THE ROLE OF THE CALCIUM-SENSING RECEPTOR IN CALCIUM-SENSITIVE PROLIFERATION AND INTRACELLULAR SIGNALING

By

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ABSTRACT

Extracellular calcium is a known modulator of proliferation and/or differentiation in numerous cell types such as keratinocytes, breast epithelial cells, and both human and Rat-1 fibroblasts. Interestingly, many of these cell types have been shown to express the calcium activated, G-protein coupled calcium-sensing receptor (CaR). Previous work in Rat-1 fibroblasts has strongly implicated the CaR as playing a central role in the proliferative response of Rat-1 fibroblasts to calcium. The work in this thesis is aimed at investigating the role of the CaR in calcium-regulated growth, and investigates the signaling mechanisms by which it might do so. Concurrent work by others and myself in this laboratory has demonstrated that both rat and human ovarian surface epithelial (OSE) cells proliferate in a calcium-sensitive manner and express functional CaR or a highly related homologue. In this thesis, I investigate whether the CaR is important for calcium-induced proliferation, utilizing previously generated rat OSE (ROSE) cell lines that stably expressed either the dominant interfering CaR-R796W mutant or a construct which expressed the CaR in the reverse orientation (Car-Rev). Thymidine incorporation and cell count assays demonstrated that, in the presence of either CaR-R796W or CaR-Rev, ROSE cells have a reduced sensitivity to calcium, requiring a higher concentration of calcium to induce increased thymidine incorporation as compared to vector transfected controls, and complete disruption of calcium-enhanced increases in cell number. This

evidence strongly supports the pivotal involvement of the CaR in calcium-induced proliferation in these cells.

In order to understand the mechanism by which the CaR might couple to proliferative responses, we investigated CaR intracellular signaling. Prior studies of mitogenically-coupled GPCRs have demonstrated an involvement of several proximal downstream kinases that mediate the downstream activation of the mitogen-activated protein kinase (MAPK) cascade, as demonstrated by extracellular-regulated kinase (ERK) activation. I have previously shown that the CaR agonists calcium and gadolinium each induced ERK activation in Rat-1 fibroblasts, and activation was sensitive to expression of the dominant-interfering CaR-R796W mutant. The work in this thesis shows that both calcium and gadolinium are capable of activating ERK in ROSE cells, and ERK-activation is inhibited by expression of CaR-R796W. The Src family of tyrosine kinases (Src) was strongly implicated as crucial to ERK activation by CaR-agonists, as the cytoplasmic tyrosine kinase inhibitor herbimycin completely inhibited ERK activation in response to calcium. Moreover, herbimycin was able to completely block calcium-induced proliferation as inferred by thymidine incorporation assays. This suggests that Src activity is required for both CaR-induced ERK activation and CaR-mediated proliferation.

In order to study CaR signaling in a defined background, CaR-transfected HEK293 cells were utilized. These studies establish that the CaR is necessary and sufficient for ERK activation by calcium in HEK293 cells, demonstrating that

ERK activation by extracellular calcium is specific to the CaR. GPCRs utilize either Ras-dependent or Ras-independent mechanisms of ERK activation, depending on the receptor and/or cell-type. The work presented here demonstrates that CaR-mediated ERK activation requires functional Ras in HEK293 cells, as demonstrated by an approximately 80-percent inhibition of ERK activation when the dominant-interfering RasN17 mutant was co-expressed with the CaR.

GPCRs can activate Ras utilizing one of several key cytoplasmic kinases. In this thesis we have implicated Src activity as a critical component in CaR-mediated ERK activation in OSE cells, and Src had likewise been implicated as important in CaR-induced ERK activation in Rat-1 fibroblasts. In contrast, we could find no evidence for the involvement of Src activity, SHC tyrosine phosphorylation, or SHC/Grb2 association in CaR-mediated ERK activation in the HEK293 system. Instead, our data strongly implicates phosphatidylinositol-3-kinase (PI3K) as a major intermediate in CaR-mediated ERK-activation in HEK293 cells, as demonstrated by the use of the PI3K-inhibitors wortmannin and LY294002. CaR-mediated ERK-activation was not sensitive to wortmannin in Rat-1 fibroblasts, indicating that PI3K involvement is a distinct feature of CaR signaling in the HEK293 cells as compared to Rat-1 cells. This difference may be a cell-type specific variation, or may reflect the fact that HEK293 cells are an epithelial cell type, whereas the OSE and Rat-1 fibroblasts are mesenchymally derived.

The work in this thesis strongly supports the importance of the CaR, utilizing a Src-dependent pathway, in the calcium-sensitive proliferation of ROSE cells. The CaR couples to the MAPK pathway, as evidenced by ERK activation in response to CaR agonists, and does so in a Src-dependent and Ras-dependent manner.

In the HEK293 cells, I have demonstrated that heterologous expression of the CaR confers calcium-sensitive ERK activation, demonstrating the necessity for CaR expression and specificity of ERK activation to the CaR. CaR mediates ERK-activation in a Ras-dependent and PI3K-dependent manner, but does not require Src activity, Shc tyrosine phosphorylation or Shc/Grb2 association. Taken together, these data demonstrate the existence of multiple distinct pathways linking the CaR to ERK-activation, depending on the cell type in which it is expressed. The usage of one or more distinct pathways suggests a potential mechanism by which activation of the CaR could couple to diverse calcium-dependent cellular responses. The CaR could thus potentially mediate both the proliferative and differentiation responses to calcium.

EXTRACELLULAR CALCIUM: A REGULATOR OF GROWTH AND DIFFERENTIATION

GENERAL INTRODUCTION

The cellular decision to remain quiescent or proliferate is critical to the organism as a whole. Once structures are established, a mechanism must be in place to retain homeostasis within that structure. In some anatomical compartments, continual proliferation and cell turnover is the rule. These include the intestinal tract, bone marrow, secretory glandular epithelia, and epidermis. Cells of the dermis and vascular endothelium are examples of cells that remain quiescent until signaled to proliferate, generally either in accommodation to stretch or in response to injury. Still other cell types, such as nerve and muscle, cannot be induced to divide once terminally differentiated; their cell number remains fixed for the lifespan of the organism. For those cells that retain proliferative capacity, there must exist mechanisms to activate and support proliferation and to prevent unregulated growth.

CALCIUM-REGULATED GROWTH

Extracellular calcium is known to have profound effects on the proliferation and differentiation of several cell types. Depending on the cell type, calcium can act as either a differentiation factor or a proliferative factor. Changes in extracellular calcium concentration are capable of modulating both the differentiation and proliferation status of keratinocytes, breast epithelial cells, and intestinal epithelial cells (Hennings *et al.*,

1980; Soule *et al.*, 1990; Yuspa *et al.*, 1989; Black and Smith, 1989) all of which proliferate maximally in media with a very low calcium concentration (approximately 0.05 mM). In contrast, increases in calcium concentration enhance the proliferation of several mesenchymally derived cell types: human dermal fibroblasts, Rat-1 fibroblasts, and the specialized mesothelial cells covering the ovary – the ovarian surface epithelium (OSE) cells (Huang *et al.*, 1995; McNeil *et al.*, 1998b; McNeil *et al.*, 1998a). In these cells, proliferation is maximal at higher calcium concentrations (1.8 mM CaCl2), that found in standard Dulbecco's Modified Eagles Medium. Reducing calcium below a threshold of 1.4 mM results in growth inhibition (Huang *et al.*, 1995; McNeil *et al.*, 1998b). Calcium can even act as a mitogen in these cells; addition of calcium to fibroblasts made quiescent by serum-starvation in low calcium media results in proliferation equal to that of cells stimulated with EGF, as measured by thymidine incorporation (McNeil, *et al.*, 1998b; Huang *et al.*, 1995).

Mouse primary keratinocytes have been used to investigate the molecular mechanisms responsible for calcium-induced differentiation. In the keratinocyte system, raising the extracellular calcium in the culturing medium from 0.05 mM to 2.0 mM triggers a terminal differentiation program resembling that in mature skin (Hennings *et al.*, 1980; Hennings and Holbrook, 1983). Using a unique ion-capture cytochemistry technique, a gradient of increasing calcium concentrations in the upper epidermal layers has been demonstrated, suggesting that calcium-regulated differentiation of keratinocytes may occur in vivo as well (Menon *et al.*, 1985). One of the most immediate (within 5 minutes) molecular events upon calcium addition is the tyrosine phosphorylation of $p62^{dok}$, the Ras-GTPase activating protein (Ras-GAP)

associated p62 (Filvaroff *et al.*, 1992). p62 ^{dok} phosphorylation is specific to calcium, as it is not induced by other keratinocyte differentiation factors such as tetradecanoylphorbol-acetate (TPA) or transforming growth factor β (Filvaroff *et al.*, 1992). Filvaroff et al. investigated the mechanism of calcium-induced p62 ^{dok} phosphorylation. Treatment of keratinocytes with calcium or the divalent cations magnesium, cobalt or nickel increases calcium influx and the intracellular calcium concentration (Hennings *et al.*, 1989; Kruszewski *et al.*, 1991; Reiss *et al.*, 1991). Activation by other divalent ions argues against a calcium influx mechanism for signaling. In addition, the increase in intracellular calcium does not appear to be responsible for p62 ^{dok} phosphorylation, since the calcium ionophores A23187 and X537A were not able to induce tyrosine phosphorylation even though intracellular calcium levels were increased (Filvaroff *et al.*, 1994). In this same study, cadherin-blocking antibodies were unable to inhibit calcium-induced p62 ^{dok} phosphorylation, demonstrating that phosphorylation is not due to a calcium-induced effect on the calcium dependent cadherin adhesion molecules. Finally, blocking calcium-induced potassium uptake with the Na+K+ATPase inhibitors harmaline and ouabain did not inhibit calcium-induced p62 phosphorylation. These data led the authors to propose that calcium may activate intracellular signals and differentiation through a "cationic receptor mechanism".

THE G PROTEIN-COUPLED CALCIUM-SENSING RECEPTOR

Recently, a Ca^{2+}-activated G protein-coupled receptor, the calcium-sensing receptor (CaR), has been described (Brown *et al.*, 1993). The CaR, by amino-acid identity and

structural homology, is most closely related to the G protein-coupled metabotropic glutamate receptor (Brown *et al.*, 1993). Although originally identified in parathyroid cells, numerous tissues other than the parathyroid gland have been shown to express the full-length CaR, CaR mRNA and/or CaR protein. These include cell-types which demonstrate calcium-sensitive proliferation such as keratinocytes, intestinal epithelial cells, OSE cells, and fibroblasts (Bikle *et al.*, 1996; Kallay *et al.*, 1997; McNeil *et al.*, 1998b; McNeil *et al.*, 1998a). The expression of the CaR in cell-types whose proliferation is calcium-sensitive suggests the possibility that the CaR may play a role in calcium-induced proliferation and/or differentiation.

Original characterization of the CaR

The CaR was first identified in parathyroid cells. The parathyroid gland is part of a complex physiological system, which maintains rigid control over Ca^{2+} homeostasis in humans and other species. The parathyroid is responsible for secreting parathormone in the face of low calcium levels, thereby recruiting the intestine, kidney and bone in raising the calcium concentration of systemic extracellular fluid. When serum calcium levels rise, parathormone release is inhibited. In order for this system to function, there must be a mechanism by which the parathyroid can 'sense' Ca^{2+} in order to respond appropriately to fluctuations.

The molecular mechanism by which parathyroid cells 'sense' calcium was unknown until the cloning of the CaR (Brown *et al.*, 1993). Parathyroid cells respond to elevated Ca^{2+} and other specific di- and tri-valent cations by ceasing parathormone secretion. This response is coupled to changes in phosphoinositide turnover and

increases in cytosolic calcium, and Brown, et al took advantage of this pharmacological characteristic for an expression-cloning strategy in Xenopus laevis oocytes. Activation of phosphatidylinositol coupled receptors, when expressed in Xenopus laevis oocytes, elicit agonist-dependent inward currents arising from phosphatidylinositol-dependent stimulation of Ca^{2+} -activated Cl^- channels. After injecting bovine parathyroid cell poly(A)+RNA into oocytes, CaR-expressing cells were identified by their response to CaR agonists as measured by inward currents. Nucleic acid hybridization techniques were then used to isolate a single 5.3 kb cDNA clone. Subsequent sequence analysis predicted a topology characteristic of the seven-transmembrane guanine nucleotide regulatory (G) protein coupled receptors (GPCRs) (Brown *et al.*, 1993; and references therein). As shown in Figure 1, the receptor is composed of three functional subunits: 1) a large amino-terminal extracellular domain containing clusters of acidic residues, 2) the characteristic membrane-spanning region comprised of seven α-helices, with intervening intracellular and extracellular loops, and 3) a cytoplasmic carboxy-terminal domain. The CaR has been isolated from human (Garrett *et al.*, 1995) and chicken parathyroid (Diaz *et al.*, 1997), both demonstrating greater than 90% amino acid identity with bovine CaR. The CaR sequence does not encode a high-affinity binding site for calcium such as an EF-hand, but instead, the negatively charged clusters in the extracellular domain are thought to bind calcium and other polycationic agonists with millimolar efficiency (Brown *et al.*, 1993).

The CaR belongs to a subgroup of the GPCR superfamily which also includes the metabotropic glutamate receptors (mGluR), whose ligand is the

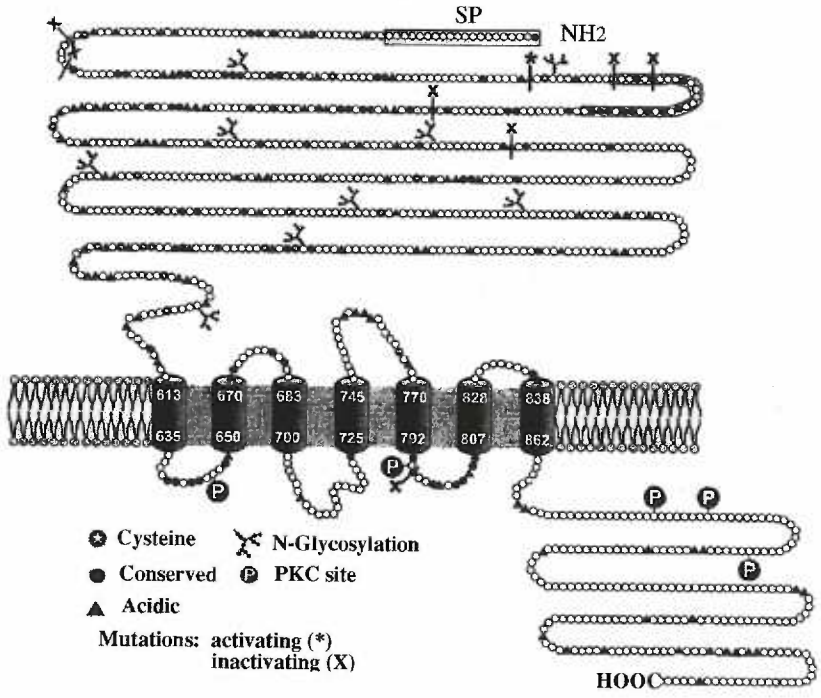

Figure 1. Predicted structure of the CaR.

excitatory neurotransmitter glutamate (Nakanishi *et al.*, 1994). These two receptors share only approximately 20% amino acid identity, but their overall topology bears great similarity, both containing very large intracellular and extracellular domains (ECD) and relatively short intracellular loops as compared to other GPCRs (Garrett *et al.*, 1995; Brown *et al.*, 1993). The overall structure of the CaR and mGluR ECDs have been shown to be similar to the bacterial periplasmic binding proteins (PBPs), which contain a bilobate structure forming a ligand binding cleft (Brown *et al.*, 1993, and references therein). These bacterial PBPs play a role in chemoreception and the response to sensory stimuli. The acquired knowledge with regard to the PBPs may be useful in further understanding of the mechanism of calcium binding by the CaR.

Tissue distribution of the CaR.

CaR expression is not restricted to the parathyroid gland. Additional full-length clones have been isolated from kidney (rat -Riccardi *et al.*, 1995; human - Aida *et al.*, 1995; rabbit - Butters Jr *et al.*, 1997), thyroid C-cells (rat - Garrett *et al.*, 1995), and brain striatum (rat - Ruat *et al.*, 1996). All of these demonstrate greater than 90% amino acid identity to the bovine parathyroid CaR. Northern analysis and RT-PCR techniques have identified other tissues and cell lines which express the CaR or a highly related homologue. These include small and large intestine (Chattopadhyay *et al.*, 1998; Kallay *et al.*, 1997), stomach (Ray *et al.*, 1997), keratinocytes (Bikle *et al.*, 1996), and numerous regions of the brain (Ruat *et al.*, 1995). Work in our lab has demonstrated expression in

Rat-1 fibroblasts (McNeil *et al.*, 1998b) and OSE cells (McNeil *et al.*, 1998a), as well. With the exception of thyroid C-cells, the physiological function of the CaR in these cell types has not been definitively demonstrated. For cell types such as nerve, breast epithelium, osteoblasts, kidney epithelium, and placenta, each of which functions in an organ where local transport and/or regulation of calcium is inherent to the function of the organ - the need for a calcium-sensing mechanism is clear and immediately suggests a possible role for the CaR. For other cell types such as keratinocytes and fibroblasts, the in vivo role of a calcium sensing mechanism is not yet apparent. It is of interest that many of these cell-types, including keratinocytes, breast epithelium, Rat-1 fibroblasts, and OSE cells, demonstrate calcium-sensitive changes in proliferation in vitro, as previously stated. This suggests the possibility that the CaR may play a role in the proliferation and/or differentiation responses induced by extracellular calcium in these cell types.

G PROTEIN-COUPLED RECEPTORS - GENERAL STRUCTURE AND FUNCTION.

The superfamily of G protein-coupled receptors (GPCRs) is comprised of structurally similar plasma membrane-associated receptors, consisting of an extracellular ligand-binding domain, a core of seven membrane-spanning α-helices with associated intervening intracellular and extracellular loops, and an intracellular carboxy-terminal signaling domain. Intracellular G protein coupling is directed by both the C-terminal domain and the intracellular loops; both loops contribute to the specificity of G protein coupling as well (Wess *et*

al., 1997; and references therein). Upon ligand binding, these receptors undergo a change in conformation, which results in the activation of associated heterotrimeric G proteins (Bourne *et al.*, 1997).

Heterotrimeric G proteins are a large family of signaling proteins which consist of three subunits - the α subunit, and the β and γ subunits which function as a single $\beta\gamma$-heterodimeric subunit. G_α subunits, of which 16 have been identified, are divided into four families: s, i/o, q, and 12/13 (Wilkie *et al.*, 1992). $G_{i/o}$ inhibits adenylyl cyclase activity and is pertussis toxin sensitive. G_s activates adenylyl cyclase, while G_q and $G_{12/13}$ activate other effectors, and each of these is insensitive to pertussis toxin (reviewed in Post and Heller Brown, 1996). Gα subunits associate with the G$\beta\gamma$ complex, and currently there are at least 11 γ subunits and 5 β subunits which have been identified (reviewed in Gutkind, *et al.* 1998). This allows for a considerable number of combinatorial possibilities among the heterotrimeric G-protein subunits.

In the inactive state, the α subunit, bound to GDP, interacts with both the receptor and the associated $\beta\gamma$ subunits. Upon stimulation by its receptor, presumably via conformational change, the Gα subunit is induced to exchange GDP for GTP, which induces both the dissociation and activity of the Gα and G$\beta\gamma$ subunits. The $\beta\gamma$ subunit has been shown to possess much greater signaling capacity than first thought. This subunit has been found to be a major contributor to GPCR-induced tyrosine phosphorylation events. Both the α-subunit and $\beta\gamma$ dimer go on to bind and modulate downstream effector

proteins, including adenylyl cyclases, phosphodiesterases, phospholipases, and kinases (Gutkind *et al.*, 1998; reviewed in Post and Heller Brown, 1996). The G_α subunit activity is self-limiting, due to its intrinsic GTPase activity which converts the active GTP-G_α into its inactive GDP-bound form. The GDP-bound subunit then re-associates with the $\beta\gamma$ subunit, and the heterotrimer complexes again with its receptor.

MITOGENIC GPCRS AND THE RAS-MAPK CASCADE

Ras: a key point of convergence for proliferative signals.

The Ras protein is a central point of convergence and a required intermediate for many extracellular signals, including those that regulate cellular proliferation and differentiation (Cai *et al.*, 1990; Deshpande and Kung, 1987; Feig and Cooper, 1988; Hagag *et al.*, 1986; Mulcahy *et al.*, 1985; Satoh *et al.*, 1992; Szeberengi *et al.*, 1990; Boguski and McCormick, 1993). Activated Ras has been shown to be essential for the proliferation of fibroblasts (Feig and Cooper, 1988; Mulcahy *et al.*, 1985). Ras is an intracellular membrane-associated GTP/GDP binding protein that is active in its GTP-bound state. Cytoplasmic guanine nucleotide exchange factors (GEFs) such as SOS1/2 and RasGRF/mCDC25 (Lowy and Willumsen, 1993) induce Ras activation by 'catalyzing' exchange of GDP for GTP, thereby activating Ras. Subsequent inactivation of Ras occurs secondary to GTPase activity, which converts GTP into GDP. Ras has a very low intrinsic GTPase-activity, but the binding of GTPase activating proteins (GAPs) such as p120GAP and neurofibromin (NF1) enhance the GTPase activity of Ras up to 1000

fold, thereby initiating Ras inactivation and assuring its regulation (Trahey and McCormick, 1987; Trahey *et al.*, 1988; Vogel *et al.*, 1988; Bollag and McCormick, 1991; Boguski and McCormick, 1993). Inhibition of GAP activity as well as activation of GEFs (Downward *et al.*, 1990) can activate Ras. Mutations which interfere with the ability of Ras to interact with GAPs render Ras constitutively activated and oncogenic.

The MAPK cascade, and other Ras effector mechanisms.

Upon activation, Ras initiates an ordered cascade of serine/threonine protein phosphorylations known as the mitogen-activated protein kinase cascade. The MAPK module consists of the activation of one of several mitogen-activated protein (MAP) kinase kinase kinases such as Raf-1, Raf-A, or Raf-B, which then activates the MAP kinase kinases, MEK1 and MEK2, which in turn activate the MAP kinases ERK1 and ERK2. ERK1 and ERK2 then translocate to the nucleus where they influence transcriptional events through phosphorylation of nuclear proteins such as transcription factors.

Activated GTP-bound Ras activates the MAPK cascade by recruiting the serine/threonine kinase Raf to the membrane. Three forms of Raf exist – Raf-1, A-Raf and B-Raf. Raf-1 is ubiquitous, while A-Raf and B-Raf expression is restricted to specific tissues – in general, steroid-responsive urogenital tissues and neuronal tissue, respectively (Barnier *et al.*, 1995; Lee *et al.*, 1996; Winer and Wolgemuth, 1995). As Raf-1 is most relevant to this thesis, from here on Raf will refer to Raf-1 only. The Ras/GTP:Raf interaction results in Raf activation by mechanisms which are still unclear but appear to involve

translocation to the plasma membrane (Marais *et al.*, 1995; Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Activated Raf then phosphorylates the MAP kinase kinases-1 and -2 (MEK), dual specificity kinases which then phosphorylate the MAP kinases extracellular regulated kinase-1 and –2 (ERK) on tandem threonine and tyrosine residues (TEY motif; Alessi *et al.*, 1994; Payne *et al.*, 1991). It is not yet apparent why there exist two isoforms of MEK and ERK, but the observation that the two forms of each enzyme are conserved among eukaryotic species would suggest that functions unique to each isoform do exist (Lewis *et al.*,1998). Phosphorylation results in activation and translocation of ERK to the nucleus (Lenormand *et al.*, 1993), resulting in the phosphorylation of transcription factors such as $p62^{TCF}$/ELK-1 and thereby regulating gene expression (Marais *et al.*, 1993; Gille *et al.*, 1995). ELK-1 joins a complex containing the serum response factor (SRF), which then binds promoter regions containing the SRE element, resulting in transcriptional activation of the downstream gene. The SRE is present in numerous gene promoter regions such as c-Fos (Treisman, 1986), an immediate early gene whose expression is thought to be involved in the progression of cells through G1 of the cell cycle. MEK/ERK activation also results in the transcriptional induction of cyclin D1 (Lavoie *et al.*, 1996), an important regulatory component of the cell-cycle control machinery. Additionally, a new putative downstream effector, MAPK interacting kinase (Mnk-1/Mnk-2) has been independently identified by two groups (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). Activated ERK induces Mnk-dependent phosphorylation of the translational

initiation factor eIF-4E, enhancing the ability of eIF-4E to bind to the 5'-cap structure of mRNAs. ERK-induced enhancement of eIF-4E activity could be responsible for enhancing translation of important gene products necessary for cell cycle progression.

Activated ERKs also phosphorylate numerous cytoplasmic proteins. These include pp90 ribosomal S6 kinase (RSK), RSK-2, RSK-3, cytosolic phospholipase A2, and the tail of the EGF receptor (Bunone *et al.*, 1996; Lin *et al.*, 1993; Sturgill *et al.*, 1988; Takashima *et al.*, 1991; Zhao *et al.*, 1995). The role of these substrate interactions in the proliferative response is unknown.

The importance of the MAP kinase cascade as a core signaling mechanism is evidenced by its evolutionary conservation. Receptor tyrosine kinase/Ras/MAP kinase signaling homologues are found in the fruit fly Drosophila melanogaster, where they control eye development (Rubin, 1991). Similarly, proper vulval development in Caenorhabditis elegans requires the same signaling scheme (Sternberg and Horvitz, 1991). Homologues involved in the regulation of cell growth in response to mating pheremones are also found in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe (Broach, 1991; Nishida and Gotoh, 1993; Tamanoi, 1988).

Ras-induced transformation.

Constitutively activated Ras mutants such as v-Ras are oncogenic in rodent fibroblasts. The full mechanism by which Ras induces transformation is not completely understood. Investigations in the rodent fibroblast system have

demonstrated that Raf is a very important effector of Ras. This is supported by the finding that Ras transformation can be greatly impaired by expression of the dominant negative mutants of Raf, or of Raf's downstream targets MEK and ERK, suggesting that the MAP kinase cascade is necessary for transformation in response to Ras (Cowley *et al.*, 1994; Khosravi-Far *et al.*, 1995; Kolch *et al.*, 1991; Qiu *et al.*, 1995; Schaap *et al.*, 1993; Westwick *et al.*, 1994). Conversely, a constitutively activated Raf mutant could overcome the loss of Ras function (Feig and Cooper, 1988; Smith *et al.*, 1986), and the Ras transformation phenotype was completely reproduced by a membrane-targeted Raf in fibroblast transformation assays (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). These findings led to the suggestion that the main function of Ras is to facilitate the activation of Raf by translocating it to the membrane.

It has since been shown that Ras-induced transformation in rodent fibroblasts requires proteins in addition to Raf/MEK/ERK. For instance, Marais et al have shown that maximal activation of Raf requires activated cytoplasmic tyrosine kinases synergizing with oncogenic Ras, such as oncogenic Src (Marais *et al.*, 1995). Phosphatidylinositol-3-kinase (PI3K) and the small GTPases Rac and Rho have been implicated as effector proteins necessary for Ras-induced transformation in several reports. In NIH3T3 fibroblasts, inhibition of Rac or Rho function by expression of the corresponding dominant interfering mutants inhibited focus formation induced by oncogenic Ras, but not by activated Raf (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). In fact, the activated Rac and Rho mutants displayed strong synergy with Raf in focus-forming assays, suggesting Raf-dependent and –independent mechanisms of Ras-

induced transformation (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). Similarly, phosphatidylinositol-3-kinase activity has been shown to be necessary for Ras induced transformation, likely due to its ability to activate Rac (Rodriguez-Viciana *et al.*, 1997).

Ras-induced transformation: the mesenchymal versus epithelial dichotomy.

Cellular transformation by Ras may occur via different mechanisms or may require signaling proteins in addition to the MAPK module, depending on the cellular background in which it is expressed. There is significant evidence in the literature that cells of differing embryonic origin, i.e. epithelial or mesenchymal, demonstrate fundamentally different biological responses to a given signal. For example, extracellular calcium can induce proliferation in cells of mesenchymal origin, such as fibroblasts and OSE cells, yet extracellular calcium induces differentiation in keratinocytes, breast epithelial cells, and intestinal epithelial cells (Huang *et al.*, 1995; McNeil *et al.*, 1998b; McNeil *et al.*, 1998a; Hennings *et al.*, 1980; Soule *et al.*, 1990; Yuspa *et al.*, 1989; Black and Smith, 1989). This may be due simply to differential expression of a receptor or intracellular signaling intermediate, or may be due to fundamental differences in signaling mechanisms or signal interpretation.

The transforming capacity of Ras and Raf differ depending on the cell system in which they are expressed. In contrast to the rodent fibroblast system, where activated Raf can transform in a manner apparently identical to Ras, constitutively activated Raf was unable to induce transformation in RIE-1 rat intestinal epithelial cells, although activated Ras was fully transforming (Oldham *et al.*, 1996). Likewise, activated Ras, but not Raf, induced transformation of IEC-6 rat intestinal and MCF-10A human mammary

epithelial cells (Oldham *et al.*, 1996). It has since been shown that a Raf-independent pathway, which requires an EGFR-autocrine loop, is necessary for Ras transformation in this epithelial cell type (Gangarosa LM, 1997). Similarly, whereas Src-transformation requires Ras function in rodent fibroblasts, in RIE-1 cells, Src can transform independent of Ras, and independent of an EGFR-autocrine loop (Oldham *et al.*, 1998). These observations suggest the existence of fundamentally different, cell-type specific mechanisms of Ras-transformation depending upon whether the cell is of epithelial or mesenchymal derivation.

Mitogenic GPCRs couple to the Ras-MAPK cascade.

Although long considered to be independent, signaling pathways activated by GPCRs and the receptor tyrosine kinases (RTKs) have been recently shown to converge in several significant aspects. In general, mitogenic GPCRs activate Ras and the components of the mitogen-associated protein kinase (MAPK) cascade, Raf, MEK, and the extracellular signal regulated kinases ERK1/2 (reviewed in Post and Heller Brown, 1996). These mitogenic GPCRs, which couple to various Gα-subtypes, include the receptors for lysophosphatidic acid (LPA) (Howe and Marshall, 1993; Hordijk *et al.*, 1994), Bombesin (Faure *et al.*, 1994), Angiotensin II (Duff *et al.*, 1992; Ishida *et al.*, 1992), thrombin (LaMorte *et al.*, 1993), and α1B-adrenergic and α2A-adrenergic (Faure *et al.*, 1994) receptors. Although activation of ERK occurs upon stimulation of mitogenic GPCRs, ERK activation may or may not be required for the resulting mitogenesis. ERK activity has been reported to be required for the mitogenic effects of thrombin stimulation in Rat-1 fibroblasts (Pages *et al.*, 1993). Yet TSH-induced mitogenesis in thyroid cells

(Lamy *et al.*, 1993) is not associated with ERK activation. Thus the role of ERK activation in GPCR-elicited mitogenesis can vary in a cell-specific fashion.

The mechanism by which GPCRs activate ERK is still under investigation. In general, ERK activation can occur either in a Ras-dependent and PKC-independent manner (Alblas *et al.*, 1993; Koch *et al.*, 1994; Luttrell *et al.*, 1995; Eguchi *et al.*, 1996; Della Rocca *et al.*, 1997), or PKC-dependent and Ras-independent manner (Hawes *et al.*, 1995; van Biesen *et al.*, 1996). An interesting exception was reported by Li et al (Li *et al.*, 1998), who found two mechanisms by which the angiotensin II receptor stimulated ERK activation in rat liver epithelial cells: a PKC-dependent and Ras-independent pathway, or a latent but equipotent Ras-dependent and PKC-independent pathway which was unmasked by PKC depletion.

The PKC-dependent and Ras-independent pathway by which GPCRs activate ERK is not well understood. G_{α} activity, rather than $G_{\beta\gamma}$ activity, appears to be pivotal as this pathway is insensitive to inhibition by the C-terminal fragment of βARK1, a competitive inhibitor of $G_{\beta\gamma}$– mediated signals (van Biesen *et al.*, 1996; Hawes *et al.*, 1995). The GTP-bound $\alpha_{q/11}$ subunit activates phosphoinositide hydrolysis by phospholipase C-β (Wu *et al.*, 1992), generating IP_3 and diacylglycerol (DAG). DAG activates protein kinase C (PKC), as does increased cytosolic calcium released from IP_3-sensitive intracellular stores. PKC activation may be responsible for Ras-independent ERK activation, as PKC has been reported to phosphorylate and activate Raf-1 (Heidecker *et al.*, 1992; Kolch *et al.*, 1993; Carroll and May, 1994). Importantly,

$G_{q/11}$-bound receptors can also activate ERK in a PKC-independent or partially independent manner (Charlesworth and Rozengurt, 1997; Crespo *et al.*, 1994) and are involved in Ras-dependent ERK activation, as well. This demonstrates that $G_{q/11}$ may have differential signaling partners as determined by the specific receptor and/or the cellular background in which it is activated. As many of the molecular events involved in GPCR activation of ERK through Ras are increasingly identified, it is becoming clear that multiple mechanisms exist for this process, and these vary in a cell-type specific fashion.

SIGNALING MECHANISMS BY WHICH GPCRS ACTIVATE THE RAS/MAPK CASCADE

Recent reports have identified several key signaling intermediates which are required for GPCR-induced Ras and ERK activation (Figure 2). Stimulation of mitogenic GPCRs results in associated tyrosine phosphorylation events similar to those seen with receptor tyrosine kinase (RTK) activation. The RTK adaptor protein Shc and associatedGrb2/SOS have been shown to be involved in GPCR signaling. In addition, three major classes of kinases, previously associated only with RTK signaling, have been implicated in GPCR signaling pathways, including MAPK activation. These are the Src-family kinases (Simonson et al., 1996; Wan et al., 1996; Dikic et al., 1996; Luttrell et al., 1996), phopsphatidylinositol-3-kinase (Hu et al., 1996; Hawes et al., 1996; Lopez-Ilasaca et al., 1997; Kranenburg et al., 1997), and the receptor tyrosine kinases such as the EGF receptor and the PDGF receptor (Daub et al., 1997; Daub et al., 1996; Eguchi et al., 1998a; Soltoff, 1998; Linseman et al., 1995). Additionally, in specialized cell types,

Important Intracellular Mediators of ERK Activation By GPCRs

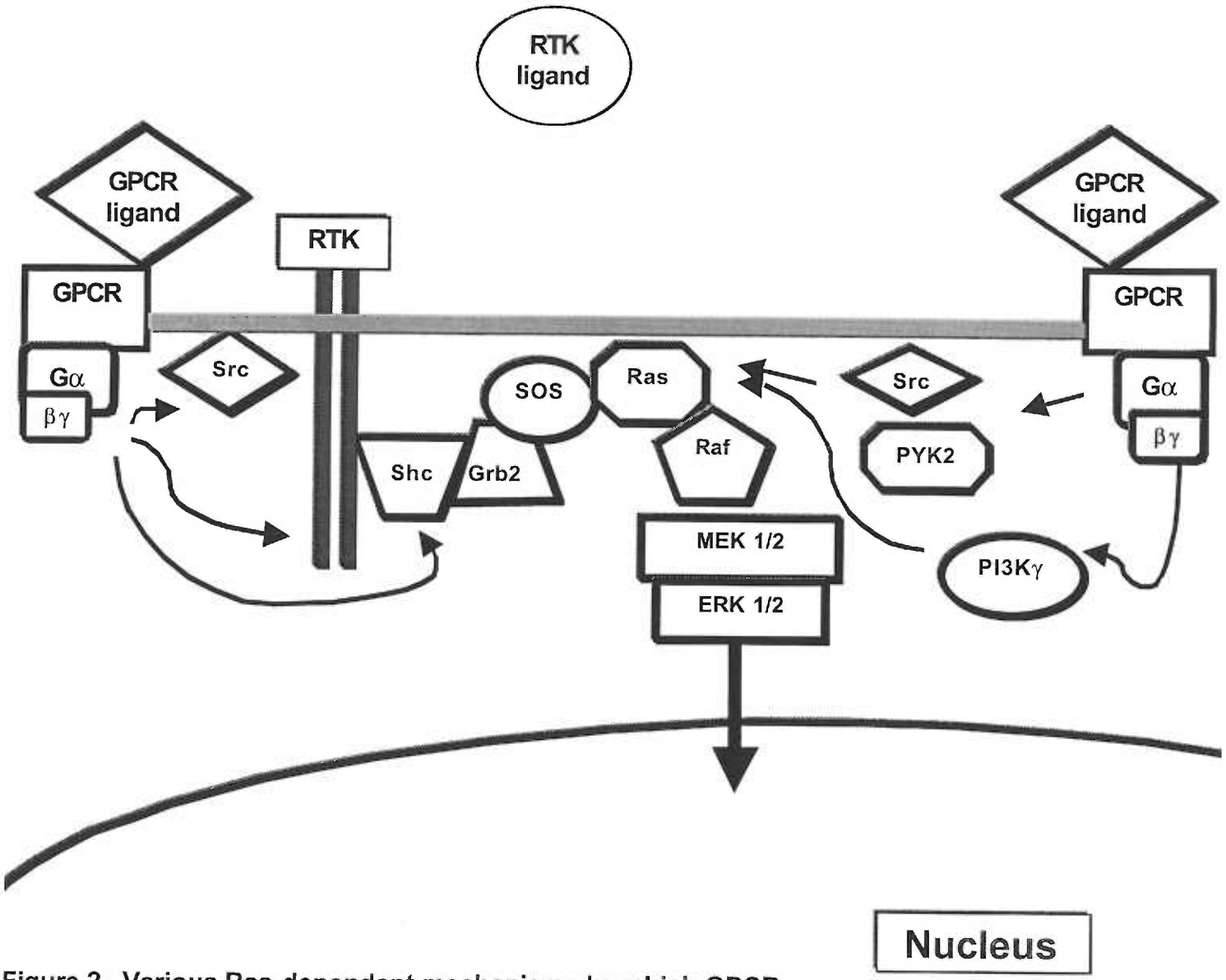

Figure 2. Various Ras-dependent mechanisms by which GPCRs couple to ERK activation.

the calcium-sensitive cytoplasmic kinase PYK-2 (also designated CADTK or RAFTK) has been implicated (Dikic et al., 1996; Soltoff, 1998).

GPCR signaling: requirement of Shc/Grb2/SOS module.

The Shc adaptor proteins have been shown to be crucial to growth regulation by the EGFR, integrins, and the G protein-coupled thrombin receptor (Chen et al., 1996b; Wary et al., 1996; Sasaoka et al., 1994). Shc binds tyrosine phosphorylated receptors such as the EGF receptor and then, upon becoming tyrosine phosphorylated, forms a complex with Grb2/SOS. This brings SOS into proximity with membrane-associated Ras. SOS is a guanine nucleotide exchange protein which activates Ras. Shc-induced association of SOS with Ras is thought to be a major mechanism by which RTKs activate Ras.

Shc exists as three isoforms – Shc p46, p52 and p66, which all derive from a single gene (reviewed in Wary *et al.*, 1996). The p52 and p46 isomers result from differential translation initiation from two proximal ATG sites. The p66 isoform is a splicing isoform containing all of the p52 sequence with an additional glycine/proline rich amino terminal region. It has not yet been well established whether there exist unique signaling capacities to each isoform or if they are functionally redundant.

Shc becomes tyrosine-phosphorylated in response to many growth-regulatory signals, including growth factor and cytokine stimulation, G-protein coupled receptor activation, and integrin-mediated adhesion (reviewed in (Bonfini *et al.*, 1996; Lev *et al.*, 1995; van Biesen *et al.*, 1995; Wary *et al.*, 1996), but the phosphorylating kinase in these systems has not been definitively identified. Shc proteins have been shown to

associate with and be phosphorylated in vitro by several receptors such as the EGFR and cytoplasmic protein kinases such as Src and FAK (Chen *et al.*, 1996; Schlaepfer *et al.*, 1998). These kinases phosphorylate Shc at Tyr-317 and/or Tyr-239, which are sites critically involved in association with the Grb-2/SOS complex (van der Geer et al., 1996; Harmer *et al.*, 1997; Schlaepfer *et al.*, 1998), consistent with an *in vivo* role in Shc regulation. The importance of Tyr317, and presumably Grb2 binding, to Shc function is demonstrated by the observation that overexpression of wild-type Shc is transforming in murine fibroblasts (Pelicci *et al.*, 1992), but Shc proteins mutated at the Tyr317 residue are unable to induce transformation (Salcini *et al.*, 1994).

Numerous GPCRs can induce Shc tyrosine phosphorylation and the formation of SHC/Grb2/SOS complexes (Chen *et al.*, 1996b; van Biesen *et al.*, 1995; Touhara *et al.*, 1995), and in some systems, the Shc/Grb2/SOS module is crucial to GPCR mitogenic signaling (Chen *et al.*, 1996b). Disruption of the Shc/Grb2/SOS module blocks ERK activation in response to G-$\beta\gamma$ overexpression in COS-7 cells (van Biesen *et al.*, 1995) and to LPA and bradykinin stimulation in PC-12 cells (Dikic *et al.*, 1996). Using expression of dominant interfering forms of the protein, Shc function was shown to be required in thrombin induced mitogenesis in CCL39 fibroblasts (Chen *et al.*, 1996b). The mechanism of Shc activation by GPCRs has not been established. Both Src and PI3K have been implicated in GPCR-induced Shc tyrosine phosphorylation and ERK activation by unknown mechanisms. It is clear, though, that GPCRs are able to utilize the Shc/Grb2/SOS module to

activate Ras, ERK and mitogenesis, although through means not completely established.

GPCR signaling: requirement of Src family kinase activity.

The Src family kinases (c-Src, Fyn, Lyn, Yes, and the distantly related SYK) are cytoplasmic tyrosine kinases which are activated by receptor tyrosine kinases, oxidative and UV stress, integrin-mediated signal transduction, and GPCRs. c-Src has been shown to be involved in the regulation of proliferation in rodent fibroblasts. The EGFR associates with c-Src (Luttrell *et al.*, 1994), and the mitogenic effects of EGF require endogenous c-Src (Roche *et al.*, 1995;Twamley-Stein *et al.*, 1993) and are enhanced by c-Src overexpression (Luttrell *et al.*, 1988). In addition, activated c-Src proto-oncogene and the oncogene v-Src are capable of inducing transformation in cells (Jove *et al.*, 1987). The critical downstream targets of the kinase remain controversial in many of these pathways. Given its importance, it was somewhat surprising that the c-Src knock-out mouse displayed only one demonstrable phenotype, osteopetrosis – a bone disorder characterized by thickened bone, a narrowed marrow space, and failure of tooth eruption (Soriano *et al.*, 1991). It has been suggested that, as in other knock-out systems, the phenotype is rescued by functional redundancy among the Src-family kinase members. One additional aspect of c-src function has also been clarified using the knock-out system. Schwartzberg et al. used a transgenic approach to introduce c-Src or the kinase deficient Src^{K295M} mutant back into src-/-osteoclasts, the bone-resorbing cells affected in the c-Src knock-out mouse (Schwartzberg *et al.*, 1997). They demonstrated that expression of the Src^{K295M} mutant rescued the phenotype completely,

in a manner identical with the wild-type c-Src. This is yet further evidence that not all pathways involving c-Src require Src kinase activity. Fibroblasts from c-src -/- mice are defective in fibronectin-induced cell-spreading, yet this phenotype can be complemented by the kinase defective c-Src as well (Kaplan *et al.*, 1994). Similarly, in fibroblasts, the c-Src-dependent tyrosine phosphorylation of $p130^{cas}$ does not require c-Src kinase activity (Schlaepfer *et al.*, 1997).

The Src-family kinases (Src) have been strongly implicated as downstream mediators of GPCR- or $G_{\beta\gamma}$-induced ERK activation (Simonson *et al.*, 1996; Wan *et al.*, 1996; Dikic *et al.*, 1996; Luttrell *et al.*, 1996; Dikic *et al.*, 1996; Lopez-Ilasaca *et al.*, 1997). In COS-7 cells, both LPA- (which acts via a G_i-coupled receptor) and Gβγ-induced ERK-activation was Src-dependent, as demonstrated by sensitivity to the expression of c-terminal Src kinase (CSK), a cytoplasmic kinase which phosphorylates and inhibits all of the Src-family kinases (Luttrell *et al.*, 1996; Nada *et al.*, 1991). The Src-dependent pathway can require other kinases and/or the Shc/Grb2/SOS module, as well. In PC-12 cells, the Src-dependent activation of ERK by LPA and bradykinin also required PYK-2 activity (Dikic *et al.*, 1996). In COS-7 cells, Lopez-Ilasaca et al demonstrated that Src-dependent Gβγ stimulation of ERK also requires PI3K activity and Shc activity (Lopez-Ilasaca *et al.*, 1997). However, the involvement of Src and Shc in LPA-induced ERK activation in COS-7 cells is contested by a report by Kranenburg et al (Kranenburg *et al.*, 1997). This report demonstrated that the inhibition of c-Src- or Shc-activities by their respective dominant interfering mutants was unable to interfere with LPA-induced ERK activation,

while inhibition of PI3K activity with wortmannin completely inhibited the ERK response to LPA. The reason for this discrepancy is unclear but may pertain to differential Src inhibition with the use of CSK expression by Luttrell et al, which inhibits all of the Src-family members (reviewed in Neet and Hunter, 1995), versus overexpression of the c-Src^{K298M} dominant interfering mutant in the work of Kranenburg et al. Overall, there is strong evidence in the literature that Src is a key player in GPCR signaling and ERK activation.

GPCR signaling: requirement of RTK transactivation.

Several groups have demonstrated transactivation, i.e. activation in the absence of ligand binding, of receptor tyrosine kinases in response to GPCR agonists. This transactivation has been shown to be required for downstream signaling in some systems. One postulated function of RTKs in GPCR signaling is to act as a scaffolding structure to which other signaling proteins may be recruited in response to GPCR activation (Luttrell *et al.*, 1997; reviewed in Pawson and Scott, 1997). Transactivated RTKs include the EGF receptor (Daub *et al.*, 1997; Daub *et al.*, 1996; Eguchi *et al.*, 1998a; Soltoff, 1998) and the PDGF receptor (Linseman *et al.*, 1995). Daub, et al. demonstrated that in Rat-1 fibroblasts, the EGF receptor was tyrosine-phosphorylated in response to stimulation with endothelin-1 (ET-1), lysophosphatidic acid (LPA), or thrombin (Daub *et al.*, 1996). Each of these GPCR-agonists have been shown to be mitogenic in Rat-1 cells (Muldoon *et al.*, 1990; van Corven *et al.*, 1989; Gupta *et al.*, 1992). In addition, stimulation with these agonists induced Shc tyrosine phosphorylation and EGFR/Shc/Grb2 association. Blockade of the EGFR either by

expression of the dominant interfering mutant lacking the cytoplasmic domain, or use of the EGFR-specific antagonist AG1478, inhibited EGFR and Shc tyrosine phosphorylation and inhibited EGFR/Shc/Grb2 association in response to GPCR ligands. These GPCR agonists also induce ERK activity, which is sensitive to EGFR blockade as well. Finally, the importance of the EGFR transactivation in the mitogenic effect of these agonists was supported by the decrease in GPCR agonist-induced DNA-synthesis, as determined by ^{3}H-thymidine incorporation, in the presence of the dominant-interfering EGFR mutant or the calcium ionophore AG1478.

GPCR-induced receptor tyrosine kinase transactivation is not specific to Rat-1 cells or to the activation of LPA, ET-1, or thrombin receptors. In rat vascular smooth muscle cells (VSMC), angiotensin II (ATII) also induced EGFR tyrosine phosphorylation and EGFR/Shc/Grb2 association, which was sensitive to AG1478 (Eguchi *et al.*, 1998b). Interestingly, in the VSMC system, ATII induced association of catalytically active c-Src with the EGFR, seen as early as 1 minute after stimulation. c-Src activation and association was not inhibited by AG1478, demonstrating that c-Src activation occurs independently and upstream of the EGFR-dependent tyrosine phosphorylation event. This result strongly implicates c-Src as a candidate for the initial phosphorylating kinase in GPCR-mediated ERK-activation. Src family members, including c-Src, are pivotal in the downstream signaling from numerous GPCRs, as discussed. This suggests that, in at least some systems, Src kinase activation is an early event in G protein activation by GPCRs. The mechanism by which Src kinases are activated by GPCRs remain unclear.

GPCR MITOGENIC SIGNALING: REQUIREMENT FOR PI3K -

General function of phosphatidylinositol-3-kinase.

The phosphatidylinositol 3-kinase (PI3K) lipid kinase family plays a central role in intracellular signaling and has been implicated in such diverse biological processes as mitogenesis (Roche *et al.*, 1995; Valius and Kazlauskas, 1993; Fantl *et al.*, 1992), protection from apoptosis (Kauffmann-Zeh *et al.*, 1996; Kennedy *et al.*, 1997; Dudek *et al.*, 1997, reviewed in King *et al.*, 1997), cytoskeletal rearrangement, migration, and protein trafficking (Kapeller and Cantley, 1994; Schu *et al.*, 1994; Shepherd *et al.*, 1996; Toker and Cantley, 1997; Vanhaesebroeck *et al.*, 1997). The mechanisms by which PI3K mediates these effects are currently being elucidated.

The PI3K family is composed of three enzyme classes based on substrate specificity. Class I enzymes phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P2 in vitro, but exhibit a preference for PI(4,5)P2 in vivo. Enzymes in this class exhibit a moderate serine/threonine kinase activity (Stoyanova *et al.*, 1997) in addition to their lipid kinase activity. Class I PI3Ks couple to important intracellular signaling pathways: Class I_A PI3Ks are involved in receptor tyrosine kinase signaling, while Class I_B enzymes are involved in GPCR-signaling pathways. Very little is known about the precise downstream effectors and mechanisms by which they are activated, but protein-protein interactions, serine/threonine kinase activity, and lipid product generation have

each been shown to be necessary for various signaling activities of Class I PI3Ks.

The PI3K p110 subunit is known as the catalytic subunit and possesses both lipid kinase and protein kinase activity. The mammalian Class I_A PI3Ks, the p110 α, β and δ isoforms, heterodimerize with a regulatory subunit such as p85α or –β, or p55α or -γ. P85-associated p110 $PI3K_\alpha$ is activated by SH2-mediated binding to tyrosine phosphorylated receptor tyrosine kinases and other signaling proteins, such as Ras, Crk, Abl, middleT/pp60c-Src complexes, and focal adhesion kinase (Fak)/integrin complexes (Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996; van der Geer *et al.*, 1994; Cantley *et al.*, 1991; Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Fantl *et al.*, 1992; Chen *et al.*, 1996a; Chen and Guan, 1994). Association of the heterodimer with tyrosine-phoshorylated receptors is thought to increase the catalytic activity of the p110 subunit, target the associated p110 catalytic subunit to tyrosine kinase signaling pathways, and bring the kinase into proximity with lipid substrates at the cell membrane (Carpenter *et al.*, 1993a; Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996).

The Class I_A heterodimers associate with Ras in a GTP-dependent manner, resulting in stimulation of PI3K lipid kinase activity *in vitro* and *in vivo* (Marte *et al.*, 1997; Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996). Klinghoffer et al demonstrated that GTP-bound Ras is required for full activation of Class I_A PI3Ks by the PDGF receptor (Klinghoffer, 1996). In COS-7

cells, PI3K acts downstream of Ras to mediate integrin-stimulated ERK activation; PI3K and Ras activity are both required, but Ras-GTP loading was unaffected by inhibitors of PI3K (King *et al.*, 1997). To what extent Class I_A PI3K activation occurs independent of Ras is not clear.

The Class I_B p110γ isoform does not associate with the p85 subunit. It has been reported to associate tightly with a p101 subunit which displays no homology to any known protein, but the functional significance of this interaction has not yet been clarified (Stephens *et al.*, 1997). PI3Kγ is known to be activated by direct binding to G-protein βγ subunits and is an important downstream effector of GPCR signaling (Leopoldt *et al.*, 1998; Lopez-Ilasaca *et al.*, 1997). Prior to being characterized, two initial observations suggested the existence of the PI3Kγ isoform: 1) the observation that GPCR activation can induce an increase in PtdIns(3,4,5)P3, a lipid product known to be generated by PI3Ks, and 2) the demonstration that wortmannin can inhibit the GPCR activation of ERK (Ferby *et al.*, 1994; Hawes *et al.*, 1996). The identification of the PI3Kγ isoform and its activation by βγ subunits led to studies by Lopez-Ilasaca et al (Lopez-Ilasaca *et al.*, 1997), which demonstrated that PI3Kγ was required for ERK activation by the G protein-coupled muscarinic receptor m2 and by βγ overexpression in COS-7 cells. Additionally, overexpression of PI3Kγ induced SHC/Grb2 association and SHC tyrosine phosphorylation, tyrosine kinase associated signaling elements which had previously been linked to Gβγ-dependent signaling (van Biesen *et al.*, 1995). These data suggest that

PI3Kγ may be an early point of convergence for the GPCR- and tyrosine kinase-cascades. The βγ stimulated PI3Kγ has since been characterized (Stephens *et al.*, 1994; Stoyanov *et al.*, 1995).

The PI3Kγ isoform, expressed as a recombinant GST-linked protein, has been shown to interact with Ras *in vitro*. While the interaction of p110α/p85α with Ras significantly activates p110α lipid kinase activity, P110γ lipid kinase activity was unaffected by interaction with Ras (Rubio *et al.*, 1997; Marte *et al.*, 1997; Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996). *In vivo* experiments were corroborative, in that simultaneous overexpression of PI3Kγ and Ras in COS-7 cells induced less than a two-fold increase in PtdIns(3,4)P2 and PtdIns(3,4,5)P3, in contrast to a strong induction of lipid kinase activity of p110α under the same conditions (approximately 30-fold increase in PtdIns(3,4)P2 and about a 10-fold increase in PtdIns(3,4,5)P3). The authors postulated that Ras may induce a GTP-dependent translocation of PI3Kγ to the plasma membrane without enhancement of lipid kinase activity (analogous to the translocation of Raf-1 by Ras (Leevers *et al.*, 1994)). The authors also acknowledged the possibility of PI3Kγ affecting Ras activity, but this was not addressed directly.

The role of D3-phosphorylated Phosphoinositides in PI3K signaling.

The Class I p110 catalytic subunits of PI3K are capable of phosphorylating inositol lipids at the D3 position of the inositol ring, thereby generating the formation of 3' phosphorylated phosphoinositides (PPIs): phosphatidylinositol 3-phosphate [PI(3)P], phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3] (Stephens *et al.*, 1993; Liscovitch and Cantley, 1994). The predominant products formed in vivo, though, are PI(3,4)P2 and PI(3,4,5)P3. Nearly absent in resting cells, PPIs are formed upon growth factor stimulation and are constitutively present at increased levels in transformed cells (Vanhaesebroeck *et al.*, 1997).

The PPIs appear to be important mediators of PI3K signaling, but little is known about the downstream effector molecules with which they interact. *In vitro* association of PI(3,4,5)P3 with the SH2 domains of Src and the p85 regulatory subunit of PI3K has been demonstrated (Rameh *et al.*, 1995), but the in vivo relevance of this finding has yet to be described. The novel PKC isoforms δ, ε and η also bind and are activated by both PI(3,4)P2 and PI(3,4,5)P3 in vitro (Palmer *et al.*, 1995; Toker *et al.*, 1994). *In vivo* relevance for a PKC-PPI interaction is suggested by the observation that the atypical isoform PKCγ requires PI3K activity in order to induce transcriptional activity from the tetradecanoyl-phorbol acetate (TPA) response element (Akimoto *et al.*, 1996). The full physiological relevance of these observations has not yet been demonstrated.

One very important effector target for the PPIs has been demonstrated to be Akt (PKB/Rac kinase; Klippel *et al.*, 1997; Franke *et al.*, 1997). Akt is a protein serine/threonine kinase which lies downstream of PI3K in signal transduction pathways activated by agonists such as platelet-derived growth factor (PDGF) (Burgering and Coffer, 1995; Franke *et al.*, 1995). Akt has been shown to be required for PI3K-dependent protection from apoptosis (Kennedy *et al.*, 1997; Kauffmann-Zeh *et al.*, 1996; Dudek *et al.*, 1997). Akt binds PI(3,4)P2 directly through its pleckstrin homology (PH) domain resulting in activation of the kinase (Franke *et al.*, 1997; Klippel *et al.*, 1997). This effect was found to be specific to PI(3,4)P2, as PI(3,4,5)P3 had no effect. Not only is Akt activated by PI(3,4)P2, but it is also activated by phosphorylation by another kinase, PtdIns(3,4,5)P3-dependent protein kinase-1 (PDK-1), which, as the name implies, is also activated by a PI3K-generated PPI (Alessi *et al.*, 1997; Franke *et al.*, 1997; Stephens *et al.*, 1998; Stokoe *et al.*, 1997). Thus, full Akt activation requires PI(3,4)P2 and PI(3,4,5) products, the first of which directly binds and activates PKB, and the latter of which binds and activates a PKB kinase, PDK-1.

PI3Ks signal by two independent mechanisms: protein kinase and lipid kinase activities.

The relative contribution of PI3K protein kinase activity and lipid kinase activity to downstream signaling is an important issue which has only recently been addressed in the literature. Studies which have looked at the requirement for

PI3K activity have used the pharmacological inhibitors wortmannin and/or LY294009 or overexpression of the catalytically inactive PI3K constructs, which interfere with PI3K function in a dominant manner. Each of these strategies block both the protein kinase and lipid kinase activities of PI3K (Carpenter *et al.*, 1993b; Dhand *et al.*, 1994; Stoyanova *et al.*, 1997). A recent study by Bondeva et al. has shed considerable light on the mechanisms of PI3K signaling in vivo (Bondeva *et al.*, 1998). They used hybrid PI3Ks engineered to separately study the protein kinase and lipid kinase activity of PI3Kγ. The PI3Kγ catalytic domain was replaced by the catalytic domains of PI3Ks from other classes, thereby changing both the lipid specificity and resultant lipid product of PI3Kγ while leaving the remaining domains intact. An additional hybrid was constructed which was unable to phosphorylate PPI and was thus unable to generate any lipid kinase products. Each of these hybrids retained full protein kinase activity. Their data demonstrate differential involvement of lipid kinase and protein kinase activities of PI3Kγ in the activation of MAPK and Akt. ERK activation by PI3Kγ expression was independent of PPI production, in that changing the identity of the lipid produced, or complete lack of phospholipid production was inconsequential to ERK activation. In contrast, expression of a PI3Kγ which lacked both protein kinase and lipid kinase activities was associated with a loss of ERK activation either in response to kinase overexpression or in response to LPA stimulation, indicating that it is the serine/threonine kinase activity of PI3Kγ which is crucial to ERK activation.

The authors also investigated the importance of Ras-association and membrane-localization of PI3Kγ. Expression of the PI3Kγ mutant PI3Kγ-Lys ^{270}-Glu (a mutation shown to inhibit Ras binding by 80%) actually activated ERK to a greater extent than wild-type PI3Kγ. Localization of PI3Kγ to the membrane by adding an isoprenylation signal actually inhibited ERK activation, due to PI3Kγ overexpression. These findings suggest that Ras association and membrane localization is not a major contributor to PI3Kγ's ability to activate ERK, in fact these data suggest a potential role for Ras as an inhibitor of PI3Kγ–induced ERK activation.

In contrast to ERK activation, Akt activation by PI3Kγ required the preservation of both wild-type lipid kinase and protein kinase activities. In addition, activation of Akt by PI3Kγ expression was only seen with a membrane- targeted PI3Kγ. This suggests that PI3K activities that are dependent upon either lipid access or interaction with other localized proteins, are necessary for Akt activation, as well as PI(3,4)P2 and PI(3,4,5)P3 production. These data clearly demonstrate a differential requirement for the lipid kinase activity and protein kinase activity of PI3Kγ in activating downstream effector molecules. In addition, there appears to be a differential requirement for membrane localization, as well.

GPCR signaling: requirement for PI3Kγ

PI3K is clearly a very early intermediate in GPCR signaling since it is activated by βγ-binding, but the subsequent downstream signaling events are not yet defined. The mechanism by which PI3Kγ mediates ERK activation is also not yet clear. It is possible that PI3Kγ may interact with and activate Ras, as PI3Kγ binds to Ras in vitro and in vivo (Rubio *et al.*, 1997), but activation of Ras by PI3Kγ has not been demonstrated. PI3Kγ was shown to mediate Gβγ-induced ERK activation in COS-7 cells in a manner which required both Shc and Src kinase activity, as well (Lopez-Ilasaca *et al.*, 1997). This effect appeared to depend on the cell type and/or method of stimulation, though, since Kranenburg et al. demonstrated that in both COS-7 cells and Rat-1s, LPA activation of ERK was PI3K-dependent but did not involve either Shc or Src activity (Kranenburg *et al.*, 1997). Thus, PI3K, like the other kinases involved in GPCR-signaling to ERK, can function in conjunction with other signaling molecules in a receptor-type and cell-type specific fashion.

It is clear that numerous mechanisms exist by which GPCRs can activate ERK. These mechanisms are likely to be both receptor-specific, and cell-type specific. A compelling question is whether the various mechanisms are outcome-specific, in terms of their biological consequences for the cell.

Is the CaR involved in calcium-regulated proliferation?

As described above, the proliferation of numerous cell types has been shown to be modulated by the concentration of extracellular calcium in vitro (Hennings *et al.*, 1980;

Soule *et al.*, 1990; Yuspa *et al.*, 1989; Black and Smith, 1989). It is of note that those cell types shown to proliferate in response to elevated calcium are of mesodermal origin, whereas those cell types which quiesce and/or differentiate in response to calcium elevations are epithelial and of ectodermal origin. Several of these calcium sensitive cell types, both of mesodermal and ectodermal origin, appear to express the G protein-coupled calcium-sensing receptor (CaR; Bikle *et al.*, 1996; Kallay *et al.*, 1997; McNeil *et al.*, 1998a; McNeil *et al.*, 1998b). Data from our laboratory have suggested a key role for the CaR in the calcium-sensitive proliferation of Rat-1 fibroblasts. It will be of interest to elucidate whether the CaR plays a role in the calcium-sensitive regulation of proliferation in cells from both categories of embryological derivation.

If the CaR plays a role in both the promotion and inhibition of growth in a cell type-specific fashion, it will be of interest to elucidate the mechanism by which one receptor can couple to such diverse biological outcomes. Several observations made with regard to GPCR signaling suggest possible mechanisms by which this may occur. GPCRs can couple to G protein subtypes in a cell type-specific manner, and it may be that the G protein subtype to which the CaR couples is cell type specific. In addition, I have presented in detail (illustrated in Figure 2) the diverse mechanisms by which GPCRs can couple to mitogenic pathways, especially the mitogen-activated protein kinase cascade, depending upon the receptor type studied or cell type in which it is studied. Thus biological outcome may also depend on which intermediate pathways are activated in a given cell type. These mechanisms may account for very different biological outcomes mediated by the CaR in response to elevated calcium.

The work in this thesis investigates the role of the CaR in the calcium-sensitive proliferation of ovarian surface epithelial cells (OSE), which, like Rat-1 fibroblasts, are of mesodermal derivation and express the CaR. Demonstrating that the CaR mediates calcium sensitive proliferation in OSE cells would suggest that this function of the CaR may be of generalized importance in calcium-responsive cell types of mesodermal derivation.

This thesis is also targeted at investigating the downstream signaling from the CaR. The downstream signaling events induced by mitogenic GPCRs are varied and complex, and depend upon the receptor and the cell type in which it they are expressed. Once we have identified the key signaling intermediates, we can then begin to make some comment on the general nature of CaR-induced intracellular signaling in cells that respond mitogenically to calcium. In addition, this information may provide clues to cell type-specific variations in CaR-mediated signaling which may result in very different biological outcomes, such as differentiation or quiescence.

Hypotheses to be tested:

1. The wild-type CaR is required for the full proliferative response of rat OSE cells to extracellular calcium.

2. In rat OSE cells, as in Rat-1 fibroblasts, Src is a required intermediary for both CaR-mediated ERK activation and resultant proliferation in response to calcium.

3. The proliferative response to extracellular calcium is mediated by a signal transduction pathway which consists of the CaR, Ras, Raf, MEK and ERK.

4. In a CaR-null background, wild-type CaR is both necessary and sufficient to convey activation of the MAP kinase cascade by extracellular calcium.

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PROLIFERATION OF OVARIAN SURFACE EPITHELIAL CELLS IN RESPONSE TO EXTRACELLULAR CALCIUM: ROLE OF CALCIUM-SENSING RECEPTOR, SRC, AND MAP KINASE

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ABSTRACT

Although ovarian surface epithelial cells are the cell type responsible for malignant ovarian carcinoma, relatively little is known about either the extracellular stimuli or intracellular signaling mechanisms responsible for regulating proliferation in these cells. We have previously demonstrated that ovarian surface epithelial (OSE) cells proliferate in response to elevation of extracellular calcium, and that OSE cells express functional calcium-sensing receptors (CaR) similar or identical to parathyroid and kidney calcium-sensing receptors. In this report we show that agonists of the calcium-sensing receptor increase the kinase activity of Src and ERK (extracellular signal regulated kinase) in rat OSE cells (ROSE cells). Calcium-induced tyrosine phosphorylation and ERK kinase activity were inhibited when a dominant negative mutant of the CaR was expressed in ROSE cells. Expression of an interfering dominant negative mutant CaR inhibited the proliferative response to elevated extracellular calcium, and inhibited CaR agonist-induced tyrosine phosphorylation and ERK activation. Disruption of the MAP kinase pathway subsequent to transfection with dominant negative mutant forms of Ras, Raf, and MKK1 also inhibited the increase in ERK kinase activity in response to agonists of the CaR, as did treatment with herbimycin, a selective inhibitor for Src family kinases. These results indicate that functional calcium-sensing receptors are required for the proliferative response of rat OSE cells to extracellular calcium, and implicate the Src kinases and Ras-dependent ERK activation as important mediators of proliferative signaling in OSE cells.

INTRODUCTION

Ovarian surface epithelial (OSE) cells represent an extension of the peritoneal mesothelium which covers the surface of the ovary as a simple cuboidal epithelium. Subjected to repeated wounding at each ovulation, these cells retain proliferative potential throughout adult life. This proliferative capacity may play an important role in the progression of these cells to malignant ovarian adenocarcinoma, as the number of ovulatory cycles experienced by a given woman is one of the major risk factors contributing to ovarian cancer (1, 2). Although some potential mitogens of OSE cells have been identified, including transforming growth factor β, GM-CSF, and lysophosphatidic acid (3, 4, 5, 6), relatively little is known about the downstream signal transduction mechanisms regulating the proliferative response of OSE cells to these mitogens. We have shown that human and rat OSE cells are sensitive to changes in extracellular calcium concentration (7). In this report we have investigated the signal transduction mechanisms linking increased extracellular calcium to increased proliferation in rat OSE cells (ROSE cells).

A novel mechanism mediating the effects of extracellular calcium on intracellular events has recently been elucidated through the cloning and characterization of a G-protein coupled, seven transmembrane domain receptor capable of specifically binding calcium, magnesium, barium and gadolinium (8, 9, 10). Activation of the CaR by calcium or other ligands results in the production of inositol trisphosphates and the release of intracellular calcium (8, 9, 10), presumably as a consequence of G-protein

mediated phospholipase C activation. In parathyroid cells, the release of intracellular calcium is associated with the inhibition of parathormone secretion (11).

Initially characterized as the calcium-sensing receptor of parathyroid cells responsible for modulating parathormone release in response to changes in plasma ionized calcium (12), the CaR is also expressed in kidney, brain, bone, gastric epithelial cells and fibroblasts (9, 10, 13, 14, 15, 16). We have shown that human OSE cells also express functional CaR, as demonstrated by both protein expression and induction of inositol trisphosphate production and intracellular calcium release in response to known agonists of the CaR (7). Although proliferative signaling from other G-protein coupled receptors has been shown to involve a variety of downstream events, including increased tyrosine phosphorylation, activation of phosphatidylinositol-3 kinase, association of Shc with receptor tyrosine kinases, and activation of the MAP kinase cascade (17, 18, 19, 20, 21), the mechanisms by which CaR activation may stimulate proliferative pathways in specialized cell types such as ovarian surface epithelium are not known. In this report we have specifically examined the effect of extracellular calcium on proliferation-associated signaling pathways in rat OSE cells, including the potential role of the MAP kinase cascade in mediating the proliferative effects of extracellular calcium. We have used a dominant negative mutant of the CaR, CaR-R796W, to disrupt CaR function and establish a mechanistic relationship between CaR activation and downstream proliferation-associated events in OSE cells. We have also used dominant negative mutant forms of Ras, Raf, and MKK1 to demonstrate that ERK activation in response to agonists of the CaR is dependent on both Ras and Raf. In

addition, we show that thymidine incorporation in response to extracellular calcium is inhibited by herbimycin and by the MKK1 inhibitor PD 98059, providing a potential link between CaR-dependent activation of ERK and Src and subsequent proliferative events.

RESULTS

Effect of the R796W mutant CaR on intracellular calcium release and thymidine incorporation in stably transfected ROSE cells.

The CaR mutant R796W, originally identified in a family with hereditary disorders of parathyroid function (22), has been shown function as an interfering mutant when co-expressed with wild type CaR in CHO cells and Rat 1 fibroblasts, inhibiting response to a variety of CaR agonists (16, 23). The ability of the CaR-R796W mutant to interfere with wild-type CaR function in ROSE cells was tested by comparing the dose response for both intracellular calcium release and proliferation in response to changes in extracellular calcium (Ca^{2+}_o) in ROSE cells stably transfected with either pcDNA3 alone, pcDNA3 expressing CaR-R796W, or pcDNA3 containing wild-type CaR sequences in reverse (anti-sense) orientation. As shown in Figure 1A, the control ROSE cells displayed a significant increase in intracellular calcium release when extracellular calcium was elevated to 2 mM to 5 mM; additional increases in intracellular calcium were observed with step-wise additions of Ca^{2+}_o to 10 mM and 30 mM. In contrast, ROSE cells expressing the R796W-CaR mutant failed to release intracellular calcium in response to challenges with 2, 5, or 10 mM; some transfected cells displayed a slight increase in intracellular calcium when Ca^{2+}_o was elevated to 30 mM. ROSE cells stably transfected with the reverse CaR showed a similar insensitivity to lower concentrations of extracellular calcium. Thus at calcium concentrations likely to be encountered in vivo (2 to 5 mM) the R796W-CaR would appear to be non-responsive, at least in terms of inducing intracellular calcium release.

In separate experiments, thymidine incorporation was measured as a function of extracellular calcium concentration in ROSE cells transfected with either the pcDNA3 vector, the R796W-mutant CaR, or the reverse CaR. ROSE cells expressing wild-type CaR showed a nearly 2-fold increase in thymidine incorporation when extracellular calcium was elevated from 0.05 mM to 0.3 mM (Figure 1B). A further increase was seen at 0.8 mM, with a plateau between 0.8 and 4.0 mM extracellular calcium. In contrast, the R796W-CaR and reverse CaR cells showed a significantly lower level of thymidine incorporation at all extracellular calcium concentrations; a 50-60% increase in thymidine incorporation was observed at 1.4 and 2.0 mM extracellular calcium. Similar results were obtained when cell proliferation was assessed using a MTT assay to estimate cell number (Figure 1C).

Effect of the R796W mutant CaR on protein tyrosine phosphorylation.

Certain G-protein coupled receptors, such as the thrombin, bombesin, muscarinic acetylcholine, and lysophosphatidic acid receptors, are known to stimulate increased cellular tyrosine phosphorylation in response to ligand binding (18, 19, 24, 25). We tested the possibility that activation of the CaR was also coupled to tyrosine phosphorylation pathways by stably transfecting rat OSE cells with either vector control DNA, wild-type CaR, R796W mutant CaR, or wild-type CaR DNA in a reversed orientation, and measuring tyrosine phosphorylation in response to the CaR agonist Gd^{3+}. Gd^{3+} was used in these experiments as a known specific agonist of the CaR which is incapable of influx through calcium channels (12, 28). As shown in Figure 2A, in vector control and wild-type CaR-transfected rat OSE cells, extracellular Gd^{3+} at 250

μM stimulated an increase in the tyrosine phosphorylation of at least three proteins of approximately 130, 68, and 62 kDa in size (lanes 6 and 7 compared to lanes 1 and 2). The increased tyrosine phosphorylation was not observed in cells transfected with either R796W-CaR, a 1:1 mix of wild-type CaR and R796W-CaR, or the reverse-orientation wild-type CaR (lanes 8, 9, and 10 compared to lanes 3, 4, and 5). This result supports the conclusion from Figure 1 that the R796W-CaR is non-functional, and also suggests that interference with the normal function of wild-type CaR by co-transfected R796W mutant CaR can block the ability of CaR agonists to stimulate tyrosine kinase activity. One likely intermediary linking activation of the CaR to increased tyrosine phosphorylation of multiple proteins is the cytoplasmic tyrosine kinase c-Src. Measurement of c-Src kinase activity in 0.05 mM Ca^{2+} and 5 min after transfer to 2 mM Ca^{2+} demonstrated a modest but statistically significant increase in c-Src kinase activity, as shown in Figure 2B. Although these data are correlative, they do suggest an association between activation of the CaR, increased tyrosine phosphorylation of some proteins, and increased Src activity.

Stimulation of ERK kinase activity in response to extracellular calcium. One of the most consistent events observed in response to proliferative stimuli is an increase in the activity of mitogen-activated protein kinases such as ERK1 and ERK2. In order to determine whether the proliferative response of OSE cells to extracellular calcium involved activation of the MAP kinase pathway, we examined the effect of elevated extracellular calcium on the activity on ERK1/2 in rat OSE cells. ROSE cells grown in medium containing 0.05 mM Ca^{2+} had low basal levels of ERK kinase activity, as

measured by either detection with phospho-specific antibodies (anti-phospho MAPK, NE Biolabs; Figure 3) or by the ability of immunoprecipitated ERK to phosphorylate GST-Elk *in vitro* (data not shown). Elevating extracellular Ca^{2+} to 2.0 mM produced a greater than 2-fold increase in ERK kinase activity in ROSE cells (Figure 3A). The ROSE cells transfected with the mutant CaR-R796W failed to increase ERK activity significantly in response to 2 mM Ca^{2+} (fold induction 1.2 $\pm$ 0.2; Figure 3B). Exposure to herbimycin, a tyrosine kinase inhibitor with selectivity for cytoplasmic tyrosine kinases, particularly c-Src (26), inhibited the ability of the wild type ROSE cells to increase ERK kinase activity in response to 2 mM calcium. Herbimycin treatment reduced the fold induction in comparison to 0.05 mM Ca^{2+}_o from 2.18 $\pm$ 0.11 to 0.65 $\pm$ 0.16 (n = 4, Figure 3A). This result suggests that c-Src, or a Src family tyrosine kinase, may be a component of the pathway leading to activation of ERK in response to agonists of the CaR. Overall, these results link the previously demonstrated proliferative effects of elevated extracellular calcium on OSE cells to increased ERK kinase activity, and furthermore suggest that functional CaR, potentially operating via a Src family intermediate, may be required for the activation of ERK by extracellular calcium.

Effect of dominant negative Ras, Raf and MKK1 on extracellular calcium-induced activation of ERK kinase. In order to determine which members of the well-characterized proliferation-associated pathway leading from Ras to Raf to the MAP kinase kinase MEK1/MKK1 were responsible for transducing signals from the CaR to ERK, we used transient transfection with appropriate interfering or dominant negative mutants to disrupt individual components of this pathway. The kinase activity of HA-

tagged ERK1 co-transfected with the interfering mutants was measured by *in vitro* kinase assays following immunoprecipitation with anti-HA antibodies; GST-Elk was used as the substrate. As shown in Figure 4, ROSE cells transfected with HA-ERK and the pcDNA3 expression vector displayed an approximately 2-fold increase in kinase activity in response to 2 mM Ca^{2+}. Co-expression of either N17-Ras, Raf301 or dnMKK1 inhibited the responses to 2 mM Ca^{2+} ($p \leq 0.05$, Figure 6). These results demonstrate that in this system, activation of ERK is dependent on both Ras and Raf. MKK1 is further implicated as a component of this pathway.

Effect of PD98059 and herbimycin on thymidine incorporation in response to extracellular calcium. If the observed increase in ERK kinase activity is mechanistically related to the proliferative effects of elevated extracellular Ca^{2+}, then treatments that inhibit ERK kinase activity should also reduce the proliferative response to extracellular Ca^{2+}. To test this possibility, the ability of 2 mM extracellular Ca^{2+} to stimulate thymidine incorporation in rat OSE cells was measured in the presence of the MKK1 kinase inhibitor PD98059 (27). In the presence of PD9805, thymidine incorporation was decreased approximately 50% – 60% compared to control cells (hatched bars compared to white bars, $p < 0.05$, Figure 5) in both low and high extracellular Ca^{2+}. PD98069 also decreased thymidine incorporation in response to polyarginine, another polyvalent cation that has been used as a specific agonist of the CaR (28). Treatment with herbimycin significantly inhibited thymidine incorporation at both 0.05 mM and 2 mM Ca^{2+}; herbimycin treatment prevented any increase in thymidine

incorporation in response to 2 mM Ca^{2+}, even in comparison to the reduced thymidine incorporation seen in herbimycin-treated cells at 0.05 mM Ca^{2+} (Figure 5, dark bars).

DISCUSSION

Ovarian surface epithelial cells are exposed to repeated bouts of proliferative stimuli *in vivo*, as each round of ovulation can be viewed as a wounding and subsequent healing of the surface epithelium. Peptide growth factors present in the follicular fluid, including TGFα and G-CSF, are thought to act as paracrine growth factors for the OSE, as normal OSE express the appropriate receptors and at least some ovarian tumor cell lines can be demonstrated to proliferate in response to these factors (3, 29, 30). We have shown in this and in a prior report (7) that normal, untransformed ovarian surface epithelial cells modulate their rate of proliferation in response to changes in extracellular calcium concentration, with human and rat OSE showing decreased thymidine incorporation at calcium concentrations below 0.8 to 1.4 mM. For human OSE, the transition calcium concentration correlates well with the difference between plasma ionized free calcium (1.1 – 1.3 mM, ref 31) and the reported value of 1.8 to 2.0 mM in follicular fluid immediately prior to ovulation (32). Thus it is conceivable that an increase in the immediate concentration of ionized calcium may be one of several proliferative stimuli prompting the process of wound repair in the ovarian surface epithelium.

In this report we have investigated the potential role of the CaR in mediating the proliferative effects of increased extracellular calcium on rat OSE cells. The loss of

proliferative responses in ROSE cells stably expressing either the interfering CaR mutant R796W or a reverse orientation (antisense) CaR strongly implicates functional CaR as playing an important role in transducing this response. The usefulness of the R796W-CaR and reverse CaR in demonstrating a requirement for normal CaR function is supported by the ability of these expression constructs to inhibit the release of calcium from intracellular stores in response to increasing extracellular calcium. The dose response to extracellular calcium for both intracellular calcium release and thymidine incorporation was similarly affected by expression of the R796W-CaR.

Measurement of downstream effector responses to increased extracellular calcium in wild type ROSE cells stimulated with 2 mM Ca^{2+}_o or 250 μM Gd^{3+} demonstrated an increase in tyrosine phosphorylation of specific proteins, Src kinase activity, and ERK kinase activity in response to both agonists of the CaR. Each of these responses is significantly blunted in ROSE cells expressing the R796W mutant CaR, further implicating functional CaR as an essential component of Ca^{2+}_o-sensitive signaling pathways in ROSE cells. Transfection of wild-type ROSE cells with interfering mutants for Ras, Raf, and MKK1 inhibited the induction of ERK activity in response to extracellular Ca^{2+}_o , suggesting that activation of ERKs in response to extracellular calcium is dependent on both Ras and Raf in ROSE cells. To our knowledge this is the first demonstration of Ras-, Raf- and MEK-dependent ERK activation in OSE cells. The importance of this pathway in sustaining basal rates of proliferation in ROSE cells is demonstrated by the substantial decrease in thymidine incorporation observed in quiescent ROSE cells treated with the MKK1/2 inhibitor PD 98059 under low calcium

conditions. PD98059 also blunted the response to 2 mM Ca^{2+}_o and the CaR agonist polyarginine, suggesting that this pathway also plays a role in the response to extracellular calcium. Although there was a net increase in thymidine incorporation when ROSE cells were stimulated with 2 mM Ca^{2+}_o or polyarginine in the presence of PD 98059, this response may reflect the limitations of using PD 98059 to inhibit events downstream of MAP kinase kinase. In the original description of PD 98059's effects on MAP kinase kinase, it was shown that certain mitogens, notably EGF and TPA, were able to induce a significant increase in p42 MAPK (ERK2) kinase activity in the presence of PD 98059, even though MAP kinase kinase activity had been reduced by over 85% (27). Thus the observation of a reduced but not abolished proliferative response to extracellular calcium in the presence of PD 98059 may reflect either a partial inhibition of MAP kinase activity under these conditions or the possibility of additional signaling pathways contributing to ERK activation and/or proliferation.

The ability of herbimycin to completely inhibit both ERK activation and thymidine incorporation in response to 2 mM Ca suggests that c-Src, or another Src family tyrosine kinase, may link CaR activation to the MAP kinase cascade and increased proliferation. Src family tyrosine kinases have been shown to be involved in downstream signaling from several GPCRs linked to ERK activation (18 – 21, 36). Although herbimycin has selectivity towards the cytoplasmic tyrosine kinases (26), due to the possibility that other kinases may be inhibited at the dose of herbimycin used, the current study only implicates the involvement of Src family tyrosine kinases. Further experiments, such as the over-expression of the c-Src terminal kinase (Csk), a physiological inhibitor of Src

family kinases, would be required to establish a mechanistic role for Src kinases in CaR downstream signaling. However, the present data are in agreement with our previous report in Rat-1 fibroblasts demonstrating that agonists of the CaR activate c-Src kinase, and that herbimycin inhibited both ERK activation and increased proliferation (16). Taken together, these findings implicate the Src family tyrosine kinases as relevant mediators of CaR-dependent signaling in cells which proliferate in response to extracellular calcium.

In conclusion, the ability of the R796W mutant CaR to inhibit multiple responses observed when normal ROSE are transitioned from low to high extracellular calcium provides a consistent set of associated data linking CaR activation to changes in tyrosine phosphorylation, ERK activity, and proliferation. Use of interfering mutants for Ras, Raf, and MKK1 suggests that ERK activation is mediated by a Ras-dependent, Raf-dependent MAP kinase cascade. Evidence implicating the CaR-dependent activation of Src and ERK in the proliferative response of ROSE cells to extracellular calcium is provided by the observed correlations between ERK kinase activity and thymidine incorporation in response to herbimycin and PD 98059. Our data strongly implicate the CaR as playing a major role in calcium-dependent proliferation of ROSE cells.

MATERIALS AND METHODS

Cell culture: Rat OSE (ROSE) cells obtained from N. Auersperg (UBC, Vancouver Canada) were cultured in Dulbecco's Modified Eagle's Medium (1.7 mM Ca^{2+}) plus 10%

calf serum (Atlanta Biologicals) at 37 C in 5% CO_2 / 95% air essentially as described in Adams et al (38). When media of defined calcium concentration were used, commercially obtained calcium-free DMEM (Life Technologies, Inc, Gaithersburg MD) was used as the starting medium, and calcium concentration was brought to the desired level by addition of sterile calcium chloride. Cells were not cultured in less than 0.05 mM $CaCl_2$.

Construction of wild-type and mutant CaR expression vectors.

pcDNA3-CaR (CaR): The full-length 3.7kb rat kidney CaR clone was excised from pCIS:CaR using XbaI-BamHI (10). This was subcloned into pBluescript II (pBS II) for ease of manipulations. The full length 3.7kb CaR from the pBS:CaR construct was excised with XbaI - XhoI and subcloned into the pcDNA3 mammalian expression vector.

pcDNA3-CaR:R796W (R796W): The R796W mutation was introduced into a 1.2 kb SphI fragment of the CaR by inverse PCR, using the oligomer pair: J5: TTG AAG GCA AAG AAG AAG CAG ATG G and J3: GTC CTG GAA GTT ACC CGA GAA CTT C. Primer J5 is identical to the published rat CaR sequence. Primer J3 introduces the arginine to tryptophan mutation at aa 796 and adds a diagnostic Ava I site. Positive colonies identified by Ava I digests were sequenced and subcloned into the pBS:CaR construct. Correct orientation was verified by Ava I digests. The full-length mutant CaR -R796W was subcloned into pcDNA3.

Transfections. Rat OSE (ROSE) cells were transfected with either pcDNA3 alone, pcDNA3-CaR, or pcDNA3-CaR-R796W using Lipofectin as previously described (39). Three independent clonal isolates wee selected from each transfection, and tested for release of intracellular calcium in response to increasing amounts of extracellular calcium, essentially as described in Figure 1. A representative clone for each different transfection vector was selected and used in subsequent experiments.

Cells were initially selected in media containing 700 μg/ml G418 (Bio-Whitaker) and stable clones were cultured in 300 μg/ml G418 to maintain selection. New cultures were started from frozen stocks every 6-8 weeks.

The pcDNA3-HA_3-ERK2 expression vector was a kind gift of D. Cohen (Portland VAMC; ref 40). The dominant MKK1 mutant was obtained from E. Krebs (41), the N17-Ras was obtained from G. Cooper (42) , and the Raf-301 mutant was obtained from U. Rapp (43, 44).

Kinase assays. *In vitro* kinase assays were conducted on immunoprecipitates as previously described (45). Confluent ROSE cells in 10 cm plates were cultured in serum-free F12 (0.3 mM Ca^{2+}) for 16 h, followed by 4 h in 0.05 mM Ca^{2+}. Cells were stimulated with the indicated concentration of Ca^{2+} or Gd^{3+} for 10 min prior to lysis in 750 μl M-TG lysis buffer [20 mM HEPES, pH 8.0, 1% Triton X-100, 10% glycerol, 2 mM Na_3VO_4, 150 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin]. Lysates were cleared by addition of 10 μl protein A agarose and centrifugation. Aliquots of cleared lysates normalized for protein content were subjected to immunoprecipitation for 1 h on ice using either anti-ERK-2 (SantaCruz Biotechnology, Santa Cruz, CA), anti-c-SRC (Upstate Biotechnology Inc, Lake Placid NY), or anti-HA (12CA5) antisera followed by addition of protein A agarose and an additional 2 h incubation at 4 C. Immunoprecipitates were recovered by centrifugation and washed once in M-TG lysis buffer, once in LiCl buffer [500 mM LiCl, 100 mM Tris HCl, pH 7.6, 0.1% Triton X-100, 1 mM DTT, 1 mM vanadate, 0.4 mM PMSF] and once in MOPS assay buffer [20 mM MOPS, pH 7.2, 20 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT, 0.2% Triton X-100]. The pellets were resuspended in 20 μl kinase assay buffer [10 mM MOPS, pH 7.2, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1% Triton X-100, 1 μCi ^{32}P-γ-ATP] and incubated at 30 C for 30 min. In kinase assays with endogenous ERK or HA-ERK, 3 μg GST-Elk1 was added as a substrate for phosphorylation. Phosphorylated proteins were resolved by SDS-PAGE in 12% acrylamide gels. Proteins were

electrophoretically transferred to PVDF membranes (Millipore) and radioactivity visualized and quantified with a Molecular Dynamics PhosphorImager and IP LabGel software. The membranes were then stripped and immunoblotted with anti-ERK1/2 antibody (Santa Cruz) to verify equal loading of protein. Src kinase assays measured phosphorylation of a synthetic peptide substrate (PTK Biotinylated Peptide Substrate 2, Promega, Madison WI), essentially as described by the manufacturer.

Incorporation of ^{3}H-thymidine. ROSE cells in 12-well plates were grown to 70 - 80% confluence in DMEM + 10% CS. Cells were then changed into serum-free Hams F-12 medium (0.3 mM Ca^{2+}) for 16 h. This medium was replaced with defined calcium media, as described in Cell Culture. ^{3}H-thymidine (1 μCi/ml) was added 18 h later, and cells harvested after 4 h thymidine incorporation. Thymidine incorporation was determined by precipitation in 10% trichloroacetic acid, solubilization in 0.2 M NaOH and liquid scintillation counting.

Immunoblotting. Proteins were size fractionated by SDS-PAGE and transferred to PVDF membrane (Immobilon P, Millipore) by electroblotting. Membranes were blocked in TTBS (0.05% Tween 20, 20 mM Tris pH 7.5, 150 mM NaCl) with 3% BSA, 0.05% NaN_3 for 1 hour at room temperature followed by overnight incubation with primary antibody at 4° C in TTBS. Membranes were washed 3 times in TTBS, incubated with appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for at least 2 hours and washed extensively in TTBS. Bands were visualized by chemiluminescence (Renaissance, DuPont NEN, Boston MA). Quantification of chemiluminescence signals was achieved using a Lumi-Imager and LumiAnalyst 2.1 software (Boehringer-Mannheim, Indianapolis IN).

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Figure 1A

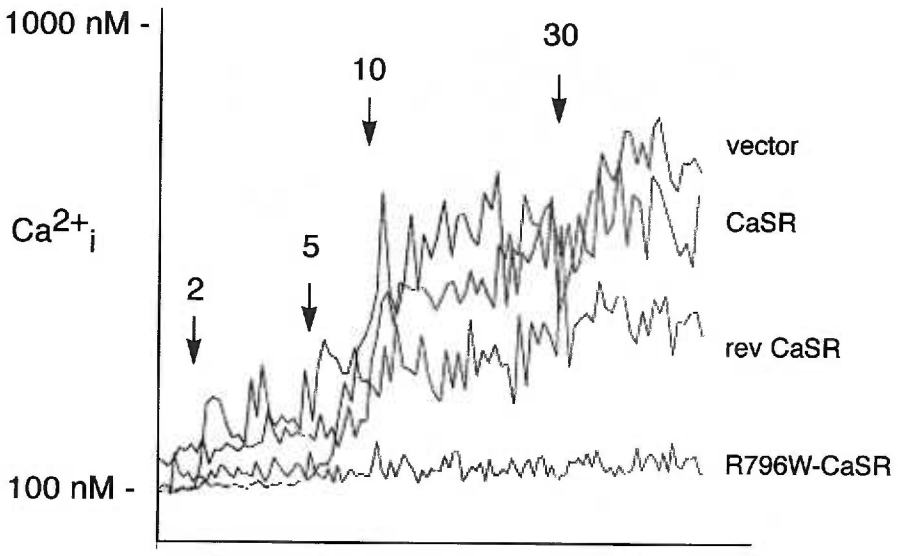
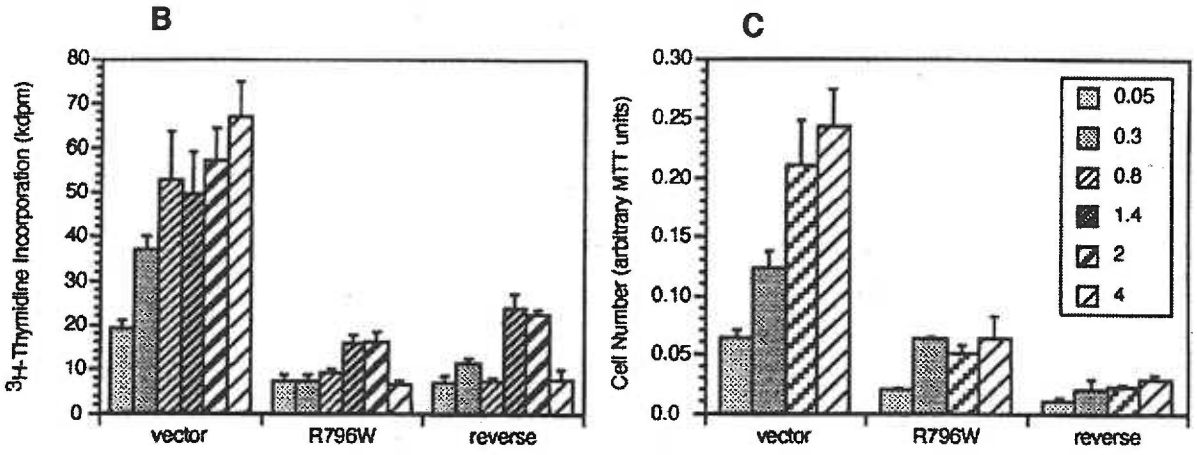


Figure 1


B
C
^{3}H-Thymidine Incorporation (kdpm)
Cell Number (arbitrary MTT units)
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0.3
0.8
1.4
2
4
vector
R796W
reverse
vector
R796W
reverse

Figure 2:

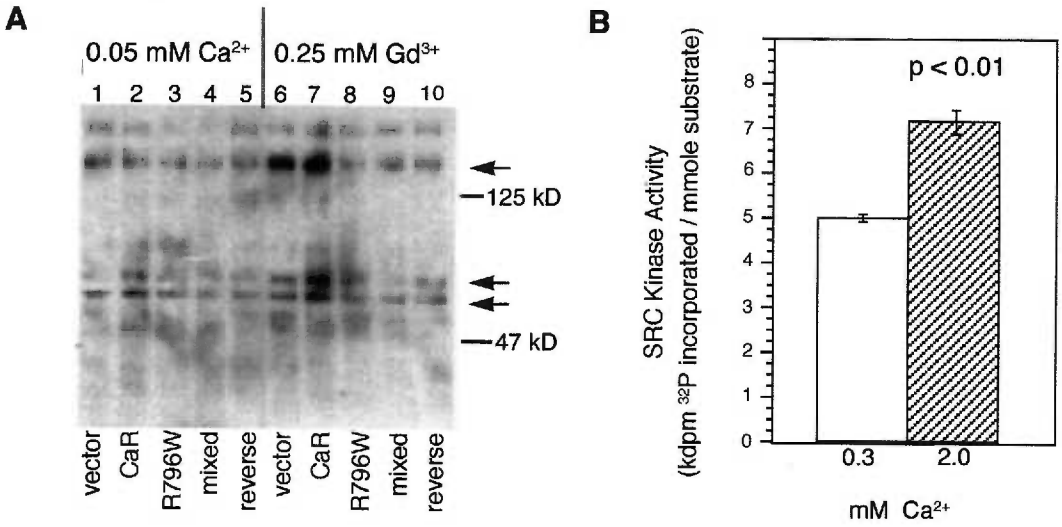
A
0.05 mM Ca2+
0.25 mM Gd3+
1 2 3 4 5 6 7 8 9 10
125 kD
47 kD
vector
CaR
R796W
mixed
reverse
vector
CaR
R796W
mixed
reverse
B
SRC Kinase Activity
(kdpm 32P incorporated / mmole substrate)
p < 0.01
0
1
2
3
4
5
6
7
8
0.3
2.0
mM Ca2+

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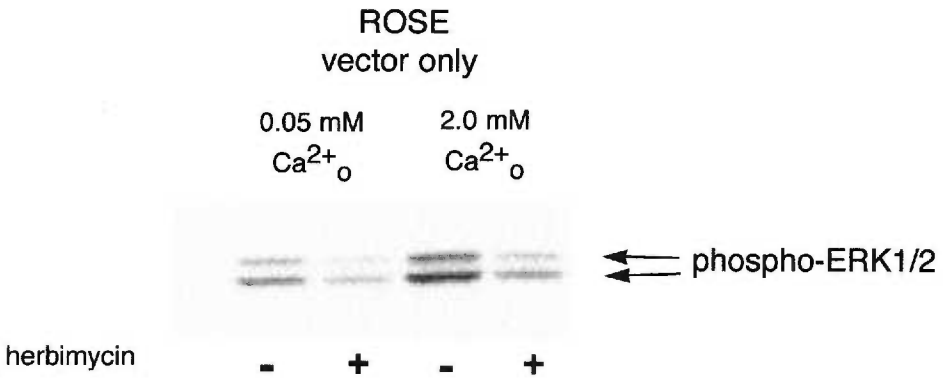
Figure 3

A:



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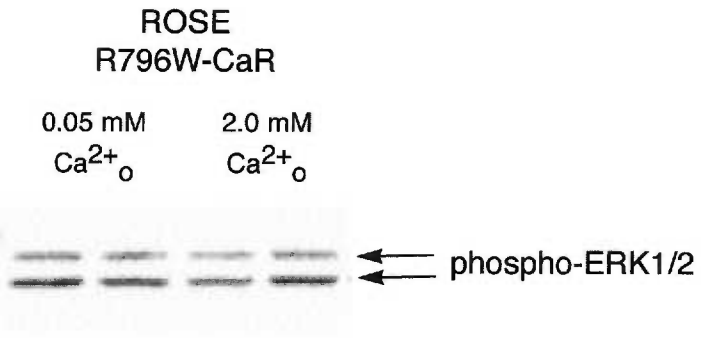

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Figure 4

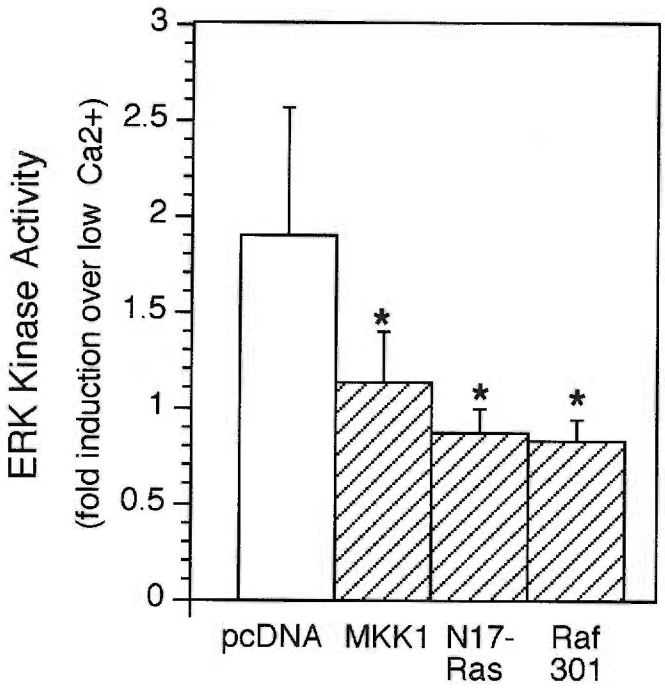

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Figure 5

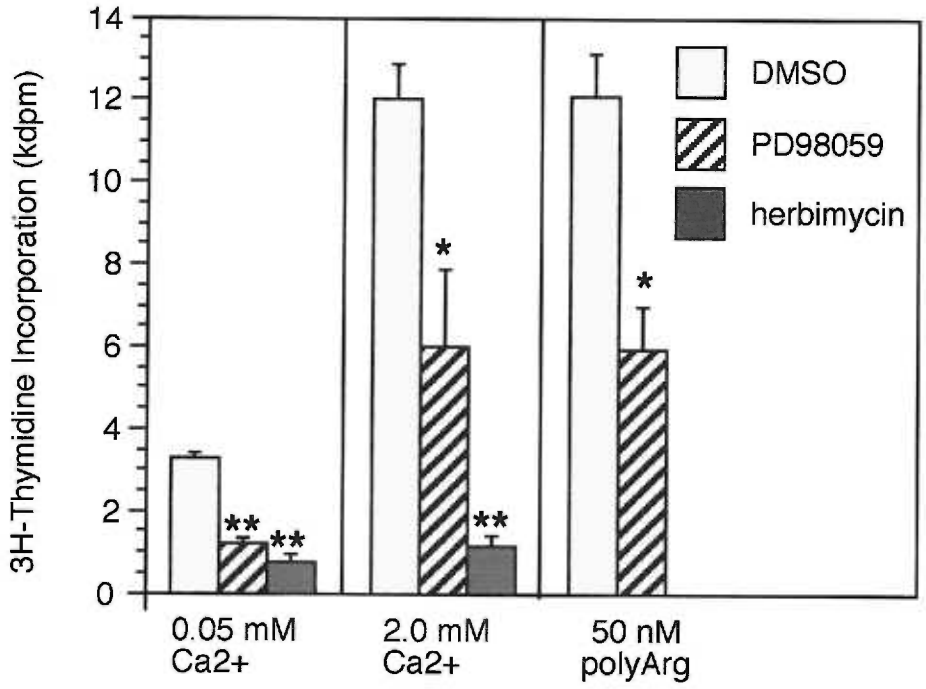

FIGURE LEGENDS

Figure 1: Dose response for extracellular calcium effects on calcium release and proliferation in ROSE cells expressing wild type and mutant CaR.

Panel A: Fura-2 measurements of intracellular calcium concentration in ROSE cells transfected with either pcDNA3 vector only, pcDNA3-CaR, pcDNA3-R796W-CaR, or pcDNA3-reverse CaR. Cells were loaded with Fura-2-AM and de-esterified in Ca^{2+}-free HBSS as described in Methods. Boluses of $CaCl_2$ were added at the times indicated to produce the indicated Ca^{2+}_o. Traces represent the average of 15 cells per field in a single representative experiment with each transfection. Similar results were obtained in three replicate experiments.

Panel B: Thymidine incorporation as a function of Ca^{2+}_o in ROSE cells transfected with pcDNA3 vector only, pcDNA3-R796W-CaR, or pcDNA3 reverse CaR. Thymidine incorporation was measured as described in Methods and results are shown as mean $\pm$ SD, n = 4. Similar results were obtained in three replicate experiments.

Panel C: Proliferation as a function of Ca^{2+}_o in ROSE cells transfected with pcDNA3 vector only, pcDNA3-R796W-CaR, or pcDNA3 reverse CaR. Cells were plated at 10^5 per 14 mm well as described in Methods. Proliferation was determined after 4 days in culture at the indicated calcium concentration using an MTT assay as described in Methods. Results are presented as mean $\pm$ SD, n = 4. Similar results were obtained in three replicate experiments.

Figure 2: Changes in protein tyrosine phosphorylation in response to agonists of the CaR. Panel A: Rat OSE cells were stably transfected with either pcDNA (vector), pcDNA-

CaR (CaR), pcDNA-CaR-R796W (R796W), a 1:1 mixture of pcDNA-CaR and pcDNA-CaR-R796W (mixed) or pcDNA-reverse CaR (reverse) and selected in 300 μg/ml G418. Selected cells were grown to confluence in DMEM (1.7 mM Ca^{2+}), then cultured in low calcium (50 μM) conditions for 4 h prior to harvesting. Parallel plates were treated with 250 μM Gd^{3+} for 5 min prior to lysis, as indicated in the right-hand lanes. Total cell lysates equalized for protein amount were size-fractionated by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The molecular weight in kDa of protein standards is indicated. Three bands which show increased tyrosine phosphorylation in response Gd^{3+} are indicated by arrows; the approximate sizes are 130 kDa, 68 kDa and 62 kDa. Coomassie staining of the membrane indicated that equivalent amounts of protein were loaded in each lane (data not shown). Panel B: Activation of c-Src in response to Gd^{3+}. Confluent plates of ROSE cells were rendered quiescent in low calcium (0.05 mM) as described in Methods. Cells were exposed to 2 mM Ca^{2+} for 5 min then immediately lysed. Src kinase activity was assayed as phosphorylation of a synthetic peptide substrate as described in Methods. Results are presented as dpm ^{32}P incorporated, mean ± SD, from three independent kinase reactions using lysates from independent plates. Similar results were obtained in replicate experiments.

Figure 3: Increased ERK2 Kinase Activity in Response to Activation of the CaR. Panel A: ROSE cells were grown in serum-free medium containing 0.05 mM Ca^{2+} for 4 h, then exposed to either 2 mM Ca^{2+} for 12 min before lysis. Whole cell lysates representing equivalent amounts of protein (200 μg) were subjected to SDS-PAGE and

immunoblotting with anti-phospho MAPK antibodies as described in Methods. Herbimycin (500 ng/ml) or DMSO as a vehicular control was added 4 h prior to stimulation where indicated. Membranes were stripped and immunoblotted with anti-ERK1/2 (Santa Cruz) to verify equivalent loading (data not shown). Panel B: ROSE cells stably transfected with expression vectors for the R796W mutant CaR as described in Methods were stimulated and processed as described in Panel A.

Figure 4: Effect of dominant negative Ras, Raf and MKK1 on ERK activation in response to agonists of the CaR. ROSE cells were co-transfected with HA-ERK and either pcDNA3, N17-Ras, Raf 301, or dn MKK1 as described in Methods. Cells were allowed to recover overnight, serum starved in 0.3 mM Ca^{2+} media overnight, then rendered quiescent in low calcium (0.05 mM) Ca^{2+}_o for 4 h. Cells were harvested after a 10 min exposure to 2 mM Ca^{2+}_o. HA-ERK was immunoprecipitated with anti-HA antibody, and the immunoprecipitates used in an *in vitro* kinase assay as described in Methods. Membranes were stripped and immunoblotted with anti-ERK1/2, and the phosphorylation of GST-Elk normalized to the amount of HA-ERK2 in each lane. Results are presented as the average fold-induction obtained in 4 independent experiments, $\pm$ SEM. Significant differences ($p < 0.05$) compared to the pcDNA3 controls are indicated with an asterisk.

Figure 5: Effect of PD98059 and herbimycin on thymidine incorporation in response to extracellular Ca^{2+}. ROSE cells at 70% confluence in 18 mm wells were rendered quiescent by growth in serum-free F12 (0.3 mM Ca^{2+}) for 24 h. Cells were changed into

fresh serum-free medium containing either 0.3 mM Ca^{2+} (open bars) or 2.0 mM Ca^{2+} (hatched bars) in the presence of either 0.5% DMSO (control), 200 ng/ml herbimycin (herbimycin) or 20 μM PD98059 (PD98059) for 18 h prior to addition of ^{3}H-thymidine (1 μCi/ml). ^{3}H-thymidine incorporation was measured after 4 h as described in McNeil et al (7). P values between paired treatments at the same Ca^{2+} concentration, plus or minus inhibitors, are indicated by asterisks; * = $P \leq 0.05$, ** = $P \leq 0.01$.

THE CALCIUM-SENSING RECEPTOR ACTIVATES THE MITOGEN ACTIVATED PROTEIN KINASE CASCADE IN A PHOSPHATIDYLINOSITOL-3-KINASE DEPENDENT MANNER IN HEK CELLS

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ABSTRACT

Extracellular calcium is a known modulator of proliferation and/or differentiation in numerous cell types such as keratinocytes, breast epithelial cells, ovarian surface epithelial cells, and both human and Rat-1 fibroblasts. Interestingly, many of these cell types have been shown to express the calcium activated, G-protein coupled Calcium-sensing Receptor CaR (CaR). The CaR has been strongly implicated as playing a central role in the proliferative response of Rat-1 fibroblasts and ovarian surface epithelial cells to calcium; expression of the non-functional CaR-R796W mutant inhibited both thymidine incorporation and activation of the proliferation associated extracellular-regulated kinase (ERK) in response to calcium. Here we utilize CaR-transfected HEK293 cells to demonstrate that functional CaR is required for calcium-induced ERK activation and to delineate the signaling pathway by which this receptor activates ERK in response to calcium. Further, we show that the CaR activates ERK in a Ras-dependent fashion in HEK293 cells, as ERK activation was blocked by the co-expression of the Ras dominant-negative mutant, Ras N17. Importantly, while Src kinase and SHC are implicated in CaR-mediated ERK activation in Rat-1 fibroblasts, we could find no evidence for the involvement of Src kinase activity, SHC tyrosine-phosphorylation, or SHC/Grb2 association in CaR-mediated ERK activation in the HEK293 system. Instead, our data strongly implicates phosphatidylinositol-3-kinase (PI3K) as a major intermediate in CaR-mediated ERK activation, as demonstrated by the use of the PI3K inhibitors wortmannin and LY294002. Thus, the CaR is sufficient for ERK activation in response to extracellular calcium and mediates ERK-activation via a Ras-dependent mechanism. We also provide strong evidence that PI3K activity is required for the full activation of ERK by the CaR, while Src and Shc are not. This report, taken together with our previously published work in Rat-1 fibroblasts, demonstrates the

existence of multiple distinct pathways linking the CaR to ERK activation. The usage of one or more distinct pathways appears to vary in a cell-type specific manner, suggesting a potential mechanism by which activation of the CaR could couple to distinct calcium-dependent cellular responses.

INTRODUCTION

Extracellular calcium is known to have profound effects on the proliferation and differentiation of several cell types. Changes in extracellular calcium concentration are capable of modulating both the differentiation and proliferation status of keratinocytes, breast epithelial cells, and intestinal epithelial cells (1-4), which proliferate maximally in media with a very low calcium concentration (approximately 0.05 mM). The proliferation status of human dermal fibroblasts, Rat-1 fibroblasts, and the specialized mesothelial cells covering the ovary - the ovarian surface epithelium (OSE cells), is also modulated by calcium (5-7). In these cells, proliferation is maximal at a higher calcium concentration (1.8 mM CaCl2), that found in standard Dulbecco's Modified Eagles Medium. Reducing calcium below a threshold of 1.4 mM results in growth inhibition (5). Calcium can even act as a mitogen in these cells: addition of calcium to fibroblasts made quiescent by serum-starvation in low-calcium media results in proliferation equal to that of cells stimulated with EGF, as measured by thymidine incorporation (5,7). The mechanism by which calcium regulates these proliferation-associated changes is incompletely understood.

Recently, a Ca^{2+}-activated G-protein coupled receptor, the Calcium-sensing Receptor (CaR), has been described (8). The CaR, by amino-acid identity and structural homology, is most closely related to the G-protein coupled metabotropic glutamate receptor. Although originally identified in parathyroid cells, numerous tissues other than the parathyroid gland have been shown to express the full-length CaR, CaR mRNA and/or CaR protein. These include cell types which demonstrate calcium-sensitive proliferation such as keratinocytes, intestinal epithelial cells, OSE cells and fibroblasts (6, 7, 9, 10). The expression of the CaR in cell types whose proliferation is calcium-sensitive suggests the possibility that the CaR may play a role in calcium-induced

proliferation and/or differentiation. In support of this hypothesis, we have demonstrated that calcium-induced thymidine incorporation in both Rat-1 fibroblasts (7) and OSE cells (6) is inhibited by the expression of the CaR mutant CaR-R796W, which is capable of interfering with normal CaR function (11). This strongly supports a role for the CaR in the calcium-induced proliferation of these two cell types.

Many G protein-coupled receptors (GPCRs) and their ligands have been implicated in cellular growth, differentiation, and oncogenesis (12). The majority of GPCRs linked to proliferation activate the classic proliferation-associated, mitogen-activated protein kinase (MAPK) cascade and the extracellular regulated kinases, ERK1/2, but the mechanism by which they do so varies (13-18). In general, GPCR-induced ERK activation is either Ras-dependent and PKC-independent (19-23), or PKC-dependent and Ras-independent (24, 25). The signaling events proximal to the receptor can differ as well, with variable involvement of signaling intermediaries such as the SHC/Grb2/SOS module (26-28), the Src kinase family kinases (27, 29-31), phosphatidylinositol-3-kinase (PI3K) (32-35), and the receptor tyrosine kinases such as the EGF receptor and the PDGF receptor (36-40). Additionally, in specialized cell types, the calcium sensitive kinase PYK-2 (also designated CADTK or RAFTK) has been implicated (27, 39). Several groups have demonstrated transactivation of receptor tyrosine kinases in response to GPCR agonists. This transactivation has shown to be required for downstream signaling in some systems. Transactivated RTKs include the EGF receptor (36-39) and the PDGF receptor (40). Thus, there are numerous proximal signaling mechanisms involved in the cross-talk between GPCR signaling and the MAPK cascade. The physiological significance of these variations has not yet been determined.

In a prior report, we demonstrated that CaR-agonists activate the MAPK cascade in Rat-1 fibroblasts in a Src kinase dependent manner (7). Moreover, ERK activation was abrogated by expression of the CaR-R796W mutant (7), demonstrating specificity of

this ERK activation to the CaR. The finding that CaR-R796W expression inhibits both calcium-induced ERK activation and calcium-induced proliferation in these cell-types strongly implicates the CaR as a central player in these calcium-sensitive responses. However, interpretation of these results is complicated by the presence of the endogenous receptor.

In this report, we express the CaR in HEK 293 cells, which are characterized as null for the CaR (41, 42). This model system allows rigorous testing of the specificity of ERK activation by extracellular calcium, and simplifies investigation of downstream signaling events. We demonstrate that calcium-induced ERK activation requires expression of functional CaR in HEK293 cells, as calcium could not induce ERK activation in wild-type cells or those transfected with the non-functional CaR-R796W mutant. This CaR-mediated ERK activation is abrogated by expression of the Ras dominant-negative mutant Ras N-17, thus demonstrating that the CaR activates ERK in a Ras-dependent manner in HEK293 cells. The down-regulation of phorbol-sensitive PKC isoforms with phorbol pretreatment does not inhibit CaR-mediated ERK activation, demonstrating that PKC is not of major importance in this pathway. Interestingly, unlike the Rat-1 fibroblast system, we found no evidence for the involvement of Src kinase activity or SHC tyrosine phosphorylation in the CaR-mediated ERK activation in HEK293 cells. In contrast, we present evidence implicating PI3K as a major component of CaR-mediated ERK activation in HEK293 cells.

MATERIALS AND METHODS

Cell culture and transfection: HEK293 cells (American Type Culture Collection) were grown in Dulbecco's Modified Eagle's medium (DMEM; 1.8mM Ca^{2+}; Gibco) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) at 37 C in 5% CO2/95% air. New thaws were started every six weeks. HEK293 cells were transiently transfected with the indicated constructs via electroporation, and then plated in DMEM supplemented with 10% FBS and allowed to recover for 24 hours. The cells were then made quiescent by culture in serum-free Hamm's F12 (0.3 mM Ca^{2+}; Gibco BRL) for 24 hours before stimulation with agonists as indicated in figure legends.

Western Blotting and Immunoprecipitation: After stimulation, the media was completely removed and the cells were lysed in ice-cold M-TG lysis buffer [20 mM HEPES, pH 8.0, 1% Triton X-100, 10% glycerol, 150 mM NaCl]. (Immediately before use, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% aprotinin were added to M-TG or other buffers as appropriate.) Lysates were cleared by centrifugation and assayed for protein concentration using the Bradford assay (BioRad, Hercules, CA). Lysates were aliquoted to contain equivalent amounts of protein and denatured in Laemmli sample buffer prior to resolution by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to PVDF (Immobilon) membranes and immunoblotted by incubation with the indicated antibodies. For immunoprecipitation, lysates normalized for protein were incubated on ice for one hour with the antibody indicated in the figure legends. Protein A/G+ agarose beads (Santa Cruz Biotechnology) were added and incubated at 4 C with agitation for 45 minutes. Immune complexes were collected by centrifugation, washed three times with M-TG lysis buffer containing inhibitors and then denatured in Laemmeli sample buffer. Following resolution by SDS-

PAGE and transfer to PVDF membranes, immunoblots were performed by incubation with the indicated antibodies, followed by incubation with the appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). Detection and quantification were performed using Enhanced ChemiLuminescence reagent (Renaissance, Dupont NEN, Boston, MA) and the Lumi-Imager and LumiAnalyst 2.1 software (Boerringer Manheim, Indianapolis, IN).

Kinase assay: *In vitro* kinase assays were conducted as previously described (7). Anti-HA (12CA5) immune complexes were obtained as above, and then washed once with M-TG lysis buffer, once with LiCl Buffer [500 mM LiCl, 100 mM Tris HCl, pH 7.6, 0.1% Triton X-100, 1 mM DTT], and once with MOPS assay buffer [20 mM MOPS, pH 7.2, 20 mM MgCl2, 2 mM EGTA, 2 mM DTT, 0.1% Triton X-100]. The pellets were resuspended in 20 μl kinase assay buffer [MOPS assay buffer with 1 μCi ^{32}P-γ-ATP and 3 μg GST-ELK as substrate] and incubated at 30 C for 30 minutes. Kinase assays were stopped with the addition of 2X Laemmeli buffer. Phosphorylated proteins were resolved by SDS-PAGE, transferred to PVDF, and phosphorylated GST-Elk visualized and quantified using the Molecular Dynamics PhosphorImager and IP LabGel software. Membranes were then immunoblotted with anti-ERK2 antibody (Santa Cruz Biotechnology) to verify equal loading of protein.

RESULTS:

Functional CaR are required for calcium induced ERK activation in HEK293 cells. In prior reports, we have shown that in Rat-1 fibroblasts, which endogenously express the CaR, stimulation with 2.0 mM calcium induces ERK kinase activation (7). Although extracellular calcium is capable of inducing cellular responses by multiple mechanisms, CaR involvement was strongly implicated by the inhibition of calcium-induced ERK activation in the presence of exogenously expressed non-functional mutant CaR-R796W. In order to rigorously test the role of the CaR in calcium-induced ERK activation, we transiently expressed the CaR in HEK293 cells, as these cells have previously been described as lacking endogenous CaR (41, 42). As shown in Figure 1A, wild-type HEK293 cells show no increase in ERK activity when shifted from low to high calcium. In contrast, HEK293 cells transfected with wild-type (but not mutant) CaR displayed a significant increase in ERK activity when shifted to high calcium. (Expression of the CaR-R796W mutant was verified by immunocytochemistry – data not shown.) The fold-induction of ERK phosphorylation in response to calcium was consistently approximately 40% of the fold-induction in response to EGF, as shown in Figure 1B. These results demonstrate that the CaR is sufficient for calcium-induced activation of the MAPK cascade in HEK293 cells. ERK activity was determined using either an in vitro kinase assay (Figure 1B) or anti-phosphoERK antibody (not shown), and equivalent results were obtained with either method.

Growth factor receptor and mitogenic GPCR-mediated ERK activation follows a typical pattern where ERK is maximally activated within five to ten minutes after receptor stimulation and returns to baseline level within several minutes after stimulation. To ascertain whether CaR-dependent ERK activation would display these characteristic kinetics, we performed a time-

course experiment. As shown in Figure 1C, ERK activation displays the expected physiological kinetics, with maximal activation at five and ten minutes and return to baseline activity by 15 minutes. Thus, the CaR activates ERK with kinetics similar to those observed after endogenous mitogenic GPCR activation in other systems.

The CaR activates ERK in a Ras-dependent manner. Recent reports demonstrate that GPCR's which are capable of activating the MAPK pathway can do so in a manner which is Ras-dependent and PKC-independent (19-23), or PKC-dependent and Ras-independent (24, 25). To analyze the involvement of Ras in linking the CaR to ERK activation, we co-expressed the dominant-negative RasN-17 mutant, along with the CaR and HA-ERK2, in HEK293 cells (Figure 2A and B). RasN-17 expression resulted in an 80% inhibition of CaR-induced HA-ERK activation, whereas ERK activation in response to the ionophore A23187 was not inhibited. This result identifies Ras as a necessary intermediate in CaR induced ERK-activation. To investigate the involvement of PKC in ERK activation by the CaR, we asked whether the CaR could stimulate ERK after down-regulation of phorbol-sensitive PKC isoforms with prolonged phorbol pretreatment (18 hours). CaR-mediated ERK activation was not inhibited by this treatment (data not shown).

Src kinase activity is not necessary for CaR mediated ERK activation. Several proximal signaling mechanisms have been reported to be capable of linking GPCRs to Ras-dependent activation of the MAPK cascade. Src kinase has been demonstrated to be involved in the Ras-dependent activation of ERK by many GPCRs (27, 29-31). In addition, we have shown that Src kinase plays a prominent role in CaR-induced ERK activation in Rat-1 fibroblasts (7). In order to determine the involvement of Src kinase in CaR-induced ERK activation in the HEK293 system, we utilized the cytoplasmic tyrosine

kinase inhibitor herbimycin (43). We were unable to demonstrate significant inhibition of ERK activation in the presence of this inhibitor, although ERK activation by LPA was diminished by 80% (Figure 3). Thus, although Src kinase can play a prominent role in GPCR-induced ERK activation in some systems and is strongly implicated as a crucial mediator of CaR-induced ERK activation in Rat-1 fibroblasts, we found no evidence of a requirement for Src kinase activity in CaR-mediated ERK activation in the HEK293 system. The enhanced ERK activation by the CaR in the presence of herbimycin suggests the possibility that Src kinase may in fact play an inhibitory role in CaR-mediated ERK activation.

PI3K is involved in CaR-induced ERK activation in HEK293 cells. Recent literature has implicated PI3K as an essential mediator of GPCR-induced ERK activation in some systems (32-35). In order to investigate the role of PI3K in ERK activation by the CaR, we utilized the PI3K-inhibitors wortmannin and LY294002. Wortmannin treatment blocked 60-80% of the ERK activation in response to the CaR at concentrations as low as 200 nM. LY294009, a more specific PI3K inhibitor, inhibited the CaR-induced ERK response by 50% at 10μM (Figure 4). This was comparable to the inhibition of LPA-stimulated ERK activation by these same compounds in control cells. Neither compound inhibited ERK activation by the calcium ionophore A23187, demonstrating the specificity for CaR-induced ERK activation. Thus, PI3K is strongly implicated as a crucial component of CaR-mediated ERK activation in the HEK system.

Neither SHC tyrosine phosphorylation nor SHC/Grb2 association is induced by CaR activation. Many mitogenic GPCRs activate SHC tyrosine phosphorylation and SHC/Grb2 association, and function of the Shc/Grb2/SOS module is required for ERK activation in several systems (26-28). To determine whether activation of the CaR is coupled to SHC tyrosine phosphorylation in HEK 293 cells, we utilized HEK293 cells

stably expressing the CaR (two independent clones were used). Immunoprecipitation of SHC followed by anti-phosphotyrosine (4G10) immunoblotting demonstrated no detectable induction of SHC tyrosine phosphorylation after 2, 5, or 12 minutes stimulation with calcium (Figure 5A). In contrast, EGF stimulation for 5 or 12 minutes resulted in the expected tyrosine phosphorylation of SHC, specifically the 42kd and 56kd isoforms (Figure 5A). Immunoprecipitation with 4G10 antibody followed by blotting with anti-SHC was also performed and equivalent results were obtained (not shown). Aliquots from each sample were separately used for anti-phosphoMAPK western blotting, demonstrating ERK activation in response to both calcium and EGF treatments (Figure 5B), thereby indicating that the CaR had indeed been stimulated. Anti-SHC immunoprecipitates were also immunoblotted with an anti-Grb2 antibody (Santa Cruz Biotechnology) to detect changes in the association of Grb2 with SHC. While EGF strongly induced SHC/Grb2 association, as indicated by co-immunoprecipitation of Grb2 with SHC, calcium stimulation did not stimulate detectable changes in SHC/Grb2 association (data not shown).

DISCUSSION

It has been recognized for some time that extracellular calcium can modulate the proliferative state of various cell types. In calcium-sensitive epithelial cells, extracellular calcium can act as a differentiation factor, as exemplified in breast epithelial cells, intestinal epithelial cells, and keratinocytes (1-4). In contrast, several mesenchymal cell types respond proliferatively to increased extracellular calcium, including Rat-1 fibroblasts and ovarian surface epithelial cells (specialized peritoneal mesothelial cells; (6, 7). Expression of the G protein-coupled Calcium-sensing Receptor has been demonstrated in these calcium-responsive epithelial and mesenchymal cell types, suggesting a possible mechanism by which extracellular calcium could effect changes in proliferation. In support of this model, we have previously demonstrated that expression of the dominant interfering mutant CaR-R796W in Rat-1 fibroblasts results in the inhibition of calcium-sensitive proliferation (7).

To further elucidate the role of the CaR in calcium-induced modulation of proliferation, we have investigated the effects of CaR activation on the proliferation-associated MAPK cascade. The MAPK cascade has been shown to be integral to cellular decisions with regard to proliferation and differentiation (44). In a previous report, we demonstrated that Rat-1 fibroblasts respond to elevation of extracellular calcium with activation of the MAP kinase ERK, and this activation was inhibited by the expression of the CaR-R796W mutant (7). While this result strongly implicates the CaR as the protein responsible for linking extracellular calcium to ERK activation, calcium is also capable of inducing cellular responses by other means, such as influx through ion channels or modulation of cadherin function. In addition, interpretation of these data is complicated by the expression of endogenous CaR. To rigorously test the specificity of the CaR in mediating calcium-induced ERK activation, we have expressed the receptor

in HEK293 cells which have been characterized as null for the CaR (41, 42). In this report, we demonstrate that expression of the CaR in this system is sufficient to confer calcium-inducible ERK activation in response to elevated extracellular calcium, and this response requires functional CaR, as calcium was unable to stimulate ERK activation in HEK293 cells expressing the non-functional interfering mutant CaR-R796W. Thus, in HEK293 cells, the activation of ERK in response to elevated extracellular calcium is specific to and requires functional CaR.

We then utilized this system to delineate the signaling mechanisms by which the CaR activates ERK. Many GPCRs activate the MAP kinase pathway and ERK. These include the lysophosphatidic acid (LPA) (13, 14), bombesin (15), angiotensin II (16, 17), alpha-thrombin (18), alpha1B-adrenergic and alpha2A-adrenergic (15) receptors. In general, GPCRs can activate ERK in either a Ras-dependent and PKC-independent (19-23), or PKC-dependent and Ras-independent (24, 25). An interesting exception was reported by Li et al. (45), who demonstrated that the angiotensin II-stimulated ERK activation in rat liver epithelial cells can occur via two mechanisms: a Ras-independent and PKC-dependent pathway, or an equipotent Ras-dependent PKC-independent pathway which was unmasked by PKC depletion. Utilizing expression of the dominant interfering Ras-N17 mutant, we demonstrate here that CaR-induced ERK activation is Ras-dependent in the HEK293 system. In addition, we demonstrate that PKC down-regulation is unable to inhibit CaR-mediated ERK activation, thus demonstrating this pathway to be PKC-independent (data not shown).

Signal transduction from GPCRs to MAPK is complex, involving variable interactions between several distinct components. Signaling proteins such as the SHC/Grb2/SOS module have been shown to be activated in conjunction with and, in some cases, required for Ras-dependent GPCR-mediated ERK activation (26-28). Three major classes of kinases have been implicated in this Ras-dependent cross-talk, as well. These are the receptor tyrosine kinases such as the EGF receptor and the

PDGF receptor, the Src family kinases, and PI3K. In addition, the involvement of the calcium-sensitive cytoplasmic kinase PYK-2 has been reported in specialized cell types (27, 39).

Several groups have demonstrated transactivation of receptor tyrosine kinases in response to GPCR agonists. These include the EGF receptor (36-39) and the PDGF receptor (40). One postulated function of these RTKs in GPCR signaling is to serve as a scaffolding structure which may serve to recruit other signaling proteins in response to GPCR activation (46) and reviewed in (47).

Another class of kinases, the Src-family kinases (c-Src, Fyn, Lyn, Yes, and the distantly related SYK), has been strongly implicated as downstream mediators of GPCR induced ERK activation (27, 29-31). The activation of ERK by several of these Src kinase-dependent systems was shown to also depend upon other signaling intermediates such as SHC/Grb2/SOS, PI3K, PYK-2 (27, 34). In a prior report, we demonstrate that agonists of the CaR induce SHC tyrosine phosphorylation, and provide strong evidence that Src is a crucial mediator of CaR-induced ERK activation in Rat-1 fibroblasts (7). However, neither SHC nor Src appear to play significant roles in CaR-induced ERK activation in HEK293 cells. Although the Src inhibitor herbimycin inhibited ERK activation by LPA, no appreciable inhibition of CaR-induced ERK activation was demonstrated, suggesting that Src activity is not required for CaR-induced ERK activation in HEK293 cells. To determine whether SHC may play a role in CaR-mediated downstream signaling, we asked whether CaR activation resulted in SHC tyrosine phosphorylation. These studies demonstrate that SHC tyrosine-phosphorylation is undetectable at 2, 5 or 12 minutes after CaR stimulation, whereas at 5 and 12 minutes after EGF stimulation, the tyrosine- phosphorylation of the p42- and p56-SHC isoforms is greatly enhanced. The lack of CaR-induced SHC tyrosine-phosphorylation strongly argues against the involvement of SHC in CaR-induced ERK activation, although it is possible that small changes in tyrosine phosphorylation below

the level of detection may be contributing to ERK activation by the CaR. To definitively demonstrate that activation of ERK by the CaR is SHC-independent it will be necessary to block SHC activity with dominant negative SHC, for example.

Phosphatidylinositol-3-kinase (PI3K) has been shown to be important in many cellular processes such as cytoskeletal rearrangements, cellular migration, mitogenesis, differentiation, and protection from apoptosis (reviewed in (48, 49). Recently, PI3K has been shown to play a role in GPCR-mediated ERK activation, as well. In vascular smooth muscle cells, the activation of alpha1-adrenergic receptors results in PI3K activation (32). In COS-7 cells, overexpression of PI3Kγ, the isoform which does not associate with the p85 regulatory subunit, results in $G_{\beta\gamma}$ -dependent ERK activation in a pathway which also requires both SHC and Src kinase activity (34). In contrast, in Rat-1 and COS-7 cells, LPA-induced ERK-activation was PI3K-dependent, but neither SHC nor Src kinase were involved (35).

In this report, we utilize the PI3K inhibitors wortmannin and LY294009 to demonstrate that the full activation of ERK by the CaR requires PI3K activity in HEK293 cells. Maximal inhibition of ERK activation was seen at concentrations of wortmannin as low as 200 nM, arguing against a non-specific effect of wortmannin on other signaling proteins. The use of the specific PI3K inhibitor LY294009 corroborates the specificity of these results to PI3K inhibition. Neither inhibitor completely abolished ERK activation. In the present study we cannot determine whether the residual ERK activation in response to the CaR is due to a second PI3K-independent pathway by which the CaR activates ERK, or due to very high levels of receptor expression. Nevertheless, PI3K is strongly implicated as playing a major role in the proximal signaling mechanism utilized for CaR-induced ERK activation in HEK293 cells.

The present study, taken together with our prior report in Rat-1 fibroblasts, demonstrates that the CaR can couple to the MAP kinase pathway in a manner that depends upon the cellular background in which it is expressed (7). Our finding that PI3K

is a prominent player in ERK activation without the co-involvement of Src kinase or the SHC/Grb2/SOS module is unusual but not without precedent. Kranenburg et al report that LPA receptor-mediated ERK activation in both Rat-1 fibroblasts and COS-7 cells is PI3K dependent but independent of Src kinase activity, SHC tyrosine phosphorylation and SHC/Grb2 association (35). Thus, it is clear that numerous mechanisms exist by which GPCRs can activate ERK. These mechanisms are likely to be both receptor-specific, and cell type-specific. A compelling question is whether the various mechanisms of ERK activation specify different biological consequences for the cell.

PI3K has been shown to be involved in multiple diverse cellular processes, including proliferation and escape from apoptosis (reviewed in (48, 49). PI3K inhibits apoptosis induced by serum-deprivation (50), UV irradiation (51), and c-Myc-expression (52) in fibroblasts. Recent evidence suggests that GPCRs may activate survival pathways mediated by PI3Kγ activation of AKT (53). In a recent report, Lin et al. found that expression of the CaR in HEK293 cells could inhibit c-Myc-induced apoptosis in a calcium-dependent manner (54). Importantly, ERK activation has also been shown to inhibit apoptosis in some systems, including apoptosis due to serum-deprivation in PC-12 cells (55) and UV-induced apoptosis of human primary neutrophils (56). Our finding that the CaR activates ERK and does so in a PI3K-dependent manner suggests a possible signaling mechanism by which CaR activation might protect HEK293 cells from c-Myc-induced apoptosis. This could be addressed by investigating whether PI3K and/or ERK activity is required for the inhibition of c-Myc-induced apoptosis in CaR-transfected HEK293 cells. Importantly, the finding of Lin et al suggests another possible role that the CaR may be playing in cellular biology, that of a survival mediator.

Extracellular calcium has recently been demonstrated to function as an extracellular ligand, acting through the CaR. This was first demonstrated in parathyroid cells by Brown et al, and extended to Rat-1 fibroblasts by work done in this laboratory (7, 8). The expression of the CaR both in cell types which proliferate in response to calcium

and in cell-types which are induced to differentiate in response to calcium suggests the possibility that the CaR may be involved in mediating either proliferation or differentiation in a cell-type specific manner. Our findings here, taken together with our earlier report of CaR-induced signaling in Rat-1 fibroblasts, strongly suggest that the CaR can couple, in a cell-type specific fashion, to divergent proximal signaling intermediates in order to activate ERK. This demonstrates heterogeneity in CaR signaling and suggests a potential mechanism by which the CaR could couple to diverse calcium-dependent biological responses.

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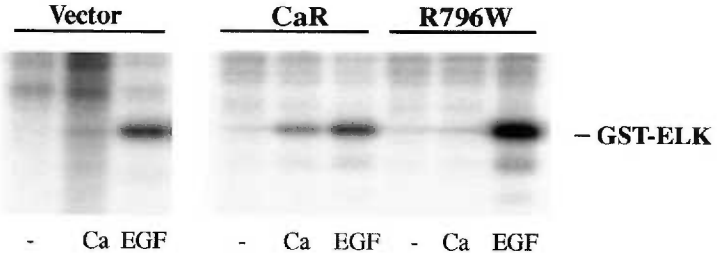
Figure 1. Hobson et al

A.



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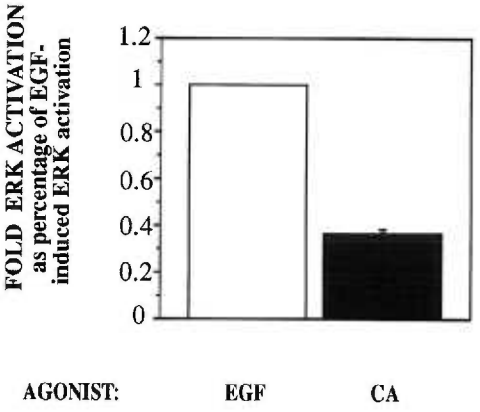

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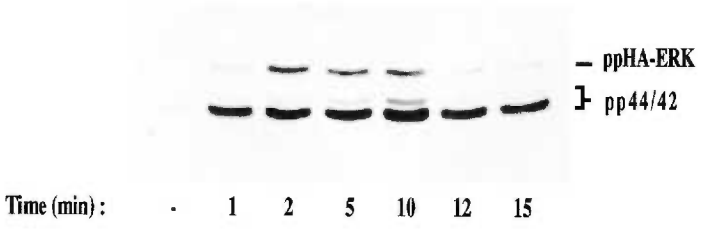
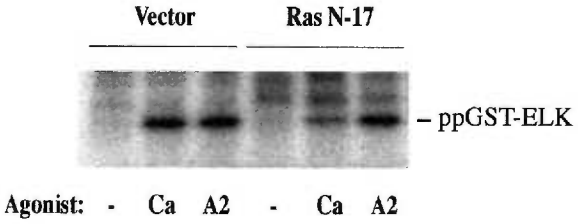


Figure 2. Hobson et al

A.



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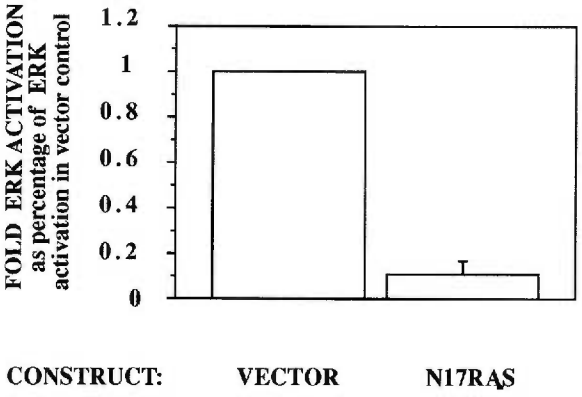

Figure 3 Hobson et al

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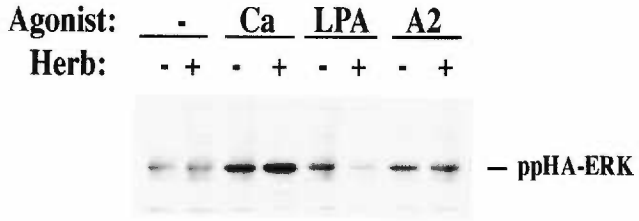

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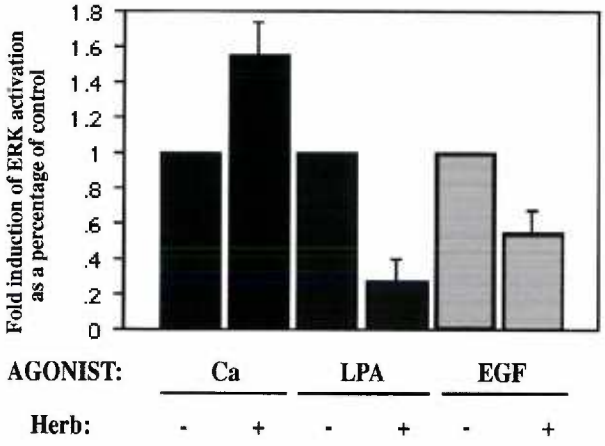
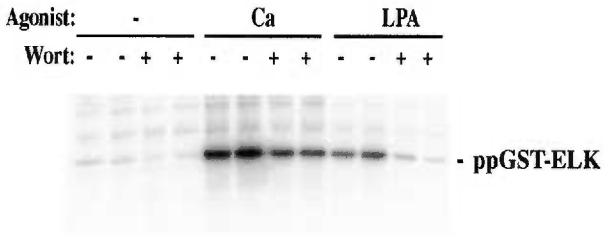


Figure 4. Hobson, et al

A.



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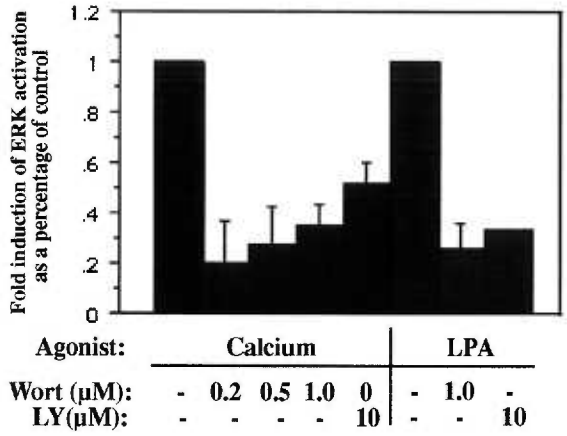
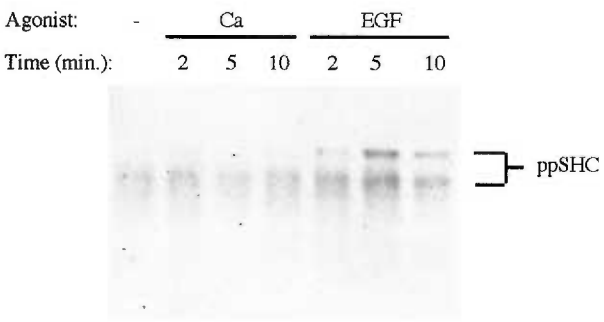


Figure 5 Hobson et al

A.



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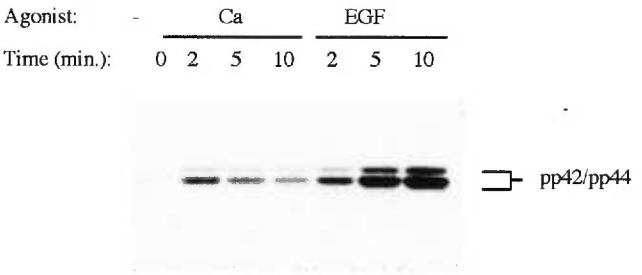

FIGURE LEGENDS

Figure 1: Calcium induces ERK activation in HEK293 cells expressing the CaR. Panel A: HEK293 cells were transiently co-transfected with HA-ERK2 and either CaR, non-functional R796W mutant, or vector. Cells were grown in the presence of serum for 24 hours after electroporation, and then serum starved in low calcium medium (Hamm's F12 - 0.3mM Ca) for 24 hours prior to stimulation. Cells were stimulated for 10 minutes with 2.0 mM CaCl, or EGF, 20 ng/ml before lysis. HA-ERK2 was immunoprecipitated from lysates normalized for protein using 12CA5 (anti-HA-tag) antibody. Immunoprecipitates were subjected to in vitro kinase assay as described in materials and methods using GST-Elk as the substrate. After separation on polyacrylamide gel, the proteins were transferred and the incorporated radioactivity quantified by PhosphorImager (Molecular Dynamics). Membranes were then immunoblotted with ERK-2 to verify equivalent loading of HA-ERK2. ERK activity was also determined the phospho-specific MAPK antibody, and equivalent results were obtained with either method.

Panel B: In each experiment, the fold-activation of ERK in response to calcium was represented as a percent of the fold-activation of ERK in response to EGF-stimulation. Data represents five independent experiments (n=5).

Panel C: Timecourse of ERK-activation by the CaR in response to calcium (2.0 mM). Total protein lysates were separated using SDS-PAGE and western blotted using anti-phosphoMAPK.

Fig. 2 - Ras activity is required for CaR-mediated ERK activation. Panel A: HEK293 cells were transiently co-transfected with the CaR and HA-ERK along with either the Ras dominant-negative mutant RasN17 or empty vector. Cells were serum starved in low calcium medium as described in Figure 1, and then stimulated with 2.0 mM calcium or the calcium ionophore A23187 (10 μM) for 5 minutes. Lysates were subjected to in vitro kinase assay with GST-Elk used as a substrate as described in Fig. 1 and Materials and Methods. Panel B: Bar graph representing the fold-activation of ERK in the presence of RasN17 as a percent of fold-activation in vector transfected control (n=4).

Figure 3. The Src kinase inhibitor herbimycin cannot inhibit CaR-mediated ERK activation. Panel A: HEK293 cells were transiently co-transfected with the CaR and HA-ERK. After serum starvation in low-calcium medium as previously described, cells were pretreated with either vehicle (DMSO) or 1uM herbimycin overnight. Cells were then stimulated with 2.0 mM $CaCl_2$ or 20ng/ml EGF for five minutes before lysis. A representative blot is shown. Panel B: Bar graph representing fold-induction of ERK in response to each agonist as a percentage of their respective vehicle treated controls (n=3, minimum).

Figure 4 – Activation of ERK2 by the CaR involves PI3K.

Panel A: HEK293 cells were transiently co-transfected with the CaR and HA-ERK. After serum- and calcium-deprivation in F-12 medium as previously described, cells were pretreated with either 0.1% DMSO or 1 μM wortmannin or 10μM LY294009 for 30

minutes, before stimulation with 2.0 mM Ca or LPA (10μM) for 5 minutes. Lysates were subjected to Western blot analysis and phosphoERK immunoblotting. The same results were obtained when samples were subjected to in vitro kinase assay as described in Fig. 1 and Materials and Methods (data not shown).

Panel B: Bar graph representing fold-induction of ERK activation in response to 2.0 mM Ca or LPA (10μM) in the presence of either wortmannin or LY294009 represented as a percentage of fold-induction of ERK activation without inhibitor (n=3).

Figure 5 – CaR activation does not induce SHC tyrosine phosphorylation.

Panel A: HEK 293 cells and HEK293 cells stably expressing the CaR were serum-and calcium-deprived in low calcium medium overnight. Cells were stimulated for 5 minutes with either 2.0 mM Ca or 10ng/ml EGF. Immunoprecipitations were performed using anti-SHC (Santa Cruz Biotechnology) and membranes were immunoblotted with the 4G10 anti-phosphotyrosine antibody. A representative blot is shown (n=3).

Panel B: Aliquots of the lysates used above were western blotted with anti-phosphoMAPK antibody to confirm CaR ERK activation in response to treatment (n=3).

DISCUSSION

The proliferation and differentiation status of numerous cell types such as keratinocytes, breast epithelial cells, fibroblasts and ovarian surface epithelial cells can be modulated independently by extracellular calcium concentration (Hennings *et al.*, 1980; Yuspa *et al.*, 1989; Soule *et al.*, 1990; McNeil *et al.*, 1998a; McNeil *et al.*, 1998b). Many of these cell types have been shown to express the calcium-activated, G protein-coupled calcium-sensing receptor (CaR) or a highly related homologue (Bikle *et al.*, 1996; Kallay *et al.*, 1997; McNeil *et al.*, 1998a; McNeil *et al.*, 1998b), and the CaR has been implicated as a key component of calcium-regulated proliferation in Rat-1 fibroblasts (McNeil *et al.*, 1998b). This thesis represents an investigation of the role of the CaR in modulating the proliferation of a calcium-sensitive cell-type, OSE, and the signaling mechanisms by which it might do so.

The proliferative status of Rat OSE cells is modulated by extracellular calcium in a manner dependent upon the CaR or a highly related CaR-homologue.

The ovarian surface epithelium is a specialized extension of the peritoneal mesothelium which covers and invests the ovary. Ovarian surface epithelial (OSE) cells are the cell type from which ovarian carcinoma is thought to derive. This single cell layer is subjected to wounding and subsequent healing at each ovulatory cycle. It has been proposed that this repeated wound healing may be an initiating factor in ovarian

carcinoma, since the principle risk for this disease is the number of ovulatory cycles experienced in a woman's lifetime (Piver *et al.*, 1991; Richardson *et al.*, 1985).

OSE cells are exposed to follicular fluid at each ovulation with the rupture of the mature follicle. OSE cells express the appropriate receptor for numerous growth factors present in follicular fluid, including TGFα and G-CSF, and respond proliferatively to these factors in culture (Berchuck *et al.*, 1990; Bauknecht *et al.*, 1989; Boocock *et al.*, 1995). Another component of follicular fluid is calcium, reported as 1.8-2.0mM immediately prior to ovulation (Schuetz and Ansiowicz, 1974). In a prior report, we have shown that human OSE cells exhibit calcium-sensitive thymidine incorporation under serum-free conditions, demonstrated by enhanced thymidine incorporation at 1.4 and 1.8 mM calcium, which is two-fold greater than incorporation at 0.3 and 0.8 mM calcium (McNeil *et al.*, 1998a). Growth curve experiments confirmed the proliferation-enhancing effects of calcium, with significantly increased cell-doubling rates at 1.4 mM calcium as compared to 0.3 and 1.0 mM calcium. The transition calcium concentration in both of these experiments parallels a transition from the ionized calcium concentration of 1.1-1.3 mM in serum to the reported follicular calcium concentration of 1.8-2.0 mM. This finding is consistent with the hypothesis that an increase in the local concentration of ionized calcium due to follicular rupture may be one of multiple proliferative stimuli initiating a wound repair process in the ovarian surface epithelium.

It is known that the proliferation and differentiation of numerous cell types is modulated by extracellular calcium, but the mechanisms involved are incompletely defined. The finding that OSE cells express the G protein-coupled calcium-sensing receptor (CaR), which is implicated in the calcium-dependent proliferation of Rat-1

fibroblasts, suggests a mechanism by which calcium might modulate proliferation in OSE cells (McNeil *et al.*, 1998b; McNeil *et al.*, 1998a). Both rat and human OSE cells were shown to express the CaR, or a highly-related homologue, utilizing 1) RT-PCR followed by sequence analysis, 2) northern analysis, and 3) both western blotting and immunoprecipitation techniques (McNeil *et al.*, 1998a). Pharmacological function was demonstrated by IP_3 generation and increased intracellular calcium in response to the CaR agonist gadolinium, responses previously shown to be characteristic for the CaR. In order to investigate the potential role of the CaR in calcium-regulated OSE growth, we sought to inhibit CaR function by expressing a dominant-interfering CaR mutant. We could then ask whether calcium-inducible proliferation remained intact or was disrupted in the absence of CaR function. This experiment would be difficult in the human OSE cells, due to their limited proliferative potential (Maines-Bandiera *et al.*, 1992; McNeil *et al.*, 1998a). Therefore, the rat OSE cell line ROSE was used. These cells are rat OSE cells which have been transformed with the Kirsten murine sarcoma virus in order to facilitate long-term culture (Adams and Auersperg, 1981). ROSE cells demonstrate a small (1.3 fold) yet statistically significant increase in thymidine incorporation when shifted from 0.3 to 0.7 or 1.4 mM calcium (McNeil *et al.*, 1998a). The lesser magnitude of response and lower calcium concentration necessary for this response may represent a species-specific difference or a difference in the calcium-response mechanisms among different cell types, but it is more likely that this difference reflects the relative levels of transformation and growth disregulation of the two cell lines. The human OSE cell lines are early passage cell lines of normal human OSE cells which have been transfected with SV40 large T antigen. The presence of the large T antigen

serves to extend the number of doublings the cells can maintain in culture (up to 25; Maines-Bandiera *et al.*, 1992; McNeil *et al.*, 1998a). These cells are not immortal and do not form tumors in nude mice. In contrast, ROSE cells are immortalized secondary to Kirsten murine sarcoma virus infection and have been cultured in 0.7 mM calcium for greater than 15 years. In addition, these cells are transformed and can form a rare type of sarcoma-like tumor when injected into nude mice. Nevertheless, they do express the CaR and exhibit calcium-regulated proliferation, albeit of lesser magnitude than the human OSE cell line used, and are therefore a suitable model system. In future experiments, primary rat OSE cells could be subjected to the same assays for comparison with the ROSE cells. This would demonstrate the calcium sensitivity of the rat OSE cells before genetic changes induced by long-term culturing and Kirsten murine sarcoma virus infection could occur, more closely approximating the situation in vivo, and clarifying the significance of the disparity between the ROSE cells and human OSE cells.

We utilized previously generated ROSE cell lines that stably express the non-functional, dominant-interfering CaR-R796W mutant or a construct with the CaR in the reverse orientation, which in prior experiments had exhibited dominant interfering properties (Bai *et al.*, 1996; McNeil *et al.*, 1998b). In thymidine incorporation assays (Chapter 1, Figure 1B), an indirect measure of proliferation, ROSE cells transfected with the empty vector demonstrated a 1.5 fold increase in thymidine incorporation at 0.8 mM calcium as compared to cells at 0.3 mM calcium. In contrast, thymidine incorporation in CaR-R796W or the CaR-Rev transfected cells was not greater at 0.8 mM calcium than 0.3 mM calcium. Higher calcium concentrations (1.4 mM) were necessary to induce an

increase in thymidine incorporation in CaR-R796W and CaR-Rev cells. The fold increase at 1.4 mM calcium was equal to the magnitude of response observed in control cells at 0.8 mM calcium. Thus, inhibition of the endogenous CaR by CaR-R796W or CaR-Rev expression resulted in a decreased sensitivity of thymidine incorporation to calcium, shifting the dose response curve to the right. Disruption of calcium responsiveness by CaR-R976W and CaR-Rev was even more apparent in a colorimetric assay for cell number, the MTT assay. The MTT assays (Chapter 1, Fig 1C) demonstrated that whereas vector transfected controls exhibited two-fold greater cell numbers after culturing for 3 days in 2.0 and 4.0 mM calcium versus 0.3mM calcium, the proliferation of CaR-R796W and CaR-Rev expressing cells was insensitive to calcium concentration. These data strongly implicate the CaR as a pivotal component of the proliferative response to calcium observed in ROSE cells.

It is not clear why the ROSE.CaR-R796W and ROSE.CaR-Rev cells responded to calcium with increased thymidine incorporation even though they clearly were not induced to proliferate, as demonstrated by the MTT assay. The reason for this disparity may lie in the nature of the two assays. Thymidine incorporation measures DNA-synthesis starting at 16 hours after experimental stimulation and extending over the subsequent four hours. This assay indicates the relative number of cells that are induced to leave Go (quiescence) and proceed through S phase. It is possible that calcium, through means other than or in addition to CaR stimulation, may induce cells to enter the cell cycle, but the MTT data would indicate that these cells do not continue to double after this first round of synthesis. The MTT assay is a more specific indicator of cellular proliferation because it reflects the change in actual cell number, and was used

to measure an accumulation of responses over 3 days, rather than a limited 'snapshot' view at 20 hours. It has been reported that cells which have been induced to differentiate often enter a single round of synthesis before they become quiescent. It is possible that in the absence of a functional CaR, ROSE cells may be induced to differentiate in response to calcium, thus demonstrating a calcium-induced thymidine incorporation without significant concomitant increase in cell numbers. Nevertheless, both wild type and vector-transfected control cells respond to calcium with both increased cell numbers and thymidine incorporation, and expression of the interfering CaR-R796W or CaR-Rev disrupts both calcium-induced thymidine incorporation and cell doubling. Expression of these constructs had no effect on thymidine incorporation or cell doubling in the presence of serum (data not shown), thus demonstrating their specific effect on calcium-mediated proliferation. These data strongly support the pivotal involvement of the CaR in calcium-induced proliferation in ROSE cells.

THE CALCIUM-SENSING RECEPTOR ACTIVATES THE MAPK CASCADE AND ERK: TWO SEPARATE RAS DEPENDENT PATHWAYS EXIST, AND THE PATHWAY USED VARIES IN A CELL-TYPE SPECIFIC MANNER.

It was reasoned that if the CaR mediates calcium-induced proliferation, receptor activation would likely couple to known proliferation-associated pathways. Prior studies of mitogenically coupled GPCRs have demonstrated an involvement of several proximal downstream kinases which mediate activation of the mitogen-activated protein kinase (MAPK) cascade, as demonstrated by extracellular-regulated kinase (ERK) activation.

Our previous work had shown that the CaR agonists calcium and gadolinium each induced ERK activation in Rat-1 and human OSE cells (McNeil *et al.*, 1998b; and data not shown). ERK activation was inhibited in Rat-1 cells stably expressing the dominant-interfering CaR-R796W mutant, indicating that ERK activation by these agonists was specific to the CaR (McNeil *et al.*, 1998b). The work in this thesis demonstrates that ROSE cells also exhibit a two-fold activation of ERK in response to the CaR agonist calcium (Chapter 1, Figure 3). The specificity of the ERK response to CaR activation was demonstrated by the ability of the CaR-R796W mutant to inhibit calcium-induced ERK-activation. This implicates ERK as a specific downstream mediator of CaR signaling and demonstrates that the CaR can couple to MAPK cascade activation in ROSE cells.

Mitogenic GPCRs utilize one or more of several intracellular kinases to mediate ERK activation, including Src, PI3K, and receptor tyrosine kinases. Previous data from our laboratory has demonstrated that agonists of the CaR induced c-Src activity in Rat-1 fibroblasts, and that inhibition of Src kinase activity with the cytoplasmic tyrosine kinase inhibitor herbimycin was effective at inhibiting both ERK activation and thymidine incorporation induced by calcium in these cells(McNeil *et al.*, 1998b). The work in this thesis implicates Src as a key signaling intermediate from the CaR in ROSE and human OSE cells. Calcium-induced ERK activation was completely inhibited by herbimycin (Chapter 1, Figure 3; and data not shown). In addition, a modest but statistically significant increase in c-Src activity was demonstrated in ROSE cells in response to calcium (Chapter 1, Figure 2B). Moreover, herbimycin was able to completely inhibit calcium-induced proliferation as inferred by thymidine incorporation

(Chapter 1, Figure 5). Thus, Src activity is implicated as a downstream intermediate which is crucial to CaR signaling and CaR-induced proliferation in ROSE cells. Herbimycin is a selective Src family inhibitor but may have non-specific effects on other cellular kinases at the concentration used (Li *et al.*, 1993). Therefore, these experiments can only strongly implicate Src involvement in CaR-mediated ERK activation and proliferation. Further studies, such as over-expression of c-terminal Src kinase (CSK) (Nada *et al.*, 1991) or a dominant interfering Src (e.g. c-src$^{K298M;}$ Snyder *et al.*, 1985) would be useful to confirm these results. Alternatively, the use of another Src-specific inhibitor, such as PP1 (Hanke *et al.*, 1996), could corroborate these findings.

To establish whether the CaR activates ERK in a Ras-dependent manner, the RasN17 dominant interfering mutant was used in transient co-transfection studies. These studies demonstrated that the RasN17 mutant was capable of completely inhibiting ERK activation in response to calcium. As expected, the dominant interfering forms of MEK (MKK1) and Raf were also effective inhibitors of ERK activation by the CaR. This indicates that the CaR-induced ERK activation is mediated by Ras-dependent activation of the MAPK pathway.

Although ERK is activated by CaR agonists, it is unclear whether ERK activity is necessary for calcium-induced proliferation. The MEK inhibitor PD 98069 has been used in prior studies investigating ERK-dependent signals, and while useful in certain scenarios, this compound has been shown to inhibit basal ERK activity and only incompletely inhibit ERK activation by strong agonists in other systems (Alessi *et al.*, 1995). Our data demonstrates an overall inhibition of basal thymidine incorporation by PD98069. This may indicate that ERK activity is essential to ROSE proliferation, but we

cannot exclude the possibility of non-specific cytotoxicity. When ROSE cells are pretreated with PD 98069, they exhibit a much reduced absolute level of thymidine incorporation in response to 2.0 mM calcium as compared to vehicle treated controls, yet the fold-induction of thymidine incorporation at 2.0 mM as compared to PD98069-treated ROSE cells at 0.3 mM was equal to that of the fold-induction in untreated controls at 2.0 versus 0.3 mM. It is formally possible that ERK may play a fundamental role in basal levels of OSE proliferation while the increase in DNA synthesis in response to extracellular calcium may reflect the activation of an additional proliferative pathway. This data does not allow us to confidently confirm or deny a critical role for ERK activation in the proliferative response to calcium in Rose cells. Further studies await the development of a potent and specific inhibitor of stimulated as opposed to basal ERK activity.

Studies of CaR-mediated ERK activation in HEK293 cells.

The investigation of CaR signaling in the ROSE cells has the advantage of known biological relevance, i.e. the cells endogenously express the receptor and respond proliferatively to calcium. Yet rigorous testing of the specificity of ERK activation and other signaling events is difficult in a system where the endogenous receptor is present and must be inhibited by means of a dominant-interfering mutant. Therefore, I chose to complement the studies of CaR signaling in ROSE cells with investigations in a CaR-null background.

HEK293 cells have been previously reported as null for the CaR (Chattopadhyay *et al.*, 1996; Kifor *et al.*, 1997). In addition, my preliminary experiments demonstrated

that ERK was not activated in these cells in response to extracellular calcium. These properties mad e HEK293 cells suitable for studies of CaR-induced signaling utilizing transient and stable transfection techniques. The work in this thesis shows that ERK is not activated in response to extracellular calcium in the vector transfected HEK293 cells (Chapter 2, Figure 1). However, both transient and stable transfection with the CaR confers calcium-inducible ERK activation in these cells. The non-functional CaR-R796W mutant was unable to mediate ERK activation in response to calcium, demonstrating the necessity of functional CaR in this response. When normalized as a percentage of EGF-induced ERK-activation, CaR induced ERK activation was reproducibly about 40% of the EGF-induced activation in five independent experiments (Chapter 2, Figure 1B). These studies establish both the specificity of and the requirement for functional CaR in the calcium-induced ERK activation observed in HEK293 cells.

GPCRs can utilize either Ras-dependent or Ras-independent mechanisms of ERK activation, depending on the receptor and/or cell-type. To investigate the involvement of Ras in CaR-mediated ERK activation, we used the RasN17 dominant interfering mutant. Co-expression of RasN17 along with the CaR in HEK293 cells resulted in an approximately 80% inhibition of ERK activation by calcium (Chapter 2, Figure 2A and B). The inhibition of calcium-induced ERK activation was specific, as RasN17 expression had no effect on ERK activation by the calcium ionophore A23187. This result indicates that ERK activation via the CaR in HEK293 cells is largely, if not completely, Ras-dependent.

GPCRs can activate Ras utilizing one of several key kinases. In this thesis, Src kinase activity is shown to be involved in CaR-mediated ERK activation in OSE cells, and had likewise been shown to be important in Rat-1 fibroblasts. However, Src activity does not appear to be an important signaling component of the CaR mediated ERK-activation in HEK293 cells. This was demonstrated by the inability of the Src inhibitor herbimycin to disrupt ERK activation by the CaR, although herbimycin fully inhibited ERK activation in response to lysophosphatidic acid in these cells (Chapter 2, Figure 3). This was a somewhat unexpected result, since Src kinase activity had been shown to be required for ERK activation by CaR agonists in Rat-1 fibroblasts (McNeil *et al.*, 1998b) and OSE cells (Chapter 1). However, other GPCRs have been reported to have variable signaling mechanisms depending on the cell type in which they are expressed. Co-transfection of the Src family inhibitor CSK with the CaR in future studies would definitively confirm or deny the Src -independence of CaR-induced ERK-activation in HEK293 cells, but it is apparent from the studies presented here that CaR-mediated ERK activation in HEK293 cells exhibits a vastly different level of sensitivity to herbimycin than that seen in Rat-1 cells. These data strongly suggest that the CaR can mediate ERK activation by at least two distinct pathways, one which is herbimycin-sensitive and the other which is not. In addition, whereas SHC tyrosine phosphorylation was induced by CaR agonists in the Rat-1 fibroblasts, we found no evidence for the involvement of the SHC/Grb2/SOS module in CaR signaling in the HEK293 cells (Chapter 2, Figure 5, and data not shown). These data further support the existence of multiple signaling mechanisms by which the CaR might mediate ERK activation and intracellular events.

Among alternative mediators linking GPCRs to ERK activation is phosphatidyl-inositol-3-kinase (PI3K). PI3K has been reported to be required for GPCR-mediated ERK activation by pathways both dependent and independent of Src. The data presented here strongly implicate PI3K activity as a crucial component of CaR-mediated ERK activation in HEK293 cells. The PI3K inhibitor wortmannin effected a 60 - 80% inhibition of CaR-mediated ERK activation at concentrations as low as 200 ng/ml (Chapter 2, Figure 4). The specificity of this result was corroborated by use of LY294002, a more specific inhibitor of PI3K, which inhibited CaR-mediated ERK-activation by 50%. Although strongly inhibitory, neither of these reagents completely blocked ERK activation by the CaR. In the present work, we cannot determine whether this is due to excessive levels of receptor expression or due to the presence of a second PI3K-independent pathway. However, these data strongly implicate PI3K as a crucial component of CaR-mediated ERK activation in HEK293 cells. Similar studies in Rat-1 cells indicated that PI3K activity is not universally required for the coupling of the CaR to the MAPK cascade in these cells since wortmannin was unable to inhibit ERK activation by CaR agonists in (data not shown). This observation supports the existence of multiple distinct pathways linking the CaR to ERK-activation, depending on the cell type in which it is expressed (Figure 3 and Table 1).

Potential mechanisms responsible for cell type-specific signaling include the variable coupling of GPCRs to specific G protein subtypes depending on the cellular background in which they are expressed. Another mechanism might be the availability or accessibility of important downstream effectors, such as cell type-specific intermediates with restricted expression among tissues, e.g. PYK-2. PYK-2 is not a

Model for ERK activation by the CaR

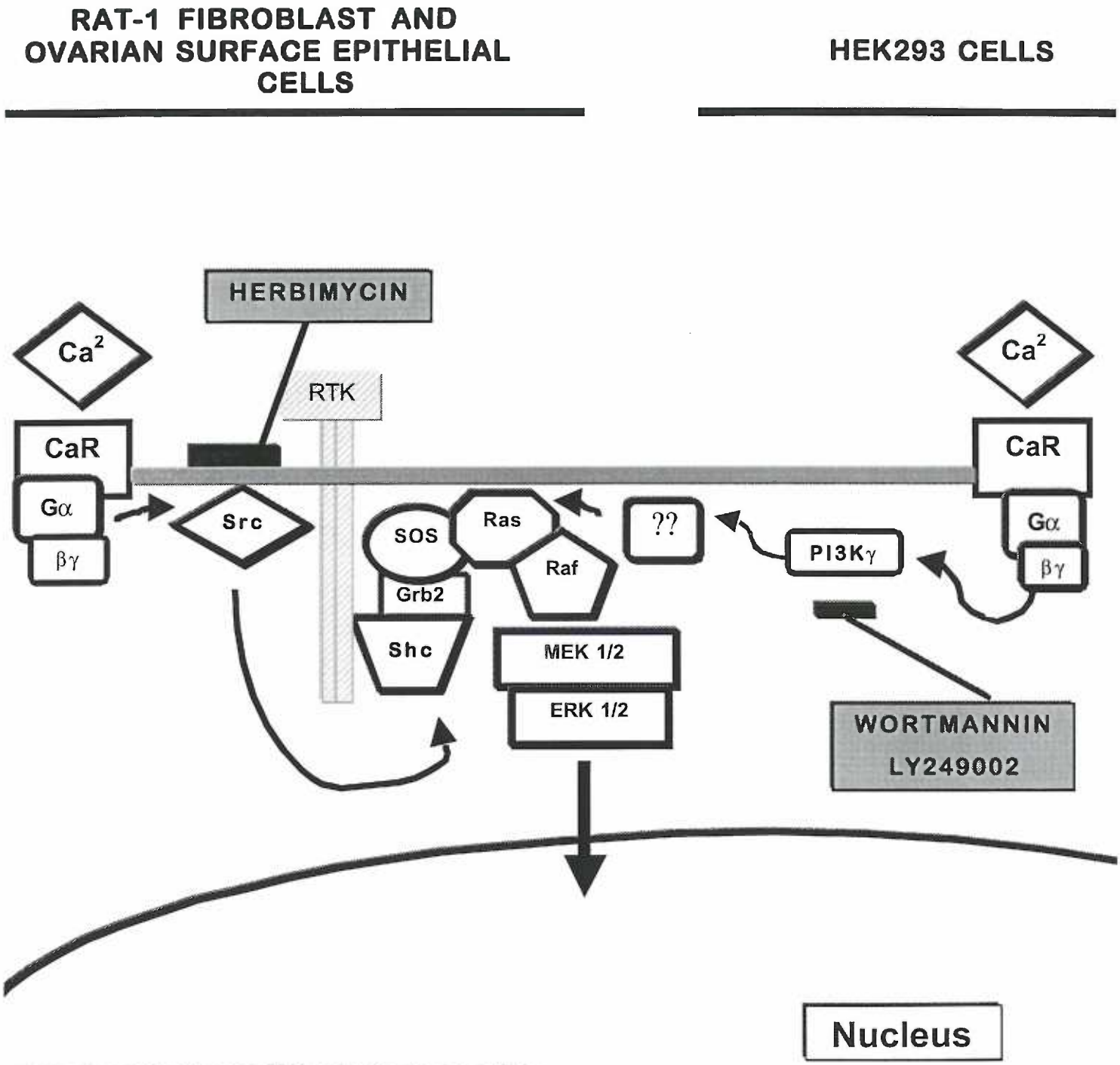

Figure 3. Mechanisms of ERK activation by the CaR in Rat-1 fibroblasts and ovarian surface epithelial cells, and in HEK293 cells.

	Rat-1	OSE	HEK293
1. Kinases implicated in CaR-induced ERK activation:	Src kinase	Src kinase	PI3K
2. Ras-dependent?	nd	Yes	Yes
3, CaR activation is coupled To Shc tyrosine-phosphorylation?	Yes	nd	No
4. CaR activation is coupled to Increased proliferation?	Yes	Yes	No*
5. Origin of CaR in studies:	Endogenous	Endogenous	Transient or stable transfection
6. Classification of cell type:	Mesenchymal	Mesothelial	unknown**

Table 1. Characteristics of CaR signaling in Rat-1 fibroblasts, ovarian surface epithelial cells, and HEK293 cells. ('nd' = not done)

* data not shown
** not indicated by American *Type Culture* Collection.

candidate in HEK293 cells since these cells do not express PYK-2. The role of receptor tyrosine kinase transactivation in CaR-mediated ERK activation has not been addressed but may also be important, since differential coupling to receptor tyrosine kinases could also account for variable signaling by GPCRs between specific cell types.

The differential signaling observed in Rat-1 and OSE cells versus HEK293 cells suggests a potential mechanism by which the CaR may specify very different biological outcomes. As previously detailed, the calcium-induced proliferative effects in those cells which are sensitive to calcium currently can be divided into two groups – epithelial cells which differentiate in response to calcium, and mesenchymally derived cells which exhibit enhanced proliferation in response to calcium. Numerous cell types of both groups have been shown to express the CaR, including small and large intestine (Chattopadhyay *et al.*, 1998; Kallay *et al.*, 1997), keratinocytes (Bikle *et al.*, 1996), Rat-1 fibroblasts (McNeil *et al.*, 1998b) and OSE cells (McNeil *et al.*, 1998a). Work from our lab and that presented in this thesis strongly supports a role for the CaR in mediating the proliferation-promoting effects in Rat-1 fibroblasts and rat ovarian surface epithelial cells. (As previously stated, ovarian surface epithelial cells are not truly epithelial cells but are mesothelial cells of mesenchymal origin.) The role of the CaR in mediating differentiation in response to calcium in epithelial cell types has not been definitively demonstrated.

Using stably transfected HEK293.CaR cells, the CaR-mediated effects of calcium on thymidine incorporation and cell growth were studied and revealed no demonstrable proliferative response to calcium (data not shown). This may be due to the transformed nature and factor-independent growth of these cells, as even 10% serum induced only a

five-fold increase in thymidine incorporation. Another explanation is that HEK293 cells lack the signaling intermediates capable of linking the CaR to proliferation, while expressing those linked to differentiation or calcium-sensitive survival. Recent literature underscores the pivotal role PI3K plays in the rescue of cells from apoptosis (Franke *et al.*, 1997). The involvement of PI3K activity in CaR-mediated downstream signals suggests the possibility that the CaR may couple to a cell survival or differentiation program rather than a proliferative one in HEK293 cells. To ask whether the CaR can mediate a calcium-sensitive decrease in the basal rate of apoptosis in HEK293 cells, the TUNEL assays could be performed on stably transfected cells. Lin et al found that expression of the CaR in HEK293 cells could inhibit c-Myc-induced apoptosis in a calcium-dependent manner (Lin *et al.*, 1998), suggesting that the CaR does communicates positively with a cell survival mechanism. It is tempting to speculate that activation of PI3K by the CaR may be a critical aspect of the anti-apoptotic effects mediated by CaR activation in these c-Myc expressing cells. The involvement of the CaR in either calcium induced proliferation, differentiation, or escape from apoptosis could best be addressed utilizing the CaR knock-out mouse (Ho, et al., 1995). By generation of CaR+/+ and CaR-/- neonatal mouse fibroblasts and keratinocytes, the involvement of the CaR in calcium-induced keratinocyte differentiation, fibroblasts proliferation, and cellular survival could be rigorously investigated. The signaling mechanisms by which the CaR might mediate these varied responses could then be delineated. The mechanism and biological importance of the differential coupling of the CaR to intracellular signals is a future direction of investigations in this laboratory. The existence of two distinct pathways by which the CaR can activate ERK suggests a

potential mechanism by which activation of the CaR could couple to diverse calcium-dependent biological responses.

The in vivo role of the CaR in OSE cells is unknown. We have speculated that the CaR may be one of several mechanisms by which OSE proliferation might be prompted after rupture of the mature follicle, due to the increased calcium concentration of follicular fluid as compared to plasma ionized calcium. It is not yet known if disregulation of the CaR might play a role in tumor initiation or progression. OSE cells are often found encrypted in the stroma of the ovary, forming small epithelial cysts, and it has been hypothesized that it is the cells incorporated in these cysts, rather than the overlying ovarian surface epithelium, which may give rise to ovarian carcinoma. The OSE cells in these cysts would likely be subjected to a very different microenvironment which may vary significantly in local calcium concentration, either within the stroma of the ovary or in the lumen of the resultant cyst. Changes in the calcium concentration of the microenvironment may play a role in the growth regulation of these encrypted cells or may signal changes in CaR expression levels. Further histological and functional studies will be needed to gain a better understanding of the CaR's role in the biology of the OSE and ovary.

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