In cultured keratinocytes, the acute increase of the extracellular calcium concentration above 0.03 mM leads to a rapid increase in intracellular calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) and inositol trisphosphate production and, subsequently, to the expression of differentiation-related genes. Previous studies demonstrated that human keratinocytes express the full-length extracellular calcium-sensing receptor (CaR) and an alternatively spliced variant lacking exon 5 and suggested their involvement in calcium regulation of keratinocyte differentiation. To understand the role of the CaR, we transfected keratinocytes with an antisense human CaR cDNA construct and examined its impact on calcium signaling and calcium-induced differentiation. The antisense CaR cDNA significantly reduced the protein level of endogenous CaRs. These cells displayed a marked reduction in the rise in [Ca\(^{2+}\)]\(_{i}\) in response to extracellular calcium or to NPS R-467, a CaR activator, whereas the ATP-evoked rise in [Ca\(^{2+}\)]\(_{i}\) was not affected. Calcium-induced inhibition of cell proliferation and calcium-stimulated expression of the differentiation markers involucrin and transglutaminase were also blocked by the antisense CaR cDNA. When cotransfected with luciferase reporter vectors containing either the involucrin or transglutaminase promoter, the antisense CaR cDNA suppressed the calcium-stimulated promoter activities. These results indicate that CaR is required for mediating calcium signaling and calcium-induced differentiation in keratinocytes.

Changes in the concentration of extracellular calcium affect the balance between proliferation and differentiation in epidermal keratinocytes (1, 2). Elevation of the extracellular calcium concentration ([Ca\(^{2+}\)]\(_{o}\)) above 0.03 mM (calcium switch) inhibits proliferation and induces the onset of terminal differentiation. One early response to the elevation of extracellular calcium is an increase in intracellular calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) (3). Blocking the rise in [Ca\(^{2+}\)]\(_{i}\) with an intracellular calcium chelator blocks the ability of extracellular calcium to induce differentiation (4). After the calcium switch, the levels of inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol also increase rapidly (5, 6). This is subsequently followed by elevated expression of differentiation-related genes such as involucrin (7) and transglutaminase (8, 9), a substrate and enzyme, respectively, required for cornified envelope formation. Previous studies (10–12) suggest the involvement of the extracellular calcium-sensing receptor (CaR) in mediating calcium signaling during keratinocyte differentiation. Activation of CaR with calcium or other polyvalent cations activates the phospholipase C signaling pathway, resulting in the generation of inositol 1,4,5-trisphosphate and the release of calcium from intracellular stores (13, 14). Human keratinocytes express the full-length CaR and an alternatively spliced variant of CaR lacking exon 5 (AltCaR) (15). Unlike the full-length CaR, AltCaR fails to mediate the acute IP\(_3\) response to [Ca\(^{2+}\)]\(_{i}\). The full-length CaR message is maximally expressed in undifferentiated keratinocytes, but its level decreases as the cells differentiate. On the other hand, the message levels of AltCaR remain relatively unchanged throughout differentiation (15). These changes in CaR expression are consistent with the reduction in [Ca\(^{2+}\)]\(_{i}\) and IP\(_3\) response to [Ca\(^{2+}\)]\(_{i}\) during differentiation (15) and further support a role for CaR in keratinocyte differentiation.

In the present study, we transfected an antisense CaR cDNA construct into undifferentiated keratinocytes to elucidate the role of CaRs in calcium sensing and calcium-induced differentiation. We report here that the full-length antisense CaR cDNA construct significantly reduced the protein level of endogenous CaRs. In these cells, the [Ca\(^{2+}\)]\(_{i}\) response to an increase in [Ca\(^{2+}\)]\(_{o}\) was inhibited, as were calcium-induced inhibition of proliferation and calcium-stimulated expression of the differentiation markers involucrin and transglutaminase. These results demonstrate that CaR-mediated calcium signaling is required for keratinocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM; Clonetics, San Diego, CA) as described previously (9). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4°C, 16 h), and primary cultures were established in KGM containing 0.07 mM calcium. First- and second-passage keratinocytes were plated in KGM containing 0.03 mM calcium and used in the experiments described below.

**Vector Construction**—To construct a vector expressing CaR antisense RNA, a 3.3-kb human CaR cDNA fragment (15) containing the entire open reading frame was subcloned in an antisense orientation into mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA), which expresses a neomycin resistance gene. The 2.2-kb fragment of the human keratinocyte transglutaminase promoter (gift from Dr. R. Rice) (17) were subcloned into pGL-3 basic vector (Promega, Madison, WI), linking them to the firefly luciferase gene to generate involucrin and transglutaminase promoter/reporter constructs (gifts from Dr. D. Ng, respectively).

**Transfection, Selection, and Luciferase Assay**—First-passage kerati-
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Keratinocytes were transfected with the CaR antisense cDNA construct or pcDNA3.1 vector using Effectene reagent (Qiagen, Chatsworth, CA) or Mirus Trans-It keratinocyte transfection reagent (PanVera Corp., Madison, WI) according to the manufacturers’ protocols. Transfected cells were then incubated in KGM containing 0.03 mM calcium for 2 days before harvest. Cells were then selected with 300 μg/ml G418 for 3 days prior to further study. At this point, membrane protein was prepared, and the level of endogenous CaR was measured by Western analysis (see below). To examine the impact of antisense CaR on the expression of differentiation marker genes, transfected cells were switched to KGM containing 1.2 mM calcium for an additional 2 days before harvest. Total RNA was isolated, and the message levels for involucrin, transglutaminase, and antisense CaR were quantitated by Northern analysis (see below). To monitor the impact of antisense CaR on the calcium-stimulated transcriptional activation of involucrin and transglutaminase genes, the antisense CaR cDNA construct was transiently transfected into second-passage keratinocytes together with either involucrin-luciferase or transglutaminase-luciferase constructs and with a β-galactosidase gene expression vector, pRSV-gal (18), used to normalize the transcription efficiency. Transfected cells were incubated overnight in KGM supplemented with 0.03 mM calcium. The next day, fresh KGM containing 0.03 or 1.2 mM CaCl₂ was added to the cultures. Cells were lysed 48 h later, and the cell extract was assayed for luciferase activity using the Luciferase Assay System (Promega) and for β-galactosidase activity using the Gaussia luciferase kit (Tropix, Inc., Bedford, MA). Chemical analysis of the luciferase activity expressed by the luciferase gene was employed to express the luciferase activity accurately. Transfected cells were infected overnight in KGM supplemented with 0.03 mM calcium. The next day, fresh KGM containing 0.03 or 1.2 mM CaCl₂ was added to the cultures. Cells were lysed 48 h later, and the cell extract was assayed for luciferase activity using the Luciferase Assay System (Promega) and for β-galactosidase activity using the Gaussia luciferase kit (Tropix, Inc., Bedford, MA). Chemical analysis of the luciferase activity expressed by the luciferase gene was employed to express the luciferase activity accurately.

**DNA Synthesis**—The effect of antisense CaR cDNA on cell proliferation was assessed by measuring the rate of DNA synthesis. Preconfluent keratinocytes were transfected with the CaR antisense cDNA construct or pcDNA3.1 vector and selected by G418 as described above. Transfected cells were trypsinized and seeded at a density of 1 × 10⁶ cells/ml. Cells were placed in a quartz cuvette, and [Ca²⁺]₀ was recorded at an absorbance of 510 nm. Intracellular calcium concentration was calculated from the ratio of emission at the two excitation wavelengths based on the formula [Ca²⁺]ᵢ = KᵢQ/R – RᵢQ/Rᵢ ROI, where Fᵢ/IFO₃₈₀₅₈₀ = Fᵢ₀/IFO₃₈₀₅₈₀ and Kᵢ for Fura-2 for Ca²⁺ is 224 nm. [Ca²⁺]ᵢ, Measurement in Suspended Keratinocytes—[Ca²⁺]ᵢ measurement of suspended keratinocytes was carried out as described previously (11). Briefly, untransfected keratinocytes were transfected with the antisense human CaR cDNA construct or pcDNA3.1 vector and selected by G418 as described above. Transfected cells were trypsinized and loaded with 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 MgCl₂, 1 mM sodium pyruvate, and 1 mg/ml glucose) containing 0.07 mM calcium. Cells were then washed and measured in buffer A containing 0.03 mM calcium before exposure to 1.2 or 5 mM calcium or to the calcimimetic compound NPS R-467 (provided by Dr. Edward P. Nemeth, NPS Pharmaceuticals, Salt Lake City, UT), a selective CaR activator (24). The cells were alternately illuminated with 340 and 380 nm light, and the fluorescence at emission wavelength 510 nm was recorded. The signals from 15–40 single cells for each measurement were recorded. Each sample was calibrated by the addition of 20 μM ionomycin (Fₙᵢₒ) followed by 20 mM EGTA/Tris, pH 8.3 (Fₙᵢᵣ), intracellular calcium concentration was calculated from the % response to elevated [Ca²⁺]₀, Fₙᵢₒ/Fₙᵢᵣ = 1000. Membrane protein samples were prepared, and the level of endogenous CaR was measured by Western blotting.

**Protein Analysis by Western Blot**—Crude plasma membranes were isolated from cultured human foreskin keratinocytes and from human embryonic kidney HEK293 cells transfected with human cDNA for full-length or splice variant CaR as described previously (15, 19). Briefly, the cells were sonicated and centrifuged at 100,000 × g for 30 min. Membrane fractions were incubated with RPA buffer containing 9 mM Hepes, pH 7.4, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 2 μg/ml aprotinin. The protein concentration in these membrane preparations was determined by the BCA Protein Assay Kit (Pierce). Membrane protein samples were electrophoresed through 5% polyacrylamide gels and electroblotted onto nitrocellulose membrane (Towbin: P.0.45 μm pore, Bedford, MA). After blocking, the blot was incubated with a polyclonal anti-CaR antibody, either ADDR or BoCaR#3 (a gift from Dr. Dolores Shoback), which reacts with both full-length human CaR and its splice variant, AltCaR, or with another polyclonal anti-CaR antibody, hCaR4/6, which specifically reacts with AltCaR. The ADDR antibody was raised against the peptide corresponding to amino acids 215–236 of the bovine parathyroid CaR (20) and subsequently incubated with fluorescein-conjugated anti-rabbit IgG. Finally, the cells on coverslips were mounted on glass slides using Gel-Mount (Biomedia, Foster City, CA) and examined with a Leica TCS NT/SP confocal microscope (Leica Microsystems, Heidelberg, Germany).

**Results**

**Expression of CaR Proteins in Human Keratinocytes**—We previously reported that human keratinocytes express the transcripts of the CaR, both the full-length form and its alternately spliced variant (AltCaR) lacking exon 5 (15). To detect the presence of CaR proteins, we analyzed membrane extracts from preconfluent human keratinocytes by Western blotting. The CaR was detected using a peptide affinity-purified polyclonal antibody against human CaR, ADDR (Fig. 1A). Although this antibody could detect both forms of CaR, the reaction with full-length CaR is stronger than that with AltCaR (Fig. 1A). ADDR specifically detected three bands of 120, 160, and 185 kDa in the human keratinocytes grown in serum-free medium containing either 0.03 or 1.2 mM calcium (Fig. 1A, left panel). ADDR also detected a minor band of 130 kDa in keratinocyte

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membranes, which was best observed on longer-exposed film (data not shown). The 160-kDa band corresponds to one of the two major glycosylated forms of CaR (with sizes of 140 and 160 kDa), whereas the 120-kDa band corresponds to the nonglycosylated form of CaR expressed in HEK293 cells transfected with cDNA for the full-length human CaR (Fig. 1A, left panel). The 130-kDa band corresponds to the single band of CaR expressed in HEK293 cells transfected with cDNA for AltCaR. The size of the 130-kDa band is consistent with the spliced variant lacking 77 amino acids of exon 5 that we have previously shown has altered glycosylation. The identity of the 185-kDa band remains unclear, although it specifically reacted with ADDR antibody. Preincubation of ADDR antibody with the specific peptide to which it was raised prevented detection of these bands (Fig. 1A, right panel). These bands were also detected by another anti-CaR antibody, BoCaR#3, which was raised against a peptide derived from the intracellular domain of CaR protein (data not shown). To confirm that the 130-kDa protein present in keratinocyte membranes is the spliced variant of human CaR, the same blot was incubated with hCaR4/6 antibody. Detection by hCaR4/6 confirmed that the 130-kDa protein present in keratinocyte membranes is the spliced variant of human CaR. The specificity of the immunostaining of these bands was confirmed by incubation with the antibodies preabsorbed with the specific peptides against which they were raised.

Transfection of the Human Antisense CaR cDNA Reduced [Ca2+]i Response to [Ca2+]o—To elucidate the role played by CaRs in calcium sensing and keratinocyte differentiation, we transfected an expression construct with full-length human CaR cDNA in the antisense orientation into undifferentiated keratinocytes. The transfectants were selected with neomycin analogue G418 in KGM containing 0.03 mM calcium for 3 days to enrich transfected cells. Total RNA and membrane proteins were then prepared and subjected to Northern and Western analyses, respectively, to examine the expression of the CaR antisense transcript and the level of endogenous CaR protein. Northern analysis using a sense strand CaR RNA as probe showed that the keratinocytes transfected with the CaR antisense cDNA construct efficiently expressed the full-length CaR antisense transcript and a low level of CaR protein (Fig. 2A, left panel). In additional to the three bands (120, 160, and 185 kDa) shown in A, a 130-kDa band (observed on longer-exposed film; data not shown) was specifically detected by ADDR in keratinocytes. Detection by hCaR4/6 confirmed that the 130-kDa protein present in keratinocyte membranes is the spliced variant of human CaR. The specificity of the immunostaining of these bands was confirmed by incubation with the antibodies preabsorbed with the specific peptides against which they were raised.

Western analysis of the membrane proteins by BoCaR#3 (Fig. 3B) or hCaR4/6 (Fig. 3C) antibody showed that the expression of both the 180-kDa full-length CaR (Fig. 3B) and its 130-kDa spliced variant (Fig. 3C) was decreased in the cells transfected with the antisense CaR construct as compared with cells transfected with empty vector. In this experiment, hCaR4/6 also detected a 115-kDa band in HEK293 cells but not in keratinocytes (Fig. 3C); however, this band is not detected by BoCaR#3 antibody.

We next evaluated the effects of the CaR antisense cDNA construct on calcium sensing by examining the [Ca2+]i response to raised [Ca2+]o, in the transfected keratinocytes. As shown in Fig. 4A, raising [Ca2+]o from 0.03 to 5 mM induced an increase in [Ca2+]i, in keratinocytes transfected with empty vector from 90 ± 9 to 803 ± 114 nM (mean ± S.D.; n = 31). Keratinocytes transfected with the CaR antisense cDNA had comparable basal [Ca2+]i levels (57 ± 6 nM; n = 27). However, these cells displayed a marked reduction in the rise of [Ca2+]i, (to 238 ± 28 nM) in response to 5 mM extracellular calcium. Because transfection of CaR antisense cDNA could not completely block CaR expression (as shown in Fig. 3), the residual CaR protein presumably accounts for the residual 35% of the [Ca2+]i response to [Ca2+]o, in these cells. The reduction of the [Ca2+]i response by antisense CaR was also observed when the [Ca2+]o was raised from 0.03 to 1.2 mM (Fig. 4B). In this experiment,
[Ca\(^{2+}\)], in keratinocytes transfected with vector increased from 79 ± 20 to 243 ± 49 nM (n = 40) in response to 1.2 mM [Ca\(^{2+}\)]\(_o\), whereas the cells transfected with the CaR antisense cDNA displayed a slow increase of [Ca\(^{2+}\)], from 75 ± 11 to 131 ± 12 nM (n = 36).

To test the specificity of the antisense CaR cDNA transfection, we examined its impact on the [Ca\(^{2+}\)] response induced by ATP through the activation of P2 purinergic receptors and by the calcimimetic NPS R-467, a positive modulator of the CaR (24). We have previously shown that extracellular ATP evoked a rapid and transient mobilization of calcium from intracellular sources in keratinocytes (25). In the current study, ATP (100 μM) induced a rapid increase in [Ca\(^{2+}\)], (from 223 ± 12 to 295 ± 21 nM; n = 21) in keratinocytes transfected with empty vector in the presence of 1.2 mM calcium (Fig. 5A).

ATP also evoked a rapid rise in [Ca\(^{2+}\)], to the same magnitude (from 106 ± 10 nM to 172 ± 18 nM; n = 15) in the cells transfected with the antisense CaR cDNA, whereas extracellular calcium failed to induce a [Ca\(^{2+}\)] response (Fig. 5A). We have previously shown that NPS R-467 raised [Ca\(^{2+}\)], and stimulated differentiation in keratinocytes (12). As shown in Fig. 5B, 1 μM NPS R-467 increased [Ca\(^{2+}\)], in the cells transfected with vector in the presence of 0.03 and 1.2 mM extracellular calcium (from 93 ± 11 to 123 ± 10 nM and from 241 ± 18 to 302 ± 19 nM, respectively; n = 15). In contrast, the NPS R-467-induced increase in [Ca\(^{2+}\)] was abolished in the cells transfected with the antisense CaR cDNA, as was the response to [Ca\(^{2+}\)], (n = 18; Fig. 5B). To compare these results with our previous studies (12), we examined the NPS R-467-elicted [Ca\(^{2+}\)] response in suspended keratinocytes. As shown in Fig. 5C, in the cells transfected with vector, 1 μM NPS R-467 increased the [Ca\(^{2+}\)], from 158 ± 40 nM (Basal) to 233 ± 20 nM (+R467) in the presence of 0.03 mM extracellular calcium. Subsequent addition of 1.2 mM extracellular calcium raised [Ca\(^{2+}\)], to 381 ± 39 nM (+R467 +Ca\(^{2+}\)). However, in the cells transfected with the antisense CaR cDNA, the ability of NPS R-467 to raise [Ca\(^{2+}\)], was blunted (an increase from 159 ± 10 to 181 ± 13 nM) at a [Ca\(^{2+}\)], of 0.03 mM, as was the response to the subsequent addition of 1.2 mM [Ca\(^{2+}\)], (increased to 219 ± 36 nM). These results demonstrate that the CaR antisense construct specifically blocks the [Ca\(^{2+}\)] response to [Ca\(^{2+}\)] mediated by endogenous CaR.

Transfection of the Human Antisense CaR cDNA Suppressed Calcium-induced Keratinocyte Differentiation—One of the key characteristics of calcium-induced keratinocyte differentiation is an inhibition of cell proliferation (1). To investigate the role of CaR in calcium-induced differentiation, we first examined the effect of antisense CaR cDNA transfection on proliferation by measuring the rate of DNA synthesis. After transfection with the CaR antisense cDNA construct or empty vector and selection by G418 in KGM containing 0.03 mM calcium for 3 days, keratinocytes were either maintained in 0.03 mM calcium or switched to 1.2 mM calcium for 24 h, and the rate of DNA synthesis was determined by \(^{3}H\)thymidine incorporation. In the keratinocytes transfected with vector, 24 h of incubation in 1.2 mM calcium resulted in a reduction of DNA synthesis to 46.6 ± 0.1% as compared with the control cells maintained in 0.03 mM calcium (Fig. 6). In contrast, DNA synthesis was minimally affected by 1.2 mM calcium (99 ± 19% of control) in the keratinocytes transfected with antisense CaR cDNA (Fig. 6).

We next examined the ability of the antisense CaR construct...
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The CaR antisense construct suppressed the acute [Ca\(^{2+}\)] response to elevated [Ca\(^{2+}\)] and a calcimimetic CaR activator, NPS R-467, but not to ATP. A and B, keratinocytes were transfected with the CaR antisense cDNA construct (anti-CaR) or pcDNA3.1 (vector) on glass coverslips, selected by G418, and loaded with Fura-2 AM as described in the Fig. 4 legend. The [Ca\(^{2+}\)] was measured in buffer A containing 0.03 mM Ca\(^{2+}\), and the response to 100 \(\mu M\) ATP (A) or 1 mM NPS R-467 (B) was then recorded in the presence or absence of 1.2 mM Ca\(^{2+}\). The trace shown in A and B represents the average [Ca\(^{2+}\)] of 15–21 individual transfected keratinocytes during recording. ATP induced an increase in [Ca\(^{2+}\)], in cells transfected with vector as well as in cells transfected with the antisense CaR cDNA. However, the [Ca\(^{2+}\)] response to [Ca\(^{2+}\)] and to NPS R-467 was abolished in keratinocytes transfected with the CaR antisense cDNA (anti-CaR). C, keratinocytes were transfected, selected by G418, trypsinized, suspended, and loaded with Fura-2 AM in buffer A as described under “Experimental Procedures.” Cells were placed in a quartz cuvette, [Ca\(^{2+}\)] was measured in buffer A containing 0.03 mM Ca\(^{2+}\), and the response to 1 mM NPS R-467 was then recorded. 1.2 mM Ca\(^{2+}\) was then added to record the response to extracellular calcium. Each data point shows the mean ± S.D. of triplicate determinations.

To block calcium induction of the differentiation markers involucrin and transglutaminase. As shown in Fig. 7, 48 h of incubation in 1.2 mM calcium significantly increased the levels of involucrin and transglutaminase mRNA in keratinocytes transfected with vector. However, the stimulation of involucrin and transglutaminase expression by calcium was blocked by the antisense CaR cDNA construct. Basal levels of involucrin and transglutaminase mRNA at 0.03 mM calcium were little affected by the antisense CaR cDNA construct.

To determine whether CaR mediates the calcium-induced transcriptional activation of the involucrin and transglutaminase genes, involucrin or transglutaminase promoter/reporter constructs were transiently cotransfected with the antisense CaR cDNA construct, and their response to calcium was then evaluated (Fig. 8). Raising [Ca\(^{2+}\)] from 0.03 to 1.2 mM induced a nearly 5-fold increase in involucrin promoter activity. Antisense CaR cDNA suppressed this increase (Fig. 8A). Similarly, transglutaminase promoter activity was increased 3-fold in response to 1.2 mM extracellular calcium, an increase that was completely blocked by the antisense CaR cDNA construct (Fig. 8B). These data indicate that the induction of both involucrin and transglutaminase by calcium requires CaR.

Conceivably the neomycin analogue G418 could affect the [Ca\(^{2+}\)] response and cell differentiation in keratinocytes by stimulating the CaR. However, our pilot experiments had shown that 500 \(\mu M\) G418, which was used to enrich transfected cells, did not raise [Ca\(^{2+}\)], in keratinocytes in the presence of 0.03 mM Ca\(^{2+}\), (data not shown). Furthermore, G418 at this concentration did not drive cultured keratinocytes toward differentiation because only very low levels of the differentiation markers involucrin and keratinocyte transglutaminase were detected in the transfected cells before exposure to 1.2 mM [Ca\(^{2+}\)], (Fig. 7). Moreover, similar results were obtained in keratinocytes transfected with a CaR antisense construct carrying a hygromycin-resistant gene and subsequently selected by 100 \(\mu g/ml\) hygromycin (data not shown).
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FIG. 8. The CaR antisense cDNA construct blocked the calcium-stimulated transcriptional activation of involucrin and transglutaminase. The CaR antisense construct (anti-CaR) or pcDNA3.1 (vector) was transiently transfected into keratinocytes along with a β-galactosidase expression vector and either an involucrin promoter/luciferase reporter construct (A) or a transglutaminase promoter/luciferase reporter construct (B) in KGM containing 0.03 mM Ca²⁺ as described under “Experimental Procedures.” Cells were incubated in 1.2 mM Ca²⁺ for 48 h before the luciferase and β-galactosidase activities were measured. The promoter activity is expressed as luciferase activity normalized to β-galactosidase activity.

DISCUSSION

Extracellular calcium tightly controls the balance between proliferation and differentiation in a number of different cell types. In mesenchymal fibroblasts (26, 27) and human ovarian surface epithelial cells (28), increased extracellular calcium promotes proliferation. It has been shown that the CaR modulates this proliferative response to extracellular calcium through activation of Src kinase and mitogen-activated protein kinase (27). However, in keratinocytes (1), breast epithelial cells (29), and intestinal epithelial cells (30), differentiation is promoted, whereas proliferation is inhibited by elevated extracellular calcium. The question this study addresses is whether the CaR is also involved in that cellular response to calcium.

We reported previously that keratinocytes express the full-length CaR and its alternatively spliced variant (15). The transcript of the full-length CaR is predominantly expressed in undifferentiated keratinocytes, and its level decreases as the cells differentiate (15). The changes in CaR expression are consistent with the finding that the [Ca²⁺]i, response to [Ca²⁺]o, is maximal in undifferentiated cells and is attenuated as the cells differentiate (15). The notion that CaR modulates the cellular response to extracellular calcium is further supported by the ability of a selective CaR activator, NPS R-467, to potentiate the [Ca²⁺]i, response to [Ca²⁺]o, and to activate the genes required for cornified envelope formation (12). In the present study, we confirmed the expression of CaR proteins in keratinocytes. Because the blockage of CaR production specifically reduced the [Ca²⁺]i, response to [Ca²⁺]o, and to NPS R-467, our results indicate that CaR mediates calcium sensing directly or indirectly in keratinocytes. Transfection of the antisense CaR cDNA also suppressed the calcium-induced inhibition of proliferation and calcium-stimulated expression of involucrin and transglutaminase genes. These results are complementary to our findings (22) in a mouse model in which the synthesis of full-length CaR was disrupted (31). The loss of the full-length CaR altered the morphologic appearance of the epidermis and resulted in a reduction in the expression of the differentiation-related gene, loricrin (22). Furthermore, epidermal keratinocytes isolated from these animals displayed a reduced [Ca²⁺]o response to [Ca²⁺]i (22). These studies indicate that CaR is one of the critical factors that modulate keratinocyte differentiation in vitro and in vivo by mediating calcium sensing and/or by coupling to other calcium signaling molecules important for proliferation and differentiation of these cells.

Immunocytochemical localization studies revealed that in keratinocytes, a low level of CaR protein is expressed on the plasma membrane, whereas its cellular localization is mostly cytoplasmic. Nonetheless, substantial cytoplasmic localization of CaR, often a perinuclear distribution, is commonly observed in other cell types, i.e. rat chondrogenic RJC.C5.18 cells (32), mouse osteoblastic MC3T3-E1 cells (33), osteoblasts and articular and growth plate chondrocytes (34), and pancreatic acinar cells (35). Currently, it is unclear whether intracellular CaR represents nascent receptor protein being processed through the biosynthetic pathway or whether it has distinct biological functions. Indeed, the possibility that CaR mediates calcium sensing not only on the plasma membrane but also within the endoplasmic reticulum has been suggested (36). Although our observations that the antisense CaR specifically reduced the [Ca²⁺]o response to [Ca²⁺]i can be understood as indicating a role for calcium sensing of extracellular calcium by the CaR, it is conceivable that antisense CaR transfection could disrupt a CaR-mediated release of calcium from intracellular stores or a CaR-mediated increase in calcium influx through calcium channels. In either case, the [Ca²⁺]i-mediated increase in [Ca²⁺]o, would be blocked by inhibition of CaR production, and differentiation would subsequently be aborted. Thus, the role of the CaR in calcium-mediated keratinocyte differentiation remains open for further investigation, but this study indicates that it has such a role.

Acknowledgments—We are grateful to Dr. Dolores Shoback for providing anti-CaR antibodies and Dr. Edward F. Nemeth for providing NPS R-467. We thank Sally Pennypacker for providing human epidermal keratinocytes.

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J. Biol. Chem. 2001, 276:41079-41085.
doi: 10.1074/jbc.M107122200 originally published online August 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107122200

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