Guanine nucleotide regulation of inositol phospholipid hydrolysis and CD3-antigen phosphorylation in permeabilized T lymphocytes

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A method of membrane permeabilization of T lymphocytes with the bacterial cytotoxin streptolysin O has allowed the effect of guanine nucleotide analogues on phosphatidylinositol metabolism and protein kinase C (PKC) activation to be investigated. The data demonstrate that, in permeabilized cells, phosphorylation of the γ subunit of the CD3 antigen can be induced in response to the PKC activator phorbol 12,13-dibutyrate, the polyclonal mitogen phytohaemagglutinin (PHA) and the stimulatory guanine nucleotide analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]). Application of a pseudo-substrate inhibitor of PKC indicated that CD3 γ -chain phosphorylation induced in response to all three agonists was mediated by PKC. PHA and GTP[S] also stimulated inositol phospholipid turnover and inositol phosphate accumulation. The kinetics and concentration-dependence of PHA-induced inositol phospholipid hydrolysis correlated with PHA-induced CD3 γ phosphorylation, suggesting that PHA may regulate CD3 γ phosphorylation via diacylglycerol produced as a consequence of inositol phospholipid hydrolysis. However, there was an inconsistency in that PHA induced greater (> 200 %) levels of inositol phospholipid turnover than did GTP[S], but much weaker (< 50 %) levels of CD3-antigen phosphorylation. There was also a discrepancy between GTP[S] effects on phosphatidylinositol turnover and PKC activation, in that the half-maximal GTP[S] concentration for inositol phosphate production and CD3 γ -chain phosphorylation was 0.75 μ M and 75 μ M respectively. Moreover, 10 μ M-GTP[S] induced maximal inositol phosphate production, but only 10 % of maximal CD3 γ -chain phosphorylation. The data are consistent with the idea that other signal-transduction pathways, in addition to those involving inositol phosphate production, exist for the regulation of PKC in T lymphocytes.

INTRODUCTION

PKC plays an important role in T-lymphocyte activation [1,2] and has been implicated in diverse early cellular responses, including a role in phosphorylating the γ subunit of the antigen-receptor/CD3-antigen complex [3,4]. The physiological regulator of PKC is thought to be diacylglycerol, which exerts its stimulatory effect by increasing the enzyme's affinity for Ca^{2+} [5,6]. Although it is established that diacylglycerol is produced as a consequence of phospholipase C-mediated metabolism of phosphatidylinositols [7], examples of receptor stimulation of PKC in the absence of phosphatidylinositol hydrolysis [8–10] can be explained by the fact that diacylglycerol is also an intermediate in the cyclic re-synthesis of phosphatidylcholine. Thus diacylglycerol can be generated by the activities of phosphatidylcholine-specific phospholipase C and phospholipase D [11]. PKC may also be activated by the non-esterified-fatty-acid products of phospholipase A_2 action on membrane phospholipids [12]. These alternative pathways present the possibility of multiple intracellular mechanisms for the control of cellular PKC activity.

In many cells, G-proteins play a role in the coupling of receptors to intracellular effector proteins, e.g. adenylate cyclase, ion channels and phospholipase C [13,14]. In T lymphocytes, G-proteins have been implicated in the

control of ornithine decarboxylase activity and exocytotic secretion [15,16], but the role of G-proteins in the regulation of cellular PKC activity in T cells is not understood. AlF_4^- , a pharmacological stimulator of G-protein in intact cells, can induce phosphatidylinositol turnover and PKC activation in murine T cell hybridomas [17]. Also, in isolated T-cell membranes, guanine nucleotides can regulate the phosphorylation of the CD3 antigen, an established substrate for PKC-mediated phosphorylation [18]. Guanine nucleotides have been shown to regulate phospholipase C-mediated hydrolysis of phosphatidylinositol, and hence cellular levels of diacylglycerol, in murine thymocytes [19]. It is not established, however, that the products of inositol lipid metabolism are the sole regulators of PKC in T cells. In particular, cell-surface receptors that induce inositol phospholipid breakdown do not necessarily activate PKC in T lymphocytes [1]. Accordingly, the fact that G-proteins regulate phosphatidylinositol metabolism in T cells does not automatically indicate that G-proteins will regulate PKC. Nevertheless there are several points where G-proteins could have an effect on cellular diacylglycerol levels, and hence on PKC activity. In this regard, G-proteins have been implicated in the regulation of phosphatidylinositol- [20] and phosphatidylcholine- [21] specific phospholipase C, phospholipase D [22] and phospholipase A_2 [23] enzymes in other cells.

Abbreviations used: PKC, protein kinase C; G-protein, guanine nucleotide binding regulatory protein; p[NH]ppG, guanosine 5'-[β - γ -imido]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio] diphosphate; PDBu, phorbol 12,13-dibutyrate; PHA, phytohaemagglutinin; PAGE, polyacrylamide-gel electrophoresis; $[\text{Ca}^{2+}]$, concentration of free calcium.

The object of the present study was to develop a system that would enable an exploration of the role of G-proteins in the intracellular pathways that regulate phosphatidylinositol metabolism and kinases such as PKC in T cells. A method of membrane permeabilization of intact cells, with the bacterial toxin streptolysin O [24], allowed access to the cytoplasm of otherwise impermeant guanine nucleotide analogues and synthetic PKC inhibitors. The effect of G-protein agonists on PKC activity was then examined by monitoring the phosphorylation of the γ subunit of the CD3 antigen. The data demonstrate that guanine nucleotides can regulate CD3 phosphorylation in permeabilized T cells and also induce phosphatidylinositol metabolism. However, evidence is presented that changes in phosphatidylinositol breakdown do not always correlate with stimulation of PKC activity.

MATERIALS AND METHODS

Materials

Purified PHA and reduced streptolysin O were obtained from Wellcome Diagnostics (Dartford, Kent, U.K.). [γ - 32 P]ATP (370 MBq/ml) and *myo*-[3 H]inositol (3.05 TBq/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). All nucleotides were from Boehringer Mannheim (Lewes, Sussex, U.K.). PDBu and other biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). The PKC pseudo-substrate prototype having the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val was synthesized as previously described and has been shown to inhibit PKC *in vitro* and in permeabilized T cells [25].

Cell culture

Human T lymphoblasts were prepared as described [3], by stimulating peripheral-blood mononuclear cells (10^6 /ml) in RPMI 1640/10% (v/v) foetal-calf serum with 5 μ g of PHA/ml for 72 h. After washing, cells were maintained in exponential growth in RPMI 1640/10% foetal-calf serum supplemented with 0.1 nM recombinant interleukin-2. Before use, cells were deprived of interleukin-2 and allowed to quiesce in the G_0/G_1 stage of the cell cycle.

Cell permeabilization

Preliminary experiments established an optimum streptolysin O concentration of 0.4 i.u./ml, at which concentration > 95% of T lymphoblasts became permeable to Trypan Blue within 1 min of addition of the toxin. Typically, T lymphoblasts were washed twice and resuspended at 5×10^7 cells/ml in permeabilization medium containing 0.4 i.u. of streptolysin O/ml, 120 mM-KCl, 30 mM-NaCl, 10 mM-Hepes (pH 7.2), 10 mM-EGTA and 10 mM-MgCl₂. The concentrations of CaCl₂ required to give free Ca²⁺ in the range of nominal 0–1 μ M at 37 °C and pH 7.2 were predicted by the computer program CHELATE, by using appropriate dissociation constants for Ca²⁺, Mg²⁺ and H⁺ [26].

Phosphorylation and immunoprecipitation

Phosphorylation of proteins in permeabilized cells was carried out by permeabilizing 2.5×10^7 cells in the presence of 100 μ M-[γ - 32 P]ATP (1 μ Ci/pmol) and the specified stimulant or inhibitor. After incubation at 37 °C for the indicated time, cells were pelleted at 8000 g for 5 s

and lysed in a buffer containing 10 mM-Tris/HCl (pH 7.4), 1% Nonidet P-40, 150 mM-NaCl, 1 mM-EDTA, 1 mM-EGTA, 20 mM-Na₄P₂O₇, 50 mM-NaF, 4 μ g of leupeptin/ml and 1 mM-benzamide, added to the cell pellet on ice. The CD3 antigen was immunoprecipitated by using the anti-CD3 antibody UCHT1 as described [3,4]. CD3 antigens were separated by SDS/PAGE (12%-acrylamide gel) under reducing conditions. Gel autoradiographs were scanned with an LKB Ultrosan XL densitometer, and 32 P incorporation into the M_r -26000 CD3 γ chain was quantified by using the Ultrosan XL internal digital integrator.

[3 H]inositol labelling and inositol phosphate determination

Cells were incubated at 2×10^7 /ml in inositol-free Eagle's medium containing 10 mM-Hepes (pH 7.4) and 1 mg of bovine serum albumin/ml with *myo*-[3 H]inositol (15 μ Ci/ 10^7 cells) for 4 h at 37 °C in a 5%-CO₂ atmosphere. Cells were washed three times in inositol-free Eagle's medium and once in permeabilization medium before resuspension at 2.5×10^7 cells in 500 μ l of permeabilization buffer containing 0.4 i.u. of streptolysin O/ml, 10 mM-LiCl, 100 μ M-ATP and the indicated stimulant. After incubation for the indicated time at 37 °C, the reaction was terminated by addition of 800 μ l of ice-cold phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl). The cells were pelleted by centrifugation (8000 g for 5 s), and the supernatant was loaded directly on to Dowex columns for analysis by anion-exchange chromatography as described previously [27].

Data presentation

The data presented were representative of at least three separate experiments performed with cells prepared from different donors.

RESULTS

Phorbol ester- and PHA-dependent phosphorylation of the CD3 γ subunit in permeabilized T cells

To establish that PKC-mediated phosphorylation of CD3 γ chains could be detected in permeabilized T lymphocytes, cells were permeabilized in medium containing [γ - 32 P]ATP in the presence or absence of the PKC activator PDBu. The CD3 antigen was then immunoprecipitated and analysed by SDS/PAGE. In repeated experiments ($n = 18$) PDBu (20 ng/ml) induced a rapid and stable phosphorylation of the M_r -26000 CD3 γ polypeptide, detectable as early as 1 min after stimulation. CD3 γ -chain phosphorylation was also induced by the synthetic diacylglycerols 1,2-diolein and 1-oleoyl-2-acetyl-glycerol (results not shown). The dependence of PDBu-stimulated phosphorylation of CD3 γ chains on [Ca²⁺] is depicted in Fig. 1(b). A substantial effect was observed at nominally zero [Ca²⁺] (60% of maximum), consistent with the fact that T cells express predominantly the β isoform of PKC, which is relatively Ca²⁺-insensitive [28,29]. Maximal CD3 γ phosphorylation occurred at 500 nM-Ca²⁺, with a slight but reproducible inhibition at 1 μ M-Ca²⁺. In the absence of other exogenous stimuli [Ca²⁺], at 500 nM and 1 μ M concentrations, was found to stimulate a relatively weak phosphorylation of CD3 γ chain. The strength of this effect varied between donor preparations, but was typically 6-fold less than CD3 γ -subunit phosphorylation induced by PDBu at 500 nM-Ca²⁺.

Fig. 1. Phosphorylation of the CD3-antigen γ subunit in permeabilized T lymphocytes

(a) Time course of CD3 γ -subunit phosphorylation. Cells were incubated with streptolysin O in the presence of 500 nM- Ca^{2+} , [γ - ^{32}P]ATP and 20 ng of PDBu/ml (▲), 10 μg of PHA/ml (■) or 100 μM -GTP[S] (●) for the time indicated. The CD3 antigen was immunoprecipitated, analysed by SDS/PAGE (left panel), and ^{32}P incorporation quantified by densitometric scan of gel autoradiographs. Those for PHA-induced phosphorylations were exposed for longer than those for GTP[S]- and PDBu-induced phosphorylations. Thus the control (C) in the PHA panel is a longer exposure of the control shown in the top panel. The level of CD3 γ phosphorylation, normalized with respect to controls in permeabilization buffer alone and expressed in arbitrary units, is shown in the right panel. (b) Ca^{2+} -dependence of CD3 γ -subunit phosphorylation. Cells were incubated for 5 min with streptolysin O in the presence of various concentrations of Ca^{2+} , [γ - ^{32}P]ATP and 20 ng of PDBu/ml (▲), 10 μg of PHA/ml (■), 100 μM -GTP[S] (●) or no addition (○).

The mitogen PHA, which interacts with multiple surface receptors, has been shown previously to induce CD3 γ -chain phosphorylation in intact T cells [4]. PHA was also found to induce rapid and stable phosphorylation of CD3 γ subunits in permeabilized T cells (Fig. 1a). As in intact cells, PHA was a less potent stimulator of CD3 γ -chain phosphorylation than PDBu (50% of maximal PDBu-induced phosphorylation). PHA did not induce detectable phosphorylation of CD3 γ chains at zero [Ca^{2+}], but was synergistic with [Ca^{2+}] up to 150 nM- Ca^{2+} . The ability to regulate CD3 γ -chain phosphorylation by [Ca^{2+}], phorbol esters and PHA indicated that it was possible to induce PKC- and receptor-mediated phosphorylation pathways in T lymphocytes permeabilized by streptolysin O.

Guanine nucleotide regulation of CD3 phosphorylation

GTP[S] is a non-hydrolysable analogue of GTP that persistently activates G-proteins [14]. In permeabilized T cells, GTP[S] proved to be a very effective stimulator of CD3 γ -chain phosphorylation. The data, representative of 20 experiments, show that 100 μM -GTP[S] induced a rapid, stable, Ca^{2+} -dependent phosphorylation of CD3 γ polypeptides (Figs. 1a and 1b). The Ca^{2+} -dependency curve of GTP[S]-induced CD3 γ -subunit phosphorylation correlated very closely with that of PHA-stimulated phosphorylation, with no response at zero and maximum synergy at 150 nM- Ca^{2+} . GTP[S] was as potent as PDBu in inducing CD3 phosphorylation, and 2–3-fold more potent than PHA (Figs. 1a and 1b).

Fig. 2. Nucleotide specificity of CD3-antigen γ -subunit phosphorylation and inhibition with a pseudo-substrate peptide in permeabilized T lymphocytes

(a) Effect of GTP and its analogues on phosphorylation of the CD3 γ subunit. Cells were incubated for 5 min with streptolysin O in the presence of 500 nM- Ca^{2+} , [γ - ^{32}P]ATP and various concentrations of GTP[S] (●), p[NH]ppG (○), GTP (□), GDP[S] (■), CTP (▲) or ITP (△). The CD3 antigen was immunoprecipitated, analysed by SDS/PAGE, and ^{32}P incorporation quantified by densitometric scan of gel autoradiographs. The level of CD3 γ -chain phosphorylation was expressed in arbitrary units. (b) Pseudo-substrate inhibition of CD3 γ -subunit phosphorylation. Cells were incubated for 5 min with streptolysin O in the presence of 500 nM- Ca^{2+} , [γ - ^{32}P]ATP and either 20 ng of PDBu/ml (○) or 100 μM -GTP[S] (●) with the indicated concentration of pseudo-substrate peptide.

The specificity of the guanine-nucleotide-induced phosphorylation was examined by permeabilizing T lymphoblasts in the presence of a panel of exogenous nucleotides at concentrations in the range 10 μM –1 mM. The data (Fig. 2a) demonstrate that only the hydrolysis-resistant analogues of GTP (GTP[S] and p[NH]ppG) induced significant phosphorylation of the CD3 γ chain, their rank order of potency and therefore their ability to stimulate G-proteins reflecting their reported activity in other systems [26,30], i.e. GTP[S] > p[NH]ppG. Half-maximal stimulation in response to GTP[S] occurred at 75 μM . GTP, GDP[S], CTP and ITP were ineffective at

inducing phosphorylation of the CD3 antigen. The absence of detectable CD3 γ phosphorylation in response to GTP could be explained by its rapid hydrolysis in permeabilized cells. Analogues of cyclic AMP did not induce CD3 γ -subunit phosphorylation in intact or permeabilized T cells (results not shown).

Evidence that GTP[S] stimulates CD3 phosphorylation via PKC

PDBu- and PHA-induced CD3 antigen phosphorylation in permeabilized T cells is mediated by PKC [25]. The identification of PKC as the mediator of CD3-

Fig. 3. Characteristics of inositol phosphate production in permeabilized T lymphocytes

(a) Kinetics of inositol phosphate production. [³H]Inositol-labelled cells were permeabilized in the presence of 500 nM-Ca²⁺ and 10 μg of PHA/ml (■) or 100 μM-GTP[S] (●). Total inositol phosphates were extracted at the indicated time and analysed by anion-exchange chromatography. Data are expressed as a percentage of the inositol phosphates produced in cells permeabilized in the presence of Ca²⁺ alone. (b) Ca²⁺-dependency of inositol phosphate production. [³H]Inositol-labelled cells were permeabilized in the presence of the indicated Ca²⁺ concentration alone (▲) or in the presence of 10 μg of PHA/ml (■) or 100 μM-GTP[S] (●). Total inositol phosphates were extracted at 10 min. Data are expressed as a percentage of the inositol phosphates produced in cells permeabilized in the presence of nominal zero Ca²⁺ alone. (c) PHA and (d) GTP[S] concentration-dependence for inositol phosphate production and CD3γ-subunit phosphorylation. For inositol phosphate determinations, [³H]inositol-labelled cells were permeabilized in the presence of 500 nM-Ca²⁺ and the indicated concentration of PHA (□) or GTP[S] (○). Total inositol phosphates were extracted at 10 min. Data are expressed as fold stimulation of inositol phosphate production and are representative of three separate experiments. For CD3 phosphorylations, cells were incubated for 10 min with streptolysin O in the presence of 500 nM-Ca²⁺, [γ-³²P]ATP and the indicated concentration of PHA (■) or GTP[S] (●). Data are expressed as fold stimulation of CD3γ phosphorylation. All data are normalized with respect to cells permeabilized in the presence of Ca²⁺ alone.

antigen phosphorylation in permeabilized cells rests heavily on the use of a novel pseudo-substrate inhibitor of PKC [31]. The 13-amino-acid peptide inhibitor, based on a sequence in the regulatory domain of the enzyme itself, acts as a competitive inhibitor of PKC with high efficiency and specificity. Application of this inhibitor to T lymphocytes has been described elsewhere, including a demonstration that the protope inhibits specifically PDBu- and PHA-induced CD3 antigen phosphorylation [25]. The data (Fig. 2b) show that the peptide inhibited both PDBu- and GTP[S]-stimulated CD3γ-chain phosphorylation in a dose-dependent manner, with half-maximal inhibition for both agonists at 10–20 μM. The phosphorylation level of non-PKC-substrate bands observed in total lysates after immunoprecipitation of the CD3 antigen was not affected by the pseudo-substrate inhibitor (result not shown).

PHA and guanine nucleotides regulate inositol phospholipid hydrolysis

Guanine nucleotides have been shown to induce phospholipase C-mediated turnover of inositol phospho-

lipids in many cells [14]. To examine possible links between the regulation of CD3 phosphorylation and the stimulation of phosphatidylinositol metabolism, T cells were labelled biosynthetically with *myo*-[³H]inositol and the effects of PHA and GTP[S] on inositol phosphate accumulation were examined. PHA and GTP[S] initiated a rapid accumulation of inositol phosphates that was maximal after 4 min (Fig. 3a). The effect of both agonists on inositol phosphate production was Ca²⁺-dependent (Fig. 3b). No induction of inositol phosphates was observed at nominal zero [Ca²⁺], and maximal inositol phosphate accumulation occurred at 150 nM-Ca²⁺. The kinetics and Ca²⁺-dependency of both GTP[S]- and PHA-induced inositol phosphate production correlates with the effects of these agonists on CD3γ-chain phosphorylation (Figs. 1, 3a and 3b). The dose/response curves of PHA for inositol phosphate production and CD3γ phosphorylation showed a half-maximal effect for both responses at 2 μg/ml (Fig. 3c). However, the concentration of GTP[S] required to induce CD3 phosphorylation was markedly different from that required to initiate inositol phosphate production (Fig. 3d). At a GTP[S]

concentration of $10\ \mu\text{M}$ there was maximal inositol phosphate production, whereas CD3 γ -chain phosphorylation was 10% of maximal. The half-maximal concentrations of GTP[S] for inositol phosphate accumulation and CD3 phosphorylation were $0.7\ \mu\text{M}$ and $75\ \mu\text{M}$ respectively (Fig. 3*d*). Further evidence of the lack of correlation between agonist effects on inositol lipid hydrolysis and CD3 phosphorylation was provided by comparing the actions of PHA and GTP[S]. It was observed consistently that PHA was more effective than GTP[S] in inducing inositol phosphate production (Fig. 3). This is in direct contrast with the relative efficiency of PHA and GTP[S] with respect to CD3 γ -chain phosphorylation (Figs. 1*a*, 1*b*, 3), where GTP[S] was more potent than PHA. To summarize this point, PHA induced approx. 8–10-fold increases in inositol phosphates (Fig. 3*c*), but 3–4-fold increases in CD3 γ -chain phosphorylation. GTP[S], however, induced 4–5-fold increases in inositol phosphates, but 7–10-fold increases in CD3 γ -chain phosphorylation (Fig. 3*d*).

DISCUSSION

The present data demonstrate that phosphorylation of the CD3 γ chain can be induced in response to PDBu, PHA and GTP[S] in streptolysin O-permeabilized T cells. The specificity of guanine nucleotide effects are consistent with the hypothesis that phosphorylation of the CD3 γ subunit in permeabilized T cells is under G-protein control. The data also indicate that GTP[S] regulates CD3-antigen phosphorylation via PKC. This conclusion is based on experiments with a specific pseudo-substrate inhibitor of PKC. Of particular relevance to the identity of the GTP[S]-regulated kinase is the observation that the inhibition dose/response curves for the PKC pseudo-substrate are identical for PDBu-, PHA- and GTP[S]-induced phosphorylation. Since the pseudo-substrate is a competitive inhibitor of PKC [25,31], the data imply that PDBu, PHA and GTP[S] regulate CD3 phosphorylation via a common kinase, PKC.

It has been proposed that CD3-antigen phosphorylation is regulated via phosphatidylinositol turnover and the production of diacylglycerol [32]. It is clear, however, that the production of diacylglycerol from phosphatidylinositol breakdown does not always result in stimulation of PKC [1,9]. In permeabilized T cells, the kinetics, Ca^{2+} -dependency and concentration curve of PHA effects on inositol phosphate production correlate with PHA-induced stimulation of CD3 γ -chain phosphorylation. Thus PHA could regulate PKC-mediated CD3 phosphorylation via inositol phospholipid turnover and consequent production of diacylglycerol. There is, however, the discrepancy that PHA induces higher levels (> 200%) of phosphatidylinositol turnover than GTP[S], but is much weaker (< 50%) than GTP[S] with respect to the induction of CD3-antigen phosphorylation. The lack of a correlation between inositol lipid hydrolysis and PKC stimulation is demonstrated further by the observation that GTP[S]-induced inositol phosphate production was maximal at $10\ \mu\text{M}$, whereas CD3 γ phosphorylation was only 10% of maximum. As GTP[S] concentrations are increased above $10\ \mu\text{M}$, PKC mediated phosphorylation increases, but there are no further increases in inositol phospholipid metabolism. It therefore appears that GTP[S] regulation of CD3 γ -chain phosphorylation is not regulated solely by the products

of inositol phosphate hydrolysis. Accordingly, the present study suggests that there is more than one G-protein-regulated mechanism for the control of PKC in T lymphocytes. Whether the differences in the GTP[S] dose/response curves for phosphatidylinositol turnover and PKC activation reflects that these pathways are controlled by G-proteins with differing affinities for GTP[S] remains to be determined.

There are several points at which agonists could influence the phosphorylation level of PKC substrates. One explanation for the discrepancy between GTP[S] and PHA effects on the phosphatidylinositol hydrolysis and CD3 γ -chain phosphorylation is that PKC may be regulated by diacylglycerol produced from the breakdown of other membrane phospholipids such as phosphatidylcholine [11]. In addition, phospholipid metabolites such as arachidonic acid, which could be regulated by G-proteins or PHA, may stimulate PKC [12]. It should also be emphasized that T cells express at least two isoenzymes of PKC, the α and β forms [28,29]. The physiological relevance of PKC heterogeneity is not known, but it is possible that the isoenzymes have different signalling requirements for activation and are differentially stimulated by PHA and GTP[S]. Finally, the possibility should be considered that cellular phosphatases may be differentially controlled by PHA and GTP[S] and thus influence the relative effects of these agonists on protein phosphorylation.

In conclusion, the present study demonstrates that phosphatidylinositol turnover can be initiated by PHA and GTP[S] in permeabilized T cells. Although the identity of the G-protein(s) that regulates phosphatidylinositol hydrolysis and its relationship to the receptors triggered by PHA (such as the T-cell antigen-receptor/CD3 complex) are not established, this method of cell permeabilization clearly offers the potential to investigate such questions. PHA and GTP[S] also induce PKC-mediated CD3-antigen phosphorylation, and the data are consistent with the model that stimulation of PKC is explained in part by agonist effects on phosphatidylinositol metabolism. It is probable, however, that GTP[S] does not regulate PKC and CD3 phosphorylation by phosphatidylinositol turnover alone, but may use alternative mechanisms or a combination of different signals. Consequently, it will be of interest to investigate whether the present data have identified a G-protein-regulated signal-transduction pathway that couples PKC to receptors that do not regulate phosphatidylinositol metabolism and that are distinct from the receptors triggered by PHA.

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