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Authors

Tu, Chia-Ling
Chang, Wenhan
Xie, Zhongjian
et al.

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Inactivation of the calcium sensing receptor inhibits E-cadherin-mediated cell-cell adhesion and calcium-induced differentiation in human epidermal keratinocytes

Chia-Ling Tu[1, 3], Wenhan Chang[1, 3], Zhongjian Xie[1, 2] and Daniel D. Bikle[1, 3]
From Endocrine Unit, Veteran Affairs Medical Center[1], Northern California Institute for Research and Education[2], and University of California, San Francisco[3], California 94121

Running title: CaR regulates E-cadherin-mediated intercellular adhesion

Address correspondence to Dr. Chia-Ling Tu, Endocrine Unit (111N), VA Medical Center, 4150 Clement Street, San Francisco, California 94121. Tel: (415) 7502089; Fax: (415) 7506929; E-mail: chia-ling.tu@ucsf.edu

Extracellular Ca^{2+} (Ca^{2+}_o)[1] is a critical regulator that promotes differentiation in epidermal keratinocytes. The calcium sensing receptor (CaR) is essential for mediating Ca^{2+} signaling during Ca^{2+}_o -induced differentiation. Inactivation of the endogenous CaR gene by adenoviral expression of a CaR antisense cDNA inhibited the Ca^{2+}_o-induced increase in intracellular free calcium (Ca^{2+}_i) and expression of terminal differentiation genes, while promoting apoptosis. Ca^{2+}_o also instigates E-cadherin-mediated cell-cell adhesion, which plays a critical role in orchestrating cellular signals mediating cell survival and differentiation. Raising Ca^{2+}_o concentration ($[Ca^{2+}]_o$) from 0.03 to 2 mM rapidly induced the co-localization of α-, β-, and p120-catenin with E-cadherin in the intercellular adherens junctions (AJs). To assess whether CaR is required for the Ca^{2+}_o-induced activation of E-cadherin signaling, we examined the impact of CaR inactivation on AJ formation. Decreased CaR expression suppressed the Ca^{2+}_o-induced AJ formation, membrane translocation, and the complex formation of E-cadherin, catenins, and the phosphatidylinositol 3-kinase (PI3K), although the expression of these proteins was not affected. The assembly of E-cadherin/catenin/PI3K complex was sensitive to the pharmacologic inhibition of Src-family tyrosine kinases, but was not affected by inhibition of Ca^{2+}_o-induced rise in Ca^{2+}_i. Inhibition of CaR expression blocked the Ca^{2+}_o-induced tyrosine phosphorylation of β-, γ- and p120-catenin, PI3K, and the tyrosine kinase Fyn, and the association of Fyn with E-cadherin and PI3K. Our results indicate that the CaR regulates cell survival and Ca^{2+}_o -induced differentiation in keratinocytes at least in part by activating the E-cadherin/PI3K pathway through a Src-family tyrosine kinase-mediated signaling.

Introduction

Keratinocytes proliferate and differentiate in cultures in a manner recapitulating the process of epidermal differentiation in vivo (1). Raising the $[Ca^{2+}]_o$ above 0.07 mM triggers an acute and then a sustained increase in Ca^{2+}_i, and the onset of terminal differentiation (2,3). Blocking the increase in Ca^{2+}_i with BAPTA, a Ca^{2+}_i chelator, inhibits the ability of Ca^{2+}_o to induce differentiation (4,5). Previous studies demonstrated an obligatory role for the CaR, a G-protein-coupled receptor, in mediating Ca^{2+} signaling and Ca^{2+}_o-induced keratinocyte differentiation (6-8). Activation of the CaR by Ca^{2+}_o stimulates phospholipase C (PLC) to convert phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglylcerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 in turn induces Ca^{2+} release from internal stores and increases Ca^{2+}_i (9). Inhibition of CaR expression led to a decline of Ca^{2+}_i pools and altered cellular Ca^{2+} handling, hence reducing the Ca^{2+}_i response to Ca^{2+}_o and impairing differentiation in keratinocytes (8).

One of the immediate cellular responses to Ca^{2+}_o in epithelial cells is the formation of cell-cell contacts, a process mediated by the adhesion molecule E-cadherin (10). E-cadherin is a major classical cadherin in keratinocytes and is expressed through out the epidermis (11). Upon Ca^{2+}_o stimulation, the extracellular portion of E-cadherin interacts with E-cadherin molecules on

the surface of neighboring cells, whereas its cytoplasmic tail interacts with β- (or γ-), α-, and p120-catenins to form the core adhesive structure of adherens junctions (AJ) (12). E-cadherin-mediated cell adhesion plays key roles in remodeling of epithelial cell-cell interaction and maintaining proper epidermal differentiation (10,13). Loss of E-cadherin in the epidermis leads to a loss of AJ and impaired terminal differentiation (14). The sequential binding of catenins physically links E-cadherin to the actin cytoskeleton and other signaling molecules, including phosphatidylinositol-3-kinase (PI3K) (10,15). Keratinocyte differentiation induced by Ca^{2+}_o necessitates the activation of PI3K. Pharmacological inhibition of PI3K blocks the expression of late differentiation markers and induces apoptosis in differentiating keratinocytes (16,17). Through interactions with the E-cadherin/catenin complex PI3K is recruited to the cell membrane, where it converts PIP_2 to phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 in turn binds and activates PLCγ1 (17), which is required for maintaining the Ca^{2+}_o-induced increase in Ca^{2+}_i crucial for keratinocyte differentiation (18). Inactivating E-cadherin function by specific antibody or blocking E-cadherin expression by siRNA prevents Ca^{2+}-induced activation of PI3K and, thus, keratinocyte differentiation (16,19). Besides E-cadherin, our recent studies demonstrated that the Ca^{2+}_o-induced recruitment of PI3K to cell membrane, activation of PI3K, and keratinocyte differentiation require β- and p120-, but not γ-catenin of PI3K. Therefore, E-cadherin-dependent cell adhesion plays a key role not only in coordinating cellular organization and movement in epidermis, but also in transducing cellular signals that influence keratinocyte differentiation.

Keratinocyte differentiation induced by Ca^{2+}_o is accompanied by increased tyrosine phosphorylation (20,21). Many studies have demonstrated that tyrosine kinase activity is necessary for the assembly of AJ and the interaction of PI3K with the E-cadherin/catenin complex. Pharmacological inhibition of tyrosine kinase perturbs the formation of AJ and prevents activation of PI3K by Ca^{2+}_o (16,22,23). In differentiating mouse keratinocytes, Ca^{2+}_o-induced assembly of E-cadherin/catenin complex and the recruitment of PI3K to E-cadherin are accompanied by tyrosine phosphorylation of β-, γ-, and p120-catenin (22). The stimulation of E-cadherin/PI3K signaling by Ca^{2+}_o involves activation of Src-family tyrosine kinases. Inhibiting Src-family tyrosine kinases effectively blocks formation of AJ (22), abolishes Ca^{2+}_o-activation of PI3K and suppresses expression of differentiation markers in keratinocytes (17). Consistent with the notion that Src family kinases control keratinocyte cell-cell adhesion, Fyn tyrosine kinase colocalizes with E-cadherin at the cell membrane (22). Additionally, Fyn-deficient keratinocytes exhibit decreased tyrosine phosphorylation of β-, γ-, and p120-catenin, abnormalities in cell adhesion (22), and compromised differentiation (21). Hence, in contrast to what has been found in transformed or mitogenically stimulated cells, tyrosine phosphorylation plays a positive role in control of cell adhesion in differentiating keratinocytes (24).

Ca^{2+}_o activates CaR-mediated Ca^{2+}_i signaling and E-cadherin-mediated cell-cell adhesion that lead to differentiation. In the present study, we investigated the involvement of CaR in the activation of E-cadherin signaling. Knocking down CaR expression blocked the Ca^{2+}_o-induced formation of AJ, the association of PI3K with the E-cadherin/catenin complex, and expression of late differentiation markers. Furthermore, the Ca^{2+}_o-induced tyrosine phosphorylation of β-, γ-, and p120-catenin and Fyn were blocked in the CaR-deficient keratinocytes. This indicates that the CaR regulates the E-cadherin/PI3K pathway via a Src-family tyrosine kinase-mediated signaling and impacts on keratinocyte differentiation.

Experiment procedures

Materials- The membrane permeable Ca^{2+} chelator 1,2-bis (2-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid-AM (BAPTA-AM), 2-aminoethoxydiphenyl borate (2-APB), and Src kinase inhibitor PP2 and its non-functional analog PP3 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Stock solutions of these compounds were prepared in DMSO. All other chemicals were purchased form Sigma-Aldrich (St. Louis, MO). All DNA constructs used in generation of adenoviruses were prepared using Qiagen Maxi-prep columns

(Chatsworth, CA) according to the manufacturer's protocol. Monoclonal antibodies (mAbs) for Bip (GRP78) and α2 integrin were obtained from BD Biosciences (Palo Alto, CA). The rabbit polyclonal antibody (Ab) for CaR, ADDR, was raised against the peptide corresponding to amino acids 215-236 of the human keratinocyte CaR (6). Rabbit polyclonal and mAbs against E-cadherin, α-, β-, γ-, and p120-catenin, c-Src, Fyn, and the regulatory subunit of PI3K, p85α, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Texas Red-conjugated phalloidin was from Invitrogen Corp. (Carlsbad, CA).

Cell Culture- Normal human keratinocytes (NHKs) were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (154CF, Cascade Biologics, Portland, OR) as described (17). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4^{o}C, 16 h), and primary cultures were established in growth medium containing 0.07 mM $CaCl_2$. Second passage keratinocytes were plated in medium containing 0.03 mM $CaCl_2$ and used in the experiments described.

Generation of adenoviral stocks and infection of keratinocytes- A replication-defective adenovirus carrying the antisense human CaR cDNA (Ad-ASCaR) and the control viruses Ad-DNR were constructed using an Adeno-X Expression System II kit (BD Biosciences) as described (8). Viral particles were collected and titered using an Adeno-X rapid titer kit (BD Biosciences) and used to infect NHKs. For inactivation of the CaR, subconfluent NHKs were infected with the Ad-ASCaR adenovirus (60 pfu/cell) in growth medium containing 0.03 mM $CaCl_2$ and cultured for 5 to 7 days before exposure to 1.2 mM $CaCl_2$ for 5-10 minutes to induce formation of AJ or for 3 days to induce differentiation. Three days after infection, the viral supernatant was replaced with fresh culture medium containing 0.03 mM $CaCl_2$. No additional adenovirus was provided after the initial infection. Control cells were infected with an adenovirus Ad-DNR.

Quantitative real-time PCR (q-PCR) analysis- The expression of late differentiation genes was determined by q-PCR. NHKs were infected with adenovirus (60 pfu/cell) in growth medium containing 0.03 mM $CaCl_2$ and cultured for 5 days before exposure to 1.2 mM $CaCl_2$ for 3 days. Total RNA were then isolated using Qiagen RNeasy RNA purification kit (Chatsworth, CA) according to the manufacturer's instructions. Equal amounts of RNA samples were reverse transcribed by M-MLV reverse transcriptase (Invitrogen Crop.) to generate cDNA. qPCR was performed on cDNA using TaqMan premixed primer/probes and reagents from Applied Biosystems (Foster City, CA) or SYBR Green primers. RNA levels of late differentiation markers in keratinocytes were normalized to mitochondrial ribosomal protein L19 for all experiments.

TUNEL staining- Keratinocyte cultures grown on glass cover slips were infected with adenovirus as described. Then cells were cultured in 0.03 or 1.2mM $CaCl_2$ for 2 days before fixation in 10% neutral buffered formalin. Apoptotic cells were detected by ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon International, Inc., Temecula, CA) according to the manufacturer's protocol. Briefly, fixed cells were washed and endogenous peroxidase activity is quenched by hydrogen peroxide. Cells were incubated with TdT enzyme in the presence of digoxigenin at 37^{o}C for 1 hour, washed, and then incubated with peroxidase-conjugated anti-digoxigenin antibody. After washing with PBS, the sections are incubated with DAB substrate for 5 min to reveal peroxidase activity. Following the color reactions, the coverslips are washed and mounted. Digital images of 10 representative fields per experimental condition were acquired and quantified using a computer-assisted program (BIOQUANT, Nashville, TN). The degree of apoptosis was presented as the number of TUNEL-positive cells per 100 cells in the field. Student's *t*-test was used for statistical analysis.

Measurement of cytosolic Ca^{2+} - The $Ca^{2+}{}_i$ responses to elevated $Ca^{2+}{}_o$was measured using a Dual-wavelength Fluorescence Imaging System (Intracellular Imaging Inc., Cincinnati, OH) as described (25). Pre-confluent keratinocytes were infected with an adenovirus carrying the antisense human CaR cDNA (Ad-ASCaR) or a control virus (Ad-DNR) on a coverslip in medium containing 0.03 mM Ca^{2+}. Five to 7 days later, cells were loaded with 5 μM Fura-2/AM (Molecular Probes,

Eugene, OR) in 0.1% Pluronic F127 in buffer A (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mg/ml sodium pyruvate, 1 mg/ml glucose) containing 0.07 mM Ca^{2+}. Cells were then washed and measured in buffer A containing 0.03 mM Ca^{2+} before exposure to 2 mM Ca^{2+}. The cells were alternately illuminated with 340 nm and 380 nm light, and the fluorescence at emission wavelength 510 nm was recorded. All experiments were performed at room temperature. The signals from 20 to 50 single cells for each measurement were recorded. Each sample was calibrated by the addition of 20 mM ionomycin (R_{max}) followed by 20 mM EGTA/Tris, pH 8.3 (R_{min}). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the ratio of emission at the two excitation wavelengths based on the formula $[Ca^{2+}]_i = K_dQ(R-R_{min})/(R_{max}-R)$, $R = F_{340}/F_{380}$, $Q = F_{min}/F_{max}$ at 380 nm, and K_d for Fura-2 for Ca^{2+} is 224 nM.

Cell lysate preparation and Immunoblotting- Total cell lysates and membrane proteins were prepared from NHKs 5 to 7 days after adenoviral infections. Keratinocytes were washed twice in PBS containing 1 mM Na_3VO_4 and lysed for 30 min on ice in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl, pH 8.0, 120 mM NaCl) supplemented with 1 mM PMSF and protease inhibitors (Complete™ protease inhibitor tablet, Roche Molecular Biochemicals, Indianapolis, IN). Total cell lysates were centrifuged for 5 min at 4°C and the supernatant was collected. Keratinocyte membrane lysates were prepared using Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's instructions. The protein concentrations in the total cell lysates and membrane lysates were determined by the BCA Protein Assay Kit (Pierce Corp., Rockford, IL). 50 μg protein samples were electrophoresed through reducing polyacrylamide gels and electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, 0.45 μm; Millipore Corp., Bedford, MA). After blocking with 5% milk in TBS (20 mM Tris- HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA), the blots were incubated at 4°C overnight with appropriate primary antibodies: polyclonal Abs against E-cadherin, α-, and p120-catenin at a dilution of 1:300, mAbs against PI3K-p85α, phosphotyrosine, β- and γ-catenin at a dilution of 1:200, polyclonal Abs against Fyn and c-Src at a dilution of 1:200, mAbs against Bip and α2 integrin at a dilution of 1:250. Subsequently the blots were incubated with appropriate HRP-conjugated anti-IgG secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature. The bound antibody was visualized using the SuperSignal West Dura Chemiluminescent Kit (Pierce Corp.) and subsequent exposure to x-ray film.

Immunoprecipitations- Total cell lysate containing 0.5-1 mg were immunoprecipitated by 3 μg of mAbs against β-, γ-, and p120-catenin, Fyn, PI3K-p85α and phosphotyrosine (Santa Cruz Biotechnology, Inc.), and 200 μg membrane proteins by mAbs to E-cadherin and PI3K-p85α (Santa Cruz Biotechnology, Inc.), followed by Sepharose-conjugated protein G (ImmunoLink Immobilized Protein G, Pierce Corp.) in 1 ml of cold NP-40 lysis buffer at 4°C with gentle tumbling overnight. Immunoprecipitates were washed 4 times in NP-40 lysis buffer, eluted, and then analyzed by Western analysis.

Immunofluorescence staining- Keratinocytes were cultured on coverslips, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeablized with 0.5% NP-40 in PBS for 5 min. After blocking with 5% goat serum in PBS/0.01% Tween-20, cells were incubated with 10 μg/ml of primary antibodies at 4°C for overnight. Subsequently cells were incubated with the appropriate fluorescein- or Texas red-conjugated secondary antibody (20 μg/ml, Molecular Probes) at room temperature for 1 h. For F-actin staining, cells were incubated with Texas Red-conjugated phalloidin at room temperature for 1h. Finally, coverslips were washed in PBS, mounted on glass slides using Gel-Mount (Biomeda, Foster City, CA) and examined with a Leica TCS NT/SP confocal microscope (Leica Microsystems, Heidelberg, Germany).

Results

Inhibition of CaR expression reduced Ca^{2+}_i response to Ca^{2+}_o, promoted apoptosis and suppressed Ca^{2+}_o-induced keratinocyte differentiation - To study the role of CaR in activation of E-cadherin signaling and Ca^{2+}_o-induced differentiation, we inhibited CaR

expression by infecting preconfluent human keratinocytes with an adenovirus carrying a full-length CaR antisense cDNA (Ad-ASCaR). Immunoblotting (**Fig 1A**) and immunostaining (**Fig 1B**) showed that Ad-ASCaR effectively blocked the endogenous CaR protein level as compared with the cells infected with a control adenovirus (Ad-DNR). To determine whether inhibition of CaR expression altered Ca^{2+} signaling, we examined its impact on Ca^{2+}_i response to Ca^{2+}_o. Consistent with our previous studies, knockdown of CaR inhibited the Ca^{2+}_o-induced increase in Ca^{2+}_i. As shown in **Fig 1C**, raising Ca^{2+}_o from 0.03 to 2 mM induced an increase in Ca^{2+}_i in keratinocytes infected with the control virus Ad-DNR from 82 $\pm$ 7 to 472 $\pm$ 40 nM (mean $\pm$ SD; n=49). Keratinocytes infected with the Ad-ASCaR virus had comparable resting Ca^{2+}_i (71 $\pm$ 12 nM; n=45), but had a marked reduction in the rise of Ca^{2+}_i (to 202 $\pm$ 31 nM) in response to 2 mM Ca^{2+}_o. As knockdown of CaR inhibited much of, but not completely blocked, the ability of Ca^{2+}_o to increase Ca^{2+}_i, other mechanisms, such as calcium channels, independent of CaR appear to play a role in mediating Ca^{2+}_o-induced Ca^{2+}_i response.

To investigate whether the change in Ca^{2+} signaling following reduction in CaR expression affected cell survival, we compared the apoptosis in keratinocytes infected with Ad-ASCaR to that in control cells infected with Ad-DNR by TUNEL staining. Quantitative analysis of TUNEL-positive cells (**Fig 2**) showed that fewer than 2% of control keratinocytes undergo apoptosis when cultured in either 0.03 or 1.2 mM Ca^{2+}_o, whereas the number of apoptotic cells increased greatly in the Ad-ASCaR-infected keratinocytes (21 $\pm$ 3 % and 13 $\pm$ 4 % in 0.03 and 1.2 mM Ca^{2+}_o, respectively), indicating an important role of CaR in cell survival.

To determine whether the decrease in CaR expression affected differentiation, we examined its impact on Ca^{2+}_o-induced expression of terminal differentiation genes by quantitative real-time PCR in keratinocytes infected with Ad-ASCaR or Ad-DNR viruses. As shown in **Fig 3**, 72 h of incubation in 1.2 mM Ca^{2+} significantly increased the mRNA levels of terminal differentiation markers loricrin, filaggrin and caspase 14 in control keratinocytes infected with Ad-DNR (4-, 2.4- and 5.5-fold increase, respectively), but failed to do so in the cells infected with Ad-ASCaR. However, neither Ca^{2+}_o nor CaR knockdown decreased keratin 14 (K14), a marker for basal layer keratinocytes. These results demonstrated that CaR regulates calcium signaling, cell survival, and differentiation in keratinocytes.

Inhibition of CaR expression blocked E-cadherin-mediated cell-cell adhesion and recruitment of PI3K to the cell membrane- One of the early cellular responses to Ca^{2+}_o in keratinocytes is the formation of the E-cadherin/catenin complex at cell-cell contacts and the linkage of this core adhesive structure to the actin cytoskeleton. To investigate whether CaR is required for Ca^{2+}_o-activation of E-cadherin signaling, we examined the impact of CaR knockdown on the assembly of the E-cadherin/catenin complex. Fluorescence immunolocalization revealed that the initiation of E-cadherin-mediated cell-cell AJ formation occurred within 5 minutes after the Ca^{2+}_o was switched from 0.03 to 2 mM in Ad-DNR-infected keratinocytes (**Fig 4A-4D**). Ca^{2+}_o promoted the co-localization of E-cadherin with α- (**Fig 4C**), β- (**Fig 4A**) and p120-catenin (**Fig 4B**), as well as actin filaments (**Fig 4D**) in AJ in control keratinocytes, while inhibition of CaR expression by Ad-ASCaR blocked the ability of Ca^{2+}_o to induce the formation of this complex.

We then tested whether inhibition of CaR expression affects the production of the components of the E-cadherin signaling pathway. Immunoblotting analyses were performed on the total cell lysates from keratinocytes infected with Ad-ASCaR or Ad-DNR and treated with or without 2 mM Ca^{2+} using antibodies against E-cadherin, α-, β-, γ-, and p120-catenins, and p85α, the regulatory subunit of PI3K. Neither CaR knockdown nor the 5-min Ca^{2+} treatment significantly altered the expression levels of these proteins (data not shown). To confirm the immunolocalization results, we assessed whether CaR knockdown influences the cell membrane localization of these proteins. Immunoblotting analyses on the plasma membrane lysates demonstrated that 5 minutes of Ca^{2+} treatment promoted translocation of E-cadherin, α-, β-, γ-, p120-catenin, and PI3K-p85α to the cell membrane in Ad-DNR-infected keratinocytes,

whereas the Ca^{2+}_o-induced membrane-association of E-cadherin, catenins and PI3K-p85α were markedly reduced in the Ad-ASCaR-infected cells (**Fig 5A**). α2-integrin was used as a control to demonstrate equal membrane protein extraction. The purity of the plasma membrane lysates was confirmed when only the antibody against the plasma membrane marker α2-integrin, but not the antibody against endoplasmic reticulum marker Bip, immunoreacted with the plasma membrane lysates.

We next carried out co-immunoprecipitation to examine whether CaR knockdown affects the complex formation of E-cadherin, catenins, and PI3K. Keratinocytes were infected with ad-DNR or ad-ASCaR then treated with 2 mM Ca^{2+} for 10 minutes. Plasma membrane lysates were incubated with a monoclonal antibody against either E-cadherin or PI3K-p85α, and precipitated with protein G-conjugated Sepharose beads. The immunoprecipitates were then analyzed for the presence of E-cadherin, α-, β-, γ-, p120-catenin, and PI3K-p85α by immunoblotting. As shown in **Fig 5B**, Ca^{2+}_o increased the complex formation of E-cadherin, α-, β-, γ- and p120-catenin, and PI3K-85α in Ad-DNR-infected keratinocytes, but it failed to do so in the Ad-ASCaR-infected cells. α2-integrin was used as a control for equal membrane protein input for immunoprecipitation. Inhibition of CaR expression suppressed the complex formation of E-cadherin, catenins and PI3K at the cell membrane, demonstrating a key role for CaR in the Ca^{2+}_o-activation of E-cadherin/PI3K pathway.

Src-family tyrosine kinases regulate the activation of E-cadherin pathway- In addition to an increase in Ca^{2+}_i, the initiation of keratinocyte differentiation is associated with increased tyrosine kinase activity. To determine whether the activation of E-cadherin pathway by Ca^{2+}_o is a downstream event of increased Ca^{2+}_i or the activation of tyrosine phosphorylation, we pretreated keratinocytes with PP2, a specific inhibitor for the Src-family non-receptor tyrosine kinase, or BAPTA and 2-APB, two agents that prevent the Ca^{2+}_o-induced increase in Ca^{2+}_i, then examined their impact on AJ formation. As shown in **Fig 6A**, fluorescence immunostaining showed that the intercellular E-cadherin-mediated adhesive structure formed after a 5-minute exposure of 2 mM Ca^{2+} in normal keratinocytes pretreated with vehicle (0.1% DMSO). Pre-treating keratinocytes with the Ca^{2+}_i chelator BAPTA (25 μM) or 2-APB (75 μM), the store-operated Ca^{2+} channel blocker and IP_3 receptor inhibitor, for 30 minutes had no effect on the Ca^{2+}_o-induced formation of the E-cadherin/catenin complex at AJ. On the other hand, pretreatment of keratinocytes with 10μM PP2 completely blocked the Ca^{2+}-induced formation of cell-cell adhesion (**Fig 6A**). The inhibitory effect of BAPTA and 2-APB on the Ca^{2+}_o-induced increase in Ca^{2+}_i was confirmed by measuring the changes of Ca^{2+}_i in response to the raised Ca^{2+}_o. Pretreatment of BAPTA or 2-APB nearly completely blocked, whereas PP2 only partially inhibited, the Ca^{2+}_o-induced rise in Ca^{2+}_i (**Fig 6B**). These results indicate that Ca^{2+}_i is not a major factor that regulates cell-cell adhesion. Furthermore, co-immunoprecipitation of the plasma membrane lysates using antibodies for E-cadherin and PI3K-85α demonstrated that PP2 effectively blocked the Ca^{2+}_o-induced recruitment of PI3K to E-cadherin, whereas neither the vehicle control (DMSO) nor PP3, the inactive analog of PP2, affected the recruitment of PI3K by E-cadherin (**Fig 6C**). Additional co-immunoprecipitation studies confirmed that neither BAPTA nor 2-APB pretreatment affected the Ca^{2+}_o-induced formation of E-cadherin/PI3K complex (data not shown). These results indicate that Src-family tyrosine kinase-mediated signaling, but not Ca^{2+}_i, plays a major role in the Ca^{2+}_o-activation of E-cadherin/PI3K signaling.

Studies of mouse keratinocytes have shown that Ca^{2+} induced tyrosine phosphorylation of β-, γ- and p120-catenin is essential for their association with E-cadherin and the activation of PI3K, and that Fyn tyrosine kinase is likely involved in this process (22,26). To investigate whether CaR regulates the Ca^{2+}_o-activation of E-cadherin-mediated cell-cell adhesion via tyrosine kinase-mediated signaling, we examined the impact of CaR knockdown on tyrosine phosphorylation of β-, γ-, and p120-catenin, and PI3K. Keratinocytes were infected with adenoviruses Ad-DNR or Ad-ASCaR, and exposed to 2 mM Ca^{2+} for 10 minutes to induce

cell junction formation. Total cell lysates were prepared and immunoprecipitated with an antibody against phosphotyrosine. Immunoprecipitates were then analyzed by immunoblotting to detect β-, γ-, p120-catenin, PI3K-p85α, and tyrosine kinases Src and Fyn. As shown in **Fig 7A**, Ca^{2+}_o increased the tyrosine phosphorylation of β-, γ-, p120-catenin, PI3K-p85α, and Fyn, but not Src, in control keratinocytes, whereas the induction of tyrosine phosphorylation of these proteins by Ca^{2+}_o was diminished in the Ad-ASCaR-infected cells. β-actin was used as a control for equal protein input for immunoprecipitation. Similar findings were obtained when the total cell lysates were immunoprecipitated separately by antibodies against β-, γ-, p120-catenin, PI3K-p85α and Fyn, and immunoprecipitates were analyzed by immunoblotting for phosphotyrosine (**Fig 7B**). Our results demonstrate that CaR controls the Ca^{2+}_o-induced cell-cell adhesion via tyrosine kinase-mediated signaling.

To further determine the role of Src and Fyn in the Ca^{2+}_o-induced cell-cell adhesion, we assessed whether these proteins associated with E-cadherin and PI3K at the cell membrane. Immunoblotting analyses on plasma membrane lysates of Ad-DNR- and Ad-ASCaR-infected keratinocytes demonstrated that Ca^{2+}_o promoted the membrane localization of Fyn, not Src, to the cell membrane in control keratinocytes, but failed to do so in the cells infected with Ad-ASCaR (**Fig 8A**). Analyses of total cell lysates showed that the expression levels of Src and Fyn were not changed by Ca^{2+} exposure or CaR knockdown (**Fig 8B**). Co-immunoprecipitation assays demonstrated that Ca^{2+}_o induced recruitment of Fyn to the E-cadherin/PI3K complex at the cell membrane in control keratinocytes (**Fig 8C**). But the Ca^{2+}_o-induction of the association of Fyn with E-cadherin and PI3K was blocked when CaR expression was inhibited by Ad-ASCaR (**Fig 8C**). However, no association of Src with E-cadherin or PI3K in the cell membrane was detected by the co-immunoprecipitation assays (data not shown). These results indicate that CaR knockdown interferes with the Ca^{2+}_o-activation of E-cadherin-mediated cell adhesion due to reduced tyrosine kinase signaling that was likely mediated by the Fyn tyrosine kinase.

Discussion

E-cadherin-mediated intercellular adhesion is critical for maintaining the tissue integrity of skin and for correct differentiation of epidermal keratinocytes. In keratinocyte cultures, Ca^{2+}_o stimulates the interaction of E-cadherin with the actin cytoskeleton via the direct binding of α-catenin to actin filaments and recruitment of other cytoskeleton-binding proteins, stabilizing the AJ and leading to stratification (27). Besides adhesion, E-cadhrin coordinates signaling events within and between keratinocytes that are crucial for cell survival and differentiation (16,19). E-cadherin interacts with the lipid kinase PI3K at the plasma membrane. PIP_3 generated by the membrane-associated PI3K activates its downstream effector Akt, which promotes differentiation and protects keratinocytes from apoptosis (16). PIP_3 also binds to and activates PLCγ1 (17), which is essential for maintaining Ca^{2+}_o-induced increase in Ca^{2+}_i and keratinocyte differentiation (18). Disruption of E-cadherin in mouse blastocysts abrogated cell aggregation (28) and in lactating mammary gland affected differentiation and caused cell death (29). Conditional ablation of E-cadherin gene in skin led to loss of AJ and altered epidermal differentiation (14,30). In this report, we demonstrated that CaR participates in the Ca^{2+}_o-activation of E-cadherin-mediated signaling. Reduction of CaR expression in keratinocytes caused severe disruption in the E-cadherin-dependent intercellular adhesion, blocked Ca^{2+}_o-induced recruitment and activation of PI3K, inhibited Ca^{2+}_o-activated Ca^{2+}_i signaling, increased premature cell death and inhibited terminal differentiation. Although Ca^{2+}_o-induced increase in Ca^{2+}_i is critical for stimulating differentiation, CaR-mediated regulation of Ca^{2+}_o-induced E-cadherin complex formation and resulting cell-cell adhesion does not require a measurable increase in Ca^{2+}_i, since neither BAPTA nor 2-APB blocked this process. Rather, our results provide evidence that CaR regulates E-cadherin dependent cell-cell interactions via a Src-family kinase-dependent signaling, which is likely mediated by Fyn.

Forming stable cadherin-dependent AJ requires tyrosine kinase activities. Three E-cadherin-associated proteins, β-, γ- and p120-catenin, are directly tyrosine phosphorylated after

Ca^{2+} stimulation, correlated with the establishment of close intercellular contact and the onset of stratification (22). Tyrosine phosphorylation of β- and γ-catenin increases the association of α-catenin with E-cadherin, increasing the strength of cell adhesion due to the bridging ability of α-catenin between the cadherin/catenin complex and the actin cytoskeleton (22). Src-family tyrosine kinases are evidently an integral part of the Ca^{2+}_o-induced E-cadherin signaling pathway. Pharmacologically inhibiting Src-family tyrosine kinases blocked formation of AJ, abolished Ca^{2+}_o-activation of PI3K (22) and suppressed expression of differentiation markers in keratinocytes (17). In CHO cells, Src is required for the recruitment of PI3K to E-cadherin. We showed here that in cultured human keratinocytes, Ca^{2+}_o activated Fyn, as evidenced by the increased self-phosphorylation, increased membrane localization of Fyn and induced association of Fyn with E-cadherin and PI3K, supporting the involvement of Fyn in the Ca^{2+}_o-activation of E-cadherin/PI3K pathway. Our results were consistent with the findings in mouse keratinocytes: Fyn was selectively activated during differentiation (21) induced by Ca^{2+}_o and was found to colocalize with E-cadherin at the cell-cell borders (22). In addition, decreased tyrosine phosphorylation of β-, γ-, and p120-catenin and abnormal cell adhesion were observed in mouse keratinocytes lacking of Fyn (22). However, Fyn is not the only kinase to be involved in E-cadherin signaling, as a combination of dominant negative *fyn* and *src* is required to block the Ca^{2+}_o-induced PI3K activation and keratinocyte differentiation (17). Whereas the skin of mice with a single *fyn* knockout mutation appears normal, the skin of mice with a concomitant disruption of the *fyn* and *src* genes shows reduced tyrosine phosphorylation of β-catenin and p120-catenin level, and impaired cell adhesion, indicating the occurrence of functional compensation within the Src kinase family (22).

The CaR couples to multiple G proteins involved in distinct signaling pathways: $G\alpha_i$ to inhibit the activity of adenylyl cyclase (31) and activate ERK (32), $G\alpha_q$ to stimulate PLC and phospholipase A2 (33), and $G\beta\gamma$ to stimulate PI3K (34). In the present study, we placed Src-family tyrosine kinase-mediated signaling downstream of CaR activation, as down-regulation of CaR in keratinocytes blocked the Ca^{2+}_o-induced activation and association of Fyn with E-cadherin and PI3K, reduced the tyrosine phosphorylation of β-, γ-, and p120-catenins, and consequently inhibited cell adhesion and E-cadherin/PI3K signaling. However, the mechanism by which the CaR regulates Src-family kinases is unclear. In mouse keratinocytes, Fyn is a downstream mediator of Rho A function in intercellular adhesion, since blocking endogenous Rho-GTPase activity inhibited Ca^{2+}_o-induced Fyn activation, tyrosine phosphorylation of β-, γ-, and p120-catenins, and AJ formation (26). Several studies demonstrated the ability of CaR to activate Rho-mediated signaling by coupling to $G\alpha_{12/13}$ (35,36) or $G\alpha_q$ (37). CaR-mediated activation of Rho requires the physical interaction of the C-terminal region of CaR with the cytoskeletal protein filamin (38,39). Filamin interacts with a Pleckstrin-homology (PH) domain of Rho guanine nucleotide exchange factor (Rho-GEF) (40) to link Gα and Rho A (41). This provides a possible mechanism by which the CaR regulates keratinocyte cell-cell adhesion.

Thus, Ca^{2+}_o regulates cell survival and induces keratinocyte differentiation by at least two pathways: First, activation of CaR by Ca^{2+}_o stimulates the PLC pathway to increase Ca^{2+}_i and activate protein kinase C and downstream signaling events. Second, via a CaR-dependent mechanism Ca^{2+}_o induces the formation of E-cadherin-mediated AJ, providing a scaffold for recruiting and activating other signaling molecules, such as PI3K, Akt and PLCγ1, that is critical for differentiation. Disruption of CaR or either pathway results in abnormal differentiation in keratinocytes, although other mechanisms are likely also involved in regulating Ca^{2+}_o-activated cellular responses.

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Footnotes

[1]*Abbreviations*: Ca^{2+}_o, extracellular Ca^{2+}; Ca^{2+}_i, intracellular Ca^{2+}; CaR, calcium sensing receptor; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; AJ, adherens junction; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-triphosphate.

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Figure legends

Figure 1. **Inhibition of CaR expression by a CaR antisense cDNA blunted the acute Ca^{2+}_i response to elevated Ca^{2+}_o.** Human keratinocytes were infected by an adenovirus carrying a full-length CaR antisense cDNA (Ad-ASCaR) or a control virus (Ad-DNR) at a titer of 60 plaque-forming units (pfu)/cells in medium containing 0.03 mM Ca^{2+}. Five days after viral infection, endogenous CaR protein levels were assessed by (A) immunoblotting of total cell lysates and by (B) fluorescence immunostaining using a polyclonal antibody against CaR. Bar = 50 μm. Infection of the Ad-ASCaR effectively decreased the expression of endogenous CaR protein as compared with cells infected with Ad-DNR. After infection with Ad-ASCaR or Ad-DNR, keratinocytes were loaded with Fura-2. (C) Ca^{2+}_i was measured before and after the addition of 2 mM Ca^{2+}. The trace shown here represents the average Ca^{2+}_i of 45-49 individual keratinocytes during recording. The results are representative of three separate experiments.

Figure 2. **Inhibition of CaR expression promoted apoptosis in keratinocytes.** Keratinocytes were cultured on glass cover slips and infected with an Ad-ASCaR or a control (Ad-DNR) adenovirus as described. Then cells were grown in 0.03 or 1.2mM $CaCl_2$ for 2 days before being fixed for *in situ* TUNEL staining. Quantitative analysis of TUNEL-positive cells was performed as described in the *Materials and Methods*. The level of apoptosis was expressed as number of TUNEL-positive cells per 100 cells in the field. The number of TUNEL-positive keratinocytes in the cultures infected with the Ad-ASCaR (black bars) was significantly higher than that in cultures infected with control Ad-DNR (white bars). * $P<0.01$. The data are representative of two independent experiments.

Figure 3. **Inhibition of CaR expression blocked the Ca^{2+}_o-stimulated expression of late differentiation genes.** Keratinocytes were infected with Ad-DNR or Ad-ASCaR adenoviruses for 5 days. Cells were either maintained in 0.03 mM or switched to 1.2 mM Ca^{2+} for 72 hours, and total RNA was collected and analyzed for loricrin, filaggrin, caspase 14, and keratin 14 (K14) by real-time qPCR. Expressions of differentiation markers are presented as ratios to mitochondrial ribosomal protein L19. Raising Ca^{2+}_o from 0.03 to 1.2 mM significantly increased the mRNA levels of loricrin, filaggrin and caspase 14 in keratinocytes infected with control virus (Ad-DNR, white bars). * $P<0.01$. The Ca^{2+}-stimulated expression of late differentiation markers was blocked in cells infected with ad-ASCaR (black bars). The results are representative of three experiments.

Figure 4. **Inhibition of CaR expression blocked the Ca^{2+}_o-induced assembly of intercellular adherens junctions.** Keratinocytes were infected with Ad-DNR (A-D) or Ad-ASCaR (E-H) adenoviruses for 5 days. Cells were then exposed to 2 mM Ca^{2+} for 5 minutes to induce formation of cell-cell contacts. Keratinocytes were stained with a polyclonal antibody against E-cadherin, and monoclonal antibodies against β- (A, E), p120- (B, F) and α-catenin (C, G), followed by the appropriate FITC- or Texas red-conjugated secondary antibody. F-actin was detected by Texas red-conjugated phalloidin (D, H). Fluorescent signals were detected with a confocal microscope. Bar = (A, B, C, E, F, G) 20 μm, (D, H) 10 μm. Green (FITC) and red (Texas red) images were superimposed, so that sites of staining overlap are visualized as yellow. Substantial colocalization of E-cadherin with α-, β- and p120-catenin, as well as actin- cytoskeleton filaments was detected in the intercellular adherens junctions in control cells infected with Ad-DNR. The Ca^{2+}_o-induced formation of intercellular contacts was markedly inhibited in the keratinocytes infected with Ad-ASCaR. Similar observations were made in three separate cell preparations.

Figure 5. **Inhibition of CaR expression blocked the Ca^{2+}_o-stimulated cell membrane localization and complex formation of E-cadherin, catenins and PI3K.** Keratinocytes were infected with Ad-DNR or Ad-ASCaR adenoviruses as described and then exposed to 2 mM Ca^{2+} for 5 min. (A) The plasma membrane lysates were extracted, and analyzed by immunoblotting with antibodies against E-cadherin,

α-, β-, γ- and p120-catenin, and p85α subunit of PI3K. (B) The plasma membrane lysates were immunoprecipitated with antibody against either E-cadherin or PI3K-p85α. The immunoprecipitates were then analyzed by immunoblotting for E-cadherin, α-, β-, γ- and p120-catenin, and PI3K-p85α. α2-integrin (a plasma membrane marker) was used as a control for equal extraction of cell membrane proteins. Ca^{2+} increased the levels and complex formations of E-cadherin, catenins, and PI3K-p85α in the plasma membrane in Ad-DNR-infected keratinocytes, but not in the Ad-ASCaR-infected cells. The data are representative of three experiments.

Figure 6. **Src-family tyrosine kinase-mediated signaling, but not Ca^{2+}_i, is required for the activation of E-cadherin-dependent intercellular adhesion by Ca^{2+}_o.** Keratinocytes grown in 0.03 mM Ca^{2+} were pretreated for 30 min with either 10 μM PP2, 25 μM BAPTA-AM, 75 μM 2-APB, or with 0.1% DMSO. (A) Keratinocytes were exposed to 2 mM Ca^{2+} for 5 minutes, stained with a polyclonal antibody against E-cadherin, and monoclonal antibody against β-catenin, followed by FITC-conjugated anti-rabbit and Texas red-conjugated anti-mouse antibodies. Bar = 20 μm. (B) Ca^{2+}_i was measured before and after the addition of 2 mM Ca^{2+}. The traces shown here represent the average Ca^{2+}_i of 31 to 55 individual cells during recording. (C) Keratinocytes were pretreated with 10 μM PP2, 10 μM PP3 or 0.1% DMSO, exposed to 2 mM Ca^{2+} for 5 min. The plasma membrane lysates were extracted, and immunoprecipitated with antibody against either E-cadherin or PI3K-p85α. The immunoprecipitates were then analyzed by immunoblotting for E-cadherin and PI3K-p85α. The E-cadherin dependent intercellular adhesion and association of PI3K with E-cadherin was sensitive to the inhibition of Src-family tyrosine kinases. These results are representative of two separate experiments.

Figure 7. **Inhibition of CaR expression diminished the Ca^{2+}_o-induced tyrosine phosphorylation of β-, γ- and p120-catenin and PI3K.** Keratinocytes were infected with Ad-DNR or Ad-ASCaR adenoviruses as described and then exposed to 2 mM Ca^{2+} for 10 min. (A) Total cell lysates were immunoprecipitated with antibody against phosphotyrosine. The immunoprecipitates were analyzed by immunoblotting for β-, γ- and p120-catenin, PI3K-p85α, and tyrosine kinases Fyn and c-Src. β-actin was used as a control for equal protein input before precipitation. (B) In a reverse approach, total cell lysates were immunoprecipitated separately with antibodies against β-, γ- and p120-catenin, PI3K-p85α, and Fyn. The immunoprecipitates were analyzed by immunoblotting for phosphotyrosine. Ca^{2+} promoted the tyrosine phosphorylation of β-, γ- and p120-catenin, PI3K-p85α, and Fyn in keratinocytes, but failed to do so when CaR expression was blocked by Ad-ASCaR. The data are representative of two experiments.

Figure 8. **Decreased expression of CaR blocked the Ca^{2+}_o-induced association of Fyn with E-cadherin and PI3K at the cell membrane.** Keratinocytes were infected with Ad-DNR or Ad-ASCaR adenoviruses as described and then exposed to 2 mM Ca^{2+} for 10 min. The plasma membrane lysates (A) and total cell lysates (B) were extracted, and analyzed by immunoblotting with antibodies against Fyn and c-Src. α2-integrin and Bip were used as controls for equal extraction of cell membrane and total lysate proteins, respectively. While the total expression levels of Fyn and Src were not affected by Ca^{2+} or decreased CaR expression, Ca^{2+} selectively increased the level of Fyn in the plasma membrane in Ad-DNR-infected keratinocytes, but not in the Ad-ASCaR-infected cells. However, Ca^{2+} did not change the localization of Src. (C) The plasma membrane lysates were immunoprecipitated with antibody against either E-cadherin or PI3K-p85α. The immunoprecipitates were analyzed by immunoblotting for the presence of Fyn. Ca^{2+} promoted the association of Fyn with E-cadherin and PI3K in the cell membrane in Ad-DNR-infected keratinocytes, but not in the Ad-ASCaR-infected cells. The data are representative of two experiments.

Figure 1

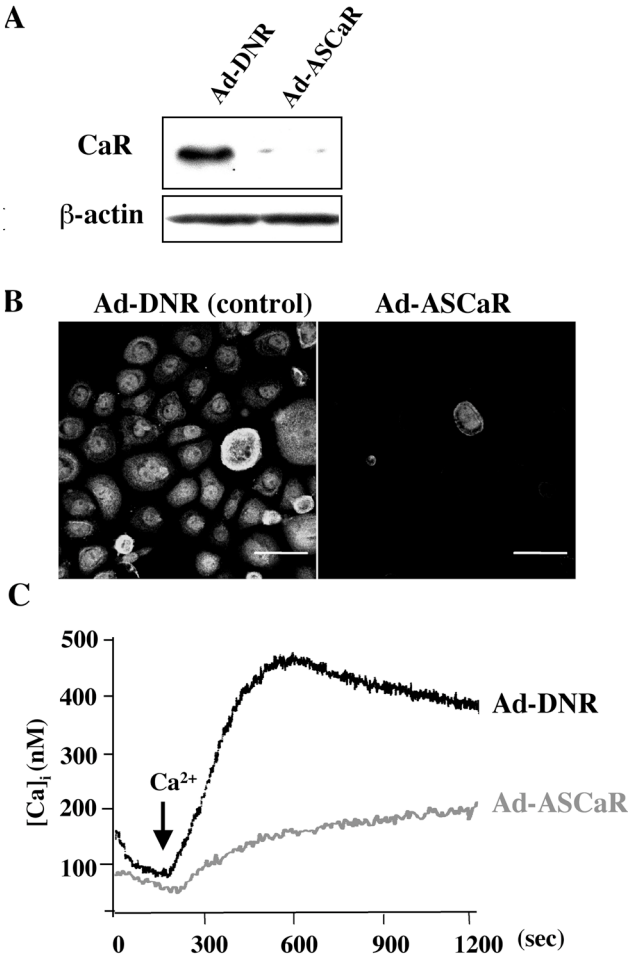

A
Ad-DNR
Ad-ASCaR
CaR
β-actin
B
Ad-DNR (control)
Ad-ASCaR
C
500
400
300
200
100
[Ca]i (nM)
Ca2+
Ad-DNR
Ad-ASCaR
0
300
600
900
1200
(sec)

Figure 2

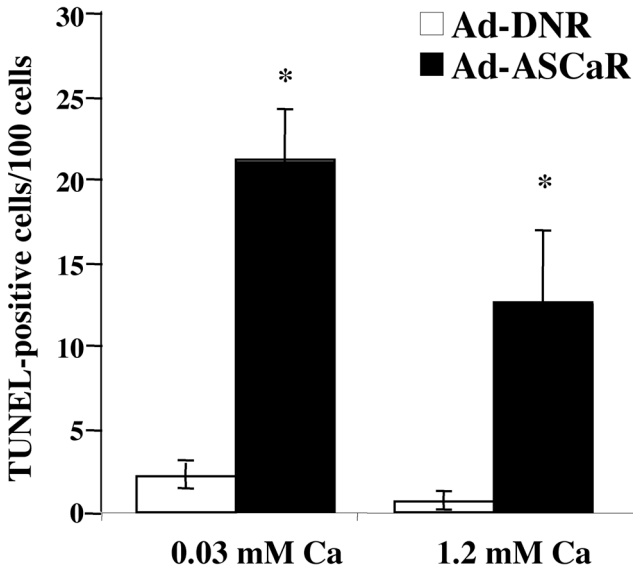

TUNEL-positive cells/100 cells
30
25
20
15
10
5
0
Ad-DNR
Ad-ASCaR
*
*
0.03 mM Ca
1.2 mM Ca

Figure 3

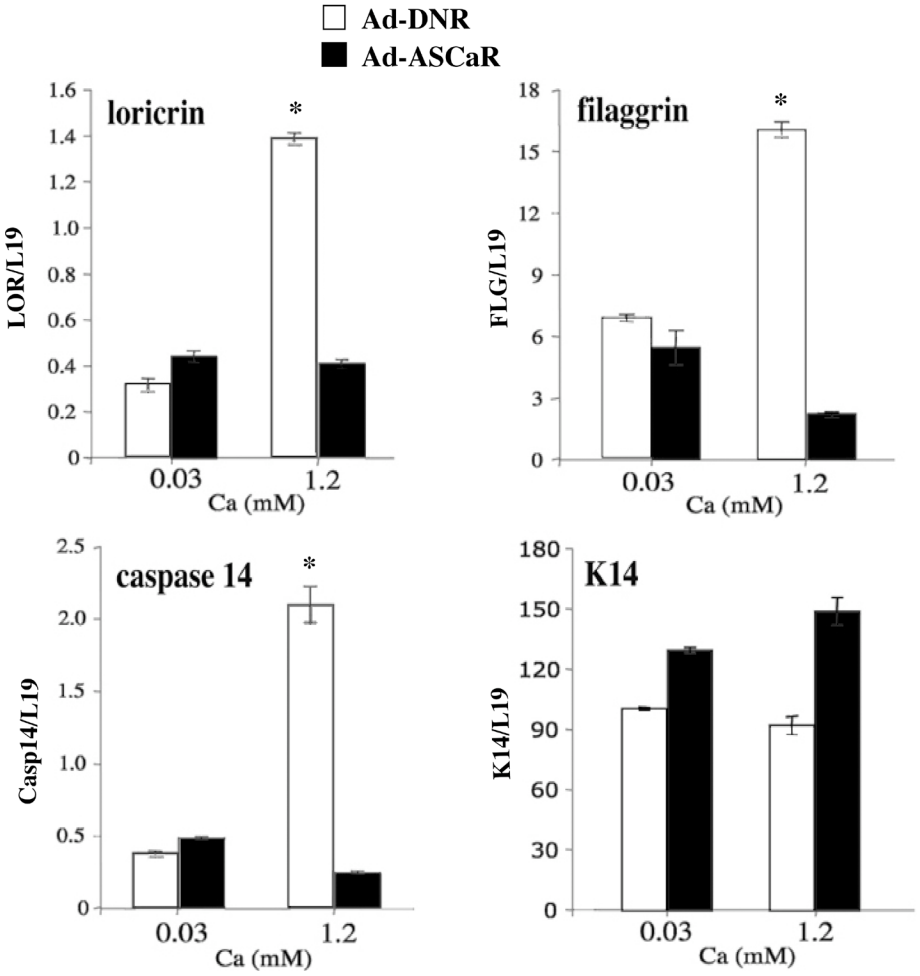

Ad-DNR
Ad-ASCaR
loricrin
LOR/L19
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
0.03
1.2
Ca (mM)
filaggrin
FLG/L19
18
15
12
9
6
3
0
*
0.03
1.2
Ca (mM)
caspase 14
Casp14/L19
2.5
2.0
1.5
1.0
0.5
0
*
0.03
1.2
Ca (mM)
K14
K14/L19
180
150
120
90
60
30
0
0.03
1.2
Ca (mM)

Figure 4

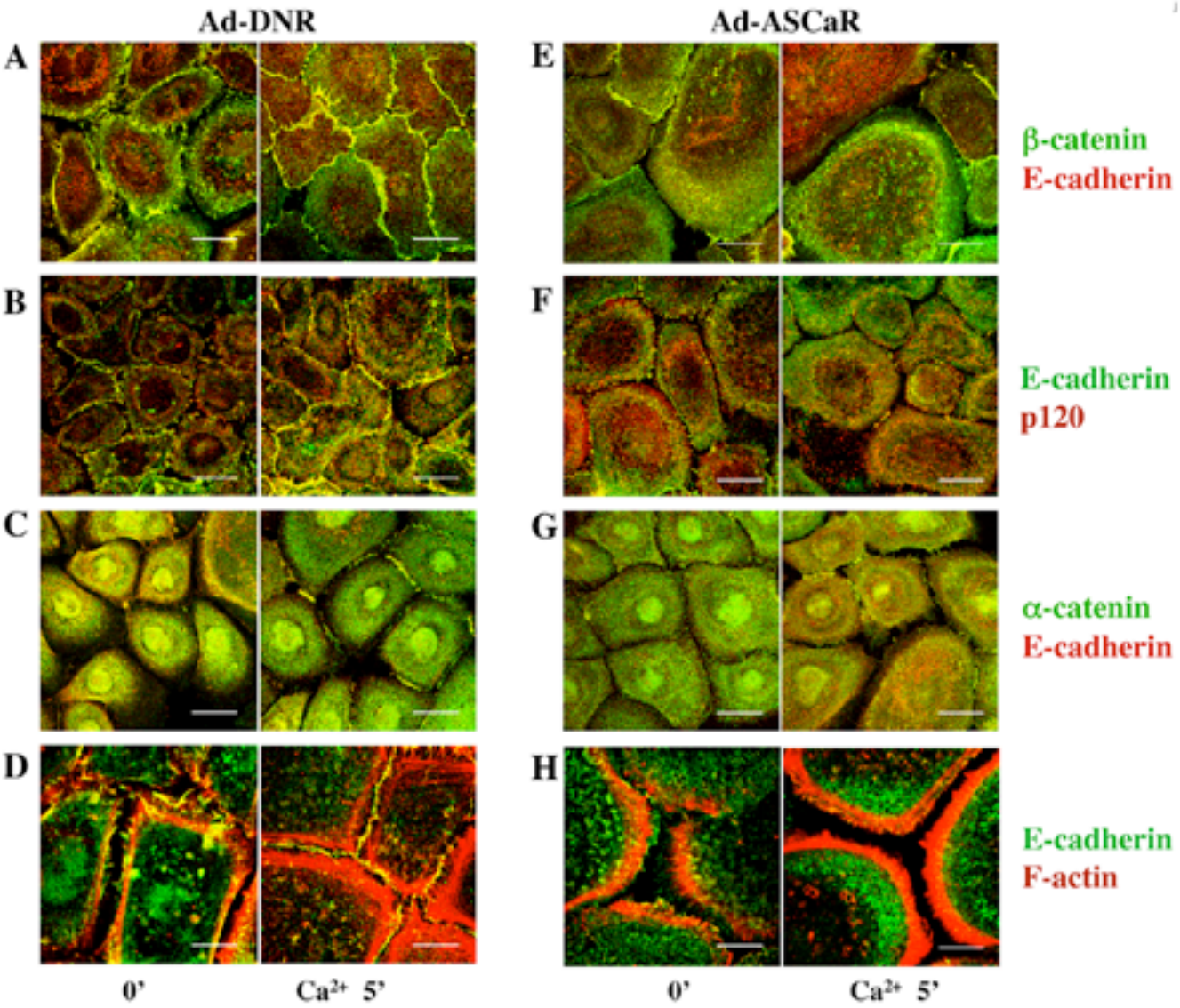

Ad-DNR
Ad-ASCaR
A
B
C
D
E
F
G
H
β-catenin
E-cadherin
E-cadherin
p120
α-catenin
E-cadherin
E-cadherin
F-actin
0'
Ca2+ 5'
0'
Ca2+ 5'

Figure 5

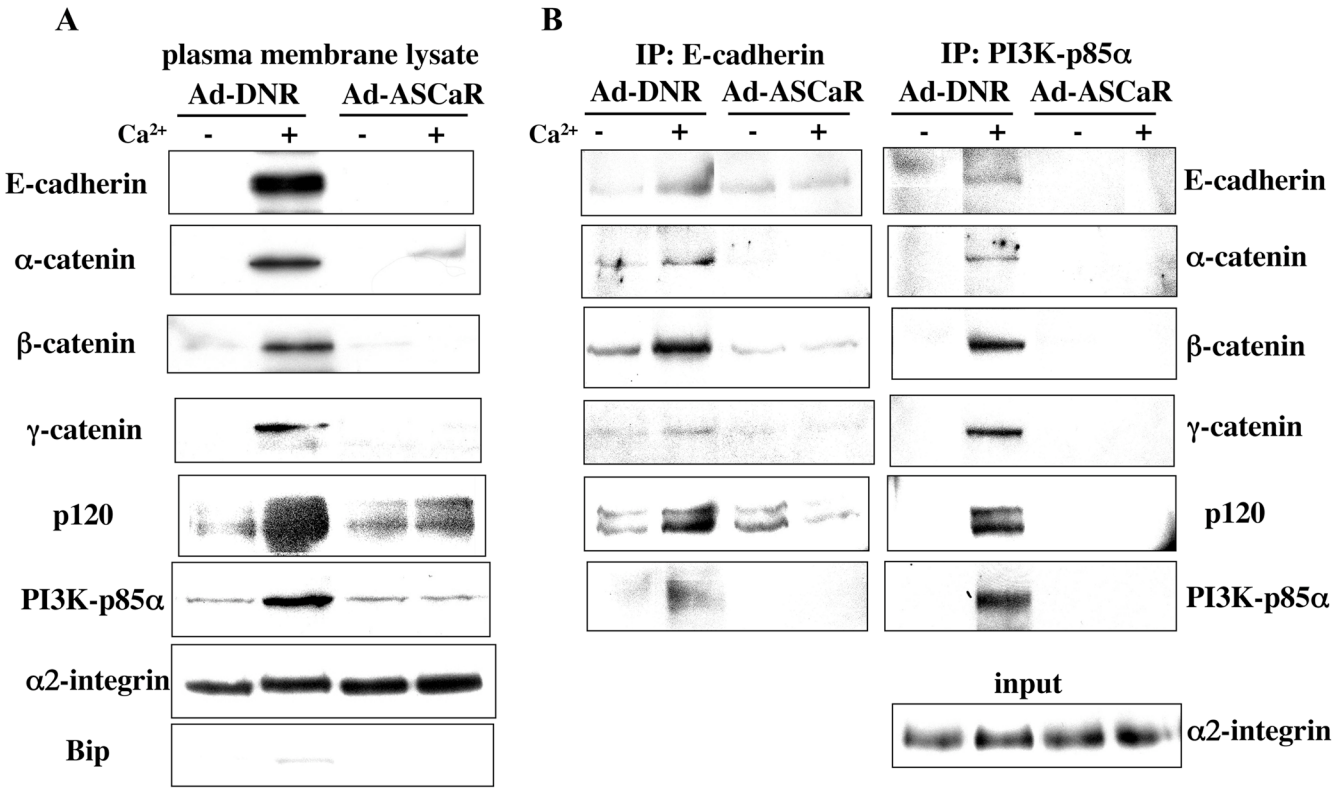

A
plasma membrane lysate
Ad-DNR
Ad-ASCaR
Ca2+ - + - +
E-cadherin
α-catenin
β-catenin
γ-catenin
p120
PI3K-p85α
α2-integrin
Bip
B
IP: E-cadherin
IP: PI3K-p85α
Ad-DNR
Ad-ASCaR
Ca2+ - + - +
E-cadherin
α-catenin
β-catenin
γ-catenin
p120
PI3K-p85α
input
α2-integrin

Figure 6

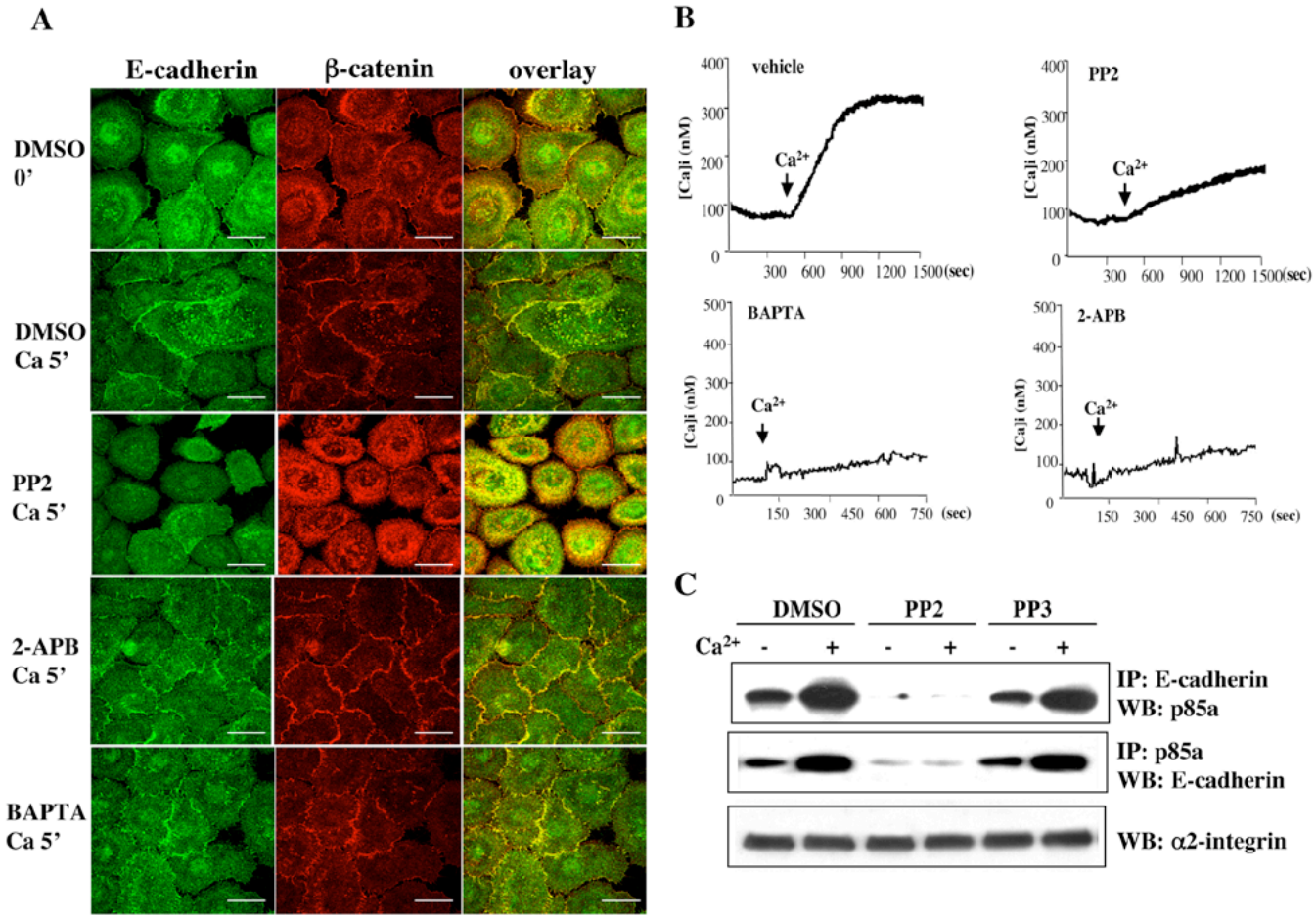

A
E-cadherin
β-catenin
overlay
DMSO 0'
DMSO Ca 5'
PP2 Ca 5'
2-APB Ca 5'
BAPTA Ca 5'
B
vehicle
PP2
BAPTA
2-APB
[Ca]i (nM)
Ca2+
(sec)
C
DMSO
PP2
PP3
Ca2+
IP: E-cadherin
WB: p85a
IP: p85a
WB: E-cadherin
WB: α2-integrin

Figure 7

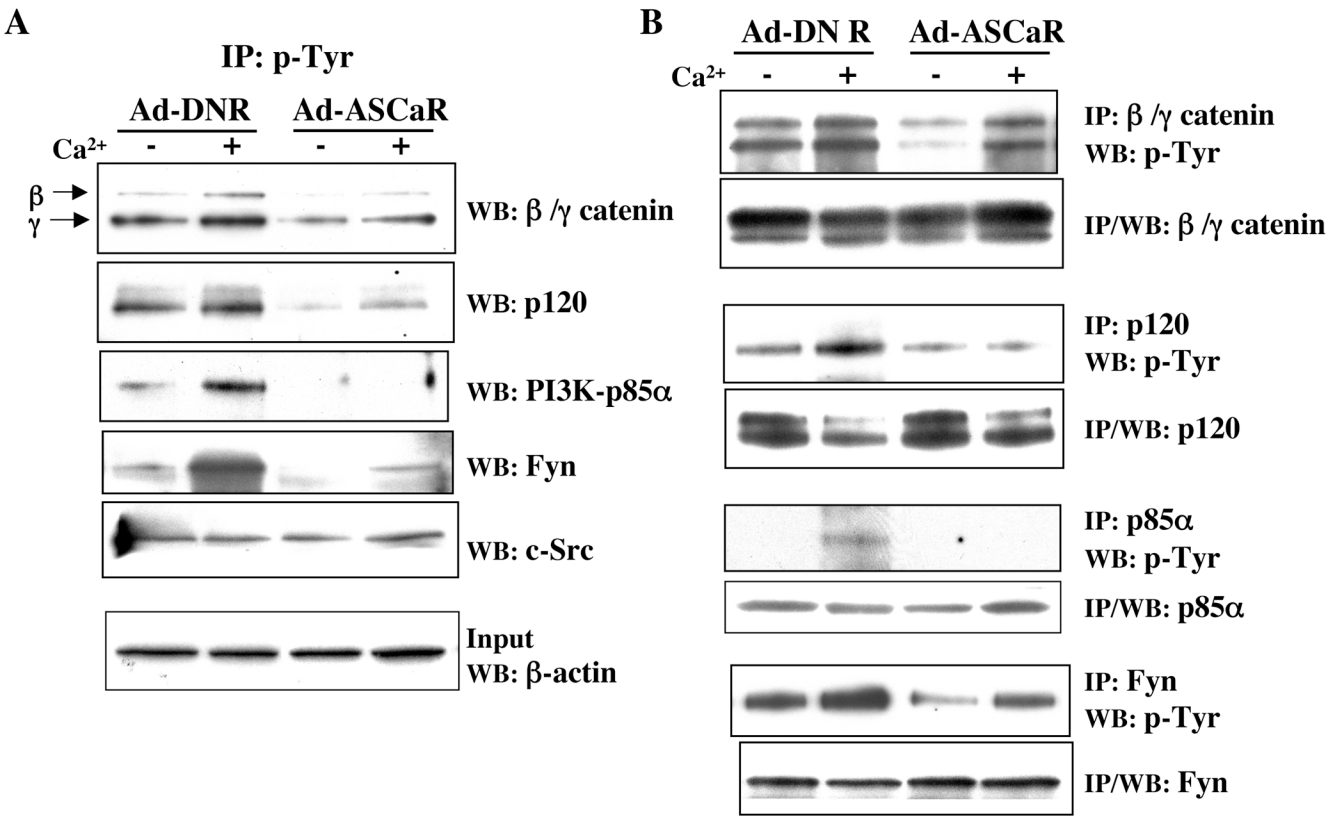
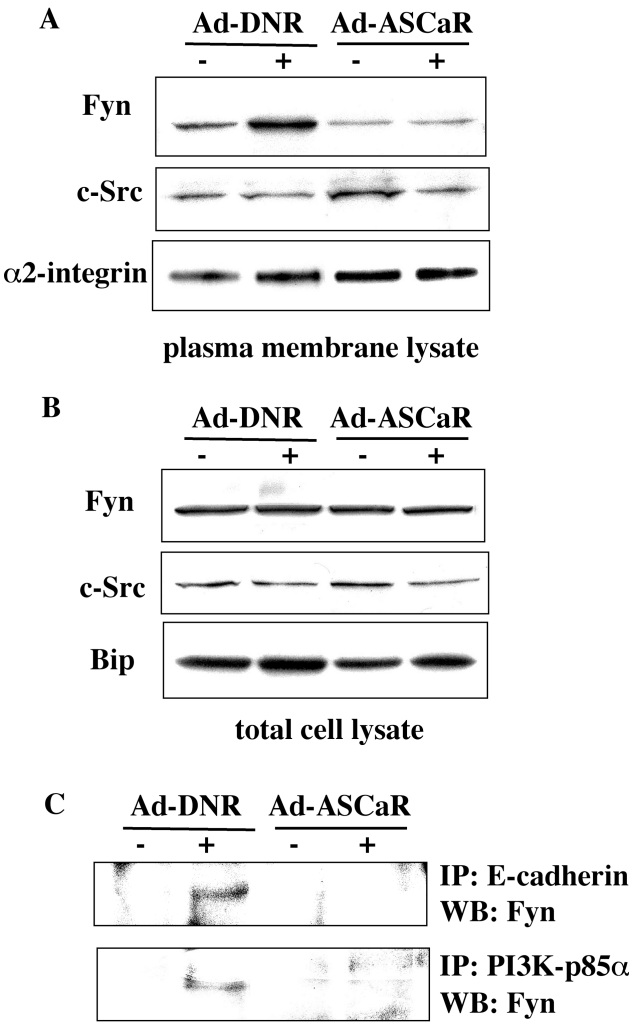

A
IP: p-Tyr
Ad-DNR
Ad-ASCaR
Ca2+ - + - +
β
γ
WB: β /γ catenin
WB: p120
WB: PI3K-p85α
WB: Fyn
WB: c-Src
Input
WB: β-actin
B
Ad-DN R
Ad-ASCaR
Ca2+ - + - +
IP: β /γ catenin
WB: p-Tyr
IP/WB: β /γ catenin
IP: p120
WB: p-Tyr
IP/WB: p120
IP: p85α
WB: p-Tyr
IP/WB: p85α
IP: Fyn
WB: p-Tyr
IP/WB: Fyn

Figure 8


A
Ad-DNR
Ad-ASCaR
- + - +
Fyn
c-Src
α2-integrin
plasma membrane lysate
B
Ad-DNR
Ad-ASCaR
- + - +
Fyn
c-Src
Bip
total cell lysate
C
Ad-DNR
Ad-ASCaR
- + - +
IP: E-cadherin
WB: Fyn
IP: PI3K-p85α
WB: Fyn