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THE USE OF FUNCTIONAL ANTAGONISM TO DETERMINE WHETHER β-ADRENOCEPTOR AGONISTS MUST HAVE A LOWER EFFICACY THAN ISOPRENALINE TO BE TRACHEA-ATRIA SELECTIVE *in vitro* IN GUINEA-PIGS

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1 The relative efficacies of three trachea-atria selective β-adrenoceptor agonists, fenoterol, Me 506 and Me 454, compared to isoprenaline, were determined on both trachea and atria of guinea-pigs.

2 On tracheal preparations the β-adrenoceptor agonists were used as functional antagonists of carbachol and a comparison of the maximum shifts in the carbachol concentration-response line produced by each of the β-adrenoceptor agonists provided a comparison of their efficacies.

3 On atrial preparations carbachol was used as a functional antagonist of the β-adrenoceptor agonists and comparison of the maximum responses to the β-adrenoceptor agonists in the presence of carbachol provided a comparison of their efficacies.

4 On trachea and atria the order of efficacy of the compounds was Me 454 > Me 506 ≥ isoprenaline = fenoterol.

5 The results indicated that high efficacy in a non-catechol β-adrenoceptor agonist is possible provided there is a favourable N-substituent group.

6 Since Me 454, Me 506 and fenoterol, which are trachea-atria selective, have efficacies equal to or greater than that of isoprenaline, which is non-selective, it is concluded that low efficacy in a compound is not essential for it to show trachea-atria selectivity *in vitro* in guinea-pigs.

Introduction

A number of β-adrenoceptor stimulants used clinically as bronchodilators produce bronchodilatation without cardiac stimulation, i.e. unlike isoprenaline they are selective. This selectivity of action can be demonstrated *in vitro* in pharmacological experiments in that higher concentrations of selective β-adrenoceptor stimulants are required to give responses on guinea-pig atria than on trachea, whereas this is not so for isoprenaline (O'Donnell & Wanstall, 1974). Lands, Luduena & Buzzo (1967) have proposed that various tissues contain different subtypes of β-adrenoceptor and the conventional view has been that potency differences observed for selective drugs in *in vitro* experiments reflect their affinities for two different receptors in trachea (β_2) and atria (β_1). This view has been challenged (Buckner & Abel, 1974; Buckner & Saini, 1975) as a result of data obtained with soterenol. The potency (EC_{50} values) of soterenol was shown to be different on trachea and atria of guinea-pig by Buckner & Abel (1974). However, EC_{50} values are not necessarily a true measure of the affinity of β-adrenoceptor agonists for the receptor (Offermeier, Dreyer & Brandt, 1972; Buckner & Saini, 1975) and from subsequent experiments Buckner & Saini (1975) concluded that soterenol had the same affinity for the receptors in trachea and atria in guinea-pig. Buckner & Saini (1975) suggested that this supported the postulate that the β-adrenoceptors in these two tissues could be of a single type, but they also made two other important points. The first was that the intrinsic activity of soterenol was less than that of isoprenaline on both trachea and atria. Although Buckner & Saini (1975) used the term intrinsic activity, the term efficacy (Stephenson, 1956) has been used in this paper. The term efficacy is equivalent to the term intrinsic activity (Furchgott, 1966). Secondly, they made the statement that the β-adrenoceptor reserve in guinea-pig trachea was approximately 20-fold greater than that in atria, although the supporting data were not at that time published.

It thus appeared that the selectivity of soterenol could not be explained in terms of different affinities for receptors but that it might result from the low

efficacy of soterenol if the receptor reserve in guinea-pig trachea was greater than that in the atria. A number of other selective β-adrenoceptor stimulants, e.g. salbutamol, terbutaline and carbuterol, have been shown to be partial agonists in some tissues, indicating that they have low efficacies, and Jenkinson (1973) envisaged that 'in general, selective β-agonists may have lower efficacies on all β-receptors than unselective compounds such as isoprenaline'. The present experiments were therefore undertaken in guinea-pig to determine whether trachea-atria selectivity could occur *in vitro* even with compounds with high efficacy. The compounds selected for study were trachea-atria selective but, although they were all full agonists, their efficacies relative to that of isoprenaline were not known.

A preliminary account of this work was given to the Australian Physiological and Pharmacological Society (O'Donnell & Wanstall, 1976b).

Methods

Female guinea-pigs (300–550 g) pretreated with reserpine (5 mg/kg, i.p., 24 h previously) were used throughout this study. All concentration-response lines were determined in the presence of metanephrine (50 μM) and phentolamine (10 μM), to block extraneuronal uptake and α-adrenoceptors respectively. These drugs were in contact with the tissue for 30 min before each concentration-response line was obtained.

Experiments on trachea

Tracheal chain preparations were set up for isotonic recording as described by O'Donnell & Wanstall (1974). The resting tension on the tissues was 500 mg. Concentration-response lines to carbachol were obtained by the cumulative method of drug addition.

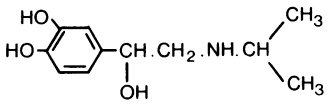
The method used on trachea to obtain efficacy (intrinsic activity) values of the compounds was that advocated by Van den Brink (1973b). A control concentration-response line to carbachol was obtained, followed by further concentration-response lines to carbachol in the presence of different and increasing concentrations of a β-adrenoceptor agonist. The β-adrenoceptor agonist was in contact with the tissue for 15 min before obtaining the next carbachol line. The maximum response to carbachol was established at the end of every line. Each response was expressed as a percentage of the maximum contraction which was calculated from the difference between the resting state at the beginning of the line in question and the maximally contracted state at the end of the control line, i.e. in the absence of the β-adrenoceptor agonist drug. Values for % maximum contraction were plotted against log concentration carbachol; from these graphs the effect of the β-adrenoceptor agonist drug on the maximum response to carbachol could be ascertained.

From each line the concentration of carbachol producing 50% of the maximum response in that particular line was also interpolated and converted to a negative log value (neg log EC_{50}). The β-adrenoceptor agonist, acting as a functional antagonist, shifted the carbachol line to a higher concentration range (i.e. decreased the neg log EC_{50}). For each concentration of β-adrenoceptor agonist, the difference between the neg log EC_{50} in the absence (control) and presence (functional antagonism) of the β-adrenoceptor agonist was obtained. This difference is referred to as the log unit shift. Mean values of log unit shift for each concentration of β-adrenoceptor agonist were obtained from several preparations. In some experiments, after a certain concentration of β-adrenoceptor agonist, no further decrease in the neg log EC_{50} value for carbachol occurred despite increasing the concentration of β-adrenoceptor agonist, i.e. a maximum value for log unit shift could be obtained (max. log unit shift). Comparison of the values for max. log unit shift for different β-adrenoceptor agonists should provide a comparison of their efficacies (Van den Brink, 1973b).

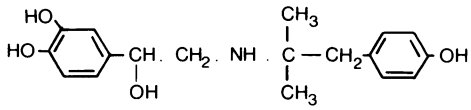
Experiments on atria

Preparations of spontaneously beating atria were set up at 37°C and atrial rate recorded as described by O'Donnell & Wanstall (1974). On these preparations cumulative concentration-response (increase in rate) lines to the β-adrenoceptor agonists were obtained in the presence of carbachol as a functional antagonist. The maximum rate of beating of the preparation was routinely obtained at the beginning of each experiment by adding a supramaximal concentration of isoprenaline (1 μM). When resting rate was restored, a concentration of carbachol was added which was sufficient to cause a depression in the maximum response to the β-adrenoceptor agonist being tested. This ranged from 100nM to 500 nM in different experiments. Carbachol decreased the resting atrial rate and this new rate was taken as the baseline rate for the next concentration-response line to β-adrenoceptor agonist. The difference between this baseline rate and the maximum rate of beating of the preparation was taken as 100% response and all other responses expressed as a percentage of that difference in order to plot log concentration-% maximum response lines.

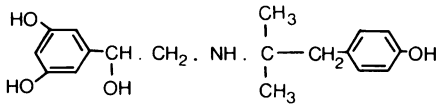
Up to 3 different β-adrenoceptor agonists could be compared on each preparation in the presence of the same concentration of carbachol. Comparison of the % maximum responses was used to compare the efficacies of the β-adrenoceptor agonists.



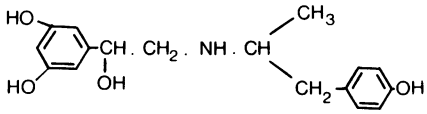
Isoprenaline



Me 454



Me 506



Fenoterol

Figure 1 Structures of isoprenaline, Me 454, Me 506 and fenoterol.

Drugs

The β-adrenoceptor agonists used were: fenoterol hydrobromide (Th 1165a, Boehringer-Ingelheim); (±)-isoprenaline sulphate (Burroughs Wellcome); Me 454 base (Boehringer-Ingelheim) and Me 506 hydrobromide (Boehringer-Ingelheim). The structures of these drugs are shown in Figure 1. Other drugs used were: carbachol (Sigma); hydrocortisone hemisuccinate sodium (Glaxo); metanephrine hydrochloride (Calbiochem); phentolamine methanesulphonate (Regitine, Ciba); propranolol hydrochloride (ICI); tropolone (Regis).

All drugs were used as pure powders. The β-adrenoceptor agonists were made up in 0.01 N HCl to give stock solutions of 10 or 100 mM. Dilutions were made in Krebs solution containing ascorbic acid (0.2 μg/ml) and kept on ice for the duration of the experiment.

Statistical analyses

The measure of the variation of the mean quoted is the standard error (s.e. mean).

Results

Selection of experimental conditions

In previous studies in tracheal preparations with intrinsic tone (O'Donnell & Wanstall, 1976a) phenoxybenzamine was used as the inhibitor of extraneuronal uptake. In the present study phenoxybenzamine was unsuitable because it antagonized the contractions to carbachol on trachea. Phenoxybenzamine has been shown to have antimuscarinic activity (Goodman & Gilman, 1975). Both metanephrine (50 μM) and hydrocortisone (50 μM) potentiated, by 2 to 2.5-fold, the responses to isoprenaline on tracheae contracted by 5 μM carbachol. However, the hydrocortisone tended to reduce the maximum contraction that could be attained with carbachol whereas metanephrine did not. The potentiation of isoprenaline produced by 200 μM metanephrine was no greater than that produced by 50 μM. Also, although tropolone (100 μM), a catechol-*O*-methyl transferase inhibitor, caused a 2.5-fold potentiation of isoprenaline, it caused no further potentiation after metanephrine (50 μM). Therefore metanephrine (50 μM) was selected as an inhibitor drug of extraneuronal uptake for inclusion in the Krebs solution. Stimulation of α-adrenoceptors was prevented by the inclusion of phentolamine (10 μM) in the Krebs solution as used by Buckner & Saini (1975). This concentration caused little change in the sensitivity of the tracheal preparations to isoprenaline. On atria, inclusion of metanephrine (50 μM) and phentolamine (10 μM) produced no marked change in sensitivity to isoprenaline but these drugs were routinely used to ensure identical experimental conditions for tracheal and atrial preparations. The potency of all the β-adrenoceptor agonists in the present study had been shown to be unaffected by cocaine (O'Donnell & Wanstall, 1974). Thus it was not considered necessary to include cocaine in the Krebs solution.

On trachea the values for neg log EC_{50} carbachol obtained using cumulative and single dose additions of the drug were not significantly different. Thus cumulative drug additions were selected. The sensitivity of tracheal preparations to carbachol did not change with repeated lines during the course of an experiment, either in the absence or in the presence of isoprenaline (50 μM), the latter situation necessitating the use of much higher concentrations of carbachol because of functional antagonism. Therefore several concentration-response lines to carbachol could be

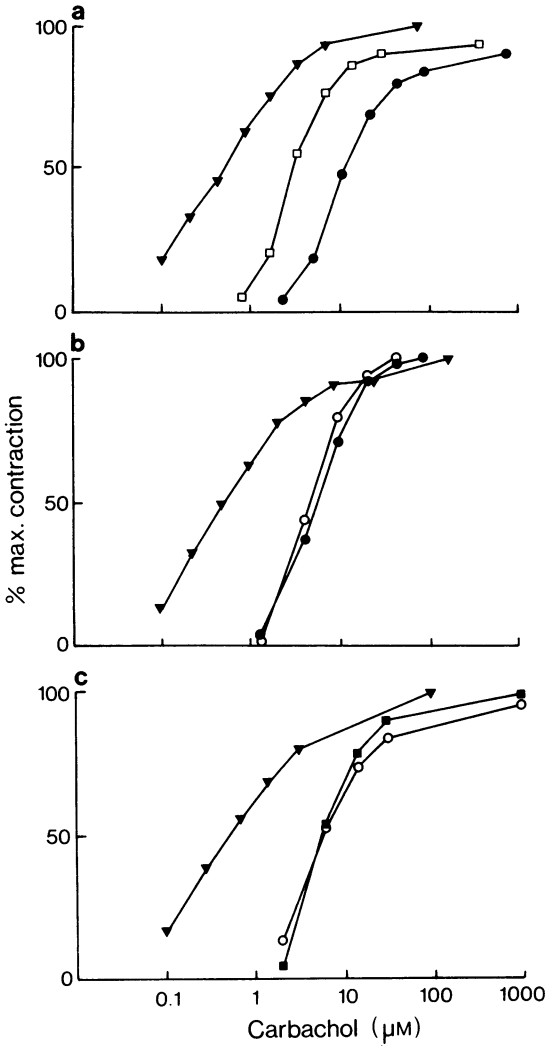


Figure 2 Functional antagonism of carbachol by isoprenaline in 3 preparations of guinea-pig isolated trachea. Concentration-response lines (with response representing % maximum contraction) to carbachol are shown in the absence (▼) and in the presence of isoprenaline 100 nM (□), 1 μM (●), 5 μM (○) and 20 μM (■). This illustrates that the maximum log unit shift in the carbachol line has been reached in experiments (b) and (c) but not in (a).

obtained on each preparation and no allowances were necessary, in the calculations, for changes in sensitivity.

Relative efficacy values on trachea

In the presence of increasing concentrations of isoprenaline, fenoterol, Me 454 or Me 506 there was an increase in the EC_{50} values for carbachol (i.e. a decrease in the neg log EC_{50} values, Table 1) until, where possible, a limiting value was reached. This is illustrated for isoprenaline in Figure 2. Isoprenaline (100 nM and 1 μM) produced progressive shifts in the carbachol concentration-response line (Figure 2a) but no further shift occurred with 5 or 20 μM isoprenaline (Figure 2b and c). Mean values ± s.e. mean of the log unit shift for isoprenaline were: 1.14 ± 0.05 (4), 1.13 ± 0.07 (15) and 1.08 ± 0.05 (26) at concentrations of 1, 5 and 20 μM respectively, with numbers of determinations in parentheses. Thus, in subsequent experiments with other β-adrenoceptor agonists either 5 μM or 20 μM isoprenaline was used to obtain a value for the maximum shift to isoprenaline.

Fenoterol (100 nM, 1 μM and 10 μM) produced progressive shifts in the carbachol line (Figure 3a) with the maximum shift occurring after both 10 and 100 μM fenoterol (Figure 3b, Tables 1 and 2). The maximum log unit shift produced by fenoterol was not significantly different from that produced by isoprenaline (Table 2). Both Me 454 and Me 506 in various concentrations caused a shift in the carbachol line (Figure 3c and d) but it was not possible to establish the maximum shift for either of these drugs. This was because, at concentrations above 100 μM Me 454 and above 1 mM Me 506, any further shift in the carbachol line was accompanied by a depression in the carbachol maximum response (39%–58% depression for Me 454 and 44% depression for Me 506). This was in contrast to fenoterol and isoprenaline for which no marked depression of the maximum response occurred with concentrations which maximally shifted the carbachol line (Table 1). Nevertheless, at 100 μM Me 454 and 1 mM Me 506 the shift produced by the agonist was already significantly greater than the maximum shift produced by isoprenaline in the same experiments (Table 2). Therefore the order of efficacies of the compounds was Me 454 > Me 506 > isoprenaline = fenoterol.

The shifts in the carbachol concentration-response line produced by isoprenaline, fenoterol, Me 454 and Me 506 were prevented if propranolol (1 μM) was present in the Krebs solution.

Relative efficacy values on atria

On atria, in the presence of a carbachol concentration which depressed the maximum response to isoprenaline and the other β-adrenoceptor agonists, the maximum response produced by Me 454 was significantly greater than that produced by isoprenaline. The maximum responses to fenoterol and Me 506 were not significantly different from that produced by isoprenaline (Figure 4). The order of efficacies of the compounds was Me 454 > Me 506 ≥ isoprenaline = fenoterol.

Discussion

At present there is no established irreversible antagonist at β-adrenoceptors available for use in studies on the relative efficacy values of β-adrenoceptor agonists. Therefore in the present study the experimental procedure used by Besse & Furchgott (1976) for estimating the relative efficacies of different agonists on α-adrenoceptors could not be used since it involved the analysis of concentration-response data before and after irreversible inactivation of a fraction of the total receptors. As an alternative procedure, functional antagonism may be used, the theoretical models for which have been put forward by Van den Brink (1973a) and verified by him for β-adrenoceptor agonists (Van den Brink, 1973b). Buckner & Saini (1975) elaborated on the principle of functional antagonism and used it to determine affinity values for β-adrenoceptor agonists in guinea-pig trachea.

In the present study functional antagonism has been used to estimate the relative efficacies for β-adrenoceptor agonists on both trachea and atria of guinea-pig. Van den Brink (1973b) described two experimental procedures, both involving functional antagonism, which could be used to reveal differences in efficacy of those β-adrenoceptor agonists which normally appear as full agonists. He suggested that the best method was that in which the β-adrenoceptor agonist, acting as the functional antagonist, caused a shift in the concentration-response lines to a muscarinic receptor agonist. Using this approach the ratio of the maximum shift produced by two β-adrenoceptor agonists is equal to the ratio of their efficacies. In the present study this method has been used in the tracheal experiments but it was not suitable for use in atrial experiments because of difficulties in obtaining concentration-response lines to carbachol on atrial rate. Therefore, with atria the alternative method was used in which the maximum responses produced by the β-adrenoceptor agonists in the presence of a muscarinic receptor agonist acting as functional antagonist, were compared. From the theoretical models proposed by Van den Brink (1973a,b) the maximum responses to β-adrenoceptor agonists in the presence of a given concentration of the muscarinic agonist will differ according to their efficacies.

These methods have been used to obtain the order of efficacies of fenoterol (Th 1165a), Me 506, Me 454 and isoprenaline. The former three drugs were selected because they were trachea-atria selective compounds and had previously been shown to give a maximum response on both trachea and atria (O'Donnell, 1972; O'Donnell & Wanstall, 1974). Also, in recent biochemical studies using adenylate cyclase assays in frog erythrocyte membranes (Mukherjee, Caron, Mullikin & Lefkowitz, 1976) Me 454 (or Cc-34) had a higher efficacy (the term intrinsic activity is used in their paper) than that of isoprenaline. In the same

Table 1 Guinea-pig isolated tracheal preparations. Values of neg log EC_{50} and % maximum contraction for carbachol in the absence and in the presence of various β-adrenoceptor agonists acting as functional antagonists.

β-Adrenoceptor agonist (functional antagonist) Compound	Concentration (μM)	neg log EC_{50} carbachol	% maximum contraction
None (control)		6.26 ± 0.02 (58)	100
Isoprenaline	1	5.18 ± 0.07 (4)	94 ± 3.0
	5	5.14 ± 0.04 (15)	91 ± 1.8
	20	5.18 ± 0.04 (26)	93 ± 1.4
Fenoterol	10	5.44 ± 0.11 (7)	98 ± 2.7
	100	5.26 ± 0.07 (9)	100 ± 3.4
Me 454	10	5.06 ± 0.06 (6)	89 ± 2.3
	100	4.62 ± 0.05 (7)†	85 ± 1.8
Me 506	100	5.20 ± 0.03(4)	97 ± 0.85
	1000	5.04 ± 0.03 (6)*	92 ± 1.7

All values are mean values ± s.e. mean.
The number of different tissue preparations is given in parentheses.
† Significantly lower than value for 10 μM Me 454 (*t*-test, $P < 0.001$); * significantly lower than value for 100 μM Me 506 (*t*-test, $0.01 > P > 0.001$).

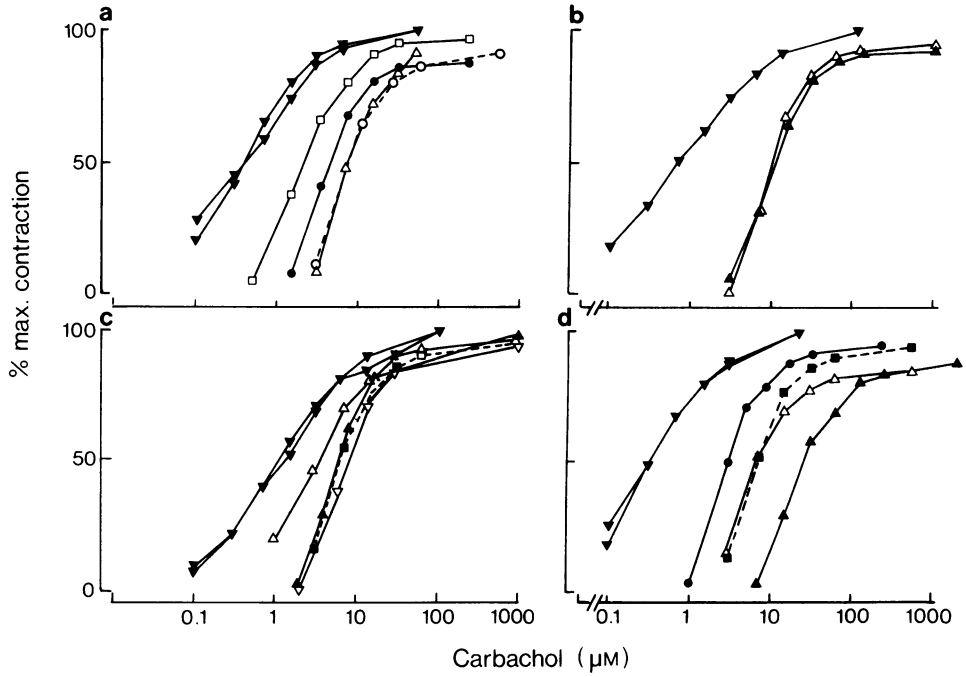


Figure 3 Functional antagonism of carbachol by fenoterol (a and b), Me 506 (c) and Me 454 (d) on 7 preparations of isolated trachea from 4 guinea-pigs. Concentration-response lines to carbachol in the absence (▼) and in the presence of various concentrations of functional antagonist are illustrated. (a) and (b) show the shift in the carbachol line by concentrations of fenoterol of 100 nM (□), 1 μM (●), 10 μM (△) and 100 μM (▲). The shift with 10 μM fenoterol is the same as that with 100 μM fenoterol and with 5 μM isoprenaline (○– – –○). (c) shows the shift in the carbachol line by concentrations of Me 506 of 10 μM (△), 100 μM (▲) and 1mM (▽). The shift with 1mM Me 506 is greater than that with 20 μM isoprenaline (■– – –■). (d) shows the shift in the carbachol line by concentrations of Me 454 of 1 μM (●), 10 μM (△) and 100 μM (▲). The shift with 100 μM Me 454 is greater than that with 20 μM isoprenaline (■– – –■).

Table 2 Guinea-pig isolated tracheal preparations. Values of log unit shift of carbachol concentration-response lines produced by various concentrations of β-adrenoceptor agonist together with the maximum log unit shift produced by isoprenaline on the same tissues.

Compound	β-Adrenoceptor agonist Concentration (μM)	log unit shift	Isoprenaline max log unit shift	Number of different tissue preparations
Fenoterol	10	0.88 ± 0.11	1.08 ± 0.07	7
	100	0.99 ± 0.05	1.03 ± 0.05	9
Me 454	10	1.24 ± 0.04	1.16 ± 0.08	5
	100	1.76 ± 0.06***	1.22 ± 0.07	7
Me 506	100	0.88 ± 0.08	0.81 ± 0.14	4
	1000	1.11 ± 0.06*	0.99 ± 0.05	6

All values are mean values ± s.e. mean.
* Significantly greater than the log unit shift with 100 μM Me 506 (Student's *t*-test) and than the max log unit shift with isoprenaline (paired *t*-test $0.05 > P > 0.01$); *** Significantly greater than the log unit shift with 10 μM Me 454 (Student's *t*-test) and than the max log unit shift with isoprenaline (paired *t*-test $P < 0.001$).

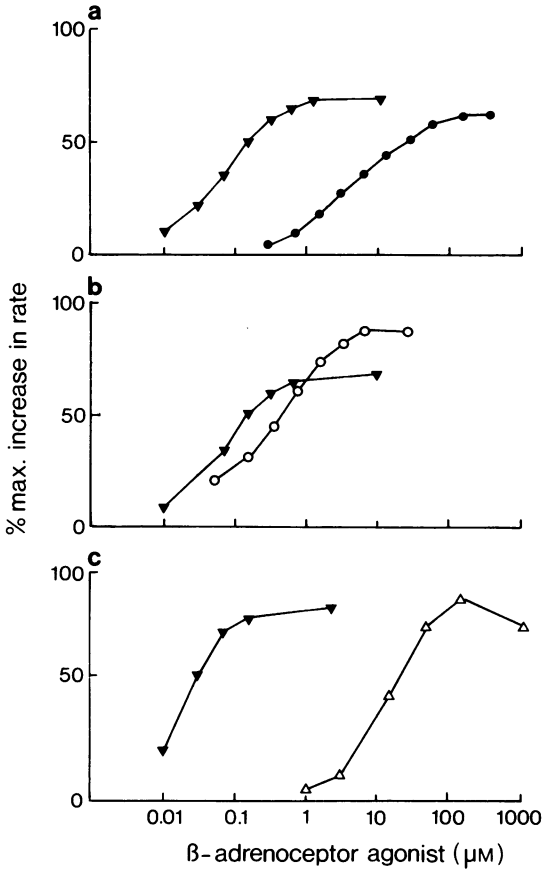


Figure 4 Three preparations of guinea-pig isolated atria. Concentration-response lines to isoprenaline (▼), fenoterol (●), Me 454 (○), and Me 506 (△) are shown. The experiments were carried out in the presence of carbachol (300 nM in (a) and (b) and 200 nM in (c)). The figure uses one typical experiment for each β-adrenoceptor agonist to illustrate the findings that the maximum response to fenoterol was not significantly different from isoprenaline ($t=0.14$, d.f. 5, paired t-test); the maximum response to Me 454 was significantly greater than that to isoprenaline ($t=4.76$, d.f. 4, $0.01>P>0.01$ paired t-test) and the maximum response to Me 506 was greater than that to isoprenaline but not significantly ($t=1.14$, d.f. 3, paired t-test).

study Cc-25 was also shown to have an efficacy greater than that of isoprenaline. A similar finding was reported by Van den Brink (1973b) in pharmacological experiments of the type described in this paper but carried out on calf trachea. Me 506 and fenoterol are resorcinolamines with the same N-alkyl substituent groups as Me 454 and Cc-25 respectively, the latter compounds being catecholamines.

In the present study the efficacies of Me 454 and Me 506 on trachea were both higher than that of isoprenaline and the efficacy of fenoterol was as high as that of isoprenaline. On the atria the efficacy of Me 454 was clearly greater than that of isoprenaline, the efficacy of Me 506 was at least equal to and possibly greater than that of isoprenaline and the efficacy of fenoterol was the same as that of isoprenaline. In other words, the three compounds examined had an efficacy at least as great as that of isoprenaline on both tissues. For Me 454, a catecholamine, it was encouraging that there was some agreement between the conclusions from the pharmacological (present study) and biochemical (Mukherjee *et al.*, 1976) approaches to efficacy (intrinsic activity) measurements. This was despite the involvement of different species and different tissues in the two experimental situations. The other two compounds examined (fenoterol and Me 506) were resorcinolamines, not catecholamines, and were not included in the study by Mukherjee *et al.* (1976). The non-catecholamines which these workers studied all had low efficacies and this led them to conclude that a catechol ring structure was necessary for full efficacy. The present pharmacological study has indicated that high efficacy is possible in the absence of a catechol structure provided that a favourable N-substituent group is present in the molecule. On the other hand, comparison of the results for Me 454 (catecholamine) and Me 506 (resorcinolamine) indicated that, if the catechol ring was also present, an even higher efficacy was feasible.

The trachea-atria selectivity of the drug soterenol may depend entirely on its low efficacy combined with a difference between the β-receptor reserves in trachea and atria since no difference was found between its affinity values on trachea and atria (Buckner & Saini, 1975). This could be the reason why soterenol was not a strikingly selective β-agonist (Buckner & Abel, 1974). Thus soterenol may not be the ideal compound to use when obtaining data from which conclusions on the similarity or difference between receptors are being made. Raper & Malta (1975) commented that the selectivity of soterenol and other similar compounds may be related more to their efficacy than to their affinity but they did not make it clear whether they were referring to low efficacy on both trachea and atria or to different efficacies on the two tissues. For other compounds which are in general more selective than soterenol, it is feasible that low efficacy and differences in tissue receptor reserves contribute to their trachea-atria selectivity but that they have, in addition, different affinities for receptors in the two tissues. For example, salbutamol is a partial agonist on guinea-pig atria (O'Donnell, 1972) and on carbachol-contracted tracheal preparations (O'Donnell & Wanstall, unpublished results) suggesting that it has a lower efficacy than iso-

prenaline, but there is also evidence, from pA_2 values, that it has a different affinity for the β-receptors in the two tissues (Raper & Malta, 1973). On the other hand the present study has shown that compounds do exist (e.g. fenoterol, Me 506 and Me 454) which are trachea-atria selective but which have efficacies at least as high as that of isoprenaline. Therefore, low efficacy cannot be invoked to explain their trachea-atria selectivity.

For those compounds without low efficacy the assumption can be made that their selectivity reflects a true difference between their affinities for the β-receptors in guinea-pig trachea and atria, although further experiments are required to confirm this. This concept is not compatible with the hypothesis that the receptor in these two tissues is of a single type (Buckner & Patil, 1971; Buckner & Abel, 1974; Buckner & Saini, 1975). The present study has also raised again the question of whether isoprenaline is really the ideal reference compound for use in pharmacological studies on receptor mechanisms in tissues containing β-receptors. We have previously illustrated how loss of isoprenaline into extraneuronal uptake sites, e.g. in the guinea-pig trachea, can influence conclusions on the selectivity of compounds (O'Donnell & Wanstall, 1976a). Now we have confirmed that isoprenaline is not the β-adrenoceptor agonist with the highest efficacy on β-adrenoceptors.

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