G-proteins are not directly involved in the CD3-antigen-mediated production of inositol phosphates in HPB-ALL T-leukaemia cells expressing phospholipase C isoforms γ_1 and β_3

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The possible involvement of G-proteins in T cell antigen-receptor complex (TCR)-mediated inositol phosphate production was investigated in HPB-ALL T-cells, which were found to express the phospholipase C γ_1 and β_3 isoforms. Cross-linking the CD3 antigen on streptolysin-O-permeabilized cells stimulated a dose-dependent increase in inositol phosphate production, as did addition of guanosine 5'-[γ -thio]triphosphate (GTP[S]) or vanadate, a phosphotyrosine phosphatase inhibitor. It was possible, therefore, that the CD3-antigen-mediated production of inositol phosphates was either via a G-protein-dependent mechanism or by stimulation of protein tyrosine phosphorylation. The CD3-induced inositol phosphate production was potentiated by addition of vanadate, but not by addition of GTP[S]. Guanosine 5'-[β -thio]diphosphate (GDP[S]) inhibited the rise in inositol phosphates induced by GTP[S], vanadate or cross-linking the CD3 antigen. The increase in

protein tyrosine phosphorylation stimulated by vanadate or the OKT3 monoclonal antibody was not observed in the presence of GDP[S], showing that in permeabilized HPB-ALL cells, GDP[S] inhibits the actions of tyrosine kinases as well as G-protein function. Addition of either ADP[S] or phenylarsine oxide inhibited CD3- and vanadate-mediated increases in both tyrosine phosphorylation and inositol phosphate production, but did not inhibit GTP[S]-stimulated inositol phosphate production. On the other hand, pretreatment of cells with phorbol 12,13-dibutyrate inhibited subsequent GTP[S]-stimulated inositol phosphate production but did not inhibit significantly inositol phosphate production stimulated by either OKT3 F(ab')₂ fragments or vanadate. Our results are consistent with the CD3 antigen stimulating inositol phosphate production by increasing the level of protein tyrosine phosphorylation, but not by activating a G-protein.

INTRODUCTION

The T-cell antigen-receptor complex (TCR) comprises an $\alpha\beta$ heterodimer that recognizes antigen, the CD3 antigen, composed of $\gamma\delta\epsilon$ -subunits, and a dimer of $\zeta\zeta$ - or $\zeta\eta$ -subunits. Recognition of foreign antigen bound to major histocompatibility complex (MHC)-encoded proteins on the surface of antigen-presenting cells initiates a series of TCR-mediated intracellular signals (Weiss and Imboden, 1987). These include increases in protein tyrosine phosphorylation, and protein kinase C activation and increases in intracellular free calcium mediated by InsP_3 (Klausner and Samelson, 1991). These signals lead ultimately to the expression of the interleukin-2 receptor and to interleukin-2 production (Smith, 1988). Phospholipase C (PLC) is thought to play an important role in the generation of these mitogenic signals by cleaving $\text{PtdIns}(4,5)\text{P}_2$, generating InsP_3 and diacylglycerol (Imboden and Stobo, 1985).

So far, eight distinct isoforms of PLC have been cloned or purified (Meldrum et al., 1991; Rhee 1991) and the existence of a further isoform has been postulated (Thomas et al., 1991). It is apparent that there is cell-specific expression of these isoforms; for example, mRNA encoding $\text{PLC}\gamma_1$ and $\text{PLC}\gamma_2$ has been detected in Jurkat T-leukaemia cells (Goldfien et al., 1991), and Park et al (1991) identified two immunologically distinct isoforms in Jurkat cells, i.e. β_1 and γ_1 .

The mechanism of regulation of the activities of many of the PLC isoforms is not known, though possible mechanisms for the activation of the $\text{PLC}\gamma_1$ and $\text{PLC}\beta$ isoforms have recently been described. $\text{PLC}\gamma_1$ is phosphorylated following stimulation of platelet-derived growth factor and epidermal growth factor (EGF) receptors (Meisenhelder et al., 1989). The phosphorylations are on both serine and tyrosine residues and, in the case of EGF-treated A431 cells, the increased tyrosine phosphorylation also results in increased catalytic activity of $\text{PLC}\gamma_1$ (Nishibe et al., 1990). There have been numerous reports of GTP or non-hydrolysable GTP analogues stimulating inositol phosphate production (Cockcroft and Gomperts, 1985; Litosch et al., 1985; Uhing et al., 1985). This form of regulation is likely to be confined to the β -family of isoforms, since β_1 is activated by a G-protein related to the G_q class of G-protein α -subunits (Blank et al., 1991; Taylor et al., 1991), or possibly to $\text{PLC}\epsilon$ (Thomas et al., 1991). Similarly a 150 kDa PLC from turkey erythrocytes is regulated by a 43 kDa protein which reacts with antisera to G_q (Waldo et al., 1991). However, the possibility of G-proteins being involved in the regulation of $\text{PLC}\gamma_1$ has been suggested by Yang et al (1991).

In T-cells the CD3-mediated increase in inositol phosphate production is preceded by an increase in the level of tyrosine phosphorylation in several proteins (June et al., 1990a), including $\text{PLC}\gamma_1$ (Weiss et al., 1991; Park et al., 1991; Secrist et al., 1991).

Abbreviations used: ADP[S], adenosine 5'-[β -thio]diphosphate; EGF, epidermal growth factor; FCS, foetal calf serum; GDP[S], guanosine 5'-[β -thio]diphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; InsP_3 , total inositol phosphates ($\text{InsP}_1 + \text{InsP}_2 + \text{InsP}_3 + \text{InsP}_4$); PAO, phenylarsine oxide; PdBu, phorbol 12,13-dibutyrate; PLC, phospholipase C; p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]diphosphate; PTPase, phosphotyrosine phosphatase; TCR, T-cell antigen-receptor complex.

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and, following CD3 stimulation, PLC γ_1 has been shown to associate with the CD3 complex (Dasgupta et al., 1992). The requirement for tyrosine phosphorylation to mediate increased inositol phosphate production is further supported by the use of tyrosine kinase inhibitors (June et al., 1990b). Similarly, cholera toxin and staurosporine inhibit receptor-induced rises in tyrosine phosphorylation and total inositol phosphate (InsP) production (Augustine et al., 1991).

T-cells contain a G-protein-regulated PLC, and this can be demonstrated by adding agents which perturb G-protein function. AIF $_4^-$ stimulates InsP $_1$ production in intact cells (O'Shea et al., 1987), and guanosine 5'-[γ -thio]triphosphate GTP[S] increases the level of inositol phosphates in permeabilized cells (Graves et al., 1990). It is possible that cross-linking the CD3 antigen might result in a G-protein-dependent stimulation of inositol phosphate production, and evidence for this comes from experiments in which addition of guanosine 5'-[β -thio]diphosphate (GDP[S]) to permeabilized murine T-helper cells, or of GDP to permeabilized human T-lymphoblasts, inhibits the CD3-mediated increases in inositol phosphate (Bonvini et al., 1991; Graves and Cantrell, 1991). Therefore evidence exists for TCR-mediated production of inositol phosphates by both tyrosine phosphorylation-dependent and G-protein-dependent mechanisms.

Previous investigations in intact T-leukaemia HPB-ALL cells have demonstrated CD3-induced protein tyrosine phosphorylation (Deans et al., 1992) and inositol phosphate production (Koretzky et al., 1990), suggesting that tyrosine phosphorylation regulates a PLC present in these cells (PLC γ_1). Potential evidence for another PLC species in these cells is inferred from the work of Koretzky et al. (1990), who transfected the muscarinic receptor, a seven-transmembrane-domain receptor coupled to a G-protein-dependent PLC (Peralta et al., 1988), into HPB-ALL cells. Addition of carbachol, a muscarinic agonist, stimulated a rise in intracellular calcium only in the cells transfected with the receptor, suggesting that the cells have a G-protein-regulated PLC. Therefore it is likely that HPB-ALL cells have at least two distinct PLC isoforms.

We now show that either GTP[S] or vanadate stimulates InsP $_1$ production in permeabilized HPB-ALL T-cells, confirming that both G-protein- and tyrosine phosphorylation-dependent processes are coupled to stimulation of InsP $_1$ production. Using a variety of inhibitors we show that compounds which inhibit tyrosine phosphorylation {such as phenylarsine oxide (PAO) and ADP[S]} in these cells inhibit CD3- and vanadate-induced increases in InsP $_1$ without inhibiting GTP[S]-mediated increases in InsP $_1$. Addition of phorbol 12,13-dibutyrate (PdBu) inhibits GTP[S]-induced increases in InsP $_1$, but does not significantly inhibit InsP $_1$ production stimulated by monoclonal antibody OKT3 F(ab') $_2$ fragments or vanadate. Our results, therefore, provide no specific evidence to support G-protein coupling of the CD3 antigen to InsP $_1$ production, and indicate that the CD3 antigen mediates increased InsP $_1$ production by elevating levels of protein tyrosine phosphorylation in HPB-ALL cells.

MATERIALS AND METHODS

Reagents

OKT3 F(ab') $_2$ (anti-CD3) was kindly provided by M. Glennie (Tenovus Research Laboratory, Southampton, U.K.), and anti-PLC antibodies were generously given by A. Carozzi and P. Parker (I.C.R.F., Lincoln's Inn Fields, London, U.K.). *myo*-[3 H]Inositol and ECL light-based detection system for proteins were from Amersham International (Amersham, Bucks., U.K.),

GDP[S] and GTP[S] were from Boehringer (Lewes, E. Sussex, U.K.) AND ADP[S], PdBu, PAO and other biochemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Cell culture

The NY subclone of the HPB-ALL T-leukaemia cell line was kindly provided by P. Beverley (I.C.R.F. Human Tumour Immunology Group, London, U.K.). The cells were maintained in RPMI 1640/3% (v/v) foetal calf serum (FCS). Cells were found to be CD3 $^+$, CD4 $^+$, CD8 $^+$, CD45 $^+$ by FACS analysis with a flow cytometer.

Inositol phosphate analysis in permeabilized cells

Cells were labelled overnight at 37 °C with *myo*-[3 H]inositol (37 kBq/ml) at a cell density of 1.5×10^6 cells/ml in *myo*-inositol-deficient RPMI-1640/2% (v/v) FCS (Gibco Ltd.). The FCS had previously been dialysed overnight against PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.2). Labelled cells were pelleted, resuspended in PBS and incubated at 37 °C for 15 min, before being harvested and added to ice-cold permeabilization buffer (details of buffer in Alexander et al., 1990). Incubations were initiated by addition of 2×10^6 cells to agonists giving a final volume of 0.2 ml and had the following composition: streptolysin O (0.4 unit/ml), 3 mM free Mg $^{2+}$, 120 mM K $^+$, 10 mM Pipes, pH 7.4, 10 mM EGTA, pH 7.4, and 150 nM free Ca $^{2+}$. Incubations were at 37 °C and were terminated by addition of 0.75 ml of chloroform/methanol (1:2, v/v). Extraction of inositol phosphates and separation on Dowex AG 1X8, formate form (Bio-Rad), was as described (Berridge et al., 1983). [3 H]Inositol and [3 H]glycerophosphoinositol were run to waste and the fraction containing InsP $_1$, InsP $_2$, InsP $_3$ and InsP $_4$ was collected, solubilized in Optiphase HiSafe 3 (Pharmacia) and the radioactivity incorporated was determined. Analysis of the phospholipids in the organic lower phase showed that, routinely, 100000 d.p.m. were present in the inositol phospholipids per assay.

Tyrosine phosphorylation assay

Cell permeabilization and incubations were the same as for InsP $_1$ determinations (described above), with the exception that incubations were terminated by addition of SDS sample buffer containing 1 mM sodium vanadate. Incubations were terminated after 3 min, as the CD3-antigen-induced tyrosine phosphorylation was maximal at 2–3 min in permeabilized cells (results not shown). The samples were boiled for 10 min, separated by SDS/PAGE (7.5% acrylamide) and the proteins then transferred to Immobilon-P (Millipore Corp.). Tyrosine phosphorylation was measured by immunoblotting with a rabbit anti-phosphotyrosine antiserum (D67) generated against polymers of alanine, threonine and phosphotyrosine chemically coupled with keyhole limpet haemocyanin as described (Kamps and Sefton, 1988). A second antibody goat anti-(rabbit-horseradish peroxidase) (Dako Ltd.) (1:500 dilution) was added, and the bound antibody was quantified with the ECL detection system. Detection was specific for phosphorylated tyrosine residues, as it could be totally inhibited by addition of 10 mM phosphotyrosine to the primary antibody incubations.

Detection of PLC isoforms

Cells were washed in PBS, lysed at 4 °C in 1% (w/v) Triton X-100, 10% (v/v) glycerol, 10 mM Hepes, pH 7.2, 1 mM EGTA,

150 mM NaCl, 1 mM phenylmethanesulphonyl fluoride, 0.7 mg/ml pepstatin, 2 mg/ml leupeptin and 10 mM benzamidine. The samples were centrifuged ($g_{\max}$, 14500 g for 2 min) and the supernatant was heated in SDS sample buffer. Aliquots equivalent to 5×10^6 cells were separated on an SDS/7.5% polyacrylamide gel. Following transfer to Immobilon-P, the membrane was incubated with antibodies (1:2000 dilution) raised against specific peptides derived from PLC isoform amino acid sequence data (A. Carozzi and P. Parker, unpublished work), and detected by the method described above for the anti-phosphotyrosine antibodies.

RESULTS

PLC isoform expression in HPB-ALL cells

PLC isoforms present in HPB-ALL cells were detected using antibodies raised against specific peptides derived from PLC-isoform amino acid sequence data (Figure 1). Antisera reacted with proteins corresponding to both PLC γ_1 (molecular mass approx. 145 kDa) and PLC β_3 (approx. 156 kDa), and the binding of antibody was blocked in both cases with the relevant antigenic peptide. A fainter band corresponding to a protein migrating with a slightly lower molecular mass was also detected with antiserum to PLC β_3 ; this could represent a proteolytic product. This band is unlikely to be the result of cross-reactivity of the antisera, as the antibodies to PLC β_3 do not cross-react with PLC γ_1 (A. Carozzi and P. Parker, personal communication).

Regulation of Inositol phosphate production

To determine how InsP_i produced is regulated in HPB-ALL cells, a method of cell permeabilization using streptolysin O was carried out (Alexander et al., 1990) to enable InsP_i production to be assayed following the addition of otherwise impermeable compounds such as GTP[S] and vanadate. As it was possible that the permeabilization procedure might uncouple the TCR from signalling pathways, the ability of cross-linked CD3 antigen to stimulate InsP_i production was assessed (Figure 2a). OKT3 F(ab') $_2$ fragments stimulated InsP_i production in a dose-dependent manner, showing that coupling of the CD3 antigen to second messenger production was still functional in permeabilized HPB-ALL cells, in agreement with findings in T-lymphoblasts (Graves and Cantrell, 1991).

GTP[S] was added to permeabilized HPB-ALL cells to investigate the G-protein regulation of InsP_i production; dose-dependent production of InsP_i was observed (Figure 2b). This confirmed that HPB-ALL cells do indeed have a G-protein-

Figure 1 Identification of PLC γ_1 and PLC β_3 in HPB-ALL cells

Cell lysates from 5×10^6 cells were separated on SDS/7.5%-PAGE and transferred to Immobilon-P. Anti-PLC antibodies were used at a 1:2000 dilution and the bound antibody was detected with goat anti-rabbit-horseradish peroxidase (1:500) in conjunction with the ECL detection system. Where indicated (+ A), antigen (1:2000 dilution of a 15 $\mu\text{g}/\text{ml}$ solution) to which the antisera had been raised was included to determine specificity of binding.

Figure 2 Dose-dependent stimulation of InsP_i production in streptolysin O-permeabilized HPB-ALL cells by OKT3, GTP[S] and vanadate

Cells prelabelled with $\text{myo-}^3\text{H}$ inositol were incubated at 37 °C with streptolysin O in an intracellular medium containing 150 nM free Ca^{2+} with the indicated additions present from $t = 0$. Incubations were terminated after 10 min, and the InsP_i extracted and determined as described in the Materials and Methods section. Data are for InsP_i production stimulated by: (a) OKT3 F(ab') $_2$; (b) GTP[S] (○), GTP[S] + 75 ng/ml OKT3 F(ab') $_2$ (●), GTP[S] + 75 ng/ml OKT3 F(ab') $_2$ minus GTP[S] (to show any potential synergy between the two signals) (■); (c) vanadate (□); vanadate + 75 ng/ml OKT3 F(ab') $_2$ (■), vanadate + 75 ng/ml OKT3 F(ab') $_2$ minus vanadate (to show any potential synergy between the two signals) (●). The average value of duplicate incubations is shown with the range, and the data are representative of three separate determinations.

regulated PLC and is the first direct demonstration of such a phenomenon in these cells. This rise in InsP_i production is not likely to be the result of a purinergic receptor coupling via a G-protein, as addition of ATP (100 μM) or ATP[S] (100 μM) to intact HPB-ALL cells did not stimulate any detectable InsP_i production (results not shown).

Addition of the phosphotyrosine phosphatase (PTPase) inhibitor vanadate to permeabilized cells resulted in a dose-dependent increase in InsP_i production (Figure 2c). Vanadate (1 μM) inhibited 85–90% of Jurkat T-cell PTPase, assayed by measuring the extent of dephosphorylation of [^{32}P]angiotensin added to permeabilized cells (results not shown). When permeabilized HPB-ALL cells were incubated in the absence of

Table 1 ATP-dependence of vanadate-stimulated InsP_i production in permeabilized HPB-ALL cells

Cells prelabelled with $\text{myo-}[^3\text{H}]\text{inositol}$ were incubated at 37°C with streptolysin O (0.4 unit/ml) in an intracellular medium containing 150 nM free Ca^{2+} with the indicated additions in the presence or absence of $1\ \mu\text{M}$ vanadate. Incubations were terminated after 5 min, and the InsP_i extracted and determined as described in the Materials and methods section. The level of radioactivity in InsP_i after 5 min in cells incubated without ATP was set at 100% (actual values 300–506 d.p.m., depending on experiment) and all other values are relative to this. Values are the means $\pm$ S.D. of three separate experiments performed in duplicate, except where experiments were replicated twice, in which case the values of each experiment are given. n.d. not determined.

Addition	Increase in InsP_i (%)	
	Control	+ Vanadate ($1\ \mu\text{M}$)
–	100 (3)	101 $\pm$ 1 (3)
25 μM ATP	n.d.	106, 114
100 μM ATP	n.d.	268, 290
500 μM ATP	138 $\pm$ 12 (3)	672 $\pm$ 103 (3)
500 μM p[NH]ppA	82, 94	95, 99
10 μM GTP[S] (no ATP)	205 $\pm$ 38 (3)	n.d.

exogenous ATP, vanadate ($1\ \mu\text{M}$) did not stimulate any InsP_i production (Table 1). As the concentration of ATP was raised to 500 μM there was a dose-dependent rise in InsP_i production in the presence of vanadate. If ATP was substituted with 500 μM adenosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppA), no vanadate-stimulated increase in InsP_i was observed. The inability of vanadate to induce InsP_i production in the absence of ATP does not reflect a lack of $\text{PtdIns}(4,5)\text{P}_2$, as GTP[S] under the same experimental conditions stimulated a 2-fold increase in InsP_i production (Table 1). The result with vanadate suggests that in unstimulated permeabilized HPB-ALL cells the actions of active tyrosine kinases in mediating InsP_i production are reversed by PTPase activity, consistent with the low level of tyrosine phosphorylation in unstimulated cells (Figure 3b) and with the low basal levels of InsP_i production (Figure 2). When the PTPase activity is inhibited with vanadate, the increase in tyrosine phosphorylation observed (Figure 3b) presumably reflects the actions of one or more active tyrosine kinases.

Ability of GTP[S] and vanadate to potentiate InsP_i production following CD3 antigen cross-linking

The data presented in Figures 2(b) and 2(c) and Table 1 show that it is possible to distinguish between G-protein- and tyrosine kinase-mediated activation pathways by the use of GTP[S] and vanadate respectively, which induce InsP_i production independently of the TCR. As the cells expressed $\text{PLC}\gamma_1$ and $\text{PLC}\beta_3$ (Figure 1), and the production of InsP_i is potentially regulated by two distinct processes, it was of interest to determine whether the CD3-antigen-induced rise in InsP_i was mediated via increased tyrosine phosphorylation (of $\text{PLC}\gamma_1$), or via G-protein coupling (possibly to $\text{PLC}\beta_3$).

The generation of InsP_i in permeabilized cells in the presence of ATP is dependent upon regeneration of $\text{PtdIns}(4,5)\text{P}_2$, so at saturating levels of agonists it is possible for the rate of $\text{PtdIns}(4,5)\text{P}_2$ formation to become rate-limiting. Under such conditions no further stimulation of $\text{PtdIns}(4,5)\text{P}_2$ breakdown would be observed. To avoid this eventuality, InsP_i production in permeabilized HPB-ALL cells was determined with a sub-maximal dose of OKT3 (75 ng/ml) in the presence of a range of

Figure 3 GDP[S] inhibits InsP_i production and tyrosine phosphorylation in permeabilized HPB-ALL cells

(a) The increases in total InsP_i stimulated by 10 μM GTP[S] ($\circ$), 2 $\mu\text{g/ml}$ OKT3 F(ab)₂ ($\bullet$) or 1 μM vanadate ($\square$) over a 10 min incubation were determined in the absence (100% stimulation) and presence of GDP[S]. Each value is the mean $\pm$ S.D. of three separate determinations each performed in duplicate. (b) Tyrosine phosphorylation was determined following addition of GDP[S] plus OKT3 F(ab)₂ (2 $\mu\text{g/ml}$) or vanadate ($1\ \mu\text{M}$). Incubations were terminated after 3 min by addition of SDS sample buffer and samples were heated for 10 min at 105°C . Proteins were separated on reduced SDS/PAGE (7.5%) and transferred to polyvinylidene difluoride membranes, and phosphotyrosine was detected with anti-phosphotyrosine antibodies as described in the Materials and methods section.

GTP[S] concentrations (Figure 2b). The increase in InsP_i production stimulated by OKT3 was additive and not synergistic with that produced by a range of GTP[S] concentrations. In contrast, when OKT3 was added to permeabilized cells in the presence of vanadate, a synergy in the production of InsP_i was observed (Figure 2c), suggesting a role for tyrosine phosphorylation in the mediation of CD3-antigen-stimulated InsP_i production in HPB-ALL cells. This synergy was most likely the result of a CD3-induced increase in the tyrosine phosphorylation of $\text{PLC}\gamma_1$ and a concomitant inhibition of $\text{PLC}\gamma_1$ dephosphorylation by vanadate. Potentiation was less marked at higher concentrations of vanadate ($1\ \mu\text{M}$), possibly due to a limiting availability of $\text{PtdIns}(4,5)\text{P}_2$.

GDP[S] inhibits InsP_i production and tyrosine phosphorylation

One way to distinguish whether a receptor couples via a G-protein is by the addition of GDP or GDP[S], which interact with G-proteins and prevent their activation. GDP[S] and GDP have been shown to inhibit the CD3-dependent production of inositol phosphates in a permeabilized murine T-helper cell clone (Bonvini et al., 1991) and in permeabilized human T-lymphocytes (Graves and Cantrell, 1991). Figure 3(a) shows that GDP[S] inhibited InsP_i production induced by OKT3 and GTP[S] in a dose-dependent manner in HPB-ALL cells, as would be expected if a receptor was coupled via a G-protein. However, GDP[S] also inhibited InsP_i production stimulated by vanadate (Figure 3a).

As vanadate mediates its effect by inhibiting PTPase activity and thereby increasing protein tyrosine phosphorylation, the effect of GDP[S] on protein tyrosine phosphorylation was determined. Figure 3(b) shows that OKT3 F(ab')₂ and vanadate both induced tyrosine phosphorylation of a 145 kDa protein, and vanadate also increased the level of phosphorylation of an 85 kDa protein, GDP[S] inhibited both CD3- and vanadate-dependent tyrosine phosphorylation in a dose-dependent manner (Figure 3b).

Effect of ADP[S] on InsP₃ production and tyrosine phosphorylation

To determine the specificity of the effect of GDP[S], permeabilized cells were incubated with similar concentrations of ADP[S] and stimulated as before (Figure 4a). ADP[S] inhibited InsP₃ production stimulated by OKT3 and vanadate, but had little effect on InsP₃ production stimulated by GTP[S]. In this respect ADP[S] was more discriminating than GDP[S], and indicated a common mechanism for CD3 and vanadate stimulation of PtdIns metabolism. The production of InsP₃ by OKT3 F(ab')₂ was more resistant to inhibition by both ADP[S] and GDP[S] than was the vanadate-induced production (Figures 3a and 4a). This was probably due to the time-dependence of the permeabilization process. All additions, including OKT3 and vanadate, were made to intact cells in ice-cold streptolysin O buffer, before immediately transferring the cells to a 37 °C water bath. Under these conditions the membranes are permeabilized as the temperature rises (Bonvini et al., 1991). Therefore OKT3 F(ab')₂ probably binds to the cells and initiates tyrosine phosphorylation and InsP₃ production prior to cell permeabilization, whereas both vanadate and added nucleotides are dependent upon the cells being permeabilized before they can enter and mediate their effects. The vanadate-stimulated InsP₃ production was inhibited by at least 20% following addition of a range of nucleotide diphosphates (CDP, IDP, TDP, all at 0.2 mM) to the permeabilized cells (results not shown).

As ADP[S] inhibited OKT3- and vanadate-stimulated InsP₃ production, the effect of ADP[S] on OKT3- and vanadate-induced tyrosine phosphorylation was assessed (Figure 4b). It was apparent that ADP[S] inhibited the tyrosine phosphorylation of both the 145 kDa and 85 kDa proteins, similar to the effects of GDP[S]. These data suggest a role for protein tyrosine phosphorylation in mediating both CD3- and vanadate-induced InsP₃ production, but are inconsistent with coupling of the TCR to InsP₃ production via a G-protein.

Effect of PAO on InsP₃ production and tyrosine phosphorylation

PAO has been reported to inhibit purified CD45 PTPase and anti-Thy-1-stimulated tyrosine phosphorylation in a murine T-cell hybridoma (Garcia-Morales et al., 1990). It has also been shown to inhibit insulin-dependent glucose uptake in adipocytes. This action was accompanied by increased tyrosine phosphorylation of a 15 kDa protein (Bernier et al., 1987). From such data PAO is considered to be a potential PTPase inhibitor. PAO forms arsenical complexes with thiol groups (Stockten and Thompson, 1946), and as PTPases have a highly conserved cysteine involved in the mechanism of dephosphorylation (Guan and Dixon, 1991), reaction of PAO with this residue could inactivate the PTPase.

In previous experiments of ours a range of PAO concentrations had been added to permeabilized Jurkat T-cells, with the finding that 20 µM PAO elicited a maximal inhibition of CD3-mediated production of InsP₃ (results not shown). In permeabilized HPB-ALL cells, PAO (20 µM) inhibited OKT3- and vanadate-induced

Figure 4 Effect of ADP[S] on InsP₃ production and tyrosine phosphorylation in permeabilized HPB-ALL cells

(a) The increases in total InsP₃ stimulated by 10 µM GTP[S] (○), 2 µg/ml OKT3 F(ab)₂ (●) or 1 µM vanadate (□) over a 10 min incubation were determined in the absence (100% stimulation) and presence of ADP[S]. Each value is the mean ± S.D. of three separate determinations each performed in duplicate. (b) Tyrosine phosphorylation was determined following addition of ADP[S] (2 mM) with OKT3 F(ab)₂ (2 µg/ml) or vanadate (1 µM). Incubations were terminated after 3 min. Other details as in Figure 3(b).

Table 2 Effect of PAO and PdBu on InsP₃ production in permeabilized HPB-ALL cells

PAO (20 µM) was added to HPB-ALL cells at the time of permeabilization, whereas cells treated with PdBu were initially incubated in HEPES-buffered Hanks' Balanced Salt Solution, pH 7.3, plus PdBu (100 ng/ml) or vehicle (0.02% dimethyl sulphoxide) for 5 min at 37 °C. Cells were pelleted (5 s in a Microfuge) washed in PBS and then resuspended in ice-cold permeabilization buffer before incubation at 37 °C for 10 min, at which time InsP₃ production was determined. Increases in InsP₃ in the presence of PAO or following PdBu pretreatment are expressed as percentages of stimulated increases over background values. Data shown are the means ± S.D. of three separate experiments performed in duplicate.

Addition	InsP ₃ (% of stimulated level)	
	PAO (20 µM)	PdBu (100 ng/ml)
OKT3 (2 µg/ml)	10 ± 5	93 ± 16
Vanadate (1 µM)	8 ± 3	103 ± 11
GTP[S] (10 µM)	105 ± 10	50 ± 5

InsP₃ production by 90%, while having no effect on GTP[S]-stimulated InsP₃ production (Table 2). This result was surprising as the effects of PAO, a putative PTPase inhibitor, were opposite and even antagonistic to those of the PTPase inhibitor, vanadate, used in this study. Garcia-Morales et al. (1990) observed that PAO inhibited Thy-1-mediated tyrosine phosphorylation and they interpreted this as evidence for involvement of a PTPase in the regulation of tyrosine kinase activity. We therefore investigated the effect of PAO on tyrosine phosphorylation and observed that PAO inhibited CD3- and vanadate-induced

Figure 5 Effects of PAO on tyrosine phosphorylation in permeabilized HPB-ALL cells

Additions were PAO (20 μ M), OKT3 F(ab)₂ (2 μ g/ml) and vanadate (1 μ M), and incubations were terminated after 3 min. Conditions were as for Figure 3(b).

increases in the level of protein phosphotyrosine (Figure 5). In the HPB-ALL cells it may be argued that PAO is inhibiting receptor function. However, in permeabilized Jurkat cells in which addition of monoclonal antibody OKT3 induced tyrosine phosphorylation of a greater number of substrates (> 10) than was seen in HPB-ALL cells, addition of PAO (20 μ M) inhibited the tyrosine phosphorylation of only a few of the proteins (results not shown). This shows that PAO does not block all receptor functions, indicating that the TCR was still functional in permeabilized HPB-ALL cells treated with this reagent. These results further suggest that CD3-mediated InsP_i production in HPB-ALL cells is associated with increased levels of tyrosine phosphorylation and not with activation of a G-protein.

Phorbol ester pretreatment inhibits GTP[S]-induced InsP_i production but does not affect tyrosine phosphorylation

PKC has been shown to phosphorylate $\text{PLC}\beta$ and this phosphorylation may prevent the phospholipase interacting with a G-protein (Ryu et al., 1990). Intact HPB-ALL cells were pretreated with PdBu (100 ng/ml) for 5 min prior to permeabilization to assess the effect of PKC-dependent phosphorylation on InsP_i production stimulated by cross-linking the CD3 antigen (Table 2). Our results show that PKC activation decreased the efficiency of G-protein coupling to PLC. No significant inhibition by PdBu of vanadate- or OKT3-induced InsP_i production was observed, whereas PdBu pretreatment inhibited the GTP[S]-stimulated rise in InsP_i by 50%. To examine the effect of PdBu on InsP_i production in intact cells, pretreatment with PdBu (100 ng/ml) was similarly carried out for 5 min in the presence of 10 mM LiCl, followed by stimulation with OKT3 F(ab')₂. In intact cells inhibition by PdBu of the OKT3-dependent rise in InsP_i was insignificant (result not shown), a similar finding to the result observed in permeabilized cells, providing further evidence that streptolysin O permeabilization does not disrupt the regulation of second messenger production.

As InsP_i production stimulated by GTP[S] in permeabilized cells was inhibited by 50% following PdBu pretreatment, presumably by inhibition of coupling of a G-protein to $\text{PLC}\beta$, then inhibition of at least 50% would be expected if OKT3 was mediating its effect through a G-protein. As the inhibition by PdBu of OKT3-mediated InsP_i production was insignificant in permeabilized cells, it is unlikely that signals initiated by CD3 antigen cross-linking are coupled via a G-protein.

The effect of PdBu on protein tyrosine phosphorylation in

Figure 6 Effect of PdBu pretreatment on tyrosine phosphorylation in permeabilized HPB-ALL cells

HPB-ALL cells in HEPES-buffered Hanks' Balanced Salts Solution, pH 7.3, were incubated with PdBu (100 ng/ml) or vehicle (0.02% dimethyl sulphoxide) for 5 min at 37 °C. Cells were pelleted (5 s in a Microfuge), washed in PBS and then resuspended in ice-cold permeabilization buffer before being incubated with the additions shown for 3 min. Cells were incubated with no further additions (C), 10 μ M GTP[S] (G), 2 μ g/ml OKT3 F(ab)₂ (O) or 1 μ M vanadate (V).

permeabilized HPB-ALL cells were examined (Figure 6). PdBu did not inhibit vanadate- or OKT3-induced tyrosine phosphorylation. The addition of PdBu induced a shift in the mobility of the 145 kDa protein to a position of apparently higher molecular mass. If this polypeptide is $\text{PLC}\gamma$, this shift may reflect the PKC-dependent phosphorylation of this enzyme (Granja et al., 1991).

DISCUSSION

One characteristic of receptors which couple to PLC via a G-protein is the synergy observed when agonist and GTP analogues are present, as demonstrated in the production of inositol phosphates from liver plasma membranes stimulated with vasopressin (Uhing et al., 1986) and carbachol-stimulated inositol phosphate production in electrically permeabilized pancreatic acinar cells (Merritt et al., 1986). This synergy in the production of inositol phosphates has not, however, been observed in T-cells. In permeabilized Jurkat cells GTP[S] was inhibitory to inositol phosphate production rather than stimulatory when added together with an anti-CD3 antibody (Augustine et al., 1991). The effects of cross-linking the CD3 antigen and adding GTP[S] to permeabilized murine T-helper cells were additive at low concentrations of GTP[S], but at higher concentrations there was no additional effect of cross-linking CD3 (Bonvini et al., 1991). We similarly did not observe any synergy in the InsP_i production stimulated by joint addition of GTP[S] and OKT3 F(ab')₂ (Figure 2b), but noted that the increases were additive. Such additivity has been observed in streptolysin O-permeabilized HL-60 cells stimulated with f-Met-Leu-Phe and GTP[S], and may be explained by agonists activating two distinct PLC isoforms or two distinct G-proteins (Stutchfield and Cockcroft, 1991).

In contrast, a synergy in the production of InsP_i was observed following the addition of OKT3 F(ab')₂ fragments and vanadate (Figure 2c). The level of protein tyrosine phosphorylation in a cell is determined by the balance between the rate of tyrosine phosphorylation and the PTPase activity. HPB-ALL cells express $\text{PLC}\gamma_1$ (Deans et al., 1992; Figure 1) which is regulated by tyrosine phosphorylation (Rhee, 1991), so addition of vanadate, a PTPase inhibitor, is likely to disturb the phosphorylation equilibrium and result in increased tyrosine phosphorylation of proteins in the absence of TCR activation (Figure 3b). One of the proteins phosphorylated by this method is probably $\text{PLC}\gamma_1$, as a rise in InsP_i was observed (Figure 2c). The increase in InsP_i

stimulated by vanadate was dependent upon the presence of ATP in the incubations (Table 1), suggesting that there is a requirement for tyrosine kinase activity and regeneration of $\text{PtdIns}(4,5)\text{P}_2$. The synergy in InsP_i production produced by addition of vanadate and OKT3 (Figure 2c) could be explained by the concomitant CD3-mediated increase in $\text{PLC}\gamma_1$ tyrosine phosphorylation and vanadate-induced blockade of its dephosphorylation, resulting in a higher rate of InsP_i production than when the two agonists were added separately.

Our results indicate the complexity of cellular signalling when a receptor can potentially activate more than one isoform of PLC. In the presence of ATP, InsP_i production can be increased by either G-protein activation of PLC or tyrosine kinase-dependent phosphorylation of $\text{PLC}\gamma_1$. Whenever it has been demonstrated that GDP or GDP[S] can inhibit CD3-antigen-induced inositol phosphate production (Figure 3a; Bonvini et al., 1991; Graves and Cantrell, 1991), ATP has been present and thus activation of tyrosine phosphorylation as well as activation of a G-protein is possible. Data (Figure 3b) demonstrating that GDP[S] inhibits CD3- and vanadate-stimulated tyrosine phosphorylation reveals that when two potential pathways can be activated the effects of inhibitors on both pathways need to be assessed.

The ability of GDP[S] to inhibit stimulation of tyrosine phosphorylation and G-protein action may explain the observation of Graves and Cantrell (1991), who showed that CD3-mediated PtdIns hydrolysis in permeabilized human T-lymphoblasts is not dependent on exogenous guanine nucleotides. However, they too observed that GDP inhibited CD3-antigen-mediated inositol phosphate production. If GDP inhibits increases in tyrosine phosphorylation in these cells, then this would support their conclusion that a G-protein is not directly involved in coupling the CD3 antigen to PLC, and is consistent with the idea that CD3-antigen-triggered increases in inositol phosphate production are mediated by changes in protein tyrosine phosphorylation.

The ability of ADP[S] to inhibit tyrosine phosphorylation in permeabilized HPB-ALL cells (Figure 4b) as well as the subsequent InsP_i production (Figure 4a), while having no effect on InsP_i production stimulated by addition of GTP[S], suggests that the effect of ADP[S] is to uncouple the TCR from the actions of one or more tyrosine kinases. Recently the ζ -chain of the TCR complex has been shown to have a GTP-binding site (Peter et al., 1992). Studies using fusion proteins made of the extracellular domain of CD8 and the intracellular domain of the ζ -chain have shown that cross-linking these proteins results in several intracellular signals, including increased tyrosine phosphorylation, inositol phosphate production and calcium mobilization (Irving and Weiss, 1991). These results suggest that the ζ -chain could play a central role in the recruiting of tyrosine kinases and/or substrates or in the activation of tyrosine kinases, especially as it has been demonstrated that in activated Jurkat T-cells $\text{PLC}\gamma_1$ is immunoprecipitated by anti-CD3 ζ Ab (Dasgupta et al., 1992). If the GTP-binding site is important in the function of the ζ -chain, then it is possible that the inhibition of CD3-induced protein tyrosine phosphorylation and inositol phosphate production by GDP[S] (Figures 3a and 3b) and ADP[S] (Figures 4a and 4b) is due to the competitive displacement of exogenous nucleotide from this site.

PAO is a hydrophobic compound, and in permeabilized cell experiments it probably accumulates in the plasma membrane where it appears to inhibit tyrosine kinase activity (Figure 5). In kinase assays *in vitro*, no effect of PAO on p56^{Lck} or p59^{fyn} activity was observed (Garcia-Morales et al., 1990). However, when added to intact T-lymphocytes, PAO has been reported to

reduce p59^{fyn} activity (Mustelin et al., 1992), suggesting that the actions of PAO on isolated kinases differ from those in intact or permeabilized cells. As the permeabilized HPB-ALL cells contain active tyrosine kinase(s) which increase the level of tyrosine phosphorylation when PTPases are inhibited by vanadate, the observation that PAO inhibits this increase in tyrosine phosphorylation (Figure 5) suggests either that there is some selectivity for these inhibitors in their substrates, or that PAO is functioning as a tyrosine kinase inhibitor.

Our data show that HPB-ALL cells express $\text{PLC}\gamma_1$ and $\text{PLC}\beta_3$ (Figure 1), and demonstrate that the production of InsP_i in these cells can either be stimulated by activation of a G-protein or by increased levels of protein tyrosine phosphorylation. We have also shown that agents which inhibit tyrosine phosphorylation (ADP[S], GDP[S], PAO) all inhibit CD3-dependent InsP_i production (Figures 3a and 4a; Table 2) without affecting G-protein-dependent InsP_i production (apart from GDP[S]). Conversely, pretreatment of the cells with PdBu inhibited G-protein-mediated InsP_i production by 50%, while not significantly affecting CD3-cross-linking-stimulated InsP_i production. These data are therefore consistent with a CD3-antigen-mediated increase in InsP_i production regulated by $\text{PLC}\gamma_1$ tyrosine phosphorylation, as reported by others (Weiss et al., 1991; Park et al., 1991; Secrist et al., 1991). However, it should be noted that the cells used in our experiments are a T-leukaemia cell line representing a clone of cells at a particular differentiation state. As such we cannot rule out the involvement of G-proteins in TCR-mediated InsP_i production in T-cells at other stages of differentiation or development. As the cells have at least one G-protein-regulated PLC isoform, it is of considerable importance to characterize the receptors that couple to these enzymes. This is especially so in light of the observation by Desai et al (1990) that stimulation of transfected human muscarinic receptors in Jurkat cells was a sufficient signal to induce interleukin-2 production and IL-2 receptor expression.

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