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Contraction of guinea-pig gallbladder: muscarinic M_3 or M_4 receptors?

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Abstract

The muscarinic receptor mediating contraction of the guinea-pig isolated gallbladder, currently being disputed to belong either to the M_3 or M_4 subtype, was characterized by subtype-preferring agonists and discriminating antagonists. Highly significant correlations of agonist potencies to contract the gallbladder, e.g., arecaidine propargyl ester, oxotremorine, 5-methylfurtrethonium > arecoline, arecaidine 2-butyne-1,4-diyl bisester > (*R*)-nipecotic acid ethyl ester > 4-[[*N*-(4-chlorophenyl)carbamyl]oxy]-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343), (*S*)-nipecotic acid ethyl ester > 4-[[*N*-(3-chlorophenyl)carbamoyl]oxy]-2-butynyltrimethylammonium chloride (McN-A-343) were found with muscarinic M_3 receptors mediating contraction of the guinea-pig ileum and vasodilation in rat perfused kidney. Functional affinities at guinea-pig gallbladder muscarinic receptors of antagonists known to distinguish between native or cloned muscarinic M_3/m3 and M_4/m4 receptors, e.g., himbacine, methoctramine, mefurtramine, tripitramine, idaverine, zamifenacin and 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepin-6-one (AQ-RA 741), were consistent with those at guinea-pig ileal muscarinic M_3 receptors but not with published data at recently defined muscarinic M_4 receptors in rabbit anococcygeus muscle or at muscarinic M_1 and M_2 receptors in rabbit vas deferens. Antagonist affinities at guinea-pig gallbladder correlated also best with published binding data on native or cloned muscarinic M_3/m3 receptors but not with those for muscarinic M_4/m4 receptors. The agonist potencies and antagonist affinities suggest that smooth muscle contraction elicited by muscarinic stimuli in guinea-pig gallbladder is mediated by functional muscarinic M_3 receptors. © 1997 Elsevier Science B.V.

Keywords: Muscarinic M_3 receptor; Muscarinic M_4 receptor; (Agonist); (Antagonist); Gallbladder; (Guinea pig)

1. Introduction

On the basis of pharmacological criteria, muscarinic receptors have been divided into at least three subtypes, termed M_1, M_2 and M_3. Additionally, molecular biological techniques have given evidence for the existence of at least five genes encoding muscarinic m1–m5 receptors that are expressed in distinct parts of the brain and body (Bonner, 1989; Hulme et al., 1990). The m1–m3 gene products correspond to the pharmacologically defined muscarinic M_1–M_3 receptors, from which muscarinic M_3 receptors mainly control salivation and intestinal motility (Brann et al., 1993; Levey, 1993). After pharmacological candidates for the muscarinic M_4 receptor have been tentatively identified in rat striatum, rabbit lung and some cell lines (Hulme et al., 1990; Lazareno et al., 1990; Waelbroeck et al., 1990; Dörje et al., 1991a,b), the inhibition of Ca-current in neuroblastoma × glioma hybrid (NG 108-15) cells (Caulfield and Brown, 1991), and the non-adrenergic, non-cholinergic (NANC) relaxation in rabbit anococcygeus muscle (Gross et al., 1995) have been suggested to represent functional methods for native muscarinic M_4 receptors, whereas a functional equivalent of the m5 gene product has yet to be identified (Buckley et al., 1989; Dörje et al., 1991b).

Cholinergic innervation controls gallbladder motility, but the nature of the muscarinic receptor subtype involved in contraction of guinea-pig is still a matter of debate (Eglen et al., 1996). Both binding and functional studies have tried to characterize muscarinic receptors in this tissue as belonging either to subtype M_3 (Von Schrenck et al., 1993, 1994) or to subtype M_4 (Kurtel et al., 1990; Oktay et al., 1993, 1995; Özkutlu et al., 1993; Akbulut et al., 1995). Particularly, the potencies of muscarinic receptor antagonists to displace binding of the ligand [^{3}H]*N*-methylscopolamine and to reverse carbachol-evoked contraction in guinea-pig gallbladder smooth muscle suggested a muscarinic M_3 receptor to be involved in these effects (Von Schrenck et al., 1993). This hypothesis was

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further reinforced by the finding that carbachol-induced contraction of this tissue is accompanied by an increase in phosphatidylinositol turnover, but is insensitive to pertussis toxin pretreatment of the animals (Von Schrenck et al., 1994). Karahan et al. (1991) reported atypical affinities of a series of antagonists at muscarinic receptors mediating contraction of the guinea-pig common bile duct, yet concluded that muscarinic M_3 receptors were involved.

On the contrary, other studies have suggested that not muscarinic M_3, but M_4 receptors mediate contraction of guinea-pig gallbladder to acetylcholine and carbachol (Kurtel et al., 1990; Oktay et al., 1993, 1995; Özkutlu et al., 1993; Akbulut et al., 1995). Analysis of [^{3}H]quinuclidinyl benzilate displacement curves with pirenzepine revealed the existence of two binding sites in this tissue, one is suggested to belong to muscarinic M_2 receptors, the other (putative muscarinic M_4 receptor) being ascribed to mediate smooth muscle contraction (Oktay et al., 1993, 1995). However, all functional experiments accompanying these binding studies mentioned were performed either with no real antagonist affinities being determined (Von Schrenck et al., 1993) or with a too small number of muscarinic receptor antagonists (Oktay et al., 1993; Özkutlu et al., 1993). Additionally, these studies either contained antagonists, namely silahexocyclium, 4-diphenylacetoxy-*N*-methylpiperidine methiodode (4-DAMP), hexahydro-sila-difenidol (HHSiD) and its *para*-fluoro derivative (*p*-F-HHSiD), which of course distinguish muscarinic M_3 from M_2 receptors, but not muscarinic M_3 from possibly relevant M_4 receptors, or were performed with but few antagonists exhibiting moderate selectivity for native or cloned muscarinic M_4/m4 over M_3/m3 receptors, i.e., pirenzepine (3-fold), 11-[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl)-5,11-dihydro-6H-pyrido-(2.3-b)(1.4)benzodiazepin-6-one (AF-DX 116) (6-fold) and methoctramine (9-fold, Table 2; for all antagonists mentioned but not listed in Table 2, references are also given in the legend of this table). Therefore, due to either missing binding affinities or a small number of only moderately discriminating antagonists used in these mentioned studies, both conclusions of either muscarinic M_3 or M_4 receptors being involved in guinea-pig gallbladder contraction are not very convincing. We have therefore re-evaluated these controversial suggestions with respect to the participation of muscarinic M_3 or M_4 receptors.

For a detailed functional characterization of the muscarinic receptor responsible for smooth muscle contraction of guinea-pig gallbladder we investigated its contractile response to various subtype-preferring agonists, e.g., arecaidine propargyl ester (APE), 5-methylfurtrethonium, arecaidine 2-butyne-1,4-diyl bisester (bis-ABE), 4-[[*N*-(4-chlorophenyl)carbamyl]oxy]-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343) and *N*-ethyl-guvacine propargyl ester (NEN-APE), the potency rank order of which at muscarinic M_3 receptors present in guinea-pig ileum and rat kidney has previously been shown to be identical, but different from that at muscarinic M_1 receptors in rabbit vas deferens and from that at muscarinic M_2 receptors in guinea-pig atrium (Eltze et al., 1993). Additionally, the affinities of a number of appropriate antagonists capable to discriminate 3–10-fold (pirenzepine, methoctramine, ±)-5,11-dihydro-1[[(2-[2-[(dipropylamino)methyl]-1-iperidinyl)ethyl]amino]carbonyl]-6H-pyrido(2,3-b)(1,4)benzodiazepin-6-one (AF-DX 384), idaverine, secoverine and zamifenacin) and more than 10-fold (himbacine, 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepin-6-one (AQ-RA 741), tripitramine and mefurtramine) between native or cloned muscarinic M_3/m3 and M_4/m4 receptors (for references, see legend of Table 2), were determined in guinea-pig gallbladder and compared with their functional affinities at muscarinic M_3 receptors in guinea-pig ileum (Eltze et al., 1993), with muscarinic M_4 receptors mediating NANC-relaxation in rabbit anococcygeus muscle (Gross et al., 1995), and with muscarinic M_1 and M_2 receptors in rabbit vas deferens (Eltze, 1988) as well with published binding affinities of the compounds at native muscarinic M_3 and M_4 receptors or cloned and stably expressed human muscarinic m3 and m4 receptors.

2. Materials and methods

2.1. Guinea-pig gallbladder strip

The gallbladder was removed from guinea-pigs (male, 400–600 g) and cut into 6 longitudinal strips (2×10 mm) which were mounted into water-jacketed organ baths at 37°C filled with 10 ml of Krebs solution of the following composition (mM): NaCl 117.6, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 11.7, gassed with a mixture of 95% O_2/5% CO_2. The preparations were fixed under a resting tension of 1 g and were allowed to equilibrate for at least 1 h before isometric contractions to cumulatively added concentrations of muscarinic receptor agonists (0.5 log increments) were performed in 45-min intervals. Force recordings by isometric transducers (Model K 30, H. Sachs Elektronik, Freiburg, Germany) were displayed on 2-channel flat-bed chart recorders (Model BD 41, Kipp and Zonen, Delft, Netherlands). pD_2 values of agonists ($-\log EC_{50}$ values of half-maximal contraction) were determined graphically from semilogarithmic plots of agonist concentration–response curves. The apparent agonist efficacies (intrinsic activity, i.a. values) were determined by comparing their maximum contractile response with that to carbachol (i.a. = 1.00). In antagonist experiments, three to five different antagonist concentrations were equilibrated with the tissue for 30 min before repeating the carbachol concentration–response curve. Generally, only two different antagonist concentrations, spaced at least 10-fold, were tested on one individual gallbladder strip.

2.2. Guinea-pig ileum, rat perfused kidney and rabbit vas deferens

Isotonic contractions of guinea-pig ileal longitudinal muscle strips to cumulatively added muscarinic receptor agonists were performed in 30 min intervals and pD_2 values were determined graphically from semilogarithmic plots. Intrinsic activities of the agonists were related to the maximal effect elicited by arecaidine propargyl ester (APE; i.a. = 1.00). For the assessment of antagonist affinity (pA_2), concentration–response curves to APE were performed in the absence and presence of the antagonists equilibrated with the tissue for 20 min (Eltze et al., 1993). Most of the agonist and antagonist data used for comparison were taken from this paper. Additionally, the agonists pilocarpine and (±)-muscarine and the antagonists tripitramine and zamifenacin were investigated.

The potency of muscarinic receptor agonists to reverse vasoconstriction evoked by cirazoline (10^{-7} M) in isolated, constant-pressure perfused rat kidney was studied using the previously described technique (Eltze et al., 1993). Briefly, once the vasoconstriction to continuously present cirazoline had stabilized, increasing doses of the test drugs (100 μl) were injected within 2 s into the renal inflow tract and the resulting percent reversal of vasoconstriction related to the maximal effect elicited by 3×10^{-7} mol APE (100%) determined. $-\log ED_{50}$ (mol) values for half-maximal reversal of vasoconstriction were calculated from non-linear regression analysis. Most agonist data used for comparison were taken from this paper. Additionally, the potency of (±)-muscarine was determined.

Antagonist affinities at muscarinic M_1 and M_2 receptors were obtained from concentration–response curves either to McN-A-343 for inhibition (muscarinic M_1 receptors) or to carbachol for potentiation (muscarinic M_2 receptors) of neurogenic contractions of the field-stimulated rabbit vas deferens in the absence and presence of antagonists equilibrated for 45 min with the tissue (Eltze, 1988). Most of the antagonist data used for comparison were taken from this paper and from Eltze et al. (1989, 1993) and Eltze and Galvan (1994). Additionally, the affinities of tripitramine and zamifenacin were determined.

2.3. Antagonist affinities and linear regressions

Schild plots were constructed to estimate the pA_2 value and the slope β of the regression line from each experimental series (Arunlakshana and Schild, 1959). The pA_2 values quoted in Table 2 were calculated from Schild plots in which the slopes of the regression lines were constrained to 1.00. In those cases where the slope of the Schild plot differed significantly from unity ($P < 0.05$), pA_2 values determined from the constrained regression lines ($\beta = 1.00$) should be regarded as approximations.

The slope β of the regression line of data comparing two sets of antagonist affinities was calculated by the least-squares method. Calculation of the correlation coefficient r and a t-test for significance of the difference of the slope from unity were performed to test for receptor identity or non-identity in different tissues.

2.4. Drugs

Arecaidine propargyl ester HBr (APE), 4-[[*N*-(3-chlorophenyl)carbamoyl]oxy]-2-butynyltrimethylammonium chloride (McN-A-343) (Research Biochemicals International, Cologne, Germany). Guvacine propargyl ester HBr (GPE), guvacoline HBr, *N*-ethyl-guvacine propargyl ester HBr (NEN-APE), 4-[[*N*-(4-chlorophenyl)carbamyl]oxy]-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343) and arecaidine 2-butyne-1,4-diyl bisester *p*-toluene sulfonate (bis-ABE) were kindly supplied by Prof. G. Lambrecht (Frankfurt am Main, Germany). Oxotremorine sesquifumarate, pilocarpine chloride were from Merck (Darmstadt, Germany). (*R*)- and (*S*)-nipecotic acid ethyl ester tartrate ((*R*)- and (*S*)-NAEE) were kindly donated by Prof. P. Krogsgaard-Larsen (Copenhagen, Denmark). Methacholine chloride (EGA Chemie, Steinheim, Germany). Pirenzepine diHCl, (±)-5,11-dihydro-1[[(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) (Prof. B. Wetzel, Thomae, Biberach, Germany). Himbacine HCl was kindly donated by Prof. W.C. Taylor (University of Sydney, Sydney, Australia). Tripitramine tetraoxalate and mefurtramine tetraoxalate were kindly supplied by Prof. C. Melchiorre (Bologna, Italy). Secoverine HCl, idaverine (Duphar, Weesp, Netherlands). Zamifenacin fumarate was kindly provided by Pfizer (Sandwich, UK). 5-Methylfurtrethonium iodide (Byk Gulden, Konstanz, Germany). All other drugs (arecoline HBr, (±)-muscarine chloride and carbamoylcholine chloride (carbachol), hexamethonium bromide and tetrodotoxin) were purchased from Sigma (Munich, Germany).

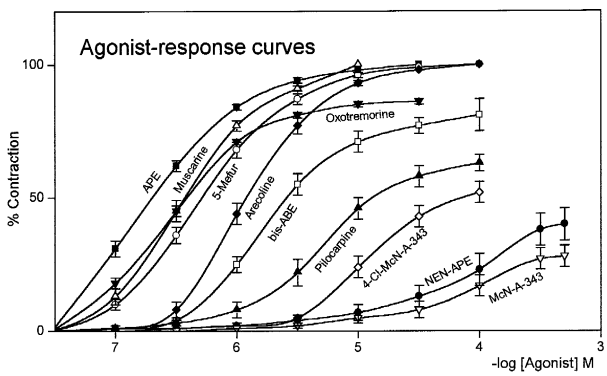

Fig. 1. Concentration–response curves for the contractile effect of muscarinic receptor agonists, with responses being expressed as a percentage of the maximum contraction elicited by carbachol (= 100%) in the isolated guinea-pig gallbladder. Given are means ± S.E.M. for $n = 18–24$. Abbreviation: 5-Mefur, 5-methylfurtrethonium.

3. Results

3.1. Guinea-pig gallbladder

3.1.1. Effect of muscarinic receptor agonists

Cumulative administration of muscarinic receptor agonists evoked sustained contractions of isolated guinea-pig gallbladder strips. In most cases, reproducible responses were obtained during the third concentration–response curve which then remained constant during the experiment. Time-matched controls by various muscarinic receptor agonists revealed no time-dependent change of sensitivity of the tissue. The maximum contractile effect produced by the most potent agonists, e.g., APE, 5-methylfurtrethonium, GPE, carbachol, methacholine and (±)-muscarine (intrinsic activity i.a. = 1.00), was not reached by oxotremorine, bis-ABE and pilocarpine (i.a. = 0.86, 0.81 and 0.63, respectively; Fig. 1). 4-Cl-McN-A-343 behaved as weak partial agonists with i.a. value of 0.52, whereas the weakest agonists, NEN-APE and McN-A-343 at 5×10^{-4} M, did not reach more than 39 and 28% of maximum respectively, the contractile effect of which could not be further increased (Fig. 1 and Table 1).

Table 1
Potencies of muscarinic receptor agonists to contract the guinea-pig gallbladder in comparison with those to contract the guinea-pig ileum and to evoke vasodilation in rat perfused kidney

Drug	pD_2 (i.a.)		Rat kidney $-\log ED_{50}$ (mol) (% max. effect)
	GP gallbladder	GP ileum	
APE	6.60 (6.1–7.1) (1.00)	7.58 (7.5–7.6) (1.00)	9.44 (8.8–9.9) (100)
Oxotremorine	6.55 (6.3–6.8) (0.86 ± 0.07)	7.39 (7.1–7.7) (1.00)	8.56 (7.7–9.4) (95 ± 5)
GPE	6.48 (6.0–6.9) (1.00)	7.13 (6.9–7.3) (1.00)	8.25 (7.2–9.3) (83 ± 15)
(±)-Muscarine	6.46 (6.0–6.9) (1.00)	7.14 (6.8–7.5) (1.00)	8.52 (7.2–9.8) (80 ± 5)
5-Methylfurtrethonium	6.29 (5.8–6.8) (1.00)	7.62 (7.5–7.7) (1.00)	8.65 (7.7–9.6) (80 ± 12)
Carbachol	6.14 (5.7–6.6) (1.00)	6.67 (6.6–6.7) (1.00)	8.55 (7.5–9.6) (80 ± 14)
Methacholine	6.02 (5.5–6.6) (1.00)	7.51 (7.2–7.8) (1.00)	8.60 (7.1–9.9) (64 ± 6)
Arecoline	5.94 (5.5–6.4) (1.00)	6.46 (6.3–6.6) (1.00)	7.77 (6.9–8.7) (77 ± 7)
Guvacoline	5.78 (5.3–6.2) (1.00)	6.43 (6.1–6.7) (1.00)	7.58 (6.4–8.7) (69 ± 6)
bis-ABE	5.76 (5.4–6.1) (0.81 ± 0.14)	6.27 (6.1–6.4) (0.89 ± 0.09)	7.70 (7.0–8.4) (79 ± 6)
(*R*)-NAEE	5.34 (4.7–5.9) (0.66 ± 0.03)	5.78 (5.7–5.9) (1.00)	6.70 (6.5–6.9) (92 ± 2)
Pilocarpine	5.34 (4.8–5.9) (0.63 ± 0.03)	5.98 (5.8–6.2) (0.96 ± 0.02)	n.t.
(*S*)-NAEE	4.98 (4.5–5.5) (0.73 ± 0.03)	5.19 (5.1–5.3) (0.93 ± 0.04)	6.46 (6.1–6.9) (73 ± 8)
4-Cl-McN-A-343	4.94 (4.5–5.4) (0.52 ± 0.14)	5.35 (5.2–5.5) (1.00)	6.82 (6.2–7.4) (63 ± 10)
NEN-APE	4.19 (3.4–5.0) (0.39 ± 0.20)	4.65 (4.4–4.9) (0.46 ± 0.08)	6.60 (6.2–7.0) (71 ± 12)
McN-A-343	4.19 (3.2–5.1) (0.28 ± 0.14)	4.96 (4.8–5.1) (0.51 ± 0.07)	6.19 (5.6–6.7) (51 ± 12)

Given are means with 95% confidence limits or S.D. of $n = 18$–24 experiments in guinea-pig gallbladder and ileum and $n = 6$–7 in rat kidney. Maximal effects in guinea-pig gallbladder and ileum (i.a. values) are related to carbachol and APE, respectively (i.a. = 1.00), and those in rat kidney to APE (100%). Most data for the agonists in guinea-pig ileum and rat kidney were taken from Eltze et al. (1993). Additionally, (±)-muscarine and pilocarpine were investigated in these tissues. n.t. = not tested.

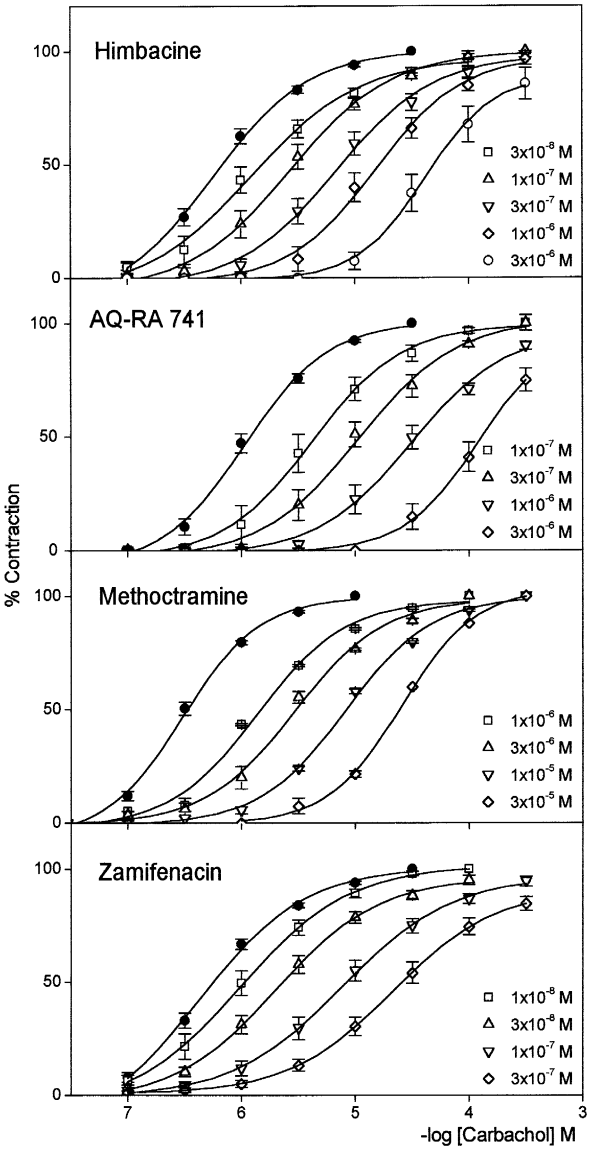

Fig. 2. Representative concentration–response curves of carbachol to evoke contraction of the isolated guinea-pig gallbladder in the absence (filled circles) or presence of increasing concentration (open symbols) of himbacine, AQ-RA 741, methoctramine and zamifenacin equilibrated with the tissue for 30 min. Given are means ± S.E.M. of $n = 6$–18 for the control and $n = 3$–9 in the presence of each concentration of the antagonists.

Table 2
pA_2 values (means ± S.E.M. with slopes β of regression lines in brackets) from constrained Schild plots ($\beta = 1.00$) for competitive antagonism at muscarinic receptors in guinea-pig gallbladder in comparison with those for muscarinic M_3 receptors in guinea-pig ileum and with their published binding constants (pK_i values, means ± S.D., with the number of pooled data in parentheses) for native or cloned human muscarinic M_3/m3 and M_4/m4 receptors

Antagonist	pA_2		Native or cloned receptors (pK_i)	
	GP gallbladder	GP ileum	M_3/m3	M_4/m4
Pirenzepine	6.64 ± 0.08 ($y = 0.89$)	6.87 ± 0.20 ($y = 0.89$)	6.83 ± 0.17 (15) (Selectivity = 3)	7.33 ± 0.26 (17)
Himbacine	7.41 ± 0.12 ($y = 0.81$) [a]	7.10 ± 0.09 ($y = 0.98$)	6.88 ± 0.28 (5) (Selectivity = 19)	8.16 ± 0.29 (6)
AF-DX 384	7.68 ± 0.09 ($y = 1.00$)	7.41 ± 0.07 ($y = 0.98$)	7.28 ± 0.48 (3) (Selectivity = 9)	8.23 ± 0.37 (5)
AQ-RA 741	7.31 ± 0.12 ($y = 0.97$)	6.97 ± 0.07 ($y = 0.87$)	6.84 ± 0.23 (6) (Selectivity = 19)	8.12 ± 0.24 (7)
Methoctramine	6.31 ± 0.09 ($y = 0.83$) [a]	6.00 ± 0.08 ($y = 1.04$)	6.38 ± 0.39 (12) (Selectivity = 9)	7.31 ± 0.59 (15)
Tripitramine	6.99 ± 0.11 ($y = 1.14$)	6.67 ± 0.09 ($y = 0.86$) [a]	6.70 ± 0.54 (5) (Selectivity = 18)	7.96 ± 0.17 (4)
Secoverine	7.95 ± 0.09 ($y = 0.95$)	7.81 ± 0.09 ($y = 0.96$)	8.05 ± 0.28 (3) (Selectivity = 5)	8.71 ± 0.53 (4)
Mefurtramine	6.48 ± 0.05 ($y = 0.76$) [a]	6.41 ± 0.03 ($y = 1.06$)	6.40 (1) (Selectivity = 13)	7.50 (1)
Idaverine	7.63 ± 0.11 ($y = 0.95$)	7.77 ± 0.04 ($y = 1.03$)	7.11 ± 0.02 (2) (Selectivity = 10)	8.13 (1)
Zamifenacin	8.13 ± 0.03 ($y = 1.17$)	8.26 ± 0.07 ($y = 0.96$)	8.13 ± 0.33 (3) (Selectivity = 0.13)	7.24 ± 0.76 (2)

The selectivity of the antagonists for muscarinic M_4/m4 over M_3/m3 receptors is also indicated. Results are the means ± S.E.M. of $n = 12$–16 in gallbladder and ileum for each pA_2 determination. Most data for the antagonists on guinea-pig ileum were taken from Eltze et al. (1989, 1993) and Eltze and Galvan (1994). Data on native or cloned and expressed human muscarinic M_3/m3 and M_4/m4 receptors were taken from published studies using rat submandibular glands and pancreas (M_3), rabbit peripheral lung, rat striatum and NG 108-15 cells (M_4), and transfected CHO and NIH 3T3 cells stably expressing cloned human muscarinic m3 and m4 receptors. Refs.: Buckley et al., 1989; Michel et al., 1989; Waelbroeck et al., 1989, 1990; Hulme et al., 1990; Lazareno et al., 1990; Caulfield and Brown, 1991; McKinney et al., 1991; Lucot et al., 1991; Miller et al., 1991; Bolden et al., 1992; Doods et al., 1993; Lazareno and Birdsall, 1993; Melchiorre et al., 1993, 1995; Maggio et al., 1994; Minarini et al., 1994; Pfaff et al., 1995; Wallis, 1995; Watson et al., 1995; Bräuner-Osborne and Brann, 1996; Eglen et al., 1996; Esqueda et al., 1996; Nunn et al., 1996.
[a] Slope β significantly different from unity ($P < 0.05$).

3.1.2. Effect of muscarinic receptor antagonists

In antagonist experiments, carbachol was used as agonist, the contractile response of which was not significantly ($p > 0.05$) affected by prior incubation for 30 min with tetrodotoxin (10^{-6} M) or hexamethonium (3×10^{-5} M), excluding the involvement of neuronal nicotinic or muscarinic receptors (not shown). The antagonists listed in Table 2 caused parallel shifts to the right of the carbachol concentration–response curve without having a contractile effect per se or affecting the maximum contraction caused by the agonist, indicating competitive antagonism at muscarinic receptors in guinea-pig gallbladder. Examples of four representative antagonists, himbacine, AQ-RA 741, methoctramine and zamifenacin are shown in Fig. 2. The regression lines for a number of antagonists, e.g., zamifenacin, AF-DX 384, idaverine, AQ-RA 741, tripitramine, pirenzepine and methoctramine, are shown in Fig. 3. Also the Schild plots for all other antagonists listed in Table 2 were linear through the concentration range tested and gave no indication of the presence of multiple subtypes of muscarinic receptors in this tissue. With the exception of himbacine, methoctramine and mefurtramine, the slopes (β) were not significantly different from unity ($P > 0.05$). The pA_2 values for the antagonists, as calculated from constrained regression lines, are listed in Table 2.

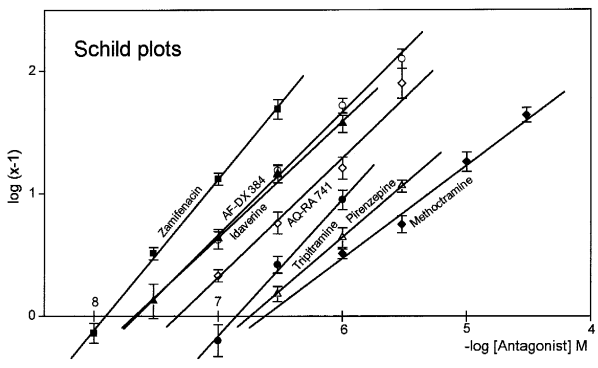

Fig. 3. Schild plots for a selection of antagonists to inhibit carbachol-evoked contractions of the isolated guinea-pig gallbladder. Given are means ± S.E.M. of $n = 6$–8.

3.2. Linear regressions

3.2.1. Comparison of agonist data for guinea-pig gallbladder with those at guinea-pig ileum and rat kidney muscarinic M_3 receptors

A highly significant correlation was found ($r = 0.953$, $P < 0.001$; $\beta = 1.03$ not significantly different from 1.00, $P > 0.05$) when we compared the pD_2 values of 16 agonists, as calculated from their potency to evoke guinea-pig gallbladder contractions, with that to contract the guinea-pig ileum (Fig. 4, top). Also a highly significant correlation was obtained by comparing these agonist potencies with respective values of the compounds to evoke vasorelaxation ($-\log ED_{50}$ mol values) in cirazoline-preconstricted rat kidney ($r = 0.920$, $P < 0.001$; $\beta = 1.07$ not significantly different from 1.00, $P > 0.05$; Fig. 4, bottom).

3.2.2. Comparison of antagonist data for guinea-pig gallbladder with functional affinities at guinea-pig ileal muscarinic M_3, rabbit anococcygeus M_4 and rabbit vas deferens M_1 and M_2 receptors

An excellent correlation was found ($r = 0.949$, $P < 0.001$; $\beta = 1.05$ not significantly different from 1.00, $P >$

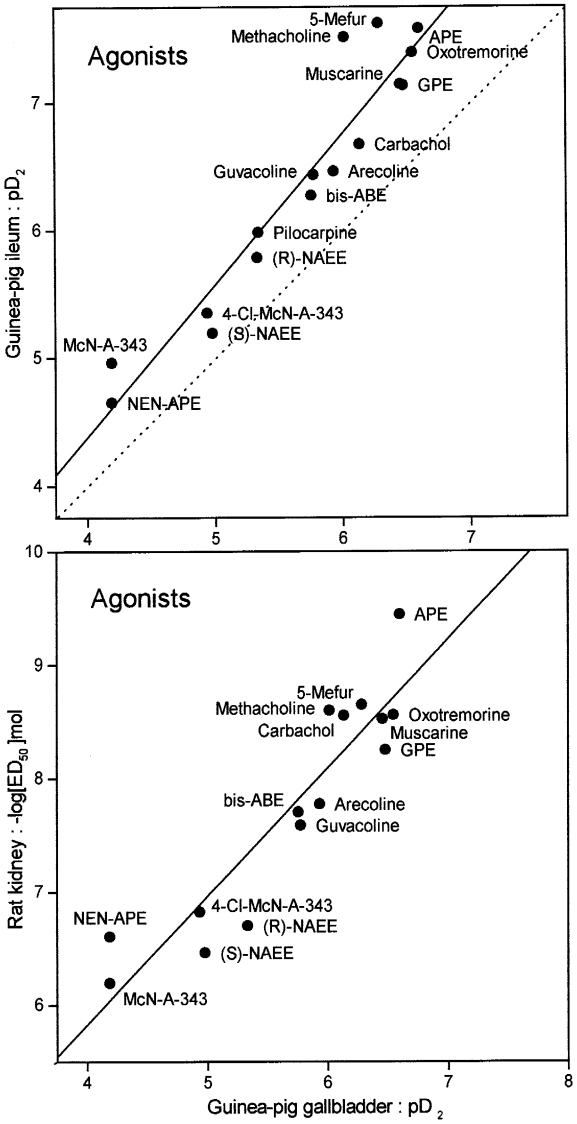

Fig. 4. Relationship between the potencies of muscarinic receptor agonists listed in Table 1 to elicit smooth muscle contraction in guinea-pig gallbladder and guinea-pig ileum (top) and to evoke vasodilation in cirazoline-preconstricted rat kidney (bottom). All agonist data from guinea-pig ileum and rat kidney used for comparison were taken from Eltze et al. (1993). Additionally, (±)-muscarine and pilocarpine were investigated. Abbreviation: 5-Mefur, 5-methylfurtrethonium.

0.05) by comparing ten antagonist affinities from guinea-pig gallbladder experiments with respective pA_2 values at muscarinic M_3 receptors in guinea-pig ileum (Fig. 5). Also an apparently good correlation resulted ($r = 0.917$, $P < 0.05$; $\beta = 0.84$, significantly different from 1.00, $P < 0.05$) when a comparison was made between affinity data of five antagonists on guinea-pig gallbladder and available functional data at muscarinic M_4 receptors on rabbit anococcygeus muscle (Gross et al., 1995), however, the regression line significantly deviates from the equality line, amounting to a factor of 3 (for pirenzepine) up to 10 (for methoctramine) for the difference in antagonist affinities in these two tissues (Fig. 5). Also affinities of antagonists in guinea-pig gallbladder muscarinic receptors did not significantly correlate with those determined at muscarinic M_1 ($r = 0.66$, $p < 0.05$; $\beta = 0.75$ significantly different from 1.00, $p < 0.01$) and M_2 receptors in rabbit vas deferens ($r = 0.41$, $p > 0.05$; $\beta = 0.59$ significantly different from 1.00, $p < 0.01$) (Fig. 6).

3.2.3. Comparison of antagonist data for guinea-pig gallbladder with binding affinities at native or cloned muscarinic M_3 / m3 and M_4 / m4 receptors

The affinities for a number of antagonists have now been measured by means of binding studies in a number of tissues endowed with either native muscarinic M_3 receptors (rat submandibular gland, rat pancreas) and muscarinic M_4 receptors (rabbit lung, rat striatum and NG 108-15 cells) or on membranes from cells (CHO, NIH 3T3) transfected with the cDNA for each of the five cloned human muscarinic m1–m5 receptors. In the present study, ten antagonists were selected from which sufficient affinity values at native muscarinic M_3 and M_4 or cloned human muscarinic m3 and m4 receptors have been published (for references, see legend of Table 2). Antagonists which display a higher affinity at native or cloned muscarinic M_4/m4 over muscarinic M_3/m3 receptors (with selectivity in brackets) are: pirenzepine (3), secoverine (5), AF-DX 384 and methoctramine (9), idaverine (10), mefurtramine

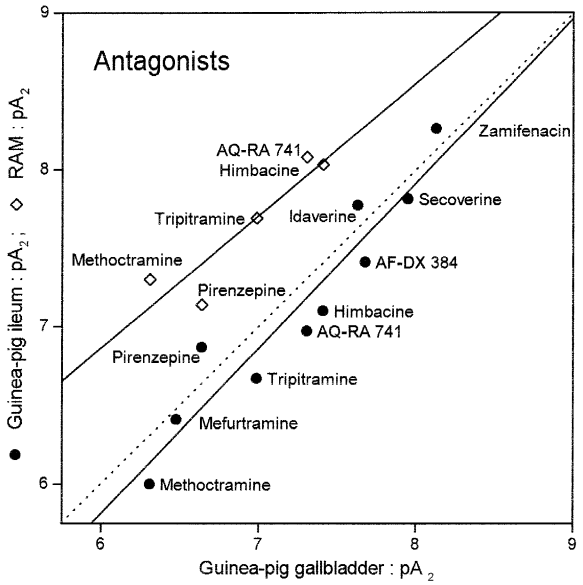

Fig. 5. Relationship between the affinities of the antagonists listed in Table 2 determined in guinea-pig gallbladder and their functional affinities at muscarinic M_3 receptors in guinea-pig ileum (Table 2) and at muscarinic M_4 receptors in rabbit anococcygeus muscle (RAM; antagonism of (+)-muscarine-evoked relaxation in histamine precontracted tissues; pA_2 values for pirenzepine = 7.14, AQ-RA 741 = 8.08, himbacine = 8.03, tripitramine = 7.69, methoctramine = 7.30, were taken from Gross et al., 1995; and personal communication). For receptor identity ($pA_2 = pA_2$), the normal regression line of the experimental data points (solid) should not deviate significantly from the depicted theoretical equality line (dotted).

(13), tripitramine (18), himbacine and AQ-RA 741 (19), or with the inverse selectivity (M_3/m3 over M_4/m4): zamifenacin (8).

An excellent correlation was found ($r = 0.898$, $P < 0.001$; $\beta = 0.87$, not significantly different from 1.00, $P > 0.05$) when we compared the antagonist affinities at guinea-pig gallbladder muscarinic receptors with their average pK_i values for native or cloned muscarinic M_3/m3 receptors (Fig. 7). However, no correlation was obtained when antagonist affinities in gallbladder were compared

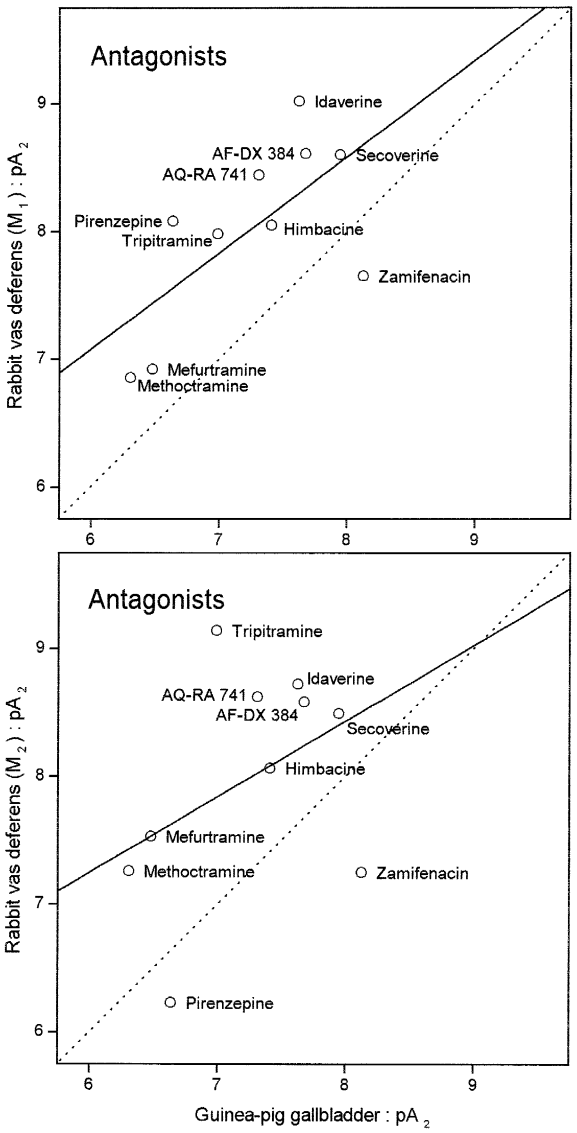

Fig. 6. Relationship between the affinities of the antagonists listed in Table 2 determined in guinea-pig gallbladder and their functional affinities at muscarinic M_1 (top) and M_2 receptors (bottom) in rabbit vas deferens. All pA_2 values from rabbit vas deferens, except for zamifenacin ($M_1 = 7.65$, $M_2 = 7.25$) and tripitramine ($M_1 = 7.98$, $M_2 = 9.14$), were taken from Eltze (1988), Eltze et al. (1989, 1993), Eltze and Galvan (1994) and Lambrecht et al. (1995). For receptor identity ($pA_2 = pA_2$), the normal regression line of the experimental data points (solid) should not deviate significantly from the depicted theoretical equality line (dotted).

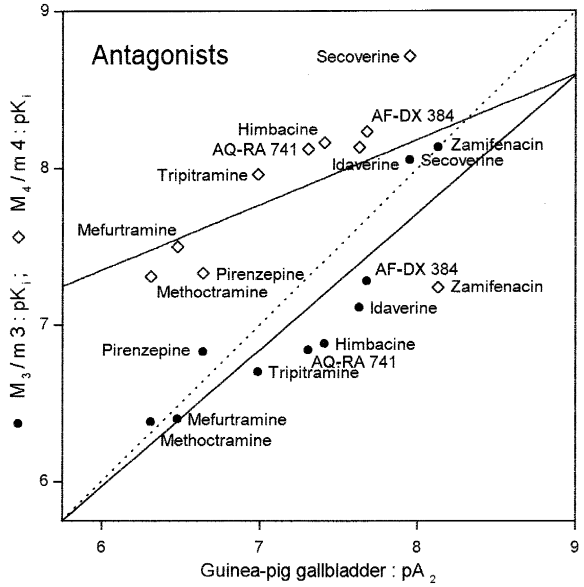

Fig. 7. Relationship between the affinities of the antagonists listed in Table 2 determined in guinea-pig gallbladder and their binding affinities at native or cloned muscarinic M_3/m3 receptors and at native or cloned muscarinic M_4/m4 receptors. For receptor identity ($pA_2 = pK_i$), the normal regression line of the experimental data points (solid) should not deviate significantly from the depicted theoretical equality line (dotted).

with respective average pK_i values at native or cloned muscarinic M_4/m4 receptors ($r = 0.526$, $P > 0.05$; $\beta = 0.41$, significantly different from 1.00, $P < 0.01$; Fig. 7).

4. Discussion

4.1. General considerations

The characterization of muscarinic receptor(s) in guinea-pig gallbladder mediating smooth muscle contraction is still a matter of dispute (Oktay et al., 1994, 1995; Von Schrenck, 1994; Eglen et al., 1996). Based on radioligand experiments with [^{3}H]*N*-methylscopolamine, a single muscarinic receptor binding site has been identified in guinea-pig gallbladder smooth muscle (Von Schrenck et al., 1993). The close agreement between the rank order of antagonist potencies to displace the radioligand from this site and to inhibit carbachol-induced smooth muscle contraction, together with the relatively high potency of silahexocyclium, suggested the involvement of muscarinic M_3 receptors in this tissue. However, careful inspection of antagonist potency rank order for inhibition of gallbladder contraction (atropine > silahexocyclium > AF-DX 384 ≥ HHSiD ≥ AF-DX 116, pirenzepine) (Von Schrenck et al., 1993) does not allow the decision of muscarinic M_3 receptors to be involved, because (a) three of these antagonists, namely atropine, silahexocyclium and HHSiD, do not discriminate between muscarinic M_3/m3 and M_4/m4 receptors, and (b) this rank order certainly fits more to native or

cloned muscarinic M_4/m4 receptors (atropine > silahexocyclium > AF-DX 384 > HHSiD ≥ pirenzepine > AF-DX 116) than to native or cloned muscarinic M_3/m3 receptors (atropine > silahexocyclium > HHSiD > AF-DX 384 > pirenzepine > AF-DX 116) (for references, see legend of Table 2). Nevertheless, typically for muscarinic M_3 receptors, the contractile response to carbachol was shown to be coupled to phosphatidylinositol 4,5-biphosphate hydrolysis as intracellular signal transduction pathway, but remained resistant to pretreatment of the animals with pertussis toxin (Von Schrenck et al., 1993, 1994). On the other hand, inhibition of adenylate cyclase by carbachol as an additional postreceptor mechanism, generally being coupled to muscarinic M_2/m2 and M_4/m4 receptor stimulation (Berridge, 1987), has recently been found in guinea-pig gallbladder smooth muscle which may complicate this scheme (Takahashi et al., 1994). The functional consequence of the fall in adenosine 3′,5′-cyclic monophosphate (cAMP) in response to carbachol is as yet unclear, possibly it could act to optimize smooth muscle contraction as hypothetized by Takahashi et al. (1994), nevertheless, it appears to be without measurable effect on muscarinic contraction of guinea-pig gallbladder (Von Schrenck et al., 1994).

The hypothesis for muscarinic M_3 receptors mediating contraction in guinea-pig gallbladder has been questioned by the finding that [^{3}H]quinuclidinyl benzilate labels two muscarinic binding sites in this tissue which are distinguishable by pirenzepine (Oktay et al., 1993, 1995). One of these sites has been ascribed to muscarinic M_2 receptors with an as yet undefined function, the other suggested to belong to muscarinic M_4 receptors mediating smooth muscle contraction (Oktay et al., 1993, 1995; Özkutlu et al., 1993). Interestingly, this would be the first example for the existence of muscarinic M_4 receptors in smooth muscle tissue, and inevitably reminds one of the similar discrepancy of suggested muscarinic M_4 receptors responsible for contraction of guinea-pig uterus (Dörje et al., 1990), which, by use of a greater number of discriminating antagonists, have later been characterized as being of the muscarinic M_2 subtype (Doods et al., 1993), as initially proposed (Eglen et al., 1989). Thus, pitfalls in receptor characterization often may be due to the small number of available functional affinity data and the use of antagonists which do not sufficiently discriminate (selectivity < 10) between the particular receptors in question. Since a final assessment of this issue in guinea-pig gallbladder seems highly warranted (Eglen et al., 1996), we decided to identify its muscarinic receptor subtype.

For this purpose, we investigated a series of subtype-preferring muscarinic receptor agonists known to show identical rank orders of potencies at muscarinic M_3 receptors present in guinea-pig ileum and rat perfused kidney, distinct from muscarinic M_1 receptors in rabbit vas deferens and muscarinic M_2 receptors in guinea-pig atrium (Eltze et al., 1993). Additionally, a number of discriminating antagonists were used, from which functional affinity data at muscarinic M_3 receptors in guinea-pig ileum (Eltze et al., 1993), muscarinic M_1 and M_2 receptors in rabbit vas deferens (Eltze, 1988) and at recently defined muscarinic M_4 receptors mediating NANC-relaxation in rabbit anococcygeus muscle (Gross et al., 1995) are available for comparison. Binding affinities of these appropriate antagonists, capable to distinguish between native muscarinic M_3 and M_4 receptors or between cloned and expressed human muscarinic m3 and m4 receptors, were also used for comparison with our functional data and taken from the literature (for references, see legend of Table 2).

4.2. Agonist studies

With the exception of McN-A-343 and NEN-APE, the agonists produced contractile effects more than 50% related to the maximal response elicited by carbachol. Their rank order of potency was: APE, oxotremorine, 5-methylfurtrethonium, methacholine, GPE > arecoline, bis-ABE, guvacoline > (*R*)-NAEE > 4-Cl-McN-A-343, (*S*)-NAEE > NEN-APE, McN-A-343. This is consistent with the potency profile of the agonists as observed (a) in contraction experiments in the guinea-pig ileum, the functional muscarinic receptor of which has been characterized as the M_3 subtype (Eglen et al., 1992), and (b) in rat perfused kidney, where endothelial muscarinic receptors of the M_3 subtype have been identified to cause vasodilation (Eltze et al., 1993). This agonist potency rank order is quite different for muscarinic M_1 receptors in rabbit vas deferens: APE > 4-Cl-McN-A-343, bis-ABE, 5-methylfurtrethonium > NEN-APE, and also for muscarinic M_2 receptors in guinea-pig left atrium: APE > bis-ABE > NEN-APE, 5-methylfurtrethonium > 4-Cl-McN-A-343 (Eltze et al., 1993). Furthermore, it is interesting to note, that potencies of muscarinic receptor agonists to contract the guinea-pig gallbladder were consistently lower than that determined in ileum obviously reflecting a considerably lower receptor reserve in gallbladder compared to ileum (Kenakin, 1987).

4.3. Antagonist studies

The affinities of a number of sufficiently discriminating antagonists at guinea-pig gallbladder smooth muscle muscarinic receptors were very close to respective values at previously determined guinea-pig ileal muscarinic M_3 receptors (Eltze et al., 1989, 1993; Eltze and Galvan, 1994). The affinity difference of ten antagonists determined in gallbladder and ileum did not exceed a factor of 2.5, suggesting that smooth muscle contractions to muscarinic stimuli in both tissues are mediated by the same muscarinic receptor, i.e., the M_3 subtype. For the series of antagonists investigated, M_4/m4 versus M_3/m3 subtype affinity difference ranged from the selective himbacine, AQ-RA 741 and tripitramine (nearly 20-fold), over the nearly unselective pirenzepine (3-fold) to zamifenacin

(0.13-fold). Thus, if the muscarinic M_4 receptor played an important functional role in mediation of carbachol-induced contraction of guinea-pig gallbladder, Schild plot slopes of less than unity might be expected (Kenakin, 1985). However, despite this range of selectivities, the slopes of Schild plots for these and most of the other compounds were not significantly different from unity which is consistent with a single subtype mediating the response rather than multiple subtypes.

In contrast, antagonist affinities at guinea-pig gallbladder muscarinic receptors were up to 10-fold lower than those determined at muscarinic M_4 receptors in rabbit anococcygeus muscle (e.g., methoctramine $pA_2 = 6.31$ versus 7.30). Since antagonist affinities at guinea-pig gallbladder muscarinic receptors did not coincide with this functional model for muscarinic M_4 receptors (Gross et al., 1995), its contraction appears unlikely to be mediated via this receptor subtype. A similar conclusion is reached by comparing pA_2 values of the antagonists in gallbladder with those derived from experiments at muscarinic M_1 and M_2 receptors located in rabbit vas deferens (Eltze, 1988).

It has been repeatedly suggested that the functional muscarinic receptor in the guinea-pig gallbladder resembles the M_4, rather than the M_3 subtype (Kurtel et al., 1990; Oktay et al., 1993, 1995; Özkutlu et al., 1993). However, most of the antagonists used in these studies are either unselective (atropine, 4-DAMP, HHSiD, *p*-F-HHSiD) in respect to affinity at muscarinic M_4/m4 versus M_3/m3 receptors (see Section 1). Thus, the conclusion of muscarinic M_4 receptors being responsible for guinea-pig gallbladder contraction is based on the affinities of only three moderately discriminating antagonists (M_4/m4 versus M_3/m3 selectivity ranging from factor 3 to 9), namely AF-DX 116 ($pA_2 = 6.6–6.7$), pirenzepine ($pA_2 = 7.8–7.9$) and methoctramine ($pA_2 = 7.6–7.7$) (Kurtel et al., 1990; Oktay et al., 1993; Özkutlu et al., 1993), the values of the latter two antagonists surprisingly agree more with respective affinities at native or cloned muscarinic M_4/m4 receptors (pirenzepine $pK_i = 7.33$, methoctramine $pK_i = 7.31$) than with native or cloned muscarinic M_3/m3 receptors (pirenzepine $pK_i = 6.83$, methoctramine $pK_i = 6.38$; see Table 2). However, in our hands, affinities of both pirenzepine ($pA_2 = 6.64$) and methoctramine ($pA_2 = 6.31$) in guinea-pig gallbladder exactly fit to values for native or cloned muscarinic M_3/m3 receptors. The reason for the inconsistency of antagonist affinities found in the present study and those reported previously remains unclear. However, in order to evaluate this discrepancy more precisely, we compared the functional affinities of a greater number of antagonists with respective binding data at native or cloned muscarinic M_3/m3 and M_4/m4 receptors, thus allowing a better decision of the receptor subtype involved in guinea-pig gallbladder contraction.

There was a very good correlation and a regression line close to the line of identity between ten antagonist affinities on guinea-pig gallbladder and their published affinity data at native or cloned muscarinic M_3/m3 receptors, suggesting that the functional muscarinic M_3 receptor mediating contraction to carbachol in guinea-pig gallbladder exhibits a pharmacological equivalency to the cloned muscarinic m3 receptor. However, the correlation obtained by utilizing the antagonist affinities at native or cloned muscarinic M_4/m4 receptors was shown to be insignificant. Thus the contraction of the guinea-pig gallbladder appears unlikely to be mediated via this receptor subtype.

4.4. Conclusions

The potency rank order of subtype-preferring muscarinic receptor agonists to evoke contraction in guinea-pig gallbladder is identical with those for native muscarinic M_3 receptors in guinea-pig ileum and rat kidney. The affinities of muscarinic receptor subtype-discriminating antagonists in guinea-pig gallbladder are in close agreement to those determined at muscarinic M_3 receptors in guinea-pig ileum but not to those determined at muscarinic M_4 receptors in rabbit anococcygeus muscle or to those at muscarinic M_1 and M_2 receptors in rabbit vas deferens. Functional antagonist affinities in guinea-pig gallbladder exhibit a pharmacological equivalency to binding affinities at native or cloned muscarinic M_3/m3 receptors, but clearly differ from those for native or cloned muscarinic M_4/m4 receptors. The present results demonstrate that the muscarinic receptor mediating smooth muscle contraction of the guinea-pig gallbladder can be best characterized as being of the M_3 subtype and thus agree to the proposal as it has been initially made for this tissue (Von Schrenck et al., 1993), whereas the hypothesis of muscarinic M_4 receptors responsible for gallbladder contraction (Kurtel et al., 1990; Oktay et al., 1993, 1995; Akbulut et al., 1995; Özkutlu et al., 1993) could not be confirmed. In light of the absent or poor selectivity of the antagonists used previously to identify the muscarinic receptor in this tissue, the present study also demonstrates the necessity of a greater number of appropriate antagonists, in this particular case capable to distinguish between muscarinic M_3 and M_4 receptors, to unequivocally characterize the receptor subtype.

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