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Parallel Observation of the Occupancy of the *Alpha*$_2$-Adrenergic Receptor in Intact Platelets and Its Ability to Inhibit the Adenylate Cyclase

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SUMMARY

The binding of the selective *alpha*$_2$-adrenergic receptor antagonist [*methyl*-^{3}H]yohimbine to intact human blood platelets was studied in parallel with an assessment of the ability of epinephrine to inhibit their accumulation of cyclic AMP in response to prostaglandin E_1 and a phosphodiesterase inhibitor. Specifically bound [^{3}H]yohimbine dissociated in a monoexponential fashion, and equilibrium binding showed 200–400 sites/platelet with a dissociation constant of 2–7 nM. The concentration of epinephrine which inhibited cyclic AMP accumulation was one-quarter of that required for an equal degree of inhibition of [^{3}H]yohimbine binding. Conversely, the concentration of yohimbine required to inhibit the action of epinephrine on the adenylate cyclase was 10 times higher than that required for an equal degree of saturation of the *alpha*$_2$-adrenoreceptor. The ability of epinephrine and the partial agonist *p*-aminoclonidine to compete with [^{3}H]yohimbine for binding was not influenced by agonist-occupation of the ADP receptor, which also inhibits the adenylate cyclase. These results are not compatible with the concept that each adenylate cyclase unit is coupled to an *alpha*$_2$-receptor, nor do they indicate that the agonist-occupied *alpha*$_2$-receptor forms a complex with the enzyme which persists for the duration of the inhibitory effect. However, it is suggested that these results are compatible with a persistent inhibition of the cyclase being induced by a brief interaction between the agonist-occupied receptor and the adenylate cyclase.

INTRODUCTION

Over the past decade a substantial effort has been mounted to explain in molecular terms how pharmacological receptors on the surface of cells influence the rate at which the membrane-bound adenylate cyclase synthesizes cyclic AMP (1–4). The majority of this work concerned receptors whose action is to activate the enzyme, and it has been clearly established that the signal transmission involves three components, the receptor (*R*), which binds agonistic hormones (*H*); a guanine nucleotide-binding protein (*N*), which binds GTP; and the catalytic unit (*C*). Although some authors have suggested that these three components are clustered in a 1:1:1 complex (e.g., ref. 2), this arrangement becomes unwieldly when we consider cells such as human blood platelets, which have eight or nine types of receptors influencing the enzyme (5). It is also not compatible with observations of the relationship between the receptor occupancy by an agonist and the resultant fractional effect on the adenylate cyclase; in a variety of preparations such experiments reveal that the effect usually exceeds the occupancy (6–9).

The floating receptor hypothesis (10, 11) both accounts for this discordance and enables multiple receptors to impinge their message on the adenylate cyclase. It postulates that a physical association between the receptor and the adenylate cyclase is brought about as a consequence of an agonist binding to the receptor. Thermodynamic analysis of this model shows that the affinity between the agonist and the complex it induces is higher than the affinity of the agonist for the freely diffusing receptor (10, 11).

A third model, described by Tolkovsky and Levitzki (12), supposes that the adenylate cyclase is switched on by a brief collision between freely diffusing agonist-occupied receptors and the catalytic unit. Scheme 1 sets out these three models in a somewhat simplified form and indicates the results that should be obtained when measurements are made of agonist binding and the corresponding effect on the adenylate cyclase.

The coupling of receptors which deactivate the adenylate cyclase has received less attention than the receptors which activate it (4). Human blood platelets have several deactivating receptors [ADP (13), *alpha*$_2$-adrenergic (14), adenosine (14), prostaglandin E_2, and endoperoxide analogues (15)]. We (16) and others (17) have recently described the use of [*methyl*-^{3}H]yohimbine as a radioligand for the *alpha*$_2$-adrenergic receptor on the

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Model I (precoupled receptors)

$$H + RNC \rightleftharpoons (HRNC) \rightleftharpoons HRNC^*$$

The fractional effect equals receptor occupancy,[2] and binding curves are "normal." The number of catalytic units that are influenced is *equal* to the number of receptors that are occupied by hormone.

Model II (floating receptors)

$$H + RN + C \rightleftharpoons HRN + C \rightleftharpoons HRNC^*$$

The fractional effect exceeds the occupancy, and because of the accumulation of *HRNC**, which (for thermodynamic reasons) must have high affinity for *H*, the binding curves are not "normal" but reflect a variable mixture of high- and low-affinity forms. The number of catalytic units influenced is *less* than the number of receptors occupied.

Model III (collision coupling)

$$H + RN + C \rightleftharpoons HRN + C \rightleftharpoons (HRNC) \rightarrow C^*$$

The fractional effect exceeds the occupancy. Because the species *HRNC* does not accumulate, binding curves merely reflect the formation of *HRN* and are therefore "normal." The number of catalytic units influenced may *exceed* the number of receptors occupied.

SCHEME 1

intact platelet. In this paper we present our observations of the binding of agonists to this receptor in the intact cell in parallel with observations of the degree of inhibition of cyclic AMP accumulation.

The experiments were designed to enable us to choose between the three models set out above. Our results suggest that the *alpha*$_2$-adrenergic receptor deactivates the adenylate cyclase by collision coupling. In the accompanying paper (18) we examine the theoretical consequences of bidirectional collision coupling, in which we assume that receptors act as catalysts regulated by external stimuli ("allozymes") to induce a change in state of the adenylate cyclase.

METHODS

Our procedures for preparing washed platelets and their use in both the [*methyl*-^{3}H]yohimbine binding assay (16) and the radiochemical estimation of their accumulation of cyclic AMP (19, 20) have been described previously in detail. Briefly stated, platelet-rich plasma was prepared from blood anticoagulated with acid-citrate-dextrose and 100 μM sodium acetyl salicylate (16). Half of this preparation was incubated with [U-^{14}C]adenine and half without (37°, 90 min). After centrifugation and resuspension in Ca^{2+}- and Mg^{2+}-free buffered saline, the platelets were incubated with [^{3}H]yohimbine and other additions as indicated at 37° for about 20 min.

The amount of [^{3}H]yohimbine bound to the receptor was then determined by diluting the platelets with EDTA-containing platelet-poor plasma [which permits the dissociation of loosely bound radioligand (16)] and centrifuging them in a micro-sedimentation tube 30 sec later. The pellet was dissolved in a scintillation cocktail (Scintiverse, Fisher Scientific Company, Pittsburgh, Pa.), using an ultrasonic bath, and was counted for tritium content (16).

The platelets prelabeled with [^{14}C]adenine were treated in the same way (including the incubation with [^{3}H]yohimbine), but instead of being diluted and centrifuged, they were incubated at 37° with prostaglandin E_1 and the phosphodiesterase inhibitor RA233[3] plus an excess of epinephrine as appropriate. EDTA was also added to prevent the induction of the release reaction by the epinephrine. 30 sec later, perchloric acid containing [^{3}H] cyclic AMP as a recovery standard was added, and the supernatant was applied to a column of Dowex 50 (H^+ form), which was eluted with water. The cyclic AMP peak was further purified by precipitation of other nucleotides with $Ba(OH)_2$ and $ZnSO_4$. The final supernatant was then counted for tritium and ^{14}C content. After correction for recovery (about 50%) and channel crossover, the [^{14}C]cyclic AMP was expressed as a percentage of total intracellular ^{14}C (19, 20).

Data are presented with curves fitted to them by the following simple procedure. It is assumed that the value observed is related to the concentration of the agent (agonist or ligand) by the equation of the Langmuir isotherm, which is mathematically identical with the Michaelis-Menton equation. We make rough estimates of the value at zero and saturating concentrations of the agent used, and of the concentration of the half-maximal effect. These values are fed into a computer program which generates the curve of the Langmuir isotherm. This curve is compared with the raw data, and appropriate changes are made in the parameters until we are satisfied with the fit. The curve is presented in the figures, and the values for the equation are given in the figure legends.

Materials. Prostaglandin E_1 was a gift from Dr. J. E. Pike, The Upjohn Company (Kalamazoo, Mich.). Yohimbine was purchased from Aldrich Chemical Company, (Milwaukee, Wisc.). RA233 and *p*-aminoclonidine were gifts from Boeringer Ingelheim, Ltd. (Ridgefield, Conn.). (−)-Epineprine hydrochloride was purchased from Sigma Chemical Company (St. Louis, Mo.). [*methyl*-^{3}H]Yohimbine (81 Ci/mmole) and [2-^{3}H]cyclic AMP were obtained from New England Nuclear Corporation (Boston, Mass.), and [U-^{14}C]adenine (291 Ci/mole) was obtained from Amersham (Arlington Heights, Ill.).

RESULTS

Kinetics of binding [^{3}H]yohimbine. In contrast to [^{3}H] dihydroergocryptine, [^{3}H]yohimbine binds to intact platelets with kinetics compatible with a simple bimolecular reaction (16, 17). Figure 1 shows the time course of

[2] Fractional effect is the influence on the adenylate cyclase of a particular concentration of agonist expressed as a fraction of the effect at a saturating concentration. Occupancy is the fraction of receptors occupied by an agonist at a particular concentration. In each case the guanine nucleotide-binding protein (*N*) is considered to be coupled to the receptor (*R*). Identical kinetic mechanisms arise if *N* is precoupled to the catalytic unit (*C*), but models in which *N* is induced to migrate from *R* to *C* when *R* binds the agonist hormone (*H*) generate complex binding curves and occupancy effect relationships (22). Since experimentally we do not observe deviations from "normal" curves (i.e., those with a shape corresponding to the Langmuir isotherm, lacking positive or negative "cooperativity"), these models are not considered further here.

[3] The abbreviation used is: RA233, 2,6-bis(diethanolamino)-4-piperidinopyrimido-[5,4-*d*]-pyrimidine.

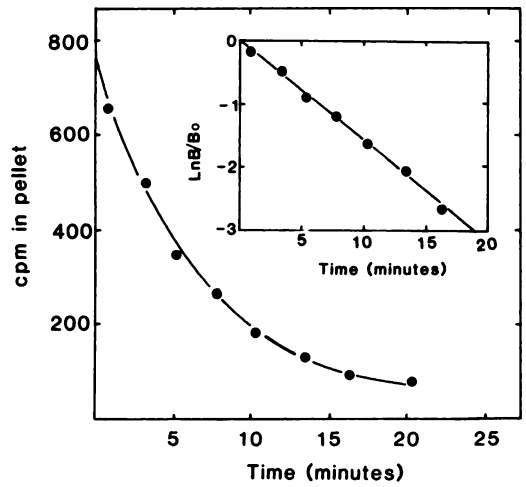

FIG. 1. *Time course of dissociation of [^{3}H]yohimbine from the platelet*

Washed platelets (2 ml) were incubated with 10 nM [^{3}H]yohimbine at 37° for 20 min. Nonradioactive yohimbine (20 μl, final concentration 10 μM) was then added. At times thereafter, as shown, 25-μl samples were diluted with 300 μl of platelet-poor plasma. One minute later the mixture was centrifuged and the radioactivity in the pellet was determined. There were approximately 700 cpm in 25 μl of supernatant of the original incubation medium. The *inset* shows a plot of the natural logarithm of B/B_0, where B and B_0 are pellet counts per minute at the time indicated and time zero, respectively, both minus nondisplaceable radioactivity (40 cpm). The slope of the straight line and the rate constant of the computed curve is 0.156 min^{-1}. On other occasions we obtained values between 0.144 and 0.200 min^{-1}.

this dissociation, and the *inset* shows that the dissociation is monoexponential. We have shown previously that this rate is not influenced by adding nonradioactive epinephrine or yohimbine (16). Equilibrium binding showed

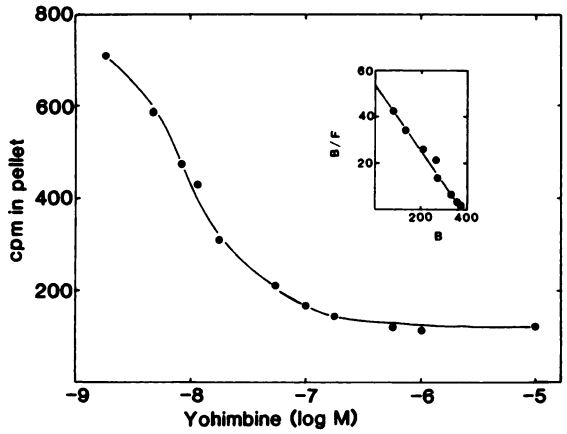

FIG. 2. *Equilibrium binding of [^{3}H]yohimbine*

Washed platelets (1.3×10^9/ml) were incubated with [^{3}H]yohimbine (2 nM) plus nonradioactive yohimbine to give the total concentration shown for 20 min at 37°. Samples (50 μl) were diluted with platelet-poor plasma and centrifuged 30 sec later. The radioactivity in the pellets is shown (means of duplicates). Nonspecific binding amounted to 120 cpm (equivalent to 2.3 μl/10^8 platelets). The *inset* shows a Scatchard plot of the data corrected for nonspecific binding. B, molecules bound per platelet; F, concentration (nanomolar). Both the straight line and the computed curve indicate results expected for 375 binding sites/platelet with a K_{diss} of 6.9 nM. At the lowest concentration of yohimbine (2 nM) about 15% was bound. On other occasions we obtained values of 305 sites with a K_{diss} of 3.1 nM and 280 sites with a K_{diss} of 5.0 nM.

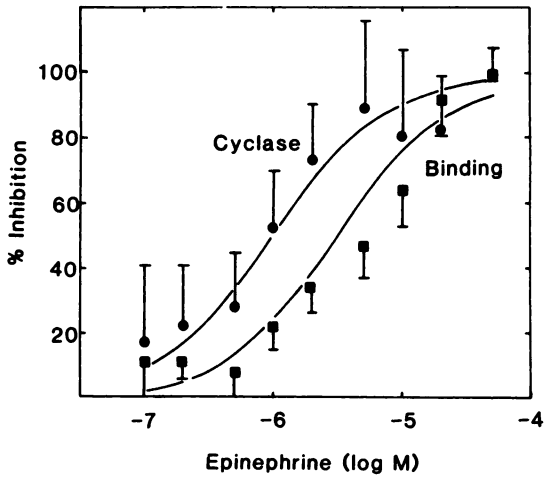

FIG. 3. *Simultaneous assay of the ability of epinephrine to displace yohimbine and to inhibit the adenylate cyclase*

Washed platelets, some of which were prelabeled with [^{14}C]adenine, were incubated with 2 nM [^{3}H]yohimbine plus the concentration of epinephrine shown for 23 min at 37°. The bound [^{3}H]yohimbine was then determined in duplicate. Simultaneously, duplicate 100-μl samples of the prelabeled platelets were incubated at 37° for 30 sec with 20 μl of a mixture giving a final concentration of prostaglandin E_1 of 10 μM, 200 μM RA233, and 10 mM EDTA; the content of [^{14}C]cyclic AMP was then determined. The means ± 1 SD results from two experiments (n = 4) are shown. Both the inhibition of [^{3}H]yohimbine binding (■) and inhibition of the accumulation of [^{14}C]cyclic AMP (●) are plotted as percentage of results in the absence and presence of an excess of epinephrine. The curves are computed for half-maximal inhibition of cyclic AMP accumulation at 1 μM and binding at 4 μM. The means of the data points on the two curves are statistically different ($p < 0.05$, unpaired t-test) at epinephrine concentrations of 1 μM, 2 μM, and 5 μM.

that platelets have 200–400 binding sites with affinity about 2–7 nM. There was no evidence for the existence of more than one site, nor for cooperativity between sites (e.g., Fig. 2).

Correlation of binding and inhibition of the adenylate cyclase. A comparison of the proportional occupancy of the *alpha*$_2$-receptor by epinephrine with the degree of inhibition of the adenylate cyclase was approached in two ways. First, platelets were incubated with 2 nM [^{3}H] yohimbine and increasing concentrations of epinephrine; the binding of yohimbine was then determined in one sample, while in a parallel sample the increase in cyclic AMP occurring after the addition of prostaglandin E_1 plus a phosphodiesterase inhibitor was measured. The pooled results of two such experiments are shown in Fig. 3. The data are normalized with respect to maximal and minimal binding and cyclic AMP levels, but not with respect to the concentration of epinephrine. Half-maximal suppression of cyclic AMP accumulation occurred at a concentration of epinephrine about one-quarter that required for half-maximal suppression of yohimbine binding.

In the second approach, platelets were incubated with [^{3}H]yohimbine plus increasing concentrations of unlabeled yohimbine. At the end of the incubation, binding was measured in one sample, and the increase in cyclic AMP was measured in the parallel sample in response to prostaglandin E_1 and the phosphodiesterase inhibitor plus or minus a saturating concentration of epinephrine.

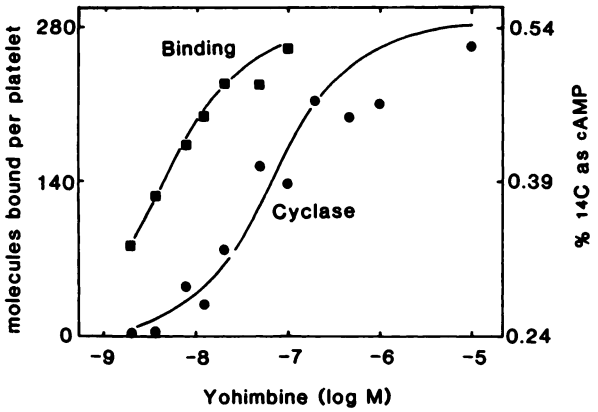

FIG. 4. *Simultaneous assay of yohimbine binding and blockade of the epinephrine effect on [^{14}C]cyclic AMP accumulation*

Washed platelets, some of which were prelabeled with [^{14}C]adenine, were incubated with [^{3}H]yohimbine plus nonradioactive yohimbine to give the concentration shown for 22 min at 37°. The binding of [^{3}H] yohimbine was then determined, and the number of molecules of yohimbine bound per platelet was calculated (■). Simultaneously, duplicate samples of prelabeled platelets were incubated for 30 sec with 10 μM prostaglandin E_1, 0.2 mM RA233, 50 μM epinephrine and 10 mM EDTA, and their content of [^{14}C]cyclic AMP was determined (●). The curves were computed assuming the ranges of effect shown and K_{diss} for yohimbine of 5.0 nM and that half-maximal relief of epinephrine's inhibition of the adenylate cyclase occurs at 50 nM yohimbine. Two other similar experiments gave values for half-maximal binding and half-maximal relief of 2 and 20 nM and 7 and 25 nM, respectively.

An example of such an experiment is shown in Fig. 4, from which it can be seen that the concentration of yohimbine needed to block the epinephrine inhibition of the adenylate cyclase by one-half was 10 times that needed to half-saturate the receptor. Note that the duration of exposure to epinephrine was the same as the time allowed for dissociation of nonspecifically bound material (30 sec). In both cases we anticipate about 10% dissociation of specifically bound material (Fig. 1), and

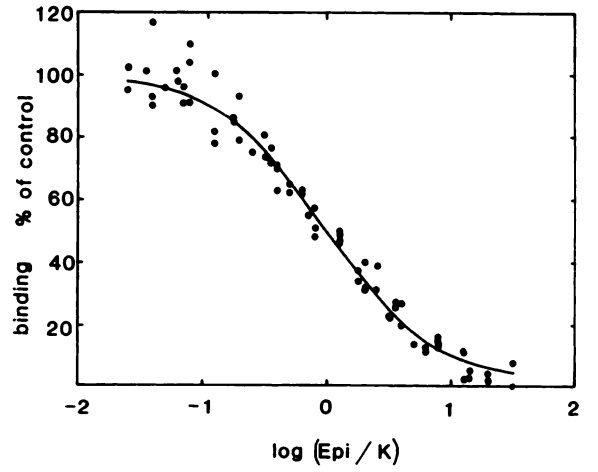

FIG. 5. *Competition by epinephrine for [^{3}H]yohimbine binding*

In each of four experiments, washed platelets were incubated with 2 nM [^{3}H]yohimbine plus epinephrine at various concentrations for 20 min. Bound radioactivity was determined, these data were plotted against the logarithm of the epinephrine concentration, and a computed curve was fitted to them. These data were then re-expressed as percentage of displaceable binding, and are plotted versus the logarithm of the epinephrine concentration divided by the concentration for 50% displacement (1.6, 5.0, 2.8, and 2.5 μM). The curve is computed.

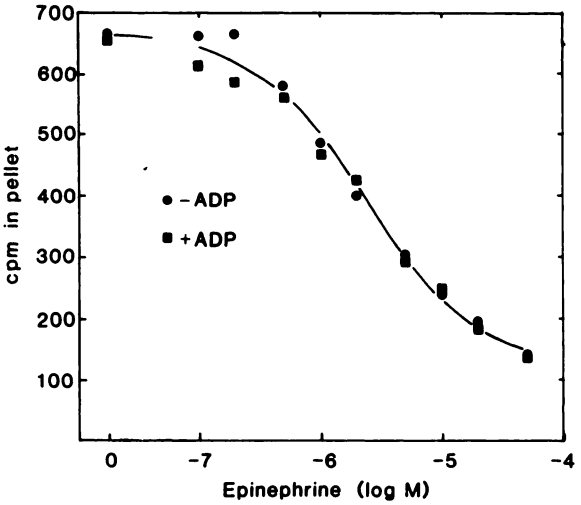

FIG. 6. *Effect of ADP on epinephrine's ability to displace [^{3}H] yohimbine*

Washed platelets were incubated with 2 nM [^{3}H]yohimbine and the concentrations of epinephrine shown with (■) or without (●) 20 μM ADP for 20 min at 37°. The platelets were diluted and centrifuged 30 sec later and the radioactivity of the pellet was determined. Each point represents the mean of duplicates. The curve was computed assuming half-maximal displacement by 2.5 μM epinephrine and nonspecifically bound radioactivity of 120 cpm.

this results in a small overestimate in the IC_{50} for blockade of the cyclase response.

Inhibition of [^{3}H]yohimbine binding by epinephrine. In several experiments we incubated platelets with a constant concentration of [^{3}H]yohimbine and increasing concentrations of epinephrine. On no occasion did we find evidence that the *$alpha_2$*-receptor presents more than one affinity for epinephrine. The data from four such experiments were normalized with respect to the concentration for half-maximal inhibition of binding, and for pellet counts in the absence and presence of an excess of epinephrine (Fig. 5). It can be seen that the data are clustered about the theoretical displacement curve cal-

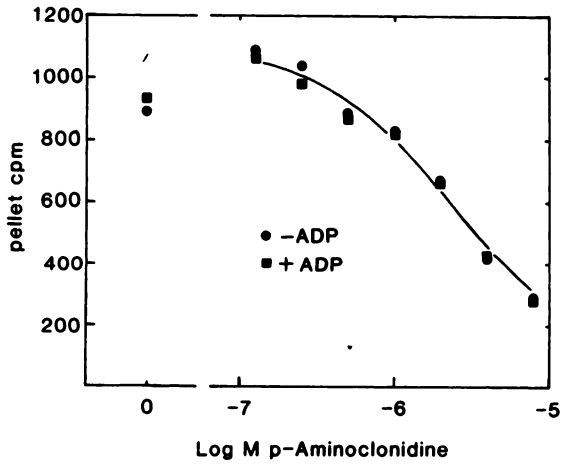

FIG. 7. *Effect of ADP on inhibition of [^{3}H]yohimbine binding by p-aminoclonidine*

The experiment was conducted as described in Fig. 6 except that epinephrine was replaced with *p*-aminoclonidine. The means of duplicates from each of two experiments are shown. The curve was computer-drawn, assuming half-maximal inhibition at 2.5 μM.

culated on the basis of simple competition for a site of uniform affinity.

Receptor interactions. Although the experiment described above appears to show simple competition for a single site, we tried to reveal the existence of high-affinity sites by blocking their formation. We did this by exposing platelets to ADP, which, like epinephrine, inhibits the adenylate cyclase. Such an experiment is shown in Fig. 6, and it can be seen that ADP did not cause the curve to be shifted to the right. We also peformed this experiment with an agonist for the *alpha*$_2$-receptor with lower intrinsic activity, namely *p*-aminoclonidine, which inhibits the adenylate cyclase of intact platelets by about 60% (21). The result (Fig. 7) was the same as with epinephrine; that is, occupation of the ADP receptor did not influence the ability of *p*-aminoclonidine to suppress the binding of [^{3}H]yohimbine.

DISCUSSION

The three models presented in Scheme 1 give rise to different predictions regarding the shape of the agonist-binding curves and the relationship between the fractional effect of an agonist and its fractional occupation of the receptor. This enables us to choose between them experimentally. When diagnostic experiments have been carried out to choose between these alternatives on intact cells, in general the data have been compatible with the collision coupling model, but these have been performed only with agonists that activate the adenylate cyclase (6–9).

Blood platelets are particularly suitable for the analysis of coupling mechanisms, because they have an adenylate cyclase that responds very rapidly to at least eight pharmacological receptors, of which four deactivate the enzyme (5). We can simultaneously measure the rate of accumulation of metabolic cyclic AMP and the occupancy of the *alpha*$_2$-adrenergic deactivating receptor in the intact cells, and thus preserve the normal architecture of the membrane components as well as the intracellular complement of ions and nucleotides.

We found that all receptors were equivalent in that they present a uniform affinity for the antagonist yohimbine and for the agonist epinephrine; there was no detectable fraction of receptors with high affinity for epinephrine. The degree of inhibition of cyclic AMP accumulation by epinephrine exceeded its occupancy of the *alpha*$_2$-receptor, and the blockade of the effect of the receptor by yohimbine was less than the occupancy of the receptor by this antagonist. These results are clearly incompatible with the precoupled model (Model I), which predicts that the fractional effect of an agonist should equal its occupancy of the receptor.

The floating receptor model (Model II) predicts that the fractional effect of an agonist will exceed its occupancy of the receptor (10, 11), but this model postulates that two species of receptor should be present: one with high affinity for the agonist corresponding to the *HRNC* complex and one with low affinity corresponding to the remaining uncoupled receptors (*HR* or *HRN*).

We found no evidence for the existence of these predicted high-affinity sites for epinephrine. Our ability to detect these sites experimentally is a function of both their abundance and the difference in the dissociation constants of the high- and low-affinity states. At saturation, epinephrine inhibits the adenylate cyclase by 60–90%, implying that the number of *HRNC* complexes in Model II must be a substantial fraction of the number of adenylate cyclase units. The number of these units in the platelet has not been determined directly, but a rough estimate can be made from the rate of cyclic AMP accumulation within platelets stimulated maximally with prostacyclin. These platelets initially synthesize cyclic AMP at a rate of about 10% of their metabolic ATP per minute (e.g., Fig. 5 of ref. 13), or 3×10^6 molecules of cyclic AMP per platelet per minute. The turnover number of a purified bacterial adenylate cyclase is reported to be about 1.4×10^3/minute (see ref. 6), suggesting that the platelet has about 2000 catalytic units/cell. Although this sort of calculation is subject to a variety of errors (see discussion of ref. 6), it suggests that the number of high-affinity *HRNC* sites predicted by Model II should be substantially higher than the resolution of our binding assay (about 20 sites/cell); thus they should be easily detectable if their affinity for epinephrine is at least 2 orders of magnitude higher than that of the uncomplexed receptor.

If the affinity difference is of lesser magnitude, however, the experimentally observed binding curve will differ only slightly from that obtained with a single site of intermediate affinity, so we also sought to demonstrate the existence of *HRNC* complexes by interfering with their formation. We did this by adding an agonist which inhibits the adenylate cyclase via another receptor, that for ADP. This maneuver is designed to take advantage of the assumption implicit in the floating receptor hypothesis; namely, that receptors with like action inhibit the adenylate cyclase by binding to the same site on the catalytic unit; it is upon this assumption that the elegance of the floating receptor model rests. Since the ADP receptor and the *alpha*$_2$-adrenergic receptor cannot both form *HRNC* complexes with the same catalytic unit, they should mutually suppress the formation of their respective high-affinity states. In fact, ADP did not influence the ability of epinephrine (or the weaker agonist, *p*-aminoclonidine) to displace yohimbine from the receptor. On the basis of these data, then, we reject the floating receptor model.

Our data are clearly compatible with the collision coupling model (Model III), which assumes that the *HRNC* complex is only transitorily formed, but results in a persistent change in the activity of the adenylate cyclase. This raises the interesting possibility that the adenylate cyclase is not only switched on by a brief interaction with an activating receptor complex as suggested by Tolkovsky and Levitzki (12), but that it is also switched off by a similar interaction with a deactivating receptor complex. Since the receptors appear to have a catalytic role in switching the adenylate cyclase between two stable states, and this catalysis is regulated by external stimuli, we refer to this possibility as the "allozyme" hypothesis. In the following paper (18) we present a mathematical analysis of the properties of this model.

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