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Characterization of the muscarinic receptor subtype involved in phosphoinositide metabolism in bovine tracheal smooth muscle

*,†,[1]A.F. Roffel, *H. Meurs, *,†C.R.S. Elzinga & *J. Zaagsma

*Department of Pharmacology & Therapeutics, †Department of Analytical Chemistry & Toxicology, University of Groningen, Antonius Deusinglaan 2, 9713 AW Groningen, The Netherlands

1 The muscarinic receptor subtype involved in the methacholine-induced enhancement of phosphoinositide metabolism in bovine tracheal smooth muscle was identified by using the M_2-selective antagonist AF-DX 116 and the M_3-selective antagonist 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide, in addition to the M_1-selective antagonist pirenzepine, in a classical Schild analysis.

2 All the antagonists shifted the methacholine dose-response curve to the right in a parallel and concentration-dependent fashion, yielding Schild plots with slopes not significantly different from unity. The pA_2 values (6.94, 6.32 and 8.54 for pirenzepine, AF-DX 116 and 4-DAMP methobromide respectively) indicate that it is the M_3 (smooth muscle/glandular), but not the M_2 (cardiac) muscarinic receptor subtype, present in this tissue, that mediates phosphoinositide turnover, in accordance with our previous contractile studies.

3 The results provide additional evidence for the involvement of phosphoinositide turnover in the pharmacomechanical coupling between muscarinic receptor stimulation and contraction in (bovine tracheal) smooth muscle.

Introduction

Enhancement of phosphoinositide (PI) turnover has been proposed as the transduction mechanism in airway (and other) smooth muscle cells involved in the coupling of muscarinic receptor stimulation and contraction (Baron *et al.*, 1984; Abdel-Latif, 1986). Thus, it has been demonstrated that muscarinic receptor stimulation results in the breakdown of membrane polyphosphoinositides and/or the formation of inositol phosphates in canine (Baron *et al.*, 1984), bovine (Grandordy *et al.*, 1986) and guinea-pig (Robertson *et al.*, 1988) tracheal smooth muscle, and very recently in human bronchial smooth muscle (Meurs *et al.*, 1989). Furthermore, it has been suggested that in canine tracheal smooth muscle inositol 1,4,5-trisphosphate is able to release Ca^{2+} from intracellular stores (Hashimoto *et al.*, 1985), which is thought to be involved in the smooth muscle contraction process (see e.g. Yousufzai *et al.*, 1987), and that the formation of this putative second messenger in bovine tracheal smooth muscle actually precedes contraction (Chilvers *et al.*, 1989). Finally, recent work from our laboratory provided evidence for a direct relationship between PI metabolism and bovine tracheal smooth muscle contraction; the abilities of three muscarinic agonists to elicit inositol phosphates accumulation and contraction were correlated (Meurs *et al.*, 1988). This study also revealed the presence of an agonist-dependent receptor (actually transduction) reserve.

Important additional evidence for the involvement of PI turnover in muscarinic receptor-mediated smooth muscle contraction might come from the pharmacological characterization of these processes with receptor subtype-selective antagonists. Indeed, both contraction in a wide variety of smooth muscle preparations (Eglen *et al.*, 1987; Konno & Takayanagi, 1986; O'Rourke *et al.*, 1987), including bovine trachea (Grandordy *et al.*, 1986; Moore *et al.*, 1988; Roffel *et al.*, 1988), and PI metabolism in a number of these preparations (Grandordy *et al.*, 1986; Akhtar *et al.*, 1987; Noronha-Blob *et al.*, 1987; Gardner *et al.*, 1988) have been shown to be mediated by muscarinic receptors with low affinity for the M_1-selective antagonist pirenzepine.

Since the further subdivision of muscarinic receptors with low affinity towards pirenzepine into M_2 (cardiac) and M_3 (smooth muscle/glandular) subtypes, by use of selective antagonists like AF-DX 116, methoctramine, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide and hexahydrosiladifenidol, it has been established that bovine tracheal smooth muscle contraction is mediated by the M_3 receptor subtype (Roffel *et al.*, 1988; Moore *et al.*, 1988). Interestingly, equilibrium (Giraldo *et al.*, 1988; Lazareno & Roberts, 1988b; Michel & Whiting, 1987; Moore *et al.*, 1988; Roffel *et al.*, 1988) and dissociation (Giraldo *et al.*, 1988; Roffel *et al.*, 1989) radioligand binding experiments, as well as molecular biology techniques (Maeda *et al.*, 1988), have demonstrated the presence of a large population of cardiac but only a small population of smooth muscle/glandular type binding sites in (among others bovine tracheal) smooth muscle membranes. Which of these muscarinic receptor subtypes mediates PI metabolism in smooth muscle is, at present, unknown. Until now, only in one study were M_2/M_3 subtype-selective muscarinic antagonists used. It was found that carbachol-induced PI breakdown in guinea-pig bladder was antagonized with high potency by AF-DX 116 ($pA_2 = 7.3$) and with low potency by hexahydrosiladifenidol ($pA_2 = 6.8$), suggesting the involvement of the M_2 (cardiac) subtype (Noronha-Blob *et al.*, 1987). However, it should be mentioned that atropine showed an anomalous low potency ($pA_2 = 7.7$).

The aim of the present study was to establish the pharmacological subtype of muscarinic receptor that mediates PI turnover in bovine tracheal smooth muscle, following the characterization of these receptors in contraction and binding studies (Roffel *et al.*, 1987; 1988), by using, in addition to pirenzepine, the M_2-selective antagonist AF-DX 116 and the M_3-selective antagonist 4-DAMP methobromide in a classical Schild analysis.

Methods

Tissue prepararation

Fresh bovine tracheae were obtained from the local slaughterhouse and transported to the laboratory within 30 min in Krebs-Henseleit (KH) buffer, at room temperature, pregassed with 95% O_2/5% CO_2; pH 7.4. Trachealis muscle was carefully dissected and smooth muscle strips (15 × 5 mm) were prepared free of mucosa and connective tissue in KH solution gassed with 95% O_2/5% CO_2 at room temperature. The strips

[1] Author for correspondence at: Dept. of Pharmacology & Therapeutics, University of Groningen.

were kept overnight at 24°C in a system in which they were continuously superfused (flow 3.9 ml min^{-1}) with gassed KH solution.

Inositol phosphates determination

Total inositol phosphates accumulation was determined essentially as described previously (Meurs *et al.*, 1988; 1989). Tracheal smooth muscle strips weighing a total of 6 g were chopped with a McIlwain tissue chopper, twice at a setting of 500 μm followed by three times at 100 μm. The tissue particles were washed four times with 45 ml KH containing 5 mM LiCl (KH/LiCl) and were then loaded with 75 μCi of [^{3}H]-inositol in 30 ml KH/LiCl for 60 min at 37°C, with gentle shaking and continuous gassing with 95% O_2/5% CO_2. After this incubation, the tissue was washed twice with KH/LiCl and was finally resuspended in 32 or 37 ml of this medium, depending on the number of dose-response curves (4–5) in the experiment. Aliquots of 450 μl of tissue suspension were then incubated with 25 μl of antagonist solutions or vehicle for 30 min at 37°C in capped tubes pregassed with 95% O_2/5% CO_2. After this preincubation, 25 μl of muscarinic agonist solutions were added, the tubes were gassed and capped again, and were incubated for another 25 min. Incubations were terminated by the addition of 500 μl ice-cold 10% (w/v) trichloroacetic acid. After the tubes had been on ice for 30 min, the precipitated protein was removed by centrifugation and 800 μl of the supernatants were extracted three times with four volumes of water-saturated diethyl ether. The extracted supernatants were then diluted with 8 ml of water and were applied to columns containing approximately 1 ml of Dowex AG 1X8 anion exchange resin (formate form). The columns were washed with 10 ml of water, followed by the sequential elution of glycerophosphoinositol with 15 ml of 5 mM disodium tetraborate/30 mM sodium formate buffer and of total inositol phosphates with four times 2 ml of 0.1 M formic acid/1.0 M ammonium formate buffer. These inositol phosphate samples were mixed with Plasmasol scintillation cocktail, 15 ml per sample, and counted for radioactivity in a Beckman LS 1800 liquid scintillation counter (35% efficiency).

Tissue protein was measured by the method of Lowry *et al.* (1951), after solubilization with 5 ml 1 N NaOH (10 min in a boiling waterbath) and subsequent neutralization with 5 ml 1 N HCl. Bovine serum albumin was used as the standard.

Data analysis

In each experiment, methacholine dose-response curves in the presence of muscarinic antagonists were related to the control dose-response curve, of which the maximum response was taken as 100%. In most experiments three or four concentrations of a muscarinic antagonist were tested and the slopes of the resulting Schild plots were used to assess competitive antagonism. When the slope did not differ significantly from unity (two-tailed Student's t test, $\alpha = 0.05$), pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log$ ([antagonist]/(dose ratio − 1)) (Mackay, 1978).

Materials

[^{3}H]-inositol (L-myo-[1,2-^{3}H(N)]) (40.8–60.8 Ci $mmol^{-1}$) was purchased from New England Nuclear (Boston, MA, U.S.A.) and methacholine from Sigmal Chemical Co. (St. Louis, MO, U.S.A.). Plasmasol was purchased from Packard Instrument B.V. (Groningen, The Netherlands). AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one) and pirenzepine were gifts from Dr Karl Thomae GmbH (Biberach an der Riss, F.R.G.); 4-DAMP (4-diphenylacetoxy-N-methylpiperidine) methobromide was a gift from Dr R.B. Barlow (Bristol, U.K.). All other chemicals were of reagent grade.

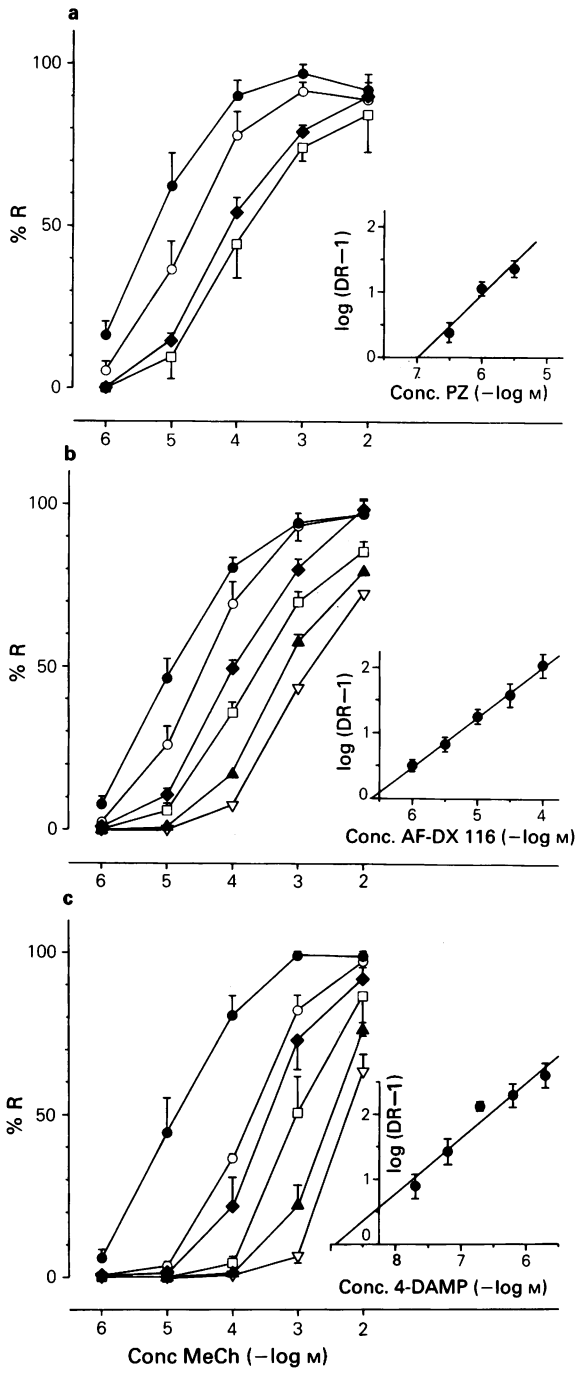

Figure 1 Dose-response curves for methacholine (MeCh)-induced phosphoinositide metabolism in bovine tracheal smooth muscle in the absence and presence of different concentrations of muscarinic antagonists, (a) pirenzepine (PZ) 0 (●) (3), 0.3 (○) (3), 1 (◆) (3) and 3 (□) (3) μM; (b) AF-DX 116 0 (●) (7), 1 (○) (4), 3 (◆) (6), 10 (□) (6), 30 (▲) (3) and 100 (▽) (3) μM; and (c) 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide 0 (●) (5), 0.02 (○) (3), 0.06 (◆) (3), 0.2 (□) (3), 0.6 (▲) (4) and 2 (▽) (4) μM. The number of determinations is given in parentheses. The insets show the corresponding Schild plots. Data points represent means of 3–7 experiments (as indicated in parentheses) each performed in duplicate. Vertical lines show s.e.mean. % R indicates responses relative to the control dose-response curves.

Results

In the absence of muscarinic antagonists, methacholine caused an 8.5 ± 0.9 fold stimulation of inositol phosphates accumulation over basal levels (2356 ± 310 d.p.m. mg^{-1} protein over 288 ± 35 d.p.m. mg^{-1} protein, with 3.7 ± 0.2 mg protein per tube) with a $-\log EC_{50}$ of 4.93 ± 0.10 (means ± s.e.mean,

Table 1 Functional potencies of selective muscarinic antagonists on methacholine induced phosphoinositide (PI) metabolism and contraction in bovine tracheal smooth muscle

	PI metabolism		*Contraction*	
	pA_2 (−log M)	*Slope*	pA_2 (−log M)	*Slope*
AF-DX 116	6.32 ± 0.06 (22)	0.81 ± 0.10 (7)	6.30 ± 0.07 (25)	1.06 ± 0.02 (4)
4-DAMP methobromide	8.54 ± 0.08 (17)	0.90 ± 0.05 (4)	9.03 ± 0.05 (20)	1.12 ± 0.06 (4)
Pirenzepine	6.94 ± 0.07 (9)	0.97 ± 0.08 (3)	6.92 ± 0.08 (30)	1.10 ± 0.05 (4)

pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log([\text{antagonist}]/(\text{dose-ratio} - 1))$ since Schild analysis revealed no significant deviations of slopes from unity, indicating competitive antagonism. Results are expressed as means ± s.e.mean with number of determinations in parentheses. Data for contraction were included for comparison and were taken from Roffel *et al.* (1988).

$n = 15$). As shown in Figure 1 the three muscarinic antagonists tested were all able to shift the methacholine dose-response curve to the right in a parallel and concentration-dependent fashion. The slopes of the Schild plots derived from these data were not significantly different from unity, indicating competitive antagonism for all three antagonists. These slopes and the pA_2 values are given in Table 1. The pA_2 values indicate that 4-DAMP methobromide was 40 times more potent than pirenzepine and 166 times more potent than AF-DX 116 in antagonizing methacholine-induced stimulation of PI metabolism in bovine tracheal smooth muscle.

Discussion

Data on the pharmacological characterization of the muscarinic receptors that mediate PI breakdown in (airway) smooth muscle are relatively scarce and most of the information available has been obtained with only pirenzepine as the selective antagonist. Pirenzepine discriminates between M_1 and M_2 but not really between cardiac and smooth muscle/glandular receptor subtypes. The pA_2 value of 6.94 found for pirenzepine in bovine tracheal smooth muscle in the present study indicates an M_2 (i.e. non-M_1) character for the muscarinic receptors involved, in full agreement with previous studies on PI metabolism in this and other smooth muscle tissues (Grandordy *et al.*, 1986; Akhtar *et al.*, 1987; Noronha-Blob *et al.*, 1987; Gardner *et al.*, 1988).

In order to establish the subtype of muscarinic receptor that mediates PI metabolism in bovine tracheal smooth muscle (cardiac (M_2) or smooth muscle/glandular (M_3)), the selective muscarinic antagonists AF-DX 116 and 4-DAMP methobromide were used. These compounds possess opposite selectivity profiles, 4-DAMP methobromide being typically 5 to 10 fold more potent than AF-DX 116 at M_2 and 200 to 500 fold more potent than AF-DX 116 at M_3 receptors. In the present study 4-DAMP methobromide was found to be 166 times more potent than AF-DX 116, suggesting the involvement of M_3 rather than M_2 receptors. The pA_2 value of 6.32 found for AF-DX 116 is within the range of 6.0–6.6 obtained for antagonism of smooth muscle contraction (Batink *et al.*, 1987; Duckles *et al.*, 1987; Lazareno & Roberts, 1988a; Moore *et al.*, 1988; Roffel *et al.*, 1988), whereas pA_2 values obtained in the heart (6.9–7.5) (Batink *et al.*, 1987; Duckles *et al.*, 1987; Micheletti *et al.*, 1987) are 4 to 16 times higher than the value we measured. Similarly, the pA_2 value of 8.54 for 4-DAMP methobromide is close to the values found on various smooth muscle preparations (8.6–9.2) (Batink *et al.*, 1987; Gater *et al.*, 1987; Eglen *et al.*, 1987; Moore *et al.*, 1988; Roffel *et al.*, 1988) but outside the range observed with cardiac preparations (7.7–8.2) (Batink *et al.*, 1987; Gater *et al.*, 1987; Lazareno & Roberts, 1988a). The conclusion that PI metabolism in bovine tracheal smooth muscle is brought about by M_3 type muscarinic receptors concords with the M_3 character of the muscarinic receptors that mediate contraction (see Roffel *et al.*, 1988 and references cited therein). As shown in Figure 2a there is a significant correlation between the pA_2 values for pirenzepine, AF-DX 116 and 4-DAMP methobromide when contraction (shown for comparison in Table 1) and PI metabolism were measured ($r = 0.9984$, $P < 0.05$). This provides more evidence that the latter process is involved in the pharmacomechanical coupling of muscarinic receptor stimulation and (airway) smooth muscle contraction. Interestingly, a significant correlation ($r = 1.0000$, $P < 0.005$) was also noticed between the pA_2 values found here for PI metabolism in bovine tracheal smooth muscle and those obtained in human SK-N-SH neuroblastoma cells (Fisher & Heacock, 1988), in accordance with the putative M_3 character of the muscarinic receptors in that cell line (Figure 2b). In this context, it should be mentioned that the neuronal SH-SY5Y clone of SK-N-SH cells was very recently found to possess a homogeneous M_3 receptor population (Lambert *et al.*, 1989).

The finding that PI metabolism in bovine tracheal smooth muscle is mediated by M_3-type muscarinic receptors is not completely unexpected. Firstly, muscarinic receptors in exocrine glands (which are typically of the M_3 subtype, as assessed in binding (Lazareno & Roberts, 1988a) and secretion (Gater *et al.*, 1987) studies) are also coupled to this second messenger system (Ek & Nahorski, 1988), although the receptor involved in PI turnover has not, as yet, been pharmacologically identified. Secondly, expression studies with different muscarinic receptor genes have shown that the putative M_3 receptor subtype (HM4, mAChR III, m3) (Barnard, 1988) couples to PI turnover (but not to adenylate cyclase) in human kidney cells (Peralta *et al.*, 1988) and NG108-15 neuroblastoma cells (Fukuda *et al.*, 1988), whereas the M_2 subtype is efficiently coupled to adenylate cyclase in human kidney cells (Peralta *et al.*, 1988), chinese hamster ovary cells (Ashkenazi *et al.*, 1987) and A9L fibroblasts (Jones *et al.*, 1988), though it can, albeit poorly, also stimulate PI turnover in the first two of these cell types.

In conclusion, it has become clear that both PI metabolism and contraction in bovine tracheal smooth muscle are medi-

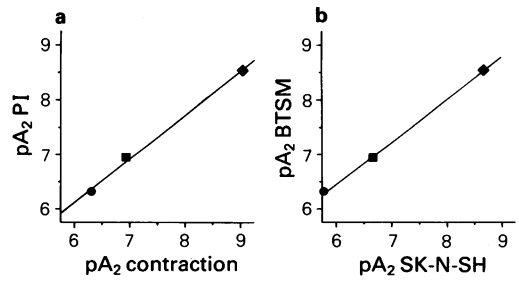

Figure 2 Correlation between the functional affinities (pA_2) of pirenzepine (■), AF-DX 116 (●), and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide (◆) for phosphoinositide (PI) metabolism and contraction in bovine tracheal smooth muscle (a) and between the functional affinities for PI metabolism in bovine tracheal smooth muscle (BTSM) and those obtained in human SK-N-SH neuroblastoma cells (Fisher & Heacock, 1988) (b). Correlation coefficients were 0.9984 and 1.0000, respectively; probability values were <0.05 and <0.005, respectively.

ated by smooth muscle/glandular type (M_3) muscarinic receptors. As an important implication, the function of the major population of cardiac type (M_2) muscarinic receptors and the transduction mechanism involved remain to be elucidated.

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