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Covalent Labeling of the β-Adrenergic Ligand-Binding Site with *para*-(Bromoacetamidyl)benzylcarazolol

A Highly Potent β-Adrenergic Affinity Label

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SUMMARY

para-(Bromoacetamidyl)benzylcarazolol (*p*BABC) was synthesized and found to be an extremely potent affinity label for β-adrenergic receptors. Its interaction with mammalian (rabbit and hamster lung) and nonmammalian (turkey and frog erythrocyte) β-adrenergic receptors was similar, displaying EC_{50} values of 400–900 pM for inhibiting ^{125}I-cyanopindolol binding to these receptors. *p*BABC reduced the number of β-adrenergic receptors in frog erythrocyte membranes, without any change in the affinity of the remaining sites for [^{125}I]iodocyanopindolol. *p*BABC has been radioiodinated. As assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, this affinity probe specifically labeled the β-adrenergic peptide of a purified preparation of hamster lung, with high efficiency (~40%) and with a pharmacological specificity characteristic of an interaction at the β_2-adrenergic receptor ligand-binding site. Comparison of the proteolyzed products derived from purified receptor labeled with [^{125}I]*p*BABC and with the photoaffinity agent [^{125}I] *p*-azidobenzylcarazolol suggested that covalent labeling of the β-adrenergic receptor by these probes occurs at similar domains of the β-adrenergic receptor. Because of the much higher level of incorporation of this affinity probe as opposed to photosensitive probes, *p*BABC should prove to be a useful tool for structural studies of purified β-adrenergic receptors.

INTRODUCTION

In recent years, the design of reversible (1, 2) and irreversible ligands has allowed major advances in the study of β-adrenergic receptor function. Irreversible probes in particular have provided important information about the binding subunit of β-adrenergic receptors (3, 4), the coupling efficiency between receptor and effector (5), the presence of "spare" β-adrenergic receptors (6), and the rate of synthesis of β-adrenergic receptors in tissues (7) and cell preparations (8). Atlas and coworkers (9) first showed that a bromoacetylated derivative of propranolol irreversibly blocked both ligand binding to turkey erythrocyte β-adrenergic receptors and epinephrine-stimulated adenylate cyclase. The tritiated form was also used in attempts to identify the protein subunits of β-adrenergic receptors in turkey erythrocytes and cultured LG muscle cells (3). However, the low affinity and specific activity of this probe made such efforts unsatisfactory. Other affinity ligands and long lasting β-adrenergic receptor antagonists have since been described which include analogs of the β-adrenergic receptor antagonists acebutolol (10), bufurolol (11), propranolol (12, 13), and alprenolol (14). All of these, however, have generally exhibited relatively low affinity for β-adrenergic receptors or have been radiolabeled only to low specific radioactivity.

Recently, we (4) and others (15, 16) have synthesized radioiodinated photoaffinity arylazide β-adrenergic receptor antagonists which covalently label membrane-bound and purified β-adrenergic receptors (17). These compounds have the advantage of possessing extremely high affinity (K_D = 10–40 pM) and specificity for β-adrenergic receptors. However, the yield of covalent photoincorporation (10–15%) limits their usefulness as tools for structural studies of purified β-adrenergic receptors. We therefore set out to develop a high affinity alkylating probe for β-adrenergic receptors which could be radioio-

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dinated and, because of its reactivity, could label the receptor covalently with increased efficiency. In this paper, we describe the characteristics of such a potent affinity label, *p*BABC,[3] which can be prepared in radioiodinated form. Inactivation of and covalent incorporation into purified β-adrenergic receptors by the radioiodinated probe occur with high efficiency and by interactions which can be specifically blocked by β-adrenergic agents.

MATERIALS AND METHODS

Materials. [^{125}I]Iodocyanopindolol was synthesized in our laboratories using the procedure described by Engel *et al.* (2). Carrier-free Na^{125}I was purchased from New England Nuclear, Boston, MA. Premixed SDS-PAGE samples (phosphorylase *b*, M_r = 94,000; bovine serum albumin, M_r = 67,000; ovalbumin, M_r = 43,000; carbonic anhydrase, M_r = 30,000; soybean trypsin inhibitor, M_r = 20,100; α-lactalbumin, M_r = 14,000) were from Pharmacia and were iodinated by the chloramine-T method of Greenwood *et al.* (17). SDS was obtained from British Drug House, Poole, England and all electrophoresis reagents were from Bio-Rad Laboratories. X-ray film XAR-5 and developing solution were from Kodak, and intensifying screens (Cronex Lightning Plus) were from DuPont. TLC plates were Scientific Products silica gel 60 F-254 containing fluorescent indicator.

(−)Alprenolol hydrochloride, L-cysteine hydrochloride, (−)isoproterenol bitartrate, (−)epinephrine bitartrate, and (−)norepinephrine bitartrate were from Sigma Chemical Co. (+)Epinephrine bitartrate, (+)norepinephrine, and (+)isoproterenol were from Sterling-Winthrop. The following drugs were kindly supplied by the indicated companies: phentolamine hydrochloride (Ciba-Geigy); haloperidol hydrochloride (McNeil Laboratories); Betaxolol hydrochloride (Synthelab); (+)alprenolol (Hässle); ICI 118551 hydrochloride (ICI, Macclesfield). The proteolytic enzymes were purchased as follows: trypsin, chymotrypsin, papain (Sigma), and *Staphylococcus aureus* V8 protease (Miles Laboratories). Digitonin was purchased from Gallard Schlessinger; and bovine serum albumin, essentially fatty acid free, was from Sigma. Other chemicals were generally from Aldrich Chemical Co. and of the highest available purity.

Synthesis of pBABC. p-Aminobenzylcarazolol, the synthesis of which has been previously described (18) (320 mg, 0.8 mmol) was reacted with bromoacetylbromide (250 mg, 1.5 mol eq) in CH_2Cl_2 (100 ml; dried over $MgSO_4$) at room temperature overnight. During this time, a white precipitate formed. HCl-saturated ether (4 ml) was then added and the precipitate was collected by vacuum filtration to give *p*BABC (hydrobromide salt, 410 mg; 85% yield). This was recrystallized from CH_3OH/CH_2Cl_2/ether (1:4:5), m.p. 159–161°. ^{1}H NMR (trimethylsilyl/CD_3OD/$CDCl_3$ (0.01:5:95): δ 7.45 (m, 8, carbazole), 7.34 (d, 2, aromatic), 7.13 (d, 2, aromatic), 4.27 (m, 3, $ROCH_2CHOH$), 4.02 (s, 2, $COCH_2Br$), 3.02 (m, 2, $RNCH_2$), 2.71 (s, 2, CH_2), and 1.11 (s, 6, CH_3).

*Radioiodination of pBABC. p*BABC (4 μg/4 μl of CH_3OH; 10 nmol) in CH_3OH/2 M sodium acetate buffer (1:1; 24 μl; pH 5.6) was treated by sequential addition with Na^{125}I (10 mCi, 4.5 nmol, >350 mCi/ml 0.1 N NaOH) and chloramine-T (4 μg in 4 μl, 15 nmol) at room temperature. The reaction was halted at 1 min with $Na_2S_2O_5$ (8 μg in 8 μl; 42 nmol) and analyzed by TLC (20% CH_3OH/CH_2Cl_2/1 mM phenol) co-spotting with *p*BABC (R_f 0.28; trace impurity, R_f 0.32). The major radioactive product was observed by autoradiography at R_f 0.30 (trace impurity, R_f 0.37). [^{125}I]*p*BABC was isolated and purified by TLC, the desired product being eluted from the band of silica with 20% CH_3OH/EtOAc. This eluate was concentrated to dryness under N_2 and reconstituted in EtOH/1 mM phenol. Samples were stored in the dark at −20°.

β-Adrenergic receptor preparations. "Purified" membranes of turkey and frog erythrocytes were prepared as previously described (19, 20). Membranes of hamster lung were prepared essentially as described previously for rabbit lung (21). Protein was determined by the method of Lowry *et al.* (22) using bovine serum albumin (Sigma) as standard. Soluble hamster lung β-adrenergic receptor, purified to homogeneity by affinity chromatography and steric exclusion high performance liquid chromatography (23), was a generous gift of J. L. Benovic, Duke University Medical Center.

Membrane β-adrenergic receptor assay. Membranes (10–20 μg of protein, 125 μl) were incubated for 90 min at 25° in a total volume of 250 μl containing 150 mM NaCl, 20 mM phosphate buffer, pH 7.2 (or in some cases 50 mM Tris-HCl, pH 7.4), ^{125}ICYP (25 μl, dissolved in 0.5% EtOH/1 mM HCl) and drugs (50 μl). *p*BABC was weighed out just before use and dissolved in dry dimethyl sulfoxide. Bound and free radioligand was separated by the addition of 5 ml of ice-cold 50 mM Tris-HCl, pH 7.2, followed by vacuum filtration on Whatman GF/C filters using a further 20 ml of washing buffer. The radioactivity of the wet filters was counted in a Packard Auto 800 gamma counter at 75% efficiency. Binding data for saturation isotherms and competition curves were computer fitted to a previously described model of binding to a single site (24). Nonspecific binding of ^{125}ICYP to β-adrenergic receptors was determined using 200 μM (−)isoproterenol.

Soluble β-adrenergic receptor assay. Soluble β-adrenergic receptors were assayed essentially as previously described (20) using the radioligand ^{125}ICYP.

[^{125}I]pBABC reaction with soluble β-adrenergic receptors. Soluble, purified hamster lung β-adrenergic receptor preparation (0.1–0.25 pmol of receptor) was incubated with [^{125}I]*p*BABC (200–400 pM) in a total volume of 0.5 ml containing 100 mM NaCl, 20 mM phosphate buffer, pH 7.2, 0.05% digitonin, and competing drugs. Incubation proceeded for 60 min at 25° in a shaking water bath. The reaction was terminated and the bromoacetyl function was "quenched" by the addition of 25 μl of 200 mM cysteine, pH 7.4. Bound and free radioligand was separated by "desalting" on Sephadex G-50 columns (20) using ice-cold 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.05% digitonin as eluting buffer. Aliquots of the 1-ml eluate were counted directly as a measure of protein-bound radioactivity, or they were lyophilized and reconstituted into 150 μl of SDS buffer (8% SDS, 10% glycerol, 25 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol) prior to SDS-PAGE.

SDS-polyacrylamide gel electrophoresis. Labeled membranes (100 μg of protein) and soluble β-adrenergic receptors were suspended in SDS sample buffer (150 μl) and centrifuged at 13,000 × *g* for 15 min at 4°, and an aliquot (50–70 μl) was applied to sample wells of a polyacrylamide slab gel (10–12%). Electrophoresis was performed according to the method of Laemmli (25) using a constant current of 3 mamp/lane. Gels were dried using a slab gel drier (Hoefer Scientific Instruments), and autoradiography was performed at −80° on the dried gels using Kodak XAR-5 film. Autoradiograms were scanned on a Zeineh scanning densitometer SL-504-XL (Biomed Instruments, Inc.) using laser light. In some cases, radioactive bands were located, cut out, and counted in a Packard Auto 800 gamma counter. Data obtained by both methods were essentially identical.

RESULTS

Synthesis of pBABC and [^{125}I]pBABC. Fig. 1 shows the synthesis of *p*-(bromoacetamidyl)benzylcarazolol from *p*AMBC following a reaction with bromoacetylbromide. The derived product was obtained by simple filtration of the hydrobromide salt formed during the reaction. Analytical evidence for the designated structure is provided in Materials and Methods. *p*-(Bromoacetamidyl)benzylcarazolol was then radioiodinated directly using carrier-free Na^{125}I and chloramine-T. This reaction sequence (route I, Fig. 1) afforded one major product, [^{125}I]*p*BABC, the binding characteristics of which are described later. Attempts to obtain [^{125}I]pBABC by

[3] The abbreviations used are: *p*BABC, *p*-(bromoacetamidyl) benzylcarazolol; ICYP, iodocyanopindolol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *p*AMBC, *p*-aminobenzylcarazolol; *p*-ABC, *p*-azidobenzylcarazolol.

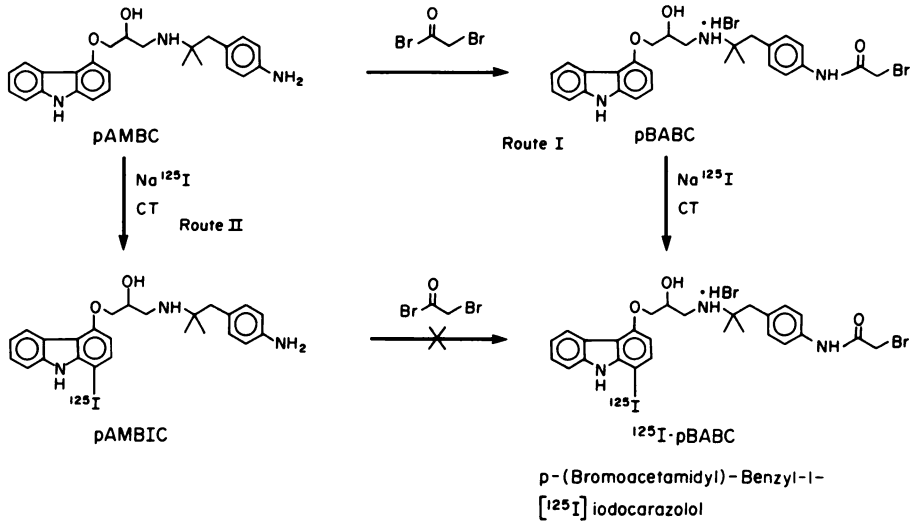

FIG. 1. *Synthesis of pBABC and [^{125}I]pBABC*

treating [^{125}I] pAMBC (prepared as described in Ref. 18) with bromoacetylbromide (route II, Fig. 1) resulted in multiple product formation.

Interaction of pBABC and its precursor with β-adrenergic receptors. The affinity label *p*BABC and its precursor *p*AMBC compete for the specific β-adrenergic receptor-binding sites as assessed by ^{125}ICYP binding to hamster lung membranes as shown in Fig. 2. After an incubation of 90 min at 25°, the ability of *p*BABC to inhibit ^{125}ICYP binding is similar to that of *p*AMBC and both are substantially more potent than (−)alprenolol (Fig. 2). The half-maximal concentration (EC_{50}) of *p*BABC causing inhibition of ^{125}ICYP binding was 0.87 nM when ^{125}ICYP was present at 125–150 pM in the assays. It should be noted that the value obtained reflects the particular incubation conditions chosen (especially time) since the true potency of an irreversible ligand should increase with time.

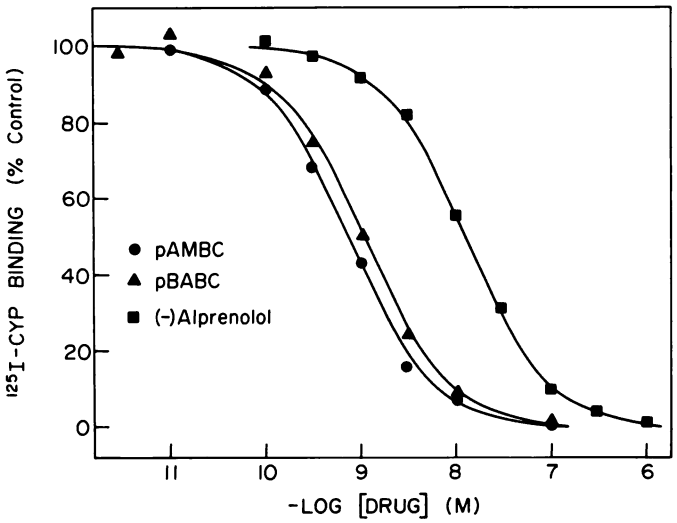

FIG. 2. *Inhibition of ^{125}ICYP binding to hamster lung membranes by various agents*

^{125}ICYP (100–150 pM) was incubated with increasing concentrations of competitors *p*AMBC (●), *p*BABC (▲), and (−)alprenolol (■) for 90 min at 25° and binding to membranes was assessed as described in Materials and Methods. 100% binding was typically 15–25 pM. These data are representative of experiments performed two to five times.

In order to determine the selectivity of *p*BABC for mammalian and nonmammalian β_1- and β_2-adrenergic receptors, inhibition of ^{125}ICYP binding to β-adrenergic receptors of a number of tissue preparations was examined (see Table 1). The apparent potencies determined for *p*BABC binding to amphibian erythrocyte and mammalian lung β_2-adrenergic receptors were not significantly different. Moreover, there was no marked difference between the apparent potency of the compound at the β_1-adrenergic receptors of turkey erythrocytes and rabbit lung. Thus, the affinity label showed no selectivity for either of the β-adrenergic receptor subtypes. Similar data were also obtained for the non-subtype-selective reversible antagonists *p*AMBC and (−)alprenolol (see Table 1).

Inactivation of β-adrenergic receptors by pBABC. Treatment of frog erythrocyte membranes with *p*BABC caused a dose-dependent loss of binding sites, which could not be recovered following an extensive washing

TABLE 1

Estimates of inhibition constants of β-adrenergic antagonists competing for ^{125}ICYP binding to mammalian and nonmammalian tissue preparations

Tissue preparation	K_i[a]		
	(−)Alprenolol	*p*AMBC	*p*BABC[b]
		nM	
β_2-Adrenergic receptors			
Frog erythrocyte	3.4 (2)	0.125 (3)	0.43 (3)
Hamster lung	2.8 (2)	0.113 (4)	0.87 (5)
β_1-Adrenergic receptors			
Turkey erythrocyte	4.2 (2)	0.104 (3)	0.45 (3)
Rabbit lung	3.9 (2)	0.267 (3)	0.91 (3)

[a] Inhibition constants were calculated using the following K_D values for ^{125}ICYP: frog erythrocyte, 28 pM; hamster lung, 18 pM; turkey erythrocyte, 22 pM; and rabbit lung, 25 pM. Number of experiments is in parentheses.

[b] Data for *p*BABC represent the concentration which inhibited the binding of ^{125}ICYP (125–150 pM in the assays) by 50% (EC_{50}) during a 90-min incubation at 25°.

protocol (Fig. 3A). Following treatment of membranes for 55 min at 25°, almost 90% of the specific β-adrenergic receptor sites were apparently inactivated by 10 nM *p*BABC. In contrast, saturating concentrations of the reversible β-adrenergic receptor antagonist (−)alprenolol failed to reduce the number of sites. That the *p*BABC-induced loss resulted from inactivation of receptors rather than persistently bound ligand was suggested by two lines of experimentation.

Treatment of *p*BABC with the nucleophilic reagent L-cysteine HCl at pH 7.2 would be expected to result in a product that would no longer contain the active alkylating bromoacetyl group and that would behave as a reversible β-adrenergic antagonist. Product formation was confirmed by TLC using a 20% CH_3OH/CH_2Cl_2/1 mM phenol solvent system (R_f values of *p*BABC and the cysteine adduct were 0.28 and 0.01, respectively). Fig. 3A shows that, when frog erythrocyte membranes were incubated with 1 nM *p*BABC preinactivated with cysteine, the loss of β-adrenergic sites was significantly attenuated. Treatment of *p*BABC with cysteine did not result in any marked change in potency for the interaction of the cysteine adduct with the β-adrenergic receptors (the EC_{50} values for *p*BABC and its cysteine product were 0.43 ± 0.1 and 0.96 ± 0.2 nM, respectively, $n = 3$; ^{125}ICYP concentrations were 130–150 pM in the assays), nor did cysteine treatment per se have any influence on β-adrenergic receptor number or the K_D of ^{125}ICYP for β-adrenergic receptors (data not shown).

Fig. 3B confirms, by saturation experiments, that the number of binding sites is significantly decreased by treatment with *p*BABC (1 and 10 nM *p*BABC decrease the number of β-adrenergic receptors by 43 and 88%, respectively), whereas 10 μM (−)alprenolol is without effect. As shown in Fig. 3B, blockade of the receptor does not significantly alter the dissociation constant (K_D) of the remaining sites for ^{125}ICYP, which is consistent with irreversible interaction of the probe with the receptor.

Irreversible binding of [^{125}I]pBABC to purified hamster lung β-adrenergic receptor. In order to assess the ability of *p*BABC to label the β-adrenergic receptor, purified preparations of the receptor from hamster lung were used. [^{125}I] pBABC binds to soluble purified hamster lung β-adrenergic receptors. Following SDS-PAGE, there is a time-dependent increase in the labeling of a protein which exhibits a $M_r = 63{,}000$ (Fig. 4A). Prior treatment of [^{125}I] pBABC with cysteine still results in receptor-bound radioligand as assessed by measuring bound ligand as described in Materials and Methods; however, none is covalently bound as shown by the absence of radioactivity following SDS-PAGE (Fig. 4A, lane 9).

The time course of covalent association of [^{125}I] pBABC is shown in Fig. 4B, and it is characterized by an apparent "lag phase" of approximately 15 min over which there is relatively slow covalent incorporation. During the succeeding 15 min, increasing incorporation occurs until a maximum is reached at approximately 45 min. Maximum covalent incorporation under these conditions represents 30–40% of the specifically bound radioligand.

Using preparations of pure receptor, the peptide la-

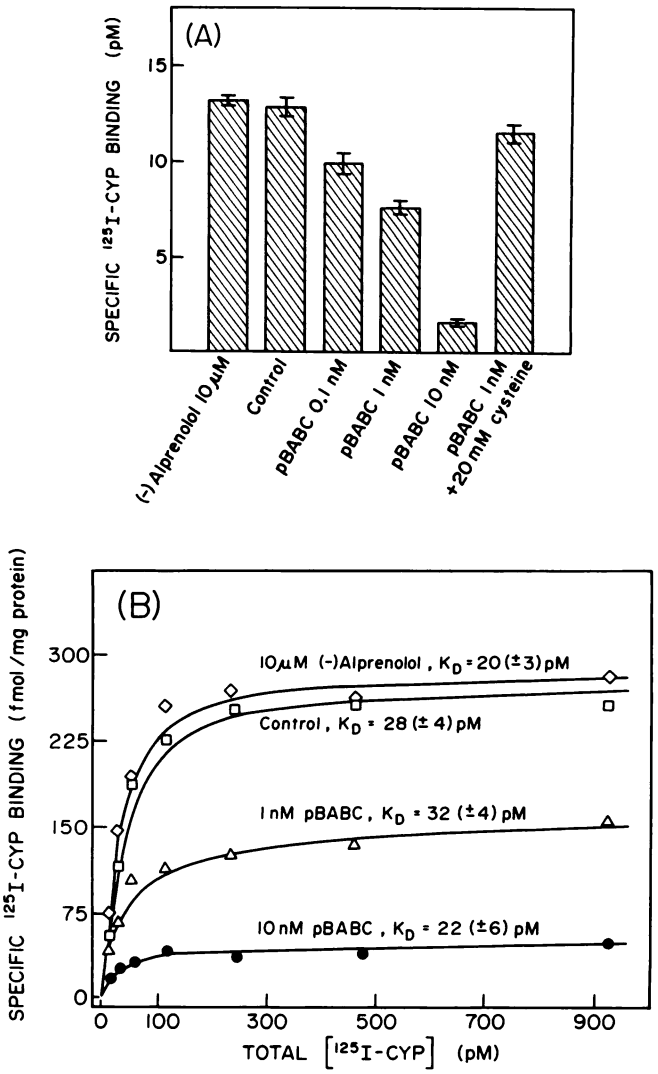

FIG. 3. *Inactivation of frog erythrocyte β-adrenergic receptors by pBABC*

A, frog erythrocyte membranes were incubated at 25° for 55 min in 50-ml centrifuge tubes containing 100 mM NaCl, 20 mM phosphate buffer, pH 7.2 (10 ml), and the indicated concentrations of drugs shown. One tube containing 1 nM *p*BABC was preincubated with 20 mM cysteine HCl, pH 7.2, at 25° for 30 min prior to membrane addition in order to deactivate the bromoacetyl moiety of *p*BABC. Following incubation, membranes were washed three times by centrifugation with 50 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 10 μM (−)alprenolol. The membranes were further incubated in 10 ml of this buffer for 17 hr at 25° using a slowly shaking water bath, and then washed three times further in alprenolol-free buffer. Final pellets were taken into 150 mM NaCl and 50 mM Tris-HCl, pH 7.4, and specific ^{125}ICYP binding was assessed as described in Materials and Methods using a saturating concentration (700–900 pM) of radioligand. Data shown are mean (± SE) of triplicate determinations of a single experiment repeated five times. B, ^{125}ICYP binding to *p*BABC-treated membranes. Membranes were treated with buffer (□), 10 μM (−)alprenolol (◇), 1 nM *p*BABC (△), and 10 nM *p*BABC (●) for 55 min at 25°, processed as described above, and assayed for specific ^{125}ICYP binding using increasing concentrations of radioligand. Results shown are representative data of experiments performed three times. Mean dissociation constants (K_D) for ^{125}ICYP are shown. Binding site maxima (femtomoles/mg of protein) were: control, 289 ± 30; alprenolol, 308 ± 16; 1 nM *p*BABC, 165 ± 22; and 10 nM *p*BABC, 35 ± 5.

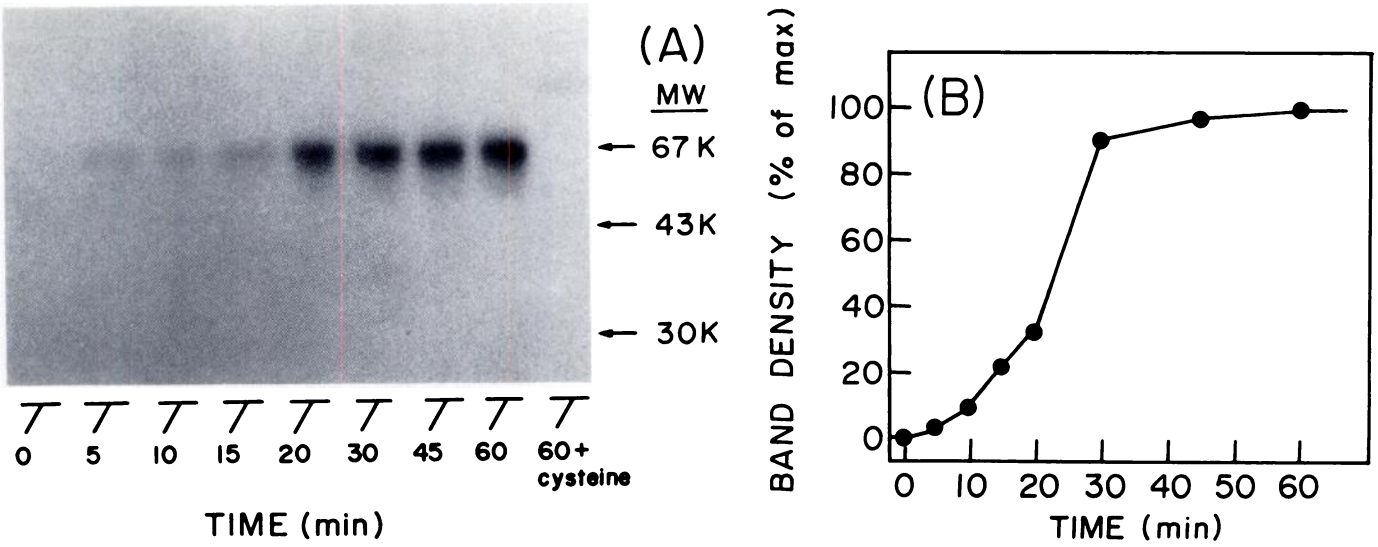

FIG. 4. *Time course of covalent interaction of [^{125}I]pBABC with purified hamster lung β-adrenergic receptors*

A, a purified hamster lung β-adrenergic receptor preparation (0.25 pmol) was incubated at 25° with [^{125}I]pBABC (200–400 pM) for the indicated times using incubation conditions described in Materials and Methods. The reaction was terminated by the addition of 25 μl of 200 mM L-cysteine HCl, pH 7.5, followed by immediate desalting on Sephadex G-50 columns. To one tube containing [^{125}I]pBABC was added 20 mM L-cysteine HCl, pH 7.4, and the contents were incubated at 25° for 30 min. Following the addition of soluble receptor, the quenched radioligand was incubated with the receptor preparation for 60 min as described above. Desalted Sephadex G-50 column eluates were processed for SDS-PAGE as described in Materials and Methods. B, autoradiograms were scanned by densitometry and band density was recorded as a measure of covalent incorporation of [^{125}I]pBABC. Band density (expressed as percentage of the maximum density observed at 60 min) is plotted versus incubation time. Points are the means of two experiments which differed by <10%.

beled by [^{125}I]pBABC was identical to that labeled nonspecifically by radioiodination with chloramine-T, or "specifically" by the photoaffinity β-adrenergic receptor antagonist [^{125}I]pABC (23). It was therefore essential to determine whether the covalent interaction of [^{125}I]pBABC with purified receptor was ligand-binding site specific. Fig. 5 demonstrates the pharmacological specificity of the labeling. Adrenergic agonists stereoselectively attenuate the labeling of the hamster lung β-adrenergic receptor and, in all cases, (−)isomers of the catecholamines are more potent than the (+)isomers (Fig. 5A). Inhibition of [^{125}I]pBABC labeling by catecholamines exhibits an order of potency: (−)isoproterenol > (−)epinephrine > (−)norepinephrine, which indicates a β_2-adrenergic receptor subtype classification of the labeled sites as originally defined by Lands *et al.* (26). Data shown in Fig. 5B for antagonists confirm the stereoselectivity and the β_2-adrenergic receptor subtype classification. Thus, (−)alprenolol is more potent than (+)alprenolol, although at the concentration used here (0.1 μM), (+)alprenolol significantly inhibited incorporation. At equimolar concentrations, the β_2-selective antagonist ICI 118551 is more potent than the β_1-selective antagonist Betaxolol. Phentolamine and haloperidol, potent α-adrenergic and dopamine receptor antagonists, are much less potent in blocking [^{125}I]pBABC labeling of the β-adrenergic receptors than if they were interacting at their respective receptors.

As one might expect, because of the extreme reactivity associated with bromoacetyl affinity ligands, [^{125}I]pBABC was not effective in specifically labeling the β-adrenergic receptor in membrane preparations. Treatment of membranes derived from frog or turkey erythrocyte and hamster lung resulted in the covalent labeling of multiple nonspecific membrane proteins rather than the specific receptor peptides (data not shown).

Comparison of [^{125}I]pBABC- and [^{125}I]pABC-labeled purified hamster lung β-adrenergic receptor by partial proteolytic digestion. It was of interest to investigate whether the domain of the receptor which was involved in covalent bond formation with [^{125}I]pBABC differed from that with the photoaffinity reagent [^{125}I]pABC. We therefore covalently labeled purified hamster lung receptor with both radioligands and exposed the labeled receptors to proteases. The peptide maps of the radioligand receptor fragments are shown in Fig. 6. Lanes 1 and 2 show β-adrenergic receptor peptides (M_r = 62,000) labeled with [^{125}I]pBABC and [^{125}I]pABC, respectively. A trace contaminant is apparent at M_r = 12,500 in the [^{125}I]pABC-labeled preparation. Treatment with trypsin results in a major peptide product at M_r = 38,000 with minor products of M_r = 22,000 (faint band) and 9,500. The pattern of tryptic products, although not identical, shows some similarities for both probes. [^{125}I]pABC-labeled receptor seems to be more extensively proteolyzed by trypsin than [^{125}I]pBABC-labeled receptor. This observation also holds for chymotrypsin and papain digestion. Treatment with *S. aureus* V8 protease results in a major product of M_r = 49,000.

A peptide fragment at M_r = 35,000 formed from [^{125}I]pBABC-labeled receptor is less apparent for the [^{125}I]pABC-labeled receptor (lane 6) although other products at M_r = 18,500 and 14,000 (faint bands) are common to both preparations. Papain causes extensive proteolysis

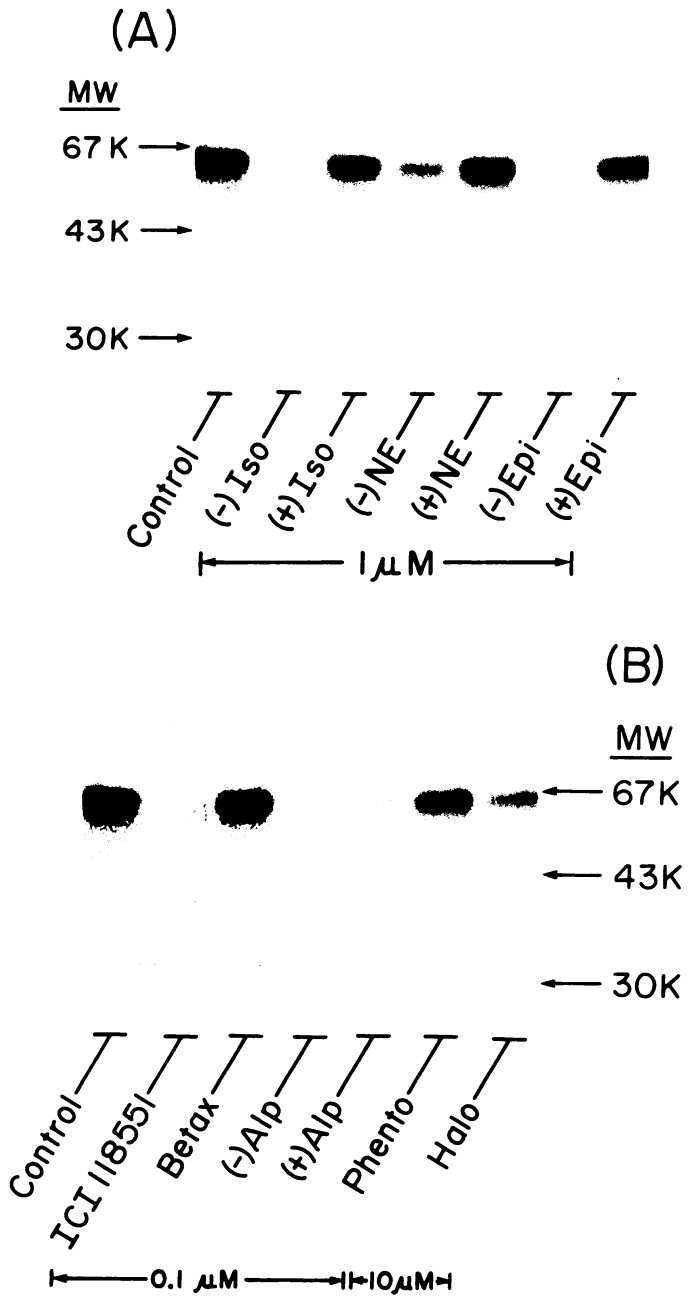

FIG. 5. *Pharmacological specificity of covalent [^{125}I]pBABC binding to purified hamster lung β-adrenergic receptors*

Purified hamster lung β-adrenergic receptors were labeled with [^{125}I] pBABC (250–300 pM) in the absence and presence of the indicated concentrations of agonists (A) or antagonists (B) during a 50–60-min incubation at 25°. The labeled receptors were processed for SDS-PAGE and autoradiography as described in Materials and Methods, using 10% polyacrylamide gels. Control, *Iso*, isoproterenol; *NE*, norepinephrine; *Epi*, epinephrine; *Betax*, Betaxolol, *Alp*, alprenolol; *Phento*, phentolamine; and *Halo*, haloperidol.

of labeled receptor and the major product has M_r = 12,000. Other radioiodinated peptides of intermediate electrophoretic mobilities, which are not apparent for [^{125}I] pABC-labeled preparations, may merely reflect the different rates of proteolysis. The patterns of chymotrypsin digestion were similar for both probes with the major proteolyzed product of M_r = 38,000. Although

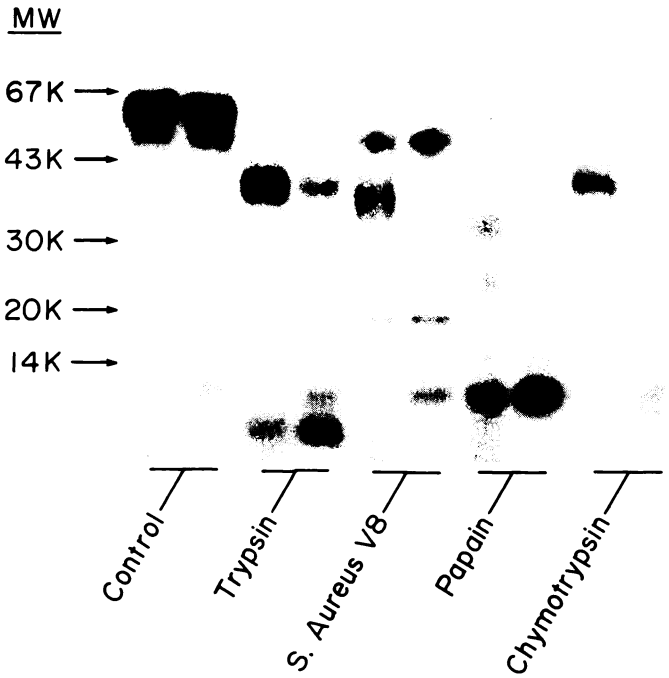

FIG. 6. *Peptide maps of [^{125}I]pBABC- and [^{125}I]pABC-labeled hamster lung β-adrenergic receptor*

Purified hamster lung β-adrenergic receptor (0.08 pmol) was labeled with [^{125}I]pBABC (60 min at 25°) as described in Materials and Methods and with [^{125}I]pABC as reported in Ref. 23. Efficiency of incorporation of [^{125}I]pABC in the receptor peptide was ~15% under the conditions used. In a medium containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.1% digitonin (30 μl), identical amounts of labeled receptor were digested for 15 min at 4° with trypsin (12.5 μg/ml), *S. aureus* V8 protease (120 μg/ml), papain (0.7 μg/ml), or chymotrypsin (50 μg/ml). Proteolysis was terminated by the addition of 3 μl of 3 mM *N*-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride. The samples were allowed to stand for 5–10 min at room temperature followed by the addition of SDS sample buffer after which the samples were electrophoresed on 12% polyacrylamide gels. [^{125}I]pBABC-labeled receptor is shown to the left of the treated pairs. Molecular weight standards are shown × 1000.

these data do not prove that both probes incorporate within the same peptide of the receptor, they suggest that incorporation occurs in the same general domain of the ligand-binding site.

DISCUSSION

This paper describes the biochemical characteristics of an affinity ligand (*p*BABC) which can interact with mammalian and nonmammalian β-adrenergic receptors with very high potency [EC_{50} = 430–910 pM for inhibition of ^{125}ICYP binding (125–150 pM)] and which can covalently label the purified hamster lung β-adrenergic receptor with an efficiency approaching 40%. It is a non-subtype-selective β-adrenergic receptor ligand with equal affinities for both β_1- and β_2-adrenergic receptors. This probe therefore has considerable advantage over the affinity ligand bromoacetylalprenolol menthane (14) which, although possessing high potency for frog eryth-

rocyte β-adrenergic receptors, has considerably reduced potency at mammalian β_1- and β_2-adrenergic receptors (7). This phenomenon, which is not shown by our carazolol derivative, probably relates to differences in the membrane preparation used or to the "atypical" nature of nonmammalian β-adrenergic receptors as defined by their affinity for certain β-adrenergic receptor antagonists (28).

Irreversible β-adrenergic receptor antagonists are normally characterized biochemically by incubation with β-adrenergic receptor preparations followed by extensive washing of the preparations and assay of β-adrenergic receptor number by ligand binding. Numerous washing protocols have been described (7, 12, 14, 27, 29–31) which vary considerably in their requirements. In view of the high affinity shown by *p*BABC for β-adrenergic receptors, the following wash procedure was followed. After incubation of the membranes with the probe *p*BABC (55 min at 25°), membranes were washed three times by centrifugation and then incubated for an extended period (17 hr at 25°) in the presence of 10 μM (−)alprenolol to prevent rebinding of any *p*BABC dissociating from the receptor. After this prolonged incubation period, membranes were washed an additional three times to remove dissociated *p*BABC and excess alprenolol and the receptors were assayed by ligand binding. The apparent loss of receptor number caused by *p*BABC was not reversed by such a washing protocol, which suggested an irreversible inactivation of receptors. Moreover, inactivation of the bromoacetyl function with cysteine resulted in a high affinity product but its ability to reduce the number of β-adrenergic receptors under the above conditions was considerably attenuated.

Whereas the data cited above are consistent with covalent interaction of the probe with the receptor, the ultimate criterion is to demonstrate direct incorporation of the labeled probe into the peptide of interest after SDS-PAGE (Figs. 4 and 5). Using purified hamster lung preparations, [^{125}I] pBABC labeled a β-adrenergic receptor peptide (M_r = 63,000) in a concentration- and time-dependent manner. The covalent interaction of [^{125}I] pBABC with purified hamster lung β-adrenergic receptor was an efficient process with up to 40% incorporation being recorded. This yield of incorporation is considerably greater than that found for photoaffinity reagents, where only 10–15% or less of the bound ligand is usually covalently incorporated into membrane-bound receptors (4, 21).

Covalent binding of [^{125}I] pBABC proceeded at pH optimum of 7.2 (data not shown) and with an unusual time course. Thus, we observed a lag phase of some 15 min followed by a more rapid rate of covalent labeling (Fig. 4B). Other workers have proposed that bromoacetyl affinity ligands bind to β-adrenergic receptors initially as reversible ligands and later as noncompetitive irreversible ligands (7), and our data agree with this postulate. Indeed, the nature of the inhibition of ventricular adenylate cyclase by bromoacetylalprenolol menthane has recently been reported to be competitive in the short term and irreversible in the long term (32).

We have also demonstrated that the interaction of [^{125}I] pBABC with purified β-adrenergic receptor occurred with the pharmacological specificity expected for an interaction with the β-adrenergic receptor ligand-binding site. Covalent labeling was inhibited stereoselectively by β-adrenergic receptor agonists and antagonists, but not by non-β-adrenergic agents. Drugs selective for β_2-adrenergic receptors were more potent inhibitors, which confirms the β_2-adrenergic receptor classification previously reported for this preparation (23).

The fact that, in membrane preparations, the use of [^{125}I] pBABC resulted in the nonspecific covalent labeling of multiple peptides underlines the high reactivity of bromoacetyl affinity probes. Clearly, affinity ligands will react rapidly with the available nucleophiles on tissue or membrane preparations and thereby decrease the free ligand concentration which is available for interaction with the receptor. This phenomenon has been noticed previously with ligands such as [^{3}H]bromoacetylalprenolol menthane (14) or [^{3}H]phenoxybenzamine (33). Moreover, reduction of binding site number by affinity ligands often requires excess concentration of the drug (14), which may relate to the loss of free drug bound to non-receptor sites. These observations imply the need for caution in the use of affinity ligands to study receptor-coupled events. Indeed, the depression of maximum contractile responses of guinea pig atria to the chloroacetyl affinity ligand Ro 03-7894 has been interpreted as evidence for noncompetitive irreversible, blocking activity of this compound (11). More recently, however, this interpretation has been questioned since, at the high concentrations used, this drug causes a nonspecific depressive effect on cardiac muscle preparations (34). Thus, the high reactivity of affinity ligands may limit their use as specific radioactive labeling probes in crude systems. However, it should not be concluded based on these difficulties that these probes are devoid of use in crude membrane systems or whole cells to assess receptor turnover and coupling. Such an irreversible compound, similar to *p*BABC, bromoacetylaminomethylpindolol, has recently been used to assess the turnover of β-adrenergic receptors in C6 glioma cells (35).

The target sites for affinity ligands are generally thought to be nucleophiles, either amino or sulfhydryl groups located at or near the ligand-binding sites of β-adrenergic receptors. Such a group, located near the alkylamine side chain-binding sites of β-adrenergic receptors of C6 glioma cells, has been reported to interact covalently with a photoactivated nitrophenyl ether derivative of propranolol (12). The carazolol derivatives *p*BABC and *p*ABC are substituted with functional "attacking" groups on a similar portion of the antagonist molecule, and they might be expected to insert into the β-adrenergic receptor at similar positions. It should be noted, however, that unlike affinity labels which can only react covalently with nucleophiles, the nitrene product of photosensitive probes such as *p*ABC can insert into any available chemical bond.

We have compared the radioliganded receptor fragments obtained following proteolysis of [^{125}I] pBABC- and [^{125}I] pABC-labeled receptors. In general, the peptide maps observed with both ligands were similar, although

not identical, which suggests similar points of covalent insertion for the two probes. Under the conditions used in these studies, [^{125}I]pABC-labeled receptor appeared more susceptible to the action of the various proteases. Clearly, further work will be necessary in order to define precisely the chemical nature of the covalent interaction of these ligands with the ligand-binding site of the β-adrenergic receptor. In the future, however, this new potent affinity ligand should prove to be a very useful compound for studies of purified β-adrenergic receptors, especially in view of the high efficiency of covalent incorporation.

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