Biochem. J. (1992) 284, 189–199 (Printed in Great Britain)

Increased intracellular cyclic AMP inhibits inositol phospholipid hydrolysis induced by perturbation of the T cell receptor/CD3 complex but not by G-protein stimulation

Association with protein kinase A-mediated phosphorylation of phospholipase C-γ1

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Modulation of inositol phospholipid (InsPL) hydrolysis in response to increasing intracellular concentrations of cyclic AMP (cAMP) was studied in a murine T helper type II (Th2) lymphocyte clone, 8-5-5. Intact 8-5-5 cells produced maximal amounts of cAMP in response to prostaglandin E_2 (PGE_2), cholera toxin (CTx) or 7β-deacetyl-7β-(γ-*N*-methylpiperazino)butyryl forskolin (dmpb-forskolin). cAMP generation reached a plateau after 5 min of treatment with dmpb-forskolin (300 μM) or PGE_2 (1 μM), but required 60 min of treatment with CTx (1 μg/ml). Preincubation of 8-5-5 cells with 1 μM-PGE_2 or 300 μM-dmpb-forskolin (10 min at 37 °C) or with 1 μg of CTx/ml (60 min at 37 °C) completely inhibited InsPL hydrolysis induced by perturbation of the T cell receptor (TCR)/CD3 complex with the monoclonal antibody 145.2C11. Preincubation with the cAMP analogue 8-bromo-cyclic AMP (8-Br-cAMP) also inhibited InsPL hydrolysis. Tetanolysin-permeabilized 8-5-5 cells produced cAMP in response to PGE_2, dmpb-forskolin and guanosine 5′-[γ-thio]triphosphate (GTP[S]), a non-cell-permeating, non-hydrolysable analogue of GTP that directly activates G-proteins. No inhibition of TCR/CD3-induced InsPL hydrolysis was observed under these conditions. InsPL hydrolysis was also unaffected when permeabilized cells were incubated with up to 10 mM-8-Br-cAMP, suggesting that permeabilized cells lost (a) soluble effector molecule(s) involved in mediating the inhibitory effect observed in intact cells. Treatment of 8-5-5 cells with dmpb-forskolin or CTx prior to permeabilization resulted in inhibition of TCR/CD3-induced InsPL hydrolysis, but did not affect InsPL hydrolysis induced via G-protein stimulation with GTP[S]. Treatment of permeabilized 8-5-5 cells with purified cAMP-dependent protein kinase (PKA) resulted in inhibition of TCR/CD3- but not GTP[S]-induced InsPL hydrolysis. This effect was associated with phosphorylation of phospholipase (PLC)-γ1 in the absence of phosphorylation of components of the TCR/CD3 complex. These results suggest that PKA-mediated phosphorylation of PLC may regulate TCR/CD3-induced InsPL hydrolysis.

INTRODUCTION

Multiple biochemical and biological events are triggered by the interaction of the T cell receptor (TCR) with processed antigens in the context of the proper major histocompatibility complex (MHC) molecules of antigen-presenting cells [1]. Aggregation of surface determinants with antibodies (Abs) directed against TCR components or those of the CD3 complex, a structure non-covalently associated with the α/β heterodimer of the TCR [2], mimics the effects of the TCR/antigen/MHC interaction. The hydrolysis of inositol-containing phospholipids (InsPL) is one of the early biochemical responses to antigen activation or TCR/CD3 perturbation, and may play a role in T lymphocyte activation [1,3,4]. This metabolic pathway depends upon the activation of a specialized enzyme, InsPL-specific phospholipase C (PLC), resulting in the generation of inositol phosphates and diacylglycerol [5]. Certain inositol phosphates [e.g. Ins(1,4,5)P_3] mobilize cellular Ca^{2+}, while diacylglycerol activates protein kinase C (PKC) [5,6], which may act synergistically in inducing T cell activation [7,8]. The mechanism coupling the TCR to PLC is not defined: a role for tyrosine kinase activity(ies) [9–11] as well as for G-proteins [12–14] have both been suggested.

Another signal transduction mechanism of relevance in T lymphocyte activation is the adenylate cyclase cyclic AMP (cAMP) pathway. Activation of adenylate cyclase in T lymphocytes may be triggered by certain autocoids [e.g. prostaglandin E_2 (PGE_2)], bacterial toxins [e.g. cholera toxin (CTx)], or pharmacological treatment with forskolin. Whereas PGE_2 activates adenylate cyclase by acting through a surface receptor [15], CTx enzymically ADP-ribosylates the stimulatory G-protein, G_s, which controls adenylate cyclase, resulting in its activation [16]. Forskolin activates adenylate cyclase by directly binding to the enzyme [17].

Elevated cAMP levels have been associated with the down-regulation of several T cell responses, including lymphokine secretion and cell proliferation (reviewed in [18]). The mechanism by which increased cAMP levels affect lymphocyte function is not completely understood; however, the inhibition may occur at early phases of cell activation. This was suggested by the

Abbreviations used: Ab, antibody; cAMP, cyclic AMP; 8-Br-cAMP, 8-bromo-cyclic AMP; CTx, cholera toxin; FBS, fetal bovine serum; dmpb-forskolin, 7β-deacetyl-7β-(γ-*N*-methylpiperazino)butyryl forskolin dihydrochloride; GAH, goat anti-hamster antibody; GTP[S], guanosine 5′-[γ-thio]triphosphate; IBMX, 3-isobutyl-1-methylxanthine; InsPL, inositol phospholipids; MAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline solution; PGE_2, prostaglandin E_2; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; TCR, T cell receptor; Th2, T helper type II.

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observation that the inhibitory effect of PGE_2 on lectin-stimulated human T lymphocyte proliferation and interleukin 2 secretion was bypassed by treatment with a PKC activator and a Ca^{2+} ionophore [19]. Similar results were obtained for the TCR-induced cytolytic activity of murine cytotoxic T lymphocytes [20]. Consequently, the effect of increased cAMP concentrations may be exerted at levels proximal to PKC activation and Ca^{2+} mobilization. In agreement with this hypothesis, inducers of cAMP have been shown to inhibit inositol phosphate generation and Ca^{2+} mobilization in response to treatment of T lymphocytes with lectins or an anti-receptor Ab [21–23].

cAMP-mediated down-regulation of ligand-induced inositol phosphate production may occur by different mechanisms: (1) decreased inositol phosphate production by either inhibition of PLC activity or alteration in receptor/PLC coupling; or (2) increased inositol phosphate catabolism by enhancement of phosphatase activity. In this study, we have investigated these possibilities by examining the effect of activation of the adenylate cyclase/cAMP pathway on the induction of InsPL hydrolysis in response to TCR/CD3 perturbation in a murine T helper type II (Th2) clone. By using permeabilized cells, we have ruled out activation of inositol phosphate phosphatases and obtained evidence indicating that the inhibitory effect of cAMP is selective for TCR/CD3-induced InsPL hydrolysis and not for G-protein-mediated activation of PLC. Inhibition of TCR/CD3-induced InsPL hydrolysis could be obtained in permeabilized cells by treatment with purified cAMP-dependent protein kinase (PKA) and was associated with the phosphorylation of membrane proteins, including the PLC isoenzyme PLC-γ1.

MATERIALS AND METHODS

Reagents

Tissue culture-grade *myo*-inositol, 3-isobutyl-1-methylxanthine (IBMX), phorbol 12-myristate 13-acetate (PMA), PGE_2, 8-bromo cyclic AMP (8-Br-cAMP), essentially fatty-acid-free BSA and the purified PKA from bovine heart (PKA holoenzyme, catalytic or regulatory subunits) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Purified PKC (a mixture of PKC isoenzymes from rat brain) was from Lipidex (Westfield, NJ, U.S.A.). CTx was from List Biological Laboratories (Campbell, CA, U.S.A.). CTx was reduced for 10 min with 1 mM-dithiothreitol before use. 7β-Deacetyl-7β-(γ-*N*-methylpiperazino)butyryl forskolin dihydrochloride (dmpb-forskolin), a forskolin derivative with increased water solubility [24], was obtained from Calbiochem (La Jolla, CA, U.S.A.). ATP and guanosine 5′-[γ-thio]triphosphate (GTP[S]), a non-hydrolysable analogue of GTP, were from Boehringer–Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Okadaic acid was obtained from LC Services Corporation (Woburn, MA, U.S.A.). Tetanolysin, a gift from Dr. W. Habig (Center for Biologics Evaluation and Research, Bethesda, MD, U.S.A.), was obtained as a partially purified preparation from a tetanus-toxin-negative mutant of *Clostridium tetani*. *myo*-[2-^{3}H]inositol (specific radioactivity 55 Ci/mmol) and [γ-^{32}P]ATP (specific radioactivity 3000 Ci/mmol) were obtained from NEN Research Products (Boston, MA, U.S.A.) or Amersham (Arlington Heights, IL, U.S.A.). All other reagents were from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.).

Anti-CD3 monoclonal antibody (MAb) and perturbation of the TCR/CD3 complex

The hamster anti-[mouse CD3 (ϵ chain)] MAb-producing hybridoma, clone 145.2C11 [25], was obtained from Dr. R. Hodes (NIH, Bethesda, MD, U.S.A.) and grown in the peritoneal cavities of athymic nude mice, and the MAb was purified from the ascitic fluid by affinity chromatography on protein A (Beckman, Fullerton, CA, U.S.A.). MAb 145.2C11 will hereafter be referred to as 2C11 MAb. In certain experiments, cells were stimulated by using 2C11 MAb which was immobilized by adsorption on to polystyrene beads (6 μm diameter; Polysciences, Inc., Warrington, PA, U.S.A.) as previously described [4]. This strategy offered optimal stimulation of InsPL hydrolysis in intact cells. Alternatively, cells (10^7/ml) were preincubated with gentle agitation for 1 h at 4 °C in medium supplemented with 15 μg of purified 2C11 MAb/ml, and washed. Aggregation of the TCR/CD3/2C11 MAb complex was then obtained by the addition of a second Ab [50 μg/ml; affinity-purified goat anti-hamster Ab (GAH); Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.). These conditions were optimal for stimulation of InsPL hydrolysis in permeabilized cells.

Th2 lymphocyte clone 8-5-5

The murine Th2 clone 8-5-5 [26] was maintained in medium consisting of RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) 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), 25 mM-Hepes buffer (all from Flow Inc., McLean, VA, U.S.A), 50 μM-β-mercaptoethanol (Sigma), 7% heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD, U.S.A.), 20 units of recombinant human interleukin 2/ml (kindly supplied by Cetus Corporation, Emeryville, CA, U.S.A.). This clone was $CD3^+$, $CD4^+$ ($L3T4^+$), $CD8^-$ ($Lyt2^-$), $Thy1.2^+$, is specific for the antigen keyhole limpet haemocyanin in the context of I^b and could act as T helper cells 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 permeabilization procedure

Permeabilization of T lymphocytes was performed in an 'intracellular' buffer (potassium glutamate/Hepes buffer) composed of 50 mM-Hepes, pH 7.15 at 37 °C (pH 7.40 at 0 °C), 100 mM-potassium glutamate, 10 mM-LiCl, 100 μM-*myo*-inositol, 7 mM-magnesium acetate, 1 mM-Mg/EGTA, 0.1 mg of BSA/ml, 5 mM-D-glucose, 5 mM-ATP, 10 mM-phosphocreatine (Sigma), 30 units of creatine kinase/ml (EC 2.7.3.2; Sigma) and 0.285 mM-$CaCl_2$, to give 100 nM free Ca^{2+} concentration at 37 °C [13]. Unless otherwise indicated, 100 μM-IBMX, an inhibitor of cAMP phosphodiesterase, was added to the buffer.

Permeabilization was obtained with reduced (10 min at room temperature with 5 mM-dithiothreitol) tetanolysin used in a ratio of 10 μl of tetanolysin stock per ml of cell suspension containing 10^8 cells. Tetanolysin binds in the cold and produces pores of 25–50 nm, in cholesterol-containing membranes upon exposure at 37 °C [27]. In all experiments cell permeabilization, measured as the percentage of cells stained by the non-permeating probes ethidium bromide or Trypan Blue, was better than 95% (results not shown).

cAMP assay

RPMI 1640 medium containing 25 mM-Hepes buffer, pH 7.40 at 25 °C, 1 mg of BSA/ml, 10 mM-LiCl, 300 μM-*myo*-inositol and 100 μM-IBMX (unless otherwise indicated) was used when cells were tested in their intact form. Potassium glutamate/Hepes buffer was used for permeabilized cells. Incubation was terminated by the addition of trichloroacetic acid and EDTA to final concentrations of 10% (w/v) and 0.5 mM respectively. Insoluble material was precipitated by centrifugation and trichloroacetic acid extracted from the supernatant with an equal volume of a mixture of trichlorotrifluoroethane (Fisher) and tri-*N*-

octylamine (Sigma) in the proportions 76:24 (v/v). cAMP was measured in the supernatant by using a radiocompetition assay (Amersham). ATP (tested at concentrations up to 5 mM) or GTP[S] (tested at concentrations of up to 1 mM) did not interfere with the assay (results not shown).

Assay of InsPL hydrolysis

InsPL hydrolysis was measured as the production of radiolabelled inositol phosphates from *myo*-[2-^{3}H]inositol-labelled 8-5-5 cells, as previously described [4]. Briefly, cells were labelled for 18–24 h at 37 °C with 15–25 μCi of *myo*-[2-^{3}H]inositol/ml. Viable cells were recovered by Ficoll–Paque (Pharmacia) gradient centrifugation, resuspended in RPMI 1640 medium (supplemented with 25 mM-Hepes buffer, pH 7.40, 1 mg of BSA/ml, 10 mM-LiCl, 300 μM-*myo*-inositol and 100 μM-IBMX, unless otherwise indicated) or potassium glutamate/Hepes buffer, dependent upon the assay. The incubation was terminated by the direct addition of ice-cold trichloroacetic acid to a final concentration of 10% (w/v). Inositol phosphates were separated by anion-exchange chromatography of the trichloroacetic acid-soluble material on AG 1-X8 resin, 100–200 mesh (Bio-Rad). The trichloroacetic acid-precipitable pellet was washed once with 5% trichloroacetic acid, dissolved in 10% Triton X-100 or 10% SDS and counted for an estimate of the radioactivity incorporated into the phospholipid pool. Radioactivity was converted to d.p.m. by using an external standard ratio, and data were normalized as the percentage of the total cell-associated radioactivity. The amount of total cellular radioactivity in all experiments ranged from 1×10^5 to 5×10^5 d.p.m./sample [using $(1.5–4) \times 10^6$ cells/sample]. Within each experiment, the amount of total cellular radioactivity was constant and was within 5% of the average for each sample.

H.p.l.c. analysis of inositol phosphates was performed as previously described [28], by using a reversed-phase column (5 μm octyldimethylsilyl; 250 mm × 4.6 mm internal diam., LC-8-DB; Supleco, Bellefonte, PA, U.S.A.) and a two-solvent system composed of a micellar mobile phase [hexadecyltrimethylammonium hydroxide (Fisher), 20 mmol/l; methanol, 2.4% (v/v); KH_2PO_4, 20 mmol/l, pH 5.80] and an eluting solution [propan-1-ol/KH_2PO_4 (80 mmol/l, pH 4.50), 1:1, v/v] coupled to in-line radioactivity detection. Peak identification and column calibration was performed against authentic radiolabelled external standards, except for Ins(1,3)P_2, whose identification was only tentative.

Phosphorylation and immunoprecipitation assays

8-5-5 cells (3×10^7/ml) were washed twice in a modified potassium glutamate/Hepes buffer without phosphocreatine and creatine kinase and containing 500 μM-ATP. Cells were resuspended in this buffer and permeabilized for 5 min at 37 °C with tetanoylysin. PMA (100 nM) was added to the positive control cells to be treated with purified PKC. After permeabilization, cells were incubated at 37 °C for 10 min with 0.5 mCi of [γ-^{32}P]-ATP/ml in the presence or absence of 150 units of either the purified catalytic subunit of PKA or purified PKC/ml. The reaction was terminated by adding cold phosphate-buffered saline (PBS) containing 1 mM-EDTA, 1 mM-EGTA, 10 mM-sodium pyrophosphate, 1 mM-sodium orthovanadate, 10 mM-NaF and 1 mg of BSA/ml. Permeabilized cells were pelleted for 10 min at 1500 *g* at 4 °C and the supernatants, representing cytoplasmic material, were discarded. Cell lysates were obtained by solubilization for 30 min at 4 °C with a lysis buffer (1 ml/3×10^7 cells) containing 1% Triton X-100 (Bio-Rad), 50 mM-Hepes/NaOH, pH 7.40, 150 mM-NaCl, 1 mg of BSA/ml, 1 mM-EDTA, 1 mM-EGTA, 10 mM-sodium pyrophosphate, 50 mM-NaF, 1 mM-sodium orthovanadate, 10 μg of leupeptin/ml (Boehringer–Mannheim), 10 μg of aproptinin/ml (Boehringer), 20 μg of benzamidine/ml, 20 μg of α-iodoacetamide/ml and 1 mM-phenylmethanesulphonyl fluoride (BRL). Solubilized material was recovered by centrifugation at 13000 *g* for 15 min at 4 °C. CD3 immunoprecipitation was performed on the cell lysates as described [29], with minor modifications, using 2C11 MAb. Briefly, supernatants were precleared with Protein A–Trisacryl beads (100 μl/ml of lysate) (Pierce, Rockford, IL, U.S.A.) and rabbit antibodies directed against hamster IgG (5 μg/ml of lysate) (Cappel, Westchester, PA, U.S.A.). A 1 ml sample of the cleared lysate was incubated for 1 h at 4 °C with 10 μg of 2C11 MAb, and then mixed for 1 h with 5 μg of rabbit anti-hamster antibodies preadsorbed on to Protein A–Trisacryl beads (100 μl/ml of lysate). Precipitable material was recovered by centrifugation (15000 *g*, 30 s), washed three times with 1 ml of lysis buffer, once with 1 ml of lysis buffer containing 0.5 M-NaCl, once with 1 ml of lysis buffer and once with 1 ml of lysis buffer lacking BSA and containing 0.1% Triton X-100. The pellets were solubilized with a modified Laemmli sample buffer [30] containing 10% SDS, 20 mM-sodium pyrophosphate, 10 mM-NaF, 1 mM-sodium orthovanadate, 62.5 mM-Tris/HCl, pH 6.8, 10% glycerol, 0.01% Bromophenol Blue and 5% β-mercaptoethanol. Samples were boiled for 5 min and analysed by SDS/PAGE [30]. Gels were dried and autoradiographed on to XAR 2 films (Kodak, Rochester, NY, U.S.A) at −80 °C.

Immunoprecipitation employing anti-PLC-γ1 Ab (a pool of MAbs described previously [31], generously provided by Dr. S. G. Rhee (National Heart, Lung, and Blood Institute, National Institute of Health, Bethesda, MD, U.S.A.) was performed as described [32], except that lysates were precleared with Protein A–Trisacryl beads. The immunoprecipitates were analysed by SDS/PAGE as above.

RESULTS

Increased cAMP production is associated with inhibition of TCR/CD3-induced InsPL hydrolysis in 8-5-5 lymphocytes

PGE_2, CTx and dmpb-forskolin stimulated cAMP production by the T lymphocyte clone 8-5-5 (Fig. 1). 8-5-5 cells produced cAMP (6–15 pmol/10^6 cells) after treatment for 5 min with either PGE_2 (1 μM) or dmpb-forskolin (300 μM). In the case of CTx (10 μg/ml) slower kinetics were observed, with cAMP production reaching a plateau at ~ 6 pmol/10^6 cells after 1 h of treatment. No increased cAMP levels were detected when 8-5-5 cells were incubated with 2C11 MAb-coated beads or IBMX alone. This lack of an effect of immobilized 2C11 MAb suggests that murine Th2 cells differ from peripheral blood human T lymphocytes or human Jurkat cells, in which CD3 perturbation induces a transient increase in cAMP [33,34].

Dose–response studies with PGE_2 showed a near-maximal effect at 3 μM-PGE_2 (Fig. 1). cAMP production in response to dmpb-forskolin did not reach a plateau at concentrations between 0.03 mM and 1 mM, the highest concentration tested. The CTx dose–response showed no effect at 0.01 μg/ml, but was maximal between 0.1 and 0.3 μg/ml.

To obtain information on the effect of cAMP inducers on InsPL hydrolysis in response to TCR/CD3 perturbation, [^{3}H]inositol-labelled 8-5-5 cells were incubated with cAMP inducers prior to perturbation of the TCR/CD3 with immobilized 2C11 MAb. Pretreatment of T cells with CTx or dmpb-forskolin resulted in complete inhibition of TCR/CD3-induced InsPL hydrolysis (Figs. 2*a* and 2*b*). Preincubation was necessary, since simultaneous addition of these agents with 2C11 MAb-coated beads did not affect InsPL hydrolysis. Similar results were

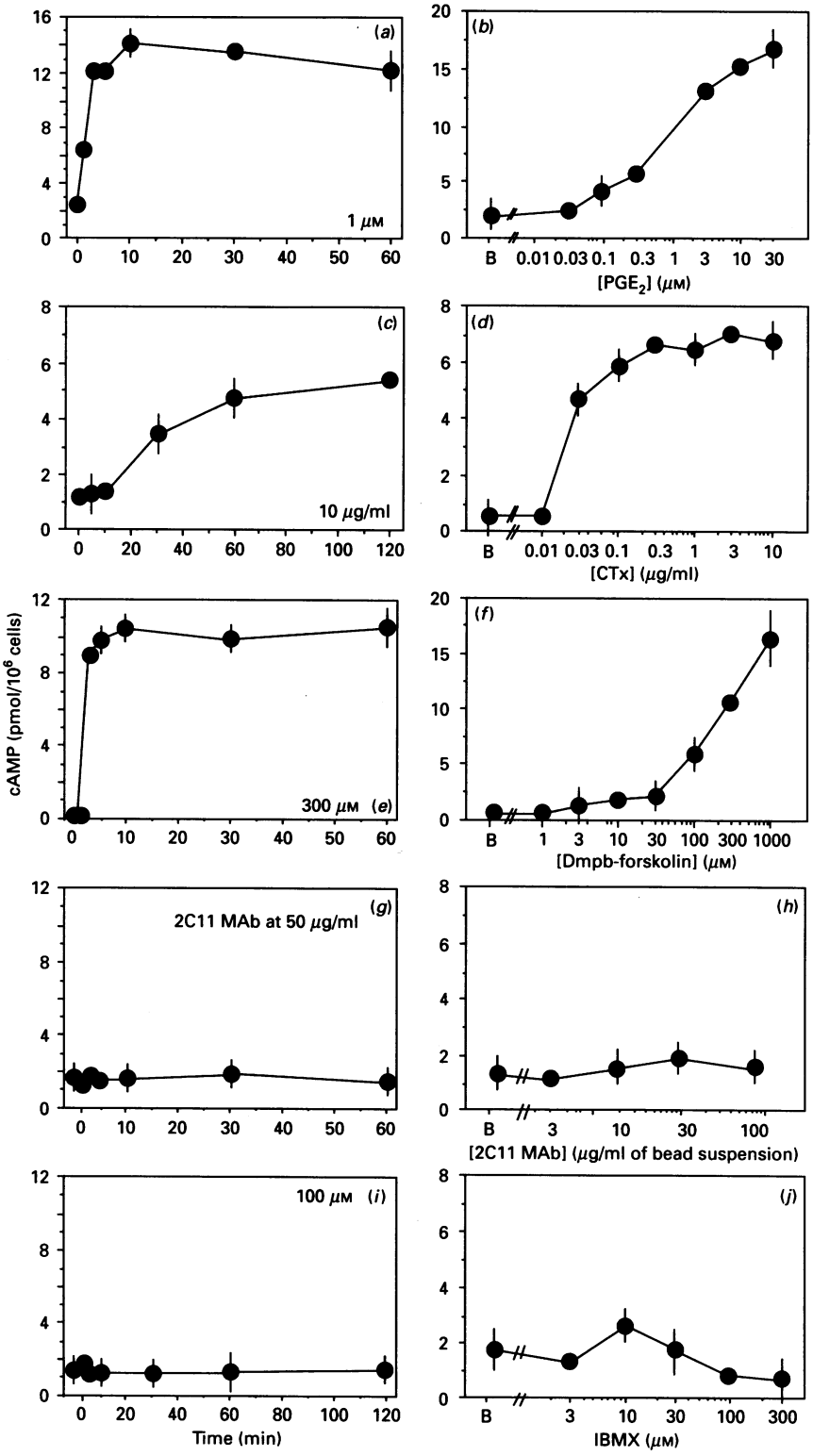

Fig. 1. 8-5-5 lymphocytes produce cAMP in response to various agonists

8-5-5 lymphocytes [(2–4) × 10^6 cells/sample] were incubated at 37 °C for the indicated times with PGE_2 (*a*, *b*), CTx (*c*, *d*), dmpb-forskolin (*e*, *f*), or 2C11 MAb which had been previously adsorbed at the indicated concentrations on to 2 × 10^8 polystyrene beads in 1 ml (2C11-beads; beads:cells = 5:1) (*g*, *h*). IBMX (100 μM) was included with all the above agents (*a–h*) or tested separately (*i*, *j*). The reaction was terminated at the indicated times by the addition of cold trichloroacetic acid. cAMP was measured as indicated in the Materials and methods section. The data on the right column refer to cAMP accumulation measured after 10 min of incubation, except for CTx which was incubated for 60 min (B = buffer control). The data shown are representative of two to three separate experiments.

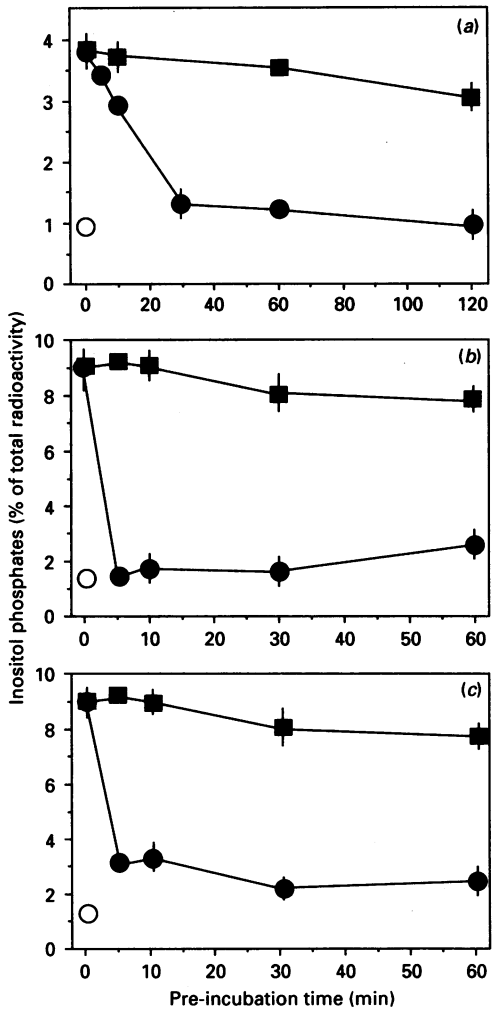

Fig. 2. Pretreatment with cAMP inducers or 8-Br-cAMP inhibits TCR/CD3-induced InsPL hydrolysis in 8-5-5 lymphocytes

myo-[^{3}H]Inositol-labelled cells [(2–4) × 10^6/sample] were preincubated at 37 °C for the indicated times with buffer alone (■) or cAMP inducers (●): (*a*) CTx (10 μg/ml), (*b*) dmpb-forskolin (300 μM) or (*c*) 8-Br-cAMP (3 mM). InsP hydrolysis was induced with 2C11-MAb immobilized on to polystyrene beads (50 μg of 2C11 MAb per 2×10^8 beads in 1 ml; beads/cells ratio = 5:1). Untreated cells stimulated with BSA-coated beads were used as negative control in all experiments (○). IBMX (100 μM) was included in all samples. The incubation was terminated after 10 min by the addition of trichloroacetic acid and the amount of inositol phosphates produced was determined as indicated in the Materials and methods section. The data shown are representative of three experiments each performed in duplicate.

obtained with PGE_2 (results not shown). A short preincubation time (less than 5 min) was sufficient to induce inhibition in the case of dmpb-forskolin (Fig. 2*b*) or PGE_2 (results not shown). A longer incubation time (30–60 min) was required to obtain complete inhibition with CTx (Fig. 2*a*). This time-dependence of the inhibitory effect of cAMP inducers was consistent with the kinetics observed for the generation of cAMP.

The cAMP analogue 8-Br-cAMP was used to further corroborate the possibility that the inhibitory effect of cAMP inducers was related to the production of the cyclic nucleotide. Total inhibition was observed after 5 min of pretreatment with 3 mM-8-Br-cAMP (Fig. 2*c*).

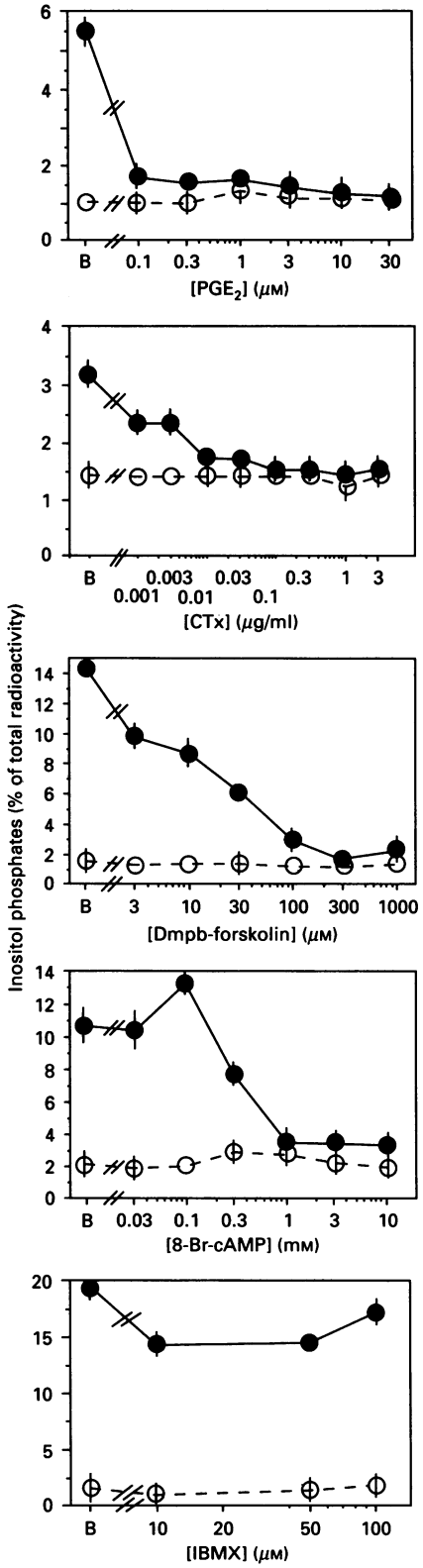

Fig. 3. Dose–response effect of cAMP inducers or 8-Br-cAMP on TCR/CD3-induced inositol phospholipid hydrolysis

8-5-5 cells [(2–4) × 10^6 cells], prelabelled with *myo*-[2-^{3}H]inositol, were incubated at 37 °C with the indicated concentrations of cAMP inducers, 8-Br-cAMP, or IBMX. Preincubation was for 30 min, except for CTx, which was preincubated for 1 h. Inositol phosphate production was stimulated with beads coated with 50 μg of 2C11 MAb per 2×10^8 beads in 1 ml and used at a bead/cell ratio of 5:1 (●). BSA-coated beads were used as control (○). The data shown are representative of two to three separate experiments. B, buffer control.

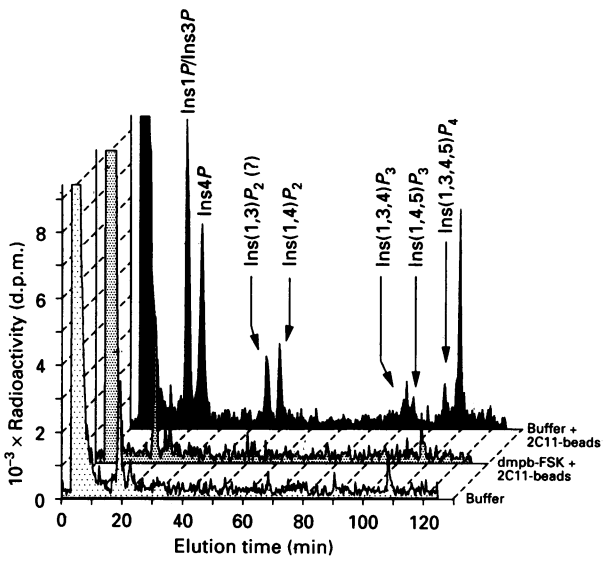

Fig. 4. H.p.l.c. analysis of inositol phosphate species produced after TCR/CD3 perturbation by 8-5-5 cells: effect of treatment with dmpb-forskolin

8-5-5 cells (6×10^6 cells), prelabelled with *myo*-[2-^{3}H]inositol, were preincubated for 10 min at 37 °C with or without 300 μM-dmpb-forskolin (dmpb-FSK). Inositol phospholipid hydrolysis was induced with 2C11-MAb immobilized on to polystyrene beads (2C11-beads) and the incubation was terminated after 10 min by the addition of cold trichloroacetic acid. After removal of trichloroacetic acid, the samples were filtered, reduced to a volume of 150 μl and injected into the h.p.l.c. apparatus. Arrows point to the elution positions of radiolabelled standards. The identification of Ins(1,3)P_2 is tentative.

All effective agents inhibited TCR/CD3-induced InsPL hydrolysis in a dose-dependent manner (Fig. 3). Concentrations of 0.1 μM-PGE_2, 0.01 μg of CTx/ml, 300 μM-dmpb-forskolin and 1 mM-8-Br-cAMP induced nearly complete inhibition. IBMX, at all concentrations tested, failed to affect InsPL hydrolysis, consistent with the absence of its effect on cAMP levels in 8-5-5 cells (Fig. 1). The effect of treatment with dmpb-forskolin on the inositol phosphate species produced was further characterized by using separation of ^{3}H-labelled inositol phosphates by reversed-phase h.p.l.c. coupled to in-line radioactivity detection. Pretreatment of 8-5-5 cells with 300 μM-dmpb-forskolin completely inhibited inositol phosphate generation, including production of the biologically relevant inositol phosphate species Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Fig. 4).

The negative effect of cAMP inducers on InsPL hydrolysis triggered by TCR/CD3 perturbation could not be explained by decreased receptor expression. Expression of the CD3 ϵ chain, which is recognized by the 2C11 MAb, was tested by treating 8-5-5 cells exposed to medium alone, CTx (10 μg/ml) or dmpb-forskolin (300 μM) with 2C11 Mab followed by staining with a fluoresceinated second Ab. Cytofluorographic analysis showed that neither CTx nor dmpb-forskolin affected CD3 expression under conditions that resulted in InsPL hydrolysis inhibition (results not shown).

cAMP production by permeabilized T helper lymphocytes

Cell permeabilization with tetanolysin was used to characterize the site of action of cAMP inducers in inhibiting TCR/CD3-induced InsPL hydrolysis. We first tested the cAMP response in permeabilized 8-5-5 cells. Tetanolysin-treated cells exposed to PGE_2 (1 μM) showed linear cAMP production for 20 min, up to a maximal level of approx. 70 pmol/10^6 cells (Fig. 5). A dose-dependent increase in cAMP production was observed with PGE_2, with a plateau at 3 μM. When dmpb-forskolin (300 μM) was used, cAMP production was virtually linear for up to 1 h of incubation, the longest time point tested, reaching approx. 100 pmol/10^6 cells. The response to dmpb-forskolin of permeabilized 8-5-5 cells did not plateau at doses up to 1 mM, the highest concentration tested.

Permeabilized cells were also challenged with the non-cell-permeating guanine nucleotide analogue GTP[S]. This non-hydrolysable GTP analogue binds stably to G-proteins, including the adenylate cyclase-stimulatory protein G_s. This nucleotide (10 μM) induced nearly linear production of cAMP for the first 30 min of incubation, reaching a level of 500 pmol/10^6 cells. The response to GTP[S] reached a plateau at $\sim$ 10 μM, and in certain experiments, as shown in Fig. 5, a decrease was observed at higher concentrations. The observed decrease may result from variable activation of inhibitory G-proteins (e.g. G_i) at higher concentrations of the nucleotide. The stimulated cAMP levels in permeabilized cells were substantially higher than those observed in intact cells. This phenomenon may be attributed to the loss of cAMP phosphodiesterase activity from permeabilized cells.

No response to CTx was observed in permeabilized cells at any time or dose tested. This finding may be explained by the possibility that cytoplasmic factor(s) essential for the toxin enzymic action may be lost upon permeabilization. This factor(s) may include NAD^+ and/or the cytoplasmic ADP-ribosylating factor [35], which is known to promote substrate ADP-ribosylation by CTx.

Similar to the observation in intact 8-5-5 cells, TCR/CD3 perturbation by the combination of 2C11 MAb plus a second Ab (GAH) also resulted in no effect on cAMP levels. IBMX alone was also ineffective (results not shown).

Soluble factor(s) are required for cAMP-mediated inhibition of TCR/CD3-induced InsPL hydrolysis

In spite of their ability to induce a sustained cAMP response in permeabilized cells, the cAMP inducers tested failed to affect TCR/CD3-mediated InsPL hydrolysis in permeabilized cells. Data from a representative experiment utilizing dmpb-forskolin are shown in Table 1. Since the cAMP produced by permeabilized 8-5-5 lymphocytes may leak out of the cells, it could be argued that insufficient nucleotide would be present to trigger a local, compartmental inhibitory response. To rule out this possibility, permeabilized cells were preincubated for 5 min with 8-Br-cAMP and then tested for their ability to respond to perturbation of the TCR/CD3 complex in terms of inositol phosphate accumulation. No inhibition of InsPL hydrolysis was observed at 10 mM-8-Br-cAMP (Table 1). Thus cAMP has no direct inhibitory effect on PLC activity or TCR/CD3/PLC coupling. The 8-Br-cAMP concentration used was in excess of that estimated for the intracellular concentration of cAMP reached by stimulated intact 8-5-5 cells. This was calculated as 106 μM from a maximal amount of cAMP of 20 pmol/10^6 cells, in a cell volume of 268×10^6 $\mu m^3/10^6$ cells (average radius 4 μm), and a relative water content of 70% [36]. We concluded that the inhibition induced by cAMP or cAMP inducers in intact cells occurred by a mechanism involving soluble effector molecule(s) (other than cAMP) which were lost upon permeabilization.

The pores produced by tetanolysin treatment (25–50 μm) probably allow the escape of several cytoplasmic components. If an intact cytoplasmic environment were required for cAMP to mediate its inhibitory effect, pretreatment of intact cells with cAMP or a cAMP inducer may result in decreased InsPL hydrolysis even after permeabilization. When intact 8-5-5 cells

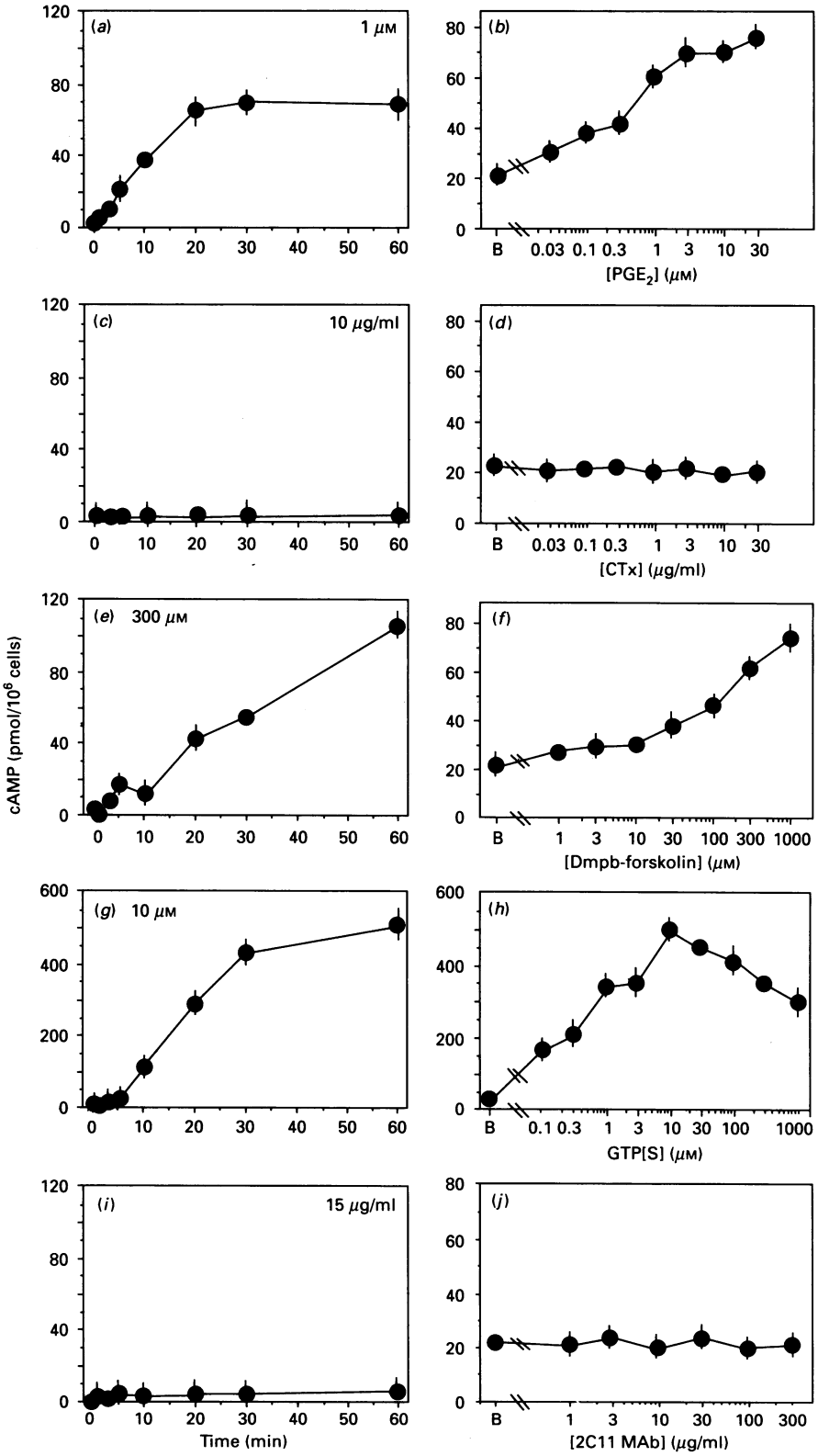

Fig. 5. cAMP response of permeabilized 8-5-5 lymphocytes

Tetanolysin-permeabilized 8-5-5 cells [(2–4) × 10^6] were incubated in potassium glutamate/Hepes buffer at 37 °C with the following agents at the concentrations shown: PGE_2 (*a*, *b*), CTx (*c*, *d*), dmpb-forskolin (*e*, *f*), GTP[S] (*g*, *h*) and 2C11 MAb (*i*, *j*). In the case of 2C11 MAb-treated cells (*i*, *j*), intact 8-5-5 cells (10^7 cells/ml) were preincubated for 1 h at 4 °C with the indicated amounts of Ab and stimulation was induced after permeabilization by the addition of a second Ab (GAH, 50 μg/ml). The reaction was terminated at the indicated times by the addition of cold trichloroacetic acid. cAMP was measured as indicated in the materials and methods section. The data on the right column refer to cAMP accumulation measured after 40 min of incubation (B, buffer control). The data shown are representative of two to three separate experiments.

Table 1. Treatment of permeabilized 8-5-5 lymphocytes with dmpb-forskolin or 8-Br-cAMP does not affect InsPL hydrolysis induced TCR/CD3 perturbation

8-5-5 cells [(2–4) × 10^6] were prelabelled with *myo*-[2-^{3}H]inositol, coated with 2C11 MAb and permeabilized with tetanolysin. Permeabilized cells were incubated with the indicated concentrations of dmpb-forskolin or 8-Br-cAMP for 5 min before stimulation of InsPL hydrolysis was induced by the addition of a second Ab (GAH). The reaction was terminated after 10 min and the inositol phosphates produced were determined. The data shown represent the inositol phosphates recovered as the percentage of total cellular radioactivity (mean ± S.E.M.) of three experiments. Each experiment was performed in duplicate.

		Inositol phosphates (%)	
Expt. no.	Pretreatment	Buffer	2C11 MAb+GAH
1	Buffer	2.9±0.6	17.2±3.6
	Dmpb-forskolin, 300 μM	2.9±0.7	17.8±3.7
2	Buffer	2.3±0.1	11.7±1.3
	8-Br-cAMP, 0.01 mM	2.4±0.2	10.5±1.2
	8-Br-cAMP, 0.1 mM	2.4±0.1	10.0±1.1
	8-Br-cAMP, 1 mM	2.6±0.2	10.6±1.1
	8-Br-cAMP, 10 mM	2.8±0.2	10.3±1.3

were pretreated with the above agents, followed by tetanolysin-mediated permeabilization, inhibition of TCR/CD3-induced InsPL hydrolysis was maintained, albeit to a lesser degree than that observed in similarly treated intact cells (Table 2).

GTP[S] induces InsPL hydrolysis in permeabilized T lymphocytes [13,14,37]. Similarly to its effect on the adenylate cyclase system, GTP[S] is likely to act on a regulatory G-protein that controls PLC activity. GTP[S] induces InsPL hydrolysis in 8-5-5 lymphocytes with an EC_{50} of between 1 and 3 μM and a maximal effect at 100–300 μM [13]. The stimulatory effect of GTP[S] on InsPL hydrolysis in permeabilized cells, which was observed in spite of its potent activation of adenylate cyclase activity, further supports the conclusion that cAMP *per se* has no effect on PLC activity. It was therefore of interest to investigate whether pretreatment of intact cells with cAMP agonists could affect GTP[S]-induced PLC activation in a manner similar to its effect on TCR/CD3-induced InsPL hydrolysis. To test this hypothesis, intact 8-5-5 cells were pretreated with CTx or dmpb-forskolin, followed by permeabilization and exposure to 300 μM-

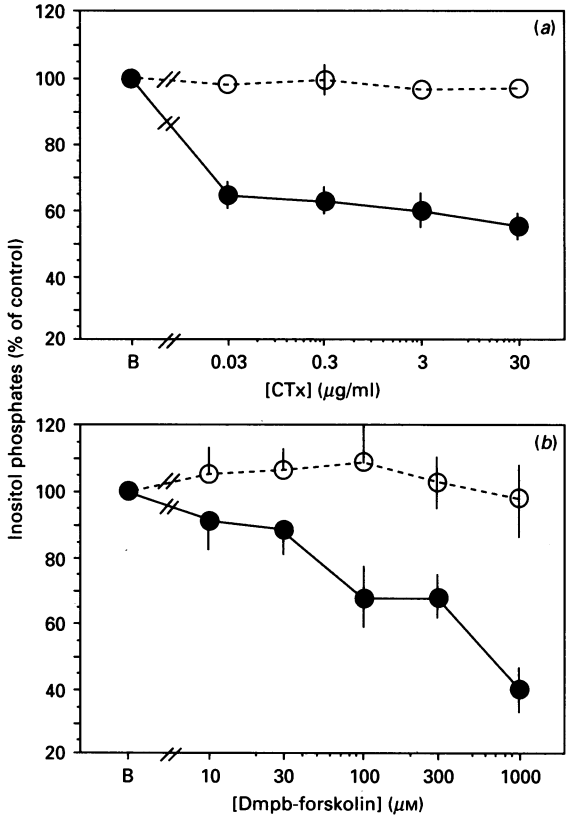

Fig. 6. Pretreatment of intact 8-5-5 cells with cAMP inducers inhibits InsPL hydrolysis produced in permeabilized cells by TCR/CD3 perturbation but not GTP[S]

8-5-5 cells [(2–4) × 10^6], prelabelled with *myo*-[2-^{3}H]inositol, were preincubated for 60 min with CTx (*a*) or for 10 min with dmpb-forskolin (*b*) at the indicated concentrations. Cells were then permeabilized with tetanolysin and stimulated for 10 min by perturbation of the TCR/CD3 complex (●) or with 300 μM-GTP[S] (○). Inositol phosphate production was determined as described in the Materials and methods section. Data are shown as percentages of the value for stimulated cells pretreated with buffer alone (control cells). The data shown represent the means ± S.E.M. of three (*a*) or four (*b*) separate experiments. Error bars were not drawn when smaller than the symbols. Inositol phosphate accumulation (as percentage of total cellular radioactivity) for stimulated cells was as follows: (*a*) 2C11 Ab plus GAH, 18.3±1.0%; GTP[S], 5.6±0.6%; (*b*) 2C11 plus GAH, 20.1±3.9%; GTP[S], 6.0±1.0%. Unstimulated (background) inositol phosphate content was equivalent to (*a*) 2.2±0.2% and (*b*) 2.9±0.2% of the total cellular radioactivity.

Table 2. Pretreatment of intact 8-5-5 cells is required for inhibition of TCR-induced InsPL hydrolysis

8-5-5 cells [(2–4) × 10^6], prelabelled with *myo*-[2-^{3}H]inositol, were incubated with the indicated concentrations of CTx, dmpb-forskolin or 8-Br-cAMP, either in their intact form or after permeabilization with tetanolysin. InsPL hydrolysis was subsequently stimulated with 2C11 MAb immobilized on to polystyrene beads in the case of intact cells, or with the combination of a 2C11 MAb coating and a second Ab. The analysis of inositol phosphates were performed as described in the Materials and methods section. The data represent the level of inhibition of inositol phosphate generation compared to the amount recovered from cells pretreated with medium alone. The data shown represent the means ± S.E.M. of three to four separate experiments. N.T., not tested.

Cell status		Inhibition of InsPL hydrolysis (%)		
Pretreatment	TCR/CD3 Perturbation	CTx (10 μg/ml)	Dmpb-forskolin (300 μM)	8-Br-cAMP (10 mM)
Intact	Intact	97.7±1.2	98.0±0.9	97.5±1.1
Intact	Permeabilized	41.9±3.6	43.7±4.9	49.7±2.5
Permeabilized	Permeabilized	N.T.	−5.1±4.5	1.6±10.2

Table 3. Treatment of permeabilized 8-5-5 lymphocytes with purified PKA inhibits InsPL hydrolysis induced by TCR/CD3 perturbation

8-5-5 cells [(2–4) × 10^6] were prelabelled with *myo*-[2-^{3}H]inositol, coated with or without 2C11 MAb and permeabilized with tetanolysin as indicated in the Materials and methods section. Permeabilized cells were preincubated for 5 min with purified PKA from bovine heart (150 units/ml) in the presence or the absence of 100 μM-8-Br-cAMP; the purified catalytic subunit (150 units/ml); or the purified regulatory subunit (150 units/ml). Cells were also pretreated with 100 μM-8-Br-cAMP alone as control (*a*) or with okadaic acid in combination with the purified catalytic subunit (*b*). Stimulation of InsPL hydrolysis was induced by the addition of 300 μM-GTP[S] or the second Ab (GAH) (50 μg/ml) to 2C11 MAb-coated cells. The reaction was terminated after 10 min and the inositol phosphates produced were determined as described in the Materials and methods section. The data shown represent the percentage inhibition of InsPL hydrolysis compared with cells pretreated with medium alone. The data in (*a*) are expressed as the means ± S.E.M. of four experiments. The data in (*b*) are from a representative experiment of three performed. Inositol phosphate accumulation of control cells, expressed as a percentage of total cellular radioactivity, was as follows: GTP[S], 6.6 ± 0.3 %; 2C11 MAb plus GAH, 14.1 ± 1.3 %. Unstimulated (background) inositol phosphate content was equivalent to 3.1 ± 0.4 % of the total cellular radioactivity.

(*a*)

	Inhibition of InsPL hydrolysis (%)				
	Medium +8-Br-cAMP	PKA holoenzyme −8-Br-cAMP	PKA holoenzyme +8-Br-cAMP	PKA catalytic subunit −8-Br-cAMP	PKA regulatory subunit −8-Br-cAMP
2C11 MAb+GAH	1.0 ± 2.0	1.9 ± 0.3	31.0 ± 5.0	30.0 ± 10.5	1.0 ± 0.6
GTP[S] (300 μM)	1.2 ± 0.3	1.3 ± 0.2	1.2 ± 0.4	2.0 ± 0.5	1.0 ± 0.5

(*b*)

	Inhibition of InsPL hydrolysis (%)	
	−Okadaic acid	+Okadaic acid
2C11 MAb+GAH	26.2 ± 2.0	73.8 ± 2.7
GTP[S] (300 μM)	−10.0 ± 8.6	4.5 ± 8.9

GTP[S] or TCR/CD3 perturbation. GTP[S]-induced InsPL hydrolysis was essentially unaffected by pretreatment with these agents (Fig. 6). Under the same experimental conditions. TCR/CD3-induced InsPL hydrolysis was inhibited. Maximal inhibition was observed at 0.3 μg of CTx/ml or 1 mM-dmpb-forskolin. These data suggest that the cAMP-induced inhibition of inositol phosphate generation was selective for receptor-mediated InsPL hydrolysis and not for G-protein-controlled PLC activity.

Treatment of permeabilized cells with purified PKA selectively inhibits TCR/CD3-induced InsPL hydrolysis

Experiments were performed to address the role of PKA in mediating the inhibitory effect of cAMP. PKA is a tetrameric cytoplasmic protein whose two catalytic subunits are normally suppressed by their association with two regulatory subunits. By binding to the regulatory subunits, cAMP frees the catalytic activity of the enzyme [38]. Preincubation of permeabilized cells for 5 min with the purified PKA from bovine heart (50 units/ml) resulted in a substantial inhibition of TCR/CD3-induced InsPL hydrolysis when cAMP was present in the incubation (Table 3). No effect was observed in the absence of cAMP. As expected, addition of the purified catalytic subunit of PKA induced similar levels of inhibition in the absence of added cAMP. The regulatory subunit of PKA was without effect. The limited effect of PKA may be due to its inability to reach sufficiently high localized concentrations of enzyme in permeabilized cells and/or to the presence of phosphoprotein phosphatases that may counteract its effect. However, the use of higher enzyme concentrations was impractical for technical and economical reasons. Addition of 150 units of the purified catalytic subunit of PKA/ml together with 1 μM-okadaic acid, a serine/threonine-specific protein phosphatase inhibitor [39], potentiated the effect of PKA on CD3-induced InsPL hydrolysis, achieving nearly complete inhibition (Table 3). Okadaic acid alone had no effect (results not shown). Under all circumstances, GTP[S]-induced InsPL hydrolysis was unaffected. These data suggest that PKA mediates the inhibitory effect of cAMP, presumably by phosphorylating a membrane-associated substrate in the pathway leading to PLC activation. Although phosphatases may limit the effect of PKA reconstitution in permeabilized cells, they may be effectively controlled by the addition of a phosphatase inhibitor.

Protein phosphorylation by PKA was studied by monitoring the incorporation of ^{32}P from [γ-^{32}P]ATP into proteins of permeabilized cells treated with the purified catalytic subunit of PKA. Reducing SDS/PAGE of proteins extracted from PKA-treated permeabilized cells revealed a pattern of phosphorylation characterized by a prominent substrate with an apparent molecular mass of 145 kDa (Fig. 7*a*). Other substrates were observed with apparent molecular masses ranging from 17 to 80 kDa. The pattern of phosphorylation was specific, since the addition of the combination of purified PKC and its activator, PMA, resulted in a different phosphorylation pattern.

The phosphorylation of components of the TCR/CD3 complex was monitored by analysing the incorporation of ^{32}P from [γ-^{32}P]ATP into detergent-solubilized proteins immunoprecipitated with 2C11 MAb. While permeabilized cells treated with PKC and PMA showed phosphorylation of 25 kDa and 20 kDa substrates, representing the murine ϵ- and γ-chains of the CD3 complex [2], no effect of PKA was observed (Fig. 7*b*). Similar results were observed for 2C11 MAb-immunoprecipitates from $^{32}PO_4^-$-labelled intact cells treated with forskolin or PGE_2 (results not shown). Anti-PLC-γ1 Ab precipitated a phosphorylated protein with an apparent molecular mass of 145 kDa, corresponding to that of PLC-γ1, from permeabilized cells treated with PKA (Fig. 7*c*). Additional phosphorylated substrates with apparent molecular masses of approx. 90 and 46 kDa were co-precipitated. The nature of these proteins is unknown. Similar phosphorylated proteins were co-precipitated by anti-PLC-γ Ab from C_6Bu1 glioma cells treated with cAMP or cAMP inducers [32]. Under these experimental conditions no phosphorylation of PLC was observed after treatment with purified PKC. Thus cAMP/PKA-mediated inhibition of TCR/CD3-induced InsPL hydrolysis is associated with the phosphorylation of PLC-γ1.

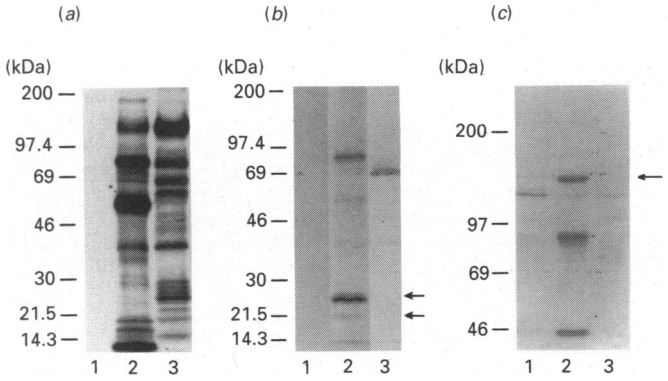

Fig. 7. Effect of treatment with purified protein kinases on the phosphorylation of cell proteins in permeabilized 8-5-5 lymphocytes

Cells (3×10^7/ml) were resuspended in the potassium glutamate/Hepes containing 500 μM-ATP and permeabilized with tetanolysin for 5 min at 37 °C. Cells to be treated with purified PKC were permeabilized in the presence of 100 nM-PMA. After permeabilization, cells were incubated for 10 min at 37 °C with 0.5 mCi of [γ-^{32}P]ATP/ml in the presence or absence of the indicated purified enzymes (150 units/ml): lane 1, no enzyme; lane 2, PKC; lane 3, PKA. The reaction was terminated by washing the permeabilized cells as described in the Materials and methods section. Cell lysates were obtained and the solubilized material was analysed by SDS/PAGE (8–16% gradient gel, *a*). Immunoprecipitation was performed on the cell lysates by using 2C11 MAb (*b*) or anti-PLC-γ1 Ab (*c*) and the phosphorylated proteins were analysed by SDS/PAGE [8–16% gradient gel (*b*) or 8% gel (*c*)]. The arrows in (*b*) point to the location of the 25 kDa and 20 kDa phosphorylated proteins, representing the murine ϵ- and γ-chains of the CD3 complex. The 90 kDa and 70 kDa bands seen after PKC or PKA treatment respectively were not identified. The location of PLC-γ1 in (*c*) is also indicated. The 90 kDa and 46 kDa phosphorylated proteins observed after PKA treatment were not identified.

DISCUSSION

The present study characterizes the cAMP-mediated inhibition of InsPL hydrolysis in a model employing intact and permeabilized Th2 lymphocytes. Treatment with agents that increase cAMP production by different mechanisms led to a dose-dependent reduction in InsPL hydrolysis in 8-5-5 lymphocytes. The inhibitory effect required pretreatment and showed a time-dependence that resembled that for cAMP production. In addition, inhibition was induced by 8-Br-cAMP, a cell-permeating cAMP analogue. To further characterize this phenomenon, we have used permeabilized cells which maintain their ability to respond to cAMP inducers or TCR/CD3 perturbation with cAMP production or InsPL hydrolysis respectively. cAMP-mediated inhibition was observed upon treatment of intact, but not permeabilized, 8-5-5 cells, suggesting a requirement for (a) soluble factor(s). The requirement for an intact cytoplasmic environment was further suggested by the observation that pretreatment of intact 8-5-5 cells with cAMP inducers followed by permeabilization maintained the inhibition of TCR/CD3-induced PLC activation, albeit to a lesser degree. Addition of purified PKA to permeabilized cells reconstituted the inhibitory effect of cAMP on TCR/CD3-induced InsPL hydrolysis.

GTP[S]-induced InsPL hydrolysis was not affected, suggesting that cAMP differentiated between TCR/CD3- and G-protein-mediated PLC activation. The distinct effects of cAMP/PKA on TCR/CD3- and GTP[S]-induced InsPL hydrolysis may imply that GTP[S] and CD3 perturbation activate different phospholipases. However, the possibility that the same enzyme may be activated by distinct mechanisms cannot be completely ruled out based on the present data. The absence of an effect on GTP[S]-induced InsPL hydrolysis under conditions where TCR/CD3-induced inositol phosphate accumulation was inhibited argues against increased inositol phosphate catabolism to account for the effect of cAMP/PKA activation. Furthermore, all our incubations were carried out in the presence of 10 mM-LiCl, an inhibitor of inositol phosphate phosphatases [40].

A salient finding of our work was that the inhibitory effect of cAMP or its inducers was lost upon permeabilization, but could be partially or totally reconstituted by treatment with purified PKA alone or in combination with a protein serine/threonine phosphatase inhibitor. It is likely that in permeabilized cells protein phosphatases may limit the effect of kinase activation; in fact, the partial inhibition of CD3-induced inositol phosphate accumulation in permeabilized cells observed after treatment of intact cells with cAMP inducers could be completely maintained if the inhibitor okadaic acid was also included (results not shown). The molecular mass of the tetrameric form of PKA (around 180 kDa) [38] is compatible with the possibility of its escaping through the pores induced by tetanolysin [27]. Since the catalytic subunit of PKA was an effective inhibitor in permeabilized cells, PKA-mediated phosphorylation of membrane-bound protein substrate(s) was considered as the likely inhibitory mechanism. This was supported by the observation that addition of purified PKA to permeabilized cells was associated with the phosphorylation of specific cell substrates, including PLC-γ1.

The inhibition by PKA of early events involved in signal transduction, such as induction of InsPL hydrolysis, may represent a form of heterologous desensitization of the TCR/CD3 complex which leads to uncoupling from PLC. This mechanism plays a role in desensitizing other receptors [41]. However, phosphorylation of components of the TCR/CD3 complex by PKA was not observed. Nevertheless, phosphorylation of PLC may also lead to functional deactivation of the pathway triggered by TCR/CD3 perturbation, by affecting either its activity or its coupling to the receptor. Since PKA-mediated phosphorylation of PLC-γ in C_6Bu1 glioma cells was not associated with decreased hydrolytic activity *in vitro* [32], the latter hypothesis is more likely to be correct. Although evidence [12–14,42–44] has suggested the involvement of a putative G-protein in TCR/CD3 coupling to PLC, more recent studies have focused on the importance of tyrosine kinases in T cell activation [9–11], and their role in the stimulation of PLC-γ isoenzymes [9,11,45]. Recent data suggesting that forskolin treatment of Jurkat cells decreases the level of tyrosine phosphorylation of PLC-γ1 induced by CD3 perturbation [46] are in agreement with our findings. One may speculate that PKA-phosphorylated PLC-γ1 may not be a suitable substrate for TCR/CD3-coupled tyrosine kinases. Permeabilized lymphocytes constitute an excellent model for testing this hypothesis.

The mechanism by which increased levels of cAMP and PKA activation affect lymphocytes at the functional level has been the subject of several studies, which have attempted to discriminate between effects at early or late stages of cell activation. Lingk *et al.* [47] have obtained evidence favouring an effect at distal levels, arguing that Ca^{2+} mobilization in response to phytohaemagglutinin was not affected in human T lymphocytes, whereas the proliferative response was substantially abrogated. These data are in contrast with those of other investigators, who have suggested that pharmacological activation of PKC and/or Ca^{2+} mobilization bypasses the cAMP-induced inhibition of human T cell proliferation or CTL effector function [19,20]. The different cellular models utilized in these studies makes it difficult to reconcile these contrasting results. Biological and biochemical differences between transformed and normal T lymphocytes or between T lymphocyte subclasses have now become apparent. Muñoz *et al.* [48] described the lack of sensitivity of Th2,

compared with Th1, lymphocytes with respect to cAMP inhibition of proliferation, lymphokine production and receptor or photo-oncogene expression. Together with these data, our findings suggest that late events may be dissociated from the down-modulation of PLC activation by cAMP described here. It is likely that both early and late events may be affected by cAMP, with different functional consequences.

We thank Dr. M. Brunswick, Dr. J. Finlayson and Dr. K. B. Seamon for reviewing the manuscript and for helpful discussion, and Dr. S. G. Rhee for the gift of anti-PLC-γ1 MAbs.

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Received 29 July 1991/28 November 1991; accepted 13 December 1991