Biochem. J. (1989) 260, 893–901 (Printed in Great Britain)

A protein kinase C pseudosubstrate peptide inhibits phosphorylation of the CD3 antigen in streptolysin-O-permeabilized human T lymphocytes

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Activation of human T lymphocytes leads to the phosphorylation of the CD3-antigen γ polypeptide. We have investigated a possible role for protein kinase C (PKC) in mediating this phosphorylation event by using T cells permeabilized with streptolysin-O in the presence of 120 mM-K^+ buffers containing Ca^{2+}–EGTA. The γ-chain was phosphorylated by [γ-^{32}P]ATP in permeabilized T lymphoblasts in the presence of phorbol 12,13-dibutyrate (Pdbu) or phytohaemagglutinin (PHA). Ca^{2+} alone in the range 0.5–1.0 μM also induced γ-chain phosphorylation in some T-lymphoblast preparations; that in Jurkat-6 cells occurred at lower concentrations (50–500 nM). Two experimental approaches were used to investigate the possible involvement of PKC. Firstly, when permeabilization was carried out in buffer lacking free Ca^{2+}, PKC was lost from the cells, and γ-chain phosphorylation could then no longer be induced on subsequent addition of Pdbu or PHA in 400 nM-Ca^{2+}, or 800 nM-Ca^{2+} alone, to permeabilized cells. However, when permeabilization was carried out in the presence of these three agents, PKC was translocated to intracellular membranes, and subsequent addition of [γ-^{32}P]ATP to these cells then resulted in γ-chain phosphorylation. In the second approach, induction of γ-chain phosphorylation by Pdbu, 1-oleoyl-2-acetylglycerol, 1,2-diolein, PHA or Ca^{2+} alone was effectively blocked by permeabilizing T cells in the presence of a PKC pseudosubstrate peptide (50 μM). Pseudosubstrate concentrations in the range 7–20 μM inhibited γ-chain phosphorylation by 50%. In contrast, addition of four other 'irrelevant' basic peptides (50 μM) did not result in detectable inhibition, and 50 μM-pseudosubstrate did not inhibit the phosphorylation of 17 other polypeptides isolated from permeabilized T cells. These data suggest that Pdbu-, 1,2-diacylglycerol-, PHA- and Ca^{2+}-induced phosphorylation of the CD3-antigen γ chain in permeabilized T cells is mediated by PKC.

INTRODUCTION

Antigen recognition and response in T lymphocytes is mediated by the antigen-receptor–CD3 complex [1]. The receptor for antigen (Ti) comprises an α–β heterodimer, associated non-covalently with the γ (M_r 26000), δ (M_r 21000) and ϵ (M_r 19000) subunits of the CD3 antigen [2]. Homologous subunits, together with two additional polypeptides (ζ and η) which also associate with Ti, have been described for the murine antigen-receptor complex [3,4]. It is widely thought that the polypeptides which associate with Ti are involved in mediating T-cell responses to antigen recognition [1,2,5]. It is therefore of interest that an immediate consequence of activation of T cells by specific antigen [6], or by agonistic antibodies against the CD2 antigen [7], is the phosphorylation of the CD3-antigen γ-chain at serine residues, and of the ζ chain at tyrosine residues [8]. Phosphorylation of the γ chain also occurs on treatment of human T cells with mitogenic lectins, phorbol esters or ionomycin [9].

The activity of PKC is known to increase during the activation of T cells via the Ti–CD3 or CD2 pathways (reviewed in [10]), since activation is accompanied by translocation of PKC to the membrane [11] and by a marked increase in phosphorylation of the M_r-80000 cytosolic protein, a known substrate for PKC [12–14]. Specific antigen, mitogenic lectins, or antibodies which bind to Ti, CD3 or CD2 lead to PtdIns turnover in T cells, with the consequent production of DAG and InsP_3 [15–17]. Since it is known that DAG activates PKC [18], and that InsP_3 increases intracellular Ca^{2+} by releasing Ca^{2+} from internal stores [19], it seems clear that PtdIns turnover represents one of the important mechanisms whereby PKC activation can occur during T-cell activation. However, direct evidence that the CD3 antigen is a substrate for PKC is not yet available, and a role for other Ca^{2+}-regulated kinase(s) is implicated by the finding that ionomycin induces γ-chain phosphorylation, yet apparently without any concomitant PKC activation [20]. Furthermore, a single serine residue in the CD3-antigen γ chain (Ser-126) is apparently phosphorylated upon activation of T cells with phorbol esters [9], whereas Ser-123 of the γ chain is preferentially phosphorylated *in vitro* by purified PKC [21]. Although these anomalous site-mapping results may be explained by the differential actions of phosphatases and/or PKC isoenzymes [10,22], such data indicate that more direct evidence is necessary to demonstrate a role for PKC in CD3-antigen phosphorylation.

In contrast with cell lines such as Swiss 3T3 fibroblasts [12] and murine T-cell hybridomas [23], it has not proved possible to remove PKC from normal human T cells by

Abbreviations used: PKC, protein kinase C; Ti, the α–β heterodimer of the T-lymphocyte antigen-receptor complex; OAG, 1-oleoyl-2-acetylglycerol; DAG, 1,2-diacylglycerol; PHA, phytohaemagglutinin; Pdbu, phorbol 12,13-dibutyrate; PAGE, polyacrylamide-gel electrophoresis.

chronic phorbol ester treatment [24]. For this reason, we have developed novel techniques using streptolysin-O-permeabilized human T cells [25] in order to investigate PKC-mediated events. The involvement of PKC was investigated by two main experimental approaches. Firstly, conditions were established whereby the presence or absence of the enzyme in permeabilized cells could be correlated with γ-chain phosphorylation. Secondly, a pseudosubstrate peptide inhibitor of PKC was used to inhibit the enzyme in permeabilized cells. The results obtained by both approaches suggest that phorbol ester-, PHA- and Ca^{2+}-induced phosphorylation of the CD3-antigen γ chain in permeabilized T cells is mediated through PKC.

MATERIALS AND METHODS

Materials

Pdbu, OAG, 1,2-diolein and other biochemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Purified PHA and streptolysin-O were from Burroughs Wellcome. The C5 monoclonal antibody against PKC (1.55 mg of protein/ml), other immunological reagents, pre-stained M_r markers and [γ-^{32}P]ATP were from Amersham International. The UCHT1 (IgG1) mouse monoclonal antibody against the CD3 antigen [26] was purified as described in ref. [9]. Peptides were synthesized as previously described [10]. Cell-culture materials were from Gibco, Paisley, Scotland, U.K. Cellulose nitrate sheets were from Schleicher and Schüll, Dassell, Germany.

Cell culture

Human T lymphoblasts were prepared by stimulating peripheral blood mononuclear cells [10^6 cells/ml of RPMI 1640/10% (v/v) foetal-calf serum] with 5 μg of PHA/ml for 72 h. After washing of cells, growth was maintained for up to 10 days by supplementing the medium every 2 days with 0.1 nM-interleukin 2, as previously described [6]. Cells were washed free of interleukin 2 for 2–4 days before experiments, in order to induce quiescence. The Jurkat 6 human T leukaemia-cell line was cultured in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (50 μg/ml) and 5% foetal-calf serum.

Buffers

The 'intracellular buffer' used during cell permeabilization contained 13.6 mM-$MgCl_2$, 94 mM-KCl, 12.5 mM-Pipes, pH 7.4, and 12.5 mM-EGTA with various concentrations of $CaCl_2$. 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. The 'chelator' computer program [27] was used to calculate $CaCl_2$ concentrations giving free Ca^{2+} in a range up to 1 μM.

Cell permeabilization

Cells were washed twice before permeabilization, firstly in phosphate-buffered saline (10 mM-sodium phosphate/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. Permeabilization was carried out at 37 °C by adding 0.1 ml of streptolysin-O (final concn. 0.4 i.u./ml) to 2×10^7 washed cells suspended in 0.4 ml of intracellular buffer. These conditions give optimal permeabilization of T cells (results not shown); variations in this protocol are specified in Figure legends. Addition of streptolysin-O entailed a 0.8 dilution factor in the intracellular buffer, such that the final concentrations of buffer components were 11 mM-$MgCl_2$, 120 mM-K^+, 10 mM-Pipes, 10 mM-EGTA and various concentrations of $CaCl_2$. After exposure to streptolysin-O, permeabilized cells were either recovered by centrifuging for 10 s at 6500 rev./min in an MSE Microcentaur instrument, or, when required for further experimentation, by centrifuging at 1200 rev./min for 5 min in a Centaur 2 instrument.

Immunoblotting

Proteins were separated by SDS/PAGE on 8% (w/v) polyacrylamide slab gels under reducing conditions as described by Laemmli [28], before being electro-transferred to 0.1 μm-pore-size nitrocellulose sheets overnight at 0.25 A. The C5 monoclonal antibody was used at a 1:100 dilution to detect PKC. Bound antibody was detected with horseradish peroxidase and diaminobenzidine as previously described [29].

Radiolabelling and immunoprecipitation

Phosphorylation of proteins in permeabilized cells was carried out by mixing 100 μCi of 2 mM-[γ-^{32}P]ATP in 50 μl of intracellular buffer with 0.1 ml of streptolysin-O before adding them to 0.35 ml portions of 2×10^7 washed cells at 37 °C, giving a final concentration of 200 μM-[γ-^{32}P]ATP in the permeabilization media. Further additions of PKC activators or inhibitors were added in a volume of 10 μl or less to the streptolysin-O/[γ-^{32}P]ATP mixture before its addition to washed cells. Permeabilization and phosphorylation were continued for 2 min before stopping the reaction by centrifuging cells at 13000 *g* for 10 s in an MSE instrument, followed by rapid removal of the supernatant, and subsequent addition to the cell pellet on ice of 1.0 ml of lysis buffer, containing 10 mM-Tris/HCl, pH 7.4, 1% Nonidet P-40, 150 mM-NaCl, 1 mM-EDTA, 1 mM-EGTA, 20 mM-pyrophosphate, 50 mM-NaF, 4 μg of leupeptin/ml and 1 mM-benzamidine. The CD3 antigen was then immunoprecipitated by using the UCHT1 antibody attached to Sepharose and separated by SDS/PAGE (non-reducing 12%-acrylamide gels) as described in ref. [6]. Analysis of the kinetics of CD3 γ-chain phosphorylation showed that maximal [^{32}P]P_i incorporation was obtained within 2–3 min (results not shown). Phosphorylated PKC was immunoprecipitated from the same lysates by first adding dithiothreitol and SDS to final concentrations of 1 mM and 0.1% (w/v) respectively, followed by 10 μl of the stock C5 antibody, 1 μl of rabbit anti-mouse IgG and 50 μl of Protein A–Sepharose with mixing for 1 h at 4 °C [30]. Immunoprecipitates were washed with 2×1 ml of the lysis buffer containing 1 mM-dithiothreitol and 0.1% SDS, before separation by SDS/PAGE (8%-acrylamide reducing gels).

^{32}P-labelled proteins were detected by autoradiography using Kodak XAR-5 or XOMAT-S X-ray film with DuPont intensifying screens. Films were scanned with an LKB UltraScan XL densitometer.

RESULTS

Characterization of streptolysin-O-permeabilized T cells

Addition of streptolysin-O to Jurkat-6 human T leukaemia cells (Fig. 1*a*) and T lymphoblasts (results not shown) led to a rapid loss of lactate dehydrogenase, which was essentially complete within 10 min, in agreement with previous work on other mammalian cells [31]. Cytosolic phosphatases leaked from the cells with a closely similar time course (results not shown). Fig 1(*b*) shows that there was also a rapid loss of PKC from Jurkat-6 cells after permeabilization in the presence of 400 nM-Ca^{2+}, so that the enzyme was detected by immunoblotting in the cytosolic fraction within 1 min, and was no longer detectable in the cell pellet after 3 min. The loss of lactate dehydrogenase and PKC therefore followed an approximately similar time course, considerably faster than the rate of loss of these enzymes from digitonin-permeabilized cells [32].

The association of PKC with the membrane fraction after treatment of cells with phorbol esters has been observed in many cell types [18,33,34]. Most studies have involved homogenization of cells before PKC assay in subcellular fractions. Fig. 1(*c*) shows that the presence of

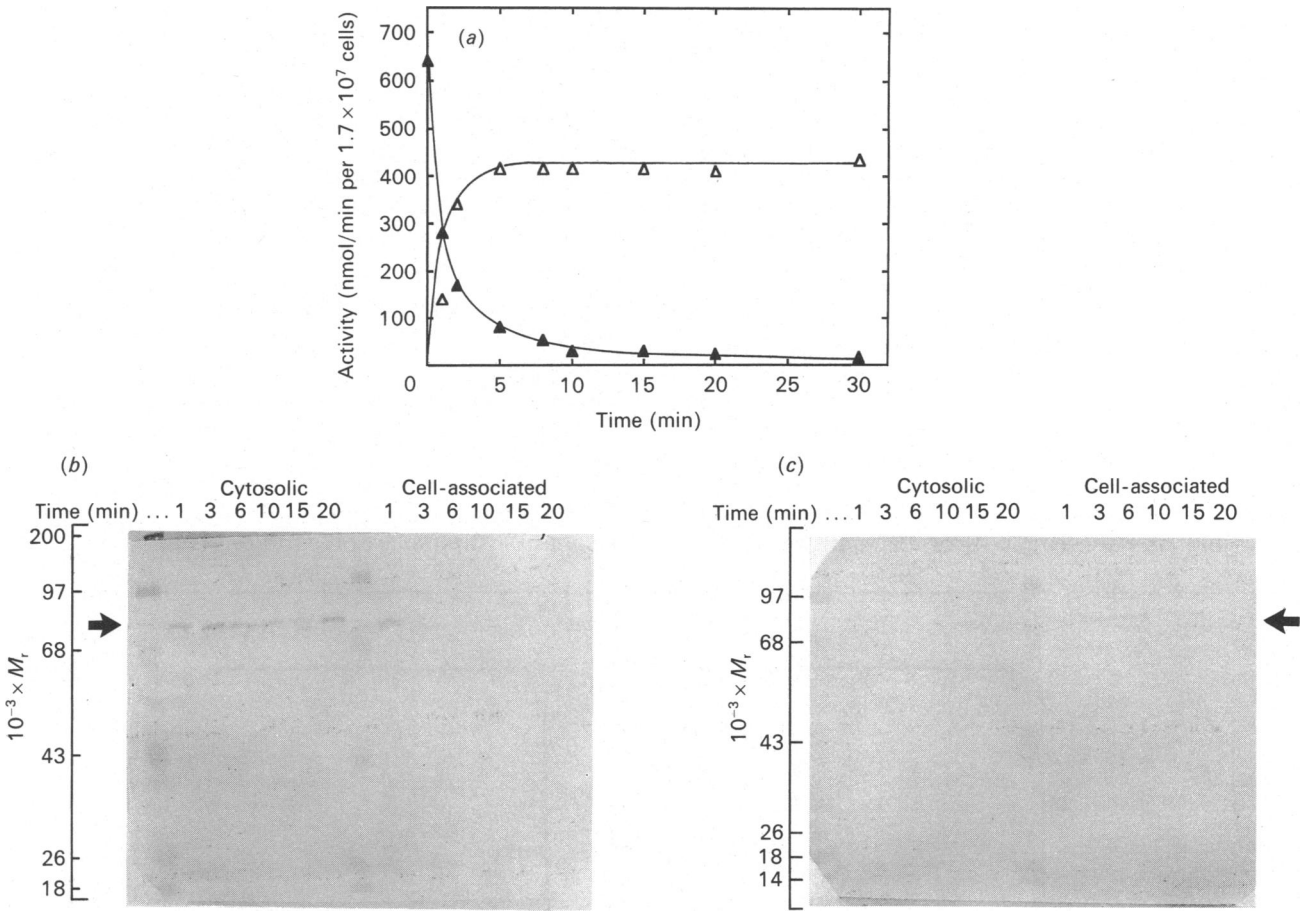

Fig. 1. Characterization of streptolysin-O-permeabilized Jurkat-6 cells

(*a*) Loss of lactate dehydrogenase after addition of streptolysin-O. Jurkat-6 cells (2.4×10^8) were washed and resuspended in 8 ml of intracellular buffer containing 80 nM Ca^{2+} (final concn.). A 0.57 ml sample (1.7×10^7 cells) was removed before addition of streptolysin-O and centrifuged for 10 s at 13000 ***g*** before removal of the supernatant and lysis of the cell pellet with 2% (v/v) Triton X-100 in ice. These samples were the zero-time controls. After initiation of permeabilization, 0.76 ml samples (1.7×10^7 cells) were removed at the times shown and centrifuged as above; the supernatant was recovered and the cell pellet resuspended in permeabilization buffer. Lactate dehydrogenase was assayed [29] in samples of untreated cells, supernatant or permeabilized cells. The values shown are means of duplicate assays. The apparent yield of lactate dehydrogenase in streptolysin-O-treated cells and supernatant combined was 70–75% of that in untreated cells. △, Cytosolic activity; ▲, cell-pellet-associated activity. (*b*) Loss of PKC after addition of streptolysin-O. Jurkat-6 cells (1.5×10^8) were washed as for Fig. 1(*a*), before suspension in 6 ml of intracellular buffer (final volume) containing 400 nM-Ca^{2+} (final concn.). After addition of streptolysin-O (0.4 i.u./ml), 1 ml samples (2.5×10^7 cells) were removed at the times shown and centrifuged as in Fig. 1(*a*). Protein from the supernatant fractions was recovered by precipitation with 80% (v/v) ethanol at −20 °C overnight. The total protein recovered in the supernatant and permeabilized cell fractions was separated by SDS/PAGE, and PKC was detected by immunoblotting as described in the Materials and methods section. A faint band of PKC was detected in the original immunoblot of the cell-associated material after exposure to streptolysin-O for 3 min. (*c*) PKC remains associated with the cell pellet after addition of Pdbu and streptolysin-O. The experimental details were as for Fig. 1(*b*), except that 100 nM-Pdbu was added together with the streptolysin-O to Jurkat-6 cells.

Pdbu during permeabilization of Jurkat-6 cells led to a striking association of PKC with the cell pellet, which persisted for at least 15 min. Results identical with those shown in Figs. 1(*b*) and 1(*c*) were also obtained with T lymphoblasts. Thus streptolysin-O permeabilization provides a convenient system for detecting PKC translocation in T cells without cell breakage. Fig. 1 also shows that permeabilized T cells could be prepared containing PKC, yet lacking in cytosolic markers.

Immunoprecipitation of ^{32}P-labelled PKC from permeabilized cells

Activated PKC has previously been shown to be autophosphorylated [35], and the enzyme is also a substrate for other endogenous kinase(s) [36]. The Western-blotting results presented in Figs. 1(*b*) and 1(*c*) were confirmed and extended by immunoprecipitating ^{32}P-labelled PKC from lysates of permeabilized cells. This technique has the advantage that PKC can be conveniently detected in the same lysates used to immunoprecipitate the radiolabelled CD3 antigen. Figs. 2 and 3 show that the presence or absence of PKC (Figs. 2*a* and 3*a*) correlated with the ability of various agents to induce CD3-antigen phosphorylation (Figs. 2*b* and 3*b*). For clarity, the results relating to PKC are described first, followed by data for the CD3 antigen. Thus, Fig. 2(*a*) shows that phosphorylated PKC remained associated with cells which were permeabilized in the presence of [γ-^{32}P]ATP and Pdbu (track 1) or PHA (track 2) with 400 nM-Ca^{2+}, or 800 nM-Ca^{2+} alone (track 3). However, when cells were permeabilized in a large excess of intracellular buffer in the nominal absence of Ca^{2+}, ^{32}P-labelled PKC could no longer be detected in the cell pellets after a subsequent phosphorylation step in the presence of Pdbu (track 4) or PHA (track 5) with 400 nM-Ca^{2+}, or 800 nM-Ca^{2+} alone (track 6). These data confirm the findings in Fig. 1(*b*), namely that PKC was readily lost from permeabilized cells, provided that the Ca^{2+} concentration in the intracellular buffer was 400 nM or less. In contrast with these results, Fig. 3(*a*) shows that when cells were permeabilized in a large excess of buffer containing 400 nM-Ca^{2+} in the presence of either Pdbu (track 4) or PHA (track 5), or containing 800 nM-Ca^{2+} alone (track 6), ^{32}P-labelled PKC was then readily detectable after a subsequent phosphorylation step. Interestingly, the incorporation of [^{32}P]P_i into PKC was markedly increased when phosphorylation was carried out in the absence of cytosol (Fig. 3*a*, cf. tracks 1–3 and 4–6). This appears to be due to the removal of cytosolic phosphatases, which has the effect of diminishing the dephosphorylation of phosphorylated proteins in washed permeabilized T cells (see refs [21,29]; also D. R. Alexander, unpublished work).

Taken together, the data of Figs. 2(*a*) and 3(*a*) demonstrate that cell permeabilization in the nominal absence of Ca^{2+} resulted in an apparent complete loss of PKC from cells, whereas permeabilization in the presence of Pdbu or PHA with 400 nM-Ca^{2+}, or 800 nM-Ca^{2+} alone, induced translocation of PKC to the membrane of permeabilized cells.

Phosphorylation of the CD3 antigen in permeabilized cells

Phosphorylation of the CD3-antigen γ chain was induced by adding Pdbu, streptolysin-O and [γ-^{32}P]ATP simultaneously to T lymphoblasts (Figs. 4*a* and 4*b*) or Jurkat-6 cells (Fig. 4*b*). Ca^{2+} alone either had no effect in T lymphoblasts (Fig. 4*a*), or in the concentration range 0.5–1.0 μM induced low levels of γ-chain phosphorylation in some T-lymphoblast preparations (Fig. 4*b*; cf. Figs. 2*b*

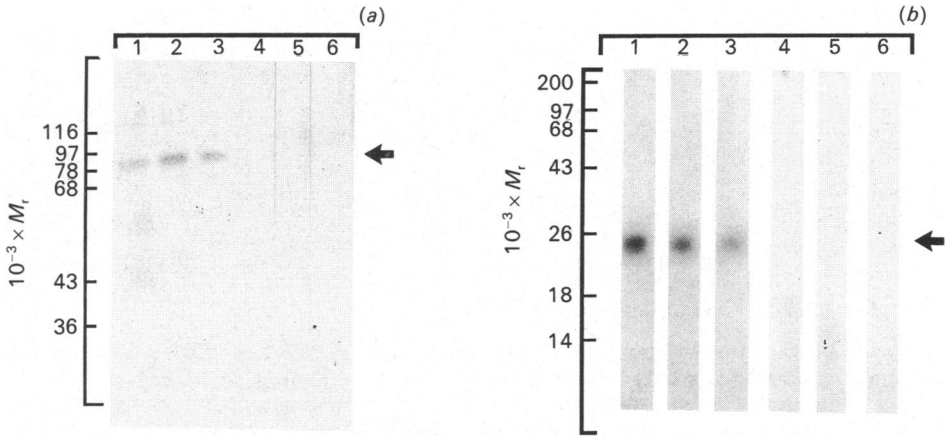

Fig. 2. Removal of PKC from permeabilized T lymphoblasts correlates with a lack of CD3-antigen γ-chain phosphorylation

(*a*) Immunoprecipitation of PKC. Washed T lymphoblasts (1.2×10^8) were separated into two samples, A and B. Cells A were permeabilized with streptolysin-O and phosphorylated under the conditions detailed below (tracks 1–3) as described in the Materials and methods section. Cells B were incubated with streptolysin-O for 10 min in 8 ml of intracellular buffer in the nominal absence of Ca^{2+}, after which cells were centrifuged at 1200 rev./min for 5 min, the supernatant was removed, and the permeabilized cells were then phosphorylated (tracks 4–6) under conditions identical with those used for tracks 1–3. Cell lysates equivalent to 2×10^7 cells were used for each track. Tracks: 1 and 4, +40 nM-Pdbu in 400 nM-Ca^{2+}; 2 and 5, +10 μg of PHA/ml in 400 nM-Ca^{2+}; 3 and 6, +800 nM-Ca^{2+} only. After phosphorylation, cell lysates were sequentially immunoprecipitated with antibodies against the CD3 antigen and PKC, as described in the Materials and methods section. The Figure shows an autoradiograph of phosphorylated PKC separated by SDS/PAGE. (*b*) Autoradiograph of an SDS/PAGE analysis of the CD3 antigen immunoprecipitated from the lysates corresponding to the same tracks shown in Fig. 2(*a*); the arrow indicates the position of the γ chain.

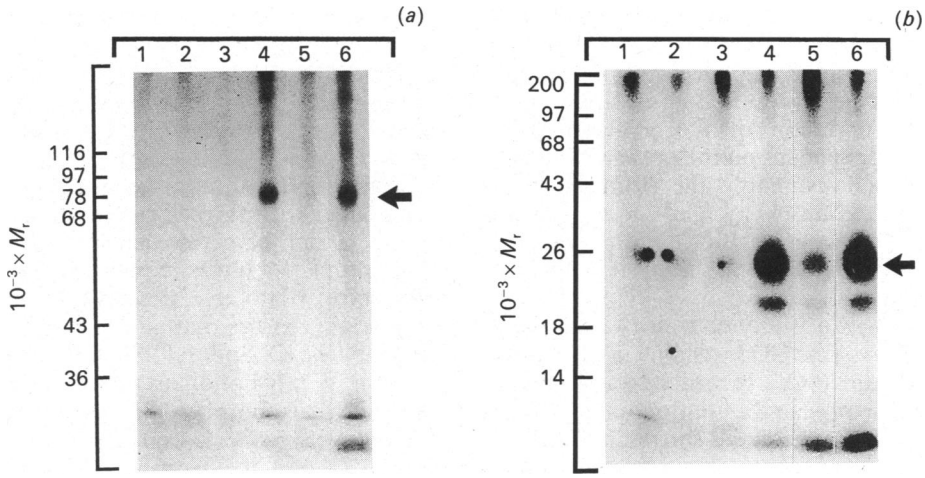

Fig. 3. Association of PKC with permeabilized T lymphoblasts correlates with CD3-antigen γ-chain phosphorylation

(*a*) Washed T lymphoblasts (1.2×10^8) were separated into four portions, A–D. Cells A (6×10^7 cells) were permeabilized with streptolysin-O and phosphorylated under the conditions detailed below (tracks 1–3) as described in the Materials and methods section. Cells B–D were resuspended in 8 ml portions of intracellular buffer containing 40 nM-Pdbu (B, track 4) or 10 μg of PHA/ml (C, track 5) in 400 nM-Ca^{2+}, or 800 nM-Ca^{2+} alone (D, track, 6), before permeabilization with streptolysin-O for 10 min. Permeabilized cells were recovered as described in Fig. 2, the supernatant was removed, and the cells were resuspended in intracellular buffer containing 400 nM-Ca^{2+} (cells B and C) or 800 nM-Ca^{2+} (cells D). Cells were then phosphorylated as shown. Cell lysates equivalent to 2×10^7 cells were used for each track. Phosphorylation conditions were as follows: track 1, +40 nM-Pdbu in 400 nM-Ca^{2+}; track 2, +10 μg of PHA/ml in 400 nM-Ca^{2+}; tracks 3 and 6, +800 nM-Ca^{2+} only; tracks 4 and 5, +400 nM-Ca^{2+} only. Cell lysates were analysed as in Fig. 2. The figure shows an autoradiograph of an SDS/PAGE gel of phosphorylated PKC (arrowed). Note that the faints bands of PKC in tracks 1–3 were more clearly visible in the original autoradiograph. The greater prominence of PKC bands in tracks 4–6 is probably not due to a higher amount of the enzyme itself, but to a higher [^{32}P]P_i incorporation owing to the absence of cytosolic phosphatases (see the text). (*b*) Autoradiograph of phosphorylated CD3 antigen immunoprecipitated from the lysates corresponding to the same tracks shown in Fig. 3(*a*); the arrow indicates the position of the γ chain. The spots in tracks 1–3 are artefacts, and do not affect the interpretation of the experiment.

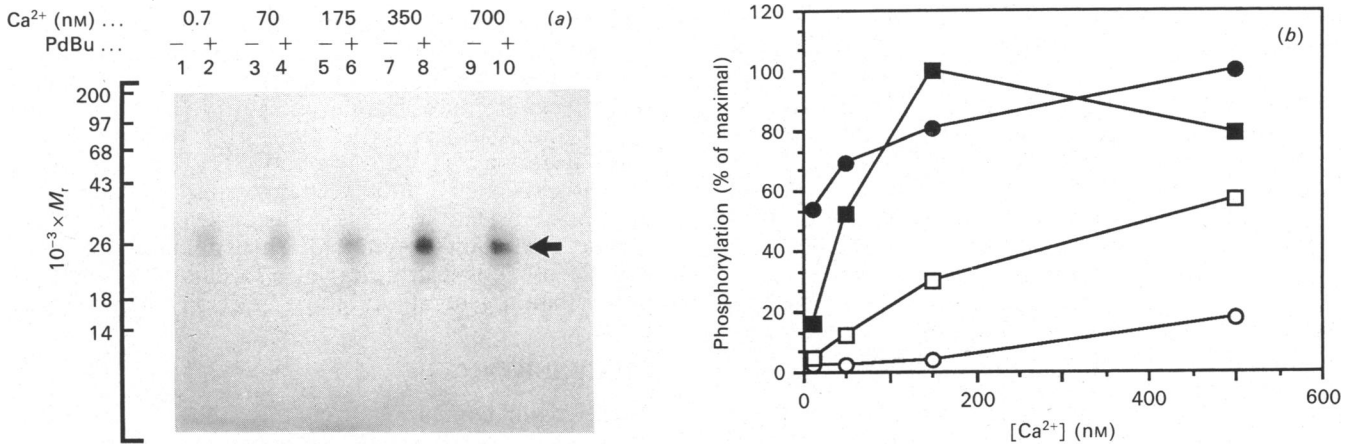

Fig. 4. Stimulation by Ca^{2+} and Pdbu of CD3 γ-chain phosphorylation in permeabilized Jurkat-6 cells and T lymphoblasts

(*a*) T lymphoblasts (2×10^8) were washed and then divided into five equal portions before resuspension in intracellular buffer to give the final free Ca^{2+} concentrations as shown. Each sample was further divided into two identical portions of 2×10^7 cells, which were permeabilized and phosphorylated in either the absence or the presence of 100 nM-Pdbu for 2 min. The Figure shows the autoradiograph of the phosphorylated CD3 antigen after immunoprecipitation and separation by SDS/PAGE; the arrow indicates the position of the γ chain. Further details are in the Materials and methods section. (*b*) Cells (1.6×10^7) were permeabilized and phosphorylated under the conditions shown in Fig. 4(*a*). The Figure shows densitometric scans of autoradiographs of the phosphorylated γ chain separated by SDS/PAGE. The data shown are representative results of one of three separate experiments. Key: ○, ●, T lymphoblasts; □, ■, Jurkat-6 cells; ○, □, no added Pdbu; ●, ■, +100 nM-Pdbu.

and 3*b*, track 3). In marked contrast with T lymphoblasts, γ-chain phosphorylation was observed in Jurkat-6 cells in the presence of Ca^{2+} alone at concentrations of 50–500 nM (Fig. 4*b*). Furthermore, Fig. 4(*b*) shows that Pdbu-induced phosphorylation of the γ chain in T lymphoblasts was less dependent on the presence of Ca^{2+} than in Jurkat-6 cells. Presumably the stimulation of Pdbu-induced phosphorylation by Ca^{2+} was due to promotion of Pdbu binding to PKC, with the concomitant translocation and activation of the enzyme [37–40].

Mitogenic lectins or Ca^{2+} alone, conceivably, may induce phosphorylation of the CD3 γ chain via activation of a kinase distinct from PKC. In order to investigate this possibility, the CD3 antigen was immunoprecipitated from the cell lysates of the experiment shown in Fig. 2(*a*). Fig. 2(*b*) shows that the γ chain was phosphorylated provided that PKC was present in the cells (cf. Figs. 2*a* and 2*b*, tracks 1–3), but, after removal of PKC (Fig. 2*a*, tracks 4–6), its phosphorylation was no longer induced by Pdbu or PHA in the presence of 400 nM-Ca^{2+}, nor by 800 nM-Ca^{2+} alone (Fig. 2*b*, tracks 4–6). Thus the presence or absence of PKC correlated with the induction or lack of induction of γ-chain phosphorylation. These data appear to exclude a role for a membrane-associated kinase in phosphorylating the γ chain under the conditions of this experiment. However, from the data of Figs. 1(*a*) and 1(*b*), it appears that under the 2 min permeabilization and phosphorylation-assay conditions of tracks 1–3 in Fig. 2, many cytosolic kinases could still be present inside the cells. In contrast, cytosolic kinases were presumably no longer present in the cells used for Fig. 2, tracks 4–6, which were permeabilized for 10 min and the supernatant was removed, before phosphorylation (see the legend to Fig. 2). Thus, in order to investigate a possible role for cytosolic kinases apart from PKC, the CD3 antigen was immunoprecipitated from the cell lysates of the experiment shown in Fig. 3(*a*). The key difference from Fig. 2 in this experiment was that the 10 min permeabilization and washing step before phosphorylation was carried out in the presence of agents shown to retain PKC inside the cells (Fig. 3*a*, tracks 4–6). Fig. 3(*b*) shows that under these conditions, phosphorylation of the γ chain was readily detected, whether induced by Pdbu (track 4) or PHA (track 5) with 400 nM-Ca^{2+}, or by 800 nM-Ca^{2+} alone (track 6). As with ^{32}P-labelled PKC, there was a marked increase of [^{32}P]P_i incorporation into the γ chain after removal of cytosol (Fig. 3*b*, cf. tracks 1–3 and 4–6), again probably due to the absence of cytosolic phosphatases. Similarly most cytosolic kinases apart from PKC were presumably lost from the cells relating to Fig. 3(*b*), tracks 4–6. This experiment therefore confirms the specific correlation between the presence or absence of PKC and the ability of Pdbu or PHA with 400 nM-Ca^{2+}, or 800 nM-Ca^{2+} alone, to induce CD3 γ-chain phosphorylation in permeabilized T lymphoblasts.

Inhibition of CD3-antigen and PKC phosphorylation by a pseudosubstrate inhibitor

It was recently reported that an 18-amino-acid peptide, derived from the regulatory domain of PKC, known as the 'pseudosubstrate prototope', is a potent inhibitor of the enzyme *in vitro* [41]. A shorter form of this peptide, with the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val, had the lowest concentration giving 50% inhibition of phosphorylation of a glycogen synthase peptide by PKC [41]. Thus this form of the pseudosubstrate was used in the present studies.

To assess the rate of entry of peptides into permeabilized cells, portions of streptolysin-O and ^{32}P-labelled peptide A3 (M_r 1671; see ref. [21]) were incubated with T lymphoblasts, and the amount of ^{32}P-labelled A3 associated with the cell pellet was measured at intervals up to 10 min of permeabilization. The results indicated that about 5 min was required for maximal entry of ^{32}P-labelled A3 into cells (results not shown).

Fig. 5(*a*) shows that addition of pseudosubstrate with streptolysin-O and [γ-^{32}P]ATP during the permeabilization of T lymphoblasts led to a marked inhibition of γ-chain phosphorylation induced by Pdbu, PHA or the diacylglycerols OAG or 1,2-diolein. Fig. 5(*b*) shows that 10–15 μM-pseudosubstrate inhibited Pdbu- or PHA-induced γ-chain phosphorylation by 50%. The phosphorylation of the γ chain induced by 800 nM-Ca^{2+} was also markedly inhibited by pseudosubstrate (Fig. 5*c*). Further studies in Jurkat-6 cells (Fig. 5*d*) showed that the extent of inhibition of Ca^{2+}-induced γ-chain phosphorylation with 10–100 μM-pseudosubstrate was comparable with that found for inhibition of Pdbu- or PHA-induced phosphorylation in T lymphoblasts (Fig. 5*b*). In contrast, higher concentrations of pseudosubstrate were required to inhibit PKC phosphorylation in Jurkat-6 cells (Fig. 5*d*). Furthermore, inhibition of phosphorylation of an unidentified polypeptide of M_r 100000, detected in PKC immunoprecipitates, only occurred at a high (100 μM) pseudosubstrate concentration (Fig. 5*d*), showing that the pseudosubstrate was selective in its action.

It was considered that inhibition of phosphorylation by the pseudosubstrate was due to non-specific effects, e.g. an action as a polycation mediated by its high percentage (39%) of basic residues. However, addition of four other 'irrelevant' peptides, each rich in basic residues (15–33%), did not cause any detectable inhibition of γ-chain phosphorylation induced by Pdbu or by 800 nM-Ca^{2+} (results not shown, and Fig. 5*c*, track 3). Furthermore, when 50 μM-pseudosubstrate was added to permeabilized Jurkat-6 cells with [γ-^{32}P]ATP, in the absence or presence of cyclic AMP and/or various concentrations of Ca^{2+}, followed by SDS/PAGE analysis of cell-associated ^{32}P-labelled proteins, no inhibition of phosphorylation of 17 distinct phosphorylated polypeptides could be detected. These data are consistent with the pseudosubstrate having a specific effect on PKC, rather than a general inhibitory effect on many kinases.

DISCUSSION

The major conclusion from this study is that Pdbu, PHA and Ca^{2+} induce CD3-antigen γ chain phosphorylation in T cells via a process mediated by PKC. This conclusion derives from data obtained by two complementary experimental approaches to the study of γ-chain phosphorylation in permeabilized cells. In the first approach, PKC was eluted from T lymphoblasts in the nominal absence of Ca^{2+} until the enzyme was no longer detectable. Under these conditions γ-chain phosphorylation was not detected when the permeabilized cells were subsequently incubated with Pdbu or PHA in 400 nM-Ca^{2+}, or with relatively high (800 nM) concentrations of Ca^{2+} alone (Fig. 2). Yet, when

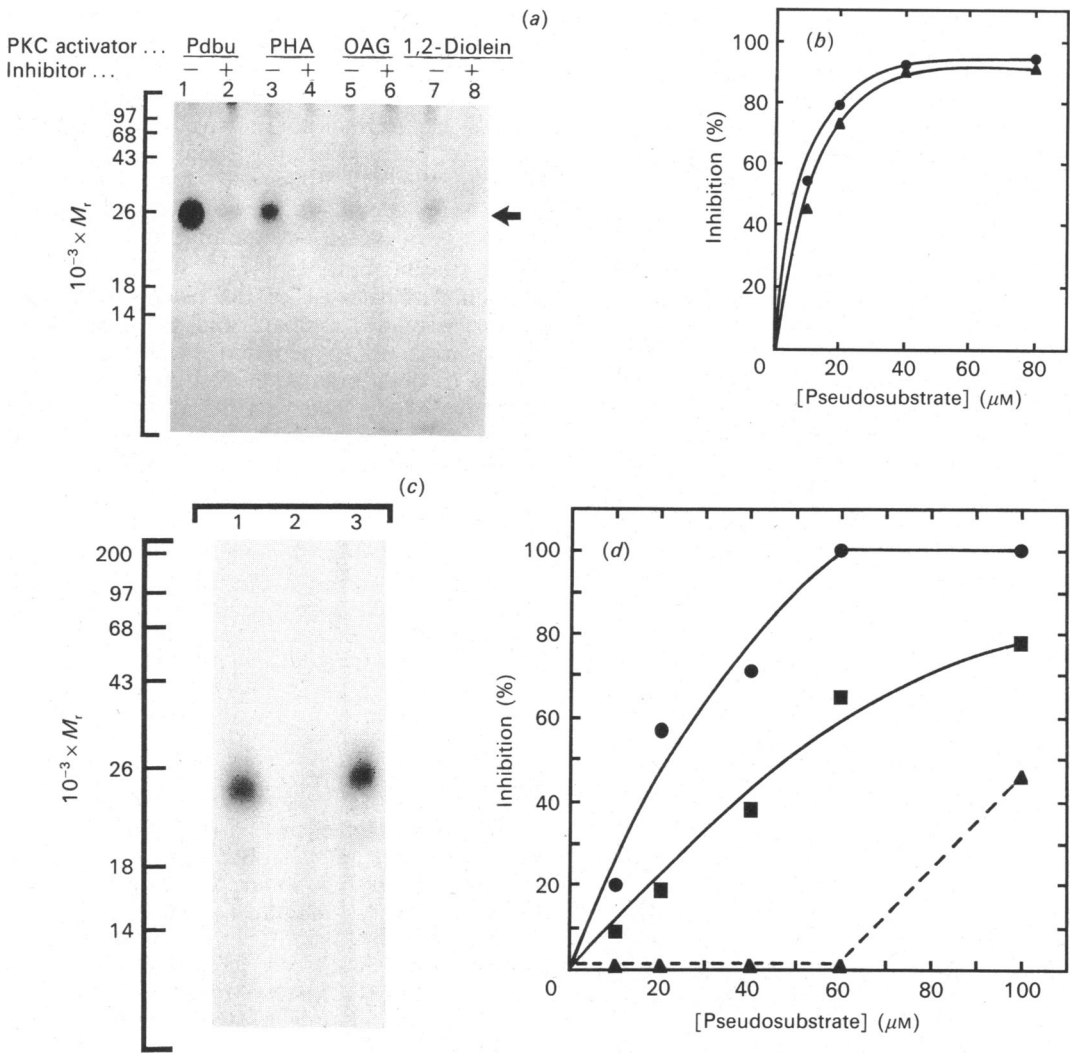

Fig. 5. Inhibition of CD3-antigen γ-chain phosphorylation by the pseudosubstrate in permeabilized T lymphoblasts and Jurkat-6 cells

(*a*) Batches of 2×10^7 T lymphoblasts were permeabilized and phosphorylated in intracellular buffer containing 400 nM-Ca^{2+} (final concn.), and the induction of γ-chain (arrowed) phosphorylation was analysed as described in the Materials and methods section. Phosphorylation was induced with 40 nM-Pdbu (tracks 1 and 2), 10 μg of PHA/ml (tracks 3 and 4), 200 nM-OAG (tracks 5 and 6) or 200 nM-1,2-diolein (tracks 7 and 8), in the absence (tracks 1, 3, 5 and 7) or presence (tracks 2, 4, 6 and 8) of 50 μM-pseudosubstrate. (*b*) Phosphorylation of the γ-chain induced by 40 nM-Pdbu (●) or 10 μg of PHA/ml (▲) in permeabilized T lymphoblasts was carried out as in Fig. 5(*a*) in the presence of the final pseudosubstrate concentrations shown. (*c*) Phosphorylation of the γ-chain induced by 800 nM-Ca^{2+} in permeabilized T lymphoblasts was carried out as in Fig. 5(*a*). Tracks: 1, no addition; 2, +50 μM-pseudosubstrate ; 3, +50 μM-protein kinase A inhibitor (15% basic residues), i.e. Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp. (*d*) Phosphorylation of permeabilized Jurkat-6 cells in the presence of 400 nM-Ca^{2+} was carried out with 2×10^7 cells per reaction. Phosphorylated γ-chain and PKC were analysed in the same cell lysates as described in the Materials and methods section. The data for this Figure were obtained from densitometric scans of autoradiographs of SDS/PAGE gels of either γ-chain (●) or PKC (■) phosphorylation. An unidentified phosphorylated M_r-100000 band in the PKC immunoprecipitates (▲) was scanned for comparison.

the permeabilization process was carried out in the presence of these three agents, PKC was retained inside the cells and phosphorylation of the γ chain could be subsequently demonstrated by addition of [γ-^{32}P]ATP (Fig. 3). Presumably PKC was both translocated to the plasma membrane and activated during the permeabilization step under the conditions used in the latter experiment [18,37–40].

In the second approach, a role for PKC was confirmed by the capacity of the pseudosubstrate peptide inhibitor to block CD3-antigen γ-chain phosphorylation induced by Pdbu, DAG, PHA or Ca^{2+} alone in both permeabilized T lymphoblasts and Jurkat-6 cells (Fig. 5). The phosphorylation levels of many other proteins remained unchanged in the presence of pseudosubstrate, supporting the findings by House & Kemp [41] that this peptide has a specific action on PKC. Indeed, on theoretical grounds a peptide sequence based on the regulatory domain of PKC itself, and acting as a competitive inhibitor [41], should demonstrate considerable specificity. The apparent pseudosubstrate concentration range required to produce 50% inhibition of γ-chain phosphorylation was 7–20 μM (Figs 5*b* and 5*d*). This is some two orders of magnitude higher than that reported

to inhibit by 50% phosphorylation *in vitro* of a peptide substrate by purified PKC [41]. However, the effective intracellular pseudosubstrate concentrations in permeabilized cells may have been considerably less than the theoretical extracellular concentrations. Furthermore, the pseudosubstrate concentration required to inhibit the enzyme will be greater when PKC phosphorylates substrates for which it has a higher affinity.

Taken together, the data from the two approaches (Figs. 2, 3 and 5) strongly suggest that PHA, or Ca^{2+} alone at higher concentrations, mediates activation of PKC and consequent phosphorylation of the γ chain in permeabilized T cells. The findings with PHA are important in that they demonstrate that key components of the transmembrane signal-transducing process must remain functional after streptolysin-O treatment. Though lectins bind to a large number of T-cell surface glycoproteins [42], there is considerable evidence that the Ti–CD3 complex plays an essential role in mitogenic-lectin-induced T-cell activation [1]. Presumably, therefore, the Ti–CD3 complex also plays a role in mediating the PHA-induced activation of PKC described here in permeabilized cells.

The results obtained by using Ca^{2+} alone (Fig. 4) were initially puzzling. Although Ca^{2+} promotes the binding of PKC to membranes, the concentrations of Ca^{2+} in the intracellular buffers used in the present work are insufficient to activate the purified enzyme [37]. Furthermore, in a study on digitonin-permeabilized chromaffin cells [32], even 10 μM-Ca^{2+} was insufficient to prevent a 50% loss of PKC from the cells. However, it appears that 50–500 nM-Ca^{2+} alone is sufficient to induce a marked increase in breakdown of inositol-containing lipids in permeabilized Jurkat-6 cells, a relatively small increase also occurring in T lymphoblasts (D. A. Cantrell, unpublished work). The DAG so produced, together with Ca^{2+}, presumably then activates PKC [18]. Though Ca^{2+} may act on a PtdIns-specific phospholipase C [43], the hydrolytic products of Ca^{2+}-stimulated phospholipase A_2 could also be involved in activating PKC [44–46]. Thus the markedly greater Ca^{2+} concentrations required to induce γ-chain phosphorylation in T lymphoblasts as compared with Jurkat-6 cells (Fig. 4*b*) may be explained by differences between these cells with respect to their Ca^{2+} requirements for mobilizing various activators of PKC. In addition, such differences could be due to the expression of distinct PKC isoenzymes in these cells, which may be differentially regulated by Ca^{2+} [47–49]. For example, it has recently been shown that Jurkat-6 cells largely express the PKC_α isoenzyme, whereas relatively more of the PKC_β isoenzyme is found in T lymphoblasts (R. Marais & D. A. Cantrell, unpublished work), and there is some evidence that PKC_β is less dependent on Ca^{2+} for its activity than is PKC_α [47].

The relationship, if any, between the ionomycin-induced phosphorylation of the CD3-antigen γ chain previously observed with T lymphoblasts *in vivo* [10] and the Ca^{2+}-induced phosphorylation reported here in permeabilized cells, remains to be clarified. Ionomycin itself does not induce γ-chain phosphorylation in permeabilized T cells (D. R. Alexander, unpublished work). The main criteria for a lack of activation of PKC by ionomycin in intact T cells have been the failure of this ionophore to induce translocation of the enzyme [50] and phosphorylation of the cytosolic M_r-19000 and -80000 proteins which have been characterized as PKC substrates [20]. In the light of the present study, it is conceivable that ionomycin promotes activation of a PKC isoenzyme in T lymphoblasts which induces γ-chain phosphorylation without detectable phosphorylation of the M_r-19000 and -80000 proteins. Indeed, Ca^{2+} ionophores have previously been noted to induce hydrolysis of inositol-containing lipids [51].

The permeabilized T-cell system described here, coupled with the use of the pseudosubstrate PKC inhibitor, provides a novel and convenient method for investigating PKC-mediated events. Further work is needed to determine whether other signal-transduction pathways which lead to CD3-antigen phosphorylation remain intact in these permeabilized cells and, if so, whether these pathways are also mediated by PKC.

We are indebted to Dr. Enrique Rozengurt and Dr. Nancy Hogg for making helpful comments on this manuscript, to Dr. Jonathan Rothbard for peptide synthesis and to Kim Richardson for typing the manuscript.

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Received 8 February 1989; accepted 9 March 1989