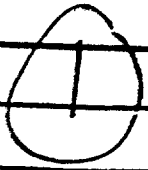


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# Bimodal Effect of Phorbol Ester on B Cell Activation

IMPLICATION FOR THE ROLE OF PROTEIN KINASE C\*

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The role of protein kinase C (PKC) in B cell activation is controversial. These studies were undertaken to determine whether protein kinase C has a stimulatory or inhibitory role in B cell activation. We found that treatment of B cells for a short period of time (30 min) with the PKC activator phorbol 12,13-dibutyrate (PDBU) primed the cells for enhanced proliferative responses to anti-immunoglobulin (anti-Ig) antibody whereas treatment for a longer period of time (3 h or more) resulted in suppression of proliferation. The enhanced proliferative response to treatment of B cells with PDBU for short periods of time was associated with inhibition of anti-Ig-stimulated increases in phosphatidyl 4,5-bisphosphate (PIP<sub>2</sub>) hydrolysis and inhibition of increases in [Ca<sup>2+</sup>]<sub>i</sub>, indicating that activation of PKC *per se* might be sufficient for enhancing B cell activation. The time-dependent effect of phorbol esters on the inhibition of B cell proliferation was found to be closely correlated with the kinetics of disappearance of PKC as measured by Western blot and by enzymatic activity but not with inhibition of [Ca<sup>2+</sup>]<sub>i</sub> and PIP<sub>2</sub>. These data demonstrate a bimodal time-dependent effect of PDBU on B cell activation and suggest that (a) the inhibitory effect of phorbol ester on anti-Ig-induced proliferation may be due to the disappearance of PKC rather than to the inhibition of PIP<sub>2</sub> and Ca<sup>2+</sup>; and (b) the early activation of PKC is a stimulatory rather than an inhibitory signal in the induction of B lymphocyte proliferation by anti-Ig.

The role of PKC<sup>1</sup> in influencing proliferation of B lymphocytes is controversial; evidence has been presented which suggests that PKC may have a stimulatory or inhibitory role. Thus, treatment of B lymphocytes with phorbol esters inhibits anti-immunoglobulin (anti-Ig)-mediated increases in phos-

phatidylinositol bisphosphate (PIP<sub>2</sub>) hydrolysis and intracellular ionized calcium [Ca<sup>2+</sup>]<sub>i</sub>, as well as cell proliferation (1-3). These results led to the suggestion that PKC has a negative regulatory effect on the early events associated with induction of mitogenesis in B lymphocytes (4, 5). Conversely, the possibility that activation of PKC in B cells may be stimulatory rather than inhibitory to anti-Ig-mediated stimulation of B cell proliferation is supported by the findings that stimulatory concentrations of anti-Ig antibody induce hydrolysis of PIP<sub>2</sub> with subsequent release of diacylglycerol (1-3, 6, 7). This intracellular mediator stimulates PKC activity (8) and induces the rapid phosphorylation of numerous cytoplasmic proteins (9, 10), enhanced expression of a number of oncogenes (11, 12), and facilitation of B cell entry into G1 or S. Some of these early events, which can also be stimulated by phorbol ester-mediated activation of PKC (13-15), appear therefore to herald cell activation rather than suppression of activation. This model of PKC-dependent B cell activation has been verified by a number of investigators who have demonstrated that B cell proliferation can be stimulated by the combination of phorbol esters and calcium ionophores (16-19). Likewise, this combination has also been shown to mimic anti-Ig-mediated inhibition of the growth of the B cell line WEHI-231 (20).

In the current study we used phorbol ester, a PKC activator, as a tool to study whether PKC has a stimulatory or inhibitory role in B cell activation. We demonstrate that phorbol ester can exert both stimulatory and inhibitory effects on B cell proliferation depending on the length of exposure of the cells to it. The implications of these observations for understanding the role of PKC in stimulation or inhibition of B cell activation are discussed.

## EXPERIMENTAL PROCEDURES

### Mice

Eight-week-old DBA/2 mice were purchased from Jackson Laboratories (Bar Harbor, ME).

### Reagents

PMA, PDBU, and ionomycin were purchased from Sigma. Monoclonal antibodies were grown as ascitic fluid in nude mice and were prepared as described previously (21). The antibodies used were: anti-Thy 1.2 (clone 30-H12) (22), anti-CD4 (clone GK1.5) (23), and anti-CD8 (clone 53-6.7) (22). The mouse monoclonal antibody against rat Ig  $\kappa$  chain Mar 18.5 (24), was produced by cells grown in tissue culture. The monoclonal antibody (H87/1) (25) with specificity for the heavy chain of IgD( $\delta$ ) was grown in nude mice and the monoclonal anti-Fc $\gamma$  receptor (24G2) (26). Dithiothreitol and leupeptin were purchased from Boehringer Mannheim. Aprotinin, pepstatin, soybean trypsin inhibitor, EGTA, EDTA, phosphatidylserine, CaCl<sub>2</sub>, and ATP were purchased from Sigma. Triton X-100 was obtained from Pierce

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<sup>1</sup> The abbreviations used in this paper are: PKC, protein kinase C; PDBU, phorbol 12,13-dibutyrate; PIP<sub>2</sub>, phosphatidyl 4,5-bisphosphate; [Ca<sup>2+</sup>]<sub>i</sub>, concentration of intracellular ionized calcium; PMA, phorbol 12-myristate 13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid; sIg, surface immunoglobulin.

Chemical Co. [ $\gamma$ - $^{32}$ P]ATP (3,000 Ci/mM) was supplied by Du Pont. Millititer filtration plates were purchased from Millipore Corp. (Boston, MA).

### Cell Cultures

**B Cell Purification**—B cells were purified as described previously (21). Briefly, single cell suspensions of spleen were washed three times in RPMI 1640 plus 10% fetal calf serum (Flow Laboratories, McLean, VA) and treated with the following antibodies: monoclonal anti-Thy 1.2 (1  $\mu$ g/ml), anti-CD4 (2.5  $\mu$ g/ml), and anti-CD8 (1:400 dilution of ascitic fluid), respectively, per  $10^7$  spleen cells for 30 min followed by treatment with newborn rabbit complement (10%) (Pel-Freez, Rogers, AR) in the presence of a 1:10 dilution of tissue culture fluid containing the anti-rat  $\kappa$  chain monoclonal antibody MAR 18.5 at 37 °C for 45 min. Small resting B cells were prepared as follows. Five ml of 50, 60, 65, and 70% Percoll were layered in a 50-ml centrifuge tube. Three ml of a spleen cell suspension that contained  $3 \times 10^8$  T-depleted spleen cells was layered over the 50% Percoll, and the tubes were placed on ice for 15 min, after which they were spun at 3,000 rpm for 15 min. The cells that banded between the 65 and 70% Percoll layers (density of 1.0815–1.086) were isolated and were used routinely in all assays.

**Cell Proliferation**—B cells were cultured for 2 days in a final volume of 0.2 ml of modified Mishell-Dutton medium in flat bottom 96-well trays (Nunc, Inter Med, Thousand Oaks, CA). [ $^3$ H]Thymidine (10  $\mu$ Ci) (Amersham Corp.) with a specific activity of 20 Ci/mmol was added to the cultures for the final 18 h, and cultures were harvested with a PhD Cell Harvester (Cambridge Technology, Watertown, MA) onto glass fiber filters. The specific incorporation of thymidine was analyzed by liquid scintillation counting, and the results are expressed as the arithmetic mean of triplicate cultures.

**Measurement of [ $Ca^{2+}$ ]**— $2.0 \times 10^7$  cells in 1 ml of HEPES-buffered RPMI with 3% fetal calf serum were loaded with 1.5  $\mu$ M indo 1 for 30 min at 37 °C. Cells were then washed and resuspended in 15 ml of HEPES-buffered RPMI with 3% fetal calf serum. Tubes with 0.5 ml of cells were prewarmed to 37 °C, and individual cells were analyzed at 300 cells/s for changes in [ $Ca^{2+}$ ], as described previously (27). Samples were analyzed for 1 min to establish an appropriate baseline after which the ligand was added at the indicated concentration ( $\mu$ g/ml) of anti- $\delta$ -dextran or unconjugated anti- $\delta$ , and analysis was continued for an additional 5 min, 30 s. The ratio of violet to blue fluorescence was converted to calcium concentration (nM) and calculated in real time for individual cells analyzed. The cumulative calcium flux of the population was determined by integration of the plot of mean [ $Ca^{2+}$ ], versus time after the cells were stimulated. Results are expressed as nM-s of calcium in stimulated cells after the subtraction of nM-s of calcium in unstimulated cells.

**Measurement of Anti-Ig-stimulated  $PIP_2$  Turnover**—For measurement of  $PIP_2$  breakdown B cells were prepared as above and cultured at  $2 \times 10^7$  cells/ml in the presence of *myo*-[ $^3$ H]inositol (20  $\mu$ Ci/ml) overnight. Cells were then washed three times, placed in polypropylene tubes at  $5 \times 10^6$ /ml at 0.2 ml/tube, in the presence of 10 mM LiCl and the anti-IgG Fc $\gamma$  receptor antibody, 24G2 (10  $\mu$ g/ml), and kept at 37 °C for 30 min. Ligands were then added, and the incubation was continued for an additional 40 min. After the incubation, the water-soluble inositol polyphosphates were isolated by the addition of 0.75 ml of chloroform:methanol (1:1) and then 0.25 ml of chloroform followed by 0.25 ml of water. Cultures were vortexed and spun at 3,000 rpm and the water phase separated and applied to a Dowex ion exchange column from which the inositol polyphosphates were eluted with 1 M sodium formate, 0.1 M formic acid (3).

**Measurement of Cellular PKC by Western Blotting**—Immunoblot analysis of PKC was performed as described previously (28). B cells ( $2.5 \times 10^6$ – $5 \times 10^6$ ) were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol, and 0.5% Nonidet-40. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and transferred electrophoretically to a nitrocellulose membrane. Immunoblot was carried out by using a 1,000-fold dilution of a goat immune serum raised against rat brain PKC (a kind gift of Dr. K. P. Huang, NIH) which reacts with all isoforms of PKC followed by a rabbit anti-goat antibody. Detection of immunoreactive bands was done with  $^{125}$ I-labeled protein A followed by autoradiography of the nitrocellulose membrane for 24 h.

**PKC Enzyme Assay**—Cell extraction was carried out as described previously by Aquino *et al.* (29).  $25 \times 10^6$  B cells/assay tube were pelleted and lysed in 200  $\mu$ l of 50 mM Tris-HCl (pH 7.5) containing

10 mM dithiothreitol, 2 mM EGTA, 1% Triton X-100, 100  $\mu$ g/ml leupeptin, 20  $\mu$ M pepstatin, 5  $\mu$ g/ml aprotinin, and 4  $\mu$ g/ml soybean trypsin inhibitor on ice for 30 min. Subsequently, the suspension was sonicated at 4 °C for 5 s and centrifuged at  $15,000 \times g$  for 6 min at 4 °C in an Eppendorf microcentrifuge.

**PKC DEAE-Sepharose Chromatography**—All procedures were carried out at 4 °C in a batchwise manner in Eppendorf tubes. A 30% suspension of DEAE-Sepharose equilibrated with Buffer A [20 mM] was prepared, 0.1 ml of this 30% suspension was pelleted in an Eppendorf tube, and 200  $\mu$ l of the soluble extract obtained as above was added for 2 min followed by vigorous vortexing. The resin was washed three times with Buffer A, and the enzyme was eluted with 100  $\mu$ l of Buffer A containing 0.3 M NaCl. Bio-Rad protein reagent was used to quantify protein in the eluate using bovine serum albumin as the standard.

Five  $\mu$ l of the DEAE-Sepharose eluate was used to assay PKC activity according to Aquino *et al.* (29).

### RESULTS

**Brief Exposure of B Cells to Phorbol Ester Enhances Anti-Ig-stimulated B Cell Proliferation**—The fact that mitogenic concentrations of anti-Ig induce the release of diacylglycerol (6, 7), a PKC activator (8), is consistent with the possibility that PKC has a stimulatory role in B cell activation. However, observations that exposure of B cells to phorbol ester inhibits early signal transduction and DNA synthesis suggest that the activation of PKC may also inhibit B cell activation (1–3). One possible explanation of this apparent discrepancy is that the low avidity, reversible interaction between diacylglycerol and PKC initiates a stimulatory rather than inhibitory effect whereas the irreversible, high avidity PKC-phorbol ester interaction initiates an inhibitory pathway. To explore this possibility we treated B cells for 30 min with the phorbol ester PDBU, which binds to PKC with a lower avidity than does PMA and can be effectively washed free from cells.<sup>2</sup> This enabled us to control the duration of PKC-phorbol ester interaction. After a 30-min culture period at 37 °C with medium or 100 ng/ml PDBU, B cells were washed extensively and placed in culture with anti- $\delta$  antibody (Table I). The 30-min PDBU pulse enhanced the proliferative response of B cells that were cultured with anti- $\delta$  antibody. Of 10 experiments of this kind, the enhancement in thymidine incorporation induced by pulse with PDBU ranged between 1.5- and 4-fold. When PDBU was left in for the duration of culture at concentrations of 100 ng/ml, there was a marked suppression of the B cell proliferative response, confirming previous reports (4, 5).

To exclude the possibility that enhancement might have resulted from the continued presence of very low concentrations of PDBU which remained after washing and which may have acted synergistically with anti-Ig to induce enhanced proliferation, we cultured B cells with anti-Ig together with PDBU at concentrations ranging from  $10^0$  to  $10^{-9}$  ng/ml PDBU and evaluated thymidine incorporation 2 days later. No enhancement was observed at any concentration of PDBU employed (data not shown). Although this finding does not exclude the possibility that low concentrations of PDBU may be sequestered in the cytoplasm and contribute to cell activation, it makes it considerably less likely. When an inactive phorbol ester (4 $\alpha$ -phorbol 12,13-dibutyrate) that does not bind to PKC was employed instead of PDBU, no enhancement or suppression of B cell stimulation was observed (not shown).

In an attempt to demonstrate that such short PDBU pulses enhance B cell stimulation via its effects on PKC, we cultured B cells together with the PKC inhibitors H-7 or staurosporine in the presence of PDBU for the 30-min pulse period. At all

<sup>2</sup> P. Blumberg, personal communication.

TABLE I

Activation of PKC for brief periods of time enhances anti-Ig-stimulated B cell proliferation

Resting B cells were cultured with PDBU (100 ng/ml) for 30 min followed by two rapid washes in large volumes of complete medium and placed in culture with an optimal mitogenic concentration of anti-IgD. The other group was cultured for the duration of the culture period with PDBU (100 ng/ml). All cultures were pulsed with [<sup>3</sup>H]thymidine (1.0 μCi/ml) at 24 h and harvested 18 h later. Thymidine incorporation was measured as described under "Experimental Procedures." Results are expressed as the arithmetic mean of triplicate wells. This is one of 10 representative experiments.

	Thymidine incorporation		
	Medium	PDBU 30-min pulse	PDBU continuous
	cpm		
Medium	719 ± 53	1,162 ± 96	564 ± 41
Anti-IgD	5,432 ± 643	11,908 ± 1260	972 ± 22

TABLE II

Effect of PDBU treatment on PIP<sub>2</sub> and [Ca<sup>2+</sup>]<sub>i</sub>

Resting B cells were cultured in medium or PDBU (100 ng/ml) for 30 min or 18 h followed by washing at 4 °C with large volumes of complete medium and then with the addition of anti-IgD for 40 min. Other B cells were cultured with PDBU for 30 min at which time anti-IgD antibody was added for an additional 40 min in the continued presence of PDBU (PDBU continuous column). Inositol phosphates were extracted as described. For analysis of intracellular ionized calcium, B cells were loaded with indo 1 and 30 min for 34 °C and treated with PDBU as described above. Analysis of mean peak [Ca<sup>2+</sup>]<sub>i</sub> was carried out as described under "Experimental Procedures."

	Medium	PDBU continuous	PDBU 30-min pulse	PDBU 18 h
		cpm water-soluble phosphoinositides		
PIP <sub>2</sub> hydrolysis				
Medium	131 ± 20	96 ± 15	104 ± 17	110 ± 15
Anti-IgD	343 ± 40	129 ± 22	246 ± 27	546 ± 25
	(nM) [Ca <sup>2+</sup> ] <sub>i</sub>			
Flow cytometric analysis of [Ca <sup>2+</sup> ] <sub>i</sub>				
Medium	125	125	125	125
Anti-IgD	715	216	461	770

concentrations tested which inhibited PKC activity there was a substantial nonspecific toxic effect on B cells. We therefore were unable to conclude definitively that PDBU mediated its effect by activation of PKC.

It may be argued that PDBU exerts its effect via potentiation of early PKC-independent events associated with B cell activation. Thus, we determined whether a 30-min pulse with PDBU stimulates the early events of B cell activation reflected by PIP<sub>2</sub> turnover and calcium mobilization. B cells were pulsed with PDBU for various times followed by extensive washing. After this pretreatment, anti-Ig-stimulated changes in [Ca<sup>2+</sup>]<sub>i</sub> and hydrolysis of PIP<sub>2</sub> were measured. Although treatment with PDBU for 30 min suppressed PIP<sub>2</sub> hydrolysis and calcium mobilization significantly the suppressive effect induced by this short exposure to PDBU was very short lived in that there was a rapid return toward control levels of stimulation when PDBU was removed and anti-Ig stimulation continued for an additional 40 min (Table II). Enhancement of anti-Ig-stimulated PIP<sub>2</sub> hydrolysis or [Ca<sup>2+</sup>]<sub>i</sub> was never observed after PDBU pulsing for times ranging from 5 to 45 min, and in fact there was always a significant suppression of these early biochemical events after short pulses with PDBU. This suggests that the stimulatory effect on B cell proliferation produced by a brief PDBU pulse is not mediated by potentiation of [Ca<sup>2+</sup>]<sub>i</sub>-related or PIP<sub>2</sub>-related events but rather is related to activation of PKC.

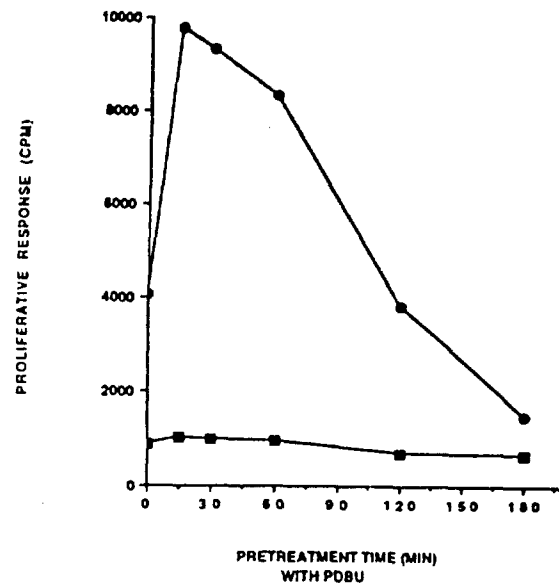


FIG. 1. B cells were cultured with medium (■—■) or with PDBU (●—●) for various times ranging from 15 to 180 min and then washed extensively in medium with 10% fetal calf serum. Cells at  $1 \times 10^5$ /well were cultured for an additional 48 h with medium only or with anti-δ antibody (10 μg/ml), and incorporation of the [<sup>3</sup>H]thymidine was measured. For cells that were cultured in medium only or PDBU only, thymidine incorporation was 275 and 210 cpm, respectively. These results represent one of four representative experiments.

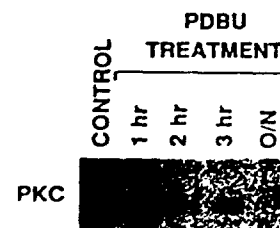


FIG. 2. Purified B cells were cultured with medium or with PDBU for 60, 120, or 180 min and overnight, lysed in 0.5% Nonidet P-40, and immunoblotted as described under "Experimental Procedures" using an antibody to PKC which reacts with all the isoforms of PKC.

TABLE III

Rapid depletion of PKC enzymatic activity after short term culture of B cells with PDBU

B cells were cultured with medium or with PDBU for 2, 3, or 18 h and PKC enzymatic activity determined as described under "Experimental Procedures." This experiment is one of three representative experiments.

B cell culture	PKC activity
Medium	1.85
PDBU (200 ng/ml) (2 h)	0.34
(3 h)	0.22
(18 h)	Undetectable

**Inhibitory Effect of PDBU on Anti-Ig-mediated Stimulation Is Correlated with Depletion of PKC but Not with Inhibition of [Ca<sup>2+</sup>]<sub>i</sub> Increases**—To determine the kinetics of the PDBU-mediated enhancement, we cultured cells for 15–180 min with 100 ng/ml PDBU followed by extensive washing and culture with anti-δ antibody (Fig. 1). Enhancement of anti-δ-induced proliferation was observed when cells were pulsed for 15–60 min with PDBU. Extending the length of the PDBU pulse to 120 min eliminated the enhancement of proliferation, and extending the length of the pulse to 180 min suppressed

TABLE IV

Phorbol ester-mediated inhibition of anti-Ig-stimulated increases in  $[Ca^{2+}]_i$ , does not suppress B cell proliferation.

Percoll-separated resting B cells were treated as follows. Group 1 was cultured for 60 min in medium; an aliquot was then stimulated with anti-IgD, and  $[Ca^{2+}]_i$  was measured. The remainder was placed in culture for 48 h, and  $[^3H]$ thymidine was measured. Group 2 was cultured with 100 ng/ml PDBU for 60 min; an aliquot of cells was then stimulated with anti-IgD in the presence of PDBU, and  $[Ca^{2+}]_i$  was measured. The remainder was cultured for 48 h with anti-IgD + PDBU, and  $[^3H]$ thymidine was measured. Group 3 was cultured with 100 ng/ml PDBU for 60 min followed by extensive washing; an aliquot of cells was then stimulated with anti-IgD, and  $[Ca^{2+}]_i$  was measured. The remainder was placed in culture with anti-IgD for 48 h and  $[^3H]$ thymidine measured. Group 4 was cultured with PDBU for 15 min prior to the addition of anti-IgD in the continued presence of PDBU for an additional 45 min. Cells were then extensively washed free of PDBU at 4 °C. An aliquot of cells was then stimulated with anti-IgD, and  $[Ca^{2+}]_i$  was measured. The remainder was placed in culture with anti-IgD for 48 h, and  $[^3H]$ thymidine was measured. Increases in  $[Ca^{2+}]_i$  are measured as nM-s, which reflects the cumulative calcium flux of the stimulated populations over a 6-min time period. This is one of three representative experiments.

	PDBU treatment			Anti-Ig-mediated stimulation of	
	0 min	15 min	60 min	$[Ca^{2+}]_i$	$[^3H]$ Thymidine incorporation
				nM-s	cpm
Group 1			Anti-Ig	39,400	8,500
Group 2	PDBU	PDBU + anti-Ig	PDBU	200	500
Group 3	PDBU	PDBU	Wash and readd anti-Ig	38,200	8,838
Group 4	PDBU	PDBU + anti-Ig	Wash and readd anti-Ig	3,500	9,430

proliferation to varying degrees (Fig. 1). To determine if the suppressive effect of a more prolonged pulse of PDBU results from PKC depletion, we cultured B cells in medium only or with PDBU for 60, 120, or 180 min and overnight and evaluated total cellular PKC by Western blotting techniques. Although there was significant diminution of cellular PKC after treatment of B cells with PDBU for 60 min there was little detectable PKC remaining after treatment for 180 min (Fig. 1).

Furthermore, when B cells were treated for 18 h with PDBU there was no significant depletion of PKC at 24 h after the removal of PDBU (not shown). To exclude the possibility that the anti-PKC antibody we were using may not have detected as yet unidentified PKC isozymes, we measured PKC enzymatic activity in B cells after a 2-, 3-, and 18-h incubation period with medium or PDBU (Table III). On average, less than 10% of enzymatic activity remained after cells were cultured for 18 h with 10 ng/ml PDBU. These data demonstrate that the kinetics of depletion of PKC is correlated with

the kinetics of the suppressive effect of PDBU on B cell activation. The implication of this observation is contradictory to the previous suggestions that the suppressive effect of PMA on B cell proliferation is due to inhibition of  $PIP_2$  and  $[Ca^{2+}]_i$ , which is mediated by activation of PKC (4, 5). In fact, we found that B cells that were depleted of PKC showed enhanced  $PIP_2$  hydrolysis and  $[Ca^{2+}]_i$  mobilization in response to anti-Ig antibody (Table II, PDBU 18 h column). In order to explore this point further we designed an approach that was aimed at inhibiting the early anti-Ig stimulated increases in  $[Ca^{2+}]_i$ , using phorbol esters and determining the effect on total DNA synthesis. Preincubation of B cells with PDBU for 15 min at 37 °C followed by the addition of anti-Ig antibody (without washing of PDBU) inhibited to almost background levels the anti-Ig-induced increases in  $[Ca^{2+}]_i$ , and in thymidine incorporation (Table IV, group 2). Anti-Ig-mediated modulation of surface immunoglobulin (sIg) was unchanged, and sIg was rapidly patched and capped under these conditions.<sup>3</sup> A parallel group of cells was treated similarly with PDBU for 15 min followed by anti-Ig (in the continued presence of PDBU) for an additional 45 min to induce modulation of sIg in the absence of elevations in  $[Ca^{2+}]_i$ , and was then washed twice at 4 °C to remove PDBU and to prevent regeneration of sIg that had been modulated. These washed

cells were divided into two groups for determination of stimulation of  $[Ca^{2+}]_i$  and thymidine incorporation after the read-dition of anti-Ig antibody in the absence of PDBU (Table IV, group 4). A greater than 90% diminution in stimulation of  $[Ca^{2+}]_i$  was observed, even in the absence of PDBU. Control groups of B cells that had been pulsed with PDBU for 60 min in the absence of Ig receptor modulation followed by washing showed nearly normal increases after anti-Ig stimulation of  $[Ca^{2+}]_i$  (Table IV, group 3), indicating that the PDBU-mediated inhibitory effect was rapidly reversible. Thus, the absence of detectable anti-Ig antibody stimulated calcium mobilization in the cells that had been treated with anti-Ig + PDBU followed by washing was not a result of residual PDBU but reflected either desensitization of B cells by prior exposure to anti-Ig or the inability of anti-Ig to stimulate large increases in  $PIP_2$  hydrolysis in B cells that had had most of their sIgD removed by prior exposure to stimulating concentrations of anti-IgD antibody. Despite the greater than 90% suppression of increase in  $[Ca^{2+}]_i$ , these B cells that had been pretreated with PDBU and anti-Ig antibody proliferated in response to anti-Ig antibody with a magnitude comparable to that of control cells. These B cells that had been pretreated with PDBU and anti-Ig antibody and recultured in the constant presence of anti- $\delta$  antibody for the duration of the culture period expressed barely detectable amounts of sIgD and could not be stimulated to show any additional increases in  $[Ca^{2+}]_i$ . Thus, any sIg-mediated  $PIP_2$ -dependent signals that might occur at later times in the response would be stimulated at very low levels, if at all. These data demonstrate that the phorbol ester-mediated inhibition of  $[Ca^{2+}]_i$  and  $PIP_2$  is not necessarily associated with suppression of proliferation, thus supporting the hypothesis that PDBU-mediated suppression of B cell activation may result from PKC depletion.

#### DISCUSSION

The consensus of the numerous studies investigating the effects of phorbol ester on B cell activation is that it inhibits anti-Ig-mediated murine splenic B cell activation (4, 5) and stimulates human B cell activation (30) by itself or in conjunction with anti-Ig (31). Although it has been demonstrated recently that PMA can also stimulate proliferation in a discrete population of murine  $Ly1^+$  B cells (32), all reports to date that study  $Ly1^+$  murine B cell activation indicate that PKC has a marked inhibitory influence on both early and late

<sup>3</sup>J. J. Mond, N. Feuerstein, C. H. Juner, A. K. Halapure, R. I. Glazer, K. Witherspoon, and M. Brunswick, personal observations.

B cell activation events. Thus, phorbol esters inhibit anti-Ig-stimulated  $\text{PIP}_2$  hydrolysis and increases in  $[\text{Ca}^{2+}]_i$  as well as the later event of B cell DNA synthesis (1-5). The data in this paper demonstrate a bimodal effect of phorbol ester on B cell activation and suggest that the ability of a phorbol ester to inhibit these early activation events may not account for its ability to inhibit anti-Ig-stimulated B cell proliferation. Thus, when surface Ig was modulated under conditions in which little increase in  $[\text{Ca}^{2+}]_i$  or in  $\text{PIP}_2$  hydrolysis was noted, the resultant sIg "sparse" B cells were stimulated to proliferate by anti-Ig with no significant diminution in their responses. This suggests that suppression of  $\text{PIP}_2$  hydrolysis and of  $[\text{Ca}^{2+}]_i$  mobilization by PDBU is not necessarily associated with inhibition of the proliferative response. In addition, we demonstrate that after prolonged exposure of B cells to PDBU there is an increase in anti-Ig-mediated elevations in  $\text{PIP}_2$  hydrolysis and  $[\text{Ca}^{2+}]_i$  without induction of proliferation. This result supports the conclusion that the inhibitory effect of PDBU on proliferation is not due to inhibition of  $[\text{Ca}^{2+}]_i$  and  $\text{PIP}_2$ . The time-dependent inhibitory effect of PDBU on B cell proliferation was associated more closely with a time-dependent decrease in PKC activity.

Our data therefore are more supportive of the hypothesis that the prolonged exposure of B cells to phorbol esters is inhibitory because PKC is reduced markedly within a relatively short time (3 h) after PDBU treatment, and anti-Ig-mediated stimulation requires that PKC be present in the B cell above a threshold level for a period greater than 3 h. Not only was PKC activation *per se* not inhibitory to B cell activation, it was stimulatory; thus, short exposure of B cells to PDBU enhanced its subsequent proliferative response to anti-Ig.

Taken together, these data suggest that phorbol esters can mediate both stimulatory and inhibitory effects on B cells. A similar phenomenon describing the effects of short or prolonged exposure of cells to PDBU has been reported in neutrophils (34). Thus, neutrophils that are primed for 30 min with PMA show enhanced fMet-Leu-Phe-stimulated superoxide generation but inhibition of  $[\text{Ca}^{2+}]_i$  (35-37). Other studies demonstrated that short pulses of neutrophils with diacylglycerol may lead to either a priming or inhibition of respiratory burst (38-40). Our data also suggest that the avidity or the duration of binding of agonists to PKC may play a critical role in determining whether stimulatory or inhibitory pathways are recruited. Thus, a short pulse with PDBU may mimic more closely the reversible binding that may be observed with binding of low concentrations of diacylglycerol to PKC, which would result in B cell activation, than the tight irreversible binding of PMA which leads to inhibition. This hypothesis is supported by two recent reports. One demonstrates that PMA but not 1-oleoyl-2-acetyl-glycerol, a physiologic PKC activator, induced the translocation of PKC from the cytoplasm to the membrane. 1-Oleoyl-2-acetyl-glycerol was only effective in synergizing with ionomycin in inducing PKC translocation (40). Furthermore, PKC translocation induced by 1-oleoyl-2-acetyl-glycerol and ionophore was a transient phenomenon as compared with the long lasting translocation mediated by PMA. A more recent report demonstrated that a short exposure of T cells to diacylglycerol analogues stimulates enhanced expression of interleukin 2 receptors whereas prolonged exposure to these analogues or to PMA leads to interleukin 2 secretion (41). These data support a recent report that suggested that the role mediated by the hydrolysis products of  $\text{PIP}_2$  *in vitro* may be quite different from that mediated by phorbol esters (42). Furthermore, it indicates the need for caution when phorbol esters are used as a tool to examine the

role of PKC in cellular activation.

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