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European Journal of Pharmacology 466 (2003) 169–180
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Activation of cytosolic phospholipase A_2 in human T-lymphocytes involves inhibitor-κB and mitogen-activated protein kinases

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Received 11 February 2003; accepted 18 February 2003

Abstract

The group IV 85 kDa cytosolic phospholipase A_2 regulates many aspects of innate immunity. However, the function of this enzyme in T-cells remains controversial. We show here that human peripheral blood lymphocytes and Jurkat cells express cytosolic phospholipase A_2 and produce prostaglandin A_2 and leukotriene B_4. Selective inhibitors of this enzyme suppressed Ca^{2+}-ionophore-, mitogen- and T-cell receptor-mediated expression of interleukin-2 at the level of transcription from the promoter. Activation of mitogen-activated protein kinases (MAPK), degradation of inhibitor-κBα and transactivation by nuclear factor-κB (NFκB) were impaired as was the antigen-, lectin- and interleukin-2-driven proliferation of T-cells in vitro. Ligands of peroxisome proliferator-activated receptor-γ (PPARγ) induced rapid phosphorylation of MAPK in human monocytic but not in Jurkat cells. These data indicated that in T-cells, eicosanoids generated upon signal-activated cytosolic phospholipase A_2 promote NFκB-dependent interleukin-2 transcription via a PPARγ-independent mechanism involving the MAPK-pathway.

Keywords: Phospholipase A_2; NFκB (nuclear factor-κB); MAP (mitogen-activated protein) kinase; Interleukin-2; T-lymphocyte

1. Introduction

The role of the 85 kDa Ca^{2+}-dependent group IV cytosolic phospholipase A_2 in signal-mediated release of arachidonic acid has been thoroughly examined in cells of the innate immunity such as monocytes, macrophages and granulocytes (Murakami et al., 1997; Capper and Marshall, 2000). Due to subsequent metabolization of arachidonic acid into eicosanoids (prostaglandins and leukotrienes) by cyclooxygenases and lipoxygenases, this enzyme, together with the 14 kDa group II secretory phospholipase A_2, is directly involved in a variety of inflammatory, autoimmune and allergic diseases (Cunningham, 1994; Sapirstein and Bonventre, 2000; Tilley et al., 2001).

Cross-linking of the T-cell receptor by antigens, mitogens or stimulatory antibodies initiates arachidonic acid turnover mainly from phosphatidylcholine implicating *sn-2* selective phospholipases A_2 (Roshak et al., 2000; Boilard and Surette, 2001). Influx of free Ca^{2+}-ions and costimulatory molecules including the CD3-complex (Cifone et al., 1995; Gilbert et al., 1996), CD28 (Los et al., 1995), CD4 (Garofalo et al., 1998) and CD2 (Le Gouvello et al., 1990) are also coupled to activation of phospholipases A_2 and lipoxygenases. More recent studies claim a role for the Ca^{2+}-independent group VI intracellular phospholipase A_2 (Roshak et al., 2000) and a CoA-independent transacylase (Boilard and Surette, 2001) in proliferation of lymphoid cells. Thus, the function of cytosolic phospholipase A_2 in the adaptive immune response remains largely controversial (Courtney et al., 1993; Feltenmark et al., 1995; Gilbert et al., 1996).

Because of their rapid secretion from cells by ATP-driven transporter molecules, eicosanoids can act in at least three different modes: (1) as paracrine mediators, they bind to G-protein-coupled receptors on target cells (Breyer et al., 2001); (2) as intracellular ligands, they target peroxisome proliferator-activated receptors (PPAR) (Rosen and Spiegelman, 2001); (3) as intracrine second messengers, they directly modulate signalling molecules such as ion channels and kinases via yet unknown molecular mechanisms (Brash, 2001; Gilmour and Mitchell, 2001; Straus and Glass, 2001).

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0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01492-4

Referring to these pleiotropic actions of eicosanoids, we were interested in the molecular consequences upon inhibition of cytosolic phospholipase A_2, the rate-limiting enzyme in eicosanoid generation in human leukocytes. We have shown previously that site-directed inhibitors and antisense oligonucleotides directed against the mRNA of this enzyme attenuated lipopolysaccharide-, Ca^{2+}-ionophore and mitogen-induced arachidonic acid release and eicosanoid production in human peripheral blood monocytes and the human monocytic cell lines THP-1 and U937 (Burgermeister et al., 1999). The in vitro synthesis of proinflammatory cytokines (interleukin-1β, tumor necrosis factor-α and interleukin-6) and chronic inflammation in vivo were also suppressed (Amandi-Burgermeister et al., 1997). We further addressed the molecular mechanism and found that phosphorylation and activation of the three subfamilies of mitogen-activated protein kinases (MAPK), namely extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 kinase, by upstream MAPK kinases (MEK) were impaired. This was followed by reduced degradation of inhibitor-κB and transcriptional activation of cytokine promoters by nuclear factor-κB (NFκB), activation protein-1 (AP-1), nuclear factor-interleukin-6 (NF-IL6) and signal-transducer-and-activator-of-transcription-1 (STAT-1) (Burgermeister et al., 2000).

These data pointed at a profound role of cytosolic phospholipase A_2 in regulation of immune response genes in monocytic cells. In the present study, we further investigated the function of this enzyme in the lymphoid subpopulation. We show that cytosolic phospholipase A_2 is expressed in T-cells and is involved in proliferation and in the MAPK- and NFκB-dependent transcription of the interleukin-2 gene.

2. Materials and methods

2.1. Reagents

All reagents used were from Sigma (Deisenhofen) and Merck (Darmstadt) if not stated otherwise. The trifluoromethylketone analogues of arachidonic acid C20:4-$COCF_3$, γ-linolenic acid C18:3-$COCF_3$ and linoleic acid C18:2-$COCF_3$ and 6,7-dihydroxy-4-(7-trifluoromethyl-quinolin-4-ylsulfanylmethyl)chromen-2-on (CF_3-CM) and (6,7-dihydroxy-2-oxo-*2H*-chromen-4-ylmethylsulfanyl)-acetate (MS-CM) were synthesized by Roche Diagnostics GmbH (Mannheim). The latter compounds and methyl-arachidonyl-fluorophosphonate (Calbiochem, Schwalbach), 15-desoxy-$\Delta^{12,14}$-prostaglandin J_2 (Calbiochem) and cyclosporine A (Novartis, Basel) were stored as 10 mg/ml stock solutions in dimethylsulfoxide at −20 °C under nitrogen. Compounds were freshly diluted in RPMI 1640 cell culture medium and added to cells with a final dimethylsulfoxide concentration < 0.1% (v/v). The monoclonal immunoglobulins (Ig) against CD3 (IgG2a, clone HIT3a) and CD28 (IgG1, clone CD28.2) were purchased from Pharmingen (Hamburg).

2.2. Cell lines

The cell lines Jurkat (human T-lymphoid), U937, THP-1 (human promonocytic) and P388D1 (mouse macrophage) were obtained from the American Type Culture Collection (ATCC, Rockville). The MUTZ3 cell line (human monocytic) was from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig).

All cell lines were cultured in RPMI 1640 medium (low endotoxin, Seromed Biochrom, Berlin) supplemented with 10% (v/v) fetal calf serum (heat-inactivated for 45 min at 56 °C, low endotoxin), 2 mM glutamine, 100 IU/ml penicillin and 20 μg/ml streptomycin (all from Gibco Life Technologies, Karlsruhe) in a humidified atmosphere at 37 °C and 7.5% CO_2.

2.3. Stimulation of a human autoreactive T-cell clone with antigen peptides

Peripheral blood mononuclear cells from human donors with a matched major histocompatibility complex haplotype (Endl et al., 1997) were seeded (2×10^6/ml) in RPMI 1640 medium with 5% (v/v) human serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and were supplemented with 10 μg/ml recombinant antigen (human glutamate decarboxylase) or with 5 μg/ml of a 20-mer peptide derived from this antigen (Roche Diagnostics GmbH). For binding of the antigen to major histocompatibility complex class II molecules, the cells were incubated for 3 h at 37 °C. Thereafter, cells were inactivated by γ-irradiation using 4.000 rad. Then, 1×10^5 of irradiated cells were seeded in 50 μl per well of a 96-well round bottom plate. The human, autoreactive T-cell clone (glutamate decarboxylase-specific from a patient with insulin-dependent diabetes mellitus) was seeded at a density of 4×10^5 cells/ml in RPMI 1640 medium. Then, 2×10^5 cells were added in 50 μl per well to the inactivated antigen-presenting cells and the cells were cocultured for 3 to 4 days at 37 °C. After 3 days, 30 U/ml interleukin-2 (human recombinant, Roche Diagnostics GmbH) in 100 μl medium per well was supplemented. Every 3 to 4 days, 100-μl medium was removed and replaced by interleukin-2-enriched medium. Thereafter, the cells were passaged every 2 to 3 days, and every 12 to 16 days, supplementation of interleukin-2 was repeated.

2.4. Enrichment of human peripheral blood lymphocytes

Human peripheral venous blood was collected and human peripheral blood mononuclear cells were enriched, as described previously (Amandi-Burgermeister et al., 1997; Burgermeister et al., 1999). Mononuclear cells were cultivated in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 1% (v/v) minimal

essential medium-vitamins (Gibco Life Technologies), 100 IU/ml penicillin and 20 μg/ml streptomycin. For removal of monocytes by adherence, cells were seeded at a density of 1×10^6 cells/ml and cultured for 1 h in polystyrole tissue culture flasks. Non-adherent peripheral blood lymphocytes were recovered and purity was greater than 95%.

2.5. Quantification of eicosanoids and cytokines

Prostaglandin E_2, leukotriene B_4 (RD Systems, Wiesbaden) and interleukin-2 (Roche Diagnostics GmbH) were determined in the supernatants of stimulated cells using enzyme-linked immunosorbent assays according to the manufacturer's instructions.

2.6. Reporter DNA construction and stable transfection of Jurkat cells

Upstream regulatory sequences of the human pro-interleukin-2 gene ($-1263/+10$ bp) were obtained by polymerase chain reaction (PCR) and ligated 5′ to the β-galactosidase gene in the reporter plasmid pNASβ (Clontech, Heidelberg). This plasmid was then cotransfected with pHMR272 coding for hygromycin resistance into Jurkat cells and positive clones were selected, as described before (Burgermeister et al., 2000).

For removal of mutants, transfected Jurkat cells were cultured frequently in presence of 1 mg/ml hygromycin B (Roche Diagnostics GmbH).

2.7. β-Galactosidase assay

Stably transfected Jurkat cells (1×10^6/ml) were stimulated in round-bottom 96-well plates. Supernatants were removed after centrifugation, and a chromogenic β-galactosidase substrate solution was added to the cell pellets, as described previously (Burgermeister et al., 2000). Alternatively, total β-galactosidase protein was determined in cell lysates from 1×10^7 Jurkat cells by immunoassay (Roche Diagnostics GmbH) according to the manufacturer's instructions.

2.8. Transient transfection of reporter plasmids

The following reporter plasmids were from Roche Diagnostics GmbH: pCMV (positive control plasmid containing a strong promoter from cytomegalovirus); $3 \times$ NFκB-pBL2 (inducible enhancer with a trimer of the NFκB-binding motif in front of a minimal promoter); pBL2 (minimal promoter from the herpes simplex virus thymidine kinase gene); pBL3 (negative control lacking promoter sequences) (Mihm et al., 1991). Cells were seeded in serum-free RPMI 1640 cell culture medium at a density of 1×10^6/ml in 6-well plates. Transient transfection was then performed by lipofection, as described before (Burgermeister et al., 2000). Twenty-four hours upon transfection, cells were stimulated for another 24 h in presence of the relevant compounds as described in Section 3.

Thereafter, cell lysates were assayed for expression of the reporter protein chloramphenicol acetyltransferase (CAT) using immunoassays according to the manufacturer's instructions (Roche Diagnostics GmbH).

2.9. Semi-quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from cells using Tripure Reagent (Roche Diagnostics GmbH). Serial ten-fold dilutions of 100 ng total RNA were reverse transcribed using a 5′-downstream primer specific for β-galactosidase according to the manufacturer's instructions (Geneamp®-Thermostable rTh-Reverse-Transcriptase RNA PCR Kit, Perkin-Elmer, Dreieich). Resulting cDNAs were amplified ($35 \times$ cycles) using the 3′-upstream primer: 5′-primer: 5′-TAACGA-CATTGGCGTAAGTG-3′; 3′-primer: 5′-AATCCGAGC-CAGTTTACCCG-3′. Equal RNA content was confirmed by using primers specific for β-actin (Stratagene, Amsterdam). Amplification products were separated by ethidium-bromide agarose gel electrophoresis. Band intensity in gel was estimated by video-based densitometry (EasyPlus device by Herolab, Wiesloch). Results were calculated from arbitrary optical density units (see Section 3).

2.10. Western blotting

Cells ($1-10 \times 10^7$) were lysed and supplemented with Laemmli-loading buffer. Denaturing polyacrylamide gel electrophoresis and Western blotting were then performed, as described previously (Burgermeister et al., 1999, 2000).

Primary antibodies used were directed against the unphosphorylated (rabbit polyclonal) and dual phosphorylated (mouse monoclonal, both from Sigma) forms of ERK-1 and -2, src tyrosine kinase (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz) and MEK-1 and -2 (rabbit polyclonal, Cell Signalling, Beverly), PPARγ (rabbit polyclonal), inhibitor-κBα (rabbit polyclonal) (all from Santa Cruz Biotechnology) and cytosolic phospholipase A_2 (rabbit polyclonal, Cayman Chemicals, Ann Arbor). Membranes were developed by enhanced chemiluminescence (Amersham, Freiburg) according to the manufacturer's instructions and exposed to X-ray films (Kodak). Bands in films were quantified by video-based densitometry.

2.11. In vitro kinase assays

The non-radioactive in vitro kinase reactions for the ERK, JNK and p38 kinases were performed according to the manufacturer's instructions (New England Biolabs, Frankfurt a.M.). As described before (Burgermeister et al., 2000), the active phosphorylated forms of MAPK were immunoprecipitated from total cell lysates (equivalent to 1×10^7 cells) and phosphorylation of the specific

substrate proteins was detected. Upon denaturing polyacrylamide gel electrophoresis Western blotting with antibodies against the phosphoforms of c-jun, elk-1 and activating transcription factor-2 (ATF-2) (Mouse monoclonal, New England Biolabs) served as an estimation of the relative kinase activities.

2.12. Cytotoxicity assays

Cell viability was monitored by flow cytometry using propidiumiodide exclusion (Amandi-Burgermeister et al., 1997) and by colorimetric assays (Burgermeister et al., 2000). Apoptotic cells were quantified applying the In Situ Cell Death Detection system (Roche Diagnostics GmbH) according to the manufacturer's instructions. In the terminal transferase-mediated dUTP nick end labelling (TUNEL) reaction free 3′-end of double-stranded DNA are labelled with fluorescein-labelled dUTP which are then detected by flow cytometry.

2.13. Proliferation assay

In order to quantify the early proliferation rate of T-lymphocytes, 0.6 to 1×10^5 cells from the autoreactive T-cell clone were cultivated for 72 h with antigen-presenting cells in 50 μl per well of a 96-well plate. Thereafter, 1 μCi [^{3}H]thymidine was added per well in a final volume of 10 μl and the plates were incubated for another 16 h. The cells were harvested on filters and the incorporated radioactivity was determined using scintillation and a β-counter. For the interleukin-2-driven proliferation the assay was performed as described in Section 2.3.

2.14. Statistics

Each experiment was performed at least three times using different blood donors or log-phase passages of cell lines. Values are expressed as means ± S.D. or S.E.M. as indicated in legends to figures. Significance was determined by Student's *t*-test and is marked by asterisks (* = $P < 0.05$, ** = $P < 0.01$, ** = $P < 0.001$).

3. Results

3.1. Expression of cytosolic phospholipase A_2 and eicosanoid formation in human T-lymphoid cells

We first examined the expression of cytosolic phospholipase A_2 in cell lysates by Western blotting. Cell lysates were prepared from human peripheral blood mononuclear cells, which had been separated by adhesion on polystyrol into the adherent monocytic and non-adherent lymphocytic subpopulations (Fig. 1A). Cell lysates were also prepared from the human T-cell line Jurkat and the human monocytic cell lines THP-1, U937, MUTZ3 and the mouse macrophage line P388D1 (Fig. 1B). All cell types examined expressed a band around 100 kDa which has been shown to represent the 85 kDa cytosolic phospholipase A_2 (Boilard and Surette, 2001).

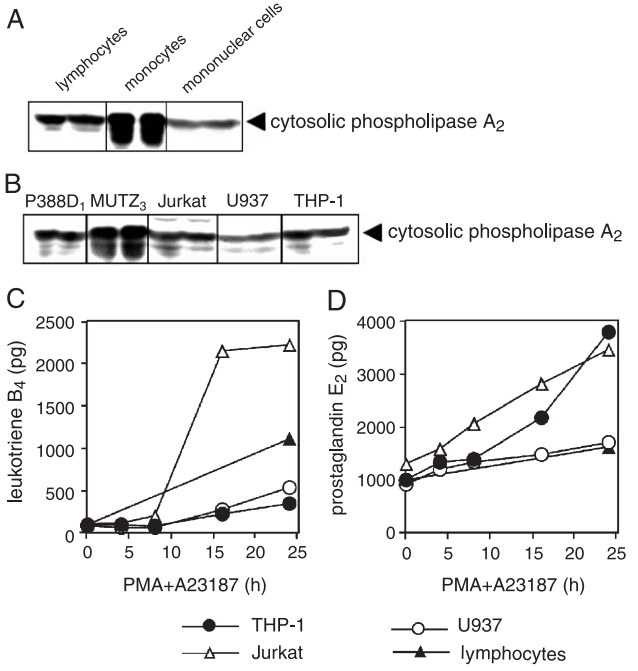

Fig. 1. Arachidonate metabolism in subpopulations of human venous blood and leukemia cell lines. (A–B) Expression of cytosolic phospholipase A_2: (A) from left, non-adherent primary blood lymphocytes, adherent monocytes and primary blood mononuclear cells; (B) from left, mouse macrophage (P388D1), monocytic (MUTZ3), T-lymphoid (Jurkat), promonocytic (U937), promonocytic (THP-1). Cell lysates were separated by denaturing polyacrylamide gel electrophoresis and Western blotting was performed with an antiserum against cytosolic phospholipase A_2. Total protein from two different passages and primary cells from two independent blood donors were loaded on the gel. (C–D) Secretion of eicosanoids. Cells were stimulated with PMA + A23187 for the times indicated and (C) leukotriene B_4 and (D) prostaglandin E_2 were quantified in the supernatants by immunoassay. Values are means in pg per 10^6 cells ($n = 3$).

We next determined the secretion of eicosanoids (Fig. 1C and D) into the cell culture medium by enzyme-linked immunosorbent assay. Non-adherent peripheral blood lymphocytes and Jurkat cells were induced to secrete significant amounts of prostaglandin E_2 (1.1–2.2 ng/10^6 cells) and leukotriene B_4 (1.6–3.4 ng/10^6 cells) upon a 24-h stimulation with a combination of 20 ng/ml phorbol-12-myristate-13-acetate (PMA) and 0.5 μg/ml Ca^{2+}-ionophore A23187. These data confirmed that T-cells secrete eicosanoids and that a functional high molecular weight phospholipase A_2 is involved in this process.

3.2. Attenuation of antigen- and mitogen-stimulated proliferation of human T-cells by inhibitors of cytosolic phospholipase A_2

Specific molecular tools are used to abrogate cytosolic phospholipase A_2 activity and prevent arachidonic acid

release and eicosanoid formation in vitro and in vivo: gene knock-out (Sapirstein and Bonventre, 2000), antisense oligonucleotides (Marshall and Roshak, 1998) and low-molecular-weight inhibitors (Street et al., 1993; Trimble et al., 1993). Here, we applied the trifluoromethylketone derivatives of arachidonic (C20:4-$COCF_3$), linolenic (C18:3-$COCF_3$) and linoleic acid (C18:2-$COCF_3$) as reversible transition state inhibitors of the catalytic serine 228 residue within the active site of enzyme (IC_{50} versus cytosolic phospholipase A_2 = 100 nm; versus intracellular phospholipase A_2 = 5 μM) (Huang et al., 1996; Roshak et al., 2000). We further synthesized a dual cytosolic/secretory phospholipase A_2 inhibitor CF_3-CM (IC_{50} versus both enzymes = 15 μM) composed of a coumarin moiety (Huang et al., 1992) and a trifluoromethyl group (Street et al., 1993).

We found previously that these inhibitors reduced eicosanoid and cytokine synthesis in monocytic cells comparable to the effect exerted by antisense oligonucleotides and without cytotoxic side effects (Amandi-Burgermeister et al., 1997; Burgermeister et al., 1999). We then probed their effect on T-cell receptor signalling. As shown before (Burgermeister et al., 1999), the trifluoromethylketones diminished PMA + A23187-induced eicosanoid production in non-adherent peripheral blood lymphocytes and Jurkat cells whereas inhibitors of secretory phospholipase A_2 were ineffective (not shown). The dual inhibitor methyl-arachidonyl-fluorophosphonate (IC_{50} versus cytosolic and intracellular phospholipases A_2 < 0.5 μM) (Lio et al., 1996) also effectively blocked eicosanoid production (not shown).

In order to further evaluate the biological consequences of phospholipase A_2 inhibition, we investigated the antigen-specific response of human T-cells (Fig. 2A and B). We used an autoreactive T-cell clone derived from a patient with insulin-dependent diabetes mellitus (Endl et al., 1997). This clone is reactive to the glutamate decarboxylase from β-islet cells of the pancreas or to stimulatory peptides derived from amino acids 270 to 283 of this antigen. Human major histocompatibility complex-compatible antigen-presenting peripheral blood mononuclear cells were loaded with the stimulatory peptides in vitro and inactivated upon γ-irradiation. These stimulator cells were then cocultured with the T-cell clone in presence of 10 μg/ml of the phospholipase A_2 inhibitors and [^{3}H]thymidine.

After 3 to 4 days, the proliferation rate of the coculture was determined by incorporation of [^{3}H]thymidine in the DNA of the T-cells. The initial antigen-dependent generation of blasts and mitoses was reduced by 95–100% in presence of the cytosolic phospholipase A_2 inhibitor C18:2-$COCF_3$ and the dual inhibitor of cytosolic and secretory phospholipase A_2 CF_3-CM (both at 10 μg/ml) compared to the vehicle-treated stimulated control cells (Fig. 2A). Subsequent restimulation with exogenous recombinant interleukin-2 for another 2 days revealed that these inhibitors also attenuated, however less pronounced, the late antigen-independent proliferation by 45–55% (Fig. 2B). In contrast, an

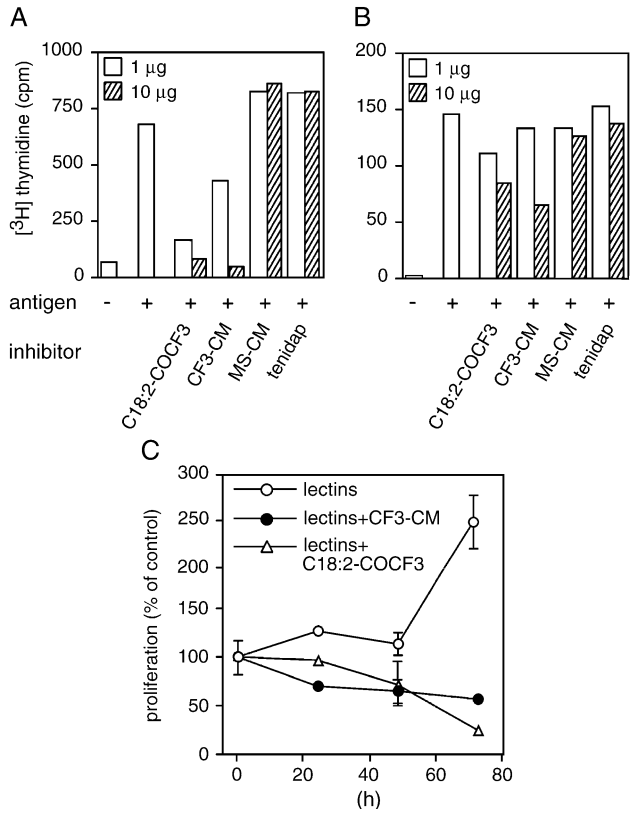

Fig. 2. Attenuation of T-cell proliferation by cytosolic phospholipase A_2 inhibitors. (A–B) Autoreactive T-cells derived from a human islet antigen-specific clone of a diabetes patient were cocultured for 3 days with peripheral blood mononuclear cells, which had been loaded with antigenic peptides, in presence of [^{3}H]thymidine and final inhibitor concentrations of 1 and 10 μg/ml. After 3 days, recombinant interleukin-2 was added for another 2 days. The amount of incorporated [^{3}H]thymidine is expressed in cpm. (A) Antigen-stimulated initial stage (3 days); (B) antigen-independent, interleukin-2-driven stage (5 days). (C) Time course of lectin-stimulated proliferation of human peripheral blood lymphocytes. Cells were stimulated with a mitogenic combination of phytohaemagglutinin and concanavalin A in presence of 10 μg/ml cytosolic phospholipase A_2-inhibitors. After 1 to 4 days, cells were subjected to a colorimetric proliferation assay as detailed under Section 2. Values are expressed as % proliferation ± S.D. (n = 3) compared to vehicle-treated stimulated control cells.

inhibitor of secretory phospholipase A_2 MS-CM (IC_{50} = 1 μM) and the dual the cyclooxygenase/lipoxygenase-inhibitor tenidap were ineffective.

In addition to antigens, mitogenic lectins trigger the mitotic bursts of lymphocytes by cross-linking the T/B-cell receptor molecule complexes. We propagated non-adherent peripheral blood lymphocytes for 3 days with a combination of phytohaemagglutinin and concanavalin A. Proliferation was measured by a colorimetric assay as detailed under Section 2 (Fig. 2C). The cytosolic phospholipase A_2 inhibitors (at 10 μg/ml) decreased the lectin-induced proliferation of lymphocytes by 74% within 48–72 h (n = 3). These results confirmed that cytosolic phospholipase A_2 is involved in the antigen- and mitogen-induced proliferation of lymphocytes. To rule out toxic effects, we determined the

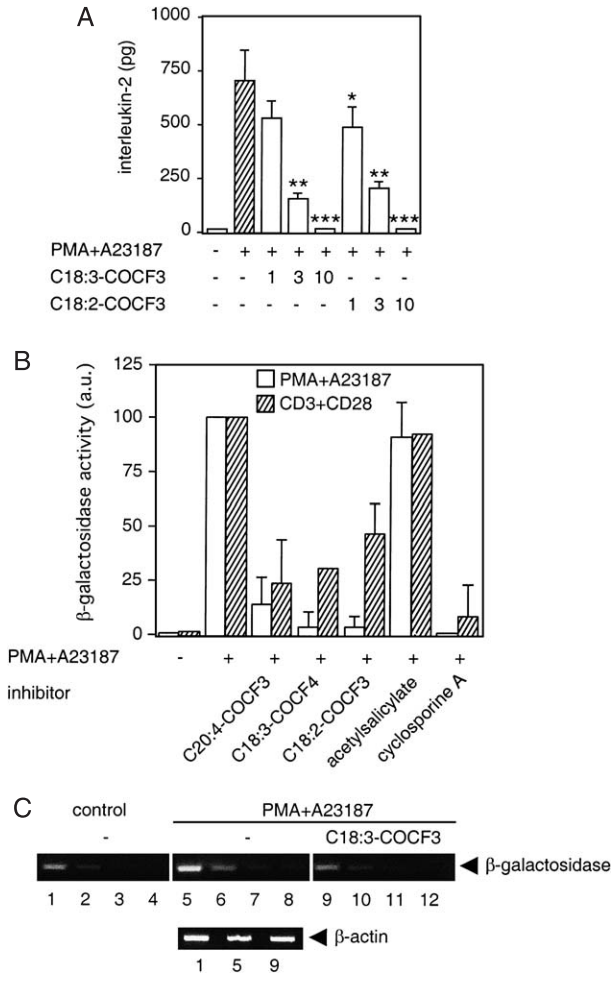

Fig. 3. Effect of cytosolic phospholipase A_2 inhibitors on interleukin-2 synthesis. (A) Secretion of interleukin-2 from human non-adherent peripheral blood lymphocytes. Cells were stimulated for 24 h with PMA + A23187 in presence of increasing concentrations of inhibitors (1, 3 and 10 μg/ml). Quantification of interleukin-2 was performed in cell lysates by immunoassay. Values are means ± S.E.M. in pg per 10^6 cells ($n = 3$). (B–C) Reporter gene expression in Jurkat cells stably transfected with the 5′-upstream regulatory sequence of the human interleukin-2 promoter. Cells were stimulated for 24 h with either PMA + A23187 or antibodies against CD3 and CD28 in presence of 10 μg/ml of the inhibitors. Cells were lysed and protein and RNA levels of β-galactosidase were determined. (B) Colorimetric assay of β-galactosidase activity: Values are means ± S.D. in % compared to the optical density resulting from stimulated control cells ($n = 3$). (C) RT-PCR of β-galactosidase RNA: Serial dilutions of total RNA (100, 50, 10 and 1 ng) were reverse transcribed and the β-galactosidase transcript was amplified by PCR and compared to the signal derived from the mRNA of β-actin (100 ng). Amplification products were visualized by ethidiumbromide agarose gel electrophoresis.

proportion of free double-strand breaks in the DNA by flow cytometry (not shown). No significant induction of cell death was observed in presence of all inhibitors used, except of C20:4-$COCF_3$, which after 24 h at a final concentration of 10 μg/ml, lead to accumulation of 47% fluorescein-labelled DNA-positive $CD3^+$ T-lymphocytes. We therefore used this inhibitor only for short-time studies.

3.3. Inhibition of cytosolic phospholipase A_2 reduces interleukin-2 secretion

Synthesis of interleukin-2 is an early characteristic in the activation of T-cells. Non-adherent peripheral blood lymphocytes were therefore stimulated for 24 h in presence of cytosolic phospholipase A_2 inhibitors with PMA + A23187. The quantification of interleukin-2 in the supernatants was performed using an immunoassay (Fig. 3A). We found that the secretion of interleukin-2 was reduced in a concentration-dependent manner by the cytosolic phospholipase A_2 inhibitors with IC_{50}-values of 4–8 μg/ml (Table 1), whereas secretory phospholipase A_2 inhibitors were ineffective (not shown). These data indicated that cytosolic phospholipase A_2 is involved in the synthesis of interleukin-2 in T-cells.

3.4. Cytosolic phospholipase A_2 inhibitors decrease mRNA transcription from the interleukin-2 promoter

We next examined the effect of cytosolic phospholipase A_2 inhibitors on the activity of the interleukin-2 promoter. A Jurkat cell line was used which was stably transfected with a plasmid containing the upstream regulatory sequence (−1263/+10 bp) of the human interleukin-2-gene in front of a β-galactosidase reporter gene. The quantification of β-galactosidase was performed in situ using a colorimetric enzyme assay (Fig. 3B). These cells were then stimulated with PMA + A23187 or antibodies against the T-cell receptor complex CD3 and the costimulatory CD28 molecule. Within 2–24 h, the cytosolic phospholipase A_2 inhibitors (at 10 μg/ml) decreased the activity of the reporter enzyme by 55–100% and 30–90%, respectively ($n = 3$). These values were comparable to the effect of the immunosuppressive agent cyclosporine A, whereas the cyclooxygenase-inhibitor acetylsalicylate and the secretory phospholipase A_2 inhibitor MS-CM were ineffective. The intracellular accumulation of β-galactosidase was already attenuated by the cytosolic phospholipase A_2 inhibitors in the first 2 h (data not shown).

Results from reverse transcription polymerase chain reaction (RT-PCR) experiments confirmed that already the initial increase in β-galactosidase RNA-transcript was decreased in the presence of cytosolic phospholipase A_2

Table 1
Inhibition of interleukin-2 secretion[a]

Compound	IC_{50} (μg/ml)
C18:3-$COCF_3$	3.5
C18:2-$COCF_3$	4.0
CF_3-CM	8.8

[a] IC_{50}-values for the inhibition of interleukin-2 secretion by cytosolic phospholipase A_2 inhibitors. Non-adherent blood lymphocytes were stimulated with PMA + A23187 for 24 h in presence of different concentrations of inhibitors (10, 1 and 0.1 μg/ml). Interleukin-2 was determined in the supernatants by immunoassay ($n = 6$).

inhibitors in a similar time range of 1–2 h after stimulation. Here, C18:2-$COCF_3$ and C20:4-$COCF_3$ exerted an inhibition of 50 ± 25% (Fig. 3C) and 70 ± 20% (not shown), respectively, and cyclosporine A an inhibition of 97 ± 5% compared to vehicle-treated control ($n = 3$).

The dual inhibitor of both intracellular and cytosolic phospholipases A_2 methyl-arachidonyl-fluorophosphonate also effectively reduced the amount of eicosanoid production after PMA + A23187 stimulation, but not when CD3 + CD28 were used as a stimulant (not shown). These findings indicate that in the physiologic context, the cytosolic, but not the intracellular phospholipase A_2, is the relevant enzyme responsible for eicosanoid-mediated events on interleukin-2 transcription.

3.5. Inhibitors of cytosolic phospholipase A_2 prevent NFκB-dependent transactivation of a minimal promoter

To measure the transactivation potential of NFκB, Jurkat cells were transiently transfected with reporter plasmids, that either harbour an inducible trimer of the cognate NFκB-binding motif or different control plasmids, as detailed in the method in Section 2.8 (Mihm et al., 1991) (Fig. 4A). Cells were then stimulated for 24 h with PMA + A23187 in presence of 10 μg/ml cytosolic phospholipase A_2 inhibitors. Quantitation of the reporter protein (CAT) was performed in cell lysates using an immunoassay. The cytosolic phospholipase A_2 inhibitors C20:4-$COCF_3$, CF_3-CM and the antioxidant pyrolidine dithiocarbamate reduced NFκB-mediated reporter protein expression by 61–100% ($n = 3$) compared to vehicle-treated stimulated control cells. The secretory phospholipase A_2 inhibitor MS-CM and the dual cyclooxygenase and lipoxygenase inhibitor tenidap and acetylsalicylate remained ineffective (not shown).

3.6. Effect of cytosolic phospholipase A_2 inhibitors on inhibitor-κB phosphorylation and degradation

As a marker of NFκB activation, we detected the degradation of inhibitor-κBα. Jurkat cells (Fig. 4B) and non-adherent peripheral blood lymphocytes (Fig. 4C) were stimulated with PMA + A23187 for 15–60 min, and the amount of inhibitor-κB in cell lysates was determined by Western blotting. Densitometric analysis of three independent experiments revealed that within 15–60 min, inhibitor-κB was completely degraded in stimulated control cells. In lysates from cells which had been treated with 10 μg/ml of the cytosolic phospholipase A_2-inhibitors, C20:4-$COCF_3$ and C18:2-$COCF_3$, the degradation was decreased to 60–0% compared to vehicle-treated PMA + A23187-stimulated control cells ($n = 3$). Pyrolidine dithiocarbamate but not tenidap was able to prevent degradation of inhibitor-κB. These data corroborated that eicosanoids generated upon stimulation of cytosolic phospholipase A_2 interact with signalling cascades that govern mobilization of NFκB.

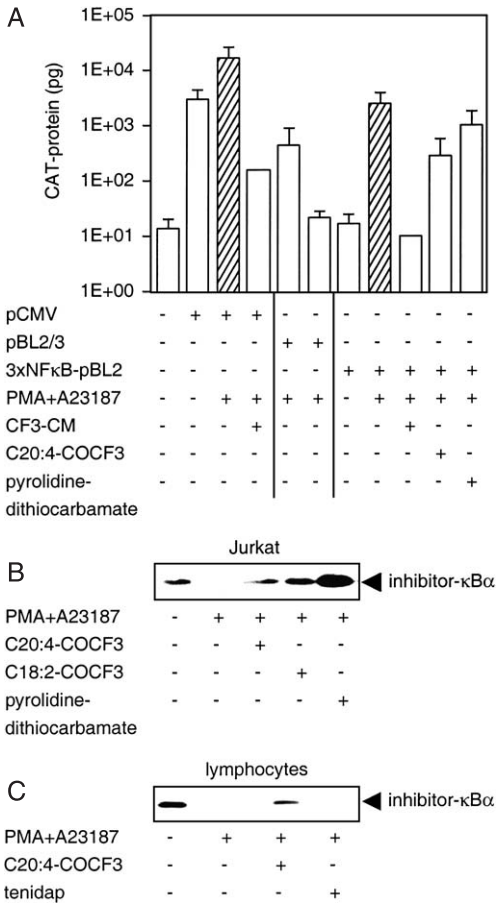

Fig. 4. Decrease in NFκB-mediated transcription upon inhibition of cytosolic phospholipase A_2. (A) Jurkat cells were transiently transfected with different reporter plasmids as indicated in Section 2.8: pCMV positive control; pBL2/3 negative controls; 3 × NFκB-pBL2 inducible enhancer with trimers of the NFκB-binding motif. Upon transfection, cells were stimulated for 24 h with PMA + A23187 in presence of 10 μg/ml inhibitors. Reporter protein (CAT) expression was measured in cell lysates by immunoassay. Values represent means ± S.E.M. in pg reporter CAT protein per mg of total protein ($n = 3$). (B–C) Phosphorylation and degradation of inhibitor-κB. (B) Jurkat cells and (C) non-adherent peripheral blood lymphocytes were stimulated for 15 min with PMA + A23187 in presence of 10 μg/ml of the inhibitors. Inhibitor-κBα protein was detected in cell lysates by denaturing polyacrylamide gel electrophoresis and Western blotting.

3.7. Cytosolic phospholipase A_2 inhibitors diminish MAPK-mediated phosphorylation of elk-1 and c-jun

MAPK are central upstream regulators of transcription factors. We therefore performed in vitro kinase activity assays. In contrast to monocytes (Burgermeister et al., 2000), no activation of p38 kinase was detectable in primary blood lymphocytes nor in Jurkat cells as measured by ATF-2 phosphorylation (not shown).

However, as summarized upon densitometric analysis, the amount of ERK- and JNK-mediated phosphorylation of their substrate proteins elk-1 and c-jun increased by 13 ± 2 upon a 15–30 min stimulation with PMA + A23187 ($n = 3$)

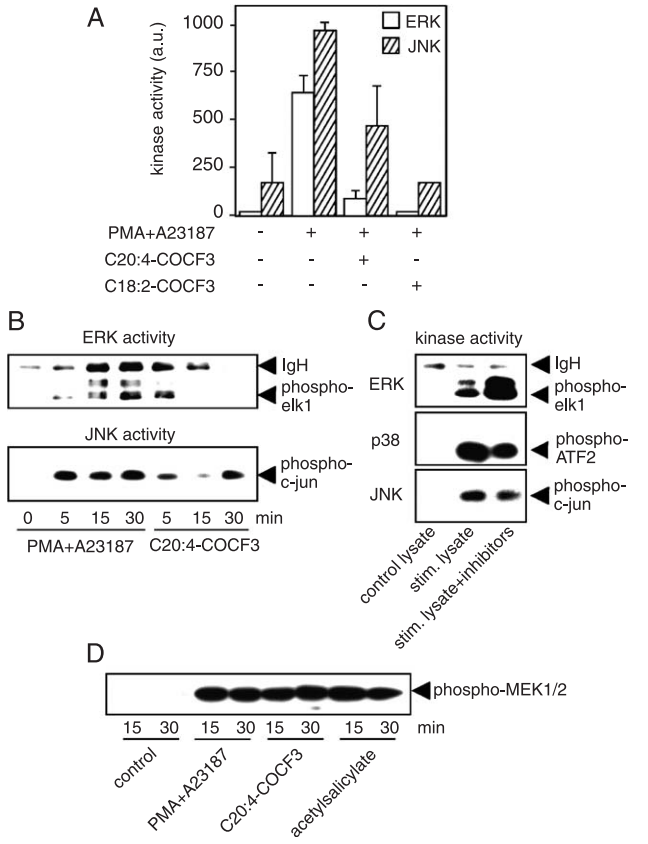

Fig. 5. In vitro kinase activity assays. (A–B) Inhibition of ERK-and JNK-activities in non-adherent peripheral blood lymphocytes and Jurkat cells. Cells were stimulated for 15 min with PMA + A23187 in presence of cytosolic phospholipase A_2-inhibitors (all at 10 μg/ml). Active ERK and JNK were immunoprecipitated from cell lysates and kinase activity was determined by phosphorylation of elk-1 and c-jun, respectively. Proteins were separated by denaturing polyacrylamide gel electrophoresis. The phosphorylated substrates and the heavy chains of the immunoglobulins (IgH) were detected by western blotting. (A) Summary of the densitometric analysis from gels from three independent experiments. Values are means ± S.D. of relative kinases activity (a.u.). (B) Inhibition of ERK-activity by 10 μg/ml C20:4-$COCF_3$ in Jurkat cells. Representative experiment. (C) Mononuclear cells were left untreated (control) or were stimulated with PMA + A23187 for 15 min. Active ERK, p38 and JNK were immunoprecipitated from lysates. A mixture of the three trifluoromethylketones (10 μg/ml each) were then added to the kinase reaction and phosphorylation of substrates was determined. (D) Detection of phosphorylated MEK-1/-2 by Western blotting. Jurkat cells were treated as in (A–B).

(Fig. 5A and B). In order to rule out that the inhibitors directly inhibit the kinase activity, we performed a modified in vitro kinase assay (Fig. 5C). Immunoprecipitates against the active phosphoforms of the kinases from cell lysates from either unstimulated or PMA + A23187-stimulated cells were incubated with the substrate protein and ATP a posteriori in presence or absence of 10 μg/ml of each trifluoromethylketone. Under these conditions, the compounds did not block the substrate phosphorylation by each of the kinases. From this result, it was evident that the inhibitors act in intact cells but remain ineffective in a cell-free condition. This conclusion was corroborated when we took immunoprecipitates from Jurkat cells and non-adherent peripheral blood lymphocytes which had been stimulated for 5–30 min with PMA + A23187 and in a priori presence of cytosolic phospholipase A_2 inhibitors (at 10 μg/ml) (Fig. 5A and B). Then, C20:4-$COCF_3$ and C18:2-$COCF_3$ reduced phosphorylation of elk-1 by 90–100% and of c-jun by 63–100% ($n = 3$). In contrast, the phosphorylation of the upstream MEK-1 and-2 (specific for ERK) (Fig. 5D) and of MEK-4 (specific for JNK) (not shown) was not impaired by the inhibitors.

3.8. Rapid phosphorylation of MAPK by ligands of PPARγ

In order to distinguish between paracrine and intracrine effects of eicosanoids, we used three classes of PPARγ-ligands: 15-desoxy-$\Delta^{12,14}$-prostaglandin J_2, ciglitizone and phenylacetate. Jurkat cells and human monocytic THP-1 cells were starved for 16 h and then stimulated for 5–60 min with different ligands (all at 10 μM) and PMA as a positive control (Fig. 6A and B). These agonists induced a rapid and

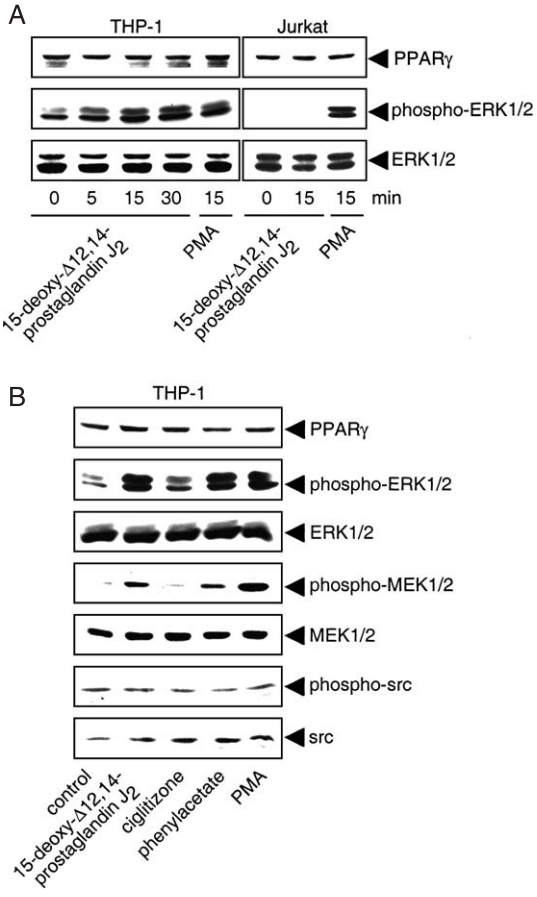

Fig. 6. Rapid phosphorylation of ERK-1/-2 and MEK-1/-2 by PPARγ-ligands. THP-1 and Jurkat cells were starved and then stimulated (A) with 15-desoxy-$\Delta^{12,14}$-prostaglandin J_2 for 5–60 min or (B) for 15 min with different ligands (all at 10 μM). PMA was used as positive control. Cell lysates were subjected to denaturing polyacrylamide gel electrophoresis and Western blotting using antibodies against the phosphorylated or unphosphorylated form of the proteins of interest.

transient 2- to 3-fold increase in phosphorylation of ERK-1 and -2 in THP-1 cells. This phosphorylation was inhibited by addition of the MEK-inhibitor PD098059 (unpublished observation). The ligands also increased the phosphorylation of MEK-1 and -2 but not of *src* tyrosine kinase. In contrast, these effects were not observed in Jurkat cells. Both cell lines expressed equal amounts of PPARγ protein.

From these results, we conclude that monocytic cells eicosanoids are involved in stimulation of MAPK in a cytosolic phospholipase A_2 and PPARγ-dependent manner. In Jurkat cells, eicosanoid-regulated MAPK-activation seems to be independent of this nuclear receptor.

4. Discussion

T-cells are target cells for monocyte-derived eicosanoids, which regulate the production of T-helper lymphokines, interferons and interleukin-2 receptors by triggering G-protein-coupled receptors (Cunningham, 1994; Breyer et al., 2001; Tilley et al., 2001). However, the importance of endogenous eicosanoids in T-cells is less understood. In the present study, we therefore investigated the molecular mechanism on how lipid mediators generated upon activation of cytosolic phospholipase A_2 regulate T-cell-specific responses. The data clearly show that inhibition of cytosolic phospholipase A_2 activity abrogated MAPK-signalling, inhibitor-κB degradation and NFκB-dependent transcription of the interleukin-2 gene as well as proliferation of human T-lymphoid cells. These findings suggest an important role for cytosolic phospholipase A_2 in regulation of the adaptive immune system.

Both in monocytic cells (Burgermeister et al., 1999, 2000) and in T-lymphoid cells central signalling pathways were impaired upon inhibition of cytosolic phospholipase A_2, emphasizing that this enzyme is integrated in the receptor-dependent (i.e. endotoxin, antigen, anti-CD3/CD28, lectin) and receptor-independent (i.e. phorbol ester and Ca^{2+}-ionophore) activation program of immune competent cells (Fig. 7). Ligation of the T-cell receptor initiates rapid recruitment of cytosolic tyrosine kinases (fyn, lck and ZAP70) followed by parallel activation of several signalling cascades (phospholipase Cγ, protein kinase C, MAPK and calcineurin). All these pathways, together with those triggered by the receptor-independent stimulants PMA and A23187, converge on activation of promitotic transcription factors such as NFκB, AP-1 and nuclear-factor-of-activated-T-cells (NFAT) (Werlen and Palmer, 2002) which leads to proliferation and interleukin-2 synthesis.

Despite these divergent signalling pathways, inhibition of cytosolic phospholipase A_2 efficiently blocked interleukin-2 synthesis and proliferation in response to various stimuli. This phenomenon suggests that this enzyme interacts with events far upstream in the T-cell receptor-signalling cascade. In contrast, inhibition of secretory phospholipase A_2 or the downstream arachidonic acid metabolizing enzymes such as

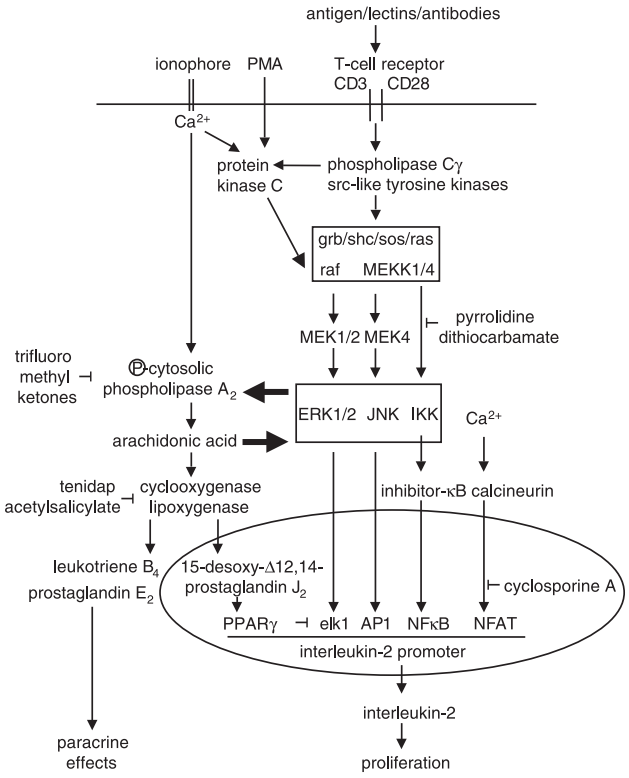

Fig. 7. Scheme of the signal transduction pathways during activation of human T-lymphoid cells by receptor-dependent and -independent stimuli. Involvement of intracellular eicosanoids generated upon stimulation of cytosolic phospholipase A_2 in regulation of MAPK and mobilization of transcription factors, which culminate in interleukin-2 transcription and proliferation of T-cells.

cyclooxygenases and lipoxygenases was ineffective. This finding, together with our results obtained from PPARγ-ligands, corroborated that endogenous, but not exogenous, production of eicosanoids is crucial for T-cell activation and that shunting of arachidonic acid towards lipoxygenase and cyclooxygenase pathways is not sufficient to block signalling pathways which lead towards interleukin-2 transcription.

The inhibitor-κB system (Anthonsen et al., 2001; Straus and Glass, 2001) and the MAPK cascade are rapidly activated upon receptor-mediated stimulation of intracrine eicosanoid production (Tournier et al., 1997; Hii et al., 1998; Paine et al., 2000; Reddy et al., 2002) or by PPARγ-ligands (Takeda et al., 2001). In these cases, eicosanoids act as second messengers independently of membrane-bound or nuclear receptors (Alexander et al., 2001; Chang and Wang, 2001). Vice versa, inhibition of cytosolic phospholipase A_2 attenuated PMA + A23187-induced ERK- and JNK-dependent phosphorylation of elk-1 and c-jun, degradation of inhibitor-κB and NFκB-dependent activation of a minimal promoter. The phospholipase A_2 inhibitors neither directly inhibited the kinase activity by steric hindrance or other unspecific means nor affected the phosphorylation status of upstream MEK. In contrast to monocytes

(Burgermeister et al., 2000), the stress/cytokine reactive p38 kinase was not responsive to stimulation in T-cells (Dong et al., 2002).

Consequently, the cytosolic phospholipase A_2 and its follow-up products shall intersect with signalling components downstream of the bifurcation of the three MAPK-pathways and the inhibitor-κB-kinase (IKK) module, which both are indispensable for efficient transcription of the interleukin-2 gene. For example, anergy of tolerant T-cells can be achieved in vivo by blockade of MAPK-activity (Li et al., 1996; Dumont et al., 1998), implicating that the therapeutical application of cytosolic phospholipase A_2 inhibitors may have immunosuppressive side effects comparable to cyclosporine A.

Synthetic ligands of PPARγ are useful tools to dissect extracellular, i.e. eicosanoid-receptor-dependent, from intracellular eicosanoid actions. This nuclear receptor is targeted by 15-desoxy-$\Delta^{12,14}$-prostaglandin J_2, thiazolidinediones and phenylacetic acids (Rosen and Spiegelman, 2001) and regulates transcription of genes involved in the immune response, lipid/carbohydrate metabolism and differentiation (Kersten et al., 2000). In monocytes, PPARγ antagonizes transcription factors that induce expression of proinflammatory cytokines. In T-cells, it attenuates proliferation by counteracting expression of interleukin-2 induced by NFAT (Yang et al., 2000; Clark, 2002). These agonists, unlike other eicosanoids such as prostaglandin D_2 and E_2, do not bind to a plasma-membrane receptor but have additional second messenger PPARγ-independent effects (e.g. 15-desoxy-$\Delta^{12,14}$-prostaglandin J_2 binds to and inhibits IKK) (Straus and Glass, 2001). Thus, we expected that prevention of PPARγ-ligand generation by blockade of cytosolic phospholipase A_2 shall derepress its immunosuppressive effects. To our surprise, the ligands evoked a rapid MEK-dependent phosphorylation of ERK-1 and -2 independently of long-term genotropic effects in cytosolic extracts of monocytic THP-1 cells but not in Jurkat cells.

These data indicated that PPARγ-ligands exert cell-specific, non-genomic effects on the MAPK cascade which are dissociated from those exerted by extracellular eicosanoids and by classical genomic effects on transcription of immune response genes.

The question remains, by which mechanisms phospholipase A_2-derived lipid mediators interact with signalling molecules? Studies using oncogenic forms of ras place cytosolic phospholipase A_2 downstream of the receptor-associated shc-grb2-sos-ras module (Jiao et al., 1998; Yoo et al., 2001). The enzyme is also phosphorylated on serine 295 by ERK, an event together with a rise in intracellular Ca^{2+}-ions strongly increases its activity (Murakami et al., 1997). PPARs are in turn regulated in their transactivating potential by ERK-mediated serine phosphorylations (Kersten et al., 2000). Thus, the modulatory effects of arachidonic acid metabolites may be caused by a positive feed forward loop between ligand-bound PPARγ, MAPK and cytosolic phospholipase A_2. A similar scenario has been described for the estrogen receptor which directly interacts and recruits caveolin (Razandi et al., 2002), shc (Song et al., 2002), src and MAPK (Kousteni et al., 2001; Migliaccio et al., 1998, 2000). However, this hypothesis has to be evinced in future research.

Acknowledgements

We are very grateful to Dr. U. Pessara for providing the reporter plasmids, to G. Lang for establishment of the stably transfected Jurkat cell line and to Dr. Friebe (Roche Diagnostics GmbH, Mannheim) for supplying the pharmacological compounds.

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