Modulation of B lymphocyte signalling by the B subunit of *Escherichia coli* heat-labile enterotoxin

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Keywords: CD25, cholera toxin, class II MHC, extracellular signal-regulated kinase, ganglioside GM₁, phosphoinositol 3-kinase, protein kinase C

Abstract

The non-toxic B subunit of *Escherichia coli* heat-labile enterotoxin (EtxB) is a potent mucosal adjuvant and immunomodulator capable of blocking autoimmune disease. These effects are linked with its ability to modulate lymphocyte populations—a feature that is dependent on binding to ubiquitously expressed cell surface receptors. Here, we demonstrate that EtxB can trigger up-regulated expression of class II MHC and CD25 on purified populations of B lymphocytes, suggesting that EtxB can directly activate biochemical signalling pathways in these cells. The nature of the intracellular signalling events was investigated. B cells cultured with EtxB, but not a non-receptor binding mutant protein, EtxB(G33D), caused the activation of the extracellular signal-regulated kinase (Erk) forms of mitogen-activated protein (MAP) kinase in a process that was dependent on MAPK/Erk kinase (MEK), phosphoinositide 3-kinase (PI3-kinase) and protein kinase C (PKC), as determined by the use of specific inhibitors. PI3-kinase was critical not only in the activation of MAP kinase but also in the up-regulation of both class II and CD25. However, MEK inhibition only partially abrogated the EtxB-mediated up-regulation of MHC class II expression and did not affect CD25 expression—findings suggesting that additional pathways downstream of PI3-kinase are involved. A role for PKC in these processes was suggested by the finding that inhibitors of PKC completely blocked EtxB-mediated CD25 up-regulation. Thus, we have shown that receptor binding by EtxB triggers multiple signalling pathways in B cells that regulate the expression of key cell surface molecules.

Introduction

The non-toxic B subunit of *Escherichia coli* heat-labile enterotoxin (EtxB) and its structural homologue from cholera toxin (CtxB) are highly stable pentameric proteins that have been used widely as immune modulators in animal models of disease. EtxB is a potent mucosal adjuvant when co-administered with viral proteins, and when given alone can block the establishment and progression of autoimmune disease. The adjuvant activities of EtxB have been clearly demonstrated with a number of antigens including herpes simplex virus (HSV) glycoproteins (1) and influenza haemagglutinin (2). In the case of HSV, intranasal administration of an EtxB/HSV glycoprotein mixture triggered strong protective immunity to ocular challenge with the virus. The ability of EtxB to modulate autoimmune disease has been demonstrated since s.c. (3), intranasal or oral (4) delivery of EtxB can prevent collagen-induced arthritis (CIA) in the DBA/1 mouse model, and intranasal EtxB can prevent insulin-dependent diabetes mellitus (IDDM) in the NOD mouse (Turcanu and Williams, unpublished). Unlike EtxB, CtxB is a relatively weak adjuvant and cannot alter the course of autoimmune disease when given alone (4,5). However, when chemically conjugated to autoantigen, CtxB is capable of profoundly reducing the dose of antigen required to trigger mucosal tolerance; a feature that has been used to block experimental allergic encephalomyelitis (6), IDDM in the NOD mouse (7) and CIA (5).

The ability of the B subunits to alter the immune response results from their binding to cell surface receptors. This is evidenced by the observation that a receptor non-binding mutant of EtxB, EtxB(G33D), fails to exert any of the effects reported for the wild-type molecule (3,4,8–10). The principal
receptor to which both EtxB and CtxB bind is the ganglioside GM₁ (11). Receptor binding involves cross-linking five GM₁ molecules through extremely tight interactions with pockets formed at the interfaces between individual B subunit monomers [overall Kd 5.7 × 10⁻¹⁰ M and 7.3 × 10⁻¹⁰ M for EtxB and CtxB respectively (12)]. In addition, both molecules bind to GD1b, and EtxB has some affinity for asialo-GM₁, lactosylceramide and certain galactoproteins (13,14). GM₁ is ubiquitously found on mammalian cells and is composed of a pentasaccharide moiety bound into the outer leaflet of the plasma membrane by a ceramide tail. There is currently no known natural ligand for GM₁; however, its function as a component of membrane raft structures has been an area of intense recent interest. Along with cholesterol, GM₁ localizes to detergent-insoluble membrane microdomains following the ligation of a number of cell surface protein receptors. These structures have been defined, in large part, by the use of CtxB as a marker and are sites where a number of key intracellular signaling molecules are localized. In particular, the Src kinases Lck, Fyn and Lyn have all been shown to preferentially associate with GM₁-rich membrane structures in lymphocytes, and cross-linking membrane-bound CtxB with specific antibodies has been shown to be capable of substituting for co-stimulation upon T cell activation (15). It is therefore likely that the modulation of the immune system that follows administration of EtxB in vivo results from signaling processes triggered as a result of GM₁ interaction.

Receptor binding by EtxB and CtxB has been shown to have a number of profound effects on individual populations of leukocytes. EtxB triggers IL-10 production by monocytes and blocks the release of IL-12 (16), a property that is also mediated by whole Ctx, but not CtxB (17). Modulation of monocyte activity in this way has been shown to lead to altered T cell differentiation following antigen presentation by treated monocytes, favoring the generation of IL-10-secreting cells with regulatory activity (16). In addition to the effects of the B subunit on monocytes, EtxB and CtxB trigger CD8 T cells to die by apoptosis (10). EtxB, but not EtxB(G33D), triggers transcriptionally dependent activation of caspase 8 and 3 in murine CD8 T cells by a process that is independent of Fas and tumor necrosis factor receptor (18). Cell death associated with DNA degradation, PARP cleavage and membrane phosphatidyl serine externalization occurs within 16-24 h as a result. The depletion of CD8 T cells from the intestinal epithelial compartment and from Peyer's patches has also been reported after oral administration of CtxB (19). The effects of B subunit–receptor interaction on B cells are in marked contrast to those observed with CD8 T cells. Both Ctx and CtxB promote isotype switching to IgM and IgA (20,21), apparently through a process involving the secretion of transforming growth factor (TGf)-β (22). In addition, the culture of spleen cells in the presence of EtxB, but not EtxB(G33D), results in the up-regulated expression of class II MHC, CD25, CD40, B7 and intracellular adhesion molecule (ICAM)-1 on B cells (8). CtxB has also been reported to enhance class II MHC expression on purified murine B cells (23).

In order to determine the biochemical processes by which EtxB triggers signaling events leading to altered cellular activation, we have investigated the consequences of receptor interaction on primary murine B cells. We report that EtxB, but not EtxB(G33D), modulates class II MHC and CD25 expression in pure B cells and causes enhanced levels of mitogen-activated protein (MAP) kinase phosphorylation. The use of specific inhibitors revealed that these processes are phosphoinositide 3-kinase (PI3-kinase)-dependent. However, inhibition of the MAP kinase pathway, MAPK/extracellular signal-regulated kinase (Erk) kinase (MEK), revealed that while MAP kinase activity contributes to regulation of MHC class II expression, other downstream events from PI3-kinase are critical in the regulation of cellular activation by EtxB. Protein kinase C (PKC) was also found to be involved in the activation of Erk and contributed to the up-regulated expression of CD25.

Methods

Recombinant preparations of EtxB and EtxB(G33D) were kindly provided by Professor T. R. Hirst (University of Bristol, UK) from stocks purified as reported previously (24). The B subunits were then run through a detoxigel column (Perbio, Tattenhall, UK) using conditions recommended by the manufacturer in order to remove endotoxin. The batches of B subunit used throughout this study were essentially lipopoly saccharide (LPS)-free, with endotoxin levels <30 U/mg protein (1 ng LPS corresponds to ~1–10 EU), as measured using Limulus amoebocyte lysate assays (Biowhittaker, Poole, UK). The anti-EtxB antibody was the kind gift of Dr Roger James (University of Leicester, UK). LPS from E. coli serotype 0127:B8 was purchased from Sigma (Poole, UK). Affinity-purified goat anti-mouse IgM (Fab')₂ fragment was obtained from Jackson ImmunoResearch (West Grove, PA). The following mAb were used for flow cytometry analysis (PharMingen, Cambridge, UK): phycocerythrin (PE)-labelled anti-CD25 (PC61) and FITC-labelled anti-I-A<sup>κ</sup> (7-16.17). 17-Amino-actinomycin D (7-AAA) was purchased from Sigma. The MEK inhibitors, PD98059 and U0126, the PI3-kinase inhibitors, Wortmannin and LY294002, and the PKC inhibitors, Go6983 and Bisindolylmaleimide I (BIM), were purchased from Calbiochem (Nottingham, UK).

Preparation and culture of primary B cells

Primary murine B cells were prepared from the spleens of female NIH mice (obtained from Harlan Olac, Bicester, UK; used between 8 and 12 weeks of age) and purified via magnetic cell sorting by negative selection using anti-CD43 magnetic beads and MACS separation columns (Miltenyi Biotec, Bisley, UK) as described previously (25). B cell preparations were 90–95% pure as determined by flow cytometry. B cells were cultured in RPMI 1640 medium supplemented with 20 mM HEPEs, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% FBS (Gibco Life Technologies, Paisley, UK). Cells were either untreated or pre-treated for 30 min with inhibitors as indicated. Cells were then stimulated with EtxB or EtxB(G33D) and cultured at 37°C in a humidified atmosphere of 95% air/5% CO₂.
Up-regulation of cell surface molecules

Following 24 or 48 h in culture (2 × 10⁶/ml), B cells were suspended in PBS supplemented with 2% FBS and 0.1% sodium azide (PBS/FBS/azide). Samples of 10⁶ cells were incubated on ice for 30 min with PE-anti-CD25 (1:200 dilution) and FITC-anti-I-A<sup>p</sup> (1:1000 dilution) in PBS/FBS/azide supplemented with 10% normal rat serum (Sigma). Cells were then washed twice in PBS/FBS/azide and 7-AAD added to a final concentration of 1 µg/ml just prior to analysis. Flow cytometry analysis was performed on a FACScan (Becton Dickinson, Oxford, UK), and data collected on 10,000 viable cells as determined by forward/side scatter parameters and 7-AAD exclusion. CellQuest software was used for collecting data and WinMDI was used for the analysis.

Immunofluorescence confocal microscopy

B cells were washed in PBS and 2 mg/ml BSA (PBS/BSA), and suspeded at 5 × 10⁶/ml prior to stimulation with 40 µg/ml EtxB at 37°C for the times indicated. The cells were then adhered to microscope slides coated with 1 mg/ml poly-L-lysine (Sigma) by incubating on ice for 10 min, and were then fixed for 4 min at 4°C in 3.7% formaldehyde in PBS and subsequently incubated in methanol at −20°C for 5 min. The fixed cells were then incubated for 1 h at room temperature with anti-EtxB antibodies (1/500 dilution in PBS/BSA) and washed 3 times in PBS/BSA prior to incubation with the FITC-conjugated goat anti-rabbit secondary antibodies (Jackson Immunoresearch) (1/100 dilution in PBS/BSA) for 30 min at room temperature. For samples cross-linked to induce patching, EtxB-stimulated cells were washed in PBS/BSA and incubated with anti-EtxB antibodies (1/100 in PBS/BSA) for 30 min. The samples were then washed twice in PBS/BSA, adhered to poly-L-lysine-coated slides, fixed and stained with secondary antibody as described above. After a final wash, all samples were mounted in 20 mM Tris–HCl, pH 8.5, 25% glycerol, and 10% Mowiol 4-88 with 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma) to reduce fading. Confocal microscopy was performed with a Leica TCS NT confocal microscope using a ×63 objective lens.

Western blot analysis

Freshly purified B cells were suspended at 1 × 10⁷/ml in serum-free complete RPMI and incubated at 37°C for 60 min prior to stimulation. Cells were either untreated or pre-treated with inhibitors as indicated. Subsequently, cells were stimulated with the indicated concentration of EtxB or EtxB(G33D), or 10 μg/ml anti-IgM F(ab′)² for the times shown. Cells that were not stimulated were incubated under the same conditions for the duration of the stimulation. Cells were lysed at 5 × 10⁶ cell equivalents/40 μl in ice-cold lysis buffer [1% (v/v) Triton X-100, 50 mM Tris–HCl, pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 10 mM sodium fluoride, 1 mM sodium molybdate, 1 mM PMSF, 2 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin and 0.7 μg/ml pepstatin]. The lysates were centrifuged at 14,000 r.p.m. and the supernatants removed and boiled in 5 × SDS sample buffer [10% (w/v) SDS, 50% (v/v) glycerol, 0.2M Tris–HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol and bromophenol blue to colour]. Proteins (10 μl sample) were resolved by SDS–PAGE on 7.5% or 10% acrylamide gels, transferred to nitrocellulose (BioRad, Hemel Hempstead, UK), and blocked in 5% BSA and 1% ovalbumin in Tris-buffered saline. Blots were incubated overnight with a 1:2000 dilution of anti-phospho-Erk antibodies (New England Biolabs, Hitchin, UK), or for 3 h with 0.1 μg/ml anti-Erk1 (Santa Cruz Biotechnology, Santa Cruz, CA) or 0.1 μg/ml 4G10 (Upstate Biotechnology, Lake Placid, NY). Goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Dako, High Wycombe, UK) were used at 0.03 μg/ml. Immunoblots were developed using the ECL system and Hyperfilm (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were stripped completely of antibodies by incubation with stripping solution [62.5 mM Tris–HCl, pH 6.7, 2% (v/v) SDS, 100 mM 2-mercaptoethanol] at 55°C for 60 min. For image analysis, blots were scanned using an Epson GT 8000 scanner. The density of the bands were calculated using Scion software.

Results

EtxB induces up-regulation of cell surface activation markers on primary murine splenic B cells

We have previously reported that the addition of EtxB to cultures of murine spleen cells results in B cell activation; as revealed by increased expression of cell surface MHC class II, CD25, B7, CD40 and ICAM-1 (8). To determine whether this was a direct effect of EtxB-receptor interaction, we examined the expression of these markers on pure populations of negatively selected primary B cells following treatment with EtxB or the non-receptor binding mutant EtxB(G33D). Following 24 and 48 h in culture, cells were washed and labelled with specific antibodies, and stained with 7-AAD for exclusion of dead cells. Incubation of primary B cells with EtxB resulted in a modest up-regulation of CD25 after 24 h and a proportion of the cells had up-regulated expression of class II MHC (Fig. 1A). By 48 h in culture, the majority of the B cells expressed increased levels of class II MHC and the proportion that were CD25<sup>+</sup> had also increased. Neither EtxB(G33D) or 10 ng/ml LPS, a concentration ~10 times that which was determined to be in the EtxB preparations, affected the expression of MHC class II or CD25. This indicates the receptor binding dependency of the effects of EtxB in these B cell cultures. The levels of up-regulated CD25 and class II MHC expression achieved after incubation of B cells with EtxB were markedly lower than those seen after BCR cross-linking. Neither EtxB or EtxB(G33D) affected the expression of CD80, CD40 or ICAM-1 in these pure B cell cultures, but increased levels of CD86 expression were observed in cultures with EtxB (data not shown).

In order to determine the effects of EtxB dose on the up-regulation of CD25 and class II, we incubated pure B cells for 48 h either alone or in the presence of increasing concentrations of EtxB (Fig. 1B). Incubation with 1 and 0.5 μg/ml EtxB induced maximal expression of MHC class II and CD25 respectively. This correlates with the saturating dose of EtxB as determined in experiments that have directly measured EtxB surface binding to B cells using B subunit specific antibodies (26).
Effects of EtxB on the induction of proximal signalling events

We hypothesized that the EtxB-mediated signalling events leading to up-regulated class II MHC and CD25 expression by B cells may be a consequence of raft formation resulting from the cross-linking of GM1 by the B subunit. Notably, raft formation has been observed after cross-linking CtxB with anti-CtxB antibodies on a variety of cell types (27–29). Therefore, B cells were stimulated with EtxB at 37°C for the times indicated (Fig. 2A, left panels) and were then fixed and stained to reveal the distribution of the B subunit on the cell membrane. Anti-EtxB antibodies clearly revealed the presence of EtxB bound to the surface of the B cells. However, even after 60 min, the staining was uniform across the surface of the cell. Additionally, when cells were incubated with EtxB on ice for 20 min and then shifted to 37°C for various times, again, no patches were observed (data not shown). Patch formation on the membrane could be induced by cross-linking bound EtxB with anti-EtxB antibodies (Fig. 2A, right panels). These data indicate that pentavalent GM1 occupancy does not trigger detectable raft formation.

Cross-linking membrane bound CtxB with antibodies on Jurkat T cells has been shown to trigger protein tyrosine kinase activity (27). In order to determine whether the addition of EtxB alone to primary B cells had a similar effect, freshly isolated B cells were either left unstimulated or stimulated with EtxB or EtxB(G33D) for various times. Cell lysates were prepared and Western blot analysis was carried out using specific anti-phosphotyrosine antibodies (Fig. 2B). Despite repeated experiments, treatment with EtxB did not induce any detectable increase in tyrosine phosphorylation. The ability of the experimental system to detect such changes was clearly illustrated since the addition of anti-IgM antibodies to cross-link the BCR led to a substantial increase in the levels of a number of tyrosine phosphorylated proteins. Thus, receptor binding by EtxB does not induce detectable lipid raft formation or tyrosine phosphorylation of cellular proteins in total anti-phosphotyrosine blots of primary B cell lysates.

EtxB induces activation of the Erk MAP kinases in primary B cells

Although proximal signalling events, including lipid raft formation and tyrosine phosphorylation of cellular proteins, could not be detected following treatment of B cells with EtxB, we investigated whether more downstream signalling events were apparent. The MAP kinases, Erk1 and Erk2, play a critical role in the regulation of cell growth and differentiation in many cell types (30). Therefore, we investigated whether EtxB could stimulate Erk activation in B cells. To become activated, the Erk kinases must be dually phosphorylated on specific threonine and tyrosine residues (31). Thus, immunoblotting with antibodies that recognize the dually phosphorylated forms of Erk1 and Erk2, play a critical role in the regulation of cell growth and differentiation in many cell types (30). Therefore, we investigated whether EtxB could stimulate Erk activation in B cells. To become activated, the Erk kinases must be dually phosphorylated on specific threonine and tyrosine residues (31). Thus, immunoblotting with antibodies that recognize the dually phosphorylated forms of Erk1 and Erk2 is a good indication of Erk activation. B cells were left untreated as a control or stimulated with EtxB or EtxB(G33D) for various times and cell lysates were immunoblotted with anti-phospho-Erk antibodies (Fig. 3A, upper
The blots were stripped and re-probed with anti-Erk antibodies to show equal loading (Fig. 3A, lower panels). The addition of EtxB, but not EtxB(G33D) led to a clear increase in the levels of phosphorylated Erk1 and Erk2 that was detectable by 5 min, and appeared to peak at 120 min after stimulation in the experiment shown. Densitometric analysis of blots from five identical experiments showed that in fact maximal Erk activation occurs by 60 min in culture with EtxB, but that this peak level is maintained until at least 4 h. Such analysis showed that EtxB triggers, on average, a 2.5-fold increase in the level of Erk activation. The level and kinetics of EtxB-induced Erk activation were in marked contrast to that seen after BCR cross-linking (Fig. 3B). The addition of anti-IgM antibodies to B cells induced a 30-fold increase in Erk activation that was maximal within 5 min, declining out to 60 min. Both EtxB and BCR signalling had a slightly more pronounced effect on levels of Erk2 phosphorylation in comparison to Erk1. The dominance of Erk2 in the response to BCR signalling is in agreement with published data (32).

Dose–response experiments revealed that Erk activation can be triggered with as little as 0.5 \( \mu \text{g/ml} \) EtxB (Fig. 3C), a dose that was sufficient to stimulate up-regulated class II MHC and CD25 expression. EtxB(G33D) did not induce Erk activation above basal levels indicating the receptor-binding dependency of these processes.

Inhibitors of MEK and PI3-kinase block EtxB-induced Erk activation

In order to test the MEK dependence of EtxB-induced Erk activation, primary B cells were pre-incubated with PD98059 before addition of the B subunit. PD98059 is a selective and potent inhibitor of the Erk-specific MAP kinase kinases MEK1 and MEK2 (33). Cell lysates were prepared after treatment with EtxB for 60 min and Western blots were performed using anti-phospho-Erk antibodies. Erk activation was inhibited in a dose-dependent manner by PD98059 (Fig. 4A) with the response to EtxB reaching basal levels with only 0.1 \( \mu \text{M} \) PD98059. Higher concentrations of the inhibitor reduced Erk activation to below basal levels. BCR-induced Erk activation was completely inhibited with 10 \( \mu \text{M} \) PD98059, which corresponds to previously reported data (32).

PD98059 has been shown to inhibit MEK1/2 with an IC \( 50 \) of \( \sim 10 \mu \text{M} \) (33) and has recently been shown to be specific for MEK1 and MEK2 in B cells up to concentrations of at least 50 \( \mu \text{M} \) (32). Pre-treatment of B cells with 50 \( \mu \text{M} \) PD98059 completely inhibited EtxB-induced Erk activation over a 2-h time course (Fig. 4B). Similar treatment of B cells with the carrier alone had no effect on control or stimulated Erk levels (data not shown).

We next investigated the involvement of PI3-kinase in the activation of Erk induced by EtxB using two structurally distinct PI3-kinase inhibitors, the microbial metabolite Wortmannin (34) and the reversible inhibitor LY294002 (35). PI3-kinase has been implicated in the regulation of Erk activation by a variety of different stimuli, and is involved in the regulation of cellular proliferation, differentiation, survival, metabolism, cytoskeletal...
reorganization and membrane trafficking (36,37). We have shown that both LY294002 and Wortmannin inhibit BCR-induced PI3-kinase activation in primary B cells (25). Thus, we next investigated the effect of these inhibitors on EtxB-induced Erk activation. Both LY294002 and Wortmannin inhibited EtxB-induced activation of Erk1 and Erk2 at 60 min in a dose-dependent manner (Fig. 5A and C); levels reaching background with as little as 0.1 \( \mu \text{M} \) LY294002 and 10 nM Wortmannin. Pre-treatment of B cells with 10 \( \mu \text{M} \) LY294002 (Fig. 5D) or 50 nM Wortmannin (Fig. 5E) was used to check that Erk inhibition was complete over a 2-h time course. As expected no Erk activation was observed during this period after EtxB treatment in the presence of the inhibitors. Both PI3-kinase inhibitors also inhibited basal levels of Erk activation and 10 \( \mu \text{M} \) LY294002 inhibited Erk phosphorylation induced following BCR cross-linking to basal levels (Fig. 5B). These data indicate that EtxB-induced Erk activation is dependent on both MEK and PI3-kinase.

**Fig. 3.** EtxB induces activation of the Erk1/2 MAP kinases in primary mouse B cells. B cells were either left untreated as a control or treated with 40 \( \mu \text{g/ml} \) EtxB or EtxB(G33D) (A) or with 10 \( \mu \text{g/ml} \) anti-IgM F(ab')2 (B) for the times indicated. (C) B cells were stimulated with the indicated concentrations of EtxB for 60 min. Cell extracts were separated by SDS-PAGE on 10% acrylamide gels and immunoblotted with anti-phospho-Erk antibodies which recognize dually phosphorylated forms of Erk1 and Erk2 (P-Erk1 and P-Erk2; upper panels). The blots were then stripped and re-probed with anti-Erk1 antibodies (which also cross-reacts with Erk2) to show equal loading between lanes (lower panels). Densitometric imaging analysis of five independent experiments (A) show the fold activation (mean ± SE) induced by EtxB (black bars) or EtxB(G33D) (open bars) compared to basal activation after normalizing for protein loading.

The role of PKC in EtxB induced Erk activation in B cells

PKC has been reported to be upstream of Erk activation following BCR cross-linking (38). Therefore, we next investigated the role of PKC in EtxB-induced Erk activation using two distinct staurosporin-derived PKC inhibitors. EtxB-induced Erk phosphorylation in primary B cells was inhibited in a dose-dependent manner by BIM, a broad spectrum PKC inhibitor (Fig. 6A). Pre-treatment with 10 nM BIM inhibited EtxB-induced Erk phosphorylation down to basal levels, with increasing concentrations reducing Erk phosphorylation to below basal levels. Pre-treatment of B cells with 1 \( \mu \text{M} \) BIM inhibited Erk phosphorylation over a 2-h time course (Fig. 6B). In addition, depletion of PKC by incubation overnight in phorbol 12-myristate 13-acetate (PMA) also completely inhibited EtxB-induced Erk phosphorylation (data not shown). In contrast, pre-treatment of primary B cells with G6983, a more selective PKC inhibitor, had little or no effect on EtxB-induced Erk activation between 100 nM and 1 \( \mu \text{M} \).
The failure of Go6983 to affect the EtxB-mediated response was consistently observed in three identical experiments. We have shown previously that 500 nM Go6983 completely inhibits BCR-induced IκBα degradation, a known PKC-dependent process (25).

Inhibition of MEK, PI3-kinase and PKC have different effects on the EtxB-induced up-regulation of MHC class II and CD25 on primary murine B cells

We next investigated the possible pathways through which EtxB may signal to induce up-regulation of MHC class II and CD25 on B cells. We first examined the effects of inhibiting the MEK/Erk pathway on EtxB-induced up-regulation of MHC class II and CD25. For these experiments we used U0126 as it is known to be more stable than PD98059 in culture. Primary B cells were either untreated or pre-treated with increasing concentrations of U0126 for 1 h before the addition of EtxB or diluent alone. After 48 h, the levels of class II MHC and CD25 were assessed by flow cytometry (Fig. 7A). U0126 partially inhibited EtxB-induced up-regulation of MHC class II, but had little or no effect on EtxB-induced expression of CD25 at concentrations up to 10 μM. The results shown represent data from five individual experiments. Incubation of B cells with DMSO carrier had no effect on control or EtxB-induced expression of the surface markers studied (data not shown).

We next examined the effects of inhibiting PI3-kinase on EtxB-induced up-regulation of MHC class II and CD25. Wortmannin, in contrast to LY294002, is unstable in aqueous solution, therefore LY294002 was used in these experiments. Contrary to the effects of inhibiting MEK on up-regulation of these surface markers, inhibiting PI3-kinase with LY294002 completely blocked EtxB-induced up-regulation of both MHC class II and CD25 (Fig. 7B). Concentrations of 0.1 μM LY294002 markedly reduced EtxB-induced up-regulation of class II, but had only a modest inhibitory effect on CD25 expression (data not shown). However, both MHC class II and CD25 expression were reduced to nearly basal levels with 5 μM LY294002. Thus, PI3-kinase-dependent pathways appear to be critically involved in the up-regulation of MHC class II and CD25 induced by EtxB.

Since the two PKC inhibitors, BIM and Go6983, had different effects on EtxB-induced Erk activation, we investigated the effects of both inhibitors on the up-regulation of MHC class II and CD25 induced by EtxB (Fig. 7C and D). Neither inhibitor affected the up-regulated expression of MHC class II induced by EtxB. However, both Go6983 and BIM had an inhibitory effect on EtxB-induced CD25 expression. CD25 expression was reduced to nearly basal levels with 500 nM Go6983 (an effect that was also observed using 250 nM Go6983; data not shown). Inhibition of EtxB-induced CD25 expression was less marked following treatment with BIM.

Thus, while the data indicate that MEK/Erk may contribute to EtxB-mediated class II MHC expression, other pathways involving PI3-kinase are also involved. CD25 up-regulation appears to occur via a distinct process, also mediated through a PI3-kinase-dependent pathway, but involving PKC as well.

Discussion

Our investigations have focused on the processes leading to B cell activation by EtxB. These data indicate that EtxB triggers the activation of Erk1 and Erk2 in a PI3-kinase- and PKC-dependent manner. Blocking PI3-kinase inhibited both the EtxB-induced phosphorylation of Erk1/2 and up-regulated expression of class II MHC and CD25 on the cell surface. The finding that inhibition of MEK, a key regulator of Erk activation, does not affect CD25 up-regulation by EtxB and only partially blocks the enhancement of class II MHC levels indicates that additional pathways downstream of PI3-kinase are involved. Inhibition of PKC blocked the up-regulation of CD25, suggesting that it plays a critical role in this pathway.

The activation of B cells by EtxB was shown to be a direct effect of receptor interaction since culture of highly purified primary murine B cells with wild-type B subunit, but not the non-receptor binding mutant, EtxB(G33D), led to up-regulated expression of class II MHC, CD25 and CD86. Our failure to
demonstrate increases in the levels of ICAM-1 and CD40 expression in these pure B cell cultures indicates that their regulation in spleen cell cultures with EtxB is an indirect effect; presumably resulting from enhanced B±T cell interactions following effects of EtxB on either population. Up-regulation of class II MHC and CD25 on B cells was evident within 24 h of culture with EtxB, but reached higher levels by 48 h. This contrasts to the ability of IFN-γ or LPS to up-regulate class II MHC expression, where the response reaches maximal levels within 24 h (data not shown). The slow response may reflect either the prolonged period required for the critical signal to pass a threshold or could indicate that the primary effect of EtxB is to trigger the production of other factors that in turn trigger the observed changes in phenotype. It is noteworthy, however, that we have failed to detect the production IFN-γ, IL-2 or TGF-β by pure B cells cultured in the presence of EtxB (data not shown).

Our findings that EtxB triggers Erk1/2 activation are the first indication of the biochemical processes that follow B subunit±receptor interaction. The enhanced phosphorylation of Erk1/2 was highly reproducible between experiments, peaking at 60 min and continuing to be raised above background for as long as 4 h. Importantly, EtxB(G33D) failed to trigger MAP kinase activation, indicating the receptor binding dependence of this signal. The EtxB-induced MAP kinase response was weak relative to the response that followed BCR cross-linking and took much longer to develop. Cross-linking the BCR triggered activation of Erk with similar kinetics as been reported previously, with maximal activation occurring within 5 min (38–40). EtxB-stimulated MAP kinase activation was shown to depend on the Erk kinases MEK1/2 since the specific inhibitors PD98059 and U0126 completely ablated the enhancement of Erk1/2 phosphorylation. This finding is consistent with current models which place MEK1/2 as the specific MAP kinase kinases directly upstream of Erk activation, and agree with findings showing that PD98059 blocks BCR-induced MAP kinase activity in DT40 chicken B cells and primary murine splenic B cells (our data; 32,41). Interestingly, the phosphorylation of Erk1/2 induced by EtxB was also blocked by the addition of the PI3-kinase inhibitors LY294002 and Wortmannin. Several reports have demonstrated inhibition of Erk activation by PI3-kinase inhibitors in response to various stimuli and in many cell types (42–47). Importantly in lymphocytes, Erk2 activation in response to TCR engagement was inhibited by over-expressing a mutated form of p85 (45,47) and Erk2 activation by anti-IgM antibodies was partially inhibited in the presence of 1 μM Wortmannin in a human B lymphoma cell line (41). In this study, LY294002 was also able to completely inhibit Erk activation following BCR cross-linking. Thus our findings indicate that PI3-kinase plays a critical role in the signalling pathway leading to Erk activation following EtxB treatment and stimulation through the BCR in murine B cells.

Activation of Erk in B cells appears to be regulated not only via the classical Ras/Raf-1/MEK cascade (48) but also via a PKC-dependent pathway. Indeed PKC appears to be essential in BCR-stimulated Erk activation since depletion of PKC or disruption of the phospholipase C (PLC)-γ2 gene, which is critical for PKC activation, in DT40 cells nearly eliminated Erk activation (38,49). PKC, a serine/threonine kinase, is capable of phosphorylating multiple substrates including Raf-1, the upstream MAP kinase kinase kinase in the Erk pathway (50). Here, EtxB-induced Erk activation also appeared to be dependent on PKC as a broad spectrum PKC inhibitor, BIM,
Gangiosides have been shown to interact with transmembrane receptor tyrosine kinases (53). Further, GM1 can interact with intracellular membrane-bound proteins, such as the Src-family kinases, facilitating their localization to lipid rafts (54). In B cells, antigen receptor cross-linking induces formation of lipid rafts enriched in GM1, as well as Lyn, Btk and PI3-kinase (55–57). Incubation of B cells with EtxB, in the absence of additional anti-EtxB antibodies, did not induce detectable patching on the surface of B cells. This does not rule out the possibility that small microdomains may be induced by the pentavalent EtxB-receptor binding, but that these microdomains may be too small to be visualized by confocal microscopy, consistent with a study that estimated that lipid rafts are <70 nm in diameter (58). Indeed, EtxB has been shown to be internalized into B cells under similar conditions to those used here (26); a process that is likely to result from the redistribution of membrane-bound EtxB molecules into caveolae-like structures.

Although it is not clear at this point how the binding of EtxB to its receptors on the surface of B cells initiates signalling events, PI3-kinase appears to play a key role in the subsequent up-regulated expression of MHC class II and CD25. To date, only the class Ia PI3-kinases have been shown to be activated in B cells, a process dependent on the tyrosine kinases Lyn and Syk (59–61). Immunoprecipitation studies of these kinases from EtxB-treated cells has failed to detect an increase in their tyrosine phosphorylation status and in vitro kinase assays using anti-phosphotyrosine antibodies to precipitate class Ia PI3-kinase failed to detect a reproducible increase in PI3-kinase activity following treatment with EtxB (data not shown). While it is likely that these findings reflect the sensitivity of the assays currently available, they may also suggest that other signalling pathways are involved. In this regard, the class Ib, II and III PI3-kinase members are also sensitive to Wortmannin and LY294002 (62).

The biochemical signals initiated by EtxB ultimately result in the up-regulated expression of MHC class II and CD25. Inhibitor studies indicated that PI3-kinase was critical in mediating these processes. While Erk activation was shown to depend on PI3-kinase, the MEK/Erk pathway appeared to be only partially involved in the up-regulation of MHC class II and not involved in expression of CD25 as revealed by the use of U0126. Thus, alternative PI3-kinase-dependent pathways are likely involved in mediating up-regulation of these surface markers. The use of G06983 and BIM indicated that PKC also plays a significant role in the up-regulation of CD25 by EtxB. The promoter of CD25 contains NF-κB DNA binding sites and we have recently shown that BCR-induced activation of the transcription factor NF-κB is dependent on both PI3-kinase and PKC in primary murine B cells (25). Preliminary results suggest that EtxB can induce increased NF-κB DNA binding activity in B cells (data not shown). Thus, a potential pathway leading to EtxB-induced up-regulated CD25 expression, mediated via PI3-kinase and PKC, through activation of the transcription factor NF-κB, could be envisioned. The pathway leading to increased expression of class II MHC by EtxB is less clear. Up-regulated class II MHC expression was dependent on PI3-kinase, was partially blocked by MEK inhibition and was unaffected by the PKC inhibitor BIM (which inhibited Erk activation). Taken together, these findings indicate that while

**Fig. 6.** Effects of PKC inhibitors on EtxB-induced Erk1/2 activation. B cells were pre-treated with the indicated concentrations of BIM (A) or G06983 (C) for 30 min and then either left unstimulated or stimulated with 40 μg/ml EtxB for 60 min. (B) Cells were pre-treated with 1 μM BIM and then stimulated with 40 μg/ml EtxB for the times indicated. Cell extracts were separated by SDS-PAGE on 10% acrylamide gels and immunoblotted with anti-phospho-Erk antibodies (upper panels), and then stripped and re-probed with anti-Erk1 antibodies (lower panels).

and depletion of PKC by overnight incubation with PMA completely inhibited up-regulated Erk phosphorylation. However, a different PKC inhibitor, G06983, did not affect EtxB-induced Erk activation. G06983 was, nevertheless, active in our hands since it was able to block EtxB-induced up-regulation of B cell CD25 expression. It is noteworthy that PKC-δ, a candidate PKC isoform for mediating BCR-induced Erk activation, is not effectively inhibited by G06983 (51). PKC-δ has been shown to become associated with the BCR complex, Syk and PLC-γ (52). It is therefore possible that the differential effects of BIM and G06983 on EtxB-mediated Erk phosphorylation are due to the involvement of PKC-δ in this pathway. A direct assessment of the activity of this and other PKC isoforms would be necessary to confirm this possibility.

How might EtxB binding to its receptors trigger these signalling processes? It is noteworthy that GM1 ganglioside does not span the plasma membrane. It is therefore likely that EtxB signalling either involves a secondary interaction with a GM1-associated transmembrane protein or results from the redistribution of GM1-associated molecules at the membrane.

**C**
PI3-kinase is critical to up-regulation of class II MHC, Erk plays only a minor role.

In conclusion, our findings provide the first report that EtxB±receptor interaction triggers biochemical signalling processes in B lymphocytes. Membrane-proximal events that are unrelated to gross membrane patching lead to PI3-kinase dependent signalling cascades that ultimately regulate the expression of key surface molecules involved in B cell activation. The regulation of B cell responsiveness mediated by these events may play a critical role in the capacity of EtxB to stimulate extremely potent systemic and mucosal antibody responses in vivo and may be involved in the capacity of EtxB to modulate autoimmune disease. A detailed in vivo analysis of the relative contributions of EtxB-mediated modulation of different cell types will be necessary to confirm this possibility. Further, elucidation of the biochemical and cell biological effects of EtxB will allow the determination of its potential as a modulator of the immune system for use in human disease.

Acknowledgements

We would like to thank Professor T. R. Hirst and Dr Martin Kenny for providing the EtxB and EtxB(G33D) that were used in these studies, and Dr Roger James for the gift of the anti-EtxB antibodies. Thanks to R. Salamond for help with flow cytometry data. This work was supported by a grant from The Wellcome Trust. N. A. W. is in receipt of a Wellcome Trust Research Leave Fellowship.

Abbreviations

7-AAD 7-aminoactinomycin D  
BIM Bisindolylmaleimide I  
CIA collagen-induced arthritis  
CIITA class II transactivator  
CtxB cholera toxin B subunit  
Erk extracellular signal-regulated kinase  
EtxB Escherichia coli heat-labile enterotoxin B subunit  
ICAM intracellular adhesion molecule  
IDDM insulin-dependent diabetes mellitus  
LPS lipopolysaccharide  
MAP mitogen-activated protein  
MEK MAP/ERK kinase  
PE phycoerythrin  
PI3-kinase phosphoinositide 3-kinase  
PKC protein kinase C  
PMA phorbol 12-myristate 13-acetate  
PLC phospholipase C  
TGF transforming growth factor

References

secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. Vaccine 16:2069.


secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. Vaccine 16:2069.


secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. Vaccine 16:2069.


secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. Vaccine 16:2069.


secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. Vaccine 16:2069.


secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. Vaccine 16:2069.


