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Human Skin Fibroblasts Express m2, m4, and m5 Subtypes of Muscarinic Acetylcholine Receptors

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Abstract Previous studies have demonstrated that muscarinic acetylcholine receptors (mAChRs) are expressed by human skin fibroblasts (HSF). We have identified the molecular subtypes of these receptors by reverse transcription-polymerase chain reaction (RT-PCR), using m1-m5 subtype-specific primers. These experiments showed that only mRNAs for m2, m4, and m5 mAChR subtypes are present in HSF. The RT-PCR products were characterized by restriction analysis and Southern blotting. Northern blot analysis showed the presence of m2 and m4 mAChR RNA. Rabbit antibodies were raised using a synthetic peptide as immunogen corresponding to the C-terminus of the m2 protein and were used to visualize fibroblast mAChRs. Cell membranes of HSF in cell culture and specimens of normal human skin had a unique staining pattern specific for anti-m2 antibody, as well as for antibodies against m4 and m5. In Western blots of fibroblast proteins, the antibodies visualized the m2 receptor at 65 kDa, m4 at 70 kDa, and m5 at 95 kDa. The function of fibroblast mAChRs was examined by measuring muscarinic effects on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Muscarine increased transiently $[Ca^{2+}]_i$ in cultured HSF. This effect could be abolished by the muscarinic antagonist atropine. Thus, the results of this study showed that HSF express m2, m4, and m5 mAChR subtypes, and that fibroblast mAChRs are coupled to the regulation of $[Ca^{2+}]_i$. J. Cell. Biochem. 74:264–277, 1999.

Key words: human skin fibroblasts; muscarinic acetylcholine receptors; reverse transcriptase-polymerase chain reaction; Northern blot; anti-peptide antibody production; Western blot; indirect immunofluorescence; intracellular free calcium concentration

Muscarinic acetylcholine receptors (mAChRs) are present in a wide variety of tissues in which they modulate important physiological responses. The mAChRs are members of a large family of receptors that mediate signal transduction by coupling with heterotrimeric guanine nucleotide-binding proteins, or G-proteins, which modulate the activity of a number of effector enzymes in addition to regulating ion channels directly, leading to increases or decreases in second messengers or changes in ion concentrations [reviewed by Nathanson, 1987]. Each mAChR subtype is a single glycoprotein molecule that spans the plasma membrane seven times, resulting in seven hydrophobic transmembrane domains (TM1-TM7) connected by three extracellular (e1-e3) and three intracellular (i1-i3) hydrophilic loops. Through molecular cloning, five human subtypes of mAChRs (m1-m5) have been identified to date [Allard et al., 1987; Bonner et al., 1987, 1988; Peralta et al., 1987a,b], all of which lack introns in their coding sequences. The amino acid sequences of these receptors are highly conserved, particularly within transmembrane domains. The region of the least homology occurs in the cytoplasmic loop i3 located between TM5 and TM6. These mAChR subtypes are generally grouped according to their functional coupling either to the mobilization of intracellular calcium (m1, m3, and m5) through the activation of phospholipase C, which results in the release of the second messenger inositol 1,4,5-trisphosphate (IP_3), or by the inhibition of adenylyl cyclase (m2 and m4), which results in the reduction of the intracellular levels of cyclic adenosine monophosphate [reviewed by Hulme et al., 1990; and Hosey, 1992].

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The different human cell types that inhabit the skin form a non-neuronal signaling network and use acetylcholine as a common cytotransmitter [Grando, 1997; Grando and Horton, 1997]. Human skin fibroblasts (HSF), which play pivotal roles in connective tissue production, physiological skin remodeling, and wound repair, are part of this network. These mesenchyme-derived cells located in the dermis express mAChRs, in common with the epithelial cells that form the epidermis. We previously demonstrated mAChRs on the cell surfaces of HSF [Grando et al., 1995] using the monoclonal antibody M35 [Andre et al., 1984], which can label all known mAChR subtypes [Carsi-Gabrenas et al., 1997]. However, previous studies of mAChRs in HSF have been a subject of some controversy. For instance, Nadi et al. [1984] reported that cultured adult HSF possess mAChRs that could be labeled by the specific, nonselective, reversible muscarinic radioligand [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB). Vestling et al. [1995] also demonstrated high levels of saturable binding of [^{3}H]QNB and the M2/M4 selective radiolabel [^{3}H]AF-DX 384 to lysed HSF membrane preparations. By contrast, Van Riper et al. [1985], Lin and Richelson [1986], and Kelsoe et al. [1986] reported that fibroblasts have only negligible amounts of muscarinic ligand binding sites. Difficulties with studying fibroblast mAChRs have been acknowledged by a number of investigators [e.g., Lennox et al., 1985].

In this study, we identified the molecular subtypes of mAChRs present in HSF by detecting mRNA encoding these receptors using the reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot techniques. To further verify and visualize the expression of mAChR subtypes in HSF, we raised subtype-specific rabbit antibodies against synthetic peptide analogues of the C-termini of the mAChR proteins and used these antibodies in Western blotting and indirect immunofluorescence (IIF) experiments. The function of the mAChRs expressed by HSF was investigated by measuring changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to the cholinergic agonist muscarine, and antagonist atropine. We report herein that HSF express the m2, m4, and m5 mAChR subtypes on their cell surface, and that activation of fibroblast mAChR leads to a transient increase in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Cell Culture

Pure cell cultures of adult HSF were established from the cells purchased from Clonetics (San Diego, CA) and grown in special fibroblast growth medium-2 supplemented with 1 ng/ml human fibroblast growth factor, 5 µg/ml insulin, 2% fetal bovine serum (FBS), and 50 µg/ml of each gentamicin and amphotericin-B (all from Clonetics) in a 5% CO_2 environment at 37°C, as described by Varani et al. [1990]. The medium was replenished every 3–4 days. Confluent cells were subcultured by detaching the monolayer with 0.01% EDTA supplemented with trypsin (0.025% final concentration) in HEPES-buffered saline solution (Clonetics). Cells of passages 3–10 were used in these studies.

Preparation of Total RNA

Total RNA was extracted from cultured HSF using the guanidinium thiocyanate-phenol-chloroform extraction procedure (Tri-Reagent, Sigma, St. Louis, MO), as previously described [Chomczynski and Sacchi, 1987]. The quality and structural integrity of RNA samples were confirmed by electrophoresis on 1.1% agarose/2.2 M formaldehyde gels containing 0.5 µg/ml ethidium bromide, and by the optical density ratio at 260 nm, as compared with that at 280 nm. Only RNA samples that displayed intact 18S and 28S ribosomal RNA (rRNA) bands and that exhibited a 260/280 ratio of >1.8 were used in the experiments.

RT-PCR of Intronless mAChR Gene Sequences

In order to remove traces of contaminating genomic DNA, total RNA was treated with 2.5 U RNase-free DNase I (Gibco BRL, Gaithersburg, MD) for 60 min at 37°C in a solution containing 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 EDTA, and the DNase I was inactivated by heating the samples for 10 min at 65°C. RT was performed from 5 µg of DNase-treated RNA samples 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, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP and 600 U RNase H-free reverse transcriptase (Superscript; Gibco-BRL) in a final volume of 60 µl. The synthesis of single-stranded cDNA was car-

ried out at 42°C for 80 min. PCR amplification of the five known subtypes of human mAChRs was directed by subtype-specific primers that were designed based on published nucleotide sequences for each of the human mAChR genes [Allard et al., 1987; Bonner et al., 1987, 1988; Peralta et al., 1987a,b]. Primer sequences, PCR product sizes, and GenBank data base accession numbers are shown in Table I. Oligonucleotide primers were synthesized by Bio-Synthesis (Lewisville, TX). The PCRs were carried out in a final volume of 50 µl containing 8 µl of the RT reaction product, 10 mM Tris-HCl, (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 1 µM each of both the sense and the corresponding antisense primers and 2.5 U *Taq* DNA polymerase (Promega, Madison, WI). The reaction mixture was first heated at 95°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/ml ethidium bromide and photographed under fluorescent ultraviolet (UV) illumination. The size of each PCR product was estimated from the migration of DNA size markers that were run concurrently (250 bp DNA ladder, Gibco-BRL). The identity of each PCR product was confirmed by Southern blotting and restriction analysis. PCR products derived from specific primers were excised from the gel, subcloned into the *Sma*I site of the vector pBluescript SK (Stratagene), sequenced, and used as labeling probes.

PCR amplification of these intronless sequences necessitated the inclusion of three additional controls. To control for the adequacy of PCR primers, RNA samples containing contaminating genomic DNA were used as a template. These samples were neither treated with DNase I nor reverse transcribed. To check DNase-treated samples for the absence of residual genomic DNA, the RT step was omitted. Finally, to ensure no carryovers from other samples at every step, blank samples consisting of reaction mixtures without RNA were routinely processed in parallel with the experimental samples.

Southern Blot Analysis

RT-PCR products, separated by agarose-gel electrophoresis, were transferred onto a Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, CA) and prehybridized for 1 h at 55°C in 20% deionized formamide, 900 mM NaCl, 6 mM EDTA, 90 mM Tris-HCl (pH 7.0), 1 mM $Na_4P_2O_7$, 1% sodium dodecyl sulfate (SDS), 0.5% nonfat milk, 5 µg/ml polyadenylic acid, and 500 µg/ml denatured salmon sperm DNA. Hybridization was performed overnight by adding ^{32}P-labeled nick-translated specific probes for each mAChR subtype (0.5×10^6 cpm/ml) simultaneously to the prehybridization solution. It was established in a series of control experiments that none of the five subtype-specific mAChR probes could cross-hybridize with other receptor subtypes (data not shown). The membrane was then washed once with 2× saline-sodium citrate (SSC; 300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 30 min at room temperature and finally with 1× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.5% SDS

TABLE I. Primers Used to Direct RT-PCR Amplification of the Human m1–m5 mAChR Subtypes[a]

Gene	Primer name	Sequence	Expected size of PCR product (bp)	GenBank database accession number
m1	SSm1	5′-AGACGCCAGGCAAAGGGGGTGG-3′	348	M35128
	ASm1	5′-CACGGGGCTTCTGGCCCTTGCC-3′		
m2	SSm2	5′-ACAAGAAGGAGCCTGTTGCCAACC-3′	438	X15264
	ASm2	5′-CAATCTTGCGGGCTACAATATTCTG-3′		
m3	SSm3	5′-GACAGAAAACTTTGTCCACCCCAC-3′	496	U29589
	ASm3	5′-AGAAGTCTTAGCTGTGTCCACGGC-3′		
m4	SSm4	5′-TCCTCAAGAGCCCACTAATGAAGC-3′	430	X15265
	ASm4	5′-TTCTTGCGCACCTGGTTGCGAGC-3′		
m5	SSm5	5′-CTCACCACCTGTAGCAGCTACCC-3′	397	M80333
	ASm5	5′-CTCTCTTTCGTTTGGTCATTTGATG-3′		

[a]RT-PCR, reverse transcription-polymerase chain reaction.

for 1 h at 65°C. X-ray films were then exposed to the membranes for 15 min.

Northern Blot Analysis

RNA isolated from cultured HSF were denatured in 10 mM MOPS, pH 7.0, 5 mM sodium acetate, 0.1 mM EDTA, 2.2 M formaldehyde, and 50% formamide at 65°C for 10 min. Samples were size-fractionated by electrophoresis on a 1.1% agarose/2.2 M formaldehyde gel in a buffer consisting of 10 mM MOPS, pH 7.0, 5 mM sodium acetate and 0.1 mM EDTA, blotted onto an Amersham Hybond-N™ filter (RPN1520N; Amersham Life Science, Arlington Heights, IL) and cross-linked by UV irradiation. Each lane contained 100 µg of total RNA. After prehybridization for 3 h at 55°C, RNA blots were hybridized for 20 h with ^{32}P-labeled nick-translated specific probes for each mAChR subtype (10^6 cpm/ml) in 50% deionized formamide, 5× SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 0.1% SDS, 10% dextran sulfate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 50 µg/ml denatured salmon sperm DNA. The filters were washed once with 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 30 min at room temperature and finally with 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) containing 0.1% SDS for 1 h at 65°C. X-ray films were exposed to the blots for 72 h [Buchli et al., 1995].

Generation of Rabbit Polyclonal Antibody to m2 mAChR

The m2 mAChR subtype-specific antiserum was generated employing the same procedures we previously used to raise rabbit polyclonal antibodies specific for the m1, m3, m4, and m5 mAChR subtypes [Ndoye et al., 1998], which were also used in this study. Briefly, a synthetic peptide analogue of the C-terminus of m2, CHYKNIGATR (residue numbers 457–466), was conjugated onto bovine thyroglobulin specifically through the Cys residue that was incorporated into the peptide structure, and the conjugate was purified before being used to immunize rabbits. The antisera produced by the rabbits were tested by enzyme-linked immunosorbent assay (ELISA) for their ability to recognize specifically the m2 receptor. Positive antisera were selected and then tested in Western blots of whole rat brain homogenate. Only antiserum that both recognized the m2 subtype in ELISA and yielded a positive Western blot was used in experiments with HSF.

IIF

The IIF experiments with cultured HSF we performed essentially as described previously [Ndoye et al., 1998] with minor modifications. Briefly, HSF were grown in six-well plates until approximately 70–80% confluent, after which the dishes were washed with phosphate-buffered saline (PBS), air dried, and kept frozen at −80°C until use. On the day of the experiment, the cells were fixed for 3 min with 3% paraformaldehyde and 7% sucrose in ice-cold PBS, and incubated overnight at 4°C with a primary anti-mAChR subtype-specific antibody diluted 1:1,000 in PBS. After washing, the cells were exposed for 1 h at room temperature to fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG antibody (Dako Corporation, Carpenteria, CA) diluted 1:30 in PBS.

Double staining experiments were performed using 4- to 8-µm sections of freshly frozen normal human neonatal foreskins following the protocol described elsewhere [Grando et al., 1995]. Briefly, fixed skin specimens were incubated overnight at 4°C with a mixture of primary antibodies: rabbit anti-mAChR subtype-specific antibody (final dilution 1:500) and mouse anti-fibroblast antibody MAS 516b (Accurate Chemical Corp., Westbury, NY) (final dilution 1:5). Excess primary antibody was removed by repeated washing, and the specimens were exposed for 1 h at room temperature to FITC-conjugated anti-rabbit IgG antibody (final dilution 1:15) and a tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG antibody (Pierce, Rockford, IL) (final dilution 1:50). All antibodies were diluted in PBS (pH 7.4). The immunofluorescence images were acquired using a computer-linked, video-monitored Axiovert 135 fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a CCD video camera (Photon Technology International, Monmouth Junction, NJ), and analyzed using an image analysis software package purchased from Signal Analytics (Vienna, VA).

Western Blot Analysis

To localize fibroblast mAChRs in Western blots, cellular proteins were obtained by lysing directly monolayers of 70–80% confluent HSF cultures with a solution containing 4% SDS, 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 of HSF cell lysates were performed by a modification of the procedures described previously [Webber et al., 1994; Ndoye et al., 1998]. Briefly, the proteins were separated on a 10% gel, electroblotted onto an Immobilon-P membranes (Millipore, Bedford, MA), and blocked overnight in 4% normal goat serum and 20% evaporated goat milk in Tris-buffered saline/Tween 20 buffer. All primary and secondary antibodies were applied in this buffer. The working dilution of primary antibodies was 1:1,000 for m2, and 1:300 for m4 and m5, and that of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was 1:2,500. To visualize antibody binding, the membranes were developed using the enhanced diaminobenzidine reaction.

Measurement of $[Ca^{2+}]_i$

HSF were plated into 2-well Lab-Tek slide chambers (Nunc, Naperville, IL) and incubated in a 5% CO_2 incubator at 37°C until reaching 50–60% confluency. The cells were then loaded with 5 μM fura-2 AM (Molecular Probes, Junction City, OR) in a calcium assay buffer consisting of 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.74 mM KH_2PO_4, 0.46 mM Na_2HPO_4, 10 mM glucose, 1.8 mM $CaCl_2$, 1 mg/ml bovine serum albumin (BSA), and 10 mM HEPES, pH 7.4, for 90 min at room temperature. After that, the cells were washed twice with the same buffer and placed in a 5% CO_2 incubator at 37°C for 30 min to recover. Ratio-imaging experiments were performed on an inverted, computer-linked, video-monitored Axiovert 135 fluorescence microscope equipped with a heating stage, an automated, computer-controlled filter wheel carrying excitation filters (10-nm bypass) of various wavelengths, and an IC-100 video camera (Photon Technology International) to transmit images from the microscope to a Power Macintosh 8500/120 computer. The data were analyzed using the IP Lab Ratio Imaging software package (Signal Analytics). The slide chamber was mounted on the heated stage of the inverted microscope, and HSF were excited sequentially at wavelengths of 340 and 380 nm, and emission was detected at 510 nm. Fluorescence was quantified by averaging pixel intensities throughout the cell at each of the two wavelengths and used to calculate the 340-nm to 380-nm ratios after background subtraction. The results of each experiment were expressed as the mean fluorescence ratio recorded at 37°C in 25 to 35 cells every 5 s, and $[Ca^{2+}]_i$ was calculated from the fluorescence ratios according to Grynkiewicz et al. [1985]. Ionomycin, 5 μM, and EGTA, 10 mM, were added at the end of each experiment to establish the maximum and minimum 340-nm to 380-nm ratios in fura-2 AM-loaded cells. Using an external calibration standard kit (Calbiochem, San Diego, CA), the effective dissociation constant for fura-2 AM was calculated to be 216 nM at 37°C, which approximates to the value previously published by Grynkiewicz et al. [1985]. The results were expressed as mean±SEM. Significance was determined using the Student's *t*-test.

RESULTS

Identification of mAChR Subtype RNAs Expressed in HSF by RT-PCR

Amplification of receptor mRNAs using subtype-specific PCR primers. In an effort to examine carefully the mAChR subtypes expressed by HSF, we performed RT-PCR experiments on total fibroblast RNA using subtype-specific primers (Table I). The primers were matched within the intracellular loop region i3 that connects the transmembrane domains TM5 and TM6. This region shows the highest degree of divergence between the mAChR subtypes; thus, each primer is unique for a particular mAChR subtype. These PCR primers amplified mRNAs encoding m2, m4, and m5 subtypes from the total RNA extracted from cultured HSF, whereas mRNAs for the m1 and m3 receptors were not detected (Fig. 1A, lanes 2–6). RNA samples were treated with DNase before the RT step. The effectiveness of the m1-m5 mAChR subtype-specific primers in directing synthesis of mAChR nucleotide sequences was verified by amplifying all five mAChR sequences from genomic DNA in a positive control experiment (Fig. 1A, lanes 8–12). Each band of interest was found to be exactly the size expected.

Because the mAChR genes do not contain introns, and the amplification product derived from genomic DNA has the same size as the cDNA PCR product, additional controls were necessary to distinguish between these signals. When the RT step was omitted, no amplification product was generated, thus demonstrat-

ing the absence of contaminating genomic DNA in the cDNA samples used (Fig. 1A, lanes 14–18). In addition, blank samples were routinely used to ensure no carryover from other samples (Fig. 1A, lanes 20–24). To confirm that the specific PCR products indeed represented authentic sequences of human m2, m4, and m5 mAChRs, the amplified DNA bands were subjected to restriction digestion, Southern blotting (see below), and sequencing.

Restriction analysis of PCR products. On the basis of known restriction sites within the PCR products, each restriction enzyme was expected to produce two or more new fragments of known size. As shown in Figure 1B, the size of each fragment generated was as predicted.

Southern blotting of PCR products. PCR products were further characterized by Southern blot hybridization with ^{32}P-labeled fragments of subcloned and sequenced DNAs that derived from PCR amplification products coding for the i3-loop region of each human mAChR subtype (Fig. 1C). The radiolabeled probes specifically hybridized to the corresponding band amplified in the PCR, thus confirming the authenticity of the amplified human mAChR sequences. As expected, only m2, m4, and m5, but not m1 or m3, sequences were present in HSF.

Analysis of mRNA Encoding mAChR Subtypes in HSF by Northern Blotting

The results of the Northern blot analysis of total RNA isolated from HSF are shown in Figure 2. Single species of mAChR mRNA of 6.0 kb for m2 and 4.8 kb for m4, respectively, were detected using ^{32}P-labeled nick-translated specific probes from the highly divergent i3 cytoplasmic loop. No band for the mRNA encoding m5 subtypes could be detected (not shown), suggesting that this RNA transcript is expressed in the cultured HSF at a level below the sensitivity of the Northern blot technique used.

Characterization of the m2 mAChR Subtype-Specific Antibody

The m2 antiserum was characterized by ELISA and Western blotting. By ELISA titration, the m2 antiserum was tested for its ability to bind to the peptide analogue used as immunogen absorbed directly onto the microtiter plate. Antibody specificity in ELISA was determined by the lack of recognition of unrelated amino acid sequences, such as the other mAChR subtype specific peptides and peptide sequences derived from other receptors. No cross-reaction with the other mAChR subtype-specific peptides or unrelated receptor peptides was found in these experiments (data not shown). Antibody specificity was also investigated in Western blots of whole rat brain homogenate. It was demonstrated that only the specific amino acid sequence used to elicit the anti-m2 antiserum blocked the binding of the antibody to the membrane-bound protein (data not shown).

Visualization of mAChR Subtypes Expressed by HSF in Cell Cultures and Skin Specimens

Although the presence of m2, m4, and m5 mAChR mRNAs in HSF strongly suggested that these cells possess mAChRs, further evidence was required to assure that these mRNAs are indeed translated into proteins. Therefore, cultures of adult HSF and cryostat sections of normal human neonatal foreskins were stained with antibodies specific for each of the five (m1-m5) mAChR subtypes. The antibody binding was visualized by IIF (Fig. 3). Rabbit antibodies specific for m2 (Fig. 3a,d), m4 (Fig. 3b,e), and m5 (Fig. 3c,f), but not for m1 or m3 (not shown), stained fibroblasts.

In cultures of HSF (Fig. 3a–c), the anti-mAChR subtype antibodies produced specific staining of the cell membranes, indicating the presence of the receptor proteins. An intense staining pattern of the HSF cytoplasmic processes was observed with all three antibodies (m2, m4, and m5). When the cells were treated with antibodies raised against the m4 and m5 receptors, the staining was also observed in the perinuclear region of the cells.

In the skin specimens (Fig. 3d–f), antibodies against m2, m4, and m5 receptors specifically stained spindle cells residing in the papillary and reticular parts of the dermis (green pseudocolor). That the cells that carry mAChRs were indeed HSF was established in double-staining experiments wherein, in addition to the rabbit anti-mAChR subtype antibody, the skin specimens were also stained with the murine anti-fibroblast antibody MAS-516b (red pseudocolor). All MAS-516b-positive cells were found to be mAChR-positive as well. The cell membrane areas enveloping spindle-like cytoplasmic processes of HSF were intensely stained for m2 (Fig. 3d). Anti-receptor antibodies, particularly m4 antibody (Fig. 3e), produced a weak,

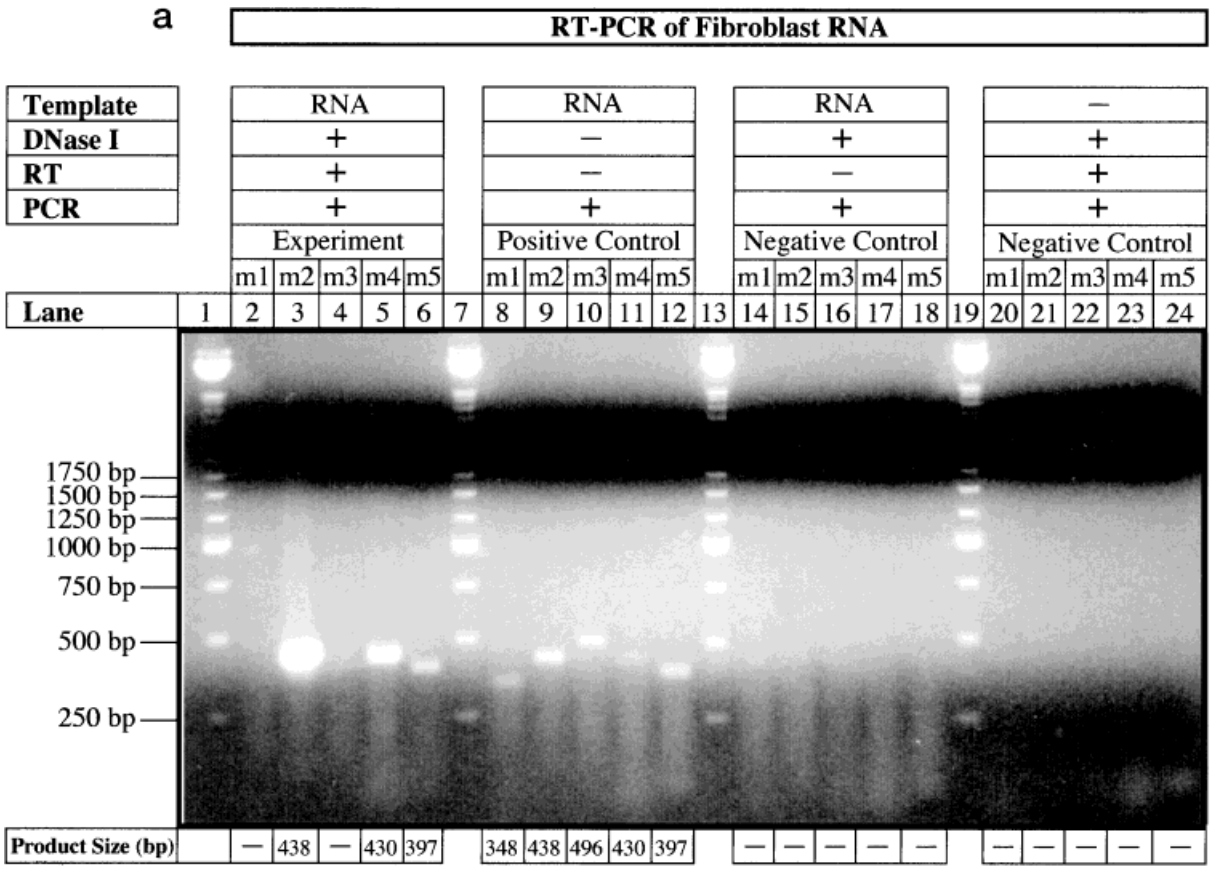

Fig. 1. Polymerase chain reaction (PCR) amplification of fibroblast mAChR subtypes. **a:** Agarose gel electrophoresis of reverse transcription (RT)-PCR products amplified from HSF RNA using primers specific for each of the five known mAChR genes amplified from: (1) DNase-treated fibroblast RNA samples that were reverse-transcribed to cDNA ("experiment"; **lanes 2–6**); (2) DNase nontreated fibroblast RNA samples that also contained genomic DNA ("positive control;" **lanes 8–12**); (3) DNase-treated fibroblast RNA samples in which the reverse transcriptase step was omitted to check for residual genomic DNA ("negative control;" **lanes 14–18**); and (4) blank sample consisting of reaction mixtures lacking RNA that was subjected to the same procedures as the experimental sample to control for template contamination at every step ("negative control;" **lanes 20–24**). The size of each amplification product was determined using a 250-bp DNA ladder standard loaded in lanes 1, 7, 13, and 19 with bands labeled in base pairs (bp). The size of each RT-PCR product is indicated at the bottom of each lane. The results shown herein were reproduced in three independent experiments. **b:** Restriction analysis of RT-PCR products. RT-PCR products were digested with restriction enzymes specific for each mAChR sequence. The resulting fragments were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. The following restriction enzymes were used: *Nco*I for ml (295- and 143-bp fragments), *Bam*HI for m2 (295- and 143-bp fragments), *Hin*dIII for m3 (281-, 158-, 47-, and 10-bp fragments), *Sac*II for m4 (258- and 172-bp fragments), and *Eco*RI for m5 (288- and 109-bp fragments). The size of each fragment is shown at the bottom of each lane. **c:** Southern blot of RT-PCR products. RT-PCR products were subjected to Southern blotting and hybridized with specific probes used to amplify each mAChR subtype (see under Materials and Methods). The blot was exposed for 15 min. The bands correspond to the expected products. The size of each product is shown at the bottom of each lane.

homogeneous staining pattern of the cell as a background against which bright dots of stain were clearly seen. The latter apparently represented clusters of mAChRs on the cell surface of HSF. The m5 subtype was localized predominantly to the perinuclear areas of the cells (Fig. 3F). No fluorescence staining could be detected in cell cultures and skin specimens incubated without primary antibody or when a receptor antiserum was preincubated with the peptide used for immunization (not shown).

Visualization of mAChR Subtypes Expressed by HSF in Western Blots

Antibodies specific to m2, m4, and m5 mAChR subtypes were used to probe total fibroblast cellular proteins resolved by SDS-PAGE. As

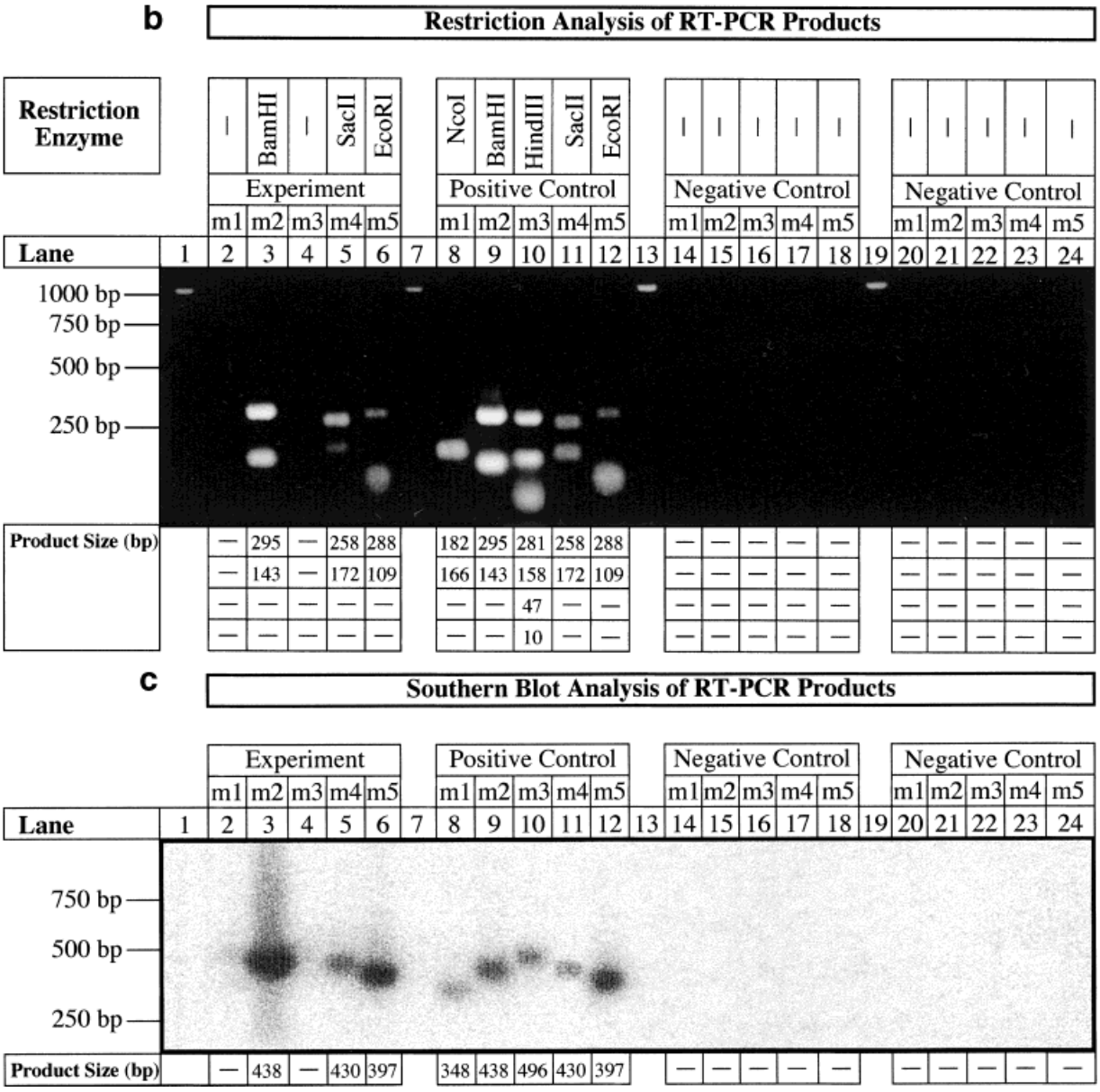

Figure 1. (Continued.)

shown in Figure 4, the m2 antibody specifically recognized a protein band with apparent molecular mass of 65 kDa, m4 antibody reacted with a 70-kDa protein, and m5 antibody visualized a protein band at 95 kDa. The m1 and m3 antibody did not react specifically with the fibroblast proteins (not shown). The specific staining observed on the Western blot membranes was abolished by preincubation of the primary antiserum with the respective peptide immunogen. No protein bands were found if the primary antibody was omitted (data not shown).

Muscarine Elicits $[Ca^{2+}]_i$ Transients in Cultured HSF

To investigate the ability of mAChRs expressed by HSF to bind muscarinic ligands and to elicit intracellular signaling, we examined changes of $[Ca^{2+}]_i$ using ratio-image analysis. Video imaging was performed in 25–35 cells loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 AM. As shown in Figure 5a, exposure of HSF to 100 µM muscarine resulted in a rapid and transient increase of $[Ca^{2+}]_i$ from the rest-

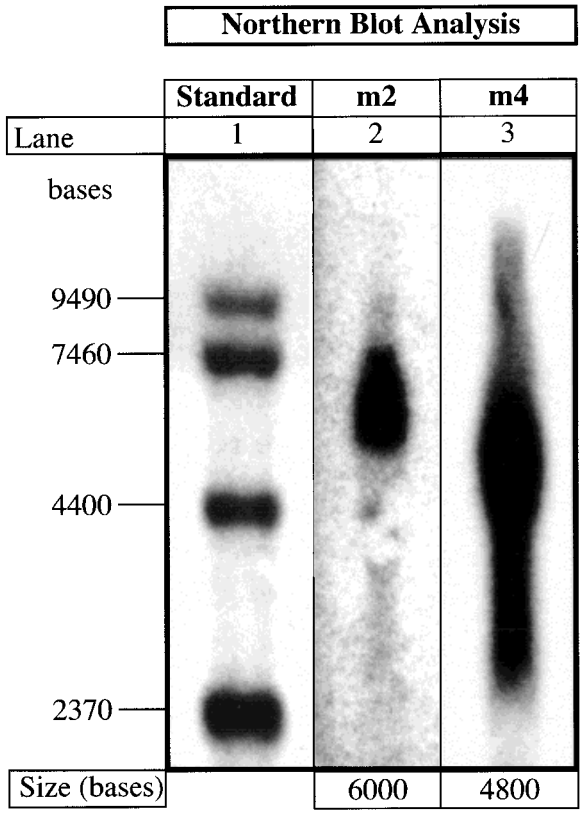

Fig. 2. Northern blot analysis of RNAs from cultured human skin fibroblasts (HSF). Blots were hybridized with ^{32}P-labeled nick-translated probes coding for the i3-loop region of each human mAChR subtype (m1-m5). No band was seen in a blot hybridized with the m5 probe (not shown). The blots were exposed to X-ray film for 72 h. The scale in the ordinate indicates kilobases, as determined using a set of RNA size markers.

ing level of 25 ± 9 nM to 279 ± 137 nM ($P < 0.05$). After the increase, $[Ca^{2+}]_i$ decreased to 66 ± 5 nM, which significantly ($P < 0.05$) exceeded the basal level, and remained at this level, forming a plateau, until muscarine was washed out (Fig. 5a). After a single exposure to muscarine, a recovery period of several minutes was needed before a new response could be obtained. The magnitude of the next response correlated directly with the length of the recovery time between the two stimulations.

Pretreating HSF with the muscarinic antagonist atropine, 10 µM, which competes with muscarine for binding to mAChRs, abolished the ability of muscarine to increase $[Ca^{2+}]_i$ in cultured HSF (Fig. 5b). When given alone to intact cells, atropine had no effect on $[Ca^{2+}]_i$ (Fig. 5b).

DISCUSSION

In this study, we demonstrated for the first time that HSF both in vivo and in vitro express the m2, m4, and m5 molecular subtypes of mAChRs, and that activation of fibroblast mAChRs elicits a transient increase in $[Ca^{2+}]_i$.

HSF are spindle-like mesenchymal cells inhabiting the dermis. These cells play an important role in the biology of the dermis owing to their ability to produce collagen, elastin, and proteoglycans to protect underlying structures, and to participate in age-related tissue remodeling and wound healing. Other types of the cells dwelling in the skin, such as epidermal keratinocytes, melanocytes and endothelial cells, all have been shown to express cholinergic enzymes and/or receptors [Iyengar, 1989; Warren, 1994; Grando, 1997]. These cholinergic molecules could allow skin cells to form a local signal transduction network with acetylcholine as a common cytotransmitter [Grando and Horton, 1996]. HSF can be a part of this nonneuronal cutaneous cholinergic network because anti-mAChR antibody [Grando et al., 1995] and cholinergic muscarinic drugs [Nadi et al., 1984; Vestling et al., 1995] have been shown to react specifically with HSF. Muscarinic cholinergic drugs alter vital functions of fibroblasts [Chew et al., 1992; Chew and Beuerman, 1993].

We sought to identify which of the five known mAChR subtypes are expressed by HSF. Because of the structural homology and pharmacological similarity of these receptors, cholinergic ligands currently available cannot clearly distinguish among the subtypes. Therefore, we identified the molecular subtypes of fibroblast mAChRs by detecting mRNA using RT-PCRs with subtype-specific primers. Since the transcribed portions of the mAChR genes lack introns, in order to distinguish between the amplification of genomic DNA and cDNA, we pretreated fibroblast RNA samples with DNase before RT. Only the m2, m4, and m5 mRNAs could be detected in HSF, which is consistent with previous findings of the M2/M4 pharmacological profile of mAChRs expressed by HSF [Vestling et al., 1995]. The sequences amplified were identical to the previously published sequences of human mAChRs [Allard et al., 1987; Bonner et al., 1987, 1988; Peralta et al., 1987a,b]. The m1 and m3 mRNAs were not detected. No PCR products were obtained when the RT step was omitted, thus demonstrating that no contaminating genomic DNA was present in our cDNA samples.

Notably, when Northern blot analysis was performed on total RNA isolated from HSF,

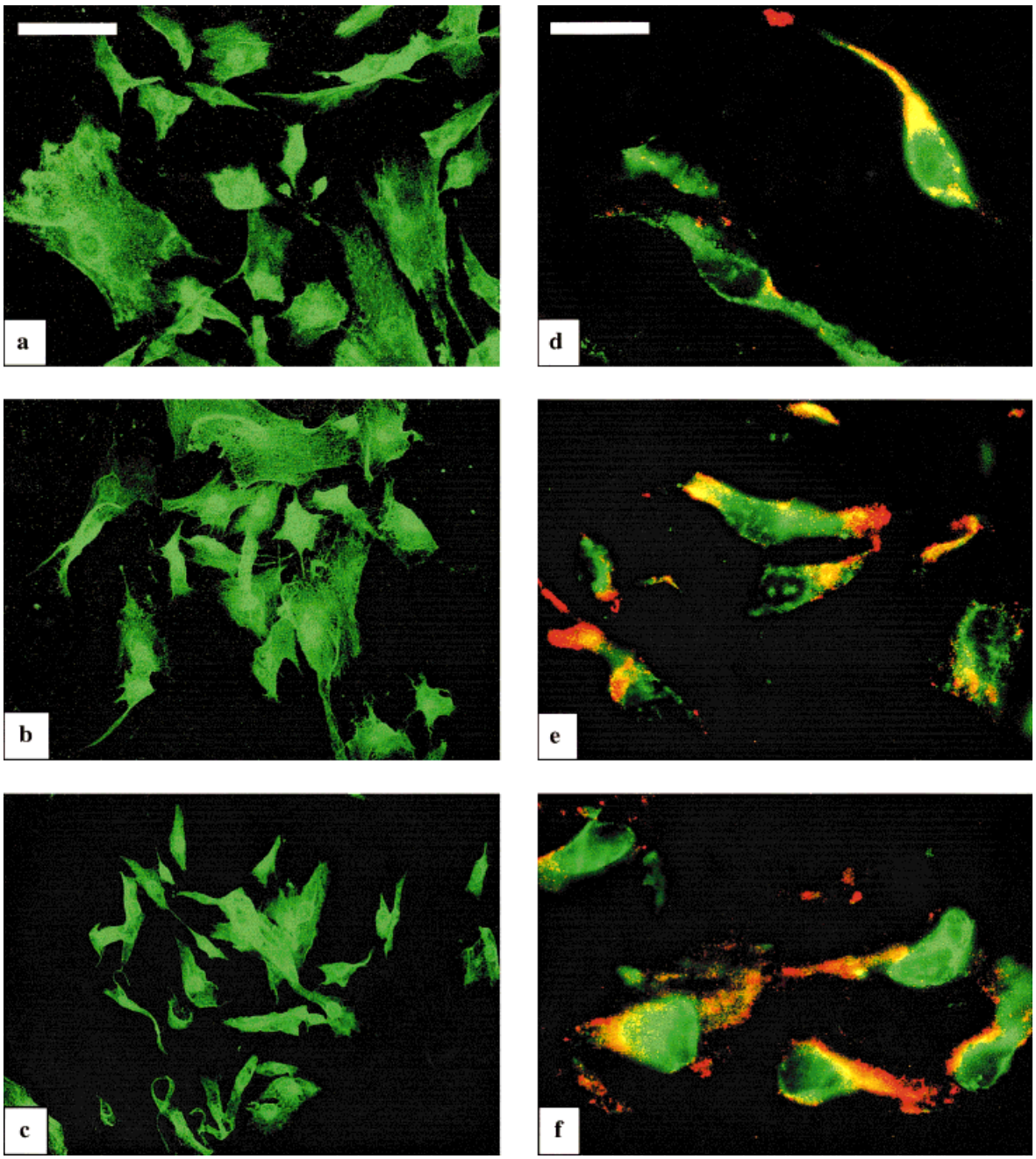

Fig. 3. Visualization of fibroblast muscarinic acetylcholine receptors (mAChRs) in cell cultures and skin specimens. Rabbit polyclonal antibodies raised to unique protein sequences of m2 (a,d), m4 (b,e), and m5 (c,f) mAChR subtypes were used to probe cultures of normal adult human skin fibroblasts (HSF) **(a–c)** and cryostat sections of fresh-frozen specimens of normal human foreskins **(d–f)** by IIF. In the skin specimens, HSF were identified by the fibroblast-specific antibody MAS 516b. Binding of anti-mAChR antibodies was visualized with FITC-labeled anti-rabbit IgG antibody (*green pseudocolor*), and binding of the MAS 516b antibody was visualized by tetramethylrhodamine isothiocyanate-labeled anti-mouse IgG antibody (*red pseudocolor*). Both preincubation of the anti-mAChR immune sera with the synthetic peptides used for immunization and omission of the primary antibody abolished fluorescent staining (not shown). Scale bars = 50 µm in a–c, and 5 µm in d–f.

single hybridization bands of 6.0 kb for m2 and 4.8 kb for m4 were observed. The size of these bands corresponds closely to the previously published mRNA sizes encoding the m2 and m4 human mAChR subtypes [Koman et al., 1990; Mak et al., 1992; Zhang et al. 1995]. Since the m5 mRNA could not be detected in Northern blots of total RNA extracted from cultured HSF, this transcript is apparently less abundant than the m2 and m4 transcripts. Thus, Northern blot

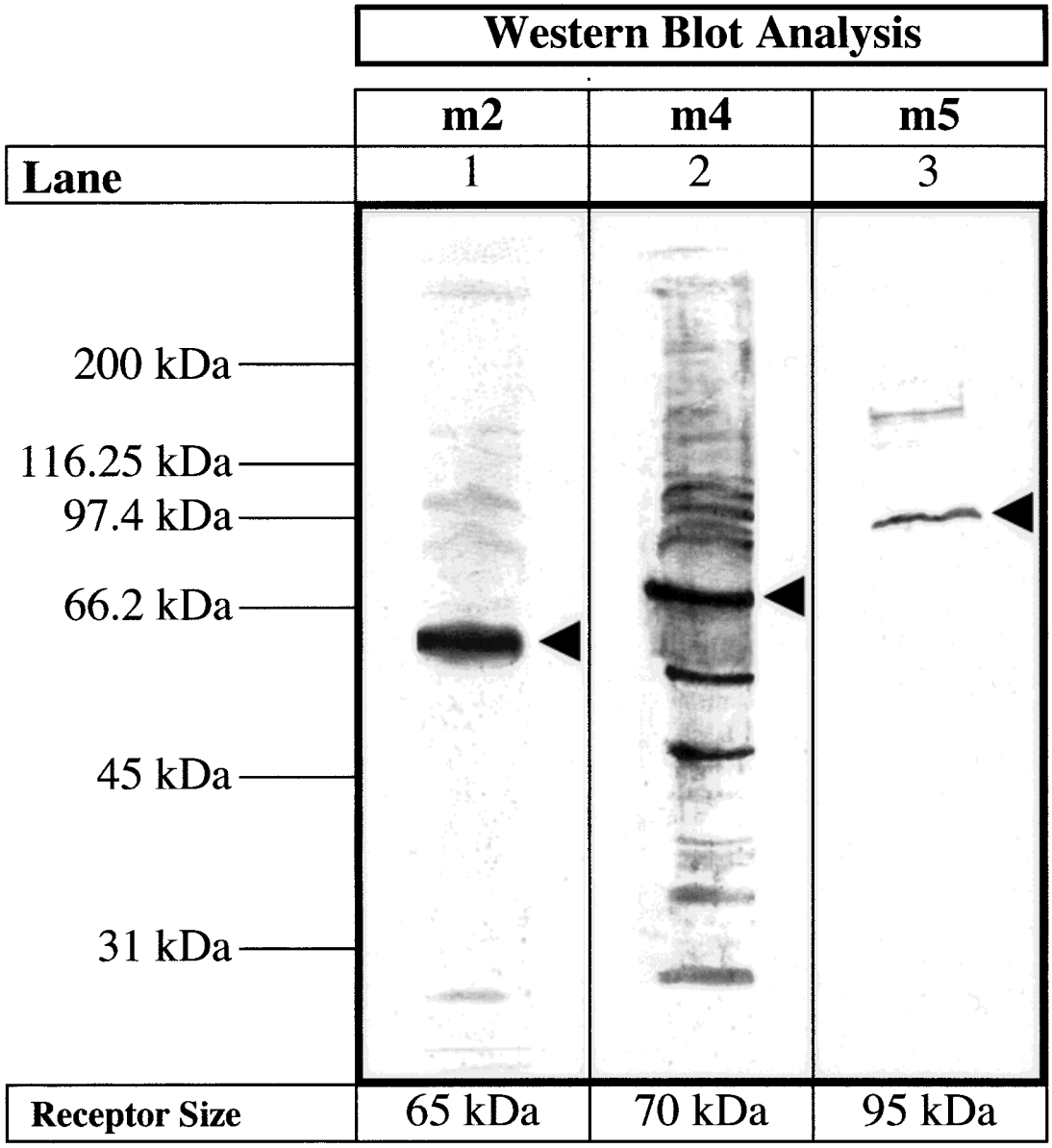

Fig. 4. Visualization of fibroblast muscarinic acetylcholine receptors (mAChRs) in Western blots. Results of a representative experiment showing protein bands recognized by rabbit polyclonal antibodies specific for m2, m4, and m5 mAChR subtypes among fibroblast proteins resolved by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as detailed under Materials and Methods. The bands did not appear in the control experiments in which immunoblotted membranes were either treated without primary antibody or in which the anti-peptide antisera were preincubated with the m2-, m4-, or m5-specific peptide used for immunization. The apparent molecular mass of each receptor protein is shown in kDa. The position and size of the molecular mass markers used are indicated at the left side.

analysis appeared not to be a sensitive enough technique to detect relatively low levels of mRNA encoding m5 in cultured HSF. The ability of the highly sensitive RT-PCR technique, as compared with the inability of the relatively insensitive Northern blot technique, to demonstrate the presence of the low-abundance mRNA for m5 has been previously reported in experiments with RNA extracted from other cell types [Drescher et al., 1992; Steel and Buckley, 1993; Wei et al., 1994]. Since different primers were used to generate the various PCR reaction products, it was not possible to assess quantitatively the relative amounts of m2, m4, and m5 transcripts by comparing the ethidium bromide-induced fluorescence.

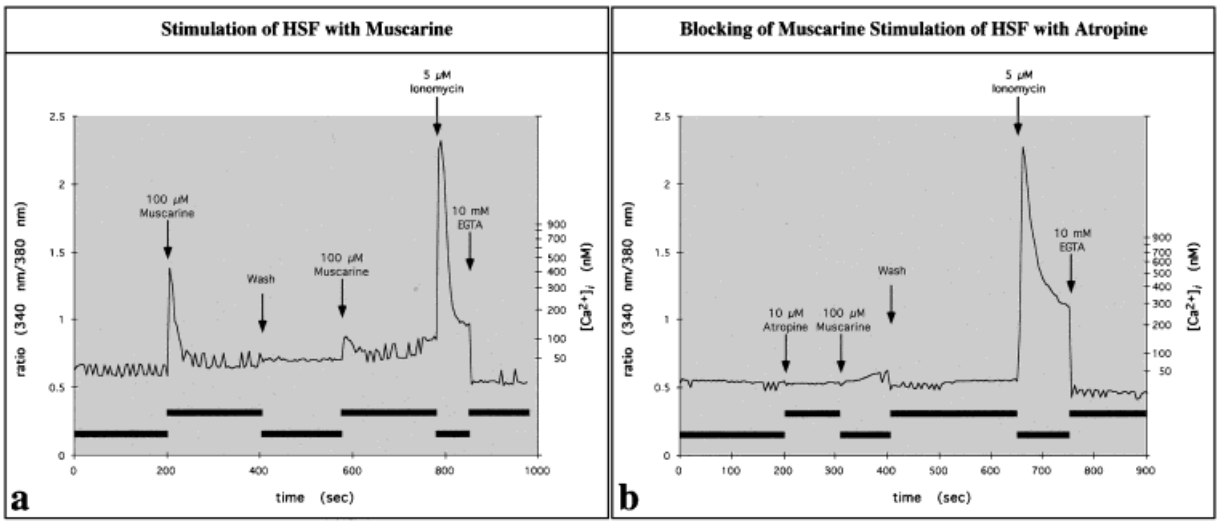

Fig. 5. Effect of muscarinic stimulation on the intracellular free Ca^{2+} concentration in human skin fibroblasts (HSF). HSF were loaded with the Ca^{2+}-sensitive dye fura-2 AM, and $[Ca^{2+}]_i$ was measured by ratio-imaging fluorescence microscopy of single cells at dual excitation wavelengths of 340 and 380 nm (see under Materials and Methods). Averaged light intensities at each of the two wavelengths were used to calculate the 340-nm/380-nm ratios after background subtraction. **a:** Changes in $[Ca^{2+}]_i$ induced by muscarine. **b:** Blocking of muscarine stimulation by atropine. Addition of drugs and wash steps are indicated with arrows. Horizontal bars, change of action. Ionomycin, 5 μM, and EGTA, 10 mM, were added at the end of each experiment to establish the maximum and minimum 340-nm/380-nm ratios. Values of $[Ca^{2+}]_i$ are expressed in nM on the right side of each panel, whereas the ratios between fluorescence recordings are shown on the left. Each trace is a representative result obtained in at least three independent experiments.

To visualize the genomic sequences of mAChR subtypes in HSF translated into proteins, we raised polyclonal antibodies against a synthetic peptide analogue of the unique amino acid sequence at the C-terminus of the human m2 mAChR. In these experiments, we also used anti-m1, m3, m4, and m5 antibodies developed previously [Ndoye et al., 1998]. The IIF staining of cultured HSF with the subtype-specific antibodies produced specific staining patterns consistent with the expression of m2, m4, and m5 receptors in these cells. The receptor proteins appeared to be randomly distributed on the cell surface of cultured HSF (Fig. 3a–c), whereas the mAChRs expressed by HSF residing in the dermis formed clusters, as suggested by the dot-like immunofluorescence pattern (Fig. 3d–f). Since fibroblast mAChRs have previously been reported to cluster in response to agonist stimulation [Raposo et al., 1987], our findings are consistent with the notion that in vitro HSF do not possess choline acetyltransferase to catalyze the synthesis of acetylcholine [Chen et al., 1978]. By contrast, free non-neuronal acetylcholine has been shown to be present in significant amounts in the skin [Klapproth et al., 1997], thus providing a natural agonist for the fibroblast mAChRs in vivo.

The antibodies that visualized mAChRs subtypes expressed by HSF in cell cultures and skin specimens reacted specifically with fibroblast proteins resolved by SDS-PAGE. Receptor molecular weights of 65 kDa for m2, 70 kDa for m4, and 95 kDa for m5 obtained in Western blot analysis match closely with the molecular masses of these subtypes found by us previously in human epidermal keratinocytes [Grando et al., 1995; Ndoye et al., 1998] and by other workers in various other cell types [Andre et al., 1984, 1988; Carsi-Gabrenas et al., 1997; McLeskey and Wojcik, 1990].

To investigate function of fibroblast mAChRs, cultured HSF were stimulated with the cholinergic agonist muscarine, and $[Ca^{2+}]_i$ was measured. Cholinergic stimulation increased transiently $[Ca^{2+}]_i$ in HSF. Recordings of changes in the $[Ca^{2+}]_i$ showed that the muscarinic response of HSF is biphasic and consists of both an initial transient peak and a secondary sustained elevation of $[Ca^{2+}]_i$. This finding is consistent with the results reported previously by Merritt and Rink [1987] and Negulescu and Machen [1988]; on this basis, the secondary sustained elevation may be attributable to Ca^{2+} influx into the cells, whereas an initial rise of $[Ca^{2+}]_i$ may be explained by the release of Ca^{2+}

from intracellular stores. Ca^{2+} signaling in nonexcitable cells is believed to be regulated via IP_3 formation and subsequent Ca^{2+} release from intracellular stores, followed by Ca^{2+} entry from the extracellular medium via Ca^{2+} channels [Putney, 1993].

Atropine antagonized the effect of muscarine on HSF, indicating that an agonist elicits a biochemical response that can be blocked by its specific antagonist. Recent findings indicate that all five mAChRs can employ Ca^{2+} as a common second messenger [Ashkenazi et al., 1987, 1989; Ishizaka et al., 1995]. However, the m2 and m4 receptors use a signaling pathway different from that coupled by the m1, m3, and m5 receptors. Since HSF have a mAChR subtype arrangement that permits activation of two different effector systems, i.e., one coupled by the even numbered mAChR subtypes (m2 and m4) and another coupled by the m5 receptor, activation of fibroblast mAChRs may simultaneously lead to several kinds of intracellular biochemical changes [Brown and Brown, 1984; McKinney and Richelson, 1986; Peralta et al., 1988]. Under these circumstances, it is hard to speculate which of the mAChR subtype(s) expressed in cultured HSF mediated effects of cholinergic drugs on $[Ca^{2+}]_i$. Further studies are needed to define specific biological functions of each mAChR subtype expressed by HSF.

In conclusion, it is becoming evident that the cells inhabiting human skin assemble an intercellular signaling network in which acetylcholine acts as a common cytotransmitter mediating communications between different cell types. A combination of molecular biological and immunohistochemical approaches with subtype-specific PCR primers and antibodies, respectively, enabled us to demonstrate the expression of m2, m4, and m5 mAChR subtypes in HSF. The fibroblast mAChR is coupled to regulation of $[Ca^{2+}]_i$, and may regulate physiological effects of free cutaneous acetylcholine on HSF.

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