Calcium-sensing receptor stimulates PTHrP release by pathways dependent on PKC, p38 MAPK, JNK, and ERK1/2 in H-500 cells

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WE HAVE PREVIOUSLY SHOWN a positive relationship between the level of extracellular calcium ([Ca²⁺]o) and the release of parathyroid hormone-related peptide (PTHrP) in rat H-500 Leydig cancer cells, as well as in breast and prostate cancer cell lines (30–32). H-500 is a xenotransplantable rat Leydig cancer cell and is a well-established model for the syndrome of humoral hypercalcemia of malignancy (HMM) (27). The calcium-sensing receptor (CaR) is a mediator of calcium-induced inhibition of the release of PTH in the parathyroid gland and has been shown to have a variety of functions, including the regulation of the cell cycle, peptide release, and apoptosis in other systems (35). The role of the CaR in the regulation of PTHrP release has been confirmed in a prostate cancer cell line, PC-3, in which adenoviral-mediated infection with a dominant negative CaR shifts the calcium dependence of PTHrP release to the right (31). In addition, PTHrP release has been shown to be mediated by the CaR in human embryonic kidney (HEK) cells stably transfected with the CaR (HEK-CaR), whereas nontransfected HEK cells, which lack endogenous CaR expression, showed no such effect (23).

Here, we have used a well-accepted HHM cell model (H-500) to investigate the CaR-mediated intracellular signaling that underlies PTHrP release. High [Ca²⁺]o, acting via the CaR, regulates PKC activity, which in turn modulates PTH release from the bovine parathyroid gland (22). In addition, involvement of the PKC pathway in [Ca²⁺]o-stimulated PTHrP release has been reported in nonsmall cell lung cancer (NCI-H727); therefore, PKC is a potential candidate for postreceptor signaling associated with CaR-induced PTHrP release from H-500 cells (6). On the other hand, the CaR stimulates MAPKs in a variety of cell types, including HEK-CaR, rat-1A fibroblasts, ovarian surface epithelial cells, and Madin-Darby canine kidney (MDCK) cells, regulating functions such as phospholipase C activation and proliferation (2, 16, 17). Because PKC regulates a variety of MAPKs, including ERK1/2, in this study we sought to identify intracellular signaling cascades that regulate CaR-mediated PTHrP release. Our data reveal that the CaR, but not the ADP receptor [a G protein-coupled receptor (GPCR) linked to Gαq11 like the CaR], induces PTHrP release. This effect is mediated by PKC as well as by PKC-independent activation of ERK1/2, p38 MAPK and its downstream activating transcription factor (ATF)-2 cascade, and the stress-
activated protein kinase activator (SEK-1). In addition, the effect of the CaR on PTHrP release is likely transcriptional, as high \([\text{Ca}^{2+}]_o\) upregulates the PTHrP transcript in the absence, but not in the presence, of actinomycin D.

**MATERIALS AND METHODS**

**Materials.** Polyclonal antisera against phosphorylated and nonphosphorylated ERK1/2, p38 MAPK, SEK1, and ATF-2 kinases were purchased from New England Biolabs (Beverly, MA). Selective inhibitors of p38 MAPK (SB-203580, MEK1 (PD-98059), JNK (SP-600125), and PKC (GF-109203X) were all obtained from Calbiochem-Novabiochem (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Biomol (Plymouth Meeting, PA). The enhanced chemiluminescence kit Supersignal was purchased from Pierce (Rockford, IL), and other reagents were from Sigma Chemical (St. Louis, MO).

**Cell culture.** The Rice H-500 rat Leydig cell tumor was obtained from the National Cancer Institute-Frederick Cancer Research and Development Center DCT Tumor Repository (Frederick, MD). Male Fischer 344 rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 200–220 g (10 wk of age) were used for all experiments. A fragment of the H-500 tumor or dispersed H-500 cells (10⁶ per rat) were implanted or injected subcutaneously, respectively, in each rat, and the tumors were allowed to grow for 8–14 days. The encapsulated tumor was then excised, rinsed several times with cell culture medium (to be described), minced into small pieces, and dispersed by repeated pipetting and several passages through a 22-gauge needle. Dispersed H-500 cells were subsequently plated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-100 g/ml streptomycin and grown at 37°C in a CO₂ atmosphere. Cells were passaged every 4 days, and dispensed by repeated pipetting and several passages through a 22-gauge needle. Dispersed H-500 cells were subsequently plated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-100 g/ml streptomycin and grown at 37°C in a humidified 5% CO₂ atmosphere. Cells were passaged every 4–5 days with 0.05% trypsin-0.53 mM EDTA and used for experimentation with local institutional guidelines.

**Northern blot analysis.** To study whether high \([\text{Ca}^{2+}]_o\), exerts an effect on the expression of PTHrP mRNA, we performed Northern blot analysis as described before (7). Briefly, cellular RNA was isolated (9) using the TRIzol reagent (Invitrogen, Carlsbad, CA) by following the manufacturer’s instructions. The recovered RNA was quantitated by spectrophotometry, and aliquots of 20 μg total RNA from low \([\text{Ca}^{2+}]_o\) (0.5 mM) or high \([\text{Ca}^{2+}]_o\) (7.5 mM) were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA so that we could document equal loading of RNA from the various experimental samples. The RNA was then blotted onto nylon membranes (Duralon; Stratagene, La Jolla, CA). Blots were hybridized with a cDNA probe for PTHrP and washed under high-stringency conditions as described previously (23). Equal loading was also confirmed by reprobing the membranes with GAPDH cDNA. Specific radioactive signals were analyzed on a Molecular Dynamics PhosphorImager (Sunnyvale, CA) with the ImageQuant program.

**Gene transfer into H-500 cells with CaR constructs.** High-efficiency gene transfer into H-500 cells was accomplished using a recombinant adeno-associated virus (rAAV)-based method. The CaR sequence with a naturally occurring, dominant negative mutation (R185Q), as well as the same vector containing the cDNA for the β-gal (BG) protein, were under the control of a cytomegavirus immediate-early, or CMV-IE, promoter element and packaged as previously described (37). The BG served as the control for nonspecific effects of rAAV infection. Cells were seeded (1,000 cells/well) in 96-well plates in 0.1 ml of growth medium and cultured overnight. About 1,000 virus particles/cell (as optimized by pilot studies) were used to infect each well. Cells were washed once with serum-free medium and grown at 37°C in a cell culture incubator. Equal volumes of RPMI 1640 containing 20% serum were added to the cells to achieve a final serum concentration of 10%. The cells were then cultured for 48 h, and experiments with basal (low, 0.5 mM) and high calcium concentrations were performed as described in subsequent sections.

**Western blot analysis.** For the determination of ERK1/2, p38 MAPK, SEK1, or ATF-2 phosphorylation, monolayers of H-500 cells were grown on six-well plates. Cells were incubated for 18 h in serum-free, Ca²⁺-free DMEM containing 4 mM l-glutamine, 0.2% BSA, and 0.5 mM CaCl₂. This medium was then removed and replaced with fresh serum-free DMEM supplemented with 7.5 mM CaCl₂ either alone or with the PKC inhibitor. The cells were also preincubated with PKC inhibitor for 0.5 h, as described in RESULTS. At the end of the incubation period, the medium was removed, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium vanadate and 25 mM NaF, and then 100 μl of ice-cold lysis buffer were added (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM glycerophosphate, and a cocktail of protease inhibitors). The protease inhibitors were aprotinin, leupeptin, soybean trypsin inhibitor, pepstatin, and calpain inhibitor (10 μg/ml of each), all from frozen stocks, as well as 100 μg/ml of Pefabloc. The sodium vanadate, NaF, and Pefabloc were freshly prepared on the day of the experiment. The cells were scraped into the lysis buffer, sonicated for 5 s, and then centrifuged at 6,000 g for 5 min at 4°C. The supernatants were frozen at −20°C. After thawing, equal amounts of supernatant protein (100 μg) were separated by SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell) and incubated with blocking solution (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.25% BSA) containing 5% dry milk for 1 h at room temperature. ERK1/2, p38 MAPK, SEK1, and ATF-2 phosphorylation was detected by immunoblotting using an 18-h incubation with 1/1,000 dilutions of rabbit polyclonal antiserum specific for phospho-ERK1/2, phospho-p38 MAPK, phospho-SEK1, or phospho-ATF-2, respectively. Blots were washed for five 15-min periods at room temperature (1% PBS, 1% Triton X-100, and 0.3% dry milk) and then incubated for 1 h with a secondary goat anti-rabbit, peroxidase-linked antiserum (1: 2,000) in blocking solution. Blots were then washed again (5 × 15 min). Bands were visualized by chemiluminescence according to the manufacturer’s protocol (Supersignal, Pierce Chemical). The same membrane was used after stripping (Restore Western Blot Stripping, Pierce) to measure nonphospho-ERK1/2, -p38 MAPK, and -SEK1. Protein concentrations were measured with the Micro BCA protein kit (Pierce).

**PTHrP release.** The effects of \([\text{Ca}^{2+}]_o\), as well as MAPK and PKC inhibitors, on PTHrP release were determined by seeding cells in 96-well plates (1 × 10⁴ cells/well) in 0.1 ml of growth medium. After 48 h, the growth medium was removed and replaced with 0.1 ml of Ca²⁺-free DMEM containing 4
mM L-glutamine, 0.2% BSA, 100 U/ml penicillin-100 µg/ml streptomycin, and 0.5 mM CaCl₂. Two hours later, this medium was removed and replaced with 0.225 ml of the same medium or that supplemented with additional CaCl₂ (to a final concentration of 2.5, 5.0, or 7.5 mM) for 6 h. In other experiments, the medium was supplemented either with the kinase inhibitors described in RESULTS or with 7.5 mM CaCl₂ together with the same inhibitors. In the experiment with ADP, the concentrations used were 10⁻⁷, 10⁻⁸, and 10⁻⁹ M. Six hours later, the conditioned medium was removed for determination of PTHrP release. The 6-h incubation time was decided upon after a time course experiment had been carried out that examined the effects of low and high calcium on PTHrP release at 4, 6, and 24 h. The fold increase of PTHrP release at high [Ca²⁺]₀ did not vary over the first 24 h. The 6-h time point was chosen for subsequent experiments because it yielded PTHrP values falling on the linear portion of the PTHrP assay, whereas at 4 and 24 h, PTHrP was at the lower or upper portion of the curve, respectively.

PTHRP was measured in conditioned medium using a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) that detects PTHrP:(1-32) with a sensitivity of ~0.3 pmol/l. PTHrP assays were initiated immediately after the conditioned medium was removed from cultures to minimize degradation of the peptide resulting from freeze-thawing and other manipulations. Standard curves of PTHrP concentrations were generated with the addition of recombinant PTHrP:(1-86) to the treatment medium used in the study (i.e., unconditioned Ca²⁺-free DMEM containing 0.5 mM CaCl₂). Calcium and MAPK inhibitors alone had no effect in the PTHrP assay.

Measurement of intracellular calcium by fluorimetry in cell populations. Coverslips with H-500 cells were loaded for 2 h at room temperature with fura 2-AM in 20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 1.25 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 0.1% BSA, and 0.1% dextrose and then were washed once with a bath solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 0.1% dextrose, and 0.1% BSA) at 37°C for 20 min. The coverslips were then placed diagonally in a thermostatted quartz cuvette containing the bath solution with a modification of the technique employed previously in this laboratory (4). In the experiment with agonists for other G protein-coupled receptors, angiotensin II was used at 10⁻⁶ M, ADP at 10⁻⁸ M, thrombin peptide agonist at 10⁻⁸ M, and carbachol at 10⁻⁴ M. The angiotensin II, ADP, thrombin peptide agonist, and carbachol were added into the bath solution. Excitation monochromators were centered at 340 and 380 nm, and emission light was collected at 510 ± 40 nm through a wide-band emission filter. The 340/380-excitation ratio of emitted light was used to estimate intracellular calcium ([Ca²⁺]₀), as described previously (4).

RESULTS

CaR mediates high [Ca²⁺]₀-induced PTHrP release in H-500 cells. High calcium (7.5 mM) increased PTHrP release to 313 ± (SE) 24% of basal release (at 0.5 mM [Ca²⁺]₀) in H-500 cells transfected with virus containing BG. This increase was substantially attenuated in cells infected with dominant negative CaR, in which high calcium increased PTHrP release only to 233 ± 24% of basal release ([Ca²⁺]₀ = 0.5 mM; P < 0.05; Fig. 1). Similar results were found at 2.5 and 5 mM [Ca²⁺]₀; the dominant negative transduced cells secreted 152 ± 11 and 194 ± 19%, respectively, of basal PTHrP release, compared with BG-infected cells, in which 2.5 and 5 mM [Ca²⁺]₀, increased PTHrP release to 170 ± 11 and 268 ± 23%, respectively, of basal release. Therefore, infection of cells with dominant negative CaR (R185Q) shifts [Ca²⁺]₀-stimulated PTHrP release to the right and downward compared with infection of cells with BG, thereby confirming that the CaR mediates the effect of high [Ca²⁺]₀ on PTHrP release in a cell model used to study HHM. As a positive control, we examined the effect of PMA on PTHrP release in both of the cells infected with BG, as well as those infected with the dominant negative CaR; in both cases, the PMA induced PTHrP secretion.

Stimulation of PTHrP release is not a generalized function of GPCR activation. Because high [Ca²⁺]₀, acting via the CaR, increased PTHrP release, we next examined whether this is a generalized effect of acti-

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Fig. 1. Effect of dominant negative calcium-sensing receptor (CaR) on calcium-stimulated parathyroid hormone-related peptide (PTHrP) secretion. H-500 cells were infected with either the dominant negative CaR or the vector expressing the β-gal (BG) protein 48 h before stimulation, as described in MATERIALS AND METHODS. Cells were then starved for 2 h in serum-free medium, and the medium was collected after incubation of cells with 0.5, 2.5, 5, or 7.5 mM [Ca²⁺]₀, or 0.5 mM [Ca²⁺]₀ + 100 nM PMA in serum-free medium for 6 h. Results represent the % increase compared with basal PTHrP release and are pooled data from 4 independent experiments. Ca²⁺-mediated PTHrP release (7.5 mM) was 228 ± (SE) 24% of basal PTHrP release in cells infected with dominant negative CaR, whereas it was 313 ± 24% of basal release in cells infected with BG (*P < 0.05 vs. BG, +P < 0.05 vs. 0.5 calcium, n = 10).
vation of any GPCR. We first assessed whether the agonists affected [Ca\(^{2+}\)]\(_i\) release by using cells loaded with the calcium-sensitive dye fura 2. Treatment of cells with ADP (10\(^{-8}\) M) produced a rapid and transient increase in [Ca\(^{2+}\)]\(_i\) in the H-500 cells, whereas [Ca\(^{2+}\)]\(_o\), angiotensin II, thrombin agonist, and carbachol had no such effect (data not shown; \(n = 3\)). This proves that the H-500 cells express a functional ADP receptor linked to the phosphoinositol (PI)-PLC system, thereby elevating [Ca\(^{2+}\)]\(_i\) in an agonist-dependent manner. We then incubated H-500 cells with increasing ADP concentrations from 10\(^{-9}\) to 10\(^{-7}\) M in medium containing basal calcium (0.5 mM). We observed that, whereas high [Ca\(^{2+}\)]\(_o\) increased PTHrP release to 492 ± 117% of basal PTHrP release (\(P < 0.05\)), ADP had no effect (Fig. 2).

**Effect of [Ca\(^{2+}\)]\(_o\) on PTHrP transcripts.** We next determined whether high [Ca\(^{2+}\)]\(_o\)-stimulated PTHrP release occurred at the level of transcription. We first pretreated the cells with actinomycin D (50 \(\mu\)g/ml) for 1 or 2 h and stimulated them with high [Ca\(^{2+}\)]\(_o\) for another 6 h, as described in MATERIALS AND METHODS. The inhibition of [Ca\(^{2+}\)]\(_o\)-stimulated PTHrP release in response to actinomycin D was dependent on the pretreatment time (Fig. 3A); i.e., the reduction in PTHrP release was more robust when cells were pretreated for 2 h than for 1 h. Expressed quantitatively, [Ca\(^{2+}\)]\(_o\)-stimulated PTHrP release was 129 ± 7 and 103 ± 2% of basal release with pretreatment for 1 and 2 h, respectively, with act D (50 \(\mu\)g/ml) for 1 or 2 h. We then performed Northern analysis of mRNA extracted from H-500 cells incubated for 6 h with high (7.5 mM) or low (0.5 mM) [Ca\(^{2+}\)]\(_o\) after a 2-h preincubation with or without actinomycin D. The PTHrP transcript was upregulated by high [Ca\(^{2+}\)]\(_o\) in the absence of actinomycin D (\(P < 0.05\)). In contrast, actinomycin D markedly downregulated the level of PTHrP mRNA, and no difference was seen between high and low calcium (Fig. 3B).

**Effects of PKC and MAPK inhibitors on high [Ca\(^{2+}\)]\(_o\)-induced PTHrP release.** To identify the signal transduction pathways involved in CaR-mediated stimulation of PTHrP release, we examined the effects on both basal ([Ca\(^{2+}\)]\(_o\) = 0.5 mM) and high calcium ([Ca\(^{2+}\)]\(_o\) = 7.5 mM) stimulated PTHrP release of a PKC inhibitor, GF-199203X (1 \(\mu\)M) (36), a MEK inhibitor, PD-98059 (10 \(\mu\)M) (11), a JNK inhibitor, SP-600125 (10 \(\mu\)M) (5), and a P38 MAPK inhibitor, SB-203580 (10 \(\mu\)M) (10). None of the four inhibitors alone had any effect on basal PTHrP release (Fig. 4). In contrast, the MEK
Fig. 4. Effect of a PKC inhibitor and MAPK inhibitors on basal and high calcium-induced PTHrP release. Cells were plated at $10^4$ cells/well in a 96-well plate, and 48 h later cells were at 70% confluence. Cells were starved for 2 h before 6-h stimulation with 0.5 or 7.5 mM [Ca$^{2+}$], with or without the 4 inhibitors: PKC inhibitor GF-109203X (1 μM; BIS), MEK inhibitor PD-98059 (10 μM; PD) (A), p38 inhibitor SB-203580 (10 μM; SB; B), JNK inhibitor SP-600125 (10 μM; SP; C). Results are pooled data from 3 independent experiments. All three compounds inhibited CaR-mediated PTHrP release by ~60%, and no additive effect was seen when either SB-203580 or PD-98059 was used together with GF-109203X. In contrast, there was an additive effect of GF-109203X and SP-600125 when administered together. See text for details (*P < 0.05; n = 9).
inhibitor, PD-98059, decreased calcium-stimulated PTHrP release from 432 ± 47 to 229 ± 25% of basal release (P < 0.05). This is a reduction by 61% in the CaR-mediated component of PTHrP release. Similar results were found with GF-109203X, SP-600125, and SB-203580; PTHrP release decreased to 237 ± 29, 251 ± 14, and 185 ± 13% of basal release, respectively, all of which were significant reductions compared with high calcium alone (P < 0.05). Either PD-98059 or SP-600125, when administered with GF-109203X, decreased PTHrP release to 26 ± 11 and 42 ± 12%, respectively, of the value observed with low calcium alone (P < 0.05).

It has been previously shown that CaR-mediated activation of ERK1/2 in HEK-CaR cells is PKC dependent (20), whereas little is known about the involvement of p38 MAPK and JNK. Here, we studied the combined effects of the PKC and p38 inhibitors on [Ca^{2+}]_o-stimulated PTHrP release from H-500 cells and found that the effects of the two inhibitors on high [Ca^{2+}]_o-induced PTHrP release were not additive (170 ± 12% of basal release). Interestingly, we observed that the JNK and PKC inhibitors together had an additive effect, because in combination, these inhibitors reduced high calcium-stimulated PTHrP release to 157 ± 23% compared with SP-600125 (251 ± 14%) or GF-109203X (300 ± 30%) alone (P < 0.05). When administered together in all possible combinations of pairs, the MAPK inhibitors did not show any additive effect on PTHrP secretion (data not shown).

High [Ca^{2+}]_o stimulates phosphorylation of MAPks. Because the MEK inhibitor PD-98059 inhibited high [Ca^{2+}]_o-stimulated PTHrP release in H-500 cells, we determined whether high [Ca^{2+}]_o activated the MEK-ERK1/2 MAPK cascade by using a specific antibody to phosphorylated ERK1/2, a MEK substrate. High [Ca^{2+}]_o promoted the phosphorylation of ERK1/2 (Fig. 5A). [Ca^{2+}]_o-induced ERK1/2 phosphorylation was delayed, reaching a maximum level between 30 and 60 min that was sustained for 2 h before starting to decline. To determine whether PKC is capable of stimulating ERK1/2, we added PMA (100 nM) to H-500 cells and measured ERK1/2 phosphorylation; as expected, PMA stimulated ERK1/2 phosphorylation at 5 min compared with high calcium. Surprisingly, however, we found that the PKC inhibitor did not block high calcium-stimulated ERK1/2 phosphorylation at 30 and 60 min, suggesting that the CaR stimulation of the ERK pathway is PKC independent.

Next, we studied the activation of p38 MAPK by using an antibody against the phosphorylated form of this kinase. A delayed and sustained phosphorylation of p38 MAPK was observed in H-500 cells in response to high [Ca^{2+}]_o (Fig. 5B); i.e., the phosphorylation reached a maximum intensity at 1 h and decreased to the basal level by 2 h. The PKC inhibitor did not reduce the calcium-induced phosphorylation of p38 MAPK at 30 and 60 min, suggesting that PKC is not upstream of p38 MAPK. Upon phosphorylation at its Thr-Gly-Tyr motif, p38 MAPK is translocated into the nucleus and activates various transcription factors. Two major substrates for phospho-p38 MAPK are ATF-2 and Elk-1. Phosphorylation of ATF-2 occurred as early as 5 min and peaked at 2 h (Fig. 5B). We therefore observed an early response and a much-delayed activation for ATF-2 (2 h compared with 1 h, as observed in the case of p38 MAPK). No phosphorylation of Elk-1 was observed after ≥2-h incubation with high [Ca^{2+}]_o (data not shown).

Because SEK1 is upstream of JNK, and we observed that the JNK inhibitor diminishes calcium-induced PTHrP release, we next investigated whether high calcium promoted phosphorylation of SEK1. Indeed, we observed that SEK1 is phosphorylated by high

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**Fig. 5.** Time course of the phosphorylation (p-) of extracellular signal-related kinase (ERK)1/2, p38, stress-activated protein kinase (SEK)1, and activating transcription factor (ATF)-2 by high [Ca^{2+}]_o. A: ERK1/2 is maximally phosphorylated at 30–60 min in response to high [Ca^{2+}]_o. PMA (100 nM) induces ERK1/2 phosphorylation at 5 min, but the PKC inhibitor GF-109203X has no effect on calcium-induced ERK1/2 phosphorylation. B: a similar time course is seen with stimulation of p38 phosphorylation by high [Ca^{2+}]_o, whereas ATF-2 is maximally phosphorylated at 2 h. The PKC inhibitor GF-109203X has no effect on calcium-induced p38 phosphorylation. C: phosphorylation of SEK1 was already maximal at 5 min and persisted for 60 min. The PKC inhibitor GF-109203X has no effect on calcium-induced SEK1 phosphorylation. Western blots are representative of ≥3 experiments.
DISCUSSION

The purpose of this study was to determine whether high [Ca\(^{2+}\)]_o-stimulated PTHrP release by H-500 Leydig cells is mediated by the CaR and to identify the signaling pathways(s) mediating this effect. It has been previously shown that transient coexpression of the mutant R185Q, a dominant negative CaR, with the wild-type (WT) CaR in HEK293 cells shifted [Ca\(^{2+}\)]_o-evoked accumulation of total inositol phosphates (IP) downward and to the right, thereby indicating a dominant negative effect on the WT CaR (3). We therefore investigated the mediatory role of the CaR in [Ca\(^{2+}\)]_o-induced PTHrP release by using high-efficiency expression of the dominant negative CaR and the BG protein utilizing the rAAV system. Recently, using a similar approach with an adenoviral vector, we observed attenuation of high [Ca\(^{2+}\)]_o-induced PTHrP release from PC-3 prostate carcinoma cells (31). The result with the H-500 cells was similar, thereby suggesting that high [Ca\(^{2+}\)]_o-induced PTHrP release from various cells is generally mediated by the CaR.

Because the CaR is a member of the GPCR family that uses G\(_{o,q11}\) for coupling to its major signaling pathway, PI-PLC, we studied whether PTHrP release could be induced by activation of other GPCRs coupled to G\(_{o,q11}\). Our data showed that ADP did not induce PTHrP release despite mobilizing [Ca\(^{2+}\)]_o, thereby suggesting that stimulation of PTHrP release is not a generalized effect of GPCR-mediated activation of PI-PLC. This specific induction of PTHrP release by the CaR has important pathophysiological implications in HMM, in which the major mediator, PTHrP, is under the control of the CaR, culminating in a vicious cycle (12). Interestingly, [Ca\(^{2+}\)]_o did not activate [Ca\(^{2+}\)]_o store release. This might be due to the low receptor density on these cells compared with parathyroid chief cells or because the CaR uses a PLC-independent signaling pathway to regulate PTHrP release. We have observed this phenomenon in other cells that are uninvolved in extracellular calcium homeostasis, such as PC-3, MDA-MB 231, and U87 cells (S. Quinn, unpublished observations).

Expression and release of PTHrP have been shown to be regulated by a variety of nuclear and membrane-bound receptor ligands. These various forms of regulation are mostly at the transcriptional level and are often exerted in a cell type-specific fashion. Much work has been carried out to identify agents that regulate PTHrP expression/release; however, relatively little is known about the mechanisms underlying these effects, particularly in relation to GPCRs. Our data show that high [Ca\(^{2+}\)]_o acting via the CaR, augments the synthesis of PTHrP, likely acting at the transcriptional level.

The postreceptor mechanisms by which the CaR stimulates PTHrP release involve activation of PKC, MEK, JNK, and p38 MAPK pathways. Regulation of PTHrP release by PKC has been reported in the NCI-H727 nonsmall lung cancer cell and in alveolar epithelial cells (6, 13). We observed that CaR-mediated induction of PTHrP release is potentiated by a PKC activator and blunted by a PKC inhibitor. In some cell systems, activation of PKC participates in the activation of the ERK1/2 cascade (15). A Ras/MEK/ERK pathway has recently been implicated in the production of PTHrP from cardiac myocytes likely acting at the transcriptional level (15). Because the Ras/PKC/MEK pathway represents a classical signaling cascade, it is therefore conceivable that the CaR acts via this pathway to induce PTHrP production at the transcriptional level. However, surprisingly, we did not detect any effects of the PKC inhibitor on [Ca\(^{2+}\)]_o-induced ERK activation. Taken together with our data on PTHrP release, where no additive effect was seen, this result suggests that the pathways are parallel and converge distal to ERK. In contrast to the rapid and transient kinetics of ERK activation observed in other cells in response to high [Ca\(^{2+}\)]_o, in H-500 cells the ERK1/2 response was both delayed and sustained. Although delayed and sustained activation of ERK1/2 has been observed in response to a variety of stimuli in many cell systems, its role(s) is conjectural (21, 29, 33). Certainly, the most important determinant of the duration of ERK activation is the balance between activating kinases and inactivating phosphatases. However, the occurrence of nuclear translocation of ERK1/2 in response to prolonged activation suggests that high [Ca\(^{2+}\)]_o-induced activation of ERK acts at the transcriptional level to increase PTHrP production. The effect of sustained activation of ERK1/2 has been demonstrated in Swiss 3T3 fibroblasts, where it led to translocation of activated ERK and was associated with proliferation (25).

p38 MAPK is a stress-activated kinase that was originally identified as the target of pyridinylimidazole compounds found to inhibit inflammatory cytokine production and cell death following cellular stress (26). Here we observed that high [Ca\(^{2+}\)]_o-induced PTHrP release was attenuated in the presence of SB-203580, a specific inhibitor of p38\(\alpha\) and -\(\beta\) subtypes. We also observed that high [Ca\(^{2+}\)]_o induced the phosphorylation of p38 MAPK. Phosphorylation activates p38 MAPK, which in turn activates ATF-2, which then acts as a transcription factor for various target genes. The rapid activation of ATF-2 observed in our studies in H-500 cells in response to high [Ca\(^{2+}\)]_o may be due to other intracellular signaling pathways that also activate ATF-2. Recently, p38 MAPK was shown to be involved in the Smad-independent induction of PTHrP release from MDA-MB-231 breast cancer cells by transforming growth factor-\(\beta\) (19). However, our results represent the first demonstration that a GPCR can regulate PTHrP production via the p38 MAPK pathway in a pathophysiological setting. Similar to the temporal pattern observed with ERK1/2 activation, we observed a delayed activation of p38 MAPK and its downstream substrate, ATF-2, by high [Ca\(^{2+}\)]_o in these...
cells. The delayed and sustained nature of p38 MAPK activation such as that found in our system has been reported in reactive murine astrocytes following the induction of seizures with kainic acid and in arteriolar smooth muscle cells after balloon injury (8, 18). In the field of GPCR agonists, the thromboxane analog U-46619 has been reported to activate p38 MAPK in platelets, an activation blocked by SB-203580 (28). However, in another study (34), the GPCR agonist adenosine stimulated IL-6 in the intestinal epithelial cell line T84. Adenosine-induced IL-6 production was mediated through cAMP-mediated activation of nuclear cAMP-responsive element-binding, CREB, protein and ATF-2. ATF-2 was activated between 1 and 3 h, which was similar to the maximal activation at 2 h in our study.

CaR activation of the JNK pathway has been shown in MDCK cells (2). Here we show that the JNK inhibitor SP-600125 reduces CaR-mediated PTHrP release and that this action is additive with the PKC inhibitor. This suggests that the PKC and JNK pathways do not converge, as seems to be the case for MEK and p38 MAPK cascades. The fact that activation of the three MAPKs is independent of PKC might be due to the interaction of the CaR with lamin A (14). Filamin A has also been shown to interact with MEK, p38, and SEK1 (24). The CaR’s potential interactions with these three MAPKs via filament A could provide a PI-PLC-PKC-independent mode of activation of these enzymes in H-500 cells. In conclusion, our data demonstrate that the CaR stimulates transcription of the PTHrP gene, thereby increasing release of PTHrP in H-500 cells. CaR-mediated PTHrP release occurs via PKC-, ERK1/2-, and p38 MAPK-dependent pathways that are activated in parallel but likely converge downstream of these protein kinases to regulate PTHrP synthesis and release. The JNK pathway, in contrast, appears to function independently of and parallel to the PKC pathway. Surprisingly, the activations of ERK1/2, SEK1, and p38 are independent of PKC.

DISCLOSURES

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