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# Characterization of muscarinic receptors mediating vasodilation in guinea-pig ileum submucosal arterioles by the use of computer-assisted videomicroscopy

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Muscarinic receptors of resistance vessels (submucosal arterioles, outside diameter 50–75 $\mu$m) from the guinea-pig small intestine were investigated in vitro using a computer-assisted videomicroscopy system (Diamtrak[®]). The muscarinic receptor which mediates vasodilation of precontracted [U-46619 (300 nM) or (−)-noradrenaline (10 $\mu$M)] arterioles was characterized with several muscarinic agonists and subtype-selective antagonists. The following agonists all produced equivalent maximum vasodilation (given in rank order of potency): acetylcholine = arecaidine propargyl ester (APE) > oxotremorine = (±)-muscarine = (±)-methacholine > carbachol > 4-[[N-(4-chlorophenyl)carbamoyl]oxy]-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343). 4-[[N-(3-Chlorophenyl)-carbamoyl]oxy]-2-butynyltrimethylammonium chloride (McN-A-343) and N-ethyl-guvacine propargyl ester (NEN-APE) produced minimal or no arteriolar vasodilation. The muscarinic antagonists pirenzepine, (±)-5,11-dihydro-11-[[[2-[2-((dipropylamino)methyl)-1-piperidinyl]ethyl]amino]-carbonyl]-6H-pyrido(2,3-b)(1,4)-benzodiazepin-6-one (AF-DX 384), 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepin-6-one (AQ-RA 741), p-fluoro-hexahydro-sila-difenidol (p-F-HHSiD), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and (R)- and (S)-hexahydro-difenidol [(R)-HHD, (S)-HHD] shifted the muscarine, methacholine or carbachol dose-response curve to the right in a competitive manner. Schild analysis of the data yielded $pA_2$ values for pirenzepine (6.74/6.9), AF-DX 384 (6.72), AQ-RA 741 (6.58), p-F-HHSiD (7.53/7.57), 4-DAMP (9.06), (R)-HHD (7.88/8.32) and (S)-HHD (5.52/5.88). Thus, it can be concluded that submucosal arterioles possess only the $M_3$ functional muscarinic receptor, the activation of which causes blood vessel dilation. The preparation described is considered to be a valuable new bioassay for pharmacological investigations of drug actions at muscarinic receptors in the peripheral vascular system.

## 1. Introduction

It is now generally accepted that three distinct muscarinic receptor subtypes can be distinguished by pharmacological means (Eglen and Whiting, 1986; Mitchelson, 1988; Levine and Birdsall, 1989; Lazareno et al., 1990; Lambrecht et al., 1989; Waelbroeck et al., 1989, 1990, 1991a). These three subtypes, termed $M_1$, $M_2$ and $M_3$ receptors, have also been shown to correspond to the structurally distinct cloned muscarinic receptors m1, m2 and m3, respectively (Akiba et al., 1988; Bon-

ner et al., 1987; Buckley et al., 1989; Dörje et al., 1991b). $M_1$ receptors appear to be located primarily on neuronal tissue, $M_2$ receptors have been most often localized in cardiac tissue, smooth muscle and lower brain areas and $M_3$ receptors are found predominantly in exocrine glands and smooth muscle (Eglen and Whiting, 1986; Mitchelson, 1988; Lambrecht et al., 1989; Waelbroeck et al., 1990; Dörje et al., 1991a). There is a candidate $M_4$ receptor, found in rat striatum (Waelbroeck et al., 1990), rabbit lung (Lazareno et al., 1990) and NG108-15 cells (Michel et al., 1989; Caulfield and Brown, 1991), which is considered to be the m4 gene product (Dörje et al., 1991a; Brann et al., 1988).

Each of the three subtypes, $M_1$, $M_2$ and $M_3$, can be identified in vascular preparations depending on the

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species, tissue and function examined. In general, $M_3$ receptors mediate EDRF-dependent relaxation, $M_1$ receptors mediate venous contraction and $M_2$ or $M_3$ receptors appear to inhibit noradrenaline overflow (Fernandes et al., 1991; for a review, see Eglen and Whiting, 1990). Recently, $M_3$ receptors have been implicated in endothelial-dependent vascular relaxation in the rabbit ear artery and in bovine coronary artery, mediating both endothelial-dependent relaxation as well as endothelial-independent vascular contraction (Duckles and Garcia-Villalon, 1990; Brunner et al., 1991a,b).

Muscarinic receptors, whose activation produces vasodilation, have also been demonstrated in resistance vessels of the gastrointestinal microcirculation, the submucosal arterioles of guinea-pig ileum (Neild et al., 1990). These submucosal arterioles are innervated by cholinergic enteric neurones and these neurones release acetylcholine to produce pronounced vasodilation. This vasodilation is presumably caused by activation of $M_3$ receptors present on these blood vessels although it is not yet known whether muscarinic receptors reside on vascular smooth muscle directly or on the vascular endothelium (Neild et al., 1990).

The present experiments were undertaken to provide a detailed pharmacological characterization of the muscarinic receptor subtype which mediates the cholinergic vasodilation in submucosal arterioles of guinea-pig ileum. The in vitro submucosal arteriolar preparation used (Neild et al., 1990) allows the study of receptor-mediated vasodilation by means of a computer-assisted videomicroscopy system without the need to isolate individual segments of the vascular tree.

## 2. Materials and methods

The pharmacological experiments were performed at two different institutes [Portland (U.S.A.) and Frankfurt (F.R.G.)] using nearly identical hardware. For technical reasons the experimental design used in Portland differed slightly from that in Frankfurt (see table 1). The data obtained in Portland are marked as 'method P' whereas those from Frankfurt are listed under 'method F'.

### 2.1. Tissue preparation

Submucosal plexus preparations were obtained from the small intestine of guinea-pigs that had been killed by a blow on the head and exsanguinated. Preparations were dissected as described previously (Neild et al., 1990). Briefly, the mucosa was stripped from segments (8–12 mm long, 5–7 mm wide) of the small intestine and the submucosal plexus was pulled away from the underlying circular smooth muscle. The plexus was

TABLE 1

Experimental conditions used in Portland and Frankfurt (method P and F) to determine muscarinic effects in guinea-pig ileum submucosal arterioles. For further details, see text.

|  | Method P | Method F |
|---|---|---|
| Buffer: | Physiological saline (mM) | Tyrode solution (mM) |
| NaCl | 126.0 | 137.00 |
| KCl | 5.0 | 2.68 |
| $CaCl_2$ | 2.5 | 1.80 |
| $MgCl_2$ | 1.2 | 1.05 |
| $NaHCO_3$ | 25.0 | 11.90 |
| $NaH_2PO_4$ | 1.2 | 0.42 |
| D-Glucose | 11.0 | 5.05 |
| Temperature: | 34–36°C | 32–34°C |
| Preconstriction: | U-46619 (285–300 nM) | Noradrenaline (10 $\mu$M) |
| Muscarinic agonist: | Muscarine/carbachol | Methacholine |

pinned onto transparent silicone rubber, mucosal surface down, in the base of a small organ bath (0.3–0.5 ml volume). The preparation consisted of arteriolar trees, the outside diameters of which ranged from 50–75 $\mu$m; tension applied to the connective tissue sheath resulted in approximately optimum-length conditions in the vascular network (Vanner et al., 1990). Physiological saline solution or Tyrode solution (for composition see table 1), gassed with 95% $O_2$–5% $CO_2$, flowed continuously at 32–36°C at 8–10 ml/min. Preparations were equilibrated for 45–60 min prior to initiation of pharmacological experiments.

### 2.2. Monitoring of arteriolar diameter

The outside diameter of submucosal arterioles was monitored with the Diamtrak* system as described previously (Neild, 1989; Neild et al., 1990). This system uses an Imaging Technology PCVisionPlus framegrabber board (Woburn, MA, U.S.A.) in an AT-compatible computer to digitize television images of outside edges of the arteriole and converts this result to an analog signal which is displayed on a conventional strip chart recorder. Resolution of the system was < 1 $\mu$m; the sampling rate was 10–20 Hz.

### 2.3. Agonist potencies

To preconstrict the vessel, method P used the thromboxane receptor agonist U-46619 at a concentration of 285–300 nM, whereas in method F (−)-noradrenaline (10 $\mu$M) was used. Both agonists produced 80–90% of the maximum contractile response. The noradrenaline solution was freshly prepared before each experiment. No other precautions were taken

to prevent oxidation of the catecholamine because of the high concentration used and the short duration of each experiment (Neild and Kotecha, 1989). All muscarinic agonists were applied to preconstricted vessels by superfusion for 2–4 min (per agonist concentration) with a 20-min washout period between applications. In initial experiments, all agonists were applied in a non-cumulative fashion with 4–6 concentrations of agonist applied to each vessel. In later experiments agonists were applied cumulatively because no significant differences were found between concentration-response curves obtained by either of these methods. The apparent potency of a muscarinic agonist is expressed as its $EC_{50}$ value, i.e. the molar concentration inducing 50% of the individual maximal vasodilation.

## 2.4. Antagonist affinities

Affinities for muscarinic antagonists were obtained by constructing concentration-response curves for agonists in the absence and then presence of two to four increasing concentrations of the antagonist. All antagonists were present for 12–15 min prior to agonist application. $EC_{50}$ values were used to construct Schild plots, and slopes were obtained by least-squares fit. When slopes were not significantly different from unity, the x-intercept ($pA_2$ value) was obtained by fitting the best line with unit slope (Tallarida and Murray, 1986).

## 2.5. Data analysis

The data are presented as means ± S.E.M. Differences between mean values were tested for statistical significance by using Student's t-test; $P < 0.05$ was accepted as being significant.

## 2.6. Drugs

The following drugs were used: acetylcholine chloride, carbachol chloride, eserine hemisulfate, hexamethonium bromide, (±)-muscarine chloride, oxotremorine sesquifumarate, sodium nitroprusside and tetrodotoxin (all from Sigma, St. Louis, U.S.A.); (−)-noradrenaline hydrochloride (Fluka, Buchs, Switzerland); 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F2_a$ (U-46619; Upjohn, Kalamazoo, U.S.A.); (±)-methacholine chloride (EGA-Chemie, Steinheim, F.R.G.); 4-[[N-(3-chlorophenyl)-carbamoyl]oxy]-2-butynyltrimethylammonium chloride (McN-A-343; Serva, Heidelberg, F.R.G.); 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; Research Biochemicals Inc., Natick, U.S.A.); pirenzepine dihydrochloride, (±)-5,11-dihydro-11-[[[2-[2-((dipropylamino)-methyl)-1-piperidinyl]ethyl]amino]carbonyl]-6H-pyrido-(2,3-b)(1,4)-benzodiazepin-6-one methanesulfonate (AF-DX 384), 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepin-6-one (AQ-RA 741), all provided by Thomae (Biberach, F.R.G.); the (R)- and (S)- enantiomers of hexahydro-difenidol ((R)-HHD and (S)-HHD; Tacke et al., 1989), (±)-p-fluoro-hexahydro-sila-difenidol (p-F-HHSiD; Tacke et al., 1991), arecaidine propargyl ester (APE; Mutschler and Hultzsch, 1973), N-ethyl-guvacine propargyl ester (NEN-APE; Wolf-Pflugmann et al., 1989) and 4-[[N-(4-chloro-phenyl)-carbamoyl]oxy]-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343; Nelson et al., 1976) were synthesized in our laboratories. The other chemicals were of reagent grade and were used as purchased.

# 3. Results

## 3.1. Agonists

Figure 1 shows typical vasodilations produced by muscarinic agonists applied in a cumulative fashion to guinea-pig ileum submucosal arteriolar preparations. Maximum dilations produced by muscarine (fig. 1A) were 84 ± 8% (n = 43) and by methacholine 88 ± 6% (n = 48) of the resting (non-constricted) outside diameter and this maximum response was not significantly different from the vasodilation produced by a supramaximal concentration of sodium nitroprusside (20 $\mu$M; vasodilation = 79 ± 6%, n = 14). All agonist effects were considered to be due to direct activation of muscarinic receptors on the submucosal arterioles, and not to possible indirect activation of submucosal cholinergic neurones innervating the blood vessel, because responses were not altered in the presence of hexamethonium (100 $\mu$M, n = 8) or tetrodotoxin (0.5–1 $\mu$M, n = 12).

Acetylcholine, muscarine, methacholine, carbachol, APE, oxotremorine and 4-Cl-McN-A-343 dilated submucosal arterioles to the same maximum (fig. 2; data not shown for methacholine); $EC_{50}$ values are shown in table 2. The acetylcholine-induced vasodilation was significantly enhanced in the presence of the cholinesterase inhibitor eserine (1 $\mu$M), which produced an approximately two-fold shift to the left in the concentration-vasodilation curve (fig. 2A). Higher concentrations of eserine (10 and 100 $\mu$M) had no further effect (n = 5). Vasodilations produced by the agonists were maintained for the duration of agonist application and were reproducible in the same arteriole for up to 5 h during repeated applications at intervals of 20 min. McN-A-343 and NEN-APE were virtually ineffective in producing arteriolar vasodilation; concentrations as high as 100 $\mu$M produced less than 10% of the vasodilation evoked by application of the other agonists (figs. 1C and 2).
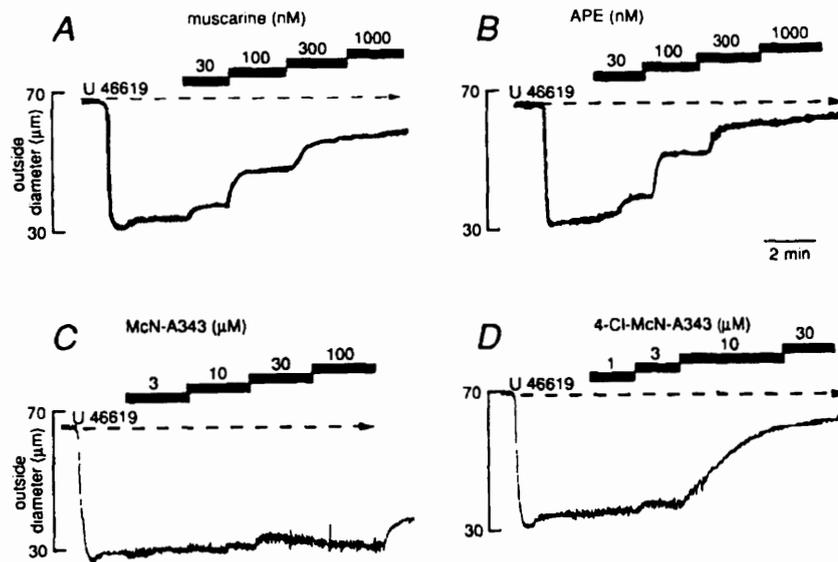
Fig. 1. Chart recordings of outside diameter of guinea-pig ileum submucosal arterioles preconstricted by 300 nM U-46619 during cumulative addition of muscarinic agonists to the superfusion solution. Vasodilations in response to muscarine (A), APE (B), McN-A-343 (C) and 4-Cl-McN-A-343 (D) are illustrated. Results in A through D obtained from different arterioles (method P).

TABLE 2

Potencies of agonists at muscarinic receptors in guinea-pig ileum submucosal arterioles and longitudinal smooth muscle. The data are presented as means ± S.E.M., the numbers of experiments are given in parentheses. i.a. = intrinsic activity.

| Agonist | Guinea-pig ileum submucosal arteriole $EC_{50}$ (nM) | | Guinea-pig ileum longitudinal smooth muscle $EC_{50}$ (nM) |
|---|---|---|---|
| | Method P | Method F | |
| Acetylcholine | 35 ± 4 (16) | – | 62 [a] |
| Muscarine | 170 ± 23 (34) | – | 250 [b] |
| Methacholine | – | 190 ± 16 (27) | 62 [c] |
| APE | 40 ± 9 (13) | 42 ± 8 (7) | 17 [d] |
| NEN-APE | – | > 100 000 (5) | inactive, $pA_2 = 6.1$ [e] |
| Carbachol | 450 ± 26 (29) | – | 100 [b] |
| Oxotremorine | 185 ± 20 (6) | – | 25 [b] |
| 4-Cl-McN-A-343 | 4900 ± 210 (9) | 18 000 ± 5 000 (6) | 4 000 [b] |
| McN-A-343 | > 100 000 (9) | > 100 000 (5) | 10 000, i.a. = 0.5 [b] |

[a] Data from Mutschler et al. (1983). [b] Data from Jokisch–Mehrling et al. (1989). [c] Lambrecht et al., unpublished data. [d] Data from Moser et al. (1989). [e] Data from Eltze et al. (1991), rat ileum.

TABLE 3

Affinity constants ($pA_2$ values) and slopes of Schild plots for antagonists at muscarinic receptors in guinea-pig ileum submucosal arterioles and longitudinal smooth muscle. The data are presented as means ± S.E.M. of 6–19 experiments.

| Antagonist | Guinea-pig ileum submucosal arteriole | | | | Guinea-pig ileum longitudinal smooth muscle $pA_2$ |
|---|---|---|---|---|---|
| | Method P | | Method F | | |
| | $pA_2$ | Slope | $pA_2$ | Slope | |
| Pirenzepine | 6.9 [a] | | 6.74 ± 0.06 | 0.95 ± 0.13 | 6.9 [b] |
| AF-DX 116 | 5.9 [a] | | – | | 6.2 [c] |
| HHSiD | 8.5 [a] | | – | | 8.0 [b] |
| AF-DX 384 | – | | 6.72 ± 0.04 | 0.95 ± 0.08 | 7.6 [c] |
| AQ-RA 741 | – | | 6.58 ± 0.05 | 1.09 ± 0.10 | 6.6 [c] |
| p-F-HHSiD | 7.57 ± 0.03 | 1.04 ± 0.11 | 7.53 ± 0.17 | 0.70 ± 0.11 | 7.8 [b] |
| (R)-HHD | 8.32 ± 0.04 | 1.04 ± 0.08 | 7.88 ± 0.10 | 0.98 ± 0.18 | 8.4 [d] |
| (S)-HHD | 5.88 ± 0.04 | 0.99 ± 0.11 | 5.52 ± 0.09 [e] | | 6.1 [d] |
| 4-DAMP | 9.06 ± 0.05 | 0.95 ± 0.06 | – | | 9.3 [f] |

[a] Data from Neild et al. (1990). [b] Data from Lambrecht et al. (1989). [c] Data from Eberlein et al. (1989). [d] Data from Feifel et al. (1990). [e] Only two concentrations of (S)-HHD were investigated due to non-specific effects of the antagonist at higher concentrations. The $pA_2$ value was therefore determined from the individual dose ratios according to Tallarida et al. (1979). [f] Data from Dörje et al. (1990).
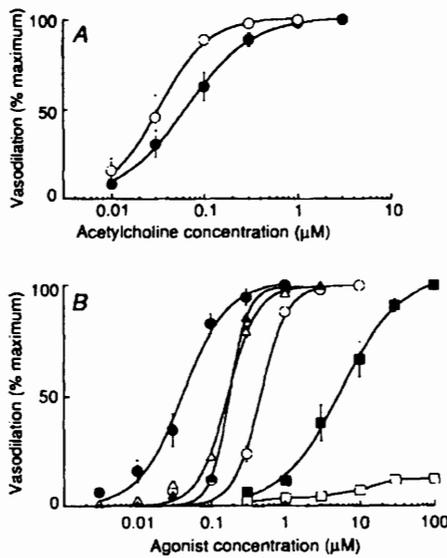
Fig. 2. Action of muscarinic agonists to dilate guinea-pig ileum submucosal arterioles (method P). (A) Concentration-dilation curves for acetylcholine in the absence (●) and presence (○) of the cholinesterase inhibitor eserine (1 μM); n = 5–10 for each point. (B) Concentration-response curves for muscarinic agonists, APE (●), muscarine (△), oxotremorine (▲), carbachol (○), 4-Cl-McN-A-343 (■) and McN-A-343 (□); n = 9–24 for each point. All points are means ± S.E.M. Some error bars were within the width of the symbols.

## 3.2. Antagonists

The antagonists investigated all shifted the agonist concentration-vasodilation curves parallel to higher concentrations without any appreciable changes of pre-
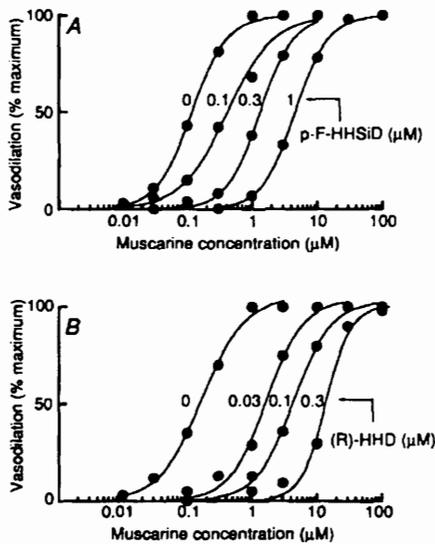


Fig. 3. Antagonism of muscarinic vasodilation by p-F-HHSiD (A) and (R)-HHD (B). Each graph shows data obtained from a single arteriole (one typical experiment), preconstricted with 300 nM U-46619 (method P).
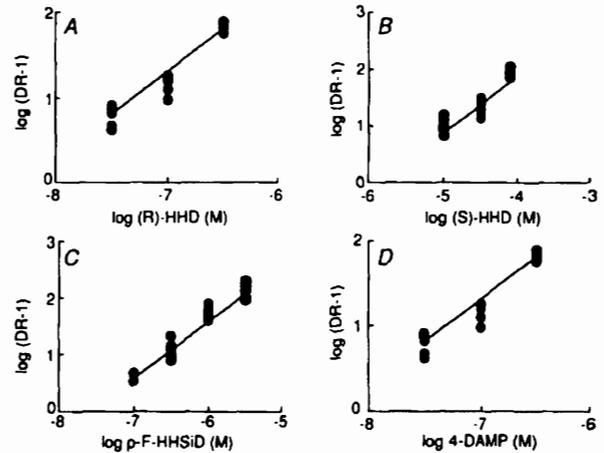


Fig. 4. Schild plots obtained for muscarinic antagonists at guinea-pig ileum submucosal arterioles preconstricted with 300 nM U-46619 using muscarine as agonist (method P). Log (dose ratio − 1) is plotted as a function of log [(R)-HHD] (A), log [(S)-HHD] (B), log [p-F-HHSiD] (C) or log [4-DAMP] (D). Lines are best fit linear regression lines, using least-squares fits.

constriction or reduction of the maximum response. This is illustrated for p-F-HHSiD and (R)-HHD in fig. 3. The effects of 4-DAMP, (R)-HHD and p-F-HHSiD were readily reversible within 20–30 min of washout even at the highest concentrations used, while those of (S)-HHD were slowly and, at the higher concentrations, only partially reversible (method P). Arunlakshana-Schild plots were linear and slopes were not significantly different from unity, except in the case of p-F-HHSiD (method F: slope = 0.7) and (S)-HHD (method P; using 10, 30, 100 and 300 μM to construct the Schild plot). However, when the highest antagonist concentration (300 μM) was omitted from the analysis, the slope was not significantly different from unity (table 3). Therefore, it was assumed that this very high concentration of (S)-HHD produced additional, nonspecific effects on submucosal arterioles. With method F is was not possible to construct Schild plots for (S)-HHD because of similar nonspecific effects occurring at concentrations higher than 10 μM, which led to unstable declining preconstrictions. Thus, only two concentrations (3 and 10 μM) were investigated and the $pA_2$ value was therefore determined from the individual dose ratios (Tallarida et al., 1979). The (R)-enantiomer of HHD displayed 250- to 500-fold higher affinity than its corresponding (S)-isomer. Schild plots for 4-DAMP, (R)-HHD, (S)-HHD and p-F-HHSiD are shown in fig. 4 (method P). Table 3 summarizes antagonist $pA_2$ values obtained from all experiments.

## 4. Discussion

Based on $pA_2$ values obtained with pirenzepine, (±)-11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-

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5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepin-6-one (AF-DX 116) and hexahydro-sila-difenidol (HHSiD) (table 3; Neild et al., 1990), we had previously suggested that submucosal arterioles of the guinea-pig ileum possess the functional $M_3$ muscarinic receptor which mediates vasodilation.

This study was designed to characterize this muscarinic receptor further, using agonists and a larger number of subtype selective antagonists including one pair of enantiomers. The experiments were carried out at two institutes; the most important differences between the methods used in Portland and Frankfurt (method P and F) were the use of (−)-noradrenaline instead of U-46619 to preconstrict the vessels and methacholine instead of muscarine or carbachol as muscarinic agonist for antagonist investigations. Furthermore there were minor differences in the concentration of $Ca^{2+}$ and $K^+$ in the buffers (1.8 vs. 2.5 mM, 2.68 vs. 5 mM, respectively; table 1). However, both methods produced the same agonist potencies and antagonist affinities (tables 2 and 3).

The results from the present study provide convincing evidence that a single type of muscarinic receptor, the $M_3$ subtype, mediates the cholinergic vasodilation in these submucosal arterioles.

The agonists used in the present experiments, with the exception of McN-A-343 and NEN-APE, acted as full agonists in producing arteriolar vasodilation. The rank order of potency was acetylcholine = APE > oxotremorine = muscarine = methacholine > carbachol > 4-Cl-McN-A-343. This is the same agonist profile as observed in functional experiments with the guinea-pig ileal longitudinal smooth muscle preparation (table 2), the functional muscarinic receptor of which has been characterized as the $M_3$ subtype. McN-A-343 was ineffective in producing vasodilation in submucosal arterioles. This is consistent with its ineffectiveness in producing endothelial-dependent relaxation in rabbit ear artery or endothelial-independent contraction in bovine coronary artery (Duckles, 1988). McN-A-343 has been proposed to act as a selective $M_1$ agonist (Jokisch-Mehrling et al., 1989) but exhibits variable responses at all muscarinic receptor subtypes, depending upon the receptor reserve of the preparation (Eglen and Whiting, 1986). The $M_2$ and $M_3$ activity reported varies from no agonistic action (Eglen et al., 1987) up to a partial agonism ($pD_2 = 4.8$ and 5.0, i.a. $= 0.5$ at both $M_2$ and $M_3$ receptors (Jokisch-Mehrling et al., 1989)). The weak agonism at $M_2$ and $M_3$ receptors of McN-A-343 might explain its very low activity at submucosal arterioles and may exclude the $M_1$ subtype for mediating the vasodilation. NEN-APE also shows an interesting activity profile at muscarinic receptors. At $M_2$ receptors it acts as a potent partial agonist (rabbit vas
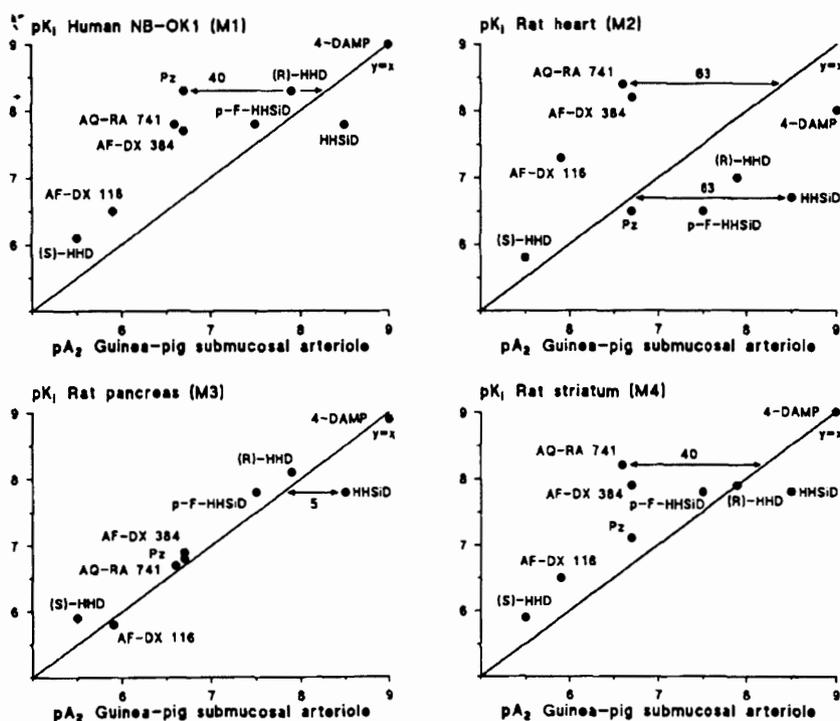
Fig. 5. Comparison of functional antagonist affinities (pA$_2$ values, table 3) obtained for pirenzepine (Pz), AF-DX 116, HHSiD, 4-DAMP, AF-DX 384, AQ-RA 741, p-F-HHSiD, as well as (R)- and (S)-HHD, at guinea-pig ileum submucosal arterioles (this study and Neild et al., 1990) to their binding affinity (pK$_i$ values) determined in radioligand binding assays at $M_1$, $M_2$, $M_3$ and $M_4$ receptors (Waelbroeck et al., 1990, 1991a,b and personal communication).

deferens: $pD_2 = 6.3$, i.a. $= 1.1$ or rat left atrium: $pD_2 = 6.6$, i.a. $= 0.8$). At $M_1$ (rabbit vas deferens) and $M_3$ (rat ileum) receptors it behaves as a competitive antagonist ($pA_2 = 5.8$ and $pA_2 = 6.1$, respectively) (Eltze et al., 1991). These findings might explain its lack of agonistic activity at muscarinic receptors in submucosal arterioles.

All antagonists investigated acted as pure competitive antagonists of the methacholine-, muscarine- or carbachol-induced vasodilation. Moreover, since the slopes of Arunlakshana–Schild regression lines did not significantly differ from unity (with exception of p-F-HHSiD, method F), the presence of a mixture of functional subtypes appears to be unlikely. Table 3 shows that there is a remarkable agreement in $pA_2$ values for the antagonists studied in submucosal arterioles and in guinea-pig ileum longitudinal smooth muscle preparations. It is also worth noting that these values are very similar to those obtained for the cloned m3 muscarinic receptor (Dörje et al., 1991b; Buckley et al., 1989; Wess et al., 1991). Figure 5 shows a comparison of the $pA_2$ values determined at submucosal arterioles and the corresponding binding affinities ($pK_i$ values) obtained in radioligand binding assays at $M_1$, $M_2$, $M_3$ and $M_4$ receptors (data from Waelbroeck et al., 1990, 1991a,b, and personal communication). For receptor identity, the data points should not significantly deviate from the theoretical equality line ($y = x$). The distance to this line (given in fig. 5 as antilog of the difference between $pA_2$ and $pK_i$ values) is a direct measure of receptor selectivity. The affinity of pirenzepine ($pA_2 = 6.74$) for the muscarinic receptor in submucosal arterioles was 40-fold lower than that observed at $M_1$ (human NB-OK1 cells) receptors, thereby excluding the presence of functional $M_1$ receptors. The evident discrimination for AQ-RA 741 (63-fold lower than observed at $M_2$ and 40-fold lower than determined at $M_4$ receptors), AF-DX 384 and HHSiD (63-fold higher than observed at $M_2$ receptors) furthermore clearly excludes $M_2$ or $M_4$ receptors from mediating the vasodilation. A highly significant correlation was found for the $M_3$ (rat pancreas) preparation only. Calculation of the eudismic index for the two enantiomers of HHD (method P: 2.44, method F: 2.36) and comparison of these values with data obtained in the same radioligand assay ($M_1$: 2.2, $M_2$: 1.2, $M_3$: 2.2, $M_4$: 2.0) also exclude the $M_2$ subtype and favour the $M_3$ subtype for mediating the vasodilation. Thus, it can be concluded that the functional muscarinic receptor mediating vasodilation in guinea-pig ileum submucosal arterioles is of the $M_3$ subtype.

Depending on species, tissue and function measured, each of the three subtypes, $M_1$, $M_2$ and $M_3$, have been identified on vascular smooth muscle. In general, $M_1$ receptors mediate venous contraction, $M_2$ or $M_3$ receptors appear to inhibit noradrenaline over-

flow and direct EDRF-dependent relaxations are mediated in the majority of preparations by $M_3$ receptors (Fernandes et al., 1991; for a review, see Eglen and Whiting, 1990). Recent studies report $M_3$ receptor-mediated relaxation of the feline middle cerebral artery (Dauphin and Hamel, 1990) and of bovine coronary artery (Brunner et al., 1991a). In addition, a study of muscarinic relaxation in the rabbit ear artery has shown $pA_2$ values for pirenzepine (6.8), AF-DX 116 (6.0) and HHSiD (8.2) (Duckles and Garcia-Villalon, 1990) similar to those determined in submucosal arterioles (this study). Therefore our results obtained with muscarinic agonists and antagonists are in good agreement with these earlier findings.

In conclusion, the present study investigated the muscarinic receptors of resistance vessels in guinea-pig ileum by the use of computer-assisted videomicroscopy. The muscarinic receptor which mediates vasodilation of the precontracted submucosal arterioles was characterized by determining the potencies of a series of muscarinic agonists and the affinities of various selective muscarinic antagonists. The results provide convincing evidence that a single type of muscarinic receptor, the $M_3$ subtype, mediates the cholinergic vasodilation in submucosal arterioles of the guinea-pig ileum. Thus, the application of computer-assisted videomicroscopy for tracking the diameter of small resistance vessels (i.e. arterioles) is a useful new method for pharmacological investigations of drug actions at muscarinic receptors in the peripheral vascular system.

## Acknowledgements

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