B Cell Receptor-induced cAMP-response Element Binding Protein Activation in B Lymphocytes Requires Novel Protein Kinase C$^d$

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RUNNING TITLE

PKCd links the BCR to CREB phosphorylation in B cells
SUMMARY

The cAMP response element-binding protein (CREB) is activated by phosphorylation on Ser133 and plays a key role in the proliferative and survival responses of mature B cells to B-cell receptor (BCR) signaling. The signal link between the BCR and CREB activation depends on a phorbol ester (PMA)-sensitive protein kinase C (PKC) activity and not protein kinase A or calmodulin kinase; however, the identity and role of the PKC(s) activity has not been elucidated.

We found the novel PKC\(\text{d}\)(nPKC\(\text{d}\)) activator, bistratene A is sufficient to induce CREB phosphorylation in murine splenic B cells. The pharmacological inhibitor Gö6976, which targets conventional PKCs and PKC\(\gamma\), has no effect on CREB phosphorylation, whereas the nPKC\(\text{d}\) inhibitor rottlerin, blocks CREB phosphorylation following BCR cross-linking. Bryostatin 1 selectively prevents nPKC\(\text{d}\) depletion by PMA when coapplied, coincident with protection of BCR-induced CREB phosphorylation. Ectopic expression of a kinase-inactive nPKC\(\text{d}\) blocks BCR-induced CREB phosphorylation in A20 B cells. In addition, BCR-induced CREB phosphorylation is significantly diminished in nPKC\(\text{d}\)-deficient splenic B cells in comparison to wild-type mice. Consistent with the essential role for Bruton’s tyrosine kinase (Btk) and phospholipase C\(\gamma\) (PLC\(\gamma\)) in mediating PKC activation, Btk- and PLC\(\gamma\)-deficient B cells display defective CREB phosphorylation by the BCR. We also found that p90 RSK directly phosphorylates CREB on Ser133 following BCR cross-linking and is positioned downstream of nPKC\(\text{d}\). Taken together, these results suggest a model in which BCR engagement leads to the phosphorylation of CREB via a signaling pathway that requires nPKC\(\text{d}\) and p90 RSK in mature B cells.
INTRODUCTION

Signaling through the B-cell antigen receptor (BCR)\(^1\) is required throughout B-cell development and peripheral maturation (1,2). BCR signaling requires the activities of multiple non-receptor protein tyrosine kinases, including Syk, Bruton's tyrosine kinase (Btk), and Src family kinases and the lipid kinase, phosphatidylinositol 3-kinase (PI3K) (3). Btk plays an integral role in transducing BCR signals, because mutations in the gene encoding \(\text{btk}\) result in the B cell deficiencies X-linked immunodeficiency (\(x\text{id}\)) in the mouse and X-linked agammaglobulinemia (XLA) in humans (reviewed in 4). The \(x\text{id}\) phenotype results from a single amino acid substitution (R28C) in the pleckstrin homology domain of Btk and is characterized by a near complete absence of peritoneal B-1a cells, defective responses \textit{in vivo} to immunization with thymus-independent type II antigen, and a block at the transitional-to-mature B cell checkpoint (4-7).

Much genetic and biochemical data support the concept that B cell activation by the BCR occurs through a signalosome in which the adaptor protein, B cell linker protein (BLNK) allows protein tyrosine kinases access to signal transduction molecules (8,9). It is envisaged in this model that BLNK is phosphorylated by Syk following BCR ligation (9-12). Btk and phospholipase C\(\text{\textgamma}2\) (PLC\(\text{\textgamma}2\)) are recruited to BLNK via their Src homology 2 domains, thereby allowing Syk and Btk to fully phosphorylate and activate PLC\(\text{\textgamma}2\) (9-14). PLC\(\text{\textgamma}2\) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (15). IP3 functions in part to mobilize calcium from intracellular compartments and by promoting extracellular calcium influx, whereas DAG binds to and activates conventional (Ca\(^{2+}\)-dependent) protein kinase C (cPKC) and novel (Ca\(^{2+}\)-independent) PKC (nPKC) isoforms (15,16).
The cAMP response element-binding protein (CREB) is a 43 kDa protein belonging to the CREB/ATF bZip family of transcription factors. It binds to a consensus TGANNTCA cAMP-response element (CRE) as a homo- or heterodimer with other CREB/ATF and activator protein-1 transcription factor members (17). Phosphorylation of CREB on Ser133 increases its association with CREB-binding protein, leading to activation of the basal transcriptional machinery (17, 18). CREB phosphorylation and CRE-dependent gene expression occurs in response to BCR cross-linking of resting B cells (19-21). Interestingly, a recent report shows that CREB binding activity is induced following BCR cross-linking is negatively regulated by IFNγ (22).

Accumulating evidence suggests that CREB plays an important role in B cell growth and survival. Phosphorylation of CREB is necessary for activation of the BCR-dependent c-fos/jun-B immediate-early growth response program (20,23). In particular, transgenic mice expressing a mutant CREB containing a serine-to-alanine substitution at position 133 exhibit impaired proliferation and survival in response to BCR cross-linking (24). In regard to the latter, CREB phosphorylation is necessary for bcl-2 gene expression in human B cells (25). In addition, CREB has been implicated in the regulation of numerous gene promoters involved in B cell function, including the 3’ enhancer, MHC class II promoter, OCA-B promoter, and human Ig proximal promoter (26-30).

Despite the importance of CREB phosphorylation in B cell function, there remain many fundamental questions concerning the mode of regulation of CREB by the BCR. One of these questions related to the role of PKC in linking the BCR to CREB phosphorylation. Nearly a decade ago a phorbol-ester (PMA) sensitive PKC activity was shown to be required for BCR-induced CREB phosphorylation in B cells (21,31). In contrast, several known CREB kinases,
including inducible protein kinase A (PKA) and calmodulin kinase II (CaMK II) activities do not contribute to CREB phosphorylation in mature B cells response to BCR cross-linking (31). While these observations suggest an important contribution of PKC to CREB phosphorylation, the identity of the PKC isoform(s) has not been established. Furthermore, the signaling components that link PKC activity to CREB phosphorylation remain to be defined. In the work described herein, we have made use of pharmacological activators and inhibitors of c/nPKC isoforms, nPKC\(-\)-deficient mice, A20 B-cells expressing a kinase-inactive nPKC\(-\), and DT40 B cell lines deficient in signalosome components to define the signaling pathways that regulate CREB phosphorylation in mature B cells. Our results show that BCR cross-linking mediates CREB phosphorylation via a signaling pathway involving nPKC\(-\) and p90 RSK.
EXPERIMENTAL PROCEDURES

Reagents and Antibodies-Anti-CREB antibodies (Abs) were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phospho(Ser32/36)Iκb, anti-nPKCδ(Thr505), anti-MSK-1 (Ser376), anti-MAPKAP kinase-2 (Thr334), and anti-p90 RSK (Thr573) Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-nPKCδ (C-17), anti-PKCα,β,γ (MC5), anti-rabbit and anti-mouse IgG-coupled horseradish peroxidase Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKCε (P9103-20C) was from United States Biologicals (Swampscott, MA). The anti-chicken IgM (M4) Ab and chicken serum were obtained from Southern Biotechnology Associates (Birmingham, AL). Bryostatin 1, U73122, and rottlerin were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Protease inhibitor cocktail, phorbol 12-myristate 13-acetate (PMA) and 4aPMA were obtained from Sigma-Aldrich, Inc (St. Louis, MO). Inhibitors were prepared in DMSO at -fold concentrations such that the final amount of DMSO in culture medium was below 0.1%. F(ab’)2 fragments of goat anti-mouse IgM or IgG (anti-Ig) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Gö6976 was purchased from CalBiochem-NovaBiochem (San Diego, CA). The Immobilon-P membrane was from Millipore (Bedford, MA). Enhanced chemiluminescence reagents were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). The rabbit complement and lympholyte M were purchased from Accurate Chemical and Scientific (Westbury, NY).

Cell Lines and Preparation of Murine B Lymphocytes- The murine sIgM+Bal17 B-lymphoma was obtained from Dr. Richard Asofsky (National Institutes of Health, Bethesda, MD). The murine A20 B-lymphoma was kindly provided by Dr. Jeffery V. Ravetch (Rockefeller
University, New York). Stable A20 cell lines were generated by culturing at a density of 5 x 10^5 cells/1 ml RPMI-1640 medium (Sigma-Aldrich, Inc.) containing 3 µg of plasmid DNA/6 µL of LipofectAMINE 2000 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol. A20 cells were stably transfected with pMTH plasmids constitutively expressing either wild-type nPKCd or a mutant nPKCd containing a substitution (K376R) in the ATP-binding site that results in a kinase-inactive nPKCd as previously described (32). Stable transfectants were selected in culture medium containing G418 (Mediatech, Herndon, VA). The murine B cell lines were maintained in RPMI-1640 medium containing 5% heat inactivated FCS (Atlanta Biologicals, Norcross, GA) in a 37°C humidified incubator at 5% CO2.

The chicken DT40 B cell lines were provided by Drs. E.A. Clark (Department of Microbiology, University of Washington, Seattle, WA) and T. Kurosaki (Riken Cell Bank, Japan) and maintained in RPMI-1640 medium containing 5% FCS, 1% chicken serum, 10 mM Hepes (pH 7.5), 2 mM L-glutamine, and 5 x 10^-5 M 2-ME (11,12).

CBA/CaJ and Balb/c mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed at Boston College. Mice were cared for and handled at all times in accordance with National Institutes of Health and institutional guidelines. Splenic B cells were purified by depletion of T cells with anti-Thy-1.2 plus rabbit complement; macrophages (and other adherent cells) were removed by plastic adherence (20). RBCs and non-viable cells were removed by sedimentation on Lympholyte M. Splenic B lymphocytes were purified from nPKCd/- mice as described previously (33); the generation of nPKCd-null mice on C57Bl/6 background has been described (33). The resulting B cell populations were cultured in RPMI-1640 medium supplemented with 10 mM Hepes, pH 7.5, 2 mM L-glutamine, 5 x10^-5 M 2-ME, 100 U/ml
penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone and 10% heat inactivated FCS.

Splenic B cells were maintained in a 37°C humidified incubator at 5% CO₂.

**Western Blotting**—B cells were solubilized in modified RIPA buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1% sodium deoxycholate, 1% NP-40, and 1% SDS) containing 1 mM PMSF, 1 mM NaF, 1 mM Na₃VO₄ and supplemented with protease inhibitor cocktail. Lysate protein was separated by electrophoresis through a 10% polyacrylamide SDS-gel and transferred to Immobilon-P membrane. The membrane was blocked in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween-20) containing 5% nonfat dry milk for 5 h and then incubated overnight (4°C) with primary Ab at 1 μg/ml in TBS-T. The membrane was washed several times in TBS-T and then incubated with a 1:2500 dilution of anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase Abs for 90 min and developed by enhanced chemiluminescence (ECL). Autoradiograms were analyzed by densitometry using a Bio-RAD GS-800 Calibrated Densitometer and Quantity One software (BioRad Laboratories, Hercules, CA).

**In Vitro Kinase Assay**—Approximately 2.0 x 10⁷ B-cells were collected by centrifugation, washed once in 1X PBS and solubilized in a lysis buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EGTA, 1mM EDTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1mM Na₃VO₄, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.2 mM PMSF. Lysates were then incubated for 18 h at 4°C with anti-phospho-p90RSK (Ser³⁸⁰) Ab that recognizes activated p90 RSK (Cell Signaling Technology). Immune complexes were collected with a 40 μl slurry of Protein A/G agarose beads and washed six times with lysis buffer. Kinase reactions were carried out at 30 °C for 30
min in 20 μl of kinase buffer (20 mM Hepes, pH 7.2, 5 mM MnCl₂, 200 μM Na₃VO₄, 10 μM ATP and 2 μg of recombinant MBP-CREB fusion protein as substrate (Biosource International, Camarillo, CA). The reactions were terminated by adding SDS-PAGE sample buffer and the products resolved by SDS-PAGE. CREB phosphorylated on Ser133 was detected by Western blotting with anti-phospho-CREB (Ser 133) Ab as described above.

In vitro nPKCδ kinase activity was measured as described above with the following modifications. Anti-nPKCδ (C-17) immune complexes obtained from B cell extracts were used to phosphorylate 1 μg histone H1 substrate in 30 μl kinase buffer supplemented with 5 μCi of [γ-32P]ATP for 15 min at 30°C. The kinase reactions were terminated by the addition of SDS-PAGE sample buffer, boiled (5 min), and separated by SDS-PAGE. The phosphorylated histone H1 was detected by autoradiography.

**Electrophoretic Mobility Shift Assay**-Nuclei were isolated by hypotonic lysis and extracted in a 450 mM NaCl buffer as described previously (20). Binding reactions were carried out in a final volume of 15 μl and contained 1.5 μg nuclear protein (extracted with 450 mM NaCl), 0.5 μg double stranded poly(dI-dC), and 10,000 cpm of DNA labeled probe. DNA probes were labeled with [γ-32P]ATP by T4 polynucleotide kinase (New England BioLabs, Beverly, MA). After 20 min (23°C), the reaction products were electrophoresed through a 5% polyacrylamide/TBE gel and subjected to autoradiography. For the analysis of nuclear extract binding to the bcl-2 promoter CRE site, the wild-type and mutant probes corresponded to 5’-GAACCGTGACGTACGCA-3’ and 5’-GAACCGTGTAATATGCAGCA-3’, respectively. B cell derived nuclear extracts containing CREB or purified recombinant CREB bind the wild-
type *bcl*-2 promoter CRE site, whereas the mutant *bcl*-2 promoter CRE site fails to bind CREB/ATF proteins (25). Supershift analysis was performed by including in the nuclear extract binding reactions approximately 1 μg of anti-CREB, anti-phospho(Ser133)CREB or non-immune Abs.
RESULTS

*BCR-induced Phosphorylation of CREB is Dependent on c/nPKCs*-We initially sought to demonstrate the requirement for a c/nPKC isoform(s) in BCR-induced CREB phosphorylation on Ser133. The PKC isoforms expressed in mouse B cells and activated by BCR-induced diacylglycerol production, include calcium-dependent cPKC -α, -βI/II, and -γ and calcium-independent nPKC -δ, -ε, and -η (33,35,36). Splenic B cells were pre-treated for 18 h with 100 ng/ml PMA in order to deplete cells of c/nPKC isoforms and then stimulated with anti-Ig or PMA. As a control for the specificity of PMA, B cells were also pre-treated for 18 h with 100 ng/ml of the inactive PMA analog, 4aPMA. Treatment of B cells with PMA (18 h) led to a block in BCR-induced CREB phosphorylation (Fig. 1A, lanes pCREB, compare M with Ig, PMA o/n). Addition of fresh PMA to parallel B cell cultures failed to increase CREB phosphorylation (Fig. 1A, lanes pCREB, compare M with P, PMA o/n). The lack of CREB phosphorylation cannot be attributed to depletion of cellular CREB by prolonged PMA treatment (Fig. 1A, lanes CREB, PMA o/n). By contrast, CREB phosphorylation was induced in 4aPMA pre-treated B cells subsequently stimulated with anti-Ig or PMA (Fig. 1A, lanes pCREB, 4aPMA o/n). To assess the efficacy of PMA as it pertains to PKC down-regulation, we found that the cellular levels of nPKCδ and nPKCε were depleted by PMA, but not 4aPMA pre-treatment (Fig 1A, lanes nPKCδ and nPKCε). Similar results were obtained for the cPKC-α, -β and -γ(data not shown).

*Effects of nPKCδ Inhibitors and Activators on BCR-induced CREB Phosphorylation in Splenic B Cells*-To identify the PKC isoform(s) contributing to BCR-induced CREB phosphorylation, several cell permeable inhibitors of PKCs were evaluated for their ability to
inhibit CREB phosphorylation. Gö6976 is a specific inhibitor of cPKCs (ID$_{50}$=2-6 nM) and PKCβ (ID$_{50}$=20 nM)(37,38). Pre-treatment of splenic B cells with Gö6976 ranging from 5 to 25 nM did not measurably inhibit anti-Ig-induced CREB phosphorylation under the conditions used (Fig. 1B, lanes pCREB). Similar results were obtained with the cPKC inhibitor, Gö6850 (data not shown). The efficacy of Gö6976 was confirmed in that it blocked anti-Ig-induced IκBα phosphorylation on Ser32/36, an event dependent, at least in part, on cPKCs (Fig. 1B, lanes pIκBα)(39). Taken together with the data in Figure 1A, these findings point to a possible role for nPKCs in linking the BCR to CREB activation.

Of the known nPKC isoforms expressed in B lymphocytes, nPKCδ exhibits the highest level of expression and is phosphorylated on regulatory residues, Tyr311 and Thr505 in response to BCR cross-linking (33,40). To determine if nPKCδ activity is necessary for BCR-induced CREB phosphorylation, splenic B cells were pre-incubated with rottlerin, a specific inhibitor of nPKCδ(41). At concentrations ranging between 10 and 20 μM, rottlerin blocked BCR-induced CREB phosphorylation in primary splenic B cells isolated from either Balb/c or CBA/CaJ mice (Fig. 1C, lanes pCREB). As a control, we found that BCR-induced nPKCδ activity was inhibited by pre-treatment of splenic B cells with 10 μM rottlerin (Fig. 1C, inset). These findings suggest that nPKCδ activity is required for BCR-induced CREB phosphorylation.

To obtain further evidence for a role of nPKCδ in CREB phosphorylation, we evaluated whether the highly selective activator of nPKCδ, bistratene A (Bis A) was sufficient to promote CREB phosphorylation. Bis A is a cyclic polyether toxin isolated from Lissoclinum bistratum and has been shown to selectively activate nPKCδ in vivo at concentrations between 50-100 nM (42). Treatment of splenic B cells with 50 nM Bis A led to a rapid and transient increase in
CREB phosphorylation (Fig. 1D, lanes pCREB). Bis A did not alter the cellular levels of β-actin protein (Fig. 1D, lanes β-actin). Bis A also induced CREB phosphorylation in splenic B cells from CBA/CaJ mice (data not shown). Thus, BCR-induced CREB phosphorylation is blocked by the nPKCδ inhibitor, rottlerin, and can be induced by selective activation of nPKCδ in the absence of BCR cross-linking.

**Bryostatin 1 Selectively Protects nPKCδ from PMA-depletion Concomitant with BCR-induced CREB Phosphorylation** - To corroborate the findings above, we evaluated the effects of bryostatin 1, a non-phorbol ester activator of PKCs, on anti-Ig-stimulated CREB phosphorylation. Numerous studies have shown that exposure of mammalian cells to bryostatin 1 at concentrations between 100 nM to 1 μM results in the selective protection of nPKCδ, but not other c/nPKC, from PMA-induced depletion when coapplied (43,44). To test whether bryostatin 1 was capable of protecting BCR-induced CREB phosphorylation following PMA-depletion of c/nPKCs, splenic B cells were pre-treated with PMA alone or with the combination of PMA and bryostatin 1. In agreement with data in Figure 1A, pre-treatment of splenic B cells with PMA alone led to a near complete block in anti-Ig-induced CREB phosphorylation at the time points examined (Fig. 2, lanes pCREB). By contrast, anti-Ig stimulated CREB phosphorylation was induced in B cells co-incubated with the combination of PMA and bryostatin 1. In data not shown, we also found that bryostatin 1 effectively protected BCR-induced CREB phosphorylation from PMA in the mature B-cell lines, A20 and Bal17. The cellular level of nPKCδ in B cells treated with the combination of PMA plus bryostatin 1 was significantly greater in comparison to PMA alone, indicating that nPKCδ levels were protected from PMA-induced depletion (Fig. 2, lanes nPKCδ). Further, the cellular levels of aPKCα, which are not
responsive to PMA-depletion, were not altered by PMA alone or the combination of PMA and bryostatin 1 (Fig. 2, lanes aPKCβ). The cellular levels cPKCs -α, -δ, and -λ in PMA plus bryostatin 1 treated B cells were actually lower in comparison to PMA alone, suggesting that these cPKC isoforms were not protected by bryostatin 1 (Fig. 2, lanes cPKCβ). These findings provide further evidence in support of a role for nPKCδ in BCR-induced CREB phosphorylation.

**BCR-induced CREB Phosphorylation is Diminished in nPKCδ-deficient Splenic B Cells**—To determine directly if nPKCδ is important in BCR-mediated phosphorylation of CREB on Ser133, we compared CREB phosphorylation in anti-Ig stimulated splenic B cells from wild-type and nPKCδ−/− mice (33). Cross-linking of the BCR on nPKCδ-deficient B cells resulted in a significantly diminished level of CREB phosphorylation in comparison to wild-type B cells (Fig. 3A, lanes pCREB). The impaired CREB phosphorylation in nPKCδ-deficient B cells cannot be attributed to a decrease in the amount of CREB protein in nPKCδ−/− B cells relative to wild-type (Fig. 3A, lanes CREB). It is noteworthy that recent reports demonstrated induction of NF-κB and NF-IL6 in response to BCR cross-linking in nPKCδ-null B cells that is comparable to wild type (33,45). Thus, impaired phosphorylation of CREB following BCR ligation in nPKCδ-null B cells does not reflect a general defect in BCR signaling to nuclear transcription factor activation.

To obtain further support for nPKCδ in the regulation of CREB phosphorylation by the BCR, we generated mature A20 B cell lines that stably express either wild type nPKCδ (nPKCδWT) or a kinase-inactive nPKCδ(nPKCδDN). The nPKCδDN recombinant protein contains an amino acid substitution (K376R) in the catalytic domain, which is necessary for ATP
binding as previously described (32). Stable expression of wild type nPKC\(\text{d}\) in A20 B cells cultured in medium alone resulted in an elevated level of CREB phosphorylation in comparison to the parental A20 B cells (Fig. 3B, lanes M, compare A20 and nPKC\(\text{d WT}\)). Nonetheless, stimulation of wild type nPKC\(\text{d}\) expressing A20 B cells with anti-Ig or PMA resulted in increased CREB phosphorylation (Fig. 3B, nPKC\(\text{d WT}\), lanes IgG and PMA, respectively). In contrast, stable expression of nPKC\(\text{d DN}\) in A20 B cells resulted in a near complete loss of PMA- and anti-Ig-induced CREB phosphorylation (Fig. 3B, nPKC\(\text{d DN}\)). The levels of total cellular CREB were relatively equal between the A20 cell lines (Fig. 3B, lanes CREB). These data support a critical role for nPKC\(\text{d}\) in linking the BCR to CREB phosphorylation.

**BCR-induced Phosphorylation of CREB Requires Btk and PLC\(\text{g 2}\)-Little is known about the regulation of CREB phosphorylation by signaling components proximal to the BCR. The results above suggest that CREB phosphorylation is dependent on nPKC\(\text{d}\). It might be expected that PLC\(\text{g 2}\) is necessary for CREB phosphorylation, given that PLC\(\text{g 2}\) activation results in the production of DAG that binds to and activates Ca\(^{2+}\)-independent nPKCs. However, nPKC\(\text{d}\) can be activated by alternative pathways some of which may occur independent of membrane translocation and PLC\(\text{g 2}\) (46,47). To test whether PLC\(\text{g 2}\) is necessary for BCR-induced CREB phosphorylation, we made use of chicken DT40 B cells deficient in PLC\(\text{g 2}\) (34,48). PLC\(\text{g 2}^{-/-}\) DT40 B cells exhibited impaired CREB phosphorylation in response to BCR cross-linking in comparison to the parental DT40 B-cell line (Fig. 4A, compare wt and PLC\(\text{g 2}^{-/-}\)). In agreement with this finding, pre-treatment of *ex vivo* splenic B cells with 1 \(\mu\)M U73122, a highly specific
PLC\(g\) inhibitor, resulted in approximately 50% reduction in BCR-induced CREB phosphorylation in comparison to non-treated anti-Ig stimulated B cells (Fig. 4B). We also found that DT40 B cells deficient in Btk, a key upstream activator of PLC\(g\), failed to induce CREB phosphorylation in response to anti-Ig (Fig. 4A, compare wt and Btk\(^{-/-}\)). DT40 B cells deficient in Syk, which contributes to PLC\(g\) activation, also failed to phosphorylate CREB in response to BCR cross-linking (Fig. 4A, compare wt and Syk\(^{-/-}\)). It should be noted that these results cannot be attributed to decreased cellular CREB or differences in protein loads for SDS-PAGE as the levels of CREB and hsp90 were similar in each of the DT40 B-cell types (Fig. 4A).

Since phosphorylation at residues in the activation loop of nPKC\(d\) is thought to play a role in determining catalytic activation and its regulation by the B-cell signalosome (33,46), we examined whether BCR-induced phosphorylation of nPKC\(d\) at the activation loop site Thr505 was impaired in mutant DT40 B cells. As shown in Fig. 4C, BCR cross-linking on the parental DT40 B-cell line induced an increase in nPKC\(d\) phosphorylation at Thr505, consistent with a previous report in mouse splenic B cells (33). In contrast, DT40 B cells deficient in Btk, PLC\(g\) or Syk exhibited a complete absence of BCR-induced phosphorylation of nPKC\(d\) on Thr505 (Fig. 4C).

**Role for p90 RSK in Linking nPKC\(d\) to BCR-mediated CREB Phosphorylation**—We next sought to define signaling components that function to link nPKC\(d\) to CREB phosphorylation. Several protein kinases have been identified that function as CREB kinases, including MSK1, PKA, p90 RSK, CaMK II/IV, and MAPKAP-K2/3 (reviewed in 49; 51-53). Previous reports ruled out direct participation of inducible PKA activity and CaMKII in mediating CREB phosphorylation in mature B lymphocytes following BCR cross-linking (20,21,31). We found that stimulation of
Bal17 or splenic B-cells with anti-Ig led to an increase in MSK-1 phosphorylation on the activation residue Ser376, however the increase in phosphorylation was not dependent on nPKCd activity (data not shown). In addition, we were unable to detect activation of MAPKAP-K2 in response to anti-Ig stimulation of either splenic B cells or the Bal17 B-cell lymphoma (data not shown) (50). With this in mind, we evaluated the activation of p90 RSK in splenic B cells and Bal17 B-cell lymphomas following BCR cross-linking.

Phosphorylation of p90 RSK on the activation residue Thr573, which has been shown to correlate with catalytic activity (54), was observed at 5, 15, and 30 min following BCR ligation in Bal17 B-cells (Fig. 5A, Bal-17). Similar results were obtained using splenic B cells (Fig. 5A, BALB/C). To determine if p90 RSK phosphorylation is dependent on nPKCd, Bal17 B cells or splenic B cells were pre-treated with rottlerin and then p90 RSK phosphorylation on Thr573 was monitored by Western blot. As shown in Figure 5B, rottlerin pre-treatment completely blocked BCR-induced p90 RSK phosphorylation at the time point examined in both splenic and Bal-17 B cells. In addition, pre-treatment of B cells with PMA (for 18 hr), which serves to deplete cellular c/nPKCs (refer to Fig. 1A), blocked BCR-induced p90 RSK phosphorylation (Fig. 5B, lanes PMA). Of note, the basal level of p90 RSK phosphorylation was elevated in splenic B cells cultured overnight in PMA. Pre-treatment with the inactive PMA analog, 4aPMA had not effect on anti-Ig-induced CREB phosphorylation in splenic B cells, although it reduced the level of anti-Ig-stimulated CREB phosphorylation in the Bal-17 B cell lymphoma (Fig. 5B, 4aPMA, lanes BALB/C and Bal-17, respectively). Consistent with these data, A20 B cells stably expressing a kinase-inactive nPKCd failed to phosphorylate p90 RSK in response to BCR engagement or PMA (Fig. 5C, lanes nPKCdDN), whereas parental A20 B cells and wild type nPKCd expressing A20 B cells exhibited inducible p90 RSK phosphorylation (Fig. 5C, A20 and
nPKC$_{WT}$). We note that stable expression of wild type nPKC in A20 B cells cultured in medium alone resulted in an elevated level of p90 RSK phosphorylation in comparison to the parental A20 B cells.

We next determine if endogenous p90 RSK was capable of directly phosphorylating CREB on Ser133. For these experiments, p90 RSK-containing immune complexes were isolated from anti-Ig stimulated Bal-17 B cells or splenic B cells and assayed for phosphorylation of a recombinant MBP-CREB fusion protein in vitro. p90 RSK-containing immune complexes isolated from B cells culture in medium alone were essentially devoid of MBP-CREB kinase activity, whereas p90 RSK-immune complexes isolated from anti-Ig stimulated B cells phosphorylated MBP-CREB on Ser133 (Fig. 5D, lanes p90$_{RSK}$). Isotype-matched non-immune IgG did not exhibit MBP-CREB kinase activity (Fig. 5D, lanes normal rabbit IgG). Importantly, pre-treatment of B cells with rottlerin effectively blocked p90 RSK-mediated CREB phosphorylation (Fig. 5D, lanes R). Taken together, these data suggest that p90 RSK phosphorylates CREB on Ser133 and functions downstream of nPKC in mature B cells.

**Requirement for nPKC in BCR-induced CREB Phosphorylation at the bcl-2 Promoter CRE Site**-The bcl-2 gene is important for B cell survival and is regulated by CREB that binds to a canonical CRE site in the bcl-2 gene promoter (25). The signaling pathway linking BCR-induced bcl-2 gene expression via CREB has not been defined; however, activation of the bcl-2 gene promoter in response to phorbol diester stimulation of B cells suggests that a c/nPKC activity is necessary (25). We sought to determine if nPKC contributes to BCR-induced CREB phosphorylation bound to the bcl-2 gene promoter CRE site. In initial experiments, nuclear extracts were isolated from control and anti-Ig stimulated B cells and nucleoprotein
binding activity specific to a probe containing the bcl-2 gene promoter CRE was examined by electrophoretic mobility shift assay (EMSA). As shown in Fig. 6A, a single nucleoprotein complex was detected with the bcl-2 promoter CRE probe. The nuclear extract binding was specific in that EMSA with a mutant bcl-2 promoter CRE probe, that fails to bind CREB/ATF proteins, was devoid of nuclear extract binding activity (Fig. 6A inset, lane Mut) (25). Supershift analysis with anti-CREB Ab using nuclear extracts from anti-Ig stimulated B cells indicate the presence of CREB in the nucleoprotein complex (Fig. 6B). Nuclear extract binding activity was supershifted by the anti-phospho(Ser133)CREB Ab (Fig. 6B). As a control, inclusion of a non-immune Ab did not inhibit or supershift the nucleoprotein complex.

To establish whether nPKC\(\beta\) was required for phosphorylation of CREB at the bcl-2 promoter CRE site, ex vivo splenic B cells were pre-incubated with varying concentrations of rottlerin and then cultured in medium alone or stimulated with anti-Ig. Treatment of B cells with rottlerin resulted in a concentration-dependent inhibition of Ser133 phosphorylation of CREB at the bcl-2 promoter CRE site, as judged by a decrease in the relative amount of anti-phospho(Ser133)CREB Ab supershifted nucleoprotein complex (Fig. 6C, lanes pCREB). The decrease in CREB phosphorylation cannot be attributed to reduced levels of CREB, as the amount of CREB bound to the bcl-2 promoter CRE was not decreased by rottlerin at the concentrations used (Fig. 6C, lanes CREB). To further demonstrate a role for nPKC\(\beta\) B cells were treated with Bis A alone for various times and then nuclear extracts were prepared and analyzed by EMSA. The data in Fig. 6D indicate that Bis A is sufficient to promote Ser133 phosphorylation of CREB bound to the bcl-2 promoter CRE site. Importantly, Bis A did not increase the total amount of CREB bound to the bcl-2 promoter CRE site (Fig. 6D, CREB).

Finally, we observed that inhibition of nPKC\(\beta\) by pre-treatment with 10 \(^{\mu}\)M rottlerin blocked the
basal level and BCR-induced $bcl-2$ expression in splenic B cells (Fig. 6E). These results are consistent with the inhibition by rottlerin of CREB Ser133 phosphorylation bound at the $bcl-2$ gene promoter CRE (Fig. 6C).
DISCUSSION

Activation of CREB by phosphorylation on Ser133 represents an early event in the signaling pathway triggered by BCR cross-linking. Phosphorylation of CREB is important for the transcription of numerous genes involved in B cell function and survival (e.g., bcl-2) (19-31). Despite the critical role that CREB plays in B cell responses, the molecular pathway(s) that link CREB Ser133 phosphorylation to the BCR remain unknown. A notable exception is an earlier report that prolonged treatment of B cells with PMA abrogated BCR-induced CREB phosphorylation, suggesting a contribution of c/n PKC activity (21). Interestingly, in contrast to non-lymphoid cells, this BCR-induced phosphorylation was determined to be independent of PKA or CaMK activities (20,21,31). In the current study, we have investigated the signaling pathway responsible for CREB phosphorylation in ex vivo splenic B cells. Evidence is provided consistent with a model wherein BCR-induced CREB phosphorylation on Ser133 is mediated by a pathway that requires the novel isoform, nPKC\(\text{d}\). The validity of this model is supported by several experimental findings. The chemical nPKC\(\text{d}\) inhibitor, rotterlin blocks anti-Ig-stimulated CREB phosphorylation. Congruent with this observation, BCR signaling was shown to activate nPKC\(\text{d}\) (herein and reference 40). We also find that bryostatin 1, a macrocyclic lactone that is used in combination with PMA to elucidate the cellular role of endogenous nPKC\(\text{d}\), protects anti-Ig stimulated CREB phosphorylation from PMA-induced down regulation concomitant with protection of nPKC\(\text{d}\) from depletion (43,44). Consistent with these findings, bistratene A, a highly selective activator of nPKC\(\text{d}\), when added to B cells in the absence of BCR cross-linking is sufficient to stimulate CREB phosphorylation.

These findings are also supported by data indicating that expression of a kinase-inactive nPKC\(\text{d}\) in mature A20 B cells completely blocks BCR-induced CREB phosphorylation on
Ser133. We also find that BCR-induced CREB phosphorylation is significantly diminished in splenic B cells isolated from \( pkc^d \)-deficient mice in comparison to wild type mice. That CREB phosphorylation in nPKC\( ^d \)-deficient splenic B cells is not completely abrogated following BCR cross-linking, suggests the existence of a second pathway that contributes, albeit minor, to CREB phosphorylation. Whether this pathway exists in normal B cells or alternatively represents a compensatory up-regulated pathway in \( pkc^d \)-deficient mice is not known. On this point, BCR-induced CREB phosphorylation was not completely abolished by rottlerin pre-treatment of normal splenic B cells. It would seem unlikely that a cPKC activity is responsible for the residual CREB phosphorylation given that the cPKC/ PKC\( ^m \) inhibitor Gö6976 did not block BCR signaling to CREB phosphorylation. Importantly, the efficacy of Gö6976 was demonstrated insofar as BCR-mediated I\( ^B \)B\( ^a \) phosphorylation was blocked by this inhibitor, an event dependent on cPKC activity (39,51). nPKC\( ^d \) but not nPKC\( ^e \) has been shown to mediate common cytokine receptor-induced CREB phosphorylation in myeloid cells (52). These findings raise the possibility that nPKC\( ^d \) may contribute, albeit minor, to BCR-induced CREB phosphorylation in mature B cells. Interestingly, expression of a kinase inactive nPKC\( ^d \) in A20 B cells resulted in a complete loss of BCR-induced CREB phosphorylation, suggesting that CREB phosphorylation is entirely dependent upon a nPKC\( ^d \) pathway in A20 B cells. The discrepancy in CREB phosphorylation between A20 B cells and \( pkc^d \)-deficient splenic B cells (wherein some CREB phosphorylation occurs in response to BCR ligation) remains unexplained, although this may reflect differences in the cell types used (i.e., primary culture (splenic B cells) and transformed (A20) B cells). Notwithstanding, the data collectively point to a critical requirement for nPKC\( ^d \) in linking the BCR to CREB Ser133 phosphorylation.
The nuclear targets of nPKC\(\beta\) in B cells are poorly characterized; however, a recent study has shown that nPKC\(\beta\) mediates NF-AT-dependent gene expression in response to BCR cross-linking (53). Wilson et al. (25) demonstrated that phosphorylation of CREB represents a necessary signaling event in the activation of the \(bcl-2\) gene promoter and that promoter activation is dependent upon a PKC activity. We find that when \textit{ex vivo} splenic B cells are pretreated with rottlerin, there is a concentration-dependent reduction in the amount of Ser133 phosphorylated CREB bound to the \(bcl-2\) gene promoter CRE site. Moreover, bistratene A pre-treatment of splenic B cells is sufficient to induce Ser133 phosphorylation of CREB bound to the \(bcl-2\) promoter CRE site. In agreement with these observations, inhibition of nPKC\(\beta\) with rottlerin blocks BCR-induced \(bcl-2\) expression. These results provide the first evidence that nPKC\(\beta\) plays a role in CREB-dependent \(bcl-2\) gene expression in mature B cells. Additional studies are currently underway to understand more fully the regulation of \(bcl-2\) gene expression by nPKC\(\beta\) in the context of BCR signaling.

Our results also implicate for the first time p90 RSK in BCR-mediated CREB phosphorylation in mature B cells. In particular, we show that endogenous p90 RSK-containing immune complexes are capable of directly phosphorylating a MBP-CREB fusion protein on Ser133. Evidence positioning p90 RSK downstream of nPKC\(\beta\) is provided by experiments in which treatment of splenic B cells with rottlerin blocks BCR-induced p90 RSK phosphorylation on the activation residue, Thr573 (54). It should be noted that although rottlerin has been implicated in the inhibition of cPKCs, much higher concentrations of rottlerin are required in comparison to nPKCs (46). Studies have shown that rottlerin blocks the kinase activity of nPKC\(\beta\) and nPKC\(\eta\); however, among these two nPKCs, only nPKC\(\beta\) is expressed in B cells (55). Although we cannot exclude a possible involvement of other rottlerin-sensitive pathways
in BCR-induced p90 RSK activation, we find that expression of a kinase-inactive nPKCd in A20 B cells results in a complete loss of BCR-induced p90RSK phosphorylation on Thr573. It is noteworthy that members of the p90 RSK family have been identified as direct CREB kinases (56). In particular, the importance of the p90 RSK member, RSK-2 in CREB phosphorylation is demonstrated by the finding that CREB phosphorylation is impaired in human fibroblasts isolated from Coffin-Lowry syndrome patients, which carry mutations in the gene encoding RSK-2 (57). Of note, the molecular link(s) between nPKCd and p90 RSK was not evaluated in this study. Earlier reports in mammalian cell types demonstrated that ERK activation results in phosphorylation of RSK2 in an nPKCd-dependent manner (56, 58,59). A recent report by Khan and co-workers (53) suggests that nPKCd may link the BCR to NF-AT activation by inducing ERK activity.

Although our results do not allow us to definitively rule out the contribution of other known CREB kinases in linking nPKCd to CREB phosphorylation following BCR cross-linking, experimental results not described herein fail to support a role for MSK-1 in this capacity. Moreover, previous work has excluded the involvement inducible PKA and CaMK II activities in the BCR-mediated CREB phosphorylation in mature B cells (20,21,31). It should be mentioned that p38 MAP kinase has also been implicated in linking nPKCd to gene transcription (55). The action of p38MAPK kinase is thought to be mediated, in part, by its downstream kinase MAPKAP kinase-2, which in turn directly phosphorylates CREB on Ser133 (60). Given the lack of inducible phosphorylation of MAPKAP kinase-2 on the activation residue Thr334 (study herein) and our previous finding that the p38 MAPK inhibitor, SB203580 does not block BCR-induced CREB phosphorylation in splenic B cells, we do not believe that components of the p38 MAPK kinase pathway contribute to CREB phosphorylation in mature B cells stimulated
via the BCR (50). This contrasts with the regulation of CREB phosphorylation in CH31 B-cell lymphomas, a model for BCR-induced growth arrest and apoptosis in immature B cells, wherein CREB phosphorylation is dependent on the p38 MAPK pathway (50). These results, taken together with data herein, suggest the interesting possibility that the BCR directs activation of CREB via different signaling pathways in developmentally distinct B-cell subsets. This situation contrasts with that of NGF-treated PC12 cells wherein both RSK and p38 MAPKAP kinase-2 pathways contribute to CREB Ser133 phosphorylation (61).

We have also provided the first insights into the requirement for individual components of the signalosome in mediating BCR-induced CREB phosphorylation in mature B cells. The absence of detectable CREB phosphorylation in PLC\textsubscript{g2}-deficient DT40 B cells, together with inhibition of BCR-induced CREB phosphorylation in normal B cells treated with the PLC\textsubscript{g2} inhibitor U73122, suggest that PLC\textsubscript{g2} is essential for BCR-mediated CREB phosphorylation. Congruent with these observations, a recent report demonstrated that U73122 prevents cytosol-to-membrane translocalization of nPKC\textsubscript{d} in response to BCR cross-linking in B cells (40). Activation of PLC\textsubscript{g2} upon BCR ligation requires membrane recruitment and tyrosine phosphorylation by Btk (11-14,48,62). In agreement with the role of Btk in regulating PLC\textsubscript{g2}, we find that Btk-deficient DT40 B cells do not exhibit BCR-induced CREB phosphorylation in comparison to wild type DT40 B cells. We also observed that BCR-induced phosphorylation of nPKC\textsubscript{d} on Thr505 is impaired in each of the mutant DT40 B cells. These findings establish that the functional integrity of the Btk/PLC\textsubscript{g2} signaling bloc is crucial for the induction of CREB phosphorylation by the BCR.

In summary, the experiments herein establish a role for nPKC\textsubscript{d} in BCR signaling to CREB. The data are consistent with a model in which BCR engagement on mature B
lymphocytes leads to the phosphorylation of CREB via a signaling pathway that requires Btk/PLCγ, nPKCδ, and p90 RSK.
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FOOTNOTES

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1The abbreviations used are: Ab, antibody; Anti-Ig, F(ab')₂ fragments of anti-mouse IgM; BCR, B-cell antigen receptor; BLNK, adaptor protein B cell linker protein; Btk, Bruton's tyrosine kinase; cPKC, conventional PKC; CREB, cAMP response element-binding protein; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MSK1, mitogen-activated stress kinase 1; nPKC, novel PKC; nPKCd, novel PKCd; PKC, protein kinase C; PLCγ, phospholipase Cγ; PMA, 12-O-tetradecanoyl phorbol-13-acetate; RSK, ribosomal S6 kinase, xid, X-linked immunodeficiency.
FIGURE LEGENDS

Fig. 1. nPKC\textsuperscript{d} activity is required for BCR-induced CREB phosphorylation. A, Splenic B cells were cultured in medium (M) with 100 ng/ml PMA or 100 ng/ml 4\textsuperscript{a}PMA for 18 h (O/N). Cells were then washed and cultured in medium alone (M) or stimulated with 12 \textsuperscript{a}g/ml anti-Ig (\textsuperscript{a}Ig) or 300 ng/ml PMA (P) for 30 min. Splenic B cells were also pre-treated with Gö6976 for 2 h (B) or rottlerin for 30 min (C) at the indicated concentrations. Cells were then cultured in medium (M) or stimulated with 12 \textsuperscript{a}g/ml anti-Ig (\textsuperscript{a}Ig) for 30 min. The inset in (C) shows anti-nPKC\textsuperscript{d}- or non-immune IgG-containing immune complexes isolated from parallel anti-Ig stimulated B cells pre-treated with 10 \textsuperscript{a}M rottlerin. The immune complexes were assayed for phosphorylation of histone H1 as described in Experimental Procedures. D, Splenic B cells were incubated in medium containing the absence (0) or presence of 50 nM bistratene A for the indicated times. Whole cell detergent extracts were prepared and Western blotted with anti-phospho-CREB(Ser133), anti-CREB, anti-phospho-I\textsuperscript{κ}B\textsuperscript{α}(Ser32/36) or anti-\textsuperscript{β}-actin Abs as indicated. To evaluate depletion of nPKCs, the membrane in A was stripped and reprobed with anti-nPKC\textsuperscript{d} or anti-nPKC\textsuperscript{e} Abs. Numerical values below (C) represent densitometric analysis of the pCREB bands and represented as fold induction above medium in relative optical units, standardized to each autoradiographic film. The data are representative of three independent experiments.

Fig. 2. Bryostatin 1 protects nPKC\textsuperscript{d} from PMA-induced depletion, coincident with recovery of anti-Ig stimulated CREB phosphorylation. Splenic B cells were cultured in medium containing 0.5 \textsuperscript{a}M PMA alone or in combination with 1.0 \textsuperscript{a}M bryostatin 1 for 18 h as described (37,43). Cells were then stimulated in the absence (0) or presence of 12 \textsuperscript{a}g/ml anti-Ig
(aIg) for 15-60 min. Whole cell detergent extracts were prepared and immunoblotted with anti-phospho CREB(Ser133) or anti-CREB Abs. The cellular levels of individual c/nPKCs were monitored by immunoblotting with a pan specific anti-cPKC-α, -β, -γ Ab, that recognizes a shared epitope (amino acids 292-317), anti-nPKCδ, or anti-aPKCζ Abs (lower band denoted by arrow). The data are representative of four independent experiments.

Fig. 3. BCR-induced CREB phosphorylation is impaired in nPKCδ-deficient B cells. A, Splenic B cells from nPKCδ−/− and wild-type mice (nPKCδ+/+) were cultured in medium alone (0) or stimulated with 12 μg/ml anti-Ig (αIg) for the indicate times (15-60 min). B, Parental A20 B cells (A20), A20 B cells stably expressing a wild-type (nPKCδWT), or kinase-inactive recombinant nPKCδ (nPKCδDN) were cultured in medium alone (M) or stimulated with 10 μg/ml F(ab')2 fragments of goat anti-mouse IgG (αIgG) or 100 ng/ml PMA for 30 min. Whole cell detergent extracts were prepared and immunoblotted with anti-phospho-CREB(Ser133) or anti-CREB Abs as indicated. C, Western blot analysis indicating the level of expression of endogenous nPKCδ (nPKCδ) and recombinant β-tagged nPKCδ (nPKCδ-tagged) in the parental A20 cell line (A20) and the A20 B cells stably expressing a wild-type (WT), or kinase-inactive (DN) recombinant nPKCδ described in B (32). Numerical values represent densitometric analysis of the pCREB bands and represented as fold induction above medium in relative optical units, standardized to each autoradiographic film.

Fig. 4. PLCγ2 and Btk are necessary for BCR-induced CREB phosphorylation. DT40 B cell lines were cultured in the absence (M) or presence of 4 μg/ml anti-chicken IgM Ab (aIg) for 30
Whole cell extracts were prepared and Western blotted with anti-phospho-CREB(Ser133), anti-CREB and anti-HSP90 Abs (in A) or anti-phospho-nPKC\(_{\text{d}}\)(Thr505) and anti-nPKC\(_{\text{d}}\) Abs (in C) as indicated. B, Splenic B cells were pre-treated with 1 \(\mu\)M U73122 for 30 min; the cells were then cultured in medium alone or stimulated with 12 \(\mu\)g/ml anti-Ig (\(\alpha\)Ig) for 30 min. Whole cell extracts were prepared and phosphorylation of CREB on Ser133 and CREB levels determined by Western blot. Numerical values represent densitometric analysis of the pCREB bands and are represented as fold induction above medium in relative optical units, standardized to each autoradiographic film.

**Fig. 5. p90 RSK activation is dependent on nPKC\(_{\text{d}}\) and contributes to CREB phosphorylation in response to BCR cross-linking.** A, Bal17 B-cell lymphomas or splenic B cells were cultured in the absence (M) or presence of anti-Ig (\(\alpha\)IgM) for the indicated times. B, Bal17 B-cell lymphomas and splenic B cells were pre-treated with PMA or 4\(\alpha\)PMA for 18 h as described in Fig. 1A or with 10 \(\mu\)M rottlerin for 30 min. Cells were then cultured in medium (M) or stimulated with anti-Ig (\(\alpha\)IgM) for 15 min. C, A20 B cells (A20), A20 B cells stably expressing a wild-type (nPKC\(_{\text{d}}\)WT), or kinase-inactive nPKC\(_{\text{d}}\)(nPKC\(_{\text{d}}\)DN) were cultured in medium alone (M) or stimulated with 10 \(\mu\)g/ml F(ab’), fragments of goat anti-mouse IgG (\(\alpha\)Ig) or 100 ng/ml PMA for 30 min. Whole cell extracts were prepared and Western blotted with anti-phospho-p90 RSK(Thr573) and anti-p90 RSK Abs as indicated. D, B cells were cultured in medium alone or stimulated with 10 \(\mu\)g/ml anti-Ig (\(\alpha\)IgM) for the indicated times (5, 15, 30 min). B cells were also pre-treated with 10 \(\mu\)M rottlerin for 30 min and then stimulated with 10 \(\mu\)g/ml anti-Ig for 15 min (R). Whole cell extracts were then prepared and immunoprecipitated with isotype-matched rabbit IgG or anti-p90 RSK Ab (\(\alpha\)-p90\(_{\text{RSK}}\)). The immune complexes were
assayed for phosphorylation of MBP-CREB fusion protein (CREB) on Ser133 as described in Experimental Procedures. Numerical values below (C) represent densitometric analysis of the bands and represented as fold induction above medium in relative optical units, standardized to each autoradiographic film.

Fig. 6. nPKCd is required for BCR-induced phosphorylation of CREB at the bcl-2 promoter CRE site. A, nuclear extracts were prepared from splenic B cells culture in media (M) alone and stimulated with anti-Ig (aIg) for the indicated times. EMSA using a 32P-labeled probe corresponding to the bcl-2 promoter CRE site was performed. P and NC denote the migration of the probe alone and the specific nucleoprotein complex, respectively. The inset denotes nuclear extract binding activity by EMSA using wild type (WT) or mutant (Mut) bcl-2 promoter CRE site containing probes. B, supershift analysis was performed by carrying out binding reactions with nuclear extracts from anti-Ig-stimulated B cells (30 min) in the presence of anti-CREB (CREB), anti-phospho(Ser133)CREB (pCREB), or non-immune (NI) Abs. C, splenic B cells were cultured in media alone (M) or pretreated with the indicated concentrations of rottlerin (30 min). Cells were then stimulated with 12 μg/ml anti-Ig (aIg) for 30 min. D, splenic B cells were culture in media alone (M) or in the presence of 100 nM bistratene A (Bis A) for the indicated times. Binding reactions with a 32P-labeled bcl-2 promoter CRE site probe were carried out in the presence of anti-CREB or anti-phospho(Ser133)CREB Abs with CREB and pCREB denoting the corresponding supershifts, respectively. NC denotes migration of the unsupershifted nucleoprotein complex. The data are representative of three independent experiments. E, splenic B cells were cultured in the absence (-) or presence (+) of 10 μM rottlerin for 30 min. Cells were then cultured in medium alone (- aIg) or stimulated with 12
g/ml anti-Ig (+ Ig) for 8 h. Whole cell extracts were prepared and Western blotted with anti-bcl-2 or anti-β-actin Abs.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
B cell receptor-induced cAMP-response element binding protein activation in B lymphocytes requires novel protein kinase C-delta
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