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TEMPERATURE EFFECTS ON α_2-ADRENERGIC RECEPTOR–G_i INTERACTIONS

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Abstract—The effect of temperature on the binding of α_2-adrenergic agonists and antagonists to human platelet membranes was studied. Equilibrium binding of the α_2 antagonist, [^{3}H]yohimbine, was affected minimally, whereas the rate of dissociation changed 40-fold over a temperature range of 5–35°. The antagonist dissociation rates were characterized by a linear Arrhenius plot and an activation energy of 20.5 kcal/mol. The equilibrium binding of the full α_2 agonist, [^{3}H]UK 14,304 [5-bromo-6-*N*-2-4,5-dihydroimidazolyl)quinoxaline tartrate] showed a 50% decrease in B_{max} at 5° as well as a 2-fold decrease in affinity. The kinetics of [^{3}H]UK 14,304 binding were affected more significantly by decreases in temperature. The agonist exhibited fast and slow phases of binding. The fast binding was minimally sensitive to temperature in the range of 0–30° with only a 6-fold change in rate. The slow binding rates changed nearly 100-fold over the same temperature range. Also, the slow rate of agonist binding was characterized by a nonlinear Arrhenius plot with a "break" at approximately 17°, which was found previously to be the phase transition temperature of platelet membrane lipids [Lohse *et al.*, *Molec. Pharmac.* **29**, 228 (1986)]. Despite the reduction of high affinity [^{3}H]UK 14,304 binding at 5°, approximately half of the binding remained sensitive to guanine nucleotides. The data are interpreted in the context of a model in which the fast agonist binding represents a bimolecular interaction of ligand with two pre-existing conformations of the α_2 receptor, one coupled to G_i and the other permanently uncoupled. The slow binding of agonist appears to require protein diffusion in the lipid membrane or a protein conformational change which is dependent on the lipid environment.

The mechanism of many membrane-bound receptors involves guanine nucleotide regulatory proteins or G-proteins§ (see Ref. 1 for review). Complex models of receptor interactions with the G-proteins have been proposed based on the equilibrium binding properties of agonists and antagonists at a variety of receptors [2–4]. It is difficult to obtain reliable estimates of rate and equilibrium constants for the individual steps in these complex models from measurements of equilibrium binding alone. This is true because such models may predict simple hyperbolic binding behavior, yet the apparent K_d is a composite function of the individual parameters in the model [3, 5, 6]. Even when agonist binding is nonhyperbolic, it is not possible to determine all of the parameters of the receptor G-protein interaction from equilibrium binding data [7]. Measurements of agonist binding *kinetics* to identify receptor conformational changes have been used with great success in the nicotinic acetylcholine receptor system from *Torpedo* [5, 8, 9]. Kinetics methods have also been used in studies of the glucagon receptor which involves a G-protein mechanism [10, 11] and for antagonist binding to muscarinic [12] and β-adrenergic receptors [13].

We have used measurements of the kinetics of α_2-agonist binding to probe the state of α_2 receptor G-protein interactions [14]. This is possible because the affinity of binding of agonists to this receptor is greatly enhanced in the presence of G_i [15–17]. Consequently, as the receptor interacts with the G-protein, agonist binding increases, providing an indirect measure of the rate and extent of receptor G-protein coupling. The time course of binding of [^{3}H]UK 14,304, a full α_2-adrenergic agonist, exhibits fast and slow components [6, 14, 18, 19]. In our recent analysis of α_2-agonist binding kinetics [14], the fast component of the binding was interpreted as the diffusion-limited interaction of ligand with both free receptor (R) and a precoupled receptor G-protein complex (RG). The existence of the precoupled complex was suggested by mathematical modelling of agonist association kinetics and further supported by pre-equilibrium dissociation in the presence of GppNHp [14]. The slow binding appeared to be due to a ligand-independent conformational change in the receptor or to the slow diffusion of the α_2-receptor and G-protein in the membrane.

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§ Abbreviations: EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; TME, 50 mM Tris Cl, 10 mM $MgCl_2$ and 1 mM EGTA, pH 7.6; GppNHp, guanosine 5′-(beta, gamma imido)triphosphate; G_i, inhibitory guanine nucleotide regulatory protein; G-protein, guanine nucleotide binding regulatory protein; and UK 14,304, 5-bromo-6-*N*-(2-4,5-dihydroimidazolyl)quinoxaline tartrate.

In this report, we tested this latter hypothesis by applying an approach that has been widely used to identify a role for protein diffusion in the function of membrane enzymes and receptors (i.e. to study the temperature dependence of enzyme or receptor activity, for review see Refs. 20, 21). β-Adrenergic receptor stimulation of adenylate cyclase shows a strong temperature dependence [22]. There was also an increase in the rate of activation of that enzyme in the presence of *cis*-vaccenic acid which modifies membrane fluidity [23].

Measurements of the thermodynamic parameters for the *equilibrium* binding of beta adrenergic ligands revealed striking differences between agonists and antagonists [24–26]. The effects of temperature on agonist and antagonist binding to D_2 dopamine receptors [27] and muscarinic cholinergic receptors [28] were also characterized, but the patterns were different from that for the beta receptor. Lohse *et al.* [29] have reported the effects of temperature on the *equilibrium* binding of α_2-adrenergic agonists and antagonists to human platelet membranes. Differences between the thermodynamic parameters for the high and low affinity agonist binding were obtained, and van't Hoff plots of both agonist and antagonist binding exhibited nonlinearities at approximately 17°, a temperature shown by those authors to correspond to a membrane phase transition detected by fluorescence methods. The interpretation of these equilibrium binding studies is somewhat difficult in that the equilibrium binding in these receptor systems involves a series of complex steps.

In the present study, we combined the use of temperature manipulations with measurements of [^{3}H]UK 14,304 binding *kinetics*. The kinetic approach provides additional information about the steps leading to the equilibrium state. We observed a pronounced reduction of the rate and amplitude of the slow GTP-sensitive agonist binding at low temperatures with a "break" in the Arrhenius plot at the platelet membrane phase transition temperature. The rates of the antagonist binding and fast agonist binding were less affected by changes in temperature and were characterized by linear Arrhenius plots. These results support our hypothesis that the fast agonist binding is limited by aqueous diffusion, whereas the slow binding depends on the interaction of the α_2 receptor with a G-protein in the membrane. A preliminary report of a portion of these results has been presented previously [30].

METHODS

Purified human platelet plasma membranes enriched in α_2 receptors were prepared as described by Neubig and Szamraj [31] and stored for up to 6 weeks at −70°. Preparations used in these studies contained approximately 400–1300 fmol [^{3}H]yohimbine binding sites/mg protein. [^{3}H]Yohimbine (75–85 Ci/mmol) and [^{3}H]UK 14,303 (77–88 Ci/mmol) were obtained from New England Nuclear.

Measurements of ligand binding at various temperatures were performed as described [6]. The pH of the TME buffer (50 mM Tris Cl, 10 mM $MgCl_2$, 1 mM EGTA) was adjusted to 7.6 at the appropriate temperature. The diluted radioligand was pre-equilibrated at the appropriate temperature prior to each experiment. [^{3}H]Yohimbine dissociation measurements were performed by incubating platelet membranes with 10 nM radioligand for 30–60 min at 23–25°, pH 7.4 to 7.8. The reaction mixture was then incubated at the indicated temperature (pH 7.6) for 10 min and dissociation was initiated by addition of 10^{-5} M unlabeled yohimbine. Aliquots of 0.1 ml were removed and diluted into 3 ml of buffer. Nonspecific binding was determined in parallel samples that had been incubated with 10^{-5} M unlabeled yohimbine prior to addition of the radioligand.

Equilibrium binding of [^{3}H]UK 14,304 was determined [6] in a reaction volume of 1 ml at the following times: 20–30 hr (5°), 20 hr (10°), 6 hr (15°), 2.5 hr (20°), 90 min (25°) and 60 min (30°). The kinetics of [^{3}H]UK 13,304 association were measured and analyzed as described [14] with the above-mentioned times used for the equilibrium values in semilogarithmic plots and nonlinear least squares fits. Semilogarithmic plots of specific binding were prepared for association kinetics by plotting ln $(B_{eq}/(B_{eq} - B_t))$ against time. B_{eq} is specific binding at equilibrium*, and B_t is specific binding at time t. Binding time courses were analyzed using EXPFIT [32], a nonlinear least squares parameter estimation program, according to either a single exponential (Eq. 1) or a double exponential (Eq. 2) model.

$$B_t = A(1 - e^{-kt}) \quad (1)$$

$$B_t = A_f(1 - e^{-k_f t}) + A_s(1 - e^{-k_s t}) \quad (2)$$

A and k are, respectively, the amount of equilibrium binding and the rate constant for the single exponential model. A_f and A_s are the amplitudes and k_f and k_s are the rates of the fast and slow binding components in the double exponential model.

Arrhenius plots of ln k vs $1/T$ [i.e. the natural logarithm of the rate of binding (time^{-1}) plotted against temperature^{-1} (degrees Kelvin^{-1})] were prepared to assess temperature effects. The linear portions of the resulting plots were fit by linear regression to calculate slopes. The activation energies (E_a) were calculated from the relation

$$d(\ln k)/d(1/T) = -E_a/R \quad (3)$$

RESULTS

[^{3}H]Yohimbine dissociation from platelet α_2 receptors was characterized by a simple exponential time course with half-times varying more than 40-fold over the temperature range 5–35° (Fig. 1). This is in marked contrast to the small changes (less than a factor of 2) in the antagonist equilibrium binding affinity previously reported by Lohse *et al.* [29] and confirmed by us (data not shown). An Arrhenius plot of the dissociation rates was linear over the temperature range studied and provided an activation energy of dissociation of 20.5 kcal/mol.

* In some experiments at 30° there was a decrease in [^{3}H]UK 14,304 binding at times longer than 30 min. In those cases, the peak binding value was used as B_{eq}.

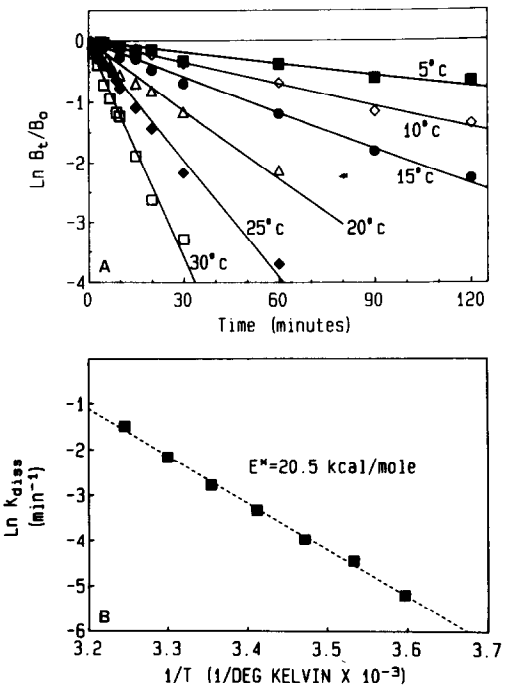

Fig. 1. Effect of temperature on yohimbine dissociation kinetics. (A) The time course of [^{3}H]yohimbine dissociation at the indicated temperatures was measured as described in Materials and Methods. A semilogarithmic plot of specific binding versus time is shown (5°, ■; 10°, ◇; 15°, ●; 20°, △; 25°, ◆; 30°, □). B_t represents specifically bound [^{3}H]yohimbine at the indicated time, and B_0 represents binding prior to initiation of dissociation. Lines are linear regressions of the transformed data. (B) An Arrhenius plot of the rates of [^{3}H]yohimbine dissociation is shown. Data are those from panel A. k_{diss} is the rate constant for antagonist dissociation and E* is the activation energy calculated from the slope of the line.

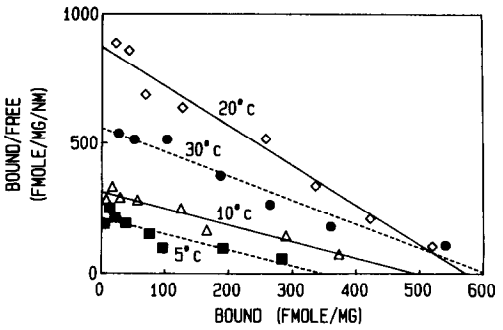

Fig. 2. Effect of temperature on [^{3}H]UK 14,304 equilibrium binding. [^{3}H]UK 14,304 binding (0.05 to 5 nM) was measured for temperatures of 5–30° as described in Materials and Methods. Scatchard transformations of specific [^{3}H]UK 14,304 binding at 5° (■), 10° (△), 20° (◇) and 30° (●) are shown. Data at 15° and 25° are omitted for clarity. Lines are linear regressions of the Scatchard transformations of the data. Data are averages of triplicate determinations from one experiment. A summary of data from four similar experiments is presented in Table 1.

* In some experiments at 30° there was a decrease in [^{3}H]UK 14,304 binding at times longer than 30 min. In those cases, the peak binding value was used as B_{eq}.

Table 1. Equilibrium binding parameters for [^{3}H]UK 14,304 at different temperatures

Temperature (°C)	B_{max} (fmol/mg)	K_d (nM)
5	210 ± 60*	1.24 ± 0.63†
10	429 ± 72†	1.03 ± 0.43†
15	473 ± 51†	0.64 ± 0.35†
20	476 ± 74†	0.74 ± 0.26†
25	341 ± 52	0.72 ± 0.13
30	415 ± 84†	0.89 ± 0.18†

Binding of 0.05 to 5 nM [^{3}H]UK 14,304 was measured at the indicated temperatures. Equilibration times were as described in Materials and Methods. B_{max} and K_d were calculated from linear least squares fits of Scatchard transformations of specific binding. The platelet membranes used in these studies were from four different preparations and contained 1000 ± 210 fmol/mg of [^{3}H]yohimbine binding sites (B_{max} at 23°). Values are means ± SEM of four determinations. Paired t-tests were done to compare B_{max} and K_d values with the control value (25°).

*,† An asterisk indicates significant differences ($P < 0.05$) and a dagger indicates nonsignificant differences ($P > 0.01$).

The binding of the full α_2 agonist [^{3}H]UK 14,304 was studied at temperatures from 0 to 30°. Binding at 35° and above could not be measured reliably because at those temperatures no plateau was observed. The equilibrium binding of [^{3}H]UK 14,304 at 5° was characterized by a B_{max} significantly lower than that observed at the higher temperatures (Fig. 2 and Table 1). This was accompanied by a 2-fold reduction in affinity at 5°. The incubations at 5° were carried out for at least 20 hr which is equal to five half-times for the slow phase of agonist binding at 5° (see Fig. 4 below). Thus, the reduction in binding was not due to an inadequate equilibrium time. To test whether receptor or radioligand inactivation during the long incubation was the cause of the lower B_{max} at 5°, we rewarmed the reaction mixture to 25° for 1 hr after an overnight incubation at 5°. Binding increased to the level seen after a 1-hr incubation at 25° alone (data not shown), indicating that receptor or radioligand inactivation was not occurring during the prolonged incubation at 5°.

Studies of [^{3}H]UK 14,304 association kinetics provide additional insights into a possible role of membrane lipids in agonist binding. At all temperatures studied, the [^{3}H]UK 14,304 association kinetics were significantly better fit by the double exponential function (F test, $P < 0.05$), as previously described at room temperature [14]. Linear and semilogarithmic plots of such data are shown in Fig. 3, along with theoretical curves obtained by non-linear least squares fits of the data to the two-exponential function (Eq. 2)*. Both the fast and slow components of the agonist binding were slowed at the lower temperatures. It appears, however, that the rate of the slow component (Fig. 3D) was affected more significantly than that of the fast component (Fig. 3C). An Arrhenius plot of the fast and slow rates illustrates this more clearly (Fig. 4). The fast rate exhibited a linear decrease (6-fold) over the temperature range of 30–0°. The calculated activation energy for this binding process was 10.6 kcal/mol.

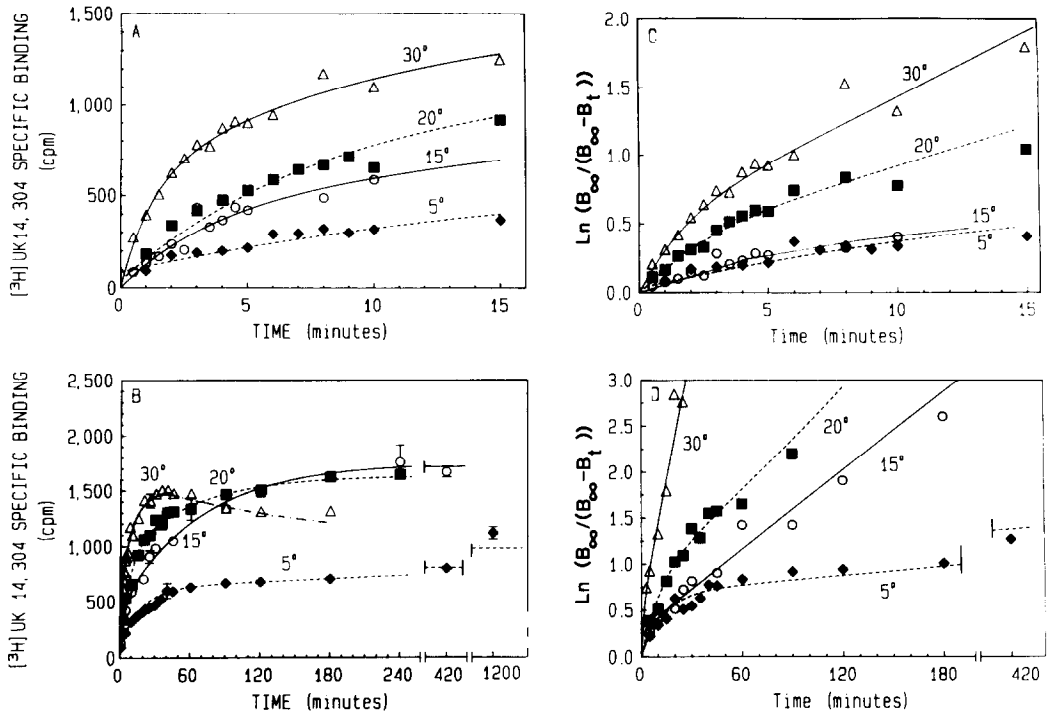

Fig. 3. Effect of temperature on kinetics of [^{3}H]UK 14,304 binding. (A and B) The time course of specific binding of 1 nM [^{3}H]UK 14,304 was measured after pre-equilibration of membrane and ligand for 5 min at the indicated temperatures (5°, ◆; 15°, ○; 20°, ■; and 30°, △). (C and D) Semilogarithmic plots of the data are shown. B_t equals specific binding at time t and B_∞ is equilibrium binding determined at the times described in Materials and Methods. Rate constants and amplitudes for the fast and slow phases of binding were determined by nonlinear least squares methods and the curves shown are theoretical plots according to Eq. 2. The data are individual determinations (0–10 min) or means of duplicate determinations (20 min or greater) from a series of experiments on a single preparation of platelet membranes. The equilibrium values were determined in triplicate. A summary of the kinetic constants from four such series is shown in Figs. 4 and 5.

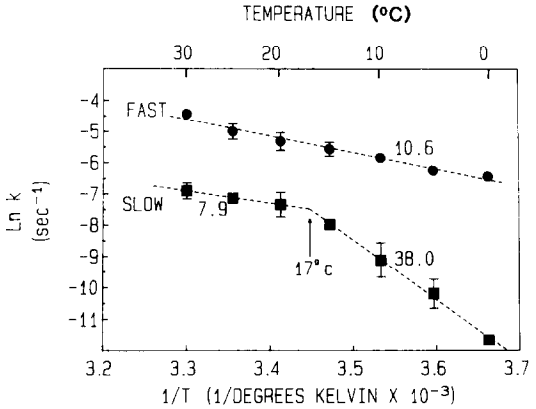

Fig. 4. Arrhenius plot of rates of [^{3}H]UK 14,304 binding. The rate constants of the fast (●) and slow (■) phases of [^{3}H]UK 14,304 binding are shown on an Arrhenius plot. The lines are linear regressions of the straight segments of the plots. The numbers adjacent to the lines are calculated activation energies in kcal/mol for that portion of the data. The arrow indicates the temperature at which the nonlinearity in the slow rates occurs. Values are mean ± SEM of four determinations except for the value at 0° which is from a single experiment.

The slow rate exhibited a nonlinear Arrhenius plot with relatively small changes in rate for temperatures from 30 to 20° but much greater changes from 20 to 0°. The activation energies calculated from the slopes of the two linear portions of the Arrhenius plots were 7.9 and 38.0 kcal/mol for the high and low temperature ranges respectively. The intersection of the two linear portions of the Arrhenius plot occurred at approximately 17°. This is the same temperature at which measurements of anilinonaphthalene sulfonate fluorescence revealed a phase transition in human platelet membrane lipids [29]. Thus, below the lipid phase transition temperature, the rate of the slow phase of agonist binding declines dramatically.

To determine which component of the [^{3}H]UK 14,304 binding accounted for the decrease in B_{max} observed in the equilibrium binding studies, we examined the magnitudes of the fast (A_f) and slow (A_s) components of the binding kinetics at different temperatures. Figure 5 shows that the magnitude of the fast phase of [^{3}H]UK 14,304 binding was only reduced slightly from 15° and 20° to 5°. In contrast, there was a 2.5- to 3-fold decrease in the magnitude of the slow binding from 15° and 20° to 5°. Thus, the decreased B_{max} at 5° was primarily due to a reduction in the amount of the slow binding component.

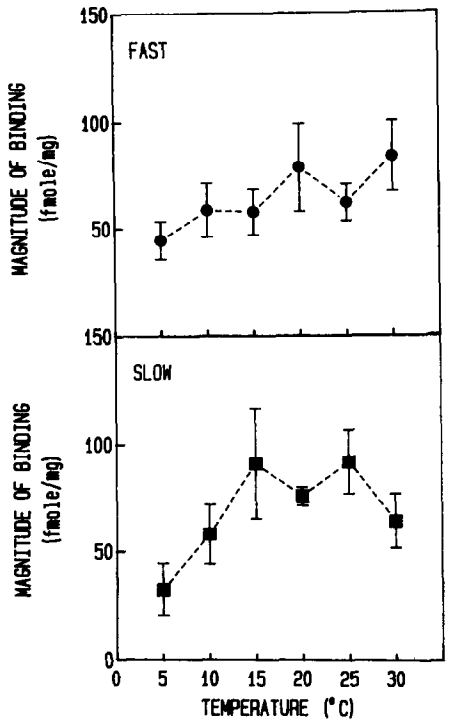

Fig. 5. Amplitudes of fast and slow [^{3}H]UK 14,304 binding. The magnitudes of the fast (●) and slow (■) [^{3}H]UK 14,304 binding determined by nonlinear least squares fits of data such as in Fig. 3 are plotted against temperature. Values are means ± SEM of four determinations.

Since low temperatures result in a decreased amount of high affinity [^{3}H]UK 13,304 binding and Lohse *et al.* [29] found a decreased sensitivity of α_2-agonist binding to guanine nucleotide, we tested the sensitivity of [^{3}H]UK 14,304 binding at 5° to the addition of 10 μM GppNHp. One nanomolar [^{3}H]UK 14,304 was incubated with membranes at 5° for 90 min, a time at which 90% of the binding is associated with the rapid component. Excess competing drug (oxymetazoline, 10^{-5} M), GppNHp (10^{-5} M) or both were added to initiate dissociation of the bound [^{3}H]UK 14,304 (Fig. 6). Although the dissociation of the [^{3}H]UK 13,304 was slower than that observed at room temperature [14], approximately 60% of the bound [^{3}H]UK 14,304 was sensitive to the guanine nucleotide. In the combined presence of cold ligand and nucleotide, more than 90% of the bound [^{3}H]UK 14,304 dissociated by 30 min. These data indicate that at 5° substantial coupling of the α_2 receptor to G_i was still present even though the slowly appearing GTP-sensitive [^{3}H]UK 14,304 binding was reduced both in rate and magnitude.

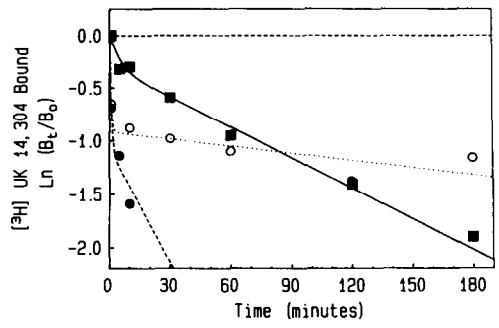

Fig. 6. Effect of GppNHp on pre-equilibrium [^{3}H]UK 14,304 binding at 5°. One nanomolar [^{3}H]UK 14,304 was incubated with platelet membranes for 90 min at 5°, and specific binding was determined in triplicate. The reaction mixture was divided into three aliquots and the following additions made: (○) 10^{-5} M GppNHp; (■) 10^{-5} M oxymetazoline; or (●) both. Specific binding was determined at the indicated times following additions and is plotted on a semilogarithmic scale. B_t is specific binding at time t after additions, and B_0 is specific binding prior to additions (960 ± 30 cpm).

DISCUSSION

Temperature changes produce dramatic alterations in the lipid phase of biological membranes [21]. In the present study we have provided evidence that there are differences in the behavior of the α_2 receptor-G_i system above and below the lipid phase transition temperature of human platelet membranes. In particular, the slow appearance of high affinity GTP-sensitive α_2 agonist binding was reduced both in rate and extent at 5°. In contrast, there was a minimal effect on either the antagonist binding or the rapid phase of [^{3}H]UK 14,304 binding. The latter consisted of both GTP-sensitive and -insensitive components ([14], and see Fig. 6). These data are consistent with our hypothesis that the slow binding of [^{3}H]UK 14,304 is due to an agonist specific diffusional interaction of the α_2 receptor with G_i in the membrane, while the fast binding represents an interaction of agonist with pre-existing receptor conformations [14].

In the analysis of Arrhenius plots, "breaks" can have multiple interpretations. It is possible that a protein conformational change rather than lipid changes could produce the nonlinear Arrhenius plot [33, 34]. However, the nonlinearity in the Arrhenius plot of our slow binding rate occurred at the same temperature as membrane alterations detected by anilinonaphthalene sulfonate fluorescence [29]. This agreement suggests that a generalized membrane phase transition is likely to be affecting our binding rather than a discrete effect on a minor protein component of the membrane such as the alpha$_2$ receptor or G_i. Both protein diffusion in a lipid membrane and a simple protein conformational change could be influenced profoundly by a lipid phase transition [21]. Thus, our results indicate that the slow appearance of high affinity [^{3}H]UK 14,304 binding, a process that appears to require the interaction with G_i, is affected significantly by the state of the membrane lipids. Processes that are limited by aqueous diffusion tend to have a much less striking dependence on temperature [35], and this appears to be the case for the fast binding of [^{3}H]UK 14,304.

Our results and those of Lohse *et al.* [29] indicate that the α_2 receptor *can* interact with G_i at 0° or 5°, but in both cases there is approximately a 50% reduction in the amount of high affinity agonist binding detected. A similar decrease in the fraction of inhibitory adenosine receptors [36] and beta-adre-

nergic receptors [26] binding agonist with high affinity has been reported for low temperatures. These data are in contrast to the results of Briggs and Lefkowitz [25] with the turkey erythrocyte β-adrenergic receptor in which no high affinity GTP-sensitive agonist binding was observed even at a temperature as high as 20°. For the α_1-adrenergic receptor which is not coupled to adenylate cyclase, the opposite result was observed [37]. High affinity α_1-agonist binding predominated at 4° and was not observed in equilibrium studies at 37°. This appeared to be a property of the receptor itself rather than the receptor–G-protein or receptor–lipid interaction since it occurred with solubilized as well as membrane-bound receptors.

Our recently described model of α_2-agonist binding kinetics in platelet membranes [14] provides one possible interpretation of the decreased B_{max} in equilibrium binding studies. In that model approximately one-third of the platelet α_2 receptors are permanently in a low affinity state unable to couple to G_i. Two thirds of the receptors bind agonist with high affinity at equilibrium; one third is coupled to G_i before agonist is present and the other third couples slowly after agonist binds to the receptor. The high affinity binding of α_2 agonists present at 0° or 5° may represent receptor precoupled to G_i, while the decreased amount of slow binding is due to an impaired ability of the receptor to couple to G_i after agonist binds. Indeed, the amount of high affinity α_2-agonist binding observed at 0° or 5° (*ca.* 30% of the number of [^{3}H]yohimbine binding sites) is in good agreement with the amount of α_2 receptor predicted to be precoupled to G_i based on the [^{3}H]UK 14,304 binding kinetics [14]. Recent biochemical data also suggest that precoupled α_2 receptor and G_i exist since some of the platelet α_2 receptor can be solubilized in a complex with a G-protein without prior incubation with agonist [38].

The dissociation kinetics of the antagonist [^{3}H]yohimbine are characterized by a linear Arrhenius plot; however, they show a more pronounced temperature dependence than that seen for the fast [^{3}H]UK 14,304 binding (E_a 20.5 kcal/mol vs 10.6 kcal/mol). Although the former are dissociation and the latter association data, this is unlikely to be the cause of the differences since the equilibrium K_d for yohimbine binding changes by less than a factor of two over the temperature range studied [29]. Consequently, the [^{3}H]yohimbine association kinetics should have very nearly the same temperature dependence as the dissociation kinetics. We have pointed out previously [14] that [^{3}H]yohimbine had a significantly slower apparent association rate constant than that observed for the fast phase of [^{3}H]UK 14,304 binding ($3 \times 10^5\ M^{-1}\ sec^{-1}$ vs $7 \times 10^6\ M^{-1}\ sec^{-1}$). The more striking temperature dependence of the yohimbine kinetics also suggests that binding of the antagonist may not be diffusion-limited even though the kinetics are formally bimolecular. Additional characterization of the yohimbine binding kinetics will be necessary to confirm this hypothesis.

Although the temperature dependence of agonist binding kinetics has not been studied for the β-adrenergic receptor, extensive studies of the kinetics of adenylate cyclase activation have been undertaken [22, 39, 40]. In turkey erythrocyte membranes, steady-state activation of adenylate cyclase by beta-adrenergic receptors exhibits a nonlinear Arrhenius plot with a break at 24°. No evidence for a change in lipid fluidity was observed at that temperature using diphenylhexatriene fluorescence polarization [39, 40]; however, with dansylphosphatidyl ethanolamine, fluorescence changes were noted at 25° that were interpreted as occurring predominantly in the inner leaflet of the plasma membrane [22]. Surprisingly, the *rate* of activation of adenylate cyclase by β-adrenergic receptors did not show a discontinuity at this temperature. Since it has recently become possible, by the use of rapid mix quench methods, to study the rate of onset of inhibition of adenylate cyclase [41, 42], it will be interesting to see whether the effects of temperature on the rate of agonist binding will be accompanied by changes in the rate of adenylate cyclase inhibition.

In summary, the effects of temperature on the kinetics of binding of the full α_2 agonist, [^{3}H]UK 14,304, provided additional evidence for the role of lipid diffusion or a lipid-dependent protein conformational change in slow agonist binding. High affinity agonist binding present at low temperatures may be due to the existence of a precoupled α_2 receptor G_i complex.

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