Changes in the concentration of extracellular calcium can affect the balance between proliferation and differentiation in several cell types, including keratinocytes, breast epithelial cells, and fibroblasts. This report demonstrates that elevation of extracellular calcium stimulates proliferation-associated signaling pathways in rat fibroblasts and implicates calcium-sensing receptors (CaR) as mediators of this response. Rat-1 fibroblasts express CaR mRNA and protein and respond to known agonists of the CaR with increased IP$_3$ production and release of intracellular calcium. Agonists of the CaR can stimulate increased c-SRC kinase activity and increased extracellular signal-regulated kinase 1/mitogen-activated protein kinase activity. Both of the increases in SRC activity and mitogen-activated protein kinase activation are blocked in the presence of a nonfunctional mutant of the CaR, R796W. Proliferation of wild-type Rat-1 cells is sensitive to changes in extracellular calcium, but expression of the nonfunctional CaR mutant or inhibition of the calcium-dependent increase in SRC kinase activity block the proliferative response to calcium. These results provide evidence of a novel signal transduction pathway modulating the response of fibroblasts to extracellular calcium and imply that calcium-sensing receptors may play a role in regulating cell growth in response to extracellular calcium, in addition to their well known function in systemic calcium homeostasis.

Extracellular calcium is a potent mediator of the balance between proliferation and differentiation in a number of different cell types. In keratinocytes, breast epithelial cells, and intestinal epithelial cells, increased extracellular calcium promotes differentiation and inhibits proliferation (1–4). In contrast, human dermal fibroblasts are growth inhibited at low extracellular calcium concentrations and proliferate in response to elevated extracellular calcium (5). In keratinocytes, elevation of extracellular calcium is associated with an increase in c-SRC kinase activity (6) and with an increase in the amount of tyrosine-phosphorylated p62 detected in immunoprecipitates with anti-p120 rasGAP antibodies (7). The potential role of calcium-sensing receptors (CaR) in mediating this response in keratinocytes is supported by evidence of CaR mRNA expression in human keratinocytes (8) and by the observation that calcium ionophore cannot mimic the effects of elevated extracellular calcium on rasGAP-associated p62 (9).

A seven-transmembrane receptor capable of sensing millimolar changes in extracellular calcium has recently been cloned from bovine parathyroid (10) and rat kidney and brain (11, 12). Activation of this receptor with Ca$_{2+}$, Mg$_{2+}$, Ba$_{2+}$, or Gd$_{3+}$ results in the generation of IP$_3$ and the release of intracellular calcium when assayed in parathyroid cells, Xenopus oocytes, or transfected Chinese hamster ovary cells (10, 13, 14). Hereditary disruptions of systemic calcium homeostasis have been mapped to mutations in the human CaR gene (15). In particular, a mutation of arginine 796 to tryptophan in the third intracellular loop of the CaR was found to cause neonatal severe hyperparathyroidism when homozygous and hypocalcemic hypercalcemia when heterozygous (15). This CaR-R796W mutant was nonfunctional when assayed for Gd$_{3+}$-stimulated intracellular calcium release in Xenopus oocytes (15) and has been characterized as a “dominant negative” mutant when co-expressed with wild-type CaR in HEK293 cells or in human parathyroid cells (16).

In this report we show that Rat-1 fibroblasts express endogenous CaR mRNA and protein and that these cells respond to the CaR agonist Gd$_{3+}$ with an increase in IP$_3$ production and intracellular calcium release. Proliferation of Rat-1 cells is sensitive to changes in extracellular calcium concentration as shown by a marked increase in thymidine incorporation at 2.0 mM as opposed to 0.3 mM extracellular calcium. Stimulation of Rat-1 cells with the CaR agonist Gd$_{3+}$ resulted in increased c-SRC kinase activity and increased ERK1 kinase activity. Each of these responses was significantly inhibited in Rat-1 cells expressing the nonfunctional CaR-R796W mutant. Furthermore, inhibition of the calcium-mediated increases in SRC and ERK1 activity prevented the calcium-stimulated increase in proliferation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Rat-1 fibroblasts were grown at 37 °C in 5% CO$_2$, 95% air in DMEM (1.7 mM Ca$^{2+}$, BioWhittaker) supplemented with 10% bovine calf serum (Hyclone) and gentamycin (10 mg/ml). New cultures were started from frozen stocks every 4–6 weeks. For experiments where calcium concentration was specified, Hams F-12 medium (0.3 mM Ca$^{2+}$, BioWhittaker) was used and the calcium concentration was adjusted with calcium.

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1 The abbreviations used are: CaR, calcium-sensing receptor; ERK, extracellular signal-regulated kinase; IP$_3$, inositol triphosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; HBSS, Hank’s buffered saline solution; DMEM, Dulbecco’s modified Eagle’s medium; kb, kilobase(s); GST, glutathione S-transferase; EGF, epidermal growth factor.
chloride as indicated. When calcium concentrations lower than 0.3 mM were used, calcium levels were buffered with 1 mM EGTA, and additional calcium was added to obtain the desired calcium concentration. Whenever serum was present at extracellular calcium concentrations below 1.7 mM, the serum was pretreated with Chelex to remove divalent cations.

**RNA Isolation and Northern Blot Analysis**

Total RNA was purified as described previously (17), size fractionated by electrophoresis in 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Nytran, Schleicher & Schuell). The hybridization probe was a 3.7-kb XbaI/BamHI fragment from the full-length rat striatal CaR cDNA clone, pcDNA3.CaR (kindly provided by S. Snyder) (12) labeled with 32P by random primer extension. Hybridization was conducted at 42 °C in a 50% formamide hybridization solution. The blot was then washed 2 times in 2 × SSC at 42 °C and 2 times in 2 × SSC at 50 °C. Bound radioactivity was detected by PhosphorImager analysis (Molecular Dynamics) following a 16 h exposure of the phosphorimage screen.

**Immunoprecipitations**

Crude plasma membranes were isolated from Rat-1 cells essentially as described by Bai et al. (16). Cells were scraped in 1 ml of homogenization buffer (50 mM Tris, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) on ice. The cells were homogenized with 15 strokes of a 1.5-ml Dounce homogenizer. At room temperature, homogenates were centrifuged at 43,000 g for 10 min. The supernatant was subjected to centrifugation at 42,000 g for 1 h to pellet the plasma membrane fragments. The resulting pellet was resolubilized in homogenization buffer with 1% Triton X-100 added.

**Immunoblotting**

Proteins were size fractionated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore) by electroblotting. Membranes were blocked in 3% bovine serum albumin, 0.05% NaN3, 1% Triton X-100, 10 mM phenylmethylsulfonyl fluoride, and 1% aprotinin at 4 °C in 2.5 % Tris-buffered saline (0.05% Tween 20, 20 mM Tris, pH 7.5, 150 mM NaCl). Membranes were washed 3 times in TTBS, incubated with appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for at least 2 h, and washed extensively in TTBS. Bands were visualized by chemiluminescence (Renaissance, NEN Life Science Products). Films from at least three independent experiments were scanned where indicated, and densitometry analysis was performed using NIH Image.

**Measurement of Intracellular Calcium**

Cells were plated on 18-mm dishes containing a central section of optical glass, and grown overnight in the appropriate culture medium containing serum. Two hours prior to Fura-2 measurement, cells were transferred into Hank's buffered salt solution containing 0.5 mM Mg2+ (Mg-HBSS). Cells were exposed to 1 µM Fura-2/AM (Molecular Probes, Eugene, OR) for 30 min at 37 °C, then the Fura-2/AM-containing medium was removed and replaced with Mg-HBSS. Cells were incubated at room temperature for 20–40 min to allow de-esterification of the Fura-2/AM and then subjected to calcium imaging. Intracellular calcium concentrations were determined from emissions triggered from the emission of eosin measured at 510 nm following excitation at 340 and 380 nm. Images were collected in an integrating CCD camera and analyzed with the Double-Wavelength InCa program (software and hardware provided as an integrated system by Intracellular Imaging, Inc., Cincinnati OH). Measurements were collected from 20–30 individual cells per field.

**Measurement of Inositol Trisphosphate Production**

Rat-1 cells grown to confluence in 10-cm dishes were transferred into serum-free DMEM containing 3 µCi/ml myo-[3H]inositol and loaded for 48 h. 16 h prior to stimulation and harvest, the cells were transferred into serum-free myo-[3H]inositol-free Ham's F-12 (0.3 mM Ca2+). Cells were stimulated by the addition of 250 mM Gd3+ in the presence of 100 mM LiCl to inhibit IP3 turnover as described previously (18). 20 min after the addition of LiCl with or without Gd3+, cells were lysed, and the inositol phosphates were extracted and fractionated on Dowex-formate columns as described previously (18).

**Construction of Wild-type and Mutant CaR Expression Vectors**

pcDNA3-CaR(CaR)—The full-length 3.7-kb rat kidney CaR clone was excised from pCIS.CaR using XbaI/BamHI (12). This was subcloned into pBluescript II for ease of manipulations. The full-length 3.7-kb rat CaR construct was purified with affini- ty BioReagents (Golden, CO). The monoclonal anti-phosphorysine antibody (4G10) was a generous gift from Brian Druker (Oregon Health & Science University). The monoclonal anti-p62 (2C4) antibody was a generous gift from Richard Roth (Stanford University).

**Transfections**

Rat-1 cells were transfected with either pcDNA3 alone, pcDNA3-CaR, or pcDNA3-CaR-R796W using Lipofectin as described previously (19). Cells were selected in medium containing 700 µg/ml G418 (BioWhittaker), and stable clones were cultured in 300 µg/ml G418 to maintain selection. New cultures were started from frozen stocks every 6–8 weeks.

**Kinase Assays**

In vitro kinase assays were conducted on immunoprecipitates as described previously (17). Confluent Rat-1 cells in 10-cm plates were cultured in serum-free Ham's F-12 (0.3 mM Ca2+) for 4 h and then brought to the indicated concentration of Ca2+ or Gd3+. Cells were stimulated for 10 min prior to lysis in 750 µl HEPES-KOH lysis buffer (20 mM HEPES-KOH, 2 mM EGTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin). Lysates were cleared by addition of 10 µl of protein A-agarose and centrifugation. Aliquots of cleared lysates normalized for protein content were subjected to immunoprecipitation overnight at 4 °C using either anti-ERK1 (Santa Cruz Biotechnology) or anti-c-SRC (Upstate Biotechnology, Inc.), followed by addition of protein A-agarose and an additional 2 h of incubation at 4 °C. Immunoprecipitates were recovered by collection and washing in HEPES-KOH buffer, once in LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM vanadate, 0.4 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin), lysates were cleared by addition of 10 µl of protein A-agarose and centrifugation. Aliquots of cleared lysates normalized for protein content were subjected to immunoprecipitation overnight at 4 °C using either anti-ERK1 (Santa Cruz Biotechnology) or anti-c-SRC (Upstate Biotechnology, Inc.), followed by addition of protein A-agarose and an additional 2 h of incubation at 4 °C. Immunoprecipitates were recovered by collection and washing in HEPES-KOH buffer, once in LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM vanadate, 0.4 mM phenylmethylsulfonyl fluoride) and once in MOPS assay buffer (20 µM MOPS, pH 7.2, 20 mM MgCl2, 2 mM EGTA, 2 mM dithiothreitol, 0.2% Triton X-100). The pellets were resuspended in 20 µl of kinase assay buffer (10 mM MOPS, pH 7.2, 20 mM MgCl2, 2 mM EGTA, 1 mM dithiothreitol, 0.1% Triton X-100, 1 µCi [γ-32P]ATP) and incubated at 30 °C for 20 min. In ERK1 kinase assays, 3 µg of GST-Elk1 was added as a substrate for phosphorylation. SRC kinase assays measured autophosphorylation of c-SRC. Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis in 12% acrylamide gels. Gels were dried, and radioactivity was visualized and quantitated with a Molecular Dynamics PhosphorImager and IP LabGel software.
Calcium Receptor-mediated Proliferative Signals

Rat-1 fibroblasts in 12-well plates were grown to 70–80% confluence then made quiescent in serum-free Ham's F-12 medium for 24 h. Where indicated, cells were either changed into high calcium medium (2.0 mM Ca\(^{2+}\); □) or changed into fresh 0.3 mM Ca\(^{2+}\) (■) or fresh 0.3 mM Ca\(^{2+}\) plus 5 ng/ml EGF (□) for 18 h prior to addition of [\(^{3}\)H]thymidine (0.1 \(\mu\)Ci/ml). Cells were harvested for 4 h after addition of [\(^{3}\)H]thymidine and thymidine incorporation determined as described under “Experimental Procedures.” The results shown are mean ± S.D. normalized to cell number, n = 4; similar results were observed in three replicate experiments.

Incorporation of [\(^{3}\)H]thymidine
Rat-1 fibroblasts in 12-well plates were grown to 70–80% confluence in DMEM + 10% calf serum. Cells were then changed into serum-free Ham’s F-12 medium (0.3 mM Ca\(^{2+}\)) for 24 h prior to the addition of either CaCl\(_{2}\) to 2.0 mM or EGF to 1 ng/ml. [\(^{3}\)H]Thymidine (0.1 \(\mu\)Ci/ml) was added 18 h later, and cells were harvested after a 4-h thymidine incorporation. Thymidine incorporation was determined by precipitation in 10% trichloroacetic acid, solubilization in 0.2 \(\mu\)l NaOH, and liquid scintillation counting.

RESULTS
Effect of Extracellular Calcium on Proliferation of Rat-1 Fibroblasts
Although the role of extracellular calcium in modulating the proliferation of keratinocytes is well established, less is known about the responsiveness of mesenchymal cell types to extracellular calcium. To determine whether Rat-1 cells resembled human diploid fibroblasts in displaying a positive mitogenic response to increasing extracellular calcium (5), we measured [\(^{3}\)H]thymidine incorporation in Rat-1 fibroblasts as a function of extracellular calcium concentration. As shown in Fig. 1, changing the extracellular calcium concentration from 0.3 to 2.0 mM produced an 8.4-fold increase in [\(^{3}\)H]thymidine incorporation in Rat-1 cells; the extent of the increase was equivalent to that observed when EGF at 0.5 ng/ml was added in the presence of 0.3 mM extracellular calcium (Fig. 1, white bar).

Expression of Functional CaR in Rat-1 Fibroblasts
Activation of calcium-sensing receptors similar or identical to those expressed in parathyroid cells, neurons, and intestinal epithelial would provide a potential mechanism for inducing proliferative signals in response to changes in extracellular calcium. To detect the presence of CaR protein, we generated a polyclonal antibody against a synthetic peptide representing amino acids 11 through 27 in the extracellular domain of the rat CaR. Antibodies generated against the same region of the rat CaR were effective in both immunohistochemical and immunoblot studies of CaR expression in the brain (12). The anti-CaR antibody was affinity purified against the synthetic peptide and used in immunoblot experiments with partially purified plasma membrane preparations from rat kidney and Rat-1 fibroblasts (Fig. 2A). The affinity purified antibodies detected an identical pattern of strongly hybridizing bands at 120–140 kDa in both kidney and fibroblasts. Rat kidney was used as a positive control as both Riccardi et al. (11) and Ruat et al. (12) have shown high levels of CaR expression in rat kidney. Preincubation with the synthetic peptide prevented detection of the putative CaR protein as shown in Fig. 2A. The anti-CaR antibody was also effective in immunohistochemical detection of CaR protein in fixed Rat-1 cells (Fig. 2B), and specificity was demonstrated by a loss of staining when excess synthetic CaR peptide was present during the antibody incubation (Fig. 2B).

The identity of the CaR immunoreactive protein was confirmed by Northern hybridization analysis and reverse transcription-polymerase chain reaction. Two bands of approximately 5.3 and 3.8 kb were observed following hybridization with a full-length CaR probe; CaR mRNA levels were not influenced by extracellular calcium concentration (data not shown). Sequence analysis of an 800-base pair reverse transcription-polymerase chain reaction product obtained from Rat-1 fibroblasts using primers flanking the conserved transmembrane domain indicated that the Rat-1 CaR was 100% identical to the rat kidney CaR between amino acids 627 and 724 (Ref 11; data not shown).

Release of Intracellular Ca\(^{2+}\) in Response to Agonists of the CaR—To determine whether the presence of CaR protein in Rat-1 cells represented the presence of functional receptors, we tested the ability of Rat-1 fibroblasts to release intracellular Ca\(^{2+}\) in response to extracellular Gd\(^{3+}\) as used by Nemeth, Brown, and others (10, 11, 15, 20) to detect functional CaR. When Rat-1 cells were exposed to 1.0 mM Gd\(^{3+}\) in Mg-HBSS, many of the cells within a given field responded with an increase in intracellular calcium of at least 3-fold over basal intracellular Ca\(^{2+}\) levels from a resting level of 100 nM to a peak of 250–300 nM (Fig. 3A). The magnitude of this response is similar to that observed following treatment with thapsigargin and is sufficient to promote calcium-dependent gene expression (17). In contrast, treatment with 1 \(\mu\)mol ionomycin produced intracellular calcium peaks of 900–1000 nM (data not shown). In six independent experiments on Rat-1 fibroblasts, 25–33% of the cells in a given field responded to extracellular Gd\(^{3+}\) with a release of intracellular Ca\(^{2+}\). Similar results were obtained in response to 2 mM Ca\(^{2+}\) (data not shown). In each of these experiments, no cells responded to addition of Mg-HBSS alone, indicating that the response was specific to the presence of Gd\(^{3+}\) or Ca\(^{2+}\).

Insitol Triphosphate Production in Response to Activation of the CaR—Activation of the CaR has been demonstrated to result in increased production of IP\(_{3}\) (10, 21). To determine whether the CaR agonist Gd\(^{3+}\) could induce IP\(_{3}\) production in rat fibroblasts, we measured IP\(_{3}\) production directly in response to 250–300 nM Gd\(^{3+}\) as described previously (18). Fig. 3B shows that stimulation with Gd\(^{3+}\) produced a significant increase (p < 0.05, n = 3) in IP\(_{3}\) production in the Rat-1 cells, documenting a second aspect of established CaR function in these cells.

Effect of CaR Activity on c-SRC Kinase Activity
Elevation of extracellular calcium levels has been associated with increased c-SRC kinase activity in keratinocytes (6). If the CaR is responsible for modulating c-SRC activity in response to changes in extracellular calcium, then an increase in SRC kinase activity should be observed in the presence of Gd\(^{3+}\), which binds and activates the CaR without passing through calcium channels. If activation of the CaR is required for Ca\(^{2+}\)- and Gd\(^{3+}\)-induced activation of SRC, expression of an interfering CaR mutant (such as the R796W mutant associated with severe neonatal hyperparathyroidism; see Refs. 16 and 22)
CaR protein expression. Panel A, immunoblot detection of CaR protein in rat kidney and fibroblasts. Fresh rat kidney and Rat-1 fibroblasts were processed for plasma membrane proteins as described previously (28). Equal amounts of protein (30 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis, blotted, and incubated with anti-CaR antibody as described under "Experimental Procedures." In the two lanes on the right, incubation with anti-CaR antibody occurred in the presence of 50 μg/ml blocking peptide. The arrow indicates the position of a strongly immunoreactive band at approximately 120 kDa. Panel B, immunohistochemical detection of CaR protein in Rat-1 fibroblasts. Rat-1 cells fixed in 4% paraformaldehyde were incubated overnight at 4 °C with a 1:1000 dilution of anti-CaSR (left panel) or anti-CaSR plus blocking peptide at 50 μg/ml (right panel). The second antibody was goat anti-rabbit conjugated to horseradish peroxidase, and 3,3'-diaminobenzidine was used as the chromophore. No staining was seen in the presence of secondary antibody alone (data not shown).

CaR expression. Functional evidence of CaR expression. Panel A, Fura-2 measurements of intracellular calcium levels were conducted as described under "Experimental Procedures." Basal calcium concentrations were determined in cells that had been cultured in calcium-free conditions (Mg-HBSS) for 2–4 h. At the times indicated by the arrows, culture medium was brought to 1 mM Gd³⁺. A test application of one-tenth volume Mg-HBSS was made as a control for nonspecific responses to agonist addition. Shown are traces from two individual Rat-1 cells showing a peak response to Gd³⁺ of approximately 300 nM intracellular calcium. Panel B, IP₃ production in response to Gd³⁺. Rat-1 cells were loaded with myo-[³²P]inositol as described under "Experimental Procedures." After 16 h in low calcium (0.3 mM) medium, cells were exposed to 250 μM Gd³⁺ for 20 min (T). LiCl (100 mM) was present for 20 min prior to cell harvest. Inositol phosphates were extracted and quantified as described (18). Results are mean ± S.D., n = 3. Similar results were obtained in three replicate experiments.

should inhibit the activation of c-SRC. We tested this possibility by measuring SRC kinase activity in the Rat-1 cells stably transfected with either pcDNA3 alone, pcDNA3-CaR, or pcDNA3-CaR-R796W. A 2- to 3-fold increase in immunoreactive CaR protein was detected in the pcDNA3-CaR and pcDNA3-CaR-R796W. A 2- to 3-fold increase in immunoreactivity by measuring SRC kinase activity in the Rat-1 cells stably transfected with either pcDNA3 alone, pcDNA3-CaR, or pcDNA3-R796W-transfected Rat-1 cells (data not shown). The results of these experiments indicate that the endogenous CaR in Rat-1 cells has the potential to activate c-SRC as a consequence of Gd³⁺ binding and that this ability is disrupted in the presence of the R796W mutant.

Effect of CaR Activity on Tyrosine Phosphorylation in Rat-1 Cells

Increased c-SRC kinase activity in response to elevated extracellular Ca²⁺ should be accompanied by an increase in the tyrosine phosphorylation of at least some c-SRC substrates. If the calcium-sensitive increase in c-SRC activity is mediated by the CaR then Gd³⁺ should be able to mimic the effects of Ca²⁺, and expression of the nonfunctional CaR mutant R796W should inhibit calcium-sensitive phosphorylations. To test these hypotheses, we analyzed changes in tyrosine phosphorylation in whole cell lysates obtained from Rat-1 cells transfected with either vector, wild-type CaR, or the R796W CaR mutant. As shown in Fig. 5A, increasing extracellular Ca²⁺ from 0.3 to 1.8 mM was associated with an increase in the tyrosine phosphorylation of proteins with apparent molecular masses of approximately 125–135, 62–65, and 41 kDa in control and CaR-transfected cells. Stimulation with Gd³⁺, rather than Ca²⁺, resulted in increased phosphorylation of the same or similar proteins (data not shown). Tyrosine phosphorylation of the 125–135- and 62–65-kDa bands was significantly inhibited in cells expressing the R796W mutant CaR (Fig. 5A). This
result suggests that the 62–65- and 125–135-kDa bands may represent proteins phosphorylated in response to the extracellular calcium-stimulated activation of SRC.

To identify the proteins showing increased tyrosine phosphorylation, we used a combination of immunoprecipitation and immunoblotting with antibodies specific for candidate proteins. When antiphosphotyrosine immunoprecipitates obtained from Rat-1 cells under low and high calcium conditions were immuno­blotted with a monoclonal anti-FAK antibody (F15020, Transduction Laboratories, Inc.), increased FAK immunoreactivity was observed in immunoprecipitates from cells treated with 2 mM Ca2+, compared with 0.3 mM Ca2+ (Fig. 5B). These data suggest that p125FAK is a potential substrate of CaR-stimulated SRC activation.

The protein band of approximately 63–65 kDa could represent any of three proteins that are known to be tyrosine phosphorylated in response to various stimuli. These three proteins are p68Sam, a known SRC substrate (23), p62dok, a supposed SRC substrate that associates with p120 rasGAP (24), and a protein of approximately 65 kDa that shows increased tyrosine phosphorylation in response to extracellular calcium (25). Immunoprecipitation with antibodies specific for p68Sam and p62dok, respectively, followed by immunoblotting with antiphosphotyrosine antibodies indicated that neither of these proteins showed an increase in tyrosine phosphorylation in response to extracellular calcium (data not shown). As no antibodies are currently available for study of the calcium-associated p65 protein, we were unable to test the phosphorylation status of this protein directly. Since the calcium-responsive p65 protein was originally identified in Rat-1 cells (25), it is possible that the 63–65-kDa protein showing CaR-dependent increases in tyrosine phosphorylation in Fig. 5A is the same protein identified by Medema et al. (25).

**CaR Activation and ERK1 Kinase Activity**

The observation of increased c-SRC kinase activity and increased tyrosine phosphorylation of specific proteins in response to elevated extracellular calcium raised the possibility that downstream proliferation-associated signaling events were also stimulated in a CaR-dependent fashion. To obtain a direct measurement of the effects of the CaR on an important proliferation-associated pathway, we measured changes in mitogen-activated protein kinase activity in response to extracellular Ca2+ and Gd3+ using in vitro kinase assays with immunoprecipitated ERK1 and GST-Elk1 as a substrate. As shown in Fig. 6, control Rat-1 cells displayed a 10- to 25-fold increase in ERK1 kinase activity in response to either 1 mM Ca2+ or 100 μM Gd3+. By comparison, EGF treatment produced a 48- to 68-fold increase in ERK1 activity. Rat-1 cells transfected with the R796W mutant CaR showed a nearly complete inhibition of ERK1 kinase activity in response to either Gd3+ or Ca2+ (Fig. 6B); ERK1 activation in response to EGF was also reduced in these cells but remained significantly higher than control values (p < 0.001). These results suggest that the extracellular calcium-dependent changes in SRC activity may be associated with activation of mitogen-activated protein kinase signaling pathways and that these pathways are disrupted in the presence of the mutant CaR. The observation of decreased ERK1 kinase activity in response to EGF in the presence of overexpressed mutant R796W-CaR suggests that cross-talk may exist between the CaR and the EGF receptor, as has been previously documented between the endothelin receptor and the EGF receptor (26, 27). It is possible that the R796W-CaR may be inhibiting the activity of intermediate proteins involved in signaling from the EGF receptor.

**Role of c-SRC in Calcium-dependent Activation of ERK1**

If the CaR-dependent activation of c-SRC is an essential component of the signal transduction pathway leading to a calcium-dependent increase in proliferation and mitogen-activated protein kinase activity in Rat-1 cells, then inhibition of c-SRC activity should significantly reduce or prevent the extracellular calcium-dependent increase in ERK1 activity and...
Calcium Receptor-mediated Proliferative Signals

Fig. 6. CaR-dependent activation of ERK1. Rat-1 cells stably transfected with either the pcDNA vector (wild-type CaR) or pcDNA-CaR-R796W (R796W) were grown to 90% confluence and then made quiescent in serum-free DMEM for 24 h. Cells were changed into low calcium medium (0.3 mM Ca$^{2+}$) for 4 h prior to addition of agonists as indicated. Cells were harvested 10 min after agonist addition, and lysates containing 200 μg protein were immunoprecipitated with anti-ERK1 (Santa Cruz Biotechnology). In vitro kinase assays using GST-Erk1 as the substrate were performed as described (17), products were resolved by SDS-polyacrylamide gel electrophoresis, and the dried gel was imaged by PhosphorImager. Treatments were as follows: control, no additions; Ca$^{2+}$, added Ca$^{2+}$ to 2 mM final concentration; Gd$^{3+}$, 100 μM Gd$^{3+}$; EGF, 10 ng/ml. Panel A, normalized PhosphorImager data to immunoprecipitated ERK1. Results represent mean and standard deviation of two independent experiments.

Rat-1 proliferation. This hypothesis was tested by measuring ERK1 kinase activity and thymidine incorporation in Rat-1 cells stimulated with 2.0 mM Ca$^{2+}$ in the presence or absence of herbimycin, a tyrosine kinase inhibitor with selectivity for c-SRC (28). As shown in Fig. 7, treatment with herbimycin (200 ng/ml) inhibited both the increase in ERK1 kinase activity (open bars) and the increase in thymidine incorporation (widely hatched bars) induced by elevated extracellular calcium. The values observed in the presence of herbimycin were approximately 20% of control values (Fig. 7). These data indicate that activation of c-SRC or a related cytoplasmic tyrosine kinase is required for the CaR-mediated activation of proliferative signals involving ERK1 activation. Neither wortmannin nor pertussis toxin could inhibit the calcium-induced activation of ERK1 (data not shown), implying that neither pertussis toxin-sensitive G proteins nor phosphatidylinositol-3 kinases play an important role in signaling from the CaR in Rat-1 cells.

To determine whether activation of ERK kinases was required for increased proliferation of Rat-1 cells in response to agonists of the CaR, we measured thymidine incorporation in Rat-1 cells as a function of extracellular calcium concentration in the presence or absence of PD98059, a specific inhibitor of the mitogen-activated protein kinase kinase MEK1 (29). PD98059 effectively inhibited the increase in the thymidine incorporation that is normally observed in response to elevated extracellular calcium (Fig. 7, closely hatched bars). In contrast, the ability of EGF to stimulate thymidine incorporation was only partially reduced in the presence of PD98059 (approximately 75% of control values). This result indicates that MEK1-dependent activation of ERK kinases is an essential component of the signaling mechanism leading from CaR activation to increased proliferation of Rat-1 cells.

DISCUSSION

In this report we provide evidence demonstrating expression of functional CaR on fibroblastic cells. The physical presence of CaR on rat fibroblasts was established by immunodetection of CaR protein with specific anti-CaR antibodies and by Northern hybridization analysis. Sequence analysis of reverse transcription-polymerase chain reaction products representing the conserved transmembrane domain indicates that the fibroblast CaR is identical in sequence to the kidney and brain CaR, at least in this subdomain. Production of IP$_3$ and release of intracellular calcium in response to extracellular Ca$^{2+}$ or Gd$^{3+}$ provided pharmacological evidence that the immunoreactive protein represented functional receptors.

We also present data bearing on the biological function of the CaR in fibroblastic cells. We show that Rat-1 cells respond to elevated extracellular Ca$^{2+}$ or Gd$^{3+}$ with an increase in the activity of proliferation-associated signaling events, including activation of the mitogen-activated protein kinase ERK1. Primary dermal fibroblasts have been shown to increase thymidine incorporation in response to elevated extracellular Ca$^{2+}$ (5); in this report we demonstrate a similar response in Rat-1 fibroblasts. These results indicate that extracellular Ca$^{2+}$ can have cell-type specific effects on proliferative pathways, stimulating increased proliferation in fibroblasts (this report and Ref. 5) while inhibiting proliferation in keratinocytes (1, 2). Opposing effects of a common agonist on fibroblasts and keratinocytes is not unknown; the ability of transforming growth factor β to stimulate proliferation in fibroblasts while inhibiting proliferation in keratinocytes (30, 31) presents an established example.
The mechanisms by which changes in extracellular Ca$^{2+}$ signal a change in the proliferation of keratinocytes have not yet been established. Exposing keratinocytes to elevated extracellular Ca$^{2+}$ is associated with an increase in the kinase activity of c-SRC and a decrease in the activity of c-YES (6, 32); the mechanism producing this response has not been established. An increase in the apparent phosphorylation of an approximately 62-kDa protein co-immunoprecipitated with anti-rasGAP antibodies has also been demonstrated and attributed to activation of a calcium-binding receptor as opposed to Ca$^{2+}$-influx (7, 9). However, these data remain correlative as it has not yet been shown that disrupting these responses to elevated Ca$^{2+}$ alters the antiproliferative response of keratinocytes to extracellular Ca$^{2+}$.

We have shown that many of these same signaling events occur in Rat-1 fibroblasts exposed to elevated extracellular Ca$^{2+}$ or Gd$^{3+}$. Specifically, we have shown that these treatments are associated with an increase in the kinase activity of c-SRC and an increase in ERK1 kinase activity. Furthermore, we have used the nonfunctional CaR mutant R796W as a tool to disrupt the function of the endogenous CaR. In addition to its identification as the genetic mutation responsible for at least one form of hereditary severe neonatal hyperparathyroidism (15), CaR-R796W has been shown to function as an interfering receptor for stimulation of CaR-mediated activation of Gi, although this remains correlative as it has not yet been shown that disrupting these responses to elevated extracellular calcium to induce chemotaxis in osteoblasts is sensitive to either wortmannin or pertussis toxin. These results support the existence of a proliferative pathway linking the CaR to activation of SRC and ERK1 and provide a potential mechanism for the known ability of extracellular calcium to modulate proliferation in a variety of cell types. 

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