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Title Page

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

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Running Title Page

Running title: CaS^{S875} inhibitory phosphorylation

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Non-standard abbreviations: ANOVA, one-way analysis of variance; AUC, area-under-the-curve; Ca^{2+}_i, intracellular Ca^{2+}; Ca^{2+}_o, extracellular calcium; CaS, calcium-sensing receptor; CaS^{WT}, wild-type CaS; DMEM, Dulbecco's modified Eagle's medium; ECD, extracellular domain; ERK1/2, extracellular signal-regulated kinase 1/2; GF109203X, bisindolylmaleimide I; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; ICD, intracellular domain, mGluR5, metabotropic glutamate receptor 5; NPS-2143, N-(2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl)-1,1-dimethyl-2-(2-nephthyl)ethylamine; PDB, protein data bank; pERK1/2, phosphorylated extracellular-signal regulated kinase 1/2; PKC, protein kinase C; PMA, phorbol 12- myristate 13-acetate; PTH, parathyroid hormone; RPMI, Roswell park memorial institute; TMD, transmembrane domain.

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Abstract

The calcium-sensing receptor (CaS) is the principal controller of extracellular calcium (Ca^{2+}_o) homeostasis and is inhibited *in vitro* and *in vivo* by protein kinase C (PKC)-mediated phosphorylation at CaS^{T888}. However, PKC inhibition enhances signalling 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 CaS^{T888} but instead with CaS^{S875}, not previously considered a PKC site. CaS^{S875A} (non-phosphorylatable) exhibited significantly enhanced Ca^{2+}_o sensitivity of both intracellular Ca^{2+} mobilisation and extracellular signal-regulated kinase 1/2 (ERK1/2) activation, whereas the phosphomimetic CaS^{S875D} mutant exhibited a loss of function. The $CaS^{S875A/T888A}$ double mutant exhibited even greater Ca^{2+}_o sensitivity than CaS^{T888A} alone, a response no longer enhanced by PKC inhibition. Finally, when expressed in CaS lacking its extracellular domain, the $CaS^{S875A/T888A}$ double mutation elicited maximal activation even under control conditions, but remained sensitive to negative allosteric modulation (NPS-2143) or Ca^{2+}_o removal. Therefore, we have now identified CaS^{S875} as the missing PKC phosphorylation site that, together with CaS^{T888}, shapes the CaS signalling that underpins Ca^{2+}_o homeostasis. Together with the inactive form of the CaS's extracellular domain, these sites attenuate Ca^{2+}_o sensitivity to attain appropriate physiological Ca^{2+}_o sensing.

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Introduction

The calcium-sensing receptor (CaS) is the principal controller of extracellular calcium (Ca^{2+}_o) homeostasis, suppressing both parathyroid hormone (PTH) secretion and renal calcium reabsorption in response to high Ca^{2+}_o 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's intracellular tail, Thr-888, Ser-895 and Ser-915 (Garrett et al., 1995; Bai et al., 1998). Previous results from this and other laboratories (Bai et al., 1998; Davies et al., 2007; Young et al., 2014) have shown that one of these residues, CaS^{T888}, represents the key phosphorylation site responsible for PKC-mediated inhibition of CaS-mediated intracellular Ca^{2+} (Ca^{2+}_i) mobilisation *in vitro*. In humans, mutation of CaS^{T888} to a non-phosphorylatable methionine produces a gain-of-function CaS resulting in autosomal dominant hypocalcaemia (Lazarus et al., 2011). Bai et al. reported that while the other four known PKC sites play little or no role in mediating PKC's functional effect, CaS^{T888} cannot be the sole determinant of the PKC effect (Bai et al., 1998). Furthermore, in the current laboratory, it was shown that CaS^{T888A} still causes Ca^{2+}_i oscillations in some cells (at least in the presence of 2 mM Ca^{2+}_o), and PKC activation with the phorbol ester PMA elicits a partial inhibitory effect in CaS^{T888A} (Davies et al., 2007) and CaS^{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 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 ($mGluR5^{S839A}$) was shown to prevent Ca^{2+}_i oscillations in HeLa cells (Kim et al., 2005). Interestingly, $mGluR5^{S839}$ does not align with CaS^{T888} or indeed with any of the known PKC consensus sequences in the CaS. Instead it aligns with CaS^{S875}, a residue not previously

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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^{S875}. Furthermore, while only 5 CaS residues were originally proposed to be PKC consensus sequences (Garrett et al., 1996), the NetPhos database (NetPhos 3.1) predicts CaS as having as many as 15 potential PKC sites, including both CaS^{S875} and CaS^{T888}.

The aim of this study therefore, was to evaluate the effect of mutating Ser-875 on CaS-mediated signalling in order 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^{WT}) 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).

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Materials and Methods

Cell Culture - Human embryonic kidney (HEK-293) cells transfected with CaS^{WT}, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Sigma-Aldrich, Gillingham, UK). To avoid cell death, gain-of-function mutant cell lines were routinely cultured in low Ca^{2+} Roswell park memorial institute (RPMI; containing 0.42 mM $CaCl_2$) instead of DMEM (containing 1.8 mM $CaCl_2$).

Mutagenesis - Mutations were introduced into the wild-type human parathyroid CaS by site-directed mutagenesis using the QuikChange® lightning site-directed mutagenesis kit in accordance with the manufacturer's instructions (Agilent Technologies Ltd, UK). HEK-293 cells were then transiently transfected with wild type or mutant receptors using FuGENE®6 (Promega, Southampton, UK). For stable expression, vectors were linearised prior to transfection and the resulting CaS-expressing cells were selected using Hygromycin (Duchefa Biochemie, Netherlands). Clonal cell lines were then established. The Rho-C-hCaS mutant was obtained from R. Mun (The Charles Perkins Centre, The University of Sydney, Australia) having been initially generated by Zhao and co-workers (1999). All mutations were subsequently verified by 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, #MA1-934). 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 12,000 xg (10 minutes, 4°C) and the supernatant solubilised in Laemmli buffer at 65°C.

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Intracellular Calcium Imaging - CaS-induced Ca^{2+}_i mobilisation was assayed by epifluorescence microscopy as described previously (Davies et al., 2007) after loading cells with Fura-2AM (Life Technologies Ltd, Paisley, UK). CaS-transfected cells were exposed to increasing concentrations of Ca^{2+}_o in Experimental buffer (20 mM HEPES (pH 7.4), 125 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5.5 mM glucose) at room temperature to compare relative Ca^{2+}_o-sensitivities between the receptors. The coverslips were mounted in a perfusion chamber (Warner Instruments, Hamden, CT) and fluorescence visualised using a Nikon Diaphot inverted microscope equipped with a digital camera charge-coupled device.

ERK1/2 Phosphorylation - Phosphorylation of extracellular signal-regulated kinase (ERK1/2) in the CaS-transfected cells was assayed as described previously (Ward et al., 2002). In brief, cells were exposed to various concentrations of Ca^{2+}_o 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 Signalling, #9106)

Data and Statistical Analysis - The data are presented as means ± SD (for pEC_{50} variance; shown as insets on the graphs), or, ± S.E.M. (for the precision of the individual responses, shown graphically). Statistical significance between pEC_{50} values (P values <0.05) was determined by Student's unpaired / paired t-test or one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's multiple comparison test as appropriate (GraphPad Prism V7). For Ca^{2+}_i assay, the area-under-the-curve (AUC) of the Fura-2 ratios (MetaFluor) for all cells in a field of view over a given time range was calculated using GraphPad Prism (V7) with resulting curves produced using a sigmoidal dose-response (variable slope) equation.

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Results

Effect of CaS^{S875A} and CaS^{S875D} phospho-mutations on Ca^{2+}_o-induced Ca^{2+}_i mobilisation. The key inhibitory PKC site in mGluR5, mGluR5^{S839}, aligns in the CaS not with the recognised inhibitory PKC site CaS^{T888} (Bai et al., 1996; Davies et al., 2007), but instead with CaS^{S875} (Supplementary Figure 1) which has not been previously considered a PKC site (Garrett et al., 1995; Bai et al., 1996). Thus, to examine the potential role of CaS^{S875} phosphorylation on receptor signalling, this residue was mutated either to a non-phosphorylatable alanine residue (CaS^{S875A}) or to a phosphomimetic aspartic acid residue (CaS^{S875D}) in CaS^{WT}. Subsequently, both mutated receptors were transiently transfected into HEK-293 cells, and CaS-induced Ca^{2+}_i mobilisation was measured in response to increasing concentrations of Ca^{2+}_o (0.5–10 mM). As shown in Figure 1, CaS^{S875A} was a gain-of-function mutation showing enhanced receptor sensitivity to Ca^{2+}_o relative to CaS^{WT} (EC_{50}, 2.3 CaS^{S875A} vs 3.5 mM CaS^{WT}; P<0.01). A similar gain of function was seen in HEK-293 cells stably expressing the CaS^{S875A} mutation (Supplementary Figures 2 and 3). In contrast, CaS^{S875D} exhibited decreased Ca^{2+}_o sensitivity relative to CaS^{WT} indicating that it is a loss-of-function mutation (EC_{50}, 4.7 CaS^{S875D} vs 3.5 mM CaS^{WT}; P<0.01; Figure 1).

CaS^{T888A} and CaS^{S875A} mutations enhance Ca^{2+}_o-induced ERK1/2 phosphorylation. The relative responses of CaS^{T888A} and CaS^{S875A} on Ca^{2+}_o-stimulated ERK1/2 phosphorylation were next compared to CaS^{WT} control responses using HEK-293 cells expressing each receptor stably. In agreement with the Ca^{2+}_i mobilisation data, CaS^{S875A} enhanced Ca^{2+}_o-induced pERK1/2 activation relative to CaS^{WT} control (EC_{50}, 1.9 CaS^{S875A} vs 3.8 mM CaS^{WT}; P<0.01; Figure 2). A similar gain of function was seen, as expected, in CaS^{T888A}-expressing cells (EC_{50}, 2.2 CaS^{T888A} vs 4.4 mM CaS^{WT}; P<0.01). Equal protein loading on the blots was confirmed by uniform β-actin expression. An equivalent gain of function for Ca^{2+}_i mobilisation by the

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CaS^{T888A} stably-expressing cells is shown in Supplementary Figure 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^{S899} phosphorylation (Grant et al., 2011; Grant et al., 2015). As such it is important to consider whether CaS^{S875} phosphorylation affects functional signalling directly or merely as a determinant of cell surface localisation. To examine this, HEK-293 cells were transiently transfected with CaS^{WT}, CaS^{S875A} and CaS^{T888A} and the membrane localisation of these receptors was then analysed using a surface biotinylation assay. As shown in Supplementary Figure 5 we saw no evidence of substantive changes in cell surface localisation in receptors lacking Ser-875 or Thr-888. Taken together however, these observations confirm that CaS^{S875A} is a gain-of-function mutation, with a similar impact on Ca^{2+}_o-stimulated signalling responses to CaS^{T888A}.

Effect of $CaS^{S875A/T888A}$ double mutation on CaS signalling. Since both CaS^{S875A} and CaS^{T888A} 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 and coworkers found that PKC inhibition could further enhance CaS^{T888A} signalling suggesting that another PKC site may contribute additional receptor modulation (Bai et al., 1996). The resulting $CaS^{S875A/T888A}$ double mutant was tested for its effect on Ca^{2+}_i mobilisation as before, in both transiently (Supplementary Figure 6) and stably-transfected (Figure 3) HEK-293 cells. In both cases, the doubly-mutated $CaS^{S875A/T888A}$ exhibited significantly lower EC_{50} values, increased sensitivity, for Ca^{2+}_o than the CaS^{T888A} mutant (stable, 1.8 $CaS^{S875A/T888A}$ vs 2.4 mM CaS^{T888A}; $P<0.01$, Figure 3). It was further noted that the $CaS^{S875A/T888A}$ double mutant completely abolished Ca^{2+}_i oscillations which CaS^{T888A} failed to do at lower Ca^{2+}_o concentrations.

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Similar to its effect on Ca^{2+}_i mobilisation, the $CaS^{S875A/T888A}$ double mutant also increased Ca^{2+}_o-sensitivity of ERK1/2 phosphorylation, compared to CaS^{T888A} alone (EC_{50}, 2.5 vs 3.2 respectively; $P<0.05$, Figure 3B), further demonstrating the additive effect of the two mutations. Overall, these observations confirm that the double mutant receptor $CaS^{S875A/T888A}$ was significantly more sensitive to Ca^{2+}_o than CaS^{T888A} alone with respect to both Ca^{2+}_i mobilisation and ERK1/2 activation.

In order to confirm that stable transfection with the double mutant receptor $CaS^{S875A/T888A}$ had not increased $G\alpha_q$-mediated Ca^{2+}_i mobilisation in a non-specific manner, the effect of carbachol on Ca^{2+}_i mobilisation was also tested. Carbachol also elicits oscillatory Ca^{2+}_i mobilisation in wild-type HEK-293 cells (Supplementary Figure 7, panel i) most likely via the muscarinic acetylcholine (M_3) receptor (Atwood et al., 2011). However, the $CaS^{S875A/T888A}$ double mutant failed to increase carbachol sensitivity for Ca^{2+}_i mobilisation suggesting that $CaS^{S875A/T888A}$ gain of function is not due to a non-specific increase in $G\alpha_q$-mediated Ca^{2+}_i mobilisation or to an artefact of transfection (Supplementary Figure 7, panels ii–iv). Indeed, carbachol responsiveness was inhibited in cells expressing the $CaS^{S875A/T888A}$ (EC_{50}, 7.5 vs 3.2 μM CaS^{WT}; $P<0.01$). The cause of this reduced carbachol responsiveness was not investigated further.

PKC inhibition fails to further enhance $CaS^{S875A/T888A}$ signalling. Having confirmed that the double mutant $CaS^{S875A/T888A}$ exhibits significantly greater Ca^{2+}_o responsiveness than for CaS^{T888A} alone, we next tested whether PKC inhibition could elicit additional signal enhancement suggestive of yet further PKC sites. For this, $CaS^{S875A/T888A}$ stably-expressing cells were treated with or without GF109203X (GFX, 250 nM), a non-specific PKC inhibitor, for 30 minutes and then co-stimulated with increasing concentrations of Ca^{2+}_o (0.5–5 mM), with Ca^{2+}_i mobilisation assayed as before (Figure 4). For comparison, CaS^{WT} and CaS^{T888A}

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cells were tested alongside one another in these experiments. As expected, GF109203X treatment significantly increased the sensitivity of both CaS^{WT} (EC_{50}; 5.6 control vs 3.3 mM GFX; P<0.001) and CaS^{T888A} (EC_{50}; 2.4 control vs 1.2 mM GFX; P<0.001) for $Ca^{2+}{}_o$. However, there was no difference in CaS-induced $Ca^{2+}{}_i$ mobilisation between $CaS^{S875A/T888A}$ double mutants treated without or with GF109203X, that is they both increased CaS sensitivity to a virtually identical extent (EC_{50}, 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^{T888A} and CaS^{S875A} mutations on Rho-C-hCaS-induced $Ca^{2+}{}_i$ mobilisation. Having established the dual inhibitory effect of CaS^{T888} and CaS^{S875} on CaS signalling, 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 activation of the CaS. In such a case, mutation of the inhibitory phosphorylation sites might enhance responsiveness even in a headless CaS.

The Rho-C-hCaS lacks most of the ECD (residues 1-599) and, therefore, contains only the 7TMD and the carboxyl-terminal ICD with a stop codon at residue 903 as described previously (Zhao et al., 1999, Supplementary Figure 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). Firstly, Rho-C-hCaS was transiently transfected into HEK-293 cells, and then the effect of increasing $Ca^{2+}{}_o$ concentrations (up to

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10 mM) on Ca^{2+}_i mobilisation was assayed as before. In contrast to CaS^{WT}, which elicits oscillatory signalling, Rho-C-hCaS elicited only transient or sustained Ca^{2+}_i mobilisation (Supplementary Figure 8B). It should be noted that no Ca^{2+}_i mobilisation is seen under these conditions in non-transfected HEK-293 cells (not shown). Moreover, the effect of PKC inhibition on Rho-C-hCaS signalling was tested. For this, HEK-293 cells transiently transfected with Rho-C-hCaS were first exposed to 3 mM Ca^{2+}_o to elicit Ca^{2+}_i mobilisation and then were co-treated with 250 nM of GF102903X. Indeed, GF102903X enhanced Rho-C-hCaS-induced Ca^{2+}_i mobilisation in response to 3 mM Ca^{2+}_o ($P<0.05$; Supplementary Figure 8C).

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

Effect of Rho-C-$hCaS^{S875A/T888A}$ double mutation on Ca^{2+}_i mobilisation. 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-$hCaS^{T888A}$ when expressed in combination. Indeed, the Rho-C-$hCaS^{S875A/T888A}$ double mutant produced maximal Ca^{2+}_i mobilisation even under baseline conditions (0.5 mM Ca^{2+}_o). This effect could be virtually abolished, however, by co-treatment with the CaS negative allosteric modulator (calcilytic) NPS-2143 (1 μM; $P<0.001$, Figure 6B). To determine whether the Rho-

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C-hCaS$^{S875A/T888A}$ double mutant is constitutively active, i.e., elicits continuous signalling even in the absence of an agonist, $Ca^{2+}{}_i$ mobilisation was tested using a buffer that was nominally free of Ca^{2+} or Mg^{2+}. As shown in Figure 6C, the Rho-C-hCaS$^{S875A/T888A}$-induced $Ca^{2+}{}_i$ mobilisation elicited in control buffer (0.5 mM Ca^{2+}) was almost abolished upon removal of $Ca^{2+}{}_o$ and $Mg^{2+}{}_o$ ($P<0.001$). Interestingly, introduction of 0.5 mM Mg^{2+} alone was sufficient to elicit cellular $Ca^{2+}{}_i$ transients in some coverslips. Subsequent introduction of 0.5 mM Ca^{2+} restored maximal activation of the Rho-C-hCaS$^{S875A/T888A}$ double mutant. Thus, although Rho-C-hCaS$^{S875A/T888A}$ was not constitutively active, it exhibited substantially enhanced sensitivity to $Ca^{2+}{}_o$ (i.e. with a presumed $EC_{50} < 0.5$ 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 signalling.

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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^{T888}, represents the key phosphorylation site responsible for PKC-mediated inhibition of CaS-mediated $Ca^{2+}{}_i$ mobilisation (Bai et al., 1998; Davies et al., 2007; Young et al., 2014). It should be noted, however, that the human CaS has in fact 54 serine and threonine residues in either its ICD or three intracellular loops, though 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 1 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 account of 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 signalling outcomes (Tobin et al., 2008; Yang et al., 2017). The barcode hypothesis was first devised by studying two GPCRs: the M_3 receptor (Butcher et al., 2011) and the β2-adrenergic receptor (Nobles et al., 2011). The

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

Consistent with the NetPhos database prediction and with 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^{S875} as a phosphorylation site in the kidney (Huttlin et al., 2010). This demonstrates that CaS^{S875} 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, Supplementary Figure 1A). Therefore, the alignment of CaS^{S875} and $mGluR5^{S839}$ suggests that they share similar regulatory roles in the modulation of receptor signalling. Moreover, the amino acid conservation of the putative Ser-875 phosphorylation site was examined in the current study across a previously published multiple sequence 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 (Supplementary Figure 1B), CaS^{S875} is well conserved within different species. Overall, the conservation of Ser-875 is consistent with the phosphorylation site prediction and with a strong functional role in CaS signalling. One important consideration regarding these phosphorylation sites is to determine whether they modulate signal transduction directly, or instead by altering CaS cell surface localisation (Breitwieser, 2015) as is apparently the case for CaS^{S899} (Grant et al., 2015). In this regard, we did not see a significant difference in the surface biotinylation of CaS^{S875A} or CaS^{T888A} vs CaS^{WT} 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 signalling, internalisation and

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desensitisation. In the meantime, however, the current data establish a clear link between residues CaS^{S875} and CaS^{T888} and CaS downstream signalling.

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

In support of this idea, Bai et al. previously showed that PKC activation by PMA reduced the responsiveness of a mutant CaS, in which all five predicted PKC sites were eliminated (Bai et al., 1998). Moreover, PMA elicited a partial inhibitory effect in cells expressing CaS^{T888A} (Davies et al., 2007) and CaS^{T888M} (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^{S875A} with its non-phosphorylatable mutation enhanced signalling whereas CaS^{S875D} with its phosphomimetic mutation inhibited signalling. Furthermore, the $CaS^{S875A/T888A}$ double mutation further enhanced both Ca^{2+}_o-induced Ca^{2+}_i mobilisation and ERK1/2 phosphorylation more than for CaS^{T888A} alone, whereas concomitant PKC inhibition had no further effect. To prove that CaS^{S875} is a PKC site and then determine the ligand sensitivity of such phosphorylation, as was done for CaS^{T888} (Davies et al., 2007; McCormick et al., 2010), it will be necessary to raise a phospho-CaS^{S875} specific antibody. However, our

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initial attempt to generate such an antibody proved unsuccessful and thus we must rely instead on the mutagenesis studies reported here, the new consensus predictions described above, the alignment to mGluR5^{S839}, 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^{S875} is a functionally important PKC site, in conjunction with CaS^{T888}. However, the possible involvement of other phosphorylation sites in the regulation of CaS function awaits determination.

That CaS$^{S875A/T888A}$ expression failed to enhance carbachol signalling supported the idea that the gain of function was CaS-specific and not an artefact of transfection. However, the observation that CaS$^{S875A/T888A}$ expression, in fact, inhibited carbachol signalling is interesting. It is proposed that receptors exist in a conformational equilibrium between inactive and active states and that G proteins have a higher affinity for the active state of the receptor (Burstein et al., 1995). Since both CaS and the M_3 muscarinic are $G\alpha_q$-coupled, it seems feasible that they compete for the same $G\alpha_q$-protein pool (Linderman, 2009) and thus perhaps $G\alpha_q$ has a sufficiently higher affinity for the hyperactive CaS$^{S875A/T888A}$ double mutant that fewer $G\alpha_q$ 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^{2+}_o, which further verified the existence of at least one Ca^{2+}_o-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^{2+}_o sensitivity to that seen for the full-length CaS^{T888A}. Although Rho-C-hCaS^{S875A} did not, on its own, enhance Ca^{2+}_o sensitivity (we observed a trend in this direction), the Rho-C-hCaS$^{S875A/T888A}$ exhibited maximal Ca^{2+}_i responsiveness even in control buffer. The response

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did not represent constitutive activity as Rho-C-hCaS$^{S875A/T888A}$ could be suppressed by both calcilytic (NPS-2143) co-treatment and by exposure to nominally Ca^{2+} / Mg^{2+}-free buffer. These findings suggest that the TMD / proximal ICD core of the CaS might be much more Ca^{2+}_o-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^{S875} as a phosphorylation site that together with CaS^{T888} acts as a negative controller of CaS signalling and maintains Ca^{2+}_o-stimulated Ca^{2+}_i oscillations that underpins CaS-mediated control of Ca^{2+}_o homeostasis.

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Authorship contributions

Participated in research design: Ward, Conigrave and Binmahfouz.

Conducted experiments*:* Binmahfouz and Centeno.

Contributed new reagents or analytic tools: Conigrave.

Performed data analysis: Binmahfouz.

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

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Footnotes

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Figure Legends

Figure 1: CaS^{S875} acts as an inhibitory phosphorylation site. Fura-2-loaded HEK-293 cells were transiently transfected with either (i) CaS^{WT} (ii) CaS^{S875A} or (iii) CaS^{S875D} then exposed to increasing Ca^{2+}_o concentrations (0.5–10 mM) to determine the effect of the putative phosphorylation site CaS^{S875} on Ca^{2+}_i mobilisation. The representative traces show the Ca^{2+}_i changes (Fura-2 ratio) in single cells from the field of view. The whole field-of-views changes in Ca^{2+}_i concentration are shown as concentration-effect curves in panel iv. **P<0.01 vs CaS^{WT} by one-way ANOVA with Dunnett's posthoc test. Data representative of three independent experiments (n≥6 coverslips) are shown.

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

Figure 3: $CaS^{S875A/T888A}$ double mutant is more sensitive to Ca^{2+}_o than CaS^{T888A} alone. A) Fura-2-loaded HEK-293 cells stably transfected with either CaS^{T888A} (i) or $CaS^{S875A/T888A}$ (ii) were stimulated with increasing Ca^{2+}_o concentrations (0.5–10 mM). The two panels show Ca^{2+}_i traces from representative single cells. The resulting concentration-effect curves are shown in iii. **P<0.01 for CaS^{T888A} vs. $CaS^{S875A/T888A}$ 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

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same cell lines as before, stimulated with various Ca^{2+}_o concentrations (0.5–5 mM) for 10 mins. The resulting concentration-effect relationship is shown to the right. ERK1/2 responses are expressed as a percentage of the CaS^{T888A} maximal response in each experiment. *P<0.05 EC_{50} values for CaS^{T888A} vs $CaS^{S875A/T888A}$ by unpaired t-test; n ≥ 6 from three independent experiments.

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

Figure 5: T888A and S875A mutations enhance Ca^{2+}_i mobilisation of the ECD-deleted mutant, Rho-C-hCaS. Fura-2-loaded HEK cells were transiently transfected with either (i) Rho-C-hCaS, (ii) Rho-C-$hCaS^{T888A}$ or (iii) Rho-C-$hCaS^{S875A}$ mutants then stimulated with increasing Ca^{2+}_o concentrations (0.5–10 mM). Representative Ca^{2+}_i traces are shown. The resulting concentration-effect curves are shown in panel iv. *P<0.05, Rho-C-hCaS vs Rho-C-$hCaS^{T888A}$; NS=not significant, Rho-C-hCaS vs Rho-C-$hCaS^{S875A}$ by one-way ANOVA with Dunnett's posthoc test; n ≥ 16 coverslips from five independent experiments.

Figure 6: Effect of $CaS^{S875A/T888A}$ double mutation on Rho-C-hCaS. A) Rho-C-$hCaS^{S875A/T888A}$ transfected HEK-293 cells exhibited maximal Ca^{2+}_i mobilisation even under control conditions (0.5 mM Ca^{2+}_o) but was substantially inhibited by the calcilytic (Cx) NPS-2143 (1 μM). *** P<0.001 by paired t-test. **B)** Rho-C-$hCaS^{S875A/T888A}$-induced Ca^{2+}_i mobilisation was then assayed in the presence or absence of 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+} with resulting responses normalised to the 0.5 mM Ca^{2+}_o 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.

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Figure 1

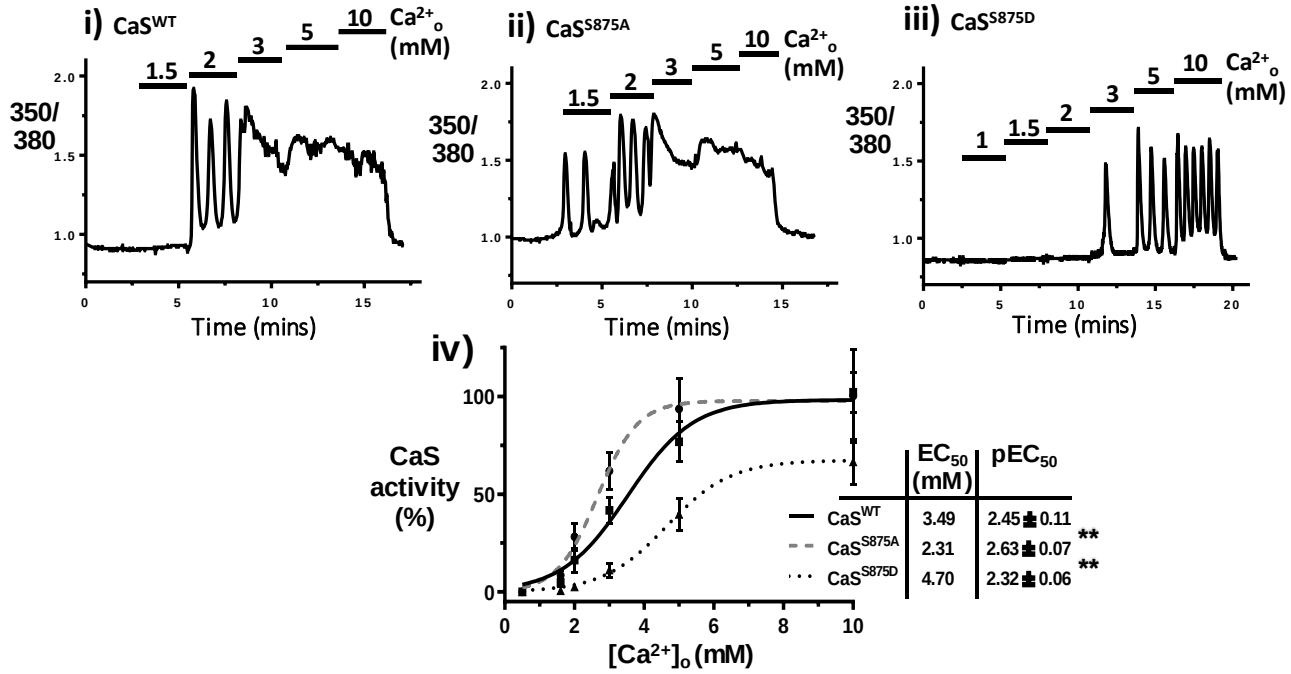

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Figure 2

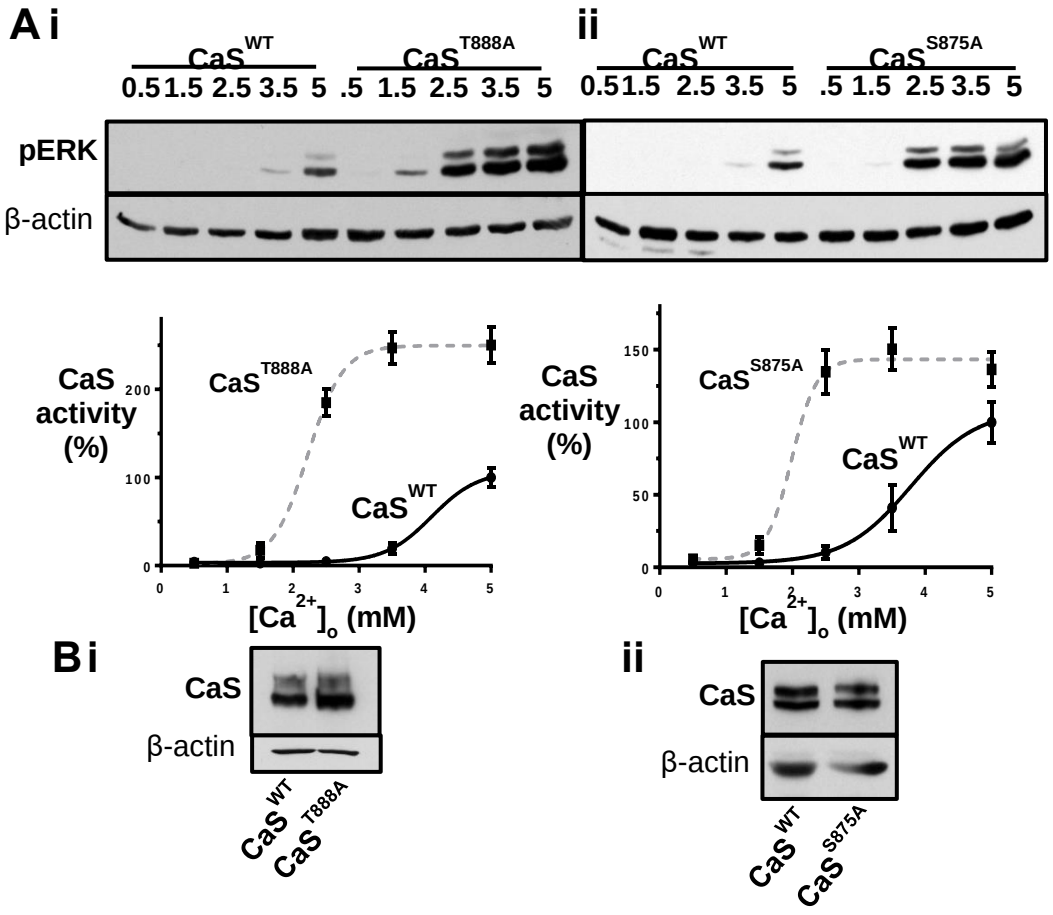

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Figure 3

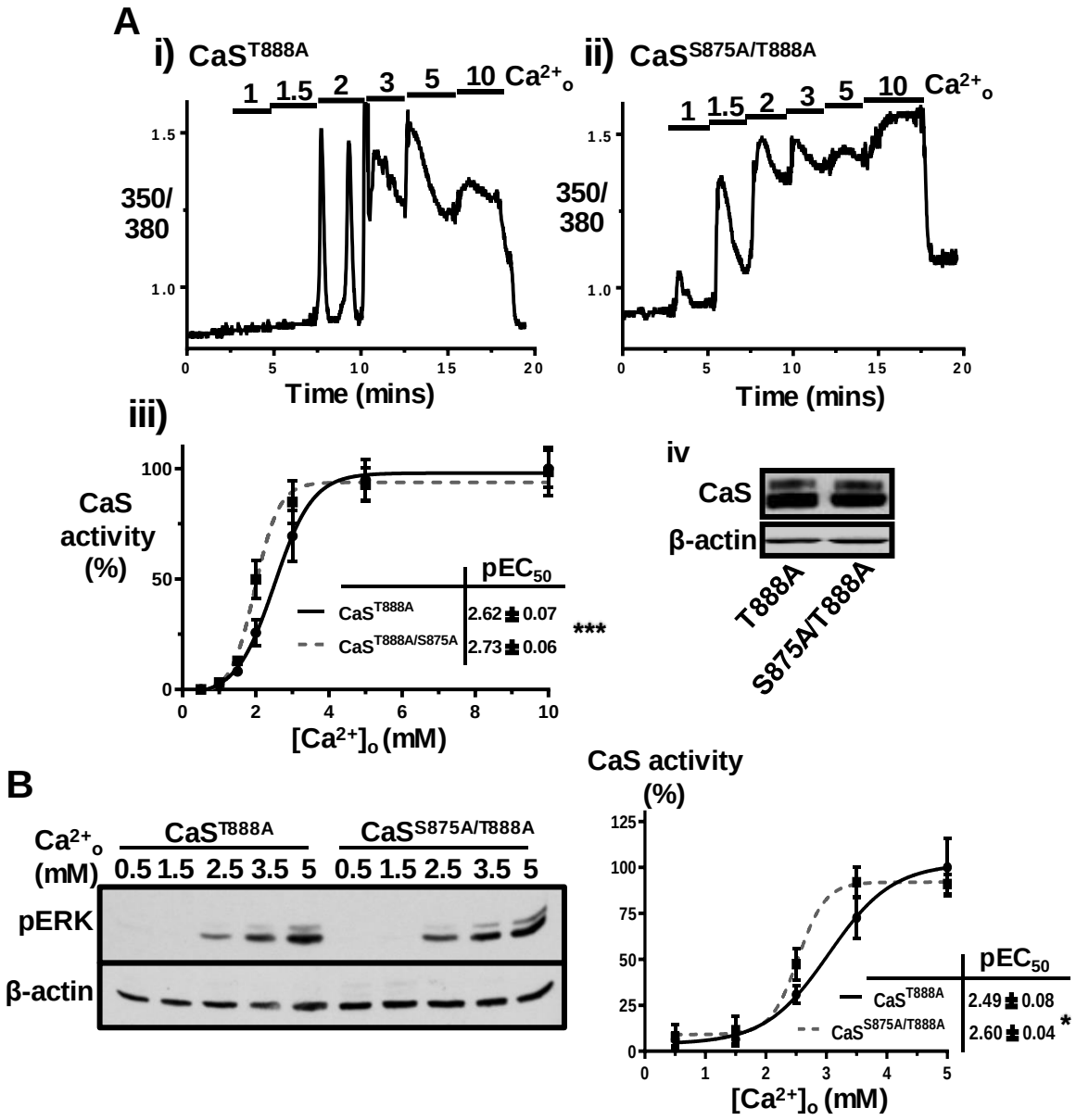

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Figure 4

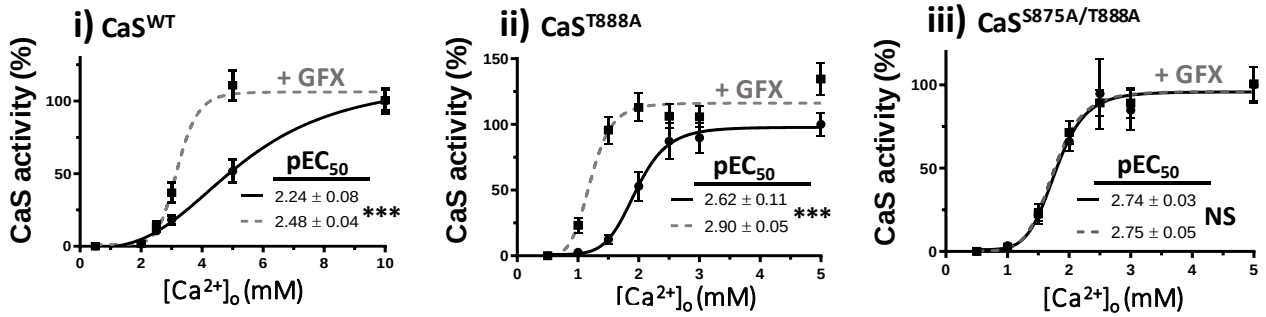

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Figure 5

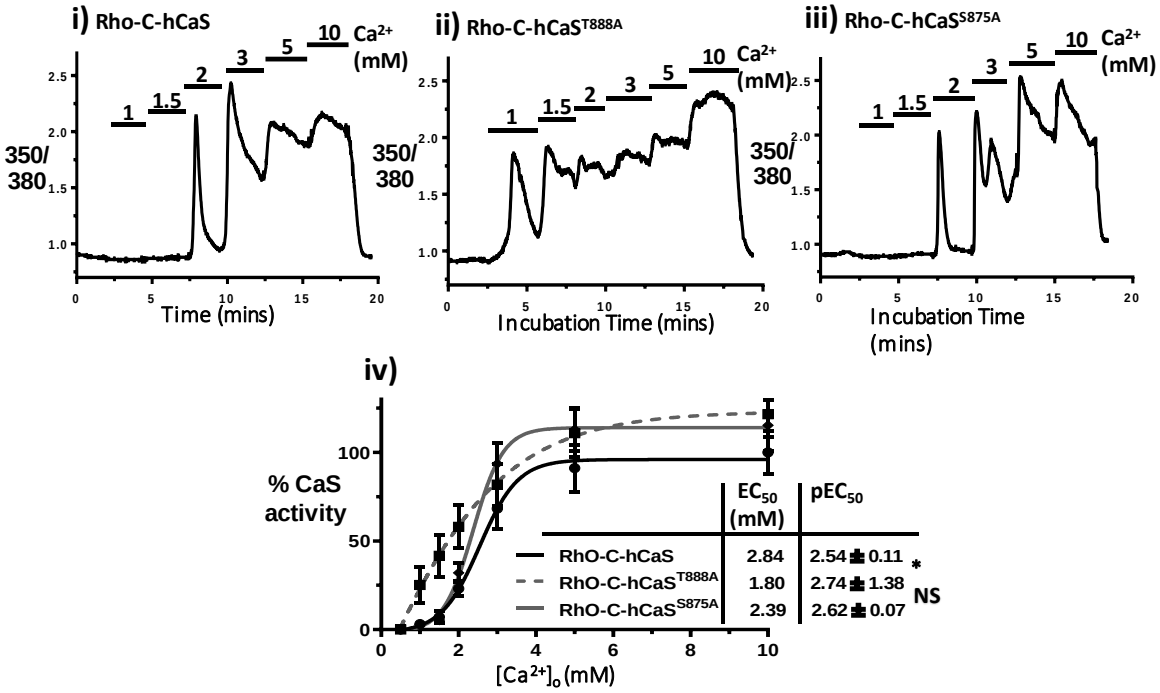

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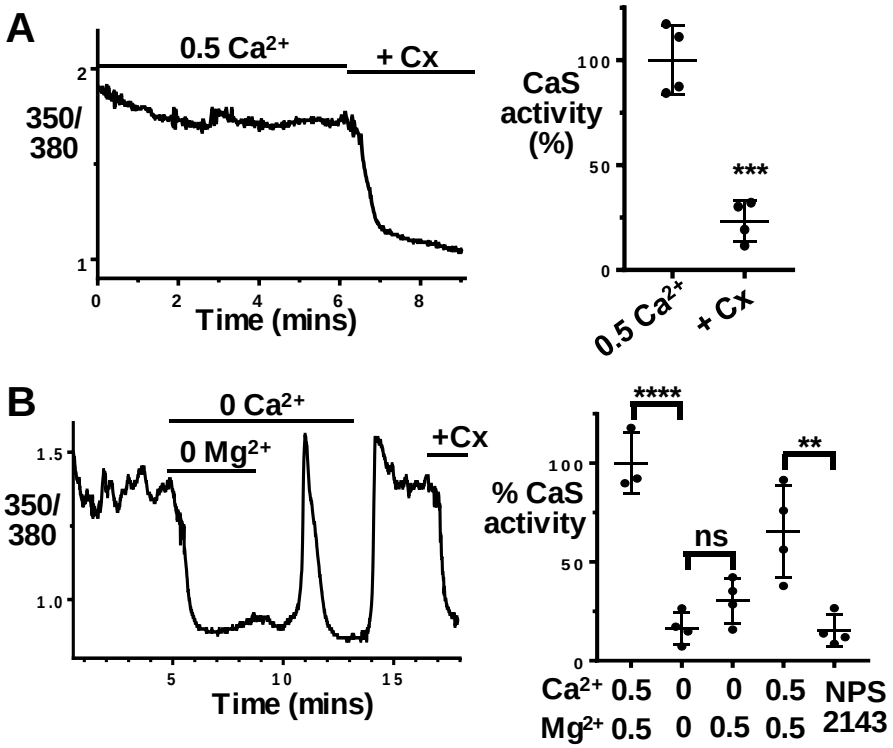
Figure 6



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