycerol.

Finally, the inability of PdBu plus ionomycin to reproduce fully the biological effects of anti-IgM may indicate that there is another second messenger that cooperates with Ca^{2+}_i and diacylglycerol to

mediate the actions of anti-IgM on WEHI-231 cells. In particular, the differences in the effects of anti-IgM and those of PdBu plus ionomycin on WEHI-231 cell volume suggest the existence of another second messenger. The size of WEHI-231 cells treated for 24 hr with PdBu plus ionomycin corresponded to the size of WEHI-231 cells in the early G_1 phase of the cell cycle (46 and unpublished observations). Cells treated with anti-IgM also arrested in G_1 phase, but they accumulated at a considerably smaller size. These results suggest that anti-IgM caused WEHI-231 cells to arrest in the G_1 phase of the cell cycle and additionally caused the cells to decrease their size, whereas cells treated with PdBu and ionomycin arrested in G_1 phase but did not undergo a further size decrease. According to this interpretation, Ca^{2+}_i and diacylglycerol may be largely responsible for the irreversible G_1 phase growth arrest of WEHI-231 cells treated with anti-IgM, whereas some other second messenger(s) may be necessary for the additional, non-cell cycle related, size decrease. This additional second messenger may also play an important role in the growth regulation. Although an impressive growth arrest can be achieved with 250 nM ionomycin plus 4-7 nM PdBu, these reagents may be elevating $[Ca^{2+}]_i$ and activating protein kinase C more than do the lower, but still fully active, doses of anti-IgM. Several anomalies in the data indicate that this may be the case. First of all, LPS prevented the growth arrest caused by anti-IgM better than it prevented the growth arrest caused by PdBu plus ionomycin. Furthermore, there is an imperfect correlation between the effects of ionomycin and various doses of anti-IgM on growth and on the sustained elevation of $[Ca^{2+}]_i$ (Fig. 1-3, 4B). Thus, even if anti-IgM-generated

Ca^{2+}_i and diacylglycerol induce a substantial growth arrest, an additional second messenger may do so as well, i.e. there may be redundancy in the growth control.

In T cell activation, there is also evidence for biological importance of a second messenger in addition to Ca^{2+}_i and diacylglycerol. In the experiments of Kaibuchi et al (52), the mitogenic response of human lymphocytes to the combination of phorbol diester and phytohemagglutinin (PHA) could not be fully reproduced with phorbol diester plus Ca^{2+}-ionophore. PHA is believed to act via the T cell antigen receptor by inducing phosphoinositide hydrolysis. Suboptimal doses of PHA were required in addition to Ca^{2+}-ionophore and phorbol diester in order to induce proliferation that was as vigorous as that seen with the optimal doses of PHA and phorbol diester. The results were interpreted as suggesting that another signal is generated by PHA.

If an additional second messenger is needed to reproduce fully the effects of antigen receptor signaling in B cells and T cells, it may come from phosphoinositide breakdown. In addition to increases in $[Ca^{2+}]_i$ and diaclyglycerol, antigen receptor-induced phosphoinositide hydrolysis generates $Ins(1,4,5)P_3$, $Ins(1,3,4)P_3$ and $InsP_4$ in WEHI-231 cells (19) and in the human T cell line Jurkat (24). Interestingly, $InsP_4$ synergizes with $InsP_3$ in the activation of sea urchin eggs (25) and in the activation of Ca^{2+}-dependent K^+ channels in acinar cells (26).

In both non-transformed mature B cells and in the immature B cell lymphoma WEHI-231, anti-IgM has been shown to induce phosphoinositide breakdown (9-11, 19). Correspondingly, in non-transformed human and

murine mature B cells, the combination of a Ca^{2+}-ionophore and a phorbol diester induces many of the effects that anti-IgM induces (28-35). The results reported here indicate that Ca^{2+}_i and diacylglycerol also play a role in mediating the effects of anti-IgM in WEHI-231 cells, yet the effects of anti-IgM on growth are opposite in the two systems: In normal B cells, anti-IgM stimulates early activation events contributing to proliferation, whereas in WEHI-231 cells, anti-IgM causes growth arrest. Either the different types of B cells possess machinery for opposite interpretations of the same anti-IgM-generated second messengers, or there are unknown second messengers which differ between the two systems.

In conclusion, we have presented evidence which suggests that Ca^{2+}_i and diacylglycerol, resulting from phosphoinositide breakdown, play a role in mediating the biological effects of anti-IgM in the B lymphoma cell line WEHI-231. The inability of PdBu plus ionomycin to completely reproduce the action of anti-IgM on these cells could be due to imperfect mimicking of the actions of the anti-IgM-generated second messengers and/or to the involvement of other second messengers that cooperate with Ca^{2+}_i and diacylglycerol in mediating the action of anti-IgM on these cells.

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Chapter Two:

Analysis of protein kinase C activation induced in WEHI-231 B lymphoma cells by anti-IgM or phorbol diester

INTRODUCTION

Binding of antigen or of anti-receptor antibodies to membrane IgM activates the phosphoinositide signal transduction pathway. Two major second messengers of this pathway are diacylglycerol, which activates protein kinase C, and calcium, which regulates the activity of many proteins via calcium-binding proteins such as calmodulin. The combination of a phorbol diester that can activate protein kinase C and a calcium ionophore were shown to partially reproduce the growth inhibitory effects of anti-IgM on the WEHI-231 B lymphoma cell line (1, i.e. Chapter 1 of Ph. D. Thesis). These results suggest that the phosphoinositide-derived second messengers diacylglycerol and calcium play roles in mediating the effects of anti-IgM on these cells. The combination of phorbol diester and calcium ionophore did not inhibit WEHI-231 growth as much as did anti-IgM, however, suggesting that either a second messenger other than diacylglycerol and calcium also mediates the growth arrest or that the mimicking reagents do not activate protein kinase C and increase cytosolic calcium similarly to anti-IgM. The effects of ionomycin and anti-IgM on the levels of cytosolic calcium were measured and were found to be similar, at least at early times (1, i.e. Chapter 1 of Ph. D. Thesis). It was also found that doses of phorbol 12,13-dibutyrate (PdBu) greater than 7 nM probably activated protein kinase C more than did stimulation via the antigen receptor, since these doses of PdBu caused cellular responses not seen with anti-IgM (1, i.e. Chapter 1 of Ph. D. Thesis). However, this was only an indirect assessment of protein kinase C activation. Thus, the goal of the experiments described here was to assess directly the extent to which anti-IgM activates protein kinase C and to determine which dose of PdBu to use to mimic the anti-IgM-induced activation.

Protein kinase C activation is associated with several biochemical events. Activation of this enzyme in many systems, including B lymphocytes (2, 3), causes it to be translocated from the cytosol to the plasma membrane. The activated enzyme will then phosphorylate exogenously added substrate proteins or synthetic substrate peptides (4). Activated protein

kinase C also phosphorylates multiple endogenous substrates. One of the most prominent phosphoproteins appearing after protein kinase C activation is an 80-87 kd heat-stable protein. This protein was first determined to be a substrate for protein kinase C in fibroblasts (5). Since then, it has been cloned (6), and the purified protein has been shown to be a substrate for purified protein kinase C (7). Phosphorylation of this protein in response to phorbol esters has been seen in T lymphocytes (8), B lymphocytes (9, 10), the WEHI-231 B cell line (9, 10), and many other tissues and cell types (11 and refs therein).

It has often been assumed that translocation of protein kinase C to the membrane reflects activation of the enzyme. Although activation clearly involves translocation, it is possible that translocation could occur without activation. Accordingly, we have used substrate phosphorylation assays to examine protein kinase C activation in WEHI-231 cells. When phosphorylation of a synthetic peptide was examined, the activation of protein kinase C by anti-IgM could not be detected above the background of constitutive activity. In contrast, doses of PdBu of 7 nM or more did cause detectable increases in peptide phosphorylation, suggesting that 7 nM PdBu activates protein kinase C more than does anti-IgM in these cells. The phosphorylation of the 80-87 kd heat-stable protein proved to be a more sensitive assay of protein kinase C activation in these cells, since increased phosphorylation of this protein could be detected by treating WEHI-231 cells with 2 nM PdBu. Anti-IgM also caused increased phosphorylation of this protein, presumably by activating protein kinase C, as had been previously suggested (9, 10). The degree of activation induced by anti-IgM was comparable to that induced by doses of PdBu from 2-4 nM. This result suggests that doses of PdBu from 2-4 nM are the most appropriate for assessing the role of diacylglycerol in mediating the growth inhibitory effects of anti-IgM on WEHI-231 cells.

MATERIALS AND METHODS

Reagents. Phorbol 12,13-dibutyrate (Sigma Chemical Co., St. Louis, MO) was stored at -20°C as a 200 μM solution in ethanol. The rat monoclonal anti-mouse IgM Bet1 was obtained and purified as previously described (12).

Cell culture. The murine B lymphoma cell line WEHI-231 was cultured as previously described (1, i.e. Chapter 1 of Ph. D. Thesis). Cells used in experiments were obtained from cultures in the exponential phase of growth with a density generally below 5 x 10^5/ml.

Phosphorylation of endogenous, heat-stable proteins. Phosphorylation of the 80-87 kd heat-stable protein was examined as previously described (8). Briefly, WEHI-231 cells were collected by centrifugation and washed twice with phosphate-free growth medium containing 20 mM HEPES. The cells (2 x 10^6/ml) were then incubated for 1 hr at 37°C in an atmosphere containing 5% CO_2 in growth media containing 20 mM HEPES, only 50 μM phosphate, and 50 μCi/ml ^{32}P (HCl-free, 8 mCi/ml, Amersham Corp., Arlington Heights, IL). The labelled cells were subsequently incubated with stimuli for various lengths of time at 37°C. Each sample (2 x 10^6 cells) was then collected by centrifugation for 30 sec in an Eppendorf model 235B micro-centrifuge, washed once by centrifugation with 1 ml ice-cold phosphate-buffered saline, and incubated for 30-60 min at 0°C with 0.2 ml lysis buffer (10 mM EDTA, 5 mM EGTA, 10 mM NaF, 1% (w/v) Triton X-100, 0.15 M NaCl, 5 mM phenylmethylsulphonylfluoride, 10 mM Tris, pH 7.5). Next, the lysed cells were boiled for 10 min, and precipitated proteins were removed by centrifugation for 10 min at 4°C in the micro-centrifuge. Soluble, heat-stable proteins were precipitated by addition of ice-cold acetone to 66% and centrifugation for 10 min at 4°C in the micro-centrifuge. After removing the supernatant, the pellet was dried under vacuum, dissolved in sample buffer (0.1 M Tris, pH 6.8, 2% SDS, 10% (w/v) glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue), and analyzed by electrophoresis on SDS-containing 8% polyacrylamide gels. Phosphorylated proteins were visualized by

autoradiography; incorporation of ^{32}P into these proteins was determined by liquid scintillation counting of the proteins after excision from the gels.

Phosphorylation of a synthetic peptide in permeabilized cells. WEHI-231 cells were first immobilized onto 96-well plates (Costar, Van Nuys, CA) with the Cell-Tak adhesive (Biopolymers, Inc., Farmington, CT). Briefly, each well was coated with Cell-Tak by incubation at room temperature for 60 min with 0.7 μg Cell-Tak in 30 μl of a solution of 67 mM sodium bicarbonate, pH 8.0. Unbound Cell-Tak was removed by rinsing the wells three times with phosphate-buffered saline. 5×10^4 WEHI-231 cells in Hanks Balanced Salt Solution (GIBCO, Santa Clara, CA) were subsequently centrifuged onto each well. The supernatant was then removed and growth medium containing 20 mM HEPES was added. Approximately 3×10^4 cells adhered to each well by this method. Cells were then warmed to 37°C and treated with various stimuli. Protein kinase C activity was subsequently measured by examining phosphorylation of the VRKRTLRRL peptide, which is derived from the major phosphorylation site of the epidermal growth factor receptor (13), as described by Heasley and Johnson (4). HPLC-purified peptide was first generously provided by Dr. Gary Johnson (National Jewish Hospital, Denver, CO) and later synthesized for us by the Biomolecular Resource Center (University of CA, San Francisco, CA). Briefly, the growth medium of stimulated cells was removed by aspiration and replaced with 40 μl reaction mix (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 1 mg/ml glucose, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM $CaCl_2$, 20 mM HEPES, pH 7.2, 50 μg/ml digitonin, 100 μM ATP, 50 μM peptide, 187.5 μCi/ml γ[^{32}P]-ATP (10 mCi/ml, >2000 Ci/mmol, Amersham), 1 mM phenylmethylsulphonylfluoride, and 5 μg/ml leupeptin). The concentration of free Ca^{2+} in the reaction mix varied between 100 and 300 nM from batch to batch of the reaction mix. Reactions proceeded for 5 min at 30°C in the microtiter wells and were terminated by addition of 10 μl 25% (w/v) trichloroacetic acid. 45 μl of each reaction were then spotted onto 2 cm x 2 cm squares of phosphocellulose

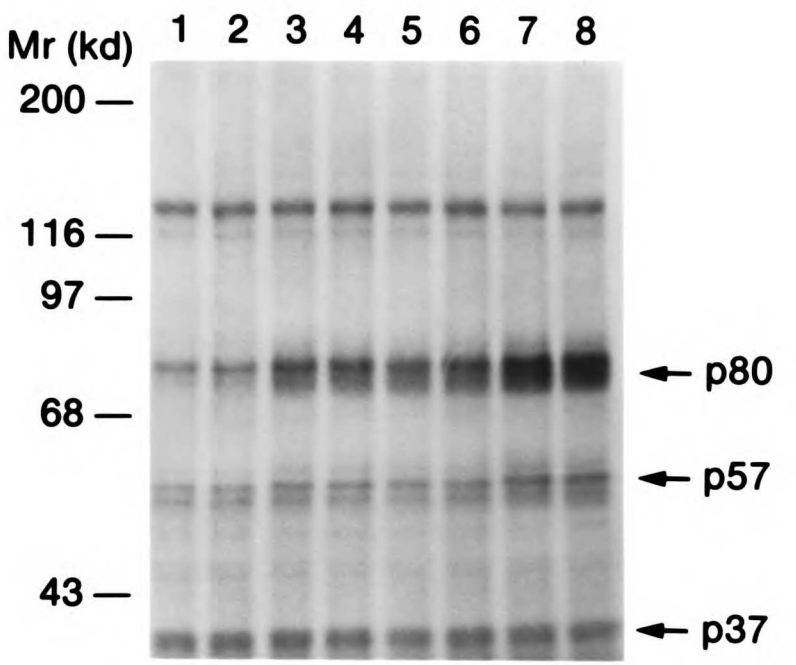
paper (Whatman P-81). The squares were immediately washed in four changes (500 ml, 2 min each) 75 mM phosphoric acid, one change (500 ml, 5 min) 75 mM sodium phosphate (pH 7.5), and one more change (500 ml, 2 min) 75 mM phosphoric acid. The squares were air-dried, and incorporation of ^{32}P into the peptide, which was bound to the squares, was determined by liquid scintillation counting. Spurious incorporation of ^{32}P into the peptide was determined by running reactions with no cells present.

RESULTS AND DISCUSSION

Protein kinase C activity in WEHI-231 cells was first measured by assaying for changes in the phosphorylation of the endogenous 80-87 kd heat-stable protein. When WEHI-231 cells were treated with anti-IgM or PdBu, phosphorylation of heat-stable proteins of M_r ~80,000 increased dramatically (Fig. 1). In unstimulated cells, a phosphorylated protein of M_r 80,000-85,000 was readily apparent. In stimulated cells, this protein exhibited increased phosphorylation, and a phosphorylated protein of M_r 75,000-80,000 also appeared. As these two bands differ very little in migration rate, they probably represent differently phosphorylated forms of the 80-87 kd heat-stable protein that has been previously described to be a substrate for protein kinase C (5, 7). Thus, phosphorylation of both of these proteins was examined, as both increased their phosphorylation in response to anti-IgM or to PdBu. Several other phosphorylated heat-stable proteins were also apparent in WEHI-231 cells, some of which did (M_r 57,000) or did not (M_r 37,000) exhibit changes in phosphorylation upon treatment with anti-IgM or PdBu (Fig. 1). The increase in phosphorylation of the 80 kd proteins could readily be measured by excising the relevant bands from the gel and determining the amount of ^{32}P incorporated. Since the phosphorylation of the 37 kd protein was not responsive to cellular activation, the incorporation of ^{32}P into this protein was used as a control to normalize for spurious differences in cell recovery between samples.

The kinetics of the phosphorylation of the 80 kd proteins in WEHI-231 cells treated with anti-IgM or PdBu was determined. The data from eight experiments are summarized in Fig. 2. The phosphorylation of the 80 kd proteins induced by 5 to 30 min of treatment with anti-IgM was best mimicked by 2-4 nM PdBu in each of these experiments. In general, however, the phosphorylation induced by 2 nM PdBu was closest to that induced by anti-IgM. Ionomycin (250 nM) did not increase the phosphorylation of the 80 kd proteins (102 $\pm$ 5% stimulation versus untreated controls, mean $\pm$ range, n=2). Also, the combination of 250 nM ionomycin and 4 nM PdBu did not induce greater phosphorylation

Figure 1. Effect of anti-IgM or PdBu on the phosphorylation of heat-stable proteins. Duplicate samples of ^{32}P-labelled WEHI-231 cells were treated for 15 min with buffer (lanes 1 and 2), 5 μg/ml of the monoclonal anti-IgM Bet1 (lanes 3 and 4), 4 nM PdBu (lanes 5 and 6), or 20 nM PdBu (lanes 7 and 8). Cellular proteins were then boiled and soluble, heat-stable proteins were analyzed by electrophoresis on SDS-containing 8% polyacrylamide gels. Labelled proteins were visualized by autoradiography. The positions of the molecular weight standards are shown at the left. The arrows indicate the positions of heat-stable proteins of M_r 80,000, 57,000 and 37,000. In this experiment, the ratio of incorporation of ^{32}P into the 80 kd proteins and into the 37 kd protein was 1.10 ± 0.01, 1.73 ± 0.03, 1.88 ± 0.07, and 2.87 ± 0.21 (mean $\pm$ range of duplicate samples) for treatment with buffer, anti-IgM, 4 nM PdBu, and 20 nM PdBu, respectively.


Mr (kd)
1 2 3 4 5 6 7 8
200
116
97
68
43
p80
p57
p37

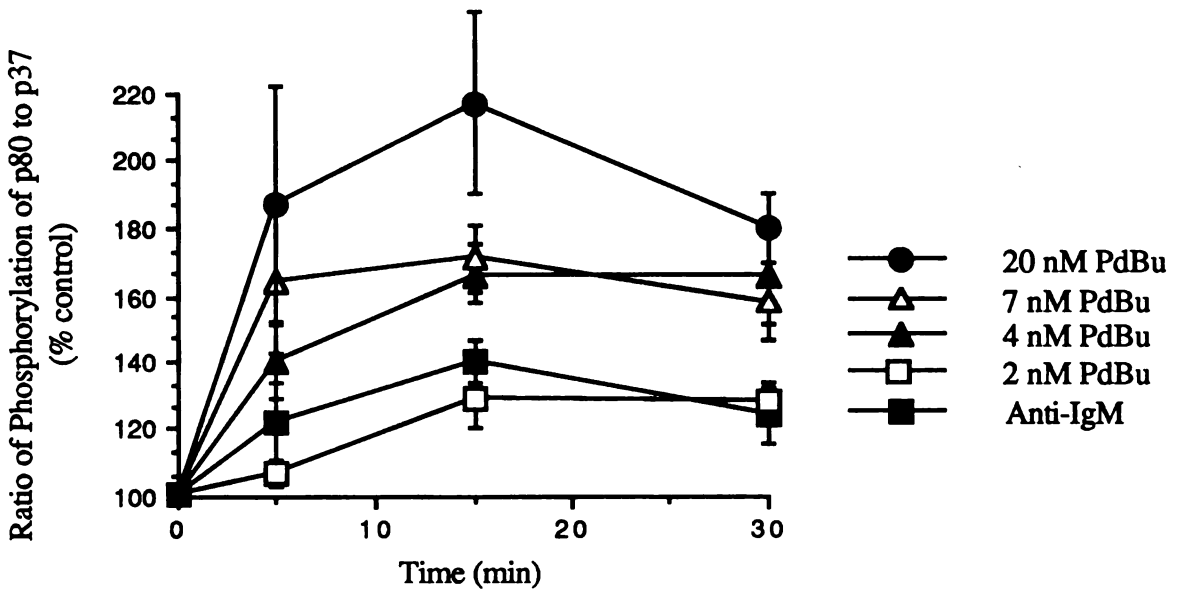


Figure 2. Kinetics of the phosphorylation of proteins of M_r ~80,000 induced by anti-IgM or PdBu. ^{32}P-labelled WEHI-231 cells were treated for various lengths of time with the indicated stimuli, and heat-stable, phosphorylated proteins were analyzed as described in the legend to Fig. 1. Proteins of ~80 kd and 37 kd were excised from the gels, and their incorporation of ^{32}P was determined. The results are presented as the ratio of phosphorylation of p80 to p37 relative to untreated controls, which is 100% x [cpm (p80 of stimulated cells)/cpm (p37 of stimulated cells]/[cpm (p80 of unstimulated cells)/cpm (p37 of unstimulated cells)]. Each timepoint had an untreated control. These data are drawn from eight experiments, and each point represents the mean ± SEM of 2-7 determinations.

of the 80 kd proteins than that obtained with 4 nM PdBu alone (ratio of stimulation with ionomycin and PdBu to stimulation with PdBu alone was 1.06 ± 0.02, mean ± range, n=2). Since 250 nM ionomycin causes a calcium increase in WEHI-231 cells similar to that induced by anti-IgM (1, i.e. Chapter 1 of Ph. D. Thesis), these results suggest that phosphorylation of the 80 kd heat-stable proteins is not sensitive to changes in calcium caused by antigen-receptor stimulation. These results also suggest that the increased levels of calcium induced by anti-IgM do not synergize with diacylglycerol to activate protein kinase C in these cells.

Protein kinase C activity was next assayed in permeabilized WEHI-231 cells by stimulating them with anti-IgM or PdBu for various lengths of time, permeabilizing them with digitonin, and examining phosphorylation of exogenously added VRKRTLRRL substrate peptide. Protein kinase C activation induced by anti-IgM could not be detected in this assay (Table I). Although 4 nM PdBu sometimes caused increased phosphorylation of this peptide, at least 7 nM PdBu was generally required for reliable detection of peptide phosphorylation. Since 80 kd protein phosphorylation could be readily detected with only 2 nM PdBu (Fig. 2), the peptide phosphorylation assay appears to be less sensitive to protein kinase C activation than the 80 kd protein phosphorylation assay, at least in these cells. There are several possible explanations for this phenomenon. First, the conditions in permeabilized cells may not reproduce the normal cellular environment very well, which could cause protein kinase C to require more phorbol diester/diacylglycerol in order to be activated. Second, protein kinase C is actually a family of several isozymes (14). The peptide is likely a substrate for many or all of the protein kinase C isozymes present in WEHI-231 cells. In contrast, the 80 kd protein may be phosphorylated in response to a subset of the protein kinase C isozymes, which could affect the relative sensitivity. Third, protein kinase C may prefer the 80 kd protein as a substrate over the exogenously added peptide because of the amounts or cellular localizations of these substrates. Finally, it is possible that the 80 kd protein is being phosphorylated by a different protein kinase. This

Table I

Use of a synthetic peptide to examine

protein kinase C activity in WEHI-231 cells

	Length of Incubation					
	2 minutes		5 minutes		10 minutes	
Stimulus	kcpm[a]	% stimulation[b]	kcpm	% stimulation	kcpm	% stimulation
Media	51 ± 0	100	53 ± 0	100	58 ± 3	100
Anti-IgM	55 ± 2	111	55 ± 0	107	57 ± 3	97
4 nM PdBu	57 ± 1	118	65 ± 6	134	64 ± 2	115
7 nM PdBu	63 ± 4	133	72 ± 1	153	80 ± 1	152
20 nM PdBu	78 ± 1	179	96 ± 3	219	86 ± 4	166

[a] WEHI-231 cells (1 x 10^5/ml) were cultured in media with 5 μg/ml of the monoclonal anti-IgM Bet1 or the indicated concentrations of PdBu for 2, 5 or 10 min. Cells were then permeabilized and protein kinase C activity was measured by incorporation of ^{32}P into the VRKRTLRRL peptide, as described in Materials and Methods. The results are presented as the incorporation of ^{32}P into the peptide induced by stimulation of duplicate cultures (mean ± range). This experiment is representative of four similar experiments.

[b] % stimulation is 100% x [cpm (stimulus) - cpm (no cells)]/[cpm (media) - cpm (no cells)]. The incorporation of ^{32}P into the peptide with no cells present was 16 ± 1 kcpm (mean ± range, n=2).

is unlikely, however, since an 80-87 kd heat-stable protein has been shown to be a substrate for protein kinase C in many other cells and tissues (5, 7, 11).

The results obtained from these two assays indicate that doses of PdBu of 7 nM activate protein kinase C more than does anti-IgM stimulation of WEHI-231 cells. Doses of 2-4 nM PdBu more closely mimicked the anti-IgM-induced phosphorylation of heat-stable 80 kd proteins in these cells. These data agree quite well with the results obtained in assays of the biological effect of PdBu on WEHI-231 cells. In the biological assays, the combination of ionomycin and doses of PdBu greater than 7 nM caused cellular responses not seen with anti-IgM (1, i.e. Chapter 1 of Ph. D. Thesis). Correspondingly, those doses of PdBu activated protein kinase C more than did anti-IgM in these two biochemical assays of protein kinase C activation.

Hornbeck and Paul (9) originally showed that treatment of WEHI-231 cells with anti-IgM caused phosphorylation of a series of proteins. These proteins were found to be a subset of the proteins that were phosphorylated in response to treatment of the cells with phorbol diester. One of these proteins was recently identified as the 80-87 kd heat-stable protein, which migrated at 68 kd under their electrophoretic conditions (10). These results strongly suggest that anti-IgM causes protein kinase C activation in these cells. Sarthou, et al. (15) have also examined protein kinase C activation in WEHI-231 cells by assaying for translocation of the enzyme from the cytosol to the membrane. They failed to observe significant translocation of protein kinase C and concluded that anti-IgM does not activate this enzyme in WEHI-231 cells. However, their method of analysis would probably not detect low levels of protein kinase C activation, since at least 1.6 nM phorbol myristate acetate was required to induce detectable translocation. WEHI-231 cells are approximately 30-fold more sensitive biologically to phorbol myristate acetate than to PdBu (Gold, Fahey and DeFranco, unpublished results), implying that at least 48 nM PdBu would be required to detect protein kinase C translocation in their system. Thus, their system clearly lacked sufficient sensitivity to detect partial activation of protein kinase C. In our experiments,

phosphorylation of heat-stable 80 kd proteins in WEHI-231 cells was readily detected with only 2 nM PdBu, and phosphorylation of a synthetic peptide was detected with 7 nM PdBu. Anti-IgM also clearly induced phosphorylation of heat-stable 80 kd proteins. This observation combined with the demonstration that anti-IgM causes production of diacylglycerol in these cells (as measured by its subsequent conversion to phosphatidic acid, 16) provide compelling evidence that anti-IgM induces protein kinase C activation in WEHI-231 cells, albeit to a limited extent. Of course, it is still possible that phosphorylation of the 80 kd proteins induced by anti-IgM is due to another protein kinase. This possibility is unlikely, however, since depletion of protein kinase C by prolonged treatment of WEHI-231 cells with phorbol myristate acetate ablated the ability of anti-IgM to stimulate phosphorylation of the 80-87 kd heat-stable protein (9, 10).

The results presented here indicate that anti-IgM activates protein kinase C in WEHI-231 cells and that doses of PdBu from 2-4 nM activate protein kinase C to a similar extent. This result confirms the validity of using PdBu to assess the role of diacylglycerol in mediating the growth inhibitory effects of anti-IgM on WEHI-231 cells (1, i.e. Chapter 1 of Ph. D. Thesis). In those experiments, it was shown that 250 nM ionomycin and anti-IgM induce similar increases in cytosolic calcium. Correspondingly, treatment of WEHI-231 cells for 24 hr with 250 nM ionomycin and 2-4 nM PdBu inhibited their growth by approximately 30-60% as compared to the 90-95% inhibition seen upon treatment with anti-IgM (1, i.e. Chapter 1 of Ph. D. Thesis). These results suggest that the natural second messengers calcium and diacylglycerol are only causing 30-60% of the growth arrest caused by anti-IgM. Although it is possible that ionomycin and PdBu cannot precisely mimic the actions of calcium and diacylglycerol, there is no evidence in favor of this formal possibility. Thus, these data strongly imply that there are other second messengers involved in mediating the effects of anti-IgM on WEHI-231 cells.

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Chapter Three:

Antigen receptor-induced cell cycle arrest in WEHI-231 B lymphoma cells depends on the duration of signalling prior to the G_1 phase restriction point

ABSTRACT

Stimulation of antigen receptors on WEHI-231 B lymphoma cells with anti-receptor antibodies (anti-IgM) causes irreversible growth arrest. This may be a model for antigen-induced tolerance to self components in the immune system. Antigen receptor stimulation also causes inositol phospholipid hydrolysis, producing diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate, which causes release of calcium from intracellular stores. We examined the growth arrest of WEHI-231 cells synchronized in various phases of the cell cycle in response to anti-IgM or to the combination of phorbol diester and calcium ionophore. These mimicking reagents were used at doses which approximated the protein kinase C activation and calcium increases caused by anti-IgM. Treatment with anti-IgM or the mimicking reagents did not lengthen the cell cycle. Instead, growth inhibition was solely the result of arrest in G_1 phase. The efficiency of G_1 arrest increased with the length of time during which the cells received signalling before reaching the G_1 phase arrest point. Maximum efficiency of arrest required approximately one full cell cycle of receptor signalling. These results imply that anti-IgM causes G_1 arrest of WEHI-231 cells by slowly affecting components required for S phase progression, rather than by rapidly inhibiting such components or by rapidly activating a suicide mechanism. Antigen receptor stimulation was twice as effective as stimulation via the mimicking reagents phorbol dibutyrate and ionomycin. Thus, although diacylglycerol and calcium probably play roles in mediating the effects of anti-IgM on WEHI-231 cells, other second messengers may also be involved.

INTRODUCTION

The antigen receptor of B lymphocytes, membrane immunoglobulin (mIg), plays an important role in regulating B cell growth. Crosslinkage of mIgM with anti-IgM antibodies causes mature, resting B cells to enter the G_1 phase of the cell cycle (9) and to become more responsive to growth factors produced by helper T cells (18). In contrast, anti-IgM causes irreversible inactivation of immature B cells. This negative response probably represents one mechanism by which tolerance to self components is achieved (29).

The murine B lymphoma cell line WEHI-231 has been extensively used to study antigen receptor regulation of B cell growth (25, 16, 17, 35, 13, 27). Anti-IgM causes irreversible growth inhibition of WEHI-231 cells (33, 4, 17). After 24 hr of treatment with anti-IgM, most of the cells have arrested in the G_1 phase of the cell cycle (35), and the cell volume has decreased to less than half that of untreated cells (14). Interestingly, the cells still exclude the dye trypan blue at this time; thus, G_1 arrest occurs before cell death. After 48 hr of treatment, however, the cells are no longer viable (17). This cell line appears to be a transformed derivative of an immature B cell, in that it has the surface phenotype and biological response expected of this developmental stage (8).

Stimulation of mIg on mature B cells (3, 7) and on WEHI-231 cells (11) induces hydrolysis of inositol phospholipids. Thus, the B cell antigen receptor is a member of a large family of receptors, including the T cell antigen receptor and many hormone receptors, which transmit signals across the cell membrane via breakdown of inositol phospholipids (40, 1). Hydrolysis of phosphatidylinositol 4,5-bisphosphate yields the second messengers diacylglycerol, which activates protein kinase C (28), and inositol 1,4,5-trisphosphate, which causes release of calcium from intracellular stores (2, 20). The importance of diacylglycerol and calcium in mediating the effects of anti-IgM on B cells has been examined by mimicking the actions of these second messengers with pharmacologic agents. WEHI-231 cells were treated with phorbol diesters and calcium ionophores such that protein kinase C was activated and intracellular free calcium ($[Ca^{2+}]_i$) was increased to

an extent similar to that occurring following treatment of the cells with anti-IgM (31, i.e. Chapter 1 of Ph. D. Thesis, and Chapter 2 of Ph. D. Thesis). The combination of these mimicking reagents inhibited the growth of WEHI-231 cells, although not as effectively as did anti-IgM (31, i.e. Chapter 1 of Ph. D. Thesis). Many of the effects of anti-IgM on mature B cells can also be reproduced with phorbol diesters and calcium ionophores (26, 34, 19, 32). These results suggest that diacylglycerol and calcium play roles in mediating the effects of anti-IgM on B cell growth, but that other second messengers may also be involved.

In order to gain more insight into how antigen receptor signalling regulates B cell growth, we set out to determine the length of signalling via the antigen receptor or via phorbol diester plus calcium ionophore that is required to arrest the growth of WEHI-231 cells. It had previously been shown that these cells arrest in the G_1 phase of the cell cycle (35). We wished to determine whether cells that had received only short periods of signalling (~2 hr) before entering G_1 phase would arrest, or whether longer periods of signalling were required. The former result would suggest that the growth arrest is due to the action of a quickly activated suicide mechanism (38), or to rapid inhibition of a component required for cell cycle progression. In contrast, a requirement for long periods of signalling would suggest that the growth arrest is due to slow increases or decreases in a component which regulates cell cycle progression, perhaps via a change in the rate of synthesis of such a component. In the experiments reported here, we found that the efficiency of anti-IgM-induced cell cycle arrest increases with the length of signalling received prior to arrival at the potential arrest point in G_1 phase. Maximum efficiency was not achieved until at least one full cell cycle of signalling had occurred. Furthermore, growth arrest mediated by the antigen receptor was approximately twice as efficient as that caused by phorbol diester plus calcium ionophore. These results confirm and extend our previous observations that these mimicking reagents cannot fully reproduce the effects of anti-IgM on WEHI-231 cells (31, i.e. Chapter 1 of Ph. D. Thesis). Thus, diacylglycerol

and calcium may not be the only second messengers which contribute to the growth arrest of WEHI-231 cells.

MATERIALS AND METHODS

Reagents. Phorbol 12,13-dibutyrate (PdBu) was obtained from Sigma Chemical Co. (St. Louis, MO) and was stored at -20°C. Ionomycin (calcium salt, stored at -80°C in DMSO), colcemid, propidium iodide, and 5-bromo 2'-deoxyuridine (BrdUrd) were obtained from Calbiochem, La Jolla, CA. The concentration of ionomycin solutions was determined by measurement of OD at 300 nm in methanol, under which conditions the extinction coefficient is 21,600 M^{-1} cm^{-1} (23). The rat monoclonal anti-mouse IgM Bet1 (hybridoma provided by Dr. J. Kung, University of Texas Health Sciences Center, San Antonio, TX) was purified from culture fluids by affinity chromatography with MOPC104E (IgM)-Sepharose.

Cell culture. The murine B lymphoma cell line WEHI-231 (39) (from Dr. N. Warner, Becton Dickinson, Mountain View, CA) was cultured in RPMI-1640 (M.A. Bioproducts, Walkersville, MD, or Cell Culture Facility, University of CA, San Francisco) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol at 37°C in an atmosphere containing 5% CO_2. Some cultures also contained either 25-50 U/ml penicillin plus 25-50 μg/ml streptomycin or 20-50 μg/ml gentamicin. Cells were periodically screened for *Mycoplasma* contamination (Mycotect Kit, GIBCO, Santa Clara, CA) and were found to be *Mycoplasma*-free. Cells used in experiments were obtained from cultures in the exponential phase of growth with a density below 5 x 10^5/ml.

Cell volume analysis. WEHI-231 cells (1 x 10^5/ml) were cultured with various reagents in a volume of 0.5-1.0 ml in 24-well flat-bottomed plates (Costar, Van Nuys, CA). 10,000 cells/sample were subsequently analyzed with a Coulter Counter and Channelyzer (Coulter Electronics, Hialeah, FL) as previously described (14).

Cell cycle status. The cell cycle status of WEHI-231 cell populations was determined by two-parameter flow cytometric analysis of total DNA content and recently incorporated BrdUrd (10). Briefly, 5-10 ml of cells at 1 x 10^5/ml were cultured for various lengths of

time, and 15 μM BrdUrd was added to the cells during the final 0.5 hr of culture. The cells were then pelleted by centrifugation, fixed by addition of 2.5-5.0 ml of cold 70% ethanol, and stored in the dark at 4°C until staining, which could be done up to 3 wk later. All incubations during the staining were performed at room temperature in the dark. 0.5-1.0 x 10^6 fixed cells were collected by centrifugation and resuspended in 1 ml of 2.5 M HCl containing 0.5% Triton X-100. After incubation for 25 min, 2.5 ml of wash buffer (0.5% Tween-20 in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline) were added to each sample, which was then centrifuged and washed twice more. Next, each sample was incubated for 30 min with 70 μl incubation buffer (wash buffer containing 1.5% Carnation Nonfat Dry Milk, w/v) containing 20 μl of the mouse anti-BrdUrd monoclonal antibody (clone B44, Becton Dickinson, Mountain View, CA) per 1 x 10^6 cells. After two washes, each sample was incubated with 3 μg of fluorescein-conjugated goat anti-mouse IgG (Fc fragment-specific, Jackson Immunoresearch Laboratories, West Grove, PA) in 0.3 ml incubation buffer for 20-30 min. The samples were subsequently washed twice, incubated in 1 ml of phosphate-buffered saline containing 10 μg/ml propidium iodide for 30-60 min, and then analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Fractions of cells in G_1, S, and G_2+M phases were determined by integration of the relevant areas of a two-dimensional plot of fluorescein fluorescence versus propidium iodide fluorescence.

Centrifugal elutriation. WEHI-231 cells in different phases of the cell cycle were isolated according to their cell volume by centrifugal elutriation at 20°C in a JE-6 elutriator rotor using a 4.5 ml elutriation chamber (Beckman Instruments, Inc., Palo Alto, CA). The cells were first collected by centrifugation and resuspended at 2 x 10^6/ml in growth medium containing 2% fetal bovine serum. 2.5-4.0 x 10^8 cells were loaded into the elutriation chamber using a centrifugal speed of 2000 RPM and a flow rate of 13 ml/min, which was maintained with a Master Flex pump (Cole-Parmer Instrument Co., Chicago, IL). Fractions were subsequently obtained by increasing the flow rate of growth

medium/2% fetal bovine serum and collecting the first 100 ml coming from the elutriation chamber. 50 ml were then eluted and discarded before collecting the next fraction. Cells that were predominantly in G_1, early S, late S, and G_2+M phases were eluted with flow rates of 16-20, 21-22, 23-26, and 33-37 ml/min, respectively, and had median cell volumes of 390-470, 530-540, 610-645, and 920-1000 μm^3, respectively.

Calculations. The time required for 50% of the elutriated cells in each fraction to undergo mitosis was determined as follows. Cell cycle length in these experiments was estimated to be 12 hr (see Results section). The length of each cell cycle phase was then calculated from the fraction of unsynchronized WEHI-231 cells in the various cell cycle phases. For example, from the data in Table I for untreated WEHI-231 cells, the lengths of G_1, early S, late S, and G_2+M phases are 2.1, 5.3, 3.5, and 1.1 hr, respectively. Finally, using these values for the lengths of the different phases, the length of time required for 50% of the cells in each fraction to divide was determined by calculating how far from mitosis the 50th percentile cell was. For this purpose, the cells within each cell cycle phase were assumed to be equally distributed throughout that phase. For example, for fraction A+B (Table I, untreated cells) the 50th percentile cell was located in early S phase 6.75 hr after elutriation. Thus, the time at which this cell would divide was calculated as follows: 6.75 hr (=length of time already passed) + 1.1 hr (=length of G_2+M phase) + 3.5 hr (=length of late S phase) + {[50% - (26% + 2%)]/59% x 5.3 hr} (=length of early S phase remaining for 50th percentile cell) = 13.3 hr (data are from Table I, untreated cells from Fraction A+B after 6.75 hr). As these calculations could be made from data obtained at several different times, a range of times required for 50% of the cells to divide was calculated for each fraction. Since experimentally-treated cells progressed through the cell cycle at the same rate as untreated cells (see Results section), the data for untreated cells were used for these calculations.

RESULTS

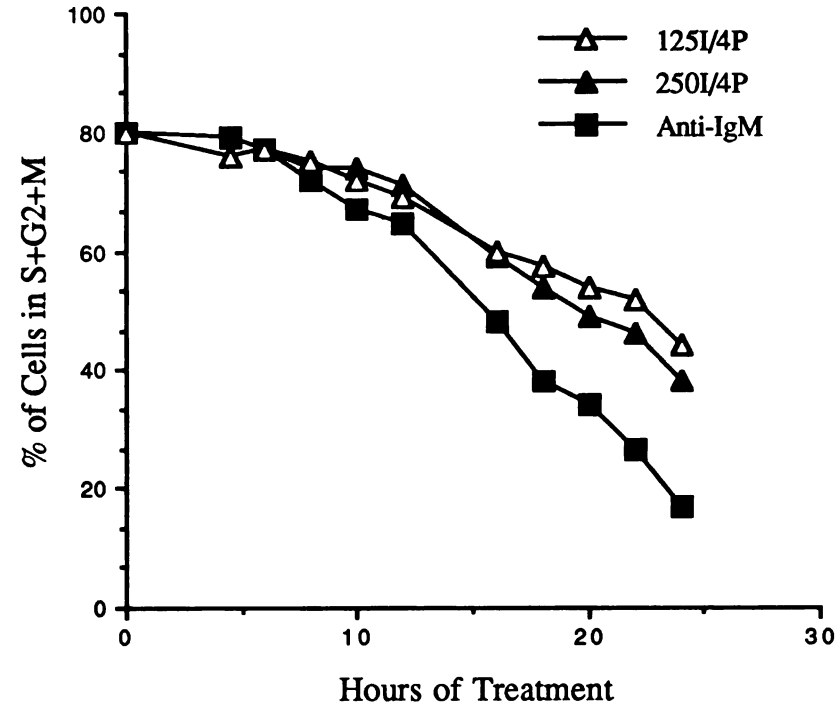
Two approaches were used to determine the length of signalling via the antigen receptor that is required to arrest WEHI-231 cells in the G_1 phase of the cell cycle. First, detailed kinetic analyses of the growth inhibition of unsynchronized cells were performed. Second, WEHI-231 cells synchronized in various phases of the cell cycle were isolated, and the onset of growth arrest was determined.

Time course of growth inhibition. It had previously been shown that approximately 90% of WEHI-231 cells are inhibited in the incorporation of [^{3}H]thymidine into their DNA by 24 hr of treatment with anti-IgM (17). In contrast, antibodies directed against cell surface structures other than mIgM (e.g. major histocompatibility proteins) do not cause growth arrest of these cells (4). Although the inhibition of thymidine incorporation clearly reflects inhibition of DNA synthesis, it could also be influenced by rates of thymidine uptake, metabolism, and biosynthesis. Thus, in order to monitor the growth inhibition of these cells more precisely, cell volume decreases and G_1 arrest were examined. These responses were found to be more sensitive indicators than [^{3}H]thymidine uptake, especially at the early stages of growth inhibition. At least 6 hr of treatment with anti-IgM were required before the arrest of cells in G_1 phase became apparent (Fig. 1A). Half-maximal G_1 arrest was achieved at 17.7 $\pm$ 0.5 hr (mean $\pm$ SEM, n=3). The cell volume decrease also followed a similar time course (Fig. 1B). This decrease, however, is due not only to G_1 arrest, but also to another cellular response, as the cells become even smaller than cells which are in G_1 phase (31, i.e. Chapter 1 of Ph. D. Thesis). Since the doubling time of WEHI-231 cells is 12-14 hr (unpublished results of Gold & DeFranco, and see below), these data imply that many of the cells do not arrest until they have received one full cell cycle of signalling via the antigen receptor before reaching the G_1 arrest point.

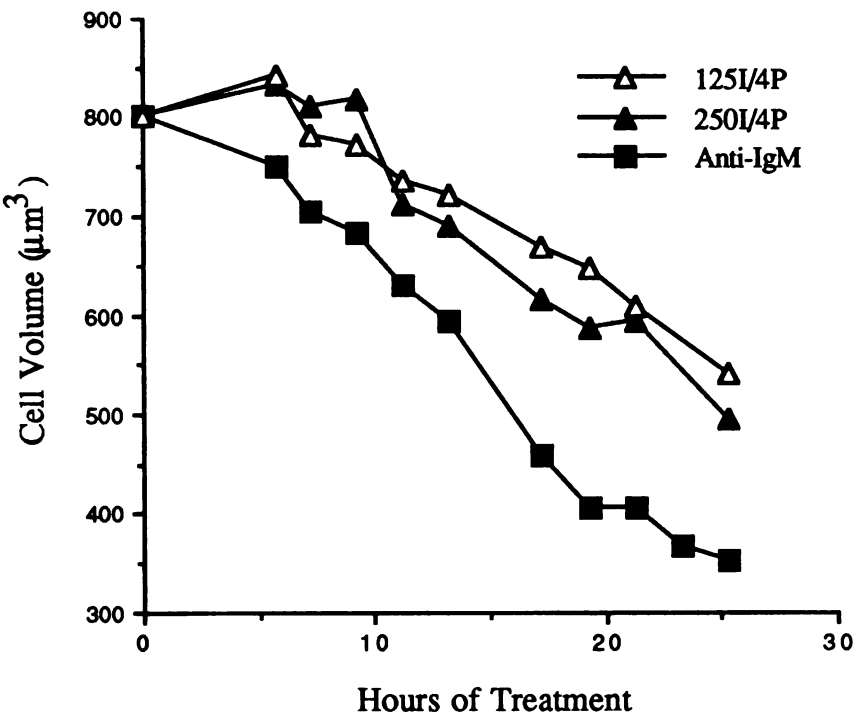
The effect of calcium ionophore and phorbol diester on growth arrest and cell size was also examined. Two doses of these mimicking reagents were used: a dose combination which was previously determined to approximate the increase in $[Ca^{2+}]_i$ and the activation

Figure 1. Kinetics of the cell volume decrease and G_1 arrest of WEHI-231 cells treated with anti-IgM or ionomycin plus PdBu. Cells at 10^5/ml were cultured in growth medium for 25 hr. 2 μg/ml of the monoclonal anti-IgM Bet1, 250 nM ionomycin plus 4 nM PdBu (250I/4P), or 125 nM ionomycin plus 4 nM PdBu (125I/4P) were added at times 0, 2, 4, 6, 8, 12, 14, 16, 18, or 20 hr of culture; cell volume and cell cycle position were then analyzed at 24 hr of culture. The cell volume histograms of the cells were determined using a Coulter Counter and Channelyzer. Cell cycle position was assessed by adding BrdUrd for 30 min to a parallel set of cultures and processing the samples as described in Materials and Methods. The experiment shown is representative of three experiments done with anti-IgM and 250I/4P and two done with 125I/4P. A) Results are presented as % of cells in $S+G_2+M$ phases. The % of untreated cells in these phases was 80 ± 2% (mean ± SD, n=6; control additions of media made at times 0, 4, 8, 12, 16, and 20 hr). B) Results are presented as median cell volume. The median cell volume of the untreated cells was 802 ± 18 μm^3 (mean ± SD, n=6; control additions of media made at times 0, 4, 8, 12, 16, and 20 hr).

A


125I/4P
250I/4P
Anti-IgM
% of Cells in S+G2+M
100
80
60
40
20
0
0
10
20
30
Hours of Treatment

B


125I/4P
250I/4P
Anti-IgM
Cell Volume (μm3)
900
800
700
600
500
400
300
0
10
20
30
Hours of Treatment

of protein kinase C induced by treatment with anti-IgM (250 nM ionomycin plus 4 nM PdBu; 31 and D. M. Page, Chapter 2 of Ph. D. Thesis) and a combination with a slightly lower dose of ionomycin for comparison (125 nM ionomycin plus 4 nM PdBu). The combination of 250 nM ionomycin and 4 nM PdBu caused less of a cell volume decrease and less of a G_1 arrest than did antigen receptor signalling at all times of treatment (Fig. 1). This result is consistent with previous data showing that these doses of PdBu and ionomycin are also less efficient at causing growth arrest after 24-48 hr of treatment (31, i.e. Chapter 1 of Ph. D. Thesis). Half-maximal G_1 arrest was achieved at 22.6 ± 1.0 hr (mean $\pm$ range) in two experiments, and in the third experiment, half-maximal G_1 arrest was still not quite achieved by 24 hr of treatment. Thus, these reagents are at least 5 hr slower than anti-IgM at inducing cell cycle arrest. The combination of 250 nM ionomycin and 4 nM PdBu also lagged 6.0 ± 1.0 hr (mean $\pm$ SEM, n=3) behind anti-IgM at inducing half of the cell volume decrease obtained by 24 hr of treatment with anti-IgM. Treatment of the cells with 125 nM ionomycin plus 4 nM PdBu was even less effective at inducing cell cycle arrest and cell volume decreases (Fig. 1). Thus, many of the cells treated with the mimicking reagents did not arrest until they had received nearly two cell cycles of this type of signalling.

Growth inhibition of synchronized cells. WEHI-231 cells treated with anti-IgM exhibited a lag of 6 hr before G_1 arrest became apparent (Fig. 1A). This result would be observed if the cells required a certain period of signalling in order for growth arrest to occur. Alternatively, if anti-IgM treatment lengthened the cell cycle, a lag would also be observed before cells began to arrest. To distinguish between these and other possibilities, we isolated WEHI-231 cells in different phases of the cell cycle and treated them with anti-IgM. The effect of this treatment on the rate at which the cells progressed through the cell cycle and the extent to which they arrested in G_1 phase was then determined.

We had previously determined that cell volume corresponds to position in the cell cycle for this cell line (data not shown, but see below). Thus, we used centrifugal elutriation, a

technique which allows for separation of cells by their cell volume, to isolate WEHI-231 cells from different phases of the cell cycle. The cell volume histograms of typical elutriated populations are shown in Fig 2. Cells from fractions A and B were small in comparison to the normal population and were shown to be mostly in the G_1 phase of the cell cycle (Table I, 0.5 hr analysis). Cells from fractions C, D, and E were progressively larger and correspondingly more advanced in the cell cycle, i.e. predominantly in early S, early and late S, and late S and G_2+M phases, respectively (Table I, 0.5 hr analyses). Thus, it was possible to obtain WEHI-231 cell populations that were concentrated in particular phases of the cell cycle.

The elutriated cell populations were treated with anti-IgM or the mimicking reagents and were analyzed at various times to determine their cell cycle position. An example of the cell cycle analysis of one of these populations is shown in Fig. 3. Cells from G_1 phase-enriched fractions similar to the A and B fractions shown in Fig. 2 were cultured in growth medium alone (Fig. 3A) or in medium containing anti-IgM (Fig. 3B) for 19 hr. They were then analyzed for cell cycle position by fluorescence analysis of DNA content and recently incorporated BrdUrd. When the data were represented as rate of DNA synthesis (anti-BrdUrd fluorescence) versus total DNA content (propidium iodide fluorescence), four main and two minor populations of cells could be distinguished. The four main populations were those in G_1 phase (box 1), early S phase (box 2), late S phase (box 3), and G_2+M phases (box 4). The distinction between early S phase and late S phase was arbitrarily set near the middle of the propidium iodide fluorescence. For untreated cells, these four populations generally comprised greater than 97% of the total cell population. For anti-IgM- and mimicking reagent-treated cells, a small population of cells with less than unit DNA content appeared (box 5; called "<G_1 phase"). These cells are probably dying and degrading their DNA after arresting in G_1 phase. Sometimes, treatment for 18-24 hr with anti-IgM or the mimicking reagents or treatment with colcemid resulted in the appearance of a population of cells that had S phase DNA content but did not incorporate BrdUrd (box 6;

Figure 2. Cell volume histograms of elutriated WEHI-231 populations. WEHI-231 cells were fractionated by centrifugal elutriation as described in Materials and Methods. The cell volume histograms of the starting (dashed line) and elutriated (solid lines) populations were determined using a Coulter Counter and Channelyzer, and each histogram represents 10,000 cells. The median cell volume of the starting population was 608 μm^3. Fractions A-E (labelled solid lines) were elutriated with flow rates of 16, 18, 21, 23, and 33 ml/min, respectively, and had median cell volumes of 413, 458, 540, 616, and 923 μm^3, respectively. Fractions of cells with median cell volumes between those of fractions D and E were also eluted with different flow rates, but they were not used in the subsequent analyses. Recovered in fractions A-E were 4.3%, 6.5%, 11.2%, 9.3%, and 5.8% of the starting population, respectively. Fractions A+B, C, D, and E were used as cell populations representing primarily G_1, early S, mid S, and G_2+M phases, respectively, and were subjected to the treatments described in Table I.

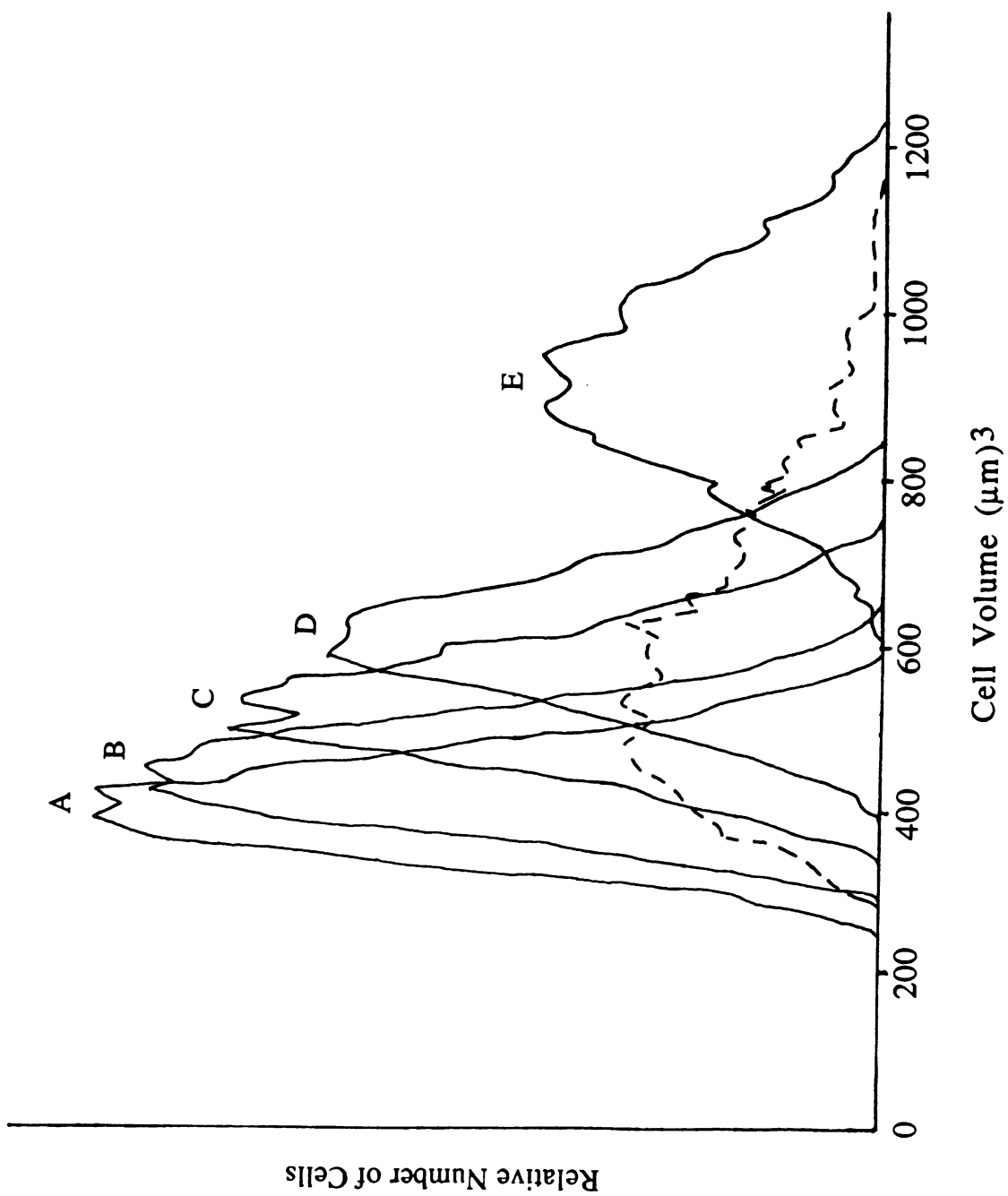
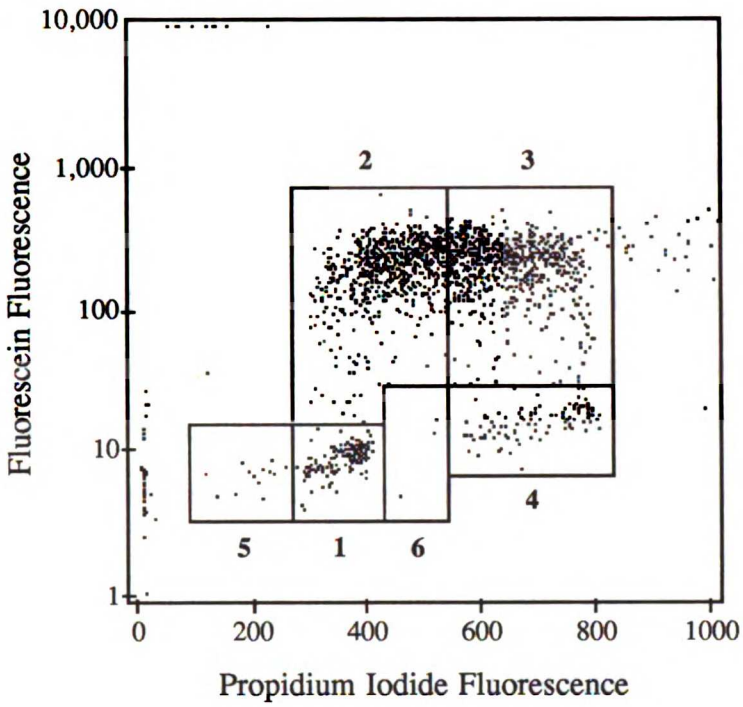

A
B
C
D
E
0
200
400
600
800
1000
1200
Cell Volume (µm)3
Relative Number of Cells

Figure 3. Cell cycle analysis of elutriated WEHI-231 cells. WEHI-231 cells were subjected to centrifugal elutriation, and two fractions were eluted which were similar to fractions A and B in Fig. 2. Their median cell volumes were 420 μm^3 and 473 μm^3, and they represented 3.7% and 5.9% of the starting population. These fractions were combined and cultured for 19.25 hr without (A) or with 2 μg/ml of the monoclonal anti-IgM Bet1 (B). BrdUrd was added during the final 30 min of culture, and cell cycle position was analyzed. Fluorescence data were collected for 10,000 cells, and BrdUrd uptake (fluorescein fluorescence) was plotted versus DNA content (propidium iodide fluorescence). Boxes 1-6 represent cells in G_1, early S, late S, G_2+M, <G_1, and non-BrdUrd-incorporating S, respectively. A) The % of untreated cells in boxes 1-6 are 9, 40, 43, 6, 1, and 1%, respectively. B) The % of anti-IgM-treated cells in boxes 1-6 are 67, 7, 10, 3, 11, and 1%, respectively.

A


10,000
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10
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Fluorescein Fluorescence
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1
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0
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400
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800
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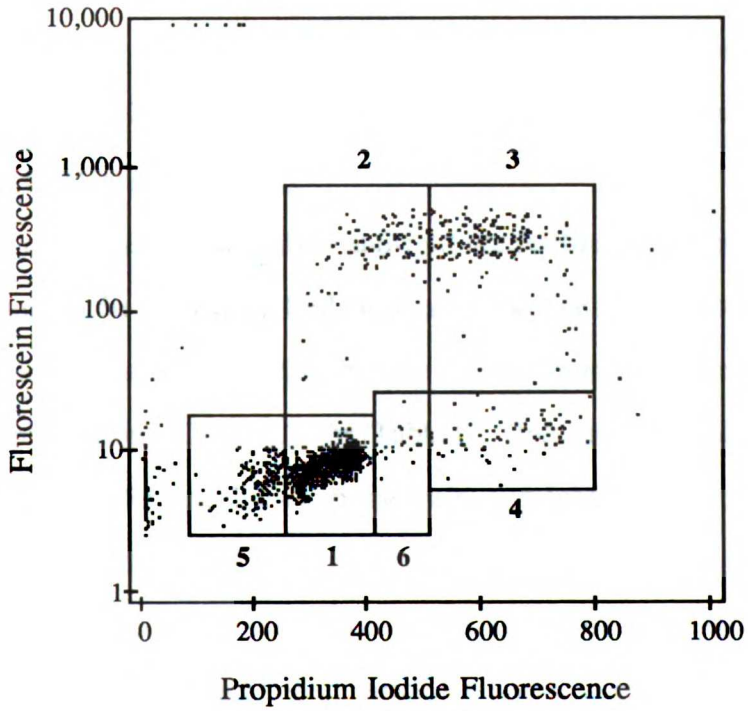
Propidium Iodide Fluorescence

B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Propidium Iodide Fluorescence

called "non-BrdUrd-incorporating S phase"). This population could account for up to 6% of the total population. These cells generally represented less than 2% of the total population, however, and were discounted unless they increased to more than 2%. Overall, the populations in boxes 1, 5, and 6 are designated in Table I as "G_1 + non-cycling," since growth arrested WEHI-231 cells are found in these states.

The elutriated populations shown in Fig. 2 were treated with anti-IgM or 250 nM ionomycin plus 4 nM PdBu and were analyzed for cell cycle progression over the next 24 hr (Table I). The elutriated cells exhibited a doubling time of 12 hr. This can be seen by comparing the cell cycle position of untreated cells at different time intervals. The proportions of the cells in the various cell cycle phases were almost exactly the same at 12 hr intervals (e.g. fraction C, 0.5 vs 12.5 hr and 6.75 vs 18.75 hr). The exception to this is fraction A+B. The cells from this fraction exited G_1 phase slowly in the first 7 hr after their isolation, but thereafter exhibited a doubling time of 12 hr. It is likely that this fraction contained some very small cells which had a long G_1 phase and were thus slow to enter S phase. Such cells are probably in an anomalous cell cycle state rather than a part of G_1 phase that all cells pass through, because the other elutriated populations exhibited a more rapid progress through G_1 phase after arriving at that stage (see data for untreated cells from fractions C, D, and E, Table I).

These data clearly show that neither anti-IgM nor PdBu and ionomycin slow down progression through the cell cycle. Cell cycle analysis of cells from each fraction revealed that the cells had similar cell cycle distributions whether or not they were treated with anti-IgM or PdBu plus ionomycin for 6.75 hr (Table I). Thus, treatment of WEHI-231 cells that were in several different cell cycle phases with anti-IgM or PdBu plus ionomycin did not lengthen their cell cycle, at least during the first 7 hr of incubation.

Cell cycle analysis of cells cultured for various lengths of time coupled with the use of colcemid, which arrests cells in M phase, made it possible to determine the proportion of cells that arrested in G_1 phase upon treatment with anti-IgM or the mimicking reagents.

Table I

Cell Cycle Position of Elutriated WEHI-231 Populations

		Cell Cycle Position[a]											
		Media				Anti-IgM				250 nM Ionomycin + 4 nM PdBu			
Elutriated Population	Culture Length (hr)	G_1+Non-Cycling	Early S	Late S	G_2 + M	G_1+Non-Cycling	Early S	Late S	G_2 + M	G_1+Non-Cycling	Early S	Late S	G_2 + M
A+B	0.5	63	30	4	3								
	6.75	13	59	26	2	16	44	38	2	17.5	63	17.5	2
	12.5	38	23	19	21	58	7	18	17	46	13	22	19
		(5.5)[b]	(7)	(26)	(62)	(21)	(5)	(22)	(52)	(8)	(13)	(33)	(46)
	18.75	12	53	31	4	82	9	6	4	51	33	11	6
	24.25	27	31	26	16	89	3	4	4	57	14	19	11
		(3)[c]	(5)	(35)	(57)	(81)	(1)	(5)	(14)	(40.5)	(15)	(20.5)	(25)
C	0.5	29	56	13	2								
	6.75	10	18	55	17	13	20	53	14	12	27	46	15
		(5)[d]	(23)	(52)	(20)	(9)	(25)	(47)	(19)	(5)	(22)	(54)	(19)
	12.5	31	53	8	8	65	23	4	8	48	33	9	10
	18.75	11	23	52	14	64	12	19	5	29	30	33	17
		(2)[e]	(16)	(55)	(27)	(61)	(11)	(17)	(12)	(23)	(26)	(34)	(17)
D	0.5	13	53	30	4								
	6.75	32	9	34	26	36	10	31	23	33	9	33	26
		(3)[d]	(7)	(40)	(50)	(4)	(8)	(38)	(50)	(4)	(6)	(42)	(48)
	12.5	15	65	16	4	51	40	7	3				
	18.75	26	15	37	22	58	10	23	10	35	17	33	16
		(2)[f]	(6)	(43)	(48)	(51)	(7)	(23)	(20)	(21)	(13)	(39)	(28)

		Cell Cycle Position[a]											
		Media				Anti-IgM				250 nM Ionomycin + 4 nM PdBu			
Elutriated Population	Culture Length (hr)	G_1+Non-Cycling	Early S	Late S	G_2 + M	G_1+Non-Cycling	Early S	Late S	G_2 + M	G_1+Non-Cycling	Early S	Late S	G_2 + M
E	0.5	14	12	31	44								
		(13)[d]	(8)	(33)	(46)								
	6.75	17	67	12	4	21	65	11	3				
	12.5	12	19	50	19	24	13	46	18				
		(3)[b]	(9)	(54)	(33)	(15)	(10)	(48)	(26)				
	18.75	15	63	17	5	75	14	6	6				
	24.25	20	24	40	16	79.5	5	9	6				
		(2)[c]	(11)	(43)	(44)	(70.5)	(4)	(10)	(16)				
WEHI-231	24.25	18	44	29	9	83	3	6	8	62	16	12	10

[a] 0.5-1 x 10^6 elutriated cells from the fractions shown in Fig. 2 or unsynchronized WEHI-231 cells were cultured for the indicated times at 1 x 10^5/ml in growth medium. Some cultures also contained 2 μg/ml of the monoclonal anti-IgM Bet1 or 250 nM ionomycin plus 4 nM PdBu. BrdUrd was added for the final 30 min of culture, and the cells were analyzed for cell cycle position as described in Materials and Methods. Results are presented as the % of cells in G_1 plus non-cycling phases of the cell cycle (<G_1 and non-BrdUrd-incorporating S) or in the cycling phases of early S, late S, or G_2+M, as described in the text.

[b-f] The numbers in parentheses represent the % of cells in the various cell cycle phases for cell populations that were treated with 200 ng/ml colcemid .

[b] Colcemid was added at 5.5 hr of culture.

[c] Colcemid was added at 17.75 hr of culture.

[d] Colcemid was added at the start of the culture.

[e] Colcemid was added at 12.75 hr of culture.

[f] Colcemid was added at 11.5 hr of culture.

For example, the percent of untreated fraction A+B cells in G_1 phase increased from 13% at 6.75 hr to 38% at 12.5 hr, presumably because cells had divided and reentered G_1 phase (Table I). This was confirmed by adding colcemid at 5.5 hr of incubation to the fraction A+B cells. In the presence of colcemid, the percent of untreated cells in G_2+M phase at 12.5 hr increased from 21% to 62%, demonstrating that many of the cells had divided by 12.5 hr (Table I). Since colcemid treatment would still allow cells to exit G_1 phase, this treatment would clear cycling cells out of G_1 phase. Thus, in theory, only growth arrested cells would remain in G_1 phase after a short incubation with colcemid. By comparing the 12.5 hr cell cycle distributions of the colcemid-treated populations that did or did not receive anti-IgM, it can be seen that 15.5-21% of anti-IgM-treated cells arrested in G_1 phase without ever entering S phase (Table I, fourth line). These cells are probably the cells with the long G_1 phase mentioned above, which are still in this phase 7 hr after their isolation even in the absence of anti-IgM. Thus, of the 63% of cells which started in G_1 phase, one-quarter to one-third of them arrested without entering S phase. In contrast, very few of the cells treated with the mimicking reagents arrested in G_1 phase without first entering S phase, suggesting that the mimicking reagents need more signalling time in which to induce G_1 arrest.

After dividing at approximately 12 hr, the fraction A+B cells treated with anti-IgM or the mimicking reagents could have either entered S phase or arrested in G_1 phase. By 19 hr of treatment, 70-82% of the anti-IgM-treated cells and 39-51% of the mimicking reagent-treated cells had arrested in G_1 phase (Table I). Since 12% of the untreated population is also present in G_1 phase, the uncertainty in these figures is due to not knowing whether 12% of the experimentally-treated cells are actually arrested in or just transiting G_1 phase. However, by adding colcemid after the first mitosis was completed but before the second mitosis had begun (i.e. at 18 hr of culture), it was possible to clear the cycling cells out of G_1 phase and obtain a more precise measurement of the proportion of cells that arrested. Thus, by comparing the colcemid-treated fraction A+B cells analyzed at 24 hr, it can be

seen that 78-81% of the anti-IgM-treated cells and 38-41% of the PdBu plus ionomycin-treated cells arrested. The 79.5 ± 1.5% (mean ± range) of anti-IgM-treated cells that arrested are composed of the 18.5 ± 3% (mean ± range) that arrested without entering S phase and about 61% that divided and then arrested. Thus, of the 81.5% of the cells that were actually cycling, 75 ± 3% of these arrested between 18 and 24 hr of treatment.

Similar analyses were conducted with elutriated cells that were isolated in later stages of the cell cycle. The fraction C cells began in G_1 and early S phases (Table I, 0.5 hr analysis). Examination of the colcemid-treated populations (6.75 hr analyses) shows that essentially none of the 29% of cells that started in G_1 phase arrested there without entering S phase. This result suggests that these cells had already passed the arrest point or that more signalling was needed before reaching that point to arrest them there. All of the fraction C cells had divided by 12.5 hr, which was earlier than the cells from fraction A+B divided, as expected from the more advanced cell cycle position of fraction C cells at the start of culture. By 12-19 hr of treatment, some of the cells that had been treated with anti-IgM or the mimicking reagents arrested in G_1 phase. Again, by adding colcemid between the first and second mitoses (i.e. at 13 hr of culture), it was determined that 59-61% of the anti-IgM-treated cells and 21-23% of the PdBu plus ionomycin-treated cells arrested after the first mitosis (Table I, 18.75 hr analyses with colcemid). It should be noted that fewer experimentally-treated cells from fraction C than from fraction A+B arrested after the first mitosis. The fraction C cells received signalling for less time before arriving at the potential G_1 arrest point than did the fraction A+B cells. Thus, the efficiency of G_1 arrest correlates with the duration of time between the start of signalling and the arrival at the arrest point in G_1 phase.

The elutriated cells from fraction D were still more advanced in the cell cycle at the time of their isolation; they were almost all in S phase (Table I, 0.5 hr analysis). As was the case with the cells from fraction C, neither treatment with anti-IgM nor PdBu plus ionomycin caused the fraction D cells that started in G_1 phase (13%) to arrest there without

entering S phase (Table I, 6.75 hr analyses with colcemid). Again, either these cells had already passed the arrest point or more signalling was needed before reaching that point to arrest them there. Fraction D cells could be seen arresting in G_1 phase after the first mitosis (12.5 hr analyses). It was shown by adding colcemid between the first and second mitosis (i.e. at 11.5 hr of culture) and examining cell cycle position at 19 hr that 49-51% of the anti-IgM-treated cells and 19-21% of the mimicking reagent-treated cells arrested. Even fewer cells from fraction D arrested in G_1 phase after their first division than did cells from fractions C or A+B. Since fraction D cells received signalling for an even shorter period of time before arriving at the arrest point than did fraction A+B or C cells, there is again a correlation between the efficiency of G_1 arrest and the duration of exposure to the growth arrest-inducing agents.

The fraction E cells were predominantly in the late S and G_2+M phases of the cell cycle at the time of their isolation (Table I, 0.5 hr analysis). 50% of this population had apparently divided by 2.0-2.7 hr of culture. By adding colcemid to the cells after they had all divided and waiting until the cycling cells had progressed out of G_1 phase, it was evident that only 12-15% of the fraction E cells treated with anti-IgM arrested after this first mitosis (12.5 hr analyses with colcemid). Thus, 2-3 hr of receptor signalling prior to division were not enough to induce efficient arrest in G_1 phase. In contrast, 68-71% of the cells arrested in G_1 phase after the second mitosis, i.e. after they had received signalling for slightly more than one full cell cycle (24.25 hr analyses with colcemid); this number includes the 12-15% that arrested after the first division. After taking into account the previously arrested cells, it was determined that the mean fraction of cycling cells that arrested after the second mitosis was $65 \pm 1.5\%$ (the error being the maximum possible range of uncertainty). This result is similar to that obtained with the fraction A+B cells that were isolated in G_1 phase and treated with anti-IgM for a full cell cycle, as $75 \pm 3\%$ of the cycling cells from that fraction arrested in G_1 phase after mitosis.

The data from three elutriation experiments were analyzed as described above. We wished to depict the relationship between the efficiency of growth arrest and the length of signalling received by the cells prior to their arrival at the G_1 arrest point. As the exact point of arrest within the 2 hr long G_1 phase is not known and as the cells are not narrowly synchronized, we estimated the length of signalling achieved before arrest to be the time required for 50% of the cycling cells to undergo mitosis. This was determined by calculating how long it would take the 50th percentile cell in each fraction to divide. For example, the 50th percentile cell in Fraction C (Table I, untreated cells) is in early S phase 0.5 hr after elutriation. Since the length of each cell cycle phase is known, it can be calculated that that cell will divide at 9.2 ± 0.8 hr of culture (mean $\pm$ maximum range of uncertainty), as described in Materials & Methods. Thus, for each elutriated cell population, the time at which 50% of the cells had divided was compared to the percent of cycling cells that arrested in G_1 phase following that mitosis. Fig. 4 illustrates the relationship between the length of exposure to anti-IgM and the efficiency of arrest in G_1 phase. The efficiency of the G_1 arrest of WEHI-231 cells increases linearly with the length of signalling received before the arrest point is reached. After a period of approximately one cell cycle, the efficiency of growth arrest induced by anti-IgM appeared to reach a plateau of 75% efficiency. Thus, antigen receptor-induced growth inhibition of WEHI-231 cells is not a rapid process.

The efficiency of growth arrest induced PdBu and ionomycin also increased linearly with the length of signalling, at least for the first 12 hr. It was not clear whether the efficiency of growth arrest induced by the mimicking reagents would continue to increase or not. The ratio of the slopes of the lines representing G_1 arrest due to PdBu plus ionomycin treatment and G_1 arrest due to anti-IgM treatment was 0.45 (Fig. 4). Thus, treatment with reagents that approximate the protein kinase C activation and increase in $[Ca^{2+}]_i$ produced by antigen receptor signalling was only half as efficient at inducing G_1 arrest as compared to antigen receptor stimulation. These results suggest that although

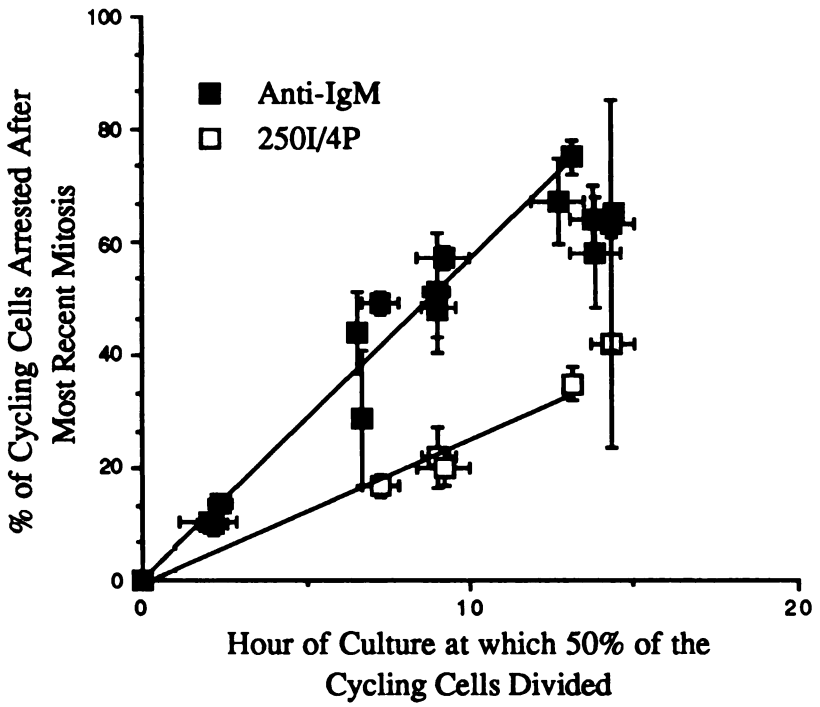

Figure 4. Efficiency of growth arrest of elutriated WEHI-231 cells increases with the duration of treatment with anti-IgM or PdBu plus ionomycin. The data shown are the percent of cells that arrest in G_1 and the non-cycling phases of the cell cycle (<G_1 and non-BrdUrd-incorporating S, as discussed in the text). The data are a compilation of results obtained in three elutriation experiments, including the one shown in Table I. The time at which 50% of the cells in each elutriated population had undergone mitosis was calculated as described in Materials and Methods. The % of cycling cells arresting is 100% x [% of cells arrested after most recent mitosis - % arrested previously]/ [100% - % of cells arrested previously]. The error bars represent the maximum range of uncertainty in these calculations. Lines were fitted to the data by linear regression analysis; the data points used were (0,0) and all points before 13 hr of treatment. The data points after 13 hr of treatment were not used because the efficiency of growth arrest appeared to level off at those times. The equations for the lines are $y = 0.19 + 5.64x$ for cells treated with anti-IgM and $y = -1.10 + 2.57x$ for cells treated with 250 nM ionomycin plus 4 nM PdBu (250I/4P).

calcium and diacylglycerol likely play roles in mediating the effects of anti-IgM on WEHI-231 cells, other second messengers may also be involved.

DISCUSSION

Stimulation of the antigen receptor on mature B lymphocytes induces cellular activation. In contrast, antigen receptor stimulation induces anergy or death in immature B cells, a response which likely represents one mechanism for the induction of tolerance to self components (29). We have examined this latter process in the WEHI-231 B lymphoma cell line, which has been proposed to be a model for antigen-induced inactivation of immature B cells (8). In particular, we examined how antigen receptor stimulation influences cell cycle progression and how it promotes the cell cycle arrest of these cells. The data obtained address the following issues: 1) the effect of antigen receptor signalling on the length of the cell cycle, 2) the duration of antigen receptor signalling required for G_1 arrest to occur, 3) the cell cycle dependence of responsiveness to this negative signalling, and 4) the role of protein kinase C activation and increases in $[Ca^{2+}]_i$ in mediating antigen receptor-induced inhibition.

Although a 6 hr lag was observed before unsynchronized WEHI-231 cells treated with anti-IgM began to arrest in G_1 phase (Fig. 1A), it was apparent that this lag was not due to lengthening of the cell cycle. The elutriated fractions that were enriched in cells that were in G_1, early S, or mid S phases (i.e. fractions A+B, C, and D, respectively, in Table I) exhibited no significant differences in their cell cycle progression for the first 7 hr of culture with or without anti-IgM. This was also true for cells treated with PdBu and ionomycin, which were used to mimic the phosphoinositide-derived second messengers diacylglycerol and calcium. Even more striking, cells from elutriated fractions that were concentrated in the late S and G_2+M phases (i.e. fraction E in Table I) progressed through a full cell cycle at the normal rate, whether or not they were treated with anti-IgM. Thus, antigen receptor or mimicking reagent stimulation of WEHI-231 cells did not slow down the cell cycle.

Instead, growth inhibition was solely the result of cell cycle arrest in G_1 phase. Interestingly, G_1 arrest was not an all or none response. Rather, the efficiency of the cell cycle arrest increased with the length of signalling the cells received before reaching the

potential G_1 arrest point (Fig. 4). The efficiency of arrest appeared to increase linearly with the duration of treatment with anti-IgM, reaching maximum efficiency after approximately one full cell cycle of signalling had occurred. Even then, only about 75% of the cells arrested, while the remaining 25% entered S phase and completed another cell cycle. Thus, for a cell population that started in G_1 phase, anti-IgM would be required for two full cell cycles in order to induce 94% of the cells to arrest. These data explain previous results showing that 90% inhibition of [^{3}H]thymidine incorporation was not obtained until 24 hr, i.e. nearly two cell cycles, of treatment with anti-IgM (17). This slow regulation of cell growth contrasts with the G_1 arrest seen upon serum starvation of fibroblasts. Only 1 hr of serum starvation was needed to induce fibroblasts in early G_1 to arrest and to enter a G_0-like state (41). The growth arrests observed in these two systems also differ in that the fibroblast arrest is reversible whereas the G_1 arrest of WEHI-231 cells is not (17). Thus, it is not surprising that the receptor signalling requirements for the two types of arrest are different.

Stimulation of WEHI-231 cells with doses of PdBu and ionomycin that approximated the protein kinase C activation and increases in $[Ca^{2+}]_i$ caused by anti-IgM (31, i.e. Chapter 1 of Ph. D. Thesis, and Chapter 2 of Ph. D. Thesis) also induced a growth inhibition that was due to G_1 phase arrest. As with anti-IgM-induced growth arrest, the efficiency of G_1 arrest increased linearly with the duration of treatment (Fig. 4). However, the mimicking reagents were only about one-half as effective at causing growth arrest as was stimulation with anti-IgM. This result confirms our previous conclusion that these reagents are partially able to replace antigen receptor signalling in WEHI-231 cells (31, i.e. Chapter 1 of Ph. D. Thesis). Thus, the natural second messengers diacylglycerol and calcium probably play roles in mediating the effects of anti-IgM on these cells. However, since the growth arrest induced by the mimicking reagents was considerably less efficient than that induced by anti-IgM, then either PdBu and ionomycin cannot effectively mimic the signals delivered by diacylglycerol and calcium, or there are other second messengers

involved in controlling the growth arrest. We favor the latter of these two possibilities because higher concentrations of the mimicking reagents are still less effective than anti-IgM at inducing growth arrest (31, i.e. Chapter 1 of Ph. D. Thesis).

Interestingly, the elutriated WEHI-231 cells appeared to be receptive to negative signalling not just in G_1 phase, but throughout the cell cycle. This is evident from the linear shape of the curve relating duration of signalling to the efficiency of G_1 arrest (Fig. 4). If negative signalling occurred only in G_1 phase, then a step-function curve would be expected, since G_1 phase comprises only 2 hr out of a cell division time of 12 hr in these cells. This issue could be addressed by examining growth arrest after removal of the signalling reagents at different points in the cell cycle. Due to difficulty in removing anti-IgM that had been bound to the cells, however, we did not undertake such experiments. Thus, the cells' receptiveness to negative signalling throughout the cell cycle is implied, but not proven, by these studies.

The antigen receptor signalling requirements for the G_1 arrest of WEHI-231 cells had previously been examined by Scott, et al (35). They obtained a WEHI-231 population that was enriched in very early G_1 phase cells by centrifugal elutriation. These cells were treated with anti-IgM for various lengths of time, after which free anti-IgM was washed away from the cells. Those authors concluded that 2 hr of antigen receptor signalling were sufficient to cause growth arrest and that the cells were only susceptible to negative signalling in the G_1 phase of the cell cycle. Clearly, our results do not support their conclusions. Although all of the reasons for these discrepancies may not be apparent, we can envisage two contributing factors: 1) The early G_1 phase cells used in the other work probably resembled the smallest population of cells we obtained (fraction A+B). These cells had an abnormally long G_1 phase in comparison to the other elutriated populations or to unelutriated cells. Unlike cells from the other elutriated populations, a significant proportion of these cells (25-33%) arrested in G_1 phase without entering the cell cycle, i.e. after only short periods of signalling. The cell population used by the other group may

have had an even longer G_1 phase and hence have been even more susceptible to early growth arrest. 2) The procedure used by the other group to remove anti-IgM probably did not remove anti-IgM bound to the cells (our unpublished observations). The bound anti-IgM would have continued to send signals to the cells, producing longer periods of signalling than just 2 hr. In any case, the current kinetic analysis of cell cycle position was more extensive, allowing for a more detailed examination of cell cycle progress and G_1 arrest.

The results reported here clearly show that the efficiency of G_1 arrest correlates with the length of signalling received by the cells before reaching the G_1 arrest restriction point. Short periods of signalling were not sufficient to cause growth arrest. When elutriated cells from the late phases of the cell cycle were treated with anti-IgM, most of these cells did not arrest in G_1 phase, even though they had received 2-3 hr of signalling via anti-IgM before mitosis occurred (Fig. 4). These results imply that anti-IgM does not cause G_1 arrest by rapidly inhibiting a component required for progression into S phase or by activating a suicide mechanism. In contrast, cytotoxic T lymphocytes and glucocorticoids induce an endogenous suicide pathway in target thymocytes (38). Since the suicide pathway activated in response to attack by cytotoxic T cells is readily apparent after only 1 hr of treatment, a "death protein(s)" could be induced in these cells. The slow induction of G_1 arrest in WEHI-231 cells by anti-IgM, on the other hand, suggests that the arrest is due to a slow increase in a component which inhibits progression into S phase, or else a slow decrease in a component required for progression into S phase.

Little is known about the intracellular events that control cell cycle progression in lymphocytes. Expression of a 40 kd nuclear protein and of prosolin were recently found to correlate with lymphocyte proliferation. Normal B cells stimulated with anti-IgM exhibited an increase in the amount of a 40 kd nuclear protein in G_1 phase, and a subsequent decrease in S phase (13). This protein was also found to decrease in WEHI-231 cells upon anti-IgM-induced G_1 arrest (13). Similarly, the expression of the 18 kd cytosolic protein

prosolin correlated with entry into or exit from proliferation in peripheral blood lymphocytes (5). Phosphorylation of prosolin also accompanied exit from the proliferative state, as had been previously observed with HL-60 promyelocytic leukemia cells (12). It is still unclear whether these proteins are important regulators of the cell cycle, or merely part of the response machinery. If they are cell cycle regulators, then it is possible that anti-IgM could arrest the growth of WEHI-231 cells by affecting the phosphorylation of prosolin and/or the levels of the 40 kd nuclear protein.

One intriguing part of the response of WEHI-231 cells to anti-IgM is the decrease in cell size. This decrease is greater than that which would be caused simply by arrest in G_1 phase, implying that the size decrease response is controlled separately from the G_1 arrest. One possibility is that the decrease in cell size actually causes the G_1 arrest. In agreement with this notion, we observed that an abnormally small, elutriated population of WEHI-231 cells (fraction A+B) arrested more rapidly in response to anti-IgM than did other elutriated populations. The relationship of cell size to cell cycle progression has been best studied in yeast, where a critical cell size must be reached in G_1 phase before cells will commit to S phase progression (15, 6). Thus, anti-IgM could cause G_1 arrest by decreasing cell size below the minimum required for progression into S phase. This model fits most of the observations in this system. For example, the 6 hr lag observed before G_1 arrest is seen (Fig. 1A) could be due to a period in which cell size has not yet decreased below the threshold for cell cycle commitment. Furthermore, treatment with the mimicking reagents was less effective at causing growth arrest and was also less effective at causing the size decrease (31, i.e. Chapter 1 of Ph. D. Thesis, and Fig. 1B). Thus, G_1 arrest in WEHI-231 cells could be a consequence of inhibiting a normal cell size increase or of causing an additional cell size decrease.

The $p34^{cdc2}$ protein has recently emerged as a central regulator of the cell cycle in yeast and higher eucaryotic cells. $p34^{cdc2}$ is a serine/threonine protein kinase that is highly conserved from yeast to man (22), and it is required for progression from G_1 phase to S

phase in budding (24) and fission yeast (30). Strikingly, the $p34^{cdc2}$ protein becomes dephosphorylated in the α-IFN-induced G_1 arrest of Daudi B lymphoma cells (37), the nitrogen starvation-induced G_1 arrest of fission yeast (36), and the serum starvation-induced arrest of fibroblasts (21). The decreased phosphorylation was also demonstrated to be accompanied by decreased kinase activity in the yeast and fibroblast cases. Thus, the kinase activity of the $p34^{cdc2}$ protein may be necessary for progression from G_1 phase to S phase. It remains to be seen whether WEHI-231 cells treated with anti-IgM exhibit dephosphorylation of the $p34^{cdc2}$ protein or a change in its kinase activity.

In summary, our analysis of the anti-IgM-induced G_1 arrest of WEHI-231 cells has shown that the efficiency of the growth arrest correlates with the length of signalling achieved before the cells reach the G_1 arrest restriction point. Maximum efficiency was not achieved until at least one full cell cycle (12 hr) of signalling had occurred, and 2-3 hr of signalling caused less than 15% of the cells to arrest their growth. In addition, signalling via the antigen receptor was approximately twice as effective as signalling via reagents which approximate the protein kinase C activation and increases in $[Ca^{2+}]_i$ caused by anti-IgM. These results therefore imply that diacylglycerol and calcium play roles in mediating the effects of anti-IgM on WEHI-231 cells, but that other second messengers may also be involved.

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Chapter Four:

Mutational analysis of the role of the phosphoinositide signaling pathway in regulating B cell growth

SUMMARY

Stimulation of the antigen receptor of WEHI-231 B lymphoma cells with anti-receptor antibodies (anti-IgM) causes phosphoinositide hydrolysis and irreversible growth arrest. A genetic approach was used to analyze the relationship of these events. Mutants of WEHI-231 cells were isolated that were resistant to anti-IgM-induced growth arrest. Five independent mutants had defects in the phosphoinositide signaling pathway. One of these appeared to be altered in a component(s) that responds to diacylglycerol and calcium, suggesting that those second messengers play roles in mediating the growth arrest. The other four mutants exhibited decreased production of second messengers, probably due to an alteration in phospholipase C. The decreased second messenger production appeared to be responsible for the growth-resistant phenotype; moreover, the limiting second messenger appeared to be calcium.

INTRODUCTION

Many different receptors transmit information across the plasma membrane by triggering inositol phospholipid hydrolysis. These receptors activate phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to yield two second messengers: diacylglycerol, which activates protein kinase C (Nishizuka, 1984), and 1,4,5-inositol trisphosphate, which causes release of calcium from intracellular stores (Berridge and Irvine, 1984). Receptors that transduce information by this mechanism include those for hormones, neurotransmitters, and growth factors (Abdel-Latif, 1986; Berridge, 1987), as well as the antigen receptors of T lymphocytes (Weiss et al., 1986) and of B lymphocytes (Bijsterbosch et al., 1985; Cambier, 1987; DeFranco, 1987). Many of these receptors, including the B lymphocyte antigen receptor (Gold et al., 1987), stimulate phospholipase C via GTP-binding proteins (Litosch et al., 1985; Martin et al., 1986; Smith et al., 1985; Imboden et al., 1986), which appear to be related to the G proteins involved in receptor signaling events that produce cAMP, degrade cGMP, and open ion channels (Gilman, 1987).

Although many receptors induce phosphoinositide hydrolysis, it has been difficult to determine if the biological events caused by receptor stimulation are mediated by this signal transduction pathway or by other receptor signaling mechanisms. In a number of systems, the relevance of the phosphoinositide signaling pathway to subsequent biological events has been established (Fain et al., 1984; Garrison et al., 1984), but in many others the link remains uncertain (Sussman et al., 1988). The work described here addresses this issue for growth regulation of B lymphocytes by the antigen receptor.

Stimulation of the B lymphocyte antigen receptor with anti-receptor antibodies, e.g.anti-immunoglobulin (anti-Ig), has different effects on proliferation depending on the developmental state of the B cell. For example, treatment of mature, resting B cells with anti-IgM causes inositol phospholipid hydrolysis (Bijsterbosch et al., 1985), entry into the G_1 phase of the cell cycle (DeFranco et al., 1982a), and in combination with T cell-derived

growth factors, proliferation and antibody secretion (Kishimoto, 1985). Conversely, anti-IgM causes inactivation (clonal anergy or clonal deletion) of immature B cells, a response which is probably one mechanism for achieving tolerance to self components (Nossal, 1983; Goodnow et al., 1988; Nemazee and Burki, 1989). Stimulation of the WEHI-231 B lymphoma cell line with anti-IgM also causes inositol phospholipid hydrolysis (Fahey and DeFranco, 1987) and irreversible growth arrest (Ralph, 1979; Jakway et al., 1986). Thus, WEHI-231 appears to be a transformed derivative of an immature B cell, in that it has the surface phenotype and biological response expected of this developmental stage (DeFranco et al., 1982b). We and others have used this cell line as a model for understanding how antigen receptor signaling regulates B cell growth.

Several groups have investigated whether the hydrolysis of inositol phospholipids induced by stimulation of the B cell antigen receptor mediates the biological effects of antigen binding. One approach used to address this issue was to assess the effect of phorbol diesters and calcium ionophores on B cell growth. These agents are used to mimic the protein kinase C activation and the increases in cytosolic calcium caused by the natural second messengers diacylglycerol and inositol 1,4,5-trisphosphate. Incubation of mature B cells with these mimicking reagents induces entry into the G_1 phase of the cell cycle (Monroe and Kass, 1985) and proliferation - either alone (Klaus et al., 1986; Rothstein et al., 1986) or in combination with the T cell-derived lymphokine interleukin-4 (Paul et al., 1986). The combination of a phorbol diester and a calcium ionophore also causes growth inhibition of WEHI-231 cells, although not as efficiently as does anti-IgM (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis; Chapter 3 of Ph. D. Thesis). These studies suggest that the phosphoinositide-derived second messengers calcium and diacylglycerol mediate the effects of anti-IgM on B lymphocytes, at least in part. Several lines of evidence, however, suggest that other second messengers are also involved. First, the growth inhibitory effects of anti-IgM on WEHI-231 cells could not be fully reproduced with phorbol diesters and calcium ionophores, even when doses of these reagents were

used which activated protein kinase C and increased calcium more than anti-IgM did (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis; Chapter 2 of Ph. D. Thesis). Second, Tisch et al. (1988) introduced a gene encoding the δ heavy chain of immunoglobulin into WEHI-231 cells, producing cells that expressed similar levels of IgD and IgM on their surface. Anti-IgM still inhibited the growth of these cells, but anti-IgD did not inhibit their growth. Stimulation with either anti-IgM or anti-IgD, however, induced comparable production of inositol phosphates by 20 min of treatment. These results suggest that the anti-IgM-induced growth inhibition of WEHI-231 cells may depend on a signal other than or in addition to inositol phospholipid hydrolysis. Several other groups have also called into question the relevance of calcium and diacylglycerol as second messengers for regulating B cell growth (Scott et al., 1987; Brunswick et al., 1989).

We have taken a somatic cell genetic approach to gain a better understanding of the mechanism of the anti-IgM-induced growth arrest in WEHI-231 cells. We have isolated 36 independent mutants of WEHI-231 that are resistant to anti-IgM-induced growth inhibition and have examined seven of them in detail. Two of these mutants expressed little or no surface antigen receptor. The other five, however, appeared to have defects in the phosphoinositide signal transduction pathway. These results suggest that the phosphoinositide-derived second messengers play important, although perhaps not sufficient, roles in mediating the effects of antigen-receptor stimulation on WEHI-231 cells.

RESULTS AND DISCUSSION

We wished to investigate the mechanism by which stimulation of the antigen receptor induces WEHI-231 B lymphoma cells to arrest their growth. Therefore, we isolated mutants of WEHI-231 cells that were resistant to anti-IgM-induced inactivation. WEHI-231 cells were mutagenized with EMS, ICR-191, or diepoxyoctane and then cultured in the presence of anti-IgM. Wild-type cells stop growing and eventually die under these conditions, whereas mutants resistant to anti-IgM would be expected to continue to grow. In this way, 36 mutants were isolated from independently mutagenized cultures. Most of the mutants obtained were partially or completely resistant to anti-IgM. The seven mutants which exhibited the greatest resistance to anti-IgM-induced growth inhibition were analyzed in greater detail for their biological and signaling responses to anti-IgM.

Effect of anti-IgM on mutant proliferation and cell volume. Treatment of WEHI-231 cells with anti-IgM for 48 hr causes essentially complete growth arrest (Jakway et al., 1986). In contrast, the seven mutants of WEHI-231 were partially (W53.1, W62.1, W88.1, and W305.1) or completely (W52.1, W303.1.5 and W306.1) resistant to the inhibition of DNA synthesis induced by 48 hr of treatment with a monoclonal anti-IgM (Fig. 1) or with a polyclonal anti-IgM (data not shown). Anti-IgM also causes WEHI-231 cells to decrease their cell volume to less than half that of untreated cells by 24 hr of treatment (Gold and DeFranco, 1987). This size decrease is partly due to arrest in the G_1 phase of the cell cycle (Scott et al., 1986). However, since the cells become smaller than G_1 phase cells from untreated populations (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis), another cellular response may also be contributing to the size decrease. The seven mutants analyzed were also quite resistant to the anti-IgM-induced size decrease (Table I). Moreover, there was a good correlation between the degree of resistance to the cell volume decrease and the degree of resistance to the inhibition of DNA synthesis in each of these mutants.

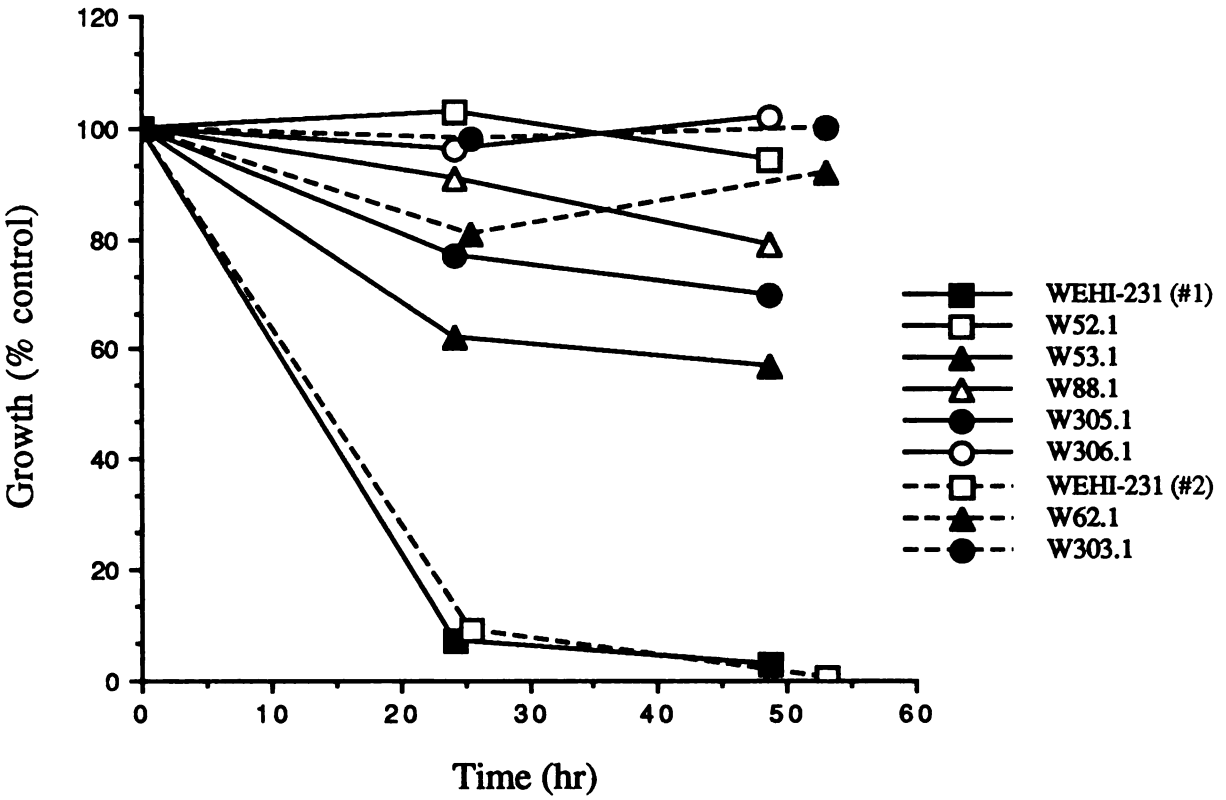


Figure 1. Effect of anti-IgM on the proliferation of mutants derived from WEHI-231 cells. Cells were cultured at 10^5/ml for 26-27 hr and at 5 x 10^4/ml for 50-54.5 hr in growth medium alone or containing 5 μg/ml of the monoclonal anti-IgM Bet1. [^{3}H]Thymidine was added for the final 2-3 hr of culture and incorporation was determined. Results are expressed as % thymidine incorporation of anti-IgM-treated cultures relative to untreated cultures. The data shown are from two separate experiments. The solid lines are from one experiment, and the dashed lines are from the other. The errors ranged from 1-13% (95% confidence limits), and the data are representative of 3 to 6 experiments performed with each mutant.

Table I

Effect of anti-IgM on cell volume

Cell Line	Median Cell Volume (% of control)[a]
WEHI-231	42 ± 1 (10)
W52.1	91 ± 5 (3)
W53.1	67 ± 2 (3)
W62.1	91 ± 3 (2)
W88.1	84 ± 2 (3)
W303.1.5	99 ± 1 (2)
W305.1	90 ± 3 (4)
W306.1	103 ± 3 (3)

[a] Cells were cultured at 10^5/ml for 22-27 hr with or without 1-5 μg/ml of the monoclonal anti-IgM Bet1. The median cell volume of each population was then determined using a Coulter Counter and Channelyzer. Results are expressed as % median cell volume ± SEM. % median cell volume is 100% x [median cell volume (anti-IgM-treated cells)/median cell volume (untreated cells)]. The median cell volumes of untreated W52.1, W53.1, W62.1, and W88.1 cells were similar to the median cell volume of untreated WEHI-231 cells, which was 722 ± 17 μm^3 (mean + SEM, n=10). The median cell volume of untreated W303.1.5 cells was ~85% of that of untreated WEHI-231 cells, and the median cell volumes of untreated W305.1 and W306.1 cells were ~115-120% of that of untreated WEHI-231 cells. The number of experiments performed is indicated in parentheses.

Expression of surface IgM by the mutants. Resistance to anti-IgM could occur by loss of expression of surface antigen receptor. Immunofluorescence studies with intact cells revealed that two of these seven mutants had decreased expression of surface IgM (Table II). W303.1.5 expressed little or no surface IgM, and W88.1 expressed less than 10% of the amount of surface IgM expressed by the wild-type cells. These mutants may express less surface IgM because they have less μ or κ chains or because they cannot transport IgM to the plasma membrane. Accordingly, expression of intracellular μ and κ chains was examined in metabolically labelled cells. W303.1.5 cells expressed normal levels of κ chain, but very little μ chain (Fig. 2A). Thus, the lack of expression of surface IgM on these cells is probably the result of a mutation that considerably decreases expression of the μ chain. This mutation could be in the μ gene itself or in a component that controls expression of the μ gene. In contrast, the levels of biosynthetically labelled μ and κ chains in W88.1 cells were similar to the levels in WEHI-231 cells. Thus, the decreased surface IgM expression in W88.1 cells is not due to a defect in the synthesis of heavy or light chains.

The transport of the μ chain to the cell surface was next examined in W88.1 cells by using endoglycosidase H (EndoH). EndoH cleaves high mannose oligosaccharides on proteins from the endoplasmic reticulum, resulting in a decrease in apparent molecular weight. Proteins that have reached or passed through the medial-Golgi, however, are EndoH-resistant (Kornfeld and Kornfeld, 1985). EndoH converted the μ chain in WEHI-231 cells from 73-80 kd forms to 58-62 kd forms, and in W88.1 cells from a 73 kd form to 58-62 kd forms (Fig. 2B). A greater proportion of the μ chain in W88.1 cells was EndoH-sensitive than was the μ chain in the wild-type cells, indicating that transport to the plasma membrane was impaired in W88.1 cells. Moreover, there appeared to be defective modification of the μ chain in these cells from the 73 kd form to the 80 kd form easily visible in WEHI-231 cells (Fig. 2, especially apparent in part A). The 80 kd form is probably a more highly glycosylated version of μ chain that is further along the transport

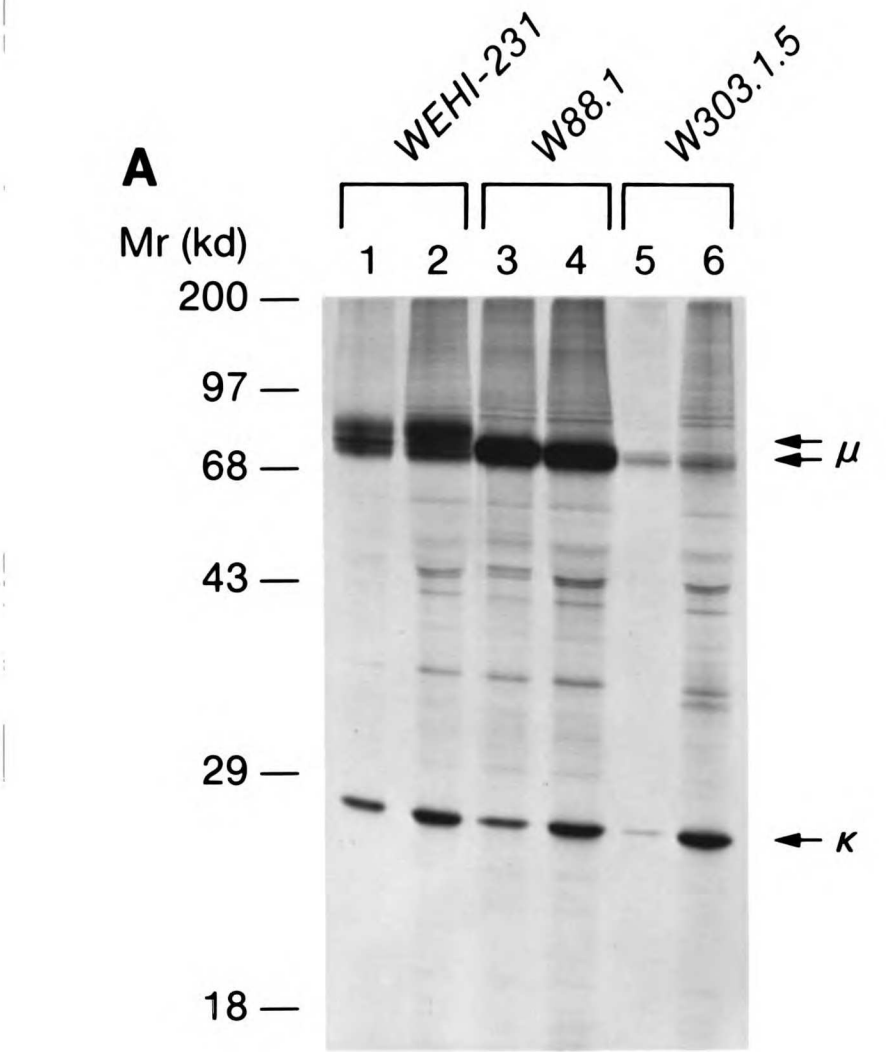
Table II

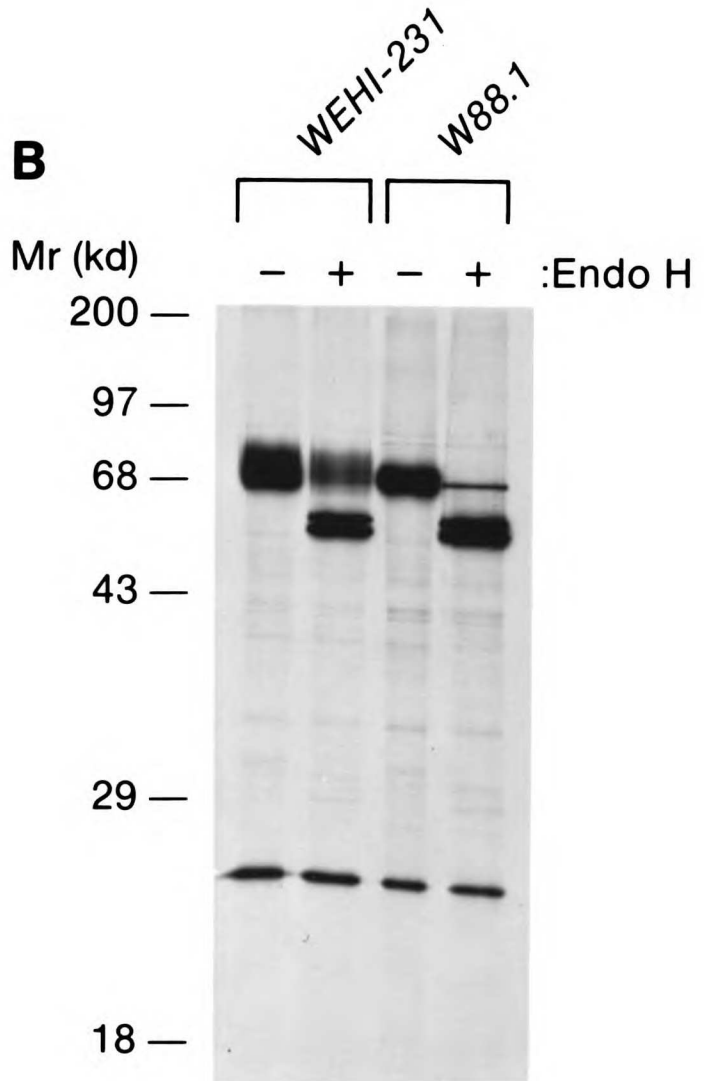
Expression of surface IgM by anti-IgM-resistant mutants of WEHI-231

Mutant Cell Line	Surface IgM Expression (% WEHI-231)[a]
W52.1	97 ± 13 (4)
W53.1	94 ± 6 (4)
W62.1	102 ± 12 (3)
W88.1	7 ± 1 (7)
W303.1.5	0.3 ± 0.1 (3)
W305.1	90 ± 4 (5)
W306.1	88 ± 8 (4)

[a] Cells were analyzed for expression of surface IgM, as described in Experimental Procedures. Results are expressed as % surface IgM ± SEM, which is {[median fluorescence (mutant cells) - median fluorescence (non-specific binding control)]/[median fluorescence (WEHI-231 cells) - median fluorescence (non-specific binding control)]} x 100%. The number of determinations averaged is indicated in parentheses.

Figure 2. Biosynthesis and glycosylation of intracellular μ and κ chains in W303.1 and W88.1 cells. A) 10^8 WEHI-231, W88.1 or W303.1.5 cells were metabolically labelled with ^{35}S, and μ and κ chains were immunoprecipitated, as described in Experimental Procedures. The immunoprecipitates were then analyzed on an SDS-polyacrylamide (12%) gel. The immunoprecipitations with anti-μ are shown in lanes 1, 3 and 5; the immunoprecipitations with anti-κ are shown in lanes 2, 4 and 6 The positions of the molecular weight standards are indicated at the left of the autoradiogram, and the arrows at the right point to the μ and κ chain bands. B) 3 x 10^7 WEHI-231 or W88.1 cells were metabolically labelled with ^{35}S, and the cell extracts were immunoprecipitated with anti-μ. Half of each immunoprecipitate was then digested with EndoH, as described in Experimental Procedures, and the products were analyzed on an SDS-polyacrylamide (12%) gel. The positions of the molecular weight standards are indicated at the left of the autoradiogram.


A
WEHI-231
W88.1
W303.1.5
Mr (kd)
1 2 3 4 5 6
200
97
68
43
29
18
μ
κ

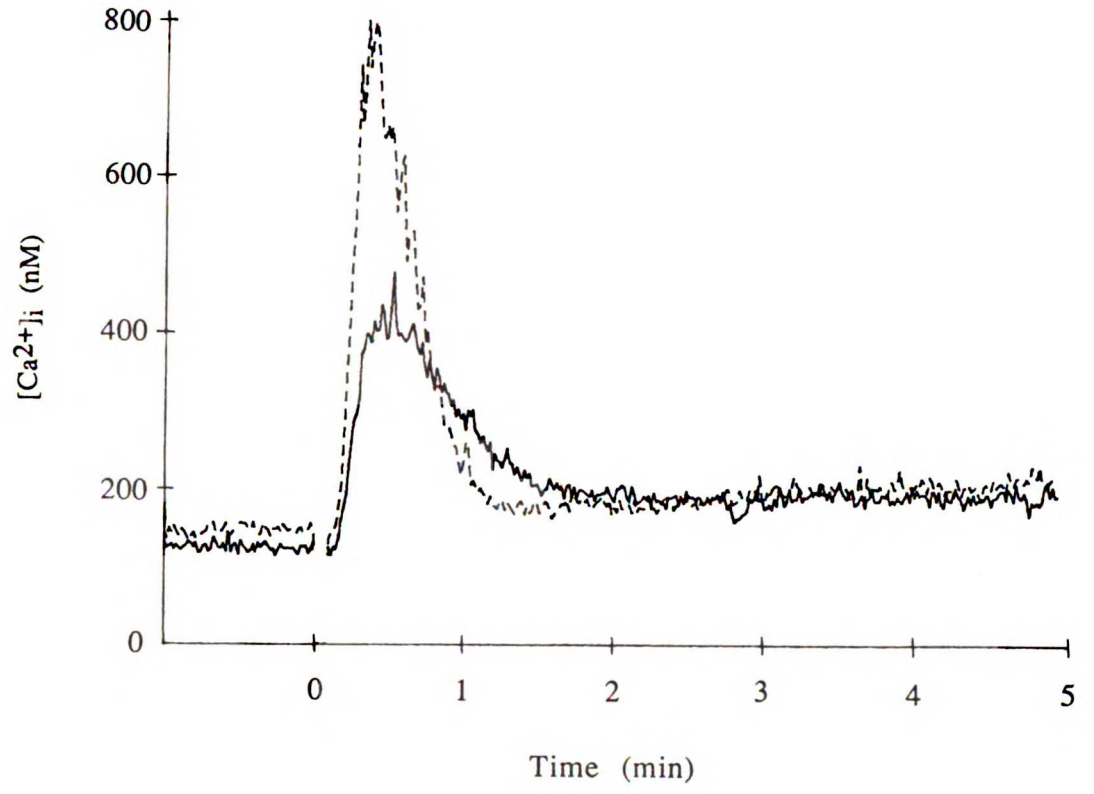

B
WEHI-231
W88.1
Mr (kd)
– + – + :Endo H
200 —
97 —
68 —
43 —
29 —
18 —

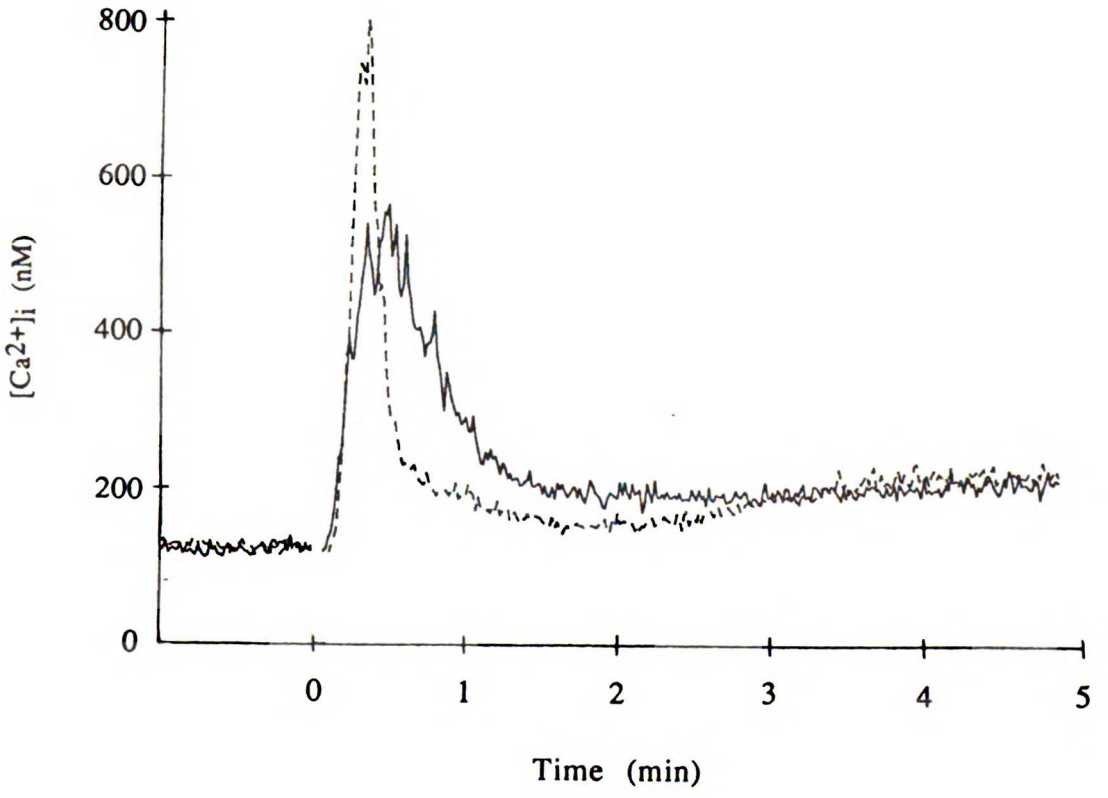
pathway. Corresponding with this notion, the 80 kd form is the form expressed on the surface, since it is the predominant form labelled by iodination of WEHI-231 cells (data not shown). A small amount of ^{125}I-labelled 80 kd μ chain was also present in W88.1 cells (data not shown), as expected from the low levels of surface IgM that are expressed on this mutant. Together, the results from the EndoH and iodination experiments suggest that the low expression of surface IgM on W88.1 cells is due to defective transport of the μ chain out of the endoplasmic reticulum or cis-Golgi to the plasma membrane. In summary, W303.1.5 cells are resistant to anti-IgM because they do not express surface antigen receptor. W88.1 cells express very little of the antigen receptor because they have a transport defect, and this low expression may explain their resistance to anti-IgM.

Effect of anti-IgM on calcium and inositol phosphate generation in the mutants. The anti-IgM-resistant phenotypes of the other five mutants were due to defects other than decreased expression of surface IgM (Table II). These mutants could have decreased generation of second messengers in response to anti-IgM. To test this possibility, the mutant and wild-type cells were analyzed for increases in intracellular free calcium ($[Ca^{2+}]_i$) (Fig. 3) and inositol phosphates (Fig. 4) in response to anti-IgM. W303.1.5 cells and W88.1 cells did not increase their $[Ca^{2+}]_i$ when treated with anti-IgM, as expected since they express little or no surface IgM (LaBaer et al., 1986, and data not shown). Correspondingly, W88.1 cells produced very little inositol phosphates in response to treatment with anti-IgM (data not shown). Thus, W88.1 is probably resistant to the growth inhibitory effects of anti-IgM because only small quantities of the second messengers are generated in response to antigen-receptor stimulation.

The other five mutants, however, exhibited quite marked increases in $[Ca^{2+}]_i$ in response to antigen-receptor stimulation. The anti-IgM-induced increase in $[Ca^{2+}]_i$ in W53.1 cells was similar to that seen in the wild-type cells (data not shown), while a greater increase in $[Ca^{2+}]_i$ was observed in W305.1 cells than that seen in the wild-type cells (Fig. 3A). Interestingly, three of the mutants (W52.1, W62.1, and W306.1) exhibited an

Figure 3. Effect of anti-IgM on $[Ca^{2+}]_i$ in anti-IgM-resistant mutants of WEHI-231 cells. The fluorescence of Indo-1-loaded cells (10^5/ml, intracellular concentrations of Indo-1 at 0.11-0.17 mM) was monitored at 37°C. Data points were collected every second and were used to determine $[Ca^{2+}]_i$ (nM). 5 μg/ml of the monoclonal anti-IgM Bet1 were added at time zero. The solid lines represent the responses of WEHI-231 cells; the dashed lines represent the responses of A) W305.1 and B) W306.1 cells. The data are representative of at least two experiments performed with each mutant.


800
600
400
200
0
[Ca2+]i (nM)
0
1
2
3
4
5
Time (min)


800
600
400
200
0
[Ca2+]i (nM)
0
1
2
3
4
5
Time (min)

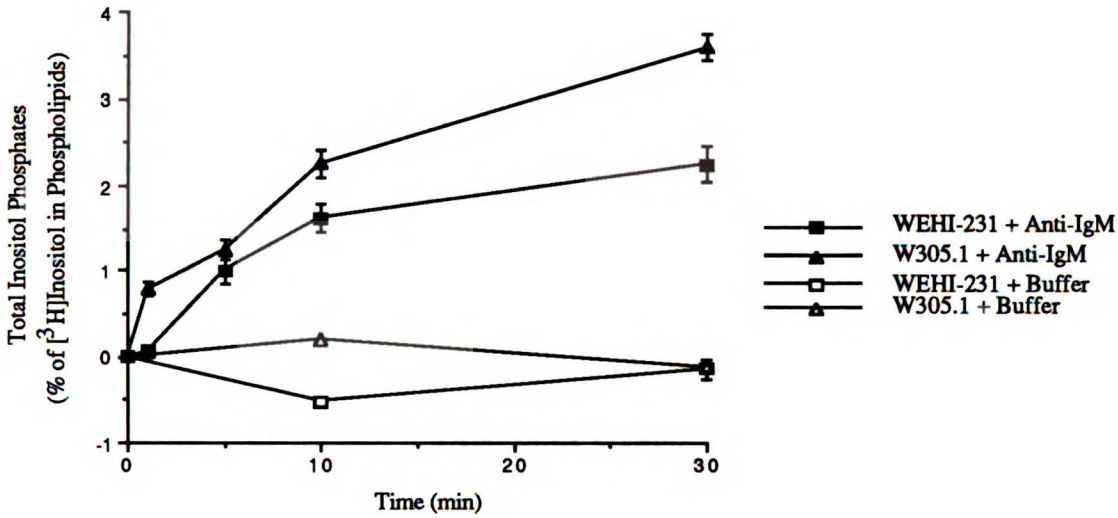
abbreviated peak increase in $[Ca^{2+}]_i$ in response to anti-IgM (Fig. 3B and data not shown). By 5 min of treatment, however, their $[Ca^{2+}]_i$ were similar to the $[Ca^{2+}]_i$ of anti-IgM-treated WEHI-231 cells at this time (Fig. 3B and data not shown). Thus, each of the five mutants with normal expression of surface IgM exhibited significant increases in $[Ca^{2+}]_i$ in response to anti-IgM, demonstrating the capacity of the antigen receptors on these cells to initiate signal transduction reactions.

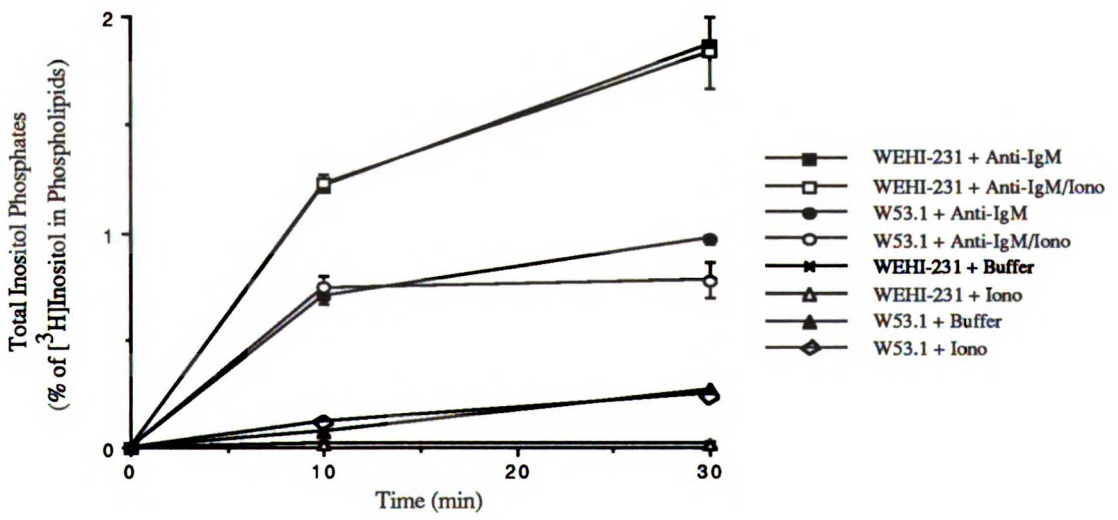
Since calcium increases could conceivably be generated under conditions of reduced inositol phospholipid hydrolysis, we examined inositol phosphate production in these mutants. Corresponding with W305.1's greater peak increase in $[Ca^{2+}]_i$ in response to anti-IgM, this mutant exhibited increased generation of inositol phosphates in response to anti-IgM (Fig. 4A). Thus, the anti-IgM-resistance of this mutant is probably not due to defective generation of phosphoinositide-derived second messengers. Instead, the mutation could lie in a component that mediates the action of the phosphoinositide-derived second messengers or it could lie in a separate signaling pathway (see below).

In contrast, mutants W52.1, W53.1, W62.1, and W306.1 each generated less inositol phosphates in response to anti-IgM than did the wild-type cells (Fig. 4B, Table VII). W53.1 and W62.1 cells had the most striking defects in inositol phosphate generation, producing less than 50% of the inositol phosphates produced by the wild-type cells after 10 min of treatment with anti-IgM. Anti-IgM also induced less inositol phosphate generation in W52.1 and W306.1 cells; however, the responses of these mutants were somewhat variable and the decreased inositol phosphate production was not reproducibly apparent until 20 min of treatment with anti-IgM (data not shown).

The decreased inositol phosphate generation in W53.1 cells was observed at all doses of anti-IgM tested (Fig. 5). The amount of inositol phosphates generated by both WEHI-231 and W53.1 cells reached a plateau at approximately 2 μg/ml of the monoclonal anti-IgM Bet1, with half-maximal generation occurring at 0.2 μg/ml. Consistent with the possibility that decreased inositol phosphate generation is responsible for the lack of

Figure 4. Effect of anti-IgM on inositol phosphate generation by anti-IgM-resistant mutants of WEHI-231 cells. [^{3}H]Inositol-labelled cells (2 x 10^6/ml) were examined for inositol phosphate generation in response to anti-IgM. Results are expressed as the increase in the % release after addition of stimulants or buffer controls. The % release is 100% x [cpm (total inositol phosphates)/cpm ([^{3}H]phospholipids)]. Each data point represents the average and range of duplicate samples; for samples with no error bars, the errors are smaller than the symbols. The data are representative of at least two experiments performed with each mutant. A) WEHI-231 and W305.1 cells were treated in growth medium/20 mM HEPES alone or containing 10 μg/ml Bet1 at 37°C. The samples treated for 30 minutes were collected by centrifugation. The incorporation of [^{3}H]inositol into the phospholipid layers was 23,000 ± 1,000 cpm and 33,000 ± 50 cpm (mean ± range, n=2) for WEHI-231 and W305.1 cells, respectively. The time zero values that were subtracted from the 1-10 min timepoints were 2.71 ± 0.11% release and 1.68 ± 0.01% release (mean ± range, n=2) for WEHI-231 and W305.1 cells, respectively. The time zero values that were subtracted from the 30 min timepoints were 2.16 ± 0.07% release and 1.52 ± 0.03% release (mean ± range, n=2) for WEHI-231 and W305.1 cells, respectively. B) WEHI-231 or W53.1 cells were treated at 37°C with 10 μg/ml Bet1, 250 nM ionomycin, or 10 μg/ml Bet1 plus 250 nM ionomycin in growth medium/20 mM HEPES. Growth medium was used to approximate the tissue culture conditions used in the other assays, such that the effect of ionomycin could be determined. The incorporation of [^{3}H]inositol into the phospholipid layers was 79,000 ± 1,000 cpm and 86,000 ± 1,000 cpm (mean ± SD, n=4) for WEHI-231 and W53.1 cells, respectively. The time zero values that were subtracted were 0.70 ± 0.04% release and 0.54 ± 0.01% release (mean ± range, n=2) for WEHI-231 and W53.1 cells, respectively.


Total Inositol Phosphates
(% of [3H]Inositol in Phospholipids)
4
3
2
1
0
-1
0
10
20
30
Time (min)
WEHI-231 + Anti-IgM
W305.1 + Anti-IgM
WEHI-231 + Buffer
W305.1 + Buffer


Total Inositol Phosphates
(% of [3H]Inositol in Phospholipids)
2
1
0
0
10
20
30
Time (min)
WEHI-231 + Anti-IgM
WEHI-231 + Anti-IgM/Iono
W53.1 + Anti-IgM
W53.1 + Anti-IgM/Iono
WEHI-231 + Buffer
WEHI-231 + Iono
W53.1 + Buffer
W53.1 + Iono

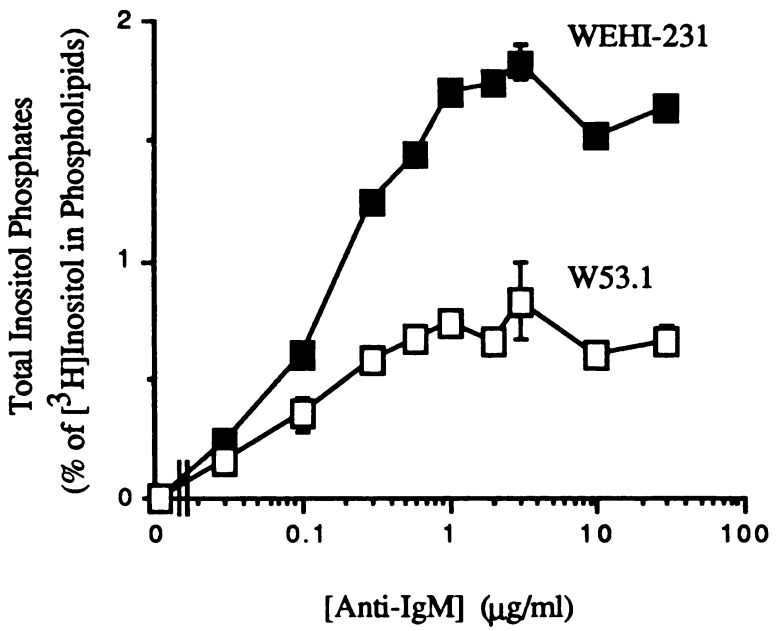


Figure 5. Effect of various doses of anti-IgM on inositol phosphate generation in WEHI-231 and W53.1 cells. [^{3}H]Inositol-labelled WEHI-231 and W53.1 cells (2 x 10^6/ml) were treated in growth medium/20 mM HEPES alone or containing the indicated doses of Bet1 for 20 min at 37°C. The results are expressed as the % release of [^{3}H]phospholipids as inositol phosphates, and each point represents the mean ± range of duplicate samples. For points with no error bars, the errors are smaller than the symbols. The % release of untreated controls was subtracted from all the measurements. The incorporation of [^{3}H]inositol into the phospholipid layers was 137,400 ± 2,800 cpm and 141,700 ± 7,000 cpm (mean ± SD, n=4) for WEHI-231 and W53.1 cells, respectively. The % release of the untreated controls was 0.66 ± 0.00% and 0.59 + 0.02% (mean ± range, n=2) for WEHI-231 and W53.1 cells, respectively. This experiment was performed twice with identical results.

growth inhibition of W53.1 cells, high doses of anti-IgM did not cause growth inhibition of W53.1 cells (data not shown).

If the maximal level of inositol phosphate production seen in the wild-type cells is required for efficient growth arrest, then the reduced levels of production seen in W53.1 and the other mutants could account for their anti-IgM-resistant phenotype. The minimum dose of the Bet1 anti-IgM necessary to cause maximal growth arrest of WEHI-231 cells was 0.1 μg/ml when the cells were cultured at 1 x 10^5/ml (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis). However, this minimum effective dose depended on the cell concentration. For example, when the cell concentration was reduced by 2-fold or 4-fold, then only one-half or one-quarter the dose of Bet1 was required to induce maximal growth arrest (data not shown). Thus, in order to compare the dose response curve for inositol phosphate production shown in Fig. 5 to the dose response curve for growth arrest, the cell concentration must be taken into account. A 20-fold higher cell density is used in the inositol phosphate assays than in the biological assays. Thus, if the biological assays could be done at the cell density employed in the experiment shown in Fig. 5, 20-fold more Bet1 would likely be required to cause growth arrest. This extrapolation implies that the minimum effective dose for growth arrest would be 2 μg/ml at that cell density. As the minimum dose of Bet1 that caused maximal inositol phosphate production was also approximately 2 μg/ml (Fig. 5), efficient growth arrest may indeed require the maximal level of inositol phosphate production seen in the wild-type cells. These results therefore suggest that W53.1 is resistant to the growth inhibitory effects of anti-IgM because this mutant does not make sufficient amounts of second messengers. Similarly, treatment of W52.1, W62.1, and W306.1 cells with 10 μg/ml Bet1 also did not cause inositol phosphate production to reach the plateau level induced in WEHI-231 cells by doses of 2-10 μg/ml Bet1 (Table VII). Thus, these mutants may also be resistant to the growth inhibition caused by anti-IgM because they do not generate enough of the phosphoinositide-derived second messengers.

In summary, all of the mutants that expressed normal levels of surface IgM could initiate phosphoinositide breakdown. However, four of them exhibited decreased inositol phosphate production. Among these four mutants, the ones with the greater defect in inositol phosphate production were not, as might be expected, the same as the ones with the greater resistance to growth arrest (see summary in Table VII). However, it should be noted that the anti-IgM-induced growth arrest of WEHI-231 cells requires signaling for many hours (Chapter 3 of Ph. D. Thesis), whereas for technical reasons, inositol phosphate production could be measured only at early times. At later times, the relative defects in phosphoinositide breakdown could change. Alternatively, there may be some other explanation for this minor discrepancy. In any case, the defective growth responses of these mutants to anti-IgM could be due to inefficient generation of diacylglycerol, calcium, and/or some other second messenger.

Analysis of G protein- and calcium-stimulated phospholipase C activity in signaling-deficient mutants. As described in the previous section, mutants W52.1, W53.1, W62.1 and W306.1 had significantly reduced inositol phosphate production in response to anti-IgM when compared to the wild-type WEHI-231 cells. We next wanted to determine the site of this defect. Crosslinking surface IgM causes release of inositol phosphates by activating a phosphoinositide-specific phospholipase C. We have previously shown that activation of phospholipase C by surface IgM involves a GTP-binding component that is probably a member of the G protein family (Gold et al., 1987). Since these mutants had normal amounts of surface IgM, their defective generation of inositol phosphates could lie in the ability of surface IgM to activate the G protein, in the ability of the G protein to activate phospholipase C, or in the phospholipase C enzyme itself. An additional possibility was that the mutants had a defect in metabolism of the inositol phospholipids that resulted in decreased production of the substrate for phospholipase C, phosphatidylinositol 4,5-bisphosphate (PIP_2). However, thin layer chromatography analysis showed that the relative amounts of [^{3}H]inositol incorporated into

phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (PIP_2) were very similar in the mutant and wild-type cells (data not shown). This result suggests that the mutants did not have alterations in the metabolism of the inositol phospholipids. Therefore, the defects in the mutants are likely to be in components involved in activation of phospholipase C by surface IgM.

We first asked whether G protein stimulation of phospholipase C activity was normal in the mutant cell lines. Cell homogenates were made from [^{3}H]inositol-labelled cells and the production of inositol phosphates was measured in response to two compounds that constitutively activate all G proteins, GTPγS and AlF_4^- (added as varying amounts of NaF in the presence of $AlCl_3$). Homogenates of [^{3}H]inositol-labelled WEHI-231 cells generated substantial amounts of inositol phosphates in response to either GTPγS or AlF_4^- (Table III). Stimulation by GTPγS was maximal at 100 μM with half-maximal activity at approximately 3 μM. Maximal stimulation by AlF_4^- occurred at approximately 20 mM NaF; 5 mM NaF usually caused about one-third maximal stimulation. AlF_4^- consistently caused greater inositol phosphate production than did saturating concentrations of GTPγS. Similar results were obtained with membrane fractions prepared from the cell homogenates (data not shown). This cell homogenate system differed from the permeabilized cell system we have used previously (Gold et al., 1987) in that anti-IgM was not required for GTPγS to cause maximal stimulation of inositol phosphate production. The preparation of cell homogenates in some way allowed the G protein to be activated by GTPγS without apparent receptor involvement. This is an advantageous property of the homogenates, in that it allowed us to examine the integrity of G protein-phospholipase C coupling independently of the receptor-G protein interaction. Similarly, AlF_4^- probably directly activates the G protein without a requirement for receptor involvement (Gilman, 1987). When G protein-stimulated inositol phosphate production was assessed in the mutant cells, we found that W53.1, W62.1, and W306.1 cells each produced less total inositol phosphates in response to either GTPγS or AlF_4^- than did the wild-type WEHI-231 cells.

Table III

G-protein stimulation of phospholipase C

in anti-IgM-resistant mutants of WEHI-231 cells[a]

		Total Inositol Phosphates (% Release)[b]		
Stimulus		WEHI-231	W62.1	W62.1/WEHI-231[e]
None		0.19 ± 0.02	-0.02 ± 0.01	
GTPγS	3 μM	0.88 ± 0.04	0.32 ± 0.02	0.49
	100 μM	1.47 ± 0.04	0.52 ± 0.02	0.42
NaF	5 mM	0.73 ± 0.08	0.15 ± 0.02	0.31
	20 mM	2.10 ± 0.12	0.28 ± 0.05	0.16
		Total Inositol Phosphates (% Release)[c]		
Stimulus		WEHI-231	W53.1	W53.1/WEHI-231[e]
None		0.09 ± 0.02	0.01 ± 0.01	
GTPγS	3 μM	0.69 ± 0.02	0.38 ± 0.01	0.62
	100 μM	1.18 ± 0.01	0.75 ± 0.01	0.68
NaF	5 mM	0.83 ± 0.01	0.31 ± 0.05	0.41
	20 mM	2.19 ± 0.09	0.85 ± 0.04	0.40
		Total Inositol Phosphates (% Release)[d]		
Stimulus		WEHI-231	W306.1	W306.1/WEHI-231[e]
None		0.01 ± 0.02	0.03 ± 0.02	
GTPγS	3 μM	0.32 ± 0.02	0.24 ± 0.01	0.68
	100 μM	0.65 ± 0.01	0.43 ± 0.03	0.63
NaF	20 mM	1.23 ± 0.05	0.62 ± 0.02	0.48

[a] Cell homogenates were incubated with the indicated stimuli for 10 min at 37°C. Stimulations with NaF also included 10 μM $AlCl_3$. Total inositol phosphate production is reported as % release (100% x cpm (total inositol phosphates)/cpm incorporated into inositol phospholipids) with time zero values subtracted. Each data point is the mean ±

range of duplicate samples, and each experiment was performed at least twice with similar results.

[b] The time zero values subtracted in this experiment were 1.74 ± 0.01% release for WEHI-231 cells and 1.08 ± 0.01% release for W62.1 cells. The incorporations into the phospholipid layers were 160,300 ± 2200 cpm (mean ± SEM, n=4) for WEHI-231 cells and 198,100 ± 2600 (mean ± SEM, n=4) for W62.1 cells.

[c] The time zero values subtracted in this experiment were 1.13 ± 0.02% release for WEHI-231 cells and 0.79 ± 0.04% release for W53.1 cells. The incorporations into the phospholipid layers were 157,200 ± 1300 cpm (mean ± SEM, n=4) for WEHI-231 cells and 168,400 ± 1200 (mean ± SEM, n=4) for W53.1 cells.

[d] The time zero values subtracted in this experiment were 0.48 ± 0.03% release for WEHI-231 cells and 0.37 ± 0.01% release for W306.1 cells. The incorporations into the phospholipid layers were 164,000 ± 1000 cpm (mean ± range, n=2) for WEHI-231 cells and 161,500 ± 4100 (mean ± range, n=2) for W306.1 cells.

[e] Ratio of the inositol phosphate response of the mutant to that of the wild-type cells. This ratio is [mutant response (stimulus) - mutant response (no stimulus)]/[WEHI-231 response (stimulus) - WEHI-231 response (no stimulus)].

A representative experiment for each mutant is shown in Table III. W62.1 had the greatest defect in G protein-stimulated phospholipase C activation, producing only 42% as much inositol phosphates as the wild-type cells in response to GTPγS and only 16% as much as the wild-type cells in response to AlF_4^-. G protein-mediated phospholipase C activation was 40-68% that of the wild-type response for W53.1 and 48-68% that of wild-type response for W306.1. The degree of the defect in G protein-mediated phospholipase C activation in cell homogenates roughly correlated with the degree of the defect in anti-IgM-stimulated phospholipase C activation in intact cells (Table VII).

The decreased ability of G protein activators to stimulate inositol phosphate production in the mutants suggested that the signaling defects in these cells are either in the G protein or in phospholipase C, as opposed to upstream components such as surface IgM or possible associated proteins. To determine whether the mutants had decreased or altered phospholipase C activity, we used supra-physiologic concentrations of calcium to activate the enzyme directly without the involvement of the G protein. In homogenates from wild-type cells, maximal activation of phospholipase C occurred at free calcium concentrations of 0.3-1 mM while half-maximal stimulation was observed at approximately 100 μM free calcium (Fig. 6). The amount of labelled inositol phosphates produced by homogenates of WEHI-231 cells in response to 1 mM free calcium was consistently 1-1.5% of the total label incorporated into the inositol phospholipids, which was very similar to the amount generated in response to 100 μM GTPγS. In contrast, phospholipase C activity in homogenates from mutants W52.1, W53.1, W62.1, and W306.1 had considerably different behavior. A representative experiment with W306.1 is shown in Fig. 6. Virtually identical results were obtained with W52.1, W53.1 and W62.1. All four of these mutants had much greater calcium-activated phospholipase C activity than did WEHI-231 cells. In addition, the phospholipase C enzymes present in the mutants were activated by much lower calcium concentrations than the enzymes in the wild-type cells. In all four mutant cell lines, maximal phospholipase C activity was observed at 100 μM calcium with

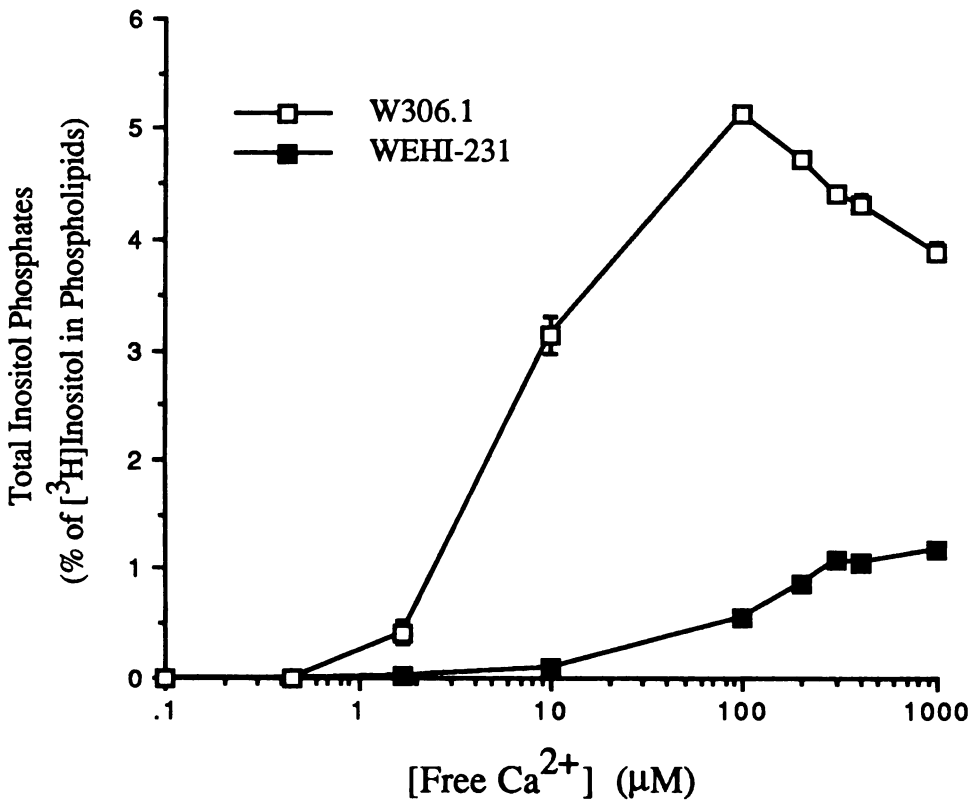


Figure 6. Ca^{2+}-stimulated phospholipase C activity in W306.1 cells. Cell homogenates were made from [^{3}H]inositol-labelled cells. These extracts were incubated with buffer containing the indicated concentrations of free Ca^{2+} (buffered with EGTA) for 10 min at 37°C, and production of total inositol phosphates was determined. The data are expressed as in Table III. The time zero values that were subtracted were 0.80 ± 0.05 % release for WEHI-231 cells and 0.55 ± 0.02 % release for W306.1 cells. The incorporation of [^{3}H]inositol into the phospholipid layers was 161,200 ± 2100 cpm (mean ± range, n=2) for WEHI-231 cells and 193,000 ± 2200 cpm (mean ± range, n=2) for W306.1 cells. The data are representative of four similar experiments.

half-maximal stimulation at approximately 6 μM calcium. It should be noted that the high phospholipase C activity in the mutant cells could have caused a depletion of substrate (PIP_2) before maximal calcium-stimulated activity could be achieved. If this were the case, the true sensitivity of the mutants' phospholipase C for calcium may not be altered. In any case, phospholipase C in these mutants clearly shows significant activity when stimulated with 2-10 μM calcium, whereas phospholipase C from the wild-type cells does not.

It is remarkable that four mutant cell lines, which were derived from independent cultures mutagenized with three different types of mutagenic agents (see Experimental Procedures), each exhibited such similar and striking biochemical phenotypes. Several hypotheses could explain the two apparently distinct alterations in the phosphoinositide signaling pathway observed in these cells. One possibility is that the mutant cell lines each have a mutation in the gene for phospholipase C that makes the enzyme much more sensitive to calcium but at the same time less responsive to activation by the G protein. A second hypothesis is that the mutant cell lines each have altered expression of two different isozymes of phospholipase C. Multiple isozymes of phospholipase C have been discovered (Rhee et al., 1989). These isozymes differ considerably in structure and could have different properties and functions. The mutant cell lines could have decreased expression of a phospholipase C isozyme that is activated by the G protein or by high concentrations of calcium, while having increased expression of a phospholipase C isozyme that is insensitive to G protein activation but highly sensitive to activation by calcium. More complicated explanations could also be advanced, such as defective metabolism of inositol phosphates which is affected by high concentrations of calcium, or defects in as yet unidentified components that regulate the activity of phospholipase C and/or G proteins. Nonetheless, it is striking that four independent mutants selected for loss of anti-IgM-induced growth arrest all have decreased surface IgM-induced production of inositol phosphates and similar alterations in phospholipase C activity. This observation

strongly argues that phosphoinositide hydrolysis plays an important role in mediating the antigen receptor-induced growth arrest of WEHI-231 cells.

We also analyzed G protein- and calcium-stimulated phospholipase C activity in several other mutants. W303.1.5, a mutant which has almost no surface IgM due to a defect in heavy chain expression (Fig. 2A), also showed greatly decreased responses to either GTPγS or AlF_4^- (Table IV). Unlike the mutants described above, W303.1.5 showed a significant decrease in calcium-stimulated phospholipase C activity. Despite the decreased activity, the calcium dose-response curve for phospholipase C in W303.1.5 was similar to that in wild-type cells, with half-maximal activity at approximately 100 μM calcium and maximal activity at 0.3-1 mM calcium. The apparent decrease in phospholipase C activity in W303.1.5 cells was not due to an alteration in inositol phospholipid metabolism since the incorporation of [^{3}H]inositol into phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (PIP_2) was identical in WEHI-231 cells and W303.1.5 cells (data not shown). Thus, it appears that W303.1.5 has decreased phospholipase C activity relative to WEHI-231 cells, in addition to a defect in μ chain production. Mutant W88.1, which has a defect in transporting IgM to the cell surface and has only 7% as much surface IgM as wild-type cells, had normal G protein- and calcium-stimulated phospholipase C activity (data not shown). Therefore, loss of IgM surface expression in W303.1.5 cells is probably not responsible for the decreased phospholipase C activity in these cells. Thus, W303.1.5 cells may have a defect in one or more phospholipase C isozymes, resulting in greatly decreased phospholipase C activity. W303.1.5 cells could therefore have two genetic lesions: one in the μ gene and one in a phospholipase C gene. Alternatively, they could have a defect in a transcription factor which controls expression of these, and possibly other, genes.

Effect of PdBu and ionomycin on mutant proliferation. The experiments described above showed that four mutants had decreased production of inositol phosphates, and one mutant (W305.1) exhibited slightly elevated production of second messengers. This latter mutant

Table IV

Decreased G-protein- and Ca^{2+}-stimulated phospholipase C activity in W303.1.5 cells

	Total Inositol Phosphates (% Release)[a]		
Stimulus	WEHI-231	W303.1.5	W303.1.5/WEHI-231[b]
None	0.05 ± 0.01	0.00 ± 0.01	
GTPγS 100 μM	0.92 ± 0.01	0.11 ± 0.01	0.12
NaF 5 mM	0.66 ± 0.05	0.09 ± 0.01	0.14
20 mM	2.11 ± 0.05	0.33 ± 0.04	0.16
Ca^{2+} 0.1 mM	0.64 ± 0.04	0.20 ± 0.01	0.31
0.3 mM	1.04 ± 0.01	0.34 ± 0.02	0.32
1.0 mM	1.28 ± 0.01	0.36 ± 0.03	0.28

[a] Cell homogenates were stimulated as indicated for 10 min at 37°C. Stimulations with NaF also included 10 μM $AlCl_3$. The production of total inositol phosphates was determined, and the data are expressed as in Table III. The time zero values that were subtracted were 0.78 ± 0.01% release for WEHI-231 cells and 0.56 ± 0.02% release for W303.1.5 cells. The incorporations into the phospholipid layers were 173,400 ± 600 cpm (mean ± range, n=2) for WEHI-231 cells and 140,800 ± 7300 cpm (mean ± range, n=2) for W303.1.5 cells. This experiment is representative of four similar experiments.

[b] Ratio of the inositol phosphate response of W303.1.5 cells to that of the wild-type WEHI-231 cells. This ratio is [W303.1.5 response (stimulus) - W303.1.5 response (no stimulus)]/[WEHI-231 response (stimulus) - WEHI-231 response (no stimulus)].

could have a defect in a component(s) that mediates the action of the second messengers. To further explore the nature of the defects in these mutants, we used a phorbol diester and a calcium ionophore in order to activate the protein kinase C and calcium second messenger pathways in the absence of phosphoinositide hydrolysis. We had previously established conditions under which phorbol 12,13-dibutyrate (PdBu) and ionomycin activated protein kinase C and increased $[Ca^{2+}]_i$ to an extent similar to that occurring following treatment of WEHI-231 cells with anti-IgM (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis; Chapter 2 of Ph. D. Thesis). This combination of 2-4 nM PdBu and 250 nM ionomycin inhibited the growth of WEHI-231 cells, although not as efficiently as did anti-IgM.

These concentrations of PdBu and ionomycin were used to investigate the integrity of the protein kinase C and calcium second messenger pathways in W305.1 cells. This mutant exhibited slightly elevated second messenger generation in response to anti-IgM (Fig. 3A, 4A) but was still resistant to the growth inhibitory effects of anti-IgM (Fig. 1, Table I). Consistent with the idea that W305.1 cells have a defect in a pathway(s) that responds to the second messengers, this mutant was significantly resistant to the growth inhibition induced by 48 hr of treatment with several combinations of PdBu and ionomycin as compared to WEHI-231 cells (Table V). It should be noted, however, that the growth of these cells was still partially inhibited by PdBu and ionomycin. This partial inhibition was evident in the first 24 hr of treatment, at which time the combination of PdBu and ionomycin inhibited W305.1 cells nearly as well as WEHI-231 cells. Moreover, W305.1 cells were not resistant to the more modest inhibition of proliferation caused by PdBu alone or ionomycin alone. Thus, the defect was delayed in effect and could not be localized to just the protein kinase C pathway or to just the calcium pathway.

Although we do not know the molecular nature of the defect in W305.1 cells, this mutant was clearly defective in its response to the doses of PdBu and ionomycin that best mimic the phosphoinositide-derived second messengers diacylglycerol and calcium. Thus, it is likely that the resistance of W305.1 to anti-IgM-induced growth arrest is due to an

Table V

Effect of anti-IgM, phorbol ester, and ionomycin on the proliferation of W305.1 cells

Additions[a]		[^{3}H]Thymidine Incorporation (% control)[b]	
		WEHI-231	W305.1
Anti-IgM		1	89
[Ionomycin] (nM)	[PdBu] (nM)		
0	2	110*[c]	111*
0	4	76	92
0	7	63	51*
125	0	83	87
125	4	20	68
125	7	17	47
250	0	63	74
250	2	23	81
250	4	8	55
250	7	8	36

[a] Cells at 5 x 10^4/ml were cultured in growth medium for 51 hr with 2 μg/ml of the monoclonal anti-IgM Bet1 or with the indicated concentrations of PdBu and ionomycin.

[b] [^{3}H]Thymidine was added for the final 3 hr of culture and incorporation was determined. The incorporations of cells with no additions (controls) were 110,000 ± 7,000 cpm and 155,000 ± 9,000 cpm (mean ± SD, n=9) for WEHI-231 and W305.1 cells, respectively. The results are expressed as % incorporation relative to control cultures. This experiment is representative of four similar experiments.

[c] An asterisk denotes an error of 8-12% (95% confidence limits); all other errors were <8%.

alteration in a growth regulatory component(s) that is responsive to both diacylglycerol and calcium. This interpretation is consistent with previous data indicating that calcium and diacylglycerol play roles in mediating the effects of anti-IgM on WEHI-231 cells (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis).

We also examined the responses to the mimicking reagents of the mutants with decreased inositol phosphate production (W52.1, W53.1, W62.1, and W306.1). W53.1 cells were slightly resistant to 2-7 nM PdBu and were more sensitive to 125-250 nM ionomycin than the wild-type cells (Table VI). Their responses to the combination of these reagents ranged from slightly resistant to more sensitive than the wild-type cells, depending on whether the response to PdBu or the response to ionomycin dominated in that particular experiment (Table VI and data not shown).

Since these mutants exhibited decreased second messenger production in response to anti-IgM, it was possible that the failure to arrest growth could result from production of limiting amounts of cytosolic calcium, diacylglycerol, and/or another second messenger. If the limiting second messenger were calcium or diacylglycerol, then addition of ionomycin or PdBu with anti-IgM could compensate for the limitation and result in growth arrest. Indeed, W53.1 cells treated with anti-IgM plus ionomycin arrested their growth as well as did the wild-type cells (Table VI). In contrast, the combination of PdBu and anti-IgM did not correct this mutant's anti-IgM-resistant phenotype. Similarly, ionomycin but not PdBu corrected the cell volume decrease response to anti-IgM (data not shown). Strikingly, all of the other mutants with decreased production of inositol phosphates (W52.1, W62.1, and W306.1) behaved in a similar fashion (data not shown). In contrast, the growth of the mutant with slightly elevated production of inositol phosphates (W305.1) was not markedly inhibited by the combination of anti-IgM and either 250 nM ionomycin or 4-7 nM PdBu (data not shown).

This striking result suggested that the increase in $[Ca^{2+}]_i$ caused by ionomycin corrected the defect in these mutants. Since all of these mutants were found to have

Table VI

Effect of anti-IgM, phorbol ester, and ionomycin on the proliferation of W53.1 cells

Additions[a]		[^{3}H]Thymidine Incorporation (% control)[b]			
[Ionomycin] (nM)	[PdBu] (nM)	WEHI-231 -Anti-IgM	WEHI-231 +Anti-IgM[a]	W53.1 -Anti-IgM	W53.1 +Anti-IgM[a]
0	0	100	26	100	60*
0	2	94*[c]	33	117**[c]	88*
0	4	65	32	104**	87*
0	7	48	30	69*	70*
125	0	83*	26	59*	10
125	4	50	27	46	22
125	7	37	27	37	25
250	0	82	21	40	7
250	2	63	24	34	12
250	4	45	27	29	18
250	7	34	27	31	27

[a] Cells at 10^5/ml were cultured in growth medium for 26.5 hr with the indicated concentrations of PdBu and ionomycin, either with or without 1 μg/ml of the monoclonal anti-IgM Bet1.

[b] [^{3}H]Thymidine was added for the final 3.5 hr of culture and incorporation was determined. The incorporations of cells with no additions (controls) were 93,000 ± 6,000 cpm and 121,000 ± 14,000 cpm (mean ± SD, n=8-9) for WEHI-231 and W53.1 cells, respectively. The results are expressed as % incorporation relative to control cultures. This experiment is representative of four similar experiments.

[c] An asterisk denotes an error of 9-15% (95% confidence limits); a double asterisk denotes an error of 16-24%. All other errors were ≤ 8%.

decreased inositol phosphate production in response to anti-IgM (Fig. 5, Table VII), the most straightforward explanation is that calcium is the second messenger whose production is limiting in these mutants, and that the use of ionomycin with anti-IgM supplies the correct amount of calcium needed to cause growth arrest. As we had observed that phospholipase C in these mutants is apparently more sensitive to calcium, another possible explanation would be that ionomycin corrected the defective responses of these mutants to anti-IgM by increasing their generation of inositol phosphates. This was found not to be the case, however. These concentrations of ionomycin neither caused inositol phosphate generation nor potentiated anti-IgM-induced inositol phosphate production in W53.1 cells (Fig. 4B). Similar results were also obtained with W52.1, W62.1 and W306.1 cells (data not shown). These results are consistent with the *in vitro* studies of phospholipase C in these mutants. The free calcium levels had to be raised to 1.7 μM or more in W52.1, W53.1, W62.1 and W306.1 cells in order to activate phospholipase C (Fig. 6 and data not shown), whereas the combination of anti-IgM and 250 nM ionomycin did not raise $[Ca^{2+}]_i$ above 1 μM (data not shown).

Although more complicated explanations for the action of ionomycin in this system cannot be excluded at this time, it is likely that ionomycin corrects the defective growth responses of these mutants to anti-IgM by elevating calcium and thereby increasing the concentration of the limiting second messenger. By raising calcium to the level required for growth arrest, ionomycin could restore the synergy between calcium and any other second messengers required to cause growth arrest. At present, however, direct evidence for this hypothesis is lacking. Although W52.1, W62.1, and W306.1 cells did exhibit abnormal calcium increases in response to anti-IgM, none of them appeared to have defective elevation of $[Ca^{2+}]_i$ from 5 min to 1 hr of treatment with anti-IgM (Fig. 3B and data not shown). We have been unable to accurately measure cytosolic calcium levels in cells treated with anti-IgM for more than 1 hr. Thus, it is still possible that the levels of calcium in these mutants decline after 1 hr of treatment. Since at least 18 hr of treatment with anti-

IgM are necessary to achieve half-maximal growth arrest of the wild-type cells (Chapter 3 of Ph. D. Thesis), it is possible that calcium is not elevated sufficiently in these mutants over the long periods of time needed to achieve efficient growth arrest and that ionomycin corrects this defect. Thus, these results provide further evidence for the relevance of the phosphoinositide signaling pathway in mediating the effect of antigen-receptor stimulation on B cell growth and suggest that calcium is an essential second messenger in mediating the anti-IgM-induced growth arrest of WEHI-231 cells.

Summary of mutant phenotypes. The phenotypes of the seven anti-IgM-resistant mutants of WEHI-231 described here are summarized in Table VII. Our interpretation of the probable sites of their defects is shown in Fig. 7. Two of these mutants had defective expression of surface antigen receptor. W303.1.5 cells were resistant to the growth inhibitory effects of anti-IgM because they did not express detectable levels of surface IgM and thus did not generate second messengers in response to anti-IgM (LaBaer et al., 1986). The lack of surface IgM appeared to be due to defective expression of the μ chain (Fig. 2A). W88.1 cells expressed less than 10% of the levels of surface IgM expressed by the wild-type cells, probably because of a defect in transport of the μ chain out of the endoplasmic reticulum or cis-Golgi (Fig. 2B). One interesting possibility is that W88.1 cells have a mutation in the *mb-1* gene, whose protein product may be required for transport of IgM to the cell surface (Hombach et al.,1988; Sakaguchi et al., 1988). Consistent with their low level of expression of surface IgM, W88.1 cells generated only low levels of inositol phosphates in response to anti-IgM (data not shown), which probably accounts for this mutant's resistance to the growth inhibitory effects of anti-IgM.

The other five mutants had defects in the phosphoinositide signal transduction pathway. W305.1 cells had normal levels of surface IgM and slightly increased production of calcium and inositol phosphates in response to anti-IgM (Fig. 3A, 4A). Although this mutant was selected for resistance to anti-IgM, it was also partially resistant to the combination of PdBu and ionomycin (Table V). This result suggests that W305.1 cells

Table VII

Properties of Anti-IgM-Resistant Mutants of WEHI-231

		Biological Responses		Signalling Responses				
				To Anti-IgM in intact cells		To GTPγS and Ca^{2+} in cell homogenates		
Cells	% surface IgM[a]	To Anti-IgM[b] (% inhibition)	To PdBu/Iono[c]	Ca^{2+}[d]	InsPs[e]	G protein[f]	Phospho-lipase C[g]	Conclusions
Wild-type	100	99 ± 1% (10)	Growth inhibited					
W303.1	0.3	-20 ± 27% (4)	ND[h]	None	ND	18 ± 2% (2)	Much less	Decreased amount of μ chain and of phospholipase C activity.
W88.1	7	30 ± 8% (3)	ND	None	2 ± 3% (2)	100% (1)	Normal	Defective transport of μ chain.
W305.1	90	23 ± 9% (5)	Partially resistant to combination.	More	163 ± 4% (2)	ND	ND	Defect probably in component(s) responsive to Ca^{2+} and diacylglycerol.
W52.1	97	4 ± 6% (4)	Close to normal.	Altered[i]	59 ± 7% (3)	77 ± 15% (2)	Ca^{2+}-sensitive	Defect probably in phospholipase C itself or in a regulatory component.
W53.1	94	50 ± 6% (4)	Ionomycin plus anti-IgM results in growth arrest.	Normal	32 ± 9% (7)	62 ± 7% (2)	"	
W62.1	102	26 ± 22% (3)		Altered	35 ± 4% (3)	46 ± 4% (2)	"	
W306.1	88	-12 ± 10% (4)		Altered	57 ± 12% (3)	58 ± 8% (4)	"	

[a] Data are from Table II.

[b] The data are presented as the % inhibition of proliferation (mean ± SD) as measured by [^{3}H]thymidine incorporation of cells treated with anti-IgM for 48-52 hr versus untreated controls. The number in parentheses is the number of experiments performed, and the results include the data in Fig. 1.

[c] Conclusion from data of Table V, Table VI, or data not shown.

[d] Conclusion from data of Fig. 3, LaBaer et al. (1986), or data not shown.

[e] The responses of the mutants are presented as a percentage of the wild-type response (mean ± SD of several experiments, as indicated in parentheses). The response measured was % release of [^{3}H]phospholipids as inositol phosphates in response to stimulation for 20-30 min with the monoclonal anti-IgM Bet1 (10 μg/ml). Data are from Fig. 4, 5, or data not shown.

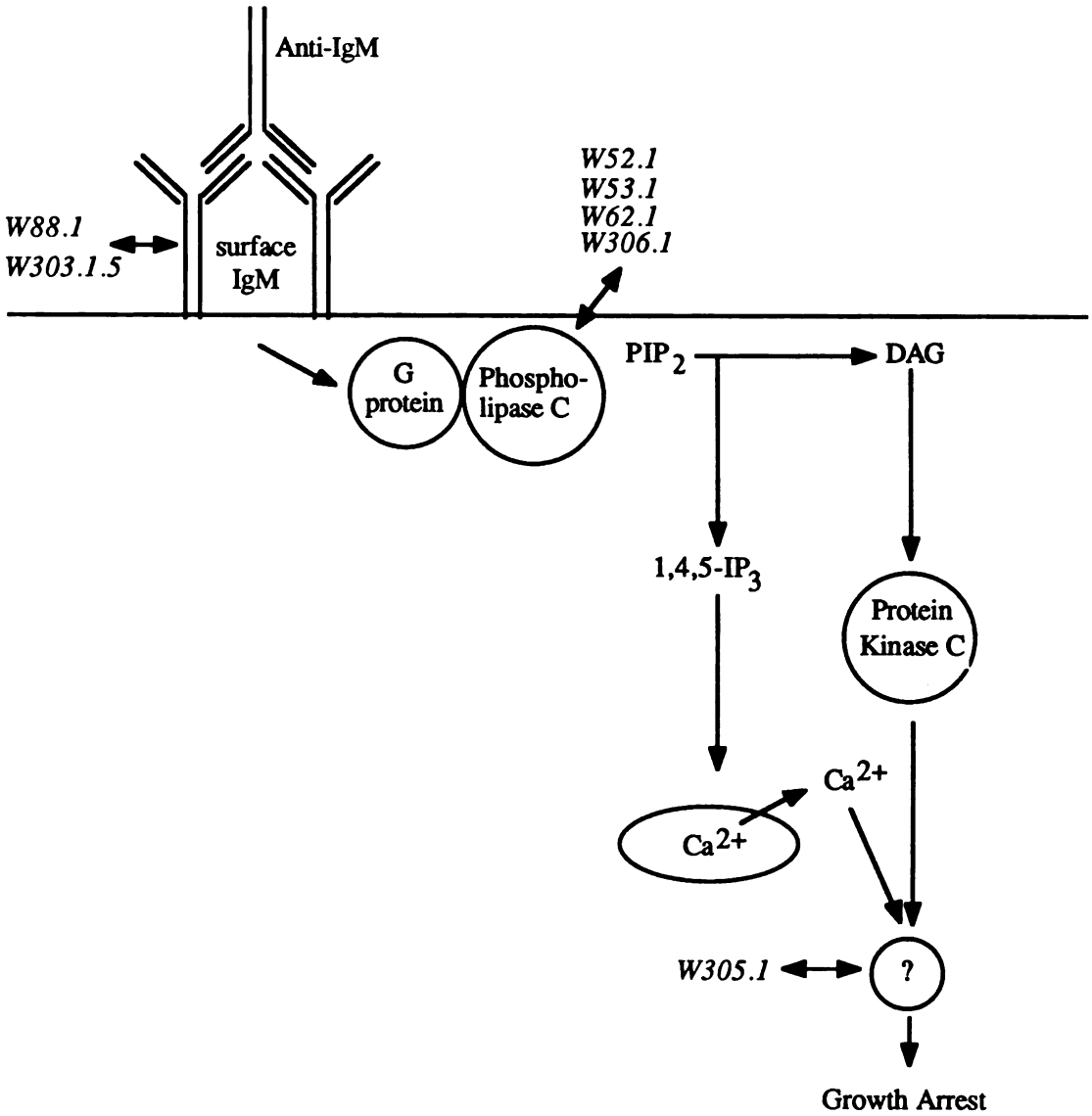
[f] The responses of the mutants are presented as a percentage of the wild-type response (mean ± SEM for n>2, or mean ± range for n=2, as indicated in parentheses). The response measured was % release of [^{3}H]phospholipids as inositol phosphates in response to stimulation for 10 min with 100 μM GTPγS. Data are from Table III, Table IV, or data not shown.

[g] Conclusion from data of Table IV, Fig. 6, or data not shown.

[h] Not determined.

[i] See Fig. 3B.

Figure 7. Sites of the defects in seven anti-IgM-resistant mutants of WEHI-231 cells. The current view of the phosphoinositide signal transduction pathway that is activated by crosslinkage of surface IgM in WEHI-231 cells is illustrated. Stimulation of surface IgM with anti-IgM antibodies activates a GTP-binding protein that subsequently activates phospholipase C. This enzyme(s) then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2), producing inositol 1,4,5-trisphosphate (1,4,5-IP3), which causes release of calcium from intracellular stores, and diacylglycerol (DAG), which activates protein kinase C. Calcium and protein kinase C can then regulate multiple cellular proteins via calmodulin-binding proteins and phosphorylation. The seven anti-IgM-resistant mutants of WEHI-231 cells analyzed appear to have defects at different sites in this signal transduction pathway. Two mutants clearly have defective expression of surface IgM; W303.1.5 has poor expression of heavy chain protein, whereas W88.1 has defective transport of IgM to the cell surface. One mutant, W305.1, appears to have a defect in a growth regulatory component(s) that responds to calcium and diacylglycerol. Four mutants, W52.1, W53.1, W62.1, and W306.1, have defective production of inositol phosphates, probably because of a defect in phospholipase C or its regulation.


Anti-IgM
W88.1
W303.1.5
surface IgM
W52.1
W53.1
W62.1
W306.1
G protein
Phospho-lipase C
PIP2
DAG
1,4,5-IP3
Protein Kinase C
Ca2+
Ca2+
W305.1
?
Growth Arrest

have an alteration in a growth regulatory component(s) that is responsive to both of the natural second messengers diacylglycerol and calcium. This alteration may be solely responsible for the anti-IgM-resistant phenotype of this mutant. However, the possibility that another mutation also occurred in these cells and that this other mutation also contributes to the anti-IgM-resistance cannot be ruled out at this time. In any case, together with the results showing that phorbol diesters and calcium ionophores mimic many of the effects of anti-IgM on WEHI-231 cells (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis), these results strongly argue that diacylglycerol and calcium are playing roles in mediating the effects of anti-IgM on these cells.

The other four mutants examined (W52.1, W53.1, W62.1, and W306.1) had normal amounts of surface IgM, but had defective generation of inositol phosphates, ranging from approximately 30% to 60% of that seen in the wild-type WEHI-231 cells after 20-30 min of treatment with anti-IgM (Table VII). The defective generation of inositol phosphates in these mutants was correlated with an alteration in phospholipase C or in a component that regulates phospholipase C; this alteration made the enzyme(s) less sensitive to G protein stimulation and more sensitive to calcium stimulation (Table VII, Fig. 6, and data not shown). Strikingly, all four of these mutants were corrected in their defective growth responses to anti-IgM by the addition of 250 nM ionomycin with anti-IgM (Table VI and data not shown). The most straightforward interpretation of this result is that these mutants fail to exhibit anti-IgM-induced growth arrest because calcium is not elevated adequately, whereas other required second messengers are produced in sufficient amounts.

Role of the phosphoinositide signaling pathway in regulating B lymphocyte growth. The role of the phosphoinositide-derived second messengers in mediating the effects of antigen-receptor stimulation on B lymphocytes has also been examined by other groups. Experiments using phorbol diesters and calcium ionophores to mimic receptor-generated diacylglycerol and calcium have suggested that both of these second messengers are important mediators of antigen receptor signaling (Monroe and Kass, 1985; Klaus et al.,

1986; Rothstein et al., 1986; Paul et al., 1986; Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis). On the other hand, Brunswick et al. (1989) found that extremely low doses of anti-Ig conjugated to dextran could activate mature, resting B cells without inducing detectable increases in cytosolic calcium. These results indicated that calcium may not be a necessary second messenger for mediating the effects of antigen-receptor stimulation on mature B cells. However, it is possible that the low doses of anti-Ig-dextran induced small or slow increases in calcium that were required for B cell activation. In agreement with this idea, Dennis et al. (1987) found that removal of extracellular calcium impaired the ability of mature, resting B cells to respond to anti-IgM. This effect was probably not due to a general deleterious effect on the B cells because they could still respond to the bacterial mitogen lipopolysaccharide, which does not trigger phosphoinositide hydrolysis in these cells. Thus, calcium is probably an important second messenger in mediating the effects of anti-IgM on mature B cells.

Although the mimicking and calcium-depletion approaches indicated that the phosphoinositide-derived second messengers are important for regulation of B cell growth by the antigen receptor, these approaches do have limitations. Calcium-depletion could have effects other than preventing elevation of cytosolic calcium. Pharmacologic mimicking reagents could activate the second messenger pathways more than do the natural second messengers. Thus, we employed a genetic approach for evaluating the role of various signaling events in mediating the biological responses of B cells. In the work described here, five out of seven anti-IgM-resistant mutants had defects in the phosphoinositide signal transduction pathway. The remaining two mutants were defective in expression of the antigen receptor. Monroe et al. (1989) also reported obtaining a mutant of WEHI-231 with defective anti-IgM-induced inositol phosphate production. The repeated isolation of anti-IgM-resistant mutants with alterations in the phosphoinositide signal transduction pathway strongly suggests that this pathway is required for mediating the growth inhibitory effects of anti-IgM on WEHI-231 cells.

It should be noted that these results do not rule out the possibility that another second messenger pathway may also be required to mediate the growth arrest. The existence of important second messengers other than calcium and diacylglycerol was suggested by the observation that the combination of phorbol diesters and ionomycin could not completely reproduce the growth inhibitory effects of anti-IgM on WEHI-231 cells (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis). Similarly, phosphoinositide breakdown and growth arrest were apparently dissociated in WEHI-231 cells expressing surface IgD from a transfected δ gene (Tisch et al., 1988). The growth of these transfected cells was not inhibited by anti-IgD, but was inhibited by anti-IgM, despite the fact that comparable production of inositol phosphates was seen in response to either anti-IgM or anti-IgD. These results can be reconciled with the ones reported here if growth inhibition of WEHI-231 cells depends on two types of signals, one provided by inositol phospholipid hydrolysis and one that is provided by a pathway activated by surface IgM but not surface IgD. The mutants we have isolated that have decreased inositol phosphate production in response to anti-IgM could still have the hypothetical second pathway intact. Indeed, since only ionomycin was required to correct these mutants' defective growth responses to anti-IgM, then a second required pathway would not be limiting in these mutants. Similarly, either diacylglycerol is not limiting or it is not essential for growth arrest, since phorbol diesters were not required with ionomycin to correct these mutants' defective growth responses to anti-IgM. In summary, the isolation of anti-IgM-resistant mutants of WEHI-231 cells with alterations in the phosphoinositide signal transduction pathway provides compelling evidence that this pathway is playing an important, although perhaps not sufficient, role in mediating the growth inhibitory effects of anti-IgM on WEHI-231 cells.

EXPERIMENTAL PROCEDURES

Reagents. Phorbol 12,13-dibutyrate (PdBu) was obtained from Sigma Chemical Co. (St. Louis, MO). The calcium salt of ionomycin (Calbiochem, La Jolla, CA) was stored in DMSO; the concentration was determined by measurement of OD at 300 nm in methanol, under which conditions the extinction coefficient is 21,600 M^{-1} cm^{-1} (Liu and Hermann, 1978). Goat anti-mouse IgM (antisera provided by Dr. R. Asofsky, LMI, NIAID, National Institutes of Health, Bethesda, MD) and the rat monoclonal anti-mouse IgM Bet1 (hybridoma provided by Dr. J. Kung, University of Texas Health Sciences Center, San Antonio, TX) were purified from antisera or culture fluid, respectively, by affinity chromatography with MOPC104E (IgM)-Sepharose. Goat anti-mouse IgM was also obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, PA (AffiniPure, μ chain-specific). The hybridoma producing the monoclonal mouse anti-rat κ MAR 18.5 was obtained from the American Tissue Culture Collection (Rockville, MD). The antibody was purified from culture fluid by affinity chromatography with Protein A-Sepharose.

Cell culture. The murine B lymphoma cell line WEHI-231 (Warner et al., 1979) (from Dr. N. Warner, Becton Dickinson, Mountain View, CA) and mutants derived from WEHI-231 were cultured in RPMI-1640 (M.A. Bioproducts, Walkersville, MD; GIBCO, Santa Clara, CA; Cell Culture Facility, University of CA, San Francisco) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT, or Applied Scientific, San Francisco, CA), 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol at 37°C in an atmosphere containing 5% CO_2. Some cultures also contained either 25-50 U/ml penicillin plus 25-50 μg/ml streptomycin or 20-50 μg/ml gentamicin. Cells were periodically screened for *Mycoplasma* contamination (Del Giudice and Hopps, 1978, or Mycotect Kit, GIBCO, Santa Clara, CA) and were found to be *Mycoplasma*-free. Cells used in experiments were obtained from cultures in the exponential phase of growth with a density generally below 5 x 10^5/ml.

Mutagenesis. Anti-IgM-resistant mutants of WEHI-231 were made by treating the wild-type cells with various mutagens for 16-24 hr. The mutagens used were ethyl methanesulfonate (EMS, Sigma Chemical Co., St. Louis, MO), which causes point mutations (Malling and de Serres, 1968), ICR-191 (PolySciences, Inc., Warrington, PA), which causes frameshift mutations (Brammar et al., 1967), and 1,2,7,8-diepoxyoctane (DEO, Aldrich Chemical Co., Milwaukee, WI), which causes small deletions and frameshift mutations (Van Duuren et al., 1967). Concentrations of mutagens were chosen such that 25-50% of the cells were killed by the mutagenesis. Anti-IgM-resistant cells were then selected by culturing the mutagenized cells in the presence of anti-IgM. Specifically, the mutants W52.1, W53.1, W62.1 and W88.1 were obtained by mutagenizing independent batches of 4-5 x 10^6 WEHI-231 cells at 5 x 10^5/ml for 16-24 hr in growth medium containing 0.02% EMS, 0.02% EMS, 0.75 μg/ml ICR-191, and 1.5 μg/ml ICR-191, respectively. The cells were then washed, cultured in growth medium for 2 days in order to allow expression of the mutations, and cultured for 3 days with 2-5 μg/ml goat anti-IgM. Surviving cells were isolated by centrifugation over Ficoll-Hypaque and then plated in 24-well plates in the presence of goat anti-IgM. Resistant colonies were cloned by limiting dilution. Mutants W303.1.5, W305.1, and W306.1, on the other hand, were placed under immediate selection after mutagenization. Independent batches of 1-2 x 10^7 WEHI-231 cells at 2-5 x 10^5/ml were treated for 16-24 hr with 0.25 ppm DEO (except for W303.1.5, which was obtained using 0.5 ppm DEO). The cells were subsequently washed and cultured in growth medium containing goat anti-IgM for 3-5 days. Surviving cells were isolated, plated in the presence of anti-IgM, and cloned as described above. When 1 x 10^9 unmutagenized WEHI-231 cells were cultured with anti-IgM, no anti-IgM-resistant colonies were obtained. Thus, since all of the mutants were derived from independently mutagenized cultures, they are all very likely to be independent mutants.

Cell proliferation analysis. Proliferation of WEHI-231 and anti-IgM-resistant mutants was assessed by measuring the incorporation of radiolabelled thymidine, as described

elsewhere (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis). Cultures were harvested with a PHD cell harvester (Cambridge Technologies, Cambridge, MA), and all determinations were performed at least in triplicate.

Cell volume analysis. WEHI-231 cells or anti-IgM-resistant mutants (1 x 10^5/ml) were cultured with various reagents in a volume of 0.5-1.0 ml in 24-well flat-bottomed plates (Costar, Van Nuys, CA). 10,000 to 15,000 cells/sample were subsequently analyzed with a Coulter Counter and Channelyzer (Coulter Electronics, Hialeah, FL) as previously described (Gold and DeFranco, 1987).

Expression of surface IgM. The amount of surface IgM present on cells was assessed by direct or indirect immunofluorescence staining, which was performed in sorter buffer (phosphate-buffered saline (PBS) containing 1% fetal calf serum and 0.1% sodium azide) at 0°C. For indirect immunofluorescence staining, 10^6 cells were incubated with 1 μg of the Bet1 rat monoclonal anti-mouse μ for 30 min. Cells were then washed twice and incubated for 30 min with 3 μg of the monoclonal mouse anti-rat κ MAR 18.5 which had been labelled with fluorescein isothiocyanate (Kung et al., 1981). For a nonspecific-binding control, WEHI-231 cells were incubated with fluorescein-labelled MAR 18.5 alone. For direct immunofluorescence staining, 0.5 x 10^6 cells were incubated in sorter buffer with 2 μg fluorescein-labelled goat anti-μ (AffiniPure, Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The nonspecific-binding control used for this procedure was the IgM-negative B lymphoma cell line 2PK3. After washing twice, cells stained by either procedure were resuspended in sorter buffer containing 4 μg/ml propidium iodide (Calbiochem, La Jolla, CA) and analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data from 5,000 to 10,000 cells were collected; dead cells were gated out by their increased propidium iodide fluorescence.

Immunoprecipitation of μ chain and κ chain from metabolically-labelled cells. 10^8 WEHI-231, W88.1, or W303.1.5 cells were cultured for 5.5 hr in 10 ml of RPMI-1640

which was depleted of methionine and cysteine and contained 10% heat-inactivated, dialyzed FCS (HyClone, Logan, UT), 2 mM glutamine, 50 μM 2-mercaptoethanol, and 1 mCi ^{35}S-Translabel (ICN Biomedicals, Irvine, CA) at 37°C in an atmosphere containing 5% CO_2. Cells were lysed in 1 ml of NDET buffer (1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4), and nuclei were removed by centrifugation at 16,000 x g for 5 min at 4°C. SDS was then added to a final concentration of 0.3%. Immunoprecipitation was subsequently performed with an excess of either affinity purified rabbit anti-mouse μ chain (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) or rabbit anti-mouse κ chain (ICN Biomedicals, Irvine, CA) and fixed protein A-containing *Staphylococcus aureus* cells (Zysorbin, Zymed Labs, So. San Francisco, CA) essentially as described by Burgess et al. (1985). The labelled proteins were analyzed on SDS-containing, 12% polyacrylamide gels, and radioactive signals were enhanced by soaking the gels in 0.5 M sodium salicylate before drying and autoradiographing.

Endoglycosidase H (EndoH) digestion. 3 x 10^7 WEHI-231 or W88.1 cells were metabolically labelled for 6 hr with ^{35}S-Translabel and cell extracts were prepared, as described above. IgM was immunoprecipitated with rabbit anti-μ chain and eluted from the fixed *Staphylococci* by boiling for 2 min in 100 mM sodium citrate, pH 5.5, 0.2% SDS. Each sample was subsequently diluted two-fold with water and divided in half. 2 mU of EndoH from *Streptomyces plicatus* (recombinant in *E. coli*, Boehringer Mannheim, Indianapolis, IN) or an equal volume of buffer was added to each half. After digestion had proceeded for 18-20 hr at 37°C, the material was analyzed by polyacrylamide gel electrophoresis, as described above.

Measurement of $[Ca^{2+}]_i$. WEHI-231 cells or anti-IgM-resistant mutants were loaded with 5-10 μM of the acetoxymethyl ester of Indo-1 (Grynkiewicz et al., 1985; Molecular Probes, Junction City, OR) per 2 x 10^7 cells/ml, as described elsewhere (Page and DeFranco, 1988, i.e. Chapter 1 of Ph. D. Thesis). After washing once, the cells were

analyzed for calcium responses to various stimuli in fluorometer medium (growth medium containing 20 mM HEPES, but lacking phenol red and pyroxidine HCl) at 37°C using a Spex F2C spectrofluorometer (excitation 334 nm, emission 400 nm). The calcium concentrations measured by Indo-1 fluorescence were determined according to the equations of Tsien, using a K_d of 250 nM as previously described (Grynkiewicz et al., 1985; LaBaer et al., 1986).

Measurement of inositol phosphate production. WEHI-231 cells or anti-IgM-resistant mutants were washed twice with PBS and then labelled at 3 x 10^6/ml for 3 hr with 15 μCi/ml [^{3}H]inositol (15 Ci/mmol, American Radiochemical Corp., St. Louis, MO), as described elsewhere (Gold and DeFranco, 1987). The labelled cells were then washed with HEPES saline (125 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 0.1% glucose, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 25 mM HEPES, pH 7.2, 0.1 % bovine serum albumin) or growth medium containing 20 mM HEPES and resuspended in the same buffer at 2 x 10^6/ml. Some reactions were performed in growth medium to approximate the tissue culture conditions used in the other assays. 1-2 ml of labelled cells were warmed for 10 min at 37°C and stimulated as described in the text. Reactions were terminated by addition of 4 volumes of methanol:chloroform (2:1), and inositol phosphates were separated from the aqueous extract on Dowex formate columns as previously described (Gold and DeFranco, 1987). All determinations were performed in duplicate. To compare inositol phosphate production by different cell lines, the amount of [^{3}H] in the inositol phosphates was divided by the amount of [^{3}H]inositol incorporated into the membrane phospholipids and is reported as % release. The amount of radioactivity in the lipids was determined by drying the organic phases of the methanol:chloroform extractions under nitrogen and measuring the radioactivity in the residues by liquid scintillation counting.

Measurement of G protein- and calcium-stimulated phospholipase C activity in cell homogenates. 1.5-3.5 x 10^8 [^{3}H]Inositol-labelled cells were washed once with ice-cold PBS and resuspended in 3-4.5 ml ice-cold hypotonic lysis buffer (5 mM NaHEPES, pH

7.2, 1 mM $MgCl_2$, 2 mM Mg-ATP, 1 mM DTT, 1 mM EGTA, 0.4 mM $CaCl_2$, free calcium approximately 100 nM). After swelling for 10 min on ice, the cells were broken with 50 strokes in an ice-cold Dounce homogenizer. The lysed cell suspension was diluted to a concentration of approximately 20 x 10^6 cell equivalents per ml, and the buffer was adjusted to the final reaction buffer (10 mM NaHEPES, pH 7.2, 116 mM KCl, 4 mM NaCl, 10 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM Mg-ATP, 1 mM DTT, 10 mM LiCl, 1 mM EGTA, 0.4 mM $CaCl_2$). The free calcium in this cytoplasm-like buffer was approximately 100 nM. After warming to 37°C for 5 min, reactions were initiated by adding 0.5 ml of the cell homogenate to pre-warmed tubes containing 0.5 ml of reaction buffer plus the various stimuli. Free calcium concentrations were manipulated by varying the concentrations of $CaCl_2$ from 0.4-2 mM in the presence of 1 mM EGTA. Free calcium concentrations of less than 1 mM were determined experimentally using the calcium-sensitive dye Indo-1, as described previously (Gold, et al., 1987). For $CaCl_2$ concentrations of 1.1 mM and greater, the [free Ca^{2+}] was assumed to be the amount of $CaCl_2$ in excess of EGTA. The reactions were stopped after 10 min by adding 4 ml of methanol:chloroform (2:1). Some reactions were stopped immediately (time zero) in order to determine the amount of inositol phosphates present at the beginning of the reaction. Productions of total inositol phosphates was quantitated as described above, except that all of the inositol phosphates were eluted together using 1 M ammonium formate in 0.1 M formic acid. The "time zero" values were subtracted from all the experimental values in order to measure only the inositol phosphates produced during the reaction. The results are reported as % release of inositol phosphates, as described above, and each data point represents the mean and range of duplicate samples. Each experiment was performed at least twice with similar results.

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CONCLUSIONS

These studies have examined the role of the phosphoinositide signal transduction pathway in mediating the growth inhibitory effects of anti-IgM on WEHI-231 B lymphoma cells. Two approaches were taken to investigate this issue. First, the effect on WEHI-231 cells of reagents which mimic the effects of two of the phosphoinositide-derived second messengers, calcium and diacylglycerol, was examined. These second messengers were mimicked with ionomycin and phorbol 12,13-dibutyrate (PdBu). Doses of these agents were chosen such that cytosolic calcium and phosphorylation of a protein kinase C substrate were increased to levels similar to those attained by treatment of the cells with anti-IgM. It was found that these doses of the mimicking reagents, i.e. 250 nM ionomycin and 2-4 nM PdBu, synergistically inhibited the growth of WEHI-231 cells. These agents, however, induced only partial (30-60%) growth inhibition as compared to the 90-95% growth inhibition caused by treatment with anti-IgM. These results suggest that although calcium and diacylglycerol are likely playing roles as second messengers in mediating the effects of anti-IgM on WEHI-231 cells, other second messengers could also be involved.

The other possible explanation for these results is that PdBu and ionomycin cannot reproduce the actions of diacylglycerol and calcium. Although this formal possibility cannot be ruled out, it is unlikely for the following reasons. First, even high doses of the mimicking reagents, which appeared to activate protein kinase C and increase calcium more than did anti-IgM, could not reproduce the anti-IgM-induced growth arrest. These results suggest that the problem is not simply one of degree, but rather that another type of second messenger combines with diacylglycerol and calcium to cause the growth arrest. Second, the cell volume decrease caused by anti-IgM appeared to be due to arrest in the G_1 phase of the cell cycle and an additional cell volume decrease, whereas the cell volume decrease caused by PdBu and ionomycin appeared to be due only to G_1 arrest. Again, this suggests that a third signal could be responsible for the additional cell volume decrease. Finally, as discussed in Chapter 4, Tisch et al. (1988) have evidence that a non-

phosphoinositide-derived second messenger is involved in the anti-IgM-induced growth inhibition of WEHI-231 cells. If this third signal exists, it is unlikely to be cAMP, since cAMP agonists do not affect the growth of WEHI-231 cells, nor does anti-IgM affect cAMP levels in these cells (unpublished results of Gold and DeFranco). Instead, this third signal could involve the opening of an ion channel, the activation of a tyrosine kinase, or even the activation of a tyrosine phosphatase.

The second approach used to investigate the mechanism of the anti-IgM-induced growth arrest was the examination of anti-IgM-resistant mutants of WEHI-231 cells. Out of 36 mutants, the seven that were the most resistant to the growth inhibitory effects of anti-IgM were analyzed. Two of these had defects in antigen receptor expression. The other five had defects in the phosphoinositide signal transduction pathway. The fact that several mutants were obtained with mutations in this pathway suggests that it is involved in mediating the growth arrest. Of these five mutants, one mutant exhibited normal or elevated production of second messengers. This mutant was resistant to the growth inhibitory effects of the combination of PdBu and ionomycin, but not to PdBu alone or ionomycin alone. These results suggest that this mutant has an alteration in a growth regulatory component(s) that is responsive to both arms of the phosphoinositide signalling pathway. The possibility that this mutant had other mutations which were responsible for its growth resistance to anti-IgM could not be excluded. Taken together with the mimicking experiments, however, these results provide compelling evidence that diacylglycerol and calcium are playing roles in mediating the anti-IgM-induced growth arrest of WEHI-231 cells. Interestingly, the response of this mutant to PdBu and ionomycin also supported the notion that doses of PdBu greater than 4 nM were stimulating the cells more than signalling via antigen receptor-generated diacylglycerol. This mutant was not as resistant to the effects of 4-7 nM PdBu plus 125-250 nM ionomycin as it was to the effects of anti-IgM. The simplest interpretation of these results is that these doses of the mimicking reagents affect cellular response pathways more than receptor-generated

diacylglycerol and calcium, and that lower doses would more faithfully mimic the receptor-generated signals. Correspondingly, the dose combination of 250 nM ionomycin plus 2 nM PdBu inhibited the cells similarly to anti-IgM at 48 hr of treatment (19 ± 6% growth inhibition with 250I/2P versus 21 ± 10% growth inhibition with anti-IgM, mean ± SD, n=4). Thus, in agreement with the direct measurements of protein kinase C activation, these results again suggested that 4-7 nM PdBu activates protein kinase C more than the natural second messenger diacylglycerol.

The other four of the five anti-IgM-resistant mutants with defects in the phosphoinositide signal transduction pathway exhibited decreased inositol phosphate production, probably due to alterations in phospholipase C. Examination of this decreased production in response to different doses of anti-IgM suggested that it was responsible for the growth resistant phenotype of these mutants. Strikingly, the use of ionomycin with anti-IgM corrected these mutants' defective growth responses to anti-IgM. Since ionomycin was shown not to be causing additional inositol phosphate release, these results suggest that ionomycin is acting by replacing calcium as a limiting second messenger. Interestingly, calcium was not decreased in these cells from 5 min to 1 hr after stimulation with anti-IgM as compared to the wild-type cells. However, it is quite possible that the decreased production of inositol phosphates in these mutants in response to anti-IgM eventually leads to a decline in calcium levels at later times. As shown in Chapter 3, at least 18 hr of signalling are required to induce half-maximal G_1 arrest of a normally growing population of WEHI-231 cells. Thus, it is reasonable to think that the calcium levels do not remain at appropriate levels long enough for anti-IgM to induce growth inhibition in these mutants. Together, these results provide strong evidence that calcium is an essential second messenger in mediating the anti-IgM-induced growth arrest of WEHI-231 cells.

As discussed in the introduction to Chapter 1, it has been shown by several investigators that the combination of a calcium ionophore and a phorbol diester will reproduce many of the effects of anti-IgM on mature, resting B cells. These results suggest

that calcium and diacylglycerol are important second messengers in mediating the effects of anti-IgM on mature B cells. Anti-IgM has opposite effects on B cells of different developmental stages: it induces proliferation of mature B cells and inactivation of immature B cells. WEHI-231 appears to be a tumor model of an immature B cell. Since calcium and diacylglycerol also appear to be important in mediating the effects of anti-IgM on WEHI-231 cells, these results suggest that anti-IgM induces production of the same second messengers in cells of different developmental stages, but that the signals are interpreted differently. Other possibilities have not been excluded, however. For example, although calcium appears to be an essential second messenger in WEHI-231 cells, perhaps diacylglycerol is more important in mature B cells. This type of differential signalling could be achieved if anti-IgM induced more hydrolysis of the inositol phospholipids that do not produce inositol 1,4,5-trisphosphate as a second messenger in mature B cells than in WEHI-231 cells. Another possibility is that anti-IgM induces different third signals in immature and mature B cells. In immature B cells, the third signal could combine with calcium and diacylglycerol to induce growth inhibition, whereas in mature B cells, a different third signal could combine with calcium and diacylglycerol to induce proliferation. The studies described here do not specifically address these points. Instead, the resolution of these hypotheses awaits the discovery of the postulated third signal and also the examination of anti-IgM-induced signal transduction in normal immature B cells, which can be isolated from neonatal mice.

In summary, the results presented here provide strong evidence that calcium and diacylglycerol play important roles in mediating the growth inhibitory effects of anti-IgM on WEHI-231 B lymphoma cells. Moreover, studies of anti-IgM-resistant mutants suggest that calcium is an essential second messenger in this process. The results obtained, however, also suggest that diacylglycerol and calcium are not sufficient to mediate the growth arrest and, thus, that another second messenger may be necessary to mediate the full effects of anti-IgM on these cells.