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A method for measuring protein kinase C activity in permeabilized T lymphocytes by using peptide substrates

Evidence for multiple pathways of kinase activation

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Activation of protein kinase C (PKC) in human T lymphocytes is an immediate consequence of mitogenic signalling via the antigen–receptor complex and CD2 antigen. In order to investigate further the signal-transduction pathways which result in PKC activation, we have established a novel PKC assay system using streptolysin-O-permeabilized T cells. Known peptide substrates of PKC were introduced into permeabilized cells in the presence of [γ-^{32}P]ATP, 3 mM-Mg^{2+} and 150 nM free Ca^{2+}. The peptide found to have the lowest background phosphorylation had the sequence Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys (peptide GS), and the phosphorylation of the peptide was increased up to 6-fold by direct activation of PKC with phorbol 12,13-dibutyrate. Induction of PKC activation with the UCHT1 antibody against the CD3 antigen, or with phytohaemagglutinin (PHA) or guanosine 5′-[γ-thio]triphosphate (GTP[S]), increased peptide-GS phosphorylation by 2–3 fold. The specificity of PKC action on peptide GS was demonstrated by blocking increases in phosphorylation with a pseudosubstrate peptide PKC inhibitor. PKC activation by this technique could be detected within 1 min of adding external ligand. Dose–response curves revealed that PHA-induced production of inositol phosphates correlated closely with PKC activities, whereas only a partial correlation between these parameters was observed with GTP[S]. Our data are consistent with the presence of more than one G-protein-mediated pathway of PKC regulation in T cells. The quantitative PKC assay system described is both simple and reproducible, and its potential application to a wide range of cell types should prove useful in further investigations of PKC activation mechanisms.

INTRODUCTION

Activation of protein kinase C (PKC) in T lymphocytes is an early consequence of antigen recognition by the antigen receptor–CD3 complex and of the binding of agonistic antibodies against the CD3 and CD2 antigens [1–6]. The subsequent phosphorylation of the CD3-antigen γ polypeptide is mediated by PKC in both murine [3] and human [5] T cells, and PKC activation also results in the phosphorylation of M_r-80000 and M_r-19000 cytosolic proteins [4]. There is strong circumstantial evidence that this observed increase in PKC activity is involved in mediating mitogenic signals during the initial activation of quiescent T lymphocytes. Firstly, quiescent T cells are induced to proliferate by a combination of ionomycin, which increases intracellular Ca^{2+}, and phorbol ester, which directly activates PKC, suggesting a role for the kinase in inducing the proliferative response [7,8]. Secondly, a close correlation has been noted between mitogenic signalling via the CD3 and CD2 pathways, and the turnover of PtdIns in T cells, with the consequent production of DAG and InsP_3, which causes a release of Ca^{2+} from internal stores [9–11]. Both DAG and Ca^{2+} are involved in promoting the translocation and activation of PKC [12]. Thirdly, expression of the cDNA for the PKC catalytic domain in T-cell leukaemia Jurkat cells results in constitutive PKC activity in the absence of phorbol ester, and the consequent activation of the c-*fos* gene enchancer [13], an event thought to be involved in the induction of the IL-2 gene [14]. Fourthly, activation of PKC by phorbol ester induces a specific subset of genes which are also induced by mitogenic signalling via Ti–CD3 [15].

Although the cumulative weight of these data indicates a role for PKC in transducing signals via Ti–CD3 and the CD2 antigen, it is not yet clear how PKC is regulated via these pathways. Investigations of PKC regulation have been hindered by the inability of present methods to provide quantitative estimates of the PKC activity without prior disruption of cells. For example, the activation of PKC in intact T cells has been monitored by measuring the phosphorylation of the M_r-80000 protein [4], a well-established substrate for PKC [16], and also the phosphorylation of the CD3-antigen γ chain [17,5]. Such methods have proved invaluable as ways of detecting PKC activation, but the techniques are inconvenient when there is a need to carry out large numbers of comparative quantitative assays on a single batch of cells. Furthermore, there is evidence that a Ca^{2+}-regulated kinase, besides PKC, is involved in the phosphorylation of the CD3-antigen γ chain [18].

We have recently established that several potential mechanisms of signal transduction remain intact after the permeabilization of T cells with streptolysin-O [5]. It has been shown that mitogenic lectins such as PHA, as well as DAG analogues and phorbol esters, can induce CD3-antigen γ-chain phosphorylation in permeabilized cells by a PKC-mediated process which can be blocked by using a PKC pseudosubstrate peptide [5]. Furthermore, evidence has also been obtained by using this system for G-protein-regulated pathways of PKC activation, which are

Abbreviations used: PKC, protein kinase C; Ti, the α–β heterodimer of the T-lymphocyte antigen–receptor complex; DAG, 1,2-diacylglycerol; PHA, phytohaemagglutinin; PdBu, phorbol 12,13-dibutyrate; PC, phosphatidylcholine; IL-2, interleukin 2; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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only partially mediated by the products of PtdIns metabolism [19]. Our previous work demonstrated that small peptides have rapid access to the interior of streptolysin-O-treated T cells [5,20]. We now show that selective peptide substrates of PKC can be introduced into such permeabilized cells, their subsequent phosphorylation providing reproducible quantitative estimates of PKC activation induced via Ti–CD3, by mitogenic lectins such as PHA, or by GTP[S] and phorbol esters. Our data also provide further evidence for the existence of multiple pathways of PKC activation in human T cells.

MATERIALS AND METHODS

Materials

PdBu and other biochemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Purified PHA and streptolysin-O were from Burroughs Wellcome, and GTP[S] was from Calbiochem. [γ-^{32}P]ATP and *myo*-[^{3}H]inositol were obtained from Amersham International. The UCHT1 (IgG1) mouse monoclonal antibody against the CD3 antigen [21] was kindly given by Professor Peter Beverley, University College and Middlesex School of Medicine, London, and was purified as in ref. [22]. Cell-culture materials were from Gibco, Paisley, Scotland, U.K. P81 phosphocellulose ion-exchange-chromatography paper was from Whatman International.

Cells

Human T lymphoblasts were prepared by stimulating peripheral-blood mononuclear cells with 5 μg of PHA/ml for 72 h, and then growing for up to 10 days in medium supplemented with 0.1 nM human recombinant IL-2, as previously described [12,17]. Cells were washed free of IL-2 and were then cultured for 2–4 days in the absence of IL-2 before experiments, to induce quiescence. Primary T lymphocytes were prepared from peripheral-blood mononuclear cells by negative selection involving monocyte adherence to plastic plates, followed by removal of B cells, NK cells, platelets and remaining monocytes by using monoclonal antibodies against cell-surface antigens and induction of cell lysis with complement, as described in ref. [23].

Peptides

Peptides were synthesized as previously described [22]. The peptide GS has the sequence Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, and its properties as a selective substrate of PKC have previously been reported [24]. Peptide PS, an analogue of the PKC pseudosubstrate inhibitor [25], has the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val, and a version of this peptide (four amino acids longer) has previously been used as a PKC substrate [26]. The pseudosubstrate PKC peptide inhibitor has been previously described [5]. Peptides were dissolved just before assays, to avoid any decline in their concentration owing to binding to glass or plastic surfaces.

Buffers

The 'intracellular buffer' used during cell permeabilization contained 5.16 mM-$MgCl_2$, 94 mM-KCl, 12.5 mM-Pipes, 12.5 mM-EGTA and 8.17 mM-$CaCl_2$; adjustment of the pH of Pipes and EGTA to 7.4 with KOH resulted in a final concentration of 150 mM-K^+ in this buffer. Concentrations of free Ca^{2+} and Mg^{2+} (187 nM and 3.75 mM respectively) were calculated by the 'Chelate' computer program, by using appropriate dissociation constants for Ca^{2+}, Mg^{2+} and H^+ [27].

PKC assay

Cells were washed twice before permeabilization, firstly in phosphate-buffered saline (10 mM-sodium phosphate buffer/150 mM-NaCl, pH 7.2) and secondly in intracellular buffer. Streptolysin-O (22 i.u.) was reconstituted from the freeze-dried powder with 11 ml of distilled water. Washed cells suspended in intracellular buffer were incubated in 1.5 ml Eppendorf tubes in 190 μl portions [(2.5–4.8) $\times$ 10^6 cells per portion] at 37 °C. Assays were started by the addition of 50 μl of streptolysin-O mixed with [γ-^{32}P]ATP (300–450 c.p.m./pmol), and peptide substrate and various activators of PKC, giving a final reaction volume of 250 μl and final concentrations of 200 μM-[γ-^{32}P]ATP and 250 μM-peptide (unless otherwise shown). The 0.8 dilution factor entailed by the addition of streptolysin-O resulted in final concentrations of buffer components of 3 mM free Mg^{2+}, 150 nM free Ca^{2+}, 120 mM-K^+, 10 mM-Pipes and 10 mM-EGTA. Reactions were continued for the times shown and stopped by the addition of 100 μl portions of 25% (w/v) trichloroacetic acid in 2 M-acetic acid. After being left for a least 10 min on ice, samples were centrifuged in an MSE Microcentaur instrument for 5 min. Two samples (80 μl each) per assay were spotted on numbered squares of P81 ion-exchange-chromatography paper, which were washed three times in 30% (v/v) acetic acid containing 1% H_3PO_4 (600–900 ml per wash for up to 90 squares) for a minimum of 10 min, and once with ethanol. The P81 squares were dried, immersed in Picofluor, and the bound radioactivity was measured in a scintillation counter. Routinely, 2×10^8 cells were used for a single experiment, assays were performed in duplicate, and a single tube containing no peptide was included for each pair of duplicate assays to estimate the 'background' phosphorylation of basic cell components which were not precipitated by 7% trichloroacetic acid/0.6 M-acetic acid, and which adhered to P81 paper. The values for such blanks were 3–10% of the values determined in the presence of peptide, and these blank values have been subtracted. Unless otherwise shown, the peptide-phosphorylation results given are the means of duplicate values, which are expressed as pmol of phosphate incorporated into peptide/time used for assay per number of cells used in assay.

Inositol phosphate determination

Cells were incubated at 2×10^7/ml in inositol-free Eagle's medium containing 10 mM-Hepes buffer, pH 7.4, and 1 mg of bovine serum albumin/ml with *myo*-[^{3}H]inositol (15 μCi/10^7 cells) for 4 h at 37 °C in a 5%-CO_2/air atmosphere. Cells were washed three times in inositol-free Eagle's medium and once in the intracellular buffer described above before resuspension of portions containing 2.5×10^7 cells in 500 μl of intracellular buffer containing 0.4 i.u. of streptolysin-O/ml, 10 mM-LiCl, 100 μM-ATP and either GTP[S] or PHA. After incubation for 8 min at 37 °C, the reaction was terminated by the addition of ice-cold phosphate-buffered saline. 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 [28].

RESULTS

Phosphorylation of peptides in permeabilized T cells

In order to establish an effective PKC assay in streptolysin-O-permeabilized cells, it was clearly necessary to use a highly selective substrate for PKC. Initially, a comparison was made between peptide GS, having a sequence derived from glycogen synthase kinase [24], and peptide PS, an analogue of the pseudosubstrate PKC inhibitor [5,25]. Fig. 1(*a*) shows that peptide GS was phosphorylated at a relatively low level when added to permeabilized quiescent T lymphoblasts in the presence of 3 mM-Mg^{2+} and 150 nM-Ca^{2+} without further additions. Upon addition of GTP[S] (a non-hydrolysable analogue of GTP that

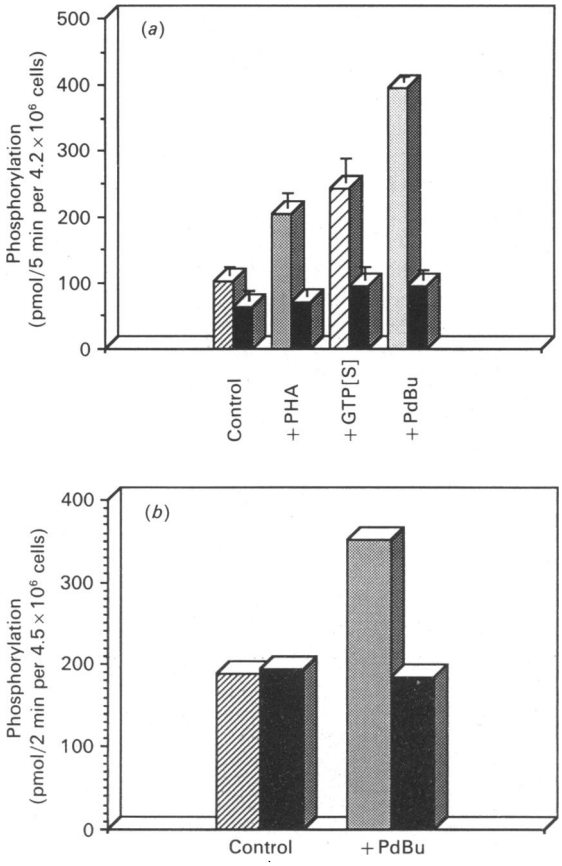

Fig. 1. Peptide phosphorylation in permeabilized T lymphoblasts

(*a*) Phosphorylation of peptide GS. Assays were carried out for 5 min with 100 μM-peptide GS, with no additions (control), 10 μg of PHA/ml, 100 μM-GTP[S] or 50 ng of PdBu/ml. Black columns indicate the respective values obtained on addition of 200 μM pseudosubstrate peptide PKC inhibitor. The values shown are the means ± S.D. of 4 assays; all assays were carried out on the same batch of cells. (*b*) Phosphorylation of peptide PS. Assays were carried out for 2 min with 250 μM peptide PS, with or without 1 mM pseudosubstrate peptide. Other details are as in (*a*).

induces prolonged activation of G-proteins), the mitogenic lectin PHA, or PdBu, all agents previously shown to activate PKC in such permeabilized cells [5,19], the level of peptide-GS phosphorylation was increased in this experiment by 2–4-fold. Since an increase in phosphorylation could be caused by the activation of kinases other than PKC, the pseudosubstrate PKC peptide, which is a competitive inhibitor of PKC activity [25], was included in the assays at a concentration twice that of substrate. In each case the phosphorylation level was decreased to values close to the control (Fig. 1*a*), indicating that the increase in phosphorylation was being mediated solely by PKC. There was also a small inhibition by pseudosubstrate of the background level of peptide GS phosphorylation, possibly reflecting a low intrinsic PKC activity under these assay conditions in the absence of specific activators. The background level of peptide-GS phosphorylation did not vary greatly between T-lymphoblast preparations. For example, in a series of five experiments carried out on different cell batches by using 10 min assays, a mean of 207 ± 50 pmol/10 min per 4.2×10^6 cells was found for the background value (range 127–250). In contrast, the PdBu-induced increment constituted a 2–6-fold increase over this background (results not shown). Presumably this activation range reflects subtle differences in the degree of quiescence of the cells or, equally likely, genetic differences in the levels of T-cell PKC expression between individual donors.

The peptide-GS assay system was also used with primary quiescent T lymphocytes (97% $CD2^+$), and a basal value of 412 pmol/10 min per 4.4×10^6 cells was found, which increased by 4.7-fold to 1935 pmol/10 min per 4.4×10^6 cells on addition of 50 ng of PdBu/ml. The extent of PdBu-induced PKC activation was therefore within the range noted for quiescent T lymphoblasts, though further work will be required to determine whether the higher absolute values obtained are typical of all primary T-cell preparations.

It was found in experiments with peptide PS that its background phosphorylation level in proportion to the PdBu-induced increment was considerably higher than that with peptide GS as substrate (Fig. 1*b*), a finding that was reproduced in five separate T-lymphoblast preparations (results not shown). Although Fig. 1(*b*) shows that PdBu induced a 1.8-fold increase in peptide-PS phosphorylation, which was decreased to baseline levels by adding the PKC pseudosubstrate inhibitor, it is apparent that this relatively low fold increase in phosphorylation makes peptide PS a less suitable substrate for PKC assays in this system than peptide GS. Thus peptide GS was used in further experiments.

Fig. 2 shows that optimal levels of peptide-GS phosphorylation induced by PHA, GTP[S] or PdBu were obtained within 5–10 min. The extent of PKC activation induced by 50 ng of PdBu/ml was consistently about 2–4-fold higher than that obtained with 10 μg of PHA/ml or 100 μM-GTP[S] (Figs. 1*a* and 2). In contrast, we have previously shown that GTP[S] is as potent as PdBu and 2–3-fold more potent than PHA in inducing PKC-mediated CD3-antigen γ-chain phosphorylation [19]. The reason(s) for this difference are not known, though it should be noted that the final free Mg^{2+} concentration was 10 mM in those previous experiments, compared with 3 mM in the present work.

Fig. 3 shows that optimal peptide-GS phosphorylation was obtained at concentrations of 50–100 μM under the conditions used. The data of Fig. 3 clearly do not allow the application of formal enzyme kinetics, since assays are dependent on multiple factors, including the time taken for agents to induce the activator(s) of PKC, the necessity for PKC translocation to the cell membrane, and the time taken for peptide substrate to enter the cells [5]. However, it may be noted that half the apparent maximal PdBu-induced PKC activity was obtained at a concentration of approx. 13 μM peptide GS, which compares with an

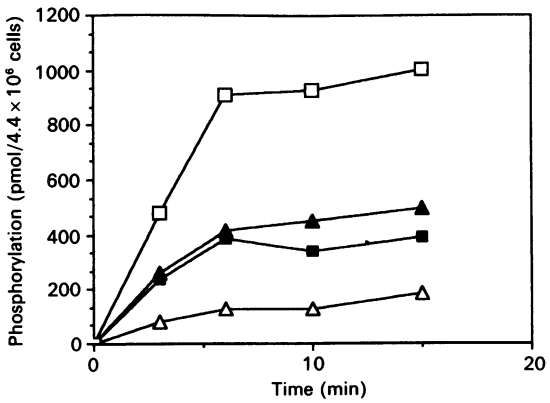

Fig. 2. Time course of phosphorylation of peptide GS in permeabilized T lymphoblasts

Assays were carried out as described in the Materials and methods section. △, No additions; ■, +10 μg of PHA/ml; ▲, +100 μM-GTP[S]; □, +50 ng of PdBu/ml.

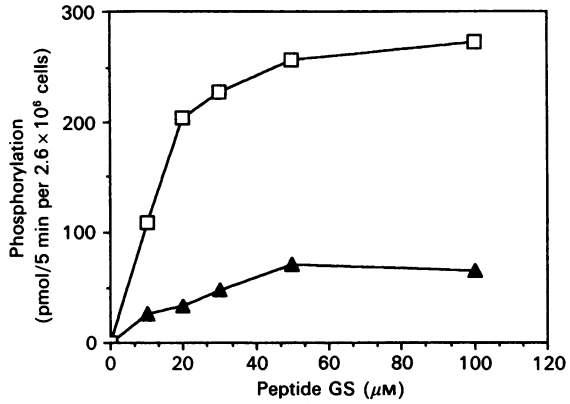

Fig. 3. Concentration-dependence of peptide-GS phosphorylation in permeabilized T lymphoblasts

Assays were carried out as described in the Materials and methods section. Assays were for 5 min: ▲, +100 μM-GTP[S]; □, +50 ng of PdBu/ml.

apparent K_m value of 4.1 μM found for this substrate with purified PKC in an assay system *in vitro* [24].

PKC activation induced via the CD3 antigen

Although mitogenic antibodies against the CD3 antigen have been previously shown to induce PKC activation in intact T cells [4], it was of considerable interest to determine whether the coupling between Ti–CD3 and PKC activation remained functional in streptolysin-O-permeabilized cells. As shown in Fig. 4, the UCHT1 monoclonal antibody induced peptide-GS phosphorylation up to levels 3-fold higher than background during a 10 min incubation. During this assay time addition of the PKC pseudosubstrate decreased the increment of UCHT1-induced phosphorylation by 70% (Fig. 4). At shorter assay times of 5 min, the pseudosubstrate inhibitor blocked UCHT1-induced peptide-GS phosphorylation by more than 95%, and further experiments showed that the addition of a further batch of pseudosubstrate at 5 min during a 10 min assay period was sufficient to block UCHT1-induced phosphorylation to the same extent (results not shown). It is therefore apparent that the increase in UCHT1-induced GS phosphorylation was entirely due to PKC activation, but that the efficiency of pseudosubstrate PKC inhibition became less over longer periods, presumably owing to the lowering of inhibitor concentration by peptide degradation and/or non-specific binding.

These data confirm that PKC is activated via the Ti–CD3 pathway, and establish that this assay system can be used to investigate the regulation of this pathway in permeabilized cells. Furthermore, a time course of UCHT1-induced PKC activation showed that peptide-GS phosphorylation increased within 1–2 min (results not shown). Since we have previously found that PKC is rapidly lost from T cells within 3 min of permeabilization with streptolysin-O in the absence of agents which activate the enzyme [5], it is clear from these results that soluble UCHT1 mediates via the antigen–receptor complex a rapid translocation and activation of PKC.

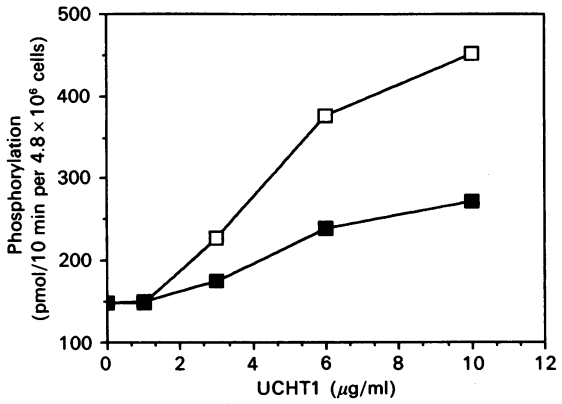

Fig. 4. CD3 monoclonal-antibody (UCHT1)-induced peptide-GS phosphorylation in permeabilized T lymphoblasts

Assays were carried out for 10 min in the presence of 250 μM peptide GS. The data are representative of three separate experiments. □, +UCHT1 alone; ■, +500 μM pseudosubstrate peptide PKC inhibitor.

Comparison of PKC activation with PtdIns metabolism

The dose–response curves in Fig. 5 demonstrate two important points. Firstly, only a partial correlation was found between the concentrations of GTP[S] which induced the production of inositol phosphates and those which activated PKC. For example, 10 μM-GTP[S] induced 90% of maximal inositol phosphate production, but only 47% of maximal PKC activation. A similar lack of correlation was also noted in previous work comparing inositol phosphate production and CD3-antigen γ-chain phosphorylation, for which the comparative values were 100% of maximal inositol phosphate production induced by 10 μM-GTP[S], compared with only 10% of maximal PKC-mediated γ-chain phosphorylation [19]. Secondly, Fig. 5 reveals an aspect of GTP[S]-induced PKC activation which was not observed in studies of γ-chain phosphorylation [19], namely that it appears to be a composite of at least two responses, an initial response over the range 0.5–5 μM-GTP[S] and a secondary response over the range 20–100 μM-GTP[S].

In contrast with the lack of correlation found between GTP[S]-induced PtdIns metabolism and PKC activation, Fig. 6 shows that the dose–response curve of PHA-induced inositol phosphate production correlated closely with that found for PKC activation.

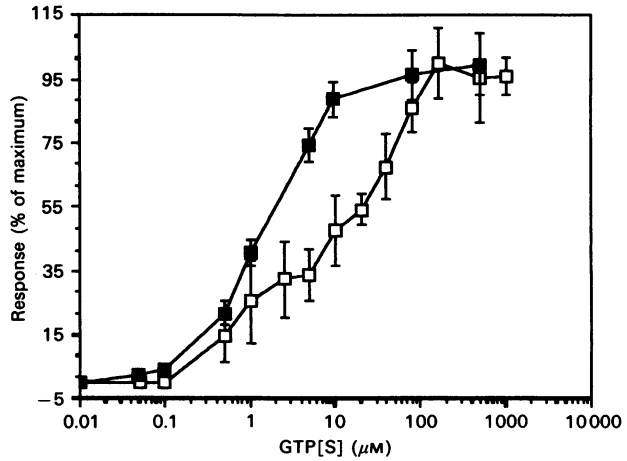

Fig. 5. Dose–response curves of GTP[S]-induced inositol phosphate production and PKC activation

Inositol phosphate production was measured as described in the Materials and methods section, and the values shown are the means ± S.D. of three determinations using two batches of cells. PKC activity was assayed at 10 min with peptide GS as substrate, and the values shown are the means ± S.D. of four determinations using two batches of cells, and 4.2×10^6 cells per assay. □, PKC; ■, inositol phosphates.

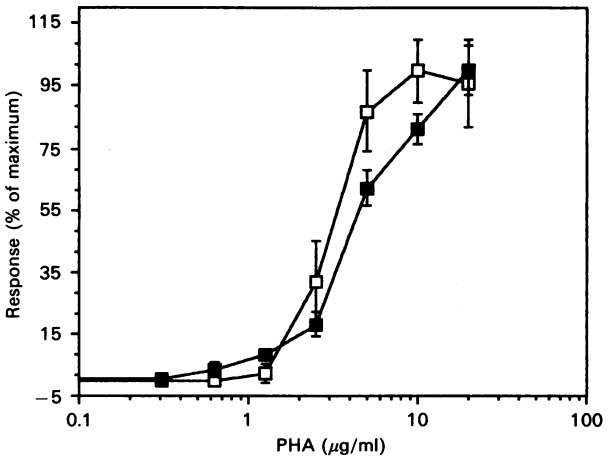

Fig. 6. Dose–response curves of PHA-induced inositol phosphate production and PKC activation

PKC activity was assayed at 10 min with peptide GS as substrate, and the values shown are the means ± S.D. of three determinations using a single batch of cells. The values for inositol production were also obtained from a single set of assays. □, PKC; ■, inositol phosphates.

These data are consistent with there being a direct relationship between the two events, and support the idea that the DAG produced as a result of PHA-induced inositol phospholipid turnover contributes to the subsequent activation of PKC. These findings are in agreement with previous studies in which a similar correlation was observed between PHA-induced inositol phosphate production and CD3-antigen γ-chain phosphorylation [19].

DISCUSSION

The main conclusion from this study is that PKC activation induced in permeabilized T cells can be rapidly and conveniently assayed by monitoring peptide-GS phosphorylation. The identity of the kinase(s) which phosphorylate(s) peptide GS in the absence of PKC activation is unknown (Fig. 1*a*). Since changes in the activity of kinases apart from PKC may occur during signal transduction, it is essential that the pseudosubstrate peptide inhibitor be used in conjunction with this assay system in order to demonstrate the specificity of PKC action (Fig. 1).

An advantage of this peptide-assay technique in permeabilized cells is that it allows quantification of rapid changes in PKC activity in response to the binding of external ligands. An essential component of this response is the translocation of PKC to intracellular membranes, since without translocation PKC is rapidly lost from streptolysin-O-permeabilized T lymphoblasts, even in the presence of 400 nM free Ca^{2+} [5]. The PKC activation noted in response to PHA (Fig. 2) and to binding of monoclonal antibody UCHT1 to the CD3 antigen (Fig. 4) therefore implies that PKC must have been translocated within 1–2 min of antibody binding in order to be available in an activated state for subsequent peptide-GS phosphorylation. A similarly rapid induction of peptide-GS phosphorylation has been noted after cross-linking of the CD3 and CD2 antigens (D. R. Alexander, unpublished work). These data are consistent with the idea that PKC plays a role at an early stage of signal transduction via the Ti–CD3 and CD2 antigen pathways.

It is also of interest that soluble UCHT1, which is non-mitogenic when added to quiescent T lymphoblasts [4], induced a marked activation of PKC in the absence of cross-linking (Fig. 4). In some T-cell preparations the extent of soluble UCHT1-induced PKC activation was 50–60% or more of that obtained with PdBu. This is in contrast with studies on CD3-antibody-induced phosphorylation of the M_r-80000 PKC substrate in T lymphoblasts [4], and with investigations into the translocation of PKC in Jurkat T cells [29], in which it was found that antibody cross-linking was required to induce levels of PKC activation comparable with those obtained with PdBu. In comparing these findings with the marked activation of PKC induced by soluble CD3 monoclonal antibody in the present study, it should be noted that the peptide-assay system is unlikely to reflect the Ca^{2+}-dependency which characterizes the activation of PKC in intact cells. This Ca^{2+}-dependency is thought to have two distinct aspects, the increase in intracellular Ca^{2+} which contributes to binding of PKC to membranes, and the synergistic action of Ca^{2+} with DAG in activating PKC once it becomes membrane-bound [30–33]. The first of these aspects is not relevant to the permeabilized cells used in the present work, since it is known that the artificially stabilized free Ca^{2+} concentration of 150 nM is insufficient to induce PKC binding to intracellular T-lymphoblast membranes [5]. Furthermore, the second aspect of Ca^{2+}-dependency is less applicable when using the peptide-assay system, since peptide substrates for PKC do not reflect the Ca^{2+}-dependency shown for polypeptide substrates. For example, little difference was found between the levels of PdBu-induced peptide-GS phosphorylation at nominally zero Ca^{2+} compared with 400 nM-Ca^{2+} (D. R. Alexander, unpublished work), in contrast with the relatively greater sensitivity to Ca^{2+} previously noted in studies of PdBu-induced CD3-antigen γ-chain phosphorylation in T lymphoblasts [5]. Thus the extent of PKC activation in the present assay presumably reflects solely the production of DAG and/or other products of lipid metabolism which activate the enzyme, in contrast with the situation in intact cells, in which PKC activation reflects both elevated Ca^{2+} levels as well as DAG production. Such differences may explain the marked effects of soluble UCHT1 in the present assay system, in contrast with its relative ineffectiveness in inducing PKC activation in intact T lymphoblasts and Jurkat cells [4,29].

A further complexity in assaying PKC activity in intact or permeabilized cells is the presence of more than one PKC isoform. At least seven isoforms of PKC have been described [34], and the expression of two of these, PKC_α and PKC_β, has recently been demonstrated in human T cells [35–37]. The expression of $PKC_{\beta 1}$, one of two β isoenzymes derived from the same gene by alternative splicing [34], is relatively high compared with PKC_α in T lymphoblasts (D. A. Cantrell & R. Marais, unpublished work). We have previously discussed the potential significance of PKC-isoform expression in T-cell function [6]. Our present data do not indicate whether peptide GS is selective as a substrate for a particular PKC isoform. However, some differences have been noted between the α, β1 and γ isoforms of PKC in their phosphorylation of various peptides [26]. Furthermore, it has been reported that a pseudosubstrate peptide analogue derived from the sequence of the PKC_ϵ isoform is an effective substrate for this enzyme, although it has little activity towards histone IIIS, a commonly used protein substrate for PKC [38]. This raises the intriguing possibility that peptide and/or polypeptide substrates could be developed which distinguish between the activities of the various PKC sub-types. The use of such substrates in the assay system described here could lead to an understanding of the regulation of individual PKC sub-types during T-cell signal transduction.

The source(s) of the DAG and/or other lipid metabolites which activate PKC in T cells remains unknown. Though the dose–response curve of PHA-induced inositol phosphate production closely parallels that of PKC activation (Fig. 6),

phospholipase C action on PTdIns is only one of several possible metabolic pathways which may lead to DAG production during the early stages of T-cell activation. This is clear from previous work [19], and also from the data of Fig. 5, which show that the level of G-protein-coupled PtdIns-phospholipase C activity correlates only partially with the observed level of PKC activation. Agonist-induced PC breakdown has been shown to be an important source of DAG in other cell types (reviewed in [39]). This may occur directly via PC-specific phospholipase C action, or indirectly via the sequential actions of phospholipase D and phosphatidate phosphohydrolase [39]. Evidence for G-protein regulation of both phospholipase D and PC-specific phospholipase C has been presented [40–43]. In addition, phospholipase A_2 action on PC may generate lyso-PC, which is then metabolized by five further steps to DAG [39]. The shape of the dose–response curve of PKC activation shown in Fig. 5 is consistent with the production of DAG from more than one source. An alternative view is that increasing concentrations of GTP[S] activate PKC via a single G-protein-coupled process, and that PKC then stimulates PC breakdown to generate more DAG, as demonstrated in other cell types [39]. However, phorbol-ester-induced PKC activation does not appear to regulate phospholipase D in purified T cells [44].

The readily measurable (one-third maximal) activation of PKC induced by 0.5–5 μM-GTP[S] (Fig. 5) was unexpected in view of the lack of PKC-mediated CD3-antigen γ-chain phosphorylation over this range [19]. The trivial explanation, that γ-chain phosphorylation in this range would be below the level of detection, is unlikely in light of these previously published data [19]. Since peptide GS presumably has access to the entire cytoplasmic interior of streptolysin-O-permeabilized T cells, it is possible that peptide phosphorylation reflects the activity of PKC irrespective of its intracellular location, whereas CD3-antigen γ-chain phosphorylation is restricted to a particular location of enzyme and/or substrate.

It is clear that considerable work remains in order to clarify the various pathways of PKC activation in human T cells, and the way in which these pathways are coupled to cell-surface receptors such as Ti–CD3 and CD2 antigen requires elucidation. The peptide-phosphorylation PKC assay in permeabilized cells that we have described should prove a useful tool in further investigations of these current enigmas.

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REFERENCES

1. Samelson, L. E., Harford, J. B., Schwartz, R. H. & Klausner, R. D. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1969–1973
2. Cantrell, D., Davies, A. A., Londei, M., Feldman, M. & Crumpton, M. J. (1987) Nature (London) **325**, 540–542
3. Patel, M. D., Samelson, L. E. & Klausner, R. D. (1987) J. Biol. Chem. **262**, 5831–5838
4. Friedrich, B., Cantrell, D. A. & Gullberg, M. (1989) Eur. J. Immunol. **19**, 17–23
5. Alexander, D. R., Hexham, J. M., Lucas, S. C., Graves, J. D., Cantrell, D. A. & Crumpton, M. J. (1989) Biochem. J. **260**, 893–901
6. Alexander, D. R. & Cantrell, D. A. (1989) Immunol. Today **10**, 200–205
7. Weiss, A., Wiskocil, R. L. & Stobo, J. D. (1984). J. Immunol. **133**, 123–129
8. Truneh, A., Albert, F., Goldstein, P. & Schmitt-Verhulst, A.-M. (1985) Nature (London) **313**, 318–320
9. Imboden, J. B. & Stobo, J. D. (1985) J. Exp. Med. **161**, 446–456
10. Imboden, J. B., Weyand, C. & Garonzy, J. (1987) J. Immunol. **138**, 1322–1324
11. Pantaleo, G., Olive, D., Poggi, A., Kozumbo, W. J., Moretta, L. & Moretta, A. (1987) Eur. J. Immunol. **17**, 55–60
12. Nishizuka, Y. (1984) Nature (London) **308**, 693–697
13. Muramatsu, M.-A., Kaibuchi, K. & Arai, K. (1989) Mol. Cell. Biol. **9**, 831–836
14. Crabtree, G. R. (1989) Science **243**, 355–361
15. Irving, S. G., June, C. H., Zipfel, P. F., Siebenlist, U. & Kelly, K. (1989) Mol. Cell. Biol. **9**, 1034–1040
16. Rodriguez-Pena, A. & Rozengurt, E. (1986) EMBO J. **5**, 77–83
17. Cantrell, D. A., Davies, A. A. & Crumpton, M. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8158–8162
18. Cantrell, D. A., Friedrich, B., Davies, A. A., Gullberg, M. & Crumpton, M. J. (1989) J. Immunol. **142**, 1626–1630
19. Graves, J. D., Lucas, S. C., Alexander, D. R. & Cantrell, D. A. (1989) Biochem. J. **265**, 407–413
20. Alexander, D. R., Hexham, J. M., Cantrell, D. A., Goris, J., Lucas, S. C., Graves, J. D. & Crumpton, M. J. (1989) Adv. Protein Phosphatases **5**, 313–331
21. Beverley, P. C. L. & Callard, R. E. (1981) Eur. J. Immunol. **11**, 329–334
22. Davies, A. A., Cantrell, D. A., Hexham, J. M., Parker, P. J., Rothbard, J. & Crumpton, M. J. (1987) J. Biol. Chem. **262**, 10918–10921
23. Wallace, D. L. & Beverley, P. C. L. (1990) Immunology **69**, 460–467
24. House, C., Wettenhall, R. E. H. & Kemp, B. E. (1987) J. Biol. Chem. **262**, 772–777
25. House, C. & Kemp, B. E. (1987) Science **238**, 1726–1728
26. Marais, R. M. & Parker, P. J. (1989) Eur. J. Biochem. **181**, 401–406
27. Barrowman, M. M., Cockcroft, S. & Gomperts, B. D. (1986) Nature (London) **319**, 504–507
28. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) Biochem. J. **212**, 473–478
29. Manger, B., Weiss, A., Imboden, J., Laing, T. & Stobo, J. D. (1987) J. Immunol. **139**, 2755–2760
30. Nishizuka, Y. (1986) Science **233**, 305–312
31. Trilivas, I. & Brown, J. H. (1989) J. Biol. Chem. **264**, 3102–3107
32. Siess, W. & Lapetina, E. G. (1988) Biochem. J. **255**, 309–318
33. Ito, T., Tanaka, T., Yoshida, T., Onoda, K., Ohta, H., Hagiwara, M., Itoh, Y., Ogura, M., Saito, H. & Hidaka, H. (1988) J. Cell Biol. **107**, 929–937
34. Nishizuka, Y. (1988) Nature (London) **334**, 661–665
35. Shearman, M. S., Berry, N., Oda, T., Ase, K., Kikkawa, U. & Nishizuka, Y. (1988) FEBS Lett. **234**, 387–391
36. Sawamura, S., Ase, K., Berry, N., Kikkawa, U., McCaffrey, P. G., Minowada, J. & Nishizuka, Y. (1989) FEBS Lett. **247**, 353–357
37. Koretzky, G. A., Wahi, M., Newton, M. E. & Weiss, A. (1989) J. Immunol. **143**, 1692–1695
38. Schaap, D., Parker, P. J., Bristol, A., Kriz, R. & Knopf, J. (1989) FEBS Lett. **243**, 351–357
39. Pelech, S. L. & Vance, D. E. (1989) Trends Biochem. Sci. **14**, 28–30
40. Irving, H. R. & Exton, J. H. (1987) J. Biol. Chem. **262**, 3440–3443
41. Bocckino, S. B., Blackmore, P. F., Wilson, P. B. & Exton, J. H. (1987) J. Biol. Chem. **262**, 15309–15315
42. Tettenborn, C. S. & Mueller, G. C. (1988) Biochem. Biophys. Res. Commun. **155**, 249–255
43. Anthes, J. C., Eckel, S., Siegel, M. I., Egan, R. W. & Billah, M. M. (1989) Biochem. Biophys. Res. Commun. **163**, 657–664
44. Kinsky, S. C., Loader, J. E. & Benedict, S. H. (1989) Biochem. Biophys. Res. Commun. **162**, 788–793

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