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Muscarinic[Q1] Cholinoceptor Activation by Pilocarpine Triggers Apoptosis in Human Skin Fibroblast Cells

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The aim of the present work was to examine the role of muscarinic acetylcholine receptors (mAChRs) on apoptosis in human skin fibroblast cells. Neonatal human skin fibroblast cultures were stimulated with pilocarpine in the presence or absence of specific antagonists. Pilocarpine stimulates apoptosis, total inositol phosphates (InsP) accumulation and nitric oxide synthase (NOS) activity. All these effects were inhibited by atropine, mustard hydrochloride (4-DAMP) and pirenzepine, indicating that M_1 and M_3 mAChRs are implicated in pilocarpine action. Pilocarpine apoptotic action is accompanied by caspase-3 and JNK activation. The intracellular pathway leading to pilocarpine-induced biological effects involved phospholipase C, calcium/calmodulin and extracellular calcium as U-73122, W-7, verapamil, BAPTA and BAPTA-AM blocked pilocarpine effects. L-NMMA, a NOS inhibitor, had no effect, indicating that the enzyme does not participate in the apoptosis phenomenon. These results may contribute to a better understanding of the modulatory role of the parasympathetic muscarinic system on the apoptotic human skin fibroblast process.

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Fibroblasts are 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. They are not a homogeneous population, even within a single tissue, but exist as subsets of cells much like tissue macrophages and dendritic cells (Phipps et al., 1999; Buckley et al., 2001). Human skin fibroblasts play pivotal roles in connective-tissue production, physiological skin remodelling and wound repair. They have also been found to be important modulators of the immune system (Fries et al., 1995; Smith et al., 1997).

Human fibroblasts express muscarinic acetylcholine receptors (mAChRs) of different subtypes (Carsi-Gabrenas et al., 1997; Buchli et al., 1999). We demonstrated that mAChRs are expressed in human skin fibroblasts and their activation by carbachol induced an increment in DNA synthesis and in CD40 expression (Casanova et al., 2006). 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_1 and M_3 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 competition-binding assay. Therefore, our results satisfy the pharmacological criteria for the coexistence of active M_1 and M_3 mAChRs in human fibroblasts. In agreement with this, Vestling et al. (1995) have identified M_1 and M_3 as the causes of agonist-stimulated DNA synthesis in fibroblasts. However, using the reverse transcription-polymerase chain reaction and Northern blot technique the m_2, m_4 and m_5 mAChR subtypes were identified human skin fibroblast surface (Buchli et al., 1999), but only negligible activity of these receptors was documented (Van Riper et al., 1985).

Fibroblasts differ, depending on the tissue origin and mAChR subtypes differ, depending on organ location. Thus, Chinese hamster lung fibroblast (CCL39) cell lines expressed predominantly high levels of human M_1 mAChR.

Fibroblasts of lung parenchyma from a pig expressed M_4 subtype in contrast to the M_3 subtype of large airways in this species (Mckenzie et al., 1992).

The mAChR subtypes are generally grouped according to their functional coupling, either (1) to mobilize intracellular calcium (M_1, M_3, M_5) through the activation of phospholipase C (PLC), which results in the release of the second messenger inositol 1,4,5-trisphosphate (IP_3) or (2) by inhibiting adenylyl cyclase (M_2, M_4), which results in reduction of the intracellular levels of cyclic adenosine monophosphate (Hulme et al., 1990). It is important to note that the same receptor may generate more than one set of intracellular second messengers and that considerable cross-talk exists between signalling cascades (Felder, 1995; Nicke et al., 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 and Robbins, 1992).

Apoptosis is a highly regulated form of cell death that is known to sculp tissues during development and maintains tissue homeostasis by eliminating unnecessary or harmful cells. Extensive research has been established the critical role of caspases, a family of cysteine proteases, in mediating the signal

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transduction and execution of apoptosis (Yi and Yuan, 2008). To programmed apoptosis, cell human requires eleven caspases. Such expansion of the caspases family might arise for dual proposed: the execution of apoptotic cell death and to carry out other cellular processes such as remodelling.

A number of G-protein coupled receptors can regulate apoptotic signalling (Dale et al., 2000; Gu et al., 2000). β-Adrenergic stimulation in cardiac myocytes promotes apoptosis via a mechanism that may involve the dephophorylation of the proapoptotic protein, Bcl-2, and, in so doing, initiate its translocation to the mitochondria (Saito et al., 2000; Zhu et al., 2001).

Among these receptors are the mAChRs, whose activation can stimulate or inhibit cell growth and apoptosis, depending on prior levels of cellular activity (Lanzafame et al., 2003).

The key role that mAChRs play in controlling cellular function rests not only in this diversity of receptor subtypes, but also in the fact that each receptor has the ability to activate a large array of interconnecting, intracellular signalling pathways (Leloup et al., 2000; Marinissen and Gutkind, 2001; De Sarno et al., 2003).

The biochemical mechanisms involved in the initiation and execution of apoptosis have been extensively studied and are now extremely well understood at the molecular level. During the course of these studies it has emerged that components of apoptotic signalling pathways are regulated by phosphorylation, and in many cases the protein kinases responsible for these modifications have been identified (Gutcher et al., 2003; Liu et al., 2005).

Little is known about the apoptotic modulator mechanisms activated by skin fibroblast mAChRs and the participation of these receptors in the phenomenon. In this work, we have demonstrated that pilocarpine, through mAChR activation of human skin fibroblasts, stimulated apoptosis. In addition, a direct functional relationship between the activation of M_1 and M_3 mAChRs and the enhancement of the apoptotic cascade in human skin fibroblasts was observed. The mechanisms underlying mAChR activation enhancement of fibroblast apoptosis involves PLC and calcium/calmodulin (CaM) activation via InsP hydrolysis.

Materials and Methods

Cell culture

Pure cell cultures of neonatal human (7 days after birth) skin fibroblast (2×10^6 cells/ml) were established from the foreskin obtained after circumcision and grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% CO_2 environment at 37°C 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 (0.1 M pH 7.2) containing (PBS): Na_2HPO_4 anhydrous 1.09 g; NaH_2PO_4 anhydrous 0.32 g; NaCl 9.00 g and distilled water 100 ml serum-free. Only cell of passages 4–6 were used in the experiments and all the experiments were carried out in serum-free medium. To identify the fibroblast experiments was carrying out to establish the incubation time and the number of cell used throughout. Human studies have been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki.

Fibroblast cell treatment

To evaluate the apoptosis, cell cultures were subjected to treatment with pilocarpine (from 1×10^{-10} to 1×10^{-6} M) for 48 h. When different non-specific (atropine 1×10^{-6} M) or specific (pirenzepine and 4-DAMP from 1×10^{-9} M to 1×10^{-6} M) mAChR antagonists and different enzymatic inhibitors: phospholipase C (PLC) (U-73122 5×10^{-6} M) and its analogue (U-73343 5×10^{-6} M), calcium calmodulin (CaM) (W-7 5×10^{-6} M), calcium flux (verapamil 1×10^{-5} M), calcium chelators (BAPTA 5×10^{-6} M, BAPTA-AM 5×10^{-6} M), JNK activity (SP 600125 1×10^{-5} M) and nitric oxide synthase (NOS) N^G-monomethyl-L-arginine (L-NMMA 1×10^{-6} M) were used, they were added 20 min before the addition of mAChR agonist pilocarpine. Controls included both untreated cells and cells incubated for 48 h with the pro-apoptotic agent actinomycin D 1×10^{-7} M.

Determination of apoptosis

TUNEL assay. We also assayed apoptosis by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) method, which examines DNA strand breaks that occurs during apoptosis, using and in situ cell death detection reagent (Roche Molecular Biochemicals, Mannheim, Germany). We performed this assay as described previously (Takada et al., 2005). Briefly 2×10^6 cells were incubated with pilocarpine from 1×10^{-10} to 1×10^{-6} M for 48 h and when the mAChR antagonist and enzymatic inhibitors were used, they were added 20 min before to pilocarpine. Then, fixed with 4% paraformaldehyde and permeabilised with 0.1% triton-X100 in 0.1% sodium citrate. After quenching endogenous peroxidase activity with 3% (v/v) H_2O_2 and rinsing in 150 mM NaCl, 10 mM Tris pH 7.4 buffer (TBS), terminal deoxynucleotidyl transferase (TdT) was subsequently applied for 1 h add 37°C in biotynylated nucleotide mix and equilibration buffer (Tris acetate pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 0.25 mM $CoCl_2$ and 24 μM biotin-dATP). The cells then blocked with stop/wash buffer, and incubated with 100 μl streptavidin-horseradish peroxidase (HRP) (1:500 dilutions) for 30 min at room temperature. Finally, after several washings in TBS, the cells were developed using 3,3′-diaminobenzidine (DAB) and counterstained with haematoxylin and eosine. Apoptotic nuclei (percent of total number) were evaluated at 100× magnification in 10 different fields of at least 100 cells in each field. Values are expressed as a percentage above control without drugs taken as 100%.

DNA fragmentation of fibroblasts. Degradation of fibroblast cell DNA was used as an index of apoptosis. Fibroblast cells (2×10^6) from each treatment were washed in phosphate buffer solution (PBS) and lysed in 10 mM Tris pH 7.4, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100 for 20 min on ice. Then, 1 ml of DNA-zol (Molecular Research Center Inc., [illegible]) was added to cells in the presence of proteinase K (100 μg/ml) for 30 min at 37°C. After centrifugation at 11000 g for 20 min at 10°C, the supernatant was collected and RNAse A (20 μg/ml) was added for 1 h at 37°C, in order to eliminate contaminating RNA. The DNA extracted was precipitated in 100% (v/v) ethanol and centrifuged at 11000 g for 20 min. Purified DNA was dissolved in 8 mM NaOH separated by electrophoresis in a 2% (w/v) agarose gel and visualised by ethidium bromide staining.

Measurement of inositol phosphates (InsP). Fibroblasts (2×10^6 cell/ml) 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) from Dupont/New England Nuclear and LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique previously described (Borda et al., 1998). Pilocarpine (1×10^{-7} M) was added 30 min before the end of the incubation period and the enzymatic inhibitors and mAChR blockers were added 20 min before the addition of pilocarpine. Water-soluble InsPs were extracted after a 120 min incubation period. 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 3,000*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) 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 myoinositol followed by 6 volumes of water and InsP were eluted with 1 M ammonium formate in 0.1 M formic acid. One ml fractions were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation (Hokin-Neaverson and Sadeghian, 1976). Results were

expressed as a percentage of the total radioactivity incorporated (1st plus 2nd peaks). In order to determine the absence of (^{3}H)-MI in the eluted peaks of InsP, chromatography in silica gel 60 F254 sheets (Merck & Co, Inc., Whitehouse Station, NJ) was performed using propan-2-ol-6,NH_4 (14:5) as the developing solvent (Hokin-Neaverson and Sadeghian, 1976). Spots were located by spraying with freshly prepared 0.1% ferric chloride in ethanol followed, after air drying with 1% sulphosalicylic acid in ethanol. To assay the radioactivity a histogram was constructed by cutting up the sheet gel, placing each sample in Triton-toluene based scintillation fluid and the counting.

Determination of nitric oxide synthase (NOS) activity

Nitric oxide synthase (NOS) activity was measured in cells (2×10^6/ml) by production of (U^{-14}C)-citrulline from (U^{-14}C)-arginine according to the procedure described by Bredt and Zinder (1999). Briefly, after 20 min preincubation in KRB solution, cells were transferred to 500 μl of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of (U^{-14}C)-arginine (0.5 μCi). Appropriate concentrations of pilocarpine were added and the cells were incubated for 20 min under 5% CO_2 in O_2 at 37°C. When pirenzepine and 4-DAMP were used, they were added 20 min before pilocarpine. Measurement of basal NOS activity in cells by the above mentioned procedure was inhibited 95% and 78% in the presence of 0.5 mM and 0.05 mM L-NMMA respectively. The results were expressed as picomol per gram of tissue wet weight (pmol/g/tissue wet wt).

Caspase-3 assay

Caspase-3 activity was measured by means of the CaspACE Assay System Fluorometric Kit (Promega Corporation, Madison, WI). Cells were initially seeded at a density of 1×10^9 in 10 cm dishes. After pilocarpine treatment for the indicated time, caspase-3 activity was measured by the leavage of the fluorometric substrate Ac-DFVD-AMC according the manufacturer's instructions. When enzymatic inhibitors were used, they were added 20 min before pilocarpine.

JNK assay

JNK activity was assessed using a recombinant protein fragment of c-Jun as an affinity ligand and substrate glutathione Stransferase (GST-c-Jun [1-79]) (Wylie et al., 1999). Cleared cell lysates were prepared exactly as described above and incubated for 60 min at 4°C with 20 μl of a 25% (v/v) slurry of glutathione ± Sepharose (Pharmacia-LKB), pre-coupled to GST-c-Jun (5 μg of protein). Beads were collected by centrifugation and washed twice in 200 μl of lysis buffer and twice in 200 μl of kinase buffer. Reactions were initiated by addition of 40 μl of kinase buffer containing 20 μM [γ-^{32}P]ATP (2.5 μCi/nmol). After incubation for 20 min at 30°C, reactions were terminated by addition of 40 μl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by PAGE through 12% acrylamide in the presence of 0.1% SDS, stained with Coomassie Blue R250 and visualized by autoradiography. Radioactivity incorporated into GST-c-Jun was quantified by liquid-scintillation counting of the excised bands.

Drugs

Pilocarpine, atropine, pirenzepine, verapamil, U-73343, L-N^G-monomethyl arginine citrate (L-NMMA), L-arginine and actinomycin D were provided by Sigma Chemical Company (St. Louis, MO); U-73122 and 4-DAMP were provided by ICN Pharmaceuticals Inc. (Costa Messa, CA); BAPTA, BAPTA-AM, W-7 and SP 600125 were provided by Tocris Biosciences (Ellisville, MO). Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted to achieve the final concentrations stated in the text.

Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P < 0.05$.

Results

We initially determined the effect of different concentrations of pilocarpine at varying times of culture (Fig. 1A). We found that pilocarpine caused a marked increase in the number of apoptotic fibroblasts, compared with the same cells without drugs, as shown by the TUNEL assay. Maximal effect of the muscarinic receptor agonist was obtained at 48 h with a concentration of 1×10^{-7} M pilocarpine. Figure 1B shows the increase in the number of apoptotic fibroblasts in the presence of increasing concentrations of pilocarpine at 48 h. It can be seen that 1×10^{-7} M pilocarpine and 48 h are critical conditions needed to reach the maximal capacity of stimulation of the agonist.

Figure 2A shows that the maximal capacity to stimulate apoptotic fibroblasts in the presence of 1×10^{-7} M pilocarpine was approximately 25% less than that obtained with 1×10^{-7} M actinomycin D. Figure 2 also shows the lack of action of 1×10^{-7} M pilocarpine, in the presence of 1×10^{-6} M atropine. Moreover, SP 600125 (1×10^{-5} M), at concentration known that inhibited JNK enzyme activity (Wylie et al., 1999) prevented the pilocarpine induced apoptosis [apoptotic nuclei % of total number (mean ± SE mean): basal 11 ± 2.0, n = 5; pilocarpine 1×10^{-7} M 76 ± 6.1, n = 5; pilocarpine plus SP 600125 1×10^{-5} M 25 ± 5.2, n = 5].

DNA fragmentation analysis in fibroblast cells treated with 1×10^{-7} M pilocarpine and 1×10^{-7} M actinomycin D yielded the same results (Fig. 2B). We have already demonstrated that mAChRs are expressed in human fibroblasts and that M_1 and M_3 subtypes are important mediators of the agonist induced biological effect, while M_2 and M_4 do not seem to have relevance (Mckenzie et al., 1992). To determine if M_1 and M_3 subtypes are responsible for the apoptotic effect of pilocarpine on skin fibroblasts, the action of pirenzepine and 4-DAMP were selectively studied. Figure 3 shows that pirenzepine (A) and 4-DAMP (B), from 1×10^{-9} M to 1×10^{-6} M, shifted to the right the stimulatory dose-response curve of pilocarpine on the number of apoptotic fibroblasts. A Schild plot shows a pA2 of 9.37 and 10.21 for pirenzepine and 4-DAMP, respectively.

To elucidate if there were changes in the post-receptor mechanisms involving PLC and NOS activities, we studied the participation of InsP and nitric oxide (NO) in the system. With this purpose, we explored the action of U-73122 (PLC inhibitor, 5×10^{-6} M), W-7 (CaM inhibitor, 5×10^{-6} M), verapamil (calcium blocker, 1×10^{-5} M) and LNMMA (NOS inhibitors, 1×10^{-6} M) on the apoptotic effect of pilocarpine. It can be seen in Figure 4 that the enzymatic inhibition of PLC, CaM and calcium mobilisation decreased the pilocarpine-increased apoptotic effect. Moreover, to identify the source of calcium in this event we used a selective calcium chelator BAPTA (5×10^{-6} M) and a cell-permeable calcium chelator BAPTA-AM (5×10^{-6} M), corroborating the role for calcium in pilocarpine-induced apoptosis [apoptotic nuclei % of total number (mean ± SE mean): basal 12 ± 2.0, n = 5; pilocarpine alone 1×10^{-7} M 78 ± 7, n = 4; pilocarpine plus BAPTA 5×10^{-6} M 17 ± 3.1, n = 5; pilocarpine plus BAPTA-AM 5×10^{-6} M 15 ± 2.2, n = 5]. On the contrary, the inhibition of NOS activity was not effective in impairing the apoptotic effect of pilocarpine. All the inhibitory agents at the concentration used had no effect on the number of apoptotic fibroblast basal values (Table 1). As control the inactive analogue for U-73122

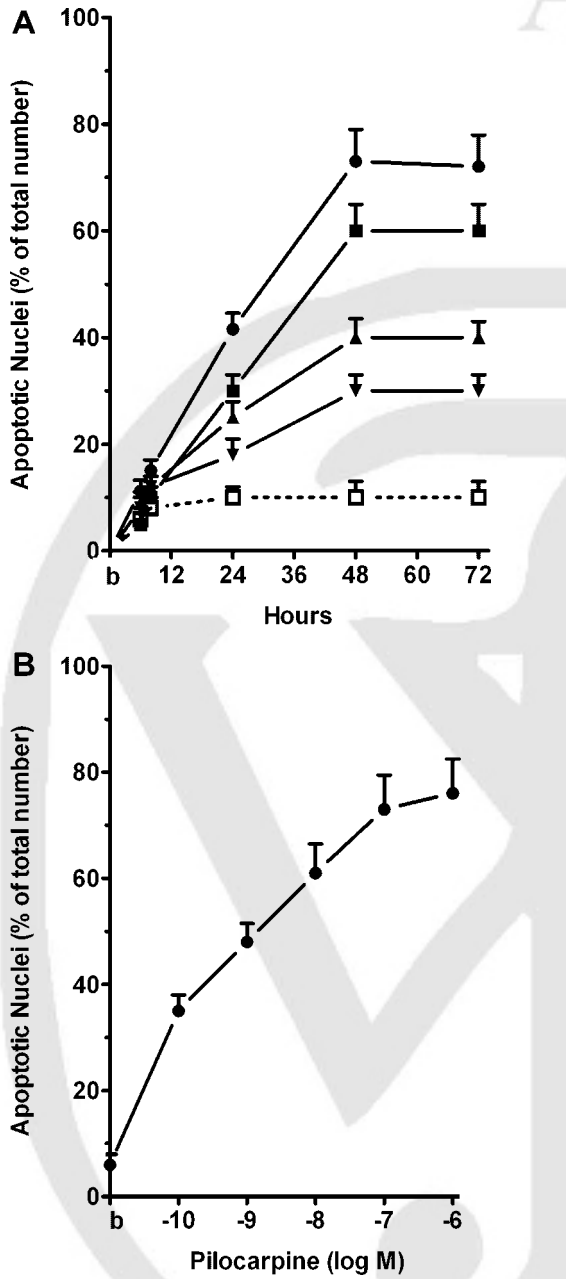

Fig. 1. Effect of pilocarpine on apoptosis of human skin fibroblast by TUNEL assay (performed as describe in material and methods). A: Time course of apoptotic effect of different concentrations of pilocarpine: 1×10^{-7} M (●—●), 1×10^{-8} M (■—■), 1×10^{-9} M (▲—▲) and 1×10^{-10} M (▼—▼). Control without drug (□- - -□) is also shown. B: Concentration-response curve of pilocarpine at 48 h of culture. Values are means ± SE mean of n = #6 in each group performed by duplicate.

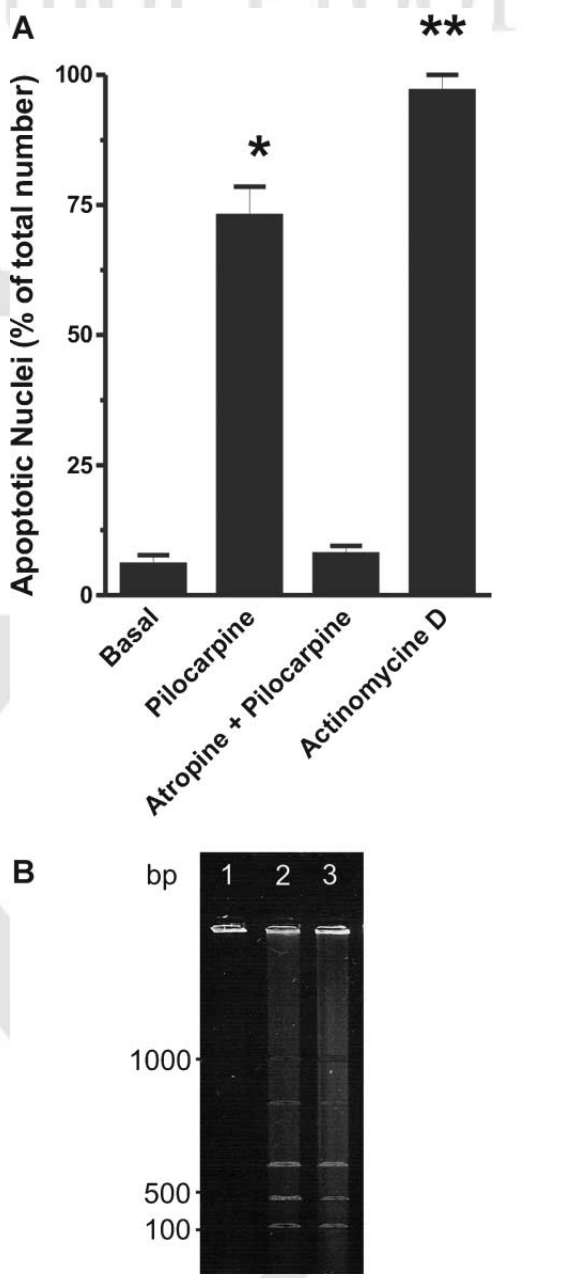

Fig. 2. A: Apoptotic effect measure by TUNEL of 1×10^{-7} M pilocarpine alone or in the presence of 1×10^{-6} M atropine. The effect of 1×10^{-7} M actinomycin D and the basal value without drug are also shown. Values are means ± SE mean of n = #5 in each group performed by duplicate. *$P < 0.0001$ between pilocarpine alone versus atropine + pilocarpine or basal values; **$P < 0.05$ between actinomycin D versus pilocarpine alone. B: Apoptotic effect measure by DNA laddering of 1×10^{-7} M pilocarpine (line 2) or 1×10^{-7} M actinomycin D (line 3) compared with untreated cells (line 1). The molecular weight standard was shown in line bp.

(U-73343 5×10^{-6} M) was uneffective in the studied system [apoptotic nuclei % of total number (X ± SE mean): pilocarpine 1×10^{-7} M 74 ± 6.1, n = 4; pilocarpine + U-73343 5×10^{-6} M 71 ± 7.2, n = 4].

To determine if the apoptotic effect of pilocarpine was related to PLC signalling, we measured InsP accumulation and NOS activity. As shown in Figure 5, pilocarpine increased InsP accumulation (A) and NOS activity (B). Pirenzepine (1×10^{-6} M) and 4-DAMP (1×10^{-6} M) shifted to the right the stimulatory dose-response curve of pilocarpine on InsP accumulation and NOS activity. As control, the histograms inserted in Figure 5 show that the pilocarpine induced InsP accumulation and NOS stimulation was prevented by U-73122 and L-NMMA at 5×10^{-6} M, respectively (Figure 5C and D). L-Arginine at 1×10^{-4} M restored the inhibitory action of

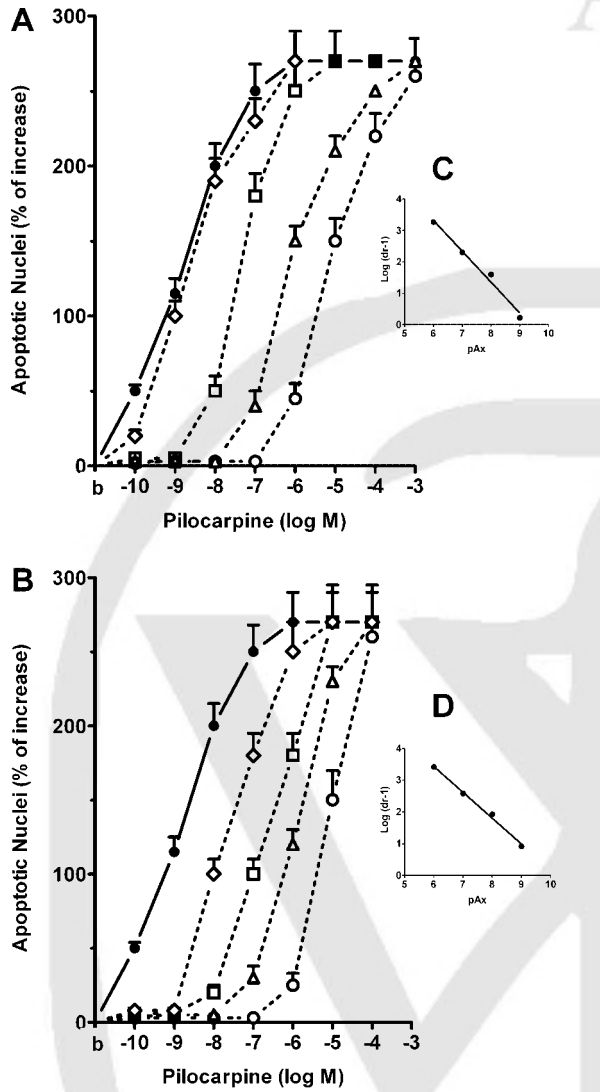

Fig. 3. Effect of 1×10^{-9} M (◇- - -◇), 1×10^{-8} M (□- - -□), 1×10^{-7} M (△- - -△) and 1×10^{-6} M (○- - -○) of pirenzepine (A) and 4-DAMP (B) on the concentration response-curve of pilocarpine (●- - -●) induced stimulation on apoptotic fibroblasts. Each point represents the means ± SE mean of n = #5 performed by duplicate. Values are expressed as a percentage of increase upon basal value (b) taken as 100%. C: Schild plots and (D) pirenzepine and 4-DAMP respectively antagonism of pilocarpine-mediated apoptotic fibroblast stimulation.

L-NMMA on pilocarpine-increase NOS activity (Fig. 5D). The close structural analogue of U-73122 (U-73343 5×10^{-6} M) failed to affect the InsP accumulation by pilocarpine (Fig. 5C).

Figure 6 demonstrates a significant correlation between the increase in apoptotic nuclei and the increase in InsP accumulation in the presence of increasing concentration of pilocarpine. On the other hand, no correlation was observed between increase in apoptotic nuclei and increase in NOS activity in the presence of increasing concentration of pilocarpine (Pearson r: 0.9376, *P* value: 0.0057, R^2: 0.8790).

To corroborate that cell death induced by pilocarpine is apoptotic we evaluated the activation of caspase-3. Figure 7 shows that pilocarpine 1×10^{-7} M at 48 h (critical conditions needed to reach the maximal apoptotic effect of the muscarinic agonist), triggered an increase in caspase-3 activity. Active caspase-3 was observed also in actinomycine D-treated cells.

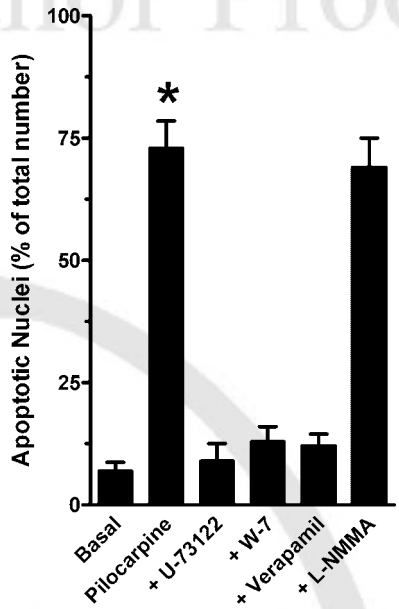

Fig. 4. Effect of 1×10^{-7} M pilocarpine alone or in the presence of U-73122 (5×10^{-6} M), W-7 (5×10^{-6} M), verapamil (1×10^{-5} M) and L-NMMA (1×10^{-6} M) on pilocarpine-induced apoptotic effect on fibroblast. Values are means ± SE mean of n = #6 in each group performed by duplicate. *$P < 0.0001$ between pilocarpine alone versus pilocarpine + U-73122, W-7 or verapamil.

The pilocarpine-induced caspase-3 increment was inhibited by U-73122 (5×10^{-6} M), W-7 (5×10^{-6} M), verapamil (1×10^{-5} M), BAPTA (5×10^{-6} M) and BAPTA-AM (5×10^{-6} M). All the inhibitory agents at the concentration used had no effect on the basal caspase-3 activity (data no shown).

To support that JNK activation is involved in pilocarpine-induced apoptosis by M_1/M_3 mediated signalling the JNK activity was assessed in the presence of the mAChR agonists. Figure 8 show that 1×10^{-7} M pilocarpine induced activation of JNK enzyme. This effect was blocked by U-73122, W-7, BAPTA and BAPTA-AM. All the inhibitory agents at the concentration used had no effect on the basal JNK activity (data no shown).

Discussion

Our studies demonstrated an enhancement of apoptosis in cultured human skin fibroblasts in response to mAChR agonist pilocarpine as assessed by both the TUNEL and DNA

TABLE 1. Influence of inhibitory agents upon number of apoptotic nuclei

Inhibitory agents	Number of apoptotic nuclei	n
Control	11 ± 1.6	10
Pirenzepine (1×10^{-6} M)	10 ± 1.7	5
4-DAMP (1×10^{-6} M)	12 ± 2.2	5
U-73122 (5×10^{-6} M)	9 ± 2.0	6
W-7 (5×10^{-6} M)	11 ± 1.9	6
L-NMMA (5×10^{-6} M)	13 ± 1.8	5
Verapamil (1×10^{-5} M)	13 ± 2.4	5
U-73343 (5×10^{-6} M)	11 ± 1.1	4
BAPTA (5×10^{-6} M)	12 ± 2.3	5
BAPTA-AM (5×10^{-6} M)	13 ± 3.1	5
SP 600125 (1×10^{-5} M)	13 ± 2.7	5

Results are means ± SE mean for control without drugs. Values showing number of apoptotic nuclei of n = number of experiments tested by duplicate.

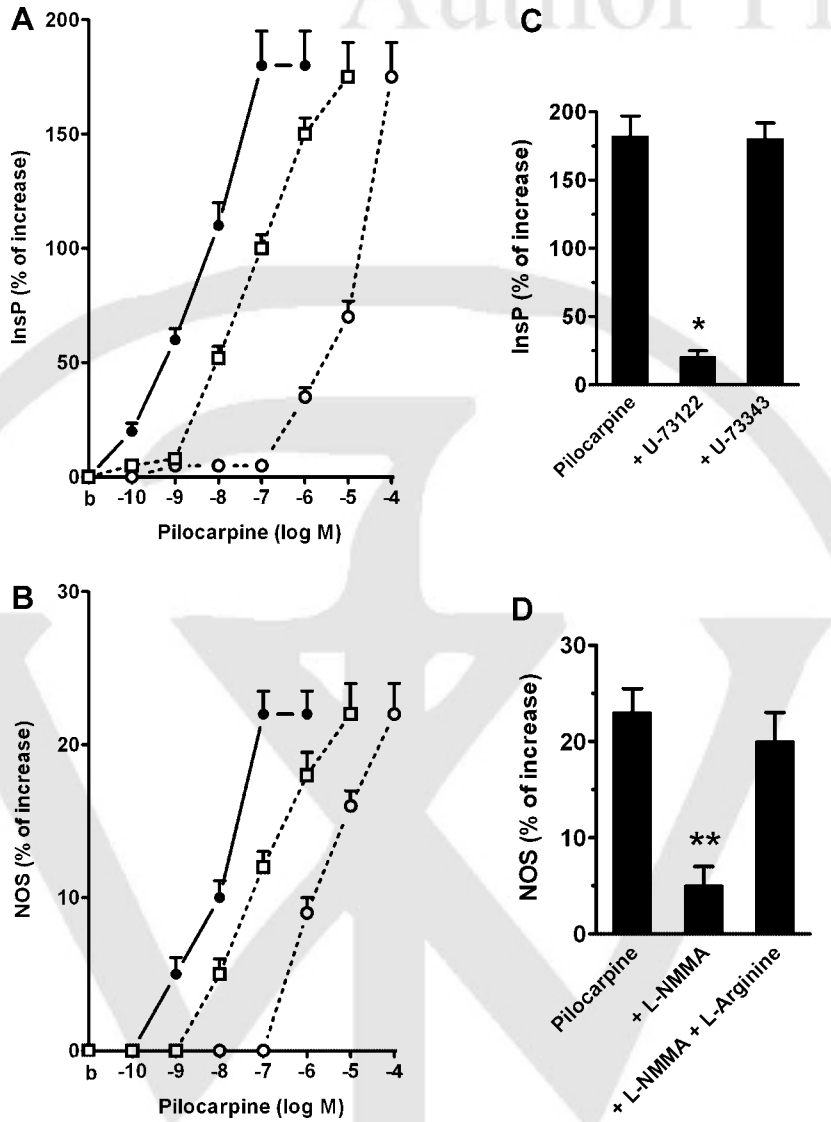

Fig. 5. Changes in (A) intracellular total inositol phosphates (InsP) and (B) NOS activity in response to incubating fibroblast in the presence of increasing concentration of pilocarpine alone (●- - -●), or in the presence of 1×10^{-6} M pirenzepine (□- - -□) or 4-DAMP (○- - -○). Histogram showing the effect of PLC inhibitor (U-73122 5×10^{-6} M) and its analogue (U-73343 5×10^{-6} M) (C); NOS inhibitor (L-NMMA 1×10^{-6} M) and L-NMMA plus L-arginine (1×10^{-4} M) (D) on pilocarpine (1×10^{-7} M). *$P < 0.0001$ between pilocarpine alone versus pilocarpine + U-73122. **$P = 0.0005$ between pilocarpine alone versus pilocarpine + L-NMMA. Values are means ± SE mean of n = #5 in each group performed by duplicate and are expressed as a percentage of increase upon basal value (b) taken as 100%.

fragmentation assays. The increase in the apoptotic process is provoked by an activation of fibroblast-mAChR M_1 and M_3 subtypes. Previously, by pharmacological analysis, we had shown that M_1 and M_3 mAChR subtypes were important mediators of mAChR agonist-induced biological effects on human skin fibroblasts (Casanova et al., 2006), while M_2 and M_4 seemed to have no relevance in these actions. Depending on the receptor subtype and the cell type in which the receptor is expressed, G-protein coupled receptors (GPCRs) can either induce apoptosis or protect cells from apoptotic stimuli. For example, β_1 adrenergic receptors in cardiac myocytes can induce apoptosis through a Gs-mediated pathway, whereas β_2 adrenergic receptors in the same cell type can protect cells through a Gi-mediated mechanism (Zhu et al., 2001). Furthermore, experimental protocols that prevent desensitisation of certain GPCRs, such as metabotropic glutamate receptors in transfected cell lines (Dale et al., 2000; Koh et al., 1991) result in unregulated GPCR signalling and stimulus-dependent apoptosis. On the contrary, M_3 mAChR in cerebellar primary neuronal cultures can protect against cell death induced by culturing in non-depolarising conditions (Yan et al., 1995).

The present result provides unequivocal evidence that in cultured human neonatal skin fibroblasts, mAChR agonist pilocarpine consistently induces apoptosis. An explanation of the discrepancy between anti apoptotic and pro apoptotic mAChR activation might be related to different cell phenotypes. This concept would be consistent with several studies that have shown that skin fibroblasts can differentiate into a new phenotype, known as myofibroblasts, in response to different stimuli (Powell et al., 1999; Urata et al., 2005). These myofibroblasts are responsible for normal wound healing. During the process they die via apoptosis (Saed and Diamond, 2002).

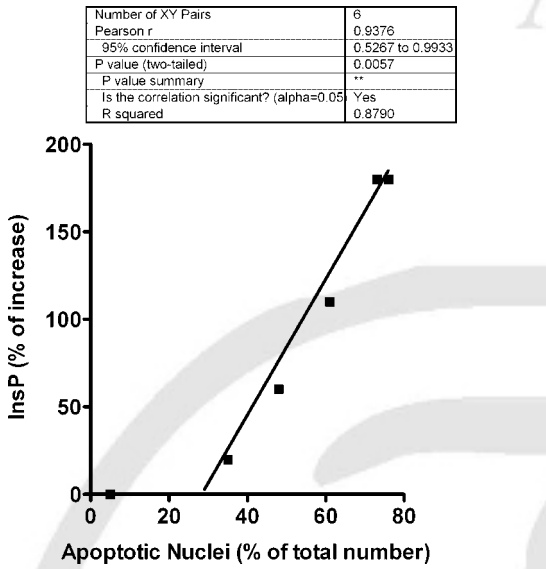

Fig. 6. Correlation in the effect of pilocarpine-induced apoptotic action and pilocarpine-increased InsP accumulation. Values are means ± SE mean of n = #5 in each group performed by duplicate.

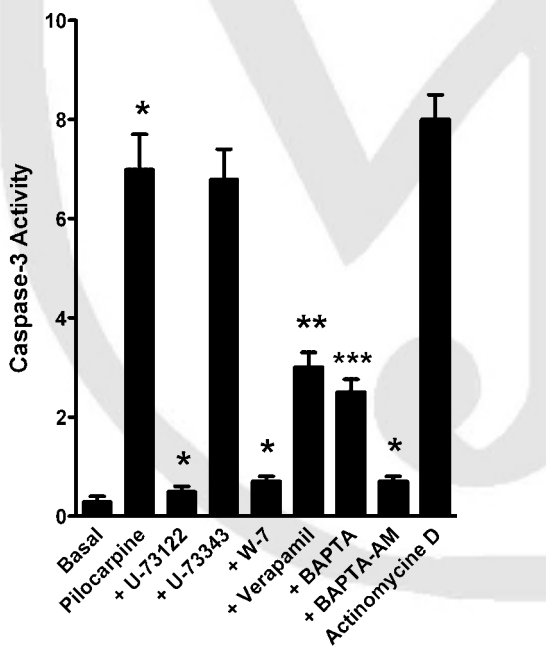

Fig. 7. Effect of pilocarpine 1×10^{-7} M alone or in the presence of U-73122 (5×10^{-6} M), U-73343 (5×10^{-6} M), W-7 (5×10^{-6} M), verapamil (1×10^{-5} M), BAPTA (5×10^{-6} M) and BAPTA-AM (5×10^{-6} M) on pilocarpine-induced increase caspase-3 activity. The actinomycine D (1×10^{-7} M) effect is also shown. Values are means ± SE mean of n = #7 in each group performed by duplicate. *$P < 0.0001$ between pilocarpine alone versus basal value or pilocarpine + U-73122, W-7 and BAPTA-AM. **$P = 0.0008$ between pilocarpine alone versus pilocarpine + verapamil. ***$P = 0.0003$ between pilocarpine alone versus pilocarpine + BAPTA.

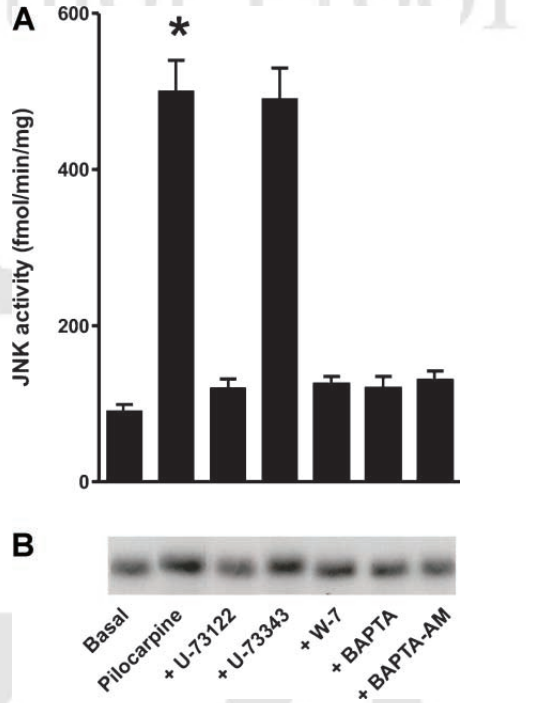

Fig. 8. A: Effect of pilocarpine 1×10^{-7} M alone or in the presence of U-73122 (5×10^{-6} M), U-73343 (5×10^{-6} M), W-7 (5×10^{-6} M), BAPTA (5×10^{-6} M) and BAPTA-AM (5×10^{-6} M) on pilocarpine-induced increase JNK activity. B: The lower portion of part A show representative autoradiograms of GST-Jun phosphorylation and activities represent the means ± SE mean for six separate determinations. Values are means ± SE mean of n = #8 in each group performed by duplicate. *$P < 0.0001$ between pilocarpine alone versus basal values or pilocarpine + U-73122, W-7, BAPTA and BAPTA-AM.

Concerning the mechanism by which pilocarpine induces apoptosis, we found agonist-enhanced InsP production to be at the same concentration as that which enabled it to increase apoptosis and a correlation between pilocarpine induced-apoptosis and pilocarpine increased InsP accumulation. Moreover, the relative potency of the receptor subtype antagonist for inhibiting pilocarpine-stimulated apoptosis was identical to its relative potency for blocking pilocarpine-induced InsP accumulation. Thus, pilocarpine's activating the M_1 and M_3 mAChRs results in InsP accumulation, which in turn, accelerates the apoptotic process. The mechanism appears to relate to an increase in intracellular calcium concentration. This initial rise of intracellular calcium may be regulated by calcium influx and by IP_3, which triggers the release of calcium from intracellular stores. The fact that inhibitors of calcium influx, PLC and CaM, prevented the pilocarpine effects indicates the participation of both enzymes in pilocarpine-promoted fibroblast apoptosis. In human renal cancer cells, *m*-3M3FBS treatment induces apoptosis via PLC signalling pathways and increases the intracellular calcium level (Jung et al., 2008). Moreover, angiotensin II stimulates cardiac fibroblast proliferation in cultured neonatal rats and stimulates apoptosis via the PLC pathway, which is accompanied by an increase in the intracellular calcium level (Vivar et al., 2008).

On the other hand, studies that report an antiapoptotic response of M_3 mAChR activation in CHO cells show that this response is not linked to the ability of the receptor to signal through the calcium/PLC pathways, but does appear to involve gene transcription and changes in the level of anti apoptotic Bcl-2 protein (Saed and Diamond, 2002). The fact that M_1

and M_3 mAChR subtypes of human skin fibroblasts regulate apoptosis and proliferation may be a key mechanism in regulating adhesion development in the postoperative healing process.

The apoptotic mechanism may involve at least two pathways; one dependent on JNK and other independent on JNK. The M_3 mAChR stimulation can activate JNK via calcium and PLC-dependent mechanisms (Wylie et al., 1999; Lanzafame et al., 2003). In our case, the M_1/M_3 mAChR activation triggers apoptosis using a pathway involving JNK activation as the pilocarpine-apoptotic action was attenuated by SP 600125, a JNK inhibitor (Wylie et al., 1999). Moreover, pilocarpine was able to stimulate JNK activity.

Our findings also demonstrated that pilocarpine activating M1 and M3 mAChR induced the activation of effector caspase-3. Both, the caspase-3 and JNK increased activity also involved receptor-mediated InsP accumulation and calcium/CaM signalling system.

In conclusion, the results of our studies have revealed that M_1 and M_3 mAChRs express in skin fibroblast cells are able to stimulate apoptotic cell death and proliferation. Both mechanisms proceed via PLC and calcium signalling pathway. The mayor challenger will be to link these observations with physiologically relevant scenarios where mAChRs may well play a key role in determining the fate of specific cell populations in intact tissues.

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