Muscarinic receptors on intact human fibroblasts. Absence of receptor activity in adult skin cells.

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Research Article

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Muscarinic Receptors on Intact Human Fibroblasts

Absence of Receptor Activity in Adult Skin Cells

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Abstract

The intact human fibroblast has been used in clinical and basic research studies of receptor-mediated control of cell function, however there is little information about the relationship between muscarinic receptor density and the regulation of cyclic AMP (cAMP) accumulation. We have compared the muscarinic receptor characteristics of both lung and skin intact fibroblasts at fetal and adult stages of development using carbachol-mediated inhibition of cAMP accumulation and the binding of [^{3}H]quinuclidinyl benzilate (QNB).

Prostaglandin E_1 (PGE_1) stimulated cAMP accumulation in all four cell lines, while carbachol inhibited cAMP accumulation only in the fetal lung, adult lung, and to a lesser extent, the fetal skin. Adult skin fibroblasts did not display significant evidence of inhibitory muscarinic receptor activity. [^{3}H]QNB binding was saturable for the fetal and adult lung, and the fetal skin, yielding K_d values of ~0.5 nM for these cell lines. B_{max} values were 360 fmol/mg for fetal skin, 660 fmol/mg for adult lung, and 876 fmol/mg for the fetal lung. Specific binding of [^{3}H]QNB to adult skin fibroblasts was found to be low and variable. Comparisons of the B_{max} values and maximal inhibitory capacities showed that the receptor density paralleled receptor activity in all cell lines. The lack of muscarinic receptor activity in the adult skin fibroblast was confirmed in several different adult skin cell lines, indicating that the adult skin fibroblast may not be an appropriate model for muscarinic receptor activity in clinical investigations.

Introduction

The human fibroblast has been useful in the clinical investigation of a variety of conditions due to its ease of access and its potential for reflecting alterations in central and systemic processes. The human fetal lung fibroblast has an adenylate cyclase system that can be stimulated by prostaglandin and inhibited by stimulation of muscarinic cholinergic receptors (1). This cell type has been used as a model system for investigations of cholinergic regulation in intact human cells (2, 3). Skin fibroblasts from individuals with pseudohypoparathyroidism have been shown to be deficient in activity of the guanine-nucleotide regulatory protein associated with the stimulation of adenylate cyclase (4, 5). It has also been demonstrated that isoproterenol-stimulated accumulation of cAMP in skin fibroblasts from diabetics is twice as high as that of controls (6). In these clinical studies, it was concluded that the changes seen in the fibroblasts were consistent with similar changes shown in other tissues of the body. More recently, Nadi et al. (7) reported the use of adult skin fibroblasts in a clinical investigation of muscarinic receptor activity in patients with a psychiatric history of major affective illness.

Previous studies in our laboratory have characterized the inhibitory coupling of the α_2 adrenergic receptor to adenylate cyclase in the human platelet, and compared receptor binding properties and cAMP production in intact cells (8). Although muscarinic-mediated inhibition of cAMP accumulation in the human fetal lung fibroblast has been well characterized in the intact cell preparation (1–3), little is known of the muscarinic binding characteristics or the size of the receptor population in these cells. We report here our findings related to the properties of muscarinic receptors on intact human fibroblasts from human lung and skin at two stages of development. These studies have compared the receptor binding characteristics and the muscarinic-mediated inhibition of cAMP accumulation in these various cell types, and have addressed the suitability of the adult skin fibroblast as a model for the muscarinic receptor in clinical investigations.

Methods

Human fetal lung fibroblasts (IMR-90), human fetal skin fibroblasts (AG4392), and adult skin fibroblasts (IMR-GM5225) were obtained from the Institute for Medical Research, Camden, NJ. Adult fibroblasts were obtained locally; skin fibroblasts (DVR, RL, MA) were obtained from 4-mm punch biopsies of the medial aspect of the upper arm, and adult lung fibroblasts were obtained from normal human lung parenchyma. Fibroblasts were isolated in a culture medium of minimal essential medium containing 10% fetal bovine serum, nonessential amino acids, 0.1 mg/ml Na pyruvate, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cultures were incubated at 37°C in an atmosphere of 5% CO_2, 95% air. For subculture of all lines, cells were dissociated with 0.25% trypsin, resuspended in complete medium without nonessential amino acids and Na pyruvate. Stock cultures were seeded 1–2 × 10^6 in 75-cm^2 flasks with 40 ml medium. Cells received from other sources were shipped as monolayers in 25-cm^2

A preliminary report of these findings was presented at the 14th Annual Meeting of the Society for Neuroscience, Los Angeles, CA, in 1984.

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flasks filled with medium and were passaged at least once before seeding for experiments.

For experiments, cells were seeded in 24-well cluster plates (2 cm^2) at a density of 2.5×10^4 per well in 1 ml medium and incubated for 1 wk. Cultures were between population doubling levels, 15–30 for fetal cells and 5–15 for adult cells. Cell densities at the time of assay ranged from 1.0×10^5 to 5.5×10^5/well.

For adenylate cyclase assays, the culture medium was removed and the cells washed with Hanks' balanced salt solution (HBSS), and 1 μCi [^{3}H]adenine was added to a final volume of 0.5 ml in each well and the cells incubated for 1 h at 37°C. After incubation, the [^{3}H]adenine was removed and the cells washed with HBSS. Final volume was 0.5 ml in each well. The reaction was initiated by the addition of prostaglandin E_1 (PGE_1)[1] and terminated 10 min later by the addition of 0.5 ml SDS stop solution (6.0 mM cAMP, 139 mM SDS, pH 7.5) and heating to 90°C for 7 min. The [^{3}H]cAMP formed was isolated using a modification of the chromatography method of Salomon et al. (9). Results are expressed as percentage conversion of ^{3}H-precursor to [^{3}H]cAMP. Recovery of [^{3}H]cAMP averaged >70%.

For intact cell radioligand binding studies, fibroblasts were grown in 24-well cluster plates as above. The cells were washed with HBSS and the reaction carried out in a final volume of 0.5 ml. [^{3}H]quinuclidinyl benzilate (QNB) was added to initiate the reaction and the cells were incubated for 1 h at 25°C. Nonspecific binding was determined in the presence of 10 μM QNB. The reaction was terminated by the removal of the [^{3}H]QNB, two rapid washings of the monolayer with cold HBSS, and the addition of 0.5 ml 0.1 N NaOH. Cells were allowed to sit for 1 h at 25°C and the contents of the wells were removed to scintillation vials, neutralized, and counted.

In most assays, all cell types were tested simultaneously, but when this was not feasible, fetal lung cells (IMR-90) were always included as a standard.

Results

The addition of PGE_1 to fibroblasts that had been prelabeled with [^{3}H]adenine resulted in the stimulation of cAMP accumulation in all four cell lines (Fig. 1 *A*). The results indicate that the skin cells were more responsive to 10 μm PGE_1 than lung cells. The results also suggest that with respect to the lung fibroblasts the fetal cells appeared relatively more sensitive than the adult, and in the skin cells the opposite was true.

The inhibition of PGE_1-stimulated cAMP accumulation in the fibroblasts was examined using the muscarinic agonist, carbachol (Fig. 1 *B*). Carbachol at 10 μm was used as a measure of maximal inhibitory activity after determining carbachol dose-response relationships for inhibition of cAMP accumulation. The results indicate that carbachol inhibited 50–60% of control cAMP accumulation in both fetal and adult lung cells, with only 20% inhibition observed in the fetal skin and insignificant inhibition noted in the adult skin. The apparent lack of inhibitory activity in adult skin fibroblasts was examined in five additional skin cell lines and the results were similar to those shown in Fig. 1.

The pharmacological specificity of this receptor-mediated inhibition of cAMP accumulation was demonstrated using the muscarinic antagonist, atropine (Table I). The addition of 10 μm atropine blocked the action of carbachol in the three fibroblast cell lines in which inhibition was demonstrated. Pharmacological specificity was further examined by comparing the ability of carbachol and five other muscarinic agonists to

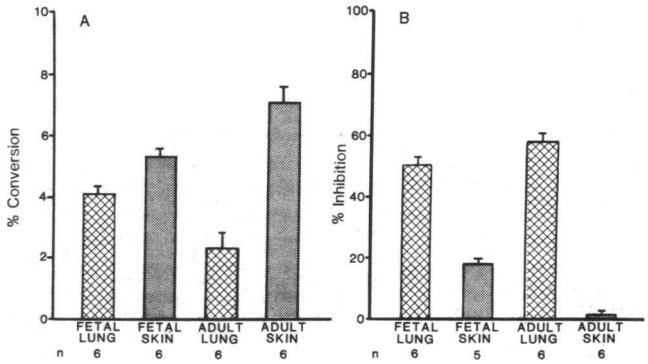

Figure 1. (*A*) Cyclic AMP accumulation in four intact human fibroblast cell lines. Cyclic AMP accumulation is expressed as percentage conversion of [^{3}H] precursor to [^{3}H]cAMP. Cells were incubated for 10 min with 10 μm PGE_1. n, Number of determinations, each determination done in triplicate; values are ±SEM. Values for basal percentage conversion (no PGE_1) are similar to those given in Table I. (*B*) Inhibition of cAMP accumulation by carbachol in four intact fibroblast cell lines. Inhibition expressed as percentage inhibition of control (no carbachol) values. Cells treated as in Fig. 1, except for the addition of cabachol. One concentration of carbachol (10 μM) was used as a measure of maximal inhibitory capacity. n, Number of determination, each performed in triplicate; values are ±SEM. Statistical comparisons (by analysis of variance). Lung vs. skin, $P < 0.01$; lung: fetal vs. adult, NS; skin: fetal vs. adult, $P < 0.01$.

inhibit PGE_1-stimulated cAMP accumulation (data not shown). The results indicated that the relative levels of inhibition shown in Fig. 1 *B* for the four cell lines were maintained for all the agonists. These findings support the conclusion that these receptors are of the muscarinic type.

The muscarinic receptors on the intact fibroblasts were also characterized by conducting saturation binding experiments using the muscarinic antagonist [^{3}H]QNB. Representative saturation curves for fetal and adult lung, and fetal skin are shown in Fig. 2. The K_d and B_{max} values from several experi-

Table I. Antagonism of Carbachol-mediated Inhibition in Intact Fibroblasts

	Percentage conversion			
	Fetal lung	Fetal skin	Adult lung	Adult skin
Basal	0.073	0.10	0.072	0.090
PGE_1	3.91	4.70	2.51	7.71
PGE_1 + carbachol	2.30	3.82	1.41	7.31
(% inhibition)	(41)	(19)	(44)	(5)
PGE_1 + atropine	3.89	5.41	2.38	9.24
PGE_1 + atropine + carbachol	3.69	5.24	2.29	8.05

Cells were incubated with 10 μM PGE_1 for 10 min. Carbachol was added at 10 μM, and atropine was added at 1 μM. The basal levels of cAMP accumulation shown here are similar to values obtained in other experiments. The values for percentage conversion in the presence of PGE_1 and the levels of carbachol-mediated inhibition were consistent with previous results.

1. *Abbreviations used in this paper:* PGE_1, prostaglandin E_1; QNB, quinuclidinyl benzilate.

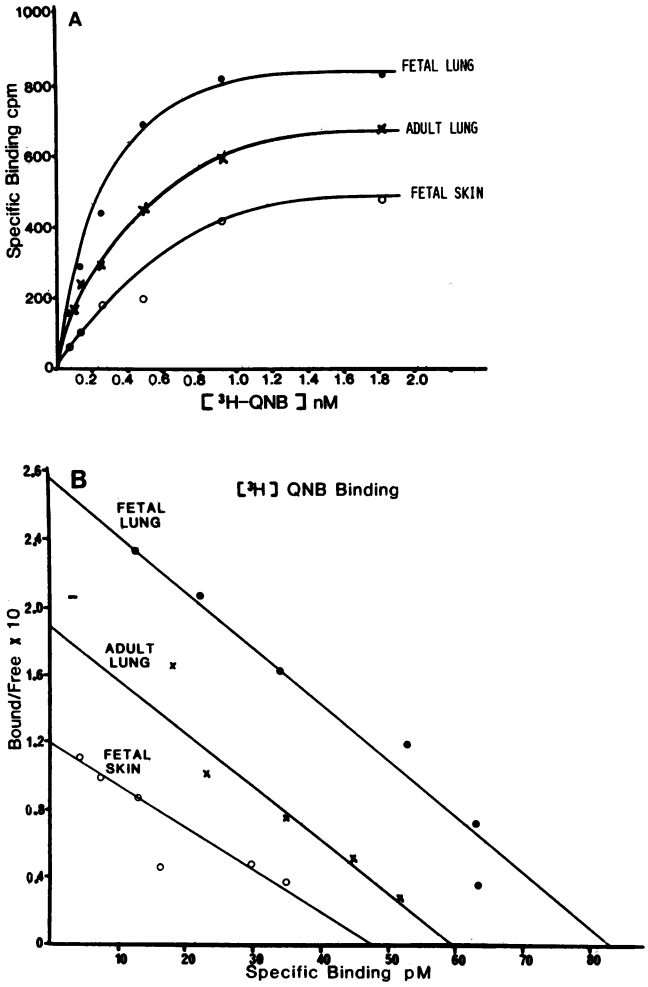

Figure 2. Saturation binding experiments using [³H]QNB in three intact fibroblast cell lines. (*A*) Representative saturation curve. Values for percentage specific binding at 0.5 nM [³H]QNB: fetal lung, 92%; adult lung, 91%; fetal skin, 74%. (*B*) Scatchard analysis of the data. K_d and B_{max} values are given in Table II.

ments are listed in Table II. The [³H]QNB binding to fetal lung, adult lung, and fetal skin cells was saturable and specific, yielding percentage specific binding values ranging from 60 to 90%. Several lines of adult skin fibroblasts were examined, and the results indicated that there was either no, or very weak, specific binding of [³H]QNB. Saturation binding experiments were performed with five other adult skin cell lines and similar results were obtained (data not shown).

One adult skin fibroblast cell line (DVR) did demonstrate some specific binding and the results of three determinations are shown in Fig. 3. Simultaneously, [³H]QNB binding to fetal lung fibroblasts was examined and the results of these determinations are also shown in Fig. 3. The binding to adult skin fibroblasts was of low specificity (Table II) and was highly variable. A least-squares regression line could be obtained, but the regression was not significant. The variability due to the regression (coefficient of determination) was 6.2% for the adult skin and 81% for the fetal lung. The data for [³H]QNB binding to this adult skin fibroblast line, thus, did not support reliable K_d and B_{max} estimates.

The K_d values for the fetal lung, adult lung, and fetal skin cells were comparable (Table II), indicating little difference in affinity for [³H]QNB. The B_{max} values, however, suggest that there are differences in the size of the receptor populations of the three cell types. The largest number of receptors was seen in the fetal and adult lung cells, the levels of which appear similar. The fetal skin cells displayed a comparatively smaller number of receptors, and adult skin receptors were negligible.

A comparison of the receptor densities of the four cell lines and their respective maximal inhibitory capacity is shown in Fig. 4. This comparison indicates that in the fetal and adult lung fibroblasts, the similarity in the number of muscarinic binding sites is reflected in the similarity of the levels of inhibition of PGE_1-stimulated cAMP accumulation. The fetal skin cells have a smaller receptor population, and the inhibitory capacity of these cells is correspondingly lower. Consistent with these results, adult skin fibroblasts display minimal capacity to inhibit cAMP accumulation and minimal specific binding of [³H]QNB.

Table II. [³H]QNB Binding to Intact Fibroblasts

Cell type	K_d	B_{max}	Sites/cell ($\times 10^5$)	Specific binding	No. cells ($\times 10^5$)	Total protein
	nM	*fmol/mg*		%		*mg*
Fetal lung (IMR 90)	0.57	876	1.41	60–94	1.5–3.9	0.05–0.08
$n = 6$	±0.09	±165	±0.54			
Adult lung (SC10)	0.45	662	1.52	64–93	1.1–2.1	0.05–0.06
$n = 4$	±0.07	±63	±0.26			
Fetal skin (AG4392)	0.51	364	0.84	46–83	1.6–1.7	0.05–0.07
$n = 4$	±0.08	±19	±0.05			
Adult skin DVR ($n = 4$)	*	*	*	8–40	1.0–2.1	0.04–0.06
RL ($n = 2$)	*	*	*	8–45	2.1–3.2	0.27–0.35
MA ($n = 3$)	*	*	*	NSB		
IMR—GM5225 ($n = 3$)	*	*	*	16–28	1.2–1.6	0.03–0.05

K_d, B_{max}, and percentage specific binding values for intact fibroblasts were determined in experiments as shown in Fig. 2. The number of cells and milligram total protein values are for individual wells on the plates. NSB, no specific binding. * Although specific binding was seen with adult skin fibroblasts, the data did not permit calculation of K_d and B_{max} values.

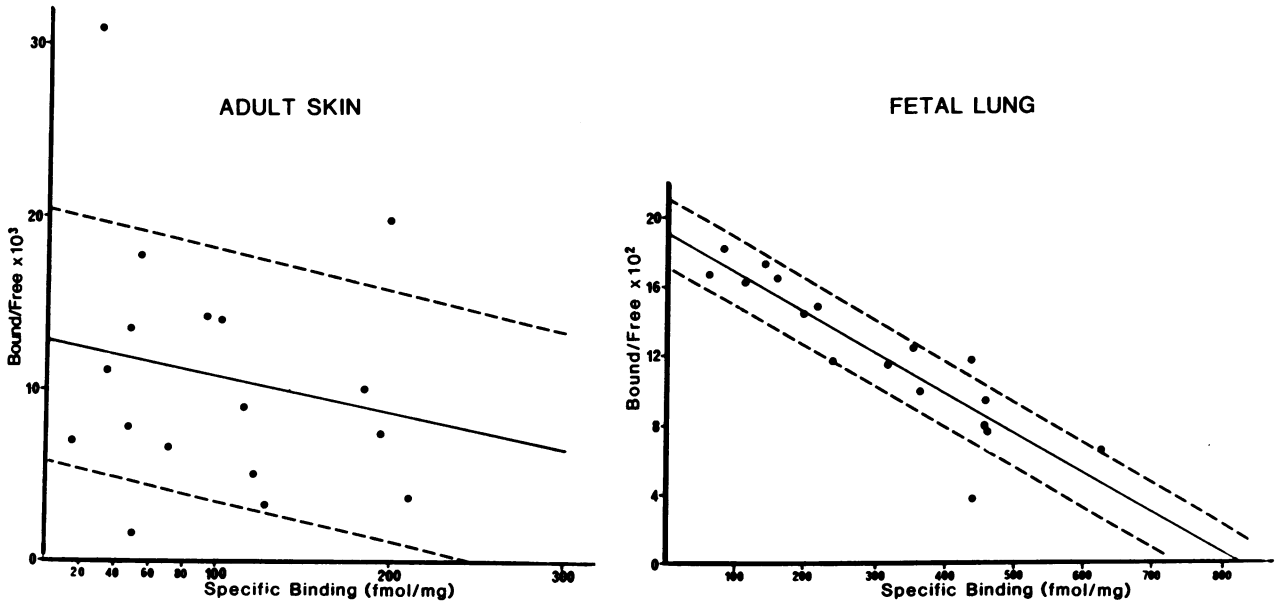

Figure 3. Scatchard plot of [^{3}H]QNB binding to adult skin fibroblasts (*left*) and fetal lung fibroblasts (*right*). Dashed line is ±1 SE of the estimate. Data is combined from three separate experiments. Binding studies for both adult skin and fetal lung cells were carried out on each experimental day. Correlation coefficients for the least-squares regression (solid line): adult skin, 0.25; fetal lung, 0.90. Data was similarly prepared for fetal skin and adult lung; correlation coefficients were 0.79 and 0.67, respectively (data not shown).

Discussion

The present study demonstrates that the adenylate cyclase in all the fibroblast cell types examined responded to stimulation by PGE_1, although different levels of cAMP accumulation were observed. The differences seen here in the levels of cAMP accumulation across cell types may be due to many factors, such as the number and sensitivity of hormone receptors, levels of phosphodiesterase, concentration of stimulating agent, etc.

The carbachol-mediated inhibition of PGE_1-stimulated cAMP accumulation reported for fetal lung cells (1–3) has been replicated in this study and has now been demonstrated for the adult lung and fetal skin fibroblasts. The levels of inhibition in the fetal and adult lung cells appear to be comparable, and the fetal skin cells displayed a smaller level of inhibition. No inhibition of PGE_1-stimulated cAMP accumulation could be demonstrated in any of six different adult skin cell lines that were tested.

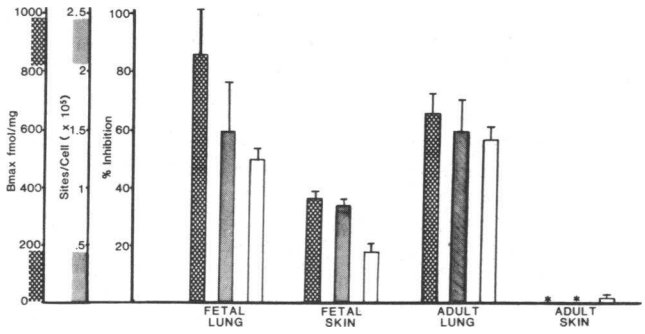

Figure 4. Comparison of B_{max} values and maximal inhibitory capacity of three intact fibroblast cell lines. Data for carbachol-mediated inhibition are from Fig. 2, and [^{3}H]QNB B_{max} values are from Table II. *Reliable values for B_{max} could not be obtained with adult skin fibroblasts.

The cholinergic receptors of the three cell types possessing measurable receptor activity display similar muscarinic pharmacological specificity. The K_d values for [^{3}H]QNB binding are also similar across the cell lines. Furthermore, the inhibitory capacity of the cells investigated appears to be paralleled by the number of receptors on the cells. These findings strengthen the role of receptor density as a determinant of inhibitory capacity, and suggests that the degree of coupling between receptor and activity may be similar in the three cell lines. In analogous studies of cholinergic receptors in the pancreas and chick flight muscles, it was shown that the receptor density changed significantly with maturation, but that the affinity (K_d) for the receptor ligand did not (10, 11). In these studies, changes in receptor number were paralleled by changes in physiological responses that were coupled or associated with the receptors.

Our investigation has shown that, under the experimental conditions used here, there is little evidence for the presence of muscarinic receptors on adult skin fibroblasts. Neither the carbachol-mediated inhibition of PGE_1-stimulated cAMP accumulation nor the binding of [^{3}H]QNB could be adequately demonstrated in the cell lines examined. The binding that was seen in some of the cell lines was of low specificity and was highly variable (Table II, Fig. 2). The data did not allow the calculation of reproducible K_d, B_{max}, or percentage inhibition values. Care was taken throughout this investigation to exclude any experimental variation that would bias the results of experiments using adult skin fibroblasts. Fibroblasts were obtained through our own facilities as well as from commercial and other research laboratories, and were treated the same as the other cell lines that gave reproducible binding of high specificity in association with inhibitory receptor activity.

Preliminary investigations in other laboratories have ex-

amined muscarinic binding in intact adult skin fibroblasts using [^{3}H]QNB as well as [^{3}H]*N*-methylscopolamine (12). Their data are consistent with our findings of a small population of binding sites in only a small percentage of adult skin cell lines. Further studies using adult skin fibroblasts have examined other cellular response systems associated with muscarinic receptors, i.e., cyclic guanosine 3',5'-monophosphate response and phosphoinositol turnover, and have also been unable to demonstrate any receptor-mediated activity (12).

The presence of few, if any, muscarinic receptors on adult skin fibroblasts is in disagreement with the report of Nadi et al. (7). They reported a cholinergic inhibition of hormone-stimulated adenylate cyclase as well as saturable and highly specific binding of [^{3}H]QNB to intact adult skin fibroblasts. We can neither confirm their findings nor account for this discrepancy by any apparent difference in cell growth or assay conditions. Our data, however, do indicate the presence of muscarinic receptors on fetal skin cells and suggest that the expression of these receptors may be lost during development. It is possible that under certain, as yet undefined conditions, receptor activity could be expressed in the adult skin fibroblast, but this must await further study.

Although the adult skin fibroblast shares properties with neuronal cells, such as a high affinity choline uptake system (13), and has been used in investigations of certain neurological disorders (14, 15), these cells would appear to be inappropriate as a system for the study of muscarinic receptors in clinical investigations. However, the finding that lung fibroblasts possess classical muscarinic receptor activity throughout maturation while the skin cell type loses this property suggests that the fibroblast may be an interesting model with which to study the ontogenetic development of the muscarinic receptor and its coupling to cellular response in a human cell system.

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