Identification and Mapping of Keratinocyte Muscarinic Acetylcholine Receptor Subtypes in Human Epidermis[1]

Assane Ndoye,[2] Rico Buchli,[2] Brandon Greenberg, Vu Thuong Nguyen, Shaheen Zia, John G. Rodriguez,* Robert J. Webber,* Monica A. Lawry, and Sergei A. Grando

Department of Dermatology, University of California, Davis, California, U.S.A.; *Research & Diagnostic Antibodies, Richmond, California, U.S.A.

Acetylcholine mediates cell-to-cell communications in the skin. Human epidermal keratinocytes respond to acetylcholine via two classes of cell-surface receptors, the nicotinic and the muscarinic cholinergic receptors. High affinity muscarinic acetylcholine receptors (mAChR) have been found on keratinocyte cell surfaces at high density. These receptors mediate effects of muscarinic drugs on keratinocyte viability, proliferation, adhesion, lateral migration, and differentiation. In this study, we investigated the molecular structure of keratinocyte mAChR and their location in human epidermis. Polymerase chain reaction amplification of cDNA sequences uniquely present within the third cytoplasmic loop of each subtype demonstrated the expression of the m1, m3, m4, and m5 mAChR subtypes. To visualize these mAChR, we raised rabbit anti-sera to synthetic peptide analogs of the carboxyl terminal regions of each subtype. The antibodies selectively bound to keratinocyte mAChR subtypes in immunoblotting membranes and epidermis, both of which could be abolished by preincubating the anti-serum with the peptide used for immunization. The immunofluorescent staining patterns produced by each antibody in the epidermis suggested that the profile of keratinocyte mAChR changes during epidermal turnover. The semiquantitative analysis of fluorescence revealed that basal cells predominantly expressed m3, prickle cells had equally high levels of m4 and m5, and granular cells mostly possessed m1. Thus, the results of this study demonstrate for the first time the presence of m1, m3, m4, and m5 mAChR in epidermal keratinocytes. Because keratinocytes express a unique combination of mAChR subtypes at each stage of their development in the epidermis, each receptor may regulate a specific cell function. Hence, a single cytotransmitter, acetylcholine, and muscarinic drugs may exert different biologic effects on keratinocytes at different stages of their maturation. ***Key words: antipeptide antibody production/immunoblotting/polymerase chain reaction amplification of intronless gene sequences/semiquantitative immunofluorescence. J Invest Dermatol 111:410–416, 1998***

Human epidermal keratinocytes are members of a non-neuroneal signaling network mediating intercellular communication in the skin in which the cytotransmitter acetylcholine (ACh) acts as a local hormone, or a cytokine (Grando and Horton, 1997). Non-neuroneal ACh is synthesized, secreted, and degraded by keratinocytes, and exerts a plethora of biologic effects on these cells because it can simultaneously activate different intracellular signal transduction cascades (reviewed by Grando, 1997). ACh binds to keratinocytes via two distinct classes of cholinergic cell-surface receptors: (i) the ACh-gated ion channels comprised of different nicotinic receptor subunits; and (ii) the G protein-coupled single-subunit transmembrane glycoproteins – the muscarinic acetylcholine receptors (mAChR) (Grando *et al*, 1995a, b). Whereas the nicotinic receptors mediate the transmembrane flow of Na^+, K^+, and Ca^{++}, the mAChR convert ACh signals into metabolic responses through the interactions of G proteins with signal transducing enzymes, leading to increases or decreases in second messengers, ion concentrations, and modulations of protein kinase activities. Muscarinic drugs have been shown to exhibit dramatic effects on keratinocyte proliferation, migration, cell-to-substrate and cell-to-cell adhesion, and differentiation. These effects may involve mAChR-dependent changes in transmembrane Ca^{++} flux and intracellular metabolism (Grando and Dahl, 1993; Grando *et al*, 1993a; Grando and Horton, 1997).

The heterogeneity of mAChR had been suggested by their variable affinity for various pharmacologic ligands, and then demonstrated by molecular cloning the genes coding for these receptors (Bonner, 1989; Mei *et al*, 1989). It has been determined that the family of mAChR is comprised of at least four *pharmacologic* (M1–M4) and five *molecular* (m1–m5) subtypes. The pharmacologic analysis demonstrated that human keratinocytes express $\approx 2.5 \times 10^5$ high affinity mAChR per cell (Grando and Dahl, 1993; Grando *et al*, 1995b), and preliminary data indicated that not all of these mAChR are identical (Grando and Horton, 1997). Whereas experiments designed to elucidate the cellular functions regulated by each *pharmacologic* subtype of keratinocyte

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Reprint requests to: Dr. Sergei A. Grando, Department of Dermatology, University of California, Davis, 4860 Y Street, Suite 3400, Sacramento, CA 95817.

Abbreviations: ACh, acetylcholine; mAChR, muscarinic acetylcholine receptor.

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[2]A. Ndoye and R. Buchli contributed equally to this work. The order of the first two authors is arbitrary.

mAChR are being conducted using subtype-specific muscarinic drugs,[1] the localization of *molecular* subtypes expressed by keratinocytes and other skin cells has been awaiting development of subtype-specific antibodies.

In this study, we established the molecular subtypes of mAChR expressed by human keratinocytes, developed subtype-specific antibodies, and mapped keratinocytes expressing each mAChR subtype to a specific epidermal layer. We found that at each stage of their maturation in epidermis, keratinocytes express different combinations of mAChR subtypes, which could allow a single cytotransmitter ACh to control selectively keratinocyte functions at different stages of cell development.

MATERIALS AND METHODS

Cell and tissue source Normal human skin samples were obtained from circumcision of neonatal foreskins and other surgical procedures (this study has been approved by the University of California Davis Human Subjects Review Committee). The skin specimens destined for immunohistochemistry were snap frozen in liquid nitrogen and stored at –80°C until use. Those skin samples destined for keratinocyte cultures were freed of fat tissue and clotted blood, rinsed in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (Gibco BRL, Gaithersburg, MD), and cut into 3–4 mm pieces. The pieces of skin were placed epidermis up into 35 mm Petri dishes (Falcon 3001, Becton Dickinson Labware, Lincoln Park, NJ) containing 2.5 ml of 0.125% trypsin (Sigma, St. Louis, MO) and 2.5 ml of minimum essential medium (Gibco BRL) supplemented to contain 50 μg gentamicin sulfate per ml, 50 μg kanamycin sulfate per ml, 10 U penicillin G per ml, 10 μg streptomycin per ml, and 5 μg amphotericin B per ml (all from Gibco BRL), and incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. The following day the epidermis was separated from the dermis and individual keratinocytes were released by rapid pipetting or vortexing for about 15 s in minimum essential medium supplemented to contain 20% heat inactivated newborn calf serum (Gibco BRL). The resultant keratinocyte suspension was inoculated into 25 cm^2 culture flasks (Corning Glass Works, Corning, NY) and cultured in serum-free keratinocyte growth medium containing 0.09 mM Ca^{++} (Gibco BRL). After cultures reached ≈70%–80% confluence, the cells were further subcultured into 75 cm^2 flasks. In some cultures, keratinocyte differentiation was stimulated by increasing the concentration of Ca^{++} in keratinocyte growth medium to 1.8 mM during the last 24–72 h of incubation.

Polymerase chain reaction (PCR) amplification of intronless sequences Cultured keratinocytes were scraped from the bottom of the flasks with a rubber policemen, and total RNA samples were prepared using the guanidium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). To remove contaminating genomic DNA, total RNA was incubated for 30 min at 37°C in a solution containing 2.5 U RNase-free DNase I (Gibco BRL) in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, and 40 U of RNase Block (Stratagene, La Jolla, CA). This reaction was stopped by adding 4 mM ethylenediamine tetraacetic acid and DNase I inactivated by heating the samples for 10 min at 65°C. The DNase-treated RNA samples (5 μg) underwent reverse transcription (RT) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 60 U RNase Block, 10 μM random decamer primers $(dN)_{10}$, 600 U RNase H-free reverse transcriptase (Superscript; Gibco BRL), and 0.5 mM each of dATP, dCTP, dGTP, and dTTP in a final volume of 60 μl. The synthesis of single-stranded cDNA was carried out for 80 min at 42°C. To amplify different members of the human mAChR gene family, we designed primers to match specific sequences of each mAChR molecular subtype (Peralta *et al*, 1987; Bonner *et al*, 1988; Bonner, 1989). The sense and anti-sense primers were: for m1, 5′-AGACGCCAGGCAAAGGGGGTGG-3′ and 5′-CACGGGGCTTCTGGCCCTTGCC-3′ (expected size 348 bp); for m2, 5′-ACAAGAAGGAGCCTGTTGCCAACC-3′ and 5′-CAATCTTGCGGGCTACAATATTCTG-3′ (438 bp); for m3, 5′-GACAGAAAACTTTGTCCACCCCAC-3′ and 5′-AGAAGTCTTAGCTGTGTCCACGGC-3′ (496 bp); for m4, 5′-TCCTCAAGAGCCCACTAATGAAGC-3′ and 5′-TTCTTGCGCACCTGGTTGCGAGC-3′ (430 bp); and for m5, 5′-CTCACCACCTGTAGCAGCTACCC-3′ and 5′-CTCTCTTTCGTTTGGTCATTTGATG-3′ (397 bp). The PCR were performed in a final volume of 50 μl containing 8 μl of the RT product, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 2.5 U Taq DNA polymerase (Promega, Madison, WI), and 1 μM each of the sense and corresponding anti-sense primer pairs. The reaction mixture was first heated at 94°C (5 min) before DNA polymerase was added. Cycling was performed at 95°C (60 s), 60°C (60 s), and 72°C (120 s) for 35 cycles. After a period of 20 cycles, the time for the elongation step at 72°C was increased (15 s) for every new cycle. The final cycle had an additional extension time of 10 min at 72°C. The PCR products were electrophoresed on 1.5% agarose gels containing 1 μg ethidium bromide per ml and photographed under fluorescent UV illumination. The size of each PCR product was estimated by using a 250 bp DNA ladder standard (Gibco BRL). To verify that the PCR products indeed represented authentic sequences of human mAChR, the appropriately sized DNA bands were subjected to restriction analysis (see *Results*), and sequenced.

The PCR amplification of intronless sequences necessitated inclusion of additional controls. To control the adequacy of PCR primers, non-DNase-treated samples containing genomic DNA were used as the template. To control for contamination of DNase-treated samples with residual genomic DNA, the RT step was omitted. Lastly, to control for template contamination at every step, an empty tube was subjected to the same procedure as all other samples.

Generation of the mAChR subtype-specific antibodies The m1, m3, m4, and m5 receptor subtype specific anti-sera were generated using synthetic peptide analogs of the carboxyl termini of these proteins by procedures previously described by us (Lark *et al*, 1995). The following peptides were used for immunization: for m1, Ac-SVHRTPSRCQ (residue numbers 451–460); for m3, CFHKRVPEQAL (579–589); for m4, CQYRNIGTAR (469–478); and for m5, CEEKLYWQGNSKLP (518–531). The peptides were conjugated onto bovine thyroglobulin specifically through the Cys residue that was incorporated into each structure, and the conjugates purified before being used to immunize groups of rabbits. The anti-serum collected from each bleed of each animal was tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies specific for the immunogen peptide. Only anti-sera that recognized a specific receptor subtype by ELISA were selected for experiments with keratinocytes.

Immunoblotting The localization of keratinocyte mAChR in western blots employed cellular proteins obtained by lysing directly monolayers of 70%–80% confluent second passage human neonatal foreskin keratinocyte cultures with a solution containing 4% sodium dodecyl sulfate, 20% glycerol, 0.02% bromophenol blue, and 200 mM dithiothreitol in 0.5 M Tris-HCl (pH 6.8). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were performed by a modification of the procedure previously described by us (Webber *et al*, 1994). Briefly, the proteins were separated on 10% gels, electroblotted onto the Immobilon-P membranes (Millipore, Bedford, MA), and blocked overnight with 4% normal goat serum and 20% evaporated goat milk in Tris-buffered saline/Tween 20 buffer, and all primary and secondary antibodies were applied in the same buffer. For blocked antibody controls, 150 nM of the peptide immunogen was added to 6.0 ml of the diluted rabbit polyclonal antibody solution, and preincubated for at least 45 min at 37°C before applying to the membrane. The blots were developed using the enhanced diaminobenzidine reaction.

Semi-quantitative immunofluorescence assays The indirect immunofluorescence experiments were performed as detailed elsewhere (Grando *et al*, 1995b), with minor modifications. Briefly, 4–8 μm sections of freshly frozen normal human skin were fixed for 3 min with phosphate-buffered saline (pH 7.35) containing 3% formaldehyde freshly prepared from paraformaldehyde and 7% sucrose, and incubated overnight at 4°C with a primary anti-mAChR subtype-specific antibody diluted 1:1000. After washing, the specimens were exposed for 1 h at room temperature to the secondary, fluorescein-isothiocyanate conjugated swine anti-rabbit IgG antibody (DAKO, Carpenteria, CA) diluted 1:30 in phosphate-buffered saline. The indirect immunofluorescence images were acquired using a computer-linked, video-monitored Axiovert 135 fluorescence microscope (Carl Zeiss, Thornwood, NY), equipped with a CCD video camera (PTI, Monmouth Junction, NJ), and analyzed with image analysis software (Signal Analytics, Vienna, VA). The relative amounts of each mAChR subtype were estimated by the previously described semiquantitative immunofluorescence assay (Zia *et al*, 1997), which is based on calculating the intensity of fluorescence pixel by pixel by dividing the summation of pixels of fluorescence intensity by the segment occupied by the pixels (i.e., a square template of fixed size that covers an area of epidermis that is equivalent to an average sized keratinocyte), and then subtracting the mean intensity of fluorescence of the background (i.e., a tissue-free segment of the same size). For each skin specimen, at least three different randomly selected segments in at least three different microscopic fields were analyzed.

Statistics The results of the quantitative assays were expressed as mean ± SD. Significance was determined using Student's t test.

[1]Zia S, Buchli R, Ndoye A, Greenberg B, Nguyen V-T, Grando SA: The m4 keratinocyte muscarinic receptor regulates cell motility and modulates intracellular calcium. *J Invest Dermatol* 110:545, 1998 (abstr.)

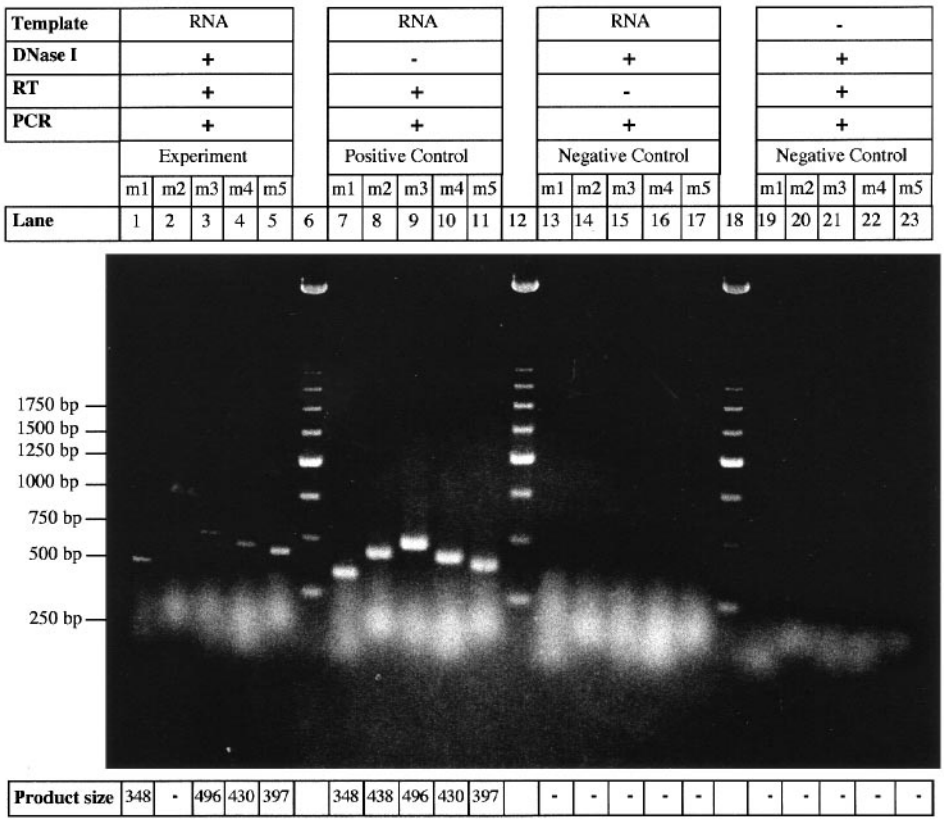

Figure 1. PCR amplification of mAChR sequences from human epidermal keratinocyte RNA. Agarose gel electrophoresis of PCR products amplified from DNase-treated keratinocyte RNA samples reverse-transcribed to cDNA (*lanes 1–5*, "experiment"), DNase nontreated keratinocyte RNA samples containing residual genomic DNA (*lanes 7–11*, "positive control"), DNase-treated keratinocyte RNA samples in which the RT step was omitted to check for residual genomic DNA (*lanes 13–17*, "negative control"), and an empty tube that was subjected to the same procedures as experimental sample to control for template contamination at every step (*lanes 19–23*, "negative control"). Five micrograms of purified total RNA extracted from keratinocytes cultured at 1.8 mM Ca^{++} were reverse-transcribed to cDNA and amplified for 35 cycles with five pairs of mAChR subtype-specific primers. The size of each amplification product was determined using the 250 bp DNA ladder standard loaded in *lanes 6*, *12*, and *18*. These results were reproduced in three independent experiments. The bands amplified from the experimental samples were subjected to the restriction enzyme analysis, and directly sequenced. Both procedures verified that the products amplified from keratinocyte cDNA contained authentic sequences of human m1, m3, m4, and m5 mAChR subtypes.

RESULTS

Human epidermal keratinocytes contain mRNA of m1, m3, m4, and m5 mAChR subtypes The expression of the genes coding for various molecular subtypes of mAChR in human keratinocytes was analyzed using RNA isolated from keratinocyte cultures. As seen in **Fig 1**, each PCR experiment included one positive and two negative controls. The positive control checked for the adequacy of PCR primer pairs, whereas the negative control verified that PCR products were amplified from reverse transcribed mRNA and not from contaminating genomic DNA.

When cDNA from keratinocyte mRNA was used in PCR experiments, the sequences corresponding to authentic m1, m3, m4, and m5, but not to m2, were amplified. These PCR yielded products with the expected size. To confirm their identity, the aliquots of PCR products were examined by restriction analysis, using Nco I (for m1), Hind III (m3), Sac II (m4), and Eco RI (m5). The actual sizes of the obtained fragments were as predicted from the DNA sequence (data not shown). The PCR products were also re-amplified and directly sequenced, further demonstrating their identity with human mAChR.

The m3 mRNA was detected after keratinocyte monolayers were fed with keratinocyte growth medium containing 1.8 mM Ca^{++} for 72 h. In keratinocytes cultured in keratinocyte growth medium containing 0.09 mM Ca^{++}, the m3 was undetectable in two independent experiments.

In positive control experiments, using non-DNAse treated samples of keratinocyte nucleic acids, PCR primers amplified sequences of all five mAChR subtypes (**Fig 1**). In these experiments, the m2 sequence was amplified from genomic DNA. Because the mAChR genes do not contain introns (Peralta *et al*, 1987; Bonner *et al*, 1988; Bonner, 1989), each amplification product derived from genomic DNA was of the same size as the corresponding amplification product derived from the cDNA.

The negative control experiments were performed to verify that our approach to distinguishing between genomic DNA and mRNA was adequate. In these experiments, the RNA samples were treated with DNase (as were the experimental RNA samples), but the RT step was omitted. As expected, no amplified product was generated either in these samples or in the other negative control samples that lacked the RNA templates (**Fig 1**).

Characterization of antibodies to keratinocyte mAChR subtypes The anti-serum to each keratinocyte mAChR subtype, m1, m3, m4, and m5, was characterized for its ability to recognize and bind to the specific amino acid sequence used to elicit its production. Each anti-serum was tested by ELISA titration for its ability to bind to the peptide analog, used as immunogen adsorbed directly onto the microtiter plate (data not shown). Antibody specificity in ELISA was determined by the lack of recognition of unrelated amino acid sequences. No cross-reaction with the other mAChR subtype specific peptides or unrelated receptor peptides was found with any of the anti-sera used in these experiments (data not shown).

Subtype-specific mAChR antibodies label keratinocyte mAChR proteins in western blots The antibodies specific to m1, m3, m4, and m5 mAChR subtypes were used to probe keratinocyte mAChR subtypes among keratinocyte proteins resolved by SDS-PAGE. As seen in **Fig 2**, all four receptors subtypes could be visualized. The m1 subtype migrated with molecular mass (*Mr*) of ≈60 kDa. Both m3 and m4 proteins appeared at the 65 kDa region, whereas the m5 subtype was visualized at ≈95 kDa (**Fig 2**).

The specificity of antibody binding to keratinocyte mAChR in western blots was determined by the ability of only the corresponding immunogen peptide to block the binding of the antibody to the membrane bound protein (data not shown). No protein bands were seen if the primary antibody was omitted (data not shown).

Localization of keratinocyte mAChR subtypes in human epidermis Antibodies to each of the four mAChR subtypes found to be expressed by keratinocytes, namely, m1, m3, m4, and m5, produced an intercellular staining pattern in normal human epidermis consistent with the presence of receptor molecules on keratinocyte cell membranes, as has been previously demonstrated by immunoelectron microscopy (Grando *et al*, 1995b). The m1 antibody selectively stained cell membranes of keratinocytes comprising the granular cell layer (**Fig 3*a***). The staining for m3 was localized to the basal cell layer

(**Fig 3*b***). In addition to the areas of cell-to-cell contact, the m3 antibody also specifically stained areas of the cell membrane covering wide cytoplasmic aprons of basal cells. The bulk of m4 immunoreactivity was seen in the prickle cell layer (**Fig 3*c***). The intensity of staining produced by the m4 antibody gradually increased from the innermost to the uppermost rows of prickle cells. The distinct intercellular, fishnet-like staining pattern produced by m5 antibody was distributed evenly throughout the epidermal layers (**Fig 3*d***).

The indirect immunofluorescence staining was abolished by pre-incubating the specific anti-serum with the synthetic peptide used to elicit the antibody (data not shown).

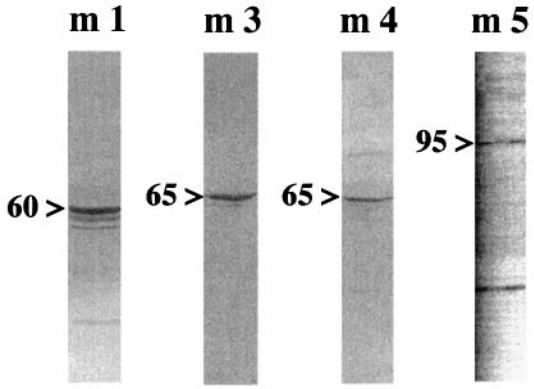

Figure 2. Visualization of mAChR subtypes in western blots of keratinocyte proteins. Results of a representative experiment showing protein bands recognized by rabbit polyclonal antibodies specific for m1, m3, m4, and m5 mAChR subtypes among keratinocyte proteins resolved by SDS-PAGE and immunoblotted as detailed in *Materials and Methods*. Before SDS-PAGE, keratinocyte monolayers were cultured for 24 h in keratinocyte growth medium containing 1.8 mM Ca^{++}. The working dilution of primary antibody was 1:250 and that of secondary, goat anti-rabbit IgG antibody was 1:2500. The bands did not appear in the control experiments, in which immunoblotted membranes were treated without primary antibody or in which the anti-peptide anti-sera were preincubated with the m1, m3, m4, and m5 specific peptides used for immunization. The apparent *Mr* of each receptor protein is shown in kDa.

Semi-quantitative analysis of mAChR subtypes expressed by keratinocytes at different stages of cell differentiation The relative amounts of each molecular subtype of mAChR expressed at various stages of keratinocyte development in epidermis were estimated by using computer-assisted analysis of the intensity of fluorescence (**Fig 4*a***). The relative amount of m1 in granular cells was found to be significantly higher ($p < 0.05$) than in less mature keratinocytes. The basal cells expressed two and three times more m3 as compared with

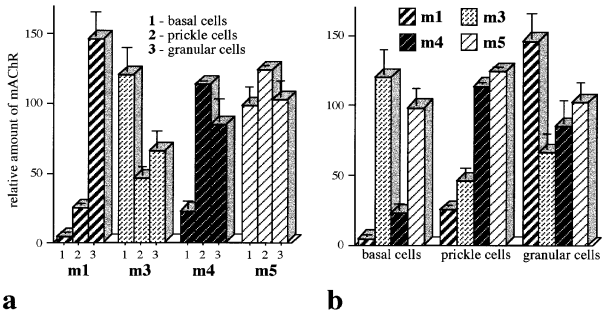

Figure 4. Relative amounts of mAChR subtypes expressed at different stages of keratinocyte development in epidermis. The indirect immunofluorescence images of normal human epidermis stained with m1, m3, m4, and m5 antibodies were acquired using a computer-linked Axiovert 135 fluorescence microscope through a CCD video camera and analyzed by image analysis software as detailed in *Materials and Methods*. The data are mean ± SD of relative amounts of mAChR subtypes computed in three different segments of three different microscopic fields on at least three skin specimens (m1 antibody, n = 3; m3 antibody, n = 4; m4 antibody, n = 4; and m5 antibody, n = 3). (*a*) The relative amounts of each mAChR subtype expressed by basal, prickle, and granular cells; (*b*) the same data reorganized to better observe the profile of mAChR subtypes expressed at each particular stage of keratinocyte development in epidermis.

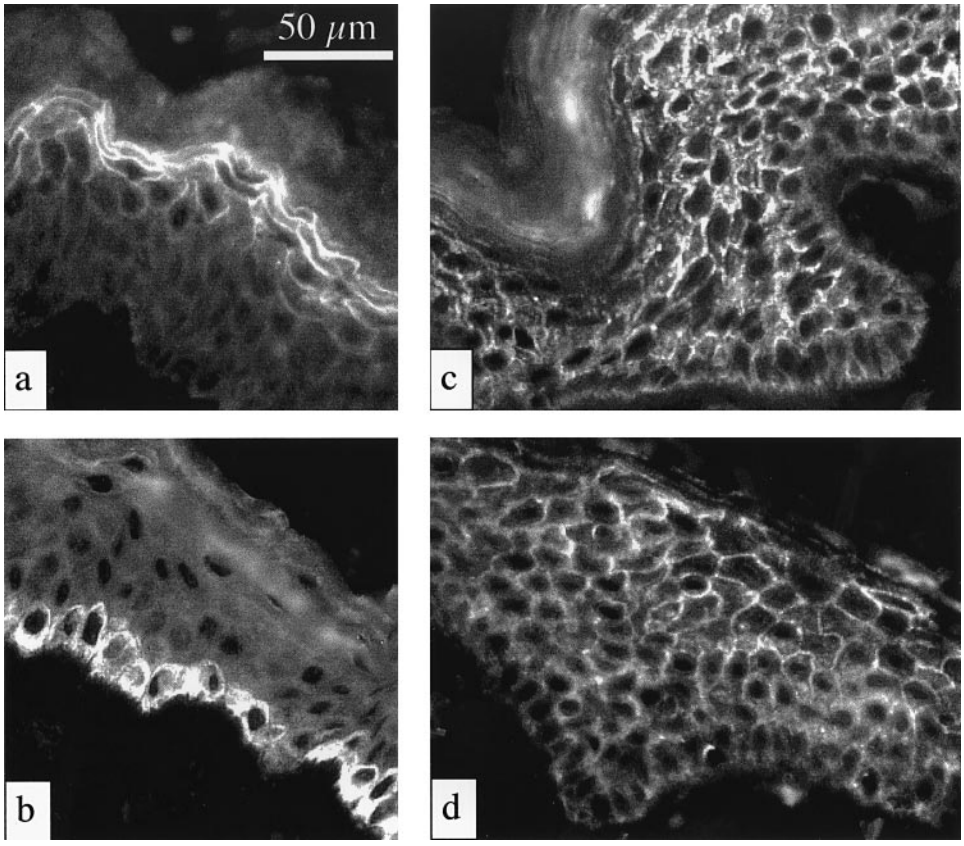

Figure 3. Immunolocalization of mAChR subtypes in human epidermis. The cryostat sections of freshly frozen normal human skin specimens were fixed and treated overnight with rabbit anti-sera raised against synthetic peptides representing the carboxyl terminus of (*a*) m1, (*b*) m3, (*c*) m4, and (*d*) m5. Binding of the primary antibodies was visualized using secondary, fluorescein-isothiocyanate-labeled anti-rabbit IgG antibody (see *Materials and Methods*). Both preincubation of the anti-peptide immune sera with the synthetic peptides used for immunization and omitting the primary antibody abolished the fluorescent staining.

granular and prickle cells, respectively. The relative amount of m4 expressed by prickle cells was significantly greater ($p < 0.05$) than that expressed by basal and granular cells. The relative amounts of m5 expressed by keratinocytes at different stages of their differentiation were rather similar.

Figure 4(*b*) illustrates the profile of mAChR subtypes at each stage of keratinocyte development in epidermis. It appears that basal cells predominantly express m3 and to a lesser extent m5. Only negligible amounts of m1 and m4 are present in basal cells. The prickle cells chiefly express m4 and m5 and significantly less m1 and m3 ($p < 0.05$). The predominant mAChR subtype expressed by granular cells is m1. Granular cells also express other mAChR subtypes in the following relative amounts: m5 > m4 > m3, all of which are significantly less as compared with the relative amount of m1 ($p < 0.05$).

DISCUSSION

In this study, we demonstrated for the first time that normal human epidermal keratinocytes both *in vivo* and *in vitro* express the m1, m3, m4, and m5 molecular subtypes of mAChR, and that the time and the level of the expression of each subtype depend upon the stage of keratinocyte differentiation.

Five mAChR genes (m1–m5) that encode distinct mAChR subtypes have been cloned (Bonner *et al*, 1987, 1988; Peralta *et al*, 1987). Because of their structural homology and pharmacologic similarity, ligand binding probes currently available do not clearly distinguish among the subtypes. Therefore, to identify the profile of keratinocyte mAChR, we used a combination of molecular biologic and immunohistochemical approaches. We screened keratinocyte RNA using primers specific for the nucleotide sequences of m1–m5 subtypes of human mAChR. Because mAChR genes do not have introns, to distinguish between amplification of genomic DNA and cDNA, we pretreated keratinocyte RNA samples with DNase I before RT. No PCR products were obtained if RT was omitted, thus demonstrating the absence of contaminating genomic DNA in our cDNA samples. The PCR products corresponding to subtypes m1, m3, m4, and m5, but not to m2, were obtained. We have shown previously that fibroblasts express m2 mRNA.[2] Therefore, because no m2 mRNA was detected in our PCR experiments, we believe that the keratinocyte cultures used as a source of RNA were free of fibroblast contamination.

To visualize keratinocytes expressing each mAChR in epidermis, we raised polyclonal antibodies against peptide sequences from the nonhomologous regions of the carboxyl terminus of m1, m3, m4, and m5. The specificity of the antibodies for the sequences used for immunization was demonstrated by ELISA with the synthetic peptides. The finding that antibody can selectively label a specific mAChR subtype that is expressed in epidermal keratinocytes was verified by western blotting of SDS-PAGE-resolved cellular proteins. The m1 was visualized at ≈60 kDa, m4 at 65 kDa region, and m5 at 95 kDa, which corresponds to the molecular weights of these subtypes found in other tissues (McLeskey and Wojcik, 1990). The immunoblotting results obtained in this study concur also with those obtained by us previously, using the M35 monoclonal antibody (Grando *et al*, 1995b), which may react with all mAChR subtypes (Carsi-Gabrenas *et al*, 1997). We reported that amongst the SDS-PAGE-resolved membrane proteins solubilized from keratinocytes cultured at low, 0.09 mM Ca^{++}, the M35 antibody labels three protein bands: a duplet at the 60–65 kDa region and single band at ≈95 kDa. Those bands apparently represented m1, m4, and m5. Although the apparent *Mr* of m3 in keratinocytes is about 65 kDa, which is smaller than that of m3 expressed in the cerebellar granule cells (McLeskey and Wojcik, 1990), the m3 band might not be detected at that time because, as demonstrated in this study, keratinocytes start to express detectable amounts of m3 only after exposure to an elevated extracellular Ca^{++}. The absence (or underexpression) of m3 in keratinocytes rapidly growing in culture is consistent with the observation that m3 is coupled to inhibition of cell proliferation in other cell types (Williams and Lennon, 1991). If m3 had the same biologic function in keratinocytes, then localization of the m3 immunoreactivity to the epidermal basal layer would indicate that constant activation of this receptor by endogenous ACh is required to control proliferation of basal cells in epidermis, stimulated via other mechanisms, and that interfering with this inhibitory pathway may lead to a hyperproliferative state.

Early studies employing the M35 antibody also demonstrated that an intercellular, pemphigus-like staining pattern of epidermal keratinocytes for mAChR could be explained by an accumulation of receptor molecules at the cell membrane areas associated with desmosomes (Grando *et al*, 1995b). An important role for mAChR in regulating cell adhesion and motility has been demonstrated in a series of *in vitro* experiments (Grando and Dahl, 1993; Grando *et al*, 1993a). In this study, antibodies to m1, m4, and m5, each produced a distinct intercellular type of staining consistent with an accumulation of these receptor subtypes at the sites of cell-to-cell attachment. Therefore, it is tempting to speculate that these mAChR subtypes are intimately involved with control of cell adhesion and motility.

The appreciable difference in the relative amounts of m1 expressed by granular cells as compared with basal and prickle cells suggests a putative relationship for this mAChR subtype with the process of terminal differentiation that is characteristic of these cells.

Although conspicuous variations in the distributions of mAChR subtypes in epidermis suggest a unique role for each receptor in leading keratinocytes through each stage of their development in epidermis, further studies are needed to define specific biologic functions of each mAChR subtype. The heterogeneity of the keratinocyte muscarinic receptor family demonstrated in this study, taken together with the pleotropic effects of ACh and muscarinic drugs on the cellular functions of human keratinocytes (reviewed by Grando, 1997), suggest that the muscarinic pathways play an important role in mediating endogenous control of epidermal homeostasis by autocrine/paracrine ACh. Variations in keratinocyte mAChR subtype throughout the development of immature basal cells into terminally differentiated granular cells are apparently genetically predetermined. An array of endogenous factors have been shown to alter the mAChR profile. In addition to steroid hormones and retinoids (Baumgartner *et al*, 1993), these include certain mediators of inflammation (Haddad *et al*, 1996a) and growth factors (Haddad *et al*, 1996b).

The expression of certain mAChR subtype(s) can herald malignant transformation, because the cholinergic receptor phenotype of malignant melanoma cells is different from that of normal melanocytes (Grando *et al*, 1995b; Kohn *et al*, 1996; Lammerding-Koeppel *et al*, 1997). The mAChR subtypes may also be selectively targeted in immune-mediated skin diseases. For instance, patients with Sjogren's syndrome develop IgG antibodies that can modify biologic effects mediated by m3 activation (Bacman *et al*, 1996). Patients with pemphigus vulgaris or foliaceus both develop antibodies capable of precipitating a putative keratinocyte cholinergic receptor labeled with the muscarinic radioligand propylbenzylilcholine mustard,[3] and muscarinic agonists can block and reverse pemphigus IgG-induced acantholysis in keratinocyte cultures (Grando and Dahl, 1993). It is also worth noting that cholinergic stimulation is deficient in both lesional and nonlesional skin in vitiligo (Elwary *et al*, 1997), which is associated with abnormal calcium uptake by both keratinocytes and melanocytes obtained from vitiliginous epidermis (Schallreuter and Pittelkow, 1988; Schallreuter-Wood *et al*, 1996).

Each of the five molecular subtypes of mAChR is preferentially coupled to a distinct signal transduction pathway. The two intracellular enzymes that are involved in signaling from mAChR subtypes are phospholipase C and adenylyl cyclase. It is well established from studies with transfected cell lines that the expression of the odd-numbered subtypes couple to pertussis toxin-*insensitive* G proteins and thereby stimulate phospholipase C. This causes the release of both phosphatidylinositol, which releases Ca^{++} from intracellular stores, and diaglycerol,

[2]Buchli R, Ndoye A, Webber R, Grando SA: Identification of m2, m4 and m5 subtypes of muscarinic receptors in human skin fibroblasts. *J Invest Dermatol* 110:607, 1998 (abstr.)

[3]Grando SA, Lee TX, Dahl MV: Antibodies to keratinocyte cholinergic receptor in pemphigus. *J Invest Dermatol* 108:560, 1997 (abstr.)

which activates protein kinases. These mAChR also stimulate arachidonic acid release, increase cyclic AMP and cyclic GMP levels, and open Ca^{++}-dependent K^+ channels. In contrast, even-numbered subtypes couple to pertussis toxin-*sensitive* G proteins and thereby inhibit (previously stimulated) adenylyl cyclase, weakly stimulate phospholipase C, open inwardly rectifying K^+ channels, inhibit calcium currents, and augment arachidonic acid release (reviewed by Hosey, 1992; Jones, 1993). It is possible, however, that a single subtype can couple to either phospholipase C or adenylyl cyclase (Caulfield, 1993).

The mAChR subtypes are distributed heterogeneously throughout the central nervous system and in the periphery. Cumulative data obtained from molecular biologic and immunohistochemical studies demonstrate that m1 is expressed in significant amounts in cerebral cortex, hippocampus, thalamus, caudate-putamen, and amygdala. The m2 subtype is found in basal forebrain, caudate-putamen, hippocampus, hypothalamus, amygdala, and pontine nuclei. The m3 mAChR is abundant in cerebral cortex, olfactory tubercle, hippocampus, thalamus, pons, and cerebellum. The m4 subtype is also seen in cerebral cortex, olfactory tubercle, hippocampus, thalamus, and caudate-putamen. The m5 is localized to hippocampus, thalamus, striatum, lateral habenula, medial mammillary nucleus, and cerebellum (reviewed by Caulfield, 1993).

In the periphery, m1 is expressed in sympathetic ganglia and submaxillary gland (Dorje *et al*, 1991), in biliary (Elsing *et al*, 1997) and prostate epithelia (Ruggieri *et al*, 1995), in zymogen cells, and, to a lesser extent, in the surface mucosal and the muscle stomach layers (Helander *et al*, 1996). The m2 subtype is found in sympathetic ganglia, ileum, uterus, heart (Dorje *et al*, 1991; Hoover *et al*, 1994), corpus cavernosum (Toselli *et al*, 1994), and detrusor and ciliary muscles (Zhang *et al*, 1995; Yamaguchi *et al*, 1996). The m3 mAChR has been localized to peripheral auditory system (Safieddine *et al*, 1996), fundic mucosa, and smooth muscle cells of the gastric tunica muscularis (Hunyady *et al*, 1996), enterocytes on rat jejunal villi (Przyborski and Levin, 1993), submaxillary gland (Dorje *et al*, 1991), striated and interlobular salivary gland duct cells (Shida *et al*, 1993), corneal and anterior lens epithelia (Gupta *et al*, 1994), as well as tracheal smooth muscle, epithelium, and blood vessels, and, to a lesser extent, bronchiolar smooth muscle (Mak *et al*, 1993). The m4 is present in bronchiolar smooth muscle and alveolar walls (Mak *et al*, 1993), and m5 is present in arterial endothelium (Phillips *et al*, 1997). Peripheral blood mononuclear cells and purified T cells express three mAChR subtypes, m3, m4, and m5 (Costa *et al*, 1995; Hellstrom-Lindahl and Nordberg, 1996), as does iris-ciliary body (Gil *et al*, 1997).

In summary, free ACh (neuroneal or non-neuroneal) is abundantly present in the surface tissues lining human mucocutaneous membranes (Grando *et al*, 1993b; Johansson and Wang, 1993; Klapproth *et al*, 1997). The diversity of mAChR expressed by keratinocytes allows this single cytotransmitter to exert different effects at various stages of cell differentiation. The cutaneous cholinergic network, however, is not limited to keratinocytes. It is becoming increasingly clear that the cells inhabiting human skin assemble an intercellular signaling network wherein ACh acts as a common cytotransmitter mediating intercellular communications. Normal human skin melanocytes (Iyengar, 1989), fibroblasts (Vestling *et al*, 1995), and endothelial cells (Gardner-Medwin *et al*, 1997), have all been found to express one or more functional elements of the cellular cholinergic system. Perhaps, the cholinergic signals generated by these non-neuroneal cells in response to environmental stimuli are recognized by the PGP 9.5-positive nerves penetrating epidermis (Grando *et al*, 1995b), thus providing a functional neurocutaneous axis of the nervous system.

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