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The antigen receptors on mature and immature T lymphocytes are coupled to phosphatidylcholine-specific phospholipase D activation

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SUMMARY

Phosphatidylcholine–phospholipase D has been proposed to play a key role in the transduction of the proliferative responses of a wide range of mitogens and growth factors. We now report that the antigen receptors on T lymphocytes derived from human tonsillar or murine splenic preparations are coupled to phosphatidylcholine (PtdCho)–phospholipase D (PLD) activation following stimulation of these T cells with anti-CD3 antibodies. However, since we also demonstrate that the antigen receptors on murine thymocytes are coupled to PtdCho–PLD activation, we propose that it is unlikely that this PLD pathway plays a central role in the transduction of T-cell proliferative responses, but rather, may be involved in either driving cells into cycle or maintaining cell cycle progression, processes required both for proliferation and activation-induced cell death. Whilst the molecular mechanisms underlying T-cell receptor (TCR)-coupling to PtdCho–PLD activation in these cells have not been fully defined, kinetics studies and experiments using pharmacological inhibitors of protein tyrosine phosphatases (PTPases) and reconstituting CD3-coupled PtdCho–PLD activity in streptolysin-O permeabilized cells, suggest that the TCR/CD3 complex, under optimal conditions of activation, may be predominantly coupled to PtdCho–PLD activation downstream of tyrosine phosphorylation of phospholipase C-γ (PLC-γ), phosphatidylinositol (PtdIns)P_2 hydrolysis, calcium mobilization and protein kinase C (PKC) activation.

INTRODUCTION

Ligation of the antigen receptors on T cells (TCR) results in the activation of TCR-associated protein tyrosine kinases (PTKs) and the rapid tyrosyl phosphorylation, recruitment and subsequent activation of key signal transducing systems such as the phosphatidylinositol-4,5-bisphosphate (PtdInsP_2)-specific phospholipase C-γ (PLC-γ). PLC-mediated hydrolysis of PtdInsP_2 generates the intracellular second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), leading to the release of calcium from intracellular stores and the transient activation of protein kinase C (PKC). Downstream signalling events reported, include the recruitment of the phosphatidylinositol-3-kinase (PI-3-K) and activation of the Ras and MAP-kinase signalling cascades leading ultimately to the activation of transcription factors regulating the growth and proliferation of the cell (reviewed in refs. 1, 2).

However, T-cell activation, via the TCR, has been shown to occur in the absence of PtdInsP_2–PLC hydrolysis[3,4] a finding consistent with the growing body of evidence that PLC-mediated hydrolysis of PtdInsP_2 may simply play a role in the transduction of early activation signals such as Na^+/H^+ exchange and induction of c-fos, c-myc and c-jun, but is not sufficient, or even necessary, to stimulate or maintain cell proliferation.[5–8] In contrast, phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho) has been implicated as playing a key role in proliferative responses of a wide range of mitogens and growth factors.[5–8] Hydrolysis of PtdCho by PLD yields phosphatidic acid (PtdOH) and choline; PtdOH, which has been shown to be an important lipid second messenger by its capacity to modulate PKC and Ras activation, can also be converted to yield other biologically active lipids such DAG (by PtdOH phosphohydrolase)[5–10] or the putative mitogen, lysophosphatidic acid which are capable of sustained PKC activation or initiating further signal transduction pathways.[5–10] Interestingly, the TCR has recently been shown to be coupled to PLD activation in the leukaemic T cell line, Jurkat, resulting in the induction of the transcription factor AP-1, a signalling event involved in the regulation of growth and proliferation of cells.[11–13]

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Abbreviations: PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12,13 myristate acetate; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PTK, protein tyrosine kinase; TCR, T cell antigen receptor.

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We now report that the antigen receptors on T lymphocytes derived from human tonsillar or murine splenic preparations are also coupled to PtdCho–PLD activation following stimulation of these T cells with anti-CD3 antibodies. In addition, we also demonstrate that the antigen receptors on murine thymocytes are coupled to PtdCho–PLD activation. Kinetics studies and experiments using pharmacological inhibitors of protein tyrosine phosphatases (PTPases) and reconstituting CD3-coupled PtdCho–PLD activity in streptolysin-O permeabilized cells suggest that the TCR/CD3 complex may be predominantly coupled to PtdCho–PLD activation downstream of tyrosine phosphorylation of PLC-γ, $PtdInsP_2$ hydrolysis, calcium mobilization and PKC activation in manner analogous to that described for the platelet derived growth factor (PDGF) receptor.[14,15]

MATERIALS AND METHODS

Reagents and cell lines

The human T lymphocyte cell line, Jurkat (J6) was maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 5% (v/v) heat inactivated fetal calf serum (FCS) at 37° in 5% CO_2/95% humidified air (all media and supplements were purchased from Gibco BRL, Paisley, UK). Antibodies from the hamster anti-mouse CD3-ε hybridoma, 145-2C11 and the mouse anti-human CD3 hybridoma, OKT3 were purified from culture supernatants by protein-A sepharose chromatography as before.[16,17]

Guanosine triphosphate (GTP)γS and guanosine diphosphate (GDP)βS were purchased from BCL, Lewes, UK. Reduced streptolysin-O (SL-O) was from Wellcome Diagnostics, Beckenham, UK. Affinity purified phytohaemagglutinin (PHA-P), lectin from *Phaseolus vulgaris* was obtained from Sigma. Sheep red blood cells were obtained from Scottish Antibody Production Unit (SAPU), Carluke, UK. Radiolabels including [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (spec. act. 213 Ci/mmol), [^{3}H]myo-inositol (spec. act. 80–120 Ci/mmol), [9,10(*n*)-^{3}H] myristic acid (spec. act. 49 Ci/mmol), [9,10(*n*)-^{3}H] oleic acid (spec. act. 10 Ci/mmol) and [1-^{14}C]stearate (60–62 mCi/mmol) were purchased from Amersham International, Amersham, UK, while [9,10(*n*)-^{3}H]palmitic acid (spec. act. 39 Ci/mmol) was purchased from NEN-Dupont (Stevenage, Herts, UK). All other reagents were obtained from Sigma (Poole, UK).

Pervanadate (5 mM) was produced by addition of H_2O_2 (final concentration of 1 mM) to sodium orthovanadate diluted in RPMI-1640 media (5 mM), followed by incubation at 22° for 15 min. Residual H_2O_2 was removed by addition of catalase (200 μg/ml). Control solutions were similarly prepared using only H_2O_2 and catalase.

Purification of T cells and thymocytes

Murine T cells and thymocytes were prepared as described previously.[18,19] Briefly, spleens from male BALB/c mice (12 weeks old) or thymic tissue from 4-week-old BALB/c mice were dispersed through stainless steel mesh. Depletion of red blood cells (RBC) was achieved by centrifugation through a Histopaque-1077 cushion (400 *g* for 15 min). B cells were removed from splenic preparations by two rounds of 'panning' with goat anti-mouse immunoglobulin antibodies (Sigma). T cell or thymocyte preparations were then further purified by centrifugation on discontinuous percoll gradients (85%/75%/50%) at 1200 *g* for 15 min.

T cells were prepared from tonsils obtained from routine surgery as described.[20] Cells were dispersed through mesh and centrifuged through a Histopaque 1077 cushion (350 *g* for 15 min) to deplete RBC. Mononuclear cells were pelleted with 2-aminoethyl-isothiouronium bromide sheep red blood cells (AET-SRBC) (for 5 min at 90 *g*) to produce a T-cell enriched preparation. FCS (1 ml) was layered onto the pellet, followed by a 30 min incubation on ice before centrifugation through a Histopaque cushion (350 *g*, 20 mins at room temperature). SRBC were lysed by addition of ammonium chloride (0·144 M) for 2 min and the resulting T-cell preparation further purified by Percoll discontinuous gradients.

Resting (harvested from 75/85% percoll interface) and '*in vivo* activated' (harvested from 75/50% percoll interface) murine thymocytes and splenic lymphocytes were stained with Quantum Red streptavidin (Sigma)-biotinylated anti-murine CD3 antibody (1 μg/ml), fluoroscein isothiocyanate (FITC)-labelled anti-murine CD4 antibody (1 μg/ml), phycoerythrin (PE)-labelled anti-murine CD8α antibody (1 μg/ml) (all obtained from Pharmingen, Cambridge Bioscience, Cambridge, UK) and FITC-labelled anti-murine immunoglobulin antibody (1 μg/ml) (Sigma) for analysis of thymocyte (>70% $CD4^+$ $CD8^+$) and T lymphocyte (>85% $CD3^+$) populations, respectively, by fluorescence-activated cell sorting (Facscan; Becton-Dickenson, Sunnyvale, CA). Enriched human tonsillar T cell (>95% $CD3^+$) populations were analysed by flow cytometry using FITC-labelled anti-human CD19 (pan-B cell marker) and PE-labelled anti-human CD3 monoclonal antibodies (mAbs) from Pharmingen, Cambridge Bioscience, Cambridge, UK.

Measurement of phospholipase D (PLD) activity

Phospholipase D (PLD) activity was measured by the transphosphatidylation assay as described previously.[5–15] Briefly, lymphocytes were resuspended in RPMI-1640 medium containing 5% (v/v) dialysed FCS (5×10^7 cells/ml) and labelled with [^{3}H]palmitic acid (1–2·5 μCi/10^6 cells) for 4 hr at 37°. Jurkat T cells were prelabelled (10^6 cells/ml) with [^{3}H]palmitic acid (5 μCi/ml) overnight. Preliminary studies demonstrated that [^{3}H]palmitate, [^{3}H]myristate, [^{3}H]oleate and [^{3}H]arachidonate were all highly preferentially incorporated into PtdCho indicating that [^{3}H]PtdBut (phosphatidylbutanol) species generated from such [^{3}H]fatty acid-labelled lymphocytes were derived from PtdCho. Following labelling, the cells were washed in RPMI-1640/10 mM HEPES/0·1% bovine serum albumin (BSA) (RHB medium), resuspended at 2×10^7 cells/ml and incubated at 37° for 15 mins in RHB medium containing butan-1-ol (0·3% final). Reactions (total volume 150 μl; 0·3% butan-1-ol, final concentration) were initiated by the addition of cells (100 μl) to stimuli in glass trident vials. Reactions were terminated by the addition of 0·75 ml chloroform : methanol (1:2 v/v). Samples were then extracted on ice for 10 min. Phase separation was achieved by the addition of 0·25 ml chloroform and 0·3 ml water followed by vortexing and centrifugation at 270 *g* for 5 mins. The lower organic phase was removed, dried down under vacuum (Jouan RC1022; Jouan Ltd, Tring, Herts, UK), and the samples redissolved in 25 μl chloroform: methanol (19:1 v/v),

containing 40 μg unlabelled phosphatidylbutanol (Lipid Products, South Nutfield, UK) as standard, and applied to pre-run, heat activated thin-layer chromatography (TLC) plates (20 × 20 cm, Silica gel 150 A grooved plates, Whatman, Kent, UK). The plates were developed in the organic phase of the solvent, ethyl acetate:2,2,4 trimethylpentane:acetic acid:water (11:5:2:10) for ≈90 min and the position of the phosphatidylbutanol product detected using iodine vapour. [^{3}H]PtdBut-containing silica indicated by the phosphatidylbutanol standard was then scraped into scintillation fluid and counted. All results were expressed as the mean of triplicate samples ± sem from single assays representative of at least three independent experiments.

Assay of PLD activity in permeabilized T cells

Briefly, Jurkat cells labelled with [^{3}H]palmitic acid as described above, were washed three times in 20 mM PIPES buffer, pH 6·8 containing 140 mM potassium glutamate, 2 mM $MgCl_2$, 5·6 mM glucose, 15 μM egtazic acid (EGTA), 1 mg/ml fatty-acid free BSA (permeabilization buffer). The cells were resuspended (10^7 cells/ml) in permeabilization buffer containing 5 mM MgATP, 1 mM phenylmethyl sulphonyl fluoride (PMSF), CLAP (1 μg/ml chymostatin, leupeptin, antipain and pepstatin A) and 0·3% butan-1-ol, permeabilized with streptolysin-O (0·4 IU/ml) for 2·5 mins at 37° and then stimulated with calcium-EGTA buffers (pCa6 or pCa7), anti-CD3 (OKT3, 1 μg/ml), 100 μM GTPγS and/or 1 mM GDPβS for the time indicated (butan-1-ol was maintained at a final concentration of 0·3% throughout all manipulations). Reactions were quenched by a two-phase separation and processed as described above.

Assay of inositol phosphate release

Jurkat cells were resuspended (10^6cells/ml) in F-10 (Ham) medium containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5% dialysed FCS and labelled with myo-[^{3}H]-inositol (1 μCi/10^6 cells) for 16 hr at 37°. The cells were washed three times and resuspended (at $1–3 \times 10^7$ cells/ml) in RHB medium, pH 7·4. Reactions were initiated by addition of cells (90 μl) to 10× stimuli (10 μl) in glass trident vials and terminated by addition of 0·94 ml chloroform:methanol (1:2 v/v) and extraction of samples on ice. Phase separation was achieved by addition of 0·31 ml chloroform and 0·31 ml water, vortexing and centrifugation. Levels of [^{3}H]inositol trisphosphate ($InsP_3$) or total [^{3}H]inositol phosphates (reaction mixture containing 10 mM LiCl) were determined by liquid scintillation counting of fractions eluted following Dowex (formate form) ion-exchange chromatography of the aqueous phase as described previously.[18]

RESULTS

The antigen receptors are coupled to PtdCho–PLD activation in immature and mature T cells

Stimulation of resting (small, high density) or '*in vivo*' activated (large, low density) human tonsillar or murine splenic T cells with anti-CD3 antibodies (human OKT3; murine 145-2C11) or the TCR-directed mitogenic lectin, PHA (PHA-P) showed that the antigen receptors were coupled to PtdCho–PLD in normal primary T cells (Fig. 1a and b and results not shown). Moreover, anti-CD3-mediated stimulation of murine thymocytes also results in PtdCho–PLD activition and [^{3}H]PtdBut formation (Fig. 1c).

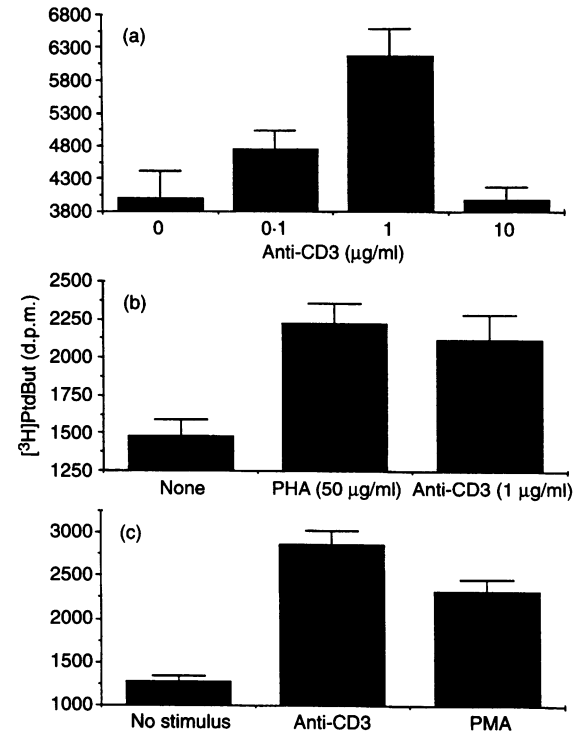

Figure 1. CD3 is coupled to PtdCho–PLD activation in human tonsillar, murine splenic T cells and murine thymocytes. (a) '*in vivo* activated' human tonsillar T cells, prelabelled with [^{3}H]palmitate, were stimulated with media alone or anti-CD3 at the concentrations (μg/ml) indicated for 30 min and (b) '*in vivo* activated' murine splenic T cells, prelabelled with [^{3}H]palmitate, were stimulated with anti-CD3 (1 μg/ml) or PHA (50 μg/ml) for 30 min. (c) murine thymocytes, prelabelled with [^{3}H]palmitate, were stimulated with anti-CD3 (1 μg/ml), PMA (1 μg/ml) or media alone for 60 min. [^{3}H]PtdBut was measured as described in the Materials and Methods.

Kinetics of the coupling of the TCR to PtdCho–PLD activation

Whilst significant stimulation of PtdCho–PLD activity can be detected at concentrations of anti-CD3 as low as 0·001 μg/ml, maximal stimulation of PtdCho–PLD activity in mature or Jurkat T cells occurs at concentrations of anti-CD3 of ≈0·1–1 μg/ml (Fig. 1, Fig. 2a,c). Higher concentrations of anti-CD3 are inhibitory not only to Jurkat T cells (maximum response typically inhibited by some 40%) but particularly, to freshly isolated T-cell preparations (maximum response typically inhibited by some 80–90%), indicating receptor desensitization/uncoupling from this pathway at high concentrations of receptor ligand (Figs 1 and 2). In contrast, significant generation of [^{3}H]InsPs, and hence activation of $PtdInsP_2$–PLC, can only be detected following stimulation with 0·1–1 μg/ml anti-CD3 (Fig. 2b,c). Moreover, stimulation of [^{3}H]InsPs does not reach maximal levels, even at concentrations of anti-CD3 as high as 10 μg/ml (Fig. 2b,c). These results demonstrate that PtdCho–PLD activity in T cells may become activated at much lower concentrations of antigen than that required for stimulation of $PtdInsP_2$–PLC, suggesting that one or more PLD pathways are likely to be activated in the absence of $PtdInsP_2$

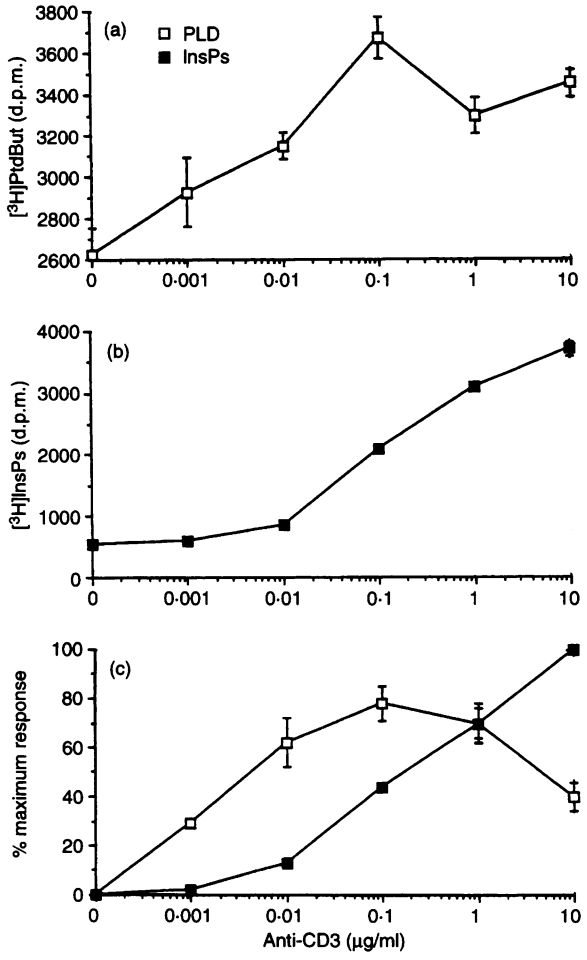

Figure 2. The antigen receptors on Jurkat T cells are differentially coupled to PtdCho–PLD and PtdInsP$_2$-PLC activation. Jurkat cells prelabelled overnight with [^{3}H]-palmitic acid (a,c) or myo-[^{3}H] inositol (b,c) were incubated with the indicated concentration of anti-CD3 for 15 min at 37°. [^{3}H]PtdBut and total [^{3}H]InsPs were measured as described in the methods section. In (c), the relative concentration dependence of CD3-coupling to PtdCho–PLD and PtdInsP$_2$-PLC activation is determined from the pooled data from a number of experiments. Results are presented as mean values ± SEM where n = 5 independent experiments for [^{3}H]PtdBut and n = 4 independent experiments for [^{3}H]InsPs, respectively.

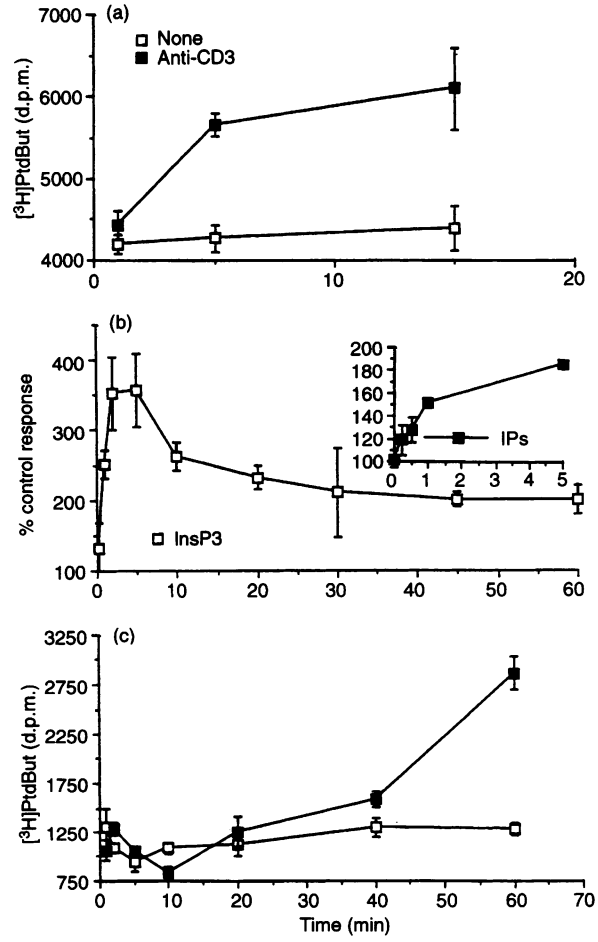

Figure 3. Time dependence of CD3 coupling to PtdCho–PLD activation in T cells and thymocytes. Jurkat cells prelabelled overnight either with [^{3}H]-palmitic acid (a) or myo-[^{3}H] inositol (b) were incubated with media alone or anti-CD3 (1 μg/ml) for the indicated time at 37°. In (c), thymocytes prelabelled with [^{3}H]palmitic acid were incubated with media alone or anti-CD3 (145–2C11, 1 μg/ml) for the indicated time. [^{3}H]PtdBut (a,c), [^{3}H]InsP$_3$ (b) and total [^{3}H]InsPs (b, insert) were measured as described in Materials and Methods.

hydrolysis and may play a role in initiating T-cell activation and/or cell cycle progression.

In mature or Jurkat T cells PtdCho–PLD activation in response to optimally stimulatory concentrations (0·1–1 μg/ml) of anti-CD3 antibodies is only detectable after 1–5 min (approx 140% control response) and maximal by 15 min (Fig. 3a and results not shown). However, stimulation of [^{3}H]inositol phosphate (IP$_3$) generation is substantial by 1 min (approx 250% of the control response) and maximal (≈350% of control response) by 2 min (Fig. 3b; main figure). Moreover, substantial stimulation (approximately 150% control response) of total [^{3}H]InsPs can also be detected within 1 min of stimulation via the TCR by the LiCl-accumulation assay. (Fig. 3b, insert). Taken together, these results suggest that some PtdCho–PLD activation occurs after, and may be dependent on, CD3-coupled hydrolysis of PtdInsP$_2$ and subsequent PKC activation. In contrast to the relatively rapid kinetics observed in mature and Jurkat T cells (maximal within 10–15 min), anti-CD3 significantly stimulates PtdCho–PLD in thymocytes only after a lag of some 20–30 min with substantial accumulation of [^{3}H]PtdBut occurring by 60 min (Fig. 3c).

The precise substrate(s) of CD3- or PKC-stimulated PtdCho–PLD(s) in Jurkat T cells have not, as yet, been defined, but experiments investigating [^{3}H]PtdBut generation from phosphatidylcholine pools prelabelled with [^{3}H]palmitate, [^{3}H]oleate and [^{3}H]myristate-labelled fatty acids (Fig. 4a) failed to show preferential hydrolysis of any such radiolabelled-phosphatidylcholine species following ligation of the TCR/CD3 complex, or via direct activation of PKC by PMA, in Jurkat T cells. In contrast, anti-CD3 or phorbol myristate (PMA) could not induce significant levels of [^{3}H]PtdBut generation from [^{3}H]arachidonate-labelled cells, despite high incorporation of [^{3}H]arachidonic acid into phosphatidylcholine in Jurkat cells (results not shown)

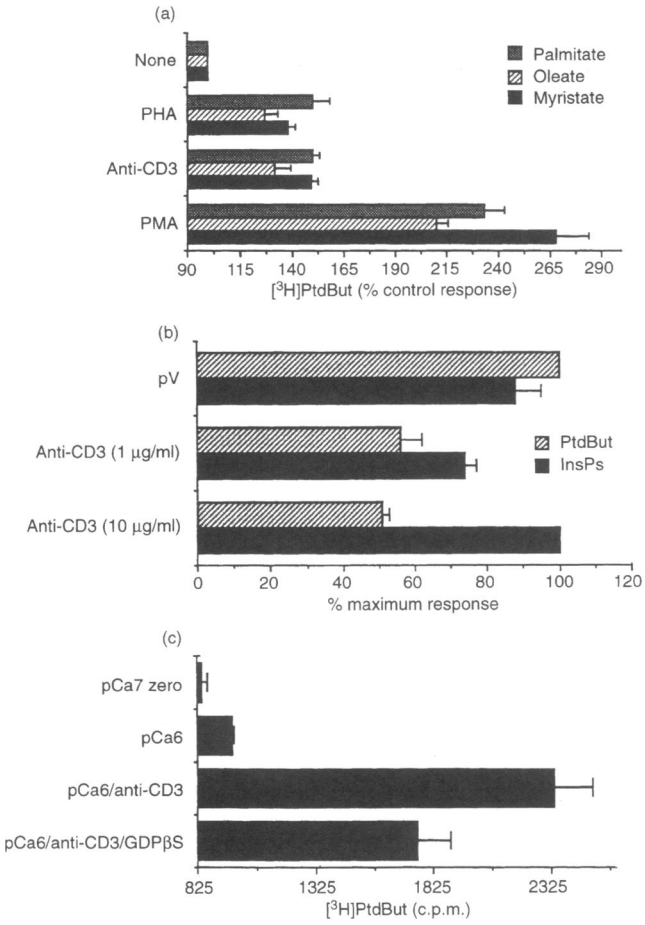

Figure 4. Characterization of TCR-coupling to PtdCho–PLD activation. In (a), Jurkat cells prelabelled overnight with [^{3}H]-palmitate, [^{3}H]oleate or [^{3}H]myristate were incubated with media alone, PHA (50 μg/ml), anti-CD3 (1 μg/ml) or PMA (1 μg/ml) for 7 min at 37°. Control values (d.p.m.) of [^{3}H]PtdBut formation in Jurkat cells incubated with media alone were: [^{3}H]palmitate, 1384±116; [^{3}H]oleate, 1028±118 and [^{3}H]myristate, 1228±85. In (b), Jurkat cells prelabelled overnight either with [^{3}H]-palmitic acid or myo-[^{3}H] inositol were incubated with pervanadate (pV, 0·5 mM) or anti-CD3 for 15 min at 37°. In (c) [^{3}H]palmitate-labelled, SL-O permeabilized Jurkat T cells were stimulated at pCa 7 (100 nM) or pCa 6 (1 μM) with buffers alone, anti-CD3 (1 μg/ml) or anti-CD3 plus GDPβS (1 mM) as indicated, for 1 min at 37°. [^{3}H]PtdBut and total [^{3}H]InsPs were measured as described in Materials and Methods.

suggesting that arachidonyl–PtdCho is not a substrate for PtdCho–PLD(s) in Jurkat T cells. Interestingly, a recent study investigating TCR-coupling to PtdCho–PLD in HPB-ALL T cells[21] showed that although [^{3}H]arachidonyl–PtdOH is generated following ligation of the TCR on [^{3}H]arachidonate-labelled HPB-ALL T cells, this PtdOH is not the result of TCR-coupling to a PLD activity: rather, such PtdOH appears to be produced by DAG kinase-conversion of DAG species resulting from coupling of the TCR to a phosphoinositide–PLC pathway(s).[21] Taken together these findings may have interesting implications concerning the role for PtdCho–PLD in T cell activation as only the steroyl–arachidonyl-species of PtdOH has been shown to prolong Ras activation by inhibiting the conversion of activated GTP-bound Ras to its inactive GDP-bound form.[5–10]

Analysis of TCR coupling to PtdCho–PLD activation

Treatment with pervanadate (0·5 mM), a compound which has been shown to stimulate early PTK-mediated signalling events in T cells by inhibiting tyrosine phosphatase (PTPase) activity,[22] stimulated [^{3}H]InsPs and [^{3}H]PtdBut generation in Jurkat cells (Fig. 4b); these results indicated that one, or more, PLD pathways in T cells are likely to be under the control of a PTK–PTPase regulatory cycle.

Further evidence of PTK-mediated coupling was provided by *in vitro* studies which showed that CD3-mediated PtdCho–PLD activation was not disrupted in streptolysin-O (SLO)-permeabilized Jurkat cells (Fig. 4c and results not shown), an approach used to demonstrate that the antigen receptors on T cells were coupled to InsPs generation in a PTK-dependent manner.[18] For example, anti-CD3 (1 μg/ml) ligation of the antigen receptors on permeabilized Jurkat cells increased the PtdCho–PLD activity observed at resting levels of calcium (100 nM; pCa7) within 1 min following stimulation (136±20%). However, whilst addition of the poorly hydrolysable GTP analogue, GTPγS could also induce activation of PtdCho–PLD at pCa7 (162±15%), suggesting that one or more G-protein-regulated PLD activities are expressed in Jurkat T cells, costimulation with anti-CD3 and GTPγS did not induce an enhanced PLD response (135±7%) relative to that observed with these stimuli, individually. Moreover, costimulation with the G-protein antagonist, GDPβS did not block this anti-CD3-stimulated PtdCho–PLD activation. Taken together, these results suggest that, at resting levels of calcium, anti-CD3-mediated coupling to PtdCho–PLD involves one or more PTKs and is independent of G-protein regulation.

Following ligation of the antigen receptors on T cells, the consequent generation of $InsP_3$ will rapidly induce a rise in intracellular calcium to micromolar levels (pCa6) in intact cells. Thus, as the kinetics of PtdCho–PLD activation at optimally stimulatory concentrations of anti-CD3 were suggestive of such PLD activation being downstream of TCR–$PtdInsP_2$–PLC coupling, reconstitution of CD3-stimulated PtdCho–PLD activity in SL-O permeabilized Jurkat T cells was also investigated at μmolar calcium concentrations (Fig. 4c and results not shown). Indeed, elevation of free calcium to the micromolar range was found to be sufficient to induce a 2·5-fold increase in PtdCho–PLD activation over a 30 min time period (results not shown), a finding consistent with the calcium-induced activation of PtdCho–PLD reported for other cell types [reviewed in ref. 6]. More importantly, costimulation of permeabilized Jurkat cells with anti-CD3 and μmolar levels of calcium was found to induce a strong synergistic activation of PtdCho–PLD which could not be enhanced by addition of exogeneous GTP analogues. However, CD3-coupled PtdCho–PLD activation could be partially abrogated (30–35%) by costimulation with GDPβS (Fig. 4c and results not shown).

DISCUSSION

It has recently been reported that the antigen receptors on Jurkat T cells are coupled to PLD activation[11–13] and that such CD3-mediated PtdCho–PLD activity may play a role in the activation of the transcription factor, AP-1, an event linked

to T-cell activation.[13] We now report that PtdCho–PLD can also be activated in T lymphocytes derived from human tonsillar or murine splenic preparations following stimulation of these T cells with similar concentrations of anti-CD3 antibodies (Fig. 1), results consistent with a role for PtdCho–PLD in T-cell activation. We have, however, found that the antigen receptors on murine thymocytes, the vast majority of which are programmed for cell death by apoptosis, are also coupled PtdCho–PLD activation (Fig. 1), perhaps suggesting that PtdCho–PLD is unlikely to play a central role in the transduction of T cell proliferative responses but rather, may be involved in either driving cells into cycle or maintaining cell cycle progression, processes required both for proliferation and activation-induced cell death. However, in contrast to the relatively rapid and biphasic kinetics observed in mature and Jurkat T cells (early phase maximal within $\approx$10 min and the second, later phase sustained for at least 60 min, ref. 11 and our results not shown), anti-CD3 significantly stimulates PtdCho–PLD in thymocytes only after a lag of some 20–30 min with substantial accumulation of [^{3}H]PtdBut occurring by 60 min (Fig. 3c). These differential kinetics of CD3-coupled PtdCho–PLD activation observed in mature T cells and murine thymocytes could therefore possibly reflect uncoupling of the antigen receptors from one or more activation-associated PtdCho–PLD pathways in immature T cells. Corroborative evidence, for such differential coupling of the TCR to PtdCho–PLD pathways in a maturation-dependent manner, may also be provided by the finding that the TCR is apparently uncoupled from PLD in HPB-ALL T cells, at least at early time points (<15 min) following ligation of the TCR.[21]

Distinct PtdCho–PLD subtypes have been identified in mammalian cells which appear to differ in their coupling to cell surface receptors, subcellular localization and phospholipid substrate specificities.[5–15] Moreover, recent evidence from *in vitro* systems has suggested the existence of at least two major classes of PtdCho–PLD activity differing in their cofactor requirements: these comprise the oleate-dependent PLD(s) and the PtdInsP_2-stimulated G-protein-dependent (ARF or Rho) enzymes.[5–15] Although the mechanisms underlying receptor coupling to these isoforms have not been defined, it has been suggested that PtdCho–PLD activation in the PDGF receptor system is downstream of PDGF-mediated tyrosine phosphorylation of PLC-γ, PtdInsP_2 hydrolysis and PKC activation.[13,14] Since the activation of the TCR/CD3 complex is predominantly regulated by *src*-related PTKs, and TCR-coupling to PtdCho–PLD has previously been shown to be abrogated by PTK and/or PKC inhibitors,[11–13] we initially considered it likely that this receptor system would be coupled in a manner analogous to this PTK–PLC-γ-dependent system. However, following a combination of kinetic, reconstitution and inhibitor studies a more complicated picture of TCR/CD3-coupling to PtdCho–PLD activation has emerged, suggesting that the TCR may be coupled to multiple PLD activities with distinct regulatory mechanisms.

Firstly, examination of the dose dependancy of CD3-mediated [^{3}H]PtdBut formation demonstrated that PtdCho–PLD was maximally activated following stimulation with anti-CD3 antibodies at concentrations of 0·1–1 μg/ml, a range suboptimal for activation of PtdInsP_2–PLC. Indeed, we found substantial activation of PtdCho–PLD activity at concentrations of anti-CD3 that did not induce detectable levels of InsPs generation, suggesting that at least one form of CD3-coupled PtdCho–PLD is stimulated independently of PtdInsP_2-hydrolysis and resultant calcium mobilization and PKC activity (Fig. 2). Further evidence for such a PTK-mediated activity is provided by our finding that CD3 is coupled to PtdCho–PLD activation even at resting levels of calcium (Fig. 4). However, kinetic, inhibitor and reconstitution studies have provided evidence that the majority of [^{3}H]Ptdbut formation occurring following ligation of the TCR with optimally stimulatory concentrations of anti-CD3 (1–10 μg/ml) is likely to occur downstream of PTK-dependent PtdInsP_2 hydrolysis, PKC activation and calcium mobilization. For example, reconstitution studies demonstrated that, at elevated levels of intracellular calcium (1 μM), anti-CD3 (1 μg/ml) appears to stimulate a much larger PLD activity. Further supporting evidence is provided by the temporal relationship of the kinetics of TCR-coupling to the PtdInsP_2–PLC and PtdCho–PLD pathways (Fig. 3) and our findings that the PTPase inhibitor, pervanadate and the PKC activator, PMA stimulates PtdCho–PLD activation. Thus our data confirm and extend earlier studies which showed that CD3-mediated [^{3}H]PtdBut formation is abrogated by PTK and PKC inhibitors, uncoupling from PtdInsP_2–PLC hydrolysis and down-regulation of PKC (refs. 11–13 and our results not shown), findings consistent with CD3-coupling to PtdCho–PLD being downstream of PTK-mediated activation of PIP$_2$–PLC-γ, calcium mobilization and PKC activation.

Finally, the finding that the CD3-stimulated PLD activity in permeabilized Jurkat T cells is not enhanced by GTPγS, but is partially abrogated by GDPβS suggested the possible involvement of a low molecular weight G-protein, such as Arf or Rho, but not a heterotrimeric G-protein, in TCR coupling to PtdCho–PLD at elevated levels of intracellular calcium. Interestingly, although Biffen *et al.*[21] found no compelling evidence of CD3-coupling to PtdCho–PLD in [^{3}H]arachidonate- or [^{32}P]-labelled HPB-ALL T cells, they clearly demonstrated a GTPγS-dependent PLD pathway similar to that reported here for Jurkat cells. Taken together with our finding that GDPβS only partially abrogates CD3-coupled PtdCho–PLD activation, these results raise the possibility that ligation of CD3, resulting in PKC activation and elevation of intracellular calcium, can stimulate the activation of more than one independently regulated PLD activity, possibly via differential activation of PKC isoforms in distinct subcellular compartments in a maturation stage-dependent manner.[5–14]

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