B cell receptor signalling in chronic lymphocytic leukaemia cells is regulated by overexpressed active protein kinase CβII

Running Title: PKCβII in CLL cells.

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Abstract

Signals through the B-cell antigen receptor (BCR) are important for the survival of chronic lymphocytic leukaemia (CLL) cells. Therefore, factors that influence these signals have important pathophysiological roles in this disease. One key mediator of BCR signalling is protein kinase C β (PKCβ), which regulates activation of I-κB kinases and deactivation of Bruton’s tyrosine kinase within the signalling pathways initiated by BCR engagement. The present study demonstrates that overexpression of the PKCβII isoform is a feature of CLL cells, and that activity of this enzyme strongly correlates with CLL-cell response to BCR engagement. Thus, intracellular Ca\(^2+\) release and increases in cell survival following BCR crosslinking were significantly greater in CLL cases with low levels, than in cases with high levels of active PKCβII. Furthermore, BCR-induced Ca\(^2+\) fluxes could be restored in CLL cases with high levels of active PKCβII by pre-treating the cells with the PKCβ-specific inhibitor LY379196. Conversely, BCR-mediated intracellular Ca\(^2+\) release could be inhibited in CLL cells with low levels of active PKCβII by pre-treatment with PKC agonist bryostatin. Taken together, these results demonstrate that overexpressed active PKCβII plays a role in the regulation and outcome of BCR signals that can be important for the progression of CLL.
Introduction:

Chronic lymphocytic leukemia (CLL) is a malignant disorder of mature B cells characterised by monoclonal expansion in blood, bone marrow, and lymphoid organs of cells arrested in the $G_0/G_1$ stage of the cell cycle. The factors involved in clonal expansion of the malignant CLL cells remain incompletely understood.

One approach to understanding CLL is to investigate the nature of intracellular signals responsible for the development and prolonged survival of the malignant cells. In this regard, signals generated by B-cell receptor (BCR) engagement are known to play an important role. A key mediator of BCR-induced signalling is protein kinase C (PKC), and, in CLL cells, this class of enzymes has been identified as a possible target of therapeutic intervention based on in vitro studies demonstrating that inhibition of these enzymes induces apoptosis. Considering the role of BCR signals in survival and clonal expansion of CLL cells and the role of PKC(s) in the signalling pathways that are induced by BCR engagement, it follows that PKC(s) may play an important role in BCR-induced survival of CLL cells.

The PKC family is divided into three subgroups; the classical which includes PKCα, βI, βII, and γ, the novel which includes PKCδ, ε, η, and θ, and the atypical which includes PKCλ, and ζ. These enzymes are activated by the presence of Ca$^{2+}$ and/or diacylglycerol, or by other activating factors, and function in an array of cellular processes that can be specific for a particular cell type. In B cells, PKCζ, PKCβ3, PKCδ, and PKCε play important roles in regulating signals generated by the BCR. With respect to CLL, active PKCδ is thought to maintain cell survival downstream of phosphoinositol 3'-kinase. Despite the potential of PKCs as therapeutic targets in CLL, little is known about the relative levels and activities...
of the different isoforms known to be expressed within the malignant cells of this disease.

In the present study, we show that PKCβII is overexpressed in CLL cells, and that the activity of this enzyme inversely correlates with CLL-cell response to BCR engagement. Therefore, by regulating BCR signals important for malignant-cell survival, PKCβII may be a key factor in CLL progression.

Materials and Methods:

Materials: Mouse monoclonal and rabbit polyclonal anti-PKCβII, monoclonal anti-PKCβI, PLCγ2, and CD40 antibodies, rabbit anti-PKCα, -PKCδ, -PKCζ, McI-1 and procaspsae 8, and horseradish peroxidase-conjugated anti-mouse, anti-rabbit, Ig antibodies were purchased from Santa Cruz Biotechnology Inc. (Insight Biotechnology Ltd, Middlesex, U.K.). Monoclonal anti-PKCδ and anti-PKCε antibodies were purchased from BD Biosciences (Oxford, U.K.). F(ab2)’ fragments of goat anti-human IgM were purchased from Jackson Immunoresearch Laboratories (Stratech, Soham, U.K.). Mouse anti-pS180-Bruton’s tyrosine kinase (Btk) and rabbit anti–Btk and –pY759-PLCγ2 antibodies were purchased from Cell Signaling Technology (New England Biolabs, Hitchin, Herts, U.K.). Purified recombinant PKCα, PKCβI, PKCβII, PKCδ, PKCε, and PKCζ proteins, and Ro32-0432 were purchased from Merck Biosciences Ltd. (Nottingham, U.K.). Purified recombinant PKCε and PKCζ proteins and mouse anti-ZAP-70 antibody were purchased from Upstate Ltd. (Milton Keynes, U.K.). Indo-1AM, and Alexa-Fluor 630 anti-mouse and anti-rabbit antibodies were purchased from Molecular Probes (InVitrogen, Paisley, U.K.). 3,3’-dihexyloxacarbocyanine iodide (DiOC₆), propidium iodide (PI) and polyHEMA were from (Gillingham U.K.). Enhanced chemiluminescence (ECL)
Purification of cells: Normal B cells were purified from buffy coats obtained from the British Transfusion Service (Liverpool, U.K.). Hairy cell (HC) leukaemia and CLL cells were obtained from peripheral blood of patients by informed consent and with the approval of the Liverpool Research Ethics Committee. The purified cells were cryopreserved. When required, the cells were thawed, resuspended in culture media and equilibrated at 37°C as described before\(^{17}\). In some experiments, the cells were further purified using anti-CD19 antibodies coupled to magnetic beads within a MiniMacs system (Miltenyi Biotech, Bisley U.K.) according to the manufacturer’s instructions. Cell purity was assessed by flow cytometry using a Becton Dickinson FACSCalibur\(^{\text{TM}}\), and was not less than 90% B cells for all the cases used.

Diagnosis of HCL (n=5 cases) and CLL (n=63 cases) were based on standard morphological, immunophenotypic and cytogenetic criteria\(^{18}\). The CLL-cell samples used in the present study were from patients selected at random from our Departmental tissue bank in which cases with high malignant-cell counts and advanced disease stage are overrepresented (supplementary Table 1).

Quantitation of PKC isoforms in cells: B lymphocytes were lysed with Buffer A (125 mM Tris pH 6.8, 5 mM EDTA, 1% SDS, 10% glycerol), sonicated to disrupt released DNA, and incubated for 10 min at 95°C. The protein concentration in the cell lysates was quantitated using a BioRad\(^{\text{TM}}\) DC protein assay kit. 10 µg protein was
loaded on to 10% SDS-PAGE gels together with known amounts of purified recombinant proteins (PKCβII, PKCβI, PKCδ, PKCα, PKCζ, and PKCε), and the separated proteins then electroblotted to Immobilon™ membranes. Membranes were blocked in 5% milk T-TBS (150mM NaCl, 25mM Tris pH 7.5, 0.1% Tween-20), and probed with anti-PKC antibodies. The membranes were further probed with second-layer antibodies conjugated with Alexa-Fluor 633 fluorochromes, or with HRP. Results were read with a Fujifilm FLA-5000 imager for measurement of immunofluorescence, or with a Fujifilm LAS-1000 for measurement of chemoluminescence following treatment of the membrane with ECL-Plus™ substrate reagents. Specific protein quantitation was achieved by comparing the immunofluorescence / chemiluminescence of PKC bands within CLL cell lysates with that of recombinant protein bands used as standards on the same Western blot, as depicted for PKCβII in Figure 1A. Protein quantities are reported as percent values of total loaded protein (10µg). Equal protein loading was validated by Western blot analysis of β-actin in the CLL cell lysates (Figure 1A).

Real Time reverse transcription-PCR analysis of PKCβ mRNA expression: Total RNA was extracted from B cells using an RNeasy® mini kit (Qiagen, Crawley U.K.) according to the manufacturers instructions. 1µg purified RNA was reverse transcribed using Molony murine leukaemia virus reverse transcriptase (Promega, Southampton U.K.) and an oligo(dT)15 primer. Purified cDNA was mixed with DyNAmo® SYBR green I qPCR master mix (Finnzymes, Espoo Finland), a consensus PKCβ forward primer (5′–TGGGGTGACAACCAAGACATTC–3′), and reverse primers specific for PKCβI (5′–TGGAGGTGTCTCTCTTGTCTC–3′) or PKCβII (5′–GTCAATAATCCTGATGACTTCCTG–3′). We used β-actin and glyceraldehyde-3-
phosphate dehydrogenase (gapdh) as internal controls, and these were amplified from each cDNA sample using the forward primer (5′–CCTCGCCTTTGCCGATCC–3′) and reverse primer (5′–GGATCTTCATGAGGTAGTCAGTC–3′) for β-actin, and the forward primer (5′–AGCCACATCGCTCAGAACAC–3′) and reverse primer (5′–GACGCATTGCTGATGATCTTG–3′) for gapdh19. All PCR reactions were performed on a DNA engine Opticon 2 system (MJ Research) under optimised, identical cycling conditions consisting of a 10 min initial denaturing step at 95°C, followed by 45 cycles of amplification (denature at 94°C for 20s, anneal at 60°C for 20s, extension at 72°C for 20s, and collect fluorescence data at 80°C). Following a final 10min extension at 72°C, a melting curve was measured from 65°C to 98°C. The PCR products were reannealed at 72°C for 10 min if they were to be analysed on agarose gels. The specificity of each of these PCR products was confirmed as a single band with the expected molecular size on agarose gels, and as a narrow peak that appeared in the melting curve when temperatures rose higher than 80°C. The transcript levels of PKCβI and PKCβII were presented as a ratio to the transcript level of gapdh.

**CLL cell viability assays:** CLL cells, at 5x10^6 cells/ml, were cultured for 5 days with RPMI 1640 plus 0.5% BSA supplemented with L-glutamine and streptomycin in 24-well culture plates (Falcon, BD Biosciences, Cowley U.K.) coated with polyHEMA. Cell viability was assessed by flow cytometry using DiOC6 to measure mitochondrial integrity, and propidium iodide incorporation (as a measure of dead cells) according to established protocol20. In some experiments BCR was crosslinked with 10µg/ml F(ab’)2 fragments of goat anti-human IgM antibody to promote CLL cell survival.
Measurement of intracellular Ca\(^{2+}\). Intracellular calcium concentration was determined by measuring fluorescence emission at 410 nm and at 485 nm during excitation at 350 nm according to published protocol\(^{21}\) using an Aminco Bowman Series 2 spectrofluorometer (Thermo Electron Corporation, U.K.) and Indo-1AM as a fluorescent ratiometric probe.

Intracellular Ca\(^{2+}\) release in CLL cells was stimulated with the addition of 20\(\mu\)g/ml goat anti-human IgM antibodies to the Indo-1-loaded cells. Fluorescence was monitored continuously, and intracellular [Ca\(^{2+}\)] was determined using the software provided with the spectrofluorometer\(^{21}\).

Determination of membrane-associated PKC\(\beta\)II in CLL cells. This was performed by subcellular fractionation to obtain membrane and cytosolic fractions as described\(^{22}\). The protein concentration of each fraction was determined and equal amounts of protein from each fraction separated by SDS-PAGE. The amount of PKC\(\beta\)II in each fraction was determined by Western blotting. The percent of membrane-associated PKC\(\beta\)II was taken as the amount of PKC\(\beta\)II in the membrane fraction divided by the amount of total PKC\(\beta\)II in both the membrane and cytosolic fractions. Purity of the membrane and cytosolic fractions was verified by Western blot analysis for CD40 (membrane fraction)\(^{23}\) and procaspase 8 (cytosolic fraction)\(^{24,25}\) (data not shown).

PKC kinase assays. 2x10\(^7\) CLL cells were resuspended into buffer B (20mM Tris pH 7.4, 2mM EDTA, 0.5mM EGTA, 1mM PMSF, 1\(\mu\)g/ml aprotinin) and incubated on ice for 30min. The cells were disrupted by passing them 10 times through a 25-gauge needle, nuclei and other cellular debris were removed by centrifugation at 1000xg for
10 min, and the supernatant was loaded on to a HiTrap™-DEAE-FF column (GE Healthcare, Little Chalfont, U.K.). The column was washed with 5ml buffer B, and the PKC containing fraction eluted from the column with buffer B containing 200mM NaCl. PKC activity was assessed in the eluate using a SignaTECT® PKC assay system according to the manufacturer’s instructions (Promega, Southampton, U.K.). PKCβ activity was calculated by subtracting PKC activity in the presence of 50nM LY379196 from PKC activity in the absence of this PKCβ inhibitor.

Statistical Analysis: Data sets were compared for statistical significance using either student’s t-test or Mann-Whitney U-test. The comparisons were performed by computer with Microsoft Excel® and SPSSv13.0® software, respectively.

Results:

Since PKC expression in CLL cells has not been fully characterised, the profile of PKC isoforms expressed in these cells was determined first and compared with PKC-isoform expression in normal peripheral blood B cells, hairy cell leukaemia cells (HCs) and a number of other mature malignant B cells.

Profiles of PKC isoforms expressed in CLL cells, HCs, and normal peripheral blood B cells. Analysis of PKC isoforms showed that CLL cells express PKCα, βI, βII, δ, ε, μ, ζ, and ι. These isoforms were found to be expressed also in HCs (cells which are similar to CLL cells in having memory B cell gene expression profiles26-28) and in normal peripheral blood B cells, but the expression levels differed between different cell types (Figure 1B). Thus, CLL cells and HCs expressed less PKCα and βI, and more PKCε than did normal B cells. Moreover, an overexpression of PKCβII clearly
distinguished CLL cells from both HCs and normal B cells (Figure 1B), as well as from mature B lymphoid malignancies such as mantle cell lymphoma, marginal zone lymphoma, follicular lymphoma, and plasma-cell leukaemia (Figure 1C).

Quantitation of PKC isoform expression in CLL cell lysates using the method depicted in Figure 1A shows that PKCβII comprises 0.53 ± 0.25% of total cellular protein (n=63). This, in comparison with other PKC isoforms (Table 1) establishes PKCβII as the dominant PKC isoform expressed in CLL cells.

**PKCβII is overexpressed in CLL cells.** PKCβII is an important mediator of BCR-induced signalling in B cells. To further explore the role of this PKC isoform in CLL cells, its expression in these cells was compared to that in HCs and normal B cells. Figure 2A shows that the amount of PKCβII protein in CLL cells is approximately 7-fold greater than in either HCs or normal B cells. The levels of PKCs in CLL cells were measured in relation to known amounts of recombinant protein and expressed as a percentage of total cellular protein to provide quantitative data for the expression of each isoform (Figure 1A). The validity of this approach was confirmed by FACS analysis of permeabilized cells showing that CLL cells express considerably more of this enzyme than do HCs (Figure 1D). Further analysis revealed a pronounced variability in expression of PKCβII in CLL cells that was not observed in either HCs or normal B cells. This variability, however, had no clear relationship with IgVH gene mutation or with CD38 expression (see supplementary Table 1); however, higher PKCβII expression levels were observed in the malignant cells from patients with 11q23 and 17p13 abnormalities (respectively responsible for ATM and p53 gene deletions in CLL) (Figure 2B, supplementary Table 1). Moreover, there was an apparent link with disease stage and tumour load at the time of sampling. Thus, cells...
from CLL cases with stage A disease had significantly lower PKCβII levels than did cells from CLL cases with stage C disease (Figure 2C, supplementary Table 1), and there was a positive relationship between PKCβII expression and white blood cell count (Figure 2D, supplementary Table 1). Taken together, these data demonstrate an overexpression of PKCβII that distinguishes CLL cells from HCs, and normal B cells, but do not provide insight into the underlying cause(s) of the variability in the expression of this protein in different CLL-cell clones.

The pkcβ gene is transcribed as a single mRNA, which is then variably spliced to yield the coding sequences for PKCβI and PKCβII. RT-PCR analysis of RNA isolated from CLL cells showed that both splice variants were present, and that mRNA for PKCβII was present in far greater quantities than was that for PKCβI (data not shown). This suggests that PKCβII is the preferred splice variant in CLL cells, a finding supported by the observation that PKCβII protein was present in 35.7±15.2 (n=4) fold greater quantities than was PKCβI.

A comparison of PKCβII mRNA expression in CLL cells, HCs, and normal B cells by quantitative RT-PCR mirrored the protein data. Figure 2E shows that PKCβII mRNA is more highly expressed in CLL cells than in HCs. In Figure 2E, PKCβII mRNA levels in normal B cells are not included because, although PKCβII mRNA was present in these cells (n=4), its expression levels were too low to be reliably quantitated using the same method that was used for CLL cells and HCs (data not shown). Taken together, the above data demonstrate that PKCβII is overexpressed in CLL cells at both the mRNA and protein levels.
PKCβII is active in CLL cells. To measure PKCβII activity in CLL cells, subcellular fractions were prepared to establish the ratio between the membrane-bound and cytosolic fractions of the total enzyme. This method was used because membrane association is an established paradigm of PKC activation. Furthermore, immune-complex kinase assays cannot be used to directly measure PKCβII activity owing to an inability of specific antibodies to reliably immunoprecipitate active enzyme (data not shown), and also to a reduction of activating lipids by the detergents used to lyse the cells during the immunoprecipitation procedure. Measurement of the ratios of membrane- and cytosolic-PKCβII clearly demonstrated that the degree of PKCβII activation varies between different CLL cases (Figure 3A and B). This variation in the degree of PKCβII activation as measured by membrane association was confirmed using a second technique where PKCβ activity was assessed in kinase assays of crude PKC extracts from CLL cells. Thus, a near perfect correlation between PKCβ kinase activity and PKCβII membrane association was observed (Figure 3C). We therefore used membrane association as a measure of PKCβII activity for the rest of this study.

In HCs, PKCβII was almost entirely membrane-associated in contrast to that in normal B cells where the enzyme was predominantly cytosolic (Figure 3B), reflecting the respective “activated” and “resting” states of these two cell types. In CLL cells, PKCβII activity was much higher than in normal B cells (p<0.001), but not all cases showed the same high degree of membrane association as observed in HCs (Figure 3B). This suggests that stimuli that activate PKCβII in some CLL cases may not be present in others.

Effect of PKCβ inhibition on CLL cell survival. Several studies have suggested that PKCs play an important role in CLL-cell cytoprotection. To determine the role of
PKCβII in CLL cell survival, we used the PKC inhibitor LY379196 at concentrations which specifically inhibit both PKCβI and PKCβII while having little effect on other PKC isozymes and protein kinases. Incubation of CLL cells for 5d with this inhibitor resulted a relatively small reduction in cell viability (Figure 4A) although the phosphorylation of Btk, a known substrate of PKCβII, was clearly reduced by this treatment (Figure 4C). In contrast, treatment of CLL cells with more general PKC inhibitors such as Ro32-0432 (Figure 4B) or safingol (data not shown) led to a much greater reduction in cell viability. These results indicate that PKCβII does not play a decisive role in protecting CLL cells from spontaneous apoptosis.

The role of PKCβII in BCR signaling. In normal B cells, the phosphorylation of Btk by PKCβII inhibits the ability of Btk to associate with the membrane resulting in the feedback inhibition of BCR signals. It was therefore important to establish whether, in CLL cells, the levels of membrane-associated Btk correlate with active PKCβII. Figure 5A shows that Btk in CLL cases with high PKCβII activity was mainly in the cytosol, whereas in CLL cases with low PKCβII activity Btk was mainly at the membrane resulting in a negative correlation between these two parameters. The main substrate of Btk in B cells is phospholipase Cγ2 (PLCγ2) which is activated by Btk through phosphorylation of tyrosine 759. Analysis of (pY759)-PLCγ2 showed a similar negative correlation with active PKCβII (Figure 5B). Taken together, these data indicate that active PKCβII in CLL cells can influence these cells through downregulation of BCR-mediated signals.

Following BCR stimulation, activated PLCγ2 hydrolyses phosphatidylinositol bisphosphate to inositol trisphosphate (IP₃) and diacylglycerol which are respectively...
required for the release of intracellular Ca\(^{2+}\) and activation of PKC(s). Since feedback inhibition of BCR signaling by overexpressed PKC\(\beta\)II would be expected to influence this process, the BCR-induced Ca\(^{2+}\)-fluxes in cells with different levels of PKC\(\beta\)II activity were examined next. Figure 6A and B show that cases with high levels of PKC\(\beta\)II activity responded to BCR crosslinking with a significantly lower Ca\(^{2+}\) flux than cases with low levels of PKC\(\beta\)II activity. This difference could not be ascribed to effects of IgV\(\mu\) mutation on the cell response because the cells used in Figure 6A contained unmutated IgV\(\mu\) genes. Also, the variation in Ca\(^{2+}\) flux could not be ascribed to differences in ZAP-70 expression, a protein known to influence BCR signaling in CLL cells\(^{35,36}\), because the CLL cases used in Figure 6A had similar levels of this enzyme (Figure 6A, inset). In addition, in CLL cases with high PKC\(\beta\)II activity, pre-treatment of the cells with 100nM LY379196 (to inhibit the activity of PKC\(\beta\)II) restored BCR-induced Ca\(^{2+}\) fluxes (Figure 6C). In contrast, in cases with low PKC\(\beta\)II activity, pre-treatment of the cells with 10nM bryostatin to activate PKC\(\beta\)II inhibited BCR-induced Ca\(^{2+}\) fluxes (Figure 6D). The fact that 100nM LY379196 inhibited the PKC\(\beta\)II-mediated serine phosphorylation of Btk (Figure 4C) shows that this compound was indeed acting to inhibit PKC\(\beta\)II in CLL cells. Taken together, these results demonstrate that overexpressed active PKC\(\beta\)II in CLL cells has an important modulatory influence on BCR signaling.

In CLL cells, BCR stimulation provides important pro-survival signals\(^1\). Therefore, to examine how the survival of CLL cells is affected by different levels of active PKC\(\beta\)II, the viability of different CLL cell clones was measured following culture with a BCR crosslinking agent. Figures 7A and B show that BCR crosslinking enhanced the survival of malignant cells from CLL cases with low levels of PKC\(\beta\)II
activity, whereas such stimulation had little or no effect on the malignant cells from CLL cases with high levels of PKCβII activity.

Mcl-1 upregulation is an important component of BCR-induced survival of CLL cells. Therefore, we next examined the effect of BCR crosslinking on Mcl-1 expression in CLL cases with high and low levels of PKCβII activity. BCR stimulation induced a 1.4±0.1 fold ($p=0.045$, n=3) increase in Mcl-1 expression in PKCβII low-activity CLL cases (Figure 7C). In contrast, in PKCβII high-activity cases, BCR crosslinking did not lead to a significant change in Mcl-1 expression (Figure 7C).

This modulation of BCR-induced survival of malignant cells by PKCβII suggests that this enzyme may influence CLL progression through its effects on BCR signaling and its outcomes.

**Discussion:**

Although it is clear that BCR signals are important for CLL-cell survival, and that PKCs may play a role in the regulation of these signals, neither the identity nor the role(s) of the involved PKC(s) were known. Therefore, the aim of the present study was to identify and characterise the PKC isozymes which participate in the regulation of BCR-mediated CLL-cell survival. The data presented here demonstrate that CLL cells overexpress PKCβII, and show an important role for this isozyme in the regulation of BCR signalling and its outcomes.

The overexpression of PKCβII in CLL cells is important for several reasons. In mature and developing B cells it is PKCδ which is the dominantly-expressed PKC isoform. Moreover, during B-cell maturation to the memory stage, which is thought to correspond to the stage of development of CLL, expression of PKCβ remains
constant\textsuperscript{40}, or may be downregulated\textsuperscript{41}. Also, the levels of PKCβII are much higher in CLL cells than in HCs which, like CLL cells, have also reached a memory-cell stage of differentiation\textsuperscript{26,28}. In diffuse large B cell lymphoma (DLBCL) high PKCβII expression identifies cases with an activated B-like phenotype\textsuperscript{42,43}; however, the overall level of \textit{pck}β gene expression in DLBCL is lower than that observed in CLL\textsuperscript{39,44}. Thus, PKCβII overexpression may be a feature which is unique to CLL and which could be used as an aid in disease diagnosis. Whether high PKCβII expression reflects the cell of origin in CLL is not known. Further studies comparing gene expression in B cells differentiating under the influence of different microenvironments will have to be performed before this question can be addressed.

Overexpression of PKCβII in CLL cells could also be a specific feature of cell malignancy. This notion is supported by observations that the clinical course of activated B-like DLBCL, in which PKCβII is overexpressed, is more aggressive than GC-like DLBCL\textsuperscript{42-45}, and by reported high PKCβII levels in cancers of the colon where it is suggested that overexpressed enzyme can promote carcinogenesis in these cells\textsuperscript{46,47}.

In CLL, expansion of the malignant cell clones is thought to involve chronic antigenic stimulation\textsuperscript{1} whereby PKCβII may contribute to this expansion through its established role in BCR and Toll-like receptor (TLR) signaling\textsuperscript{3,4,7,8,48-50}. PKCβII initially participates in positive BCR-induced signalling for cell proliferation and survival\textsuperscript{51}, but is also an important component of feedback inhibition of such signalling through a mechanism involving phosphorylation-induced dissociation of Btk from the cell membrane\textsuperscript{3}. Therefore, we examined the correlation between the levels of active PKCβII in CLL cells and cell signals thought to originate from BCR. This showed that constitutively active PKCβII levels negatively correlate with levels...
of membrane-associated Btk and with tyrosine phosphorylation of its substrate
PLCγ2. The notion that PKCβII may be influencing CLL cells through
downregulation of BCR-mediated signals is further supported by its inhibitory effects
on BCR-induced Ca^{2+} release and cell survival. Thus, CLL cases with high levels of
active PKCβII responded to BCR crosslinking with a significantly lower Ca^{2+}-flux
than did cases with low levels of PKCβII activity. Moreover, BCR crosslinking had a
greater pro-survival effect on CLL cells with low PKCβII activity than on cells with
high PKCβII activity. The decrease in pro-survival effect of IgM crosslinking
observed in cases with high PKCβII activity levels could be explained by the
pronounced feedback inhibition of BCR signaling seen under these circumstances. A
possibility that differences in BCR responsiveness reflected an abnormality upstream
of PKCβII was excluded by the demonstration that the defect in BCR-induced Ca^{2+}
release was overcome by inhibiting PKCβII with LY379196, and that high Ca^{2+}
fluxes in CLL cases with low levels of active PKCβII were reduced when this enzyme
was activated by bryostatin. Taken together, these results demonstrate that active
PKCβII in CLL cells has a regulatory influence on BCR signalling and its outcomes,
and provide a mechanistic explanation for frequently observed variation in BCR-
induced Ca^{2+} release and survival.

The heterogeneity of CLL-cell responses to BCR-induced signals is well
documented, and may involve both the induction of cell death as well as
a variable degree of cell protection from spontaneous apoptosis. The
heterogeneity in response to BCR stimulation is related to the degrees of CD38 and
ZAP-70 expression, and the degree of mutation of IgVH genes. Furthermore, one report has shown that ZAP-70 expression enhances IgM signalling
in CLL cells. Here we show that by causing feedback inhibition of upstream signals,
PKCβII has a ZAP-70-independent effect on the cell responses to BCR stimulation. This effect was clearly not due to variation in ZAP-70 because expression levels of this kinase were equal in the CLL cases with high and low levels of PKCβII activity (Figure 6A). Thus, heterogeneity in the levels of expression and activation of PKCβII in CLL observed in the present study could at least partially explain the differences in the effects of BCR stimulation on CLL-cell survival.

In the present study we show that, unlike other PKC isoforms, PKCβII does not play a decisive role in the cytoprotection of CLL cells from spontaneous apoptosis. Thus, inhibition of PKCβII activity with LY379196 had only a minimal effect on survival of unstimulated CLL cells whereas the effects of more general PKC inhibitors were more profound. In addition, we confirmed that one of the candidate survival factors induced by BCR crosslinking is Mcl-1. The upregulation of this survival factor was also suppressed in CLL cases with high PKCβII activity.

It is currently believed that survival of malignant CLL cells, like that of normal B-1 cells and T cells, depends on a low affinity interaction of BCR with self antigens. However, when drawing a parallel between normal mature B cells and CLL cells, additional properties unique to the malignant cells must be considered. Thus, in contrast to quiescent normal B cells, CLL cells are thought to be subjected to chronic antigenic stimulation. Moreover, unlike normal B cells, antigen-stimulated CLL cells fail to undergo terminal differentiation into antibody-secreting cells. Although turnover studies show that malignant CLL cells have a relatively limited life span, the balance between cell proliferation and cell death favours clonal expansion of these cells. Therefore, BCR signals, known to protect CLL cells from apoptosis, are likely to play an important role in the progression of CLL. In this light, the results of the present study support a hypothesis where, in CLL cells, overexpressed and activated
PKCβII functions as a feedback switch that keeps cell activation below the potentially pro-apoptotic level responsible for negative cell selection. Below this level BCR signalling is involved in cell rescue which is also kept in balance by PKCβII in a way that activation of this overexpressed enzyme may limit these pro-survival signals. Presumably the expansion of the malignant cell clone is then principally driven by proliferative and cell-rescue stimuli mainly originating from sources other than BCR. The likely candidates are receptor tyrosine kinases for autocrine growth factors such as VEGF60, TNF receptor families including CD4061 and BAFF receptor62, and receptors for cytokines such as IL463. Our observation that PKCβII is more highly expressed in the malignant cells from patients with advanced disease suggests that these other factors rather than BCR provide signals for disease progression at the late stages of CLL.

In conclusion, the present study demonstrates that PKCβII is overexpressed in CLL cells, and that the activity level of this enzyme is inversely related to the ability of these cells to respond to BCR stimulation. Considering that antigenic stimulation is a major factor involved in selection, clonal expansion and survival of CLL cells, the results of the present study suggest an important role for PKCβII in the modulation of the consequences of this stimulation.

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References


Tables:

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<td>0.021 ± 0.0088</td>
<td>5</td>
</tr>
<tr>
<td>PKCμ</td>
<td>0.12 ± 0.047</td>
<td>8</td>
</tr>
<tr>
<td>PKCζ</td>
<td>0.0097 ± 0.0036</td>
<td>6</td>
</tr>
<tr>
<td>PKCι</td>
<td>0.0082 ± 0.0035</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 1: *PKC expression in CLL cells.* Expression levels of various PKC isoforms in CLL cells presented as the percentage fraction of total cellular protein.
Figure Legends.

Figure 1 Profile of PKC isoform expression in mature B lymphocytes. 
A.) Quantitation of PKCβII expression in CLL cells by Western blotting. Equal amounts of protein were used from each CLL case and the intensity of the PKCβII-reactive band was compared with that generated from known amounts of recombinant PKCβII. β-actin is used to indicate equal protein loading. B.) Western blot analysis of PKC isoform expression by CLL, HCL, and CD19-purified normal B cells. Equal amounts of protein were analysed for each PKC isoform and cell type. This figure is representative of 3 separate experiments using different cases. C.) Western blot analysis of PKCβII expression in CLL cells, mantle cell lymphoma cells (MCL), marginal zone lymphoma cells (MZL), follicular lymphoma cells (FC), and plasma cell leukaemia cells (PCL). Equal amounts of protein were loaded for each cell type. D.) Flow cytometric analysis of PKCβII expression in permeabilised CLL and HCL cells. This figure is representative of 2 separate experiments using different cases of CLL and HCL.

Figure 2 PKCβII is overexpressed by CLL cells. Quantitative analysis of PKCβII protein and mRNA expression. A.) Analysis of PKCβII protein in CLL, HCL, and CD19-purified normal B cells. B.) Analysis of PKCβII protein in CLL cells from patients with the indicated chromosomal abnormalities. 11q23 and 17p13 deletions were combined because these abnormalities are typically associated with bad disease. 13q14 deletion and trisomy 12 chromosomal abnormalities were grouped together with normal karyotypes because these abnormalities are not associated with bad disease. C.) Analysis of PKCβII protein in CLL cells from patients with different disease stages at the time of sampling. D.) Analysis of PKCβII protein in relation to white blood cell count. In parts A – D PKCβII protein levels are expressed as a percentage of total cellular protein. E.) Analysis of PKCβII mRNA by quantitative RT-PCR in CLL cells and HCs. The results are expressed relative to β-actin mRNA in the same cell. Tests for statistical significance in this Figure were performed using a Mann-Whitney U test.
Figure 3 PKCβII is active in CLL cells. Subcellular fractions from CLL, HCL, and
CD19-purified normal B cells were isolated using ultracentrifugation. A.) Western
blot analysis of the distribution of PKCβII between membrane (M) and cytosol (C)
fractions in the malignant cells from representative CLL cases. B.) Quantitative
representation of membrane-association of PKCβII in CLL cells (n=25), HCL cells
(n=5), and normal B cells (n=3). Results are presented as the ratio of the band density
of PKCβII in the membrane fraction over the combined densities within the
membrane and cytosolic fractions. Tests for statistical significance were performed
using a Mann-Whitney U test. C.) Correlation between PKCβII membrane-
association in CLL cells with in-vitro kinase assay of enzyme activity in crude
preparations of total cellular PKC.

Figure 4 Effect of PKC inhibition on CLL cell survival. CLL cells were treated with
the indicated inhibitors and measured for viability. Viability of the treated cells was
normalised to that of untreated cells and is reported as a percentage thereof. A.)
Viability of CLL cells incubated with 1µM LY379196 for 5 days. (n=15) B.) Viability
of CLL cells incubated with 10µM Ro32-0432 for 3 days. (n=4) Tests for statistical
significance were performed using student’s t-test for paired data. C.) pS<sup>180</sup>-Btk was
immunoprecipitated from RIPA lysates of CLL cells incubated in the presence or
absence of 100nM LY379196. Immunoprecipitated proteins and whole cell lysates
were separated by SDS-PAGE, and Western blots were probed with anti-Btk
antibodies. CLL A, B, and C are new cases which are not represented in
supplementary Table 1.

Figure 5 Levels of active PKCβII correlate with membrane-associated Btk and with
pY<sup>759</sup>-phosphorylated PLCγ2. A.) Plot of membrane-associated Btk against
membrane-associated (active) PKCβII in CLL cells (n=12). The percent membrane
association of Btk in CLL cells was determined from membrane and cytosolic
subcellular fractions as described for PKCβII. B.) Plot of pY<sup>759</sup>-PLCγ2 against
membrane-associated (active) PKCβII in CLL cells (n=12). The degree of pY<sup>759</sup>-
PLCγ2 was determined in Western blot analysis of whole cell lysates, taking the ratio
of the densities of the bands recognised by antibodies specific for non-phosphorylated PLCγ2 and for pY759-PLCγ2. The results are reported in arbitrary units.

**Figure 6 PKCβII activity regulates BCR-induced Ca²⁺ release in CLL cells.** A.) CLL cases with high and low PKCβII activity (defined, respectively, as containing amounts of active PKCβII greater or less than one standard deviation from the mean value) were stimulated with 20µg/ml anti-IgM antibody (BCR-XL) and intracellular Ca²⁺ release measured using the dye Indo-1. Comparison of BCR-induced Ca²⁺ release in CLL cases with high and low levels of active PKCβII. Western blot analysis of ZAP-70 expression in these cases is shown in the inset. CLL21 is the CLL case with low PKCβII activity. CLL50 is the CLL case with high PKCβII activity. B.) Comparison of peak Ca²⁺ levels during BCR stimulation of CLL cases with high (n=5) and low (n=5) levels of active PKCβII. C.) 1h pre-treatment with 100nM LY379196 restores BCR-induced Ca²⁺ release in CLL cases with high levels of active PKCβII. D.) 30min pre-treatment with 10nM bryostatin suppresses BCR-induced Ca²⁺ release in CLL cases with low levels of active PKCβII. Parts C and D are representative examples of 3 separate experiments using cells from different patients.

**Figure 7 PKCβII activity regulates BCR-induced survival of CLL cells.** Cells from the same CLL cases with high and low PKCβII activity as in Figure 6 were incubated with 10µg/ml F(ab')₂ fragments of goat anti-human IgM. A.) Aliquots of stimulated (dashed line) and control (solid line) CLL cells were taken at 1d intervals, stained with PI and DiOC₆ to determine viability using flow cytometry with a fixed time setting of 30s to quantitate the number of cells per unit volume. The data presented in each graph is representative of a single experiment using 4 CLL cases with high and 4 CLL cases with low levels of PKCβII activity. Each experiment was done in triplicate. B.) Percentage difference in cell viability between stimulated and control cells with high and low PKCβII activity CLL cases following BCR stimulation. Cell viability was measured at day 3 of culture. Tests for statistical significance were performed using a Mann-Whitney U test (n=5). C.) Influence of BCR crosslinking (BCR-XL) and byrostatin on Mcl-1 expression in CLL cells with different levels of PKCβII activity (representative Western blots of experiments with cells from three
CLL cases with high and three CLL cases with low levels of PKCβII activity) CLL cells were stimulated with 10nM bryostatin or with BCR crosslinking for 24h.
Figure 1

A
PKCβII protein standards
1600ng 400ng 100ng CLL-21 CLL-12 CLL-16 CLL-50 CLL-17
82kD PKCβII
49kD β-actin

B
CLL HCL B cell
PKCα
PKCβI
PKCβII
PKCγ
PKCe
PKCμ
PKCζ
PKCε
PKCι

C
CLL MCL MZL FL PCL

D
WB:PKCβII

Figure 2

A
PKCβII Expression
CLL cells HCL cells Normal B cells
(n=63) (n=5) (n=13)
P<0.001 P<0.001

B
PKCβII Expression
11q23 + 17p13 Normal + 13q14 + tri 12
(n=12) (n=98)
P<0.001

C
PKCβII Expression
Stage A Stage B Stage C
(n=20) (n=11) (n=10)
P=0.006

D
White Blood Cell Count
PKCβII Expression

E
Relative PKCβII Expression
CLL (n=11) HCL (n=4)
P<0.001
Figure 3

A

B

C

Figure 4

A

B

C
Figure 5

A

B
B cell receptor signalling in chronic lymphocytic leukaemia cells is regulated by overexpressed active protein kinase C βI

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