ACCELERATED PUBLICATION

Protein kinase Cβ is critical for the metabolic switch to glycolysis following B-cell antigen receptor engagement

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INTRODUCTION

B-cell homeostasis is critical for maintaining adaptive immunity and to avoid autoimmune and immunodeficiency diseases. In keeping with this, B-cells are dependent upon extrinsic signals to maintain survival during development in the bone marrow and the periphery and for cellular homeostasis. Responses of B-cells to antigens are associated with the activation of PKC (protein kinase C), which comprise a subfamily of 11 closely related serine/threonine kinase isoforms [1]. A key challenge is to identify PKC isoform-specific substrates and to elucidate their physiological significance in the context of B-cell antigenic responses. Various genetic and biochemical studies suggest distinct biological functions for several PKC isoforms in B-cells [1–3]. PKCδ plays a role in the regulation of B-cell responses to self-antigens [2]. In contrast, mice deficient in PKCβ (PKCβ−/−) exhibit impaired TI-2 (T-independent type 2) responses and poor survival in the absence of stimuli [3]. Ex vivo proliferative responses of PKCβ−/−-splenic B-cells following BCR engagement are significantly reduced in comparison with PKCβ+/+ B-cells [3,4]. IL-4 (interleukin 4) promotes PKCβ−/− B-cell survival and increases the proportion of dividing cells, suggesting that accelerated death contributes to impaired proliferative responses to BCR (B-cell antigen receptor) ligation [3].

An emerging body of evidence suggests that extrinsic signals support lymphocyte survival, in part, by maintaining the cellular metabolism associated with general housekeeping functions [5–10]. Notably, naïve lymphocytes require glucose uptake and metabolism to maintain survival and function in both ex vivo primary cultures and in vivo [7–11]. Antigen receptor cross-linking is accompanied by increased bioenergetic and biosynthetic demands and is characterized by a metabolic switch to aerobic glycolysis; however, the nature of this regulation and the signaling pathways involved are poorly defined in lymphocytes [12–15]. Interestingly, B-cell lymphomas exhibit a high rate of aerobic glycolysis similar to that of activated primary B-cells [11]. In the present study, we used combined pharmacological and genetic approaches to test directly the requirement for PKCβ in the BCR-mediated switch to glycolysis. The results described in the present paper establish a definitive role for PKCβ in the metabolic switch to glycolysis following BCR engagement of naïve B-cells.

MATERIALS AND METHODS

Reagents

Anti-GLUT1 (glucose transporter 1) Ab (antibody) was from Research Diagnostics. Anti-PKCβ Ab was from Santa Cruz Biotechnology. Anti-MEK1/2 (mitogen-activated protein kinase/extracellular-signal-related kinase kinase 1/2) Ab was from Cell Signaling Technologies. F(ab)′, fragments of anti-Ig [goat anti-(mouse IgM)] and FITC-labelled goat anti-rabbit secondary Ab were from Jackson ImmunoResearch Laboratories. 2DG (2-deoxyglucose) and Gö6976 were from Calbiochem/Novabiochem. Alexa Fluor® 546-conjugated CTB (cholera toxin subunit B) was from Molecular Probes/Invitrogen. CD40L (CD40 ligand) was prepared and used as described in [16].

Abbreviations used: Ab, antibody; anti-Ig, goat anti-(mouse IgM); BCR, B-cell antigen receptor; Btk, Bruton’s tyrosine kinase; CD40L, CD40 ligand; CTB, cholera toxin subunit B; 2DG, 2-deoxyglucose; GLUT1, glucose transporter 1; HK2, hexokinase 2; PFK1, phosphofructokinase 1; PGAM1, phosphoglycerate mutase 1; PKC, protein kinase C; PKM2, pyruvate kinase M2; PPP, pentose phosphate pathway; RT, reverse transcription; xid, X-linked immunodeficiency.

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Mice and B-cell isolation

BALB/cByJ and xid (X-linked immunodeficiency) mice were from Taconic Farms. C57BL/6 x 129 x 1/SvJ and mice deficient in PKCδ/II were obtained from Dr George King (Dana Faber Cancer Institute, Boston, MA, U.S.A.); PKCβ−/− mice were obtained from Dr Thomas Rothstein (The Feinstein Institute for Medical Research, Manhasset, NY, U.S.A.). Mice were cared for and handled at all times in accordance with National Institutes of Health guidelines; vertebrate animal studies have been reviewed and approved by IACUC (Institutional Animal Care and Use Committee) at Boston College. Splenic B-cells from mice at 8–12 weeks of age were purified via negative selection (Miltenyi Biotec) [8]; small dense B-cells were isolated following centrifugation through a discontinuous Percoll gradient and cultured RPMI 1640 medium plus 10% (v/v) fetal bovine serum [14]. Western blot analysis confirmed depletion of PKCβ in splenic B-cells from PKCβ/II−/− mice (Supplementary Figure S1 at http://www.biochemj.org/bj/448/bj4480165add.htm).

Glucose utilization assay

B-cell cultures (10⁶ cells/0.5 ml) were incubated with [5-3H]glucose (GE Healthcare) for 90 min. Cells (100 µl) were then removed and placed in 1.5 ml microcentrifuge tubes containing 50 µl of 0.2 M HCl. [3H]Water was separated from unmetabolized [5-3H]glucose by evaporation diffusion (25°C) for 48 h as described in [14,17]. The amount of diffused and non-diffused tritium was quantified by liquid-scintillation spectrometry and compared with vials containing [5-3H]glucose only and [3H]water only.

Indirect immunofluorescence

B-cells were centrifuged on to glass slides using a Cytospin (Thermo Electron) and incubated with 5 µg/ml Alexa Fluor® 546–CTB for 30 min at 22°C. Slides were washed once with PBS and incubated in 3.7% (v/v) formaldehyde for 20 min (22°C). Cells were permeabilized in 0.5% (v/v) Triton X-100 for 5 min and then blocked with 2% (w/v) BSA at 22°C for 30 min. Slides were incubated at 22°C for 1 h with anti-GLUT1 rabbit polyclonal Ab, followed by an FITC-conjugated goat anti-rabbit IgG as described in [14]. Following several washes with PBS, the slides were mounted with Aqua Polymount and analysed by confocal microscopy.

RT (reverse transcription)–PCR

Glycolytic gene transcripts were quantified as described by Faber et al. [18]. Total RNA was isolated using the RNeasy mini RNA isolation kit (Qiagen), following the manufacturer’s protocol. Following DNase I treatment, 2 µg of RNA was reverse-transcribed to cDNA using MMLV (Moloney murine leukaemia virus) reverse transcriptase (Ambion). Real-time PCR was performed with the SYBR Green Supermix on an iCycler with iQ5 Real-time PCR detection system (Bio-Rad Laboratories). Amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 58°C for 1 min. The following primers were used: PKF1_fwd, 5′-TCCGAGGAGGCCGTCTTGA-3′, and PKF1_rev, 5′-TGAG-GCTACATTGCAAGTTGC-3′, for phosphofructokinase 1; PKM2_fwd, 5′-GCCGCTGACATTGACTC-3′, and PKM2_rev, 5′-AGCCGAGCCATCTCATTCC-3′, for phosphoglycerate kinase 1; HK2_fwd, 5′-TGGAGATTTCTAGGGCGTTC-3′, and HK2_rev, 5′-CATCCCGAGTGTACCTCACAA-3′, for hexokinase 2; PGAM1_fwd, 5′-AGAGCAGTGCCTTTCGGAAT-3′, and PGAM1_rev, 5′-TCCATGAGCTTCTTGAGAGG-3′, for phosphoglycerate mutase 1; and β2MG_fwd, 5′-CACCCGCTCTACATTGAAATA-3′, and β2MG_rev, 5′-CATGCTTAACTCTGAGGCGT-3′, for β2-microglobulin.

RESULTS AND DISCUSSION

To test the hypothesis that PKCβ is essential in BCR-induced glycolytic metabolism, we first evaluated the expression of GLUT1, which is a ubiquitously expressed facilitated glucose transporter induced on the cell surface upon B- and T-cell activation and is necessary to promote glycolytic flux [12,14,19]. Both wild-type and PKCβ−/− quiescent B-cells exhibit GLUT1 expression as visualized by immunofluorescence (Figure 1A). Incubation of wild-type and PKCβ−/− splenic B-cells with anti-Ig results in increased GLUT1 expression (Figure 1A). We also examined GLUT1 expression by Western blot analysis in isolated plasma membrane fractions. GLUT1 expression increases in wild-type and PKCβ−/− B-cells following BCR engagement (Figure 1B). As a positive control for these studies, CD40L treatment also increases GLUT1 expression in both wild-type and PKCβ−/− B-cells (Figure 1B).

With this in mind, we evaluated whether a similar defect in BCR-activated glycolytic flux is also observed in xid mice, which have a point mutation in the pleckstrin homology domain of Btk (Bruton’s tyrosine kinase), impairing BCR-mediated glycolytic flux in Btk−/− B-cells (results not shown). In contrast with the immunodeficiency phenotype of PKCδ−/− mice, the novel PKC isoform PKCδ−/− mice behave in a similar manner to that observed in xid mice, which have a point mutation in the pleckstrin homology domain of Btk (Bruton’s tyrosine kinase), supporting the well-established functional co-operation within an integrated signalling pathway between Btk and PKCδ. These results suggest that PKCδ is essential in coupling the BCR to increase glycolytic metabolism.

The immune phenotype of PKCβ−/− mice is similar to that observed in xid mice, which have a point mutation in the pleckstrin homology domain of Btk (Bruton’s tyrosine kinase), supporting the well-established functional co-operation within an integrated signalling pathway between Btk and PKCβ (20,21). With this in mind, we evaluated whether a similar defect in BCR-induced glycolysis is present in B-cells from xid mice. Anti-Ig stimulation of wild-type C57BL/6 B-cells increases glycolysis, whereas B-cells from xid mice exhibit a significantly lower level of glycolytic induction (Figure 2C). Of note, we also observed impaired BCR-mediated glycolytic flux in Btk−/− splenic B-cells (results not shown). In contrast with the immunodeficiency observed in PKCβ−/− mice, the novel PKCδ isoform PKCδ plays a non-redundant role in the regulation of B-cell tolerance [2,22]. To evaluate whether PKCδ−/− B-cells exhibit altered glycolytic flux in response to BCR engagement, B-cells were cultured in medium alone or with anti-Ig (Figure 2D). In contrast with PKCβ−/− B-cells, cross-linking the BCR on PKCδ−/− B-cells increases glycolysis (6-fold above unstimulated B-cells), whereas anti-Ig stimulated wild-type B-cells exhibit a 9-fold increase in glycolysis (Figure 2D).

To understand further how PKCβ regulates BCR-induced glycolysis, we compared the expression of several glycolytic genes. The results reveal a diverse impact of PKCβ-deficiency on glycolytic gene expression. The induction of HK2 mRNA is impaired following BCR engagement in PKCβ−/− B-cells (Figure 3, panel HK2). Moreover, the increase expression of HKII protein in response to BCR engagement in PKCβ−/− B-cells is impaired following BCR cross-linking (Supplementary Figure
A role for PKCβ in BCR-induced glycolysis

**Figure 1** Expression of GLUT1 in splenic B-cells of PKCβ−/− and PKCβ+/+ mice

(A) B-cells from PKCβ+/+ (wild-type) and PKCβ−/− mice were cultured in medium alone or stimulated with 10 μg/ml anti-Ig (αIg) for 12 h. B-cells were incubated with anti-GLUT1 Ab, followed by FITC-labelled goat anti-rabbit secondary Ab (upper panels); B-cells were also stained with Alexa Fluor® 546-conjugated CTB (lower panels) in order to detect plasma membrane GM1 ganglioside as described in [14]. Analysis was carried out with a Leica TCS SP2 confocal laser-scanning microscope with a ×100/0.53 numerical aperture objective. Leica confocal software V2.61 was used to acquire the images. Images are representative of three independent experiments. (B) Parallel B-cells were cultured in the absence (M) or presence of 10 μg/ml anti-Ig (αIg) or CD40L; plasma membranes were then isolated, detergent lysates were prepared, and equivalent amounts of protein (30 μg) were examined by Western blotting for GLUT1 expression [14,25].

S2 at http://www.biochemj.org/bj/448/bj4480165add.htm). The induction of PKM2 and PGAM1 mRNAs are also impaired following BCR engagement in PKCβ−/− B-cells (Figure 3, panels PKM2 and PGAM1), whereas the induction of PFK1 in PKCβ−/− B-cells is not significantly altered compared with wild-type B-cells (Figure 3, panel PFK1).

To test the importance of glycolysis induced via cross-linking of the BCR, we cultured B-cells in the presence and absence of 2DG, an inhibitor of the glycolytic pathway. 2DG inhibits HK2, the first rate-limiting enzyme of glycolysis. In control studies, treatment of anti-Ig-stimulated B-cells from BALB/cByJ mice with 2DG reduces glycolytic flux, as expected (Table 1). Moreover, 2DG significantly reduces B-cell viability despite the presence of anti-Ig in the culture medium (Table 1). Consistent with these findings, B-cells treated with iodoacetamide, which inactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, also exhibit decreased cell viability despite signalling from the BCR (Supplementary Table S1 at http://www.biochemj.org/bj/448/bj4480165add.htm) [23].

In the present study, we found evidence that PKCβ expression is necessary for the metabolic switch to glycolysis following BCR engagement of naïve B-cells. The results also suggest that Btk is required for glycolytic induction following BCR cross-linking. The findings suggest that the BCR-mediated increase in glycolysis results, at least in part, from PKCβ-dependent up-regulation of several key glycolytic genes, including HK2 and PKM2; HK2 is the first rate-limiting enzyme of the glycolytic pathway. Interestingly, the induction of PFK1 gene expression was not significantly impaired in PKCβ−/− splenic B-cells; PFK1 catalyses the committed step of glycolysis with conversion of fructose 6-phosphate and ATP into fructose 1,6-bisphosphate and ADP. In contrast with the findings with PKCβ, which is necessary for survival and growth stimulation, our results fail to implicate PKCδ in BCR-induced glycolytic flux. PKCδ functions as a critical negative regulator in activated B-cells inasmuch as ablation of PKCδ−/− in mice is associated with a specific loss of peripheral B-cell tolerance [2,22]. Taken together, the findings

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**Table 1** Inhibition of glycolysis reduces ex vivo splenic B-cell viability in the presence of BCR cross-linking

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glycolysis (nmol/10⁶ cells per h)</th>
<th>B-cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without 2DG</td>
<td>With 2DG (1 mM)</td>
</tr>
<tr>
<td>B-cells, medium</td>
<td>12.9 ± 0.6</td>
<td>11.1 ± 0.3</td>
</tr>
<tr>
<td>B-cells + anti-Ig</td>
<td>65.5 ± 0.3</td>
<td>18.5 ± 0.7</td>
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Figure 2  Impaired up-regulation of glycolysis in PKCβ−/− B-cells but not PKCδ−/− B-cells after BCR cross-linking

(A) B-cells from wild-type (open bars) or PKCβ−/− (closed bars) mice were cultured in medium alone or stimulated with 10 μg/ml anti-Ig or CD40L. (B) B-cells obtained from control BALB/cByJ mice were cultured in medium alone or stimulated with 10 μg/ml anti-Ig. The B-cells were also cultured in the absence (closed bars) or presence of an equal volume of DMSO (open bars) or 100 nM Gö6976 (hatched bar). Note that Gö6976 was dissolved in DMSO. (C) B-cells from wild-type (closed bars) or xid (open bars) mice were cultured in medium alone or stimulated with 10 μg/ml anti-Ig. (D) B-cells from wild-type (open bars) or PKCδ−/− (closed bars) mice were cultured in medium alone or stimulated with 10 μg/ml anti-Ig. In all panels, B-cells were cultured for 8.5 h and then the medium was supplemented for 90 min with [5-3H]glucose and glycolysis was measured as described in the Materials and methods section. Results are means ± S.D. of triplicate measurements, representative of three independent experiments. Cell viability was greater than 85% for all experimental conditions.

Figure 3  Reduced expression of several glycolytic genes after BCR cross-linking on PKCβ−/− splenic B-cells

Glycolytic gene transcripts (HK2, PKM2, PFK1 and PGAM1) were measured by RT–PCR and the amounts normalized to β2-microglobulin as described in the Materials and methods section. B-cells from wild-type (open bars) or PKCβ−/− (closed bars) mice were evaluated. Results are means ± S.D. of triplicate measurements, representative of three independent experiments.
suggest distinct biological roles for specific PKC isoforms (i.e. PKCβ) in regulating glucose energy metabolism.

Our findings also indicate that glycolysis is required to maintain survival of \textit{ex vivo} B-cells stimulated via the BCR. We base this conclusion on the use of 2DG, which inhibits HK2 and is thus widely used to evaluate the biological significance of glycolysis in mammalian cells and recently in the differentiation of TH17 (T-helper 17) and Treg (regulatory T-) cells [24]. We are aware that inhibition of HK2 with 2DG may prevent glucose 6-phosphate metabolism by the PPP (pentose phosphate pathway). However, we have reported previously that glucose 6-phosphate flux through the PPP is minimal during the first 24 h following BCR engagement [14]. Moreover, pre-treatment of naïve B-cells with inhibitors of the PPP do not measurably decrease B-cell viability in response to BCR engagement (F.A. Dufort, unpublished work). Along these lines, we cannot rule out a potential secondary effect of 2DG on mitochondrial oxidative metabolism.

It is noteworthy that cross-linking the BCR also promotes B-cell growth. At present, we cannot rule out an additional role for PKCβ-dependent glycolysis in the growth of B-cells, which occurs in response to BCR ligation. A major function of glycolysis in activated lymphocytes is to support \textit{de novo} macromolecular synthesis necessary for growth and proliferation [11]. Nevertheless, these results provide the first evidence of an essential role for PKCβ in the metabolic switch to glycolysis following BCR engagement of naïve B-cells. Moreover, our findings suggest that the regulation of glycolysis by PKCβ contributes to the well-documented role for PKCβ in BCR-mediated B-cell survival. Finally, the results may provide new therapies in the form of targeting glycolytic enzymes in combination with PKCβ for immune disorders characterized by dysregulated glycolysis and elevated PKCβ activity, such as observed in a subset of diffuse large B-cell lymphomas [1].

AUTHOR CONTRIBUTION

Derek Blair and Fay Dufort assisted in the design of experiments, performed all of the experiments and interpreted the data. Thomas Chiles conceived of the study, assisted in the design of experiments and wrote the paper.

ACKNOWLEDGEMENTS

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REFERENCES

SUPPLEMENTARY ONLINE DATA

Protein kinase Cβ is critical for the metabolic switch to glycolysis following B-cell antigen receptor engagement

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Figure S1 Expression of PKCβ in wild-type but not PKCβ−/−-deficient splenic B-cells

Splenic B-cells from PKCβ+/+ (wt) and PKCβ−/− (-/-) mice were isolated and whole-cell detergent-soluble extracts were prepared. Equivalent amounts of protein (10 μg) were then examined by Western blot analysis for PKCβ (upper panel) expression as described in [1]; the blot was then stripped and re-probed for expression of hsp90 (heat-shock protein 90) (lower panel). The molecular mass in kDa is indicated on the left-hand side.

Figure S2 Increased expression of HK2 in wild-type but not PKCβ−/−-deficient splenic B-cells following BCR cross-linking

B-cells from PKCβ+/+ (wt) and PKCβ−/− (-/-) mice were isolated and cultured in the absence (M) or presence of 10 μg/ml anti-Ig (αIg). Whole-cell detergent-soluble extracts were prepared, and equivalent amounts of protein (30 μg) were then examined by Western blot analysis for HK2 expression (upper panels) as described in [1]; the blot was then stripped and re-probed for expression of MEK1/2 (mitogen-activated protein kinase/extracellular-signal-related kinase kinase 1/2) (lower panels). The molecular mass in kDa is indicated on the left-hand side.

REFERENCE


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Table S1 Iodoacetamide (IAA)-mediated inhibition of glycolysis reduces ex vivo splenic B cell viability

Glycolysis: B-cells from control BALB/cByJ mice were stimulated with 10 μg/ml anti-Ig for 24 h. Glycolysis was then measured for 90 min in the absence or presence of 10 μM IAA as described in the Materials and methods section of the main text. Results are means ± S.D. of triplicate measurements. Viability: B-cells from control BALB/cByJ mice were stimulated with 10 μg/ml anti-Ig for 24 h in the absence and presence of 10 μM IAA. Viability was determined by propidium iodide staining by flow cytometry. Results are representative of three independent experiments.

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<tr>
<td></td>
<td>Without IAA</td>
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<tr>
<td>B cells + anti-Ig</td>
<td>65.6 ± 0.2</td>
<td>25.8 ± 0.4</td>
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