Agonist-Induced Isomerization of the α_1-Adrenergic Receptor: Kinetic Analysis Using Broken-Cell and Solubilized Preparations†

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ABSTRACT: The affinity of agonists but not antagonists at hepatic membrane α_1-adrenergic receptors is temperature dependent; a 100-fold higher affinity is observed at 4 °C than at 37 °C. The relationship between these two agonist affinity states was investigated by using a strategy that allows the kinetics of this transition to be examined under equilibrium conditions. When competition assays are performed at 37 °C for varying intervals and the reaction mixture is then rapidly cooled by freezing, allowed to thaw, and further equilibrated at 4 °C, a rapid and progressive decrease ($t_{1/2}$ of 1–2 min) in agonist affinity occurs, the extent of which is directly related to the incubation time at 37 °C. This decrease in agonist affinity is sustained as long as agonist is present but can be reversed by its subsequent removal. In contrast, no change in affinity is seen in identical experiments when antagonists are employed as the competing ligand. High-affinity binding of agonists is also demonstrated in short-term nonequilibrium experiments, indicating that the low-temperature incubations do not induce, but rather stabilize, a receptor conformation of high affinity for agonists. These findings suggest that the predominantly low-affinity binding of agonists to α_1-adrenergic receptors demonstrated in equilibrium studies at physiological temperatures may be the result of a ligand-driven decrease in affinity. Since the transition in receptor affinity for agonists occurs not only in broken-cell preparations but also after detergent solubilization of the membrane receptor, it most likely is due to an agonist-induced change in the conformation of the receptor protein per se. As the transition to a state of lower agonist affinity appears to occur more slowly than functional effects associated with receptor activation (phosphorylase *a* activation), it may represent a rapid form of receptor desensitization.

Radioligand binding studies have revealed marked differences between agonists and antagonists in their behavior as competitive ligands (Weiland et al., 1979; Abrahamson & Molinoff, 1984). In contrast to antagonist binding, the interaction of agonists with adrenergic receptors is not explained by a simple mass action relationship. Most adrenergic receptor types have been shown to exist in multiple affinity states for agonists, where guanine nucleotide sensitive high-affinity binding sites appear to represent the effector-coupled entities. At α_1-adrenergic receptors, which are typically not coupled to adenylate cyclase, the existence of a high-affinity state of the receptor is controversial. Most authors had initially reported uniform low-affinity binding of agonists to α_1-adrenergic receptors.

An intrinsic difference between an antagonist and an agonist has also been shown from thermodynamic studies of agonist binding to β-adrenergic receptors (Weiland et al., 1979, 1980). While the interactions of antagonists are largely entropy driven, the binding of agonists is associated with a large decrease in enthalpy, possibly representing a specific agonist-induced change in receptor conformation. This agonist-induced conformational change may be a link in the chain of events leading to the activation of adenylate cyclase.

When the ability of α_1-adrenergic agents to elicit or inhibit a functional effect is compared with its dissociation constant (K_D) calculated from radioligand binding studies, agonists again behave in an anomalous fashion, as compared to antagonists (El Refai et al., 1979; Preiksaitis et al., 1982; Kunos et al., 1983). The inhibitory constants for antagonists obtained in functional assays in liver correlate well with their dissociation constants calculated from radioligand binding studies carried out in membrane preparations. In contrast, agonists demonstrate lower affinity in radioligand binding studies than would have been predicted from functional assays.

This report focuses on the potential mechanism underlying this discrepancy. We have previously reported that the affinity of agonists at α_1-adrenergic receptors is markedly higher in *intact* cells when competition assays are performed at 4 °C or under initial velocity conditions at 37 °C (Schwarz et al., 1985a). We now extend these observations to broken-cell and solubilized receptor preparations and investigate the kinetics of the transition to a receptor configuration of lower affinity for agonists. "Pulsed" incubation of the reaction mixture (membranes, [^{3}H]prazosin, and competing ligand) at 37 °C for progressively longer intervals, followed by rapid freezing, thawing, and further equilibration at 4 °C, results in a rapid, progressive, and sustained decrease in agonist but not antagonist affinity.

Materials and Methods

[^{3}H]Prazosin (80.9 Ci/mmol) was purchased from New England Nuclear. (−)-Epinephrine was purchased from Sigma Corp. and prepared in buffer containing 0.7 mM ascorbic acid. Phentolamine and prazosin were gifts of Ciba-Geigy, Summit,

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NJ, and Pfizer Corp., Groton, CT, respectively. Gpp(NH)p was purchased from Boehringer Mannheim, Indianapolis, IN. All other reagents were purchased from Sigma (St. Louis, MO).

Isolation of Hepatocytes and Membrane Preparation. Female Sprague-Dawley rats (180–240 g) were purchased from Charles River laboratories and maintained on standard Purina laboratory chow. Isolated hepatocytes and a crude membrane fraction were prepared as previously described (Schwarz et al., 1985b). Protein determination was performed according to the method of Lowry et al. (1951).

Functional Studies with Isolated Hepatocytes. Determination of phosphorylase *a* activity was performed according to Stalmans and Hers (1975), as previously described (Schwarz et al., 1985b).

Radioligand Binding Assay. A typical binding assay employed membranes (200–600 μg of membrane protein) and competing ligands in a total volume of 300 μL in 10-mM sodium phosphate buffer containing NaCl (150 mM) and $MgCl_2$ (1 mM). Incubations were terminated by vacuum filtration with a Millipore apparatus onto glass fiber filters (Schleicher & Schuell no. 32; Keene, NH) and rapid washing with 4 × 4 mL of buffer (25 °C). Incubations at 37 °C were performed in a water bath for 30 min. When binding assays were performed at 4 °C, competing ligands (labeled and unlabeled) were pipetted into reagent tubes and cooled for 15 min. Membranes were equally cooled for 15 min before addition to the incubation mixture. Incubation at 4 °C was carried out for 150 min on a rotatory shaker (American Rotator V, 150 cpm). The total amounts of "specific" binding (80–95% of total binding) and "nonspecific" binding were not influenced by the incubation temperature.

For nonequilibrium binding studies, a total assay of 1 mL was employed. Incubations were started by adding membranes (800 μL) to the competing ligands (200 μL), followed by 5 s of low-speed vortexing and rapid filtration after 30 s of incubation at 37 °C. In experiments where incubations were performed for varying time intervals at 37 °C, several identical sets of 36 tubes, each of which represented one entire competition assay (in triplicate), containing equal amounts of [^{3}H]prazosin, (0.6–0.8 nM) and increasing concentrations of competing ligand (100 μL) were prepared and initially warmed to 37 °C. Assays were started by adding membranes (200 μL) previously warmed to 37 °C followed by vortexing for 5 s. Every tube of such a set of 36 was incubated at 37 °C for exactly the same time (60 s, 120 s, and 30 min). The incubation mixture was then rapidly cooled by individually rapidly freezing each tube in liquid nitrogen. Samples were then allowed to thaw at 4 °C and to further equilibrate for an additional 150 min at this temperature. In control experiments, membranes (200 μL) were first snap-frozen in liquid nitrogen. Competing ligands were then added (100 μL), and incubations were continued at 4 °C for 150 min. In these studies, nonspecific binding was routinely less than 20% of total binding. The total amount of ligand specifically bound was identical within a group of experiments and thus independent of the different incubation protocols.

Solubilization of α_1-Adrenergic Receptors. Liver cell membranes were solubilized as previously reported (Graham et al., 1985) with minor modifications. Membranes were agitated for 45 min at 25 °C in phosphate buffer containing 0.5% digitonin (Sigma) and subsequently centrifuged for 45 min at 36000*g*. Aliquots of the supernatant (400 μL) were incubated with [^{3}H]prazosin (5 nM) and increasing concentrations of (−)-epinephrine in a total assay volume of 500 μL for 60 min at 37 °C or for 12 h at 4 °C. Precipitation was achieved by subsequent addition of 1-mL aliquots of ice-cold bovine γ-globulin (1 mg/mL) and polyethylene glycol (25%) solutions. Filters were presoaked and reactions terminated by washing with ice-cold 8% polyethylene glycol solution (3 × 4 mL).

Data Analysis. Dissociation constants determined from equilibrium binding studies were calculated from the untransformed radioligand binding data (triplicate determinations) by using a mass action law based weighted nonlinear curve-fitting program, LIGAND (Munson & Rodbard, 1980). Statistical analyses were performed according to the extra sum of squares principle (Rodbard, 1974). "Goodness of fit" for several models of the ligand–receptor interaction was compared with the F test (Snedecor & Cochran, 1967). Experiments were first calculated individually. Subsequently, groups of experiments (three to five) from different tissue preparations were fitted simultaneously in a single calculation step using correction factors for different receptor concentrations, as described by Munson & Rodbard (1980). A two-site model was assumed when the "fit" for a model involving two binding sites was statistically better than that for a one-site model ($p < 0.05$). To test for differences in the parameter estimates between two data series, one set of data was first calculated separately. The parameter(s) obtained was (were) then constrained during the analysis of the second set of data. A significant difference was established when the fit for the analysis with the constrained parameter(s) resulted in a significantly greater residual variance ($p < 0.05$ by F test) than the best fit without constrained parameters.

RESULTS

Effect of Temperature on the Affinity of α_1-Adrenergic Ligands. As previously demonstrated (Schwarz et al., 1985b), binding of the α_1-adrenergic antagonist [^{3}H]prazosin to membranes prepared from isolated hepatocytes was reversible and of appropriate specificity. Analysis of saturation experiments conducted at 37 °C (30-min incubations) indicated that the binding of [^{3}H]prazosin occurred to a single class of homogeneous sites (B_{max} = 131 ± 12 fmol/mg of protein) with a dissociation constant (K_D) of 105 ± 20 pM.

In contrast, competition assays at 37 °C using the agonist (−)-epinephrine were shallow (Hill coefficient = 0.6–0.85) (Figure 1). When analyzed with LIGAND, the results were best described ($p < 0.001$) by a two-component model in which (−)-epinephrine competed for the majority (65–90%) of [^{3}H]prazosin binding sites with a dissociation constant (K_D) of 2200 ± 300 nM. The remaining fraction of sites (10–35%) exhibited a 100-fold higher affinity (K_H = 20 ± 5 nM[for the agonist. A similar proportion of high- and low-affinity sites for agonists was also detected in whole cell binding assays employing intact isolated hepatocytes (Schwarz et al., 1985b). When incubations were performed in the presence of 0.1 mM Gpp(NH)p, the resultant curves now indicated exclusive interaction of (−)-epinephrine with low-affinity sites (Figure 1). This is in agreement with previous reports (El Refai et al., 1979; Goodhardt et al., 1982; Lynch et al., 1985) that guanine nucleotides convert α_1-adrenergic receptors to a single state of low affinity for agonists.

When equilibrium competition assays were performed at 4 °C (150-min incubations), (−)-epinephrine was approximately 100-fold more potent as a competing ligand than at 37 °C (Figure 1). The increase in affinity observed at 4 °C was unchanged even with prolonged incubations (12 h). The dissociation constant for (−)-epinephrine in studies performed at 4 °C was similar to that calculated for the high-affinity sites

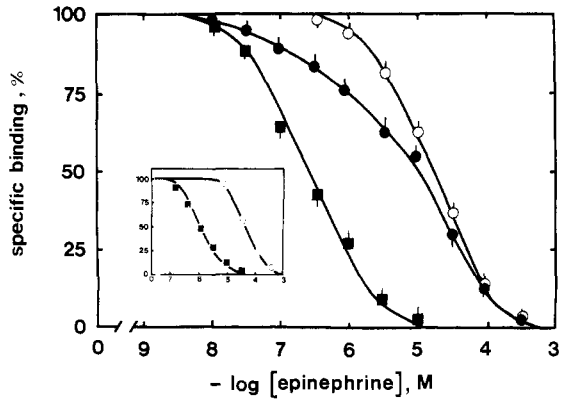

FIGURE 1: Competition for [^{3}H]prazosin binding by (−)-epinephrine in membranes prepared from isolated hepatocytes and in solubilized receptor preparations. Data are depicted as percent of maximally bound [^{3}H]prazosin at each concentration of competing ligand. Nonlinear regression analysis with LIGAND was performed by using the untransformed data from three individual experiments as described under Materials and Methods. Data points are depicted ± SEM. [^{3}H]Prazosin (0.5–0.8 nM) was incubated with increasing concentrations of (−)-epinephrine for 30 min at 37 °C in the presence (○) or in the absence (●) of Gpp(NH)p (0.1 mM) or at 4 °C (■) for 150 min. Inset: Competition for [^{3}H]prazosin binding by (−)-epinephrine in solubilized liver cell membranes. Solubilization was performed as described under Materials and Methods. [^{3}H]Prazosin (5 nM) was incubated with increasing concentrations of (−)-epinephrine for 60 min (○) at 37 °C or at 4 °C (●) for 12 h.

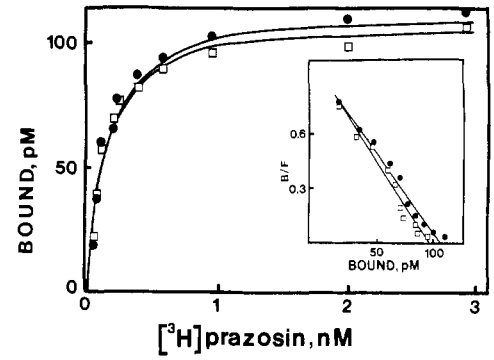

FIGURE 2: Equilibrium binding of [^{3}H]prazosin to liver cell membranes at 37 and 4 °C. [^{3}H]Prazosin (0.02–3 nM) was incubated with membranes prepared from isolated hepatocytes as described under Materials and Methods at 37 °C for 30 min (●) and at 4 °C for 150 min (□). The depicted experiment is representative of a series of n = 4. Inset: Scatchard plots of the data.

at 37 °C (24 ± 4 vs. 20 ± 5 nM). However, the high-affinity agonist binding sites accounted for only 10–35% of the total at 37 °C, whereas the majority of sites were of low affinity with a dissociation constant of 2200 nM. Similar results were obtained with (−)-norepinephrine. The virtual identity of the dissociation constants at 4 °C, and for the small percentage of high-affinity sites identified at 37 °C, can be reconciled with a two-state model of the receptor for agonists, where the receptor is "locked" in the high-affinity configuration when incubations are performed at the low temperature. This locking of the high-affinity configuration in the cold is further demonstrated by the inability of Gpp(NH)p to decrease the affinity for agonists when studies are performed at 4 °C, in agreement with the findings reported by Lynch et al. (1985).

The temperature dependency of agonist affinity was also examined in solubilized receptor preparations. When competition experiments were performed at 37 °C for 1 h using a solubilized receptor preparation, uniform low-affinity binding of (−)-epinephrine to a single affinity state was observed (K_D = 6100 ± 500 nM; Figure 1, inset), whereas high-affinity binding (K_D = 40 ± 6 nM) was seen when incubations were performed at 4 °C (12 h).

In contrast to the results obtained with agonists, the affinity of antagonists was not affected by temperature. Similar dissociation constants for [^{3}H]prazosin were determined from Scatchard plots of saturation binding studies performed at 4 and 37 °C (105 ± 20 vs. 112 ± 23 pM, n = 4, p = ns) (Figure 2). There was also no statistical difference in the number of binding sites detected at the two temperatures (131 ± 12 vs. 125 ± 13 fmol/mg of protein), in agreement with previous reports (Lynch et al., 1985; Sladeczek et al., 1983).

To investigate possible reversibility of the effects of temperature on agonist affinity, the reaction mixtures for an entire competition assay were first incubated at 37 °C for 30 min. Subsequent cooling and equilibration at 4 °C, even for prolonged intervals (20 h), did not result in a leftward shift in the agonist competition curve. This finding indicates persistence of predominantly low-affinity binding when agonist is maintained throughout the incubation period and is in agreement with similar studies with β-adrenergic receptors (Insel & Sanda, 1979). However, when membranes were exposed to (−)-epinephrine at 37 °C (10^{-3} M, 30 min) and then extensively washed to remove the agonist, high-affinity binding could again be demonstrated in a subsequent competition assay performed exclusively at 4 °C (data now shown). To exclude the possibility that an accelerated breakdown of (−)-epinephrine at 37 °C accounts for the observed changes in affinity, membranes were initially incubated with (−)-epinephrine (10^{-3} M) for 30 min at 37 °C. (−)-Epinephrine was then separated from the membranes by centrifugation and the supernatant employed in a subsequent competition assay at 4 °C. Identical competition curves were observed in studies with dilutions from this stock solution as those obtained with fresh (−)-epinephrine.

Kinetics of the Transition to a Low-Affinity State for Agonists. The previous studies suggested that a sustained decrease in agonist affinity occurs when equilibrium studies are performed at 37 °C and that this decrease can be prevented when incubations are performed at low temperatures. A strategy was then devised to monitor the kinetics of this transition. The reaction mixtures for entire competition assays were incubated at 37 °C for varying time intervals, rapidly frozen, and subsequently thawed and equilibrated at 4 °C for 150 min. Increasing the incubation time of the membranes with the agonist–[^{3}H]antagonist mixture at 37 °C prior to rapid freezing and equilibration at 4 °C for 150 min led to a rapid and progressive rightward shift of the competition curve for (−)-epinephrine (Figure 3). While less than 15% low-affinity sites were present after 0.5 min of incubation at 37 °C, these sites accounted for 27 ± 4% (1 min) and 54 ± 5% (2 min) of all sites upon further incubation at this temperature. The $t_{1/2}$ for the transition to predominantly low-affinity agonist binding was 1.4 min. In contrast to these findings with (−)-epinephrine, no significant change in affinity was observed when identical experiments were performed with a competing antagonist such as phentolamine (Figure 4). Binding of the competing antagonist strictly followed a mass action relationship for a single-site model.

The protocol employed in these studies did not per se alter the binding characteristics of the radioligand. When equilibrium saturation experiments were performed with [^{3}H]-prazosin using an identical protocol (incubation at 37 °C followed by rapid freezing and equilibration at 4 °C), both the affinity of [^{3}H]prazosin and the amount of specific and nonspecific binding of the radioligand did not change (data not shown).

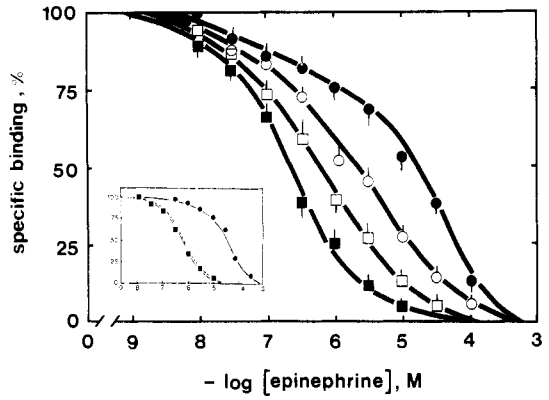

FIGURE 3: Effect of increasing incubation time at physiological temperatures on agonist affinity. [^{3}H]Prazosin was incubated with membranes and increasing concentrations of (−)-epinephrine at 4 °C (150 min) (■) or at 37 °C for 60 s (□), 120 s (○), and 30 min (●). Each sample was exposed at 37 °C for the exact time indicated, then rapidly frozen, and further equilibrated for 150 min at 4 °C as described under Materials and Methods. Nonlinear regression analysis with LIGAND was performed from triplicate determinations of [^{3}H]prazosin binding. Data are expressed as percent [^{3}H]prazosin ± SEM bound at the indicated concentration of (−)-epinephrine (n = 3). Inset: Hepatocyte membranes were rapidly cooled and then incubated at 4 °C for 150 min (■) or incubated at 37 °C for 30 min (●) prior to equilibration at 4 °C. Upon prolonged incubation (480 min) at 4 °C (□), the shift in affinity was unaltered.

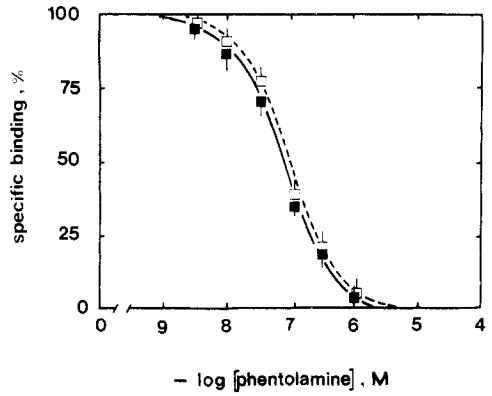

FIGURE 4: Effect of increasing incubation time at physiological temperatures on antagonist affinity. [^{3}H]Prazosin (0.7 nM) was incubated with increasing concentrations of the α-adrenergic antagonist phentolamine and liver cell membranes at 4 °C for 150 min (■) or at 37 °C for 30 min (□) followed by subsequent rapid freezing, thawing, and equilibration at 4 °C for 150 min. Data are expressed as the means ± SEM from three experiments.

Determination of Agonist Affinity Using Nonequilibrium Initial Velocity Conditions. In functional assays employing isolated hepatocytes, near-maximal α_1-adrenoceptor-mediated stimulation of glycogenolysis was elicited within a period of 30 s (Figure 5), in agreement with previous studies (Studer & Borle, 1983; Aggerbeck et al., 1980). Thus, the receptor-coupled response may be triggered at low levels of receptor occupancy, before equilibrium is reached. On the basis of this observation, the affinity of the α_1-adrenergic receptor for (−)-epinephrine was, therefore, also determined under nonequilibrium conditions, using short incubation periods.

When incubations were terminated by filtration after 30 s at 37 °C, specific binding [binding inhibitable by 10^{-4} M (−)-epinephrine] was 50–75%, compared to 75–90% at equilibrium. At this time, 6–11% of the amount of specific binding at equilibrium was detected. Thus, initial velocity conditions can be assumed. (−)-Epinephrine inhibited the binding of the radioligand with an IC_{50} of 105 ± 7 nM (n = 4), which is 100-fold lower than that determined in equilibrium competition assays conducted for 30 min (Figure 6).

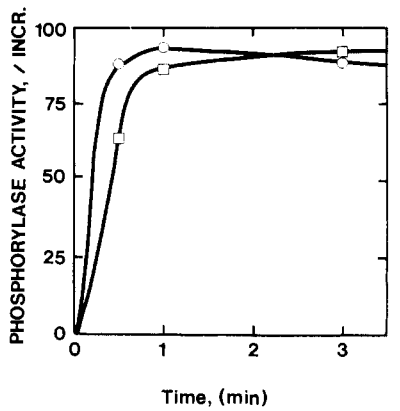

FIGURE 5: Time course of activation of glycogen–phosphorylase *a* with α_1-adrenergic agonists in isolated rat hepatocytes. Isolated hepatocytes were incubated with (−)-epinephrine (10^{-5} M, ○) and phenylephrine (10^{-5} M, □) for increasing time intervals at 37 °C in the presence of propranolol (10^{-5} M) and then rapidly frozen for determination of glycogen–phosphorylase *a* activity (Schwarz et al., 1985b).

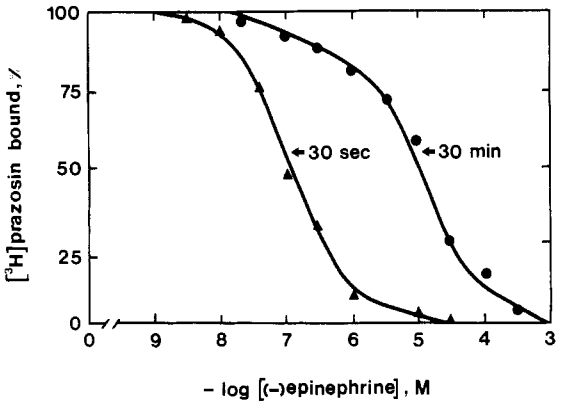

FIGURE 6: Competition for [^{3}H]prazosin binding by (−)-epinephrine at 30 s and 30 min of incubation at 37 °C. [^{3}H]Prazosin (0.4–0.7 nM) was incubated with membranes and increasing concentrations of (−)-epinephrine. Assays were rapidly terminated by vacuum filtration after 30 s (▲) or 30 min (●) of incubation at 37 °C. Points depicted are the means of triplicate determinations derived from an individual experiment, which was repeated 4 times. The concentration of [^{3}H]prazosin was 0.4 nM, and the dissociation constant for [^{3}H]prazosin in this preparation was 240 pM. The amount of specifically bound [^{3}H]prazosin was 70 fmol (9000 cpm) at 30 min and 6.3 fmol (700 cpm) at 30 s.

The same experimental approach was also employed in studies with the α-adrenergic antagonist phentolamine. The apparent affinity of phentolamine was only minimally altered when determined in short-term incubations, as compared to equilibrium studies (45 ± 6 vs. 76 ± 6 nM). In contrast to the results obtained with agonists, the resulting curves were steep and uniphasic at all incubation times (n_H = 0.96–1.05) and shifted slightly to the right with increasing incubation time, approaching equilibrium conditions, at which time the apparent affinity depends on the radioligand concentration (Cheng & Prusoff, 1973).

Relationship between Affinity for Agonists and α_1-Adrenergic Receptor Mediated Functional Responses. To relate the dissociation constants of (−)-epinephrine to an effector signal in the same tissue, dose–response curves for glycogen–phosphorylase *a* stimulation via α_1-adrenergic receptors were performed in isolated hepatocytes (Figure 7). The EC_{50} in these functional studies was 24 ± 3 nM and thus in good agreement with the receptor occupancy levels predicted by binding to the high-affinity sites (K_D = 20–40 nM). In con-

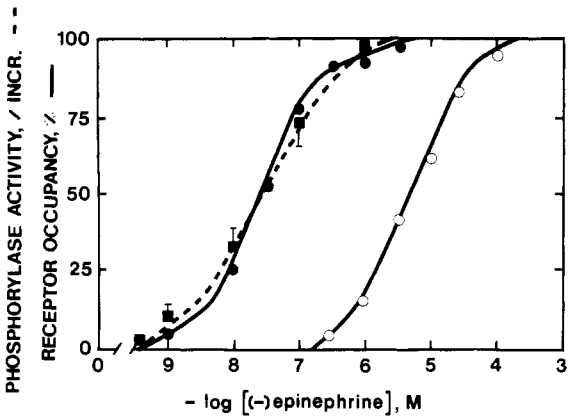

FIGURE 7: Comparison between the effects of (−)-epinephrine on glycogen–phosphorylase *a* activation via α_1-adrenergic receptors and the predicted receptor occupancy levels for binding of (−)-epinephrine to high- and low-affinity sites. Determination of glycogen–phosphorylase *a* activity in the presence of propranolol (10^{-5} M) was performed as described (Schwarz et al., 1985b). Basal glycogen–phosphorylase *a* activity was 15.1 ± 2.5 nmol min^{-1} (mg of protein)$^{-1}$. Data are expressed as percent increase over basal activity (■). Receptor occupancy levels for high- [K_D = 19 nM (●)] and low-affinity [K_D = 2000 nM (○)] sites for (−)-epinephrine were calculated for the different concentrations of (−)-epinephrine from the following relationship: % occupancy = [H]/([H] + K_D), where [H] is the concentration of (−)-epinephrine (Goldstein et al., 1974).

trast, a marked discrepancy between the functional studies and the radioligand studies is evident when the receptor occupancy levels for (−)-epinephrine binding are calculated for the low-affinity sites (K_D = 2000–10 000 nM).

Discussion

The discrepancy between the potency of (−)-epinephrine in eliciting functional responses via α_1-adrenergic receptors, such as activation of glycogen–phosphorylase *a*, and its low affinity for these same receptors as determined in competition experiments with radiolabeled antagonists has been the subject of a number of studies (El Refai et al., 1979; Goodhardt et al., 1982; Lynch et al., 1985; Hoffman et al., 1981, 1982). One group has postulated that the conventionally used radiolabeled antagonists might predominantly (though not exclusively) identify a site other than the physiologically relevant hormone binding site (El Refai et al., 1979). While the conclusions of this study have been criticized (Hoffman et al., 1981, 1982), the interactions of agonists with α_1-adrenergic receptors remain incompletely understood. Several laboratories have presented preliminary evidence for the existence of two interconvertible affinity "states" for agonists in liver (Goodhardt et al., 1982; Lynch et al., 1985), while others observed only low-affinity binding of agonists in competition assays with the specific α_1-antagonist [^{3}H]prazosin (Hoffman et al., 1981, 1982).

The findings of the present study suggest that the predominant low-affinity binding of α_1-adrenergic agonists observed at equilibrium is due to a rapid isomerization of the high-affinity agonist binding site to a low-affinity configuration. The transition from high- to low-affinity agonist binding was demonstrated by two techniques. First, transient high-affinity binding of agonists was documented in nonequilibrium initial velocity experiments, where agonists, but not antagonists, competed with a 100-fold greater potency for specific binding of the α_1-antagonist [^{3}H]prazosin than that observed in equilibrium experiments carried out at 37 °C. The theoretical basis of this initial velocity approach for determining the binding affinities of rapidly equilibrating ligands has been presented by Toews et al. (1983). At sufficiently early time points when [RL] ≪ [R][L], the IC_{50} provides a valid estimate of the dissociation constant.

Second, an equilibrium approach to investigate the transition from high- to low-affinity binding of agonists was developed. We had initially observed that the receptor is locked in a high-affinity configuration when incubations are conducted in the cold and that incubation at 37 °C results in a sustained decrease in affinity. On the basis of these observations, the following protocol was developed. Reaction mixtures for entire competition assays were first incubated for progressively longer intervals at physiological temperatures, then snap-frozen, and allowed to thaw and equilibrate at 4 °C. A rapid decrease in agonist affinity was observed, the extent of which was directly related to the time of the initial incubation at 37 °. In contrast, no change in affinity was noted in identical studies with competing antagonists. These results suggest that a better approximation of the affinity of the physiologically relevant form of the α_1-adrenergic receptor for an agonist can be obtained when competition binding experiments are performed at 4 °C or under initial velocity conditions. High-affinity binding of agonists can even be demonstrated with these techniques when exclusively low-affinity agonist binding is observed at 37 °C such as in membrane prepared in the absence of ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Lynch et al., 1985). Moreover, the affinity of the receptor for agonists determined with the latter methods is in agreement with the K_{act} for glycogen–phosphorylase *a* activation in isolated hepatocytes. The validity of this comparison between functional data obtained in intact cells and radioligand binding data obtained in membrane preparations is apparent from our previous radioligand studies in intact hepatocytes (Schwarz et al., 1985b). Identical values for the affinity of (−)-epinephrine at high- and low-affinity sites at 37 °C (K_H = 15 ± 2 nM, 30%; K_L = 2000 ± 200 nM, 70%) or at 4 °C (K_H = 22 ± 4 nM, 100%) were found in these studies, indicating that the two distinct affinity states for agonists demonstrated in intact hepatocytes are fully preserved in membrane preparations.

The existence of spare receptors could also account for the discrepancy whereby epinephrine is 100-fold more potent in functional studies, as compared to its affinity in radioligand binding studies conducted at 37 °C. However, previous studies have provided evidence against a receptor reserve in this tissue (Kunos et al., 1983; Lynch et al., 1983).

Transient high-affinity binding of agonists was first observed by Pitman and Molinoff at β-adrenergic receptors in intact cells (Pitman & Molinoff, 1980), where the low affinity of agonists observed in equilibrium competition binding experiments contrasted with their relative potency in eliciting an increase in cyclic AMP. This phenomenon has since been studied in greater detail (Toews et al., 1983; Toews & Perkins, 1984; Hoyer et al., 1984; Insel et al., 1984). The agonist-induced decrease in affinity also occurs in cells devoid of a functional coupling protein (G_s) and thus appears to be a property of the receptor molecule itself (Hoyer et al., 1984; Insel et al., 1984). Identical high-affinity binding of agonists to intact cells was also demonstrated when equilibrium competition assays were conducted at 4 °C, indicating that both maneuvers (nonequilibrium binding and incubation at low temperatures) may be adequate to characterize the initial, physiologically relevant hormone–receptor interaction (Toews et al., 1983). We initially observed a similar phenomenon at α_1-adrenergic receptors in intact hepatocytes (Schwarz et al., 1985a). Subsequently, as demonstrated in this report, we could obtain similar findings even in a broken-cell preparation. Thus,

the large decrease in agonist affinity is apparently not a consequence of changes in receptor metabolism but more likely reflects a change at the level of the plasma membrane, possibly of the receptor molecule itself. This interpretation is also supported by our finding that distinct temperature-dependent affinity states for agonists are preserved even following detergent solubilization of the receptor. Thus, the temperature dependency of agonist affinity is preserved when the receptor is removed from its native milieu within the plasma membrane.

As (−)-epinephrine generates a near-maximal α_1-adrenergic stimulation of glycogenolysis in liver cells within 30 s, it is likely that the physiologically relevant hormone–receptor interaction occurs within this time period. This is also evident from studies designed to examine early steps in receptor-coupled signal transduction in this tissue. Following α_1-adrenergic stimulation, a maximal increase in intracellular free Ca^{2+} concentration in isolated hepatocyes (Charest et al., 1983) or efflux of Ca^{2+} from perfused liver (Reinhart et al., 1982) occurs within 10 s. When incubations of hepatocyte membranes with (−)-epinephrine and [^{3}H]prazosin at physiological temperatures were terminated after 30 s, the receptor still exhibits a high affinity for agonists. Kinetic analysis of the transition of the receptor to a state of predominantly low affinity for agonists indicates that the affinity changes rapidly with a $t_{1/2}$ of 1–2 min. However, this transition of the affinity state probably does not precede signal transduction per se, which occurs within the first seconds of the hormone–receptor interaction in liver cells. Thus, the change in affinity of the agonist binding site described here likely does not reflect the initial event in the process of signal transduction, as suggested by Weiland et al. (1979) based on thermodynamic analysis of ligand binding to β-adrenergic receptors.

It is likely that agonist binding initiates a transmembrane signal by effecting a conformational change in a portion of the receptor molecule that allows it to couple in a productive manner with its effector machinery. Our results suggest that this same process, triggered by agonist binding, also promotes a change in the configuration of the ligand binding site itself to a state of a 100-fold lower affinity for agonists. By facilitating the dissociation of receptor-bound agonist, this mechanism may permit a more rapid attenuation of receptor-mediated functional responses.

However, the exact mechanism underlying this rapid time- and temperature-dependent transition in agonist affinity will require further clarification. It is not possible to distinguish from the equilibrium binding data alone whether the conversion of sites via a ligand-induced conformational change is the sole mechanism underlying this phenomenon or whether site–site interactions (negative homotropic cooperativity) are contributing to the decline in agonist affinity. In fact, the initial velocity studies might be interpreted as suggesting that the latter mechanism is operative. However, if the α_1-adrenergic receptor contains only a single hormone binding subunit (Graham et al., 1982), then the demonstration of two agonist affinity states in a solubilized receptor preparation would argue against a cooperative interaction.

Registry No. (−)-Epinephrine, 51-43-4; glycogen phosphorylase, 9035-74-9.

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