Calcium-Sensing Receptor-Mediated ERK1/2 Activation Requires Gαi2-Coupling and Dynamin-Independent Receptor Internalization

Deborah M. Holstein†, Kelly A. Berg‡, L.M. Fredrik Leeb-Lundberg‡, Merle S. Olson†, Christine Saunders*
*Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232-6600
Departments of Biochemistry‡ and Pharmacology‡
University of Texas Health Science Center at San Antonio
San Antonio, Texas 78229-3900

Running Title: Calcium-Sensing Receptor and MAP kinase

Correspondence to: Christine Saunders, Ph.D.
Department of Pharmacology
Vanderbilt University Medical Center
417B Preston Research Building
Nashville, TN 37232-6600
Phone: (615) 936-3771
Fax: (615) 322-6379
E-mail: christine.saunders@vanderbilt.edu
The calcium-sensing receptor (CaR) recently has been shown to activate MAP kinase (ERK1/2) in various cell types as well as in heterologous expression systems. In this study, we show that the CaR agonist NPS R-467 (1 µM), which does not activate the CaR by itself, robustly activates ERK1/2 in the presence of a low concentration of Ca$^{2+}$ (0.5 mM CaCl$_2$) in HEK cells permanently expressing the human CaR (HEK-hCaR). Ca$^{2+}$ (4 mM) also activates ERK1/2, but with differing kinetics. CaR-dependent ERK1/2 activation begins to desensitize to 4 mM Ca$^{2+}$ after 10 minutes, whereas there is no desensitization to NPS R-467/CaCl$_2$ as late as 4 hours. Moreover, recovery from desensitization occurs as rapidly as 30 minutes with 4 mM CaCl$_2$. Pretreatment of HEK-hCaR cells with concanavalin A (250 µg/ml) to block CaR internalization completely eliminated the NPS R-467/CaCl$_2$-mediated ERK1/2 activation, but did not block the 2 minute time point of 4 mM Ca$^{2+}$-mediated ERK1/2 activation. Neither dominant-negative dynamin (K44A) nor dominant-negative β-arrestin inhibited ERK1/2 activation by either CaR agonist treatment, suggesting that CaR-elicited ERK1/2 signaling occurs via a dynamin-independent pathway. Pertussis toxin pretreatment partially attenuated the 4 mM Ca$^{2+}$-ERK1/2 activation; this attenuated activity was completely restored by co-expression of the Ga$_{i2}^{C351I}$, but not Ga$_{i1}^{C351I}$ or Ga$_{i3}^{C351I}$ G proteins, PTX insensitive G protein mutants. Taken together, these data suggest that both 4 mM Ca$^{2+}$ and NPS R-467/CaCl$_2$ activate ERK1/2 via distinguishable pathways in HEK-hCaR cells, and may represent a nexus to differentially regulate differentiation vs. proliferation via CaR activation.
INTRODUCTION

The extracellular calcium-sensing receptor (CaR) was cloned using mRNA from the bovine parathyroid gland in 1993 (1). Since then, CaR expression has been described in other organs as well, including the thyroid, kidney (2,3), brain (3), intestine (3), testis, lung (except in mice) (4,5) and pancreas (6) as well as in lens epithelial cells (7), keratinocytes (8), fibroblasts, osteoblasts (9,10) and osteoclasts (11). The CaR is part of the GPCR family C, all of which have large extracellular domains responsible for ligand binding. Other class C GPCR include the metabotropic glutamate receptors, GABA_B receptors, and a subset of pheromone, olfactory and taste receptors.

The CaR is activated by millimolar calcium (as well as some other cations such as Mg^{2+}, Ba^{2+}, Gd^{3+}) and polyamines (e.g. spermine and neomycin) (12). The CaR activates phospholipase C to produce inositol 1,4,5-trisphosphate (IP_3) which mobilizes intracellular calcium stores to increase intracellular calcium (13). The CaR-mediated PI-PLC activation in parathyroid cells and HEK-hCaR has been reported to be pertussis toxin-insensitive and therefore G_{q/11}-mediated (14). The CaR also has been reported to activate K^+ channels (15) and produce a pertussis toxin-sensitive (G_i) inhibition of agonist-stimulated cAMP accumulation in some cells (16). These findings suggest that the CaR can couple to more than one population of G proteins.

The mitogen-activated protein kinase, MAP kinase, pathways have been described to regulate a number of cell processes such as cell growth, differentiation, apoptosis, oncogenetic transformation and enzyme activity (17). These include the extracellular signal-regulated kinases (ERKs) that are stimulated by agonist binding to GPCR and tyrosine kinase growth factor receptors, as well as by mechanical stress in many cell types. The CaR has been shown to
activate MAP kinases in a number of different cell types, including ERK1/2 in fibroblasts (18), osteoblasts (10) and their respective cell lines (9), parathyroid cells (19), ovarian surface epithelia (20,21), and in a variety of cultured cell lines (19,22-28). Ca²⁺-stimulated activation of ERK1/2 by way of the CaR has been reported to occur via both pertussis toxin-sensitive (G_i) and -insensitive (G_q/11) pathways (19). It is possible that the mechanisms, and the physiologic outcomes, differ depending on whether CaR-stimulated ERK1/2 activation is mediated via G_i or G_q/11.

The CaR specific agonist, NPS R-467, is a phenylalkylamine calcimimetic, which activates the CaR by allosterically increasing the affinity of the CaR for calcium (29,30). It has been determined that the dose-response curve for the ability of calcium to activate down-stream signaling cascades (intracellular calcium mobilization, ERK1/2 activation) in cells expressing the CaR is shifted to the left at least 10 fold in the presence of NPS R-467 (31). Despite the fact that numerous reports in the past several years have described CaR-mediated ERK1/2 activation and responses in several cells, little is know about the kinetics and dynamics by which ERK1/2 activation occurs. Furthermore, most have used polyvalent cations as the agonist instead of specific calcimimetics such as NPS R-467 and NPS R-568. The present studies report the novel finding that the properties of CaR-mediated ERK1/2 activation in HEK-CaR cells differ depending on the ligand stimulating the GPCR, NPS R-467/CaCl₂ or 4 mM Ca²⁺ alone. Our results show that high extracellular calcium causes ERK1/2 desensitization whereas stimulation with NPS R-467 does not. We also demonstrate that, independent of the agonist, CaR-mediated ERK1/2 activation is dependent on receptor internalization, which occurs via a dynamin- and β-arrestin-independent mechanism. Furthermore, we show for the first time that Gα_i2, but not Gα_i1 or Gα_i3, is responsible for the pertussis toxin-sensitive component of the 4 mM calcium-
mediated ERK1/2 activation. Thus, there appears to be a unique pharmacology for activation of ERK1/2 by the CaR that might lead to unraveling the pleiotropic mechanisms for this signaling protein.
MATERIALS AND METHODS

Cell Culture – A clonal HEK-293 cell line stably expressing the human CaR (transfected with the cDNA for the human parathyroid calcium-sensing receptor) was kindly donated by NPS Pharmaceuticals. Cells were grown in high glucose DMEM (Gibco #11965-092) containing 10% fetal bovine serum, 0.8 mM L-glutamine (Gibco #25030-081), 200 µg/ml hygromycin B (Boehringer-Mannheim #843555, 50 mg/ml), and Penn/strep (Life Technologies, cat# 15140). The human wild-type and K44A dominant negative dynamin I were kindly provided by Dr. Marc Caron, and the rat wild-type and dominant negative β-arrestin 2 were provided by Dr. Jeff Benovic.

MAP kinase stimulation – Activation of MAP kinase was assessed as previously described for HEK 293 cells (32) with a few alterations. Clonal lines were plated in 35 mm plastic dishes (Falcon) at 50% confluency and grown until reaching 70-80% confluency. The cells were serum-deprived overnight the day before the experiment. On the day of the experiment, the cell medium was removed and replaced with High Salt Glucose Buffer (HSGB) for 1.5 – 2 hours due to the potential contributions of the calcium in the cell culture medium. HSGB contains: 10 mM Hepes, pH 7.4, 140 mM NaCl, 4 mM KCl, 2 mM MgSO₄, 1 mM KH₂PO₄, and 10 mM glucose. After the pretreatment period (either concanavalin A or pertussis toxin), cells were stimulated with either 4 mM CaCl₂ or 1 µM NPS R-467 with 0.5 mM CaCl₂. For comparisons of the kinetics of ERK1/2 activation between 4 mM Ca²⁺ vs. the NPS agonist, experiments were done on the same day and with cells from the same passage number. After the indicated times, the drug medium solution was removed, rinsed once with PBS, and the cells lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% w/v SDS; 10% glycerol; 50 mM DTT) supplemented
with 1 mM sodium orthovanadate (Sigma), 10 U/ml leupeptin (Sigma), and 10 U/ml aprotinin (Bayer Corp., Kankakee, Ill). The lysates were transferred to an Eppendorf tube on ice, sonicated for 20 seconds, then placed in a heating block at 95°C for 5 minutes. The lysates were then centrifuged in a microcentrifuge at room temperature for 5 minutes to remove debris. The supernatants were assayed in Bio-Rad’s protein assay for relative protein concentration, and equivalent amounts of protein were loaded on a 10% SDS-polyacrylamide gel for electrophoresis and transfer onto nitrocellulose. MAP kinase (ERK1/2) activation was evaluated using an antibody which recognizes dually phosphorylated (Thr/Tyr) MAP kinase (Promega catalog #V6671) and normalized to total MAP kinase using an antibody which recognizes MAP kinase regardless of its phosphorylation state (NEB catalog #9102). The enhanced chemiluminescence (ECL) images were scanned into Adobe Photoshop with a HP Scanjet 6200C scanner.

**Western analyses for the CaR:** Lysates of the HEK-hCaR cells were prepared as described above, except without sodium orthovanadate. The lysate was centrifuged at 2000 rpm for 15 minutes, and the postnuclear supernatant was centrifuged at 13,500 rpm in a microcentrifuge for 30 minutes. The pellet from this spin was then resuspended in lysis buffer and comparable amounts of protein were used as determined by the Bradford protein assay. About 20 µg of the cellular membrane fraction was subjected to SDS-PAGE (10% gel). Proteins in the gel were transferred to a nitrocellulose, after which the membrane was blocked (4% BSA, 0.1% Tween 20, 0.1% gelatin in PBS) for one hour at room temperature. The anti-CaR antibody (rabbit anti-CaR antibody, Affinity Bioreagents, Golden, CO; catalogue # PA1-934) was then added at a dilution of 1:1000 in blocking buffer for 2 hours at room temperature. The membrane was washed with rinse buffer (10% goat serum and 0.2% Triton X-100 in PBS) 3 x 15 minutes, followed by incubation with goat anti-rabbit IgG conjugated to horseradish
peroxidase (1:5000 for 1 H at room temperature). After washing (3 x 15 minutes), the membrane was treated with ECL reagent (Amersham), covered in plastic wrap, and exposed to film to visualize the ECL signal.

**Blocking Receptor Internalization**

*Concanavalin A* - Cells were pretreated for 30 minutes with 250 µg/ml concanavalin A made up fresh in HSGB 10 minutes before use. EGF receptor-mediated ERK1/2 activation (25 ng EGF/ml for 5 minutes) was used as a positive control to demonstrate that the concanavalin A was able to block this signaling pathway of this tyrosine-kinase receptor pathway as previously demonstrated (33).

*Co-expression of Wild-type or K44A Dominant Negative Dynamin, or Wild-type or Dominant Negative β-arrestin 2* – The HEK-hCaR cells plated in 35 mm dishes were transiently transfected with either human wild-type or K44A dominant negative dynamin I in pCB1, or with wild-type or dominant negative β-arrestin 2 using the Polyfect® Transfection Reagent (Qiagen, catalogue # 301105) according to the handbook directions. Forty-eight hours after transfection, the cells were stimulated with agonists, and ERK1/2 activation was determined as described above. To confirm positive expression of both forms of transfected dynamin, membranes from both wild-type and K44A transfected cells were harvested, subjected to SDS-PAGE and Western blot analyses using an antibody directed against dynamin (Transduction Laboratories, Lexington, KY; catalogue # D25520) and compared to untransfected control cells.
Co-expression of PTX-resistant Gαi subunits – The cDNAs of individual pertussis-toxin insensitive Gαi subunits were transiently co-expressed using the Polyfect® Transfection Reagent as described above. The cDNA constructs that have the C351I mutation render these subunits (Gαi1, Gαi2 and Gαi3) insensitive to PTX treatment, as described previously (34).

Immunofluorescence analysis and confocal microscopy - All steps were done at room temperature while rocking on a rotating platform. The coverslips containing the cells (50 to 70% confluent, plated in 24 well plates) were washed twice with phosphate buffered saline (PBS: 154 mM NaCl, 11 mM Na2HPO4, 2.7 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, pH 7.4) fortified with extra calcium and magnesium (1.0 mM CaCl2, 0.5 mM MgCl2) constituting PBS/CM to facilitate the cells adhering to the poly-lysine coated glass coverslips. They were then fixed for 15 minutes with 4% paraformaldehyde made up fresh in PBS. Remaining paraformaldehyde was quenched with 50 mM NH4Cl made up fresh in PBS for 15 minutes. The cells were then rinsed twice with PBS/CM, permeabilized in 0.2% Triton X-100 made up in PBS for 10 minutes and blocked with 10% normal goat serum (NGS) diluted in PBS for one hour. The blocking solution was then aspirated off and the cells rinsed once with PBS. The coverslips were incubated with the primary antibody (rabbit anti-CaR antibody) at a dilution of 1:150 in 5% NGS for one hour. The primary antibody was aspirated off and the cells washed three times with PBS containing 0.05% Triton X-100 (PBST) for 10 minutes per incubation. After washing, the cells were incubated with secondary antibody (goat anti-rabbit Cy3, Jackson Immunolaboratories) diluted 1:500 in PBST for one hour. The secondary antibody was removed and the cells washed three times with PBST (10 minute incubations each), and once with PBS. Coverslips were then mounted onto slides with Crystal Mount (Biomeda Corp., Foster City, CA) and allowed to dry. Confocal microscopy
was performed using a BioRad MRC1024 (BioRad Laboratories, Hercules, CA) confocal imaging system operated via a Nikon Diaphot (Nikon Inc., Garden City, NY) inverted microscope. Sample illumination was via a Krypton-Argon laser using 568 nm excitation and a 598 ± 40 nm emission filter. Lasersharp acquisition software (BioRad) allowed for z-resolution imaging at either 0.5 or 1 µm increments.
RESULTS

ERK1/2 is activated by CaR agonists – Four mM Ca\(^{2+}\) rapidly and robustly activate ERK1/2 in HEK-293 cells which express the human CaR (Mr \~120-140\) kDa due to differential glycosylation states, Fig. 1A, and immunofluorescence, Fig. 1B). The lack of ERK1/2 activation in HEK-293 cells not heterologously expressing the CaR (Fig. 1A) indicates that the ERK1/2 signaling in this cell line (19) is CaR-mediated. Interestingly, the time course of ERK1/2 activation was delayed when NPS R-467 was used to activate CaR when compared to activation by 4 mM Ca\(^{2+}\). As seen in Fig. 1B, 1 \(\mu\)M NPS R-467 in the presence of 0.5 mM CaCl\(_2\) did not achieve maximal activity until 10 minutes after presenting the stimulus to the cells, instead of the usual 2 minute maximal responses seen with 4 mM CaCl\(_2\) alone. The NPS R-467 agonist did not activate ERK1/2 activity in cells not expressing the CaR (Fig. 1B), nor in the absence of added CaCl\(_2\) (data not shown).

Lack of ERK1/2 desensitization in HEK-hCaR cells with the NPS R-467 agonist vs. Ca\(^{2+}\) alone – Consistently, we observed that 4 mM Ca\(^{2+}\) stimulated ERK1/2 was reduced at 60 minutes to a level slightly above baseline, and down to baseline by 4 hours (Fig. 2A). In contrast, there was no significant desensitization to CaR stimulation of ERK1/2 phosphorylation when 1 \(\mu\)M NPS R-467 in the presence of 0.5 mM CaCl\(_2\) were used as agonists, even as late as 240 minutes after addition of stimulus (Fig. 2B). A densitometric analysis of the data obtained from four to six separate experiments comparing the 10 and 60 minute time points for ERK1/2 activation in figure 1B reveals that there is significant desensitization with the endogenous ligand, calcium, for the CaR, whereas there is no desensitization at 60 minutes with NPS R-467. The desensitization observed with 4 mM calcium undergoes rather rapid resensitization (Fig. 2C).
Thus, a 10 minute stimulation with 4 mM CaCl₂ alone, followed by a 30 minute wash period, demonstrated a full recovery of calcium to activate ERK1/2, with the same kinetics as before prior agonist exposure. These findings suggest that this GPCR rapidly recovers from desensitization, perhaps by a mechanism involving rapid recycling.

Blocking Receptor Internalization Inhibits CaR Agonist-mediated ERK1/2 Activation – Numerous studies have shown that some GPCR require internalization to activate ERK1/2. To determine whether CaR endocytosis is required for ERK1/2 activation, we exploited several independent experimental strategies used to inhibit recruitment to clathrin-coated pits (pretreatment of concanavalin A, which prevents internalization of membrane-associated receptors by stabilizing the surface integrity due to tetravalent lectin contacts (35)) and dynamin and/or arrestin-dependent internalization (co-expression with either dominant negative (K44A) dynamin 1 or dominant negative β-arrestin). Treatment with concanavalin A revealed additional differences in CaR activation by 4 mM Ca²⁺ vs. activation by NPS R-467/CaCl₂. As seen in Fig. 3A, a thirty minute pretreatment of the HEK-hCaR with 250 µg/ml concanavalin A completely eliminates the NPS R-467/0.5 mM CaCl₂-induced ERK1/2 activation. Hypertonic sucrose pretreatment, which also perturbs the clathrin-coated pit internalization pathway, also blocked the ability of CaR to activate ERK1/2 when stimulate with NPS-R467/CaCl₂ (data not shown). When the HEK-hCaR cells were stimulated with 4 mM CaCl₂, pretreatment with concanavalin A only eliminated the later time points of ERK1/2 activation (Fig. 3B), whereas the 2 minute time point of ERK1/2 activation remained intact. This observation may represent a bifurcation in the CaR-mediated ERK1/2 signaling pathway in HEK-hCaR cells, since 2 minutes is sufficient to robustly activate ERK1/2 with calcium alone, yet may not be ample time for agonist-elicited
receptor loss from the cell surface. It may also be indicative of a type of receptor “cross-talk” with calcium channels. The functional efficacy of the concanavalin A treatment to block CaR endocytosis is seen in the confocal images in Fig. 3B, where the intracellular pool of CaR present at steady-state is no longer as prevalent, as well as in its ability to block EGF receptor-mediated ERK1/2 activation on the same blot (Fig. 3A), as the ability of concanavalin A to block the tyrosine kinase receptor-mediated activation of ERK1/2 has been demonstrated in some systems (33,36). These findings suggest that CaR internalization is partially required for ERK1/2 activation and further suggest the possibility that like other GPCR, CaR activation of ERK1/2 by NPS-R467/CaCl2 and the later wave of 4 mM Ca2+ activation may involve receptor cross-talk with the receptor tyrosine kinase signaling pathway.

Dynamin is a 100 kDa GTPase that binds to clathrin-coated pits where it oligomerizes around the neck of the budding clathrin vesicles. A point mutant form of dynamin, K44A, is a dominant negative form of the wild-type in that it interferes with the regular dynamics of dynamin and thereby inhibits vesicular endocytosis (37). Co-expression of dominant negative dynamin (K44A) was also explored as a tool to evaluate the dependence of internalization for agonist-elicited ERK1/2 activation. As seen in Fig. 4A, overexpression of dynamin K44A (as confirmed by Western blot analysis using an antibody against dynamin, bottom blot) did not eliminate 4 mM CaCl2-mediated ERK1/2 activation in HEK-hCaR. The ability of dynamin K44A to interfere with the normal internalization processes was confirmed by its ability to block EGF receptor-mediated ERK1/2 activation, as has been previously described by others (38,39), and argue against a need for EGF receptor signaling cross-talk to achieve CaR activation of ERK1/2. (The lack of total elimination of EGF receptor-mediated ERK1/2 activation can be attributed to the 70% transfection efficiency of this procedure (40)). When HEK-hCaR cells co-

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expressed with dynamin K44A were stimulated with the NPS R-467 agonist with 0.5 mM CaCl₂, ERK1/2 activation was also not blocked (Fig. 4B). Overexpression of dominant negative β-arrestin 2, a protein known for its roles in both receptor desensitization and internalization and which serves as a scaffolding protein the ERK and the c-jun N-terminal kinase 3 (JNK3) cascade, also did not interfere with the ability of CaR agonist to activate ERK1/2. Stimulation of CaR with NPS R-467/CaCl₂ (Fig. 4C) or with 4 mM Ca²⁺ (not shown) was indistinguishable in HEK-hCaR cells transfected with either wild-type β-arrestin 2 or dominant negative β-arrestin 2. However, dominant negative β-arrestin 2 did block EGF-stimulated ERK1/2 activation. Lastly, as seen in Figure 4D, expression of K44A or dominant negative dynamin did not effect stimulation with NPS R-467 as late as 4 hours, time points at which the allosteric modulator has its most distinguishing effects.

*The Pertussis Toxin-Sensitive Activation of ERK1/2 by CaR is restored by Gα₁₂, but not by Gα₁₁ or Gα₁₃*- Numerous GPCR mediate MAP kinase activation by coupling to the Gᵢ family of G proteins, and are therefore sensitive to pertussis toxin pretreatment. Pertussis toxin (PTX) catalyzes the selective ADP-ribosylation of Gᵢₒ proteins and eliminates their functional interactions with cognate GPCR. Kifor et al. have already demonstrated that pretreatment of HEK-hCaR cells with PTX attenuated high calcium-mediated ERK1/2 activation by 60% (19). ERK1/2 activation by NPS R-467 with low CaCl₂ was also partially blocked by overnight pretreatment with 200 ng/ml PTX (Fig. 5), demonstrating that independent of the agonist used, ERK1/2 partially couples to Gᵢ proteins. Moreover, overnight treatment of the HEK-hCaR with PTX did not alter the steady-state localization of CaR as assessed by confocal microscopy (Fig. 5), and thus could not be responsible for the altered ERK1/2 signaling. Thus, CaR-induced
phosphorylation of ERK1/2 is mediated by two distinct signaling pathways, one of which is pertussis toxin-sensitive and therefore $G_i$-mediated, and one which is pertussis toxin-insensitive and presumably $G_{q/11}$-mediated (19).

We were interested in determining which $G_i$ protein ($G_{\alpha i_1}$, $G_{\alpha i_2}$, or $G_{\alpha i_3}$) specifically contributed to ERK1/2 activation via CaR. Thus, we transiently expressed PTX-resistant forms of $G_{\alpha i_1}$, $G_{\alpha i_2}$, or $G_{\alpha i_3}$ (34), in the HEK-hCaR cells and evaluated their ability to restore ERK1/2 activation in PTX-treated cells. As seen in Fig. 6A, only overexpression of $G_{\alpha i_2}^{C351I}$ was able to restore the 4 mM CaCl$_2$-mediated ERK1/2 activation to control conditions, consequently overriding the effect of PTX treatment of the cells to eliminate the function of endogenous $G_i$ proteins. Neither $G_{\alpha i_1}^{C351I}$ (Fig. 6B) nor $G_{\alpha i_3}^{C351I}$ (Fig. 6C) were able to restore CaR activation of ERK1/2. We have no molecular explanation for why $G_{\alpha i_1}^{C351I}$ and $G_{\alpha i_3}^{C351I}$ appear to have a trend for further reducing the PTX-insensitive ERK activation at 10 minutes. Taken together, however, the data support the interpretation that $G_{\alpha i_2}$ mediates the PTX-sensitive component of the CaR-ERK1/2 pathway.
DISCUSSION

There is little information on how CaR-mediated ERK1/2 activation occurs and how it is linked to trafficking. In this study, we demonstrate that NPS R-467-mediated ERK1/2 activation via the CaR does not appear to undergo desensitization compared to the activity induced by high concentration of Ca$^{2+}$ alone. We also show that CaR agonist-mediated ERK1/2 activation is dependent on the ability of this GPCR to undergo internalization. Furthermore, we provide the first demonstration that the PTX-sensitive, CaR-mediated ERK1/2 activation requires coupling to the G$\alpha_{i2}$ subtype of G$\iota$ proteins, specifically.

The reason for the difference in the kinetics of ERK1/2 activation, and specifically the differences in desensitization, between the two agonists for the CaR, calcium vs. NPS R-467, as observed in Fig. 2, is not immediately apparent. One interpretation might be that calcium is a more general agonist than NPS R-467, and therefore the observed calcium desensitization might represent transactivation with a cation channel or tyrosine kinase receptor, whereas the NPS ligand does not. However, treatments which eliminate effects of EGF on ERK1/2 activation, such as expression of dominant negative forms of dynamin or $\beta$-arrestin, perturb EGF receptor-mediated ERK1/2 phosphorylation without perturbing stimulation by the CaR, thus arguing against this cross-talk paradigm, at least for tyrosine kinase receptors. The relevance of the fact that ERK1/2 does not desensitize, even after 4 hours of stimulation with NPS R-467, is that the CaR-stimulated ERK pathway might be connected to two different physiologic consequences for ERK, the NPS-sustained ERK activation to proliferative signaling, the high calcium ERK pathway to differentiation and gene transcription. If the high calcium-CaR conformation is able to compartmentalize some of its signaling partners to different microdomains than the allosteric
modulator, then this might afford a mechanism for the differences in the kinetics of ERK1/2 dephosphorylation and inactivation.

Resensitization of GPCR following short-term (seconds to minutes) desensitization has been shown to involve receptor recycling. Mechanisms which attenuate the GPCR’s signaling events are crucial to the maintenance of their ability to respond to agonists over time. The ability of calcium-stimulated ERK1/2 phosphorylation to recover (resensitize) after desensitization (Fig. 2C) observed after a 30 minute agonist wash out period suggest that a rapid recycling of CaR back to the cell surface following 4 mM Ca\textsuperscript{2+} stimulation may also underlie resensitization of this CaR. The fact that the resensitization occurs within 30 minutes suggests a need for CaR to be readily available for access to its agonist, as has also recently been found for the neurokinin-1 receptor (41). Our findings suggest the value of determining if recycling of the CaR is altered in disease states where circulating levels of calcium are not normally regulated, such as in familial hypocalciuric hypercalcemia, as such altered trafficking of the CaR would afford a plausible mechanism for this phenotype observed in patients with missense mutations in the CaR gene.

Within the last decade, many reports have shown that GPCR internalization is necessary for ERK activation, including the \(\alpha\) and \(\beta\)-adrenergic receptors (39), some muscarinic receptor subtypes (42), the opioid receptors (43) as well as others. Still other groups have shown that GPCR-mediated ERK activation can be internalization independent, including the \(\alpha2\)-adrenergic receptors (32), some muscarinic receptors (44), the B2 bradykinin receptor (45), some opioid receptor subtypes (46) and more. Our data provide evidence that CaR internalization is important for ERK1/2 activation, but occurs via a pathway that is dynamin- or arrestin-independent.

A possible explanation as to why only partial elimination of ERK1/2 activation with concanavalin A is observed in cells stimulated with 4 mM CaCl\(_2\), but not with NPS-R467, is that
4 mM Ca\(^{2+}\) may evoke activation of other cellular pathways. Although 4 mM Ca\(^{2+}\) stimulated ERK1/2 activation is dependent on CaR expression (Fig. 1A), occupancy of CaR by calcium in the absence of NPS \textit{R}-467 may elicit transactivation of a channel. For example, Ye \textit{et al} have also demonstrated that numerous polycationic CaR agonists, including neomycin and calcium, activate nonselective cation channels in HEK293 cells stably expressing CaR, but not in untransfected HEK293 cells (47). Thus, the observed kinetics of calcium-stimulated ERK1/2 activation might represent “cross-talk” with another signaling partner, one which is resistant to general internalization inhibitors.

A lack of an apparent reliance on dynamin for agonist-induced ERK1/2 activation and yet a dependence on the ability of the receptor to undergo internalization may be related to the previously described association of CaR with caveolae. The work of Edward Brown and colleagues has shown the CaR to reside in caveolin-rich membrane domains of parathyroid cells, the localization of which might dictate some of the signaling pathways this GPCR couples to, particularly since CaR in the parathyroid caveolae colocalizes with G\(_{q/11}\) (48). Furthermore, during the course of our studies, they showed that the carboxy tail of CaR binds to filamin-A (a potential scaffolding protein which also binds to caveolin-1 as well as several players of the MAP kinase family), and that disruption of the CaR-filamin-A interaction in HEK-293 cells expressing CaR greatly attenuates CaR-mediated ERK1/2 activation with calcium (49). This latter study suggests, albeit indirectly, that a component of calcium-induced CaR-mediated ERK1/2 activation takes place in caveolae. However, it may not be mutually exclusive from a dynamin or clathrin-coated pit-dependent mechanism, especially since a component of the ERK1/2 activation is G\(_i\)-dependent and also in light of our observed different kinetics of activation (Fig. 2), depending on the ligand being used.
We (Figs. 5 and 6) and others (19) have shown that PTX pretreatment eliminates part of the CaR-stimulated ERK1/2 phosphorylation. Thus, we were interested in determining which $G_i$ subunit might be responsible for the PTX-sensitive component of the CaR-mediated ERK1/2 activation. By using a transient expression system for PTX-insensitive $G_{\alpha_i}$ subunits, we found that $G_{\alpha_i2}$, but not $G_{\alpha_i1}$ or $G_{\alpha_i3}$, was responsible for the $G_i$-mediated ERK1/2 activation since only the $G_{\alpha_i2}^{C351I}$ was able to restore PTX-attenuated CaR-mediated ERK1/2 phosphorylation (Fig. 6). In an elegant series of experiments in 1995, Bourne and colleagues used this exact strategy in CHO cells, where they demonstrated that the $G_{\alpha_i2}$ subunit is responsible for activating the MAP kinase cascade in these cells as well (50). This novel finding may prove to be a very useful tool in the future for selectively activating one part of the signaling network of CaR-mediated events over another.

Our data provide strong evidence for two different ERK1/2 activation pathways in response to the CaR, depending on whether calcium or the calcimimetic NPS R-467 are used. Since there is mounting evidence that the CaR plays a role in the regulation of cell proliferation (51-53), the pharmacologically distinct mechanisms of CaR-mediated ERK1/2 activation may represent cellular mechanisms to differentially regulate the temporal and spatial activity of MAP kinases, thereby determining the consequences of this GPCR stimulation with respect to transcriptional activation, cell proliferation and apoptosis.
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ABBREVIATIONS:

CaR, calcium-sensing receptor; MAP kinase, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinases; con A, concanavalin A; HEK-293 cells, human embryonic kidney 293 cells; GPCR, G protein-coupled receptors; PTX, pertussis toxin.
REFERENCES


FOOTNOTES

The current address of Dr. L.M. Fredrik Leeb-Lundberg is: Division of Molecular Neurobiology, Wallenberg Neuroscience Center, Lund University, BMC, A12, SE-22184 Lund, Sweden.
FIGURE LEGENDS

1. Agonists for the CaR mediate ERK1/2 activation in the CaR-expressing cell line, HEK-hCaR. A. HEK-hCaR cells were serum-starved overnight and pretreated with HSGB as described in “Methods”. A high concentration of CaCl₂ (4 mM) activates ERK1/2 in the HEK-hCaR cell line, but not in untransfected HEK-293 cells, for the indicated times. Depicted under the immunoblot for p-ERK1/2 is a Western blot of the CaR expression in HEK-hCaR cells. The GPCR is not present in untransfected HEK-293 cells. B. The CaR agonist NPS R-467 functions allosterically in the presence of lower CaCl₂ concentrations (0.5 mM) to mediate ERK1/2 activation for the indicated times. The agonist has no effect on ERK1/2 activity in cells not expressing the CaR, despite the ability of EGF (25 ng/ml for 5 minutes) to activate ERK1/2 in these cells due to the endogenously-expressed EGF receptor. Shown beneath the blot are confocal microscopy images of the CaR taken with a 60x objective in the x-y plane (top panels), and then zoomed in 4 times with the 60x objective. No immunostaining is evident in the HEK-293 cells not expressing CaR.

2. Desensitization of CaR-mediated ERK1/2 activation: CaCl₂ vs. NPS R-467. A. Immunoblots of ERK1/2 activation for 4 mM CaCl₂ vs. 1 µM NPS R-467 in the presence of 0.5 mM CaCl₂ shows desensitization for only the 4 mM CaCl₂ stimulation. These are representative blots of four experiments. The top panels represent ECL images of Western blots of dually phosphorylated (active) ERK1/2, the bottom panels of total ERK1/2 enzyme protein. The bar graph shows the compiled data from these four experiments using NIH image for pixel quantification of the immunoblots. p < 0.05 as assessed by one-way ANOVA. B. Immunoblots
of ERK1/2 activation for 1 µM NPS R-467 in the presence of 0.5 mM CaCl₂ show a longer peak of sustained activity compared to CaCl₂ alone, with a slow decrease at 4 hours. These are representative blots of three experiments. C. CaR-mediated ERK1/2 activity resensitizes rapidly after previous exposure to agonist. HEK-CaR cells are stimulated for 1-60 minutes in the first four lanes, and 10 minutes in the last four lanes. Then, lanes 1-4 are harvested, whereas lanes 5-8 go through a 30 minute wash after their 10 minute agonist exposure, followed by a restimulation with agonist for 0-60 minutes, as indicated. The kinetics of ERK1/2 activation after prior exposure to 10 minutes of 4 mM CaCl₂ are not different than under control conditions. This is a representative of one of three experiments.

3. Concanavlin A and sucrose inhibit the ability of the CaR to activate ERK1/2. A. HEK-hCaR cells were pretreated for 30 minutes or not with 250 µg/ml concanavalin A made up fresh in HSGB 10 minutes before use. After the pretreatment period, cells were stimulated with CaR agonist, 4 mM CaCl₂, for the indicated time points. EGF (25 ng/ml for 5 minutes) was used as a control to demonstrate that EGF receptor-mediated ERK1/2 activation in these cells was inhibited by pretreatment with con A. The blot is representative of five independent experiments. B. HEK-hCaR cells were pretreated or not with concanavalin A as described above. Cells were then stimulated with the CaR agonist, NPS R-467, for the indicated times as shown. Cells were harvested and assayed for phosphorylated (top panel) and total (bottom panel) ERK1/2 as described. The blot is representative of three independent experiments. The confocal microscopy images are of HEK-hCaR cells in the absence and presence of 30 minutes concanavalin A pretreatment. The intracellular pool of CaR usually observed under steady-state conditions is no longer present after 30 minutes con A treatment, demonstrating its ability to
interfere with the internalization of CaR in these cells. The images in the xy plane were taken with the 60x objective and then zoomed in 4x.

4. **Neither dominant negative dynamin (K44A) nor dominant negative β-arrestin 2 eliminate CaR-mediated ERK1/2 activation in HEK-hCaR cells.** The HEK-hCaR cells were transiently transfected with either K44A dynamin or vector as described under “Experimental Procedures”. Cells were then stimulated with either A. 4 mM CaCl₂ or B. 1 μM NPS R-467 in the presence of 0.5 mM CaCl₂ for the indicated time points. Cells were harvested and assayed for phosphorylated (top panel) and total (middle panel) ERK1/2 as described. The top part of the blot (bottom panel) was cut off from the bottom and probed for over-expression of dynamin with an anti-dynamin antibody. C. The HEK-hCaR cells were transiently transfected with either wild-type β-arrestin or dominant-negative β-arrestin as described under “Experimental Procedures”. Cells were then stimulated with 1 μM NPS R-467 in the presence of 0.5 mM CaCl₂ for the indicated time points, harvested and probed for ERK1/2 activity as above. This blot is a representative of three experiments. For both transfection with K44A and dominant negative β-arrestin, EGF (25 ng/ml for 5 minutes) was used as an internal control to demonstrate functional ability to significantly attenuate EGF receptor-mediated ERK1/2 activation. D. The HEK-hCaR cells were transiently transfected with either vector, K44A, or dominant-negative β-arrestin as described above, and stimulated for longer time points with 1 μM NPS R-467 in the presence of 0.5 mM CaCl₂. The blot is a representative of four experiments.

5. **HEK-hCaR-mediated ERK1/2 activation by NPS R-467 partially couples to Gi.** HEK-hCaR cells were pretreated or not with 200 ng/ml pertussis toxin overnight, and then stimulated
with 1 µM NPS R-467/0.5 mM CaCl₂ at the indicated time points. Cells were harvested and assayed for phosphorylated (top panel) and total (bottom panel) ERK1/2 as described. The confocal microscopy images are of HEK-hCaR cells in the absence and presence of PTX pretreatment (18 hours). The images in the xy plane were taken with the 60x objective and then zoomed in 4x. The bar graph shows the compiled data from experiments using NIH image for pixel quantification of the immunoblots, and expressed a percentage of ERK1/2 activation remaining after pertussis toxin treatment (% PTX insensitive).

6. HEK-hCaR-mediated ERK1/2 activation is partially dependent on Gᵢ coupling, specifically to Gᵢ₂. HEK-hCaR cells were transiently transfected with PTX-insensitive Gαᵢ₁₋₃ (A-C) cDNA on day one, pretreated or not with 200 ng/ml pertussis toxin overnight on day two, and then stimulated with agonist (4 mM CaCl₂) at the indicated time points and harvested on day three. Cells were harvested and assayed for phosphorylated (top panel) and total (bottom panel) ERK1/2 as described. The bottom panels represent Gαᵢ₁₋₃ protein, which is overexpressed in the samples which were transfected with the mutant, PTX-insensitive forms of the G proteins, Gαᵢ₁^{C351I}, Gαᵢ₂^{C351I}, and Gαᵢ₃^{C351I}. The bar graphs for each mutant show the compiled data at 10 minutes stimulation with high calcium, and are expressed as a percentage of ERK1/2 activation remaining after pertussis toxin treatment (% PTX insensitive) in the absence or presence of the respective G protein mutant. Only expression of Gαᵢ₂^{C351I} was able to restore ERK1/2 activation after PTX treatment (* = p < 0.05).
A. 4 mM CaCl₂

Stimulation (min): 0 2 10 0 2 10

p-ERK1/2

untransfected HEK-293 cells

HEK-hCaR

CaR

B. EGF

1 μM NPS R-467 and 0.5 mM CaCl₂

Stimulation (min): 0 2 10

p-ERK1/2

untransfected cells

HEK-293 cells

HEK-hCaR

60X zoom xy
A. 

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<thead>
<tr>
<th>CaCl₂ (mM)</th>
<th>NPS R-467 (mM)</th>
<th>Stimulation (min):</th>
<th>p-ERK1/2</th>
<th>ERK1/2</th>
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B. 

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C. 

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All 10 minute stimulation, wash (30 min), variable re-stimulation
A.

1 μM NPS R-467 and 0.5 mM CaCl₂

Stimulation (min): 0 2 10 60 0 2 10 60

no conA + conA

B.

4 mM CaCl₂ + EGF

Stimulation (min): 0 2 10 60 0 2 10 60

no conA + conA
A. 4 mM CaCl$_2$ (min): 0 2 10 0 2 10

- p-ERK1/2
- ERK1/2
- anti-dynamin

vector K44A dynamin

B. 1 µM NPS/ 0.5 mM CaCl$_2$ (min): 0 2 10 0 2 10

- p-ERK1/2
- ERK1/2
- anti-dynamin

vector K44A dynamin

C. 1 µM NPS/ 0.5 mM CaCl$_2$ (min): 0 2 10 0 2 10

- p-ERK1/2
- ERK1/2

WT β-arrestin DN β-arrestin

EGF

D. 1 µM NPS/ 0.5 mM CaCl$_2$ (min): 0 60 120 180 240

vector K44A dynamin DN β-arrestin

- p-ERK1/2
- ERK1/2
1 μM NPS R-467/0.5 mM CaCl₂

Stimulation (min): 0 2 10 60 0 2 10 60

p-ERK1/2

ERK1/2

control + PTX control + PTX

Stimulation with NPS R-467 (min)

ERK1/2 Activation (% PTX insensitive)

n=5 n=6 n=2 n=2
A.

4 mM CaCl₂ (min): 0 2 10 60 0 2 10 60 0 2 10 60

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<th>ERK1/2</th>
<th>anti Gα₁₂</th>
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control + PTX + PTX + Gα₁₂ C₃₅₁₁

% PTX-insensitive ERK activation at 10 min.

B.

4 mM CaCl₂ (min): 0 2 10 60 0 2 10 60 0 2 10 60

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control + PTX + PTX + Gα₁₁ C₃₅₁₁

% PTX-insensitive ERK activation at 10 min.

C.

4 mM CaCl₂ (min): 0 2 10 60 0 2 10 60 0 2 10 60

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control + PTX + PTX + Gα₁₃ C₃₅₁₁

% PTX-insensitive ERK activation at 10 min.
Calcium-sensing receptor-mediated ERK1/2 activation requires Gαi-coupling and dynamin-independent receptor internalization
Deborah M. Holstein, Kelly A. Berg, L.M. Fredrik Leeb-Lundberg, Merle S. Olson and Christine Saunders

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