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FUNCTIONAL ANTAGONISM AS A MEANS OF DETERMINING DISSOCIATION CONSTANTS AND RELATIVE EFFICACIES OF SYMPATHOMIMETIC AMINES IN GUINEA-PIG ISOLATED ATRIA

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1 The positive inotropic and chronotropic responses to sympathomimetic amines were examined in guinea-pig isolated atria.

2 The order of potency measured from EC_{50} values was isoprenaline > orciprenaline > salbutamol ⩾ fenoterol > terbutaline. Terbutaline and salbutamol were partial agonists on rate and together with orciprenaline and fenoterol also on tension responses.

3 Functional antagonism by carbachol caused a rightwards shift of the dose-response curve and depression of the maximum response. The rate maxima for orciprenaline, fenoterol and terbutaline were above that of isoprenaline. All the tension maxima were below isoprenaline.

4 Dissociation constants (K_A) and relative efficacies (e_r) were determined by analogy with irreversible antagonism.

5 The relative orders of affinity (K_A) were isoprenaline > orciprenaline > fenoterol > salbutamol > terbutaline. Affinities were identical on rate and tension.

6 The relative efficacies were all greater than isoprenaline for rate responses. On tension they were the same or less than isoprenaline.

7 The implications of these results are discussed, in particular the fact that a partial agonist has a greater efficacy than a full agonist.

Introduction

The question whether the β-adrenoceptors mediating the positive inotropic and chronotropic responses of the heart are identical has been examined by the use of both antagonists and agonists. Evidence from antagonist studies is conflicting since there are reports of preferential antagonism of both the chronotropic (Dreyer & Offermeier, 1975) and inotropic responses (Fenyvesi, 1972). A small selectivity for the inotropic response was also observed by Blinks (1967), but he did not consider this was sufficient for subclassification of the β-adrenoceptors. This conclusion has been confirmed by several studies demonstrating identical antagonism of the inotropic and chronotropic responses (Bristow & Green, 1970; Horii, Kawada, Takeda & Imai, 1974; Lumley & Broadly, 1975; 1977; Imbs, LeClerc, Mann, Miesch, Schwartz, Velly & Wermuth, 1977).

Studies using agonists have yielded several examples of selectivity for either the rate or force of contraction. Preferential chronotropic activity was shown to be exerted by soterenol and salbutamol (Farmer, Kennedy, Levy & Marshall, 1970) and by OPC-2009 (Yabuuchi, 1977) in guinea-pig isolated atria. Furthermore, the latter agent when employed as an antagonist, preferentially antagonized the positive chronotropic responses (Yabuuchi, 1977). In contrast, dopamine (Goldberg, 1972), dobutamine (Tuttle & Mills, 1975) and thyronamine (Boissier, Giudicelli, Larno & Advenier, 1973) have reported inotropic selectivity. Another manifestation of selectivity is that the maximum responses of partial agonists relative to isoprenaline are greater for the chronotropic responses than for the inotropic responses (Lumley & Broadley, 1977).

These studies have all expressed the potency of the agonists in terms of their EC_{50} values or the maxima. However, this measure is not sufficient for characterization of the receptors by the use of agonists, the individual contributions of both the affinity (measured as the dissociation constant) and the relative efficacy must be determined (Jenkinson, 1973; Triggle & Triggle, 1976). The methods available for calculating the dissociation constant of a full agonist are by the use of an irreversible antagonist (Furchgott

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& Bursztyn, 1967) and by functional antagonism (Van den Brink, 1973; Buckner & Saini, 1975). In the present paper we have applied the latter method to several full and partial agonists at β-adrenoceptors of isolated left and right guinea-pig atria and have extended it to determine additionally their relative efficacies.

A preliminary account of some of this work has been given to the British Pharmacological Society (Broadley & Nicholson, 1978).

Methods

Isolated atrial preparations

Guinea-pigs of either sex and weight range 300 to 500 g were killed by a blow on the head. Separated left and right atria were mounted on a combined perspex tissue holder and electrode as described previously (Broadley & Lumley, 1977). They were suspended in an organ bath (50 ml) containing Krebs-bicarbonate solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2 2H_2O$ 1.9, $NaHCO_3$ 25, $MgSO_4 7H_2O$ 1.2, glucose 11.7, $NaH_2PO_4 2H_2O$ 1.2) gassed with 5% CO_2 in O_2 and maintained at 38 ± 0.5°C. Each atrium was attached by means of a cotton thread to an isometric transducer (Devices, Type UF1, 28 g sensitivity range), and the tension developed recorded on a Devices M19 polygraph. An initial diastolic tension of 0.5 to 0.8 g was applied to both atria. Records of force of contraction were obtained from the left atria driven at a constant rate of 2 Hz with square wave pulses (5 ms and threshold voltage plus 50%) delivered by an SRI stimulator (Type 6053). Rate of contraction was recorded from the spontaneously beating right atria by means of a ratemeter (Devices, Type 2751) triggered by the tension signal. All preparations were allowed to stabilize for 30 min during which time several changes of bathing medium were made, before experiments were started.

Experimental protocol

Different experimental designs were adopted for calculation of EC_{50} values and for functional antagonism.

Calculation of EC_{50} values Two preliminary cumulative dose-response curves to isoprenaline were obtained, followed by a curve for the agonist under test. Small changes in sensitivity arising from repeating dose-response curves were corrected for by performing control experiments. These included three dose-response curves to isoprenaline. The mean ($n = 4$) total rate or total tension at each concentration on the third curve of control experiments was expressed as a fraction of the second curve. These factors were then applied to the second isoprenaline curve of the test experiments. The increases in rate and tension were obtained by subtracting the resting levels immediately before the dose-response curve. The resting level for isoprenaline was first corrected from the control experiments. The mean ($n = 4$) increases in rate and tension were then expressed as a percentage of the corrected isoprenaline maximum increases for plotting purposes. The EC_{50} values were calculated by plotting the increases in rate and tension of each agonist from individual experiments as a percentage of *their own* maxima. The geometric mean EC_{50} value was then calculated.

Functional antagonism Functional antagonism of each agonist was compared with that of isoprenaline in the same preparation. A cumulative dose-response curve to the agonist was constructed after a single curve to isoprenaline. Carbachol was then added to the organ bath in a concentration (2×10^{-7} M) that was found in preliminary experiments to exert a negative inotropic and chronotropic effect that was sustained for the remainder of the experiment. Dose-response curves to isoprenaline and then the test agonist were repeated in the presence of carbachol. Control experiments were performed for each agonist without the intervention of carbachol. The pre-carbachol dose-response curves were corrected from control experiments as before. The corrected increases in rate and tension before carbachol and the values obtained in its presence were expressed as a percentage of the maximum possible increase. This was obtained by subtracting the resting level from the corrected pre-carbachol isoprenaline maximum total rate or tension. These individual dose-response curves were used to determine the dissociation constants.

Calculation of dissociation constants (K_A)

The functional interaction between the cholinoceptor and β-adrenoceptor agonist occurs at a level beyond receptor activation. A progressively larger fraction of adrenoceptors must be occupied in order to overcome the stimulus generated through cholinoceptor activation. The adrenoceptor reserve is insufficient to cope with the cholinoceptor stimulus. Once this reserve is exhausted a reduction of the maximum attainable response ensues (Van den Brink, 1973). The dose-response curve is therefore displaced in a fashion resembling irreversible antagonism.

The principles derived for irreversible antagonism (Furchgott, 1966; Furchgott & Bursztyn, 1967; Besse & Furchgott, 1976) can be extended to functional antagonism (Buckner & Saini, 1975). Although one

of Furchgott's (1972) assumptions in deriving the equations was that the antagonist alters the response only by reducing the concentration of receptors, the situation with functional antagonism is analogous. Receptor occlusion is replaced by occlusion of the stimulus generated, by one of opposite direction. This is assumed to occur without affecting the relationship between stimulus and effect in the adrenergic system thereby fulfilling another important criterion. The equation of Furchgott (1966),

$$\frac{1}{[A]} = \frac{1-q}{q[A']} + \frac{1}{q[K_A]}$$

was therefore applied to the results.

The equieffective molar concentration of each agonist obtained in the absence [A] and presence [A'] of carbachol was obtained from individual corrected dose-response curves. The reciprocal values were plotted as 1/A against 1/A' and from the calculated regression line, the dissociation constant K_A = (slope − 1)/intercept.

Calculation of relative efficacy (e_r)

Each agonist was compared with isoprenaline in the same preparation by replotting the mean dose-response data obtained prior to application of carbachol. The uncorrected responses to isoprenaline and the test agonist, corrected as described for the preliminary study, were used. These were expressed as a percentage of the corrected isoprenaline maximum response and plotted against the negative logarithm of the fraction of receptors occupied (RA/Rt) by each concentration [A]. This value was obtained for each concentration [A] by substitution of the K_A value (obtained from the *same* experiment) into the equation RA/Rt = [A]/K_A + [A]. This equation is derived from basic concepts of drug-receptor interactions (see, Stephenson, 1956; Furchgott, 1972) and is therefore independent of the fact that functional antagonism was used to determine the dissociation constant (K_A). The relative efficacy (e_r) was the antilogarithm of the distance along the RA/Rt axis between the agonist curve and the corresponding isoprenaline reference (Furchgott & Bursztyn, 1967; Besse & Furchgott, 1976).

Drugs

Carbachol (Halewood Chemicals), (±)-fenoterol hydrobromide (Boehringer Ingelheim, (−)-isoprenaline bitartrate dihydrate (Ward Blenkinsop), (±)-orciprenaline sulphate (Boehringer Ingelheim), (±)-salbutamol sulphate (Allen & Hanburys) and (±)-terbutaline sulphate (A.B. Draco) were used.

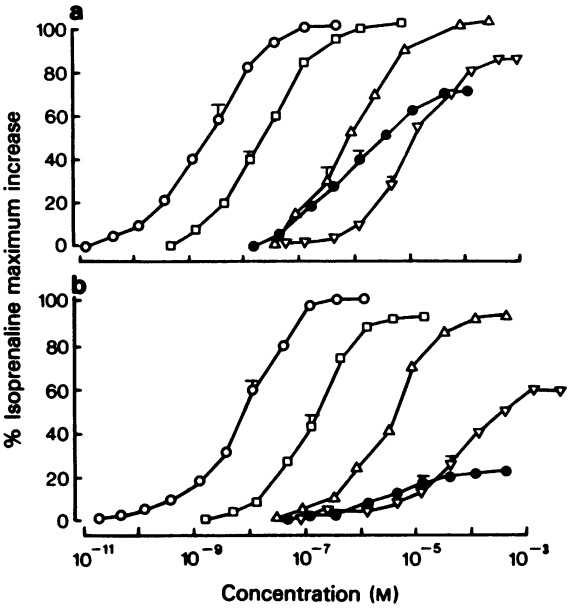

Figure 1 Positive chronotropic (a) and positive inotropic (b) responses of guinea-pig isolated atria to orciprenaline (□), fenoterol (△), terbutaline (▽) and salbutamol (●) compared with isoprenaline (○). Mean (n = 4) cumulative dose-response curves to each agonist are plotted as a percentage of the corrected isoprenaline maximum response as described in the text. The isoprenaline curve represents the average from the experiments using all of the agonists.

Results

The sympathomimetic amines orciprenaline, fenoterol, terbutaline and salbutamol were compared individually with isoprenaline and the mean (n = 4) dose-response curves for the positive chronotropic and inotropic responses are shown in Figures 1a and 1b respectively. Their relative orders of potency, calculated from the geometric mean EC_{50} values (Table 1), were isoprenaline > orciprenaline > salbutamol ≥ fenoterol > terbutaline on both rate and tension responses. The maximum rate responses to terbutaline (84.4 ± 4.8%) and salbutamol (69.6 ± 1.9%) were significantly less ($P < 0.05$) than those of isoprenaline, orciprenaline and fenoterol; which did not differ significantly from each other. The maximum tension responses to orciprenaline (92.0 ± 0.3%), fenoterol (90.2 ± 0.9%), terbutaline (60.2 ± 2.6%) and salbutamol (20.0 ± 2.1%) were significantly less ($P < 0.05$) than that to isoprenaline and were significantly less than the corresponding rate values. Furthermore, rate selectivity of each agonist was apparent from the EC_{50} values.

Table 1 EC_{50} values, dissociation constants (K_A) and efficacies relative to isoprenaline (e_r) for the positive chronotropic and inotropic responses of guinea-pig isolated atria to β-adrenoceptor agonists. Potency and K_A of each agonist are also expressed relative to isoprenaline

	Positive chronotropic responses					*Positive inotropic responses*				
	EC_{50} (M)	*Relative potency*	*Dissociation constant (K_A)*	*Relative K_A*	*Relative efficacy*	*EC_{50} (M)*	*Relative potency*	*Dissociation constant (K_A)*	*Relative K_A*	*Relative efficacy*
Isoprenaline	$3.0 \pm 1.4 \times 10^{-9}$	100	$6.7 \pm 1.8 \times 10^{-8}$	100	1	$9.5 \pm 1.3 \times 10^{-9}$	100	$9.6 \pm 2.0 \times 10^{-8}$	100	1
Orciprenaline	$2.5 \pm 1.4 \times 10^{-7}$	1.2	$1.2 \pm 0.8 \times 10^{-6}$	5.6	1.14	$8.5 \pm 1.4 \times 10^{-7}$	1.12	$4.9 \pm 2.7 \times 10^{-6}$	1.96	0.97
Salbutamol	$1.0 \pm 0.2 \times 10^{-6}$	0.3	$4.2 \pm 1.7 \times 10^{-4}$	0.02	9.55	$2.7 \pm 1.6 \times 10^{-6}$	0.35	$4.7 \pm 2.3 \times 10^{-4}$	0.02	0.78
Fenoterol	$1.0 \pm 0.14 \times 10^{-6}$	0.3	$1.3 \pm 0.9 \times 10^{-4}$	0.05	1.32	$4.3 \pm 1.1 \times 10^{-6}$	0.22	$1.4 \pm 1.1 \times 10^{-4}$	0.07	1.0
Terbutaline	$4.0 \pm 1.2 \times 10^{-6}$	0.075	$1.1 \pm 0.3 \times 10^{-3}$	0.006	1.33	$7.6 \pm 0.1 \times 10^{-5}$	0.013	$7.7 \pm 3.1 \times 10^{-4}$	0.013	0.21

Isoprenaline was given an arbitrary value of 100 for determining relative potency and K_A and a value of 1 for relative efficacy.

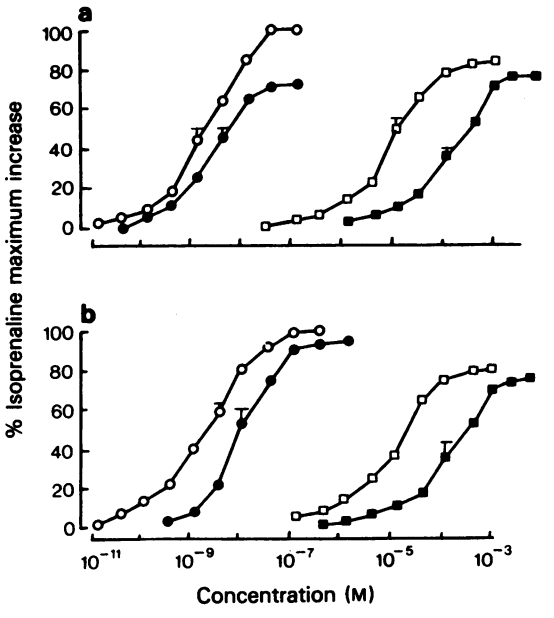

Figure 2 Functional antagonism by carbachol (2×10^{-7} M) of the positive chronotropic (a) and positive inotropic (b) responses of guinea-pig isolated atria. Mean ($n = 4$) cumulative dose-response curves to isoprenaline (O) and terbutaline (□) were constructed before (open symbols) and in the presence of (closed symbols) carbachol. Method of plotting and correcting the dose-response curve is described in the text.

The functional antagonism by carbachol is illustrated by plotting the mean dose-response curves to terbutaline and isoprenaline before and in the presence of carbachol for both the positive chronotropic (Figure 2a) and inotropic (Figure 2b) responses. The curves for both agonists were displaced to the right and the maxima depressed by the carbachol. However, the maximum rate response to terbutaline was now greater than that to isoprenaline. This was also the case with orciprenaline and fenoterol. In contrast, the tension maxima for all the agonists were depressed below that of isoprenaline.

The equiactive molar concentrations of isoprenaline and terbutaline obtained from the above mean data in the absence and presence of carbachol were plotted as their reciprocals for both rate (Figure 3a and b) and tension (Figure 3c and d) to yield straight line graphs. The dissociation constants (K_A) were derived from individual experiments and the mean values for these agonists and for orciprenaline, fenoterol and salbutamol are shown in Table 1. Their orders of affinity (dissociation constants) relative to isoprenaline were isoprenaline > orciprenaline > fenoterol > salbutamol > terbutaline on both rate and tension. The affinity values did not differ significantly ($P > 0.05$) between rate and tension for any agonist examined, as determined by Student's t test.

Calculation of the relative efficacy values (e_r) is illustrated by use of the data shown above for terbutaline and isoprenaline. The pre-carbachol dose-response curves were replotted as the response against $-\log RA/Rt$ for both rate (Figure 4a) and tension (Figure 4b). Terbutaline had a greater efficacy than isoprenaline for rate responses (1.33), whereas for tension responses (0.21) it was less than isoprenaline. The values for the other agonists were similarly greater on rate than isoprenaline, and on tension they were less except for fenoterol which had an identical efficacy to isoprenaline (Table 1).

Discussion

Functional antagonism has been applied to the determination of the dissociation constants of a range of sympathomimetic amines for the β-adrenoceptors mediating the positive inotropic and chronotropic responses of guinea-pig isolated atria. The values yielded by this method were identical for these two responses irrespective of the agonist employed. This confirms the observation of Buckner, Torphy & Costa (1978) in which soterenol and isoprenaline were applied to the same technique. This finding is also consistent with the reports that show the affinity (pA_2) values of competitive antagonists to be identical for the rate and tension responses (Blinks, 1967; Bristow & Green, 1970; Horii *et al.*, 1974; Lumley & Broadley, 1975; 1977). It can therefore be concluded that the rate selectivity of the agonists observed here and elsewhere (Lumley & Broadley, 1977) from their EC_{50} values and the maxima of partial agonists, is not due to affinity differences.

The orders of potency of the agonists were also identical for the positive inotropic and chronotropic responses. The potency was measured as the EC_{50} and it is well known that the potency order of sympathomimetic amines can be modified by factors affecting their concentration in the vicinity of the receptor. For example, in untreated guinea-pig isolated atria the order of activity of the classical catecholamines is isoprenaline, adrenaline, noradrenaline. However, if neuronal uptake is inhibited by cocaine the order becomes isoprenaline, noradrenaline, adrenaline (Furchgott, 1967), which is characteristic of adrenoceptors of the β_2-type (Furchgott, 1972). Although neuronal uptake is unlikely to occur with isoprenaline (Burgen & Iversen, 1965) and therefore the structurally related agonists used here, other factors such as catechol-*O*-methyl transferase (COMT),

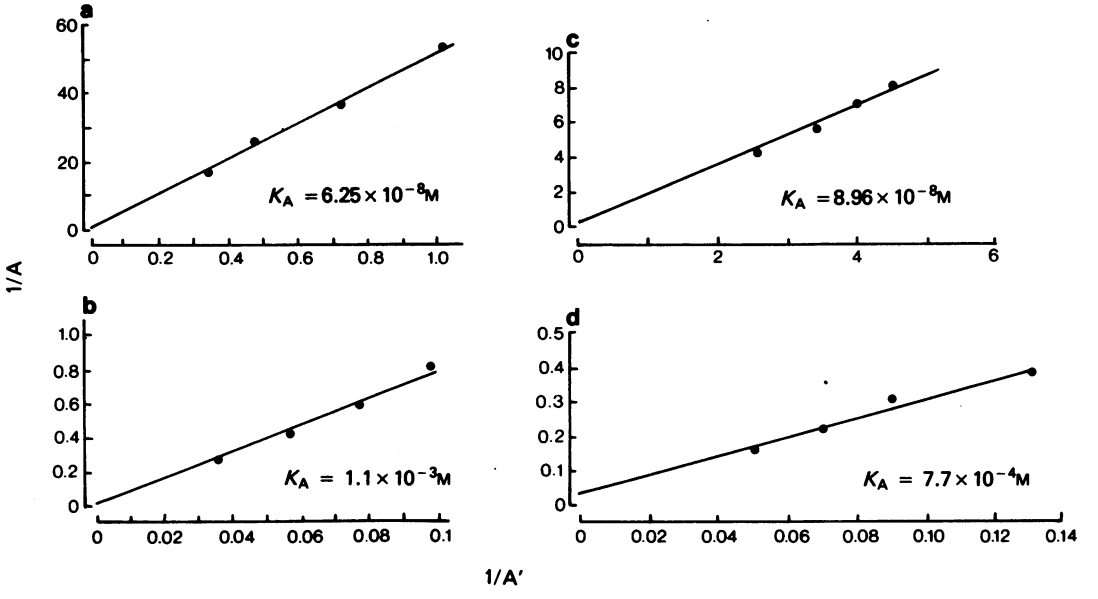

Figure 3 Double reciprocal plots for the equiactive molar concentrations of isoprenaline (a and c) and terbutaline (b and d) before (1/A) and after (1/A') carbachol. The values were obtained from the mean (n = 4) dose-response curves for the positive chronotropic (a and b) and positive inotropic (c and d) responses of guinea-pig isolated atria. Dissociation constants (K_A) were calculated as (slope − 1)/intercept from each regression line.

extraneuronal uptake or indirect sympathomimetic activity were considered.

Indirect activity was eliminated since super- rather than sub-sensitivity to isoprenaline, terbutaline and salbutamol occurs with reserpine pretreatment (Broadley & Lumley, 1977). Extraneuronal uptake and COMT are possible sources of interference, especially as COMT would destroy only isoprenaline, being the sole catecholamine. In view of this, preliminary functional antagonism experiments had been performed with isoprenaline and orciprenaline as the agonists in the presence of tropolone and metanephrine as inhibitors of COMT and extraneuronal uptake respectively. The K_A values yielded by these studies were not different from those obtained under the conditions described in the current results (unpublished observations). Furthermore, it has been demonstrated in these laboratories that tropolone and metanephrine (and phenoxybenzamine) have negligible potentiating effect upon isoprenaline or orciprenaline dose-response curves of guinea-pig isolated atria (Broadley & Duncan, 1977; Duncan, 1978). Therefore extraneuronal uptake and COMT do not appear to play an important role in modifying the responses to these sympathomimetic amines in this tissue. We were also influenced by the fact that tropolone during the course of these prolonged experiments (four dose-response curves) accelerated the deterioration of the preparation. It is worth noting that Buckner *et al.* (1978) included tropolone in their studies with rat atria, but avoided any deterioration by constructing only one dose-response curve in each preparation. As a consequence, they were unable to determine the affinities relative to isoprenaline in the same preparation and also the relative efficacies. Nevertheless, the K_A values for isoprenaline obtained in the present study were of the same order as those of Buckner *et al.* (1978). It was therefore decided that controlling for these factors was an unnecessary complication in this case and that doing so would not have altered the results and conclusions.

The orders of relative potency of the five agonists measured from their EC_{50} values were similar to their orders of relative affinities, except for the interchange of salbutamol and fenoterol. The potency of an agonist is a function of many factors, the affinity and efficacy being major determinants of the stimulus to produce the response (Jenkinson, 1973; Triggle & Triggle, 1976). The possibility exists that the exchange between salbutamol and fenoterol arises from efficacy differences. Salbutamol certainly possessed a greater efficacy relative to isoprenaline than did fenoterol for the rate response; however, for tension responses the value was less than that for fenoterol.

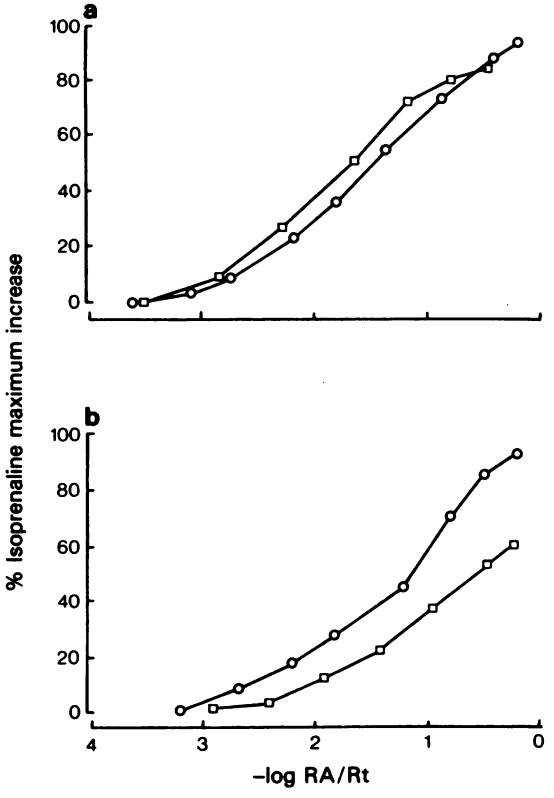

Figure 4 Replotted mean ($n = 4$) cumulative dose-response curves for the positive chronotropic (a) and positive inotropic (b) responses of guinea-pig isolated atria to isoprenaline (○) and terbutaline (□) before carbachol. Response (as percentage of the isoprenaline maximum) is plotted against $-\log RA/Rt$ for each agonist concentration [A], where $RA/Rt = [A]/K_A + [A]$ (see text). Relative efficacy (e_r) was the antilogarithm of the distance between curves along the abscissa.

The most surprising finding regarding the relative efficacy values was that all the agonists examined had a greater efficacy than isoprenaline for the rate responses, in spite of their low affinities. This conclusion was also suggessted by the dose-response curves to orciprenaline, fenoterol and terbutaline in the presence of carbachol which exhibited greater maxima than for isoprenaline. This confirms the observations of O'Donnell & Wanstall (1977) who also showed a greater maximum for the catecholamine derivative Me454 on the rate response of guinea-pig atria in the presence of carbachol.

We considered the possibility that factors other than a greater relative efficacy might have yielded an erroneous result. For example, an antimuscarinic property of the agonists at the higher concentrations would reverse carbachol and raise the maximum. However this could be confidently discounted on several grounds. One might expect biphasic dose-response curves and complete reversal to the same maximum as before carbachol. But most convincingly, such a property should affect both the rate and tension responses. This latter argument would dispel most doubts that another property gave rise to this greater maximum and we were left with the conclusion that these β-adrenoceptor agonists had a greater efficacy than isoprenaline for the positive chronotropic responses. It was difficult to reconcile this with the failure by the partial agonists to produce the same rate maximum as isoprenaline. If these agonists occupied all the receptors at their maxima, how could they not produce a maximum response the same as isoprenaline? Admittedly their affinities were low and diffusion barriers may impede total receptor occupation. It is also conceivable that the efficacy of isoprenaline is not in fact as high as one would expect for such a potent agonist and that the natural transmitters noradrenaline and adrenaline may have much higher efficacies.

The fact that the efficacies of these partial agonists were greater than isoprenaline for the positive chronotropic responses, but less for the positive inotropic responses might explain their higher rate response maxima. Whether this also accounts for the rate selectivity of all sympathomimetic amines remains to be established since it is not valid to compare different tissues where receptor populations are not identical. Neither is it valid to compare different responses such as rate and tension changes where the stimulus-response relationship may vary.

The present study has examined the activity of a range of β-adrenoceptor agonists in terms of their potencies, affinities and relative efficacies their orders of activity varied depending upon the measure used. This differs from the interaction with α-adrenoceptors of rabbit aorta where the potency and affinity were shown by a similar three-way analysis to run parallel (Besse & Furchgott, 1976). In the guinea-pig atria, potency alone is not a sufficient measure for characterizing the interaction of agonists with the β-adrenoceptor. The comparison of the positive inotropic and chronotropic responses has shown that the orders of affinity for the receptors are the same for these two parameters. This suggests that the receptors are identical. However, the orders of relative efficacies were virtually reversed.

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