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Muscarinic cholinoceptor activation modulates DNA synthesis and CD40 expression in fibroblast cells

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Summary

1 The aim of the present work was to examine the role of muscarinic acetylcholine receptors (mAChR) on DNA synthesis and CD40 expression in human fibroblast cells. Neonatal human skin fibroblast cultures were stimulated with carbachol in presence or absence of specific antagonists and the following parameters were measured: identification of mAChR subtypes, DNA synthesis, inositol phosphates (InsP) production and CD40 expression.

2 Human fibroblasts express mAChR with K_d 0.47 ± 0.11 nM and B_{max} 236 ± 22 fmol mg protein^{-1}. Carbachol stimulates DNA synthesis, InsP and the expression of CD40. All these effects were inhibited by atropine, mustard hydrochloride (4-DAMP) and pirenzepine but not by AF-DX 116 and tropicamide, indicating that M_3 and M_1 mAChR are implicated in carbachol action. The relative K_i of the antagonists obtained by competition binding assay was in parallel to the relative potency for blocking both carbachol-stimulated InsP accumulation and DNA synthesis.

3 The intracellular pathway leading to carbachol-induced biological effects involved phospholipase C and calcium/calmodulin, as U-73122 and trifluoroperazine blocked carbachol effects, respectively. Calphostin C, a protein kinase C inhibitor, had no effect, indicating that this enzyme does not participate in the system.

4 These results may contribute to a better understanding of the modulatory role of the parasympathetic muscarinic system on normal human fibroblast function.

Keywords: culture assay, inositol phosphates, cholinoceptors, phospholipase C, CD40 protein, radioligand binding

Introduction

Fibroblasts have been considered important connective tissue cells that construct a supporting framework crucial for tissue integrity and repair. Furthermore, fibroblasts from different anatomical regions display characteristic phenotypes and are not a homogeneous population even within a single tissue but exist as subsets of cells much like tissue macrophages and dendritic cells (Phipps, Borrello & Blieden, 1999; Buckley *et al.*, 2001). Human skin fibroblasts play pivotal roles in connective tissue production, physiological skin remodelling and wound repair and also, they were found to be important modulators of the immune system (Fries *et al.*, 1995; Smith, Smith, Blieden & Phipps, 1997).

Human skin fibroblasts express muscarinic acetylcholine receptors (mAChR) of different subtypes (Carsi-Gabrenas, Van Der-Zee, Luiten & Potter, 1997). Nevertheless, it has been reported that adult skin fibroblasts have only negligible amount of muscarinic ligand binding sites (Van Riper, Absher & Lenox, 1985; Kelsoe *et al.*, 1986; Lin & Richelson, 1986). However, using the reverse transcription-polymerase chain reaction and Northern blot technique the m_2, m_4 and m_5 mAChR subtypes on human skin fibroblast surface were identified (Buchli *et al.*, 1999).

The mAChR subtypes are generally grouped according to their functional coupling either to mobilization of intracellular calcium (m_1, m_3, m_5) through the activation of phospholipase C (PLC), which results in the release of the second messenger

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inositol 1,4,5-trisphosphate (IP_3) or by inhibition of adenylyl cyclase (m_2, m_4), which results in the reduction of the intracellular levels of cyclic adenosine monophosphate (Hulme, Birdshall & Buckley, 1990; Hosey, 1992). It is important to note that the same receptor may generate more than one set of intracellular second messengers and considerable cross-talk exists between signalling cascades (Felder, 1995; Nicke, Detjen & Logsdon, 1999). The ability of these receptors to either stimulate or inhibit cell growth has been attributed to differences in the cell models, but the real mechanisms involved in these cell type-dependent differences in growth response are unknown (Gutkind, Novotny, Brann & Robbins, 1991; Gutkind & Robbins, 1992).

CD40 is a type I cell surface receptor glycoprotein, which is constitutively expressed by many cells of haematopoietic (Stamenkovic, Clark & Seed, 1989) and by non-haematopoietic (Hollenbaugh *et al.*, 1995) origin. Significant progress has been made in characterizing the functional importance of CD40 expression on B or T cells. Non-immune cells have been shown to express CD40 both *in vivo* (Fries *et al.*, 1995) and *in vitro* (Armitage *et al.*, 1992). However, the role of the CD40 signalling system in the physiology of these cells is controversial. Yellin *et al.* (1995) demonstrated that CD40 was ligated to proliferation signals, to regulation of adhesion molecules, as well as to cytokine secretion by synovial fibroblasts. This cell has the capacity to participate in the histopathological changes associated with chronic inflammatory diseases, such as rheumatoid arthritis (Arend & Dayer, 1990) and periodontitis (Schroeder & Page, 1972). Also, expression of CD40 has been reported on gingival fibroblast (Fries *et al.*, 1995), in which this molecule may play an immunopathogenetic role in periodontitis by increasing the inflammatory responses of these cells, regulating the secretion of inflammatory cytokines (IL-6/IL-8) (Bartold & Haynes, 1991; Dongari-Bagtzoglou & Ebersole, 1996). Additionally, human fibroblast CD40 from periodontal tissues induces the production of matrix metalloproteinases (Malik, Greenfield, Wahl & Kiener, 1996; Mach, Eschonbeck, Bonnefoyd, Pober & Libby, 1997; Schonbeck *et al.*, 1997).

However, little is known concerning the growth stimulatory mechanisms activated by fibroblast mAChR and the participation of these receptors in the expression of fibroblast CD40 antigen. Moreover, the activation of mAChR subtypes (M_3 and M_1) can trigger an increment of DNA synthesis. The mechanisms underlying mAChR activation triggered CD40 overexpression, and enhancement of fibroblast proliferation involve PLC and calcium/calmodulin (CaM) activation via inositol phosphates (InsP) hydrolysis. In this work, we demonstrated that human skin fibroblasts express CD40 protein. In addition, a direct functional relationship between the activation of mAChR and the enhancement of the CD40 expression in human fibroblasts was observed. The results may provide new insight into the cholinergic cellular growth and CD40 expression and regulation.

Materials and Methods

Cell culture

Pure cell cultures of neonatal human skin fibroblasts were established from the foreskin obtained after circumcision and grown in Dulbecco's modified Eagle's medium (Gibco, Invitrogen Corporation, Carlsead, CA, USA) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) 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 sub-cultured by detaching the monolayer with 0.25% trypsin in phosphate-buffered saline solution (PBS). Cells of passages 6–8 were used throughout.

Measurement of DNA synthesis

DNA synthesis was estimated by measurement of [^{3}H]-thymidine (Dupont/New England Nuclear, Boston, MA, USA) incorporation into trichloroacetic acid (TCA) – precipitable material. The muscarinic actions of carbachol were evaluated in cells (1×10^6 cell ml^{-1}) that had been serum-starved for 8 h. Carbachol, over a range of concentrations, was 8 h in contact with the cells. When cholinergic antagonistic drugs or enzymatic inhibitors were used, they were added 10 min before carbachol. The [^{3}H]-thymidine (0.1 μCi ml^{-1}) was added during the last 2 h. Cells were precipitated twice with ice-cold 10% TCA and fibroblasts were then removed with trypsin/PBS and radioactivity was determined by liquid scintillation counting. Cells that had been serum-starved for 8 h without drugs were used as control (basal value). The cell cycle distribution of the fibroblasts used were in S phase and the percentage of confluence at the time of study was about 92%.

Binding assay

For the radioligand binding assays, cells were grown to confluence in tissue culture dishes, quantitatively harvested and collected by means of low-speed centrifugation. The cellular pellet was resuspended in five volumes of 10 mM potassium phosphate, 1 mM $MgCl_2$, 0.25 M sucrose pH 7.5 (buffer A), supplemented with 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 2 μg ml^{-1} leupeptin and 1 μM pepstatin A, and homogenized in an Ultraturrax at 4 °C. Then, the homogenate was

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centrifuged twice for 10 min at 3000 *g*, then at 10 000 *g* and 40 000 *g* at 4 °C for 15 and 90 min respectively. The resulting pellets were resuspended in 50 mM phosphate buffer supplemented with 0.1 mM PMSF, 2 μg ml^{-1} leupeptin and 1 μM pepstatin A pH 7.5 (buffer B). Receptor ligand binding was performed as described previously (Borda, Cremaschi & Sterin-Borda, 1999). Aliquots of the membrane suspension (50 μg protein) were incubated with increasing concentrations of tritium quinuclydinyl benzilate (0.1–2 nM) ([^{3}H]-QNB) (Dupont/New England Nuclear; Sp.Ac. 48.6 Ci $mmol^{-1}$) for 60 min at 25 °C in a total volume of 150 μl of buffer B. Binding was stopped by adding 2 ml ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 12 ml of ice-cold buffer, transferred into vials containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. Non-specific binding was determined in the presence of 1 μM atropine and never exceeded 10% of total binding. Bound radioactivity was lower than 10% of total counts. For competition binding assays, membranes were incubated with about 0.51 nM [^{3}H]-QNB and increasing concentrations of different cholinoceptor antagonists were added and the experiments were then carried out as described above.

Flow cytometric analysis

The analysis of the expression of cell surface CD40 protein was performed using fluorescein isothiocyanate-conjugated monoclonal antibodies (MoAbs) to human CD40 or isotype-matched controls (Becton Dickinson, Franklin Lakes, NJ, USA) in a standard staining procedure. Briefly, fibroblasts (1×10^6) were incubated or not incubated with carbachol (1×10^{-9} M) during 90 min. When atropine (mAChR antagonist) and U-73122 (PLC inhibitor) were used, they were added 10 min before carbachol. Then, the cells with the antibody were washed with PBS containing 2.5% FBS, fixed in 0.5% paraformaldehyde and analysed with a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson), with appropriate forward and side scatter adjustment for human skin fibroblast cells. Mean fluorescence intensity values obtained by staining with specific MoAbs were corrected by the subtraction of background values (isotype-matched control MoAb).

Measurement of inositol phosphates (InsP)

Fibroblasts (1×10^6 cell ml^{-1}) were incubated for 120 min in 0.5 ml of Krebs–Ringer bicarbonate (KRB) gassed with 5% CO_2 in O_2 with 1 mCi [*myo*-^{3}H]-inositol ([^{3}H]-MI) (Sp.Act. 15 Ci $mmol^{-1}$) from Dupont/New England Nuclear and LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique previously described (Borda, Genaro, Sterin-Borda & Cremaschi, 1998). Carbachol was added 30 min before the end of the incubation period and the blockers were added 30 min before the addition of carbachol. Water-soluble IPs was extracted after 120 min incubation. Tissues were washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v/v) to stop the reaction. Then, chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3000 *g* for 10 min and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7-ml column of Bio-Rad AG (Formate Form) 1 × 8 anion-exchange resin (100–200 mesh) (Bio-Rad, Richmond, CA, USA) suspended in 0.1 M formic acid that had been previously washed with 10 mM Tris-formic pH 7.4. The resin was then washed with 20 volumes of 5 mM *myo*-inositol followed by six volumes of water and InsP was eluted with 1 M ammonium formate in 0.1 M formic acid. One millilitre fractions were recovered and radioactivity was determined by scintillation counting. The results corresponding to the area of the second peak, were expressed as absolute values of area units under the curve per milligram of wet weight tissue (area mg^{-1} tissue wet wt^{-1}) following the criteria of Simpson's equation. In order to confirm the absence of [^{3}H]-MI in the eluted peaks of total InsP, chromatography on silica gel 60 F 254 sheets (Merck, Darmstadt, Germany) was performed as previously described (Borda *et al.*, 1998).

Drugs

Carbachol, pirenzepine, AF-DX 116, atropine, trifluoroperazine (TFP) and calphostin C (Sigma Chemical Company, Saint Louis, MO, USA); U-73122, 4-DAMP and tropicamide (ICN Pharmaceuticals Inc., Costa Messa, CA, USA). Stock solutions were freshly prepared in the corresponding buffers.

Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) and *post hoc* test (Dunnett's Method and Student–Newman–Keuls test) were employed when pair-wise multiple comparison procedure was necessary. Differences between mean values were considered significant if $P < 0.05$.

Results

Radioligand binding procedure: Scatchard plots and K_i values

To verify the mAChR expression in human fibroblasts, radioligand binding studies were performed

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labelling cell membranes with specific radioligand [^{3}H]-QNB. Binding of [^{3}H]-QNB to human fibroblast membranes was a saturable process to a single class of binding sites. The equilibrium parameters calculated from Scatchard plots were: K_d 0.47 ± 0.11 nM and B_{max} 236 ± 22 fmol mg protein^{-1} (Fig. 1). The relative potencies of selective antagonists indicate a predominant expression of M_3 and M_1 mAChR subtypes. The K_i values were: atropine 1.02×10^{-8} M, 4-DAMP 1.12×10^{-8} M, pirenzepine 1.27×10^{-8} M, AF-DX 116 6.01×10^{-6} M and tropicamide 4.37×10^{-6} M.

Fibroblast cultures: stimulatory responses to carbachol

To study DNA synthesis induced by carbachol, a mAChR agonist, human skin fibroblasts were treated with different carbachol concentrations. As can be seen in Fig. 2a, carbachol increased thymidine incorporation in a concentration-dependent manner, with a maximal effects at 1×10^{-6} M (approximately 20-fold increase). Atropine (1×10^{-6} M) inhibited the effect on carbachol-mAChR activation upon DNA synthesis (Histogram, Fig. 2b).

To determine the subtype of mAChR (M_1–M_4) responsible for carbachol stimulation on DNA synthesis, the respective antagonists pirenzepine (M_1), 4-DAMP (M_3), AF-DX 116 (M_2) and tropicamide (M_4), were selectively studied. Fig. 3a,b shows that M_1 and M_3 antagonists induced a significant inhibition upon the carbachol action on fibroblasts with a right wards shift of the concentration–response curves to carbachol (pA_2: pirenzepine 8.72; 4-DAMP 8.25). On the contrary, neither AF-DX 116 nor tropicamide modified carbachol effect (Fig. 3c and d). It is important to

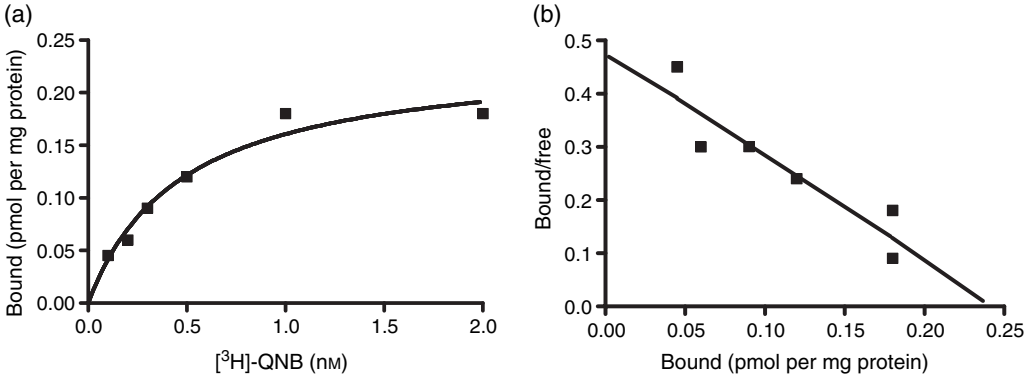

Figure 1 Binding of [^{3}H]-QNB to fibroblast membranes that were prepared as referred in Materials and methods. (a) Membranes (50 μg protein) were incubated with increasing concentrations of the radioligand [^{3}H]-QNB. (b) Scatchard plot for calculation of equilibrium parameters (B_{max} and K_d) which was analysed with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) programs. Data show one representative experiment from five separate experiments performed in duplicate.

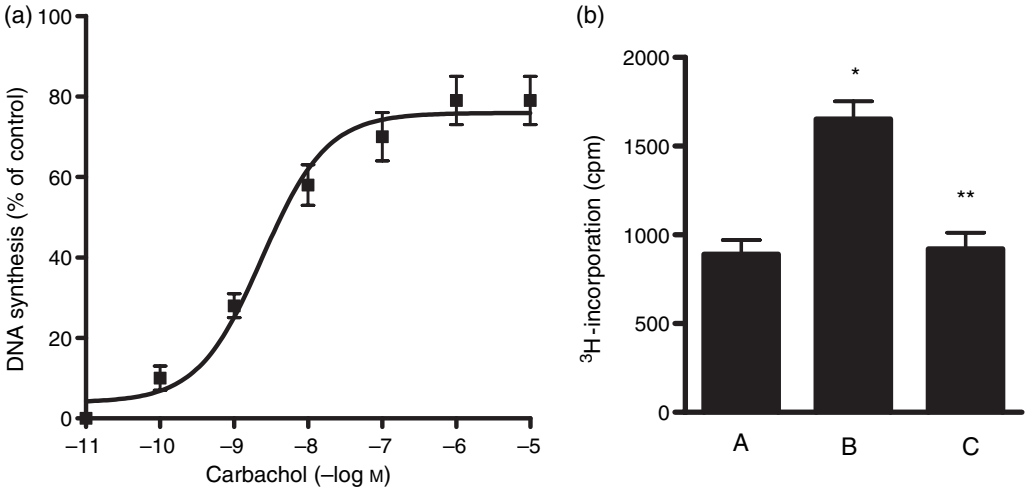

Figure 2 Concentration–response curve of carbachol upon human skin fibroblast DNA synthesis measured by [^{3}H]-thymidine incorporation. (a) Each concentration of carbachol reacted with 1×10^6 cells during 8 h; (b) A: basal values, B: effect of 1×10^{-9} M carbachol, C: effect of 1×10^{-6} M atropine + 1×10^{-9} M carbachol. Results are expressed as percentage of change before the addition of each concentrations of carbachol. Values are mean ± SEM of seven experiments in each group performed by triplicate. *Differ significantly from A with $P < 0.001$; **differ significantly from B with $P < 0.001$.

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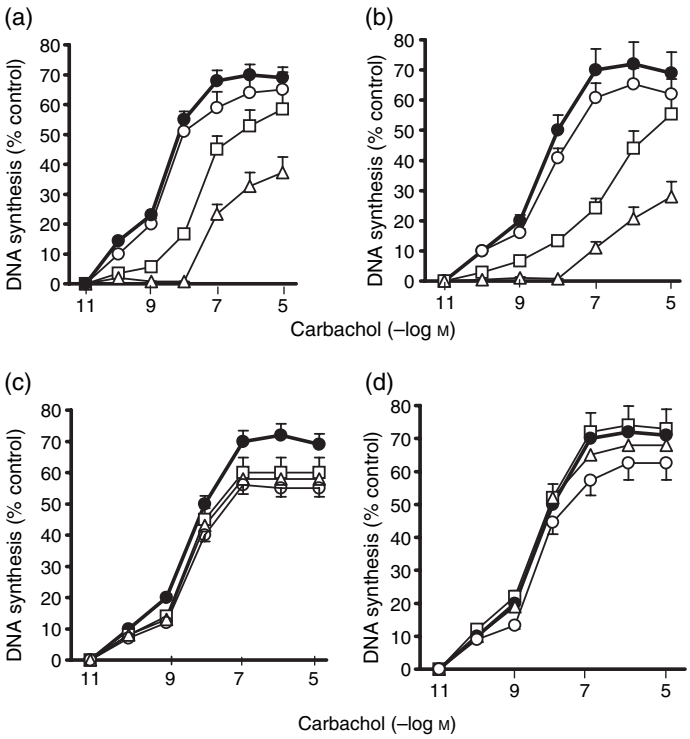

Figure 3 Effects of different mAChR subtype antagonists on carbachol-stimulated fibroblast DNA synthes effect. Effect of (a) pirenzepine, (b) 4-DAMP, (c) AF-DX 116 and (d) tropicamide. Dose–response curves of carbachol alone (●) or in the presence of 1×10^{-8} M (○), 1×10^{-7} M (□) and 1×10^{-6} M (△) of different muscarinic cholinergic antagonists are shown. Values are mean ± SEM of seven experiments in each group performed by triplicate.

note that all the cholinergic inhibitors at the concentration used did not modify basal values (Table 1).

To assess if there were changes in the post-receptor mechanisms involving G protein, we studied the action of PLC, protein kinase C (PKC) and CaM inhibitors on carbachol-stimulated fibroblast DNA synthesis. With this purpose, we employed U-73122 (5×10^{-6} M), calphostin C (1×10^{-8} M) and TFP (5×10^{-6} M) respectively. As can be seen in Fig. 4, inhibition of PLC and CaM prevented the stimulatory effect of carbachol-induced DNA synthesis. PKC inhibition by calphostin C did not modify the stimulatory action of carbachol, ruling out the PKC participation in the system. The concentrations used for the enzymatic inhibitory agents, were those known to specifically block PLC (Smallridge, Kiang, Gist, Feim & Galloway, 1992), PKC (Bruns, 1991) and CaM (Scharff & Foder, 1984) respectively. Also, the inhibitors alone *per se* did not modify basal values (Table 1).

To elucidate whether CD40 expression resulted in functional postsynaptic mAChR activation by carbachol, we measured the CD40 expression in human skin fibroblasts cultured alone or in the

Table 1 Influence of inhibitory agents upon basal values of ^{3}H-incorporation

Inhibitors	^{3}H-incorporation (cpm)	No. of experiments
None	958 ± 44	16
Atropine	956 ± 55	9
Pirenzepine	955 ± 52	7
4-DAMP	960 ± 63	7
AF-DX 116	959 ± 51	7
Tropicamide	962 ± 72	7
U-73122	958 ± 32	5
Trifluoroperazine	964 ± 73	5
Calphostin C	954 ± 68	5

Results are mean ± SEM.

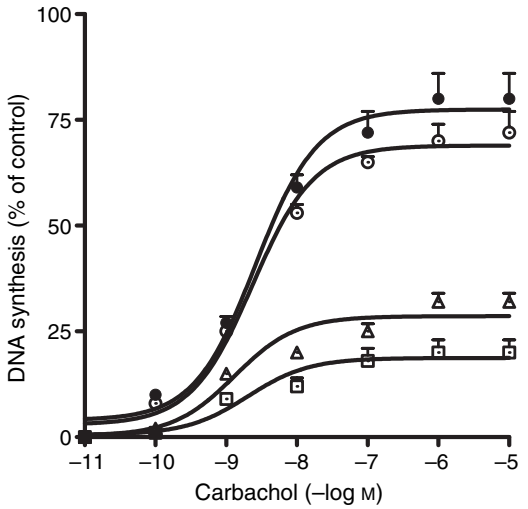

Figure 4 Effect of enzymatic inhibitors on carbachol dose–response curve for increased fibroblast DNA synthesis. Carbachol alone (●) or in the presence of U-73122 5×10^{-6} M (□) or TFP 1×10^{-6} M (△) or calphostin C 1×10^{-8} M (○). Values are mean ± SEM of five experiments performed by triplicate. Differ significantly from basal value with $P < 0.001$.

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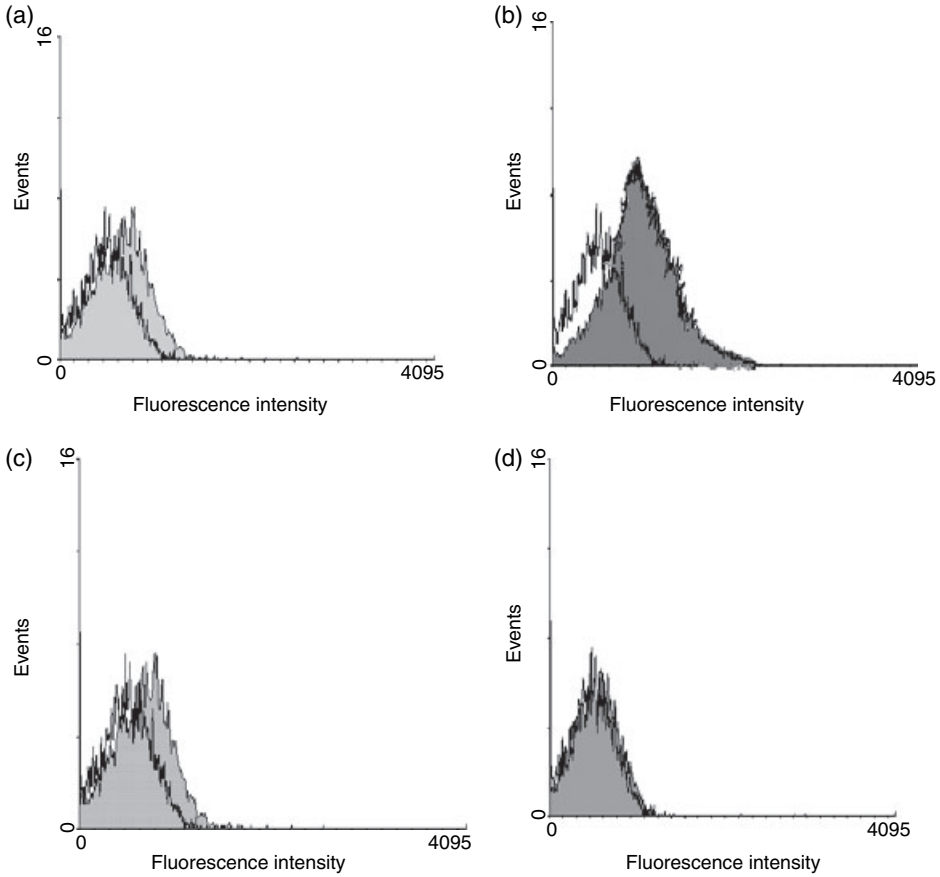

Figure 5 CD40 expression by human skin fibroblasts. FACS profiles of one representative culture from a total of 20 tested (five in each group) are shown. Fibroblast cultures were stained with anti CD40 or isotype-matched control MoAb as described in Materials and methods. Basal (without carbachol) (a); in the presence of 1×10^{-9} M carbachol (b), or 1×10^{-6} M atropine + 1×10^{-9} M carbachol (c), or 5×10^{-6} M U-73122 + 1×10^{-9} M carbachol (d). Values are mean ± SEM of five experiments in each group performed by triplicate.

presence of 1×10^{-9} M carbachol. As shown in Fig. 5a, fibroblasts cultured in medium alone (basal) showed a moderate expression of CD40 which was significantly enhanced in 1×10^{-9} M carbachol-treated cells (Fig. 5b). When atropine (1×10^{-6} M) (Fig. 5c) or U-73122 (5×10^{-6} M) (Fig. 5d) were added 10 min prior carbachol addition, the increment in the CD40 expression was blocked. There were no differences in the level of CD40 expression between fibroblasts cultured alone scanned or in the presence of 1×10^{-6} M atropine and 5×10^{-6} M U-73122 (data not shown). The basal values of positive cells was 18 ± 2% ($n = 5$), in presence of carbachol alone was 48 ± 3% ($n = 5$), in presence of carbachol plus atropine 20 ± 2% ($n = 5$) and in the presence of carbachol plus U-73122 15 ± 3% ($n = 8$). These results point to the participation of mAChR and PLC in carbachol CD40 overexpression.

To assess if carbachol activates InsP hydrolysis in fibroblasts, total InsP was measured. As can be seen in Fig. 6, the basal values of InsP production in response to 1×10^{-9} M carbachol were increased significantly. The mAChR antagonists atropine, 4-DAMP and pirenzepine, all at 1×10^{-6} M and PLC inhibitor U-73122 (5×10^{-6} M) prevented carbachol-induced InsP accumulation (Fig. 6a). On the contrary, AF-DX 116 (M_2) and tropicamide (M_4) were without action in the study system (Fig. 6b).

Discussion

In the current study, we demonstrated that muscarinic cholinergic receptors are expressed in human skin fibroblasts and their activation by carbachol induced an increment in DNA synthesis and in CD40 expression.

The pharmacological analysis with different mAChR antagonists allowed us to identify which of the known mAChR subtypes are active in human skin fibroblasts. Data showed that M_3 and M_1 subtypes are important mediators of carbachol induced biological effects, while M_2 and M_4 seem to have no relevance in these actions. This finding is consistent with the respective K_i of the antagonists obtained by the competition binding assay. Therefore, our results satisfy the pharmacological criteria

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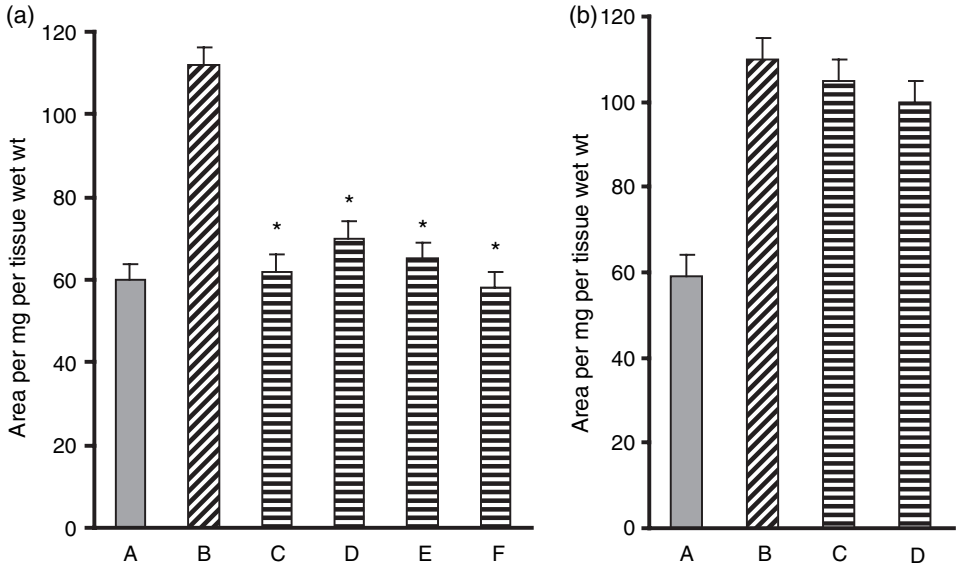

Figure 6 Carbachol action on the production of inositol phosphates (InsP). (a) A: basal values, B: carbachol alone 1×10^{-9} M, C: atropine 1×10^{-6} M + carbachol 1×10^{-9} M, D: pirenzepine 1×10^{-6} M + carbachol 1×10^{-9} M, E: 4-DAMP 1×10^{-6} M + carbachol 1×10^{-9} M and F: U-73122 1×10^{-6} M + carbachol 1×10^{-9} M; (b) A: basal values, B: carbachol alone 1×10^{-9} M, C: AF-DX 116 1×10^{-6} M + carbachol 1×10^{-9} M and D: tropicamide 1×10^{-6} M + carbachol 1×10^{-9} M. Values are mean ± SEM of five experiments in each group. *Differ significantly from B with $P < 0.001$.

for the coexistence of active M_3 and M_1 mAChR in human fibroblasts. In agreement with this, Vestling *et al.* (1995) have identified M_3 and M_1 as the cause of agonist-stimulated DNA synthesis in fibroblasts. It is of noted, that fibroblasts are different depending on the tissue origin and the mAChR subtypes expressed differ with organ location. Thus, Chinese hamster lung fibroblast (CCL39) cell lines predominantly expressed high levels of human M_1 mAChR (Mckenzie, Seuwen & Pouyssegur, 1992). Fibroblasts of lung parenchyma from pig expressed M_4 subtype in contrast to the M_3 subtype of large airways in this species (Mckenzie *et al.*, 1992).

Concerning the mechanism by which carbachol stimulated DNA synthesis, we found that the agonist enhanced InsP production at the same concentration at which it is able to increase DNA synthesis. Moreover, the relative potency of receptor subtype antagonist for inhibiting carbachol-stimulated DNA synthesis was identical to their relative potency for blocking carbachol-induced InsP accumulation. Thus, carbachol activating M_3 and M_1 mAChR results in InsP accumulation, that in turn, increased fibroblast DNA synthesis. The mechanism appears to relate to an increase in intracellular calcium concentration. This initial rise of intracellular calcium may be regulated by IP_3 that triggers the release of calcium from intracellular stores (Putney, 1993). The fact that inhibitors of PLC and CaM prevented the carbachol effects indicated the participation of both enzymes in carbachol-promoted fibroblast DNA synthesis and InsP production. On the contrary, PKC activity appeared not to participate in carbachol effects.

That human skin fibroblasts have predominant expression of M_3 and M_1 mAChR subtypes could confer on them the ability to convert into a contractile type of fibroblasts. Fibroblasts undergoing transformation to myofibroblasts play important structural roles in the connective tissue during wound healing as well as in inflammatory responses (Urata, Nishimura, Hirase & Yokoyama, 2005).

Additionally, we presented here evidence that the activation of cholinoceptors not only is able to stimulate fibroblast DNA synthesis, but also increased the expression of immunoregulatory proteins, such as, CD40. In fact, carbachol induced an increment in the expression of CD40 that was blocked by atropine, supporting the notion that fibroblast overexpression of CD40 antigen is intrinsically activated by the parasympathetic muscarinic system. The mechanism by which carbachol increased CD40 expression, involves the participation of mAChR messengers InsP as demonstrated by the inhibitory effect of U-73122 on carbachol-triggered overexpression of CD40. There is evidence that the activation and the increment in the CD40 expression in glandular epithelial cells are associated with the induction of various immunoregulatory and apoptosis-related proteins and/or with the presence of different cytokines in normal physiology and abnormal pathology (Wassenaar, Reinhardus, Thepent, Abraham-Inpijn & Kievits, 1995). Also, CD40 expression is upregulated by cytokines that are

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produced in inflammatory lesions (Takahashi *et al.*, 1994; Tsai, Ho & Chen, 1995; Dimitriou, Kapsogeorgou, Moutsopoulos & Manoussakis, 2002).

Taken together, our results suggest that the overexpression of CD40 and the stimulation of DNA synthesis by carbachol established a modulatory role of parasympathetic muscarinic system on normal human skin fibroblasts. CD40 expression directly or indirectly is associated with different pathological conditions, such as, periodontitis (Dongari-Bagtzoglou, Warren, Bretón & Ebersole, 1997), bone and cartilage resorption (Mundy, 1991), granuloma formation (Poli *et al.*, 1994), autoimmune and allergy diseases (Wassenaar *et al.*, 1999; Brouty-Boyé, Pottin-Clémenceau, Doucet, Jasmin & Azzarone, 2000) and even in peri-implantitis and chronic inflamed periodontium (Schroeder & Page, 1972) providing a rationale for up-modulation of CD40-mAChR-mediated signalling as etiopathogenetic factor(s). Overall, these findings support the concept that stimulation of the fibroblast's DNA synthesis and CD40 expression, probably are, one among other factor(s), control and participate in inflammation and fibrosis through fibroblast-mAChR activation.

Acknowledgments

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