Molecular Biology of Adrenergic Receptors in the Rat and Frog Central Nervous System

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SUMMARY Recent developments in the characterization of the adrenergic receptors have led to the identification and purification of the binding subunits of the various catecholamine receptors. β-Adrenergic receptors have been identified in a wide variety of tissues by photoaffinity labeling with the antagonist \( \text{\textsuperscript{125I}} \text{P}-\text{azidoazobenzylrazolol} \) and have been purified to apparent homogeneity from several of these tissues. Thus, β₁- and β₂-adrenergic receptor binding sites appear to reside on peptides with molecular weights of 60,000 to 65,000. The α₁-adrenergic receptor binding subunit has been identified in several peripheral tissues by photoaffinity labeling with a newly developed probe (4-amino-6,7-dimethoxy-2-[4(5(\text{\textsuperscript{125I}})-iodo-4-azidophenyl) pentanoyl]-1-piperazinyl]-quinazoline, or \( \text{\textsuperscript{125I}} \text{APDQ} \)). This binding site resides on a peptide with a molecular weight of 80,000. These techniques have been applied to the elucidation of the binding subunit structure of these receptors in the rat central nervous system with the result that β₁-, β₂-, and α₁-adrenergic binding sites appear to reside on peptides of similar molecular weight to those identified in peripheral tissues (i.e., 60,000-65,000 and 80,000). Using immunocytochemical techniques with antibodies raised to the frog erythrocyte, β-adrenergic receptors were identified at the light microscopic level in regions of the rat and frog brain previously found by ligand binding and autoradiography to be richest in β-adrenergic receptors. At the electron microscopic level, β-receptor immunoreactivity was found throughout dendritic processes with local accumulations at certain postsynaptic sites. This finding is consistent with the idea that the density of the receptors might be significantly increased at postsynaptic junctions of adrenergic neurons. (Hypertension 6 (Suppl II): II-22-II-27, 1984)

KEY WORDS • photoaffinity probes • β-adrenergic receptor antibodies • affinity chromatography

**During** the last decade considerable progress has been made in our understanding of the mechanisms by which hormones and neurotransmitters act through specific receptors to elicit biochemical and physiological responses in their target tissues. Many of these data have come as a result of the development of ligand binding techniques. These techniques have provided investigators with the ability to identify and quantitate receptors. In addition, they have permitted correlation of the pharmacological properties of these receptors with the properties of the biochemical or physiological responses elicited by these hormones or neurotransmitters. In the adrenergic systems, the properties of β₁- and β₂-adrenergic receptor subtypes have been found to correlate well with the ability of catecholamines to stimulate adenylate cyclase in numerous tissues. \(^1\) \(^2\) Binding of ligands to the α₁-adrenergic receptors has been shown to correlate with the ability of catecholamines to inhibit stimulation of adenylate cyclase in certain tissues. \(^3\) \(^4\) Binding of ligands to α₂-adrenergic receptors in liver and vascular smooth muscle has been shown to correlate with the physiological effects of catecholamines on these tissues. The exact biochemical mechanisms involved in the signal transfer for this receptor, however, have not been entirely elucidated, although changes in cellular calcium and membrane phospholipid metabolism...
have been implicated.\(^5\)\(^6\) Because these various receptor subtypes all bind the same catecholamines, the question of whether \(\alpha\)- and \(\beta\)-adrenergic receptors are structurally related molecules has been of long-standing interest in the field of adrenergic pharmacology. Until recently, however, the molecular characteristics of these receptors had not been examined.

Several approaches lend themselves to the biochemical characterization of receptors. Because receptors bind ligands with a high degree of specificity and selectivity, they represent ideal proteins to be purified by affinity chromatography. Moreover, because of this specificity and high affinity of binding, specific affinity and photoaffinity probes can be designed by which the receptors can be covalently tagged and identified. Finally, the availability of purified preparations of receptor should allow the development of antibodies against the purified proteins for use in the further characterization of the receptors.

In fact, these approaches have proved successful in the characterization of the adrenergic receptors from various sources. Affinity chromatography procedures for the purification of \(\beta_1\), \(\beta_2\), \(\alpha_1\), and \(\alpha_2\)-adrenergic receptors have been reported.\(^1\)\(^-\)\(^10\) With these procedures the \(\beta\)-adrenergic receptor from several tissues has been purified to apparent homogeneity.\(^1\)\(^1\)\(^-\)\(^14\) Similary, purification of the \(\alpha_1\)-adrenergic receptor from rat liver plasma membranes also has been reported\(^10\) with affinity chromatography. Moreover, high-affinity, high-specific-radioactivity, photoaffinity probes specific for \(\alpha\)- and \(\beta\)-adrenergic receptors have been developed.\(^1\)\(^5\)\(^-\)\(^10\) The availability of purified \(\beta\)-adrenergic receptor preparations has led recently to the development of antibodies against the frog erythrocyte \(\beta\)-adrenergic receptor.\(^2\)\(^1\) These results underscore the feasibility of using the various approaches just described in the characterization of the various receptors for catecholamines.

Although these procedures have been developed for the characterization of the various catecholamine receptors, the original studies were done almost exclusively with peripheral tissues. Very little information is actually available on the molecular characteristics of these receptors in the central nervous system. In this paper we will attempt to compare the subunit structure of \(\alpha_1\)- and \(\beta\)-adrenergic receptors of the brain with that of peripheral tissues as revealed by photoaffinity labeling. In addition, we will show that by using anti-\(\beta\)-adrenergic receptor antibodies and immunocytochemistry, the \(\beta\)-adrenergic receptor of the brain can be localized in postsynaptic clusters.

### Identification of Adrenergic Receptors in Rat Brain by Photoaffinity Labeling

Visualization of receptors for hormones and neurotransmitters in tissue or crude membrane preparations is a complex problem as receptors are present in the plasma membrane in very low concentration. Thus, for a potential receptor probe to be usable it should: (1) have high affinity and selectivity for the receptor site, (2) be labeled to high-specific radioactivity (preferably with iodine), and (3) have a suitable chemically reactive group capable of incorporating into a polypeptide chain. The photoaffinity probes that have been developed recently for the labeling of \(\alpha\)- and \(\beta\)-adrenergic receptors all possess these properties. Thus, it has become possible to identify receptors in crude membrane preparations even in the absence of any purification of the receptors themselves.

In the central nervous system, the four subtypes of receptors for catecholamines have been demonstrated by ligand binding\(^2\)\(^2\)\(^-\)\(^4\) and autoradiography.\(^2\)\(^5\)\(^-\)\(^2\)\(^6\) Little information is available, however, about the molecular structures of these receptors. Thus, we have used the \(\alpha\)- and \(\beta\)-adrenergic photoaffinity probes developed in our laboratories: 4-amino-6,7-dimethoxy-2[5(3\(^{125}\)I]-ido-4-azidophenyl] pentanol]-1-piperazinyl] quinazoline, or \([^{125}\text{I}]\text{APDQ}\), and \([^{125}\text{I}]\text{p-azidobenzylcarazolol}\), or \([^{125}\text{I}]\text{pPABC}\), to attempt to probe the structure of these receptors in the brain.

### Labeling of the \(\beta\)-Adrenergic Receptors

Membranes from rat cerebellum and cortex were prepared in the following manner. Brains were removed and bathed in ice-cold 0.32 M sucrose, 1 mM EDTA. Cerebellum and cerebral cortex were dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose, 1 mM EDTA in a glass teflon homogenizer. The homogenate was centrifuged at 500 g for 10 minutes at 0° C to 4° C (Sorvall SS-34 rotor, Ivan Sorvall, Inc., Norwalk, CT). The supernatant was recentrifuged at 12,000 g for 20 minutes. The pellet obtained was discarded and the supernatant centrifuged at 158,000 g for 45 minutes (Beckman Ti-45; Beckman Instruments, Fullerton, CA). The resulting pellet (membrane fraction) was washed twice by centrifugation at 40,000 g for 15 minutes with 150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride (pH 7.5), resuspended in the same buffer, and stored at –80° C until used.

Membranes (2–3 mg of protein; equivalent to 400–600 fmol of \(\beta\)-adrenergic receptor) were incubated in 50-ml polycarbonate tubes in a total volume of 25 ml (16–24 pM receptor concentration) for 90 minutes at 25° C with 50 to 100 pM \([^{125}\text{I}]\text{pABC}\) under dim light in the presence and absence of 10 \(\mu\)M alprenolol. The membranes were washed by centrifugation at 40,000 g for 15 minutes in cold incubation buffer that contained 0.5% bovine serum albumin (essentially fatty-acid free), resuspended in 15 ml of bovine-serum-albumin-free buffer, and photolyzed. Labeled membranes were dissolved in 10% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, 25 mM Tris-hydrochloride (pH 6.8) and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli.\(^2\)\(^7\) After the electrophoresis the gels were dried and exposed at –70° C for 1 to 3 days with Kodak XAR film (Kodak, Rochester, N.Y.) with intensifying screens (Lightning Plus; du Pont Co., Wilmington, DE); the films were developed manually according to Kodak instructions.
As shown in Figure 1, when a microsomal preparation of either rat cerebellum or cerebral cortex was incubated with the antagonist \[^{125}\text{I}]\text{pABC a peptide with a molecular weight of 60,000 was labeled, as revealed by SDS-PAGE autoradiography of the membrane samples.}^{19}\] The covalent labeling of this band was blocked by the presence of 10 \(\mu\text{M}\) alprenolol in the membrane incubation. Because the cerebellum contains almost exclusively \(\beta_2\)-adrenergic receptors\(^{28}\) and cerebral cortex contains predominantly \(\beta_1\)-adrenergic receptors,\(^{29}\) it is reasonable to suggest that the peptides labeled by \[^{125}\text{I}]\text{pABC represent respectively the \(\beta_2\)- and \(\beta_1\)-adrenergic receptors in these two regions of the brain. The peptides labeled in the brain by the photoaffinity probe \[^{125}\text{I}]\text{pABC seem to have molecular weights similar to the \(\beta\)-adrenergic receptor peptides identified or purified from peripheral tissues. Recent studies have shown that the cardiac \(\beta_1\)-adrenergic receptor binding site of many species (including humans) resides on a peptide with a molecular weight of 62,000,\(^{30}\) while the \(\beta_2\)-adrenergic receptor from mammalian lung has been characterized as a peptide with a molecular weight of 64,000.\(^{30-32}\) Determination of the extent of homology among the receptor peptides from different tissues or from the two different subtypes will have to await further characterization of these proteins.

**Labeling of the \(\alpha_1\)-Adrenergic Receptor**

Membranes were prepared as described in the previous section. Membranes (2–3 mg of protein, 400–600 fmol of total receptor) were then incubated for 90 minutes at 25°C with 50 to 100 pM \[^{125}\text{I}]\text{APDQ in a total volume of 25 ml in the dark alone or with prazosin 10 \(\mu\text{M}\); phentolamine, 10 \(\mu\text{M}\); yohimbine, 10 \(\mu\text{M}\); (-)epinephrine, 30 \(\mu\text{M}\); (+)epinephrine, 30 \(\mu\text{M}\); (-)norepinephrine, 30 \(\mu\text{M}\); or (-)isoproterenol 30 \(\mu\text{M}\), washed, and photolyzed as described previously.\(^{19}\) Other conditions were as described in the previous section.

As shown in Figure 2, incubation of a cerebral cortex microsomal preparation that is rich in \(\alpha_1\)-adrenergic receptors with the \(\alpha_1\)-adrenergic photoaffinity probe \[^{125}\text{I}]\text{APDQ\(^{15}\) leads to the specific incorporation of the labeled ligand into a major peptide with a molec-
ular weight of 79,000. As can be seen in Figure 2
incorporation of this high-affinity probe also can be
observed in lower-molecular-weight peptides (54,000
and 42,000); however, only the peptide with a molecu-
lar weight of 79,000 was labeled with the appropriate
specificity. Thus, at 0.1 μM concentrations, the α2-
selective antagonist prazosin was much more potent
than was the α1-selective agent yohimbine in blocking
covalent incorporation of [125I]APDQ. (−)Epineph-
rine and (−)norepinephrine were equally effective at
30 μM but (−)isoproterenol was ineffective. The
(+)-isomer of epinephrine was slightly less potent than
its (−)-isomer, as expected for an α1-adrenergic recep-
tor. A similar peptide (Mw = 80,000) has been identi-
fied by photoaffinity labeling of rat liver plasma mem-
brane15 as well as of several other tissues that contain
α1-adrenergic receptors (Dickinson and Leeb-Lund-
berg, unpublished observations). Recently, Kunos et
al.22 have reported the identification of a peptide with a
molecular weight of 80,000 with the properties of an
α1-adrenergic binding site by covalently labeling rat
liver plasma membranes with the alkylating agent
[3H]-phenoxybenzamine. In this study, another pep-
tide with a molecular weight of 58,000 also was la-
beled specifically but was thought to represent a pro-
teolytically generated fragment from the larger
receptor peptide. These data agree with our own find-
ings in rat liver with [125I]APDQ where similar lower-
molecular-weight peptides could be evidenced.15
These peptides, which still contain an intact ligand
binding site, may be similar to the peptide (Mw = 59,000)
purified by Graham et al.10 from rat liver membranes
with affinity chromatography.

Immunocytochemical Localization of
β-Adrenergic Receptors

As mentioned previously the presence of β-adren-
ergic receptors in various regions of the brain was
documented originally by ligand binding stud-
ies.23, 24, 29 Palacios and Kuhar25 later confirmed and
extended these findings by performing light micro-
scopic autoradiographic localization of these receptors
with radiolabeled antagonists; however, these tech-
niques are of limited resolution and can only be used
to map the regional distribution of these receptors in
the brain. Although numerous studies that used biochemi-
cal fractionation techniques or selective neurotoxins
have suggested that β-adrenergic receptors in the brain
may be associated with postsynaptic neurons (re-
viewed in25), it previously has not been possible to test
this hypothesis directly.

The availability of antibodies capable of specifically
recognizing the β-adrenergic receptor21 has allowed the
examination of its morphological localization. Im-
munocytochemical localization in frog and rat brain
with the antibodies and secondary peroxidase-antiper-
oxidase staining revealed immunoreactivity in the
cerebellum, hippocampus, and neostriatum, regions
that have been shown by ligand binding22, 23 or autora-
diography25 to be the areas where the β-receptors are
most concentrated. No labeling was detected in brain
sections incubated with receptor-adsorbed antiserum or
preimmune serum. Moreover, in laminated regions of
the cerebellar cortex, receptor immunoreactiv-
ity was localized in the outer molecular layer with
concentrations in distal regions of Purkinje cell den-
drites and dendritic spines but not in cell bodies.21
Thus, at the light microscopic level, the distribution of
β-adrenergic receptor immunoreactivity was the same
as the localization of β-adrenergic receptors docu-
mented previously by other techniques.21

To determine the localization of the receptors at a
higher resolution, the specific immunoreactivity of the
dendrites was examined at the electron microscopic
level. Brain sections were prepared as described in
Strader et al.21 Adjacent sections were incubated at
4°C for 24 hours with antiserum to the receptor at a
1:100 dilution or with antiserum absorbed with partial-
ly purified receptor. The sections were then washed
and treated sequentially with a 1:50 dilution of goat
antirabbit immunoglobulin, a 1:100 dilution of peroxi-
dase-antiperoxidase, and 3,3'-diaminobenzidine-hy-
drogen peroxide. Samples were prepared for electron
microscopy and examined with a Philips 201 electron
microscope (Philips Co., Gildelampenfabriken-Eind-
hoen, The Netherlands). The results are taken from
Strader et al.11

Figure 3 shows sections of frog hippocampus and rat
cerebellum. In frog hippocampus (Figure 3A) β-
adrenergic receptor immunoreactivity was observed
through the cytoplasm of the cell and on the cell mem-
brane. The highest concentration of receptor was locat-
ed on regions of the membrane directly opposite the
synaptic cleft (identified by the characteristic structure
of presynaptic vesicles). In rat cerebellum (Figure 3B)
a section of a Purkinje cell dendrite also revealed accu-
cumulations of receptor immunoreactivity in areas of
synaptic contact. In larger fields the distribution of
these postsynaptic clusters of receptor immunoreactiv-
ity was comparable to the distribution of specific ca-
teholaminergic nerve terminals, as assessed by the
immunolocalization of tyrosine hydroxylase.23 Thus,
in brain, β-adrenergic receptors appear to be clustered
at the postsynaptic junctions of adrenergic neurons.
This finding represents the first demonstration of the
localization of the β-adrenergic receptor at the high
resolution afforded by the electron microscope.

Discussion

Receptors for catecholamines are almost ubiquitous
in the body as responsiveness to catecholamines can be
demonstrated in virtually every tissue. Stimulation or
blockade of these receptors is important in the therapy
of many prevalent human illnesses. Thus, the mecha-
nisms by which these receptors mediate their effects is
of primary interest. To elucidate the complete mecha-
nisms of action of these hormones/neurotransmitters,
however, it will be essential eventually to characterize
biochemically the receptors and their effector systems.
As reviewed in this paper, methods are now becoming
available to accomplish this endeavor for the various catecholamine receptors. Affinity chromatography and photoaffinity-labeling techniques have been developed for α- and β-adrenergic receptors,22-26 and β1-, and β2-, as well as α1-adrenergic receptors have been purified to apparent homogeneity.16-18

As reported in this paper, with the technique of photoaffinity labeling it appears that β1- and β2-adrenergic receptor binding sites in the central nervous system reside on peptides of molecular weight similar to those peptides that have been identified or purified from peripheral tissues. Whether these peptides represent products from the same gene cannot be determined at this time; however, a recent study comparing mammalian peripheral β1- and β2-receptors by peptide mapping of photoaffinity-labeled peptides suggest that β1- and β2-peptides, while having similar molecular weights, show significant differences.35

The α1-adrenergic receptor of the cerebral cortex also appears to reside on a peptide of molecular weight (80,000) similar to that from peripheral tissues, as evidenced by photoaffinity labeling of α1-adrenergic receptors. In both brain and peripheral tissues α1- and β-adrenergic receptors appear to reside on peptides of completely different molecular weights (62,000-64,000 versus 80,000). Whether these α1- and β-adrenergic receptors show any degree of homology will have to await further structural characterization of the purified proteins.

As reviewed in this paper, the availability of an antibody to the frog erythrocyte β2-adrenergic receptor has permitted immunocytochemical localization of the β-receptors in the brain. It has been demonstrated in both frog and rat brain that β1-adrenergic receptors are clustered at the postsynaptic junctions of adrenergic neurons.

References

Figure 3. Electron microscopic × 20,000 immunocytochemical localization of specific immunoreactivity to the β-adrenergic receptor in dendrites. A. Frog hippocampus. B. Rat cerebellum. Arrows indicate peroxidase-reaction product accumulation at postsynaptic sites, t = terminal; bars = 1.00 μm.


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