Biochemical Pharmacology, Vol. 35, No. 10, pp. 1663–1669, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

AFFINITY LABELING OF THE DDT$_1$ MF-2 CELL α_1-ADRENERGIC RECEPTOR WITH [^{3}H]PHENOXYBENZAMINE

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(*Received* 20 *May* 1985; *accepted* 24 *October* 1985)

Abstract—In this study, we used phenoxybenzamine to label the α_1-adrenergic receptor of a smooth muscle cell line. Our results demonstrate a dose-dependent occupancy of α_1-adrenergic receptors by phenoxybenzamine determined by competition for the [^{3}H]prazosin binding site. Following incorporation of [^{3}H]phenoxybenzamine, partially purified membranes were solubilized and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Despite numerous Coomassie blue-stained bands, only three bands, $M_r = 80{,}000 \pm 500$, $M_r = 33{,}000 \pm 2{,}000$, and $M_r = 21{,}000 \pm 400$ (N = 4), were labeled with [^{3}H]phenoxybenzamine as determined by autofluorography. Incorporation of [^{3}H]phenoxybenzamine into the $M_r = 80{,}000$ band, but not the $M_r = 33{,}000$ and $M_r = 21{,}000$ bands, was affected by adrenergic agonists and antagonists in a manner consistent with an α_1-adrenergic interaction. Labeling of the $M_r = 33{,}000$ and $M_r = 21{,}000$ bands was partially blocked by phenoxybenzamine. We conclude that [^{3}H]phenoxybenzamine can be used as an affinity probe for the α_1-adrenergic receptor and that the ligand binding site of the α_1-adrenergic receptor resides in a $M_r = 80{,}000$ protein.

Stimulation of α_1-adrenergic receptors has been associated with several physiological events including contraction of smooth muscle [1, 2] and stimulation of glucose output from the liver [3, 4]. It is thought that α_1-adrenergic responses are mediated by calcium [5], although the precise molecular mechanisms involved are not completely understood. One possible approach to this problem would be the isolation of each of the cellular components involved in an α_1-adrenergic response, the ultimate goal being reconstitution of the functional complex in an artificial membrane. This has been accomplished for the acetylcholine receptor–sodium channel complex [6], but the constituents of the α_1-adrenergic receptor–calcium mobilization complex have eluded purification to this point.

In this report, we have utilized [^{3}H]phenoxybenzamine to label covalently the α_1-adrenergic receptor of the DDT$_1$ MF-2 cell. Phenoxybenzamine, a β-haloalkylamine, is an irreversible antagonist of α_1-adrenergic mediated events [7]. However, its usefulness as a probe for the α_1-adrenergic receptor has been questioned because of demonstrated interactions with α_2-adrenergic [8], histamine [9], dopamine [10], and muscarinic cholinergic membrane receptors [11]. The DDT$_1$ MF-2 cell line used in this study was derived from a leiomyosarcoma of a Syrian hamster vas deferens [12] and has been demonstrated to possess α_1-adrenergic receptors. α_2-Adrenergic receptors are not detectable in this cell line [13, 14].

Our results demonstrate a dose-dependent blockade of DDT$_1$ MF-2 cell α_1-adrenergic receptors with phenoxybenzamine. Furthermore, SDS-PAGE§ of DDT$_1$ MF-2 cell membranes labeled with [^{3}H]phenoxybenzamine indicates that the binding site of the DDT$_1$ MF-2 cell α_1-adrenergic receptor resides on a peptide with an apparent molecular weight under reducing conditions of 80,000. These findings support the use of [^{3}H]phenoxybenzamine as a probe for more detailed studies of the molecular characteristics of the DDT$_1$ MF-2 cell α_1-adrenergic receptor.

MATERIALS AND METHODS

Chemicals used. [^{3}H]Prazosin (sp. act. = 80.9 Ci/mmole) and [^{3}H]phenoxybenzamine (sp. act. = 55.8 Ci/mmole) were obtained from the New England Nuclear Corp. Phenoxybenzamine HCl was a gift from Smith, Kline & French Laboratories. Other adrenergic agonists and antagonists were obtained from sources previously mentioned [13, 14]. Hydrofluor and Autofluor were purchased from National Diagnostics. Molecular weight standards were purchased from Bio-Rad Laboratories. Other chemicals were reagent grade or better obtained from commercial sources.

Membrane preparation. DDT$_1$ MF-2 cells were grown in suspension culture as previously described [13]. Cells were harvested, centrifuged at 800 *g* for

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§ Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; [^{125}I]APDQ, 4-amino-6,7- dimethoxy- 2- [4- [5- (4- azido- 3- [^{125}I]iodophenyl)-pentanoyl]-1-piperazinyl]quinazoline; and [^{125}I]APD, 2-[4-(4-azido-3-[^{125}I]iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline.

10 min, and resuspended in 50 mM Tris·HCl, pH 7.4, at 4° (all further operations are at 4°). Following centrifugation at 800 *g* for 10 min and resuspension in 50 mM Tris·HCl, pH 7.4, cells were disrupted in a Wheaton glass–teflon homogenizer (ten strokes). Further disruption was accomplished by sonication (six bursts of 5-sec duration at 1-min intervals) using a Branson Biosonic Sonicator (1 cm tip, setting of 60). A partially purified membrane preparation was obtained by differential centrifugation as follows. The supernatant fraction resulting from a centrifugation at 600 *g* for 10 min to remove nuclei and intact cells was centrifuged at 30,000 *g* for 15 min. The resulting membrane pellet was washed once with 50 mM Tris·HCl, pH 7.4, and again centrifuged at 30,000 *g* for 15 min. Partially purified membranes were resuspended in 50 mM Tris·HCl, pH 7.4, and protein was determined [15] using bovine serum albumin as the standard. Membranes were always used the day of preparation.

Radioligand assays. Binding of [^{3}H]prazosin to partially purified membranes prepared from DDT_1 MF-2 cells was carried out in polypropylene tubes in a total volume of 250 μl. [^{3}H]Prazosin was diluted in 50 mM Tris·HCl, pH 7.4, containing 20% ethanol and was added to assay tubes in a 25 μl volume. In saturation experiments, concentrations ranged from 0.049 to 2.42 nM. Phentolamine, used to define nonspecific binding, was dissolved in 1.0 mM HCl and was added to assay tubes in a 25 μl volume at a final concentration of 10 μM. Membrane preparations were added to assay tubes in a 200 μl volume to initiate the assay. Protein concentration was either 0.16 mg/ml or 0.08 mg/ml in the assay tubes. Incubations were carried out at 30° for 15 min at which time bound and free [^{3}H]prazosin were separated by vacuum filtration through Whatman GF/C filters. Radioactivity associated with filters was determined with a Packard model 460C scintillation counter at 45% efficiency.

Blockade of α_1-adrenergic receptors with phenoxybenzamine. In 50-ml polypropylene centrifuge tubes, 1 mg of membrane protein and either 25 μl of 1.0 mM HCl or 25 μl of phenoxybenzamine dissolved in 1.0 mM HCl were combined in a 2.5 ml volume with 150 mM NaCl, 5 mM EDTA, 50 mM Tris·HCl, pH 7.5 (Buffer A), as the buffer. The incubation was carried out for 20 min at 25° in the dark. To remove free phenoxybenzamine, 35 ml of 0.5% BSA in Buffer A was added and tubes were centrifuged at 30,000 *g* for 10 min twice with resuspension of the pellet between centrifugations. The pellet was resuspended in Buffer A, centrifuged at 30,000 *g* for 10 min, and the pellet resuspended in 1.0 ml of Buffer A. Protein concentration was determined [15], and [^{3}H]prazosin radioligand assays were carried out as described above.

Receptor labeling with [^{3}H]phenoxybenzamine. The procedure was the same as described in the preceding section except that adrenergic competitors were suspended in 1.0 mM HCl and added in a 25 μl volume, and the final pellet was suspended in 150 μl of Buffer A. Protein concentration was determined [15] using bovine serum albumin as the standard prior to SDS-PAGE.

SDS-PAGE and autofluorography. Samples in SDS sample buffer were heated to 100° for 2 min prior to electrophoresis on 5–15% gradient pore gels [16]. Molecular weight standards were phosphorylase B, M_r = 92,500; bovine serum albumin, M_r = 66,200; ovalbumin, M_r = 45,000; soybean trypsin inhibitor, M_r = 21,300; and lysozyme, M_r = 14,400. Visualization of protein was achieved by staining gels with 0.25% Coomassie Brilliant Blue R250. For autofluorography, gels were immersed in Autofluor for 1 hr, dried for 1.5 hr under vacuum, and exposed to Kodak AR X-OMAT film at −76° for 3 weeks in cassettes with Cronex intensifying screens. Densitometric tracings of autofluorographs were made with an E-C model 910 Transmission Densitometer. A light aperture of 0.1 × 3 mm, a wavelength of 540 nm, and a scanning speed of 1.1 inch/min were used.

Calculations. Results are presented as the mean ± S.E. with the number of determinations in parentheses. Molecular weights were determined from a plot of the relative mobility of standards with known molecular weight versus the log of their molecular weight.

RESULTS

Prior to the phenoxybenzamine-labeling experiments, saturation analysis with [^{3}H]prazosin was carried out on aliquots of each of the six membrane preparations used in this study. Consistent with previously published results [17], a single saturable site was observed with a [^{3}H]prazosin dissociation constant of 0.28 ± 0.06 nM (N = 6) and a binding site concentration of 1049 ± 185 fmoles/mg protein (N = 6). Specific binding was 93 ± 2% (N = 6) with added [^{3}H]prazosin equal to 0.1 nM and 82 ± 4% (N = 6) with added [^{3}H]prazosin equal to 3.0 nM. Specific binding of [^{3}H]prazosin to DDT_1MF-2 cell membranes was linear at protein concentrations ranging from 0.05 to 0.2 mg/ml (data not shown).

Preincubation of DDT_1 MF-2 membranes with phenoxybenzamine followed by extensive washing to remove unbound phenoxybenzamine resulted in a dose-related reduction in α_1-adrenergic receptor number with no change in the [^{3}H]prazosin dissociation constant (Fig. 1). A small (approximately 16%) loss in [^{3}H]prazosin binding sites was observed in control membranes that were not incubated with phenoxybenzamine yet were taken through the washing procedure. Therefore, results in Fig. 2 are presented as a percentage of the binding to washed membranes not exposed to phenoxybenzamine. Approximately 50% of the α_1-adrenergic receptors were blocked with 10 nM phenoxybenzamine (Fig. 2). Increasing the concentration of phenoxybenzamine to 100 nM resulted in approximately 95% blockade of the α_1-adrenergic receptors (Fig. 2).

A representative Coomassie blue-stained gel of DDT_1 MF-2 cell membranes is shown in Fig. 3. The corresponding autofluorograph along with densitometric tracings of each lane are shown in Fig. 4. Despite numerous Coomassie blue-stained bands (Fig. 3), only three bands were labeled by [^{3}H]-phenoxybenzamine (Lanes A and B, Fig. 4). The calculated molecular weights from four separate

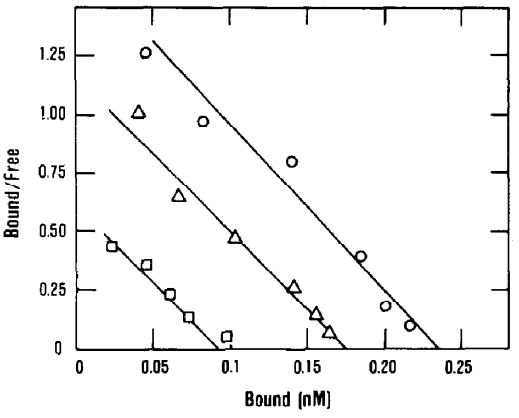

Fig. 1. Scatchard analysis of [^{3}H]prazosin binding to DDT$_1$ MF-2 cell membranes previously incubated with phenoxybenzamine. Membranes (1 mg) were incubated with either vehicle (○), 2.0 nM phenoxybenzamine (△), or 10 nM phenoxybenzamine (□) for 20 min at 25° as described in the text. The membranes were washed, protein concentration was determined [15], and [^{3}H]prazosin binding assays were carried out as described in the text.

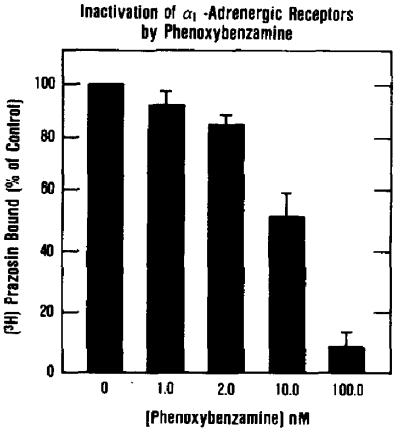

Fig. 2. Inhibition of [^{3}H]prazosin binding to DDT$_1$ MF-2 cell membranes by phenoxybenzamine. Membranes (1 mg) were incubated with either vehicle or the indicated concentration of phenoxybenzamine for 20 min at 25° as described in the text. The membranes were washed, protein concentration was determined [15], and [^{3}H]prazosin binding assays were carried out as described in the text. Results are reported as the means ± S.E. (N = 5).

experiments were 80,000 ± 500, 33,000 ± 2,000 and 21,000 ± 400. No other bands with incorporated radioactivity were observed (N = 4).

Incorporation of radioactivity into the M_r = 80,000 band was affected by adrenergic agonists and antagonists in a manner consistent with an α_1-adrenergic interaction. For instance, incorporation of radioactivity into the M_r = 80,000 band was blocked by 0.1 μM prazosin (Lane C), an α_1-selective antagonist, but was not affected by 0.1 μM yohimbine (Lane D), an α_2-selective antagonist. The biologically active (−)-stereoisomer of epinephrine at 30 μM (Lane E) blocked incorporation of radioactivity into the M_r = 80,000 band, whereas the biologically inactive (+)-stereoisomer of epinephrine at 30 μM (Lane F) partially blocked incorporation

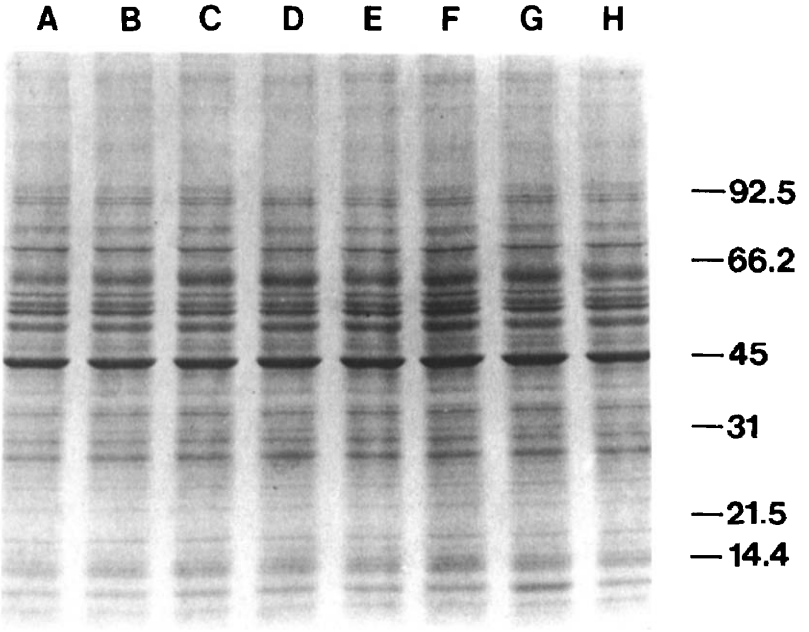

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie blue for protein visualization. Membranes (1 mg) were incubated for 20 min at 25° with 6.5 nM [^{3}H]-phenoxybenzamine and either vehicle (Lanes A and B); 0.1 μM prazosin (Lane C); 0.1 μM yohimbine (Lane D); 30 μM (−)-epinephrine (Lane E); 30 μM (+)-epinephrine (Lane F); 30 μM (−)-norepinephrine (Lane G); and 30 μM (−)-isoproterenol (Lane H). Following a washing procedure to remove free [^{3}H]phenoxybenzamine, membranes were prepared for SDS-PAGE as described in the text. Forty-nine micrograms of protein was applied to each lane. This experiment was repeated four times with identical results.

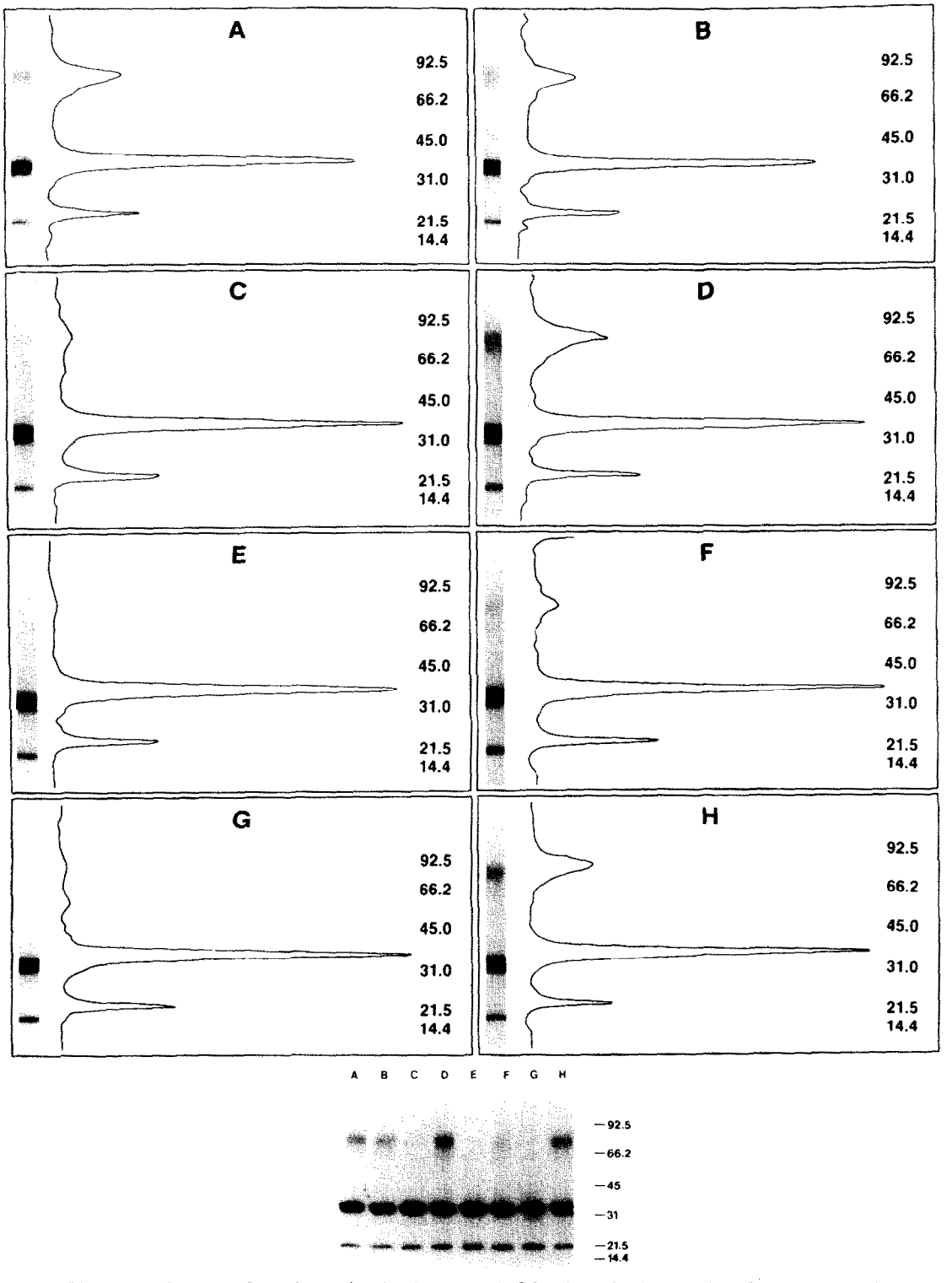

Fig. 4. Autofluorography and scanning densitometry of SDS polyacrylamide gel. Autofluorography and scanning densitometry of the SDS-polyacrylamide gel from Fig. 3 were performed as described in the text. Treatments by Lane are given in the legend to Fig. 3. This experiment was repeated four times with identical results.

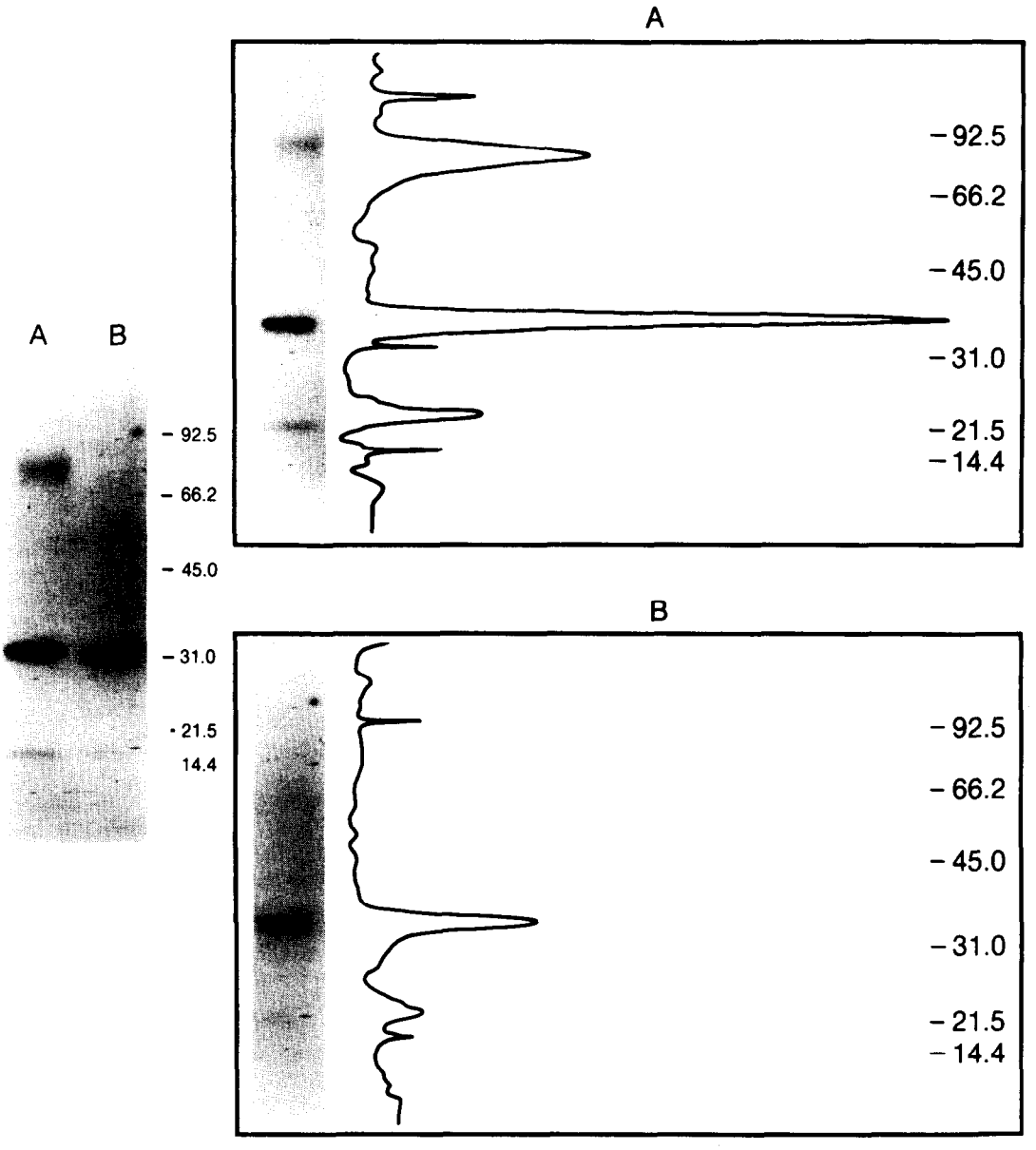

Fig. 5. Effect of unlabeled phenoxybenzamine on [^{3}H]phenoxybenzamine incorporation into DDT$_1$ MF-2 cell membranes. Membranes (1 mg) were incubated for 20 min at 25° with 2.2 nM phenoxybenzamine and either vehicle (Lane A) or 0.1 μM phenoxybenzamine (Lane B). Following a washing procedure to remove free [^{3}H]phenoxybenzamine, membranes were prepared for SDS-PAGE. Autofluorography and scanning densitometry were performed as described in the text. Thirty-six micrograms of protein was applied to each lane. This experiment was performed three times with identical results.

of radioactivity into the $M_r = 80{,}000$ band. (−)-Norepinephrine at 30 μM (Lane G) blocked incorporation of radioactivity into the $M_r = 80{,}000$ band and 30 μM (−)-isoproterenol (Lane H), a β-adrenergic agonist, did not affect labeling of the $M_r = 80{,}000$ band.

The incorporation of radioactivity into the $M_r = 33{,}000$ and $M_r = 21{,}000$ bands was not affected by the adrenergic agonists and antagonists used above. Typically, however, 0.1 μM phenoxybenzamine, besides blocking incorporation of radioactivity into the $M_r = 80{,}000$ band, also partially reduced incorporation of radioactivity into the $M_r = 33{,}000$ and $M_r = 21{,}000$ bands (Fig. 5).

DISCUSSION

Phenoxybenzamine and related β-haloalkylamines were first noted for their prolonged adrenergic antagonism which was suggested to be due to irreversible blockade of the receptor [7]. Subsequently, phenoxybenzamine was demonstrated to form a covalent bond with the receptor following an initial competitive interaction [18]. Despite extensive use of

phenoxybenzamine as an α-adrenergic antagonist for a number of years, controversy exists concerning its pharmacological selectivity. Phenoxybenzamine has been shown to be a more potent inactivator of α_1-adrenergic receptors compared to α_2-adrenergic receptors [19]. However, in addition to interaction with α-adrenergic receptors, phenoxybenzamine has been shown to interact with muscarinic cholinergic receptors [11], H_1 histamine receptors [9], and dopamine receptors [10]. Most recently, [^{3}H]phenoxybenzamine has been used to identify the binding site subunit of the α_2-adrenergic receptor [20].

The results of our study indicate that [^{3}H]phenoxybenzamine can be used as an affinity probe for the DDT_1 MF-2 cell α_1-adrenergic receptor. Under the experimental conditions employed in this study, [^{3}H]phenoxybenzamine consistently labeled three proteins of which only one, $M_r = 80{,}000$, displayed pharmacological characteristics consistent with those of the α_1-adrenergic receptor. Specifically, only adrenergic agents known to interact with α_1-adrenergic receptors blocked incorporation of [^{3}H]phenoxybenzamine into the $M_r = 80{,}000$ peptide, while the β-adrenergic agonist, (−)-isoproterenol, and the α_2-adrenergic selective antagonist, yohimbine, were unable to prevent labeling of the $M_r = 80{,}000$ peptide.

Although our data were the first demonstrating that phenoxybenzamine is an affinity ligand for the α_1-adrenergic receptor of smooth muscle, [^{3}H]-phenoxybenzamine has been used previously in attempts to label and characterize α_1-adrenergic receptors in rat liver. Initially, Guellaen *et al.* [21] reported partial purification of a $M_r = 44{,}800$ protein by SDS-PAGE that bound [^{3}H]phenoxybenzamine. Kunos *et al.* [22] observed two proteins that were labeled by [^{3}H]phenoxybenzamine, $M_r = 80{,}000$ and $M_r = 58{,}000$, as determined by SDS-PAGE. Prazosin blocked incorporation of [^{3}H]phenoxybenzamine into the $M_r = 80{,}000$ and $M_r = 58{,}000$ proteins; no other adrenergic agents were used to conclusively demonstrate specificity of the labeled proteins.

More recently, two photoaffinity ligands have been used to characterize the molecular properties of the α_1-receptor in a variety of tissues. Both ligands are structurally similar to prazosin and both are α_1-selective. 4-Amino-6,7-dimethoxy-2-[4-[5-(4-azido-3 - [^{125}I]iodophenyl)pentanoyl] - 1 - piperazinyl] quinazoline ([^{125}I]APDQ) has been shown to label a $M_r = 79{,}000$ protein in cerebral cortex with pharmacological characteristics of the α_1-adrenergic receptor [23]. In liver, at least five proteins ($M_r = 80{,}000$, $M_r = 52{,}000$, $M_r = 42{,}000$, $M_r = 34{,}000$, and $M_r = 16{,}000$) were seen on autoradiograms following SDS-PAGE of [^{125}I]APDQ-labeled membranes [24]. Only the $M_r = 59{,}000$ protein was labeled in the presence of 0.1 μM prazosin, suggesting that this protein was non-specifically labeled by [^{125}I]APDQ. The smaller molecular weight proteins were thought to be proteolytic fragments of the $M_r = 80{,}000$ protein. Seidman *et al.* [25] have used 2-[4-(4-azido-3-[^{125}I]-iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline ([^{125}I]APD) to label the α_1-adrenergic receptor in rat liver membranes. SDS-PAGE of [^{125}I]APD-labeled membranes results in five labeled proteins: $M_r = 77{,}000$, $M_r = 68{,}000$, $M_r = 59{,}000$, $M_r = 42{,}000$ and $M_r = 31{,}000$. The $M_r = 42{,}000$ and $M_r = 31{,}000$ proteins did not display pharmacological specificity expected of an α_1-adrenergic receptor. Finally, our laboratory has published evidence that [^{125}I]APD specifically labels a $M_r = 81{,}000 \pm 2{,}000$ (N = 4) protein in DDT_1 MF-2 cell membranes using the same experimental procedures described in this report [17].

However, using [^{3}H]phenoxybenzamine, two additional proteins, $M_r = 33{,}000$ and $M_r = 21{,}000$, were observed to be labeled in DDT_1 MF-2 cells. Of the adrenergic agonists and antagonists tested, only 0.1 μM phenoxybenzamine blocked labeling of these peptides. At the present time, the identity of these lower molecular weight proteins is not clear. However, they do not appear to be identical to any of the low molecular peptides labeled in liver membranes as observed by Leeb-Lundberg *et al.* [24] and Seidman *et al.* [25]. For instance, we have shown that [^{125}I]APD does not label smaller molecular weight peptides in DDT_1 MF-2 cell membranes under conditions identical to these used in the current study [17]. Thus, it appears that the $M_r = 33{,}000$ and $M_r = 21{,}000$ peptides labeled by [^{3}H]-phenoxybenzamine are not proteolytic products of the α_1-adrenergic receptor. Further, of the adrenergic agonists and antagonists tested, only phenoxybenzamine blocked labeling of the $M_r = 33{,}000$ and $M_r = 21{,}000$ peptides by [^{3}H]phenoxybenzamine. In addition, from the studies of Guellaen *et al.* [21] and Kunos *et al.* [22], [^{3}H]phenoxybenzamine does not appear to label liver membrane peptides in this molecular weight range. Therefore, it appears that the $M_r = 33{,}000$ and $M_r = 16{,}000$ peptides labeled by [^{3}H]-phenoxybenzamine in DDT_1 MF-2 membranes are not fragments of the binding site containing portion of the α_1-adrenergic receptor. The identity of these proteins will require additional experimentation.

In summary, we have presented evidence which indicates that [^{3}H]phenoxybenzamine can be used successfully as an affinity probe for the α_1-adrenergic receptor. Our report is the first to demonstrate α_1-adrenergic specificity of a peptide covalently labeled with [^{3}H]phenoxybenzamine using known adrenergic agonists and antagonists. Additional studies are underway aimed toward establishing the functional properties of the $M_r = 80{,}000$ protein in mediating α_1-adrenergic response.

Acknowledgements—We acknowledge the technical assistance of Robert Waldron, Steward MacLeod and Timothy Taylor, and the secretarial assistance of Mrs. Patricia Johnson and Ms. Yvonne Rodriguez. This work was supported by National Institutes of Health Grant GM 30669.

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