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Receptor-activity-modifying proteins are required for forward trafficking of the calcium-sensing receptor to the plasma membrane

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Summary

The calcium-sensing receptor (CaSR) is a class III G-protein-coupled receptor (GPCR) that responds to changes in extracellular calcium concentration and plays a crucial role in calcium homeostasis. The mechanisms controlling CaSR trafficking and surface expression are largely unknown. Using a CaSR tagged with the pH-sensitive GFP super-ecliptic pHluorin (SEP-CaSR), we show that delivery of the GPCR to the cell surface is dependent on receptor-activity-modifying proteins (RAMPs). We demonstrate that SEP-CaSRs are retained in the endoplasmic reticulum (ER) in COS7 cells that do not contain endogenous RAMPs whereas they are delivered to the plasma membrane in HEK 293 cells that do express RAMP1. Coexpression of RAMP1 or RAMP3, but not RAMP2, in COS7 cells was sufficient to target the CaSR to the cell surface. RAMP1 and RAMP3 colocalised and coimmunoprecipitated with the CaSR suggesting that these proteins associate within the cell. Our results indicate that RAMP expression promotes the forward trafficking of the GPCR from the ER to the Golgi apparatus and results in mature CaSR glycosylation, which is not observed in RAMP-deficient cells. Finally, silencing of RAMP1 in the endogenously expressing HEK293 cells using siRNA resulted in altered CaSR traffic. Taken together, our results show that the association with RAMPs is necessary and sufficient to transfer the immature CaSR retained in the ER towards the Golgi where it becomes fully glycosylated prior to delivery to the plasma membrane and demonstrate a role for RAMPs in the trafficking of a class III GPCR.

Keywords

Calcium-sensing receptor; Receptor-activity-modifying protein; siRNA; pHluorin; GPCR

Introduction

G-protein-coupled receptors transduce extracellular stimuli into intracellular signals and constitute the largest and most diverse family of receptor proteins (Bockaert and Pin, 1999). The calcium-sensing receptor is a GPCR that detects subtle changes in the extracellular calcium concentration ($[Ca^{2+}]_{out}$) and acts to maintain systemic calcium homeostasis (Bouschet and Henley, 2005; Brown and MacLeod, 2001; Hofer and Brown, 2003). It was isolated by functional screening from the bovine parathyroid gland, an organ that secretes parathyroid hormone in response to variations in $[Ca^{2+}]_{out}$ (Brown et al., 1993). Importantly, CaSR mutations have been linked to hypercalcaemia and hypocalcaemia (Brown and

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MacLeod, 2001; Hofer and Brown, 2003; Pollak et al., 1993; Pollak et al., 1994). The CaSR is a class III GPCR that shares significant homology with metabotropic receptors for glutamate and γ-aminobutyric acid (mgluRs and $GABA_BRs$, respectively) (Brown et al., 1993). The CaSR regulates a wide range of additional cellular processes, such as proliferation (Mailland et al., 1997), differentiation (Komuves et al., 2002), apoptosis (Lin et al., 1998; Lorget et al., 2000), secretion (Liu et al., 2003) and chemotaxis (Olszak et al., 2000) through the activation of a pleiotropic signalling cascade, including activation of G_q, $G_{i/o}$, G12/13 and MAPK (Hofer and Brown, 2003; Ward, 2004).

Receptor-activity-modifying proteins are single-transmembrane proteins expressed in numerous tissues and cell types (McLatchie et al., 1998; Morfis et al., 2003). Human RAMP1 was isolated during a search for the receptor for calcitonin gene-related peptide (CGRP). The expression cloning strategy yielded a single cDNA that permitted response to CGRP, leading to the subsequent activation of the cAMP-activated chloride channel, CFTR (McLatchie et al., 1998). Two other RAMPs, RAMP2 and RAMP3, were subsequently identified by database searching (McLatchie et al., 1998) and, so far, no additional RAMPs have been reported. Interestingly, whereas RAMP1 transports the calcitonin receptor-like receptor (CRLR) to the plasma membrane and then remains associated with it to function as a terminally glycosylated CGRP receptor, RAMPs 2 and 3 transfer the CRLR to the cell surface to generate receptors that are preferentially selective for adrenomedullin (AM) (McLatchie et al., 1998; Morfis et al., 2003). RAMP1, RAMP2 and RAMP3 share a similar basic domain structure, with 56% sequence similarity but only 31% amino acid sequence identity (McLatchie et al., 1998; Morfis et al., 2003). Recently, using RAMP translocation to the cell surface as a marker of RAMP-receptor interaction, six class II GPCRs were identified as RAMP binding partners (Christopoulos et al., 2003) suggesting that RAMPs may interact with a wide variety of GPCRs (Morfis et al., 2003). To date, however, no class III GPCRs have been identified as RAMP interactors.

In this study, we have investigated CaSR surface expression using a receptor tagged with the modified eGFP reporter molecule super-ecliptic pHluorin (SEP) in two clonal cell lines. With standard GFP tags, it is not possible to distinguish proteins close to the plasma membrane from those that are truly surface expressed. The pH-sensitive GFP, pHluorin (Ashby et al., 2004b; Miesenbock et al., 1998) provides an elegant way to circumvent this problem as regions of membrane protein that are destined to be extracellular are located in the acidic lumen of secretory vesicles (Mellman et al., 1986). The reporter molecule used here is an enhanced version of pHluorin (SEP) that yields brighter and more stable fluorescence. Therefore, CaSR tagged with SEP at the N-terminus (SEP-CaSR) displays little fluorescence inside the cell but gives a bright fluorescent signal upon surface expression. In HEK293 cells, recombinant SEP-CaSR is robustly expressed at the surface whereas in COS7 cells no surface expression of SEP-CaSR was detected. We demonstrate that the difference in SEP-CaSR traffic in these cells is due to the absence of endogenous RAMPs in COS7 cells as coexpression of SEP-CaSR and RAMP1 or RAMP3 results in their association and in surface targeting of the GPCR. Furthermore, we show that RAMPs act to allow forward trafficking of the SEP-CaSR from the ER to the Golgi and, in so doing, facilitate mature glycosylation of SEP-CaSR. Finally, we have observed that traffic of CaSR is altered in HEK293 cells transfected with siRNA targeting RAMP1. Our results thus demonstrate, for the first time, that RAMPs are involved in the trafficking of group III GPCRs and specifically that they regulate the plasma membrane delivery of the CaSR.

Materials and Methods

Materials

The mouse monoclonal anti-CaSR antibody ADD (Goldsmith et al., 1997) was kindly provided by NPS Pharmaceuticals (Salt Lake, USA) and Allen Spiegel (NIH, Bethesda, USA). Rabbit anti-giantin and anti-calnexin antibodies (Covance) were a kind gift of David Stephens (Bristol University, UK). Mouse anti-Myc (9E10), anti-haemagglutinin (HA) (12CA5) antibodies were from Roche and the rabbit anti-GFP from Chemicon. The Alexa 655 anti-GFP antibody was from Molecular Probes. Peroxidase-linked secondary antibodies were from Amersham. Minimal interspecies cross-reaction secondary antibodies coupled to Cy2, Cy3 and Cy5 fluorophores were from Jackson Immunoresearch laboratories. All reagents were of analytical grade.

Generation of the super-ecliptic pHluorin-CaSR (SEP-CaSR) construct

The super-ecliptic pHluorin (SEP) pH-sensitive variant of eGFP was inserted in-frame downstream of the signal peptide sequence of rat CaSR as follows. A *Hin*dIII restriction site was inserted between the Asp23 and Gln24 codons of the CaSR (amino acid number 1 being the initiating methionine) using the PCR overlap extension method and the pRK5-CaSR plasmid [CaSR receptor cDNA into the expression plasmid pRK5 (a kind gift from Solomon Snyder, Johns Hopkins, Baltimore, USA) (Ruat et al., 1995)]. In parallel, the SEP was amplified and flanked with *Hin*dIII sites. Finally, the sequence encoding the SEP was ligated in the newly created *Hin*dIII site on the CaSR. This resulted in the insertion of the SEP amino acid sequence after the Asp23. The resulting plasmid was named pRK5-SEP-CaSR and the integrity of the entire construct was checked by sequencing (Oxford Sequencing Facility).

Cell culture, cDNA transfection and plasmids

Human embryonic kidney (HEK) 293 and COS7 (CV-1 cells transformed with an origin-defective mutant SV40) cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell transfections were performed using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen) with plasmids encoding SEP-CaSR (see above) and Myc-RAMP1, HA-RAMP2 and HA-RAMP3 (a generous gift of Patrick Sexton, Melbourne, Australia). As an internal control, to adjust the cDNA levels and the translation efficiency between the different conditions, cells were transfected with the plasmid encoding the chloramphenicol acetyl transferase (CAT) (a kind gift of Laurent Journot, CNRS, Montpellier, France). Cells were seeded on 6-cm dishes and transfected with 200 ng pRK5-SEP-CaSR and with 4 μg plasmid encoding the RAMP of interest (or CAT as a control) in the combination indicated in the figure legends. Cells were used 48 hours after transfection. For immunofluorescence experiments, cells were transferred onto collagen-coated glass coverslips the day after transfection.

siRNA synthesis, labelling and transfection

Chemically synthesised, double-stranded siRNAs with 19-nucleotide duplex RNA and 2-nucleotide dTdT overhangs were purchased from Ambion and Qiagen. Two sequences corresponding to human RAMP1 were used as targets for siRNAs: GGGACGTGACCTTGACTTA (siRAMP1 #1) and GTTCCAGGTAGACATGGAG (siRAMP1 #2) (Ambion). One unrelated sequence TTCTCCGAACGTGTCACGT that shows no significant homology to any human known gene (analysed using BLAST search) was used as a control and purchased from Qiagen. siRNAs were labelled with Cy3 and

transfected into cells according to the manufacturers' instructions (Ambion and Mirus, respectively).

Immunofluorescence of fixed cells

For surface staining, living cells were rinsed in Earle's low calcium media (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, 25 mM HEPES pH 7.4, 0.9 g D-glucose) to reduce CaSR activity, and incubated with rabbit anti-GFP (for surface SEP-CaSR) and/or mouse anti-HA (for surface HA-RAMP3) in Earle's low calcium buffer supplemented with 2% BSA for 15 minutes at room temperature. After a quick washing step, cells were fixed for 5 minutes in a 4% paraformaldehyde solution without permeabilisation and stained with the appropriate secondary antibody (coupled to Cy3 and/or Cy5). To stain internal proteins, cells were fixed for an additional 20 minutes in a 4% paraformaldehyde solution before permeabilisation with 10% horse serum, 0.1% Triton X-100 in PBS. Cells were subsequently incubated with antibodies as described in the figure legends (antibodies to GFP, Myc, HA, giantin or calnexin) and incubated with the appropriate secondary antibodies coupled to Cy2, Cy3 or Cy5. Samples were analysed by confocal laser-scanning microscopy using a Zeiss LSM510 META confocal system. Experiments were performed three times for each condition and at least 20 cells were imaged per experiment.

Live-cell confocal imaging

All live imaging experiments were performed at 37°C using a 63× water-immersion objective, and cells were continually perfused at 2 ml/minute with pre-heated solutions. Bathing solutions were adjusted to appropriate pH using either NaOH or HCl. When NH_4Cl was used to collapse pH gradients, 50 mM NH_4Cl was added to Earle's buffer in place of equimolar NaCl. Time duration for each condition was 2 minutes. For calcium imaging experiments, cells were loaded with Fura Red-AM (Molecular probes, 5 μM in Earle's buffer) for 1 hour at 37°C before performing experiments. Temporal analysis of fluorescence is shown as changes compared with resting (initial) levels. Regions for analysis were selected manually and positioned to account for any small movements of the cell during the experiment.

RNA preparation and RT-PCR

Total RNA was isolated using Tri reagent as described by the manufacturer (Sigma). RT reactions were performed on 5 μg total RNA using the Promega RT kit. The RT enzyme was omitted in the RT(−) sample to estimate contamination by genomic DNA. Primer sequences against human RAMPs were as described (Moreno et al., 2002) and the pair of primers against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was: 5′-CCCTTCATTGACCTCAACTACATGGT-3′ and 5′-GAGGGGCCATCCACAGTCTTCTG-3′. Reaction mixtures were assembled according to the PCR kit manufacturer (Roche). The PCR amplification conditions were: hot start at 94°C for 5 minutes, denaturation at 94°C for 60 seconds, annealing at 52°C for 60 seconds and extension at 72°C for 60 seconds. After 32 cycles, a final extension at 72°C for 5 minutes was performed. The amplified products were resolved on 1.8% agarose gels.

Surface biotinylation experiments

Living cells were biotinylated using the membrane-impermeant sulfo-NHS-SS-biotin (Sigma, 0.1 mg/ml in PBS) for 15 minutes on ice as described previously (Martin and Henley, 2004). Briefly, cells were rinsed with ice-cold PBS, then with NH_4Cl (50 mM in PBS) for 10 minutes and solubilised in cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS, 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma) and 0.1 mM PMSF. Sonicated lysates were clarified by centrifugation at

12,000 ***g*** for 15 minutes. To isolate biotinylated proteins, equal amounts of supernatant proteins (400 μg) were incubated with streptavidin-agarose beads (Sigma) overnight at 4°C on a rotating wheel. Precipitates were washed three times with lysis buffer and biotinylated proteins were eluted from beads using Laemmli buffer. Finally, biotinylated proteins were subjected to western blotting. As a control, 20 μg total protein were resolved in parallel. The specificity of surface biotinylation was confirmed by the fact that β-actin and Akt, two intracellular proteins, were not present in the surface fraction but were present in the totals (not shown). Bands were quantified using ImageJ 1.30 software (NIH, USA) and normalised to the total receptor fraction. Data were analysed for statistical significance using the GraphPad Prism software (GraphPad software) and are expressed as mean±s.e.m. Unpaired Student's *t*-tests were performed with a Newman-Keuls post-test for multiple comparison data sets.

Coimmunoprecipitation experiments

600 μg protein prepared as above from transfected cells were sequentially incubated at 4°C on a rotating wheel with 2 μg antibody (mouse monoclonal anti-HA, anti-Myc or anti-GFP) for 1 hour, then with protein G-agarose beads (Sigma) overnight. Immunoprecipitates were washed three times with lysis buffer and proteins were eluted from beads using Laemmli buffer. Finally, eluted proteins were subjected to western blotting. As a control, 20 μg total protein (input) were resolved in parallel.

Deglycosylation treatments

Deglycosylation of transfected cell extracts was carried out with the enzymes Endonuclease H or PNGase F (Roche). 30 μg protein were incubated for 16 hours at 37°C in 60 μl lysis buffer at either pH 5.5 (Endo H) or pH 7.5 (PNGase F) supplemented with 4 U Endo H or 4 U PNGase F. Sample incubation in the absence of enzyme was performed as a control. Reactions were stopped by addition of Laemmli sample buffer and heating the samples at 37°C for 15 minutes.

Western blotting

Cell lysates resuspended in Laemmli sample buffer were heated at 37°C for 15 minutes, proteins were resolved by SDS-PAGE, blotted onto nitrocellulose membrane and probed with appropriate primary antibodies. The nitrocellulose was subsequently incubated with anti-rabbit or anti-mouse horseradish peroxidase conjugate (1:5000) for 60 minutes and developed using the Pico chemiluminescence kit (Pierce).

Results

Functional characterisation of super-eclipitic pHluorin-tagged CaSR (SEP-CaSR), a tool to track CaSR cell surface expression

We tagged the CaSR with the pH-sensitive eGFP molecule super-ecliptic pHluorin (SEP), which is essentially nonfluorescent at pH values of ≤6.0 and its brightness increases with pH values up to 8.5 (Ashby et al., 2004b). Plasma membrane-targeted proteins are delivered to the surface via the secretory pathway in vesicular compartments that have an acidic lumen (Mellman et al., 1986). Likewise, endocytosed plasma membrane forms vesicles that are rapidly acidified (Mellman et al., 1986). SEP was inserted in the extracellular N-terminal domain of CaSR, which orientates inside the vesicle lumen (Fig. 1A). Live confocal imaging of SEP-CaSR-transfected HEK293 cells verified that the construct is visible mainly at the cell surface (Fig. 1B). There was a marked reversible decrease in fluorescence in extracellular media at pH 6, and a dramatic increase in total fluorescence in the presence of

NH_4Cl that collapses proton gradients and equilibrates the intracellular pH to the external buffer (pH 7.4).

In western blots, SEP-CaSR was recognised by both the anti-CaSR (ADD) and the anti-GFP antibodies (Fig. 1C). SEP-CaSR was present as two major forms. A doublet at molecular masses ~150 and ~175 kDa, which correspond to monomeric forms of the SEP-CaSR in partial and fully glycosylated states as reported previously for wild-type CaSR (Ray et al., 1997). The second form was of a much higher mass corresponding to stable SDS-resistant homodimers, a property required for CaSR functionality (Bai et al., 1998). These results confirm that SEP-CaSR is expressed at the expected molecular size, forms dimers, and is surface expressed in HEK293 cells.

We next tested whether SEP-CaSR was functional by measuring intracellular calcium mobilisation, one of the main features of native CaSR (Brown and MacLeod, 2001; Hofer and Brown, 2003). SEP-CaSR-transfected HEK293 cells were loaded with Fura Red-AM, a cell-permeant dye whose fluorescence decreases as intracellular calcium rises, and changes in the dye fluorescence were monitored by live confocal microscopy. Following CaSR stimulation by increasing the extracellular calcium concentration from 1 mM to 5 mM $CaCl_2$, we observed a significant oscillating decrease in Fura Red fluorescence in transfected cells but not in non-transfected neighbouring cells. These results demonstrate that SEP-CaSR forms functional receptors.

CaSR is surface expressed in HEK293 but not in COS7 cells

To investigate further the surface expression of SEP-CaSR in HEK293 cells, surface labelling experiments were performed using a rabbit anti-GFP antibody followed by a Cy3-anti-rabbit antibody. The SEP-CaSR observed close to the plasma membrane is truly surface expressed (Fig. 2A). No antibody staining was detected in non-transfected cells.

In contrast to HEK293 cells, when SEP-CaSR was expressed in COS7 cells no surface expression was detected by SEP fluorescence or by immunocytochemistry (Fig. 2B). Rather, fluorescence was restricted to an intracellular compartment. The fact that bright fluorescence was observed inside COS7 cells indicates that the compartment has a relatively neutral pH and that there is abundant SEP-CaSR present. As the compartment in the secretory pathway with the least acidic luminal pH is the ER (Mellman et al., 1986), these results suggest that SEP-CaSR is efficiently expressed in COS7 cells but that it is not able to exit the ER.

To determine if there was any surface expression of SEP-CaSR in COS7 cells we performed surface biotinylation. Two days after transfection, surface proteins were biotinylated and isolated by incubation with streptavidin linked to Sepharose beads. The amounts of surface and total SEP-CaSR were analysed by western blotting with anti-GFP antibody. Consistent with the fluorescence data, the receptor was not detected at the surface of COS7 cells but was abundant in the surface fraction of HEK293 cells (Fig. 2C). Significantly, the monomeric fully glycosylated form of the receptor (molecular mass, 175 kDa) was missing in COS7 cells extracts, indicating incomplete glycosylation.

It has been reported previously that RAMPs are expressed in HEK293 (McLatchie et al., 1998) but not in COS7 cells (Buhlmann et al., 1999). RAMPs are a family of single-transmembrane molecules involved in trafficking and cell surface delivery of several GPCRs (Morfis et al., 2003). There have however, been no previous reports of RAMP association with class III GPCRs. To establish the expression of endogenous RAMPs in our COS7 and HEK293 cell lines we performed RT-PCR using primer pairs directed against RAMP1, RAMP2 or RAMP3 (Moreno et al., 2002). RAMP1 was expressed in HEK293 but not COS7 cells and RAMP2 and RAMP3 were not detected in either cell line (Fig. 2D). Consistent

with published data (Buhlmann et al., 1999; McLatchie et al., 1998), our results confirm that RAMP1 is only expressed in HEK293 cells. These data suggest that the absence of RAMP1 could account for the deficient CaSR surface delivery in COS7 cells.

RAMP1 and RAMP3, but not RAMP2, promote cell surface delivery of the CaSR

We next transfected COS7 cells with SEP-CaSR together with excess Myc-RAMP1, HA-RAMP2, HA-RAMP3 or CAT as a control (1/20 plasmid ratio) and measured SEP-CaSR surface expression by biotinylation. SEP-CaSR was absent from the cell surface in control COS7 cells coexpressing CAT or RAMP2 but there was robust SEP-CaSR surface expression in cells coexpressing RAMP1 and RAMP3 (Fig. 3A,B). Surface labelling of SEP-CaSRs with anti-GFP antibody (Fig. 3C) was entirely consistent with the biotinylation data showing that SEP-CaSR reached the surface of COS7 cells that coexpressed HA-RAMP3 or Myc-RAMP1, but not HA-RAMP2 (not shown) or CAT (control). The surface expression of untagged CaSR was also promoted by RAMP3, indicating that the observed effect of RAMPs on SEP-CaSR is not an artefact due to the tagging of the GPCR with SEP (supplementary material Fig. S1). These results show that RAMP1 or RAMP3 expression is sufficient to promote plasma membrane expression of SEP-CaSR. Furthermore, they demonstrate CaSR-RAMP specificity as RAMP2 was ineffective in these assays.

Subcellular colocalisation of CaSR and RAMPs

RAMPs and their receptor partners form stable complexes that originate in the endoplasmic reticulum (ER) and Golgi apparatus. These complexes are maintained during the processes of translocation to the cell surface, agonist activation, internalisation and trafficking to lysosomes (Hilairet et al., 2001; Morfis et al., 2003). We investigated the level of colocalisation of RAMP SEP-CaSR by immunocytochemistry and confocal imaging. There were extensive overlapping distributions for CaSR and the RAMPs (stained with antibodies against GFP and Myc or HA respectively) inside the cells with defined areas of marked colocalisation (Fig. 4A; purple colour in right-hand panels). In parallel, we assessed the possibility that SEP-CaSR and RAMP3 colocalised at the cell surface. We used RAMP3 as it was most efficient at promoting CaSR surface delivery (Fig. 3). After expression for 48 hours, living cells were simultaneously incubated with antibodies against SEP-CaSR and HA-RAMP3 (rabbit anti-GFP and mouse anti-HA respectively), labelled with corresponding secondary antibodies (conjugated with Cy5 and Cy3 respectively) and imaged by confocal microscopy. Both CaSR and RAMP3 were robustly surface expressed with some areas of defined colocalisation. There was no surface expression of CaSR in the absence of RAMP (Fig. 4B, middle panels) and no antibody staining was detected in untransfected cells (Fig. 4B, upper panels).

The CaSR interacts with RAMP1 and RAMP3

The colocalisation experiments indicate that the GPCR and the single-transmembrane proteins reside in close vicinity within the cell. This prompted us to investigate whether these proteins associate. To test the hypothesis of an interaction between CaSR and RAMPs, we transfected COS7 cells with plasmids encoding SEP-CaSR and Myc-RAMP1 or HA-RAMP3. By using antibodies against the GFP epitope (to isolate proteins present in complex with the CaSR), we were able to immunoprecipitate Myc-RAMP1 (Fig. 5A, lane 3) and HA-RAMP3 (Fig. 5B, lane 3). Remarkably, Myc-RAMP1 was not retrieved in precipitates when the GFP antibody or the SEP-CaSR coding plasmids were omitted (Fig. 5A, lanes 4 and 5), indicating that the precipitation of Myc-RAMP1 occurs in a CaSR-dependent manner. Similarly, HA-RAMP3 was found to be absent from the immunoprecipitates when the SEP-CaSR plasmid was omitted during transfection (Fig. 5B, lane 4). In reciprocal experiments, by using antibodies against the HA epitope (to detect proteins in complex with HA-RAMP3), we were able to coimmunoprecipate the CaSR (Fig. 5C). These biochemical data

confirm the microscopy colocalisation experiments and demonstrate that both RAMP1 and RAMP3 interact with the CaSR when overexpressed in COS7 cells.

RAMP3 allows CaSR translocation from the endoplasmic reticulum to the Golgi apparatus

In the absence of RAMPs SEP-CaSR was retained in an intracellular compartment that we inferred to be the ER (Fig. 2B). Furthermore, in cells that express RAMPs, either endogenously or exogenously, we observed a higher molecular mass SEP-CaSR band consistent with RAMP-dependent post-translational modifications. As RAMPs are known to modulate GPCR glycosylation (Bouvier, 2001; Morfis et al., 2003) we performed dual-labelling experiments for SEP-CaSR in the presence or absence of RAMP3 with markers for the ER and the Golgi apparatus: two intracellular organelles crucial for the glycosylation maturation process. In the absence of RAMP3 (Fig. 6a-h), CaSRs were highly localised with the ER marker calnexin (Fig. 6a-d) with negligible staining in the Golgi apparatus labelled by giantin (Fig. 6e-h). By contrast, when coexpressed with RAMP3, high levels of both SEP-CaSR and RAMP3 were in the giantin-positive Golgi compartment (Fig. 6i-o). Similar data were obtained when CaSR subcellular localisation was estimated by staining with an anti-GFP antibody (not shown). These results suggest that RAMPs promote the trafficking of the SEP-CaSR from the ER to the Golgi.

RAMP3 promotes CaSR glycosylation

We next directly tested the effect of RAMP3 expression on SEP-CaSR glycosylation. In COS7 cell total extract we observed a major species of 150 kDa, which probably represents the core-glycosylated immature form of SEP-CaSR (Ray et al., 1997). In the presence of RAMP3, however, an additional 175 kDa species was observed (Fig. 7). This form corresponding to the mature glycosylated SEP-CaSR, was detected both in the total extract (Fig. 7) and at the cell surface (Fig. 2A-C). We determined the glycosylation status of both forms by in vitro deglycosylation with Endo H, which digests glycoproteins when carbohydrates are incompletely processed and PNGase-F, which removes all N-linked carbohydrates. As expected, in COS7 cells, when SEP-CaSR was expressed alone (Fig. 7, −RAMP), the 150-kDa form was reduced to a 130-kDa form by treatments with Endo H and PNGase F; the Endo H sensitivity confirming that this species represents an immature form of the CaSR that has not yet trafficked from the ER to the Golgi (Bai et al., 1998; Ray et al., 1997). By contrast, the 175-kDa form obtained by RAMP3 coexpression (Fig. 7, +RAMP), which was detected at the cell surface (Fig. 3A), was sensitive to PNGase F but resistant to Endo H, indicating that it corresponds to the mature glycosylated receptor. Importantly, in vitro deglycosylation experiments on cell extracts from HEK293 cells transfected with SEP-CaSR alone yielded the same pattern as COS7 cells cotransfected with SEP-CaSR and RAMP3 (Fig. 7). Taken together, these results indicate that RAMP3 promotes SEP-CaSR transit from the ER to the Golgi apparatus where the receptor is terminally glycosylated prior to delivery to the plasma membrane.

Silencing RAMP1 expression impairs CaSR traffic in HEK293 cells

Our results indicate that RAMP1 and RAMP3 are sufficient to promote CaSR traffic to the plasma membrane. To test the hypothesis that RAMPs are not only sufficient but also required for adequate receptor traffic, RAMP1 expression was silenced in the endogenously expressing HEK293 cells using siRNAs. We selected two specific sequences targeting human RAMP1 to generate siRNAs (named siRAMP1 #1 and siRAMP1 #2, see Materials and Methods). When transfected in HEK293 cells, the siRAMP1 #2 effectively blocked RAMP1 expression (Fig. 8A). By contrast, the siRAMP1 #1 or a control siRNA targeting an unrelated sequence were ineffective.

We next determined the effect of these siRNAs on CaSR traffic (Fig. 8B). HEK293 cells were transfected with SEP-CaSR in conjunction with Cy3-labelled siRNAs (to indicate the siRNA-expressing cells) and surface CaSR was labelled using an Alexa 655 anti-GFP antibody. We observed no effect of the control siRNA or of the inactive siRNA used as a control (siRAMP1 #1, Fig. 8A) on CaSR traffic with receptors present both in the cytoplasm and at the plasma membrane. By marked contrast, in the cells transfected with siRAMP1 #2, which interferes with RAMP1 expression (Fig. 8A), the receptor was found to be abundant in the cell body, forming vacuoles, and poorly expressed at the plasma membrane as we observed in COS7 cells (Figs 2, 3, 6) confirming the necessary role of RAMP in CaSR trafficking.

Discussion

The pH-sensitive GFP tag pHluorin has been used to monitor the lateral diffusion and dynamic removal of AMPA-type ionotropic glutamate receptors from the synapse (Ashby et al., 2004a) but there have been no previous reports using this label to monitor the trafficking properties of a GPCR. Our data demonstrate that SEP-CaSRs are delivered to the plasma membrane of HEK293 cells but not COS7 cells (Fig. 2). Further investigation of this cell-type-specific phenotype revealed that the difference is attributable to the endogenous expression of RAMP1 in HEK293 cells (Fig. 2). Coexpression of RAMP1 or RAMP3 in COS7 cells was sufficient to promote the cell surface delivery of the CaSR (Fig. 3). The mechanism of action of RAMP involves release of SEP-CaSR retained to the Golgi where it is fully glycosylated (Figs 6, 7) prior to delivery to the cell surface (see model, Fig. 9). These results indicate a pivotal role for RAMPs in the trafficking of a class III GPCR to the plasma membrane.

Dynamic interactions between GPCRs and regulatory molecules control all aspects of receptor trafficking and signal transduction properties (Tan et al., 2004). To date, several CaSR transduction properties have been shown to be modulated through binding to partner proteins in addition to heterotrimeric G proteins. For example, a direct interaction between the CaSR and filamin-A is necessary for CaSR agonist-evoked stimulation of the MAPK pathway (Awata et al., 2001; Hjalm et al., 2001) and RhoA activation (Pi et al., 2002). Here, we show that RAMP1 and RAMP3 can be found associated with the CaSR (Figs 4, 5) and that they modulate its forward trafficking. The fact that RAMP2 did not promote targeting of the receptor to the plasma membrane (Fig. 3) indicates a specificity of action within this family of single-transmembrane proteins and is consistent with some previous observations. For example, all RAMPs interact with the CRLR (McLatchie et al., 1998) whereas only RAMP2 interacts with the glucagon receptor (Christopoulos et al., 2003). It is interesting to note that we were unable to detect the inefficient RAMP2 in the HEK293 cells used in this study in contrast to previous reports using the same cell line (McLatchie et al., 1998).

RAMP3 and SEP-CaSR show a degree of colocalisation both in the cell body and at the plasma membrane (Fig. 4) consistent with the fact that two proteins forming a complex (Fig. 5) that traffics to the cell surface as described for RAMP1-CRLR complexes (Hilairet et al., 2001; McLatchie et al., 1998). In the absence of RAMPs, the SEP-CaSR is retained in the ER in an immature core-glycosylated form (Figs 2, 7). By analogy with other GPCR-RAMP interactions we hypothesise that CaSRs can form a complex with endogenous or exogenous RAMP1 or RAMP3 to facilitate ER exit and translocation to the Golgi where it is terminally glycosylated and trafficked to the cell surface (Fig. 9). This model suggests that an as yet unidentified ER retention/retrieval site present in the CaSR is masked by RAMP binding thereby liberating the receptor from its anchor in the ER.

The CaSR can be added to the growing list of GPCRs, including CRLR, AM (McLatchie et al., 1998) and VPAC1 receptors (Christopoulos et al., 2003), whose properties are modulated by RAMPs. Importantly, the CaSR is the first member of the class III GPCR family that has been shown to interact with RAMPs. Whether these findings can be extended to other class III GPCRs remains to be investigated. Several binding partners involved in the traffic of group III receptors have been identified for $GABA_BRs$ and mGluRs. For example, surface expression of functional $GABA_BRs$ requires $GABA_BR1$-$GABA_BR2$ heterodimerisation mediated in part via a C-terminal coiled-coil interaction (Bouvier, 2001). The dendritic and axonal targeting of mGluR5 involves the association of the receptor with the EVH1 (ENA/VASP homology) domain of homer (Fagni et al., 2002). Although no exhaustive screens have been reported, the fact that neither of these interacting domains is present in the CaSR infers that RAMPs may fulfil these trafficking roles for this receptor.

As RAMP1 or RAMP3 expression is both sufficient (Figs 2-7) and necessary (Fig. 8) to promote expression of CaSR at the plasma membrane it is likely that the level of expression of RAMPs determines the presence or absence of functional CaSRs. An attractive possibility is that CaSRs may be expressed and retained or released from the ER depending on the level of available RAMP. Significantly, marked changes in RAMP expression have been reported during certain pathophysiological situations (Udawela et al., 2004). In particular, renal RAMP1 mRNA is dramatically increased in an obstructive nephropathy model (Nagae et al., 2000). The functional consequences of this increase are currently unclear but may lead to a rise in CaSR surface expression. Similarly, the expression of RAMP1 and RAMP3 is increased in rats with chronic heart failure (Cueille et al., 2002). The CaSR is expressed in cardiac tissue and myocytes where its activation triggers intracellular calcium mobilisation (Wang et al., 2003). Given the central role played by calcium signalling in the regulation of the balance between apoptosis and survival (Berridge et al., 2000), enhanced CaSR surface expression could modify the equilibrium between these two states.

RAMPs were originally discovered as proteins regulating both the transport and ligand specificity of the CRLR (McLatchie et al., 1998). The CaSR is known to be activated by a number of diverse agonists including calcium, neomycin, β-amyloid peptide and L-phenylalanine (for a review, see Hofer and Brown, 2003). One intriguing possibility is that RAMPs may be involved in regulating the selectivity of CaSRs for these agonists.

In summary, we have shown a previously unsuspected role for RAMPs in the forwarding trafficking of the CaSR. Our results indicate that RAMP1 or RAMP3 facilitate entry of CaSRs into the secretory pathway by releasing them from the ER to allow the translocation to the Golgi where they undergo terminal glycosylation and surface expression. This previously unsuspected role for RAMPs opens a new avenue of research for this clinically important but still poorly understood receptor and may have wider implications for other group III GPCRs.

Supplementary Material

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Acknowledgments

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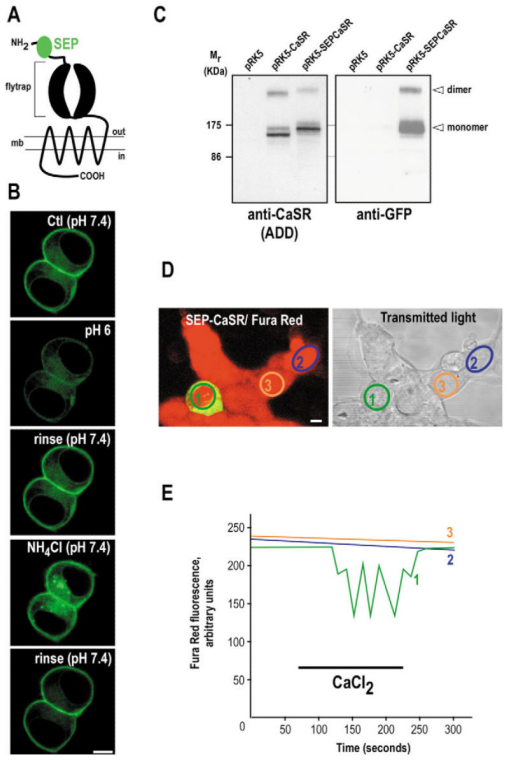

Fig. 1.

Functional characterisation of SEP-CaSR. (A) Schematic representation of super-ecliptic pHluorin-tagged calcium-sensing receptor (SEP-CaSR). The plasma membrane and the flytrap domain are indicated. (B) Live confocal imaging of typical SEP-CaSR fluorescence distribution in two cultured HEK cells transfected with SEP-CaSR. Note that fluorescence is mainly visible at the plasma membrane. SEP-CaSR surface expression was assessed by monitoring fluorescence intensity in response to pH changes: reducing extracellular pH from pH 7.4 (Control, Ctl) to pH 6 causes a decrease in fluorescence as surface SEP-CaSR is eclipsed. Surface fluorescence is restored following a rinse with the pH 7.4 control solution. Application of a solution at pH 7.4 containing NH_4Cl (50 mM), which rapidly equilibrates cellular pH levels, causes a sharp increase as all the SEP-CaSR fluorescence in the cell is revealed. The intracellular fluorescence disappears following a rinse with the control solution. (C) Immunoblot of SEP-CaSR. Cell extracts (~20 μg) from HEK cells expressing pRK5 (empty vector, as a control), pRK5-CaSR or pRK5-SEP-CaSR were run on an 8% polyacrylamide gel and probed with anti-CaSR ADD antibody (left panel) or with anti-GFP antibody (right panel). Position of molecular mass markers is indicated and arrowheads indicate the putative monomeric and homodimeric forms of SEP-CaSR. (D) Intracellular calcium concentration is increased by SEP-CaSR activation. HEK293 cells were transfected with SEP-CaSR (green) and loaded with the calcium-sensitive dye Fura Red (red). Merged areas appear yellow. The corresponding transmitted light image is shown. (E) Graph of the time courses of fluorescence from the regions selected in the fluorescence and transmitted light images in D. Stimulation with 5 mM $CaCl_2$ causes oscillations in the Fura Red fluorescence intensity, which corresponds to $[Ca^{2+}]_i$ oscillations, in the SEP-CaSR-expressing cell. The data are representative of three independent experiments. Bar, 10 μm.

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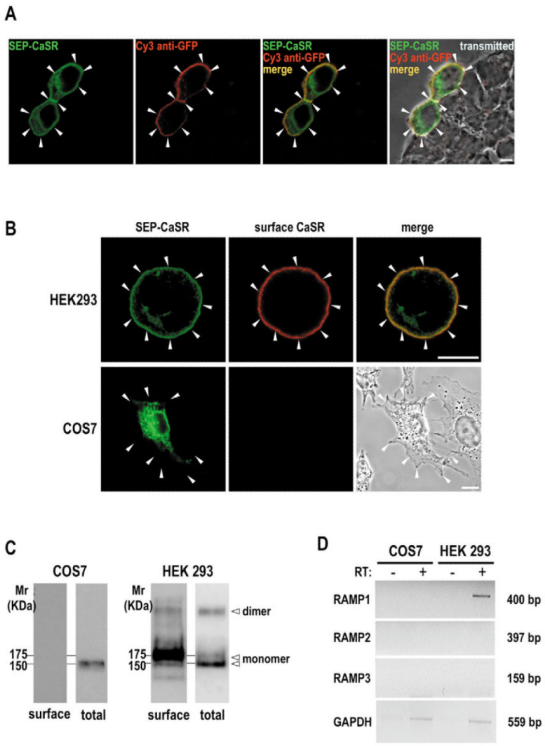

Fig. 2.

Cell-type-specific CaSR surface expression. (A) SEP-CaSR expression at the plasma membrane. Surface CaSR was labelled without permeabilisation in HEK293 cells expressing SEP-CaSR using a rabbit anti-GFP antibody followed by a Cy3 anti-rabbit antibody. Colocalisation of the SEP-CaSR fluorescence (green) and surface-labelled SEP-CaSR (Cy3, red) is revealed in yellow. A corresponding transmitted light image is shown to highlight the localisation of SEP-CaSR at the plasma membrane (arrowheads). Representative micrographs of five independent experiments are shown. (B) Confocal images showing differential CaSR distribution in HEK293 and COS7 cells expressing SEP-CaSR. Surface CaSR was labelled without permeabilisation using a rabbit anti-GFP antibody followed by a Cy3 anti-rabbit antibody. The overlay of total (green, left) and surface SEP-CaSR (Cy3, red, centre) is revealed in yellow (right). Arrowheads indicate the plasma membrane. A corresponding transmitted light image is shown for COS7 cells to highlight the lack of staining at the plasma membrane. Representative micrographs of three independent experiments are shown. (C) Differential CaSR cell surface expression assessed by surface biotinylation experiments. Surface proteins from COS7 and HEK293 cells transfected with SEP-CaSR were isolated by surface biotinylation as described in the Methods and the total and surface CaSR populations revealed by western blotting using an antibody against GFP. Representative blots of four independent experiments are shown. (D) Cell-type-restricted RAMP mRNA expression. RT-PCR amplifications of RAMP1, RAMP2 and RAMP3 mRNA were performed on samples from HEK293 and COS7 cell cultures and resolved on an agarose gel. A similar expression pattern was obtained in all the preparations tested (three independent experiments). Before PCR amplification, samples were (+) or not (−) submitted to reverse transcriptase (RT) treatment to estimate DNA contamination. GAPDH was used as an internal control and sizes of expected products are indicated. Bar, 5 μm (A); 10 μm (B).

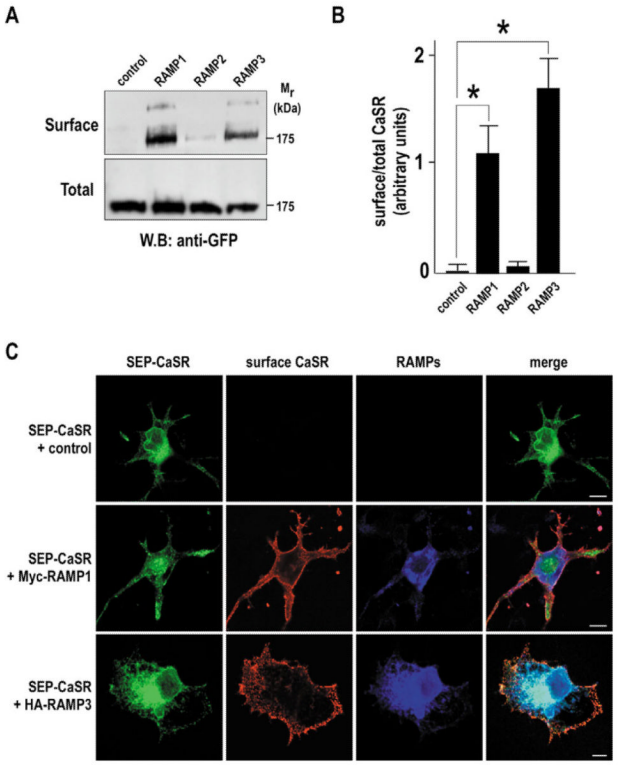

Fig. 3.

RAMP1 and RAMP3, but not RAMP2, promote cell surface delivery of the CaSR. (A) Effect of RAMPs on CaSR surface expression assessed by surface biotinylation. COS7 cells were transfected with SEP-CaSR in combination with a control vector (encoding CAT) or vectors encoding RAMP1, RAMP2 or RAMP3. 48 hours post-transfection, the total and cell surface CaSR populations were determined as described in Fig. 2 legend and Materials and Methods. A representative western blot of four independent experiments is shown. (B) Densitometric analysis of the effect of RAMPs on CaSR surface expression measured by biotinylation assays as illustrated in A. Results are the mean±s.e.m. of four independent experiments. *$P \leq 0.001$ compared with levels in the control (Student's *t*-test). (C) Effect of RAMPs on CaSR surface expression assessed by immunofluorescence. COS7 were transfected with plasmids encoding SEP-CaSR and Myc-RAMP1, HA-RAMP2, HA-RAMP3 or CAT (as a control). Cells were then stained for surface CaSR using sequentially rabbit anti-GFP and Cy3 anti-rabbit antibodies. Cells were then permeabilised and Myc-RAMP1, HA-RAMP2 or HA-RAMP3 were labelled using mouse anti-Myc or anti-HA antibodies followed by Cy5 anti-mouse antibodies. Surface CaSR and RAMPs appear in red and blue respectively. A transmitted light image is shown for the control condition. Images are representative of three independent experiments. Bar, 10 μm.

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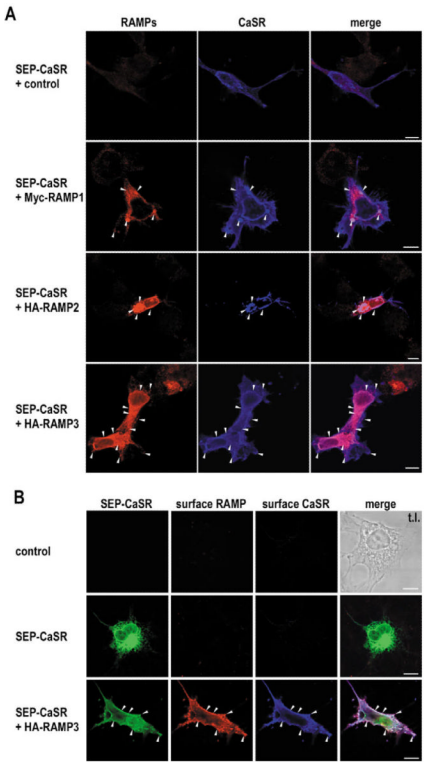

Fig. 4.

Internal and surface colocalisation experiments of CaSR with RAMPs. (A) COS7 were transfected with plasmids encoding SEP-CaSR and Myc-RAMP1, HA-RAMP2, HA-RAMP3 or CAT (as a control). 48 hours post-transfection, cells were stained in permeabilised conditions (see methods) for CaSR and RAMPs using respectively rabbit anti-GFP and mouse anti-HA or Myc antibodies followed by Cy5 anti-rabbit or Cy3 anti-mouse antibodies. Colocalisation regions of RAMPs (red) and CaSR (blue) appear in purple and are indicated by arrowheads. Images are representative of three independent experiments. (B) Surface colocalisation of CaSR and RAMP3. COS7 cells were transfected with either control plasmid (upper panels) or SEP-CaSR in the absence (middle panels) or presence (lower panels) of HA-RAMP3. Cells were incubated in non-permeabilised conditions with anti-GFP and anti-HA antibodies followed by Cy5 anti-rabbit and Cy3 anti-mouse antibodies to label surface CaSR and surface RAMP3 respectively (see Materials and Methods). Regions of colocalisation between surface CaSR (blue) and surface RAMP (red) appear purple and triple colocalisation areas (white) are indicated by arrowheads. Images are representative of three independent experiments. Bar, 10 μm.

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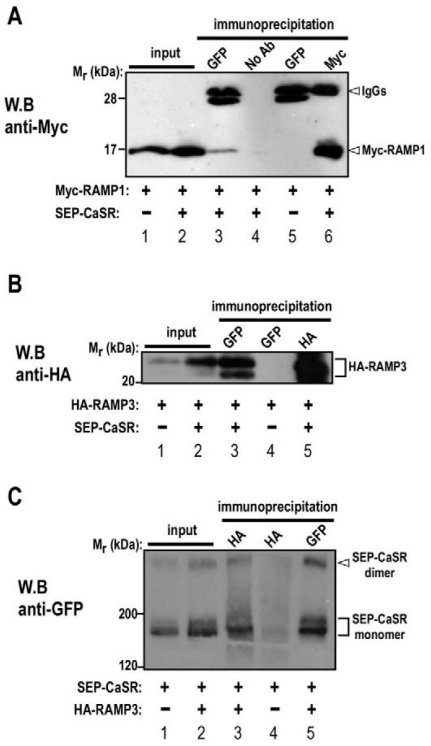

Fig. 5.

The CaSR associates with RAMP1 and RAMP3. COS7 cells were transfected with SEP-CaSR in combination with Myc-RAMP1 and HA-RAMP3 as indicated. 48 hours post-transfection, cells were lysed and 600 μg of proteins were immunoprecipitated with monoclonal anti-HA, anti-Myc or anti-GFP antibodies. (A) RAMP1 is present in CaSR immunoprecipitates. Western blot (W.B.) with anti-Myc antibody (to detect Myc-RAMP1) of total proteins (input, lanes 1 and 2) and of immunoprecipitates isolated by incubation with indicated antibodies (lanes 3-6). Myc-RAMP1 was retrieved in anti-GFP immunoprecipitates prepared from COS7 cells expressing both the SEP-CaSR and Myc-RAMP1 (lane 3) but not when the anti-GFP antibody or the SEP-CaSR coding plasmid were omitted (lanes 4 and 5 respectively), indicating that the binding of Myc-RAMP1 occurs in a CaSR-dependent manner. Immunoprecipitation using anti-Myc antibodies indicates the expected size of immunoprecipitated Myc-RAMP1 (lane 6). (B) RAMP3 is present in CaSR immunoprecipitates. Immunoblot using anti-HA antibody (to detect HA-RAMP3) of total proteins (input, lanes 1 and 2) and of immunoprecipitates (lanes 3-5). RAMP3 was retrieved in anti-GFP immunoprecipitates prepared from COS7 cells expressing both the SEP-CaSR and HA-RAMP3 (lane 3) but not when the SEP-CaSR plasmid was omitted (lane 4), indicating that the binding of the HA-RAMP3 occurs in a CaSR-dependent manner. A positive control immunoprecipitate using anti-HA antibodies was loaded to indicate the expected size of HA-RAMP3 (lane 5). (C) The CaSR is present in RAMP3 precipitates. Western blot using anti-GFP antibody (to detect SEP-CaSR) of total proteins (input, lanes 1 and 2) and immunoprecipitates (lanes 3-5). The CaSR was retrieved in anti-HA immunoprecipitates prepared from COS7 cells expressing both the SEP-CaSR and HA-RAMP3 (lane 3) but not when HA-RAMP3 plasmid was omitted (lane 4), indicating that the binding of the CaSR occurs in a RAMP3-dependent manner. Immunoprecipitation using anti-GFP antibodies indicates the expected size of immunoprecipitated SEP-CaSR (lane 5). The monomeric and dimeric SEP-CaSR forms are indicated.

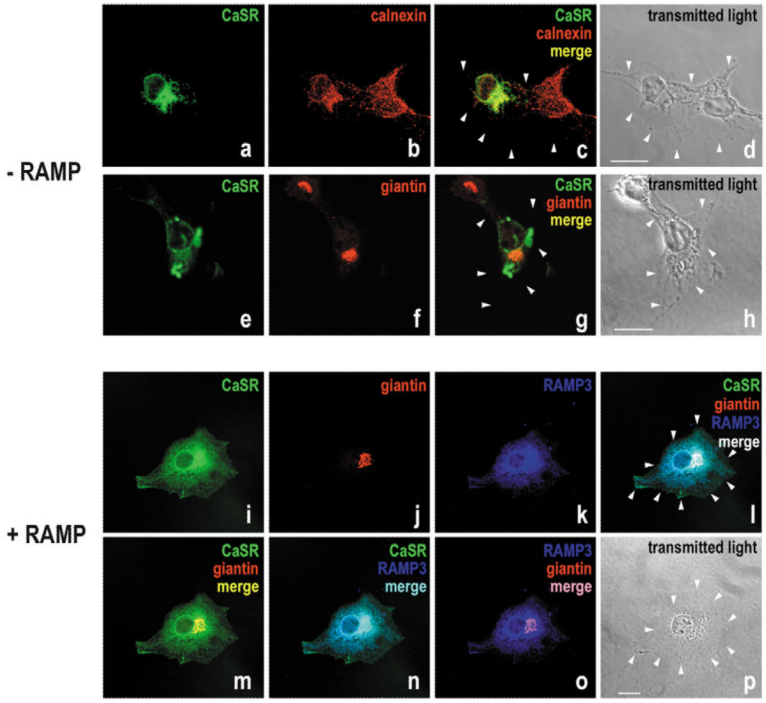

Fig. 6.

RAMP stimulates the traffic of the CaSR from the ER to the Golgi apparatus. Subcellular localisation of the SEP-CaSR in the ER and the Golgi apparatus in COS7 cells expressing the receptor in the absence (upper panels, a-h) or in the presence of RAMP (HA-RAMP3, lower panels, i-o). Cells were fixed and the ER and the Golgi apparatus were stained with antibodies directed against protein markers calnexin and giantin, respectively. In parallel, cells were stained with HA antibody to label HA-RAMP3 and a final wash with a solution at pH of 7.4 containing 50 mM NH_4Cl was performed in order to illuminate the entire SEP-CaSR population (as shown in Fig. 1B). The colors expected for merged areas are indicated. Images are representative of three independent experiments. Bar, 20 μm.

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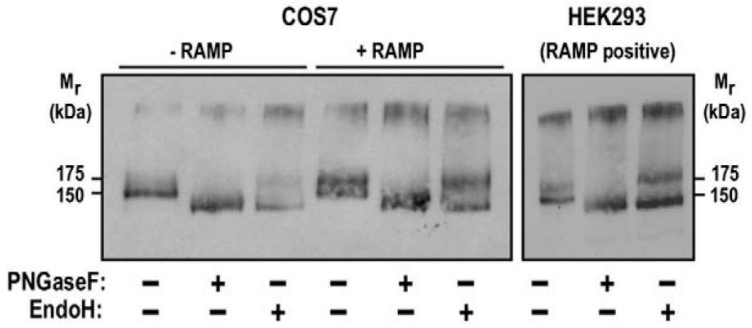

Fig. 7.

RAMPs promote terminal glycosylation of the CaSR. Proteins (30 μg) from HEK293 and COS7 cells expressing SEP-CaSR in the absence or presence of HA-RAMP3 were deglycosylated by incubation with Endo-H or PNGase-F. The samples were subsequently resolved by SDS-PAGE and the different states of glycosylation of the CaSR were revealed by probing with an anti-GFP antibody. The presence or absence of enzyme treatment are respectively indicated by the + and − signs. Gels shown are representative of three independent experiments.

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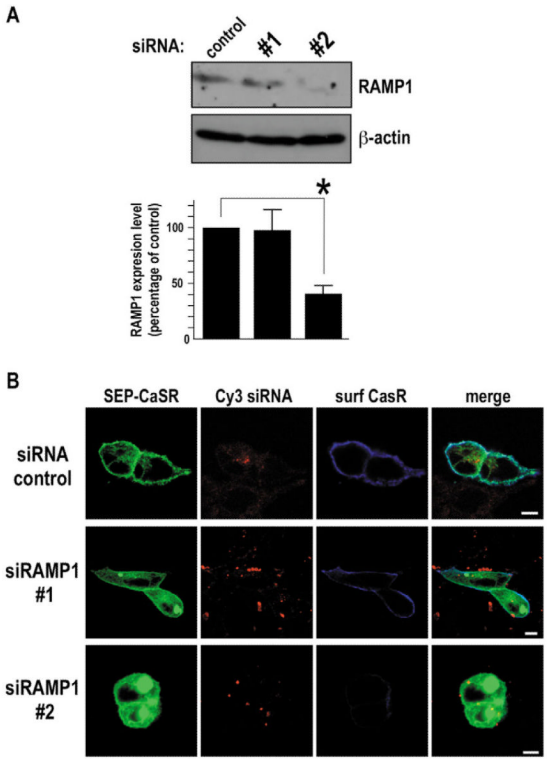

Fig. 8.

Depletion of RAMP1 in HEK293 cells alters CaSR traffic. (A) Silencing of RAMP1 using siRNA. HEK293 cells were transfected with 20 nM siRNAs (control, siRAMP1# 1, siRAMP1 #2, see Materials and Methods) and 500 ng Myc-RAMP1. Whole cell lysates were prepared 48 hours after transfection. The immunoblot was probed with anti-Myc antibody and reprobed with anti-β-actin antibody to ensure equal loading. Films were scanned and quantified using the image J software. A significant decrease of RAMP1 expression (**P*<0.05) was observed with siRAMP1 #2 compared to the expression level in the control. The blots shown are representative of three independent experiments. (B) Silencing RAMP1 alters CaSR traffic. HEK293 cells were transfected with 20 nM Cy3-labelled siRNA against RAMP1 (siRAMP1 #1 and #2) or control siRNAs together with 200 ng pf pRK5-SEP-CaSR. 48 hours after transfection, the surface expressed SEP-CaSR population was labelled with Alexa 655 anti-GFP antibody, cells were fixed and imaging was performed as described in Materials and Methods. SEP-CaSR appears green, Cy3-siRNAs red and surface CaSR blue. Bar, 5 μm.

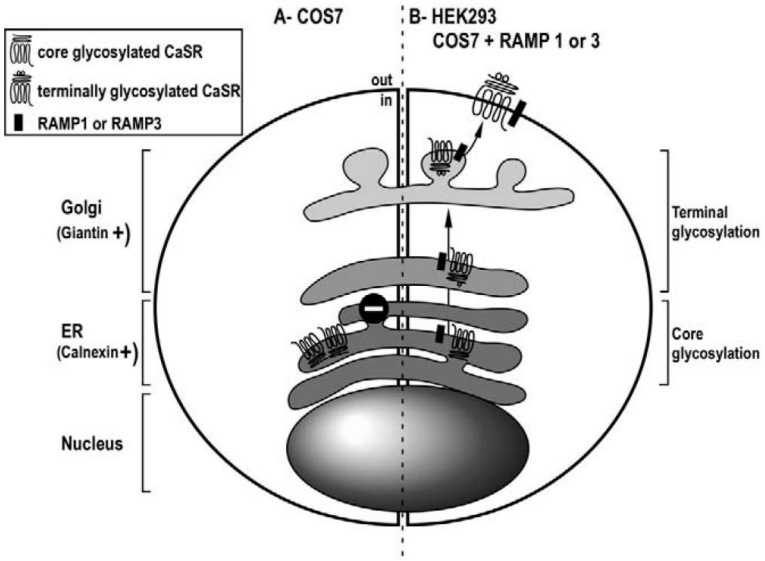

Fig. 9.

Proposed model for RAMP regulation of CaSR trafficking. (A) In the absence of RAMPs (COS7 cells), the CaSR is retained (stop sign) in the endoplasmic reticulum (calnexin-positive compartment) in its incompletely processed, core-glycosylated form. (B) By contrast, in cells expressing RAMP endogenously (such as HEK293 cells), or exogenously (such as RAMP-transfected COS7 cells), the CaSR in association with RAMP1 or RAMP3 bypasses the ER retention and reaches the Golgi apparatus (giantin-positive compartment) where it is terminally glycosylated before being delivered to the cell surface.