In hypocalcaemia, elevated parathyroid hormone transitorily down-regulates the kidney vitamin D receptor, which returns to normal levels with the rise in serum extracellular calcium $[Ca^{2+}]_e$. In this study, we investigated the mechanism that underlies VDR increase in kidney in association with elevated $[Ca^{2+}]_e$. Examination of MAP kinase signals in a proximal tubule human kidney (HK-2G) epithelial cell line showed that treatment of $[Ca^{2+}]_e$ in the culture medium elevated phosphorylation of both ERK and p38 MAPKs. Blockade of p38 phosphorylation with SB203580 or SB202190 in turn abolished $[Ca^{2+}]_e$-mediated activation of VDR promoter. We also demonstrate that si-RNA knock down of p38c completely diminished high $[Ca^{2+}]_e$-mediated VDR induction. Direct CaSR involvement was demonstrated by using an si-RNA of CaSR that impeded $[Ca^{2+}]_e$-mediated induction of VDR. In conclusion, a high extracellular $[Ca^{2+}]_e$ concentration in the physiological range is capable of directly increasing renal proximal VDR expression, and the induction mechanism requires activation of the CaSR and signal mediation by the p38c MAP kinase pathway.

Extracellular ionized calcium $[Ca^{2+}]_e$ is a critical mediator of cell signaling for the storage and release of both parathyroid hormone (PTH) and calcitonin. Homeostasis of $[Ca^{2+}]_e$ is important to proper neuromuscular contractions, cellular integrity, and the deposition of mineral in skeletal structures (1). A multi-organ system coordinates and maintains $[Ca^{2+}]_e$ homeostasis within a narrow physiological range (2). Activation of an extracellular calcium sensing receptor (CaSR) when $[Ca^{2+}]_e$ concentrations are high maintains the storage form of PTH in the parathyroid gland (3, 4). Release of PTH from the parathyroid gland is a direct result of a decrease in serum $[Ca^{2+}]_e$ (5, 6). PTH is a potent stimulus of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) biosynthesis in the kidney proximal convoluted tubule by its induction of 1α-hydroxylation (7). Resultant increase in $[Ca^{2+}]_e$, by 1,25(OH)$_2$D$_3$ regulates PTH at two levels; where CaSR signaling shuts down PTH secretion (8, 9) and 1,25(OH)$_2$D$_3$ through transactivation of its nuclear receptor (VDR) inhibits pre-pro-PTH transcription (10).

$[Ca^{2+}]_e$ is also a potent mediator of the balance between cellular proliferation and differentiation while VDR mediates biological functions of 1,25(OH)$_2$D$_3$ and is expressed in many different cell types (11). Past work has focused on the role of VDR in mineral homeostasis, with VDR activators used mainly to treat hyperparathyroidism secondary to chronic kidney disease (12). A more recent perspective includes the use of vitamin D analogs in combination with calcimimetics and phosphate binders in the management of hyperparathyroid disorders (13). It is therefore important to identify the mechanisms whereby CaSR signaling and VDR activation coincide in the tissues that co-express these two receptors. We previously reported for the first time that $[Ca^{2+}]_e$ is a direct regulator of VDR in proximal human kidney HK-2G cells (14). The present study was designed to better explore the mechanism by which $[Ca^{2+}]_e$ influences VDR increase in these cells.

PTH exerts several actions in the renal proximal tubule that include control of phosphate transport and induction of 1,25(OH)$_2$D$_3$-1α-hydroxylase (CYP27B1) mRNA (15–17). The receptor for PTH is another member of the GPCR family capable of coordinating bidirectional signaling through pathways involving adenylyl cyclase or activation of protein kinase C depending on the concentration of PTH and the specific cell type (18, 19). In proximal tubule epithelial cells, PTH stimulates 1α-hydroxylation, mediates 1,25(OH)$_2$D$_3$-24-hydroxylase (CYP24) down-regulation and represses 1,25(OH)$_2$D$_3$-receptor (VDR) transcription all by increasing cAMP and activating the VDR.

In addition to its more well studied effects in the distal tubule, reports demonstrate that CaSR also is expressed in the proximal tubule suggesting that $[Ca^{2+}]_e$, plays an as yet undefined role in proximal kidney physiology (23, 24). Studies of inherited disorders of calcium sensing have established the central role of CaSR in calcium homeostasis (25). Dysregulation of CaSR may
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cause an irregular pattern of PTH secretion and excessive tubular calcium reabsorption (26). Because PTH indirectly influences [Ca\(^{2+}\)]\(_e\), homeostasis largely through regulated synthesis of 1,25(OH)\(_2\)D\(_3\) in the kidney proximal tubule (27, 28), we asked the question of whether [Ca\(^{2+}\)]\(_e\) plays a direct role in the compensatory down-regulation of the vitamin D system in the proximal tubule.

The effect of [Ca\(^{2+}\)]\(_e\) on renal vitamin metabolism has been addressed both \textit{in vitro} (29–31) and \textit{in vivo} (32) by studying the regulation patterns of CYP27B1 or CYP24. The direct role of [Ca\(^{2+}\)]\(_e\) in cell culture is minimal, but a correlation has been seen between the rise in serum [Ca\(^{2+}\)]\(_e\), and the suppression of CYP27B1 (32). The CaSR has been shown to be involved in multiple mechanisms that include mitogen activated protein kinase (MAPK) intermediates and release of stored intracellular Ca [Ca\(^{2+}\)]\(_i\), (33). These pathways are known for their roles in mediating increases in VDR (34, 35) and renal VDR is a key determinant for the reciprocal regulation of CYP27B1 and CYP24 by PTH (36). It is therefore plausible to hypothesize that [Ca\(^{2+}\)]\(_e\) is counter-regulatory to the effects of PTH in the proximal tubule by inducing VDR up-regulation (14).

In the present study, the mechanism regarding high [Ca\(^{2+}\)]\(_e\)-mediated VDR up-regulation in HK-2G cells was explored. We found this effect to be associated with rapid activation of p38 MAPK and more specifically phosphorylation of p38α MAPK, but not the ERK MAPK signaling pathways. Thus, specific intracellular targets of [Ca\(^{2+}\)]\(_e\) in proximal tubule epithelial cells were confirmed, establishing [Ca\(^{2+}\)]\(_e\) as a potential trigger for the counter-regulatory effects by interaction with its CaSR.

EXPERIMENTAL PROCEDURES

Materials—DMEM/F12 with normal calcium (1.8 mM) was purchased from (Invitrogen, Carlsbad, CA). The antibiotic G418 was obtained from Cellgro (Mediatech Inc., Herndon, VA). Antibodies purchased were α-tubulin, (Sigma-Aldrich), VDR, phospho-EGF, phospho-p38, p38/8 MAPK, protein A/G-garoside beads (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ATF2, phospho-MKK3/6, p38α MAPK, immobilized phospho-p38 MAPK (Thr180/Tyr182) mouse mAb (Cell Signaling Technologies, Danvers, MA), CaSR (Affinity BioReagents, Golden, CO) EDTA, Triton X-100, β-glycerophosphate, and Na\(_2\)VO\(_4\) (Sigma). Selective inhibitors of MAPK Kinase-1 (PD98059, U0126), p38 MAPK (SB203580, SB202190), phospholipase C (U73122) were purchased from Calbiochem-Novabiochem (EMD Biosciences, San Diego, CA). SB202190 was purchased from BIOMOL international (Plymouth Meeting, PA). The enhanced chemiluminescence kit, employing the SuperSignal West Pico substrate, was purchased from Pierce Biotechnology Inc. Lysis buffer and protease inhibitors were obtained from Pierce.

Cell Culture—The human proximal kidney cell type (HK-2) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). This cell line was originally developed for the purpose of a well differentiated proximal kidney cell line derived from human adult kidney (37). To establish a human renal proximal cell model to study the mechanism by which PTH and high [Ca\(^{2+}\)]\(_e\) influences VDR gene expression, HK-2 cells were further stably transfected with human PTHR1 and selected in G418 and made a stable clone (HK-2G) to study VDR and CYP27B1 expression in response to PTH (14). Cells were maintained in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and grown at 37 °C in a humidified 5% CO\(_2\) atmosphere. Cells were passaged every 4–5 days with 0.05% Versene and used for experimentation within 10 passages. Experiments were started by switching the medium to non-supplemented synthetic Ketaminocyte serum-free medium (Invitrogen) containing a minimal calcium concentration (0.02 mM), and antibiotics. In this medium, the cells could be manipulated with changes in calcium concentration. No effect on cell growth or viability during the experimental periods used were observed at concentrations of [Ca\(^{2+}\)]\(_e\), at or below 3 mM. Treatments were as described in figure legends using calcium chloride as the active agent for [Ca\(^{2+}\)]\(_e\) delivery. In addition to HK-2G cells, the study used the human proximal cell line HKC-8 obtained from Dr. Loraine Racusen, murine proximal MPCt and distal DKC-9 cells provided by Dr. Peter Friedman and human MG-63 osteosarcoma cells purchased from the ATCC.

Transfection and Luciferase Assay—For transient transfection experiments, HK-2G cells were seeded in 6-well dishes in DMEM/F12 plus 10% fetal bovine serum (1.5 × 10\(^5\) cells/well, ~80% confluent) and incubated overnight. The next day cells were transfected with 1 μg of hVDR promoter fused to luciferase (from Dr. Sylvia Christakos (38) and 500 ng of β-galactosidase and 4 μl of Lipofectamine (Invitrogen) in 1 ml of serum deficient DMEM/F12 per well. At 3 h post-transfection, media containing 10% fetal bovine serum replaced the transfection medium for the next 24 h. The following day, cells were serum-starved in DMEM/F12 overnight and pretreated for an hour with the MEK inhibitor, PD98059 (10 μM) or the p38 MAPK inhibitor, SB203580 or SB202190 (10 μM). Following incubations with the inhibitors, cells were treated with or without [Ca\(^{2+}\)]\(_e\). Lysates for luciferase assays were extracted 6 h after Ca treatment. The cells were washed in phosphate-buffered saline and lysed in 150 μl of lysis buffer (1% Triton X-100, 15 mM MgSO\(_4\), 4 mM EGTA, 1 mM diithiothreitol, 25 mM glycyl-glycine) on ice. Extracted samples were subjected to a luciferase assay system (Promega, Madison, WI) in triplicate and measured using a luminometer (Applied Biosystem TR17, Foster City, CA). The β-galactosidase measurements were performed on a Spectramax plus 384 (Molecular Devices, Sunnyvale, CA). Luciferase activity was normalized to β-galactosidase activity.

For siRNA experiments, HK-2G cells were seeded in complete medium without antibiotics the day before the experiment in 6-well plates at a density of 1.5 × 10\(^5\) cells per well on the day of the experiment. Cells were transfected with 100 nM SMARTpool PLUS siRNA, specifically targeting either CaSR or p38α, along with scrambled SMARTpool PLUS siControl (Dharmacon Inc., Lafayette, CA) using DharmaFECT 1 (Dharmacon Inc.) diluted in Opti-MEM I (Invitrogen) for 24 h. Transfectants were then incubated with fresh complete medium for a total time period of 48 or 72 h before protein extraction. For other experiments, siRNA transfected HK-2G cells were grown in complete medium for 48 h and serum-starved overnight, stimulated with or without [Ca\(^{2+}\)]\(_e\) for 12 h.
Fat milk in TBST (20 mM Tris-HCl, 137 mM NaCl, and 0.05% pro tease inhibitor mixture as described previously (39) with slight modification. Briefly, aliquots of cleared lyses containing 1 mg total proteins were pre-cleared with protein A/G-agarose beads and incubated with 2 µg of p38, p38α, p38β, phospho-p38 antibodies overnight with gentle rocking at 4 °C. Immune complexes were precipitated with protein A/G-agarose beads. For other experiments, protein lyses were incubated with immobilized phospho-p38 MAPK (Thr180/Tyr182) mouse mAb beads overnight, and immune complexes containing beads were isolated by centrifugation. Washed and precipitated protein/bead complexes were boiled with 2× SDS sample buffer and spun down the beads. The sample was loaded without beads on a 10% SDS-PAGE for Western blot analysis.

Western Blot Analysis—For the determination of ERK1/2 and p38 phosphorylation and VDR protein regulation, monolayers of HK-2G cells were grown on 6-well plates up to 80–90% confluent. Cells were incubated for 18 h in serum-free DMEM/F12 medium with normal (1.2 mM) calcium. This medium was removed and replaced with same medium supplemented with a variable Ca concentration or the 3 mM [Ca2+]e treatment as outlined in each experiment. Inhibitors were treated an hour before [Ca2+]e treatment. At the end of incubation period, media was removed and the cells were washed with ice-cold phosphate-buffered saline and then lysed in MPER lysis buffer (Pierce) containing 10 µl/ml of protease inhibitor mixture (Pierce). Cell lyses were centrifuged at 14,000 × g to separate cell protein from cell debris. The soluble protein content was measured by a Bradford assay (Bio-Rad) and was stored at −70 °C. After thawing, the cell lyses were combined with an equal volume of Laemmli sample buffer (Bio-Rad) heated at 95 °C, and separated using a 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred electrotherically to nitrocellulose membranes (Bio-Rad). After transfer, the membranes were blocked with 5% powdered non-fat milk in TBST (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween 20) pH 8.0 for 1 h at room temperature.

ERK1/2 and p38 MAPK phosphorylation and VDR induction were detected by immunoblotting using an overnight incubation with 1:1,000 dilution of rabbit/mouse monoclonal/polyclonal antisemur specific for phospho-ERK1/2, phospho-p38 MAPK and VDR. Blots were then washed in TBST 4 times for 10 min each at room temperature and then incubated at 1:10,000 for 1 h with Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted in 5% nonfat milk in TBSST. The membranes were washed with TBST again four times for 10 min each. After washing the membranes, bands were visualized by chemiluminescence according to the manufacturer’s protocol for SuperSignal West Pico detection (Pierce). The same membrane was used after 30 min stripping for determination of β-tubulin band as loading control.

Statistical Analysis—Results were expressed as the mean ± S.E., and significance was determined by analysis with two tailed Student’s t test. Values sharing the superscript are significant at p < 0.05.

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**RESULTS**

**HK-2G Cell Model**—In HK-2G cells, VDR protein is increased by a 3 mM dose of [Ca2+]e at 1 h post-treatment, then peak at 12 h and be sustained beyond 24 h (14). VDR induction at 12 h was selected for the following experiments. The regulation of VDR in response to elevated [Ca2+]e was examined in two additional proximal cell models (HK-2G plus human HKC-8 and murine MPCT), a distal cell model (murine DKC-8) and an osteoblast cell model (human MG-63). Interestingly, HK-2G cells had the greatest response among the proximal cell models, whereas the DKC-8 distal cells and MG-63 osteoblast cells both displayed VDR decreases following 3 mM [Ca2+]e exposure (Fig. 1).

High [Ca2+]e Activates Phosphorylation of ERK1/2 MAPK in HK-2G Cells—It has been shown that high levels of [Ca2+]e causes rapid protein tyrosine phosphorylation and activation of ERK1/2 in both primary cells of the parathyroid gland and HEK-293 cells transfected with CaSR (33). The MAPKs are pleiotropic regulators of numerous cellular activities. It was our interest to examine the involvement of MAPKs in high [Ca2+]e-mediated VDR up-regulation in HK-2G cells, in which CaSR was found to be endogenously expressed. To identify the signal transduction pathways involved in VDR up-regulation, we first examined whether ERK1/2 is phosphorylated by high [Ca2+]e in HK-2G cells. Fig. 2 showed that high [Ca2+]e increased phosphorylation of ERK1/2 in a time (Fig. 2A) and dose-dependent manner (Fig. 2C), as assayed by use of a phospho-ERK1/2 specific polyclonal antibody. Fig. 2, B and D depict the fold increase in ERK1/2 phosphorylation in the time and dose-dependent experiments. The results of this experiment demonstrated that 3 mM [Ca2+]e caused a rapid activation of pERK1/2 at 2.5-fold at 10 min, which remain elevated at 30 min. However, [Ca2+]e treatment of 4 mM and higher affected cell viability.

High [Ca2+]e Stimulates Phosphorylation of p38 MAPK in HK-2G Cells—It was previously shown that high [Ca2+]e (4.5 mM) or addition of the polycationic CaSR agonists, gadolinium (Gd3+) (25 µM), or neomycin (300 µM) or spermine (1 mM), each can stimulate phosphorylation of both ERK1/2 and p38 MAPKs, but not JNK, as assessed using phosphospecific antibodies to the respective MAPKs in mouse osteoblastic

**FIGURE 1. The cell specificity for [Ca2+]e-mediated VDR regulation was tested in proximal and distal kidney cells and in osteoblasts.** Proximal tubule epithelial cells were represented by human HK-2G and HKC-8 lines and the mouse MPCT proximal cell line. Mouse DKC-8 cells were used to represent a distal tubule epithelial type and human osteoblast-like MG-63 cells were employed as a non-renal cell model. In each case, cells were first serum-deprived in synthetic keratinocyte SFM for 16 h to reduce the exposure to serum Ca and mediators of intracellular signals. Then the cells were exposed to a 3 mM [Ca2+]e treatment. VDR protein levels were analyzed by Western blot following 12 h exposure to [Ca2+]e. The subsequent figures explain how the specific dose and time parameters were derived. Well to well loading was controlled by stripping and re-probing the blot using β-tubulin.
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**FIGURE 2. High [Ca\(^{2+}\)]\(_{e}\) activates phosphorylation of ERK1/2 MAPK in HK-2G cells.** A, serum-deprived HK-2G cells were incubated for the times indicated in the presence of 3 mM [Ca\(^{2+}\)]\(_{e}\) and ERK1/2 phosphorylation (P-ERK1/2) was measured by Western blot using the phospho-ERK1/2-specific antiserum. Anti-ERK2 was used as the loading control. B, blots were scanned and P-ERK1/2 bands were normalized with loading control (ERK2) and plotted as fold increase with the control level set at 1. C and D, dose response of [Ca\(^{2+}\)]\(_{e}\) (0–4 mM) for 10 min on the activation P-ERK1/2 in HK-2G cells (n = 2 blots). Equal amounts of protein from the whole cell lysates were analyzed in Western blots with anti P-ERK1/2. ERK2 was used as the loading control.

**FIGURE 3. High [Ca\(^{2+}\)]\(_{e}\) stimulates phosphorylation of p38 MAPK in HK-2G cells.** A, serum-deprived HK-2G cells were incubated for the times indicated in the presence of 3 mM [Ca\(^{2+}\)]\(_{e}\), and p38 MAPK phosphorylation (P-p38) was measured by Western blot with the phospho-p38 MAPK-specific antiserum. Anti-ERK2 was used as the loading control. B, blots were scanned and P-p38 MAPK bands were normalized with loading control and plotted as fold increase with control level set at 1 (*, p < 0.05; n = 3 blots). C and D, dose response of [Ca\(^{2+}\)]\(_{e}\) (0–4 mM) for 0.5 min on the activation P-p38 MAPK in HK-2G cells. These results clearly demonstrate that pretreatment of the SB203580 inhibitor abolished [Ca\(^{2+}\)]\(_{e}\)-mediated activation of phospho-p38, and furthermore diminished high [Ca\(^{2+}\)]\(_{e}\]-mediated VDR up-regulation in HK-2G cells (Fig. 4C). These results clearly indicate the involvement of the p38 MAPK pathway in [Ca\(^{2+}\)]\(_{e}\)-mediated VDR up-regulation in HK-2G cells. To check if this regulation also occurs at the level of transcription of VDR, transfection experiments were done with minus 1500 to plus 60 bp (−1500/+60) of the hVDR promoter fused to a luciferase gene (38) and subjected to the same treatments. Luciferase assay results revealed that pretreatment of SB203580 had a significant inhibitory effect both alone and further on high [Ca\(^{2+}\)]\(_{e}\]-mediated VDR gene expression (Fig. 4D). These data plainly demonstrate that high [Ca\(^{2+}\)]\(_{e}\) activates multiple MAPKs, but only activation of p38 MAPK is involved in VDR up-regulation, which occurred at the level of VDR gene transcription in HK-2G cells.

MC3T3-E1 cell line (40). Therefore, we next examined whether p38 MAPK was activated by high [Ca\(^{2+}\)]\(_{e}\) in HK-2G cells. Fig. 3 showed that high [Ca\(^{2+}\)]\(_{e}\) increased phosphorylation of p38 MAPK in a time (Fig. 3, A and B) and dose-dependent manner (Fig. 3, C and D), as assayed by use of a phospho-p38 MAPK-specific polyclonal antibody. The results demonstrate that 3 mM [Ca\(^{2+}\)]\(_{e}\) treatment caused a rapid and cyclical activation of p38 MAPK ~2–3-fold at 0.5 min that subsided at 1 min and rose again at 5 and 10 min time intervals (Fig. 3, A and B). Fig. 3, C and D demonstrate that 2–3 mM of [Ca\(^{2+}\)]\(_{e}\) elicited a highly significant increase in p38 MAPK phosphorylation (p < 0.001), while the effect at 4 mM of [Ca\(^{2+}\)]\(_{e}\) was abrogated.

**Effect of Inhibitors of Phospho-ERK and Phospho-p38 in Assessing [Ca\(^{2+}\)]\(_{e}\)-mediated VDR Induction—** Selective inhibitors of phospho-ERK were utilized to test if calcium-mediated increase of VDR is linked to ERK phosphorylation. The results of this experiment demonstrated that pretreatment of ERK1/2 inhibitors PD98059 (Fig. 4A) and U0126 (Fig. 4B) completely abolished 3 mM [Ca\(^{2+}\)]\(_{e}\)-activated phospho-ERK1/2 but not VDR in HK-2G cells. These results clearly demonstrated that although high [Ca\(^{2+}\)]\(_{e}\) activates phospho-ERK1/2, this effect is not responsible for VDR up-regulation in HK-2G cells, indicating that another MAPK pathway might be involved in this process.

Next, to confirm further that VDR activation by high [Ca\(^{2+}\)]\(_{e}\) directly involves the p38 MAPK pathway, a similar experiment was done using the selective inhibitor of the p38 MAPK pathway, SB203580. The results of this experiment demonstrated that pretreatment of the SB203580 inhibitor abrogated [Ca\(^{2+}\)]\(_{e}\)-mediated activation of phospho-p38, and furthermore diminished high [Ca\(^{2+}\)]\(_{e}\]-mediated VDR up-regulation in HK-2G cells (Fig. 4C). These results clearly indicate the involvement of the p38 MAPK pathway in [Ca\(^{2+}\)]\(_{e}\)-mediated VDR up-regulation in HK-2G cells. To check if this regulation also occurs at the level of transcription of VDR, transfection experiments were done with minus 1500 to plus 60 bp (−1500/+60) of the hVDR promoter fused to a luciferase gene (38) and subjected to the same treatments. Luciferase assay results revealed that pretreatment of SB203580 had a significant inhibitory effect both alone and further on
and declined at 30 min following high \([Ca^{2+}]_e\) treatment. Results also show that ATF2, a downstream effector molecule of p38 MAPK activation, had detectable phosphorylation at 0.5 min and sustained this elevated state of phosphorylation out to 10 min (Fig. 5, C and D).

**CaSR Is Involved in High \([Ca^{2+}]_e\)-mediated Up-regulation of VDR in HK-2G Cells**—In a previous study it was observed that HK-2G cells express CaSR endogenously, and we showed that VDR could be increased in these cells by treatment of 20 mM HK-2G cells (Fig. 6). Western blot results confirm that the CaSR agonist, Gd\(^{3+}\), dose-dependently activates VDR in HK-2G cells (Fig. 6A).

In several cell types, activation of MAPK via G-protein-coupled receptors has been reported to be sensitive to pertussis toxin (PTX) (41). We therefore examined the effects of PTX on high \([Ca^{2+}]_e\)-induced VDR activation in HK-2G cells. Serum-starved cells were preincubated with PTX for 2 h both in presence and absence of \([Ca^{2+}]_e\). Fig. 6B demonstrates that PTX did not inhibit VDR induction. This supports the involvement of the G\(_{q/11}\) coupling system in CaSR activated MAPK cascade in HK-2G cells. To further characterize the signaling mechanism that underlies CaSR-mediated VDR activation, we treated HK-2G cells with the phospholipase C (PLC) inhibitor, U73122. Pretreatment with 1 \(\mu M\) of U73122 disrupted the catalytic activity of PLC (data not shown) and blocked the induction of VDR both in the presence and absence of high \([Ca^{2+}]_e\) (Fig. 6C).

**siCaSR Abrogates High \([Ca^{2+}]_e\)-mediated Up-regulation of VDR in HK-2G Cells**—To test for a mediatory role of the CaSR in activating VDR, we used different approaches to down-regulate endogenous expression of CaSR in HK-2G cells. Endogenous CaSR expression was significantly down regulated by transfection with SMARTpool PLUS siRNA specifically targeting CaSR as demonstrated by Western blot analysis with anti-CaSR antibodies in Fig. 7A. A time course experiment showed that 72 h of treatment of siCaSR had a down-regulation effect of ~70% of endogenous CaSR expression compared with a siControl. To further confirm that VDR activation was mediated by CaSR, we examined the effects of siCaSR on VDR both in presence and absence of high \([Ca^{2+}]_e\) treatment. The results in Fig. 7B demonstrated that high \([Ca^{2+}]_e\) stimulates VDR in wild type as well as by a scrambled siControl construct transfected in HK-2G cells, and furthermore, siCaSR dramatically diminished up-regulation of VDR mediated by high \([Ca^{2+}]_e\) treatment.

Accordingly, as it was observed that gadolinium potently induced VDR up-regulation via CaSR (Fig. 6A), it was confirmed that the gadolinium-mediated VDR up-regulation could also be diminished by knocking down CaSR using siRNA targeting CaSR. Fig. 7C clearly demonstrates that VDR induction was profoundly inhibited when endogenous expression of CaSR in HK-2G cells was knocked-down compared with a scrambled siControl treatment. Surprisingly, down-regulation of endogenous expression of CaSR caused an increase in phosphorylation of ERK1/2, that we were not able to explain.

**Activation of p38\(\alpha\) Not p38\(\beta\) MAPK Associated with High \([Ca^{2+}]_e\)-mediated VDR Up-regulation in HK-2G Cells**—To further confirm that high \([Ca^{2+}]_e\)-mediated VDR up-regulation in HK-2G cells is associated with activation of specific isoform of p38 MAPK, we used a selective p38\(\alpha\) and p38\(\beta\) MAPK specific pharmacological agent, SB202190 (42). Results demonstrated that pretreatment of this agent completely abolished \([Ca^{2+}]_e\)-mediated VDR up-regulation in HK-2G cells (Fig. 8A), indicating that activation of one or both p38\(\alpha\)
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**FIGURE 6.** The polycationic CaSR agonist, gadolinium (Gd\(^{3+}\)), up-regulates VDR in HK-2G cells. Serum-starved HK-2G cells were treated with the indicated concentration of Gd\(^{3+}\) for 12 h. Whole cell proteins were extracted and Western blot was done using anti-VDR, anti-β-tubulin antibodies (A). Serum-starved HK-2G cells were preincubated with or without 100 ng/ml PTX for 1 h and treated with 3 mM [Ca\(^{2+}\)]\(_e\), for 12 h. VDR protein was assessed by Western blot using an anti-VDR antibody (B). In C, the PLC inhibitor (U73122) was found to block high [Ca\(^{2+}\)]\(_e\)-mediated up-regulation of VDR in HK-2G cells. Overnight serum-deprived HK-2G cells were pretreated with or with out 1 μM U73122 for 1 h and then treated in the presence and absence of 3 mM [Ca\(^{2+}\)]\(_e\), for 12 h. Equal amounts of protein were analyzed by Western blot using anti-VDR antibody. Anti-β-tubulin was used as the loading control.

**FIGURE 7.** siRNA targeted specifically to CaSR blocks high [Ca\(^{2+}\)]\(_e\)-mediated up-regulation of VDR in HK-2G cells. A, HK-2G cells were transfected with 100 nM SMARTpool Plus siCaSR or 100 nM scrambled SMARTpool siRNA control (Dharmacon Inc.) using DharmaFECT 1 (Dharmacon Inc.) diluted in Opti-MEM I (Invitrogen) for 24 h, and transfectants were incubated with fresh complete medium for total time period of 48 and 72 h. Following siRNA treatment whole cell lysates were analyzed by Western blot. Membranes were probed with primary antibody against CaSR. Stripped blots were re-probed with β-tubulin as loading control. B, 48 h siRNA-transfected HK2G cells from A, were serum-deprived overnight, stimulated with or without [Ca\(^{2+}\)]\(_e\), for 12 h. Western blot was done using VDR monoclonal antibody and β-tubulin was used as loading control. In C, knocking down endogenous expression of CaSR diminished gadolinium (Gd\(^{3+}\))-mediated VDR up-regulation. HK-2G cells were transfected with 100 nM SMARTpool Plus siRNA targeted to CaSR or scrambled siControl for 24 h. Following transfection cells were serum-deprived for 16 h and treated with 3 mM [Ca\(^{2+}\)]\(_e\), or Gd\(^{3+}\) (100 ng/ml) for 12 h. Whole cell lysates were prepared, and equal amounts of protein were used for Western blot analysis. Blots were probed with monoclonal antibodies raised against VDR, phospho ERK1/2, and phospho-p38. Equal loading was checked by re-probing the same blots with either β-tubulin or ERK2 antibodies, as indicated.

In this study we sought to determine whether high [Ca\(^{2+}\)]\(_e\) treatment could stimulate VDR in a kidney proximal epithelial cell model and to identify the receptor and signaling pathways mediating this effect. Here, the data suggest that VDR is a potential on/off switch for regulating CYP27B1. The proximal tubule is the main site of regulation CYP27B1 expression and synthesis of 1,25(OH)\(_2\)D\(_3\) (43, 44). PTH regulates its effects on CYP27B1 by elevating cAMP and activation of CREB followed by additional transcriptional modification at the CYP27B1 promoter (20, 45). Loss of VDR by PTH probably relieves repression of CYP27B1 and this loss would appear to be corrected by the eventual rise in [Ca\(^{2+}\)]\(_e\], and CaSR-mediated activation of p38 in renal proximal cells.

It is also well established that 1,25(OH)\(_2\)D\(_3\) activates its own break down by induction of CYP24 and p38β MAPK isoforms are involved in [Ca\(^{2+}\)]\(_e\)-mediated VDR up-regulation. Additional experiments were done to determine if a specific single isoform of p38 MAPK is activated in response to high [Ca\(^{2+}\)]\(_e\), in HK-2G cells. Total p38 MAPK was first immunoprecipitated with a pan p38 antibody, and immunoblotted with antibodies specific for p38α and p38β (Fig. 8B). This showed that HK-2G cells expressed both isoforms of p38 and [Ca\(^{2+}\)]\(_e\), treatment did not affect the total level of either p38α or p38β. The increase in p38α phosphorylation in high [Ca\(^{2+}\)]\(_e\)-activated cells was confirmed by immunoprecipitation with specific antibodies against p38α or p38β and subsequent immunoblot analysis with phospho-p38 antibody (Fig. 8C). We further confirmed that high [Ca\(^{2+}\)]\(_e\), treatment can induce the specific activation of p38α MAPK in HK-2G cells. In this experiment, total phospho-p38 was immunoprecipitated using immobilized phospho-p38 MAPK (Thr180/Tyr182) mouse mAb followed by an immunoblot with antibodies specific for p38α and p38β (Fig. 8D). These data clearly demonstrated that high [Ca\(^{2+}\)]\(_e\) treatment activates p38α, but not p38β MAPK and that this activation of p38α is linked to up-regulation of VDR HK-2G cells.

si-p38α MAPK Abrogates High [Ca\(^{2+}\)]\(_e\)-mediated Up-regulation VDR in HK-2G Cells—To elucidate the regulation of VDR signaling by p38α MAPK isoforms in HK-2G cells, we inhibited p38α expression by using siRNA. High [Ca\(^{2+}\)]\(_e\) does not affect the level of total p38α in siControl cells, but expression of total p38α was abrogated in si-p38α-treated cells (Fig. 8E, middle panel). As expected, high [Ca\(^{2+}\)]\(_e\)-mediated up-regulation of VDR was inhibited in si-p38α-treated cells (Fig. 8E). These data illustrate that activation of p38α, not p38β MAPK, is associated with high [Ca\(^{2+}\)]\(_e\)-mediated VDR up-regulation in HK-2G cells.

**DISCUSSION**

In this study we sought to determine whether high [Ca\(^{2+}\)]\(_e\) treatment could stimulate VDR in a kidney proximal epithelial cell model and to identify the receptor and signaling pathways mediating this effect. Here, the data suggest that VDR is a potential on/off switch for regulating CYP27B1. The proximal tubule is the main site of regulation CYP27B1 expression and synthesis of 1,25(OH)\(_2\)D\(_3\) (43, 44). PTH regulates its effects on CYP27B1 by elevating cAMP and activation of CREB followed by additional transcriptional modification at the CYP27B1 promoter (20, 45). Loss of VDR by PTH probably relieves repression of CYP27B1 and this loss would appear to be corrected by the eventual rise in [Ca\(^{2+}\)]\(_e\], and CaSR-mediated activation of p38 in renal proximal cells.

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Renal p38α Activation by [Ca\(^{2+}\)]\(_e\) Increases VDR

expression (30) and simultaneously down-regulation of CYP27B1 expression (30, 46). The molecular details of these events are just beginning to unfold, but both of these effects by 1,25(OH)\(_2\)D\(_3\) require VDR (47). In the case of CYP27B1, the regulation by 1,25(OH)\(_2\)D\(_3\) might be indirect because a classical VDRE is absent in the first 1500 bp of CYP27B1 promoter, while suppressive effects on this promoter segment still occur via ligand-mediated transrepression via a novel negative VDRE (48). The mechanism for the transrepressive regulation appears to involve recruitment of acetylated histones and methylation (49). The phosphaturic molecule, fibroblast growth factor-23 (FGF-23), is a secondary repressive mechanism on CYP27B1, where FGF-23 induction from bone tissue is under 1,25(OH)\(_2\)D\(_3\) control (50). Based on this finding and the data of this study, Ca/VDR/1,25(OH)\(_2\)D\(_3\) and FGF-23 represent distinct mechanisms for controlling CYP27B1. Whether or not [Ca\(^{2+}\)]\(_e\) can directly repress CYP27B1 will require more data.

The HK-2G model used in this study was previously demonstrated to be responsive to PTH, which down-regulated VDR (14). This prior study suggested that high [Ca\(^{2+}\)]\(_e\) treatment in HK-2G cells is counter-regulatory to the effects of PTH on VDR. These in vitro changes in VDR mirror the exact scenario that is reflective of in vivo renal VDR regulation that occur during hypo- and hypercalcemia conditions (47, 51, 52). It will be interesting to examine the dual impact of Ca versus PTH signaling in HK-2G cells with regard to molecular components of renal vitamin D metabolism.

Importantly, the concentration of [Ca\(^{2+}\)]\(_e\) used to study signaling and VDR induction in HK-2G cells was within the physiological range of 3 mM indicating that activation of CaSR was likely. Furthermore, use of calcium channel blockers did not preclude the [Ca\(^{2+}\)]\(_e\)-mediated regulation of VDR (data not shown), strengthening the hypothesis for CaSR involvement. CaSR activation in other cell models has been demonstrated to result in ERK phosphorylation that is tied to effects of [Ca\(^{2+}\)]\(_e\) on targets in the central nervous system and cell survival and proliferation (53–56). Thus, it was reasonable to examine this signaling pathway in the context of VDR regulation. The results in Figs. 3 and 5 demonstrate that [Ca\(^{2+}\)]\(_e\)-mediated ERK phosphorylation does occur in the HK-2G cells, but blocking ERK phosphorylation failed to confirm it plays a direct role in VDR up-regulation, as was the case with p38 phosphorylation. Nevertheless, ERK phosphorylation seems to have some involvement with regulating HK-2G cell VDR levels since levels of phospho-ERK are dramatically increased when CaSR is knocked-down by siRNA treatment (Fig. 8). We also acquired data (not shown) indicating an up-regulation of VDR promoter in the presence of the PD98059 inhibitor of phospho-ERK, but these results cannot be explained in the framework of the present study.

The most significant effect of [Ca\(^{2+}\)]\(_e\) in the HK-2G model was rapid and highly significant phospho-activation of p38 as that was critical to the induction of VDR (see Figs. 3C and 4D). Numerous reports also demonstrate the ability of CaSR activation to lead to an increase of phospho-p38 (56–58) that results in changes in PThrP release during humoral hypercalcemia, for example. As well, the repression of VDR promoter activity by treatment with SB203580 emphasizes an important role of phospho-p38 to maintain constitutive VDR expression in the HK-2G cell type. We further dissected the p38 MAPK signaling in regulating high [Ca\(^{2+}\)]\(_e\)-mediated VDR up-regulation in HK-2G cells. Using another specific pharmacological inhibitor that affects both p38α, p38β isoforms, SB202190; and using an siRNA specifically targeting p38α, we demonstrated that p38α, not the p38β MAPK, is involved in high [Ca\(^{2+}\)]\(_e\)-mediated VDR regulation in HK-2G cells.

SB inhibitors are pyridinyl imidazole compounds have been widely used in investigation of the biological function of p38 MAPK (30). These compounds are also well known for their ability to inhibit TGFβ receptor activation (59). TGFβ is an important regulator of 1,25(OH)\(_2\)D\(_3\)-mediated VDR trans-
Renal p38α Activation by \([\text{Ca}{}^{2+}]_e\) Increases VDR

activation on target genes (60). SB203580 belongs to a class of pyridinyl imidazoles that inhibits the stress-activated protein (SAP) kinases SAPK2a/p38 and SAPK2b/p38 beta 2 but not other mitogen-activated protein kinase family members. As with inhibitors of other protein kinases, SB203580 binds in the ATP-binding pocket of SAPK2a/p38α. The type I TGFβ receptor, which has serine at the position equivalent to Thr106 of SAPK2a/p38α and SAPK2b/p38β, is inhibited by SB203580. In the present study, 1,25(OH)2D3 effects were not studied and treatment of HK-2G cells with the SB inhibitors was only used to demonstrate the involvement of p38 in \([\text{Ca}{}^{2+}]_e\)-mediated increase in VDR. Control of this effect by \([\text{Ca}{}^{2+}]_e\) was confirmed to involve transcriptional regulation of VDR itself. We showed that activation of the upstream intermediate of p38, MKK3/6, and p38-mediated downstream transcriptional control by ATF2 were both involved. Thus, we can rule out the interference of TGFβ in \([\text{Ca}{}^{2+}]_e\)-mediated VDR gene expression.

During this study, we did not have access to pharmaceutical CaSR inhibitors or the CaSR mimetic drugs; we therefore used siRNA to knock down CaSR expression to examine its intermediary effect on VDR induction. A SMARTpool PLUS siRNA specifically and effectively targeted the CaSR in HK-2G cells and dramatically inhibited CaSR protein expression. This molecular tool, which includes a combination of 3 siRNA constructs, was successfully utilized to demonstrate the involvement of CaSR in both the \([\text{Ca}{}^{2+}]^2_\text{C}^2\) and \([\text{Ca}{}^{2+}]_e\)-mediated inductions of VDR in proximal cells, respectively (see Fig. 7C). Further study will be needed to determine which actual si-construct(s) was responsible for the interference signal against CaSR. A recent report published by Rodriguez et al., (61) demonstrated a positive inductive action of the calcimimetic R-568 on VDR levels in the parathyroid gland (PTG). Given that Garfia et al., (62) have shown a positive correlation of PTG VDR with exposure to \([\text{Ca}{}^{2+}]_e\) in the PTG and an inverse regulation of VDR with blood PTH in PTG, it is now plausible to predict that the proximal convoluted tubule and the PTG share common effectors for coupling of G-protein, respectively, for Ca and PTH signaling in these cell types. These findings provide strong rationale for the use of potent calcimimetic drugs that specifically target the CaSR for treatment of chronic kidney disease and severe secondary hyperparathyroidism. Caution should be applied if it can be determined that CaSR activation leads to weakened 1,25(OH)2D3 responsiveness in distal convoluted tubules, bone cells and extrarenal tissues where \([\text{Ca}{}^{2+}]_e\) signaling may decrease cellular VDR concentrations (see Fig. 1). Osteoblasts were previously reported to express functional CaSR, and also to sense fluctuations in local \([\text{Ca}{}^{2+}]_e\), resulting in induced osteoblast activity (63).

Additionally, the present study indicates that the CaSR utilizes \(G_{\text{q/11}}\) for coupling to its major signaling pathways. The data in Fig. 7 demonstrated that knocking down endogenous CaSR, resulted in suppression of VDR in response to high \([\text{Ca}{}^{2+}]_e\) while activating CaSR with gadolinium, a known polycationic agonist of CaSR, increased VDR protein levels. This confirms a direct role of CaSR to mediate \([\text{Ca}{}^{2+}]_e\) induced up-regulation of VDR in proximal kidney cells. Thus in a high \([\text{Ca}{}^{2+}]_e\) condition (Fig. 9), CaSR couples to the \(G_{\text{q/11}}\) system, leading to PLC activation and phosphorylation of p38α MAPK, which in turn may activate ATF2 to promote the gene expression of VDR. This last step is currently under investigation (Fig. 9).

In conclusion, high extracellular \([\text{Ca}{}^{2+}]_e\) plays a critical role in up-regulating renal proximal cell VDR through a mechanism that involves activation of p38α MAPK. The data herein demonstrate that CaSR signaling is key to the mechanism of VDR increase in HK-2G cells. Our data suggest that serum \([\text{Ca}{}^{2+}]_e\) concentrations may be counter-regulatory to actions of PTH in renal proximal cells.

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