Characterization of muscarinic receptors mediating vasodilation in rat perfused kidney

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The muscarinic receptor mediating vasodilation of resistance vessels in the rat isolated, constant-pressure perfused kidney (preconstriction by \(10^{-7} \) M cirazoline) was characterized by subtype-preferring agonists and selective antagonists. The agonists produced vasodilation with the following rank order of potency: arecaidine propargyl ester (APE) > 5-methylfurthetronium = methacholine = oxotremorine > (S)-aceclidine > arecaidine 2-butyne-1,4-diyl bisester > 4-Cl-McN-A-343 = (R)-nipecotic acid ethyl ester = (R)-aceclidine = (S)-nipecotic acid ethyl ester > McN-A-343. Agonist-induced vasodilation disappeared after destruction of the endothelium with detergent. Highly significant correlations of agonist potencies for vasodilation were found between rat kidney and guinea-pig ileum submucosal arterioles as well as agonist potencies at smooth muscle muscarinic M_3 receptors of the guinea-pig ileum. The rank order of antagonist potencies (4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) > (R)-hexahydro-difenidol - hexahydro-sila-difenidol > pirenzepine - p-fluoro-hexahydro-sila-difenidol - himbacine - AF-DX 384 - AQ-RA 741 > (S)-hexahydro-difenidol) to attenuate vasodilation to APE in rat kidney, correlated significantly with affinities at M_3 receptors in submucosal arterioles and in smooth muscle of the guinea-pig ileum, but differed from those at M_1 and M_2 receptors in rabbit vas deferens. The agonist and antagonist potencies suggest that vasodilation elicited by muscarinic stimuli in endothelium-intact rat renal vasculature is mediated by functional muscarinic M_3 receptors.

Kidney (perfused, rat); Muscarinic receptor agonists; Muscarinic receptor antagonists; Arterioles (submucosal); Ileum; Atrium; Vas deferens; Videomicroscopy (guinea pig)

1. Introduction

Muscarinic receptors have been classified on the basis of pharmacological evidence into at least three subtypes termed M_1, M_2 and M_3 (Hulme et al., 1990). Molecular biological evidence indicates that at least five genes exist that encode m_1-m_5 receptors (Hulme et al., 1990). The m_1-m_3 gene products correspond to the M_1-M_3 receptors (Hulme et al., 1990; Waelderbroeck et al., 1990; Dörje et al., 1991b), whilst pharmacological candidates for the M_4 receptor have also been tentatively identified in rat striatum, rabbit lung and some cell lines (Hulme et al., 1990; Lazareno et al., 1990; Waelderbroeck et al., 1990; Dörje et al., 1991a). A functional equivalent of the m_5 gene product has yet to be identified.

Each of the three subtypes, M_1, M_2 and M_3, has been demonstrated to exist in vascular preparations from different species depending on the tissue and function examined (for a recent review, see Eglen and Whiting, 1990). In general, M_3 receptors mediate endothelium-dependent relaxation, whereas M_1 receptor stimulation causes contraction of e.g. canine saphenous and femoral veins. Stimulation of prejunctional M_2 receptors appears to inhibit sympathetic outflow in vascular preparations from a number of anatomical locations from different species. Contraction may also occur due to stimulation of M_3 (e.g. in rabbit aorta; Jaiswal et al., 1991) and M_2 receptors (e.g. bovine and porcine coronary artery; Eglen and Whiting, 1990).

Studies on vascular resistance in the rat isolated perfused kidney have provided evidence that nitric oxide (NO) mediates the vasodilator response to acetylcholine (Bhardwaj and Moore, 1988; Burton et al., 1990). Muscarinic receptor sites in rat kidney labelled with \(^3\)Hquintulidinyl benzilate (\(^3\)HQNB) were
2. Materials and methods

2.1. Rat isolated perfused kidney

The experiments were performed on kidneys taken from normotensive rats (Sprague-Dawley, male, 400–450 g, Wiga, Sulzfeld, Germany), similarly to the method described previously (Eltze et al., 1991). Briefly, after the aorta adjacent to the left renal artery had been cannulated and the abdominal vena cava cut, the kidney was removed and perfused at a constant pressure of 100 cm H₂O with prewarmed (37°C) Tyrode solution of the following composition (mM): NaCl 137.0, KCl 2.7, CaCl₂ 1.25, MgCl₂ 1.1, NaHCO₃ 12.0, NaH₂PO₄ 0.42, Ca-EDTA 0.026, glucose 5.6, additionally containing L-arginine hydrochloride 0.03 and indomethacin 0.01, gassed with a mixture of 95% O₂–5% CO₂. The prerenal perfusate flow was measured continuously using an electromagnetic flowmeter.

2.1.1. Agonist potency

Each experiment consisted of a 40-min equilibration period during which renal perfusate flow stabilized at 17.4 ± 3.6 ml/min (mean ± S.D., n = 51). Following this period, a second reservoir containing the vasoconstrictor agent, cirazoline (10⁻⁷ M), which was an appropriate concentration to reduce renal perfusate flow between 62–83% (resulting flow = 4.8 ± 1.9 ml/min; mean ± S.D., n = 51), was connected via a three-way stopcock to continuously perfuse the kidney. Once the resulting constriction had stabilized, increasing doses of the muscarinic agonists (100 μl aqueous bolus) were injected within 2 s into the renal inflow tract and the resulting vasodilatation was recorded. The -log ED₅₀ values (mol) were determined graphically from semilogarithmic plots. At the end of drug administration the maximal effect was determined by bolus injection of 3 x 10⁻⁷ mol arecaidine propargyl ester (APE). The perfusion was then changed to Tyrode solution without vasoconstrictor. The kidneys were repeatedly constricted in cycles of 45 min. Each preparation was used to evaluate the responses of the renal vasculature to maximally six different agonists in random order, provided that (a) the vasoconstriction in response to 10⁻⁷ M cirazoline could be exactly reproduced and (b) the vasodilator response to bolus injections of 3 x 10⁻⁷ mol APE remained stable. No significant time-dependent changes in the vascular responses to the agonists could be detected.

2.1.2. Removal of endothelium

Vascular endothelium was removed by perfusion of the kidney for 5 min with Tyrode solution containing 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS, 0.2 mg/ml) similar to the method described by Bhardwaj and Moore (1988). The degree of endothelial damage was verified functionally by the inability of bolus injections of muscarinic agonists to elicit a vasodilatation of more than 7% in cirazoline-preconstricted (10⁻⁷ M) organs. Functional integrity of the vascular smooth muscle after CHAPS treatment was investigated with the endothelium-independent vasodilator, nitroprusside (10⁻⁷ mol).

2.1.3. Antagonist potency

The increase in renal flow due to bolus injections of 5 x 10⁻⁹ mol APE (= ED₅₀ of its own maximal effect) during perfusion of the kidneys with cirazoline (10⁻⁷ M) in the absence of the muscarinic antagonists was taken to be 100%. The percentage inhibition of this effect in the presence of increasing concentrations of the antagonists perfused together with cirazoline for 20 min was calculated. Thus, complete individual concentration-response curves for an antagonist were generated in five or six kidneys, enabling calculation of an EC₅₀ value, i.e., the molar concentration for half-maximal antivasodilator effect.

2.2. Guinea-pig isolated ileum submucosal arterioles

Submucosal plexus preparations were obtained from the small intestine of guinea-pigs as described previously (Bungardt et al., 1992). Briefly, the preparations were perfused continuously at 8–10 ml/min with Tyrode solution gassed with 95% O₂–5% CO₂ at 32–36°C (composition, see under 2.3). The outside diameter of
2.3. Guinea-pig isolated left atrium and ileum

Guinea-pigs (300-400 g) of either sex were killed by cervical dislocation. The removed left atrium was set up in a 6-ml organ bath under 0.5 g tension in oxygenated (95% O₂-5% CO₂) Tyrode solution at 32°C composed of (mM): NaCl 137.0, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.05, NaHCO₃ 11.9, NaH₂PO₄ 0.42 and glucose 5.6, and was paced electrically (2 Hz, 3 ms, 5 V) by means of platinum electrodes. The negative inotropic effect of the muscarinic agonists was measured as change in isometric tension and pD₂ values (−log EC₅₀) were calculated. In antagonist studies, APE was used as agonist, the antagonists being equilibrated with the tissue for at least 30 min (Feifel et al., 1990).

Strips of ileal longitudinal smooth muscle suspended in Tyrode solution (composition as used for the atrium) were maintained at 32°C and gassed with 95% O₂-5% CO₂ under a resting load of 0.5 g (Feifel et al., 1990). Isometric contractions were elicited by cumulative addition of muscarinic agonists at 30-min intervals and pD₂ values (−log EC₅₀) were determined graphically from semi-logarithmic plots. For assessment of antagonist affinity, concentration-response curves to acetylcholine or APE were calculated by the method of linear regression by the method of least squares, to estimate the slope of the regression lines (Arunlakshana and Schild, 1959). pA₂ values were obtained from Schild plots in which the slopes of the regression lines were constrained to 1.00 (Tallarida et al., 1979). In those few cases (table 2) where the slopes differ significantly from unity (P < 0.05), the pA₂ values should be regarded as a purely experimental quantity.

2.4. Rabbit vas deferens

Male New Zealand white rabbits were killed by i.v. injection of pentobarbital sodium (60 mg/kg) and exsanguination. Vasa deferentia were removed and divided into four segments, two prostatic portions of 1 cm and two epididymal portions each approximately 1.5 cm in length. Each tissue was folded in two over a platinum electrode in a 10-ml organ bath and connected via a thread to a force-displacement transducer. A second platinum ring electrode was placed at the top of the bathing fluid for continuous field stimulation (0.05 Hz, 0.5 ms, 30 V). The bathing fluid (composition in mM: NaCl 118.0, KCl 4.7, CaCl₂ 2.5, MgSO₄ 0.6, KH₂PO₄ 1.2, NaHCO₃ 25.0, glucose 11.1, yohimbine 0.001) was maintained at 31°C and gassed with 95% O₂-5% CO₂. A resting tension of 0.75 g was applied and neurogenic twitch contractions were measured isometrically and recorded on a multi-channel recorder (Eltze, 1988).

Agonist potencies (pD₂ values) and apparent efficacies (i.a. values) at muscarinic M₁ receptors in rabbit vas deferens were determined on close prostatic segments suspended in the nutrient solution mentioned above, with the exception that the CaCl₂ concentration was reduced to 1.8 mM. 4-Cl-McN-A-343 was used as reference agonist.

Antagonists affinities for M₁ and M₂ receptors were obtained from concentration-response curves either to McN-A-343 for inhibition (M₁ receptors) or to carbachol for potentiation (M₂ receptors) of neurogenic twitch contractions in the absence and presence of antagonists (45-min exposure time) (Eltze, 1988).

2.5. Data Analysis

For the assessment of antagonist affinity in rabbit vas deferens and guinea-pig atrium and ileum, EC₅₀ values of agonists were determined for the control and the antagonist-shifted concentration-response curves using at least four different antagonist concentrations (log interval = 0.48). Each concentration of an antagonist was tested 3-4 times and the ratios of agonist molar EC₅₀ values obtained in the absence and presence of antagonists were calculated. Schild plots were made, using linear regression by the method of least squares, to estimate the slope of the regression lines (Eltze, 1988). pA₂ values were obtained from Schild plots in which the slopes of the regression lines were constrained to 1.00 (Tallarida et al., 1979). In those few cases (table 2) where the slopes differ significantly from unity (P < 0.05), the pA₂ values should be regarded as a purely experimental quantity.

2.6. Linear Regressions

Linear regressions were calculated by the method of least squares in order to determine the correlation coefficient r and the slope of regression β. Significance of differences of the slope from unity was assessed with t-tests.

2.7. Drugs

Arecaidine propargyl ester hydrobromide (APE), guvacine propargyl ester hydrobromide (GPE), guvacine hydrobromide, N-ethyl-guvacine propargyl ester hydrobromide (NEN-APE), arecaidine 2-butyne-1,4-diyi bistere p-toluene sulfonate (bis-ABE), (R)-
aceclidine perchlorate and (S)-aceclidine hydrobromide, 4-[[N-(4-chlorophenyl)carbamoyl]oxy]-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343), hexahydro-sila-difenidol hydrochloride (HHSiD) and its p-fluoro analogue (p-F-HHSiD), (R)- and (S)-enantiomers of hexahydro-difenidol hydrochloride [(R)-HHD and (S)-HHD] were synthesized in our laboratories. Oxtremorine sesquifumarate (Merck, Darmstadt, Germany). (R)- and (S)-nipecotic acid ethyl ester tartrate ((R)- and (S)-NAEE) were kindly donated by Prof. Krogsgaard-Larsen (Copenhagen, Denmark). Nuvenzepine hydrochloride (Dompe Pharmaceuticals, Milan, Italy). Mequitamium iodide (Laboratori Guidotti, Pisa, Italy). 4-[[N-(3-Chlorophenyl)carbamoyl]oxy]-2-butynyltrimethylammonium chloride (McN-A-343) was purchased from RBI (Cologne, Germany). (±)-Methacholine chloride (EGA Chemie, Steinheim, Germany). Pirenzepine dihydrochloride, (±)-6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11H-dibenzo-[b,e]diazepin-11-one hydrochloride (UH-AH 37), (±)-5,11-dihydro-11-[[2-[(dipropylamino)methyl]-1-piperidiny]ethyl]aminocarbonyl]-6H-pyrido[2,3-b](1,4)-benzodiazepin-6-one (AF-DX 384), 11-[[4-(4-diethylamino)butyl]-1-piperidiny]acetyl]-5,11-dihydro-6H-pyrido[2,3-b](1,4)-benzodiazepin-6-one (AQ-RA 741) (Prof. B. Wetzel, Thomae, Biberach, Germany). Him-
bacine hydrochloride was kindly donated by Prof. W.C. Taylor (University of Sydney, Australia). Ida verine (Duphar BV, Weesp, The Netherlands). (+)-Telenzapine dihydrochloride, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), 5-methylfurfur thionium iodide (MeFur) (Byk Gulden, Konstanz, Germany). Cirazoline hydrochloride (Synthélabo, Paris, France). Disodium nitroprusside (Schwarz, Monheim, Germany). Rac-Cromakalim (SmithKline Beecham, U.K.). All other drugs (arecoline hydrobromide, (+)-muscarine chloride, carbamoycholine chloride (carbachol), hexamethonium bromide, tetrodotoxin, atropine sulfate, papaverine hydrochloride and 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate (CHAPS)) were purchased from Sigma (Munich, Germany).

3. Results

3.1. Rat perfused kidney

3.1.1. Effect of drugs on non-preconstricted kidneys

Without vasoconstrictor stimuli, bolus injection of papaverine (up to $10^{-6}$ mol) proved to be ineffective. Injection of other smooth muscle relaxants, e.g. nitroprusside (up to $10^{-6}$ mol) and cromakalim (up to $10^{-7}$ mol) into the renal inflow tract did not increase perfusion flow of the kidneys by more than 5%, indicating an essential absence of intrinsic vascular tone. None of the muscarinic agonists and antagonists listed in tables 1 and 2, respectively, when injected into the renal inflow tract up to $10^{-7}$ mol, enhanced or diminished basal flow by more than 7%.

<table>
<thead>
<tr>
<th>Drug Rad kidney</th>
<th>$-\log ED_{50}$ (mol)</th>
<th>$pD_2$ (i.a.)</th>
<th>$M_3$ (GPI)</th>
<th>$M_1$ (RVD)</th>
<th>$M_2$ (GPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) APE</td>
<td>9.44 (8.8-9.9)</td>
<td>7.58 (7.5-7.6)</td>
<td>7.74 (7.5-7.9)</td>
<td>8.19 (8.1-8.3)</td>
<td></td>
</tr>
<tr>
<td>(2) MeFur</td>
<td>8.65 (7.7-9.6)</td>
<td>7.62 (7.5-7.7)</td>
<td>6.89 (6.7-7.1)</td>
<td>6.46 (6.4-6.5)</td>
<td></td>
</tr>
<tr>
<td>(3) Methacholine</td>
<td>8.86 (7.1-9.9)</td>
<td>7.51 (7.2-7.8)</td>
<td>6.51 (6.2-6.8)</td>
<td>7.08 (7.0-7.2)</td>
<td></td>
</tr>
<tr>
<td>(4) Oxotremorine</td>
<td>8.56 (7.7-9.4)</td>
<td>7.39 (7.1-7.7)</td>
<td>7.77 (7.7-7.9)</td>
<td>7.65 (7.6-7.8)</td>
<td></td>
</tr>
<tr>
<td>(5) Carbachol</td>
<td>8.55 (7.5-9.6)</td>
<td>6.67 (6.6-6.7)</td>
<td>6.77 (6.6-6.9)</td>
<td>6.90 (6.6-7.0)</td>
<td></td>
</tr>
<tr>
<td>(6) GPE</td>
<td>8.25 (7.2-9.3)</td>
<td>7.13 (6.9-7.3)</td>
<td>7.28 (7.1-7.4)</td>
<td>6.97 (6.8-7.1)</td>
<td></td>
</tr>
<tr>
<td>(7) (S)-Aceclidine</td>
<td>8.15 (7.3-9.0)</td>
<td>6.52 (6.4-6.6)</td>
<td>6.60 (6.5-6.7)</td>
<td>6.48 (6.4-6.6)</td>
<td></td>
</tr>
<tr>
<td>(8) Arecoline</td>
<td>7.77 (6.9-8.7)</td>
<td>6.46 (6.3-6.6)</td>
<td>6.69 (6.6-6.8)</td>
<td>6.84 (6.6-6.9)</td>
<td></td>
</tr>
<tr>
<td>(9) bis-ABE</td>
<td>7.70 (7.0-8.4)</td>
<td>6.27 (6.1-6.4)</td>
<td>6.94 (6.8-7.1)</td>
<td>7.10 (7.0-7.2)</td>
<td></td>
</tr>
<tr>
<td>(10) Guvacoline</td>
<td>7.58 (6.4-8.7)</td>
<td>6.43 (6.1-6.7)</td>
<td>5.95 (5.9-6.0)</td>
<td>6.65 (6.5-6.7)</td>
<td></td>
</tr>
<tr>
<td>(11) 4-Ci-McN-A-343</td>
<td>6.82 (6.2-7.4)</td>
<td>5.35 (5.2-5.5)</td>
<td>7.06 (7.0-7.1)</td>
<td>5.26 (5.1-5.4)</td>
<td></td>
</tr>
<tr>
<td>(12) (R)-NAEE</td>
<td>6.70 (6.5-6.9)</td>
<td>5.78 (5.7-5.9)</td>
<td>inactive</td>
<td>5.82 (5.7-5.9)</td>
<td></td>
</tr>
<tr>
<td>(13) NEN-APE</td>
<td>6.60 (6.2-7.0)</td>
<td>4.65 (4.4-4.9)</td>
<td>4.65 (4.4-4.9)</td>
<td>6.84 (6.4-6.6)</td>
<td></td>
</tr>
<tr>
<td>(14) (R)-Aceclidine</td>
<td>6.47 (6.2-6.7)</td>
<td>5.29 (5.2-5.4)</td>
<td>5.07 (5.0-5.2)</td>
<td>5.00 (5.4-5.6)</td>
<td></td>
</tr>
<tr>
<td>(15) (S)-NAEE</td>
<td>6.46 (6.1-6.9)</td>
<td>5.19 (5.1-5.3)</td>
<td>inactive</td>
<td>4.77 (4.7-4.8)</td>
<td></td>
</tr>
<tr>
<td>(16) McN-A-343</td>
<td>6.19 (5.6-6.7)</td>
<td>4.96 (4.8-5.1)</td>
<td>6.57 (6.5-6.7)</td>
<td>4.87 (4.5-5.3)</td>
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</tr>
</tbody>
</table>
3.1.2. Effect of muscarinic agonists on kidneys preconstricted by cirazoline

During vasoconstriction induced by cirazoline \(10^{-7}\) M, which evoked a stable and reproducible decrease in renal flow of 72 \(\pm\) 11% (mean \(\pm\) S.D., \(n = 51\); resulting perfusion flow between 2.9 and 6.7 ml/min), injections of increasing doses of e.g. APE \(10^{-12} - 3 \times 10^{-7}\) mol, MeFur \(10^{-11} - 2 \times 10^{-7}\) mol, bis-ABE \(10^{-10} - 3 \times 10^{-7}\) mol and 4-Cl-McN-A-343 \(10^{-9} - 3 \times 10^{-7}\) mol caused a dose-dependent and reversible dilation in the rat kidney (fig. 1). Related to the maximal vasodilation by \(3 \times 10^{-7}\) mol APE (defined as 100%), which itself reversed the cirazoline-induced flow reduction by 67 \(\pm\) 17% (mean \(\pm\) S.O., \(n = 51\)), high doses of the other agents only partially reached this value, i.e. those producing more than 80% vasodilation were oxotremorine, (S)-aceclidine, (R)-NAEE, MeFur, GPE, bis-ABE and carbachol, those not reaching 80% were (S)-NAEE, arecoline, guvacoline, methacholine, 4-Cl-McN-A-343, NEN-APE and McN-A-343. The ED\(_{50}\) values of the agonists are listed in table 1.

The dose-response curves of the potent agonists, e.g. APE, MeFur and carbachol, to evoke vasodilation were more shallow than those obtained for the weaker agonists, e.g. 4-Cl-McN-A-343, (R)-NAEE and NEN-APE. One possible explanation could be that steady state conditions after injection of the lower doses of the agonists are less closely attained than at higher doses during perfusion flow. Additionally, it must be kept in mind that muscarinic agonist-induced vasodilation per se might promote agonist removal from the kidney during increased perfusion flow.

3.1.3. Effect of CHAPS

Administration of CHAPS-containing (0.2 mg/ml) Tyrode solution for 5 min reduced basal perfusion flow from 18.6 \(\pm\) 0.4 ml/min to 15.4 \(\pm\) 0.7 ml/min (mean \(\pm\) S.D., \(n = 6\)). No vasoconstrictor or vasodilator effect of bolus injections of APE, MeFur, 4-Cl-McN-A-343 and methacholine (up to \(3 \times 10^{-7}\) mol) was observed after perfusion of the kidney with CHAPS. The reduction in perfusion flow in response to continuous cirazoline administration \(10^{-7}\) M was slightly enhanced from 5.2 \(\pm\) 2.2 (control) to 4.4 \(\pm\) 1.2 ml/min following CHAPS administration (means \(\pm\) S.D., \(n = 6\)). In kidneys so treated, APE, MeFur, 4-Cl-McN-A-343 and methacholine (up to \(3 \times 10^{-7}\) mol) did not evoke vasodilation by more than 5% of the values observed for the agonists in CHAPS-untreated organs and no vasoconstrictor response to these muscarinic agonists was seen. In contrast, the vasodilator response to nitroprusside \(10^{-7}\) mol remained unchanged by CHAPS treatment (increase in perfusion flow by approximately 75% prior to and after CHAPS administration).

3.1.4. Effect of muscarinic antagonists

The antagonists did not significantly alter the vasoconstriction due to cirazoline \(10^{-7}\) M), but attenuated the vasodilation due to APE \(5 \times 10^{-9}\) mol). Concentration-response curves of the antagonists are shown in fig. 2. In most cases, high concentrations of the antagonists abolished the effect of APE by more than 80%. The \(-\log EC_{50}\) (M) values for the antagonists are summarized in table 2.

The concentration-response curves for UH-AH 37, telenzepine, p-F-HHSiD and (S)-HHD were steeper than those for the other antagonists investigated. Assuming that a steady equilibrium was reached for all antagonists during the 20-min perfusion period, the steep concentration-response curves of UH-AH 37, telenzepine, p-F-HHSiD and (S)-HHD might be explained by their weak displacement from muscarinic receptors due to bolus injection of the agonist APE because of slow off kinetics.

3.2. Guinea-pig ileum submucosal arterioles

MeFur and bis-ABE, applied in a cumulative fashion to guinea-pig ileum submucosal arteriolar preparations produced concentration-dependent dilation of the
(−)-noradrenaline-preconstricted vessel (10^{-5} \text{ M}). These responses were resistant to blockade by tetrodotoxin (10^{-6} \text{ M}) and hexamethonium (10^{-4} \text{ M}), thereby confirming the direct activation of muscarinic receptors on the submucosal arteriole. Both agonists produced a maximal vasodilation of 85 ± 4\% (MeFur; n = 6; mean ± S.E.M.) and 86 ± 3\% (bis-ABE; n = 5; mean ± S.E.M.), respectively, with EC_{50} values (± S.E.M.) of 0.24 ± 0.07 × 10^{-6} \text{ M} (n = 6) and 1.59 ± 0.29 × 10^{-6} \text{ M} (n = 5).

3.3. Potency of agonists at muscarinic M_{1}, M_{2} and M_{3} receptors

All agonist responses were characterized as muscarinic in nature in that their activities were blocked by pirenzepine (10^{-7} \text{ M}) in rabbit vas deferens, AQ-RA 741 (3 × 10^{-7} \text{ M}) in guinea-pig atrium and p-F-HHSiD (5 × 10^{-7} \text{ M}) in guinea-pig ileum. Tetrodotoxin (10^{-6} \text{ M}) and hexamethonium (10^{-4} \text{ M}) did not block agonist activities in guinea-pig atrium and ileum.

When close prostatic segments of the rabbit vas deferens were used, electrical field stimulation elicited phasic contractions of the 'rapid twitch' type, which could be inhibited concentration dependently by muscarinic agonists, e.g. APE, MeFur, bis-ABE, McN-A-343, 4-Cl-McN-A-343, oxotremorine, carbachol, (S)- and (R)-aceclidine (not shown), acting at inhibitory prejunctional M_{1} receptors (Eltze, 1988). Except for the partial agonist, (R)-aceclidine (i.e. 0.86), all other compounds behaved as full agonists. In contrast, NEN-APE, (R)- and (S)-NAEE were inactive at M_{1} receptors in rabbit vas deferens and behaved as competitive antagonists. The potencies (pD_{2} values) and apparent efficacies (i.a. values) obtained for the agonists at M_{1} receptors are listed in table 1.

**TABLE 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Rat kidney</th>
<th>pA_{2}</th>
<th>M_{1} (RVD)</th>
<th>M_{2} (RVD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>– log EC_{50} (M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Atropine</td>
<td>8.42 ± 0.08</td>
<td>8.99 ± 0.07</td>
<td>9.53 ± 0.06</td>
<td>9.05 ± 0.11</td>
</tr>
<tr>
<td>(2) 4-DAMP</td>
<td>8.30 ± 0.09</td>
<td>8.83 ± 0.17 ^c</td>
<td>9.12 ± 0.08 ^b</td>
<td>8.16 ± 0.10 ^b</td>
</tr>
<tr>
<td>(3) (R)-HHD</td>
<td>7.51 ± 0.06</td>
<td>8.35 ± 0.04 ^d</td>
<td>8.71 ± 0.05 ^c</td>
<td>6.83 ± 0.07</td>
</tr>
<tr>
<td>(4) HHSiD</td>
<td>7.26 ± 0.08</td>
<td>7.76 ± 0.06</td>
<td>7.92 ± 0.07 ^a</td>
<td>6.54 ± 0.07 ^a</td>
</tr>
<tr>
<td>(5) UH-AH 37</td>
<td>6.96 ± 0.05</td>
<td>8.07 ± 0.08 ^e</td>
<td>8.64 ± 0.08 ^c</td>
<td>7.15 ± 0.12 ^c</td>
</tr>
<tr>
<td>(6) Telenzepine</td>
<td>6.90 ± 0.04</td>
<td>7.87 ± 0.08</td>
<td>8.86 ± 0.06 ^a</td>
<td>7.51 ± 0.09 ^a</td>
</tr>
<tr>
<td>(7) Mequitamium</td>
<td>6.72 ± 0.11</td>
<td>8.14 ± 0.04</td>
<td>8.68 ± 0.08</td>
<td>8.36 ± 0.06</td>
</tr>
<tr>
<td>(8) Pirenzepine</td>
<td>6.19 ± 0.09</td>
<td>6.87 ± 0.20 ^e</td>
<td>8.08 ± 0.04</td>
<td>6.23 ± 0.08 ^e</td>
</tr>
<tr>
<td>(9) Nuvenzepine</td>
<td>6.12 ± 0.07</td>
<td>7.03 ± 0.09 ^e</td>
<td>7.74 ± 0.08 ^c</td>
<td>6.63 ± 0.10 ^c</td>
</tr>
<tr>
<td>(10) p-F-HHSiD</td>
<td>6.00 ± 0.07</td>
<td>7.49 ± 0.09 ^e</td>
<td>6.77 ± 0.07 ^c</td>
<td>6.09 ± 0.11 ^c</td>
</tr>
<tr>
<td>(11) Himbacine</td>
<td>5.89 ± 0.05</td>
<td>7.10 ± 0.06</td>
<td>8.05 ± 0.10 ^a</td>
<td>8.06 ± 0.09 ^a</td>
</tr>
<tr>
<td>(12) Iveridine</td>
<td>5.72 ± 0.11</td>
<td>7.77 ± 0.04</td>
<td>9.02 ± 0.11</td>
<td>8.72 ± 0.05</td>
</tr>
<tr>
<td>(13) AF-DX 384</td>
<td>5.72 ± 0.09</td>
<td>7.41 ± 0.07</td>
<td>8.61 ± 0.09</td>
<td>8.58 ± 0.10</td>
</tr>
<tr>
<td>(14) AQ-RA 741</td>
<td>5.40 ± 0.10</td>
<td>8.97 ± 0.07</td>
<td>8.44 ± 0.06</td>
<td>8.62 ± 0.09</td>
</tr>
<tr>
<td>(15) (S)-HHD</td>
<td>4.64 ± 0.11</td>
<td>6.07 ± 0.05 ^d</td>
<td>5.97 ± 0.04 ^d</td>
<td>5.25 ± 0.04</td>
</tr>
</tbody>
</table>

Similarly, the agonists caused negative inotropy due to stimulation of muscarinic M₂ receptors in left atrium, and smooth muscle contraction due to stimulation of M₃ receptors in ileum of the guinea-pig. Potencies (pD₂ values) and apparent efficacies (i.a. values) of the agonists at cardiac M₂ and smooth muscle M₃ receptors are also listed in table 1.

Comparison of data obtained at different muscarinic receptor subtypes (table 1) shows that most compounds did not discriminate between M₁, M₂ and M₃ receptors. However, APE was the most potent agonist, possessing a small preference for M₂ receptors (Lambrecht et al., 1993). The bivalent ligand, bis-ABE (Moser et al., 1990), exhibited high potency at M₁ and M₂ and a 5- and 6-fold lower potency at M₃ receptors, respectively (M₂ = M₁ > M₃). A different potency rank order was found for MeFur (M₃ > M₁ > M₂), methacholine (M₃ > M₂ > M₁) as well as McN-A-343 and its 4-chloro derivative (M₃ > M₂ > M₁) (Lambrecht et al., 1993). The (R)- and (S)-enantiomers of NAEE (Zorn et al., 1987) were non-selective agonists at M₂ and M₃ receptors (eudismic ratios = 11.2 and 3.9, respectively), but competitive antagonists at M₁ receptors (eudismic ratio...
3.9. NEN-APE was a potent partial agonist at \( M_2 \) receptors (pD\(_2\) = 6.48) but about 100-fold less potent at ileal \( M_3 \) receptors (pD\(_2\) = 4.65), and behaved as a competitive antagonist at \( M_1 \) receptors (pA\(_2\) = 5.81) (Wolf-Pflugmann et al., 1989). Fig. 3 summarizes graphically the results obtained with these subtype-prefering muscarinic agonists.

3.4. Affinity of antagonists at muscarinic \( M_1, M_2 \) and \( M_3 \) receptors

Neurogenic contractions of the rabbit isolated vas deferens to field stimulation could be inhibited concentration dependently by the \( M_1 \)-selective agonist, McN-A-343 (10\(^{-7}\)-2 × 10\(^{-6}\) M) (Lambrecht et al., 1993), or potentiated by carbachol (10\(^{-8}\)-2 × 10\(^{-7}\) M) (Eltze, 1988). Both effects were antagonized in a surmountable fashion by the muscarinic antagonists tested in the present study, and the pA\(_2\) values of the compounds at inhibitory prejunctional \( M_1 \) and excitatory postjunctional \( M_2 \) receptors (Eltze, 1988) are listed in table 2. Among the antagonists investigated, 4-DAMP, pirenzepine, telenzepine, UH-AH 37, HHSiD, (R)-HHD and nuvenzepine clearly showed a preference (factor \( \geq 10 \)) for \( M_1 \) receptors, whereas atropine, himbacine, mequitamium, p-F-HHSiD, AQ-RA 741, AF-DX 384 and (S)-HHD antagonized \( M_1 \) and \( M_2 \) receptor-mediated responses with similar potency (factor < 5; table 2). The affinity of idaverine appeared to be equally high for \( M_1 \) and \( M_2 \) receptors in rabbit vas deferens. All antagonists inhibited the contractions of the guinea-pig ileum evoked by acetylcholine or APE. There was a concentration-dependent parallel shift to the right of the agonist concentration-response curves without either basal tension or maximum responses being affected. The antagonists showed quite wide variations in their antimuscarinic potencies at smooth muscle \( M_1 \) receptors, their pA\(_2\) values differing by up to three orders of magnitude (table 2).

In all cases, the Schild plots were linear and most slopes of the regression lines were not significantly different from unity (P > 0.05) (table 2), indicating simple competitive antagonism at the three muscarinic receptor subtypes.

3.5. Linear regressions

In the first instance, we compared the potency of a number of agonists and antagonists at muscarinic receptors in rat kidney with their effects in resistance vessels of the submucosal arterioles of the guinea-pig small intestine. In the latter preparation, only functional muscarinic \( M_3 \) receptors have been demonstrated, the activation of which causes vasodilation (Bungardt et al., 1992). An excellent correlation resulted (r = 0.988, P < 0.001; \( \beta = 0.98 \) not significantly different from unity, P > 0.05) on comparison of the potencies of seven agonists (APE > methacholine = oxotremorine = MeFur = carbachol > bis-ABE > 4-CI-McN-A-343) to evoke vasodilation in both vascular preparations (fig. 4, top). There was also a highly significant correlation (r = 0.9498, P < 0.001; \( \beta = 1.00 \) not significantly different from unity, P > 0.05) when the potencies of eight antagonists to inhibit APE-induced vasodilation in rat kidney were compared with...
their pA₂ values for M₃ receptor antagonist in guinea-pig submucosal arterioles (fig. 4, bottom).

Comparing the potencies of muscarinic agonists to attenuate cirazoline-induced renal vasoconstriction with the potencies from contraction studies with muscarinic M₄ receptors in guinea-pig ileum (table 1), a highly significant correlation resulted (fig. 5, top; r = 0.95, P < 0.001; β = 0.95 not significantly different from unity, P > 0.001). Weak correlations were obtained when comparing agonist potencies in rat kidney with the respective pD₂ values from experiments on M₂ receptors in guinea-pig atrium (fig. 5, middle; r = 0.87, P < 0.001; β = 0.90 not significantly different from unity, P > 0.05) or on M₁ receptors in rabbit vas deferens (fig. 5, bottom; r = 0.61, P < 0.01; β = 0.81 significantly different from unity, P < 0.01).

A highly significant correlation also resulted from comparison of -log EC₅₀ values of the antagonists derived from the rat kidney experiments with their respective pA₂ values at muscarinic M₃ receptors in guinea-pig ileum (fig. 6, top; r = 0.91, P < 0.001; β = 1.24 significantly different from unity, P < 0.05). In contrast, no significant correlation was obtained between the -log EC₅₀ values of the antagonists from the rat kidney experiments and pA₂ values of the antagonists at M₁ receptors in rabbit vas deferens (fig. 6, middle; r = 0.29, P > 0.05; β = 0.27 significantly different from unity, P < 0.001). A weak correlation resulted when the antagonist potencies in rat kidney were compared with M₁ receptor affinities in rabbit vas deferens (fig. 6, bottom; r = 0.56, P < 0.01; β = 0.76 significantly different from unity, P < 0.05).

4. Discussion

4.1. General considerations

In the past, the existence of a cholinergic system within the kidney was the object of controversy (Di Bona, 1982). While there is now strong evidence for muscarinic receptors in the kidney (Garg, 1992), the potential source of acetylcholine in this organ is less clear. Specific binding of [³H]QNB has been demonstrated in rat kidney membranes. The majority of these renal muscarinic binding sites were suggested to be primarily localized postsynaptically within the smooth muscle of the renal vascular tree and to a lesser extent within cortical and medullary tubules (Yamada et al., 1986). In addition, cholinergic markers such as acetylcholinesterase, choline acetyltransferase and a high-affinity choline uptake system, have been suggested to be present in mammalian kidney (Pirola et al., 1989). Recently, muscarinic M₁ binding sites in rat kidney cortex have been identified, probably located in vascular tissue (Blankesteijn et al., 1993).

In vitro studies on the (−)-noradrenaline-preconstricted, constant-flow perfused rat kidney suggested that vasodilator muscarinic receptors mediate the endothelium-dependent decrease in perfusion pressure (Bhardwaj and Moore, 1988). The present study was therefore designed to further characterize this muscarinic receptor, using a series of subtype-prefering agonists and antagonists, including three pairs of enantiomers: (R)- and (S)-aceclidine (Lambrecht, 1976), -NAEE (Zorn et al., 1987) and -HHD (Feifel et al., 1990). The results obtained provide convincing evidence that a single functional muscarinic receptor, the M₃ subtype, mediates muscarinic vasodilation in rat renal resistance vessels.

4.2. Agonist studies

Among the agonists investigated in the three tissues containing M₁ (rabbit vas deferens), M₂ (guinea-pig left atrium) and M₃ receptors (guinea-pig ileum), the functional subtype involved can be characterized by means of four agonists exhibiting different rank orders of potencies (fig. 3). M₁: 4-Cl-McN-A-343 = bis-ABE = MeFur ≪ NEN-APE; M₂: bis-ABE > MeFur > 4-Cl-McN-A-343; M₃: MeFur > bis-ABE > 4-Cl-McN-A-343 > NEN-APE.

The agonists tested in the present study, dose dependently increased perfusion flow in rat kidney previously preconstricted by the continuous presence of cirazoline. With the exception of McN-A-343, they produced maximal effects between 63 and 96% (related to the maximal vasodilation elicited by APE; fig. 1). The rank order of potency was: APE > MeFur = methacholine = oxotremorine = carbachol > GPE = (S)-aceclidine > arecoline = bis-ABE = guvacoline > 4-Cl-McN-A-343 = (R)-NAEE = NEN-APE = (R)-aceclidine = (S)-NAEE > MeN-A-343. This is consistent with the potency profile of the agonists (a) in the guinea-pig submucosal arteriolar preparation (fig. 4), where only M₃ subtype vasodilator muscarinic receptors have been demonstrated (Bungardt et al., 1992), and (b) as observed in contraction experiments with the longitudinal smooth muscle preparation of the guinea-pig ileum (fig. 5), the functional muscarinic receptor of which has been characterized as the M₃ subtype (Eglen et al., 1992). These results suggest that muscarinic M₃ receptors mediate the vasodilation in rat kidney.

4.3. Antagonist studies

Besides some unselective antagonists, i.e. atropine, mequitamium (Renzetti et al., 1990; this study) and (S)-HHD (Feifel et al., 1990) which did not discriminate between muscarinic M₁, M₂ and M₃ receptor subtypes, pirenzepine (Hulme et al., 1990), telenzepine...
values) obtained in functional experiments with two reversibly abolished the vasorelaxant response to bolus consistent with the presence of functional M1 and M2 receptors in rat kidney. Thus it can be concluded also from the antagonist characteristics that the functional muscarinic receptor mediating vasodilation in rat perfused kidney is of the M3 subtype.

4.4. Possible localization and function of renovascular muscarinic receptors

In the rat isolated perfused kidney, (−)-noradrenaline constricts both preglomerular and postglomerular vessels, but only constriction of the former is sensitive to the Ca²⁺ channel antagonist, nisoldipine (Loutzenhisler et al., 1984). The α₁-adrenoceptor agonist, cirazoline, used in the present study has been shown to evoke pressor responses in the pithed rat which depend on both entry of extracellular Ca²⁺ and the release of Ca²⁺ from intracellular sources (Nichols and Ruffolo, 1986). Although it is reasonable to suggest that cirazoline may also exert renal microvascular actions at sites similar to (−)-noradrenaline, the localization of renovascular dilation produced by stimulation of M3 receptors cannot be deduced from our experiments. However, it has been shown previously by vessel diameter observations via television microscopy in the split hydrenephrotic rat kidney preparation that acetylcholine produced significant dilation of all preglomerular vessels (arcuate and interlobular arteries and afferent arterioles) and postglomerular efferent arterioles, its effect being greater at afferent than at efferent arterioles near the glomerulus (Fleming et al., 1987). Such a dilator pattern in response to acetylcholine is consistent with reports that it substantially increases renal blood flow but has little or no effect, on glomerular filtration rate (Burton et al., 1990). A surprising observation was that hemoglobin inhibited the effect of acetylcholine only in the efferent, but not in the afferent arteriole. Hence, acetylcholine appears to release nitric oxide (NO) only in the efferent arteriole and a different relaxing factor in the afferent arteriole (Loutzenhisler et al., 1990).

In anaesthetized rats, endogenous or acetylcholine-stimulated release of NO may play an important role in the local regulation of renal cortical blood flow (Walder et al., 1991). Infusion of acetylcholine into the renal artery of anaesthetized rats increased only cortical flow, while medullary interstitial infusion increased both cortical and papillary blood flow. Only papillary blood flow was selectively reduced by inhibition of NO synthesis, thus indicating a role in the control of papillary blood flow by local production of NO in the renal medulla. A selective reduction in papillary blood flow by M3 receptor stimulation may therefore be associated with sodium and water retention (Mattson et al., 1992).

4.5. Summary and conclusion

In summary, the present study investigated the muscarinic receptors in resistance vessels of rat isolated perfused kidney. The muscarinic receptor which mediates endothelium-dependent vasodilation of the cirazo-
line-preconstricted renal vasculature was characterized by determining the potencies of a series of subtype-preferring agonists and the affinities of various selective antagonists. The results provide convincing evidence that a single muscarinic receptor, the M₂ subtype, mediates muscarinic vasodilation in rat kidney, which disappears after destruction of the endothelium by means of detergent. In endothelium-denuded rat kidney, muscarinic agonists can evoke any vascular effect at all.

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