

Muscarinic contraction in isolated guinea-pig trachea and antagonism by noradrenaline

A. Walland, R. Hammer

Muscarinic contraction in isolated guinea-pig trachea and antagonism by noradrenaline. A. Walland, R. Hammer. ©ERS Journals Ltd 1997.

ABSTRACT: In contrast to other muscarinic agonists, WAL 2014 FU does not induce bronchospasm in laboratory animals. The present investigation was intended to test the hypothesis that this is due to a particular susceptibility of the drug's effect to antagonism by catecholamines, as a result of partial M₃-agonism.

The tonic activity of the muscarinic agonists, aceclidine, arecoline, carbachol, McN-A-343, RS 86, thiopilocarpine and WAL 2014 FU, was tested in groups of isolated tracheal muscle of the guinea-pig. Susceptibility to functional antagonism by β -adrenoceptor stimulation was measured by the displacement of the concentration-force curves by 3 μ M noradrenaline.

Evaluation of the concentration-force relationship revealed differences in potency and intrinsic activity (carbachol=100%) ranging from 114% for arecoline to 36% for thiopilocarpine (WAL 2014 FU=63%). The catecholamine increased the concentration of agonist which induced 5% of the maximum effect achievable (EC₀₅) values fivefold (carbachol) to more than 4,680 fold (thiopilocarpine) (WAL 2014 FU: 2,860 fold). Regression analysis between the intrinsic activity of the seven compounds and the antagonistic effect of noradrenaline revealed a significant correlation (Spearman correlation coefficient (r_s) = -0.79; $p=0.036$). Inhibition of the effects of endogenous catecholamines by β -adrenolysis with 50 μ M toliprolol increased the maximal contraction induced by 1 mM WAL 2014 FU, but did not affect maximal contraction induced by 30 μ M arecoline. Pretreatment with 0.3–1.0 mM dibutyrylcyclic adenosine monophosphate (DBcAMP) shifted the concentration-response curves of arecoline, WAL 2014 FU and thiopilocarpine in a similar manner to noradrenaline.

The results exclude an important contribution of adenylate cyclase-coupled M₂-receptors to the susceptibility of tracheal contraction by muscarinic agonists to functional antagonism by noradrenaline, but emphasize the importance of intrinsic activity at the M₃-receptors. The pronounced susceptibility of WAL 2014 FU-induced contraction to functional antagonism by β -adrenoceptor activation provides an explanation for the failure of the drug to induce bronchospasm *in vivo*.

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Memory impairment in patients with Alzheimer's disease seems to be causally related to the loss of cholinergic neurons in the forebrain. Cholinergic replacement therapy has been repeatedly attempted with substances exerting muscarinic agonistic actions; however, full exploration of the therapeutic efficacy of this principle has been limited by side-effects [1]. Recently, the muscarinic agonist, WAL 2014 FU, has been introduced as a new candidate for cholinergic replacement therapy, with an action profile in experimental pharmacology that promises less side-effects [3].

The existence and physiological importance of muscarinic receptor subtypes is generally recognized and reliable pharmacological methods for drug classification are available [2]. Investigations in isolated tissues characterized WAL 2014 FU as a full muscarinic agonist at M₁-receptors but as a partial agonist at M₂- and M₃-receptors. Moreover, the compound showed a preference for the M₁-receptor. In anaesthetized guinea-pigs, the intravenous injection of WAL 2014 FU, in contrast to

Dept of Research, Boehringer Ingelheim KG, Ingelheim, Germany.

Correspondence: A. Walland
Boehringer Ingelheim KG
Dept of Biological Research
General Pharmacology
55216 Ingelheim
Germany

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This paper is dedicated to Prof. Dr Dr Ernst Mutschler on the occasion of his 65th birthday.

arecoline, did not cause an increase in airway resistance [3].

This discrepancy raised the question of why the muscarinic agonist, WAL 2014 FU, is devoid of bronchospastic activity *in vivo*. Muscarinic agonists exert bronchospasm by activating M₃-receptors at the bronchial smooth muscle. On the other hand, activation of M₁-receptors at sympathetic ganglia induces functional antagonism by liberated catecholamines *via* bronchial β_2 -adrenoceptors. As both compounds induced pressor effects in pithed rats, the bronchospastic effect of arecoline *in vivo* [3] does not appear to be due to a lack of sympathetic ganglionic activation. It, therefore, seems that the bronchospastic effects of arecoline are more resistant to antagonism by endogenous catecholamines than those of WAL 2014 FU.

Earlier studies by GRANDORDY *et al.* [4] and GUNST *et al.* [5] in isolated bovine and canine tracheal muscle revealed an appreciable receptor reserve for acetylcholine and carbachol but little reserve for McN-A-343. In

isolated guinea-pig tracheal muscle, the contractile effects of three muscarinic agonists showed increasing susceptibility to antagonism by isoprenaline, in the order: methacholine, oxotremorine and McN-A-343. The maximum effect on phosphoinositide metabolism in bovine trachea decreased in the same order. However, the maximal contractile effect was highest with oxotremorine in muscles of both species [6, 7]. These results prompted us to reinvestigate the relationship between intrinsic activity and susceptibility to antagonism by β -adrenoceptor activation in isolated tracheal muscle with a greater number of muscarinic agonists, and WAL 2014 FU in particular.

Materials and methods

Animals

Guinea-pigs (Chbb:DHP) of both sexes, weighing 300–500 g, were obtained from Dr Karl Thomae GmbH (Biberach, Germany).

Preparation and recording

The animals were stunned by a blow to the neck and exsanguinated by carotid transection. The trachea was removed and placed in Tyrode's solution, which had the following composition (mM): NaCl 137.0; KCl 2.7; CaCl₂ 1.8; MgCl₂ 0.3; NaHCO₃ 11.9; Na₂HPO₄ 0.4; and glucose 15.0; and was aerated with 95% O₂ and 5% CO₂ at 37°C. The trachea was cut into four rings comprising four tracheal cartilages each. Strips were obtained by cutting the cartilage opposite to the muscle layer. The cartilaginous ends of the preparation were fixed with surgical cement to perspex chips (8 × 8 × 1 mm), which were provided with central holes for mounting in the force transducer system of the organ bath filled with aerated Tyrode's solution at 37°C. The contractions of four preparations in response to the cumulative addition of muscarinic agonists were recorded isometrically with 15 mN preload on a Recomed 4-channel polygraph (Hellige, Freiburg, Germany) by means of a TF 3V3 force transducer (Hellige).

Experimental protocol

Potency and intrinsic activity of the agonists. After allowing the preparations 20–30 min for adaptation, muscarinic agonists were added at their individual threshold concentrations, which were determined in preliminary experiments. The drug concentration was increased cumulatively in steps by tripling the concentration at 10 min intervals, until an increase in concentration did not produce any further detectable increase in force. Usually 10 min sufficed to obtain equilibrium, however, in some cases 12–15 min had to be allowed for stabilization of effects. The experiments finished with the addition of the supramaximally effective concentration of 300 μ M carbachol, which is more than 1,000 fold the concentration needed to induce 50% of the maximum effect achievable (EC₅₀).

Shift of concentration-response curves by noradrenaline. After a period of adaptation, maximally effective concentrations of the muscarinic agonists were added for calibration of the individual response. Following wash-out and complete relaxation, a concentration of 3 μ M noradrenaline was established in the organ bath and 10 min later the concentration-response manoeuvre was started with a muscarinic agonist.

Shift of concentration-response curves by dibutyrylcyclic adenosine monophosphate (DBcAMP). After a period of adaptation, 300 μ M carbachol was added for calibration of the individual response. Following wash-out and complete relaxation, concentrations of 0.3 or 1 mM DBcAMP were established in the organ bath, and 10 min later concentration-response manoeuvres were started with the muscarinic agonists arecoline, WAL 2014 FU and thiopilocarpine, respectively.

Importance of endogenous catecholamines. After stabilization of tracheal muscle preparations, maximally effective concentrations of either 1 mM WAL 2014 FU or 30 μ M arecoline were added to the organ bath. As soon as the spasm in response to these supramaximally effective concentrations of the muscarinic agonists reached a stable plateau, 50 μ M of the β -adrenolytic compound, toliprolol, was added. The same experimental protocol was used for testing 5 μ M toliprolol in contractions elicited by 3 μ M and 1 mM WAL 2014 FU in six preparations each.

Evaluation and statistics

Increases in force development in response to muscarinic compounds were expressed as a percentage of individual maximal response to 300 μ M carbachol. Data were expressed either as mean \pm SEM or as median and maximum/minimum. Sigmoid concentration-response curves were obtained by fitting the individual data by means of the computer program Graph PAD Inplot, Version 3.1 (Graph PAD Software, San Diego, CA, USA). This calculation is based on a Michaelis-Menten equation with Hill-coefficient and the method of least squares. Final curves were calculated by using the medians of EC₅₀, maximum response and Hill-coefficient obtained from a series of experiments.

The susceptibility to functional antagonism by noradrenaline was evaluated by comparing the concentrations of the muscarinic agonist which induced 5% of the achievable maximum effect (EC₀₅) in the presence and the absence of the catecholamine. The regression between the antagonistic effect of noradrenaline, expressed as a shift of the EC₀₅ values, and the intrinsic activity, with respect to carbachol, of the muscarinic agonists tested was evaluated by calculation of a Spearman correlation coefficient (r_s) and testing for significance from zero. The effects of toliprolol were tested with the paired t-test.

Chemicals and drugs

Chemicals for the preparation of the medium were all of analytical reagent grade. WAL 2014 FU ((R)-3-(2-propynyl)oxo-1-azabicyclo[2,2,2]octane-2-butene-diacid(E)

Table 1. – Force development in isolated tracheal muscle from guinea-pigs in response to cumulative addition of muscarinic agonists

Compound	Concentration range μM	n	pD_2 -value	Intrinsic activity
Aceclidine	0.01–30	7	6.52 (6.11/6.74)	107 (100/111)
Arecoline	0.01–30	7	6.49 (6.34/6.98)	114 (102/118)
Carbachol	0.01–30	6	6.70 (6.50/6.78)	101 (99/102)
McN-A-343	0.1–300	6	5.29 (5.09/5.68)	58 (49/60)
RS 86	0.01–30	5	6.66 (6.34/7.65)	90 (88/101)
Thiopilocarpine	0.1–100	8	5.30 (4.85/5.67)	36 (16/52)
WAL 2014 FU	1–3000	6	5.17 (4.81/5.62)	63 (37/80)

Intrinsic activity is expressed as a percentage of the effect of 300 μM carbachol. Values are presented as median and minimum/maximum from individual curve fitting in parenthesis. n: number of preparations; pD_2 : negative log of EC_{50} ; EC_{50} : concentration of agonist inducing 50% of the maximum effect achievable.

salt), arecoline, RS 86 [8], thiopilocarpine and toliprolol were from Boehringer Ingelheim KG (Ingelheim, Germany). Carbachol (carbamoylcholine chloride) was purchased from E. Merck (Darmstadt, Germany), Aceclidine from Chibret (Munich, Germany), noradrenaline, DBcAMP ($\text{N}^6,2'$ -O-dibutyryl adenosine 3':5'-cyclic monophosphate) and McN-A-343 (4-(N-[3-chlorophenyl] carbamoyloxy)-2-butyryltrimethylammonium chloride) from Sigma (St. Louis, MO, USA).

Results

Potency and intrinsic activity of muscarinic agonists

As can be seen from table 1 and figure 1, the potency of the seven muscarinic agonists investigated was quite variable. Carbachol, for instance, was 34 times more potent than WAL 2014 FU. Similarly the agonists were different in intrinsic activity, arecoline showing the highest intrinsic activity and thiopilocarpine the least, *i.e.* 114 and 36% of the intrinsic activity of carbachol.

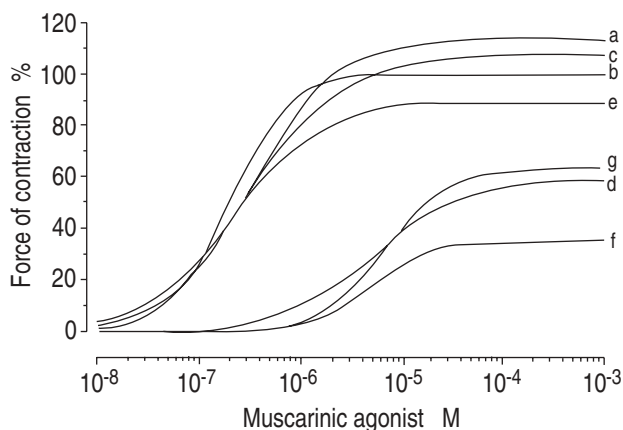


Fig. 1. – Comparison of the concentration-dependence of the force development in isolated tracheal muscle of the guinea-pig by cumulative addition of muscarinic agonists. Force is expressed as a percentage of the force development in response to 300 μM carbachol (100%). Curves were calculated from the medians shown in table 1. a: arecoline; b: aceclidine; c: carbachol; d: McN-A-343; e: RS 86; f: thiopilocarpine; g: WAL 2014 FU.

Antagonism by noradrenaline

Noradrenaline, at a concentration of 3 μM exerted functional antagonism and shifted the concentration-contraction curves of all the muscarinic agonists to the right, although the extent was variable. Examples are given in figures 2 and 3, showing concentration-response curves of arecoline and WAL 2014 FU and the different effect of pretreatment with noradrenaline. In the case of WAL 2014 FU, the curve was shifted into the millimo-

lar range, thus preventing full exploration of the relationship between concentration and effect. The antagonistic effect of noradrenaline was most pronounced in the investigation with thiopilocarpine. The effect of 3 mM

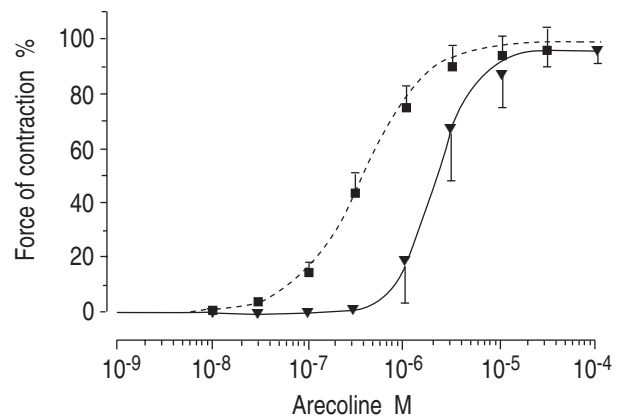


Fig. 2. – Concentration-dependence of contraction induced by cumulative additions of arecoline in isolated tracheal muscle of the guinea-pig without ($n=7$) and with ($n=8$) noradrenaline pretreatment. The ordinate shows the force as a percentage of the maximum effect of the muscarinic agonist. The curves were calculated from the medians from individual sigmoid curve fitting. Points represent mean \pm SEM. —: no pretreatment; —: plus noradrenaline (3 μM).

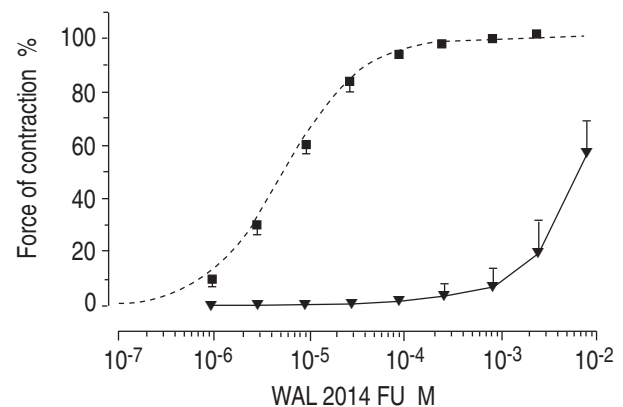


Fig. 3. – Concentration-dependence of contraction induced by cumulative addition of WAL 2014 FU in isolated tracheal muscle of the guinea-pig without ($n=6$) and with ($n=6$) noradrenaline pretreatment. The ordinate shows the force as the percentage of the maximum effect of the muscarinic agonist. The control curve was calculated from the medians from individual sigmoid curve fitting. Points represent mean \pm SEM. —: no pretreatment; —: plus noradrenaline (3 μM).

Table 2. – Force development in groups of isolated tracheal muscle from guinea-pigs in response to cumulative addition of muscarinic agonists without and in the presence of 3 μ M noradrenaline

	log EC ₀₅						Intrinsic activity			
	Control			+3 μ M noradrenaline			Difference	Ranking order	% carbachol = 100%	Ranking order
	n	Median	Max/min	n	Median	Max/min				
Aceclidine	7	-7.91	-6.95/-8.41	8	-6.16	-5.74/-6.41	1.75	4	107	2
Arecoline	6	-7.45	-7.35/-7.50	8	-6.24	-6.11/-6.42	1.21	2	114	1
Carbachol	6	-7.50	-7.35/-7.86	8	-6.77	-6.48/-7.04	0.73	1	100	3
McN-A-343	6	-6.63	-6.38/-7.40	6	-3.11	>-2.52/-3.42	3.52	6	58	6
RS 86	5	-7.91	-7.61/-10.43	6	-6.22	-5.81/-6.72	1.69	3	90	4
Thiopilocarpine	8	-6.19	-5.62/-7.01	3	>-2.52	>-2.52/>-2.52	>3.67	7	36	7
WAL 2014 FU	6	-6.39	-5.91/-6.54	6	-2.93	-2.64/-3.83	3.46	5	63	5

The concentration of the agonist which induced 5% of the maximum effect achievable (EC₀₅) was evaluated by individual curve fitting. Intrinsic activity is expressed as a percentage of the effect of 300 μ M carbachol. n: number of preparations. The ranking orders of the shift in EC₀₅ by noradrenaline and the intrinsic activity of the seven drugs are related by a Spearman correlation coefficient (r_s) of -0.79 ($p=0.036$).

thiopilocarpine was completely suppressed by 3 μ M noradrenaline. The quantitative evaluation of the displacement of the concentration-response curves at the 5% level of the maximum effect of the muscarinic agonists by 3 μ M noradrenaline is presented in table 2, together with the intrinsic activities. The nonparametric calculation of a correlation between intrinsic activity and displacement of the 5% response by 3 μ M noradrenaline revealed a Spearman correlation coefficient (r_s) of -0.79 ($p=0.0362$). This result means that the resistance of the muscarinic contraction of the trachea to relaxation by β -adrenoceptor stimulation increases with the intrinsic activity of the muscarinic agonists.

Antagonism by DBcAMP

Investigation of an antagonism between the tracheal effects of arecoline, thiopilocarpine and WAL 2014 FU by DBcAMP, at concentrations of 0.3 and 1 mM revealed positive results. As for the experiments with noradrenaline, arecoline had the greatest effects (fig. 4a) and the effects of thiopilocarpine (fig. 4c) were least resis-

tant to antagonism. Whilst 1 mM DBcAMP displaced the concentration-response curve of arecoline moderately and hardly suppressed the plateau, 0.3 mM sufficed to inhibit the effects of thiopilocarpine completely. The results with WAL 2014 FU indicate a displacement of the curve and a reduction of the maximum effects by 0.3 mM DBcAMP and complete inhibition of the effects by 1 mM (fig. 4b).

The importance of endogenous catecholamines

The addition of 50 μ M of the β -adrenolytic compound toliprolol to tracheal muscle in the state of maximal contraction induced by 1 mM WAL 2014 FU resulted in a small further increase in tension in all preparations. Maximal contraction induced by 30 μ M arecoline, however, was not influenced by toliprolol (fig. 5). Compatible statistically significant increases in force of 18.7 and 18.2% were obtained with 5 μ M toliprolol (which produces approximately 95% receptor occupancy) in submaximal and maximal contractions induced by 3 μ M and 1 mM WAL 2014 FU, respectively, in six preparations each.

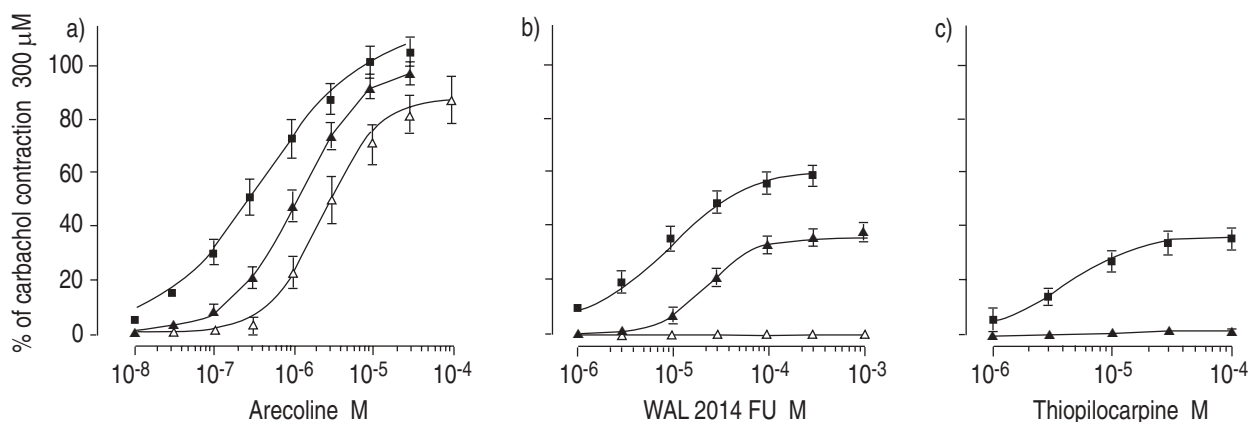


Fig. 4. – Concentration-dependence of contraction induced by cumulative addition of arecoline, WAL 2014 FU or thiopilocarpine, without and with pretreatment with 0.3 or 1 mM dibutyryladenosine 3':5'-cyclic monophosphate (DBcAMP). Each curve was calculated from the medians obtained by sigmoid curve fitting of the forces developed in six preparations. Forces were expressed as a percentage of the individual response to 300 μ M carbachol. Points represent mean \pm SEM. ■ : no pretreatment; ▲ : plus DBcAMP 0.3 mM, Δ: plus DBcAMP 1 mM.

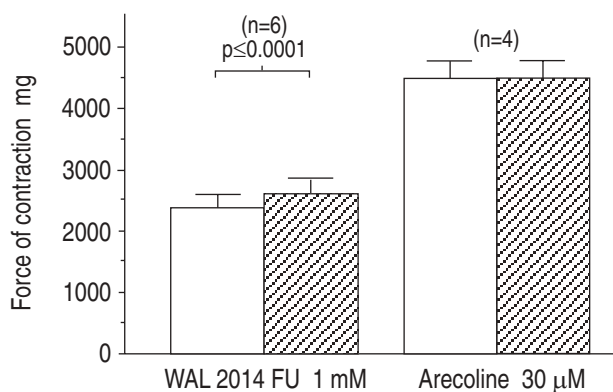


Fig. 5. — Force of contraction developed in isolated tracheal muscle in response to maximally effective concentrations of WAL 2014 FU and arecoline (□), in comparison to the force after the addition of 50 µM toliprolol (▨). Values are presented as mean ± SEM. Statistical comparison by paired t-test.

Discussion

The seven muscarinic agonists, tested in isolated tracheal muscle of the guinea-pig for spastic activity, covered the wide range of 36 to 114% intrinsic activity (carbachol = 100%). Arecoline, aceclidine and carbachol developed full intrinsic activity, while WAL 2014 FU, McN-A-343 and, in particular, thiopilocarpine were partial agonists. RS 86, a compound intended for treatment of Alzheimer's disease [8], exerted nearly full agonistic activity. It is peculiar, but probably merely accidental, that the four full agonists were also the most potent compounds as compared to the three partial agonists (fig. 1 and table 1).

According to investigations with fluorescence microscopy, the trachea of the guinea-pig is innervated by adrenergic nerves [9]. Therefore, noradrenaline seemed particularly suitable for the quantitative analysis of the functional antagonism of muscarinic contraction in the tracheal smooth muscle by β -adrenoceptor stimulation. The shift of the EC_{05} by 3 µM noradrenaline varied considerably within the agonists. Five (carbachol) to >4,680 fold (thiopilocarpine) increases in EC_{05} were observed. WAL 2014 FU, with a factor of 2,860, was one of the compounds which were most effectively antagonized by noradrenaline (table 2). The statistically significant correlation between intrinsic activity and the antagonistic effect of noradrenaline suggested a causal relationship between these parameters.

Evidence of a sympathetic innervation of the tracheal muscle of the guinea-pig is also derived from *in vitro* experiments with field stimulation and pharmacological characterization of the mediator [10]. However, the detection of catecholamine leakage from unexcited sympathetic nerve endings in the tracheal muscle by pharmacological means (fig. 5) appears to be new. It is remarkable that the minute amounts of catecholamines that leak from tracheal muscle suffice to have a clear influence on WAL 2014 FU-induced maximal contraction.

The molecular mechanisms of the functional antagonism between drugs utilizing the tracheal M_3 -receptor and the β_2 -adrenoceptor have not been fully elucidated [11, 12]. However, synthesis of cyclic adenosine monophosphate in response to β -adrenoceptor activation ap-

pears to play a crucial role by induction of inactivation of calcium-calmodulin-myosine light chain kinase by phosphorylation [11]. In guinea-pig trachea, M_2 - and M_3 -receptor populations are approximately equal (see ROFFEL *et al.* [13]). The M_2 -receptors are linked to the adenylate cyclase by an inhibitory G-protein. Therefore WATSON and EGLIN [14] speculated that the susceptibility to relaxation by β -agonists might also depend on the extent of the M_2 -receptor stimulation by the muscarinic spasmogen. Experimental evidence in support of this idea is based on the observation that the relaxant potency of isoprenaline against the bronchospastic effect of SDZENS 163, an M_2 -antagonist and M_3 -agonist, is greater than against an equipotent mixed agonist. However, SDZENS 163 is a partial M_3 -agonist [14].

DBcAMP mimics the effects of β -adrenoceptor activation but is not influenced by M_2 -receptor activation. The experiments with arecoline, WAL 2014 FU, thiopilocarpine and DBcAMP (fig. 4) revealed similar results to those with noradrenaline (table 2), and confirmed the importance of intrinsic activity at M_3 -receptors for susceptibility to functional antagonism. Results from experiments with selective muscarinic antagonists also deny an essential role of M_2 -receptors in functional antagonism in tracheal muscle [13].

In conclusion, our results show that the intrinsic activity of muscarinic agonists determines the susceptibility of their bronchospastic effects to antagonism by β -adrenoceptor activation. WAL 2014 FU proved to be a partial agonist, whose tonic effects in isolated tracheal muscle from guinea-pigs are easily antagonized by noradrenaline. This prominent susceptibility to antagonism by catecholamines is an indispensable prerequisite for the explanation of the failure of WAL 2014 FU to induce bronchospasm in the guinea-pig [3]. If this hypothesis is correct, WAL 2014 FU should cause bronchospasm in animals with β -blockade. This is indeed the case and will be reported in another paper.

In contrast to other muscarinic agonists [1], single oral doses of 40–140 mg WAL 2014 FU are devoid of bronchospastic activity in healthy volunteers but stimulate salivary secretion [15]. Information on the contractile potency as well as the intrinsic activity of WAL 2014 FU and its proneness to antagonism by β -agonists in isolated human tracheal muscle, however, is lacking.

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