Agonist Interactions at Hepatic α_1- and β-Adrenergic Receptors: Affinity-State Regulation by Guanine Nucleotides and Temperature†

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ABSTRACT: We investigated the binding characteristics of agonists to α_1- and β-adrenergic receptors of (a) intact liver cells, (b) broken rat liver cell membranes, and (c) detergent-solubilized preparations under varying experimental conditions, focusing on the different "states" of the receptor for agonists and the regulation of these states by temperature and guanine nucleotides. While only low-affinity binding of agonists to both receptor subtypes was evident in studies performed at 37 °C with solubilized preparations, biphasic competition curves for agonists were observed in both intact cells and membrane preparations; the majority of sites were of low affinity. In membrane preparations, the nonhydrolyzable GTP analogue Gpp(NH)p caused a rightward shift of agonist competition curves and a loss of high-affinity binding. These results are consistent with the involvement of guanine nucleotide binding proteins in both α_1- and β-adrenergic transduction pathways. When competition studies were performed at 4 °C, receptor sites existed predominantly in the high-affinity configuration, in intact cells and membranes, as well as in soluble preparations. In contrast to the studies conducted at 37 °C, no Gpp(NH)p-induced conversion to the lower affinity state could be demonstrated in studies performed with membrane preparations at 4 °C. Thus, the high-affinity state of α_1- and β-adrenergic receptors is stabilized at 4 °C in intact cells, membranes, and soluble preparations. After incubations had been performed at 37 °C, high-affinity binding of agonists could not be restored by subsequent incubation at 4 °C. As previously reported for the β-adrenergic receptor [Weiland, G. A., Minnemann, K. P., & Molinoff, P. B. (1979) *Nature (London) 281*, 114–117], the magnitude of the temperature shift in agonist affinity correlated with the functional efficacy of adrenergic agents at α_1-adrenergic receptors (agonist > partial agonist > antagonist) and thus seems to reflect an agonist-induced conformational change at the receptor binding site, per se.

The molecular basis of the interaction of adrenergic agonists with their receptors is incompletely understood. As the affinity of the receptors for agonists may be a functional regulator of catecholamine-induced responses, the binding characteristics of agonists have been the focus of attention in previous radioligand binding studies. The binding of agonists to β-adrenergic receptors has been most extensively studied. In membrane preparations, agonist competition curves are complex; it has been proposed that this reflects the presence of interconvertible affinity "states" of the receptor for agonists (DeLean et al., 1980). Receptor affinity for agonists is selectively influenced by several factors, such as guanine nucleotides and temperature, whereas the affinity for antagonists is not. While the effects of guanine nucleotides have been interpreted as being due to an "uncoupling" of receptors from the guanine nucleotide binding regulatory proteins (DeLean et al., 1980), the marked temperature dependency of agonist affinity was interpreted as reflecting the different thermodynamic behavior of the agonist–receptor as opposed to the antagonist–receptor interaction (Weiland et al., 1979, 1980). It is not clear, however, how the presence of different affinity states of the receptor relates to thermodynamic analysis of agonist–receptor interactions. As shown by Weiland et al. (1980) and in this paper, the proportions of high- and low-affinity sites are temperature sensitive.

We have previously demonstrated that binding of agonists to α_1- and β-adrenergic receptors is complex and subject to regulation by guanine nucleotides at physiological incubation temperatures (Schwarz et al., 1985b). Moreover, we demonstrated that agonists bind initially to α_1-adrenergic receptors with high affinity in both intact cells (Schwarz et al., 1985a) and broken cell preparations (Schwarz et al., 1986). In the present study, the affinity of hepatic α_1- and β_2-adrenergic receptors for agonists and its regulation by the nonhydrolyzable GTP analogue Gpp(NH)p and temperature were examined in more detail in three different experimental models of decreasing complexity: (a) intact rat heptocytes; (b) rat hepatocyte plasma membranes; and (c) detergent-solubilized receptor preparations.

The rat hepatocyte was employed in these studies, as in this tissue both α_1- and β-adrenergic receptors are involved in the activation of glycogenolysis, with the α_1 response prevailing (Schwarz et al., 1985b). While both receptor subtypes react with the same agonists (epinephrine and norepinephrine) and lead to the same functional effect (glycogenolysis), their signal transduction systems within the plasma membrane are different. α_1-Adrenergic receptors are likely linked to an as yet unidentified phosphodiesterase, which generates diacylglycerol and 1,4,5-*myo*-inositol, the second messenger molecule involved in intracellular Ca^{2+} mobilization (Homcy & Graham, 1985). β-Adrenergic receptors, in contrast, are linked to adenylate cyclase via the stimulatory GTP protein (Homcy & Graham, 1985). It has been observed that temperature (Adams &

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Jarrott, 1985; Wikberg et al., 1983) and guanine nucleotides (Hoffman et al., 1981, 1982) affect agonist binding to the two receptor types differentially. In simultaneously investigating both α_1- and β_2-adrenergic receptors, we addressed the question of whether the difference in the coupling mechanisms linking these receptors to their common cellular response reflects a fundamental difference in the way that these two receptors behave in response to agonist binding. The findings of this study indicate that the modulation of agonist binding at both α_1- and β-adrenergic receptors by GTP analogues and temperature is qualitatively and quantitatively similar.

Materials and Methods

Isolation of Hepatocytes and Membrane Preparation. Female Sprague-Dawley rats (180–240 g) were purchased from Charles River Farm and maintained on 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).

Solubilization of α_1- and β-Adrenergic Receptors. α_1-Adrenergic receptors from liver cell membranes were solubilized with a minor modification of our previously published method (Schwarz et al., 1986). Membranes (3 mg/mL) were stirred with 10 mM phosphate buffer/150 mM NaCl (pH 7.4) containing 0.5% digitonin (Sigma) at 25 °C for 45 min. For β-adrenergic receptors, membranes (10 mg/mL) were stirred for 120 min (25 °C) in 10 mM phosphate buffer/0.1 M NaCl (pH 7.4) containing 1% digitonin. The membranes were subsequently centrifuged for 30 min at 100000*g*; 400-μL aliquots of the supernatant were used for radioligand studies. For binding studies with [^{125}I]cyanopindolol ([^{125}I]CYP), the supernatant was diluted 1:10.

Radioligand Binding Assay. A typical binding assay employed 200 000 cells and [^{3}H]prazosin (0.02–4 nM) with and without phentolamine (10^{-5} M) or [^{125}I]cyanopindolol (0.01–2 nM) with and without alprenolol (10^{-5} M) in a total volume of 300 μL. For membrane studies, a protein concentration of 100–300 μg was used. Studies in intact cells and membrane preparations were both performed in 10 mM sodium phosphate buffer containing NaCl (154 mM) and $MgCl_2$ (1 mM). Competition assays involving agonists were conducted in the presence of 0.1 mM ascorbic acid. Incubations were performed for 30 min at 37 °C or for 150 min at 4 °C. As demonstrated previously (Schwarz et al., 1986), these incubation conditions permit binding of the involved ligands to reach equilibrium. When binding was performed at 4 °C, competing ligands (labeled and unlabeled) were pipetted into reagent tubes and thoroughly cooled for 15 min. Membranes or cells were equally cooled for 15 min before addition to the incubation mixture. Incubations were terminated by vacuum filtration using a Millipore apparatus onto glass fiber filters (Schleicher & Schuell type 32; Keene, NH) and rapid washing with 4 × 4 mL of ice-cold buffer. When soluble preparations were used, 400 μL of the supernatant was incubated with competing ligands and [^{3}H]prazosin (5 nM) or [^{125}I]CYP (0.2 nM) for 60 min at 37 °C or for 12 h at 4 °C. Proteins were precipitated by subsequent addition of 1-mL aliquots of ice-cold bovine γ-globulin (1 mg/mL) and polyethylene glycol (25%). Filters were placed in 10 mL of scintillation fluid (Hydrofluor) and counted in a liquid scintillation counter with an efficiency of 56% (tritium) or by direct counting in a γ spectrometer (Micromedic) at 80% efficiency (^{125}I).

Data Analysis. The nontransformed radioligand binding data (triplicate determinations) were evaluated with the mass action based, weighted, nonlinear curve-fitting procedure LIGAND (Munson & Rodbard, 1980). Statistical analysis was performed according to the "extra sum of squares" principle (Rodbard, 1974). "Goodness of fit" for several models of 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 et al. (1980).

Chemicals. [^{3}H]Prazosin and [^{125}I]cyanopindolol were purchased from New England Nuclear. Gpp(NH)p was obtained from Sigma Chemical Co. The following were gifts: Clonidine and inanidine, Boehringer Ingelheim International; ST 587 NI, Siegfried Pharmaceuticals.

Results

Binding of Radiolabeled Adrenergic Antagonists to Intact Rat Hepatocytes. We have previously demonstrated that the preparation of rat hepatocytes employed here retains the ability to respond functionally to adrenergic stimulation (Schwarz et al., 1985b). Catecholamines induce an increase in glycogenolysis by activation of phosphorylase *a*. This effect is mainly elicited via α_1-adrenoceptors, though a minor response to β-adrenergic stimulation was also present (up to 30% of that achieved with α_1-adrenergic stimulation).

As previously reported, α_1- and β-adrenergic receptors were identified by the specific antagonists [^{3}H]prazosin and [^{125}I]CYP, respectively (Schwarz et al., 1985b). In equilibrium saturation experiments, binding of [^{3}H]prazosin to intact isolated hepatocytes was of appropriate specificity, reversible, saturable (93 000 receptor sites per cell), and of high affinity (K_D = 125 ± 10 pM, n = 15). The β-adrenergic antagonist [^{125}I]CYP also bound to intact isolated hepatocytes in a saturable and reversible manner with a dissociation constant of 66 ± 6 pM (n = 12). Scatchard analysis indicated interaction with a single class of binding sites ($p < 0.001$) with a capacity of 2000 receptors per cell. Both radioligands bound with virtually identical dissociation constants to liver cell membranes (data not shown), where a mean density of 114 ± 15 fmol/mg for [^{3}H]prazosin binding sites was calculated. The affinity of the adrenergic receptors for the radioligands and the rank order of potency for a series of competing adrenergic ligands were preserved in solubilized receptor preparations. Additionally, with solubilized preparations, the dissociation constants both for radiolabeled antagonists and for competing unlabeled antagonists were not altered by variations in the incubation temperature (4 vs. 37 °C) (data not shown).

Binding of Agonists to Intact Hepatocytes. When competition assays of both radiolabeled antagonists and various competing antagonists were performed in intact hepatocytes or membrane preparations, the results were always in good agreement with computer-generated predictions for interaction with a single class of binding sites according to the mass action law. In contrast, agonists exhibited complex binding behavior. For the sake of clarity, this report presents mainly data derived from competition studies using (−)-epinephrine with [^{3}H]-prazosin, and (−)-isoproterenol with [^{125}I]CYP, although similar results were obtained with other full agonists at both receptor subtypes.

Analysis of competition assays performed at 37 °C, employing (−)-epinephrine and [^{3}H]prazosin, indicated the presence of two different binding sites ($p < 0.001$) (Figure 1, panel A). (−)-Epinephrine bound to the majority of sites (73 ± 6%) with a dissociation constant of 3.8 ± 0.5 μM, and

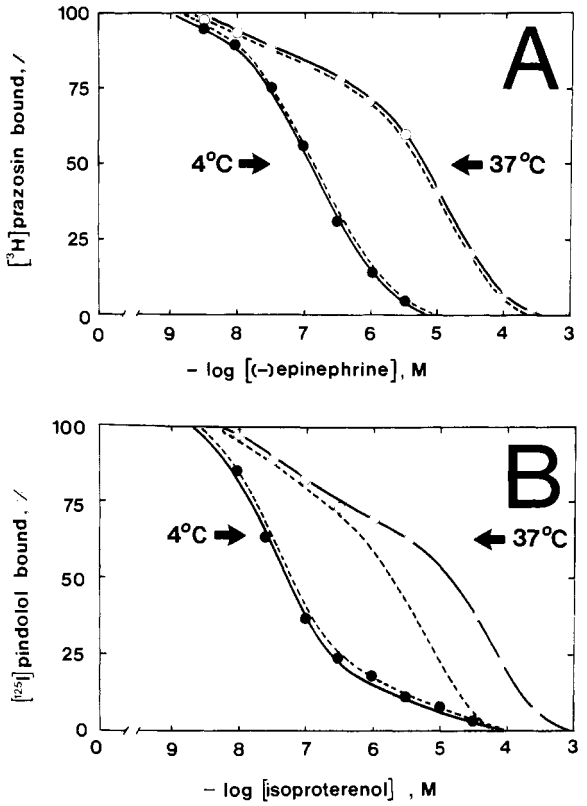

FIGURE 1: Regulation of agonist affinity for adrenergic agonists by temperature in intact hepatocytes and broken cell preparations. Isolated hepatocytes (10^5/mL) or membranes prepared from the hepatocytes were incubated with (A) [^{3}H]prazosin (0.5 nM) and increasing concentrations of (−)-epinephrine or (B) [^{125}I]cyanopindolol (0.1 nM) and increasing concentrations of (−)-isoproterenol. Incubations were carried out at 37 °C for 45 min or at 4 °C for 180 min. The broken lines represent the curves obtained in broken cell preparations. Results shown are the means of triplicate determinations for each point and are representative of at least four independent experiments for each radioligand.

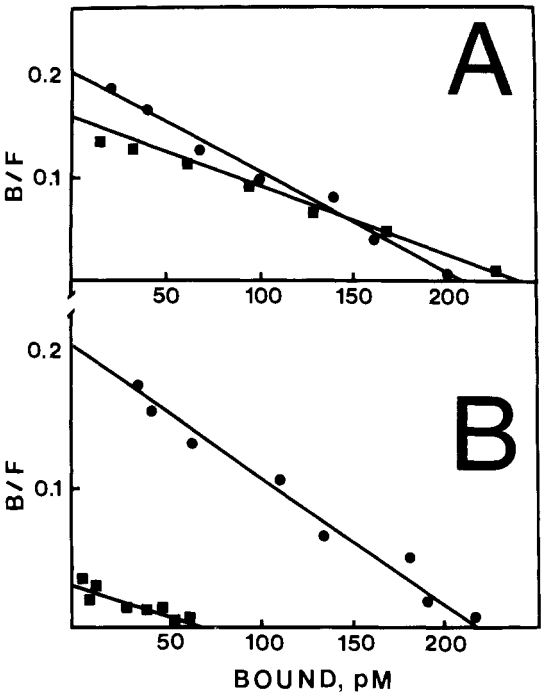

FIGURE 2: Trapping of agonist at α_1-adrenergic receptors by induction of the high-affinity state. Isolated hepatocytes (10^6/mL) were initially incubated with buffer (●) or (−)-epinephrine (10^{-6} M) (■) for 30 min at 37 °C (A) or for 180 min at 4 °C (B). Incubations were terminated by adding a large excess of ice-cold buffer. Subsequently, cells were washed 2 times with ice-cold buffer. Equilibrium saturation binding studies were then carried out at 4 °C (180 min) with increasing doses of [^{3}H]prazosin, in the presence or absence of phentolamine (10^{-5} M). Shown are the "Scatchard plots" derived from the equilibrium binding data, which were determined by computer analysis using LIGAND, as detailed under Materials and Methods. Results shown are the means of triplicate determinations for each point and are representative of at least three independent experiments for each temperature.

to a minor fraction (24.4 ± 3%) with a higher affinity (K_D = 20 ± 4 nM). The β-adrenergic receptor exhibited similar agonist binding characteristics (Figure 1, panel B). At the binding sites labeled with [^{125}I]CYP, the β-adrenergic agonist (−)-isoproterenol also bound to two different sites with affinities of 23 ± 4 nM (21 ± 2%) and 57 ± 2 μM (79 ± 5%).

When incubations with [^{3}H]prazosin and (−)-epinephrine were conducted at 4 °C, the competing agonist was almost 100-fold more potent in competing for binding with the radioligand (Figure 1, panel A). The dissociation constant determined in these studies (22 ± 3 nM) is in good agreement with that obtained for the high-affinity sites at 37 °C, where they constituted only a minority of sites. Goodness of fit was best for a model involving a single binding site for (−)-epinephrine. The affinity of β-adrenergic receptors for isoproterenol was also enhanced by performing incubations at 4 °C. Competition curves were best fit by a two-site model, with 85 ± 7% of the binding sites being in the high-affinity state (K_D = 17 ± 3 nM).

We next investigated whether the decrease in agonist affinity resulting from incubation at 37 °C can be reversed by subsequent cooling to 4 °C. In these studies, two sets of cells were initially exposed to (−)-epinephrine (10^{-6} M) or buffer at 37 and 4 °C. Subsequently, both batches were washed repeatedly with ice-cold buffer and used in equilibrium saturation binding studies (150 min at 4 °C). As shown in Figure 2, when the initial incubation was performed at 4 °C, the number of binding sites identified with [^{3}H]prazosin in the (−)-epinephrine-treated cells was markedly reduced, as compared to that in the buffer-treated controls. However, the affinity of the receptors for the radioligand was unaltered. In contrast, a full complement of binding sites could be identified in cells initially incubated at 37 °C in the presence of (−)-epinephrine.

At β-adrenergic receptors, the ability to modulate the affinity for a ligand with changes in temperature has been shown to correlate with the functional efficacy of the ligand (Weiland et al., 1979). Thus, marked temperature-induced changes in receptor affinity are observed with full agonists, an intermediate response with partial agonists, and no changes with antagonists. To investigate if a similar correlation exists for α_1-adrenergic receptors, the temperature sensitivity of agonist affinity was evaluated for a series of partial agonists. Clonidine is a partial agonist at α_1-adrenergic receptors in liver and induces 30–50% of the increase in glycogen phosphorylase *a* that can be achieved with full agonists. For this ligand, receptor affinity increases by a factor of 10 when incubations are conducted at low temperatures (Figure 3), as compared to an approximately 100-fold increase obtained with the full agonists (−)-epinephrine and (−)-norepinephrine. Similarly, the partial agonists ST 587 NI and inanidine showed smaller temperature-induced shifts than those obtained with full agonists (Table I). The specificity of this response for α-adrenergic ligands is demonstrated by the fact that with the β-adrenergic agonist (−)-isoproterenol, no temperature-induced shift in affinity was observed at the sites identified with [^{3}H]prazosin (Figure 3). By contrast, a 100-fold increase in affinity was observed with (−)-isoproterenol in the same

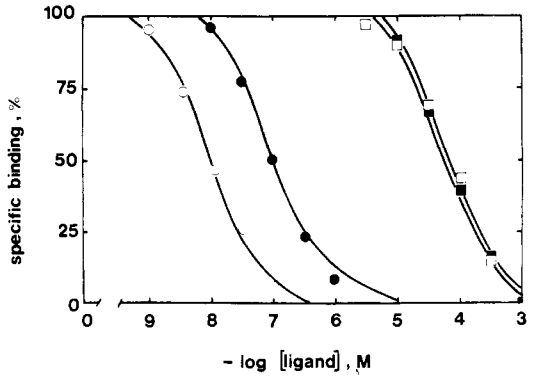

FIGURE 3: Temperature sensitivity of the binding of agonists to α_1-adrenergic receptors of intact hepatocytes. Isolated hepatocytes (10^5/mL) were incubated with [^{3}H]prazosin (0.5 nM) and increasing concentrations of clonidine (○, ●) or (−)-isoproterenol (□, ■). Incubations were carried out at 37 °C for 45 min (●) or at 4 °C for 180 min (○). Values shown are the means of triplicate determinations, and the results are representative of two to four independent experiments for each agonist.

Table I: Temperature Sensitivity of the Affinity of α_1-Adrenergic Receptors for a Variety of Adrenergic Ligands, As Indicated by the Ratio of the Dissociation Constants (K_D) Determined at 37 and 4 °C[a]

ligand	K_D(37 °C)/K_D(4 °C)	ligand	K_D(37 °C)/K_D(4 °C)
(−)-epinephrine[b]	93 ± 6	inanidine	9 ± 4
(−)-norephinephrine[b]	90 ± 8	(−)-isoproterenol	2 ± 0.5
clonidine	11 ± 7	prazosin	1 ± 1
ST 587 NI	13 ± 3	phentolamine	3 ± 2

[a] Isolated hepatocytes (10^5 mL) were incubated with [^{3}H]prazocin (0.5 nM) and increasing concentrations of competing ligands at 37 °C (45 min) or 4 °C (180 min). The dissociation constants at both temperatures were calculated by LIGAND (Munson & Rodbard, 1980). Results shown are the means ± SE for two to six studies at each temperature for each ligand. [b] For these full agonists, which gave shallow competition curves with 37 °C incubations that could best be modeled to two components of high and low affinity, the K_D values to obtain the ratios shown were the dissociation constants calculated for the low-affinity sites.

preparation at the sites labeled with [^{125}I]CYP (Figure 1B).

Binding of Agonists to Rat Hepatocyte Membrane Preparations. In agreement with our previous studies, competition studies with the agonist (−)-epinephrine at α_1-adrenergic receptors conducted at physiological temperatures revealed two-component binding (Figure 4A), indicating interaction of (−)-epinephrine with high-affinity sites (K_D = 24 ± 5 nM, 24%) and low-affinity sites (K_D = 2.5 ± 3 μM, 76%). As shown in Figure 1, the resulting curves from experiments performed with both intact cells and broken cell preparations yielded identical results. In the presence of the nonhydrolyzable GTP analogue Gpp(NH)p (0.1 mM), the slope of the competition curve approached unity and binding occurred exclusively to a single class of low-affinity sites.

As in intact cell preparations, the affinity of (−)-epinephrine was almost 100-fold higher at low incubation temperatures, and the results were best fit by a single-component model (Figure 4A). The dissociation constant at this temperature was again virtually identical with that obtained at the high-affinity sites demonstrated at higher temperatures. In contrast to the results obtained at 37 °C, Gpp(NH)p did not alter the affinity for agonists.

At β-adrenergic receptors, (−)-isoproterenol interacted with two distinct binding sites ($p < 0.001$, Figure 4B) with dissociation constants of 2 μM (55–85%) and 6 nM (15–45%) (37

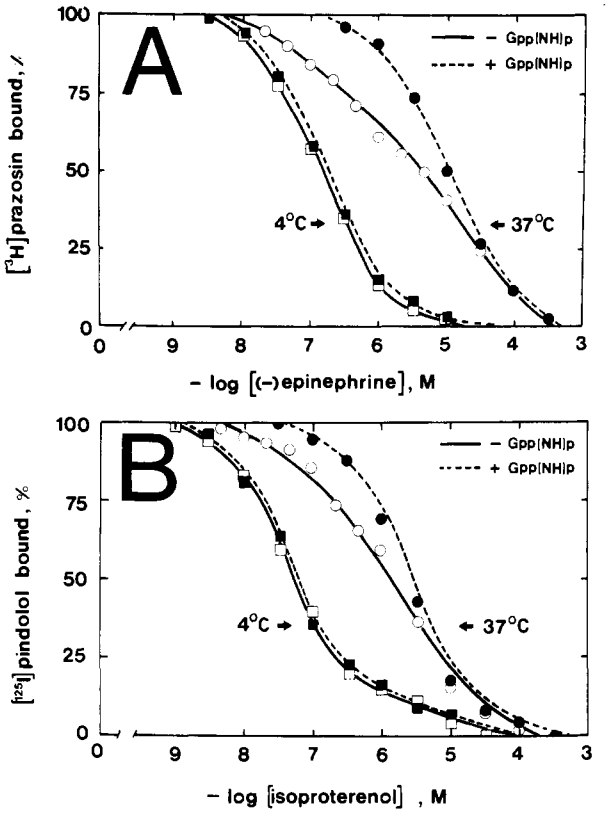

FIGURE 4: Effects of temperature and Gpp(NH)p on binding of agonists to α_1- and β_2-adrenergic receptors in hepatic membranes. Hepatic membranes were incubated with (A) [^{3}H]prazosin (0.5 nM) and increasing concentrations of (−)-epinephrine or (B) [^{125}I]-cyanopindolol (0.1 nM) and increasing concentrations of (−)-isoproterenol. Incubations were carried out at 37 °C for 45 min or at 4 °C for 180 min in the presence (broken lines) or absence (solid lines) of Gpp(NH)p (10^{-4} M). Values shown are the means of triplicate determinations, and the results are representative of at least four independent studies for each perturbation.

°C, 60 min). The low-affinity site observed in these studies was, nevertheless, of higher affinity than that determined in intact cells (K_D = 2 vs. 67 μM). This finding contrasts with the results obtained at α_1-adrenergic receptors, where virtually identical affinities were observed in intact cells and membrane preparations. When incubations were performed in the presence of Gpp(NH)p (0.1 mM), goodness of fit was best for interaction of (−)-isoproterenol with a single class of low-affinity sites (K_D = 2 μM). As with intact cells, binding of (−)-isoproterenol displayed a marked temperature dependency. At 4 °C, the affinity of (−)-isoproterenol was approximately 100-fold higher than at 37 °C.

Temperature-Dependent Effects of Gpp(NH)p on Agonist Affinity in Hepatocyte Membranes. When competition experiments were performed with hepatocyte membranes at 25 °C, as compared to 37 °C, competition curves were shallower, and statistical analysis indicated that the percentage of high-affinity sites was increased (39% vs. 24%, $p < 0.01$). However, the affinity at both low- and high-affinity sites was not significantly altered. Such an experiment using [^{125}I]CYP and (−)-isoproterenol is depicted in Figure 5. The increase of high-affinity sites at 25 °C was observed at both α_1- and β-adrenergic receptors. In the presence of Gpp(NH)p, a complete conversion of receptors to the low-affinity configuration could be demonstrated. Thus, the Gpp(NH)p-induced shift is greater at the lower temperature. However, upon further reduction of the incubation temperature to 4 °C and incubation for 150 min, Gpp(NH)p could no longer alter agonist binding (Figure 5C).

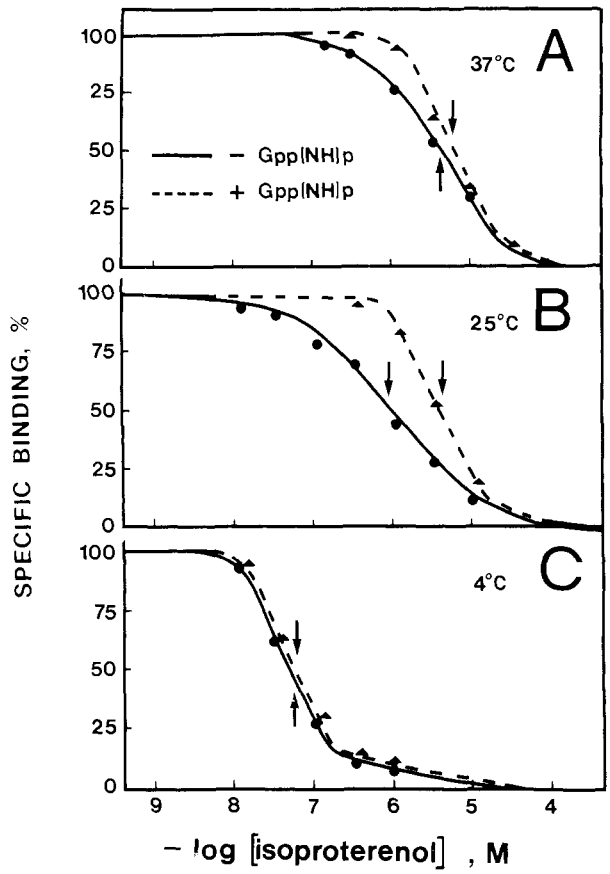

FIGURE 5: Effect of temperature on agonist binding and modulation of agonist binding by Gpp(NH)p. Hepatic membranes were incubated with [^{125}I]cyanopindolol (0.1 nM) and increasing concentrations of (−)-isoproterenol in the presence (broken lines) and absence (solid lines) of Gpp(NH)p (10^{-4} M) at 37 °C for 45 min (A), at 25 °C for 45 min (B), and at 4 °C for 180 min (C). Values shown are the means of triplicate determinations, and the results are representative of at least four studies at each temperature.

Binding of Agonists to Solubilized Receptor Preparations. No high-affinity sites for agonists were observed in experiments performed at 37 °C with solubilized receptor preparations (Figure 6). The affinity of agonists was in good agreement with that calculated for the low-affinity sites in membranes and not affected by the presence of Gpp(NH)p. At 4 °C, the affinity of agonists at both solubilized α_1- and β-receptors was again markedly higher and close to the values obtained for the high-affinity sites in membrane preparations.

Discussion

α_1- and β_2-Adrenergic Receptors Exist in Two Configurations in Intact Cells. The molecular basis and functional significance of the complex binding behavior of agonists are not fully understood. At β-adrenergic receptors, it has been proposed that high-affinity sites for agonists may account for the actions of catecholamines (DeLean et al., 1980). We have previously presented evidence for the functional role of the high-affinity binding component of the α_1-adrenergic receptor population as well (Corvera et al., 1986). According to the ternary complex model proposed by DeLean et al. (1980), high-affinity binding sites for adrenergic agonists represent an agonist-induced ternary complex of hormone, receptor, and a regulatory GTP binding protein (G protein). Binding of GTP would then lead to an uncoupling of the receptor from the G protein, leaving the receptor in a low-affinity conformation. As endogenous GTP is presumably present in sufficient quantities in intact cells to prevent the accumulation

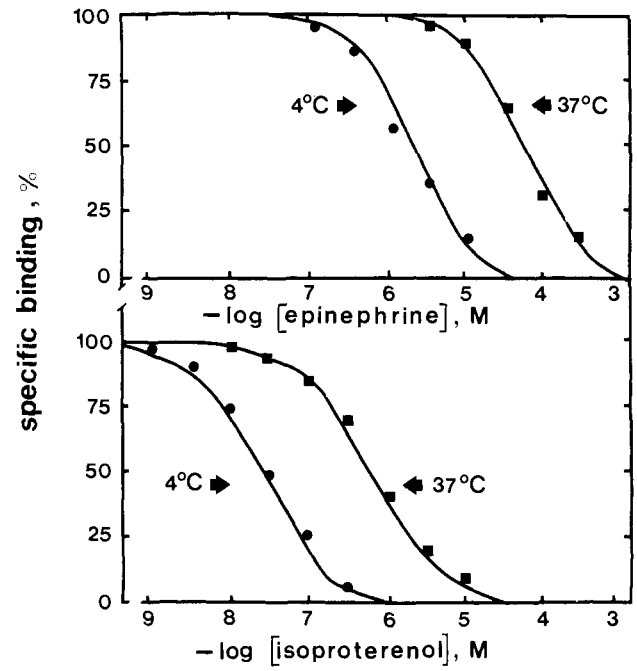

FIGURE 6: Temperature sensitivity of agonist binding to α_1- and β_2-adrenergic receptors in solubilized preparations. Solubilized hepatic membranes were incubated with [^{3}H]prazosin (5 nM) and increasing concentrations of (−)-epinephrine (upper panel) or with [^{125}I]-cyanopindolol (0.2 nM) and increasing concentrations of (−)-isoproterenol (lower panel). Incubations were carried out at 37 °C for 45 min or at 4 °C for 180 min. Values are the means of triplicate determinations, and results are representative of four independent experiments for each radioligand.

of high-affinity sites, only low- and no high-affinity sites should be detectable in this system.

In contrast to these considerations, we observed a fraction of both α_1- and β-adrenergic binding sites which bound agonists with high affinity in intact cells. The interpretation of these results, that is, the presence of high-affinity sites in intact cells, remains to be clarified. It is possible that availability of GTP may be limiting in this particular cellular model. Alternatively, it may be reasoned that the high-affinity configuration prevails in the resting state of the receptor and that binding of agonist leads to an isomerization of the receptor binding site to a state of lower affinity. Experimental support for the latter mechanism has been presented by our group (Schwarz et al., 1985a, 1986) and by others (Toews et al., 1983; Hoyer et al., 1984).

Differences in Agonist Binding to α_1- and β_2-Adrenergic Receptors between Membrane Preparations and Intact Cells. In our studies, differences in agonist binding in intact cells as compared to membrane preparations were evident only at β-adrenergic receptors. The affinity for agonists at the low-affinity sites was 30-fold lower in intact cells than in membrane preparations, which is in agreement with the findings of other investigators (Insel & Sanda, 1979; Pitman & Molinoff, 1980). Previous studies have suggested that the lower affinity of agonists in intact cells may be due to an agonist-induced sequestration of receptors away from the cell surface to a different cellular compartment [for a review, see Harden (1983)]. In contrast, no difference in agonist binding to intact cells and membrane preparations was observed at α_1-adrenergic receptors. The dissociation constants for agonists obtained in membrane preparations and intact cells were virtually identical. We have previously suggested that this may be related to the finding that no functional desensitization of α_1-adrenergic receptors is demonstrable in hepatocytes (Morgan et al., 1983).

Effects of Guanine Nucleotides on Agonist Binding in Membrane Preparations. In membrane preparations, Gpp(NH)p causes a rightward shift of agonist competition curves at both α_1- and β_2-adrenergic receptors. While this was expected for β-receptors, contradictory results have been presented with respect to the modulation of agonist binding by guanine nucleotides at α_1-adrenergic receptors (Schwarz et al., 1985b; Hoffman et al., 1982; Lowry et al., 1951; Goodhardt et al., 1982; Lynch et al., 1985). Our observation that Gpp(NH)p converts agonist binding sites at α_1-adrenergic receptors to a state of lower affinity suggests that, in analogy to the β-adrenergic system, a ternary complex of agonist, receptor, and an as yet unidentified G protein may be involved in the signal transduction pathway of this adrenergic receptor subtype.

Temperature Dependency of both Agonist Binding and the Gpp(NH)p-Induced Conversion to a State of Lower Affinity. Agonists at both α_1- and β-adrenergic receptors display a markedly higher affinity at lower temperatures in intact hepatocytes, liver cell membranes, and solubilized membrane preparations. In contrast, antagonist binding was not affected by temperature. The temperature-induced shift in agonist affinity thus seems to be an unifying feature of all adrenergic receptor subtypes and has been attributed to the fundamental difference in the way agonists react with receptors, as compared to antagonists. This concept is further supported by our findings with partial agonists. At α_1-adrenergic receptors, the temperature-induced shift in affinity for partial agonists is less than that for full agonists, and no shift is observed with antagonists. Also, the affinity for the β-adrenergic agonist (−)-isoproterenol is not affected by temperature at [^{3}H]prazosin binding sites, whereas a 100-fold shift is observed with this agonist in competition studies with [^{125}I]CYP. Thus, for an agonist in a given system, the functional efficacy of the ligand appears to be predictive of the degree of temperature-induced shift in affinity.

When studies at β-adrenergic receptors were performed at 25 °C as compared to 37 °C in the absence of Gpp(NH)p, agonist competition curves were shallower due to a greater proportion of receptors being in the high-affinity state, a finding that is in agreement with the observations of Weiland et al. (1980). However, in the presence of Gpp(NH)p, the competition curves obtained from studies at both incubation temperatures were uniphasic and the IC_{50} values for the competing agonist not significantly different. Thus, the degree of rightward shift in the agonist competition curve produced by Gpp(NH)p is quantitatively greater at 25 °C than at 37 °C, because the proportion of high-affinity sites available for conversion to the low-affinity state is greater at the lower temperature. Nevertheless, there appears to be a threshold temperature below which guanine nucleotides are no longer able to modulate the affinity state for agonists. This is evidenced by the finding that with 4 °C incubations, agonist affinity at both β- and α_1-adrenergic receptors was not altered by the presence of Gpp(NH)p. Similar findings with low incubation temperatures have been reported at dopamine receptors by Watanabe et al. (1985) and at α_1-adrenergic receptors by Lynch et al. (1985).

The predominant affinity state for agonists at 4 °C at both α_1- and β-adrenergic receptors is virtually identical with the dissociation constants calculated for the high-affinity sites determined at 37 °C, even though at this latter temperature the high-affinity sites constitute only a small fraction of the total population. While it is difficult to prove that the high-affinity configuration observed at 4 °C represents the same binding site configuration as that induced physiologically by the interaction of receptor and G protein, it seems unlikely that both perturbations should produce almost identical receptor recognition properties with the same excellent fit for agonists.

We previously demonstrated, using competition studies, that although the agonist-driven decrease in receptor affinity that occurs at 37 °C is sustained as long as agonist is present, it can be reversed and agonist affinity again locked in the high-affinity state by subsequent removal of agonist and incubation at 4 °C (Schwarz et al., 1986). In the present investigation, the ability of low incubation temperatures to lock α_1-adrenergic receptors in the high-affinity state for agonists was further evaluated in equilibrium binding studies with intact hepatocytes. As shown in Figure 2, if the cells were exposed to (−)-epinephrine at 4 °C and the low temperature then maintained during washing to remove unbound ligand, the number of binding sites subsequently identified with [^{3}H]-prazosin was markedly reduced. This reduction in the number of binding sites is most likely due to retention of agonist promoted by the induction of the high-affinity state with the 4 °C incubation, since the affinity for the radioligand [^{3}H]-prazosin was unaltered. Moreover, a full complement of binding sites could be identified in parallel studies in which cells were exposed to agonist at 37 °C and then cooled and washed at 4 °C.

A similar phenomenon of "trapping" of an agonist by induction of the high-affinity configuration with deoxycholate has also been reported for β-adrenergic receptors (Neufeld et al., 1983). We speculate, therefore, that the initial high-affinity binding of agonists is sustained at 4 °C as an agonist-induced conformational change leading to a lower affinity for agonists is inhibited. This interpretation is also supported by initial velocity studies, which demonstrated that the affinity of both α_1- and β-adrenergic receptors is initially high (Schwarz et al., 1985a, 1986; Toews et al., 1983; Hoyer et al., 1984). However, the exact mechanism whereby the receptors are locked in the high-affinity configuration at 4 °C remains to be determined.

In previous studies, the temperature dependency of agonist binding to several receptor systems has been analyzed in thermodynamic terms (Weiland et al., 1979, 1980; Zahniser & Molinoff, 1983; Speth et al., 1979; Lohse et al., 1986; Moehler & Richards, 1981). It was observed that the temperature dependency of agonist binding can be explained by a decrease in enthalpy, whereas the binding of antagonists appeared to be largely entropy driven. In related studies of the temperature dependence of agonist—but not antagonist—affinity, Speth et al. (1979) and Lohse et al. (1986) observed break points in the van't Hoff plots of agonist binding to benzodiazepine and α_2-adrenergic receptors, respectively. As the break points coincided with the phase transition of the plasma membrane, it was suggested that the physical state of the membrane may differentially regulate the affinity for agonists as opposed to antagonists. Such a mechanism, however, cannot explain the temperature sensitivity of agonist affinity that we observed in solubilized preparations. We speculate, therefore, that a model whereby agonists produce a temperature-dependent change in the conformation of the receptor protein itself, as suggested for the benzodiazepine receptor (Moehler & Richards, 1981), is most compatible with the available data. Such a mechanism also provides an explanation for our previous finding of high-affinity agonist binding when determined by initial velocity studies performed at 37 °C (Schwarz et al., 1986) and

of the irreversibility of the decrease in agonist affinity, providing that agonist is present before cooling, a finding that has been similarly observed by Insel and Sanda (1979) at β-adrenergic receptors.

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CORRECTIONS

Outside–Inside Translocation of Aminophospholipids in the Human Erythrocyte Membrane Is Mediated by a Specific Enzyme, by Alain Zachowski,* Edith Favre, Sophie Cribier, Paulette Hervé, and Philippe F. Devaux, Volume 25, Number 9, May 6, 1986, pages 2585–2590.

Page 2585. On line 11 of the Abstract, a ratio of $\simeq 1/34$ should read a ratio of $\simeq 1/9.4$.

Page 2589. In column 1, lines 43–53 should be replaced with the following: The apparent K_m of the spin-labeled PE is given by the expression

$$K_{m,app}^{(0,2)PE} = K_m^{(0,2)PE}(1 + [PE]/K_m^{PE} + [PS]/K_m^{PS})$$

where [PE] and [PS] are the concentrations of endogenous PE and PS on the outer layer of the erythrocytes and K_m^{PE} and K_m^{PS} are the K_m values of the endogenous lipids for the translocase. A similar expression is obtained for $K_{m,app}^{(0,2)PS}$. Finally, the relative affinity of the spin-labeled aminophospholipids for the translocase is equal to the ratio of the apparent K_m, so that

$$K_m^{(0,2)PS}/K_m^{(0,2)PE} \simeq 1/9.4$$

Raman and Infrared Spectra of Cytochrome *c* Peroxidase–Carbon Monoxide Adducts in Alternative Conformational States, by Giulietta Smulevich, Ruby Evangelista-Kirkup, Ann English, and Thomas G. Spiro*, Volume 25, Number 15, July 29, 1986, pages 4426–4430.

Page 4427. In Figure 2, the spectrum labels a and c should be interchanged.