Residue 826 in the Calcium-Sensing Receptor (CaR) is implicated in the response to calcium and to R-568 calcimimetic compound.

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ABSTRACT

Within the extracellular loops of the seven transmembrane domain of the calcium-sensing receptor (CaR) there is a region (I819-E837) relevant for calcimimetic activity. As the naturally occurring variant Ala826Thr is within this important region, it may be postulated that this change may influence the CaR response to calcium and R-568.

Human embryonic kidney (HEK-293) cells transiently transfected with three different human CaR (wild type [A826], variant allele [T826] and artificial mutant [W826]) were used to test the ability of calcium alone or in combination with the calcimimetics R-568 to modulate CaR activity. CaR activation was detected by flow cytometry using a fluorescent probe. Intracellular calcium changes were measured in response to changes in extracellular calcium alone or with different R-568 concentrations.

The change of the alanine in the 826 position (A826) for threonine (T826) worsened calcium sensitivity, increasing EC_{50} value from 2.34±0.48 mM (A826, wild type) to 2.96±0.75 mM (T826) ($p<0.05$). The T826 receptor reached similar response with 1 µM R-568 compared with the wild type receptor. On the contrary, the artificial introduction of a tryptophan in the same position (W826) did not affect calcium sensitivity (EC_{50}= 2.64±0.81 mM) but reduced the ability of the receptor to respond to R-568.

The results demonstrate the importance of the 826 residue in the CaR the response to calcium and calcimimetics. Since A826T change was described as a natural variant, the differences in calcium and calcimimetic response observed between both alleles could have potential clinical impact.

Key words: PTH, Calcium-sensing receptor, calcimimetics, HEK-293, Site-directed mutagenesis.

INTRODUCTION

The calcium-sensing receptor (CaR) plays an essential role in extracellular calcium ion homeostasis by regulating the rate of parathyroid hormone (PTH) secretion from parathyroid cells and the rate of calcium reabsorption by the kidney in response to extracellular Ca^{2+} [1]. The CaR is a member of the G protein-coupled receptors (GPCR) family 3, characterized by a large amino-terminal extracellular domain (ECD). The ECD, more precisely some areas of the Venus flytrap (VFT) domain, are considered the main Ca^{2+} binding region [1-3] although a possible binding site in the seven transmembrane (7TM) domain has also been postulated [4, 5].

Most of the gain or loss of function mutations in the CaR, cause of several disorders of calcium homeostasis [1, 5-7], are localized in the ECD, although several have also been identified within the area surrounding the last 3 transmembrane helixes and the loops of the 7TM domain [8-10].

Calcimimetic compounds, including the phenylalkylamine type II compounds R-568 and cinacalcet HCl, are allosteric CaR modulators that activate the parathyroid cell CaR to inhibit PTH secretion [11-16]. Cinacalcet HCl, Sensipar®/Mimpara®, with the same mechanism of action but improved metabolic profile compared with R-568 [17] is currently being used for the treatment of secondary hyperparathyroidism [18, 19]. However, not all patients treated with cinacalcet HCl respond equally to the same dose of calcimimetic [18-20]; these differences could be partly explained by changes in the activity of the calcimimetic on the CaR due to genetic variants of the receptor.

Although the exact calcimimetic binding sites have not been definitively established, some studies have demonstrated that the extracellular loops of the 7TM domain of the CaR, and more precisely the region I819-E837, are relevant for R-568 activity [4, 9, 21, 22]. Within this region, amino acid position 837 is critical for CaR responsiveness to the positive allosteric modulator [9, 21-24].

The residue 826 is within this important region, thus, changes in this particular residue may influence the CaR response to calcimimetics that bind loop 3 of the 7TM

domain of the receptor [25]. The study investigates the effect of CaR 826 residue in response to calcium and the calcimimetic R-568.

MATERIAL AND METHODS

Site-directed mutagenesis of human CaR (CaR)

The full length wild type CaR had been cloned into the pcDNA 3.1 expression vector (*Invitrogen™*, Carlsbad, CA, USA) [7]. Site directed mutagenesis was performed using the single-tube Megaprimer approach [26]. Briefly, this method involves two rounds of PCR that utilize two flanking primers with significantly different melting temperatures (Tm) and one internal mutagenic primer containing the desired base substitution. While the first PCR round is performed at low annealing temperature using the mutagenic primer and the low Tm flanking primer, the second PCR reaction is performed at high annealing temperature using the high Tm flanking primer and the product of the first PCR round as primer, favoring the selective synthesis of the PCR product with the desired mutation. With this method two different CaR were created that differed in the 826 residue and an artificial mutant containing the bulky amino acid tryptophan (W826). Like the wild type CaR, the other two receptors were cloned into the pcDNA 3.1 expression vector.

The sequences of the three CaR inserts cloned were confirmed by automated DNA sequencing using dRhodamine terminator cycle sequencing kit and ABI-PRISM 310 DNA sequencer (Applied Byosistems, Foster City, CA, USA).

Transient transfection of wild type and mutant CaRs in HEK-293 cells

Human embryonic kidney cells (HEK-293, ATCC-LGC No. CLR-1573, *Promochem*, Teddington, UK) were cultured in DMEM-F12HAM (*Sigma-Aldrich,* St. Louis, MO, USA) with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin-sulphate (all from *Biochrom AG*, Berlin, Germany) at 37ºC in a humidified atmosphere with 5% CO_2.

Transfections were performed in 10 cm diameter culture plates of non-confluent HEK-293 cells using 10µg of plasmid DNA for each transfection. Cells were transfected with three different constructs: wild type (A826), polymorphic allele (T826) and mutated allele (W826), using the calcium-phosphate method [27].

CaR expression

CaR expression was determined by Western-blot using an anti-CaR polyclonal antibody (kindly provided by Drs. A. Brown and E. Slatopolsky from Washington University School of Medicine, St. Louis, Missouri;1:1000) [28]. Aliquots containing 40 µg of total protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane following a standard protocol [27]. Chromogenic detection was performed with Pearce ECL Western blotting Substrate (*Pierce Biotechnology Inc.*, Rockford, IL, USA).

Transfection efficiency and CaR localization were determined by immunocytochemistry using the same anti-CaR polyclonal antibody (dilution 1:1000) [28]. $5x10^5$ HEK-293 cells were fixed in 4% formalin-PBS *(Sigma-Aldrich*) on a glass slide. Staining was carried out as previously described [29] and the presence of CaR was positive in approximately 70% of transfected cells.

CaR activation Assay

CaR activation was assessed 48 hours after transfection. The fluorescent probe Fluo-3/acetoxymethylester (Fluo-3/AM, *Invitrogen™*) was used to monitor the intracellular calcium ($[Ca^{2+}]_I$) response in transfected cells upon exposure to increasing levels of $CaCl_2$ (0.5-16.0 mM) and $CaCl_2$ (0.5-16.0 mM) + R-568 (0.1 µM, and 1 µM), or its less active enantiomer S-568 (1 µM). Transfected cells were washed and re-suspended in a calcium-free buffer without antibiotics (118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, buffered at pH 7.3 with 20 mM Hepes) and loaded firstly with 0.5 µM fluo-3 and secondly with 0.25 µg/mL of the vital stain 7-amino-actinomycin D (7-AAD,

Beckton Dickinson, Mountain View, CA, USA) [30]. The Fluo-3 fluorescent emission (505 nm) was detected with a FACScan flow cytometer (*Becton Dickinson*) 3-5 seconds after the cells were stimulated with different calcium concentrations. Only viable and functional transfected cells were used for CaR response experiments. For each individual stimulus, 2 000 gated cells were acquired and the Cell Quest software (*Becton Dickinson*) was used to determine the peak mean fluorescence intensity of the transient response.

Calcium response curves were studied in the three different constructs. Ten different experiments with separate transfections were performed for each construct. Calcium response curves in the presence of different concentrations of R-568 were also performed for the three constructs. Before the cells were exposed to the increasing calcium concentrations, cells were incubated for 10 minutes with the calcimimetic compound [14, 31]. Five different experiments with separate transfections were performed for each calcimimetic concentration in every construct. Data were normalized against the activation obtained with the wild type receptor in 16 mM calcium in order to obtain the maximal receptor activation. Using non-linear regression, the data were fitted to a logarithmic curve to calculate the EC_{50} values (extracellular calcium concentration required to reach 50% of the maximal response) for calcium alone or in the presence of the calcimimetic compound with the three receptors: wild type (A826), polymorphic allele (T826) and artificial mutant (W826).

Statistical analysis

The statistical analyses were performed using ANOVA and Student's t-tests. Statistical signification was considered with $p< 0.05$.

RESULTS

Characterization of CaR-expressing HEK-293 cells

Prior to the activation studies, we determined the presence of CaR protein in transfected HEK-293 cells and its location in the cell membranes. Western-blot analysis showed a high CaR expression (Figure 1A). The band pattern consisted of a double band, the high mannose intracellular form of the receptor (lower band at ~130 kDa) and the fully glycosylated cell surface-expressed form of the receptor (the upper band at ~150 kDa) [32]. No significant differences in CaR expression levels were observed among the three different constructs (wild type, T826, and W826). As expected, no CaR expression was found in non-transfected cells (Figure 1A).

Moreover, immunocytochemical staining against CaR showed that 70% of transfected cells expressed CaR correctly localized at the cell membrane with no differences between the different constructs (Figure 1B).

CaR response to extracellular calcium and calcimimetics

Initial experiments evaluated CaR response to calcium in a wide range of concentrations ($CaCl_2$ 0.5-16.0 mM) in the three different constructs. We found a calcium concentration-dependent effect, with a maximal activation at 16.0 mM calcium. Data were then normalized against the maximal response observed in the wild type receptor (A286).

No significant differences were observed in maximal CaR activation between the wild type receptor (A826, 100%) and the variant allele receptor (T826, 102%), while a slight reduction in the maximal response was observed in the artificial mutant receptor (W826, 83%) (Figure 2). When CaR sensitivity to calcium was analyzed (EC_{50}), the variant allele receptor (T826) showed a significant reduction, i.e. higher EC_{50} value (Table 1). Conversely, the mutant receptor (W826) showed a non-significant increase in the EC_{50} value compared to the wild type receptor (Table 1).

The response to calcium curves were also performed in wild type, T826 and W826 receptors in the presence of two different R-568 concentrations (0.1 µM and 1 µM). In the wild type receptor, a slight decrease in maximal activation was observed (92%) (Figure 3A), although no significant changes were observed in the EC_{50} value (Table 1) when 0.1 µM R-568 was added. By contrast, the addition of 1µM R-568 did not show any difference in maximal activation (105%) (Figure 3A), while it increased CaR sensitivity, causing a significant left-shift in the EC_{50} value from 2.34 mM to 1.70 mM (Table 1).

Maximal activation was slightly reduced in the T826 variant when 0.1 µM was added (93%) while the addition of 1 µM R-568 did not modify the maximal activation (99%) (Figure 3B). The addition of 1µM R-568 decreased EC_{50} values (Table 1).

Finally, in the mutant variant (W826), addition of 0.1 µM R-568 did not modify the maximal activation produced by calcium, although 1 µM R-568 slightly increased it by a 9% (82% calcium alone, 86% calcium + R-568 0.1 µM, and 91% calcium + R-568 1 µM) (Figure 3C). No significant changes were observed in EC_{50} values for calcium with the two R-568 concentrations tested, though the addition of 1 µM R-568 showed the lowest EC_{50} value of the series (Table 1).

The use of lower R-568 concentrations (0.02 µM) did not modify either maximal activation or sensitivity (EC_{50}) in any of the three constructs studied (data not shown). Likewise, the addition of higher concentrations (5 µM) did not produce further change either in maximal activation or calcium EC_{50} values in any of the constructs (data not shown). As expected, the less active enantiomer S-568 (1 µM) neither modified the maximal activation nor the calcium EC_{50} in the three constructs (Table 1).

DISCUSSION

The region between helixes 6 and 7 of the 7TM domain of the CaR, and specifically residue 837, is critical for calcimimetic binding and response [9, 21-24]. Within this important region, a genetic variant has been described at amino acid 826 [25]. The present study shows, for the first time, that this particular residue of the CaR may be implicated in the response to calcium and calcimimetics.

We found that the wild type allele, which is the alanine in the 826 position, showed an EC_{50} for calcium similar to that previously reported [33]. By contrast, the CaR with the less frequent allele (T826) presented a significantly higher EC_{50} value. This difference cannot be explained by different cell surface expression levels of CaR since the likely variation in the receptor expression might affect maximal response to extracellular calcium but not EC_{50} values [34].

The region from I819 to E837 of the CaR has been described as an important region for the response to extracellular calcium. Most of the naturally occurring or artificial mutations in this region produced an increase in the sensitivity of CaR to calcium (activating effect) [9]. However, within this region, it has also been described that the substitution of threonine 828 for alanine produced an inactivating effect on the receptor, and a drastic inactivation when proline 823 was changed for alanine [9]. In our study, the substitution of alanine 826 for threonine also produced an inactivating effect.

An additional construct was also created at the 826 position in order to confirm the likely role of this residue in calcium response. To this end, we introduced the bulky amino acid tryptophan (W826) that could greatly modify the 3D structure of the receptor. The introduction of this amino acid did not modify the CaR sensitivity to calcium, although a slight reduction in maximal receptor activation was found.

These results suggest that the effect of the threonine in the 826 position on CaR sensitivity could be explained in terms of polarity of the amino acid and not in terms of size. Threonine presents a polar radical with a hydroxyl group while both alanine and

tryptophan present hydrophobic radicals, the first smaller and the latter bigger than the threonine radical. Hu et al [9] found no effect on CaR response when alanine in the 826 position was substituted for serine, so the tiny difference between serine and threonine radicals would be enough to affect CaR response to calcium.

According to our results, it could be speculated that the presence of the polar amino acid threonine in the 826 position of the CaR could worsen calcium binding to the receptor or lead to a greater destabilization of the 3D structure than the presence of the tryptophan, a bulky but hydrophobic amino acid. Once we found that the 826 residue seems to be an important factor for calcium sensing, we speculated if the A826T amino acidic change described in this position of the CaR could be also implicated in different response to calcimimetics.

As expected, the response to calcium in the wild type receptor was modified in the presence of 1 µM R-568, reducing EC_{50} value without modifying maximal receptor activation. The same effect was previously observed by other authors [9, 22, 24, 31], although an increase in maximal receptor activation has also been described [23]. We could not find any effect on the response to calcium when we tested lower R-568 concentrations (0.1 µM), which supported previous findings using the same experimental approach [31, 35].

The CaR with the less frequent allele (T826) behaved similarly to the wild type receptor when exposed to R-568. The sensitivity to calcium of both receptors in the presence of 1 µM R-568 was comparable, showing similar EC_{50} values. Furthermore, and as for the wild type receptor, the addition of 1µM R-568 did not modify maximal receptor activation in the T826 receptor. On the contrary, the presence of tryptophan in the 826 position of the CaR reduced the ability of the receptor to significantly respond to R-568 in a wide range of concentrations (0.02-5 µM, the effect of 0.1 and 1 µM R-568 is shown in table 1). This different behavior of the CaR in response to calcium and/or calcimimetics with different amino acids in the same position has been reported previously in other areas important for the CaR functionality [9, 24].

The results of our study demonstrate that the presence of a threonine in the 826 position of the CaR protein (T826) impairs the response to calcium although it does not modify the response to calcimimetics. On the contrary, the introduction of the bulky amino acid tryptophan in the same residue does not affect the response to calcium but reduced the ability of the receptor to respond to R-568. These findings support the concept that the 826 residue, located in the extracellular loop of the 7TM domain of the CaR could be relevant for calcium and calcimimetic binding and response. Further clinical and epidemiological studies should elucidate if differences between these two polymorphic alleles may explain some of the differences in response observed in chronic kidney disease patients receiving calcimimetics [18, 19].

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Table 1: EC_{50} values (mM) for calcium and for calcium combined with 0.1 µM or 1.0 µM R-568 and 1.0 µM S-568 for the three different CaR constructs (wild type, T826 and W826) transiently transfected in HEK-293 cells. EC_{50} values were calculated fitting the data to a logarithmic curve.

		WILD TYPE*	T826*	W826
Calcium		2.34±0.48	2.96±0.75**	2.64±0.81
Calcium + R-568	0.1 µM	2.37±0.53	2.45±0.63	2.45±0.80
	1 µM	1.70±0.35#	1.97±0.31#	2.28±0.25
Calcium + S-568	1 µM	2.62±0.36	2.43±0.17	2.53±0.79

*p<0.05, ANOVA. # p<0.03 vs calcium alone, Student t-test. **p<0.05 vs wild type, Student's t-test.

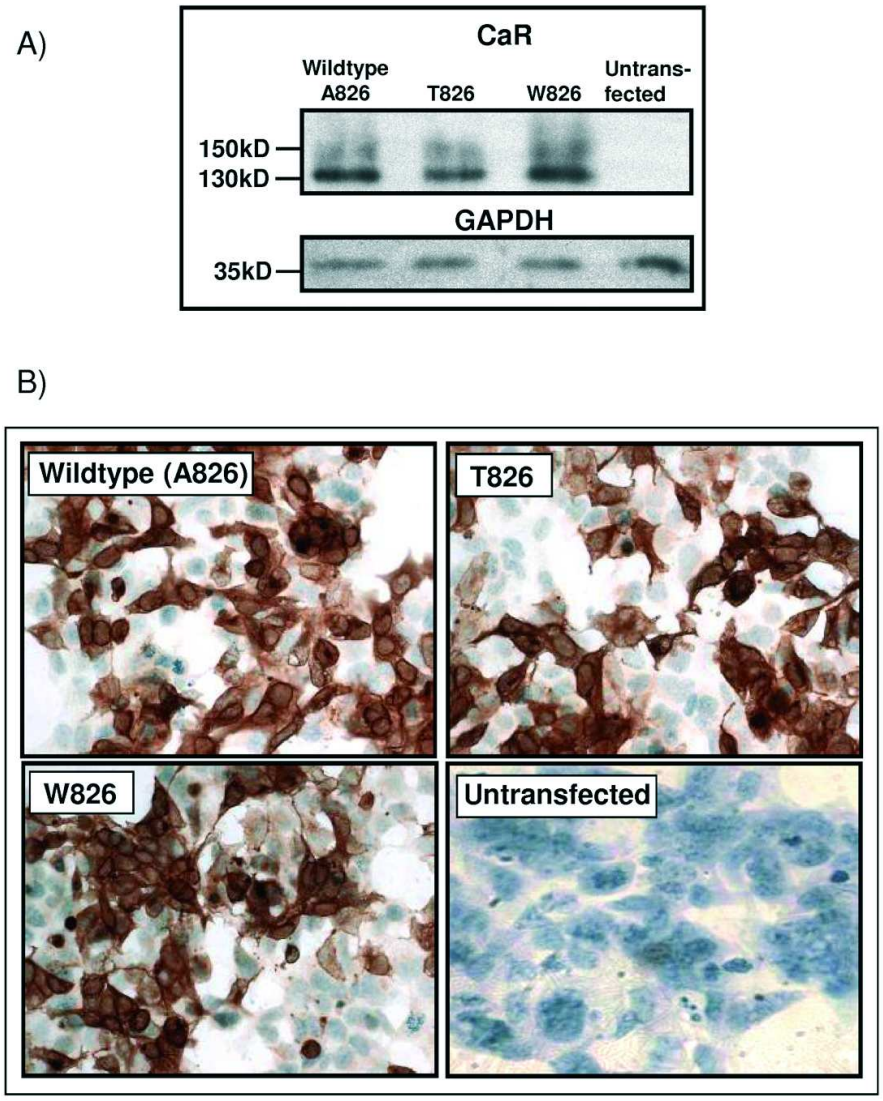

Figure 1: CaR expression. A) Western Blot analysis of CaR content in transiently transfected (wild type, T826 and W826) and non-transfected HEK-293 cells (whole cell extracts). GAPDH was used as a loading control. B) Immunocytochemical staining for CaR in HEK-293 cells transiently transfected with wild-type, T826 (polymorphic allele), and W826 (artificial mutant) CaRs. Haematoxylin counterstaining. Magnification X40.
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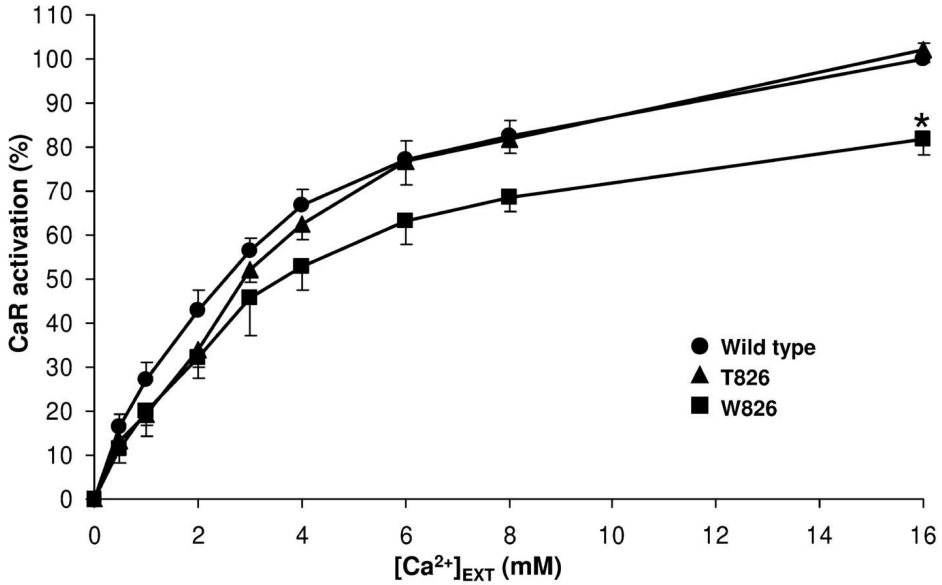

Figure 2: CaR activation in response to extracellular calcium in HEK-293 cells transiently transfected with three different constructs: wild type (circles), T826 polymorphic allele (triangles), and W826 artificial mutant (squares). Fluorescence values were normalized against the maximal wild type receptor activation at 16 mM extracellular calcium. Values represent the mean±standard deviation. Each curve contains 10 replicates with different transfections each one. *p<0.05 compared with the wild type.
64x42mm (600 x 600 DPI)

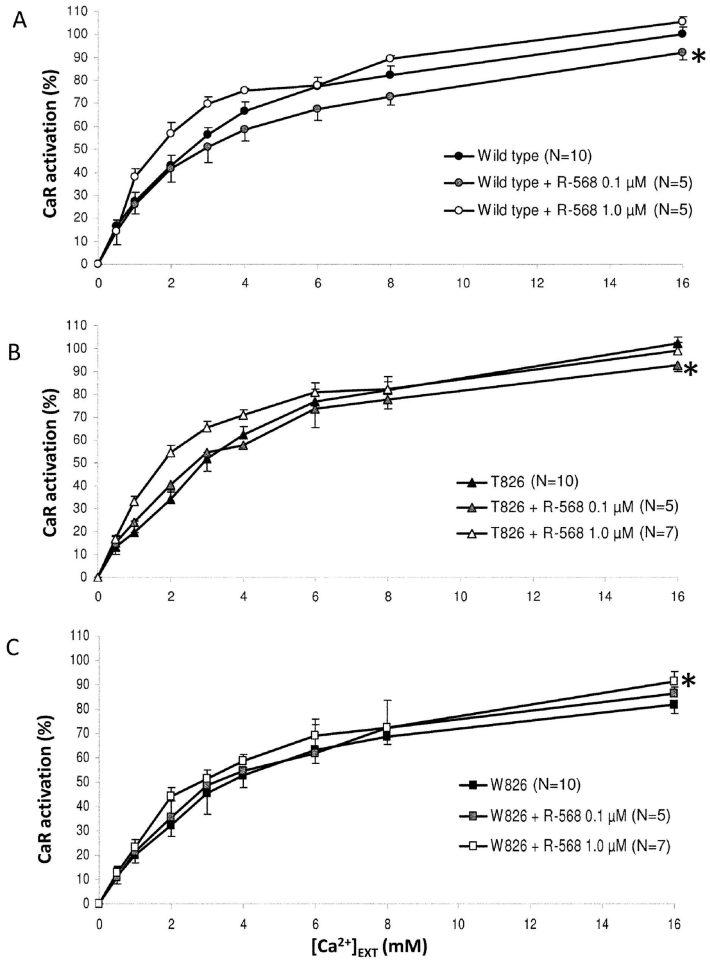

Figure 3: CaR activation in response to extracellular calcium alone or calcium in presence of two different R-568 concentrations (0.1 µM and 1.0 µM) in HEK-293 cells transiently transfected with three different constructs: A) Wild type, B) T826 polymorphic allele, and C) W826 artificial mutant. Fluorescence values were normalized against the maximal wild type receptor activation at 16 mM extracellular calcium alone in all cases. Values represent the mean±standard deviation of at least 5 different experiments from different transfections. *p<0.05 compared with calcium alone. The number of replicates for each curve (different transfections) is shown in parenthesis.
95x134mm (600 x 600 DPI)