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Some evidence for the extraneuronal uptake of rimiterol and its metabolism by catechol-*O*-methyltransferase in guinea-pig trachealis smooth muscle cells

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Rimiterol is a sympathomimetic bronchodilator amine with selectivity for β_2-adrenoceptors (Carney et al 1971; Bowman & Rodger 1972; Marlin & Turner 1975; O'Donnell & Wanstall 1977). Like isoprenaline, but unlike most of the other clinically used sympathomimetic bronchodilators, rimiterol is a catecholamine and hence is a substrate for catechol-*O*-methyltransferase (COMT). However, it was not clear whether extraneuronal uptake exposed rimiterol to intracellular COMT in tissues, particularly in the smooth muscle of the airways. In the present study, extraneuronal uptake and subsequent *O*-methylation of rimiterol has been demonstrated in trachealis smooth muscle cells in the guinea-pig, using a fluorescence histochemical technique which has previously been used in similar studies on noradrenaline, adrenaline and isoprenaline (O'Donnell & Saar 1978; Anning et al 1979; Bryan & O'Donnell 1979, 1980a).

Materials and methods

Adult, female guinea-pigs (300–450 g) were pretreated with 50 mg kg^{-1} 6-hydroxydopamine intravenously 24 h before the experiment. The animals were killed by a blow on the head and the tracheae removed. These were cut into up to 12 rings, but the laryngeal and bronchial rings were discarded (Bryan & O'Donnell 1980b). The rings were washed for 30 min in two changes, each of 10 ml, of Krebs solution aerated with 95% O_2 and 5% CO_2. Unless otherwise indicated, 100 μM U-0521 was included in the second Krebs wash solution and for the remainder of the experiment, to inhibit COMT (Bryan & O'Donnell 1979). Tissues were then incubated for the specified time in Krebs solution only or in Krebs solution containing the required concentration of rimiterol. For this stage the Krebs solution contained an additional 0·57 mM ascorbic acid. The tissues were then washed at 0–1 °C for 30 min to remove extracellular and loosely bound, but not intracellular, catecholamine (Bryan & O'Donnell 1980b). When an extraneuronal uptake inhibitor was used (metanephrine, corticosterone) this was included in the Krebs solution throughout all stages of the experiment.

Tracheal rings were prepared for fluorescence histochemistry by the Falck-Hillarp technique (Falck 1962) with the tissues exposed to formaldehyde gas at 80 °C for 3 h, unless otherwise specified. Subsequent processing of the tissues and measurement of fluorescence intensities with a Leitz MPV microphotometer, were carried out as described by Bryan & O'Donnell (1979, 1980b). Fluorescence intensities were measured in areas of trachealis smooth muscle 2·5 μm square. These values, in arbitrary units, were corrected for background fluorescence, measured in the relevant control tissue from the same animal.

Protein model experiments. Aqueous solutions of 2% bovine serum albumin containing no amine or the specified concentration of amine were prepared. Spots (10 μl) of these solutions were placed on microscope slides with an Agla micrometer syringe (Wellcome Reagents Ltd), air-dried, then heated at 50 °C for 15 min. Care was taken to ensure that the spots were as close as possible to the same thickness. The slides were then exposed to formaldehyde gas at 80 °C for the required time. Liquid paraffin was used as the mounting medium and the fluorescence intensities were measured in 5 separate areas, each 2·5 μm square, in the central region of each of 4 spots.

Drugs and solutions used. The drugs and materials used were: bovine serum albumin (Commonwealth Serum Laboratories), corticosterone (Sigma), 3′,4′-dihydroxy-2-methylpropiophenone (U-0521, Upjohn), 6-hydroxydopamine hydrobromide (Sigma), (±)-isoprenaline sulphate (Sigma), (±)-metanephrine hydrochloride (Calbiochem), rimiterol hydrobromide (Riker).

The Krebs solution contained (in mM) NaCl 114, KCl 4·7, $CaCl_2$ 2·5, KH_2PO_4 1·2, $MgSO_4$ 1·2, $NaHCO_3$ 25, glucose 11·7, ascorbic acid 1·14 and was aerated throughout all experiments with 95% O_2 and 5% CO_2. The solutions of 6-hydroxydopamine and U-0521 were prepared as described by Bryan & O'Donnell (1980a).

Treatment of data. All mean values are shown with the standard error of the mean (s.e.). The significance of differences was assessed by Student's *t*-test or paired *t*-test as indicated in the text or Tables.

Results

Demonstration of formaldehyde-induced fluorescence of rimiterol. In an experiment with bovine serum albumin

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models fluorescence intensities in the spots containing rimiterol were significantly greater than in controls (no amine). With 3 h exposure to formaldehyde, the fluorescence intensity of rimiterol was approximately 60% of the fluorescence intensity of isoprenaline, at the same concentration. The experiments with bovine serum albumin indicated that a 5 h exposure to formaldehyde gave brighter fluorescence of rimiterol than a 3 h exposure. However, in guinea-pig trachealis smooth muscle cells, a 5 h exposure to formaldehyde significantly increases the background fluorescence, compared with that seen in tissues exposed for only 3 h. Thus, an exposure time to formaldehyde of 3 h was selected for experiments on the uptake of rimiterol in tissues.

Tests for the specificity of the rimiterol fluorescence were carried out using the method of Corrodi et al (1964) as described by Anning et al (1979). The fluorescence was quenched if tissue sections were treated with water but could be regenerated on heating the slide. The fluorescence was relatively resistant to 90% isopropyl alcohol but was removed when the sections were treated for 5 min with 0·1% sodium borohydride in 90% isopropyl alcohol, thoroughly washed with solvent, and then dehydrated at 50 °C for 30 min. Re-exposure of the tissue sections to formaldehyde at 80 °C for 3 h regenerated the fluorescence.

Effect of inhibition of COMT and effect of metanephrine on rimiterol fluorescence in guinea-pig trachealis smooth muscle cells. Inhibition of COMT with 100 μm U-0521 caused a small but significant increase in the fluorescence intensity values measured in the trachealis smooth muscle cells in tissues which had been incubated in 50 μM rimiterol (Table 1). The increase (in absolute values) was no greater with 500 μM rimiterol (68 arbitrary fluorescence units) than with 50 μM rimiterol (78 arbitrary units) indicating that, at 50 μM rimiterol, COMT was already fully saturated. Metanephrine (500 μM), an extraneuronal uptake inhibitor, caused a significant reduction in fluorescence values (for both 50 and 200 μM rimiterol).

Kinetic analysis of rimiterol uptake and of its inhibition by corticosterone. Using 80 μM rimiterol (in the presence of 100 μM U-0521), uptake of rimiterol by trachealis smooth muscle cells was measured at incubation times of 2, 5, 10, 20 and 30 min. Uptake occurred at a constant rate up to 20 min and then decreased at 30 min. An incubation time of 10 min in rimiterol was used in the subsequent initial rate study.

Table 1. Effect of inhibition of catechol-*O*-methyltransferase by U-0521 on rimiterol fluorescence in guinea-pig trachealis smooth muscle cells.

	Corrected fluorescence intensity values (arbitrary units)†	
	No U-0521	100 μM U-0521
50 μM rimiterol	50·4 ± 6·7	128·0 ± 15·9*
500 μM rimiterol	636·1 ± 19·8	703·6 ± 49·3

† Incubation time in rimiterol was 30 min. Values represent mean ± s.e. of measurements taken from tracheal segments from 5 guinea-pigs.

* Significant increase in fluorescence compared with the no U-0521 values (paired *t*-test). $0·05 > P > 0·1$.

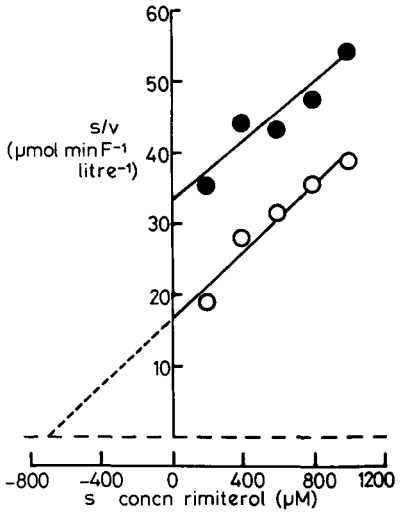

FIG. 1. Kinetic analysis of the extraneuronal uptake of rimiterol in guinea-pig trachealis smooth muscle cells in the absence (○) and in the presence (●) of 10 μM corticosterone. Mean initial rates of rimiterol uptake (v) were obtained from tissues from 10 guinea-pigs and used to calculate s/v (μmol min F^{-1}litre^{-1}, where F is arbitrary fluorescence units). The regression lines were then obtained by linear least squares regression analysis.

Initial rates of uptake (v) of rimiterol into trachealis smooth muscle cells were determined for rimiterol concentrations (s) of 200, 400, 600, 800 and 1000 μM (in the presence of 100 μM U-0521) in tissues treated with no extraneuronal uptake inhibitor and in tissues from the same animals treated with 10 μM corticosterone. Plots of s/v against s (Fig. 1) were analysed by a linear least squares regression analysis (Snedecor & Cochran 1980). The uptake of rimiterol appeared to be saturable in that it obeyed Michaelis-Menten saturation kinetics and the uptake of rimiterol was reduced by corticosterone.

With no corticosterone present, the plot of s/v versus s was linear (correlation coefficient 0·9734) and had an ordinate intercept significantly greater than zero. An estimate of the half saturation constant (K_m) of rimiterol for the uptake was obtained from this kinetic data. The mean K_m (obtained from the K_m values determined for each animal by the method of Marquardt (1963) as outlined by Bryan & O'Donnell 1981), was 1111 ± 178·2 μM (n = 10 animals). Thus the affinity of rimiterol for this uptake was significantly lower than that previously determined for isoprenaline, which had a K_m of 273 ± 12·1 μM (Bryan & O'Donnell 1980a).

The V_{max} for rimiterol was 53·40 ± 5·21 F min^{-1} (where F is arbitrary fluorescence units).

In the presence of 10 μM corticosterone, the uptake of rimiterol was reduced but the plot of s/v versus s was still linear (correlation coefficient 0·9457) and it was also parallel to the line in the absence of inhibitor (no significant difference between slopes, Snedecor & Cochran 1980). This provided evidence that corticosterone produced a reversible, and probably competitive, inhibition of rimiterol uptake.

Discussion

The conversion of a number of amines to a fluorophore by condensation with formaldehyde in a Pictet-Spengler reaction has been described by Corrodi & Jonsson (1967) and, from the chemical structure of rimiterol, we predicted that it should undergo this reaction. A formaldehyde-induced fluorescent product of rimiterol has been demonstrated in the present study in bovine serum albumin model experiments. The specificity of the fluorescence was established in that it was quenched by water and reduced by sodium borohydride. In sections of guinea-pig trachea which had been incubated in rimiterol, there was an increase in fluorescence in the trachealis smooth muscle cells, indicating that rimiterol had entered these cells. The intensity of this fluorescence was enhanced by U-0521, an inhibitor of COMT. This result indicated that the rimiterol which entered these cells could be metabolized by intracellular COMT, as has previously been described for isoprenaline (Bryan & O'Donnell 1979).

Evidence was obtained that the transport of rimiterol into these cells occurred by an uptake with characteristics similar to the extraneuronal uptake of isoprenaline, i.e. rimiterol uptake was inhibited by metanephrine and corticosterone. Furthermore, by examining the initial rate kinetics of the uptake of rimiterol and its inhibition by corticosterone, it was shown that corticosterone produced a reversible, competitive, inhibition of rimiterol uptake, as described previously for adrenaline in the same cells (Bryan & O'Donnell 1981). However, because of the low affinity of rimiterol for extraneuronal uptake (vide infra) it was not possible to get a reliable estimate of the K_i value for corticosterone with rimiterol as substrate, for comparison with the value obtained with adrenaline as substrate (Bryan & O'Donnell 1981).

The initial rates of rimiterol uptake fitted a model of one saturable transport process obeying Michaelis-Menten kinetics. However, the K_m of rimiterol (1111 μM) was 4 and 7 times higher than the values for isoprenaline and adrenaline respectively (Bryan & O'Donnell 1980a), i.e. rimiterol had a lower affinity for extraneuronal uptake. The reproducibility of values of K_m between animals was not as good as occurred when using isoprenaline, adrenaline or noradrenaline as substrate (Bryan & O'Donnell 1980a). This may have been due to the high K_m of rimiterol and the fact that the concentrations of amine selected for the kinetic study did not include sufficient concentrations greater than that of the K_m, i.e. the experimental conditions were not ideal for obtaining an accurate estimate of the K_m of rimiterol. Even after allowing for the different intensities of the fluorophores of rimiterol and isoprenaline (60:100), the V_{max} value for rimiterol uptake obtained in this study was significantly lower than that previously reported for isoprenaline, adrenaline or noradrenaline. This indicated that the extraneuronal site in guinea-pig trachealis smooth muscle cells had a low capacity to accumulate rimiterol.

Garland et al (1981) have recently demonstrated that guinea-pig trachealis smooth muscle can *O*-methylate isoprenaline by a high affinity, saturable extraneuronal *O*-methylating system. It is also known that inhibitors of extraneuronal uptake, e.g. phenoxybenzamine, cause potentiation of isoprenaline responses on guinea-pig isolated tracheal chain preparations (O'Donnell & Wanstall 1976). The observation that rimiterol responses on guinea-pig tracheal chain preparations were not potentiated by phenoxybenzamine (O'Donnell & Wanstall 1976) may suggest that the extraneuronal *O*-methylating activity of the trachea for rimiterol is much less than that for isoprenaline. Results with fluorescence histochemistry give no direct kinetic evidence on the *O*-methylation of catecholamines in a tissue but some data obtained in the present study could support the above conclusion on the poor extraneuronal *O*-methylation of rimiterol in this tissue. In tissues incubated in 50 μM rimiterol, COMT appeared to be metabolizing rimiterol at its maximum rate in that the increase in fluorescence produced by U-0521 was no greater at 500 than at 50 μM rimiterol. Even allowing for the slightly less intense fluorescence of rimiterol compared with isoprenaline, this maximum rate of *O*-methylation of rimiterol was much lower than that for the same concentration of isoprenaline (compare an increase in fluorescence intensity by U-0521 of 78 arbitrary units for 50 μM rimiterol (present study) with a value of 500 arbitrary units for 50 μM isoprenaline reported by Bryan & O'Donnell (1979)).

Thus, this study is the first direct demonstration that catecholamines, other than isoprenaline, adrenaline and noradrenaline, can utilize extraneuronal metabolizing systems in specific cells. It has provided also direct in vitro evidence that rimiterol, like isoprenaline, is a substrate for extraneuronal uptake and that this relates to its metabolism by COMT, at least in smooth muscle cells of the larger airways in guinea-pigs. However, extraneuronal *O*-methylation is probably of less functional importance for rimiterol than for isoprenaline. Direct evidence for the extraneuronal uptake of rimiterol has previously been lacking although Shenfield et al (1976) concluded, from plasma and urine levels of rimiterol and its metabolites, that rimiterol, administered directly into the human lung at diagnostic bronchoscopy, could be taken up and metabolized by COMT in the lung.

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A preferential blocking effect of oxprenolol on α_1-adrenoceptors in the rat

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It is now widely accepted that α-adrenoceptors can be subdivided into α_1 and α_2 subtypes (Berthelsen & Pettinger 1977). While α_1-adrenoceptors are located postsynaptically, α_2-adrenoceptors are situated not only at presynaptic nerve terminals (Langer 1977; Starke 1977) but also postsynaptically, at least in the vascular smooth muscle (Docherty et al 1979; Drew & Whiting 1979; Timmermans & Van Zwieten 1980). The β-blocking agent oxprenolol appears to have some α-adrenoceptor antagonist properties (Mazurkiewicz-Kwilecki 1970; Law et al 1978; Vila et al 1978; Roselló et al 1978). We have investigated the effects of oxprenolol on the α-adrenoceptor subtypes in several rat preparations. The in vitro studies were carried out on the field-stimulated vas deferens and the anococcygeus muscle of the rat, and the in vivo study was in the pithed rat.

Methods

The entire vasa deferentia from male Sprague-Dawley rats, 300–325 g, were removed and set up in isolated organ baths containing 20 ml of Krebs solution as modified by Huković (1961). The solution was maintained at 32 ± 0·5°C and gassed with 95% O_2-5% CO_2.

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Platinum ring electrodes were placed above and below the preparation and continuous field stimulation was by means of an Ealing stimulator (0·1 Hz, 3 ms and 20–30 V). The responses of the preparation against 0·5 g tension were recorded by means of an isotonic transducer on an Omniscribe pen recorder. Xylazine was used as α_2-adrenoceptor agonist (Docherty & McGrath 1980), and when the twitch contractions of the vas became stable, cumulative concentration-response curves of the inhibitory effect of the agonist were obtained. As a full recovery after washout of the agonist was difficult to obtain, one vas deferens was used as a control while in the other the activity of xylazine was evaluated 5 min after the addition of oxprenolol. Only two concentrations of antagonist could be used (3×10^{-5} and 1×10^{-4} mol litre^{-1}), and the pA_2 was determined according to the method described by van Rossum (1963). To rule out effects mediated by β-adrenoceptors, experiments were carried out in presence of 1×10^{-7} mol litre^{-1} of propranolol.

The postsynaptic α_1-adrenergic blocking activity of oxprenolol was evaluated on the isolated anococcygeus muscle as suggested by Doxey et al (1977). The muscle from male Sprague-Dawley rats, 250–300 g, was dissected as described by Gillespie (1972). The tissue was set