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METABOLISM OF ALPHA- AND BETA-ADRENERGIC RECEPTORS IN VITRO AND IN VIVO

Lawrence C. Mahan

Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892

Ruth M. McKernan and Paul A. Insel

Division of Pharmacology, Department of Medicine, University of California at San Diego, La Jolla, California 92093

INTRODUCTION

In the past decade the development of radioligand-binding techniques for membrane receptors has been a major advance in molecular pharmacology. With these techniques investigators have been able to study drug and neurotransmitter receptors as discrete molecular entities and have described changes in receptor number and affinity in different settings. A variety of drugs, diseases, and physiologic states are associated with increased (up-regulation) or decreased (down-regulation) numbers of receptors for catecholamines (alpha- and beta-adrenergic receptors) in target cells. In spite of the voluminous literature that describes these changes, our understanding of the cellular and molecular mechanisms that mediate alterations in receptor expression is limited. These alterations in the steady-state expression of receptors on target cells must represent perturbations in one or more steps of the metabolism or turnover of receptors. (The schematic model shown in Figure 1 incorporates some general features of this turnover.) Although many parts of the scheme have not been well characterized for adrenergic receptors, this model forms a basis for examining how receptor number changes in target cells. Alterations in receptor number must result from changes in the rate of receptor appearance and/or disappearance from the plasma membrane. Recep-

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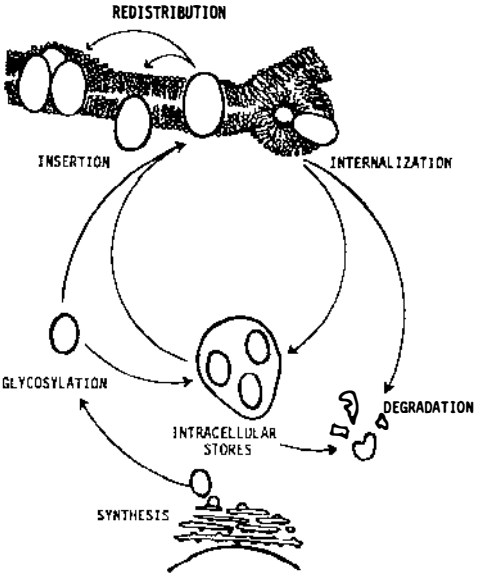

Figure 1 General features of receptor metabolism in cells.

tors appearance includes receptor synthesis, intracellular processing and transport, and membrane insertion or reutilization (recycling) of receptors from intracellular pools. Receptor disappearance includes removal from the cell surface, loss of binding function, and degradation of primary structure.

Adrenergic receptors, which recognize the endogenous catecholamines, epinephrine and norepinephrine, can be divided into four subtypes—alpha$_1$, alpha$_2$, beta$_1$, and beta$_2$—based on agonist and antagonist potencies for a variety of physiological responses (1–3). Although their precise structures are not yet known, each subtype of adrenergic receptor appears to be a distinct integral membrane glycoprotein with subunit molecular weight in mammalian cells between 60,000 and 80,000 (4, 5). Whereas beta$_1$- and beta$_2$-adrenergic receptors both activate adenylate cyclase and stimulate generation of the second messenger cAMP, signal transduction by alpha$_1$- and alpha$_2$-adrenergic receptors may occur through a variety of effector systems. These include inhibition of adenylate cyclase (alpha$_2$), Ca^{2+} mobilization (alpha$_1$ and alpha$_2$), phosphatidylinositol hydrolysis (alpha$_1$), enhancement of Na^+ or K^+ flux (alpha$_1$ and alpha$_2$), and arachidonic acid release (alpha$_1$ and alpha$_2$) (6, 7). Despite such functional heterogeneity, all adrenergic receptors are thought to act via interaction with one or more transduction proteins (the guanine nucleotide–binding (G or N) proteins), which couple the receptors to their respective effector mechanisms.

The extent to which the similarities and differences between adrenergic

receptors are reflected in aspects of their cellular metabolism has yet to be elucidated. Thus, whereas much is known about adrenergic receptors and their coupling mechanisms, comparatively little is known of their turnover. In contrast, with other hormone receptors (e.g. insulin and epidermal growth factor receptors), considerably more is known about the receptor turnover than about coupling mechanisms. As discussed below, the limited understanding of the turnover of adrenergic receptors results partly from the use of indirect techniques to assess adrenergic receptor metabolism.

METHODS FOR THE STUDY OF RECEPTOR METABOLISM

A number of methodological approaches exist for the study of receptor metabolism in cells in vitro. Few of these methods can be successfully applied to the study of receptor turnover in vivo. Ideally, when measuring receptor metabolism, the tools used to make the measurements should not interfere with the metabolism of the receptors themselves.

Direct Approaches

Antibodies to receptors can often be used to identify the receptor protein. This method does not require structural integrity of the agonist- or antagonist-binding site. Use of antibodies is a preferred method because immature or partially degraded forms of the receptor can be identified (8). Two approaches to localizing and quantitating receptors are the use of radiolabeled antireceptor antibodies or the labeling of cells with radioactive amino acids and identification of receptors by immunoprecipitation or immunoblotting methods with unlabeled antibodies. Unfortunately, few antibodies for adrenergic receptors exist that can be used for these types of studies.

Isotopically heavy (^{15}N, ^{13}C, ^{2}H) amino acids have been used to study rates of receptor synthesis and degradation in cultured cells. In these studies the proportions of solubilized receptors were determined in "heavy" and "light" fractions on density gradients prepared from labeled cells (9–11). Aside from the considerable expense of heavy amino acids and potential problems of developing appropriate labeling, solubilization, and density gradient methods, these amino acids can have cytotoxic effects on some cells (11).

For classes of receptors other than adrenergic receptors, investigators have combined morphological approaches with biochemical methods to examine receptor metabolism (12). Receptors have been detected in intracellular organelles involved in receptor processing using fluorescently labeled agonists or antibodies tagged with fluorescent or electron-dense markers, or using irreversible binding of radiolabeled agonist or antagonist probes containing covalently reactive groups (chemically or photochemically activatable). This

approach has been hindered by the relatively small number of adrenergic receptors expressed on most cells (typically $< 2 \times 10^4$/cell) and the lack of high affinity agonists or other probes that would allow receptors to be studied during the entire turnover process. Decreases in binding affinity for agonists commonly occur after beta- and alpha-adrenergic receptors encounter agonists. These changes make it difficult to use agonists to follow receptors through pathways of intracellular processing. In addition, it has not been shown that agonists are internalized along with adrenergic receptors and thus labeling of receptors with agonists may not be an appropriate approach for tracking receptors.

Indirect Approaches

Measuring receptor metabolism indirectly requires radiolabeled agonists or antagonists of high specific activity and specificity for the receptor studied. To estimate the rate(s) of receptor disappearance, cells or tissues are treated with inhibitors of protein synthesis, and the subsequent loss of receptors is measured by radioligand-binding methods. This technique has several shortcomings: (*a*) protein synthesis inhibitors may themselves alter receptor metabolism; (*b*) it cannot easily distinguish reutilization of receptors from intracellular pools; (*c*) use of agonists and antagonists to identify receptors may contribute to changes in receptor appearance and disappearance, since they can down-regulate or up-regulate receptors, respectively; (*d*) receptors cannot be measured before the binding site is formed (or in the correct conformation) or after the binding site is lost (or conformationally modified); and (*e*) some radiolabeled antagonists cross the cell membrane and thus receptors identified in studies with intact cells may include plasma membrane receptors (presumably functional) as well as receptors in intracellular pools.

Another indirect approach well suited for studies of metabolism of certain classes of receptors is the analysis of the reappearance of receptors (by radioligand-binding assays) after treatment of cells or tissues with antagonists capable of irreversible binding to receptors. There are a number of such alpha- and beta-adrenergic antagonists, and this approach has been the one most widely used to study metabolism of adrenergic receptors. Antagonists of high pharmacological specificity are preferred so that low concentrations can be used to achieve substantial blockade of receptors on target cells while facilitating removal of unreacted reagent after treatment. One critical assumption with the use of irreversible blocking agents is that covalently modified receptors do not themselves alter the normal expression of reappearing receptors. As described below, analysis of the kinetics of reappearance of receptors after blockade can provide insights into the potential cellular mechanisms that regulate expression of adrenergic receptors.

Kinetic Analysis of Receptor Metabolism

Kinetic analyses of receptor turnover have been performed for a number of receptors including alpha- and beta-adrenergic receptors (13–18). These studies allow one to define rates and rate constants that characterize general features of receptor metabolism. Generally, two criteria must be met before an adequate analysis can be made: (*a*) receptor production should be constant during the period of study and (*b*) the degradation of receptors, at any time, should be proportional to the concentration of these receptors in the cell. These criteria have been validated for a number of receptor systems, including those for insulin (9), epidermal growth factor (10), and acetylcholine (13). Thus, the kinetics of receptor synthesis and degradation (appearance and disappearance in binding studies) can be described by

$$R(t) = \frac{k_a}{k_d}(1 - \exp^{-k_d t}) + R_0 \exp^{-k_d t}, \qquad 1.$$

where $R(t)$ represents receptor number at time t, k_a, and k_d are the rate of receptor appearance and the rate constant of receptor disappearance, respectively, and R_0 is the initial number of receptors at the start of an experiment (9).

One experimental approach is to measure directly either k_a or k_d and then to derive the other value from the steady-state relationship when receptor levels, R_{ss}, are constant and $R_{ss} = k_a/k_d$. Thus, for example, k_d can be ascertained from the rate of loss of receptor binding sites when cells are treated with a protein synthesis inhibitor. Receptor half-life is then defined as $0.693/k_d$.

An alternative approach is to measure the rate of receptor reappearance after receptors have been blocked with an irreversible antagonist. Analysis of such data by nonlinear regression methods can yield unique values for k_a or k_d and a value for R_{ss} that should be equivalent to the steady-state number of receptors in untreated controls. This approach is probably best applied to studies with cells in vitro. When these analyses are performed on confluent cells that are not dividing, the values obtained apply to a single cell. In continuously growing cultures, cells in different phases of the cell cycle contain different levels of receptors, since as a first approximation, receptor synthesis must lead to a doubling in receptor number during one generation time prior to mitosis. Therefore one must take into account cell growth during the course of such studies, described by

$$C(t) = C_0 \exp^{k_c t}, \qquad 2.$$

where k_c is the rate of cell growth and C_0 is the initial cell density. When

equations for receptor turnover and cell growth are combined (17), total receptor production is expressed as

$$R_T(t) = [(k_a C_0)/(k_c + k_d)]\exp^{k_c t} + [R_0 - (k_a C_0)/(k_c + k_d)]\exp^{-k_d t}, \quad 3.$$

and values for k_a and k_d can then be obtained by nonlinear regression analysis. Use of this equation to obtain unique solutions of k_a and k_d is difficult when both variables are unknown. Total receptor production is a function of two independent exponential processes with disparate rate constants and is later heavily contributed to by cell growth. Thus a reasonable fit of data can be obtained by a series of different values for the two parameters, each combination of which yields an equivalent value for R_{ss}. Precise determination of k_a and k_d requires extensive data in the initial phase of receptor reappearance, where the signal-to-noise ratio is lowest. A more manageable rearrangment of this equation to receptors/cell (i.e. dividing by the cell growth equation) gives an equation similiar to that for confluent cultures except it reflects the influence of the cell growth rate constant:

$$R(t) = [k_a/(k_c + k_d)]\exp^{-(k_c + k_d)t} + (R_0/C_0)\exp^{-(k_c + k_d)t}. \quad 4.$$

Accurate measurement of k_c in treated cells is necessary for deriving values for receptor appearance and disappearance. In addition, no alterations should occur in cell growth or distribution in cell cycle phases, since such alterations could influence the calculation of receptor number per average cell. Because this analysis describes the average cell in a continuously growing culture, measurements of receptor expression during the cell cycle in a synchronized population of cells can provide a more detailed description of changes that occur in a single cell.

Receptor metabolism studied in the absence of agonists is often termed "basal" metabolism. Experiments in vitro therefore require the use of serum-free media or serum screened for negligible levels of catecholamines. Studies of basal metabolism of receptors in vivo entail assessment of receptor metabolism in the presence of endogenous levels of tissue and plasma catecholamines in animals. Prolonged agonist or antagonist interaction with adrenergic receptors alters receptor expression on a number of target cells. These alterations are perhaps best understood with a knowledge of the basal metabolism of receptors. To date few kinetic analyses have been used to derive rates and rate constants for adrenergic receptor appearance and disappearance, respectively. The fairly straightforward nature of the approach should facilitate its application both in vitro and in vivo.

IN VITRO STUDIES OF ADRENERGIC RECEPTOR METABOLISM

Alpha-Adrenergic Receptors

Most studies on the metabolism of alpha-adrenergic receptors have involved the use of irreversible blockade of receptors, most commonly by administration of the antagonist phenoxybenzamine (POB), although *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) has also been used (19, 20). Subsequent reappearance of receptors has been assessed using radiolabeled antagonists such as [^{3}H]prazosin for alpha$_1$-receptors or [^{3}H]yohimbine, [^{3}H]rauwolscine, and [^{3}H]clonidine for alpha$_2$-receptors.

Both POB and EEDQ are somewhat nonspecific. POB alkylates proteins through an unstable ethyleniminium ion that may irreversibly couple to negatively charged groups on the receptor protein (19). EEDQ efficiently couples to carboxylic acids either at the C-terminus or through an acidic amino acid, and is used as a coupling agent in peptide synthesis (21). Hence, both compounds rely on the accessibility of suitable chemical groups at, or near the site at which, the ligands bind to a given receptor. POB is reportedly more selective for alpha$_1$- than for alpha$_2$-adrenergic receptors (22, 23), whereas EEDQ appears more selective for alpha$_2$-adrenergic receptors (24). Unfortunately, both these drugs can bind to several classes of receptors in addition to alpha-adrenergic receptors. POB blocks dopaminergic (25, 26), cholinergic (27), gamma–aminobutyric acid (28), and opiate (29) receptors. EEDQ is similarly promiscuous and blocks binding to dopaminergic (30, 31), serotonergic (24), and cholinergic receptors and to various enzymes (32). The radioligand used to quantitate receptors provides some specificity in measuring receptor metabolism. However, different receptor systems may interact, and thus events measured in a given system could include both direct effects as well as indirect changes produced by interacting receptor systems. Also, if an extensive number of proteins are inactivated by POB or EEDQ, the altered protein synthesis in the cell may influence the metabolic turnover of a given receptor.

There are only a few reports on in vitro metabolism of alpha-adrenergic receptors (Table 1). One limiting factor has been that few established cell lines express sufficiently high numbers of alpha$_1$- or alpha$_2$-receptors. Assessment of reappearance of alpha$_1$-receptors after POB treatment has been reported for the BC_3H1 muscle cell line (18, 33). Other cell lines expressing high numbers of alpha$_1$-adrenergic receptors, DDT_1 cells derived from smooth muscle (34), and MDCK cells derived from renal tubular epithelium (35) are now available as useful models.

Table 1 Turnover of alpha- and beta-adrenergic receptors: in vitro studies

Cell type	$T_{1/2}$ (hr)[a]	Comment	Ref.
Alpha-receptors: Basal			
BC_3H1 muscle	24	Confluent	18, 23
Hepatocytes	18	Loss in primary culture	36
Down-regulation			
BC_3H1 muscle	3	Recovery CHX[d] sensitive	18
MDCK renal	8		37
Beta-receptors: Basal			
BC_3H1 muscle	n.d.[b]	Preconfluent	18
	170–200	Confluent	
C6 glioma	>150	Confluent	48
1321N1 astrocytoma	>100	Preconfluent	51
VA lung	25–30	Confluent	49
S49 lymphoma	30	Continuous growth	17
Down-regulation			
BC_3H1 muscle	1	Recovery CHX sensitive	18
S49 lymphoma	3	Recovery CHX sensitive	55, 58
C6 glioma	2	Down-regulation CHX sensitive Recovery CHX insensitive	48, 57
1321N1 astrocytoma	n.m[c]	Recovery CHX/TUN insensitive in preconfluent cells	45, 51
	n.m (<12)	Recovery CHX sensitive, TUN insensitive in postconfluent cells	

[a]receptor half-life, calculated (k_d) or estimated for basal data; estimated half-life for down-regulation data.
[b]n.d. = not detectable
[c]n.m. = measured.
[d]CHX = cycloheximide; TUN = tunicamycin.

Alpha$_1$-adrenergic receptor number increases in BC_3H1 cells as a function of cell growth. At confluence, cells maintain a steady-state level of 200–250 fmol receptor/mg membrane protein. Measurements of receptor number in confluent cells after treatment with cycloheximide or POB yielded an identical estimate for receptor half-life of ~24 hr (18, 33). This estimate of receptor turnover is similar to that reported for alpha$_1$-receptor disappearance ($t_{1/2}$ = 18 hr) in hepatocytes that lose receptors in primary culture (36). Reappearance of receptors in BC_3H1 cells after POB treatment was inhibited by treatment of cells with cycloheximide, indicating that reappearance was dependent upon protein synthesis (presumably receptor protein) (33).

Incubation of target cells with catecholamine agonists promotes a down-regulation of alpha$_1$-adrenergic receptor number (18, 37). In BC_3H1 cells, this decrease in receptor number occurred at a rate substantially faster ($t_{1/2}$ = 3 hr) than that obtained for basal turnover of receptors. This enhanced loss of

receptors was unaffected by cycloheximide, implying that an increase in receptor disappearance, and not a decrease in receptor appearance, accounted for down-regulation of receptor number (18). Following removal of agonists, $alpha_1$-receptors in BC_3H1 cells returned to control levels within 10 hr, a rate considerably faster than that obtained after POB treatment of cells (18, 33). This result suggests either that receptors down-regulated by agonists are endocytosed but not degraded and are then recycled from an intracellular site, or that agonist treatment increased a rate-limiting step in new receptor synthesis, processing, or insertion. Recovery of down-regulated receptors was inhibited by cycloheximide, indicating that protein synthesis is required. Recovery of functional response (phosphatidyl-inositol turnover) closely paralleled the recovery of $alpha_1$-receptors in these cells after POB or agonist treatment (18). Thus, those receptors measured during the period of reappearance were probably located on the plasma membrane.

Although several cell lines have been reported to express $alpha_2$-adrenergic receptors [including NG108-15, rodent neuroblastoma–glioma hybrid cells (38); HT29, human adenocarcinoma cells (39); HEL, human erythroleukemia cells (40); and RINm5F, rat insulin-secreting cells (41)], studies examining metabolism of these receptors have not been reported.

Beta-Adrenergic Receptors

Most data on the cellular metabolism of beta-adrenergic receptors result from studies using inhibition of protein synthesis or irreversible blockade of receptors in cultured cell lines. Receptors have been identified using radiolabeled antagonists such as [^{3}H]dihydroalprenolol (DHA), [^{125}I]iodocyanopindolol (IYCP), [^{125}I]iodopindolol (IPIN), or [^{125}I]iodohydroxypindolol (IHYP). The pindolol antagonists, IPIN and ICYP, bind with high affinity (pM) and low nonspecific binding (<20%) to both $beta_1$- and $beta_2$-receptors in membrane preparations or in intact cells (42–45). As detailed below, the irreversible antagonists are derivatives of propranolol, alprenolol, or pindolol that undergo alkylation reactions with neighboring thiol, amino, thioether, or imidazole groups on the receptor (46).

Basal rates of beta-receptor turnover have been measured in many cell lines that reach confluence in culture. In BC_3H1 muscle cells, turnover of $beta_2$-receptors virtually ceased in confluent cells (18). In preconfluent cultures, receptor appearance paralleled cell growth and receptor turnover was negligible. Estimated half-lives of receptors in confluent cells were 170–200 hr. These half-lives were calculated from rate constants of disappearance in experiments using either inhibition of protein synthesis or irreversible inactivation of receptors with the antagonist *N*-[2-hydroxy-3-(1-naphthoxy)-propyl]-N'-bromoacetylethylenediamine (NHNP-NBE). This slow rate of receptor disappearance was associated with a very slow receptor appearance

rate: <15% of control levels of receptors accumulated over 72 hr. These properties of beta-receptor turnover differ markedly from those for $alpha_1$-receptors on the same cell (Table 1).

In C_6 glioma cells, which contain predominantly $beta_1$-receptors, similar results were obtained. In preconfluent cells synchronized by double thymidine block, receptor number increased continuously as cells moved from the G_1 through the S phase of the cell cycle (47). By contrast, beta-receptor turnover was negligible in confluent cells ($t_{1/2}$>150 hr); few receptors reappeared after treatment of cells with the irreversible antagonist bromoacetylaminomethyl-pindolol (Br-AAM-pindolol) (48). This reappearance was caused by a transient stimulation of cell growth and was completely blocked by cycloheximide.

In human embryonic lung (VA) cells, turnover of $beta_2$-receptors was more rapid (49). The half-life of reappearance of receptors after blockade with NHNP-NBE was 25–30 hr. However, in this study the steady-state level of receptors attained after blockade was only 60% of that of control cells, which makes this estimate of receptor turnover difficult to interpret. The protein synthesis inhibitor, puromycin, blocked the reappearance of receptors, but more interestingly, receptors reappeared at a faster rate in cells treated with glucocorticoids.

Beta-receptor expression in another cell line that reaches confluence in culture, 1321N1 human astrocytoma cells, is biphasic, i.e. receptor number increases prior to, but then decreases after, confluency is reached (45, 50). The initial receptor expression (80–100 sites/cell/hr) was consistent with cell growth and dependent on receptor glycosylation. Preconfluent cultures treated with cycloheximide lost receptors very slowly ($t_{1/2}$>100 hr) (51). At confluence, receptor number reached a peak of ~4000 sites/cell and then decreased over 48 hr to 800–1000 sites/cell. This slower receptor expression in confluent cultures (~20 sites/cell/hr) was insensitive to inhibition of glycosylation, thus indicating differences in beta-receptor metabolism in pre- and postconfluent cultures.

In a continuously growing cell line, the S49 T-lymphoma, that grows in suspension culture, turnover of $beta_2$-receptors was measured after receptor blockade with the irreversible antagonist, bromoacetylalprenololmenthane (BAAM) and compared to receptor expression during the cell division cycle (17). Receptors appeared at a continuous rate of 75 sites/cell/hr in synchronized cells that maintained an average of 1200–1400 sites/cell when grown in asynchronous culture. This average number of receptors reflects the distribution of cells within the cell cycle phases (G_1, S, G_2/M). Receptors reappeared on cells treated with BAAM to steady-state levels. When analyzed according to Equation 1, a receptor half-life of ~30 hr was obtained. This estimate of turnover (0.023 hr^{-1}) is less than the value predicted by Equation

4, or $k_c + k_d$. The minimum half-life of receptors would equal the rate constant for S49 cell doubling, $k_c = 0.041$ hr^{-1} if receptor turnover was negligible ($k_d = 0$). (This point was not noted in the report of these data.) Although BAAM produced no apparent alteration in cell growth or cell cycle phase distribution, it may have slowed receptor appearance, possibly through interactions with intracellular receptor pools.

Incubation of cells in culture with beta-adrenergic agonists markedly reduces receptor number as measured by radioligand-binding assays. This agonist-induced down-regulation is distinct from and preceded by a rapid onset of cellular refractoriness, or desensitization, in which total receptor number is unchanged (52–54). During this early phase of refractoriness, receptors have become uncoupled from adenylate cyclase and may be localized in cellular compartments distinct from the plasma membrane. In several cell types studied thus far, agonist-promoted down-regulation of beta-receptors occurs more quickly than basal turnover (Table 1). The estimated half-life of receptor down-regulation in these studies is typically 1–4 hr.

Down-regulation of receptors could result from a marked decrease in receptor appearance or an increase in receptor disappearance. In BC_3H1 cells inclusion of cycloheximide had no effect on the rate of agonist-induced down-regulation, suggesting that accelerated clearance (or degradation) of receptors, not decreased synthesis, was the underlying mechanism. Similarly, in S49 cells a decrease in receptor appearance alone could not account for the kinetics of down-regulation (17, 55). In contrast, down-regulation of receptor number by agonists in C6 and C6-2B glioma cells was inhibited by cycloheximide, although in these cells down-regulation is induced by elevation of intracellular cAMP (56, 57). This type of down-regulation is distinct from that observed in studies using clonal variants of wild-type S49 cells that have lesions in the coupling of receptors to adenylate cyclase and the activation of cAMP-dependent protein kinase. Both the rate and extent of down-regulation of receptors by agonists were dependent upon receptor interaction with the alpha subunit of G_s but not upon activation of adenylate cyclase, generation of cAMP, or phosphorylation by cAMP-dependent protein kinase (55). Studies of agonist-induced down-regulation of beta-receptors have not produced direct evidence for the degradation of the primary structure of receptors.

In two cell lines, BC_3H1 and S49, recovery of beta-receptors following down-regulation by agonists occurred more rapidly than could be accounted for by basal receptor appearance or cell growth. Following a 16-hr exposure to epinephrine, receptor levels on BC_3H1 cells returned to control cell levels after 10 hr, following an initial 2-hr lag (18). In S49 cells, in which ~80% of the receptors were lost by an overnight exposure to isoproterenol, receptors recovered fully by 10–12 hr (55, 58). Recovery in both cell types was

inhibited by cycloheximide (18, 58). Interestingly, recovery of receptors from down-regulation occurred at a similiar rate in wild-type S49 cells and in several clonal variants, suggesting that in contrast to down-regulation, recovery was independent of G_s, cAMP, or of cAMP-dependent protein phosphorylation.

Recovery of beta-receptors differed in pre- and postconfluent cultures of 1321N1 cells subjected to agonist-induced down-regulation. In preconfluent cultures, receptors recovered at a rate similiar to the rate of receptor expression in untreated cells. Also in preconfluent cultures, recovery was not inhibited by cycloheximide or the glycosylation inhibitor, tunicamycin, suggesting that recycling of receptors may occur (45, 51). In contrast, the rate of recovery of receptors in postconfluent cultures was rapid and occurred during a period when receptor levels in untreated cells fell more than 50%. Inclusion of cycloheximide, but not tunicamycin, inhibited recovery, suggesting that recovery in postconfluent cultures requires protein synthesis but not receptor glycosylation. Subsequent studies using the heavy amino acid/density-shift method (11), demonstrated clearly that receptors were newly synthesized during recovery from down-regulation in postconfluent cells. Thus both basal metabolism and agonist-altered metabolism of beta-receptors appear to be regulated differently in pre- and postconfluent 1321N1 cells.

In studies on postconfluent C6 glioma cells, the rate of recovery of receptors following down-regulation was faster than that of basal expression of receptors (48). When cells were incubated with Br-AAM-pindolol prior to down-regulation, and receptor reappearance was assessed, however, the resulting increased rate of recovery was not due to an increase in the rate of receptor appearance. In addition, this recovery process was insensitive to cycloheximide. Thus agonists may promote a recycling of beta-adrenergic receptors in this system. The cellular compartment in which down-regulated receptors reside and from which they recycle has not yet been identified in either C6 cells or 1321N1 cells.

IN VIVO STUDIES OF ADRENERGIC RECEPTOR METABOLISM

Alpha-Adrenergic Receptors

More data are available on the metabolism of alpha-adrenergic receptors in vivo than in vitro. To date, most studies have involved administration of POB or EEDQ to rats or rabbits. Reappearance of either $alpha_1$- or $alpha_2$-adrenergic receptors and function has subsequently been measured in peripheral and CNS tissues (Table 2).

In rats treated with POB, reappearance of $alpha_1$-receptors in liver occurred with a half-life of 42 hr (59). Recovery paralleled the return of $alpha_1$-

Table 2 Turnover of alpha- and beta-adrenergic receptors: in vivo studies

Tissue	$T_{1/2}$	Comment	Ref.
Alpha$_1$-receptors: Basal			
Rat			
salivary gland	33 hr	Reappearance cycloheximide sensitive	23
liver	42 hr	Recovery of function parallels receptor reappearance	59
cerebral cortex	5–6 day		22
	8 day	Young rats	
	15 day	Aged rats	62
Rabbit			
spleen	86 hr	Functional recovery (vascular) precedes receptor reappearance	60
cerebral cortex	11 day		61
brainstem	13 day		61
Alpha$_2$-receptors: Basal			
Rat			
cerebral cortex	4–5 day	Differential recovery of functional response	20, 62
	10–14 hr	30% blockade of receptors; no inhibition of function	22, 64
Rabbit			
spleen	38 hr	Vascular response parallels receptor reappearance	60
cerebral cortex	6 day	CNS-mediated function	61
brain stem	4–5 day	precedes receptor reappearance	
Beta-receptors			
Rat			
heart (beta$_1$)	100 hr	Young rats; basal	73
	350 hr	Old rats; basal	74
lung (beta$_2$)	320 hr	Young rats; basal	73
	550 hr	Old rats; basal	74
renal cortex	45 hr	Beta$_1$ recovery from down-regulation	76
	18 hr	Beta$_2$ recovery from down-regulation	
	12 hr	Down-regulation: beta$_1$ and beta$_2$	

adrenergic function, as measured by phenylephrine-stimulated glucose release ($t_{1/2}$ = 49 hr) and $^{45}Ca^{2+}$ efflux ($t_{1/2}$ = 38 hr) in tissue slices. In rat submaxillary glands (23), kinetic analysis of receptor reappearance yielded a similiar value for the half-life of alpha$_1$-receptors ($t_{1/2}$ = 33 hr). In contrast, alpha$_1$-receptors in rabbit spleen reappeared more slowly ($t_{1/2}$ = 86 hr) after POB administration (60). In these studies, however, alpha$_1$-mediated pressor response to phenylephrine and contraction of renal arteries by norepinephrine

recovered more quickly ($t_{1/2}$ = 22 hr and 10 hr, respectively). Aside from the potential for tissue-specific differences in the metabolism of alpha$_1$-adrenergic receptors, these results demonstrate the potential difficulty in predicting alterations in function from changes in receptor number in vivo.

Studies thus far in CNS tissues suggest that alpha$_1$-receptors in the brain are more metabolically stable than those in peripheral tissues. After treatment of rabbits with POB, reappearance of alpha$_1$-receptors was slow; observed half-lives for reappearance were 10–11 days in cortex and 13 days in brain stem (61). In rats treated with POB, cortical alpha$_1$-receptors reappeared with a half-life of 5–6 days (22). Results from similar treatments of young (3-months-old) and aged (24-months-old) rats, and measurements of reappearance of [^{3}H]prazosin-binding sites in cerebral cortex and hypothalamus, extend these findings (62). Reappearance of 50% of the number of receptors in controls took approximately 8 days in young rats, but 15 days in old rats. The high lipid content of the brain could serve as a depot for POB, a lipophilic compound, and could potentially affect receptor reappearance. However analysis of the concentration and rate of disappearance of labeled POB in brains from young and old rats were identical and did not account for the differences in rates of reappearance observed in these studies. Thus a decreased turnover of alpha$_1$-receptors in the brain, especially in aged animals, may reflect the generally slower rate of protein synthesis and turnover in brain than in peripheral tissues (63).

Similiar studies have examined the metabolism of alpha$_2$-adrenergic receptors in vivo. In rabbits treated with POB, reappearance of [^{3}H]-clonidine binding sites in the spleen occurred with a $t_{1/2}$ of 38 hr; this rate is in good agreement with the recovery of maximum pressor response to administration of guanabenz, an alpha$_2$-selective agonist (60). The turnover of alpha$_2$-receptors in the CNS is slower and similar to results observed for alpha$_1$-receptors. Analysis of the kinetics of reappearance of alpha$_2$-receptors in cortex and brain stem from rabbits yielded estimates for receptor half-life of 6.1 and 4.6 days, respectively (61). Recovery of central alpha$_2$-mediated depressor response to intracisternally administered clonidine, however, occurred with a half-life of 2.4 days. Comparable estimates of 4–5 days were obtained for the half-life of reappearance of alpha$_2$-receptors in rat cortex (20, 62). Agonist (UK-14,304)-mediated inhibition of release of preloaded [^{3}H]norepinephrine in rat cortical slices (20) recovered more quickly ($t_{1/2}$ = 2.4 days), although alpha$_2$-mediated inhibition of [^{3}H]serotonin release paralleled the reappearance of receptors ($t_{1/2}$ = 4.6 days). These results cannot distinguish, however, whether inhibitory alpha$_2$-receptors exhibit different rates of turnover on noradrenergic and serotonergic neurons or that these inhibitory responses have different receptor occupancy requirements.

An exception to the slow turnover of alpha$_2$-receptors in the CNS has been

reported for cortical tissue from rats administered POB (22, 64). In this study, the half-life of reappearance was 10–12 hrs; however, only 25–30% blockade was achieved and no change was observed in clonidine-mediated inhibition of release of preloaded [^{3}H]norepinephrine from synaptosome preparations. Theoretically the extent of blockade does not alter estimates of receptor turnover in a homogeneous population of receptors. This study suggests that cortex may contain subpopulations of alpha$_2$-receptors with different rates of turnover and/or accessibility to POB, however dose-dependent effects on the rate of reappearance of adrenergic receptors after irreversible blockade have been reported in vivo (23), whereas these effects are not observed in vitro (15, 17). Of note, reappearance of alpha-adrenergic receptors in vivo has been blocked in animals treated with inhibitors of protein synthesis such as 5-fluorouracil (61) and cycloheximide (23).

An alternative approach to the study of the metabolism of adrenergic receptors *in vivo* is to examine the recovery of receptor binding sites and function after down-regulation of receptor number. Receptor down-regulation has been indirectly elicited by administration of drugs that elevate plasma levels of catecholamines, such as antidepressants (65–67) and steroids (68), or by denervation (69). In many cases it has been difficult to determine whether elvated catecholamines or other compensatory responses are responsible for changes in receptor number. Administration of agonists in vivo has been successfully used to elicit down-regulation of alpha$_1$-adrenergic receptors. Plasma concentrations of catecholamines have been elevated either by intraveneous administration or by implantation of catecholamine-secreting pheochromocytomas (69–71). Studies of the influence of agonists on receptor turnover, however, have not been performed. This approach has not proved useful for the study of alpha$_2$-receptors in vivo. Agonist-induced down-regulation of these receptors has rarely been observed in tissues thus far examined (72). It is conceivable that after exposure to agoinsts, alpha$_2$-receptors become uncoupled from functional response, and receptors are translocated or sequestered in the membrane, but the binding site remains intact. Thus, as discussed earlier, such changes might not be detected in subsequent radioligand-binding studies.

Beta-Adrenergic Receptors

A limited number of studies have examined the in vivo metabolism of beta-adrenergic receptors. These studies have investigated the reappearance of receptors following either irreversible blockade or treatment with agonists.

Reappearance of [^{3}H]DHA binding to beta-receptors in heart (>beta$_1$) and lung (>beta$_2$) was measured in young rats (1 month old) after treatment with BAAM, which caused an ~90% reduction in receptor number (73). Receptors reached control levels after 8 days ($t_{1/2}$~100 hr) in heart and 27 days

($t_{1/2}$~320 hr) in lung. In a similar series of experiments, reappearance of beta-receptors in the heart and lung was compared in young (1 month old) and senescent (27 month old) rats (74). Reappearance was markedly slower in both tissues from senescent rats ($t_{1/2}$ of ~350 hr and ~550 hr for heart and lung, respectively). Although BAAM is lipid soluble and could potentially partition in fat tissues or cross the blood-brain barrier, no evidence of residual BAAM in membrane preparations was detected in radioligand binding studies. In studies of blockade of beta-receptors by BAAM in guinea-pig lung, reappearance of antagonist binding sites preceded that of high affinity agonist binding sites and airway responsiveness to beta-agonists (75).

Subtype-selective alterations in the number of beta-adrenergic receptors occur in various tissues from rats implanted with norepinephrine-secreting pheochromocytomas or catecholamine-infusing osmotic minipumps (70, 71). In these studies plasma catecholamines were markedly elevated (>50-fold). Using continuous infusion and removal of the agonist isoproterenol, rates of $beta_1$- and $beta_2$-receptor appearance and disappearance were determined in rat renal cortical membranes, which contain 70% $beta_1$- and 30% $beta_2$-subtype (76). Infusion rates of 50–110 μg/kg/hr for 72 hr reduced total beta-receptor number by 50%. A similar reduction in both $beta_1$- and $beta_2$-subtypes (40% and 50%, respectively) was observed. Agonist-induced rate constants of receptor disappearance for both subtypes were identical, yielding an estimate for receptor half-life of 12 hr. $Beta_1$-receptors recovered more slowly ($t_{1/2}$ = 45 hr) from down-regulation, however, than did $beta_2$-receptors ($t_{1/2}$ = 18 hr). These data were analyzed according to Equation 1 to estimate k_a and k_d. The faster recovery observed for $beta_2$-receptors was associated with a more than a twofold elevation in both the rate of receptor appearance and rate constant of receptor disappearance compared to values observed for $beta_1$-receptors. Although limited in number, these in vivo studies suggest that basal turnover of beta-adrenergic receptors is slow and exhibits age dependency similiar to that observed for alpha-adrenergic receptors. By analogy with in vitro studies, exposure of tissues to elevated concentrations of agonists may accelerate the cellular metabolism of beta-adrenergic receptors.

SUMMARY AND CONCLUSIONS

Despite considerable evidence that changes in number of adrenergic receptors can occur under various conditions, knowledge of the mechanisms mediating these changes is still rudimentary. As discussed, indirect approaches emphasizing the kinetics of receptor turnover have been the principal means of investigation. These indirect methods, which depend on the ability of a radioligand to detect the receptors, are limited by several factors. Even so, the

data obtained using indirect approaches, in particular on various model systems in cell culture, lead to several conclusions:

1. Both $alpha_1$- and beta-adrenergic receptors are metabolized rather slowly in vitro under basal conditions, in the absence of exposure to agonists. Typical half-lives are >20 hr, a turnover that is slower than that of several other classes of neurotransmitter and hormone receptors (9, 10, 12, 16). Moreover, $alpha_1$-adrenergic receptors and beta-adrenergic receptors can have substantially different half-lives, even when expressed on the same cell.
2. In view of the relatively slow rate of disappearance of adrenergic receptors under basal conditions, settings in which receptor number increases are almost certainly to result from increases in one or more of the factors that contribute to the rate of receptor appearance on the plasma membrane.
3. Treatment of cells with agonists markedly shortens the half-life of $alpha_1$- and beta-adrenergic receptors. This shortened half-life results primarily from an enhanced loss of receptors from the plasma membrane, and not from agonist-induced attenuation of receptor appearance. In fact, data acquired from studies of receptor recovery after agonist-induced down-regulation suggest that rates of receptor reappearance are markedly enhanced through either receptor recycling or an increase in receptor synthesis.
4. Limited studies conducted in vivo yield qualitatively similar results to those observed in in vitro studies of the metabolism of adrenergic receptors. In general, adrenergic receptors in the CNS turn over more slowly than those in peripheral tissues.

These conclusions help to highlight the many aspects of metabolism of adrenergic receptors that are as yet unknown, including identification and characterization of the cellular machinery responsible for receptor metabolism, elucidation of the molecular events that control metabolism, and assessment of how drugs and other factors influence these events. Future studies are likely to be based on the development of new methodology with antireceptor antibodies, receptor cDNA's, and improved morphological methods (autoradiography, immunohistochemistry, etc). Application of these techniques should help provide the biochemical and morphologic answers to the numerous unresolved aspects of adrenergic receptor metabolism.

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