Photoaffinity label for the $\alpha_1$-adrenergic receptor: Synthesis and effects on membrane and affinity-purified receptors

HANS-JÜRGEN HESS*, ROBERT M. GRAHAM†, AND CHARLES J. HOMCY‡

*Pfizer Central Research, Groton, Connecticut 06340; and †Cellular and Molecular Research Laboratory, Cardiac Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

Communicated by Frederic M. Richards, December 13, 1983

ABSTRACT An azide analog, 2-[4-(4-azidobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP59,430), of the highly selective $\alpha_1$-adrenergic receptor antagonist prazosin was synthesized and its effects on rat hepatic membrane and affinity-purified $\alpha_1$-adrenergic receptor preparations were examined. CP59,430 behaved as a competitive antagonist before photolysis. When the membrane or purified preparations pretreated with CP59,430 were irradiated with UV light, CP59,430 behaved as a noncompetitive antagonist. Labeling of membrane $\alpha_1$-adrenergic receptors was irreversible; repeated dialysis or washing could not reverse the photolysis-induced inactivation by CP59,430, whereas dialysis completely reversed the antagonism by the same concentration of the label prior to photolysis. Additionally, photolabeling of purified receptors was resistant to Sephadex G-50 chromatography, whereas in the absence of photolysis the same concentration of CP59,430 or prazosin (10 $\mu$M) could be readily removed by this procedure. CP59,430 appears to label specifically only $\alpha_1$-adrenergic receptors because prazosin protected the membrane and purified receptors from photolysis-induced inactivation by CP59,430. Furthermore, specific [$^3$H]dihydroalprenolol and [$^3$H]yohimbine binding to membrane $\beta$- and $\alpha_1$-adrenergic receptors, respectively, was unchanged by CP59,430 at 1 $\mu$M, a concentration that decreased specific [$^3$H]prazosin binding to $\alpha_1$-adrenergic receptors by 72%. In additional studies, the photolysis-induced receptor inactivation by CP59,430 remained unchanged in the presence of the scavenger p-aminobenzoic acid. It is likely, therefore, that receptor labeling by CP59,430 occurs via a true photoaffinity mechanism. CP59,430, which specifically and irreversibly labels the $\alpha_1$-adrenergic receptor after photolysis, should thus be uniquely valuable for the molecular characterization of this receptor.

While considerable progress has been made with the isolation, purification, and molecular characterization of a number of hormone and drug receptors (1–3), studies of the $\alpha$-adrenergic receptor have been hampered by the lack of suitable techniques for the isolation and characterization of the minute quantities of this receptor present in most cells. In recent years, photoaffinity labels for several hormone and drug receptors have been developed and have aided greatly in the identification and biophysical characterization of these receptors (4–7). Such photoaffinity probes offer the advantage over affinity labels of being completely reversible until photactivated. Reliable affinity constants can thus be obtained, and the labels can be characterized prior to their photolysis and concomitant irreversible binding. We have previously described the development and application of an affinity matrix synthesized with an analog of the highly selective $\alpha_1$-adrenergic receptor antagonist prazosin, which permitted purification of the $\alpha_1$-adrenergic receptor (8–10). To further aid in the molecular characterization of this receptor, a specific photoaffinity label has been developed. We report here on the synthesis of this compound, 2-[4-(4-azidobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP59,430), which is also an analog of prazosin [2-[4-(2-formyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline], and on its characterization as a photoaffinity label for the membrane-bound and purified $\alpha_1$-adrenergic receptor.

MATERIALS AND METHODS

Materials. [$^3$H]Yohimbine was purchased from New England Nuclear and p-aminobenzoic acid from Sigma. The sources of all other reagents and compounds were as described (8).

Synthesis of CP59,430. A solution of 2-[1-piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (838 mg, 2.90 mmol) prepared as described (11) and the N-hydroxy succinimide ester of 4-azidobenzoic acid (12) (830 mg, 3.19 mmol) in 10 mL of dimethylformamide was stirred in the dark for 18 hr. The reaction mixture was diluted with ethyl acetate and filtered. The filtrate was then washed successively with water and brine and dried with MgSO$_4$. The MgSO$_4$ was filtered and the filtrate was concentrated. The resulting concentrate was chromatographed on 50 g of silica gel (70–230 mesh), with ethyl acetate as the eluant, to furnish 500 mg (40%) of CP59,430 as a cream-colored solid, mp 193–195°C (TLC, CH$_2$Cl$_2$/MeOH, 8:1 (vol/vol), $R_f = 0.4$), which in turn was recrystallized [mp 195–196°C (decomposition)] from ethanol.

Preparation of Membranes and Receptor Purification. Rat hepatic membranes were prepared by a modification of the technique of Williams et al. (13). Briefly, after removal, the liver was placed into ice-cold buffer (buffer A, 0.25 M sucrose/1 mM MgCl$_2$/5 mM Tris-HCl pH 7.1 at room temperature). The liver was then minced and homogenized in buffer A (10 mL/g of liver) with 10 strokes of a Dounce tissue grinder. The homogenate was filtered twice through four layers of cheesecloth and centrifuged at 400 × g for 30 min at 4°C, and the supernatant was recentrifuged at 28,000 × g for 15 min at 4°C. The resulting pellet was washed six times by resuspension in ice-cold buffer (buffer B, 5 mM MgCl$_2$/5 mM EDTA/100 mM Tris-HCl, pH 7.1 at room temperature). The resulting pellet was resuspended in buffer B, rapidly frozen in an acetone/dry ice bath, and stored in liquid N$_2$. Prior to use the membranes were rapidly thawed in a 25°C bath and washed twice by resuspension and centrifugation at 28,000 × g in buffer B. Membranes prepared by this technique had binding characteristics identical to those of membranes prepared by the method of Ray (14) as described previously (8); however, the yield of membrane protein

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: CP59,430, 2-[4-(4-azidobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline.
Effect of irradiated for 23.22.
Calculated
ysis.
Neither light
in
binding
membranes and
purified
products,
(254 nm)
membrane-bound
receptors were
described
membrane
and purified
receptors
enhanced.
A1-Adrenergic
receptor. The
ability of CP59,430
to interact
with the
membrane
and purified
receptors
was characterized
as shown in Figs.
2 and 3. In
competition
binding
experiments
with 
[3H]prazosin,
CP59,430 was
found to interact
with the
membrane
and purified
receptor
with relatively
high affinity
(Kd = 8.5 ± 2.4 \times 10^{-8} \text{M}, n = 4, membrane; Kd = 6.3 ±
1.5 \times 10^{-8} \text{M}, n = 3, purified). This
affinity was less
than that of the
parent compound,
prazosin (Kd = 5.8 \times 10^{-10} \text{M}
and 1.6 \times 10^{-9} \text{M}, respectively). However,
like prazosin,
CP59,430 retains
selectivity,
because it
competes
potently
for 
[3H]prazosin
binding
to the membrane
or purified
receptors
than does the
a2-selective
agonist
drauzolsine
(Kd = 14 \times 10^{-9} \text{M}
and 87 \times 10^{-9} \text{M}, respectively)
(2).
Photoysis
of CP59,430
in buffer
alone
did not
convert
it to a
product
of higher
affinity.
Scatchard analysis
of
equilibrium
binding
studies
revealed
that,
prior
to
photoysis,
CP59,430

RESULTS

Structure and Properties of CP59,430. The structure
of CP59,430 (Fig. 1) was confirmed by
elemental and NMR
analysis.
Calculated
for
C_{25}H_{20}N_{10}O_{7}C_{6}H_{5}OH: C, 57.48; H, 5.87;
N, 23.32.
Found: C, 57.41; H, 5.92; N, 23.22.
The NMR spectrum
[(\text{CHCl}_3)\delta: 7.48 (2H, doublet, aromatic); 7.09 (2H,
doublet, aromatic); 6.92 (1H, singlet, aromatic); 6.90
(1H, singlet, aromatic); 5.2 (2H, singlet, NH_2); 3.97
(3H, singlet, OCH_3); 3.93
(3H, singlet, OCH_3); 3.70 (3H, multiplet, aliphatic)]
was recorded on a
Bruker
WM250
spectrometer.
The UV-visible
spectrum
[(\text{CHCl}_3) \lambda_{\text{max}}, 255, 340; \varepsilon, 60,400, 6,600] was
recorded
on a Cary 15
spectrophotometer
in
matched
quartz
vessels
with
water
in
the
reference
cell.
After
photolysis
(Fig. 1)
there
was
a
fall in the
shoulder
at 265 nm,
which corresponds
to
the
wavelength
of
maximal
absorption
of the
p-azidobenzoic
acid
moiety
of
CP59,430.
This
change
in
the
UV
spectrum
was
already
apparent
after
photolysis
for 5
min
with
little
additional
change
after 15
min.
The
shoulder
at
265 nm
was
not
apparent
in
the
UV
absorption
spectrum
of
the
parent
compound
prazosin,
and
there
was
no
change
in
the
UV
spectrum
of
this
compound
after
15
min
of
photolysis
(Fig. 1 Inset).

Interaction
of CP59,430
with
the
Membrane
and Purified
a2-Adrenergic
Receptor. The ability
of
CP59,430
to
bind
to
the
membrane
and
purified
a2-adrenergic
receptor
was characterized
as shown in Figs.
2 and 3. In
competition
binding
experiments
with 
[3H]prazosin,
CP59,430 was
found to interact
with the
membrane
and purified
receptor
with relatively
high affinity
(Kd = 8.5 ± 2.4 \times 10^{-8} \text{M}, n = 4, membrane; Kd = 6.3 ±
1.5 \times 10^{-8} \text{M}, n = 3, purified). This
affinity was less
than that of the
parent compound,
prazosin (Kd = 5.8 \times 10^{-10} \text{M}
and 1.6 \times 10^{-9} \text{M}, respectively). However,
like prazosin,
CP59,430 retains
selectivity,
because it
competes
potently
for 
[3H]prazosin
binding
to the membrane
or purified
receptors
than does the
a2-selective
agonist
drauzolsine
(Kd = 14 \times 10^{-9} \text{M}
and 87 \times 10^{-9} \text{M}, respectively)
(2).
Photoysis
of CP59,430
in buffer
alone
did not
convert
it to a
product
of higher
affinity.
Scatchard analysis
of
equilibrium
binding
studies
revealed
that,
prior
to
photoysis,
CP59,430

(11 mg/g
wet
weight
of
liver)
was markedly
enhanced.
Protein
concentrations
were determined by the
method
of
Lowry
et
al.
(15).

a2-Adrenergic
receptors
were purified,
after
solubilization
of
the
membrane-bound
receptors
with
digitonin,
by
affinity
cromatography
using
CP57,609-agarose
as
described
(8). A 100-
to
200-fold
purification
was
achieved
by
this
procedure.
The
specific
activity
of
this
affinity-purified
preparation
was
30–40
pmol/mg.

Binding Assays. Binding
of
the
radioligands
to the
hepatic
membranes
and
to
the
purified
receptor
preparations
was
determined
as described
(8).
Specific
[3H]prazosin
and
[3H]yohimbine
binding
referred
to
that
fraction
of
binding
inhibited
by
10
\mu M
phenotamine.
Specific
[3H]dihydroalprenolol
binding
referred
to
that
fraction
of
the
binding
inhibited
by
0.1
mM
(-)-isoproterenol.

Photolysis. Membranes
or
the
purified
receptor
preparations
were
incubated
with
circular
CP59,430
to
the
desired
concentration
in
buffer
B
(see
figure
legends
for
details)
and
then
placed
in
quartz
vessels.
Photolysis
was
initiated
by
exposure
to
ultraviolet
light
2–4
cm
from
the
source
for
15
min;
the
source
was
a
short-wavelength
(254
nm)
UV
lamp
(UVS-11
Mineral-
light; Ultraviolet
Products,
San
Gabriel,
CA).
Similar
results
were
obtained
with
a
UVSL
long-wavelength
lamp
(Ultraviolet
Products).
Neither
light
source
affected
either
the
membrane
or
purified
receptor
activity
after
15
min
of
exposure.

![Graph](attachment:graph.png)

FIG. 1. Effect
of
photolysis
on
the
UV
absorption
spectrum
of
CP59,430
(M_w, 434.46). A
5 \mu M
solution
of
CP59,430
in
buffer
B
plus
0.1%
digitonin
was
irradiated
for
various
periods
of
time
with
a
UVSL
lamp,
and
the
absorption
spectra
were
recorded
on
a
Cary
15
spectrophotometer.
(Inset)
Effect
of
15
min
of
photolysis
on
the
UV
absorption
spectrum
of
5
\mu M
prazosin
(M_w, 382.42).
produced competitive inhibition of [3H]prazosin binding because the apparent affinity of the radioligand was reduced without loss of maximal binding capacity (Fig. 3).

**Specificity of CP59,430 Binding.** To examine further the specificity of CP59,430 binding, the ability of the compound to inhibit [3H]dihydralpranolol binding to the hepatic membrane α1-adrenergic receptor and [3H]yohimbine binding to the membrane α2-adrenergic receptor was also investigated. As can be seen in Table 1, at a concentration of CP59,430 (1 μM) that inhibited 72% of specific [3H]prazosin binding to the α1-adrenergic receptor, binding of [3H]dihydralpranolol and [3H]yohimbine at saturating concentrations and at concentrations approximating their dissociation constants remained unchanged.

**Covalent Interactions of CP59,430 with the Membrane and Purified α1-Adrenergic Receptor.** Photoysis-induced covalent linkage of CP59,430 to membrane α1-adrenergic receptors was examined in two ways. First, hepatic membranes were incubated with CP59,430 for 90 min and then washed three times with buffer B to remove nonspecifically bound label. The membrane-bound CP59,430 was then photolyzed for 15 min and used in equilibrium binding studies. These studies were carried out at 37°C for 3 hr to enhance the dissociation of CP59,430 not covalently linked to the receptor. As shown in Fig. 4B, Scatchard

![Figure 2](image2.png)  
**FIG. 2.** Competition by prazosin (C), CP59,430 before photolysis (C), and CP59,430 after photolysis in buffer alone for 15 min (A), and rauwolfscine (D) for [3H]prazosin (0.8–1.0 nM) binding to the hepatic membrane (A) or purified (B) α1-adrenergic receptor. Studies were performed by incubating membranes (100 μl) suspended in buffer B or purified receptor preparations (400 μl) in buffer B plus 0.1% digitonin with [3H]prazosin (25 or 50 μl, respectively) and buffer or various concentrations of prazosin (A, B) or CP59,430 (C, D) at 25°C for 60 min. Unbound radioligand was separated by suction filtration as described (8). Apparent dissociation constants (Kd) for the drugs were calculated according to the Cheng and Prusoff analysis (16). Results shown are representative of three similar studies with each drug.

![Figure 3](image3.png)  
**FIG. 3.** Scatchard analysis (17) of equilibrium binding studies with rat hepatic membrane (A) or purified (B) α1-adrenergic receptor preparations performed in the absence (C) or presence (D) of 1 μM CP59,430. Aliquots of membranes (100 μl, 1 mg of protein per ml) in buffer B or purified receptor preparations (400 μl) in buffer B plus 0.1% digitonin were incubated with increasing concentrations of [3H]prazosin (0.1–6.0 nM) in a total volume of 150 μl (membrane) or 500 μl (purified) at 25°C for 60 min. The concentration of [3H]prazosin bound was determined as that fraction of total binding inhibited by 10 μM phentolamine. The Kd and Bmax (maximal binding) values were determined from the slope and x intercept of the linear regression lines (r > 0.95 in each case) relating [3H]prazosin specifically bound (abscess) to the ratio of bound to free (ordinate). Results are the means of triplicate determinations and are representative of two similar studies. Bmax values in the absence and presence of CP59,430 are 177 and 187 (A) and 92 and 94 (B) fmol/ml, respectively. Corresponding Kd values are 1.0 and 2.4 (A) and 1.6 and 2.7 (B) × 10^-9 M.

![Figure 4](image4.png)  
**FIG. 4.** Photoinactivation of membrane-bound α1-adrenergic receptors with CP59,430. (A) Five 10-ml aliquots of rat hepatic membrane suspension (1 mg of protein per ml) in buffer B were prepared by adding buffer CP59,430 (1 mM), prazosin (0.5 mM), or both drugs to the final concentrations indicated. The mixtures were incubated for 90 min at 25°C and then washed three times with buffer B (50 ml) at 4°C by pelleting (28,000 × g) and then resuspending the membranes. After resuspension to the original volume the aliquots were photolyzed and then extensively dialyzed (five 4-liter exchanges, 8 hr each), to allow complete dissociation of tightly but not covalently bound CP59,430. The remaining α1-adrenergic receptor activity was then determined by saturation binding assay. Error bars represent + SEM; h indicates photosynthesis. (B) Scatchard analysis of equilibrium binding studies with rat hepatic membranes. Aliquote of membrane suspension (5 ml, 1 mg of protein per ml) in buffer B were prepared by adding buffer CP59,430 (5 μM final concentration). The mixtures were then incubated for 90 min at 25°C and washed and photolyzed as indicated above. Equilibrium binding studies were then performed as described for Fig. 3 except that the incubations were performed at 37°C for 3 hr to promote the dissociation of any tightly, but not covalently, bound CP59,430. Results shown are the means of triplicate determinations. The Bmax values for control (○) and CP59,430 (□) were 149 and 48 fmol/ml, respectively, and the Kd values were 1.4 and 1.0 × 10^-9 M.

**Table 1.** Competition by CP59,430 for binding to rat hepatic membrane β-([3H]dihydralpranolol), α2- ([3H]yohimbine), and α1-([3H]prazosin) adrenergic receptors.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Conc., nM</th>
<th>Control, CP59,430</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Dihydralpranolol</td>
<td>4</td>
<td>246 ± 21</td>
<td>283 ± 35</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>73 ± 16</td>
<td>98 ± 19</td>
</tr>
<tr>
<td>[3H]Yohimbine</td>
<td>20</td>
<td>424 ± 36</td>
<td>414 ± 29</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>181 ± 18</td>
<td>173 ± 12</td>
</tr>
<tr>
<td>[3H]Prazosin</td>
<td>5</td>
<td>322 ± 17</td>
<td>91 ± 6</td>
</tr>
</tbody>
</table>

Membranes (100 μl) containing 1.2 mg of protein per ml in buffer B were incubated in a total volume of 150 μl with buffer (25 μl) or CP59,430 (25 μl, 1 μM) and a [3H]-labeled ligand (25 μl) at the concentration (final) shown for 60 min at 25°C. Unbound radioligand was then separated by suction filtration as described (8). Specific binding was determined as described in the text. Results shown are the means ± SEMs of three studies performed in duplicate.

*P < 0.001 versus control.
analysis of the binding data indicates noncompetitive inactivation as the $B_{max}$ was reduced by 67% without a change in the affinity of the residual binding sites. Second, aliquots of membranes (1 mg of protein per ml) were exposed to a high concentration of CP59,430 (1 mM), extensively washed, photolyzed, and then dialyzed against buffer B (five 4-liter exchanges, 8 hr each). Subsequent measurement of $\alpha_1$-adrenergic receptors by saturation binding assay employing $[^3H]$prazosin revealed a 77% loss of receptor-binding sites as compared to control membranes treated in an identical manner but without CP59,430 preincubation or photolysis (Fig. 4A). Preincubation of the membranes with CP59,430, without photolysis, followed by the same extensive washing and dialysis protocol, resulted in a 15% loss of binding sites as compared with control. There was also a 20% loss of sites in the membranes that were photolyzed after preincubation with CP59,430 and a high concentration of prazosin (500 nM). Prazosin was included in this sample to protect the binding sites from photoactivation by CP59,430. Additionally, membranes that were preincubated with prazosin and then subjected to photolysis showed no loss of receptor-binding sites. The small amounts of receptor inactivation observed in the absence of photolysis (15%), or in the presence of a high concentration of prazosin (500 nM) may be due to nonphotochemically induced incorporation of CP59,430 or to incomplete protection from ambient light during the experiment. It should be noted that in these experiments the membranes were exposed to a high concentration of CP59,430 to assess the maximal degree of photoinactivation. In additional studies in which the membranes were exposed to a lower concentration of CP59,430 (1 nM) and then photolyzed, a 59% loss of receptor-binding sites was observed. However, in this situation additional preincubation with prazosin (500 nM) completely prevented photoactivation by the azide compound.

To ensure further that the loss of binding sites observed in the above studies was due to covalent incorporation of CP59,430 (1 mM), the remaining membranes were heated at 60°C for 3 hr to inactivate the binding sites and then washed with three 30-ml portions of buffer B to remove any CP59,430 that may have been tightly (but not covalently) bound and thus did not dissociate despite dialysis. After this procedure no specific $[^3H]$-prazosin binding activity could be detected in the membranes. The presence of CP59,430 remaining in the membranes was then assessed by radioimmunoassay using $[^3H]$prazosin as the radioligand and antibodies developed against another prazosin analog (CP57,609, 2-(4-(2-succinyl)piperazin-1-yl)-4-amino-6,7-

dimethoxyquinazoline) covalently linked to albumin, as described (18). This antiserum binds CP59,430 and prazosin with similar affinity (data not shown). In these studies only the membranes photolyzed after preincubation with CP59,430, 430 alone significantly inhibited $[^3H]$prazosin binding by the antiserum.

Photoactivation by CP59,430 of affinity-purified $\alpha_1$-adrenergic receptors is shown in Table 2. Aliquots of the purified preparation were exposed to CP59,430 (1 nM) for 90 min, photolyzed for 15 min, and then chromatographed on a Sephadex G-50 column. Subsequent measurements of specific $[^3H]$prazosin binding in identical aliquots of the void volume revealed a 67% loss of receptor-binding sites compared to the purified receptor preparation treated in an identical fashion with CP59,430 but without photolysis. Moreover, preincubation with prazosin (10 nM) completely protected the purified receptors from photoactivation by CP59,430, whereas no loss of receptor-binding activity was observed in the aliquot treated with buffer alone or in that photolyzed after preincubation with prazosin.

To investigate the mechanism of photoaffinity labeling by CP59,430, the effect of the scavenger p-aminobenzoic acid on CP59,430-induced inactivation of affinity-purified receptor binding sites was additionally studied, as shown in Table 3. Aliquots of the purified preparation were exposed to CP59,430 (1 nM), p-aminobenzoic acid (10 nM), or both agents for 90 min, photolyzed for 15 min, and then chromatographed on a Sephadex G-50 column, as described for the studies shown in Table 2. Subsequent quantitation of specific $[^3H]$prazosin (5 nM) binding revealed that p-aminobenzoic acid alone did not alter specific $[^3H]$prazosin binding. Moreover, p-aminobenzoic acid did not reduce or prevent the photoactivation by CP59,430.

### Table 3. Effect of p-aminobenzoic acid on photolysis-induced $\alpha_1$-adrenergic receptor inactivation by CP59,430

<table>
<thead>
<tr>
<th>Additions/treatment</th>
<th>Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP59,430 (1 μM)</td>
<td>PABA (10 μM)</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
</tr>
</tbody>
</table>

Aliquots of affinity-purified receptor preparation containing specific $[^3H]$prazosin (5 nM) binding activity at 0.12 pmol/ml were exposed to buffer B plus 0.1% digitonin, CP59,430, p-aminobenzoic acid (PABA), or both, as indicated, treated as detailed for Table 2, and then assayed for specific $[^3H]$prazosin binding. Results are the means ± SEMs for two studies determined in triplicate.

*P < 0.01 versus aliquot 1.

**DISCUSSION**

In the present report the synthesis and characteristics of an azide analog (CP59,430) of the highly selective $\alpha_1$-adrenergic antagonist prazosin have been described. Several lines of evidence suggest that this compound binds specifically and with high affinity to both membrane and purified $\alpha_1$-adrenergic receptors and that photoactivation results in covalent interaction of the ligand with the receptor-binding site. First, competitive inhibition studies and equilibrium binding experiments demonstrate that before photolysis CP59,430 competitively inhibits $[^3H]$prazosin binding to the $\alpha_1$-adrenergic receptor (Figs. 2 and 3). The affinity of CP59,430 determined in these studies is less than that of the parent compound, prazosin, but 200- to 300-fold greater than that of the $\alpha_2$-selective antagonist rauwolscine.
(Fig. 2). Second, in membrane preparations no inhibition of 
[3H]dihydralpranolol binding to the β-adrenergic receptor or 
[3H]yohimbine binding to the α2-adrenergic receptors is demonstra-
table when the membranes are exposed to a concentration of 
CP59,430 that inhibits 72% of specific [3H]prazosin binding to 
the α1-adrenergic receptor (Table 1). Third, evidence that coval-
ent attachment of CP59,430 occurs with photolysis is provided 
by the demonstration that receptor inactivation after photolysis 
in the presence of CP59,430 is resistant to extensive dialysis or 
Sephadex G-50 chromatography. Fourth, the receptors in both 
the membrane and purified preparations can be protected from 
photoactivation by the α1-selective antagonist prazosin. Fifth, 
the degree of photoactivation with CP59,430 is dose related. 
Finally, the observation that CP59,430 can be detected by 
radioimmunoassay only in the membranes photolyzed after 
exposure to the compound suggests that the loss of receptor-binding 
sites is due specifically to covalent incorporation of the 
photoaffinity probe. Thus, the interaction of CP59,430 with 
the membrane and purified α1-adrenergic receptor fulfills the 
essential criteria that need to be satisfied in order to demonstrate 
that covalent affinity labeling of a receptor has occurred (19): (i) 
the demonstration of irreversible or long-lasting inactivation of 
the receptor-binding sites; (ii) the stoichiometric correlation of 
the irreversible effect and the covalent incorporation of an affin-
ity label; and (iii) the specific protection against this effect by 
the receptor's natural ligand or a competitor.

As reported with other receptor-specific photoaffinity labels, 
complete photoactivation of the α1-adrenergic receptor by 
CP59,430 could not be achieved. Thus, only 60–80% of the 
α1-adrenergic receptor-binding sites could be inactivated despite 
the use of CP59,430 in a concentration that prior to photolysis 
completely inhibited specific [3H]prazosin binding. This may be 
due, in part, to incomplete photolysis resulting from the tur-
bidity of the membrane suspension. In addition, it is likely that 
due to the relatively long half-life of aryltrimethanes (10−5 sec) (20), 
a fraction of the photoactivated CP59,430 bound at the receptor 
may have reacted preferentially with solvent rather than with 
the binding site. Thermal inactivation (21) or photoactivation 
(22) of some of the CP59,430 compound may also have occurred 
during the photolysis procedure. Nevertheless, the extent of 
receptor inactivation produced by CP59,430 compares favorably 
with that observed with other photoaffinity labels, some of 
which have been estimated to label only 1–2% of the total num-
ber of receptors (23).

The possibility that CP59,430 reacts with buffer to generate 
a species of much higher affinity that would mimic an irre-
versible antagonist, similar to that described by Schmelch et al. 
(24), Erez et al. (25), and Kenakin and Black (26), can be elim-
inated by the experiments described in Fig. 2. Thus, the affinity 
of CP59,430 photolyzed in buffer alone was unchanged from that 
observed with the native ligand. Moreover, photoactivation of 
the receptor-binding sites by CP59,430 could not be reversed 
by extensive dialysis or chromatography. After photolysis, 
CP59,430 could be detected in the membranes by radioimmu-
noassay despite heat inactivation of the binding sites and 
extensive washing.

Studies with the scavenger p-aminobenzoic acid (Table 3) 
suggest that CP59,430 labels α1-adrenergic receptor sites by a 
true photoaffinity labeling reaction (27), because the photo-
inactivation by CP59,430 was unaltered by p-aminobenzoic acid. 
This mechanism contrasts with that proposed for "pseudo" 
photoaffinity labeling in which a photolytic intermediate, formed 
in solution, may react with solvent or with added scavenger, as 
well as with the receptor protein. High concentrations of a suitable 
scavenger, such as p-aminobenzoic acid, would thus be ex-
pected to reduce or prevent photoinactivation with this mode of 
photoaffinity labeling.

Taken together, the findings of the present study indicate that 
CP59,430 is a specific photoaffinity label for the α2-adrenergic 
receptor. It should be possible to radiolabel CP59,430 by in-
corporation of a tritium probe into the azide compound at an 
early stage of its synthesis. We have reported initial progress 
with the affinity purification of the α2-adrenergic receptor (8– 
10), and a radiolabeled photoaffinity ligand may provide an ad-
ditional tool for its isolation and for the characterization of its 
biophysical properties in solution.

We thank Dr. E. Haber for the opportunity to perform these studies 
and for valuable discussions; Dr. A. G. Gilman for reviewing the manu-
script; P. K. Shah, D. Bussolotti, B. Taylor, D. Egan, and T. Gandler 
for technical assistance; R. Rubin for editorial assistance; and W. Deaner 
for typing the manuscript. R. M. G. and C. J. H. are Established 
Investigators, American Heart Association (Grants AHA 82-240 and 80-145, 
respectively). These studies were supported in part by National Insti-
tutes of Health Grant HL-10959-07, a Biomedical Research Support Grant 
(2-S60 RR07175-05) and a grant from DNAX, Inc., and were performed in 
part while R. M. G. was on sabbatical leave from the Depts. of Phar-
macology and Internal Medicine, Univ. of Texas Health Science Center 
Dallas, TX.

Chem. 255, 11442–11447.
Chem. 236, 5262–5269.
77, 4449–4453.
5. Carney, D. H., Glenn, K. C., Cunningham, D. D., Das, M., Fox, 
76, 81–85.
Chem. 253, 1743–1745.
Acad. Sci. USA 79, 2196–2190.
Clin. Res. 30, 482A (abstr.).
Chem. 257, 15174–15181.
J. Biol. Chem. 249, 3350–3362.
Chem. 251, 6015–6023.
3108.
Hypertension 4, 118–118.
12066.
366.