# Role of ceramide in $Ca^{2+}$-sensing receptor-induced apoptosis

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**Abstract** **Increased extracellular $Ca^{2+}$ ($[Ca^{2+}]_o$) can damage tissues, but the molecular mechanisms by which this occurs are poorly defined. Using HEK 293 cell lines that stably overexpress the $Ca^{2+}$-sensing receptor (CaR), a G protein-coupled receptor, we demonstrate that activation of the CaR leads to apoptosis, which was determined by nuclear condensation, DNA fragmentation, caspase-3 activation, and increased cytosolic cytochrome *c*. This CaR-induced apoptotic pathway is initiated by CaR-induced accumulation of ceramide which plays an important role in inducing cell death signals by distinct G protein-independent signaling pathways. Pretreatment of wild-type CaR-expressing cells with pertussis toxin inhibited CaR-induced [$^3$H]ceramide formation, c-Jun phosphorylation, and caspase-3 activation. The ceramide accumulation, c-Jun phosphorylation, and caspase-3 activation by the CaR can be abolished by sphingomyelinase and ceramide synthase inhibitors in different time frames. Cells that express a nonfunctional mutant CaR that were exposed to the same levels of $[Ca^{2+}]_o$ showed no evidence of activation of the apoptotic pathway. In conclusion, we report the involvement of the CaR in stimulating programmed cell death via a pathway involving GTP binding protein alpha subunit ($G\alpha_i$)-dependent ceramide accumulation, activation of stress-activated protein kinase/c-Jun N-terminal kinase, c-Jun phosphorylation, caspase-3 activation, and DNA cleavage.**—Wu, Z., R. Tandon, J. Ziembicki, J. Nagano, K. M. Hujer, R. T. Miller, and C. Huang. **Role of ceramide in $Ca^{2+}$-sensing receptor-induced apoptosis. *J. Lipid Res.* 2005.** 46: **1396–1404.**

**Supplementary key words** stress-activated protein kinase/c-Jun N-terminal kinase • HEK 293 cell • G protein-coupled receptor

The $Ca^{2+}$-sensing receptor (CaR) is a G protein-coupled receptor (GPCR) that plays a crucial role in the regulation of parathyroid hormone synthesis and secretion in the parathyroid and $Na^+$, $Cl^-$, $Ca^{2+}$, and water transport in the kidney (1). Mutations of the human CaR gene cause disorders of mineral-ion homeostasis and lead to two different types of human disease: inactivating mutations cause familial hypocalciuric hypercalcemia (the heterozygous state) and neonatal severe hyperparathyroidism (the homozygous state) (2); and activating mutations cause autosomal dominant hypocalcemia (3, 4). In the kidney, the receptor mediates the adverse renal effects of hypercalcemia, including reducing $Na^+$, $K^+$, $Ca^{2+}$, $Cl^-$, and water reabsorption, reducing the renal response to vasopressin, aquaporin expression, vasoconstriction, and hypertension and leading to chronic renal insufficiency (4–6). In addition to sensing extracellular $Ca^{2+}$ ($[Ca^{2+}]_o$) under physiological conditions, the CaR also responds to $Pb^{2+}$. The $EC_{50}$ for $Pb^{2+}$ may be as low as 100 μM at physiological levels of $[Ca^{2+}]_o$ (7), so that an increased body lead burden could cause chronic renal insufficiency via activation of this receptor (8). Thus, either mutations of the CaR or exposure to heavy metals cause renal cell damage and injury that can perturb their morphology and function. The eventual end point of this process is cell death, which suggests that the CaR could be involved in the regulation of renal epithelial cell apoptosis.

Apoptosis plays a major role in embryonic development and tissue homeostasis. However, apoptosis induced by cytotoxic agents, ultraviolet (UV) and γ irradiation, oxidative stress, and long-term agonist stimulation can lead to pathological changes such as renal insufficiency and acute renal failure (9–11). A characteristic feature of acute toxic renal cell injury is partial degradation of membrane lipid constituents and generation of signaling molecules. One recent study showed that renal apoptosis was directly related to endogenous ceramide production in response to chronic unilateral ureteral obstruction in the rat (9). Ceramide belongs to a group of sphingosine-based lipid second messenger molecules that are involved in the regula-

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Abbreviations: $[Ca^{2+}]_i$, intracellular $Ca^{2+}$; $[Ca^{2+}]_o$, extracellular $Ca^{2+}$; CaR, $Ca^{2+}$-sensing receptor; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase kinase 1.

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tion of proliferation, cell cycle arrest, differentiation, and programmed cell death (12–14). A variety of exogenous stimuli, such as cytokines, heat shock, growth factors, tumor necrosis factor, vitamin D, and UV and γ irradiation, acting through distinct G protein-independent signaling pathways, are able to stimulate sphingomyelin degradation and ceramide production through the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway to induce apoptosis (12–15); however, recent evidence shows that agonists acting through GPCRs may also affect sphingolipid metabolism (16) or the SAPK/JNK signaling pathway (17). Although diverse forms of renal injury elicit ceramide accumulation, the coupling of the CaR to ceramide production, SAPK/JNK activation, and apoptosis has not been reported.

The mitogen-activated protein kinase (MAPK) superfamily includes three major pathways: the extracellular signal-regulated kinase (ERK) 1/2 pathway, the SAPK/JNK pathway, and the p38 MAPK pathway (18, 19). The activation of MAPK pathways mediates a variety of cellular responses ranging from cell proliferation to differentiation and apoptosis, depending on the stimulus, the cell type, and the conditions (18–21). Increasing evidence indicates that the SAPK/JNK pathway plays an important role in triggering cell apoptosis (21–25). The CaR acting via $G\alpha_i$ has been shown to activate ERK1/2 in a variety of cell lines, including fibroblasts, osteoblasts, parathyroid cells, HEK 293 cells, and MDCK cells (1). In in vitro kinase assays using glutathione *S*-transferase (GST)-c-Jun as the substrate, the CaR stimulates JNK through a pertussis toxin-sensitive G protein pathway in MDCK cells (26). Recently, one report showed that the CaR also activates p38 MAPK in the regulation of a $Ca^{2+}$-activated $K^+$ channel (27). Here, we report that the CaR induces cell apoptosis via a signaling pathway involving $G\alpha_i$-dependent ceramide accumulation, SAPK/JNK activation, c-Jun phosphorylation, caspase-3 activation, and DNA cleavage.

## MATERIALS AND METHODS

### Materials

All chemicals were purchased from Sigma Chemicals or Fisher Scientific unless specified otherwise. The monoclonal anti-CaR antibody and HEK 293 cells that stably overexpress the hemagglutinin-tagged wild-type CaR ($CaR^{WT}$) and the hemagglutinin-tagged nonfunctional mutant CaR ($CaR^{Mut}$; point mutation Arg-796 to Trp leads to a blunted response to $[Ca^{2+}]_o$) were described previously (7). G418 sulfate and cell culture reagents were purchased from Life Technologies. [$^3$H]palmitic acid (43 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. Monensin and fumonisin $B_1$ were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Monensin was prepared in stock solutions of ethanol. SuperSignal West Pico chemiluminescent substrate and BCA protein assay reagent were obtained from Pierce. The polyclonal antibodies against phospho-c-Jun (Ser-73), phospho-SAPK/JNK (Thr-183/Tyr-185), phospho-SAPK/ERK kinase 1 [SEK1/mitogen-activated protein kinase kinase 4 (MKK4); Thr-261], and total ERK1/2 were supplied by New England Biolabs (Beverly, MA). The ceramide standards were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The monoclonal anti-cytochrome *c* (A-8) and anti-caspase-3 (E-8) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell prelabeling, treatment, and measurement

HEK 293 cells that are undetectable for endogenous CaR by immunoblotting were stably overexpressed in $CaR^{WT}$ or $CaR^{Mut}$ and maintained in DMEM supplemented with 10% FBS, 5 U/ml penicillin, 5 μg/ml streptomycin, and 0.2 mg/ml G418. The cells were cultured on six-well plates and prelabeled with ~1 μCi/ml [$^3$H]palmitic acid in 1 ml of 5% FBS-DMEM overnight. To measure [$^3$H]ceramide production, the cultures were starved in 0.5% FBS-DMEM containing 20 mM HEPES (pH 7.4) for 27 h and then treated with 5 mM $CaCl_2$ for the last 0–24 h. In some experiments in which the effect of inhibitors on CaR-stimulated ceramide formation was determined, after radiolabeling, the cells were starved in 0.5% FBS-DMEM in the presence or absence of 30 μM monensin, 25 μM fumonisin $B_1$, 15 μM desipramine chloride, or 15 μM SP600125 for 27 h and treated for the last 0–24 h with 5 mM $CaCl_2$. In the experiments with pertussis toxin treatment, HEK 293 cells that stably overexpressed $CaR^{WT}$ were prelabeled with [$^3$H]palmitic acid for 1 day in the presence or absence of 100 ng/ml pertussis toxin. The cells were starved in 0.5% FBS-DMEM for 27 h and treated with 5 mM $CaCl_2$ for the last 0–24 h. The reaction was terminated by adding 0.6 ml of cold 2% acetic acid in methanol, and total cellular lipids were extracted with chloroform, 2% acetic acid in methanol, and water (6:6:5.5, v/v). [$^3$H]ceramide was resolved from the total cellular lipids by TLC and identified by comigration with commercial standards in a solvent system containing chloroform-methanol-ammonium hydroxide (200:25:2.5, v/v) for ceramide. The standards were visualized with iodine vapor, and the areas corresponding to ceramide were scraped into scintillation vials and quantitated by liquid scintillation spectrometry. The cellular proteins from lipid extraction samples were washed with acetone once, air dried, resuspended in 1× loading buffer without bromphenol blue, and cooked at >95°C for 5 min, and the protein concentration was normalized for immunoblotting.

### Immunoblotting

HEK 293 cells that stably overexpress $CaR^{WT}$ or $CaR^{Mut}$ were starved in 0.5% FBS-DMEM for 27 h and stimulated with 5 mM $CaCl_2$ for the last 0–24 h. The cultures were harvested in a buffer containing 20 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, and protease inhibitors and homogenized with 30 strokes of a Dounce homogenizer. Homogenates were centrifuged at 1,500 rpm for 10 min at 4°C to yield a postnuclear pellet. The resulting supernatants were centrifuged at 15,000 rpm for 1 h at 4°C to yield crude membrane and cytosol fractions. Protein concentrations were determined using the BCA protein assay reagent with BSA as the standard and then adjusted to the same concentration. The samples were subjected to 11% SDS-PAGE, processed for immunoblotting with the antibodies indicated, and visualized with enhanced chemiluminescence. The cytosol fraction was used for immunoblot assay of cytosolic cytochrome *c*. Immunoblot films were scanned and bands were quantified using Scion Image software. The data were analyzed for significance using one-way repeated-measure ANOVA followed by Tukey's test for comparison of the same treatment of $CaR^{WT}$-expressing cells with $CaR^{Mut}$-expressing cells (28).

### Nuclear staining

HEK 293 cells at 40–50% confluence that stably overexpress either $CaR^{WT}$ or $CaR^{Mut}$ were grown on glass cover slips for 2 days. Cells were starved in 0.5% FBS-DMEM for 27 h and stimulated with or without 5 mM $CaCl_2$ for the last 24 h. The cover slips were fixed with cold acetone for 20 min. After fixation, the cover slips

were stained for 5 min with 200 nM 4′,6-diamino-phenylindole (DAPI), washed with 1× PBS three times, and then observed using a fluorescence microscope (Zeiss model LSM-5 Pascal), and images were collected using the Axiovert 200 program (Zeiss).

### Measurement of DNA fragmentation

HEK 293 cells that stably overexpress either $CaR^{WT}$ or $CaR^{Mut}$ were starved in 0.5% FBS-DMEM for 27 h and stimulated with $CaCl_2$ as indicated. After appropriate treatment, the cells (both floating and adherent) were harvested and centrifuged at 1,500 *g* for 2 min, and the pellets were resuspended in 0.5 ml of lysis buffer containing 5 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100 and placed on ice for 15 min. The samples were then centrifuged at 12,000 *g* for 20 min, and the supernatant containing DNA cleavage products in the same amount of cellular proteins was precipitated overnight using isopropyl alcohol. The samples were centrifuged at 14,500 rpm for 20 min. Pellets were resuspended in Tris-EDTA buffer and digested with 0.2 mg/ml proteinase K and 1 mg/ml RNase A for 60 min at 48°C. DNA fragments were separated on a 1.5% agarose gel, visualized with ethidium bromide, and photographed using the Bio-Rad image system.

## RESULTS

### $[Ca^{2+}]_o$ induces apoptosis in HEK 293 cells that overexpress $CaR^{WT}$

The signature events of apoptosis are cell shrinkage, nuclear condensation, caspase activation, and DNA cleavage. The biochemical feature of DNA cleavage is the onset of endonuclease cleavage of cellular DNA into a nucleosome ladder (22, 25, 29). To study this, we examined the effect of $[Ca^{2+}]_o$ on apoptosis in CaR-expressing HEK 293 cells. **Figure 1A**, **B** show that $[Ca^{2+}]_o$ induced a typical apoptotic DNA ladder in a time- and dose-dependent manner in $CaR^{WT}$-expressing cells but not in $CaR^{Mut}$-expressing cells. $[Ca^{2+}]_o$-induced nuclear changes were also evaluated using DAPI staining and fluorescence microscopy. Exposure of $CaR^{WT}$-expressing cells to 5 mM $[Ca^{2+}]_o$ for 24 h showed typical apoptotic nuclei, exhibiting highly fluorescent, condensed chromatin, whereas untreated cells showed uniform nuclear staining, indicating that these cells were healthy and the nuclei were intact (Fig. 1C). Next, we determined whether the CaR stimulates caspase activation, which plays a central role in the execution of apoptosis. Immunoblot analysis of $CaR^{WT}$-expressing HEK 293 cells treated with 5 mM $[Ca^{2+}]_o$ for different times showed induction of the apoptotic protease caspase-3 cleavage and increased cytochrome *c* in the cytosol, whereas $[Ca^{2+}]_o$ did not activate caspase-3 or increase cytosolic cytochrome *c* in $CaR^{Mut}$-expressing cells (**Fig. 2**).

### The CaR activates the SAPK/JNK pathway

Recently, increasing evidence has shown that SAPK/JNK may play an important role in triggering apoptosis in response to UV and γ irradiation, cytotoxic agents, oxidative stress, and inflammatory cytokines (22, 23). The CaR plays a central role in the regulation of MAPK signaling cascades (1, 26, 27). To test the hypothesis that CaR-induced apoptosis may involve the stimulation of SAPK/JNK, we examined the effect of the CaR on this signaling pathway. As shown in **Fig. 3B**, the CaR stimulated the phosphorylation of SAPK/JNK in a time-dependent manner, whereas the phosphorylation of SAPK/JNK was not significantly changed in $CaR^{Mut}$-expressing cells (Fig. 3A).

Earlier studies also showed that the SAPK/JNK pathway

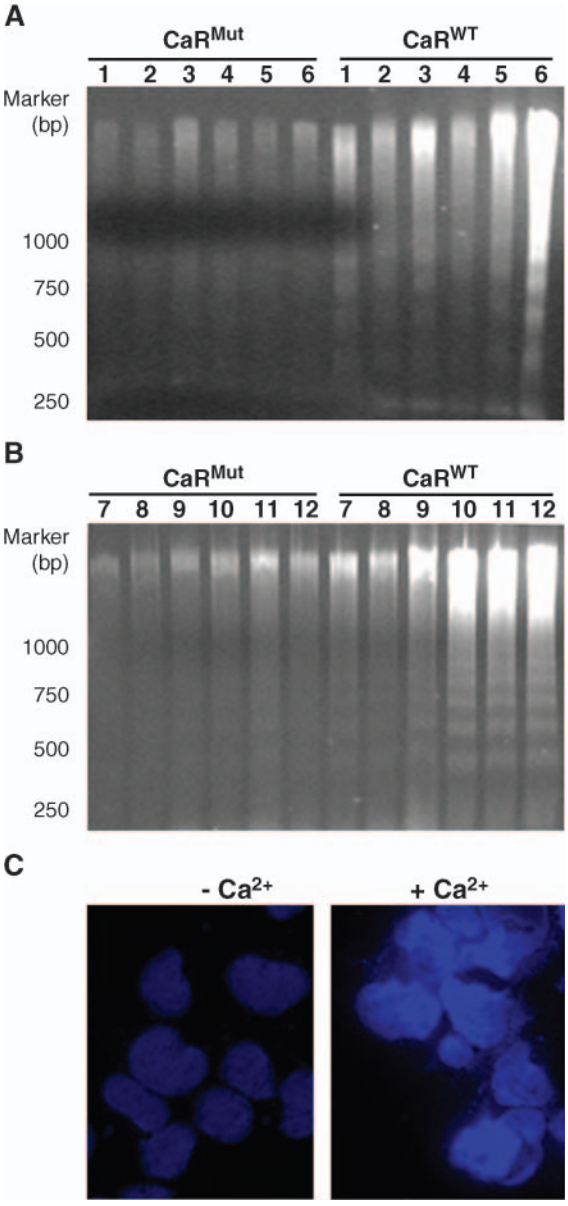


**Fig. 1.** Effect of the $Ca^{2+}$-sensing receptor (CaR) on apoptosis in HEK 293 cells. HEK 293 cells that stably overexpress the wild-type CaR ($CaR^{WT}$) or the mutant CaR ($CaR^{Mut}$) were starved and stimulated with 5 mM $CaCl_2$ for 0 min (lane 1), 5 min (lane 2), 1 h (lane 3), 3 h (lane 4), 6 h (lane 5), and 24 h (lane 6) (A) or with 0.5 mM (lane 7), 1 mM (lane 8), 2.5 mM (lane 9), 5 mM (lane 10), 7.5 mM (lane 11), and 10 mM $CaCl_2$ (lane 12) for 24 h (B). After appropriate treatment, the cells (both floating and adherent) were collected and lysed to prepare total DNA. The samples were separated on a 1.5% agarose gel. The results represent duplicates from five separate experiments. C: $CaR^{WT}$-expressing HEK 293 cells were cultured on cover slips for 2–3 days. The cells were starved and treated with or without 5 mM $CaCl_2$ for 24 h. The cells were stained with 200 nM 4′,6-diamino-phenylindole and photographed using a fluorescence microscope.

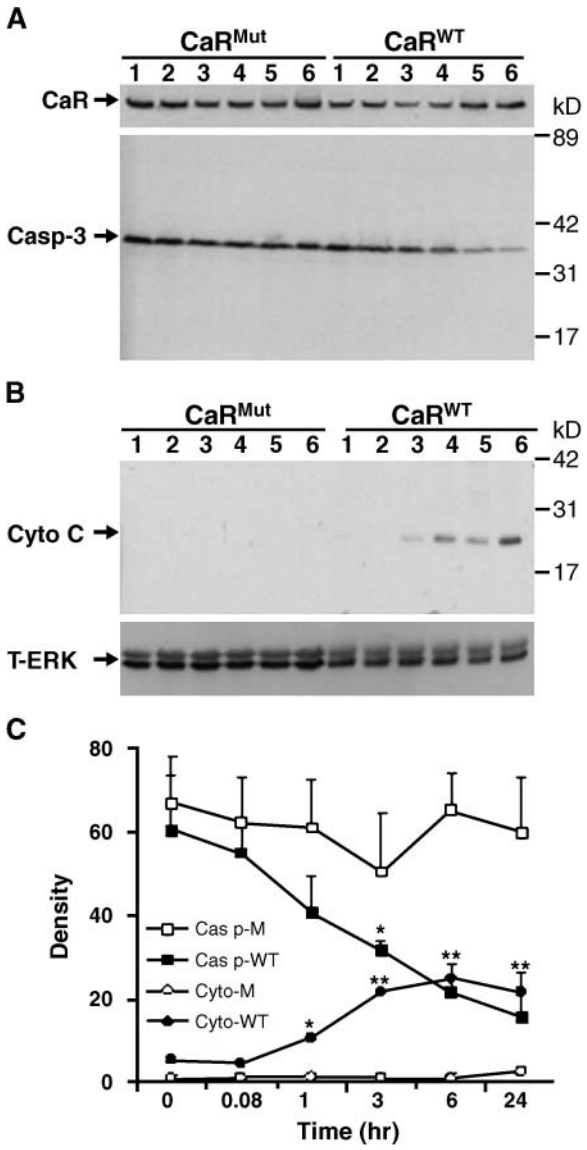


**Fig. 2.** The CaR activates caspase-3 and increases cytosolic cytochrome *c*. HEK 293 cells that stably overexpress $CaR^{WT}$ or $CaR^{Mut}$ were starved, incubated with 5 mM $CaCl_2$ for different periods of time, and then fractionated to obtain the pellet and cytosol. Equal amounts of cellular protein from the pellet for caspase-3 and CaR (A) and cytosol for cytochrome *c* and extracellular signal-regulated kinase (ERK; B) were subjected to 11% SDS-PAGE and processed for immunoblotting using antibodies against caspase-3 (Casp-3) and cytochrome *c* (Cyto C). T-ERK, total ERK. C: Immunoblot films taken from three individual experiments with duplicate samples were scanned, and the optical densities of the caspase-3 and cytochrome *c* bands were calculated using Scion Image software. The optical density values of the caspase-3 (Casp-WT) and cytochrome *c* (Cyto-WT) bands in $CaR^{WT}$-expressing cells were statistically different from those in $CaR^{Mut}$-expressing cells (Casp-M and Cyto-M). $P > 0.05$ unless indicated otherwise: * $P < 0.05$ and ** $P < 0.01$ by ANOVA. Error bars represent standard deviation.

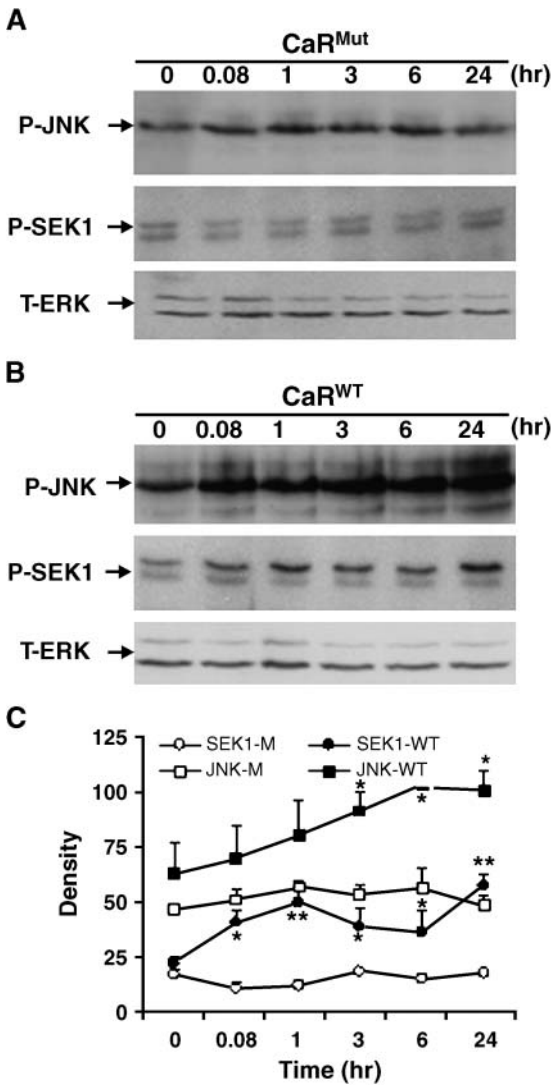


**Fig. 3.** The CaR activates stress-activated protein kinase/ERK kinase 1 (SEK1) and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in HEK 293 cells. HEK 293 cells that stably overexpress $CaR^{Mut}$ (A) or $CaR^{WT}$ (B) were starved, incubated with 5 mM $CaCl_2$ for different periods of time, and then fractionated to obtain the postnuclear pellet. Equal amounts of cellular protein from the postnuclear pellet were subjected to 11% SDS-PAGE and processed for immunoblotting using antibodies against phospho-SEK1 (P-SEK1), phospho-SAPK/JNK (P-JNK), and total ERK (T-ERK). C: Immunoblot films taken from three individual experiments with duplicate samples were scanned, and the optical densities of the phospho-SAPK/JNK and phospho-SEK1 bands were calculated using Scion Image software. The optical density values of the phospho-SAPK/JNK (JNK-WT) and phospho-SEK1 (SEK1-WT) bands in $CaR^{WT}$-expressing cells were statistically different from those in $CaR^{Mut}$-expressing cells (JNK-M and SEK1-M). $P > 0.05$ unless indicated otherwise: * $P < 0.05$ and ** $P < 0.01$ by ANOVA. Error bars represent standard deviation.

involves an orderly activation of proteins: SEK1, SAPK/JNK, and c-Jun (24, 25). SEK1 is an upstream kinase of SAPK/JNK, so we investigated the effect of the CaR on SEK1 activation in HEK 293 cells that stably overexpress the CaR. SEK1 is activated upon the phosphorylation of Thr-261. Using a polyclonal antibody against phospho-SEK1/MKK4 in immunoblot analysis, the activation of SEK1/MKK4 was assayed at various time points. In $[Ca^{2+}]_o$-stimulated HEK 293 cells that stably overexpress $CaR^{WT}$ but not $CaR^{Mut}$, activation of SEK1/MKK4 was detected as early as 5 min and persisted for up to 24 h (Fig. 3A, B).

SAPK/JNK is responsible for mediating Ser-63/73 phosphorylation of c-Jun in response to a variety of cellular stimuli, and this phosphorylation is important for c-Jun-dependent transcriptional activity (30). Increasing evidence indicates that c-Jun plays a pivotal role in cell differentiation and apoptosis induced by multiple agents (22–25, 29, 30). To confirm that CaR-activated SAPK/JNK leads to an increase in phosphorylation of c-Jun, we measured the specific phospho-c-Jun immunoreactive band in $[Ca^{2+}]_o$-treated HEK 293 cells that stably overexpress either $CaR^{WT}$ or $CaR^{Mut}$. Treatment of $CaR^{WT}$-expressing cells with $[Ca^{2+}]_o$ resulted in a 4-fold induction of phospho-c-Jun at 1 h and a sustained 6-fold induction from 3 to 24 h. However, only an increase of ~1-fold was found in $CaR^{Mut}$-expressing cells (**Fig. 4**). Compared with the cells that express the nonfunctional $CaR^{Mut}$, our data demonstrate that the CaR stimulates the SAPK/JNK signaling pathway.

### The CaR induces ceramide accumulation and apoptosis via a $G\alpha_i$-dependent pathway

Earlier studies showed that treating cells with short forms ($C_2$ or $C_6$) of ceramide and endogenous ceramide induced by cytokines, growth factors, vitamin D, and UV and γ irradiation causes cell growth arrest and promotes apoptosis (12–14). Possible downstream targets of ceramide in cellular signaling systems include ceramide-associated protein kinase, protein kinase C, SAPK/JNK, and ceramide-associated protein phosphatases as well as phospholipases (13, 14). Recently, increasing evidence has shown that endogenous ceramide generated by different stress signals can stimulate JNK activity (22, 23). We recently reported that the CaR can stimulate the generation of several lipid second messengers (7, 28, 31). To study the effect of the CaR on ceramide accumulation and the resulting ceramide that stimulates the SAPK/JNK pathway, HEK 293 cell lines that stably overexpress either $CaR^{WT}$ or $CaR^{Mut}$ were prelabeled with [$^3$H]palmitic acid overnight and stimulated with 5 mM $CaCl_2$ for various periods of time (0–24 h). **Figure 5A** shows that $[Ca^{2+}]_o$ in-

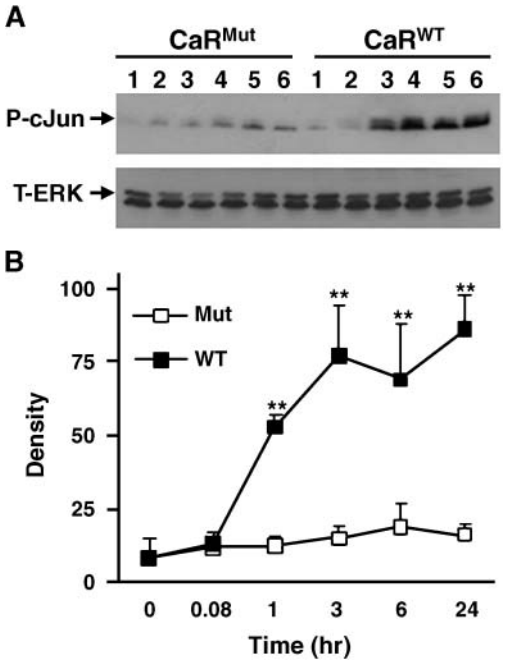


**Fig. 4.** The CaR stimulates phosphorylation of c-Jun in HEK 293 cells. A: HEK 293 cells that stably overexpress the CaR were starved, incubated with 5 mM $CaCl_2$ for different periods of time, and then fractionated to obtain the postnuclear pellet. Equal amounts of cellular protein were subjected to 11% SDS-PAGE and processed for immunoblotting using an anti-phospho-c-Jun antibody. T-ERK, total ERK. B: Immunoblot films taken from seven individual experiments were scanned, and the optical densities of the phospho-c-Jun bands were measured using Scion Image software. The optical density values of the phospho-c-Jun bands in $CaR^{WT}$-expressing cells (WT) were statistically different from those in $CaR^{Mut}$-expressing cells (Mut). $P > 0.05$ unless indicated otherwise: ** $P < 0.01$ by ANOVA. Error bars represent standard deviation.

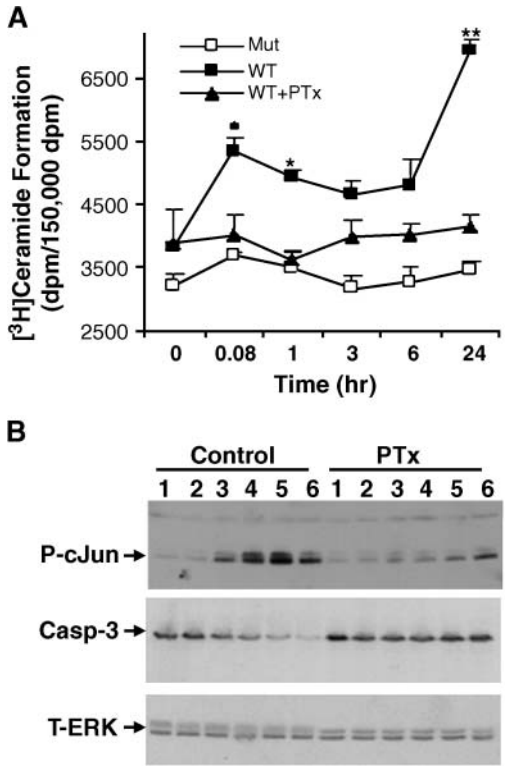


**Fig. 5.** Effect of pertussis toxin (PTx) on CaR-induced ceramide formation, c-Jun phosphorylation, and caspase-3 cleavage. A: HEK 293 cells that stably overexpress the CaR were prelabeled with [$^3$H]palmitic acid for 24 h with or without 100 ng/ml PTx. The cells were then starved and treated with 5 mM $CaCl_2$ for the times shown. Total cellular lipids were extracted, and [$^3$H]ceramide was separated from total lipids by TLC and quantitated using liquid scintillation counting. The ceramide results represent averages of three experiments performed with duplicate or triplicate samples. The values for [$^3$H]ceramide in $CaR^{WT}$-expressing cells (WT) were statistically different from those in $CaR^{Mut}$-expressing cells (Mut). $P > 0.05$ unless indicated otherwise: * $P < 0.05$ and ** $P < 0.01$ by ANOVA. B: The cellular proteins from the lipid-extracted samples were washed with acetone, air dried, and resuspended in 1× loading buffer without bromphenol blue, and the concentration of protein was measured. Equal amounts of cellular protein were subjected to 11% SDS-PAGE and processed for immunoblotting using antibodies against phospho-c-Jun (P-cJun), caspase-3 (Casp-3), and total ERK (T-ERK). The immunoblot shown is representative of four gels. Error bars represent standard deviation.

duced a time-dependent increase in [$^3$H]ceramide accumulation in HEK 293 cells that stably overexpress CaR$^{WT}$ but not CaR$^{Mut}$. Pretreatment of CaR$^{WT}$-expressing cells with 100 ng/ml pertussis toxin blocked the formation of ceramide. We further determined whether the CaR induces apoptotic processes via a $G\alpha_i$-mediated signaling pathway. The cellular proteins from lipid extraction samples were processed for immunoblotting. Figure 5B shows that pertussis toxin blocked both c-Jun phosphorylation and caspase-3 activation. These data indicate that the CaR induces renal epithelial cell apoptosis via a $G\alpha_i$-dependent pathway.

### Effect of ceramide accumulation on apoptosis

Ceramide as a lipid second messenger is a common intermediate of many apoptotic pathways. Our data indicate that the CaR stimulates the accumulation of ceramide and the activation of SAPK/JNK and caspase-3. To test whether c-Jun is phosphorylated and caspase-3 is cleaved upon $[Ca^{2+}]_o$-induced ceramide stimulation, we used two common inhibitors: monensin for sphingomyelinase and fumonisin $B_1$ for ceramide synthase. Monensin, an inhibitor of the proton pump that blocks endosome/lysosome acidification and inhibits pH optima sphingomyelinase, prevents ceramide generation in rat hepatocytes (32) and blocks alkyl-lysophospholipid-induced apoptosis in S49 cells (33). As shown in **Fig. 6A**, monensin reduced the formation of ceramide at early time points of CaR stimulation, which corresponds to the inhibition of sphingomyelinase, whereas fumonisin $B_1$ blocked ceramide synthase at later time points. After extraction of total lipids, cellular proteins in the same samples were dissolved, measured, and processed for immunoblotting using antibodies against phospho-c-Jun and caspase-3. Monensin significantly inhibited the phosphorylation of c-Jun and caspase-3 cleavage; however, fumonisin $B_1$ only partially reduced CaR-stimulated phospho-c-Jun and caspase-3 cleavage (Fig. 6B). To confirm the effect of CaR-induced ceramide on apoptosis, CaR$^{WT}$-expressing cells were prelabeled with [$^3$H]palmitic acid overnight, starved for 27 h in the presence or absence of either 15 μM desipramine (another sphingomyelinase inhibitor) (34, 35) or 15 μM SP600125 (a JNK inhibitor), and then stimulated with 5 mM $[Ca^{2+}]_o$ for different time periods. The samples were analyzed for ceramide accumulation, c-Jun phosphorylation, and caspase-3 cleavage. **Figure 7** showed that desipramine blocked ceramide formation, c-Jun phosphorylation, and caspase-3 cleavage, whereas SP600125 only abolished the phospho-c-Jun signal and caspase-3 cleavage.

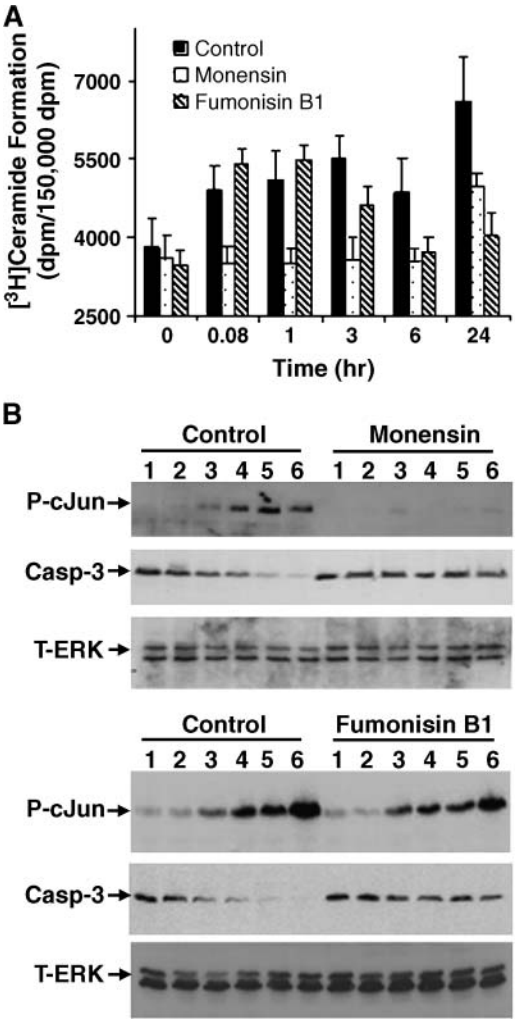


**Fig. 6.** Effect of CaR-induced ceramide formation on c-Jun phosphorylation and caspase-3 cleavage. A: HEK 293 cells that stably overexpress CaR$^{WT}$ were prelabeled with [$^3$H]palmitic acid overnight and starved for 27 h with or without 30 μM monensin or 25 μM fumonisin $B_1$. The cells were treated with 5 mM $CaCl_2$ for the last 0–24 h. Total cellular lipids were extracted, and [$^3$H]ceramide was separated from total lipids by TLC and quantitated using liquid scintillation counting. The ceramide results represent means of two separate experiments performed with triplicate samples. B: The cellular protein samples from lipid extractions were washed with acetone, air dried, and resuspended in 1× loading buffer without bromphenol blue, and the protein concentration was measured. Equal amounts of cellular protein were subjected to 11% SDS-PAGE and processed for immunoblotting using antibodies against phospho-c-Jun (P-cJun), caspase-3 (Casp-3), and total ERK (T-ERK). The immunoblot shown is representative of five gels. Error bars represent standard deviation.

## DISCUSSION

Studies by others indicate that the CaR can regulate either proliferation or differentiation in different cell lines (1). Fibroblasts (36), osteoblasts (37), and myeloma cells (38) proliferate in response to short-term increases in $[Ca^{2+}]_o$, whereas in keratinocytes (39), breast epithelial cells (40), colon carcinomas (41), and parathyroid cells (1), exposure to $[Ca^{2+}]_o$ inhibits cell proliferation and induces cell differentiation. In AT-3 prostate carcinoma cells, increased $[Ca^{2+}]_o$ prevents Sindbis virus-induced apoptosis (42). However, apoptosis directly regulated by the CaR has not been reported. Here, we present evidence that $[Ca^{2+}]_o$ induces programmed cell death in HEK 293 cells that stably overexpress CaR$^{WT}$ but not CaR$^{Mut}$, which contains a point mutation in the third intracellular loop and leads to a blunted response to $[Ca^{2+}]_o$ to serve as a negative control. The apoptotic changes observed include cell

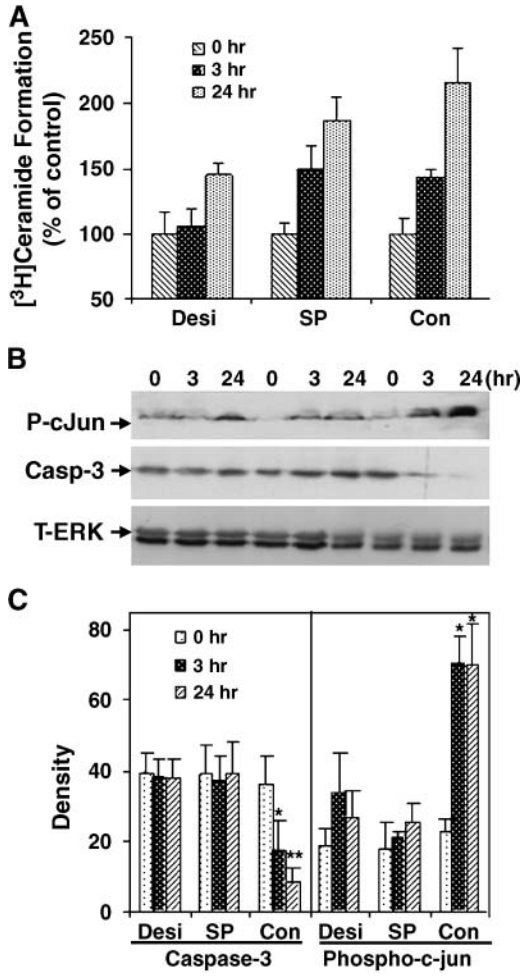


**Fig. 7.** Effect of c-Jun phosphorylation on CaR-induced apoptosis. A: HEK 293 cells that stably overexpress $CaR^{WT}$ were prelabeled with [$^3$H]palmitic acid for 24 h with or without 15 μM desipramine (Desi) or 15 μM SP600125 (SP). The cells were then starved and treated with 5 mM $CaCl_2$ for the times shown. Total cellular lipids were extracted, and [$^3$H]ceramide was separated from total lipids by TLC and quantitated using liquid scintillation counting. The ceramide results represent averages of three experiments performed with duplicate samples. Con, control. B: The cellular proteins from the lipid-extracted samples were processed for immunoblotting using antibodies against phospho-c-Jun (P-cJun), caspase-3 (Casp-3), and total ERK (T-ERK). The immunoblot shown is representative of six gels. C: Immunoblot films were scanned, and the optical densities of the phospho-c-Jun and caspase-3 bands were measured using Scion Image software. The optical density values in the control were statistically different from those in the treatment samples. $P > 0.05$ unless indicated otherwise: * $P < 0.05$ and ** $P < 0.01$ by ANOVA. Error bars represent standard deviation.

shrinkage, nuclear condensation, caspase activation, and DNA cleavage and are similar to those induced by cytotoxic agents, UV and γ irradiation, or oxidative stress signals, which activate sphingomyelinases and generate ceramide (12–15). In many but not all cases, ceramide is associated with apoptosis (12–14). For instance, increasing cellular ceramide has been shown to trigger the proliferation of fibroblasts (43) or the differentiation of keratinocytes (44) and to initiate programmed cell death (9, 21, 22). It has long been recognized that plasma membrane phospholipids play crucial roles in cell signaling events. We recently reported that the CaR stimulates phospholipase $A_2$, C, and D and phosphatidylinositol kinases (28, 30) that are involved in the metabolism of membrane phospholipids and the generation of lipid signaling molecules. The central observation of this report is that the CaR induces ceramide accumulation and that the resulting ceramide acting as a second messenger regulates programmed cell death in renal cells.

In many cell lines, treatment of cells with membrane-permeable and soluble short-chain ($C_2$ or $C_6$) ceramide leads to an increase in c-Jun expression and phosphorylation as well as cell apoptosis (12–14). Recently, increasing evidence has shown that various agonists, including tumor necrosis factor, $H_2O_2$, UV and γ irradiation, hypoxia, stress, heat shock, and death receptor activation, induce cellular ceramide accumulation, stimulate SAPK/JNK activity, and cause programmed cell death (45). Progressive renal apoptosis in obstructive nephropathy parallels increases in endogenous cellular ceramide (9). Increased ceramide levels are also found during the induction phase of ischemic and nephrotoxic acute renal failure (10, 11). These data suggest that natural long-chain ceramide, like synthetic short-chain ceramide, could also induce cell apoptosis. In cells, ceramide accumulates via sphingomyelin hydrolysis by sphingomyelinase and de novo synthesis by ceramide synthase. To demonstrate whether ceramide is a mediator of CaR-mediated apoptosis in CaR-expressing cells, we pretreated them with inhibitors of sphingolipid metabolism and ceramide generation (monensin and desipramine for sphingomyelinase, fumoninsin $B_1$ for ceramide synthase) to block the formation of CaR-induced ceramide and measured c-Jun phosphorylation and caspase-3 activation. Although earlier data showed that the cells treated with either monensin or desipramine have some significant and different side effects, including inhibition of the proton pump, membrane trafficking, inhibition of transporter, and lysosomal destruction (32–35), the similar effect of these two compounds on CaR-induced apoptosis could be attributable to the specific inhibition of sphingomyelinase and probably not to other causes. Our results demonstrate that CaR-induced ceramide plays a crucial role in the execution of apoptosis.

In the last few years, several laboratories have shown that GPCRs, such as the dopamine receptor (46), β-adrenergic receptor (47), and somatostatin receptor (48), trigger apoptosis through the activation of SAPK/JNK, but little information is available for GPCR-regulated sphingomyelin turnover and ceramide generation (17). Our data provide evidence that the generation of ceramide by the CaR sequentially stimulates SEK1 and SAPK/JNK activities, c-Jun phosphorylation, caspase activation, and DNA fragmentation. These data fill in the gap between GPCRs and SAPK/JNK in the apoptotic signaling cascade. On the other hand, although we do not know the molecular mechanism by which severe gain-of-function mutations of human CaR lead to reduced renal function, ceramide-apoptotic signaling provides a testable hypothesis for further study.

The CaR couples to multiple G proteins involved in distinct signaling pathways: $G\alpha_i$ to inhibit the activity of adenylyl cyclase (1), $G\alpha_q$ to stimulate phospholipase C and phospholipase $A_2$ (31), $G\beta\gamma$ to stimulate phosphatidylinositol 3-kinase (49), and $G\alpha_{12/13}$ to stimulate phospholipase D (28) and possibly phosphatidylinositol 4-kinase (50). In the regulation of MAPKs, CaR-$G\alpha_i$ coupling has been shown to activate ERK1/2 in a variety of cell lines, such as fibroblasts, osteoblasts, parathyroid cells, HEK 293 cells, and MDCK cells (1, 28, 30, 51). Recently, one report showed that the CaR activates p38 MAPK in the regulation of a $Ca^{2+}$-activated $K^+$ channel (27). Here, we report that the CaR induces ceramide accumulation to stimulate SAPK/JNK activity and caspase-3 activation (Figs. 2, 3), and treatment of cells with pertussis toxin prevents both ceramide accumulation and caspase-3 cleavage (Fig. 5) in HEK 293 cells. This indicates that the CaR induces apoptosis via a $G\alpha_i$-dependent signaling pathway. Using GST-c-$Jun_{1-79}$ as a substrate and analyzing kinase activity in vitro, similar results, that the CaR stimulates JNK through a pertussis toxin-sensitive G protein pathway, were also observed in MDCK cells (26). The regulation of MAPK activities is a fundamental cellular process in cell proliferation, differentiation, and apoptosis that is essential for embryonic development and tissue homeostasis (52). However, pathological stimuli, such as heat shock, UV and $\gamma$ irradiation, tumor necrosis factor, growth factor withdrawal, and long-term agonist stimulation, such as in patients with activated $CaR^{Mut}$, cause inappropriate regulation leading to pathophysiological changes, including the perturbation of cell morphology and function.

The CaR stimulates phospholipase C to hydrolyze phosphatidylinositol bisphosphate and release inositol trisphosphate. The increase of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) by inositol trisphosphate could play an important role in many $Ca^{2+}$-dependent signaling pathways, including apoptosis, but we do not think that it is important in this case. Phospholipase C is activated by a Gαq-dependent mechanism that is insensitive to pertussis toxin (31), whereas ceramide production, c-Jun phosphorylation, and caspase cleavage are inhibited by pertussis toxin, a characteristic of a Gαi-dependent pathway. On the other hand, although we did not determine the long-term effects of activation of the CaR on $[Ca^{2+}]_i$, in short-term studies, $[Ca^{2+}]_i$ returns to baseline over 2–5 min (31). In many other studies using this nonfunctional mutant CaR, increased $[Ca^{2+}]_o$ does not activate ERK, phospholipase C, phospholipase $A_2$, or phospholipase D (1, 7, 28, 31). $CaR^{Mut}$-expressing cells did not show evidence of apoptosis or activation of the apoptotic pathway despite experiencing the same level of $[Ca^{2+}]_o$, suggesting that this pathway requires the CaR and does not simply represent a toxic effect of $[Ca^{2+}]_o$.

In summary, we present evidence that the CaR induces the apoptotic process in HEK 293 cells. The molecular mechanism involved in these events has been extensively characterized. $[Ca^{2+}]_o$ initially induces the accumulation of ceramide, the resulting ceramide stimulates SEK1/MKK4 and SAPK/JNK activation, the activated SAPK/JNK phosphorylates c-Jun, and the phospho-c-Jun induces caspase activation, DNA fragmentation, and apoptosis. This process uses a $G\alpha_i$-dependent pathway. The wide tissue distribution of the CaR and its regulation of multiple signaling pathways suggest that the receptor may play important and different roles in different tissues and cells under physiological and pathophysiological conditions.

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