B-cell antigen receptor activates transcription factors NFAT (nuclear factor of activated T-cells) and NF-κB (nuclear factor κB) via a mechanism that involves diacylglycerol

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Abstract

Engagement of the B-cell antigen receptor (BCR) induces the activation of various transcription factors, including NFAT (nuclear factor of activated T-cells) and NF-κB (nuclear factor κB), which participate in long-term biological responses such as proliferation, survival and differentiation of B-lymphocytes. We addressed the biochemical basis of this process using the DT40 chicken B-cell lymphoma. We discovered that Bruton's tyrosine kinase (BTK) and phospholipase C-γ2 (PLC-γ2) are required to activate NFAT and NF-κB, and to produce the lipid second messenger diacylglycerol in response to BCR cross-linking. Therefore the functional integrity of the BTK/PLC-γ2/diacylglycerol signalling axis is crucial for BCR-directed activation of both transcription factors NFAT and NF-κB.

Introduction

The biochemical signals that emanate from the B-cell antigen receptor (BCR) are critical for the physiological state of B-lymphocytes. Indeed, they modulate gene expression that participates in various long-term biological responses, such as survival, proliferation, differentiation and apoptosis [1]. B-cell activation requires the recruitment of multiple enzymes to the plasma membrane, including Bruton's tyrosine kinase (BTK) and phospholipase C-γ2 (PLC-γ2), to form a multicomponent signalling complex termed the 'BCR signalosome' [2]. In B-cells, it is well established that one of the main targets of the signalosome is PLC-γ2 [3]. Upon activation, PLC-γ2 hydrolyses phosphatidylinositol bisphosphate (PtdInsP_2) to produce two lipid second messengers (LSMs), DAG (diacylglycerol) and inositol 1,4,5-trisphosphate (InsP_3) [4]. We discovered that BCR directs the activation of two essential transcription factors, NFAT (nuclear factor of activated T-cells) and NF-κB (nuclear factor κB), via a molecular mechanism that involves BTK and PLC-γ2 [5–7]. The production of DAG in B-cells represents an essential event that has been poorly understood as compared with that of InsP_3. However, DAG is a physiological activator of PKC (protein kinase C) that plays a key role in B-cell development. Understanding the precise signalling pathways that are involved in the generation and the modulation of DAG is of fundamental importance, because its relative levels may determine the BCR-directed biological outcomes.

Key words: B lymphocyte, Bruton's tyrosine kinase, diacylglycerol, nuclear factor of activated T-cells (NFAT), nuclear factor κB (NF-κB), phospholipase C-γ2.

Abbreviations used: BCR, B-cell antigen receptor; BTK, Bruton's tyrosine kinase; PLC-γ2, phospholipase C-γ2; LSM, lipid second messenger; DAG, diacylglycerol; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor κB; PKC, protein kinase C; MG, monoacylglycerol; NEFA, non-esterified fatty acid; TG, triacylglycerol.

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Materials and methods

Cells and reagents

The chicken DT40 B-cell line, which is used for studies of BCR signalling, was made deficient of BTK by homologous recombination (DT40.BTK). DT40.BTK cells reconstituted with BTK (DT40.BTKR) and DT40 cells made deficient of PLC-γ2 (DT40.PLC-γ2), were kindly given by Dr Tomohiro Kurosaki (Riken Cell Bank, Tsukuba, Japan; [8,9]). Cells were maintained in RPMI medium with 10 % (v/v) fetal calf serum, 1 % chicken serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine and penicillin/streptomycin at 39°C in 5% CO_2.

Lipid analysis

DT40, DT40.BTK, DT40.PLC-γ2 and DT40.BTKR cells were labelled with [^{3}H]palmitic acid (1 μCi/ml) as the fatty acid precursor. Labelled cells were washed twice with PBS and cultured for 10 h in low-serum (0.5–1.0%) RPMI 1640 medium. Cells (5×10^6 per stimulation) were washed and stimulated with anti-IgM antibodies (M4 supernatant; 1:1, v/v) for 5 min. The reactions were stopped by the addition of cold PBS. The cells were centrifuged and the pellet was used for lipid extraction [10]. Lipids were separated by TLC in one dimension using double development, and DAG was quantified as described previously [7]. Briefly, plates were first developed to the 'half-way stage' with the upper phase of the mixture of ethyl acetate/iso-octane/acetic acid/water (13:2:3:10, by vol.). Phosphatidic acid (PtdOH) was then separated from the neutral lipids. The second TLC was performed with the mixture isopropyl ether/acetic acid (96:4, v/v), inducing the separation of neutral lipids, namely

Figure 1 | BTK is a key regulator of DAG production in BCR-activated DT40 cells

[^{3}H]Palmitic acid-labelled DT40, DT40.PLC-γ2, DT40.BTK and DT40.BTKR cells were stimulated through the BCR (black bars) for 5 min, or incubated with vehicle (white bars). The production of DAG is expressed as a percentage of total radioactivity in lipids, and represents the average ± S.E.M. for three independent experiments performed in triplicate.

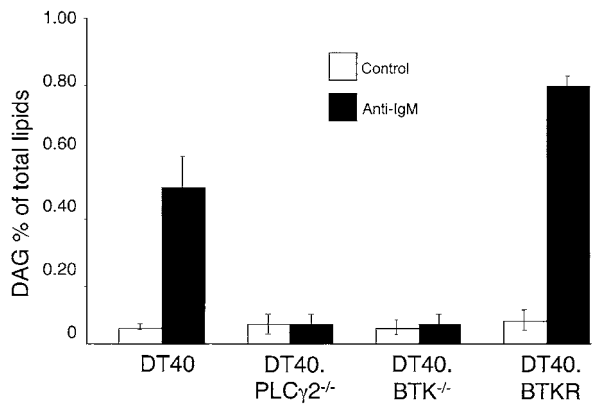
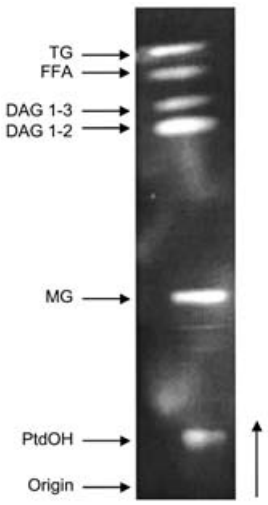


Figure 2 | Separation of lipids by TLC

Shown is the separation of the lipids of interest [PtdOH, MG, DAG, NEFA (free fatty acids; FFA) and TG]. TLC was performed in one dimension with a double development system, as described in the Materials and methods section. 'DAG 1-3' and 'DAG 1-2' refer to 1,3-DAG and 1,2-DAG respectively.



monoacylglycerols (MGs), isomers of DAG, non-esterified fatty acids (NEFAs) and triacylglycerols (TGs).

Results and discussion

We recently discovered that the tyrosine kinase BTK and a lipid metabolizer, PLC-γ2, are essential for the activation of two transcription factors, NFAT and NF-κB, in response to BCR stimulation [5–7]. The involvement of PLC-γ2 suggests a role for LSMs in the regulation of these transcription factors. Therefore, we investigated the underlying mechanism of LSM production, with a particular emphasis on DAG.

Here, we show that 5 min stimulation of DT40 B-cells by BCR engagement induces the production of DAG (Figure 1). As expected, BCR-dependent production of DAG was not detected in DT40.PLC-γ2 cells [7]. Although the latter finding is in agreement with a requirement for PLC in the formation of LSMs, a block in this process was unexpected in DT40.BTK cells under similar stimulatory conditions, since BTK is only partially responsible for activation of PLC-γ2 catalytic function [8]. These results suggest that the full activation of PLC-γ2 function and discernible production of DAG is BTK-dependent. To test the physiological relevance of BTK in this process, DT40.BTK cells were transfected with cDNA encoding human BTK protein, and cells were stimulated through the BCR for 5 min. Ectopic expression of human BTK in DT40.BTK cells rescued BCR-induced production of DAG as a function of the amount of expressed BTK (Figure 1). As such, BTK acts as a pivotal regulatory enzyme for the formation of a LSM, namely DAG.

Analysis of the steady-state levels of DAG alone does not reflect the total production, because DAG is subject to a dynamic metabolism, as observed in activated human platelets and Jurkat T-cells [11,12]. Therefore, for systematic studies of DAG metabolism in B-cells, we developed an experimental procedure that permits monitoring of the levels of PtdOH, the product of DAG kinase activity, and MG and NEFA, which are generated upon the activation of DAG lipase from DAG. In addition, we are able to evaluate the level of TG that represents an alternative pool for DAG (Figure 2). In contrast with the level of DAG, the levels of other LSMs and metabolites were not significantly modified in response to BCR cross-linking in B-cells (results not shown). Therefore neither PtdOH nor NEFA participates in the modulation of downstream events, such as PKC activation.

Further studies are required to determine the precise contribution of BTK, and perhaps other protein tyrosine kinases, in the kinetics of BCR-directed DAG production. Pharmacological inhibitors of DAG kinase (R59949) and DAG lipase (RHC-80267) are commercially available. Therefore our protocol will serve as a judicious tool to unravel the levels of LSMs that may direct the fate of B-lymphocytes.

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