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Signals transduced through the B cell Ag receptor (BCR) drive B cell development. However, BCR-induced responses are developmentally regulated; immature B cells are tolerated following antigenic exposure while mature B cells are triggered to proliferate and differentiate. This differential responsiveness allows for the negative selection of self-reactive immature B cells while simultaneously allowing for clonal expansion of mature B cells in response to foreign Ags. Intrinsic differences in BCR-induced signal transduction at various stages of development may account for this functional dichotomy. We had previously demonstrated that the BCR-induced proliferation of mature B cells is accompanied by an increase in intracellular calcium levels and polyphosphoinositide bis phosphate (PIP$_2$) hydrolysis. In contrast, immature B cells that undergo BCR-induced apoptosis increase intracellular calcium in the relative absence of PIP$_2$ hydrolysis. Since PIP$_2$ hydrolysis leads to the generation of diacylglycerol, a cofactor for protein kinase C (PKC) activation, these data suggested that an “imbalance” in BCR-induced signal transduction resulting from a relative inability to activate PKC may play a role in the susceptibility of immature B cells to BCR-induced apoptosis. In support of this hypothesis, we demonstrate that PKC activation can rescue immature B cells from BCR-induced apoptosis. Furthermore, the susceptibility of immature B cells to BCR-induced apoptosis is recapitulated in mature B cells that are either PKC depleted or are stimulated in the presence of PKC inhibitors, suggesting that an uncoupling of PKC activation from BCR-induced signaling is responsible for the apoptotic response of immature B cells.


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throughout B cell development, signals transduced through the B cell Ag receptor (BCR) play an important role in regulating B cell maturation (reviewed in Refs. 1–5). However, BCR (or pre-BCR)-induced signals can lead to dramatically different functional responses, depending on the maturational stage of the B cell. For instance, although both immature and mature B cells express the mature, Ag-binding form of the BCR, immature B cells undergo negative selection in response to receptor ligation while mature B cells are induced to proliferate and secrete Ig. Immature B cells in the bone marrow that have just begun to express surface (s)IgM, as well as late-immature or transitional-stage B cells that have recently emigrated from the bone marrow to the spleen and that express high levels of sIgM and low levels of sIgD, are sensitive to this tolerization process (6–8). The sensitivity of immature B cells to tolerization following antigenic exposure is thought to be critical for the maintenance of immunological self-tolerance. Immature B cells are thought to be tolerated by a number of mechanisms including clonal anergy (9), receptor editing (10–12), competition for follicular niches (13, 14), and clonal deletion (3, 15). The number of newly generated immature B cells greatly exceeds that of mature B cells (16, 17), suggesting that a vast majority of these cells may undergo Ag-mediated negative selection. Although the relative susceptibility of immature B cells to tolerance induction has been appreciated for many years, the molecular events that distinguish an activation signal in mature cells from a tolerogenic signal in immature cells have yet to be elucidated.

According to the original model of Bretscher and Cohn (18), B lymphocytes stimulated through their Ag receptor (signal 1) in the absence of T cell help (signal 2) would be tolerated. The relative lack of T cells in the bone marrow that could provide a “second signal” to immature B cells exposed to self Ag suggested that the negative selection of immature B cells occurred because of a lack of T cell help. However, lack of T cell help does not totally account for the relative inability of immature lymphocytes to proliferate in response to Ag; highly purified populations of mature B cells proliferate in response to anti-Ig stimulation in vitro in the absence of T cell help, while immature B cells readily undergo apoptosis under the same stimulatory conditions (19–21). However, addition of cytokines, such as IL-4, or ligation of CD40 makes it possible to rescue immature lymphocytes from anti-Ig-induced death, indicating that the availability of T cell help can certainly influence the outcome of negative selection (19, 22, 23). It has also been suggested that the dichotomy in responses elicited by BCR engagement in immature (IgMhighIgDdull) vs mature (IgMlowIgDhigh) B cells could be due to differential signaling through sIgM and sIgD (24). However, we have recently reported that ligation of IgD on immature, transitional B cells readily induces apoptosis and fails to protect from anti-IgM-induced apoptosis (20), indicating that the differential responses of immature and mature B cells cannot be attributed to a “protective” signaling mechanism transduced through sIgD. These results are consistent
with studies done in transgenic mice that express either anti-hen egg lysozyme IgM or IgD (25). In these studies, exposure to Ag led to efficient tolerization in both IgM-only and IgD-only expressing mice, conclusively demonstrating that signaling through IgD does not protect immature B cells from negative selection. Taken together, these observations suggest that the differential sensitivity to negative selection of the immature and mature B lymphocyte must be due to intrinsic, developmentally regulated differences in the Ag-receptor-mediated signal transduction pathways.

Ag-receptor ligation of mature B cells leads to the activation of multiple signaling pathways, including those involving tyrosine kinases and phosphatases, Ras and mitogen-activated protein kinases (MAPKs), and phosphoinositide-3 kinase (26). Activation of the src family kinases following BCR engagement leads to the eventual tyrosine phosphorylation and subsequent activation of phospholipase C γ (27–29), resulting in the breakdown of polyphosphoinositide bis phosphate (PIP2) into the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG) (30, 31). IP3 is believed to be responsible, at least in part, for the release of calcium from intracellular stores, while DAG can activate a subset of PKC isoenzymes, including the conventional isoenzymes, PKC-α, -β, and -γ and the “novel” isoenzymes, PKC-δ and PKC-ε (32). While it is not known what roles individual PKC isoenzymes may play in B cell activation, it is clear that they are not completely interchangeable. PKC-β-deficient mice exhibit a phenotype that is similar to the xid immunodeficient mouse strain in that they display a reduced primary response to T cell-dependent Ags, as well as a decreased response to T cell-independent type II Ags (33).

Although these mice presumably express other PKC isoenzymes, they appear to be unable to replace PKC-β in signaling through the BCR, thus demonstrating a unique requirement for PKC-β in B cell activation.

Previous studies from our laboratory have demonstrated that although mature B cells respond to BCR cross-linking by increasing PIP2 hydrolysis and elevating intracellular-free calcium levels, immature B cells increase intracellular calcium levels in the relative absence of PIP2 hydrolysis (34, 35). Such findings suggest that while DAG-responsive PKC isoenzymes may be activated in mature B cells following slg cross-linking, they are unlikely to be activated in response to BCR-induced signal transduction in immature B cells. These findings led us to propose a hypothesis in which an imbalance in BCR-induced signal transduction due to the relative inability of immature B cells to activate DAG-responsive PKC isoenzymes may be responsible for the BCR-induced apoptosis of immature B cells. In this report, we present evidence that suggests that activation of PKC can rescue immature B cells from BCR-induced apoptosis. Furthermore, mature B cells that are either depleted of PKC or are stimulated in the presence of PKC inhibitors are rendered susceptible to BCR-induced apoptosis, suggesting that a relative inability to activate PKC following BCR engagement is responsible for the apoptotic response. These results suggest that an uncoupling of PKC from BCR-induced signal transduction may, therefore, play an integral role in the negative selection of developing B lymphocytes.

Materials and Methods

Animals and reagents

All mice used in experiments were BALB/c, obtained from The Jackson Laboratory (Bar Harbor, ME), and maintained in our colony. For autoreconstitution studies, adult mice 8–10 wk of age were subjected to 500 rad of whole-body γ irradiation, then the peripheral lymphoid compartments were allowed to reconstitute over a period of 13–15 days, as described (7). F(ab′)2 fragments of polyclonal rabbit anti-mouse IgM were created in our laboratory and are described previously (36). PMA, phorbol dibutyrate (PDBu), 4α- and 4β-phorbol diecanoate (PDD), and thapsigargin were purchased from Sigma (St. Louis, MO), and ionomycin was purchased from Calbiochem (SanDiego, CA). Polyconal anti-PKcα, -PKcβ, -PKcδ, and -PKcε were purchased from Life Technologies (Gaithersburg, MD). 12-deoxyphorbol-13-phenylacetate 20-acetate (DOPPA), a PKc-β1-specific activator was purchased from Biomol Research Labs (Plymouth Meeting, PA), while the PKC inhibitors bisindolylmaleimide I and Ro-32-0432 were purchased from Calbiochem.

B lymphocyte purification

Spleenic B cells were prepared as previously described (19). Briefly, adult mice were killed by cervical dislocation at 8–10 wk of age. Spleens were removed aseptically and placed in HBSS + 2% FCS. Single cell suspensions were prepared by grinding spleens through the frosted ends of glass slides. To remove T cells, the cells were treated with anti-Thy-1.2 (HO-13-4) (37), rabbit complement, and DNase I. BRC were lysed by treatment with 150 mM EGTA, 50 mM Tris (pH 7.4), and the cells were washed two more times with 50/75% Percoll gradient (Pharmacia Biotech, Piscataway, NJ). B cells from autoreconstituting mice were isolated in an analogous manner. In autoreconstituting mice, B cell populations are regenerated in a relatively synchronous wave (7, 8). Late-stage immature or transitional-stage B cells (IgMhigh, IgDlow, B220int, heat-stable Ag (HSA)high) can be found in the spleens of autoreconstituting mice as early as day 13 and persist as long as day 18. After this time, the cells begin to acquire the phenotypic characteristics of mature B cells (IgMhi, IgDlo, B220hi, HSAlo) and after day 21, respond functionally as do mature cells (L.B.K., unpublished observation).

Apoptosis assay

Purified B cells were cultured under various conditions overnight in either sterile 2-ml click-cap tubes or 96-well plates. When utilized, PKC inhibitors were added 1.5 min before the addition of the stimulant. After 14–18 h in the case of immature B cells or 24 h in the case of mature B cells, the cells were harvested and washed in PBS containing 2% FCS. Cells were fixed and permeabilized by resuspending in 70% ethanol made in 1× PBS and incubating at −20°C for at least 2 h. After incubation, the cells were washed twice with PBS + 2% FCS and finally resuspended in a staining solution made in 1× PBS containing 50 µg/ml of RNAse A, 0.01% sodium azide, and 10 µg/ml of propidium iodide (PI). The cells were stained at 4°C overnight and analyzed by flow cytometry on a Becton Dickinson (Mountain View, CA) FACScan using LYSYS II software. The mean percentages of apoptotic cells and the SEs for each culture condition were determined and significance assessed by the Student’s two-tailed t test.

Measurement of intracellular calcium

Purified B cells were washed in HBSS without phenol red and supplemented with 2% FCS (HBSS + 2% FCS). For loading, cells were resuspended in HBSS without phenol red and supplemented with 10% FCS (HBSS + 10% FCS). Fura-2 acetoxy methyl ester (Molecular Probes, Eugene, OR) was added to a final concentration of 1 µM from a 1 mM stock made in DMSO. Cells were incubated in light tight tubes for 30 min at 30°C with occasional inversion. Cells were then washed twice with HBSS + 2% FCS and finally resuspended at a concentration of 3.3 × 106/ Artifact 1× 107/ml in HBSS (pH 8.5), 150 mM NaCl, 0.05% Tween 20) plus 2% BSA to block nonspecific binding. Membranes were washed twice with TBST and probed with the appropriate Ab solutions for 90 min, then washed three times.
times in TBST, followed by secondary incubation with the appropriate horseradish peroxidase-conjugated reagents. Three washes with TBST were conducted before detection with the enhanced chemiluminescence system (Amer sham, Arlington Heights, IL).

Modified TUNEL assay

In some cases, the frequency of apoptotic cells was quantitated using a modified TUNEL assay (38). Briefly, \(1\times10^6\) cells were fixed in 2.5% formaldehyde in 1:1 PBS. The fixed cells were permeabilized in 0.2 ml of 0.1% Triton/0.1% citrate for 2 min on ice. After washing, cells were incubated in 50 \(\mu\)l of nick translation buffer (500 mM Tris (pH 7.5)/100 mM MgSO\(_4\)/1 mM DTT), 0.03 \(\mu\)l of fluorescein-dUTP (1 nmol/\(\mu\)l; Boehringer Mannheim, Indianapolis, IN), 1 \(\mu\)l of dTTP (0.7 nmol/\(\mu\)l), 1 \(\mu\)l each of dATP, dCTP, dGTP (1 mmol/\(\mu\)l), and 0.1 \(\mu\)l of DNA polymerase (5 U/\(\mu\)l) from Boehringer Mannheim (Indianapolis, IN), and incubated for at least 1 h at 37°C. Cells were then washed and analyzed by flow cytometry on a Becton Dickinson FACScan.

Results

To further investigate the role of BCR-initiated signal transduction in immature B cell negative selection, we have utilized an in vitro model system, which we have previously described (19, 20), that makes use of an immature population of B cells purified from the spleens of adult mice after sublethal irradiation (7, 8). Immature B cells obtained from these autoreconstituting mice are phenotypically immature (IgM\(^{hi}\), IgD\(^{lo}\), B220\(^{int}\), HSA\(^{hi}\)) and respond identically to immature B cells purified from either neonatal spleen or adult bone marrow (20, 19). This homogeneous population of immature B cells is devoid of pre-B cells and mature B cells found in neonatal spleen and adult bone marrow, respectively. Furthermore, unlike the immature B cells purified from either bone marrow or neonatal spleen, immature B cells from autoreconstituting mice can be isolated without positive selection on anti-Ig panning plates. Since large numbers of unmanipulated immature B cells can be obtained, autoreconstituting B cells are an ideal system for analyzing biochemical events responsible for tolerogenic signal transduction. The immature B cells isolated from autoreconstituting mice exist as a small population in normal adult spleen and represent a “transitional” stage of development in which cells are subject to negative selection in vivo (6). In this in vitro system, apoptosis is used as an indicator of negative selection.

Immature B cells undergo apoptosis in response to BCR cross-linking in vitro

Purified populations (>85% B220\(^{+}\)IgM\(^{+}\)) of either immature B cells obtained from the spleens of day 14 autoreconstituting mice or mature B cells isolated from the spleens of normal adult mice were incubated in the absence or presence of 50 \(\mu\)g/ml of rabbit anti-mouse IgM F(ab')\(_2\) fragments (anti-Ig). Cells were harvested, fixed, and permeabilized in cold ethanol, stained with PI, and assayed by flow cytometry. The frequency of apoptotic cells was assessed by the number of cells with subdiploid amounts of DNA.

Activation of PKC prevents apoptosis induced by anti-Ig

Mature B cells that proliferate in response to BCR engagement increase intracellular calcium levels and hydrolyze PIP\(_2\) in response to BCR cross-linking. In contrast, immature B cells are susceptible to BCR-induced apoptosis and respond to specific Ag-receptor signaling in the absence of detectable PIP\(_2\) hydrolysis (35). Based on these results, a hypothesis was proposed that stated that an “imbalance” in BCR-induced signal transduction in immature B cells in which the activation of DAG-dependent conventional and novel PKC isoenzymes (39, 40) might be compromised, may result in their enhanced sensitivity to BCR-induced apoptosis. If the inability to activate PKC in response to BCR cross-linking was directly linked to the induction of apoptosis, it would suggest that pharmacologic activation of PKC in immature B lymphocytes should protect them from anti-Ig-induced death. To test this hypothesis, isolated immature B cells were cultured with the phorbol ester, PMA, which bypasses the Ag receptor and activates DAG-dependent PKC isoenzymes directly (40). Late-stage immature B cells were cultured for 18 h in the presence of medium alone, anti-IgM F(ab')\(_2\) fragments (50 \(\mu\)g/ml), PMA (10 ng/ml) or the combination of anti-Ig and PMA. After culture, the cells were harvested and assayed for relative DNA content. As shown in Fig. 2, activation of PKC in the immature B cell population resulted in

![FIGURE 1. Immature B cells undergo apoptosis in response to BCR cross-linking in vitro. Purified immature B cells (A and B) or mature B cells (C and D) were cultured for 16–18 h in the absence (A and C) or presence (B and D) of 50 \(\mu\)g/ml of rabbit anti-mouse IgM F(ab')\(_2\) fragments (anti-Ig). Cells were harvested, fixed, and permeabilized in cold ethanol, stained with PI, and assayed by flow cytometry. The frequency of apoptotic cells was assessed by the number of cells with subdiploid amounts of DNA.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.120.1.2657)
significant protection from anti-IgM-induced apoptosis. The ability of phorbol esters to activate PKC is critical for the observed response; in contrast to the biologically active stereoisomer 4β-PDD, which efficiently rescued the cells, the inactive stereoisomer 4α-PDD, did not (Fig. 2). These observations suggest that while BCR-induced signaling events can lead to cell death, activation of PKC is sufficient to rescue the cell from apoptosis.

The relative inability of PKC-β-deficient mice to proliferate to anti-Ig suggested that activation of this particular conventional PKC isoenzyme may play an important role in transducing a positive signal following BCR cross-linking. Based on these observations, we next addressed the question of whether the specific activation of PKC-β would rescue immature B cells from BCR-induced apoptosis. For these experiments, DOPPA, a selective activator of PKC-β in vitro, was utilized (41). As shown in Fig. 2B, addition of DOPPA rescues immature B cells from BCR-induced apoptosis in a dose-specific manner and at doses that correspond well to the amount required for PKC-β activation in vitro (41). These results suggest that PKC-β activation may play an important role in rescuing immature B cells from BCR-induced apoptosis. However, it must be pointed out that while some groups have observed that DOPPA maintains its selective activation of PKC-β in vivo (42, 43), other groups have noted the activation of other PKC isoenzymes, perhaps due to the deacetylation of DOPPA (44). Even with this caveat, these data support the hypothesis that the inability to activate PKC (and perhaps PKC-β) upon ligation of the BCR is mechanistically involved in the negative selection of immature B cells in response to BCR engagement.

Depletion of PKC in mature splenic B lymphocytes renders them susceptible to anti-Ig-mediated negative selection

The relative inability of immature B cells to increase PIP₂ hydrolysis in response to BCR ligation, coupled with the ability of PMA to rescue immature B cells from BCR-induced apoptosis, suggested that BCR-induced activation of PKC may be necessary to prevent anti-Ig-induced death. Therefore, we next determined if PKC-depleted mature B cells are susceptible to BCR-induced apoptosis. Chronic exposure to high doses (100 ng/ml) of the phorbol ester PDBu results in a depletion of PKC activity, at least in part, through an increased rate of degradation of the enzyme (45). Previous studies have demonstrated that primary splenic B cells are susceptible to PKC depletion and that PKC-depleted B cells are no longer able to proliferate in response to anti-Ig (46, 47). PDBu was utilized in these studies because it is effective in depleting PKC, yet it is less hydrophobic than PMA and, thus, can be removed from the cultures by washing (48). PKC depletion in PDBu- or mock-treated cultures were monitored by Western blot analysis (Fig. 3). The two conventional, phorbol ester-responsive isoenzymes of PKC expressed in B cells, PKC-α and -β, are efficiently depleted in PDBu-treated cells, while the two novel isoenzymes, PKC-ε and -δ, are also depleted, although PKC-ε is depleted somewhat less efficiently (Fig. 3). As predicted, the level of expression of the lower m.w. phorbol ester-insensitive PKC-ζ was decreased only slightly (49), while a higher m.w. PKC isoenzyme detected by this particular anti-PKC-ζ antiserum (previously reported to be a partially characterized phorbol ester-sensitive isoenzyme (50, 51)) was also depleted by PDBu. As can also be seen, PKC levels in unstimulated immature and mature cells were quite similar, making it unlikely that differential regulation of PKC activity would occur at the level of expression of the individual isoenzymes.

To assess the role of PKC activation in the prevention of Ag-receptor-mediated apoptosis, the ability of anti-Ig to induce apoptosis in PKC-depleted mature B cells was determined. Mature-stage B cells were cultured overnight with or without 100 ng/ml of PDBu. After overnight treatment, the cells were washed extensively and subjected to an apoptosis analysis in which the B cells were recultured with medium alone, anti-Ig, anti-Ig and PMA, or anti-Ig and 4α-PDD. After an 8-h incubation, the cells were harvested and subjected to a modified TUNEL analysis. While mock-depleted B cells did not show increases in the percentages of apoptotic cells in any of the culture conditions, a strikingly different result was observed with the PDBu-treated B cells. After PKC-depletion, mature B cells were specifically susceptible to anti-Ig-mediated apoptosis (Fig. 4). These data suggest that an uncoupling of PKC activation from BCR-induced signal transduction is sufficient to render mature B cells sensitive to anti-Ig-induced apoptosis. Interestingly, PMA was still capable of rescuing the cells from anti-Ig-induced apoptosis, perhaps because of its ability to
activate the very low residual amounts of the conventional isoenzymes (PKC-α and -β) or the more substantial amounts of the novel phorbol ester-responsive isoenzymes, PKC-ε and -δ (Fig. 4). Thus, it appears that the BCR-induced activation of PKC in PDBu-treated mature B cells is insufficient to protect them from BCR-induced cell death. However, activation of PKC by PMA, leading to either more efficient activation of PKC or to the activation of a subset of PKC isoenzymes that are not normally coupled to BCR-induced signal transduction events, is sufficient to protect PKC-depleted mature B cells from apoptosis. The hypothesis that only a subset of PMA-inducible PKC isoenzymes (perhaps the conventional isoenzymes PKC-α and -β that are efficiently depleted following PDBu treatment) are normally coupled to BCR signal transduction is substantiated by the nonredundant nature of the PKC-β isoenzyme in mature B cell signal transduction observed in PKC-β-deficient mice (33). Interestingly, mature B cells from PKC-β-deficient mice proliferate normally in response to phorbol ester and calcium ionophore, suggesting that while other PKC isoenzymes are capable of promoting a proliferative signal, only PKC-β is activated to induce proliferation following BCR ligation.

**PKC inhibitors render mature B cells susceptible to cell death following BCR engagement and block PMA-induced protection of immature B cells from BCR-induced apoptosis**

While phorbol ester treatment depletes conventional and novel PKC isoenzymes in mature B cells, this depletion is preceded by activation of these phorbol ester-responsive isoenzymes. To assure that this prior PKC activation was not rendering mature B cells susceptible to BCR-induced apoptosis, we utilized two PKC inhibitors, bisindolylmaleimide I, an inhibitor of conventional and novel PKC isoenzymes (52), and Ro-32-0432, an inhibitor with a greater selectivity for the conventional isoenzymes, PKC-α and -β (53). Addition of these inhibitors to mature B cells 15 min before stimulation with anti-Ig lead to the induction of apoptosis (Fig. 5A). This enhanced cell death was not due to toxic effects because the frequency of apoptosis in unstimulated or LPS-stimulated mature B cells was not affected (Fig. 5A).

The causative role of PKC in preventing the BCR-induced apoptosis of immature B cells was also addressed using the PKC inhibitors. In this case, the ability of PMA to protect immature B cells from BCR-induced apoptosis was assessed in the presence or absence of bisindolylmaleimide or Ro-32-0432. Prior addition of the PKC inhibitors substantially blocked the PMA-induced protection of immature B cells from BCR-induced apoptosis (Fig. 5B). Again, these inhibitors were relatively nontoxic, as the frequency of apoptotic cells in unstimulated or LPS-stimulated immature B cells was relatively unaffected (Fig. 5B). The ability of PKC inhibitors to reduce PMA-induced protection of immature B cells from BCR-induced apoptosis and render mature B cells sensitive to BCR-induced apoptosis greatly supports our hypothesis that PKC plays an active role in preventing BCR-induced apoptosis and that PKC activation is differentially coupled to BCR-induced signal transduction events in immature and mature B cells.

**Phorbol ester-induced receptor desensitization does not totally account for the ability of PMA to rescue cells from BCR-induced death**

It has previously been shown that phorbol ester treatment of mature B cells can result in a decrease in calcium influx and PIP₂ hydrolysis in response to subsequent BCR ligation (54–57), an effect known as receptor desensitization. This suggested that PMA may be rescuing anti-Ig-stimulated immature B cells by reducing the early BCR-induced signal transduction events following BCR ligation. Since calcium influx is one of the earliest of these events, we monitored the effect of PMA on BCR-induced calcium influxes. Fura 2-AM-loaded immature B cells were prestimulated with medium, PMA, or 4α-PDD for 5 min and then stimulated with 50 μg/ml of anti-Ig. As shown in Fig. 6A, acute stimulation with the active compound PMA could indeed reduce BCR-induced increases in intracellular calcium levels in immature B cells, whereas the inactive 4α-PDD did not. This data confirmed studies by others showing that PMA can desensitize the BCR with respect to its coupling to calcium flux when it is present at the onset of BCR stimulation.

Since PMA is capable of rescuing mature, PKC-depleted B cells from anti-Ig-induced apoptosis, we next assessed whether PMA
was capable of desensitizing the BCR in these cells. While PMA efficiently reduced the BCR-induced increase in intracellular calcium in mock-treated B cells (Fig. 6B), it did not alter the BCR-induced calcium flux in PDBu-treated cells at all (Fig. 6C), indicating that the ability of PMA to rescue B cells from BCR-induced death does not result from receptor desensitization.

Discussion

While mature B cells proliferate in response to BCR engagement, immature and transitional B cells undergo apoptosis in response to anti-Ig stimulation in vitro (19–21) and are especially sensitive to negative selection in vivo. This developmentally regulated difference in functional phenotype is associated with a unique intracellular signaling response in each cell type; mature B cells increase PIP2 hydrolysis and elevate intracellular calcium levels in response to sIg cross-linking, while immature B cells increase intracellular calcium levels in the relative absence of increased PIP2 hydrolysis (35). Since PIP2 hydrolysis is known to lead to PKC activation, we hypothesized that BCR-induced signal transduction events that are uncoupled from PKC activation would lead to negative selection. The ability of PMA, a pharmacologic activator of PKC, to rescue immature B cells from BCR-induced apoptosis supported our hypothesis that activation of PKC is essential for preventing the induction of apoptosis following BCR ligation. Furthermore, the ability of PKC inhibitors to block the PMA-induced protection suggested that PMA was indeed protecting immature B cells from BCR-induced apoptosis by activation of PKC.

The finding that mature B cells that have either been depleted of PKC or are stimulated with anti-Ig in the presence of PKC inhibitors are rendered sensitive to BCR-induced apoptosis strengthened our hypothesis that an “imbalance” in BCR-induced signal transduction events resulting from a relative inability to activate PKC play a role in the induction of B cell apoptosis following receptor engagement. Specifically, PDBu treatment of splenic B cells, which results in markedly reduced levels of PKC-α and -β and PKC-δ expression, renders them susceptible to anti-Ig-induced apoptosis, suggesting that uncoupling of the conventional, and perhaps novel, PKC isoenzymes from BCR-induced activation events plays a critical role in this process. The ability of PMA to rescue PKC-α- and PKC-β-depleted mature B cells from anti-Ig-induced death suggests that perhaps the novel DAG-responsive isoenzyme of PKC (PKC-ε) is still efficiently activated by phorbol ester and is capable of rescuing the cells from cell death. The relative inability of the residual amounts of DAG-responsive PKC isoenzymes found in PDBu-treated mature B cells to rescue mature B cells from apoptosis following BCR engagement may reflect either the inefficient coupling of a subset of these isoenzymes to the BCR or to the more efficient activation of these isoenzymes by PMA.

FIGURE 5. PKC inhibitors render mature B cells susceptible to cell death following BCR engagement and block PMA-induced protection of immature B cells from BCR-induced apoptosis. Mature B cells (A) and immature B cells (B) were precultured for 15 min in the presence or absence of the PKC inhibitors bisindolylmaleimide I (bis) at 750 nM or Ro-32-0432 (Ro) at 2.5 μM. Anti-Ig (50 μg/ml) in the presence or absence of PMA (10 ng/ml) or LPS (25 μg/ml) was then added where indicated. After 14–16 h for immature B cells and 24 h for mature B cells, cells were harvested and assessed for the frequency of apoptotic cells with subdiploid levels of DNA by PI staining and analysis by flow cytometry.
FIGURE 6. Phorbol ester-induced receptor desensitization does not totally account for the ability of PMA to rescue cells from BCR-induced death. Fura 2-AM-loaded immature B cells (A) were incubated for 1 min, stimulated with either medium, PMA, or 4α-PDD for 5 additional minutes and then stimulated with anti-Ig (50 μg/ml). Intracellular calcium levels were assessed by spectrometry as described. Mature B cells cultured overnight in the absence (B, mock depleted) or presence of PDBu (C, PDBu depleted) were washed extensively and loaded with Fura-2AM. These cells were then incubated, as described above, in the absence or presence of PMA (10 ng/ml) for 5 min and stimulated with 50 μg/ml of anti-Ig, and intracellular calcium levels were assessed. Stimuli were loaded at the times indicated by the arrows.

Support for the suggestion that anti-Ig may activate a subset of PKC isoenzymes, while PMA may activate all conventional and novel PKC isoenzymes, comes from the observation that the induction of the immediate early gene, egr-1 is markedly higher following stimulation with PMA relative to induction by cross-linking sIg (59, 60). In addition, the dramatic effect on B cell function observed in PKC-β-deficient mice indicates that PKC-β is directly coupled to the BCR and that other PKC isoenzymes are not able to compensate for its function following receptor engagement although they are capable of doing so when activated pharmacologically (33). The relative inability of PKC-β-deficient mice to mount a response to a T-independent type II Ag or an efficient primary response to a T-dependent Ag in vivo or to proliferate to anti-Ig in vitro suggests that the activation of PKC-β is specifically regulated by BCR engagement and is critical for B cell proliferation. It remains to be tested whether this lack of response is due to inefficient signal transduction, an inability to proliferate, or to induced apoptosis, as our results might suggest.

Stimulation with PMA did not result in BCR down-regulation on immature B cells (data not shown), ruling out one trivial explanation for why PMA might be able to rescue immature B cells from BCR-induced apoptosis. However, short-term stimulation with PMA is known to desensitize the Ag receptor of mature B cells and prevent the induction of both calcium flux and PIP₂ hydrolysis (54–57) through an, as yet, unknown mechanism. While this phenomenon could potentially explain the ability of PMA to rescue immature B cells from BCR-induced death, it appears to only partially account for the ability of PKC activation to inhibit apoptosis. First, PMA can reverse the apoptosis-inducing effects of both ionomycin and thapsigargin, agents that increase intracellular calcium levels, but do so by bypassing the BCR (data not shown). Second, mature B cells that have been treated overnight with PDBu are susceptible to BCR-induced death and can be rescued by PMA, but PMA does not desensitize the BCR in these cells. Thus, PKC activation appears to exert its effects at points downstream of the BCR as well as at the level of receptor desensitization. It is also possible that PKC activation may engage cell survival pathways. For instance, both Bcl-2 and Bad, a molecule that can heterodimerize with Bcl-x and promote cell death, can be regulated at the level of phosphorylation (61, 62).

The mechanisms by which primary immature B cells undergo apoptosis stand in apparent contrast to those observed in cell lines such as WEHI-231 and DT40 that are thought to serve as model systems for the negative selection of immature B cells. In these systems, death is associated with the activation of phospholipase C γ, leading to an increase in both PKC activity and intracellular calcium levels (34, 54, 63). It is not clear why the two systems differ, but it may reflect differences between primary cells that have not yet entered the cell cycle compared with actively proliferating tumor cells.

Briefly summarized, the data presented here and in previous reports (35) have demonstrated that cross-linking the Ag receptor of immature B cells initiates intracellular biochemical changes. Thus, the induction of apoptosis is not associated with the absence of signal transduction in immature B cells, but rather it is an active process that is initiated by BCR cross-linking and subsequent signaling events. Immature B cells have a specific Ag-receptor-associated signaling phenotype; cross-linking the BCR leads to increases in intracellular calcium concentrations in the absence of PIP₂ hydrolysis (35). The ability of PMA treatment to inhibit the anti-IgM mediated apoptosis, as well the sensitivity of PKC-depleted mature splenic B cells to anti-Ig-induced apoptosis, implicates the activation of PKC with protection from anti-Ig-induced apoptosis. Future efforts will focus on the identification of the specific PKC isoenzymes activated by stimulation through the Ag receptor and characterizing the specific downstream actions of PKC that mediate the inhibition of anti-Ig-mediated apoptosis and, thus, regulate the process of negative selection.

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References


