Analysis of Prostaglandin E_2 Effect on T Lymphocyte Activation

Abrogation of Prostaglandin E_2 Inhibitory Effect by the Tumor Promotor 12.0 Tetradecanoyl Phorbol-13 Acetate

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Abstract

We have investigated the inhibitory potential of prostaglandin E_2 (PGE_2) with respect to intracellular messengers implicated in the signaling system of T-lymphocyte activation pathway. Using the fluorescent indicator Quin 2, it is demonstrated that PGE_2 inhibits the increase in cytosolic-free calcium concentration $[Ca^{2+}]_i$. Reconstitution of calcium mobilization in the presence of PGE_2 by the calcium ionophore A23187 results in a partial restoration of both interleukin 2 (IL2) production and cell proliferation and has no effect on the inhibition of transferrin receptor expression. In contrast, the treatment of cell cultures with the tumor promotor 12.0 tetra decanoyl phorbol-13-acetate (TPA) abrogates the suppressor activity of PGE_2. When T lymphocyte stimulation is provided by the combination of A23187 and TPA, the PGE_2 inhibitory effect does not occur. These data also indicate that the down regulation of transferrin receptor by PGE_2 is proximal to protein kinase C activation and is not associated with decreased expression of the functional IL2 receptor.

Introduction

Prostaglandin E2 (PGE_2)[1] is a potent chemical transmitter of intercellular and intracellular signals that mediate a diversity of physiologic and pathologic cell functions (1). Several studies have revealed that PGE_2 at physiologic concentrations induces a profound inhibition of T-lymphocyte activation and proliferation after in vitro stimulation with phytohemagglutinin (PHA) (2, 3). This inhibition has been shown to be associated with a parallel increase in intracellular levels of cyclic adenosine monophosphate (cAMP) (4) but the relationship of this cyclic nucleotide and the molecular events involved in the cellular activation process is still poorly understood.

We have previously demonstrated that PGE_2 and increased intracellular concentrations of cAMP inhibit the production and secretion of the lymphocytotropic growth regulating peptide interleukin 2 (IL2), and the signal for proliferation provided by the expression of transferrin receptor (5, 6). In the murine system, it was reported that PGE_2 down regulates Ia antigens on antigen-presenting cells (7, 8).

Ligand binding to its specific membrane receptor results in the generation of a signal that is transmitted across the plasma membrane. This interaction promotes a rapid turnover of phospholipids in lymphocyte membranes. The enhanced turnover is related to early events that trigger cellular activation including elevation of intracellular-free calcium $[Ca^{2+}]_i$ and phosphorylation of cytoplasmic and nuclear proteins that trigger the lymphokine cascade and the sequential gene expression of activation antigens. Several lines of evidence support the concept that increase in $[Ca^{2+}]_i$ is an essential activation signal toward the commitment of cells to DNA synthesis (9, 10). $[Ca^{2+}]_i$ simultaneously results from the stimulated influx across the plasma membrane as well as the release from intracellular stores most likely from the endoplasmic reticulum. Protein kinase C (PKC) a Ca^{2+} and phospholipid-dependent enzyme, has also been identified as an intracellular signaling system in T-lymphocyte activation. This enzyme is selectively activated by diacylglycerol (11), but can also be stimulated in vitro by its structural analogue, the tumor-promotor phorbol ester 12.0 tetradecanoyl-phorbol 13 acetate (TPA) (12). PKC activation appears to act synergistically with increases in $[Ca^{2+}]_i$ to mediate the responses to extracellular ligands (13, 14). Recently, it has been reported that IL2-receptor interaction with IL2 stimulates a rapid and transient redistribution of PKC from the cytosol to the plasma membrane and that TPA induces PKC transposition in an analogous manner (15).

In the present study we have investigated the regulatory effect of PGE_2 on T cell activation with respect to calcium mobilization and activation of PKC. Our results indicate that the PGE_2 immunosuppressive effect on T cell activation involves predominantly PKC and support the involvement of PGE_2 in the modulation of this enzymatic activation pathway.

Methods

Peripheral blood mononuclear leukocytes (PBL)

PBL were prepared from heparinized venous blood of normal healthy donors. The PBL were isolated by Ficoll-Hypaque density gradient centrifugation (Lymphoprep, Accurate Chemical and Scientific Corp., Hicksville, NY). Cells were washed twice in Hanks' balanced salt solution (Gibco, Grand Island, NY) and then resuspended in RPMI 1640 (Gibco) that had been supplemented with 25 mM Hepes, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) supplemented RPMI 1640).

Enriched T cell preparation. Enrichment for T lymphocytes was performed by filtration of PBL through a nylon wool column as described earlier (16). The cell population obtained contains between 3 and 5% peroxidase-positive monocytes.

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1. *Abbreviations used in this paper:* CT, cholera toxin; CTLL, cytotoxic T lymphocytes; ISO, isoproterenol; k_d, dissociation constant; PGE_2, prostaglandin E_2; $PGF_2\alpha$, prostaglandin $F_2\alpha$; PHA, phytohemagglutinin; PKC, protein kinase C; TPA, tetra decanoyl phorbol-13-acetate.

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Experiments described in this study were performed with enriched T cells in order to decrease endogenous PGE production by either B cells or excess contaminating number of monocytes. It was previously shown that this method for T cell enrichment provides a cell preparation in which monocyte-derived PGE_2 has a minimal effect on T cells, while retaining sufficient monocytes for induction of mitogen activation (5, 15). The PGE synthetase inhibitor, indomethacin, could not be used in these experiments since this drug is not a specific PG synthetase inhibitor and has actions other than on PG synthesis (i.e., inhibition of cAMP-dependent protein kinase and blocking of active uptake of PGE_2 in cells) (18).

T lymphocyte proliferation assay. Enriched T cells were tested for their proliferative response to PHA. Cells (1×10^6 cells/ml) resuspended in supplemented RPMI 1640 with 20% pooled human serum were distributed into round-bottomed microtiter plates (NUNC, Vanguards, Neptune, NJ) (100 μl) in the presence or absence of the indicated drugs and PHA. After 3 d incubation at 37°C in 5% CO_2, humidified atmosphere, the cultures were pulsed with 2 μCi of tritiated thymidine ([^{3}H]TdR); 80.3 Ci/mol; (New England Nuclear, Boston, MA). Plates were reincubated for 6 h at 37°C and then harvested onto filter paper with a Skatron harvesting apparatus (Flow Laboratories, Walkersville, MD). Thymidine incorporation was measured in a beta scintillation counter (Packard Instrument Co., Downers Grove, IL) and the results from triplicate wells were expressed as mean counts per minute ±SE.

Assay for IL2 activity in supernatants. For the IL2 assay, 4000 murine IL2-dependent cytotoxic T-lymphocytes (CTLL) were grown in the presence of $\log_2$ dilution of putative IL2-containing medium in 96-well microtiter plates (Costar Data Packaging, Cambridge, MA). The total volume in each well was 0.2 ml; 24 h later, 0.5 μCi of [^{3}H]TdR (20 Ci/mmol, New England Nuclear) was added to each well. After 4 h, the cells were harvested on glass fiber strips, and [^{3}H]TdR incorporation was measured in a liquid scintillation counter (Packard Instrument Co.). 1 U/ml of IL2 was defined as the quantity of IL2 released in 48-h culture medium conditioned by rat spleen cells (1×10^6/ml) stimulated by concanavalin A (19).

IL2 production. Briefly, enriched T-lymphocytes were stimulated with PHA. Supernatants were collected after 48 h of culture, centrifuged at 1,000 *g* for 10 min, sterilized by filtration (45-μm pore size, Millipore Filter Corp., Bedford, MA) and were stored at 4°C until assayed.

$^{45}Ca^{2+}$ Uptake. Calcium influx was performed by measuring incorporation of $^{45}Ca^{2+}$ during lymphocyte activation. Enriched T cells at 4×10^6/ml were preincubated in medium alone or in medium containing the test reagent (e.g., PGE_2 or $PGF_2\alpha$) in 24-well plates for 60 min at 37°C in 5% CO_2 humidified atmosphere. After the preincubation, PHA or A23187 and $^{45}CaCl_2$ (New England Nuclear) (1 μCi/ml) were added to the plates. After an additional 30-min incubation, cells were transferred in aliquots from each culture, to microtiter plates and were harvested onto fiberglas filters. Filters were transferred to glass vials containing scintillant (Aquasol) and the radioactivity was determined in a scintillation counter (Packard Instrument Co.).

Measurement of $[Ca^{2+}]_i$. The use of intracellularly trapped Ca^{2+} indicator, Quin 2, to measure $[Ca^{2+}]$ in lymphocytes was performed as described by Tsien (20). Quin 2 acetoxymethyl ester (30 μM) was added to a cell suspension containing 0.5×10^8 cells per ml and incubated for 20 min. The suspension was then diluted 10-fold and incubated for a further 60 min. After loading, the cells were centrifuged and resuspended in fresh RPMI 1640 at 10^7 per ml and kept at room temperature. Shortly before fluorescence measurements, 10^7 cells were washed once by spinning for 15 s at 15,000 *g* in a microcentrifuge, and resuspended in a volume of 2 ml of simplified medium without phenol red (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM glucose, 10 mM Na Hepes, pH 7.4) and transferred to cuvette thermostated to 37°C. Quin 2 fluorescence was recorded with Perkin Elmer LS5 spectrofluorimeter (Analytical Instruments, Norwalk, CT). Excitation and emission wavelength were 339 nm (5 nm slit) 492 nm (10 nm slit). $[Ca^{2+}]_i$ Was calculated by the method of Tsien et al. (20) according to the equation $[Ca^{2+}]_i = k_d \cdot [(F - F_{min})/(F_{max} - F)]$nM, where F_{max} was determined for each condition by measuring fluorescence after the cells were lysed by addition of 1% Triton X (Sigma Chemical Co.). F_{min} was determined by fluorescence after the addition of ethylenebis-(oxyethylenenitzilo) tetraacetic acid (EGTA) 200 mM pH 12 (Sigma Chemical Co.). The effective dissociation constant, k_d, of Quin 2 has been reported as 115 nM (21).

Quantitation of IL2 receptor

As described previously (22) serial dilutions of ^{125}I-IL2 and ^{3}H-anti-Tac IgG were incubated in 1.5-ml Eppendorf micro test tubes at 37°C with 1.0 to 1.5×10^6 cells in a total volume of 100 μl RPMI 1640, 10 mg/ml bovine serum albumin (BSA). (For the anti-Tac assay, the buffer also contained 100 μg/ml UPC10, an IgG2a murine monoclonal, to block Fc binding). After 8 min (IL2) or 20 min (anti-Tac) incubation, the tubes were placed on ice and 1 ml ice-cold RPMI 1640-BSA was added to each. The tubes were then spun for 20 s in a (Beckman 12, Beckman Instruments, Inc., Fullerton, CA) microfuge at 12,000 rpm. The supernatant was transferred to counting vials for determination of the level of unbound ligand. The cell pellet was resuspended in 100 μl RPMI 1640-BSA and layered over 200 μl of a mixture of 81% silicone oil and 19% paraffin oil in 400 μl Bio-Rad micro test tubes (Bio-Rad Laboratories, Richmond, CA). The tubes were spun 2 min at 12,000 rpm in the Beckman 12 microfuge. The tips of the tubes containing the cell pellet were cut off and transferred to counting vials. The ^{125}I-IL2 was determined using a gamma counter (Quatro, LKB Instruments, Gaithersburg, MD). The cell pellet containing the ^{3}H-anti Tac was resuspended with 200 μl PBS in 20-ml glass scintillation vials followed by solubilization of the cells with 200 μl 1% sodium dodecyl sulfate (SDS) and addition of 10 ml scintillation fluid. All results were adjusted for counting efficiency (78% for bound and free ^{125}I; 45 and 39% for bound and free ^{3}H, respectively).

The specific activity of the ^{125}I-IL2 was 7.5×10^5 dpm/pmol and its initial concentration in the assay was 200 pM. The specific activity of the ^{3}H-anti Tac was 8.3×10^4 dpm/pmol and its initial concentration in the assay was 4 nM.

Indirect immunofluorescent staining of cells and analysis with the fluorescence activated cell sorter. Indirect immunofluorescence was performed by incubating 1×10^6 cells with the monoclonal antibodies at the appropriate dilution for 30 min at 4°C, washing three times in phosphate-buffered saline (PBS) with 1% BSA and 0.02% sodium azide (PBS-BSA-azide). The cells were then incubated with a 1/40 dilution of affinity-purified goat anti-mouse IgG, $F(ab')_2$ fluoroisothiocyanate (FITC) (Cappel Laboratories, Cochranville, PA) for an additional 30 min at 4°C. After extensive washing in the PBS-BSA-azide buffer, cells were resuspended in 1-ml PBS and examined using a fluorescence-activated cell sorter (EPICS-C, Coulter Electronics, Hialeah, FL).

Fluorescence data were collected on 1×10^4 viable cells, as determined by forward light scatter intensity. Background fluorescence was determined by using diluted normal mouse serum instead of monoclonal antibody. Percentage of stained cells was obtained from computed histograms.

Reagents. PHA (purified HA 16/17) was obtained from Wellcome Research Laboratories, Beckenham, England. PGE_2 and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) purchased from Upjohn Co. (Kalamazoo, MI) were dissolved in 95% ethanol at a concentration of 10 mg/ml and stored at −70°C. Final ethanol concentrations in the culture did not exceed 0.1% and had no effect on the cell cultures. Dibutyryl cAMP (dBcAMP), cholera toxin (CT), TPA, and isoprotenerol (ISO), were obtained from Sigma Chemical Co. A23187 was purchased from Boehringer Mannheim, GmbH, FRG); and Quin 2 AM from (Calbiochem Behring Corp., La Jolla, CA). Culture medium was used for all dilutions.

Monoclonal antibodies. Monoclonal anti-Tac antibody was kindly supplied by Dr. W. C. Greene, National Cancer Institute. Anti-Tac reacts with human IL2 receptor on activated T cells (23). OKT9, which detects the human transferrin receptor (24) was purchased from Ortho Pharmaceutical Corp. (Raritan, NJ).

Results

Abrogation of PGE_2 inhibitory effect by TPA

Because PKC activation has been proposed in several cellular studies to mediate the responses of extracellular ligands including proliferation of T cells, we asked whether PGE_2 inhibitory effect interferes with the enzymatic activation pathway.

We have used resting T cells because they do not differ in their level of activation as measured by their expression of IL2 receptor and also because T cell lines or clones have been previously stimulated and committed to cycle. We also have shown that PGE_2 exerts its effect on cells in active cell cycle (3, 6) and that activated cells have a different sensitivity to suppression by PGE_2.

Enriched T cells are cultured in the presence of PHA and increasing concentrations of PGE_2 (i.e., 10^{-10} to 10^{-6} M). Both T cell proliferation and IL2 production are inhibited in a dose-dependent manner as previously shown (3, 5, 6).

Fig. 1 *A* shows that addition of TPA at a concentration which by itself does not stimulate resting T cells (2.5 μg/ml) to the culture can overcome the PGE_2-induced inhibitory effect on T-lymphocyte proliferation, suggesting a probable interference of PGE_2 with PKC activation. Although addition of TPA to a PHA-stimulated culture in the presence of PGE_2 elicits full restoration of thymidine incorporation, it only partially restores the level of IL2 production in PGE_2-treated cultures compared with the normal level obtained in untreated cultures (Fig. 1 *B*).

Effect of PGE_2 on free-cytoplasmic Ca^{2+}. Mitogen-stimulated intracellular-free calcium increase, is well documented as a trigger for the intracellular activation process in T lymphocytes (9, 10). Therefore, we investigated the effects of PGE_2 on Ca^{2+} flux and intracellular Ca^{2+} mobilization, a key step in the cytoplasmic signaling system.

Changes in intracellular-free calcium $[Ca^{2+}]_i$ was investigated by measurement of $^{45}Ca^{2+}$ uptake, as well as by the Quin 2 assay. As shown in Table I, PHA-stimulation of T lymphocytes resulted in a marked calcium influx (3,930–4,320 cpm) compared with unstimulated cells (830–1,350 cpm). Addition of PGE_2 at a concentration that inhibits lymphoproliferation (10^{-7} M) resulted in a decreased $^{45}Ca^{2+}$ uptake by PHA-stimulated lymphocytes (2,080–2,290 cpm). T cell stimulation with the calcium ionophore A23187 as a positive control for Ca^{2+} influx resulted in a relatively high uptake (6,210–8,025 cpm); however,

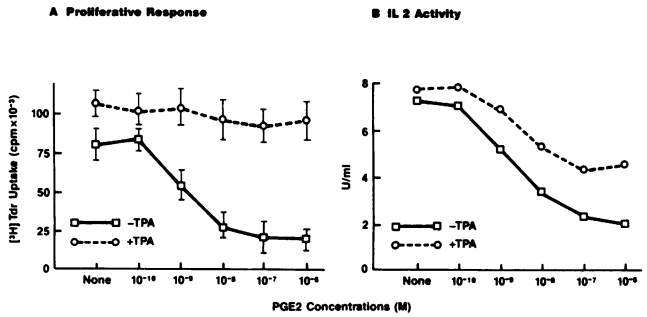

Figure 1. Effect of TPA (2.5 ng/ml) on PGE_2-treated PHA-stimulated cultures. Thymidine incorporation (*A*) and IL2 production (*B*) were determined as described in legend to Fig. 2. [^{3}H]TdR incorporation in control cultures were: medium alone (340 cpm) and medium plus TPA (910 cpm).

Table I. Effect of PGE_2 on $^{45}Ca^{2+}$ Uptake in T Lymphocytes after PHA Stimulation

Cell treatment	Experiment I	Experiment II	Experiment III
	cpm±SEM	*cpm±SEM*	*cpm±SEM*
Medium	830	1065	1350
PHA	3930 (100)*	4320 (100)	4130 (100)
PGE_2+PHA	2290 (42)	2110 (52)	2080 (50)
$PGF_2\alpha$ + PHA	4480	4990	4270
A23187	6210 (100)	8025 (100)	7435 (100)
PGE_2+A23187	5630 (91)	7490 (93)	7100 (90)

Enriched T cells were preincubated for 1 h in medium alone or in medium containing PGE_2 (10^{-7} M), $PGF_2\alpha$ (10^{-7} M). $^{45}CaCl_2$ was then added (1 μCi/ml) together with PHA (0.5 μg/ml) or A23187 (100 ng/ml). After 1 additional h of incubation, cells were harvested and the $^{45}Ca^{2+}$ uptake was determined in triplicate aliquots.

* Numbers indicated in parentheses are percentages of uptake compared with the positive control culture (100%) set up in the absence of PGE_2.

no appreciable change in $^{45}Ca^{2+}$ occurred when PGE_2 was added (5,630–7,490 cpm). $PGF_2\alpha$, which has no direct effect on T cell activation, was also tested and was found not to affect the $^{45}Ca^{2+}$ uptake. We additionally tested the PHA-induced $[Ca^{2+}]_i$ increase in cells treated with PGE_2 at a concentration that inhibits T cell proliferation (10^{-7} M). The fluorescent indicator Quin 2 was used for these determinations. As shown in Fig. 2 the baseline of $[Ca^{2+}]_i$ in nonstimulated Quin 2-loaded T lymphocytes is 98 nM. After addition of PHA, a substantial increase in $[Ca^{2+}]_i$ (172 nM) was observed within 2 min. In contrast, preincubation of T lymphocytes in the presence of PGE_2 before PHA stimulation resulted in a substantially lower level in $[Ca^{2+}]_i$ (126 nM). $PGF_2\alpha$ had no effect on this parameter confirming the specific effect of PGE_2 (data not shown).

Effects of calcium ionophore A23187 on PGE_2-treated T lymphocytes. If the major mechanism by which PGE_2 inhibits IL2 production and cell proliferation by T cells is mediated via an alteration of calcium mobilization, it would be expected that calcium influx induced by the calcium ionophore A23187 would

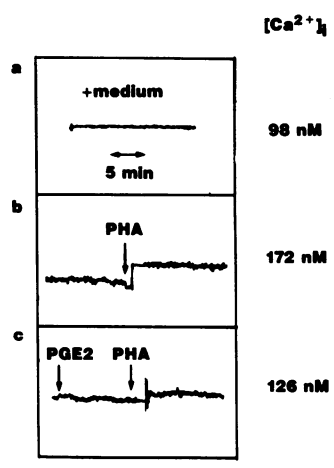

Figure 2. Fluorimeter traces illustrating the effect of PGE_2 on calcium mobilization in PHA-stimulated enriched T cells. Quin 2 loaded-enriched T lymphocytes (5×10^6) were resuspended in buffered saline solution in a cuvette maintained at 37°C and continuously stirred during analysis. $[Ca^{2+}]_i$ was calculated as described in Methods. (*a*) Baseline internal calcium level of resting cells before addition of PHA. (*b*) Effect on $[Ca^{2+}]_i$ by PHA (0.5 μg/10^6 cells) added at the time point indicated by arrow. (*c*) PHA (0.5 μg) response of PGE_2 (10^{-7} M) pretreated cells.

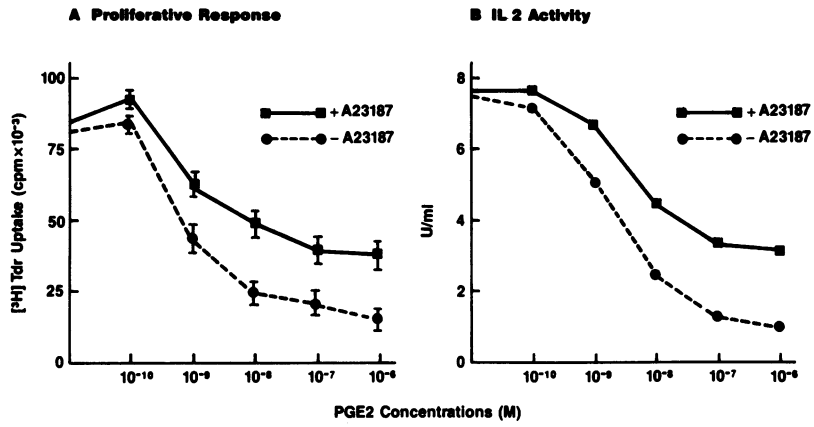

Figure 3. Effect of Ca^{2+} ionophore A23187 on PHA-induced cell proliferation and IL2 production in the presence of PGE_2. Enriched T cells were cultured in RPMI 1640 supplemented with 15% human serum. PHA (0.5 μg) and PGE_2 at concentrations indicated were added simultaneously at the initiation of the culture. (*A*) Thymidine incorporation in the presence or absence of A23187 (100 ng/ml) results are the mean±SEM of triplicate. Cell proliferation was determined by [^{3}H]TdR uptake during the last 6 h of 3 d incubation. Bars indicate SE. (*B*) Supernatants of A23187-treated or untreated cultures were collected after 48 h and tested for IL2 activity in the CTLL assay. [^{3}H]TdR incorporation in unstimulated control cultures were: medium alone (520 cpm) and medium plus A23187 (1,300 cpm).

restore these events to normal. A23187 was utilized at a concentration that is not mitogenic (100 ng/ml), but which leads to increase in $[Ca^{2+}]_i$ (data not shown). A23187 was added to PHA-activated T-cells in the presence or absence of varying concentrations of PGE_2. The results summarized in Fig. 3 demonstrate that cell proliferation measured by thymidine incorporation (Fig. 3 *A*) and IL2 production (Fig. 3 *B*) of PHA-stimulated T cells were only partially restored after addition of A23187 in PGE_2-treated cultures. It is also shown that partial restoration of the proliferative response parallels the partial restoration of IL2 production. These data suggest that PGE_2 may exert its regulatory activity, at least in part, by interfering with calcium mobilization but do not support an exclusive role for Ca^{2+} in the inhibitory effect of this compound.

Abrogation of PGE_2 inhibitory effect upon A23187 and TPA costimulation on PGE_2-treated T lymphocytes. It is demonstrated in Table II that when PHA is used as a mitogen, PGE_2 inhibits the IL2 production and the subsequent T cell proliferation in a concentration-dependent way. However, under conditions where stimulation of T lymphocytes is provided by the combination of TPA (2.5 ng/ml) and A23187 (100 ng/ml), proliferation is not blocked by PGE_2 at concentrations that did effectively inhibit the PHA-induced T cell proliferative response. Since these cells escape the immunosuppressive effect of PGE_2 the results would suggest that PGE_2 interferes with the endogenous T cell activation pathway involving both of the common transmembrane signals: Ca^{2+} mobilization and PKC activation.

Differential effects of TPA and A23187 on transferrin receptor expression by PGE_2-treated T-lymphocytes. One mechanism by which PGE_2 modulates T cell proliferation is by inducing a down regulation of the transferrin receptor expression in PHA-stimulated culture (6). We, therefore, examined if Ca^{2+} mobilization and PKC activation are involved in this phenomenon.

As shown in Fig. 4 using OKT9 antibody for detection of the transferrin receptor, addition of PGE_2 (10^{-7} M) to PHA-stimulated culture induces a down regulation of transferrin receptor expression (67 vs. 28%). Addition of A23187 (100 ng/ml) under those conditions has no effect on the expression of this receptor, which remains inhibited (72 vs. 34%). When TPA (2.5 ng/ml) was added to the culture a complete restoration of the expression of transferrin receptor was achieved (84 vs. 78%). Moreover, the inhibitory effect of PGE_2 on transferrin receptor expression observed when PHA is used as stimulator is not observed when the cellular activation is triggered by costimulation with A23187 and TPA (80 vs. 79%). These results are compatible with the concept that down regulation of transferrin receptor by PGE_2 involves modulation of PKC activity.

Effect of PGE_2 upon high affinity IL2 binding. In earlier

Table II. Lack of PGE_2 Effect on TPA and Calcium Ionophore-induced Lymphocyte Proliferation and IL2 Production

	Stimulus			
	PHA		TPA + A23187	
Cell treatment	Thymidine incorporation	IL2 activity	Thymidine incorporation	IL2 activity
	cpm	*U/ml*	*cpm*	*U/ml*
Medium	92,130±4,160* (116)‡	7.5	123,600±5,120* (106)‡	8.8
PGE_2 (10^{-10} M)	107,600±3,600 (100)	7.3	117,080±4,200 (100)	7.0
PGE_2 (10^{-9} M)	49,185±2,360 (47)	5.6	115,300±4,990 (98)	7.2
PGE_2 (10^{-8} M)	32,900±1,980 (64)	3.2	121,950±5,060 (104)	6.8
PGE_2 (10^{-7} M)	26,300±1,420 (72)	2.4	126,500±4,380 (108)	6.2
PGE_2 (10^{-6} M)	29,720±1,760 (68)	2.1	118,000±3,950 (100)	6.5

Enriched T cells were activated for 72 h by PHA (0.5 μg/ml) or combination of TPA (2.5 ng/ml) plus A23187 (100 ng/ml) in the presence of the indicated concentrations of PGE_2. Thymidine incorporation and IL2 production were determined as described in the legend to Fig. 2. * ± Denotes SE of mean cpm. ‡ Numbers in parentheses indicate percentage response compared with positive control culture (100%).

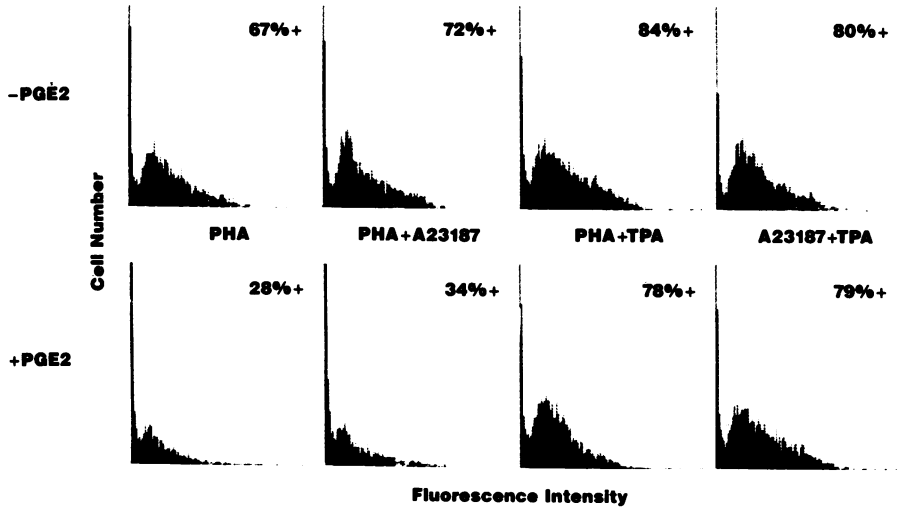

Figure 4. Fluorescence activated cell sorter analysis (FACS) of transferrin receptor expression of PHA-stimulated enriched T cells treated with TPA (2.5 ng/ml) or A23187 (100 ng/ml) in the presence or absence of PGE_2 (10^{-7} M). After 72 h of incubation, cells were prepared for FACS analysis as indicated in Methods, and stained with OKT9 antibody. 1×10^4 live cells were analyzed. Percentage of immunofluorescent positive cells were determined by analysis of the area below the curves. The background staining of nonspecific binding (5%) obtained with normal mouse serum was substrated.

studies, we demonstrated using cytofluorographic analysis with an anti-Tac monoclonal antibody that PGE_2 had no inhibitory effect on the expression of IL2 receptor (6). Two classes of IL2 receptors have been defined according to their binding affinity for IL2 and both are recognized by anti-Tac (25, 26). However, only binding of IL2 to high affinity receptors have been reported to correlate with the magnitude of the proliferative response of T cells in vitro (25, 26). Therefore, it was possible that PGE_2 interfered specifically with the expression of the functional high affinity IL2 receptor that was ultimately responsible for the down regulation of transferrin receptor. To address this possibility, a binding assay using labeled IL2 was performed. As shown in Table III, concentration of PGE_2 that induced optimal inhibition of T cell proliferation induces a decrease in the number of the high affinity IL2 receptor (720 sites vs. 1,470 sites/cell) compared to untreated cultures. Furthermore, Scatchard analysis (Fig. 5) indicated that the receptor ligand affinity remained essentially constant in the presence or absence of PGE_2. Addition of exogenous IL2 to PGE_2-treated cultures was accompanied by full recovery of high affinity IL2 receptor, a significant enhancement of Tac epitope expression and partial restoration of T cell proliferation, while expression of transferrin receptor remained diminished (data not shown). These data suggests that the suppressive effect of PGE_2 on high affinity IL2 receptors is not the predominant cause for the down regulation of transferrin receptor and the inhibition of T cell proliferation.

Antagonistic effects of TPA and cAMP elevating agents on T cell activation. The effects of PGE_2 and cAMP elevating agents CT, and ISO on T cell proliferation and transferrin receptor expression were tested in the absence and in the presence of TPA at a concentration that by itself had no effect on lymphocyte activation.

As shown in Table IV, in the absence of TPA, PGE_2, CT, ISO, or dibutyryl cAMP inhibits T cell proliferation and transferrin receptor expression while PGF_2 is found to have no regulatory effect. Under the same culture conditions, after addition of TPA (2.5 ng/ml) a complete loss of the inhibitory capacity of these pharmacologic agents with respect to thymidine incorporation and transferrin receptor expression is observed.

Discussion

In previous studies, we have demonstrated that PGE_2 affects PHA-induced T-cell proliferation at least at two distinct levels:

Table III. Effect of PGE_2 upon High Affinity IL2 Binding on PHA-stimulated T Cells

Cell treatment*	High affinity IL2 receptor		Anti-Tac binding	High affinity IL2-binding sites as a percentage of anti-Tac sites	Thymidine incorporation
	Sites per cell‡	Estimated kD§	Sites per cell‖		
		$\times 10^{-12}$ M		%	cpm¶
Medium	1,470	11.3	11,900	(12.4)	82,700
PGE_2	720	9.1	8,050	(8.9)	23,500
PGE_2 + IL2	1,550	6.1	36,600	(4.2)	49,100

Enriched T cells (10^6/ml) were incubated with 0.5 μg of PHA for 72 h and specific binding of IL2 and anti-Tac was determined. * PGE, 10^{-7} M IL2, 10 U/ml of highly purified IL2 (36). ‡ High affinity binding sites per cell as determined by Scatchard analysis performed on 72 h PHA-stimulated cells. § Dissociation constants were estimated from Scatchard analysis. ‖ ^{3}H-anti-Tac binding as described in Methods. ¶ [^{3}H]Thymidine uptake was measured after 3 d. The standard deviation of each mean value were within 15%.

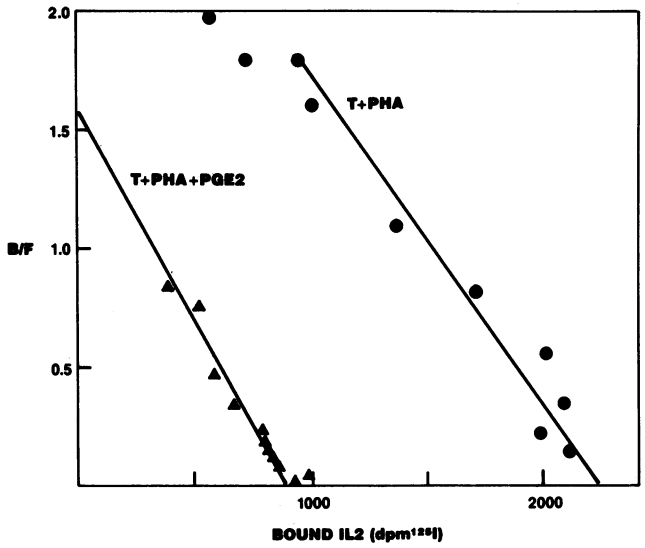

Figure 5. Scatchard plots from the [^{125}I] IL2 binding data illustrated in Table III. B/F, Bound IL2/free IL2.

inhibition of IL2 production and down regulation of transferrin receptor (6). The experiments reported here confirm and extend these findings. Our studies demonstrate that PGE_2 affect both $[Ca^2]_i$ and PKC activation. It is known that $[Ca^{2+}]_i$ serves as a messenger in initiation of T cell activation and that PHA increases Ca^{2+} influx and $[Ca^{2+}]_i$ mobilization (9, 10, 27, 28). Our data demonstrate that PGE_2 treatment of T lymphocytes reduces both Ca^{2+} and $[Ca^{2+}]_i$ mobilization. Although the calcium ionophore A23187 eliminated the PGE_2-induced inhibition of Ca^{2+} uptake and $[Ca^{2+}]_i$, it only partially restored T cell proliferation

Table IV. Abrogation of cAMP Elevating Agents' Inhibiting Effect on PHA-induced T Cell Proliferation in the Presence of TPA

	−TPA		+TPA	
Cell treatment*	Thymidine incorporation‡	% Transferrin receptor§ positive cells	Thymidine incorporation	% Transferrin receptor positive cells
	cpm		*cpm*	
Medium	138,600±6,270	67 (100)‖	177,050±7,800	71 (100)
PGE_2	34,900±2,115	29 (43)	147,660±6,190	63 (89)
CT	68,100±3,010	34 (51)	162,350±6,280	68 (96)
ISO	63,420±2,970	31 (46)	149,900±7,340	63 (89)
dBcAMP	48,720±2,540	27 (40)	147,420±5,080	59 (83)
$PGF_{2\alpha}$	147,000±7,190	70 (104)	189,160±6,320	74 (104)

* Enriched T cells were cultured in the presence of PHA (0.5 μg/ml) in the presence of PGE_2 (10^{-7} M), CT (25 ng/ml), ISO (10^{-4} M), $PGF_{2\alpha}$ (10^{-7} M) and tested with or without TPA (2.5 ng/ml).
‡ Thymidine incorporation was performed as described in Fig. 2.
§ Percentage of reactive cells after OKT9 monoclonal-antibody staining and FACS analysis determined as described in the legend to Fig. 4.
‖ Numbers in parentheses are percentages of response compared with the positive PHA-stimulated control culture (100%).

and IL2 production (Fig. 2). A similar, partial restoration of PGE_2 inhibition of PHA-stimulated T cell proliferative response can be achieved by addition of exogenous IL2 (6). Our results would suggest that the effect of PGE_2 on $[Ca^{2+}]_i$ results in inhibition of IL2 production, and support the observation that increase in $[Ca^{2+}]_i$ is an integral event in the induction of IL2 production and subsequent T-cell proliferation during mitogenic activation (29, 30). While A23187 only partially restored T cell proliferation, TPA treatment fully restored the mitogenic response. $[Ca^{2+}]_i$ has been reported to have a dual effect on PKC: membrane binding and enzyme activation (31). Tsien and Nishizuka have, however, reported that activation via the PKC pathway is separate from and synergistic to those activated via increase in $[Ca^{2+}]_i$ (10, 14). Furthermore, it has been reported that IL2 binding to its receptor induce PKC transposition from the cytosol to the cell plasma membrane (15). These observations, as well as our results, suggest that PGE_2 exert additional effects on PKC activity beyond the effects mediated via $[Ca^{2+}]_i$.

While TPA completely restored mitogenic T cell proliferation of PGE_2-treated cells, the IL2 activity in the culture supernatants, however, remained diminished. These seemingly contradictory observations can be explained if TPA at the concentration used was able to restore IL2 production to a level sufficient to induce a full proliferative response. Although IL2 is an essential requirement for T cell proliferation, it should be noted that the dose-response curve of T cell proliferation reaches a plateau at a finite IL2 concentration. It is also possible that in the presence of TPA, less IL2 is required to induce optimal T cell proliferation. Finally, TPA stimulation could involve an IL2-independent PKC-related proliferative pathway as has been suggested (32). It is in the present study demonstrated that PGE_2 exerts a dual effect on PHA-induced T cell activation: alteration of $[Ca^{2+}]_i$ and inhibition of PKC activation pathway resulting in inhibition of cell proliferation. If, however, the endogenous T cell activation pathway is triggered by costimulation with TPA and A23187, PGE_2 has no effect on cell proliferation. This further reinforces the PGE_2 involvement with the intracellular events regulating T cell activation following PHA stimulation.

Our studies also indicate that the mechanism that accounts for down regulation of transferrin receptor by PGE_2 primarily involve the PKC-activation pathway. While TPA treatment of PHA-stimulated T lymphocytes in the presence of PGE_2 resulted in a restoration of normal expression of transferrin receptor, A23187 had no effect on this receptor. If, however, the stimulation was provided by the combination of TPA and A23187, PGE_2 had no inhibitory effect on transferrin receptor expression. Again, another essential event in PHA-induced T cell activation is controlled by PGE_2.

It has been shown that the interaction of IL2 with its receptors induce the expression of transferrin receptor (33). As few as 100–200 molecules IL2 bound per cell is, however, sufficient to induce maximal T cell response (34). Our studies demonstrate that PGE_2 inhibit transferrin receptor expression as well as reduce the number of high affinity IL2 receptors. However, the decrease in IL2 receptors is not sufficient to result in inhibition of cell proliferation. Furthermore, exogenous IL2 will restore only IL2 receptor expression and has no effect on transferrin receptor expression. In contrast, TPA will restore both IL2 receptor and transferrin receptor expression. These results suggest that IL2 and TPA activation have different regulatory effects on the in-

duction of these receptors and that PGE_2 does not affect the signaling pathway controlled by TPA.

Modulation of PKC activity by PGE_2 appears to involve cAMP. Our results clearly indicate that adenylate cyclase activators, in the presence of TPA, lose their inhibitory capacity on T cell proliferation and transferrin receptor expression. These data are in agreement with the concept that PHA-induced activation of PKC might be controlled by cAMP through activation of PKA, which blocks the PKC activity (10). It has also been reported that increased cAMP induce inhibition of diacylglycerol production, and thereby inhibition of PKC (14, 35). However, in our system, addition of diacylglycerol did not abolish the effect of PGE_2 (data not shown).

The mechanism by which PGE_2 affects PKC activity and at which level of the enzyme activation process PGE_2 operates are presently unknown. Our study would indicate that PGE_2 could regulate the translocation of PKC from the cytosol to the cell membrane. Preliminary studies would suggest this mechanism (Piau, J. P., and S. Chouaib, unpublished observations). In contrast, TPA directly activates PKC, and thereby bypasses the effect of the second messenger generated following PHA stimulation. Our observations illustrate how biochemical defects in the signal transduction system correlate with phenotypic and functional abnormalities of T cell response and could explain why functional T cell defects occur in diseases associated with abnormal levels of PGE_2 (37, 38).

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