The adaptor protein 14-3-3 binds to the calcium-sensing receptor and attenuates receptor-mediated Rho kinase signalling

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INTRODUCTION

The CaR (calcium-sensing receptor) is a class C GPCR (G-protein-coupled receptor) that is highly expressed in the parathyroid gland and renal tubules where it regulates parathyroid hormone secretion and calcium excretion respectively [1,2]. The human CaR protein (1078 amino acids) is composed of a large extracellular N-terminal ligand-binding domain (610 residues), a signature seven-transmembrane domain and an intracellular C-terminal tail (215 residues) [3]. In addition to Ca	extsuperscript{2+}, agonists of the CaR include other di- and tri-valent cations as well as polypeptides, polyamines and aminoglycoside antibiotics [1,2,4]. The CaR is also modulated by ionic strength and pH as well as amino acids, γ-glutamyl peptides and various classes of synthetic calcimimetics and calcilytics [4–6]. Upon ligand binding, the receptor couples to three distinct classes of heterotrimeric G-proteins, Gαq, Gαo, and G12α, in a tissue- and/or ligand-specific manner. This leads to activation of phospholipase C and release of Ca	extsuperscript{2+} ions from intracellular stores, activation of protein kinases including PKC (protein kinase C), Akt and the MAPKs (mitogen-activated protein kinases) p38, ERK (extracellular signal-regulated kinase) 1/2 and JNK (c-Jun N-terminal kinase), kinases including PKC (protein kinase C), Akt and the MAPKs (mitogen-activated protein kinases) p38, ERK (extracellular signal-regulated kinase) 1/2 and JNK (c-Jun N-terminal kinase), as well as inhibition of adenylyl cyclase [2,4,7]. The CaR also stimulates SRE (serum-response element) activity downstream of Gq and the monomeric G-protein Rho [8], and through G12α the receptor can modulate Rho-dependent phospholipase D activation and L-amino acid-induced Ca	extsuperscript{2+} mobilization [9,10] (for reviews of CaR-mediated signalling, see [2,4,7]). Downstream of its signalling pathways, the CaR regulates critical cellular processes including secretion, apoptosis, chemotactic responses, cell proliferation, cytoskeletal rearrangements, ion channel activity, the control of gene expression and cell differentiation [2,4].

The CaR intracellular tail comprises amino acids 865–1078 and contains important determinants for cell-surface expression and activation of signalling pathways [1,2]. The membrane-proximal region of the tail is essential for biological activity with critical residues identified for expression between residues 865 and 877, and those for signalling between residues 877 and 888 [1,11,12]. The remaining tail is dispensable, as exemplified by the impact of a large in-frame deletion (residues 895–1075) which exhibits gain-of-function compared with the wild-type CaR [13]. There are three predicted PKC phosphorylation sites in the CaR tail, Thr	extsuperscript{885}, Ser	extsuperscript{905} and Ser	extsuperscript{915}, and phosphorylation at Thr	extsuperscript{885} uncouples the receptor from phosphorylidyinositol-specific phospholipase C and intracellular Ca	extsuperscript{2+} release [3,14]. The CaR tail also houses the ER (endoplasmic reticulum) retention signal RR and RKR located at amino acids 890 and 891, and 896–898 respectively [15,16]. In addition to G-protein coupling,
CaR-dependent signalling also requires interactions with binding partners including filamin [17,18], a 250 kDa actin-binding protein that anchors cell-surface receptors to the cytoskeleton [19]. Filamin is essential for CaR-mediated activation of the ERK1/2 pathway [17,18] and for optimal CaR-mediated SRE activity via the Rho pathway, providing a scaffold for the G_{q,12}-dependent activation of RhOA via Rho-nucleotide-exchange factor [8].

In addition to filamin and PKC, a number of other proteins bind to the intracellular tail of the CaR, including the inwardly rectifying K^{+} channels Kir4.1 and Kir4.2 [20], the cargo receptor p24A [21], and proteins of the ubiquitin degradation system such as the E3 ubiquitin ligase dorfin [22] and deubiquitinating enzyme AMSH [associated molecule with the SH3 (Src homology 3) domain of STAM (signal-transducing adaptor molecule)] [23]. With a view to identifying novel binding partners of the CaR tail, we embarked on a yeast two-hybrid screen using the full-length CaR tail as bait with a cDNA library derived from a mammalian expression vectors pcDNA3–EGFP and pcDNA3.1 (EMLC.1) [24], which has been used successfully in other screens [25,26]. We identified the 14-3-3 adaptor protein as a binding partner of the CaR tail. The 14-3-3 proteins are ubiquitously expressed chaperones that play a key role in facilitating diverse biological processes that include the regulation of cell signalling events and cellular trafficking [27,28]. In the present paper we show that two isoforms of 14-3-3 bind to the membrane proximal region of the CaR and negatively regulate CaR-dependent SRE activation.

**MATERIALS AND METHODS**

**Antibodies and chemicals**

The antibodies used in the present study were from the following sources: anti-GFP (green fluorescent protein) (rabbit polyclonal), anti-(14-3-3θ) (C-17, rabbit polyclonal), anti-(14-3-3ζ) (C-16, rabbit polyclonal) and anti-(filamin 1) (mouse monoclonal) were from Santa Cruz Biototechnology; anti-FLAG (mouse monoclonal), anti-α-tubulin (mouse monoclonal) and HRP (horseradish peroxidase)-conjugated goat anti-mouse antibody were from Sigma–Aldrich; the anti-CaR ADD antibody (mouse monoclonal) was from Affinity Bioreagents; anti-PDI (protein disulphide isomerase) (rabbit polyclonal) was from Stressgen Bioreagents; anti-GFP (green fluorescent protein) (rabbit polyclonal), anti-ERK1/2 (rabbit polyclonal), anti-(phospho-ERK1/2) (rabbit polyclonal) and anti-(filamin 1) (mouse monoclonal) were from Promega; anti-ERK1/2 (rabbit polyclonal) and HRP-conjugated goat anti-rabbit antibody were from Promega; anti-PDI (rabbit polyclonal) and anti-ERK1/2 (rabbit polyclonal) was from Cell Signaling Technology and Alexa Fluor® 546 goat anti-mouse and Alexa Fluor 647 goat anti-rabbit antibodies were from Molecular Probes, Invitrogen Life Technologies. Chemicals were of laboratory grade and purchased from Sigma–Aldrich unless otherwise specified.

**Plasmids and yeast two-hybrid library**

A pVP16 cDNA library of an EMLC.1 mouse pluripotent haemopoietic cell line [24], kindly donated by Dr Shickwann Tsai (Fred Hutchinson Cancer Research Center, Seattle, U.S.A.) was further amplified for use in the present study. Construction of the library using NotI-linkered inserts and the LexA-based screening procedure in which reconstitution of a transcriptional transactivator is able to activate His and LacZ reporter genes, have been described previously [24,25,29]. For selection purposes, the pVP16 library vector carries the Leu gene.

The bait construct used for the library screen (pBTM116-CaR[865–1078]) was generated by PCR amplification of the human CaR intracellular tail (amino acids 865–1078) from a template described previously [30], and insertion of the CaR tail into the unique SalI site of the bait vector pBTM116 to create a Lex-A DNA-binding fusion. This vector also carries the Trp1 gene. Bait deletion constructs for use in yeast two-hybrid mapping studies, pBTM116-CaR[865–922], pBTM116-CaR[865–898], pBTM116-CaR[899–922] and pBTM116-CaR[923–1078], were similarly generated.

For other studies, the full-length human 14-3-3θ protein was recovered from the osteosarcoma cell line Saos-2 by RT (reverse transcription)–PCR, verified by sequence analysis, then cloned via the EcoRV and NotI restriction enzyme sites into the vector pcDNA3-EGFP (enhanced GFP) version 1 (supplied by Professor Karin Eidne, Western Australian Institute for Medical Research, Nedlands, Australia) for expression as an N-terminally tagged EGFP fusion protein (pcDNA3-EGFP–14-3-3θ) and into the vector pGEX-4T-1 (GE Healthcare Life Sciences) using the Sall and NotI sites for expression as a GST (glutathione transferase) fusion protein (pGEX-4T-1–14-3-3θ). Untagged 14-3-3θ (pCDNA3.1–14-3-3θ) was created by subcloning 14-3-3θ into pcDNA3.1 using the EcoRI and NotI sites. In addition, a truncated version of another 14-3-3 isoform, 14-3-3ζ, was found to bind to the CaR in the yeast two-hybrid screen. Full-length human 14-3-3ζ was amplified from Saos-2 cells by RT–PCR and cloned into the pVP16 yeast two hybrid vector and into the mammalian expression vectors pcDNA3–EGFP and pcDNA3.1 essentially as described for the 14-3-3θ isoform.

The C-terminally FLAG-tagged CaR construct (pcDNA3.1–CaR–FLAG) has been described previously [30]. For cell-surface-expression assays, an extracellular domain, FLAG-tagged CaR with the FLAG tag inserted between amino acids 371 and 372, was generated and cloned into the pcDNA3.1 vector at the KpnI and XbaI sites (pcDNA3.1–exFLAG–CaR). In order to express His-tagged CaR tail, the human CaR intracellular tail (amino acids 865–1078) was cloned into the Ndel and Sall sites of the pET-28a expression vector by PCR amplification of the CaR tail from a pET15b-CaR tail construct using primers with engineered Ndel and Sall sites. The SRE–Luc (luciferase) reporter plasmid, pSRE–Luc, was donated by Professor Jeffrey E. Pessin (Albert Einstein College of Medicine, New York, U.S.A.).

**Yeast two-hybrid library screening, verification of positive interactions and deletion mapping studies**

The library screen was performed using the lithium acetate method by sequential transformation of library plasmid DNA into the L40 yeast strain pretransformed with the CaR tail bait, essentially as described previously [29]. Following library transformation, cells were amplified for 6 h, plated on to selection medium [84.6 mM succinic acid, 150 mM sodium hydroxide, 543 mM adenine hemisulphate (pH 5.8) with agar, 2% glucose and 1 x yeast nitrogen base (NH₄)₂SO₄] deficient in tryptophan, leucine, histidine, lysine and uracil [29], and examined for the appearance of cotransformants exhibiting CaR-tail-dependent transactivation of the His reporter gene. Potential interacting clones were confirmed by examining LacZ reporter gene activity using a β-galactosidase colony lift assay, comparing known positive and negative cotransformation interactions: pBTM116-CyP40[185–370] + pVP16-Hsp90[520–724] (where Hsp is heat-shock protein) and pBTM116-ARL-E1 + pVP16-Hsp90[520–724] respectively [25,29,31]. Library insert DNA from positive colonies was amplified by PCR and categorized by size and restriction enzyme profiling using the HaeIII site. Plasmid DNA from unique clones was ‘rescued’ by transformation into Escherichia coli HB101 and plating on to M9 plates deficient in leucine [25,29,31]. For final verification of interaction, bait and rescued library plasmid DNA were cotransformed into...
yeast, plated on to selection medium and examined for His and LacZ reporter gene activation. Verified library inserts were sequenced and basic local alignment searches performed to establish sequence identity. For yeast two-hybrid mapping studies, plasmid DNA of deletion constructs of the CaR tail and full-length 14-3-3 isoforms were cotransformed into yeast, plated on to selection medium, and co-transformants were examined for LacZ reporter gene activity. Times to colour development were compared with positive and negative controls to determine the relative strength of reporter activation between each CaR tail deletion and 14-3-3.

**Cells, cell culture and transfections**

HEK (human embryonic kidney)-293 cells were obtained from Professor Karin Eidne (Western Australian Institute for Medical Research, Nedlands, Australia) and HEK-293 cells stably expressing the CaR (HEK-293/CaR) were those used in previous studies [32]. COS-1 cells and the A7 melanoma cell line were obtained from the A.T.C.C. and M2 cells were donated by Professor Fumihiko Nakamura (Brigham and Women's Hospital, Boston, MA, U.S.A.). All cells were propagated in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum) and antibiotics as described previously [30]. In addition, for the HEK-293/CaR and A7 cells, G418 sulfate (Sigma–Aldrich) at 100 μg/ml and 500 μg/ml respectively, was included in the medium to maintain the stable expression of CaR and filamin in these cell lines. Cell monolayers were transfected at 40–60% confluence using Lipofectamine™ 2000 (Invitrogen) as described previously [30].

**Western blot analysis and co-immunoprecipitation studies**

Following transfection, cells were washed, lysed in cell lysis buffer [20 mM Tris/HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA and 1% (v/v) Triton X-100] containing iodoacetamide and protease inhibitors, and the extracted protein was quantified and subjected to Western blot analysis as described previously [30]. Specifically, for co-immunoprecipitations, EGFP-14-3-3 (3 μg) and wild-type or mutant CaR–FLAG (5 μg) were co-transfected into COS-1 or HEK-293 cells and lysed 48 h later. Lysate protein (2 mg) was then pre-cleared with 40 μl of GammaBind™ G Sepharose beads (GE Healthcare Life Sciences) for 1 h at 4 °C. For EGFP–14-3-3 pull-downs, pre-cleared lysate was mixed by rotation overnight at 4°C with 6 μg of a rabbit polyclonal anti-GFP antibody, after which the antibody–protein complex was mixed by rotation for 4 h at 4°C with 40 μl of fresh Sepharose beads. The beads were then washed six times with cell lysis buffer (with the omission of iodoacetamide) and the bound protein was eluted with 40 μl of SDS sample buffer containing 2-mercaptoethanol. Proteins separated by SDS/PAGE (7.5% gel) were blotted and examined for co-immunoprecipitated CaR–FLAG using anti-FLAG M2 monoclonal antibody as described previously [30]. Reciprocal CaR–FLAG pull-downs were performed in essentially the same way, except that lysate containing 1.25 mg of protein was pre-cleared and mixed overnight with 5 μg of anti-FLAG M2 antibody prior to binding on to the beads. Proteins were separated on a PAGE gel (10%) and, for immunodetection, a rabbit polyclonal anti-EGFP antibody (dilution 1:1000) was used as the primary antibody. Note that the CaR is expressed in two forms: a mature form modified with complex carbohydrates (150 kDa) and an immature form modified with high mannose carbohydrates (130 kDa) [1,2].

**In vitro pull-down studies**

A GST fusion protein of 14-3-3 was expressed in the E. coli BL21 codon (+) cells and purified by affinity chromatography using glutathione–Sepharose 4B beads (GE Healthcare Life Sciences) as described previously [33] except that the 14-3-3 protein attached to the beads was cleaved from its GST moiety by thrombin cleavage. The CaR tail was expressed as an His6 fusion protein in the E. coli BL21 codon (+) cells and purified by affinity chromatography using Ni-NTA (Ni2+–nitriilotriacetic acid)–agarose beads in an 8 M urea denaturation/renaturation procedure essentially as described previously [18] with the inclusion of phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM 2-glycerophosphate) as well as protease inhibitors in the buffers. For the pull-down experiments, 32 μg of purified 14-3-3 protein was mixed by rotation overnight at 4°C with an equimolar ratio of purified His6-tagged CaR tail (approximately 50 μl of beads) in renaturation buffer (buffer B) [25 mM imidazole, 0.2% Triton X-100, 2.5 mM 2-mercaptoethanol, 1 mM sodium orthovanadate, 10 mM 2-glycerophosphate all dissolved in PBS (pH 7.4)] after which time, beads (on ice) were washed five times in buffer B then five times in buffer B without Triton X-100. The beads were then resuspended in 50 μl of SDS sample buffer containing 2-mercaptoethanol and boiled for 10 min. Samples were centrifuged and proteins in the supernatant were separated by SDS/PAGE (12% gel) and stained with Coomassie Brilliant Blue.

**Site-directed mutagenesis**

The pcDNA3.1-CaR–FLAG construct was used as a template for the construction of various CaR tail mutants, pcDNA3.1-CaR–FLAG-S895A, in which the serine residue at amino acid 895 was mutated to alanine; pcDNA3.1-CaR–FLAG-ΔRRSNSVS, in which a putative 14-3-3 consensus binding site, encompassing amino acids 890–895, was deleted; and pcDNA3.1-CaR–FLAG-RKR/AAA in which amino acids 896–898 encompassing an ER-retention motif (RKR) were each mutated to alanine. Mutations were generated using the QuikChange® site-directed mutagenesis kit (Stratagene). The part of the CaR tail containing the mutation and bound by the restriction enzymes Smal and Xbal was sequenced and, once authenticated, inserted via a cassette into Smal- and XbaI-digested wild-type pcDNA1-CaR–FLAG [30], then subcloned into wild-type pcDNA3.1-CaR–FLAG using the unique HindIII and Xbal sites.

**Confocal microscopy**

HEK-293/CaR cells were transfected with EGFP–14-3-3 (or EGFP alone) and 24 h later seeded on to poly-l-lysine-coated coverslips and incubated overnight. The next day, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 30 min, then blocked (10% goat serum and 1% BSA in PBS) prior to incubation for 1 h in fresh blocking solution with primary antibody to detect the CaR (1 μg/ml of ADD mouse monoclonal antibody and a 1:750 dilution of a rabbit anti-PDI polyclonal primary antibody to identify the ER). EGFP–14-3-3 was detected by the fluorescence emitted from its EGFP tag. All incubations and washes were performed at room temperature (23°C). Following washing, cells were incubated for 45 min with secondary antibody in fresh blocking solution (1:400 dilution of goat anti-mouse Alexa Fluor® 546 and 1:400 dilution goat anti-rabbit Alexa Fluor® 647 antibodies for detection of CaR and PDI respectively). Cells were mounted on to glass slides using low-flare mounting medium, sealed with nail polish and examined under a Nikon 60× NA (numerical aperture) 1.4 oil-immersion objective.
lens using a Bio-Rad Laboratories MRC-1000/1024 UV confocal laser-scanning microscope with excitation wavelengths of either 488 nm (FITC), 543 nm (tetramethyl rhodamine isothiocyanate) or 633 nm (far-red).

**ERK1/2 assay**

The ERK1/2 assay was based on an assay described previously [34]. HEK-293/CaR cells in 25 cm² flasks were transfected with either EGFP–14-3-3, untagged 14-3-3 or vector alone, and 24 h later seeded on to poly-L-lysine-coated 24-well plates. The following day, cells were serum-starved by incubating overnight in DMEM containing 1.5 mM Ca²⁺ and 0.2 % BSA, and the next day for 30 min in MBS (physiological saline solution) containing 0.5 mM Ca²⁺ and 0.2 % BSA. The cells were then left unstimulated or stimulated with 1, 2 or 4 mM Ca²⁺ in PSS containing 0.1 % BSA for 5 min. The reaction was terminated by the addition of ice-cold PBS to the cells which were then lysed in 100 μl of MAPK lysis buffer [150 mM NaCl, 24.7 mM NaF, 50 mM 2-glycerophosphate, 2 mM sodium orthovanadate, 1 mM dithiothreitol and 2 mM EDTA in 20 mM Tris/HCl (pH 7.4) with 10 % glycerol, 1 % (v/v) Triton X-100 and protease inhibitors] and centrifuged (15,800 g for 3 min at 23 °C), and the protein in the supernatant quantified. Per treatment, 20 μg of protein was separated on a 10 % PAGE gel and examined by Western blot analysis for phosphorylated ERK1/2 using a rabbit polyclonal anti-phospho-ERK1/2 antibody at a dilution of 1:1000 for 1 h followed by HRP-conjugated goat anti-rabbit IgG at a dilution of 1:10,000 for 1 h. Blocking solution and diluent for the antibodies consisted of 5 % BSA in TBS containing 0.2 % Tween 20, otherwise immunodetection was as described previously [30]. For examination of total ERK1/2 expression, the membrane was stripped, blocked and incubated with rabbit polyclonal anti-ERK1/2 antibody at a dilution of 1:5000 for 1 h followed by HRP-conjugated goat anti-rabbit IgG at a dilution of 1:10,000 for 1 h with blocking solution/antibody diluent as described for the detection of phosphorylated ERK1/2.

To examine the effect of 14-3-3ζ depletion on CaR-mediated SRE activity, HEK-293/CaR cells were transfected with an siRNA oligonucleotide primer specific for 14-3-3ζ (or a control oligonucleotide primer), as described in the previous section, and 24 h later were transfected with psRE–Luc. The following day, the cells were grown in DMEM containing 0.5 mM Ca²⁺, 0.1 % BSA and antibiotics overnight in poly-L-lysine-coated six-well plates in readiness for the Luc reporter assay performed as described above. The siRNA primer specific for 14-3-3ζ resulted in over 50 % knockdown of 14-3-3ζ compared with the control primer (Supplementary Figure S1B).

**ELISA–based cell-surface-expression assay**

The assay was based on that described previously [35]. HEK-293 cells were transfected with exFLAG–CaR and after a 4 h incubation seeded into poly-L-lysine-coated six-well plates. The following day, cells were transfected with either untagged 14-3-3 or vector alone and 48 h later were incubated for 90 min at 4 °C with DMEM containing 10 % FBS and 1:2000 dilution (≈2.5 μg/ml) mouse M2 anti-FLAG antibody. Cells were then detached by gently washing in ice-cold PBS and incubated for 1 h at 4 °C in DMEM containing 10 % FBS and 1:5000 dilution HRP-conjugated goat anti-mouse IgG. The cells were then washed and centrifuged (200 g for 2 min at 4 °C), and the cell pellet was resuspended in 200 μl of TMB (3,3′,5,5′-tetramethylbenzidine) liquid peroxidase substrate for ELISA (Sigma–Aldrich) for 20 min at room temperature in the dark. Cells were centrifuged (200 g for 5 min at 23 °C) and 50 μl of the supernatant was transferred (in triplicate) into a 96-well microtitre plate and the reaction stopped by adding HCl (final concentration 0.5 M). Absorbance was read at wavelength of 450 nm using a POLARstar Optima microplate reader. Background readings were ascertained using cells transfected with empty vector alone.

**Densitometry and statistical analyses**

For the ERK1/2 assay Western blots, bands were scanned using a Scanjet 6200C scanner (Hewlett Packard) and densities measured using Scion Image software (Scion Corporation) with correction for background. Phospho-ERK1/2 values were expressed relative to total ERK1/2 and for each experiment the phospho-ERK1/2 values for 0 mM Ca²⁺/empty vector were assigned a value of ‘1’ and other treatment values expressed relative to this.

For ELISA–based cell surface expression and SRE activation studies, mean absorbance and luciferase activity data were analysed via mixed effects models, with each experiment treated as a random effect, and transfection and stimulation conditions as fixed effects. Graphical results are presented as means ± S.E.M. ERK1/2 densities were evaluated in a similar way, with calcium dosage and 14-3-3ζ expression treated as fixed effects. All statistical analyses were conducted in PASW 18 and results assessed at the 5 % significance level.
RESULTS

14-3-3 protein binds to the membrane proximal region of the CaR intracellular tail

Full-length murine 14-3-3ζ protein was identified as a binding partner for the human CaR intracellular tail (Figure 1). The interaction in yeast was verified by co-transformation of rescued library plasmid and CaR tail bait plasmid into L40 yeast and observation of LacZ reporter gene activation using a colony lift assay. Amino acid sequence alignments of the murine and human isoforms of 14-3-3ζ showed a single conservative amino acid difference at amino acid 143, with a glutamate residue in the murine sequence exchanged for aspartate in the human sequence. The near identities of the two isoforms of 14-3-3ζ supported the notion that they would bind identically to the human CaR tail, hence mapping studies using human CaR tail deletions as bait were performed using the murine 14-3-3ζ clone in LacZ reporter gene assays. These studies showed that the 14-3-3ζ protein bound to the proximal membrane region of the CaR tail (amino acid residues 865–922), but not to a juxtaposed C-terminal region (amino acids 923–1078) (Figure 1). The interaction with CaR peptide 865–922 was comparable with that with the full-length CaR tail, indicating that the membrane-proximal region contains the entire binding site. Neither of the two subfragments of the membrane proximal region (amino acids 865–898 and 899–922) was positive in the LacZ reporter assays, suggesting that residues from both subfragments are required for interaction with 14-3-3ζ. In addition, a truncated version of another 14-3-3 isoform, 14-3-3ζε, was identified as a binding partner in the yeast two-hybrid screen. Deletion mapping studies showed that the full-length human 14-3-3ζ isoform bound to the same membrane-proximal region of the CaR as described for 14-3-3ζ (results not shown).

For subsequent studies, the human DNA sequence for 14-3-3ζ was recovered by RT–PCR from Saos-2 cells and cloned into bacterial and mammalian expression vectors (see the Materials and methods section).

14-3-3ζ and the CaR interact directly in pull-down studies in vitro and interact in mammalian cells

To determine whether the 14-3-3ζ protein directly interacts with the CaR tail peptide in vitro, we performed pull-down studies with purified 14-3-3ζ and CaR tail proteins. Bacterially expressed 14-3-3ζ fused to GST was purified by affinity chromatography using glutathione–Sepharose 4B beads, cleaved of its GST moiety and then examined in pull-down experiments with a bacterially expressed His-tagged CaR tail protein that was purified using Ni-NTA beads. It was found that 14-3-3ζ directly interacted with the CaR tail immobilized on Ni-NTA beads (Figure 2, right-hand lane) and no binding of 14-3-3ζ was detected in the absence of the CaR tail protein (Figure 2, middle lane).

In order to determine whether the 14-3-3ζ–CaR interaction observed in yeast also occurs in mammalian cells, we performed co-immunoprecipitation studies. Human full-length 14-3-3ζ, cloned into the vector pcDNA3–EGFP for expression as an EGFP-tagged protein and pcDNA3.1 containing the full-length CaR for expression as a FLAG-tagged protein (CaR–FLAG), were co-expressed in COS-1 or HEK-293 cells. Lysates from co-transfected HEK-293 cells were immunoprecipitated with anti-FLAG antibody to pull down the CaR, 14-3-3ζ was immunoprecipitated with anti-FLAG antibody to pull down the CaR, 14-3-3ζ was found to co-immunoprecipitate (Figure 3A, top panel). In a reciprocal experiment performed in COS-1 cells, when the lysate was immunoprecipitated with anti-CaR antibody to pull down the CaR, 14-3-3ζ was found to co-immunoprecipitate (Figure 3B, top panel). There was negligible non-specific binding of either FLAG–CaR or EGFP–14-3-3ζ to the GammaBind™ G Sepharose beads used for immunoprecipitation (Figure 3A, top panel, and Figure 3B, top panel respectively). The results demonstrate that the CaR and 14-3-3ζ interact in mammalian COS-1 and HEK-293 cells. The same results were obtained in co-immunoprecipitation studies with CaR–FLAG and EGFP–14-3-3ζ ε (not shown).

Binding of 14-3-3ζ to the CaR does not require a 14-3-3 consensus binding sequence in the CaR tail and is independent of PKC phosphorylation

It has been shown previously that 14-3-3 proteins bind to some protein partners via a phosphorylated serine recognition motif, RSx(S/P) [27]. A recognized variant, the so-called serine-rich motif, RXmXXSx(S) (in which at least one serine residue is phosphorylated) [27], is present in the membrane-proximal region of the CaR tail (RRSNVS; residues 890–895) [3]. Therefore we performed experiments to assess its significance in...
14-3-3 protein binding, including the impact of CaR S895A on the ability of 14-3-3 to co-immunoprecipitate the CaR in HEK-293 cells (Figure 3A, top panel), as well as deletion of the entire consensus motif on the ability of mutant and wild-type CaRs to co-immunoprecipitate 14-3-3 in COS-1 cells (Figure 3B, top panel). The results demonstrated that the PKC phosphorylation site at Ser895 and the associated 14-3-3 interaction. Finally, we assessed the potential significance of other PKC phosphorylation sites including Thr888 and Ser915 in the CaR's 14-3-3-binding region on the 14-3-3 consensus binding site in the CaR tail are not required for PKC phosphorylation of the receptor.

14-3-3 and the CaR co-localize in the ER in HEK-293 cells

In order to determine whether 14-3-3 and the CaR co-localize in HEK-293 cells, we expressed EGFP–14-3-3 in HEK-293/CaR cells and used confocal fluorescence microscopy to determine the locations of: (i) the CaR using the anti-CaR ADD antibody in conjunction with an Alexa Fluor® 546-labelled secondary antibody, and (ii) EGFP–14-3-3 via its EGFP tag. The ER was identified using antibody against PDI in conjunction with an Alexa Fluor® 647-labelled secondary antibody. The CaR predominantly localized in the ER, but was also detected in other regions of the cytoplasm and the plasma membrane in some cells (Figure 5, compare panels 1 and 2). EGFP–14-3-3 also localized primarily in the ER in those cells in which it was expressed (Figure 5, compare upper panels 2 and 3). Although a quantitative assessment could not be made, 14-3-3 appeared to have no effect on the pattern of CaR expression (Figure 5, compare CaR localization in cells expressing and not expressing EGFP–14-3-3). These experiments were repeated using EGFP–14-3-3 in place of EGFP–14-3-3 with the same pattern of co-localization with the CaR observed (results not shown).

Overexpression of 14-3-3ζ (but not 14-3-3φ) reduces CaR cell-surface expression, but not through disruption of binding to an RKR retention motif

On the basis of the results of the co-localization studies it was hypothesized that 14-3-3 might play a role in post-translational processing of the CaR in the ER; for example, by masking two established ER-retention motifs (RR, residues 890 and 891, and RKR, residues 896–898) in the CaR tail [16,36–38]. In order to determine whether 14-3-3 specifically binds to the RKR retention motif on the CaR, the RKR residues were each mutated to alanine (AAA) and the impact on binding with 14-3-3 was determined in co-immunoprecipitation experiments (Figure 6). Disrupting the

Figure 3  Wild-type CaR–FLAG and EGFP–14-3-3φ immunoprecipitate, but a putative 14-3-3 consensus binding site on the CaR tail is not required for CaR–14-3-3φ interaction

(A) Effect of mutating the serine phosphorylation site on the consensus binding sequence: lysate protein from HEK-293 cells, transfected with EGFP–14-3-3φ and either CaR–FLAG (wild-type) or CaR–FLAG-S895A (mutant), was immunoprecipitated with an anti-GFP antibody to pull down EGFP–14-3-3φ and examined for co-association of wild-type or mutant CaR using anti-FLAG antibody. (B) Effect of deleting the entire consensus binding sequence: lysate protein from COS-1 cells transfected with EGFP–14-3-3φ and either CaR–FLAG (wild-type) or CaR–FLAG-ΔRSSNVS (deletion mutant), was immunoprecipitated with an anti-FLAG antibody to pull down wild-type or mutant CaR–FLAG and examined for co-association of 14-3-3φ using anti-GFP antibody. For both (A) and (B), the expression levels of EGFP–14-3-3φ and CaR–FLAG (wild-type and mutant) are shown in the middle and lower panels. Blots are representative of three separate experiments for both (A) and (B). The molecular mass is given in kDa on the left-hand side. IP: immunoprecipitation; WB: Western blot.

Figure 4 Neither PKC inhibition nor activation affects CaR–14-3-3φ interaction

(A) Effect of PKC inhibitor (GFX109203X): COS-1 cells transfected with EGFP–14-3-3φ (middle panel) and CaR–FLAG (bottom panel) were treated 48 h later with either vehicle or 2 μM GFX109203X for 1 h prior to lysis and immunoprecipitation of protein with anti-FLAG antibody to pull down CaR–FLAG. Co-associated 14-3-3φ was examined using anti-GFP antibody (top panel). (B) Effect of PKC activator (PMA): COS-1 cells transfected with EGFP–14-3-3φ and CaR–FLAG were treated 48 h later with either vehicle or 100 nM PMA for 1 h prior to lysis and immunoprecipitation with anti-FLAG antibody and detection of co-associated 14-3-3φ (top panel). For both (A) and (B), the middle and bottom panels show expression levels for EGFP–14-3-3φ and CaR–FLAG respectively in untreated and treated lysates. Results are representative of two separate experiments. The molecular mass is given in kDa on the left-hand side. IP: immunoprecipitation; WB: Western blot.
RKR motif had no effect on the binding affinity of either 14-3-3ζ or 14-3-3ζ for the CaR. Nevertheless, it is possible that binding of 14-3-3 to an adjacent region of the CaR may still mask either the RKR motif or upstream RR motif leading to reduced ER retention and forward trafficking to the cell surface [36–38]. To investigate whether overexpression of 14-3-3 affects CaR cell-surface expression, we transiently transfected exFLAG–CaR in the presence of untagged 14-3-3ζ or 14-3-3ζ (or an empty vector control) and performed an intact cell-surface-expression assay using an anti-FLAG antibody. Untagged 14-3-3 isoforms were used for these studies to avoid any interference from fusion tags. Surprisingly, overexpression of 14-3-3ζ significantly reduced CaR cell-surface expression, whereas 14-3-3ζ overexpression, although showing a similar trend, did not significantly reduce CaR cell-surface expression (Figure 7A). Western blot analysis demonstrated an abundance of 14-3-3ζ and, to a lesser extent, 14-3-3ζ over endogenously expressed levels in cells transfected in parallel with those used for the cell surface expression studies (Figure 7B).

Altered expression of 14-3-3ζ and 14-3-3ζ has no effect on CaR-mediated ERK1/2 activation

The region of the CaR tail to which 14-3-3ζ binds mediates various cell signalling events [2,4,7]. In particular, filamin-dependent ERK1/2 activation requires a predicted α-helix proximal to the membrane (residues 868–879) [39], which lies within the 14-3-3-binding domain. Therefore we investigated the impact of 14-3-3ζ overexpression on CaR-mediated ERK1/2 activation (Figure 8). Elevated Ca²⁺ stimulated ERK1/2 phosphorylation as expected; however, at any given Ca²⁺ concentration, there was no appreciable difference in phosphorylated ERK1/2 or in total ERK1/2 between the control cells and cells overexpressing 14-3-3ζ (Figure 8A). Furthermore, when blots from all three experiments were examined by densitometry and the data were pooled, there was no significant difference (P < 0.05) between the mean relative phosphorylated ERK1/2 values of control and overexpressed 14-3-3ζ at any Ca²⁺ concentration including that for 4 mM Ca²⁺ (P = 0.8346) (Figure 8B). Expression of EGFP–14-3-3ζ was confirmed in HEK-293/CaR cells concurrently transfected with EGFP–14-3-3ζ (Figure 8C). Since these experiments were performed with EGFP-tagged 14-3-3ζ, we repeated the experiment with overexpressed untagged 14-3-3ζ to confirm that there was no interference from the tag. As with the tagged 14-3-3ζ experiments, there was no difference in phospo-ERK1/2 activity between control and overexpressed untagged 14-3-3ζ (results not shown). Analogous experiments were performed with untagged 14-3-3ζ with the same outcome (results not shown). In addition, owing to a previously defined role for 14-3-3ζ in MAPK signalling [40], we investigated the effect of transient knockdown of 14-3-3ζ on CaR-mediated ERK1/2 activation in HEK-293/CaR cells using a specific 14-3-3ζ siRNA oligonucleotide primer. No difference in Ca²⁺-dependent activation of ERK1/2 was observed compared to a negative siRNA oligonucleotide control (results not shown).

Overexpression of 14-3-3ζ and 14-3-3ζ attenuates CaR-mediated SRE activation, but depletion of 14-3-3ζ has no effect

Elevated Ca²⁺ activates SRE in CaR-expressing HEK-293 cells via a Rho-dependent pathway that is blocked by expression of a CaR C-terminal minigene encoding residues 906–980 [8]. Since this region overlaps the CaR 14-3-3ζ-binding site (residues 865–922; Figure 1), we investigated whether overexpression of 14-3-3ζ might modulate extracellular Ca²⁺-induced SRE activation in CaR-expressing HEK-293 cells transfected with pSRE–Luc (Figure 9A, HEK-293/CaR). We used untagged isoforms of 14-3-3 for these overexpression studies to avoid any possible interference from tags. Compared with control HEK-293/CaR cells, those in which 14-3-3ζ was overexpressed exhibited a greater than 50% reduction in 5 mM Ca²⁺-induced, SRE-mediated Luc expression (P < 0.05; Figure 9A, HEK-293/CaR, compare ‘I’ and ‘II’). Overexpression of 14-3-3ζ had a similar suppressive effect (results not shown).

Since optimal CaR-mediated SRE activation is dependent upon filamin [8], we next investigated whether the inhibitory effect of 14-3-3ζ might be modulated by filamin, by comparing the impact of elevated Ca²⁺ (5 mM) on SRE activation in CaR and 14-3-3ζ-transfected M2 cells, which do not express filamin, with CaR and 14-3-3ζ-transfected A7 cells, an M2 clone that stably expresses filamin [41]. No difference in the effect of 14-3-3ζ was observed in these two cells types (Figure 9A, compare M2 and A7). A similar outcome was observed for 14-3-3ζ (results not shown). In control experiments, the expression of filamin in A7 cells and its absence in M2 cells was confirmed by Western blotting (Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410995add.htm). However,
Figure 6  Disruption of the RKR retention motif on the CaR has no effect on 14-3-3 binding

Lysate protein from COS-1 cells, transfected with EGFP–14-3-3ζ or EGFP–14-3-3ζ, and either CaR–FLAG (wild-type) or CaR–FLAG-RKR/AAA (mutant), was immunoprecipitated with anti-FLAG antibody to pull down wild-type or mutant CaR and examined for co-associated 14-3-3ζ or 14-3-3ζ using anti-GFP antibody (top panel). Expression levels of EGFP–14-3-3ζ isoforms and CaR–FLAG (wild-type and mutant) in the lysates are shown in the middle and bottom panels respectively. Blots are representative of three separate experiments. The molecular mass is given in kDa on the left-hand side. IP, immunoprecipitation; WB, Western blot.

DISCUSSION

In the present study, a yeast two-hybrid screen revealed that two isoforms of the 14-3-3ζ adaptor protein, 14-3-3ζ and 14-3-3ζ, are binding partners of the CaR intracellular tail and EGFP-tagged forms of these two 14-3-3ζ isoforms were found to interact with CaR–FLAG when overexpressed in mammalian cells. Previous studies have shown that the CaR interacts with endogenous levels of 14-3-3ζ using a pan antibody to immunoprecipitate 14-3-3ζ [16]. There are seven mammalian isoforms of 14-3-3ζ (β, γ, ε, η, σ, θ and ξ), with the θ and ξ isoforms being phylogenetically closely related with approximately 80% amino acid identity [45]. It is therefore not surprising that they both bound to the same membrane proximal region of the CaR tail, exhibited similar patterns of co-localization and co-immunoprecipitation with the CaR, and suppressed CaR-dependent activation of SRE. The 14-3-3ζ isoforms are differentially expressed in tissues and, consequently, appear to play distinct roles in mammalian biology [28,45]. It was from rat brain that 14-3-3ζ was first cloned [46] and subsequently mouse testis in which it plays a role in the control of spermatogenesis via the regulation of Sertoli cell adhesion [47]. Interestingly, H-500 Leydig cell (testicular tumor) growth is controlled in part by the pituitary tumor-transforming gene which is up-regulated by the induction of high extracellular Ca2+ mediated by the CaR [48]. Whether 14-3-3ζ binding to the CaR is required for the control of Leydig and/or Sertoli cell fate or function remains to be determined. The 14-3-3ζ isoform, on the other hand, is widely expressed and plays roles in numerous cellular events [28]. For example, it is an important adaptor protein in the MAPK and Rho family signalling pathways and regulates the cytoskeleton [27,40,42,43].

In many cases, 14-3-3ζ protein binding requires the phosphorylation of specific serine or threonine residues in consensus binding sites on target proteins [27,28]. The location of a 14-3-3ζ phospho-serine recognition motif [27] within the CaR tail-binding region prompted us to examine its significance for 14-3-3ζ binding. Surprisingly, neither mutation of the putative phosphorylation target Ser895 to alanine nor deletion of the entire CaR tail-binding region impaired 14-3-3ζ binding. In addition, we excluded a role for PKC phosphorylation in 14-3-3ζ binding to the CaR since neither the PKC inhibitor GFX109203X nor the PKC activator PMA influenced the 14-3-3ζ–CaR interaction. The results suggest that the CaR may fall into the category of target proteins that do not require phosphorylation for 14-3-3ζ binding, although there is recent evidence that the PKA site at Ser899 could influence 14-3-3ζ binding.

Figure 7  14-3-3ζ overexpression reduces CaR cell-surface expression

(A) HEK-293 cells were transfected with exFLAG–CaR in a single flask and several hours later distributed into six-well plates. Cells were transfected the next day with pcDNA3.1 alone (1), untagged 14-3-3ζ (2) or untagged 14-3-3ζ (3). Post-transfection (48 h), the cells were examined for cell surface expressed CaR, as described in the Materials and methods section, using anti-FLAG primary antibody, HRP-conjugated goat anti-mouse secondary antibody and colour detection with TMB substrate. After stopping the reaction with HCl, the absorbance was measured at 450 nm. Results are means ± S.E.M. from three separate experiments, each performed in triplicate. Absorbance values with different superscripts are significantly different (P<0.05). (B) Confirmation of overexpressed (i) 14-3-3ζ or (ii) 14-3-3ζζ by comparison of cells transfected with vector alone (−) or with untagged 14-3-3ζζ or 14-3-3ζζ (−). Extracellularly FLAG-tagged CaR-expressing cells were transfected concurrently with those used for the cell surface expression assay and lysates were examined 48 h post-transfection by Western blot (WB) analysis using anti-14-3-3ζζ or anti-14-3-3ζζ antibody. Dividing lines in the composite images in (ii) signify lanes grouped from different parts of the same gel. Blots are representative of three experiments performed. The molecular mass is given in kDa on the left-hand side.

filamin expression in A7 cells was considerably less than that detected in HEK-293/CaR cells. Since 14-3-3ζζ is reported to play a key role in Rho signalling [42–44], we also examined the effect of transient knockdown of 14-3-3ζζ on SRE reporter activation in HEK-293/CaR cells using a specific 14-3-3ζζ siRNA oligonucleotide primer. There was no difference in SRE reporter activation compared with a negative siRNA oligonucleotide control (results not shown).
recognition [16]. A number of target proteins bind to 14-3-3 proteins independently of phosphorylation [27]. In one example, inositol polyphosphate 5-phosphatase bound 14-3-3 with high affinity in the unphosphorylated state despite the presence of a 14-3-3-phospho-serine consensus binding motif [49]. In addition, the γ-aminobutyric acid B receptor, which, like the CaR, belongs to GPCR family C, binds to 14-3-3ζ, in a manner that is independent of PKA, PKC or Ca²⁺/calmodulin-dependent protein kinase II phosphorylation [50].

14-3-3 proteins form homodimers or heterodimers with two peptide-binding pockets [27,28,45]. As a result, 14-3-3 dimers can alter the conformational state of a single target protein, or may bind two separate proteins, thereby mediating the formation of protein networks [27,28,45]. This versatility allows 14-3-3 to play diverse functional roles in cell biology, including the modulation of cytoskeletal organization, adhesion, survival and cell-cycle control through interactions with key signalling enzymes such as MAPK, Rho GTPase and Cdc25 (cell division cycle 25) [27,28,40,42–44]. For example, 14-3-3ζ binds cRaf-1 and is critical for its activation [27,40]. It also regulates Rho GTPase by disabling the Rho GEF AKAP (A-kinase-anchoring protein)–Lbc [44]. In view of these findings, and since the membrane-proximal region of the CaR tail to which 14-4-3ζ and 14-3-3ζ bind is also required for the activation of ERK1/2 and Rho-dependent signalling [8,39], we investigated the impact of overexpression or siRNA knockdown of 14-3-3ζ and/or 14-3-3ζ on ERK1/2 and Rho-mediated SRE activation in HEK-293/CaR cells. We found that overexpression of either 14-3-3ζ or 14-3-3ζ attenuated CaR-mediated SRE activation through the Rho pathway, but had no effect on ERK1/2 activation. In addition, when 14-3-3ζ was depleted, there was no effect on ERK1/2 activation or, surprisingly, SRE activation. Although overexpression of 14-3-3ζ did not lead to enhancement of ERK1/2 activation as might have been expected, it is possible that it acts downstream of the elements in the Gi₁₁ and G₁₁ pathways that control ERK1/2 and that its endogenous level of expression is optimal for ERK1/2 activation. The failure of the knockdown strategy to disturb CaR-dependent control of SRE may have arisen from persisting expression of 14-3-3ζ and/or functional redundancy between different 14-3-3 isoforms.

Attenuation of CaR-mediated SRE activation by 14-3-3 overexpression may have arisen from the masking of crucial CaR interaction sites for proteins, such as Rho GTPase, that are required for Rho-mediated SRE activation. Alternatively, excess 14-3-3 might suppress RhoGEF as demonstrated for 14-3-3ζ control of RhoGEF Lfc [51]. In addition, the apparent negative influence of 14-3-3 overexpression on CaR expression at the plasma membrane (at least in the case of 14-3-3ζ; Figure 7) might negatively affect CaR-mediated SRE activity. Filamin has been shown to bind to 14-3-3 in activated T-cells [52] and is important for optimal CaR-mediated SRE activity in HEK-293 cells [8]. Although analyses in M2 cells, which do not express filamin, and the A7 subclone of M2, which stably expresses it, would appear at first to exclude a role for filamin in the attenuation of CaR-mediated SRE activation by 14-3-3 (Figure 9A, compare M2 and A7), we cannot exclude the possibility that filamin levels in A7 cells are suboptimal for achieving a discernible modulating effect (Supplementary Figure S2B).
cells stably expressing the CaR. Trafficking of some cell-surface proteins from the ER to the plasma membrane is promoted by 14-3-3 proteins by masking ER-retention signals in their binding partners [36,38]. In two examples, 14-3-3 binding masks a dibasic retention motif in the KCNK3 K⁺ channel [37] and a tribasic RKR motif in the α-subunit of the KATP channel Kir6.2 [38], thereby disrupting their binding to the vesicle COPI (coat protein I) involved in protein retrieval in the ER. A recent study by Stepanchick et al. [16] has found that the RKR motif of the CaR (amino acids 896–898) is a genuine retention motif with conversion of RKR into AAA, leading to plasma membrane abundance. The RKR motif forms part of an arginine-rich region (residues 890–898), which also includes a dibasic RR motif (residues 890 and 891) that is regulated by phosphorylation at Ser899 by PKA. 14-3-3 proteins have been shown to bind the arginine-rich region with binding considerably reduced when both the RR and RKR sites were converted into alanine residues, suggesting that 14-3-3 interaction. The reduced plasma membrane expression of the CaR resulting from overexpression of 14-3-3ζ is consistent with the observations of Stepanchick et al. [16], but is contrary to the currently held view that 14-3-3 proteins competitively inhibit the binding of COPI to arginine-based motifs to allow release of target protein from the ER and trafficking to the plasma membrane [36–38]. Clearly, further investigation is required to elucidate the role played by 14-3-3 proteins in CaR trafficking.

The 14-3-3 isoforms play key roles in reorganization of the actin cytoskeleton through their regulatory effects on Rho signalling [42–44,51] and the CaR itself initiates cytoskeletal changes through Rho kinase [53]. This raises the possibility that the 14-3-3 association with the CaR may be required for inducing these cytoskeletal changes, a subject worthy of further investigation. In conclusion, the present study demonstrates that two 14-3-3 isoforms, 14-3-3θ and 14-3-3ζ, bind to the membrane-proximal region of the CaR tail, and, when overexpressed, selectively attenuate CaR-mediated SRE activation via the Rho pathway. In addition, 14-3-3ζ reduces plasma membrane expression of the CaR.

**AUTHOR CONTRIBUTION**

Bryan Ward and Thomas Ratajczak devised and supervised the project with input from Ajanthy Arulpragasam, Evan Ingley and Arthur Conigrave. Experimental work was performed largely by Ajanthy Arulpragasam with input from Aaron Magno. Evan Ingley amplified the cDNA library used for the yeast two-hybrid screen. Suzanne Brown performed the statistical analyses. The paper was written by Bryan Ward with suggested comments from all other authors.

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REFERENCES


10 Roy, D., Young, S. H., Yuan, J., Slice, L. and Rozengurt, E. (2005) Amino acid-stimulated Ca\(^{2+}\) oscillations produced by the Ca\(^{2+}\) -sensing receptor are mediated by a phospholipase C\(_{\text{in1d}}\)/inositol 1,4,5-triphosphate-independent pathway that requires G\(_{\text{q/11}}\), rho, filamin-A, and the actin cytoskeleton. J. Biol. Chem. 280, 22875–22882


14 Bai, M., Trivedi, S., Lane, C. R., Yang, Q., Quinn, S. J. and Brown, E. M. (1996) Protein kinase C phosphorylation of threonine at position 888 in Ca\(^{2+}\) -sensing receptor (CaR) inhibits coupling to Ca\(^{2+}\) store release. J. Biol. Chem. 271, 21267–21275


20 Bai, M., Trivedi, S., Lane, C. R., Yang, Q., Quinn, S. J. and Brown, E. M. (1996) Protein kinase C phosphorylation of threonine at position 888 in Ca\(^{2+}\) -sensing receptor (CaR) inhibits coupling to Ca\(^{2+}\) store release. J. Biol. Chem. 271, 21267–21275


Calcium-sensing receptor induces messenger ribonucleic acid of human securin pituitary tumor transforming gene, in rat testicular cancer. Endocrinology 144, 5188–5193


SUPPLEMENTARY ONLINE DATA

The adaptor protein 14-3-3 binds to the calcium-sensitive receptor and attenuates receptor-mediated Rho kinase signalling

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Figure S1 Level of 14-3-3ζ knockdown achieved following transfection of a siRNA oligonucleotide primer specific to 14-3-3ζ in HEK-293/CaR cells

HEK-293/CaR cells were used for experiments examining the effect of 14-3-3ζ depletion on CaR-mediated ERK1/2 activity (A) and CaR-mediated SRE activity (B). Protein expression was examined 48 or 72 h after transfection for ERK1/2 and SRE-Luc assays respectively, to coincide with the time post-transfection that each assay was performed. Western blots (WB) were performed using an antibody specific against 14-3-3ζ. Control experiments used a validated negative oligonucleotide primer.

Figure S2 Levels of expression of filamin 1 in M2, A7, HEK-293 and HEK-293/CaR cells

Western blot (WB) analyses demonstrating the absence of filamin 1 in M2 compared with HEK-293 cells (A) and comparative levels of filamin 1 expression in A7 and HEK-293/CaR cells. Western blots were performed using equivalent amounts of protein [50 μg in (A) and 35 μg in (B)] in conjunction with an anti-filamin 1 monoclonal antibody.

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