The calcium-sensing receptor couples to $G\alpha_s$ and regulates PTHrP and ACTH secretion in pituitary cells

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Abstract

The calcium-sensing receptor (CaR or CASR as listed in the MGI Database) is a G protein-coupled receptor that binds and signals in response to extracellular calcium and other polycations. It is highly expressed on parathyroid and kidney cells, where it participates in the regulation of systemic calcium homeostasis. It is also expressed on many other cell types and is involved in a wide array of biological functions such as cell growth and differentiation, ion transport, and hormone secretion. It has been described to couple to several different G proteins including $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. Recently, it has also been shown to stimulate cAMP production by coupling to $G\alpha_s$ in immortalized or malignant breast cells. The CaR is expressed on cells in the anterior pituitary and had previously

been described to stimulate cAMP production in these cells. In this report, we examined signaling from the CaR in murine pituitary corticotroph-derived, AtT-20 cells. We found that CaR activation led to the stimulation of cAMP production, and PTH-related protein (PTHrP or PTHLH as listed in the MGI Database) and ACTH secretion from these cells. Furthermore, manipulation of cAMP levels was able to modulate PTHrP and ACTH secretion independent of changes in extracellular calcium. Finally, we demonstrated that the CaR couples to $G\alpha_s$ in AtT-20 cells. Therefore, in pituitary corticotroph-like cells, as in breast cancer cells, the CaR utilizes $G\alpha_s$ and activates cAMP production to stimulate hormone secretion.

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Introduction

The extracellular calcium-sensing receptor (CaR or CASR as listed in the MGI Database) is a seven-transmembrane-spanning, G protein-coupled cell-surface receptor (GPCR) that binds and signals in response to ionized calcium and other cations or cationic compounds such as magnesium, gadolinium, or neomycin (Brown & MacLeod 2001, Tfelt-Hansen & Brown 2005, Brown 2007). It was initially discovered as a parathyroid calcium sensor and subsequently was shown to be required for the normal regulation of calcium metabolism (Brown *et al.* 1993, Chattopadhyay & Brown 2006). The CaR is prominently expressed in the parathyroid glands and the kidney, where it functions in the regulation of parathyroid hormone (PTH) secretion and renal calcium handling respectively (Quarles 2003, Chattopadhyay & Brown 2006). However, the CaR is also expressed in many other organs and participates in the regulation of a variety of processes such as fluid and ion transport, cellular proliferation, differentiation, and apoptosis, and hormone secretion (Brown & MacLeod 2001, Tfelt-Hansen & Brown 2005, Brown 2007).

As with other GPCRs, signaling from the CaR is initiated by activation of heterotrimeric G proteins. Most literature has suggested that the CaR couples primarily to $G\alpha_{i/o}$ and $G\alpha_{q/11}$ (Brown & MacLeod 2001, Hofer & Brown 2003,

Ward 2004). Signaling pathways downstream of the receptor appear to vary somewhat in different cell types. In HEK cells transfected with the CaR and in parathyroid cells, activation of the receptor stimulates phospholipase C (PLC) activity and generates inositol trisphosphate (IP_3) and intracellular calcium transients (Brown & MacLeod 2001, Hofer & Brown 2003, Ward 2004). This, in turn, has been described to result in activation of mitogen-activated protein kinase cascades involving the extracellular signal-regulated kinases 1 and 2, p38 and the stress-activated kinase, cJun N-kinase (Kifor *et al.* 2001, Corbetta *et al.* 2002, Ward 2004). Activation of this pathway has been shown to be important for the ability of CaR signaling to inhibit PTH secretion from dispersed parathyroid cells (Kifor *et al.* 2001, Corbetta *et al.* 2002). Stimulation of the CaR also acts via $G\alpha_i$ to inhibit adenylyl cyclase and reduce cAMP levels in several cell types, although this has not been thought to be a major contributor to the regulation of PTH secretion (Hofer & Brown 2003, Ward 2004). We have recently shown that inhibition of cAMP is important for the ability of calcium to inhibit PTH-related protein (PTHrP or PTHLH as listed in the MGI Database) secretion from normal mouse mammary epithelial cells (Mamillapalli *et al.* 2008). Furthermore, we also demonstrated that in immortalized mammary epithelial cells or in human breast cancer cells, the CaR switches from coupling to $G\alpha_i$ to

coupling to $G\alpha_s$ (Mamillapalli *et al.* 2008). This causes a reversal of the biological effects of calcium such that activation of the CaR stimulates, instead of inhibits, PTHrP secretion. Thus, the CaR activates distinct signaling events in different cells, at least in part, by utilizing different G proteins, including coupling to either $G\alpha_i$ or $G\alpha_s$, depending on the setting.

PTHrP is a peptide growth factor originally described to cause humoral hypercalcemia of malignancy (Strewler 2000, Wysolmerski 2008). It is produced by many different normal cells and has important local functions in diverse sites such as the skeleton, vasculature, teeth, and mammary gland (Strewler 2000, Wysolmerski 2008). In normal mammary epithelial cells and in breast cancer cell lines, CaR signaling regulates PTHrP production (Sanders *et al.* 2000, VanHouten *et al.* 2004, Mamillapalli *et al.* 2008). The CaR has also been shown to modulate PTHrP production in astrocytes, osteoblasts, cytotrophoblasts, ovarian surface epithelial cells, and HEK 293 cells transfected with the CaR (Chattopadhyay 2006). Therefore, PTHrP is responsive to changes in extracellular calcium in many sites. Both PTHrP and the CaR are expressed in the normal pituitary gland, pituitary tumors, and pituitary cell lines, although their biological function(s) in pituitary cells is not clear (Ikeda *et al.* 1988, Asa *et al.* 1990, Fraser *et al.* 1991, Ito *et al.* 1993, Holt *et al.* 1994, Emanuel *et al.* 1996, Danks *et al.* 1997, Ferry *et al.* 1997, Romoli *et al.* 1999, Loretz 2008). Previous studies have shown that the stimulation of corticotroph, somatotroph, and melanotroph cell lines with extracellular calcium increased, rather than decreased, intracellular cAMP levels (Emanuel *et al.* 1996, Romoli *et al.* 1999, Zivadinovic *et al.* 2002, van den Hurk *et al.* 2008). Given the recent data showing that the CaR couples to $G\alpha_s$ in breast cancer cells (Mamillapalli *et al.* 2008), we asked if the CaR might also couple to $G\alpha_s$ in anterior pituitary cells and stimulate PTHrP production by increasing intracellular cAMP. In this report, we demonstrate this to be the case. Activation of the CaR in murine pituitary corticotroph-derived, AtT-20, cells activated $G\alpha_s$, increased cAMP production, and stimulated PTHrP and ACTH secretion.

Materials and Methods

Materials

Ham's F12 medium, calcium-free DMEM, gentamycin, disperse, NuPAGE LDS sample buffer, CaR siRNAs, and TRIzol were purchased from Invitrogen Life Technologies. Forskolin, H89, $N^6,2$ -O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (Bt_2cAMP), 3-isobutyl-1-methylxanthine, guanosine 5'-diphosphate sodium salt (GDP), carbachol, and guanosine 5'-triphosphate sodium salt hydrate (GTP) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA, USA). [^{35}S]GTP γ S was from Perkin-Elmer Life Sciences (Boston, MA, USA), and ACTH RIA kits were

obtained from MP Biomedicals, LLC (Orangeburg, NY, USA). PTHrP IRMA kits were from Diagnostic Systems Laboratories (Webster, TX, USA), and correlate-enzyme immunoassay (EIA) direct cAMP assay kits were from Assay Designs (Ann Arbor, MI, USA). Polyvinylidene difluoride (PVDF) membrane came from Fisher Scientific (Atlanta, GA, USA). Antibodies recognizing $G\alpha_s$ (anti- $G\alpha_s$) and both $G\alpha_q$ and $G\alpha_{11}$ (anti- $G\alpha_{q/11}$) were purchased from Upstate Biotechnology (Temecula, CA, USA), while the anti- β -actin antibody was obtained from Cell Signaling Technologies (Danvers, MA, USA). Goat anti-mouse secondary antibody was obtained from Jackson ImmunoResearch (West Grove, PA, USA). The Message Clean system was obtained from Gen Hunter (Nashville, TN, USA), and Brilliant SYBR-Green quantitative real time (QRT)-PCR Master Mix kit was purchased from Stratagene (La Jolla, CA, USA). Protease inhibitors and the enhanced chemiluminescence kit, employing the SuperSignal West Pico substrate, were purchased from Pierce Chemical (Rockford, IL, USA). The PLC inhibitor, U-73122, and the protein kinase C (PKC) inhibitor, Go6976, were purchased from Sigma-Aldrich.

Cell culture

AtT-20/LAF1 (AtT-20) cells (American Type Culture Collection, Manassas, VA, USA) were grown in Ham's F12 or DMEM medium supplemented with 15% FBS, 2.5 μ g/ml amphotericin B, and 50 μ g/ml gentamycin in a 5% CO₂ atmosphere at 37 °C. In preparation for experiments, the media was switched to DMEM containing 0.1 mM calcium, 0.2% BSA, and antibiotics, and the final calcium content was adjusted to the desired concentration with the addition of calcium chloride.

cAMP assay

AtT-20 cells were seeded into six-well plates. After reaching 70–80% confluence, cells were switched to media containing 2.5 mM calcium, 5 mM calcium, or the polycationic agonist neomycin (300 μ M). Cells were incubated at 37 °C for 30 min and washed with cold PBS. Cell lysates were prepared with 0.1 M HCl, and cAMP was measured in cell lysates by EIA as per the manufacturer's instructions. In additional experiments, cells were preincubated with forskolin at varying doses for 30 min before additional calcium or neomycin was added. The amount of cAMP was corrected for the total protein content of each individual lysate.

ACTH secretion

AtT-20 cells were seeded into 24-well plates and treated with 2.5 mM calcium, 5 mM calcium, or 300 μ M neomycin for 90 min at 37 °C. ACTH was measured in samples of conditioned media immediately or after storage at –80 °C using a commercial RIA (MP Biomedicals, LLC). In other experiments, cells were preincubated with varying doses of

forskolin (0.1–10 μ M), Bt_2cAMP (1 mM), the protein kinase A (PKA) inhibitor, H89 (10 μ M), the PLC inhibitor, U73122 (10 μ M), or the PKC inhibitor, Go6976 (10 nM), for 1–2 h before the addition of $CaCl_2$ (5 mM) or neomycin for 90 min. ACTH levels were normalized to the total cellular protein from each well.

Measurement of PTHrP secretion

AtT-20 cells were seeded into 12-well plates at a density of 10^6 cells per well. After 48 h, the cells were treated with 2.5 mM calcium, 5 mM calcium, or 300 μ M neomycin. Conditioned medium was removed after 16 h, and PTHrP (1–86) was measured using a two-site IRMA following the manufacturer's instructions (Diagnostic Systems Laboratories). PTHrP levels were normalized to the total cellular protein from each well. In other experiments, cells were preincubated with varying doses of forskolin (0.1–10 μ M), Bt_2cAMP (1 mM), the PKA inhibitor, H89 (10 μ M), the PLC inhibitor, U73122 (10 μ M), or the PKC inhibitor, Go6976 (10 nM), for 1–2 h before the addition of $CaCl_2$ (5 mM) or neomycin.

Western blot analysis

Cells were washed with ice-cold PBS and then lysed in 400 μ l Nonidet-40 lysis buffer (20 mM Tris base, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 with protease inhibitors). Cell lysates were centrifuged at 14 000 g to pellet debris, and protein concentrations were measured using Bio-Rad protein assay reagent. Protein samples (25 μ g) were resolved on 12% SDS-PAGE minigels and electroblotted onto PVDF membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% Tween, washed, and incubated overnight at 4 °C with polyclonal anti- $G\alpha_s$ antibody (1:500). The membranes were washed and then incubated with goat anti-rabbit IgG conjugated to HRP (1:5000), and signals were detected using a SuperSignal West Pico detection kit. The same membrane was stripped and blotted again for β -actin protein with anti- β -actin antibody.

Quantitative real time RT-PCR

Total RNA was isolated from cells 48 h posttransfection using TRIzol as per the manufacturer's instructions. Any contaminating DNA was removed using the Message Clean system. The Brilliant SYBR-Green QRT-PCR Master Mix kit was used to perform one-step QRT-PCRs, in triplicate, using 20 ng RNA with a reaction volume of 20 μ l on the Opticon 2 DNA Engine (MJR, Waltham, MA, USA). Reverse transcription (RT) was carried out for 30 min at 50 °C, followed by 40 cycles consisting of 30 s at 95 °C and 1 min at 60 °C. The glyceraldehyde-3-phosphate dehydrogenase primers were as follows: forward, 5'-CGTCCCGTAGACAAAATGGT-3'; reverse, 5'-TCAATGAAGGGGTCGTTGAT-3', and the CaR primers were forward, 5'-ATGTTTGGCTACTGTTTGG-3'; reverse, 5'-CAGAGCCTTGAGAC GGTGT-3'.

For each experimental sample, a control without reverse transcriptase was run to verify that the amplification product arose from cDNA and not from genomic DNA. The relative expression levels, normalized to glyceraldehyde-3-phosphate dehydrogenase, were determined using the comparative C_t method (also known as the $2^{-\Delta\Delta C_t}$ method; Applied Biosystems 1997, Mamillapalli *et al.* 2008). CaR mRNA levels were expressed as the fold suppression in CaR siRNA-transfected cells relative to basal expression in nontransfected cells.

[35 S]GTP γ S binding assay

Coupling of $G\alpha_s$ or $G\alpha_{q/11}$ to the CaR was assessed using the [35 S]GTP γ S binding assay as described previously (Mamillapalli *et al.* 2008). Briefly, cell membranes were prepared from actively growing AtT-20 cells. The cells were washed with 10 mM Tris-maleate buffer, pH 7.4, scraped into a 6 ml aliquot of Tris-maleate buffer, and passed 15 times through an 18-gauge needle. The resultant cell lysate was centrifuged at 5000 g for 10 min, and the supernatant was then centrifuged at 100 000 g for 30 min to pellet the membranes. The membrane pellet was resuspended in 10 mM Tris-maleate buffer and passed through a 22-gauge needle. Membrane protein concentrations were assessed using the Bio-Rad protein assay reagent. Membrane preparations were used immediately or were frozen at –80 °C for later use. The assay reaction mixture contained membrane protein (850 μ g) and 360 nM [35 S]GTP γ S in 50 μ l of 50 mM Tris-HCl, pH 7.4, 2.8 mM $MgCl_2$, 100 mM NaCl, and 1 μ M GDP. The reaction was initiated by adding 5 mM $CaCl_2$, 300 μ M neomycin, or 100 μ M carbachol for 30 min at 30 °C, and the reaction was terminated by adding 600 μ l of ice-cold immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40, 20 μ g/ml aprotinin, 100 μ M GDP, and 100 μ M GTP), followed by a 30-min incubation on an orbital shaker at 4 °C. After incubation, the reaction contents were centrifuged at 14 000 g , and the supernatant was transferred to 110 μ l of a previously prepared anti- $G\alpha_s$ or anti- $G\alpha_{q/11}$ antibody-protein A-Sepharose mixture. The resulting mixtures were incubated for 1 h at 4 °C on an orbital shaker, and immunoprecipitates were pelleted by centrifugation at 4000 g for 2 min at 4 °C. The pellet was washed thrice, boiled for 1 min in 500 μ l of 0.5% SDS, and transferred to scintillation vials containing 4 ml scintillation liquid. Samples were analyzed in triplicate on a scintillation counter (Beckman Coulter, LS5000 TA and TD Liquid Scintillation System, Fullerton, CA, USA). Nonimmune serum was used to control for nonspecific binding of [35 S]GTP γ S.

Silencing of CaR expression

AtT-20 cells were grown to 70% confluence and then transfected with Casr Stealth Select RNAi (MSS202652 or MSS202653, 260 pmol/well; Invitrogen) using

Lipofectamine-2000 (7.8 μ l/well) according to the manufacturer's protocol. The Stealth RNAi Negative Control Med GC (Invitrogen) was transfected in parallel as a control in all experiments. To verify knockdown of CaR mRNA, total cellular RNA was isolated from cells 48 h posttransfection and subjected to RT-PCR as described above. cAMP, PTHrP, and ACTH levels were assayed at 48 h posttransfection as described above.

Statistical analysis

Each experiment was performed at least three times, and each individual experiment was performed in triplicate. However, although there were nine or more replicates, statistical analysis was performed using the number of completely independent experiments. Data are expressed as means $\pm$ the S.E.M. Statistical significance was determined using one-way ANOVA with the Newman-Keuls multiple comparisons test. All statistical analyses were carried out using Graph Pad Prism 4.00 for Macintosh (Graph-Pad Software for Science Inc., San Diego, CA, USA).

Results

CaR activation increases cAMP levels and stimulates PTHrP and ACTH secretion in AtT-20 cells

We first treated pituitary, AtT-20 cells with increasing doses of calcium and the type I calcimimetic agent, neomycin, and measured cAMP production and PTHrP secretion. AtT-20 cells are murine corticotroph-derived cells previously shown to express the CaR (Emanuel *et al.* 1996). As shown in Fig. 1A, exposure of these cells to increasing doses of extracellular calcium (from 0.1 to 5.0 mM) for 30 min caused a dose-dependent rise in intracellular cAMP. Treatment with 300 μ M neomycin at 0.1 mM calcium also increased cAMP levels, suggesting that the CaR mediated the effects of calcium on cAMP concentrations. The pituitary is known to produce PTHrP (Ikeda *et al.* 1988, Asa *et al.* 1990, Ito *et al.* 1993, Holt *et al.* 1994, Danks *et al.* 1997), so we next measured PTHrP secretion by AtT-20 cells treated with extracellular calcium or neomycin. PTHrP levels in conditioned media mirrored the changes in cAMP. As shown in Fig. 1B, increasing doses of extracellular calcium led to a progressive increase in PTHrP

Figure 1 Activation of the CaR stimulates cAMP accumulation and PTHrP and ACTH secretion. (A) Intracellular cAMP levels in AtT-20 cells treated with 0.1, 2.5, or 5 mM extracellular calcium and neomycin (300 μ M). (B) PTHrP levels were increased in conditioned media from cells treated with 2.5 or 5 mM calcium or neomycin. (C) ACTH levels were increased in conditioned media from cells treated with extracellular calcium or neomycin. Bars represent the means $\pm$ S.E.M. of three independent experiments. Statistical significance ($P < 0.05$) is noted on the graphs: a denotes a significant difference compared with 0.1 mM calcium, b denotes a significant difference compared with 2.5 mM calcium, and c denotes a significant difference as compared with 5 mM calcium.

secretion by these cells as did treatment with neomycin. Finally, previous reports had demonstrated that CaR activation increases ACTH secretion by pituitary cells (Emanuel *et al.* 1996, Ferry *et al.* 1997). Therefore, we also measured ACTH secretion by these cells and confirmed that increasing doses of calcium and treatment with a calcimimetic agent triggered ACTH secretion by AtT-20 cells in a manner identical to the changes we observed for cAMP and PTHrP (Fig. 1C).

Elevations in cAMP stimulate PTHrP and ACTH secretion from AtT-20 cells

In order to determine if CaR activation stimulated PTHrP and ACTH secretion by causing an increase in cAMP, we next asked if elevated cAMP levels would stimulate PTHrP and ACTH secretion independent of changes in extracellular calcium. We used forskolin, a pharmacologic activator of adenylyl cyclase to increase cAMP levels and also treated cells with dibutyryl-cAMP (dBcAMP), a cell-permeable synthetic analog of cAMP that is resistant to degradation by phosphodiesterases (Ryan & Heidrick 1974, Insel & Ostrom 2003, Mamillapalli *et al.* 2008). As expected, forskolin led to a dose-dependent increase in cAMP levels in AtT-20 cells at 0.1 mM calcium (Fig. 2A). Figure 2B demonstrates that treating AtT-20 cells with increasing doses of forskolin stimulated the secretion of PTHrP into the media, mimicking the effects of treatment with high calcium or calcimimetics. There was not a one-to-one correlation between cAMP and PTHrP levels, and at higher doses of forskolin, the relative increase in PTHrP secretion leveled off. Nonetheless, increasing cAMP concentrations led to a progressive increase in PTHrP secretion. Treatment with forskolin had similar effects on ACTH release from these cells (Fig. 2C). Treating cells with dBcAMP at 0.1 mM calcium also stimulated PTHrP and ACTH secretion (Supplementary Figure 1, see section on supplementary data given at the end of this article). These data suggest that cAMP may mediate the actions of the CaR on PTHrP and ACTH secretion within these cells.

Figure 2 Manipulation of cAMP levels alters PTHrP and ACTH secretion in AtT-20 cells. (A) Intracellular levels of cAMP from AtT-20 cells treated with varying doses of forskolin (0.1–10 μM) in the presence of 0.1 mM calcium. (B and C) Secretion of PTHrP (B) and ACTH (C) into conditioned media in response to varying doses of forskolin at 0.1 mM calcium. Control denotes 0.1 mM calcium without forskolin. Raising cAMP levels independent of changes in extracellular calcium stimulated both PTHrP and ACTH secretion by AtT-20 cells in a dose-dependent fashion. In all panels, bars represent the mean $\pm$ S.E.M. of three individual experiments. Statistical significance ($P < 0.01$) is denoted on the graphs: a denotes a significant difference compared with control, b denotes a significant difference as compared with 0.1 μM forskolin, c denotes a significant difference compared with 1.0 μM forskolin, d denotes a significant difference compared with 2.5 μM forskolin, e denotes a significant difference compared with 5 μM forskolin, and f denotes a significant difference compared with 10 μM forskolin.

Inhibition of PKA blocks the effects of CaR activation on ACTH and PTHrP secretion

Many of the effects of cAMP are mediated through activation of PKA (Taylor *et al.* 2005). Therefore, in order to confirm that cAMP contributes to the effects of CaR signaling on PTHrP and ACTH secretion, we asked if inhibition of PKA activity could block PTHrP and/or ACTH release in response to calcium or neomycin. For this purpose, we treated AtT-20 cells with the selective PKA inhibitor, H89 (Taylor *et al.* 2005, Mamillapalli *et al.* 2008). Treatment with H89 lowered basal levels of PTHrP and ACTH secretion only slightly at 0.1 mM calcium. However, H89 effectively blocked the stimulation of PTHrP (Fig. 3A) and ACTH (Fig. 3B) secretion by high calcium (5.0 mM) or neomycin. Activation of the CaR receptor has previously been shown to trigger phosphoinositol accumulation and to trigger increases in intracellular calcium concentrations in these cells (Emanuel *et al.* 1996). Either of these second messenger systems might contribute to or synergize with cAMP in the regulation of PTHrP and/or ACTH secretion from these cells. Therefore, we also examined the effects of the PLC inhibitor, U73122, and the PKC inhibitor, Go6976. As shown in Supplementary Figure 2, see section on supplementary data given at the end of this article neither inhibition of PLC nor inhibition of PKC prevented the secretion of PTHrP or ACTH in response to calcium or neomycin, suggesting that these pathways are not required for the effects of calcium on their secretion. These data demonstrate that the cAMP/PKA pathway regulates ACTH and PTHrP secretion from these cells, and suggest that CaR activation stimulates ACTH and PTHrP production in a cAMP/PKA-dependent fashion.

The CaR mediates the effects of calcium and neomycin on cAMP levels and PTHrP and ACTH secretion

The fact that neomycin mimicked high extracellular calcium in stimulating cAMP, PTHrP, and ACTH levels, suggested that the CaR mediates these effects. In order to validate this conclusion, we examined the effects of calcium and neomycin on these parameters after 'knocking down' CaR expression in AtT-20 cells using siRNA directed against the CaR (Mamillapalli *et al.* 2008). As shown in Fig. 4A, transfection of untreated cells with two different siRNAs directed specifically against the CaR reduced the basal levels of CaR mRNA by ~75%. In contrast, a nonspecific control siRNA resulted in only a 6% decrease in CaR mRNA levels (Fig. 4A). As shown in Fig. 4B, the CaR-specific siRNAs had no effect on baseline cAMP levels but they resulted in a significant inhibition of cAMP accumulation in response to either 5 mM calcium or neomycin. This was also associated with the ablation of the effects of 5 mM calcium and neomycin on PTHrP (Fig. 4C) and ACTH (Fig. 4D) secretion. In contrast, transfection of the nonspecific control

Figure 3 cAMP/PKA signaling mediates the effects of CaR activation on PTHrP and ACTH secretion. (A) PTHrP levels in conditioned media harvested from AtT-20 cells pretreated with the PKA inhibitor, H89 (10 μ M), prior to exposure to high calcium (5 mM) or neomycin. H89 lowered basal rates of PTHrP secretion and blocked the response to high calcium or neomycin. (B) ACTH levels in conditioned media from cells pretreated with H89 (10 μ M), prior to exposure to high calcium (5 mM) or neomycin. As with PTHrP, inhibition of PKA lowered basal secretion of ACTH and blocked the effects of calcium or neomycin. Data are means $\pm$ S.E.M. from three independent experiments. Statistical significance ($P < 0.01$ or lower) is noted on the graph: a denotes a significant difference as compared with 0.1 mM calcium, b denotes a significant difference as compared with 5 mM calcium, and c denotes a significant difference as compared with neomycin.

siRNA had little effect on cAMP, PTHrP or ACTH production. Since two independent CaR-specific siRNA gave the same result and since the control siRNA did not, it is unlikely that these results are related to any nonspecific,

Figure 4 Knockdown of *CaR* mRNA expression inhibits the effects of high calcium and neomycin on cAMP, PTHrP, and ACTH. (A) Measurement of *CaR* mRNA levels by quantitative real-time RT-PCR in AtT-20 cells transfected with two different, specific siRNA (CaR-siRNA-652 and CaR-siRNA-653) against the *CaR* as well as with a control, nonspecific siRNA. CaR-specific siRNAs caused approximately a 75% reduction in *CaR* mRNA levels as compared with the untransfected cells. Bars represent the mean $\pm$ s.e.m. of three individual experiments. *Denotes significant differences between CaR-siRNAs (652 and 653) versus untransfected cells ($P < 0.001$). (B, C, and D) show the effects of these *CaR* siRNAs on intracellular cAMP and PTHrP and ACTH secretion respectively, in response to *CaR* activation by high calcium (5 mM) and neomycin. *CaR* activation failed to increase cAMP, PTHrP or ACTH levels in cells transfected with specific CaR-siRNAs (652 and 653). However, nontransfected cells and cells transfected with nonspecific CaR-siRNA (siRNA-control) demonstrated the expected stimulation of cAMP, ACTH, and PTHrP levels in response to 5 mM calcium or neomycin. Bars represent the mean $\pm$ s.e.m. of three independent experiments. *Denotes significant differences between CaR-siRNAs (652 and 653) versus nontransfected cells ($P < 0.001$).

off-target effects. Therefore, these data confirm that the *CaR* mediates the effects of calcium and neomycin on cAMP, PTHrP, and ACTH production by AtT-20 cells.

The *CaR* couples to $G\alpha_s$ in AtT-20 cells

We had previously demonstrated that the *CaR* signals through $G\alpha_s$ and stimulates adenylyl cyclase in immortalized mouse mammary epithelial cells and in human breast cancer cells (Mamillapalli *et al.* 2008). In order to determine if the *CaR* also couples to $G\alpha_s$ in AtT-20 cells, we first confirmed that these cells express this particular G protein. As expected, western blot analysis showed that AtT-20 cells express ample $G\alpha_s$ protein (not shown). Next, we performed the [35 S]GTP γ S binding assay as described previously (Mamillapalli *et al.* 2008). This assay relies on activation of the *CaR* *in vitro* in the presence of a 35 S-labeled, phosphatase-resistant GTP derivative, GTP γ S. The G protein of interest is subsequently immunoprecipitated, and the amount of

radioactivity within the immunoprecipitate is an index of activation of that particular G protein in response to receptor activation. Consistent with the lack of effects of the PLC and PKC inhibitors, we found that the *CaR* did not couple to either $G\alpha_q$ or $G\alpha_{11}$ in AtT-20 cells (Supplementary Figure 3, see section on supplementary data given at the end of this article). However, as shown in Fig. 5, activation of the *CaR* led to increased association of [35 S]GTP γ S with $G\alpha_s$. Little [35 S]GTP γ S was incorporated into anti- $G\alpha_s$ immunoprecipitates in the presence of 0.1 mM calcium but incorporation increased approximately fourfold in response to 5 mM calcium and 3.2-fold in response to neomycin. The incorporation of radioactivity into immunoprecipitates using nonimmune serum served as a control for nonspecific binding of [35 S]GTP γ S. These results demonstrate that the *CaR* couples to $G\alpha_s$ in AtT-20 cells, and suggest that treatment of these cells with calcium or calcimimetics stimulates adenylyl cyclase resulting in increased cAMP levels, which, in turn, stimulate PTHrP and ACTH secretion.

Figure 5 The CaR couples to $G\alpha_s$ in AtT-20 cells. Results of [³⁵S]GTPγS binding assays from AtT-20 cell membranes using an anti- $G\alpha_s$ -specific antibody. As shown in the figure, activation of the CaR with 5 mM calcium or neomycin triggered [³⁵S]GTPγS binding to $G\alpha_s$. Nonspecific binding of [³⁵S]GTPγS was assessed by using nonimmune serum (N. I. serum) in control immunoprecipitations. Bars represent the mean $\pm$ S.E.M. of three individual experiments. Statistical significance is denoted on the graphs.

Discussion

Our results confirm that increasing extracellular calcium concentrations stimulate cAMP production in the murine pituitary corticotroph-derived, AtT-20, cell line. Calcium also stimulates PTHrP and ACTH secretion from these cells. The CaR mediates these effects of extracellular calcium because they are mimicked by neomycin and are inhibited by specific, siRNA-induced knockdown of *CaR* mRNA expression. In addition, we demonstrated that raising cAMP levels, independent from CaR activation, led to the secretion of both PTHrP and ACTH by AtT-20 cells, and that blocking PKA activity prevented the secretion of these peptides in response to calcium or neomycin. Therefore, cAMP mediates the effects of CaR activation on the secretion of both PTHrP and ACTH. Finally, we demonstrated that the CaR couples to $G\alpha_s$ in AtT-20 cells, suggesting that the activation of cAMP production by $G\alpha_s$ may be an important pathway through which the CaR modulates neuropeptide release by some pituitary cells.

CaR mRNA and/or protein expression has been detected in the anterior pituitaries of humans, rodents, and fish, and in AtT-20 cells (Emanuel *et al.* 1996, Mitsuma *et al.* 1999, Zivadinovic *et al.* 2002, Loretz 2008). Previous studies on downstream signaling events in pituitary cells have documented that CaR activation stimulates intracellular calcium transients in AtT-20 cells, in cells cultured from rat and *Xenopus* pituitaries, and in cells cultured from human pituitary

adenomas (Emanuel *et al.* 1996, Ferry *et al.* 1997, Romoli *et al.* 1999, Zivadinovic *et al.* 2002, van den Hurk *et al.* 2008). In addition to calcium transients, activation of the receptor in AtT-20 cells was reported to cause a pertussis toxin-sensitive accumulation of phosphoinositides, suggesting that at least some signaling pathways are mediated through activation of $G\alpha_i$ (Emanuel *et al.* 1996). Interestingly, although increases in intracellular calcium in response to CaR activation have been described to be pertussis toxin insensitive in cells from human growth hormone-secreting pituitary adenomas, we could not document coupling of the CaR to $G\alpha_q$ or $G\alpha_{11}$ in AtT-20 cells. It is not clear if this represents differences between pituitary cell types or if it suggests differences in the nature of CaR-mediated intracellular calcium mobilization in pituitary cells as compared to parathyroid or other cell types.

Activation of the CaR has also been shown to stimulate cAMP accumulation in pituitary cells (Emanuel *et al.* 1996, Romoli *et al.* 1999, Zivadinovic *et al.* 2002, van den Hurk *et al.* 2008). Our experiments both confirm and extend these prior data by showing that the CaR couples to $G\alpha_s$ in AtT-20 cells, and that the cAMP-PKA pathway is necessary and sufficient to elicit PTHrP and ACTH secretion in response to CaR activation. These results are similar to our recent findings demonstrating that the CaR couples to $G\alpha_s$ in immortalized murine mammary epithelial cells and in human breast cancer cells, and that activation of cAMP/PKA signaling mediates the effects of the CaR on stimulating PTHrP secretion from these cells (Mamillapalli *et al.* 2008). They are also consistent with the observation that the accumulation of cAMP in response to extracellular calcium was not inhibited by pertussis toxin in AtT-20 cells (Emanuel *et al.* 1996). While seemingly contradictory from a cAMP standpoint, simultaneous coupling of GPCRs to both $G\alpha_s$ and $G\alpha_i$ has been reported for dopamine D1, opioid, and prostacyclin receptors (Sidhu *et al.* 1991, Cruciani *et al.* 1993, Lawler *et al.* 2001). Moreover, when chimeric A(1)/A(2A) adenosine receptors designed to couple to both $G\alpha_s$ and $G\alpha_i$ were studied in HEK 293 cells, $G\alpha_s$ -mediated stimulation of adenylyl cyclase predominated over $G\alpha_i$ -mediated inhibition of adenylyl cyclase (Tucker *et al.* 2000). Therefore, in AtT-20 cells, it is possible that CaR activation leads to activation of both $G\alpha_s$ and $G\alpha_i$, and that $G\alpha_s$ stimulates adenylyl cyclase, while $G\alpha_i$ activates PLC/calcium transients and/or other downstream events.

Data from this and previous studies demonstrate that extracellular calcium is a potent stimulus for ACTH secretion (Emanuel *et al.* 1996, Fuleihan *et al.* 1996, Ferry *et al.* 1997). The fact that this effect is mimicked by neomycin and other calcimimetics, and the fact that it is blocked by siRNA-mediated knockdown of the CaR clearly demonstrate that calcium acts through the CaR to trigger ACTH secretion. Furthermore, inhibition of this effect by H89 suggests that CaR signaling stimulates ACTH release by augmenting intracellular cAMP, a known secretagogue for ACTH (King & Baertschi 1990). While it is not clear if our results are applicable to normal pituitary corticotrophs, it is interesting

to note that elevations in systemic calcium levels have been shown to induce ACTH release in intact human subjects (Fuleihan *et al.* 1996). This may also be the case in fish, since circulating levels of calcium correlate with circulating levels of corticosteroids (Abbink *et al.* 2004). The fish pituitary expresses the CaR and calcium-mediated release of cortisol is thought to participate in the regulation of calcium economy (Abbink *et al.* 2004, Loretz 2008). The biological significance of calcium-regulated ACTH release is less clear in mammals. It may be a vestige of the ancestral regulation of calcium metabolism by the pituitary gland. Interestingly, some data suggest that polyamines, which are potent CaR agonists, can stimulate ACTH release from the pituitary gland (Yamamori *et al.* 2001, Cheng *et al.* 2004). Moreover, there is diurnal variation in polyamine levels in the pituitary, raising the intriguing possibility that signaling through the CaR might be involved in modulating ACTH secretion in a circadian pattern (Yamamori *et al.* 2001).

Cells in the anterior pituitary have also been noted to produce PTHrP. Its expression has been documented by either *in situ* hybridization or immunohistochemistry in the pituitaries of mice, rats, frogs, fish, and humans (Ikeda *et al.* 1988, Asa *et al.* 1990, Ito *et al.* 1993, Danks *et al.* 1997). It is also produced by human pituitary adenomas and has been measured in the venous drainage of the human anterior pituitary in a patient undergoing petrosal sinus sampling (Ikeda *et al.* 1988, Asa *et al.* 1990, Ito *et al.* 1993, Hofle *et al.* 2001). Very little is known about the regulation of PTHrP expression, the specific cell types that normally secrete it, or its function in pituitary cells. In a rat pituitary tumor cell line, PTHrP gene expression was shown to be controlled by estrogen (Holt *et al.* 1994). Our data demonstrate that PTHrP is also regulated by the CaR through a cAMP/PKA pathway. The PTHrP gene contains a cAMP response element and previous data have shown that the CaR controls PTHrP production in normal and malignant breast cells through the regulation of cAMP levels (Zajac *et al.* 1989, Chilco *et al.* 1998, Mamillapalli *et al.* 2008). However, the biological function of PTHrP and the reasons for its regulation by extracellular calcium in the pituitary remain obscure. As with ACTH, it has been suggested that PTHrP secretion from the pituitary is involved in the regulation of systemic calcium metabolism in fish (Fraser *et al.* 1991, Abbink *et al.* 2004, 2006, Loretz 2008). In mammals, data also suggest that PTHrP can affect the growth and vascularity of malignant pituitary tumor cells transplanted into rodents (Akino *et al.* 1996, 2000). However, more work will be needed to determine the biological function of PTHrP in the pituitary and whether it is regulated by the CaR in the intact gland.

In summary, in this study we demonstrate that the CaR modulates PTHrP and ACTH secretion from mouse pituitary corticotroph-derived AtT-20 cells by regulating cAMP production and coupling to $G\alpha_s$. We recently demonstrated that the CaR controls cAMP and PTHrP secretion in an opposing fashion in normal versus malignant breast cells by switching its use of $G\alpha_i$ to $G\alpha_s$ (Mamillapalli *et al.* 2008).

Prior to this previous report, the CaR had only been described to couple to $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ pathways (Arthur *et al.* 1997, Hofer & Brown 2003, Huang *et al.* 2004, Ward 2004, Gerbino *et al.* 2005, Rey *et al.* 2005). However, AtT-20 cells represent a second example of the receptor coupling to $G\alpha_s$. Therefore, $G\alpha_s$ should be added to the potential repertoire of G proteins mediating signaling from the CaR. Furthermore, since AtT-20 cells activate both $G\alpha_s$ and $G\alpha_i$ pathways, these cells may be useful for studying the regulatory mechanisms that mediate G protein usage by the CaR.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JOE-09-0183>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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