


# Identification of Serine-875 as an Inhibitory Phosphorylation Site in the Calcium-Sensing Receptor<sup>SI</sup>

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## ABSTRACT

The calcium-sensing receptor (CaS) is the principal controller of extracellular calcium ( $\text{Ca}_o^{2+}$ ) homeostasis and is inhibited in vitro and in vivo by protein kinase C (PKC)-mediated phosphorylation at  $\text{CaS}^{\text{T888}}$ . However, PKC inhibition enhances signaling even in CaSs lacking Thr-888, suggesting that an additional inhibitory site exists. An apparently equivalent PKC regulatory site in metabotropic glutamate receptor 5 (Ser-839) aligns not with  $\text{CaS}^{\text{T888}}$  but instead with  $\text{CaS}^{\text{S875}}$ , which was not previously considered to be a PKC site.  $\text{CaS}^{\text{S875A}}$  (nonphosphorylatable) exhibited significantly enhanced  $\text{Ca}_o^{2+}$  sensitivity of both intracellular  $\text{Ca}^{2+}$  mobilization and extracellular signal-regulated kinase 1/2 activation, whereas the phosphomimetic  $\text{CaS}^{\text{S875D}}$  mutant exhibited a loss of function. The  $\text{CaS}^{\text{S875A/T888A}}$  double mutant exhibited even greater  $\text{Ca}_o^{2+}$  sensitivity than  $\text{CaS}^{\text{T888A}}$  alone, a response no longer enhanced by PKC inhibition. Finally, when expressed in CaS

lacking its extracellular domain, the  $\text{CaS}^{\text{S875A/T888A}}$  double mutation elicited maximal activation even under control conditions, but remained sensitive to negative allosteric modulation [N-(2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl)-1,1-dimethyl-2-(2-naphthyl)ethylamine] or  $\text{Ca}_o^{2+}$  removal. Therefore, we have now identified  $\text{CaS}^{\text{S875}}$  as the missing PKC phosphorylation site that, together with  $\text{CaS}^{\text{T888}}$ , shapes the CaS signaling that underpins  $\text{Ca}_o^{2+}$  homeostasis. Together with the inactive form of the CaS extracellular domain, these sites attenuate  $\text{Ca}_o^{2+}$  sensitivity to attain appropriate physiologic  $\text{Ca}_o^{2+}$  sensing.

## SIGNIFICANCE STATEMENT

Serine-875 represents the missing inhibitory PKC phosphorylation site in CaS that in tandem with Thr-888 controls receptor activity.

## Introduction

The calcium-sensing receptor (CaS) is the principal controller of extracellular calcium ( $\text{Ca}_o^{2+}$ ) homeostasis, suppressing both parathyroid hormone secretion and renal calcium reabsorption in response to high  $\text{Ca}_o^{2+}$  concentration. When first cloned, CaS was predicted to contain five protein kinase C (PKC) consensus sequences: two in the first and third intracellular loops, Thr-646 and Ser-794; and three in the CaS intracellular tail, Thr-888, Ser-895, and Ser-915 (Garrett et al., 1995; Bai et al., 1998). Previous results from this laboratory and others (Bai et al., 1998; Davies et al., 2007; Young et al., 2014) have shown that one of these residues,  $\text{CaS}^{\text{T888}}$ , represents the key phosphorylation site responsible for PKC-mediated inhibition of CaS-mediated intracellular  $\text{Ca}^{2+}$  ( $\text{Ca}_i^{2+}$ ) mobilization in vitro. In humans, mutation of  $\text{CaS}^{\text{T888}}$  to a nonphosphorylatable methionine produces a

gain-of-function CaS, resulting in autosomal dominant hypocalcaemia (Lazarus et al., 2011). Bai et al. (1998) reported that while the other four known PKC sites play little or no role in mediating PKC's functional effect,  $\text{CaS}^{\text{T888}}$  cannot be the sole determinant of the PKC effect. Furthermore, in the current laboratory, it was shown that  $\text{CaS}^{\text{T888A}}$  still causes  $\text{Ca}_i^{2+}$  oscillations in some cells (at least in the presence of 2 mM  $\text{Ca}_o^{2+}$ ), and PKC activation with the phorbol ester phorbol 12-myristate 13-acetate (PMA) elicits a partial inhibitory effect in  $\text{CaS}^{\text{T888A}}$  (Davies et al., 2007) and  $\text{CaS}^{\text{T888M}}$  (Lazarus et al., 2011) expressing cells. Together, these findings have suggested the existence of an additional PKC site in CaS.

The idea of PKC-mediated inhibition of class C G protein-coupled receptors (GPCRs) came initially not from CaS but from the structurally homologous metabotropic glutamate receptor 5 (mGluR5) (Dale et al., 2001; Hermans and Challiss, 2001), with Ser-839 being shown to be the most likely mediator of the PKC response (Kim et al., 2005). Indeed, mutation of Ser-839 in mGluR5 to alanine (mGluR5<sup>S839A</sup>) was shown to prevent  $\text{Ca}_i^{2+}$  oscillations in HeLa cells (Kim et al., 2005). Interestingly, mGluR5<sup>S839</sup> does not align with  $\text{CaS}^{\text{T888}}$  or indeed with any of the known PKC consensus sequences in the CaS. Instead, it aligns with  $\text{CaS}^{\text{S875}}$ , a residue not

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**ABBREVIATIONS:**  $\text{Ca}_i^{2+}$ , intracellular  $\text{Ca}^{2+}$ ;  $\text{Ca}_o^{2+}$ , extracellular  $\text{Ca}^{2+}$ ; CaS, calcium-sensing receptor;  $\text{CaS}^{\text{WT}}$ , wild-type calcium-sensing receptor; ECD, extracellular domain; ERK1/2, extracellular signal-regulated kinase 1/2; GFX, GF109203X (bisindolylmaleimide I); GPCR, G protein-coupled receptor; HEK-293, human embryonic kidney 293; ICD, intracellular domain, mGluR5, metabotropic glutamate receptor 5; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TMD, transmembrane domain.

previously considered likely to be a PKC site. In support of this idea, Huttlin et al. (2010) reported murine phospho-proteomic data that included evidence of phosphorylation at CaS<sup>S875</sup>. Furthermore, while only five CaS residues were originally proposed to be PKC consensus sequences (Garrett et al., 1995), the NetPhos database (NetPhos 3.1 Server; <http://www.cbs.dtu.dk/services/NetPhos-3.1/>) predicts CaS as having as many as 15 potential PKC sites, including both CaS<sup>S875</sup> and CaS<sup>T888</sup>.

Therefore, the aim of this study was to evaluate the effect of mutating Ser-875 on CaS-mediated signaling to assess whether it likely represents the missing PKC site (Bai et al., 1998) in the intracellular domain (ICD). This was investigated both in wild-type CaS (CaS<sup>WT</sup>) and also in a CaS construct (Rho-C-hCaS) lacking most of the extracellular domain [ECD], residues 1–599], thus leaving just the seven-transmembrane domain (TMD) and N-terminal ICD as described previously (Zhao et al., 1999).

## Materials and Methods

**Cell Culture.** Human embryonic kidney 293 (HEK-293) cells transfected with CaS<sup>WT</sup> were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, Gillingham, UK). To avoid cell death, gain-of-function mutant cell lines were routinely cultured in low Ca<sup>2+</sup> RPMI (containing 0.42 mM CaCl<sub>2</sub>) instead of Dulbecco's modified Eagle's medium (containing 1.8 mM CaCl<sub>2</sub>).

**Mutagenesis.** Mutations were introduced into the wild-type human parathyroid CaS by site-directed mutagenesis using the QuickChange lightning site-directed mutagenesis kit in accordance with the manufacturer's instructions (Agilent Technologies Ltd., Cheadle, UK). HEK-293 cells were then transiently transfected with wild-type or mutant receptors using FuGENE6 (Promega, Southampton, UK). For stable expression, vectors were linearized prior to transfection and the resulting CaS-expressing cells were selected using Hygromycin (Duchefa Biochemie). Clonal cell lines were then established. The Rho-C-hCaS mutant was obtained from R. Mun (The Charles Perkins Centre, The University of Sydney, Camperdown, Australia) having been initially generated by Zhao et al. (1999). All mutations were subsequently verified by the DNA sequencing facility at The University of Manchester.

**Immunoblotting.** CaS expression was demonstrated by immunoblotting as described previously (Ward et al., 1998) using an anti-CaS mouse monoclonal antibody (ADD; amino acids 214–235 of human CaS; Fisher Scientific, Loughborough, UK). In brief, cells were lysed on ice in a detergent-containing HEPES buffer supplemented with protease inhibitors and 1 mM *N*-ethylmaleimide. The lysate was centrifuged at 12,000g (for 10 minutes at 4°C) and the supernatant was solubilized in Laemmli buffer at 65°C.

**Intracellular Calcium Imaging.** CaS-induced Ca<sub>i</sub><sup>2+</sup> mobilization was assayed by epifluorescence microscopy as described previously (Davies et al., 2007) after loading cells with Fura-2 AM (Life Technologies Ltd., Paisley, UK). CaS-transfected cells were exposed to increasing concentrations of Ca<sub>o</sub><sup>2+</sup> in experimental buffer [20 mM HEPES (pH 7.4), 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 5.5 mM glucose] at room temperature to compare relative Ca<sub>o</sub><sup>2+</sup> sensitivities between the receptors. The coverslips were mounted in a perfusion chamber (Warner Instruments, Hamden, CT) and fluorescence visualized using a Nikon Diaphot inverted microscope equipped with a digital camera charge-coupled device.

**Extracellular Signal-Regulated Kinase 1/2 Phosphorylation.** Extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in CaS-transfected cells was assayed as described previously (Ward et al., 2002). In brief, cells were exposed to various concentrations of Ca<sub>o</sub><sup>2+</sup> in experimental buffer at 37°C for 10 minutes prior to lysis on ice

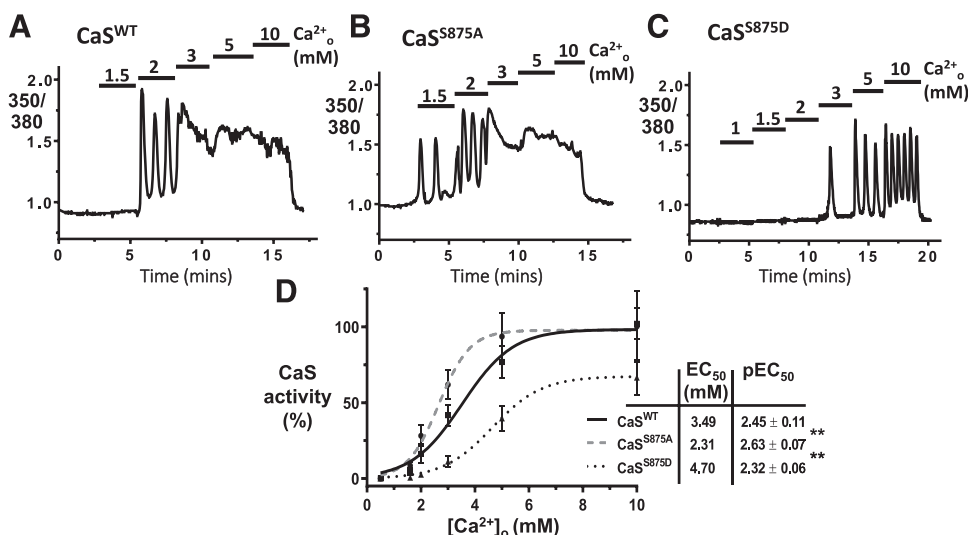
in detergent-containing buffer supplemented with protease and phosphatase inhibitors. ERK1/2 phosphorylation was then determined by semiquantitative immunoblotting using a phosphospecific antibody (Cell Signaling Technology).

**Data and Statistical Analysis.** The data are presented as mean ± S.D. (for pEC<sub>50</sub> variance; shown as insets in the graphs) or mean ± S.E.M. (for precision of the individual responses, shown graphically). Statistical significance between pEC<sub>50</sub> values (*P* values < 0.05) was determined by Student's unpaired/paired *t* test or one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons test as appropriate (GraphPad Prism version 7). For the Ca<sub>i</sub><sup>2+</sup> assay, the area under the curve of the Fura-2 ratios (MetaFluor) for all cells in a field of view over a given time range was calculated using GraphPad Prism (version 7) with resulting curves produced using a sigmoidal dose-response (variable slope) equation.

## Results

**Effect of CaS<sup>S875A</sup> and CaS<sup>S875D</sup> Phospho-Mutations on Ca<sub>o</sub><sup>2+</sup>-Induced Ca<sub>i</sub><sup>2+</sup> Mobilization.** The key inhibitory PKC site in mGluR5 (mGluR5<sup>S839</sup>) aligns in the CaS not with the recognized inhibitory PKC site CaS<sup>T888</sup> (Bai et al., 1996; Davies et al., 2007) but instead with CaS<sup>S875</sup> (Supplemental Fig. 1), which has not been previously considered to be a PKC site (Garrett et al., 1995; Bai et al., 1996). Thus, to examine the potential role of CaS<sup>S875</sup> phosphorylation on receptor signaling, this residue was mutated either to a nonphosphorylatable alanine residue (CaS<sup>S875A</sup>) or to a phosphomimetic aspartic acid residue (CaS<sup>S875D</sup>) in CaS<sup>WT</sup>. Subsequently, both mutated receptors were transiently transfected into HEK-293 cells, and CaS-induced Ca<sub>i</sub><sup>2+</sup> mobilization was measured in response to increasing concentrations of Ca<sub>o</sub><sup>2+</sup> (0.5–10 mM). As shown in Fig. 1, CaS<sup>S875A</sup> was a gain-of-function mutation showing enhanced receptor sensitivity to Ca<sub>o</sub><sup>2+</sup> relative to CaS<sup>WT</sup> (EC<sub>50</sub>, 2.3 CaS<sup>S875A</sup> vs. 3.5 mM CaS<sup>WT</sup>; *P* < 0.01). A similar gain of function was seen in HEK-293 cells stably expressing the CaS<sup>S875A</sup> mutation (Supplemental Figs. 2 and 3). In contrast, CaS<sup>S875D</sup> exhibited decreased Ca<sub>o</sub><sup>2+</sup> sensitivity relative to CaS<sup>WT</sup>, indicating that it is a loss-of-function mutation (EC<sub>50</sub>, 4.7 CaS<sup>S875D</sup> vs. 3.5 mM CaS<sup>WT</sup>; *P* < 0.01) (Fig. 1).

**CaS<sup>T888A</sup> and CaS<sup>S875A</sup> Mutations Enhance Ca<sub>o</sub><sup>2+</sup>-Induced ERK1/2 Phosphorylation.** The relative responses of CaS<sup>T888A</sup> and CaS<sup>S875A</sup> on Ca<sub>o</sub><sup>2+</sup>-stimulated ERK1/2 phosphorylation were next compared with CaS<sup>WT</sup> control responses using HEK-293 cells expressing each receptor stably. In agreement with the Ca<sub>i</sub><sup>2+</sup> mobilization data, CaS<sup>S875A</sup> enhanced Ca<sub>o</sub><sup>2+</sup>-induced phosphorylated ERK1/2 activation relative to CaS<sup>WT</sup> control (EC<sub>50</sub>, 1.9 CaS<sup>S875A</sup> vs. 3.8 mM CaS<sup>WT</sup>; *P* < 0.01) (Fig. 2). A similar gain of function was seen, as expected, in CaS<sup>T888A</sup>-expressing cells (EC<sub>50</sub>, 2.2 CaS<sup>T888A</sup> vs. 4.4 mM CaS<sup>WT</sup>; *P* < 0.01). Equal protein loading on the blots was confirmed by uniform β-actin expression. An equivalent gain of function for Ca<sub>i</sub><sup>2+</sup> mobilization by the CaS<sup>T888A</sup> stably expressing cells is shown in Supplemental Fig. 4. There is evidence that forward trafficking of the CaS can be modulated by its association with 14-3-3 protein under the control of CaS<sup>S899</sup> phosphorylation (Grant et al., 2011, 2015). As such, it is important to consider whether CaS<sup>S875</sup> phosphorylation affects functional signaling directly or merely as a determinant of cell surface localization. To examine this, HEK-293 cells were transiently transfected with CaS<sup>WT</sup>, CaS<sup>S875A</sup>, and CaS<sup>T888A</sup>, and the membrane localization of



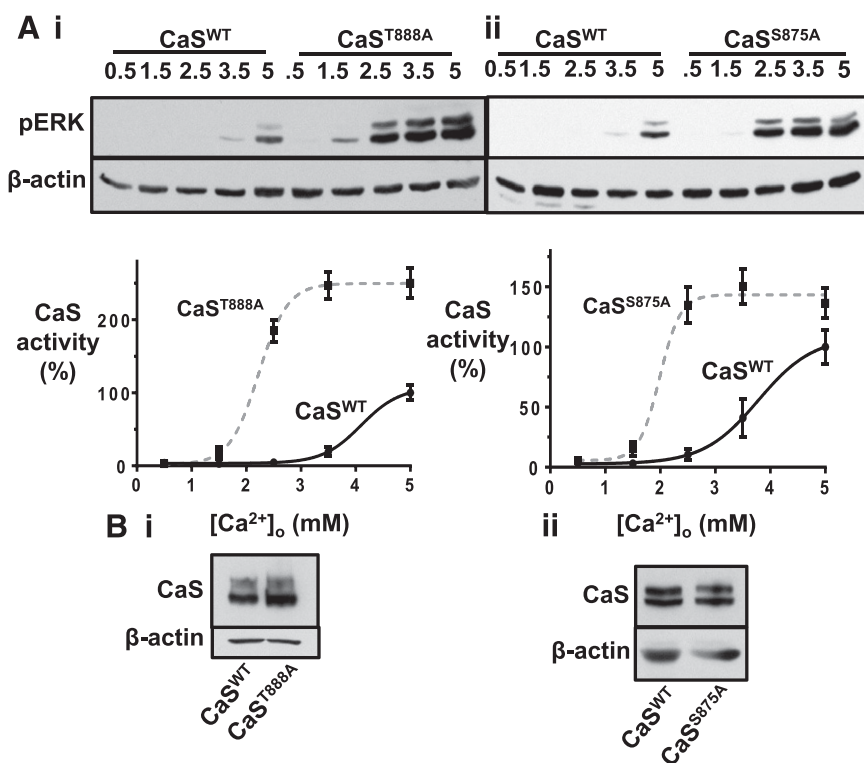
**Fig. 1.** CaS<sup>S875</sup> acts as an inhibitory phosphorylation site. Fura-2-loaded HEK-293 cells were transiently transfected with either (A) CaS<sup>WT</sup> (B) CaS<sup>S875A</sup> or (C) CaS<sup>S875D</sup>, and then exposed to increasing Ca<sub>o</sub><sup>2+</sup> concentrations (0.5–10 mM) to determine the effect of the putative phosphorylation site CaS<sup>S875</sup> on Ca<sub>o</sub><sup>2+</sup> mobilization. The representative traces show the Ca<sub>o</sub><sup>2+</sup> changes (Fura-2 ratio) in single cells from the field of view. The whole field-of-view changes in Ca<sub>o</sub><sup>2+</sup> concentration are shown as concentration-effect curves in (D). \*\**P* < 0.01 vs. CaS<sup>WT</sup> by one-way ANOVA with Dunnett's post hoc test. Data are representative of three independent experiments (*n* ≥ 6 coverslips).

these receptors was then analyzed using a surface biotinylation assay. As shown in Supplemental Fig. 5 we saw no evidence of substantive changes in cell surface localization in receptors lacking Ser-875 or Thr-888. However, taken together these observations confirm that CaS<sup>S875A</sup> is a gain-of-function mutation, with a similar impact on Ca<sub>o</sub><sup>2+</sup>-stimulated signaling responses to CaS<sup>T888A</sup>.

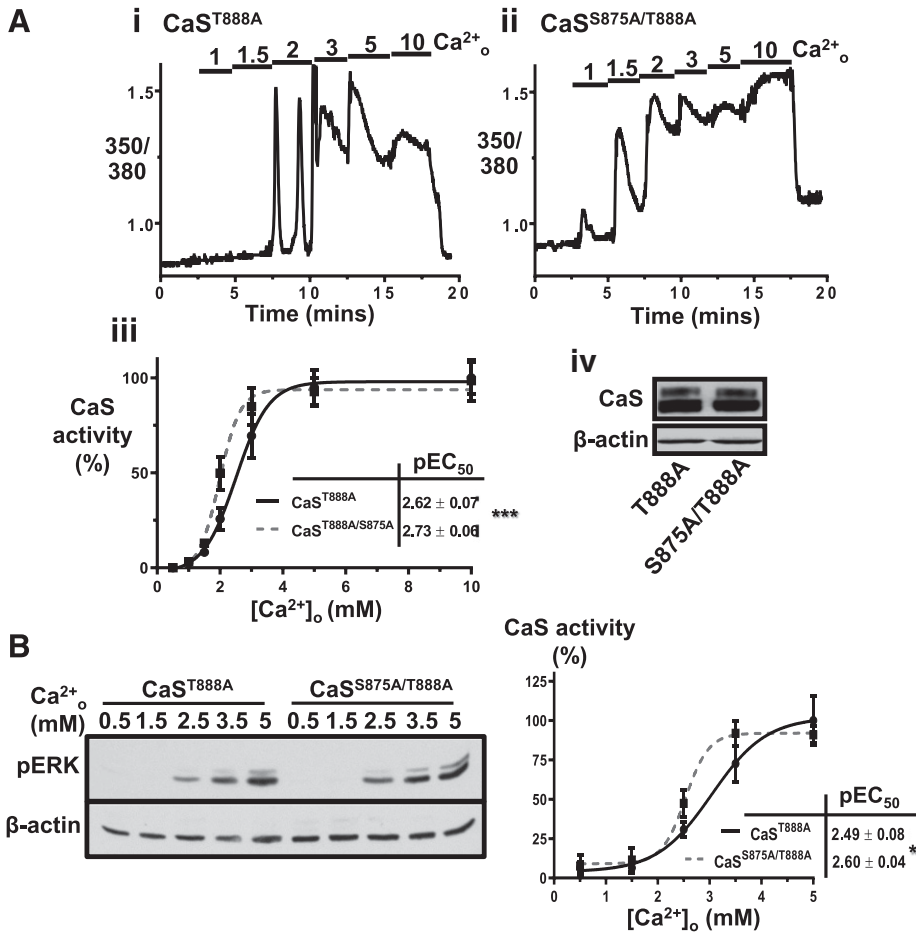
**Effect of CaS<sup>S875A/T888A</sup> Double Mutation on CaS Signaling.** Since both CaS<sup>S875A</sup> and CaS<sup>T888A</sup> exhibited gain of function by two different experimental readouts, it was next decided to introduce both mutations in the same receptor to test whether their effects are additive. This question was asked since Bai et al. (1996) found that PKC inhibition could further enhance CaS<sup>T888A</sup> signaling, suggesting that another

PKC site may contribute additional receptor modulation. The resulting CaS<sup>S875A/T888A</sup> double mutant was tested for its effect on Ca<sub>i</sub><sup>2+</sup> mobilization as previously detailed in both transiently (Supplemental Fig. 6) and stably transfected (Fig. 3) HEK-293 cells. In both cases, the doubly mutated CaS<sup>S875A/T888A</sup> exhibited significantly lower EC<sub>50</sub> values and increased sensitivity for Ca<sub>o</sub><sup>2+</sup> than for the CaS<sup>T888A</sup> mutant (stable, 1.8 CaS<sup>S875A/T888A</sup> vs. 2.4 mM CaS<sup>T888A</sup>; *P* < 0.01) (Fig. 3). It was further noted that the CaS<sup>S875A/T888A</sup> double mutant completely abolished Ca<sub>o</sub><sup>2+</sup> oscillations, which CaS<sup>T888A</sup> failed to do at lower Ca<sub>o</sub><sup>2+</sup> concentrations.

Similar to its effect on Ca<sub>i</sub><sup>2+</sup> mobilization, the CaS<sup>S875A/T888A</sup> double mutant also increased Ca<sub>o</sub><sup>2+</sup> sensitivity of ERK1/2 phosphorylation compared with CaS<sup>T888A</sup> alone (EC<sub>50</sub>, 2.5 vs.



**Fig. 2.** CaS<sup>T888A</sup> and CaS<sup>S875A</sup> mutations increase CaS-induced ERK1/2 phosphorylation. (A) HEK-293 cells were stably transfected with either CaS<sup>WT</sup>, CaS<sup>T888A</sup> (i) or CaS<sup>S875A</sup> (ii), and then stimulated with various Ca<sub>o</sub><sup>2+</sup> concentrations (0.5–5 mM) for 10 minutes to determine the effect of mutating the two phosphorylation sites on extracellular signal-regulated kinase (pERK) activation. Representative western blots indicating ERK1/2 phosphorylation, together with β-actin loading control, are shown above the resulting concentration-effect curves in each graph. ERK1/2 responses are expressed as a percentage of CaS<sup>WT</sup> maximal response in each experiment. \*\**P* < 0.01 CaS<sup>T888A</sup> vs. CaS<sup>WT</sup> (*n* = 6 from three independent experiments) and CaS<sup>S875A</sup> vs. CaS<sup>WT</sup> (*n* = 7 from three independent experiments) pEC<sub>50</sub> values by unpaired *t* test, *n* ≥ 8 from three independent experiments. (B) CaS immunoblots showing similar receptor abundance between cell lines, with their protein loading equivalence confirmed by β-actin expression.



**Fig. 3.** CaS<sup>S875A/T888A</sup> double mutant is more sensitive to Ca<sub>o</sub><sup>2+</sup> than CaS<sup>T888A</sup> alone. (A) Fura-2-loaded HEK-293 cells stably transfected with either CaS<sup>T888A</sup> (i) or CaS<sup>S875A/T888A</sup> (ii) were stimulated with increasing Ca<sub>o</sub><sup>2+</sup> concentrations (0.5–10 mM). The two panels show Ca<sub>i</sub><sup>2+</sup> traces from representative single cells. The resulting concentration-effect curves are shown in (iii). \*\**P* < 0.01 for CaS<sup>T888A</sup> vs. CaS<sup>S875A/T888A</sup> by unpaired *t* test; *n* ≥ 9 coverslips from three independent experiments. Equivalence of CaS abundance between cell lines is shown in (iv), with β-actin loading control. (B) Representative western blots showing ERK1/2 phosphorylation and β-actin immunoreactivity in the same cell lines as before, stimulated with various Ca<sub>o</sub><sup>2+</sup> concentrations (0.5–5 mM) for 10 minutes. The resulting concentration-effect relationship is shown on the right. ERK1/2 responses are expressed as a percentage of CaS<sup>T888A</sup> maximal response in each experiment. \**P* < 0.05 EC<sub>50</sub> values for CaS<sup>T888A</sup> vs. CaS<sup>S875A/T888A</sup> by unpaired *t* test; *n* ≥ 6 from three independent experiments.

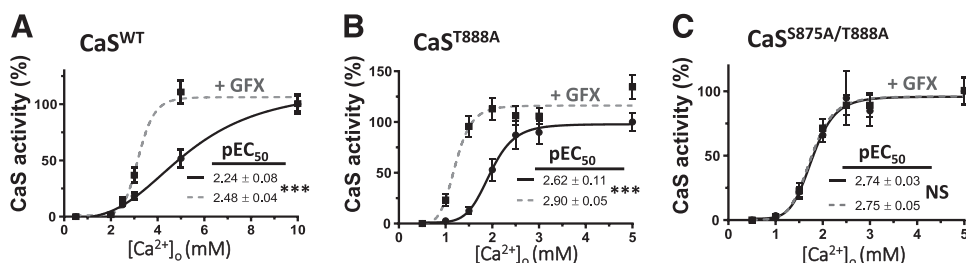
3.2 respectively; *P* < 0.05) (Fig. 3B), further demonstrating the additive effect of the two mutations. Overall, these observations confirm that the double mutant receptor CaS<sup>S875A/T888A</sup> was significantly more sensitive to Ca<sub>o</sub><sup>2+</sup> than CaS<sup>T888A</sup> alone with respect to both Ca<sub>i</sub><sup>2+</sup> mobilization and ERK1/2 activation.

To confirm that stable transfection with the double mutant receptor CaS<sup>S875A/T888A</sup> had not increased Gα<sub>q</sub>-mediated Ca<sub>i</sub><sup>2+</sup> mobilization in a nonspecific manner, the effect of carbachol on Ca<sub>i</sub><sup>2+</sup> mobilization was also tested. Carbachol also elicits oscillatory Ca<sub>i</sub><sup>2+</sup> mobilization in wild-type HEK-293 cells (Supplemental Fig. 7i), most likely via the muscarinic acetylcholine receptor (Atwood et al., 2011). However, the CaS<sup>S875A/T888A</sup> double mutant failed to increase carbachol sensitivity for Ca<sub>i</sub><sup>2+</sup> mobilization, suggesting that CaS<sup>S875A/T888A</sup> gain of function is not due to a nonspecific increase in Gα<sub>q</sub>-mediated Ca<sub>i</sub><sup>2+</sup> mobilization or an artifact of transfection (Supplemental Fig. 7, ii–iv). Indeed, carbachol responsiveness was inhibited in cells expressing CaS<sup>S875A/T888A</sup> (EC<sub>50</sub>, 7.5 vs. 3.2 μM CaS<sup>WT</sup>; *P* < 0.01). The cause of this reduced carbachol responsiveness was not investigated further.

**PKC Inhibition Fails to Further Enhance CaS<sup>S875A/T888A</sup> Signaling.** Having confirmed that the double mutant CaS<sup>S875A/T888A</sup> exhibits significantly greater Ca<sub>o</sub><sup>2+</sup> responsiveness than for CaS<sup>T888A</sup> alone, we next tested whether PKC inhibition could elicit additional signal enhancement suggestive of yet further PKC sites. For this, CaS<sup>S875A/T888A</sup> stably expressing cells were treated with or without bisindolylmaleimide

I [GF109203X (GFX), 250 nM], a nonspecific PKC inhibitor, for 30 minutes, and then co-stimulated with increasing concentrations of Ca<sub>o</sub><sup>2+</sup> (0.5–5 mM), with Ca<sub>i</sub><sup>2+</sup> mobilization assayed as previously described (Fig. 4). For comparison, CaS<sup>WT</sup> and CaS<sup>T888A</sup> cells were tested alongside one another in these experiments. As expected, GF109203X treatment significantly increased the sensitivity of both CaS<sup>WT</sup> (EC<sub>50</sub>; 5.6 control vs. 3.3 mM GFX; *P* < 0.001) and CaS<sup>T888A</sup> (EC<sub>50</sub>; 2.4 control vs. 1.2 mM GFX; *P* < 0.001) for Ca<sub>o</sub><sup>2+</sup>. However, there was no difference in CaS-induced Ca<sub>i</sub><sup>2+</sup> mobilization between CaS<sup>S875A/T888A</sup> double mutants treated without or with GF109203X; that is, they both increased CaS sensitivity to a virtually identical extent (EC<sub>50</sub>, 1.8 control vs. 1.7 mM GFX; *P* = 0.86). These data suggest that the positive effect of PKC inhibition can be replaced entirely by alanine mutation of both inhibitory phosphorylation sites, Ser-875 and Thr-888, thus indicating that Ser-875 is another PKC site.

**Effect of CaS<sup>T888A</sup> and CaS<sup>S875A</sup> Mutations on Rho-C-hCaS-Induced Ca<sub>i</sub><sup>2+</sup> Mobilization.** Having established the dual inhibitory effect of CaS<sup>T888A</sup> and CaS<sup>S875A</sup> on CaS signaling, the role of both phosphorylation sites was next tested in the context of a CaS construct lacking the entire ECD (Rho-C-hCaS) to assess their inhibitory effects on the TMD core of the receptor. The hypothesis was that if the ECD of the CaS is responsible for ligand-dependent activation (Bräuner-Osborne et al., 1999; Geng et al., 2016; Zhang et al., 2016), then relieving the PKC-mediated inhibitory constraints on the receptor might result in ligand-independent



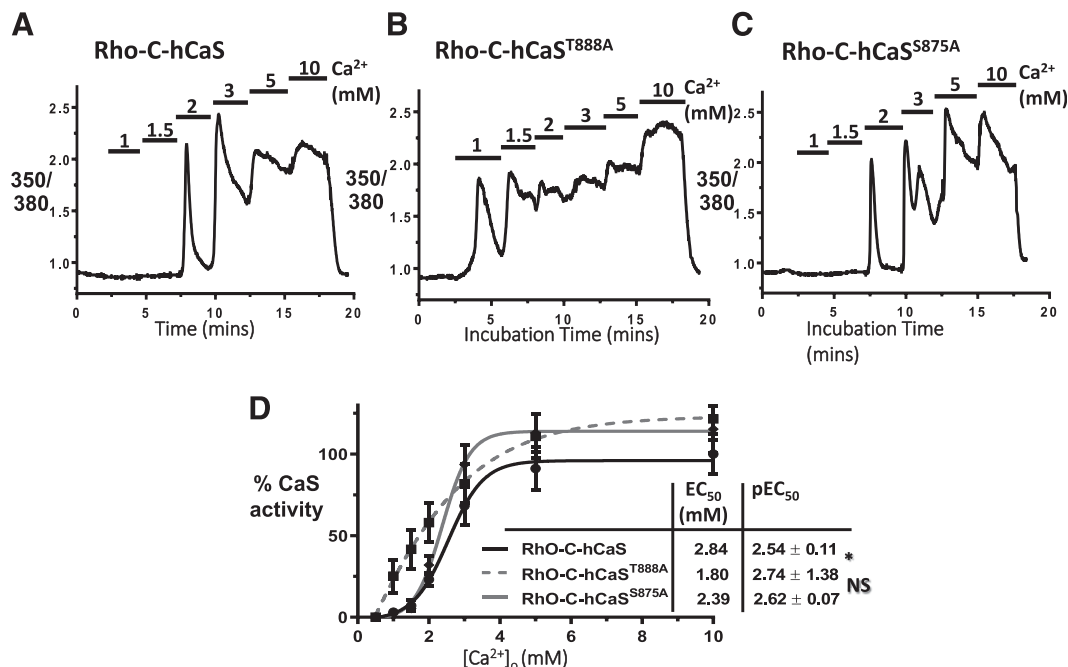
**Fig. 4.** PKC inhibition does not further enhance CaS<sup>S875A/T888A</sup> responses. Fura-2-loaded HEK-293 cells stably transfected with either CaS<sup>WT</sup> (A), CaS<sup>T888A</sup> (B), or CaS<sup>S875A/T888A</sup> (C) were stimulated with increasing concentrations of Ca<sub>o</sub><sup>2+</sup> in the absence or presence of the PKC inhibitor GF109203X (GFX, 250 nM). The concentration-effect curves show significantly enhanced Ca<sub>o</sub><sup>2+</sup> sensitivity following GF109203X treatment in CaS<sup>WT</sup> and CaS<sup>T888A</sup> cells but not in CaS<sup>S875A/T888A</sup> double mutant cells. \*\*\**P* < 0.001, NS, not significant for control vs. GF109203X by unpaired *t* test. *n* ≥ 6 coverslips from two independent experiments.

activation of the CaS. In such a case, mutation of the inhibitory phosphorylation sites might enhance responsiveness even in a headless CaS.

Rho-C-hCaS lacks most of the ECD (residues 1–599); therefore, it contains only the 7TMD and the carboxyl-terminal ICD with a stop codon at residue 903 as described previously (Zhao et al., 1999) (Supplemental Fig. 8A). To facilitate cell surface expression, the start of this truncated CaS was fused to 20 amino acid residues of the N-terminus of bovine rhodopsin (MNGTEGPNFYVPFSNKTGVV). First, Rho-C-hCaS was transiently transfected into HEK-293 cells, and then the effect of increasing Ca<sub>o</sub><sup>2+</sup> concentrations (up to 10 mM) on Ca<sub>i</sub><sup>2+</sup> mobilization was assayed as previously described. In contrast to CaS<sup>WT</sup>, which elicits oscillatory signaling, Rho-C-hCaS elicited only transient or sustained Ca<sub>i</sub><sup>2+</sup> mobilization (Supplemental Fig. 8B). It should be noted that no Ca<sub>i</sub><sup>2+</sup> mobilization was seen under these conditions in nontransfected HEK-293 cells (data not shown). Moreover, the

effect of PKC inhibition on Rho-C-hCaS signaling was tested. For this, HEK-293 cells transiently transfected with Rho-C-hCaS were first exposed to 3 mM Ca<sub>o</sub><sup>2+</sup> to elicit Ca<sub>i</sub><sup>2+</sup> mobilization and then cotreated with 250 nM of GF102903X. Indeed, GF102903X enhanced Rho-C-hCaS-induced Ca<sub>i</sub><sup>2+</sup> mobilization in response to 3 mM Ca<sub>o</sub><sup>2+</sup> (*P* < 0.05) (Supplemental Fig. 8C).

Next, it was examined whether mutating Thr-888 and/or Ser-875 to alanine can also increase Rho-C-hCaS responsiveness. Compared with the Rho-C-hCaS control, the Rho-C-hCaS<sup>T888A</sup> mutant was more sensitive to Ca<sub>o</sub><sup>2+</sup> (EC<sub>50</sub>, 1.8 Rho-C-hCaS<sup>T888A</sup> vs. 2.8 mM Rho-C-hCaS; *P* < 0.05) (Fig. 5A), similar to the effect of T888A in the full-length CaS (Supplemental Fig. 4). In contrast, Rho-C-hCaS<sup>S875A</sup> did not exhibit significantly enhanced receptor responsiveness compared with Rho-C-hCaS (EC<sub>50</sub>, 2.4 Rho-C-hCaS<sup>S875A</sup> vs. 2.8 mM Rho-C-hCaS; *P* = 0.18). Therefore, when expressed alone in the headless receptor, only T888A exhibits greater Ca<sub>o</sub><sup>2+</sup> sensitivity.



**Fig. 5.** T888A and S875A mutations enhance Ca<sub>i</sub><sup>2+</sup> mobilization of the ECD-deleted mutant, Rho-C-hCaS. Fura-2-loaded human embryonic kidney cells were transiently transfected with either Rho-C-hCaS (A), Rho-C-hCaS<sup>T888A</sup> (B), or Rho-C-hCaS<sup>S875A</sup> (C) mutants, and then stimulated with increasing Ca<sub>o</sub><sup>2+</sup> concentrations (0.5–10 mM). Representative Ca<sub>i</sub><sup>2+</sup> traces are shown. The resulting concentration-effect curves are shown in (D). \**P* < 0.05, Rho-C-hCaS vs. Rho-C-hCaS<sup>T888A</sup>, NS, not significant, Rho-C-hCaS vs. Rho-C-hCaS<sup>S875A</sup> by one-way ANOVA with Dunnett's post hoc test; *n* ≥ 16 coverslips from five independent experiments.

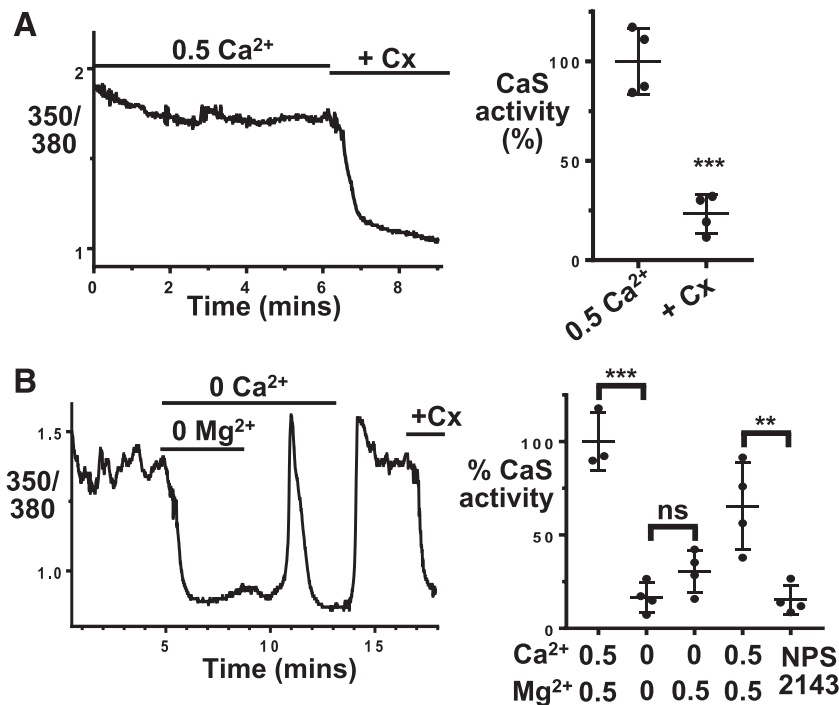
**Effect of Rho-C-hCaS<sup>S875A/T888A</sup> Double Mutation on Ca<sub>i</sub><sup>2+</sup> Mobilization.** Finally, despite the S875A mutation having no significant effect on Rho-C-hCaS responsiveness on its own, we investigated whether this mutation may potentiate the enhanced responsiveness seen with Rho-C-hCaS<sup>T888A</sup> when expressed in combination. Indeed, the Rho-C-hCaS<sup>S875A/T888A</sup> double mutant produced maximal Ca<sub>i</sub><sup>2+</sup> mobilization even under baseline conditions (0.5 mM Ca<sub>o</sub><sup>2+</sup>). However, this effect could be virtually abolished by cotreatment with the CaS negative allosteric modulator (calcilytic) *N*-(2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl)-1,1-dimethyl-2-(2-naphthyl)ethylamine (1 μM; *P* < 0.001) (Fig. 6B). To determine whether the Rho-C-hCaS<sup>S875A/T888A</sup> double mutant is constitutively active, i.e., elicits continuous signaling even in the absence of an agonist, Ca<sub>i</sub><sup>2+</sup> mobilization was tested using a buffer that was nominally free of Ca<sup>2+</sup> or Mg<sup>2+</sup>. As shown in Fig. 6, the Rho-C-hCaS<sup>S875A/T888A</sup>-induced Ca<sub>i</sub><sup>2+</sup> mobilization elicited in control buffer (0.5 mM Ca<sup>2+</sup>) was almost abolished upon removal of Ca<sub>o</sub><sup>2+</sup> and extracellular Mg<sup>2+</sup> (*P* < 0.001). Interestingly, introduction of 0.5 mM Mg<sup>2+</sup> alone was sufficient to elicit cellular Ca<sub>i</sub><sup>2+</sup> transients in some coverslips. Subsequent introduction of 0.5 mM Ca<sup>2+</sup> restored maximal activation of the Rho-C-hCaS<sup>S875A/T888A</sup> double mutant. Thus, although Rho-C-hCaS<sup>S875A/T888A</sup> was not constitutively active, it exhibited substantially enhanced sensitivity to Ca<sub>o</sub><sup>2+</sup> (i.e., with a presumed EC<sub>50</sub> < 0.5 mM) despite the absence of an ECD. Together, these data demonstrate the importance of PKC-mediated Ser-875 and Thr-888 phosphorylation in the control of CaS signaling.

## Discussion

Initial sequence analysis of the human CaS predicted that only five of the intracellular serine/threonine residues were likely to be PKC phosphorylation sites: two in the first and

third intracellular loops (Thr-646 and Ser-794) and three in the ICD (Thr-888, Ser-895, and Ser-915) of the receptor (Garrett et al., 1995; Bai et al., 1998). Previous results have shown that one of these, CaS<sup>T888</sup>, represents the key phosphorylation site responsible for PKC-mediated inhibition of CaS-mediated Ca<sub>i</sub><sup>2+</sup> mobilization (Bai et al., 1998; Davies et al., 2007; Young et al., 2014). However, it should be noted that the human CaS has in fact 54 serine and threonine residues in either its ICD or three intracellular loops, although no tyrosine residues (Garrett et al., 1995). The NetPhos database predicts that 40 of these sites reach the threshold for being potential phosphorylation sites for at least one out of a panel of 17 protein kinases (<http://www.cbs.dtu.dk/services/NetPhos-3.1/>). Interestingly, all of the serine/threonine residues in the juxtamembrane region of the ICD (residues 863–920) are predicted to be phosphorylation sites for one protein kinase or another, whereas in the later carboxyl-terminus few such residues are likely phosphorylation sites. In addition, this database predicts not five PKC sites in the CaS but 15 (including Ser-875), although it should also be noted that these predictions are based on primary sequence and do not take into account site accessibility or location. Nevertheless, current consensus site prediction databases indicate much greater scope for CaS phosphorylation than was considered when CaS was first cloned.

It has been suggested that where GPCRs have a variety of potential kinase consensus sequences, the precise pattern of phosphorylation on any given receptor could be quite different in different cellular contexts. The so-called phospho-barcode hypothesis posits that different patterns of phosphorylation could elicit distinct downstream signaling outcomes (Tobin et al., 2008; Yang et al., 2017). The barcode hypothesis was first devised by studying two GPCRs: the muscarinic acetylcholine receptor (Butcher et al., 2011) and the β<sub>2</sub>-adrenergic receptor (Nobles et al., 2011). The present findings indicate



**Fig. 6.** Effect of CaS<sup>S875A/T888A</sup> double mutation on Rho-C-hCaS. (A) Rho-C-hCaS<sup>S875A/T888A</sup> transfected HEK-293 cells exhibited maximal Ca<sub>i</sub><sup>2+</sup> mobilization even under control conditions (0.5 mM Ca<sub>o</sub><sup>2+</sup>) but was substantially inhibited by calcilytic (Cx) *N*-(2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl)-1,1-dimethyl-2-(2-naphthyl)ethylamine (NPS-2143) (1 μM). \*\*\**P* < 0.001 by paired *t* test. (B) Rho-C-hCaS<sup>S875A/T888A</sup>-induced Ca<sub>i</sub><sup>2+</sup> mobilization was then assayed in the presence or absence of 0.5 mM Ca<sub>o</sub><sup>2+</sup> and 0.5 mM Mg<sub>o</sub><sup>2+</sup> with resulting responses normalized to the 0.5 mM Ca<sub>o</sub><sup>2+</sup> response. \*\**P* < 0.01; \*\*\**P* < 0.001 and NS, not significant by one-way ANOVA with Tukey's post hoc test; *n* ≥ 4 coverslips from two independent experiments.

that the CaS may also exhibit distinct phospho-barcodes in different cell types and/or following exposure to biased agonists or cotreatments with other GPCR agonists.

Consistent with the NetPhos database prediction and the current data, a phospho-proteomic study using mass spectrometry to detect phosphorylation sites in nine different organs from 3-week-old male mice has previously determined CaS<sup>S875</sup> as a phosphorylation site in the kidney (Huttlin et al., 2010). This demonstrates that CaS<sup>S875</sup> is capable of being phosphorylated in vivo. In addition, sequence alignment analysis of the ICDs of the CaS with the structurally homologous mGluR5 confirms that CaS Ser-875 is equivalent to the principal PKC phosphorylation site in mGluR5, namely, Ser-839 (MEGA-X software) (Supplemental Fig. 1A). Therefore, the alignment of CaS<sup>S875</sup> and mGluR5<sup>S839</sup> suggests that they share similar regulatory roles in the modulation of receptor signaling. Moreover, the amino acid conservation of the putative Ser-875 phosphorylation site was examined in the current study across a previously published multiple sequences alignment of 51 different vertebrate CaS species (Herberger and Loretz, 2013) using the free WebLogo analysis website (<https://weblogo.berkeley.edu/logo.cgi>). As reflected by the high WebLogo bit scores (Supplemental Fig. 1B), CaS<sup>S875</sup> is well conserved within different species. Overall, the conservation of Ser-875 is consistent with the phosphorylation site prediction and the strong functional role in CaS signaling. One important consideration regarding these phosphorylation sites is to determine whether they modulate signal transduction directly or instead by altering CaS cell surface localization (Breitwieser, 2013), as is apparently the case for CaS<sup>S899</sup> (Grant et al., 2015). In this regard, we did not see a significant difference in the surface biotinylation of CaS<sup>S875A</sup> or CaS<sup>T888A</sup> versus CaS<sup>WT</sup> receptors. Nevertheless, it would be helpful to have a more thorough analysis of the effect of these and other CaS phospho-site point mutations on receptor maturation, forward trafficking, agonist-driven insertional signaling, internalization, and desensitization. However, in the meantime the current data establish a clear link between residues CaS<sup>S875</sup> and CaS<sup>T888</sup> and CaS downstream signaling.

In agreement with our previous study (Davies et al., 2007), CaS<sup>T888A</sup> elicits more sustained Ca<sub>i</sub><sup>2+</sup> mobilization than CaS<sup>WT</sup>. However, at lower Ca<sub>o</sub><sup>2+</sup> concentrations (1.5–2.5 mM) Ca<sub>i</sub><sup>2+</sup> oscillations continued in some CaS<sup>T888A</sup>-expressing cells. Young et al. (2002) did not observe such oscillations in CaS<sup>T888A</sup>-expressing cells, although only 3 mM Ca<sub>o</sub><sup>2+</sup> was used to stimulate the receptor in that study, and in our experiments 3 mM Ca<sub>o</sub><sup>2+</sup> also elicited only sustained Ca<sub>i</sub><sup>2+</sup> mobilization with CaS<sup>T888A</sup>. Significantly, the persistence of Ca<sub>i</sub><sup>2+</sup> oscillations in CaS<sup>T888A</sup>-transfected cells suggests that this site alone cannot be the exclusive phosphorylation location controlling Ca<sub>i</sub><sup>2+</sup> oscillations (Davies et al., 2007), meaning that an additional signaling determinant is required.

In support of this idea, Bai et al. (1998) previously showed that PKC activation by PMA reduced the responsiveness of a mutant CaS, in which all five predicted PKC sites were eliminated. Moreover, PMA elicited a partial inhibitory effect in cells expressing CaS<sup>T888A</sup> (Davies et al., 2007) and CaS<sup>T888M</sup> (Lazarus et al., 2011). Because PMA increases the phosphorylation of serine/threonine residues, these preserved inhibitory effects could be explained by the presence of an additional, previously unidentified PKC site(s) on the CaS.

As such, the current results indicate that Ser-875 is the previously unidentified PKC site. Specifically, CaS<sup>S875A</sup> with its nonphosphorylatable mutation enhanced signaling, whereas CaS<sup>S875D</sup> with its phosphomimetic mutation inhibited signaling. Furthermore, the CaS<sup>S875A/T888A</sup> double mutation further enhanced both Ca<sub>o</sub><sup>2+</sup>-induced Ca<sub>i</sub><sup>2+</sup> mobilization and ERK1/2 phosphorylation more than for CaS<sup>T888A</sup> alone, whereas concomitant PKC inhibition had no further effect. To prove that CaS<sup>S875</sup> is a PKC site and then determine the ligand sensitivity of such phosphorylation, as was done for CaS<sup>T888</sup> (Davies et al., 2007; McCormick et al., 2010), it will be necessary to raise a phospho-CaS<sup>S875</sup>-specific antibody. However, our initial attempt to generate such an antibody proved unsuccessful, and thus we must rely instead on the mutagenesis studies reported herein, the new consensus predictions described previously, the alignment to mGluR5<sup>S839</sup>, and the previous murine proteomic data (Huttlin et al., 2010). On balance, we would argue that the simplest explanation of the current data is that CaS<sup>S875</sup> is a functionally important PKC site, in conjunction with CaS<sup>T888</sup>. However, the possible involvement of other phosphorylation sites in the regulation of CaS function awaits determination.

That CaS<sup>S875A/T888A</sup> expression failed to enhance carbachol signaling supported the idea that the gain of function was CaS specific and not an artifact of transfection. However, the observation that CaS<sup>S875A/T888A</sup> expression, in fact, inhibited carbachol signaling is interesting. It is proposed that receptors exist in a conformational equilibrium between inactive and active states, and that G proteins have higher affinity for the active state of the receptor (Burstein et al., 1995). Since both CaS and the muscarinic acetylcholine receptor are G<sub>αq</sub> coupled, it seems feasible that they compete for the same G<sub>αq</sub>-protein pool (Linderman, 2009), and thus perhaps G<sub>αq</sub> has sufficiently higher affinity for the hyperactive CaS<sup>S875A/T888A</sup> double mutant, such that fewer G<sub>αq</sub> proteins were available to elicit the carbachol response. This issue requires further study.

Consistent with Mun et al. (2004), the Rho-C-hCaS mutant was sensitive to increasing concentrations of Ca<sub>o</sub><sup>2+</sup>, which further verified the existence of at least one Ca<sub>o</sub><sup>2+</sup>-binding site in the 7TMD of the CaS (Hammerland et al., 1999; Hu et al., 2002; Ray and Northup, 2002). This confirmation of the functional activity of the Rho-C-hCaS mutant allowed the determination of the role of PKC phosphorylation sites in a functionally active CaS that was not subject to control by the ECD. Introducing the mutation T888A to Rho-C-hCaS increased its Ca<sub>o</sub><sup>2+</sup> sensitivity to that seen for the full-length CaS<sup>T888A</sup>. Although Rho-C-hCaS<sup>S875A</sup> did not, on its own, enhance Ca<sub>o</sub><sup>2+</sup> sensitivity (we observed a trend in this direction), the Rho-C-hCaS<sup>S875A/T888A</sup> exhibited maximal Ca<sub>i</sub><sup>2+</sup> responsiveness even in control buffer. The response did not represent constitutive activity since Rho-C-hCaS<sup>S875A/T888A</sup> could be suppressed by both calcilytic [N-(2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl)-1,1-dimethyl-2-(2-naphthyl)ethylamine] cotreatment and exposure to nominally Ca<sub>o</sub><sup>2+</sup>/Mg<sub>o</sub><sup>2+</sup>-free buffer. These findings suggest that the TMD/proximal ICD core of the CaS might be much more Ca<sub>o</sub><sup>2+</sup> sensitive than is generally appreciated, and that the inactive form of the ECD and activation of the two PKC phosphorylation sites arrest its responsiveness. In summary, the present study has identified CaS<sup>S875</sup> as a phosphorylation site that together with CaS<sup>T888</sup> acts as a negative controller of CaS signaling and maintains

Ca<sub>o</sub><sup>2+</sup>-stimulated Ca<sub>i</sub><sup>2+</sup> oscillations that underpin CaS-mediated control of Ca<sub>o</sub><sup>2+</sup> homeostasis.

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#### Authorship Contributions

*Participated in research design:* Binmahfouz, Conigrave, Ward.

*Conducted experiments:* Binmahfouz, Centeno.

*Contributed new reagents or analytic tools:* Conigrave.

*Performed data analysis:* Binmahfouz.

*Wrote or contributed to the writing of the manuscript:* Binmahfouz, Conigrave, Ward.

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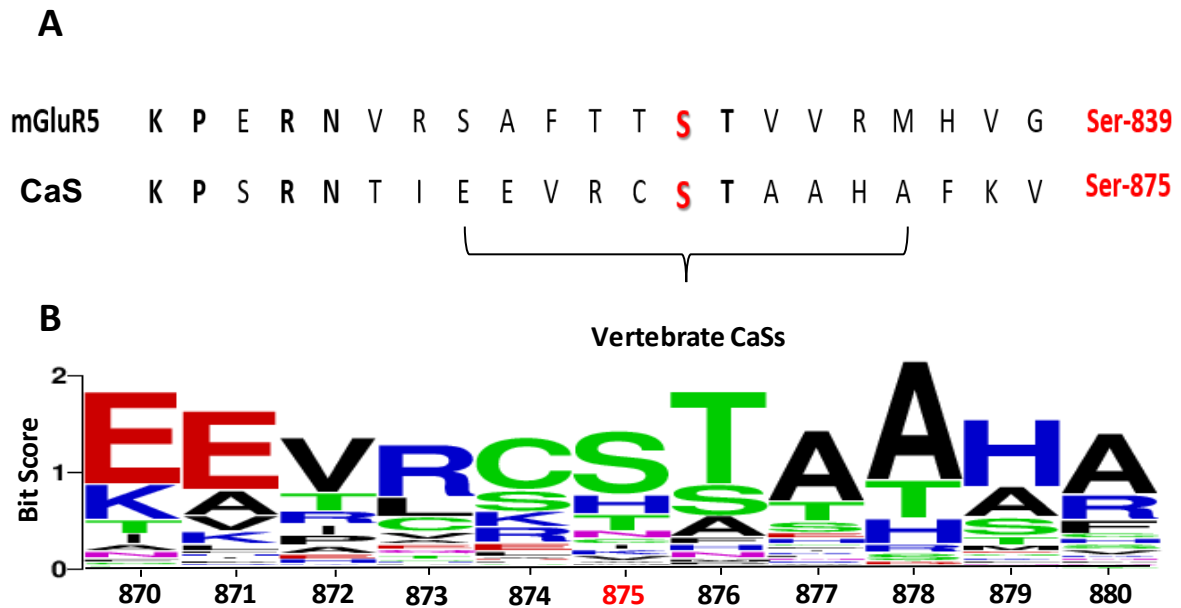
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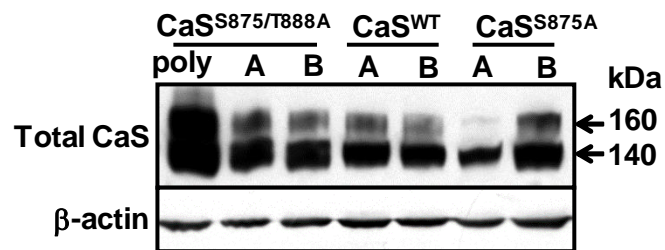
## SUPPLEMENTAL DATA

### Identification of Serine-875 as an Inhibitory Phosphorylation Site in the Calcium-Sensing Receptor

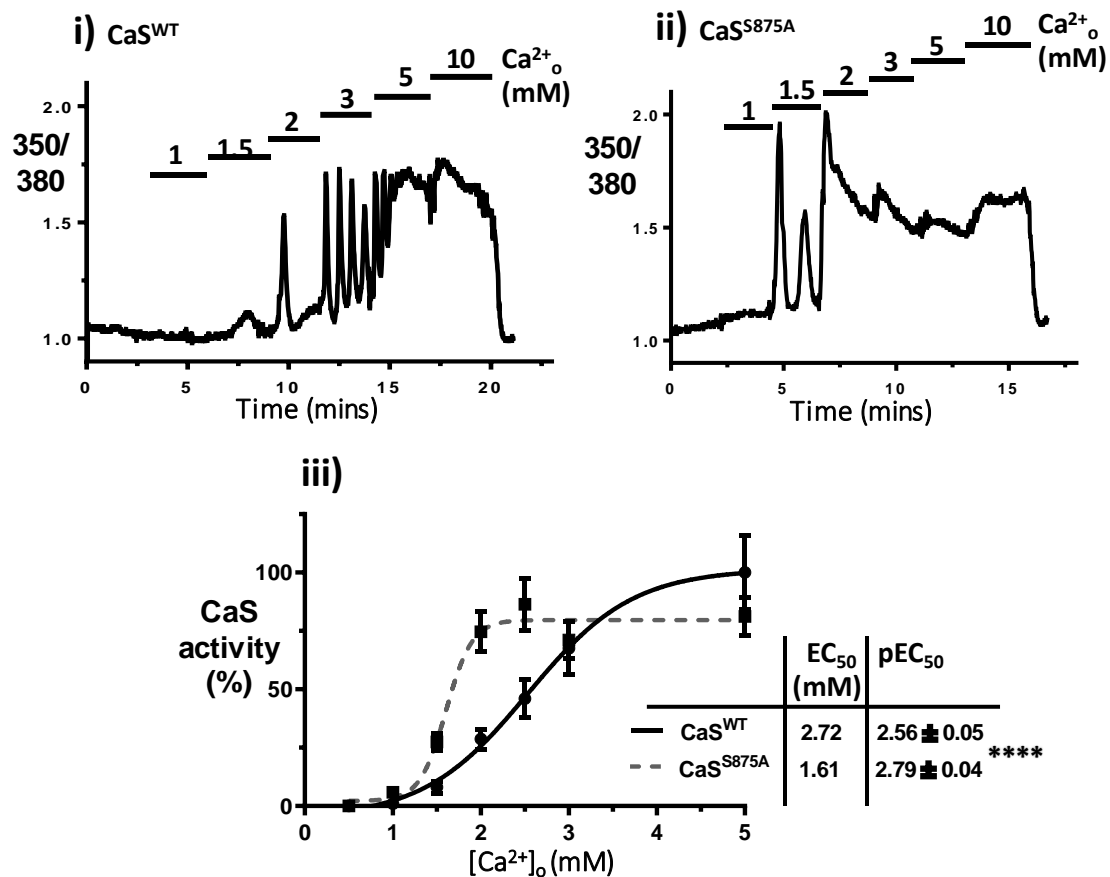
Lenah S Binmahfouz, Patricia P Centeno, Arthur D Conigrave and Donald T Ward



**Supplementary Figure 1: Sequence alignment of the CaS and mGluR5 intracellular domains.** **A)** Sequence alignment of the juxtamembrane regions of the CaS and mGluR5 intracellular domains indicates that the putative PKC phosphorylation site Ser-839 in mGluR5 aligns with Ser-875 in CaS (serine residues shown in red). **B)** Weblogo multiple sequence alignment of Ser-875 phosphorylation site. The multiple sequence alignment of the vertebrate CaSs obtained from (Herberger and Loretz, 2013) is showing amino acid residues surrounding Ser-875 phosphorylation site at positions  $-5$  to  $+5$ . The calculated bit scores indicate an overall high score for the respective amino acids reflecting their conservation and stabilisation among different species.

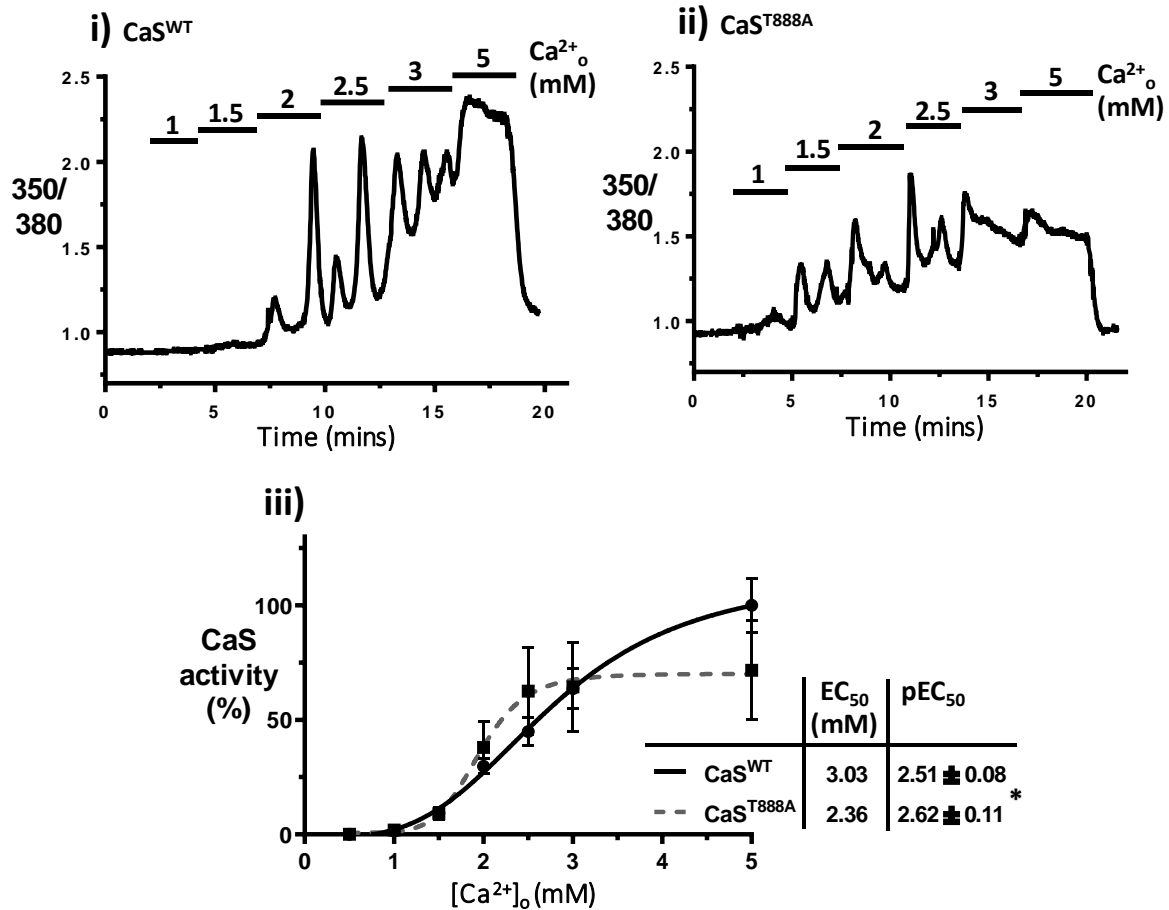


**Supplementary Figure 2: Western blot analysis of CaS in HEK-293 cells stably transfected with either wild-type or mutant CaS cDNAs.** Wild-type HEK-293 cells were transfected with linearised vectors encoding CaS<sup>WT</sup>, CaS<sup>S875A</sup> and CaS<sup>S875A/T888A</sup> then selected with 400 µg/ml hygromycin for two weeks. Single clones from the stable polyclonal pool were then expanded for an additional three weeks under hygromycin maintenance dose (200 µg/ml). Representative western blots show robust CaS expression in lysed cells from either the initial stable polyclone (poly) or from two individual monoclonal cell lines (A & B). Clonal cell lines that expressed mutant receptors at levels close to the CaS<sup>WT</sup>-expressing cells were selected for subsequent studies (clone A, CaS<sup>WT</sup>; clone B, CaS<sup>S875A</sup>; clone A, CaS<sup>S875A/T888A</sup>). The CaS immunoreactivity (ADD monoclonal anti-CaS antibody) included the distinctive immature 140-kDa and mature 160-kDa CaS proteins. β-actin was included as a loading control.

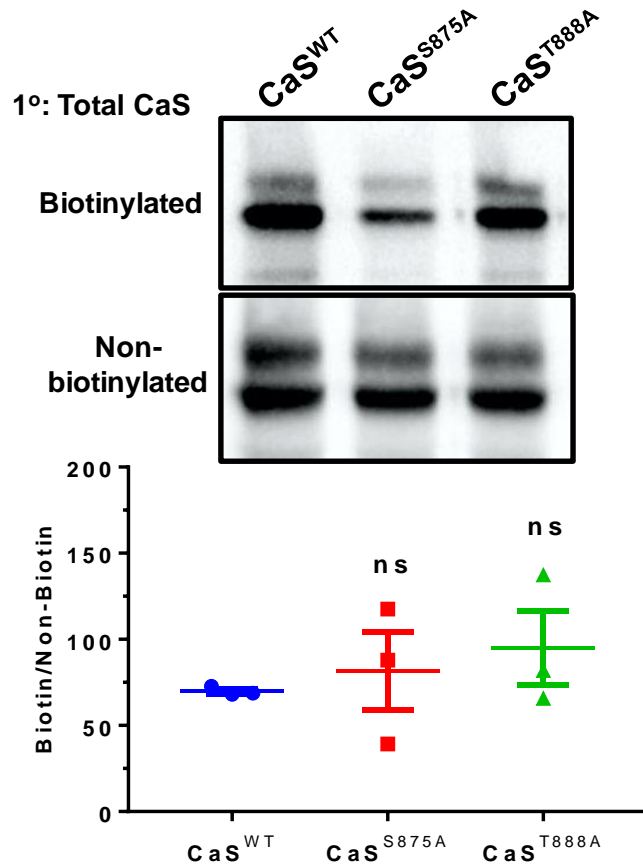


**Supplementary Figure 3: Effect of CaS<sup>S875A</sup> mutation on Ca<sup>2+</sup><sub>i</sub> mobilisation following stable transfection into HEK-293 cells.** Fura-2-loaded HEK-293 cells stably expressing either CaS<sup>WT</sup> (i) or CaS<sup>S875A</sup> (ii) were stimulated with increasing Ca<sup>2+</sup><sub>o</sub> concentrations (0.5–10 mM) and Ca<sup>2+</sup><sub>i</sub> assayed. Representative Ca<sup>2+</sup><sub>i</sub> traces are shown (i and ii) with the resulting concentration-effect curves shown in panel iii. \*\*\*P<0.001 for pEC<sub>50</sub>s of CaS<sup>S875A</sup> vs CaS<sup>WT</sup> by unpaired t-test; n ≥ 12 coverslips from three independent experiments.

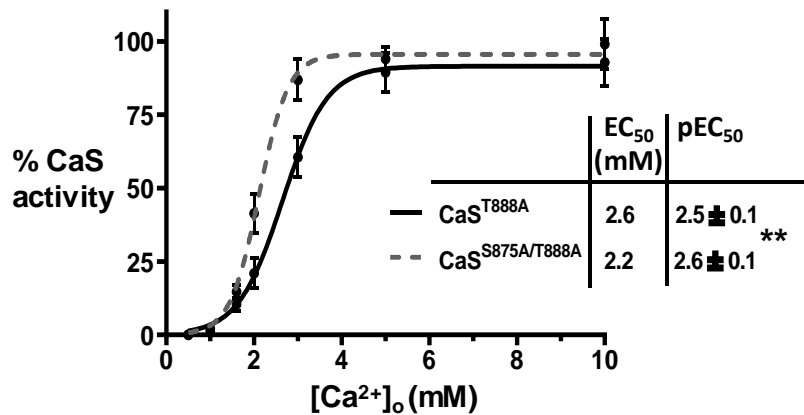
A



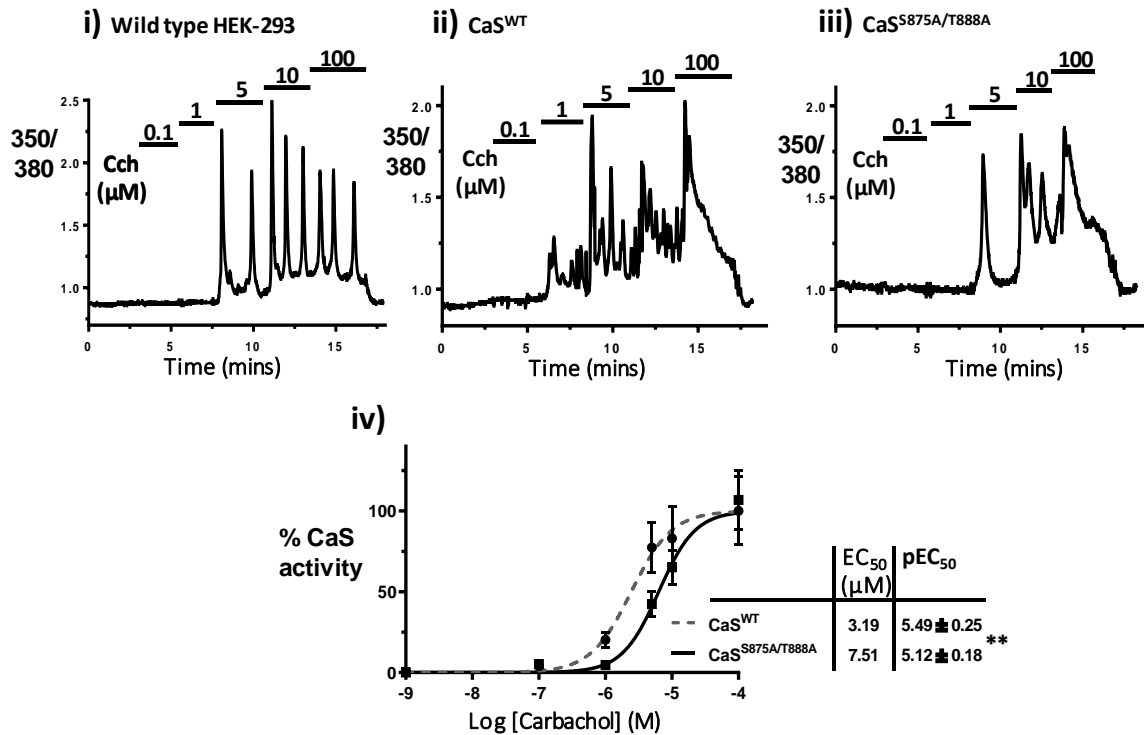
**Supplementary Figure 4: CaS<sup>T888A</sup> exhibits enhanced Ca<sup>2+</sup><sub>i</sub> mobilisation.** Fura-2-loaded HEK-293 cells stably expressing either CaS<sup>WT</sup> (i) or CaS<sup>T888A</sup> (ii) were stimulated with increasing Ca<sup>2+</sup><sub>o</sub> concentrations (0.5–5 mM) and Ca<sup>2+</sup><sub>i</sub> assayed. Representative Ca<sup>2+</sup><sub>i</sub> traces are shown (i and ii) with the resulting concentration-effect curves shown in panel iii. \*P<0.05 for pEC<sub>50</sub>s of CaS<sup>T888A</sup> vs CaS<sup>WT</sup> by unpaired t-test; n ≥ 6 coverslips from three independent experiments.



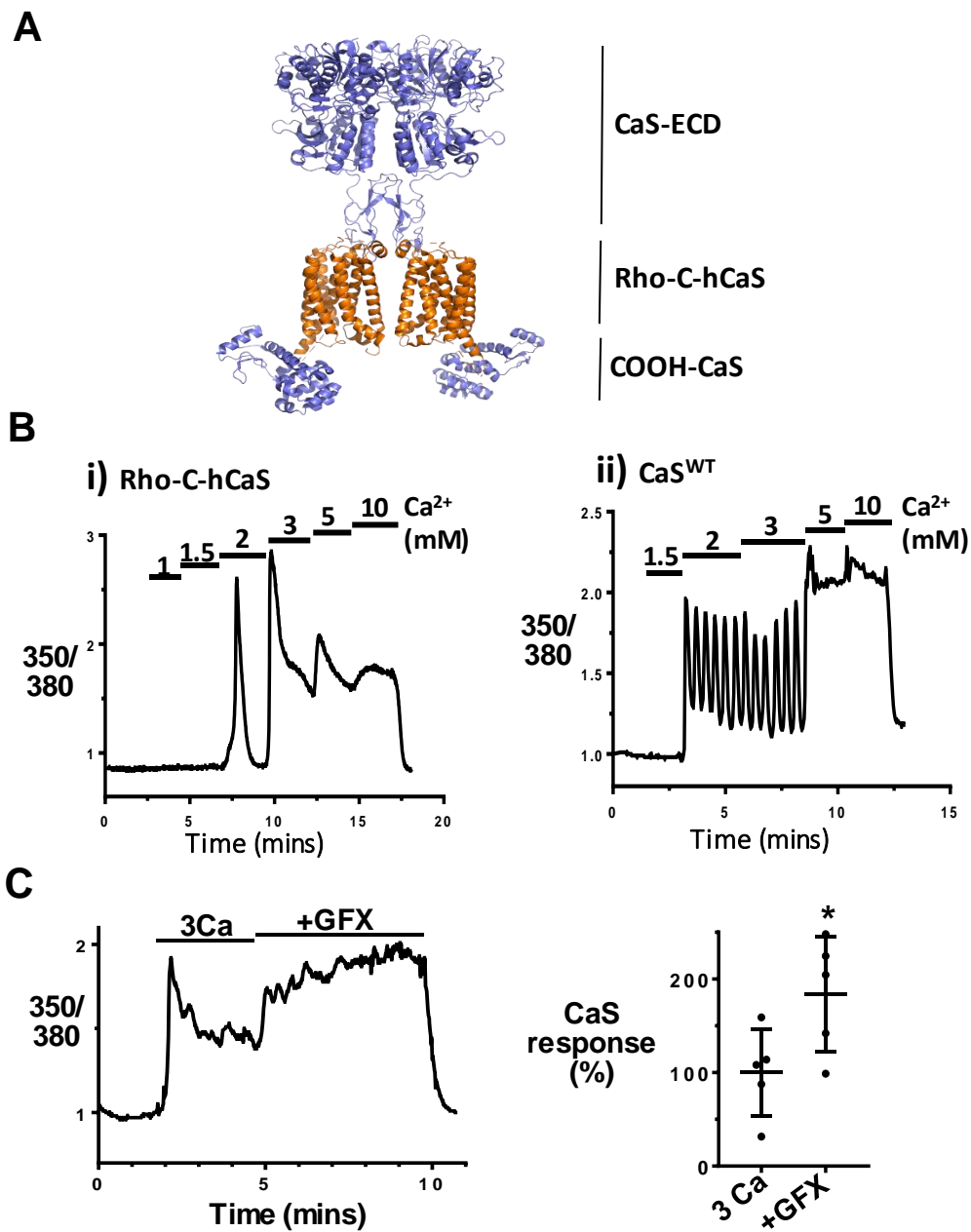
**Supplementary Figure 5: Relative membrane localisation of receptor mutants CaS<sup>S875A</sup> and CaS<sup>T888A</sup> following transient transfection in HEK-293 cells.** HEK-293 cells were transiently transfected with either CaS<sup>S875A</sup> or CaS<sup>T888A</sup> and then transferred to 35 mm dishes. Cell surface CaR expression was determined using the Pierce Cell Surface Protein Isolation Kit (Fisher Scientific) according to the manufacturer's instructions. Sulfo-NHS-SS-Biotin-labelled cell surface proteins were collected using NeutrAvidin Agarose beads. There were no gross or statistically significant differences in membrane localization between the 3 receptors (P=0.55 RM-ANOVA). Data are shown graphically as mean ± SEM and include individual data points.



**Supplementary Figure 6: Effect of CaS<sup>S875A/T888A</sup> double mutation on Ca<sup>2+</sup><sub>i</sub> mobilisation following transient transfection into HEK-293 cells.** Fura-2-loaded HEK-293 cells were transiently transfected with either CaS<sup>T888A</sup> or CaS<sup>S875A/T888A</sup>, then stimulated with increasing Ca<sup>2+</sup><sub>o</sub> concentrations (0.5–10 mM) and their Ca<sup>2+</sup><sub>i</sub> content assayed. The concentration-effect curves show the significantly enhanced Ca<sup>2+</sup><sub>o</sub> sensitivity in the CaS<sup>S875A/T888A</sup> double mutant cells. \*\*P<0.01 pEC<sub>50</sub> CaS<sup>T888A</sup> vs CaS<sup>S875A/T888A</sup> by unpaired t-test; n ≥ 12 coverslips from three independent experiments.



**Supplemental Figure 7: CaS<sup>S875A/T888A</sup> double mutation does not enhance muscarinic receptor responsiveness.** Fura-2-loaded HEK-293 cells stably transfected with either CaS<sup>WT</sup> (ii) or CaS<sup>S875A/T888A</sup> (iii) were stimulated with increasing concentrations of carbachol (0.1-100 μM; Cch) and their Ca<sup>2+</sup><sub>i</sub> content assayed. The response to carbachol in wild-type HEK-293 cells is shown in panel i. The Ca<sup>2+</sup><sub>o</sub> sensitivity of CaS<sup>S875A/T888A</sup> was not increased but was, in fact, decreased. \*\*P<0.01 pEC<sub>50</sub> vs CaS<sup>WT</sup> by unpaired t-test. Results are representative of a minimum of nine coverslips from three independent experiments.



**Supplemental Figure 8: Ca<sup>2+</sup><sub>i</sub> mobilisation response for the ECD truncated mutant, Rho-C-hCaS.**

**A)** Homology model of the full CaS structure. The ECD is adapted from the crystallography data of CaS (Geng et al., 2016, PDB: 5K5S). TMD and ICD are adapted from the crystallography data of mGluR5 (Doré et al., 2014, PDB:4009). The portion of the CaS that is Rho-C-hCaS is shown in orange.

**B)** Fura-2-loaded HEK cells transiently transfected with Rho-C-hCaS were stimulated with increasing Ca<sup>2+</sup><sub>o</sub> concentrations and then assayed for their Ca<sup>2+</sup><sub>i</sub> content (i). N=8 coverslips from three independent



transfections. The response of CaS<sup>WT</sup>-expressing cells to the same treatment is shown for comparison in panel ii. C) Rho-C-hCaS-transfected HEK cells were exposed to 3 mM Ca<sup>2+</sup><sub>o</sub> then cotreated with the PKC inhibitor GF109203X (250 nM; GFX). The response to GFX (area-under-the-curve) was normalised to the initial 3 mM Ca<sup>2+</sup><sub>o</sub> response. \*P<0.05 by paired t-test; n = 5 coverslips from two independent transfections.