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Antigen Receptor-Induced Signal Transduction Imbalances Associated with the Negative Selection of Immature B Cells

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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 tolerized 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 (PIP2) hydrolysis. In contrast, immature B cells that undergo BCR-induced apoptosis increase intracellular calcium in the relative absence of PIP2 hydrolysis. Since PIP2 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.

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 Cγ (27–29), resulting in the breakdown of polyphosphoinositide bis phosphate (PIP$_2$) into the second messengers inositol triphosphate (IP$_3$) and diacylglycerol (DAG) (30, 31). IP$_3$ 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, PKCα, -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 PIP$_2$ hydrolysis and elevating intracellular-free calcium levels, immature B cells increase intracellular calcium levels in the relative absence of PIP$_2$ hydrolysis (34, 35). Such findings 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 y 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 didecanoate (PDD), and thapsigargin were purchased from Sigma (St. Louis, MO), and ionomycin was purchased from Calbiochem (SanDiego, CA). Polyconal anti-PKCα, -PKCδ, -PKCe, -PKCɛ, and -PKCζ were purchased from Life Technologies (Gaithersburg, MD). 12-deoxyxypophorol 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**

Splenic 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-Thy1.2 (HO-13-4) (37), rabbit complement, and DNase I. RBC were lysed by treatment with red blood cell lysis buffer. Cells were washed four times with PBS containing 10% FCS, and finally resuspended in RPMI containing 10% FCS 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 LYSIS II software. The mean percentages of apoptotic B 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 cultured under various conditions overnight in either sterile 2-ml click-cap tubes or 96-well plates. When utilized, PKC inhibitors were added 15 min before the addition of the stimulant. After 3 h, cells were washed twice with PBS containing 10% FCS. Single cell suspensions were 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 PBS containing 10% FCS and resuspended at a concentration of 3.3 × 10$^8$/ml in 1 ml of HBSS containing 10 μg/ml of propidium iodide (PI). The cells were stained at 4°C overnight and analyzed by flow cytometry on a Becton Dickinson fluoroluminescence spectrometer (model LS50B; Perkin-Elmer, Norwalk, CT). Excitation was at 430 and 480 nm with emission monitored at 510 nM. Fluorometric analysis of dyed cells was performed using a luminescence spectrometer (model LS50B; Perkin-Elmer, Norwalk, CT). Lysates were microcentrifuged at 4°C for 10 min to remove the Triton X-100 insoluble fraction. Lysates were then diluted in 10 mM Tris (pH 8), 137 mM NaCl, 0.05% Tween 20 plus 2% BSA and spun at 10,000 × g for 10 min at 4°C to remove the Triton X-100 insoluble fraction.
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 µg/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.

**Modified TUNEL assay**

In some cases, the frequency of apoptotic cells was quantitated using a modified TUNEL assay (38). Briefly, 1 x 10^6 cells were fixed in 2.5% formaldehyde in 1 X 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 µl of nick translation reagent, which consists of 5 µl nick translation buffer (500 mM Tris (pH 7.5)/100 mM MgSO_4/1 mM DTT), 0.03 µl of fluorescein-dUTP (1 nmol/µl; Boehringer Mannheim, Indianapolis, IN), 1 µl of dTTP (0.7 nmol/µl), 1 µl each of dATP, dCTP, dGTP (1 mmol/µl), and 0.1 µl of DNA polymerase (5 U/µl) 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. The detection of apoptotic cells using this assay closely correlates with results obtained using a modified TUNEL technique that detects nicked DNA (38) (data not shown). In contrast, overnight culture of mature splenic B cells in the presence of anti-Ig resulted in only a marginal increase in the frequency of apoptotic cells over that observed in unstimulated cultures (Figs. 1, A and B). The detection of apoptotic cells using this assay was also performed using a modified TUNEL technique that detects nicked DNA (38) (data not shown). In contrast, overnight culture of mature splenic B cells in the presence of anti-Ig resulted in only a marginal increase in the frequency of apoptotic cells over that observed in unstimulated cultures (Figs. 1, A and B). These results reconfirm that, in the absence of T cell help, immature and mature B cells respond differentially to BCR signaling and that immature B cells are susceptible to BCR-induced apoptosis, a mechanism that may account for their deletion in vivo following exposure to self Ag.

**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 phospholipid, 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 µg/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...
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-β (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 led 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 PI(3,4,5)P3 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 influx. Fura 2-AM-loaded immature B cells were prestimulated with medium, PMA, or 4a-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 4a-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 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 immature 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.
plation 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


