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Pharmacological characterization of muscarinic receptor subtypes mediating vasoconstriction of human umbilical vein

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1 The present study attempted to pharmacologically characterize the muscarinic receptor subtypes mediating contraction of human umbilical vein (HUV).

2 HUV rings were mounted in organ baths and concentration–response curves were constructed for acetylcholine (ACh) (pEC_{50}: 6.16 ± 0.04; maximum response $80.00 \pm 1.98\%$ of the responses induced by serotonin 10 μM). The absence of endothelium did not modify the contractile responses of ACh in this tissue.

3 The role of cholinesterases was evaluated: neither neostigmine (acetylcholinesterase inhibitor) nor iso-OMPA (butyrylcholinesterase inhibitor) modified ACh responses. When both enzymes were simultaneously inhibited, a significantly but little potentiation was observed (control: pEC_{50} 6.33 ± 0.03; double inhibition: pEC_{50} 6.57 ± 0.05).

4 Atropine, nonselective muscarinic receptors antagonist, inhibited ACh-induced contraction (pK_B 9.67). The muscarinic receptors antagonists pirenzepine (M_1), methoctramine (M_2) and pFHHSiD (M_3) also antagonized responses to ACh. The affinity values estimated for these antagonists against responses evoked by ACh were 7.58, 6.78 and 7.94, respectively. On the other hand, PD 102807 (M_4 selective muscarinic receptors antagonist) was ineffective against ACh-induced contraction.

5 In presence of a blocking concentration of pirenzepine, pFHHSiFD produced an additional antagonism activity on ACh-induced responses.

6 The M_1 muscarinic receptors agonist McN-A-343 produced similar maximum but less potent responses than ACh in HUV. The calculated pA_2 for pirenzepine against McN-A-343 induced responses was 8.54.

7 In conclusion, the data obtained in this study demonstrate the role of M_1 muscarinic receptor subtypes and suggest the involvement of M_3 muscarinic receptor subtypes in ACh-induced vasoconstriction in HUV rings. In addition, the vasomotor activity evoked by ACh does not seem to be modulated by endothelial factors, and their enzymatic degradation appears to have little functional relevance in this tissue.

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChAT, cholineacetyltransferase; HUV, human umbilical vein; 5-HT, serotonin

Introduction

At present, the muscarinic receptors have been classified into five subtypes (M_1–M_5) encoded by five distinct genes identified by molecular cloning methods (Caulfield & Birdsall, 1998). Although the principal effect of acetylcholine (ACh) in most vascular beds is endothelium-dependent vasodilatation; it can also produce direct vascular smooth muscle contraction. Moreover, the endothelium removal may increase the potency of vasoconstrictor responses evoked by ACh (Pesic *et al*., 2002). The muscarinic receptor subtypes involved in blood vessels ACh-induced contraction vary in several vascular tissues, depending upon anatomic location and species (Eglen *et al*., 1996). ACh-induced vasoconstriction is mediated *via* stimulation of M_3 receptor subtypes in human pulmonary artery (Norel *et al*., 1996), perforating branch of human internal mammary artery (Pesic *et al*., 2002), bovine coronary artery (Duckles & Garcia-Villalon, 1990) and pig coronary arteries (Jaiswal *et al*., 1991). The M_1 muscarinic receptor subtypes have been associated with vasoconstriction of cat cerebral artery (Dauphin *et al*., 1991), canine saphenous vein (O'Rourke & Vanhoutte, 1987) and mouse cerebral arterioles (Shimizu *et al*., 1993).

ACh enzymatic degradation occurs through the action of two cholinesterases, butyrylcholinesterase (BChE) and acetylcholinesterase (AChE). BChE is mainly found in plasma and interstice of different tissues (Norel *et al*., 1993), whereas AChE is commonly known to be present in cholinergic neuronal synapses.

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The human umbilical vein (HUV) transports oxygen and nutrients from the placenta to the fetus; therefore, a normal HUV blood flow is crucial for its growth. Human umbilical vessels are devoid of autonomic innervation; hence, its tone regulation is mainly attributed to humoral factors acting locally (Reilly & Russell, 1977; Fox & Khong, 1990). Altura *et al*. (1972) described, in this tissue, the action of many vasoactive substances such as ACh, serotonin (5-HT), bradykinin and histamine, among others. Furthermore, they suggested that ACh produced a vasoconstrictor effect through the stimulation of muscarinic receptors.

The aim of the present study was to characterize pharmacologically the muscarinic receptor subtype(s) mediating contraction of isolated HUV rings.

Methods

HUV preparations

Approximately 15–35 cm segments were excised from human umbilical cords ($n = 306$) midway between the placenta and newborn. These cords were collected from healthy and normotensive patients after full-term vaginal or caesarean deliveries. Written informed consent was obtained from each parturient. Cords were immediately placed in modified Krebs' solution at 4°C of the following mM composition: NaCl 119, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.0, EDTA 0.004, D-glucose 11.

Functional studies

Usually within 3 h after delivery, the veins (internal diameter approximately 5 mm) were carefully dissected from Wharton's jelly using microdissecting instruments and cut into rings of approximately 3 mm width. The preparations were then suspended in 10 ml organ bath and stretched with an initial tension of $3–5 \times g$ as described previously (Errasti *et al*., 1999). Changes in tension were measured with Grass isometric force transducer (FT-03C; Grass Instruments, Quincy, MA, U.S.A.) and displayed on Grass polygraphs (Model 7D). During the equilibration period, Krebs' solution was maintained at 37°C at pH 7.4 by constant bubbling with 95% O_2/5% CO_2. The bath solution was replaced every 15 min with fresh warmed Krebs.

After 70 min of equilibration period, each preparation was contracted with 40 mM KCl and washed. This procedure was designed to test the functional state of the tissue. Optimal passive tension was adjusted throughout the equilibration period. Experiments were performed in parallel in rings from the same tissue.

Concentration–response curves to ACh and McN-A-343 (M_1 muscarinic receptor agonist) were obtained after a 150-min equilibration period by cumulative addition of agonists in 0.25 $\log_{10}$ increments. 5-HT (10 μM) was applied at the end of each experiment in order to determinate the tissue maximum response (Sardi *et al*., 1997). In this study, 98 of 306 (32.02%) HUVs did not show cholinergic response.

In some experiments, the endothelium was mechanically removed by gently rubbing the inner side of HUV rings with a roughened steel rod.

To evaluate the possible effect of cholinesterases on the vasoconstriction evoked by ACh in HUV, some rings were incubated for 30 min before the concentration–response curves to ACh with either Krebs' solution or Krebs' solution containing iso–OMPA (BChE inhibitor) or neostigmine (AChE inhibitor).

Different muscarinic antagonists were applied 60 min before the cumulative addition of agonists. These antagonists bind preferentially to the muscarinic receptors indicated between parentheses: atropine (nonselective), pirenzepine (M_1), methoctramine (M_2), pFHHSiD (M_3) and PD 102807 (M_4). To evaluate possible unspecific or toxic effects of pFHHSiD, concentration–responses curves for the unrelated agonist 5-HT were constructed in presence of that compound (60 min before the cumulative addition of agonist).

Expression of results and statistical analysis

All data are expressed as mean ± s.e.m. The number of experiments (n) is denoted as r/v, where r represents number of rings and v number of veins. Each vein was obtained from a different umbilical cord and typically four or eight rings of each vein were employed. Responses are expressed as g of developed contraction or percentage of maximum response to 10 μM 5-HT at the end of each experiment. The concentration–response curves were fitted to a four-parameter logistic model, where estimates of EC_{50} value, the agonist concentration that produces 50% of the maximum and the slope factor (n_H), were obtained using ALLFIT (DeLean *et al*., 1978). The EC_{50} were transformed into pEC_{50} ($-\log_{10} EC_{50}$). Agonist log concentration ratio (r) was determined by subtracting the pEC_{50} value of the agonist in the presence of the antagonist from the pEC_{50} in the control preparation. When criteria for competitive antagonism were satisfied, that is the antagonist produced a parallel rightward shift of the agonist curve without attenuation in the maximum response, antagonist pA_2 values and slopes of Schild's regressions were calculated as described by Arunlakshana & Schild (1959). In those cases where the slope of the Schild's plot was not significantly different from unity, the regression was recalculated with Schild's slope constrained to unity (Neubig *et al*., 2003) and the affinity value obtained was then referred as pK_B. In the case of pirenzepine (0.17 μM) vs ACh, pirenzepine (0.17 μM) vs McN-A-343 and pFHHSiD (0.03 μM) vs ACh, antagonist affinities were obtained by 'single concentration' analysis (assuming a Schild's regression slope of 1) according to the equation: $pA_2 = -\log$ [Antagonist] + $\log(r-1)$ (Lachnit *et al*., 1997; Rogines-Velo *et al*., 2002a). Statistical analysis was performed by means of paired or unpaired Student's t-test and one-way analysis of variance followed by Tukey's post-test, when appropiate. P-values lower than 0.05 were taken to indicate significant differences.

Terms and equations are as recommended by the IUPHAR Committee on Receptor Nomenclature and Drug Classification (Neubig *et al*., 2003).

Chemicals

The following compounds were used for functional studies: ACh, McN-A-343, neostigmine, tetraisopropylpyrophosphoramide (iso-OMPA), atropine, pirenzepine and methoctramine from Sigma Chemical Company (St Louis, MO, U.S.A.); (±)-*p*-fluoro-hexahydro-sila-difenidol hydrochloride (pFHHSiD) and 5-HT creatine sulphate complex from Research Biochemical Incorporated (Natrick, MA, U.S.A.); PD 102807 from

Tocris (Ellisvilee, U.S.A.) Preparation of all stock solutions (except PD 102807) and their subsequent dilutions were performed in bidistilled water. PD 102807 was dissolved in dimethylsulfoxide (DMSO). The final concentrations of DMSO in the bath solutions were always less than 1%. Control trials were performed in the presence of corresponding concentration of DMSO to rule out any possible nonspecific action of this solvent on tonus or contractility of the preparation. Stock solutions were stored frozen in aliquots, thawed and diluted daily. All concentrations of drugs are expressed as final concentration in the organ bath.

Results

Vasoconstrictor effect of ACh in HUV rings

After a 150-min incubation period, ACh produced a concentration-related contraction of HUV rings. The absence of endothelium did not modify the contractile responses to ACh in HUV rings (intact endothelium: pEC_{50} 6.16 ± 0.04, maximum response $80.00 \pm 1.98\%$, $n = 9/9$; removed endothelium: pEC_{50} 6.27 ± 0.04, maximum response $81.22 \pm 2.13\%$, $n = 10/10$).

Pretreatment with neostigmine (AChE inhibitor, $10\,\mu$M) or iso-OMPA (BChE inhibitor, $100\,\mu$M) did not reveal an additional higher potency or an increase in maximum responses induced by ACh in HUV rings (control: pEC_{50} 6.04 ± 0.10, maximum response $92.00 \pm 5.27\%$, $n = 7/7$; neostigmine $10\,\mu$M: pEC_{50} 6.21 ± 0.09, maximum response $95.34 \pm 4.45\%$, $n = 7/7$; control: pEC_{50} 6.28 ± 0.03, maximum response $85.80 \pm 2.25\%$, $n = 5/5$; iso-OMPA $100\,\mu$M: pEC_{50} 6.25 ± 0.06, maximum response $88.98 \pm 3.13\%$, $n = 5/5$). However, in tissues exposed to both inhibitors simultaneously, the concentration–response curves to ACh yielded a potency approximately two-fold higher when compared to control HUV rings (control: pEC_{50} 6.33 ± 0.03, $n = 4/4$; iso-OMPA $100\,\mu$M + neostigmine $10\,\mu$M: pEC_{50} 6.57 ± 0.05, $P < 0.01$, $n = 4/4$), without modifying maximum responses (control: $84.84 \pm 1.93\%$, $n = 4/4$; iso-OMPA $100\,\mu$M + neostigmine $10\,\mu$M: $91.85 \pm 2.99\%$, $n = 4/4$).

Antagonism of atropine on the vasoconstriction evoked by ACh in HUV rings

The nonselective muscarinic receptors antagonist atropine was used to determinate if the contractile response to ACh could be attributed to muscarinic receptor stimulation. Increasing concentrations of atropine (1, 3 and 10 nM) produced a parallel rightward shift of the ACh concentration–response curves without affecting the maximum response, indicative of competitive antagonism (Figure 1a; Table 1). Analysis of the data by Schild's regression showed a slope (0.94 ± 0.30), which was not significantly different from unity, and a pA_2 value of 9.75, yielding a pK_B value from constrained Schild's plots of 9.67 ± 0.12 ($n = 25/11$; Figure 1b).

Antagonism of pirenzepine, methoctramine or pFHHSiD on the vasoconstriction evoked by ACh in HUV rings

To evaluate the contribution of different subtypes of muscarinic receptors on the contractile response to ACh in HUV, M_1 (pirenzepine), M_2 (methoctramine) and M_3 (pFHHSiD) muscarinic receptors antagonists were employed.

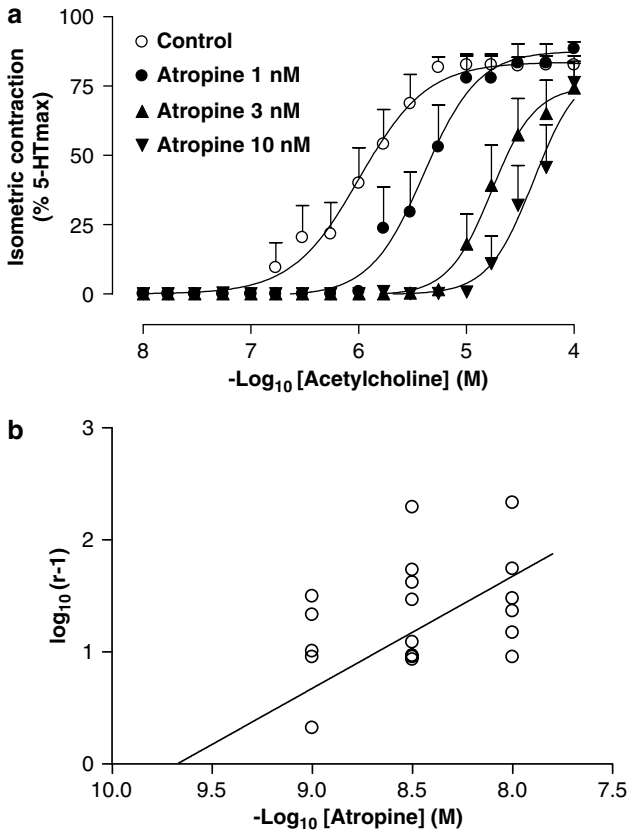

Figure 1 Antagonism of ACh by atropine in HUV. (a) Concentration–response curves to ACh on control HUV rings ($n = 11/11$) and on tissues previously exposed to atropine for 1 h (1 nM, $n = 8/8$; 3 nM, $n = 9/9$; 10 nM, $n = 8/8$). Each symbol represents the mean and vertical lines represent s.e.m. (b) Schild's plot for atropine vs ACh was constructed with concentration ratios from individual experiments.

Pirenzepine (0.17, 0.3 and $0.55\,\mu$M) reduced ACh pEC_{50} without attenuating maximum responses (Figure 2; Table 1). As pirenzepine 0.3 or $0.55\,\mu$M did not produce any further shift of concentration–response curves to ACh, a calculated pA_2 of 7.58 was obtained with the lowest blocking concentration ($0.17\,\mu$M, $n = 9/9$). In the presence of methoctramine (0.3, 3 and $10\,\mu$M), the concentration–response curves to ACh were displaced to the right in a parallel manner. Maximum responses to ACh were not attenuated by methoctramine (Figure 3a; Table 1). Data analyzed by Schild's regression showed a slope (0.98 ± 0.15), which was not significantly different from unity, and a pA_2 value of 6.79 (Figure 3b). When Schild's slope was constrained to unity, a pK_B value of 6.78 ± 0.09 was obtained ($n = 49/35$).

Whereas the exposure to pFHHSiD (0.03 and $0.3\,\mu$M) produced a parallel rightward shift of ACh concentration–response curves, pFHHSiD $1\,\mu$M clearly did not (Figure 4; Table 1). Taking into account that pFHHSiD $1\,\mu$M produced a significant reduction of the maximum response to ACh without affecting the maximum response to 5-HT ($10\,\mu$M) at the end of each experiment (control, 14.09 ± 1.35 g; treated 14.79 ± 2.29 g; $n = 8/8$), a calculated pA_2 of 7.94 was obtained with the lowest blocking concentration ($0.03\,\mu$M, $n = 10/10$).

Table 1 Effect of muscarinic receptor antagonists on the vasoconstriction induced by ACh of isolated HUV rings

	Concentration (μM)	*pEC_{50}*	*Maximum responses (% of 5-HT_{max})*	n_H
Control		5.99±0.06	83.61±2.27	1.38±0.25
Atropine	0.001	5.39±0.05[b]	87.62±2.57	1.72±0.31
	0.003	4.76±0.05[b]	74.97±3.11	2.15±0.53
	0.01	4.38±0.04[b]	81.86±3.28	2.04±0.38
Control		6.14±0.06	77.55±1.73	1.34±0.23
pFHHSiD	0.03	5.66±0.07[a]	76.75±2.67	1.34±0.28
	0.3	5.13±0.09[b]	72.88±3.70	1.17±0.27
	1	4.08±0.03[b]	44.29±2.10[b]	5.26±1.78[b]
Control		5.96±0.07	87.28±3.68	0.90±0.15
Pirenzepine	0.17	4.99±0.06[b]	79.65±5.43	1.63±0.34
	0.3	4.80±0.10[b]	83.49±10.36	1.62±0.10
	0.55	4.73±0.07[b]	83.93±8.06	2.67±0.65
Control		5.69±0.04	84.48±1.50	1.24±0.13
Methoctramine	0.3	5.43±0.05[a]	84.10±2.04	1.32±0.18
	3	4.64±0.07[b]	85.63±3.89	1.09±0.19
	10	3.95±0.11[b]	93.39±9.78	1.06±0.21
Control		6.11±0.07	81.18±3.35	1.86±0.48
PD 102807	0.1	5.96±0.06	83.15±3.68	1.78±0.46
	1	5.95±0.07	75.40±3.23	1.87±0.49

Effect of muscarinic receptor antagonists (treated) or Krebs' solution (control) on ACh-induced vasoconstriction of isolated HUV rings. Values are mean±s.e.m. Each pEC_{50} value for treated tissues was compared with the corresponding control value. The maximum responses obtained with ACh are expressed as percent of the contraction induced by 5-HT (10 μM) at the end of each experiment. [a]$P<0.01$, [b]$P<0.001$.

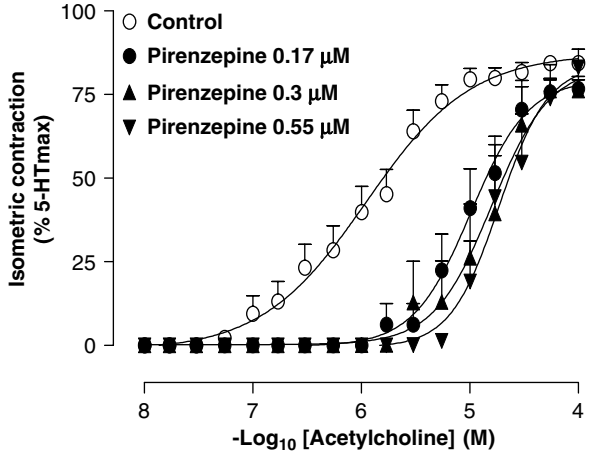

Figure 2 Antagonism of ACh by pirenzepine in HUV. Concentration–response curves to ACh on control HUV rings ($n=29/29$) and on tissues previously exposed to pirenzepine for 1 h (0.17 μM, $n=9/9$; 0.3 μM, $n=6/6$; 0.55 μM, $n=7/7$). Each symbol represents the mean and vertical lines represent s.e.m.

Lack of antagonism of PD 102807 on the vasoconstriction evoked by ACh in HUV rings

Neither pEC_{50} nor maximum responses to ACh were modified by treatment with the selective M_4 muscarinic receptors antagonist PD 102807 (0.1 μM, $n=8/8$; 1 μM, $n=6/6$) (Table 1).

Antagonism of simultaneous exposure to pirenzepine plus pFHHSiD on the vasoconstriction evoked by ACh in HUV rings

Simultaneous exposure to pirenzepine (0.17 μM) and pFHHSiD (0.03 μM) produced a parallel rightward shift of

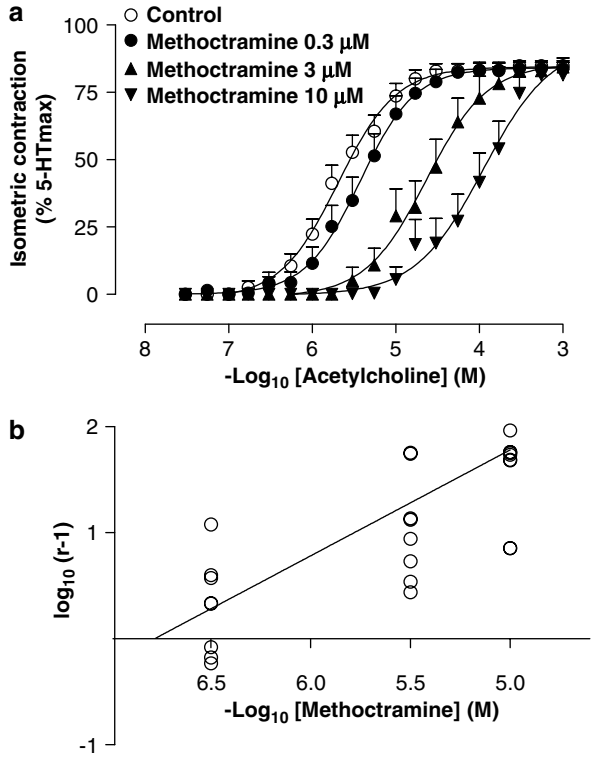

Figure 3 Antagonism of ACh by methoctramine in HUV. (a) Concentration–response curves to ACh on control HUV rings ($n=35/35$) and on tissues previously exposed to methoctramine for 1 h (0.3 μM, $n=22/22$; 3 μM, $n=15/15$; 10 μM, $n=12/12$). Each symbol represents the mean and vertical lines represent s.e.m. (b) Schild's plot for methoctramine vs ACh was constructed with concentration ratios from individual experiments.

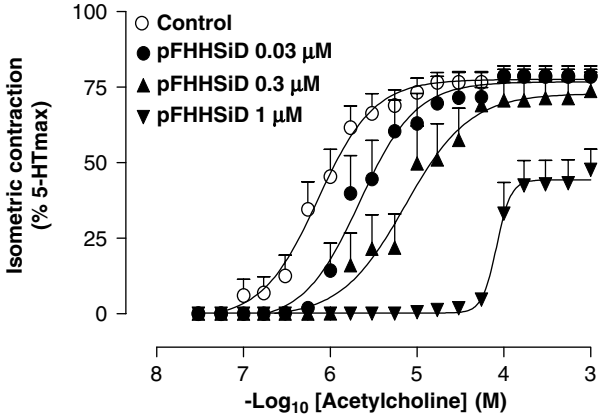

Figure 4 Antagonism of ACh by pFHHSiD in HUV. Concentration–response curves to ACh on control HUV rings ($n=17/17$) and on tissues previously exposed to pFHHSiD for 1 h (0.03 μM, $n=9/9$; 0.3 μM, $n=10/10$; 1 μM, $n=6/6$). Each symbol represents the mean and vertical lines represent s.e.m.

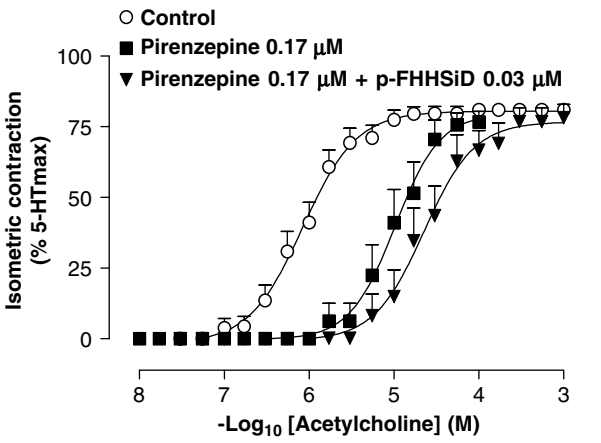

Figure 5 Antagonism of ACh by pirenzepine plus pFHHSiD or pirenzepine alone in HUV. Concentration–response curves to ACh on control HUV rings ($n=21/21$) and on tissues previously exposed to pirenzepine (0.17 μM) plus pFHHSiD (0.03 μM, $n=12/12$) or pirenzepine alone (0.17 μM, $n=9/9$) for 1 h. Each symbol represents the mean and vertical lines represent s.e.m.

the ACh concentration–response curves grater than the blocking effect produced by pirenzepine (0.17 μM) alone (control: pEC_{50} 6.08 $\pm$ 0.05, n_H 1.37 $\pm$ 0.17, $n=21/21$; pirenzepine: pEC_{50} 4.99 $\pm$ 0.06, n_H 1.63 $\pm$ 0.34, $n=9/9$; pirenzepine + pFHHSiD: pEC_{50} 4.65 $\pm$ 0.06, n_H 1.47 $\pm$ 0.28, $n=12/12$; $P<0.05$) (Figure 5). The maximum responses to ACh were not modified (control: 80.49 $\pm$ 1.37%, $n=21/21$; pirenzepine: 79.65 $\pm$ 5.43%, $n=9/9$; pirenzepine + pFHHSiD: 76.76 $\pm$ 3.44%, $n=12/12$) (Figure 5).

Vasoconstrictor effect of McN-A-343 in HUV rings

McN-A-343 as well as ACh produced concentration-related contraction curves of HUV rings. McN-A-343, M_1 muscarinic receptors agonist, was less potent but not less effective than ACh (Figure 6). The maximum response for McN-A-343 was 91.09 $\pm$ 4.84% and for ACh was 84.99 $\pm$ 5.23%. Furthermore,

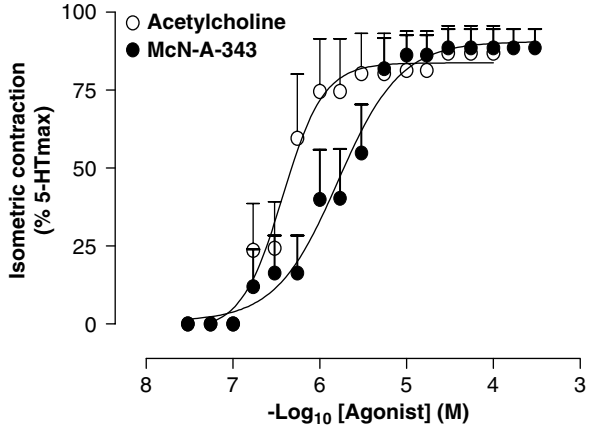

Figure 6 Concentration–response curves to ACh ($n=5/5$) or McN-A-343 ($n=5/5$) on HUV rings. Each symbol represents the mean and vertical lines represent s.e.m.

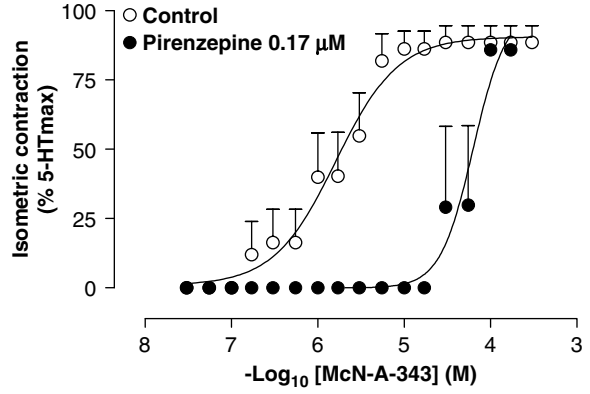

Figure 7 Antagonism of McN-A-343 by pirenzepine in HUV. Concentration–response curves to McN-A-343 on control HUV rings ($n=5/5$) and on tissues previously exposed to pirenzepine for 1 h (0.17 μM, $n=5/5$). Each symbol represents the mean and vertical lines represent s.e.m.

the pEC_{50} of McN-A-343 was 5.91 $\pm$ 0.13, whereas the pEC_{50} for ACh was 6.37 $\pm$ 0.11 ($P<0.05$, $n=5/5$).

Concentration–response curves to McN-A-343 obtained in presence of pirenzepine (0.17 μM) yield a pEC_{50} value of 4.20 $\pm$ 0.12 and a maximum response value of 99.24 $\pm$ 2.73% ($P<0.01$, $n=5/5$). A calculated pA_2 value of 8.54 was obtained (Figure 7).

Discussion

There are many evidences that support a widespread expression of the cholinergic system in non-neuronal human cells, which are able to synthesize, release and metabolize ACh. In the last decades, several works demonstrated the role of non-neuronal ACh as a local cellular signaling molecule regulating multiple cell functions (Wessler *et al.*, 1998). At present, the presence of essential elements of the non-neuronal cholinergic system such as choline acetyltransferase (ChAT), synthesis of ACh and the vesicular ACh transporter in HUV endothelial

cells has been demonstrated (Kirkpatrick *et al*., 2001; 2003). Moreover, the human placenta contains both AChE and ChAT, and the release of ACh into the fetal circulation from perfused single placental cotyledon and whole placenta has been detected (Rama Sastry, 1997). As umbilical and placental vessels lack autonomic innervation, the regulation of its vascular tone depends on vasoactive substances that are locally produced or conveyed through the blood stream (Reilly & Russell, 1977; Fox & Khong, 1990). Taking into consideration the results obtained in present study, non-neuronal ACh from placenta and/or HUV could be involved in fetal circulation regulation, *via* autocrine/paracrine mechanisms, in physiological or patophysiological conditions.

ACh induces relaxation or contraction of blood vessels depending on the stimulation of muscarinic receptors located on endothelium or smooth muscle cells, respectively. In the perforating branch of human internal mammary artery, ACh induces endothelium-dependent relaxation on arterial rings precontracted with phenylephrine (Pesic *et al*., 2001), and produces a concentration-dependent contraction on basal tone only when endothelium was denuded (Pesic *et al*., 2002). In the present study, ACh-induced vasoconstriction of control HUV rings was not influenced by endothelium. Similar results were reported in human umbilical artery (Yoshikawa & Chiba, 1991). Our results are in accordance with previous studies employing a bioassay cascade perfusion technique, which showed that ACh did not release endothelium-derived relaxing factor in HUV (Van de Voorde *et al*., 1987; Chaudhuri *et al*., 1991).

The vasoconstrictor efficacy obtained with ACh in HUV rings was slightly smaller than the maximum responses elicited by 5-HT in this tissue, indicating a full agonism. The pEC_{50} value estimated for ACh in HUV rings was similar to those described for other human tissues (Walch *et al*., 2000; Pesic *et al*., 2002). In HUV, Altura *et al*. (1972) have reported that ACh responses had lower efficacy and potency than those obtained in the present study. However, Altura *et al*. (1972) used HUV segments cut longitudinally and helically into strips, in contrast to the present work where the vessels were cut into rings. Histologically, HUV contains two layers of smooth muscle, one longitudinal and the other circular (Spivack, 1946). Thus, when a functional study is performed using ring preparations, the circular layer is the one evaluated, whereas the longitudinal layer is tested using longitudinal strips segments and both layers using helical strips segments. In previous reports, significant differences in potency or efficacy of several vasoactive agents were observed between rings and strips preparation of HUV (Rogines-Velo *et al*., 2002b; Daray *et al*., 2003). Hence, the anatomical differences described regarding the tissue preparations employed provides an explanation for the pharmacological differences observed between the results published by Altura *et al*. (1972) and our study. Taking into account the functional role of the circular layer of HUV in the *'in vivo'* vasoconstriction, ring vessels preparation could be more reliable for a pharmacological study of human umbilical vessels.

ACh can be hydrolyzed by two different types of cholinesterases namely AChE and BChE. The lack of potentiation of ACh-induced vasoconstriction in HUV when each enzyme was individually inhibited indicates that cholinesterases do not play a primordial role in the biological inactivation of ACh in this tissue. Only when both enzymes were simultaneously inhibited a significantly but little potentiation was observed. Nevertheless, we considered that the concurrent inhibition of ACh enzymatic metabolization was not necessary for a reliable pharmacological characterization of the receptor population involved in ACh-elicited responses in HUV.

Altura *et al*. (1972) described the existence of muscarinic receptors in isolated HUV strips. In the present study, atropine, a nonsubtype selective muscarinic receptors antagonist, competitively inhibited contractile responses induced by ACh in HUV rings, indicating that muscarinic receptors are involved in this effect. Furthermore, the pK_B value for atropine (9.67) was in the same order of magnitude as those estimated in other human smooth muscle with muscarinic receptors: bronchial smooth muscle (pK_B 9.1; Watson *et al*., 1995), detrusor cultured smooth muscle cells (calculated pA_2 9.4; Harriss *et al*., 1995), detrusor smooth muscle (pA_2 9.26; Miyamae *et al*., 2003).

Pirenzepine (selective M_1 muscarinic receptors antagonist) exhibited a calculated pA_2 value (7.58) against ACh-induced responses that was not different from the affinity values expected in presence of an M_1 muscarinic receptors*:* human recombinant M_1 muscarinic receptors (pK_i 8.0; Eglen *et al*., 1996), human vas deferens smooth muscle (pA_2 7.39; Miranda *et al*., 1992), human pulmonary veins (pK_B 7.89; Walch *et al*., 2000) and perforating branch of human internal mammary artery (pA_2 7.74; Pesic *et al*., 2001). Nevertheless, the characterization of M_1 muscarinic receptors on the basis of pirenzepine affinity has been questioned since it was proposed that this antagonist is not able to distinguish between M_1 and M_4 muscarinic receptors (Budriesi *et al*., 2001). In HUV rings, PD 102807 (selective M_4 muscarinic receptors antagonist) employed in concentrations 30–100-fold higher than affinities values previously reported for M_4 muscarinic receptors (Olianas & Onali, 1999; Böhme *et al*., 2002) was ineffective against ACh-induced vasoconstriction, suggesting that the blocking profile of pirenzepine could only be attributed to M_1 muscarinic receptors antagonism. Further, McN-A-343 (M_1 muscarinic receptors agonist) produced a similar maximum although less potent responses than ACh, and pirenzepine antagonized this vasoactive effect with high potency (calculated pA_2 8.54). Taken together, these previous results show strong pharmacological evidence of M_1 muscarinic receptor subtypes promoting vasoconstriction responses in HUV rings. To our knowledge, this is the first study to report contractile responses through the stimulation of M_1 muscarinic receptors in human vascular tissue.

However, the lack of further shift in the ACh concentration–response curves by increasing concentrations of pirenzepine is inconsistent with a simple competitive antagonist at a single receptor site. Further, the difference between the calculated pA_2 values obtained with pirenzepine against ACh (7.58) than against McN-A-343 (8.54) provides additional evidence of the involvement of an heterogeneous muscarinic receptors population in HUV. Taking into account that in human blood vessels ACh-induced vasoconstriction has classically been described to be mediated by M_3 muscarinic receptor subtypes (Walch *et al*., 2001), we considered interesting to examine their possible presence in HUV. The calculated pA_2 value (7.94) obtained with pFHHSiD (usually used as selective M_3 muscarinic receptors antagonist) against ACh-induced contraction was similar to those determined previously for human recombinant M_3 muscarinic receptors (pK_i 7.5; Eglen *et al*., 1996) and in

smooth muscle with functional M_3 muscarinic receptors such as human uterine artery (pK_B 8.17; Jovanovic *et al*., 1994), human detrusor smooth muscle cells (calculated pA_2 7.4; Harriss *et al*., 1995) and perforating branch of human internal mammary artery (pA_2 8.02; Pesic *et al*., 2002). Moreover, the fact that ACh-induced responses in presence of 0.17 μM pirenzepine could be blocked even further by 0.03 μM pFHHSiD, but not by higher concentration of pirenzepine, is in agreement with an heterogeneous population of muscarinic receptors in HUV and support the involvement of M_3 muscarinic receptor subtypes in this tissue.

On the other hand, the finding that the antagonism effect of pFHHSiD on ACh-induced vasoconstriction enhances with increasing concentration, unlike pirenzepine behavior, may be explained by the difficulty of this compound to distinguish between M_1 and M_3 muscarinic receptor subtypes (Eglen *et al*., 1996).

The lack of blocking effect of pFHHSiD (0.03 and 0.3 μM) on the vasoconstriction evoked by 5-HT, an unrelated agonist, provides evidence for the selectivity of this antagonist to muscarinic receptors in this tissue (data not shown).

The affinity value of methoctramine (selective M_2 muscarinic receptors antagonist) against ACh (6.78) was relatively low in contrast to previous studies (human recombinant M_2 muscarinic receptors: pK_i 7.6, Eglen *et al*. (1996); human colon circular muscle: pK_i 8.03, Mansfield *et al*. (2003), human detrusor: pK_i 7.41, Mansfield *et al*. (2005)), suggesting that M_2 muscarinic receptor subtypes were hardly involved in the contraction of these vessels.

In summary, the data obtained in this study demonstrates the role of M_1 muscarinic receptor subtypes and suggest the involvement of M_3 muscarinic receptor subtypes in ACh-induced vasoconstriction in HUV rings. In addition, the vasomotor activity evoked by ACh does not seem to be modulated by endothelial factors, and their enzymatic degradation appears to have little functional relevance in this tissue.

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