Heterogeneity of muscarinic receptors in lamb isolated coronary resistance arteries

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1 *In vitro* experiments in a microvascular myograph were designed to characterize postjunctional muscarinic receptors producing contraction both in the presence and absence of the endothelium in coronary resistance arteries (normalized diameter of 150–450 µm), isolated from the left ventricle of hearts from 3–6 month old lambs. Preferential muscarinic receptor antagonists were used to determine the receptor subtype: pirenzepine (M₁ receptor), AFDX 116 (M₂ receptor), 4-DAMP and pFHHSiD (M₃ receptor).

2 The rank order of potency for muscarinic agonist-induced increases in tension in endothelium-intact preparations was oxotremorine-M = methacholine = acetylcholine (ACh) > carbachol. Removal of the endothelium increased the potency of ACh, but this procedure did not change either the sensitivity or maximal response to carbachol.

3 The contractile response to ACh was reproducible. Incubation with 3×10^{-7} – 3×10^{-6} M pirenzepine induced non-parallel rightward shifts and depressed the maximum of the concentration-response curve to ACh in endothelium-intact arteries. The slope by Schild analysis was 2.9 ± 0.8 ($P < 0.05$, $n = 7$). Atropine, AFDX 116, 4-DAMP and pFHHSiD produced parallel rightward shifts of the curves to ACh and the slopes of the Schild plots were not significantly different from unity. The pK_B values for the antagonists from plots constrained to unity in endothelium-intact segments were: atropine (9.4), 4-DAMP (9.0), pFHHSiD (7.9) and AFDX 116 (6.2).

4 In endothelium-denuded arteries, pirenzepine, AFDX 116 and pFHHSiD caused concentration-dependent, parallel rightward displacements of the concentration-response curves to ACh and the slopes of the Schild plots were not significantly different from unity. The plots constrained to a slope of unity gave the following pK_B values: pFHHSiD (8.7), pirenzepine (7.5) and AFDX 116 (6.2).

5 In the presence of the endothelium, low concentrations of pirenzepine (10^{-9} – 10^{-7} M) produced leftward shifts of the ACh concentration-response curves. This potentiating effect of pirenzepine was reversed by endothelial cell removal. In preparations precontracted with the thromboxane-mimetic, U46619, the putative M₁-selective agonist, McN-A-343, induced a biphasic relaxation with log IC₅₀ of 8.53 ± 0.14 and 5.02 ± 0.08 for the first and second phase of the relaxation, respectively, and maximal relaxations of $22.8 \pm 4.3\%$ and $41.1 \pm 5.4\%$ ($n = 16$). McN-A-343 relaxed the vessels in the presence of 10^{-7} M pFHHSiD and 3×10^{-7} M AFDX 116, but not after incubation with 10^{-9} M pirenzepine.

6 It is concluded from the pK_B values for the antagonists used, that contraction induced by ACh in lamb coronary resistance arteries, in either the presence or the absence of the endothelium, is mediated via the M₃ subtype of muscarinic receptors, while muscarinic receptors of another subtype at the endothelium seem to modulate the contractile response to ACh.

Keywords: Coronary resistance arteries (lamb); acetylcholine; muscarinic receptors

Introduction

The cholinergic innervation is thought to play an important role in the distribution of myocardial blood flow, even though large interspecies differences have been reported (Kalsner, 1985). Vagal stimulation produced coronary vasoconstriction in the rat heart (Van Charldorp *et al.*, 1987; Bogner *et al.*, 1990), and periarterial electrical stimulation of cattle isolated perfused heart (Kalsner, 1979) or human myocardial slabs *in vitro* induced an atropine-sensitive vasoconstriction (Kalsner, 1989). In contrast, efferent stimulation of the vagal nerves or activation of reflex mechanisms in the dog produced coronary vasodilatation (Feigl, 1969; Hackett *et al.*, 1972; Ito *et al.*, 1985; Van Winkle & Feigl, 1989).

Infusion of acetylcholine (ACh) *in vivo* in the coronary circulation revealed that ACh has a dual action, causing vasodilatation at low and vasoconstriction at high doses in the baboon (Van Winkle & Feigl, 1989; Knight *et al.*, 1991), cattle and man (Hodgson & Marshall, 1989; Knight *et al.*,

1991), vasodilatation in dogs (Van Winkle & Feigl, 1989; Knight *et al.*, 1991), and only vasoconstriction in the porcine coronary circulation (Kawamura *et al.*, 1989; Cowan & McKenzie, 1990).

In vitro studies of isolated coronary resistance arteries, which excluded the influence of myocardial oxygen consumption and metabolic regulation of coronary blood flow, show the same species differences to cholinergic stimulation as *in vivo* studies. ACh relaxes precontracted rat, rabbit and dog coronary resistance arteries (Nyborg, 1990; Angus *et al.*, 1991; Simonsen *et al.*, 1992), while it induces atropine-sensitive contraction of small porcine arteries *in vitro* (Nakayama *et al.*, 1988; Angus *et al.*, 1991; Tschudi *et al.*, 1991). In human small coronary arteries isolated from the atrial appendage, ACh induces contraction, even in arteries with a functional endothelium, assessed by a relaxation to substance P (Angus *et al.*, 1991).

Although it has been recently demonstrated that muscarinic receptor subtypes mediate myocardial blood flow redistribution (Pelc *et al.*, 1988), there are only a few studies concerned with muscarinic receptor characterization, and

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most of these have been carried out in large coronary arteries. Genomic cloning studies have identified 5 distinct human gene products for muscarinic receptors, named from m_1 - m_5 (Buckley *et al.*, 1989). In functional pharmacological studies, the muscarinic receptors have been classified into three subtypes M_1 , M_2 and M_3 , which can be differentiated on the basis of their affinity for different antagonists (Eglen & Whiting, 1990; Hulme *et al.*, 1990). In large epicardial bovine and pig coronary arterial segments, the contractile response to cholinergic stimulation was found to be mediated through M_3 -receptors located on the smooth muscle (Duckles, 1988; 1990; Van Charldorp & Van Zwieten, 1989; Duckles & Garcia-Villalon, 1990; Entzeroth *et al.*, 1990; Brunner *et al.*, 1991b), and the endothelium-dependent relaxation of bovine coronary arteries was shown also to be mediated through an M_3 -receptor (Brunner *et al.*, 1991a).

The aim of the present study was to characterize, by the use of preferential receptor antagonists, the receptor subtype mediating the contractile response to ACh in coronary resistance arteries in a species, the lamb, where this contractile response predominates.

Methods

Dissection and mounting

Hearts from 3–6 month old lambs were obtained at the local slaughterhouse. The hearts were excised immediately after the lambs were killed, and placed in ice cold physiological salt solution (PSS) to reduce cardiac metabolism. Throughout the subsequent dissection, the hearts were bathed in cold PSS (4°C) of the following composition (mmol l⁻¹): NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.17, CaCl₂ 1.5, ethylenediaminetetraacetic acid (EDTA) 0.026 and glucose 5.5. The solution was gassed with 5% CO₂ in O₂ to maintain pH at 7.4.

Third to fourth order small subepicardial arteries of the left anterior descending coronary artery (LAD), located close to the apex of the left ventricle were dissected as previously described for rat and rabbit distal coronary arteries (Prieto *et al.*, 1991; Simonsen *et al.*, 1992). Segments (ca. 2 mm long) of the small vessels were subsequently mounted as ring preparations on two 40 µm wires on an isometric double myograph by fixing one of the wires to a force transducer and the second wire to a length displacement device (Mulvany & Nyborg, 1980). The vessels were allowed to equilibrate in PSS, 37°C, pH 7.4 for about 30 min. The relation between resting wall tension and internal circumference was determined, and from this the internal circumference L_{100} corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ* was calculated (Mulvany & Halpern, 1977). The vessels were set to the internal circumference L_1 , given by $L_1 = 0.9 \times L_{100}$. Preliminary experiments showed that the force development is close to maximal at this internal circumference. The effective internal lumen diameter was determined as $l_1 = L_1/\pi$.

Experimental procedure

The following procedure was performed for each small coronary artery to ensure functional integrity of the vessel segments: (1) After normalization, the contractile ability of the vessels was tested by stimulating the arterial rings with K-PSS (equivalent to PSS but NaCl exchanged with KCl on an equimolar basis giving a final concentration of 123.7 mM K⁺) until reproducible responses were recorded. (2) The presence of spontaneous tonus was measured as the difference between the baseline just after normalization compared to the baseline obtained after exchange of PSS for Ca²⁺-free PSS (similar to PSS except that CaCl₂ was omitted and replaced with 10⁻⁴ M EGTA) or application of 10⁻⁴ M papaverine as the last step of the protocol. (3) Intact

endothelium was evaluated by inducing a stable contraction either with 3×10^{-7} M ACh or $1-3 \times 10^{-7}$ M U46619 and then adding either $3-5 \times 10^{-6}$ M calcium-ionophore, A23187, or 3×10^{-8} M substance P. Relaxation greater than 50% was taken as evidence for endothelial integrity and segments with a relaxation of less than 50% were discarded.

All the responses to ACh were evoked in normal PSS, and control experiments (see Results) showed no tachyphylaxis of the preparation to this drug. Preliminary experiments were performed to test whether the nicotonic antagonist, hexamethonium, the inhibitor of acetylcholinesterase, neostigmine, the α -adrenoceptor antagonist, phentolamine, or the β -adrenoceptor antagonist, propranolol, altered the response to ACh in the coronary resistance arteries.

Cumulative concentration-response curves to various cholinergic agonists were obtained by increasing the organ bath concentration in half-log increments. To obtain comparable parameters among vessel segments the maximum response of the cholinergic agonists was expressed relative to the maximal contractile capacity, E_{max} , for each vessel. E_{max} was determined by measuring the increase in wall tension when the vessel is maximally contracted with K-PSS to which 10^{-5} M ACh was added. This procedure was performed either at the beginning or end of the experiment.

In another set of experiments, a possible relaxant effect of ACh was examined by inducing a tone with $1-3 \times 10^{-7}$ M U46619 and after a plateau was reached, a concentration-response curve to ACh (10^{-9} – 10^{-7} M) was constructed in vessels in which endothelial cell integrity was confirmed by relaxation to $1-5 \times 10^{-6}$ M of the calcium ionophore, A23187.

In experiments examining the effects of the muscarinic antagonist, atropine (10^{-10} – 10^{-8} M), pirenzepine (3×10^{-7} – 3×10^{-6} M), AFDX 116 (3×10^{-7} – 3×10^{-6} M), 4-DAMP (3×10^{-9} – 3×10^{-8} M), and pFHHSiD (3×10^{-8} – 3×10^{-7} M), a first, cumulative concentration-response curve to ACh in the absence of antagonist was obtained. This served as a control curve for the following curves. After washing, the vessel was incubated with the antagonist for 30 min and a new concentration-response curve to ACh was constructed in the presence of the antagonist. The ACh concentration-response curves were repeated in the presence of three different concentrations of antagonist for each preparation, since previous reproducibility studies revealed no significant differences among four consecutive concentration-response curves to ACh.

The role of the vascular endothelium on the effect of both muscarinic agonists (ACh and carbachol) and antagonists (pirenzepine, AFDX 116 and pFHHSiD) was studied in parallel experiments where the endothelium was mechanically removed with the arterial segment *in situ* in the myocardium, by passing a human hair through the vessel lumen and then rubbing it back and forth across the lumen several times. Thereafter, mounting, normalization and agonist and antagonist studies were performed as described above for endothelium-intact segments. Lack of functional endothelium was confirmed by the abolition of relaxation to 3×10^{-8} M substance P, and less than 10% relaxation to $3-5 \times 10^{-6}$ M A23187 in precontracted vessels.

In addition, experiments were performed to clarify whether inhibitory muscarinic receptors were located on the endothelium. In a first set of experiments with endothelium-intact segments, two cumulative concentration-response curves to ACh were obtained; the first in the absence and the second in the presence of a low concentration (10^{-9} – 10^{-7} M) of pirenzepine. In a second set of experiments, a cumulative concentration-response curve to ACh was constructed in vessels with intact endothelium, the functional presence of which was checked by precontracting with 3×10^{-7} M ACh and relaxing with $1-5 \times 10^{-6}$ M A23187. The endothelium was mechanically removed as described above, the vessels were allowed to equilibrate in PSS for 20 min and were then stimulated twice with K-PSS. After washout, two consecutive

concentration-response curves for ACh were constructed, one in the absence and one in the presence of a low single dose of pirenzepine. This protocol was carried out for 10^{-9} and 10^{-7} M pirenzepine.

Furthermore, a possible relaxant effect for the M_1 -selective muscarinic agonist McN-A-343 was examined by inducing a tone with $1-3 \times 10^{-7}$ M U46619, and once a plateau was reached a concentration-response curve to McN-A-343 (10^{-9} – 3×10^{-4} M) was constructed in vessels with intact endothelium. The specificity of this relaxant effect was checked by incubation of the preparation for 30 min with a single dose of either pirenzepine (10^{-9} M), AFDX 116 (3×10^{-7} M) or pFHHSiD (10^{-7} M) before a new concentration-response curve was repeated.

Analysis of results

The mechanical responses of the vessels were measured as force and expressed as active wall tension, δT , which is the increase in measured force, δF , divided by twice the segment length (Mulvany & Halpern, 1977). Using a computer programme (GraphPad, Institute for Scientific Information, San Diego, California, U.S.A.), the concentration-response curves to the cholinergic agonists were fitted to the classical 'Hill-equation': $R/R_{\max} = A(M)^n / (A(M)^n + EC_{50}(M)^n)$, where $R/R_{\max}$ is the relative response to the effective concentration of drug, $A(M)$, and $EC_{50}(M)$ is the concentration of agonist required to give half maximal vessel response ($R_{\max}$), when $A(M)$ and $EC_{50}(M)$ are given in molar concentrations. n is a curve fitting parameter or 'Hill-coefficient'. The same computer programme was used for the calculation of IC_{50} values for ACh and McN-A-343 in precontracted vessels. EC_{50} values are expressed as the negative log molar concentration, $pD_2 = -\log(EC_{50})$. The relative potency, R_p , of the cholinergic agonists was calculated as the ratio of equi-active concentrations taking the response to ACh in endothelium-intact preparations as reference.

For the assessment of the effect of the cholinergic antagonists at least three different concentrations of the antagonist were examined. Since four consecutive concentration-response curves to ACh could be performed in one vessel (see Results), a maximum of three concentrations of the antagonist were studied per coronary ring and the results compared to those obtained in a first concentration-response curve to ACh performed in the absence of antagonist. The pA_2 values of the competitive antagonists, defined as the negative logarithm of the molar antagonist concentration, in the presence of which twice the original agonist concentration is needed for the original effect, was determined by plotting $\log(CR-1)$ against $\log$ antagonist concentration, where CR is the concentration-ratio of the agonist in the absence and presence of antagonist (Arunlakshana & Schild, 1959). Concentration-ratios (CR) were calculated at the EC_{50} level. The slope and the pA_2 value were determined by least square fitting of a regression line to the points. Antagonism was considered to be competitive if there was not more than a 10% reduction in the maximum response and if the 95% confidence limits for the slope of the Schild plot overlapped unity. Providing that the regression of the Schild plot is linear and the slope is not significantly different from unity, the antagonist is in equilibrium with the receptor and the pA_2 -value, which is the intercept along the abscissa scale of the Schild plot is equal to the negative logarithm of the equilibrium dissociation constant for the antagonist: $pA_2 = -\log K_B = pK_B$, and in this case the points were constrained to a slope of unity and a more precise value of the pK_B was calculated (Tallarida & Muray, 1984).

Drugs

Acetylcholine HCl (Merck); AFDX 116 (11-(2-((diethylamino)methyl)-1-piperidinylacetyl)-5,11-dihydro-6H-pyrido-(2,3-b)-(1,4)-benzodiazepin-6-one) (Dr Karl Thomae GmbH);

atropine sulphate (Merck); carbachol (carbamoylcholine HCl, Sigma); methacholine (acetyl- β -methylcholine, Sigma); 4-DAMP (4-diphenyl-acetoxy-*N*-methyl piperidine methiodide) (courtesy of Dr R.B. Barlow, Bristol, U.K.); hexamethonium bromide (Serva); McN-A-343 ((4-hydroxy-2-butynyl)-1-trimethylammonium *m*-chlorocarbimilate chloride, Sigma); oxotremorine-M (ICN Biochemicals), pFHHSiD (*para*-fluoro-hexahydro-sila-diphenidol, Research Biochemicals Incorporated, UK); pirenzepine HCl (Dr Karl Thomae GmbH); calcium-ionophore (A23187), phentolamine HCl, ($\pm$)-propranolol HCl and substance P (Sigma). Most drugs were prepared daily in distilled water except for the calcium-ionophore, A23187, where a 10^{-3} M stock solution was prepared in dimethylsulphoxide and further diluted in PSS. Previous experiments showed that the solvents had no effect on the preparations at the concentrations applied. Concentrations of drugs were expressed as the final organ chamber concentrations (M).

Statistical analysis

The results are expressed as means $\pm$ s.e.mean. Significant differences between means were assessed by Student's two-tailed *t* test. pD_2 values of the agonists were analysed according to one-way analysis of variance and Bonferroni method as an *a posteriori* test (Wallenstein *et al.*, 1980). Probability levels under 5% were considered significant.

Results

The endothelium-intact lamb coronary resistance arteries investigated in the present study had an internal effective lumen diameter, $l_1 = 314 \pm 10 \mu\text{m}$ (172 vessels from 86 animals). Initially, successive stimulations with K-PSS resulted in increasing contractions, but the responses to the third and fourth stimulation with K-PSS were not significantly different, being $0.82 \pm 0.19 \text{ Nm}^{-1}$ and $0.86 \pm 0.22 \text{ Nm}^{-1}$ ($n = 6$), respectively. Therefore, the contractile ability of the vessels was tested by three stimulations with K-PSS at the beginning of each experiment. All the lamb

Figure 1 Isometric force recordings showing concentration-dependent contractions to acetylcholine (ACh) in a lamb isolated coronary resistance artery with an effective lumen diameter, $l_1 = 248 \mu\text{m}$. (a–c) Endothelium-intact segment stimulated with 125 mM potassium (K-PSS) (a), response to increasing cumulative concentrations of ACh (b), and relaxation to 10^{-7} M substance P (SP), and $1-5 \times 10^{-6}$ M calcium-ionophore A23187 (c). (d–f) The same vessel after removal of the endothelium with a $40 \mu\text{m}$ thick wire (see Methods). (d) The response to K-PSS of the artery is apparently diminished if the increase in basal myogenic tone induced by endothelial denuding is not considered (see Results). (e) The vessel responds to lower concentrations of ACh, and (f) the relaxations to substance P and A23187 are abolished. Numbers show the point at which each drug addition was made and the corresponding cumulative concentration of the drug in the tissue bath, expressed as the log molar concentration. W, wash.

Figure 2 Concentration-response curves to cholinergic agonists inducing contraction in lamb coronary resistance arteries. (a) Concentration-response curves in endothelium-intact segments to acetylcholine (O), methacholine (□), carbachol (Δ), McN-A-343 (◇), and oxotremorine M (×) are shown. (b) Concentration-response curves to acetylcholine (●) and carbachol (▲) in endothelium-denuded preparations. The contractile effect is expressed as a percentage of the response evoked by stimulation with 10^{-5} M ACh in K-PSS ($E_{\max}$). Values are means $\pm$ s.e.mean ($n = 6-14$ preparations).

coronary resistance arteries considered as endothelium-intact and preconstricted with 10^{-6} M ACh relaxed transiently to 3×10^{-8} – 10^{-7} M of substance P ($n = 8$, Figure 1), while $3-5 \times 10^{-6}$ M of the calcium-ionophore, A23187, produced a sustained relaxation with a maximum of $79.80 \pm 2.08\%$ ($n = 172$) of the preconstriction (Figure 1). Spontaneous con-

traction measured as the relaxation over the base-line tension either after addition of 10^{-4} M papaverine or by exchange of the bath solution, PSS, for calcium-free PSS was $0.21 \pm 0.02 \text{ Nm}^{-1}$ in the endothelium-intact coronary resistance arteries. The maximal contractile capacity, $E_{\max}$ (see Methods), of the endothelium-intact vessels was $2.67 \pm 0.16 \text{ Nm}^{-1}$ ($n = 54$).

In 31 lamb coronary resistance arteries with an internal diameter $l_i = 321 \pm 17 \mu\text{m}$, the endothelium was mechanically removed, evaluated as an abolished relaxation to substance P ($n = 24$) and a maximum relaxation of less than 10% to A23187 ($n = 31$). This procedure did not alter the response to the third stimulation with K-PSS, being $1.67 \pm 0.16 \text{ Nm}^{-1}$ ($n = 31$) compared to endothelium-intact preparations: $1.51 \pm 0.08 \text{ Nm}^{-1}$ ($n = 172$). The spontaneous myogenic tone measured by the application of 10^{-4} M papaverine was $0.35 \pm 0.07 \text{ Nm}^{-1}$ ($n = 18$, $P < 0.05$ compared to endothelium-intact segments, Student's *t* test). $E_{\max}$ of the endothelium-denuded arteries was $2.44 \pm 0.37 \text{ Nm}^{-1}$ ($n = 14$).

The response of coronary resistance arteries to ACh

ACh (10^{-8} – 10^{-4} M) induced a concentration-dependent contraction in endothelium-intact lamb coronary resistance arteries (Figure 1) with a maximum response which corresponded to the $E_{\max}$ of the vessel (Figure 2). In a set of experiments where the preparations were preconstricted with $1-3 \times 10^{-7}$ M U46619, ACh induced relaxation only in 3 out of 18 lamb coronary resistance arteries examined, with a half maximal inhibition $\log \text{IC}_{50} = 8.43 \pm 0.09$ ($n = 3$) and a maximal relaxation of $49 \pm 17\%$ of the preconstriction induced with U46619, and constriction in the other 15 vessels. All the 18 vessel segments relaxed to $1-5 \times 10^{-6}$ M A23187, with a maximum relaxation of $73 \pm 9\%$ ($n = 18$). There was no correlation between normalized internal diameter (150–800 μm), the A23187-induced relaxation and the effect of ACh in the preconstricted resistance arteries.

Agonist study

The order for the relative potency of the muscarinic agonist-induced increases in tension was oxotremorine-M = methacholine = acetylcholine > carbachol, while, apart from oxotremorine-M, there were no significant differences in the maximal responses to these agonists in endothelium-intact preparations (Table 1). The muscarinic agonist McN-A-343 alone did not evoke any significant response (Figure 2), but in the presence of 10 mM K^+ in PSS it did evoke a contraction that corresponded to $23.3 \pm 5.6\%$ of the $E_{\max}$ (Figure 3a).

Table 1 Contractile responses to acetylcholine compared to other cholinergic agonists in endothelium-intact (+E) and endothelium-denuded (–E) lamb coronary resistance arteries

	n	pD_2	T (Nm^{-1})	Slope	Rp	l_i (μm)
+ E						
Acetylcholine	11	6.31 ± 0.11	2.14 ± 0.24	1.42 ± 0.09	1.0	276 ± 25
Methacholine	7	6.49 ± 0.19	2.33 ± 0.30	0.78 ± 0.10	1.0	281 ± 30
Carbachol	12	$5.75 \pm 0.20^{a,b}$	1.70 ± 0.39	1.16 ± 0.15	6.7	323 ± 19
McN-A-343 ⁺	9	$4.93 \pm 0.09^{a,b,c}$	$0.59 \pm 0.17^{a,b,c}$	1.70 ± 0.27	248	381 ± 32
Oxotremorine M	6	$6.33 \pm 0.11^{c,d}$	$3.04 \pm 0.34^{a,c,d}$	1.34 ± 0.11	0.6	276 ± 23
– E						
Acetylcholine	14	$7.03 \pm 0.07^*$	2.61 ± 0.37	1.1 ± 0.06	0.2	296 ± 27
Carbachol	8	5.78 ± 0.33	1.42 ± 0.19	2.3 ± 0.4	4.9	316 ± 27

Values are mean $\pm$ s.e.mean. *n*, number of vessels from at least 3–4 animals. T is the maximal response obtained with the respective substance. The slope as determined from the Hill equation. l_i , normalized internal diameter of the coronary arterial segments at which the experiments were performed (see Methods). The relative potency, Rp is the ratio of equiactive concentrations, using the response to acetylcholine in endothelium-intact arteries as reference. ⁺The concentration-response curves to McN-A-343 were performed in presence of 10 mM K^+ in PSS.

Significantly different parameter, tested by the Bonferroni Method: ^a $P < 0.05$, versus acetylcholine; ^b $P < 0.05$ versus methacholine; ^c $P < 0.05$ versus carbachol; ^d $P < 0.05$ versus McN-A-343.

*Significantly different parameter compared to endothelium-intact preparations ($P < 0.05$, Student's *t* test).

Figure 3 Cumulative concentration-response curves to the M_1 -selective cholinergic agonist, McN-A-343, in endothelium-intact preparations. (a) McN-A-343 added alone does not produce significant contraction (○), but in presence of 10 mM K^+ in PSS it induces a contractile response (●). (b) The contractile response to McN-A-343 in 10 mM K^+ in PSS (●) is blocked by 10^{-7} M pFHHSiD, an M_3 -selective antagonist (■). The contractile effect is expressed as a percentage of the response evoked by stimulation with 10^{-5} M ACh in K-PSS (E_{max}). Each point is the mean $\pm$ s.e. mean of 5–14 vessels.

Mechanical removal of the endothelium significantly shifted to the left the concentration-response curves to ACh, while the maximum response to ACh was unaltered expressed relative to E_{max} . The endothelium did not change significantly either the sensitivity or the maximal contraction

to carbachol in lamb coronary resistance arteries, although the relative potency for this agonist was increased (Table 1).

The generation of four consecutive concentration-response curves at 40 min intervals did not reveal tachyphylaxis to ACh. The pD_2 values for the first, second, third and fourth concentration-response curves were: 6.56 ± 0.14 , 6.68 ± 0.20 , 6.81 ± 0.25 , and 6.56 ± 0.23 , respectively, and the maximal responses were: 2.31 ± 0.38 , 2.33 ± 0.37 , 2.40 ± 0.39 , 2.29 ± 0.40 Nm^{-1} , respectively ($n = 7$). Prior incubation with either the nicotinic antagonist, hexamethonium (10^{-4} M), the α -adrenoceptor antagonist, phentolamine (10^{-6} M), the β -adrenoceptor antagonist, propranolol (10^{-5} M), or the inhibitor of acetylcholinesterase, neostigmine (10^{-7} M) did not alter the contractile concentration-dependent responses to ACh. However, the muscarinic antagonist, atropine (10^{-10} – 10^{-8} M) competitively antagonized the response to ACh. Schild analysis for atropine against ACh gave a straight line with a slope, which was not significantly different from unity (Table 2), and the constrained plot revealed an intercept with the abscissa, $pK_B = 9.46 \pm 0.09$ ($n = 3$).

Antagonism of the contractile response to ACh with selective muscarinic antagonists

In endothelium-intact preparations, the M_1 -selective antagonist, pirenzepine (3×10^{-7} – 3×10^{-6} M) inhibited, in a non-competitive manner, the concentration-response curves to ACh in lamb coronary resistance arteries, producing non-parallel shifts of the curves and depressing the maximal response to ACh (Figure 4a). The Hill slope for the ACh concentration-response curve in absence of pirenzepine was 1.20 ± 0.06 ($n = 8$), and in presence of 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M pirenzepine the slopes were 0.80 ± 0.13 ($P < 0.05$, paired t test), 0.78 ± 0.14 ($P < 0.05$) and 0.65 ± 0.14 ($P < 0.05$), respectively. Schild analysis gave a straight line with a slope which significantly differed from unity: 2.87 ± 0.78 ($P < 0.05$, $n = 8$). Non-competitive antagonism evoked by pirenzepine was not agonist-dependent, since 3×10^{-7} M– 3×10^{-6} M pirenzepine also displaced the concentration-response curve for methacholine in endothelium-intact segments in a non-competitive manner, giving a straight line with a slope significantly different from unity: 2.33 ± 0.10 ($P < 0.05$, $n = 6$). The regression line intersected the abscissae at 6.41 ± 0.05 . However, after mechanical removal of the endothelium determined by a lack of relaxation to A23187, pirenzepine produced parallel rightwards shifts of the ACh concentration-response curves and Schild analysis gave a slope not significantly different from unity (Table 2, Figure 4b,c). The line of the plot intersected the abscissa at $pA_2 = 7.49 \pm 0.15$ ($n = 13$).

Table 2 Potencies of various antagonists in endothelium-intact (+E) and endothelium-denuded (–E) lamb coronary resistance arteries isolated from the left ventricle, determined by their ability to inhibit the acetylcholine-induced contraction of vascular segments

	n	pA_2	pK_B	Slope	l_1 (μm)
+ E					
Atropine	3	9.43 ± 0.17	9.46 ± 0.09	1.06 ± 0.18	212 ± 40
Pirenzepine	8	$6.58 \pm 0.04^*$	—	$2.9 \pm 0.8^*$	320 ± 28
AFDX 116	6	6.04 ± 0.25	6.15 ± 0.10	1.12 ± 0.25	273 ± 40
4-DAMP	6	9.11 ± 0.15	9.01 ± 0.08	0.91 ± 0.18	295 ± 25
pFHHSiD	7	7.56 ± 0.08	7.85 ± 0.05	1.33 ± 0.19	382 ± 24
– E					
Pirenzepine	13	7.49 ± 0.15	7.51 ± 0.06	1.02 ± 0.12	361 ± 22
AFDX 116	6	6.14 ± 0.16	6.18 ± 0.05	1.03 ± 0.14	291 ± 34
pFHHSiD	6	8.24 ± 0.19	8.65 ± 0.08	1.33 ± 0.20	300 ± 50

The values are expressed as means $\pm$ s.e. mean. n , number of vessels. pA_2 , intercept at the abscissae of the regression line in Schild plots. pK_B , the negative logarithm of the equilibrium dissociation constant of antagonist-receptor complex, calculated from a plot constrained to a slope of unity.

*Apparent pA_2 value (not real) due to the non competitive nature of the antagonism calculated for comparative interest. l_1 , normalized internal diameter of the coronary arterial segments at which the experiments were performed (see Methods).

*Slopes of the Schild plots significantly different from unity ($P < 0.05$).

Figure 4 Cumulative concentration-response curves to acetylcholine (ACh) in lamb coronary arteries: (a) with (+E, open symbols) and (b) without (-E, closed symbols) endothelium in absence (O, ●) and presence of increasing concentrations of the M_1 -selective muscarinic antagonist, pirenzepine: 10^{-7} M (×), 3×10^{-7} M (□, ■), 10^{-6} M (Δ, ▲), and 3×10^{-6} M (◇, ◆). (c) The Schild regressions for the effect of pirenzepine on concentration-response curves to ACh in endothelium-intact (O) and endothelium-denuded (●) lamb coronary arteries.

Figure 5 Cumulative concentration-response curves to acetylcholine (ACh) in lamb coronary arteries: (a) with (+E, open symbols) and (b) without (-E, closed symbols) endothelium in absence (O, ●) and presence of increasing concentrations of the M_3 -selective muscarinic antagonist, pFHHSiD: 3×10^{-8} M (□, ■), 10^{-7} M (Δ, ▲), 3×10^{-7} M (◇, ◆), and 10^{-6} M (×). (c) The Schild regressions for the effect of pFHHSiD on concentration-response curves to ACh in endothelium-intact (O) and endothelium-denuded (●) lamb coronary arteries. The pA_2 values in endothelium-intact and denuded vessels are 7.56 ± 0.08 ($n = 6$) and 8.24 ± 0.19 ($n = 6$), respectively.

Table 3 Low concentrations of pirenzepine (10^{-9} – 10^{-7} M) caused leftward shifts in acetylcholine concentration-response curves in endothelium-intact (+E), but not in endothelium-denuded (-E) lamb coronary resistance arteries

	+E			-E		
	Absent	pD_2 with antagonist Present	log shift	Absent	pD_2 with antagonist Present	log shift
10^{-9} M	$6.29 \pm 0.09(6)$	$7.30 \pm 0.25(6)^*$	$1.01 \pm 0.21(6)$	$6.58 \pm 0.16(4)$	$6.37 \pm 0.13(4)$	$-0.21 \pm 0.08(4)$
10^{-8} M	$6.16 \pm 0.20(6)$	$6.69 \pm 0.20(6)^*$	$0.53 \pm 0.11(6)$	—	—	—
10^{-7} M	$6.22 \pm 0.15(6)$	$8.18 \pm 0.60(6)^*$	$1.96 \pm 0.52(6)$	$6.93 \pm 0.19(5)$	$6.30 \pm 0.12(5)^*$	$-0.63 \pm 0.22(5)$

Values are mean $\pm$ s.e.mean. (n), number of vessels examined.

*Statistically significant shift compared to control of the concentration-response curve to acetylcholine caused by pirenzepine ($P < 0.05$, paired t test).

In endothelium-intact coronary resistance arteries, the M_2 -selective antagonist, AFDX 116, and the M_3 -selective antagonists, 4-DAMP and pFHHSiD competitively antagonized the response to ACh producing a parallel shift to the right of the ACh concentration-response curve (Figure 5, Table 2). Schild plot of the three antagonists to the ACh concentration-response curves gave straight lines with slopes not different from unity, and the plots were constrained to unity giving pK_B -values for AFDX 116 6.15 ± 0.10 , 4-DAMP 9.01 ± 0.08 and pFHHSiD 7.85 ± 0.05 (Table 2). pFHHSiD (10^{-7} M) also displaced the contractile response evoked by McN-A-343 in the presence of 10 mM K^+ in PSS (Figure 3b).

Endothelial cell removal did not alter the parallel rightward displacements induced by AFDX 116 on the concentration-response curves for ACh (Table 2), but shifted to the left the Schild plot for the M_3 -selective antagonist, pFHHSiD (Figure 5c).

Endothelium-dependent modulation of the contractile response to ACh

In endothelium-intact preparations incubation with low concentrations of pirenzepine (10^{-9} – 10^{-7} M) induced leftward shifts of the contractile concentration-response curves compared to a first ACh concentration-response curve in the absence of antagonist (Table 3).

In a second set of experiments to clarify whether inhibitory muscarinic receptors are present on the endothelium (see Methods), mechanical endothelial cell removal caused a leftwards shift ($\delta pD_2 = 0.57 \pm 0.12$, $n = 9$, $P < 0.05$, paired t test) of the concentration-response curve to ACh. Incubation with 10^{-9} M or 10^{-7} M pirenzepine did not further potentiate the response to ACh of endothelium-denuded segments (Table 3).

In lamb coronary resistance arteries precontracted with 3×10^{-7} M U46619, the putative M_1 -selective agonist, McN-A-343, produced a biphasic relaxation (Figure 6). From 10^{-9} – 10^{-7} M McN-A-343 produced a weak relaxation with an extrapolated $\log IC_{50} = 8.53 \pm 0.14$ and a maximum relaxation of $22.8 \pm 4.3\%$, and from 3×10^{-6} – 10^{-4} M, it relaxed the vessel further with an extrapolated $\log IC_{50} = 5.02 \pm 0.08$ and a maximum relaxation of $41.1 \pm 5.4\%$ ($n = 16$). In endothelium-denuded segments McN-A-343 only produced further constriction ($n = 2$). The relaxation induced by McN-A-343 was not abolished by incubation with either the M_3 -selective antagonist, pFHHSiD (10^{-7} M, $n = 6$), or the M_2 -selective antagonist, AFDX 116 (3×10^{-7} M, $n = 4$), in concentrations supposed to block the M_3 and M_2 -receptors, respectively. However, the presence of a low concentration of

pirenzepine (10^{-9} M) inhibited the relaxation to McN-A-343 ($n = 5$, Figure 6), while 5×10^{-6} M A23187 relaxed the vessels by $92.3 \pm 1.3\%$ ($n = 5$).

Discussion

The contractile response to ACh predominates in lamb coronary arteries, as has been previously reported for the proximal parts of the coronary circulation in this species (Kalsner, 1985). However, the findings that ACh relaxed a small percentage of precontracted preparations and endothelial cell removal potentiated the contractile response to ACh (Figure 1, Table 1) suggest that the endothelium might modulate the contractile response to ACh in lamb coronary resistance arteries.

The present study was designed to characterize the contractile postsynaptic muscarinic receptor, using agonists and subtype selective antagonists. The agonists used in the present experiments, with the exception of McN-A-343 acted as full agonists in producing contraction of the coronary resistance arteries. ACh was equipotent with metacholine and oxotremorine-M, but more potent than carbachol in inducing contractile responses in both endothelium-intact and denuded lamb coronary resistance arteries. These results agree with other studies on muscarinic receptors suggesting that an M_3 -subtype of receptor is involved in ACh-induced contraction of the bovine coronary artery (Duckles, 1988; Duckles & Garcia-Villalon, 1990). McN-A-343 alone did not induce any motor response in the coronary resistance arteries, a finding similar to that reported for large coronary arteries (Duckles, 1988). However, in the presence of a low concentration of potassium in PSS, it elicited a weak motor response with a potency ($pD_2 = 4.9$) similar to that found for relaxation of the rabbit ear artery (Duckles, 1988). McN-A-343 was proposed as a selective M_1 agonist (Hammer & Giachetti, 1982; Jokisch-Mehrling *et al.*, 1989), but it exhibits variable responses at all muscarinic receptor subtypes, depending upon the receptor reserve of the preparation (Eglen *et al.*, 1987). Thus, the activity of McN-A-343 was reported to vary from no agonist activity up to a partial agonism at M_2 and M_3 receptors (Eglen *et al.*, 1987; Jokisch-Mehrling *et al.*, 1989). The weak agonism at M_2 and M_3 receptors might exclude the M_1 subtype for mediating the contractile response to ACh. However, the lack of reliable selective muscarinic agonists questions the use of agonists for receptor identification. Therefore, based on our results with agonists, it is not convenient to define the subtype of muscarinic receptors mediating contraction in the lamb coronary resistance arteries.

In endothelium-denuded lamb coronary resistance arteries all the muscarinic antagonists investigated acted as pure competitive antagonists of the ACh-induced contraction. Moreover, in the absence of the endothelium the slopes of Arunlakshana-Schild regression lines did not significantly differ from unity, which suggests that the contraction to ACh is mediated by a homogeneous muscarinic receptor population at the medial smooth muscle.

In endothelium-denuded coronary resistance arteries the affinity for pirenzepine ($pK_B = 7.5$) is not comparable to its high affinity (7.7–8.1) at M_1 cortical binding sites or to the low affinity (6.5–6.7) at M_2 cardiac receptors, but it is similar to the intermediate affinity (6.8–7.5) reported for the M_3 -subclass of receptors in visceral and vascular smooth muscle and glands (Eglen & Whiting, 1985; Delmendo *et al.*, 1989; Hulme *et al.*, 1990; Dauphin & Hamel, 1990; Brunner *et al.*, 1991b). This suggests that M_1 muscarinic receptors are not mediating the contractile response to ACh in lamb coronary resistance arteries. Furthermore, the weak potency of AFDX 116 does not support a role for the M_2 receptor subtype in the contractile response to ACh, but rather a site pharmacologically coincident with an M_3 muscarinic receptor. The potent parallel displacement of the concentration-

Figure 6 Concentration-response curves to the M_1 -selective cholinergic agonist, McN-A-343, in endothelium-intact lamb coronary arterial segments contracted with 3×10^{-7} M U46619. The concentration-response curves were performed in the absence (O) and the presence (●) of 10^{-9} M pirenzepine. Each point is the mean $\pm$ s.e. mean of 5–16 vessels.

response curves to ACh in the presence of the M_3 receptor antagonists, 4-DAMP and pFHHSiD favours the M_3 subtype for mediating the contraction. Although 4-DAMP was shown to possess equal affinity for the M_1 and M_3 receptor subtypes (Delmendo *et al.*, 1988; Hulme *et al.*, 1990), pFHHSiD showed high affinity with good correlation for a M_3 receptor subtype (7.8–8.2; Lambrecht *et al.*, 1988; Eglen *et al.*, 1990; Duckles, 1990).

Therefore, both the affinities and the order of potency of the muscarinic antagonists (pFHHSiD > pirenzepine > AFDX 116) in endothelium-denuded preparations suggest that the muscarinic receptor mediating the contractile effect of ACh in lamb coronary resistance arteries seems to belong to the M_3 -subclass. This finding agrees with other functional *in vitro* studies on bovine (Duckles, 1990; Duckles & Garcia-Villalon, 1990; Brunner *et al.*, 1991b) and porcine large coronary arteries (Van Charldorp & Van Zwieten, 1989), and also with *in vivo* studies on the canine coronary circulation, where AFDX 116 showed low affinity, and pirenzepine intermediate affinity for the muscarinic receptors mediating vasoconstriction to infused ACh (Pelc *et al.*, 1988).

In the presence of the endothelium, both the responses to muscarinic agonists and the action of the antagonists, were rather different from those obtained in endothelium-denuded arteries. ACh was less potent in producing contraction in the presence than in the absence of the endothelium, while the response to carbachol was unaltered. Similar differential changes in the contractile response to ACh and carbachol upon removal of the epithelium were observed in the guinea-pig trachea, where these results were ascribed to differences in the abilities of these choline esters to diffuse to the muscarinic receptor sites in the epithelium intact preparations (Morrison & Vanhoutte, 1992). The endothelium might act as a barrier for ACh in lamb coronary arteries. If the barrier capability of the endothelium became saturated at higher concentrations of agonist, then the equilibrium state of agonist, antagonist and receptor population would be changed and the slope of the Arunlakshana-Schild plot would be expected to be less than unity (Kenakin, 1987). However, in the presence of the endothelium, the slope of the Arunlakshana-Schild plot for pirenzepine was greater than unity and antagonism produced by AFDX 116 on the ACh-induced contraction was unaltered by removal of the endothelium. This suggests that the leftwards shift of the contractile response to ACh in absence of the endothelium cannot be ascribed to the endothelium functioning as a diffusion barrier for ACh.

In some aspects, however, the present results could be compared with those recently published by Alosachie & Godfraind (1988) on the role of the vascular endothelium in the interaction of α -adrenoceptor agonists and antagonists in the rat aorta. These authors reported that α_1 -adrenoceptor antagonists acted as non-competitive antagonists against noradrenaline in the presence of endothelium and as competitive antagonists after endothelial cell removal. In contrast, the α_2 -adrenoceptor antagonist, yohimbine, and the mixed antagonist, phentolamine, acted as competitive antagonists both in the presence and absence of the endothelium, although the pA_2 values for the latter two substances were lower in vessels with intact endothelium. In the present study, pirenzepine acted as a non-competitive antagonist against ACh in the presence of the endothelium and as a competitive antagonist when the endothelium was removed, while both AFDX 116 and pFHHSiD acted as competitive antagonists in arteries both with and without endothelium, although the pK_B for pFHHSiD was underestimated in endothelium-intact preparations (Figure 5). Alosachie & Godfraind (1988) suggested that their findings might be ascribed to the continuous basal release of EDRF acting as an endogenous functional antagonist, reducing the apparent efficacy of the adrenoceptor agonists and hence leading to the non-competitive antagonism of prazosin against noradrenaline in endothelium-intact preparations. We

have likewise observed in the present study an increase in basal tension after endothelial cell removal which might indicate abolition of the basal release of EDRF, as earlier demonstrated for coronary resistance arteries (Prieto *et al.*, 1991; Tschudi *et al.*, 1991; Simonsen *et al.*, 1992).

However, an alternative explanation of the present results might be that ACh is activating a heterogeneous muscarinic receptor population with opposing effects in the lamb coronary resistance arteries, contractile M_3 receptors at the smooth muscle and inhibitory muscarinic receptors producing endothelium-dependent relaxation. Thus, there might be a receptor-mediated release of EDRF that functions as an endogenous antagonist, reducing the efficacy of ACh and leading to the non surmountable antagonism of pirenzepine against the contractile response to ACh in endothelium-intact preparations. Indeed, the contractile response to ACh is potentiated in the presence of low concentrations of pirenzepine, an effect which is reversed by endothelial cell removal (Table 3). The latter finding supports the concept that ACh might be activating receptors with opposing effects, since occupation by pirenzepine of a small part of the inhibitory receptors produces a significant leftward shift of the concentration-response curve to ACh. The lack of a change in the Schild graphs for AFDX 116 in coronary arteries with and without endothelium, despite the evidence for a heterogeneous population of muscarinic receptors obtained from the results with pirenzepine, might be ascribed to the low selectivity of this antagonist for M_1 and M_3 receptors. However, further investigation is needed to clarify the leftward shift in the Schild plot and the unmasking of an underestimated pK_B value for pFHHSiD against ACh, induced by endothelial cell removal in the coronary resistance arteries. This finding could be due to several possible factors such as a temporal disequilibrium of the antagonist-receptor complex with the endothelium as a diffusion barrier to the compound, or that pFHHSiD possess affinity for membrane sites other than muscarinic receptors in the presence of endothelium, which could result in an artifactually low pK_B value (Kenakin, 1987). Finally, this shift might also be caused by removal of either basal or of agonist-induced EDRF release, in the latter case implicating the presence of a heterogeneous muscarinic receptor population in endothelium-intact vessels.

Additional evidence for a heterogeneous muscarinic receptor population comes from the relaxation induced by the putative M_1 -selective agonist, McN-A-343, in lamb coronary resistance arteries. This relaxation to McN-A-343 was inhibited in the presence of a low concentration of the M_1 -selective antagonist, pirenzepine but not by pFHHSiD or AFDX 116, known to block M_3 and M_2 receptors, respectively. The concentration of pirenzepine inhibiting the relaxation to McN-A-343 was coincident with that inducing potentiation of the ACh concentration-response curves (Table 3). These findings, together with the demonstration of a contractile effect of McN-A-343, suggest that ACh is not the only agonist activating a heterogeneous population of muscarinic receptors with opposing effects, but also that McN-A-343 is able to produce both relaxation and contraction in lamb coronary arteries. The present results therefore suggest that an inhibitory muscarinic receptor population at the endothelium, probably of the M_1 -subclass, modulates the contractile response to ACh elicited through the M_3 -receptors at the smooth muscle layer in the lamb coronary resistance arteries.

The present findings that the endothelial receptors might belong to the M_1 -subclass and the contractile receptor at the smooth muscle of the lamb coronary resistance arteries to the muscarinic M_3 -subclass agree with *in vivo* studies in the canine coronary circulation (Pelc *et al.*, 1988). In contrast, the endothelial receptor that mediates relaxation in bovine large coronary arteries seems to belong to the M_3 -subtype (Brunner *et al.*, 1991a). However, apart from species differences, there might be muscarinic receptor heterogeneity

not only between the endothelial layer and smooth muscle layer in the distal parts of the circulation, but also between proximal and distal parts of the coronary vascular tree (Nyborg *et al.*, 1991).

In summary, the present study suggests that coronary resistance arteries have heterogeneous populations of muscarinic receptors located at both the smooth muscle and endothelium causing contraction and the release of EDRF, respectively. The affinities of the muscarinic antagonists for the receptor at the smooth muscle layer suggest that ACh-induced contraction is mediated through M_3 -receptors, while

muscarinic receptors probably of another subtype or subtypes at the endothelium seem to modulate the contractile response to ACh.

We thank Elena Ordaz and Manolo Perales for technical assistance and Dr Miguel Isla for helpful discussions. The authors wish to express their thanks to Dr Barlow, Bristol, U.K., for the gift of 4-DAMP and Dr Karl Thomae, GmbH, for the gift of AFDX 116, and the Madrid Municipal Slaughterhouse for kindly supplying fresh tissue. U.S. was supported by a grant from Universidad Complutense de Madrid. Participating laboratories in Eurad.

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(Received September 11, 1992

Revised February 26, 1993

Accepted March 17, 1993)