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IN VITRO STUDY OF β-ADRENERGIC RECEPTORS

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INTRODUCTION

Norepinephrine is synthesized and stored in nerve terminals in both the peripheral and central nervous systems. The release of norepinephrine from adrenergic nerves results in the modulation of a variety of cellular processes. The postsynaptic events resulting from norepinephrine release are initiated through interaction with specific membrane receptors. Interaction of norepinephrine with its specific receptor is only the first step in a complicated and incompletely understood series of events. The molecular configuration of adrenergic receptors determines the structural specificity and activity of both agonists and antagonists. Furthermore, changes in the density or properties of adrenergic receptors may be a mechanism for regulating the ultimate effects of catecholamines. Most of our knowledge of postsynaptic adrenergic mechanisms comes from studies either in whole animals or with isolated organ systems. Studies of this type can provide information only on the final events resulting from the interaction of catecholamines with adrenergic receptors. They cannot provide an understanding of the molecular interactions that occur when the receptor recognizes a specific agonist. This chapter deals with our present knowledge of the β-adrenergic receptor system. Emphasis is placed on the events that occur at or immediately distal to the receptor. In particular, the information that has accumulated during the past few years through the use of direct binding assays of β-adrenergic receptors is discussed. Several reviews of various aspects of this subject have recently appeared (1-7).

Classification of Adrenergic Receptors

The existence of multiple types of adrenergic receptors was first suggested by the results of Dale (8). He demonstrated that some of the effects of epinephrine or of sympathetic nerve stimulation were antagonised by ergot alkaloids while other responses were unaffected. In 1948, Ahlquist (9) divided adrenergic responses into two classes based on the effects elicited by a series of catecholamines. The first

class of receptors, called alpha (α) were stimulated by agonists with the potency order of epinephrine > norepinephrine > α-methylnorepinephrine > α-methyl-epinephrine > isoproterenol. Stimulation of these receptors led to contraction of the nictitating membrane, to vasoconstriction, and to contraction of smooth muscle in the uterus and ureter. The second type of adrenergic receptor, called beta (β), responded with the agonist specificity of isoproterenol > epinephrine > α-methyl-epinephrine > α-methyl-norepinephrine > norepinephrine. Stimulation of these receptors resulted in positive cardiac inotropy and chronotropy, vasodilation, and relaxation of uterine smooth muscle. This classification of adrenergic receptors has been extended to include most effects mediated by catecholamines. The responses characteristic of α- and β-adrenergic receptors have been further defined through the use of specific antagonists (1, 2, 10–13). For instance, β-adrenergic responses can be divided into the subclasses β_1 and β_2 (14, 15). Epinephrine and norepinephrine are approximately equipotent activators of β_1 receptors which are preferentially inhibited by practolol (16). Epinephrine is more potent than norepinephrine in activating β_2 receptors which are inhibited by butoxamine (17, 18). A variety of antagonists including propranolol, alprenolol, and pindolol do not distinguish between β_1 and β_2 receptors (19, 20).

Relationship of Adrenergic Receptors to cAMP Formation

Sutherland and co-workers (21, 22) first introduced the concept that adenosine 3', 5'-monophosphate (cAMP) serves as a second messenger for the action of a variety of hormones. It soon became apparent to these and other workers that cAMP served as a mediator of some of the actions of catecholamines (21–23). Specifically, those actions of catecholamines mediated through β-adrenergic receptors result in increased intracellular levels of cAMP. Therefore, activation of the catalytic unit of adenylate cyclase by catecholamines can serve as a measure of β-adrenergic receptor function and permits an indirect biochemical characterization of these receptors. With the exception of the central nervous system, α-adrenergic receptor stimulation does not increase cAMP levels (22). In the brain, activation of receptors with the pharmacological characteristics of α-adrenergic receptors results in increased intracellular levels of cAMP (24–28). Therefore, norepinephrine-stimulated cAMP formation in the brain reflects the stimulation of both α- and β-adrenergic receptors.

Characterization of Direct Binding Assays for β-Adrenergic Receptors

The most direct way to characterize a receptor is to assess the interaction of a specific ligand with the receptor. On the other hand, measurement of other parameters, such as catecholamine-stimulated adenylate cyclase activity, includes several events distal to the interaction of the amine with the receptor. By directly assaying the receptor, the chemistry of the initial events involved in β-adrenergic receptor–mediated responses can be more clearly approached. Also, changes in the properties of the β-adrenergic receptor itself may play a role in regulating cell responsiveness. This possibility can be most easily examined using direct in vitro assays. And finally, by serving as a means through which β-adrenergic receptors can be followed during solubilization, isolation, and reconstitution studies, this type of assay should ulti-

mately permit elucidation of the mechanism through which receptor occupation leads to the activation of adenylate cyclase.

Once a suitable ligand has been found, the technical aspects of establishing direct binding assays are relatively simple. There are several well-accepted methods for separating free radioactive ligand from ligand that has been specifically bound to the receptor. Equilibrium dialysis (29, 30), centrifugation (31–33), and filtration (34–37) techniques have all been used successfully in assays of β-adrenergic receptors. A greater concentration of receptors is required for equilibrium dialysis than for the other methods of quantifying bound ligand. The latter two systems offer several additional advantages since they are less time consuming than is equilibrium dialysis, and washing procedures can often be used to remove nonspecifically bound or occluded ligand from the filter or pellet. When a procedure is used that includes washing the sample, some consideration of the rate of dissociation of ligand from the receptor is required. It is theoretically possible that a wash procedure will cause dissociation of specifically bound ligand. In some cases, dissociation of specifically bound ligand has been minimized
cases, it has been advantageous to use buffer at 37° to remove ligand that is nonspecifically bound (34, 35). It is impossible to know a priori whether a given separation procedure will be appropriate. In any case, the use of a ligand with a high affinity will increase the probability that the ligand will have a slow rate of dissociation from its receptor.

Consideration of the properties of catecholamine-stimulated adenylate cyclase activity has led to the development of a set of expected criteria to characterize the in vitro binding of a reversible β-adrenergic receptor ligand to the receptor (38, 39). Thus, the binding of the radioactively labeled ligand should show saturability since only a finite number of receptors exist. If there are no spare receptors (38, 40) and a one-to-one relationship between β-adrenergic receptors and adenylate cyclase exists, the dissociation constant (K_d) of an agonist determined by inhibition of binding should be equal to the EC_{50} value determined by measuring the activation of adenylate cyclase. If spare receptors exist, the K_d should be greater than the EC_{50}. The K_d values for antagonists, determined by inhibition of binding, should be equal to the values determined from the inhibition of catecholamine-stimulated adenylate cyclase. This should be true irrespective of the existence of spare receptors. When a reversible β-adrenergic receptor ligand is used, the bound radiolabeled compound should be completely dissociable. It is important to demonstrate that a ligand that has dissociated from its binding site is chemically unchanged and will bind to membranes with properties identical with those of fresh ligand. The ratio of the kinetically derived rate constants of dissociation (k_2) and association (k_1) should be equal to the dissociation constant (K_d) determined at equilibrium ($k_2/k_1 = K_d$). Compounds that affect adenylate cyclase activity through interaction with β-adrenergic receptors should inhibit the binding of the radioligand. Conversely, compounds that are inactive at β-adrenergic receptors should not inhibit the binding of a radioligand. Finally, the binding of a ligand to the β-adrenergic receptor should be preferentially inhibited by stereoisomers having the R configuration at the β-carbon of the ethanolamine side chain. In general, this corresponds

to an optical rotation in the levorotatory direction (41). Both agonist-induced activation of adenylate cyclase and inhibition of catecholamine-stimulated adenylate cyclase exhibit stereospecificity.

In any in vitro assay of a receptor, it is necessary to identify specific binding of ligand to the receptor as opposed to binding to other sites. Nonspecific bindings may include hydrophobic and hydrophilic interactions with various membrane constituents. In experiments with whole cells, the uptake of ligand must also be considered (38). To define nonspecific binding, assays are carried out in the presence of a competing β-adrenergic receptor antagonist or agonist at a concentration that will occupy essentially all of the receptor sites. In this situation, specific binding of ligand will be equal to the amount of radioactivity bound in the absence of competing ligand minus the amount of radioactivity bound in its presence. In general, when assays are carried out under conditions such that the concentrations of radioactive ligand and of receptors are both well below the K_d value for the radioactive ligand, a concentration of a competing ligand which is one hundred times its K_d value should provide a satisfactory estimate of nonspecific binding. On the other hand, when the concentration of either the radioactive ligand or of receptors approaches or exceeds the K_d value for the ligand, then a higher concentration of competing ligand will be necessary to occupy all or most of the receptor sites. The concentration can be calculated easily from the Michaelis-Menten equation (42). The need for a quantitative definition of nonspecific binding arises from the fact that too high a concentration of a competing ligand may not only block specific binding of radioligand to the β-adrenergic receptor but may also compete for nonspecific sites (38, 43).

There are other points that should be considered when establishing an in vitro assay for β-adrenergic receptors. The receptors should be found on cells that have been shown by anatomical and physiological studies to be innervated by noradrenergic neurons. With only a few exceptions (44–47), adenylate cyclase has been found to be associated with the plasma membranes of cells (4, 22). Since β-adrenergic receptors seem to be functionally connected with adenylate cyclase its distribution should parallel the distribution of plasma membrane markers, including catecholamine-stimulated adenylate cyclase.

IN VITRO ASSAYS OF β-ADRENERGIC RECEPTORS

The Use of Catecholamine Agonists

The earliest attempts to establish an in vitro binding assay for β-adrenergic receptors utilized the interaction of tritiated catecholamines with membrane preparations enriched in catecholamine-sensitive adenylate cyclase. Several tissue sources were investigated. Marinetti and co-workers (48–51) as well as Leray et al (52) studied the binding of ^{3}H-epinephrine to rat liver plasma membranes. These investigators showed that the binding was saturable and that other catecholamines were potent inhibitors of the binding. However, the binding was also inhibited by a variety of catechol-containing compounds that were not active at β-adrenergic receptors.

Also, stereospecificity was not seen, and bound radioactivity did not dissociate after the addition of strong acid. Potent β-adrenergic receptor antagonists, such as propranolol, had little effect on binding even at very high concentration (0.1 mM). Lefkowitz and co-workers investigated the interaction of ^{3}H-norepinephrine with cardiac microsomes (53–55), myocardial cells grown in tissue culture (56), and fat cell membranes (57). Inhibition of the binding of ^{3}H-norepinephrine by agonists did not show stereospecificity although binding was inhibited by a variety of catechol-containing compounds. Propranolol, at a concentration of 0.1 mM, caused only a 40% inhibition of norepinephrine binding (53, 56). Acid reversed the binding to cardiac microsomes, but the addition of a large excess of unlabeled norepinephrine did not cause the dissociation of bound ligand (55). Bilezikian & Aurbach (58, 59) as well as Schramm et al (60) studied the binding of tritiated catecholamines to turkey erythrocyte membranes, which are a rich source of β-adrenergic receptors. Binding was inhibited by several catechol-containing compounds but no evidence of stereospecificity was observed. High affinity β-adrenergic receptor antagonists did not inhibit binding. In contrast to studies with other tissues, almost total dissociation of bound ^{3}H-catecholamine from turkey erythrocytes was observed on dilution of the incubation mixture (58, 61).The concentration of binding sites determined for turkey erythrocyte ghosts ranged from 55 to 300 pmoles/mg of protein while the maximum stimulation of adenylate cyclase by isoproterenol was only 4.5 to 18 pmoles cAMP formed per milligram of protein per minute (58–60). Thus, it appeared from these studies that either the turnover number of adenylate cyclase was extremely low or there were a surprisingly high number of spare receptors.

Several reports have appeared that question the conclusion that the binding of catecholamines to membrane fragments is related to β-adrenergic receptors (61–65). The binding of ^{3}H-catecholamines to heart (61–63, 65), liver (61), adipocyte (61), skeletal muscle (65), and turkey erythrocyte membranes (61) as well as to membranes from several cell lines grown in tissue culture (62) have been studied. Binding was inhibited by a large number of catechol-containing compounds, was not affected by potent β-adrenergic receptor antagonists, and did not show stereospecificity. With a few exceptions (58, 61, 65), the binding of ^{3}H-catecholamines was almost entirely irreversible. Cuatrecasas et al (61) proposed that the binding was related to the enzyme catechol-O-methyltransferase (COMT), some of which is membrane bound (61, 66). This enzyme methylates the phenolic oxygen of a variety of catechols, and it does not show stereoselectivity. Several inhibitors of COMT inhibited the binding of catecholamines (61), and S-adenosylmethionine, a cosubstrate required for COMT activity, increased the rate of binding of ^{3}H-norepinephrine to liver and heart microsomes. An analogue of S-adenosylmethionine, S-adenosylhomocysteine, decreased the amount of binding and also decreased the activity of COMT in liver microsomes. It is important to note that several of the COMT inhibitors studied by Cuatrecasas et al (61) were catechols. At least three groups of investigators have argued against the involvement of COMT in the binding of ^{3}H-catecholamines. Two noncatechol inhibitors of COMT had no effect on the binding of ^{3}H-epinephrine to membranes derived from rat heart (63). Although syringic acid and syringaldehyde are potent inhibitors of COMT activity, they had

little effect on the binding (67). In addition, the subcellular distribution of binding activity and COMT activity were markedly different (68). It was also reported that the pH optimum for COMT differed from that of the binding reaction (68).

Maguire et al (62) showed that inhibition of oxidation by agents such as sodium metabisulfite, sodium ascorbate, EDTA, and 1,2- or 1,4-dihydroxybenzene inhibited the binding of ^{3}H-norepinephrine. Saturation of the reaction media with nitrogen caused a reduction in the rate of binding of ^{3}H-catecholamine to rat glioma cells (62). The divalent cations Mn^{2+}, Co^{2+}, and Fe^{2+} reversed the inhibitory effect of EDTA on the binding (62). Along these same lines, Wolfe et al (63) showed that the time course of oxidation of ^{3}H-epinephrine was very similar to the time course of binding to rat heart preparations. In addition, ^{3}H-epinephrine bound to bovine serum albumin with properties that were qualitatively and quantitatively similar to those of membranes derived from rat heart (63). Cuatrecasas et al (61) and Wolfe et al (63) reported that the rate of binding of ^{3}H-catecholamines was increased after tissues were boiled or stored at 4° or –22°. This increase in the rate of binding was temporally associated with an increase in the rate of tissue-catalyzed oxidation of ^{3}H-epinephrine (63). The mechanism by which storage of tissues enhances the rate of oxidation of epinephrine was not established. It may reflect the release or exposure of divalent cations associated with various tissue constituents. Finally, it was demonstrated that the addition of 30 μM pyrocatechol delayed the onset of binding of ^{3}H-epinephrine to heart membranes. Simultaneous measurements of the rate of oxidation of ^{3}H-epinephrine revealed that pyrocatechol also delayed the onset of oxidation of epinephrine (63). The two reactions occurred with an identical time course. Maguire et al (62) and Wolfe et al (63) concluded that the binding of catecholamines involves a nonspecific, probably covalent, interaction of oxidized degradation products of catechols with unspecified tissue constituents.

Lacombe & Hanoune (69) studied the binding of ^{3}H-epinephrine to liver plasma membranes in the presence of 1 mM EDTA. Similarly, Pairault & Laudat (70) studied the binding of ^{3}H-norepinephrine to membranes from rat adipocytes in the presence of 1 mM EGTA and 0.5 mM pyrocatechol. Inclusion of pyrocatechol or a chelating agent decreased the oxidation of catecholamines. In the presence of these agents the residual binding of ^{3}H-catecholamine observed was more susceptible to inhibition by propranolol than was the binding in their absence. Although the residual binding was inhibited by catecholamines, no stereospecificity was observed in these studies.

A recent report by Malchoff & Marinetti (71) described stereospecific inhibition of ^{3}H-isoproterenol binding to intact chicken erythrocytes. Specific binding was inhibited by a low concentration of propranolol (80 nM). It is likely that the high affinity of isoproterenol for β-adrenergic receptors in chicken erythrocytes permitted these workers to observe stereospecific binding. In addition, they reported that there was a dramatic increase in the amount of catecholamine bound if the reaction was performed on lysed cells. However, they were unable to demonstrate stereospecific binding in preparations of broken cells.

The studies with agonists described above suggested that in vitro assays of β-adrenergic receptors require a ligand that has a high affinity for these receptors, that

can be labeled to a high specific activity, and that is resistant to oxidation. The use of a ligand with a high affinity labeled to high specific activity makes possible the assay of receptors at a low concentration of ligand. Theoretically, this should increase the proportion of total binding that is associated and with β-adrenergic receptors.

The Use of β-Adrenergic Receptor Antagonists

Many β-adrenergic receptor antagonists have affinities that are two to three orders of magnitude greater than those of catecholamine agonists. However, initial attempts to develop an in vitro β-adrenergic receptor binding assay using tritiated propranolol were unsuccessful (72, 73). These failures may have been due to the use of myocardial membranes. Levitzki et al showed that the binding of ^{3}H-propranolol (4.3 Ci/mmole) to turkey erythrocyte membranes was inhibited in a stereospecific manner by β-adrenergic receptor agonists and antagonists (29, 31). The dissociation constants of various β-adrenergic receptor ligands as determined by their ability to inhibit ^{3}H-propranolol binding were in good agreement with values obtained from measurements of adenylate cyclase activity (31, 74). Approximately 0.8 pmoles of binding sites per milligram of protein were found which was equivalent to 550 binding sites per erythrocyte (see Table 1).

More recently, Nahorski (75), using ^{3}H-propranolol with a specific activity of 21 Ci/mmol, reported stereospecifically displaceable binding to membranes from chick cerebral cortex. There were approximately 0.23 pmoles of binding sites per milligram of protein. Binding was rapid and equilibrium was reached within 2 min.

Table 1 Properties of β-adrenergic receptor binding

Tissue	Species	Ligand	K_d (nm)	pmole/mg	K_1 (M^{-1}min^{-1})	K_1 (min^{-1})	Reference
Erythrocyte	Turkey	Prop	2.5	0.90	—	—	29
		IHYP	0.020–0.025	0.2–0.3	5.4×10^{10}[a]	0.54[a]	95
	Frog	DHA	5–10	0.35	—	—	76
	Rat	IHYP	0.025	0.15	—	—	108
Heart	Dog	DHA	11	0.35	—	—	33
	Rat	IHYP	1.4	0.16	3×10^{7}[a]	4.75×10^{-2}[a]	35
Cerebral cortex	Rat	DHA	6.7	0.2	—	—	87
	Rat	IHYP	1–1.5	0.3–0.35	9.8×10^{7}[a]	4.5×10^{-2}[a]	43
	Rat	DHA	1.3	0.3	1.2×10^{8}[a]	0.1[b]	36
	Chick	Prop	11	0.23	—	—	75
Liver	Rat	IHYP	2–3	0.039	1.2×10^{6}[a]	1.2×10^{-2}[a]	126
Adipocytes	Rat	DHA	10–20	0.24	2.4×10^{7}[a]	0.294	37
Lymphocytes	Human	DHA	9	0.075	—	—	88
Pineal	Rat	DHA	18	0.6	—	—	91
Cells in culture							
Glioma (C6TG1A)	Rat	IHYP	0.25	0.075	1×10^{8}[c]	1.7×10^{-2}[c]	34
Glioma (VA2)	Rat	IHYP	0.015	0.15	—	3.5×10^{-3}[c]	34
Astrocytoma	Human	IHYP	0.6–2	0.3–3	—	8×10^{-3}[a]	101
Lymphoma	Mouse	IHYP	0.033	0.028	—	6×10^{-3}[c]	107

[a] = 37°.
[b] = 23°.
[c] = 30°.

Dissociation of bound ligand, initiated by adding excess (0.2 mM) isoproterenol, occurred with a half-time of 45 sec. One consistent problem in the studies reporting the successful use of ^{3}H-propranolol (29, 31, 74, 75) was the large amount of nonspecific binding of ligand. The low percentage of specific binding of ^{3}H-propranolol (20–45%) caused problems in quantitative studies of β-adrenergic receptors.

Lefkowitz and co-workers (32, 76, 77) reduced the unsaturated carbon-carbon double bond of (–)-alprenolol with palladium and tritium gas to obtain ^{3}H-dihydroalprenolol (DHA) with a specific activity of 17–33 Ci/mmole. The binding of DHA to frog erythrocyte membranes has been extensively characterized (32, 76, 78–86). It was inhibited stereospecifically by both β-adrenergic receptor agonists and antagonists. The binding of DHA to β-adrenergic receptors of frog erythrocyte membranes was rapid and equilibrium was reached within five min at 37° (76). Binding was reversible on addition of 10 μM propranolol or following dilution (76, 79). The half-time for dissociation at 37° was less than 30 sec. Binding was saturable with between 1000 and 2000 sites per erythrocyte. The dissociation constants of β-adrenergic receptor agonists and antagonists were determined from studies of the inhibition of DHA binding. These values were in good agreement with those obtained in studies of catecholamine-sensitive adenylate cyclase activity (76,81). Compounds not affecting catecholamine-sensitive adenylate cyclase had no effect on the binding of DHA (76).

Tritiated DHA has been used to characterize β-adrenergic receptors of several other tissues including heart (33), brain (36, 87), lymphocytes (88), pineal organ (89–91), and adipocytes (37). The properties of the binding of DHA in these systems are generally similar to those described for frog erythrocytes.

In experiments with DHA the concentration of radioligand is high relative to its K_d. Therefore, the concentration of a competing ligand that inhibits 50% of the specific binding of DHA will be greater than its true K_d value (92). In this case both the K_d of the radioligand and its concentration are needed to calculate the true K_d value for a competing ligand (81, 92, 93).

Using a slightly different approach, Aurbach et al (94) established a direct binding assay for β-adrenergic receptors in turkey erythrocytes. This group used [^{125}I]iodohydroxybenzylpindolol (IHYP),
tagonist, which can be purified to achieve a nearly theoretical specific activity of 2200 Ci/mmole. The binding of IHYP was inhibited in a stereoselective manner by both β-adrenergic receptor agonists and antagonists. The binding of IHYP to various tissues has been characterized. Brown et al (95, 96), using turkey erythrocyte membranes, showed that there were approximately 0.3 pmoles of binding sites per milligram of protein. This value corresponded to 400–600 sites per cell, agreeing well with the value obtained by Levitzki et al (29) using ^{3}H-propranolol as a ligand. Dissociation constants for β-adrenergic receptor agonists and antagonists were determined both by the inhibition of IHYP binding and by the measurement of catecholamine-sensitive adenylate cyclase activity. The K_d values determined by the two methods were in good agreement for antagonists. However, the K_d values for agonists, as determined by inhibition of IHYP binding were 2 to 10 times smaller

than the apparent K_d values determined for the activation of adenylate cyclase (96). This discrepancy was discussed in terms of the ability of 5'-guanylimidodiphosphate (GMPPNP) to increase the apparent affinity of agonists for adenylate cyclase without affecting the binding of either agonists or antagonists to β-adrenergic receptors (96; cf section on guanyl nucleotides below).

Harden et al (35, 97) characterized β-adrenergic receptors in rat heart using IHYP. The binding of IHYP was prevented in a stereospecific manner by both agonists and antagonists and was unaffected by compounds that are not active at these receptors. There were approximately 0.2 pmoles of binding sites per milligram of protein. Approximately 40 min were required for the binding of radioligand to reach equilibrium. This slow rate was discussed in terms of the low concentrations of IHYP and of receptor used. Dissociation constants of antagonists determined by binding and by measuring adenylate cyclase activity were in good agreement. The relative order of potencies for agonists as determined by the two methods were also in good agreement. However, the absolute values of dissociation constants for agonists determined by inhibition of IHYP binding were 10–100 times less than the apparent K_d values for agonists determined from activation of adenylate cyclase. The authors suggested that homogenization resulted in a perturbation of the β-adrenergic receptor-adenylate cyclase system distal to the formation of the hormone-receptor complex (22, 35, 98–100). Additional evidence consistent with this conclusion was obtained in a study of human EH118 astrocytoma cells (101). In this study the K_d value for isoproterenol in intact cells was the same whether determined in studies of IHYP binding or in studies in which cAMP accumulation in response to isoproterenol was measured. Homogenization of the cells did not affect the K_d for isoproterenol determined by IHYP binding, but the EC_{50} determined from measurements of adenylate cyclase activity was increased approximately tenfold after homogenization.

Sporn & Molinoff (43) have characterized the binding of IHYP to membranes from rat cerebral cortex, caudate nucleus, and cerebellum. The density of sites was lower in the cerebellum than in the other two regions examined. Binding was saturable and reversible and was inhibited stereospecifically by β-adrenergic receptor ligands. These authors found that the inclusion of high concentrations of phentolamine or phenoxybenzamine (up to 0.1 mM) greatly reduced the amount of nonspecifically bound IHYP without affecting specifically bound ligand. This effect of α-adrenergic receptor antagonists was not seen in experiments with heart or liver membranes (T. K. Harden, B. B. Wolfe, and P. B. Molinoff, unpublished observations). Phentolamine (0.1 mM) inhibited specific binding in experiments with membranes from hypothalamus and brain stem and thus it could not be used with these tissues. The effect of phentolamine and phenoxybenzamine did not appear to reflect interaction with α-adrenergic receptors since (+) and (–) phenoxybenzamine were equipotent and since very high concentrations of drug were required. A similar effect of phentolamine on the small amount of nonspecific binding of IHYP to turkey erythrocyte membranes has been reported by Brown and co-workers (96).

Alexander et al (87) and Bylund & Snyder (36) have studied β-adrenergic receptors in various regions of rat brain using DHA. The properties of the binding were

similar to those reported in studies of the binding of DHA to membranes from frog erythrocytes. A high density of binding sites was found in the cortex and caudate nucleus. The concentration of sites was lower in the hypothalamus and brain stem. The high density of sites in the caudate (see also 43) is surprising since there are few if any noradrenergic terminals in the caudate (102). It is not likely that the binding of DHA or IHYP in the caudate reflects the presence of a large number of dopamine receptors since dopamine was a very weak inhibitor of both DHA (36, 87) and IHYP binding (43). The presence of non-neuronal elements, which may contain these receptors (28, 103, 104), could account for the presence of binding sites in the caudate. Skolnick & Daly (105) and Markstein & Wagner (106) have recently pointed out an additional complicating factor in studying adrenergic receptors in the brain. In these studies alprenolol (105) and pindolol (106) were shown to inhibit both the α- and β-adrenergic receptor–mediated increases in brain cyclic AMP levels.

Maguire et al (34) used IHYP to study β-adrenergic receptors of several cell lines grown in tissue culture. Two types of high affinity binding sites for IHYP were found in experiments with rat glioma (C6TG1A) cells. However, binding of IHYP to only one of the sites was stereospecifically inhibited by β-adrenergic receptor ligands. This site had the properties expected of a β-adrenergic receptor. Using crude membrane preparations these workers found approximately 0.075 pmoles of binding sites per milligram of protein, corresponding to 4000 binding sites per cell. The dissociation constant determined by equilibrium binding (0.25 nM) was in good agreement with the dissociation constant (0.17 nM) determined from kinetic measurements of k_1 and k_2. Cell lines that did not respond to catecholamines with an increase in cAMP concentration did not show specific IHYP binding to β-adrenergic receptors. Conversely, stereospecifically displaceable IHYP binding was observed in cell lines that were responsive to catecholamines.

Recently Insel and co-workers (107) have suggested that the synthesis of β-adrenergic receptors and of adenylate cyclase are controlled by separate genes. With S-49 mouse lymphoma cells grown in tissue culture, it was shown that wild-type S-49 cells generated cAMP in response to catecholamines and possessed IHYP binding sites similar to those described by Maguire et al (34) for rat glioma cells. A clone of S-49 cells was isolated that did not respond to isoproterenol, PGE_1, or sodium fluoride by accumulating cAMP, indicating that these cells do not possess adenylate cyclase. However, these cells did contain IHYP binding sites which appeared identical with those of wild-type S-49 cells.

The idea that the synthesis of β-adrenergic receptors and catecholamine-sensitive adenylate cyclase are independently controlled has also been investigated in several studies of rat erythrocytes (108–110). In these reports both mature erythrocytes and reticulocyte-enriched preparations produced by the injection of phenylhydrazine or acetylphenylhydrazine were studied. After these treatments, approximately 90% of the cells in the blood were reticulocytes. Charness et al (108) reported that membranes obtained from reticulocyte-enriched preparations produced nine times more cAMP in response to a high concentration of isoproterenol than did membranes from mature red cells, but they possessed only 40% more IHYP binding sites. These

results suggested that β-adrenergic receptors and catecholamine-stimulated adenylate cyclase activity can vary independently. On the other hand, Spiegel et al (109) reported that there was a fourfold increase in both binding sites and isoproterenol-induced cAMP accumulation in cells from acetylphenylhydrazine-pretreated animals. These authors concluded that the loss of catecholamine responsiveness as erythrocytes mature involves a concomitant loss of β-adrenergic receptors and the catalytic function of the adenylate cyclase system. However, Bilezikian & Spiegel (110) have recently reported that as reticulocytes mature, catecholamine-sensitive adenylate cyclase activity decreases more rapidly than does the concentration of β-adrenergic receptors. The authors suggested that maturation of reticulocytes is associated with an uncoupling of the β-adrenergic receptor from its biological effect.

Effects of Guanine Nucleotides

Guanine nucleotides modulate both basal and hormone-sensitive adenylate cyclase activity in a variety of systems (111–115). The naturally occurring nucleotide, GTP, increases the efficacy of catecholamines for the stimulation of adenylate cyclase activity (114). On the other hand, the synthetic analogue, 5'-guanylimidodiphosphate (GMPPNP), increases both the efficacy and the potency of catecholamines for the stimulation of adenylate cyclase activity (112, 113). It has been suggested that these nucleotides bind to a regulatory site that can affect either the interaction of agonist with the receptor or the coupling of activated receptors to the catalytic subunit of adenylate cyclase. Recently, Spiegel et al (116) and Brown et al (96) showed that incubation of turkey erythrocyte membranes with GMPPNP and various β-adrenergic receptor agonists resulted in a ten-fold decrease in the EC_{50} value for the stimulation of adenylate cyclase by catecholamines. In the presence of GMPPNP, the EC_{50} values for agonists were in good agreement with the K_d values determined by inhibition of IHYP binding. In the absence of GMPPNP, the EC_{50} values were one order of magnitude greater than the K_d values. There is substantial evidence that the effects of GMPPNP are irreversible (112, 113, 115). It is therefore difficult to interpret the effects of this agent on catecholamine-stimulated adenylate cyclase activity.

Several laboratories have reported that the presence of GMPPNP does not affect the K_d values for β-adrenergic receptor antagonists, as determined from direct-binding studies (74, 84, 96, 117). However, conflicting reports have appeared on the ability of GMPPNP to affect the K_d values of agonists. Brown et al (96) reported that GMPPNP had no effect on the ability of catecholamines to compete with IHYP for binding sites on turkey erythrocyte membranes. Maguire et al (117) have studied the effects of guanine nucleotides on the binding of IHYP to membranes of various cell lines grown in tissue culture, and more recently Lefkowitz et al (84) have studied their effects on the binding of DHA to frog erythrocyte membranes. The latter two groups of investigators reported that in the presence of GTP, GDP, or GMPPNP, the potency of agonists as determined in binding studies was decreased by approximately one order of magnitude. This subject is discussed in more detail by Maguire et al (6).

Irreversible β-Adrenergic Receptor Ligands

Irreversible blockers of muscarinic and nicotinic cholinergic receptors and α-adrenergic receptors have proved to be very useful tools in pharmacology (13, 118–121). Several irreversible β-adrenergic receptor ligands have recently been described. However, none of these compounds has been radiolabeled and tested in an in vitro assay for these receptors.

In a recent report by Takayanagi and co-workers (122), β-adrenergic receptors mediating relaxation of the guinea pig taenia coli were photoaffinity labeled with (–)-isoproterenol or 2-(2-hydroxy-3-isopropylaminopropoxy)iodobenzene. These compounds acted reversibly in the absence of photoactivation, and their interaction with the receptor was blocked by propranolol. Taenia coli were irradiated for 25 min in the presence of 6 μM (–)-isoproterenol. The tissue was then washed, and the ability of isoproterenol to cause relaxation of the taenia was determined. After irradiation the potency of isoproterenol was decreased by 20-fold although the efficacy was not altered. The authors concluded that there are a substantial number of spare receptors in this tissue. It is possible that a higher concentration of ligand or repeated irradiation would yield a decrease in the maximal response of the tissue and thus permit a quantitative estimate of the number of spare receptors. Photoaffinity inactivation together with one of the in vitro assays for β-adrenergic receptors described above could yield interesting and important data regarding the presence and number of spare receptors.

Atlas and co-workers have prepared irreversible β-adrenergic receptor blockers by bromoacetylation of free amines (123, 124). The parent compounds were reversible antagonists with dissociation constants ranging from 0.2 μM to 0.1 mM. The bromoacetyl derivatives inhibited the effect of (–)-epinephrine on adenylate cyclase activity without affecting fluoride-stimulated adenylate cyclase activity. One of the compounds, a bromoacetyl derivative of propranolol, was suggested to be an irreversible antagonist of β-adrenergic receptors of turkey erythrocyte membranes since epinephrine-sensitive enzyme activity and the capacity to bind ^{3}H-propranolol were not regained after 5 washes of the membranes (123, 124). Both (–)-epinephrine and (–)-propranolol protected receptors from the irreversible effect of the affinity label. The authors discussed the potential usefulness of irreversible ligands for the purification of β-adrenergic receptors. It should be noted, however, that bromoacetylation markedly reduced the potency of several of the antagonists studied. This may be a problem in developing an in vitro assay for β-adrenergic receptors since a low affinity irreversible ligand is likely to label nonreceptor sites in addition to β-adrenergic receptors.

Erez and his collaborators (125) have studied the ability of practolol and chloropractolol to antagonize the chronotropic response to isoproterenol seen in rat atria. The blocking actions of practolol were easily reversed, while the effects of chloropractolol were reversed only by 50% after 30 washes during a period of 30 min. The authors suggested that these results reflected an irreversible action of chloropractolol. However, IHYP, which acts reversibly (34, 35, 95), exhibits rates of dissociation in liver (126) and astrocytoma cells (101) that are similar to that reported for

chloropractolol. It therefore seems likely that chloropractolol is simply a high affinity reversible antagonist with a relatively slow rate of dissociation.

A potentially exciting report has appeared describing the development of a specific antibody to β-adrenergic receptors. Wrenn & Haber (127) immunized rabbits with a deoxycholate-solubilized fraction obtained from dog heart. Serum isolated from immunized rabbits inhibited the specific binding of ^{3}H-propranolol as well as isoproterenol-stimulated adenylate cyclase activity. Preimmune serum had no effect. The inhibition appeared to be specific for the β-adrenergic receptor since serum from immunized rabbits had no effect on fluoride, GMPPNP, or glucagon-stimulated adenylate cyclase activity.

IN VITRO PROPERTIES OF β-ADRENERGIC RECEPTORS

Cooperativity of Ligand Receptor Interactions

Several laboratories have analyzed β-adrenergic receptor binding data to determine whether site-site interactions exist. Limbird et al (79) have suggested that negatively cooperative interactions occur between β-adrenergic receptors of frog erythrocyte membranes. In these studies, the time course of dissociation of DHA from its binding sites was determined. Dissociation was initiated either by a 100-fold dilution or by the addition of excess unlabeled alprenolol together with dilution. The rate of dissociation in the presence of alprenolol was greater than in its absence. Saturation data plotted by the method of Hill (128) gave a slope of 0.82, which is consistent with the existence of negative site-site interactions. A greater degree of negative cooperativity (Hill coefficient of 0.65) has been reported in a study of DHA binding to rat adipocytes (37). Maguire et al (34) in studies of cell lines maintained in culture reported Hill coefficients of about 0.8. Brown et al (95), studying the binding of IHYP to turkey erythrocyte membranes concluded from the linearity of their Scatchard plots (129) and from the agreement of K_d values determined kinetically and thermodynamically that there are no cooperative interactions among the β-adrenergic receptors of turkey erythrocyte membranes. In studies of β-adrenergic receptors of rat cerebral cortex, Hill coefficients of 1.01 (36) and 1.02 (43) were observed. Hill coefficients of 0.95 to 1.02 have been found in rat liver and heart (B. B. Wolfe, T. K. Harden, and P. B. Molinoff, unpublished observations). The difference in the results obtained in various laboratories probably reflects the differences in the tissues used.

Localization of β-Adrenergic Receptors

The anatomical and cellular complexity of a number of organs makes it difficult to interpret the results obtained when homogenates are assayed for their content of receptors. For example, in the brain β-adrenergic receptors may be associated with both neuronal (28, 130, 131) and non-neuronal elements (28, 103, 104). One way to circumvent these complexities is through the use of histological techniques. There are several possible approaches for the direct localization of β-adrenergic receptors. Melamed and his collaborators (132) have recently reported experiments carried out with a fluorescent compound, 9-amino-acridinpropranolol. This agent is a potent

(K_d = 3 X 10^{-8}M) inhibitor of epinephrine-stimulated adenylate cyclase activity. Following the intravenous administration of this compound, a well-defined yellow fluorescent band was seen in the cerebellar cortex in the region of the Purkinje cell layer. The highest density of fluorescence was observed on the apical dendrites of Purkinje cells. Stereospecific blockade of binding of the fluorescent ligand was demonstrated in experiments with (–) and (+)-propranolol.

Another approach to the localization of β-adrenergic receptors involves the use of the compound IHYP. This compound has a high affinity for β-adrenergic receptors and a slow rate of dissociation. Bylund et al (133) found that following the intravenous administration of IHYP to rats, radioactivity was found in the heart, brain, and lung. Binding was prevented by the prior administration of isoproterenol or propranolol. The eventual goal of these studies is to establish conditions for the autoradiographic localization of β-adrenergic receptors. A second aspect of this approach involves the labeling of cryostat tissue sections in vitro rather than injecting the ligand intravenously. This technique has been successful in preliminary experiments with adrenalectomized rat liver (J. Sporn, unpublished observations).

A third approach, which is of potential applicability to the study of β-adrenergic receptors, involves the development of fluorescein or peroxidase coupled antibodies. There has been one report of the production of an antibody to the β-adrenergic receptor (127). The immunochemical techniques needed to couple an antibody to a visual marker have been described in a variety of systems. Visualization of an antibody complex should offer no unique problems in terms of its applicability to the study of β-adrenergic receptors.

β-Adrenergic receptors are thought to be located on plasma membranes of cells since catecholamine-stimulated adenylate cyclase activity is largely restricted to these membranes (22, 134). Evidence that β-adrenergic receptors exist on the outer surface of cell membranes has been provided by experiments utilizing catecholamines immobilized on either glass beads or amino acid polymers (135, 136).

The subcellular localization of β-adrenergic receptors has been investigated in several different tissues using direct binding assays. Wolfe et al (126) found that β-adrenergic receptors copurified with fluoride-stimulated adenylate cyclase activity during subcellular fractionation of rat liver. Williams et al (37) demonstrated that DHA binding sites are associated with the plasma membrane fraction of rat adipocytes. In other studies (36, 137), DHA binding sites in rat cerebral cortex were associated with a synaptosomal fraction. The DHA binding sites in cortex copurified with the specific uptake mechanism for ^{3}H-norepinephrine. Unlike the results obtained with membranes from mammalian liver, β-adrenergic receptors in the brain did not copurify with fluoride-sensitive adenylate cyclase activity on continuous sucrose gradients (137).

Effect of Membrane-Altering Agents

The mechanism by which the interaction of catecholamines with β-adrenergic receptors leads to the activation of adenylate cyclase is not yet understood. Several approaches may lead to an understanding of these events. The effects of specific lipid perturbing agents on the sensitivity of adenylate cyclase to various hormones have

been investigated (138–140). Recently, the effects of these and other agents on β-adrenergic receptors and catecholamine-sensitive adenylate cyclase of frog erythrocyte membranes have been studied (85). Several agents including phospholipases A, C, and D and amphotericin B led to a decreased ability of catecholamines to stimulate adenylate cyclase activity. A dose-dependent decrease in hormone sensitivity was paralleled by a similar decrease in the specific binding of DHA. On the other hand, the polyene antibiotic filipin decreased the ability of catecholamines to stimulate adenylate cyclase activity without significantly altering DHA binding. It was suggested that filipin acted to "uncouple" receptors from catalytic sites. A role for proteins in β-adrenergic receptor sites was suggested by a decrease in DHA binding after treatment with heat, urea, or proteolytic enzymes. Furthermore, it was suggested that both hydrophobic and hydrophilic residues were necessary for the binding of DHA to β-adrenergic receptors (7).

Solubilization of β-Adrenergic Receptors

Solubilization of the receptor is the first step in the eventual isolation and chemical characterization of the active site. Through the use of detergents, adenylate cyclase has been solubilized from a number of membrane sources (141–143). Although there has been one report of the solubilization of adenylate cyclase in a catecholamine-sensitive state (144), in most instances the soluble enzyme was no longer sensitive to hormone stimulation. From these studies, it was concluded that the loss of sensitivity to hormones after solubilization was due to a loss of protein or lipid moieties that normally exist as part of or in close association with receptors and adenylate cyclase in the plasma membrane.

A means of quantitating solubilized β-adrenergic receptors is a necessary prerequisite for their eventual purification. Recently, an in vitro assay for solubilized β-adrenergic receptors has been reported (30, 77). The plant glycoside, digitonin, was used to solubilize β-adrenergic receptors from frog erythrocyte membranes (30, 77). Equilibrium dialysis and Sephadex column chromatography were utilized to measure the binding of DHA to solubilized β-adrenergic receptors, which retain the properties of the membrane-associated receptor. Saturability, stereospecificity, and the expected effects of various pharmacological agents were observed. The solubilized β-adrenergic receptor had an apparent molecular weight of 130,000–150,000 as determined by Sepharose 6B gel chromatography. The ability to bind DHA was lost at temperatures above 4°. High concentrations of EDTA appeared to stabilize the binding sites which were denatured by guanidine hydrochloride, urea, trypsin, or phospholipase A.

REGULATION OF β-ADRENERGIC RECEPTORS

Changes in the Sensitivity of the β-Adrenergic Receptor-Adenylate Cyclase System

EFFECTS OF DENERVATION The phenomenon of denervation supersensitivity has been described in a number of systems. If the normal afferent input is removed,

the response to an appropriate transmitter is often enhanced (145). Supersensitivity has been described for the peripheral cholinergic (146–149) and adrenergic nervous systems (150–153). In the adrenergic nervous system, supersensitive responses involve two separate phenomena (150–153). There is an acute presynaptic component corresponding to the loss of the ability of the tissue to take up and inactivate catecholamines. There is also a slowly developing postsynaptic component which appears to involve changes at or beyond the site at which receptor interactions take place. Increased postjunctional responsiveness to catecholamines has been demonstrated in a number of organs after surgical, chemical, or environmental alteration of their adrenergic input. For example, an increase in catecholamine-sensitive adenylate cyclase activity was seen after the surgical denervation of dog heart (154). An increase in catecholamine-sensitive enzyme activity was also observed in homogenates of rat pineal organ after superior cervical ganglionectomy (155, 156) or after chronic exposure to light (89, 156). Chronic denervation of the rat pineal organ in vivo either by the administration of 6-hydroxydopamine or after superior cervical ganglionectomy resulted in an enhanced accumulation of cAMP in response to norepinephrine in pineal organs cultured in vitro (157).

In the mammalian brain, several investigators have reported the development of supersensitive responses to catecholamines after depletion of norepinephrine stores in nerve terminals. For example, an increase in cAMP accumulation in response to catecholamines was observed in slices of cerebral cortex following the intraventricular administration of 6-hydroxydopamine to adult rats (158–162). The subcutaneous administration of 6-hydroxydopamine to newborn rats also resulted in a marked depletion of central catecholamine stores and in an enhanced responsiveness of the adenylate cyclase system (153, 163–165). Several groups of investigators have reported that the administration of reserpine results in an enhanced accumulation of cAMP in several regions of the brain in response to a maximally effective concentration of norepinephrine (166–169). On the other hand, Palmer et al (170) observed an increase in the potency of norepinephrine after the administration of reserpine. Unilateral lesions of the medial forebrain bundle resulted in increased accumulation of cAMP in response to norepinephrine in ipsilateral rat cortical slices compared to the accumulation on the contralateral side (171).

DESENSITIZATION OF β-ADRENERGIC RECEPTOR-MEDIATED RESPONSES

Decreases in responsiveness as a consequence of exposure to catecholamines have been described in a number of systems. A decrease in the ability of isoproterenol to induce N-acetylserotonintransferase activity in cultured pineal organs was observed following the in vivo administration of isoproterenol in oil (172). In addition, injection of norepinephrine in oil prevented the development of the increased response to catecholamines seen in the pineal organ after superior cervical ganglionectomy (157). It has recently been reported that long-term treatment of rats with desipramine and other tricyclic antidepressants (173–175), or with inhibitors of monoamine oxidase (176), resulted in a 50–70% decrease in the maximal response to norepinephrine observed in slices of rat cerebral cortex. Kakiuchi & Rall (177) reported that cAMP synthesis is initially stimulated by norepinephrine but that

subsequent exposure of slices of rabbit cerebellar cortex to catecholamines does not result in a rise in cAMP levels. Daly and his collaborators have observed the same phenomenon with slices of cerebral cortex from guinea pig (178) and rat (25). It should be noted that the enhanced accumulation of cAMP in guinea pig cerebral cortex is due almost entirely to α-adrenergic receptors (24, 27, 28, 179). In a recent report by Skolnick et al (180) no refractoriness to norepinephrine was seen in experiments carried out with cortical slices from several species of rat after repeated exposure of slices to norepinephrine.

There have been a number of in vitro studies with homogeneous cell systems in which catecholamine-induced loss of cell responsiveness has been demonstrated. Incubation of lymphoid (181) or fat cells (182) with catecholamines resulted in a loss of responsiveness to a subsequent challenge with catecholamines. The same phenomenon was observed when frog erythrocytes (78, 80, 82, 83, 86), human leucocytes (183), or macrophages (184) were incubated with catecholamines. Several cultured cell lines show similar properties of agonist-induced desensitization. Cultured fibroblasts (185, 186), astrocytoma cells (187–189), and glioma cells (190) are all susceptible to catecholamine-induced refractoriness.

Changes in postsynaptic responsiveness to catecholamines could reflect alterations at any of a number of sites, including (*a*) changes in the affinity of catecholamines for their postsynaptic receptor sites, (*b*) alterations in the amount of adenylate cyclase, (*c*) increases or decreases in the concentration of receptors, (*d*) alteration in the efficiency of coupling of activated receptors to the catalytic units of adenylate cyclase, or, (*e*) changes in the activity of cyclic nucleotide phosphodiesterases. Alternatively, in at least one study the appearance of an endogenous inhibitor of a hormone receptor has been implicated in the regulation of cellular responsiveness (182). Within the past two years, evidence has begun to accumulate in several systems, which implicates changes in receptor density in the modulation of responsiveness to β-adrenergic receptor agonists.

β-ADRENERGIC RECEPTORS AND SUPERSENSITIVITY The process of supersensitivity has been studied as it relates to β-adrenergic receptors in several systems. In all systems thus far examined, increased responsiveness as demonstrated by enhanced cAMP synthesis has been accompanied by an increase in the concentration of β-adrenergic receptors.

Liver The response of rat liver adenylate cyclase to catecholamines is enhanced as a result of adrenalectomy (126, 191, 192). To determine the role of β-adrenergic receptors in this process, the properties and density of β-adrenergic receptors in rat liver were determined after adrenalectomy using IHYP as a radioligand (126). The kinetics of IHYP binding and the affinities of IHYP, propranolol, and various catecholamines were unchanged. In contrast, the concentration of β-adrenergic receptors was increased three to five fold in liver from adrenalectomized rats. The magnitude of this increase was similar to that observed when catecholamine-stimulated adenylate cyclase activity was measured in homogenates of liver from adrenalectomized rats. Both the increase in receptors and the increase in catechola-

mine-responsiveness were reversed by the administration of glucocorticoids in vivo. It was suggested that the increase in the density of β-adrenergic receptors following adrenalectomy may be a compensatory response of the liver to the impairment of the normal hormonal regulation of carbohydrate metabolism.

Cerebral cortex Changes in catecholamine responsiveness in mammalian brain occur as a result of a number of physiological and pharmacological manipulations. As described above, intraventricular injection of 6-hydroxydopamine results in an enhanced response to catecholamines in the rat cerebral cortex (158–162). One week after the administration of this neurotoxin, there was an 80% greater accumulation of cAMP in treated animals in response to isoproterenol than in sham-treated controls. Using IHYP as a ligand the role of β-adrenergic receptors in this increased responsiveness has been assessed (162). Treatment with 6-hydroxydopamine resulted in a 31% increase in the density of β-adrenergic receptors in the cerebral cortex (162). During the first two days following 6-hydroxydopamine administration there was no change either in the efficacy of isoproterenol to stimulate cAMP accumulation or in the density of β-adrenergic receptors. Both parameters then began to increase reaching maximal levels by day 8 (193). An obvious disparity existed between the magnitude of the change in responsiveness and the change in receptor density. The administration of 6-hydroxydopamine also decreased phosphodiesterase activity by about 25% (160). This change may contribute to the greater change in cAMP accumulation than in the density of β-adrenergic receptors. The results suggest that 6-hydroxydopamine has multiple effects in the cerebral cortex. However, changes in receptor concentration after chemical sympathectomy appear to account for some of the observed increase in catecholamine responsiveness.

Harden et al (194) have used the developing rat brain as a model to investigate the relationship between catecholamine responsiveness and the individual components of the β-adrenergic receptor/adenylate cyclase system. The affinities of agonists and antagonists for β-adrenergic receptors as determined by IHYP binding were the same in young and old animals. Specific binding of IHYP to β-adrenergic receptors in rat cerebral cortex was barely detectable during the first week after birth (194). Between days seven and thirteen, there was a rapid increase in the density of receptors. Adult levels were reached by the end of the second week after birth. The time course of development of catecholamine responsiveness was similar to that of β-adrenergic receptors (194, 195). Catecholamine-stimulated cAMP accumulation in slices of cerebral cortex was negligible during the first week after birth. There was a rapid increase in the ability of isoproterenol to stimulate cAMP accumulation during the second week after birth, and maximal responsiveness was attained by day fourteen (194, 195). The development of fluoride-stimulated adenylate cyclase activity in the cerebral cortex differed greatly from the development of β-adrenergic receptors and catecholamine responsiveness. Enzyme activity was 25–35% of adult levels at birth and developed gradually thereafter, reaching adult levels over the next three weeks (194, 195). The authors suggested that it is the appearance of the β-adrenergic receptor in rat cerebral cortex that allows the expression of catechola-

mine-sensitive adenylate cyclase activity (194). Norepinephrine stores (194, 196) and dopamine-β-hydroxylase (196) activity were 10–20% of adult levels at birth and developed with a gradual time course, reaching adult levels over the next two months. Thus, there was no correlation between the time course of development of presynaptic markers for noradrenergic nerve terminals and the postsynaptic development of β-adrenergic receptors and catecholamine responsiveness.

The administration of 6-hydroxydopamine to newborn rats prior to the development of the blood brain barrier prevents the development of noradrenergic terminals in the cerebral cortex as evidenced by a 90–95% reduction in norepinephrine levels (197). However, the time course of the ontogeny of β-adrenergic receptors was not altered by drug treatment, indicating that presynaptic input is not essential for the appearance of these receptors (197). The accumulation of cAMP in response to isoproterenol and the density of β-adrenergic receptors were both increased by 45–75% in drug-treated animals. These changes were seen in animals ranging in age from 6 to 45 days.

Pineal organ As noted above, the rat pineal organ has been extensively utilized as a model system to investigate the influence of presynaptic sympathetic nerves on the responsiveness of a postsynaptic target organ. Axelrod and co-workers have recently evaluated the contribution of β-adrenergic receptors to changes in responsiveness of the cAMP generating system. A circadian rhythm in the sensitivity of the cAMP generating system to catecholamines has been reported (90, 158). This diurnal rhythm in responsiveness was correlated with changes in the concentration of DHA binding sites (90). Furthermore, adenylate cyclase activity in homogenates from light-adapted rats was more responsive to catecholamines than was enzyme from rats kept in the dark overnight (89, 156). The changes in catecholamine responsiveness of adenylate cyclase were paralleled by similar changes in the concentration of β-adrenergic receptors (89). The administration of cycloheximide had no effect on the alterations in catecholamine responsiveness or in receptor density, which suggested that these changes do not require the synthesis of new receptors (89, 90).

Role of β-adrenergic receptors in catecholamine-induced refractoriness Lefkowitz and co-workers have correlated decreases in postsynaptic responsiveness with alterations in the concentration of β-adrenergic receptors. The chronic administration of catecholamines to frogs led to a decrease in adenylate cyclase activity in erythrocyte membranes in response to a subsequent in vitro challenge with isoproterenol (78, 86). The same refractoriness to catecholamines was seen if the erythrocytes were isolated and incubated in vitro with isoproterenol (80, 82). Both in vivo and in vitro desensitization was accompanied by decreased levels of β-adrenergic receptors. Furthermore, the decreases in isoproterenol-stimulated adenylate cyclase activity and in the density of β-adrenergic receptors were of similar magnitude. Cycloheximide had no effect either on the desensitization process or on the subsequent reappearance of sensitivity (86). Since the alterations in receptor concentration and catecholamine responsiveness appeared to be independent of

protein synthesis, these workers suggested that desensitization of the β-adrenergic receptor/adenylate cyclase system involves "inactivation" and subsequent "reactivation" of existing receptor molecules. If this hypothesis is correct, then DHA interacts only with receptors in the functional or active configuration. The molecular basis for an alteration in existing β-adrenergic receptor molecules remains to be elucidated.

Mukherjee & Lefkowitz (83) described catecholamine-induced desensitization in a purified membrane preparation from frog erythrocytes. The desensitized receptors in this cell-free system were rapidly reactivated by exposure of the membranes to guanine nucleotides. Although the effect was most clearly seen with GTP and GMPPNP, the sensitivity of the membrane receptors was also restored by exposure to ATP. It was suggested that nucleotides induce a conformational change in adenylate cyclase which leads in turn to a conformational change in β-adrenergic receptors and to the resultant reappearance of sensitivity to catecholamines.

Perkins and co-workers (187–189, 198) have studied hormone induced refractoriness in a number of cell lines originally derived from a human astrocytoma. EH118MG astrocytoma cells showed both agonist-specific and agonist-nonspecific desensitization (198). Agonist specific refractoriness was rapid in onset. Only 20–30% of the normal response was present after incubation with 10 μM isoproterenol for 30 min (198). The role of β-adrenergic receptors in these changes has been investigated (199). Although there was a 70% decrease in isoproterenol responsiveness, the change in the density of β-adrenergic receptors was only 17%. When cells were incubated with 10 μM PGE_1 for 2 hr and then challenged with isoproterenol, there was a 50% decrease in the amount of cAMP that accumulated in the presence of 10 μM isoproterenol (198). Under these conditions there was no change in the density of β-adrenergic receptors. When cells which had been preincubated with PGE_1 for 2 hr were subsequently incubated with 10 μM isoproterenol for an additional 2 hr, a further 20% loss in responsiveness to isoproterenol occurred. This loss was accompanied by a 20% decrease in the density of β-adrenergic receptors (199). The studies with PGE_1 indicate that 50% of the response to isoproterenol can be lost by a mechanism not involving β-adrenergic receptors. The results with astrocytoma cells together with those with erythrocytes (78, 80, 82, 83, 86) suggest that desensitization to catecholamines may occur by one of several different mechanisms. Previous studies with cells in culture also support this premise. Although the recovery of responsiveness after desensitization to catecholamines in human diploid fibroblasts in culture was dependent on protein synthesis (186), the desensitization process in these fibroblasts was unaffected by inhibitors of protein synthesis. On the other hand, in RGC6 rat glioma cells inhibition of protein synthesis affected the development of desensitization to norepinephrine (190), but did not influence the recovery process after desensitization had taken place. With astrocytoma cells neither the onset nor the recovery of responsiveness following desensitization was affected by cycloheximide (188, 189).

Shear et al (200) studied agonist-induced desensitization in several lines of S-49 mouse lymphoma cells, including genetic variants with specific defects in the pathway of cAMP generation and function. In wild-type S-49 cells cAMP accumulation

in response to isoproterenol reached a maximum within 30 min and then fell rapidly. Approximately 40–50% of the IHYP binding sites were lost following incubation with isoproterenol, which suggested that changes in β-adrenergic receptor density were involved in the desensitization process. Similar studies carried out with a clone of S-49 cells devoid of cAMP-dependent protein kinase activity yielded identical findings. Since an increase in phosphodiesterase activity was not seen following an increase in cAMP levels in kinase-deficient cells, induction of phosphodiesterase cannot account for the refractoriness. In addition, since exposure to isoproterenol led to a decrease in the density of receptors and a decrease in responsiveness to isoproterenol in kinase-deficient cells, cAMP-dependent protein kinase does not appear to be required for either of these effects. When cAMP content was increased by exposing cells to PGE_1 in the absence of isoproterenol, no decrease in either the density of IHYP binding sites or the activity of isoproterenol-stimulated adenylate cyclase was seen. Also, when an S-49 clone that lacks hormone responsive adenylate cyclase activity but still possesses a normal density of β-adrenergic receptors was incubated with isoproterenol or with isoproterenol and dibutyryl cAMP, no decrease in the density of β-adrenergic receptors was seen. Thus, receptor occupancy alone, or in combination with elevated cAMP levels is not sufficient to cause loss of responsiveness or a decrease in the density of β-adrenergic receptors. In addition, it was concluded that in this system adenylate cyclase must be present for desensitization to occur.

CONCLUSION

Progress in the study of β-adrenergic receptors has been very rapid in the last few years. From initial, largely unsuccessful, attempts to use catecholamine agonists in direct assays of β-adrenergic receptors, attention has moved to the use of high affinity antagonists labeled to high specific activity with either ^3H or ^{125}I. The use of these compounds has led to the development of valid in vitro assays for these receptors in a wide variety of mammalian and nonmammalian tissues. These assays are now being used in experiments designed to increase our understanding of the mechanisms through which cellular processes are regulated.

Attention over the next several years is likely to focus on the purification of these receptors. One report of the solubilization of β-adrenergic receptors has appeared. With affinity chromatography and other techniques, it should be possible to obtain samples of the binding component of what may turn out to be a complex, multicomponent system.

An understanding of the mechanisms by which the interaction of a catecholamine with its receptor can lead to the activation of adenylate cyclase is of primary interest to many investigators. Elucidation of this problem will be greatly facilitated by the future availability of purified receptor. It may then be possible to incorporate the binding material into membranes of either defined or complex composition. This approach should provide information as to the function of the receptor/enzyme complex at the molecular level.

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