In Vivo Characterization of Vasodilating Muscarinic-Receptor Subtypes in Humans

Tobias A. Bruning, Maarten G.C. Hendriks, Peter C. Chang, Eugenie A.P. Kuypers, Pieter A. van Zwieten

Abstract The role of muscarinic (M)-receptor subtypes in the regulation of vascular tone has not yet been defined in humans. To analyze the role of M-receptor subtypes in the forearm resistance vasculature of normotensive volunteers (n=20), we infused acetylcholine (ACh) and methacholine (MCh) in the presence of saline and the antagonists atropine (nonselective), pirenzepine (M1 selective), and AF-DX 116 (M2 selective), using automated R-wave-triggered venous occlusion plethysmography. Schild analysis was applied by calculating plasma concentrations of the infused compounds and determining EC50 values. ACh and MCh both caused dose-dependent vasodilatation, with EC50 values of 537 and 52 nmol/L, respectively. The apparent 10-fold higher potency of MCh compared with ACh may be explained by rapid degradation of ACh in plasma. The concentration-response curve of MCh was shifted to the right by atropine, pirenzepine, and AF-DX 116, with apparent pA2 values of 8.03±0.03, 6.71±0.08, and 5.32±0.05, respectively, and slopes not different from unity. The present technique enabled us to perform M-receptor characterization by Schild analysis in humans. The affinity constants and rank order of potency—atropine>pirenzepine>AF-DX 116—suggest that cholinergic vasodilatation in this vascular bed is predominantly mediated by the M2-receptor subtype. The EC50 value of MCh and the pA2 values of pirenzepine and AF-DX 116 are comparable to values reported for in vitro experiments. (Circ Res. 1994;74:912-919.)

Key Words • muscarinic receptors • parasympathetic nervous system • human forearm • venous occlusion plethysmography

The recent discovery of selective antagonists of muscarinic (M)-receptors has greatly facilitated the research on cholinergic vascular responses. M-receptors are divided in three pharmacologically functional subtypes, namely, M1, M2, and M3 (for reviews, see References 1 through 3). In addition, the existence of a fourth subtype (M4) has recently been submitted.4-6 By means of molecular biologic cloning techniques, five molecular subtypes (m1 through m5) have been identified and analyzed. Three of these cloned subtypes (m1, m2, and m3) correspond to the functional receptors M1, M2, and M3, whereas the functional relevance of the m4 and m5 structures remains to be established.7

Animal and in vitro studies have shown that M1-receptors are present on sympathetic ganglia, where they inhibit the release of norepinephrine.8 Central M2-receptors can also be found in the hippocampus and cerebral cortex.9 In the heart, M2-receptors reduce contractility and heart rate (HR) and induce contraction of the coronary arteries.9 Activation of M3-receptor subtypes, which are present in glandular tissue and the intestine, causes an increase in secretion and contraction of smooth muscle.10 In several vascular beds, M3-receptors have been shown to induce endothelium-dependent vasodilatation, a process that, at least in part, is dependent on the release of endothelium-derived relaxing factor (EDRF), now known to be identical with nitric oxide (NO).10,11

Since the introduction of M-receptor antagonists with a certain degree of selectivity for one or more of these M-receptor subtypes, a major body of evidence has emerged supporting the heterogeneity of M-receptors. Accordingly, pirenzepine is considered to be selective for M1-receptors (M1>M3>M2),12,13 whereas 11-[[2-[(diethylamino)methyl]-1-piperidinyl][acetetyl]-5,11-dihydroxy-6H-pyrido[2,3-b][1,4]benzodiazepin-6-on (AF-DX 116, M2>M3>M1) displays a certain degree of cardioselectivity and is assumed to be selective for the M2-receptor subtype.14 Moderate selectivity for the M3-receptor subtype, which is known to occur in certain types of vascular smooth muscle, has been submitted for experimental compounds such as 4-diphenylacetoxo-N-methylpiperidine methobromide (4-DAMP)3,4,15 and p-fluoro-hexahydro[laflideny]l (p-FHHSiD).15

The M3-receptor subtype has been demonstrated in vitro in several types of large conduit arteries of different animal species15 and has been shown to mediate vasodilatation, presumably via the release of EDRF (NO). In addition, Hendriks et al16 have recently shown that the M3-receptor subtype is also present in rat mesenteric resistance arteries and is responsible for the cholinergic vasodilator response. Its characteristics are the same in resistance vessels and conduit arteries.16

In the resistance vessels of the human forearm, the L-arginine/NO pathway has been shown to be responsible, at least in part, for the vasodilator response to acetylcholine (ACh).17,18

Until now, little evidence has been presented concerning the character and functionality of M-receptor subtypes in human resistance vasculature. For this reason, we analyzed the M-receptor subtypes mediating cholinergic vasodilatation in resistance vessels of the human forearm.
Accordingly, we established the influence of various competitive antagonists, such as atropine, pirenzepine, and AF-DX 116, on the vasodilatation induced by methacholine (MCh). The pharmacodynamic effects were quantified as concentration-response curves (CRCs), and for the first time, in humans and in vivo we applied the Schild analysis to characterize the role of M-receptor subtypes involved in cholinergic vasodilatation.

**Subjects and Methods**

**Subjects**

The present study was undertaken in 20 healthy male volunteers (mean age, 24 years; range, 19 to 31 years). Their medical history, physical examination, and routine laboratory tests did not show any abnormalities.

Twelve hours before the study, the subjects refrained from smoking, alcohol, and caffeine-containing beverages. The protocol was approved by the medical ethics committee of the Leiden University Hospital, and informed consent was obtained from all subjects.

**Procedures**

All experiments were performed in a quiet room kept at 22°C to 24°C. During the experiments, the subjects were in the supine position with both forearms stabilized slightly above the level of the heart. After local anesthesia of the skin with 1% lignocaine, the brachial artery of the nondominant arm was cannulated in the cubital fossa. The cannula (Autocath 1453.13, Plastimed, Saint-Leu-la-Forêt, France) was used for infusion of drugs with a constant-rate infusion pump (No. 22, Harvard Apparatus, Ltd, Edenbridge, Kent, England) and for intra-arterial recording of the blood pressure (BP) with a Statham P23Id pressure transducer (Gould Inc, Oxnard, Calif). HR was derived from a continuously recorded one-lead ECG. The forearm blood flow (FBF) in both arms was measured at 15-second intervals by R-wave–triggered venous occlusion plethysmography (EC-2 plethysmograph, Hokanson Inc, Issaquah, Wash) by use of mercury-in-silastic strain gauges and a rapid cuff infulator (Hokanson E-10). Tracings of the ECG, BP, and FBF were directly recorded on a polygraph (Mingograph 803, Siemens-Elema, Stockholm, Sweden). A personal computer (model AT3, IBM, Armonk, NY) extended by an analog-digital converter (model DT 2801, Data Translation Inc, Marlborough, Mass) was used for R-wave–triggered control of the rapid cuff inflator and for on-line analysis of FBF, intra-arterial BP, and HR.20,21 Forearm vascular resistance (FVR) was derived from each separate FBF measurement and the mean arterial blood pressure (MAP) values of the concomitantly recorded intra-arterial BP. During FBF measurements, both hands were excluded from the circulation by small wrist cuffs, which were inflated to 40 mm Hg above systolic blood pressure (SBP). Baseline recordings were started 1 minute after inflation of these cuffs. Forearm and hand volumes were measured by water displacement.

The experiments started at least 45 minutes after the cannulation of the brachial artery. The total duration of the study was between 5 and 7 hours for each subject. Between the various infusions, the wrist cuffs were deflated, and sufficient time (40 to 60 minutes) was allowed for FBF to return to baseline levels.

**Drugs and Solutions**

The following compounds were infused into the brachial artery: ACh HCl (OPG, Utrecht, The Netherlands), MCh HBr (Bruswchwig, Amsterdam, The Netherlands), atropine sulfate (Bufla, Uitgeest, The Netherlands), pirenzepine 2-hydrochloride (gift from Dr Karl Thomae, Biberach a/d Riss, Germany), and AF-DX 116 (gift from Dr Karl Thomae).

All drugs were dissolved in 0.9% saline. All commercially obtained compounds were analyzed before use. All solutions were prepared aseptically from sterile stock solutions and ampules on the day of the study and stored at 4°C until used.

**Study Protocol**

Twenty subjects participated in the present investigations; they were divided into three groups (group I [n=8] and groups II and III [both n=6]). Fig 1 summarizes the general design of the study protocol.

Control infusions of the agonists were always performed together with a continuous infusion of vehicle (0.9% saline at 0.4 mL/min). All cumulative-dose infusions lasted 16 minutes and consisted of four dose steps of 4 minutes each. This interval of 4 minutes proved sufficient to allow FBF to reach steady state. The concomitant continuous infusions of either vehicle or antagonist lasted 21 minutes and started 5 minutes before the cumulative-dose infusions. The control infusions of the agonists in the presence of vehicle always preceded the experiments with the antagonists. The experiments with the antagonists were performed in order of increasing dosage.

Baseline values were recorded during 3 minutes before each experiment. The average values of FBF, FVR, HR, and intra-arterial BP, obtained from six consecutive recordings during the last 1.5 minutes of each infusion step, were used for analysis.

In the present study we investigated the potencies of the M-receptor antagonists pirenzepine (M₃) and AF-DX 116 (M₃) in three different groups of normotensive volunteers. Atropine, a nonselective M-receptor antagonist, was used for comparison.
Group I

We constructed a dose-response curve (DRC) for the effects of the endogenous M-receptor agonist ACh (1 to 1000 ng · kg⁻¹ · min⁻¹ intra-arterially). Subsequently, we constructed a DRC for MCh (0.1 to 100 ng · kg⁻¹ · min⁻¹ intra-arterially), which is known to be a more stable compound in plasma, since it is much less susceptible to degradation by cholinesterase. The control infusion with MCh was given twice in succession to validate the length of the "washout interval" of the agonist used. Subsequently, we repeated the DRCs with MCh (dose range, 0.1 to 1000 ng · kg⁻¹ · min⁻¹ intra-arterially) in the presence of continuous-dose infusions of the nonselective M-receptor antagonist atropine (0.6, 6, and 60 ng · kg⁻¹ · min⁻¹ intra-arterially).

Group II

We investigated the potency of the M₁-receptor antagonist pirenzepine by constructing DRCs for MCh (dose range, 0.1 to 1000 ng · kg⁻¹ · min⁻¹ intra-arterially) in the presence of vehicle (control) and with continuous-dose infusions of pirenzepine (8, 80, and 800 ng · kg⁻¹ · min⁻¹ intra-arterially). The experiments were always performed with increasing doses of antagonist to prevent "carryover" effects between experiments. We repeated the infusion with MCh in the presence of vehicle after the experiments with antagonists to exclude possible time-dependent variations.

Group III

The potency of the M₁-receptor antagonist AF-DX 116 (80, 800, and 8000 ng · kg⁻¹ · min⁻¹ intra-arterially) was assessed in a manner similar to that in group II (see Fig 1). Again, two control DRCs with MCh were constructed, one before the experiments with the antagonist and one at the end of the day, at least 60 minutes after the last experiment with AF-DX 116.

Calculations

The average of six consecutive FBF measurements, made in the last 1.5 minutes of each dose step, was used for further analysis. Plasma concentrations (Cplasma, in micromoles per liter) of the drugs infused were calculated from the rate of drug infusion (IR, in nanograms per kilogram per minute), body weight (W, in kilograms), hematocrit (Ht), forearm volume (V, in milliliters), FBF (in milliliters per 100 mL per minute), and molecular weight (MW, in daltons):

\[ C_{\text{plasma}} = \frac{\text{IR} \cdot \text{W}}{(1 - \text{Ht}) \cdot \text{FBF} \cdot \text{V} \cdot \text{MW}} \cdot 100 \]

The average calculated plasma concentrations and corresponding average relative FVR values were used to construct CRCs by means of a curve-fitting computer program (Graphpad Software, San Diego, Calif) based on the following relation:

\[ E = E_{\text{max}} \cdot \left( \frac{[A]^p}{(A^p + EC_{50})} \right) \]

where E is the effect (percent change in FVR) observed with a calculated agonist concentration ([A], in log moles per liter), \( E_{\text{max}} \) (percent change in FVR) is the maximally attainable effect, EC50 (in negative log moles per liter) is the apparent concentration at which the half-maximal effect is seen, and the exponent P describes the slope of the relation (Hill coefficient). Schild analysis was performed using the EC50 values and calculated plasma concentrations of the antagonists. The latter was calculated in two ways: (1) by use of individual baseline FBF and (2) by use of the estimated antagonist concentration ([B]) at the EC50 of the CRC. By plotting log[A] against the corresponding log[B] for each dose step of the cumulative dose-response curve, the [B] value corresponding to the EC50 value of the CRC can be derived. Apparent affinity constants (pA2 values) were obtained graphically as the x intercept of the regression of log(CR−1) against the log[B], where the concentration ratio (CR) represents the EC50 value of the agonist CRC in the presence of the antagonist divided by the EC50 value of the agonist CRC in the presence of vehicle. If the regression of log(CR−1) on log[B] is linear and has a slope of unity, this provides presumptive evidence that the antagonism is competitive. The dissociation constant Kd was derived from the equation \( \log K_d = \log[B] - \log(CR - 1) \) (Mackay).

Because the antagonist concentrations were calculated using two different approaches, two pA2 and pKd values will be presented for each agonist.

Statistics

Results are given as mean±SEM. FBF and FVR values are expressed as percent changes from baseline (see Fig 1). The error in the calculated concentrations in the CRCs is determined by averaging all individually calculated plasma concentrations, resulting in mean concentrations with abscissa-oriented SEM limits, given horizontally. Student’s t test and Wilcoxon’s signed-rank test for matched pairs and ANOVA were used to evaluate the statistical significance of the data. Values of P<.05 were regarded as significant.

Results

Clinical and baseline hemodynamic characteristics of the subjects are given in Table 1. Baseline values of FBF established in the various experiments were all in the same range. During administration of AF-DX 116, an increase in HR was observed, which became statistically significant at the highest intra-arterial dose (8000 ng · kg⁻¹ · min⁻¹, 35±6%, P<.01). In all other experiments, small and nonsignificant changes in HR, FBF, and FVR in the control arm were observed. MAP tended to decrease slightly (≤6%) during the series of successive infusion steps (Table 2). MAP was not significantly different between the various infusions.

Cumulative-Dose Infusions of ACh and MCh

Both nonselective M-receptor agonists ACh (dose range, 1 to 1000 ng · kg⁻¹ · min⁻¹ intra-arterially; group I) and MCh (dose range, 0.1 to 100 ng · kg⁻¹ · min⁻¹ intra-arterially; groups II, II, and III) dose-dependently decreased FVR (see Fig 2 and Table 3). In group I, MCh was a 10-fold more potent vasodilator than its endogenous counterpart ACh (P<.001, Fig 2 and Table 3). In group I, subsequent control infusions of the agonist MCh showed similar vasodilator responses (Fig 2 and Table 3). In groups II and III, the single cumulative-dose infusion with MCh was repeated at the end of the experiments, and the EC50 values were not different from the same infusion performed at the beginning of the day (Table 3).

Cumulative-Dose Infusions of MCh in the Presence of Atropine

The nonselective M-receptor antagonist atropine (0.6, 6, and 60 ng · kg⁻¹ · min⁻¹ intra-arterially; group I) caused a shift of the concentration-response curves of MCh to the right (Fig 3). Atropine (calculated plasma concentrations, 0.01 to 1 μmol/L) was the most potent antagonist of the MCh-induced vasodilatation. This is reflected by its apparent pA2 value of 8.03±0.03, which amounted to 8.35±0.06 after correcting for the increase in FBF (see Table 4). The slope of the regression line was not significantly different from unity, thus indicating competitive antagonism (see Table 4 and Fig 4).
### TABLE 1. Clinical and Hemodynamic Characteristics of the Three Groups of Subjects Studied

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group I (n=8)</th>
<th>Group II (n=6)</th>
<th>Group III (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>25±1</td>
<td>22±1</td>
<td>23±1</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.82±0.02</td>
<td>1.88±0.01</td>
<td>1.88±0.03</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76±2</td>
<td>79±3</td>
<td>80±5</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>131±3</td>
<td>133±4</td>
<td>135±2</td>
</tr>
<tr>
<td>Diastolic</td>
<td>65±3</td>
<td>66±3</td>
<td>67±1</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>65±4</td>
<td>69±5</td>
<td>65±4</td>
</tr>
<tr>
<td>FBF, mL · 100 mL⁻¹ · min⁻¹</td>
<td>2.39±0.43</td>
<td>2.80±0.60</td>
<td>2.72±0.47</td>
</tr>
<tr>
<td>FVR, mm Hg/(mL · 100 mL⁻¹ · min⁻¹)</td>
<td>48.7±9.9</td>
<td>42.0±9.8</td>
<td>42.8±11.7</td>
</tr>
<tr>
<td>Forearm volume, mL</td>
<td>1144±39</td>
<td>1180±30</td>
<td>1230±90</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.44±0.01</td>
<td>0.44±0.01</td>
<td>0.45±0.01</td>
</tr>
</tbody>
</table>

bpm indicates beats per minute; FBF, forearm blood flow; and FVR, forearm vascular resistance. Values are mean±SEM. The baseline hemodynamic characteristics were determined at least 45 minutes after the cannulation of the brachial artery and before the first experiment. During this period, the subjects were at rest and remained in the supine position.

### TABLE 2. Mean Intra-arterial Pressure Before (Basal) and During Infusions 1 and 2

<table>
<thead>
<tr>
<th>Mean Intra-arterial Pressure, mm Hg</th>
<th>Infusion 1</th>
<th>Dose I</th>
<th>Dose II</th>
<th>Dose III</th>
<th>Dose IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>Basal</td>
<td>Basal</td>
<td>Basal</td>
<td>Basal</td>
<td>Basal</td>
</tr>
<tr>
<td>Group I (n=8)</td>
<td>Saline</td>
<td>87±2</td>
<td>86±3</td>
<td>86±3</td>
<td>87±2</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>86±2</td>
<td>85±2</td>
<td>84±2</td>
<td>86±2</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>88±3</td>
<td>87±2</td>
<td>86±2</td>
<td>87±3</td>
</tr>
<tr>
<td></td>
<td>ATR, ng · kg⁻¹ · min⁻¹</td>
<td>0.6</td>
<td>89±2</td>
<td>86±3</td>
<td>85±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>89±2</td>
<td>87±3</td>
<td>88±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>87±2</td>
<td>87±2</td>
<td>86±3</td>
</tr>
<tr>
<td>Group II (n=6)</td>
<td>Saline</td>
<td>88±4</td>
<td>87±4</td>
<td>87±4</td>
<td>88±4</td>
</tr>
<tr>
<td></td>
<td>PIR, ng · kg⁻¹ · min⁻¹</td>
<td>8</td>
<td>91±3</td>
<td>90±3</td>
<td>89±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>92±3</td>
<td>89±3</td>
<td>90±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>91±4</td>
<td>89±3</td>
<td>89±4</td>
</tr>
<tr>
<td>Group III (n=6)</td>
<td>Saline</td>
<td>90±1</td>
<td>88±2</td>
<td>87±1</td>
<td>87±1</td>
</tr>
<tr>
<td></td>
<td>AF-DX, ng · kg⁻¹ · min⁻¹</td>
<td>80</td>
<td>89±1</td>
<td>87±1</td>
<td>86±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>89±2</td>
<td>87±2</td>
<td>87±2</td>
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<tr>
<td></td>
<td></td>
<td>8000</td>
<td>88±2</td>
<td>88±2</td>
<td>88±1</td>
</tr>
</tbody>
</table>

ATR indicates atropine; PIR, pirenzepine; and AF-DX, muscarinic-receptor antagonist AF-DX 116 (for details, see text and Fig 1). Values are mean±SEM. The relative changes in mean arterial blood pressure compared with baseline (infusion 1) all were ≤6%.
Cumulative-Dose Infusions of MCh in the Presence of Pirenzepine

Similar to atropine, the M2-selective antagonist pirenzepine (8, 80, and 800 ng·kg\(^{-1}\)·min\(^{-1}\) intra-arterially; group II) caused a shift to the right of the CRCs for the MCh-induced vasodilatation without affecting the maximal vasodilator response (Fig 3). Pirenzepine (calculated plasma concentrations, 0.1 to 10 \(\mu\)mol/L) was less potent than atropine, with an apparent pA\(_2\) value of 6.71±0.08 (7.07±0.09 after correcting for the increase in FBF; see Table 4), and a slope of the regression line not different from unity (Table 4 and Fig 4).

Cumulative-Dose Infusions of MCh in the Presence of AF-DX 116

AF-DX 116 (80, 800, and 8000 ng·kg\(^{-1}\)·min\(^{-1}\) intra-arterially; group III), a cardioselective compound known to display preference for M2-receptors, proved to be the weakest competitive antagonist of MCh-mediated vasodilatation (calculated plasma concentrations, 1 to 100 \(\mu\)mol/L; Fig 3). Schild regression analysis yielded a pA\(_2\) value of 5.32±0.05 (5.65±0.07 after correcting for the increase in FBF) with a slope that did not differ from unity (Table 4 and Fig 4).

None of the M-receptor antagonists used in the present investigations caused any changes in FBF or FVR. Accordingly, the rank order of potency for the three M-receptor antagonists used appears to be atropine>pirenzepine>AF-DX 116 (see Table 4).

The progressive steepening of the CRCs can be explained by the fact that the plasma concentration of the antagonist, which is infused in a fixed dose, is progressively decreased by the dose-dependent vasodilatation caused by MCh. A reduction in FVR by half (ie, EC\(_{50}\)) implies an increase in FBF by a factor of 2, provided that MAP remains constant. Therefore, we have chosen a second approach that provides corrected affinity values for the concentration of antagonist at the FBF that would have occurred at EC\(_{50}\) (see “Subjects and Methods”). A reduction of the effective antagonist concentration at EC\(_{50}\) by a log step of 0.3 thus results in affinity values that are 0.3 of a log step higher (Table 4).

Discussion

The present investigations indicate that functional M-receptors are present in the vascular bed of the human forearm and mediate vasodilatation in response to infused cholinergic agonists. We found indirect evidence of a major role involving the M2-receptor in this particular model of human peripheral resistance vessels,\(^{10,21}\) which are of importance in the regulation of BP and are possible targets for antihypertensive treatment. The application of Schild analysis,\(^{10,22}\) to define the existence and functional relevance of M-receptor subtypes, represents a novel pharmacologic approach for this particular in vivo model.

Antagonists with a certain degree of selectivity have been frequently used to characterize M-receptors, and they are more useful for this purpose than M-receptor agonists, those available being rather unselective. In vitro studies have shown that the affinities of the nonsel ective agonists ACh and MCh for M-receptors are similar.\(^{10}\) In the present investigation, however, we found that ACh is 10-fold less potent than is MCh. We presume that this is the result of the rapid degradation of ACh by cholinesterase present in plasma. Therefore, we used MCh as the reference M-receptor agonist in the present study. The EC\(_{50}\) values found for MCh were practically equal for the three groups of normotensive volunteers investigated (Table 3), which confirms the value of this agonist as a pharmacologic tool. In addition, the EC\(_{50}\) values

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**Table 3.** EC\(_{50}\) Values of the Control Dose-Response Curves With the Agonists Acetylcholine and Methacholine

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Group I (n=8)</th>
<th>Group II (n=6)</th>
<th>Group III (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>6.27±0.06</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MCh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.28±0.03</td>
<td>7.62±0.05</td>
<td>7.48±0.01</td>
</tr>
<tr>
<td>Repeated</td>
<td>7.38±0.03</td>
<td>7.41±0.08</td>
<td>7.47±0.08</td>
</tr>
</tbody>
</table>

ACh indicates acetylcholine; MCh, methacholine; and ND, not done (for details, see text and Fig 1). Values are mean±SEM.

In group I, the EC\(_{50}\) values of ACh were significantly different from the EC\(_{50}\) values of MCh (\(P<.001\)). There were no significant differences between the EC\(_{50}\) values of MCh within the groups.
found for MCh (52 nmol/L) are comparable to data obtained in experiments in the perfused mesenteric bed of the rat (44 nmol/L)\(^1\). Both ACh\(^2\)\(^-\)\(^4\)\(^-\) and MCh\(^5\)\(^-\)\(^7\)\(^-\) have been used by several groups to investigate endothelium-dependent vasodilator responses in the vascular bed of the human forearm. Angus and Lew\(^8\) have recently reviewed the application of ACh as an endothelium-dependent vasodilator and have suggested that the use of a stable choline ester would be preferable because of the relative instability of ACh in an in vivo situation. Therefore, we suggest that a stable cholinergic agonist, such as MCh, seems more appropriate for clinical studies.

The MCh-induced vasodilatation was analyzed by means of a series of selective M-receptor antagonists. Since the M\(_1\)-receptor-specific antagonists such as 4-DAMP and p-FHHSID are not available for human use at present, we compared the apparent selectivity of pirenzepine (M\(_1\)) and AF-DX 116 (M\(_3\)) to that of atropine as a nonselective reference.\(^9\) The use of these antagonists with a high affinity for the M\(_1\) and M\(_3\) receptor subtypes has provided insight into the subclass of receptors that are involved in the MCh-induced vasodilatation in the human forearm resistance vessels.

The M-Receptor Antagonists Atropine, Pirenzepine, and AF-DX 116

The nonselective M-receptor antagonist atropine proved the most potent in our investigations. The apparent pA\(_2\) value obtained in the present study (8.03±0.03) is somewhat lower than that found in the rat thoracic aorta (8.90)\(^1\)\(^2\) and the rat mesenteric vascular bed (9.86).\(^1\)\(^6\)

The M\(_3\)-selective antagonist pirenzepine exhibited only a moderate affinity (pA\(_2\), 6.71±0.08) for the M-receptor mediating vasodilatation in the vascular bed of the human forearm. The apparent affinity of pirenzepine found in the present study is more in agreement with the affinities found for the M\(_1\)-receptor subtype in the thoracic aorta of the Wistar rat by Choo et al\(^1\)\(^3\) (pA\(_2\), 6.75) and in the isolated rat pulmonary artery (pA\(_2\), 6.96).\(^1\)\(^4\) This suggests that it is not the M\(_1\)-receptor subtype that is primarily responsible for the cholinergic

### Table 4. Affinity Values of the Antagonists Atropine, Pirenzepine, and AF-DX 116 as Derived From Schild Analysis, Using Antagonist Concentrations Calculated From Baseline Forearm Blood Flow Values and From Forearm Blood Flow Values Estimated at EC\(_{50}\)

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Baseline pA(_2)</th>
<th>Baseline pK(_b)</th>
<th>Slope of the Regression Baseline</th>
<th>Slope of the Regression EC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>8.03±0.03</td>
<td>8.33±0.06</td>
<td>1.02±0.02</td>
<td>1.18±0.07</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>6.71±0.08</td>
<td>7.07±0.09</td>
<td>0.98±0.08</td>
<td>0.96±0.09</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>5.32±0.05</td>
<td>5.65±0.07</td>
<td>1.09±0.07</td>
<td>1.08±0.08</td>
</tr>
</tbody>
</table>

Values are mean±SEM. None of the slopes calculated by Schild regression were different from unity (for details, see text and Fig 4).
vasodilatation found in our experiments. The affinity of pirenzepine for the M1-receptor subtype present in the rat submandibular gland\(^4\) and the affinities found by Dauphin and Hamel\(^5\) and Eglen and Whiting\(^6\) (pA\(_2\), 7.52 and 7.57, respectively) for the M1-receptor subtype on the vascular endothelium of the cat middle cerebral artery and rabbit thoracic aorta somewhat differ from the data established in the present study.

The cardioselective (M\(_2\)) antagonist AF-DX 116 was the only compound that caused a systemic hemodynamic effect by increasing HR (reflecting a vagolytic action), whereas it exhibited a low affinity toward the receptor mediating vasodilatation (pA\(_2\), 5.32±0.05). The weak antagonistic potency of AF-DX 116 and the discrepancy observed between its affinity for the receptor mediating vasodilatation and its original affinity for the M2-receptor (pA\(_2\), 7.05)\(^4\) indicate that the M2-receptor subtype does not play an important role in MCh-induced vasodilatation.

**Schild Analysis in the Vascular Bed of the Human Forearm**

The human forearm has been a well-established model of peripheral resistance vessels and has proved useful in pharmacologic studies.\(^{18,20,21}\) The observed decrease in MAP in the course of the experiments consistently resulted from a decrease in SBP. HR and diastolic blood pressure (DBP) did not change, nor did the FVR of the contralateral (control) arm. The decrease in SBP without changes in DBP and HR is probably best explained by alterations in the BP waveform caused by changes in arterial compliance\(^{37,38}\) or by a local Venturi phenomenon at the intra-arterial catheter tip caused by the high local blood flow velocity, which is most pronounced during systole.\(^{21,39}\) Since it is possible to quantify FBF precisely by using in situ calibration against volume flows\(^{20}\) and to calculate plasma concentrations of compounds infused into the brachial artery, it is possible to use quantitative pharmacologic techniques, such as Schild analysis.\(^{19,22}\)

We observed lower pA\(_2\) values for all antagonists used, when compared with in vitro data for animal vascular tissues from the literature.\(^{10,16}\) This may be explained, at least partly, by a nonspecific loss of the compounds due to plasma protein binding, by uptake and degradation mechanisms, or by both. In addition, differences in equilibration conditions for the antagonist with the receptor caused by, for instance, distribution in the different tissue compartments may result in lower pA\(_2\) values.\(^{22}\) In our experiments, the time given for equilibration had to be limited to 5 minutes per dose for practical reasons. Another explanation may be that in the case of competitive antagonism, in which the agonist and the antagonist compete for the same recognition sites on the receptor, Schild analysis using baseline FBF to calculate the concentration of the antagonist may provide underestimated pA\(_2\) values, since the FBF at the EC\(_50\) of the CRC (concentration versus FVR) has increased approximately twofold compared with baseline. Schild analysis using calculated concentrations of the antagonist as estimated at the EC\(_50\) of the CRC resulted in pA\(_2\) values of approximately one-third log step (twofold) greater magnitude (Table 4). These pA\(_2\) values are more in agreement with the literature. The relative rank order of the antagonists, however, remains unaltered and does not affect our conclusion with respect to the involvement of the M1-receptor in cholinergic vasodilatation.

**The M1-Receptor Appears to Predominate in Cholinergic Vasodilatation in the Human Forearm**

Pirenzepine has been reported to display a high affinity for the M1-receptor subtype (pK\(_i\), 8.20; as established by radioligand-binding studies, see Reference 40) and a low affinity for the M1-receptor (pK\(_i\), 6.65) and the M1-receptor (pK\(_i\), 6.86) in systems of cloned human receptors.\(^{41}\) AF-DX 116 is known to possess some affinity for the M1-receptor (pK\(_i\), 6.09), which is intermediate between its affinity for M2-receptor (pK\(_i\), 6.71) and M3-receptor (pK\(_i\), 5.30) subtypes.\(^{4}\) The weak potencies of these two compounds observed in the present study argue against a major role for the involvement of the M1- and M2-receptor subtypes in MCh-induced vasodilatation in the vascular bed of the human forearm, although their involvement cannot be completely excluded by the present investigations.

The rank order of potency (atropine>pirenzepine>AF-DX 116) and the low affinities of pirenzepine and AF-DX 116 that we observed suggest that it is predominantly the M1-receptor that triggers vasodilatation. Since a functional role for the M1- and M2-receptor subtypes has as yet not been established, the involvement of the M1-receptor can only be postulated by inference. For ethical reasons, we could not perform human experiments with the few and moderately selective M1-receptor antagonists available (p-FHHSD and 4-DAMP).

Cholinergic vasodilatation can be mediated by presynaptic inhibition of norepinephrine release from sympathetic nerve endings (M1-receptor mediated\(^9\)) and by activation of the L-arginine/NO pathway (M1-receptor mediated\(^8\)). Since our data indicate that an important role for the M1-receptor is unlikely, it seems reasonable to assume that the vasodilator response is mainly medi-
ated by M₃-receptors, probably by stimulating the L-arginine/NO pathway. The fact that none of the M-receptor antagonists used caused a change in FBF or FVR suggests that at rest there is no basal cholinergic tone present in the vascular bed of the forearm. The apparent pA₂ values mentioned should not be interpreted as an absolute measure for receptor affinity and can only be considered as an estimate of the relative potencies of the compounds used.

To our knowledge, a functional role for M-receptor subtypes mediating vasodilatation in response to infused cholinergic agonists in the forearm has not yet been demonstrated. Moreover, characterization of the M-receptor subtype mediating cholinergic vasodilatation by use of competitive antagonists and Schild analysis in an in vivo human experimental system is novel. Cholinergic vasodilatation in this vascular bed is mediated primarily by the M₂-receptor subtype.

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