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THE β-ADRENOCEPTOR OF THE HUMAN LYMPHOCYTE AND HUMAN LUNG PARENCHYMA

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1 The response of the β-adrenoceptors of human lymphocytes to selective agonists and antagonists has been studied quantitatively by measuring changes in cyclic adenosine-3′,5′-monophosphate (cyclic AMP) levels.

2 The receptor was activated by isoprenaline and by salbutamol, and blocked by propranolol but not by practolol. A similar pattern of response was obtained with fragments of human lung tissue.

3 The mean value for pA_2 for propranolol was 8.34 and for practolol was 3.95.

4 These findings indicate that the lymphocyte β-adrenoceptor is a β_2-receptor and support the validity of using lymphocytes to study β-adrenoceptor function in bronchial asthma. It may also be of use in the evaluation of selective β_2-blocking drugs in man.

Introduction

There is a growing awareness of the need to study disease mechanisms at a cellular level. Work with isolated organs or tissue slices represent moves in that direction, though many artifacts are inherent in their preparation, and their application to man is very limited. Cellular elements of peripheral blood have therefore a great heuristic appeal since they possess receptors specific to a number of endogenous compounds, and they can be isolated repeatedly and with relative ease, even during periods of severe illness. They therefore provide a useful tool for studying a number of cellular mechanisms. Following the discovery of adenylate cyclase in sonicated leucocytes (Scott, 1970) it has been found that these cells possess both α- and β-adrenoceptors (Logsdon, Middleton & Coffey, 1972), histamine receptors (Bourne, Melmon & Lichtenstein, 1971) and prostaglandin E_1 receptors (Bourne & Melmon, 1971). Insulin (Gavin, Mann, Buell & Roth, 1972; Gavin, Roth, Neville, De Meyts & Buell, 1974) and growth hormone receptors (Archer, Gorden, Gavin, Lesniak & Roth, 1973) have also been described.

Szentivanyi, as early as 1962, postulated an inherent defect of the β-adrenoceptor in atopic diseases, including bronchial asthma (see Szentivanyi, 1968). The early work based on this concept used measurements of metabolic and cardiovascular responses to sympathomimetic agents or exercise in intact subjects (Cookson & Reed, 1963; Lockey, Glennon & Reed, 1967; Inoue, 1967; Middleton & Finke, 1968) and the results appeared to support the hypothesis. However, it is not possible to conduct a pharmacologically 'clean' experiment in intact subjects and it would be impossible to conduct such studies on a serial basis throughout severe asthmatic attacks. There has therefore been considerable enthusiasm for the use of leucocytes, either as a mixed population of cells or as purified lymphocytes, since the technique was first suggested by Smith & Parker (1970). Several studies (Logsdon *et al.*, 1972; Parker & Smith, 1972; Alston, Patel & Kerr, 1974; Gillespie, Valentine & Lichtenstein, 1974) have been reported. These authors have all assumed that the β-receptor of the leucocyte is identical with that in the lung. Before further effort is expended on this model, it is desirable to establish some measure of identity between the two receptors in terms of Land's classification (Lands, Arnold, McAuliff, Luduena & Brown, 1967) into β_1 (e.g. myocardial) and β_2 (e.g. bronchial and vascular smooth muscle) receptors. This paper assesses the lymphocyte β-receptor in terms of its responsiveness to the non-selective and β_2-selective agonists, isoprenaline and salbutamol respectively. Antagonism of isoprenaline with a non-selective and a β_1-selective adrenoceptor blocking drug (propranolol and practolol respectively) has also been studied. In addition, preliminary data on the responsiveness of the β-adrenoceptors in lung parenchyma are presented.

Methods

Pharmacological response was quantitated by measuring changes in the tissue levels of cyclic-3′,5′-adenosine monophosphate (cyclic AMP). A range of agonist concentrations has been used in the

lymphocyte studies to provide complete dose-response curves. A range of concentrations of propranolol and practolol has also been used so that the β-receptor could be characterized in terms of the pA_2 values for these antagonists (Schild, 1947; Arunlakshana & Schild, 1959). No attempt has been made to determine a pA_2 in peripheral lung tissue as many different cell types are present, including mast cells and bronchiolar and vascular smooth muscle cells, which may possess different types of β-receptor.

Cell separation

Lymphocytes were prepared from heparinized venous blood by a modification (Harris & Ukaejiofo, 1970) of Böyum's (1968) technique which produced a cell population of approximately 90–95% lymphocytes. The majority of the remaining cells were monocytes with very few granulocytes. Blood (60 ml) was centrifuged at 250 *g* for 20 min at 15°C (MSE Mistral 4L refrigerated centrifuge), to permit removal of platelet-rich plasma. This was replaced with an equivalent volume of Hanks Balanced Salt Solution (HBSS, Burroughs Wellcome Ltd) previously gassed with 95% O_2 and 5% CO_2 and adjusted to pH 7.4. Twelve ml aliquots of this 'blood' were carefully layered onto 10 ml Lymphoprep (Nyegaard & Co) and then centrifuged at 400 *g* for 25 min at 15°C. The lymphocytes accumulated at the interface between the two liquids. This layer was harvested, diluted with phosphate buffered saline (Dubelcco's, Oxoid Ltd) at pH 7.4, and then centrifuged at 1000 *g* for 20 min at 15°C. The supernatant was discarded and the cell pellet was gently resuspended in HBSS. The addition of 10% foetal calf serum (Burroughs Wellcome Ltd) to all the media in order to reduce lymphocyte adhesiveness was essential to ensure an adequate yield of cells. Cells were counted in a Coulter Cell Counter. Cell concentrations of 1×10^6 to 3×10^6 cells/ml produced a linear response with respect to cell numbers, so the suspension was diluted to give a final concentration of 2×10^6 cells/ml when all drug solutions had been added. Bovine serum albumin was added to give a final concentration of 0.3 mg/ml and the suspension was buffered with Tris HCl (5 mmol/litre).

Human lung

Tissue was obtained from a patient undergoing lobectomy for bronchiectasis. Normal lung tissue was separated from the diseased area, dissected free of visible bronchi and pleura, and chopped into small pieces. Fragments amounting to 200 mg wet weight were added to each tube and thereafter treated in the same way as lymphocytes except that no protein was added to the incubation mixture. Response was expressed as pmol cyclic AMP per mg protein, the protein being estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Drugs

The final concentrations of the drugs used (and their sources) were as follows: theophylline 10^{-2} mol/l (BDH Ltd); (±)-isoprenaline sulphate 10^{-8} to 10^{-4} mol/l (Sigma Ltd); (±)-salbutamol sulphate 10^{-8} to 10^{-4} mol/l (a gift from Allen & Hanbury Ltd); (±)-propranolol hydrochloride 10^{-9} to 10^{-4} mol/l and (±)-practolol 5×10^{-5} mol/l (both gifts from ICI Ltd).

Ascorbic acid (1 mg/ml in stock solutions) was used as an antioxidant for isoprenaline, and was also added to salbutamol for the sake of consistency.

Cell incubation

The incubations were performed in a final volume of 2 ml. To each tube 1 ml of Hanks BSS containing theophylline (2×10^{-2} mol/l) was added, and the tube was placed in a water bath at 37°C. The drugs were then added in volumes of 0.1 ml, the concentrations of each being such that when diluted to 2 ml, the required final concentrations (see above) would be achieved. Finally 1 ml lymphocytes (4×10^6 cells/ml) was gently added to each tube, thereby giving a final cell concentration of 2×10^6 ml. The tubes were gently shaken after the addition and from time to time during incubation to ensure uniform dispersal of the lymphocytes. The incubation was terminated after 15 min by plunging the tubes into boiling water for 5 minutes. Following this, 0.18 pmol [^{3}H]-cyclic AMP (5000 ct/min, New England Nuclear Corp) was added to permit estimation of recovery after purification. The cells were homogenized and centrifuged, and the supernatant applied to 0.5×2.0 cm columns of Dowex AG 1×8 (formate form, 200 to 400 mesh, Biorad Labs). The columns were eluted with 10 ml water, which was discarded. Cyclic AMP was then washed from the columns with 12 ml 2 N formic acid which did not elute cyclic-3′,5′-guanosine monophosphate (cyclic GMP). The eluate was lyophilized, and the residue was dissolved in 1 ml phosphate-citrate buffer (pH 6.5). Recovery was estimated by counting a 50 µl aliquot of this solution, and further aliquots were used in the cyclic AMP assay described below.

Cyclic AMP assay

A protein binding assay was chosen in preference to a method which depends on measuring the conversion of [^{3}H]-adenosine to [^{3}H]-cyclic AMP, since the latter gives no data on basal cyclic AMP levels.

The method was modified from that of Gilman (1970). The binding protein was extracted from rabbit skeletal muscle, using a protamine-sepharose affinity

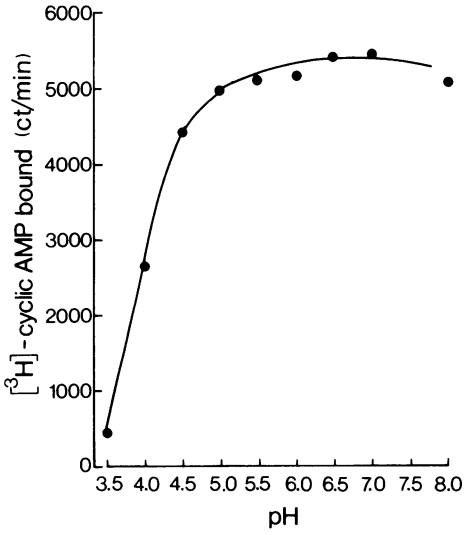

Figure 1 Binding of cyclic AMP by protein kinase from rabbit skeletal muscle over a pH range of 3.5 to 8.0.

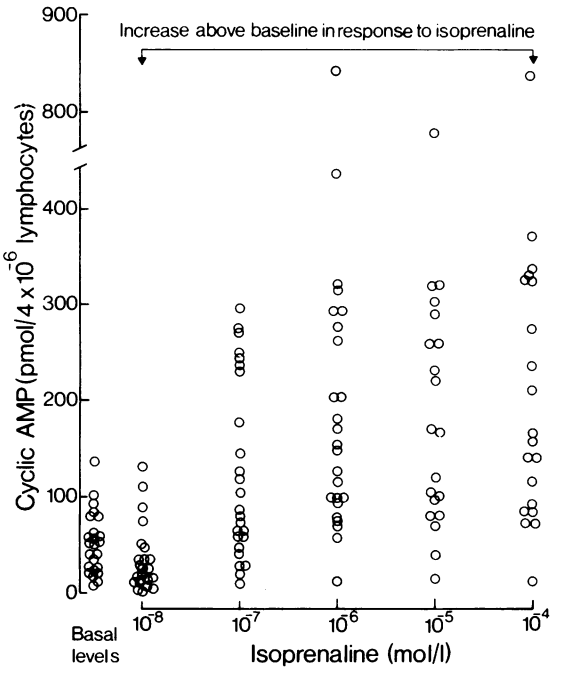

Figure 2 Basal level of cyclic AMP (pmol per 4×10^6 lymphocytes) and increase above this level in response to a 15 min incubation with isoprenaline (10^{-8} to 10^{-4} mol/l) at 37°C. Observations made on 8 normal subjects studied on 1–5 separate occasions.

chromatography column (Johnson & Schultz, personal communication). The binding protein was eluted with a 0–2 molar gradient of NaCl, dialysed, and after the addition of bovine serum albumin (1 mg/ml), frozen in 1 ml aliquots at −20°C. Under these conditions it showed no loss of activity for more than 2 years. This protein differed from that described by Gilman in that its ability to bind cyclic AMP was very pH-sensitive, being maximal over a range of pH 5.5 to 7.5 (Figure 1). All assays were performed at pH 6.5.

Each assay tube contained 50 µl phosphate-citrate buffer (pH 6.5) containing known amounts of unlabelled cyclic AMP (0 to 10 pmol) or the experimental material derived from the incubations. To each tube was added 0.5 pmol [^{3}H]-cyclic AMP in 10 µl water (10 µl water alone added to blank tubes), and 50 µl of the binding protein (which had been further diluted with bovine serum albumin). The contents were thoroughly mixed, and allowed to stand in ice for 3 hours. At the end of this time, the incubation mixture was passed through a millipore filter (Millipore HAWP 02400 filter; 0.45 µm pore size) which was then rinsed with ice cold 20 mmolar phosphate buffer (pH 6.0). The filter was dissolved in 1 ml 2-ethoxyethanol in a counting vial, and after the addition of 10 ml Instagel (Packard), the samples were counted in a Packard 3375 spectrometer. Each assay measurement was performed in duplicate. As counting efficiency was very similar for all samples, no correction was made for quenching.

The calibration curve generated at each assay was linear over the range 0.5 to 10.0 pmol. The sensitivity of the assay permitted measurement of cyclic AMP levels as low as 0.1 pmol per assay tube. With known amounts of unlabelled cyclic AMP, the assay was found to be accurate to within ±10%. Other nucleotides were tested for their ability to interfere with this assay. Adenosine triphosphate and adenosine diphosphate were without effect. Cyclic GMP in concentrations 100 times higher than cyclic AMP did interfere with binding, but since it was separated from cyclic AMP by the chromatographic purification the resultant assay may be regarded as specific for cyclic AMP. Estimation of cyclic AMP levels from the calibration curve, and all other calculations involved in this assay were performed on a Digital PDP8/I computer.

Results

Responsiveness to isoprenaline varied widely both within and between subjects. Figure 2 shows the basal levels of cyclic AMP and the increases seen at each isoprenaline concentration used. The data are derived from 8 normal subjects studied on 24 separate

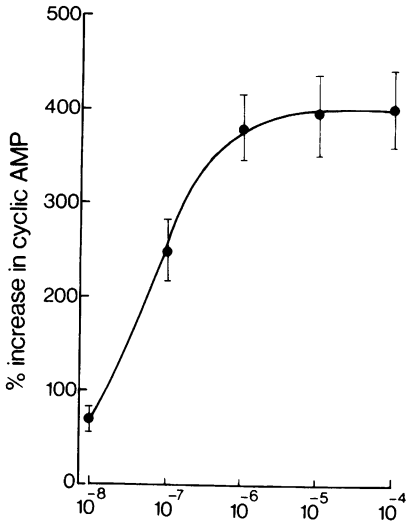

Figure 3 Percentage increase in cyclic AMP above baseline values in lymphocytes from 8 normal subjects in response to isoprenaline (same studies as shown in Figure 2). Mean values are plotted; vertical lines show s.e. mean.

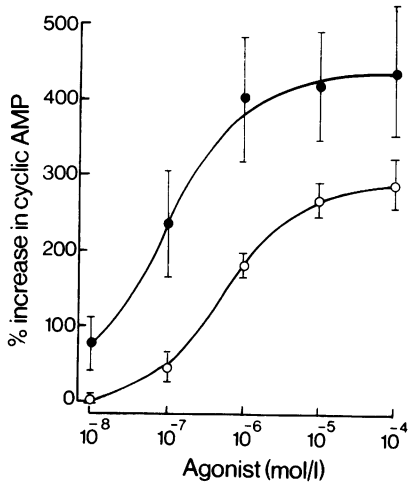

Figure 4 Percentage increase in cyclic AMP in lymphocytes from 2 normal subjects after 15 min incubation at 37°C with isoprenaline (●) or salbutamol (○).

occasions. (In some studies there were insufficient cells to examine the response to every dose in the range employed.) Cyclic AMP levels (expressed as pmol (± s.e.) per 4×10^6 cells) increased from 48 ± 5.3 to 232×25.9, in the presence of isoprenaline 10^{-4} mol/l, an increase of $388 \pm 41.8\%$. The cells

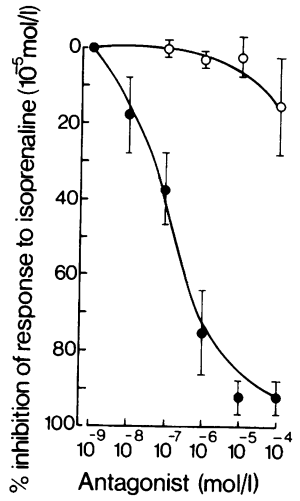

Figure 5 Antagonism of the increase in cyclic AMP produced by isoprenaline (10^{-5} mol/l) produced by propranolol (●) and practolol (○) in the concentrations shown.

with the lowest basal levels of cyclic AMP also showed the lowest increases, and *vice versa*, so the data have been analysed in terms of percentage changes, and the resulting dose-response curve for these 8 subjects is shown in Figure 3.

The response to lymphocytes from 2 normal subjects to salbutamol over the same range of concentrations has been examined (Figure 4). This drug produces a dose-response curve with a similar configuration to that of isoprenaline obtained in the same two subjects. Comparison of the two drugs where the curves are roughly parallel indicates that isoprenaline is about 9–15 times more potent, dose for dose, than salbutamol. In addition, salbutamol has only partial agonist activity, producing only a $295 \pm 26\%$ increase in cyclic AMP as compared with $428 \pm 66\%$ for isoprenaline.

The comparison between a non-selective (propranolol) and a selective β-blocking drug (practolol) is shown in Figure 5. These data represent observations in 4 normal subjects. The effects of isoprenaline on cyclic AMP levels were progressively antagonized by propranolol, whereas practolol was without effect below 10^{-5} mol/litre. No valid dose-ratio can be derived from these data but at a concentration where practolol is beginning to produce some β-blockade (5×10^{-5} mol/l) a potency ratio in the region of 5000:1 is apparent.

In order to characterize the receptors further, pA_2 values for propranolol and practolol have been derived from Schild plots (Schild, 1947) using cells from 3 normal subjects, and also lymphocytes from a patient with chronic lymphatic leukaemia (Figure 6a,b). The

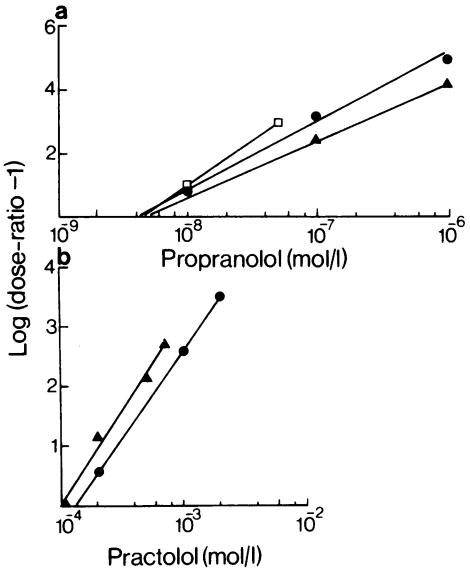

Figure 6 (a) Schild plot showing the inhibitory effect of propranolol on response to isoprenaline in lymphocytes from 2 normal subjects (□ and ●) and one with chronic lymphatic leukaemia (△). Values for pA_2 and slope of plot respectively are as follows: (□) 8.38; 2.2; (○) 8.34; 2.0; (△) 8.32; 1.8. (b) Schild plot showing the inhibitory effect of practolol on response to isoprenaline in lymphocytes from 2 normal subjects. Values for pA_2 and slope of plot respectively are as follows:- (▲) 4.0; 3.2; (●) 3.89; 2.6.

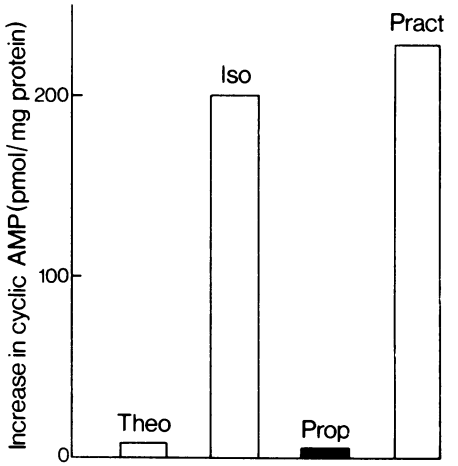

Figure 7 Cyclic AMP response (pmol/mg protein) to theophylline (Theo), isoprenaline plus theophylline (Iso), isoprenaline and theophylline plus propranolol (Prop) and isoprenaline and theophylline plus practolol (Pract). Concentrations used were: theophylline 10^{-2} mol/l; isoprenaline 10^{-4} mol/l; propranolol 10^{-4} mol/l and practolol 10^{-4} mol/l. Incubations were for 15 min at 37°C.

values ranged from 8.32 to 8.38 for propranolol, and 3.89 to 4.00 for practolol. Values obtained in other tissues in several laboratories are given in Table 1 for comparison.

β-Receptors in lung parenchyma appear to respond in the same way as those of lymphocytes with respect to propranolol and practolol. Figure 7 shows the response of duplicate samples of lung tissue to isoprenaline (10^{-4} mol/l), isoprenaline plus propranolol (10^{-4} mol/l of each drug) and isoprenaline plus practolol (10^{-4} mol/l of each drug). Propranolol virtually abolished the effect of isoprenaline, whereas with practolol there is a slight further increase, from 196.6 to 232.0 pmol cyclic AMP per mg protein. No attempt has been made to determine pA_2 values for lung parenchyma because of the heterogeneous cell population involved.

Table 1 pA_2 values for propranolol and practolol obtained in other laboratories

Tissue	*Propranolol*	*Practolol*	*Reference*
Guinea-pig atria	8.70		Blinks, 1967
	8.02		Moore & O'Donnell, 1970
	7.98		Bassett, 1971
	8.71		Conolly, 1972
	8.32	6.49	Barrett *et al.*, 1973
Guinea-pig trachea	8.02		Moore & O'Donnell, 1970
	7.93		Bassett, 1971
	8.46	4.26	Barrett, Carter, Fitzgerald, Hull & Le Count, 1973
Human bronchus	6.65	4.29	Hedges & Turner, 1971

Discussion

The subdivision of adrenoceptors into two broad categories, α and β, first suggested by Ahlquist (1948), is now well established. There is much less agreement on the validity of the further subdivision of β-adrenoceptors into β_1 and β_2 subgroups (Lands *et al.*, 1967) principally because there seem to be too many receptors which will not fit neatly into either group (Bristow, Sherrod & Green, 1970; Lefkowitz, 1975).

Nevertheless, it remains a clinically useful concept since it has been possible to develop relatively cardioselective (β_1) adrenoceptor antagonists such as practolol, atenolol (ICI 66082, Tenormin), and metoprolol, which may be used to treat angina or hypertension in many, though not all, patients with obstructive airways disease. Conversely selective β_2-agonists such as salbutamol and terbutaline are being used increasingly in the treatment of bronchial asthma, since they produce the same bronchodilatation as isoprenaline but with much less tachycardia.

It is desirable to measure the results of receptor stimulation as directly as possible, and in this respect the identification of cyclic AMP as an intracellular 'second messenger' has been of great value. However, for cyclic AMP to provide a reliable index of receptor stimulation from one experiment to another, a homogeneous cell population must be studied, and in an attempt to achieve this, we decided to use lymphocytes, which have higher levels of adenylate cyclase activity than granulocytes. However, lymphocyte separation on a density gradient, used here because of its advantages of speed and simplicity, may result in a selective loss of T lymphocytes (Thompson & Vaughan Smith, personal communication). Bach (1975) has shown in the mouse that T and B lymphocytes differ markedly from one another in respect of adenylate cyclase activity and this may well explain the pattern of variability described above. Since cells with low basal activity respond least well to β stimulants and *vice versa*, it was felt preferable to analyse the data as proportional (percentage) increase in cyclic AMP.

We have shown that the lymphocyte β-adrenoceptor is readily blocked by (±)-propranolol, but appears to be far less sensitive to the effect of practolol. The same pattern of susceptibility to the different β-blockers is seen in lung tissue.

This difference in potency between practolol and propranolol is, like that reported by Dunlop & Shanks (1968), greater than the difference observed by Vaughan Williams, Bagwell & Singh (1973) in anaesthetized dogs. The latter group reported a potency ratio of 75.9:1 in favour of propranolol when examining blockade of isoprenaline-induced vasodilatation. The difference between the results of Vaughan Williams *et al.* and those reported here may in part be due to species difference. However, it is possible that differences in the experimental conditions used also played a part. In the present study, the cells were suspended in an aqueous medium (a relatively polar solvent system), whereas in the dog studies the tissues were bathed with blood, which has a high lipid content. Practolol is far more polar than propranolol, and in experiments using aqueous media, it will preferentially partition into the medium rather than into the lipid of the cell membrane, and this may hinder access to the receptors. For the lipid soluble propranolol the reverse will be the case, tending thereby to exaggerate the already substantial difference in the potency of the two drugs with respect to the β_2-receptor. The pA_2 values for both propranolol and practolol correspond closely to those obtained by most other authors and conform to the pattern which would be predicted for a tissue possessing a $\beta_2$receptor. The slopes of the Schild plots are greater than unity (the expected value for a true competitive antagonism). There is no obvious explanation for this. Both propranolol and practolol have been shown in other tissues to exert only a simple competitive antagonism, and studies with another β-blocking drug, alprenolol, in human lymphocytes (Williams, Snyderman & Lefkowitz, 1976) indicate that β-blockade is a reversible competitive antagonism in this cell model.

The response to salbutamol is in keeping with the pattern of response to the antagonist drugs. Salbutamol stimulated the lymphocyte β-adrenoceptor, being about one tenth as active as isoprenaline. These findings correspond closely to those of Cullum, Farmer, Jack & Levy (1969) who reported a 10:1 potency ratio between isoprenaline and salbutamol on guinea-pig tracheal chain. These authors found in contrast a 2000:1 potency ratio with respect to cardiac β-receptors in the same species. In the lymphocyte, unlike tracheal smooth muscle, salbutamol acts as a partial agonist.

On the basis of these studies the lymphocyte adrenoceptor may (within the limitations of this classification) be categorized as β_2 and in this respect is valid as a model for studying β-receptor function in asthma.

Assessment of β_2-adrenoceptor function by non-invasive methods is not easy. However, the lymphocyte offers a very accessible receptor system and as such may be a useful tool for measuring this, and for assessing the selectivity of β-adrenoceptor antagonists (and possibly also selective drugs of other types) in man.

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