J. Pharm. Pharmac., 1978, **30**, 96–100 *Received June* 8, 1977

A comparison of the binding constant (K_D) of ^{125}I-labelled 3-(4-iodophenoxy)-1-isopropylamino-propan-2-ol obtained on β-adrenoceptors in guinea-pig myocardial membranes, with its dissociation constants (K_B) obtained on guinea-pig isolated atria and trachea

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The dissociation constant of binding (K_D) of ^{125}I-labelled 3-(4-iodophenoxy)-1-isopropyl-aminopropan-2-ol (IIP) to guinea-pig myocardial membrane preparations was $2{\cdot}2 \times 10^{-8}$M. In pharmacological experiments with the non-labelled material and 60 min contact time, IIP produced a parallel shift in the orciprenaline concentration-response line on guinea-pig isolated tracheal and atrial preparations. The dissociation constant (K_B) of IIP was $2{\cdot}9 \times 10^{-8}$M on atria (pA_2 7·54) and $3{\cdot}3 \times 10^{-8}$M on trachea (pA_2 7·48). These values indicate that IIP is not a selective β-adrenoceptor blocking drug. In addition, agreement was found between the affinity constant of this antagonist for β-adrenoceptors as determined by a direct binding study and an indirect pharmacological study.

Recently, the synthesis and purification of a new β-adrenoceptor antagonist containing an iodine atom which was labelled to high specific activity with ^{125}I, has been described (Bobik, Woodcock, & others, 1977). The binding of this compound, 3-(4-iodophenoxy)-1-isopropylaminopropan-2-ol (IIP), to rat myocardial membranes was studied in order to establish its properties as a ligand for β-adrenoceptors (Woodcock, Bobik & others, 1976). The compound was shown to bind with high affinity to a single class of binding sites in rat myocardial membranes using conventional criteria for binding experiments. These sites were considered to represent β-adrenoceptors. However, only preliminary experiments, *in vivo* in rabbits, were carried out with the non-radiolabelled compound to establish, pharmacologically, that the compound was a β-adrenoceptor antagonist.

Thus the aim of the present study was to obtain the affinity of IIP for β-adrenoceptors using direct binding studies and to compare this with the value obtained in a pharmacological study in the same species, the guinea-pig. The binding studies with ^{125}I-IIP were carried out on membrane fractions from guinea-pig heart suspended in the same physiological buffer solution as was used in the pharmacological experiments. The pharmacological experiments were carried out on guinea-pig isolated atria, a tissue proposed to contain β_1-adrenoceptors, and the method of Arunlakshana & Schild (1959) was used to obtain the dissociation constant (K_B). Experiments were also carried out on isolated tracheal chain preparations, a preparation proposed to contain β_2-adrenoceptors, to examine whether IIP showed selectivity as a β-adrenoceptor blocking drug.

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MATERIALS AND METHODS

(a) *Pharmacological study*

Female guinea-pigs, ~450 g, pretreated with reserpine (5 mg kg^{-1}, i.p.) 24 h before the experiment, were killed by a blow on the head and the trachea and atria removed. Four ring tracheal chains were set up at 500 mg tension as described by Chahl & O'Donnell (1967) and spontaneously beating atria were set up and atrial rate recorded as described by O'Donnell & Wanstall (1974). All preparations were in Krebs solution aerated with 5% CO_2 in oxygen at 37°. Tracheal preparations were contracted with carbachol as was used by Buckner, Birnbaum & O'Connor (1974).

On both preparations the cumulative addition of orciprenaline was used as described by van Rossum (1963). A cumulative concentration-response line to orciprenaline was obtained before and in the presence of the blocking drug, IIP. IIP was added to the

tissue bath, in the appropriate concentration, 60 min before obtaining the orciprenaline line and, on tracheal preparations, the carbachol (1×10^{-6} M) was also added during the last 10 min to contract the preparation. One or two concentrations of IIP were tested on each preparation.

Responses to orciprenaline were calculated as a percentage of the maximum response to orciprenaline. The EC50 value (concentration of orciprenaline required to produce a 50% maximum response) was interpolated from each log concentration—% maximum response line. Concentration ratios were calculated from antilog [(log EC50 with IIP)—(log EC50 without IIP)]. Log (concentration ratio—1) was plotted against log molar concentration IIP and the line of best fit through all the points from the various experiments calculated by a linear least squares regression. The slope, standard error of the slope, and comparison of slopes employed methods described in Snedecor & Cochran (1967). The intercept on the log concentration axis represented log dissociation constant (K_B) of the antagonist IIP, and pA_2 was obtained from this using the formula negative log $K_B = pA_2$.

(b) *Biochemical study*

Preparation of membrane preparation from guinea-pig heart. Female guinea-pigs, ~600 g, were killed by intraperitoneal injection of 60 mg of Nembutal. The hearts were removed immediately and placed in ice cold saline. After removal of blood vessels and epicardial fat, atria and ventricles were minced with scissors and homogenized in 3–5 volumes of buffer (10% Krebs 0·25 M sucrose) using a 'Polytron' homogenizer (10 s, speed setting 4). The homogenate was centrifuged at 10 000 *g* for 10 min at 5°, and the resulting supernatant layered over 5 ml of buffer (10% Krebs 0·5 M sucrose) in 15 ml centrifuge tubes and centrifuged at 30 000 *g* for 30 min at 5°. The pellet was resuspended in Krebs buffer at 0°, using a Dounce all-glass homogenizer, to a final protein concentration of 5–10 mg ml^{-1}. Protein concentration was determined by the method of Lowry, Rosebrough & others (1951) using bovine serum albumin as standard.

Binding of ^{125}*I-IIP to myocardial membranes.* Incubation mixtures contained 50 μl of membrane preparation and 50 μl of Krebs buffer containing increasing concentrations of ^{125}I-IIP. Incubation was carried out at 37° for 10 min, by which time equilibrium had been attained. At the end of the incubation, four 20 μl samples were removed from each tube and carefully pipetted onto the sides of 500 μl polypropylene micro-centrifuge tubes which contained 200 μl of Krebs buffer. Care was taken that the two solutions did not contact. The tubes were centrifuged for 1 min in a Beckman Microfuge 152. After aspiration of the supernatant, the tubes were washed with 500 μl of Krebs buffer and tips containing the pellet were cut off and their radioactivity counted using a Packard Autogamma Scintillation Counter. Non specific binding of ^{125}I-IIP defined as non displaceable binding, was determined for each incubation by using parallel incubations containing excess β-adrenoceptor blocking drug (10^{-5} M prindolol). Specific binding refers to the difference between total and non-displaceable binding and this was plotted against concentration ^{125}I-IIP. These data were used to calculate the dissociation constant of binding ($K_{D(37°)}$) using the method described by Scatchard (1949).

(c) *Drugs and solutions used*

The Krebs solution used in both the pharmacological and binding studies contained: (g litre^{-1}) NaCl 6·6, KCl 0·35, $CaCl_2$, 0·28, KH_2PO_4 0·162, $MgSO_4 \cdot 7H_2O$ 0·29, $NaHCO_3$ 2·10, Glucose 2·1, ascorbic acid 0·2.

3-(4-Iodophenoxy)-1-isopropylaminopropan-2-ol and the ^{125}I-labelled material were synthesized as described by Bobik & others (1977). For pharmacological studies a 10^{-3} M stock solution of IIP was prepared by dissolving the base in 0·01 N HCl. Dilutions were then made in Krebs solution.

Other drugs used were: carbachol (Sigma); orciprenaline sulphate (Boehringer-Ingelheim); reserpine (Serpasil ampoules, Ciba).

RESULTS

β-Adrenoceptor blocking potency of IIP on isolated trachea and atria

IIP produced a parallel shift of the orciprenaline log concentration-response line to a higher concentration range on both trachea and atria. In all the experiments on atria, addition of IIP caused some decrease in resting atrial rate. This ranged from 10 to 45 beats min^{-1} in different experiments depending on the concentration of IIP. Thus, for each concentration-response line, the 100% response to orciprenaline was taken as the difference between the maximum atrial rate obtained in the line and the atrial rate after addition of IIP. The maximum achieved to orciprenaline was not significantly reduced after concentrations of IIP used (up to 3×10^{-6} M) on either trachea or atria. Analysis of the data by the

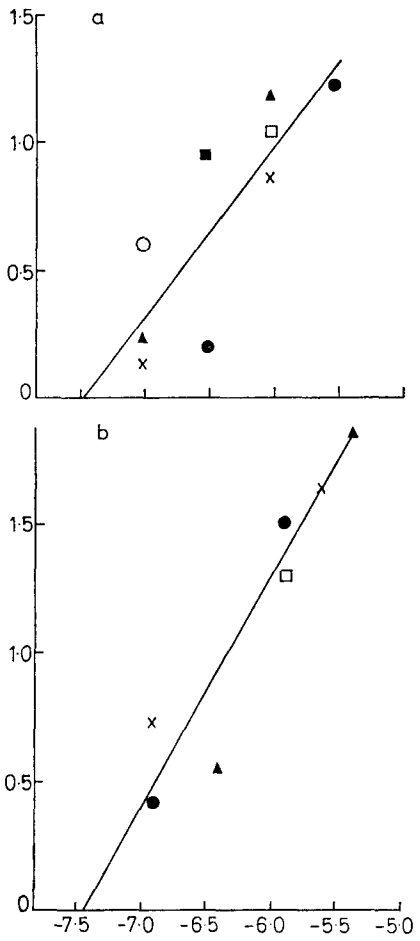

FIG. 1. Arunlakshana & Schild (1959) plots for IIP on guinea-pig trachea (a) and atria (b), using orciprenaline as agonist and 60 min contact time with IIP. The points represent values from 6 tracheal preparations and 4 atrial preparations as indicated by different symbols. Ordinate: Log (Concn ratio—1). Abscissa: Log concn IIP.

method of Arunlakshana & Schild (1959) gave regression lines with slopes of 0·90 ± 0·14 (standard error) on atria and of 0·65 ± 0·17 on trachea (Fig. 1). There was no significant difference between these two slopes nor did they differ significantly from 1·0. The dissociation constant (K_B) of IIP was $2·9 \times 10^{-8}$ M on atria (pA_2 7·54) and $3·3 \times 10^{-8}$ M on trachea (pA_2 7.48).

Binding of ^{125}I-IIP to myocardial membranes

^{125}I-IIP bound in a dose related manner to guinea-pig myocardial membranes over the concentration range 1×10^{-9} to 1×10^{-7} M (Fig. 2). Analysis of the data

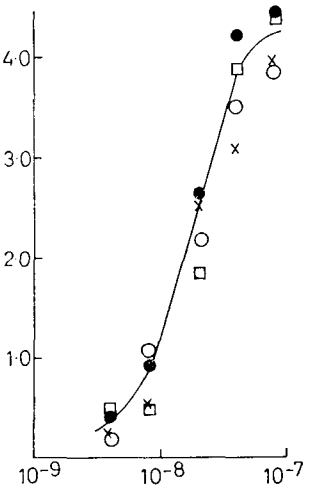

FIG. 2. Binding of ^{125}I-IIP to guinea-pig myocardial membranes. All points represent the mean of quadruplicate determinations and are the results from 4 guinea-pigs as indicated by different symbols. Ordinate: Counts min^{-1} bound mg^{-1} protein ($\times 10^{-4}$). Abscissa: ^{125}I-IIP (M).

using a Scatchard plot (Fig. 3) gave a regression line with a correlation coefficient of −0·94 and the dissociation constant of binding (K_D) as $2·2 \times 10^{-8}$ M.

DISCUSSION

3-(4-Iodophenoxy)-1-isopropylaminopropan-2-ol (IIP) is a β-adrenoceptor antagonist containing an iodine atom. This compound has been prepared radioactively labelled to high specific activity with ^{125}I and used to detect β-adrenoceptors *in vitro* (Woodcock & others, 1976). In the present experiments a combined biochemical (binding study on

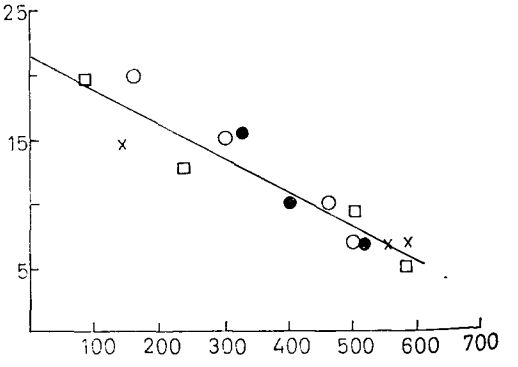

FIG. 3. Binding of ^{125}I-IIP to guinea-pig myocardial membranes. Scatchard analysis of the data presented in Fig. 2. Ordinate: Bound/free ($\times 10^{-6}$). Abscissa: ^{125}I-IIP bound mg^{-1} protein (f mol).

membrane fractions) and pharmacological (on isolated tissue preparations) study of the affinity of this drug for β-adrenoceptors has been carried out.

To obtain the best estimate of the dissociation constant (K_B) for IIP, using the approach of Arunlakshana & Schild (1959), the pharmacological experiments were designed to comply, as far as possible, with the conditions outlined by Furchgott (1967, 1972) for isolated tissue experiments. Thus preparations were taken from reserpinized animals to avoid catecholamine release and orciprenaline was selected as the agonist drug so that no inhibitor drugs need be included in the Krebs solution. Orciprenaline has no α-adrenoceptor stimulant effects (O'Donnell & Wanstall, 1974) and its responses are not modified by neuronal or extraneuronal uptake in the preparations used (O'Donnell & Wanstall, 1974, 1976). The contact time of 60 min used for IIP has been found to be sufficient for equilibrium to be reached for other β-adrenoceptor antagonists (Furchgott, 1972).

The antagonist drug, IIP, gave a parallel shift in the orciprenaline concentration-response line which was assumed to reflect competition with orciprenaline for β-adrenoceptors. However, the method of Arunlakshana & Schild (1959) provides a check on whether the results are consistent with the model of competitive antagonism, i.e. the slope of the regression line should approach 1·0. The slopes obtained from results on both atria and trachea were less than 1·0 although not significantly. There are other studies with β-adrenoceptor antagonists on trachea in which the slopes of the Schild plot have been less than 1·0 even though the antagonist produced a parallel shift of the agonist line e.g. guinea-pig (Patil, 1968; Levy & Wasserman, 1970), rabbit (Bristow, Sherrod & Green, 1970). The reason for the low slope on trachea in the present experiments is unclear but the experiments were carried out on carbachol-contracted preparations and the slope may have been affected if IIP should have any antimuscarinic activity. The slope for the atrial experiments may have been affected by the depression of resting atrial rate produced by IIP, but this is an effect produced by other β-adrenoceptor blocking drugs above certain concentrations (Blinks, 1967). It thus remains a possibility that IIP may not be a simple, reversible competitive antagonist but may have a more complex action.

Despite the low values obtained for the slopes of the Schild plots, the K_B values for IIP on trachea (contains β_2-adrenoceptors) and atria (β_1-adrenoceptors) were almost identical suggesting that IIP is a non-selective β-adrenoceptor blocking drug. Also, the values for the dissociation constants for IIP obtained in these pharmacological experiments were in very close agreement with the value for K_D of $2{\cdot}2 \times 10^{-8}$ M obtained in the present biochemical experiments on guinea-pig cardiac membrane preparations. However, it should be noted that the K_D value for IIP in the present experiments was almost 5 times greater than that previously reported for rat myocardial membranes (4×10^{-9} M), a value which was in excellent agreement with that calculated from inhibition of isoprenaline stimulated adenylate cyclase on the same preparation (Woodcock & others, 1976). The reason for this difference could be a species difference or an influence of the ionic strength of the suspending media on affinity determinations, a problem which has been observed in polypeptide hormone binding (Moore & Wolff, 1974). The buffer used for resuspending the pellet in the study on rat membrane preparations lacked sodium and contained very high magnesium concentrations (Woodcock & others, 1976).

Lefkowitz (1975) commented that the ability of a β-adrenoceptor antagonist to compete for occupancy of the receptor binding site should parallel biological activity as an antagonist. Also, reasonable quantitative agreement should be found between affinity constants of the antagonist for receptors as determined by direct binding studies and indirect pharmacological studies. The present study has gone some way towards showing this agreement for a new ^{125}I-labelled, β-adrenoceptor antagonist of high specific activity, which may be used in the identification of β-adrenoceptors.

Acknowledgments

This work was supported by grants from the National Health and Medical Research Council of Australia (to SRO'D) and from the Life Insurance Medical Research Fund of Australia (to EAW). This aid is gratefully acknowledged.

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