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Effects of Temperature and Membrane Phase Transitions on Ligand Binding to α_2-Receptors of Human Platelets

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SUMMARY

The binding of agonists and antagonists to α_2-adrenergic receptors of human platelets was studied. The receptors showed homogeneous affinities for antagonists but two affinity states for the agonist (−)-epinephrine, which were modulated by guanine nucleotides. Van't Hoff plots of antagonist binding had a break point at about 18° and considerable diversity between 18° and 0°. Agonist binding to both affinity states showed a similar break point; agonist binding to the high affinity state was characterized by a large entropy component compared to the low affinity state. This entropy component was reduced at higher concentrations of sodium, indicating that it may be due to liberation of sodium ions. Measurements of the fluorescence of 1-anilin-8-naphthalenesulfonate showed thermotropic phase transitions of the platelet membranes at about 17°. The transition temperature was decreased to about 12° by addition of 10 mM octanoic acid. Octanoic acid also shifted the break points of the van't Hoff plot of antagonist and low affinity agonist binding from 18° to 12°. High affinity agonist binding, however, remained unchanged. It is concluded that agonist-specific thermodynamic characteristics of ligand binding to α_2-receptors of human platelets can only be investigated by regarding differences between high and low affinity agonist binding. These differences include an entropy increase upon ligand binding, which is in part due to enhanced liberation of sodium ions, and a loss of sensitivity to fluidity changes in the outer layer of the plasma membrane.

The binding of various ligands to their receptors has been shown to be a temperature-dependent process. Although this dependence has been observed in many of the earlier studies, the thermodynamic parameters of this process have only recently been used to characterize the interaction of different ligands with a given receptor. With this approach Weiland *et al.* (1) investigated agonist and antagonist binding to the β-adrenergic receptor in order to study the question of why agonist occupation of a receptor leads to a response and antagonist occupation does not. They described fundamental differences between agonist and antagonist binding, the agonist binding being enthalpy driven and the antagonist binding, entropy driven. Similar differences have been observed in other receptor systems (2, 3).

However, these analyses do not take into account the existence of different states of agonist affinity which have been described for most receptors coupled to adenylate cyclase. These differences seem to be induced by coupling of the receptor to the guanine nucleotide-binding proteins (stimulatory or inhibitory), which leads to a considerable increase of the affinity for agonists (4). The two affinity states can be discriminated by use of curve fitting to competition or saturation curves (5).

An analysis of the temperature dependence of agonist binding to the two affinity states of the A_1 (R_i) adenosine receptor showed very marked thermodynamic differences between the binding to the two affinity states (6). Agonist binding to the low affinity state was enthalpy driven in a manner similar to that of antagonist binding, whereas agonist binding to the high affinity state was entropy driven and thus clearly different from the former. A possible explanation is that the thermodynamic differences are due to the interaction of the receptor with N_i and that they do not depend only on the ligand-receptor interaction.

The present study was undertaken to see whether these findings are true also for other receptors coupled to adenylate cyclase. The model chosen was the α_2-adrenergic receptor of human platelets. Human platelets appear to have adrenergic receptors of the α_2-subtype which mediate an inhibition of adenylate cyclase and induce aggregation. The receptors have been identified in binding studies with various radioligands of which [^{3}H]yohimbine appears to be the most useful (7). As has been reported for a variety of adenylate cyclase-coupled receptors, the α_2-receptor of human platelets seems to exist in two affinity states for agonists, the high affinity state probably representing a complex of receptor and N_i protein (8). The high affinity state is promoted by Mg^{2+} and other divalent cations and the low affinity state by guanine nucleotides (8). The aim of the present study was to describe the temperature depend-

ABBREVIATIONS: N_i, the inhibitory guanine nucleotide-binding protein; GppNHp, 5′-guanylylimidodiphosphate; ANS, 1-anilino-8-naphthalenesulfonate; EDTA, ethylenediaminetetraacetic acid.

ence of the binding of antagonists and of agonists to the two affinity states, in order to elucidate the mechanisms involved in receptor activation, and the role of the physical state of the plasma membrane in this process.

Experimental Procedures

Materials

[^{3}H]Yohimbine (specific activity 85.5 Ci/mmol) was obtained from New England Nuclear GmbH (Dreieich, Federal Republic of Germany). Phentolamine methane sulfonate and tolazoline hydrochloride were kindly provided by CIBA-Geigy (Wehr, Federal Republic of Germany). Rauwolscine hydrochloride was from Roth AG (Karlsruhe, Federal Republic of Germany); yohimbine hydrochloride and epinephrine hydrochloride were from Sigma GmbH (München, Federal Republic of Germany). GppNHp was purchased from Boehringer Mannheim (Mannheim, Federal Republic of Germany) and ANS was purchased from Serva (Heidelberg, Federal Republic of Germany). All other chemicals were from standard commercial sources.

Methods

Preparation of human platelet membranes. Fresh platelet-rich plasma was obtained from a local blood bank. Residual erythrocytes and leukocytes were removed by two centrifugation steps at 400 × g for 10 min. Membranes were prepared in a manner analogous to that described by Hoffman *et al.* (8). The platelets were washed three times in 150 mM NaCl, 50 mM Tris-HCl, 20 mM EDTA, pH 7.4, with centrifugations at 16,000 × g for 15 min at room temperature. The final pellets were homogenized in 5 mM Tris-HCl, 5 mM EDTA, pH 7.5, at 4° with 30 strokes of a glass-Teflon homogenizer, frozen in liquid nitrogen, and stored at −80°.

Before their use in the binding assay, the membranes were thawed, homogenized as above, and washed three times with the same hypotonic buffer with centrifugations at 39,000 × g for 15 min at 4°. The pellet was finally resuspended in 50 mM Tris-HCl, 10 mM $MgCl_2$ at pH 7.5. Protein was measured according to the method of Lowry *et al.* (9).

Binding assay. Binding of [^{3}H]yohimbine (in general, 2 nM) was done in a total volume of 250 μl. Tris-HCl (50 mM), 10 mM $MgCl_2$, pH 7.5, was used as a buffer system. The incubation was started by addition of approximately 100 μg of membrane protein and lasted from 20 min at 37° to 240 min at 0°. Steady state was assured under all conditions by using these incubation times. The reactions were terminated by rapid filtration through Whatman GF/B glass-fiber filters followed by two washes with 4 ml of ice-cold incubation buffer. Nonspecific binding was measured in the presence of 10 μM phentolamine.

ANS fluorescence measurement. The fluorescence of ANS in the presence of platelet membranes (400 μg/ml) was measured essentially as described by Träuble (10). It was recorded at varying temperatures spectrofluorometrically with an excitation and emission wave length (width) of 380 nm (±5) and 480 nm (±10).

Data Analysis

Steady state binding data were analyzed by a nonlinear regression modeling method providing estimates of K_D or K_i and B_{max} values (5). If states of high and low affinity are observed, K_H and K_L denote the respective K_D or K_i values, and R_H and R_L represent the percentage of receptors in the high or low affinity state. Improvement of the fit assuming two states was considered significant if p values of less than 0.001 were obtained in an F test. Kinetic data were fitted by nonlinear regression to the equations described (6).

Thermodynamic parameters were calculated from van't Hoff plots as described by Weiland *et al.* (1) using the following equations: (a) $\Delta G^\circ = -R \cdot T \cdot \ln K_A$, (b) $\Delta H^\circ = -a \cdot R$, and (c) $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$, where ΔG° (kcal/mol) is the Gibbs free energy change, ΔH° (kcal/mol), the enthalpy change, and ΔS° (cal/mol·deg), the entropy change; a is the slope of the van't Hoff plot and R is the gas constant (1.99 cal/mol·deg). Linear regressions with one or more components were fitted to van't Hoff plots and fluorescence measurement plots as described by Livingstone and Schachter (11). This allows the determination of best fits and break points.

Results

The binding of [^{3}H]yohimbine to human platelet membranes was saturable with a single component at all temperatures (Table 1). No changes of the number of receptor sites were found at different temperatures, but the affinity of the receptors for [^{3}H]yohimbine varied as a function of the temperature. The K_D values were clearly higher at the extremes of the temperature scale. GppNHp (100 μM) had no effect on either B_{max} or K_D values at any of the temperatures studied (data not shown), which is in agreement with reports from other groups (7, 8). When Mg^{2+} was omitted from the incubation buffer, the marked influence of temperature on the K_D values was almost abolished. The K_D values ranged from 1.5 nM at 0° to 1.1 nM at 18°. In addition, at higher temperatures, binding in the absence of Mg^{2+} began to decline relatively early (20 min at 37°) and rapidly (20%/hr at 37°); this probably indicates thermal inactivation of the receptor. For these reasons, Mg^{2+} was present in all other experiments.

The K_D values for [^{3}H]yohimbine were used to evaluate competition of various α_2-receptor antagonists for [^{3}H]yohimbine binding. Fig. 1 shows the competition curves of phentolamine at various temperatures. It demonstrates the higher affinity of this compound at lower temperatures. In each case the competition curves were monophasic, again confirming the notion that the α_2-receptor does not show heterogeneity for antagonists. GppNHp (100 μM) had no effect on the competition curve of phentolamine or any other antagonist. Consistently, a plateau was achieved at concentrations of phentolamine above 0.5 μM. Thus, 10 μM phentolamine could be used at all temperatures to define the nonspecific binding of [^{3}H]yohimbine.

The competition data were quantitatively analyzed by constructing van't Hoff plots for various antagonists (Fig. 2). These plots show two features: for all compounds the data were best fitted by a two-component model, indicating a break point at about 18°, whereas the lines are similar in the temperature range between 37° and 18° and they show considerable divergence between 18° and 0°. These differences can be seen in the thermodynamic parameters calculated for 30° and 10° (Table 2). At higher temperatures the binding of all antagonists was largely enthalpy driven, with small or even unfavorable (tolazoline) entropy components. At lower temperatures the binding

TABLE 1

Binding parameters for saturation of [^{3}H]yohimbine binding to platelet membranes

Human platelet membranes were incubated with 0.2–20 nM [^{3}H]yohimbine as described under Experimental Procedures (Methods). K_D values (95% confidence limits) and B_{max} values (± SE) were calculated by nonlinear curve fitting to the saturation curves. Only one state of homogeneous affinity was detected. Data are from three separate experiments.

Temperature	K_D	B_{max}
°C	nM	fmol/mg protein
0	5.21 (4.26–6.38)	245.2 ± 15.2
12	2.33 (1.93–2.82)	237.4 ± 11.7
25	2.34 (1.74–3.14)	238.9 ± 30.1
37	3.95 (3.24–4.81)	219.9 ± 12.6

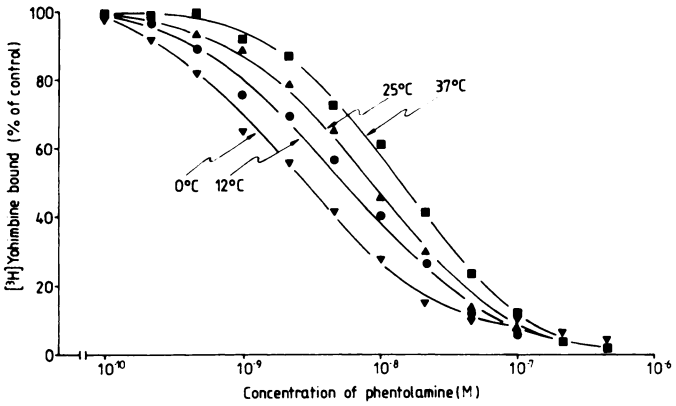

Fig. 1. Competition for [^{3}H]yohimbine binding to human platelet membranes by phentolamine. Incubations using 2 nM [^{3}H]yohimbine were carried out at varying temperatures, and K_i values were calculated by nonlinear curve fitting as described under Experimental Procedures (Methods). All curves were monophasic, and the K_i values for phentolamine at the respective temperatures were: 2.3 nM (0°), 2.7 nM (12°), 4.6 nM (25°), and 9.0 nM (37°). Values are the means of three separate experiments.

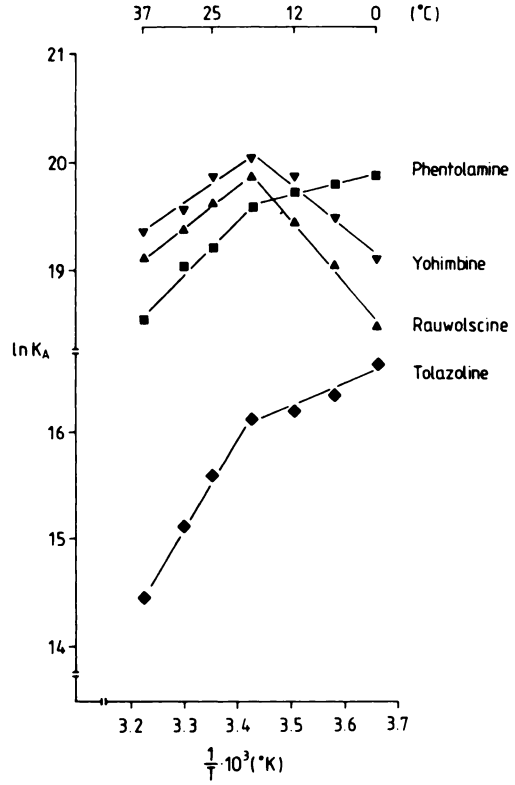

Fig. 2. van't Hoff plots for antagonist binding to α_2-receptors of human platelets. Competition experiments using 2 nM [^{3}H]yohimbine were carried out at various temperatures and analyzed as described in the legend to Fig. 1. K_A values are $1/K_i$ for the respective antagonists at the indicated temperatures. Linear regressions to the data points were performed as described under Experimental Procedures (Methods). Values are the means of three separate experiments for each data point.

of all antagonists was largely entropy driven; there remained a negative (favorable) change in enthalpy for tolazoline and phentolamine, whereas yohimbine and rauwolscine had an unfavorable positive enthalpy change.

A similar analysis was also performed for the binding of the agonist (−)-epinephrine. Fig. 3 shows the competition for [^{3}H]yohimbine binding by (−)-epinephrine at 0° and 18.5°. In the

TABLE 2

Thermodynamic parameters of antagonist binding to human platelet α_2-receptors

K_i values were obtained by nonlinear curve fitting of competition curves against [^{3}H]yohimbine at various temperatures. Changes in free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) of binding at 10° and 30° were calculated from the van't Hoff plots (Fig. 2) as described under Experimental Procedures (Methods).

	$\Delta G°$	$\Delta H°$	$\Delta S°$
	kcal/mol		*cal/mol·deg*
At 10°			
Yohimbine	−11.1	+8.0	+67.4
Rauwolscine	−10.9	+11.5	+79.1
Phentolamine	−11.1	−2.6	+30.2
Tolazoline	−9.2	−4.8	+15.6
At 30°			
Yohimbine	−11.8	−6.4	+17.7
Rauwolscine	−11.7	−7.3	+14.5
Phentolamine	−11.4	−9.8	+5.4
Tolazoline	−9.1	−16.8	−25.3

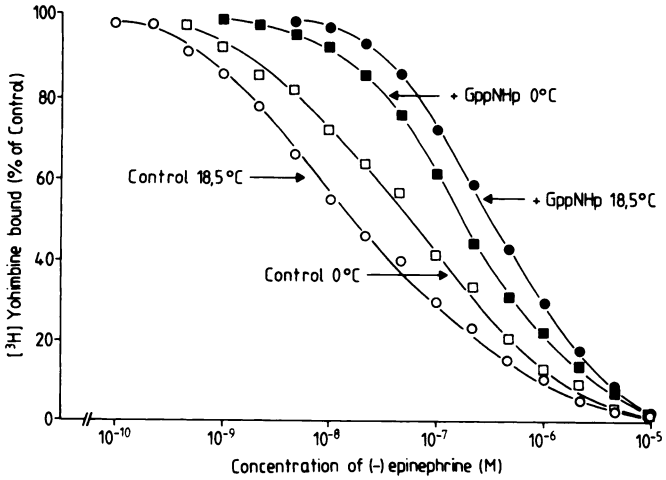

Fig. 3. Competition for [^{3}H]yohimbine binding to human platelet membranes by (−)-epinephrine. Incubations using 2 nM [^{3}H]yohimbine were carried out at 0° and 18.5° in the absence and presence of 100 μM GppNHp as described under Experimental Procedures (Methods). Nonlinear curve fitting gave best fits assuming two affinity states for (−)-epinephrine in the absence and one in the presence of GppNHp, and the following estimates were obtained: At 0°: without GppNHp, K_H 4.1 nM, K_L 146 nM, R_H 37%, R_L 63%; with GppNHp, K_L 151 nM, R_L 100%. At 18.5°: without GppNHp, K_H 1.7 nM, K_L 146 nM, R_H 61%, R_L 39%; with GppNHp, K_L 159 nM, R_L 100%. Values are the means of five separate experiments.

absence of GppNHp, the competition curves were biphasic at both temperatures, and data were best fitted to a model assuming two affinity states for the agonist. In the presence of 100 μM GppNHp, a homogeneous population of receptors with low affinity for (−)-epinephrine was seen. The K_i values for the latter agree reasonably well with the K_i values for the low affinity state in the absence of GppNHp, suggesting that GppNHp induces the low affinity state of the α_2-receptor (8). An increase in temperature from 0° to 18.5° decreased the K_H with little change of the K_L. The proportion of the receptors in the high affinity state was lower at low temperatures.

These effects were also studied at various temperatures (Table 3; see Fig. 7, *open symbols*). Again, the data were best fitted by assuming two components and a break point at about 18°, both for the high and the low affinity states. The thermodynamic parameters calculated from the plot (Table 3) showed that the binding of (−)-epinephrine to both affinity states was

TABLE 3

Thermodynamic parameters of (−)-epinephrine binding to human platelet α_2-receptors

K_i values were obtained by nonlinear curve fitting of competition curves against [^{3}H]yohimbine at various temperatures. Changes in free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) of binding at 10° and 30° were calculated from the van't Hoff plots (*open symbols* in Fig. 7) as described under Experimental Procedures (Methods).

	$\Delta G°$	$\Delta H°$	$\Delta S°$
	kcal/mol		*cal/mol·deg*
At 10°			
High affinity state	−11.2	+6.3	+61.8
Low affinity state	−8.8	−0.3	+30.1
At 30°			
High affinity state	−11.8	−9.7	+6.7
Low affinity state	−9.0	−11.6	−8.9

largely entropy driven at 10° and enthalpy driven at 30°. At 10° there was an unfavorable positive enthalpy change for the high affinity state, but there remained a favorable negative enthalpy change for the low affinity state. At 30° a positive entropy component contributed to the binding reaction to the high affinity state, which was not seen for the low affinity state.

Thus, high affinity agonist binding differs from low affinity agonist binding by an entropy gain. We then examined the question of whether this gain is caused by a liberation of cations during the binding reaction. The most likely candidate would be the sodium ion which is known to affect agonist binding to α_2-receptors (12, 13). Assuming this liberation, the binding reaction for a hormone H and a receptor complex R would be: $H + R \rightleftharpoons HR' + n \cdot Na^+$. Then the logarithm of the observed association constant, K_A, would depend on the sodium concentration: $\log K_A = \log K_A' - n \cdot \log[Na^+]$, with K_A' representing the association constant without the sodium effect.

Fig. 4 shows that, maintaining a constant ionic strength with the sodium substitute *N*-methyl-D-glucamine—which practically does not affect agonist binding to α_2-receptors (12)—this dependence is indeed observed and is adequately fitted by the above equation. This gives an n value of 0.7 for the high affinity and 0.2 for the low affinity agonist binding. The entropy increase $\Delta S°$ for the high affinity binding would be 0 for a sodium concentration of 300 mM. The contribution of this effect to the binding reaction can be calculated from the van't Hoff equation using the K_A' values. This leads to the estimate that a third of the high affinity-specific entropy gain is due to the liberation of sodium ions (difference of $\Delta S°$ values of high and low affinity binding of 18 cal/mol·deg for the K_A values compared to 12 cal/mol·deg for the K_A' values).

The occurrence of a break point in the van't Hoff plots of all ligands at about 18° suggests that the binding to α_2-receptors might be subject to membrane thermotropic phase transitions. The temperature at which these transitions occur can be lowered by addition of agents such as short chain fatty acids, with an optimum of seven to eight C-atoms (14, 15). These transitions can be investigated by measuring the fluorescence of ANS (10, 16). The presence of low concentrations of ANS (10 μM) does not appear to alter phase transitions, whereas high concentrations (>1 mM) do (14). Fig. 5 shows the break points of the ANS fluorescence with platelet membranes in the absence and presence of 10 mM octanoic aicd. The data were best fitted by assuming two components, and break points were observed

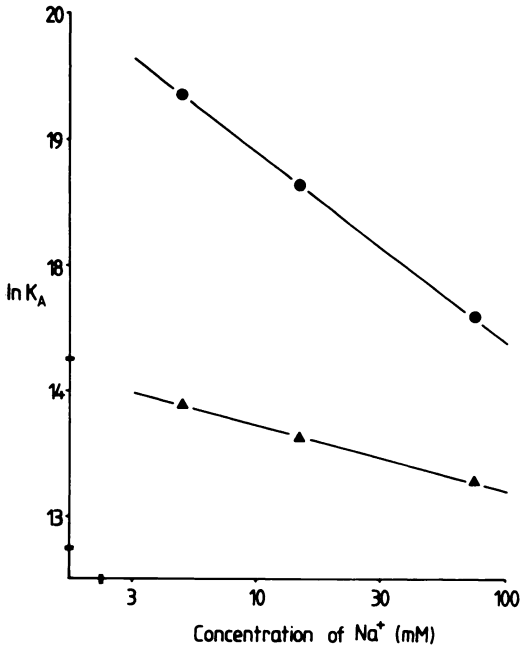

Fig. 4. Effects of Na^+ on the association constants for (−)-epinephrine binding to α_2-receptors of human platelets. Competition experiments using 2 nM [^{3}H]yohimbine were carried out at different concentrations of NaCl at 21°. Ionic strength was held constant by including *N*-methyl-D-glucamine (adjusted to pH 7.4) so that the sum of the concentrations of these two cations was always 75 mM. K_D values for [^{3}H]yohimbine were determined in saturation experiments under these conditions and were used to calculate K_H and K_L values as described for Fig. 3. K_A values are $1/K_H$ and $1/K_L$, respectively. Data represent means from three experiments and were fitted by linear regression. The slopes of the regression lines are 0.2 for the low (▲) and 0.7 for the high (●) affinity binding.

at about 17° in the absence and 12° in the presence of 10 mM octanoic acid. The same observations were made in the absence of Mg^{2+}.

If the break points in the van't Hoff plots of ligand binding were due to phase transitions reported by the ANS fluorescence measurement, then they should be shifted to lower temperatures by addition of octanoic acid. Fig. 6 shows that the presence of 10 mM octanoic acid shifts the break point for yohimbine from about 18° to about 12°. The peak of the K_A values is somewhat lower in the presence of octanoic acid. A similar effect of octanoic acid is seen for (−)-epinephrine binding to the low affinity state (Fig. 7). Again, a shift of the break point from about 18° to about 12° is observed. No such shift of the break point is observed for high affinity (−)-epinephrine binding. Here the break point occurs at about 18° both in the presence and in the absence of octanoic acid. The high affinity K_A values are more reduced by octanoic acid than are the low affinity K_A values. Thus, octanoic acid shifts the break points of antagonist and low affinity agonist binding in a manner similar to that of the break point of ANS fluorescence; high affinity agonist binding, however, is not susceptible to these changes.

Discussion

The investigation of the thermodynamics of ligand binding to receptors is thought to give insights into the fundamental differences between agonists and antagonists, which are responsible for their opposite effects on a given response (1). In the present study we used this approach to study the interaction

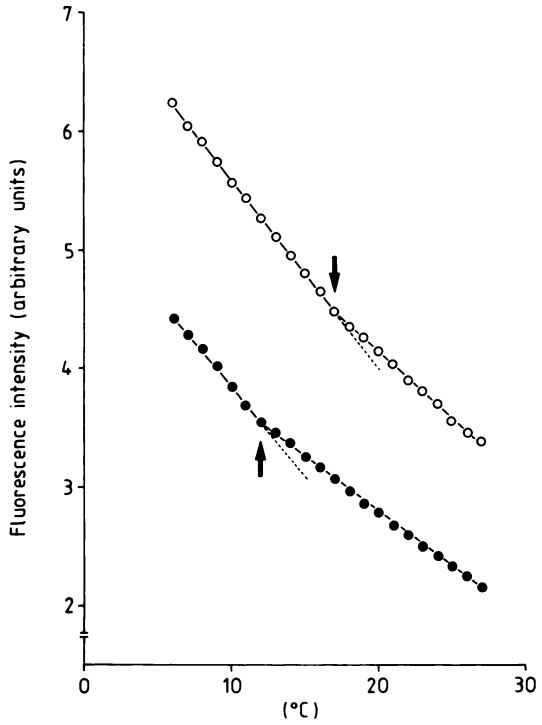

Fig. 5. ANS fluorescence measurements of human platelet membranes. Fluorescence of 10 μM ANS in the presence of 400 μg of protein/ml of human platelet membranes was recorded in arbitrary units as described under Experimental Procedures (Methods) in the absence (○) and presence (●) of 10 mM octanoic acid. Break points were determined as described under Experimental Procedures (Methods) and occurred at 17° in the absence and at 12° in the presence of 10 mM octanoic acid (*arrows*). Data are the means of two separate experiments.

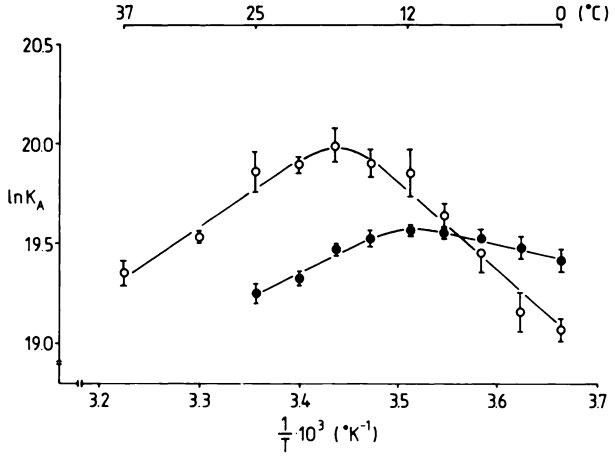

Fig. 6. Effects of octanoic acid on the van't Hoff plot of yohimbine binding. Saturation studies with [^{3}H]yohimbine were carried out at different temperatures and analyzed as described for Figs. 1 and 2. *Open symbols* indicate the absence and *solid symbols* the presence of 10 mM octanoic acid (adjusted to pH 7.4). Data represent means and SE of three experiments for each data point.

of ligands with the α_2-receptor of human platelets. Our results support the hypothesis that this receptor exists in a high and a low affinity state for agonists being modulated by guanine nucleotides and divalent cations (8). Antagonists have the same affinity for the two states and, therefore, antagonist binding is not influenced by guanine nucleotides. U'Prichard *et al.* (17) have postulated a model with three affinity states for agonists;

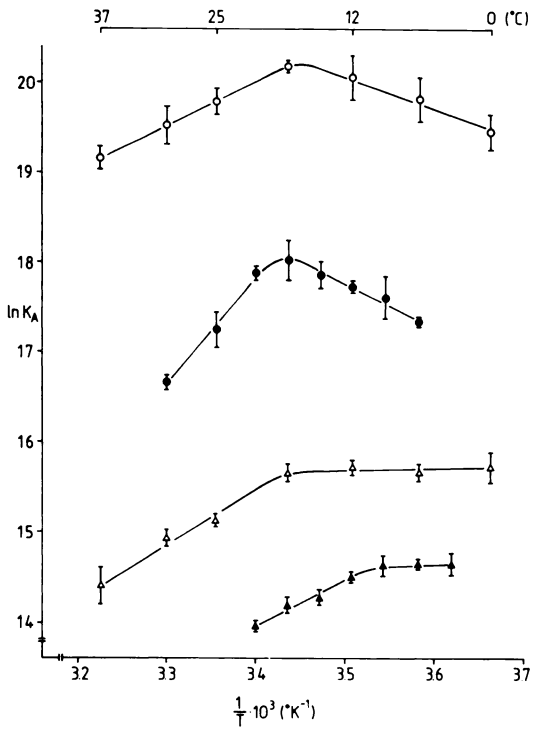

Fig. 7. Effects of octanoic acid on the van't Hoff plots of (−)-epinephrine binding. Competition experiments using 2 nM [^{3}H]yohimbine were carried out and analyzed as described for Fig. 3. *Open symbols* indicate the absence and *solid symbols* the presence of 10 mM octanoic acid (adjusted to pH 7.4). ○, ●: high affinity state; △, ▲: low affinity state. K_A values are $1/K_H$ and $1/K_L$, respectively. Data represent the means and SE of three to five experiments for each data point.

however, in no case could we significantly improve the fitting of agonist binding data by assuming a third component.

Changes in temperature do not affect the total number of binding sites. However, the effects on ligand affinities for the α_2-receptor appear to be more complex than in most other receptor systems examined so far. This is due to two facts: 1) the existence of break points in the van't Hoff plots of both agonists and antagonists and 2) the heterogeneity of antagonist van't Hoff plots. Given the heterogeneous characteristics of antagonist binding, a comparison with agonist binding seems impossible, and agonist-specific changes cannot be deduced from such a comparison. A similar situation has been reported for the benzodiazepine receptor (18). Therefore, the results obtained for the β-receptor (1) do not appear to be generally applicable.

Differences between agonist and antagonist binding have been interpreted as being due to isomerization and/or coupling steps subsequent to agonist binding (1, 19). Prevention of these subsequent steps should allow an analysis of agonist-specific changes without the hazard of using another ligand. This can be done in the case of adenylate cyclase-coupled receptors by an analysis of high and low affinity states of the receptor (6). Compared to the low affinity state, agonist binding to the high affinity state is characterized by an important entropy component. Similar results have also been found for the A_1 (R_i) adenosine receptor (6). Such an entropy increase appears surprising at first sight because high affinity agonist binding is thought to result in a more "ordered" ligand-receptor-N_i protein complex. Thus, the entropy gain must stem from additional processes such as effects of the solute or of ions. Since changes

of the solute result in loss of binding activity, we have studied only the role of ions. The results may be interpreted as follows. Sodium ions appear to be associated with the receptor (or sites linked to the receptor). They dissociate in part upon agonist binding, and this effect is more pronounced for high affinity agonist binding ($n = 0.7$) than for low affinity agonist binding ($n = 0.2$). The resultant entropy gain can be roughly estimated to contribute a third of the difference of binding energies between high and low affinity binding. The increased release of sodium for high affinity binding explains the observation of Michel *et al.* (13) that high affinity binding is influenced to a larger degree than low affinity binding by raising the concentration of sodium. The site of sodium action cannot be determined from the present study. The effect on low affinity agonist binding suggests that sodium liberation upon agonist binding occurs independently of N_i (20). The enhanced liberation upon high affinity binding may be due to either liberation from a site on N_i (21) or to enhancement of liberation from a site regulated by coupling to N_i.

Break points in the van't Hoff plots of ligand-receptor interactions have not been observed for most receptors, although they are a common finding in enzymology. One exception is the benzodiazepine receptor, where a break point is also seen at about 18° for benzodiazepines (22) but not for the antagonist Ro 15-1788 (19). This break point is interpreted as being due to an agonist-specific isomerization step by Möhler and Richards (19), and as being due to a membrane phase transition by Speth *et al.* (23). Glossmann and Hornung (24) observed a discontinuity of the van't Hoff plot of clonidine binding to the α_2-receptor of rat brain at 30°, which they interpreted as evidence for a thermal denaturation. There is indeed some loss of binding at high temperatures, but this cannot account quantitatively for the occurrence of a break point.

Instead, our data support the hypothesis that the break points are due to membrane thermotropic phase transitions (23). Using the ANS fluorescence method, such a transition can be observed in the temperature range of the break point of ligand binding. A shift of the transition temperature from 17° to 12° by addition of 10 mM octanoic acid is followed by a shift of the break point of antagonist binding. A similar shift is also seen for low affinity agonist binding, whereas the break point of high affinity agonist binding remains unchanged. Anionic compounds such as octanoic acid appear to selectively depress the phase transition temperature of the outer half of the plasma membrane (14, 25). These effects are reported by ANS, which interacts with the polar headgroups of the lipids (26). Thus, antagonist and low affinity agonist binding are influenced by alterations occurring in the outer half of the plasma membrane. High affinity agonist binding is insensitive to such alterations, probably by coupling the receptor to N_i. The resulting transmembrane complex appears to be little influenced by the outer half of the membrane. Several membrane proteins directed to the outside have been found to be influenced by phase transitions of the outer half of the plasma membrane (27, 28). Formation of the high affinity agonist state frees the α_2-receptor from such a regulation. This would allow selective regulation of agonist versus antagonist effects via the physical state of the plasma membrane.

The temperature dependence of [^{3}H]yohimbine binding is much less pronounced in the absence of Mg^{2+}. However, the phase transitions reported by ANS were not altered by the removal of Mg^{2+}. This suggests that the coupling of the receptor to phase transitions of the plasma membrane may require Mg^{2+}. Since Mg^{2+} is also needed to give stable binding conditions, we have not attempted a more detailed study of this phenomenon.

To summarize, our data indicate that a comparison of the thermodynamics of antagonist and agonist binding to α_2-receptors of human platelets does not allow the detection of agonist-specific characteristics. Instead, we attempted to find agonist-specific characteristics by comparing high and low affinity agonist binding. Two such characteristics emerged: an entropy gain which is responsible for the high affinity and which may in part be caused by enhanced liberation of sodium ions, and, second, an insensitivity to alterations of phase transitions in the outer half of the plasma membrane. The latter shows that agonist binding may be differentially regulated by the physical state of the plasma membrane.

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References

1. Weiland, G. A., K. P. Minnemann, and P. B. Molinoff. Fundamental difference between the molecular interactions of agonists and antagonists with the β-adrenergic receptor. *Nature (Lond.)* **281**:114–117 (1979).
2. Zahniser, N. R., and P. B. Molinoff. Thermodynamic differences between agonist and antagonist interactions with binding sites for [^{3}H]spiroperidol in rat striatum. *Mol. Pharmacol.* **23**:303–309 (1982).
3. Murphy, K. M. M., and S. H. Snyder. Heterogeneity of adenosine A_1 receptor binding in brain tissue. *Mol. Pharmacol.* **22**:250–257 (1982).
4. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.)* **284**:17–22 (1980).
5. De Lean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).
6. Lohse, M. J., V. Lenschow, and U. Schwabe. Two affinity states of R_i adenosine receptors in brain membranes. Analysis of guanine nucleotide and temperature effects on radioligand binding. *Mol. Pharmacol.* **26**:1–9 (1984).
7. Motulsky, H. J., S. J. Shattil, and P. A. Insel. Characterization of α_2-adrenergic receptors on human platelets using [^{3}H]yohimbine. *Biochem. Biophys. Res. Commun.* **97**:1562–1570 (1980).
8. Hoffman, B. B., T. Michel, T. B. Brenneman, and R. J. Lefkowitz. Interactions of agonists with platelet α_2-adrenergic receptors. Endocrinology **110**:926–932 (1982).
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
10. Träuble, H. Phasenumwandlungen in Lipiden. Mögliche Schaltprozesse in biologischen Membranen. *Naturwissenschaften* **6**:277–284 (1971).
11. Livingstone, C. J., and D. Schachter. Lipid dynamics and lipid-protein interactions in rat hepatocyte plasma membranes. *J. Biol. Chem* **255**:10902–10908 (1980).
12. Limbird, L. E., J. L. Speck, and S. K. Smith. Sodium ion modulates agonist and antagonist interactions with the human platelet alpha$_2$-adrenergic receptor in membrane and solubilized preparation. *Mol. Pharmacol.* **21**:609–617 (1982).
13. Michel, T., B. B. Hoffman, and R. J. Lefkowitz. Differential regulation of the α_2-adrenergic receptor by Na^+ and guanine nucleotides. *Nature (Lond.)* **288**:709–711 (1980).
14. Jain, M. K., and N. M. Wu. Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer. III. Phase transition in lipid bilayer. *J. Membr. Biol.* **34**:157–201 (1977).
15. Perlman, B. J., and D. B. Goldstein. Membrane-disordering potency and anticonvulsant action of valproic acid and other short-chain fatty acids. *Mol. Pharmacol.* **26**:83–89 (1984).
16. Zimmer, G. Fluidity of cell membranes in the presence of some drugs and inhibitors, in *Biomembranes* (M. Kates and L. A. Manson, eds.), Vol. 12. Plenum Press, New York, 169–203 (1984).
17. U'Prichard, D. C., J. C. Mitrius, D. J. Kahn, and B. D. Perry. The α_2-adrenergic receptor: multiple affinity states and regulation of a receptor inversely coupled to adenylate cyclase, in *Molecular Pharmacology of Neurotransmitter Receptors* (T. Segawa, H. I. Yamamura, and K. Kuriyama, eds.). Raven Press, New York, 53–72 (1983).
18. Doble, A. Comparative thermodynamics of benzodiazepine receptor ligand interactions in rat neuronal membranes. *J. Neurochem.* **40**:1605–1612 (1983).

19. Möhler, H., and J. G. Richards. Agonist and antagonist benzodiazepine receptor interaction *in vitro*. *Nature (Lond.)* **294**:763–765 (1981).
20. Limbird, L. E., T. M. Connolly, J. D. Sweatt, and H. D. Uderman. Human platelet α_2-adrenergic receptors: effect of Na^+ on interaction with the adenylate cyclase system and on epinephrine-stimulated platelet secretion. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:235–242 (1985).
21. Jakobs, K. H., M. Minuth, K. Aktories, and G. Schultz. Inhibition of adenylate cyclase. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:137–150 (1985).
22. Braestrup, C., and R. F. Squires. Specific benzodiazepine receptors in rat brain characterized by high-affinity [^{3}H]diazepam binding. *Proc. Natl. Acad. Sci, USA* **74**:3805–3809 (1977).
23. Speth, R. C., G. J. Wastek, and H. I. Yamamura. Benzodiazepine receptors: temperature dependence of [^{3}H]flunitrazepam binding. *Life Sci.* **24**:351–358 (1979).
24. Glossmann, H., and R. Hornung. Alpha$_2$ adrenoceptors in rat brain. *Naunyn-Schmiedebergs Arch. Pharmacol.* **314**:101–109 (1980).
25. Houslay, M. D., I. Dipple, and L. M. Gordon. Phenobarbital selectively modulates the glucagon-stimulated activity of adenylate cyclase by depressing the lipid phase separation occurring in the outer half of the bilayer of liver plasma membranes. *Biochem. J.* **197**:675–681 (1981).
26. Haynes, D. H., and H. Staerk. 1-Anilino-8-naphtalenesulfonate: a fluorescent probe of membrane surface structure, composition and mobility. *J. Membr. Biol.* **17**:313–340 (1974).
27. Shinitzky, M. Membrane fluidity and receptor function, in *Biomembranes* (M. Kates and L. A. Manson, eds.), Vol. 12. Plenum Press, New York, 585–601 (1984).
28. Dipple, I., L. M. Gordon, and M. D. Houslay. The activity of 5′-nucleotidase on liver plasma membranes is affected by the increase in bilayer fluidity achieved by anionic drugs but not by cationic drugs. *J. Biol. Chem.* **257**:1811–1815 (1982).

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