Calcimimetic R-568 effects on activity of R990G polymorphism of calcium-sensing receptor

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Abstract

Previous studies have demonstrated a gain-of-function of the calcium-sensing receptor (CASR) gene R990G polymorphism. In this study, activation of the R990G CASR stably transfected in HEK-293 (HEK-990G) cells compared with that of the common variant (HEK-wild-type (WT)) by increasing concentrations of CaCl2 or calcimimetic R-568 caused significantly higher intracellular free calcium concentration ([Ca2+]i) and lower Ca-EC50. Moreover, the [Ca2+]i oscillation percentage was higher with a larger sinusoidal pattern in HEK-990G. R-568 induced a shift of the oscillatory events from 4 to 2 mmol/l extracellular calcium concentration in HEK-990G cells and increased the sinusoidal oscillation percentage in comparison with HEK-WT. Preincubation with thapsigargin or phospholipase C inhibitors completely prevented oscillations in both cell lines, consistent with the involvement of the inositol trisphosphate pathway, while protein kinase C inhibitor prevented oscillations in HEK-WT cells only. Finally, CaCl2 and R-568 caused a significant increase in p44/42 extracellular signaling-regulated kinase phosphorylation, with the mean Ca-EC50 values being significantly lower in HEK-990G. Our findings demonstrated that the 990G allele is associated with high sensitivity to R-568, which provided new evidence for differences in CASR signaling.

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Introduction

The calcium-sensing receptor (CASR) is a G-protein-coupled membrane receptor involved in the regulation of cellular calcium metabolism. The receptor senses changes in extracellular calcium concentration ([Ca2+]o) and activates different intracellular pathways (Ward 2004, Tfelt-Hansen & Brown 2005, Huang & Miller 2007, Lorenz et al. 2007). In particular, CASR activation triggers calcium mobilization from intracellular stores through inositol trisphosphate (IP3) receptors and activation of the mitogen activated protein kinase (MAPK) cascade through p44/42 extracellular signaling-regulated kinase (ERK) phosphorylation. These events are facilitated by the scaffolding protein filamin A. The changes in intracellular free calcium concentration ([Ca2+]i) induced by CASR activation show an oscillation pattern (Breitwieser & Gama 2001) that has been proposed to play a key role in regulating various pathways, i.e. Ca2+ /calmodulin-dependent protein kinase II (Soderling et al. 2001), protein kinase C (PKC; Oancea & Meyer 1998, Mogami et al. 2003, Bartlett et al. 2005), RAS, and the ERK cascade (Kupzig et al. 2005). Moreover, calcium oscillations have been postulated to dynamically increase the cross talk between multiple signaling pathways, allowing the cell to encode more information and generate complex responses.

At the systemic level, CASR mediates the [Ca2+]o-induced inhibition of parathyroid hormone (PTH) secretion in the parathyroid gland and calcium reabsorption in the kidney. Many rare CASR mutations have been described in humans. Patients with loss-of-function mutations are characterized by hypocalciuria and hypercalcemia, while those with gain-of-function mutations show high urinary calcium excretion and hypocalcemia (Bai et al. 1996, Egbuna & Brown 2008). The intracellular tail of the receptor has three nonconservative polymorphisms, i.e. A986S, R990G, and Q1011E. We previously found an association between the R990G polymorphism and primary hypercalciuria in patients with and without kidney stones (Vezzoli et al. 2002, 2007). Moreover, we demonstrated that transfection of the 990G variant in HEK-293 cells induced a gain-of-function of the receptor (Vezzoli et al. 2007). In agreement with the
in vitro phenotype, patients with primary or secondary hyperparathyroidism (PHPT and SHPT) with the 990G allele were found to have lower levels of serum PTH (Yamauchi et al. 2001, Scillitani et al. 2004, Corbetta et al. 2007a, Wang et al. 2007) and ionized calcium (Yano et al. 2000). Accordingly, the 990R allele was found to be associated with higher levels of both PTH and ionized calcium in comparison with 990G carriers in healthy Chinese women (Yokoyama et al. 2002). Although most studies obtained in different populations confirmed the gain-of-function of the CASR 990G allele, a few studies failed to find this association (Harding et al. 2006, Kelly et al. 2006).

Calcimimetic drugs are allosteric modulators, which enhance CASR sensitivity to extracellular calcium (Nemeth 2004, Trivedi et al. 2008). In particular, it has been demonstrated that one of these compounds, R-568, at nanomolar concentrations increased \([\text{Ca}^{2+}]_i\) concentrations in bovine parathyroid cells and inhibited PTH secretion, with these effects being detectable only in the presence of extracellular calcium (Nemeth et al. 1998). Based on these properties, the calcimimetic agent AMG073 (cinacalcet) has been introduced for the treatment of patients with SHPT and very recently approved for patients with PHPT (Block et al. 2004, Sajid-Crockett et al. 2008). However, the response to cinacalcet is highly variable, probably related to different nutritional or genetic background. Interestingly, a recent report by Rothe et al. (2005) showed that SHPT patients carrying the CASR990G allele were more responsive to treatment with calcimimetic compounds than patients without this allele. The aim of this study was to define in detail the impact of the R990G polymorphism on the receptor activity by examining at which level of the IP3 signaling pathway the two CASR variant proteins differ as well as investigating whether the two CASR variant proteins have a different response to treatment with the calcimimetic R-568. To address these issues, we used HEK-293 cells stably transfected with 990R or 990G alleles (Vezzoli et al. 2007) and investigated the cellular functions at different levels.

Colonies were selected after 1–2 weeks, and two clones (HEK-WT cells and HEK-990G cells) were confirmed to express similar levels of the CASR protein by western blotting with monoclonal anti-CASR antibody (Affinity Bioreagents, Golden, CO, USA) as described (Vezzoli et al. 2007), whereas no expression was found in cells transfected with empty plasmid (HEK-vector cells). Cells were grown in DMEM medium (Sigma Chemicals), supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, and 1 mg/ml geneticin (Sigma Chemicals), under standard conditions (5% CO2, 37 °C).

Fluorescence measurements of \([\text{Ca}^{2+}]_i\), in whole cell population

\([\text{Ca}^{2+}]_i\) was measured in CASR-expressing cells (about 5 × 105 cells/ml for each experiment). Cells were loaded with 1 μM fura-2 AM (Sigma Chemicals), at 37 °C for 45 min, as described previously (Soldati et al. 1999, Vezzoli et al. 2007). The transfected cells with empty vector or untransfected HEK-293 cells were tested as controls. The calcimimetic compound R-568, kindly provided by Amgen Inc. (Thousand Oaks, CA, USA), was added at the concentration range of 0–2 μmol/l just before the beginning of fluorescence measurements. \([\text{Ca}^{2+}]_i\) was increased stepwise by addition of CaCl2 at the following concentrations: 0-6, 0-8, 1-2, 1-8, 3-6, 4-6, 6-6, 8-6, 10-6, and 12-6 mmol/l (Pearce et al. 1996). The \([\text{Ca}^{2+}]_i\) was calculated from the ratio of the fluorescence emission recorded at the two-excitation wavelengths, as described previously (Grynkiewicz et al. 1985, Soldati et al. 1999). Transient responses of \([\text{Ca}^{2+}]_i\) were normalized according to Pearce et al. (1996) and Vezzoli et al. (2007). The effective \([\text{Ca}^{2+}]_i\), giving one-half of the maximal response (Ca-EC50) was calculated for HEK-WT and HEK-990G. In other experiments, the cells maintained at the same \([\text{Ca}^{2+}]_i\), concentration of 1 mmol/l were added stepwise with R-568 from 0-001 to 10 μmol/l and EC50 was calculated (R-568-EC50).

Fluorescence measurements of \([\text{Ca}^{2+}]_i\), in single cells

Cells were seeded on glass coverslips (1 × 105, each) and after 3 days were incubated at 37 °C for 20 min with fura-2 AM (1 μmol/l, Sigma Chemicals) in a Krebs–Ringer–HEPES solution (KRH) having a minimal salt formulation (Gama et al. 1997). The coverslip with fura-2 loaded cells was mounted on a 37 °C thermostatted (TC-202 A, Medical System Corporation, New York, NY, USA) perfusion chamber (PDMI-2, Medical System Corporation) placed on a microscope stage (TE 200, Nikon, Tokyo, Japan) equipped with a High-Speed Dynamic Video Imaging System, Quanticell 700 (Applied Imaging, Sunderland, UK). When required, materials and methods

Cell culture and transfection

Human embryonic kidney cells HEK-293 (Cell Bank, Istituto Scientifico Tumori, Genoa, Italy), which do not express endogenous CASR, were transfected with polymorphic CASR (990G), kindly provided by Dr Jianxin Hu (NIH, Bethesda, MD, USA), or wild-type (WT) CASR (990R), obtained by site-directed mutagenesis, as we have described previously (Vezzoli et al. 2007). Briefly, HEK-293 cells stably expressing human CASR were selected by geneticin (Sigma Chemicals).
the calcimimetic R-568 (0·01, 0·05, and 0·1 mmol/l) was added after the fura-2 loading in the KRH, buffering the cells. Cells were stimulated with increasing CaCl₂ (0·5, 1, 2, and 4 mmol/l). According to Grynkiewicz et al. (1985), background subtracted ratio images (340/380 nm) were collected at 510 nm fluorescence emission through a ×40 oil immersion objective and were used to calculate the [Ca²⁺]ᵢ within the cells. All the experiments used 80–100 cells/optical field. Responsive cells were identified as cells responding to any stimulation with [Ca²⁺]ᵢ increments equal or above 20 nmol/l (Miedlich et al. 2002). The mean single maximum [Ca²⁺]ᵢ rise was calculated for each single cell by difference between baseline and peak value after any stimulation and averaged for all the analyzed cells. Oscillations were identified as three successive fluctuations in the [Ca²⁺]ᵢ after the first main cellular peak. Sinusoidal oscillations in [Ca²⁺]ᵢ are defined as regular fluctuations observed on top of an elevated level of calcium; thus, they represent subsequent calcium increases, after the main peak response, that do not return to the baseline. Transient oscillations are defined as discrete transients of [Ca²⁺]ᵢ due to baseline spiking behavior (Berridge & Potter 1990). The frequency of the oscillatory phenomena was determined counting the number of oscillatory events/min.

Inhibitors and activators of CASR signaling pathways

The sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor thapsigargin (Sigma Chemicals), the phospholipase C (PLC) inhibitor U73122 (Sigma Chemicals), the PKC activator phorbol 12,13-dibutyrate (PDBu; Sigma Chemicals), and the PKC inhibitor Ro-318220 (Sigma Chemicals) were prepared as stock solution in dimethylsulfoxide (DMSO) and diluted at the desired concentration in the KRH, buffering the cells before starting the experiment. A preincubation was performed before CaCl₂ stimulation as following: 5 min for 1 mmol/l thapsigargin, 10 min for 10 mmol/l U73122, 5 min for PDBu at the different concentrations used (0·01, 0·05, and 0·1 mmol/l). In the case of Ro-318220, cells were seeded on collagen-coated glass coverslips, preincubated with different Ro-318220 concentrations (0·025, 1·25, and 2·5 mmol/l) for 60 min and fura-2 was added in the last 20 min. At 2·5 mmol/l both types of cells showed marked signs of suffering. Therefore, this dose was eliminated by the experiments.

Determination of ERK1/2 phosphorylation

For the determination of p44/42 ERK1/2 phosphorylated proteins, monolayers of serum-starved transfected or nontransfected HEK-293 cells were incubated at 37 °C in serum-free medium containing 0·2% w/v BSA with varying levels of CaCl₂ (0·5–15 mmol/l) and of R-568 (0·01, 0·05, and 0·1 mmol/l) as described previously (Corbetta et al. 2002). Briefly, at the end of the incubation period (10 min), cell lysates were obtained by using lysis buffer supplemented with 0·1 mmol/l sodium vanadate, 1 mmol/l phenylmethylsulphonyl fluoride, 10 mmol/l benzamidine, 2 μg/ml leupeptine, and complete phosphatase inhibitor cocktail (Roche Diagnostics). In all, 40 μg proteins were separated by 10% w/v SDS-PAGE, analyzed by western blotting with an anti-phospho-p44/42 ERK polyclonal antibody (Cell Signalling Technology, Beverly, MA, USA) diluted 1:2000, and detected by a chemiluminescent method (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA). Quantitative comparisons of the ERK1/2 phosphorylation were performed using a CanonScan 4400F and a TotalLab version 2.01 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). Nitrocellulose membranes were then stripped off antibodies and reprobed using an anti-α-actin antibody (Sigma Chemicals) and an anti-β-actin antibody (Sigma Chemicals) as appropriate, with a significance level of P<0·05. For fluorescence measurements, mean values were obtained from at least five to eight independent experiments. For ERK phosphorylation determination, experiments were repeated at least thrice.

Results

Effect of [Ca²⁺]₀ and calcimimetic R-568 on CASR function in whole cell population experiments

HEK-293 cells stably expressing WT CASR gene (HEK-WT) or the mutated one (HEK-990G) were stimulated with CaCl₂ in a stepwise manner (range: 0·6–12·6 mmol/l) in the absence or presence of calcimimetic R-568 at different concentrations (from 0 to 2·0 mmol/l) and the change of [Ca²⁺]ᵢ was continuously monitored. Figure 1 shows the ability of R-568 (0·1 mmol/l) to potentiate [Ca²⁺]ᵢ rises elicited by CaCl₂ in HEK-WT. In absence of the drug, HEK-990G cells had a lower Ca-EC₅₀ than HEK-WT (Table 1). When R-568 was added at low concentrations, the mutated receptor was more active than the WT
Calcimimetic R-568 and CASR polymorphism

Single-cell experiments: effect of \([Ca^{2+}]_o\)

Activation of CASR followed by a single or repeated spikes of \([Ca^{2+}]_o\) is dependent on \([Ca^{2+}]_o\) and this phenomenon can be monitored only in single-cell experiments. HEK-WT and HEK-990G cells were stimulated with stepwise increases of agents at concentrations effective in the whole cell population experiments described in the previous paragraph. In these experiments, various parameters, such as percentage of responsive cells, heights of \([Ca^{2+}]_i\) rise, oscillation type, oscillation frequency, were evaluated. As shown in Figs 3 and 4, \([Ca^{2+}]_i\) rises triggered by 1 and 2 mmol/l CaCl\(_2\) in HEK-990G cells were markedly higher than those elicited in HEK-WT, while both cell lines reached the maximal \([Ca^{2+}]_i\) peak at 4 mmol/l CaCl\(_2\). Moreover, the percentage of HEK-990G cells responsive to 1 and 2 mmol/l CaCl\(_2\) was dramatically higher in comparison with the percentage of responsive HEK-WT cells (Fig. 4). At 4 mmol/l CaCl\(_2\), almost all the cells of the two cell lines responded to the agonist (Figs 3 and 4). After the exposure to 4 mmol/l CaCl\(_2\), both cell lines showed spontaneous oscillations. HEK-990G cells showed a higher percentage of total oscillating cells and a larger sinusoidal pattern in comparison with HEK-WT cells, while HEK-vector cells did not respond to the extracellular stimulus (Fig. 3 and Table 2). These data confirm the increased sensitivity of CASR 990G to physiological concentrations of extra-cellular calcium.

Table 1

Calcimimetic R-568 effect on \([Ca^{2+}]_i\) concentration in HEK-WT and HEK-990G cells

<table>
<thead>
<tr>
<th>R-568 (µmol/l)</th>
<th>EC(_{50}) HEK-WT</th>
<th>EC(_{50}) HEK-990G</th>
<th>P value (Student's t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4-49±0-15</td>
<td>2-69±0-11</td>
<td>0-0001</td>
</tr>
<tr>
<td>0-01</td>
<td>4-31±0-09</td>
<td>2-72±0-20</td>
<td>0-0001</td>
</tr>
<tr>
<td>0-05</td>
<td>3-38±0-12</td>
<td>2-85±0-14</td>
<td>0-0087</td>
</tr>
<tr>
<td>0-1</td>
<td>2-40±0-13</td>
<td>2-10±0-22</td>
<td>NS</td>
</tr>
<tr>
<td>0-5</td>
<td>1-77±0-23</td>
<td>1-48±0-15</td>
<td>NS</td>
</tr>
<tr>
<td>1</td>
<td>1-38±0-07</td>
<td>1-44±0-05</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>1-41±0-06</td>
<td>1-24±0-10</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not statistically significant.
HEK-990G compared to HEK-WT cells (Fig. 4); and 3) the appearance of the oscillations already at 2 mmol/l CaCl$_2$ in the presence of 0.05 and 0.1 mmol/l R-568 (Table 2).

The exposure to R-568 at different concentrations determined changes in the oscillation pattern in HEK-WT and particularly in HEK-990G cells. The proportion of oscillating HEK-990G cells at 0.01 mmol/l R-568 was higher than that of HEK-WT, while the difference disappeared with increasing calcimimetic concentration (Table 2). Analyzing the oscillation pattern, although the proportion of transient oscillations was similar in both cell lines at all R-568 concentrations, the proportion of sinusoidal oscillations was higher in HEK-990G at 0.01 mmol/l R-568, accounting for the observed difference in the overall proportion of oscillating cells. The difference disappeared with increasing R-568 concentrations (Table 2). ANOVA analysis showed that increasing R-568 concentration in both cell lines reduced transient oscillations, while sinusoidal oscillations gradually increased in HEK-WT cells only (Table 2). Therefore, the oscillation pattern shifted in a large percentage of cells from a transient to a sinusoidal one at 4 mmol/l CaCl$_2$ with higher R-568 concentrations (Table 2). Finally, the oscillation frequency was similar in the two cell lines in all the experimental conditions (3.46 ± 0.17 and 3.03 ± 0.09 oscillation/min, for HEK-WT and HEK-990G cells respectively). Hence, all these data indicate that HEK-990G cells were more responsive to the R-568 than HEK-WT cells.

Effect of inhibitors and activators of CASR signaling pathways

The effects of inhibitors and activators of some components of the CASR signaling pathways were analyzed by single-cell experiments. The pretreatment with 1 µmol/l SERCA inhibitor thapsigargin, which rapidly depletes intracellular Ca$^{2+}$ stores, completely blocked oscillations in both cell lines (Fig. 5A and B). The same effect was obtained with the PLC inhibitor U73122 (10 µmol/l; Fig. 5C and D). These results indicate that [Ca$^{2+}$]$_i$ oscillations depend on the intracellular calcium pools and IP$_3$ pathway.

The pretreatment with the PKC inhibitor Ro 31-8220 at 1.25 mmol/l concentration differentially affected
Percentage of transient oscillating HEK-990G cells, loaded with fura-2, were stimulated by [Ca\textsuperscript{2+}]\textsubscript{i} in the presence of different concentrations of R-568. HEK-WT and HEK-990G cells were assayed in single-cell experiments. Mean values of [Ca\textsuperscript{2+}]\textsubscript{i} rise in the presence or absence of calcimimetic R-568 in single-cell experiments. A tested lower dose of Ro 31-8220 (0.625 µmol/l) drastically reduced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in presence of 4 mmol/l CaCl\textsubscript{2}, with no differences between the two cell lines (Fig. 6C and D).

Control experiments were also performed on HEK-WT and HEK-990G cells with DMSO, used as vehicle of the inhibitors and activators, and no cell responses were monitored (data not shown).

**Effect of CASR activation by [Ca\textsuperscript{2+}]\textsubscript{o} and calcimimetic R-568 on p44/42 ERK phosphorylation**

Exposure to CaCl\textsubscript{2} (from 0.5 to 1.0 mmol/l) caused no effect on p44/42 ERK phosphorylation in HEK-WT cells, while it induced an 80% increase in p44/42 ERK phosphorylation in HEK-990G cells. The maximal p44/42 ERK phosphorylation response was observed at 5.0 mmol/l CaCl\textsubscript{2} in both cell lines, while no further increase was observed at CaCl\textsubscript{2} up to 15 mmol/l (Fig. 7 and data not shown). The mean Ca-EC\textsubscript{50} value was lower in HEK-990G (0.93±0.20 mmol/l) than in HEK-WT cells (2.46±0.83 mmol/l, P=0.046; Fig. 7B). HEK-293 cells or cells transfected with empty plasmid showed no change in p44/42 ERK phosphorylation at any [Ca\textsuperscript{2+}]\textsubscript{o} tested (data not shown).

The effect of R-568 on CASR-induced increase in p44/42 ERK phosphorylation was tested. In HEK-990G cells, the lowest R-568 concentration (0.01 µmol/l) was able to induce the maximal p44/42 ERK phosphorylation at 3 mmol/l CaCl\textsubscript{2} (Fig. 8A and B), while in HEK-WT cells a leftward shift of the dose–response curve was observed by increasing R-568 concentrations to 0.05 and 0.1 µmol/l (Fig. 8B). The mean Ca-EC\textsubscript{50} values in presence of 0.01 µmol/l R-568 were significantly lower in HEK-990G than in HEK-WT (3.18±0.06 vs 1.79±0.01 mmol/l respectively, P<0.002; Table 3). In HEK-990G cells, the Ca-EC\textsubscript{50} values were not further reduced by increasing R-568 concentrations to 0.05 and 0.1 µmol/l, while in HEK-WT, Ca-EC\textsubscript{50} values showed a trend to reduce by increasing drug concentration from 0.01 to 0.05 µmol/l R-568 that became significant at 0.1 µmol/l R-568 (Fig. 8 and Table 3).

![Figure 4 Percentage of responsive cells and [Ca\textsuperscript{2+}]\textsubscript{i} rise (nmol/l) in the presence of different concentrations of R-568. HEK-WT and HEK-990G cells, loaded with fura-2, were stimulated by [Ca\textsuperscript{2+}]\textsubscript{o} in the presence of different concentrations of R-568 in single-cell experiments. Mean values of [Ca\textsuperscript{2+}]\textsubscript{i} rise (A) and percentage of responsive cells (B) were calculated as described in the 'Materials and methods' section. *P<0.05 between HEK-WT and HEK-990G cells.](image-url)

### Table 2 Percentage of oscillating cells in the presence of different R-568 concentrations

<table>
<thead>
<tr>
<th>R-568 (µmol/l)</th>
<th>0</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HEK-WT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of oscillating cells</td>
<td>59.52±5.93</td>
<td>79.15±5.42\textsuperscript{a}</td>
<td>35.85±8.04</td>
<td>78.74±4.66\textsuperscript{b}</td>
</tr>
<tr>
<td>Percentage of transient oscillating cells</td>
<td>52.65±5.98</td>
<td>55.46±5.72</td>
<td>24.71±5.01</td>
<td>59.19±10.79</td>
</tr>
<tr>
<td>Percentage of sinusoidal oscillating cells</td>
<td>8.07±2.67</td>
<td>24.39±4.89\textsuperscript{p}</td>
<td>11.4±4.11</td>
<td>38.83±6.30\textsuperscript{d}</td>
</tr>
<tr>
<td><strong>HEK-990G</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of oscillating cells</td>
<td>56.9±5.33</td>
<td>76.25±5.42\textsuperscript{b}</td>
<td>32.6±8.04</td>
<td>78.4±4.66\textsuperscript{b}</td>
</tr>
<tr>
<td>Percentage of transient oscillating cells</td>
<td>51.0±5.98</td>
<td>51.6±5.72</td>
<td>21.7±5.01</td>
<td>57.9±10.79</td>
</tr>
<tr>
<td>Percentage of sinusoidal oscillating cells</td>
<td>8.0±2.67</td>
<td>23.9±4.89\textsuperscript{p}</td>
<td>11.4±4.11</td>
<td>38.8±6.30\textsuperscript{d}</td>
</tr>
</tbody>
</table>

Student’s t-test for HEK-990G vs HEK-WT, P values: \textsuperscript{a}0.0216, \textsuperscript{b}0.0036, \textsuperscript{p}0.0095, \textsuperscript{d}0.0065. One-way ANOVA for HEK-WT, P values: \textsuperscript{a}0.0003, \textsuperscript{b}0.0002.

One-way ANOVA for HEK-990G, P values: \textsuperscript{a}0.0027.

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Discussion

Our study confirms that the R990G polymorphic CASR is more sensitive than the WT receptor to extracellular calcium and provides new evidence for an increased sensitivity of this receptor to the calcimimetic R-568. This study investigated in detail several intracellular events generated by CASR activation. First, as calcium oscillations have been postulated to dynamically increase the cross talk between multiple signaling pathways to generate complex responses, the oscillatory patterns of $[Ca^{2+}]_i$ elicited by CaCl$_2$ and the calcimimetic were extensively studied in single-cell experiments. Two different oscillatory patterns were described: 1) transient oscillations, with a baseline spiking behavior and 2) sinusoidal oscillations, with an elevated-line spiking. Both patterns, which may occur in the same cell in response to different stimuli or to changes in agonist concentration, have been hypothesized to generate different signaling pathways (Rey et al. 2005). In agreement with previous studies (Breitwieser & Gama 2001, Young & Rozengurt 2002, Rey et al. 2005), HEK-293 cells transfected with the human WT CASR responded to increases in $[Ca^{2+}]_o$ to physiological level (2 mmol/l) with a single transient $Ca^{2+}$ spike and to higher $[Ca^{2+}]_o$ concentration with $[Ca^{2+}]_i$ oscillations. In agreement with our previous results obtained in whole cell population (Vezzoli et al. 2007), the single cells transfected with the CASR variant 990G allele were more sensitive to extracellular calcium. The calcimimetic R-568 had a profound effect on the oscillatory pattern. In fact, increasing R-568 concentrations reduced transient oscillations in both cell lines, while sinusoidal oscillations gradually increased in HEK-WT cells. At variance, HEK-990G cells display a significant percentage of sinusoidal oscillations also in the absence of R-568, a percentage not statistically modified by the increasing concentration of R-568. It is worth noting that the two oscillatory patterns are related to different mechanisms of activation. Transient oscillations seem to be controlled by activation of phosphoinositide pathway and are influenced by agonist concentrations and phorbol esters (PKC activators; Berridge & Potter 1990), while sinusoidal oscillations seem to result from a negative feedback loop involving inhibition of IP$_3$ production by PKC (Bird et al. 1993). Moreover, Bird et al. stated that full activation or inhibition of PKC is incompatible with sinusoidal oscillations.

Therefore, it can be speculated a difference in the level of PKC activity between HEK-WT and HEK-990G cells, a difference that becomes important when the calcimimetic R-568 is administered to cells. The increase in the percentage of sinusoidal oscillations in
HEK-WT cells due to the calcimimetic R-568 could be ascribed to a pharmacological modification of PKC activity, resulting in the modulation of this activity toward a fluctuating level able to affect IP₃ production and [Ca²⁺]ᵢ. This hypothesis, as well as its pharmacological significance, deserves further investigation.

As far as the oscillation frequency was concerned, this was similar in HEK-WT and HEK-990G cells and consistent with the reported values (Young et al. 2002, Szekely et al. 2009). The dose-dependent increase in oscillation frequencies reported previously was obtained at high calcimimetic concentrations (1–10 μM; Miedlich et al. 2002).

Second, we investigated some components of the pathways involved in [Ca²⁺]ᵢ oscillations. Indeed, the exact mechanisms responsible for inducing, maintaining, and ending of [Ca²⁺]ᵢ oscillations are still largely unknown. The major pathway seems to involve calcium influx and efflux mediated by PLC and IP₃, implicating intracellular stores. In this study, both SERCA and PLC inhibitors, which act at two different levels of the IP₃ pathway, were effective in preventing the oscillatory behavior.

Figure 7 Effect of increasing [Ca²⁺]₀ on p44/42 ERK phosphorylation. (A) Representative immunoblots of phosphorylated and total p44/42 extracellular signaling-regulated kinase (ERK) in HEK-WT (upper set) and HEK-990G (lower set) stimulated by increasing CaCl₂ concentrations. (B) Dose–response curve of p44/42 ERK phosphorylation. The response was normalized to the maximal effect obtained by CaCl₂ and the percentage of phosphorylation was subtracted to the basal level at 0.5 mmol/l CaCl₂. Values shown are means ± S.E.M. of three replicates. The dotted lines indicate the mean values of Ca-EC₅₀.* P = 0.01 between HEK-WT and HEK-990G cells.
responses in both cell lines, indicating that this pathway is required for \([\text{Ca}^{2+}]_i\) oscillations.

However, other regulating mechanisms have been hypothesized to explain \([\text{Ca}^{2+}]_i\) oscillations, such as the negative feedback due to PKC-induced phosphorylation at Thr\(^{888}\) residue of CASR intracellular domain. Young & Rozengurt (2002) and Young et al. (2002) found that PKC had a negative effect on \([\text{Ca}^{2+}]_i\) oscillations mediated by the CASR, both with inactive and activated PKC. In fact, the T888A CASR mutant, which is not phosphorylated by PKC at this residue, reduced \([\text{Ca}^{2+}]_i\) oscillations. However, the PKC activator PDBu produced the same reduction on the WT CASR. In agreement with these data, our study suggests that PKC plays a critical role in \([\text{Ca}^{2+}]_i\) oscillations elicited by the increase of \([\text{Ca}^{2+}]_o\) in HEK-WT. In fact, both the PKC inhibitor Ro 31-8220 and PKC activator PDBu significantly reduced oscillatory responses triggered by \([\text{Ca}^{2+}]_o\) increases, without affecting \([\text{Ca}^{2+}]_i\) peaks. Conversely, in HEK-990G cells, while the activator PDBu resulted in an almost complete inhibition of oscillations, the PKC inhibitor Ro 31-8220 seemed not to influence the presence of oscillations. These data suggest that a subsidiary mechanism, not operative for the WT receptor, might maintain the oscillatory response of CASR 990G in the absence of PKC. Moreover, we can speculate that calcium transients remained active in presence of PKC modulators because they are triggered by the PLC–IP\(_3\) pathway, upstream PKC (Young & Rozengurt 2002). Studies on CASR and PKC isoforms demonstrated that both conventional and novel PKC isoforms are activated by \([\text{Ca}^{2+}]_o\) increases (Remy et al. 2007) and PKC-specific inhibitors did not completely abolish ERK1/2 phosphorylation (Sakwe et al. 2004). Novel PKCs (\(\delta, \varepsilon, \eta, \) and \(\theta\)) are activated by \([\text{Ca}^{2+}]_i\) and diacyl glycerol, whereas conventional PKCs (\(\alpha, \beta, \) and \(\gamma\)) are activated only by \([\text{Ca}^{2+}]_i\), so we can speculate that the oscillatory response of CASR 990G could require only one PKC group (Oancea & Meyer 1998, Young et al. 2002). Our data do not answer this question but demonstrate the shift of PKC pathway in HEK-990G cells. Further experimental work will be necessary to

Table 3 Calcinimetic R-568 effect on p44/42 extracellular signaling-regulated kinase phosphorylation in HEK-WT and HEK-990G cells

<table>
<thead>
<tr>
<th>R-568 ((\mu)mol/l)</th>
<th>EC(_{50}) HEK-WT</th>
<th>EC(_{50}) HEK-990G</th>
<th>(P) value (Student’s (t)-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-01</td>
<td>3.18±0.06</td>
<td>1.79±0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>0-05</td>
<td>2.13±0.45</td>
<td>1.48±0.50</td>
<td>NS</td>
</tr>
<tr>
<td>0-1</td>
<td>2.48±0.02</td>
<td>2.22±1.23</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not statistically significant.
fully understand the interaction between PKC isoforms and CASR in \([\text{Ca}^{2+}]_o\)-evoked \([\text{Ca}^{2+}]_i\) oscillations.

Finally, we investigated components of the pathways involved in long-term effects of CASR activation. It is well known that CASR contributes to regulation of cellular processes, including cell proliferation, differentiation, and apoptosis through activation of the ERK cascade (Hofer & Brown 2003). It has been demonstrated that the ERK pathway is involved in the modulation of PTH secretion (Corbetta et al. 2002) and growth factor-induced cyclin D1 expression (Corbetta et al. 2007b) in human parathyroid cells. CASR-mediated activation of MAPK signaling requires direct interaction of the CASR carboxyl terminal with the cytoskeleton scaffolding protein filamin A (Awata et al. 2001, Hjalm et al. 2001). Analyses by yeast two-hybrid have identified strong interactions between filamin A and the CASR carboxyl terminus from residues 907 to 997 (Awata et al. 2001) and 972 to 1031 (Hjalm et al. 2001), suggesting that the minimal interaction domain is likely to be between residues 972 and 997. Indeed, MAPK signaling is dependent on the presence of an \(\alpha\)-helix (residues 868–879) shown previously to be involved in G-protein-mediated signaling. Furthermore, the interaction with filamin A stabilizes CASR and attenuates its degradation rate, facilitating MAPK signaling (Zhang & Breitwieser 2005). Intensive mutagenesis suggests that the domain proximal to transmembrane helix 7 within the carboxyl terminus is involved in CASR-mediated activation of \(G_\alpha_q\), PKC-mediated desensitization, signaling to phosphatidylinositol PLC, and induction and maintenance of the intracellular \([\text{Ca}^{2+}]_i\) oscillations (Gama & Brietwieser 1999, Miedlich et al. 2002). In this study, we first demonstrated that the 990G polymorphic variant of the CASR activated p44/42 ERK phosphorylation in the short term (10 min). Moreover, the 990G allele was associated with a higher sensitivity to extracellular \([\text{Ca}^{2+}]_o\) for the short-term activation of the p44/42 ERK pathway in comparison with the WT receptor. The potent CASR agonist R-568 efficiently increased the sensitivity of the WT CASR in terms of p44/42 ERK phosphorylation. The efficient activation of the 990G CASR in terms of p44/42 ERK phosphorylation has been observed even at lower R-568 concentrations. The binding site for R-568 has been identified within the transmembrane domain of human CASR (Holstein et al. 2004). R-568 has been demonstrated to stabilize WT and mutant CASR favoring the active receptor conformations (Miedlich et al. 2004). Our data were in line with previous observation of Huang & Breitwieser (2007) who demonstrated that gain-of-function mutations increased the stability and activity of the receptor and that R-568 failed to further stabilize those mutants. R990G polymorphism was demonstrated to result in a gain-of-function (Vezzoli et al. 2007), so the changes in \([\text{Ca}^{2+}]_i\) response, oscillation pattern, and p44/42 ERK phosphorylation level, showed in this study, can be involved in a conformational checkpoint. We are tempted to speculate that the 990G activates an alternative pathway not modified by the R-568 action or maximally activated in basal conditions. Indeed, \([\text{Ca}^{2+}]_o\) and calcimimetic activation of ERK via separate pathways in HEK-WT cells had been suggested previously and the authors speculated that it might represent a nexus to differentially regulate cell differentiation vs proliferation via CASR activation (Sakwe et al. 2004).

In conclusion, our study confirms that the R990G polymorphic CASR is more sensitive than the WT receptor to extracellular calcium and provides new evidence for an increased sensitivity of this receptor to the calcimimetic R-568. Moreover, we found some different behaviors in cells expressing the WT or the mutant receptor, such as the insensitivity of HEK-990G cells to the PKC inhibitor, which could be a critical point in the CASR signaling. Admittedly, these data have been obtained in HEK-293 cells, a cell model extensively used for investigating CASR activity, due to the significant number of similarities with parathyroid cells. Therefore, the present data should be confirmed in a more physiological system, i.e. primary cell lines expressing CASR variants. If confirmed, these data might be relevant for future pharmacogenomic studies aimed to predict different responses to treatment in individuals with or without R990G allele, as reported in small series (Rothe et al. 2005). In this respect, our findings may be the rationale behind a clinical study assessing the therapeutic response to calcimimetic, such as cinacalcet, in patients carrying the R990G polymorphic CASR.

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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