THE CONTRIBUTION OF EXTRANEURONAL UPTAKE TO THE TRACHEA-BLOOD VESSEL SELECTIVITY OF β-ADRENOCEPTOR STIMULANTS *in vitro* IN GUINEA-PIGS

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1   The potencies relative to isoprenaline of isoetharine, tertiary butyl noradrenaline, salbutamol, orciprenaline, Me 506, rimiterol, fenoterol, carbuterol and terbutaline on isolated preparations of guinea-pig trachea and blood vessels (perfused hind limb) were determined. All the compounds were selective for trachea and selectivity values, i.e. relative potency on trachea divided by relative potency on hind limb, ranged from 2.3 to 21.4.

2   Responses to isoprenaline (the reference compound), tertiary butyl noradrenaline and isoetharine were potentiated on trachea by 50 μM phenoxybenzamine (PHB) and by other inhibitors of extraneuronal uptake (ENU). Under these conditions the selectivity values of all the compounds was close to unity.

3   Selectivity values were also close to unity if they were calculated from data obtained without ENU inhibition, provided that only those compounds not potentiated by PHB on trachea were used.

4   It is proposed that the trachea-blood vessel selectivity shown by β-adrenoceptor stimulants can be caused by the influence of ENU upon them, rather than by their ability to distinguish between two β₂-adrenoceptors.

5   The suggestion that differences exist between β₂-adrenoceptors in respiratory and vascular smooth muscle is not supported by the *in vitro* experiments described.

Introduction

In a previous study the potencies relative to isoprenaline of a number of resorcinolamines were markedly higher on the isolated trachea than on the isolated perfused hind limb of guinea-pigs i.e. the compounds showed trachea/blood vessel selectivity (O'Donnell & Wanstall, 1974). Although it was tempting to suggest that this selectivity indicated a difference between adrenoceptors previously classified as being the same (β_2-adrenoceptors), other explanations for this observed selectivity, which did not require subdivision of β_2-adrenoceptors, were cited. The results described in the present paper provide support for the explanation that trachea/blood vessel selectivity *in vitro* reflects an influence of extraneuronal uptake on the potency of compounds rather than an ability of compounds to distinguish between two receptor types.

Methods

Isolated tracheal chain and perfused hind limb preparations

Tracheal chain preparations (relaxation) and isolated perfused hind limb preparations (vasodilatation) were set up as described by O'Donnell and Wanstall (1974). On each preparation maximum responses to isoprenaline were obtained and responses expressed as a percentage of that maximum.

Inhibition of extraneuronal uptake

(i) *On trachea.* The inhibitors of extraneuronal uptake tested were hydrocortisone (50 μM), deoxycorticosterone acetate (DOCA, 5 μM and 20 μM), metanephrine (10 μM and 100 μM) and phenoxybenzamine (PHB, 1 μM, 10 μM, 50 μM and 100 μM). Hydrocortisone, DOCA or metanephrine was added to the Krebs solution bathing the tissue 30 min before the start of the estimation of a concentration-response line and remained in contact with the tissue whilst the concentration-response line was obtained. Phenoxybenzamine was in contact with the tissue for 15, 30, 60 or 90 minutes. The tissue was then washed thoroughly for 10 min and the subsequent concentration-response line obtained in PHB-free Krebs solution.

(ii) *On hind limb.* Hind limb preparations were examined after perfusion with Krebs solution containing PHB (10 μM and 100 μM), hydrocortisone (10 μM) or metanephrine (10 μM). Perfusion times

varied from 10 to 40 min in different experiments. After PHB treatment, but not after hydrocortisone and metanephrine, perfusion was carried out in drug-free Krebs solution.

Treatment of data

(i) *Relative Potency*. Concentration-response or dose-response lines to isoprenaline and to at least one test compound were obtained on each of several preparations, using a randomized order of addition of the compounds. The log EC_{50} or ED_{50} (concentration or dose producing 50% maximum response) was then interpolated for the test compound and for isoprenaline. The potency of the test compound relative to that of isoprenaline (assigned a value of 100) was then calculated from the following formula:

Relative potency $= 100 \times$ antilog [mean (neg log $EC_{50_{test}} -$ neg log $EC_{50_{iso}}$)]

(ii) *Selectivity for trachea*. The relative potency value on the trachea was divided by that on the hind limb blood vessels. Compounds showing selectivity for the trachea gave high values, but a selectivity value approaching 1 indicated selectivity for neither tissue.
(iii) *Potentiation by phenoxybenzamine*. Concentration-response lines to each compound were obtained before and after treatment with phenoxybenzamine. From a minimum of 4 experiments a value for potentiation was obtained from the formula:

Potentiation $=$ mean (neg log $EC_{50_{after PHB}} -$ neg log $EC_{50_{before PHB}}$)

The antilog of this mean value gave the factor by which the EC_{50} was reduced after PHB treatment.

Drugs

The β-adrenoceptor stimulants examined were: carbuterol hydrochloride (Smith, Kline & French); fenoterol hydrobromide (Th1165a, Boehringer-Ingelheim); isoetharine hydrochloride (Sterling-Winthrop); $(\pm)$-isoprenaline sulphate (Burroughs Wellcome); Me 506 hydrobromide (Boehringer-Ingelheim); orciprenaline sulphate (Boehringer-Ingelheim); rimiterol hydrobromide (Riker); salbutamol base (Allen and Hanbury); terbutaline hydrochloride (Me 501, Boehringer-Ingelheim); terbutaline sulphate (Astra); $(\pm)$-tertiary butyl noradrenaline (Sterling-Winthrop). These drugs were donated by the various companies named.

Other drugs used were: deoxycorticosterone acetate (BDH); hydrocortisone hemisuccinate sodium (Glaxo); metanephrine hydrochloride (Calbiochem); phenoxybenzamine hydrochloride (Smith, Kline & French).

All drugs were obtained as pure powders. The β-adrenoceptor stimulants were made up in 0.01 N HCl to give stock solutions of 10 mM. Dilutions were made in Krebs solution containing ascorbic acid (0.2 μg/ml) and kept on ice for the duration of each experiment. Phenoxybenzamine was dissolved in 95% ethanol containing 0.001 ml 10 N HCl/ml to give a 100 mM stock solution. Dilutions were made in Krebs solution.

Statistical analyses

The measure of variation of the mean quoted is the standard error (s.e.). Mean relative potency values are quoted with 95% confidence limits. A paired t-test was used to assess the significance of the difference between paired log EC_{50} values.

Results

Inhibition of extraneuronal uptake (ENU)

Of the ENU inhibitors tried, phenoxybenzamine was the only drug which did not cause marked relaxation of tracheal preparations. The responses to isoprenaline on trachea were potentiated after 30 min contact of the tissue with 10 μM, 50 μM or 100 μM PHB but not after 1 μM. The potentiation was the same after either 50 μM or 100 μM and maximum potentiation was achieved after 30 minutes. Thus 50 μM PHB for 30 min was used in the tracheal experiments. The potentiation of isoprenaline produced by this treatment was maintained unchanged for several hours after removal of free PHB. Responses to seven of the β-adrenoceptor stimulants examined were not significantly affected by PHB whereas responses to the other three compounds were significantly potentiated (Table 1) i.e. isoprenaline (5.2-fold), tertiary butyl noradrenaline (2.3-fold) and isoetharine (2.0-fold). This could be taken as evidence that the pharmacological responses to three compounds used in this study are modified by loss into ENU sites.

In only 4 of the experiments with other ENU inhibitors was the relaxation produced by the inhibitor small enough to allow concentration-response lines to isoprenaline to be obtained. In these 4 experiments a potentiation of isoprenaline was observed of similar magnitude to that produced by PHB i.e. 6.8-fold with hydrocortisone (50 μM), 4.4-fold with DOCA (5 μM), 5.5-fold with DOCA (20 μM) and 4.8-fold with metanephrine (10 μM). This potentiation was not maintained when the inhibitor was washed out.

No data on hind limb preparations treated with ENU inhibitors were obtained since preparations became unresponsive after perfusion with the ENU inhibitors tried.

Selectivity for trachea

In the initial series of experiments an ENU inhibitor was not used and, for each of the test compounds, the

relative potency value on trachea was higher than that on the hind limb i.e. they all had selectivity values greater than 1. Six of the test compounds had values greater than 10 (Table 2, Column 4). Since the responses of the trachea to the reference compound isoprenaline, as well as to two of the test compounds, had been shown to be modified by PHB, all relative potency values on trachea (and hence all selectivity values) would be altered if the influence of ENU was excluded. Calculation of selectivity values using data obtained from experiments in which an ENU inhibitor was present was not possible because no data could be obtained on hind limb preparations after ENU inhibitors. An alternative approach was to use the original data but to omit from the analysis those compounds whose responses were modified by ENU (i.e. potentiated by PHB) i.e. isoetharine, tertiary butyl noradrenaline and isoprenaline. This approach required the replacement of isoprenaline by another reference compound. When relative potency and selectivity values of the remaining seven compounds were recalculated with respect to any of these seven compounds as 100 on both tissues, the relative potency values on trachea and hind limb were similar and selectivity values were not sufficiently different

from unity to suggest different receptors in the two tissues. Table 3 illustrates such a calculation using orciprenaline as 100 on both tissues. It can be seen that the selectivity values lie within the range 0.4 to 2.1 compared to the range of 2.3 to 21.4 shown in Table 2 when isoprenaline is the reference compound.

It is interesting to note that the relative potency values obtained on trachea in the series of experiments in which ENU was inhibited by phenoxybenzamine (Table 2, Column 2) were very similar to those obtained on hind limb preparations in which ENU had not been inhibited (Table 2, Column 3). If these relative potency values were used to calculate selectivity, values close to unity resulted (Table 2, Column 5) resembling those obtained in Table 3.

Discussion

A difference between the relative potency values of a series of agonist or antagonist compounds on two tissues is frequently taken as one line of evidence for the receptors in those two tissues being different. Furchgott (1972) has defined a set of optimal conditions which should be met before

Table 1 Potentiation of β-adrenoceptor stimulants by phenoxybenzamine (PHB) (50 μM for 30 min, then PHB-free Krebs) on trachea

Compound	Mean potentiation* ± s.e.	Antilog potentiation
Isoprenaline	0.72 ± 0.04‖ (27)†	5.2
Tertiary butyl noradrenaline	0.36 ± 0.05§ (5)	2.3
Isoetharine	0.31 ± 0.08‡ (5)	2.0
Orciprenaline	0.08 ± 0.10 (5)	1.2
Salbutamol	0.07 ± 0.12 (4)	1.2
Terbutaline	0.06 ± 0.12 (4)	1.1
Carbuterol	0.05 ± 0.07 (4)	1.1
Fenoterol	−0.02 ± 0.08 (5)	0.96
Rimiterol	−0.11 ± 0.10 (4)	0.78
Me 506	−0.14 ± 0.08 (4)	0.72

* mean [neg log EC_{50} after PHB − neg log EC_{50} before PHB] ; † number of paired observations; ‡ $0.05 > P > 0.01$; § $0.01 > P > 0.001$; ‖ $P < 0.001$ (paired t test in all cases).

pharmacological observations, such as relative potency values of agonists, are used as the basis for the, characterization of receptors. In studies involving sympathomimetic amines, optimal conditions would not exist if loss of compounds into neuronal and/or extraneuronal uptake sites is influencing the concentration in the receptor biophase. Several workers have reported that the potencies of certain compounds, as either β-adrenoceptor agonists or antagonists, were different in respiratory and vascular smooth muscle i.e. the compounds were selective. From this they have suggested that differences exist between β-adrenoceptors in these two tissues (Bristow, Sherrod & Green, 1970; Wardell, Colella, Shetzline & Fowler, 1974; Wasserman & Levy, 1974). Although isoprenaline is used as the reference compound in these and most other studies on β-adrenoceptors and is known to have an affinity for extraneuronal uptake (ENU), the influence of ENU is rarely excluded. In our previous study (O'Donnell & Wanstall, 1974) ENU was not inhibited and therefore, although we observed a 10 to 20-fold difference between the relative potencies of some resorcinolamines on tracheal and perfused hind limb preparations, this could not be taken as evidence that the β-

adrenoceptors in trachea and hind limb blood vessels were different. In the present study the differences between the relative potencies of an extended series of compounds (and hence their trachea/blood vessel selectivity) disappeared when the influence of ENU was excluded. This suggested that the observed

Table 3 Relative potencies and selectivity for trachea of those β-adrenoceptor stimulants which were not potentiated by phenoxybenzamine.

Compound	Relative potency* Trachea	Hind Limb	Selectivity
Orciprenaline	100	100	1.0
Salbutamol	500	1230	0.4
Me 506	739	686	1.1
Rimiterol	1045	933	1.1
Fenoterol	3540	3010	1.2
Carbuterol	274	171	1.6
Terbutaline	274	133	2.1

* Results from Table 2 (Cols 1 and 3) recalculated with orciprenaline as reference compound (assigned a value of 100).

Table 2 Relative potencies and selectivity for trachea of β-adrenoceptor stimulants on trachea and on hind limb blood vessels.

Compound	Relative potencies Trachea (T) No PHB	PHB	Hind limb blood vessels (BV)	Selectivity for trachea (T÷BV) Using T (no PHB)	Using T (PHB)
Isoprenaline*	100	100	100	1.0	1.0
Isoetharine	52.5(5)† (32–87)‡	18.6(5) (15–28)	22.9(5) (18–30)	2.3	0.8
Tertiary butyl noradrenaline	468(5) (251–871)	155(5) (79–302)	178(3) (66–479)	2.6	0.9
Salbutamol	55.0(5) (22–130)	14.5(5) (6–38)	12.9(5) (7–23)	4.3	1.1
Orciprenaline	11.0(5) (5–22)	1.91(5) (0.9–4.1)	1.05(5) (0.61–1.8)	10.5	1.8
Me 506	81.3(6) (43–155)	7.08(4) (5.6–8.9)	7.24(3) (2.2–24)	11.3	1.0
Rimiterol	115(5) (50–263)	6.76(5) (3–16)	9.77(5) (6–16)	11.8	0.7
Fenoterol	389(6) (141–1072)	60.3(6) (48–76)	31.6(5) (22–45)	12.3	1.9
Carbuterol	30.2(5) (17–54)	6.61(6) (3.3–13)	1.78(5) (1.3–2.5)	17.0	3.7
Terbutaline	30.2(5) (22–42)	3.80(5) (1.8–8.1)	1.41(5) (0.6–3.2)	21.4	2.7

* =reference compound; † =number of observations; ‡ =95% confidence limits.

selectivity was not necessarily related to receptor differences but could be explained by implicating ENU.

An important assumption made in this paper is that the potentiation of isoprenaline by PHB on trachea results from the inhibition of ENU. A number of observations would support this assumption. Foster (1967, 1969) showed that the potentiation of responses to isoprenaline on trachea correlated with inhibition of the uptake and retention of [^{3}H]-isoprenaline by tracheal tissue. O'Donnell & Saar (1972) have also shown, using fluorescence histochemistry, that PHB prevents extraneuronal accumulation of noradrenaline in guinea-pig trachea. Other drugs described as ENU inhibitors were shown in the present study to cause potentiation of responses to isoprenaline and the magnitudes of these potentiations were similar to those seen after PHB. The lack of potentiation of orciprenaline and other compounds used in the present study eliminates the possibility that PHB caused a non-specific sensitization of β-adrenoceptors.

If ENU is to explain the selectivity it is necessary to postulate that, in guinea-pigs, pharmacological responses to compounds which have an affinity for ENU, e.g. isoprenaline, are influenced by ENU to different extents in trachea and blood vessels. Responses to isoprenaline were modified by ENU inhibitors on trachea but data could not be obtained on hind limb preparations. Thus we have no direct evidence for a differential effect of ENU on responses in these two tissues. Nevertheless it is feasible that vascular responses in guinea-pigs are not modified by ENU inhibitors since atrial responses to isoprenaline are not modified by ENU inhibitors (unpublished observations; Woppel & Trendelenburg, 1973). Also, vascular smooth muscle of guinea-pig, unlike that of rabbit, rat and mouse, cannot accumulate and retain extraneuronal fluorescence after incubation in noradrenaline (Gillespie & Muir, 1970). Much reduced values for selectivity were obtained in the present study, not only when the influence of ENU was excluded on both tissues (by choosing the appropriate compounds) but also when it was excluded only on trachea (by using PHB to inhibit ENU). These observations could be taken as indirect evidence that ENU has little effect in modifying responses on hind limb vessels. Also, they do not support the suggestion that differences exist between β_2-adrenoceptors in respiratory and vascular smooth muscle.

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