NEGATIVE CROSSTALK BETWEEN THE CALCIUM SENSING RECEPTOR AND β-CATENIN SIGNALING SYSTEMS IN COLONIC EPITHELIUM*

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Here we examined the role of the extracellular Ca\textsuperscript{2+}-sensing receptor (CaSR) in the control of colonic epithelial cell proliferation in vivo and changes in β-catenin triggered by CaSR stimulation in human colonic epithelial cells in vitro. The in vivo studies, using a novel Casr intestinal-specific knock out mouse, indicate that the genetic ablation of the Casr leads to hyperproliferation of colonic epithelial cells, expansion of the proliferative zone, changes in crypt structure and enhanced β-catenin nuclear localization. The in vitro results indicate that stimulation of the CaSR, by Ca\textsuperscript{2+} or by the calcimimetic R-568, produced a striking and time-dependent decrease in the phosphorylation of β-catenin at Ser-552 and Ser-675, two amino acid residues that promote β-catenin transcriptional activity. The reduced phosphorylation of β-catenin coincided with a decline in its nuclear localization and a marked redistribution to the plasma membrane. Furthermore, CaSR stimulation promoted a downregulation of β-catenin mediated transcriptional activation. These studies demonstrate that signaling pathways emanating from the CaSR control colonic epithelial cells proliferation in vivo and suggest that the mechanism involves regulation of β-catenin phosphorylation.

Signaling pathways activated by heterotrimeric GTP-binding (G) protein-coupled receptors (GPCRs) expressed in the gastrointestinal (GI) tract play a critical role in the regulation of multiple functions of the digestive system, including cell proliferation, differentiation, inflammation and promotion of colorectal cancer (CRC) (1-8). In particular, the canonical Wingless (Wnt)/β-catenin signaling pathway, which is activated after Wnt binds its receptor complex consisting of the GPCR Frizzled and a co-receptor (9), has emerged as a key regulator of genes that control intestinal cell proliferation and differentiation (10-19). It is also recognized that β-catenin signaling is regulated via Wnt-independent pathways, including phosphorylation of Ser-552 and Ser-675 (20-22). Despite the fundamental importance of the β-catenin pathway in normal and abnormal regulation of the GI tract, including pathogenesis like inflammatory bowel diseases and CRC, the signaling and crosstalk mechanisms involved remain incompletely understood.

The extracellular Ca\textsuperscript{2+}-sensing receptor (CaSR), a member of the C family of heptahelical GPCRs, was originally cloned from parathyroid chief cells (23). Inactivating and activating mutations of the CaSR in humans (24) and genetic disruption of the CaSR gene in mice (25) established that the CaSR functions in the control of Ca\textsuperscript{2+} homeostasis. The CaSR is also present in many other tissues not directly involved in the control of Ca\textsuperscript{2+} homeostasis (26) including both surface and crypt epithelial cells in rodent and human colon (27). Interestingly, the expression of the CaSR is greatly reduced or completely lost in CRC (28-30) suggesting that signaling pathways activated by this receptor negatively control cellular proliferation and CRC development. Indeed, we previously demonstrated that CaSR stimulation inhibits the proliferation of cells derived from normal human colon mucosal epithelium and from human colon carcinomas (31). Furthermore, CaSR stimulation suppresses β-catenin-mediated transcriptional activity in colon carcinoma-derived cell lines (32,33). However, the precise cause-effect relationship of these in vitro observations is not clear. Furthermore, no data is available indicating whether CaSR signaling regulates the proliferation of epithelial cells in the intact colon.

The results presented here show, for the first time, that genetic ablation of the Casr leads to hyperproliferation of colonic epithelial cells, enhanced β-catenin nuclear localization, expansion of the proliferative zone and changes in crypt architecture. Mechanistic studies with cells derived from normal human colon mucosal epithelium demonstrate that CaSR stimulation promotes a decrease in the phosphorylation of β-catenin at Ser-552 and Ser-675 that coincided with its redistribution to the plasma membrane and β-catenin-mediated transcriptional down-regulation. Overall, these studies indicate that CaSR signaling negatively controls colonic epithelial cell proliferation in vivo and suggest that this occurs through a mechanism that involves β-catenin phosphorylation.

**EXPERIMENTAL PROCEDURES**

*Generation and genotyping of conditional Casr knock out mice*

Mice with knock out of Casr genes specifically in intestinal epithelial cells were generated by breeding Casr<sup>floX</sup> mice (34) with transgenic mice expressing Cre-re-
combining under the control of the villin 1 promoter (VilCre, The Jackson Laboratory). We routinely bred male homozygous KO (vilCre/Casr^{flx/flx}) mice, which carry the Cre transgene and both floxed-CaSR alleles, with female Casr^{flx/flx} that carry two floxed-CaSR alleles but no Cre transgene, to produce the cohorts of vilCre/Casr^{flx/flx} and Casr^{flx/flx} mice used in this study. Mouse genotypes were determined by PCR analyses of genomic DNAs from tail snips with primers for the Cre transgene (Cre-1: GCAAAAAGGGCTTCTAGCGTTCC; Cre-2: CTTCTTTCACTATCCAGTTCCG), which amplified a ~500-bp cDNA and the P3U (TGTGACGGAAAACATACTGC) and P3L (CGAGTACAGGCTTTGATGC) primer set for the loxP sequence at the 3' end of exon 7, which amplified a 284-bp DNA fragment from wild-type alleles and a 167-bp DNA fragment from floxed Casr alleles (34). To verify tissue-specific gene excision, genomic DNA was isolated from the tissues specified and was then subjected to PCR analysis with the P4 (CCTCGAACAAGGACACTTAATCGG)/P3L (CGAGTACAGGCTTTGATGC) primer set, which amplified a 284-bp DNA fragment from the Casr gene allele after the excision of exon 7 (34). Homozygous vilCre/Casr^{flx/flx} mice were born in the expected Mendelian ratios and they are grossly normal compared to their control Casr^{flx/flx} littermates. All mice were maintained under standard conditions with free access to food and water under protocols approved by the Animal Care Sub-committee, San Francisco Department of Veterans Affairs Medical Center.

**Tissue fixation, immunohistochemistry and morphometry of conditional Casr knock out mice colon**

Tissue fixation was performed as previously described (34) in animals sacrificed at four to six weeks of age. Fixed colons of males and females mice were embedded in paraffin blocks and 4 μm sections were cut and stained with hematoxylin/eosin. For immunohistochemistry, deparaffinized sections were used. Unmasking was carried out by steaming the sections for 20 min. After inhibition of endogenous peroxidase with hydrogen peroxide and incubation with normal goat serum, a primary monoclonal rat anti-mouse Ki67 antibody was applied. After washing, a secondary biotinylated goat anti-rat IgG antibody was applied and the sections were then incubated with avidin/biotinylated horseradish peroxidase. Development was done with diaminobenzidine nickel or 3-amino-9-ethylcarbazole substrate kits for peroxidase (SK 4100 and SK 4200, Vector Laboratories). Sections were then counterstained with hematoxylin and mounted routinely (35). Hematoxylin- and eosin-stained histological sections were analyzed to determine the effect of CaSR expression deficiency on tissue architecture. Briefly, 20 full-length, longitudinally cut crypts from 5 Casr KO and 5 control littermates were analyzed for crypt height (μm) and number of cells per crypt height. Cross-section of crypts (20/mouse) was used to determine the average crypt diameter (μm) and circumference (in number of cells). These data were used to calculate cell size (crypt height in μm/crypt height in cell number) and estimate the total cells per crypt (mean cells per crypt column × mean crypt circumference). Data from KO and control littermates were represented as mean ± SEM and compared by unpaired Student’s t test. To examine the nuclear content of β-catenin, transversal sections of the distal colon from 3 Casr KO mice and 3 control littermates were stained with a rabbit primary antibody against β-catenin followed by secondary biotinylated goat anti-rabbit IgG antibody and avidin/biotinylated horseradish peroxidase (see above) and counterstaining with hematoxylin. Images acquired using a 2048 x 2048 active pixels Spot Pursuit CCD Camera (Diagnostic Instruments, Inc) were recombined into RG (β-catenin) and B (hematoxylin) color planes and analyzed using the imaging software SP2 V 4.0 (Carls Zeiss Microimaging GmbH). A region of interest (ROI) was used to define the nuclear compartment and the average pixel intensity of β-catenin (RG channels) and hematoxylin (B channel) signals from each ROI was analyzed using SigmaPlot v 9 (Systat Software Inc.). Values represent the mean pixel intensity (MI) ± SEM of β-catenin/hematoxylin and they were compared by Student’s t test.

**cDNA constructs and luciferase reporter vectors**

A vector containing a β-catenin human cDNA (NM_001904) was obtained from Origene (SC107921). Site-directed mutagenesis of this β-catenin cDNA was used to generate one BamHI site 7 nucleotides upstream of the first nucleotide of the initiation codon and another BamHI site immediately downstream of the last nucleotide of the stop codon. The cDNA encoding β-catenin was isolated by BamHI digestion and sub-cloned into the BamHI site of pDsRed-Express-C1 (BD Biosciences). The obtained construct, pRFP-β-catenin, was verified by DNA sequence analysis and the product of expression analyzed by Western blot using a β-catenin monoclonal antibody (BD Transduction Laboratories).

The Firefly luciferase reporter vector of β-catenin mediated transcriptional activation M50 Super 8x TOPFlash (36) and the control plasmid M51 Super 8x FOPFlash, which has mutant TCF/LEF binding sites, were obtained from Addgene (Addgene plasmids 12456 and 12457, respectively). The constitutive luciferase activity generated by the Renilla luciferase reporter vector pHRGTK (Promega Corp.) was used to normalize for transfection efficiency (37).

**Cell culture, Western blot and indirect immunofluorescence**

NCMiCaSR cells is an immortalized cell line derived from normal human colon mucosal epithelium with functional APC and β-catenin proteins (38) that was established and maintained as previously described (31). This cell line expresses the CaSR under the control of a tetracycline inducible promoter (31). Western blot analysis was performed as reported (31,39) and the signals detected with a luminescent image analyzer were normalized by total β-catenin and α-
tubulin and represent the results of three independent experiments. Indirect immunofluorescence was performed as previously reported (31,39) using rabbit anti-β-catenin phospho-Ser-552 and a mouse monoclonal anti-total β-catenin as primary antibodies and AlexaFluor 488 chicken anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG as secondary antibodies. Images were acquired with a 2048 x 2048 active pixels Spot Pursuit CCD Camera (Diagnostic Instruments, Inc) and regions of interest (ROI) corresponding to the nuclear and plasma membrane (cell’s periphery) were defined using the imaging software SP2 V 4.0 (Carl Zeiss Microimaging GmbH). Quantification of the mean pixel intensity of the Red (phosphoSer-552 β-catenin) and Green (β-catenin) channels in the ROI was also determined using the imaging software SP2 V 4.0 software. Values represent the mean pixel intensity (MI) ± SEM and they were compared by Student’s t test.

**Transient transfection of cDNAs encoding RFP-β-catenin and luciferase reporter vectors**

For live NCMiCaSR cells analysis, cells were plated onto 15-mm No. 1 round glass coverslips inside 33-mm dishes at 1 x 10^5 cells/dish and transfected 18-20 h later with 0.4 µg/dish of pRFP-β-catenin or pRFP (pDsRed-Express-C1). Transfections were carried out in Opti-MEM using Lipofectamine Plus according to the manufacturer’s suggested conditions (Invitrogen). After 5 h, the transfection mixture was replaced with DMEM containing 10% fetal bovine serum and supplemented with CaCl2 to a final concentration of 1.4 mM, with or without 0.5 µg/ml of doxycycline to induce CaSR expression. The transfected cells were incubated 18 h before real-time imaging, a time we previously found optimum to detect very low levels of fluorescent-tagged proteins expression (39).

For report vector assays, NCMiCaSR cells were plated in 33-mm dishes at 1 x 10^5 cells/dish and cotransfected as indicated above with 1 µg pTOPFlash/0.2 µg phRGTK per dish or 1 µg pFOPFlash/0.2 µg phRGTK per dish. Five hours later, the transfection mixture was replaced with DMEM containing 10% fetal bovine serum and supplemented with CaCl2 to a final concentration of 1.4 mM, 5 mM or 100 nM R-568 and 1.4 mM CaCl2 without or with 0.5 µg/ml of doxycycline to induce CaSR expression. The reporter activity of the vectors was assayed 24 h post-transfection using a Turner Design luminometer and a dual luciferase assay system as recommended by the manufacturer (Promega Corp.). The luciferase activity values are the result of three independent experiments and correspond to: (pTOPFlash/phRGTK - pFOPFlash/phRGTK) ± SEM. The obtained values were compared by Student’s t test.

**Real-time imaging of RFP-β-catenin intracellular distribution**

In order to maintain a constant temperature of 37°C during the experimental procedures, NCMiCaSR cells grown on the 15-mm glass coverslips were mounted in a perfusion chamber (RC-25 Warner Instrument Corporation) in an epifluorescence microscope (31). The cells were perfused with a saline solution containing 138 mM NaCl, 4 mM NaHCO3, 0.3 mM Na2HPO4, 5 mM KCl, 0.3 mM KH2PO4, 1.4 mM CaCl2, 0.5 mM MgCl2, 0.4 mM MgSO4, 5.6 mM D-glucose, 20 mM HEPES, pH: 7.4 that was preheated at 37°C by a SH-27B solution in-line heater (Warner Instrument Corporation). In order to stimulate the CaSR, the CaCl2 concentration in the perfusion solution was raised to 5 mM during the indicated times. Images were captured as previously described (39). Quantification of RFP-β-catenin plasma membrane was determined as described above.

**Antibodies and kinase inhibitors**

Antibodies were obtained from: GE Healthcare, horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG; BD Transduction, anti β-catenin mouse monoclonal antibody; Invitrogen, AlexaFluor 488 chicken anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG; Cell Signaling Technology, rabbit anti-phospho β-catenin Ser-552, rabbit anti-phospho β-catenin Ser-675 and rabbit anti β-catenin; DakoCytomation, monoclonal rat anti-mouse Ki-67 antigen, clon TEC-3; Vector Laboratories, biotinylated goat anti-rat IgG. The protein kinase A and Akt inhibitors, KT 5720 and GSK 690693, respectively, were obtained from Tocris Bioscience.

**RESULTS**

**Generation of an intestinal-specific deficient Casr mouse**

The phenotype of a generalized Casr knockout (KO) (Castr^+/−) mouse is characterized by markedly elevated serum Ca^2+ and parathyroid hormone levels, parathyroid hyperplasia, bone abnormalities, retarded growth, premature death and incomplete gene excision (25,34). Therefore, that animal model is inadequate to examine the role of CaSR signaling in the proliferation of colonial epithelial cells in vivo. Accordingly, we used a recently developed floxed Casr mouse (34) to specifically delete the Casr in the GI tract by crossing mice with loxP-flanked exon 7 of the Casr (Casr^fl/fl) with mice expressing Cre recombinase under the control of the mouse villin 1 promoter (Castr^−/−Cre). Villin, an actin bundling protein, is expressed in every cell of the intestinal epithelium from duodenum through colon (40).

PCR analysis of genomic DNA extracted from vil-Cre/Castr^fl/fl (Castr KO mouse) and control littermates (animals with two floxed Casr alleles and no vilCre transgene) using the P4/P3L primers exemplifies the successful excision of exon 7 of the Casr mouse as demonstrated by the detection of a PCR product of 284 pb only in the Casr KO mouse (Fig. 1). The excision of exon 7, which renders a non-functional Casr gene, we performed qPCR on RNA extracted from intestinal epithelia and the results showed...
knockdown of CaSR RNA expression in the homozygous Casr KO mice vs controls littermates (see Supplemental Data, Fig. 1).

**Characterization of colonic crypts proliferation and morphometry in intestinal-specific deficient Casr mouse**

If CaSR signaling negatively regulates the proliferation of normal epithelial cells in the colonic crypts, the genetic ablation of this receptor should lead to an increase in their rate of proliferation. As a first step to examine this prediction, colon distal sections of Casr KO and control littermates were fixed and processed for immunohistochemistry using a Ki-67 antibody, a widely used marker of cellular proliferation (44). Results obtained after examining colonic crypts (at least 20 crypts per animal) of Casr KO (n=5) and control littermates (n=5) showed that the proportion of proliferating cells in the Casr KO was markedly higher than in control animals. Specifically, the percentage of proliferating cells in Casr KO mice (mean ± SEM) (52.76 ± 1.3) was 54% higher than in control animals (34.27 ± 0.9) with a statistically significant increase (p < 0.0001). Representative histological sections showing Ki-67 staining indicate that the proliferating crypt cells in the Casr KO mice extended well beyond the transiting region in the mid-crypt (Fig. 2 A).

The binding of the Wnt ligand to its receptor promotes the stabilization and nuclear translocation of β-catenin, a key intestinal regulator of genes involved in the control of cell proliferation and differentiation (45,46). In view of the results presented in Fig. 2 A indicating that CaSR signaling negatively regulates proliferation, we examined whether the genetic ablation of the Casr enhanced the nuclear localization of β-catenin. Representative images of β-catenin staining in colon distal transversal midsections obtained from equivalent locations suggested that the absence of Casr enhanced the nuclear localization of β-catenin (Fig. 2 B). Quantitative analysis of the nuclear content of β-catenin of at least 180 colonocytes of Casr KO mice (n=3) and 180 colonocytes of control littermates (n=3) indicated that the relative concentration of β-catenin in Casr KO (mean ± SEM) (106.1 ± 0.57) was 20% higher than in control animals (88.2 ± 0.64) with a statistically significant increase (p < 0.0001) (Fig. 2 B).

In the GI tract normal cell numbers are maintained by balancing rates of cell proliferation, differentiation, migration, and apoptosis. If increases in proliferative rates are not precisely balanced by the rate of cell removal (e. g. by increase in apoptosis) we should expect a change in the size of the crypts. To examine whether Casr deficiency promoted a change in the colonic crypt architecture, as suggested by the results presented in Fig. 2 A, we performed a quantitative morphometric analysis of the size and total number of epithelial cells in the crypts (47). Accordingly, we measured crypt height (in μm and cell number) and crypt circumference (in μm and cell number) in histological sections of Casr KO mice and control littermates. The data presented in Fig. 2 C indicates that absence of Casr promotes a dramatic increase in the crypt height and in the total number of cells per crypt that is statistically significant without any changes in cell size (see also Supplemental Data, Table 1). Thus, these results show, for the first time, that the genetic ablation of the Casr leads to in vivo hyperproliferation of colonic epithelial cells, expansion of the proliferative zone, changes in crypt structure and to an increase of nuclear β-catenin content.

**β-Catenin Ser-552 phosphorylation regulation by the CaSR and Akt**

The preceding results demonstrating a striking increase in colonic epithelial cell proliferation and enlargement of the crypt in the Casr KO mouse prompted studies to elucidate the mechanism(s) involved. Given the pivotal role of the canonical Wnt/β-catenin signaling pathway in promoting intestinal epithelial proliferation and differentiation (45,46) and the enhanced nuclear accumulation of β-catenin in Casr KO mice, we hypothesized that a crosstalk between CaSR and β-catenin was taking place in colonic epithelial cells.

Phosphorylation of β-catenin plays a fundamental role in its regulation. For example, the sequential phosphorylation of β-catenin in its N-terminal domain at Ser-45 by Casein Kinase 1 (CK1) and Thr-41/Ser-37/Ser-33 by Glycogen Synthase Kinase 3 (GSK3), targets β-catenin for proteosomal degradation. In contrast, β-catenin phosphorylation at Ser-552 and Ser-675 promotes its dissociation from cell-cell contacts, nuclear translocation and transcriptional activity upregulation (20-22). Accordingly, we examined whether CaSR signaling affected β-catenin Ser-552 phosphorylation in human colon-derived epithelial NCMiCaSR cells (31). This cell line was established by infecting NCM-460 cells, an immortalized cell line derived from normal human colon mucosal epithelium (48), with a retrovirus encoding the human CaSR under the control of a Tet promoter (31).

NCMiCaSR cells incubated for 16 h with or without 0.1 μg/ml doxycycline, a member of the tetracycline antibiotics group, were challenged with 5.0 mM Ca²⁺ and lysed at the indicated times. Lysates were analyzed by Western blot using an antibody that specifically detects the phosphorylation of Ser-552 in human CaSR under the control of a Tet promoter (31).

NCMiCaSR cells incubated for 16 h with or without 0.1 μg/ml doxycycline, a member of the tetracycline antibiotics group, were challenged with 5.0 mM Ca²⁺ and lysed at the indicated times. Lysates were analyzed by Western blot using an antibody that specifically detects the phosphorylation of Ser-552 in human CaSR under the control of a Tet promoter (31).
β-catenin as result of CaSR stimulation (Fig. 3 A). No changes were detected in the level of β-catenin phosphorylation at Ser-45, Thr-41, Ser-37 or Ser-33 in NCMiCaSR cells exposed to 5 mM Ca^{2+} or 100 nM R-568 (data not shown). Thus, these results indicate that the CaSR stimulation activates a novel signaling pathway that inhibits β-catenin phosphorylation at Ser-552 in human-colon derived epithelial cells.

The amino acid sequence of β-catenin surrounding Ser-552 is a consensus site for Akt and experimental evidence indicates that Akt leads to the phosphorylation of this residue (20). Furthermore, a recent study implicated β-catenin phosphorylation at Ser-552 via PI3K/Akt as a major mechanism potentiating Wnt signaling in inflammation-induced dysplastic transformation in the colon (50). While these recent findings indicate that Akt phosphorylates β-catenin at Ser-552 in the setting of inflammation, the role of Akt in CaSR-induced β-catenin dephosphorylation at Ser-552 is unknown. Furthermore, recent results using breast and prostate cancer cell lines suggested that modulation of the Akt signaling pathway does not change β-catenin transcriptional activation (51). Accordingly, we determined whether Akt mediates β-catenin Ser-552 phosphorylation in colon-derived epithelial cells. NCMiCaSR cells were incubated with 2.5 µM of the Akt kinase inhibitor GSK 690693 (52,53) for 4 h and lysates analyzed by Western blot using the β-catenin phospho Ser-552 antibody. The results presented in Fig. 3 B show that GSK 690693 promoted over a 50% inhibition in the phosphorylation of β-catenin Ser-552. In contrast, inhibition of cAMP-dependent protein kinase A (PKA) using the selective inhibitor KT 5720 (54-56) only induced a 15% inhibition on Ser-552 phosphorylation (Fig. 3 B), even at a concentration as high as 30 µM (data not shown).

Tyrosine kinase receptors ligands, like epidermal growth factor (EGF) are potent activators of Akt. If CaSR signaling interferes with Akt, we expected that CaSR stimulation should inhibit Akt-mediated Ser-552 phosphorylation in response to EGF. Therefore, NCMiCaSR cells were stimulated with 5mM Ca^{2+} for 4 h and then challenged with EGF (10 ng/ml) for 10 min and lysates examined by Western blot using the β-catenin phospho Ser-552 antibody. As Fig. 3 C shows, EGF promoted over a 50% increase over the basal level of β-catenin Ser-552 phosphorylation that was completely abrogated in response to CaSR stimulation. No reduction in β-catenin Ser-552 phosphorylation in response to EGF stimulation was detected after Ca^{2+} challenge when the CaSR expression was not induced (data not shown). Taken together, these results indicate that Akt is a major player in β-catenin Ser-552 phosphorylation in colon-derived epithelial and that Akt-mediated Ser-552 phosphorylation is inhibited by CaSR signaling.

Next, we determined whether CaSR stimulation decreases the phosphorylation of β-catenin Ser-675 in colon-derived epithelial cells using a phospho-specific antibody (Supplemental Data, Fig. 2). As occurred with β-catenin Ser-552, the results indicated that Ca^{2+}-mediated CaSR stimulation induced a time-dependent decrease in Ser-675 phosphorylation that was similar to that detected after challenging the cells with 100 nM R-568, i. e. ≈ 60 % (Fig. 4). No significant change in the level of β-catenin phosphorylation at Ser-675 was detected in NCMiCaSR cells exposed to 5 mM Ca^{2+} or 100 nM R-568 in the absence of CaSR expression. In addition, no changes in the level of β-catenin phosphorylation at Ser-552 or Ser-675 were detected in the parental NCM-460 cells exposed to 5 mM Ca^{2+} or 100 nM R-568 (not shown). Thus, these results indicate that the CaSR stimulation activates a novel signaling pathway that promotes a decrease in β-catenin phosphorylation at Ser-552 and Ser-675 in human-colon derived epithelial cells.

**β-Catenin intracellular distribution regulation by the CaSR**

Recent evidence indicates that β-catenin phosphorylation is also involved in the regulation of its intracellular distribution (57). For example, phosphorylation at Ser-552 and Ser-675 has been proposed to drive β-catenin from the plasma membrane to the nuclear compartment by promoting its dissociation from cell-cell contacts (20,21,50). Accordingly, and based on the results presented in Figs. 3 & 4, we hypothesized that the decrease observed in β-catenin Ser-552 and Ser-675 phosphorylation after CaSR stimulation should promote the plasma membrane translocation of β-catenin. In order to test this hypothesis, NCMiCaSR cells expressing the CaSR were exposed to Ca^{2+} or challenged with the calcimimetic R-568 for 1 h and processed for indirect immunofluorescence as previously described (39). As illustrated by representative cells displayed in Fig. 5, the distribution of β-catenin phosphorylated at Ser-552 in NCMiCaSR cells in unstimulated control cells expressing (panel A) or not (panel G) the CaSR was predominantly nuclear (arrows). In agreement with the results presented in Fig. 3 A, the expression and stimulation of the CaSR, either by 5.0 mM Ca^{2+} (panel B) or by 100 nM R-568 (panel C), induced a decrease in the reactivity of the phospho Ser-552 antibody throughout the cell, with special emphasis in the nuclear compartment (arrows). Quantitative analysis of the nuclear content of β-catenin phosphorylated at Ser-552 in cells expressing the CaSR (n=30) indicated that its relative content in control cells (mean ± SEM) (115.73 ± 5.14) underwent a 25% reduction in response to Ca^{2+} (87.4 ± 3.89) or R-568 stimulation (86.5 ± 2.3) (Fig 5). No changes in Ser-552 antibody reactivity or signal localization were observed in NCMiCaSR cells irrespective of the treatment, i. e. Ca^{2+} or R-568, when the CaSR expression was not induced (panels H & I). In agreement with these observations, quantitative analysis of the nuclear content of β-catenin phosphorylated at Ser-552 in cells that did not express the CaSR indicated that its relative content in control cells was not significantly affected despite Ca^{2+} or R-568 challenge (see also Supplemental Data, Table 2). Thus, these results further reinforced the conclusion that signaling pathways emanating from the CaSR are associated to a decrease in β-catenin Ser-552 phosphorylation.
In order to determine whether CaSR stimulation promotes the plasma membrane translocation of β-catenin, the cells were co-stained with a mouse monoclonal antibody that recognizes β-catenin independently of its phosphorylation (Fig. 5). This antibody revealed that β-catenin was not only present in the nucleus but also in the cytoplasm and plasma membrane in unstimulated control cells expressing or not the CaSR (panels D & J, respectively). In contrast, this antibody revealed that CaSR stimulation either by Ca\textsuperscript{2+} (panel E, arrows) or by the allosteric activator R-568 (panel F, arrows) promoted a reduction in the nuclear localization of β-catenin accompanied by a substantial redistribution to the plasma membrane as demonstrated by its prominent localization at intercellular plasma membrane junctions. Quantitative analysis of cells expressing the CaSR (n=20) indicated that the relative content of β-catenin in the plasma membrane underwent an increase of over a 30% or 25% in response to Ca\textsuperscript{2+} (110.89 ± 8.1) or R-568 stimulation (105.8 ± 4.43), respectively, compared to control cells (83.14 ± 6.8) (Fig. 5). Importantly, no changes in β-catenin distribution were detected after Ca\textsuperscript{2+} or R-568 challenge when the CaSR expression was not induced (panels K & L, respectively). Quantitative analysis of cells that did not express the CaSR also indicated that Ca\textsuperscript{2+} or R-568 challenge did not significantly modify plasma membrane β-catenin compared to control cells (see also Supplemental Data, Table 2).

Further support for the observation that CaSR stimulation promotes the plasma membrane translocation of β-catenin was obtained by examining the intracellular distribution of a chimeric protein between the red fluorescent protein (RFP) from Discosoma sp. fused to the N-terminus of β-catenin. As illustrated by the representative images in Fig. 6 A, RFP-β-catenin expressed in unstimulated NCMiCaSR cells was distributed throughout the cytosol with very little fluorescent signal localized to the plasma membrane. Real-time imaging revealed that CaSR stimulation induced the translocation of RFP-β-catenin to the plasma membrane, causing a localized fluorescence at the cell periphery. Quantitative analysis of RFP-β-catenin translocated to the plasma membrane (n=10) indicated an increase of over a 30% in response to Ca\textsuperscript{2+} stimulation (122 ± 7.2) compared to control cells (91.2 ± 7.7) (p < 0.01). No change in RFP-β-catenin intracellular distribution in response to 5 mM Ca\textsuperscript{2+} was detected in NCMiCaSR cells when the expression of the CaSR was not induced. Furthermore, no change in the intracellular distribution of the RFP moiety was detected in NCMiCaSR cells after Ca\textsuperscript{2+} challenging despite the expression of the CaSR. Therefore, our results demonstrate that the CaSR signaling is associated to a reduced phosphorylation of β-catenin that coincided with a decline in its nuclear localization and redistribution to the plasma membrane.

**CaSR stimulation downregulates β-catenin mediated transcriptional activation**

Because the transcription of β-catenin targeted genes requires its nuclear localization, we hypothesized that the plasma membrane translocation of β-catenin in response to CaSR stimulation should down-regulates β-catenin mediated transcriptional activation. To examine this hypothesis, we determined the effect of CaSR stimulation on β-catenin transcriptional activity using the pTOPFlash reporter vector (36). As Fig. 6 B shows, stimulation of NCMiCaSR cells with either 5 mM Ca\textsuperscript{2+} or 100 nM R-568 inhibited β-catenin-mediated transcription. Importantly, no inhibition in β-catenin-mediated transcription was detected under the same experimental conditions in the absence of CaSR expression. β-Catenin-mediated transcription was equivalent in NCMiCaSR cells expressing or not the CaSR in the presence of 1.4 mM Ca\textsuperscript{2+}.

**CONCLUDING REMARKS**

A major physiological role of the CaSR, which was originally cloned from parathyroid chief cells (23), is to correct small changes in extracellular Ca\textsuperscript{2+} concentration by regulating parathyroid hormone secretion (24,25). The fact that this GPCR recognize other ligands (23) and that it is expressed in many tissues and organs not involved in the control of Ca\textsuperscript{2+} homeostasis, including the entire GI tract (34,58,59), suggests that the CaSR plays important roles in the regulation of other cellular functions. Nevertheless, no animal model was available to investigate this hypothesis.

The results presented in this study using a recently developed intestinal-specific deficient Casr mouse show that CaSR signaling regulates the proliferation of colonic intestinal epithelial cells in vivo. Specifically, we found that the absence of a functional CaSR is associated to hyperproliferation of epithelial cells in the intact colon, to enhanced β-catenin nuclear localization and to a significant change in the crypt structure including up to a 50% height increase. We also found that in contrast to control littermates, the proliferative zone in the Casr KO mice extended beyond the base of the colonic crypts into the upper third of the colonic crypt. An increased rate of proliferation and a shift of the proliferative zone have both been shown to occur with increased frequency in patients at increased risk of developing CRC (60-63). A variety of dietary factors profoundly influence the proliferation and differentiation of intestinal epithelial cells. In this context, dietary Ca\textsuperscript{2+} has generated considerable attention since clinical trials support an inverse association between Ca\textsuperscript{2+} supplementation (1,200 to 2,000 mg/d) and the recurrence of colorectal adenoma (64-66). It is tempting to speculate that the reported chemopreventive properties of dietary Ca\textsuperscript{2+} are the result of the activation of antiproliferative pathways regulated by the CaSR in colonic epithelial cells.

The binding of the Wnt ligand to its receptor promotes the stabilization and nuclear translocation of β-catenin, a key intestinal regulator of genes involved in the control of cell proliferation and differentiation, including cyclin D1, c-Myc and Lgr5 (45,46). The importance of this pathway in intestinal homeostasis is further emphasized by the fact that over 90% of CRC are associated with deregulated β-catenin
signaling (67). Phosphorylation cascades that are dependent and independent of Wnt play a critical role in the control of β-catenin stability, intracellular distribution and transcriptional activity (20-22,45,50,68,69) implying that β-catenin is a central point of convergence and integration in multiple signal transduction pathways. In support of this hypothesis, the results presented here indicate that an inhibitory crosstalk that regulates β-catenin phosphorylation exist between CaSR signaling and Akt in colon-derived epithelial cells.

When located at the plasma membrane β-catenin interacts with E-cadherin, thereby blocking its nuclear localization, transcriptional activity and the expression of canonical Wnt/β-catenin target genes. Thus, nuclear import/export of β-catenin represents a crucial step in regulating signaling competent β-catenin. Phosphorylation of β-catenin also plays an important role in its intracellular distribution by regulating its interaction with other proteins. In particular, β-catenin phosphorylation at Ser-552 and Ser-675 promotes its dissociation from cell-cell contacts, its nuclear translocation and transcriptional activity (20,21,50). The results presented here show that CaSR stimulation is associated to a plasma membrane redistribution of β-catenin and to a down-regulation of its transcriptional activity. Although the precise mechanism mediating these effects needs further investigation, it is very likely that β-catenin Ser-552 and Ser-675 reduced phosphorylation in response to CaSR signaling affects its nuclear/cytoplasmic shuttling.

To our knowledge, this article provides the first in vivo evidence that CaSR signaling negatively controls colonic epithelial cells proliferation and suggest that this occurs by a mechanism that involves regulation of β-catenin Ser-552 and Ser-675 phosphorylation.

REFERENCES


FOOTNOTES
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The abbreviations used are: CaSR, extracellular calcium sensing receptor; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; GI, gastrointestinal; CRC, colorectal cancer; Wnt, wingless; KO, knockout; CK1, Casein Kinase 1; GSK3, Glycogen Synthase Kinase 3; PKA, cAMP-dependent protein kinase A.

FIGURE LEGENDS

Fig. 1. Mouse tissue-specific deletion of the Casr (A) Colons were fixed and processed for immunocytochemistry using a Ki-67 antibody as described under Experimental Procedures. Ki-67 positive cells were quantified in a sample size of 5 mice/group with at least 20 crypts examined per mouse. Data from KO and control littermates were represented as mean of the % of Ki-67 positive cells ± SEM and compared by unpaired Student’s t test (**p< 0.0001). Bars: 10 µm. Quantification of nuclear β-catenin in colonocytes of Casr KO (KO) mice (B) Same level cross-sections of colonic crypts were processed for immunocytochemistry using a β-catenin rabbit antibody as described under Experimental Procedures. Quantification was performed as described under Experimental Procedures in a sample size of 3 mice/group with at least 60 cells examined
per animal. Values represent the mean pixel intensity (MI) of nuclear β-catenin ± SEM and they were compared by unpaired Student’s t test (**p< 0.0001). **Morphometric analysis of Casr KO colonic crypts (C)** Full-length, longitudinally cut crypts (at least 20 per mouse) from 5 Casr KO and 5 control littermates were analyzed for crypt height (µm) and number of cells per crypt height. Cross-section of crypts (20/mouse) was used to determine the average crypt diameter (µm) and circumference (in number of cells). These data were used to calculate cell size (crypt height in µm/crypt height in cell number) and estimate the total cells per crypt (mean cells per crypt column × mean crypt circumference). Data from KO and control littermates were represented as mean ± SEM and compared by unpaired Student’s t test (**p< 0.0001).

**Fig. 3. CaSR-mediated β-catenin Ser-552 phosphorylation in colonic-epithelial cells (A)** NCMiCaR cells incubated without (-) or with (+) the CaSR inducer doxycycline (0.1 µg/ml) were challenged with 5 mM Ca²⁺ or 100 nM R-568 in a background of 1.4 mM Ca²⁺, lysed at the indicated times and analyzed by Western blot using rabbit antibodies against phospho-Ser552 (pSer552) and total β-catenin. Signals were detected with a luminescent image analyzer and quantified as described under Experimental Procedures. Bars: SD. **Akt-mediated β-catenin Ser-552 phosphorylation in colonic epithelial cells (B)** NCMiCaSR cells were incubated with 2.5 µM of the Akt kinase inhibitor GSK 690693 or with 3.0 µM of the PKA inhibitor KT 5720 for 4 h and lysates analyzed by Western blot using rabbit antibodies against phospho-Ser552 and total β-catenin. Signals were detected with a luminescent image analyzer and quantified as described under Experimental Procedures. Bars: SD. **Akt-mediated β-catenin Ser-552 phosphorylation inhibition by the CaSR (C)** NCMiCaSR cells incubated with (+) doxycycline (0.1 µg/ml) were stimulated with 5 mM Ca ²⁺ for 4 h and then challenged with EGF (10 ng/ml) for 10 min. Cells were lyzed and lysates examined by Western blot using rabbit antibodies against phospho-Ser552 and total β-catenin. Signals were detected with a luminescent image analyzer and quantified as described under Experimental Procedures. Bars: SD.

**Fig. 4 CaSR-mediated β-catenin Ser-675 phosphorylation** NCMiCaR cells incubated without (-) or with (+) doxycycline (0.1 µg/ml) were challenged with 5 mM Ca²⁺ or 100 nM R-568 in a background of 1.4 mM Ca²⁺, lysed at the indicated times and analyzed by Western blot using rabbit antibodies against phospho-Ser-675 (pSer675) and total β-catenin. Signals were detected with a luminescent image analyzer and quantified as described under Experimental Procedures. Bars: SD.

**Fig. 5. CaSR-mediated β-catenin Ser-552 phosphorylation and plasma membrane localization** NCMiCaR cells incubated without (-) or with (+) doxycycline (0.1 µg/ml) were challenged with 5.0 mM Ca²⁺ or 100 nM R-568 in a background of 1.4 mM Ca²⁺ for 1h and processed for immunofluorescence using a rabbit phospho-Ser552 antibody and mouse monoclonal antibody anti-total β-catenin. The images displayed are representative of 90% of the population of cells. Bars: 10 µm. Quantification was performed as described under Experimental Procedures. The values represent the mean pixel intensity (MI) ± SEM and they were compared by unpaired Student’s t test (**p< 0.005, *<0.036). **Fig. 6. Real-time analysis of β-catenin intracellular distribution in response to CaSR stimulation (A)** NCMiCaR cells expressing or not the CaSR were transfected with a construct encoding RFP-β-catenin or RFP as indicated under Experimental Procedures. The intracellular distribution of RFP-β-catenin and RFP in response to 5 mM Ca²⁺-mediated CaSR stimulation was examined in real-time with an epifluorescence microscope. Quantification was performed as described under Experimental Procedures. The values represent the mean pixel intensity (MI) ± SEM and they were compared by unpaired Student’s t test (*<0.01). Bar 10 µm. **CaSR-mediated β-catenin transcripational activity regulation (B)** NCMiCaR cells not expressing (Control) or expressing the CaSR were challenged with 5 mM Ca²⁺ or 100 nM R-568 and 1.4 mM Ca²⁺ and the luciferase activity determined as described under Experimental Procedures. The luciferase activity values are the result of three independent experiments ± SEM and they were compared by unpaired Student’s t test (*p< 0.01).
**FIGURE 5**

CaSR expression induction

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<th>Ca(^{2+})</th>
<th>R-568</th>
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β-catenin

- pSer552
- (total)

**FIGURE 6**

CaSR expression

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<td><img src="imageC" alt="RFP" /></td>
<td><img src="imageD" alt="Control" /></td>
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</tbody>
</table>

Plasma membrane β-catenin (MI)

- Ca\(^{2+}\)
- Control

Luciferase activity (%)

- R-568
- Ca\(^{2+}\)
- Control

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