A role for guanine-nucleotide-binding proteins in mediating T-cell-receptor coupling to inositol phospholipid hydrolysis in a murine T-helper (type II) lymphocyte clone

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Perturbation of the T-cell receptor (TCR) complex is followed by the rapid hydrolysis of inositol phospholipids (InsPL) by phospholipase C (PLC), producing diacylglycerol and inositol phosphates, which act as second messengers in signal transduction. The mechanism coupling the TCR to InsPL hydrolysis is not clearly defined, and no information is available on this mechanism in the CD4⁺ helper subset of T-lymphocytes (Th). We have tested the hypothesis that guanine-nucleotide-binding proteins (G-proteins) may couple the TCR to PLC in a murine Th type II (Th2) cell clone. Cell permeabilization with streptolysin O (SLO) or tetanolysin (TL) was used to allow membrane-impermeable nucleotides access to intracellular sites of action. Exposure of permeabilized Th2 cells to guanosine 5'-[γ-thio]triphosphate (GTPγS), a non-hydrolysable GTP analogue, resulted in a 2.1–2.5-fold increase in inositol phosphate generation. Similarly, perturbation of the TCR with the monoclonal antibody 145.2C11 (directed against the ε-chain of the CD3 component of the TCR) resulted in a 3.1–4.2-fold increase in InsPL hydrolysis by permeabilized cells. Both lysins were similarly effective in allowing GTPγS induction of InsPL hydrolysis, but TL-permeabilized cells responded better to TCR perturbation than SLO-treated cells. A role for G-proteins in TCR coupling to PLC was further supported by the inhibition of TCR-induced InsPL hydrolysis by guanosine 5'-[β-thio]diphosphate (GDPβS), a guanine nucleotide analogue that inhibits G-protein function. ATP was required for TCR-mediated InsPL hydrolysis, and potentiated GTPγS-induced hydrolysis. Other nucleotides (i.e. CTP, GDP, GTP, ITP) did not affect the response. These data indicate that G-proteins may contribute to the regulation of PLC activation in Th2 cells, coupling it to the TCR.

INTRODUCTION

The interaction of the T-cell receptor (TCR) with processed antigens or specific immobilized antibodies is accompanied by the hydrolysis of inositol phospholipids (InsPL) by a phospholipase C (PLC) [1,2], yielding a variety of inositol phosphates and diacylglycerol [3]. Certain inositol phosphates can mobilize Ca²⁺ from either intracellular or extracellular compartments, and diacylglycerol can activate protein kinase C. This sequence of events is a general feature of Ca²⁺-mobilizing receptors, such as those of hormones, neurotransmitters and certain growth factors [3].

Treatment of T-cells with Ca²⁺ ionophores and activators of protein kinase C elicits responses similar to that of TCR activation [4,5], further supporting a role for these pathways in T-cell activation. However, a variant of a murine T-cell hybridoma was obtained which responded to TCR perturbation with lymphokine production and growth inhibition in the absence of detectable InsPL hydrolysis or Ca²⁺ mobilization [6]. Although this suggests that other signal-transduction pathways may be important in the activation of T-lymphocytes [7–9], a knowledge of PLC regulation by the TCR remains a crucial aspect in understanding TCR signal transduction.

Receptors that elicit responses in other immunological systems

may mobilize Ca²⁺ or activate InsPL hydrolysis via specific guanine-nucleotide-binding proteins (G-proteins) (for a review on G-proteins, see ref. [10]). These receptors include those responsible for the response of neutrophils to chemotactic ligands [11,12] or the stimulation of B-lymphocytes triggered via the immunoglobulin receptor [13,14]. With regard to T-lymphocytes, G-proteins have been implicated in the secretory response of cytotoxic T-cells [15], the activation of ornithine decarboxylase in human peripheral-blood T-cells [16], the release of interleukin-2 by Jurkat T-cell leukaemia [17] or a T-cell hybridoma [18], Ca²⁺ mobilization in thymocytes [19] and the activation of InsPL metabolism in a T-cell hybridoma [20], Jurkat cells [17,21], and peripheral-blood lymphocytes [22].

Data are, however, scarce with regard to the role of G-proteins in coupling TCR to an effector function or to InsPL hydrolysis in particular. Furthermore, no information is available on this subject for the CD4⁺ 'helper' subset of T lymphocytes (Th cells), which recognizes antigens in the context of MHC class II products. In the murine system, two types of CD4⁺ Th cells have been described, based on the spectrum of lymphokine produced [23]. Th type I (Th1) lymphocytes produce IL-2 and IFN-γ, while Th type II (Th2) secrete IL-4, IL-5 and IL-6. We have studied the regulation of InsPL hydrolysis in murine Th2 cells utilizing cell permeabilization by bacterial cytolysins and guanine nucleotide

Abbreviations used: FBS, fetal bovine serum; GDPβS, guanosine 5'-[β-thio]diphosphate; GTPγS, guanosine 5'-[γ-thio]triphosphate; HBSS, Hanks balanced salt solution; InsPL, inositol phospholipids; MoAb, monoclonal antibody(ies); PBS, phosphate-buffered saline [KCl (0.2 g/litre)/NaCl (8 g/litre)/KH₂PO₄ (0.2 g/litre)/Na₂HPO₄ · 7H₂O (2.16 g/litre), pH 7.4]; PLC, phospholipase C; SLO, streptolysin O; TCR, T-cell receptor; Th, the CD4⁺ T-'helper' lymphocyte subset; TL, tetanolysin; IL, interleukin; IFN, interferon; MHC, major histocompatibility.

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analogues that stably bind to G-proteins. The G-protein (or G-proteins) involved in coupling PLC to Ca^{2+} -mobilizing receptors has yet to be identified. In the absence of direct isolation and identification of the protein(s) (generally referred to as G_p), the alternative strategy used here may provide important clues regarding its (their) existence and functional properties. Here we present a characterization of the model and we provide evidence that TCR-mediated regulation of InsPL hydrolysis in Th2 cells involves G-proteins.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium was obtained from Gibco (Grand Island, N.Y., U.S.A.). All other cell-culture reagents were from Flow (McLean, VA, U.S.A.). Heat-inactivated, fetal bovine serum (FBS) was obtained from M.A. Bioproducts (Walkersville, MD, U.S.A.). Tissue culture-grade *myo*-inositol and bovine albumin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guanosine 5'-[β -thio]diphosphate (GDP β S) and guanosine 5'-[γ -thio]triphosphate (GTP γ S) were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). All other reagents were obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). *myo*-[2- ^3H]inositol (sp. radioactivity 55 Ci/mmol) was obtained from NEN Research Products (Boston, MA, U.S.A.) or Amersham (Arlington Heights, IL, U.S.A.). Reduced streptolysin O (SLO) was obtained from Wellcome Diagnostic, Dartford, Kent, U.K. Tetanolysin (TL) (a partially purified preparation from a tetanus-toxin negative mutant of *Clostridium tetani*) was a gift of Dr. W. H. Habig (CBER, Bethesda, MD, U.S.A.). The hamster anti-(mouse CD3) monoclonal-antibody (MoAb)-producing hybridoma, clone 145.2C11 [24], was obtained from Dr. R. J. Hodes (NIH, Bethesda, MD, U.S.A.), grown in the peritoneal cavity of pristane-primed nude mice, and the antibody was purified by affinity chromatography (145.2C11 MoAb will hereafter be referred to as '2C11 MoAb'). This MoAb is directed against the ϵ -chain of the CD3 component of the TCR. In certain experiments 2C11 MoAb was immobilized by adsorption on to polystyrene beads (6 μm diameter; Polysciences, Warrington, PA, U.S.A.) as previously described [1]. Affinity-purified goat anti-[hamster (H- and L-chain)] antibody was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.).

T-helper lymphocyte clones

The murine Th2 clone, 8-5-5 [25], was maintained in medium consisting of RPMI 1640 supplemented with 100 units of penicillin/ml, 100 μg of streptomycin/ml, 2 mM-L-glutamine, 1 mM-sodium pyruvate, non-essential amino acids (1:100 dilution), 50 μM - β -mercaptoethanol, 25 mM-Hepes buffer, 5% heat-inactivated fetal bovine serum, 20 units of recombinant human IL-2 (kindly supplied by Cetus Corporation, Emeryville, CA, U.S.A.)/ml, as well as 50 μg of keyhole-limpet haemocyanin/ml as the antigen. Irradiated [33 Gray (3300 rad)] syngeneic spleen cells served as antigen-presenting feeder cells. To avoid possible interference from the feeder cell populations, 15 days before experimental use, cells were expanded in recombinant IL-2 alone. 8-5-5 cells grew in interleukin-2 alone at a rate similar to that observed under normal culture conditions. Cells used for experimental purposes were collected between 7 and 10 days after IL-2 addition. Clone 8-5-5 was CD4^+ (L3T4^+), CD8^+ (Lyt2^+), Thy 1.2^+ , responded specifically to the antigen, keyhole-limpet haemocyanin, and could act as helper T-lymphocyte in promoting an MHC-restricted, antigen-dependent, IgG antibody response from 2,4,6-trinitrophenyl-primed B cells in an 'in vitro' culture system (results not shown).

Cell labelling, permeabilization procedure and assay of inositol phospholipid hydrolysis

Cells were washed free of inositol-containing medium and labelled under continuous agitation for 18–24 h at 37 °C, 5% CO_2 atmosphere in a humidified incubator at a concentration of 10^7 /ml in RPMI 1640 medium lacking *myo*-inositol and supplemented with 10% dialysed FBS, 25 mM-Hepes buffer, pH 7.45 at 20 °C, 2 mM-L-glutamine, 1 mM-sodium pyruvate, 50 units of penicillin/ml, 50 μg of streptomycin/ml, 20 units of recombinant IL-2/ml and 10–25 μCi of *myo*-[2- ^3H]inositol/ml. At the end of the incubation, viable cells were recovered by Ficoll–Paque (Pharmacia, Piscataway, NJ, U.S.A.) gradient centrifugation (460 g for 20 min at room temperature).

When stimulation of the TCR was to be performed in permeabilized cells, 10^7 cells/ml were pre-incubated with gentle agitation for 1 h at 4 °C in medium supplemented with 15 μg of purified 2C11 MoAb/ml. Unbound 2C11 MoAb was removed by washing. For assay of permeabilized T-lymphocytes, cells were re-suspended at a concentration of $(1.5\text{--}2.5) \times 10^7$ /ml in a buffer (potassium glutamate/Hepes buffer) composed of 20 mM-Hepes, pH 7.15, at 37 °C (pH 7.40 at 4 °C), 138 mM-potassium glutamate, 7 mM-magnesium acetate, 1 mM-MgEGTA, 0.1 mg of bovine albumin/ml, 10 mM-D-glucose, 5 mM-ATP and 0.285 mM- CaCl_2 to give a free Ca^{2+} concentration of 100 nM at 37 °C. The formulation of the Ca^{2+} /Mg $^{2+}$ /EGTA buffer employed was calculated using a modification of the computer program CHELATE (kindly provided by Dr. M. A. Beaven, NIH, Bethesda, MD, U.S.A.), which was based on the algorithm of Perrin & Sayce [26]. The EGTA equilibrium quotients for Ca^{2+} , Mg^{2+} and H^+ were obtained from Sillen & Martell [27] and Martell & Smith [28], calculated for 37 °C using the values for the enthalpy and entropy of complexation. Reduced SLO or TL (10 min at room temperature with 1 mM-dithiothreitol) was used as permeabilizing agent in amounts of 0.3 units of SLO/ml or TL stock (10 μl /ml of cell suspension), and kept on ice for 10 min. These lysins bind to membrane cholesterol in the cold without affecting membrane integrity and form pores of 20–50 nm diameter when the temperature is raised [29,30]. The cell suspension was then diluted fivefold with potassium glutamate/Hepes buffer, divided into portions (500 μl /sample), incubated for 3 min at 37 °C, and the stimuli added in a small volume (10–25 μl) of pre-warmed potassium glutamate/Hepes buffer. In all experiments, cell permeabilization, measured as the percentage of cells stained by the non-permeating nucleic acid fluorochrome, ethidium bromide, was better than 95% with either lysin.

When tested in their intact form, washed 8-5-5 cells [$(1.5\text{--}2.5) \times 10^6$ cells/sample in HBSS containing 25 mM-Hepes buffer, pH 7.45 at 20 °C, 1 mg of bovine albumin/ml, 10 mM- LiCl , 300 μM -*myo*-inositol, 1 mM- MgCl_2 , and 1 mM- CaCl_2] were stimulated with bead-immobilized 2C11 MoAb.

The incubation was terminated by the direct addition of ice-cold trichloroacetic acid (final concn. 10%, w/v). The supernatant was recovered for analysis of the inositol phosphates by anionic exchange chromatography using AG 1-X8 resin (100–200 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) as previously described [1]. Column elution was calibrated with authentic radiolabelled standards. Homogeneity of the inositol phosphate standards was routinely checked by reversed-phase h.p.l.c. [31].

The washed trichloroacetic acid-precipitable pellet was dissolved in 0.75 ml of 10% Triton X-100 and counted for radioactivity. The Triton X-100-soluble radioactivity from [^3H]inositol-labelled, trichloroacetic-acid-precipitable material is equivalent to the amount of radioactivity recovered by chloroform extraction of cellular lipids (E. Bonvini & K. E. DeBell, unpublished work).

Data were expressed as the percentage of total cellular radio-

activity, computed as the sum of the trichloroacetic acid-soluble and -insoluble radioactivity. The amount of total cellular radioactivity ranged from 10^5 to 10^6 d.p.m./sample [$(1.5-2.5) \times 10^6$ cells, all experiments]. Within each experiment, the amount of total cellular radioactivity was constant and was within 5% of the average for each sample.

Extraction and chromatographic analysis of inositol phospholipids

myo-[^3H]Inositol-labelled cellular lipids were extracted from the washed trichloroacetic acid precipitate by a modification of the procedure described by Folch *et al.* [32]. Briefly, the pellets were re-suspended into 0.25 ml of water and 0.75 ml of water-saturated chloroform/methanol (1:2, v/v). Partition into two phases was obtained by the addition of 0.25 ml of 0.4 M-HCl and 0.25 ml of chloroform. The lower chloroform phase was re-extracted by the addition of 0.5 ml of a mixture of methanol and 0.4 M-HCl acid in a 1:1 ratio (v/v). The extracted material was applied to a 20 cm $\times$ 20 cm, pre-lined, silica gel [15 nm (150 Å) pore size; 250 μm thickness] t.l.c. plates (LK5 plates, Whatman Chemical Separation, Inc., Clifton, NJ, U.S.A.), pre-impregnated with boric acid (2%, w/v). Plates were first developed for two-thirds their length with chloroform. Phospholipid separation was obtained by re-developing the plates for their full length with chloroform/methanol/aq. 4 M- NH_3 (75:58:17, by vol.). Plates were scanned for radioactivity with a radioactivity scanner (System 200 Imaging Scanner; Bioscan, Washington, DC, U.S.A.).

Fig. 1. Effect of GTP γ S and TCR perturbation on inositol phosphate generation by Th2 cells permeabilized with either SLO (■) or TL (□)

Th2 8-5-5 cells were labelled with *myo*-[$^2\text{-}^3\text{H}$]inositol as described in the Materials and methods section. Washed cells [$(1.5-2.5) \times 10^7$ cells/ml] were permeabilized with SLO (0.3 unit/ml) or TL (1:100 dilution) in potassium glutamate/Hepes buffer. GTP γ S was added at 300 μM . TCR stimulation was achieved by treating 2C11 MoAb-coated cells (15 $\mu\text{g}/\text{ml}$, 1 h at 4 °C) with a goat anti-hamster antibody (GaH, 50 $\mu\text{g}/\text{ml}$), as described. The incubation was terminated after 15 min and the total inositol phosphate content of each sample was determined. Bars represent mean values. Vertical lines above the bars represent the 95% upper confidence limit; *n* is the number of experiments for each group. The total amount of cellular radioactivity ranged from 10^5 to 10^6 d.p.m./sample, depending on the experiment. Within each experiment, the total cellular radioactivity of each sample was constant. *P* values were calculated with an unpaired *t*-test, using equal or unequal variance models as required by the data.

RESULTS

Perturbation of the TCR by a specific antibody or exposure to GTP γ S induces InsPL hydrolysis in permeabilized T-lymphocytes

Permeabilization with bacterial lysins disrupts cellular compartmentalization and allows control of the intracellular

Fig. 2. Dose-response effect of GTP γ S on inositol phosphate generation by permeabilized Th2 cells

(a) Th2 8-5-5 cells were labelled with *myo*-[$^2\text{-}^3\text{H}$]inositol as described in the Materials and methods section. Cells [$(1.5-3.0) \times 10^6$ cells/sample] were permeabilized with TL (1:100 dilution) in potassium glutamate/Hepes buffer. GTP γ S was added at the indicated concentrations. Each data point represents the mean for two to six different experiments. Lines through the points represent the differences between the smallest and largest value observed. Lines were not drawn when smaller than the symbol. (b) Labelled Th2 cells ($10^7/\text{ml}$) were pre-coated with 15 μg of 2C11 antibody/ml, washed, permeabilized with SLO (0.3 unit/ml), and treated with buffer alone (○, control, unstimulated cells) or a goat anti-hamster antibody (GaH, 50 $\mu\text{g}/\text{ml}$) (●, TCR-stimulated cells), in the presence of the indicated concentrations of GTP γ S. The incubation was terminated after 15 min and the total inositol phosphate content of each sample determined as described in the Materials and methods section. The experiment was performed in duplicate. Each point represents the average. Vertical lines through each point represent the differences between duplicate values.

Fig. 3. Comparison of the time-dependent generation of inositol phosphates by intact or permeabilized cells

Th2 8-5-5 cells were labelled with *myo*-[2-³H]inositol and washed. In experiment 1, intact cells (10⁷/ml) were incubated in HBSS medium (supplemented with 10 mM-LiCl, 1 mM-CaCl₂ and 1 mM-MgCl₂) with 2C11 MoAb-coated polystyrene beads (6 µm diameter) at a 5:1 bead:cell ratio. Beads and cells were pelleted by centrifugation and the incubation at 37 °C started. Permeabilized cells were obtained by exposure to SLO (0.3 unit/ml) in potassium glutamate/Hepes buffer and treated with 100 µM-GTPγS. In expt. 2, an aliquot of labelled Th2 cells was incubated with 2C11 antibody (15 µg/ml) for 1 h at 4 °C. Antibody-coated and uncoated cells were permeabilized with TL (1:100 dilution) in potassium glutamate/Hepes buffer. GTPγS (300 µM) or a goat anti-hamster antibody (GaH, 50 µg/ml) was added at zero time. The incubation was terminated at the time points indicated, and the amount of radioactivity for each inositol phosphate species was assessed as indicated in the Materials and methods section. Each point represents a single determination. The data shown are representative of three different experiments.

milieu by the bathing solutions. Under these conditions, guanine nucleotides, which normally do not cross the cell membrane, may gain access to intracellular sites of action. Initial characterization of the model was obtained by studying the InsPL hydrolysis response of the T-helper cell clone, 8-5-5, to the guanine nucleotide analogue GTPγS, and comparing it with that induced by TCR perturbation. TCR perturbation was achieved by aggregating the surface receptor of T cells pre-coated with the

hamster anti-TCR 2C11 MoAb with an excess (50 µg/ml) of a purified goat anti-hamster antibody. T-cells were permeabilized with either SLO or TL, in a potassium glutamate/Hepes buffer formulated to provide Mg²⁺/ATP, a constant pH of 7.15 at 37 °C, and a buffered free Ca²⁺ concentration of 100 nM.

Addition of GTPγS (Fig. 1) to permeabilized 8-5-5 cells induced a significant accumulation of inositol phosphates when compared with cells exposed to buffer alone. There was no

Fig. 4. Effect of GDPβS on inositol phosphate generation in response to TCR perturbation or GTPγS in permeabilized Th2 cells

myo-[2-³H]inositol-labelled and washed Th2 8-5-5 cells (2.5×10^7 /ml) were permeabilized with TL (1:100 dilution) in potassium glutamate/Hepes buffer containing the indicated concentrations of GDPβS. After 6 min of incubation at 37 °C, GTPγS (○, 300 μM) or a goat anti-hamster antibody (●, GaH, 50 μg/ml) was added and the incubation continued for an additional 15 min. Total inositol phosphate content of each sample was determined as described in the Materials and methods section. Each value represents the average of duplicates. Vertical lines through each point represent the differences between duplicate values. Vertical lines were not drawn when smaller than the symbol. The experiment shown is representative of three performed. ■, Buffer control.

difference in the magnitude of the response between SLO- and TL-treated cells, indicating that the two lysins were similarly effective in allowing access to the nucleotide. Permeabilized cells also responded to TCR perturbation with significantly higher levels of InsPL hydrolysis when compared with buffer-treated cells. However, TL-permeabilized cells responded significantly better to TCR perturbation than SLO-treated cells. Although we do not have a clear interpretation for this difference, we should note that the TL used was obtained as a partially purified preparation, whereas SLO was only available as a crude extract. Non-specific toxic effects by the SLO preparation may be considered as a possible explanation. The response to TCR perturbation was significantly better than that to GTPγS, independently of the lysin employed. A greater response to antibody perturbation of the TCR compared with GTPγS was anticipated, since access to the intracellular environment was not required for the former stimulation. There was notable variation in the magnitude of this difference, which tended to be less pronounced (sometimes absent) when SLO was the permeabilizing agent. Simply coating the cells with 2C11 MoAb resulted in a small, albeit detectable, increase in inositol phosphate accumulation in permeabilized cells (10–50 % increase above the uncoated control in multiple experiments, results not shown), presumably owing to the bivalent nature of the antibody. Treatment with goat anti-hamster antibody of cells not coated with 2C11 antibody was without effect (results not shown).

A maximal effect of GTPγS was observed at 100–300 μM with a half-maximal response between 1 and 3 μM (Fig. 2a). A similar dose–response to GTPγS was observed when permeabilized cells were simultaneously activated by perturbation of the TCR mediated by the combination of 2C11 antibody coating followed

Fig. 5. Nucleotide requirement for inositol phosphate generation induced by GTPγS or TCR perturbation in permeabilized Th2 cells

myo-[2-³H]inositol-labelled and washed Th2 8-5-5 cells (2×10^7 /ml) were permeabilized with TL (1:100 dilution) in potassium glutamate/Hepes buffer containing 5 mM of the indicated nucleotide. GTPγS (■, 100 μM) or a goat anti-hamster antibody (□, GaH, 50 μg/ml) was added to medium- or 2C11 antibody-coated cells respectively and the incubation continued for an additional 15 min. Total inositol phosphate content of each sample was determined as described in the Materials and methods section. Each value represents the average for duplicates. The bars represent the differences between the mean value and the highest replicate. The data shown are representative of three separate experiments, each performed in duplicate. ■, Buffer control.

by receptor aggregation with a goat anti-hamster antibody (Fig. 2b). TCR perturbation induced InsPL hydrolysis in the absence of added guanine nucleotide. Lack of a GTP requirement is not unprecedented in ligand-induced InsPL hydrolysis in permeabilized cells [13,33]. It has been suggested that sufficient GTP may be trapped within permeabilized cells to permit ligand-mediated activation [13].

The relative amount of different inositol phosphate species produced in permeabilized cells stimulated by TCR perturbation of GTPγS was compared to that obtained in TCR-stimulated, intact cells (Fig. 3). Whereas the inositol polyphosphates were only transiently present in intact cells, all three species increased nearly linearly for up to 10 min in permeabilized cells. Inositol monophosphate isomers accumulated in intact cells as well. This was likely to reflect blockade of the InsP phosphatase by Li⁺ present in the incubation media [34]. It is likely that inositol phosphate interconversion may be limited in permeabilized cells, owing to the loss of intracellular compartmentalization. Thus the species produced may represent, at least for the major part, the products of direct hydrolysis of the corresponding phospholipid. Alternatively, the relative abundance of ATP in the incubation buffer may increase the availability of polyphosphoinositide precursor(s), thus resulting in a more linear kinetics. No differences were observed between GTPγS- and TCR-induced InsPL hydrolysis in the pattern of phosphate species generated.

Effect of GDPβS on InsPL hydrolysis

The ability of GTPγS to stimulate InsPL hydrolysis strongly suggested that a G-protein regulates PLC activity in permeabilized lymphocytes. GDPβS is a synthetic guanine nucleotide analogue that cannot be phosphorylated [35]. Pre-incubation of permeabilized cells for 10 min with GDPβS inhibited InsPL hydrolysis in response to TCR perturbation in a dose–response

Fig. 6. Effect of varying the ATP concentration on the [^3H]inositol phosphate generation and [^3H]InsPL content of permeabilized Th2 cells stimulated with GTP γ S or by TCR perturbation

myo-[2- ^3H]inositol-labelled Th2 8-5-5 cells were washed and permeabilized with TL (1:100 dilution). GTP γ S (300 μM) or a goat anti-hamster antibody (GaH, 50 $\mu\text{g}/\text{ml}$) was added to medium- or 2C11 antibody-coated cells, respectively (2.5×10^6 cells/sample). The incubation was terminated after 15 min, and the amount of radioactivity for each inositol phosphate metabolite was assessed as indicated in the Materials and methods section (a). Cell lipids were extracted from the washed trichloroacetic acid-precipitable material and the inositol-containing phospholipids analysed by t.l.c. as described in the text (b). The data in (a) and (b) are from a single experiment. The data in (a) are representative of three separate experiments; the data in (b) represent two separate experiments.

fashion (Fig. 4). Complete inhibition was observed at 10 mM-GDP β S, with 50% inhibition between 1 and 3 mM. GDP β S was less effective in inhibiting inositol phosphate production stimulated by GTP γ S. This was expected, owing to competition between the two non-hydrolysable nucleotides.

Nucleotide requirement for InsPL hydrolysis in permeabilized cells

The presence of ATP potentiated the generation of inositol phosphates in response to GTP γ S, and was essential for the

response to TCR perturbation (Fig. 5). ATP alone did not induce appreciable inositol phosphate accumulation. None of the other nucleotides tested supported InsPL hydrolysis in response to TCR perturbation. Specifically, GTP did not substitute for ATP when cells were stimulated by TCR perturbation, ruling out a role for ATP conversion into GTP in sustaining InsPL hydrolysis. Addition of GTP in combination with ATP did not further potentiate GTP γ S- or TCR-induced InsPL hydrolysis (results not shown).

To further explore the role of ATP in TCR- or GTP γ S-

induced InsPL hydrolysis in permeabilized lymphocytes, the nucleotide was added in graded concentrations and its effect in supporting the generation of the inositol phosphates evaluated. Steady-state levels of ATP were maintained by the addition of a regenerating system composed of 10 mM-creatine phosphate and 30 units of creatine kinase/ml to the incubation buffer. InsP_3 and InsP_2 were the inositol phosphate species most dependent upon the presence of ATP, and were almost undetectable in the absence of the nucleotide. InsP was also substantially decreased in the absence of the nucleotide (Fig. 6a). Synthesis of the precursor phospholipids, $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$, from PtdIns via ATP-dependent phosphorylation may explain the effect on the generation of the inositol polyphosphates (Fig. 6b). In fact, increasing ATP levels were associated with increased amounts of PtdInsP and PtdInsP_2 in unstimulated permeabilized cells. Addition of $\text{GTP}\gamma\text{S}$ or TCR perturbation was accompanied by increased polyphosphoinositide synthesis, which depended upon the presence of ATP. However, InsP accumulation was also dependent on ATP, albeit partially.

DISCUSSION

The data presented support the conclusion that InsPL hydrolysis in a Th2 clone is regulated by a G-protein. The non-hydrolysable guanine nucleotide analogue $\text{GTP}\gamma\text{S}$ induced InsPL hydrolysis in permeabilized T-lymphocytes. In addition, $\text{GDP}\beta\text{S}$ blocked TCR-mediated activation of InsPL hydrolysis in a dose-dependent manner. Hence, this protein (or proteins) may couple an InsPL-specific PLC to the TCR. Induction of InsPL hydrolysis via TCR perturbation or by $\text{GTP}\beta\text{S}$ resulted in the production of all species of inositol phosphates, but differed in their dependence on ATP, which was required for the former, but potentiated the latter.

Indirect evidence for a role of G-protein(s) in controlling PLC activation in certain T-lymphocytes was initially obtained by O'Shea *et al.* [20]. These investigators observed that AIF_4^- induced InsPL hydrolysis and inositol phosphate accumulation in a T-cell hybridoma. AIF_3 activates G-proteins by forming a GTP-like complex with bound GDP [10,36]. However, the indiscriminate activation of G-proteins may limit its use as an investigational tool. A problem in evaluating AIF_4^- -induced InsPL hydrolysis is the potential negative effect of cyclic AMP [37], which is generated by the concomitant stimulation of the adenylate cyclase by the regulatory G-protein, G_s . Hasegawa-Sasaki *et al.* [21] observed that the response of Jurkat cells to AIF_4^- increased when the cells were permeabilized. Under these conditions, the cyclic AMP produced may not exert any effect, as it escapes from the intracellular compartments. Hasegawa-Sasaki *et al.* also showed that Jurkat cells permeabilized with *Pseudomonas* cytotoxin responded, albeit modestly, to $\text{GTP}\gamma\text{S}$ with InsPL hydrolysis [21]. It is possible that the small pores (< 1.5 nm) caused by *Pseudomonas* cytotoxin [38] may not have allowed complete access to the nucleotide. Other authors have reported activation by $\text{GTP}\gamma\text{S}$ of InsPL hydrolysis in Jurkat cells [17] or human peripheral-blood T-cells [22], using ADP- or lysolethicin-mediated permeabilization respectively. Additional indirect evidence supporting a role of G-proteins in T-lymphocyte activation was obtained by using depletion of intracellular GTP with mycophenolic acid [39] or by inducing ornithine decarboxylase activity [16], Ca^{2+} mobilization [19] or secretion of lysosomal enzymes [15] with GTP analogues. Recently, Graves *et al.* [40] reported a study of InsPL hydrolysis and protein kinase C activation by GTP analogues in permeabilized human T-lymphoblasts. In addition to $\text{GTP}\gamma\text{S}$ activation of PLC, the inhibitory effect of $\text{GDP}\beta\text{S}$ on TCR-induced InsPL hydrolysis

documented here consistently supports the hypothesis of G-protein coupling of the TCR to PLC.

Imboden *et al.* [41] have previously reported inhibition of TCR-induced InsPL hydrolysis by cholera toxin in Jurkat cells in the absence of detectable cyclic AMP production, suggesting that a G-protein substrate that is covalently modified by this toxin may couple the TCR to PLC. Although Gardner *et al.* [42] caution against drawing conclusions from cholera toxin-mediated inhibition of receptor-induced InsPL hydrolysis, our data support the conclusions of Imboden *et al.* [41].

Previous studies have shown that ATP and guanine nucleotides may activate purified PLC isoenzymes *in vitro* [43]. Therefore the addition of nucleotides (such as guanine nucleotide analogues) to permeabilized cells may directly activate PLC rather than a G-protein. Two observations argue against this: direct PLC activation requires the presence of nucleotides in the millimolar range [43], whereas InsPL hydrolysis in permeabilized T-cells was observed with micromolar concentrations of $\text{GTP}\gamma\text{S}$. Addition of nucleotides other than $\text{GTP}\gamma\text{S}$ to permeabilized Th2 cells did not induce appreciable inositol phosphate accumulation. The induction of InsPL hydrolysis in Th2 cells by a non-hydrolysable analogue of GTP indicates that an element possessing intrinsic GTPase activity is involved. The inhibition of TCR-induced inositol phosphate accumulation by $\text{GDP}\beta\text{S}$ is further evidence of G-protein involvement. The concentrations of $\text{GDP}\beta\text{S}$ used to inhibit signalling were substantially in agreement with those reported by Gold *et al.* [13] for the inhibition of PLC activation by surface IgM in B lymphocytes. In that report, a maximum of 70% inhibition was observed at 2 mM- $\text{GDP}\beta\text{S}$, the highest concentration tested. The inhibition by $\text{GDP}\beta\text{S}$ we observed was not owing to a non-specific toxic effect, but appeared to be owing to the ability of $\text{GDP}\beta\text{S}$ to compete for a guanine-nucleotide-binding site. In fact, $\text{GTP}\gamma\text{S}$ -induced InsPL hydrolysis was only marginally affected, as for B-lymphocytes [13].

A characteristic of InsPL hydrolysis in permeabilized Th2 cells was its ATP-dependence. Synthesis of $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ from PtdIns explains the ATP requirement for inositol polyphosphate generation by permeabilized cells. A similar finding was reported by Best [44] in permeabilized pancreatic islets. However, ATP may act at one or more additional levels in the regulation of PLC in Th2 cells. This is also suggested by the observation that ATP also supported inositol monophosphate generation (i.e. PtdIns hydrolysis). Cockcroft *et al.* [45], observing that ATP sustained $\text{GTP}\gamma\text{S}$ -induced InsPL hydrolysis and secretion by permeabilized mast cells, speculated on a role of ATP as a phosphoryl donor in protein kinase-catalysed reactions. Although protein kinase C was the suggested effector, we question whether this enzyme, which is mostly cytoplasmic in resting cells [3], may have leaked out of the cells upon permeabilization.

Recently, it has been suggested that tyrosine kinase activation may precede TCR-induced InsPL hydrolysis [46] and evidence has been provided suggesting that tyrosine kinase activity may be required for activation of PLC after TCR perturbation [47]. Furthermore, treatment of responsive cells with epidermal growth factor or platelet-derived growth factor may induce phosphorylation of PLC by a tyrosine kinase [48,49]. In addition, Nishibe *et al.* [50] have shown that the tyrosine kinase of the epidermal growth factor receptor may phosphorylate PLC. Although these studies failed to show that PLC phosphorylation resulted in its activation *in vitro*, the role of this PLC modification in the intracellular environment remains unknown.

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