Molecular Targets for Glucocorticoids in Macrophages:

Cytosolic Phospholipase A_2 and Cytokine Formation

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Abstract
Cytosolic phospholipase A_2 ($cPLA_2$) is an important modulator of inflammatory responses that is present in macrophages as well as other cell types. The synthetic glucocorticoid dexamethasone (dex) was found to reduce the expression of $cPLA_2$ and further to inhibit the activation of $cPLA_2$. The stimuli PMA (a phorbol ester), zymosan or the protein phosphatase inhibitor okadaic acid caused activation of $cPLA_2$. After treatment with dex, okadaic acid, but not PMA or zymosan, was able to up-regulate $cPLA_2$ activity. Phosphorylation of $cPLA_2$ occurred on multiple sites. Upon stimulation with PMA or bacteria, the most C-terminal fragment (residues 698-749) of $cPLA_2$ was the most heavily phosphorylated. Neither was the expression, nor the activation of, the mitogen-activated protein kinases (MAPKs) affected to the same extent as the $cPLA_2$ activity. Although a minor inhibition of the zymosan-induced MAPK activation was observed, our results suggest that dex-mediated inhibition of $cPLA_2$ activation is exerted down-stream of the MAPKs.

The effect of dex on the formation of two inflammatory cytokines, IL-1β and TNF-α, was also investigated. Treatment with dex inhibited bacteria-induced IL-1β expression primarily at the transcriptional level whereas TNF-α expression was only partially inhibited at the level of transcription but further inhibited at the translational level. The translational inhibition after dex treatment was overcome by okadaic acid. Also the cleavage processing of TNF-α was modified by okadaic acid. The degree of dex-mediated inhibition of the different TNF-α forms varied.

In conclusion, dex inhibited the synthesis of $cPLA_2$. Studies with okadaic acid revealed protein phosphatases to be likely targets for dex both in the inhibition of $cPLA_2$ activation and in inhibition of TNF-α translation.

Key words:
cytosolic phospholipase A_2, dexamethasone, glucocorticoids, macrophages, protein kinase C, mitogen-activated protein kinases, TNF-α, IL-1β, okadaic acid, protein phosphatases, signal transduction

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LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numeral:

I. Gewert, K. and Sundler, R. (1995) Dexamethasone down-regulates the 85 kDa phospholipase A_2 in mouse macrophages and suppresses its activation. *Biochem J* **307:** 499-504.

II. Wijkander, J., Gewert, K., Svensson, U., Holst, E. and Sundler, R. (1997) Multiple C-terminal serine phosphorylation accompanies both protein kinase C-dependent and –independent activation of cytosolic 85 kDa phospholipase A_2 in macrophages. *Biochem J* **325:** 405-410.

III. Gewert, K., Hiller, G. and Sundler, R. Effects of dexamethasone on ERK, JNK and p38 MAPK in mouse macrophages. Implications for the regulation of 85 kDa cytosolic phospholipase A_2. (submitted for publication)

IV. Gewert, K., Svensson, U., Andersson, K., Holst, E. and Sundler, R. (1999) Dexamethasone differentially regulates cytokine transcription and translation in macrophages responding to bacteria or okadaic acid. *Cell Signal* **11:**665-670.

ABBREVIATIONS

AP-1	activator protein-1
ATF-2	activating transcription factor-2
dex	dexamethasone
ERK	extracellular signal-regulated kinase
G-protein	guanine-nucleotide-binding protein
GR	glucocorticoid receptor
GRE	glucocorticoid response element
IκB	inhibitory protein κB
IL	interleukin
JNK	c-jun N-terminal kinase
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
NFκB	nuclear factor-κB
PI-3 kinase	phosphatidylinositol-3 kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
$cPLA_2$	cytosolic phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
TNF	tumour necrosis factor

ABSTRACT

The aim of this investigation has been to study mechanisms by which glucocorticoids inhibit inflammatory processes *in vitro*. Type IV cytosolic 85 kDa phospholipase A_2 ($cPLA_2$) is an important modulator of inflammatory responses that is present in macrophages as well as in other cell types. In macrophages, the synthetic glucocorticoid dexamethasone (dex) was found to reduce the expression of $cPLA_2$ and further to inhibit the activation of $cPLA_2$. Stimulation with phorbol 12-myristate 13-acetate (PMA), zymosan or the protein phosphatase inhibitor okadaic acid increased the $cPLA_2$ activity. After treatment with dex, okadaic acid, but not PMA or zymosan, was able to up-regulate $cPLA_2$ activity. These results indicate that the effect of okadaic acid was exerted at, or downstream of, the dex-sensitive step(s). Phosphorylation of $cPLA_2$ occurred on multiple sites. Upon stimulation with PMA or bacteria, the most C-terminal fragment (residues 698-749) of $cPLA_2$ was the most heavily phosphorylated. We did not find any evidence for down-regulation of protein kinase C isoforms after dex treatment. Neither was the expression, nor the activation of, the mitogen-activated protein kinases (MAP kinases), extracellular signal- regulated kinase-2 (ERK-2) or p38, affected to the same extent as the $cPLA_2$ activity. Although a minor inhibition of the zymosan-induced activation of the MAP kinases was observed, our results suggest that dex-mediated inhibition of $cPLA_2$ activation is exerted down-stream of the MAP kinases.

The effect of dex on the formation of two other inflammatory mediators in macrophages, the cytokines interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α) was also investigated. Treatment with dex inhibited bacteria-induced IL-1β expression primarily at the transcriptional level whereas TNF-α expression was only partially inhibited at the level of transcription but further inhibited at the translational level. The translational inhibition after dex treatment was overcome by okadaic acid. Also the post-translational processing of TNF-α was modified by okadaic acid. TNF-α was found both in the cleaved

and uncleaved form after okadaic acid treatment. The different TNF-α forms exhibited different sensitivity to dex treatment. Based on these data, we suggest that the effect of dex on TNF-α translation is mediated by an okadaic acid-sensitive protein phosphatase.

In conclusion, dex inhibits the synthesis of $cPLA_2$. Studies with okadaic acid revealed protein phosphatases to be likely targets for dex both in the inhibition of $cPLA_2$ activation and in inhibition of TNF-α translation.

INTRODUCTION

MACROPHAGES

Macrophages are white blood cells defending the host against infection by ingesting invading microorganisms. The process, by which large particles are taken up, is called phagocytosis. Upon activation, macrophages are able to present antigens to T lymphocytes and other cell types (Adams and Hamilton 1992). This process is specific and macrophages even distinguish between bacterial and mammalian DNA (Sester *et al.* 1999). Macrophages originate from stem cell precursors in the bone marrow and circulate in the blood as monocytes. In the absence of inflammatory stimuli monocytes enter tissues and become resident macrophages (Gordon *et al.* 1992). Differentiation to mature macrophages is associated with phenotypical, functional and biochemical changes. Resident macrophages have low secretory and microbicidal activity. In contrast, activated macrophages produce many enzymes and cytokines including IL-1 and TNF (Dinarello 1991). At least 60 different receptors have been found on the macrophage and it is able to secrete more than 100 different molecules (reviewed in Rappolee and Werb 1989). A prerequisite for this investigation is that macrophages possess glucocorticoid receptors that specifically bind both natural glucocorticoids and the synthetic steroid dexamethasone (dex) (Werb *et al.* 1978).

GLUCOCORTICOIDS

Biological effects

Glucocorticoids are stress hormones released upon stimulation of the immune system via a neuro-endocrine-immune network. As the name implies, one major function of glucocorticoids is concerned with glucose metabolism but a number of other metabolic processes are also regulated by glucocorticoids. In this investigation the anti-inflammatory action is in focus. Glucocorticoids are important in preventing the immune system from over-reacting.
Glucocorticoids are widely used both as anti-inflammatory and immuno-

suppressive drugs. The free concentration of the endogenous glucocorticoid cortisol has been reported to be 12-43 nM (Lentjes *et al.* 1993). The main proportion of natural glucocorticoids in circulation is bound to transcortin or albumin.

Biological mechanisms

Glucocorticoids bind to the glucocorticoid receptor (GR), a specific intracellular receptor. Although the GR is a phosphoprotein, the importance of phosphorylation for regulation of GR is still under debate (Burnstein and Cidlowski 1993, Weigel 1996). In the absence of glucocorticoids, the GR is held in an inactive state by heat shock proteins (see Welch and Winfield 1992, Polla *et al.* 1993 for review). The classic view was that free GR was localised to the cytoplasm but the cellular distribution of GR is now a controversial issue. Upon binding of glucocorticoid to GR, heat shock proteins are released and the glucocorticoid-GR complex can translocate to the nucleus. In the nucleus, the GR interacts with a specific DNA sequence named glucocorticoid response elements (GRE). The GRE often consists of a consensus sequence, an inverted repeat separated by three nucleotides, and is often situated in the vicinity of gene promoters. The GR acts as a ligand-activated transcription factor and induces or represses the activity of the target promoter (**Fig. 1**). In this way, a great number of genes encoding cytokines and cellular regulatory elements are regulated by glucocorticoids in a tissue-specific manner. The binding of a transcription factor to its responsive DNA sequence can sterically inhibit the binding of other transcription factors nearby. This creates combinational possibilities of interaction between different transcription factors. For example, dex inhibits DNA-binding of CREB (cAMP responsive element binding protein) (Adcock *et al.* 1995), a transcription factor regulating many genes. In addition, DNA-independent mechanisms have been observed such as glucocorticoid-induced down-regulation of the transcription factor AP-1 by direct protein-protein binding (Jonat *et al.* 1990). RelA, (a possible constituent of NFκB, see below) and GR have been observed to repress each

other's activation (Caldenhoven *et al.* 1995). Direct interaction has been suggested also between glucocorticoid and NFκB (van der Saag *et al.* 1996). In addition, different modes of post-transcriptional regulation have been reported after dex treatment, for example mRNA destabilisation (Ristimäki *et al.* 1996).

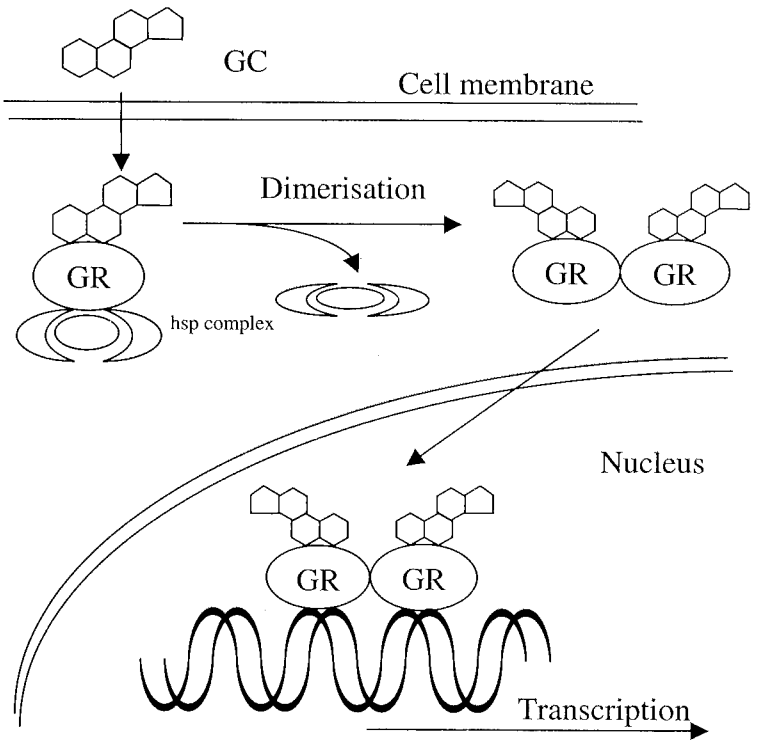


Figure 1. ***Mechanism of transcriptional regulation by glucocorticoids.*** GC; glucocorticoids, hsp; heat shock protein.

One protein that has been suggested to participate in the regulation of glucocorticoid actions is macrophage migration inhibitory factor (MIF). The release of this pro-inflammatory factor is regulated by glucocorticoids. The suggested role of MIF is to prevent excessive effects of glucocorticoids (Calandra *et al.* 1995, Donnelly and Bucala 1997). MIF has been found to cause phosphorylation of the protein kinase ERK and to lead to activation of $cPLA_2$ in a fibroblast cell line (Mitchell *et al.* 1999).

Dexamethasone

Dexamethasone (dex) is a synthetic glucocorticoid (**Fig. 2**) used as a pharmacological agent. The GR binds cortisol with a dissociation constant of about 30 nM while the affinity for dex is 5 to 10 times higher (Munck *et al.* 1990).

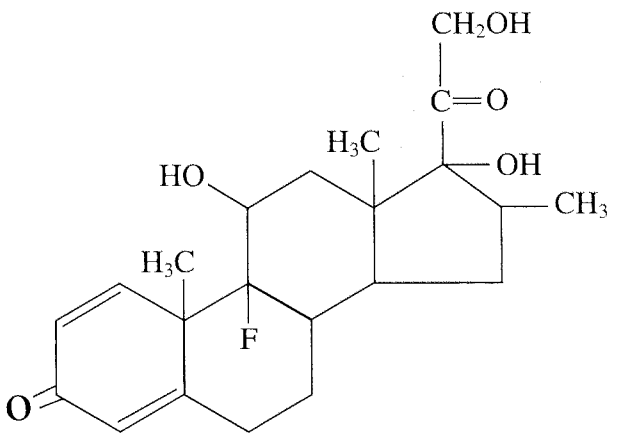
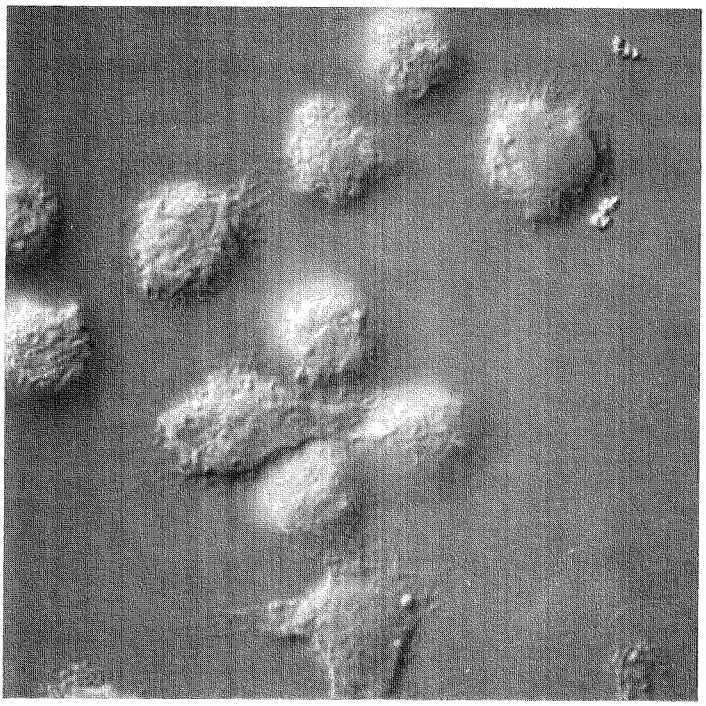


Figure 2. ***Structure of dexamethasone.***

Dex inhibits phenotypic changes associated with differentiation (Baybutt and Holsboer 1990). Most obvious is the difference in the number of spike-like extensions protruding from untreated and dex-treated macrophages (**Fig. 3**). Importantly, phagocytic activity of peritoneal macrophages is not affected by dex treatment (Nakamura *et al.* 1996).

Annexins

The annexin superfamily consists of calcium- and phospholipid-binding proteins. The synthesis of annexins is induced by glucocorticoids in some cell types (reviewed by Flower and Rothwell 1994) but not in others (reviewed by van der Velden 1998). Antisera against annexin I has been reported to impair glucocorticoid-mediated suppression of oedema and cell migration (Draeger 1999). On the other hand, no significant difference in annexin concentration



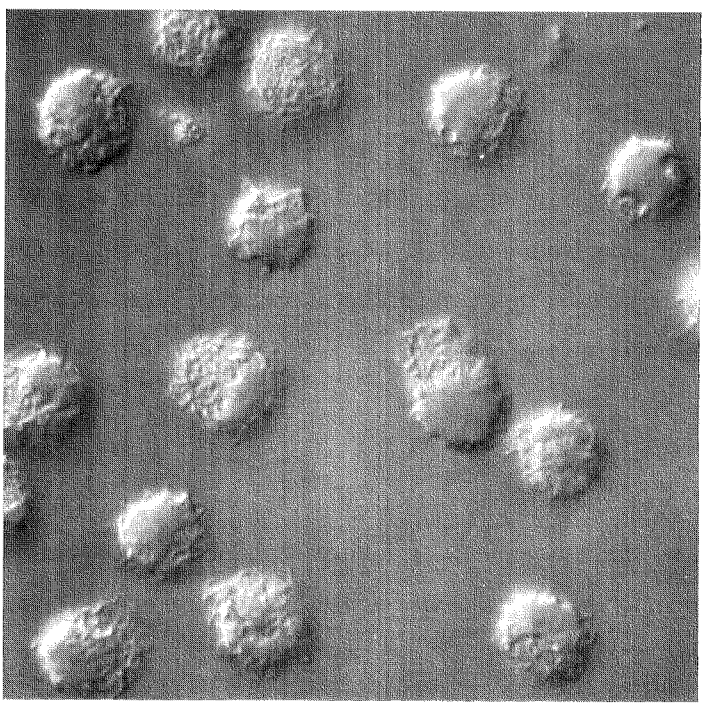


Figure 3. Left page: The top micrograph shows mouse peritoneal macrophages cultured overnight. In the bottom micrograph macrophages cultured overnight in the presence of 100 nM dex are shown. Images were recorded on an inverted fluorescence microscope using Nomarski optics. The microscope was equipped with a 100x objective. Courtesy of Hans Tapper.

in bronchoalveolar lavage fluid was observed between asthmatic patients receiving inhaled glucocorticoids and those who did not (van Hal *et al.* 1996). Annexins were originally proposed to specifically interact with PLA_2 (Flower 1988) and although this concept has still not been excluded (Kim *et al.* 1994) we have not been able to find any support for this model (paper I). However, annexins have been shown to inhibit PLA_2 in a substrate concentration-dependent manner. This suggests substrate depletion to be a result of phospholipid/annexin complexation instead of a specific interaction with PLA_2 (Davidson *et al.* 1987, Bastian *et al.* 1993).

CELLULAR SIGNALLING

One general control mechanism in cells is phosphorylation of proteins. Protein kinases are responsible for the phosphorylation of proteins while phosphatases act to dephosphorylate proteins (for reviews, see Hunter 1995, Wera and Hemmings 1995). Proteins are phosphorylated on serine, threonine or tyrosine residues.

The first step of many signal chains is the binding of a stimulus to its receptor. Examples of receptors participating in phagocytosis are Fc receptors, complement receptors and receptors recognising carbohydrates (Riches *et al.* 1988, Stahl 1992). Activation of these receptors often results in tyrosine phosphorylation of the receptors followed by tyrosine phosphorylation of a number of cellular proteins.

Guanine-nucleotide-binding proteins, G-proteins, transduce signals between receptors and effectors such as phospholipase C (PLC) or adenylyl cyclase (reviewed by Neer 1995). One class of G-proteins are the monomeric "small-molecular mass G-proteins" including signalling molecules such as Ras and Rho (reviewed in Rebollo and Martinez 1999, Denhardt 1996). Another class, the trimeric G-proteins, consists of an α subunit and a $\beta\gamma$ dimer. There are multiple isoforms of each subunit. The Gα and G$\beta\gamma$ subunits separately activate target proteins. The use of pertussis toxin, acting on some Gα subfamilies, has revealed activation of $cPLA_2$ to be dependent on G-proteins (Murray-Whelan *et al.* 1995). G-proteins have earlier been suggested to interact with PLA_2 directly (Xing and Mattera 1992), but this might be an indirect effect, mediated via, for example, regulation of PKC or a MAP kinase since G-protein coupled receptors are known to activate such pathways (Luttrell *et al.* 1997).

Phosphatidylinositol 3-kinase (PI-3 kinase) generates lipid products e.g. phosphatidylinositol 3,4,5-trisphosphate (PIP_3), first found to be important for metabolic control and mitogenic signalling. However, PI-3 kinase is involved in many signal transduction events such as activation of MAP kinase kinases, PKC, and phosphatases (reviewed in Wymann and Pirola 1998, Shepherd *et al.* 1998). In addition, PI-3 kinase has been shown to be important for $cPLA_2$ activation (Hiller *et al.*, manuscript). Interestingly, there are reports showing increased PI-3 kinase activity in glucocorticoid resistant cells, suggesting that PI-3 kinase can confer resistance to cytostatic drugs (Krasil´nikov *et al.* 1999). PI-3 kinase is of importance for activation and translocation of PLC (Gratacap *et al.* 1998). PLC generates two important products; inositol trisphosphate that releases Ca^{2+} from intracellular stores and diacylglycerol that activates PKC. Both PI-3 kinase (Giorgino *et al.* 1997) and PLC (Gewert *et al.* 1996) are partially inhibited by dex. Not only PLC but also phospholipase D (PLD) is able to regulate cellular signalling to arachidonic acid release through formation of phosphatidic acid. Phosphatidic acid can be converted by

phosphatidate phosphorylase to diacylglycerol. In undifferentiated cells, this pathway is important for the regulation of $cPLA_2$ (Burke *et al.* 1999).

Protein kinase C

PKC is a family of phospholipid-dependent serine/threonine kinases. The family consists of different isoenzymes divided into groups based on sensitivity to calcium and diacylglycerol (see Nishizuka 1984, Nishizuka 1992, Mellor and Parker 1998 for reviews). $cPLA_2$ activity in mouse macrophages is stimulated by both PKC-dependent and -independent mechanisms (Wijkander and Sundler 1989a, Svensson *et al.* 1993). It is controversial whether PKC is able to cause *direct* activation of $cPLA_2$ or not. In a report where phosphorylation by PKC was observed, peptide mapping revealed the phosphorylation sites to be distinct from those phosphorylated by ERK (Nemenoff *et al.* 1993). The increase in $cPLA_2$ activity following phosphorylation with PKC was less than that observed after phosphorylation with ERK (Nemenoff *et al.* 1993) or none at all (Wijkander and Sundler 1991, Lin *et al.* 1993). However, PKC is a component in the signalling chains leading to activation of $cPLA_2$. PKC-α has been reported involved in arachidonic acid release in kidney cells (Godson *et al.* 1990), while prostaglandin synthesis in mesangial cells has been suggested to be dependent on PKC-ε (Huwiler *et al.* 1991). Two isoenzymes, PKC-δ and PKC-ε, have been hypothesised to participate in the cross-talk between $sPLA_2$ and $cPLA_2$ in mesangial cells (Huwiler *et al.* 1997).

Protein phosphatases

The family of phosphatases that dephosphorylate serine and threonine residues in mammals include four major types based on the type of catalytic subunit: PP1, PP2A, PP2B and PP2C.

PP1 regulates different functions such as glycogen metabolism, muscle contraction and mitosis. Many different hormones including glucocorticoids regulate the activity of PP1 (reviewed in Bollen and Stalmans 1992).

A single catalytic subunit and a regulatory unit called PR65 associating to other regulatory subunits existing in multiple isoforms form the structure of PP2A. This phosphatase has been suggested to be very important in regulation of

protein kinase cascades. Kinases such as PKCα and IκB kinase are inactivated by PP2A (Millward *et al.* 1999). The most well known inhibitor of PP2A is okadaic acid, first identified as a tumour promoter. Okadaic acid is a polyether produced by dinoflagellates. This compound is the major toxic component causing seafood poisoning (Hardie *et al.* 1991). Okadaic acid inhibits PP2A *in vitro* with an IC_{50} value of 0.1 nM. However, treatment of intact cells requires much higher concentrations and under such conditions okadaic acid is specific for PP2A and the closely related phosphatases PP4 and PP5 at concentrations up to 1 μM since it is a 100-fold less potent as inhibitor of PP1 (Millward *et al.* 1999). Interestingly, okadaic acid produces changes in gene transcription through inducing transcription factors such as AP-1 (Rosenberger and Bowden 1996). PP4 is a "PP2A-like" enzyme with the same sensitivity to okadaic acid as PP2A. It has been suggested to participate in microtubule organisation. PP5 is another "PP2A-like" okadaic acid sensitive phosphatase (IC_{50}: 1-10nM). Arachidonic acid and other lipid compounds activate this phosphatase. PP5 has been found associated with hsp90 in GR complexes and is consequently a candidate in glucocorticoid signalling (Chen *et al.* 1996). A third "PP2A-like" phosphatase is PP6, a phosphatase involved in cell cycle regulation (reviewed by Cohen 1997).

PP2B (also called calcineurin or Ca^{2+}-calmodulin-regulated protein phosphatase) dephosphorylates a transcription factor called NF-AT which is of importance for T-lymphocyte activation. Two important immunosuppressive drugs, cyclosporin and FK506, exert their effects by inhibiting PP2B (reviewed in Wera and Hemmings 1995). PP2C, another protein phosphatase existing in several isoforms, is a candidate for dephosphorylation of kinases upstream of p38 and JNK-1 (Hanada *et al.* 1998).

The tyrosine phosphatase superfamily encompasses the tyrosine-specific phosphatases, dual specificity phosphatases and the low-molecular-weight phosphatases. Structurally they share a phosphate-binding loop. Although a very small portion of all phosphoproteins is phosphorylated on tyrosine residues, the tyrosine kinases and tyrosine phosphatases are very important

since they participate in the regulation of signal transduction pathways and different cell functions. After oncogenic transformation an increase in tyrosine phosphorylation can be observed. More than 100 protein tyrosine phosphatases have been identified (reviewed in Zhang 1998). A widely used tyrosine phosphatase inhibitor is vanadate (Gordon 1991). The dual specificity phosphatases, working on Tyr and Ser/Thr residues, are further divided into three groups. One group of interest is the MAP kinase phosphatases (see below).

MAP kinase cascades

A great number of stimuli have been shown to activate the MAP kinases or stress-activated protein kinases (SAPKs) (for review, see Cohen 1997). Tyrosine kinase receptors or G-protein-linked receptors mediate this activation. G-proteins, through activation of ras, can activate MAP kinases (Crespo *et al.* 1994, Faure *et al.* 1994). Three different MAPK/SAPK cascades, ERK, p38 and JNK, have been described (**Fig. 4**). All three have been suggested as potential activators of $cPLA_2$ (see below).

ERK-1 and ERK-2 are also called p44 MAPK and p42 MAPK. Polypeptide growth factors, PMA and hormones acting through G-proteins have been shown to activate ERK-2. $cPLA_2$ is one target protein for ERK-2. Another one is the p90rsk protein which is able to phosphorylate c-fos and thereby can be involved in AP-1-regulated gene transcription (Denhardt 1996). A dual tyrosine and threonine specific MAP kinase kinase called MEK is a direct upstream activator of MAP kinase (Wu *et al.* 1993). This MAP kinase kinase is in turn regulated by kinases of the Raf family. PKC has been reported to induce phosphorylation of Raf-1 (Kolch *et al.* 1993). Protein tyrosine phosphatases and dual specific phosphatases (e.g. MKP-1) have been found to dephosphorylate and thereby inactivate MAP kinases (Sun *et al.* 1993, van Vactor *et al.* 1998).

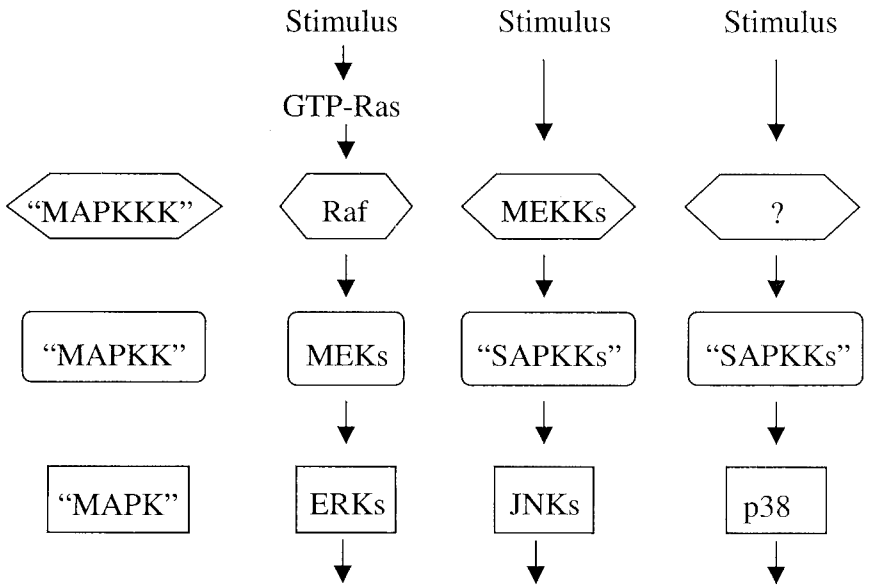


Figure 4. ***Mitogen and stress-activated protein kinase (MAPK and SAPK) pathways.***

p38 and JNK are activated by cytokines and environmental stress and are therefore called stress-activated protein kinases. p38 is phosphorylated upon stimulation with e.g. lipopolysaccharide (LPS), TNF and platelet activating factor (PAF) (Nahas *et al.* 1996). Two different MAP kinase kinases (or SAPKKs) have been found to phosphorylate p38 (Cohen 1997). Targets for p38 include kinases such as MAPK-activated protein kinase-2, and transcription factors, for example ATF-2 (Jiang *et al.* 1996) and MEF2C (Han *et al.* 1997).

The MAP kinase kinases (or SAPKKs) MKK4 or MKK7 activate JNK by dual phosphorylation of the kinase on Thr and Tyr residues. In turn JNK regulates transcription factors such as NFAT4 and c-Jun (reviewed in Schaeffer and Weber 1999). JNK has been found also to potentiate inflammatory responses by decreasing activation of GR (Rogatsky *et al.* 1998). Different isoforms of MAPK phosphatases have been found that can inactivate JNK (Ip and Davis 1998).

Tools for studying signal transduction used in this investigation

The stimuli used in paper I-IV are (**Fig. 5**): i) **Zymosan** a carbohydrate-rich cell wall component from the yeast *Saccharomyces cerevisiae*. The two predominant carbohydrates in zymosan are α-mannan and β-glucan polymers. Zymosan can bind to cell-surface receptors via β-glucan (Riggi and Di Luzio1961, Daum and Rohrbach 1992, Tapper and Sundler 1995) but whether this receptor is identical to complement receptor 3 or not is controversial (Czop and Kay 1991, Thornton *et al.* 1996). Recently, it was reported that another receptor, the toll-like receptor-2, is crucial for zymosan-induced TNF-α production (Underhill *et al.* 1999). ii) **LPS**, lipopolysaccharide, consisting of outer membrane components of Gram-negative bacteria. LPS-induced signalling has been found to occur both independently of and dependent on glycosyl-phosphatidyl (GPI)-anchored CD14 receptors in murine macrophages (Perera *et al.* 1997). Recently, in a human kidney cell line, the toll-like receptor-4 was reported to act as a co-receptor for CD14 (Chow *et al.* 1999). Binding of zymosan or LPS is followed by activation of phospholipase C (Emilsson and Sundler 1986, Moscat *et al.* 1987). Hydrolysis of phospholipids by PLC results in generation of diacylglycerol leading to activation of PKC and of inositol 1,4,5-trisphosphate with subsequent increase in intracellular free calcium (Dieter and Fitzke 1993). The involvement of PKC has been confirmed by the inability of zymosan to induce arachidonic acid release after PKC down-regulation (Wijkander and Sundler 1989a, Huwiler and Pfeilschifter 1993). Tyrosine kinases are triggered by zymosan (Green *et al.* 1992) and tyrosine phosphorylation of ERK has been observed after zymosan treatment (Qiu and Leslie 1994). iii) In paper IV we used clinical isolates of **bacteria** from female patients with genital infections (Holst 1987). These bacteria bind to receptors on the cell surface resulting in activation and phosphorylation of $cPLA_2$ (Svensson *et al.* 1993). Stimulation with certain other bacteria has been found to increase the activity of ERK and p38 (Hiller and Sundler 1999). iv) **PMA,** which is the abbreviation for phorbol-myristate acetate, is a phorbol ester with tumour-promoting properties (Fürstenberger *et al.* 1981) known to activate

PKC by mimicking diacylglycerol. Dex has been shown not to affect the binding of PMA to PKC (Driedger and Blumberg 1980). v) **Okadaic acid** inhibits certain protein phosphatases (see section phosphatases) (Bialojan and Takai 1988), resulting in increased phosphorylation of many phosphoproteins (Haystead *et al.* 1989). vi) **A23187**, a specific calcium ionophore (Pressman 1976), causes a rise in cytosolic Ca^{2+} that results in activation of many Ca^{2+}-regulated proteins including calmodulin, calcineurin B, PKC and PLA_2 (Clapham 1995).

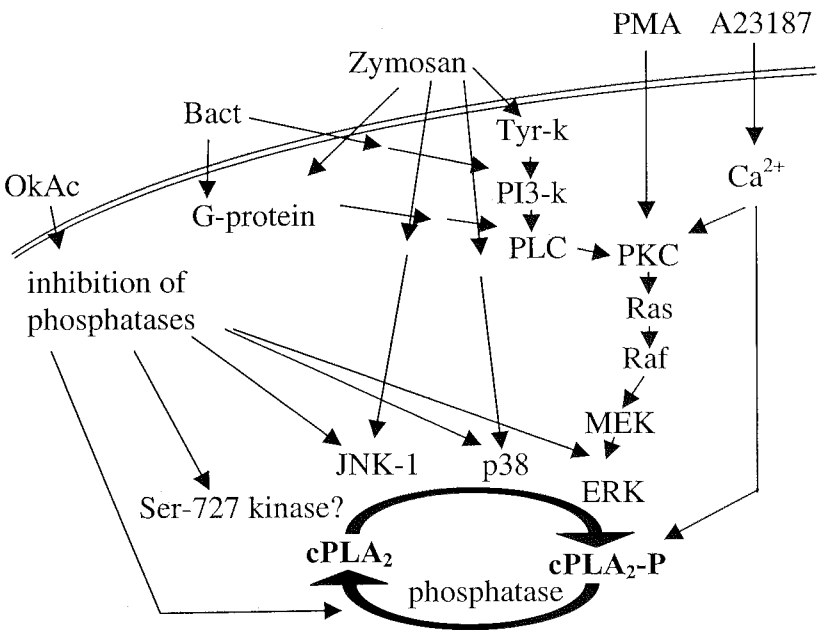


Figure 5. ***Overview of the studied signalling pathways involved in the activation of cPLA$_2$.*** bact; bacteria, OkAc; okadaic acid, PI3-k; phosphatidylinositol 3-kinase, Tyr-k; tyrosine kinase.

PHOSPHOLIPASE A_2

The PLA_2 enzymes hydrolyse the sn-2 fatty acyl bond of phospholipids and this generates free fatty acids and lysophospholipids. PLA_2 is of importance in degradation of dietary phospholipids, destruction of bacteria and in the pathology of inflammatory diseases. PLA_2 is positioned at the top of the

arachidonic acid cascade, producing inflammatory mediators such as prostaglandins and leukotrienes.

The PLA_2s have been divided into several groups according to structure, sequence, localisation and need for divalent metal ions (reviewed by Dennis 1997, Maxey and MacDonald 1998). The Ca^{2+}-dependence of different PLA_2s varies from independence (Hazen *et al.*1990), e.g. group VI, VII VIII, XII and XIII, to mM requirement, e.g. group IA, IB, IIA, IIB, IIC, IID (Ishizaki *et al.* 1999), III, V and X. Type VII, VIII and XI exhibit PAF-acetylhydrolase activity. Some PLA_2s are found in venom of animals such as bees (type III) and different snakes (type IA, IIA, IIB, VIII and IX). Others are found in human body fluids, for example type IB PLA_2 best known as a pancreatic enzyme and type IIA found in synovial fluid. Different PLA_2s can be present in the same cell at the same time, having different functions in cell signalling (Balsinde and Dennis 1996) and being differentially regulated (Bolognese *et al.* 1995).

Secretory PLA_2s ($sPLA_2$) are involved in many inflammatory diseases including acute pancreatitis, septic shock, rheumatoid arthritis, pulmonary diseases and inflammatory bowel diseases. Elevated levels of PLA_2s have also been observed postoperatively, at labour and during viral infections (for review, see Nevalainen and Grönroos 1997). Recently, $sPLA_2$ was found to prevent HIV entry into host cells (Fenard 1999). Low molecular weight secretory PLA_2s exist in different isoforms in mammals. Group II was early found in inflammatory exudates (Kramer *et al.* 1989) and is suggested to mediate agonist-induced release of arachidonic acid in many cell types. In a number of inbred mouse strains, the group IIA $sPLA_2$ gene is naturally disrupted by a frame-shift mutation. No obvious phenotypic changes or effects on viability have been observed in these widely used mice (Kennedy *et al.* 1995). In the peritoneal mouse macrophages used in our studies, mRNA for group II PLA_2 was not detected but instead mRNA for group V was found to be inducible (Wijkander and Andersson, unpublished results). The presence of group V and lack of group IIA has earlier been demonstrated in the mouse

macrophage-like cell line P388D1 (Balboa *et al.* 1996). Henceforth, the group II and group V PLA_2s will be referred to as $sPLA_2$. Glucocorticoids have been observed to inhibit cytokine-induced synthesis of $sPLA_2$ as well as $sPLA_2$ activity (van den Bosch 1997).

A number of calcium independent PLA_2s ($iPLA_2$) have been described. One suggested role for cytosolic calcium-independent PLA_2 in $P388D_1$ macrophages is to replenish cellular arachidonic acid by esterification into phospholipids (Balsinde and Dennis 1996). However, an 88 kDa $iPLA_2$ has been found to be of importance for leukotriene synthesis in human granulocytes (Larsson Forsell *et al.* 1998). Macrophage spreading has been shown to be dependent on both $cPLA_2$ and $iPLA_2$ (Teslenko *et al.* 1997).

$cPLA_2$

$cPLA_2$ is expressed ubiquitously in all cells except for in mature T and B lymphocytes (for review see Clark *et al.* 1995, Leslie 1997). Type IV 85 kDa cytosolic phospholipase A_2 was cloned by Clark *et al.* (1991) and Sharp *et al.* (1991). $cPLA_2$ preferentially cleaves phospholipids containing arachidonic acid (Wijkander and Sundler 1989b, Diez and Mong 1990, Sundler *et al.* 1994). The importance of $cPLA_2$ has been studied in knock-out mice. These mice showed abnormalities in reproduction and a decreased production of prostaglandins and leukotrienes (Uozumi *et al.* 1997, Bonventre *et al.* 1997). The $cPLA_2$ deficient mice suffered less from allergic symptoms (Uozumi *et al.* 1997), indicating an important role for $cPLA_2$ in allergic responses. In addition, $cPLA_2$ has been found to have a role in conditions such as schizophrenia, Alzheimer´s disease, cirrhosis of the liver, diabetic nephropathy and in activation of hormones, growth factors and cytokines (reviewed by Bonventre 1999). The catalytic center of $cPLA_2$ contains a serine residue, Ser-228, essential for enzyme activity (Sharp *et al.* 1991). This was confirmed when the crystal structure of $cPLA_2$ was solved in 1999 by Dessen *et al.* Translocation of $cPLA_2$ from the cytosol to membranes is essential for its activation (Channon and Leslie 1990,

Diez and Mong 1990, Wijkander and Sundler 1992). Immunofluorescence studies have revealed translocated $cPLA_2$ to be situated at the nuclear envelope and on cytoplasmic membrane structures thought to be the endoplasmic reticulum (Schievella *et al.* 1995). This translocation is regulated by Ca^{2+} in the nanomolar to micromolar range. The membrane-binding properties of $cPLA_2$ are mediated by the Ca^{2+}-dependent phospholipid binding (CalB) domain, a region with a β-sandwich structure containing two calcium binding sites (Perisic *et al.* 1998). The CalB domain shows homology to the CII domain of PKC (Clark *et al.* 1995).

$cPLA_2$ has now been further divided into subgroups. The classical $cPLA_2$ has been given the post-script α since another form of $cPLA_2$ called $cPLA_2\beta$ was found in cerebellum and pancreas (Pickard *et al.* 1999) and a calcium-independent $cPLA_2$ named $cPLA_2$-γ was found in heart and skeletal muscle (Underwood *et al.* 1998). A naturally occurring inactive variant of $cPLA_2$ has also been discovered in mononuclear leukocytes and polymorphonuclear leukocytes (Gordon *et al.* 1996).

Regulation of $cPLA_2$

$cPLA_2$ is constitutively expressed at low level (Miyashita *et al.*1995). The most studied process regulating $cPLA_2$ is post-translational phosphorylation although PMA and a number of other stimuli have been shown also to increase the half-life of $cPLA_2$ mRNA (Tay *et al.* 1994). In 1993, Nemenoff *et al.* found $cPLA_2$ to be phosphorylated by ERK-2 and Lin *et al.* (1993) identified Ser-505 as the phosphorylation site responsible for increased catalytic activity. The phosphorylation of Ser-505 was confirmed in cells over-expressing $cPLA_2$ (Abdullah *et al.* 1995). Phosphorylation of multiple sites of $cPLA_2$ has been observed by us (paper II) as well as by others (Qiu *et al.* 1993, de Carvalho *et al.* 1996, Börsch-Haubold *et al.* 1998). Two additional MAP kinases have been suggested to be responsible for the phosphorylation of $cPLA_2$, namely, p38 in thrombin-stimulated platelets and in human neutrophils (Kramer *et al.* 1996,

Waterman *et al.* 1996) and JNK in a human thrombin-stimulated astrocytoma cell line (Hernández *et al.* 1997). Dephosphorylation of $cPLA_2$ has been studied in polymorphonuclear leukocytes and was found to be exerted by both okadaic acid-sensitive and -insensitive phosphatases (Gordon *et al.* 1996).

Glucocorticoids and $cPLA_2$

The cloning of the human $cPLA_2$ promoter revealed the presence of two glucocorticoid responsive elements (Wu *et al.* 1994). The relevance of these has not been established.

Dex did not affect the basal expression of $cPLA_2$ in rat 3YI fibroblasts (Kuwata *et al.* 1998). However, a slight reduction of the constitutive $cPLA_2$ was observed in HeLa cells (Hoeck *et al.* 1993). IL-1β-induced $cPLA_2$ mRNA is inhibited by dex in various cell types (Schalkwijk *et al.* 1993, Angel *et al.* 1994, Newton *et al.* 1997).

Cross-talk

Both low molecular weight $sPLA_2$ and $cPLA_2$ may be expressed in the same cells (Marshall and Roshak 1993). In contrast to group IIA PLA_2, which is localised to secretory granules, group V PLA_2 has been found associated with cellular membranes in mouse bone marrow-derived mast cells (Bingham III *et al.* 1999). The authors conclude that the localisation of group V would facilitate its interaction with translocated $cPLA_2$. In various cell types, arachidonic acid release induced by $sPLA_2$ has been suggested to be mediated via $cPLA_2$ (Wijkander *et al.* 1995, Balsinde *et al.* 1998, Huwiler *et al.* 1997). On the other hand, the use of an inhibitor specific for $cPLA_2$ has been shown to decrease $sPLA_2$ expression (Kuwata *et al.* 1998). Addition of exogenous $sPLA_2$ has been shown to result in activation of ERK (Sugiura *et al.* 1995), as well as JNK and p38 (Hernández *et al.* 1998), suggesting the existence of cross-talk also between PLA_2 and MAP kinases.

Arachidonic acid metabolism and eicosanoid formation

Activation of PLA_2 leads to release of arachidonic acid from phospholipids. Arachidonic acid is the precursor of eicosanoids including prostaglandins, thromboxanes and leukotrienes (**Fig. 6**).

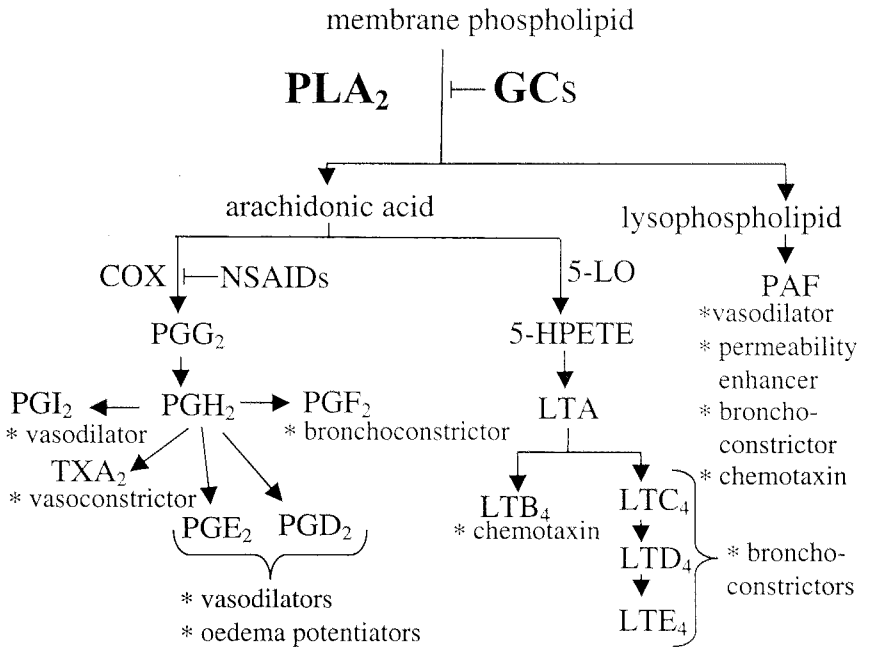


Figure 6. ***The synthesis of eicosanoids from arachidonic acid.*** Sites of action of anti-inflammatory drugs are indicated. GC; glucocorticoids, COX; cyclooxygenase, NSAIDs; non-steroidal anti-inflammatory drugs, PG; prostaglandin, PGI_2; prostacyclin, LT; leukotriene, TX; thromboxane, PAF; platelet activating factor; 5-LO; 5-lipoxygenase, HETE; hydroxyeicosatetra-enoic acid

Arachidonic acid is further metabolized via two main pathways yielding prostaglandins and thromboxanes by the action of cyclooxygenase and leukotrienes by the action of lipoxygenases. There are at least two forms of cyclooxygenase, cyclooxygenase I (COX-1), that is constitutively expressed, and cyclooxygenase II (COX-2) that can be induced by different stimuli such as cytokines (Raz *et al.* 1988). Dex has been shown to inhibit expression of IL-

1-induced cyclooxygenase-2 mRNA in various cell types (Angel *et al.* 1994, Geng *et al.* 1995, Ristimäki *et al.* 1996). The anti- inflammatory effect of non-steroidal anti-inflammatory drugs (such as aspirin, indomethacin and ibuprofen) is exerted through inhibition of cyclooxygenase although aspirin-induced inhibition of the enzyme IκB kinase-β has recently been reported (Yin *et al.* 1998). *In vitro,* glucocorticoids inhibit the formation of prostaglandins and leukotrienes (Russo-Marie *et al.* 1979, de Caterina *et al.* 1993). *In vivo,* glucocorticoids affect both the contractile function of smooth muscles and have effects on formation of inflammatory mediators such as cytokines and arachidonic acid metabolites (Shindo *et al.* 1998, Hirst and Lee 1998). The relative importance of these parameters is not fully established and is lively debated. Prostaglandin E_2 (PGE_2) has been found to be an inhibitor of TNF formation via elevation of cAMP. Vial *et al.* (1998) have suggested that this inhibition leads to down-regulation of $sPLA_2$ synthesis. Another group of potent inflammatory mediators is the leukotrienes formed from arachidonic acid by the enzyme 5-lipoxygenase. In macrophages co-localisation of $cPLA_2$ and 5-lipoxygenase was found after stimulation with A23187 (Peters-Golden and McNish 1993). In human monocytes, $cPLA_2$ has been suggested to be important in prostaglandin synthesis but less important in leukotriene synthesis (Marshall *et al.* 1997). In murine $P388D_1$ macrophages Balsinde *et al.* (1999) have presented a model in which $cPLA_2$ is necessary for activation of $sPLA_2$ and this enzyme is in turn necessary for cyclooxygenase-2 expression. In addition to being responsible for the liberation of arachidonic acid, PLA_2 generates lysophospholipids (reviewed by Snyder 1995) including the precursor of platelet activating factor (PAF), another important inflammatory factor.

CYTOKINES

Cytokines are proteins secreted by a cell into the extracellular fluid where they exert their effects on the same cell or on neighbouring cells by interacting with

specific receptors. Two cytokines released by activated macrophages have been studied in this investigation, TNF-α and IL-1β. These two cytokines have many properties in common. For example, both induce synthesis of prostaglandins, elevate acute phase reactants and are involved in septic shock. Diacylglycerol and ceramide have been suggested to participate in mediation of cellular responses induced by IL-1 or TNF (Schütze *et al.* 1994). Pretreatment with TNF or IL-1 results in hyporesponsiveness (Wallach *et al.* 1988). Both TNF-α and IL-1β activate the transcription factors AP-1 and NFκB. Possible targets for NFκB are numerous genes containing κB sites including TNF-α and IL-1β (Adcock *et al.* 1995). In many aspects, the effect of these two cytokines together is synergistic (Rosenwasser 1998).

Tumour necrosis factor-α

Tumour necrosis factor was first identified as an oncolytic protein. In addition to the cytotoxic/cytostatic effect, TNF plays a key role in inflammation. There are two forms of TNF-α. The molecular weight of the membrane-bound form is 26 kDa and that of the released form is 17 kDa. These two forms might kill cells in different ways. Instead of being secreted, the membrane-bound form can act by cell to cell contact (Kriegler *et al.* 1988). The 26 kDa form has a 76 (human) or 79 (mouse) amino acid N-terminal sequence which is absent in the secreted form. This sequence is not cleaved in the rough endoplasmic reticulum like typical signal sequences (Kriegler *et al.* 1988). The 17 kDa form of TNF-α is proteolytically released from its membrane-bound precursor. At least two enzymes have been suggested to perform this cleavage, one serine proteinase (Scuderi 1989, Kim *et al.* 1993) and a metalloproteinase that was recently cloned (Black *et al.* 1997, Moss *et al.* 1997) called TNF-α-converting enzyme (McGeehan *et al.* 1994, Gearing *et al.* 1994).

The signalling cascade leading to activation of TNF-α has been most thoroughly studied after LPS-challenge. In Kupffer cells, the LPS-induced activation of TNF-α has been suggested to include internalisation of LPS,

endosome acidification and an elevation in intracellular Ca^{2+} with a parallel activation of protein tyrosine kinases followed by activation of PKC (Lichtman *et al.* 1998). Other reports support the importance of Ca^{2+} and PKC for TNF-α production (Nakata and Hide 1998). In RAW 264.7 cells, JNK was found to be necessary for LPS-induced translation of TNF-α (Swantek *et al.* 1997). In the same study, dex was shown to inhibit LPS-induced JNK activity. Mice lacking a p38 substrate called MAPK-activated protein kinase-2 show a severe defect in translation of TNF-α (Kotlyarov *et al.* 1999). TNF-α specifically binds to two different receptors present on the cell surface of virtually all cell types, including macrophages (Riches *et al.* 1996). The receptors, TNF-R55 and TNF-R75, are named after their molecular mass of 55 kDa and 75 kDa respectively. The TNF-α gene sequence does not contain any GRE (Joyce *et al.* 1997). Instead, as mentioned above, direct interaction between glucocorticoids and transcription factors such as NFκB and AP-1 has been suggested as an explanation for the repressive effects of glucocorticoids on TNF-α. In addition to being activated by the transcription factor NFκB, TNF-α activates NFκB which is able to activate gene transcription of a large number of proteins (Adcock *et al.* 1995). NFκB is a dimeric protein consisting of a DNA-binding subunit, NFκB1 (p50), and a transactivator, RelA (p65), or other proteins of the rel family (Grigoriadis *et al.* 1996). IκBα is an inhibitor of NFκB. This inhibitory protein specifically binds to NFκB and the formed complex is not able to translocate to the nucleus. Treatment with TNF-α or okadaic acid results in phosphorylation of IκB leading to degradation of this inhibitory protein (Sun *et al.* 1995) (**Fig. 7**). The kinase phosphorylating IκB has been found to phosphorylate the p65 subunit of NFκB as well (Sakurai *et al.* 1999). Dex has been shown to induce synthesis of IκBα and thereby inhibit cytokine secretion in some cell types (Scheinman *et al.* 1995, Auphan *et al.* 1995). However, in endothelial cells, this model was not supported (Brostjan *et al.* 1996, Newton *et al.* 1998). Non-steroid inhibitors of p38 have been shown to exert their effects at the post-transcriptional level (Lee and Young 1996).

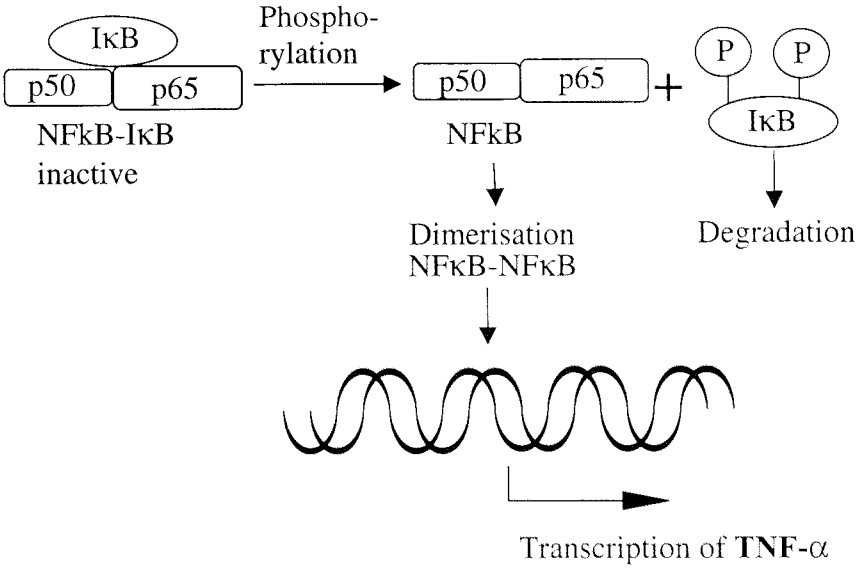


Figure 7. ***Activation of NFκB and its induction of gene expression.***

In some cell lines, $cPLA_2$ has an important role in mediating effects of TNF such as ceramide accumulation and cytotoxicity (Hayakawa *et al.* 1993, Jayadev *et al.* 1997). TNF-α-induced $cPLA_2$ activity can be inhibited by dex in Hep-2 cells (Goppelt-Struebe and Rehfeldt 1992). Dex has been found to exert this effect at the mRNA level in HeLa cells (Hoeck *et al.* 1993).

Interleukin-1β

Although the name interleukin means "between leukocytes", cells other than leukocytes produce IL-1. IL-1 has been implicated in the induction of fever, sleep, and inflammatory conditions such as asthma, arthritis, colitis and insulin-dependent diabetes mellitus. However, IL-1 also exerts protective effects in for example bacterial infections and cancer (see Rosenwasser 1998 for review). There are two forms of IL-1, α and β, binding to the same receptor (reviewed by Dinarello 1991) and resulting in very similar effects (Svensson *et al.* 1995). Only IL-1β was studied in paper IV. IL-1β is induced by LPS, Gram-positive

cell wall components, TNF, IL-1 itself (Dinarello 1991), okadaic acid (Sung and Walters 1993) and PMA (Bensi *et al.* 1990). In various cell types, PKC has been found to be important for IL-1β expression. PKC activation results in activation of the transcription factor activator protein (AP-1) consisting of a fos/jun heterodimer or jun homodimers (Palkama *et al.* 1993). AP-1 has been found to be necessary for expression of IL-1β, at least under certain conditions (Ritzenthaler and Roman 1998) (**Fig. 8**). Also, a binding site for NFκB is present in the IL-1β gene (Clark 1986). The 31 kDa precursor of IL-1β is cleaved by IL-1β-converting enzyme (ICE) yielding the 17 kDa form of IL-1β (Thornberry *et al.* 1992).

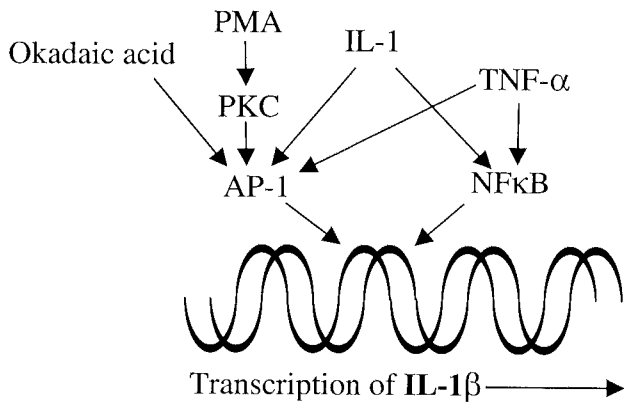


Figure 8. ***Summary of alternative pathways leading to expression of IL-1β.***

Cell injury has been associated with release of IL-1β (Hogquist *et al.* 1991). There is a well-documented inhibitory effect of dex on LPS-induced IL-1 transcription (Lew *et al.* 1988). Dex has also been shown to affect IL-1β-converting enzyme (ICE) activity (Layé *et al.* 1996) and to upregulate IL-1 receptor numbers (Shieh *et al.* 1993).

IL-1 activates the transcription factors AP-1 and NFκB. A recently identified kinase called TAK1 is able to activate both NIK, the kinase responsible for NFκB activation, and the JNK-1 pathway, resulting in activation of AP-1

(Ninomiya-Tsuji *et al.* 1999). PI-3 kinase has also been suggested to be involved in the activation of AP-1 and NFκB (Reddy *et al.* 1997). Another feature that IL-1β shares with TNF-α is the ability to phosphorylate IκB, resulting in the degradation of this inhibitory protein (Newton *et al.* 1998). As is the case with TNF-α, IL-1β has also been shown to induce $cPLA_2$ in various cell types (Hulkower *et al.* 1992, Jackson *et al.* 1993, Schalkwijk *et al.* 1992).

PRESENT INVESTIGATION

The aim of this study was to map signalling pathways in inflammatory cascades and how they can be modulated by glucocorticoids.

There are many steps in the signal chain leading from receptor activation by stimulus to induction of $cPLA_2$ and release of arachidonic acid with subsequent formation of pro-inflammatory leukotrienes and prostaglandins. All these steps are possible targets for glucocorticoids. The production of every single molecule in these cascades can be regulated at the level of transcription, translation or post-translation. We found that the glucocorticoid dex inhibited both the expression of $cPLA_2$ mRNA and specifically inhibited the activation of $cPLA_2$. Dex also inhibited other proteins important for signalling in inflammatory responses. The numerous actions of glucocorticoids are probably a prerequisite for the fine-tuning of the regulation of the immune response.

Some of the cellular events that we considered the most important have been examined in this work, and more specifically the following questions were considered:

i) Time and dose dependence for dex-mediated inhibition of the activation of $cPLA_2$ and arachidonic acid release.

ii) Effect of dex on the level of expression of $cPLA_2$, as mRNA and protein.

iii) Identification of signalling pathways by the use of different stimuli and inhibitors.

iv) Mapping of phosphorylation sites on $cPLA_2$.

v) Effect of dex on MAP kinases and protein kinase C.

vi) Effect of dex on cytokines for which $cPLA_2$ is of critical importance or which are of importance for $cPLA_2$ activity.

Dexamethasone down-regulates the 85 kDa cPLA$_2$ in mouse macrophages and suppresses its activation.

We started by studying general effects of dex. Cell viability after dex treatment was assessed by three different methods since dex affects apoptosis in some cell types; induction (Harmon *et al.* 1979) as well as inhibition of TNF-α-induced apoptosis has been reported (Costas *et al.* 1996). Lactate dehydrogenase release was measured spectrophotometrically, trypan blue exclusion was monitored and DNA was studied to confirm the absence of DNA fragmentation. None of these three parameters indicated cytotoxicity upon treatment with up to 100 nM dex for 20 hours. The binding of zymosan particles to macrophages was only slightly decreased after dex treatment (Gewert *et al.* 1996) and did not account for the inhibitory effects observed below.
We used size-dependent gel chromatography in order to find out if any inhibitor was associated with cPLA$_2$ after dex treatment or if cPLA$_2$ was proteolytically cleaved. Cytosolic fractions from peritoneal mouse macrophages revealed that cPLA$_2$ activity eluted identically, whether the cells were treated with dex or not, indicating that no such changes were induced by dex.
Glucocorticoids are known to have effects on protein levels *in vivo* as well as *in vitro* (Wool and Weinshelbaum 1960, Norton and Munck 1980). Therefore, we treated cells with 10 nM dex for 20 h and found that the total cellular protein level was not affected significantly as it remained at 87±7% of that in control cells. The effect of dex on protein synthesis, measured by ^{35}S-methionine incorporation, was more pronounced, being reduced to 59±3% under the same conditions. A general decrease in $^{32}PO_4$-labelling of phosphoproteins was also observed after dex treatment, whereas total ATP was unaffected.

The inhibitory effects of dex on arachidonic acid release and cPLA$_2$ activity evolved slowly and were time- and dose-dependent. Treatment with dex (10 nM for 20h) reduced the unstimulated cPLA$_2$ activity to 1/3 of control level. A

corresponding reduction in $cPLA_2$ protein level was observed by Western blotting. The stimuli PMA, zymosan and okadaic acid were used to study up-regulation of $cPLA_2$ activity. After treatment with dex, the ability of PMA or zymosan but not of okadaic acid to up-regulate this activity was impaired. These results indicate that the effect of okadaic acid was exerted at, or downstream of, the dex-sensitive step(s). In addition to inhibit the synthesis of $cPLA_2$, the okadaic acid data indicates that dex exerts another inhibitory effect on $cPLA_2$. These results support a mechanism of action where dex induces the synthesis of phosphatases (Zor *et al.* 1990). These authors based their hypothesis on the reduction in protein phosphorylation after dex treatment as detected by autoradiography of $^{32}PO_4$-labelled cells. Glucocorticoids have also been shown to induce acid phosphatase activity in RBL cells (Her *et al.* 1991), alkaline phosphatase in human breast cancer cells (Chang *et al.* 1994) and tyrosine phosphatase activity in murine erythroleukaemic cells (Hammond *et al.* 1989). A similar mechanism has been suggested for glucocorticoid-mediated induction of a phosphatase inhibiting Ca^{2+}/calmodulin-dependent kinase in T cells (Paliogianni *et al.* 1995). Another strategy has been postulated for glucocorticoid-induced elevation of a phosphatase regulating glycogen metabolism: glucocorticoids in this system are suggested to induce the synthesis of a protein that relieves the phosphatase from inhibition (Laloux *et al.* 1983).

Multiple C-terminal serine phosphorylation accompanies both PKC-dependent and –independent activation of cytosolic 85 kDa PLA_2 in macrophages.

The aim of this paper was to map the major phosphorylation sites on $cPLA_2$ in PMA-stimulated cells. Phosphorylation was observed exclusively on serine residues. Like others (Lin *et al.* 1992, Börsch-Haubold *et al.* 1998), we were not able to detect any increase in phosphorylation on threonine or tyrosine

residues. However, tyrosine phosphorylation has been observed after stimulation with LPS or TGF-α in other cell types (Shankavaram *et al.* 1998, Kast *et al.* 1993). The $cPLA_2$ sequence is 749 amino acids long. CNBr cleavage resulted in five fragments with increased ^{32}P-labelling. The most C-terminal fragment (residues 698-749) was the most heavily labelled when PMA or bacteria was used as a stimulus. This fragment was further cleaved by trypsin treatment. Analysis of the peaks obtained in this way revealed four ^{32}P-labelled fragments. One of these was more heavily labelled compared to the others. These results are in agreement with others who have found multiple phosphorylation sites in mouse peritoneal macrophages (Qiu *et al.* 1993). de Carvalho *et al.* have identified the four most heavily phosphorylated amino acids in okadaic acid-induced $cPLA_2$ to be Ser-437, Ser-454 (not present in mice), Ser-505 and Ser-727. In this study, okadaic acid in contrast to other stimuli caused a 4.5 fold increase in phosphorylation of Ser-727 (de Carvalho *et al.* 1996). Another study (Börsch-Haubold *et al.* 1998) also showed multiple phosphorylation sites including Ser-505 and Ser-727 after stimulation of $cPLA_2$ with a number of different physiological agonists. However, phosphorylation of Ser-727 has been found not to be responsible for the electromobility shift of $cPLA_2$ since mutation of this amino acid did not prevent a gel shift of $cPLA_2$ (Gijón *et al.* 1999) and hence the role of Ser-727 is unknown.

In order to further examine the target for glucocorticoids in the signalling leading to $cPLA_2$ activation we studied the effects of dex on bacteria-induced arachidonic acid release and activation of $cPLA_2$. Activation of $cPLA_2$ by certain genital bacteria has earlier been shown to be PKC-independent (Svensson *et al.* 1991). In paper I, we showed that dex suppressed the PMA-induced $cPLA_2$ activation. Since dex equally well suppressed bacteria-induced $cPLA_2$ activation, we conclude that this action of dex is located down-stream of PKC.

Dexamethasone partially inhibits the activation of JNK-1 but not ERK-2 or p38 MAPK in mouse macrophages. Implications for the activation of 85 kDa cPLA$_2$.

By using cycloheximide, a protein synthesis inhibitor, information on the dependence on protein synthesis for different inhibitory functions of dex was obtained. The effects of cycloheximide in combination with dex were studied with regard to arachidonate release, cPLA$_2$ activation and formation of cPLA$_2$. A dose of cycloheximide could be titrated, that caused an almost complete reversion of dex inhibition of these parameters. In conclusion, the effects of dex depended on protein synthesis which implies that the effects are mediated via the glucocorticoid receptor. This is consistent with other data based on the use of the glucocorticoid antagonist RU38486 which was able to block the inhibitory effect of dex on zymosan-induced arachidonate release (Gewert, unpublished results). Both the basal and the LPS-induced expression of cPLA$_2$ mRNA were inhibited by dex treatment.

cPLA$_2$ can be seen migrating as a doublet on SDS-polyacrylamide gels. Phosphorylation of cPLA$_2$ causes the enzyme to migrate more slowly while phosphatase treatment results in accumulation of the lower band (Lin *et al.* 1992). This phenomenon is called an electrophoretic mobility shift. Since phosphorylation leads to activation of the enzyme, it is generally accepted that a shift in mobility corresponds to an activation of the enzyme. In agreement with others (Qiu *et al.* 1998), we found that the mobility shift of cPLA$_2$ caused by okadaic acid was greater than that caused by PMA or zymosan. In paper II we found PMA to induce phosphorylation of multiple sites on cPLA$_2$. The most obvious explanation for the shift phenomenon seen after okadaic acid treatment is phosphorylation of additional sites although Gijón *et al.* have not been able to find support for this hypothesis in mutation studies (Gijón *et al.* 1999).

The possibility that dex targeted the level or activation of MAP kinases, of which ERK-2 and p38 recently have been implicated in cPLA$_2$ activation and

JNK-1 in the transcriptional activation of pro-inflammatory cytokine genes, was also addressed.

In contrast to okadaic acid-induced ERK activity, PMA- or zymosan-stimulated activation of ERK has been found PKC-dependent (Qiu and Leslie 1994). Phosphorylation of ERK, p38 and JNK can be studied by Western blotting with a primary antibody directed against the phosphorylated form of these kinases. Dex treatment did not affect PMA-induced phosphorylation of these MAP kinases. However, a decrease in zymosan-induced phosphorylation of the studied MAP kinases was observed after dex treatment. Immunoprecipitation followed by activity assays showed that also zymosan-induced activity of the MAP kinases but not PMA-induced activity was sensitive to dex treatment. Neither was there any pronounced inhibitory effect of dex on okadaic acid-induced MAP kinase activation (not shown). Whatever stimulus used, the amount of ERK or p38 protein was not decreased by dex. In the case of JNK, we also observed a somewhat lowered amount of protein after dex treatment. The possibility that this contributes to the dex-mediated inhibition of cytokine expression at the transcriptional level requires further examination.

In line with these results is a report in which extremely high doses of dex for a short time inhibits LPS-induced JNK activity but not that of p38, ERK, ERK or the MAP kinase kinases MEK3, MEK4 or MEK6 (Swantek *et al.* 1997). Our results suggest that dex-mediated inhibition of $cPLA_2$ activation is exerted down-stream of ERK or p38.

Dexamethasone differentially regulates cytokine transcription and translation in macrophages responding to bacteria or okadaic acid

This study was performed to evaluate the ability of dex to affect bacteria- or okadaic acid-induced production of the cytokines TNF-α and IL-1β. No difference was found between Gram-negative and Gram-positive bacteria with regard to induction of IL-1β or TNF-α mRNA and protein, or the sensitivity of

these responses to dex. Treatment with dex inhibited bacteria-induced IL-1β primarily at the mRNA level. Dex has been reported to exert its inhibitory effect on IL-1β mRNA by decreasing its stability (Amano *et al.* 1992), but very interestingly a regulatory region containing a negative GRE was recently found in the human IL-1β gene (Zhang *et al.* 1997). In contrast to IL-1β, bacteria-induced TNF-α was only partially inhibited at the mRNA level. The formation of bacteria-induced TNF-α protein was, however, almost completely inhibited due to further inhibition at the translational or post-translational level.
Overnight incubation with dex resulted in a minor inhibition of okadaic acid-induced IL-1β at the transcriptional level. Therefore, it is interesting to note that dex severely inhibited okadaic acid-induced formation of IL-1β as detected by Western blot. This inhibition was much more pronounced than the dex-mediated inhibitions of stimulus-induced formation of $cPLA_2$ protein discussed in paper I and III.
Treatment with okadaic acid increases IL-1β transcription and results in an increase in IL-1β-converting enzyme in human monocytes (Sung and Walters 1993). However, the IL-1β-converting enzyme does not seem to be active in resident macrophages since we, as well as others (Hogquist *et al.* 1991), were only able to detect the pro-form of IL-1β.

Dex has long been known to inhibit TNF-α gene transcription and the secretion of the 17 kDa form of LPS-stimulated TNF-α protein into the culture medium (Beutler 1986). However, dex has been reported not to inhibit the cell surface form of LPS-induced TNF-α (Chaudhri 1997).

Transcriptional activation of TNF-α induced by okadaic acid was inhibited by dex to about the same extent as that induced by bacteria. Western blots revealed that okadaic acid affected the processing of TNF-α. Sung *et al.* (1992) have observed an increase in TNF-α mRNA stability and transcription rate

after okadaic acid stimulation followed by a modulation of TNF-α processing similar to our observations.

TNF-α was found in both the cleaved and uncleaved form after okadaic acid treatment. The processed form was found both in culture medium and to some extent cell-associated. Treatment with dex for 20 h before addition of okadaic acid resulted in differential inhibition of TNF-α with the cell-associated 26 kDa form of TNF-α being more severely inhibited than the cell-associated 17 kDa form. In the culture medium, inhibition of the 17 kDa form of TNF-α corresponded to the inhibition of the 26 kDa cell-associated TNF-α. However, the total formation of TNF-α protein was only partially inhibited, suggesting that okadaic acid over-rides the dex-mediated translational inhibition. These results indicate that the inhibitory effect of dex on TNF-α translation is mediated by one or more okadaic acid-sensitive protein phosphatase(s).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Glucocorticoids are extremely potent anti-inflammatory drugs. Unfortunately, when used pharmacologically in high doses, side effects appear as does resistance to glucocorticoids. Consequently there is a need for development of more specific anti-inflammatory drugs. Such research requires knowledge about the mechanism of glucocorticoid action.

We found that the synthetic glucocorticoid dexamethasone reduced the expression of $cPLA_2$ and further inhibited the activation of $cPLA_2$. A summary of possible targets for glucocorticoids is shown if **Fig. 9**. Treatment with dex was also found to inhibit expression of the cytokine IL-1β primarily at the transcriptional level whereas another cytokine, TNF-α, was only partially inhibited at the level of transcription but further inhibited at the translational level.

In conclusion, the anti-inflammatory effects of glucocorticoids result from a great number of actions. The multitudinous actions can be explained by the fact that binding of glucocorticoids to its responsive DNA inhibits the binding of other transcription factors with regulating properties. We have found that the interference of dex with okadaic acid-sensitive phosphatases is of critical importance both for the activation of $cPLA_2$ and for the formation and processing of TNF-α. The role of protein phosphatases in the interaction between glucocorticoids and $cPLA_2$, as well as in the translational regulation of TNF-α, is worth further investigation in the development of anti-inflammatory pharmacological drugs for the future.

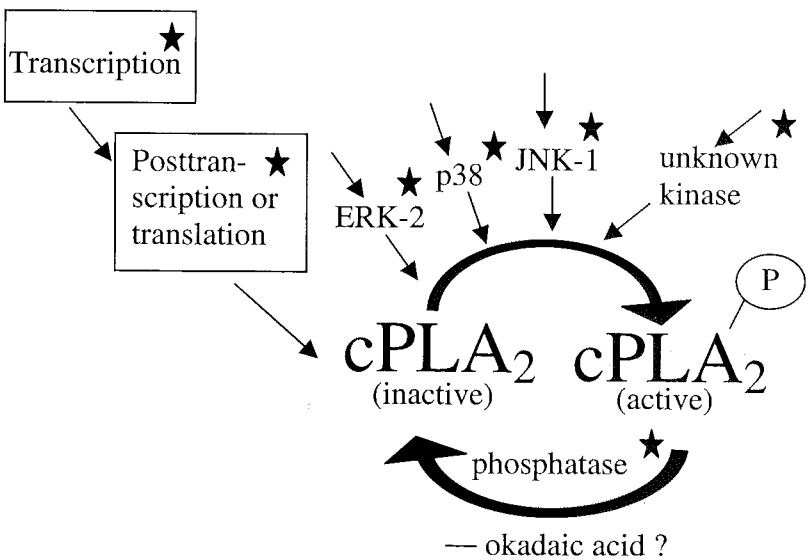


Figure 9. ***Possible targets for dex*** denoted by ★.

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EN STUDIE AV HUR GLUKOKORTIKOIDER HÄMMAR INFLAMMATION

Populärvetenskaplig sammanfattning på svenska

Glukokortikosteroider, varav kortison är den mest kända, är hormoner som reglerar många funktioner i kroppen och även används som läkemedel för att dämpa inflammatoriska tillstånd som t.ex. astma och reumatiska sjukdomar. Glukokortikosteroider är mycket potenta läkemedel och används flitigt med mycket goda effekter. Vid långvarigt systemiskt bruk av höga doser förekommer tyvärr biverkningar. Det är därför av stort intresse att klargöra glukokortikosteroidernas verkningsmekanismer för att utifrån dessa fakta kunna framställa mer specifika läkemedel med färre biverkningar. I denna studie har vi använt dexametason som är en syntetisk glukokortikosteroid.

Effekterna av dexametason har studerats på celler som kallas makrofager. Makrofager är en sorts vita blodkroppar som deltar i kroppens försvar mot främmande organismer. Vid aktivering av makrofager är fosfolipas A_2 det enzym som reglerar hastigheten med vilken olika inflammationssubstanser såsom prostaglandiner och leukotriener bildas. Cytokiner är ett annat exempel på substanser som bildas och frisätts av makrofager och medverkar vid inflammationer.

I den första studien fann vi att dexametason hämmar både bildningen av proteinet fosfolipas A_2 ($cPLA_2$) och dess aktivitet. Många proteiners aktivitet regleras genom fosforylering (ett s.k. kinas kopplar på en fosfatgrupp på en aminosyra) respektive defosforylering (borttagning av fosfatgrupp som utförs av fosfatas). Vi fann att vi genom att tillsätta en substans som hämmar fosfataser (okadainsyra) kan upphäva effekten av dexametason. Detta indikerar att hämningen av $cPLA_2$ aktiviteten eventuellt sker genom induktion av ett fosfatas. Vi har också arbetat med att kartlägga vilka aminosyror det är som

fosforyleras vid aktivering av $cPLA_2$. Tre olika kinaser har tidigare föreslagits fosforylera $cPLA_2$. Våra data ger inte något stöd för att dexametason skulle hämma något av dessa i samma utsträckning som $cPLA_2$ hämmas.

Dex påverkar också cytokiner. Vi har valt att studera främst interleukin-1 och tumour necrosis factor vilka är två cytokiner med stor betydelse vid inflammatoriska tillstånd. Regleringen av cytokiner är viktig eftersom de behövs för att kroppen ska kunna bekämpa infektioner och även cancer, men överaktivering av cytokiner kan ha dödlig utgång. Vi fann att dexametason hämmar bildningen av dessa cytokiner. De båda cytokinerna hämmas under olika betingelser och i olika steg i processen som leder fram till det färdiga proteinet. Även för regleringen av cytokiner med dexametason fann vi att fosfataser är av stor betydelse.

Sammanfattningsvis hämmas långt ifrån alla proteiner som är involverade vid inflammationer av dexametason. Dock utövade glukokortikosteroiderna en kraftfullt hämmande effekt på flera olika viktiga proteiner varav ett är $cPLA_2$. En sådan komplex verkan är med all sannolikhet av stor betydelse för finjusteringen av balansen i kroppens immunsvar.

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I

Dexamethasone down-regulates the 85 kDa phospholipase A_2 in mouse macrophages and suppresses its activation

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We have studied the effects of dexamethasone (dex) (i) on the level of the arachidonate-mobilizing phospholipase A_2 (PLA_2-85) in macrophages, (ii) on the stimulus-induced activation of this enzyme, and (iii) on the stimulus-induced release of arachidonate. Treatment of macrophages with 10 nM dex led to progressive reduction of PLA_2-85 down to approx. 35% of control levels in 20 h in the absence of stimuli. This was accompanied by a partial inhibition of calcium-ionophore-induced arachidonate release. In contrast, the ability of zymosan or phorbol ester to cause both persistent activation of PLA_2-85 and arachidonate release was greatly reduced or abolished. However, the protein phosphatase inhibitor okadaic acid, previously shown to cause enhanced phosphorylation and persistent activation of PLA_2-85, was still able to exert this effect on the dex-suppressed PLA_2-85. This suggests that the effect of okadaic acid was exerted at, or downstream of, the dex-sensitive step(s). Treatment with dex also led to inhibition of the characteristic changes in phosphoprotein labelling induced by phorbol ester or zymosan. However, phorbol-dibutyrate-binding isoforms of protein kinase C were not severely down-regulated. Thus dex was found to down-regulate PLA_2-85 and, in addition, to affect one or more component(s) in the signal chain that normally leads to its activation. However, okadaic acid retained the ability to cause activation of PLA_2-85.

INTRODUCTION

Synthetic glucocorticoids, such as dexamethasone (dex), are potent anti-inflammatory agents that also inhibit the formation of eicosanoids. The latter is long since known to be due to inhibition of the mobilization of arachidonate [1], but the mechanism for this effect has remained unclear. It was previously associated with the induction of proteins called lipocortins, the classic view being that lipocortins inhibited the enzymic activity of phospholipase $A_2$and that this inhibition could be alleviated by stimulus-induced phosphorylation of lipocortin [1,2]. However, lipocortins/calpactins have been shown to be members of a family of proteins [2,3] which can bind to anionic phospholipids and actin filaments in a calcium-dependent manner and which inhibit phospholipase A_2 only under certain conditions and then by sequestration of its substrate rather than by interaction with the enzyme [4,5]. Furthermore, lipocortin I was found to be present in considerable amounts in resident mouse macrophages, but did not undergo phosphorylation upon stimulation with 4β-phorbol 12-myristate 13-acetate (PMA) [6]. This indicated that the protein kinase C (PKC)-mediated activation of arachidonate release was not due to phosphorylation and inactivation of lipocortin I.

More recently, an intracellular 85 kDa phospholipase A_2 (PLA_2-85) has been isolated and characterized (reviewed in [7,8]). This enzyme has been implicated in the mobilization of the eicosanoid precursor arachidonate in mouse macrophages [9,10]. The mechanisms that have hitherto shown potential to regulate PLA_2-85 include changes in the cytosolic Ca^{2+} level [11–13] and phosphorylation cascades, both dependent [14] and independent [15,16] of PKC. In the present paper we investigate the effects of the glucocorticoid analogue dex on the basal level of PLA_2-85 and on the signalling for its activation, as well as the effects on the stimulus-induced release of arachidonate in mouse macrophages.

EXPERIMENTAL

Materials

Cell culture reagents were from Flow Laboratories. All other chemicals were from Sigma Chemical (St. Louis, MI, U.S.A.), except for general laboratory reagents which were of the highest quality available. Polyclonal rabbit antibodies raised against recombinant PLA_2-85 were obtained from Genetics Institute Inc., Cambridge, MA, U.S.A.

Arachidonate release

Peritoneal macrophages were isolated and cultured by a modification of the method described previously [17]. Peritoneal cells were collected from female mice (NMRI, Bommice, Denmark) in Medium 199 containing serum (1%, v/v) and heparin (20 units/ml). The cells were allowed to adhere to plastic 12-well tissue-culture dishes. The number of adherent cells per well was approximately 1.0×10^6. After 2 h incubation non-adherent cells were removed by washing with Dulbecco's phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}. The adherent macrophages were kept in culture for 20 h in Medium 199 containing 10% (v/v) serum at 37 °C in a humified atmosphere of 5% CO_2

Abbreviations used: dex, dexamethasone; DMSO, dimethyl sulphoxide; PLA_2-85, intracellular 85 kDa phospholipase A_2; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; PDBu, 4β-phorbol 12,13-dibutyrate; TNF-α, tumour necrosis factor-α; IL-1, interleukin-1; TGFβ, transforming growth factor β; M-CSF, macrophage colony-stimulating factor.

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in air. During this culture period the cells were labelled with 1 μCi of [^{3}H]arachidonate (Amersham International; 212 Ci/mmol) per well, and, when indicated, dex was included in the medium. Dex was kept as a stock solution in dimethyl sulphoxide (DMSO) and for each experiment was freshly diluted in medium. The final concentration of DMSO never exceeded 0.1 %. The presence of dex did not affect labelling with [^{3}H]arachidonate as seen after 20 h. On the second day the cells were washed three times with PBS and Medium 199 was added for 30 min. Medium was removed and replaced with fresh Medium 199 to which appropriate stimulus, zymosan (200 μg/ml), A23187 (1 μM), PMA (100 nM) or okadaic acid (0.5 μM; Bio-Zac, New Boston St., Woburn, MA, U.S.A.) was added for the time indicated. The dishes were put on ice, the medium was taken off and the cells were scraped in 0.1 % Triton X-100 (w/v). Both the tubes containing medium and the tubes containing cell extract were centrifuged for 5 min at 2500 *g*. The release of incorporated [^{3}H]arachidonate was determined from the radioactivity in the culture medium and the total radioactivity in cell extract and medium.

Dex by itself showed no inhibitory effect on the basal release of ^{3}H-labelled arachidonate from unstimulated peritoneal macrophages, neither did it affect uptake and esterification during the labelling period. However, a dose-dependent alteration in cellular morphology was observed after treatment with dex, in that the proportion of well-spread cells with spike-like extensions was reduced. Since no increase in lactate dehydrogenase in the medium was detected, hypothetical membrane alterations would be limited.

PLA_2 activity

PLA_2-85 was assayed according to the method described previously [9]. 1-Stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine (Amersham International; 80 pmol/incubation) in the form of sonicated vesicles was used as substrate. Aliquots of the cytosol fraction containing PLA_2-85 were incubated in the presence of substrate, 1.4 mM $CaCl_2$ (approx. 400 μM free Ca^{2+}) and BSA (0.19 mg/ml) in a buffer containing 80 mM KCl, 10 mM Hepes and 1 mM EDTA, pH 7.4. The final volume was adjusted to 525 μl. After incubation for 30 min at 37 °C the reaction was stopped by the addition of 1.5 ml of chloroform/methanol/HCl (20:10:1, by vol.), to which 0.05 % 2,6-di-t-butyl-*p*-cresol and carrier lipids had been added. After centrifugation the lipid phase was applied to a silicic acid column equilibrated in chloroform. Free fatty acids were eluted with 1.0 ml of chloroform and phospholipids were eluted with 2.5 ml of methanol.

Western-blot analysis of PLA_2-85

Macrophages (1.5×10^7 cells/25 cm^2 flask) were cultured as described above. Half the volume of whole-cell extracts prepared in Laemmli sample buffer supplemented with 100 mM dithioerythritol were boiled for 5 min and electrophoresed on SDS/PAGE (8 % acrylamide). The gel was equilibrated in transfer buffer [18] and the proteins transferred to a 0.2 μm pore size nitrocellulose membrane. Gelatin (1 %, w/v; for 2 h) was used to block the membrane. A 1/500 dilution of rabbit polyclonal antibodies against PLA_2-85 was incubated for 14 h with the membrane. After extensive washing, bound antibodies were detected with ^{125}I-labelled goat anti-(rabbit IgG) antibodies (0.5×10^6 c.p.m./ml). The blot was dried and thereafter analysed and quantified by digital imaging (Fujix Bas 2000).

Incorporation of [^{35}S]methionine

Isolation and culture of macrophages was performed as described above with the exception that serum was excluded from the medium. After 10 h incubation with appropriate concentrations of dex, [^{35}S]methionine (Amersham International; 31 μCi/well) was included in the medium. After another 10 h incubation in the presence of both dex and [^{35}S]methionine the dishes were put on ice, the medium was taken off and the cells were washed three times in PBS and then either scraped in 1.0 % (w/v) Triton X-100 or precipitated with 12 % trichloroacetic acid.

[^{3}H]4β-Phorbol 12,13-dibutyrate (PDBu) binding

Macrophage cultures were incubated in the presence or absence of dex as described above. After washing on the second day 5 pmol (approx. 2×10^5 d.p.m.) [^{3}H]PDBu (NEN, Bedford, MA, U.S.A.) was added to each well. A concentration of 3 μM unlabelled PDBu was used for corrrection for non-specific binding of [^{3}H]PDBu. [^{3}H]PDBu was allowed to bind for 2 h at 4 °C. The cells were washed three times with PBS containing 0.1 % BSA. The cells were then scraped off in 1 % Triton X-100 and the radioactivity was determined in the medium and in the cell extract.

[$^{32}PO_4$]Phosphoprotein labelling, electrophoresis and autoradiography

Each culture, in phosphate-free medium, was exposed to $^{32}PO_4$ (50 μCi) for 90 min or 4 h as indicated. Phosphorylation patterns in whole-cell extracts were studied after autoradiography of SDS/PAGE gels (12 % acrylamide), as previously described [6]. After electrophoresis at constant current (16 mA) for about 18 h, the gels were fixed, stained with Coomassie Blue R250, destained and dried under vacuum. The dried gels were exposed to X-ray film (Amersham, International) for time periods ranging from 4 h to 48 h. The intensity of bands on autoradiograms, and Coomassie Blue-stained and dried gels, were quantified using a video densitometer (MAKAB, Gothenburg).

Level and $^{32}PO_4$-labelling of ATP

Macrophage cultures were isolated, cultured and exposed to $^{32}PO_4$ for 4 h as described above. The cells were scraped off the dishes in 40 mM KH_2PO_4, pH 2.8. After sonication for 30 s the homogenate was centrifuged for 45 min at 100000 *g* to obtain a supernatant and a pellet. The supernatant was then analysed by h.p.l.c. [19] using a linear gradient from 40 mM KH_2PO_4, pH 2.8, to 0.5 M KH_2PO_4/0.8 M KCl, pH 2.7. The flow was adjusted to 1.0 ml/min. A radioisotope detector (Model 171 Radioisotope Detector, Beckman Instruments, U.S.A.) was used to determine the $^{32}PO_4$-labelling of ATP while the amount of ATP was determined from the monitored absorbance at 254 nm.

Other methods

Gel chromatography was run on a Sephadex G-200 superfine column as described previously [9]. Protein determination was performed according to Bradford [20], on cell lysates scraped in 0.1 % (w/v) Triton X-100, with the same amount of the detergent also in standard samples.

RESULTS

Dex down-regulated PLA_2-85

Treatment of mouse macrophages with 10 nM dex for 20 h was found to reduce the activity of PLA_2-85 to 36 ± 2 %

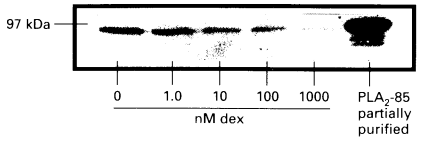

Figure 1 Reduction of the level of PLA_2-85 by dex

Macrophages were cultured for 20 h in the absence or in the presence of dex as indicated. Then whole-cell extracts were used for Western-blot analysis as described in the Experimental section. The right-hand lane is a positive control containing 125 ng of partially purified PLA_2-85. The density of the control culture was set to 100%. After treatment with 1.0, 10, 100 or 1000 nM dex the density of the bands, as revealed by image quantification, corresponded to 75, 37, 23 and 15% of control respectively.

Table 1 Effect of dex on cellular protein and the incorporation of [^{35}S]methionine

Macrophages were treated with dex for 20 h. The protein level in control cells was approx. 4 μg/culture. During the 20 h incubation [^{35}S]methionine was included in the medium for the last 10 h. The radioactivity in the culture medium and the cell extract was determined. In control cells approx. 0.5% of the [^{35}S]methionine added was incorporated. Data are means $\pm$ S.E.M. ($n = 3$–7). N.s.: not significantly different from control (i.e. $P > 0.05$).

Dex (nM)	Cellular protein (% of control)	[^{35}S]Methionine incorporation (% of control)
0.1	97 $\pm$ 3 (n.s.)	93 $\pm$ 4 (n.s.)
1.0	93 $\pm$ 3 (n.s.)	79 $\pm$ 4 ($P < 0.05$)
10	87 $\pm$ 7 (n.s.)	59 $\pm$ 3 ($P < 0.01$)
100	78 $\pm$ 4 ($P < 0.01$)	53 $\pm$ 4 ($P < 0.01$)
1000	83 $\pm$ 5 ($P < 0.02$)	41 $\pm$ 8 ($P < 0.02$)

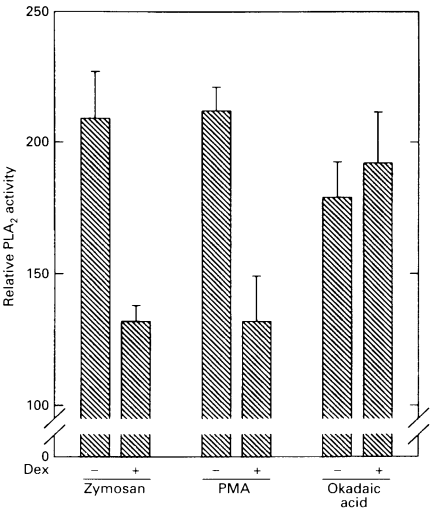

Figure 2 Effects of dex on PMA-, zymosan- or okadaic acid-induced activation of PLA2-85

Macrophages were first cultured for 20 h in the absence or presence of dex (10 nM). The cells were then treated with zymosan (200 μg/ml for 30 min), PMA (100 nM for 15 min) or okadaic acid (0.5 μM for 90 min) as indicated. A cytosolic fraction was prepared and PLA_2-85 was assayed as described in the Experimental section. Please note that the activity of PLA_2-85 in non-stimulated cells, both in control cultures and those down-regulated with dex, has been set to 100%. Data are means $\pm$ S.E.M. ($n = 3$ or 4).

(mean $\pm$ S.E.M., $n = 27$) of control level. A similar reduction of PLA_2-85 was observed by Western blotting (Figure 1), without the appearance of any other immunoreactive protein band. These effects were selective since the total protein content in macrophages preincubated in the presence of 10 nM dex was not significantly reduced (Table 1). A statistically significant, although limited, reduction in total protein level was seen only at $\geqslant 0.1$ μM dex. The effect of dex on the rate of protein synthesis during the latter half of the 20 h period, determined as [^{35}S]methionine incorporation, is also shown in Table 1.

When cytosolic fractions from untreated cultures and from cultures treated with dex (10 nM for 20 h) were subjected to gel chromatography, the activity of PLA_2-85 was found to elute in precisely the same position whether the cells had been treated with dex or not (results not shown). Furthermore, the peak from dex-treated cells showed the same reduction in activity as that determined in samples of unfractionated cytosol. Thus, we found no evidence for any inhibitor(s) that could be separated away by this type of chromatography, nor for higher-molecular-mass complexes dissociating in the enzyme assay. Neither could we find evidence for proteolytic modification of PLA_2-85 into lower-molecular-mass forms with detectable enzyme activity or immunoreactivity.

Dex differentially inhibited the persistent activation of PLA_2-85

Treatment of macrophages with zymosan or PMA in the absence of dex up-regulated the activity of PLA_2-85 to 209 and 212% of control respectively (Figure 2), essentially in agreement with previous findings [9,14]. However, after treatment with dex (10 nM for 20 h), neither zymosan nor PMA was able to up-regulate the activity of PLA_2-85 to any comparable extent. This would indicate either that the down-regulated enzyme was unable to undergo persistent activation, or that the signalling to such activation was also affected by dex.

The protein phosphatase inhibitor okadaic acid has previously been shown to cause phosphorylation and activation of PLA_2-85 in macrophages, as well as release of arachidonate [15]. As shown in Figure 2, okadaic acid was able to cause such activation of PLA_2-85 equally well in dex-treated and untreated macrophages, with an increase to approx. 180% of control. Thus, the relative inability of zymosan and PMA to cause persistent activation of PLA_2-85 in dex-treated cells does not appear to be due to any defect in the down-regulated enzyme, but is more likely to be due to attenuation of the signal for its activation. We therefore decided to investigate whether dex caused any changes in the phosphoprotein pattern in resting macrophages and whether it affected the characteristic changes in this pattern induced by zymosan or PMA [6].

Dex caused a dose-dependent decrease in protein phosphorylation

A rather general reduction of protein phosphorylation after treatment with dex has previously been observed in RBL-2H3 cells [21], but not in HeLa cells [22]. Our autoradiograms clearly showed a general decrease in the $^{32}PO_4$-labelling of phospho-

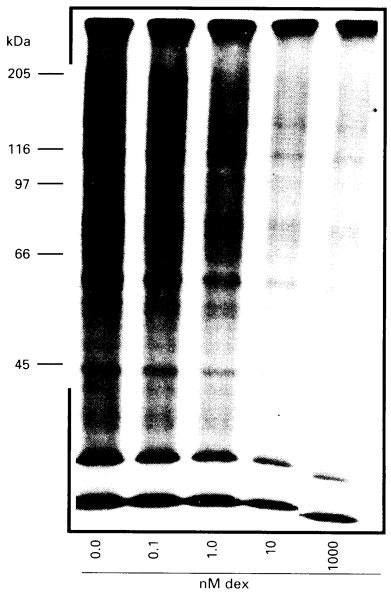

Figure 3 Effect of dex on basal phosphoprotein labelling with $^{32}PO_4$

Dex, as indicated, was included in the culture medium for 20 h. $^{32}PO_4$ (100 μCi) was added to each well for the last 90 min. Cell lysates were then prepared and processed for SDS/PAGE as described in the Experimental section. The experiment shown is representative of three separate experiments.

Table 2 Effect of dex on total ATP content and on [$^{32}PO_4$]ATP

Cells were labelled with $^{32}PO_4$ for 4 h (see the Experimental section). H.p.l.c. was performed on cell extracts from control cells and cells treated with dex for 20 h. Total ATP in the cytosolic fraction was determined as absorbance at 254 nm. Data are means $\pm$ S.E.M. ($n = 3$). Abbreviation: n.s., not significantly different from control (i.e. $P > 0.05$).

Dex (nM)	Total ATP (% of control)	[^{32}P]ATP (% of control)
0.1	98 $\pm$ 3 (n.s.)	86 $\pm$ 1 ($P < 0.01$)
1.0	100 $\pm$ 9 (n.s.)	73 $\pm$ 4 ($P < 0.05$)
10	101 $\pm$ 8 (n.s.)	67 $\pm$ 2 ($P < 0.01$)
100	92 $\pm$ 10 (n.s.)	53 $\pm$ 2 ($P < 0.01$)
1000	88 $\pm$ 3 (n.s.)	49 $\pm$ 5 ($P < 0.01$)

proteins in resting macrophages as the concentration of dex was increased (Figure 3). However, analysis of the level and $^{32}PO_4$-labelling of ATP showed that the latter was reduced to a similar extent by dex (Table 2), while the level was hardly affected at all. This may explain the general reduction of phosphoprotein labelling in dex-treated cells. Furthermore, both the difference in labelling of ATP and the difference in basal phosphoprotein labelling could be abrogated by extending the labelling time with $^{32}PO_4$ for dex-treated cells from 90 min to 4 h (see Figure 4). In agreement with previous results [6], increased phosphorylation of

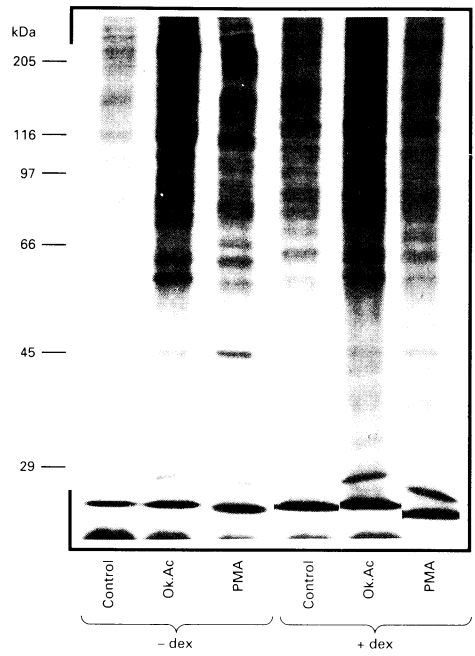

Figure 4 Effect of dex on the changes in phosphoprotein labelling induced by PMA and okadaic acid

Macrophages were cultured for 20 h in the absence (left three lanes) or presence (right three lanes) of dex (10 nM) and were labelled with $^{32}PO_4$ (50 μCi) for the last 90 min (left three lanes) or 4 h (right three lanes) respectively. The cells were treated with PMA (100 nM for 15 min) or okadaic acid (0.5 μM for 90 min) as indicated, during the latter part of the labelling period. Cell lysates were then prepared and processed for SDS/PAGE as described in the Experimental section. Densitometric analysis of a number of bands on autoradiograms showed that PMA increased their labelling 2.5- to 5.0-fold (minus dex, 90 min labelling), and 1.1- to 1.6-fold (plus dex, 4 h labelling) respectively. Okadaic acid enhanced the labelling of the corresponding bands 3.2- to 6.0-fold (minus dex) and 4.5- to 7.2-fold (plus dex) respectively. The experiment shown is representative of three separate experiments, all of which were subjected to densitometric analysis.

many protein bands, including a prominent 45 kDa band, was seen in cells stimulated with zymosan (results not shown) or PMA (Figure 4), while in dex-treated cells (10 nM dex for 20 h) stimulation with PMA resulted in a much smaller effect (Figure 4). In accordance with results obtained by Haystead et al. [23] an increase in the phosphorylation of many bands was observed after treatment with 0.5 μM okadaic acid for 90 min. Furthermore, the phosphorylation of a band at approx. 60 kDa appeared to be selectively enhanced after treatment with okadaic acid. Addition of okadaic acid after preincubation with dex (10 nM, 20 h) resulted in a similar increase in phosphorylation as that obtained in untreated cells (Figure 4).

The dex-induced reduction in phosphorylation of the 45 kDa band and other bands in response to PMA raised the question of whether dex caused down-regulation of phorbol-ester-sensitive isoforms of PKC. However, the specific binding of [^{3}H]PDBu decreased only to about the same degree as the total protein level

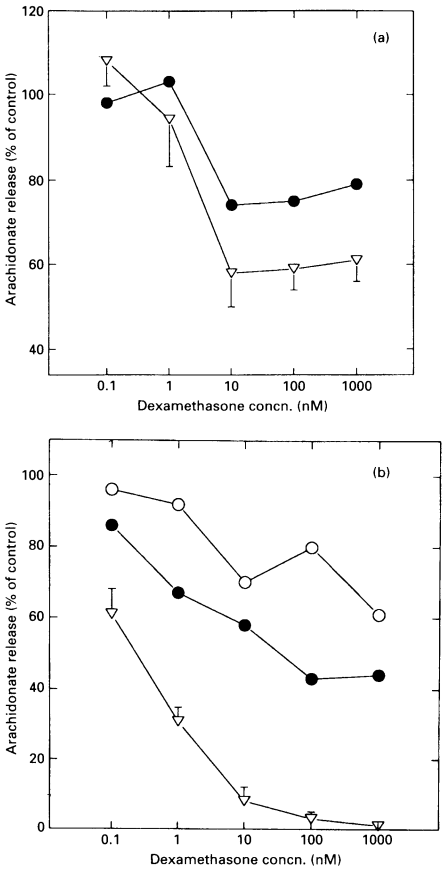

Figure 5 Effect of dex on stimulus-induced release of [^{3}H]arachidonate

Dex, as indicated, was included in the culture medium for 4 (○), 12 (●) or 20 (▽) h. The cells were then stimulated with (**a**) A23187 (1 μM) for 30 min, or (**b**) zymosan (200 μg/ml) for 30 min. The results are means (+S.E.M. where indicated) from 3–9 independent experiments. The net release in cells not treated with dex was set to 100%. It amounted to 19.5 ± 0.7 ($n = 13$) percent of total cell-associated [^{3}H]arachidonate in the case of A23187, and 16.7 ± 1.2 ($n = 7$) in the case of zymosan.

upon treatment with dex (78% of control at 10 nM dex for 20 h and 74% of control at 1.0 μM dex for 20 h; $n = 2$). This modest decrease in specific binding of [^{3}H]PDBu argues against the possibility that down-regulation of PKC was the cause of the reduction in protein phosphorylation.

Dex differentially inhibited stimulus-induced release of arachidonate

Finally we studied the effect of dex on the release of [^{3}H]arachidonate in prelabelled macrophages stimulated with the calcium ionophore A23187, zymosan (Figure 5) or okadaic acid. After prolonged incubation with dex ($\geq$ 10 nM), a partial inhibition of the ionophore-induced arachidonate release became evident (Figure 5a). However, the inhibition did not exceed 50% even after 20 h of treatment with dex. In contrast, dex proved to be much more efficient at inhibiting zymosan-induced arachidonate release (Figure 5b). Treatment with dex (10 nM–1.0 μM) for 20 h resulted in 90–98% inhibition of the zymosan-induced release of arachidonate. This inhibition developed progressively with time, being limited at 4 h and approx. half-maximal at 12 h. Macrophages were also treated with okadaic acid (0.5 μM) for 90 min after 20 h incubation with or without increasing concentrations of dex. A partial inhibition of the okadaic acid-induced release of arachidonate was observed, which amounted to 50% at 10 nM dex and approx. 65% at 0.1–1.0 μM dex.

DISCUSSION

One of the findings presented here is that the synthetic glucocorticoid dex down-regulates PLA_2-85 in resting mouse macrophages. By a combination of Western blotting analysis and activity measurements we could virtually exclude that this was due to either proteolytic modification of the enzyme, or to induction of an inhibitory protein. Neither was it paralleled by any similar reduction in total cellular protein. It was also, from the outset, unlikely that it could be caused by a reduction in the activity of PLA_2-85 due to dephosphorylation of the enzyme, as *in vitro* dephosphorylation of PLA_2-85 from unstimulated macrophages causes a much smaller reduction in activity [14]. Instead, it may be due to inhibition of the formation of PLA_2-85 at the transcriptional or post-transcriptional level, or an enhanced degradation. These possibilities need to be further investigated.

In favour of regulation at the level of biosynthesis are the recent findings that PLA_2-85 can be induced by cytokines such as tumour necrosis factor-α (TNF-α) [22,24], interleukin-1α (IL-1α) [25], IL-1β [26–28], transforming growth factor β2 (TGFβ2) [26] and macrophage colony-stimulating factor (M-CSF) [29], and that, at least in the cases of TNF-α, IL-1α and IL-1β, this induction can be abolished by dex [22,24,25,28]. However, in contrast to our findings in primary macrophage cultures, the basal level of PLA_2-85 was only slightly, or not at all, reduced by dex in the cell lines, or the mesangial cells, employed in these studies.

The release of arachidonate induced by ionophore A23187, zymosan or okadaic acid was inhibited to different degrees by the 20 h pretreatment with dex. Besides the effect on the level of PLA_2-85 and effects on its persistent activation (see below), dex could also cause apparent inhibition of arachidonate release by exerting inhibitory effects on the cyclo-oxygenase and/or 5-lipoxygenase pathways of arachidonate metabolism, or by enhancing reacylating mechanisms. However, it is well known that ionophore A23187 and zymosan both yield prostaglandin E_2 and leukotriene C_4 as major eicosanoids in mouse peritoneal macrophages, while PMA causes selective formation of cyclo-oxygenase metabolites, primarily prostaglandin E_2 [30,31]. We found also that okadaic acid caused selective formation of prostaglandin E_2 and that dex treatment (10 nM for 20 h) inhibited arachidonate release and prostaglandin E_2 formation to a similar extent (by 50 and 66% respectively). Thus, we find no simple relationship between the differential inhibition of arachidonate release caused by dex and possible inhibitory effects on either the cyclo-oxygenase or the 5-lipoxygenase pathways. Nor would we expect that a dex-dependent enhancement of arachidonate reacylation could lead to such differential effects.

The partial inhibition of the calcium-ionophore-induced release of arachidonate after treatment of macrophages with dex

might instead primarily reflect the reduced level of PLA_2-85. Ca^{2+} can bind *in vitro* to PLA_2-85 and cause its translocation to membranes [11–13] and the ionophore A23187 can, in macrophages, cause arachidonate release without a significant persistent activation of PLA_2-85 (J. Wijkander and R. Sundler, unpublished work). On the other hand, the release of arachidonate induced by zymosan is virtually inhibited by prior treatment with dex. By *in vitro* assay of PLA_2-85 we provide evidence that the rapid persistent activation of PLA_2-85 that normally occurs in response to zymosan [14] is impaired in dex-treated macrophages. The up-regulation induced by PMA is similarly impaired. This is consistent with the earlier finding that zymosan-induced arachidonate release is largely, although not exclusively, dependent on phorbol-ester-sensitive isoforms of PKC [6].

It has recently been demonstrated that the rapid up-regulation of PLA_2-85 in response not only to zymosan or PMA [14] and bacteria [15] in macrophages, but also to other agonists in other cells [32,33], is due to regulatory phosphorylation of the enzyme. It was also recently demonstrated that the protein Ser/Thr phosphatase inhibitor okadaic acid, with selectivity for phosphatases of type 1 and 2a, itself caused enhanced phosphorylation and up-regulation of the activity of PLA_2-85, as well as arachidonate release, in macrophages [15]. It is therefore of interest that this effect on PLA_2-85, as shown here, was not impaired by prior treatment with dex. This suggests either that the dex-induced attenuation of signalling to persistent activation of PLA_2-85 is overcome by the phosphatase inhibitor, or that its site of action is downstream of the dex-sensitive step(s).

Finally we should emphasize that both effects of dex demonstrated here, i.e. on the level of PLA_2-85 and on the signalling for its activation, are slow in onset and take several hours to become discernible. This could be one reason why no immediate effect of dex on TNFα-induced phosphorylation and activation of PLA_2-85 was observed in HeLa cells [22].

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II

Multiple C-terminal serine phosphorylation accompanies both protein kinase C-dependent and -independent activation of cytosolic 85 kDa phospholipase A_2 in macrophages

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Exposure of mouse macrophages to either phorbol ester or certain bacteria was previously shown to cause increased phosphorylation of the cytosolic 85 kDa phospholipase A_2 as well as a stable increase in its catalytic activity. We have now attempted to map the major phosphorylation sites on the enzyme in such cells. Phosphorylation occurred on serine residues without a detectable increase in either phosphothreonine or phosphotyrosine. After CNBr cleavage five fragments showed increased ^{32}P labelling. Among those the most heavily labelled fragment was identified as the most C-terminal (residues 698–749), containing six serine residues. This was true whether phorbol ester or bacteria, causing protein kinase C-independent phospholipase A_2 activation, was used as stimulus. The heavy phosphorylation of the most C-terminal fragment and an analysis of tryptic peptides derived from it suggested that more than one of the six serine residues became phosphorylated. Smaller increases also occurred in other CNBr-cleaved fragments from the C-terminal part of the protein, including that carrying Ser-505, a known target of the mitogen-activated protein kinase ERK-2 (extracellular-signal regulated kinase). Dexamethasone treatment (1–100 nM for 20 h), which was earlier shown to dose-dependently down-regulate the 85 kDa phospholipase A_2 and its activation by phorbol ester and zymosan, was here shown also to counteract the protein kinase C-independent activation and arachidonate release elicited by bacteria. It remains to be determined whether all phosphorylation sites are equally affected under those conditions.

INTRODUCTION

The earliest step in the arachidonate cascade is that in which arachidonate is mobilized from its stores in membrane phospholipids. In macrophages there is considerable evidence [1–5] that this step is executed by the intracellular 85 kDa arachidonate-preferring phospholipase A_2 (PLA_2-85) [6–8]. This enzyme can be regulated by changes in $[Ca^{2+}]$ at submicromolar level [2,7,9–12] and by changes in phosphorylation [2–4,13,14]. Our earlier studies have shown that the latter activation can be achieved via one or more phorbol ester-sensitive isoforms of protein kinase C [1,2,15,16]. Evidence was also provided that the regulatory phosphorylation(s) is/are not performed directly by such a kinase [7]. Furthermore a signalling pathway independent of protein kinase C, by which certain bacteria cause increased phosphorylation and activation of PLA_2-85, was also demonstrated in macrophages [4]. In other cell types, too, signalling initiated by agents such as tumour necrosis factor α [17], thrombin [14] and epidermal growth factor [18] have been shown to lead to increased phosphorylation and activation of PLA_2-85; studies *in vitro* as well as in a transfected cell line have suggested that phosphorylation of Ser-505 by the mitogen-activated protein (MAP) kinase ERK-2 (extracellular-signal regulated kinase) accounts for this activation (reviewed in [19]).

The present report identifies serine residues in the C-terminal region of PLA_2-85, distinct from Ser-505, as being the most heavily phosphorylated in macrophages responding to either phorbol ester or bacteria. It is also demonstrated by present and previous [20] data that dexamethasone treatment attenuates or abolishes arachidonate release and PLA_2-85 activation elicited by both types of agent.

EXPERIMENTAL

Isolation, culture and ^{32}P-labelling of macrophages

Resident macrophages were isolated from peritoneal cells obtained from female outbred NMRI mice (ALAB, Stockholm, Sweden, or Bommice, Copenhagen, Denmark) by adherence to plastic 80 cm^2 tissue culture flasks, with approx. 3.6×10^7 peritoneal cells per flask. The cells were incubated in an atmosphere of air/CO_2 (19:1); non-adherent cells were removed 2 h after plating, as described previously [15]. To each flask was added 10 ml of Medium 199 containing 10% (v/v) heat-inactivated fetal bovine serum, and the cells were cultured for 18–20 h. The cells were thereafter washed with Dulbecco's PBS and equilibrated for 30 min in 10 ml of serum-free Medium 199, before being washed and transferred to phosphate-free medium [16] (10 ml per flask). After the addition of $^{32}PO_4$ (30 mCi per flask), labelling was continued for 90 min. Where indicated, cultures were exposed during the latter part of the labelling period to either PMA (150 nM for 10 min) or bacteria (10^7/ml for 60 min), specified in each case and obtained as described previously [4]. At the end of the experiment the cells were washed five times with Dulbecco's PBS and were then scraped off and homogenized in

Abbreviations used: MAP kinase, mitogen-activated protein kinase/microtubule-associated protein 2 kinase (p44/p42; ERK-1/ERK-2); PLA_2-85, 85 kDa cytosolic phospholipase A_2 (also called $cPLA_2$).

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buffer consisting of 80 mM KCl, 2.5 mM dithioerythritol, 1 mM EDTA, 10 mM Hepes, 10 mM NaF and 100 μM ammonium vanadate, pH 7.4 (buffer A).

Cell fractionation and isolation of PLA_2-85

Homogenate from two flasks was combined and centrifuged at 700 *g* for 10 min and the resulting supernatant was further centrifuged at 100000 *g* for 60 min to obtain a cytosol fraction and a membrane pellet. To the cytosol fraction was then added 10% (v/v) glycerol. The cytosol fraction was run on either a column (1 cm × 48 cm) of Sephadex G-200 superfine (Pharmacia), equilibrated in buffer B [buffer A containing 10% (v/v) glycerol but lacking dithioerythritol], or a Mono Q HR 5/5 column (Pharmacia), eluted with a non-linear gradient of NaCl (0–0.4 M) in buffer B. Aliquots of the cytosol fraction or of fractions from the gel, or ion-exchange, chromatography were assayed for PLA_2-85 activity (see below). Fractions containing PLA_2-85 were combined and subjected to hydrophobic interaction chromatography on a phenyl-Superose HR 5/5 column (Pharmacia), as described [7]. In this and the preceding chromatographic step, tubes used to collect fractions were first filled and preincubated with a solution of BSA (1 mg/ml) for 20 h; 0.1 mg of albumin was then added to each tube before use, to minimize losses of PLA_2-85 by adsorption. The recovery of PLA_2-85 enzyme activity was at least 80% in each of the chromatographic steps. From relevant fractions after the hydrophobic interaction chromatography aliquots were taken for analysis by SDS/PAGE [6.5% (w/v) acrylamide gel] [21] and autoradiography, to assess the phosphoprotein purity of PLA_2-85 (see Figure 1, upper panel), while the major part of the fractions were combined and subjected to precipitation with trichloroacetic acid.

Separation and analysis of CNBr-cleaved fragments from PLA_2-85

Non-labelled PLA_2-85, isolated from J774 cells as described previously [7], was cleaved by treatment with CNBr [1% (w/v) CNBr in formic acid/water (7:3, v/v) for 20 h]. Fragments were separated by HPLC (System Gold, Beckman) in reverse-phase mode on a 4 mm × 250 mm LiChrocart RP-18 column (Merck) with a linear gradient (0–70%) of acetonitrile in trifluoroacetic acid/water (1:2000, v/v). After 10 initial fractions of 0.9 ml had been collected, the fraction volume was 0.3 ml. The N-terminal sequence of a number of separated peptides was then determined by automated sequential Edman degradation (BM unit, Lund University, Lund, Sweden) to identify their position within the protein. Some of these sequences have been briefly reported earlier [7]. Trichloroacetic acid-precipitated, ^{32}P-labelled PLA_2-85 from macrophages, carried through the two-step purification procedure described above, was also cleaved and the fragments were separated by the same procedure. ^{32}P-labelled CNBr fragments were further characterized by SDS/PAGE [16% (w/v) acrylamide gel] followed by phosphorimager analysis (FUJI Bas 2000) or autoradiography. The recovery of trichloroacetic acid-precipitable ^{32}P through CNBr cleavage and reverse-phase HPLC was 60–75%.

Analysis of tryptic peptides and phospho amino acids

Tryptic peptides were prepared from some of the ^{32}P-labelled CNBr fragments by treatment with trypsin [80 μg in 0.1 M NH_4HCO_3/acetonitrile (8:2, v/v) for 20 h at 37 °C]. These peptides were separated by reverse-phase HPLC as described above (except that fractions of 0.5 ml were collected throughout) and their ^{32}P content was determined. The recovery of radioactivity was at least 80%.

For identification of phospho amino acids, trichloroacetic acid-precipitated, ^{32}P-labelled PLA_2-85 (with added phosphoserine, phosphothreonine and phosphotyrosine) was hydrolysed in 6 M HCl at 105 °C for 2.5 h, under vacuum. After freeze-drying, separation was achieved by isocratic HPLC on a strong-anion-exchange column (4 mm × 250 mm Nucleosil 5SB, Macherey-Nagel), with 40 mM KH_2PO_4, pH 2.8, as eluent. This is a modification of a method described previously [22] and led to excellent separation not only of the three phospho amino acids, but also between each of them and PO_4 (see Figure 1, lower panel).

Assay of PLA_2-85

PLA_2-85 was assayed by the method described earlier [2]. 1-Stearoyl-2[1-^{14}C]arachidonoyl phosphatidylcholine (Amersham International; 80–100 pmol per incubation) was used as substrate. Aliquots of the cytosol fraction containing PLA_2-85 were incubated in the presence of substrate, 1.4 mM $CaCl_2$ (approx. 400 μM free Ca^{2+}) and BSA (0.19 mg/ml) in a buffer containing 80 mM KCl, 10 mM Hepes and 1 mM EDTA, pH 7.4. The final volume was adjusted to 525 μl. After incubation for 30 min at 37 °C the reaction was stopped by the addition of 1.5 ml of chloroform/methanol/HCl (2:1:0.1, by vol.), to which 0.05% carrier lipids had been added. After centrifugation the lipid phase was applied to a silicic acid column equilibrated in chloroform. Non-esterified fatty acids were eluted with 1.0 ml of chloroform, and phospholipids with 2.5 ml of methanol.

Release of [^{3}H]arachidonate

During an overnight incubation of approx. 3×10^6 peritoneal macrophages per well, in 6-well 35 mm culture dishes, they were labelled with 0.5–1.0 μCi [^{3}H]arachidonic acid (Amersham International; 212 Ci/mmol) per well and, when indicated, dexamethasone was included in the medium. At the end of the experiment the culture medium was collected and the cells were scraped off the dish in 0.1% (w/v) Triton X-100. The collected medium and the cell extract were centrifuged for 5 min at 2500 *g*. The release of incorporated [^{3}H]arachidonate was determined from the radioactivity in the culture medium and the total radioactivity in the cell extract and medium, and was corrected for the basal release in control cultures. Dexamethasone by itself showed no detectable effect on the incorporation of [^{3}H]arachidonate or the low (at most 2%) basal release in unstimulated cultures. Protein was determined by the method of Bradford [23], with BSA as standard.

RESULTS AND DISCUSSION

It was demonstrated previously that short-term treatment of macrophages with either PMA or zymosan leads to persistent activation of PLA_2-85 as well as an increase in its phosphorylation. Both of these effects could be reversed by phosphatase treatment of the enzyme, strongly indicating that the increase in phosphorylation was responsible for the increase in catalytic activity [2]. It was also shown in the same study that PLA_2-85 can be obtained by a two-step purification procedure as a virtually homogeneous phosphoprotein from a cytosolic fraction of ^{32}P-labelled peritoneal macrophages [2]. Figure 1 (upper panel) also confirms that the modified procedure employed in most of the current experiments yielded a single radiolabelled phosphoprotein, co-eluting with PLA_2 activity (results not shown) and co-migrating exactly with PLA_2-85 on SDS/PAGE as a 100 kDa protein.

Phospho amino acid analysis of PLA_2-85 from ^{32}P-labelled,

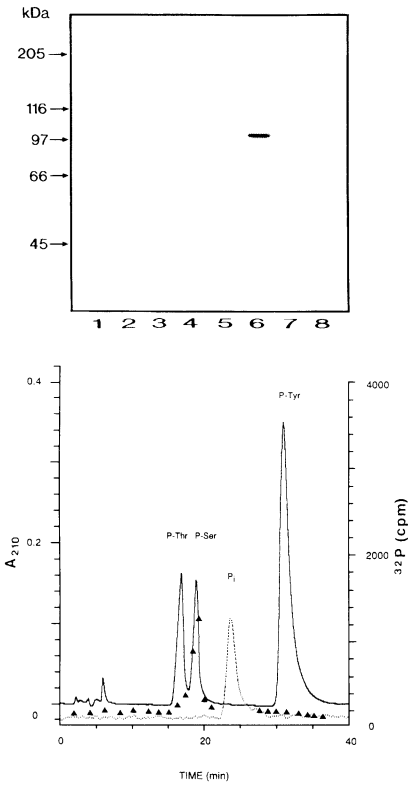

Figure 1 Phosphoprotein purity and phospho amino acid analysis of PLA_2-85

Upper panel: PLA_2-85 was purified from cytosol fractions prepared from untreated (lanes 1–4) or PMA-stimulated (lanes 5–8), ^{32}P-labelled macrophage cultures, by consecutive Mono Q and phenyl-Superose chromatography (see the Experimental section). Four consecutive phenyl-Superose fractions, containing the peak of PLA_2-85 activity, were analysed by SDS/PAGE [6.5% (w/v) acrylamide gel] and autoradiography. Lower panel: a mixture of phosphothreonine (P-Thr), phosphoserine (P-Ser), phosphotyrosine (P-Tyr) and [^{32}P]P_i (P_i) was subjected to isocratic ion-exchange HPLC as described in the Experimental section. Absorbance at 210 nm (solid line) and radioactivity (dotted line) were monitored continuously. Radioactivity data for collected fractions from a second run, where the sample contained phospho amino acid standards and a hydrolysate of isolated PLA_2-85 from ^{32}P-labelled and PMA-stimulated macrophages, are shown superimposed (▲).

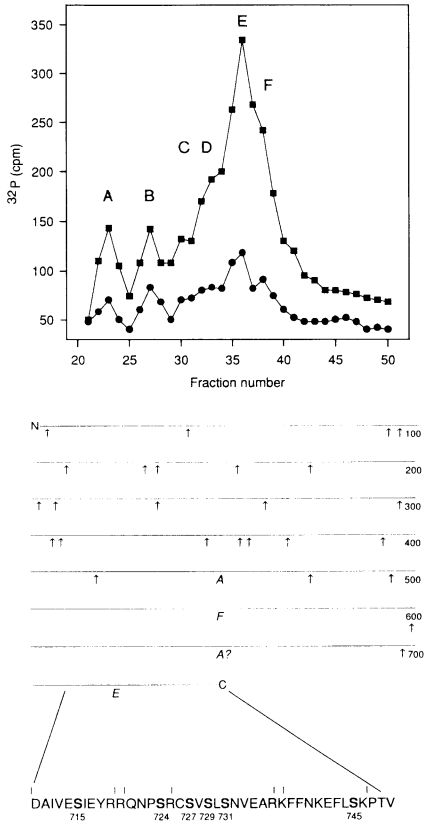

Figure 2 ^{32}P content of CNBr fragments from PLA_2-85 and their positions in the linearized protein

Upper panel: PLA_2-85 was isolated from ^{32}P-labelled control (●) or PMA-stimulated (■) macrophages as described in the Experimental section. After CNBr cleavage fragments were separated by reverse-phase HPLC. Peaks are labelled A–F. Lower panel: a schematic representation of the primary structure of mouse PLA_2-85 [6] indicating CNBr cleavage sites (arrows). The labelling of some of the CNBr fragments (A, E, F) corresponds to peaks in the upper panel. The amino acid sequence of most of fragment E, encompassing the six C-terminal serine residues, which constitute major phosphorylation sites, is also depicted. Here the bars indicate tryptic cleavage sites. Residues 698–709 contain no serine and were therefore omitted from the lower panel.

PMA-stimulated cells showed that phosphorylation occurred on serine residues, with no detectable labelling of either phosphotyrosine or phosphothreonine residues (Figure 1, lower panel). To gain information on the number of phosphorylation sites and their localization, CNBr fragments from PLA_2-85 were prepared. From the amino acid sequence of the mouse enzyme [6] we can deduce that 25 fragments and one free serine residue would be formed. Of the fragments, 18 would carry one or more serine residues. Two of these fragments, both from the C-terminal part, consist of approx. 100 amino acid residues, whereas all others would be 53 residues long or less. CNBr fragments of PLA_2-85 isolated from J774 cells and from ^{32}P-labelled macrophages were both separated identically. The former were identified by N-terminal sequencing; the latter were analysed by SDS/PAGE or subjected to further cleavage.

As shown in Figure 2 (upper panel), six ^{32}P-labelled peaks were more or less well resolved by reverse-phase HPLC. Peak E was by far the largest in terms of ^{32}P content, but seemed unusually broad for a single homogeneous fragment. However, N-terminal sequencing revealed that the most C-terminal fragment in PLA_2-85 (residues 698–749) eluted broadly over several fractions and in

Table 1 SDS/PAGE and phosphorimager analysis of CNBr fragments

Selected fractions from the chromatography shown in Figure 2 (upper panel), in which CNBr fragments from ^{32}P-labelled PLA_2-85 from control and PMA-stimulated cultures were separated, were analysed by SDS/PAGE [16% (w/v) acrylamide gel] as described in the Experimental section; the gels were then dried and subjected to phosphorimager analysis. The apparent size of bands in the peak fractions, as judged from their electrophoretic mobility, were: Fraction 23 (lower band), 4 kDa; (upper band), 12 kDa; fraction 27, 6.5 kDa; fraction 36, 4.5 kDa; fraction 38, 15 kDa. Data show band intensities (arbitrary units) and represent means ± S.E.M. ($n = 3$).

			Band intensity (arbitrary units)	
Peak	Fraction	Apparent size (kDa)	Control	PMA
A	23 (lower)	4	25 ± 1	94 ± 6
	23 (upper)	12	90 ± 20	60 ± 1
B	27	6.5	16 ± 10	129 ± 22
E	36	4.5	75 ± 13	703 ± 98
F	38	15	148 ± 16	395 ± 11

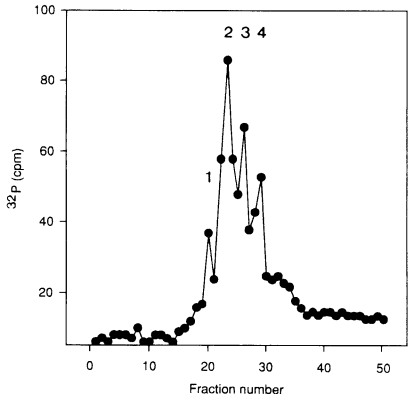

Figure 3 Analysis of ^{32}P-containing tryptic peptides from the most heavily labelled CNBr fragment

Tryptic peptides were prepared from fractions 34–36 [peak E, Figure 2 (upper panel); PMA-stimulated sample] and were then separated by reverse-phase HPLC as described in the Experimental section. Peaks obtained are numbered 1–4. The results shown are representative of three separate experiments.

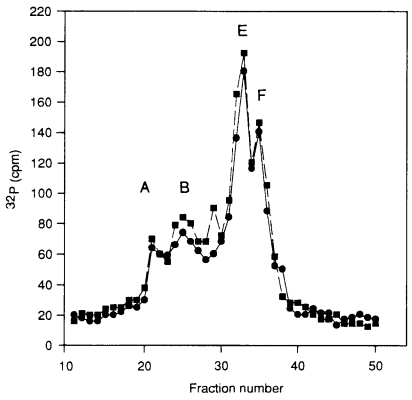

Figure 4 A comparison of protein kinase C-dependent and -independent phosphorylation patterns of PLA_2-85

PLA_2-85 was isolated from ^{32}P-labelled macrophages stimulated with either PMA (■, broken line) or bacteria (*G. vaginalis*) (●, solid line), as described in the Experimental section. After CNBr cleavage fragments were separated by reverse-phase HPLC. Peaks are labelled as in Figure 2.

precisely the same position as peak E. Furthermore peak F was found to co-elute precisely, and as a sharp peak, with one of the two big CNBr fragments from the C-terminal part (residues 494–598). Sequence data also showed that one additional fragment from the C-terminal part of PLA_2-85 (residues 418–467) co-eluted with peak A. This is indicated schematically in Figure 2 (lower panel).

The separated peaks of CNBr fragments (Figure 2, upper panel) were then analysed by SDS/PAGE and phosphorimager analysis (Table 1). Peak A was found to contain two fragments differing in size, whereas peaks B, E and F showed one predominant band each. The size of the most C-terminal fragment (peak E; 6 kDa according to amino acid sequence) corresponded quite well to the migration of the major ^{32}P-labelled band on the gel, whereas the fragment in peak F migrated slightly more slowly than its expected size (12 kDa). The faster-moving band in peak A is very likely to represent fragment 418–467, in accordance with sequence data, whereas the slower-moving band is suspected, but not proved, to be fragment 600–696, the only additional fragment of appropriate size (Figure 2, lower panel). The former increased somewhat in response to PMA, whereas the latter band showed no increase in phosphorylation (Table 1). It is also evident that the most pronounced increases occurred in peaks B and E. Peak B presented as a single band whose identity is uncertain, whereas the sometimes less distinct and also less reproducible peaks C and D showed no distinct bands in the appropriate size range, and therefore might be the result of incomplete CNBr cleavage.

Peak E, containing the C-terminal fragment (698–749), was further cleaved by trypsin treatment. Such treatment would be expected to generate four serine-containing tryptic peptides (Figure 2, lower panel), three of which would contain a single serine residue and the fourth would carry three such residues. Analysis of the tryptic peptides led to the separation of four ^{32}P-labelled peaks, one of which was clearly more heavily labelled than the others (Figure 3). This would be consistent with the phosphorylation of more than one of the six C-terminal serine residues in PLA_2-85 (Figure 2, lower panel).

So far, only the phosphorylation pattern associated with protein kinase C-mediated activation had been investigated. Because certain bacteria, on interaction with macrophages, cause the activation of PLA_2-85 via signalling independent of phorbol ester-sensitive isoforms of protein kinase C [4], we decided to compare the phosphorylation pattern elicited by such activation with that elicited by PMA. Stimulation by either a Gram-positive (*Gardnerella vaginalis*) or a Gram-negative (*Fusobacterium nucleatum*) bacterial species was found to give results very similar to those obtained with PMA; i.e. only serine phosphorylation could be demonstrated, and the C-terminal CNBr fragment (peak E) showed the same predominance with respect to ^{32}P labelling (Figure 4). Thus great similarity in ^{32}P-labelling of

Table 2 Inhibition by dexamethasone of the activation of PLA_2-85 in response to bacteria

Macrophages cultured for 20 h in the presence or absence of 10 nM dexamethasone (Dex) were exposed to bacteria (10^7/ml) for 45 min. After the removal of medium, cells from sets of three dishes were combined, homogenized and fractionated into a membrane pellet and a cytosol fraction. The cytosol fraction was then assayed for PLA_2-85 activity and the protein content was determined as described in the Experimental section. PLA_2-85 activity in non-treated cells (control, −Dex), which amounted to 27.6 ± 2.9 pmol/30 min per mg of protein, was set to 100% and the results represent means ± S.E.M. for four separate experiments.

	PLA_2-85 activity (% of control)	
	−Dex	+Dex
Control	100	36 ± 14
F. nucleatum	166 ± 23	38 ± 16
P. acnes	175 ± 19	34 ± 4

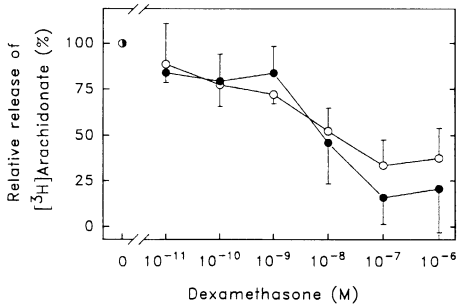

Figure 5 Dose-dependent inhibition by dexamethasone of arachidonate release in response to bacteria

Macrophages were incubated with dexamethasone for 18–20 h and were then exposed to either *F. nucleatum* (●) or *P. acnes* (○) at 10^7 bacteria/ml for 60 min. The release of radiolabel, corrected for the release in control cultures not exposed to bacteria, is expressed as a percentage of the release in the absence of dexamethasone. The latter amounted to approx. 15% of total cellular ^3H, or 21200 ± 2800 d.p.m./mg of protein (mean ± S.E.M.; $n = 7$). The results shown are means with positive or negative S.E.M. for seven separate experiments.

CNBr fragments in cells responding to PMA and bacteria respectively was a striking feature despite the clear difference in dependence on protein kinase C. This of course raises the possibility of converging signal chains. Additional evidence, consistent with such converging chains, was obtained by using macrophages pretreated for 20 h with dexamethasone. We have previously shown that this treatment not only down-regulates the PLA_2-85 protein but also greatly impairs its protein kinase C-mediated activation [20], with only very minor effects on the phorbol ester-binding isoforms of this kinase. Table 2 shows that the protein kinase C-independent activation elicited by bacteria was also severely inhibited. Furthermore, as shown in Figure 5, treatment with dexamethasone markedly and dose-dependently reduced the release of arachidonate elicited from intact cells by both a Gram-negative (*F. nucleatum*) and a Gram-positive (*Propionibacterium acnes*) bacterial species. These findings therefore suggest that the target for dexamethasone might be a downstream event involved in both PMA- and bacteria-induced signalling.

Previous studies have led to the proposal that Ser-505, surrounded by a consensus sequence for the p42/p44 proline-directed MAP kinases, would constitute a critical phosphorylation site for the regulation of PLA_2-85 [24,25]. Ser-505 is located in peak F, as one of 11 serine residues in this fragment. The same fragment also contains Ser-542, which is surrounded by basic residues and therefore is a potential substrate for other kinases such as protein kinase C. Peak F does indeed show increased labelling in PMA-stimulated cells, but it is also labelled in resting cells. Leslie and co-workers [3] previously provided evidence for multiple phosphorylation sites on PLA_2-85 in mouse macrophages, findings that are fully compatible with the results presented here. However, no information was provided in the earlier study [3] with regard to the localization of the sites. Our results indicate that the most prominent phosphorylation events occur among the six C-terminal serine residues. In addition the other fragments that we have identified and that show increased ^{32}P labelling in stimulated cells emanate from the C-terminal half of the protein. Recently, serine residues were mapped that show increased phosphorylation in response to the protein phosphatase inhibitor okadaic acid in recombinant human PLA_2-85 expressed in insect cells [26]. They were identified as Ser-437, Ser-454, Ser-505 and Ser-727. Interestingly, all of them are on CNBr fragments that in our study on mouse macrophages showed increased ^{32}P labelling in response to agonist stimulation; i.e. Ser-437 (peak A), Ser-505 (peak F) and Ser-727 (peak E). Ser-454 is not present in the mouse enzyme. Finally, whereas a severalfold increase in the phosphorylation of a single serine residue (Ser-727) among the six most C-terminal ones was reported for the recombinant human enzyme [26], our results suggest that several of these residues act as phosphorylation sites in mouse macrophages (compare Figures 2 (lower panel) and 3).

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III

Effects of dexamethasone on ERK, JNK and p38 MAPK in mouse macrophages. Implications for the regulation of 85 kDa cytosolic phospholipase A_2.

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In mouse macrophages arachidonate mobilisation in response to several stimuli is severely inhibited by prolonged (16-20 h) treatment with nanomolar dexamethasone (dex). It was earlier shown that this inhibition was accompanied by a dual effect on $cPLA_2$; down-regulation of the enzyme protein and inhibition of its activation. We now report, by the use of cycloheximide that a dose of this protein synthesis inhibitor could be titrated that caused an almost complete reversion of the inhibitory effects of dex. These results indicate that the effects depend on new protein synthesis, in consistency with other data indicating that the effects are mediated via the glucocorticoid receptor. Northern blot results showed pronounced down-regulation of $cPLA_2$ also at the level of its mRNA.

The possibility that dex targeted the level or activation of one or more of the three mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), p38 or c-Jun N-terminal kinase (JNK), was also addressed. While the level of these MAPKs and their phorbol myristate acetate (PMA)-induced activation were unaffected by dex there was a partial inhibition of their zymosan-induced activation. However, this inhibition was not as pronounced as the dex-mediated inhibition of $cPLA_2$ activation. These data were confirmed by western blot using antibodies against the phosphorylated forms of ERK, p38 and JNK. The results suggest that dex-mediated inhibition of PMA-induced $cPLA_2$ activation is exerted down-stream of the MAPK, while the partial inhibition of the zymosan-induced activation may be explained by effects exerted more up-stream. Thus, the MAPKs investigated here do not appear to be main targets for the inhibitory effects of dex on $cPLA_2$ activation.

Abbreviations: dex, dexamethasone; **cPLA$_2$:** 85 kDa cytosolic phospholipase A$_2$, **chx:** cycloheximide, **ERK**: extracellular signal-regulated kinase, **JNK**: c-Jun N-terminal kinase, **MAPK**: mitogen-activated protein kinase, **PMA**: phorbol myristate acetate, **SDS-PAGE**: sodium dodecyl sulphate polyacrylamide gel electrophoresis

INTRODUCTION

The 85 kDa cytosolic phospholipase A$_2$ (cPLA$_2$) is constitutively expressed in most cell types studied and has been found to be important for stimulus-induced release of arachidonate in macrophages [1-2]. Liberation of arachidonate from membrane phospholipids is since long regarded as a rate-limiting step in the formation of pro-inflammatory eicosanoids. The importance of cPLA$_2$ in macrophages was confirmed when the production of prostaglandin E$_2$ and leukotrienes in stimulated peritoneal macrophages from cPLA$_2$ knock-out mice was found to be abolished [3-4].

Dex, a synthetic glucocorticoid with anti-inflammatory properties, has been shown to down-regulate, or counteract the induction of, cPLA$_2$ [5-8]. Normally glucocorticoids act by binding to their receptors in the cytoplasm and entering the nucleus where they bind to DNA at so called glucocorticoid response elements (GREs) resulting in the induction or repression of gene transcription. The binding to GREs may also interfere with the binding of other important transcription factors such as activator protein-1 (AP-1) or nuclear factor κB (NFκB) to the DNA [9-10]. Glucocorticoids have also been proposed to exert their effects by direct protein-protein interaction [11-12] or by destabilisation of mRNA [13-14]. Glucocorticoids can induce the expression of proteins called lipocortins/annexins [15]. These proteins have been suggested to inhibit PLA$_2$ but have later been shown to interact not with PLA$_2$ itself but with its substrate [16].

It was earlier shown that dex reduces the level of cPLA$_2$ protein and severely inhibits its activation [8]. We now report that dex inhibits cPLA$_2$ mRNA expression in mouse peritoneal macrophages and this may underlie the down-regulation of the protein.

The MAPKs ERK [17-18] and p38 [19-20] have both been implicated in cPLA$_2$ activation. Also JNK [21] has been discussed in this context. In macrophages, bacteria-

and zymosan-induced activation of ERK and p38 has been shown to play a crucial role in the activation of $cPLA_2$ [22]. We therefore investigated whether these kinases might be targets for the inhibitory effect of dex on $cPLA_2$ activation.

MATERIALS AND METHODS

Reagents

$cPLA_2$ primers for PCR was kindly provided by UNIGEN Center for Molecular Biology, Trondheim, Norway. Rabbit polyclonal antibodies against $cPLA_2$ was a generous gift from Genetics Institute, Cambridge, USA. Antibodies against ERK-2, p38, JNK-1 and phosphorylated form of ERK-2 were bought from SDS, Santa Cruz, USA. The phosphorylated forms of p38 and JNK-2 were detected with antibodies from New England Biolabs, Beverly, USA and Promega, Madison, USA, respectively.

Cell culture

Resident macrophages were isolated from peritoneal cells obtained from female outbred NMRI mice (Bom-Mice, Denmark) by adherence to either 25 cm^2 culture flasks (Nunclon, Nunc) or plastic 10 cm^2 culture dishes (Costar). The cells were incubated in an atmosphere of 5% CO_2 in air and non-adherent cells were removed 2h after plating as earlier described [23]. To each flask or dish was then added Medium 199 containing 10% heat-inactivated foetal bovine serum and the cells were cultured for 16-20 h with or without dex and chx. The cells were then washed with Dulbecco's phosphate buffered saline (PBS) and equilibrated for 30 min in serum-free Medium 199 before the start of the experiment. At the end of the experiment, the culture medium was removed.

Preparation of RNA

Isolation of total cellular RNA was made with the use of RNeasy total RNA kit as described by the manufacturer (Qiagen, Chatsworth, USA).

Northern analysis

Equal aliquots of RNA were separated on a formaldehyde-1.2% agarose gel. To visualise the RNA load in each lane ethidium bromide was added to the gel. The gel was subsequently blotted onto Hybond-N filter (Amersham) with 20x SSC (SSC; 1 x SSC= 150 mM NaCl and 50 mM sodium citrate, pH 7.0) by capillary transfer. The blots were linked in an UV-crosslinker and thereafter pre-hybridised and hybridised in ULTRAhyb solution (Ambion, Austin, USA) according to the manufacturer´s description. The probe used was the product from PCR run with primers with the following sequences: 5´GAG TTT TGG GAG TTT CTG GC (bp 1316-1335) and 3´ATG GCA GGT TAA ATG TGA GC (bp 1777-1758). The probe was labelled with [^{32}P]-dCTP by random priming to a specific activity of approx. 10^9 cpm/μg DNA. After hybridisation, blots were washed twice at 68°C for 10 min in 2x SSC, 0.1% SDS followed by two washes in 0.2 x SSC, 0.1% SDS for 15 min at 68° C and additional washing overnight at room temperature in 0.2 x SSC, 0.1% SDS. The blots were then dried and analysed by digital imaging (Fujix Bas 2000). In order to be able to normalise each sample, the blots were re-hybridised with β-actin probe.

Release of [^{3}H]arachidonate

During the overnight incubation the cells were labelled with 0.5-1.0 μCi [^{3}H] arachidonic acid (Amersham International; 212 Ci / mmol) per well and, when indicated, dex was included in the medium. At the end of the experiment, the culture medium was collected and the cells were scraped off the dish in 0.1 % (w/v) Triton X-100. The collected medium and the cell extract were centrifuged for 5 min at 2500 x g. The release of arachidonate from cellular phospholipids was determined by liquid scintillation counting and expressed as a percentage of total recovered radioactivity.

Assay of cPLA$_2$

cPLA$_2$ was assayed according to the method described earlier [1]. 1-Stearoyl-2[1-^{14}C] arachidonoyl phosphatidylcholine (Amersham International; 80 pmol /incubation) was used as substrate. Aliquots of the cytosol fraction containing cPLA$_2$ were incubated in

the presence of substrate, 1.4 mM $CaCl_2$ (approx. 400 μM free Ca^{2+}) and BSA (0.19 mg /ml) in a buffer containing 80 mM KCl, 10 mM Hepes and 1 mM EDTA, pH 7.4. The final volume was adjusted to 525 μl. After incubation for 30 min at 37 °C the reaction was stopped by the addition of 1.5 ml of chloroform/methanol/HCl (2:1:0.1, by vol.), to which 0.05% carrier lipids had been added. After centrifugation the lipid phase was applied to a silicic acid column equilibrated in chloroform. Free fatty acids were eluted with 1.0 ml chloroform and phospholipids were eluted with 2.5 ml methanol.

ERK, JNK and p38 activity

After stimulation cells (9 x 10^6 cells/sample) were washed with cold PBS and lysed in buffer containing Hepes 50 mM, EDTA 1 mM, DTE 50 mM, triton 1% (w/v), β-glycerophosphate 50 mM, Na_3VO_4 0.1 mM and okadaic acid 0.25 μM, pH 7.4. A cytosolic fraction was obtained by centrifugation (10^5 x g, 30 min). The supernatant was immunoprecipitated by shaking at 4°C for 2h with the corresponding antibody (2.5 μg) conjugated with protein A sepharose. After several washes (in a buffer containing 80 mM KCl, 10 mM Hepes, 1 mM EDTA, 2.5 mM DTE, 25 mM β-glycerophosphate, 10 mM NaF and 0.1 mM ammonium vanadate) the activity of the immunoprecipitated MAPK was determined using myelin basic protein (10 μg/incubation) as substrate for ERK, c-jun (2.0 μg/incubation) for JNK or ATF-2 (2.0 μg/incubation) for p38. The immunoprecipitates were incubated with 40 mM $MgCl_2$, 3 mM EDTA, 4 μM ATP and 1 μCi [γ-^{32}P] ATP for 30 min at 37°C. The reaction was stopped by the addition of Laemmli sample buffer [24] and the samples were run on SDS-PAGE (ERK: 12% polyacrylamide, JNK: 10% or p38: 8%). The anti-body used for immunoprecipitation of JNK-1 cross-reacts with JNK-2 and the antibody used for ERK-2 cross-reacts with ERK-1. The radioactivity incorporated into the substrate was visualised by autoradiography and quantified using video densitometry.

Incorporation of [^{35}S] Met

Isolation and culture of macrophages was performed as described above with the exception that serum was excluded from the medium. After 10 h incubation with appropriate concentration of chx, [^{35}S]Met (Amersham International; 31 μCi/well) was included in the medium. After another 10 h incubation in the presence of both dex and [^{35}S]Met the dishes were put on ice, the medium was taken off and the cells were washed three times in PBS and then either scraped in 1.0% (w/v) Triton X-100 or precipitated with 12 % trichloroacetic acid.

Level and $^{32}PO_4$ labelling of ATP

Macrophage cultures were isolated, cultured and exposed to $^{32}PO_4$ for 4h as described above. The cells were scraped off the dishes in 40 mM KH_2PO_4, pH 2.8. After sonication for 30 s the homogenate was centrifuged for 45 min at 10^5 x g to obtain a supernatant and a pellet. The supernatant was then analysed by h.p.l.c. [25] using a linear gradient from 40 mM KH_2PO_4, pH 2.8 to 0.5 M KH_2PO_4/0.8 M KCl, pH 2.7. The flow was adjusted to 1.0 ml/min. A radioisotope detector (Model 171 Radioisotope Detector, Beckman Instruments, USA) was used to determine the $^{32}PO_4$ labelling of ATP while the amount of ATP was determined from the monitored absorbance at 254 nm.

Western analysis

Equal aliquots of whole cell extracts prepared in Laemmli sample buffer were boiled for 5 min and electrophoresed on SDS-PAGE. Gels were then equilibrated in transfer buffer [26] and the samples transferred to a nitrocellulose (Amersham, Buckinghamshire, England) or a PVDF (Millipore, Bedford, USA) membrane. The membrane was first blocked with 3 % gelatine and was then incubated for 12-14h with a 1/2000 dilution of primary antibody. Bound antibodies were probed with secondary horseradish peroxidase-conjugated antibodies and detected with the supersignal chemiluminescent method (Pierce, Rockford, USA).

RESULTS

Reversal of inhibitory dex effects by cycloheximide

As previously reported, pretreatment of macrophages with dex led to a dose- and time-dependent inhibition of arachidonate release in response to PMA and zymosan [8]. This effect of dex was mediated by the glucocorticoid receptor, as it was abolished by the simultaneous addition of a molar excess of the receptor antagonist RU 38486 (not shown). We now report evidence that the effect also depends on an intact protein synthesis, as it was counteracted by the protein synthesis inhibitor cycloheximide (chx; Fig. 1). Doses of chx could be titrated that caused significant reversal of the inhibitory effect of dex on arachidonate release and rather extensive inhibition of total protein synthesis, determined by [^{35}S]methionine incorporation (Table 1). The doses used caused no apparent cytotoxicity, as judged from the lack of lactate dehydrogenase release or, more stringently, by maintenance of the cellular level of ATP (Table 1). Similar doses of chx were also found to counteract the inhibitory effect of dex on zymosan-induced cPLA$_2$ activation (Fig. 2). The protein level in control cells was approx. 4 µg/culture as determined by the method of Bradford [27]. In cultures treated with chx (0.5 µg/ml for 16-20 h) the cellular protein was reduced to 70 ± 22 % (mean ± SEM, n=3) of that in untreated cells.

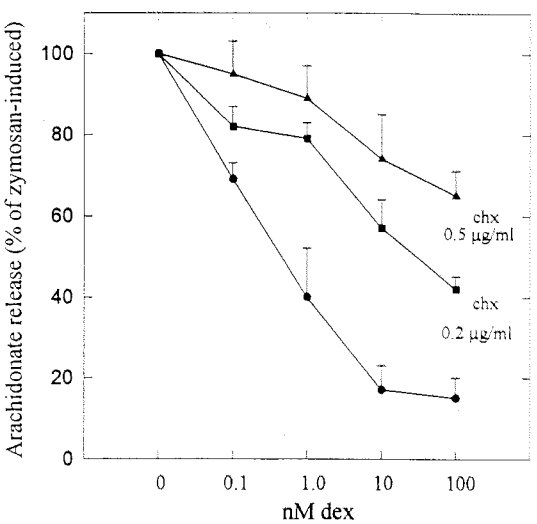

Figure 1. Reversion of dex-inhibited arachidonate release by chx. Macrophages (3x10^6 cells/well) were incubated with dex, concentrations as indicated, for 16-20h. Chx was included in medium for 16-20h. The cells were stimulated with zymosan (0.2 mg/ml, 1h). The release of radiolabel, corrected for the release in control cultures not exposed to zymosan, is expressed as percentage of the release in the absence of dex. The latter amounted to 15±5 % of total cellular [^{3}H]arachidonic acid. Data are means ± SEM, n=5.

TABLE I

Effect of chx on total ATP content, [$^{32}PO_4$]ATP and on [^{35}S]Met incorporation

Total ATP content and [$^{32}PO_4$]ATP: Cells were labelled with $^{32}PO_4$ for 4 h (see Experimental). High pressure liquid chromatography was performed on cell extracts from control cells and cells treated with chx for 20 h. Total ATP in the cytosolic fraction was determined as absorbance at 254 nm. Data are means ± SEM (n=3). *[^{35}S]Met incorporation:* during the 20 h incubation [^{35}S]Met was included in the medium for the last 10 h. The radioactivity in the culture medium and the cell extract was determined. In control cells approx. 0.5 % of the [^{35}S]Met added was incorporated. Data are means ± SEM (n=4). N.D.: not determined.

Dex (nM)	Chx (μg/ml)	Total ATP (% of control)	[^{32}P]ATP (% of control)	[^{35}S]Met incorp. (% of control)
10	-	96 ± 5	69 ± 3	59 ± 3
-	0.5	124 ± 4	65 ± 4	18 ± 6
10	0.5	100 ± 2	56 ± 4	N.D.

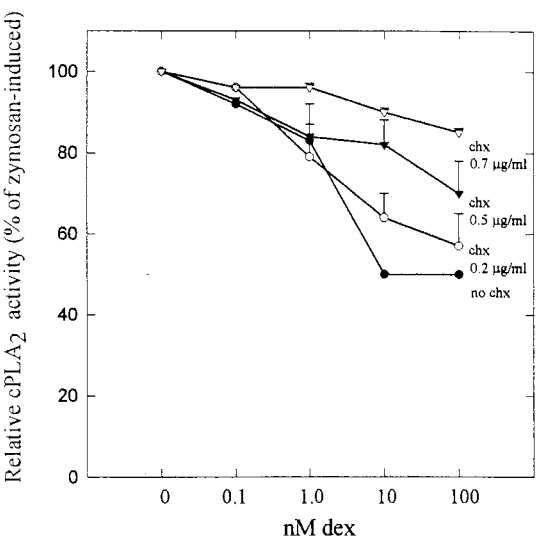

Figure 2. Reversion of dex-inhibited cPLA$_2$ activity by chx

Macrophages (3x10^6 cells/well) were incubated with dex, concentrations as indicated, for 16-20h. Chx was included in the medium for 16-20h. Data are means ± SEM, n=5.

Effect of dex on the expression of $cPLA_2$ mRNA and on stimulus-induced $cPLA_2$ gel shift

The previously reported down-regulation of macrophage $cPLA_2$ by dex [8] could in principle be due to interference with a number of different processes, including i) $cPLA_2$ gene transcription, ii) $cPLA_2$ mRNA turnover or iii) translation, or iv) an increased turn-over/degradation of the $cPLA_2$ protein. As shown in Figure 3 and determined by Northern blot analysis, dex inhibited the basal as well as the LPS-induced expression of $cPLA_2$ mRNA. Quantitative analysis showed inhibition to 14 ± 7 and 14 ± 8 % of control, respectively (mean ± SEM, n = 3). This pronounced down-regulation, whether due to inhibited transcription or increased mRNA turn-over, may well account for the reduced level of $cPLA_2$ protein alluded to above.

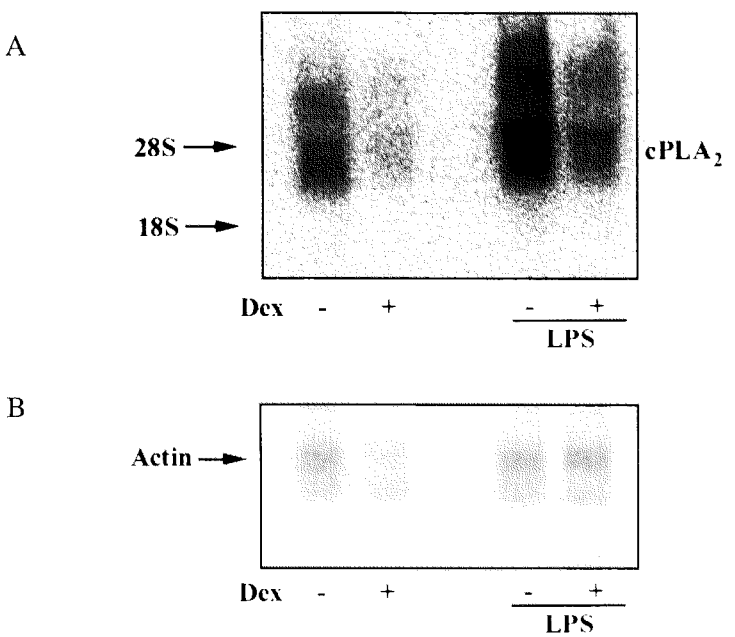

Figure 3. Effect of dex on expression of $cPLA_2$ mRNA

Macrophages (1.5 x 10^7 cells / flask) were cultured for 16-20 h in the absence or presence of dex (10 nM) and LPS (1 μg/ml) as indicated. Northern blot analysis was performed as described in Materials and methods. Representative Northern blots showing mRNA for $cPLA_2$ (A) and corresponding β-actin (B) from three independent experiments.

Phosphorylation of $cPLA_2$ is accompanied by an electrophoretic mobility shift. In unstimulated macrophages both the unphosphorylated and the phosphorylated band are detectable (Fig. 4). After stimulation with zymosan or PMA a complete gel shift was obtained. Surprisingly, after pretreatment with dex the remaining $cPLA_2$ still underwent mobility shift in response to both PMA and zymosan (Fig. 4). These results indicate that the electrophoretic mobility shift, which presumably reflects phosphorylation of Ser-505 by ERK-2, is unaffected by dex, which is consistent with its limited effect on ERK activation. However, the lack of upregulation of $cPLA_2$ catalytic activity, shown earlier [8], indicates that such upregulation requires additional phosphorylation(s) and that these are targeted by dex.

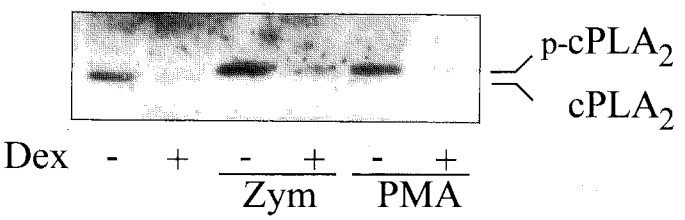

Figure 4. Effect of dex on $cPLA_2$ protein and mobility shift

Macrophages were cultured for 16-20 h in the absence or presence of dex (10 nM). After stimulation with zymosan (zym; 0.2 mg/ml, 1h) or PMA (100 nM, 1h) the culture medium was removed and a cell lysate was prepared. Aliquots thereof were then subjected to 12% SDS-PAGE followed by Western blot analysis as described in Materials and methods. This experiment is representative of twenty similar experiments.

Effects of dex on MAPK activation and phosphorylation

Activation of ERK was measured as the ability to phosphorylate MBP after immunoprecipitation with antibodies against ERK-2. Since cross-reactions between different isoforms of ERK can not be excluded we will from now on only use the designation ERK. Dex did not inhibit the PMA-induced activation of ERK. Pre-incubation with dex before addition of PMA resulted in 108 ± 4 % (mean ± SEM; n=4) of the activity obtained with PMA alone. No difference in effect of dex on ERK

activation was observed in the interval of 2 to 15 min of stimulation with PMA (data not shown). ERK activation was not detectable in unstimulated cells. However, zymosan-induced ERK activation was inhibited by pre-treatment with dex. The activation after dex treatment was found to be 54 ± 8 % (mean ± SEM; n=5) of that in cells treated with zymosan only (Fig. 5A). In order to confirm data from kinase activity measurements, complementary experiments using antibodies against the phosphorylated forms of MAPK were performed. Using such antibodies directed at phosphorylated ERK-2, Western blot revealed both PMA and zymosan to induce phosphorylation of ERK. The zymosan-induced phosphorylation of ERK was partially inhibited by dex, while that induced by PMA was not (Fig. 5B).

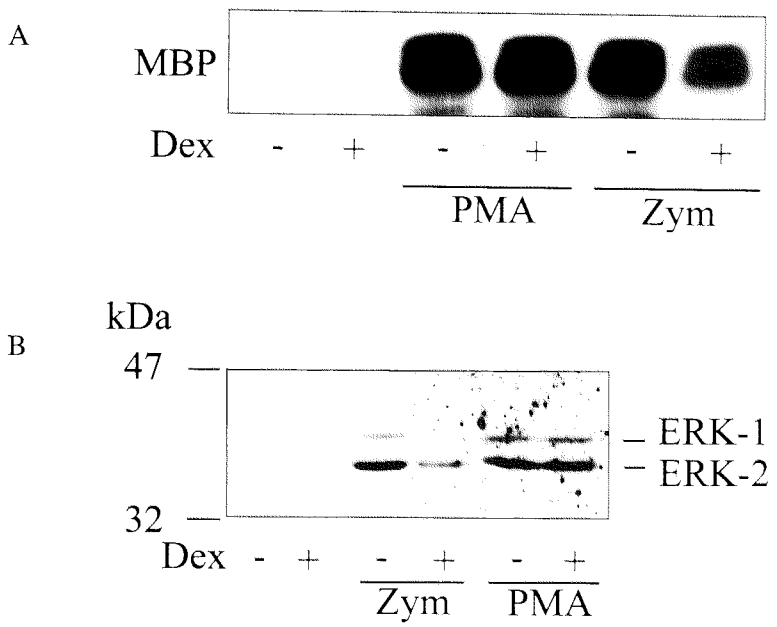

Figure 5. Effect of dex on ERK activation

Macrophages were cultured for 16-20h in the absence or presence of dex (10 nM). The cells were stimulated with PMA (100 nM, 15 min) or zymosan (zym; 0.2 mg/ml, 1h). (A) Activity assay after immunoprecipitation with ERK-2 antibodies. (B)Western blot analysis was performed with antibodies against the phosphorylated form of ERK-2. All experiments were subjected to digital imaging analysis and are representative of three similar experiments.

Dex did not inhibit the PMA-induced increase in kinase activity of p38 (Fig. 6A). Pre-incubation with dex before addition of PMA resulted in 117 ± 22 % of the activation

Dex did not inhibit the PMA-induced increase in kinase activity of p38 (Fig. 6A). Pre-incubation with dex before addition of PMA resulted in 117 ± 22 % of the activation obtained with PMA only, (mean ± SEM; n=3). A moderate inhibition (to 69 ± 7 %; mean ± SEM; n=5) was observed when zymosan was used as stimulus. Similarly, the zymosan-induced phosphorylation of p38 was partially inhibited by dex (Fig. 6B).

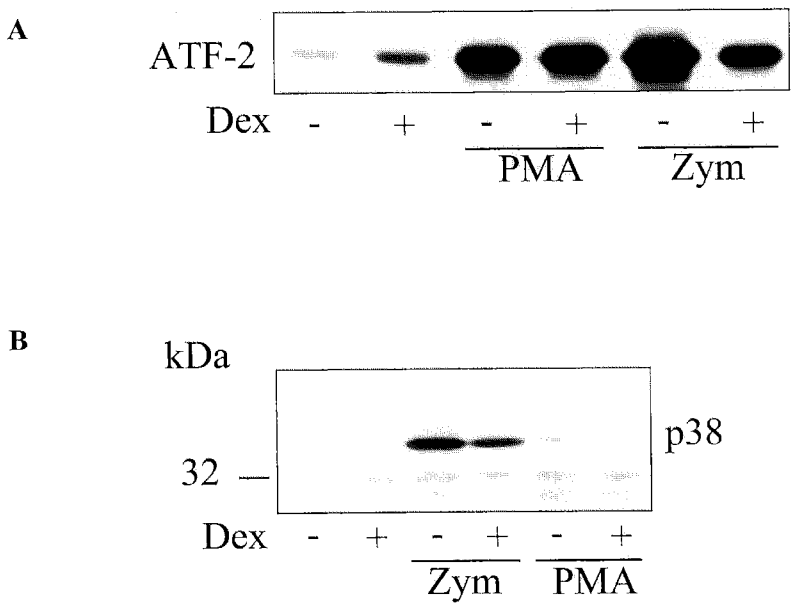

Figure 6. Effect of dex on p38 activation

Macrophages were cultured for 16-20h in the absence or presence of dex (10 nM). The cells were stimulated with PMA (100 nM, 15 min) or zymosan (zym; 0.2 mg/ml, 1h) as indicated below. (A) Activity assay was performed as described in Materials and methods. An 8-20 fold increase in activity was found after stimulation. (B) Western blot analysis was performed using antibodies against the phosphorylated form of p38 as described in Materials and methods. This experiment is representative of three similar experiments.

Antibodies against JNK-1 were used in the immunoprecipitation preceding kinase activity measurements using c-jun as substrate. This antibody probably cross-reacts with other isoforms of JNK. PMA did not induce activation of JNK. Pre-incubation with dex before addition of zymosan resulted in 54 ± 9 % (mean ± SEM; n=3) of the activity obtained with zymosan alone. (Fig. 7A). Dex severely inhibited zymosan-induced phosphorylation of JNK while PMA treatment did not result in increased phosphorylation of this kinase (Fig. 7B). Inhibitory effects of dex on JNK has been observed by others as well [28-29]. The discrepancy between the results from kinase activity measurements and the Western blot might be due to the use of antibodies against JNK-1 in the kinase activity studies, while antibodies against the phosphorylated form of JNK were raised against JNK-2.

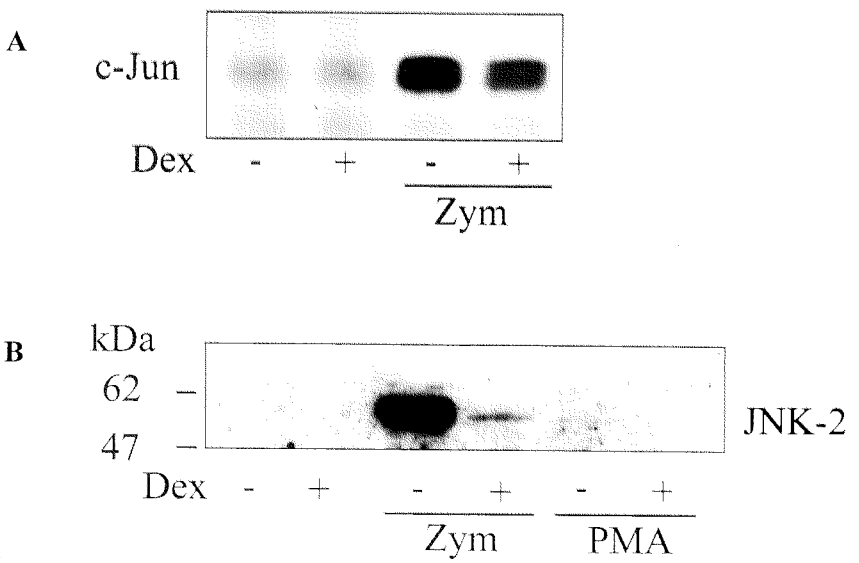

Figure 7. Effect of dex on JNK activation

Macrophages were cultured for 16-20h in the absence or presence of dex (10 nM). The cells were stimulated with zymosan (zym; 0.2 mg/ml, 1h) as indicated below. (A) Activity assay was performed using antibodies against JNK-1 in the immunoprecipitation as described in Materials and methods. (B) Western blot analysis with antibodies against the phosphorylated form of JNK-2 was performed as described in Materials and methods. All experiments were subjected to digital imaging analysis. This experiment is representative of three similar experiments.

DISCUSSION

It was aleady earlier reported that dex causes down-regulation of $cPLA_2$ in mouse macrophages [8]. The present study shows that this can be accounted for by either inhibition of $cPLA_2$ gene transcription or increased turn-over of its mRNA, as the mRNA was found to be even more down-regulated than the $cPLA_2$ protein. The almost complete reversion of zymosan-stimulated $cPLA_2$ activity shown here to be exerted by chx indicates that the suppressed activation of $cPLA_2$, the second effect of dex on this enzyme, was restored. The reversal of the effects of dex by chx indicates that they depended on synthesis of new protein(s).

Increased phosphorylation of multiple serine residues in $cPLA_2$ occurs upon activation [30-31], but only phosphorylation on Ser-505 has been found to be responsible for the shift in $cPLA_2$ electrophoretic mobility [17, 32]. This phosphorylation has also been assumed to cause upregulation of its catalytic activity. The present and previous [8] data, showing dissociation of mobility shift and activation, indicate that additional phosphorylation(s) is/are necessary for activation and that these, rather than that causing mobility shift, are targeted by dex. However, the identity of such phosphorylation site(s) is presently unclear. Unfortunately, the expression in insect cells of serine mutated forms of $cPLA_2$ or forms with C-terminal truncations did not provide clear answers as to the regulatory role of the phosphorylation of individual serine residues [32].

Although both ERK and p38 have been found to be essential for activation of $cPLA_2$ [22], neither of them appeared to be main targets for the inhibitory effect of dex in this study, in consistency with other data [28]. The inhibitory effect of dex on $cPLA_2$ activation must instead be exerted down-stream of these MAPKs, although we found that dex partially inhibited zymosan-induced activation. However, it was earlier reported that dex leads to partial inhibition of zymosan-induced phospholipase C activation [33] and this may well account for the partial inhibition of MAPK.

Furthermore, recent data demonstrate that phosphatidylinositol 3-kinase is positioned up-stream of phospholipase C [Hiller *et al.*, unpublished results] and therefore could constitute an alternative target for the partial inhibition. Indeed, phosphatidylinositol 3-kinase has been found to be a target for dex [34]. In conclusion, the present study demonstrates that the transcriptional regulation of $cPLA_2$ and the regulatory phosphorylation of this enzyme are main targets for dex, rather than the expression or activation of MAPKs.

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Dexamethasone Differentially Regulates Cytokine Transcription and Translation in Macrophages Responding to Bacteria or Okadaic Acid

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ABSTRACT. Many microorganisms and microbial products induce expression of pro-inflammatory cytokines such as interleukin-1 (IL-1α/β) and tumour necrosis factor-α (TNF-α) in macrophages, primarily by transcriptional activation. We show here, by using mouse macrophages in primary culture, that pre-treatment with dexamethasone inhibits bacteria-induced IL-1β expression as mRNA and cellular pro-IL-1β in parallel, consistent with an effect primarily on transcriptional activation. In contrast, the expression of TNF-α mRNA was only partly inhibited despite virtually complete inhibition of TNF-α protein formation. Furthermore, the selective induction of primarily cell-associated 26,000 M, pro-TNF-α by the protein phosphatase inhibitor okadaic acid also was partly inhibited at the mRNA level by dexamethasone, whereas additional translational inhibition appeared to be lacking. This latter finding is reminiscent of earlier findings regarding signalling to activation of cytosolic phospholipase A_2, which is sensitive to dexamethasone when elicited by bacteria, but not when elicited by okadaic acid. The present results raise the possibility that the inhibitory effect of dexamethasone on TNF-α translation, but not on transcriptional activation, is mediated by one or more okadaic acid-sensitive protein phosphatases. CELL SIGNAL 11;9:665–670, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. Interleukin-1, Tumour necrosis factor-α, Glucocorticoid, Bacteria, Macrophage, Okadaic acid, Dexamethasone

INTRODUCTION

Macrophages are major protective cells against invasion of a host by microorganisms. As an early response to an encounter with microorganisms, these cells produce mediators that induce and regulate the ensuing inflammation. Among the wide array of macrophage-derived mediators are prominent pro-inflammatory cytokines such as TNF-α, IL-1β and eicosanoids. Early studies on TNF-α induction gave contradictory results concerning whether activation by LPS is primarily at the transcriptional or translational level, but it now seems to have been finally settled that transcriptional activation predominates, although translational activation also occurs [1].

Glucocorticoids, in particular the synthetic analogue dexamethasone, have in several studies been found to inhibit the cytokine response in monocytes [2, 3], macrophages [4] and related cell lines. However, with regard to both IL-1 and TNF-α, uncertainty about the mechanism and specific target for this inhibition appeared early on, because modulation of transcription [2, 5–7], mRNA stability [6, 8], translation [9, 10] or post-translational processing [9] were suggested as major targets. Specific mechanisms for transcriptional inhibition were proposed, such as sequestration of activator protein-1 (AP-1) by direct interaction with glucocorticoid receptor [11] or of nuclear factor kappa B (NFκB) by induction of inhibitory protein kappa B (IκB) formation [12]. However, the latter of these mechanisms may not be universal [13], and the former may instead be indirect and due to competition for common co-activators, such as p300/CBP and related histone acetylating proteins [14, 15]. Because this latter effect would be rapid, whereas several anti-inflammatory effects of glucocorticoids take time to develop and depend on novel protein synthesis, some mechanistic uncertainty still remains. Dexamethasone was earlier shown to inhibit eicosanoid formation in macrophages at least in part by preventing the phosphorylation and activation of cytosolic 85,000 M, phospholipase A_2 [16, 17]. Interestingly, however, such activation elicited by the protein phosphatase inhibitor okadaic acid was not inhibited by dexamethasone [16]. Because okadaic acid is capable of causing apparently selective expression of TNF-α mRNA in macrophages [18, 19], it became of special interest to assess whether this response is sensitive to dexamethasone.

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Abbreviations: IL-1–interleukin-1; TNF–tumor necrosis factor; LPS–bacterial lipopolysaccharide; *P. acnes–Propionibacterium acnes*; *F. nucleatum–Fusobacterium nucleatum*; *P. anaerobius–Peptostreptococcus anaerobius*.

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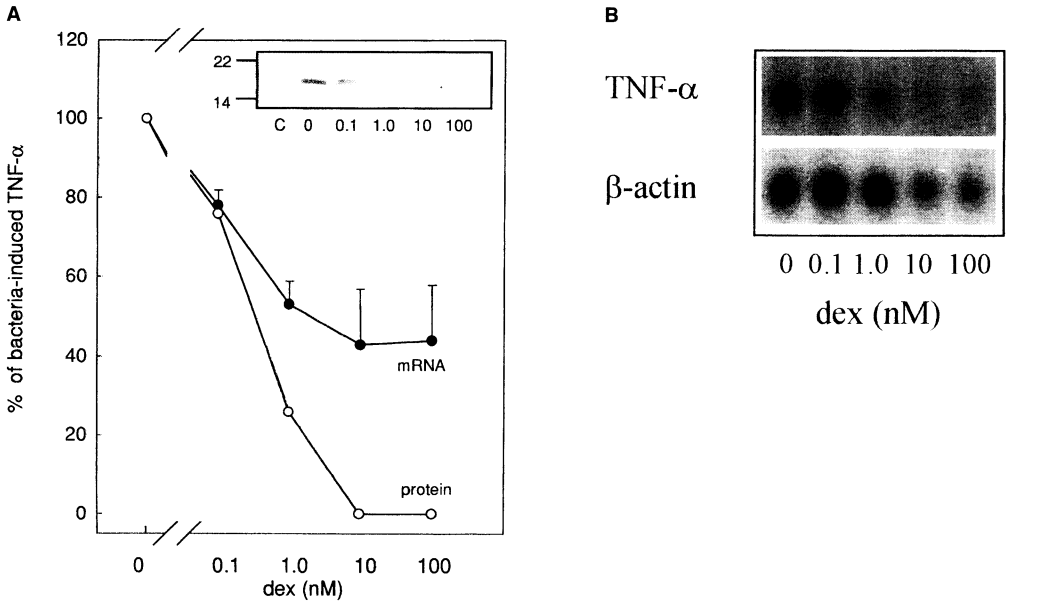

FIGURE 1. Effect of dexamethasone on TNF-α mRNA and protein expression in macrophages activated by bacteria. Macrophages (12×10^6 cells/flask) were incubated with dexamethasone (dex), concentration as indicated, for 20 h and were then exposed to *F. nucleatum* (2×10^8/mL) for 90 min (Northern blot) or 4 h (Western blot). (A) Northern blots (●) from at least three separate experiments were analysed by digital imaging and normalised to β-actin in the same lane. Data shown (mean ± S.E.M) are expressed as the percentage of the TNF-α mRNA signal in cultures without dexamethasone. Western blot (○) analysis of a trichloroacetic acid precipitate from the culture medium (see inset) was carried out as described in the Materials and Methods section. Mean values for two separate experiments are plotted. (B) Representative Northern blots showing mRNA for TNF-α and β-actin, respectively.

In the present study, the effect of dexamethasone on bacteria-induced expression of IL-1β in macrophages was found to be predominantly at the level of transcription, whereas that on TNF-α expression also entailed effects at the level of translation. Okadaic acid induced expression of TNF-α primarily as the cell-associated 26,000 M_r precursor, and the transcriptional activation showed sensitivity to dexamethasone similar to that in response to bacteria, whereas inhibition at the translational level was less pronounced.

MATERIALS AND METHODS

Cell Culture

Resident macrophages were isolated from peritoneal cells obtained from female outbred NMRI mice (Bom-Mice, Copenhagen, Denmark) by adherence to 25 cm^2 culture flasks (Nunclon, Nunc). The cells were incubated in an atmosphere of 5% CO_2 in air and non-adherent cells were removed 2 h after plating, as earlier described [20]. To each flask was then added Medium 199 containing 10% heat-inactivated foetal bovine serum, and the cells were cultured for 20 h with or without dexamethasone. The cells were then washed with Dulbecco's phosphate buffered saline (PBS) and equilibrated for 30 min in serum-free Medium 199 before the start of the experiment. In all experiments with dexamethasone pre-treatment, the compound was also included in the medium during the experiment. At the end of the experiment, the culture medium was removed, and the cells were washed with PBS.

As revealed by Trypan blue exclusion, more than 95% of the cells were viable at the end of the experiments and also after long time exposure to okadaic acid (0.25 μM, 6 h).

Reagents

Bacteria, obtained as earlier described [21], were added as a suspension in Ca^{2+}/Mg^{2+}-free PBS. Dexamethasone was purchased from Sigma (St. Louis, MO, USA) and was either dissolved in dimethyl sulphoxide and added in volumes of 0.1% of total medium or dissolved in Medium 199. Okadaic acid was bought from LC Laboratories (Woburn, MA, USA). Synthetic oligonucleotide probes (Clontech Laboratories Inc., USA) with the following sequences were used for Northern blotting; TNFα: 5′-GCC GTT GGC CAG GAG GGC GTT GGC GCG CTG-3′, IL-1β: 5′-AGC TTT CAG CTC ATA TGG GTC CGA CAG CAC-3′ and β-actin: 5′-GGG TGT TGA AGG TCT CAA ACATGA TCT GGG-3′.

Preparation of RNA

Isolation of total cellular RNA was made with the use of RNeasy total RNA kit as described by the manufacturer (Qiagen, Chatsworth, CA, USA).

Northern Analysis

Equal aliquots of RNA were separated on a formaldehyde-1.2% agarose gel. To visualise the RNA load in each lane of the gel; ethidium bromide was added to the RNA sample before application onto the gel. The gel was subsequently blotted onto Hybond-N filter (Amersham) with 20 × SSC (SSC; 1 × SSC = 150 mM NaCl and 50 mM sodium citrate, pH 7.0) by capillary transfer. The blots were linked in a UV-crosslinker and thereafter prehybridised for 12–16 h at 42°C in 50% formamide, 1% SDS, 5 × SSPE (SSPE; 1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA, pH 7.4), 1 × Denhardt's solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinyl pyrrolidone), 0.5 mg/mL denaturated salmon sperm DNA, pH 7.4. Oligonucleotide probes were labelled with $\gamma[^{32}P]$ATP by 5′ labelling to a specific activity of approximately 10^8-10^9 cpm/μg DNA. The filters were hybridised at a concentration of 5×10^6 cpm/mL at 42°C for 12–16 h in hybridisation solution. After hybridisation, blots were washed at room temperature for 5–10 min in 2 × SSC, 0.1% SDS followed by additional washes in 0.2 × SSC, 0.1% SDS for 15–30 min at 42°C. The blots were then dried and analysed by digital imaging (Fujix Bas 2000). Autoradiographs of blots in some experiments were quantified by scanning with video densitometry, and OD values were determined for each sample and normalised to values obtained for β-actin expression on the same blot. Multiple exposures were used on each blot to ensure that the exposure was in the linear range of film sensitivity. In all experiments, the blots were re-utilised and re-hybridised with different oligonucleotides.

Western Analysis of Cytokines

Equal aliquots of whole cell extracts prepared in Laemmli sample buffer were boiled for 5 min and electrophoresed on a 12% (IL-1β) or 10–18% (TNF-α) SDS-PAGE [22]. After a 5-min centrifugation at 2500 × *g*, the cell culture medium, supplemented with bovine serum albumin, was adjusted to 6% trichloroacetic acid followed by incubation on ice and centrifugation at 12,000 × *g* for 30 min. The precipitate was suspended in sample buffer and treated as described for the cell extract. Gels were then equilibrated in transfer buffer [23], and the samples were transferred to a 0.2 μm nitrocellulose membrane. The membrane was first blocked with 3% gelatin and was then incubated for 14–18 h with a 1/2000 dilution of purified goat antibodies against either IL-1β or TNF-α (R&D systems, Minneapolis, MN, USA). Bound antibodies were probed with horseradish peroxidase-conjugated anti-goat antibodies and detected with the supersignal chemiluminescent method (Pierce, Rockford, IL, USA).

RESULTS AND DISCUSSION

Effects of Dexamethasone on TNF-α and IL-1β Expression in Response to Bacteria

As a starting point, we assessed the effect of dexamethasone on the expression of IL-1β and TNF-α, induced by Gram-positive (*Propionibacterium acnes* or *Peptostreptococcus anaerobius*) or Gram-negative (*Fusobacterium nucleatum*) bacteria. In cultures pre-treated with dexamethasone, the level of mRNA for TNF-α (Fig. 1) and IL-1β (Fig. 2) after stimulation with bacteria decreased in a dose-dependent manner. In regard to IL-1β mRNA, the inhibition was half-maximal at 1 nM dexamethasone and very pronounced at 10–100 nM. However, the expression of TNF-α mRNA was not reduced to the same extent as for IL-1β. As shown in Fig. 1, there was only an approximately 55% reduction at 10–100 nM dexamethasone. Furthermore, pre-treatment of the cells with dexamethasone for only 1 h was not sufficient to cause any detectable effect on the cytokine mRNA response (not shown).

To investigate whether dexamethasone also exerted effects at the translational level, we performed Western blot analysis on the cell lysate and the culture medium. When analysing the cell lysate, we could easily detect 35,000 M_r pro-IL-1β after 4-h stimulation with either *P. anaerobius*, *P. acnes* or *F. nucleatum*, but any proteolytically processed forms of IL-1β were not detectable either in the cell lysate or in the culture medium. As shown in Fig. 2, the bacteria-induced production of pro-IL-1β was virtually completely reversed after treatment with 10–100 nM dexamethasone. This was the case whether Gram-positive or Gram-negative bacteria were used as stimulus. It is also clear from this Fig. 2 that the dose dependence for inhibition was quite similar for the expression of IL-1β at both the mRNA and (pro-) protein level. By use of the sensitive chemiluminescence detection procedure, cell-associated pro-IL-1β could also be detected in unstimulated macrophages (Fig. 3). This basal expression was extremely sensitive to dexamethasone, being inhibited with an IC_{50} value of 0.01 nM (Fig. 3); that is, at least one order of magnitude below that for bacteria-induced pro-IL-1β (Fig. 2). It is therefore likely that this expression is suppressed under *in vivo* conditions.

In contrast with the case for IL-1β, TNF-α was found exclusively as mature 17,000 M_r TNF-α in the culture medium. The generation of TNF-α in response to bacteria was virtually completely inhibited after treatment with 10–100 nM dexamethasone (Fig. 1). This inhibition was, thus, much more pronounced than that on the level of mRNA, and it was not exerted at the final processing step, because there was no detectable accumulation of immunoreactive TNF-α, or its precursor in the cell lysate (not shown).

Effects of Dexamethasone on Cytokine Expression in Response to Okadaic Acid

As reported earlier [18, 19], exposure of mouse macrophages to okadaic acid results in expression of TNF-α mRNA. In macrophage cultures pre-treated with increasing doses of dexamethasone, the level of TNF-α mRNA induced by okadaic acid was found to decrease in a dose-dependent manner (Fig. 4). However, the inhibitory effect of dexamethasone was only partial and amounted to approximately 45% at 100 nM dexamethasone. It therefore resembled the effect on bacteria-induced TNF-α mRNA (Fig. 1), but, in contrast with the complete inhibition of bacteria-induced TNF-α protein, that induced by okadaic acid was inhibited only to an extent similar to that for the mRNA (Fig. 4). The most straightforward interpretation of these results is that dexamethasone inhibits bacteria-

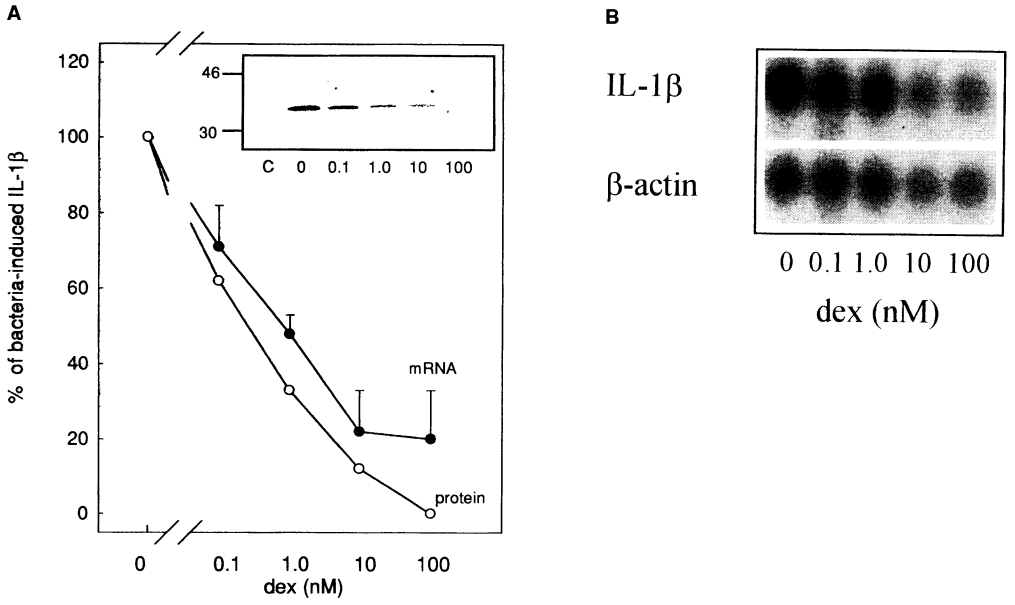

FIGURE 2. Effect of dexamethasone on IL-1β mRNA and pro-IL-1β protein expression in macrophages activated by bacteria. Macrophages (12×10^6 cells/flask) were incubated with dexamethasone (dex), concentration as indicated, for 18–20 h and were then exposed to *F. nucleatum* (2×10^8/mL) for 90 min (Northern blot) or 4 h (Western blot). (A) Northern blots (●) with the use of probe for IL-1β mRNA were carried out, normalised and expressed as in Fig. 1. Western blot (○) analysis of cell lysate (see inset) was carried out as described in the Materials and Methods section. Mean values for three separate experiments, determined by digital imaging, are plotted. (B) Representative Northern blots showing mRNA for IL-1β and β-actin, respectively.

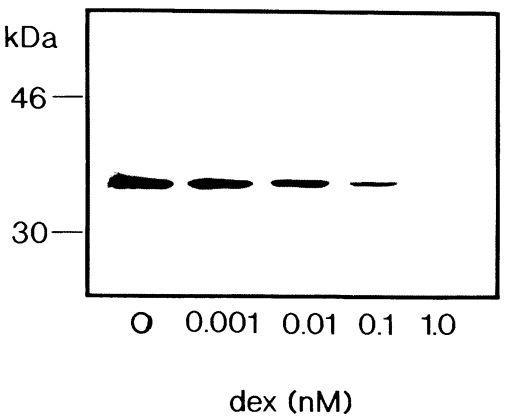

FIGURE 3. Inhibition by dexamethasone of pro-IL-1β formation in unstimulated macrophages. Macrophages were cultured for 20 h in the absence or presence of dexamethasone (dex), as indicated. Western blot analysis was performed as described in the Materials and Methods section. The results shown are representative of four separate, identically performed experiments.

induced TNF-α expression at both the transcriptional and the translational level, while inhibiting the response to okadaic acid only, or primarily, at the transcriptional level. In human cells, a phosphorylation-mediated regulation of TNF and IL-1 expression at the translational level was linked to the human p38 analogue of MAP kinase [24], but it is not known whether this also applies to mouse macrophages or whether the mechanism may be targeted by glucocorticoids.

As heretofore noted, TNF-α is found virtually exclusively in the culture medium as the 17,000 M_r processed form in cultured macrophages responding to bacteria. This also holds for a number of other stimuli, including LPS. However, treatment with okadaic acid resulted in a different distribution of immunoreactive TNF-α with only approximately 28% released into the culture medium and the remaining 72% still cell associated. The same distribution was seen when cells were stimulated with LPS in the presence of okadaic acid (not shown). Of the cell-associated TNF-α, 24 ± 7% (mean ± S.E.M., n = 4) was in the cleaved form, and the remaining 76% was in the unprocessed form. Treatment with increasing doses of dexamethasone (1–100 nM) for 20 h before addition of okadaic acid resulted in a differential inhibition of these forms of TNF-α, with the cell-associated 26,000 M_r form of TNF-α being partly inhibited and the cell-associated 17,000 M_r form not affected at all (Fig. 5). TNF-α in the culture medium was inhib-

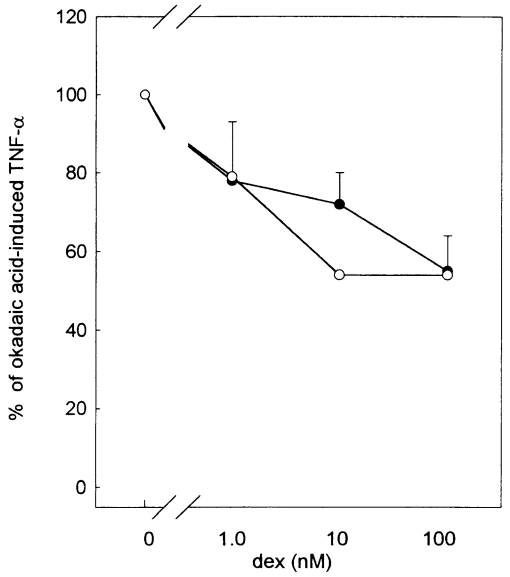

FIGURE 4. Effect of dexamethasone on okadaic acid-induced TNF-α mRNA and protein. Macrophages (3×10^6 cells/flask) were incubated without or with the indicated concentration of dexamethasone (dex) for 20 h before exposure to okadaic acid (0.25 μM) for 4 h (Northern blotting) or 6 h (Western blotting). Northern and Western analyses were performed as described in the Materials and Methods section. The value for cells treated with okadaic acid (without dexamethasone) was set to 100%. (●) TNF-α mRNA levels, normalised to β-actin in the same lane. (○) Total immunoreactive TNF-α protein was determined from Western blots (culture medium *plus* cell lysate) as described in the legend for Fig. 1.

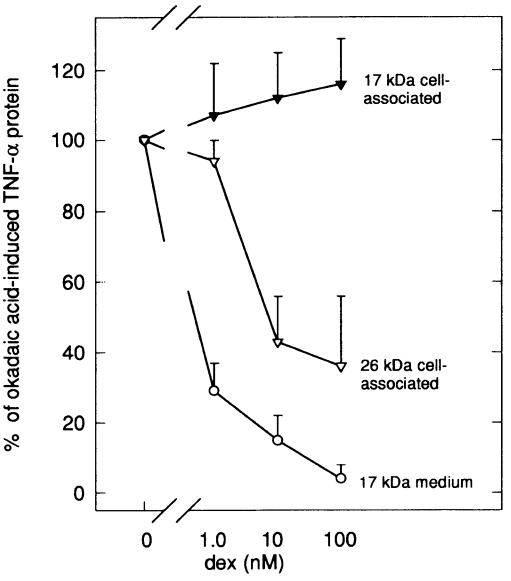

FIGURE 5. Effect of dexamethasone on okadaic acid-induced 17,000 and 26,000 M_r TNF-α. Macrophages cultured for 20 h in the absence or presence of dexamethasone (dex), as indicated, were exposed to okadaic acid (0.25 μM) for 6 h. At the end of the experiment, the culture medium was precipitated with trichloroacetic and a cell lysate was prepared. Aliquots of both were subjected to SDS-PAGE and Western blotting as described in Materials and Methods. The value for cells treated with okadaic acid (without dexamethasone) was set to 100%. Data shown are mean ± S.E.M. from three to five separate experiments.

ited with higher potency and to a somewhat higher degree than the 26,000 M_r cell-associated TNF-α (Fig. 5).

To our knowledge, the induction of TNF-α by okadaic acid in murine cells has not been previously reported. In human monocytes, okadaic acid induces both TNF-α and IL-1β mRNA [25] and the expression of primarily cell-associated 26,000 M_r TNF-α. Thus, both transcriptional activation of the TNF-α gene and inhibition of the processing of the translation product occurs similarly in mouse macrophages and human monocytes. A human cell line (Mono Mac 6) appears to be constitutively blocked in TNF-α processing because it responds to LPS by production of 26,000 M_r cell-associated TNF-α [26]. Interestingly, processing could be elicited by simultaneous treatment with phorbol ester, which also initiates proteolytic shedding of several other cell surface proteins, including cytokine receptors [27]. With the use of Mono Mac 6 cells and HeLa cells transfected with a less-cleavable mutant form of TNF-α, phosphorylated serine residues were identified in the 26,000 M_r protein, presumably in the cytoplasmic part [28]. Whether such phosphorylation directly inhibits proteolytic processing or affects intracellular transport necessary for processing remains unknown. The processing is primarily carried out by a specific metalloprotease [29, 30], but, according to an *in vitro* study [31], the same cleavage can also be executed by the serine protease proteinase-3. We found, surprisingly, 17,000 M_r TNF-α associated with the okadaic acid-treated cells (Fig. 5). The explanation for this finding is currently not known. TNF-α, which can bind to heparin [32] and most likely to cell surface proteoglycans, could adsorb from the medium, but the cell-associated 17,000 M_r form was not reduced in dexamethasone-treated cells, although that in the medium was virtually eliminated (Fig. 5). Additionally, it was not observed in cells stimulated by bacteria or LPS, although much more 17,000 M_r TNF-α is released under those conditions. Alternatively, part of the retained 26,000 M_r protein could be re-routed to granules and be processed either by the normal metalloprotease or by proteinase-3. Clearly, the process giving rise to cellular 17,000 M_r TNF-α is not sensitive to dexamethasone.

Finally, it is interesting to note that the translational inhibition exerted by dexamethasone on bacteria-induced TNF-α formation (Fig. 1) appears to be overcome when

okadaic acid is the inducing agent (Fig. 4). This effect is reminiscent of the inhibitory effect of dexamethasone on phosphorylation and activation of the cytosolic 85,000 M_r phospholipase A_2 [16, 17], which is also overcome by okadaic acid [16]. As mentioned earlier, the p38 analogue of mitogen-activated protein (MAP) kinase was implicated in translational regulation of TNF-α in human cells [24]. However, our studies on p38 in mouse macrophages do not support the possibility that this kinase is targeted by dexamethasone (K. Gewert and R. Sundler, manuscript in preparation). Consequently other potential targets, including okadaic acid-sensitive protein phosphatases, will have to be considered in future work.

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