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Agonist-induced Desensitization of the β-Adrenergic Receptor-linked Adenylate Cyclase

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I. Introduction

THE β-adrenergic receptor-linked adenylate cyclase has provided a model system for the study of hormonal regulation over the past two decades. Studies of this system in a variety of tissues have revealed that one of its prominent and apparently ubiquitous features is the capacity to undergo agonist-induced desensitization† during exposure of intact cells to catecholamines and other hormones. Elevation of cyclic AMP levels in intact cells by agonists is transient with nucleotide levels usually returning to near basal concentrations within 30 to 60 min despite the continued presence of active hormone. Viewed another way, exposure of cells to a β-adrenergic receptor agonist followed by washing and rechallenge results in a reduced responsiveness of the cyclic AMP generating system to the agonist upon the second exposure.

Recent methodological advances in the identification and quantification of the component proteins of the β-adrenergic receptor-linked adenylate cyclase have led to the proposal of models for describing the events involved in the coupling of hormone-receptor interaction to the activation of adenylate cyclase. As a result of the enhanced knowledge of the structure of this system and with methodology available for the quantification of not only the individual components of the system, but also the interaction of components, there has been significant progress in the last few years in understanding the molecular basis of agonist-induced desensitization mediated through β-adrenergic receptors. In this review, I will attempt to describe several of these advances. Since the discussion will focus mainly on the molecular aspects of this topic, most of the literature that is considered will be reports that have appeared in the last 5 years.‡ Thus, many of the historically important early studies that described the phenomenon, but (because of a lack of

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† In addition to "desensitization," the terms tachyphylaxis and refractoriness also have been used to describe the same phenomenon. In this review, these terms will be used interchangeably to indicate that an initial exposure of a cell to an agonist results in a reduced capacity of the cell to respond upon a second challenge. No specific mechanism for loss of responsiveness is implied in their usage.

‡ Several other reviews have addressed various aspects of this topic (19, 102, 141, 190, 275).

availability of suitable methodology) did not greatly advance knowledge of the molecular basis of agonist-induced desensitization, will not be examined in detail. Also, agonist-induced desensitization of other receptors (particularly, peptide hormone receptors) that are linked to adenylate cyclase will not be reviewed [see Catt et al. (29)]. The greatest progress in this area of research has occurred through use of simple systems that involve homogeneous cell populations isolated from a variety of sources or maintained in culture. However, ample evidence exists for the occurrence of similar phenomena in vivo. In light of the important role of the β-adrenergic receptor system both in normal physiology and in therapeutics, the relevance of the results obtained in model systems to those obtained with experimental animals and man will be explored.

II. Modification of Hormonal Responsiveness by Mechanisms Unrelated to Reduction in Nucleotide Synthesis

Agonist-induced changes in responsiveness of the cyclic AMP system could involve alterations at any one of several steps involved in the regulation of cellular levels of nucleotide. The most straightforward, and apparently the most important modification, occurs as a reduction in the rate of cyclic AMP synthesis. However, cyclic AMP levels also are regulated by degradation of the nucleotide catalyzed by phosphodiesterase (247, 264) and by egress of cyclic AMP from the cell (17, 44, 132). Evidence for the rapid hormonal regulation of both the degradation (163, 180) and egress (17) of cyclic AMP has recently been reported. However, the contribution of similar phenomena to losses in hormone responsiveness during short-term exposure of target cells to catecholamines has not been examined extensively. In those instances where it has been studied, egress of nucleotide has been an unimportant factor in regulating nucleotide levels during catecholamine-induced desensitization. In contrast, agonist-induced alterations in phosphodiesterase activity may have importance in regulating hormonal responsiveness, particularly after long-term exposure of cells to agonists.

Increases in phosphodiesterase activity after chronic exposure of several cell types to catecholamines have been widely reported (14, 16, 185, 219, 220, 247, 257). This effect is apparently equivalent to the phenomenon reported initially by Manganiello and Vaughan (159) and D'Armiento et al. (42). That is, elevation of cyclic AMP levels in fibroblasts by chronic addition of either prostaglandin E_1 (PGE_1), a phosphodiesterase inhibitor, or dibutyryl cyclic AMP resulted in an increase in phosphodiesterase activity. Since this elevation in enzyme activity did not occur in the presence of inhibitors of protein or RNA synthesis (42, 159) or in a S49 lymphoma cell variant that lacks cyclic-AMP-dependent protein kinase (14), it was suggested that a cyclic-AMP-mediated induction of the synthesis of phosphodiesterase occurs. Although catecholamine-induced elevation of intracellular cyclic AMP also results in an increase in phosphodiesterase activity, this effect has been observed only after several hours exposure of cells to agonist (16, 185, 219, 220, 239). Thus, in most cases an increase in phosphodiesterase activity has been unimportant in the mechanism of short-term desensitization to the effects of catecholamines. The extent of contribution of changes in phosphodiesterase activity to the diminution in responsiveness after long-term exposure of cells to catecholamines has not been well worked out. In many cell types this phenomenon is apparently of little or no importance (49, 239); in others, arguments can be made for a contribution of enhanced phosphodiesterase activity after long-term (2 to 6 hours) incubation of cells with catecholamine. However, even in cells where there is an induction of phosphodiesterase, modifications in components of the β-adrenergic receptor-linked adenylate cyclase probably account for most of the reduction in hormonal responsiveness. One caveat to this conclusion involves the method of assay of phosphodiesterase. That is, in most studies enzyme activity has been measured in homogenates from desensitized cells. Such an approach introduces the possibility that an altered enzyme activity that is of importance in the intact cell will be lost due to the vagaries of cell lysis. Perkins and coworkers (123, 239) and Butcher and coworkers (8) have carried out kinetic analyses of phosphodiesterase activity in intact astrocytoma cells and WI-38 fibroblasts to rule out this possibility. However, this approach has not been taken in most studies of catecholamine-induced desensitization in other cell types.

In summary, although agonist-induced increases of phosphodiesterase activity occur in several systems, this effect is not ubiquitous and apparently is not involved in the desensitization to catecholamines that occurs within minutes of exposure of cells to agonist. Furthermore, in those tissues where long-term incubation with catecholamine results in an increase in phosphodiesterase activity, the reduction in hormone responsiveness would of necessity be hormone-receptor nonselective (i.e. refractoriness would occur to any hormone that activates adenylate cyclase), and the refractoriness would not be apparent in cell-free assays of adenylate cyclase activity. In light of these considerations, this review will principally focus on catecholamine-induced alterations in components of the synthetic mechanism of the cyclic AMP system.

III. Structure of the Catecholamine-responsive Adenylate Cyclase

Progress in understanding the mechanism of agonist-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase system has been dependent on the development of knowledge of the molecular basis of hormone-mediated activation of adenylate cyclase. Fortunately, over the past 6 years progress has been made

in the understanding of this system such that catecholamine-induced activation of adenylate cyclase can now be described in molecular terms. These methodological and conceptual advances involving the adenylate cyclase system have been rapidly applied to studies of agonist-induced desensitization. With these points in mind, it is realized that examination of the molecular basis of agonist-induced desensitization of adenylate cyclase requires an appreciation of the structure and function of this system. For more exhaustive consideration of this topic the reader is referred to several excellent reviews (145, 209, 228, 243). However, for the purposes of this discussion, it is important that several salient features of the adenylate cyclase system be reviewed here.

First, a variety of approaches have established that the β-adrenergic receptor-linked adenylate cyclase is comprised of at least three proteins. These are: 1) the cell surface receptor (R); 2) the guanine nucleotide binding protein (G/F), which serves a regulatory function in coupling agonist-receptor interaction to activation of the enzyme; and 3) the catalytic protein (C). The identification and characterization of these components has involved a variety of approaches. The conclusion that the β-adrenergic receptor and adenylate cyclase were individual proteins could be implied from indirect evidence obtained in studies of genetic mutants of cloned tumor cells (113, 121), in examinations of the ontogenetic regulation of the β-adrenergic receptor-linked adenylate cyclase (31, 87, 91), and in cell fusion experiments (186, 196, 215, 216). Direct evidence for this idea was obtained by using chromatographic and density gradient techniques to separate β-adrenergic receptor binding activity from adenylate cyclase activity and to demonstrate that the separated proteins exhibit distinct hydrodynamic properties (83, 148, 258). Knowledge that the stimulation of adenylate cyclase by hormones was GTP-dependent (205, 211) eventually led to the further resolution of the adenylate cyclase into two proteins, one expressing catalytic activity and one expressing GTP-regulatory activity. Pfeuffer (191, 192) was able to partially resolve these activities with GTP-sepharose chromatography. The availability of genetic variants of the murine S49 lymphoma cell line allowed further delineation of these components by Gilman, Ross, and coworkers (106, 208–210, 234) and Bourne and coworkers (122, 126, 178). That is, hormone-, guanine nucleotide-, and fluoride-stimulated adenylate cyclase activities were not measurable in membranes from the cyc⁻ S49 lymphoma cell mutant. However, a detergent extract from membranes of wild type S49 lymphoma cells (which are fully competent in these activities) conferred these three activities when reconstituted with cyc⁻ membranes. On the basis of these experiments, it was proposed that the adenylate cyclase consisted of a heat labile, N-ethylmaleimide-sensitive protein (C) and a thermostable protein (G/F) that conferred guanine nucleotide and fluoride-stimulated activities to cyc⁻ membranes. G/F has now been purified to apparent homogeneity by Northup et al. (184) using conventional chromatographic procedures and an assay for activity based on its capacity to reconstitute fluoride-stimulated activity into cyc⁻ membranes.

Significant advances also have been made in understanding the mechanism of interaction of these components. Many laboratories have demonstrated that in well washed membranes agonists bind to β-adrenergic receptors in a high affinity, slowly dissociable complex (48, 95, 131, 140, 152, 153, 211, 268, 270). This complex apparently involves an association of hormone (H), R, and G/F, and serves as an obligatory intermediate through which hormones stimulate the rate of activation of adenylate cyclase by guanine nucleotides (48, 227, 231). Antagonists do not induce the functional association of R and G/F and therefore, do not activate adenylate cyclase. However, evidence for the interaction of antagonist-occupied β-adrenergic receptors with G/F has been reported recently (273). The H·R·G/F complex is responsible for the high affinity agonist binding observed in competition binding experiments with radiolabeled antagonists. Similarly, the agonist [^{3}H]hydroxybenzylisoproterenol ([^{3}H]HBI) binds in a tight complex to β-adrenergic receptors from several sources (95, 270). In the presence of guanine nucleotides the complex rapidly dissociates and can be measured as a decrease in [^{3}H]HBI binding (95, 270) or as a decrease in the apparent affinity of agonists in competition binding experiments with radiolabeled antagonists (140, 153). Thus, the formation of this high affinity complex and the extent of effects of guanine nucleotides on agonist binding can be taken as a measure of the capacity for "coupling" between R and G/F. High affinity agonist binding is lost in β-adrenergic receptor/adenylate cyclase systems that are genetically deficient in G/F (84, 152, 211) or are chemically uncoupled (48, 107, 140, 230). As will be discussed below, catecholamine-induced uncoupling of the adenylate cyclase system during desensitization also results in a decrease in the extent of high affinity agonist binding (63, 89, 115, 266). The catalytic protein appears to be unnecessary for the normal interaction of R and G/F since a cell line (HC-1 hepatoma cells) deficient in C still exhibits high affinity, GTP-sensitive agonist binding (210, 241).

Interaction of R and G/F also can be demonstrated directly. Limbird, Lefkowitz, and coworkers have shown that incubation of erythrocyte membranes with [^{3}H]HBI (but not antagonists) increases the apparent size of the solubilized β-adrenergic receptor as determined by gel exclusion chromatography (146, 147, 149). G/F (which was identified by [^{32}P]ADP-ribosylation catalyzed by cholera toxin; see below) coeluted with [^{3}H]HBI (146). [^{3}H]HBI did not coelute with G/F if preincubations were carried out with receptor antagonists or GTP before solubilization.

It has been proposed that the activity expressed by the hormone-responsive adenylate cyclase is determined by

the steady-state level of a GTP·G/F·C complex (25, 27, 209, 228, 243). Since this complex exhibits a short lifetime in the face of a GTPase activity (25, 27, 193) that appears to be inherent in G/F, any situation that decreases the rate of hydrolysis of GTP in the active complex serves to maintain an elevated steady state level of this activated species. For example, a maximally activated system can be generated by the substitution of the stable analogs, Gpp(NH)p or GTPγS, for GTP (211, 217). These analogs in effect block the inactivation reaction since they are resistant to hydrolysis by the GTPase. In many systems catecholamines have no effect on the maximally activated enzyme in the presence of stable GTP analogs (211, 241). However, they do increase the rate of activation of the system by stable analogs (211, 217, 241), since they accelerate the dissociation of GDP from G/F and/or accelerate the rate of association of GTP analogs with G/F (28, 195).

In the presence of NAD^+, cholera toxin catalyzes the ADP-ribosylation of a protein that by several criteria appears to be G/F (26, 27, 74, 122, 126). Subsequent to ADP-ribosylation, GTPase activity of G/F is greatly reduced (27) and the kinetics of activation of the system in the presence of GTP become similar to those observed with nonhydrolyzable GTP analogs (120).

It has become clear that interaction of agonists with several receptors (e.g. muscarinic cholinergic, α_2-adrenergic, opiate, adenosine) results in an inhibition rather than an activation of adenylate cyclase (11, 117–119, 151, 175). Although this inhibition is GTP-dependent (10, 117, 119, 151), recent evidence suggests that coupling of these inhibitory receptors to adenylate cyclase occurs through a guanine nucleotide binding protein that is distinct from G/F (88, 169, 204, 225).

Based on the adenylate cyclase regulatory cycle originally proposed by Cassel et al. (25), a model (Fig. 1) for hormonal activation of adenylate cyclase can be described. Although the model is speculative, it incorporates most of the ideas discussed above and depicts steps in the transmembrane signalling between β-adrenergic receptors and adenylate cyclase that may be modified during catecholamine-induced desensitization. In the absence of H, the system is maintained in a basal state with GDP associated with G/F. The combination of H with R results in the formation of the H·R·G/F complex discussed above. The model emphasizes the "catalytic" role of the receptor in producing the active enzyme species through the exchange of GTP for GDP on G/F. The nature of this exchange of GTP for GDP in relation to the formation of the H·R·G/F complex is poorly understood. Nonetheless, in the presence of GTP, H·R·G/F is rapidly converted to HR + G/F·GTP. The active enzyme complex, GTP·G/F·C is then formed from G/F·GTP + C. Thus, R and C apparently do not interact directly, but rather, transmembrane coupling of signal between these two proteins is effected by a "shuttle" function of the coupling protein, G/F. The lifetime of

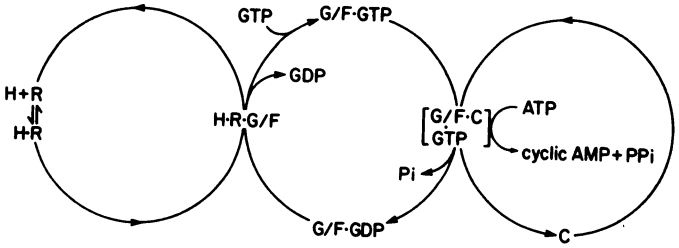

FIG. 1. Model for hormonal activation of adenylate cyclase. This model describes the hypothetical interactions of a catecholamine (H), the β-adrenergic receptor (R), the guanine nucleotide binding protein (G/F), the catalytic protein (C), and guanine nucleotides (GDP and GTP). The model indicates that R·H binds to G/F thereby resulting in the release of free GDP and the formation of H·R·G/F. The formation of the H·R·G/F complex apparently stabilizes the binding of H to R and thus increases the apparent binding affinity of H. In addition, the formation of H·R·G/F is apparently rate-limiting in the activation process and, in the presence of GTP, H·R·G/F is rapidly converted to G/F·GTP and H·R. Thus, the role of the β-adrenergic receptor is to effect the conversion of G/F·GDP to G/F·GTP. Once formed, G/F·GTP interacts with C to form the enzymically active complex C·G/F·GTP. The lifetime of the active complex is determined by the activity of a GTPase which hydrolyzes the bound GTP to release P_i with the resultant regeneration of inactive C and G/F·GDP. In the absence of GTP, addition of H leads to the formation of H·R·G/F in amounts sufficient to change the apparent K_d of the system for H. Thus, in the absence of GTP, agonists exhibit binding characteristic of interaction at two sites (R and R·G/F). Upon addition of GTP to membranes, the amount of H·R·G/F would be small because its formation is postulated to be the rate limiting step in the intact system. Under these conditions, agonists would exhibit binding properties characteristic of the reaction $R + H \rightleftharpoons RH$, namely low affinity binding to a single binding site. [Modified from Y.-F. Su, T. K. Harden, and J. P. Perkins, J . Biol. Chem. **255:** 7410–7419, 1980.]

GTP·G/F·C is regulated by the activity of the GTPase which apparently is associated with G/F. The GTPase converts GTP·G/F·C back to the ground state, liberating inactive C and P_i. The resulting GDP·G/F is then free to traverse the cycle again in the presence of hormone and GTP. Therefore, the cycle consists of an "on" reaction which is catalyzed by agonist-occupied R and an "off" reaction which is an expression of the GTPase. Under steady-state conditions the extent of activation of adenylate cyclase depends on the rate of the "on" reaction which in turn depends on the amount of HR. A more detailed statement of this basic model has been reported by Ross and Gilman (209), Limbird (145), Swillens and Dumont (243), and Stadel et al. (228). Also, several points of contention concerning the validity of such a model are discussed by Birnbaumer and Iyengar (10).

IV. General Properties of Catecholamine-induced Desensitization

In a study with rabbit cerebellar slices, Kakiuchi and Rall (124) first reported that exposure of target cells to catecholamines and other hormones resulted in a rapid rise followed by a decline in intracellular levels of cyclic AMP. Rechallenge of the slices with agonist resulted in a cyclic AMP response that was reduced compared to

that of the initial exposure. The ubiquity of this phenomenon was confirmed by many reports during the period of 1971 to 1976 that illustrated the occurrence of catecholamine-induced desensitization in a variety of other cell types (16, 37, 39, 49, 67, 69, 70, 100, 105, 137, 156, 181, 202).

Although the mechanism of catecholamine-induced desensitization was not forthcoming from these studies, several possibilities were ruled out. Loss of responsiveness was not due to: 1) destruction of agonist during hormonal challenge; 2) increased rate of egress of cyclic AMP into the extracellular medium; 3) an increase in phosphodiesterase activity, or 4) secretion into the medium of an inhibitor of hormone action. However, there is a notable exception to the last point: an inhibitory factor was produced by fat cells during receptor stimulation by epinephrine, ACTH, or glucagon (62, 99–101). The time course of appearance of this factor correlated with the time course of loss of hormonal responsiveness. Desensitization was to all hormone activators of adenylate cyclase irrespective of the receptor that was stimulated initially. Indeed, the formation of the factor was apparently in response to elevation of intracellular cyclic AMP levels since the addition of dibutyryl cyclic AMP also promoted its formation. This "feedback regulator" of hormone action does not appear to be produced by other cells.

Several properties of catecholamine-induced desensitization could be generalized from the early studies of this phenomenon. Preincubation of cells with agonists resulted in loss of hormonal responsiveness; incubation with antagonists produced no effect. Antagonists of β-adrenergic receptors blocked catecholamine-induced desensitization but had no effect on prostaglandin-induced desensitization. During short-term incubation with hormone, a good correlation was observed between efficacy for stimulation of adenylate cyclase and the extent of induction of desensitization, i.e. partial agonists induced "partial" desensitization. Deviation from this relationship during long-term incubation of cells with partial agonists is discussed below. The concentration-effect curve for induction of refractoriness by catecholamines was similar to that for elevation of cyclic AMP levels. Taken together, these results led to the conclusion that catecholamine-induced desensitization was the result of the normal interaction of agonists with β-adrenergic receptors.

The work of Kakiuchi and Rall (124) suggested that hormone-specific desensitization occurred in rabbit cerebellar slices. That is, norepinephrine induced a loss of responsiveness to catecholamines, but not to histamine. This conclusion was supported by some of the early studies of desensitization in cultured or isolated cell preparations. For example, Franklin and Foster (67) reported that preincubation of human diploid fibroblasts with either isoproterenol or PGE_1 resulted in desensitization to the inducing, but not the opposite, agonist. Similar conclusions were made by Remold-O'Donnell (202) in studies of adenylate cyclase activity in membranes derived from macrophages previously incubated with epinephrine or PGE_1. In contrast, Newcombe et al. (181) observed that preincubation of human synoviocytes with one hormone (epinephrine or PGE_1) resulted in a reduced capacity of the second hormone to elevate cyclic AMP levels. However, the extent of loss of responsiveness to the second hormone was not as great as to the agonist used in the first incubation.

V. Heterologous Desensitization

A. Distinction between Heterologous and Homologous Desensitization in Intact Cell Studies

Perkins and coworkers (238, 239) were the first to examine in detail the hormone-specific nature and kinetic properties of desensitization of the cyclic AMP generating system. Using 1321N1 human astrocytoma cells these investigators demonstrated that agonists acting through either β-adrenergic or prostaglandin receptors induced desensitization. Loss of responsiveness to the inducing agonist occurred with a $T_{1/2}$ of less than 30 min; hormone responsiveness was less than 10% of control after preincubation for 3 hours. At short times of incubation the loss of responsiveness was hormone-specific. That is, incubation of 1321N1 cells with β-adrenergic receptor agonists only resulted in a loss of responsiveness to catecholamines and incubation with PGE_1 only resulted in a loss of prostaglandin receptor responsiveness. However, when incubation times were extended past 30 min, exposure of cells to catecholamine resulted in a partial diminution of responsiveness to PGE_1 and vice versa. The extent to which hormone-nonspecific loss of responsiveness occurred (50% to 70% loss) was never as great as the almost complete loss of responsiveness to the inducing hormone. These results led Perkins and coworkers (238, 239) to propose that at least two phenomena accounted for agonist-induced desensitization in 1321N1 cells. The term "homologous" desensitization was applied to designate the type of desensitization observed when cells were rechallenged with the same agonist used in the initial exposure; "heterologous" desensitization referred to the situation where reexposure was to an alternate agonist.* Although the mechanism of neither type of agonist-induced desensitization was de-

* "Homologous" desensitization has had more than one meaning as used by workers in this field. In this review, the term will be used simply to indicate that cells previously incubated with a given agonist are rechallenged with the same agonist. No mechanistic meaning is implied in this usage. Indeed, homologous desensitization defined in this way may consist of only hormone-specific mechanisms in some cell types and both hormone-specific and hormone-nonspecific mechanisms in other cell types. "Heterologous" desensitization is also an operational term to denote a reduction in apparent responsiveness that occurs when cells are incubated with a given hormone then rechallenged with a second hormone. Although heterologous desensitization is equivalent to "agonist-nonspecific" desensitization, it may occur by more than one mechanism.

lineated in these studies, it was shown that incubation of 1321N1 cells with dibutyryl cyclic AMP induced refractoriness to both norepinephrine and PGE_1, and the time course of occurrence and extent of loss of responsiveness was the same as that of heterologous desensitization. Thus, heterologous desensitization was proposed to occur through a cyclic AMP-mediated reduction in adenylate cyclase activity. As was discussed above these workers ruled out any contribution of a cyclic-AMP-induced change in phosphodiesterase activity during the first 2 hours of desensitization (239). These studies (238, 239) and a subsequent study with another (EH118MG) cloned human astrocytoma cell line (123) led Perkins and coworkers to propose that homologous desensitization was composed of both the cyclic-AMP-mediated phenomenon that occurs during heterologous desensitization and hormone-specific phenomena that are unrelated to elevation of cyclic AMP. The nature of these hormone-specific modifications is considered below.

The conclusions made by Perkins and coworkers have been supported by studies of other intact cells. For example, Harbon and coworkers (244, 252) have reported that exposure of rat myometrium to either isoproterenol or PGE_2 results in a rapid loss of the intact cell cyclic AMP response to the inducing agonist followed by a loss of responsiveness to the alternate agonist (252). The time course and extent of occurrence of these phenomena were remarkably similar to those observed in cultured astrocytoma cells. Also, the fact that incubation of myometrium with a phosphodiesterase inhibitor resulted in refractoriness to both β-adrenergic and prostaglandin receptor agonists suggests that the heterologous type desensitization was mediated by cyclic AMP. Krall and coworkers (135, 256) have shown that isoproterenol- and prostaglandin-induced desensitization of human lymphocytes occurs by both agonist-specific and agonist-nonspecific mechanisms. Again, the heterologous desensitization was slower occurring and less extensive than homologous desensitization. The occurrence of both homologous and heterologous desensitization in the same cell has been reported for several other tissues (6, 28, 93, 94, 189, 244).

Brooker and coworkers (49, 182, 183, 246) have reported extensive studies of catecholamine-induced desensitization in C6-2B rat glioma cells. Although C6-2B cells only express one hormone receptor that is coupled in a stimulatory manner to adenylate cyclase, several lines of evidence indicate that catecholamines induce a "nonspecific" desensitization in these cells. In contrast to several other cells (137, 167, 238) where the occurrence of catecholamine-induced desensitization is unaffected by protein synthesis inhibitors, desensitization in C6-2B cells is substantially reduced in the absence of protein synthesis (182, 183, 246). As with heterologous desensitization in human astrocytoma cells (238) catecholamine-induced desensitization of C6-2B cells occurs secondarily to elevation of intracellular cyclic AMP levels. Catecholamines (49, 246), cyclic AMP analogs (246), cholera toxin (182, 183), and phosphodiesterase inhibitors (246) all induce desensitization. Moreover, the desensitization induced by a submaximal concentration of isoproterenol is potentiated by a phosphodiesterase inhibitor (246). The results of Brooker and coworkers have been duplicated by Koschel (134) who reported that elevation of intracellular cyclic AMP levels in C6 cells by cholera toxin or a phosphodiesterase inhibitor markedly decreased responsiveness to catecholamines. The desensitization that is induced by catecholamines in C6-2B cells by elevation of cyclic AMP levels is unrelated to phosphodiesterase. That is, although long-term elevation of cyclic AMP levels in several C6 glioma cell clones results in the induction of an increase in phosphodiesterase activity (16, 219, 220, 257), this does not occur in the 2B clone (49).

Loss of responsiveness during incubation of C6-2B cells with catecholamines occurred with a $T_{1/2}$ of approximately 1 hour; after 5 hours of incubation responsiveness was reduced by greater than 90% (49, 246). Almost no recovery of hormone responsiveness was observed over a period of hours if cells were desensitized and then incubated in agonist-free medium (246). In contrast, if agonist-free medium containing the protein synthesis inhibitor cycloheximide was added to desensitized cells, responsiveness rapidly recovered (246). Thus, it has been proposed that desensitization of C6-2B cells involves the synthesis of a "refractoriness protein" (246) and that this protein exhibits a high rate of turnover. Addition of actinomycin D to desensitized cultures did not result in a reversal of desensitization as was the case with cycloheximide (246). However, actinomycin D prevented desensitization if present during the first hour of incubation with catecholamine. If cells were first incubated in the presence of catecholamine and cycloheximide, then switched to drug-free medium, a reduced hormone-responsiveness developed relative to that in cells maintained in the presence of both catecholamine and cycloheximide (246). Taken together, these results suggest that incubation of C6-2B cells with catecholamine initiates cellular transcriptional events whose ultimate expression is hormonal refractoriness. Once these processes have been initiated, the inhibition of further RNA synthesis is without effect due to the synthesis of a long-lived messenger RNA. However, due to the rapid turnover of the protein product of this message, hormonal responsiveness can be returned rapidly to control levels and back to desensitized levels simply by the addition or removal from the medium of a protein synthesis inhibitor. To date, the proposed mediator of the desensitization process in C6-2B cells has not been isolated.

As was initially pointed out by Brooker and coworkers (246) the extent of desensitization induced by dibutyryl cyclic AMP is not as great as that induced by catechol-

amines, and while the effects of dibutyryl cyclic AMP in C6-2B cells are blocked completely by inhibitors of protein synthesis, the desensitization induced by catecholamines is only partially blocked. Such results led these workers to propose that the mechanism discussed above may not account entirely for the desensitization induced by catecholamines in these cells (246). Thus, the predominant mechanism in C6-2B cells involves a nonspecific type of desensitization that appears to occur secondarily to the cyclic AMP-mediated induction of a "refractoriness protein"; as will be discussed below, a second mechanism that does not involve cyclic AMP or protein synthesis occurs in these cells. This process involves a hormone-specific type mechanism and may be the major mechanism of catecholamine-induced desensitization in other C6 glioma cell clones.

B. Analysis of Adenylate Cyclase during Heterologous Desensitization

Examination of adenylate cyclase in cell free preparations from desensitized cells initially was hampered by the low sensitivity of available assays and the inability to retain sufficient hormone responsiveness upon cell lysis. However, the availability of the sensitive enzyme assay developed by Salomon et al. (214) and the advancement of knowledge of the properties of this enzyme system have allowed analyses of adenylate cyclase under conditions where either agonist-nonspecific or agonist-specific alteration of whole cell responsiveness has occurred.

Incubation of 1321N1 (238, 241) or EH118MG (123) human astrocytoma cells with catecholamines for either short or long periods results in a reduction in catecholamine-stimulated adenylate cyclase activity in cell free preparations but no reproducible alteration in either basal or PGE_1-, fluoride-, or GppNHp-stimulated adenylate cyclase activity. Thus, in the astrocytoma cell lines where the kinetics of heterologous and homologous desensitization were first extensively characterized, it has been impossible to study heterologous desensitization upon cell lysis. It is not known whether such a result is due to reversal upon cell lysis of a cyclic-AMP-induced alteration in the activity of adenylate cyclase or to the existence of a soluble intracellular factor that is lost on breaking the cell. As is discussed in detail below, analysis of hormone-specific desensitization of adenylate cyclase is very straightforward in cell-free preparations from these cells. That is, hormone-specific desensitization apparently is completely stable to cell lysis, while heterologous desensitization is not observed in cell-free preparations.

As with astrocytoma cells, only a decrement in catecholamine-stimulated adenylate cyclase activity was observed in membranes prepared subsequent to incubation of WI-38 fibroblasts with isoproterenol (38). However, incubation of these cells with PGE_1 resulted not only in a reduction in PGE_1-stimulated adenylate cyclase activity, but also a reduction in basal activity and fluoride-, GppNHp-, and epinephrine-stimulated activities.* Thus, in contrast to astrocytoma cells the hormone-nonspecific component of at least PGE_1-induced desensitization is measurable in cell-free preparations from WI-38 fibroblasts. Whether such a result indicates that heterologous desensitization proceeds by different mechanisms in fibroblasts versus astrocytoma cells is not clear. The facts that both epinephrine and PGE_1 elevate cyclic AMP levels in WI-38 fibroblasts but only PGE_1 induces a nonspecific type of desensitization suggest that elevation of cyclic AMP levels alone is insufficient to induce heterologous desensitization in these cells. The reduction in all activity measurements of adenylate cyclase, and in particular the loss of GppNHp-stimulated activity, led Clark and Butcher (38) to propose that the locus of the PGE_1-induced heterologous desensitization was on G/F. Such a conclusion has been supported by Kassis and Fishman (127). These workers found that G/F solubilized from PGE_1-desensitized WI-38 fibroblasts was less effective than native G/F in the reconstitution of hormone responsive activity into cyc^- membranes. Also, the hydrodynamic properties of a solubilized activity purportedly representing a G/F·C complex was altered after desensitization. These results led to the proposal that heterologous desensitization involves a lesion in G/F such that it serves as an "uncoupled" or less efficient promoter of catalytic activity. Such results should be interpreted with caution, however, since the lack of quantitative solubilization and/or reconstitution of G/F leaves open the possibility of misleading results. Nonetheless, the indication to date is that the heterologous desensitization induced by PGE_1 in WI-38 fibroblasts proceeds with properties very different from those observed in human astrocytoma and C6-2B glioma cells. It is induced only by PGE_1 (38, 127), its rate of occurrence ($T_{½}$ = 2 to 3 min) is much faster than in astrocytoma cells ($T_{½}$ = 30 min) and C6-2B cells ($T_{½}$ = 1 hour), and it can be observed in cell-free assays of adenylate cyclase.

Although extensive time-effect relationships have not been reported, catecholamine-induced desensitization of erythrocytes obtained from pigeon (110, 224), turkey (103, 229), or rat (267), appears to occur with properties very similar to those observed during PGE_1-induced heterologous desensitization of WI-38 fibroblasts. Hudson and Johnson (110) reported that a 4-hour incubation of pigeon erythrocytes with isoproterenol resulted in a 40%, 60%, 70%, and 40% reduction in basal, isoproterenol-

* A similar phenomenon has been observed in studies of agonist-induced desensitization of adenylate cyclase in rat ovarian membranes (93, 94). Treatment of rats with human chorionic gonadotropin resulted in a time-dependent loss of both luteinizing hormone- and epinephrine-stimulated adenylate cyclase activities, whereas treatment of rats with epinephrine resulted only in a loss of catecholamine-stimulated activity.

stimulated, GTPγS-stimulated, and fluoride-stimulated enzyme activities, respectively. Using the same preparation, Simpson and Pfeuffer (224) found that a 2.5-hour incubation of erythrocytes with isoproterenol resulted in a 70% loss of catecholamine-stimulated activity but only a 22% and 19% loss of fluoride-stimulated and GppNHp-stimulated activities, respectively. Long-term (5 hours) incubation of turkey erythrocytes with isoproterenol was shown by Hoffman et al. (103) to result in a 40% loss of isoproterenol-stimulated adenylate cyclase activity and a small (8% to 20%) but significant loss of both fluoride- and GppNHp-stimulated activities. Finally, long-term (24 hours) treatment of rats with isoproterenol subsequent to induction of reticulocytosis with 1-acetyl-2-phenylhydrazine resulted in a 50% reduction in basal and in isoproterenol-, GppNHp-, and fluoride-stimulated adenylate cyclase activities in membranes prepared from red blood cells (267). Thus, incubation of erythrocytes from at least three sources has resulted in partial losses of all activity parameters of adenylate cyclase. The mediator of these alterations has not been identified. However, incubation of pigeon erythrocytes with dibutyryl cyclic AMP and a phosphodiesterase inhibitor caused a partial desensitization (224). Also, incubation of turkey erythrocytes with a cyclic AMP analog induced a functional "uncoupling" of the β-adrenergic receptor from adenylate cyclase as determined by loss of high affinity agonist binding and a reduction in the extent of effect of GppNHp on agonist affinity in competition binding assays with a radiolabeled antagonist (229). A small reduction in fluoride-stimulated activity also was induced by the cyclic AMP analog (229). As with WI-38 fibroblasts, the concomitant reduction of all activity parameters of adenylate cyclase and the loss of capacity to form a high affinity H·R·G/F complex after desensitization of erythrocytes (229, 267) are again suggestive of a modification in the function of G/F. Hudson and Johnson (110) have reported that G/F extracted from desensitized pigeon erythrocytes is equieffective to G/F from control membranes in reconstituting adenylate cyclase activities into cyc^- membranes. However, as discussed above, this type of experiment could be misleading due to the lack of quantitative extraction and reconstitution of G/F or due to the reversal of alterations in this protein. On the basis of conformational analysis of G/F by partial tryptic digestion and peptide mapping, these workers proposed that heterologous desensitization in pigeon erythrocytes results from an alteration that affects both receptor-G/F coupling and the exchange of GTP or other nucleotides for GDP on G/F. The lag in the rate of activation of adenylate cyclase by GppNHp in desensitized pigeon erythrocyte membranes (224) also is consistent with a modification in GDP exchange.

Less extensive analyses of adenylate cyclase in cell-free preparations have been carried out after heterologous desensitization of C6-2B rat glioma cells. Terasaki et al. (246) reported that basal and isoproterenol-stimulated adenylate cyclase activities were lost during desensitization of C6-2B cells with a time course similar to that observed for hormone responsiveness measured in intact cells. However, fluoride-stimulated activity remained unaltered. In other studies with the C6-2B clone, marked reductions in isoproterenol-stimulated, but not other, adenylate cyclase activity was observed after 10 to 240 min of incubation of cells with catecholamine (189). Thus, as was discussed concerning desensitization of human astrocytoma cells, heterologous desensitization in C6-2B cells is not readily observed in cell-free analyses of adenylate cyclase from refractory cells. A similar conclusion can be made concerning heterologous desensitization in another C6 cell clone, since Koschel (134) reported that a 4.5-hour incubation of cells with a phosphodiesterase inhibitor reduced intact cell responsiveness to isoproterenol by 75% but had no effect in NaF- or GppNHp-stimulated adenylate cyclase activity in homogenates from desensitized cells. The isoproterenol-induced desensitization in several other clones of C6 glioma cells apparently is mostly hormone-specific (63, 104) and, thus, results obtained in studies of adenylate cyclase after desensitization of these cells probably are not directly applicable to a discussion of heterologous desensitization.

C. Overview of Heterologous Desensitization

In summary, catecholamine-induced heterologous desensitization of the adenylate cyclase system is a well documented phenomenon that occurs in a variety of cell types. In contrast to the situation observed during catecholamine-specific desensitization (see below) changes in receptor number apparently are not involved in the alteration in adenylate cyclase responsiveness during heterologous desensitization (85, 103, 123, 224, 246, 267). Based on kinetic considerations and the role of protein synthesis in its occurrence, it is probable that the same mechanism of heterologous desensitization does not occur in all cell types. Data from two astrocytoma cells lines and C6-2B rat glioma cells indicate that the phenomenon cannot be observed readily upon lysis of these cells. Such a result is consistent with the idea that a covalent modification of components of the adenylate cyclase system either does not occur, or is very rapidly reversed upon cell lysis. Loss of a soluble component upon breaking of the cell could explain these data. In the case of C6-2B cells such a conclusion is attractive since heterologous desensitization in these cells has been proposed to occur subsequent to the synthesis of a "refractoriness protein" which could exist as a soluble intracellular component whose effect would be lost upon cell lysis. Unfortunately, the results to date (in particular the inability to analyze the lesion in membrane assays of adenylate cyclase from these cells) do not allow the identification of the component or process in the adenylate cyclase regulatory cycle that is modified during the process of desensitization. Apparently, the modification

would occur either in some function of G/F or in catalytic activity per se since the heterologous nature of the effect makes it unlikely that receptor modification occurs. On the basis of the time course of occurrence of heterologous desensitization, its dependence on elevation of intracellular cyclic AMP, and the inability to retain the modified state after cell lysis, hormone nonspecific desensitization in human astrocytoma cells is very similar to that observed in C6-2B glioma cells. However, this event occurs in the absence of protein synthesis in one cell type and apparently requires protein synthesis in the other. Thus, if a common intracellular mediator is involved in C6-2B and astrocytoma cells, in the latter cell type only functional activation, not synthesis, of this mediator is required. The extent to which this mechanism of hormonal regulation occurs in other cell types is not known. In several cell types (e.g. frog erythrocytes, S49 lymphoma cells) where catecholamine-specific desensitization has been studied extensively, hormone responsiveness has been examined only in cell-free preparations. Thus, heterologous desensitization of the type observed in astrocytoma or C6-2B glioma cells could be a significant factor in regulating hormonal responsiveness in these cells, but due to reversal upon cell lysis, it would not be observed.

It is likely that a second mechanism accounts for heterologous desensitization in red blood cells from several species. That is, the modification in these cells is stable to cell lysis and likely involves a covalent modification of G/F. Whether this phenomenon involves a cyclic-AMP-dependent or independent phosphorylation or some other type of modification (methylation, etc.) is not yet known. The recently developed capacity to probe the status of G/F by a variety of techniques should provide more definitive answers concerning the molecular basis of heterologous desensitization of adenylate cyclase in erythrocytes.

VI. Hormone-specific Desensitization

A. Examination of Adenylate Cyclase and β-Adrenergic Receptors in Cell-free Preparations

As was discussed above, catecholamine-specific desensitization of adenylate cyclase has been shown to occur in intact cell studies of a variety of tissues. Aurbach and coworkers (4), Lefkowitz and coworkers (139), and Levitzki and coworkers (144) reported in 1974 that the radiolabeled antagonists, [^{125}I]hydroxybenzylpindolol ([^{125}I]IHYP), [^{3}H]dihydroalprenolol ([^{3}H]DHA), and [^{3}H]propranolol, could be used to quantify β-adrenergic receptors in membrane preparations from erythrocytes. The subsequent demonstration (9, 21, 23, 58, 90, 154, 232, 272) that these and other radioligands could be used to analyze receptors in a variety of preparations soon resulted in direct analyses of the β-adrenergic receptor during catecholamine-induced desensitization. Such an approach seemed especially appropriate since the most likely lesion during catecholamine-specific desensitization would be at the level of the β-adrenergic receptor.

Lefkowitz and coworkers (172) were the first to examine the relationship between changes in β-adrenergic receptors and catecholamine-induced desensitization. Treatment of frogs with isoproterenol for 24 hours resulted in a 75% reduction in catecholamine-stimulated adenylate cyclase activity in erythrocyte membranes; no change was observed in basal or fluoride-stimulated activity indicating that a hormone-specific modification occurred. The loss of hormone responsiveness was accompanied by a similar decrease in the number of β-adrenergic receptors as measured by [^{3}H]DHA (172, 173). No change was observed in the affinity of the radioligand. Incubation of frog erythrocytes in vitro with catecholamines resulted in similar changes in the β-adrenergic receptor/adenylate cyclase system (164). A subsequent study by this group (165) illustrated that the time course of loss of β-adrenergic receptors as measured by [^{3}H]DHA was similar to the time course of loss of isoproterenol-stimulated adenylate cyclase activity. Maximal reductions in both measurements were observed in 2 to 4 hours; the $T_{1/2}$ of occurrence was approximately 45 min in each case. Again, desensitization was hormone-specific at all times studied, since basal and fluoride- and PGE_1-stimulated adenylate cyclase activities were not altered. The concentration-effect curve of isoproterenol for inducing a reduction in catecholamine responsiveness was the same as for induction of receptor loss (165, 173). Each of these concentration-effect curves was similar to that for activation of adenylate cyclase. As would be expected for a β_2-adrenergic receptor-linked adenylate cyclase, isoproterenol was much more potent than norepinephrine in inducing these alterations either in vivo (173) or in vitro (165).

In contrast to the results obtained with frog erythrocytes, Shear et al. (221) reported that incubation of S49 lymphoma cells for 2 hours with isoproterenol resulted in a 60 to 80% loss of isoproterenol-stimulated adenylate cyclase activity in membrane preparations but only a 25% to 45% reduction in the number of β-adrenergic receptors as measured by [^{125}I]HYP binding. Similar results were subsequently reported by Johnson et al. (123) who illustrated that incubation of EH118MG astrocytoma cells with isoproterenol resulted in an 80 to 90% loss of catecholamine responsiveness of intact cells, a 65% loss of isoproterenol-stimulated adenylate cyclase activity, and only a 20% decrease in the number of β-adrenergic receptors. Although incubation of cells with PGE_1 resulted in a 60% loss of catecholamine-stimulated cyclic AMP accumulation, no change in β-adrenergic receptor number or catecholamine-stimulated adenylate cyclase activity occurred. Therefore, homologous desensitization was associated with a partial reduction in the number of receptors; heterologous desensitization occurred with no change in receptor number.

Thus, these early studies of β-adrenergic receptors

during catecholamine-specific desensitization presented somewhat conflicting results, with a good correlation between loss of hormone responsiveness and decrement in receptor number occurring in frog erythrocytes and much greater losses in hormone responsiveness than receptor number occurring in S49 lymphoma and astrocytoma cells. Detailed kinetic analyses of the adenylate cyclase system during catecholamine-specific desensitization has clarified some of these differences.

Su et al. (240, 241) demonstrated that incubation of 1321N1 astrocytoma cells with isoproterenol resulted in a rapid ($T_{½}$ ~ 2 to 3 min) loss of catecholamine-stimulated adenylate cyclase activity to an essentially new steady-state level that was 40% to 50% of control. No alteration in basal or PGE_1-, fluoride-, or GppNHp-stimulated enzyme activity occurred during this time or after prolonged incubation with catecholamine. Also, little change occurred in the number of β-adrenergic receptors measured in homogenates or in a membrane fraction following short-term incubation with isoproterenol. However, β-adrenergic receptors were rapidly uncoupled during incubation of 1321N1 cells with isoproterenol since the extent of high affinity binding of agonist was markedly reduced, as was the extent of effects of guanine nucleotides on agonist binding (89). Thus, it was proposed that an uncoupling of β-adrenergic receptors from adenylate cyclase accounted for the short-term desensitization in astrocytoma cells (89, 240, 241). Recovery from this state was rapid with responsiveness (and high affinity binding of agonists) returning to control levels with a $T_{½}$ of 7 min subsequent to removal of catecholamine from the incubation medium. The concentration effect curve for induction of the uncoupled state was the same as that for activation of adenylate cyclase. Furthermore, partial agonists were partially effective in inducing this phenomenon (241).

Although little change in receptor number occurred in homogenates or membrane preparations during short-term incubation of astrocytoma cells with isoproterenol, extended incubation resulted in a progressive loss of receptors such that after 12 hours of incubation receptor level was less than 20% of the initial value (240, 241). The diminution in catecholamine-stimulated adenylate cyclase activity was much greater than receptor loss during the first several hours of incubation of astrocytoma cells with isoproterenol; after 6 to 12 hours the decrement in both parameters was similar. Furthermore, the degree of recovery of catecholamine-stimulated activity after removal of isoproterenol from the medium was governed by the extent to which receptors had been lost (241). For example, if after several hours of incubation with isoproterenol an 80% loss of catecholamine-stimulated adenylate cyclase had occurred but only a 35% loss of receptors, removal of isoproterenol from the medium would result in a rapid ($T_{½}$ ~ 7 min) recovery of catecholamine-stimulated adenylate cyclase activity to the level (65% of control) to which receptors had been lost. Hormone-stimulated enzyme activity and receptor number then recovered slowly over a period of hours. Taken together, these results led to the proposal that incubation of astrocytoma cells with catecholamines rapidly results in the conversion of the β-adrenergic receptor-linked adenylate cyclase to an uncoupled state in which receptors are not capable of activating adenylate cyclase. With longer incubation receptors are converted to a form that is not detectable by radioligand binding. Recovery from the uncoupled state is rapid; recovery from the situation in which there is a loss of measurable receptors is very slow. Thus, the straightforward relationships discussed above concerning the concentration of agonist necessary for induction of desensitization versus the concentration necessary for activation of adenylate cyclase no longer applied with extended incubation of cells with agonist [i.e. the very slow reversal of the receptor loss reaction during incubation with catecholamines serves to shift the concentration-effect curve for desensitization to the left with time (240, 241)]. Indeed, concentrations of isoproterenol that produce no discernible elevation of cyclic AMP levels in astrocytoma cells will, nonetheless, lead to loss of receptors and catecholamine-stimulated adenylate cyclase activity if the time of incubation is extended over 12 to 24 hours (240). The same relationship appears to apply for other cell types. For example, Insel and coworkers (43, 50) have demonstrated that the very small level of catecholamine present in fetal calf serum used in cell growth medium is sufficient to significantly reduce the level of β-adrenergic receptors and catecholamine responsiveness of S49 lymphoma cells. Similarly, partial agonists that produced only "partial" desensitization of fibroblasts (71) or astrocytoma cells (241) during short-term incubation induced essentially complete desensitization after extended incubation.

Analysis of catecholamine-specific desensitization in frog erythrocytes has led to conclusions similar to those obtained in studies of cultured astrocytoma cells. For example, loss of binding of the β-adrenergic receptor agonist, [^{3}H]HBI, occurred to a greater extent than did the loss of binding of the radiolabeled antagonist, [^{3}H]DHA (265, 266). Thus, while [^{3}H]DHA binding was reduced by only 35% after 5 hours of incubation of frog erythrocytes with isoproterenol, [^{3}H]HBI binding and isoproterenol-stimulated adenylate cyclase activity were reduced by 67% and 69%, respectively. Since the capacity to observe [^{3}H]HBI binding depends on the interaction of the β-adrenergic receptor with G/F, these data can be interpreted as representing an uncoupling of the β-adrenergic receptor from G/F in addition to a loss of receptors (i.e. the loss of [^{3}H]HBI binding sites exceeded the loss of [^{3}H]DHA binding sites by approximately 2-fold). The uncoupled state also was apparent in isoproterenol competition curves generated with [^{3}H]DHA (266). Computer-based analysis of these curves indicated that, although there was little change in the apparent affinity of isoproterenol for interaction at the high and

low affinity states, the percentage of agonist-receptor complexes existing in the high affinity state was reduced from 81% to 51% after incubation of cells with isoproterenol for 3 hours (131).

The idea that incubation of cells with catecholamines results in a rapid uncoupling of β-adrenergic receptors from adenylate cyclase followed by a loss of measurable receptors from the cell has been supported by many studies. Although incubation of the C6-2B glioma cell clone with catecholamines mainly results in heterologous type desensitization (246), Bockaert and coworkers (104) have shown that incubation of a different C6 cell clone with catecholamine results in a rapid loss of isoproterenol-stimulated adenylate cyclase activity followed by a loss in the number of β-adrenergic receptors. Consistent with the results of others it was concluded that an initial uncoupling of the β-adrenergic receptor system was followed by a loss of receptors from the cell (104). Similar conclusions also were made by Fishman et al. (64) with either low passage or high passage C6 glioma cells. Using the C6-2B glioma cell clone, Perkins and Frederich (189) concluded that a significant amount of catecholamine-specific desensitization occurred in addition to heterologous desensitization; the properties of this hormone-specific desensitization were very similar to that previously observed in astrocytoma cells (241) and frog erythrocytes (266). The same conclusion has been made concerning catecholamine-specific desensitization in rat myometrium (244). Finally, the properties of hormone-specific desensitization in purified astroglia cultured from neonatal rat cerebral cortex (60) are very similar to that observed in tumor cell lines of supposed glial origin.

Wild-type S49 lymphoma cells also undergo catecholamine-specific desensitization with properties similar to those reported for other cells. As discussed previously, Shear et al. (221) reported that a 2-hour incubation of these cells with catecholamine resulted in a large decrease in catecholamine-stimulated adenylate cyclase activity with a much smaller decrement in β-adrenergic receptor number. Time course studies carried out by Su et al. (241) illustrated that incubation of S49 cells with isoproterenol resulted in a rapid (within 15 min) loss of catecholamine-stimulated adenylate cyclase activity to a new level that was only 30% to 45% of control. Receptor number decreased with a much slower time course. Similar results were reported by Iyengar et al. (115), who demonstrated that, although receptor number did not decrease during a 15-min incubation of cells with isoproterenol, the capacity of isoproterenol to form a high affinity binding complex was reduced. Rashidbaigi et al. (199) have reported that not only does long-term incubation of wild-type S49 lymphoma cells with a β-adrenergic receptor agonist result in a 90% loss of [^{125}I]HYP binding sites, but also, the photoaffinity labeling by [^{125}I] azidobenzylpindolol of a peptide tentatively identified on SDS-gels as the β-adrenergic receptor is almost totally lost.

Although the properties of β-adrenergic receptors have not been determined directly in cultured baby hamster kidney fibroblasts, catecholamine-specific desensitization in these cells appears to proceed by essentially the same kinetic properties as described for other cell lines (167). As was shown in astrocytoma cells, recovery from desensitization in these cells was rapid and complete following short-term incubation with catecholamine, but much less complete if the time of incubation with agonist was extended.

Thus, as opposed to the situation concerning heterologous desensitization where it has been difficult to ascribe a single mechanism that would readily incorporate the data from all systems that have been studied, catecholamine-specific desensitization appears to proceed by a common mechanism in a variety of systems. The subtle differences that have been reported among these systems most likely reflect both real differences in the rates and extent of occurrence of the reactions involved in desensitization and less straightforward differences in methodology. With the comfort of the idea that a common series of steps may apply to catecholamine-specific desensitization in all tissues, it has been possible to proceed with experiments designed to determine the molecular bases of these alterations that were initially characterized from a kinetic point of view

B. Localization of the Lesion in Catecholamine-specific Desensitization

Shear et al. (221) were the first to take advantage of genetic mutants of the adenylate cyclase system in an attempt to probe the mechanism of catecholamine-specific desensitization. As previously discussed, wild-type S49 lymphoma cells, which express a normal β-adrenergic receptor-linked adenylate cyclase, undergo catecholamine-specific desensitization with properties similar to those of other cells (59, 115, 221, 241). Also, incubation of an S49 cell variant that lacks cyclic-AMP-dependent protein kinase yielded similar results, thereby indicating that a competent protein kinase system is not necessary for the occurrence of this type of desensitization (221). Finally, incubation of a mutant (cyc$^-$) lacking G/F with isoproterenol or with isoproterenol plus dibutyryl cyclic AMP for 2.5 hours had no effect on the number of β-adrenergic receptors (221); under similar conditions there was a 25% to 45% reduction of binding sites in wild-type cells (221). Therefore, Shear et al. (221) concluded that receptor occupancy alone or occupancy in the presence of elevated cyclic AMP levels was insufficient for the agonist-induced down regulation of receptors. This conclusion was supported subsequently by Su et al. (241) who showed that even long-term (24 hours) incubation of cyc$^-$ cells with isoproterenol failed to reduce the number of β-adrenergic receptors. Since the cyc$^-$ mutant lacks G/F and as a consequence, agonist-induced receptor-G/F coupling cannot occur, it was concluded that coupling per se may be a necessary step for

the induction of receptor loss. Experiments with the HC-1 hepatoma line, which expresses β-adrenergic receptors and a functional G/F but not C, supported this conclusion (241). That is, incubation of these cells with isoproterenol resulted in a loss of β-adrenergic receptors with a time course similar to that observed in cells with fully competent adenylate cyclase systems. Interestingly, these cells apparently undergo the initial uncoupling reaction although it cannot be measured directly since they lack catalytic activity. That is, short-term (15 min) incubation of HC-1 cells with isoproterenol resulted in a decrease in high affinity agonist binding and reduced the effects of guanine nucleotides on agonist binding measured in competition binding experiments with [^{125}I]HYP (241). Taken together these studies suggested that for agonist-induced receptor loss to occur it is necessary for agonist-induced coupling of R to G/F to occur. Elevation of cyclic AMP levels or the presence of a functional catalytic protein were apparently not necessary.

By using group specific reagents, Lefkowitz and coworkers (230, 266) arrived at a somewhat different conclusion. Preincubation of cells with N′,N′-dicyclohexylcarbodiimide, an agent that purportedly selectively uncouples β-adrenergic receptors from G/F, also prevented the eventual agonist-induced loss of receptors (266). This result is consistent with those described above for other cells. However, treatment of frog erythrocytes with N-ethylmalemide to supposedly inactivate the catalytic protein also prevented agonist-induced loss of receptors (230). Thus, it was concluded that productive coupling of G/F to C, not receptor-G/F coupling alone, was necessary to initiate the desensitization process. These results must be interpreted with caution, however, due to the high potential for other effects of N′,N′-dicyclohexylcarbodiimide and N-ethylmaleimide unrelated to modification of the adenylate cyclase system.

Although coupling of β-adrenergic receptors to G/F appears necessary for catecholamine-induced loss of receptors, such may not be the case for the initial uncoupling reaction. Su et al. (241) and Wessels et al. (266) concluded on the basis of mostly indirect evidence that in order for catecholamines to induce the uncoupling reaction, it was first necessary for them to promote formation of the complex of agonist, receptor, and G/F; i.e. this complex would serve as a precursor for both agonist-induced activation and agonist-induced uncoupling of the adenylate cyclase system. However, on the basis of results obtained in a novel series of experiments by Green and Clark (79, 80) this does not appear to be the case. Cyc$^-$ cells were incubated with isoproterenol for 15 to 20 min. The reconstitution of hormone responsiveness was then tested in membranes from the cyc$^-$ cells by using native G/F obtained from wild-type S49 lymphoma cells. Catecholamine responsiveness was much less in membranes from cyc$^-$ cells preincubated with agonist than in membranes from control cyc$^-$ cells. This apparent desensitization of cyc$^-$ cells proceeded with a time course and agonist concentration dependence essentially identical with that for wild-type cells (80); reversal of these phenomena during incubation of desensitized cyc$^-$ and wild-type cells in agonist-free medium also exhibited similar time courses (80). Thus, it was concluded that the uncoupling reaction occurred independently of a functional G/F. These data have tentatively localized the lesion to the β-adrenergic receptor, not G/F, since desensitization occurred in the absence of the coupling moeity. However, the conclusion that cyc$^-$ cells do not express any of the activities inherent in G/F may prove to be inaccurate. Such conclusions have been based on 1) lack of hormone responsiveness in membranes from these cells, 2) lack of a cholera toxin substrate, and 3) functional reconstitution of the appropriate activities in cyc$^-$ membranes by a variety of sources of G/F. Preliminary evidence from Gilman's laboratory (63) has suggested recently that the lesion in cyc$^-$ membranes may not be so straightforward as had been assumed. That is, based on work involving antibodies to purified G/F, it has been proposed that cyc$^-$ membranes do indeed lack the 45,000 dalton subunit of G/F, but surprisingly, these membranes apparently express the 35,000 dalton subunit of G/F. Although the function of the 35,000 dalton subunit has not been resolved, its proposed presence in cyc$^-$ cells adds at least a level of uncertainty to the interpretation of the results obtained during desensitization of cyc$^-$ cells.

Pike and Lefkowitz (194) also concluded that the β-adrenergic receptor was modified during desensitization on the basis of experiments in which components of the β-adrenergic receptor-linked adenylate cyclase of control and desensitized frog erythrocytes were differentially modified by group-specific reagents, and then cell fusion was accomplished with Sendai virus. The fusion of β-adrenergic receptors from desensitized cells with G/F and C from normal cells resulted in a reduced response to catecholamine. In contrast, β-adrenergic receptors from normal cells fused with G/F and C from desensitized cells were capable of eliciting a normal response. Reilly and Blecher (201) have obtained similar results with cell fusion hybrids from normal and desensitized hepatocytes.

Results of several studies have been published that support the idea that G/F is unaltered during catecholamine-specific desensitization. For example, Iyengar et al. (115) showed that G/F extracted from membranes obtained from desensitized wild-type S49 lymphoma cells fully reconstituted hormone responsiveness to cyc$^-$ membranes. Similarly, although native G/F extracted from wild-type S49 cells reconstituted hormone responsiveness to membranes from cyc$^-$ cells, hormone-responsiveness was not reconstituted to membranes from wild-type cells that had been desensitized by preincubation with isoproterenol. Although arguments could be made concerning possible reversibility of changes in G/F during

the process of solubilization and reconstitution, kinetic analyses of G/F after short-term desensitization support the general conclusions obtained in the reconstitution experiments. For example, in a variety of tissues it has been shown that GppNHp-stimulated adenylate cyclase activity is unchanged during catecholamine-specific desensitization (64, 115, 241). Moreover, the rate of activation of the enzyme by GppNHp alone is not altered, but stimulation of the rate of activation by β-adrenergic receptor agonists, but not PGE_1, is reduced (241).

One potential approach for defining the molecular lesion responsible for catecholamine-specific desensitization involves the induction of this process in cell-free preparations. Some success in this regard has been reported for desensitization of adenylate cyclase induced by luteinizing hormone in a broken cell preparation from pig Graafian follicles (13, 111) or rat ovarian plasma membranes (61), induced by lysine vasopressin in pig kidney membranes (213), and induced by glucagon in rat liver plasma membranes (116). However, only one report of successful induction of catecholamine-specific desensitization in a cell-free preparation has been published. Anderson and Jaworski (3) demonstrated that incubation of crude membranes from normal rat kidney cells (NRK-S) with isoproterenol resulted in a loss of catecholamine-stimulated adenylate cyclase activity; basal and PGE_1-stimulated activities were unaltered. This process exhibited an absolute requirement for ATP, GTP, and Mg^{++}. Increasing concentrations of Mg^{++} in the presence of ATP and GTP led to refractoriness even in the absence of isoproterenol. One common feature of the occurrence of desensitization in broken cell preparations is an apparent requirement for phosphorylating conditions. However, on the basis of a lack of effect of phosphatases on the desensitized state in NRK-S cells, Levitzki and Atlas (143) hypothesized that a modulator protein may itself be a substrate for a phosphorylation-dephosphorylation reaction, but the reaction that it catalyzes which results in desensitization of the adenylate cyclase sytem is not a phosphorylation reaction. The lack of an obvious requirement for cyclic AMP eliminates the possible involvement of a cyclic-AMP-dependent protein kinase and is consistent with the lack of an apparent role for cyclic AMP in the occurrence of catecholamine-specific desensitization in intact cells. To date no analysis of the status of the β-adrenergic receptor during induction of desensitization in a cell-free preparation has been reported. Furthermore, direct correlations between the properties of cell-free desensitization and that occurring in intact cells have not been made. Clearly, further work will be required to establish whether a valid model for catecholamine-specific desensitization can be developed in a cell-free preparation.

In summary, a large amount of evidence indicates that short-term incubation of a variety of target cells with catecholamines results in a modification of the β-adrenergic receptor/adenylate cyclase system so that agonists no longer promote coupling of the components of this system to effect a normal increase in catalytic activity. The modification responsible for this altered function apparently occurs at the level of the β-adrenergic receptor. The nature of the modification is not yet known although it is attractive to propose that a phosphorylation/dephosphorylation or methylation/demethylation reaction is involved. In recent studies, several receptors for neurotransmitters and hormones have been shown to serve as substrates for phosphorylation reactions (40, 78, 128). The apparent stability of the lesion to cell lysis and membrane preparation, together with recently developed methodology for purification (24, 57, 222, 258) of the β-adrenergic receptor and its identification during gel electrophoresis (18, 57, 138, 198, 222), should provide an opportunity for further defining the molecular nature of the modification during short-term catecholamine-specific desensitization.

C. Evidence for a Change in the Membrane Form of β-Adrenergic Receptors during Catecholamine-induced Desensitization

Knowledge of the occurrence of peptide-hormone-induced receptor clustering in coated pits followed by internalization of hormone-receptor complexes into cytosolic vesicles (15, 188) has led to interest in the possibility that such a process is involved in catecholamine-induced desensitization. Indeed, several lines of evidence now support the idea that a similar mechanism is operational in the β-adrenergic receptor system. This information has been attained by relatively indirect biochemical investigations since methodology has not been available for morphological visualization of β-adrenergic receptors.

The first evidence in support of the idea of catecholamine-induced internalization of β-adrenergic receptors came from the work of Chuang and Costa (33). Incubation of frog erythrocytes with isoproterenol resulted in the appearance of β-adrenergic receptors in a supernatant fraction generated by centrifuging cell lysates at 30,000 × *g* for 20 min. The binding properties of this recognition site were the same as that for membrane-associated β-adrenergic receptors (33) with the exception that guanine nucleotides no longer reduced agonist binding affinity for the supernatant form of the receptor (36). The concentration dependence for agonist and the agonist selectivity were the same for induction of the supernatant form of the receptor as for the induction of receptor loss from the membrane fraction. β-Adrenergic receptor antagonists blocked the agonist-induced loss of β-adrenergic receptors from the membrane fraction and the appearance of receptors in the "cytosol" fraction (36). Reversal of these changes in the form of the β-adrenergic receptor was very slow (36). That is, incubation of erythrocytes in agonist-free medium subsequent to a 2-hour incubation with isoproterenol resulted in only a partial recovery of membrane receptors over a 45-

hour period with a somewhat greater disappearance of receptors from the supernatant fraction. Although this recognition site appearing in the supernatant has been discussed as a "soluble internalized" form of the β-adrenergic receptor, the exact nature of this binding activity is still not known. Its identification has been based on the arbitrary choice of a 30,000 × *g* centrifugation step to define particulate versus soluble binding sites. If this activity does represent internalized receptors, then the most likely form of these sites would be in a vesicular structure. It is not yet clear why even at early times of incubation no more than 20% to 25% of the receptors that are lost from the plasma membrane are recovered in the soluble form. Such results could be explained by: 1) problems in fully quantifying all of the receptors in this form; 2) rapid equilibrium between the soluble form and yet another form that is not measurable by current methodology; or 3) loss of receptors from the plasma membrane to a form (degraded receptors?) that is not detectable by radioligand binding and whose formation is unrelated to the mechanism through which the soluble receptors are generated. In support of the contention that these are internalized receptors, a variety of compounds that have been shown to inhibit the surface mobility, internalization, or cellular processing of several peptide hormone receptors also reduced the appearance of β-adrenergic receptors in the cytosolic fraction during incubation of frog erythrocytes with isoproterenol (34). Thus, low temperature, dinitrophenol, methylamine, concanavalin A, and cordycepin, all decreased the capacity of catecholamines to induce the appearance of soluble receptors. Furthermore, subcellular fractionation of erythrocytes subsequent to incubation with isoproterenol revealed a population of receptors that was associated with markers for lysosomes (35); this fraction of receptors was increased by pretreatment of cells with the lysomotropic agent chloroquine before desensitization.

Harden et al. (86) also have reported results that are consistent with a rapid change in the membrane form of β-adrenergic receptors during catecholamine-induced desensitization. Isoproterenol- and fluoride-stimulated adenylate cyclase activities and β-adrenergic receptors were recovered in a single peak (at approximately 45% sucrose) with other markers of the plasma membrane after sucrose density gradient centrifugation of lysates from astrocytoma cells (1321N1). In contrast receptors from desensitized (15 min with isoproterenol) cells migrated as two peaks, one at light (20% to 25%) sucrose densities and one that was equivalent to the peak from control lysates. The "light vesicle" peak of receptors generated during desensitization exhibited agonist binding properties consistent with those of uncoupled receptors. That is only low affinity agonist binding was observed, and there was no effect of GTP on binding affinity (86). Little adenylate cyclase activity was found with the light vesicle population of receptors. Thus, the partially uncoupled population of β-adrenergic receptors that can be observed in competition binding experiments with lysates after short-term desensitization (89, 241) can be resolved by sucrose density gradients into a population that is completely uncoupled on the basis of binding criteria and into a population that is "normal" in that it is still associated with the plasma membrane, still activates adenylate cyclase, and still expresses agonist binding properties of "coupled" receptors (86). Incubation of astrocytoma cells with isoproterenol did not change the sucrose density gradient distribution of muscarinic cholinergic receptors or adenylate cyclase (262), thus suggesting selectivity in the change in membrane form of the β-adrenergic receptor. The time course of appearance and disappearance of the new receptor form upon addition or removal of isoproterenol from the medium was similar to the time course for loss and recovery of isoproterenol-stimulated adenylate cyclase activity (86). Whether the alteration in physical form of the receptor is causal in the uncoupling process, or rather, occurs as a rapid sequela of another lesion is not known. However, several pieces of information support the latter proposal. First, detailed time courses indicated that a lag of 1 to 2 min occurs before the conversion of receptors to the altered membrane form (262). No lag was observed in the time course of loss of catecholamine-stimulated adenylate cyclase activity (241, 262). Furthermore, preincubation of cells with concanavalin A before short-term desensitization blocked the appearance of the light vesicle receptors without blocking the diminution in catecholamine-stimulated adenylate cyclase activity (262). Thus, the uncoupling reaction apparently can occur in the absence of a change in the membrane form of β-adrenergic receptors.

A change in the membrane form of β-adrenergic receptors also occurs during catecholamine-specific desensitization of C6-2B and C6-BD12 glioma cells (189). The properties of this modification in terms of equilibrium density of modified receptors on sucrose density gradients and time course of appearance of the "light vesicle" receptors relative to the loss of catecholamine-stimulated adenylate cyclase activity were very similar to those observed during short-term desensitization of human astrocytoma cells.

Several other laboratories have examined indirectly the mechanism of receptor changes during catecholamine-induced desensitization. For example, the enzyme transglutaminase has been proposed to be involved in the process of clustering and internalization of peptide hormone receptors (188). Dansylcadaverine, an inhibitor of transglutaminase, blocked catecholamine-induced loss of receptors in BHK cells (200). Mallorga et al. (158) used a different approach to examine the mechanism of desensitization in C6 glioma cells. A 2-hour incubation with isoproterenol resulted in an 80% loss of intact cell cyclic AMP response to catecholamine and a 20% to 30% decrement in the number of β-adrenergic receptors. Concomitant with these alterations, an increased activity of

phospholipase A_2 and turnover of methylated phospholipids occurred. Quinacrine, which inhibits phospholipase A_2, also blocked the decreases in responsiveness and receptor number. Thus, phospholipase A_2 was proposed to be involved in the regulation of the β-adrenergic receptor system during desensitization (158). In light of the large amount of heterologous densensitization that occurs in C6 cells (246), it is unclear whether quinacrine attenuates both hormone-specific and nonspecific desensitization or only one of these phenomena in C6 cells.

The role of the cytoskeleton in agonist-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase also has been examined. Colchicine and vinblastine, which are inhibitors of microtubule polymerization, significantly reduced the extent of catecholamine-induced and catecholamine- or PGE_1-induced desensitization in Ehrlich ascites tumor cells (136) and myeloid leukemic cells (223), respectively. Unfortunately, the type of desensitization occurring in these cells was not defined. Interestingly, colchicine reduced the extent of PGE_2-induced desensitization in rat myometrium, but had no effect on isoproterenol-induced desensitization.* These results suggest that two receptor types that are coupled to a common set of catalytic units may, nonetheless, be differentially regulated by cytoskeletal elements. Finally, cytochalasin B, which disrupts microfilaments, also decreases the extent of catecholamine-induced desensitization in S49 lymphoma cells (112). The specific step in the desensitization process that is modified by inhibition of microtubule or microfilament function has not been defined in any of these studies.

D. Analysis of the β-Adrenergic Receptor by Intact Cell Binding Assays during Agonist-induced Desensitization

Most analyses of the β-adrenergic receptor during agonist-induced desensitization have been carried out with broken cell preparations. Obviously, there are several compelling reasons to extend this evaluation to studies of the receptor in situ. Although only a few direct studies of the β-adrenergic receptor on intact cells have been reported, this work has provided important information concerning the status of the β-adrenergic receptor during incubation of cells with catecholamines. For example, intact cell binding assays have provided evidence that the light vesicle receptors that occur during short-term desensitization of 1321N1 astrocytoma cells may be an internalized vesicular form of the β-adrenergic receptor. If 1321N1 cells were incubated with isoproterenol for 15 min then rapidly cooled and washed free of agonist, the specific binding of [^{125}I]pindolol in an intact cell binding assay at 4°C was decreased by 50% to 70% (249). Since [^{125}I]pindolol apparently measures only surface receptors under these conditions, these data are evidence of a conversion of a significant proportion of receptors into a sequestered (internalized?) location. Furthermore, the same result was obtained when cells were desensitized, washed free of agonist with ice cold medium, lysed, and assayed for receptors with [^{125}I]pindolol at 4°C. Such a result would be expected if receptors are internalized into inside-out vesicles and again [^{125}I]pindolol measures only surface receptors at 4°C (249). Finally, when [^{125}I]pindolol was used to measure receptors on sucrose density gradients from desensitized cells, both receptors in the light vesicle and in the plasma membrane fractions were detected at 37°C, but only receptors in the plasma membrane fraction were measured in a 4°C assay (249). Taken together, these data suggest that: 1) agonists induce an internalization of β-adrenergic receptors in 1321N1 astrocytoma cells, and 2) this internalization step is a very rapid sequela of the initial uncoupling reaction.

Staehelin and coworkers (232, 233) have reported similar results with the hydrophilic radioligand, [^{3}H]CGP-12177. Whereas lipophilic ligands such as [^{125}I]HYP and [^{3}H]DHA apparently detect both cell surface and intracellular β-adrenergic receptors in an intact cell assay at 37°C, [^{3}H]CGP-12177 apparently labels only surface receptors. By using this methodology it was shown (232) that incubation of S49 lymphoma cells with isoproterenol results in a very rapid ($T_{1/2} \sim 10$ min) loss of approximately 50% of [^{3}H]CGP-12177 binding activity but a much slower loss of [^{3}H]DHA binding activity (20% loss after 1 hour; 50% loss after 4 hours). Similar discrepancies between the extent of loss of [^{3}H]CGP-12177 binding versus that for [^{3}H]DHA were observed in C6 glioma (232, 233) and WEHI7 (233) lymphoma cells. Rapid recovery of the receptors lost to detection by [^{3}H]CGP-12177 during incubation of S49 lymphoma cells with isoproterenol was attained upon removal of agonist from the medium (232). Further evidence for the occurrence of an internalized vesicular form of the β-adrenergic receptor has been obtained with this hydrophilic radioligand in studies of the sucrose density gradient distribution of receptors after desensitization of C6-2B glioma cells. That is, receptors appearing in the light vesicle fraction after desensitization were not detectable by [^{3}H]CGP-12177 binding whereas those occurring in the plasma membrane fraction were readily detected (98). Such a result would be expected if the receptors in the light vesicle fraction were in internalized, inside-out structures.

One previously puzzling finding with intact cell binding assays of β-adrenergic receptors may be explainable on the basis of agonist-induced modification of this system. Several laboratories reported that the apparent binding affinity of β-adrenergic receptor agonists, as measured by their capacity to inhibit radiolabelled antagonist binding to intact cells, was markedly lower than their apparent affinity for stimulation of cyclic AMP accumulation (114, 174, 197, 245). Since this discrepancy was 300- to 4000-fold it seemed unlikely that the presence of guanine nucleotides in intact cells (and the resulting lack of a significant proportion of the receptors in an

* S. Harbon, personal communication.

agonist-induced high affinity form) could account for this phenomenon. The first hint that the low agonist affinity in intact cell binding assays was a result of an agonist-induced modification of the receptor came from the work of Pittman and Molinoff (197). These workers reported that while a low concentration of isoproterenol inhibited [^{125}I]HYP binding during the first minute of incubation with L6 muscle cells, continuation of the incubation to equilibrium (60 min) resulted in a situation wherein the low concentration of agonist no longer inhibited radioligand binding. Thus, it was suggested that agonists rapidly convert the receptors of intact cells into a state that exhibits low affinity for agonists (197).

This conclusion of Pittman and Molinoff (197) has been confirmed and extended by Toews et al. (248). These workers have adapted an approach for nonequilibrium binding of [^{125}I]pindolol at short times of incubation to obtain K_d values for agonists. By using this method, high affinity binding of agonists to β-adrenergic receptors on 1321N1 astrocytoma cells and C6-2B glioma cells was demonstrated. The K_d values determined for agonists were in good agreement with the K_{act} values for the same agonists for stimulation of cyclic AMP accumulation. With increasing time of incubation of cells with agonist, the apparent binding affinities of isoproterenol and epinephrine were reduced by approximately 400-fold. The concentration dependence for the induction of the low affinity form of the receptor and the time course for its formation and reversal were similar to those for the agonist-induced loss of catecholamine-stimulated cyclic AMP synthesis (248). Molinoff and coworkers (202a) also have extended their initial observations by illustrating that the apparent binding affinity of isoproterenol for β-adrenergic receptors on intact S49 cells is 20- to 50-fold greater when determined in 1-min competition binding assays than in assays involving a 30-min incubation.

The nature of the agonist-induced low affinity form of the receptor observed in intact cell binding assays is still not known, but it seems likely that it is an expression of one or more of the steps involved in catecholamine-induced desensitization. First, the receptors in this form are likely to be uncoupled in regard to their interaction with G/F. Whether such an alteration would result in a measurable difference in apparent binding affinity of agonists in intact cell assays is not known. Second, the receptors in the low affinity form are also likely to represent internalized receptors. In support of this idea, predominantly high affinity agonist binding is observed in intact cell binding assays carried out at 4°C (249). It is likely that under this condition only surface receptors would be measured with [^{125}I]pindolol (see discussion above) and furthermore, agonist-induced internalization of receptors would not occur. If the low affinity receptor form does indeed represent internalized receptors, assays carried out with [^{125}I]pindolol or other lipophilic ligands at 37°C present several problems in interpretation. That is, a radioligand likely will detect both the cell surface and internalized receptor forms but its "partition" between these states of the receptor is unknown. Furthermore, catecholamines will be partially charged at physiological pH, and thus "partition" among these receptor forms is likely to occur. Such a situation would likely generate binding conditions that severely deviate from the assumption of law of mass action interaction with a single site. Indeed, complicated competition binding curves have been reported for agonists in intact cell binding assays (174, 248). It has recently been demonstrated that concanavalin A partially blocks the occurrence of the low affinity form of the receptor.* As discussed above, concanavalin A also blocks the agonist-induced appearance of receptors in the light vesicle receptor population (262), but not the agonist-induced loss of catecholamine-stimulated adenylate cyclase activity (262). These data support the idea that the low affinity population of receptors detected in intact cell binding assays is not simply an expression of the uncoupling reaction, but rather, may be an expression of receptors in an internalized form. Clearly, more information will be necessary to resolve unambiguously the significance of this low affinity form of the β-adrenergic receptor.

E. Cellular Processing of β-Adrenergic Receptors during Long-Term Exposure to Catecholamines

As has been discussed above, long-term incubation of a variety of cell types with catecholamines results in a loss of measurable β-adrenergic receptors. In several tissues this process has occurred over a period of hours rather than the almost instantaneous occurrence of an agonist-induced loss of catecholamine-stimulated adenylate cyclase activity. However, several points should be considered regarding the time relationship of receptor loss. First, the rate of receptor loss simply may be much faster in some cell types than in others. Also, if an agonist-induced internalization of β-adrenergic receptors occurs, care must be taken in interpreting changes in receptor number if a membrane fraction is utilized; i.e. a 20,000 to 40,000 × g centrifugation step may not sediment receptors that are in an internalized vesicle form. Thus, if receptors are only measured in the particulate fraction after a centrifugation step, conclusions may be reached concerning receptor loss that are invalid. In the case of frog erythrocytes at least a portion of the receptors are rapidly converted by catecholamines to a form that is not sedimented at 30,000 × g. In contrast, the "light vesicle" form of the β-adrenergic receptor that is induced during short-term incubation of 1321N1 astrocytoma cells (86) with catecholamines is sedimented during centrifugation at 35,000 × g for 20 min (262). Thus, in assays of receptors either in homogenates or in particulate preparations there is little loss of binding activity during short-term desensitization (89, 240, 241).

* M. L. Toews, J. P. Perkins, and T. K. Harden, unpublished observations.

However, it would be misleading to refer to this situation as one in which there is no loss of receptors, since certainly in a functional sense they are lost due to the fact that at least half of the receptors are no longer associated with the plasma membrane or adenylate cyclase. Nonetheless, these receptors are measurable by standard radioligand binding assays and can rapidly regain their capacity to couple to adenylate cyclase upon removal of catecholamine from the medium. In light of these considerations it is difficult to differentiate in the literature between situations in which catecholamines have induced a change in the membrane form of β-adrenergic receptors and situations where true loss of measurable receptors has occurred (i.e. receptors have usually been measured in a 20,000 to 40,000 × *g* pellet). In a situation (1321N1 astrocytoma cells) where such distinctions have been made, however, there is a clear temporal sequence of events that involves the following: activation of receptors → uncoupling of receptors → change in membrane form of receptors (? internalization) → loss of radiolabelled antagonist binding activity. Whether this is indeed a sequential series of events is problematic. Also, the rate constants between each step could be such that no distinction between the uncoupling step and receptor internalization could be made (see discussion above) or the internalized receptors could be rapidly converted to a form no longer detectable by radioligand binding.

As discussed above, long-term incubation of the G/F-deficient mutant, cyc^-, with catecholamines failed to result in loss of radiolabelled antagonist binding (221, 241) while short-term incubation of these cells with agonist resulted in a lesion (probably on the β-adrenergic receptor) such that normal catecholamine responsiveness could not be reconstituted (79, 80). If the latter modification is an accurate measure of the uncoupling reaction that occurs in other cell types, these data suggest that the uncoupling reaction alone is not sufficient to initiate the series of events that eventually results in receptor loss. The sequence of events leading to receptor loss may either: 1) require an effective agonist-induced coupling of receptors to G/F, or 2) occur through a series of steps unrelated to the initial uncoupling reaction. Alternatively, in addition to the G/F deficiency cyc^- cells may express a lesion that precludes normal cellular processing of β-adrenergic receptors. Interestingly, Rashidbaigi et al. (199) recently demonstrated that short-term (15 to 30 min) incubation of cyc^- cells with epinephrine results in a 25% to 30% loss of β-adrenergic receptors from a purified plasma membrane fraction with no further loss occurring over the next 24 hours. One interpretation of such results is that an agonist-induced internalization of β-adrenergic receptors also occurs in cyc^- cells, but that the reaction(s) leading to eventual loss of measurable receptors from the cell does not take place.

As discussed above, a role for cyclic AMP in the induction of the modifications that comprise catecholamine-specific desensitization has been ruled out in many studies. However, Moylan et al. (171) have recently reported that long-term (12 to 24 hour) elevation of cyclic AMP levels in C6-2B rat glioma cells results in a 30% to 40% loss of measurable β-adrenergic receptors. Although it could be argued that C6-2B cells present a special circumstance since these cells exhibit a prominent cyclic-AMP-induced heterologous type of desensitization (246), Moylan et al. (171) also reported that a 24-hour incubation of HC-1 hepatoma cells with dibutyryl cyclic AMP plus a phosphodiesterase inhibitor results in a 50% loss of receptors. Although HC-1 hepatoma cells lack the catalytic moiety of adenylate cyclase, they still undergo modifications during incubation with isoproterenol that are consistent with the occurrence of catecholamine-specific desensitization (241). Thus, to the extent that these two cell lines are representative of the properties of other cell types, the expression of β-adrenergic receptors may in part be regulated by intracellular cyclic AMP levels. The physiological significance of such a phenomenon is open to question, however, since in most cell types long-term elevation of cyclic AMP levels is not possible during exposure to catecholamines due to the occurrence of the desensitization process.

The fate of receptors that are lost from the cell in a measurable sense during incubation with catecholamines has not been studied comprehensively. The most extensive studies have been reported for 1321N1 astrocytoma cells. In these cells, agonists rapidly convert the receptor to a form that, as discussed above, most likely represents internalized vesicles. Measurable receptors are then lost from the cell over a period of hours (240, 241). However, the relative proportion of receptors appearing in the plasma membrane versus light vesicle form over this period did not change (262), which suggests that a rapid equilibrium is maintained with regard to receptors in these two forms. During a 12- to 24-hour incubation with isoproterenol, greater than 90% of the measurable binding sites were lost from the cell. In preconfluent 1321N1 cell cultures, the loss of β-adrenergic receptors was completely reversed upon removal of isoproterenol with receptor density returning to control levels with a $T_{1/2}$ of approximately 12 hours (53, 54). Addition of cycloheximide at a concentration that inhibited protein synthesis by greater than 90% and receptor accumulation during cell growth did not prevent the recovery of receptors (53). In contrast to the situation in preconfluent cultures, recovery of receptors in confluent cultures after agonist-induced loss was to only 60% to 70% of control. Moreover, the recovery process was completely blocked by cycloheximide (53). These experiments thus suggested that long-term exposure of preconfluent 1321N1 cell cultures to catecholamine resulted in the conversion of the β-adrenergic receptor to a form that was undetectable by radiolabelled antagonist binding but that, nonetheless, retained its primary amino acid structure. In contrast, receptor recovery after long-term exposure of post-

confluent cultures to catecholamine apparently is dependent on protein synthesis. These results have been corroborated in two ways. First, the conclusions concerning receptor processing in preconfluent cultures were supported through the use of the inhibitor of protein glycosylation, tunicamycin (54). As was the case with cycloheximide, tunicamycin blocked receptor accumulation during cell growth but did not block the recovery of lost receptors after desensitization of preconfluent cultures (54). Again, a conclusion from such results is that receptors are converted with no change in primary amino acid structure to a form no longer detectable by radioligand binding, then back to a form that is detectable by such methodology. In contrast, experiments with heavy isotope (^{2}H, ^{13}C, ^{15}N) amino acids have permitted unambiguous conclusions concerning the necessity of new receptor synthesis for recovery of lost receptors in postconfluent cultures. That is, if cells were incubated for 24 hours with isoproterenol and then switched to agonist-free growth medium containing heavy isotope (^{2}H, ^{13}C, ^{15}N) amino acids, the receptors that eventually recovered to control levels exhibited a marked increase in receptor mass (54); i.e. new receptors were synthesized with the heavy amino acids during the recovery process. Unfortunately, it has not proven possible to use this approach to study receptor recovery in preconfluent cultures, since these dividing cells do not tolerate the heavy amino acid mixture (54).

Whether these phenomena in 1321N1 cells are relevant to β-adrenergic receptor regulation in other cell types is not known. Recovery from catecholamine-induced desensitization has been shown to be (69, 167) or not be (137, 238) affected by inhibition of protein synthesis. Unfortunately, it cannot be identified with certainty which step in the desensitization process was studied in these reports. For example, the desensitization process or recovery of responsiveness were unaffected in Ehrlich ascites cells by cycloheximide (137). Since the effects were studied after only 1 hour of incubation with catecholamine, it is likely that only early events in the process of desensitization were studied in these experiments. Recovery of β-adrenergic receptors in frog erythrocytes in vivo following agonist-induced down regulation of receptor number also was not affected by protein synthesis inhibition with cycloheximide (173). Since receptors were measured in a 30,000 × *g* pellet and agonists apparently induce a form of receptors that is not sedimentable under these conditions (33), it is not clear what part of the receptor recovery process was measured in these experiments. That is, do these data represent recycling of receptors from an internalized pool of receptors or recovery from a form like that in astrocytoma cells that was not detectable by radioligand binding in any cellular location. Although receptors per se were not measured, protein synthesis inhibition during long-term catecholamine-induced desensitization of BHK cells resulted in less extensive loss of isoproterenol-stimulated adenylate cyclase activity and more rapid and complete recovery of responsiveness once cells were placed in agonist-free medium (167). One interpretation of these data is that inhibition of protein synthesis prevents a slowly reversible step (receptor degradation?) from occurring during incubation of BHK cells with catecholamine. Indeed, Homburger et al. (104) have shown that pretreatment of C6 glioma cells with cycloheximide for 15 hours blocks the agonist-induced loss of β-adrenergic receptors. Finally, in one of the early studies of desensitization Franklin et al. (69) showed that after long-term (24 hours) desensitization of human diploid fibroblasts with isoproterenol, recovery of hormone responsiveness during incubation of cells in agonist-free medium was blocked by protein synthesis inhibitors.

F. Overview of Catecholamine-specific Desensitization

While several different mechanisms may account for heterologous desensitization in catecholamine-responsive cells, this may not be the case for catecholamine-specific desensitization. The different results obtained in studies of catecholamine-specific desensitization can be explained for the most part by different experimental approaches or by differences in the rate or extent of occurrence of the separate events that are responsible for this type of refractoriness. Thus, a unifying model can be described that accounts for most of the reported results. Though speculative, this model attempts to coalesce the chiefly biochemical evidence obtained in studies of the β-adrenergic receptor system with the more extensive knowledge of other hormone (mostly polypeptide) receptor systems wherein the cellular fate of receptors has been examined both morphologically and biochemically. The schematic (Fig. 2) depicts the receptor in a native form on the cell surface. This protein is generated by normal ribosomal synthesis and is probably further modified [in particular, its oligosaccharide content (109)] and sorted (212) in the Golgi apparatus before its ultimate insertion into the plasma membrane. Once in the plasma membrane it is free to interact normally with hormone agonists (H) and G/F. However, this native form of the receptor (R) is rapidly converted to another form (R_U) upon interaction with agonists. Covalent modification of the receptor protein appears to be the most likely explanation for step 2 in this sequence of events of desensitization. R_U will not couple with G/F but is still measurable by radioligand binding techniques. Subsequent to modification of R to R_U the receptor is selectively clustered (step 3) and internalized (step 4) into vesicular structures with the ligand binding site facing inward. Direct evidence for a clustering and internalization event is not available. Nonetheless, in analogy with the peptide-receptor systems this is a likely means of converting the receptor from a surface to an intracellular form. The relationship of steps 3 and 4 to step 2 is not known. However, the most parsimonious interpretation of results thus far is that the internalized vesicular form

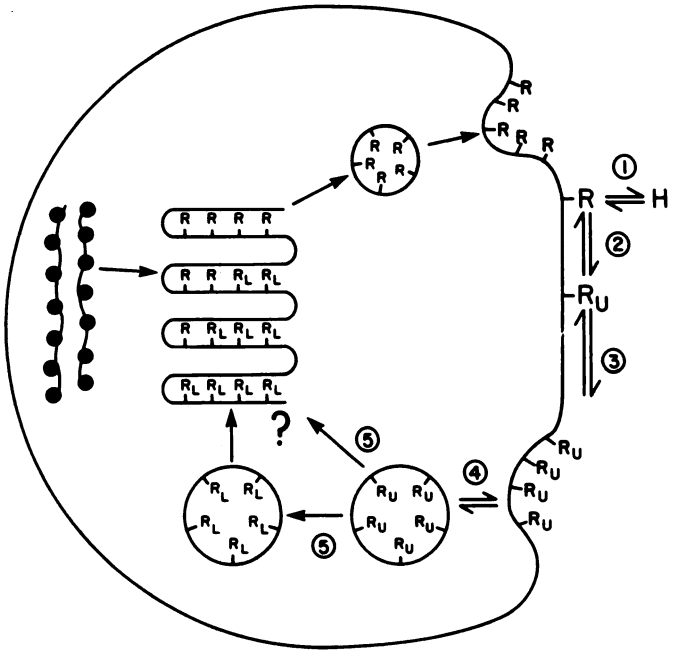

FIG. 2. Hypothetical model for the cellular processing of β-adrenergic receptors during catecholamine-specific desensitization. This scheme depicts the normal synthesis and insertion of receptors (R) into the plasma membrane after which they are free to interact (reaction 1) with β-adrenergic receptor agonists (H). Not only does this interaction result in the activation of adenylate cyclase by the series of events indicated in Fig. 1, but the receptor is converted (reaction 2) rapidly to a form (R_U) that no longer will couple to G/F. The model suggests that the uncoupled receptors are rapidly clustered (reaction 3) and internalized (reaction 4) into vesicular structures with the receptor binding site facing inward. Until this point, all of the reactions are apparently rapidly reversible and the receptor is detectable by normal radiolabelled antagonist binding techniques. However, with continued exposure of the target cell to agonist, the receptor is converted (reaction 5) to a form that is not detectable by radioligand binding techniques. This reaction may involve fusion of vesicles with lysosomal structures and degradation of the receptor polypeptide. Alternatively, the receptor polypeptide may be modified in such a way as to lose its binding activity yet retain its primary amino acid structure. Irrespective of the mechanism, reversal from this step is very slow and probably requires either the synthesis of new receptor protein or the activation of the modified protein by processing through the Golgi apparatus.

is generated as a rapid sequela of the uncoupling reaction. The subsequent fate of receptors in the internalized form is not known. Clearly, step 4 is a reversible reaction and R_U can return to the cell surface upon reduction of the extracellular concentration of agonist. While there would appear to be a dynamic equilibrium between steps 1, 2, 3, and 4, step 5 is quasi-irreversible. As pointed out earlier, there is no solid evidence that step 5 occurs as a consecution of step 4, but this seems a reasonable assumption. In some cell types step 5 may involve fusion of receptor-containing vesicles with lysosomal structures and subsequent degradation of the receptor protein. Alternatively, evidence from at least one cell type suggests that the receptor protein may be modified in such a way that binding activity is lost while the primary amino acid structure of the protein is conserved. In any case, recovery from this event is slow. In the first situation synthesis of new receptor protein would be required; in the second case the receptor apparently is processed (through the Golgi network?) in such a way as to regenerate functional activity.

Although biochemical analysis of the β-adrenergic receptor system during agonist-induced desensitization will allow further refining of these ideas, unequivocal conclusions concerning these putative mechanisms may require other approaches. The most attractive methodology would involve means whereby the receptor could be visualized. However, such an approach has proved difficult for a variety of reasons. The most useful ligands in this regard would be receptor agonists that not only could be visualized, but that also would induce this series of events. The chemical lability of the catecholamines as well as their low receptor affinity relative to several peptide hormones has retarded the development of this type of methdology. Alternatively, since there is no unambiguous evidence to indicate that an agonist-receptor complex rather than just the receptor per se is internalized, antagonist molecules substituted with suitable moieties for visualization might prove very useful. Evidence for significant advances in this regard has been reported (96, 162). The affinity and sensitivity to detection of such ligands is crucial in light of the very low level (1,000 to 10,000/cell) of β-adrenergic receptors on most cells.

Alternative approaches could be equally valuable. For example, antibodies that selectively recognized separate determinants of the receptor polypeptide would be highly useful (41, 72, 237, 277). This point is of particular importance (i.e. after step 5 in Fig. 2) for studies of the fate of the receptor subsequent to modification of the binding site. There is encouraging information suggesting that by using a combination of biochemical, morphological, and immunological approaches, definition of the molecular bases of each of the steps involved in catecholamine-specific desensitization is ultimately possible.

VII. Physiological Significance of Catecholamine-induced Desensitization

Detailed in vivo correlates of the mechanisms of catecholamine-induced desensitization that were discussed above are currently lacking. More germane to the consideration of this topic, however, is the question of whether similar events even occur in vivo. An examination of the recent literature permits this concern to be dispelled. There is no question that analogous adaptive mechanisms occur in vivo, and these phenomena are of major physiological and pharmacological importance. An extensive discussion of all of the reports in support of this contention will not be recounted here. However, an attempt will be made to identify situations wherein the mechanisms discussed above may be highly relevant and to place the results obtained in both model and intact animal systems into a working perspective. The modification of components of the β-adrenergic receptor-linked

adenylate cyclase system that occurs subsequent to alterations in glucocorticoid (142, 274) or thyroid hormone (157, 271) levels in vivo will not be considered. The reader also should refer to the reviews by Trendelenburg (253, 254) and by Fleming (66) concerning other aspects of the modification of cellular responsiveness to catecholamines. In short, the present consideration is by no means intended to exclude other mechanisms (e.g. changes in agonist disposition, modification of postsynaptic structures that are unrelated to the β-adrenergic receptor-linked adenylate cyclase, etc.) that may be of major importance in determining the final response of target cells to catecholamines. However, in light of the apparently great importance of adaptations in components of the adenylate cyclase system in determining cellular responsiveness and in the consideration of space limitations, the discussion of physiological relevance will be biased in the direction of the β-adrenergic receptor-linked adenylate cyclase system. For the most part, only representative publications concerning a given point will be considered.

There are several factors that complicate a direct comparison between data of in vivo origin and work carried out with isolated cell systems. An obvious problem concerns analysis of changes in cellular responsiveness at short times during experimentation. Whereas a desired concentration of agonist can be rapidly attained and then maintained in experiments with homogeneous cells on a culture dish or in a test tube, this is obviously not the case for an intact animal. Furthermore, the homogeneous cell system in vitro can be sampled at early times of exposure to an agonist; this is less feasible with tissues in vivo. Fear of reversal of a phenomenon of interest is less a concern in vitro than in vivo. Thus, most in vivo systems are not very accessible to the types of analyses that have been carried out with cells maintained in vitro. In most situations where catecholamine availability has been altered experimentally in vivo, the analyses have been carried out after an increase or decrease in receptor stimulation that spanned hours rather than minutes. Thus, there are few in vivo correlates involving biochemical analyses of the β-adrenergic receptor system during short-term modification of function. The difficulty in analyses of the kinetics of in vivo modification is increased further by the instability of catecholamines, by cellular heterogeneity, and by compensatory alterations in the release or action of other neurotransmitters or hormones.

One of the best characterized systems involving adaptive alterations of the β-adrenergic receptor-linked adenylate cyclase is the rat pineal gland. Indole metabolism in the pineal is regulated by a circadian rhythm that is controlled by the light-induced regulation of release of norepinephrine from the sympathetic nerve terminals innervating this structure (5). Subsequent to sympathetic dennervation there was an enhanced capacity of isoproterenol to stimulate cyclic AMP accumulation in intact glands (46, 47, 235) or in cell-free measurements of adenylate cyclase activity (22, 263). Treatment of rats with isoproterenol (130) or incubation of cultured glands with catecholamines (235) reduced the responsiveness of the cyclic AMP system to catecholamines. Maintenance of animals in constant light for 24 hours (a condition that greatly reduces sympathetic activity to the gland) produced results very similar to those of denervation (130, 263). Conversely, maintenance of animals in the dark for 24 hours (which results in enhanced sympathetic activity) resulted in an apparent desensitization of the β-adrenergic receptor-linked adenylate cyclase (130). Changes in β-adrenergic receptor number paralleled the alterations in hormone responsiveness under all of these conditions. Indeed, the extent of change in catecholamine-stimulated adenylate cyclase activity was very similar to the amount of change in receptor number in a variety of experimental paradigms (22, 130, 170). Whether these results indicate that hormonal responsiveness of the pineal adenylate cyclase is solely regulated by alterations in receptor number is not known. However, results from several experiments suggest that this view may be simplistic since basal and cholera toxin- and fluoride-stimulated adenylate cyclase activities also were changed during the adaptation process (130, 279).

Irrespective of the exact mechanism of regulation of responsiveness in the pineal, it is apparent that this regulatory mechanism has important physiological implications. That is, changes in postsynaptic responsiveness are important in the diurnal regulation of indole metabolism. During a 12-hour light cycle, sympathetic activity is reduced and there is a progressive increase in the number of β-adrenergic receptors and response of adenylate cyclase to catecholamines in the pineal (207). At the beginning of the following 12-hour dark cycle sympathetic activity is greatly increased and, because of a high level of β-adrenergic receptors and catecholamine responsiveness, large increases in cyclic AMP levels occur which result in large increases in melatonin synthesis (5, 45, 46, 206, 207). Norepinephrine-induced desensitization begins to occur during this dark phase. By the end of the 12-hour dark cycle the β-adrenergic receptor linked-adenylate cyclase is at a low level of catecholamine responsiveness due to the modifications discussed above.

A wide range of studies have indicated that drug-induced modifications in the β-adrenergic receptor/adenylate cyclase system occur in mammalian brain. In several cases, these modifications in receptor responsiveness have been implicated in the pharmacological effects produced by a clinically useful agent. Reduction in norepinephrine availability at the level of brain β-adrenergic receptors has been shown to enhance responsiveness of neural cyclic-AMP-generating systems to catecholamines. Such effects occurred after treatment of rats or mice with reserpine (51, 269) or 6-hydroxydopamine (108, 187) and after denervation (52). In several instances

these increases in responsiveness have been correlated with an elevation in the level of β-adrenergic receptors (19, 92, 166, 176, 226). In contrast, chronic administration of tricyclic antidepressants or monoamine oxidase inhibitors, which would be expected to elevate norepinephrine concentrations at the level of postsynaptic receptors, results in a progressive decrease in catecholamine responsiveness of the brain adenylate cyclase system (218, 242, 259, 260). A similar decrease in receptor number has been shown to occur coincidentally (7, 276). Interestingly, the slow time course of adaptive changes in the β-adrenergic receptor/adenylate cyclase system correspond more closely to the positive psychiatric effects of these drugs than does the initial effect on neurotransmitter uptake or metabolism (218, 242, 276). Thus, it has been proposed that the clinical efficacy of antidepressants may in part be based on their capacity to induce adaptive changes in postsynaptic responsiveness (242). Alternatively, the therapeutically positive effects of these drugs may evolve from a whole series of events, one of which is changes in postsynaptic responsiveness, that ultimately result in a "resetting" of behaviorally important synapses in the brain.

Evidence for adaptive phenomena in peripheral tissues also has been reported. For example, infusion of humans with catecholamines or long-term therapy of asthmatics results in a reduced responsiveness to catecholamines of the adenylate cyclase of lymphocytes isolated from these patients (81, 168). Krall and coworkers (135, 256) have not only shown that the properties of catecholamine-induced desensitization in isolated human lymphocytes are similar to those observed in tumor cells and frog erythrocytes, but both homologous and heterologous types of desensitization could be induced in lymphocytes in vivo during infusion of humans with isoproterenol. These changes in hormone responsiveness over a 1-hour period apparently occurred in the absence of a decrement in receptor number (135). However, Galant et al. (73) have illustrated that the β-adrenergic receptors on polymorphonuclear lymphocytes from either normal or asthmatic subjects were greatly reduced in number after 6 days of treatment with the β-adrenergic receptor agonist, terbutaline. This result has been supported by Aarons et al. (2) who demonstrated that chronic treatment of humans with either terbutaline or ephedrine reduced receptor number in lymphocytes isolated from these patients. At least in the case of one of these agonists (terbutaline), the adaptive response in terms of decrement in receptor number was temporally correlated with an adaptive change in the cardiovascular response to posture manipulation. Whether the agonist-induced alterations in lymphocytes that occur in vivo are reflective of similar changes in solid tissues has not been conclusively established, particularly in man. However, as is discussed below strong arguments can be made for this being the case.

One exception to the general finding that agonists induce a decrease in receptor number is the report by Tohmeh and Cryer (250) that infusion of humans with isoproterenol or epinephrine resulted in a 2-fold increase in the number of β-adrenergic receptors on mononuclear leukocytes during the first 30 min; extended infusion (4 to 6 hours) resulted in a large decrease in receptor number. The significance of this result is not known, particularly since it has not been confirmed in more easily controlled in vitro experiments with the same cell population. However, an agonist-induced increase in receptor number potentially could be related to the observation that phospholipid methylation unmasks "cryptic" β-adrenergic receptors in rat reticulocytes (236).

Agonist-induced desensitization of the response of cardiac (30, 77, 129, 133, 155, 160, 255, 278) and smooth (55, 65, 68, 97, 150, 179) muscle to catecholamines is a well known phenomenon. Many reports in the last few years have implicated modifications of the β-adrenergic receptor-linked adenylate cyclase in the mechanism of these adaptive changes in physiological response to catecholamines. For example, Marsh and coworkers (160) have carried out a series of experiments designed to examine the correlation between changes in the inotropic responsiveness of chick embryo ventricle to catecholamines and alterations in the β-adrenergic receptor-linked adenylate cyclase. Incubation of chick ventricle in vitro with isoproterenol for 30 min resulted in a 65% decrease in the contractile response to catecholamine; no modification of the inotropic response to Ca^{++} was observed. The diminution in catecholamine responsiveness was accompanied by a 61% reduction in isoproterenol-stimulated adenylate cyclase activity. Since basal and NaF- and GppNHp-stimulated adenylate cyclase activities and the number of β-adrenergic receptors were not significantly altered after incubation of ventricles with catecholamine, it was concluded that an agonist-induced uncoupling of the β-adrenergic receptor-linked adenylate cyclase occurred. Furthermore, it was proposed that this modification, which was similar to that observed during catecholamine-specific desensitization of tumor cells, may be responsible for the modified physiological response. These workers also have reported that incubation of cultured chick ventricular cells with isoproterenol results in a rapid loss of contractile response to catecholamines with no change occurring in the inotropic response to Ca^{++} (161). Bobik et al. (12) have examined the β-adrenergic receptor-linked adenylate cyclase during incubation of cultured chick ventricular cells with isoproterenol. The capacity of isoproterenol to elevate cyclic AMP levels in intact cells was lost with a time course similar to that reported by Marsh et al. (161) for contractile response to catecholamine. While no loss of β-adrenergic receptors was detectable after 1 hour of incubation of cultured ventricular cells with isoproterenol, receptor number was decreased by 80% after 16 hours of incubation. Again, these results are reminiscent of the changes in the β-adrenergic receptor-linked aden-

ylate cyclase that occur during catecholamine-specific desensitization of several tumor cell lines and frog erythrocytes.

Administration of isoproterenol to rats also reduced responsiveness of cardiac adenylate cyclase to catecholamines (32, 105, 255) and reduced the number of cardiac β-adrenergic receptors (30, 251, 255, 278). Although temporal comparison of the reduction in adenylate cyclase responsiveness to receptor decrement has not been reported, receptor changes have been evident only after a week of treatment, whereas at least in one study, adenylate cyclase responsiveness was significantly reduced within a day (32). Evidence from at least two studies suggests that heterologous modification of adenylate cyclase may occur in that fluoride(32, 255), GppNHp- (32, 255), glucagon- (32), and secretin- (32) stimulated activities all were reduced in addition to isoproterenol-stimulated activity. Unfortunately, no studies to date have correlated changes in cardiac responsiveness to short-term changes in the β-adrenergic receptor/adenylate cyclase during in vivo administration of catecholamine. That is, inotropic and chronotropic sensitivity of the heart to catecholamines is greatly reduced within 2 hours of infusion of catecholamine (30), yet most studies of the adenylate cyclase system have involved longer times of infusion. The report (30) that receptor number per se in homogenates is not reduced at a time of infusion with isoproterenol at which the physiological response to catecholamines is greatly modified, clearly does not exclude the occurrence of major alterations in the β-adrenergic receptor-linked adenylate cyclase that are not expressed as measurable receptor loss.

Elevation of circulating catecholamine levels by chronic hypoxia also results in an attenuated heart rate response to catecholamines (155). Maintenance of rats for 4 weeks under hypoxic conditions reduced the number of β-adrenergic receptors in the heart by 50% (261). The reduction in receptors did not occur in animals treated with a low dose of propranolol during chronic hypoxia. The decrement in receptor number was accompanied by similar decreases in basal and isoproterenol-stimulated adenylate cyclase activities. Fluoride-stimulated activity was reduced by 30% in heart membranes from hypoxic animals. Thus, as was shown with chronic administration of isoproterenol to rats (30, 32, 251, 255, 278) reductions in the expression of components of the β-adrenergic receptor-linked adenylate cyclase occur as a result of alterations in the release of endogenous catecholamines. Again, decrements in basal and fluoride-stimulated activities suggest that an agonist-nonspecific modification of adenylate cyclase may occur in addition to the agonist-specific decrement in β-adrenergic receptors.

Biochemical evidence for an enhanced level of β-adrenergic receptors subsequent to interruption of sympathetic innervation of peripheral structures also has been reported (20, 75, 76). Such results may have important clinical implications regarding withdrawal syndromes that occur after chronic drug treatment (177). For example, a compensatory increase in β-adrenergic receptors occurred in a variety of tissues during propranolol treatment (1, 75). At the moment of withdrawal of the drug the postsynaptic receptor system would exist in a hyperresponsive state and adrenergic hyperfunction could occur as a consequence. Aarons et al. (1) have reported a significant correlation between the elevated levels of β-adrenergic receptors on lymphocytes and an increased heart rate response to standing during withdrawal of humans from chronic propranolol treatment.

Although the changes discussed above mainly occur as alterations in receptor number or changes in the efficacy of catecholamines for activation of adenylate cyclase, the net physiological effect of such modifications cannot be predicted a priori. However, the most logical assumption is that these alterations would result in a change in the apparent *affinity* of catecholamines for stimulation of a physiological process. The reasoning for such an assumption is as follows.

Most cyclic-AMP-mediated physiological events operate at a maximal level at cyclic AMP levels far below that attainable by the stimulation of β-adrenergic or other receptors (203). Thus, "spare" receptors exist in the sense that maximal occupancy of the receptors is not necessary to elicit a maximal biological response, i.e. sufficient elevation of cyclic AMP levels occurs at an agonist concentration that submaximally activates adenylate cyclase. In a situation where catecholamine-induced desensitization has occurred, the efficacy of agonists for elevation of intracellular cyclic AMP levels will be reduced. Therefore, occupancy of a higher percentage of the receptors will be necessary to produce a given elevation of cyclic AMP levels. The net result would be a shift to the right of the concentration-effect curve for agonist with no change in the maximal effect attainable. Obviously, the "spareness" of the system will dictate the extent to which catecholamine-induced desensitization can occur before the occurrence of a decrease in the maximal physiological response attainable by saturating concentrations of agonists. Using similar arguments it can be concluded that adaptive increases in the number of receptors or in catecholamine-stimulated adenylate cyclase activity will result in a shift to the left of the concentration effect curve for production of a physiological effect.

Measurements of the contractile response of smooth muscle and inotropic and chronotropic response of cardiac muscle after prior exposure of these tissues to catecholamines support the argument made above. For example, prior exposure of isolated rat (150) or guinea-pig (55) tracheal smooth muscle to catecholamines resulted in a large shift to the right of the concentration-effect curve for β-adrenergic receptor agonist-induced relaxation. Although these results have been interpreted as representing an agonist-induced change in the affinity of receptors for agonists (150), the lack of direct evidence

supporting this contention makes the explanation put forth above a much more plausible one.

Chang et al. (30) reported that infusion of rats with isoproterenol for 2 hours resulted in a 10-fold shift to the right of the concentration-effect curve for isoproterenol-induced increase in heart rate and contraction. Similar results were reported by Yamaguchi et al. (278) and Tse et al. (255) after treatment of rats by chronic injection with high doses of isoproterenol. Finally, Kaumann and Birnbaumer (129) reported that exposure to isoproterenol of kitten atria maintained in a tissue bath resulted in a 6- and 15-fold shift to the right of the concentration-effect curve of isoproterenol for positive chronotropic and inotropic effects. In membranes prepared from the desensitized kitten atria there was little change in the apparent affinity of isoproterenol for stimulation of adenylate cyclase, but there was a 50% reduction in its efficacy. Consistent with the points made above, these workers concluded that maximal physiological effects of catecholamines on the heart can be attained with submaximal activation of adenylate cyclase (129).

The work of Staehelin and coworkers (56) clearly points out these relationships between catecholamine-induced desensitization and physiological effects in a tumor cell line. Maximal cyclic-AMP-dependent activation of phosphorylase by catecholamines in C6 glioma cells occurred at a concentration of agonist that produced only 6% of the maximally attainable elevation of cyclic AMP levels. Subsequent to a 3-hour incubation of cells with isoproterenol, the efficacy of isoproterenol for elevation of cyclic AMP levels was reduced by 62% with no change occurring in the apparent affinity of agonist. In contrast, isoproterenol induced full activation of phosphorylase subsequent to desensitization, but the concentration-effect curve for agonist was shifted 15-fold to the right.

As pointed out earlier, this discussion of modifications in the β-adrenergic receptor-linked adenylate cyclase system in relation to adaptive changes in target cell responsiveness to catecholamines is not intended to exclude the role of modification of other cellular mechanisms. Indeed, regulation at the level of the receptor-linked adenylate cyclase may only represent the first level of adaptive responses directed at maintaining homeostasis. Nonetheless, major modifications of the hormone responsive adenylate cyclase system occur in model systems as well as in vivo. As argued above these modifications apparently have physiological consequences. Continued elucidation of the structure and function of the β-adrenergic receptor-linked adenylate cyclase and its modification during changes in agonist availability should provide further insight into the mechanism of regulation of cellular function by catecholamines.

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