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PERSISTENT β-ADRENOCEPTOR BLOCKADE WITH ALKYLATING PINDOLOL (BIM) IN GUINEA-PIG LEFT ATRIA AND TRACHEA

PETER MOLENAAR,* FRASER RUSSELL,* JOSEPH PITHA† and ROGER SUMMERS*‡

*Department of Pharmacology, University of Melbourne, Parkville, Victoria, 3052, Australia, and †National Institute on Aging/GRC, National Institutes of Health, Francis Scott Key Medical Center, Baltimore, MD 21224, U.S.A.

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Abstract—The actions of alkylating pindolol (N^8-bromoacetyl-N^1-3′-(4-indolyloxy)-2′-hydroxypropyl-[*z*]-1,8-diamino-*p*-menthane; BIM) have been examined on β-adrenoceptors in guinea-pig left atria and trachea. In organ bath experiments, addition of BIM (≥0.1 μM), followed by washout, produced concentration-dependent rightward shifts of the dose–response curve to cumulative additions of (−)-isoprenaline and oxymethylene–isoprenaline and reductions in the maximal response. The "apparent" pA_2 value for BIM was 9.23 ± 0.20 (slope = 0.98 ± 0.20). Changes in the maximal density of β-adrenoceptor binding sites were determined in guinea-pig left atrial membranes using [^{125}I]-cyanopindolol. BIM (0.1, 1.0 and 10 μM) produced 14, 23 and 41% reductions in B_{max} with no change in K_D. The binding of [^{125}I]-BIM to guinea-pig left atrial membranes had a high non-specific binding component and a pseudo-Hill coefficient less than unity. The "apparent" K_D value of [^{125}I]-BIM at β-adrenoceptor binding sites was 85.7 ± 57.9 pM.

Compounds which combine irreversibly with receptors are important pharmacological tools with wide potential applications. The first compound to be developed and characterized was dibenamine [1–5] which alkylates α-adrenoceptors and muscarinic, 5-HT and histamine receptors and this compound and phenoxybenzamine have been used to calculate dissociation constants for agonists and study the receptor reserve in tissues. The development of compounds which combine irreversibly with the β-adrenoceptor has been less successful. NHNP–NBE [6–8], chloropractolol [9] and Ro 03-7894 [10–12] although initially thought to have appropriate properties have since been shown to be unsuitable [13–17]. Photo-affinity labels have the desirable feature of high affinity and specificity for β-adrenoceptors [18–20], but the yield of covalent photo-incorporation is low. A more successful approach is the incorporation of the highly reactive bromoacetyl group into an antagonist with high affinity for the β-adrenoceptor using a suitable linker. The prototype compound was bromoacetylalprenololmenthane (BAAM) [21–24]. Since then derivatives of pindolol, cyanopindolol [25–27] and benzylcarazolol [28] have been synthesized with this reactive moiety. Bromoacetylalprenololmenthane can be tritiated but the latter compounds can be radio-iodinated. In this paper we describe the properties of BIM, a derivative of pindolol with a bromoacetyl residue and 1,8-diamino-*p*-menthane linker in organ bath and receptor binding studies (Fig. 1).

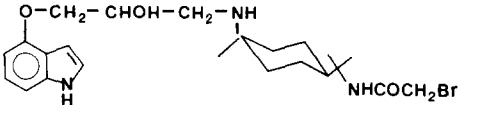

Fig. 1. Structure of alkylating pindolol (BIM).

MATERIALS AND METHODS

Organ bath studies. Left atria and trachea from guinea pigs pre-treated with reserpine (1 mg/kg i.p. 18 hr before experiment) were suspended under 0.5 *g* tension in an organ bath containing Krebs solution (NaCl 118.5, KCl 4.7, $CaCl_2$ 1.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, glucose 11.7, NaH_2PO_4 1.2, EDTA 0.1 and ascorbic acid 0.1 mmol/l) aerated with 5% CO_2 in O_2 and maintained at 37°. Changes in isometric tension were recorded using a Grass FTO3C transducer coupled to a Grass 7P1 pre-amplifier. Left atria were driven at a frequency of 2.5 Hz with square wave pulses of 1 msec duration at 1.5 times the threshold voltage using a Grass S6 stimulator. Tone in tracheal preparations was produced with carbachol (0.5 μM). Interference from α-adrenoceptor mediated effects, neuronal and extraneuronal uptake mechanisms was prevented by pretreatment with phenoxybenzamine (50 μM) [29] while left atria in which histamine was used as an agonist and tracheal preparations were bathed in a Krebs solution containing cocaine (10 μM), corticosterone (100 μM) and phentolamine (10 μM).

In each tissue, three control cumulative concentration–response curves were established to

‡ To whom correspondence should be addressed.

either (−)-isoprenaline, oxymethylene–isoprenaline, calcium or histamine at 30 min intervals. In tissues where the effects of oxymethylene–isoprenaline were monitored, two concentration–response curves to isoprenaline were established before a control curve to oxymethylene–isoprenaline. Tissues were then exposed to one concentration of BIM (0.1, 1 or 10 μM) for 90 min followed by six washes in 30 min before a curve to an agonist was established.

An estimate of the "apparent" affinity of BIM at β-adrenoceptors was made by assessing the rightward shift of cumulative concentration–response curves to (−)-isoprenaline in the absence and presence of BIM. Left atria were incubated with one concentration of BIM for 90 min before another agonist concentration–response curve was established. This procedure was repeated after a 100-fold higher concentration of BIM. Dose-ratios (DR) for half-maximal responses to (−)-isoprenaline in the absence and presence of BIM were then calculated. Linear regression analysis [30] of the plot of log(DR-1) vs log molar concentration of antagonist [A] [31] was performed to calculate the apparent pA_2 value for BIM.

Changes in tension (g) were measured above resting tension for a series of concentrations of agonist. Concentration–response curves were then plotted as a percentage of the maximum response to agonist determined immediately prior to test. Time controls were performed following an identical protocol but omitting BIM. Corrections were not required for (−)-isoprenaline, oxymethylene–isoprenaline or Ca^{2+} but were applied for histamine.

Binding studies. Left atrial tissues used in control organ bath experiments and those exposed to (−)-isoprenaline or oxymethylene–isoprenaline were washed for 30 min, rapidly frozen in isopentane previously cooled in liquid N_2, and stored at −190° before use.

In other experiments, left atrial tissues from reserpinized guinea pigs were exposed to phenoxybenzamine (50 μM) as described above. One concentration of BIM was added to the organ bath for 90 min followed by six changes of the incubation medium in 30 min. Tissues were then frozen and stored before use.

(−)[^{125}I]-cyanopindolol (CYP) was used to determine the proportion of β-adrenoceptor binding sites irreversibly blocked by BIM in homogenates of guinea-pig left atria using the procedure described previously [32].

A similar procedure was used with [^{125}I]-BIM except for a 20-min incubation period instead of 70 min to reduce non-specific binding.

Data was analysed using EBDA to perform preliminary Scatchard, Hill and Hofstee analyses and create a file for LIGAND [33] which was used to obtain final parameter estimates.

Radio-iodination of (−)-cyanopindolol and BIM. Na[^{125}I] was used to radio-iodinate (−)-CYP and BIM as described previously [34].

Drugs used. The drugs used were: alkylating pindolol (BIM) N^8-(bromoacetyl)-N^1-(3′-(4-indolyloxy)-2-hydroxy-propyl)-(*z*)-1,8-diamino-*p*-menthane; (−)-isoprenaline bitartrate (Sterling-Winthrop, Ermington, Australia); oxymethylene–isoprenaline ((±)-(1-isopropylamino-3-(3,4-dihydroxyphenoxy)-2-propanol) oxalate) (gift from Dr. E. Malta); carbachol (Sigma, St Louis, MO); (−)-propranolol (Imperial Chemical Industries, Macclesfield, U.K.); Na^{125}I (Amersham International, Bucks, U.K.); (−)-cyanopindolol (Sandoz, Basle); (−)-[^{125}I]-CYP was prepared from (−)-CYP and Na ^{125}I as previously described [34]; guanosine triphosphate (Boehringer Mannheim, F.R.G.); phenoxybenzamine hydrochloride (Smith Kline and French, Philadelphia, PA); reserpine (Serpasil®, Ciba-Geigy, Pendle Hill, Australia).

Stock solutions (20 mM) of BIM were prepared in methanol which show no detectable decomposition after 3 months (Pitha unpublished observations), (10 mM) (−)-isoprenaline and oxymethylene–isoprenaline in 0.01 M HCl and the remaining drugs in

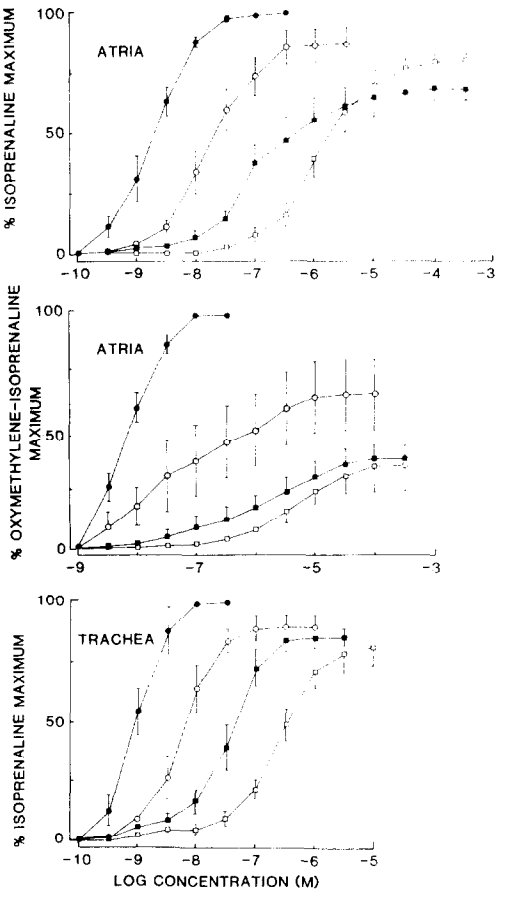

Fig. 2. The effects of BIM on responses in guinea-pig atria and trachea. Mean cumulative concentration–response curves for the positive inotropic responses to (−)-isoprenaline and oxymethylene–isoprenaline are shown in electrically driven guinea-pig left atria and relaxant responses to (−)-isoprenaline in carbachol (0.5 μM) contracted guinea-pig trachea in the absence (●) or presence of 0.1 (○), 1.0 (■) or 10 μM (□) BIM. Responses to each agonist are expressed as a percentage of the maximum response obtained immediately prior to addition of BIM. Oxymethylene–isoprenaline was a partial agonist and produced 77 ± 16% (N = 15) of the maximal response to (−)-isoprenaline. Points show mean values ± SE from three to five individual experiments.

distilled water. Dilutions were made using Krebs buffer containing 1 mM ascorbic acid. All other chemicals were of analytical grade.

RESULTS

Organ bath studies

(−)-Isoprenaline and oxymethylene–isoprenaline increased the force of contraction of electrically driven guinea-pig left atria. Oxymethylene–isoprenaline was a partial agonist which produced 77% of the maximal response produced by (−)-isoprenaline (SE = 16%, N = 15). These responses are mediated predominantly through β_1-adrenoceptors [29]. BIM had no intrinsic activity in left atria at concentrations up to 10 μM.

BIM (0.1, 1.0, 10 μM) produced concentration-dependent rightward shifts of cumulative concentration–response curves to (−)-isoprenaline in left atria and reductions in the maximal response (Fig. 2). These effects were only evident at concentrations of 0.1 μM or higher. Greater shifts to the right and reductions in the maximal response were produced on the positive inotropic effects of the partial agonist oxymethylene isoprenaline than those to (−)-isoprenaline (Fig. 2). Table 1 shows pD_2 values for (−)-isoprenaline and oxymethylene–isoprenaline, and the effects of BIM on the rightward shifts and depressions of the maximal response to the agonists.

The effect of BIM on β_1- and β_2-adrenoceptors was tested on (−)-isoprenaline induced relaxation of carbachol (0.5 μM) contracted guinea-pig trachea. BIM (0.1, 1.0, 10 μM) had no statistical effect on contractions produced by carbachol (1.68 ± 0.51 g, pre-BIM; 1.51 ± 0.39 g, post-BIM, N = 13; $P \geq 0.05$, Student's *t*-test). Relaxation to (−)-isoprenaline in trachea (Fig. 2) is mediated through both β_1- and β_2-adrenoceptors [35, 36]. BIM produced rightward shifts to (−)-isoprenaline and reductions in the maximal response which were less than in atria. Table 1 shows the effects of BIM on pD_2 values for (−)-isoprenaline and quantitates the rightward shifts and reductions in the maximal response.

The effects of BIM were also examined on the positive inotropic responses produced by Ca^{2+} and histamine. Both of these agonists produced positive inotropic responses in guinea-pig isolated left atria (Fig. 3). Responses to histamine were characterized by tachyphylaxis and corrected to compensate for this. BIM at concentrations up to 10 μM was ineffective against responses to either Ca^{2+} or histamine.

Positive inotropic responses to (−)-isoprenaline were examined in atria continually exposed to BIM. The "apparent" affinity of BIM was assessed by calculating a "pA_2" value [31] and was 9.23 ± 0.20 (slope = 0.98 ± 0.20). This is only an "apparent pA_2 value" since BIM reduced the maximal inotropic response to (−)-isoprenaline, indicating non-competitive antagonism [31].

Effects of BIM on [^{125}I]-CYP binding to atrial homogenates

The effects of BIM on the maximal density of β-adrenoceptors determined by [^{125}I]-CYP was examined. Left atria were set up in an organ bath and exposed to phenoxybenzamine 50 μM, washed and incubated with BIM. The K_D for [^{125}I]-CYP binding was similar in tissues whether or not they were exposed to BIM (0.1, 1 and 10 μM) but B_{max} values were reduced 14, 23 and 41%. The pseudo-Hill coefficients for [^{125}I]-CYP in each tissue were not markedly different from unity (Table 2).

The number of free β-adrenoceptors was also examined in left atria used to test responses to

Table 1. Effects of BIM on positive inotropic responses to (−)-isoprenaline and oxymethylene–isoprenaline in electrically driven guinea-pig left atria and on the relaxant effects of (−)-isoprenaline in carbachol (0.5 μM) contracted guinea-pig trachea

BIM concentration (μM)	pD_{2a}	pD_{2b}	DR	*f*	N
(−)-Isoprenaline			Atria		
0.1	8.67 ± 0.10	7.72 ± 0.19	11 ± 4	0.86 ± 0.06	5
1.0	8.57 ± 0.14	6.72 ± 0.38	146 ± 82	0.70 ± 0.06	5
10	8.62 ± 0.08	5.94 ± 0.19	606 ± 247	0.82 ± 0.03	5
Oxymethylene–isoprenaline					
0.1	8.28 ± 0.09	6.95 ± 0.45	66 ± 46	0.66 ± 0.15	4
1.0	8.20 ± 0.07	5.77 ± 0.28	383 ± 144	0.38 ± 0.06	4
10	8.23 ± 0.06	5.37 ± 0.04	734 ± 84	0.35 ± 0.09	3
			Trachea		
(−)-Isoprenaline					
0.1	9.07 ± 0.04	8.35 ± 0.19	6 ± 3	0.90 ± 0.05	4
1.0	9.12 ± 0.09	7.43 ± 0.13	53 ± 21	0.86 ± 0.05	4
10	9.05 ± 0.17	6.62 ± 0.11	300 ± 150	0.82 ± 0.09	5

For each agonist, pD_2 values (negative log EC_{50} for half maximal responses) are given in the absence of (pD_{2a}) and in the presence of 0.1, 1.0 or 10 μM BIM (pD_{2b}). Shifts to the right are indicated by the dose ratio (DR = EC_{50b}/EC_{50a}). Also shown are the reductions in the maximal response expressed as a fraction (*f*) of the response prior to BIM = 1.

Values given are the mean ± SE from N experiments.

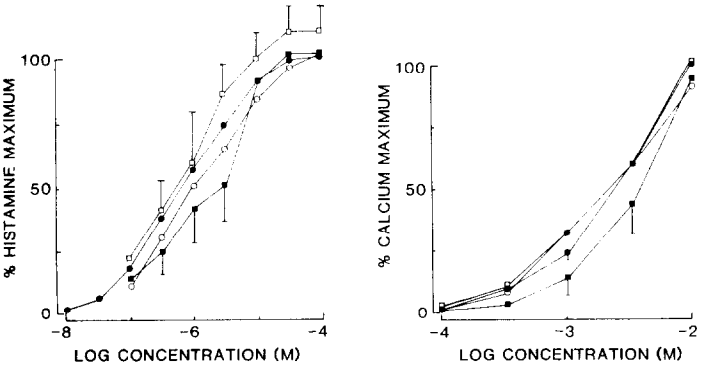

Fig. 3. Specificity of BIM for β-adrenoceptors. Mean cumulative concentration–response curves are shown for the positive inotropic responses to histamine and calcium added to Krebs bathing solution in electrically driven guinea-pig left atria in the absence (●) or presence of 0.1 (○), 1.0 (■) or 10 μM (□) BIM. The response to each agonist is expressed as a percentage of its own maximum response. Responses to both agonists were unaffected by BIM. Points show mean values ± SE from four to five individual experiments.

(−)-isoprenaline and oxymethylene–isoprenaline. In atria exposed to (−)-isoprenaline there was a loss of β-adrenoceptors (no agonist, 712; Iso, 489 fmol/g wet weight). Treatment with BIM (0.1 μM) partly prevented this effect (Iso, 489; Iso + 0.1 μM BIM, 566 fmol/g wet weight). Higher concentrations of BIM reduced β-adrenoceptor number. A similar but less pronounced effect was also observed in atria treated with oxymethylene–isoprenaline and 0.1 μM BIM, but again higher concentrations produced a reduction in sites labelled by [^{125}I]-CYP. Table 2 shows the K_D, nH and B_{max} values for [^{125}I]-CYP binding in left atria treated with BIM and exposed to no agonist, and those exposed to either (−)-isoprenaline or both (−)-isoprenaline and oxymethylene–isoprenaline. Figure 4 shows representative examples of Scatchard plots from each treatment group.

Binding of [^{125}I]-*BIM to atrial homogenates*

Non-specific binding formed a large component of the binding of [^{125}I]-BIM to guinea-pig left atrial membranes. Specific binding of [^{125}I]-BIM ranged from 17 to 38% at 10 pM and 2 to 7% at 1 nM. The pseudo-Hill coefficient was less than unity (0.78 ± 0.07, N = 4) suggesting multiple sites of interaction or a complex interaction with the β-adrenoceptor. The "apparent" K_D for [^{125}I]-BIM (85.7 ± 57.9 pM) was approximately seven times higher than that for cold BIM. The B_{max} was 864 ± 461 fmol/g wet weight tissue. A representative saturation isotherm and Scatchard transformation are shown in Fig. 5.

DISCUSSION

BIM is a product of the incorporation of a 1,8-diamino-*p*-menthane linker between the highly reactive bromoacetyl residue and the high affinity β-adrenoceptor antagonist pindolol [26]. This study examines its ability to irreversibly block β-adrenoceptors.

BIM had high "apparent" affinity for the β-adrenoceptor. Although antagonism of inotropic

Table 2. The effect of BIM on [^{125}I]-CYP binding to guinea-pig left atrial membranes

BIM conc. (μM)	No agonist			(−)-Isoprenaline			Oxymethylene–isoprenaline		
	K_D	nH	B_{max}	K_D	nH	B_{max}	K_D	nH	B_{max}
—	23.5 (6.3)	0.89 (0.13)	712 (72)	26.5 (5.9)	0.92 (0.07)	489 (112)	32.5 (6.7)	0.94 (0.06)	721 (70)
0.1	34.2 (12.6)	0.99 (0.04)	609 (55)	23.5 (5.4)	0.88 (0.09)	566 (99)	59.3 (16.6)	0.94 (0.10)	768 (63)
1.0	25.8 (2.7)	0.87 (0.12)	548 (49)	35.1 (15.7)	0.89 (0.15)	301 (5)	39.8 (12.6)	0.88 (0.08)	387 (47)
10	22.4 (5.4)	0.92 (0.01)	417 (139)	40.0 (8.9)	0.92 (0.01)	321 (66)	45.2 (3.1)	0.97 (0.09)	349 (68)

K_D (pmol/l), pseudo-Hill coefficients and B_{max} values (fmol/g wet weight) are given for [^{125}I]-CYP binding to guinea-pig left atria exposed to no agonist, (−)-isoprenaline or (−)-isoprenaline and oxymethylene-isoprenaline in the absence and presence of 0.1, 1.0 and 10 μM BIM.

Values are mean (±SE) from three to five individual experiments.

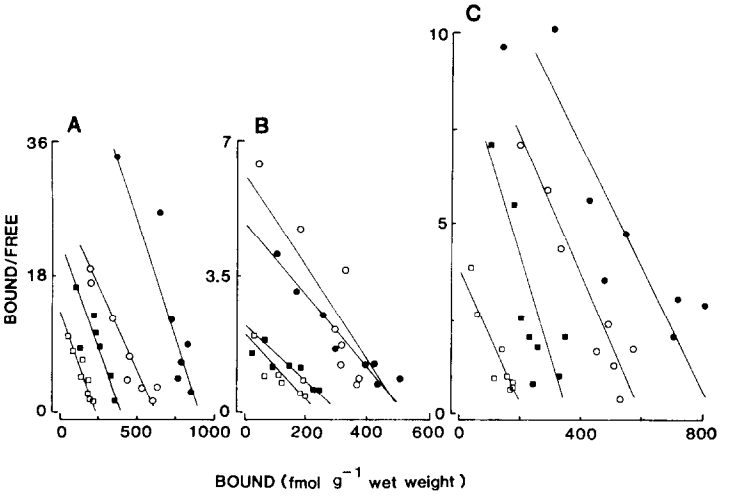

Fig. 4. Effects of BIM on binding of [^{125}I]-CYP to β-adrenoceptors in guinea-pig left atrial membranes. Tissues were exposed to no agonist (A), or four exposures to (−)-isoprenaline (B) or two exposures to (−)-isoprenaline followed by two exposures to oxymethylene–isoprenaline (C). Representative Scatchard plots are shown in the absence (●) of and in the presence of 0.1 (○), 1.0 (■) or 10 μM (□) BIM. Note a concentration dependent reduction in the maximal density of β-adrenoceptor binding sites. The pseudo-Hill coefficients for [^{125}I]-CYP were not different from unity.

responses to (−)-isoprenaline in guinea-pig left atria by BIM was non-competitive an "apparent" pA_2 value (9.23) could be calculated. This value is similar to that determined in competition binding experiments with [^{3}H]dihydroalprenolol in membranes from rat heart [26]. This may, however, represent an overestimation of the true affinity since the dissociation constant is determined by the ratio of the "off" and "on" rate constants ($K_D = K_{off}/K_{on}$) and for compounds which form an irreversible bond with the receptor K_{off} approaches zero and decreases the magnitude of K_D. This was evident in organ bath experiments where it was necessary to expose tissues to BIM at concentrations greater than 0.1 μM to produce rightward shifts in concentration–response curves and reductions in the maximal response to β-adrenoceptor agonists. These experiments demonstrate a persistent blockade of β-adrenoceptors by BIM. These actions were specific for the β-adrenoceptor since the same pretreatment did not affect responses to histamine or calcium. In receptor binding studies, it has been shown that BIM does not compete for [^{3}H]prazosin or [^{3}H]yohimbine binding [26]. In contrast, BIM at concentrations of 0.1, 1.0 and 10 μM produced 14, 23 and 41% reductions in the maximal density of sites labelled by [^{125}I]-CYP. A similar pattern was obtained with the bromoacetamido derivative of betaxolol which had an "apparent" pA_2 value of 7.66 against (−)-isoprenaline in spontaneously beating guinea-pig right atria, yet required a concentration of 10 μM to reduce the maximal response to isoprenaline by 19% [37]. The effect of this concentration on B_{max} was not determined in this tissue but in rat cortex it produced

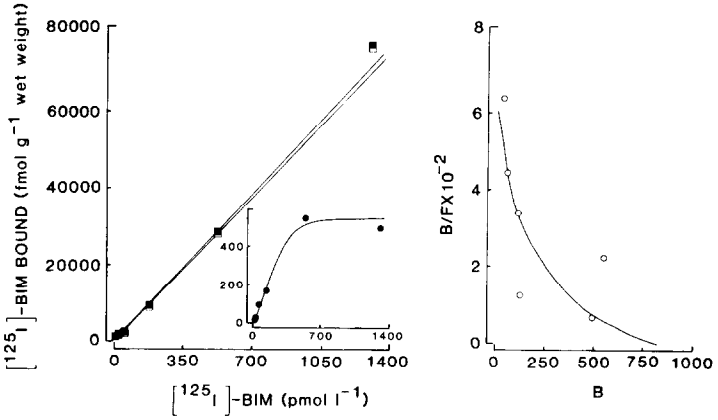

Fig. 5. Binding of [^{125}I]-BIM (10–1300 pM) to guinea-pig left atrial membranes. Shown are total (■), non-specific (□), and specific binding (●) (inset) and the corresponding Scatchard transformation of the binding data showing specifically bound [^{125}I]-BIM (B) vs the ratio of bound/free (B/F × 10^{-2}). In this representative experiment the K_D for [^{125}I]-BIM was 184 pM and B_{max} 861 fmol/g wet weight tissue.

a 60–70% reduction in binding sites. In order to reduce the maximal response to (−)-isoprenaline in guinea-pig atria it is necessary to inactivate a large fraction of the β-adrenoceptor pool as these tissues have a large receptor reserve. (−)-Isoprenaline can produce 50% of its maximal inotropic effect in guinea-pig left atria by occupying less than 5% of the total population of β-adrenoceptors [32]. This effect may be further enhanced by recycling of receptors from previously inaccessible intracellular locations after washout of the inactivating agent [24]. It is interesting to note that the potency of BIM in reducing isoprenaline stimulated adenylate cyclase activity in rat heart homogenates is greater (K_i = 5 nM for 50% reduction) [26] probably due to the close coupling between β-adrenoceptors and adenylate cyclase [38, 39].

The explanation for the relative ineffectiveness of BIM in guinea-pig atria based on the presence of a large receptor reserve would be strengthened if the drug is more effective at blocking responses to partial agonists which by definition occupy a larger fraction of the receptors to produce a response than full agonists. The effects of BIM were compared on responses to a full agonist (−)-isoprenaline and a partial agonist oxymethylene–isoprenaline [40]. Equal concentrations of BIM produced greater shifts to the right and reductions in maximal responses to oxymethylene-isoprenaline than it did to (−)-isoprenaline. However, the shape of the cumulative concentration–response curve to oxymethylene–isoprenaline was not characteristic of a partial agonist in the presence of an irreversible antagonist that inactivates α-, muscarinic or histamine receptors [4, 5, 41, 42]. In these systems, the EC_{50} of the partial agonist is a reflection of the affinity for the receptor and receptor inactivation produces reductions in the maximal response without appreciable rightward shifts. The results of the experiments in this study demonstrate persistent blockade with BIM but also, under the conditions adopted for organ bath experiments, indicate that high concentrations of agonists can compete with BIM for β-adrenoceptors.

Binding experiments with [^{125}I]-BIM were characterized by high non-specific binding and pseudo-Hill coefficients less than unity. However the B_{max} of [^{125}I]-BIM binding was 864 ± 461 fmol/g wet weight which was similar to that observed with [^{125}I]-CYP in control experiments (712 ± 72 fmol/g wet weight). Thus [^{125}I]-BIM labels a similar number of β-adrenoceptors to [^{125}I]-CYP. The "apparent" affinity of [^{125}I]-BIM was similar to that of cold BIM. A shorter incubation time (20 min) was used than for [^{125}I]-CYP (70 min) to reduce non-specific binding. High non-specific binding is also a problem with [^{125}I]-*para* - (bromoacetamidyl)benzylcarazolol in membrane preparations but not in purified receptor preparations and it was suggested that this compound forms covalent bonds with a number of membrane proteins unrelated to the receptor [28]. An alternative suggestion is that bromoacetylamino derivatives of β-adrenoceptor antagonists may combine with a lipid molecule closely associated with the β-adrenoceptor [43].

It has been demonstrated that BIM combines with the β-adrenoceptor in guinea-pig atria and trachea and produces persistent receptor blockade. The compound will be useful in studies designed to examine the properties of the β-adrenoceptor. However, the usefulness of [^{125}I]-BIM is limited by high non-specific binding and further work in this area is necessary.

REFERENCES

1. Harvey SC and Nickerson M, The chemical transformations of dibenamine and dibenzyline and biological activity. *J Pharmacol Exp Ther* **109**: 328–339, 1953.
2. Furchgott RF, Dibenamine blockade in strips of rabbit aorta and its use in differentiating receptors. *J Pharmacol Exp Ther* **111**: 265–284, 1954.
3. Furchgott RF, The pharmacology of vascular smooth muscle. *Pharmacol Rev* **7**: 183–265, 1955.
4. Furchgott RF, The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor–agonist complexes. In: *Advances in Drug Research* (Eds. Harper NJ and Simmonds AB), pp. 21–55. Academic Press, London, 1966.
5. Furchgott RF and Bursztyn P, Comparison of dissociation constants and of relative efficacies of selective agonists acting on parasympathetic receptors. *Ann NY Acad Sci* **144**: 882–899, 1967.
6. Atlas D and Levitzki A, An irreversible blocker for the β-adrenoceptor. *Biochem Biophys Res Commun* **69**: 397–403, 1976.
7. Atlas D, Steer ML and Levitzki A, Affinity label for the β-adrenoceptor in turkey erythrocytes. *Proc Natl Acad Sci USA* **73**: 1921–1925, 1979.
8. Venter JC, High efficiency coupling between beta-adrenergic receptors and cardiac contractility: direct evidence for "spare" beta-adrenergic receptors. *Mol Pharmacol* **16**: 429–440, 1979.
9. Erez N, Weinstock M, Cohen F and Shtacher G, Potential probe for the isolation of the β-adrenoceptor, chloropractolol. *Nature (Lond)* **255**: 635–636, 1975.
10. Nicholson CD and Broadley KJ, Irreversible β-adrenoceptor blockade of atrial rate and tension responses. *Eur J Pharmacol* **52**: 259–269, 1978.
11. Broadley KJ and Nicholson CD, Dissociation constants of isoprenaline and orciprenaline and their relative efficacies on guinea-pig isolated atria determined by the use of an irreversible β-adrenoceptor antagonist. *Br J Pharmacol* **72**: 635–643, 1981.
12. Rankin A and Broadley KJ, Comparison of the apparent irreversible β-adrenoceptor antagonist Ro 03-7894 with propranolol in cardiac ventricular muscle by pharmacological and radioligand binding techniques. *Biochem Pharmacol* **31**: 1325–1332, 1982.
13. Kenakin TP and Black JW, Can chloropractolol alkylate β-adrenoceptors? *Nature (Lond)* **265**: 365–366, 1977.
14. Kenakin TP and Black JW, The pharmacological classification of practolol and chloropractolol. *Mol Pharmacol* **14**: 607–623, 1978.
15. Minneman KP and Mowry CB, Interactions of putative irreversible antagonists with β_1- and β_2-adrenergic receptors. *Biochem Pharmacol* **35**: 857–864, 1986.
16. Baker SP and Posner P, The effect of the beta-adrenoceptor antagonist Ro 03-7894 on rat atrial tension development and (−)-[^{3}H]dihydroalprenolol binding to cardiac and lung membranes. *Life Sci* **33**: 459–466, 1983.
17. Krstew E, McPherson GA, Malta E, Molenaar P and Raper C, Is Ro 03-7894 an irreversible antagonist at β-adrenoceptor sites? *Br J Pharmacol* **82**: 501–508, 1984.

18. Lavin TM, Heald SL, Jeffs PW, Schorr RG, Lefkowitz RJ and Caron MG, Photoaffinity labelling of the β-adrenoceptor. *J Biol Chem* **256**: 11944–11950, 1982.
19. Rashidbaigi A and Ruoho AE, Iodoazidobenzylpindolol a photoaffinity probe for the β-adrenergic receptor. *Proc Natl Acad Sci USA* **78**: 1609–1613, 1981.
20. Burgermeister W, Hekman M and Helmreich EJM, Photoaffinity labeling of the β-adrenergic receptor with azide derivatives of iodocyanopindolol. *J Biol Chem* **257**: 5306–5311, 1982.
21. Pitha J, Zjawiony J, Nasrin N, Lefkowitz RJ and Caron MG, Potent beta-adrenergic antagonist possessing chemically reactive group. *Life Sci* **27**: 1791–1798, 1980.
22. Pitha J, Hughes BA, Kusiak JW, Dax EM and Baker SP, Regeneration of β-adrenergic receptors in senescent rats: a study using an irreversible binding antagonist. *Proc Natl Acad Sci USA* **79**: 4424–4427, 1982.
23. Baker SP and Pitha J, Irreversible blockade of beta-adrenoceptors and their recovery in the rat heart and lung *in vivo*. *J Pharmacol Exp Ther* **220**: 247–251, 1982.
24. Kusiak JW and Pitha J, Inhibition of cardiac adenylate cyclase by an irreversible beta-adrenergic blocker. *Biochem Pharmacol* **33**: 483–485, 1984.
25. Homburger V, Gozlan H, Bouhelal R, Lucas M and Bockaert J, Irreversible blockade of β-adrenergic receptors with a bromoacetyl derivative of pindolol. *Naunyn Schmiedebergs Arch Pharmacol* **328**: 279–287, 1985.
26. Pitha J, Buchowiecki W, Milecki J and Kusiak JW, Affinity labels for β-adrenoceptors: preparation and properties of alkylating β-blockers derived from indole. *J Med Chem* **30**: 612–615, 1987.
27. Kusiak JW and Pitha J, A bromoacetylated derivative of cyanopindolol: an irreversible antagonist at rat beta-adrenoceptors. *Life Sci* **41**: 15–23, 1987.
28. Dickinson KEJ, Heald SL, Jeffs PW, Lefkowitz RJ and Caron MG, Covalent labeling of the β-adrenergic ligand binding site with para-(bromoacetamidyl)benzyl carazolol. A highly potent β-adrenergic affinity label. *Mol Pharmacol* **27**: 499–506, 1985.
29. Molenaar P and Summers RJ, Characterization of β_1- and β_2-adrenoceptors in guinea-pig atrium: functional and receptor binding studies. *J Pharmacol Exp Ther* **241**: 1041–1047, 1987.
30. Tallarida RJ and Murray RB, *Manual of Pharmacological Calculations with Computer Programs*. Springer, New York, 1981.
31. Arunlakshana I and Schild HO, Some quantitative uses of drug antagonists. *Br J Pharmacol Chemother* **14**: 48–58, 1959.
32. McPherson GA, Malta E, Molenaar P and Raper C, The affinity and efficacy of the selective β_1-adrenoceptor stimulant RO363 at β_1- and β_2-adrenoceptor sites. *Br J Pharmacol* **82**: 897–904, 1984.
33. McPherson GA, *KINETIC, EBDA, LIGAND and LOWRY: A Collection of Computer Programs for the Analysis of Radioligand Binding Experiments*. Elsevier, London, 1986.
34. Lew R and Summers RJ, Autoradiographic localization of β-adrenoceptor subtypes in guinea-pig kidney. *Br J Pharmacol* **85**: 341–348, 1985.
35. Furchgott RF, Post-synaptic adrenergic receptor mechanisms in vascular smooth muscle. In: *Vascular Neuroeffector Mechanisms* (Ed. Bevan JA), pp. 131–142. Karger, Basel, 1976.
36. O'Donnell SR and Wanstall JC, The importance of choice of agonists in studies designed to predict β_1:β_2-adrenoceptor selectivity of antagonists from pA_2 values on guinea-pig trachea and atria. *Naunyn Schmiedebergs Arch Pharmacol* **308**: 183–190, 1979.
37. Amlaiky N and Leclerc G, Synthesis and irreversible β-adrenergic blockade with a bromoacetamido derivative of betaxolol. *J Pharm Sci* **74**: 1117–1119, 1985.
38. Liang BT and Molinoff PB, Beta-adrenergic subtypes in the atria: evidence for close coupling of beta-1 and beta-2 adrenergic receptors to adenylate cyclase. *J Pharmacol Exp Ther* **238**: 886–892, 1986.
39. Waelbroeck M, Taton G, Delhaye M, Chatelain P, Camus JC, Pochet R, Leclerc JL, DeSmet JM, Robberecht P and Christophe J, The human heart beta-adrenergic receptors—II. Coupling of $beta_2$-adrenergic receptors with the adenylate cyclase system. *Mol Pharmacol* **24**: 174–182, 1983.
40. Molenaar P, McPherson GA, Malta E and Raper C, The influence of molecular structure on the affinity and efficacy of some β-adrenoceptor agonists. *Naunyn Schmiedebergs Arch Pharmacol* **331**: 240–246, 1985.
41. Ruffolo RR, Jr, Important concepts of theory. *J Auton Pharmacol* **2**: 277–295, 1982.
42. Furchgott RF, The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In: *Handbook of Experimental Pharmacology* (Eds. Blashko H and Muscholl E), pp. 283–335. Springer, Berlin, 1972.
43. Chorev M, Feigenbaum A, Keenan AK, Gilon C and Levitzki A, *N*-Bromoacetyl-amino-cyanopindolol: a highly potent beta-adrenergic affinity label blocks irreversibly a non-protein component tightly associated with the receptor. *Eur J Biochem* **146**: 9–14, 1985.