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BINDING OF [^{3}H]-*p*-AMINOCLONIDINE TO α_2-ADRENOCEPTOR STATES PLUS A NON-ADRENERGIC SITE ON HUMAN PLATELET PLASMA MEMBRANES

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Abstract—Characterization of the binding of [^{3}H]*p*-aminoclonidine ([^{3}H]PAC) to purified plasma membranes from human platelets has revealed multiple binding sites. [^{3}H]PAC identified site-1 in the picomolar affinity range (site-1 K_D estimates ranged from 13 to 94 pM). Site-1 displayed a rank order of competition by various compounds for [^{3}H]PAC, indicative of an α_2-adrenoceptor, and was sensitive to 0.1 mM GTP. [^{3}H]PAC also identified a second site with nanomolar affinity (site-2 K_D estimates ranged from 0.7 to 1.7 nM). In the presence of 0.1 mM GTP, site-2 was not diminished significantly. Also in contrast to site-1, site-2 displayed low affinity for yohimbine (YOH), (−)-epinephrine and (−)-norepinephrine (NE). Therefore, site-2 could not be an active α_2-adrenoceptor; instead it had properties similar to a previously reported imidazoline-preferring binding site. A third site (site-3) bound [^{3}H]PAC with a K_D for site-3 of 26.6 ± 10.0 nM (SD). Site-3 had a rank order of competition by various compounds for 5 nM [^{3}H]yohimbine ([^{3}H]YOH) binding which was indicative of an α_2-adrenoceptor. (−)-NE competed for 5 nM [^{3}H]YOH binding at two sites: site-1 K_i = 32 pM, site-3 K_i = 239 nM. Treatment with 0.1 mM GTP completely removed site-1 and transferred the competitive binding of (−)-NE to low affinity (K_i = 437 nM). Thus, site-3 appears to be a free α_2-adrenoceptor. B_{max} estimates for untreated membranes, derived from simultaneous multi-experiment curve-fitting analyses, were site-1 = 36 ± 29 fmol/mg plasma membrane protein, site-2 = 95 ± 34 fmol/mg and site-3 = 154 ± 35 fmol/mg. We are the first to report a site for [^{3}H]PAC binding on platelets (site-2) with properties uncharacteristic of an adrenoceptor. This observation appears to be due to our use of purified plasma membrane and low ionic strength buffer. These studies relate to reports of increased binding of [^{3}H]PAC to platelets from depressed patients.

An α_2-adrenoceptor obtained from human platelets has been purified and sequenced [1]; many of the characteristics of this receptor are similar to those reported in brain [2, 3]. Accordingly, platelet α_2-adrenoceptors have been proposed to be a possible index of the status of these receptors in human brain. Platelet adrenoceptors have been studied in depressive illness, panic disorder, heroin addiction, schizophrenia, eating disorders, Parkinson's disease, orthostatic hypotension, type A behavior, premenstrual dysphoria, post-partum depression, and borderline personality disorder (reviewed in Refs. 4–9).

Until recently, brain α_2-adrenoceptors were believed to be a single molecular species, identical to those on platelets [2, 10]. It is now known that there are three genes encoding α_2-adrenoceptors [1, 11, 12]. In brain, two α_2-adrenoceptor subtypes have been identified [13, 14]. In addition, two to four "affinity states" of receptor binding have been proposed [15–17]. The brain contains at least type 2A and 2C α-adrenoceptors [12]. α_{2A}-Adrenoceptors predominate over type 2C in frontal cortex [12–14]. In addition, a non-adrenergic imidazoline-preferring site with some "α_2-adrenoceptor-like" binding properties has been detected in cerebral cortex and is abundant in human, rat, and bovine brain stem [18, 29]. "Non-adrenergic" binding sites for the α_2-adrenergic antagonist idazoxan have also been reported in brain [20, 21] and platelets [22, 23]. By pharmacological comparison, platelets contain the α_{2A}-adrenoceptor subtype [14]. However, Neubig *et al.* [24] presented evidence that [^{3}H]*p*-aminoclonidine ([^{3}H]PAC),†† an α_2 partial agonist, binds to three sites in platelets. Moreover, platelet α_2-adrenoceptor binding appears to be heterogeneous and not explainable by affinity states alone [24]. Clare *et al.* [25] have suggested that different α_2-adrenoceptor subtypes mediate adenylate cyclase inhibition and epinephrine-stimulated aggregatory responses in human platelets. α_{2A}-Adrenoceptors exist in two GTP-sensitive "states" dependent on the degree of coupling to an inhibitory GTP binding protein (G_i) [16, 26].

Radioligand binding to α_2-adrenoceptors using

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†† Abbreviations: ^{3}H, tritium; PAC, *p*-aminoclonidine; CLON, clonidine, YOH, yohimbine; (−)-NE, (−)-norepinephrine; GTP, guanosine triphosphate; G_i, GTP binding regulatory protein; E, exponential ($\times 10^{nth}$); *K*, experimental binding affinity parameter; and SEE, standard error of the estimates.

[^{3}H]clonidine ([^{3}H]CLON) and [^{3}H]PAC has been *uniquely* informative in studies of depressive illness. Chronic administration of desipramine resulted in *down-regulation* of [^{3}H]CLON binding in several rat brain regions which paralleled the time course of the efficacy of the antidepressant drug in depressed patients [27]. Down-regulation of [^{3}H]CLON binding was also reported after electroconvulsive [7, 28] and lithium carbonate [29] treatments in brains of experimental animals. Long-term antidepressant drug treatment of patients also resulted in down-regulation of platelet [^{3}H]CLON binding sites [4]. However, most studies reported no antidepressant drug effect on receptor number in rat brain as detected using α_2-adrenoceptor *antagonists* for radioligand binding [28, 30]. Additional evidence for an abnormality in CLON binding sites in depression includes the following: [^{3}H]CLON binding is elevated in prefrontal cerebral cortices from suicide victims which were identified retrospectively as being depressed [31]. Clonidine infusion into the locus coeruleus of rats reverses "depressive" symptomatology in the learned helplessness model of depression [32]. The number of [^{3}H]CLON and [^{3}H]PAC binding sites is elevated in platelets from depressed patients [4–7, 33–36]. By contrast, the number of ^{3}H-antagonist binding sites is not altered in platelets from depressed patients compared to healthy subjects [5, 7].

As described above, there has been a need to characterize precisely the multiple binding sites for [^{3}H]PAC on platelets. Also, the proper interpretation of previous data with depressed patients will require identification of the binding sites for imidazoline compounds. Therefore, we have characterized the sites for [^{3}H]PAC on platelet purified plasma membranes.

MATERIALS AND METHODS

Samples. All studies were performed with 1 to 3-day outdated platelet concentrates obtained from the Red Cross because we did not detect significant differences in [^{3}H]PAC binding between indated and outdated concentrates: [^{3}H]PAC binding with indated platelets revealed $K_{D1} = 31 \pm 21$ pM (SD) [$B_{max1} = 10.5 \pm 0.5$ fmol/mg (SD)] and $K_{D2} = 3.9 \pm 0.7$ nM ($B_{max2} = 153 \pm 11.0$ fmol/mg) *versus* with outdated platelets $K_{D1} = 38 \pm 30$ pM ($B_{max1} = 19.1 \pm 12.6$ fmol/mg) and $K_{D2} = 2.1 \pm 1.3$ nM ($B_{max2} = 266 \pm 123$ fmol/mg). To minimize variability, the concentrates were pooled before preparing plasma membranes. Experiments were performed on eighteen different pooled sources collected over a 4-year period. Each pool was derived from 3 to 24 donor bags. After purification of the plasma membranes from each pool, the samples were stored at −80° for no more than 3 months before binding experiments were performed. No effect of storage in the freezer was noted over 3 months. Repeated measures of Scatchard plots and of [^{3}H]PAC kinetics utilized different pooled donor sources. However, in situations requiring a comparison of conditions (i.e. drug competition experiments with [^{3}H]PAC, or in experiments to calculate K_D from rate association/dissociation measurements, or in experiments to compare the effects of GTP), we utilized a common donor source of platelets.

Materials. [^{3}H]*p*-Aminoclonidine ([^{3}H]PAC, sp. act. 45.6 Ci/mmol) and [^{3}H]yohimbine ([^{3}H]YOH, sp. act. 75 Ci/mmol) were purchased from New England Nuclear. The purity of the radioligands was verified by TLC and was >90%. The following drugs were gifts of pharmaceutical companies: clonidine (Boehringer, Ingelheim), ketanserin (Janssen Pharmaceuticals), (+)-norepinephrine (Sterling-Winthrop), prazosin (Pfizer). Other drugs and chemicals were purchased commercially (Sigma Chemical Co., St. Louis, MO). Nanopure water (≥17.0 MΩ resistance/cm) was used in all experiments (Barnstead Corp., Boston, MA). Schleicher & Schuell (Keene, NH) No. 30 glass fiber filters were used in the binding assays. Drug solutions were made daily.

Isolation of platelets and purification of plasma membranes. Intact platelets were washed and prepared prior to lysis as described previously [8, 33]. Platelets were counted and sized using a Coulter ZM counter and a model 256 channelizer. The mean platelet volumes did not differ significantly between the donor sources: 5.08 ± 0.37 μm^3 (SD), establishing that all pools contained a comparable homogeneous proportion of unclumped platelets. There was also minimal red and white blood cell contamination in these preparations, representing 2.2 ± 0.4% (SD) and 0.58 ± 0.20% (SD), respectively, of the total cell counts.

Plasma membranes were purified by a slight modification of the procedure of Neubig *et al.* [24] and Neubig and Szamraj [37] (see "Method D (iii)" of Ref. 8). Briefly, platelets were mixed with the protease inhibitor, phenylmethylsulfonyl fluoride to a final concentration of 100 μM, and then quickly frozen. Thawed platelets were sonicated and centrifuged to pellet. The pellets were frozen quickly at −80°, thawed, resuspended in 22 mM sodium-HEPES, pH 7.4, and recentrifuged. This process of freeze–thawing and resuspending the pellets was repeated twice. Lysates were layered over a discontinuous 14.5% and 34% sucrose gradient which was centrifuged at 105,000 *g* for 90 min using an SW-28 rotor in an L-88 ultracentrifuge (Beckman Corp., Fullerton CA). The interface layer was recovered and suspended in ice-cold water. The membranes were pelleted by a second spin at 105,000 *g* and the purified plasma membranes were resuspended in 10 mM sodium-HEPES, 8 mM $MgCl_2$, 1 mM ethyleneglycolbis(aminoethylether)-tetra-acetate (EGTA), pH 7.6 (at room temperature). An aliquot was removed for protein determination using the Lowry procedure as modified [38] with the biuret reagent (Sigma Chemical Co.). The remaining sample was stored at −80°. The protein concentration of these preparations was 2.26 ± 0.6 mg/mL (mean ± SD).

Radioligand binding conditions. Each assay tube consisted of the radioligand and 0.1 mg protein of platelet plasma membranes in 1 mL of 3.3 mM sodium-HEPES, 0.64 mM $MgCl_2$, 0.08 mM EGTA, 0.1 mM sodium-ascorbate, pH 7.4, at 21°. Non-specific binding was measured in identical tubes

containing a final concentration of 10^{-5} M of either clonidine or (−)-norepinephrine. Standard filtration techniques were used as previously described [8, 33]. In each experiment, simultaneous analysis of filter blank radioligand binding was performed to rule out artificial filter binding. Radioactivity was counted at approximately 42% efficiency by liquid scintillation counting. Enough counts were accumulated to obtain a counting error of <5%.

In competition studies most drugs were prepared as a 10^{-3} M stock solution in water. However, clonidine, (+)-norepinephrine, and (+)-propranolol were dissolved in 0.1 mM HCl. Ketanserin, UK-14,304, prazosin, and serotonin were dissolved as stock solutions in 50% ethanol/0.1 mM HCl with heating to 37°. Haloperidol was dissolved as a stock solution in absolute methanol at a 10^{-2} M concentration. After the drugs were dissolved, they were further diluted with water such that the concentration of ethanol or methanol did not exceed 0.05% in the binding assays.

In an earlier study [8], we demonstrated that specific [^{3}H]PAC binding to platelet plasma membranes increased linearly at 21° in proportion with protein concentrations in the range of 0.05 to 0.2 mg protein/mL assay. Therefore, we chose to perform most binding studies with 0.1 mg protein/mL of reaction mixture. Specific binding of 0.4 nM [^{3}H]PAC was 67 ± 10% (SD) of total binding as defined in the presence of 10 μM clonidine.

Data analysis. The association and dissociation rates of [^{3}H]PAC binding data were analyzed by the KINETIC program using specific binding as adapted by McPherson [39] for the IBM PC. The saturation and competition data were analyzed by EBDA and LIGAND computer programs [39]. We first utilized the rigorous technique of Kermode [40] in which the data for total binding (specific and non-specific) are fitted to a model comprising the specific binding sites, together with a non-specific binding component. The use of this strategy reduces the likelihood of finding heterogeneity where none exists and thus provides a rigorous test for the existence of heterogeneity [40]. A normal distribution of error was assumed. The saturation data were first modeled to a one-site fit. The data were then modeled to a two-site fit as recommended by Munson and Rodbard [41]. A three-site model was also attempted using saturation data. In all cases, the preferred model was selected by a statistical F test. The binding parameters for [^{3}H]PAC, as derived from seven saturation binding experiments, were used as starting parameters in analyzing the competition curves. With each [^{3}H]PAC competition experiment, eight models were tested as follows: (i) one site PAC, one site competitor; (ii) one site PAC, distinguished into two sites by competitor; (iii) two sites PAC, recognized as one site by competitor; (iv) two sites PAC, distinguished into two sites by competitor; (v) two sites PAC and the competitor recognizes PAC-site 1 only ($K22 = 0$) ($K22$ refers to the binding affinity parameter at the second, lower affinity PAC binding site, for the unlabeled competitor); (vi) two sites PAC and the competitor recognizes only PAC site-2 ($K21 = 0$) ($K21$ refers to the binding affinity parameter at the first, high affinity PAC binding site, for the unlabeled competitor); (vii) two sites PAC, distinguished into three sites by competitor (same total B_{max}); and (viii) the final model varied with the competitor according to which of the preceding fits was preferred resulting in three sites PAC and one, two or three sites competitor (same total B_{max} for PAC and competitor).

In addition to the [^{3}H]PAC saturation experiments and unlabeled YOH competitions of [^{3}H]PAC, we also performed [^{3}H]YOH saturation experiments and competitions of [^{3}H]YOH. The [^{3}H]YOH competition by unlabeled PAC provided high enough PAC concentrations (up to 10^{-4} M) to measure the K_i values for high and low affinity PAC binding. This matrix of five experiments ([^{3}H]PAC Scatchard plots, two concentrations of [^{3}H]PAC competition plots, [^{3}H]YOH Scatchard plots, and [^{3}H]YOH competition plots) was repeated three times on different batches of purified plasma membranes from platelets. Each matrix set consisted of at least 96 experimental points, and each was analyzed simultaneously by the LIGAND computer program [39]. In addition to the eight models mentioned above, we also tested the following models on each matrix; (i) three sites PAC, and YOH recognized only PAC sites 2 and 3 ($K21 = 0$), (ii) three sites PAC, and YOH recognized only PAC sites 1 and 2, ($K21 = 0$), (iii) three sites PAC, and YOH recognized only PAC sites 1 and 3 ($K22 = 0$), (iv) three sites PAC, and YOH distinguished a combination of two sites equally ($K21 = K22$), (v) three sites PAC, and YOH distinguished a different combination of two sites equally ($K22 = K23$), (vi) three sites PAC, and YOH distinguished yet a different combination of two sites equally ($K21 = K23$), (vii) three sites PAC, and YOH recognized all sites equally ($K21 = K22 = K23$), and (viii) three sites PAC, and YOH also distinguished the three sites. Similar simultaneous matrix analyses were performed for analysis of [^{3}H]PAC Scatchard plots and (−)-NE competition plots as well as for [^{3}H]YOH experiments plus 0.1 mM GTP. The computer-estimated binding parameters are expressed as the mean ± standard error of the estimates (SEE), unless otherwise stated.

RESULTS

Binding of [^{3}H]PAC to purified plasma membranes was observed to be maximally displaced by excess clonidine or yohimbine, but a substantial amount of specific binding was not displaced by (−)-NE. In Table 1, platelet membrane's non-specific binding of 0.3 nM [^{3}H]PAC was determined with 10^{-5} M of either CLON or (−)-NE. As can be seen, CLON displaced more [^{3}H]PAC binding than (−)-NE. Moreover, CLON displaced more [^{3}H]PAC than (−)-NE whether added before or after equilibrium binding was achieved (Table 1). In the presence of 0.1 mM GTP, most of the (−)-NE-displaceable binding was lost, while roughly 47% of the CLON-displaceable binding remained. Full competition curves with these compounds are shown in Fig. 3, also suggesting that a substantial amount of specific [^{3}H]PAC binding was not displaceable by (−)-NE.

To characterize the [^{3}H]PAC binding sites, association and dissociation experiments of specific

Table 1. Comparison of non-specific binding of 0.3 nM [^{3}H]*p*-aminoclonidine using (−)-NE or clonidine to displace binding

Condition	[^{3}H]PAC bound (fmol/mg protein)			Percent displaced binding	
	total	displaced (specific) binding			
		Plus (−)-NE (site-1)	Plus CLON (sites-1 and 2)	Plus (−)-NE (site-1)	Plus CLON (sites-1 and 2)
At equilibrium	29.1 ± 4.7	12.0 ± 1.2	16.1 ± 1.7*	41.9 ± 7.5	56.2 ± 9.8*
40-min Dissociation after equilibrium	28.7 ± 3.1	10.7 ± 0.3	14.5 ± 0.6*	38.0 ± 6.8	51.0 ± 5.9*
Equilibrium + GTP	21.7 ± 8.0	2.2 ± 1.8	6.8 ± 3.2†	10.4 ± 7.0	32.1 ± 9.8*

"At equilibrium" indicates that binding of 0.2 mg protein/2 mL was allowed to incubate with radioligand and unlabeled competitor (10^{-5} M) at 21° for 40 min prior to filtration. The "40-min dissociation" was initiated as described under Figs. 1B and 5; there was a 40-min equilibration with radioligand, then unlabeled competitor (10^{-5} M) was added, and after an additional 40 min the reactions were terminated. "Equilibrium + GTP" indicates identical binding as the "at equilibrium" conditions except in the presence of 0.1 mM GTP. "Displaced (specific) binding" refers to the binding remaining after subtraction of cpm remaining in the presence of competitor from total binding. Each experimental point was determined in triplicate. All three conditions were tested on each batch of platelet membranes. Values are means ± SD (N = 4).

* Statistically different from "Plus (−)-NE" ($P < 0.003$), using paired Student's *t*-test.

† Statistically different from "Plus (−)-NE" ($P = 0.01$), using paired Student's *t*-test.

binding of [^{3}H]PAC to purified plasma membranes at 21° were performed (Fig. 1). The data in Fig. 1A combines two, 20-point association time-course studies performed using a [^{3}H]PAC concentration of 0.27 nM. [^{3}H]PAC rapidly associated to its binding sites reaching an apparent steady state by 25 min that remained unchanged for at least 80 min. Non-specific binding, as determined with 10^{-5} M CLON, remained unchanged throughout the experimental time course (from 2 to 80 min). The association data was fitted best by a two-site rather than a one-site ($P = 0.002$) or a three-site ($P = 0.0001$) model [39]. This analysis yielded a $k_{obs1} = 0.143 \pm 0.083\ min^{-1}$ and a $k_{obs2} = 2.000 \pm 1.840\ min^{-1}$. In this computer model the site with the faster on-rate represented 21% of the binding sites. We also measured the rate of dissociation from equilibrium binding conditions (60 min at 21°) using 0.3 nM [^{3}H]PAC, after the addition of 10^{-2} M CLON, which resulted in a final concentration of 10^{-4} M unlabeled CLON. These data are shown in Fig. 1B. Computer-assisted analysis of three, 20-point dissociation experiments with 0.3 nM [^{3}H]PAC was again suggestive of a two-site fit of the data ($P = 0.03$). This analysis yielded a $k_{-11} = 0.032 \pm 0.007\ min^{-1}$ (SD) and a $k_{-12} = 1.13 \pm 0.33\ min^{-1}$ (SD) (Fig. 1). After 80 min, virtually all specific binding of [^{3}H]PAC was reversible by 10^{-4} M clonidine. The site with the faster off-rate was modeled to comprise 35% of the total specific binding at the 0.3 nM concentration of radioligand. Thus, based on the proportion of binding sites, it appeared that the site with a faster on-rate might also display a faster off-rate.

To determine more accurately which kinetic sites was comparable between the 0.27 nM [^{3}H]PAC association and the 0.3 nM [^{3}H]PAC dissociation experiments, we performed rate association studies at a lower (0.023 nM) and a higher (0.5 nM) concentration of [^{3}H]PAC. A new source of platelet plasma membranes was utilized for these additional experiments. At the 0.023 nM concentration of [^{3}H]PAC, we observed that a preponderance of the binding was slow associating ($k_{obs1} = 0.045 \pm 0.010\ min^{-1}$, 68% of the two sites; $k_{obs2} = 0.61 \pm 0.11\ min^{-1}$, 32% of the two sites), suggesting again that the slow association site represented the higher affinity binding site. Conversely, at 0.5 nM [^{3}H]PAC, a fast association site appeared to predominate ($k_{obs1} = 0.473 \pm 0.208\ min^{-1}$; one-site fit preferred). This suggested that the fast association site represented the lower affinity binding site. In additional experiments with a different pool of Red Cross platelets, we measured the clonidine rate of dissociation at 0.1 nM [^{3}H]PAC. In the 0.1 nM [^{3}H]PAC experiment, the slower dissociating site again predominated (80% of the two sites), suggesting again that the slower dissociating site represented a higher affinity binding site and the faster dissociating site represented a lower affinity binding site.

Using the kinetic parameters from the 0.27 nM [^{3}H]PAC rate associations and the 0.3 nM rate dissociations, we calculated K_D values equal to 0.060 nM (site-1) and 1.27 nM (site-2) for [^{3}H]PAC. We also analyzed rate association and dissociation experiments at 0.1 nM [^{3}H]PAC with fewer data points. The K_D values determined from the latter experiments were generally confirmatory (calculated $K_{D1} = 0.094$ nM and $K_{D2} = 1.72$ nM for data from 0.1 nM [^{3}H]PAC rate experiments).

Saturation studies were next performed utilizing between 0.01 and 8 nM [^{3}H]PAC and a 1-hr incubation at 21°. A 60-min incubation was deemed to be sufficient since in the above rate association experiments with 0.023 nM [^{3}H]PAC, the binding reached equilibrium within 60 min. Also, a 60-min

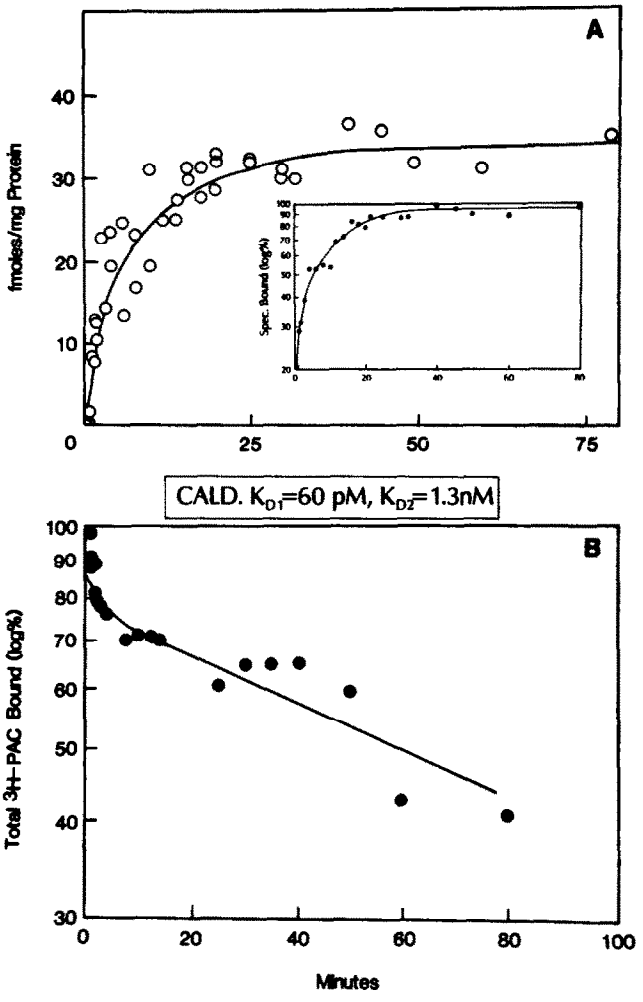

Fig. 1. Rate kinetic studies of specific [^{3}H]PAC binding to platelet purified plasma membranes. Rate association studies (panel A) were performed at 0.27 nM [^{3}H]PAC by placing assay ingredients in test tubes and incubating for the times shown at 21°. For comparison to panel B, the data are redrawn in the insert on a log scale. Rate dissociation studies (panel B) were performed at 0.3 nM [^{3}H]PAC. In the dissociation study, the assay ingredients were incubated for 1 hr at 21° (steady state) and then clonidine was added in a concentrated form so that the volume change was less than 1%, but the final concentration of clonidine was 100 μM. The reaction was then allowed to proceed for the times shown. Each experimental point was determined in duplicate and each experiment was repeated twice with similar results from different platelet membrane preparations. Total binding (panel B) at zero time was 325 ± 68 cpm. Non-specific binding (with 10^{-4} M clonidine) was unchanged throughout the experiment, averaging 20.7 fmol/mg protein of bound [^{3}H]PAC.

incubation allows tight agonist complexes to reach equilibrium [42]. The results demonstrated that [^{3}H]PAC binds to at least two high affinity sites in purified plasma membranes from platelets (Fig. 2). Seven Scatchard plots, each from different Red Cross platelet sources and similar to the one shown in Fig. 2B, were analyzed by the LIGAND computer program [39]. Both the non-specific binding and a scaling factor for each experiment were computed as independent parameters as previously recommended [40, 41]. A two-site fit was preferred over a one-site model ($P = 0.0001$) and over a three-site model ($P < 0.00005$). The estimated binding parameters for site-1 were $K_{D1} = 38 \pm 30$ pM (SD) with a $B_{max1} = 19.1 \pm 12.6$ fmol/mg protein (SD). The estimated kinetic parameters for site-2 were $K_{D2} = 2.1 \pm 1.3$ nM (SD) with a $B_{max2} = 266 \pm 123$ fmol/mg protein (SD). The percent of total binding occupied by site-1 varied between these seven sources of platelets from 2.2 to 14.6% (6.8 ± 4.4%, mean ± SD). Scatchard plot analyses were also performed in the presence of 0.1 mM GTP. GTP completely eliminated specific [^{3}H]PAC binding to site-1 (N = 3 experiments, 23 points each, data not shown). This may be explained if site-1 is a G_i-coupled adrenoceptor state. However, in the presence of GTP, a nanomolar K_D affinity site for [^{3}H]PAC appeared to be retained but the data gave large error estimates ($K_A = 3.6 \pm 2.8$ E8 M, $K_D = 2.7$ nM; $B_{max} = 146 \pm 109$ fmol/mg protein). The

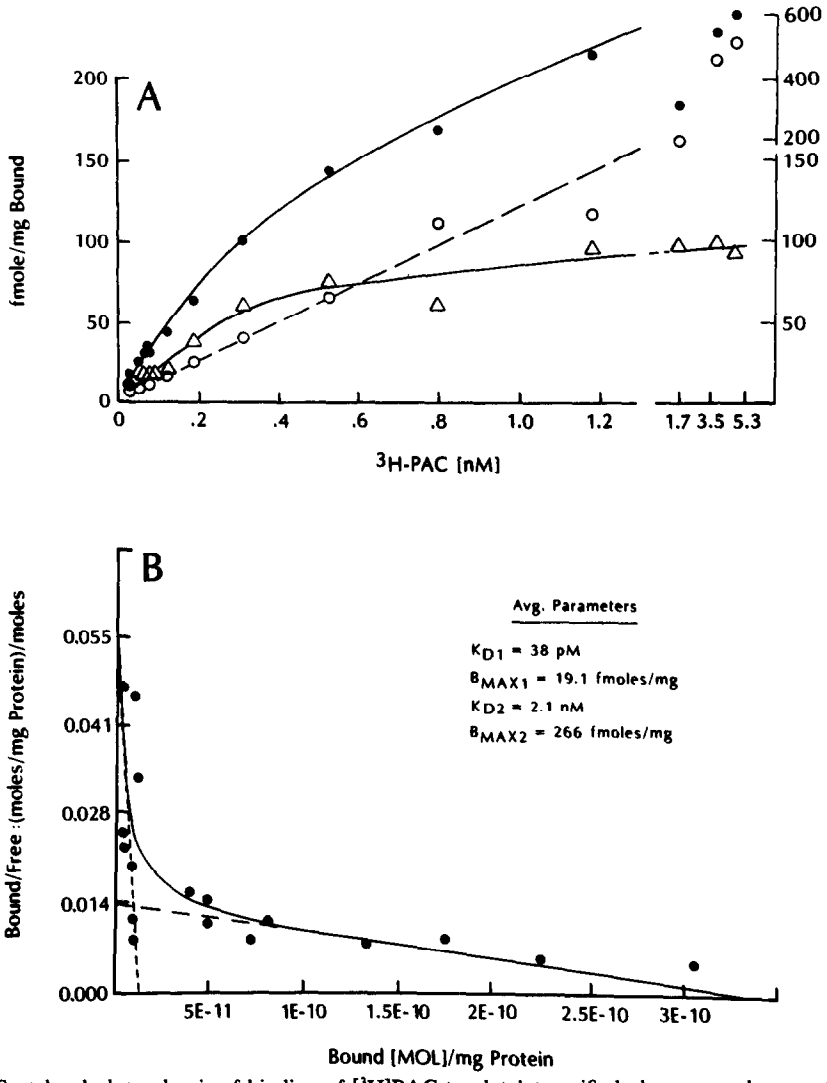

Fig. 2. Scatchard plot anlaysis of binding of [^{3}H]PAC to platelet purified plasma membranes. (A) A single representative saturation experiment is shown. Reactions contained 0.1 mg protein/mL and were incubated at 21° for 60 min. A total of 15 points were analyzed in duplicate in the experiment shown. Closed circles (●) represent total binding and open circles (○) represent non-specific binding, measured with 10^{-5} M nonradioactive clonidine. The open triangles (△) represent specific binding of [^{3}H]PAC after subtracting non-specific binding from total binding. The scale was interrupted above 1.2 nM [^{3}H]PAC in order to show, on the same graph, that binding appeared saturable. The percent specific binding was about 25% at the highest [^{3}H]PAC concentrations, whereas at low concentrations it was consistently above 50%. (B) The data from a saturation experiment similar to the one in panel A were transformed to Scatchard analysis and the curve was drawn using LIGAND, as described under Materials and Methods [39]. E = exponential ($\times 10^{nth}$).

data could not be modeled to two sites in the presence of GTP.

We next studied competition of 0.1 and 0.8 nM [^{3}H]PAC binding by non-radioactive compounds under equilibrium binding conditions. We calculated (using the laws of mass action and our parameter estimates from Scatchard plots) that at a concentration of 0.1 nM [^{3}H]PAC, approximately 53% of the specific binding capacity would occur at site-1 and 47% would be to site-2. At the 0.8 nM concentration of [^{3}H]PAC, we calculated that only approximately 20% of the specific binding capacity should be due to site-1 and 80% should be due to site-2. A concentration of 0.1 nM [^{3}H]PAC in a 2-mL reaction volume yielded sufficient bound cpm for two-site analyses; 177 ± 13 total cpm (SD) with an average percent specific binding of 72.3 ± 10.5% (SD). Since our saturation studies had indicated that the B_{max} of site-1 was much less than site-2, we could identify site-1 and site-2 by their relative B_{max} estimates from the computer analysis of the 0.1 nM [^{3}H]PAC competition experiments (Table 2). Neither the 0.1 nor 0.8 nM concentration of [^{3}H]PAC had significant binding at a lower affinity PAC site,

Table 2. Competition by various drugs for 0.1 nM [^{3}H]PAC binding to purified plasma membranes from Red Cross platelet concentrates

	Site 1	Site 2	P value
Competitor	K_i (M)	K_i (M)	2 sites vs 1 site
Clonidine	1.1E-11	1.7E-8	0.03
(−)-NE*	2.6E-10	1.6E-5	0.02
Yohimbine†	1.2E-9	2.6E-7	0.0001
UK-14,304	1.7E-9	1.7E-9	1.00
(−)-Epinephrine	4.2E-9	5.0E-6‡	0.009
Dopamine	1.1E-7	2.9E-7‡	0.0001
(+)-NE	1.5E-7	1.1E-6‡	0.0001
Haloperidol	2.7E-7	1.1E-5	0.042
Ketanserin	8.8E-7	1.1E-5	0.005
Prazosin	1.9E-6	5.4E.6‡	0.306
(−)-Isoproterenol	3.8E-6	3.8E-6	1.00
Serotonin	6.8E-6	6.8E.6‡	1.00
d,l-Propranolol	2.5E-5	2.5E-5	1.00

Each competition experiment was minimally performed with 25 concentrations of competitor (between 10^{-13} and 10^{-4} M) against 0.1 nM [^{3}H]PAC for binding to platelet plasma membranes. Membranes were obtained from out-dated Red Cross concentrates. Incubations were for 1 hr at 21° at a protein concentration of 0.2 mg/2 mL (see Materials and Methods). The binding at each concentration of competitor was determined in duplicate. A minimum of 120 cpm of total binding was obtained with an average of 72% specific binding at 0.1 nM [^{3}H]PAC versus 10^{-5} M clonidine. Enough counts were accumulated to achieve a counting efficiency of less than 5%. Each curve was analyzed for one and two sites using the LIGAND computer program [39] and the statistically preferred fit is given. Experimental binding affinity parameters are expressed in exponential molar units (E = $\times 10^{nth}$). In most cases, the assignments to site-1 and site-2 were based on the estimated B_{max} values: at 0.1 nM [^{3}H]PAC, site-1 was always assigned to a computed B_{max} in the competition curves which was less than 30% of the total binding (see exceptions below). Non-specific binding was computed for each competitor versus [^{3}H]PAC, by the computer. The computer-generated coefficients of variation for the competitor binding affinities (average of the coefficients of variation for each experiment for both K_A values) were: clonidine ($K_A \pm 70\%$), (−)-NE ($K_A \pm 0.1\%$), yohimbine ($K_A \pm 48\%$), UK-14,304 ($K_A \pm 43\%$), (−)-epinephrine ($K_A \pm 77\%$), dopamine ($K_A \pm 61\%$), (+)-NE ($K_A \pm 69\%$), haloperidol ($K_A \pm 191\%$), ketanserin ($K_A \pm 30\%$), prazosin ($K_A \pm 199\%$), (−)-isoproterenol ($K_A \pm 73\%$), serotonin ($K_A \pm 93\%$), and *d,l*-propranolol ($K_A \pm 63\%$).

* In (−)-NE competition experiments at 0.1 nM [^{3}H]PAC, the curves suggested only one site (K_i = 2.8 E-8 M). However, (−)-NE competition experiments at 0.8 nM [^{3}H]PAC indicated a second site. Both sites were verified statistically when experiments were analyzed simultaneously on LIGAND [39]. Thus, the data shown are from a simultaneous computer analysis employing both 0.1 nM [^{3}H]PAC and 0.8 nM [^{3}H]PAC competition experiments with (−)-NE having a total of 96 experimental points.

† In yohimbine competition experiments at 0.1 nM [^{3}H]PAC, the curves indicated two sites (K_i = 6.7 E-10 and K_{i2} = 2.6 E-7); however, it was difficult to be certain of the assignments (49% site-1, 51% site-2). Therefore, additional experiments were performed, and the data shown are from a simultaneous computer analysis employing yohimbine competition curves against 0.1 nM [^{3}H]PAC and 0.8 nM [^{3}H]PAC, as well as 0.1 to 6 nM [^{3}H]yohimbine Scatchard plot experiments (N = 6). This evaluation utilized 294 experimental points.

‡ These binding curves appeared to not have reached complete displacement at a competitor concentration of 10^{-4} M. Thus, the site-2 parameters for these drugs may be imprecise. The actual affinities may be lower than determined.

which, as described later, exists with a K_D of approximately 30 nM.

Competition curves of 0.1 nM [^{3}H]PAC binding yielded data compatible with two competitor binding affinities ($P \leq 0.05$ vs one site only) for seven of the thirteen non-radioactive drugs tested (Fig. 3). In this analysis, the starting parameters for the computer iterations were those from the best-fitted values from the previous seven [^{3}H]PAC Scatchard plots. Thereafter, all [^{3}H]PAC parameters ($K11$ and $K12$), the competitor parameters ($K21$ and $K22$) and the maximal binding ($R1$ and $R2$) were derived by the LIGAND program. In every case, the two sites for [^{3}H]PAC ($K11$ and $K12$) were retained by the best fitted model within the expected ranges of affinity and with site-1 representing a minority of the binding sites ($R1 \leq 30\%$ of total binding). For the six competitor drugs which appeared to distinguish only one [^{3}H]PAC site, a two-site *radioligand* model was still preferred but those competitors appeared

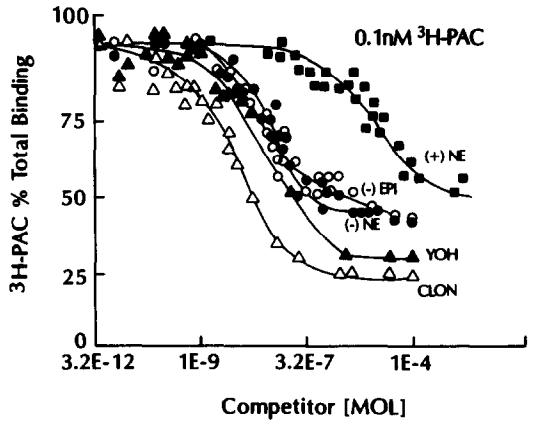

Fig. 3. Competition by various drugs for 0.1 nM [^{3}H]PAC binding to platelet purified plasma membranes. Studies were performed by placing increasing concentrations of competitor in test tubes containing 0.1 nM [^{3}H]PAC and incubating for 1 hr at 21° for each assay prior to terminating the reaction by rapid filtration. Each point was determined in duplicate. The competitors were: (−)-NE, (+)-NE, (−)-epinephrine (−)-EPI, yohimbine, and clonidine. Shown are representative experiments (see Table 2). Total cpm bound for all experiments were as follows: (−)-NE, 195 ± 21; (+)-NE, 228 ± 74; (−)-epinephrine, 200 ± 65; yohimbine, 155 ± 14; and clonidine, 142 ± 6. Curves were computer drawn using LIGAND [39].

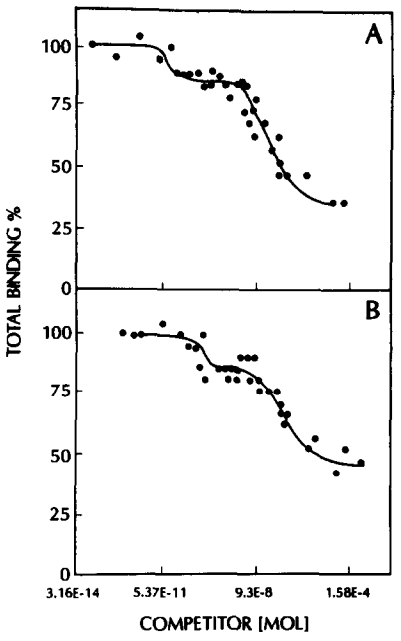

Fig. 4. Competition by unlabeled *p*-aminoclonidine for 5 nM [^{3}H]yohimbine binding to platelet purified plasma membranes in the presence and absence of GTP. Studies were performed by placing increasing concentrations of unlabeled *p*-aminoclonidine in test tubes containing 5 nM [^{3}H]yohimbine and incubating for 1 hr at 21° prior to terminating the reaction by rapid filtration. A 5 nM concentration of [^{3}H]yohimbine was chosen since this was reported to be above the K_D for yohimbine [24]. Each point was determined in duplicate. Each experiment was done four times with comparable results. Key: (A) untreated membranes; and (B) membranes treated with 0.1 mM GTP before (30 min) and throughout binding. Total cpm bound were 3305 ± 402 in the absence of GTP and 3255 ± 1252 in the presence of GTP. Curves were computer drawn using LIGAND [39].

to displace both of the [^{3}H]PAC sites with indistinguishable affinity (i.e. $K21 = K22$ was preferred over $K21$ or $K22 = 0, P \leq 0.05$). Therefore, in all experiments, the two-site [^{3}H]PAC model was preferred. Three-site models were not preferred.

As noted before, maximal competitive binding of 0.1 nM [^{3}H]PAC was obtained using clonidine as a displacing agent (Fig. 3). In these experiments clonidine displaced 30% more of the 0.1 nM [^{3}H]-PAC binding than did (−)-NE. Table 2 lists the K_i values for the competitors in the apparent rank order of potency at [^{3}H]PAC site-1 and site-2. The rank order of potency at site-1 was clonidine > (−)-NE > yohimbine > UK-14,304 > (−)-epinephrine > dopamine = (+)-NE > haloperidol > ketanserin > prazosin > (−)-isoproterenol > serotonin > *d,l*-propranolol. The rank order of potency for binding to site-1 is compatible with an α_2-adrenoceptor [24]. However, the rank order of potency at site-2 was notably different: UK-14,304 > clonidine > yohimbine = dopamine > (+)-NE > (−)-isoproterenol > (−)-epinephrine = prazosin > serotonin > haloperidol = ketanserin = (−)-NE > *d,l*-propranolol.

A similar computer-fitting analysis was performed for displacements of 0.8 nM [^{3}H]PAC. Each competition curve with 0.8 nM [^{3}H]PAC comprised a minimum of 30 competitor concentrations. The rank order of potency for the 0.8 nM [^{3}H]PAC analysis of site-2 was clonidine ($K_i = 30$ nM) > (−)-epinephrine ($K_i = 100$ nM) > (+)-NE ($K_i = 4.6\ \mu$M) > prazosin ($K_i = 7.0\ \mu$M) > (−)-NE ($K_i = 16\ \mu$M). As with the 0.1 nM [^{3}H]PAC competition curves (Table 2), site-2 displayed relatively low affinity for (−)-NE and (−)-epinephrine, compared to α_2-adrenoceptors [14].

To further investigate the relationship between the binding sites, we performed [^{3}H]YOH saturation binding analyses. [^{3}H]YOH Scatchard plots were linear over a range of 0.1–6 nM, as previously reported [24, 43]. In three separate Red Cross samples, the kinetic parameters for [^{3}H]YOH were $K_D = 0.62 \pm 0.21$ nM (mean ± SD) and $B_{max} = 319 \pm 197$ fmol/mg protein (mean ± SD). The [^{3}H]YOH affinity reasonably matched that determined for site-1 from [^{3}H]PAC displacements with unlabeled YOH (Table 2). However, the B_{max} for [^{3}H]YOH binding (319 fmol/mg) was higher than that previously determined in our [^{3}H]PAC Scatchard analyses of either site-1 or site-2. Our previous data (Table 2) had demonstrated that yohimbine would not be expected to bind significantly to site-2 in a range of 0.1 to 6 nM, due to its low affinity at site-2. Thus, it seemed unlikely that site-2 would be involved in obtaining such a high B_{max} for 0.1 to 6 nM [^{3}H]YOH. However, as described below, [^{3}H]YOH bound with high affinity to an additional site (PAC site-3). (As shown later in

Table 3. Simultaneous analysis of competition and Scatchard experiments for PAC, yohimbine and (−)-NE binding to purified plasma membranes from platelets

Data files co-analyzed	No. of membrane batches	Ligand	Parameter estimates
[^{3}H]PAC Scatchards [^{3}H]PAC vs YOH [^{3}H]YOH vs PAC [^{3}H]YOH Scatchards	3	PAC	K_D site-1 = 12.7 E-12 ± 8.8 E-12 K_D site-2 = 7.4 E-10 ± 3.7 E-10 M K_D site-3 = 50.5 E-9 ± 8.1 E-9 M
		YOH	K_D site-1 = 1.2 E-9 ± 0.6 E-9 M K_D site-2 = 2.6 E-7 ± 2.6 E-7 M K_D site-3 = 6.6 E-10 ± 0.02 E-10 M
		Both	percent (site-1/total) = 12.7 ± 10.2% percent (site-2/total) = 33.3 ± 12.0% percent (site-3/total) = 54.0 ± 12.4%
[^{3}H]PAC Scatchards, [^{3}H]PAC vs (−)-NE	2	PAC	K_D site-1 = 13.3 E-12 M (1.6 E-12–25.0 E-12) K_D site-2 = 8.3 E-10 M (9.5 E-10–7.1 E-10) K_D site-3 = 12.2 E-9 M (6.2 E-9–18.2 E-9)
		(−)-NE	K_i site-1 = 1.7 E-10 M (0.7 E-10–2.6 E-10) K_i site-2 = 7.0 E-3 M (1.4 E-2–1.6 E-5) K_i site-3 = 1.1 E-9 M (0.5 E-9–1.6 E-9)
		Both	percent (site-1/total) = 0.9% (0.8–1.0) percent (site-2/total) = 13.2% (20.3–6.1) percent (site-3/total) = 85.9% (78.9–92.9)

The "Data files co-analyzed" heading refers to the listed experimental data files that were analyzed simultaneously using the LIGAND computer program [39]. Each of the experiments listed was performed on every batch of platelets. "No. of membrane batches" refers to the number of platelet membrane batches that were analyzed separately. The preferred parameter estimates were all $P \leq 0.05$ over other fits. Experimental binding affinity parameters are expressed in exponential molar units (E = $\times 10^{nth}$). The data are derived from calculating the arithmetic mean for the results from each batch. When No. of sets = 3, the data are reported as mean ± SEM, whereas when No. of sets = 2, the data are reported as mean with the range given in parentheses. "[^{3}H]PAC vs YOH" represents competition binding curves with 10^{-12} to 10^{-4} M unlabeled YOH at two fixed [^{3}H]PAC concentrations of 0.1 and 0.8 nM. Similarly, "[^{3}H]PAC vs (−)-NE" experiments were performed at both 0.1 and 0.8 nM [^{3}H]PAC covering the same range of (−)-NE concentrations. "[^{3}H]YOH vs PAC" represents competition binding curves with 10^{-12} to 10^{-4} M unlabeled PAC at a fixed [^{3}H]YOH concentration of 5.0 nM. "[^{3}H]YOH Scatchards" were performed with 0.1 to 6 nM [^{3}H]YOH. Each experiment within the co-analyzed sets was a multiple point experiment, as in Figs. 2–4, and each point was determined in duplicate. Each set included at least 96 experimental points.

extended experiments with 4–120 nM [^{3}H]PAC, because of the lower affinity of site-3 for PAC, site-3 would have been unrepresented in the aforementioned [^{3}H]PAC saturation studies as performed up to a concentration of only 8 nM [^{3}H]-PAC.) Thus, the parameters derived from [^{3}H]YOH Scatchard plots were as predicted if the affinity constants (K_D) of [^{3}H]YOH binding were 0.62 nM to PAC, site-1, 260 nM to PAC site-2 (see Table 2), and 0.62 nM to site-3.

To further compare [^{3}H]YOH and [^{3}H]PAC binding sites, we next performed 5 nM [^{3}H]YOH competition experiments with non-radioactive PAC. Four experiments, each of more than 40 points, were performed in duplicate (Fig. 4A). These experiments allowed us to probe for the existence of a low affinity binding site for PAC having a reported K_i between 20 and 380 nm [15, 24]. Computer-assisted analysis using a one-site [^{3}H]YOH model (as determined by our [^{3}H]YOH Scatchard plots) indicated that the competition by unlabeled PAC was well fit by a two-site PAC model ($P < 0.0005$ over one site). The two-site PAC model yielded K_i parameters for displacements by PAC of [^{3}H]YOH, which were $K_{i1} = 20.6 \pm 9.6$ pM and $K_{i3} = 31.2 \pm 1.4$ nM. The B_{max} values were 129 ± 24 fmol/mg for the first site, and 396 ± 111 fmol/mg for the second site. These experiments utilized different batches of Red Cross platelets than were used for the [^{3}H]YOH Scatchard plots, and this may explain the overall higher B_{max} values. However, the relatively higher percent of binding to site-1 should not be compared to our previous [^{3}H]PAC Scatchard plots. Those saturation experiments would have measured the ratio of site-1 to site-2. However, these 5 nM [^{3}H]YOH competition experiments with PAC measured the ratios of site-1 to -3, since site-2 would not be bound with substantial affinity at 5 nM [^{3}H]YOH (Table 2). Finally, the cumulative B_{max} data demonstrated that PAC displaces all of the [^{3}H]YOH binding sites.

The following experimental data sets were analyzed simultaneously by LIGAND [41]: 0.01 to 8 nM [^{3}H]PAC saturation binding data, 0.1 nM [^{3}H]PAC and 0.8 nM [^{3}H]PAC competition experiments with unlabeled YOH, 5 nM [^{3}H]YOH competition experiments with unlabeled PAC, and

Table 4. Comparison of human brain and platelet [^{3}H]p-aminoclonidine binding studies

Tissue source/prep.	Site 1		Site 2		Site 3		Authors and reference
	K_D (nM)	B_{max} (fmol/mg protein)	K_D (nM)	B_{max} (fmol/mg protein)	K_D (nM)	B_{max} (fmol/mg protein)	
Human/WL frontal cortex	0.05	8.3 ± 5.3	1.3	75 ± 30	ND	ND	Andorn and Carlson*
Human/PPM platelets	0.013 ± 0.009	36 ± 29	0.7 ± 0.4	95 ± 34	51.0 ± 8.0	154 ± 35	Present findings

Parameters represent the mean ± SD as reported for multiple analyses performed with different tissue sources. Each study utilized computer-assisted curve modeling. The number of samples analyzed were 3 for the study by Andorn and Carlson, and 7 for our study. Our three-site data were derived from the three experimental sets utilizing [^{3}H]PAC and [^{3}H]YOH, described in the legend of Table 3. Our site-3 data represent the low affinity PAC K_i for displacement of 5 nM [^{3}H]YOH. The SEM were not reported for the K_D values reported by Andorn and Carlson. Abbreviations: WL = washed lysates; PPM = purified plasma membranes; and ND = not determined. The designation of sites 1, 2, and 3 was based on comparable K_D and B_{max} values between studies.

*Andorn AC and Carlson MA, The complexities of alpha$_2$-adrenergic ligand interaction with specific binding sites in normal human cortex and cortex from presumptive suicide victims. Abstract presented at the Annual Meeting of the American College of Neuropsychopharmacology, Washington, DC, Dec. 8–12, 1986.

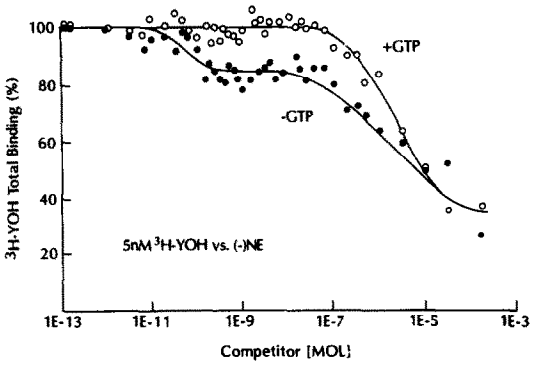

Fig. 5. Competition of unlabeled (−)-NE for 5.0 nM [^{3}H]yohimbine binding to platelet purified plasma membranes in the presence and absence of GTP. Studies were performed as in Fig. 4 except for using (−)-NE for competition. Each point was determined in duplicate. Closed circles (●) represent untreated membranes. Open circles (○) represent membranes in the presence of 0.1 mM GTP. Total cpm bound were 3442 ± 14. Curves were computer drawn using LIGAND [39].

0.1–6 nM [^{3}H]YOH saturation binding data. We assembled three such sets of experiments each set deriving from different membrane preparations and composed of at least 100 experimental points analyzed in duplicate. The results of co-analysis shown in Table 3 (first category) display the mean ± SEM for the preferred binding parameters obtained from these three sets of experiments. The results demonstrate three PAC binding sites that have parameters roughly comparable to those determined previously (compare Table 3) with Scatchard plot and rate analysis data). Our B_{max} values are listed in Table 4. The parameters were preferred over all simpler models ($P \leq 0.05$). In all models tested, the preferred parameters involved high affinity YOH binding to only site-1 and site-3. YOH displayed a roughly equal binding affinity (estimated $K_D = 0.66$ to 1.2 nM) for both site-1 and site-3. Binding of YOH to site-2 was estimated with $K_D = 260$ nM.

Similarly, we performed multi-experiment computer analyses which merged [^{3}H]PAC Scatchard plot binding data with 0.1 and 0.8 nM [^{3}H]PAC competition experiments using (−)-NE as competitor (Table 3, second category). In these analyses with (−)-NE, the best fit was with multiple [^{3}H]PAC sites (Table 3, second category). Most of the computer-generated estimates were comparable to the previous PAC estimates. Again, the preferred model for (−)-NE competition indicated a very low affinity of (−)-NE for PAC site-2 (highest $K_{i2} = 16{,}000$ nM), while (−)-NE competed for [^{3}H]PAC with high affinity at PAC site-1 ($K_i = 0.17$ nM). These multi-experiment analyses shown in Table 3 are consistent with the analyses of individual competition experiments shown in Table 2.

To measure the effects of GTP, we next exposed platelet membranes to 0.1 mM GTP and performed [^{3}H]YOH saturation binding experiments (Scatchard

analysis) and 5 nM [^{3}H]YOH competition experiments with unlabeled PAC. The competition experiments (N = 4) were performed to determine which of the PAC displaceable sites were GTP sensitive. GTP had no effect on [^{3}H]YOH binding parameters as determined by [^{3}H]YOH Scatchard plot analysis. In two experiments in the absence of GTP, the [^{3}H]YOH K_D was 0.45 to 0.58 nM with B_{max} = 170–244 fmol/mg protein, compared to K_D = 0.48–0.49 nM with B_{max} = 180–254 fmol/mg protein in the presence of GTP. However, GTP induced substantial changes in the competition curves of [^{3}H]YOH by unlabeled PAC (Fig. 4B). GTP resulted in a loss of PAC binding site-1 (K_i = 20.6 pM, see above), but caused the appearance of additional displacement near 1 nM PAC. Based on the PAC binding with a K_i similar to site-2, it seemed possible that GTP converted PAC site-1 to PAC site-2. However, that would not fit with our previous findings since yohimbine (or [^{3}H]YOH) had not shown high enough affinity for site-2 in the absence of GTP to be substantially bound by 5 nM [^{3}H]YOH.

To further explore the GTP effect we performed competition experiments with (−)-NE against 5 nM [^{3}H]yohimbine in the presence and absence of 0.1 mM GTP. In the absence of GTP, the results (Fig. 5, closed circles) were in agreement with our previous findings, that there was one site with high affinity for (−)-NE (apparently site-1 based on K_i). The putative site-1 had (−)-NE parameters that were compatible with our previous experiments: K_{i1} = 32.2 pM, B_{max1} = 48.5 ± 12.2 fmol/mg protein (calculated to be 20.6% of total binding sites). There was also a lower affinity site of competition by (−)-NE of [^{3}H]YOH binding, which, based on the percent of sites, may represent PAC site-3 (comparable to Table 3). The (−)-NE parameters for the putative site-3 were K_{A3} = 4.2 ± 1.2 E6 (K_{i3} = 239 nM) and B_{max3} = 187.5 ± 10.2 fmol/mg. As predicted by our previous experiments, GTP again completely removed site-1 (Fig. 5). In the presence of GTP, the data could only be modeled to one site (P = 0.322 vs two sites). The (−)-NE parameters in the presence of GTP were K_A = 2.3 E6 ± 4.2 E5 (K_i = 437 nM), B_{max} = 330 ± 15.9 fmol/mg. Thus, GTP appeared to have transferred the competitive binding of (−)-NE from site-1 to site-3 affinity. It may be deduced from K_i and K_D values in Table 3 and Fig. 5, that the "GTP-formed site-3" displays the following rank order of potency: yohimbine > PAC > (−)-NE.

To characterize site-3 we also performed extended saturation binding experiments, using from 4 to 120 nM [^{3}H]PAC. Non-specific binding was measured in tubes containing 10^{-5} M (−)-NE in addition to the radioligand. Over this range of concentrations the total bound cpm ranged from 1,560 to 25,600 per mL, but the amount of non-specific binding was high. The percent of [^{3}H]PAC total binding that was competed for by 10^{-5} M (−)-NE was only 22.4 ± 3.09% at 4 nM [^{3}H]PAC, and was 11.9 ± 4.8% at 120 nM [^{3}H]PAC (N = 3). The majority of the non-specific binding in

Table 5. Competition by various drugs for 5 nM [^{3}H]YOH binding to purified plasma membranes from Red Cross platelet concentrates

	Site 1	Site 3	P value
Competitor	K_i (M)	K_i (M)	2 sites vs 1 site
Yohimbine	5E-10	5E-10	1.00
Phentolamine	3.7E-7	2.7E-9	0.02
p-Aminoclonidine	2.1E-11	3.1E-8	0.01
(−)-NE	3.2E-11	2.4E-7	0.01
(−)-Epinephrine	1.8E-10	5.3E-7	0.01
Serotonin	1.3E-6	1.3E-6	1.0
Ketanserin	1.5E-6	1.5E-6	1.0
Prazosin	1.6E-6	1.6E-6	1.0
(−)-Isoproterenol	5.8E-5	5.8E-5	1.0

Each competitor concentration was minimally performed with 38 concentrations of competitor (between 10^{-13} and 10^{-4} M) against 5.0 nM [^{3}H]YOH for binding to platelet plasma membranes. Membranes were obtained from out-dated Red Cross platelets. Incubations were for 1 hr at a protein concentration of 0.05 mg protein/0.5 mL (see Materials and Methods). Total binding was 2860 ± 635 cpm (SD), and non-specific binding was 877 ± 448 cpm (SD). Each curve was analyzed for one and two sites using the LIGAND computer program [39], and the statistically preferred fit is given. Experimental binding parameters are expressed in exponential molar units (E = $\times 10^{nth}$). Assignments to site-1 and site-3 were based on the estimated B_{max} values: site-1 averaged 26.6 ± 11.2% (SD) of the total specific binding in the four cases where two sites were obtained. Non-specific binding was computed for each competitor versus [^{3}H]YOH, by the computer. The computer-generated coefficients of variation for the competitor binding affinities (average of the coefficients of variation for both K_A values) were: yohimbine (K_A ± 61.5%), phentolamine (K_A ± 68.0%), *p*-aminoclonidine (K_A ± 12.8%), (−)-NE (K_A ± 33.0%), (−)-epinephrine (K_A ± 61.6%), serotonin (K_A ± 22.5%), ketanserin (K_A ± 20.6%), prazosin (K_A ± 18.9%), and (−)-isoproterenol (K_A ± 19.4%).

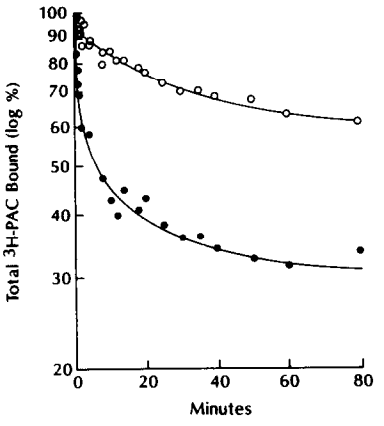

Fig. 6. Rate dissociation of specific [3H]PAC binding after addition of either clonidine or (−)-NE. [3H]PAC (0.3 nM) was incubated for 1 hr at 21° with 0.2 mg protein/2 mL to reach steady state. Thereafter, clonidine (●) or (−)-NE (○) was added in a concentrated form to reach a final concentration of 10^{-4} M (as in Fig. 1B). The reactions were then allowed to proceed for the times shown. Each experimental point was determined in duplicate and each experiment was repeated twice using whe same membrane source. Total binding at zero time was 395 ± 103 cpm. The average values are plotted.

these experiments was attributed to high filter binding: filter blanks were 74.6 ± 16.4% of the non-specific binding at 4 nM [3H]PAC and 66.0 ± 18.8% of the non-specific binding at the highest concentration of [3H]PAC. Despite the uncertainties inherent in data with such high non-specific binding, we obtained binding parameters in reasonable agreement with our previous studies of site-3. The computer-generated site-3 parameters were K_A = 3.76 ± 1.67 E7 (K_D = 26.6 nM) and B_{max} = 316 ± 98 fmol/mg protein. These data compared well with the binding parameters for PAC competition for 5 nM [3H]YOH (Fig. 4A) where the K_i for site-3 was 31.2 nM and the B_{max} was 396 fmol/mg protein. Therefore, we performed additional 5 nM [3H]YOH competition experiments to determine the affinity of various drugs for site-3. The results are presented in Table 5. The rank order for competition was yohimbine > phentolamine > PAC > (−)-NE > (−)-epinephrine > serotonin = ketanserin = prazosin > isoproterenol. This suggested that site-3 is an α_2-adrenoceptor.

It thus appeared that (−)-NE bound with low affinity to PAC site-2. To confirm this, we performed [3H]PAC rate dissociation experiments to compare CLON or (−)-NE as the displacing agents. From our earlier [3H]PAC rate dissociation analyses (Fig. 1B) we determined that the slow dissociation site represented PAC site-1 and the fast dissociation site represented PAC site-2. [3H]PAC (0.3 nM) was allowed to reach equilibrium binding at 21° for 40 min, following which the binding reactions were made to 10^{-4} M with either CLON or (−)-NE and the displacement of binding was followed over the next 80 min. As can be seen in Fig. 6, the displacement by CLON was clearly biphasic, whereas the displacement induced by (−)-NE appeared to be monophasic. CLON displaced 25% more binding than (−)-NE. Moreover, the difference in binding was due to the absence in fast dissociation binding, not seen with (−)-NE. In this experiment, CLON dissociation was modeled to two sites with k_{-11} = 0.037 ± 0.007 min^{-1} and k_{-12} = 0.897 ± 0.680 min^{-1} (P = 0.03 over one site). In contrast, (−)-NE dissociation was not well modeled by two sites, and all the binding was relatively slow dissociating (k_{-11} = 0.022 ± 0.008 min^{-1} and k_{-12} = 0.067 ± 0.166 min^{-1} (P = 0.13 over one site, N = 2). This difference in [3H]PAC dissociation rates by CLON and (−)-NE further confirmed that (−)-NE has low affinity for PAC site-2.

DISCUSSION

We have identified multiple binding sites for [3H]PAC on purified plasma membranes from human platelets. Site-1 had the highest affinity for [3H]PAC (K_D = 13 pM) and appears to be a GTP-sensitive α_2-adrenoceptor (Table 2). Site-3 had the lowest affinity (K_D = 50 nM) and appears to be an uncoupled α_2-adrenoceptor since it is increased in the presence of GTP in proportion to the GTP-induced loss of site-1 (Fig. 5). Site-2 also had high affinity for [3H]PAC (K_D = 0.7 nM) but, since it had much lower affinity for both (−)-NE and (−)-epinephrine, it is probably not an adrenoceptor (Table 2).

We are the first to report a non-adrenergic binding site (site-2) for [3H]PAC on purified plasma membranes from human platelets. Michel *et al.* [22] reported non-adrenergic [3H]idazoxan binding sites on human platelets, but those sites were localized to intracellular membranes and the ligand specificity was not the same as our site-2 [23]. Because our binding conditions were unique, previous investigators may have failed to detect site-2 for several reasons. First, we utilized more highly purified plasma membrane preparations than most previous investigators. Second, our binding conditions were extended to very low radioligand concentrations to distinguish both sites [8, 24]. Finally, Paris *et al.* [43] have shown that replacement of Tris–Mg^{2+} buffer by glycylglycine buffer provokes Scatchard plot curvature for [3H]UK-14,304 binding. This suggests that buffer choice (in our case hypotonic HEPES with low magnesium) may also be crucial to site-2 binding.

Our characterization of site-2 is similar to a norepinephrine-insensitive binding site with high affinity for imidazoline compounds, first observed in bovine brain [18]. That "imidazoline preferring site" was reported to have a nearly identical affinity for [3H]PAC as our site-2. It appears to be a receptor which mediates the antihypertensive effects of clonidine [44] and may also regulate catecholamine release [45]. In preliminary studies we have observed that 10 μM imidazoline-4-acetic acid competed for half the CLON displaceable binding of 0.4 nM [3H]PAC. This is significant since imidazole-4-acetic acid was found to be relatively selective for the imidazoline binding site in bovine brain [18]. An endogenous "clonidine-displacing substance" (CDS)

has been isolated from bovine brain which preferentially binds to the imidazoline binding site and is active both in centrally regulating arterial blood pressure [44, 46] and in peripherally stimulating smooth muscle contraction [47]. A site with similar properties was also described in rabbit kidney [48]. A CDS has been detected in plasma [49], and CDS competes for [^{3}H]rauwolscine-labeled α_2-adrenoceptors on platelets and inhibits epinephrine-induced platelet aggregation [50].

Our detection of a "non-adrenergic" site for PAC on platelet plasma membranes may explain novel effects of clonidine concerning platelet aggregation. Clonidine alone does not stimulate aggregation. However, CLON is a *potentiator* of the aggregation response to ADP, vasopressin, serotonin, collagen, thrombin and arachidonate, but it is an *inhibitor* of the aggregation response to adrenaline [25]. Those data might be explained if the CLON potentiation of aggregation and the CLON inhibition of epinephrine-induced aggregation were acting through different receptors, such as site-1 and site-2. In addition, CLON-related compounds were reported [51] to inhibit adenylate cyclase activity in platelets, which was *not* antagonized by yohimbine and was still observed in the absence of GTP.

A [^{3}H]PAC binding site-1 (Table 3) with estimated K_D in the low picomolar affinity range was verified in our [^{3}H]PAC rate association, rate dissociation, saturation analysis and competition binding experiments (Figs. 1–5, Tables 2 and 3) as well as [^{3}H]YOH competition experiments. Neubig *et al.* [52] and Gantzos and Neubig [53] have presented evidence for a site similar to our PAC site-1, which was distinguished by the agonist [^{3}H]UK-14,304. In their reports, [^{3}H]UK-14,304 bound with low affinity (K_D = 10 nM) and high affinity (K_D = 0.3–0.6 nM) to platelet plasma membranes. The low affinity binding dissociation rate was $k_{-1} = 1.02\ \text{min}^{-1}$. The high affinity binding site was divided into fast ($k_{-3} = 0.0258\ \text{min}^{-1}$) and slow ($K_{-2} = 6.6\ \text{min}^{-1}$) dissociation of agonist binding. The slow phase of high affinity binding was unique in that both the rate constants and amplitudes were relatively independent of ligand concentration [52]. That slow phase of association of high affinity binding was reported to be nearly 17-fold more sensitive to temperature change in the range of 0–30° than the fast phase of high affinity binding [53]. Also, the slow phase of high affinity agonist binding was characterized by a nonlinear Arrhenius plot with a "break" at approximately 17°, the phase transition temperature of platelet membrane lipids [53]. Therefore, Neubig *et al.* [52] proposed the following model: (a) approximately one-third of the α_2 adrenoceptors bind agonist with low affinity and are unable to couple with a guanine nucleotide binding protein (G_i protein), (b) approximately one-third are coupled to the G_i protein prior to agonist binding, and (c) the remainder of adrenoceptors interact with the G_i protein after high affinity agonist binding by "slow" diffusion in the lipid membrane or by a protein conformational change which is dependent on the lipid environment. Based on this model, our results at first raised the possibility that [^{3}H]PAC, in contrast to [^{3}H]UK-14,304, may be able to distinguish all three of these binding states based on affinity. However, the very low affinity of site-2 for (−)-NE (Tables 2 and 3) would not fit the three-state model of Neubig *et al.* [52]. Therefore, PAC site-2 cannot be the same as the "pre-coupled" adrenoceptor as proposed by Neubig *et al.* [52].

The imidazoline binding site is reported to be less sensitive to GTP than α_2 binding sites [50, 54]. Using Scatchard plot analysis of [^{3}H]PAC binding, we determined that GTP abolished [^{3}H]PAC binding to site-1. However, site-2 was still substantially present after GTP treatment, although a decreased affinity for PAC was noted in the presence of GTP as measured by [^{3}H]PAC Scatchard plot analysis (site-2 with GTP, K_D = 2.7 nM) (see also Table 2). A decreased affinity of [^{3}H]PAC has been described previously for binding of PAC in the presence of GTP [24]. Thus, PAC site-2 appears to be relatively GTP insensitive, and therefore again incompatible with a pre-coupled adrenoceptor. Instead, it shows properties in common with the imidazoline binding site in brain [18].

Studies of GTP treatments (Figs. 4 and 5) indicated the loss of site-1. As measured by PAC competition for 5 nM [^{3}H]YOH, the GTP-induced loss of site-1 correlated with a shift from picomolar affinity to an affinity of 0.67 nM (K_i) (Fig. 4B). This was intermediate between PAC site-1 (K_i = 21.6 pM) and PAC site-3 (K_i = 31.2 nM). In contrast, as measured by (−)-NE competition for 5 nM [^{3}H]YOH, the GTP-induced loss of site-1 correlated with a shift of binding to a much lower affinity of (−)-NE at K_i = 437 nM (Fig. 5). The (−)-NE competition data, therefore, clearly showed a shift from site-1 to site-3. To explain the discrepancy between PAC and (−)-NE, we might speculate that the conversion from site-1 to site-3 may involve an intermediate state which in the case of PAC may be relatively stable, since PAC is only a partial agonist. Norepinephrine on the other hand, being a full agonist, may be potent enough to destabilize any intermediate.

Prior to beginning this study, one of us (A.C.A.) performed studies on binding to human prefrontal cortical homogenates using the partial agonist [^{3}H]PAC; the data have been reported in preliminary form.* Herein we performed our platelet studies using the identical reaction conditions as described in that study for brain [3,*]. Initially we found that washed platelet *lysates* displayed a low level of [^{3}H]PAC binding, making a poor comparison to brain homogenates [8]. Now, however, using purified platelet plasma membranes, [^{3}H]PAC binding parameters were found to be highly comparable to those observed in human prefrontal cortex (Table 4). This provides evidence that platelets and cerebral cortex may possess similar binding sites. Neubig and Szamraj [37] presented evidence that the failure to detect high affinity binding in platelet lysates is due

* Andorn AC and Carlson MA, The complexities of alpha$_2$-adrenergic ligand interaction with specific binding sites in normal human cortex and cortex from presumptive suicide victims. Abstract presented at the Annual Meeting of the American College of Neuropsychopharmacology, Washington, DC, 8–12 Dec 1986.

to the presence of an inhibitor of [^{3}H]PAC binding which is washed-out in sucrose gradient preparations of plasma membranes. Kim and Neubig [16] have indicated that this inhibitor is GTP. Neubig *et al.* further showed that guanine nucleotides lower the affinity of PAC binding [24]. Another possible inhibitor may be (−)-NE since (−)-NE is washed out of platelet membranes slowly at 21° [42, 43]. We found that additional membrane washes at 37°, as well as washes in the presence of GTP (prior to membrane isolation), did not raise [^{3}H]PAC binding further, suggesting that our membranes were free of inhibitor.

Finally, we cannot definitely rule out that a small portion of our binding may be derived from low levels of contaminating white blood cells in our platelet preparations. α_2-Adrenoceptor binding sites have not been reported on red blood cells. However, lymphocytes were reported to possess high and low affinity [^{3}H]YOH binding with $K_{D1} = 2.4$ nM ($B_{max} =$ 5 fmol/mg protein) and $K_{D2} = 139$ nM ($B_{max} =$ 81 fmol/mg protein) [55]. The K_D value of the second site for [^{3}H]YOH compared well with the K_i value we observed for YOH at site-2 (Table 2). Based on 0.58% white blood cell contaminate in our preparations, and knowing the reported average size of platelets, B cells, and T cells [56], and the [^{3}H]YOH B_{max} on lymphocytes, we calculated that lymphocyte contamination may account for approximately 11–24% (median = 18%) of the total [^{3}H]YOH binding sites observed in our assays.

In summary, we have observed multiple binding sites for [^{3}H]PAC on purified plasma membranes from platelet preparations. PAC site-1 had the highest affinity and had properties consistent with a GTP-sensitive, coupled state of α_2-adrenoceptor. As described by Neubig *et al.* [52] and Gantzos and Neubig [53] for UK-14,304, our PAC site-1 could be composed of fast- and slow-associating components. However, PAC site-2 had properties inconsistent with a typical α_2-adrenoceptor since it displayed low affinity for (−)-NE and (−)-epinephrine. Instead, site-2 had properties in common with an imidazoline binding site [18]. In contrast, PAC site-3, as measured by competition studies with [^{3}H]yohimbine, displayed a rank order of affinity for various drugs which was consistent with an α_2-adrenoceptor. Since PAC site-3 was relatively GTP insensitive (Fig. 5), it probably represents the uncoupled α_2-adrenoceptor.

It is interesting to note that in studies of depressive illness, changes in the level of binding have been reported with [^{3}H]CLON and [^{3}H]PAC but *not* with [^{3}H]YOH binding in human tissue [5–7]. We have demonstrated previously increased [^{3}H]PAC binding to both PAC site-1 and site-2 in depressed patients [33, 34]. In light of our current results, it appears that in depressive illness there may be an elevation in both coupled α_2-adrenoceptors and in putative imidazoline binding sites.

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