AN IRREVERSIBLE BLOCKER FOR THE ß-ADRENERGIC RECEPTOR

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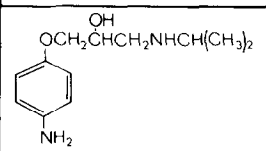
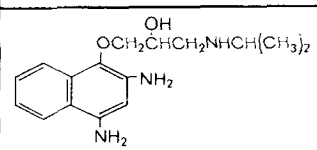
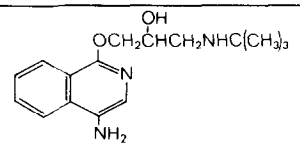
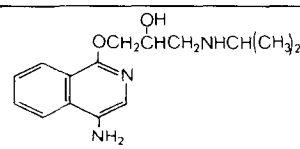
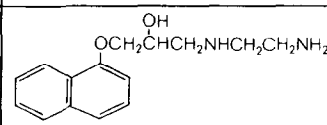
SUMMARY New reversible blockers for the ß-adrenergic receptor have been synthesized. All the compounds possess free amine(s) residues which have been bromoacetylated. The N-bromoacetyl derivatives were also found to be potent ß-blockers. One of these bromoacetyl derivatives: N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl-ethylenediamine is shown to inhibit irreversibly l-epinephrine-dependent adenylate cyclase from turkey erythrocytes, whereas it has no effect on the fluoride-dependent activity of the enzyme. This compound also eliminates the specific [^{3}H]-propranolol binding to the ß-receptors. These findings suggest that the compound N-(2-hydroxy-3-naphthoxy-propyl)-N'-bromoacetyl-ethylenediamine is a potent ß-receptor-directed affinity label.

INTRODUCTION Reversible ß-adrenergic blockers have recently been introduced as probes for monitoring ß-receptors in a number of cell types (1-7). The availability of an irreversible ß-blocker may advance our understanding of the ß-receptor from the structural point of view, and may enable one to design procedures to purify the receptor. An irreversible ß-blocker can also be instrumental in the characterization of the receptor properties in its native membrane.

MATERIALS AND METHODS

α-[^{32}P] ATP and [^{3}H] cAMP were purchased from the Radiochemical Centre, Amersham, England. [^{3}H]-dl-propranolol (4.3 Ci/mmole, over 99% pure) was obtained from the Israel Atomic Energy Commission (Negev). Creatine phosphokinase, creatine phosphate, theophylline, l-epinephrine and dl-propranolol were purchased from Sigma. Protein was determined according to Lowry et al. (8) with bovine serum albumin as the standard. Turkey erythrocyte membranes were prepared according to the method described previously (9). The adenylate cyclase activity was assayed according to the method of Salomon et al. (10). [^{3}H]-propranolol binding was measured as des-

Table I. New Reversible ß-Blockers

n°	structure of free amine analogue	K displacement of ^{3}H-propranolol binding (M)	K activity for adenylate cyclase (M)
I		—	$(8\pm1)\times10^{-6}$
II		$(7.5\pm1)\times10^{-6}$	$(1.5\pm1)\times10^{-6}$
III		$(1\pm1)\times10^{-4}$	—
IV		$(5\pm1)\times10^{-5}$	—
V		$(2\pm1)\times10^{-7}$	$(2.5\pm1)\times10^{-7}$

cribed earlier (1-3). The amine analogues of propranolol (Table I) and their corresponding bromoacetyl derivatives (Table II) were synthesized in our laboratory (to be published). The synthesis of N-(2-hydroxy-3-naphthoxypropyl)-ethylenediamine and N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine were described in detail elsewhere (11).

RESULTS

Reversible ß-blockers. A number of reversible ß-blockers have been synthesized (Table I) and tested for their potency to inhibit l-catecholamine-dependent adenylate cyclase activity as well as for their potency to displace the specifically bound [^{3}H]-propranolol. In both types of experiments the compounds shown in Table I were found to behave as competitive

TABLE II

THE CONCENTRATIONS OF BROMOACETYL DERIVATIVES REQUIRED FOR 50% EFFECT

Bromoacetyl derivative	$D_{0.5}$ (M) for [^{3}H]-propranolol displacement	$D_{0.5}$ (M) for l-epinephrine activity
I	-	$(1.5 \pm 1) \times 10^{-4}$
II	$(3 \pm 1) \times 10^{-4}$	$(2 \pm 2) \times 10^{-4}$
III	$(1.70 \pm 0.5) \times 10^{-4}$	-
IV	$(2 \pm 1) \times 10^{-4}$	-
V	$(2.5 \pm 1) \times 10^{-5}$	$(1.0 \pm 1.0) \times 10^{-5}$

The concentration of [^{3}H]-propranolol in the binding assay was 1.0×10^{-8} M (or 4 times its dissociation constant) and the concentration of l-epinephrine in the adenylate cyclase assay was 2.5×10^{-4} M (or 25 times its dissociation constant).

inhibitors. The dissociation constants for these ß-blockers to the ß-receptor were calculated both from the kinetic studies as described earlier (1-3) and from the displacement of [^{3}H]-propranolol in binding studies, also by procedures developed in our laboratory (1-3). The results summarized in Table I show that the dissociation constants obtained by the two independent methods match closely.

Irreversible ß-blockers. The bromoacetyl derivatives of the compounds shown in Table I were all found to be potent blockers of the l-epinephrine-dependent activity (Table II), but without any effect on the fluoride-stimulated activity of the enzyme. The most effective compound (Table II), N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine, was found to block irreversibly the l-epinephrine-stimulated activity (Figure 1), since extensive washings did not restore the hormone-dependent activity. However, the fluoride-stimulated activity was not affected. Figure 1 also shows that a much higher degree of receptor modification is required to cause a significant reduction in the hormone plus GppNHp activity than those required to inhibit enzyme activity induced by the hormone alone.

GppNHp - guanylylimidodiphosphate.

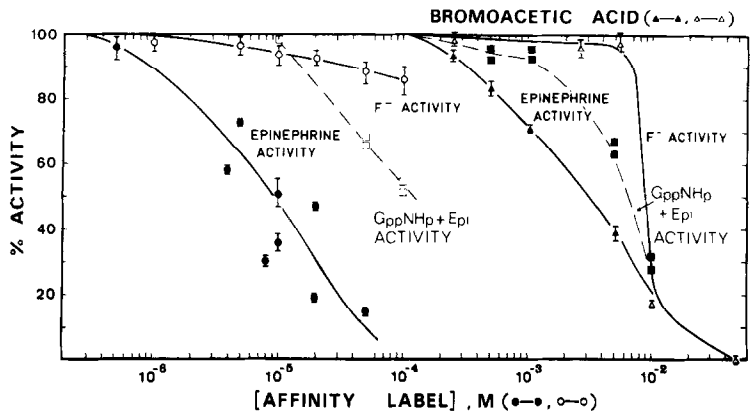


Figure 1. The Irreversible Inhibition of l-Epinephrine-Dependent Adenylate Cyclase Activity by N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine. Membranes were preincubated for 15 min at room temperature (25°) in 0.03 M sodium phosphate buffer pH 7.4 containing 2 mM $MgCl_2$ and 1 mM EDTA, at various concentrations of the affinity label, N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine (compound V, Table II). After incubation with the affinity label, the mixtures were diluted ten-fold in 0.05 M Tris buffer, pH 7.4 containing 2 mM EDTA and 1 mM $MgCl_2$ centrifuged at 23,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in the Tris-EDTA buffer. This washing procedure was repeated four times. Then the pellet was resuspended in the Tris-Mg-EDTA buffer and assayed for adenylate cyclase activity as described in Materials and Methods. In parallel the membranes were treated with bromoacetic acid or with N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl-amine in a similar fashion as described above for the affinity label. The inactivation of adenylate cyclase by N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine and by bromoacetic acid are depicted in the figure. It can be seen that the treatment of the membranes with N-(2-hyxroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine results in a complete and irreversible loss of l-epinephrine dependent activity, whereas the fluoride dependent activity remains essentially intact. When the effect of the affinity label was examined on GppNHp + epinephrine-stimulated activity 10^{-4} M GppNHp was included in the assay (13). The absolute specific activities of the adenylate cyclase were as follows: l-epinephrine, 80 pmoles cAMP/mg/min; fluoride 500 pmoles cAMP/mg/min; l-epinephrine + GppNHp, 1100 pmoles cAMP/mg/min.

The significance of this finding is discussed below.

The compound N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine was also found to inhibit irreversibly [^{3}H]-propranolol bindings. Membranes treated with 10^{-5} M affinity label and washed as described in Figure 1 bind 0.1 ± 0.1 pmole [^{3}H]-propranolol per mg membrane protein as compared to 1.3 ± 0.1 pmole [^{3}H]-propranolol per mg protein in untreated membranes.

The other bromoacetyl derivatives shown in Table II were found to be less effective than N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine by an order of magnitude.

DISCUSSION

The compound N-(2-hydroxy-3-naphthoxypropyl)-N'-bromoacetyl ethylenediamine was found to be a very potent irreversible blocker of the ß-adrenergic receptor in turkey erythrocyte ghosts. The compound blocks irreversibly both the l-epinephrine-dependent adenylate cyclase activity as well as the specific [^{3}H]-propranolol binding to the ß-receptor. Among the compounds tested (Table II) it was found to be the most potent irreversible ß-blocker although the other compounds are also effective (D. Atlas, unpublished). The reaction of the affinity label with the ß-receptor is extremely fast (Atlas and Levitzki, in preparation) and therefore, a detailed kinetic analysis of the labelling reaction must await further experimentation.

Bromoacetyl compounds such as N-bromoacetylnorepinephrine have the same effect as bromoacetic acid (Figure 1) and therefore represent the effect of non-specific alkylating agents. These two non-specific compounds which possess an active bromine, affect the adenylate cyclase system only at much higher concentrations than the ß-receptor-directed bromoacetyl derivative (Figure 1).

The fluoride-stimulated activity is unaffected by the affinity label (Figure 1), confirming the general view that fluoride activates adenylate cyclase by a receptor-independent mechanism (12). The inhibition of GppNHp + hormone activity by the affinity label occurs at a much higher degree of modification than the l-epinephrine-stimulated activity (Figure 1). It can be seen (Figure 1) that when almost complete inactivation of the l-epinephrine-stimulated activity occurs, only partial inactivation of the GppNHp + hormone activity is observed. It has been shown that the role of epinephrine in the synergistic activation of adenylate cyclase by GppNHp and hormone is a catalytic one (13). We have therefore argued (13) that a stoichiometric ratio between the number of hormone receptors and the number of GppNHp regulatory sites is not essential. Our present findings strongly support this view. Current studies in our laboratory,

which involve the new affinity labels, are devoted to three main directions: (a) the synthesis of a radioactive affinity label in the aim to characterize the chemical nature of the ß-receptor; (b) the use of the affinity label as a tool to establish whether ß-adrenergic systems contain "spare receptors" and (c) to help establish the stoichiometry between ß-receptors and the regulatory guanyl nucleotide binding sites.

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