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STUDY OF SIGNAL TRANSDUCTION PATHWAYS
OF PHORBOL ESTER-RESPONSIVE GENES

A thesis submitted to the
School of Graduate Studies
University of Ottawa

In partial fulfillment of the
requirements for the Degree of
Master of Science,
Department of Microbiology and Immunology,
School of Medicine.

by

Dorina G. Motora

Dorina G. Motora, Ottawa, Canada, 1989

à mes parents

ABSTRACT

When quiescent mouse fibroblasts are treated with serum or growth factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or the tumor promoter phorbol myristate acetate (PMA), a nuclear proto-oncogene c-fos is rapidly induced (Cochran et al. 1984, Greenberg and Ziff 1984, Kruijer et al. 1984, Muller et al. 1984a, Bravo et al. 1985). The fact that c-fos gene transcription can be detected within 5 min of treatment with agents that stimulate cell division (PDGF, phorbol esters) or differentation, makes it one of the earliest known transcriptional responses to a stimulant. c-Fos expression is transient within cells since the maximal level of mature mRNA is attained 20-30 min after stimulation and declines thereafter to reach a low level by 60 min. The observed induction is at the level of transcription, as measured by nuclear run-off transcription (Greenberg and Ziff 1984). It was suggested early that fos might be important for the passage of cells from the resting state into the actively dividing phases of the cell cycle and for differentiation.

Many changes have been observed to occur within seconds after binding of polypeptide growth factors to their membrane receptors. These include: stimulation of phosphatidylinositol-bis-phosphate (PIP_2) turnover, changes in intracellular Na^+, H^+,Ca^{2+} levels, cyclic nucleotide levels and protein phosphorylation. Two "second messengers" are produced by PIP_2 turnover: inositol trisphosphate and diacylglycerol. Inositol trisphosphate causes a transient rise in cytosolic calcium by release from an intracellular store; diacylglycerol is probably the endogenous activator of protein kinase C (PKC), an

enzyme(s) important in several cellular processes including growth control. Some of these pathways can be bypassed by phorbol ester-type tumor promoters like PMA which activates PKC directly and then triggers the expression of genes such as c-fos and c-myc. There are a multitude of agents that have been reported to induce c-fos gene expression. Biochemical details of the signaling pathways that lead from receptor binding of a growth factor or mitogen to the transcriptional activation of specific genes in the nucleus are incomplete.

To study the early biochemical events involved in the induction of c-fos, we have used confluent cultures of Balb/c-3T3 cells and have combined the treatment of phorbol ester, PMA (phorbol myristate acetate), with inhibitors of various pathways thought to play a role in the transduction of mitogenic signals from the membrane to the nucleus. One of the drugs tested was indomethacin (INDO), a non-steroidal anti-inflammatory agent. At concentrations above those used to inhibit prostaglandin synthesis, it inhibited substantially the induction of c-fos and other genes. Its mode of action is here proven not to be a non-specific toxicity effect, as shown by:

1. Reversibility of its inhibition; PMA induces expression of the gene equally well in cells preincubated for 1 hr with INDO, followed by removal of the drug.

2. PMA-stimulated [2-^{3}H]-deoxyglucose uptake was not inhibited by INDO.

3. Reversibility of growth inhibition by INDO has been documented in fibroblasts; we have shown that under the conditions used, INDO (at similar concentrations) has a reversible effect on cell growth.

Two other early PMA-inducible genes are also inhibited by INDO: c-myc, a gene which peaks at 60 min, and EGR (early growth response gene), a gene induced maximally 0.5-1 hr after stimulation with PMA. Both are inhibited by indomethacin under similar conditions.

Inhibition of these PMA inducible genes by non cytotoxic concentrations of indomethacin occurs when the drug is added 15 min before the phorbol ester. In the case of c-fos, inhibition was seen when INDO was added as late as 20 min after PMA.

The most recent observations indicate that the inhibition is temporary. In fact, c-fos and EGR mRNA levels were seen to rise, after an initial delay of 60-90 min, to near control levels. My suggestion is that the delay in the onset of specific mRNA synthesis is due to the action of INDO on the phosphorylation of a nuclear factor by a protein kinase. After the initial delay this factor is slowly builds up, interacts with the nuclear complex formed by AP-1 and p39 (thought to be the product of c-jun) and drives transcription of c-fos proto-oncogene to levels seen in cells treated without INDO.

The mode of action of INDO was consistent and reproducible throughout the study and may prove useful in exploring further the biochemical mechanisms of signal transduction.

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ABBREVIATIONS

CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
CLS	ATP Bioluminescence reagent
DMEM	Dulbecco's modified Eagle medium
EGF	epidermal growth factor
EGR	early growth-response gene
EtOH	ethanol
EtSH	mercaptoethanol
FGF	fibroblast growth factor
FBS	fetal bovine serum
HTCS	heat-treated calf serum
INDO	indomethacin
IP_3	inositol 1,4,5-trisphosphate
NaOAc	sodium acetate
HPDGF	platelet-derived growth factor
PGE_1	prostaglandin E_1
PIP_2	phosphatidylinositol bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
TCA	trichloro-acetic acid
SDS	sodium dodecyl sulfate

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I. INTRODUCTION

1. Oncogenes

Retroviral oncogenes

Retroviral oncogenes can induce the formation of tumors. There are more than twenty known retroviral oncogenes, each of which encodes a protein; an improved understanding of the biochemical action of retroviral oncogenes is adding to our understanding of neoplastic growth. At least nine of these viral oncogenes (v-abl, v-erb B, v-ets, v-mos, v-myb, v-myc, v-H-ras, v K-ras, v-sis) have normal cellular counterparts (proto-oncogenes) (Bishop 1987).

Viral oncogenes are thought to be derived from these normal cellular genes. Oncogenic retroviruses often have powerful promoters which cause sustained and abundant expression of a near-normal or more extensively mutated transduced proto-oncogenes. The capacity of oncogenic retroviruses to induce tumors may be explained by the expression of these viral oncogenes.

Proto-oncogenes

These genes are often cellular homologues of viral oncogenes. They exert a controlling influence on the molecular events causing the proliferation of normal cells. Increasing evidence supports this concept. For example:

a) Recombinant forms of cellular proto-oncogenes present in acutely transforming retroviruses act as dominant replication-stimulating agents (Denhardt et al. 1986).

b) Proto-oncogenes play a fundamental role in normal cellular metabolism, as suggested by evolutionary conservation (Bishop et al. 1987).

c) Numerous studies correlate neoplasia with perturbations of the proto-oncogenes (translocation, gene amplification, DNA methylation changes and point mutations), (Alexander et al. 1986, Denhardt et al. 1986).

d) Differential expression of some proto-oncogenes during embryonic development and tissue repair or remodeling implicates them in proliferative processes (Lockwood et al. 1987).

e) There are very significant homologies between proteins encoded by certain viral oncogenes and normal cellular growth factors or receptors for growth factors (Weinstein 1987).

f) The expression of at least five proto-oncogenes (c-myc, c-fos, c-ras, c-myb and p53) is enhanced in proliferating cells before the onset of DNA synthesis and therefore may regulate molecular events occurring at the beginning of the cell cycle (Denhardt et al. 1986).

2. Proto-oncogenes and Growth Control

Since the discovery of the cell cycle and of cell-cycle regulated genes (including proto-oncogenes), many investigators have focused on the study of their regulation, in the hope of determining the mechanics of cell replication and gene expression in eukaryotes.

Gene products of the src, abl, fps, insulin receptor and PDGF receptor families have been reported to have protein kinase activity (Denhardt et al. 1986).

Genes encoding several cell-phase restricted proteins have been cloned, the assumption being that they will provide answers as to the regulation of the cell cycle at the molecular level. The expression of cell-cycle regulated genes can be induced when quiescent mammalian cells (cells in resting, G_0 phase) are stimulated to reenter the cell cycle. The family of genes induced when cells are rendered competent to replicate their DNA in response to PDGF, is called the "competence gene" class. It includes the proto-oncogenes c-fos and c-myc, which are induced rapidly and transiently in response to growth factors and mitogens (Rozengurt 1986).

Since the c-fos gene is expressed as early as 5 min after stimulation of membrane receptors with growth factors or mitogens, it is a good candidate gene to study early signal transduction mechanisms.

3. The c-Fos Proto-oncogene

i. Origin and structure

In 1966, a murine osteosarcoma virus complex was isolated by Finkel, Biskis, and Jinkins (hence the name FBJ-MSV) from a tumor that arose spontaneously on the thoracic spine and ribs of a 260-day old CF1/An1 mouse. Injection of an osteosarcoma extract into newborn mice lead to development of bony tumors (Finkel et al. 1966). FBJ-MSV proviral DNA contains 4026 nucleotides, including two long terminal repeats (LTRs) of 617 nucleotides each, 1,639 nucleotides of acquired cellular sequences (c-fos), and a portion of the viral envelope gene. In cells transformed by FBJ-MSV, a phosphoprotein with an apparent MW of 55,000 (p55) was identified as the transforming protein (Curran et al.

1982). The sequences in the c-fos gene that are homologous to those in the v-fos gene are interrupted by four regions of nonhomology, three of which represent bona fide introns (Deschamps et al. 1985). A 104 bp deletion in the v-fos gene distinguishes it from the c-fos gene; this region was probably lost during biogenesis of v-fos. A 56 bp DNA segment approximately 300 bp upstream of the human c-fos mRNA cap site contains a sequence element with enhancer-like properties that is essential for polypeptide mitogen induction of c-fos mRNA levels (ibid).

Protein products of c-fos and v-fos are nuclear proteins (Curran et al. 1983), similar in size (380 and 381 amino acids respectively) but which differ at their carboxy-terminus due to 48 amino acids that are encoded in a different reading frame in c-fos. Both the viral and cellular fos proteins are post-translationally modified by phosphorylation (Curran et al. 1984, Kruijer et al. 1984).

The fos protein complex and several fos-related antigens bind directly or indirectly to a common sequence element that is similar to the consensus binding site for HeLa cell activator protein-1 (AP-1). This element is present in a negative regulatory sequence in the differentiation-sensitive adipocyte gene aP2, in a transcriptional enhancer for the Gibbon ape leukemia virus, in a region of the human immunodeficiency virus (HIV) long terminal repeat (partially characterized as a negative regulatory element) and the human metallothionein promoter (Franza et al. 1988).

The fos protein shares some identities with DNA binding domains of known transcriptional activators, like GCN4, a yeast DNA-binding protein that regulates expression of amino acid synthesizing enzymes and v-jun, a cellular insert of the avian sarcoma virus 17; there is also a

marginal degree of sequence similarity to c-myc proteins (Sassone-Corsi et al. 1988b). It is thought that those common protein regions bind DNA in a sequence-specific manner, exerting a regulatory function on specific promoter elements.

A recent report (Sassone-Corsi et al. 1988b) demonstrates that the c-fos protein itself specifically represses the induction of c-fos gene by serum. In this case, only the c-fos serum-inducible promoter elements are the molecular targets of repression; deletion analysis of a fos-CAT fusion plasmid has identified the region between -404 and -220 to be the target for trans-repression. In vitro binding studies indicate that this promoter element repressed by the c-fos protein _in vivo_ is associated with a nucleoprotein complex that contains the products of c-fos and c-jun and the transcriptional factor AP-1 .

The consensus sequence TGAGTCA with dyad symmetry required for AP-1 binding is different from the sequence in the dyad symmetry element, DSE, identified for c-fos induction by PMA; this suggests that the same inducer may recognize different sequences in the activation of various genes.

It is thought that upon binding to target sequences (such as the AP-1 site), the protein complex formed by fos, fos-associated proteins and p39 may modulate transcription via specific regulatory elements in the control regions of target genes and affect short-term and long-term responses to extracellular stimuli (Rauscher at al. 1988, Distel at al. 1987, Franza et al. 1988).

ii. Expression

Proto-oncogene c-fos is an inducible gene, which shows a complex pattern of tissue-, cell type- and growth stage-specific expression. During mouse prenatal development, c-fos transcripts accumulate to maximal levels in late gestational extraembryonal membranes (amnion, yolk sac) at day 18 (Muller et al., 1982), in placenta and mid-gestational fetal liver (Muller et al. 1984a) and, after birth, in whole bone marrow (Muller et al. 1984b). Expression of c-fos in the amnion is stage-specific in that c-fos mRNA levels are low during mid-gestation (around day 10) and increase as development proceeds, suggesting a correlation with cellular differentiation processes. Among hematopoietic cells high levels of c-fos mRNA have been detected only in mast cells (Conscience et al. 1986), differentiated neutrophils, blood monocytes and mature macrophages (Muller et al. 1984b, Gonda et al. 1984). These cell types express c-fos at high levels in the normal environment of the organism. Many cultured cell types are capable of expressing c-fos following exposure to mitogenic stimuli.

iii. Induced expression

a) Inducers

c-Fos gene expression has been shown to be induced in numerous cell types in response to a variety of growth factors and mitogens. In human epithelial carcinoma cells (A431) it is induced within a couple of minutes after addition of EGF (Bravo et al. 1985). When HL60 cells, (human promyelocytic leukemia cells) are induced to differentiate into macrophages in response to phorbol esters, they express high levels of c-fos (Kreipe et al. 1986); the same is also observed in U937 human

monocytic cells induced to macrophage differentiation (Forsbeck et al. 1985). Nerve growth factor stimulation of rat pheochromocytoma cells (PC 12) results in induced expression of c-fos (Morgan and Curran 1986, Greenberg et al. 1985). Treatment of G_0 arrested human foreskin fibroblasts (WI-38) with PMA induces c-fos gene expression to maximal levels after 30 min (Calabretta and Mercer 1987).

A multitude of quiescent mouse fibroblast cell types have shown that rapid c-fos expression is attained 30 min following fetal calf serum or growth factor stimulation. In quiescent Swiss 3T3 cells treated with EGF or insulin, fos is induced maximally after 20 min (Lim et al. 1987). After PDGF, FGF or PGE_1 treatment, Swiss 3T3 cells express maximal levels of fos within 45-60 min (Tsuda et al. 1986).

In quiescent NIH 3T3 cells, PDGF or FGF cause maximal induction of fos at 20 min, the mRNA then declines to undetectable levels after 60 min (Muller et al. 1984a, Kruijer et al. 1984). Bravo and his collaborators (1986) have shown serum inducibility of c-fos mRNA during the S phase of the cell cycle. The tumor promoter PMA, induces fos to maximal expression after 30 min (Rabin et al. 1983). In quiescent Balb/c-3T3 cells, c-fos attained its highest level of expression 30 min after PDGF stimulation (Cochran et al. 1984), and 40 min after addition of serum (Lau and Nathans 1987).

Thus, PMA appears to be a universal inducer of c-fos mRNA expression since it has been shown to be equally effective in various cell types including fibroblasts, epithelial cells, lymphoid cells and myeloid precursors.

b) Possible pathways involved in the transduction of signal from the membrane to the nucleus following growth factor or mitogen stimulation

Addition of PDGF and other growth factors to quiescent cells results in a cascade of early events; the first to occur is a rapid increase in Ca^{2+}, Na^{+}, K^{+}, H^{+} flux across the plasma membrane. It was described that PDGF and EGF induce rapid phosphoinositide turnover resulting in both protein kinase C activation and Ca^{2+} mobilization (as early as 15 sec after addition) in Swiss 3T3 cells, whereas cAMP and Ca^{2+} are responsible for PGE_1-induced increase of fos mRNA in Swiss 3T3 cells (Habenicht et al. 1981, Berridge et al. 1984a, Tsuda et al. 1985, Rozengurt et al. 1983).

Ca^{2+} is released from intracellular stores by activation of the plasma membrane Ca^{2+} dependent adenosinetriphosphatase (Ca^{2+}-ATP-ase). Its mobilization is mediated by inositol 1,4,5 trisphosphate (IP_3), a second messenger in the action of growth factors and hormones. IP_3 is formed as a result of increased hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane by phospholipase C. Another product of this reaction is diacylglycerol (DAG), a potent activator of the Ca^{2+}-sensitive, phospholipid-dependent PKC. When activated, PKC leads directly or indirectly to increased Na^{+}/H^{+} antiport, resulting in increased intracellular pH, increased Na^{+} influx and Na^{+}/K^{+} pump activation (Rozengurt 1986).

The liberation of arachidonic acid can occur directly from phospholipids (particularly phosphatidylcholine and phosphatidyl-ethanolamine) by the action of phospholipase A_2, or from PIP_2 through two consecutive reactions catalyzed by phospholipase C and

diacylglycerol lipase. The arachidonate that is liberated in any of these pathways may be rapidly converted to various biologically active metabolites: prostaglandins, thromboxanes and leukotrienes. Prostaglandins can interact with the receptors producing cAMP and constitute a feedback control of cellular functions and proliferation by down-regulation of the phospholipases.

These are early signals in the membrane and cytosol, and may play an important role in the biochemical route leading to mitogenesis and stimulation of early nuclear events, such as induction of the family of competence genes including the c-fos and c-myc proto-oncogenes. Some of the pathways thought to be important in signal transduction are summarized in diagram 1, adapted from Weinstein 1987.

iv. Levels of regulation of c-fos gene expression

Control of cytoplasmic mRNA levels can occur at different levels: transcription, processing and transport, stability of mRNA and post-translational regulation. In the case of c-fos proto-oncogene several sequences have been determined to play an important role in its transcriptional regulation as well as messenger stability.

a) 5' Elements regulating transcription

The TATA box. The sequence located in front of the gene, between -25 and -32 bp is required for proper initiation and orientation of transcription.

Upstream elements required for basal transcription. An element containing the consensus sequence TGACGTA/T, located between -57 to -63 in the human, mouse and chicken c-fos genes regulates the basal

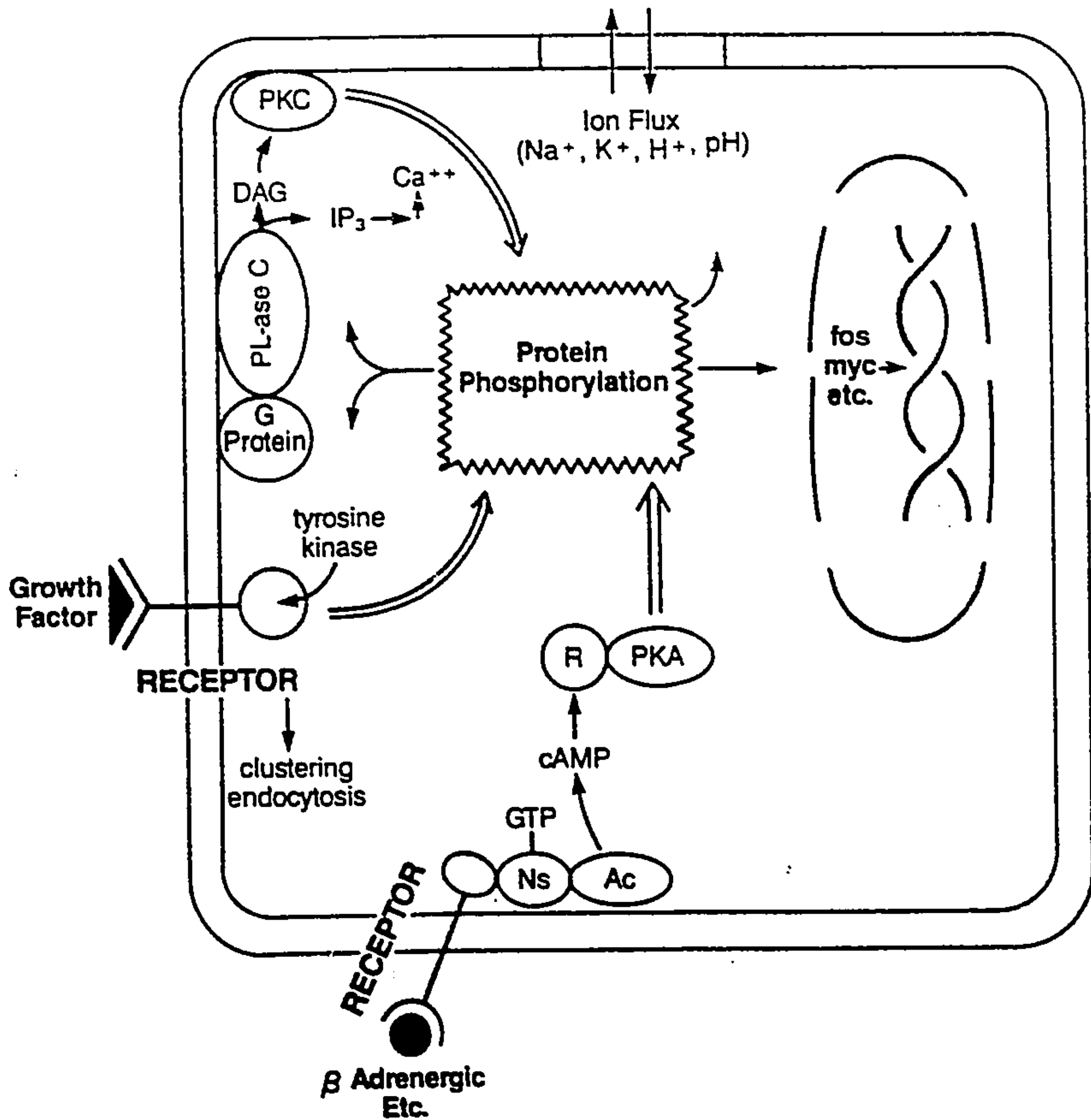


Diagram 1 - Possible pathways involved in signal transduction, from Weinstein 1987.

expression of the gene. If this region is deleted, the transcription level is reduced by 10-fold (Fisch et al. 1987). This sequence in the mouse c-fos gene is protected from DNase I digestion, following incubation with nuclear extracts (Gilman et al. 1986), suggesting the promotes the expression of the gene. Binding of a nuclear factor to this enhancer sequence is not sufficient for the induction of the gene, but rather it requires the cooperation of downstream factors for optimal induction of c-fos expression. Sequence homology studies indicate a strong resemblance with the consensus sequence of cAMP-regulated promoters (Montminy et al. 1986) and the upstream promoter of several adenovirus genes.

A second element required for basal transcription of the human c-fos gene is a direct repeat located between -76 and -97; deletion of this element decreases transcription 5- to 10-fold (Fisch et al. 1987).

Inducible elements. The 5' enhancer region situated between -297 and -317, comprises a region with dyad symmetry, termed DSE. This region is required for induction of the c-fos gene with serum, PMA and EGF (Prywes and Roeder 1986, Greenberg et al. 1987). The DSE is conserved among chicken, mouse, and human genes and is similar to sequences in the enhancers of polyoma virus, SV40, and the Moloney murine leukemia virus LTR. There are also other sequences involved in promoting the basal level of expression but they are not neccessarily conserved between the mouse and human c-fos genes (Fisch et al. 1987).

b) Importance of 3' sequences

Although both v- and c-fos proteins can transform fibroblasts, transformation by c-fos requires removal of a 67 bp AT-rich region from

the gene's 3' non-coding sequence (Meijlink et al. 1985). This region contains multiple AUUUA motifs that destabilize the mRNA (Shaw and Kamen 1986); its removal prolongs substantially the half-life of the mRNA (Treisman 1985). Therefore one level of regulation of the c-fos gene product is at the posttranscriptional level, involving messenger stability.

c) Post-translational regulation

After transcriptional activation, c-fos mRNA is processed and transported to the cytoplasm, where it is translated into the c-fos protein. The protein undergoes phosphorylation in the cytoplasm before being conveyed to the nucleus (Curran and Morgan 1988). Almost immediately upon entry into the nucleus, the fos protein forms a complex with p39 and rapidly undergoes extensive post-translational modifications of an unknown nature (ibid.).

A recent report establishes that the nuclear transcription factor AP-1 and the fos-associated p39 are in fact the same. This conclusion relies on several lines of evidence: both AP-1 proteins from mouse and human cells have an apparent molecular mass of 39,000 daltons which is similar to that of p39, a 39,000 dalton protein that can be immunoprecipitated with anti-jun (anti-AP-1) antibodies, two dimensional tryptic peptide maps of ^{35}S-labeled p39 and jun proteins are identical, sequence identity is 98% and p39 was shown to bind to a canonical AP-1 site, TGACTCA (Rauscher et al. 1988). The role of the fos protein in the transcription complex formed with AP-1 is still a subject of speculation, but several possibilities were suggested. The AP-1 binding site being symmetrical, it is possible that binding of the fos protein

may facilitate the formation of dimers and stabilize the structure. Fos could also be needed by this DNA-binding complex for efficient transcriptional activation or it may be implicated in post-translational modification of AP-1 (Sassone-Corsi et al. 1988b).

4. Other PMA-inducible Early Genes

i. c-Myc

Another member of the group of competence genes is the c-myc proto-oncogene. It is induced in response to a variety of factors such as: growth factors (EGF and PDGF) in human fibroblasts (Paulsson et al. 1987), serum or PDGF in resting Balb/c-3T3 cells (Lau and Nathans 1987), EGF or calcium ionophore in density-arrested Balb/c-3T3 cells (Ran et al. 1986) and bombesin in quiescent Swiss 3T3 cells (Bravo et al. 1987). Like c-fos, this gene is induced rapidly. Maximal c-myc mRNA levels are attained 60 min after treatment of quiescent fibroblasts with serum or growth factors. It has a biphasic induction of expression, reaching a second smaller peak at 6-10 hours and returns to the basal level of expression at 18 hours (Curran et al. 1983). Since both c-fos and c-myc encode nuclear proteins (Curran et al. 1984, Abrams et al. 1982, Persson and Leder 1984), it is plausible that their transient expression may play a role in the transduction of the mitogenic signal to the nucleus.

ii. EGR

The EGR-1 is an early response gene having fos-like induction kinetics in several tissue types, such as fibroblasts, epithelial cells and lymphocytes after mitogenic stimulation. Although EGR-1 and c-fos

proteins are structurally unrelated, both are localized in the nucleus, where they probably regulate transcriptional events. Like c-fos, transcription of EGR-1 occurs rapidly following treatment with various stimuli (mitogens, differentiation factors, PMA); maximal expression of the EGR-1 gene (transcript size is 3.7 kb) occurs 0.5 hr after stimulation with PMA (Sukhatme et al. 1988). The temporal modulation of expression of EGR-1 and the presence of zinc finger motifs in its primary structure suggest that it may function as part of a regulatory cascade of gene interactions (ibid).

5. Statement of Objectives

The objective of this project is to explore the intervening steps between membrane receptor stimulation and the subsequent induction of expression of genes early after mitogenic stimulation. Due to the fact that regulation of these genes is intricate, we attempted to simplify the system by using a protein kinase C activator (PMA) to induce these genes and probed subsequent steps using inhibitors which might interfere with the signal transduction process.

Three genes which are induced by PMA treatment were used for this study: c-fos, c-myc and EGR-1.

II. MATERIALS AND METHODS

1. Conditions of Cell Culture and Treatment with PMA and Various Inhibitors

i. Preparation of growth-arrested cells

a) Quiescent Swiss 3T6 cells

On day 0, 0.7×10^6 cells were seeded per 100 mm Petri dish, with 15 mL DMEM + 7% fetal bovine serum. The cell surface was rinsed twice with DMEM containing no serum on day 1, then 15 mL DMEM + 0.3% FBS were added. On day 3, the medium was removed and 15 mL DMEM + 0.3% FBS were added. Cells were used on day 4.

b) Confluent Balb/c-3T3 cells (clone A 31)

Cells were seeded at 0.6×10^6 cells per 100 mm dish on day 0, using 15 mL DMEM + 10% HTCS, and incubated for 5 days. Then, the medium was removed gently by suction and 15 mL DMEM + 10% HTCS were added. On day 7 cells were confluent.

ii. Conditions for cell treatment

Cells were incubated during short-term treatment with drugs in a balanced salt solution lacking Ca^{2+} and containing 5 mM glucose. The different media used in experiments are listed in the Appendix. G_0 arrested cells (serum quiesced or grown to confluence) were taken out of the incubator and the growth media was carefully removed so as not to disturb the cells. The monolayer was washed gently once with 10 mL prewarmed (37°C) medium and 10 mL treatment medium containing the

experimental drug was added. In experiments where INDO was used, cells were preincubated in medium with or without INDO for 15 min and then PMA was added. The concentration of PMA used throughout this study was 10^{-6} M unless otherwise stated. The final concentration of DMSO, the solvent for PMA was 0.1%.

At different times after treatment, cells were harvested for RNA extraction.

2. RNA Extraction Procedure

The technique used is as described by Birnboim (1988). In brief, after experimental treatments, medium was removed and the cell monolayer was dissolved in RES, a solution that contains chaotropic agents that disrupt the lipid bilayer (LiCl, SDS and urea), a strong chelator (CDTA), protein denaturants (SDS and urea) and a buffer (sodium citrate) (Appendix). Proteins were digested by incubating the cell lysate with proteinase K (50 μg/mL). The partially purified nucleic acid material was then concentrated by ethanol precipitation. DNA and tRNA were separated from RNA by precipitation of the latter with LiCl at 0°C. After two washes with EtOH, the RNA was resuspended in RNA buffer. RNA concentration was calculated from the absorbance of the sample at 260nm using a spectrophotometer (Perkin-Elmer, lambda 5 UV-Visible spectrophotometer, 1 OD_{260} unit= 42-45 μg/mL RNA). Other details are given in Birnboim (1988).

3. Purification of Human Lymphocytes

The method used was essentially that described in the Pharmacia instruction manual for the use of Ficoll-Paque gradients.

To 50 mL freshly collected blood was added 50 mL of balanced salt solution. 3 mL of Ficoll-Paque (d = 1.077 g/mL) was added to each of 25 siliconized conical pyrex tubes (capacity 15 mL). Onto each Ficoll-Paque cushion was carefully layered 4.0 mL of the initial blood dilution and each gradient was centrifuged (Sorvall RC-5B refrigerated centrifuge, SS-34 rotor) at 400xg for 25 min (4°C). Using a "right angle" Pasteur pipet, as much as possible of the plasma phase (top yellow layer) was removed, trying at the same time not to disturb the lymphocyte layer (turbid band). The lymphocyte-rich layer was removed and transferred to a fresh pyrex tube; 3 volumes of medium IV were added, the contents were mixed and centrifuged for 10 min as above. The supernatant was removed with an aspirator and the lymphocyte pellets were suspended in 1-2 mL of salt solution (the type of salt solution used in each experiment is specified in the Results section). The recovery of cells was estimated by counting in a Coulter Counter. Purity (proportion of lymphocytes) was determined microscopically, as described below. Lymphocytes were then incubated as described in Results, in 15 mL siliconized tubes at approximately 2×10^6 M cells/mL.

4. Cytospinning and Differential Staining

A Shandon cytospin filter was used to deposit cells onto a glass slide for staining. The cell suspension was loaded in the sample holder

(0.5 mL containing 5,000 cells) and spun for 5 min at 400xg. The cells were then fixed by dipping the slide into methanol (MeOH) for 5 sec, then in BDH Wright's staining solution for 5 sec, followed by washing in water, then drying. A tiny drop of immersion oil was added to the slide and the cells were covered with a cover slip. The purity was then estimated by counting the cells of interest.

5. RNA Extraction Procedure from White Blood Cells

The following procedure was used for 25 x 10^6 white blood cells. Cells were suspended in 200 μL solution I containing Ca^{2+} (0.8 mM), glucose (5 mM) and lysed with 4 mL RES 1 (see Appendix), containing proteinase K (50 μg/mL). After incubation at 50°C for 30 min, the cells were chilled on ice (2 min); 0.26 mL of 2 M NaOAc and 2 volumes of EtOH were added. Samples were held for 20 min at -20°C, then centrifuged at 12000xg, 10 min, at 0°C. The EtOH was drained off and the pellet was dissolved in 0.4 mL RES-1 and transferred to 1.5 mL conical Sarstedt tube. 100 μg/mL proteinase K was added and samples were incubated 30 min at 50°C. Extraction with 50 μL phenol/chloroform (1 g/1 mL) was done by vortexing repeatedly over a 2 min period, then centrifuging 5 min in a micro-centrifuge. The supernatant was removed and the phenol/chloroform phase was back-extracted with 100 μL RES-1. The aqueous phases were combined. The aqueous phase was extracted with 50 μL chloroform (vortexed 20 sec.) and centrifuged for 2 min. The supernatant was transferred to a Sarstaedt conical 1.5 mL tube. 0.5 mL of LiCl/EtOH and 4 μL of 2 N acetic acid were added. Samples were left at 0°C overnight. Each tube was centrifuged for 2 min at room

temperature and the supernatant (containing DNA and tRNA) was discarded. Samples were centrifuged briefly and the last few μL of supernatant were carefully removed. The pellet was dissolved in 100 μL CT solution and the RNA was again precipitated with LiCl (100 μL LiCl/EtOH and 2 μL 2 M NaOAC) at 0°C overnight (see Appendix). Samples were centrifuged 2 min, the supernatant was removed and the pellet was dissolved in 50 μL CT solution. Another LiCl/EtOH precipitation was performed: 100 μL LiCl/EtOH + 2.5 μL 2 M NaOAC were added, samples were precipitated 5 min at -70°C 15 min at -20°C and spun. The supernatant was discarded, the pellet dissolved in an appropriate buffer and stored at -20°C.

6. Northern Blot Procedure

i. Denaturation of RNA samples

10 μL of a given RNA sample (dissolved in CT) was denatured by addition of: 5 μL formaldehyde/phosphate (100 μL HCHO, 6.6 μL 1 M sodium phosphate pH 6.8, 94 μL formamide) and 5 μL formamide. Incubation was carried out at 55°C, for 30 min. After chilling on ice for 2 min, the samples were loaded onto a denaturing agarose gel (1.5 mm thick gel containing 0.2 M formaldehyde, 1.2% agarose, 2 mM CDTA and 20 mM sodium phosphate (pH 6.8) and electrophoresed, using a Sturdier Vertical Slab Gel Unit, 1.5 hours at 150 V (Model SE 400, Hoefer Scientific Instruments).

ii. Transfer of RNA from agarose gel to Hybond-N membrane

a) Blotting method

RNA transfer was carried out after the bromophenol blue dye marker had migrated approximately 9.5 cm. Blotting transfer of RNA involves

passing a flow of buffer solution (10xSSC) from a reservoir through the gel in contact with a sheet of nylon membrane (Hybond-N, Amersham), by capillary action so that RNA fragments are eluted from the gel and are deposited onto the membrane. The transfer was generally allowed to progress for 2 hr. The RNA was fixed by drying the filter membrane at 80°C (30 min) and cross-linking under UV light (8 min). After hybridization with radioactive probes the filters were stained with methylene blue. The procedure involved a 5 min wash in 95% EtOH to remove SDS present in hybridization washes, a 5 min wash in 0.5% acetic acid, followed by 15 min staining using 0.1% methylene blue in 50% ethanol. Removal of excess dye was done by destaining for 15-20 min under running warm water. The position of the ribosomal RNA was then marked on the autoradiogram for reference as a size marker.

b) Vacuum method

In the last few experiments transfer was done using a vacuum blotting system (Vacublot, American Bionetics). Transfer of RNA from the gel to a Hybond-N membrane was performed in 10xSSC, under a vacuum of 3.92 kPa (40 cm H_2O), for 20-30 min.

c) Source of plasmids

Fos-1 plasmid was from T.Curran, constructed from a fragment of the FBJ murine osteosarcoma virus inserted in pBR322; its size is 5.6 kbp (Curran et al. 1982).

pSVC-myc-1, obtained from R.Weinberg, was originally constructed by insertion of two c-myc exons (size 9.5 kb) of MOPC 315 plasmacytoma into pSV2 vector (Land et al. 1983, Shen-Ong et al. 1982).

pEGR #332, the plasmid used for EGR-1 probing consisted of a 700bp unique sequence, inserted into pUC13. It was obtained from V.Sukhatme (Sukhatme et al. 1988).

d) Procedure for nicking of plasmid DNA probe

Approx 8 μL of plasmid DNA (1.0 μg) in CT solution (see Appendix) were mixed in a 0.5 mL Eppendorf tube. The plasmid was nicked by boiling at 100°C for 5 min. The sample was chilled (0°C) briefly, then centrifuged 5 sec in a microcentrifuge. NaOH treatment, to denature and cleave at apurinic sites, was then performed: 2 μL 0.5N NaOH were added and incubation was done at 37°C overnight or 30 min at 50°C. Samples were centrifuged for 2 min, then 40 μL of sterile water was added such that the final DNA concentration was 20 ng/μL.

e) Labelling procedure

The oligonucleotide-primer method was used (Feinberg and Vogelstein 1983). Details are given in the Appendix.

f) Monitoring incorporation

2.5 hr after the start of the reaction, a small amount of the labelling mix ($\leq$ 1 μL) was precipitated with 1 N HCl in the presence of carrier RNA (Appendix). The precipitate containing labelled probe was separated from the ^{32}P-labelled dCTP centrifugation. The acid-soluble and acid precipitable fractions were separated and counted in the 1219 Rackbeta Liquid Scintillation Counter.

g) Termination of labelling reaction

5 μL of 10 mM CDTA and 5 μL of 5% SDS were added to each reaction mixture.

iii. Prehybridization and hybridization procedure

The prehybridization solution contained 5 mg/mL yeast RNA, 6xSSC, 5xDenhardt's solution (see the Appendix) and 0.5% SDS in a total volume of 10 mL. Prehybridization of filters was done in 12x15 cm dishes containing 10 mL prewarmed solution, at 65°C, for 30 min.

The hybridization solution contained 2.5 mg/mL yeast RNA, 6xSSC, 5xDenhardt's solution, 0.5% SDS and 5% Dextran sulfate in a final volume of 10 mL.

After prehybridization, the filters (1-2) were transferred to a BRL polypropylene bag, 3 of the sides were tightly sealed and hybridization solution containing the probe of interest was introduced into the bag with a Pasteur pipet. The last side was then sealed and hybridization was allowed to proceed at 62°C for at least 12 hr.

iv. Removal of radioactive probe: filter washing

After hybridization, the probe was removed from the incubation bag with a Pasteur pipet and transferred to a Pyrex tube (capacity 50 mL) for further use. The filter was washed in the bag twice with 2xSSC/0.1% SDS at room temperature; the wash-solution was then removed from the bag and the filter was transferred to a container filled with 25 mL fresh wash solution. The filter was washed twice in this solution (approximately 3 min for each wash), then washed twice in 25 mL of 0.16xSSC/10 mM Na citrate/0.1% SDS at room temperature (3 min for each

wash). The last washes were repeated in a shaking waterbath with the solution warmed to 62°C. A final rinse was done with 25 mL of 2xSSC/0.1% SDS.

The filter was dried for 20 min in an 80°C oven, followed by exposure to Kodak (XAR.5) X-ray film (-70°C, 4-24 hr).

7. ATP Measurements of PMA and INDO-treated Balb/c-3T3 Cells

Multiple cultures of confluent Balb/c-3T3 cells, grown in a 24-well plate, were preincubated with INDO for 15 min and then PMA was added for 30 min. Quadruplicates of each treatment were used. At the end of this incubation, hot TCPX (5 mM Tris HCl, 1 mM CDTA, 1 mM sodium phosphate pH 6.8) was added in the 1A - 6A direction. The TCPX extract was then briefly heated (37°C), then chilled. To a 20 μL sample of each extract was added an equal volume of CLS (Bioluminescence) reagent (Boehringer-Mannheim). After careful mixing, an aliquot of each sample was counted in a liquid scintillation counter.

8. Measurement of ^{3}H-Thymidine Incorporation

On day 1, cells were seeded at 10^4 cells/well in a 24-well plate. On day 2, INDO and the appropriate control (in medium I) were added; 1 μCi of ^{3}H-thymidine was added to one third of the wells. On day 3, incorporated ^{3}H-thymidine was counted (acid-precipitable fraction); another third were washed with prewarmed medium I, exposed to ^{3}H-thymidine for 24 hr and then counted. At the end of each period of incubation with ^{3}H-thymidine, the cells were lysed with 500 μL NaOH

(0.25 M). 80% of that lysate was centrifuged, then resuspended in 500 μL NaOH (0.25 M) and centrifuged. 400 μL of the nucleotide pellet was counted in a liquid scintillation counter. Each experiment included controls (one third of the wells) for the efficiency of radioactivity removal.

9. Growth Assessment Following Treatment with INDO

Balb/c-3T3 cells were seeded at 10^5 cells/mL in a 60 mm dish on day 1. INDO additions were as in section 8., above. Growth of cells was assessed by counting the total number of cells/plate. Counts were done on day 3 and 4.

10. Measurement of ^{3}H-Uridine Incorporation

The experiment was performed with confluent Balb/c-3T3 cells cultured in 8 10cm plates. Treatment medium V containing INDO (1.0 mM) or sodium acetate (1.0 mM, as a control) was incubated overnight at 37°C in 5% CO_2 to allow temperature and pH equilibration. The cells were washed once with prewarmed medium and incubated 15 min with INDO containing-, or control- media. PMA (10^{-6}M) and ^{3}H-uridine (50μCi, with a specific activity of 21 Ci/mmol) were then added concomitantly. After a 30 min incubation at 37°C, in a 5% CO_2 atmosphere, cells were washed with ice-cold PBS, then SDS (final concentration 0.1%) was added for the lysis of cells. A 1 mL aliquot of the lysate was counted, to represent the total cell-associated radioactivity. Another mL of lysate was precipitated with TCA (10%) for 1hr, washed twice with TCA, then

resolubilized in 500 μL NaOH (0.25 M) at 37°C for 30 min. 400 μL of this material was counted and constitutes the fraction of ^{3}H-uridine that was incorporated into RNA. The difference between total cell-associated and TCA-precipitable counts are cell soluble counts (unincorporated cell associated radioactivity). Correction for the specific activity of the radioactive pools was done by dividing the precipitable fraction by the soluble one.

11. Measurement of [2-^{3}H]-Deoxyglucose Uptake

Confluent Balb/c-3T3 cells, cultured in a 24-well plate were pre-incubated with INDO (15 min) then incubated with PMA in medium I containing glucose. 75 min after addition of PMA, the medium was removed, cells were washed once in prewarmed medium I without glucose, and further incubated in this medium with PMA, INDO, PMA + INDO, or PMA + [2-^{3}H]-deoxyglucose (0.5 μCi/well, specific activity 30-60 Ci/mmol). After 15 min incubation, the medium was removed, cells were washed once in medium I, then lysed with SDS (final concentration 0.1%). An aliquot (750μL) was counted directly by scintillation counting.

III. RESULTS

1. Preliminary Results

i. Choice of labels for probes: comparison of biotin and ^{32}P-labelled DNA probes for Northern analysis

Biotin-labelled pfos-1 probes were used in preliminary experiments to determine their suitability for Northern analysis. This type of probe has the advantage of stability and avoids the use of radioisotopes. Our laboratory had experience with this technique prior to my arrival. c-Fos mRNA could readily be detectable in some experiments but the bands were not readily quantified and the backgrounds were variable. Backgrounds were very sensitive to handling of the filter during transfer, washing and drying. ^{32}P-labelled probes, although shorter lived and more hazardous, proved to be more reproducible and allowed better quantitation of mRNA by densitometry. All subsequent experiments were carried out using ^{32}P-radiolabelled probes.

ii. Choice of cells and method of growth arrest

The mouse fibroblast cell types used for this study were Swiss albino 3T6 and Balb/c-3T3. The qualities required for our study were ease of culturing and reproducibility of the cultures, good induction of c-fos mRNA by mitogen and good yield of RNA.

Swiss albino 3T6 cells were rendered quiescent by 2-3 days of serum starvation. Such G_0-arrested cells were found to be inducible for c-fos mRNA by PMA. These cells yield a low cell number per plate (less

than $2x10^6$ cells) and secrete hyaluronic acid and collagen, which appeared to contaminate RNA samples and form insoluble complexes, which reduced RNA recovery. A stronger expression of the c-fos gene following induction was obtained in Balb/c-3T3 contact-inhibited cells. The higher cell number/plate led to a higher cell recovery. 60-200 μg/plate was obtained from Balb/c-3T3 cells whereas 16-50 μg RNA/plate was recovered from Swiss 3T6 cells. Therefore, after the preliminary experiments, further work was carried out with Balb/c-3T3 cells unless specified otherwise.

iii. Use of inhibitors

In order to investigate the signal transduction pathway for induction of c-fos mRNA in growth arrested cells, we induced the expression of the gene with the phorbol ester PMA for 30 min and added various inhibitors concomitantly. Detection of c-fos messenger RNA in these early experiments was done using biotin-labelled probes. Among the inhibitors used were active oxygen scavengers, ionophores, metabolic inhibitors and prostaglandin pathway inhibitors. No clear inhibitory or stimulatory effect could be detected with the following: ETYA (a cyclo-oxygenase pathway inhibitor, Goodman and Gilman 1985), gossypol (an inhibitor of oxidative processes, Qian and Wang 1984), catechol (a compound that reacts with superoxide anion; its rate constant is $1x10^9$ at pH 7, Halliwell and Gutteridge 1985). Slight stimulation over PMA-induced levels was obtained with NDGA (an inhibitor of the oxygenation reaction in the lipoxygenase pathway, Pryor 1982), arachidonic acid and vanadate (an inhibitor of phosphoprotein phosphatase, Earp et al. 1983).

In two experiments out of three there was inhibition of c-fos mRNA induction with A23187 (a calcium ionophore, Pressman 1976), used at a

final concentration of 3 μM (Vincentini et al. 1985); the inhibition disappeared in the presence of the chelator EGTA, and addition of extracellular Ca^{2+} with A23187 did not induce c-fos.

Partial inhibition was recorded with mellitin (a phospholipase activator), bromophenacyl bromide (BPB, an inactivator of phospholipase A_2), cumene hydroperoxide (an oxidant), superoxide dismutase (SOD) and sodium fluoride.

The most consistent and significant inhibition ($\geq$80%) was obtained with indomethacin (INDO). Like all nonsteroidal anti-inflammatory drugs, it has a lipophilic group (benzoyl group), and an acetic acid side chain with a pK_a value of 4.5 (Bayer and Beaven 1981). The fact that INDO action is pH dependent (as discussed later), can be accounted for by this acidic moiety which loses its positive charge in acidic medium, allowing more efficient uptake into the cell (diagram 2).

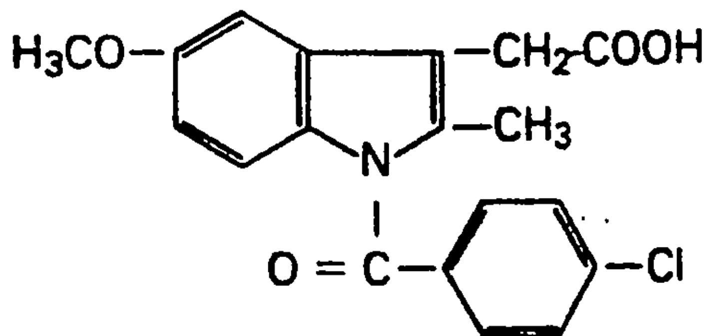


Diagram 2 - Structure of indomethacin.

At concentrations higher than those conventionally used for inhibiting the cyclooxygenase pathway, this drug significantly inhibited the induction of c-fos by PMA. This result was the most unexpected and

because of the consistency in the inhibition pattern, we decided to pursue the study of this inhibition in Balb/c-3T3 cells.

2. Conditions Chosen for Subsequent Studies

i. Treatment medium and conditions for induction of c-fos mRNA

The standard mitogen stimulant for induction of c-fos mRNA synthesis was PMA at 10^{-6}M. Initial experiments were conducted in a simple defined salt solution buffered at pH 7.4, medium I (see Appendix). Confluent cells in a 10 cm plate were washed, then incubated for treatment in medium I at 37°C, in an atmosphere of 10% CO_2, in the absence of phenol red pH indicator. It was subsequently noted that the pH dropped to about 6.7 at the end of an hour under these conditions, although c-fos mRNA was readily induced by PMA.

ii. Indomethacin effect on c-fos mRNA levels

Fig. 1 shows inhibition of c-fos mRNA induction in medium I; 50 μM INDO inhibited slightly whereas 100 μM inhibited significantly.

iii. Use of other treatment media for PMA induction and INDO inhibition

A 1986 report indicated that INDO inhibits the "A" system of amino acid transport in BRLT rat hepatocyte cultures with an IC_{50} (concentration needed for 50% inhibition) at pH 7.0 and 7.4 which is higher than at pH 6.5 (Seng and Bayer 1986). This suggested that INDO action or uptake is very pH dependent and therefore I analyzed the effect of INDO in different media with better pH control. The Northern

Fig. 1: INDO INHIBITS c-Fos mRNA INDUCTION BY PMA IN MEDIUM 1. Growth-arrested Balb/c-3T3 cells were treated with INDO (-15 min) and PMA, the RNA was extracted 30 min after the addition of PMA (unless otherwise stated), run on a denaturing agarose gel, blotted and probed with the specified ^{32}P-labelled plasmid DNA.

Exp I:
- A: Control (no addition)
- B: PMA
- C: PMA + 50μM INDO

Exp II:
- A: Control (no addition)
- B: PMA
- C: PMA + 100μM INDO

In this and subsequent figures, the position of 28S rRNA is indicated by the upper brackets and the position of 18S rRNA is indicated by the lower brackets. rRNA was detected by methylene blue staining of filters following exposure to X-ray film.

I II

A B C A B C

blots in Fig. 2 compare the degree of inhibition by 0.25 - 1 mM INDO of PMA induction of c-fos mRNA synthesis in solutions IV and V, where pH is better controlled, at pH 7.0 and 7.3. Inhibition at pH 7.0 increased with concentration, reaching almost 100% at 1 mM INDO. At pH 7.3, inhibition at 1 mM INDO was strong in one experiment and weaker in a second.

More physiological control of intracellular pH occurs in the presence of CO_2. Cells were further treated with PMA with or without INDO in medium V (pH 7.2 and incubated in 5% CO_2), conditions under which the pH does not decrease below 7.0. 150-500 μM INDO did not have any inhibitory effect but as shown in Fig. 3, 1 mM INDO inhibited $\geq$80%.

Most of the following experiments were done at pH 7.0 in air (medium IV), but some recent ones used medium V which utilized $NaHCO_3/CO_2$ buffers to control pH.

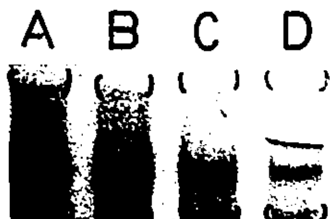
3. Indomethacin in Other Cell Types

It was interesting to determine if INDO could inhibit c-fos mRNA induction by PMA equally well in other cells, under conditions similar to those used in Balb/c-3T3 (medium IV). In Swiss 3T6 cultures, 0.5 mM had no effect but 1 mM inhibited the gene strongly (Fig. 4,I). The inhibitory effect of INDO on induced c-fos expression was similar in human lymphocytes, where 1 mM INDO strongly inhibited (Fig. 4,II). In U937 cells the induction of c-fos by PMA was weaker than in mouse fibroblasts. However, it appears that 1 mM INDO was also able to inhibit the induction of the gene (Fig. 5).

Fig. 2: STUDY ON INHIBITORY CONCENTRATION OF INDO AT pH 7.0 AND 7.3 IN TWO SEPARATE EXPERIMENTS (I and II)
The cell treatment and RNA analysis were done as described in Fig. 1

A: PMA, pH 7.0
B: PMA + 0.25 mM INDO, pH 7.0
C: PMA + 0.5 mM INDO, pH 7.0
D: PMA + 1.0 mM INDO, pH 7.0
E: PMA, pH 7.3
F: PMA + 0.25 mM INDO, pH 7.3
G: PMA + 0.5 mM INDO, pH 7.3
H: PMA + 1.0 mM INDO, pH 7.3

I.



II.

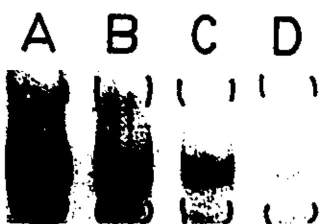


Fig. 3: INHIBITION BY 1 mM INDO IN MEDIUM IV (5% CO_2, pH 7.2)
The cell treatment and RNA analysis were done as described in Fig. 1

A: Control (no addition)
B: PMA
C: PMA + INDO

A B C

Fig. 4: INHIBITORY EFFECT OF INDO ON INDUCED c-Fos mRNA EXPRESSION IN DIFFERENT CELL TYPES
The cell treatment and RNA analysis were done as described in Fig. 1

I. IN SWISS 3T6 CELLS:
- A: Control: no addition
- B: PMA
- C: PMA + 0.5 mM INDO
- D: PMA + 1.0 mM INDO

II. IN HUMAN LYMPHOCYTES:
- A: Control: no addition
- B: PMA
- C: PMA + 0.25 mM INDO
- D: PMA + 0.5 mM INDO
- E: PMA + 1.0 mM INDO

I. A B C D

II. A B C D E

Fig. 5: STUDY ON INDO INHIBITION OF c-Fos INDUCED EXPRESSION IN U937 CELLS
The cell treatment and RNA analysis were done as described in Fig. 1

Exp I, A: Control
B: INDO (1 mM)
C: PMA (62 nM)
D: PMA (62 nM) + INDO (1mM)

Exp II, A: PMA
B: PMA + 0.25 mM INDO
C: PMA + 0.5 mM INDO
D: PMA + 1.0 mM INDO

I. A B C D

( ) ( ) ( ) ( )

( ) ( ) ( ) ( )

II. A B C D

( ) ( ) ( ) ( )

4. Indomethacin Inhibits Other Early PMA-Inducible Genes

In medium V (pH 7.2, 5% CO_2), 1 mM INDO inhibited the induction of EGR (early growth responsive gene), a PMA inducible gene maximally expressed at 30-90 min after induction in Balb/c-3T3 cells (Fig. 6,I). Inhibition clearly occurred, although c-fos was inhibited more strongly when another aliquot of the same RNA samples was tested (see Fig. 3).

Strong inhibition by INDO was also observed for c-myc mRNA. Fig. 6,II shows that, at 60 min, the point of maximal induction of this gene by PMA, INDO (1mM) almost completely inhibited expression of c-myc.

5. Indomethacin in Complete Medium

Several experiments indicated that in complete medium (DMEM + 10% FCS), 1 mM INDO did not inhibit the PMA induced expression of c-fos, whereas 2 mM almost completely inhibited c-fos mRNA expression (Fig. 7). A higher INDO concentration was needed for the same degree of inhibition presumably because the drug binds to serum proteins (Owen et al. 1983). 1 mM INDO actually appeared to stimulate the synthesis of c-fos mRNA, an effect that was seen in several experiments, but has not definitely been confirmed.

6. Investigation of the Mechanisms of Action of Indomethacin

INDO has been reported to inhibit the Na^+/H^+ exchanger (Owen and Villereal 1983). To determine whether this action of INDO was responsible for the inhibition of c-fos expression, a known inhibitor of

Fig. 6: INDOMETHACIN EFFECT ON OTHER PMA-INDUCIBLE GENES IN MEDIUM V (pH 7.2, 5% CO_2)
The cell treatment (Balb/c-3T3 cells) and RNA analysis were done as described in Fig. 1

Exp I : EGR
A: Control (30 min)
B: PMA (30 min)
C: PMA + INDO 1mM (preincubated 15 min)

Exp II: c-myc
A: Control
B: PMA (60 min)
C: PMA + INDO 1mM (preincubated 15 min)

I. A B C

II. A B C

Fig. 7: INDOMETHACIN INHIBITION OF PMA-INDUCED c-Fos mRNA EXPRESSION IN COMPLETE MEDIUM
The cell treatment and RNA analysis were done as described in Fig. 1

(I and II are separate experiments)

A: Control (no addition)
B: PMA (30 min)
C: PMA + 1 mM INDO
D: PMA + 2 mM INDO

I. A B C D

II. A B C D

the Na^+/H^+ antiporter, amiloride, was used (Besterman et al. 1985). At 2 mM, a commonly used concentration, amiloride completely inhibited c-fos induction by PMA (Fig. 8,I). However, recently the specificity of amiloride has been questioned. It has been reported to have other effects on cells, such as inhibition of protein kinase C and DNA and protein synthesis (Besterman et al. 1985, Leffert et al. 1982, Lubin et al. 1982, L'Allemain et al. 1984).

Therefore, we decided to explore the role of the Na^+/H^+ antiporter mechanism in another way. Cells were treated with PMA in a medium where Na^+ was omitted and replaced with an equimolar concentration of choline. As seen in Fig. 8,II, c-fos was as strongly induced by PMA in choline-HEPES medium in which Na^+-H^+ exchange cannot occur (Fig. 8,II lanes B), as in a Na^+ containing medium (Fig. 8,I lane B). In another experiment, we compared the induction of c-fos mRNA in choline-HEPES medium and a Na^+ containing solution, under similar conditions of pH and CO_2 and found that c-fos mRNA levels were similar whether or not Na^+ was present in the medium, (Fig. 9). It can be therefore concluded that the Na^+/H^+ exchange is not directly involved in the induction of c-fos by PMA or inhibition of this induction by INDO.

7. Indications That the Indomethacin Effect is not Due to Irreversible Cell Toxicity

i. Reversibility of INDO inhibition of c-fos mRNA induction

The specificity of the INDO effect on c-fos, c-myc and EGR mRNA induction was examined by carrying out several types of short-term experiments. To rule out the possibility that the observed inhibition

Fig. 8: STUDY ON THE ROLE OF THE Na^+/H^+ PUMP IN c-Fos mRNA INDUCTION
The cell treatment and RNA analysis were done as described in Fig. 1

I. INHIBITION BY AMILORIDE IN MEDIUM III
A: Control
B: PMA
C: Amiloride (2mM)
D: PMA + amiloride

II. INDUCTION BY PMA IN CHOLINE-HEPES MEDIUM, IN AIR
A: Control
B: PMA

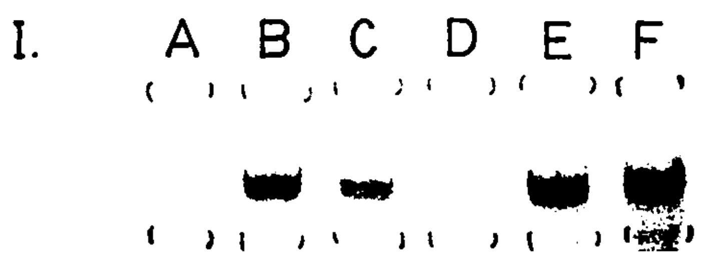
I. A B C D

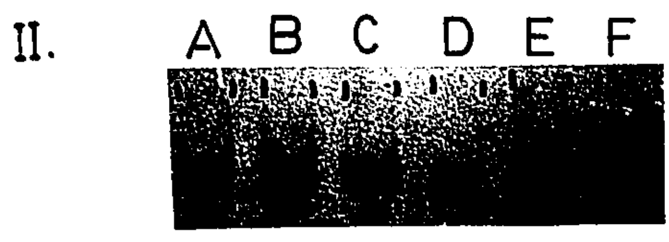
II. A B A A B B

Fig. 9: COMPARISON OF PMA-INDUCED c-Fos mRNA IN CHOLINE-HEPES AT pH 7.0 AND 7.3
The cell treatment and RNA analysis were done as described in Fig. 1

(I and II are separate experiments.)

A: Choline-Hepes pH 7.0
B: Choline-Hepes pH 7.0 + PMA
C: Medium IV, pH 7.0 + PMA
D: Choline-Hepes pH 7.3
E: Choline-Hepes pH 7.3
F: Medium IV, pH 7.3 + PMA


I.
A B C D E F


II.
A B C D E F

was a result of "generalized cell toxicity" or gross cell membrane or mitochondrial damage, we determined whether the effect of INDO was readily reversible.

One protocol for the study of induction of c-fos mRNA involved a 15 min preincubation of cells with 150 μM INDO in medium I, then addition of PMA and further incubation for 30 min in the continued presence of INDO. To demonstrate that inhibition by INDO was reversible in medium I, cells were preincubated for 75 min with or without 150 μM INDO, the cells were then washed with fresh medium, PMA was added and incubation was continued for 30 min. In two independent experiments the induction of c-fos in cells pretreated with 150 μM INDO was almost as strong as in control cells preincubated without INDO (Fig. 10,I and II).

Two separate experiments suggest that under medium IV (pH 7.0, in air), the induction of c-fos by PMA after INDO treatment and removal was somewhat weaker than the PMA induction alone (Fig. 10,III). The effect of INDO was at least partially reversible in all experiments. It is possible that in experiment III, the wash-out of INDO was less complete than in I and II.

ii. Reversibility of INDO inhibition of DNA synthesis and growth

It has previously been shown that 0.4 mM INDO can exert a cytostatic, as opposed to a cytotoxic effect on human fibroblasts (Bayer et al. 1980). Its effect on DNA synthesis and growth (as determined by 24 hr pulse with ^{3}H-thymidine) was determined in Balb/c-3T3 cells (Fig. 11). Cells were incubated in medium IV, containing 0 (control), 1 and 2 mM INDO and ^{3}H-thymidine at -24 hr. The amount of radioactivity incorporated into DNA was monitored 24 hr later (Fig. 11, t=0 hr); the

Fig. 10: REVERSIBILITY OF c-Fos mRNA INHIBITION UNDER DIFFERENT CONDITIONS

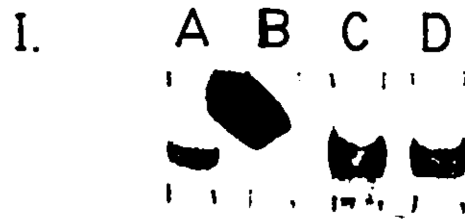
The cell treatment in this experiment includes a 60 min incubation with INDO and 30 min with PMA, subsequent steps were done as described in Fig. 1

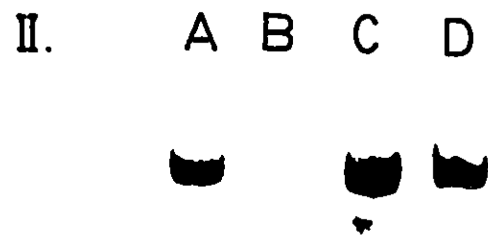
I and II: 150 μM INDO (condition I):

A: No addition 60 min, PMA 30 min
B: INDO 60 min, PMA 30 min
C: No addition 60 min, wash, PMA 30 min
D: INDO 60 min, wash, PMA 30 min

III: 1.0 mM INDO (condition IV):

A: No addition 15 min, PMA 30 min
B: INDO 15 min, PMA 30 min
C: No addition 60 min, PMA 30 min
D: INDO 60 min, wash, PMA 30 min


I.
A B C D


II.
A B C D

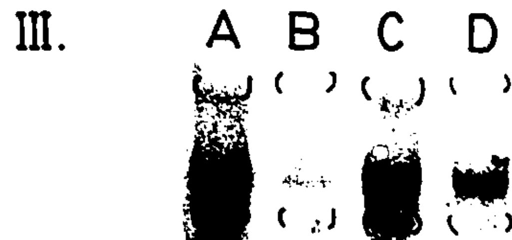

III.
A B C D

Fig. 11: REVERSIBILITY OF THE INHIBITION OF DNA SYNTHESIS (A) AND GROWTH (B) FOLLOWING TREATMENT WITH INDO (CONDITION I)

A: Cells were plated at a density of 10^4 cells/well in 24-well plates at -48 hr. Drugs were added at t=0 hr, they were removed by washing at t=24 hr. At t=0 and 24 hr cells were pulsed with ^{3}H-thymidine.

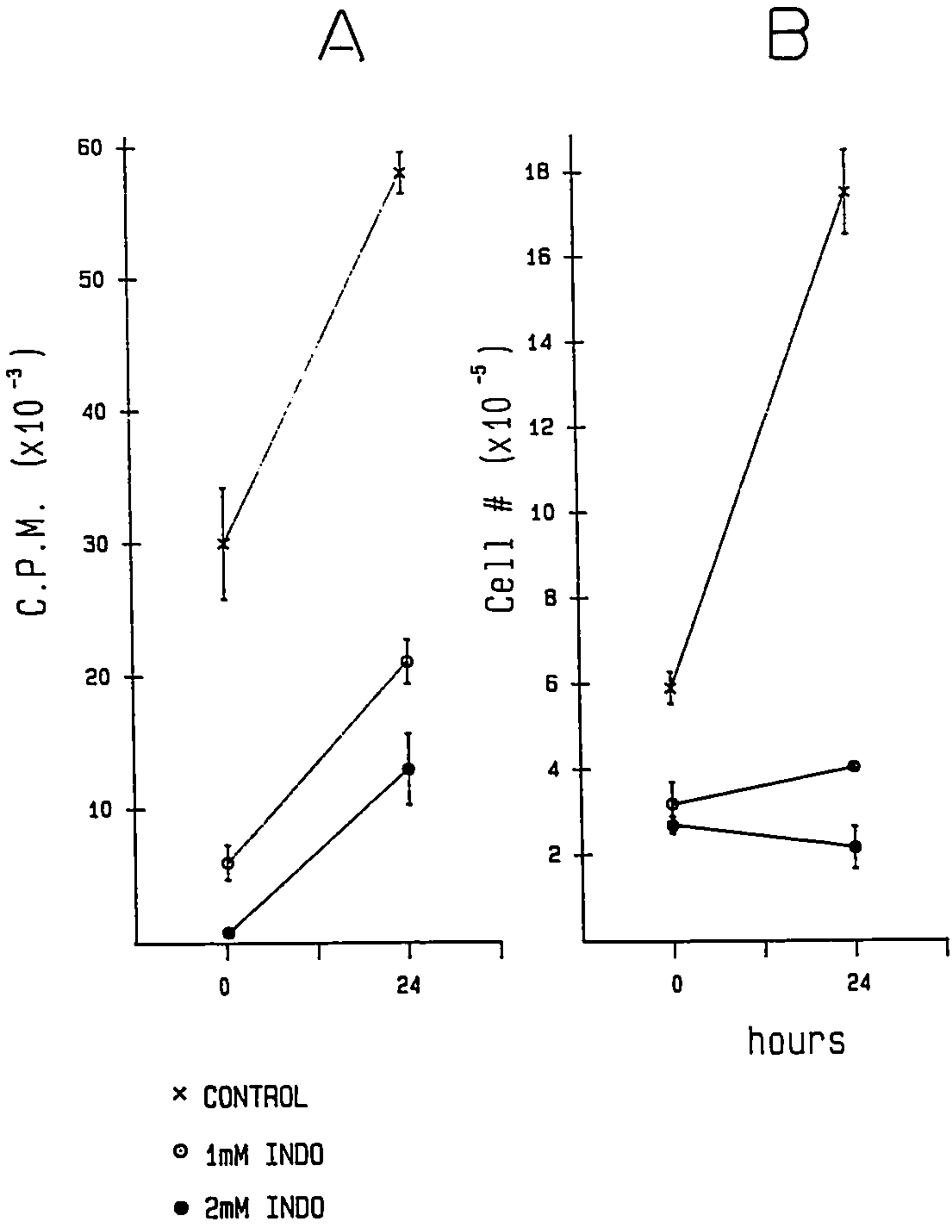
X=0 Effect of control, 1 mM and 2 mM INDO on basal DNA levels,(expressed as counts per min) after 24 hr pulse with ^{3}H-thymidine

X=24 DNA levels after removal of INDO and incubation in medium I for 24 hr

B: Cells were plated at a density of 10^5 cells/60 mm dish at -48 hr. Treatment was similar as in A, except for the pulse.

X=0 Effect of control solution, 1 mM and 2 mM INDO on cell number after 24 hr incubation

X=24 Cell number 24 hr after removal of INDO.Partial reversibility of growth after incubation with 1 mM INDO



A
B
60
50
40
30
20
10
C.P.M. (x10⁻³)
18
16
14
12
10
8
6
4
2
Cell # (x10⁻⁵)
0
24
0
24
hours
× CONTROL
○ 1mM INDO
● 2mM INDO

INDO was removed by washing cells with solution IV and incubation was continued for another 24 hr in the presence of ^{3}H-thymidine (Fig. 11, t=24 hr). After 24 hr incubation with 1 and 2 mM INDO, DNA synthesis was inhibited by 80% and more than 85% over control levels (Fig. 11,A t=0). When INDO was removed and incorporation of ^{3}H-thymidine monitored for another 24 hr, the levels went up from 30,000 cpm to 58,000 cpm in the control, from 6,000 cpm to 22,000 cpm in cells treated with 1 mM INDO, and from 2,000 to 13,000 cpm in cells treated with 2 mM INDO, which suggests that DNA synthesis recovered partially after removal cf INDO.

A similar experiment analyzed the effect of the same INDO concentrations on cell growth, as determined by changes in cell number (Fig. 11,B). Incubation with 1 and 2 mM INDO caused 50 and 58% inhibition, respectively, of cell growth after 24 hr incubation compared to cells grown without INDO. After removal of INDO and incubation in medium IV for 24 hr further, cell growth was partially restored in cultures that had previously been exposed to 1 mM INDO (from $3x10^5$ cells to $4x10^5$ cells) compared to the controls, in which the cell number increased three fold.

Considering measurements of DNA synthesis and cell growth, it seems that incubation with 1 mM INDO for 24 hr, removal of INDO and continued incubation for another 24 hr partially restores DNA synthesis and cell growth. After 24 hr incubation with 1 mM INDO, DNA synthesis was depressed by no more than 30%; removal of the drug and subsequent incubation under physiological conditions allowed partial recovery after the initial inhibition (Fig. 11,A). Cells synthesized DNA slowly after

treatment with 2 mM INDO but cell growth at this INDO concentration remained virtually unchanged.

iii. Indomethacin effect on ATP levels and RNA synthesis

Measurement of ATP levels can be a useful monitor of cellular toxicity, particularly of damage to mitochondria or leakiness of the plasma membrane. The histogram in Fig. 12 shows that incubation with INDO for 45 min and PMA for 30 min did not cause more than 34% reduction of ATP levels (10% in one experiment and 34% in another) over levels in untreated cells, while treatment with INDO alone or PMA alone did not reduce ATP levels more than 10%.

Overall RNA synthesis could conceivably be inhibited by depletion of ATP or nucleoside triphosphate pools or inhibition of RNA polymerase II itself. To observe if this was occurring, short-term RNA synthesis was monitored by following ^{3}H-uridine incorporation into acid-insoluble material.

Although early experiments indicated no inhibition of ^{3}H-uridine incorporation, more recent experiments indicated that there was indeed 90% inhibition of cell associated total radioactivity (Fig. 13,A). When examined in more detail, it was found that cell-associated acid-soluble counts were markedly decreased (Fig. 13,C), leading to a decrease in acid-precipitable counts (Fig. 13,B).

When the specific activity of the radioactive pools was normalized by calculating the amount of ^{3}H-uridine incorporated into RNA (by precipitation with trichloroacetic acid) as a function of the total intracellular radioactivity, it was clear that ^{3}H-uridine uptake is inhibited much more than the incorporation of ^{3}H-uridine triphosphate

Fig. 12: INDOMETHACIN 150μM EFFECT ON ATP LEVELS (CONDITION I) IN TWO INDEPENDENT EXPERIMENTS (A and B)
Cell treatment was done as described in Fig. 1, after 30 min incubation with PMA, bioluminescence reagent was added and an aliquot was counted (for details, see Materials and Methods).

(The "PMA" histogram represents the average of 4 separate counts; the "PMA+INDO" histogram represents the average of 8 separate counts)

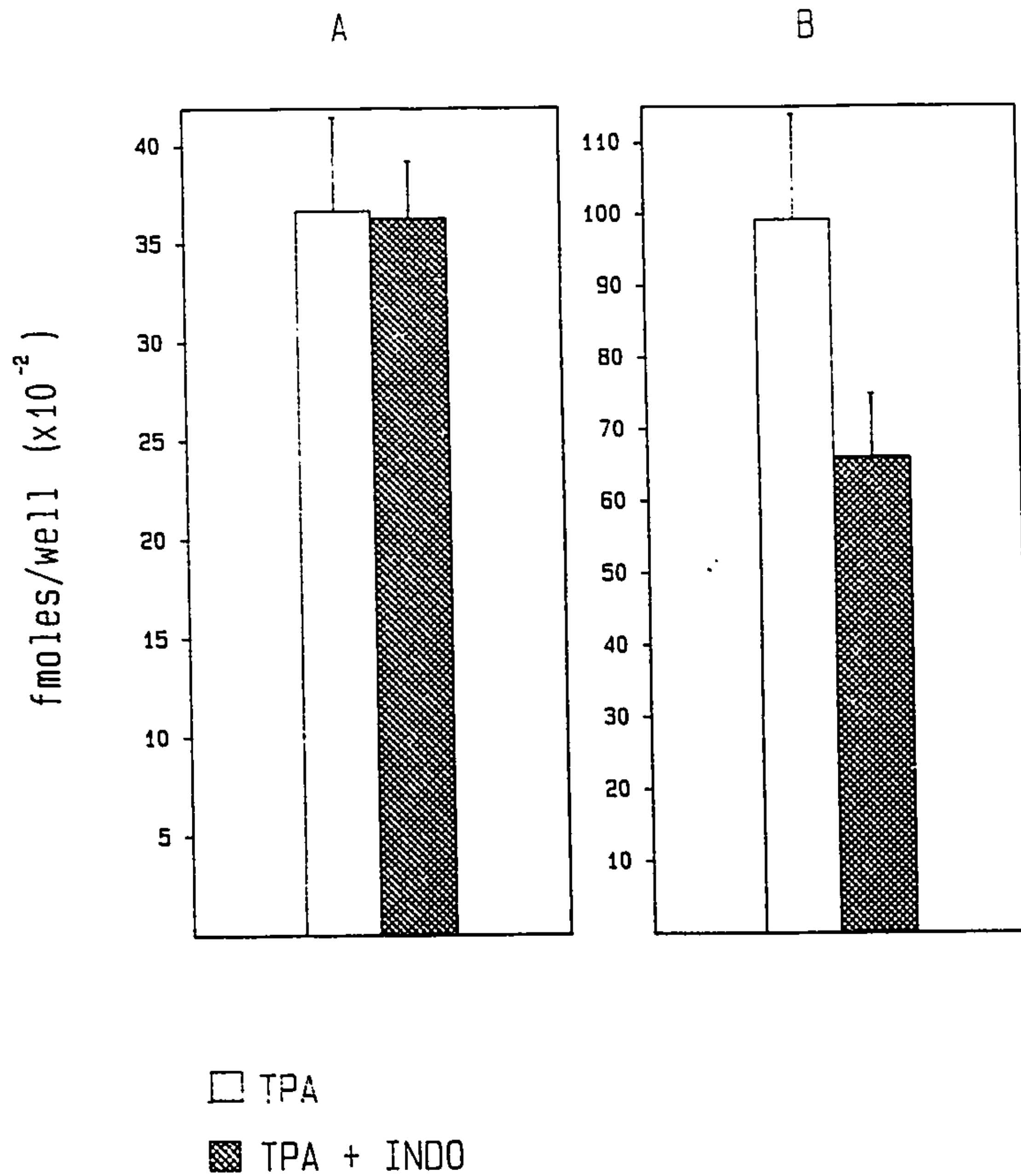
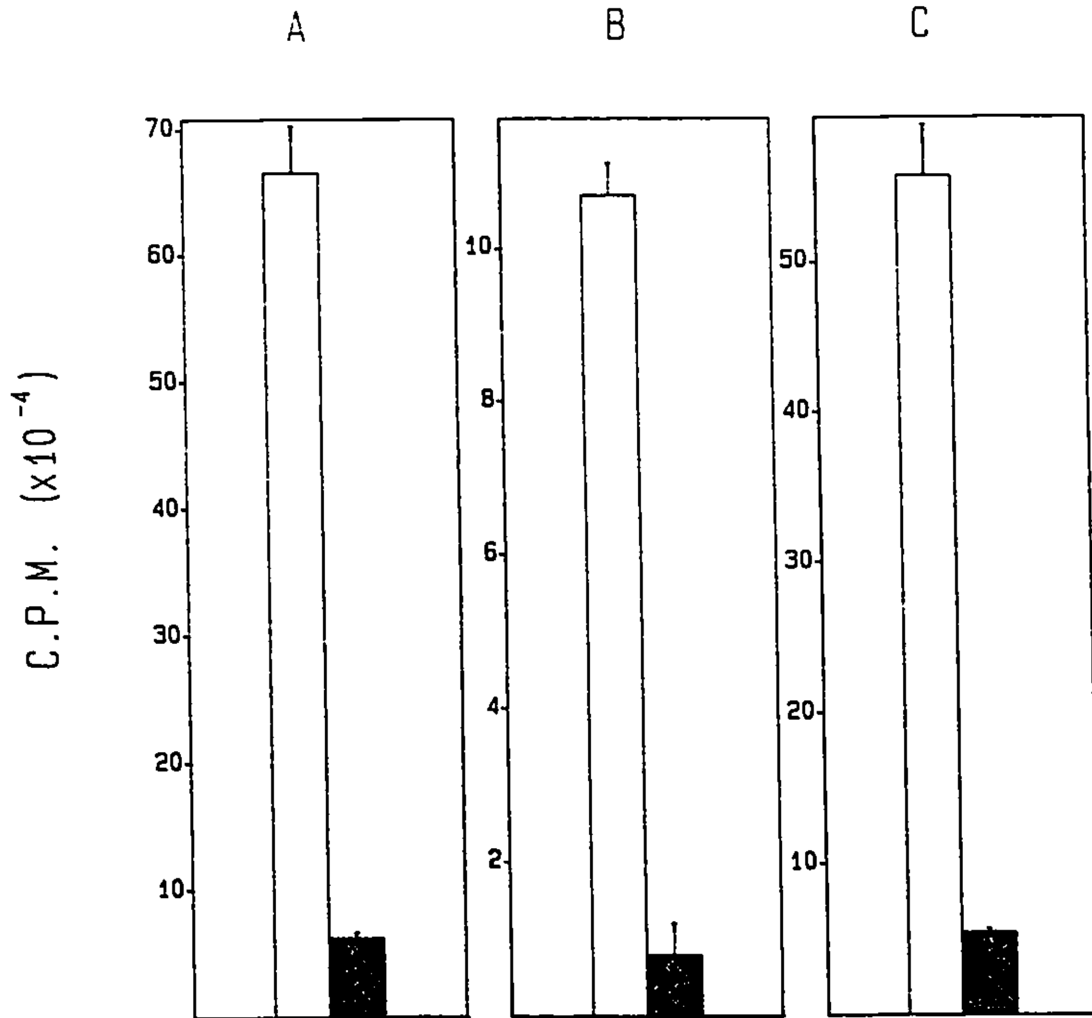

A
B
fmoles/well (x10^{-2})
40
35
30
25
20
15
10
5
110
100
90
80
70
60
50
40
30
20
10
TPA
TPA + INDO

Fig. 13: INHIBITION BY 1mM INDO IN MEDIUM IV OF:

A: CELL-ASSOCIATED ^{3}H-URIDINE ACTIVITY
B: ^{3}H-URIDINE INCORPORATION INTO RNA
C: UNINCORPORATED CELL-ASSOCIATED COUNTS

Cell treatment and analysis of the distribution of radioactive material were done as described in Materials and Methods. Each histogram bar represents the average of 4 independent counts.


A
B
C
C.P.M. (x10^-4)
70
60
50
40
30
20
10
10
8
6
4
2
50
40
30
20
10
TPA
TPA + INDO

into RNA (Table 1). This degree of inhibition of RNA synthesis (25-30%) was much less than the 80-90% inhibition of c-fos mRNA observed under similar conditions.

iv. Indomethacin likely does not interfere with PMA activation of protein kinase C

To determine whether inhibition by INDO might be due to interference in the activation of protein kinase C by PMA, we used an indirect approach. The effect of INDO on PMA-induced stimulation of [2-^{3}H]-deoxyglucose uptake, a process presumed to involve protein kinase C, was determined (Lee and Weinstein 1979). There is a lag time of 1 hr after PMA addition before the stimulation becomes evident. To maintain conditions as close as possible to those used for the study of fos induction, the following protocol was used: confluent cells were preincubated in medium I (containing glucose) with and without PMA for 60 min. The monolayer was then washed with a glucose-free medium and incubated with and without INDO for 15 min. [2-^{3}H]-Deoxyglucose was added for 15 min, after which the cells were washed and the amount of cell-associated radioactivity was determined.

The normalized histogram in Fig. 14 indicates that [2-^{3}H]-deoxyglucose uptake was stimulated 2 fold on exposure to PMA and that inclusion of INDO led to a further stimulation, not an inhibition. This experiment indicates that INDO likely does not inhibit activation of protein kinase C by PMA. Multiple species of protein kinase C are now known (Hunter 1987), so INDO could theoretically inhibit the PKC involved in the fos pathway and not the 2-deoxyglucose stimulation pathway.

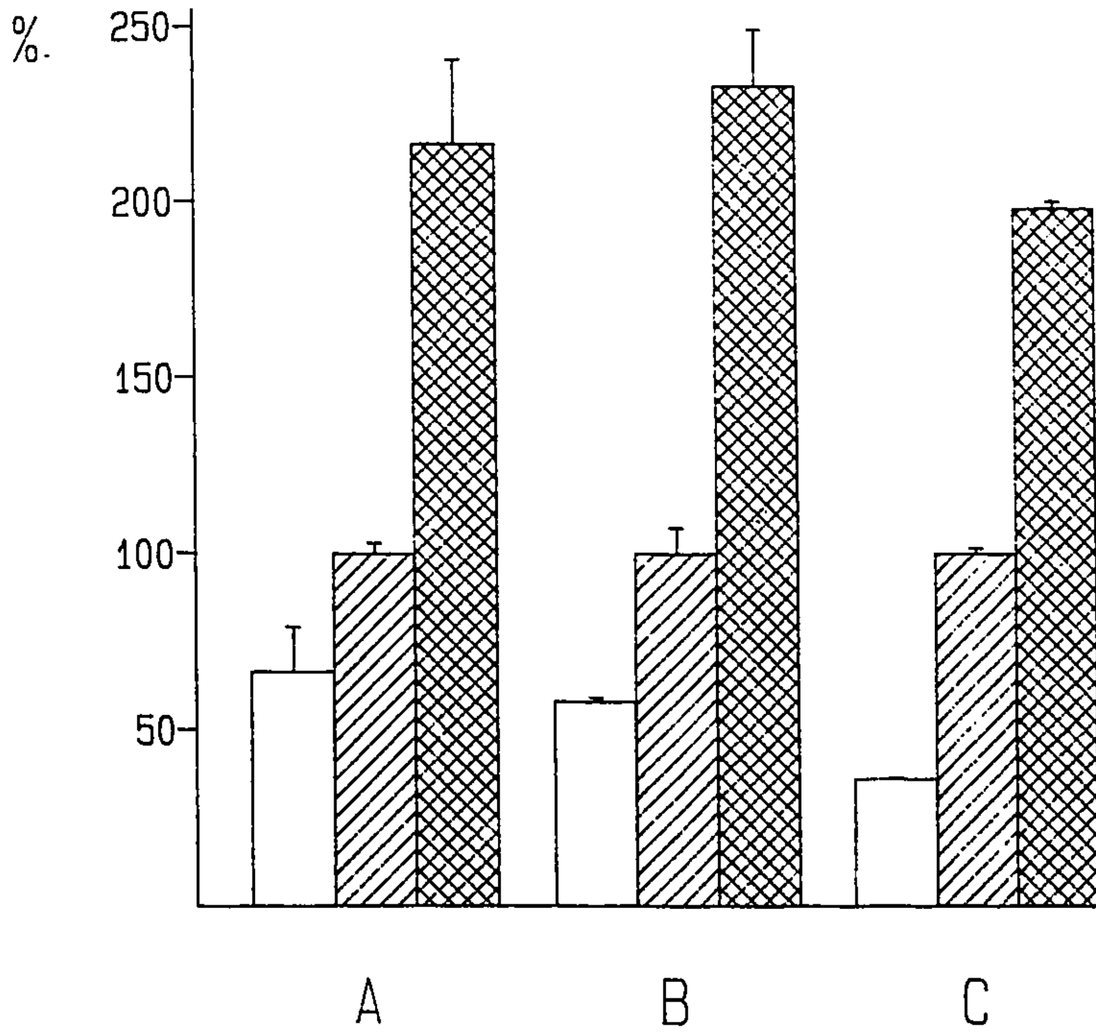
Table 1: EVIDENCE FOR INDO INHIBITION OF ^{3}H-URIDINE UPTAKE.
^{3}H-URIDINE CPM:

ADDITION	CELL ASSOCIATED (av. ± SEM)*	PRECIPITABLE (av. ± SEM)*	SOLUBLE (av. ± SEM)*	PRECIPITABLE/ SOLUBLE
TPA	66.6 ± 3.6 (100)	10.8 ± 0.4 (100)	55.8 ± 3.3 (100)	19.5% ± 0.18
TPA+ INDO	6.3 ± 0.3 (9.4)	0.82 ± 0.1 (7.4)	5.5 ± 1.7 (9.8)	14.7% ± 0.04

* averages are expressed as counts per min x 10^{-4}

Fig. 14: ENHANCED [2-^{3}H]-DEOXYGLUCOSE UPTAKE BY 10^{-6} M PMA AND PMA + 1 mM INDO: EVIDENCE FOR NON-INVOLVEMENT OF PROTEIN KINASE C
Cell treatment was performed as described in Fig. 1, [2-^{3}H]-Deoxyglucose was added for 15 min at the end of the 30 min incubation with PMA. Cell lysis and counting were done as described in Materials and Methods.

(The results represent averages of 4 counts in each of 3 separate experiments).



CONTROL

TPA

TPA + INDO

8. Delayed Addition

Although the precise mode of action of INDO has not been elucidated, I decided to explore further its use as a possible tool for the investigation of the signal transduction process. Inhibition of c-fos (and other genes) occurred when the cells were preincubated with INDO for 15 min, followed by a 30 min treatment with PMA. I decided to test how long after protein kinase C activation by PMA a response to INDO could be detected. This might provide some clues as to the signals involved in the transduction process between protein kinase C and gene induction and the mode of action of INDO.

Other workers have shown that the c-fos primary transcript is detected as early as 5-10 min after stimulation with PMA, as demonstrated by nuclear run-off experiments in NIH 3T3 cells (Rabin et al. 1983).

Fig. 15 shows the early kinetics of cytoplasmic fos mRNA induction by PMA in Balb/c-3T3 cells: after 20 min of stimulation with PMA the fos gene is starting to be expressed and at 25 min the induction is strong. A peak is reached at 30 min and levels decline thereafter (not shown). The following experiments analyze the kinetics of c-fos mRNA when INDO was added at the specified time after PMA; the RNA was prepared in all cases 30 min after treatment with PMA.

In one set of experiments, 1 mM INDO addition was delayed 2, 4, or 6 min after PMA addition (Fig. 16); c-fos mRNA induction was still inhibited. This lead to the question: how late after PMA addition could we still inhibit fos expression with INDO?

Fig. 15: TIME-COURSE OF c-Fos mRNA INDUCTION FOLLOWING ADDITION OF PMA (MEDIUM I)
Cells were treated with PMA for the times specified below. RNA extraction and analysis were done as described in Fig. 1

A: PMA 5 min
B: PMA 15 min
C: PMA 20 min
D: PMA 25 min

A B C D

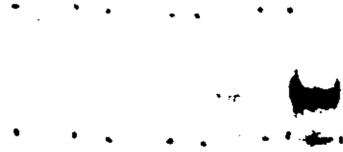


Fig. 16: EFFECT OF DELAYED ADDITION OF 1 mM INDO ON c-Fos mRNA LEVELS, AS MEASURED 30 min AFTER PMA STIMULATION
Cell treatment and RNA analysis were done as described in Fig. 1, "t" specifies time after the addition of PMA.

A: PMA
B: PMA + INDO t=0 min
C: PMA + INDO t=2 min
D: PMA + INDO t=4 min
E: PMA + INDO t=6 min

A B C D E

INDO (1 mM) was then added 15 min before, concomitantly and 5, 10, 15 min after PMA addition. As shown in Fig. 17,I, INDO inhibited strongly at all points, although the induction was variable in control cells at different time points. In another experiment (Fig. 17,II) INDO inhibited strongly at 15 min post-PMA but much less at 20 min post-PMA.

Strong inhibition of c-fos mRNA was seen when INDO was added as late as 15 min after PMA stimulation; when added at 20 min, some increase in mRNA was seen. It seems that it might take 5 min for INDO to act because levels of fos mRNA at t=20 in PMA induced cells (where the message starts to rise) are comparable to t=25 in cells treated with PMA + INDO (Fig. 17,I (i) and (ii); Fig. 17,II (i) and (ii), where time points are from the same experiment). One possibility is that INDO could be limiting the accumulation of a trans-acting factor required for the expression of the gene.

i. Indomethacin appears to delay, not inhibit, the induction of c-fos and c-myc mRNA

Fig. 18,I represents a time course after addition of PMA with or without 1 mM INDO in medium IV. PMA induced c-fos expression strongly at 30 min; at 60 min levels were slightly more elevated in this particular example. Although INDO inhibited almost completely at 30 min, the fos message rose at 60 min and rose even further at 90 min, to a level as high or higher than in controls.

c-Myc mRNA was induced to maximal levels 60 min after PMA stimulation although the signal was fairly weak in this experiment, it then declined. When treated with INDO, there was strong inhibition at

Fig. 17: EFFECT OF DELAYED ADDITION OF INDO AFTER PMA, ON THE LEVELS OF c-Fos mRNA (MEDIUM IV)
Cell treatment and RNA analysis were done as specified in Fig. 1, "t" specifies time after addition of PMA. .

I and II are results from different experiments
i): PMA + Control (Na acetate added instead of INDO) time-course
ii): PMA + INDO time-course

A: PMA alone
B: t=-15 min
C: t= 0 min
D: t= 5 min
E: t= 10 min
F: t= 15 min
G: t= 20 min

I. A B C D E F

i)

ii)

II. A D E F G

i)

ii)

Fig. 18: STUDY OF THE EFFECT OF 1 mM INDO ON THE TIME-COURSE OF TWO PMA-INDUCIBLE GENES:
Cell treatment with specified agents was done for the time specified by "t". In ii), cells were preincubated with INDO for 15 min prior to the addition of PMA. RNA analysis was done as described in Fig. 1

I: c-fos
II: c-myc

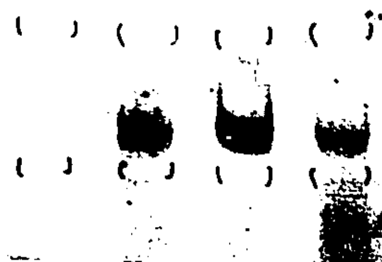
i) Time-course of PMA + Control(Na acetate)
ii) Time-course of PMA + INDO

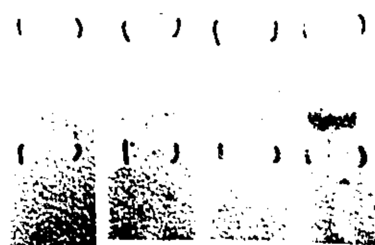
A: No addition
B: t=30 min
C: t=60 min
D: t=90 min

I. II.

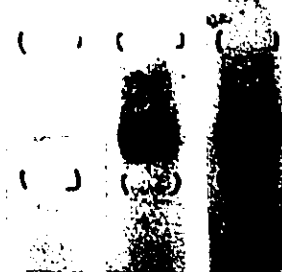
A B C D A B C D

i)





ii)



60 min. However, at 90 min c-myc mRNA levels were very high in the INDO + PMA sample (Fig. 18,II).

Although there was some degree of variation in the amount of messenger induced, in both these experiments INDO allowed messenger levels to rise after an initial inhibition, suggesting that the whole induction curve was shifted in time but induction of the respective gene could still occur. A similar result was obtained in at least 6 experiments.

ii. Evidence that INDO delays peak of expression of another PMA-inducible gene

RNA was extracted from cells treated for 1, 2, and 3 hours with PMA ± INDO, then transferred and probed with fos and EGR specific probes (Fig. 19). The PMA induced expression of fos was high at 1 hr and then declined to almost non-detectable levels; in PMA + INDO samples, mRNA levels increased until 2 hr and then declined.

A similar effect was observed also for EGR, whose expression was high at 1 hr, continued to rise until 2 hr after PMA and declined thereafter. In the presence of INDO, EGR mRNA levels were initially low but continued to rise at 2 and 3 hr (Fig. 19, I and II).

After completion of my experimental work, several experiments carried out by Dr. H.C. Birnboim and Dr. R.G. Liteplo confirmed that INDO did indeed delay the appearance of c-fos mRNA and EGR mRNA.

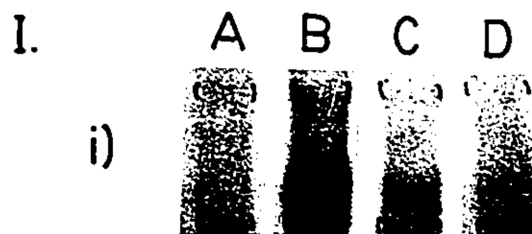
Thus, my observations have opened up an interesting area for exploration of signal transduction of 3 different PMA-inducible genes.

Fig. 19: INDOMETHACIN DELAYED EXPRESSION OF TWO PMA-INDUCIBLE GENES
Cells were treated concomitantly with PMA and INDO, then harvested at the times specified below. RNA analysis was done as described in Fig. 1

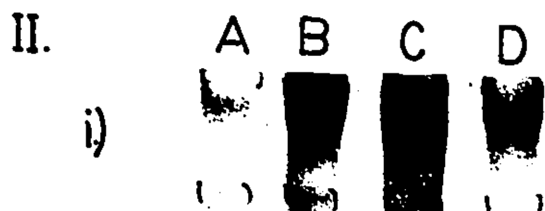
I. c-fos
II. EGR

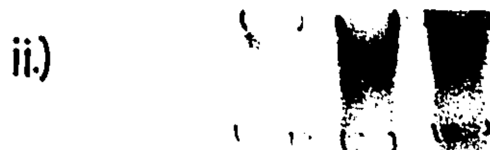
i) - INDO
ii) + INDO

A: No addition
B: t= 60 min
C: t=120 min
D: t=180 min


I.
A B C D
i)

ii)


II.
A B C D
i)


ii.)

IV. DISCUSSION

1. Importance of the Study of Nuclear Oncogenes and Genes Induced Early in the Cell Cycle

The protein products of proto-oncogenes c-myc, c-myb and c-fos have been localized predominantly in the nucleus (Rozengurt 1986). In view of the oncogenicity of the retroviral forms of these genes, it is expected that they have central roles in the nuclear events involved in cellular proliferation and differentiation.

The expression of c-myc and c-fos proto-oncogenes increases sharply, soon after stimulation of various quiescent cell types, including fibroblasts and lymphocytes (Kelly et al. 1983, Greenberg and Ziff 1984, Muller et al. 1984a). The c-fos gene is induced more rapidly and is more transient than c-myc, although even the latter declines before the onset of DNA synthesis.

Studies on these genes have established that both their RNAs and proteins have short half-lives: 10-20 min for c-myc mRNA, 40 min for c-fos mRNA and 20-30 min for c-myc protein, 2 hr for c-fos protein. (Eisenman and Thompson 1986). Such rapid metabolism suggests that these genes are under stringent control and that their expression mediates other signals essential for cell processes such as the progression of the cell through the G_0-G_1 phase of the cell cycle.

Stimulation of G_0 arrested fibroblast cells with specific growth factors or serum is associated with a number of very rapid biochemical events, including changes in intracellular Ca^{2+}, Na^+, pH, cyclic nucleotide levels and phosphorylation of proteins (Rozengurt 1986

Nishizuka 1986, Berridge and Irvine 1984b). A consequence of these rapid intracellular changes is the activation of transcription of several genes such as: c-fos, c-myc, EGR and MEP (major excreted protein). One of these early genes, namely c-fos, is transcribed within 10-15 min of PMA stimulation of NIH 3T3 cells, as shown by nuclear run-on experiments (Rabin et al. 1986). Some or all of the early changes occurring in the cell following serum, growth factor or phorbol ester addition could be involved in the transduction of the signal from the membrane to the nucleus, triggering the expression cascade: initiation of transcription, messenger capping, polyadenylation, splicing, then export to the cytoplasm and translation.

2. Approach Used to Investigate the Early Signal Transduction Mechanism

Early in my study, several drugs were tested for their capacity to inhibit the expression of PMA-induced genes. Many of the agents used gave variable results, perhaps partly due to our unfamiliarity with some of the techniques and partly to the difficulty in obtaining quantitative results using biotinylated probes. INDO, an inhibitor of the cyclo-oxygenase pathway of prostaglandin synthesis had a consistent inhibitory effect both on the induction of c-fos and c-myc mRNA synthesis. INDO, a methylated indole derivative, was introduced in 1963 for its anti-inflammatory effect. Although INDO is widely used and is effective as an anti-inflammatory agent and inhibitor of the prostaglandin-forming cyclooxygenase, it has adverse _in vivo_ effects in man, such as inhibition of the motility of polymorphonuclear leukocytes, uncoupling

of oxidative phosphorylation and depression of the biosynthesis of mucopolysaccharides (Goodman et al. 1985).

Our study was conceived as a pharmacological approach to the study of signal transduction and due to the fact that high concentrations of INDO were used, potential side-reactions had to be considered.

3. Role of pH_i

In early experiments, weakly buffered salt solutions were used as the treatment medium and incubation was carried out in a CO_2 incubator. The pH was found to drop about 0.5-0.7 pH units under these conditions. In later experiments, the pH was better controlled either by using an air incubator or by using $NaHCO_3/CO_2$ buffers. Results indicated that the inhibitory effect of INDO was stronger at lower pH (about 6.7) compared to the physiological pH (7.2-7.4). In early experiments, performed in Balb/c 3T3 cells at pH 6.7, the concentration of INDO required for $\geq$80% inhibition of c-fos induction was 100-150 μM. Under later conditions, where the incubation media were buffered at 7.0-7.3, 0.5-1 mM INDO was necessary for the same degree of inhibition.

Quiescent cells have been shown to possess less buffering capacity than non-quiescent ones (Bright et al. 1987). In my experiments, cells were incubated for at least 45 min after the first addition of drug, and by that time the pH might have dropped to 6.7 or lower in weakly buffered media in a CO_2 atmosphere. It is then understandable that higher INDO concentrations are needed for the same inhibitory effect when experiments are performed in media where the pH was under stringent control than when the pH was allowed to drop, if one assumes that the

uncharged form of the drug is the species which enters the cell. Most probably INDO, whose pK_a is 4.5, is taken up more efficiently at lower pH. Note that c-fos mRNA was strongly induced by the phorbol ester PMA, in different media in a pH range of 6.7-7.3, which indicates that the lower pH was not severely toxic for cells.

In a recent review article by Madshus (1988), it is stressed that the intracellular pH must be strictly controlled since many processes are pH-dependent: many enzymes have pH optima, the efficiency of contractile elements, the conductivity of ion channels, control of the cell cycle and proliferative capacity of cells. The importance of Na^+/HCO_3^- symport as a pH regulatory mechanism is also documented but since such studies have been done in specialized cells (basolateral membrane of the proximal tubule of salamander, monkey kidney epithelial cells, proximal tubular cells of the rat) it is not known if such transport occurs also in other cell types and if it is essential for membrane transport of acid (H^+). Considering these reports, we chose to include bicarbonate in the treatment medium and incubate in a CO_2 atmosphere, such that conditions were as close as possible to the ideal physiological state. A strong induction of the gene by PMA and a strong inhibitory effect of INDO was observed also in complete medium and 5% CO_2, conditions where the effect of the drug is equally reversible.

4. Indomethacin and Metabolic Pathways

Multiple effects of INDO have been reported in the literature. In an attempt to determine via which mechanism it inhibits the PMA-induced

activation of several early genes, we used other inhibitors of those pathways or analogs.

INDO likely does not inhibit the PMA induced expression of c-fos, c-myc and EGR (early growth response gene) by innibiting the prostaglandin synthetase, since the concentrations used were 10-100x higher than the concentrations reported to inhibit the cyclo-oxygenase pathway (10 μM).

i. INDO and the Na^+/H^+ pump

One of the actions of INDO is blockage of serum-stimulated Na^+/H^+ exchange in a dose-dependent manner (K_i=0.34 mM). The onset of action of INDO is rapid (2 min) and the inhibition of Na^+ influx is readily reversed (within 5 min) (Owen and Villereal 1983). Our investigations of this pathway started with the use of an inhibitor of Na^+/H^+ exchange, amiloride. Amiloride was found to inhibit the PMA-induced expression of c-fos as strongly as INDO. However, we were unable to conclude from this result that the Na^+/H^+ pump plays a role in signal transduction for the activation of c-fos, because amiloride has been reported to have other inhibitory effects. Among these is inhibition of protein synthesis at micromolar concentrations in quiescent Swiss 3T3 cells (Lubin et al. 1982), DNA synthesis inhibition in rat hepatocytes at concentrations that also inhibit protein synthesis (Leffert et al. 1982), and inhibition of protein phosphorylation by protein kinase C in the human leukemic line HL-60, at concentrations that inhibit the Na^+/H^+ exchange (Besterman et al. 1985). To explore the requirement of extracellular Na^+ for c-fos induction, we chose to treat cells with PMA in a medium devoid of sodium. Our results prove that this pathway is

not involved in the induced expression of c-fos proto-oncogene since induction of the gene by PMA in Na^+-free medium is as strong as in Na^+-containing medium.

ii. INDO and amino acid transport

INDO is able to inhibit the high affinity component of α-amino isobutyric acid (AIB) and α-(methylamino)-isobutyric acid (MeAIB) transport, the "A" system in rat hepatocyte cultures, at concentrations that inhibit cell growth (Bayer et al. 1980). Further studies have shown that, in addition to inhibiting basal rates of transport, INDO, through a similar mechanism, suppresses completely the increase in activity of the A system which normally occurs when cells are deprived of amino acids or stimulated with a mitogen (Bayer et al. 1981). This effect of INDO develops slowly and therefore is probably not the initial action of the drug. We found that treatment of growth arrested cells with PMA and PMA + INDO in complete medium in an atmosphere of 5% CO_2 did not influence the pattern of induction/inhibition of c-fos and EGR mRNA. INDO has the capacity to suppress the activity of the A system when no amino acids are present in the medium (our regular treatment media were devoid of amino acids), but the presence of amino acids in our treatment medium (condition V), did not have an affect on its ability to inhibit several genes. Therefore it does not seem to be the key event in the regulation of PMA-induced gene expression.

iii. INDO and protein kinases

Protein phosphorylation, an intracellular signal, is used for a variety of cellular mechanisms. One of them is transmembrane signal

transduction; some protein kinases are able to transduce signals from external stimuli: they either span the plasma membrane or are associated with its inner face. Other such mechanisms are protein synthesis and regulation of transcription factor activity. The patterns of cellular protein phosphorylation induced by PDGF, phorbol esters and cAMP suggest an intriguing connection. cAMP and PMA show synergistic phosphorylation of proteins in Chinese hamster ovary cells (Lockwood et al. 1987). In quiescent 3T3 fibroblasts, both PMA and PDGF rapidly stimulate phosphorylation of an 80,000-dalton protein (Rozengurt et al. 1983). Both growth factors and PMA stimulate phosphorylation of tyrosine residues of a 42,000-dalton protein in chick embryo fibroblasts (Gilmore and Martin 1983). In addition to these common protein phosphorylations, both cAMP and PMA induce unique phosphoprotein changes; hence all of these systems may function, in part, via phosphorylation of a common set of endogenous cellular substrates.

Cyclic AMP, by activation of protein kinase A (PKA), might induce phosphorylation alterations in components of the phosphoinositide pathway such as the lipid kinases or the PIP_2 phosphodiesterase (phospholipase C). This would result in altered activity of protein kinase C. PKA might also directly phosphorylate, and thereby regulate, growth factor receptors such as that for the sis oncogene product (PDGF). There are also protein kinases regulated by changes in Ca^{2+} concentration through the agency of calmodulin (Weinstein 1987).

PKC, activated by phosphoinositide turnover or phorbol esters, is believed to phosphorylate protein components of the cytoskeleton and the cAMP pathway to influence growth and differentiation (ibid.). In our study we investigated the possibility that after PMA activation of PKC,

addition of INDO could down-regulate or inhibit signal transduction to the nucleus by interfering with a phosphorylation step that is under PKC control. [2-^{3}H]-Deoxyglucose uptake studies have indicated that INDO did not inhibit PMA-induced stimulation of its uptake which is presumed to work through this kinase (Lee and Weinstein 1979, O'Brien 1982, Nelson and Murray 1986, Buttler-Gralla and Herschman 1983, Copley et al. 1983).

In the last few years, four mammalian protein kinase C genes have been identified, one of which gives rise to two alternately spliced mRNAs encoding proteins with different C-termini; now, seven distinct protein kinase C isozymes have been assigned to this subfamily (Coussens et al. 1986, Knopf et al. 1986, Ohno et al. 1987, Housey et al. 1987). Thus, the picture is becoming more complicated by the existence of multiple PKC's. Our experiments only prove that the particular kinase C that has increased [2-^{3}H]-deoxyglucose uptake following PMA addition is not involved in the inhibition of c-fos mRNA by INDO. In fact treatment with PMA and INDO seems to have an additive stimulatory effect on this PKC, suggesting that INDO can in fact also act as a stimulating agent. It is conceivable that another PK might be involved in the inhibition of this PMA-inducible event.

It is also known that most protein kinases have multiple substrates and these are still under investigation. Their identification and the determination of their function will lead to an understanding of the biochemical processes by which cAMP and PKC act as to regulate the cellular circuitry, from signal transduction to oncogene expression. Recently it was reported that 2-aminopurine (2AP), an inhibitor of the heme regulated and dsRNA-dependent eukaryotic

initiation factor (eIF)-2α kinases inhibits induction of β-interferon, c-fos and c-myc genes by virus or poly(I)-poly(C) in human MG63 cells, Balb/c-3T3 cells and HeLa cells. The authors state that different factors must be required for the induction of these genes, since they are not coordinately regulated by the same inducers in most of the cell lines examined (Zinn et al. 1988). Similarly, INDO could delay the appearance of c-fos mRNA by limiting the accumulation of a transacting factor and/or its phosphorylation, required for the PMA-induced response. More elaborate studies are needed to pursue the avenue that signal transduction for PMA-induced gene regulation is mediated by a protein kinase.

INDO was shown to have a complete inhibitory effect on cAMP-dependent protein kinases of different origin as well as on cAMP-independent protein kinases, at a concentration of 1 mM (Goueli and Ahmed 1980). The same concentration of INDO also inhibits the phosphorylation of the beta-subunit of the solubilized adipocyte insulin receptor by 60% (Knight et al. 1987) and blocks the biosynthesis of vesicular stomatitis virus (VSV) at the levels of primary transcription, RNA replication and protein synthesis (Mukherjee and Simpson 1985). Inhibition by INDO of PMA-induced genes may be due to its effect on phosphorylation of an unknown factor by a protein kinase. It may be possible, in future experiments, to determine if phosphorylation of a specific *trans*-acting factor (protein) is inhibited by INDO and whether this is important in the regulation of transcription of the genes studied.

iv. INDO and possible modes of action

PMA is known to induce transcription of several genes. Inhibition of mRNA synthesis by INDO is likely to be at the level of initiation of transcription. However, nuclear run-on experiments would need to be done to establish this with certainty. The possibility that there is faulty processing or transport of pre-mRNA is unlikely since my Northern blot experiments did not give any indication of accumulation of high molecular weight precursors or intermediate species (see fig 15 and 18).

We saw that inhibition of induced c-fos mRNA due to addition of INDO occurs up to 20 min following PMA treatment. Was reappearance of mRNA 25 min later due to metabolism of the drug within the cell? It does not seem so, since INDO was able to inhibit c-myc induced mRNA after 60 min. Moreover, the same concentration of INDO (1 mM) allows one PMA-inducible gene to be expressed at 120 min after an initial inhibition (c-fos, see Fig. 19,I,C) while another PMA-inducible gene is still inhibited at that time-point (EGR, see Fig. 19,II,C). Further studies will have to establish whether the timing of inhibition by INDO of different genes is due to alternate induction pathways.

Studies on c-fos gene expression have revealed the existence of positive and negative transcription factors involved in the regulation of this gene. A positive regulatory factor is the DSE-binding protein required for PMA-induction of c-fos (Treisman et al. 1985). Recently, it was shown that fos protein itself is involved in the repression of its own transcription and can also exert a positive trans-regulatory function on the PMA-responsive element (Sassone-Corsi et al. 1988a). In my study, it seems that there may be a fine balance between positive and negative factors. The inhibition of PMA-induced expression of c-fos by

INDO could occur by enhancing a negative regulatory factor, or by interfering with a positive factor. I postulate that regulation of such factors is controlled by their phosphorylation and that INDO perturbs their phosphorylation level. The delay in c-fos and EGR gene expression caused by INDO may indicate a perturbation of the phosphorylation levels of <u>trans</u>-acting factors, such that the genes are initially inhibited and subsequently stimulated.

The temporary inhibition by INDO could be explained in two different ways. One possibility is inhibition of some step or product essential for the induction of PMA-responsive genes, along the signal transduction pathway (by one of the mechanisms described above). The other possibility is that appearance of messenger RNA after an initial inhibition is due to activation of another pathway that can be stimulated by PMA, bypassing the first blockade. In either case, if the phosphorylation of a factor by a protein kinase is involved, it could be explored by analyzing the phosphorylation pattern of proteins on a 2-dimensional gel before and after reappearance of messenger.

Signal transduction mechanisms are still a "black box" because of the multiple levels of complexity involved: activation of certain protein kinases involve Ca^{2+}; others involve cAMP, growth factors or hormones. Recent studies have revealed several isoforms of protein kinases which could interact among themselves or in a more elaborate fashion complicating even further the whole picture. We are therefore contemplating a fascinating and complex circuit whose interconnections remain to be deciphered. Further research will hopefully unravel the "secrets" of signal transduction and open new avenues to cancer research.

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APPENDIX

1. Salt solutions used for cell treatment with various drugs:

a) Earles salt solutions:

Modified medium	I	II	III	IV	V
Hepes	10 mM	10 mM	0	10 mM	25 mM
KCl	5 mM	5 mM	5 mM	5 mM	0
$MgSO_4x7H_2O$	0.8mM	0.8mM	0.8mM	0.8mM	0
NaCl	137 mM	137 mM	137 mM	116 mM	0
$NaHCO_3$	0	0	0	26 mM	24 mM
Na_2HPO_4	0	0	8.5mM	0	0
atmosphere CO_2	10%	5%	5%	0	5%
pH	7.4	ND	ND	7.0	7.2
Dulbecco's minimal essential medium	0	0	0	0	+

b) Choline-Hepes medium:

Choline chloride	137 mM
KCl	5 mM
$MgSO_4x7H_2O$	0.8mM
Hepes	10 mM
atmosphere CO_2	0
pH	7.0 (or adjusted to 7.3)

2. Solutions used in the RNA extraction procedure:

a) RES solution

0.5 M LiCl

1.0 M Urea

20 mM Na citrate

5 mM CDTA

0.25% SDS

The pH of the solution was adjusted to 6.8 and filtered through 0.45 μM filter before SDS addition.

b) CT

1.0 mM CDTA

10 mM tris.HCl pH 7.5

3. Oligo primer labelling method- ^{32}P-CTP:

A.Stock reagents

1.8M HEPES (pH 6.5 with NaOH) -stored at -20°C

Hexanucleotide mix (Pharmacia pd(N)6) -stored at -20°C

50 A_{260} U/mL in salt solution

dNTPs, were prepared as follows:

"10 mg" each of dNTPs from Pharmacia/PL.Dissolved in 160-180 μL of 10 mM CDTA(pH 7), calculated to give 100 mM solution.

B.Working reagents

- BSA (BRL cat #5561 UA) 10 mg/mL in H20
- Solution OLB: mix 200 μL HEPES + 200 μL hexanucleotide mix.Stored at -20°C.
- Polymerase I -Klenow fragment (Pharmacia,cat #27-0928-19)(1unit/μL)
- Carrier RNA (1mg/mL ribosomal RNA in 0.5 M NaCl)

  ^{32} P-dCTP (5'α-32P)(Amersham) 10μCi/μL.
- Reagents for ^{32}P-dCTP labelling:

dGTP, dTTP, dATP	20 μM of each
^{32}P-dCTP	50 μCi
Sterile water (for a final volume of 50 μL)	
OLB solution	1/5 vol
BSA	20 μg/mL
EtSH (140 mM)	14 mM
Nicked, denatured DNA(50 ng)	20 μg/mL.
Mix gently,centrifuge 5sec	
Polymerase I	40 units/mL.
Mix gently,centrifuge 5sec.	
Final volume: 100μL.	

Incorporation of ^{32}P-dCTP (50μCi), was allowed to progress at room temperature (21°C).