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Apparent "Down-Regulation" of Human Platelet *Alpha*$_2$-Adrenergic Receptors Is Due to Retained Agonist

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SUMMARY

Platelets that have been incubated with (−)-epinephrine exhibit diminished responsiveness. It has been suggested that this desensitization results from "down-regulation" (loss in number) of *alpha*-adrenergic receptors. We have tested this hypothesis by examining binding of [^{3}H]yohimbine, an *alpha*$_2$-adrenergic antagonist, to platelets that had been preincubated *in vitro* with (−)-epinephrine. Binding of 10 nM [^{3}H]yohimbine to intact platelets was decreased in platelets preincubated with (−)-epinephrine, but this decrease occurred only in platelets incubated with ≥10 μM (−)-epinephrine, a concentration far in excess of physiological concentrations. Incubation of platelets with 100 μM (−)-epinephrine for 4 hr reduced the binding of [^{3}H]yohimbine (10 nM) by 25%, and incubation for 16–22 hr reduced binding by 53%. Because platelets can transport and store (−)-epinephrine, we performed experiments to determine whether the decreased radioligand binding to receptors was due to true down-regulation or was due to competition by retained (−)-epinephrine. The maximal number of [^{3}H]yohimbine binding sites did not change in platelets incubated for 4 hr with 100 μM (−)-epinephrine, but this incubation increased the apparent dissociation constant for [^{3}H]yohimbine from 2.9 nM to 5.3 nM ($n = 5$; $p < 0.001$). Membranes were prepared from the (−)-epinephrine-preincubated platelets to facilitate removal of retained (−)-epinephrine; in the presence of Na^+ and GTP (to reduce the affinity of (−)-epinephrine for the receptor), [^{3}H]yohimbine bound identically to these membranes and to membranes from control platelets. To estimate the amount of retained (−)-epinephrine, we incubated platelets with 100 μM (−)-epinephrine and a tracer quantity of ^{3}H-labeled (−)-epinephrine and determined that ~1% of the radioactivity remained with the washed platelets. Finally, we measured the affinity of (−)-epinephrine for [^{3}H]yohimbine sites in membranes prepared from platelets preincubated with (−)-epinephrine and found that it was similar to that of control platelets. Thus incubation of platelets with (−)-epinephrine changes neither the number nor the affinity of platelet *alpha*$_2$-adrenergic receptors. The decrease in [^{3}H]yohimbine binding following incubation of platelets with epinephrine results from epinephrine retained by intact platelets and platelet membranes.

INTRODUCTION

There is considerable evidence that hormone-specific or homologous desensitization involves alterations in the affinity or number of drug and hormone receptors (1). The decrease in receptor number produced by homologous hormones and drugs has been termed "down-regulation." Such regulation of receptors, which has been invoked as an important feedback mechanism modulating drug responses, has been observed in a variety of hormone and neurotransmitter systems (1, 2). For example, catecholamine-specific desensitization of *beta*-adrenergic receptors coupled to adenylate cyclase has been extensively documented and studied both *in vivo* and *in vitro* (2–7).

By contrast, there is a relative paucity of information regarding possible mechanisms of desensitization of the *alpha*-adrenergic receptor, although desensitization is readily detected at these receptors in several systems. Strittmatter *et al.* (8) have shown that the rapid desensitization of the *alpha*-adrenergic receptor of the rat parotid gland depends on membrane voltage and is associated with a decrease in [^{3}H]DHE[3] binding. Colucci *et*

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[3] The abbreviations used are: DHE, dihydroergocryptine; PRP, platelet-rich plasma; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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al. (9) reported that mesenteric artery preparations from rats treated with (−)-epinephrine for several days had fewer numbers of ^{3}H-labeled WB 4101 (*alpha*$_1$-adrenergic) binding sites than did preparations from untreated rats. In examining desensitization of the human platelet *alpha*-adrenergic receptor, Cooper *et al.* (10) observed that incubation of platelets with (−)-epinephrine for 4 hr decreased both (−)-epinephrine-stimulated platelet aggregation and [^{3}H]DHE binding; these authors concluded that the desensitization was caused by down-regulation of platelet *alpha*-adrenergic receptors.

Because we were intrigued by these observations, we set out to use human platelets as a model system with which to examine agonist-mediated down-regulation of *alpha*-adrenergic receptors. Because *alpha*-adrenergic receptors on human platelets are exclusively of the *alpha*$_2$ subclass (11, 12) and because we recently found that [^{3}H]yohimbine was a useful radioligand for examining these receptors in both intact platelets and platelet membranes (13), we performed studies designed to characterize more fully down-regulation on human platelets. Our findings indicate that incubation with (−)-epinephrine does *not* down regulate the *alpha*$_2$-adrenergic receptors of human platelets.

MATERIALS AND METHODS

Drugs and chemicals.[^{3}H]Yohimbine (~80 Ci/mmole) and ^{3}H-labeled(−)-epinephrine (78 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). (−)-Epinephrine bitartrate and (+)-epinephrine bitartrate were gifts from Sterling-Winthrop (New York, N. Y.). Phentolamine mesylate was a gift from Ciba-Geigy (Summit, N. J.). Phenylephrine HCl and GTP were obtained from Sigma Chemical Company (St. Louis, Mo.). Dulbecco's modified Eagle's medium was obtained from Grand Island Biological Company (Grand Island, N. Y.). All other chemicals were of reagent grade.

Platelet preparation and incubation. Peripheral venous blood was drawn from healthy men and women, aged 22–44, who had taken no medication for at least 1 week and who gave voluntary informed consent for participation in the study. Blood was drawn into 60-ml plastic syringes containing 6 ml of 3.8% sodium citrate and was immediately centrifuged for 20 min at 300 × *g*. In most experiments, the resultant PRP was then incubated for 4 hr with 100 μM (−)-epinephrine in a polypropylene test tube, which was inverted several times during the incubation. The (−)-epinephrine was prepared in HCl so that the final HCl concentration in PRP was 1 mM, and the same concentration was included with the control incubations. Antioxidants were not included in the incubation mixture because previous work has shown that catecholamines are only very slowly degraded in plasma (14). Following incubation, the platelet suspensions were centrifuged at 5000 × *g* at 4° for 10 min and washed four times. Washed platelets were then resuspended at 25° in fresh incubation buffer containing 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, and 0.8 mM ascorbic acid (pH 7.5). In other experiments, PRP was incubated overnight (16–22 hr) at 37° in Dulbecco's modified Eagle's medium (PRP/Dulbecco's modified Eagle's medium, 1:2) to which penicillin (final concentration 50 IU/ml) and streptomycin (final concentration 50 μg/ml) had been added; the platelets were then washed as described above. Platelets were counted in a Coulter counter ZBI using a 70-μm aperture tube.

Platelet membrane preparation. In experiments in which platelet membranes were used, the washed platelet suspension was resuspended in an ice-cold buffer containing 5 mM Tris-HCl and 0.5 mM EDTA (pH 7.5), and was homogenized using 12 strokes of a "no-clearance" Teflon-glass homogenizer (Kontes Company, Vineland, N. J., Models K886031 and K885752) as previously described (15). The homogenate was then centrifuged at 27,000 × *g* for 10 min at 4°, the pellet was washed two additional times in this buffer, and the membranes were resuspended in ice-cold 50 mM Tris buffer (pH 7.5) containing 0.5 mM EGTA and 8 mM $MgCl_2$. This membrane preparation was used immediately for binding experiments. Electron microscopy of this preparation showed a heterogeneous mixture of vesicles ranging from 0.2 to 2 μm (average ~0.5 μm) in diameter (data not shown). The concentration of protein in this membrane preparation was assayed by the method of Lowry *et al.* (16), using bovine serum albumin standards.

[^{3}H]Yohimbine binding. Platelets or platelet membranes were incubated with [^{3}H]yohimbine in a total volume of 0.25 ml at 25° for 30 min in 16 × 105 mm polypropylene (Sarstadt No. 580) tubes, using the buffers described above. We terminated the binding reactions by adding 10 ml of incubation buffer (25°) and immediately filtering the contents of the tubes over glass fiber filters (Whatman GF/C). The test tubes and filters were washed with an additional 10 ml of buffer, and the radioactivity retained on the filter was determined in a liquid scintillation counting system which counted ^{3}H at an efficiency of 32%. Filtering and washing required less than 30 sec and were performed using a Millipore filtration manifold. Specific binding was defined as binding that could be competed for by 10 μM phentolamine. In general, nonspecific binding was less than 30% of total [^{3}H]yohimbine binding with both intact platelets and platelet membranes (12, 13).

Statistics. Results are reported as means ± standard error; statistical significance was calculated with two-tailed *t*-tests.

RESULTS

Effects of incubation of platelets with agonists on subsequent [^{3}H]yohimbine binding to intact platelets. In initial experiments, we incubated freshly prepared PRP with 100 μM (−)-epinephrine and examined the change in [^{3}H]yohimbine binding as a function of time. Although we readily detected a significant decrease in binding after 4 hr, shorter incubation periods produced less reproducible decreases in [^{3}H]yohimbine binding (data not shown). Thus, incubations of 4 hr were routinely used for most subsequent experiments; this is the same incubation time that was used in an earlier report of desensitization of platelets (10).

We next incubated PRP for 4 hr with varying concentrations of (−)-epinephrine and assessed the change in *alpha*$_2$-adrenergic receptors by incubating washed platelets with 10 nM [^{3}H]yohimbine. This concentration of

[^{3}H]yohimbine was chosen because it was 4-fold greater than the K_D of [^{3}H]yohimbine in human platelets (12), because it demonstrated a high ratio of specific to nonspecific binding, and because using a single concentration of [^{3}H]yohimbine minimized the amount of blood needed. No decrease in [^{3}H]yohimbine binding occurred after incubations of platelets for 4 hr with <10 μM (−)-epinephrine, a modest decrease in [^{3}H]yohimbine binding was noted with 10 μM (−)-epinephrine, and a larger and more consistent decrease (25 ± 9%) was observed at 100 μM (−)-epinephrine (Fig. 1). When PRP was incubated overnight (16–22 hr) with (−)-epinephrine, we found a greater decrease in [^{3}H]yohimbine binding than occurred after 4 hr (53% versus 25%), but the concentration of (−)-epinephrine required was similar to that observed in the shorter incubations (Fig. 1).

The decrease in [^{3}H]yohimbine binding produced by incubating platelets with (−)-epinephrine was stereoselective: incubations with (+)-epinephrine (the enantiomer which is not active physiologically) failed to decrease subsequent [^{3}H]yohimbine binding (Fig. 2). Incubation of platelets with 100 μM phenylephrine, a synthetic *alpha*$_1$-adrenergic agent that is a partial agonist in platelets (18), decreased subsequent binding of [^{3}H]yohimbine to washed platelets to an extent (24 ± 3%) similar to that produced by incubation of platelets with 100 μM (−)-epinephrine.

Although incubation of platelets for several hours with (−)-epinephrine decreased subsequent [^{3}H]yohimbine binding, our initial studies did not allow us to distinguish between decreased receptor affinity and decreased receptor number (down-regulation). To help resolve this, we incubated platelets with 100 μM (−)-epinephrine for 4 hr and quantitated the receptors by determining binding isotherms of [^{3}H]yohimbine. As Fig. 3 shows, the expected binding isotherm in "true" down-regulation is characterized by a decrease in maximal binding without a change in affinity of the radioligand. In contrast, "pseudo" down-regulation, which would occur if agonist were retained by the cells, would be characterized by a decrease in apparent affinity without a change in maximal binding. A representative result of the binding isotherm that we obtained with platelets preincubated with 100 μM epinephrine for 4 hr is shown in Fig. 4 and pooled data from five such experiments are shown in Fig. 5. The maximal number of binding sites per platelet was 272 ± 7 in control platelets and 300 ± 28 in the platelets incubated with (−)-epinephrine ($p > 0.05$). The apparent K_D for [^{3}H]yohimbine was 2.9 ± 0.2 nM in control platelets and 5.3 ± 0.4 nM in the platelets preincubated with (−)-epinephrine for 4 hr ($p < 0.001$). Thus, preincubation of platelets with (−)-epinephrine decreased the apparent affinity (and thus increased the K_D) of the receptors for [^{3}H]yohimbine without decreasing their number. As noted above, this is the result expected if (−)-epinephrine had been inadequately washed from the platelets.

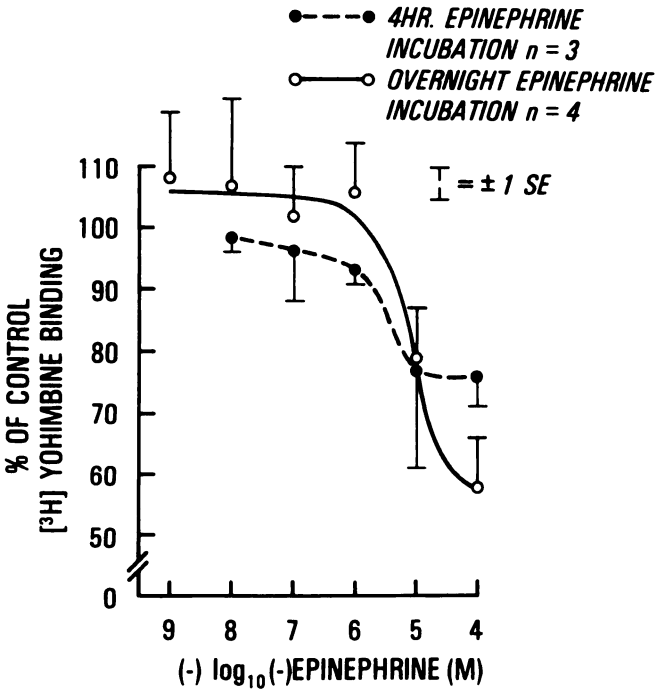

FIG. 1. *Effect of preincubation with (−)-epinephrine on [^{3}H]yohimbine binding to intact platelets*

PRP was incubated at 37° with various concentrations of (−)-epinephrine for 4 hr (○) or for 16–22 hr (●). Platelets were then washed, and the specific binding of 10 nM [^{3}H]yohimbine was determined. Each result was compared with results for control platelets incubated without (−)-epinephrine. The results shown are the mean of three or four experiments performed in triplicate. Note that the physiological plasma (−)-epinephrine concentration is less than 10 nM (ref. 17).

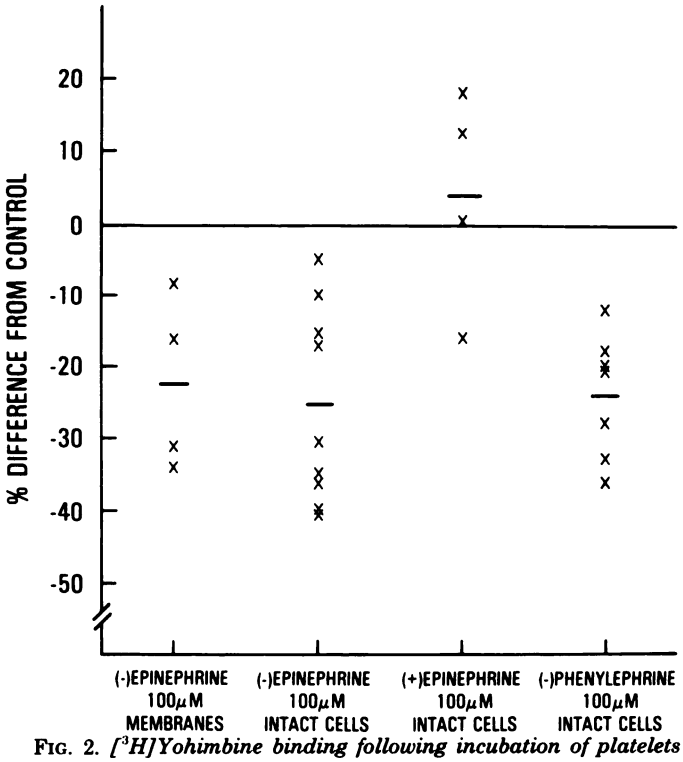

FIG. 2. *[^{3}H]Yohimbine binding following incubation of platelets with (−)-epinephrine, (+)-epinephrine, or phenylephrine*

PRP was incubated with 100 μM concentrations of each drug at 37° for 4 hr. The platelets were then washed and the specific binding of 10 nM [^{3}H]yohimbine was determined and compared with that of platelets incubated without any drug. The *left panel* shows the results of [^{3}H]-yohimbine binding to membranes prepared from platelets that had been incubated with 100 μM (−)-epinephrine. The binding with membranes was determined in the same isotonic buffer used for intact platelets.

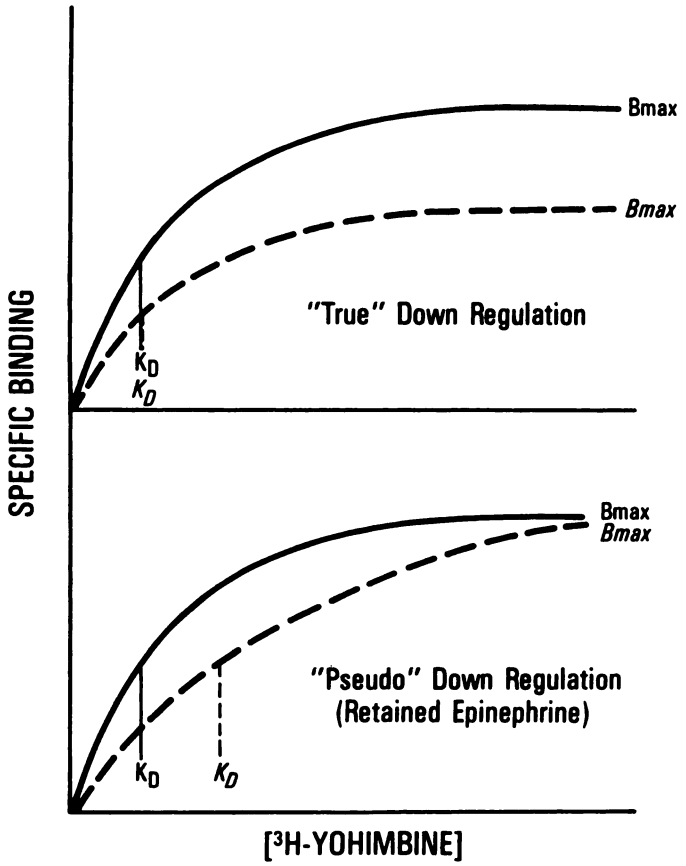

FIG. 3. *Expected changes in binding isotherms due to "true" down-regulation (top) or "pseudo" down-regulation due to retained agonist (bottom)*

In "true" down-regulation, maximal binding capacity is decreased but the K_D is unaltered. With "pseudo" down-regulation caused by retained agonist competing for receptors, more radioligand must be used to bind any given fraction of the receptors. Therefore the apparent K_D is increased whereas the maximal binding capacity (B_{max}) is unchanged.

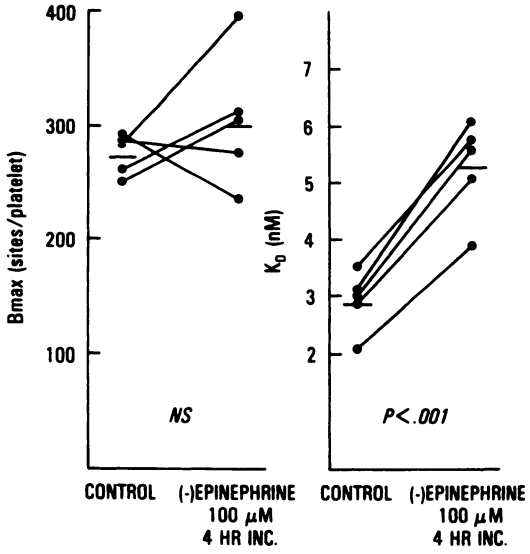

FIG. 5. *Pooled data from binding isotherms of [^{3}H]yohimbine and control or (−)-epinephrine-preincubated platelets*

Experiments were performed as described in the legend to Fig. 4 for (−)-epinephrine-preincubated and control platelets. Data are shown for five experiments; in each, the [^{3}H]yohimbine binding to (−)-epinephrine-preincubated and control platelets was performed in parallel.

Effects of incubation of platelets with (−)-epinephrine on subsequent [^{3}H]yohimbine binding to platelet membranes. Although the results with intact platelets suggested that true down-regulation had not occurred when platelets were incubated with agonists *in vitro*, it was conceivable that decreases in receptor number were transient and thus rapidly reversed under the conditions used to assay [^{3}H]yohimbine binding in intact platelets. Moreover, the results in intact platelets suggested that (−)-epinephrine retained by the platelets might account for the decreased [^{3}H]yohimbine binding. To diminish such retention we prepared membranes from the platelets. We

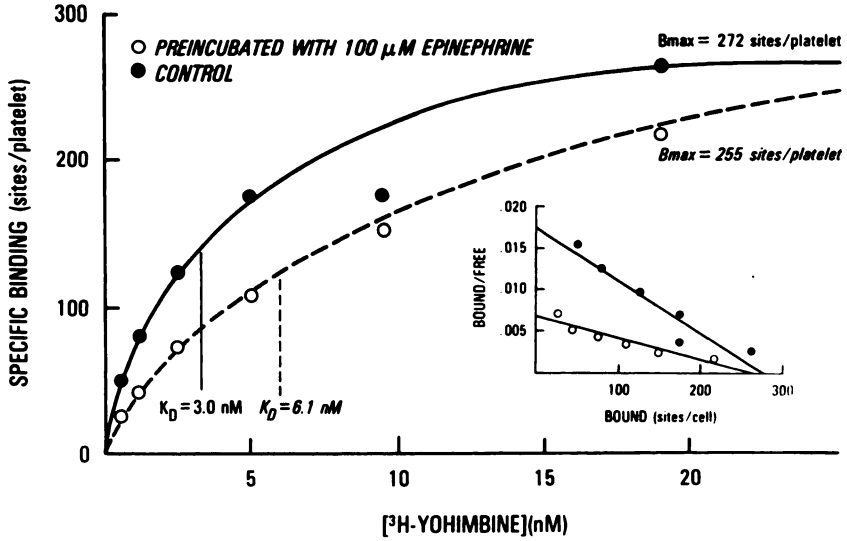

FIG. 4. *Typical binding isotherms of [^{3}H]yohimbine with control (●) and (−)-epinephrine-exposed (○) platelets*

Platelets were preincubated with or without 100 μM (−)-epinephrine for 4 hr and then washed four times. Binding was performed with intact platelets using concentrations of [^{3}H]yohimbine ranging from 0.5 to 20 nM. The specific binding was analyzed by the method of Scatchard (*inset*) in order to determine the maximal binding and the dissociation constant of [^{3}H]yohimbine. This experiment is one of the five shown in Fig. 5.

initially observed that membranes prepared from platelets incubated with (−)-epinephrine showed a decrease in [^{3}H]yohimbine (10 nM) binding that was similar (23 ± 10%) to the decline observed in the intact platelets (25 ± 9%) from which the membranes were derived (Fig. 2).

In order to determine whether retained (−)-epinephrine might explain the decreased [^{3}H]yohimbine binding in those membranes, we examined the effect of adding NaCl and GTP to membranes derived from (−)-epinephrine-preincubated platelets. The rationale for these experiments is the well-documented effect of Na^+ and GTP in decreasing the affinity of agonists for *alpha*-adrenergic receptors in platelets (13, 19, 20). We reasoned that, if retained (−)-epinephrine caused the decreased [^{3}H]yohimbine binding, then the addition of Na^+ and GTP would decrease the affinity of the receptors for the retained (−)-epinephrine and thereby would allow more [^{3}H]yohimbine to bind. Accordingly, platelets were incubated using 100 μM (−)-epinephrine, and [^{3}H]yohimbine binding was assessed using both intact cells and platelet membranes. [^{3}H]Yohimbine binding to membranes was determined both in the absence and in the presence of GTP and Na^+ ($n = 6$). In the intact platelets, the decline in [^{3}H]yohimbine (10 nM) binding was 25 ± 5%. The decline was similar in membranes assayed in the absence of GTP and Na^+ [33 ± 10% (Fig. 6)]. However, in the presence of added GTP (0.1 mM) and Na^+ (100 mM), [^{3}H]yohimbine binding in membranes from epinephrine-preincubated platelets was similar to binding of control platelets (Fig. 6). Binding isotherms of membranes from (−)-epinephrine-preincubated platelets were similar to those of intact platelets: the B_{max} was unchanged compared with control platelet membranes but the K_D was several-fold higher. When binding was performed in the presence of Na^+ and GTP there was no difference between membranes from control and (−)-epinephrine-preincubated platelets (data not shown).

Preincubation with ^{3}H-labeled (−)-epinephrine. To estimate the amount of (−)-epinephrine retained in our experimental protocols we carried out experiments using ^{3}H-labeled (−)-epinephrine. Intact platelets were incubated for 4 hr with 100 μM (−)-epinephrine plus a tracer amount (20,000–40,000 cpm) of ^{3}H-labeled (−)-epinephrine. Platelets were washed four times using the same procedure as in the binding experiments, and the radioactivity retained by the platelets was counted and expressed as a percentage of the added counts. After a 4-hr incubation, the amount of radioactivity retained ranged from 0.5 to 1.5% of the added radioactivity with a mean of 1.1% (four experiments).

Determination of the affinity of (−)-epinephrine for the alpha$_2$-adrenergic receptors. By analogy with recent observations with *beta*-adrenergic receptors (21, 22), it seemed possible that preincubation of platelets with an agonist might decrease the affinity of receptors for agonists without altering receptor number. We therefore determined the affinity of (−)-epinephrine for the *alpha$_2$*-adrenergic receptors in membranes prepared from platelets that had been preincubated either with or without 100 μM epinephrine. When tested in the presence of Na^+ and GTP, (−)-epinephrine competed identically for [^{3}H] yohimbine binding sites in membranes prepared from

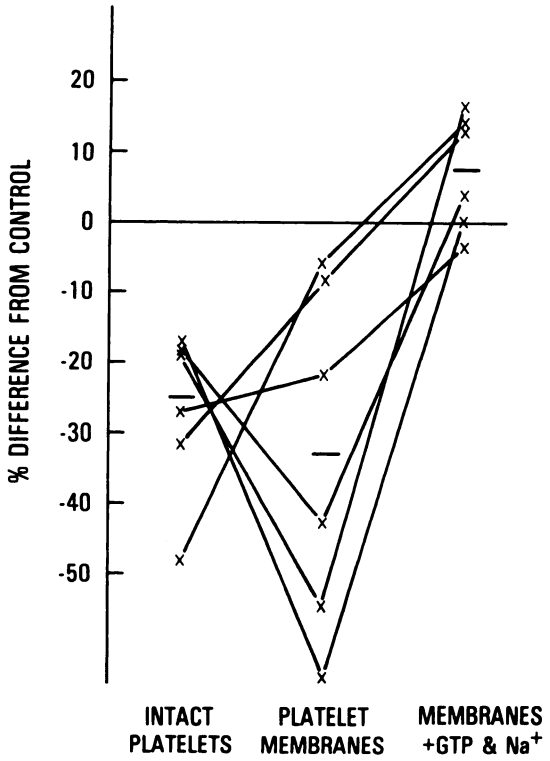

FIG. 6. *Specific binding of [^{3}H]yohimbine to platelets and platelet membranes after preincubation with (−)-epinephrine*

Platelets were incubated with or without 100 μM (−)-epinephrine for 4 hr and then washed four times. Some of the platelets were used intact (*left panel*) and the rest were used to prepare platelet membranes as described under Materials and Methods. For the [^{3}H]yohimbine binding the membranes were incubated in a buffer containing 50 mM Tris, 5 mM EGTA, and 8 mM $MgCl_2$ (*middle panel*) or in the same buffer with the addition of 100 mM NaCl and 100 μM GTP (*right panel*). The specific binding (expressed as sites per intact platelet or fentomoles per milligram of membrane protein) was compared with the specific binding of platelets (from the same individual) that had been preincubated without (−)-epinephrine.

(−)-epinephrine-preincubated and control platelets (data not shown). In contrast, when tested in the absence of Na^+ and GTP, (−)-epinephrine competed for [^{3}H]yohimbine binding sites differently when membranes prepared from (−)-epinephrine-preincubated platelets were compared with membranes prepared from control platelets, but only at concentrations of (−)-epinephrine less than 0.1 μM (Fig. 7). Our interpretation of these curves is discussed below.

DISCUSSION

Previous radioligand binding studies of desensitization to catecholamines have focused largely on the adenylate cyclase system coupled to *beta*-adrenergic receptors (1–7, 21, 22). We have examined a less well-studied *alpha*-adrenergic system, platelet *alpha$_2$*-adrenergic receptors, because functional desensitization has been reported at these receptors (10) and because these receptors are also coupled to adenylate cyclase, albeit to an inhibition of this enzyme (19, 23–25).

In the present study, we utilized the *alpha$_2$*-selective antagonist [^{3}H]yohimbine for assessing the regulation of *alpha*-adrenergic receptors in human platelets. We have

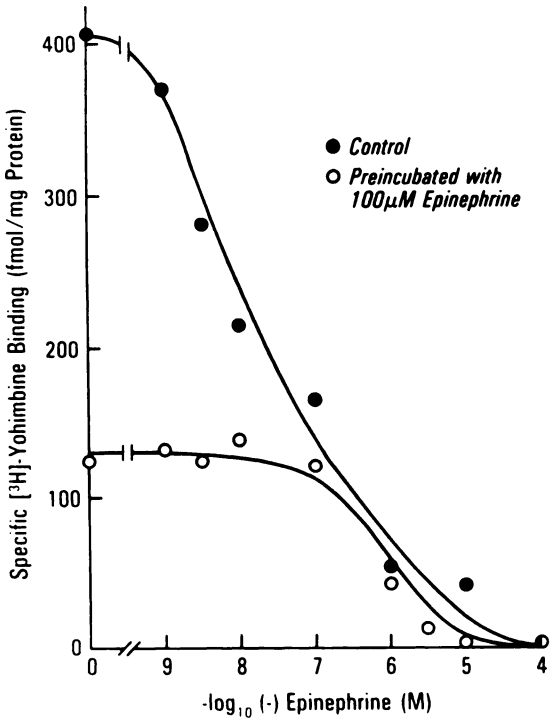

FIG. 7. *Competition of (−)-epinephrine for [^{3}H]yohimbine binding in membranes prepared from platelets preincubated with (○) and without (●) (−)-epinephrine*

PRP was incubated with 100 μM (−)-epinephrine for 4 hr, the platelets were washed, and membranes were prepared. Binding was performed with 7.1 nM [^{3}H]yohimbine plus added (−)-epinephrine at the indicated concentrations.

previously confirmed that all *alpha*-adrenergic receptors on platelets are of the *alpha*$_2$ subtype (11, 12) and that [^{3}H]yohimbine binds to these receptors on platelets and platelet membranes with high affinity (K_D of 2.7 nM) (12, 13). In the only previous report of desensitization of platelet *alpha*$_2$-adrenergic receptors (10), the investigators used the radioligand [^{3}H]DHE. We have found that [^{3}H]DHE binds to more sites on platelets and platelet membranes than does [^{3}H]yohimbine (13). Since [^{3}H]-yohimbine is more selective for *alpha*$_2$-adrenergic receptors and provides results that are much more reproducible in our hands than does [^{3}H]DHE, we have used [^{3}H]-yohimbine in the current study.

Our initial experiments indicated that incubation of platelets with (−)-epinephrine decreased subsequent [^{3}H]yohimbine binding. We observed only a modest decrease at 10 μM (−)-epinephrine and more striking decreases at 100 μM (−)-epinephrine (Fig. 1). A resting individual has a plasma concentration of (−)-epinephrine of about 1 nM and this rises to 10 nM with vigorous exercise; a patient with a pheochromocytoma has levels of (−)-epinephrine up to 1 μM (17). The concentration of (−)-epinephrine needed to produce a decline in [^{3}H]yohimbine binding in platelets is thus grossly in excess of physiological and even pathological levels. Similarly, Horne *et al.* (26) have reported in a recent abstract that incubation of platelets with 10 μM (−)-epinephrine does not alter subsequent [^{3}H]DHE binding, whereas incubation with 100 μM (−)-epinephrine causes a 70% loss in the specific binding of this radioligand. Also, Strittmatter *et al.* (8) found inhibition of [^{3}H]DHE binding in rat parotid cells but only after incubation with >1 μM (−)-epinephrine. Taken together, these observations suggest that, even if down-regulation of *alpha*-adrenergic receptors occurs, it requires elevated, nonphysiological (−)-epinephrine concentrations. However, in view of our other findings, retained hormone might account, at least in part, for the apparent decrease in receptor number in these previous reports.

Human platelets take up and store (−)-epinephrine. In our experiments with ^{3}H-labeled (−)-epinephrine we found that ~1% of the radioactivity was retained after incubation with 100 μM epinephrine. Could this be enough retained epinephrine to explain the decrease in [^{3}H]yohimbine binding in our studies? Born and Smith (27) have reported that following incubation of platelets with ^{3}H-labeled (−)-epinephrine, about one-half of the retained radioactivity is native epinephrine and the rest represents metabolites; about one-half of the retained (−)-epinephrine (~25% of the retained radioactivity) later diffused out of the platelets. In our experiments about 1% of the added radioactivity was retained by platelets incubated with ^{3}H-labeled (−)-epinephrine and 100 μM epinephrine. According to the findings of Born and Smith, it follows that about 0.25% of the epinephrine is potentially available as native drug for receptor interaction after the incubation and washes. Because the final suspension of platelets is concentrated 4-fold relative to the volume of the original PRP, we expect that the remaining concentration of (−)-epinephrine present in our binding assays would be ~1 μM (100 μM × 0.25% × 4). We have shown that the dissociation constant of (−)-epinephrine for [^{3}H]yohimbine sites is also about 1 μM (12, 13). Therefore, it is theoretically possible for enough (−)-epinephrine to be retained by the platelets during the incubations to explain the subsequent decrease in [^{3}H]yohimbine binding.

To determine whether retained epinephrine actually explained the decreased [^{3}H]yohimbine binding, we derived binding isotherms using intact washed platelets that had been incubated with 100 μM (−)-epinephrine for 4 hr. In contrast to the report of Cooper *et al.* (10), who found a decrease in the maximal number of binding sites with no change in the dissociation constant for [^{3}H]DHE, we observed no change in the maximal number of binding sites with an almost 2-fold increase in the dissociation constant for [^{3}H]yohimbine (Figs. 4 and 5). Thus our results are consistent with the hypothesis that retained agonist accounts for the decrease in [^{3}H]yohimbine binding. The binding isotherms also explain the results obtained when platelets were incubated with a single concentration of [^{3}H]yohimbine, because an increase in the dissociation constant of the radioligand would reduce the binding of [^{3}H]yohimbine at subsaturating radioligand concentrations. The reason for the discrepancy between our data and those of Cooper *et al.* (10) is uncertain but could in part be accounted for by the relative lack of specificity of the radioligand ([^{3}H]DHE) used in their studies (13).

Previously we have shown that the addition of Na^+ and GTP synergistically decreased the affinity of (−)-

epinephrine for [^{3}H]yohimbine binding sites of human platelet membranes; the resulting affinity of (−)-epinephrine in membranes was similar to that observed with intact platelets (12). In light of these observations, we reasoned that, if the decreased [^{3}H]yohimbine binding demonstrated after preincubation of platelets with (−)-epinephrine was the result of competition by retained (−)-epinephrine, lowering the affinity of the receptor for the (−)-epinephrine by the addition of both Na^+ and GTP should reverse this decrease. If, on the other hand, true down-regulation had occurred, the reduced [^{3}H]yohimbine binding would persist. We found (Fig. 6) that in the presence of Na^+ and GTP there was no difference between [^{3}H]yohimbine binding to the membranes prepared from platelets that had been preincubated with (−)-epinephrine and those that had not. These observations support the hypothesis that the apparent down-regulation observed with platelets preincubated with 100 μM (−)-epinephrine is the consequence of retained hormone.

Although we found no change in receptor number following incubation of platelets with (−)-epinephrine, we wondered whether the affinity of these receptors for (−)-epinephrine might be decreased. Others have reported that an "uncoupling" of *beta*-adrenergic receptors from adenylate cyclase is accompanied by a decrease in the affinity of these receptors for agonists, as determined by competition of agonists for radiolabeled antagonist binding sites (21, 22). In membranes prepared from (−)-epinephrine-preincubated platelets we expected that both the retained (−)-epinephrine and the (−)-epinephrine added in the binding assay would compete for [^{3}H]-yohimbine binding sites. We predicted that low concentrations of (−)-epinephrine added during binding assays would have a negligible effect compared with the effect of retained (−)-epinephrine, whereas retained epinephrine would have a negligible effect when high concentrations of (−)-epinephrine were added in the binding assays. Under these circumstances, and assuming no change in the affinity of the receptors for (−)-epinephrine, the "left half" of the (−)-epinephrine competition curve would exhibit diminished [^{3}H]yohimbine binding (caused by the retained (−)-epinephrine) compared with control, and the "right half" of the binding curve would be the same as the control. The point at which the curves for control and (−)-epinephrine-preincubated platelets merged would give an approximate estimate of the effective concentration of retained (−)-epinephrine.

Our results (Fig. 7) match this prediction. Membranes from (−)-epinephrine-preincubated platelets exhibited decreased [^{3}H]yohimbine binding, which did not change until the amount of added (−)-epinephrine was greater than ~0.1 μM. With concentrations of added (−)-epinephrine exceeding 0.1 μM, the binding curves for the (−)-epinephrine-preincubated and control platelets were the same. Thus the affinity of the receptors for (−)-epinephrine seemed to be unchanged in the membranes from the (−)-epinephrine-preincubated platelets. A more quantitative analysis is not possible because we do not know precisely the concentration of (−)-epinephrine that was retained and available. It is important to note that the manner in which the data are plotted may influence one's conclusions about changes in agonist affinity. Commonly, one plots competition of agonist for radioligand binding as "percentage of maximal specific binding," but this may not be appropriate when the maximal specific binding changes as a result of retained drug as it did here. We therefore expressed our data in absolute terms (Fig. 7) so that the change in maximal binding was not obscured.

In summary, to determine whether incubation of platelets with epinephrine down-regulates *alpha*$_2$-adrenergic receptors, we did five types of experiments. We first examined the binding of a single concentration of [^{3}H]-yohimbine, a selective *alpha*$_2$ antagonist, and found decreased binding in platelets that had been preincubated with (−)-epinephrine but only at nonphysiological (−)-epinephrine concentrations. To assess the degree to which our washing procedure removed 100 μM (−)-epinephrine, we measured the retention of ^{3}H-labeled (−)-epinephrine and found that even after four washes about 1% of the radioactivity remained with the platelets. We did two series of experiments to determine whether this retained epinephrine could explain the decreased yohimbine binding. Binding isotherms revealed that the receptor number was unchanged after (−)-epinephrine preincubation but that the apparent K_D was nearly doubled. We also examined membranes prepared from platelets preincubated either with or without epinephrine and found that under conditions designed to decrease the affinity of (−)-epinephrine the binding of [^{3}H]yohimbine was identical with both membrane preparations. These results can be explained by the effects of retained (−)-epinephrine; we found no evidence of down-regulation of platelet *alpha*$_2$-adrenergic receptors. Moreover, we found that the affinity of platelet *alpha*$_2$-receptors for (−)-epinephrine was essentially unaltered after preincubation of the platelets with epinephrine. Other investigators have found a decrease in aggregation following exposure of platelets to (−)-epinephrine (10). Since we find no evidence of down-regulation of *alpha*$_2$-adrenergic receptors, other mechanisms must be involved in this *alpha*-adrenergic desensitization.

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REFERENCES

1. Catt, K. J., J. P. Harwood, G. Aguilera, and M. L. Dufau. Hormonal regulation of peptide receptors and target cell responses. *Nature (Lond.)* **280**:109–116 (1979).
2. Lefkowitz, R. J., M. R. Wessels, and J. M. Stadel. Hormones, receptors and cyclic AMP: their role in target cell refractoriness. *Curr. Topics Cell Regul.* **17**:205–230 (1980).
3. Perkins, J. P., G. L. Johnson, and T. K. Harden. Drug-induced modification of the responsiveness of adenylate cyclase to hormones. *Adv. Cyclic Nucleotide Res.* **9**:19–32 (1978).
4. Shear, M., P. A. Insel, K. L. Melmon, and P. Coffino. Agonist-specific refractoriness induced by isoproterenol: studies with mutant cells. *J. Biol. Chem.* **251**:7572–7576 (1976).
5. Chuang, D., W. J. Kinnier, L. Farber, and E. Costa. A biochemical study of receptor internalization during β-adrenergic receptor desensitization of frog erythrocytes. *Mol. Pharmacol.* **18**:348–355 (1980).
6. Tohmeh, J. F., and P. E. Cryer. Biphasic adrenergic modulation of beta-adrenergic receptors in man: agonist-induced early increment and late decrement in beta-adrenergic receptor number. *J. Clin. Invest.* **65**:836–840 (1980).
7. Galant, S. P., L. Duriseti, S. Underwood, S. Allred, and P. A. Insel. Beta-adrenergic receptors of polymorphonuclear particulates in bronchial asthma. *J. Clin. Invest.* **65**:577–585 (1980).

8. Strittmatter, W. J., J. N. Davis, and R. J. Lefkowitz. Alpha-adrenergic receptors in rat parotid cells. II. Desensitization of receptor binding sites and potassium release. *J. Biol. Chem.* **252**:5478–5482 (1977).
9. Colucci, W. S., M. A. Gimbrone, Jr., and R. W. Alexander. Regulation of the postsynaptic alpha-adrenergic receptor in rat mesenteric artery: effects of chemical sympathectomy and epinephrine treatment. *Circ. Res.* **48**:104–111 (1981).
10. Cooper, B., R. I. Handin, L. H. Young, and R. W. Alexander. Agonist regulation of the human platelet alpha-adrenergic receptor. *Nature* (*Lond.*) **274**:703–706 (1978).
11. Hoffman, B. B., D. Mullikin-Kilpatrick, and R. J. Lefkowitz. Heterogeneity of radioligand binding to alpha-adrenergic receptors. *J. Biol. Chem.* **255**:4645–4652 (1980).
12. Motulsky, H. J., and P. A. Insel. Binding of [^{3}H]yohimbine and [^{3}H]prazosin demonstrates that platelet alpha-adrenergic receptors are of the alpha$_2$-subtype. *Clin. Res.* **29**:275A (1981).
13. Motulsky, H. J., S. J. Shattil, and P. A. Insel. Characterization of alpha$_2$-adrenergic receptors on human platelets using [^{3}H]yohimbine. *Biochem. Biophys. Res. Commun.* **97**:1562–1570 (1980).
14. Petterson, J., E. Hussi, and J. Jänne. Stability of human plasma catecholamines. *Scand. J. Clin. Lab. Invest.* **40**:297–303 (1980).
15. Shattil, S. J., M. McDonough, J. Turnbull, and P. A. Insel. Characterization of *alpha*-adrenergic receptors in human platelets using [^{3}H]clonidine. *Mol. Pharmacol.* **19**:179–183 (1981).
16. 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).
17. Koch, G., U. Johansson, and E. Arvidsson. Radioenzymatic determination of (−)-epinephrine, norepinephrine and dopamine in 0.1 ml plasma samples: plasma catecholamine response to submaximal and near maximal exercise. *J. Clin. Chem. Clin. Biochem.* **18**:367–372 (1980).
18. Jacobs, K. H. Synthetic alpha-adrenergic agonists are potent alpha-adrenergic blockers in human platelets. *Nature* (*Lond.*) **274**:819–820 (1978).
19. Tsai, B. S., and R. J. Lefkowitz. Agonist-specific effects of guanine nucleotides on *alpha*-adrenergic receptors in human platelets. *Mol. Pharmacol.* **16**:61–68 (1979).
20. Michel, T., B. B. Hoffman, and R. J. Lefkowitz. Differential regulation of the alpha$_2$-adrenergic receptor by Na^+ and guanine nucleotides. *Nature* (*Lond.*) **288**:709–711 (1980).
21. Su, Y., T. K. Harden, and J. P. Perkins. Catecholamine-specific desensitization of adenylate cyclase. *J. Biol. Chem.* **255**:7410–7419 (1980).
22. Wessels, M. R., D. Mullikin, and R. J. Lefkowitz. Selective alteration in high affinity agonist binding: a mechanism of *beta*-adrenergic receptor desensitization. *Mol. Pharmacol.* **16**:10–20 (1979).
23. Jakobs, K. H., W. Saur, and G. Schültz. Reduction of adenylate cyclase activity in lysates of human platelets by the alpha-adrenergic component of epinephrine. *J. Cyclic Nucleotide Res.* **2**:381–392 (1976).
24. Insel, P. A., P. Nirenberg, J. Turnbull, and S. J. Shattil. Relationships between membrane cholesterol, α-adrenergic receptors and platelet function. *Biochemistry* **17**:5269–5274 (1978).
25. Steer, M. L., and A. Wood. Regulation of human platelet adenylate cyclase by epinephrine, prostaglandin E, and guanine nucleotides. *J. Biol. Chem.* **254**:10791–10797 (1979).
26. Horne, W. C., R. I. Handin, and R. W. Alexander. Desensitization of (−)epinephrine-induced platelet aggregation—possible uncoupling of the platelet alpha-adrenergic receptor. Circulation (*Suppl. III*) **62**:335 (1980).
27. Born, G. V. R., and J. B. Smith. Uptake, metabolism and release of [^{3}H] adrenaline by human platelets. *Br. J. Pharmacol.* **39**:765–778 (1970).

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