Biochemical Pharmacology, Vol. 35, No. 5, pp. 857–864, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

INTERACTIONS OF PUTATIVELY IRREVERSIBLE ANTAGONISTS WITH β_1- AND β_2-ADRENERGIC RECEPTORS*

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(*Received* 17 *April* 1985; *accepted* 16 *August* 1985)

Abstract—Three compounds that have been suggested to irreversibly inactivate β-adrenergic receptors were studied: NHNPNBE [*N*-(2-hydroxy-3-[1-napthoxy]-propyl)-*N*-bromoacetylethylenediamine], BAAM (bromoacetylalprenololmenthane), and Ro 3-7894 [1-(5-chloracetylaminobenzfuran-2-yl)-2-isopropylaminoethanol]. Membranes of rat cerebral cortex were used as a source of predominantly β_1-adrenergic receptors and membranes of rat cerebellum were used as a source of predominantly β_2-adrenergic receptors. β-Adrenergic receptor binding sites were studied by Scatchard analysis of saturation isotherms of specific [^{125}I]-pindolol ([^{125}I]PIN) binding. NHNPNBE added to the incubation medium competitively inhibited specific [^{125}I]PIN binding in both cerebellum and cerebral cortex with K_I values of 1–2 μM in each tissue. After washout of membranes pretreated with NHNPNBE for 30 min at 37°, no loss of specific [^{125}I]PIN binding sites was observed in either cerebellum or cortex except at very high concentrations (30–100 μM). Ro 3-7894 caused a simple competitive inhibition of specific [^{125}I]PIN binding in rat cerebellar membranes with a K_I of approximately 14 μM, an effect which was reversed completely by washing. In cerebral cortex, Ro 3-7894 added to the incubation medium apparently decreased the density of [^{125}I]PIN binding sites with an IC_{50} around 1 μM. This effect was reversed after washing the membranes twice. However, in the presence of Ro 3-7894 some Scatchard plots showed a slight curvature. Further saturation of the [^{125}I]PIN binding sites in cerebral cortex showed that the inhibition by Ro 3-7894 was competitive but with a high- and low-affinity component, consistent with Ro 3-7894 being a β_1-selective competitive antagonist. Ro 3-7894 was also β_1-selective in other tissues. BAAM added to the incubation medium competitively inhibited specific [^{125}I]PIN binding in both cerebellum and cortex with K_I values of 0.006 to 0.03 μM, but was about 5-fold more potent in cerebellum. After treatment of membranes with higher concentrations of BAAM for 30 min at 37° and washing twice, there was a dose-dependent decrease in the density of specific [^{125}I]PIN binding sites with IC_{50} values of approximately 0.3 μM in both tissues. Similar effects were observed in rat heart. These data suggest that NHNPNBE is a simple competitive antagonist at both β_1- and β_2-adrenergic receptors except at very high concentrations. Ro 3-7894 is a β_1-selective competitive antagonist with no apparent irreversible effects. BAAM is a slightly β_2-selective competitive antagonist which can also irreversibly decrease the density of both β_1- and β_2-adrenergic receptor binding sites at relatively low concentrations.

Drugs which covalently inactivate receptor binding sites are useful tools in studying the pharmacological properties of receptors in isolated tissues. Since many tissues contain a large receptor reserve for activating a maximal tissue response, it is necessary to progressively inactivate receptor binding sites to determine the extent of the receptor reserve and the true potencies and efficacies of agonists at the receptor site. β-Haloalkylamine drugs, such as phenoxybenzamine and dibenamine, are widely used to covalently inactivate α-adrenergic, histaminergic, muscarinic cholinergic and other receptor types [1–3]. Much is known about receptor reserves, equilibrium affinity constants, and efficacies for agonists at these receptors in a variety of tissues [4–6].

Due to the lack of suitable irreversible antagonists for inactivating β-adrenergic receptors, much less is known about the extent of receptor reserves and equilibrium activation constants of agonists in activating these receptors in isolated tissues. Although it is possible to effectively reduce functional β-adrenergic receptor density in isolated tissues by functional antagonism [7] or agonist-induced desensitization [8, 9], these approaches are not always satisfactory. It would be preferable to have highly reactive specific covalent antagonists to actually reduce the density of receptor binding sites.

A number of compounds have been reported to directly reduce the density of β-adrenergic receptors in various tissues. These include slowly reversible antagonists such as FM 24 [10] and aminobenzylpropranolol [11] which form an apparently stable but noncovalent complex with the receptor binding site. Photoaffinity labels which become highly reactive when exposed to ultraviolet light can be used to inactivate β-adrenergic receptors [12–15], and *para*-azidocarazolol has recently been used successfully to label β-adrenergic receptors prior to solubilization [16]. However photoaffinity labels are not entirely suitable for use in isolated tissues where the tissue cannot always be uniformly exposed to light for a constant period of time.

Selective high-affinity antagonists which have highly reactive groups incorporated into the molecule have been synthesized as irreversible alkylating

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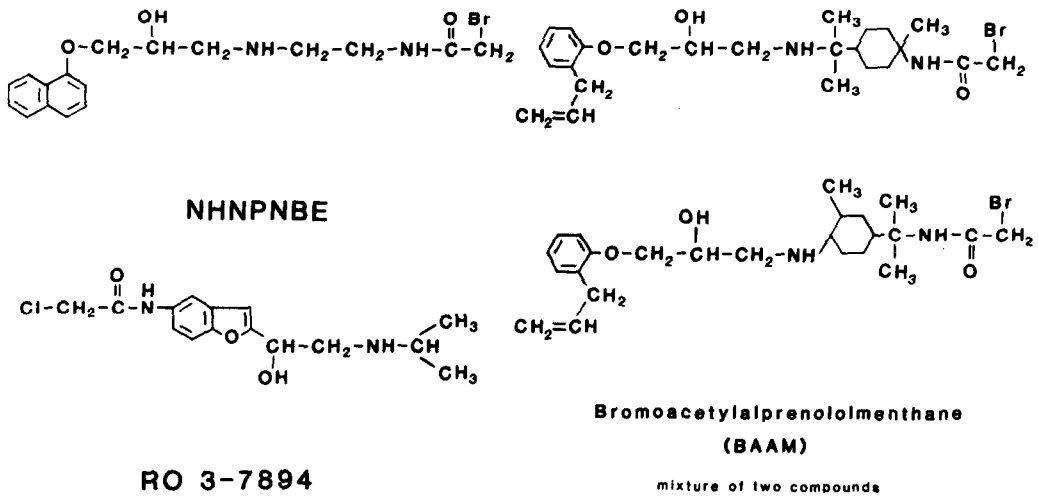

Fig. 1. Structures of NHNPNBE, Ro 3-7894 and BAAM.

agents for β-adrenergic receptors. These include NHNPNBE* [17], Ro 3-7894 [18], and BAAM [19]. In this manuscript we examine the interactions of these compounds with [^{125}I]-pindolol binding to β_1- and β_2-adrenergic receptors in membrane preparations from various tissues, to determine whether they will be useful as irreversible β-adrenergic receptor blocking drugs for studies in isolated tissues. The structures of the compounds studied are shown in Fig. 1.

MATERIALS AND METHODS

Drugs. The drugs used were obtained from the following sources: (-)-pindolol, Sandoz, Basel; NHNPNBE (NBE-propranolol) [*N*-(2-hydroxy-3-[1-napthoxy] - propyl) - *N* - bromoacetylethylenedi - amine], Sigma Chemical Co., St. Louis, MO; Ro 3-7894 [1-(5-chloracetylaminobenzfuran-2-yl)-2-isopropylaminoethanol], Roche Products Ltd., Welwyn Garden City, Hertfordshire; and BAAM (bromoacetylalprenololmenthane), Dr. Josef Pitha, National Institute on Aging, Baltimore, MD. All other chemicals were of reagent quality.

Preparation of tissue. Male Sprague–Dawley rats (200–400 g) were killed by decapitation. Various tissues were dissected and homogenized with a Brinkmann Polytron in 20 vol. of 20 mM $NaPO_4$ buffer (pH 7.6) containing 154 mM NaCl ("phosphate-salt buffer"), centrifuged at 20,000 *g* for 10 min in a refrigerated centrifuge, and resuspended in 20 vol. of phosphate-salt buffer. Cardiac ventricle, vas deferens and lung were filtered through two layers of surgical gauze to remove connective tissue fragments. For pretreatment with drugs, each tissue was aliquoted into six equal 2-ml fractions, and each fraction was incubated at 37° for 30 min with a different concentration of drug. At the end of the incubation, the tubes were diluted with 20 ml of cold buffer and immediately centrifuged at 20,000 *g* for 10 min in a refrigerated centrifuge. The supernatant fractions were discarded and the pellets were resuspended in 30 ml of phosphate-salt buffer, centrifuged again, and the pellets resuspended in 1.8 ml of phosphate-salt buffer for [^{125}I]PIN binding assays.

Radioligand binding. (-)-Pindolol was radioiodinated to theoretical specific activity as described by Barovsky and Brooker [20] and stored for up to 1 month at −20°. Tissue preparation (100 μl) (30–100 μg protein) was incubated with [^{125}I]PIN (40–800 pM), 20 mM $NaPO_4$ (pH 7.6), 154 mM NaCl, and 100 μM guanosine 5′-triphosphate in the absence or presence of competing drugs at 37° for 20 min. The reaction was stopped by addition of 10 ml of 10 mM Tris Cl (pH 7.4) at room temperature, and samples were rapidly filtered over glass fiber filters (Schleicher & Schuell, no. 30) under vacuum. Each filter was washed with 10 ml of 10 mM Tris Cl immediately after filtration, dried, and radioactivity measured in a gamma counter. Specific binding was calculated as the difference between binding in the absence and in the presence of 50 μM l-isoproterenol.

Saturation isotherms of equilibrium binding were analyzed by unweighted linear regression by the method of Scatchard [21]. Protein was determined by the method of Bradford [22].

Calculation of K_I *values from competitive inhibition of Scatchard plots.* For competitive inhibition [23]

$$K_D = \frac{K_{app}}{1 + (I/K_I)}$$

where K_D = actual radioligand affinity constant; K_{app} = apparent radioligand affinity constant from Scatchard plot in presence of inhibitor; I = concentration of inhibitor; and K_I = affinity constant for competitive inhibitor. This equation can be rearranged to

$$\frac{K_{app}}{K_D} = \frac{1}{K_I}[I] + 1.$$

* Abbreviations: NHNPNBE, *N*-(2-hydroxy-3-[1-napthoxy]-propyl)-*N*-bromoacetylethylenediamine; BAAM, bromoacetylalprenololmenthane; Ro 3-7894, 1-(5-chloracetylaminobenzfuran - 2 - yl) - 2 - isopropylaminoethanol; DHA, dihydroalprenolol; and [^{125}I]PIN, [^{125}I]pindolol.

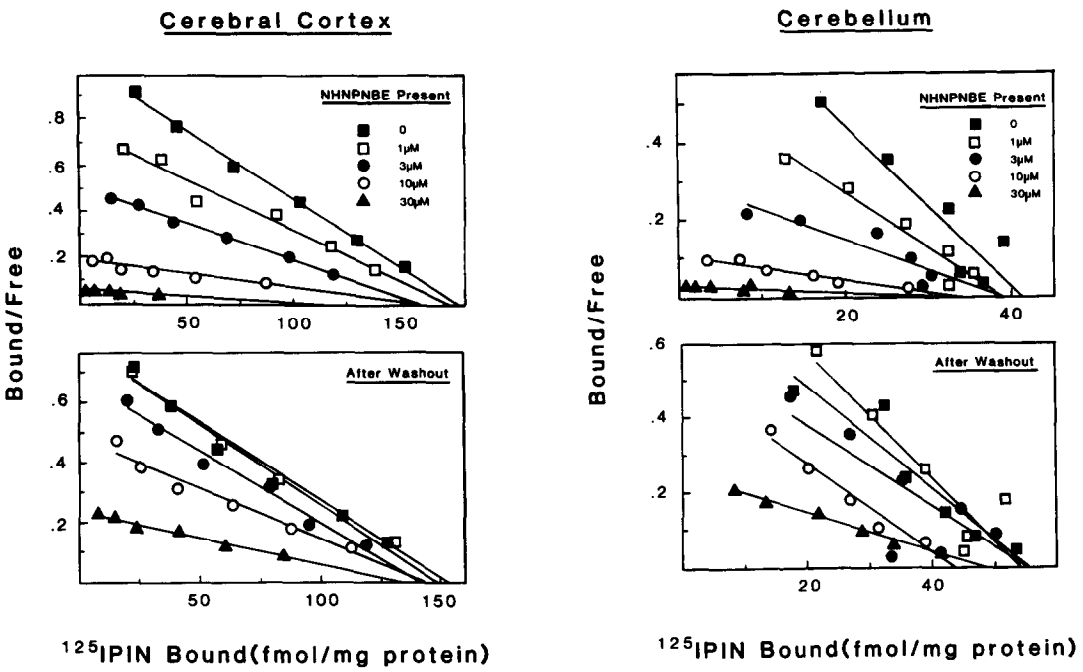

Fig. 2. Effect of NHNPNBE on Scatchard plots of saturation isotherms for specific [^{125}I]PIN binding to membranes from rat cerebral cortex (left) and cerebellum (right). In the top panels, NHNPNBE at the indicated concentrations was added directly to the incubation mixture for the radioligand binding assay, and the incubation was performed immediately as described. In the bottom panels, identical tissue aliquots were pretreated with the indicated concentration of NHNPNBE for 30 min at 37°, diluted with 20 ml of cold buffer, and centrifuged at 20,000 *g* for 10 min. Pellets were resuspended in 30 ml of cold buffer and centrifuged again. Final pellets were resuspended in 1.8 ml of cold buffer and [^{125}I]PIN binding was determined. Each point is the average of data obtained from experiments on 3 different days. Lines were fitted by nonweighted linear regression.

Therefore, plotting the ratio of the apparent radioligand affinity constant in the presence of the inhibitor and the actual radioligand affinity constant versus the concentration of inhibitor gives a straight line with a slope which is the inverse of the K_I for the inhibitor with a y-intercept of 1 as an internal check.

RESULTS

Effect of NHNPNBE. The effect of NHNPNBE on [^{125}I]PIN binding to membranes from rat cerebral cortex (mainly β_1-adrenergic receptors) and cerebellum (mainly β_2-adrenergic receptors) is shown in Fig. 2. When added directly to the incubation medium for the radioligand binding assay, NHNPNBE acted as a simple competitive antagonist of specific [^{125}I]PIN binding in both cerebral cortex and cerebellum (Fig. 2, top). Increasing concentrations of NHNPNBE caused a progressive decrease in slope of the Scatchard plots of specific [^{125}I]PIN binding corresponding to an increase in apparent K_D for the radioligand. There was little evidence for a change in the apparent density of specific [^{125}I]PIN binding sites (B_{max}) in the presence of NHNPNBE. When the membranes were pretreated with NHNPNBE for 30 min at 37° and washed twice, there was still little apparent loss of binding site density, although some loss was seen at higher concentrations. There was apparently residual competitive drug remaining in the tissue after washout, as evidenced by persistent shifts in slope of the Scatchard plots in both tissues after treatment with NHNPNBE and two subsequent washes (Fig. 2, bottom).

Effect of Ro 3-7894. When added directly to the incubation medium, increasing concentrations of Ro 3-7894 (0.3 to 30 μM) caused a progressive apparent loss of binding site density in rat cerebral cortex, with little or no change in the slope of the Scatchard plot (Fig. 3, top left). However, a curvature to the Scatchard plots was sometimes apparent in the presence of Ro 3-7894. In rat cerebellum, higher concentrations of Ro 3-7894 were needed to inhibit specific [^{125}I]PIN binding (3–100 μM) and the inhibition was mainly, although not exclusively, competitive (Fig. 3, top right). Both the apparently noncompetitive inhibition in cerebral cortex and the competitive inhibition in cerebellum disappeared completely after removal of free Ro 3-7894 by washing the membranes twice (Fig. 3, bottom). Almost identical results of an apparent noncompetitive inhibition of specific [^{125}I]PIN binding and complete reversal by washout of free drug was observed when membranes of rat heart (also mainly β_1-adrenergic receptors) were used as the tissue source (data not shown).

Since some of the Scatchard plots in cerebral cor-

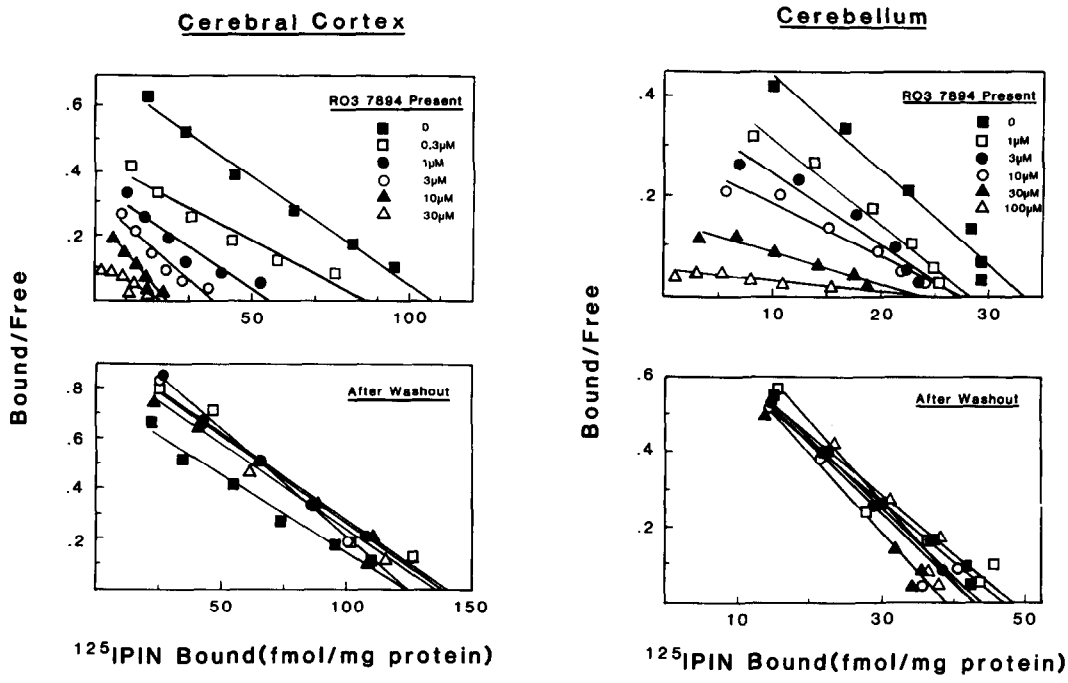

Fig. 3. Effect of Ro 3-7894 on Scatchard plots of saturation isotherms of specific [^{125}I]PIN binding to membranes from rat cerebral cortex (left) and cerebellum (right). Experimental details are identical to Fig. 2, except that Ro 3-7894 was the drug under study. Each point is the average of data from experiments on 3 different days.

tex showed a slight curvature in the presence of Ro 3-7894, experiments were performed to further saturate the β-adrenergic receptors with higher concentrations of [^{125}I]PIN . Scatchard plots of saturation isotherms of specific [^{125}I]PIN binding, up to concentrations of 3.6 nM [^{125}I]PIN (30 times the K_D), in the absence and presence of Ro 3-7894 are shown in Fig. 4. The inhibition by Ro 3-7894 was not noncompetitive, but consisted of two components with different K_i values. Since cerebral cortex and heart both contain small (15–30%) but substantial proportions of β_2-adrenergic receptors [24], these data are consistent with Ro 3-7894 being a β_1-selective competitive antagonist.

Tissue specificity of Ro 3-7894. Dose–response curves for inhibition of specific [^{125}I]PIN binding by Ro 3-7894 were performed in two predominantly β_1-adrenergic receptor containing tissues (rat cerebral cortex and cardiac ventricle) and three predominantly β_2-adrenergic receptor containing tissues (rat lung, cerebellum and vas deferens). Ro 3-7894 was about 20-fold more potent in inhibiting specific [^{125}I]-PIN binding in heart and cerebral cortex than in lung, cerebellum, or vas deferens (Fig. 5).

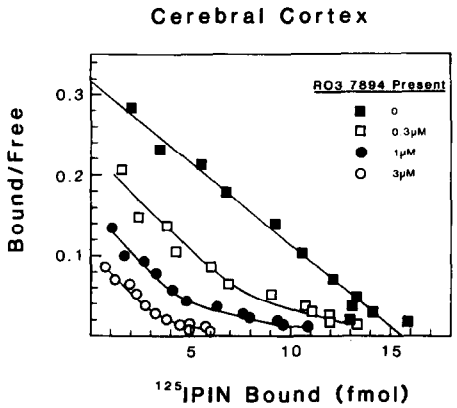

Fig. 4. Effect of Ro 3-7894 in cerebral cortex with greater saturation of [^{125}I]PIN binding. Ro 3-7894 was present at the indicated concentrations during the radioligand binding assay. Each point is the mean of triplicate concentrations from a single experiment. Data are representative of two different experiments.

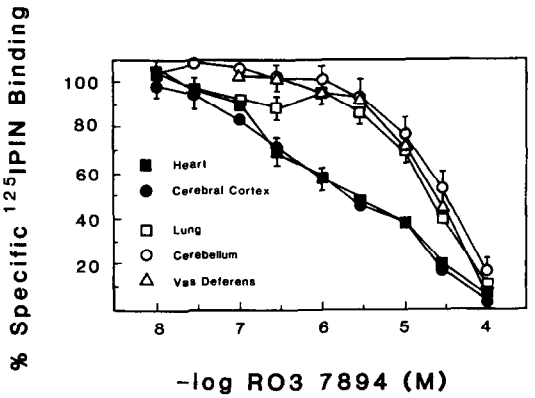

Fig. 5. Inhibition of specific [^{125}I]PIN binding in various tissues by Ro 3-7894. [^{125}I]PIN concentration ranged from 45 to 55 pM. Each point is the mean ±S.E.M. of data from three different experiments.

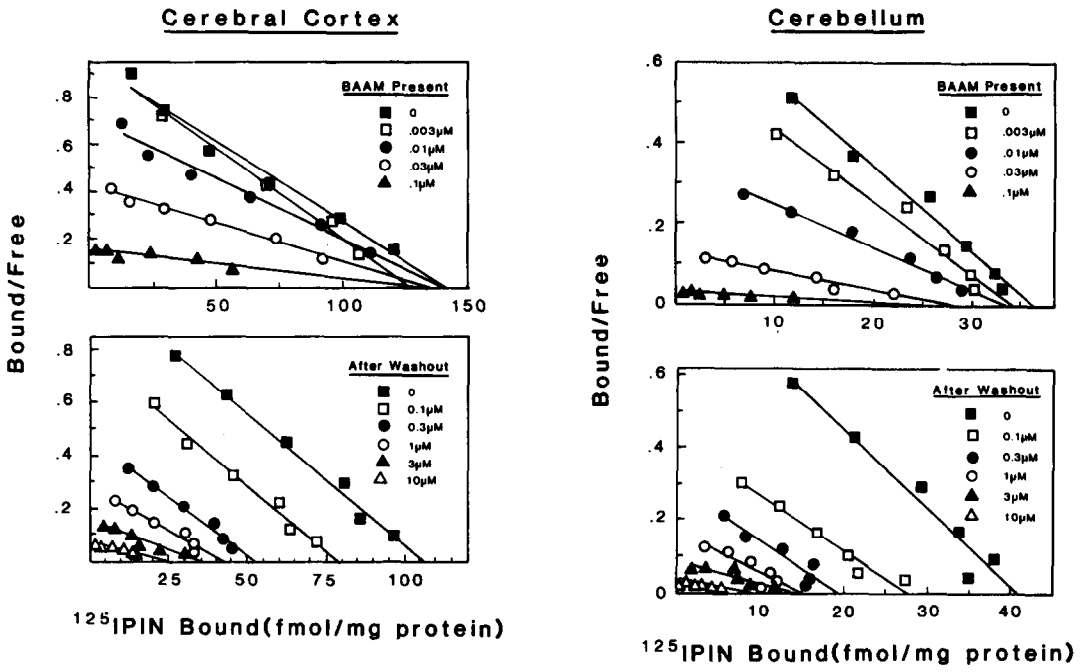

Fig. 6. Effect of BAAM on Scatchard plots of saturation isotherms of specific [^{125}I]PIN binding to membranes from rat cerebral cortex (left) and cerebellum (right). Experimental details are identical to Fig. 2, except that BAAM was the drug under study. Each point is the average of data from experiments on 3 different days.

Effect of BAAM. When BAAM was added directly to the incubation medium for the radioligand binding assay, it acted as a simple competitive antagonist of specific [^{125}I]PIN binding in both cerebral cortex and cerebellum (Fig. 6, top). Increasing concentrations of BAAM (0.003 to 0.1 μM) caused a decrease in slope of the Scatchard plots corresponding to an increase in apparent K_D for the radioligand. There was little evidence for a change in the density of specific [^{125}I]PIN binding sites with these concentrations of BAAM. However, pretreatment of membranes with higher concentrations of BAAM (0.1 to 10 μM) for 30 min at 37° and subsequent washout of free drug, caused a progressive loss of binding sites for [^{125}I]PIN in both cerebral cortex and cerebellum, with little effect on the apparent K_D for [^{125}I]PIN (Fig. 6, bottom). Similar results were obtained when membranes from rat heart were used as the tissue source (data not shown). Saturation studies with very high concentrations of [^{125}I]PIN in cerebral cortex showed that the loss of [^{125}I]PIN binding sites caused by BAAM was not due to a biphasic competitive inhibition (Fig. 7). To determine whether the effects of BAAM were irreversible, homogenates of cerebral cortex were pretreated with or without 1 μM BAAM for 30 min at 37° and then washed. Scatchard analyses of saturation isotherms for specific [^{125}I]PIN binding were then performed either immediately after the BAAM inactivation, or after storing the tissue in the refrigerator for 24 hr. There was no recovery of binding sites in the BAAM-treated tissues 24 hr after treatment (control 88.5 ± 3.01 and 86.6 ± 8.58 fmoles/mg protein measured immediately or 24 hr later respectively; BAAM-treated 35.8 ± 2.40 and 40.3 ± 4.18 fmoles/mg protein assayed immediately or 24 hr later respectively).

K_I *values for drugs.* Calculation of K_I values for each drug (Fig. 8) in affecting the slope of [^{125}I]PIN Scatchard plots shows that NHNPNBE was a competitive inhibitor with similar affinities (1–2 μM) for both β_1- and β_2-adrenergic receptors; BAAM was a much more potent competitive inhibitor (0.006 to 0.03 μM) with a slight (5-fold) β_2-selectivity, while

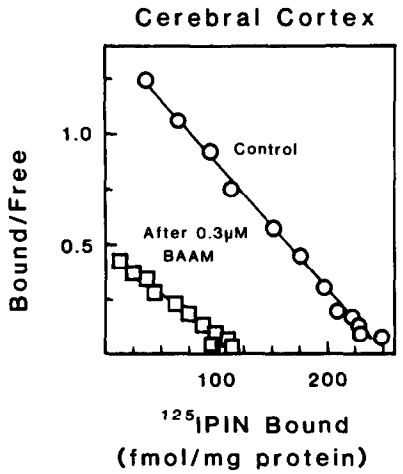

Fig. 7. Effect of BAAM in cerebral cortex with greater saturation of [^{125}I]PIN binding. Tissues were incubated with or without 0.3 μM BAAM for 30 min at 37° and washed as described in the legend of Fig. 2. Each point is the average of triplicate determinations from a single experiment.

Ro 3-7894 was a competitive inhibitor in cerebellum with a K_I of 14 μM. Due to biphasic Scatchard plots in cerebral cortex in the presence of Ro 3-7894, no apparent K_I for β_1-adrenergic receptors was calculated.

DISCUSSION

In this study we have examined the effects of NHNPNBE, Ro 3-7894, and BAAM on specific [^{125}I]PIN binding to membranes prepared from rat cerebral cortex and cerebellum. Rat cerebral cortex contains predominantly (80–85%) β_1-adrenergic receptors, and the cerebellum from rats of this age range contains predominantly (90–95%) β_2-adrenergic receptors [24, 25]. Although neither of these tissues contains homogeneous populations of a β-adrenergic receptor subtype, the ready availability of large amounts of tissue, ease of tissue preparation, and the high density of receptors in these tissues make them suitable for screening drugs. Effects on [^{125}I]PIN binding to membranes from cerebral cortex should reflect mainly effects on β_1-adrenergic receptors and effects on cerebellum should reflect mainly effects on β_2-adrenergic receptors.

NHNPNBE was shown by Atlas and Levitzki [17] and Atlas *et al.* [26] to inhibit [^{3}H]-propranolol binding and epinephrine-stimulated adenylate cyclase activity in turkey erythrocyte membranes in doses from 0.1 to 100 μM. This inhibition was resistant to removal of free drug by washing; however, saturation curves for [^{3}H]-propranolol binding or activation of adenylate cyclase by epinephrine were not reported. Therefore, it is not possible to conclude whether NHNPNBE was reducing β-adrenergic receptor density in those experiments. Venter [27] treated cat ventricle strips with 0.1 to 100 μM NHNPNBE, and found a reduction in [^{125}I]-iodohydroxybenzylpindolol binding and a shift to the right for isoproterenol-induced inotropic effects *in vitro*. Again, although the effects of NHNPNBE were resistant to washout, saturation curves for radioligand binding were not reported so it is not possible to conclude whether the inhibition observed was competitive or noncompetitive. In agreement with Atlas *et al.* [26], we found that NHNPNBE inhibited β-adrenergic receptors with an affinity constant of around 2 μM; however, saturation isotherms showed that this inhibition was due to a reduction in the affinity of the radioligand for the receptor rather than a loss of receptor binding sites. We also found that the inhibition by NHNPNBE was resistant to washout. This could be due to a persistent decrease in the affinity of the receptors by covalent modification, or due to sequestration of the drug in the tissue such that repeated washing does not effectively remove free drug from the receptor. The data for NHNPNBE in Fig. 8 can clearly be fitted to a straight line with a y-intercept of one, as would be predicted by the equation for competitive inhibition. It is therefore likely that NHNPNBE is a competitive antagonist at lower concentrations, which is resistant to washout due to its structural characteristics. Tolkovsky and Levitzki [28] did report saturation curves for specific [^{125}I]-iodohydroxybenzylpindolol binding to turkey erythrocyte membranes following treatment with 17–230 μM NHNPNBE, and found a dose-dependent decrease in receptor binding sites. We also found a 20–40% decrease in the density of specific [^{125}I]PIN binding sites after treatment of membranes from cerebral cortex and cerebellum with 30–100 μM NHNPNBE, although these high doses resulted in substantial changes in the slopes of [^{125}I]PIN Scatchard plots in these tissues even after washing. Such a low potency in decreasing receptor density would make it difficult to distinguish an irreversible reduction in receptor binding sites from competitive inhibition by residual drug in functional experiments on intact tissues.

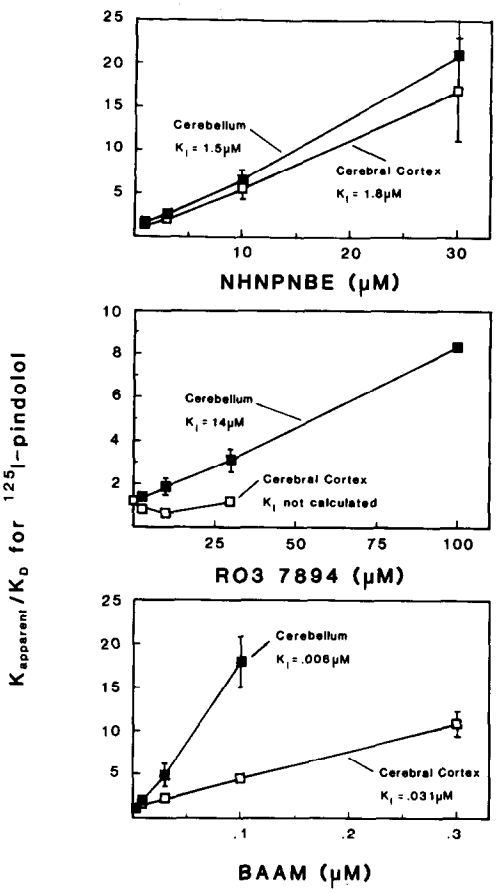

Fig. 8. Effects of drugs on the apparent K_D for [^{125}I]PIN in Scatchard plots of saturation isotherms. K_{app} values were determined as the negative reciprocal of the slope of Scatchard plots. Each point is the mean ±S.E.M. of data from experiments on 3 different days.

Ro 3-7894 was introduced by Nicholson and Broadley [18] as a halogenated derivative of a competitive β-adrenergic receptor antagonist. These authors reported that Ro 3-7894 affected rate and tension responses to isoproterenol in guinea pig atria in a manner consistent with irreversible noncompetitive antagonism. This inhibition was characterized by a depression of the maximum response to isoproterenol which was not restored by subsequent washout. In subsequent studies this compound was used to inactivate receptor reserves and calculate agonist affinity constants for β-adrenergic receptors

in various cardiac preparations under a variety of conditions [29–32]. In these studies, Ro 3-7894 was applied to isolated cardiac preparations for a short period of time and the tissue then washed extensively (up to 3 hr). Residual effects of the drug treatment after this washout period were considered to be due to irreversible β-adrenergic receptor blockade. Rankin and Broadley [33] found that Ro 3-7894 decreased the apparent density of specific [^{3}H]-dihydroalprenolol binding sites in membranes from guinea pig ventricular muscle, and that this effect was resistant to washout.

Two recent studies have obtained results inconsistent with an irreversible blockade of β-adrenergic receptor binding sites by Ro 3-7894. Baker and Posner [34] found that Ro 3-7894 had depressant effects on cardiac tension which were resistant to washout, but that these effects were not specific to β-adrenergic receptor mediated effects and the response to calcium was also depressed. Baker and Posner [34] also found no change in [^{3}H]dihydroalprenolol binding sites in rat heart after either *in vitro* or *in vivo* treatment with Ro 3-7894 and washout of free drug. Krstew *et al.* [35] also found washout-resistant nonspecific depressant effects of Ro 3-7894 on guinea pig left atrial preparations, and found no evidence for irreversible blockade of β-adrenergic receptors by this drug in guinea pig uterus or trachea. Krstew *et al.* [35] also found no change in the density of specific [^{125}I]iodocyanopindolol binding sites in guinea pig left atria following treatment with Ro 3-7894 and subsequent washout.

Ro 3-7894 appeared to be a simple competitive antagonist at β_2-adrenergic receptors in rat cerebellum. However, in our initial experiments it appeared that Ro 3-7894 noncompetitively inhibited [^{125}I]PIN binding in membranes of rat cerebral cortex, but that this inhibition was reversed completely by relatively minimal washing. The slight curvature of the Scatchard plots in the presence of Ro 3-7894 suggested that further saturation of the [^{125}I]PIN binding sites might reveal a competitive inhibition. When higher concentrations of [^{125}I]PIN were used to more completely saturate the receptors in the cerebral cortex, it became apparent that Ro 3-7894 was not noncompetitively antagonizing the receptors. Rather, the inhibition by Ro 3-7894 was apparently biphasic, with different affinities for two different binding sites. Since rat cerebral cortex contains a small (15%–20%) but significant proportion of β_2-adrenergic receptors [24], these results suggest that Ro 3-7894 is a competitive antagonist at both β_1- and β_2-adrenergic receptors, but that it is substantially more potent at β_1-adrenergic receptors. This is supported by the experiments which showed that Ro 3-7894 was more potent in competing for specific [^{125}I]PIN binding sites in the rat cerebral cortex and heart (which contain predominantly β_1-adrenergic receptors) than in rat vas deferens, cerebellum or lung (which contain predominantly β_2-adrenergic receptors). The apparent noncompetitive inhibition observed in studies of cerebral cortex where less complete saturation was achieved is caused by the high potency of Ro 3-7894 in competitively blocking the β_1-receptors in concentrations which do not affect the β_2-receptors, resulting in biphasic Scatchard plots. Rankin and Broadley [33] also reported that Ro 3-7894 inhibited specific [^{3}H]DHA binding to β-adrenergic receptors in guinea pig heart in an apparently noncompetitive manner when added to the radioligand incubation medium. These authors did not attempt to saturate their β-adrenergic receptor binding sites and, therefore, could not rule out a biphasic inhibition such as that observed here. However, Rankin and Broadley [33] found that the apparently noncompetitive effect of Ro 3-7894 on [^{3}H]DHA binding to guinea pig heart was not reversed by washing. The reason for this discrepancy is unknown, although the data in the previous study were characterized by a very high and quite variable K_D for [^{3}H]-dihydroalprenolol binding to β-adrenergic receptors (1.8 to 12.1 nM), which made it difficult to obtain saturation of radioligand binding in many experiments. We have not examined the effect of Ro 3-7894 on guinea pig ventricular membranes; however, we have obtained data on [^{125}I]PIN binding to rat ventricular membranes (data not shown), confirming our results in cerebral cortex.

The ready reversibility of the effects of Ro 3-7894 on specific [^{125}I]PIN binding is not consistent with the previously reported persistent effects of the compound in isolated tissue preparations after extensive washing. It is possible, however, that the drug is sequestered in intact tissues and is not washed out as readily as it is from a membrane preparation, or that the persistent effects are not selective for β-adrenergic receptor mediated responses [34, 35].

BAAM is a derivative of the potent β-adrenergic receptor antagonist alprenolol which possesses a highly reactive chemical grouping. BAAM has been shown to be very potent in competing for β-adrenergic receptor binding sites in membranes from frog erythrocytes [19] and rat heart and lung [36, 37]. Pretreatment of membranes with 0.2 to 1 μM BAAM [19, 36], or injection of 9–70 mg/kg BAAM *in vivo* causes a persistent loss of β-adrenergic receptor binding sites in heart and lung [38] as measured by saturation curves for [^{3}H]dihydroalprenolol binding, suggesting that it is an irreversible antagonist at β-adrenergic receptors. Pretreatment of cardiac membranes with BAAM noncompetitively inhibits the ability of isoproterenol to activate adenylate cyclase [39]. In agreement with previous results [19, 36], we found that BAAM potently competed for specific [^{125}I]PIN binding sites in both cerebral cortex and cerebellum. K_I values for competitively inhibiting specific [^{125}I]PIN binding were 5 nM in cerebellum and 30 nM in cerebral cortex, suggesting a slight selectivity of BAAM for β_2-adrenergic receptors. Baker and Pitha [36] also reported a slight β_2-selectivity for BAAM. We found that substantially higher concentrations of BAAM were needed to decrease the density of [^{125}I]PIN binding sites than to competitively inhibit specific [^{125}I]PIN binding. Even so, substantial loss of sites was observed after pretreatment with as little as 0.1 μM BAAM for 30 min at 37°, suggesting that this drug may be useful in alkylating β-adrenergic receptors in isolated tissue preparations.

Overall, our results suggest that BAAM will be the most useful compound for irreversibly inactivating β-adrenergic receptors in isolated tissue preparations,

since it persistently decreases receptor density at nanomolar concentrations. Neither NHNPNBE nor Ro 3-7894 appears to cause persistent decreases in either β_1- or β_2-adrenergic receptor density at concentrations which might be useful in isolated organ studies.

Acknowledgements—We thank Dr. Josef Pitha for supplying us with BAAM, and Dr. Peter Sorter and P. Fellner of Roche for Ro 3-7894.

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