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INVESTIGATION OF Ca^{2+} SIGNAL TRANSDUCTION PATHWAY(S) IN HUMAN FIBROBLASTS

By

Shixia Huang

A DISSERTATION

Submitted to
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ABSTRACT

INVESTIGATION OF Ca^{2+} SIGNAL TRANSDUCTION PATHWAY(S) IN HUMAN FIBROBLASTS

By

Shixia Huang

In serum-free medium containing serum replacements but totally lacking in protein growth factors, diploid human fibroblasts remained quiescent if the extracellular Ca^{2+} concentration was only 0.1 mM. However, when the Ca^{2+} concentration in this medium was increased to 1 mM, the cells replicated as rapidly as they do in medium supplemented with protein growth factors. When quiescent cells in medium with only 0.1 mM Ca^{2+} were exposed to 1 mM or 10 mM Ca^{2+} or 100 ng/ml EGF, the 42 kDa and 44 kDa forms of MAPK were rapidly activated, as demonstrated by a characteristic electrophoretic mobility shift of these proteins and by their enhanced ability to phosphorylate myelin basic protein (MBP). Analysis of fractions from Mono Q anion-exchange chromatography of lysates of cells exposed to 10 mM Ca^{2+} or 100 ng/ml EGF revealed a peak of MBP phosphorylation activity that coeluted with p42 and p44 MAPK as shown by immunoblot analysis. Activation of MAPK by extracellular Ca^{2+} was dose dependent and biphasic, with a peak of activation at 5-10 min after exposure, followed by a period of sustained activation of MAPK at a lower level. This pattern has been shown (Vouret-Craviari et al. [1993], Biochem J. **289**, 209) to correlate with the re-entry of mammalian cells into the cell cycle.

We then investigated the pathway of Ca^{2+}-induced MAPK activation. We found the exposure of human fibroblasts to extracellular Ca^{2+} increased cytosolic inositol (1, 4, 5)-

trisphosphate level and caused a transient rise in the concentration of intracellular Ca^{2+}. Ca^{2+}-induced MAPK activation was partially abolished by treatment of the cells with pertussis toxin which inhibits certain species of G proteins. It was also decreased by treatment with thapsigargin which depletes intracellular Ca^{2+} stores, by exposure to phorbol 12-myristyl 13-acetate that downregulates protein kinase C (PKC), by treatment with calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCL and calmidazolium, as well as by exposure of the cells to lanthanum, a Ca^{2+} channel inhibitor. We found that the c-raf-1 protein was not phosphorylated after Ca^{2+} stimulation, and the Ca^{2+} sensing receptor (Brown et al. [1993] Nature 366, 575-580) was not involved in mediating MAPK activation or cell growth. These results suggest that extracellular Ca^{2+} stimulates MAPK activation through a pathway(s) involving a pertussis toxin sensitive G protein, phospholipase C, intracellular Ca^{2+}, calmodulin, and PKC.

This work is dedicated to

my husband: Yi Li

my daughter: Grace (DanDan)

my unborn baby

my father: Jia Gu Huang

my mother: Dong Xiu Li

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Finally, I am thankful for the support and help of my husband Yi. I want to thank my daughter Grace (DanDan) who provides me with so much love and fun and makes my life so enjoyable, and my unborn baby whose cooperation has made the writing of my dissertation possible in a relatively short time. Special thanks also go to my parents whose love and encouragement are very important to me.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER II

CHAPTER III

ABBREVIATIONS

BSA	bovine serum albumin
CaM1	calmodulin 1
CaMK	calmodulin dependent protein kinase
cAMP	cyclic adenosine 3', 5'-phosphate
cdks	cyclin dependent kinases
CR1	conserved region 1 in raf kinase
CR2	conserved region 2 in raf kinase
CR3	conserved region 3 in raf kinase
DAG	diacylglycerol
EGF	epidermal growth factor
EGFR	EGF receptor
ER	endoplasmic reticulum
FBS	fetal bovine serum
FGFs	fibroblast growth factors
GAP	GTPase activating protein
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
G proteins	guanine nucleotide binding proteins
IGFs	insulin-like growth factors
IP3	inositol (1, 4, 5)-trisphosphate
IP4	inositol (1, 3, 4, 5)-tetrakisphosphate

IRS	insulin receptor substrate
JAK	Janus kinase
MAP-2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MEK	MAPK kinase
NGF	nerve growth factor
NMDA	N-Methyl-D-aspartate
PDGF	platelet-derived growth factor
PI3 kinase	phosphotidylinosite 3 kinase
PIP2	phosphatidylinositol 4, 5-bisphosphate
PLC	phospholipase C
PKC	protein kinase C
PMA	phorbol 12-myristyl 13-acetate
PTH	parathyroid hormone
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2	src homology 2
SH3	src homology 3
STAT	signal transducer and activator of transcription
TGFβ	tumor growth factor β
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCL
W-12	N-(4-aminobutyl)-2-naphthalenesulfonamide

INTRODUCTION

It has been known since the 1970's that Ca^{2+} is required for normal mammalian cells to grow in culture (1-4). The first evidence of Ca^{2+} acting as a growth promoter came from a study with mouse 3T3 fibroblasts by Dulbecco and Belkington (5), in which they showed that increasing the concentration of Ca^{2+} above the physiological level (1.8 mM) promoted DNA synthesis.

Previous colleagues (6,7) in Carcinogenesis Laboratory at Michigan State University have shown that human fibroblasts grow in a serum free medium supplemented with 1.0 mM Ca^{2+} as rapidly as in this medium with serum or growth factors when supplemented with the serum replacements, and in the presence of insulin as the only protein growth factor. Although normal fibroblast cells require 1-2 mM Ca^{2+} for their growth, studies (6-8) have shown that fibroblasts transformed in culture or derived from fibrosarcomas are able to replicate in culture in serum free medium containing 0.1 mM Ca^{2+} or at a lower concentration, but no protein growth factors. This indicates that these cells have some alteration in the pathway by which Ca^{2+} drives growth. Since the mechanism of Ca^{2+}-induced cell growth is not understood, thus studying the mechanism of Ca^{2+}-induced cell growth may provide insight into the transformation process. Therefore, my studies concentrated on the elucidation of the mechanism(s) of Ca^{2+}-driven growth in normal human fibroblasts.

It is well known that protein growth factors,, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, thrombin, nerve growth factor, and bombesin, bind to specific receptors on the external cell surface, and initiate phosphorylation cascades through the activation of either tyrosine kinase-linked or guanine nucleotide

binding protein (G protein)-coupled receptors, and one of the key events in these cascades is the activation of mitogen-activated protein kinase (MAPK) by phosphorylation (9-15). Several closely related MAPK isoforms, referred to by their molecular masses, i.e., p40, p42, p44, and p54 (16), have been identified in various cell types. In human fibroblasts, it has been shown that EGF (11), tumor necrosis factor (13,17,18) and interleukin (18) can cause activation of p42 and/or p44 MAPKs. The activation of MAPK occurs when activated MAPK kinase phosphorylates the tyrosine and serine/threonine residues of MAPK (19). This phosphorylation can be detected because it results in a mobility shift of MAPK in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The activated MAPK can, in turn, phosphorylate microtubule-associated protein (MAP-2) (20), myelin basic protein (MBP) (21), ribosomal S6 kinase (22), the EGF receptor (23), phospholipase A2 (24), and the nuclear transcriptional factors c-myc (25,26), c-fos, and c-jun (23,27).

In mouse 3T3 fibroblasts in culture, it has been shown that extracelluar Ca^{2+} stimulates DNA synthesis (28-33) and c-fos expression (30-32,34) at a level similar to that induced by PDGF (31,32). Furthermore, Epstein et al. (31,32) showed that both PDGF-induced and Ca^{2+}-induced c-fos expression is dependent on protein kinase C (PKC). However, unlike PDGF, Ca^{2+} did not induce the phosphorylation of the PDGF receptor or the raf protein, indicating that the pathways which result in similar gene transcription are, at least in part, independent.

The objectives of the present work were (1) to demonstrate that human fibroblasts can grow in a medium containing 1.0 mM Ca^{2+} and serum replacements but no protein growth factors and to determine whether Ca^{2+} stimulates the activation of MAPK; (2) to investigate the involvement of various intracellular components in the pathway of Ca^{2+}-

induced MAPK activation; (3) to investigate the involvement of certain membrane elements, such as the Ca^{2+}-sensing receptor of Brown et al. (35) in the pathway of Ca^{2+}-induced MAPK activation and cell growth.

Chapter I reviews the literature regarding extracellular Ca^{2+} as the first messenger, growth factor pathways, and Ca^{2+} signaling in mammalian cells. Chapter II consists of a manuscript published in the **Biochemical Journal** , 310, 881-885, 1995. It describes the research I carried which demonstrates that in normal human fibroblasts extracellular Ca^{2+} can stimulate the activation of MAPK and cell growth in the absence of protein growth factors, and correlates the MAPK activation with the growth of these cells under such conditions; Chapter III consists of a manuscript to be submitted to the **Biochemical Journal**. It describes the research I carried which demonstrates the pathways involved in extracellular Ca^{2+}-induced MAPK activation.

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CHAPTER I LITERATURE REVIEW

A. Extracellular Ca^{2+} as the first messenger

1. Extracellular Ca^{2+} and cell growth

In normal resting mammalian cells, the cytosolic free Ca^{2+} concentration is maintained at 10-100 nM (1), in contrast, the extracellular free Ca^{2+} concentration in blood and tissue culture medium, is between 1-2 mM. "The importance of intracellular Ca^{2+} in regulating cell function is well recognized. Not less important, but less well understood, is the fundamental role played by extracellular Ca^{2+} in the modulation of cell function " (2). Studies in the 1970s' have shown that there is a strict requirement of 1-2 mM extracellular Ca^{2+} (physiological level) for the growth of chicken (3-5), mouse (6), and human (7) fibroblasts in culture. However, the mechanism(s) which set this Ca^{2+} requirement for normal fibroblasts to grow in culture is not clear yet.

A study by Dulbecco and Elkington (6) in 1975 showed that when added to resting cultures of mouse 3T3 fibroblasts in serum-free medium, extracellular Ca^{2+} concentrations above 1.8 mM induced DNA synthesis and cell growth. Additionally, they showed that the growth rate in 5.4 mM Ca^{2+} was similar to that observed after the addition of 10% serum. They also found that the effect of Ca^{2+} on cell growth was synergistic with that of serum. This was the first report showing that Ca^{2+} itself acted as a growth promoter. Several reports (8-15) support and expand these findings and suggest some insight into the mechanisms by which Ca^{2+}-induced cell growth in 3T3 mouse fibroblasts. These will be discussed in detail below.

Hazelton et al. (7) showed that physiological levels of extracellular Ca^{2+} were required for human lung fibroblasts (WI-38) to enter S phase. When Ca^{2+} in the medium was reduced to 0.06 mM or lower, a substantial fraction of WI-38 cells were prevented from entering S phase. When the Ca^{2+} concentration in the medium was increased to 2 mM, the cells entered S phase. Their study also showed that, in contrast to normal WI-38 cells, SV-40 transformed WI-38 cells did not require Ca^{2+} to enter S phase. Since then, another group (16) showed similar Ca^{2+} requirements (1-2 mM) for the growth of human lung fibroblasts (WI-38 and IMR90) in medium containing serum pretreated with chelex to remove the Ca^{2+}, but not for that of their simian virus 40 (SV40) transformed counterparts. In 1986, Praeger and Cristofalo (17,18) showed that WI-38 cells can grow in a serum free medium with Ca^{2+} (5 mM) or epidermal growth factor (EGF) in the presence of insulin, dexamethasone and transferrin. They also showed that combining suboptimal doses of EGF and Ca^{2+} resulted in an additive effect on cell growth.

In 1977, Pledger et. al. (19) showed that serum contains two types of growth promoting agents: competence factors, e. g., platelet-derived growth factor (PDGF), and progression factors, e. g., insulin-like growth factors (IGFs). These factors function synergistically to promote growth of BALB/c 3T3 mouse fibroblasts (19,20). Quiescent 3T3 cells exposed briefly to PDGF become "competent" to replicate their DNA and divide; however, these "competent" cells did not progress efficiently into S phase unless they were incubated throughout G_0/G_1 with medium containing plasma which contains progression factor (19). Studies by Rubin and Samui (9) and Bowen Pope and Rubin (10) suggest that Ca^{2+} stimulated cell growth by forming precipitates with inorganic pyrophosphate or orthophosphate. In their studies, they observed that Ca^{2+}-stimulated DNA synthesis

correlated with the concentration of inorganic orthophosphate or pyrophosphate in the medium, as well as the turbidity of the medium. They proposed that precipitates of Ca^{2+}-phosphate complexes acted in a manner similar to macromolecular stimulants such as hormones and interacted with the plasma membrane. In 1979, Stiles et al. (20) found that $Ca_3(PO_4)_2$, like PDGF, acted early in the cell cycle as a competence factor, but did not act as a progression factor. Mitchell et al. (11) and Cheung et al. (12) found that basic calcium phosphate crystals, like PDGF, induced c-fos and c-myc gene expression. These above studies were carried out in mouse 3T3 fibroblasts using DME medium with 5% platelet poor plasma which contained the progression factors necessary for mouse fibroblasts to enter the cell cycle after stimulation by competence growth factors, such as PDGF. The distinct role of competence and progression factors has been demonstrated in mouse 3T3 fibroblasts (19,20) and lymphocytes (21). Human fibroblasts appear to need only competence growth factor such as PDGF or EGF for their growth. One explanation for this is that human fibroblasts synthesize IGF-1 (22) during cell proliferation, and this IGF-1 may act as a progression factor. It is also known that during proliferation, human fibroblasts secrete fibroblast growth factor (FGF), and FGF itself is a mitogen for fibroblasts (23). It has been suggested that FGF may act in a positive feedback loop to induce proliferation (23). Sugimoto et al. (24) showed that in osteoblasts, cell growth is stimulated by high concentration of Ca^{2+}, and that was mediated by IGF-1.

In the Carcinogenesis Laboratory of Michigan State University, Morgan et al. (25) showed that diploid human foreskin fibroblasts can replicate rapidly in serum-free McM medium, a modified version of MCDB 110 (26), if supplied with designated serum replacements and with insulin as the only protein growth factor, instead of insulin and EGF

as described by Ryan et al. (27). In this medium, supplemented with serum replacements factors normal fibroblasts could grow for their entire in vitro life time (McCormick et al., unpublished results). The McM medium used in these studies contained 1.0 mM Ca^{2+} (25,28). Morgan et al. (25) also showed that normal human fibroblasts did not replicate if the Ca^{2+} concentration was reduced from 1 mM to 0.1 mM, suggesting that extracellular Ca^{2+} stimulates cell growth.

2. Extracellular Ca^{2+} and other functions

In addition to its involvement in cell proliferation, extracellular Ca^{2+} has also been shown to function in hormonal secretion (29) and cell differentiation (30-35). Studies (30,32-34) showed that mouse and human keratinocytes in culture proliferated in a medium with a low Ca^{2+} concentration (0.05-0.1 mM), and when the Ca^{2+} concentration was increased to 1.4-2.0 mM, these cells underwent terminal differentiation.

In parathyroid cells, the decrease of extracellular Ca^{2+} induces the secretion of parathyroid hormone (PTH) (29,36). Extracellular Ca^{2+} also negatively regulates the growth of parathyroid cells (36). Sakaguchi (36) found that the acidic fibroblast growth factor autocrine system was a mediator of Ca^{2+}-regulated parathyroid cell growth. Expression of both the mRNA and peptide of acidic FGF was suppressed by increasing the extracellular Ca^{2+} concentration.

Kidney cells, osteoclasts, and placental cells also have the ability to sense changes in the extracellular Ca^{2+} concentration (29). Osteoclasts, the cells that resorb bone during the skeletal remodeling cycle, recognize and respond to changes in the extracellular Ca^{2+} concentration in a manner similar to that of parathyroid cells. Increasing the concentration

of extracellular Ca^{2+} reduces the expression of podosomes, which contribute to cellular attachment to bone, and also decreases their bone resorptive activity (29,37). In placental cells, high concentrations of extracellular Ca^{2+} inhibit the secretion of the polypeptide hormone, placental lactogen, from placental fragments (38,39) as well as from dispersed or enriched cultures of trophoblastic cells prepared from placenta.

B. Growth factor pathways

1. Growth factors and their receptors

Cells exchange growth and differentiation signals through growth factors and hormones (40,41). Most growth factors and hormones are unable to pass the hydrophobic cell membrane. Instead, they exert their effects by binding to specific receptor proteins on the cell surface of the target cells (41). These cell surface receptors bind to the signal molecule (e.g., a protein growth factor) and convert the extracellular signal into intracellular events that affect the behavior of target cells. Cell surface receptor proteins are divided into three classes: enzyme-linked receptors, GTP binding protein (G protein)-coupled receptors, and ion channel-linked receptors (42). Ion channel-linked receptors, which are also known as transmitter-gated ion channels, mediate their signals by neurotransmitters that are involved in rapid synaptic signaling between electrically excited cells. Growth factors or hormones, which promote cell growth in their target cells, exert their effects through the activation of either enzyme-linked or G protein-coupled receptors (43).

With enzyme-linked receptors, the extracellular binding of a ligand causes the

activation of receptors that function directly as enzymes, such as tyrosine kinases, or are associated with enzymes (42,44). Most of the receptors in this family have an extracellular domain with ligand binding sites, a single transmembrane domain, and an intracellular domain with catalytic sites (41,42). Growth factors that have this kind of receptor include EGF, PDGF, FGF, and IGF, etc. (41). The majority of receptors of this type are protein kinases, or are associated with protein kinases, each of which phosphorylates specific sets of proteins in a target cell. Most of protein tyrosine kinase receptors undergo dimerization upon ligand binding (41). After dimerization, these receptors undergo autophosphorylation, and in turn, phosphorylate downstream kinases which are associated with the receptor molecules by their src homology 2 (SH2) domain (45,46).

G protein-coupled receptors bind to their ligands, and transmit a signal through a group of G proteins, which in turn regulate downstream effectors, such as enzymes and ion channels (42,44). All G protein-coupled receptors have an extracellular domain which binds to the ligand, a common seven transmembrane domain, which is structurally distinct from the single transmembrane domain of the enzyme-linked receptors, and an intracellular domain which interacts with G proteins (42). Growth factors or hormones which bind to this kind of receptor include bradykinin, thrombin, bombesin, interleukin 8, parathyroid hormone, somatostatin, A2 adenosine, etc. (29,44,47). The G protein-coupled receptors respond to the binding of a specific ligand by undergoing a conformational change. This allows the intracellular domain of the receptor to interact with a specific type(s) of G proteins. If a receptor can interact with only one subtype of G protein, it will activate effectors only through this G protein, and the response will be relatively focused. On the other hand, if a receptor interacts with several G-proteins, each of the G proteins will interact

with one or more effectors, and the response will be more diffuse since it will involve several different pathways. The function of G proteins will be discussed in section 2.2.

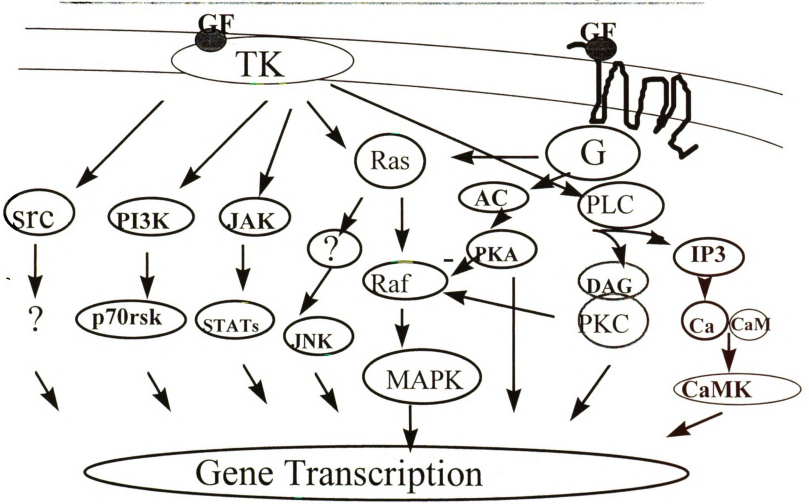
2. Intracellular pathways

2.1 Overview of the pathways upon growth factor stimulation

Growth factors or hormones promote cell proliferation by binding and activating their specific receptors, mainly through either tyrosine kinase-linked receptors, or G protein-coupled receptors, and the activation of these receptors initiates one or more phosphorylation cascade(s) (48-54) (see figure on next page and detail description below).

Pathways involving the tyrosine kinase-linked receptors The activation of receptor tyrosine kinases activates downstream effectors. As described above, the binding of a ligand to a receptor induces dimerization and autophosphorylation of the receptor . The individual phosphotyrosine residues located in the cytoplasmic domains of receptors serve as highly selected binding sites that interact with specific cytoplasmic molecules (55). A common feature of many signaling molecules that are known to bind to receptor tyrosine kinases with high affinity is that they contain src homology 2 (SH2) domains, regions of about 100 amino acids that have homology with the noncatalytic region present in the c-src proto-oncoprotein (56-58). A large number of proteins containing SH2 domains, and the specific sequences that these proteins recognize and bind to, have been identified (57,58). The SH2 domain of the p85 subunit of phosphoinositide 3 kinase (PI3 kinase) has been shown to associate with the human PDGF β receptor via tyrosines 740 and 751, which lie in

Growth Factor Pathways



the kinase insert regions of this receptor (59), with the murine colony-stimulating factor-1 (CSF-1) receptor via tyrosine 721 (60), and with several phosphorylated tyrosines on the insulin receptor substrate-1 (IRS-1) (61). All of these tyrosine residues lie within the consensus sequence of tyrosine x x methionine (YxxM) (where x can be a wide range of possible residues), which is important for PI3 kinase binding (62). Other SH2 domain-binding sites have been identified on the human PDGF β-receptor. These include tyrosine 771 in the kinase insert region, the site for interaction with ras GTPase activating protein (rasGAP) (63), tyrosine 1020 in the C-terminal tail for interaction with phospholipase C γ (PLCγ) (64), tyrosine 1009, the binding site for the phosphotyrosine phosphatase, syp (65), and tyrosine 579 and 581 in the juxamembrane segment N-terminal to the catalytic domain, as the binding site for src family protein tyrosine kinases (66). EGF receptors (EGFR) bind PLCγ with high affinity through the SH2 domain. It has been shown that the activated EGFR associates with Shc protein in vivo, most likely through its SH2 domain (67). The Shc protein serves as an adaptor protein which mediates the function of ras through other proteins such as Grb2 and Son of sevenless (Sos) (62). GAP and its associated proteins p62 and p190 become tyrosine and serine phosphorylated following stimulation with EGF (68), although a physical complex between GAP and the EGFR has not been identified. The in vitro studies have detected complexes between isolated GAP SH2 domains and the C-tail domain of the EGFR (69). Activation of PI3 kinase by the EGFR differs depending on the cell type (55). In pheochromocytoma cell line PC12, PI3 kinase is activated by the EGFR with kinetics comparable to those of other growth factor receptors (70). The EGFR has also been shown to function through G proteins (71). In human epidermoid carcinoma A431 cells, growth is inhibited by EGF, and EGF treatment of these cells induces tyrosine

phosphorylation and activation of signal transducers and activators of transcription (STATs) (72), a family of proteins that once activated, translocate from the cytoplasm to the nucleus and bind to DNA (73,74). The activation of STATs is mediated by another family of proteins called Janus kinases (JAK) that have been shown to associate with the EGFR (75). Many immediate early responsive genes including the cyclin-dependent kinase inhibitor p21 are regulated by JAK-STATs (75).

The observation that a single receptor can interact with such a diverse range of signaling molecules demonstrates the diversity of the pathways that ligand binding can induce. For example, the association and regulation of PDGF receptor or EGF receptor with PI3 kinase, rasGAP, src family, PLC, JAK-STATs, etc. leads to the involvement of many pathways or components after ligand binding. The PI3 kinase has been shown to activate p70 ribosomal s6 kinase (p70rsk) (21). RasGAP is a regulator of the ras protein which interacts with raf, and leads to MAPK activation which activates pp90rsk or directly regulates c-jun/fos transcription (76), or the activation of ras can activate jun kinase (JNK) by some unknown intermediators (76). The activation of the src/yes/fyn family leads to myc gene transcription (77). The activation of PLCγ hydrolyzes phophotidylinositol bisphosphate (PIP2) to generate diacylglycerol (DAG) and inositol (1, 4, 5)-trisphosphate (IP3). DAG in turn activates PKC and IP3 induces intracellular Ca^{2+} release (21). Many of these pathways crosstalk with each other through certain proteins. For example, the activation of PKC and the elevation of intracellular Ca^{2+} lead to MAPK activation (50,53). The activation of PI3 kinase has been shown to activate MAPK through the ras pathway (78). The activation of src has been shown to activate the ras/MAPK pathway (78), and JAK-STATs are connected with MAPK in lymphocytes (79).

Pathways involving the G protein-coupled receptors: The activated G protein-coupled receptors interact with various types of G proteins (44), and G proteins, in turn, interact with their specific downstream effectors (44,78,80-82), mainly through, PLC, cyclic adenosine 3', 5'-phosphate (cAMP), ras/raf, and ion channels, depending on the type of G proteins involved.

One important feature of the G protein-coupled receptors is that the same receptor may couple to different G proteins in different cell types (80). For example, bradykinin-activated PLC is fully inhibited by pertussis toxin in monkey renal cells (MDCK)(80,83,84), is partially inhibited by pertussis toxin in mouse NIH3T3 fibroblasts (80,85,86), and is not inhibited by pertussis toxin in human epidermoid carcinoma (A431) cells (80,87,88). The types of G proteins involved could reflect the availability of specific G protein in the cell type studied, or the G protein pools that can be accessed by the activated receptor in that cell type upon stimulation (80). Also, multiple pathways can be generated by varying the concentration of the ligand (89). For example, in pancreatic acinar tissue, Trimble et al. (90) observed that a low concentration of secretin stimulated adenylate cyclase, but a high concentration stimulated the activation of PLC to hydrolyze PIP2, leading to the generation of IP3 and induces Ca^{2+} mobilization. Chabre et al. (91) suggested that these two pathways were mediated by different G proteins upon stimulation by different concentrations of the ligand, i. e., that G_s directly activates adenylate cyclase and most likely a member of the G_q class regulates the stimulation of PLC. Alternatively, Birnbaumer (92) suggested that the stimulation of PLC could also be caused by beta gamma subunits of G_s , which generate an additional signal but only at higher concentration of the ligands.

2.2 G proteins

G proteins consist of three polypeptides, an α subunit, a β subunit, and a γ subunit (44,93). The α subunit binds and hydrolyzes guanosine 5'-triphosphate (GTP) to guanosine 5'-diphosphate (GDP), and the β and γ subunits form a dimer. When GDP is bound, the α subunit associates with the βγ subunits to form an inactive heterotrimer that binds to the receptor. Both α and βγ subunits can bind to the receptor (44). When a growth hormone or growth factor binds to a receptor, the receptor becomes activated and undergoes a conformational change. The GDP bound α subunit responds to this conformational change in the receptor and also changes its conformation, which decreases the affinity of the α subunit to GDP ; GDP then disassociates from this subunit, and is replaced with GTP. Once GTP is bound, the α subunit assumes its active conformation and dissociates from the receptor and the βγ subunit (44).

All isoforms of α subunits are GTPases. However the GTP hydrolysis rate differs from one type of the α subunit to another (94,95). The free α and βγ subunits each activate their target effectors. There are over 20 different G protein α subunits in mammalian cells (44). The proteins are divided into four major classes (families) based on their amino acid sequence similarity: as α_s, α_i, α_q, and α_{12} (44) . Each of these classes has several members, for example, α_s and α_{olf} are in the α_s family, α_{i-1}, α_{i-2},α_o, α_t are in the α_i family, α_q ,α_{11} ,α_{14}, and α_{16} are in the α_q family, and α_{12}, α_{13} are in the α_{12} family (44). A specific G protein activates its specific downstream effectors. Some types of G proteins or α subunits can be modified by cholera toxin or pertussis toxin (44). The α_s family, which activates adenylate cyclase and regulates ion channels upon stimulation, can be modified by cholera toxin. The α_i family of

G proteins, which activates cyclic GTP, phosphodiesterase, or PLC (44,80), inhibits adenylate cyclase, or regulates K^+ and Ca^{2+} channels, can be modified by pertussis toxin. α_q and α_{12} are insensitive to either of these two toxins. In general α_q activates PLC, and α_{12} regulates Na^+/K^+ exchange (81).

In addition to the signals generated by Gα subunits, Gβγ subunits can also generate signals (82,96-98). Ito et al. (99) showed that overexpression of Gβγ stimulated the phosphorylation of MAPK and enhanced the kinase activity of c-raf-1, and coexpression of dominant negative ras inhibited Gβγ-induced MAPK phosphorylation. This indicates that ras/raf/MAPK pathway can be activated by Gβγ. It has been shown that adenylate cyclase has a binding site for the Gβγ subunits (100). Hawes et al. (78) showed that Gβγ mediated MAPK activation through a pathway involving PI3 kinase, and that the activation of PI3 kinase in this pathway was upstream of Sos/ras. They also showed that Gβγ was able to activate proteins of the src family, which in turn, activate Shc/Grb2 and Sos, and mediate the activation of ras/ raf/MAPK pathway. Krapivinsky et al. (101) showed that Gβγ regulated the K+ channel by directly binding to it.

2.3 Phospholipase `C

As described above, both the tyrosine kinase-linked receptors and G protein-coupled receptors can activate PLC. This enzyme is responsible for hydrolyzation of the inositol lipid, such as PIP2 , and generation of two important intracellular second messengers (43,102), DAG, and IP3. DAG is responsible for activating the isoenzymes of PKC (103). On the endoplasmic reticulum, IP3 binds with the IP3 receptor which releases Ca^{2+} from intracellular stores. The release of intracellular Ca^{2+} is responsible for many cellular events.

The main isoforms of PLC are PLCβ, PLCγ, PLCδ, and PLCε (80,104). Each one of these represents a family of closely related molecules. Each of the subtypes is designated by adding Arabic numerals after the Greek letters such as PLCγ1 and PLCγ2 (104). PLCγ has an approximate molecular weight of 150 kDa, and PLCβ and PLC δ have a molecular weight of 85 kDa. When assayed in vitro with pure substrate, PLCβ, γ, and δ have been shown to hydrolyze both phosphotidylinositide (PI) and PIP2 (80). The hydrolysis of PIP2 occurs at micromolar concentration of Ca^{2+}, and that of PI occurs at millimolar Ca^{2+} concentration (80). The PLCε forms have molecular weights of 85-88 kDa, and can only hydrolyze PIP2 but not PI when assayed in vitro (105-107), but no sequence data has yet been obtained on molecules of this family. The previously identified PLCα (80), which has molecular weights of 57-70 kDa and shares no homology with other isoforms of PLC, is now known to be a placental protein disulfide isomerase (108,109), and therefore is no longer classified as a PLC. Although the overall amino acid sequence similarity between different PLC isoforms is low, there is two regions of homology, region X, 150 amino acids, and region Y, 240 amino acids, which are shared by PLCβ, PLCγ, and PLCδ (80,104). Among the three isoforms, there are about 60% identity in region X and 40% identity in region Y (80,110). These regions seem likely to constitute the catalytic domain.

The various isoforms of PLC are regulated differently. PLCγ is regulated by tyrosine phosphorylation (80,111). It contains SH2 and SH3 domains. The SH2 domain targets the molecule to tyrosine phosphorylated sequences present in other proteins such as tyrosine kinase-linked receptors for PDGF, EGF, etc.. When activated, both PDGF and EGF receptors have been shown to be associated with PLCγ (45,46) by their SH2 domain and to phosphorylate PLCγ1 on its tyrosine and serine residues (112-115). However, some other

receptors with tyrosine kinase activity, such as the insulin receptor and the CSF-1 receptor, do not phosphorylate PLCγ1 (46,116).

PLCβ is regulated by G protein-coupled receptors (80). Evidence suggests that the G proteins involved in regulating PLCβ include two distinct types: pertussis toxin sensitive and pertussis toxin insensitive (104). The α subunits of the G_q class of G proteins have been demonstrated to be specific activators of the PLCβ isoform (117). The specificity of the interaction between different Gα subunits and PLC has been assessed by introducing cDNAs corresponding to various Gα subunits into the monkey kidney cell line Cos-7 and measuring the amount of inositol phosphates formed after stimulation with ALF_4^- (104,118). Transfection of cells with the $G\alpha_q$ or $G\alpha_{11}$ cDNA results in a marked increase in inositol phosphate formation. Cotransfection of $G\alpha_q$ (or $G\alpha_{11}$) cDNA and PLC-β1 cDNA causes even higher levels of inositol phosphate formation. The relative ability of members of the G_q subfamily to activate PLCβ isozymes has also been determined. Membranes from Cos-7 cells transfected with cDNAs corresponding to $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, or $G\alpha_{16}$ have been reconstituted with purified PLCβ1 or PLCβ2 in the presence of GTPγS (104). All four members of the $G\alpha_q$ subfamily were found to stimulate PLCβ1, with $G\alpha_q$ or $G\alpha_{11}$ being most effective. $G\alpha_{16}$ was found to activate PLCβ2 most effectively, while $G\alpha_q$, $G\alpha_{11}$, or $G\alpha_{14}$ showed much less stimulation. Therefore, there appears to be a specificity in the interaction of different members for the G_q subfamily with different PLCβ effectors. This specificity may be important in generating tissue- or receptor- specific responses in vivo.

Other PLC regulators include Ca^{2+} and PKC. It has been shown that Ca^{2+} affects PLC activation either directly or by modulating the receptor mediated responses (80). An

increase of intracellular Ca^{2+} appears to stimulate PLC activity (80). However, there is very little information about which isoforms are Ca^{2+}-regulated or whether there is a specific isoform of PLC that is only sensitive to Ca^{2+}. PLCγ1 can be negatively regulated by PKC (80,119). When Jurkat cells were treated with PMA, PLCγ was shown to be phosphorylated at Ser-1248. This phosphorylation did not allow the tyrosine phosphorylation of this protein, and therefore, negatively regulated its activity (119).

2.4 Protein kinase C

PKC has been shown to be activated by various growth factors and hormones (53,120). Multiple isoforms have been isolated, and different cell lines or tissues have been found to express different isoforms (121). Furthermore, different stimuli or second messengers may determine which PKC isoforms become activated (121). The PKC isoforms are divided into three groups (121-123): conventional or classic PKC(cPKC) which includes α, βI, βII, and γ, new PKC or novel PKC (nPKC) which includes δ, ε, η, and θ, and atypical PKCs(aPKC) which includes ζ and λ. It has been shown that PKCα, βI, βII, δ, ε, and ζ are ubiquitously distributed, e. g., in brain, lung, spleen, thymus and skin (121). PKCγ has been found to be exclusively expressed in the central nervous system. And PKCη is strongly expressed in skin and lung. PKCθ is predominantly expressed in skeletal muscle. PKCα, δ, ε, and ζ, have been shown to be expressed in both mouse NIH3T3 fibroblasts and rat6 fibroblasts (121,124,125).

PKCs have a protein kinase domain which is conserved in all PKC isoforms, and a regulatory domain which shows different structures in different groups of PKCs (126). In the regulatory domain, all PKCs have one (aPKC) or two (cPKC, nPKC) cysteine-rich

sequences called the C1 region. This region is the putative membrane binding region (122), through which DAG activates PKC (121). The cPKC has a C2 region which contains many acidic amino acids and is thought to participate in Ca^{2+} binding (121,122) even though there is no sequence motif representing a known Ca^{2+} binding site (such as an E-F hand). The nPKC and aPKC lack the C2 region. The activation of nPKCs is dependent on DAG but not Ca^{2+}. Phosphatidylcholine (PtdCho)/PLC or Phospholipase D hydrolysis of PtdCho leading to the generation of DAG only will activate this group of PKCs (123). The activation of aPKCs is not dependent on either Ca^{2+} or DAG. They can be regulated by the PtdIns(3,4,5)P3 pathway or the ceramide pathway (123). There is sufficient and convincing evidence (121) showing that the activation of cPKC requires: the generation of DAG and IP3 from plasma membrane-associated PIP2 by the activation of PLC, the release of Ca^{2+} from the intracellular Ca^{2+} store by IP3 stimulation, the binding of Ca^{2+} to the C2 region of PKC, and subsequent translocation of PKC to the plasma membrane where it is activated by DAG through its C1 region. The activation of cPKC also requires phosphatidylserine as a cofactor which is constitutively present in the membrane (121).

Phorbol ester mimicks the action of DAG to activate PKC (121). Activation of cPKC by phorbol ester does not require Ca^{2+}, however the presence of Ca^{2+} will lower the concentration of phorbol ester required for the full activation of PKC (121). Phorbol ester treatment leads to the activation of PKC in various cells including Chinese hamster ovary (CHO) cell line (103), mouse NIH 3T3 cell line (121), Swiss 3T3 cell line (53), and human fibroblasts (127,128). In these cell lines, prolonged treatment with phorbol ester downregulates the activation of many PKC isoforms (53,121). The same PKC isoform will have a different PMA sensitivity in different cell lines. For example, PKCα in most cell lines

is PMA sensitive, but in pituitary gland cells (GH4C1), this isoform is not downregulated upon treatment with PMA (121,129). PKCβ and δ are PMA sensitive in most cell lines, whereas the sensitivity of PKCε and PKCζ to PMA is quite controversial (121). Different PKC isoforms have been overexpressed in different cell lines (121). The overexpression of PKC isoforms is correlated with an enhanced expression of the nuclear transcriptional factors such as c-jun, c-fos, and c-myc, and leads to the abnormal growth of the cells (121). In rat 6 fibroblasts (130), the overexpression of PKCβI causes the cells to grow in a disorganized manner and to be able to form colonies in soft agar. Rat 6 cells overexpressing PKCβI are more susceptible to transformation by tranfecting an activated H-ras oncogene compared to non-PKC expressing cells (131).

Activation of different isoforms of PKC can phosphorylate downstream effectors in vivo. In CHO cells, overexpression of PKCα or PKCδ leads to MAPK activation (103). It has also been shown that the activation of PKC mediates c-raf kinase activation through direct phosphorylation (132,133). Studies with fibroblasts overexpressing PKC have suggested that PKC functions in an agonist-stimulating pathway leading to the activation of phospholipase D or phospholipase A2 (122). The intracellular 85 kDa phospholipase A2 contains consensus phosphorylation sites for PKC and MAPK (122,134). However, activation of PKC alone does not appear to be sufficient. Intermediary proteins such as phospholipase A2-activating protein (135,136) may take part in activating this enzyme.

2.5 Raf

Growth factors, such as PDGF, activate downstream kinases, such as PKC, raf, and MAPK, through phosphorylation (120,130,133). The viral-raf (v-raf) oncogene was

originally identified as the transforming gene of a murine sarcoma virus strain 3611 (137). The protooncogenes c-raf (138) and A-raf were identified (139) by screening a mouse spleen cDNA library with v-raf DNA as a probe. There are three active raf genes have been found in human, mouse, and chicken, A-raf-1, B-raf, and c-raf-1 (140). They are dispersed over different chromosomes and have been mapped to sites that are frequently altered in human tumors (141,142). The raf genes encode cytosolic phosphoproteins of similar size: c-raf-1, 74 kDa; A-raf-1, 68 kDa; B-raf, 73 kDa; and these raf proteins function as serine/threonine-specific protein kinases (140,142). All raf proteins share three highly conserved regions (CR1, CR2, CR3) embedded in variable sequences (143). The catalytic domain of the raf kinases resides in CR3, whereas CR1 and CR2 function as the regulatory part of the raf protein molecules (143-145). These raf proteins are differentially expressed in tissues (142): c-raf-1 is ubiquitously expressed, A-raf-1 is predominantly expressed in urogenital tissues (139), and B-raf is most abundant in cerebrum and testis.

These kinases are inactive in quiescent cells, and can be activated by growth factors. B-raf has been shown to be activated in mammalian pheochromocytoma PC12 cells after stimulation by nerve growth factor (146,147). C-raf-1 kinase is positively-regulated by activated growth factor receptor kinases in a variety of fibroblastic (140,148), epithelial (149), and lymphoid cells (150,151). Raf kinases are located in the cytosol of unstimulated cells. Growth factors induce ras activation which causes binding to the raf protein (140). Thus, binding by ras translocates raf to the plasma membrane which is necessary, but not sufficient, for the activation of raf kinase activity (152). Activation of raf kinase activity involves phosphorylation of serine/threonine residues on raf, and in the case of c-raf-1, of additional tyrosine residues by non-receptor tyrosine kinases (152,153). In the case of B-raf,

but not c-raf-1, phosphorylation and activation can be observed in vitro using crude preparations of the kinase and farnesylated ras (154). The identity of a specific kinase activator of raf remains unknown (155). PKC has been suggested as a candidate, since studies show that PKC mediates c-raf-1 kinase activation by direct phosphorylation (132,133).

The activation of raf kinase leads to a characteristic shift in apparent molecular weight upon phosphorylation (13,150). A-raf shares several functional properties with c-raf-1 including transforming activity, stimulation of the raf/MAPK pathway, and the ability of dominant negative versions to functionally block ras signaling (156). Once activated, raf kinases phosphorylate the MAPK kinase (MEK) on its serine residues resulting in its activation (155). Two serine residues of MEK at positions 218 and 222 become phosphorylated, and either is sufficient for activation (157). MEK is a dual specificity threonine/tyrosine kinase, which when catalytically active, phosphorylates and activates the MAPK. Unlike raf and MEK, MAPKs have numerous substrates, including p90 ribosomal S6 kinase, cytoplamic phospholipase A2, c-jun, c-foc, c-myc, etc. (155). Raf-1 may also directly regulate the activation of the cell cycle through control of the mammalian Cdc25 phosphatase (158). The Cdc25 family of phosphatases activates many cyclin-dependent kinases (cdks) by dephosphorylation of conserved threonine and tyrosine residues (155). Active cdks control progression through each phase of the cell cycle. Raf-1 has been shown to physically complex with human Cdc25 in vivo and in vitro (2296). Immunoprecipitates of active, baculovirus-produced raf-1 protein phosphorylate and activate the phosphatase activity of recombinant Cdc25 (158). However, it is not clear whether Cdc25 is phosphorylated by raf-1 or another associated kinase such as MEK or MAPK (155).

2.6. Intracellular Ca^{2+}

The cytosolic concentrations of free Ca^{2+} in normal resting mammalian cells are very low (0.1-1.0 uM) compared with the extracellular levels (1.0-2.0 mM) (29). Consequently, the entry of a small quantity of Ca^{2+} into cells by Ca^{2+} channels in plasma membranes or the release of Ca^{2+} from intracellular stores will result in a sharp increase in free cytosolic Ca^{2+} concentrations. The sudden rise in cytosolic Ca^{2+} is believed to play an important role in cell proliferation (159). This idea is supported by the observation that mitogens, such as serum (16,160), PDGF (159,161,162), bradykinin (163,164), and bombesin (165), induce an intracellular Ca^{2+} increase (within seconds and which lasts for minutes) as well as cell proliferation in normal cells such as fibroblasts and muscle cells. By blocking Ca^{2+} influx with lanthanum, Estacion and Mordan (159) showed that the selective inhibition of the sustained Ca^{2+} increase completely inhibited the progression of mouse fibroblasts into S phase upon PDGF exposure. Direct evidence showing that intracellular Ca^{2+} is linked to cell growth has been obtained in studies by Gill and colleagues (166,167). Their studies showed that the intracellular Ca^{2+} pool depletion by thapsigargin arrested hamster smooth muscle cells in culture in a G_o-like quiescent state and blocked cell growth (see below for detail).

Ca^{2+} channels There are two types of Ca^{2+} channels in plasma membrane: voltage-sensitive and voltage-insensitive (47). In general, in excitable cells, such as neuronal and muscle cells, predominantly exhibit voltage sensitive calcium channels that open after membrane depolarization (168,169). There are three types of voltage sensitive calcium channels: L, T, and N type. N type Ca^{2+} channels are shown to be present exclusively in neuronal cells. L and T type Ca^{2+} channels are shown to be present in other types of cells,

including mouse and human fibroblasts as demonstrated by the patch-clamp technique which measures the conductance pattern of the cells (159,161,170). In nonexcitable cells, such as fibroblasts and epithelial cells, Ca^{2+} not only passes through the L and T types of Ca^{2+} channels (159,161,170), but also a family of poorly characterized, voltage insensitive calcium channels (169,171), which have been classified into three general groups: 1) receptor-operated calcium channels, 2) second messenger-operated calcium channels, and 3) depletion-operated calcium channels. Regulation of the first group has been shown to be independent of intracellular Ca^{2+} and some other second messengers (172). For example, muscarinic receptors can regulate these Ca^{2+} channels (169), and the structural elements in the receptor plays a role in its regulation (172). Stimulation of the second group of channels can be directly regulated by intracellular calcium, IP3, and possibly IP4. The third group of channels open after IP3-mediated depletion of intracellular stores and provide a source of calcium for refilling the stores. Regulation of calcium influx through this group of channels is triggered by the depletion of intracellular calcium and may involve a calcium influx factor released from the endoplasmic reticulum that stimulates the opening of low conductance calcium channels (168,169)

It has been shown that Ca^{2+} entry through the different channels regulates gene expression differently (173). Bading et al. (173) demonstrated that N-Methyl-D-aspartate (NMDA) receptors and L-type Ca^{2+} channels, the two major sites of Ca^{2+} entry into hippocampal neuronal cells, transmit signals to the nucleus and regulate gene transcription through two distinct Ca^{2+} signaling pathways. These investigators found that the L-type Ca^{2+} channels triggered Ca^{2+} entry and then initiated c-fos gene expression through the Ca^{2+} response element and/or the cAMP response element on the c-fos promoter region between

base pairs +1 and -222. Gene expression of c-fos was dependent on Ca^{2+}-calmodulin-dependent protein kinase (CaMK) (173). On the other hand, NMDA receptor Ca^{2+} channels induced the expression of c-fos through the serum response element of the c-fos promoter region located on or near base pair -300. In this case, gene expression did not depend on CaMK. These investigators also found that a specific L-type channel blocker or a NMDA receptor antagonist completely abolished c-fos expression (173).

Intracellular Ca^{2+} pools There are multiple intracellular Ca^{2+} pools present in endoplasmic reticulum of various cell systems (174): the IP3 sensitive pool and the IP3 insensitive pool, both of which are sensitive to thapsigargin, and a third pool is unresponsive to IP3 and insensitive to thapsigargin. The thapsigargin sensitive pools (pools 1 and 2) contain 75% of the total Ca^{2+} , and the third pool contains about 25% (174). Baumgarten et al. (175) showed that human foreskin fibroblasts also has three different intracellular Ca^{2+} pools: an IP3 sensitive, thapsigargin sensitive pool; an IP3 insensitive, thapsigargin sensitive pool; and an ionomycin sensitive pool which is not sensitive to either IP3 or thapsigargin.

Studies by Gill and colleagues (166,167) have shown that thapsigargin depletes the intracellular Ca^{2+} stores (pools) by specific inhibition of the endoplasmic reticulum (ER) Ca^{2+} -ATPase, and in turn, will deplete the source of intracellular Ca^{2+} for these pools. They found that the depletion of those Ca^{2+} pools resulted in the arrest of cells in G_0 phase (166,174), and thapsigargin suppressed the expression of the endoplasmic reticulum Ca^{2+} pump protein (176).

2.7 Calmodulin

The function of Ca^{2+} is mediated by its binding proteins. There are two different kinds of Ca^{2+} binding proteins, trigger proteins and buffer proteins (168). Buffer proteins, such as calsequestrin, simply bind Ca^{2+} as its concentration increases within cells to buffer or lower free Ca^{2+} levels since too high a free Ca^{2+} concentration is toxic to cells (168). Trigger proteins, on another hand, change their conformation upon binding Ca^{2+} and modulate effector molecules such as enzyme and ion channels. The trigger proteins include calmodulin, calcineurin, phospholipase A2, PLC,, etc. (168). Calmodulin acts as the primary mediator of Ca^{2+}-dependent signaling in eukaryotic nonmuscle and smooth muscle cells by serving as a high affinity intracellular receptor (177,178). It has been shown that calmodulin is required for progression at specific points of the cell cycle in mammalian cells (179,180) and that it affects cell proliferation in variety of cell types (1,181). For example, the selective pharmacological inhibitors of calmodulin kinase, KN-62 and KN-93, have been shown to prevent quiescent WS-1 human fibroblasts from reaching S phase upon serum stimulation (181,182). Takuwa et al. (16) showed that the potent calmodulin antagonists calmidazolium and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl (W-7) but not its inactive analogue N-(4-aminobutyl)-2-naphthalenesulfonamide (W-12), strongly inhibit serum-induced DNA synthesis in normal human fibroblasts (16) and human vascular endothelial cells (183). In neuronal cells, it has been shown (173) that the increase in intracellular Ca^{2+} caused by Ca^{2+} entry modulates the activation of calmodulin kinases (CaMK), such as CaMK2 or CaMK 4, regulating the expression of their downstream genes. Calmodulin is highly conserved throughout the eukaryotic kingdoms (184). It has a molecular weight of approximately 17 kDa and a high percentage of acidic amino acids. A distinctive property of calmodulin and of other related calcium-binding proteins is that they undergo a mobility

shift when run on a SDS-PAGE in the presence of Ca^{2+} (184). Calmodulin has been shown to be encoded by several genes in vertebrates. Three calmodulin cDNAs (CaM1, CaM2, and CaM3) have been cloned from rat and human, and multiple genes have been identified in many other species (178,184,185). These cDNAs all encode identical calmodulin proteins. Although all three mRNA transcripts are expressed in all mammalian tissues and cultured cells, the expression level of each calmodulin gene has been shown to vary according to the cell type and is modified by the concentration of extracellular growth factors, such as nerve growth factor (186). In mouse C127 cells, it has been shown (178) that all three calmodulin genes are expressed, but that only the CaM2 mRNA levels show significant changes as cells progress through the cell cycle. These results indicate that the calmodulin genes may be differentially regulated during the cell cycle.

In mammalian cells, the 148 amino acid calmodulin protein has a dumbbell-shape structure with two Ca^{2+} binding sites in each half of the molecule (178). Except for the budding yeast calmodulin which only binds three Ca^{2+} ions, calmodulins from all other species have four highly conserved "EF-hand" Ca^{2+}-binding sites, like those first described in the crystal structure of parvabumin (178,187). When intracellular Ca^{2+} increases, it binds to calmodulin, and induces a conformational change in the molecule which exposes its hydrophobic patches that are involved in interaction with and activation of its target enzymes (177,184,188,189). Without the conformational alterations, calmodulin does not bind to most of its various targets (184). More than 20 enzymes can be regulated by calmodulin (178,188,190,191). These enzymes include cyclic 3', 5'-nucleotide phosphodiesterase (178,192,193), adenylate cyclase (194,195), (Ca^{2+}- Mg^{2+})ATPase (196,197), the cardiac microsomal calcium transporter (197), IP3 kinase (178,198,199), calmodulin-dependent

protein kinases, such as myosin light chain kinase (178,200) and the multifunctional calmodulin-dependent protein kinase (201,202) as well as calmodulin-dependent protein phosphatase (calcineurin) (203-205).

Ca^{2+} has been shown to be absolutely required for all enzyme activating functions of vertebrate calmodulin (178). However, the Ca^{2+} requirement can be altered in vitro by different concentrations of calmodulin. Increasing the calmodulin concentration can decrease the amount of Ca^{2+} required to activate calmodulin-dependent enzymes. Also, increasing the Ca^{2+} concentration can decrease the amount of calmodulin required for the activation of calmodulin-dependent enzymes (178). These results indicate that in vitro Ca^{2+} and calmodulin cooperatively regulate the function of the target protein. Transformed cells typically have elevated calmodulin levels and exhibit the ability to grow in Ca^{2+}-deficient medium which does not allow the growth of their nontransformed counterparts (178). Because it has not yet been possible to replace all three active endogenous calmodulin genes with a single inducible calmodulin gene, the relationship between the calmodulin concentration and the Ca^{2+} requirement for cell growth has remained unclear. It has been shown that the growth of *A. nidulans* cells is dependent on the concentration of calmodulin and Ca^{2+} as in mammalian cells (206). Using *A. nidulans* as a model system, it has been shown (178) that increasing the calmodulin concentration allows these cells to grow at a very low extracellular Ca^{2+} concentration (10-fold lower than the noninduced or normal state). These data indicate that a cooperative regulation exists inside cells between Ca^{2+} and calmodulin, and this may provide a possible explanation for why cells that are transformed and have elevated calmodulin levels can proliferate in a Ca^{2+}-deficient medium (178).

2.8 MAPK

As described above, protein growth factors such as EGF, PDGF, insulin, thrombin, nerve growth factor (NGF), and bombesin, can initiate a phosphorylation cascade in various cell types through the activation of either tyrosine kinase-linked or G protein-coupled receptors, and one of the key events in this cascade is the activation of MAPK by phosphorylation (49-54,207,208). Several closely related MAPK isoforms, usually referred to by their molecular masses, i.e., p40, p42, p44, and p54 (209), have been identified in various cell types. In human fibroblasts, it has been shown that EGF (50), tumor necrosis factor (52,210,211) and interleukin (211) can cause activation of p42 and/or p44 MAPKs. MAPK kinase activates MAPK by phosphorylation on its tyrosine and serine/threonine residues (212), which results in a mobility shift of MAPK proteins when analyzed by SDS-PAGE.

The activated MAPK can, in turn, phosphorylate microtubule-associated protein (MAP-2) (213), myelin basic protein (MBP) (214), ribosomal S6 kinase (215), the EGF receptor (216), phospholipase A2 (217), and the nuclear transcriptional factors c-myc (218,219), and c-fos and c-jun (216,220). Pouysségur and his colleagues (221,222) showed that in quiescent Chinese hamster fibroblast cell line CCL39, MAPK was activated by thrombin or basic fibroblast growth factor and that the activation of MAPK (as detected by an immunocomplex kinase assay using MBP as substrate) exhibited a biphasic pattern i.e., a rapid increase in activity followed by a rapid decrease and then a sustained level of lower activity. They proposed that this sustained activity was required for the re-entry of these cells from G_0 into the cell cycle (222), i.e., for triggering the proliferative response, because

when they treated CCL39 cells with a thrombin analog instead of thrombin or with carbachol, the second phase of MAPK activation was not observed and no DNA synthesis took place.

Other investigators have demonstrated that the sustained activation of MAPK is associated with proliferation in fibroblasts (223-225). In addition, Pagès et al. (226) and Frost et al. (227), using MAPK antisense RNA and/or MAPK kinase-deficient mutants to suppress MAPK activation, showed that MAPK activation was required for activation of transcriptional factors (227) and for rodent fibroblast proliferation (226).

In other cell systems, continuous MAPK activation could lead to the opposite cellular behavior. In pheochromocytoma cell line PC12, cellular responses are determined by the duration of MAPK activation. In these cells, it has been shown that sustained MAPK activation is associated with differentiation, whereas transient MAPK activation is related to proliferation (228). Continuous treatment of PC12 cells with FGF or NGF leads to outgrowth of neurites and eventual cessation of cell division, whereas treatment with EGF leads to a proliferative signal (229). Many signal transduction events have been found to be shared between differentiation and proliferation in PC12 cells (230). However, notable quantitative differences were found between differentiation and proliferative signals. In PC12 cells, NGF stimulation results in a persistent elevation of rasGTP, whereas EGF produces only a short lived rise in rasGTP (231). MAPK activation is sustained for several hours following NGF stimulation, but it is short lived after EGF stimulation (229,232). In PC12 cells, it has been found that sustained MAPK activation is always associated with translocation of MAPK to the nucleus (229,232-234), whereas transient activation does not lead to nuclear translocation. Transient activation will, therefore, have very different

consequences for gene expression compared with sustained activation (2314). In this way, it is believed that cells can use transient and sustained activation of MAPK to determine a variety of responses (228).

C. Ca^{2+} signaling in mammalian cells

1. Intracellular components involved in Ca^{2+} signaling and cell differentiation/hormonal secretion

As described in "Section A", extracellular Ca^{2+} can serve as the first messenger to regulate cell differentiation, hormonal secretion, and cell proliferation in different cell systems (29-35,235). The numerous intracellular components or pathways involved in these processes have been found using different approaches (29-31,34,235)

In keratinocytes in culture, the addition of Ca^{2+} to the medium rapidly increases the intracellular phosphoinositide turnover, intracellular Ca^{2+} levels (30,34), and tyrosine phosphorylation of proteins (30,31). The rasGAP has been shown to be specifically affected during Ca^{2+}-induced differentiation (31). Upon Ca^{2+} treatment, GAP associates with tyrosine-phosphorylated proteins and translocates to the membrane (31). Also, a p62 protein, which is associated with GAP, has been found to be heavily phosphorylated on tyrosine both in membrane and cytosolic fractions (31). This protein is only phosphorylated by Ca^{2+} stimulation, and not via growth factor stimulation, such as EGF or TGF-β (30,31). Ca^{2+} does not induce the tyrosine phosphorylation of PLCγ and PI3 kinase, which are activated upon EGF stimulation in this cell type (31). Filvaroff et al. (30) suggested that Ca^{2+}-induced p62 phosphorylation, and possibly differentiation, was mediated by a Ca^{2+} receptor. Denning et

al. (235) showed that the induction of keratinocyte differentiation markers by Ca^{2+} was mediated by specific protein isozymes. In the cultured murine cells they studied (235), they detected PKC isoforms by immunoblotting (PKCα, δ, ε, ζ). They observed that PKCα, δ, and ε were translocated from the soluble fraction to particulate fraction during Ca^{2+} -induced differentiation, suggesting that the induction of keratinocyte differentiation by Ca^{2+} results in the activation of specific PKC isoforms (235). In parathyroid cells, the increase of extracellular Ca^{2+} concentration decreases PTH release (29). Studies (236,237) showed that stepwise increases in the extracellular Ca^{2+} concentration produced corresponding increases in intracellular Ca^{2+} concentration in these cells. There was a close inverse relationship between the high extracellular Ca^{2+}-induced intracellular Ca^{2+} increase and the accompanying decreases in PTH release (236,237), although it is not clear yet whether the intracellular Ca^{2+} change is the cause of the decrease in PTH release. However, other studies (29,238) showed that increasing the intracellular Ca^{2+} directly by treating cells with Ca^{2+} ionophores, such as A23187, was associated with inhibition of PTH secretion. Extracellular Ca^{2+} also stimulates the accumulation of inositol monophosphate, inositol bisphosphate, IP3, and IP-4 (239). It is believed that the increases of IP3 mediates, at least partially, the intracellular Ca^{2+} elevation evoked by extracellular Ca^{2+} (240). Shoback et al. (239) showed that Ca^{2+} ionophore, ionomycin, which raises the intracellular Ca^{2+} directly to a level equivalent to that observed at high extracellular Ca^{2+} concentration, had little or no effect on the accumulation of inositol phosphates (29). And the addition of extracellular Ca^{2+} to parathyroid cells increases IP3 level, which has been shown to be through the activation of PLC, and is mediated by a G protein-coupled receptor (29,241). In addition, extracellular Ca^{2+} inhibits the receptor-mediated cAMP increase. The decrease of cAMP level upon Ca^{2+} treatment is

mediated by enhanced adenylate cyclase activity (2,29,242). In osteoclasts, extracellular Ca^{2+} stimulates Ca^{2+} release from Ca^{2+} entry channels in the plasma membrane which can be blocked by lanthanum (37) and from intracellular stores. A Ca^{2+} receptor is suggested to be involved in the increase of intracellular Ca^{2+} in this cell type (37). They showed that in osteoclast cells, extracellular Ca^{2+} induced intracellular Ca^{2+} elevation, which leads to cytoskeletal changes affecting cell adhesion and decreasing the bone resorptive activity (37).

2. Intracellular components involved in Ca^{2+} stimulated growth pathway

A few groups have studied the mechanism of Ca^{2+}-induced cell growth. Studies by Rubin and Samui (9) and Bowen Pope and Rubin (10) showed that in mouse fibroblasts the Ca^{2+} and phosphate precipitate can serve as the primary signal to promote cell growth. In their studies, they observed that Ca^{2+}-stimulated DNA synthesis was correlated with the concentration of inorganic orthophosphate or pyrophosphate in the medium, as well as the turbidity of the medium. They proposed that precipitates of Ca^{2+}-phosphate complexes acted in a manner similar to macromolecular stimulants (such as hormones) and interacted with the plasma membrane. Most recently, Epstein et al. (13) and Epstein (14) showed that calcium chloride mimicked PDGF in its ability to stimulate DNA synthesis and to induce c-fos gene expression in mouse 3T3 fibroblasts. They showed that both PDGF-induced and Ca^{2+}-induced c-fos expression is dependent on PKC, but unlike PDGF, Ca^{2+} does not induce the phosphorylation of the PDGF receptor and the raf protein, indicating that Ca^{2+} and PDGF have distinct pathways resulting in similar gene transcription.

3. Ca^{2+} sensing receptor and its signaling

In 1993, Brown et al. (241) cloned a Ca^{2+}-sensing receptor from bovine parathyroid, and subsequently, the human Ca^{2+}-sensing receptor was cloned (243). This receptor possesses an extracellular domain of about 613 amino acids, a membrane domain which contains seven membrane-spanning segments, and an intracellular domain of about 222 amino acids) (241,244). The seven membrane-spanning domain is the characteristic of the superfamily of G-protein coupled receptors as described above in section B2.2. This protein is about 120 kDa (241,244) and it has been shown to be expressed both in tissues known to be involved in Ca^{2+} homeostasis (e.g., parathyroid, C-cells, and kidney) and in other cells that do not directly regulate systemic Ca^{2+} (e.g., brain, neuronal, and smooth muscle cells) (2). The Ca^{2+}-sensing receptor has been shown to transmit signals from outside the cell to induce cellular responses, such as to increase cytosolic Ca^{2+} and IP3 levels when expressed in xenopus oocyte (241). The rise of intracellular Ca^{2+} is sustained by the Ca^{2+} influx through Ca^{2+} channels in the plasma membrane (2). And also, the expression of this receptor cDNA in xenopus oocyte has been shown to activate PLC (241,244,245) which will hydrolyze PIP2 to generate IP3 and induce intracellular Ca^{2+} elevation. The activation is dependent on a G protein (244,246) which is sensitive to pertussis toxin in xenopus oocyte (241). However, the elevation of IP3 and intracellular Ca^{2+} in parathyroid cells is not dependent on pertussis toxin sensitive G protein (2,29), indicating that this receptor couples to a different subfamily of G protein in these different cell types (241)

It has been shown that human diseases, such as familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism, result from mutations in this Ca^{2+}-sensing receptor (247-249). Various mutants of this receptor have been generated to study the function of this

protein (249-252). The results from these studies confirm that the mutation of this receptor is responsible for various human diseases.

In parathyroid cells in culture, it has been shown that the reduction or loss of Ca^{2+} responsiveness is related to a marked reduction in expression of extracellular Ca^{2+}-sensing receptor (253,254). Recently, knockout mouse lacking Ca^{2+}-sensing receptor (255) has been generated and it has the characteristics similar to those of humans with a mutation in this receptor. The heterozygous mice (CaR+/-) are analogous to humans with familial hypocalciuric hypercalcemia and have modest elevations of serum calcium, magnesium and parathyroid hormone levels, as well as hypocalciuria. The CaR-/- mice are analogous to humans with neonatal severe hyperparathyroidism. They have markedly elevated serum calcium and parathyroid hormone levels, parathyroid hyperplasia, bone abnormalities, as well as retarded growth (255). This suggests that Ca^{2+} receptor mutations cause these human disorders by reducing the number of functional receptor molecules on the cell surface (255).

4. Other possible Ca^{2+}-sensing receptors

In parathyroid cells, extracellular Ca^{2+} not only induces the intracellular IP3 and Ca^{2+} increase, but also inhibits receptor-mediated increases in cAMP (2,29). For example, dopamine-stimulated cAMP is inhibited by extracellular Ca^{2+} (29). This inhibition can be abolished by pertussis toxin in parathyroid cells (29), indicating that a pertussis toxin-sensitive G protein is involved in this process (2,29). Such an inhibiting effect on cAMP is also observed in the thick ascending limb cell from mouse kidney (256). The decrease of cAMP level upon Ca^{2+} treatment is due to the enhanced adenylate cyclase activity (2,29,242), which is activated via a pertussis toxin-sensitive G protein. This G protein apparently differs

from that which mediates the Ca^{2+}-induced elevation of intracellular IP3 and intracellular Ca^{2+}. The receptor coupled to this pertussis toxin-sensitive G protein in parathyroid cells is either the same receptor cloned by Brown et al.(2) which couples to two different G protein subfamilies simultaneously (2), or could be another receptor which senses extracellular Ca^{2+} and mediates this action (29).

By using monoclonal antibodies, Juhlin et al. (257-259) identified an ~500 kDa membrane protein on proximal tubular cells, cytotrophoblast cells of human placenta. Exposure of these cells to extracellular Ca^{2+} (2) induces cellular responses, such as regulation of 1-hydroxylation of 25-hydroxyvitamin D in the proximal tubule and changes in circulating PTH levels. The fact that the Ca^{2+}-sensing receptor cloned by Brown et al. from bovine parathyroid has not been found to be expressed in these cells (2) suggests that the 500 kDa protein is another type of Ca^{2+} sensing receptor. Recently, Lundgren et al. (260) isolated a 2.8 kb cDNA which contains an open reading frame encoding 236 amino acids, and which represents part of the gene form the above mentioned 500 kDa protein. Using this cDNA as probe, a 15 kb transcript was identified in human kidney, placenta, and parathyroid (260). Sequence analysis of the 2.8 kb cDNA showed that this protein is a member of the low-density lipoprotein receptor superfamily and that it is closely related to Heyman nephritogenic protein, a kidney tubule glycoprotein, with Ca^{2+} binding activity (260,261). The Heyman nephritogenic protein has been cloned and sequenced recently (262). It is the human variant of principal kidney autoantigen causing Heyman membranous glomerulonephritis in rats (262). The deduced 4655 amino acid residues give a calculated molecular weight of 519.6 kDa for the mature protein. The primary structure reveals that it contains an huge extracellular region (about 4400 amino acids), a single transmembrane-

spanning domain of 23 amino acids, and an intracellular C-terminal domain of 209 amino acids (262). This protein contains three types of cysteine-rich repeats characteristic of the low density lipoprotein receptor (LDLR) superfamily (262), and its intracellular domain contains several SH3 recognition motifs, one SH2 recognition motif for the P85 regulatory subunit of PI3 kinase, and additional recognition sites for PKC, casein kinase II, and cAMP/cGMP dependent protein kinase (262). It is not yet known whether this protein, Heyman nephritogenic protein, or the 500 kDa protein identified by Juhlin et al. (257-259), function as Ca^{2+} sensors. Subsequent functional studies should resolve this question.

In addition to specific Ca^{2+} sensing receptors, other known growth factor receptors could also mediate the function of extracellular Ca^{2+} and generate intracellular signals. For example, it has been shown that the FGF receptor has a Ca^{2+} binding motif in its acid box, which binds Ca^{2+} as shown by a Ca^{2+} blotting technique, and the Ca^{2+} binding is not observed in a mutagenized form of the receptor that lacks the acidic box region (263). The mechanism(s) by which Ca^{2+} directly modulates the activities of various cell types (2), such as keratinocytes (15), cultured mammary cells (264), and intestinal goblet cells (265) still needs to be identified.

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CHAPTER II

Extracellular Ca^{2+} stimulates the activation of mitogen-activated protein kinase and cell growth in human fibroblasts

Shixia Huang, Veronica M. Maher, and J. Justin McCormick*

Carcinogenesis Laboratory- Fee Hall

Department of Microbiology and Department of Biochemistry

The Cancer Center

Michigan State University, East Lansing, MI 48824

ABSTRACT

In serum-free medium containing serum replacements but totally lacking in protein growth factors, diploid human fibroblasts remained quiescent if the extracellular Ca^{2+} concentration was only 0.1 mM. However, when the Ca^{2+} concentration in this medium was increased to 1 mM, the cells replicated as rapidly as they do in medium supplemented with protein growth factors. When quiescent cells in medium with only 0.1 mM Ca^{2+} were exposed to 1 mM or 10 mM Ca^{2+} or 100 ng/ml EGF, the 42 kDa and 44 kDa forms of MAPK were rapidly activated, as demonstrated by a characteristic electrophoretic mobility shift of these proteins and by their enhanced ability to phosphorylate myelin basic protein (MBP). Analysis of fractions from Mono Q anion-exchange chromatography of lysates of cells exposed to 10 mM Ca^{2+} or 100 ng/ml EGF revealed a peak of MBP phosphorylation activity that coeluted with p42 and p44 MAPK as shown by immunoblot analysis. Activation of MAPK by extracellular Ca^{2+} was dose dependent and biphasic, with a peak of activation at 5-10 min after exposure, followed by a period of sustained activation of MAPK at a lower level. This pattern has been shown (Vouret-Craviari et al. 1993, Biochem J. **289**, 209) to correlate with the re-entry of mammalian cells into the cell cycle.

ABBREVIATIONS

EGF, epidermal growth factor; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; SCS, supplemented calf serum.

Running Title: Extracellular Ca^{2+} stimulates MAPK and cell growth

INTRODUCTION

It is known that human fibroblasts in culture will replicate in medium containing specific protein growth factors (1), and that Ca^{2+} is an important factor in cell metabolism (2). Studies show that such protein growth factors as EGF, platelet-derived growth factor, insulin, thrombin, nerve growth factor, tumor necrosis factor, and bombesin, can initiate a phosphorylation cascade in various cell types, including human fibroblasts, and that one of the key events of this cascade is the activation of MAPK by phosphorylation (3-9). Several closely related MAPK isoforms, referred to by their molecular masses, i.e., p40, p42, p44, and p54 (10), have been identified in various cell types. In human fibroblasts, EGF (5), tumor necrosis factor (7,11) and interleukin (11) have been shown to cause activation of p42 and/or p44 MAPKs. Activated MAPK can, in turn, phosphorylate MBP, microtubule-associated protein 2, the EGF receptor, phospholipase A2, and the nuclear transcriptional factors c-myc, c-fos, c-jun (10, 12, 13).

Morgan et al. (14) in this laboratory showed that diploid human fibroblasts can replicate rapidly in serum-free McM medium (15), a modified version of MCDB 110 (1), if supplied with the serum replacements, e.g., lipids, iron, attachment factors, etc., specified by Ryan et al. (15) and with insulin as the only protein growth factor, instead of the two growth factors specified by Ryan et al. (15), e.g., insulin and EGF. McM medium, as ordinarily prepared (15), contains 1 mM Ca^{2+}. Morgan et al. (14) also showed that the cells do not replicate if the Ca^{2+} concentration is reduced from 1 mM to 0.1 mM, suggesting that

extracellular Ca^{2+} stimulates cell growth. We tested this hypothesis by removing all protein growth factors from the medium and seeing if Ca^{2+} alone could cause the cells to replicate for an extended period. Since MAPK is known to integrate the signals from various growth factor pathways, we also tested whether Ca^{2+} causes the activation of MAPK in these cells as protein growth factors do. The answer to both questions was positive. In the total absence of protein growth factors, extracellular Ca^{2+} supported growth of the cells during the two weeks of the experiment. When quiescent cells were stimulated with Ca^{2+} or EGF, MAPK activation was observed as demonstrated by a characteristic shift in the electrophoretic mobility of MAPK and an enhanced ability of MAPK to phosphorylate MBP.

MATERIALS AND METHODS

Materials

MBP, EGF, and ß-glycerophosphate were purchased from Sigma (St. Louis, MO). Polyclonal anti-MAPK antibody Ab283 (from rabbit) was kindly provided by Dr. Marsha R. Rosner of the University of Chicago. Monoclonal anti-MAPK antibodies were purchased from Chemicon (Temecula, CA) and Zymed Laboratories (South San Francisco, CA). HRP-linked goat anti-mouse IgG was obtained from BioRad Laboratories (Richmond, CA) and HRP-conjugated goat anti-rabbit IgG was from Boehringer Mannheim Corp. (Indianapolis, IN).

Cells and cell culture

Diploid human fibroblast cell lines LG1 and SL80, derived in this laboratory from neonatal foreskins, were used at cell population doubling 15 to 28. The *in vitro* life span of LG1 cells is 40 population doublings, that of SL80 cells is 80 population doublings. Cells were routinely grown in Eagle's minimum essential medium supplemented with 0.2 mM aspartic acid, 1.0 mM sodium pyruvate, and 0.2 mM serine, 10% supplemented calf serum (SCS) (Hyclone Laboratories, Logan UT) penicillin (100 units/ml), streptomycin (100 µg/ml), and hydrocortisone (1 µg/ml) (complete medium) at 37°C in humidified incubator with 5% CO_2.

Media for testing for growth stimulation

To assay for growth stimulation we used McM medium (15), a modified version of MCDB 110 (1) which was developed for the continuous proliferation of human fibroblasts. We used the serum replacements specified by Ryan et al. (15) but omitting the protein growth factors, i.e., EGF and insulin. The medium was prepared to contain 0.1 mM or 1 mM Ca^{2+} as indicated.

Preparation of cell lysates

Cells were grown in 100-mm diameter dishes in complete medium until subconfluent or confluent as desired. To make the cells quiescent, the medium was replaced for 24 to 30 h with McM medium containing serum replacements but no protein growth factors and with the Ca^{2+} concentration reduced to 0.1 mM or to zero where indicated. Unless otherwise indicated, increased Ca^{2+} or EGF was then added directly to the medium on the cells with a gentle swirling, and the incubation at 37°C was continued for the indicated time. To harvest the cells, the medium was removed, the cell sheet was quickly washed twice with Ca^{2+}-free, Mg^{2+}-free PBS, and the cells were lysed in 0.3 to 0.5 ml of buffer composed of 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, and 1 mM phenylmethylsulphonylfluoride, 5 mM EDTA, 0.15 U/ml aprotinin, and 1 mM sodium orthovanadate. The cells were collected with a rubber scraper and kept on ice for 20 min. The lysates were centrifuged for 10 min at 4°C in a microfuge, and the protein concentration of the supernatant was determined using a Bicinchroninic Acid Protein Assay kit (Pierce Chemical Co., Rockford, IL) with BSA as the protein standard. The cell lysates were used immediately after preparation or stored at -80°C until used.

Immunoblot Analysis of MAPK

Cell lysates containing 25 μg of protein were dissolved in buffer containing 0.05 M Tris-HCl, pH 6.9, 0.72 M mercaptoethanol, 9% glycerol, 2.3% SDS and 0.1% Bromphenol blue (sample buffer), and the proteins were separated by 10% SDS-PAGE, and transferred from the gels onto Immobilon PVDF transfer membranes (Millipore Corp., Bedford, MA) overnight at 35 mA, and the blots were blocked by incubating for 1 h at room temperature in Tris-buffered saline, pH 7.6, (20 mM Tris-HCl, 137 mM NaCl) containing 0.1% Tween 20 (v/v) and 5% nonfat dry milk (w/v). To test for MAPK, the membranes were incubated for 1 h with the indicated anti-MAPK antibodies in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk, washed several times in this Tris-buffered saline, and then incubated with goat anti-rabbit IgG or goat anti-mouse IgG as desired. Enhanced chemiluminescence (Amersham, Arlington Heights, IL) was used as a detection system according to the manufacturer's instructions.

MBP phosphorylation as an assay for MAPK activity

This was assayed essentially as described previously (16). Briefly, cell lysates containing 2.5 μg of protein were incubated with MBP at 0.5 mg/ml for 10 min at room temperature in a final volume of 20 μl containing 18 mM HEPES, pH 7.4, 10 mM magnesium acetate, and 2 μCi [γ-^{32}P]ATP (2-10 Ci/mmol) (DuPont, New England Nuclear Research Products, Boston, MA). The reaction was stopped by the addition of 5X sample

buffer. After 5 min at 95°C, proteins were separated on a 10% SDS-PAGE. The dried gel was used to expose Kodak X-Omat film and a phosphor screen. The phosphor screen was scanned and the amount of MBP phosphorylation was quantified using a PhosphorImager (Model 400B, Molecular Dynamics, Sunnyvale, CA) according to the manufacturer's instructions.

Purification of MAPK by mono Q column chromatography

Cell lysates from Ca^{2+}-stimulated or EGF-stimulated cells or untreated cells were prepared and normalized for protein concentration. MAPK was purified by Mono Q anion-exchange chromatography as described (17). Before purification, lysates were thawed and centrifuged for 5 min in a microfuge, and the supernatant containing 1.5 to 2 mg of protein was diluted 2-fold in column buffer, which contains 50 mM ß-glycerophosphate, pH 7.2, 100 μM sodium orthovanadate, 1 mM EGTA, and 1 mM dithiothreitol. The diluted sample was filtered through a 0.2 μm filter and loaded onto a Mono Q HR5/5 column FPLC (Pharmacia, Piscataway, NJ) (1.5 ml bed volume) at a flow rate of 0.4 ml/min. Using a linear gradient of 0 to 0.4 M NaCl to elute, 1 ml fractions were collected. Aliquots of 10.5 μl were assayed for MAPK activity using the MBP phosphorylation assay, and aliquots of 110 μl were assayed by immunoblot analysis, as described above.

RESULTS AND DISCUSSION

Evidence that extracellular Ca^{2+} stimulates cell growth

To determine if extracellular Ca^{2+} could stimulate diploid human fibroblasts to replicate in the total absence of protein growth factors, we plated LG1 and SL80 cells in McM medium containing 0.1 mM Ca^{2+} and 1% SCS. The next day, the number of attached cells was determined, and the medium was replaced with McM medium containing the serum replacements of Ryan et al. (15) but minus the protein growth factors. The rate of replication of the cells in this medium supplemented with Ca^{2+} or EGF or 10% SCS is shown in Figure 1. Under the most stringent condition, 0.1 mM Ca^{2+}, the number of cells increased only slightly or at most doubled. In the presence of 1 mM Ca^{2+}, the LG1 cells doubled every 48 h, and the SL80 cells every 36 h. This rate of growth was as fast or faster than the cells' rate of growth in medium containing EGF (10 ng/ml), and in the case of SL80 cells, almost as fast as their rate with 10% SCS. Higher concentrations of EGF do not increase the growth rate, these results indicate that 1 mM Ca^{2+} stimulates human fibroblasts to proliferate as they do in response to protein growth factors.

MAPK activation as a result of Ca^{2+} or EGF stimulation

To determine whether MAPK is involved in activation of the signal pathway induced by extracellular Ca^{2+} in human fibroblasts, we treated LG1 and SL80 cells for 5 min with 1 mM Ca^{2+}, 10 mM Ca^{2+}, or 100 ng/ml EGF, and assayed the cell lysates for evidence of MAPK activation using immunoblot analysis. Protein growth factors are typically used at

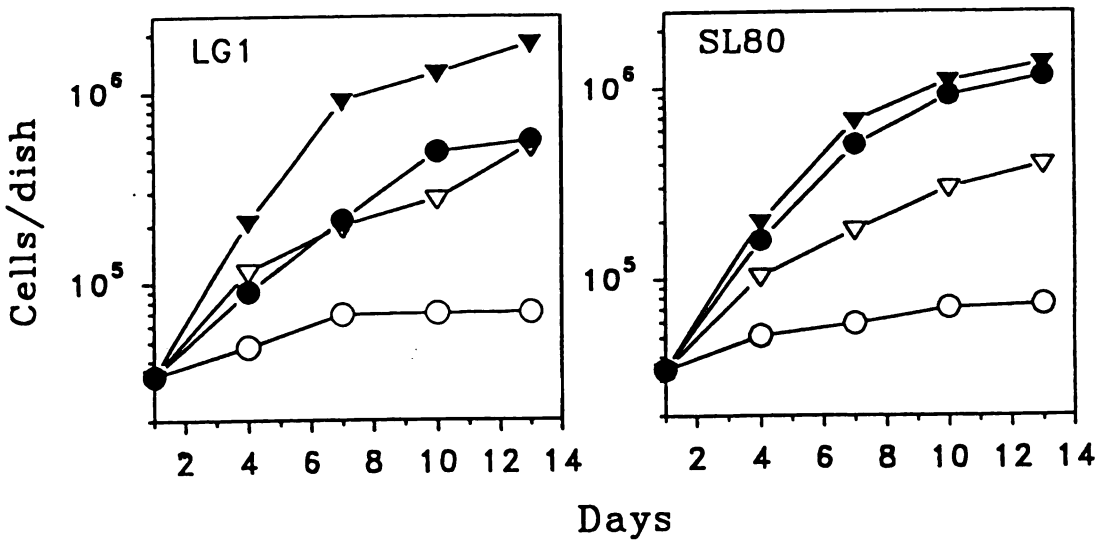

Figure 1 Growth stimulating activity of Ca^{2+}

Cells were plated into 60 mm-diameter dishes in McM medium containing 0.1 mM Ca^{2+} and 1% SCS. The next day (day 1), the number of attached cells was determined, the medium was replaced with McM medium containing the serum replacements of Ryan et al. (15) minus insulin and EGF, but modified as follows: 0.1 mM Ca^{2+} (○); 1 mM Ca^{2+} (●); 0.1 mM Ca^{2+} with 10 ng/ml EGF (▽); 0.1 mM Ca^{2+} with 10% SCS (▼). The cells were refed with appropriate medium on day 4, 7, and 10, and the number of cells in four dishes each time for each condition was determined on day 4, 7, 10 and 14. Similar results were obtained in two separate experiments.

~10 higher concentrations in MAPK assays than in growth studies. We used anti-MAPK polyclonal antibody Ab283 which was previously shown to detect both the 42 and 44 kDa isoforms of MAPK (5). With the cell lysates prepared from untreated cells, this antibody revealed two bands with molecular masses of approximately 42 and 44 kDa (lane 1 of Figure 2A and 2B). With cell lysates prepared from cells exposed for 5 min to 1 mM or 10 mM Ca^{2+} or to 100 ng/ml EGF (lanes 2, 3, and 4 of Figure 2A and 2B), slower moving bands were observed, one just above the 42 kDa band and one just above the 44 kDa band. This molecular weight shift is characteristic of the phosphorylated form of the MAPK isoforms (18, 19). The percentage of each MAPK isoform that traveled more slowly was greater with lysate from cells exposed to 10 mM Ca^{2+} or 100ng/ml EGF than from cells exposed to 1 mM Ca^{2+}. We also stripped the membrane using 62.5 mM Tris-HCl, pH 6.9, 2% SDS, and 0.1 M mercaptoethanol for 30 min at 50°C and reprobed it with monoclonal antibodies that recognize only the 42 kDa MAPK in human cell lines. The results indicated that the lower bands are the p42 form of MAPK (data not shown).

To determine whether this phosphorylation of MAPK resulted in a molecule with kinase activity, the cell lysates were also tested as described for their ability to phosphorylate MBP . As shown in Figure 2C and 2D, Ca^{2+} or EGF increased the degree of MBP phosphorylation. The degree of MBP phosphorylation produced by lysates from cells stimulated with 10 mM Ca^{2+} was much greater than that from cells given 1 mM Ca^{2+}, and approximately equal to that from the cells treated with 100ng/ml EGF.

The activation of MAPK by Ca^{2+} or 100ng/ml EGF was also evaluated on Mono Q column-purified MAPK from the cell lysates. The lysates from untreated cells or from cells

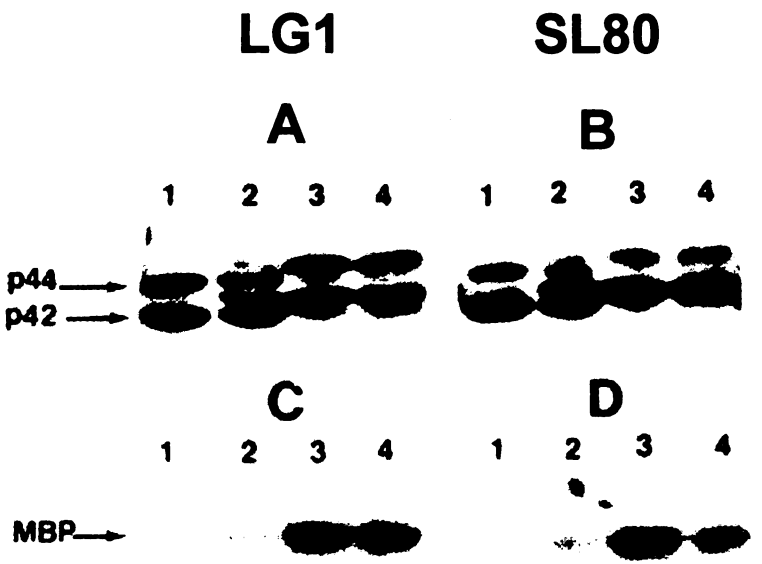

Figure 2 MAPK activation as a result of Ca^{2+} or EGF stimulation

Cells were grown to confluence in Eagle's medium with 10% SCS. The medium was changed to McM medium (containing 0.1 mM Ca^{2+}) with the serum replacements of Ryan et al. (15) but no protein growth factors. After 24 h, Ca^{2+} or EGF was added directly to the medium at the indicated concentrations. And 5 min later, cell lysates were prepared. Panels *A, B,*: immunoblot analysis of cell lysates. Proteins in cell lysates were separated by SDS-PAGE, and subjected to immunoblot analysis probed with anti-MAPK antiserum Ab283. Panels *C, D,*: phosphorylation of MBP by cell lysates. Samples of cell lysates were mixed with MBP and [γ-^{32}P]ATP for 10 min, and assayed as described. *Lane 1*, control, i.e. 0.1 mM Ca^{2+}; *lane 2*, 1 mM Ca^{2+}; *lane 3*, 10 mM Ca^{2+}; *lane 4*, 100 ng/ml EGF. The results shown is the representative for three independently performed experiments.

exposed to 10 mM Ca^{2+} or EGF were fractionated by chromatography, and aliquots of fractions 8-20 were assayed for the ability to phosphorylate MBP (Figure 3). For LG1 cells, stimulated with either Ca^{2+} or EGF, high activity was observed in fractions 12 and 13, but not in the corresponding fractions from untreated cells. For SL80 cells, high activity was found in fractions 13 and 14 with EGF, and fraction 14 with Ca^{2+}. The presence of MAPK in those specific fractions and its absence from other nearby fractions was demonstrated by immunoblotting and probing with the polyclonal anti-MAPK antibody that recognizes both isoforms (Figure 4). The two forms coeluted. Note that in the fractions from the Ca^{2+}-stimulated or EGF-stimulated cells, there was a shift in the electrophoretic mobility of the MAPK isoforms. Coelution of these two isoforms on Mono Q column has been previously reported (20) in a study using a similar volume of eluent. When a much larger volume of the eluent was used, the 42 kDa and 44 kDa isoforms showed a better separation, although some fractions still contained both isoforms (21). As shown in Figure 3, the control sample from SL80 cells showed a small peak of phosphorylated MBP in fraction 10. However, the immunoblot analysis showed no detectable MAPK in that fraction (data not shown), and use of a second assay for MAPK activity (microtube-associated protein 2 phosphorylation) also showed no such activity in this fraction (data not shown).

To test whether albumin or other factors used as the serum replacements played a role in the activation of MAPK, we also assayed for Ca^{2+}-induced MAPK activation in confluent cells that had been incubated overnight in McM medium containing 0.1 mM Ca^{2+}, but no serum replacements, as well as in confluent cells similarly incubated overnight but then incubated for two additional hours in phosphate-buffered saline. Under both

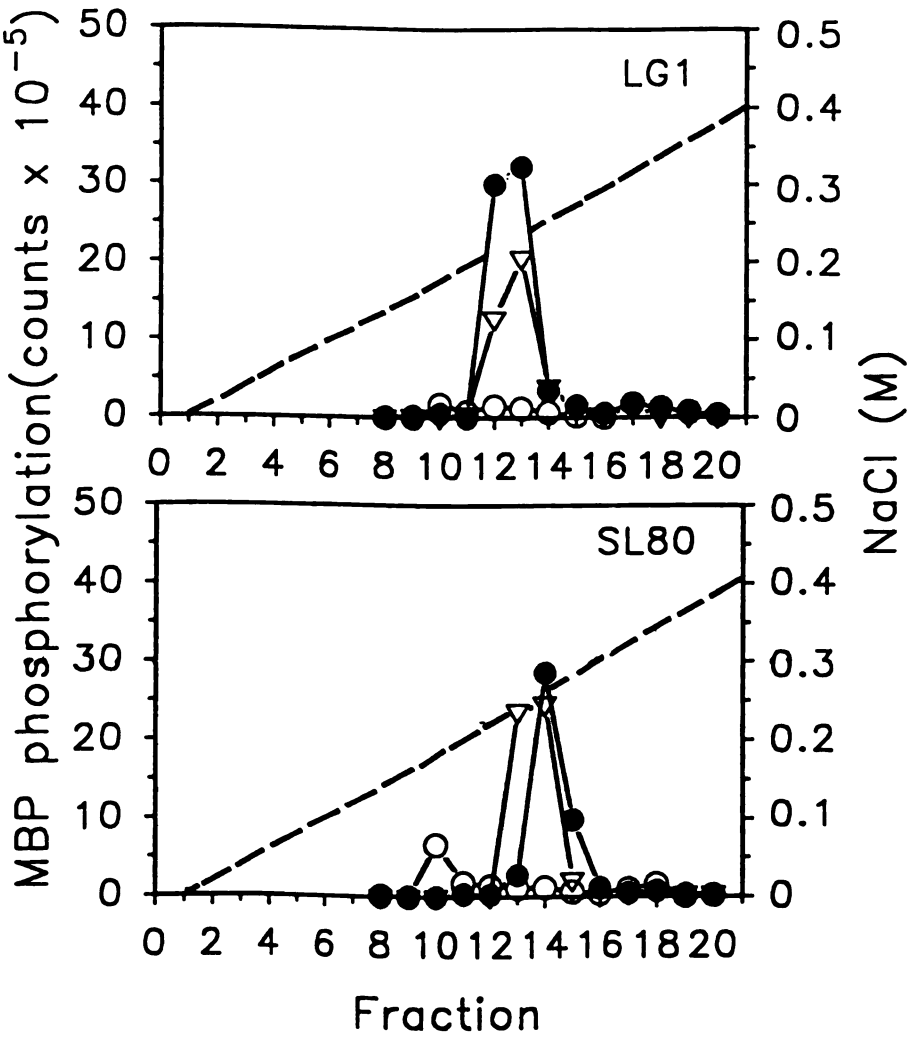

Figure 3 Assay of mono Q fractions for ability to phosphorylate MBP

Cell lysates were prepared as described from cells exposed to 10 mM Ca^{2+} (●) or 100 ng/ml EGF (▽) for 5 min or left untreated (○). Lysate protein (1.5 to 2 mg) was diluted by column buffer and loaded onto a Mono Q column. The protein was eluted by a linear NaCl gradient, and 1 ml fractions were collected. Aliquots of fraction 8 through 20 were assayed for MBP phosphorylation. The salt gradient is denoted by the broken line. Similar results were obtained in two experiments.

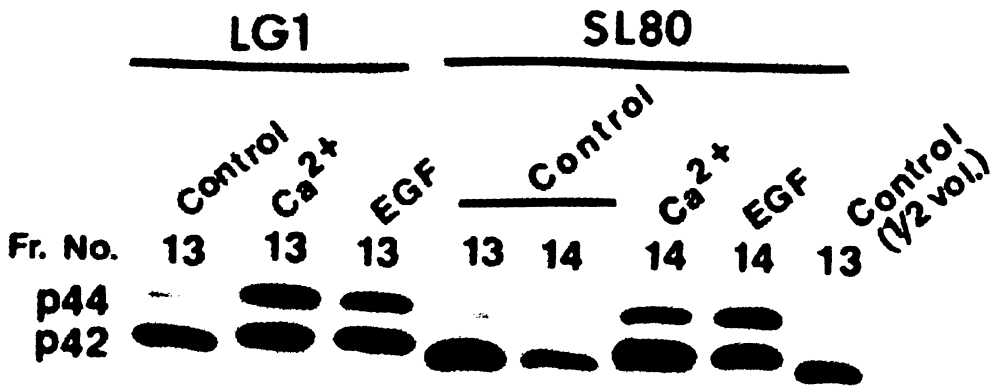

Figure 4 Immunoblot analysis of Mono Q fractions

Mono Q fractions were obtained as shown in Figure 3. Aliquots of the designated fractions were subjected to immunoblot analysis and probed as described with anti-MAPK antibody. The results shown is the representative for three experiments.

circumstances, when 10 mM Ca^{2+} was added to the solution on the cells, MAPK activation was observed (data not shown).

To determine if there was a dose-response relationship for Ca^{2+}- induced MAPK activation, quiescent LG1 and SL80 cells in McM medium lacking Ca^{2+} and supplemented with serum replacements, but without protein growth factors, were exposed for 5 min to various concentrations of Ca^{2+}. Cell lysates were prepared and analyzed for their ability to phosphorylate MBP. A dose response was observed with both cell lines (Figure 5). At high concentrations of Ca^{2+}, MAPK activation would be expected to exhibit saturation; however, at concentrations of 50 mM Ca^{2+} or higher, a precipitate formed, making it impossible to extend the curve.

Time course for Ca^{2+}-induced MAPK activation

Pouysségur and his colleagues showed that in quiescent Chinese hamster fibroblast (cell line CCL39), MAPK is activated by thrombin or basic fibroblast growth factor and that this activation exhibits a biphasic pattern i.e., a rapid increase in activity followed by a rapid decrease and then a sustained level of lower activity (22, 23). They proposed that this sustained activity is required for the re-entry of these cells from G_0 into the cell cycle (23), i.e., for triggering the proliferative response, because when they treated CCL39 cells with a thrombin analog instead of thrombin or with carbachol, the second phase of MAPK activation was not observed and no DNA synthesis took place. To see if Ca^{2+}-stimulated MAPK activation in human fibroblasts also exhibits such a biphasic pattern, we carried out a time course for Ca^{2+} stimulation, using phosphorylation of MBP as a measure of MAPK

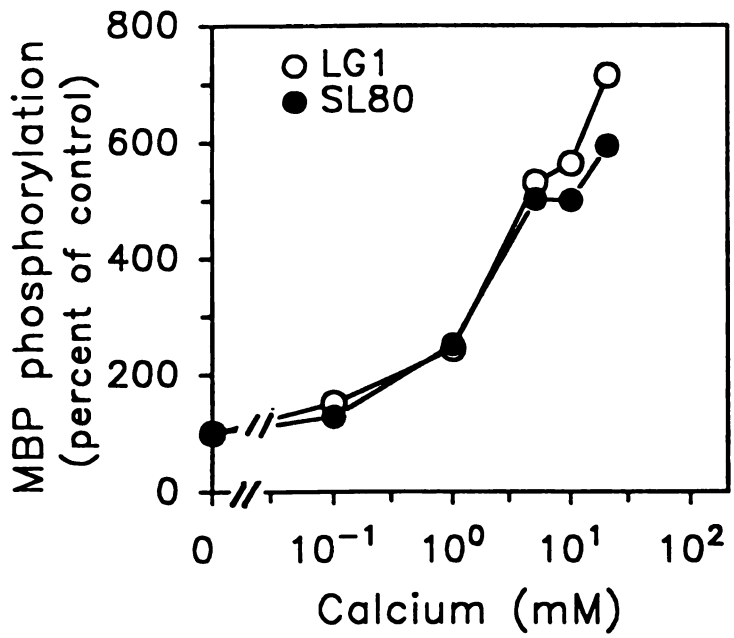

Figure 5 Dose-response of Ca^{2+}-stimulated MAPK activity

Cells were grown to confluence and the medium was changed to McM medium with serum replacements of Ryan et al. (15) but no protein growth factors, and modified to be without Ca^{2+}. After 24 h, the indicated concentration of Ca^{2+} was applied. After 5 min, cell lysates were prepared and assayed for MBP phosphorylation. Similar results were obtained in two separate experiments.

activation. The results are shown in Figure 6. Exposure of LG1 and SL80 cells to 10 mM Ca^{2+} rapidly activated MAPK, with a maximum peak of activation occurring at 5 to 10 min. After the initial burst of activation, a second wave of sustained activation was observed in both cell lines. The level of activation of MAPK did not come down to the basal level in either cell line during the 180 min of stimulation. This sustained activation, along with our finding that in the total absence of protein growth factors, Ca^{2+} induces growth of human fibroblasts (Figure 1), suggests that Ca^{2+} stimulates cell proliferation by activating MAPK in a biphasic manner. Recently, Frost et al. (24) and Pagès et al. (25), using MAPK antisense RNA and/or MAPK kinase-deficient mutants to suppress MAPK activation, showed that MAPK activation is required for activation of transcriptional factors (24) and for rodent fibroblast proliferation (25).

In summary, as far as we can determine, our study is the first to show that in the total absence of protein growth factors extracellular Ca^{2+} can stimulate sustained growth of diploid human fibroblasts and can induce the activation of MAPK. In the absence of protein growth factors, the 42 kDa and 44 kDa isoforms of MAPK were both activated, as they are by protein growth factors. A study by Chao et al (5) indicates that various pathways can lead to MAPK activation in human fibroblasts. For example, EGF-mediated activation does not require intracellular Ca^{2+}, whereas thapsigargin-mediated activation depends on intracellular Ca^{2+}. We intend to determine whether extracellular Ca^{2+} stimulates MAPK activation by one of these pathways or by some alternative mechanism.

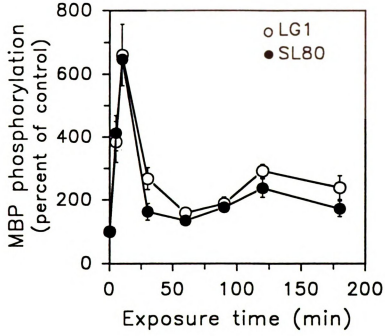

Figure 6 Time course for Ca^{2+}-induced MAPK activity

The medium on cells in exponential growth was changed to the McM medium with serum replacements of Ryan et al. (15) but no protein growth factors and only 0.1 mM Ca^{2+}. After 24 to 30 h, the Ca^{2+} concentration was increased to 10 mM for the indicated time. Cell lysates were prepared and assayed for MBP phosphorylation. Results are expressed as means ± S.E.M. of triplicate determinations. Similar results were obtained in two separate experiments.

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CHAPTER III

Characterization of the second messenger pathway of extracellular Ca^{2+}-induced mitogen-activated protein kinase activation in human fibroblasts

Shixia Huang, Veronica M. Maher, and J. Justin McCormick*

Carcinogenesis Laboratory- Fee Hall

Department of Microbiology and Department of Biochemistry

The Cancer Center

Michigan State University, East Lansing, MI 48824

ABSTRACT

Human fibroblasts in culture will grow in serum free medium containing serum replacement factors but without protein growth factors as long as the Ca^{2+} level is 1.0-2.0 mM. When the Ca^{2+} is reduced to 0.1 mM, the cells stop cycling but can be reinduced to cycle by raising the Ca^{2+} level. Mitogen-activated protein kinase (MAPK) is activated in this process just as it is by protein growth factors (Huang et al. [1995] Biochemical J. 310, 881-885). We now report that the exposure of human fibroblasts to extracellular Ca^{2+} increased cytosolic inositol (1, 4, 5)-trisphosphate level and caused a transient rise in the concentration of intracellular Ca^{2+}. Ca^{2+}-induced MAPK activation was partially abolished by treatment of the cells with pertussis toxin which inhibits certain species of G proteins. It was also decreased by treatment with thapsigargin which depletes intracellular Ca^{2+} stores, by exposure to phorbol 12-myristyl 13-acetate that downregulates protein kinase C (PKC), by treatment with calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCL and calmidazolium, as well as by exposure of the cells to lanthanum, a Ca^{2+} channel inhibitor. We found that the c-raf-1 protein was not phosphorylated after Ca^{2+} stimulation, and the Ca^{2+} sensing receptor (Brown et al. [1993] Nature 366, 575-580) was not involved in mediating MAPK activation or cell growth. These results suggest that extracellular Ca^{2+} stimulates MAPK activation through a pathway(s) involving a pertussis toxin sensitive G protein, phospholipase C, intracellular Ca^{2+}, calmodulin, and PKC.

ABBREVIATIONS

DAG, diacylglycerol; EGF, epidermal growth factor; FBS, fetal bovine serum; IP3, inositol (1, 4, 5)-trisphosphate; MAP-2, microtubule-associated protein 2; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PIP2, phosphatidylinositol bisphosphate; PLC, phospholipase C; PKC, protein kinase C; PMA, phorbol 12-myristyl 13-acetate; SCS, supplemented calf serum; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCL; W-12, N-(4-aminobutyl)-2-naphthalenesulfonamide.

INTRODUCTION

It is well known that the extracellular Ca^{2+} concentration can regulate hormonal secretion [1] and cell differentiation [2-4] in some cell types. For example, mouse and human keratinocyte cells in culture [3-5] proliferate in a medium containing Ca^{2+} which is 5-40 times (0.05-0.1 mM) lower than the physiological level (1.0-2.0 mM), but when the Ca^{2+} concentration of the medium is increase to the physiological range, these cells undergo terminal differentiation. Exposure of these cells to medium with physiological levels of Ca^{2+} causes a rapid increase in phosphoinositide turnover and intracellular Ca^{2+} levels [2,3] and the phosphorylation of tyrosine in various proteins [4].

Mesenchymal cells exhibit the opposite response. Normal human [6], mouse [7], and chicken [8-10] fibroblasts in culture stop proliferating when placed in a medium with reduced Ca^{2+} (0.01-0.1 mM). However, when the Ca^{2+} concentration is increased to the physiological level, the cells proliferate. We recently showed [11] that when one exposes human fibroblasts to medium containing Ca^{2+} at physiological levels of or higher but lacking protein growth factors, mitogen-activated protein kinase (MAPK) activation is induced and this correlates with cell growth.

In mouse 3T3 fibroblasts in culture, it has been shown that extracelluar Ca^{2+} stimulates DNA synthesis [12-17] and c-fos expression [14-16,18] similar to that induced by platelet-derived growth factor (PDGF) [15,16]. Furthermore, Epstein et al [15,16] showed that both PDGF-induced and Ca^{2+}-induced c-fos expression is dependent on protein kinase C (PKC). However, Ca^{2+} does not induce the phosphorylation of the PDGF receptor and the raf protein as PDGF does, indicating that the pathways which result in similar gene

transcription are at least in part independent.

As is well known, protein growth factors, such as epidermal growth factor (EGF), PDGF, insulin, thrombin, nerve growth factor, and bombesin, bind to specific receptors on the external cell surface, and initiate a phosphorylation cascade through the activation of either tyrosine kinase-linked or G protein-coupled receptors [19-25]. One of the key events in this cascade is the activation of MAPK by phosphorylation. Two of the well understood pathways leading to MAPK activation from various growth factor stimulation are the ras/raf pathway [19], and phospholipase C (PLC) pathway [26,27]. The activation of PLC, by either type of activated receptor, causes hydrolysis of the inositol lipid-phosphatidylinositol bisphosphate (PIP2), and generates the intracellular second messengers [26,27], diacylglycerol (DAG), and inositol (1, 4, 5)-trisphosphate (IP3). DAG is responsible for activating PKC isoenzymes [28], which leads to the activation of many other kinases including c-raf [29,30] and MAPK [31]. IP3 binds to the IP3 receptor on the endoplasmic reticulum to release Ca^{2+} from intracellular stores [27], and the free Ca^{2+} is responsible for the induction of many cellular events, including MAPK activation [21]. Intracellular Ca^{2+} exerts its effects by binding to proteins such as calmodulin [32].

How human fibroblasts "sense" the concentration of extracellular Ca^{2+} is unknown. One possibility is that there is some type of receptor on the external cell membrane analogous to the receptors for protein growth factors. Recently, Brown et al. [33] cloned a Ca^{2+}-sensing receptor from bovine parathyroid [33], and subsequently, the human homolog of that receptor was cloned [34]. This receptor, which couples to G protein (s), has been shown to mediate the release of the extracellular Ca^{2+}-regulating parathyroid hormone. Once the receptor is stimulated by extracellular Ca^{2+}, it increases the intracellular level of Ca^{2+} and

IP3 [33]. A knockout mouse lacking this receptor has recently been generated by Ho et al [35]. Homozygous Ca^{2+} receptor-deficient mice exhibit severe neonatal hyperparathyroidism, markedly elevated serum levels of Ca^{2+} and parathyroid hormone, parathyroid hyperplasia, bone abnormalities, and retarded growth [35].

Our interest was in determining how exposure to physiological levels of Ca^{2+} is able to cause the growth of human fibroblasts in culture. This is of particular importance since, unlike their normal counterparts, human fibrosarcoma-derived fibroblasts [36] and human fibroblasts malignantly transformed in culture [37,38] are able to grow in medium with a reduced Ca^{2+} concentration, even in the absence of protein growth factors. This suggests that some aberration in this pathway plays a causal role in the transformation of these cells.

Since exposure of mouse fibroblasts to high concentrations of extracellular Ca^{2+} stimulates c-fos transcription via PKC [15], we first examined the possible involvement of PKC in Ca^{2+} -induced MAPK activation in human fibroblasts. Intracellular Ca^{2+} measurements were also carried out because studies showed that extracellular Ca^{2+} regulates intracellular Ca^{2+} elevation in other cell system [1-3]. Our finding evidence of the involvement of PKC and intracellular Ca^{2+} led us to examine their downstream effectors c-raf-1 and calmodulin respectively, and their upstream activators, PLC, G protein, and the possible involvement of the Ca^{2+} sensing receptor. The results showed that the addition of extracellular Ca^{2+} activated MAPK through a pertussis toxin-sensitive G protein, and led to the elevation of intracellular IP3 and Ca^{2+}. The activation of MAPK by extracellular Ca^{2+} is dependent on PKC, intracellular Ca^{2+}, and calmodulin, but is not dependent on c-raf-1, or the Ca^{2+}-sensing receptor we examined.

MATERIALS AND METHODS

Materials

EGF, PDGF-BB, lys-bradykinin, thapsigargin, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCL (W-7), N-(4-aminobutyl)-2-naphthalenesulfonamide (W-12), calmidazolium (compound R24571), nifedipine, verapamil, lanthanum chloride were purchased from Sigma (St. Louis, MO, USA). Pertussis toxin was from Calbiolchem (La Jolla, CA, USA). Phorbol 12-myristyl 13-acetate (PMA) (from Calbiolchem) was a gift from Dr. David DeWitt of Michigan State University. Indo-1-Am, cell permeate, and indo-1, cell impermeate, were from Molecular Probe (Eugene, OR, USA). Ionomycin (free acid) and the IP3 Biotrak Radioimmunoassay System Kit (TRK 1000) were from Amersham Life Science Inc. (Arlington Heights, IL, USA). Lab-tek, 4-well chambered coverglass (Nuct#136420) was from VWR Scientific (Chicago, IL, USA). Monoclonal anti-MAPK antibodies were purchased from Chemicon (Temecula, CA, USA) and Zymed Laboratories (South San Francisco, CA, USA). Monoclonal anti-c-raf-1 antibody was from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase-linked goat anti-mouse IgG was obtained from Bio-Rad Laboratories (Richmond, CA, USA). Supplemented calf serum (SCS) and fetal bovine serum (FBS) were from Hyclone Laboratories (Logan, UT, USA).

Cells and cell culture

Diploid human fibroblast cell line LG1, derived in this laboratory from neonatal foreskin, was used in this study. Cells were routinely grown in Eagle's minimum essential medium supplemented with 0.2 mM aspartic acid, 1.0 mM sodium pyruvate, and 0.2 mM

serine, 10% supplemented calf serum (SCS), penicillin (100 units/ml), and streptomycin (100 μg/ml) (complete medium) at 37°C in an humidified incubator containing 5% CO_2. For studies on the effects of various growth conditions, medium on confluent or subconfluent cells was changed to modified McM medium, a derivative of MCDB-110 medium [39], made with 0.1 mM Ca^{2+} and supplemented with serum replacements [40] but without any protein growth factors [11]. This medium will be referred as serum free medium.

Isolation and culture of mouse skin fibroblasts

Pregnant female mice (with Ca^{2+}-sensing receptor status of +/- that had previously been mated with +/- male mice) were kindly provided by Dr. Christine Seidman of Harvard Medical School [35]. When the litter was born, newborn to 4 day-old mice were sacrificed with CO_2 and washed with 70% ethanol. The skin was removed to a 60 mm dish and washed twice in PBS, then transferred to a 50 ml sterile glass flask and minced finely with scissors. Ten ml of 0.24% trypsin was added to the flask containing the mouse skin, and the tissue was incubated at 37°C for 30 min to 1 hour, and shaken every 5 min. The suspension was then centrifuged at 400xg for 4 min to remove tissue fragments, and the turbid supernatant containing single cells was transferred to a 15 ml tube containing 4 ml Eagle's medium with 20% SCS and centrifuged at 2000xg for 10 min. The pellet was suspended in 3 ml Eagles medium containing 20% FBS, penicillin (600 units/ml), and streptomycin (600 μg/ml). To prevent possible bacteria contamination, a concentration of penicillin and streptomycin six times higher than that normally used in cell culture medium was used. Cells were counted and plated at 4×10^5 to 8×10^5 per 100 mm dish in the same medium, and incubated at 37°C in a humidified incubator containing 5% CO_2. The next day, medium in each dish was

removed, and cells were washed with warm Eagles medium once, and fresh medium, i. e., Eagles medium containing 20% FBS and the noted high concentration of antibiotics, was added into each dish. About 5 days later, confluent cells in each dish were trypsinized and expanded in Eagles medium with 10% FBS, and regular penicillin and streptomycin concentration (100 μg/ml).

Media for testing for growth stimulation of mouse fibroblasts

The same serum free medium that we employed previously for studying growth stimulation in human fibroblasts [11], was used for mouse skin fibroblasts. With growth stimulation assays, serum free medium was used with no additives, i. e., at 0.1 mM Ca^{2+}, or with 1 mM Ca^{2+}, or supplemented with 10% FBS.

Preparation of cell lysates

Cells were grown in 60 or 100 mm-diameter dishes in complete medium until confluent. Then the medium was replaced for 24-30 hours with serum free medium. Unless otherwise indicated, increased Ca^{2+} or EGF or other reagents were added directly to the medium on the cells. Cells treated with different reagents or left untreated were washed twice in cold Ca^{2+} -free, Mg^{2+} -free PBS, and the cell lysates were prepared as described previously [11].

Immunoblot analysis of MAPK (Western Blot)

Cell lysates containing 25 μg of protein were dissolved in sample buffer as previously described [11], proteins were separated by 10% SDS/PAGE, and then transferred

to Immobilon transfer membrane (Millipore Corp., Bedford, MA, USA). Immunoblot analysis was carried out as described [11]. Briefly, the blots were blocked by incubating for 1 h at room temperature in Tris-buffered saline, pH 7.6, (20 mM Tris-HCl, 137 mM NaCl) containing 0.1% Tween 20 (v/v) and 5% nonfat dry milk (w/v). To test for MAPK, the membranes were incubated for 1 h with the indicated anti-MAPK antibodies in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk, washed several times in this Tris-buffered saline, and then incubated with goat anti-mouse IgG as desired. Enhanced chemiluminescence (Amersham, Arlington Heights, IL) was used as a detection system according to the manufacturer's instructions.

Microtubule-associated protein 2 (MAP-2) phosphorylation assay (MAPK activity assay)

MAP-2 was purified from pig brain essentially as described [24]. The activity was assayed as described [11] but using MAP-2 as substrate instead of myelin basic protein . Briefly, cell lysates containing 2.5 μg proteins were incubated with MAP-2 (0.5 mg/ml), and [γ-^{32}P]ATP for 10 min at room temperature, the reaction was stopped by the addition of 5X sample buffer (1x sample buffer contains 0.05 M Tris-HCl, pH6.9, 0.72 M mercaptoethanol, 9% glycerol, 2.3% SDS and 0.1% Bromphenol blue). The proteins in the reaction were separated on a 7.5% SDS-PAGE, and gels were dried and exposed to Kodak X-Omat film to visualize the amount of MAP-2 phosphorylation.

Intracellular Ca^{2+} measurement

The intracellular Ca^{2+} concentration was measured as described [41] both in the

resting cells and cells incubated with extracellular Ca^{2+} , lys-bradykinin, ionomycin, thapsigargin or EGF. Briefly, cells were cultured in Lab-Tek coverslip chambers and incubated in serum free medium (with 0.1 Ca^{2+}) for 24 hours. The cells were then loaded with a Ca^{2+} sensitive fluorescent dye, indo-1-AM (2 μM) in the same medium by incubating them for 1 hour at 37^0C. To remove the extracellular dye, cells were then washed three times with McM medium (0.1 mM Ca^{2+}) modified to contain 20 mM Hepes but without phenol red. They were then incubated in this medium until the beginning of the measurement (within 2 hours). The concentration of free Ca^{2+} in the cells loaded with indo-1-AM was calculated from data obtained with an ACAS 570 Interactive Laser Cytometer (Meridian Instruments, Okemos, MI, USA). An argon ion laser beam was led to the specimen on an inverted microscope, and emission from the illuminated spot on the sample was directed to a sensitive photomultiplier tube. The image pairs were captured at 8-second intervals. All image analysis experiments were conducted at room temperature. The ratio of the intensities of fluorescent emission at 405 nm and 530 nm (since the UV/Blue cube was used) with excitation at 355 nm was measured. The free Ca^{2+} concentrations in each single cell was calculated by comparing the ratio of the emissions to that generated in a standard curve. The standard curve was produced by mixing the indo-1, cell impermeate, with varying amounts of added Ca^{2+} in a physiological buffer containing EGTA as described [42].

Cytosolic IP3 measurement

Cytosolic IP3 was measured by using IP3 binding protein [43] from IP3 Biotrak Radioimmunoassay System Kit (TRK 1000) (Amersham Life Science Inc.). Samples were prepared essentially as described in the manufacturer's instruction booklet with some

modifications. Briefly, cells were grown to confluence in 60 mm dishes in complete medium, and then incubated in serum free medium for overnight, treated with 10 mM Ca^{2+} for 1 min, or with 50 ng/ml bradykinin for 0.5 or 1 min, or left untreated. At the end of treatment, medium was aspirated and 0.5 ml of 10% cold trichloroacetic acid (v/v) was added to each dish, and samples in each dish were scraped by rubber policeman and transferred to an eppendorf tube. The precipitates were sedimented by centrifugation at 2100xg in microcentrifuge for 15 min at 4^0 C. The supernatant containing IP3 was transferred to a 15 ml falcon blue cap tube and extracted three times with 10 volumes of water-saturated diethyl ether. IP3 extracts were neutralized by titration to pH7.0-8.0 using $NaHCO_3$. IP3 concentrations were measured and calculated from the parallel standard curve made according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Evidence that activation of MAPK by extracellular Ca^{2+} is partially inhibited by PMA

Many protein growth factors stimulate the activation of PKC [24,44], which leads to the activation of downstream kinases, including MAPK [31]. Since in mouse fibroblasts, extracellular Ca^{2+} induces c-fos expression in a PKC-dependent manner [15], we first tested the involvement of PKC in the Ca^{2+} -induced activation of MAPK in human fibroblasts.

Exposure human fibroblasts, as well as other types of cells, to PMA for a few minutes has been shown to activate PKC [45], and a long exposure to PMA downregulates PKC [20,24,45,46]. To determine whether PKC mediates the Ca^{2+}-induced MAPK activation pathway in human fibroblasts, confluent LG1 cells were incubated in medium overnight with or without 100 nM PMA , then treated for 5 min with Ca^{2+}, EGF, or fresh PMA, or left untreated. Cell lysates were assayed for MAPK activation. Incubation of cells with PMA for 5 min induced MAPK activation, as shown by the mobility shift of the p42 MAPK(Figure 1) and detected by an activity assay using MAP-2 as the substrate for phosphorylation by MAPK (data not shown). These data indicated that in human fibroblasts, MAPK activation is mediated by PKC. Down-regulation of the PMA-sensitive PKC by overnight exposure of the cells to with PMA completely abolished the effect of subsequent PMA treatment (Figure 1), indicating that PKC is completely downregulated by PMA . Overnight treatment of cells with PMA partially abolished Ca^{2+} -induced MAPK activation, but had no effect on EGF-induced MAPK activation, as shown by the p42 MAPK mobility shift (Figure 1) and detected by MAP-2 phosphorylation assay (data not shown). These results indicated that in human fibroblasts extracellular Ca^{2+}-induced MAPK activation is

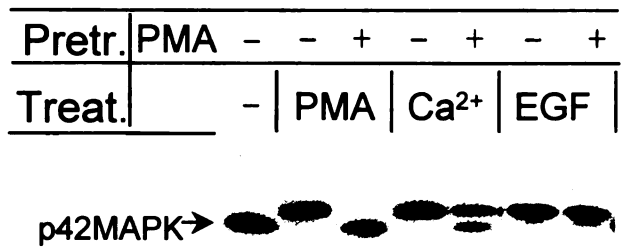

Figure 1 Activation of MAPK by extracellular Ca^{2+} is partially inhibited by PMA. Cells were grown to confluence in Eagle's medium with 10% SCS. The medium was changed to serum free medium (as described) and with or without PMA (100 nM). After 24-30 hours, cells were incubated with 100 nM PMA, 10 mM Ca^{2+}, or 100 ng/ml EGF for 5 min, or left untreated. Cell lysates were prepared and subject to immunoblot analysis with anti-MAPK monoclonal antibody. The results shown are representative of three experiments.

partially dependent on PKC activation, whereas, EGF-induced MAPK activation is independent of PKC activation. Since PKC is completely downregulated by the overnight PMA treatment, these data suggest that there are at least two independent pathways for Ca^{2+} -induced MAPK activation, one dependent on a PMA-sensitive PKC, and one independent of this PKC.

Evidence that extracellular Ca^{2+} does not induce c-raf-1 phosphorylation

Growth factors such as PDGF activate downstream kinases, such as PKC, raf-1, and MAPK, by phosphorylation [30,44,47]. It has been shown that the activation of PKC mediates c-raf-1 kinase activation by direct phosphorylation [29,30]. The activated form can be detected after the separation of cellular proteins by SDS-PAGE since the phosphorylated form migrates more slowly [30]. To determine whether c-raf-1 is in the pathway of extracellular Ca^{2+} -induced MAPK activation, we determined the phosphorylation status of c-raf-1 in human fibroblasts before and after stimulation with Ca^{2+}, EGF, or PDGF. A slower moving form of c-raf-1(Figure 2) was observed in lysates from cells treated with PDGF, when compared with that from untreated cells (Figure 2), indicating that in human fibroblasts phosphorylation of c-raf-1 is induced by PDGF. No mobility shift of c-raf-1 was observed in lysates from cells treated with extracellular Ca^{2+} or EGF, indicating that extracellular Ca^{2+} does not act via a pathway involving c-raf-1. This conclusion agrees with that of Epstein [15], who showed that in mouse 3T3 cells, c-raf-1 is not activated by extracellular Ca^{2+} but is activated by PDGF.

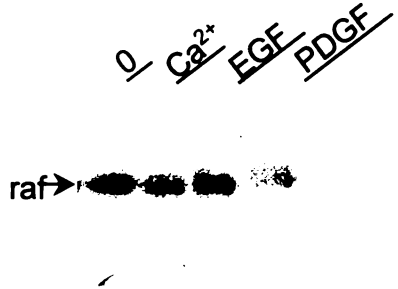

Figure 2 Extracellular Ca^{2+} does not induce c-raf-1 phosphorylation. Cells grown to confluent were incubated in serum free medium for 24 hours, and then treated with 10 mM Ca^{2+} or 100 ng/ml EGF for 5 min, or 40 ng/ml PDGF-BB for 10 min, or left untreated. Cell lysates were prepared and subject to immunoblot analysis using anti-c-raf-1 antibody following a 7.5% SDS-PAGE to separate the proteins. The results shown are representative of three independently performed experiments.

Evidence that extracellular Ca^{2+} induces intracellular Ca^{2+} mobilization in human fibroblasts

Intracellular Ca^{2+} has been shown to be an important second messenger in cell proliferation driven by protein growth factors and growth hormones [48-50]. Gill and colleagues [49,50] have shown that intracellular Ca^{2+} pools are directly linked to cell growth in hamster smooth muscle cells since when these investigators depleted the intracellular Ca^{2+} pools, the cells were arrested in G_0 phase. To determine whether intracellular Ca^{2+} is mobilized as a result of incubating human fibroblasts with extracellular Ca^{2+}, the intracellular Ca^{2+} concentration was measured in resting cells and cells incubated with Ca^{2+}, lys-bradykinin, ionomycin, or EGF, using indo-1 microflurometry with an ACAS fluorescent microscopy. Ionomycin, a calcium ionophore, and bradykinin, a protein mitogen which mediates its signal by binding to a receptor, were used as positive controls since it has been shown that the treatment of cells with these agents causes a transient increase in the free intracellular Ca^{2+} concentration [21] . As shown in Figure 3, cells treated with bradykinin (B) and ionomycin (C) exhibited a transient increase in the concentration of intracellular Ca^{2+}, which lasted about 60 seconds. After the addition of Ca^{2+}, intracellular Ca^{2+} concentration was increased quickly (Figure 3, D) to a level similar to that caused by ionomycin and bradykinin, and the transient lasted for about 180 seconds. No obvious intracellular Ca^{2+} transient was observed after EGF stimulation (Figure 3, E). This result is in agreement with a previous study in human fibroblasts that showed that when cells were pretreated with EGTA to chelate the extracellular Ca^{2+} in the medium [51] there was no noticeable elevation of the concentration of intracellular Ca^{2+} upon EGF stimulation.

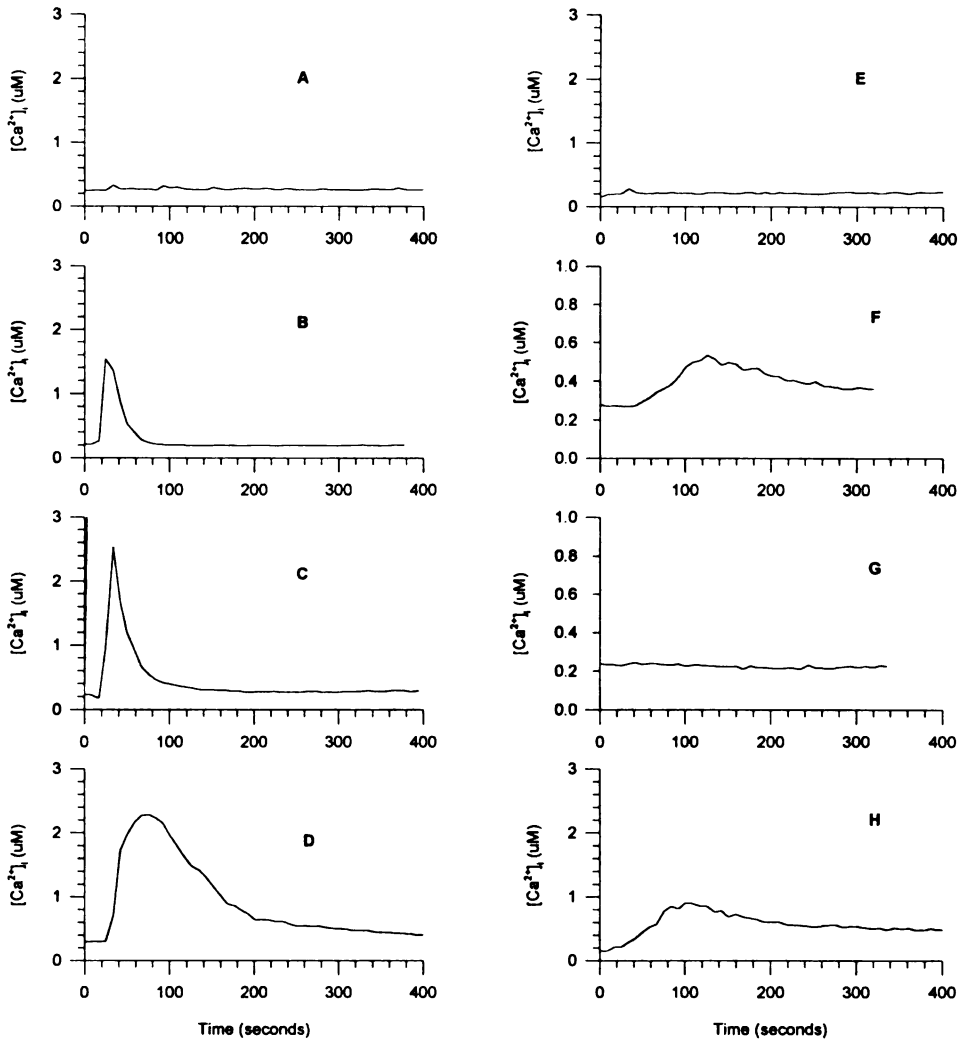

Figure 3 Intracellular Ca^{2+} mobilization after Ca^{2+} stimulation. Subconfluent cells incubated in serum free medium overnight were preloaded with indo-1-Am for 1 hour, washed and placed in McM medium containing 0.1 mM Ca^{2+} without phenol red but with Hepes. Indo-1 microflurometry were carried out using A CAS fluorescent microscope as described in "Material and method". Intracellular Ca^{2+} concentrations were measured immediately following the addition of 0.1 mM Ca^{2+} medium as control (A), 50 ng/ml lys-bradykinin (B), 1.5 μg/ml ionomycin (C), 10 mM Ca^{2+}(D), 100 ng/ml EGF (E), or 1 μg/ml thapsigargin (F), or measured after cells were pretreated with 1 μg/ml thapsigargin for 24-30 hours, then treated with 1 μg/ml thapsigargin (G), or 10 mM Ca^{2+}(H). Each curve is the data taken from a single cell of 3-5 cells measured in each experiment, and each experiment was repeated two to three times with similar results.

Evidence that the mobilization of intracellular Ca^{2+} transient by extracellular Ca^{2+} is partially abolished by thapsigargin

Because Gill and colleagues [50] have found that in mouse muscle cells, thapsigargin depletes the intracellular Ca^{2+} stores (pools) by specific inhibition of the endoplasmic reticulum (ER) Ca^{2+} -ATPase, and that this results in the arrest of the cells in G_0 phase [49,50], we examined the effect of thapsigargin treatment on Ca^{2+}-induced intracellular Ca^{2+} mobilization in human fibroblasts. We first measured the intracellular Ca^{2+} concentration in cells after 1 μg/ml thapsigargin treatment. To test whether prolonged treatment with thapsigargin depletes intracellular Ca^{2+} store, cells were incubated in medium with thapsigargin for 24-30 hours, and intracellular Ca^{2+} concentration was measured after the addition of thapsigargin. The addition of thapsigargin to human fibroblasts increased the intracellular Ca^{2+} concentration (Figure 3, F). The profile we obtained was similar to that showed by Chao et al. using human fibroblasts [21]. Prolonged treatment with thapsigargin totally blocked the thapsigargin-increased intracellular Ca^{2+} concentration (Figure 3, G). These data indicated that at the concentration used for the pretreatment, 1 μg/ml, the thapsigargin-sensitive Ca^{2+} pools are completely depleted.

We then tested whether Ca^{2+} from the thapsigargin-sensitive stores is responsible for the intracellular Ca^{2+} increase after stimulation with extracellular Ca^{2+}. Cells were pretreated with thapsigargin for 24-30 hours, and intracellular Ca^{2+} concentration was measured after the addition of extracellular Ca^{2+}. We found that thapsigargin pretreatment partially abolished the transient increase of intracellular Ca^{2+} concentration (Figure 3, H) that was normally seen after stimulation with extracellular Ca^{2+} (Figure 3, D) This indicated that the thapsigargin-sensitive Ca^{2+} stores are partially responsible for the Ca^{2+} increase after Ca^{2+}

stimulation.

Previous reports suggest that the depletion of intracellular Ca^{2+} pools triggers Ca^{2+} entry across the plasma membrane by a process known as "capacitative Ca^{2+} entry" [32,52]. It is the Ca^{2+} from both the intracellular Ca^{2+} stores and from the Ca^{2+} entry channel [32] that accounts for the longer lasting increase in intracellular Ca^{2+} concentration after stimulation of the cells by various growth factors including PDGF [32,48]. The fact that we observed a longer lasting transient increase in intracellular Ca^{2+} concentration after extracellular Ca^{2+} stimulation (Figure 3, D) than we saw in cells treated with ionomycin (Figure 3 C) or bradykinin (Figure 3, B) in low Ca^{2+} medium, and that this Ca^{2+} increase can be abolished only partially by thapsigargin pretreatment (Figure 3, H), suggests that, after extracellular Ca^{2+} stimulation, in addition to that Ca^{2+} from thapsigargin-sensitive intracellular Ca^{2+} stores, Ca^{2+} either from channels or from thapsigargin-insensitive stores, contributes to the transient intracellular Ca^{2+} mobilization we observed. It is known that there is more than one type of intracellular Ca^{2+} store in the endoplasm reticulum membrane [53,54] and that the thapsigargin-sensitive stores account for only 75% of the stored Ca^{2+} [53].

Evidence that activation of MAPK by extracellular Ca^{2+} is partially inhibited by thapsigargin

To test whether in human fibroblasts MAPK activation is mediated by intracellular Ca^{2+} from the Ca^{2+} stores, we tested the effect of thapsigargin treatment on Ca^{2+}-induced MAPK activation. Cells were treated with thapsigargin for 24-30 hours, and then stimulated with Ca^{2+}, or with EGF, or were left untreated. Cell lysates were assayed for MAPK activation by immunoblot analysis. We found that thapsigargin partially inhibited the p42

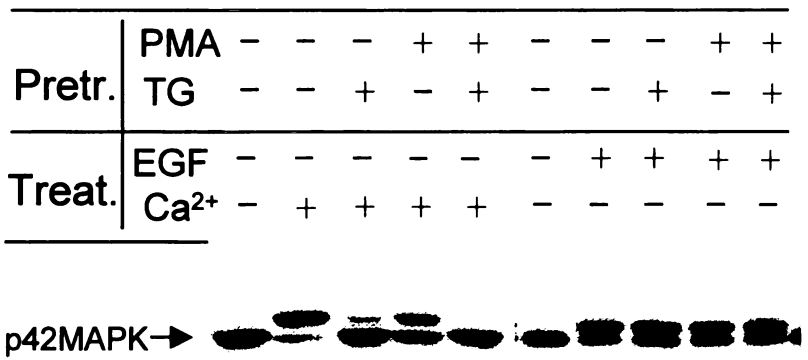

Figure 4 The effect of thapsigargin, or PMA and thapsigargin together, on Ca^{2+}- or EGF-induced MAPK activation Cells grown to confluent were incubated in serum free medium for 24-30 hours in the absence or presence of thapsigargin (1 μg/ml) or PMA (100 nM), or the presence of both thapsigargin (1 μg/ml) and PMA (100 nM). The cells in each set of conditions were then treated with 10 mM Ca^{2+} or 100 ng/ml EGF for 5 min, or left untreated. Cell lysates were subject to immunoblot analysis with anti-MAPK antibody. Similar results were obtained in three separate experiments.

MAPK mobility shift induced by extracellular Ca^{2+}, but did not inhibit the mobility shift induced by EGF (Figure 4). These data indicate that intracellular Ca^{2+} mobilization from the thapsigargin-sensitive Ca^{2+} stores lies, at least in part, in the pathway between extracellular Ca^{2+} and MAPK activation, but not in the pathway between EGF and MAPK activation.

To determine whether the PMA-sensitive PKC and intracellular Ca^{2+} from the thapsigargin-sensitive stores are in the same or a different pathway for extracellular Ca^{2+}-induced MAPK activation, cells were pretreated with PMA and thapsigargin together and then stimulated with Ca^{2+} for 5 min and lysates were subjected to immunoblot analysis using anti p42 MAPK antibody. The pretreatment with PMA and thapsigargin resulted in an almost complete inhibition of Ca^{2+}-induced MAPK mobility shift (Figure 4), the inhibition was greater than by either of the inhibitors given alone. This indicates that PMA-sensitive PKC and thapsigargin-sensitive intracellular Ca^{2+} stores lie, at least partially, in two distinct pathways. EGF-induced MAPK activation is not affected by either PMA or thapsigargin, indicating that EGF does not act through PKC activation or mobilization of the thapsigargin-sensitive intracellular Ca^{2+} stores.

Evidence that activation of MAPK by extracellular Ca^{2+} is abolished by the channel blocker lanthanum, but not by the channel blockers verapamil or nifedipine

Growth factors such as PDGF evoke transient increase in intracellular Ca^{2+} concentration through mobilization of intracellular Ca^{2+} stores and Ca^{2+} entry by channels [48]. Ca^{2+} channel antagonists have been shown to decrease the rate of growth of many cell types [48,55]. Estacion and Mordan [48] showed that in mouse fibroblasts the selective inhibition of the sustained increase in Ca^{2+} concentration by blocking Ca^{2+} influx with

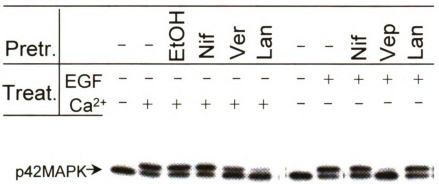

Figure 5 Effect of Ca^{2+} channel blockers on Ca^{2+} or EGF-induced MAP K activation. Cells grown to confluent were incubated in serum free medium for 24 hours, the cells were then treated with Ca^{2+} channel blockers nifedipine (50 μM), verapamil (250 μM), or lanthanum (100 μM) for 0.5-1 hour, or left untreated, and then exposed to 10 mM Ca^{2+}, or 100ng/ml EGF for 5 min, or left untreated. Cell lysates were subject to immunoblot analysis with anti-MAPK antibody. Similar results were obtained in two different experiments.

lanthanum completely inhibits progression of the cells into S phase upon exposure to PDGF. To test whether Ca^{2+} entry through Ca^{2+} channels plays a role in the Ca^{2+} -induced MAPK activation, we pretreated cells with Ca^{2+} channel blockers nifedipine, which blocks L type voltage sensitive Ca^{2+} channels, verapamil, which blocks voltage sensitive channels nonspecifically, or lanthanum, a nonspecific channel blocker [56], for 30-60 min. The three populations of cells were then exposed to Ca^{2+} or EGF for 5 min, or were left untreated. Cell lysates were assayed for MAPK mobility shift by immunoblot analysis. The results (Figure 5) show that Ca^{2+}-induced MAPK mobility shift was partially inhibited by lanthanum , but that there was no obvious effect as a result of verapamil or nifedipine treatment. The inhibition effect by lanthanum suggests that Ca^{2+} entry through Ca^{2+} channels plays a role in extracellular Ca^{2+}-induced MAPK activation pathway. Although the mechanism by which lanthanum acts as a channel blocker is as yet not clear, it has been suggested that as a poly cation, it may bind to the channels, preventing Ca^{2+} entry [57].

EGF-induced MAPK activation was not abolished by lanthanum or nifidipine, but surprisingly, it was abolished by verapamil (Figure 5). Since no changes in intracellular Ca^{2+} concentration were observed upon EGF stimulation (Figure 3, E), this effect was not expected. In human meningioma derived cells, it has been shown that Ca^{2+} channel blockers, such as verapamil, block cell growth driven by growth factors such as EGF and PDGF [55,58], although no intracellular Ca^{2+} change is observed upon stimulation by growth factors [55]. Verapamil has been shown to bind to muscarinic receptors or bind and inhibit the ryanodine receptor channel of the sarcoplasmic reticulum [59]. Therefore, the effect of verapamil on EGF-induced MAPK activation pathway might result from its interference with other signal transduction components.

Evidence that activation of MAPK by extracellular Ca^{2+} is partially abolished by calmodulin antagonists

Since we found that the increase of intracellular Ca^{2+} concentration appears to play a role in Ca^{2+}-induced MAPK activation pathway, we examined possible downstream effectors of the intracellular Ca^{2+}. The increased concentration of intracellular Ca^{2+} binds to Ca^{2+} binding proteins to exert its effect. These proteins include calmodulin, calcineurin, and phospholipase A2, etc. [32]. Calmodulin has been shown to affect cell replication in variety of cell types [60,61]. For example, the selective pharmacological inhibitor of calmodulin kinase, KN-62, prevents quiescent WS-1 human fibroblasts from reaching S phase upon serum stimulation [61,62]. Takuwa et al. [63] have shown that the potent calmodulin antagonists calmidazolium and W-7, but not its inactive analogue W-12, strongly inhibit serum-induced DNA synthesis in normal human fibroblasts [63] and human vascular endothelial cells [64]. Furthermore, a study on neuronal cells [65] showed that the transient increase of intracellular Ca^{2+} concentration via Ca^{2+} entry channels modulates the activation of calmodulin kinases (CaMK2 or CaMK 4) to regulate the expression of their downstream genes.

Using calmodulin antagonist W-7 to inhibit the effect of calmodulin, we tested the role of calmodulin in the Ca^{2+} - or EGF-induced MAPK activation pathway. Cells were pretreated with W-7 at 30 μM, a concentration which has been shown to be effective in human fibroblasts [63], and then stimulated with Ca^{2+} or EGF, and the p42 MAPK activation was analyzed by immunoblot analysis. W-7 partially abolished both the Ca^{2+} - and the EGF-induced MAPK mobility shift (Figure 6). To determine whether the effect of W-7 is specific for calmodulin, we also pretreated cells with calmidazolium or W-12, and then exposed them

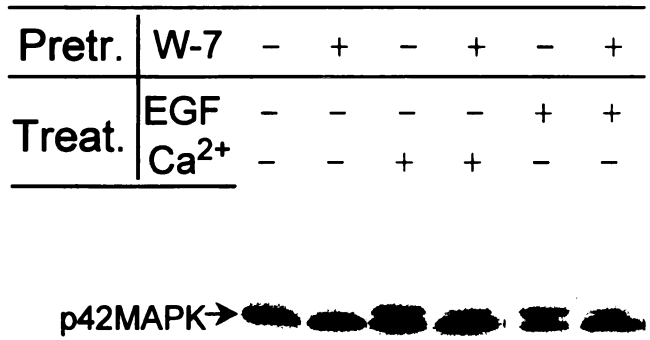

Figure 6 Effect of calmodulin antagonist on Ca^{2+} or EGF-induced MAPK activation. Cells grown to confluent were incubated in serum free medium for 24 hours, the cells were then treated with calmodulin antagonist W-7 (30 μM) for 1 hour, and then exposed to Ca^{2+} or EGF for 5 min or left untreated. Cell lysates were subject to immunoblot analysis with anti-MAPK antibody. Similar results were obtained in two independent experiments.

to Ca^{2+} or EGF. We found that, like W-7, calmidazolium (15 μM) partially abolished both Ca^{2+} and EGF-induced MAPK shift (data not shown), whereas W-12 (90 μM), the inactive analogue of W-7, had no obvious effect on Ca^{2+}- or EGF-induced MAPK activation (data not shown).

Since two different calmodulin antagonists show a similar effect, and W-12, the inactive analog of W-7, which has approximately 10 times lower affinity for calmodulin than W-7 [63,64,66,67], shows no obvious effect on MAPK activation, we conclude with Takuwa et al. [63,64] that the effect of calmodulin antagonists is very likely based on their antagonism against calmodulin. Lu and Means conclude in a review [68] that Ca^{2+} is an absolute requirement for all enzyme activating functions of vertebrate calmodulin. This supports our suggestion that calmodulin mediates the function of intracellular Ca^{2+} after extracellular Ca^{2+} stimulation. We have no ready explanation for the fact that EGF-induced MAPK activation is also abolished by calmodulin antagonists, although no obvious intracellular Ca^{2+} elevation was observed after EGF stimulation (Figure 3). It is also possible that calmodulin antagonists have effects on other components in the signal transduction pathway, although such a mechanism is not supported by W-12 data.

The role of PLC

In mammalian cells, protein growth factors activate G protein-coupled receptors or tyrosine kinase-linked receptors, which stimulate the production of DAG and IP3 through the activation of PLC [69]. DAG activates PKC, whereas IP3 increases cytosolic Ca^{2+} level by triggering the release of Ca^{2+} from intracellular stores. These two receptor-dependent mechanisms are coupled to different isoforms of the PLC family. G protein-coupled

receptors activate PLCβ [69-72]. The tyrosine kinase-linked receptors act through PLCγ1 by tyrosine phosphorylation [70,73]. In human and mouse keratinocytes and bovine parathyroid cells, extracellular Ca^{2+} has been shown to activate PLC and produce DAG and IP3, which in turn, activate PKC and elevate intracellular Ca^{2+} levels [2,3]. The fact that our results show that both PKC activation and intracellular Ca^{2+} elevation from intracellular Ca^{2+} stores are involved in the extracelluar Ca^{2+}-induced MAPK activation pathway, suggests that PLC is involved in the activation of PKC and elevation of intracellular Ca^{2+}. To test this hypothesis, we measured PLC activity by the accumulation of IP3 in cells [74], using an IP3 binding protein [33]. Figure 7 shows that IP-3 level was elevated 8-10-fold after 1 min of Ca^{2+} stimulation, and 5-fold after bradykinin stimulation for 30 seconds, and 3-fold after 1 min stimulation. The increased level found after bradykinin treatment is similar to that shown in other studies with human fibroblasts [75,76]. Bradykinin is known to function through the G protein-coupled bradykinin receptor and activate PLCβ to generate IP3 and DAG [72,77]. The elevation of IP3 after Ca^{2+} stimulation suggests that PLC is involved.

Since PLCγ has been shown to be phosphorylated on its tyrosine residue upon activation [70,73], we tested its involvement by immunoprecipitation followed by immunoblot analysis. Human fibroblast LG1 cells were treated with Ca^{2+} (Figure 8, lane 2), or EGF (Figure 8, lane 3), or left untreated (Figure 8, lane 1), and the cell lysates were immunoprecipitated with anti-PLCγ antibody as described [78]. Proteins were subjected to SDS-PAGE, transferred to a membrane, and probed with anti-phosphotyrosine antibody for immunoblot analysis. Human epidermoid carcinoma A431 cells which are known to exhibit PLCγ activity when treated with EGF [78] were used as a positive control. Our results show that EGF induced PLCγ tyrosine phosphorylation in A431 cells (Figure 8, lane 5). No

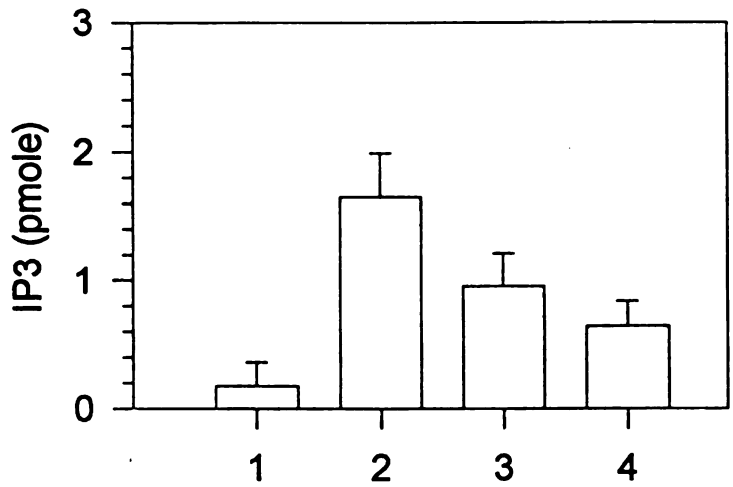

Figure 7 Cytosolic level of IP3 was increased after Ca^{2+} stimulation. IP3 was measured as described in "Material and methods" in cells with no treatment (1), or cells treated with 10 mM Ca^{2+} for 1 min (2), or 50 ng/ml bradykinin for 0.5 min (3) or 1 min (4). Values are means ±S.E.M. of four observations.

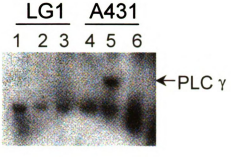

Figure 8 Effect of extracellular Ca^{2+} on PLCγ phosphorylation. LG1 cells (lanes 1-3) or A431 cells (lanes 4-6) grown to confluence were incubated in serum free medium for 24 hours, and then treated with Ca^{2+} (lane 2) or EGF (lanes 3, 5, 6) for 5 min, or left untreated (lanes 1, 4). Cell lysates were prepared and immunoprecipitated with anti-PLCγ antibody (lanes 1-5) or nonspecific antibody (lane 6), then subjected to immunoblot analysis with anti-phosphotyrosine antibody. Similar results were obtained in two separate experiments.

tyrosine phosphorylation of this protein was observed upon Ca^{2+} (Figure 8, lane 2) or EGF (Figure 8, lane 3) stimulation in human fibroblasts. This indicates that PLCγ is not involved in Ca^{2+} -induced MAPK activation pathway and that some other PLC isoform, such as PLCβ, is involved.

Evidence that MAPK activation by extracellular Ca^{2+} is partially inhibited by pertussis toxin

Because of the increase of cytosolic level of IP3 after Ca^{2+}stimulation, which suggests the involvement of PLC, we hypothesized that PLC might be activated by a G protein. Based on their sensitivity to pertussis toxin, G proteins are divided into two categories [70]: pertussis toxin-sensitive and -insensitive G-protein. Therefore, we tested the effect of pretreatment with pertussis toxin on Ca^{2+}-induced MAPK activation. Cells were pretreated with 0.5 μg/ml pertussis toxin overnight, and then stimulated with Ca^{2+} or EGF, and their lysates were assayed for MAPK activation. Pertussis toxin partially abolished the MAPK mobility shift caused by extracellular Ca^{2+}, but had no effect upon EGF stimulation (Figure 9). This suggests that Ca^{2+} -induced MAPK activation is at least partially dependent on a pertussis toxin sensitive G-protein, whereas EGF-induced MAPK activation is independent of this type of G-protein. These data indicating the involvement of a pertussis toxin sensitive G protein, which has been shown to be one of the major activators of PLC, and the data provided above indicating the involvement of PKC, which can be activated by DAG, along with the IP3 elevation after Ca^{2+} stimulation, strongly suggest that PLC is involved.

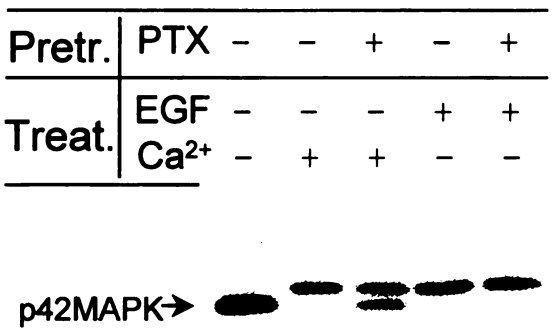

Figure 9 The activation of MAPK by extracellular Ca^{2+} is partially inhibited by pertussis toxin. Cells grown to confluent were incubated in serum free medium with or without pertussis toxin (0.5 μg/ml) for 24 hours, and then exposed to Ca^{2+} or EGF for 5 min, or left untreated. Cell lysates were subject to immunoblot analysis with anti-MAPK antibody. Similar results were obtained in three independent experiments.

Evidence that the Ca^{2+} sensing receptor of Brown et al. [33] is not involved in extracelluar Ca^{2+}-induced cell growth or MAPK activation in mouse skin fibroblasts

How the fibroblasts sense the extracellular Ca^{2+} concentration is unknown. It could be that Ca^{2+} channels open when the extracellular Ca^{2+} reaches a critical threshold, as suggested by the inhibition effect of lanthanum. On the other hand, the involvement of a G protein suggests that there may be Ca^{2+} specific receptors on the external cell surface, analogous to protein growth factor receptors, and that these are involved in this process. Just such a Ca^{2+}-sensing receptor has been cloned from bovine parathyroid cells and has been shown to mediate the elevation of intracellular Ca^{2+} and IP3 [33]. Mice lacking this receptor show a dramatically retarded growth [35], which suggests that this receptor may play a role in growth control. Therefore, we tested the possible involvement of this receptor in regulating cell growth and MAPK activation by using knockout mice lacking this Ca^{2+} sensing receptor [35]. After determining that mouse skin fibroblasts exhibit the same ability as human skin fibroblasts to grow in serum free medium with 1 mM Ca^{2+} and to induce MAPK activation after Ca^{2+} stimulation (data not shown), we tested whether skin fibroblasts from mice lacking this receptor exhibit any deficiency in growth or MAPK activation after Ca^{2+} stimulation, when compared with their wild type or heterozygous counterparts. We found that all mouse skin fibroblasts, regardless of their receptor status (+/+, +/-, or -/-), had the same ability to grow in the medium containing 1 mM Ca^{2+} or supplemented with 10% FBS (data not shown). Also 10 mM extracellular Ca^{2+} or 10%FBS induced a MAPK mobility shift in these mouse fibroblasts to the same degree, regardless of the status of the Ca^{2+} sensing receptor (Figure 10). These results indicate that this Ca^{2+} sensing receptor is not involved in Ca^{2+}-induced cell growth or MAPK activation in mouse fibroblasts. Since

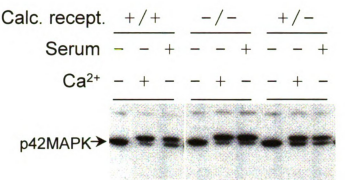

Figure 10 MAPK activation by extracellular Ca^{2+} or FBS in mouse skin fibroblasts. Mouse skin fibroblasts were prepared and cultured as described in "Material and methods". Confluent cells which were +/+, +/-, -/- for the Ca^{2+} sensing receptor (calc. recept.) were incubated in serum free medium overnight, and then exposed to 10 mM Ca^{2+}, or 10% FBS, or left untreated. Cell lysates were subject to immunoblot analysis with anti-MAPK antibody. Similar results were obtained in two experiments.

the mouse fibroblasts exhibit the same response to extracellular Ca^{2+} as human fibroblasts as judged by growth and MAPK activation assays, we conclude that it is unlikely that this Ca^{2+} receptor to be involved in Ca^{2+}-induced cell growth or MAPK activation in human fibroblasts.

Based on the results we obtained, we propose that in human fibroblasts extracellular Ca^{2+}, through a mechanism as yet unknown, activates a G protein and this in turn activates PLC, generating DAG and IP3, which in turn activates PKC and transiently increases the intracellular Ca^{2+} levels. The intracellular Ca^{2+} binds to calmodulin and, in turn, exerts its function on MAPK directly or indirectly. We realize that cellular responses are complicated, and that multiple pathways exist, so that components we found to be involved in human fibroblasts might function in a different sequence, e.g., intracellular Ca^{2+} might activate PLC or PKC, as reported for other systems [70]. Cross-talk between pathways could further complicate the issue. However, the most interesting question is how the extracellular Ca^{2+} is able to generate a signal within the cells. Although we found that the Ca^{2+}-sensing receptor cloned by Brown et el. [33], which mediates the intracellular IP3 and intracellular Ca^{2+} elevation in parathyroid cells, and does so using a pertussis toxin-insensitive G protein [1], is not involved in this process in skin fibroblasts, the probable involvement of a pertussis toxin sensitive G protein suggests that there is another receptor that mediates the Ca^{2+}-induced cellular responses in human fibroblasts. One candidate is the FGF receptor, since it has been shown to have a Ca^{2+} binding motif in its acid box [79], and it is present in human fibroblasts [80,81]. Furthermore, in neuronal cells, the FGF receptor mediated signals have been shown to be dependent on a pertussis toxin sensitive G proteins [82]. In parathyroid cells, an increase in extracellular Ca^{2+} also inhibits receptor-mediated increases in cAMP

[1,83,84] which can be abolished by pertussis toxin [1], indicating that a pertussis toxin sensitive G protein is involved in this process [1,84]. This G protein apparently differs from that which mediates the Ca^{2+}-induced intracellular IP3 and intracellular Ca^{2+} elevation, as the latter responses have been shown to be pertussis toxin-insensitive in parathyroid cells [1,83]. The receptor that couples to a pertussis toxin-sensitive G protein in parathyroid cells could be the receptor cloned by Brown et al. [33], if, as suggested, it simultaneously couples to two different G protein subfamilies [84] or it could be another, as yet unknown, receptor which senses the extracellular Ca^{2+} concentration and mediates a signal [1]. It has been shown that exposure of kidney proximal tubular cells to extracellular Ca^{2+} [84] induces cellular responses, such as regulation of the concentration of 1-hydroxylation of 25-hydroxyvitamin D in the proximal tubule, and changes in circulating PTH levels. The Ca^{2+}-sensing receptor cloned from bovine parathyroid has not been found to be expressed in these kidney cells [84]. However, Juhlin et al. [85-87], using monoclonal antibodies have identified an ~500 kDa membrane protein in kidney proximal tubular cells and cytotrophoblasts. Sequence analysis of a 2.8 kb cDNA, isolated by Lundgren et al.[88], that represents a fragment of the gene for the 500 kDa protein indicates that the protein is a member of the low-density lipoprotein receptor superfamily and is closely related to Heyman nephritogenic protein, a kidney tubule glycoprotein with Ca^{2+}-binding activity [88-90]. It is not yet known whether this protein functions as a Ca^{2+} sensor. Functional studies of this protein should resolve this question [84] .

Although a membrane-bound Ca^{2+} receptor could function as a sensor of the external Ca^{2+} concentration, alternative mechanisms cannot be excluded. For example, the inhibition effect by lanthanum suggests that extracellular Ca^{2+} may directly operates a channel which

senses the extracellular Ca^{2+} concentration. Nevertheless, the present study provides information on some of the mechanisms which must be integrated into our understanding of how extracelluar Ca^{2+} acts as a growth promoter, and suggests a number of specific hypothesis that can be tested.

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