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Phorbol ester synergizes with Ca^{2+} ionophore in activation of protein kinase C (PKC)α and PKCβ isoenzymes in human T cells and in induction of related cellular functions

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SUMMARY

Studies described herein were designed to examine the effects of 12-O-tetradecanoyl phorbol-13-acetate (TPA), and a Ca^{2+} ionophore (ionomycin), singly or in combination, on the activation and expression of the Ca^{2+}-dependent protein kinase C (PKC) isoenzymes (α, β and γ) at the protein and messenger RNA (mRNA) levels in T cells. These two agents induce the activation and proliferation of T lymphocytes by mimicking the action of inositol phospholipid-derived second messengers normally generated by triggering of the antigen-specific T-cell receptor (TcR)/CD3 complex. TPA-induced T-cell proliferation, expression of interleukin-2 receptor-alpha subunit (IL-2Rα) and transferrin receptor, CD3 down-regulation and, lastly, the cytosol-to-membrane PKC translocation (determined by an enzymatic assay or by immunoblotting with a cross-reactive anti-PKC peptide antibody) were all facilitated by ionomycin. Immunoblots with isoenzyme-specific anti-PKC monoclonal antibodies demonstrated expression of immunoreactive PKCα, PKCβ and PKCγ proteins that were translocated to the membrane upon TPA plus ionomycin stimulation. Resting T cells expressed abundant levels of mRNA for PKCα and PKCβ, but very low levels (relative to brain) of PKCγ. TPA increased by two- to threefold the expression of PKCβ, but not of PKCα or PKCγ, mRNA within 12 hr of stimulation. Ionomycin synergized with TPA in increasing the expression of PKCα and PKCβ mRNA. The two agents also synergized in inducing expression of additional activation/growth-associated genes, namely the c-*myc* protooncogene, ornithine decarboxylase (ODC) and IL-2Rα. Ionomycin alone was inactive (or marginally active) in all of these assays. The translocation of distinct Ca^{2+}-dependent PKC isoenzymes to the membrane and the up-regulation of PKCα and β mRNA suggest that at least these two isoenzymes are involved in discrete steps of the pathway leading to T-cell activation and proliferation. Moreover, the combined effects of TPA and ionomycin on T-cell function and cell-surface antigen expression appear to be due, at least in part, to their synergistic activation of distinct PKC isoenzyme(s).

INTRODUCTION

Triggering of the T-cell receptor (TcR)/CD3 complex stimulates a cascade of biochemical events that transduce the signal across the cell membrane and initiate intracellular processes leading to activation and proliferation (reviewed in ref. 1). One signal transduction pathway coupled to this receptor complex involves a phospholipase C species that hydrolyses phosphatidylinositol 4,5-bisphosphate and generates two distinct intracellular second messengers. One of these, diacylglycerol, activates protein kinase C (PKC),[2,3] and the second, inositol 1,4,5-trisphosphate, releases Ca^{2+} from the endoplasmic reticulum, thereby increasing the intracellular concentration of free Ca^{2+}.[4,5] The relative contribution of each of the two second messengers has been analysed by using phorbol esters such as TPA that mimic diacylglycerol by directly binding to, and activating, PKC,[6] and/or Ca^{2+} ionophores that stimulate Ca^{2+} influx.[7] 12-O-tetradecanoyl phorbol-13-acetate (TPA) treatment (and, hence, PKC activation) is sufficient for induction of the IL-2Rα,[8,9] but synthesis and secretion of interleukin-2 (IL-2) as well as IL-2-dependent T-cell proliferation, require an additional signal that can be provided by Ca^{2+} ionophores.[10-12] However, the exact biochemical basis for the synergistic action of TPA and Ca^{2+}

Abbreviations: IL-2, interleukin-2; IL-2Rα, IL-2 receptor-alpha (p55) chain; ODC, ornithine decarboxylase; PBL, peripheral blood lymphocytes; PHA, phytohaemagglutinin; TPA, 12-O-tetradecanoyl phorbol-13-acetate; PKC, protein kinase C; TcR, antigen-specific T-cell receptor.

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ionophores is unknown, as is the role of individual PKC isoenzymes[13 20] during the activation process.

The present study was designed to test whether ionomycin can augment the TPA-mediated stimulation of PKC enzymatic activity and, secondly, to analyse the effects of these two agents or their combination on the immunoreactive, Ca^{2+}-dependent, PKC species (α, β and γ) and on expression of the corresponding genes, using PKC-specific antibodies and cDNA probes. Here, we show that ionomycin, which in itself was inactive, synergized with TPA to induce T-cell proliferation and modulate the expression of several cell-surface antigens. These effects were paralleled by a synergistic increase in the translocation of enzymatically active or immunoreactive PKC from the cytosol to the membrane, and in the selective expression of some PKC genes, as well as other genes expressed during T-cell activation.

MATERIALS AND METHODS

Reagents

Histopaque-1077, TPA, phenylmethyl sulphonlfluoride (PMSF), soybean trypsin inhibitor, dimelthyl sulphoxide (DMSO), bovine serum albumin (BSA) (fraction V), trichloroacetic acid (TCA), ATP, phosphatidylserine, 1,2-diolein and H1 histone (type III-S) were purchased from Sigma Chemical Co. (St Louis, MO). PHA was from Gibco (Grand Island, NY). Triton X-100 and Tris were from BioRad Laboratories (Richmond, CA), DE52 cellulose and GF/F filters were from Whatman (Maidstone, U.K.), leupeptin was from Calbiochem (La Jolla, CA), aprotinin from Mobay Chemicals (New York, NY), nitrocellulose sheets from Schleicher & Schuell, human recombinant IL-2 was a gift from Hoffman LaRoche (Nutley, NJ), and [γ-^{32}P]ATP (specific activity 7000 Ci/mmol) from ICN Radiochemicals (Irvine, CA).

Cell culture

Peripheral blood lymphocytes (PBL) were prepared by Histopaque gradient centrifugation of heparinized blood from healthy volunteers. Cells were cultured in RPMI-1640 (MA Bioproducts, Walkersville, MD) supplemented with 5% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Flow Laboratories, McLean, VA), 10 mM HEPES (Irvine Scientific, Santa Ana, CA) and 5×10^{-5} M 2-mercaptoethanol (2-ME) (Sigma) in 150 cm^2 growth-area tissue culture flasks (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) in an atmosphere of 10% CO_2. Cells were stimulated with 0·5% (v/v) phytohaemagglutinin (PHA). Human recombinant IL-2 (10 U/ml) was added to the flasks on the third day. After an additional 3 days, cells were washed and cultured for 18 hr in the absence of PHA or IL-2. At this stage, the cells were >90% CD3$^+$ and 10–20% IL-2Rα^+.

Immunofluorescence and FACS analysis

Cell-surface marker expression was detected by incubation with biotinylated anti-CD3 (Leu-4) followed by avidin-conjugated fluorescein isothiocyanate (FITC) (both from Becton Dickinson Monoclonal Antibody Center, Mountain View, CA), or with the anti-human IL-2Rα antibody, Tac (a gift from Dr T. A. Waldmann, National Cancer Institute, Bethesda, MD) or OKT9 (anti-transferrin receptor antibody, Ortho Diagnostic Systems, Raritan, NJ), followed by FITC-conjugated rabbit anti-mouse Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands). Following incubation with each reagent for 30 min at 4°, cells were washed and analysed on a FACStar (Becton Dickinson, Sunnyvale, CA).

PKC isolation and enzymatic assay

PBL cytosolic and particulate fractions were prepared and PKC was partially purified by one-step elution from DE52 ion-exchange columns as described previously.[21] Active fractions were pooled, dialysed and stored at −20°. For immunoblot analyses, PKC-containing fractions were concentrated using Centricon-30 microconcentrators (Amicon, Danvers, MA). Net PKC activity was quantitated by measuring ^{32}P transfer from [γ-^{32}P]ATP into H1 histone,[21] and expressed as pmol of ^{32}P incorporated per 1×10^6 cells/min. Duplicate samples were used for each point.

Anti-PKC antibodies

The preparation and characterization of a rabbit anti-PKC peptide antibody cross-reactive with three PKC isoforms, namely, α, β and γ, was as described previously.[22] Monoclonal antibodies specific for PKCα, β and γ isoenzymes (MC-3a, MC-2a and MC-1a, respectively) were purchased from Seikagaku America, Inc. (St Petersburg, FL).

Immunoblotting

Concentrated cytosol and membrane fractions of unstimulated or stimulated T cells were fractionated by SDS–PAGE on 10% gels and electrophoretically transferred to nitrocellulose membranes.[22] Blocked membranes were incubated (2 hr at 37°) with affinity-purified anti-peptide (5 μg/ml) or monoclonal (1 μg/ml) antibody. Immunoblots were developed by treatment with a 1:1000 dilution of biotinylated goat anti-rabbit or anti-mouse IgG (Cappel, Westchester, PA) followed by ^{125}I-labelled streptavidin and autoradiography at −70° with an intensifying screen.

Slot-blot RNA analysis

Total RNA was isolated by the guanidinium/cesium chloride method[23] and quantitated spectrophotometrically at 260 nm. RNA (5 μg/slot) was applied to nitrocellulose sheets using a slot-blot microfiltration apparatus (BioRad). Following a prehybridization step, the RNA was hybridized (16 hr at 42°) with 1–3×10^6 c.p.m. of labelled probe/ml of buffer H (40% formamide, 0·05 M sodium phosphate, pH 7·5, 3 × SSC, 5 × Denhardt's reagent) containing 10% dextran sulphate (Pharmacia, Uppsala, Sweden). cDNA probes were labelled to a specific radioactivity of $1\text{–}4 \times 10^8$ c.p.m./μg by nick translation in the presence of ([α-^{32}P]dCTP, 3000 Ci/mmol). After hybridization, nitrocellulose sheets were washed in buffer H for 1 hr at 42°, followed by two washes with 2 × SSC–0·1% SDS at 50° for a total of 1 hr. Sheets were then dried and autoradiographed at −70° using Kodak XAR-5 X-ray film and intensifying screens. Autoradiograms were scanned in a laser densitometer (Ultro-Scan XL, Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The cDNA probes were kindly provided by the following investigators: PKCα, PKCβ and PKCγ[13] by Dr A. Ullrich (Genentech, Inc., South San Francisco, CA); mouse ornithine decarboxylase (ODC)[24] by Dr P. Coffino (University of California, San Francisco, CA); murine c-*myc* and β_2 microglobulin by Dr M. Haas (University of California at San Diego, CA). A full-length human IL-2Rα cDNA probe was cloned in our laboratory from mitogen-stimulated leukaemic Jurkat cells.[25]

RESULTS

Ionomycin synergizes with TPA in induction of T-cell proliferation and modulation of cell-surface receptors

An enriched human T-cell population was prepared by stimulating PBL with PHA followed by expansion of the cells in the presence of IL-2. The cells were subsequently cultured for 1–2 days in the absence of mitogen or IL-2, until their proliferation rate and IL-2Rα expression declined. Over 94% of the cells thus obtained were $CD2^+CD3^+$ and ≤15% expressed IL-2Rα. We first tested the effects of ionomycin and/or TPA on responses known to be closely associated with PKC activation. Ionomycin by itself stimulated a weak T-cell proliferation (23×10^3 versus $14{\cdot}7 \times 10^3$ c.p.m. of tritiated thymidine uptake in control cultures), whereas TPA induced a considerable mitogenic response ($55{\cdot}5 \times 10^3$ c.p.m. stimulation index of 3·8). When the two agents were combined, a clear synergistic effect was observed, namely, $139{\cdot}7 \times 10^3$ c.p.m. (stimulation index of 9·5). Similarly, this combination increased the percentage of IL-$2R\alpha^+$ and transferrin receptor positive cells and the surface density of these receptors by two- to sixfold compared to the levels induced by TPA alone. Thus, the percentages of receptor-positive cells after 2 days in cultures stimulated with nothing, TPA, ionomycin, or TPA plus ionomycin, were: IL-2Rα—11, 35, 13 and 88%; transferrin receptor—3, 10, 2 and 63%, respectively. TPA (but not ionomycin) down-regulated expression of the CD3 complex (from 89 to 18% $CD3^+$ cells), and this effect was slightly augmented by ionomycin (not shown). These results are in accordance with numerous previous studies (e.g. ref. 21; reviewed in ref. 1).

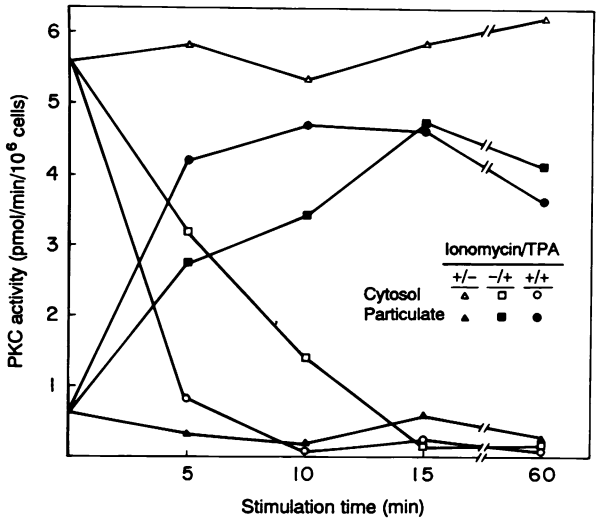

Figure 1. Ionomycin synergizes with TPA to induce PKC translocation. Human T cells (40×10^6/group) were stimulated with ionomycin (200 ng/ml), TPA (10 ng/ml) or a mixture of the two. Cells were extracted at the time intervals indicated, and cytosolic or membrane-associated PKC was partially purified by DE52 chromatography. Enzymatic activity was determined on 50-μl samples (1×10^6 cell equivalents). This experiment is representative of two other experiments.

Ionomycin synergizes with TPA in the induction of PKC redistribution

The synergistic effect of ionomycin on TPA-induced T-cell responses could reflect increased PKC activation (for example, by increasing the enzyme's affinity for TPA), and/or activation of other Ca^{2+}-dependent enzymes, that, in addition to PKC, are essential for induction of various cellular activation processes. To address the first possibility, we stimulated T cells with ionomycin and/or TPA for different time periods, and determined PKC activity in DE52-purified cytosol and membrane fractions. T-cell activation by mitogens and anti-receptor antibodies is known to be accompanied by a rapid and transient PKC translocation to the membrane, whereas TPA induces a complete and long-lasting translocation.[3,21,26–30] Over 90% of the total cellular PKC activity was located in the cytosol fraction prior to stimulation (Fig. 1), and distribution of PKC between the cytosol and membrane was not affected by ionomycin. In contrast, TPA induced a rapid cytosol-to-membrane PKC translocation that was complete within 15 min and lasted for at least 60 min. This translocation was further enhanced by ionomycin, as seen particularly 5 and 10 min after stimulation (Fig. 1), in agreement with our earlier report using leukaemic Jurkat T cells.[21] As an example, 5 min after stimulation, the distribution of PKC activity between cytosol and membrane was 54 and 46%, respectively, after TPA treatment, and 16 versus 84% after combined treatment with TPA plus ionomycin. These results suggested that ionomycin synergizes with TPA to stimulate redistribution of PKC enzymatic activity.

Next, we ascertained the effects of these treatments on the distribution of immunoreactive PKC. When extracts of unstimulated T cells were immunoblotted with anti-PKC-1 antibodies, which cross-react with PKCα, β and γ,[22] two protein bands in the molecular weight range of 72,000–80,000 were observed in the cytosol or membrane fractions (Fig. 2, lanes 1, 4). Following 10 min of TPA stimulation, the upper band disappeared from the cytosol (lane 2), and an apparently corresponding band now appeared in the membrane fraction

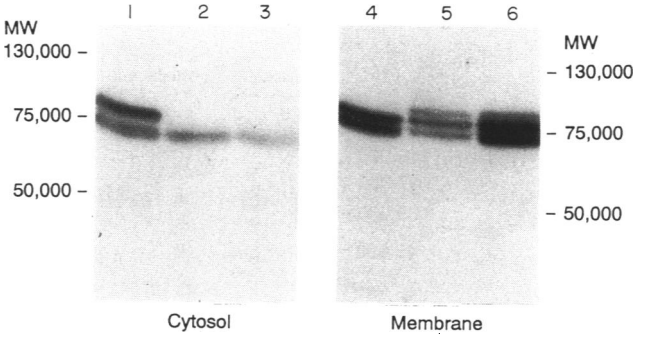

Figure 2. The synergistic effect of ionomycin and TPA on cellular distribution of immunoreactive PKC proteins. Untreated human T cells (lanes 1 and 4) or cells stimulated with TPA (10 ng/ml) in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of ionomycin (200 ng/ml) were lysed 10 min post-stimulation, and PKC from the cytosol or membrane fractions was extracted and partially purified on DE52 columns. Denatured cellular proteins (100 μg/lane) were resolved by SDS–PAGE and immunoblotted with affinity-purified anti-PKC-1 antibodies (5 μg/ml). Autoradiography was for 16 hr at −70° using an intensifying screen.

(lane 5, upper band). Stimulation of the cells with a mixture of TPA and ionomycin (lanes 3, 6) caused a similar qualitative change to that caused by TPA alone. The significance of the quantitative difference in signal intensity between lanes 5 and 6 is unclear; it may represent a smaller quantity of protein inadvertently loaded in this lane, since this difference was not seen in other experiments. As suggested by a recent report that documented a similar expression pattern,[31] the three immunoreactive bands may represent differentially phosphorylated and activated forms of PKC. These results indicate that TPA induces translocation of an 80,000 MW immunoreactive PKC from the cytosol to the membrane. The correlation between the translocation of PKC enzymatic activity and immunoreactive protein(s) eliminates, to a large extent, the possibility that the change in cellular location of enzymatically active PKC is caused by translocation of a potential PKC inhibitor from the membrane to the cytosol.

To characterize further the PKC species translocated from the cytosol to the membrane upon stimulation, a similar immunoblot analysis was performed using three different monoclonal antibodies specific for PKCα, PKCβ or PKCγ.[32] All three PKC isoenzymes were detected in T cells (Fig. 3), but PKCγ was considerably less abundant as indicated by the long exposure time (3 days) required to generate a detectable signal. The three species were all translocated from the cytosol to the membrane upon TPA (but not ionomycin) treatment (Fig. 3). The lack of detectable synergism between ionomycin and TPA in this experiment, as well as the one shown in Fig. 2, is most probably due to the longer stimulation period (10–15 min) that resulted in a near maximal effect induced by TPA alone (as seen in Fig. 1). The significance of the apparent inhibition of TPA-induced PKC translocation by ionomycin [compare lanes b versus d in Fig. 3a and b] is unclear and was not seen in other experiments.

The effect of TPA and ionomycin on expression of PKC genes

We next determined whether T-cell stimulation with TPA, in the absence or presence of ionomycin, affects expresison of specific PKC genes. Steady-state mRNA levels of individual PKC genes were measured in T cells, before or after stimulation for different time periods, by hybridization with cDNA probes specific for PKCα, PKCβ or PKCγ.

The results (Fig. 4) indicated that human T cells express mRNA corresponding to the three (α, β and γ) PKC genes. Comparison with an equal amount of total RNA from mouse brain by densitometric scanning of the autoradiograms shown in Fig. 4 revealed that the cells expressed, prior to restimulation, 40, 400 and 15% of the α, β and γ mRNA levels found in brain. Parenthetically, the low-level expression of PKCγ in T cells is in agreement with a recent report,[33] and may explain why its T-cell expression was not detected in some earlier studies. To facilitate quantitation, the slot-blot autoradiographic results are also depicted in parallel as intensities of the different hybridization signals based on densitometric scanning (Fig. 4). Ionomycin alone had little, if any, effect on expression of the PKC genes. TPA, on the other hand, augmented the expression PKCβ mRNA that peaked 12 hr after stimulation, but had minimal effect on the expression of α or γ mRNA. The effect of TPA was increased by co-stimulation with ionomycin. A clear three- to fourfold increase in PKCα mRNA that peaked after 12 hr was evident (Fig. 4a), and the level of increase in β mRNA was now higher than that induced by TPA alone (Fig. 4b), peaking at 24 hr post-stimulation and declining gradually over the next 24–48 hr to baseline levels. The combination of TPA plus ionomycin had no detectable effect on the expression of PKCγ mRNA (Fig. 4c). Thus, similar to the synergistic action of TPA plus ionomycin on functional and phenotypic aspects of T-cell activation, or on PKC translocation (Figs 1–3), a similar synergism was seen at the level of mRNA expression, although at a later time-point relative to PKC translocation.

Induction of c-*myc*, ODC and IL-2Rα genes by TPA and/or ionomycin

We next evaluated the effects of ionomycin and/or TPA on the expression of other genes known to be induced during the early stages of T-cell activation, namely, IL-2Rα, ODC and the c-*myc*

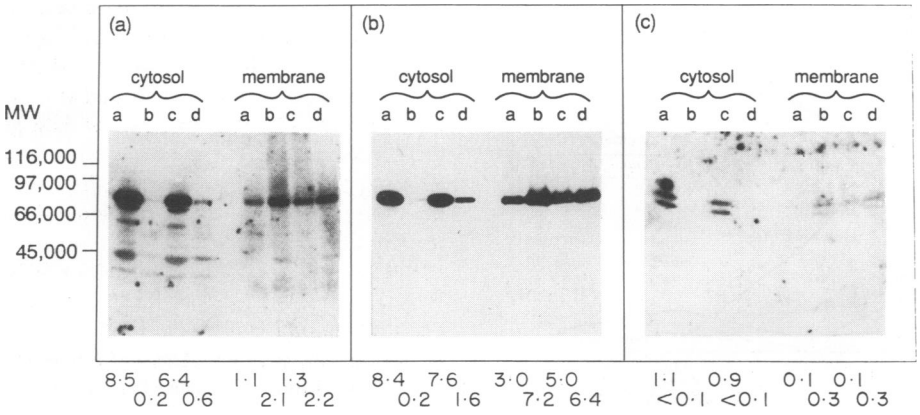

Figure 3. The effect of ionomycin ± TPA on cellular distribution of PKCα (a), β (b) or γ (c). Unstimulated human T cells (lane a) or cells stimulated for 15 min with TPA (10 ng/ml, lanes b), ionomycin (200 ng/ml, lanes c) or TPA plus ionomycin (lanes d) were lysed, and cytocol and membrane extracts were immunoblotted with MC-3a (PKCα), MC-2a (PKCβ) or MC-1a (PKCγ) monoclonal antibodies. Immunoreactive bands were visualized as in Fig. 2. Autoradiography was for 6 hr (PKCβ) or 3 days (PKCα, PKCγ) at $-70°$ with an intensifying screen. A 6 hr autoradiography revealed a clear PKCα signal in the cytosol only, and no detectable PKCγ signal (not shown). The numbers at bottom represent the integrated surface area (in absorbance units) of the peak representing the $\approx$80,000 MW PKC signal, as determined by densitometric scanning [the numbers in panel (c) correspond to the upper band].

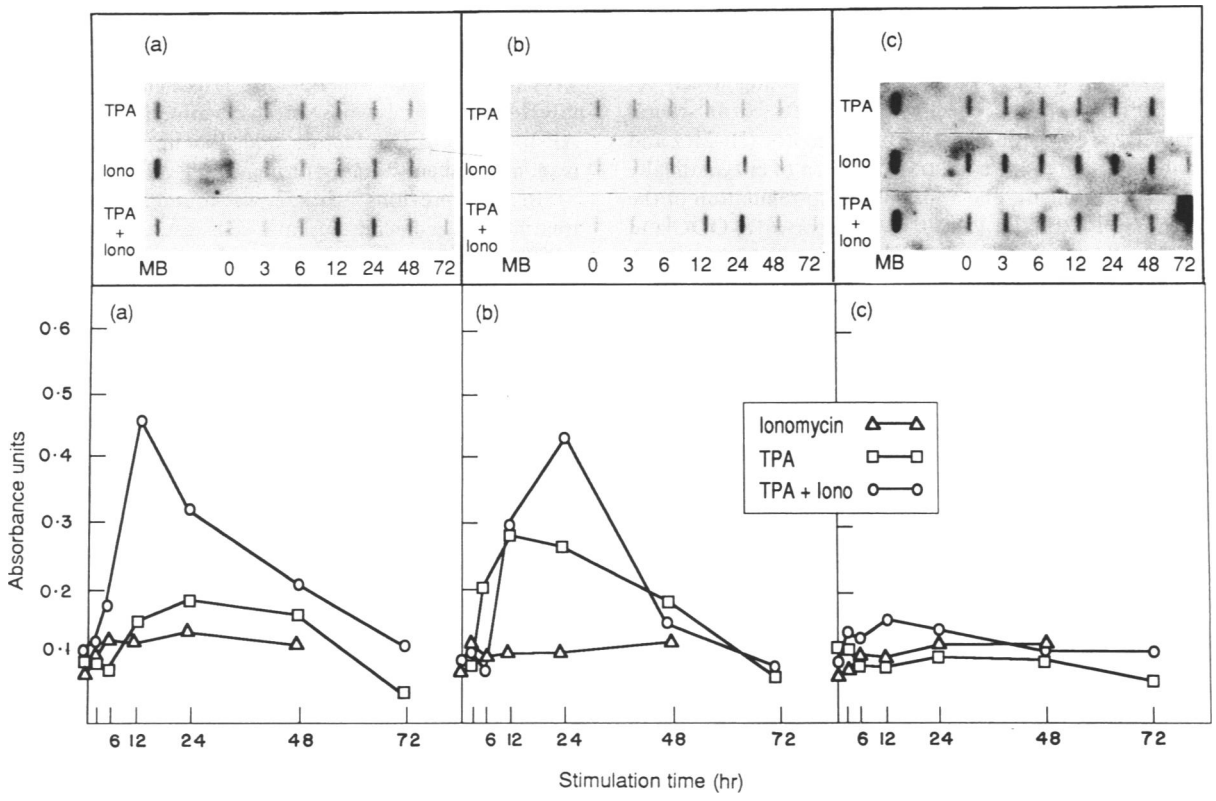

Figure 4. The effect of TPA and ionomycin on expression of PKC genes. T cells were stimulated with TPA (10 ng/ml) (□), ionomycin (200 ng/ml) (△) or a mixture of the two (○), total RNA was isolated at the indicated time intervals and hybridized (5 μg/slot) with ^{32}P-labelled nick-translated cDNA probes specific for PKCα (a), PKCβ (b) or PKCγ (c). Autoradiograms of slot-blots shown in the top panels were scanned by laser densitometry and relative signal intensity was plotted against a linear time scale, assigning arbitrary values of 0·1 to those obtained at time zero (bottom panels). MB, mouse brain.

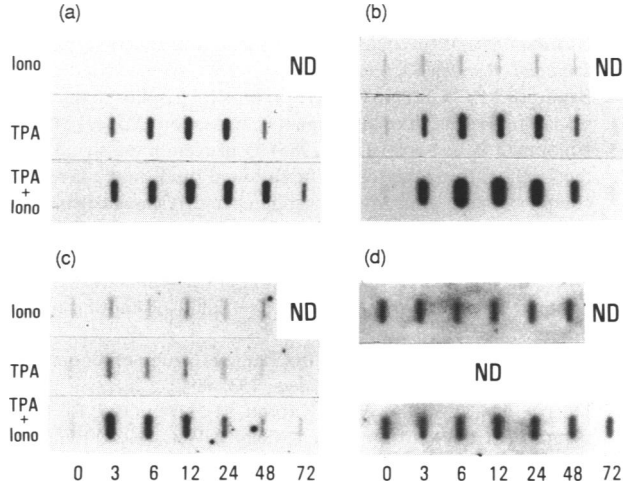

Figure 5. The effect of TPA and ionomycin on expression of IL-2Rα (a), ODC (b) and c-*myc* (c) genes. Nitrocellulose filters of slot-blots shown in Fig. 4 were stripped and reprobed with ^{32}P-labelled nick-translated probes specific for the indicated genes. The same blots were also probed with a β_2 microglobulin probe (d). ND, not done.

protooncogene. Slot-blot analysis of RNA from preactivated T cells showed very weak or no detectable levels of mRNA for c-*myc*, ODC or IL-2Rα (Fig. 5). Ionomycin alone had no effect on IL-2Rα (Fig. 5a) or ODC (Fig. 5b) mRNA levels, and induced a minor increase in c-*myc* mRNA (Fig. 5c). By contrast, TPA increased expression of the three genes, evident at the earliest time-point examined. Expression of c-*myc* mRNA peaked 3 hr post-stimulation (14-fold increase), while that of IL-2Rα and ODC peaked at ≈6–12 hr (47- and 16-fold, respectively, at 12 hr). The increase in the expression of these genes was much more pronounced when ionomycin was combined with TPA, although the kinetics of increased mRNA expression was not affected by the co-stimulation. By densitometric scanning, the increased mRNA expression (relative to unstimulated cells) was 62-fold for IL-2Rα (12 hr), 58-fold for ODC (6 hr), and 43-fold for c-*myc* (3 hr). Identical slot-blots hybridized with a β_2 microglobulin cDNA probe verified that similar quantities of RNA were applied to all slots in the experiments shown in Figs 4 and 5. The maximum change in β_2 microglobulin mRNA expression was recorded at 12 hr post-stimulation with TPA plus ionomycin, and represents a 1·35-fold increase over unstimulated cells (Fig. 5d).

DISCUSSION

In the present study, we used human T lymphocytes to test the effects of TPA and/or ionomycin on the expression and

translocation of three PKC isoforms at the protein and mRNA levels. Ionomycin, which in itself was inactive (or had minimal effects), clearly synergized with TPA in affecting T-cell responses. These synergistic effects were manifested at several levels, namely, proliferation, cell-surface receptor (IL-2Rα and transferrin receptor) expression, translocation of enzymatically active PKC to the membrane and, finally, up-regulation of the genes encoding PKCα and PKCβ, as well as c-*myc*, ODC and IL-2Rα. This synergism was not evident in immunoblots (Figs 2, 3) because at the time-points evaluated (10–15 min), TPA alone caused maximal effects. The correlation among these different parameters suggests that the synergism between TPA and ionomycin in stimulating T-cell proliferation, a late response, is accounted for, at least in part, by the synergistic effect of these two agents on the early response of PKC activation. However, these synergistic responses, including the enhanced expression of PKC mRNA, could also reflect the contribution of additional, Ca^{2+}/calmodulin-dependent kinases and 'cross-talk' between such kinases and PKC (reviewed in ref. 1).

The early translocation of enzymatically active or immunoreactive PKC to the membrane after treatment with TPA, either alone or in combination with ionomycin, was followed by a later increase in the expression of the genes encoding PKCα and PKCβ, but not PKCγ. Similar increases in mRNA were induced by a mitogen, PHA,[34] that triggers the inositol phospholipid second messenger system in T cells in a dependent manner. The translocation of PKC to the membrane stimulated by TPA (and probably by surface-binding ligands) is accompanied by increased degradation as well as increased *de novo* synthesis of PKC.[35-37] Our findings support this notion by indicating that an increase in PKC mRNA is also a part of this response. The stimulation of PKC gene transcription would then lead to *de novo* synthesis of the enzyme. The three- to fourfold increase in PKC mRNA, as compared to the much greater increase in the expression of other activation-associated genes (IL-2Rα, ODC, c-*myc*) is not surprising, since PKC genes are expressed constitutively at considerable levels (Fig. 4), whereas expression of the IL-2Rα, ODC and c-*myc* genes was minimal or undetectable before stimulation (Fig. 5). However, the increase showed some selectivity in that TPA alone increased the expression of only PKCβ mRNA, whereas the combination of TPA and ionomycin increased, in addition, the expression of PKCα mRNA. In contrast, although PKCγ was also expressed in T cells at low abundance (relative to brain), there was no detectable change in its mRNA upon cellular activation, suggesting that this isoenzyme does not play an important role in the cascade of activation events. The selective increase in PKC mRNA is in agreement with recent studies,[34,38] as well as with our previous finding of a selective PKC down-regulation in TPA-treated Jurkat cells,[22] and lends support to the notion that PKC isoenzymes may be regulated in a differential manner by second messengers, and may participate in discrete steps of the signal transduction pathway.

The mechanisms that transduce PKC-mediated signals into the nucleus are not clear, although PKC activators, such as TPA, were found to activate nuclear transcription factors that regulate gene expression by binding to their common enhancer elements.[39-42] The synergistic effects of ionomycin and TPA on PKC activation and on transcription of several growth-regulating genes suggest that the two agents, via their effect on PKC, also synergize in induction of transcription factors, thereby augmenting transcription of genes that possess the appropriate response elements. This notion is supported by findings that TPA and ionomycin synergize in: (a) induction of c-*fos* and c-*jun* mRNA,[42] two genes whose products dimerize to form the active AP-1 transcription factor; and (b) up-regulation of TPA response element-binding proteins in T cells.[43]

In our previous work,[22] we found that chronic TPA treatment of leukaemic Jurkat T cells resulted in a complete loss of CD2 and CD3 from the cell surface and inability to express IL-2Rα upon mitogen stimulation. The same treatment also led to selective reduction of PKCα, suggesting that expression of these cell-surface molecules is regulated by this particular isoenzyme. Taken together, our results suggest that TPA activates selected PKC isoenzymes in T cells, and that ionomycin, although inactive alone, synergizes with TPA in activation of these enzymes and in affecting other, more remote, events, such as transcription of genes that play an important role in T-cell activation and growth.

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