The effect of varying carbachol concentration on the slope of Schild plots of selective β-adrenoceptor antagonists in the carbachol-contracted guinea-pig trachea

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The effect of varying the level of smooth muscle tone induced by carbachol on the Schild analysis of atenolol (β_1-selective) and ICI 118,551 (β_2-selective) with salbutamol as agonist, on the guinea-pig tracheal preparation has been examined. When 10^{-6} M carbachol was used to induce near-maximal smooth muscle tone, Schild plot slopes for atenolol and ICI 118,551 were less than 1. Slopes of Schild plots for both drugs were equivalent to 1 when 10^{-7} M carbachol was used to produce approximately half-maximal smooth muscle tone. Depletion of neuronal noradrenaline by prior treatment with reserpine had no effect on the Schild analysis. Salbutamol produced maximal relaxation and was more potent when tone was induced with 10^{-7} M carbachol, but was less effective at 10^{-6} M carbachol. Pretreatment with reserpine increased the potency of salbutamol at each concentration of carbachol. The results suggest that either the level of smooth muscle tone or an unknown effect associated with a high level of smooth muscle tone induced by carbachol may contribute to low slope values of Schild plots of selective β-adrenoceptor antagonists in the carbachol-contracted guinea-pig trachea. The carbachol-contracted guinea-pig trachea can be used to determine theoretically valid pA_2 values for selective β-adrenoceptor antagonists as long as substantially less than a maximal level of smooth muscle tone is induced by carbachol.

The guinea-pig trachea has frequently been used as a model to determine the potency of β-adrenoceptor antagonists at the β_2-adrenoceptor by utilizing the pA_2 method of Arunlakshana & Schild (1959). However, the relevancy of comparing pA_2 values with the equilibrium dissociation constants has been questioned because slopes of the Schild plots were often less than 1 for presumably competitive antagonists. Several studies have demonstrated that when the non-selective β-adrenoceptor agonist, isoprenaline, is used to relax tracheal preparations, slope values were generally less than 1 for selective antagonists, e.g. ICI 118,551, butoxamine, practolol, and atenolol (Levy & Wilkenfeld 1969; Imbs et al 1977; O'Donnell et al 1980), whereas slope values for non-selective antagonists, e.g. propranolol and sotalol, were equivalent to 1 (Furchgott et al 1973; Buckner et al 1974; O'Donnell & Wanstall 1980). Previous studies (Furchgott 1976, 1977; O'Donnell & Wanstall 1979) have suggested that the guinea-pig trachea contains β_1- and β_2-adrenoceptors, both of which mediate smooth muscle relaxation. Furchgott (1976) hypothesized that the Schild plot for a selective antagonist in this situation would be less

than 1 because interaction of a non-selective β-adrenoceptor agonist with both receptor subtypes would obscure the competitive nature of the selective antagonist with the receptor subtype of interest. Thus, a selective β_2-adrenoceptor agonist should theoretically resolve the problem of obtaining low slope values when the guinea-pig trachea is used to assess the potency of β-adrenoceptor antagonists against β_2-adrenoceptors.

O'Donnell & Wanstall (1980) have systematically performed Schild analyses of selective β-adrenoceptor antagonists using both a selective β_2-adrenoceptor agonist, fenoterol, and a non-selective β-adrenoceptor agonist, isoprenaline in both the carbachol-contracted tracheal preparation and the intrinsic tone tracheal preparation. It was found that the slopes of Schild plots were less than 1 in carbachol-contracted preparations, and equivalent to 1 in intrinsic tone preparations, regardless of whether a selective or non-selective agonist was used. These differences cannot entirely be attributed to the presence of a heterogenous population of β-adrenoceptors unless it is assumed that isoprenaline interacts selectively with β_2-adrenoceptors in the intrinsic tone preparation and that fenoterol interacts with both β_1- and β_2-adrenoceptors in the

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carbachol-contracted preparation. Alternatively, it may be that carbachol is producing a secondary effect which causes the slopes to be less than 1. The previous investigations by O'Donnell & Wanstall utilized trachea in which smooth muscle tone was induced with 10^{-6} M carbachol, a concentration which produces a near-maximal contraction. It was the purpose of the present investigation to determine whether varying the level of tone induced in the trachea by varying the carbachol concentration would affect the Schild analysis of the selective β-adrenoceptor antagonist, ICI 188,551 (β_2) and atenolol (β_1), using salbutamol, a selective β_2-adrenoceptor agonist to relax the preparations. Furthermore, any possible effects of neuronal noradrenaline release were examined by experiments on trachea taken from both reserpinized and non-reserpinized animals.

MATERIALS AND METHODS

Male, Hartley guinea-pigs (400–600 g) were killed by a sharp blow to the head followed quickly by exsanguination. Tracheae and right atria were removed and placed in oxygenated Krebs-Henseleit solution (room temp. approx. 22 °C) of the following composition (mM): NaCl 118; KCl 4·7; MgSO$_4$ 1·2; CaCl$_2$ 2·5; KH$_2$PO$_4$ 1·2; NaHCO$_3$ 25; glucose 11; disodium ethylenediaminetetraacetic acid 0·027; and pH 7·4. In some experiments guinea-pigs were given reserpine (5 mg kg^{-1}, i.p.) 20–24 h before the experiment. Tracheae were cleaned of extraneous connective tissue and cut into spiral strips as described by Constantine (1965). Two tracheal strips were obtained per animal. Tracheal strips and spontaneously beating right atria were suspended in double-jacketed organ baths filled with 20 ml of Krebs-Henseleit solution (37 °C). Tracheae and right atria were placed under an initial resting tension of 4 g and 1 g, respectively. Changes in isometric tension were measured with Grass FT03 Force-displacement transducers connected to a Grass Model 7D polygraph.

After an equilibration period of 45 min, tracheae, were incubated with carbachol (10^{-7} or 10^{-6} M) to induce tone and when this had stabilized (20–30 min), the first cumulative concentration-response curve to salbutamol was constructed. After a thorough washout (60–90 min), either atenolol or ICI 118,551 was added to the baths and 30 min later carbachol. After a further 30 min, the second cumulative concentration-response curve to salbutamol was constructed. Inhibitors of potential sites of loss for salbutamol, e.g. neuronal uptake, non-neuronal

uptake, or α-receptors, were not included in the present investigation because salbutamol, a non-catechol, exhibits low affinity for uptake processes and α-receptors (O'Donnell & Wanstall 1976; Jones et al 1975). All results were expressed as percent of the maximum relaxation obtained from the first salbutamol concentration-response curve.

The potency of β-adrenoceptor antagonists was assessed according to Arunlakshana & Schild (1959). Dose ratios (EC50 of the first concentration-response curve divided by the EC50 of the second concentration-response curve) were determined at different concentrations of antagonist. A plot of the log (dose ratio−1) versus the log concentration of antagonist was constructed and a regression line determined by the method of least squares. The pA$_2$ was determined from the regression line as the absolute value of the intercept on the x-axis.

The drugs used were: carbachol and papaverine (Sigma); atenolol and ICI 118,551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) (ICI); salbutamol (albuterol) from Schering. Drugs were dissolved and diluted in either 0·9% NaCl (saline) or double distilled water and freshly prepared daily.

RESULTS

From preliminary experiments, concentrations of 10^{-7} and 10^{-6} M carbachol were chosen to induce tracheal tone since these produced approximately half-maximal (45 ± 2%) and near-maximal (91 ± 1%, n = 12) contractions, respectively. To determine the effectiveness of reserpinization, the positive chronotropic effects of tyramine, an agent that displaces neuronal noradrenaline, was examined on spontaneously beating right atria taken from reserpinized or non-reserpinized guinea-pigs. The ability of tyramine to produce a maximal positive chronotropic effect was decreased by 87 ± 5% in atria taken from reserpinized animals (n = 3).

Potential time-dependent changes in agonist potency were monitored by control experiments in which both the first and second salbutamol concentration-response curves were run in the absence of antagonist (Fig. 1). There was an increase in the potency of salbutamol in the second concentration-response curve (0·09 log units, 23%) which was statistically significant ($P < 0.05$, paired t-test). Dose-ratios for antagonists were corrected for this increase in sensitivity using the factor, 1·23.

Schild plots for atenolol and ICI 118,551 obtained using salbutamol as an agonist with smooth muscle tone induced by 10^{-7} and 10^{-6} M carbachol in tissues
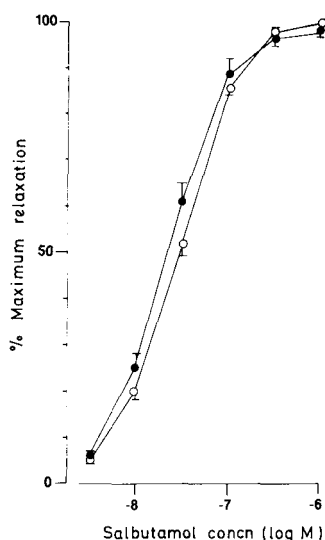
FIG. 1. Consecutive cumulative concentration-response curves for salbutamol (O, 1st curve; ●, 2nd curve) in carbachol-contracted (10^{-7} M) guinea-pig trachea. There was a slight increase in the sensitivity of salbutamol in the second concentration response curve (0·09 log units, 23%) which was statistically significant ($P < 0.05$, paired t-test). Consequently, dose-ratios for antagonists were corrected for this by the factor, 1·23. Results are expressed as the % maximum salbutamol-induced relaxation of the first concentration-response curve.

from reserpinized and non-reserpinized animals are shown in Fig. 2. The data from regression analysis of the plots are summarized in Table 1. When 10^{-7} M carbachol was used to induce tone, slopes for both atenolol and ICI 118,551 were not significantly different from 1. But when 10^{-6} M carbachol was used to induce tone, slope values were significantly less than 1. Reserpinization had no effect on the results at either concentration of carbachol. The lower slope values obtained with 10^{-6} M carbachol resulted in pA_2 values significantly higher than those obtained with 10^{-7} M carbachol-contracted preparations. The results suggest that higher concentrations of carbachol may produce an effect which contributes to the low slope values of Schild analysis.

It has been demonstrated that the potency of β-adrenoceptor agonists is decreased as tracheal tone is increased with higher concentrations of muscarinic cholinergic agonists (Van den Brink 1973) and that reserpine can induce tissue supersensitivity to β-adrenoceptors (Fleming & Trendelenburg 1961). To determine whether the potency of salbutamol was affected by the different concentrations of carbachol or reserpinization, the EC50 values of the first salbutamol concentration-response curves were compared under the four different experimental conditions (Table 2). The higher concentration of carbachol significantly decreased the potency of
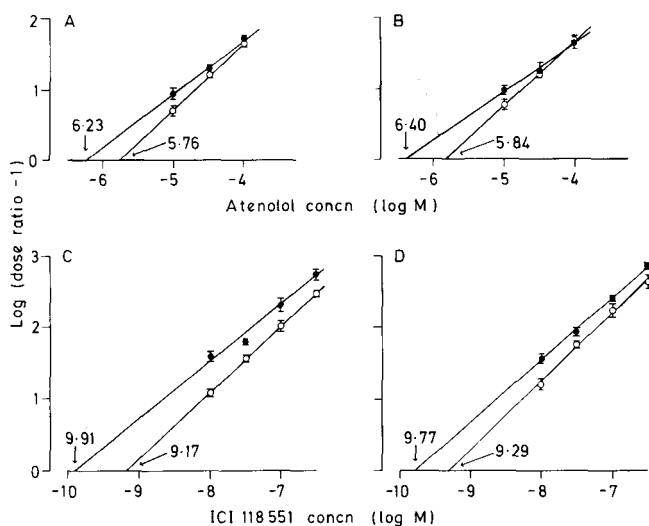

FIG. 2. Schild plots of the antagonism of salbutamol-induced relaxation of carbachol (10^{-7} M, O——O, (10^6 M, ●——●) contracted guinea-pig tracheae from (B,D) reserpinized (5 mg kg^{-1} reserpine; i.p., 20–24 h before the experiment) and (A,C) non-reserpinized animals by atenolol and ICI 118,551. The results are summarized in Table 2.

Table 1. Slopes from Schild Plots for atenolol and ICI 118,551 in carbachol-contracted (10^{-7} and 10^{-6} M) guinea-pig tracheae from reserpinized and non-reserpinized animals using salbutamol as the β_2-selective adrenoceptor agonist.

Atenolol	10^{-7} M Carbachol		10^{-6} M Carbachol	
	Non-reserpinized	Reserpinized[a]	Non-reserpinized	Reserpinized[a]
pA_2[b]	5·76	5·84	6·23[e]	6·40[e]
(95% CL)	(5·50–6·02)	(5·49–6·19)	(5·77–6·69)	(5·79–7·01)
Slope[c]	0·96	0·91	0·75[f]	0·68[f]
(95% CL)	(0·83–1·09)	(0·74–1·08)	(0·59–0·91)	(0·45–0·91)
n[d]	8	6	7	5
ICI 118,551				
pA_2[b]	9·17	9·29	9·91[e]	9·77[e]
(95% CL)	(8·95–9·39)	(9·11–9·47)	(9·69–10·13)	(9·48–10·09)
Slope[c]	0·94	0·96	0·80[f]	0·87[f]
(95% CL)	(0·85–1·03)	(0·85–1·07)	(0·72–0·88)	(0·78–0·96)
n[d]	10	7	6	7

[a] Guinea-pigs were pretreated with 5 mg kg^{-1} reserpine, i.p., 20–24 h before experiment.
[b] Mean and 95% confidence limits (95% CL) of pA_2 values derived from individual Schild plots by regression analysis.
[c] Mean and 95% confidence limits (95% CL) of slope values derived from individual Schild plots by regression analysis.
[d] n = number of Schild plots used to determine summary statistics of pA_2 and slope values.
[e] pA_2 value significantly different from corresponding value obtained when 10^{-7} M carbachol was used to induce tone ($P < 0.05$, t-test).
[f] Slope significantly less than one ($P < 0.05$).

Table 2. Potency of salbutamol-induced smooth muscle relaxation in carbachol-contracted (10^{-7} and 10^{-6} M) guinea-pig tracheae from reserpinized and non-reserpinized animals.

	Salbutamol EC50 (nM)[a]	
	10^{-7} M Carbachol	10^{-6} M Carbachol
Non-reserpinized		
$\bar{X}$[b]	28·8	89·1[d]
(95% CL)[b]	(25·7–32·4)	(74·1–107·2)
n[b]	48	42
Reserpinized[c]		
$\bar{X}$[b]	20·0[e]	44·7[d,e]
(94% CL)[b]	(16·6–24·0)	(39·8–50·1)
n[b]	39	48

[a] Concentration of salbutamol that produced 50% of the maximal relaxation induced by salbutamol.
[b] Mean ($\bar{X}$) and 95% confidence limits (95% CL) of the EC50 values for salbutamol. n = number of tracheae per group.
[c] Guinea-pigs were pretreated with reserpine (5 mg kg^{-1} i.p.) 20–24 h before experiment.
[d] Significantly different from EC50 value of 10^{-7} M carbachol-contracted tracheae ($P < 0.05$, t-test).
[e] Significantly different from EC50 value of tracheal preparation from non-reserpinized guinea-pigs ($P < 0.05$, t-test).

salbutamol in tracheal preparations from both reserpinized and non-reserpinized animals. Reserpinization produced a slight, but significant increase in potency of salbutamol at each concentration of carbachol. It has also been shown that, in addition to

decreasing the potency of β-adrenoceptor agonists, sufficiently high levels of smooth muscle tone induced by a cholinergic agonist result in the inability of β-adrenoceptor agonists to produce a maximal relaxation, i.e. exhibit partial agonist activity (Van den Brink 1973). To determine whether salbutamol was a full or partial agonist in the present investigation, 3×10^{-4} M papaverine was added to induce a maximal relaxation following the maximally effective concentration of salbutamol in the second salbutamol concentration-response curve in some experiments. When 10^{-7} M carbachol was used to induce tone, salbutamol was consistently a full agonist in both reserpinized and non-reserpinized preparations, producing greater than 99% (range 92–100%, n = 56) of the maximum relaxation produced by papaverine. When 10^{-6} M carbachol was used to induce tone, salbutamol was a partial agonist, producing 81 ± 1% of maximal relaxation (range 64–95%, n = 60) in non-reserpinized and 88 ± 1% of maximal relaxation (range 69–95%, n = 40) in reserpinized preparations. The slightly less partial agonist propensity of salbutamol in reserpinized preparations may be due to the increased potency of salbutamol in these preparations. Thus, low slope values of Schild plots for atenolol and ICI 118,551 in the present investigation were associated with smooth muscle tone induced with 10^{-6} M carbachol, conditions under which salbutamol is a partial agonist and slightly less potent than when tone was induced by 10^{-7} M carbachol.

DISCUSSION

Previous investigations have demonstrated that slopes of Schild plots less than 1 have been obtained when the potency of presumably competitive β-adrenoceptor antagonists in the guinea-pig trachea were being determined (Levy & Wilkenfeld 1969; Imbs et al 1977; O'Donnell & Wanstall 1980). Furchgott (1976) suggested that the presence of a heterogenous population of β-adrenoceptor subtypes could account for Schild plot slopes being less than 1, particularly when selective antagonists are used with non-selective agonists. O'Donnell & Wanstall (1979) suggested that factors other than a heterogenous β-adrenoceptor population may be important, because it was found that the slopes of the Schild plots were less than 1 in carbachol-contracted tracheal preparations and equivalent to 1 in intrinsic tone preparations regardless of whether a selective or non-selective agonist was used.

Theoretically, simple functional antagonism of adrenergic receptor response due to smooth muscle

tone induced by a muscarinic agonist should not affect the slope or location of Schild plots for β-adrenoceptor antagonists (Ohashi 1976). The results of the present investigation, however, suggest that either the level of smooth muscle tone, or an unknown effect associated with a high level of smooth muscle tone induced by carbachol may be contributing to low slope values in carbachol-contracted preparations. The use of 10^{-6} M carbachol to induce smooth muscle tone in the present investigation resulted in Schild plot slopes less than 1 for the selective β-adrenoceptor antagonists, ICI 118,551 and atenolol, when the β_2-selective agonist salbutamol was used. This finding is similar to the results of O'Donnell & Wanstall (1980), who used the selective β-adrenoceptor antagonists, atenolol and butoxamine, with the selective β_2-adrenoceptor agonist, fenoterol, on tracheal preparations contracted with 10^{-6} M carbachol. When we used 10^{-7} M carbachol to induce smooth muscle tone, however, the Schild plot slopes were equal to 1 as were those of the intrinsic tone preparation of O'Donnell & Wanstall (1980). Therefore, a factor contributing to low slope values in the carbachol-contracted trachea may be the extent of the tone, rather than the presence of any tone, induced by carbachol.

The reasons for the differences in slope values at different levels of tracheal tone induced by carbachol are unclear. One possibility is that at high concentrations, carbachol may be interacting with nicotinic receptors and causing the neuronal release of a substance that interferes with the Schild analysis. If this is so, it is unlikely that the neuronal release of noradrenaline is involved since reserpinization had no effect on Schild analysis when either concentration of carbachol was used. Furthermore, if the neuronal release of a non-adrenergic substance were contributing to the low slope values, it would be expected that Schild plot slopes for all antagonists would be less than 1, but propranolol and sotalol, both non-selective antagonists, yield a slope equivalent to 1 in the carbachol-contracted guinea-pig trachea (Furchgott et al 1973; Buckner et al 1974; O'Donnell & Wanstall 1980).

Another possible factor may be the decreasing potency of β-agonists as the level of tracheal tone is increased. The sensitivity to such agonists is inversely related to the level of tone induced by a muscarinic receptor agonist, and at sufficiently high levels of smooth muscle tone, a β-adrenoceptor agonist may be incapable of producing a maximal level of relaxation (Van den Brink 1973). In the present study, salbutamol was less potent at 10^{-6} M

carbachol and appeared to be a full agonist when tone was induced by 10^{-7} M carbachol, but a partial agonist with 10^{-6} M carbachol. It may be that as the smooth muscle tone increased, the agonist progressively interacted with a greater proportion of β_1-adrenoceptors, and consequently, behaved as a non-selective agonist. This would account for the observation that non-selective antagonists exhibit Schild plot slopes equivalent to 1 in carbachol-contracted preparations (Furchgott et al 1973; Buckner et al 1974; O'Donnell & Wanstall 1980), because the dose-ratios for non-selective antagonists should be the same regardless of the proportion of β_1- or β_2-adrenoceptors contributing to the smooth muscle relaxation (Furchgott 1976). This line of reasoning would not account for the observation that selective antagonists exhibited a Schild plot slope equal to 1 in intrinsic tone preparations when the non-selective agonist isoprenaline was used (O'Donnell & Wanstall 1980), unless it is presumed that isoprenaline interacts selectively with β_2-adrenoceptors in the intrinsic tone preparation. To date, no experimental evidence to support this has been put forward.

Clearly, questions remain concerning the use of the guinea-pig trachea for Schild analysis of β-adrenoceptor antagonists. Indeed, the results of O'Donnell & Wanstall (1980) and the present study have suggested that the carbachol-contracted guinea-pig trachea may not be a reliable model to test the affinity of selective β-adrenoceptor antagonists when 10^{-6} M carbachol is used to induce tone. Nevertheless, from a practical basis the results of the present investigation demonstrate that the carbachol-contracted guinea-pig trachea can be used to determine pA_2 values for selective β-adrenoceptor antagonists as long as substantially less than a maximal level of smooth muscle tone is induced by carbachol. Taylor (1982) also obtained slope values equal to 1 for selective β-adrenoceptor antagonists in the carbachol-contracted ($2 \cdot 5 \times 10^{-7}$ M) trachea using terbutaline as a selective β_2-adrenoceptor agonist. However, these experiments were run in the presence of practolol at a concentration designed to selectively block β_1-adrenoceptors. Our present results suggest that the blockade of β_1-adrenoceptors is not necessary when 10^{-7} M carbachol is used to induce smooth muscle tone and salbutamol is used to induce relaxation. Furthermore, the experiments were conducted in the absence of inhibitors of neuronal or non-neuronal uptake, or inhibitors of α-adrenoceptors. The addition of compounds to block these sites is generally recommended to ensure

that the agonist interacts predominantly with the receptor of interest (Furchgott 1972). Significant interaction of agonist with neuronal or non-neuronal uptake sites or α-receptors could theoretically result in Schild plot slopes different from 1. Salbutamol, however, is a non-catechol and exhibits very low affinity for uptake sites or α-adrenoceptors (O'Donnell & Wanstall 1976; Jones et al 1975). Indeed, O'Donnell & Wanstall (1980) found that when the non-catechol β-adrenoceptor agonist, fenoterol, was used to relax tracheal preparations, the slopes of Schild plots for butoxamine were less than 1 in the carbachol-contracted trachea (10^{-6} M carbachol) in both the absence and presence of uptake inhibitors. Therefore, there is no theoretical basis which would suggest that the addition of uptake inhibitors or α-antagonists would significantly change the results of the present investigation.

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