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AFFINITY LABELLING OF α-ADRENOCEPTORS IN INTACT LIVER CELLS BY [^{3}H]PHENOXYBENZAMINE

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SUMMARY : [^{3}H]phenoxybenzamine of high specific activity (5.3 Ci/mmol) was synthesized and its binding to isolated, viable rat liver cells was studied. Phentolamine suppressible binding of [^{3}H]phenoxybenzamine was irreversible and saturable (EC50: 10 nM, b_{max}: 200 fmol/mg wet cell weight). Competition-inhibition studies showed structural and stereoselectivity compatible with α-receptors. The IC50 of unlabelled phenoxybenzamine to reduce specific binding (9 nM) or to block adrenaline-induced phosphorylase activation in the same cells (2 nM) was similar, whereas the IC50 of agonists to suppress binding was higher than their EC50's for phosphorylase activation. The results represent the first example of labelling α-adrenoceptors in intact liver cells. The sites labelled by [^{3}H]phenoxybenzamine mediate the block of phosphorylase activation by α-adrenoceptor antagonists. However, the relationship of these sites to receptors that mediate responses to physiological, low concentrations of catecholamines remains to be clarified.

INTRODUCTION

The use of reversible radioligands has allowed the detection of α-adrenoceptor binding sites in subcellular tissue fragments. Recent studies with [^{3}H]dihydroergocryptine ([^{3}H]DHEC) indicated an unusually high density of specific binding sites in plasma membranes from rat liver (1,2). However, the functional relevance of these binding sites can only be established if they are identified in the same intact cell preparation, which can be used to measure physiological responses of the liver. In our laboratory, attempts to detect specific [^{3}H]DHEC binding in intact rat liver cells have been unsuccessful, probably because of the rapid and high capcity uptake of the ligand by the intact cells. An irreversible antagonist could be used to label α-adrenoceptors in intact cells, because the unbound ligand could be removed by disruption and washing of cells without losing label covalently

Abbreviations: [^{3}H]POB = [^{3}H]phenoxybenzamine; [^{3}H]DHEC = [^{3}H]dihydroergocryptine; TCA = trichloroacetic acid.

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bound to receptors. Phenoxybenzamine (POB) is a potent, irreversible antagonist of α-adrenoceptors (3). Previous attempts to label α-adrenoceptors by [^{3}H]POB were not successful because of the low specific activity of the compound (< 50 mCi/mmol, ref. 4,5). Using a different synthetic approach we have achieved a specific activity of 5.3 Ci/mmol. The results presented show that in intact, viable rat liver cells [^{3}H]POB (1-100 nM) binds to stereoselective sites with properties similar to α-adrenoceptors.

MATERIALS AND METHODS

Synthesis of [^{3}H]POB: Steps of the synthesis are illustrated in Fig. 1. N-(phenoxyisopropyl)-N-(3,4-dichlorobenzyl)-β-chloroethylamine (SKF 803, ref. 6) was dissolved in ethanol and converted into the ethanolamine form by the addition of NaOH. The benzylic chlorides were then replaced with ^{3}H at New England Nuclear Co., by catalytic hydrogenation with tritium gas. To ensure complete dechloridation, hydrogenation was continued for one hour after the stoichiometric amount of $^3H_2$ had been consumed. Further steps of the synthesis were done in our laboratory. The N-(phenoxyisopropyl)-N-(benzyl-3,4 [^{3}H]-ethanolamine was purified by passage through a silica gel G column, eluted with methylene chloride. Nucleophilic substitution with thionyl chloride was then done according to a published procedure (6). [^{3}H]POB HCl was recrystallized twice from ether/ethanol and from methylenechloride/heptane. The light tan crystals were dissolved in acidified ethanol (0.1 N HCl) and stored at -20°C as a 10^{-3}M stock solution. The specific activity of the compound was 5.3 Ci/mmol. Thin layer chromatography on silica gel G in heptane:chloroform:methanol (50:35:15) and radioscanning yielded a single peak containing 92% of all radioactivity. The R_f value of 0.71 was identical to the R_f of commercial unlabelled POB (SK&F). The biological activity of the compound, tested at 1 and 3 nM as block of noradrenaline-induced contractions of rat aortic strips, was similar to that of commercial POB. A similar synthesis from a monobromobenzyl analog of POB has been reported (7).

Preparation of liver cells: Hepatocytes were isolated from male Sprague-Dawley rats (300-350 g), by the method of Berry & Friend (8) with some modifications detailed elsewhere (9). Viability of the cells, tested by exclusion of trypan blue, was routinely > 90%. Cells were suspended in Krebs-Henseleit buffer (pH 7.4) containing 1.5% gelatin (Difco), and were kept on ice until use. Wet weights were determined by centrifuging a 1 ml aliquot of cell suspension for 2 min at 200 x g.

Binding assay: Cells were divided into 1 ml aliquots containing 1 mg wet weight of cells. Aliquots were placed in glass test tubes in a shaker bath at 37°C under an atmosphere of 5% CO_2 in O_2. Competing ligands were added 6 min and [^{3}H]POB 12 min after the start of incubation. Incubation with [^{3}H]POB was for 10 min unless stated otherwise. Incubation was terminated by rapid centrifugation of cells (30 s, 2,000 g), aspiration of the supernatant, and addition of 3 ml 10% trichloroacetic acid to the pellet was dissolved in 0.5 ml NCS tissue solubilizer and, after addition of a toluene based cocktail, radioactivity was measured by liquid scintillation spectrometry at 45-50% efficiency. Efficiency was determined for each sample by the channels ratio method. Values are expressed as fmol [^{3}H]POB per mg wet cell weight. One mg cell weight corresponds to approximately 2 x 10^5 cells. Specific binding of [^{3}H]POB was defined as the difference between total binding and non-specific binding determined in the presence of 10^{-5}M phentolamine. All determinations were done in triplicate. Specific binding re-

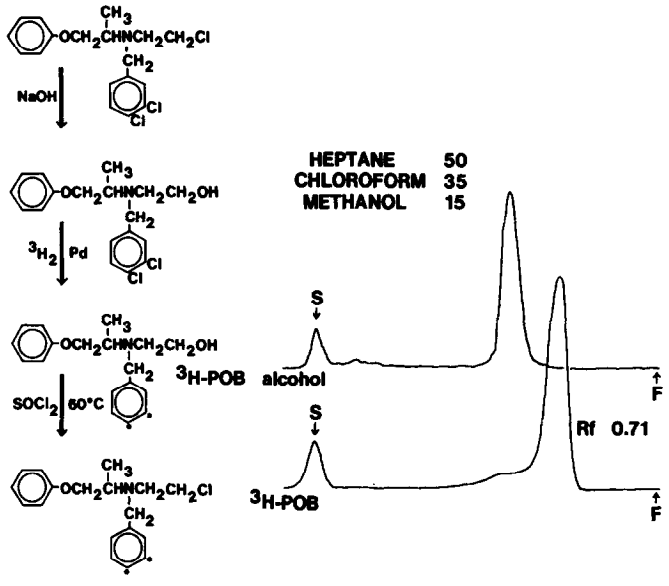

FIG. 1 Synthesis of [^{3}H]POB. Asterisks on benzyl rings indicate the position of tritium. Note the different position of the peak on the radioscan of the thin layer chromatogram of [^{3}H]POB alcohol and [^{3}H]POB. For further explanations see Methods section.

presented 40-55% of total binding at 1 nM [^{3}H]POB, and this decreased gradually with increasing [^{3}H]POB concentrations to 5-10% at 100 nM. The actual cpm values for total and non-specific binding and the % suppressions in a representative experiment are shown in the legend of Fig. 2.

Phosphorylase assay: The protocol for experiments where phosphorylase a activity was measured is detailed in the legend of Fig. 4. Phosphorylase a activity was measured by the incorporation of ^{14}C glucose-1-P into glycogen, as described in detail elsewhere (9).

RESULTS

The phentolamine suppressible 'specific' binding of [^{3}H]POB was linear with cell concentration up to 2 mg cells/ml. At higher cell concentrations uptake of [^{3}H]POB by cells resulted in a rapid and substantial reduction in free ligand concentration, which reduced specific binding. Therefore, binding of [^{3}H]POB under different conditions was tested at a cell concentration of 1 mg wet wt/ml buffer. Specific binding of [^{3}H]POB was irreversible: there was no difference in specific binding between cells precipitated immediately after incubation with [^{3}H]POB and cells resuspended in fresh buffer for 30 min following incubation and before precipitation with TCA. Varying the time of incubation between 2 and 25 min indicated that

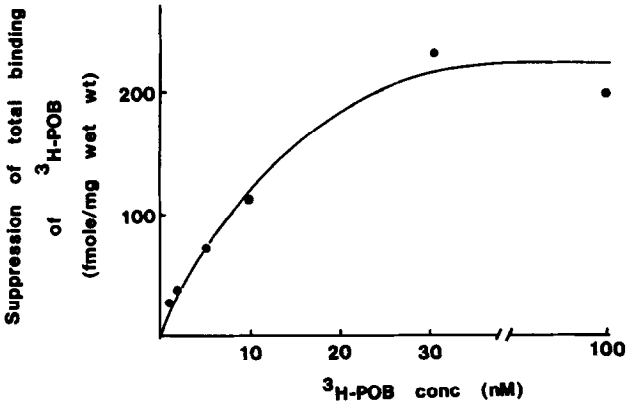

FIG. 2 Saturable binding of [^{3}H]POB to intact rat hepatocytes. Points represent means of triplicate determinations from a representative experiment. Each assay tube contained 1 mg wet weight of cells. Total and non-specific binding (mean cpm ± SE) and percent suppression at various concentrations of [^{3}H]POB were as follows: 1 nM: 348 ± 42, 171 ± 2, 51%; 2 nM: 512 ± 31, 294 ± 8, 44%; 5 nM: 1187 ± 26, 739 ± 14, 38%; 10 nM: 2309 ± 82, 1638 ± 64, 29%; 30 nM: 5558 ± 166, 4210 ± 155, 24%; 100 nM: 15222 ± 207, 14131 ± 448, 7%. Similar results were obtained in three separate experiments.

specific binding reached a maximum at 8 min and started to decline after 16 min. The eventual decline in specific binding is as expected, since the non-equilibrium antagonist will gradually displace the competitive ligand from the receptor site.

Specific binding was saturable (Fig. 2). Half-maximal binding occurred at 10 nM and the amount bound at saturation was approximately 200 fmol/mg wet cell wt. Figure 3 illustrates the effects of various competing ligands on the binding of 3 nM [^{3}H]POB. Phentolamine suppressed binding with an IC50 of 20 nM, whereas the β-receptor antagonist propranolol, the muscarinic antagonist atropine, the serotonin antagonist methysergide and the antihistamine pyrilamine were at least 3 orders of magnitude less potent (A). The potency order of agonists was l-adrenaline > l-noradrenaline > l-phenylephrine > l-isoproterenol, as expected for α-adrenoceptors. Maximal suppression by antagonists was consistently higher than maximal stereoselective suppression by agonists. The reason for this difference is not clear, although a more rapid displacement of agonists than antagonists by POB from the receptor may be a factor. A similar difference is evidence in a recent study of [^{3}H]POB

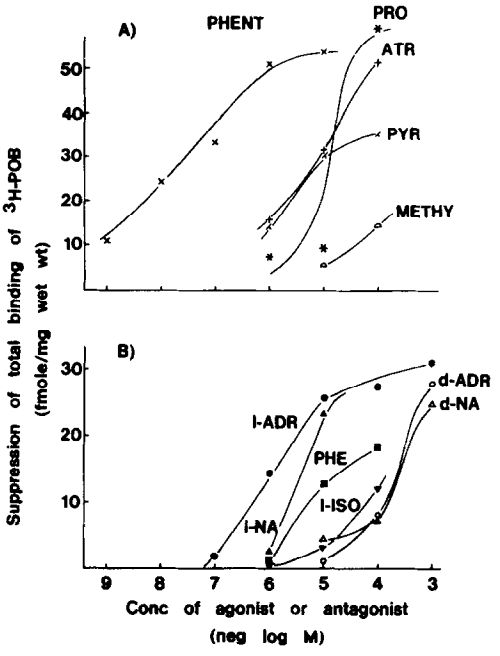

FIG. 3 Inhibition of [^{3}H]POB binding by receptor antagonists (A) and agonists (B). The concentration of [^{3}H]POB in the assays was 3 nM, and [^{3}H]POB was added to the cells 6 min after the addition of the competing ligand. Points represent the mean of triplicate determinations from 2 to 6 separate experiments. The competing ligands were phentolamine (x), dl-propranolol (*), atropine (+), pyrilamine (⊿), methysergide (⏢), l-adrenaline (●), l-noradrenaline (▲), l-phenylephrine (■), l-isoproterenol (▼), d-adrenaline (○), and d-noradrenaline (△).

binding to purified liver membranes (10), which indicates that it cannot be accounted for by differential uptake or metabolism of ligands by the intact liver cells.

Figure 4 illustrates the correlation between the pharmacological blocking effect and the binding of POB. Cells were preincubated with various concentrations of unlabelled POB. After resuspension in fresh buffer, the binding of 3 nM [^{3}H]POB and the phosphorylase activating effect of adrenaline were tested in the control and POB pretreated samples. POB pretreatment caused a concentration-dependent decrease in both binding and the maximal phosphorylase response to adrenaline. The IC50 of POB for the two effects was similar (9 and 2 nM, respectively). The low concentration of POB required to block α-receptors in the present experiments is similar to that required in many other tissues (3), but apparently contradicts findings where

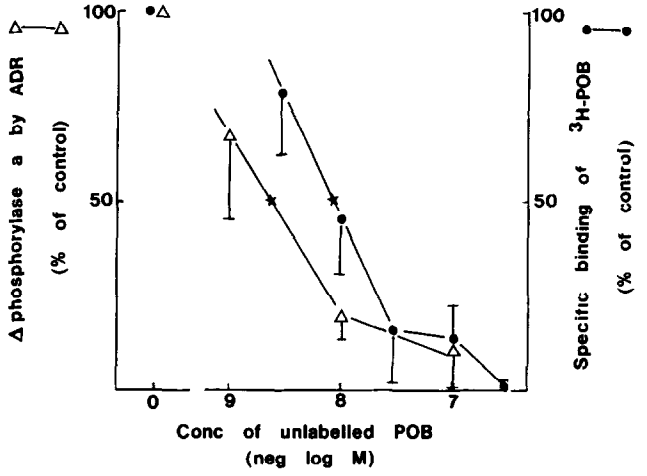

FIG. 4 The effect of preincubation of hepatocytes with unlabelled phenoxybenzamine on the specific binding of [^{3}H]POB and on the activation of phosphorylase by adrenaline. 200 ml aliquots of cell suspension (1 mg cell/ml) were preincubated with or without unlabelled POB for 30 min (37°C, 5% CO_2 in O_2). The cells were then centrifuged (75 g, 1 min) and resuspended in fresh buffer to test [^{3}H]POB binding (1 mg cell/ml), or phosphorylase *a* activity (40 mg cell/ml). The binding of 3 nM [^{3}H]POB in the presence or absence of 10^{-5}M phentolamine was assayed as described in Methods. Specific binding in the POB pretreated samples is expressed as % of binding in the control sample not incubated with POB. For assay of phosphorylase *a*, 1 ml aliquots of the resuspended cells were again incubated for 30 min (37°C, 5% CO_2 in O_2). In each pretreatment group, one aliquot served as control, and 3 other aliquots received 10^{-7}, 10^{-6}, 10^{-5}M l-adrenaline, respectively. Three min after the addition of the adrenaline, the cells were rapidly centrifuged (2,000 g, 30 s), the supernatant aspirated, and the pellet homogenized in 1 ml of ice-cold assay buffer (9). Phosphorylase *a* in the supernatant was assayed in duplicate, as detailed elsewhere (9). Maximal adrenaline-induced increase of enzyme activity in the POB-pretreated cells is expressed as % of the maximal increase in the control sample not exposed to POB. Means and their standard errors from three separate experiments are shown. Asterisks indicate the concentration of unlabelled POB producing half-maximal suppression of binding or phosphorylase activation.

unusually high concentrations of α-receptor antagonists were needed to block metabolic responses of the liver (11,12,13). This difference could be due to the fact that in earlier studies liver slices or high concentrations of liver cells (ca. 40 mg per ml) were used. The high capacity of these cells to take up antagonists from the medium results in a reduced concentration of the antagonist at the receptor site (11). When we preincubated unlabelled POB with aliquots containing a high concentration of cells (40 mg/ml) and then measured [^{3}H]POB binding and phosphorylase activation by adrenaline, much higher concentrations of POB were required to block both effects (400 and 1,000 nM, respectively) than after incubation of POB with a low cell concentration.

DISCUSSION

The present report is the first demonstration of affinity labelling of α-adrenoceptors in intact liver cells. Selectivity of labelling was made possible by the use of [^{3}H]POB with high specific activity which allowed detection of significant specific binding at nanomolar concentrations of the labelling drug. At this low concentration, [^{3}H]POB does not label other receptors that it is known to block at higher concentrations in other tissues (3). We are unaware of any other procedure which allows the labelling of α-adrenoceptors in intact liver cells, a preparation in which a physiological response can be measured and compared with binding data obtained under identical conditions. Such a comparison showed that the potency of unlabelled POB to suppress [^{3}H]POB binding and to block adrenaline-induced phosphorylase activation is similar, suggesting that [^{3}H]POB binding is to the α-adrenoceptor.

The relative potencies and stereoselectivity of agonists were as expected for the α-adrenoceptor; however, the absolute potency of agonists in suppressing [^{3}H]POB binding is lower than their potency in producing various physiological responses. l-Adrenaline has been the most extensively used agonist in studies of metabolic responses of rat liver cells, and its EC50 ranges from 20 to 200 nM (9, 14-18). This is 10-100 times lower than the IC50 of adrenaline for suppressing [^{3}H]POB binding in the present experiments (2 μM). Guellaen and Hanoune have recently found that [^{3}H]POB binding sites in purified liver cell membranes also had low affinity for agonists (10). El Refai *et al.* have identified two classes of α-adrenoceptor sites in rat liver membranes (19): one class of sites has high affinity for agonists and is preferentially labelled by [^{3}H] catecholamines, whereas the second class of sites, preferentially labelled by [^{3}H]DHEC, has low affinity for agonists but a high affinity for antagonists. Agonist and antagonist α-adrenoceptor sites with properties similar to those in liver membranes have also been identified in rat brain tissue (20). The results of El Refai *et al.* (19) indicate that

the sites with high affinity for agonists probably represent the physiological α-adrenoceptor in the liver. The physiological role of the sites preferentially labelled with [^{3}H]DHEC could not be identified, although several possibilities, including a relationship to functional α-adrenoceptors, have been suggested (19). While the sites labelled with [^{3}H]POB in the present study resemble those identified with [^{3}H]DHEC in liver membranes (1,2,19), we find that these sites do influence the α-receptor mediated response. Testing the functional relevance of binding sites was made possible by the measurement of binding and block of the response in the same, intact cell preparation. The observed good correlation between the absolute potency of POB for these two parameters (fig. 4) indicates that the binding sites are involved in blocking adrenaline-induced phosphorylase activation. The irreversible nature of binding facilitates solubilization and characterization of the binding site (21). However, as discussed above, the relationship of these [^{3}H]POB binding sites to sites which recognize physiological, low concentrations of catecholamines is unclear and its elucidation requires further study.

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