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α_2-Adrenergic Receptors in the Human Cell Line, HT29

Characterization with the Full Agonist Radioligand [^{3}H]UK-14,304 and Inhibition of Adenylate Cyclase

JOHN T. TURNER, CARLA RAY-PRENGER, AND DAVID B. BYLUND

Department of Pharmacology, University of Missouri School of Medicine, Columbia, Missouri 65212

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SUMMARY

We have characterized the α_2-adrenergic receptor in membranes from the human colonic adenocarcinoma cell line, HT29, using the recently introduced α_2-agonist 5-bromo-6-[2-imidazolin-2-yl-amino]quinoxaline ([^{3}H]UK-14,304), two other radioligands, and a series of adrenergic agonists and antagonists. We also investigated α_2-agonist inhibition of HT29 cell adenylate cyclase and reversal of inhibition by α-adrenergic antagonists. [^{3}H] Yohimbine saturation experiments indicated a single class of sites with a K_D of 0.61 nM which agreed with the kinetically determined K_D of 0.62 nM. Computer analysis of kinetic and saturation experiments with [^{3}H]UK-14,304 revealed two classes of sites. From the saturation data, one site had high affinity for the radioligand (0.14 nM) and comprised 33% of the total number of sites, whereas the other site had lower affinity (6.1 nM). The total number of sites labeled by [^{3}H]UK-14,304 (360 fmol/mg of protein) was approximately equal to the number of sites labeled by [^{3}H]yohimbine (330 fmol/mg), whereas [^{3}H]*para*-aminoclonidine labeled fewer sites of a single class. Rank order potencies of adrenergic agonists and antagonists obtained from competition binding assays indicated that: (*1*) the same receptors were labeled by the three radioligands, and (*2*) the receptors were of the α_2 subtype. UK-14,304 and epinephrine inhibited forskolin- and vasoactive intestinal peptide-stimulated adenylate cyclase in a dose-dependent manner up to 32%. Inhibition of the enzyme was reversed by yohimbine and, less potently, by phentolamine and prazosin in a dose-dependent manner. The HT29 cell line appears to be a useful model system for the investigation of the regulation and mechanism of action of α_2-adrenergic receptors in human tissues.

INTRODUCTION

α_2-Adrenergic receptors, their mechanism of action, and their regulation are currently subjects of intense interest. Two limitations in studying human α_2-receptors have been the lack of a human cell line in which these receptors have been fully characterized and a suitable radiolabeled full agonist at α_2-receptors.

Although most studies of human α_2-adrenergic receptors have been in the platelet, this cell type has several limitations (1). These include substantial variations in receptor density from individual to individual (2) and the inability to study receptor regulation (3). The rodent hybrid cell line NG108-15 has been used to characterize α_2-receptors (4). However, differences between rodent and human α_2-receptors have been observed (5). These differences underscore the need for a cell line of human origin as a source of α_2-receptors. The human colonic adenocarcinoma cell line, HT29, was established by Fogh and Trempe (6). Previous communications indicate that these cells have α_2-adrenergic receptors which can be labeled by the α_2-antagonist [^{3}H]yohimbine (7) and by the α_2 partial agonist [^{3}H]clonidine (8). Furthermore, the cells can be grown in defined media (9), suggesting their utility in studies of receptor regulation. The HT29 cell line also possesses a VIP[1]-stimulable adenylate cyclase system (10) that may be coupled to glycogenolysis (11). Thus, this cell line may prove to be a useful system for characterizing α_2-receptors and for studying their mechanism of action, as well as the regulation of both α_2-receptors and cellular processes that they modulate.

A second limitation in studying α_2-adrenergic receptors and actions in human and other tissues has been the lack of a suitable radiolabeled agonist. Initial studies

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[1] The abbreviations used are: VIP, vasoactive intestinal peptide; PAC, *para*-aminoclonidine; UK-14,304, 5-bromo-6-[2-imidazolin-2-yl-amino]quinoxaline; EDTA, ethylenediaminetetraacetate.

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of agonist binding utilized either [^{3}H]epinephrine or [^{3}H] norepinephrine and were only partially successful due to the instability and difficulty of use of these radioligands (1). Subsequent investigations with the partial agonists clonidine and PAC suffered from the less than full intrinsic activity of these imidazolines (1). A more recently introduced aryl-imidazoline, UK-14,304, has been reported to act as a full agonist at α_2-receptors in platelets (12). Loftus *et al.* (13, 14) have characterized the binding of the tritiated analogue [^{3}H]UK-14,304 in rat brain.

In this paper we report the results of studies designed to: (*1*) characterize the HT29 cell α_2-receptor using radioligand binding studies; (*2*) investigate α_2-regulation of adenylate cyclase in HT29 cells; and (*3*) assess the recently introduced imidazoline UK-14,304 and its tritiated analogue as agonists at α_2-receptors.

MATERIALS AND METHODS

Cell culture and preparation of membranes. HT29 cells, passage 187, from a human colonic adenocarcinoma, were generously provided by J. Fogh, Sloan-Kettering Institute (Rye, NY). Cells were grown routinely in Dulbecco's modified Eagle's medium with high glucose supplemented with 5% (v/v) fetal calf serum and 5% (v/v) newborn calf serum in 150-mm-diameter disposable tissue culture dishes in a humidified atmosphere of 5% CO_2:95% air. Cells were subcultured with 0.25% (w/v) trypsin and seeded at low density, with confluence being reached in about 7 days. Medium was replaced every 3–4 days. Confluent dishes were washed once with phosphate-buffered saline, pH 7.4, and then harvested with a rubber policeman. Cells were pelleted by centrifugation (1,000 × *g*, 5 min), resuspended in 25–30 ml of 50 mM Tris-HCl, pH 8.0, and homogenized with a Tissumizer (Tekmar Co., Cincinnati, OH) for 30 sec at setting 90. The pellet obtained by centrifugation for 10 min at 49,000 × *g* was washed once by resuspension in Tris-HCl followed by a second centrifugation. Unless indicated otherwise, the pellet, a crude particulate fraction, was resuspended in either 25 mM glycylglycine buffer, pH 7.6, or 25 mM Tris-HCl buffer, pH 7.6, for radioligand binding and adenylate cyclase assays, respectively. Protein was measured by the method of Lowry *et al.* (15). Cells from passages 193 to 214 were utilized for the experiments described herein.

Radioligand binding assays. The binding protocol was essentially the same as that reported previously (16). The radioligands used were [^{3}H] yohimbine, [^{3}H]PAC, and [^{3}H]UK-14,304. For saturation experiments, total binding was determined with one set of incubation tubes which contained 500 μl of membrane suspension (about 100 μg of membrane protein) and 20 μl of the appropriate concentration of the radioligand diluted in 5 mN HCl. To a parallel set of incubation tubes, 10 μl of (−)-norepinephrine (final concentration: 10 μM for [^{3}H]yohimbine and [^{3}H] PAC; 100 μM for [^{3}H]UK-14,304) were added to determine nonspecific binding. Specific binding was calculated as the difference between total and nonspecific binding. In certain experiments, as indicated in the figure and table legends, NaCl (final concentration, 30 mM) $MgCl_2$ (1 mM), or a combination of GTP (0.1 mM) and Na_2 EDTA (1 mM) was added to the incubation tubes. After a 30-min ([^{3}H]yohimbine or [^{3}H] PAC) or 90-min ([^{3}H]UK-14,304) incubation at 23°, the suspensions were filtered through GF/B glass fiber filter strips (Whatman, Inc., Clifton, NJ), using a 24-sample manifold (Brandel Cell Harvester, Biomedical Research and Development, Gaithersburg, MD). The tubes and filters were washed twice with 5 ml of ice-cold Tris-HCl, and the radioactivity retained on the filter was determined by scintillation spectroscopy. The K_D and B_{max} values were calculated from a computer-assisted nonlinear regression of the data plotted as bound versus free ligand.

For inhibition experiments, a fixed concentration of radioligand (20 μl) and various concentrations of unlabeled drug (5–10 μl) were added to 500 μl of the membrane suspension. GTP (0.1 mM) and Na_2 EDTA (1 mM) were added to the incubation tubes in specified experiments. Inhibition data were analyzed by probit transformation, and the IC_{50} (the inhibitor concentration at which specific binding is reduced 50%) and the Hill coefficient, n_H, were determined. In selected experiments, saturation and inhibition data were analyzed by computerized curve fitting to one-site and two-site models (PROC NLIN, SAS Institute, Cary, NC). An *F*-test was used to determine whether the two-site model provided a significantly better fit of the data as compared to the one-site model (17). IC_{50} values were used to calculate the equilibrium dissociation constant (K_i) of the unlabeled ligand, from $K_i = IC_{50}/(1 + F/K_D)$, where F is the radioligand concentration.

For association experiments, 500 μl of membrane suspension and a fixed concentration of radioligand were incubated with or without 10 μM or 100 μM norepinephrine for various times from 1 to 90 min. The data were linearized by plotting the natural log of the amount specifically bound at steady state divided by that amount minus the amount bound at discrete times less than the steady state time, $\ln(B_e/(B_e - B))$, versus time. The slope of this line is the pseudo-first order association rate constant, $k_{ob}(min^{-1})$. The dissociation rate constant, $k_{-1}(min^{-1})$, was determined by preincubating membrane suspension with a fixed concentration of radioligand for 30 min, then adding 10 μl of norepinephrine (10 or 100 μM final concentration) and incubating for various additional times from 0 to 90 min. A plot of $\ln B/B_o$ versus time, where B_o is the amount of radioligand bound just before the addition of norepinephrine and B is the amount bound at any time thereafter, yields a line the slope of which is $-k_{-1}$. The association rate constant, k_{+1} ($M^{-1}min^{-1}$), is determined from $k_{+1} = (k_{ob} - k_{-1})/L_t$, where L_t is the radioligand concentration. The equilibrium dissociation constant, K_D, equals k_{-1}/k_{+1} (18). Dissociation data from experiments with [^{3}H]UK-14,304 were resolved as one, two, and three sites using the CSTRIP program of Sedman and Wagner (19). The goodness of fit was evaluated with the F statistic. Estimates of the pseudo-first order association rate constants for the two receptor classes as well as estimates of the proportion of sites of each class were made using the program of J. Kurz (Department of Pharmacology, University of North Carolina) based on the equations derived by Aranyi (20).

Adenylate cyclase assay. Adenylate cyclase activity in the crude particulate fraction was measured by the method described by Nickols *et al.* (21). Briefly, 50 to 100 μg of protein were incubated with 1.2 mM [α-^{32}P]ATP in 75 μl of a medium containing 50 mM Tris-HCl (pH 7.6), 6.7 mM $MgCl_2$, 25 mM creatine phosphate, 5 units/ml of creatine phosphokinase, 1 mM cyclic AMP, 10 μM GTP, 30 mM NaCl, 0.7 mM isobutylmethylxanthine, and 2 mg/ml of bovine serum albumin. Drugs were added in 5 μl of 5 mN HCl or, for forskolin, in 50% dimethylsulfoxide or 70% ethanol. The incubation was for 10 min at 30°. Dowex-50 alumina chromatography (22) was used to separate [^{32}P]cyclic AMP with [^{3}H]cyclic AMP as an internal standard for measuring recovery. Tests for statistical significance were done by analysis of variance followed by the Studentized range test (23).

Materials. The following compounds were purchased from Sigma Chemical Company (St. Louis, MO): yohimbine hydrochloride, (−)-norepinephrine (+)-bitartrate, (−)-epinephrine (+)-bitartrate, guanosine 5′-triphosphate, and disodium ethylenediaminetetraacetic acid. PAC was purchased from Research Biochemicals, Inc. (Wayland, MA). VIP was obtained from Peninsula Laboratories (Belmont, CA) and forskolin was obtained from Calbiochem-Behring (La Jolla, CA). Dulbeccos modified Eagle's medium was purchased from KC Biologicals, Inc. (Lenexa, KS).

The following drugs were graciously donated by the respective companies: prazosin and UK-14,304-18, Pfizer, Inc. (Groton, CT); regitine hydrochloride (phentolamine), CIBA-Geigy Corp. (Summit, NJ); (+)-epinephrine bitartrate, Sterling-Winthrop Research Institute (Rensselaer, NY); and oxymetazoline hydrochloride, Schering Corporation (Kenilworth, NJ).

The radioligands [^{3}H]yohimbine (83 Ci/mmol) and [^{3}H]PAC (41 Ci/mmol) as well as [^{3}H]cyclic AMP (40 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). [α-^{32}P]Adenosine-5′-triphosphate (10–25 Ci/mmol) was purchased from ICN Radiochem-

icals (Irvine, CA). [^{3}H]UK-14,304 (84 Ci/mmol) was generously donated by Dr. S. Hurt of New England Nuclear.

RESULTS

Kinetic Analyses

A concentration of HT29 membrane suspension on the linear portion of the tissue concentration curve for the three radioligands was selected for further experiments. Association and dissociation curves for [^{3}H]yohimbine were monophasic and gave forward (k_{+1}) and reverse (k_{-1}) rate constants of 0.16 ± 0.03 $nM^{-1}min^{-1}$ and 0.097 ± 0.009 min^{-1} (mean ± SEM, n = 3), respectively. The ratio of these constants gave a kinetically derived equilibrium dissociation constant of 0.62 ± 0.05 nM.

Kinetic experiments with [^{3}H]UK-14,304 were more complex. For dissociation experiments, plots of ln B/B_o versus time were curvilinear and, assuming two classes of non-interacting sites, were analyzed according to the formula, Bound = $B_o{}^H\exp(-k_{-1}{}^H t) + B_o{}^L\exp(-k_{-1}{}^L t)$ (where $B_o{}^H$ and $B_o{}^L$ are the concentrations of the two receptor classes labeled by radioligand and $k_{-1}{}^H$ and $k_{-1}{}^L$ are the respective dissociation rate constants) using the exponential stripping program, CSTRIP, of Sedman and Wagner (19). One of three experiments analyzed by this method is shown in the *upper panel* of Fig. 1. For the three experiments, in which the [^{3}H]UK-14,304 concentration was 1.1 ± 0.1 nM, 38 ± 2% of the labeled receptors exhibited a dissociation rate constant of 0.81 ± 0.19 min^{-1} (the low affinity site; $t_{1/2}$ = 1.0 ± 0.3 min) whereas the rate for the remaining 62 ± 2% was 0.0115 ± 0.0008 min^{-1} (the high affinity site; $t_{1/2}$ = 61 ± 4 min). In each experiment, the fit of the data to the two-site model was significantly better than to a one-site model, based on the F statistic, α = 0.01. A three-site model was not better than a two-site model. Based on the computer-derived dissociation constants, at 60 min of incubation with competing ligand (total incubation time, 90 min), 31% of the total specific binding should remain. For the three experiments, the mean percentage of the observed specific binding in this time range was 31 ± 2%. Experiments involving longer incubation times have not provided definitive proof that there is not a small irreversible binding component. It is not clear whether this is due to a loss of viability of the assay system at very long incubation times (>2 hr) or to a bona fide irreversible ligand-receptor interaction.

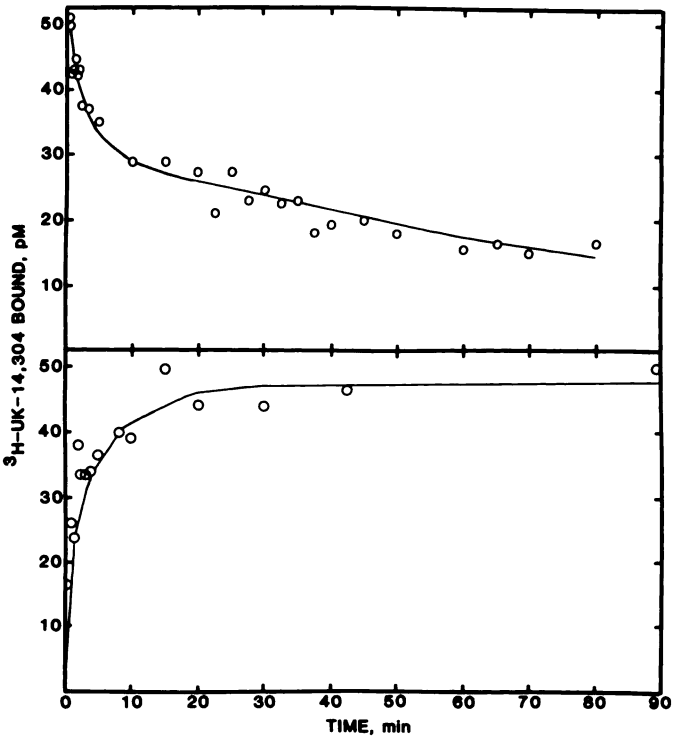

FIG. 1. *Kinetics of [^{3}H]UK-14,304 binding in HT29 cell membranes* Upper panel, dissociation of specifically bound [^{3}H]UK-14,304 at various times after a 30-min preincubation with 0.89 nM [^{3}H]UK-14,304. At time zero, 10 μM norepinephrine was added and the amount of specific binding at various times was determined. The dissociation rate constants, $k_{-1}{}^H$ and $k_{-1}{}^L$ were obtained as described in Materials and Methods. The *points* shown are the means of duplicates from a single experiment which was repeated twice. Lower panel, specific binding of [^{3}H]UK-14,304 at various times after the addition of 0.78 nM [^{3}H]UK-14,304. The *curve* shown was based on estimates determined by fitting data from a similar experiment to a two independent sites model as described in Materials and Methods and in Results.

Association experiments plotted as ln $(B_e/B_e - B)$ versus time were also curvilinear, and binding at the low affinity site was too rapid to accurately measure directly. However, theoretical curves calculated as the sum of the independent pseudo-first order rate constants for the two classes of sites and plotted as specific binding versus time were compared to experimentally obtained curves. The theoretical curves were generated by utilizing the experimentally determined k_{-1} values and varying the two k_{+1} values and the percentage of total sites contributed by the two classes. Estimates of these parameters were made using data from one experiment and, then, to determine the reproducibility of our results, data generated using these estimates were compared to actual data from a different experiment (Fig. 1, *lower panel*). A third experiment yielded similar agreement for the fit of the theoretical and experimental values. The following constants gave the curve shown: $k_{+1}{}^H$, 16 × 10^{-5} $pM^{-1}min^{-1}$; $k_{+1}{}^L$, 40 × 10^{-5} $pM^{-1}min^{-1}$; B^L_{max}/B^H_{max} = 3. Thus, the estimated K_D values from these kinetic analyses (k_{-1}/k_{+1}) were $K_D{}^H$ = 70 pM and $K_D{}^L$ = 2030 pM.

Saturation Analyses

Saturation binding experiments with all three ligands are summarized in Table 1 with a typical curve for each ligand shown in Figure 2. Rosenthal plots of [^{3}H]yohimbine binding over a 250-fold concentration range (0.022 to 5.5 nM) cearly indicated an interaction with a single class of sites. The K_D value of [^{3}H]yohimbine binding to HT29 cell membranes obtained from saturation analysis, 0.61 ± 0.03 nM, agreed well with the kinetically determined K_D value of 0.62 nM. For [^{3}H]UK-14,304, the Rosenthal plots of the saturation data were curvilinear, indicating the possibility of multiple classes of binding sites or negative cooperativity. Using computerized nonlinear least squares curve fitting, the [^{3}H]UK-14,304 binding data over a 400-fold concentration range better fit a two-site model compared to a one-site model. The

TABLE 1

Summary of HT29 α_2 saturation experiments

HT29 cell membranes were suspended in glycylglycine buffer (25 mM, pH 7.6) and used in saturation assays with the radioligands listed. Data were resolved as either one site (yohimbine, PAC) or two sites (UK-14,304), and the K_D and B_{max} values are expressed as the mean ± the standard error for the number of experiments indicated (n).

Radioligand	n	K_D	B_{max}
		nM	*fmol/mg protein*
Yohimbine	12	0.61 ± 0.03	330 ± 40
PAC	6	1.0 ± 0.2	160 ± 20
UK-14,304			
High affinity site	7	0.14 ± 0.02	120 ± 20
Low affinity site	7	6.1 ± 1.5	240 ± 30

total number of sites labeled by [^{3}H]UK-14,304 was similar to the B_{max} value of [^{3}H]yohimbine binding. [^{3}H]PAC saturation data were less consistent than those of the other two ligands. Although there was some evidence of curvilinearity, the [^{3}H]PAC binding data were not better fit by the two-site model and, thus, the single-site model was used, which indicated that fewer sites were labeled by [^{3}H]PAC than by the other two radioligands.

As indicated in Table 2, the [^{3}H]yohimbine K_D value was dependent on buffer composition and the presence of $MgCl_2$. Receptor affinity was about 3- to 4-fold lower in Tris-HCl buffer as compared with glycylglycine, and the presence of 10 mM $MgCl_2$ further reduced receptor affinity, about 4-fold in glycylglycine and about 3-fold in Tris-HCl. The total effect of using Tris-HCl buffer with $MgCl_2$ was, therefore, a 10-fold reduction in receptor affinity. In Tris-HCl buffer with 10 mM $MgCl_2$, the receptor density was 73% of that seen with glycylglycine buffer without $MgCl_2$.

The effects of Mg^{2+}, as well as Na^+ and GTP/EDTA on [^{3}H]UK-14,304 saturation curves were also examined (Fig. 3). In the absence of these modulators, as indicated above, [^{3}H]UK-14,304 saturation curves resolved best as two sites. The addition of 1 mM $MgCl_2$ in three experiments resulted in curves that did not model better as two sites compared to one site. The K_D value of the single site was 260 ± 30 pM with a density of 240 ± 30 fmol/mg of protein. Although making the results much more variable and decreasing specific binding, the presence of 30 mM NaCl resulted in a marked decrease in the number of high affinity sites (17 ± 8 fmol/mg of protein, n = 3) with no change in K_D value (150 ± 70 pM) while affecting the low affinity site much less (K_D, 3.2 ± 1.2 nM; B_{max}, 170 ± 40 fmol/mg of protein). Finally, the combination of GTP (0.1 mM) and EDTA (1 mM) essentially eliminated [^{3}H]UK-14,304 binding in HT29 membranes, thus prohibiting quantitation of their effects on [^{3}H]UK-14,304 saturation curves.

Inhibition Analyses

Fig. 4 is the composite of three separate antagonist competition experiments against [^{3}H]yohimbine (*upper panel*) and [^{3}H]UK-14,304 (*lower panel*). Against both radioligands, yohimbine was much more potent than prazosin, with phentolamine displaying an intermediate potency. Yohimbine was slightly more potent than oxymetazoline against [^{3}H]yohimbine and slightly less potent against [^{3}H]UK-14,304. The inhibitor equilibrium dissociation constant, K_i, of yohimbine in inhibiting [^{3}H]yohimbine binding was 0.6 nM which was in good agreement with the K_D of the radioligand. The Hill coefficients were near unity for antagonist inhibition of [^{3}H]yohimbine binding, whereas those observed for inhibition of [^{3}H]UK-14,304 binding were uniformly less than 1.0 (Table 3). The same antagonist rank order potency was observed in competition experiments with [^{3}H]PAC. Low Hill coefficients were also seen in competition experi-

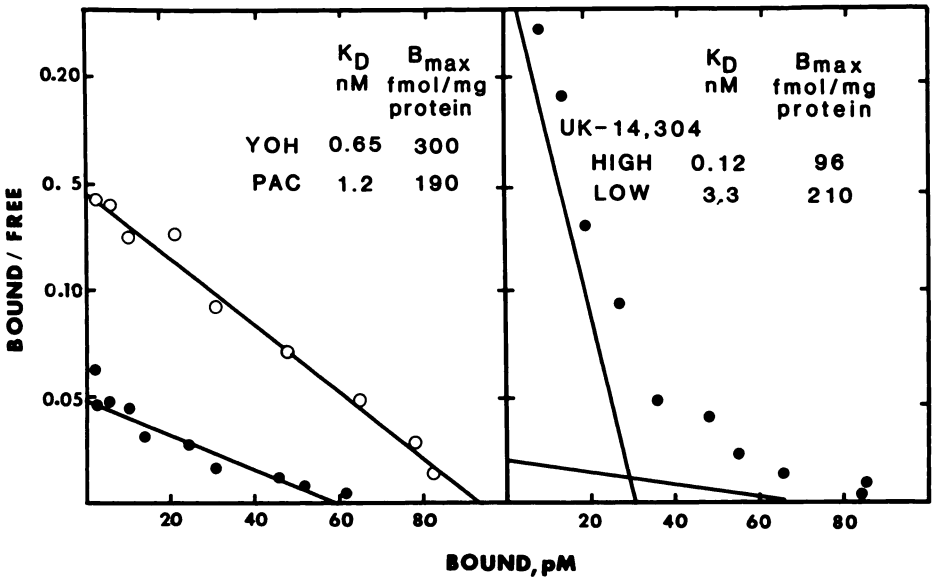

FIG. 2. *Rosenthal plots of ^{3}H-ligand binding in HT29 cell membranes*

Points shown are from a single experiment for each radioligand, performed in duplicate. These experiments were repeated at least five times. Ranges of radioligand concentrations (in nanomolar concentration) were: [^{3}H]yohimbine, 0.022–5.5 (*left panel*, ○); [^{3}H]PAC, 0.035–13 (*left panel*, ●); [^{3}H]UK-14,304, 0.044–19 (*right panel*).

TABLE 2

Effects of buffer composition on [^{3}H]yohimbine binding in HT29 membranes

HT29 cell membranes were suspended in the various buffers listed and used in saturation binding assays with [^{3}H]yohimbine. K_D and B_{max} values for each experiment were calculated and are expressed as the mean ± the standard error for the number of experiments indicated (*n*).

Buffer[a]	$MgCl_2$	K_D	B_{max}	*n*
	mM	*nM*	*fmol/mg protein*	
Glycylglycine	0	0.61 ± 0.03	330 ± 40	12
Glycylglycine	10	2.5 ± 0.2	278 ± 15	3
Tris-HCl	0	2.2 ± 0.3	250 ± 50	4
Tris-HCl	10	6.1 ± 0.6	240 ± 140	2

[a] At 25 mM, pH 7.6.

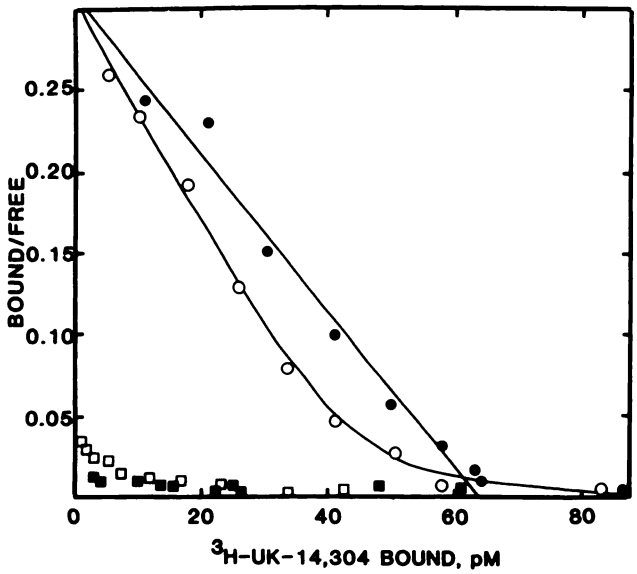

FIG. 3. *Effects of Mg^{2+}, Na^+, and GTP/EDTA on [^{3}H]UK-14,304 saturation curves in HT29 cell membranes*

Saturation experiments were performed in the absence (○) or presence of $MgCl_2$ (1 mM, ●), NaCl (30 mM, □), or GTP (100 μM) and EDTA (1 mM, ■). *Points* shown are from a single experiment done in duplicate and repeated twice. For this experiment the binding constants were:

Addition	K_D^H	B_{max}^H	K_D^L	B_{max}^L
	pM	*fmol/mg*	*pM*	*fmol/mg*
None	140	200	7200	220
$MgCl_2$	210	300		
NaCl	190	5	5500	240
GTP/EDTA		Not measurable		

ments with adrenergic agonists against all three radioligands. The rank order potency was UK-14,304 > PAC > (−)-epinephrine > (−)-norepinephrine > (+)-epinephrine against [^{3}H]yohimbine and [^{3}H]UK-14,304 (Fig. 5). PAC and (−)-epinephrine were nearly equipotent against [^{3}H]PAC (Table 3).

We further investigated the inhibition of [^{3}H]yohimbine binding by norepinephrine, UK-14,304, and PAC using 21 different concentrations of inhibitor and analyzing the results by computer-assisted curve fitting for

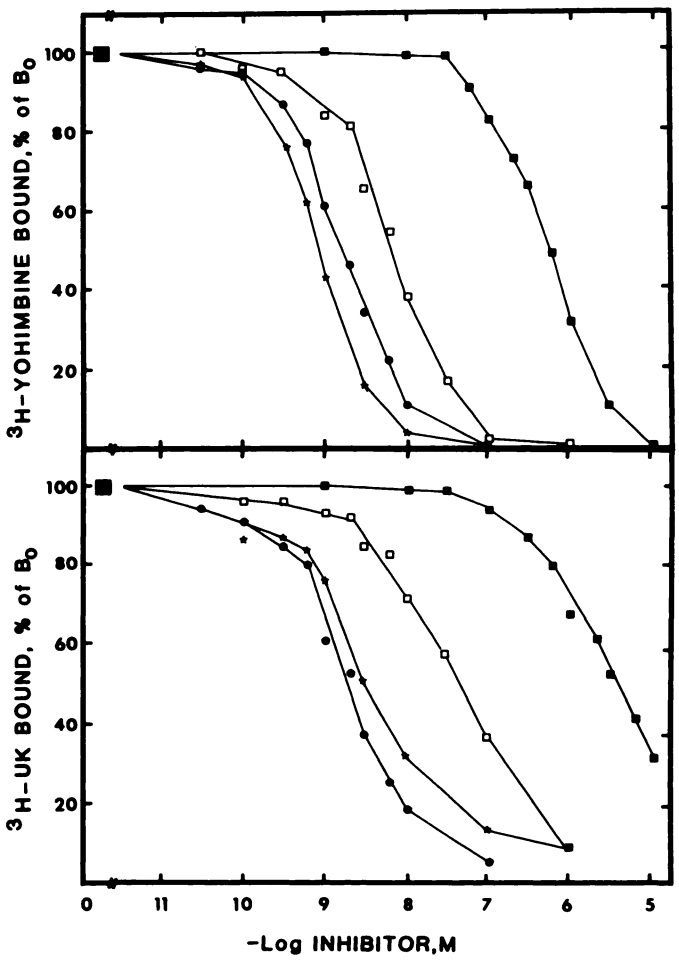

FIG. 4. *α-Adrenergic antagonist inhibition of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding in HT29 cell membranes*

Competition assays were done with the following antagonists: yohimbine (★), oxymetazoline (●), phentolamine (□), and prazosin (■) against 0.28–0.40 nM [^{3}H]yohimbine (*upper panel*) or 0.92–1.13 nM [^{3}H]UK-14,304 (*lower panel*). *Points* shown are the means of three experiments done in duplicate. Standard errors of the means were rarely greater than 10%.

one-site and two-site models. Norepinephrine and UK-14,304 inhibition data, as predicted by the low (0.5–0.7) Hill coefficients, consistently better fit a two-site model ($p < 0.01$) compared to a one-site model. The K_i values obtained for UK-14,304 at the two sites, 0.28 ± 0.06 nM and 12 ± 2 nM, are in the same range as the K_D values derived from saturation experiments (Table 1). As indicated in Table 3, for both norepinephrine and UK-14,304, about half of the receptors are of high affinity and half of low affinity. This contrasts with the two-site fit of [^{3}H]UK-14,304 saturation data, which indicated that 33% of the total receptors labeled were of high affinity (Table 1). Similar inhibition experiments in which the assay buffer included 0.1 mM GTP and 1.0 mM EDTA were also performed. In experiments of UK-14,304 inhibition of [^{3}H]yohimbine binding in the presence of GTP and EDTA, the percentage of sites in the high affinity form was reduced from 50% to 23% with no change in affinities (Table 3). A similar shift observed with norepinephrine was accompanied by a decrease in affinity

TABLE 3

Summary of HT29 α_2 inhibition experiments

IC_{50} values were determined from log-probit analyses of inhibition curves using 10 competitor concentrations except for norepinephrine, UK-14,304, and PAC versus [^{3}H]yohimbine where 21 concentrations were used. Each value is the mean ± the standard error of three experiments performed in duplicate.

Radioligand	Yohimbine			UK-14,304		PAC		
	IC_{50}	n_H	K_i	IC_{50}	n_H	IC_{50}	n_H	K_i
	nM		*nM*	*nM*		*nM*		
Antagonists								
Yohimbine	0.9 ± 0.4	1.1 ± 0.1	0.6 ± 0.3	4.1 ± 0.5	0.8 ± 0.2	2.5 ± 0.6	0.8 ± 0.1	1.2 ± 0.1
Oxymetazoline	1.7 ± 0.3	1.1 ± 0.1	1.1 ± 0.2	1.9 ± 0.3	0.9 ± 0.2	1.3 ± 0.3	1.0 ± 0.1	0.68 ± 0.2
Phentolamine	6.8 ± 0.5	1.0 ± 0.1	4.4 ± 0.3	49 ± 8	0.7 ± 0.1	30 ± 5	0.9 ± 0.1	16 ± 4
Prazosin	520 ± 20	1.1 ± 0.1	340 ± 20	3400 ± 600	0.8 ± 0.1	3900 ± 900	0.8 ± 0.1	1900 ± 200

	Yohimbine					UK-14,304		PAC	
	IC_{50}	n_H	K_i^H	K_i^L	%H[a]	IC_{50}	n_H	IC_{50}	n_H
	nM		*nM*			*nM*		*nM*	
Agonists									
(−)-EPI	15 ± 1	0.5 ± 0.1	ND[b]	ND	ND	3.0 ± 0.4	0.8 ± 0.1	1.4 ± 0.3	0.8 ± 0.1
(+)-EPI	230 ± 60	0.5 ± 0.1	ND	ND	ND	55 ± 6	0.8 ± 0.1	28 ± 6	0.7 ± 0.1
NOREPI	47 ± 3	0.5 ± 0.1	3.9 ± 1.7	250 ± 60	48 ± 8	9.3 ± 1.2	0.7 ± 0.1	8.4 ± 2.1	0.7 ± 0.1
+GTP & EDTA[c]	270 ± 60	0.7 ± 0.1	26 ± 4	400 ± 30	33 ± 3				
UK-14,304	2.3 ± 0.3	0.6 ± 0.1	0.28 ± 0.06	12 ± 2	50 ± 6	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
+GTP & EDTA	12 ± 1	0.8 ± 0.1	0.29 ± 0.14	14 ± 3	23 ± 6				
PAC	5.0 ± 1.0	0.7 ± 0.1	4.0 ± 0.9			2.3 ± 0.6	0.7 ± 0.1	1.9 ± 0.6	0.9 ± 0.1
+GTP & EDTA	13 ± 2	1.0 ± 0.1	8.2 ± 0.6						

[a] Percentage of total sites with the higher affinity.

[b] ND, not determined.

[c] Concentrations used were 0.1 mM GTP and 1.0 mM EDTA.

at the high affinity site. As was the case with [^{3}H]PAC saturation data, the competition of [^{3}H]yohimbine binding by PAC did not model better as two sites.

Inhibition of adenylate cyclase. Both VIP and forskolin stimulate HT29 cell adenylate cyclase in a dose-dependent manner.[2] The concentrations used for the results presented here (10 or 20 nM VIP; 10 μM forskolin) are from the middle portion of the dose response curves. The stimulation of cyclic AMP production by both VIP and forskolin is partially inhibited by epinephrine (in the presence of equimolar propranolol to block β-adrenergic stimulation) and by UK-14,304. Fig. 6 shows that the epinephrine inhibition of forskolin stimulation is dose dependent, with a maximal reduction of activity to 68 ± 5% of that seen in the absence of adrenergic agonist. UK-14,304 was as efficacious and at least as potent as epinephrine in inhibiting adenylate cyclase activity. For the experiments assessing reversal of adenylate cyclase inhibition, an agonist (UK-14,304 or epinephrine plus propranolol) concentration of 3 μM was chosen. The results of experiments assessing the dose-dependent effect of yohimbine, phentolamine, and prazosin on agonist inhibition of VIP-stimulated adenylate cyclase are shown in Fig. 7. Of the three antagonists, yohimbine (EC_{50} = 0.3 μM) was the most potent in reversing agonist inhibition of the enzyme with phentolamine about 7-fold and prazosin about 190-fold less potent.

DISCUSSION

To our knowledge, the HT29 cell line is the first cell line of human origin in which the α_2-adrenergic receptor has been characterized. Furthermore, [^{3}H]UK-14,304 and HT29 cells appear to be useful tools for investigating α_2-adrenergic receptors. The binding of [^{3}H]UK-14,304, as well as [^{3}H]PAC and [^{3}H]yohimbine, was rapid, reversible, and saturable. The rank order potencies of both agonists and antagonists in inhibiting [^{3}H]UK-14,304, [^{3}H]yohimbine, and [^{3}H]PAC binding were similar, indicating that all three radioligands label α_2-adrenergic receptors in HT29 cells. In assays utilizing Tris-HCl buffer containing 10 mM $MgCl_2$, our results (Table 2) for the affinity and density of sites for [^{3}H]yohimbine agree with those of Carpene *et al.* (7), who reported the K_D and B_{max} values to be 6.3 nM and 224 fmol/mg of protein, respectively. We have found, however, that binding is enhanced by the exclusion of $MgCl_2$ and by the use of glycylglycine buffer. These steps produced an overall 10-fold increase in receptor affinity accompanied by a moderate (36%) increase in receptor density. The ratio of potency for yohimbine and prazosin inhibition of [^{3}H] yohimbine binding in Tris-HCl with $MgCl_2$ as reported by Carpene *et al.* (7) is 300. This is similar to the value of 580 we obtained in glycylglycine without $MgCl_2$, indicating that the shift in affinity was not a property of yohimbine alone. Similar buffer and $MgCl_2$ effects have been observed in the human platelet (2).

The inhibition of adenylate cyclase by epinephrine in HT29 cells is similar to that observed by Laburthe *et al.* (24) in membranes from rat intestinal epithelial cells. In both studies, maximal inhibition of the stimulated enzyme is about 30–40%. Using maximally stimulatory concentrations of prostaglandin E, or VIP in intestinal

[2] J. T. Turner and D. B. Bylund, manuscript in preparation.

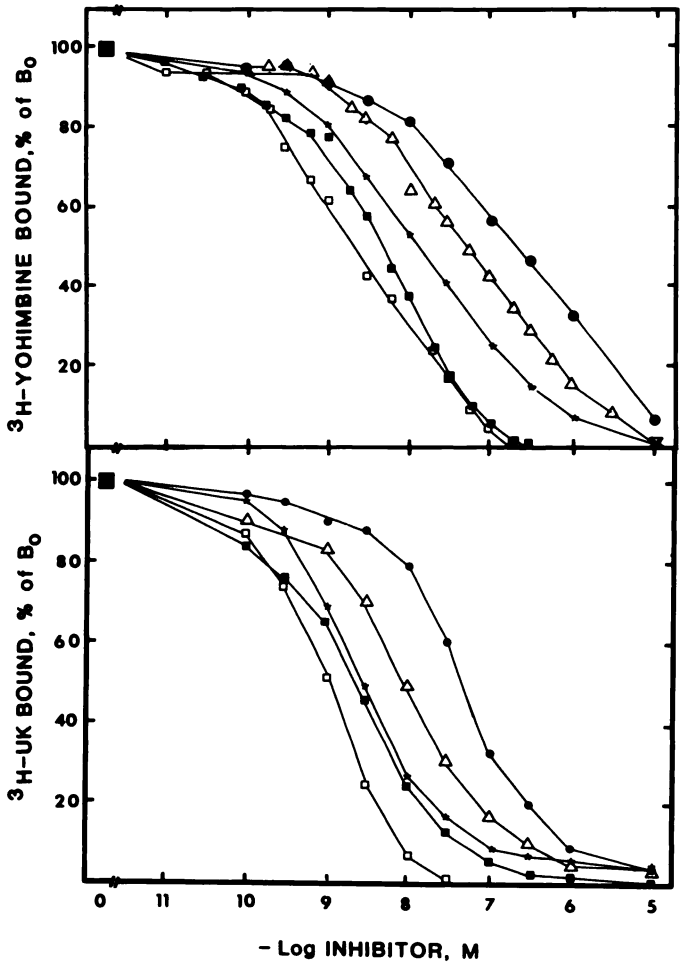

FIG. 5. *Adrenergic agonist inhibition of [^{3}H]yohimbine and [^{3}H] UK-14,304 binding in HT29 cell membranes*

Competition assays were done with the following agonists: UK-14,304 (□), PAC (■), (−)-epinephrine (★), norepinephrine (△), and (+)-epinephrine (●) against 0.29–0.49 nM [^{3}H]yohimbine (*upper panel*) or 1.01–10.7 nM [^{3}H]UK-14,304 (*lower panel*). *Points* shown are the means of three experiments done in duplicate. Standard errors of the means were rarely greater than 10%.

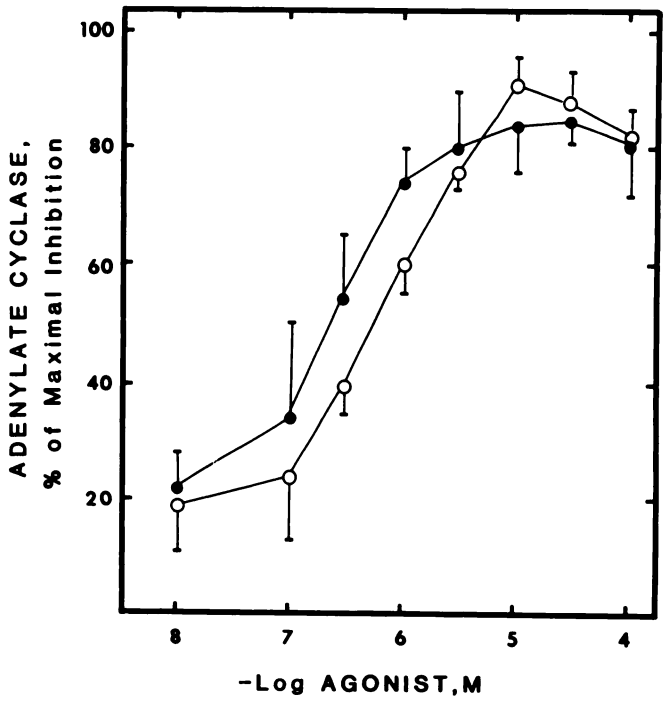

FIG. 6. *UK-14,304 and epinephrine dose-dependent inhibition of forskolin-stimulated adenylate cyclase in HT29 cell membranes*

Adenylate cyclase activity was determined in membranes incubated with 10 μM forskolin and various concentrations of UK-14,304 (●) and epinephrine (○). The data are expressed as the percentage of inhibition based on the maximal inhibition observed in each experiment. The maximal inhibition was 32 ± 5% (n = 4) of the activity with forskolin alone. Each *point* shown is the mean ± standard error of three (UK-14,304) or four (epinephrine) experiments done in duplicate.

epithelial cells isolated from rats, Nakaki *et al.* (25) observed no significant inhibition of cyclic AMP accumulation by 1 μM clonidine or 10 μM epinephrine plus pindolol. In the present study the degree of inhibition could not be increased by changing the concentration of forskolin or VIP (data not shown). UK-14,304, which exhibited similar potency and efficacy as compared to epinephrine in inhibiting HT29 adenylate cyclase (Fig. 6), also inhibits cyclase in platelets (14). The limited degree of α_2-agonist inhibition of enzyme activity makes measurement of antagonist reversal of the inhibition very difficult. Nonetheless, our results indicate dose dependence of the reversal as well as a potency order of an α_2 character and potency ratios (phentolamine/yohimbine, 7; prazosin/yohimbine, 190) of the same magnitude as observed in binding assays (Table 3). Measurements of intact cell cyclic AMP levels are currently being made and will be compared with intact cell radioligand binding.

One current model to describe agonist and antagonist binding to the α_2-receptor and the regulation of this process by GTP and Mg^{2+} is the two-state model of Hoffman and Lefkowitz (26). This model is analogous to that developed for the β-adrenergic receptor and is based mainly on data from the human platelet. Our studies in another non-rodent mammalian species (pig) but not in a rodent species (rat) are consistent with this model (27). According to the two-state model, there are low and high affinity states of the receptor for α_2-adrenergic agonists. Antagonists bind to both states with equal affinity. These two states are in an equilibrium which GTP can shift to favor the formation of the low affinity agonist state and which Mg^{2+} can shift to favor the high affinity agonist state. The results from this study using a human cell line generally support this model. The antagonist radioligand ([^{3}H]yohimbine) labeled a single site in saturation curves, whereas the agonist radioligand [^{3}H]UK-14,304 data fit a two-site model better. [^{3}H]UK-14,304 saturation experiments suggest at least a partial shift of receptors to the high affinity form (120 fmol/mg without Mg^{2+}, 240 fmol/mg with 1 mM Mg^{2+}, although the statistically significant better fit to a two-site model is lost in the presence of Mg^{2+}. The binding of [^{3}H]UK-14,304 in the presence of 100 μM GTP is essentially abolished. However, in inhibition experiments using [^{3}H]yohimbine, in which antagonists bind with a single affinity whereas agonists, including UK-14,304, bind to high and low affinity sites, GTP decreases the affinity (increases the

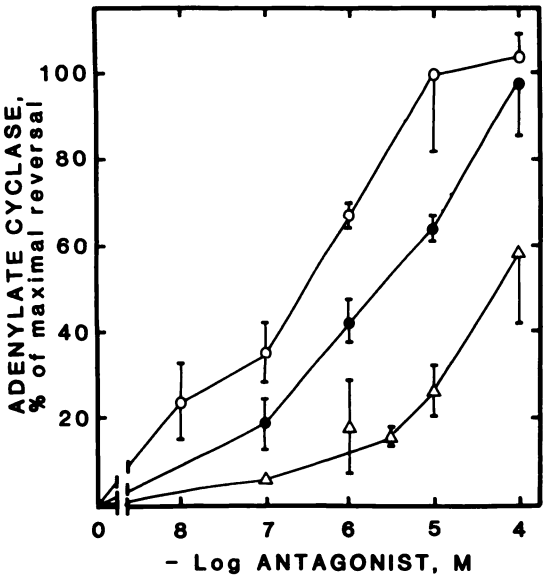

FIG. 7. *α-Adrenergic antagonist reversal of UK-14,304 or epinephrine inhibition of VIP-stimulated adenylate cyclase in HT29 cell membranes*

Adenylate cyclase activity was determined in membranes incubated with 10 or 20 nM VIP, 3 μM UK-14,304, or epinephrine and the indicated concentrations of yohimbine (○), phentolamine (●), or prazosin (△). Each *point* shown is the mean of at least three separate experiments done in triplicate except for prazosin at 1×10^{-7} M (one experiment) and 1×10^{-4} M (two experiments). For this series of experiments, enzyme activities (pmol/mg/min) were: basal, 7.9 ± 1.2 and VIP stimulated, 98 ± 19. Maximal inhibition was 20 ± 4%.

IC_{50}) of agonists by increasing the percentage of low affinity sites (Table 3).

The inclusion of 30 mM NaCl in [^{3}H]UK-14,304 saturation experiments has the primary effect of decreasing the number of high affinity sites labeled. This result is consistent with the shift to the right of epinephrine inhibition curves versus [^{3}H]yohimbine in human platelets (28) and may reflect either a loss of high affinity sites or a conversion of these sites to a low affinity state. Our results from three experiments, although suggesting the former possibility, are sufficiently variable to prohibit making a firm conclusion. The exclusion of the 30 mM NaCl present in HT29 adenylate cyclase assays had no effect on basal, VIP- or forskolin-stimulated, or UK-14,304-inhibited enzyme activity (results not shown).

Several observations support the view that UK-14,304 is a full agonist in HT29 cells. First, UK-14,304 had the same maximal effect as epinephrine in inhibiting forskolin-stimulated adenylate cyclase activity. Second, [^{3}H]UK-14,304 saturation experiments (Fig. 3B) were consistent with the labeling of two sites or states. By contrast, the [^{3}H]PAC saturation data modeled as a single site. Similarly, Loftus *et al.* (14) reported that [^{3}H]UK-14,304 labeled α_2-receptors in rat cerebral cortical membranes in a biphasic manner. Third, inhibition by both norepinephrine and UK-14,304, but not the partial agonist PAC, of [^{3}H]yohimbine binding (Table 3) was consistent with a two-site model. Fourth, the addition of GTP and EDTA to [^{3}H]yohimbine competition assays resulted in an increase in the IC_{50} value of 5.5-fold for norepinephrine and UK-14,304, but only 2.6-fold for PAC. For UK-14,304 this increase was due to an apparent shift in about 25% of the receptors from high affinity to low affinity. A 7-fold decrease in norepinephrine potency at the high affinity site in [^{3}H]yohimbine competition curves was observed, whereas no significant changes for norepinephrine at the low affinity site or for UK-14,304 at either site were seen. The reason for, or the importance of, this effect is not clear but is similar to that observed by Hoffman *et al.* (29) for epinephrine at platelet α_2-receptors.

[^{3}H]UK-14,304 was superior to [^{3}H]PAC in that, with [^{3}H]UK-14,304, experimental data were less erratic, the same total number of sites was labeled compared with [^{3}H]yohimbine, and the [^{3}H]UK-14,304 data consistently fit a two-site model whereas the [^{3}H]PAC data did not. As indicated in Table 1, and in agreement with Bouscarel *et al.* (8) using [^{3}H]clonidine, [^{3}H]PAC labels about 50% of the sites labeled by [^{3}H]yohimbine in HT29 cells. It is a general observation that the imidazoline partial agonists clonidine and PAC label fewer receptors than are labeled by [^{3}H]yohimbine or by catecholamine agonists (1). Thus, it is interesting that [^{3}H]UK-14,304, another imidazoline, labels the same number of sites as [^{3}H]yohimbine. A correlation between intrinsic activity of agonists and the number of high affinity sites labeled has been suggested, but experiments by Hoffman *et al.* (29) do not support this idea.

We have previously emphasized the pharmacologic heterogeneity of α_2-adrenergic receptors and have suggested the possibility of receptor subtypes (5). The evidence for α_2-receptor heterogeneity is based on three observations: the affinity of [^{3}H]yohimbine is higher in non-rodent mammalian species as compared to rodents; oxymetazoline is more potent in non-rodents; and prazosin is more potent (relative to yohimbine) at rodent as compared to non-rodent α_2-receptors. The potency ratio of yohimbine to prazosin in inhibiting [^{3}H]yohimbine binding in HT29 cells is similar to the ratio found in human platelet and human adipocyte and in other non-rodent mammalian cells and tissues (200–500) (reviewed in Ref. 5). In tissues from rodents, this value is much lower (4–20), and in the single example of a rodent cell line (1) the ratio is 5. These results expand the observation of differences in α_2-adrenergic receptors between rodent and non-rodent mammalian species to a homogeneous human cell line population.

Although the HT29 cells are transformed cells, their high degree of similarity to normal intestinal epithelial cells in terms of receptor complement and responsiveness to VIP and α_2-adrenergic agents, similar adenylate cyclase regulation, and end point responses such as glycogenolysis support the idea that the regulation of many processes in HT29 cells is similar to regulation in normal cells (11). The availability of a homogeneous cell line of human origin should prove useful in the study of the regulation and mechanism of action of α_2-receptors.

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REFERENCES

1. Bylund, D. B., and D. C. U'Prichard. Characterization of α_1- and α_2-adrenergic receptors. *Int. Rev. Neurobiol.* **24**:343–431 (1983).
2. Jones, S. B., D. B. Bylund, C. A. Rieser, W. O. Shekim, J. A. Byer, and G. W. Carr. α_2-Adrenergic receptor binding in human platelets: alterations during the menstrual cycle. *Clin. Pharmacol. Ther.* **34**:90–96 (1983).
3. Karliner, J. S., H. J. Motulsky, and P. A. Insel. Apparent "down-regulation" of human platelet alpha$_2$-adrenergic receptors is due to retained agonist. *Mol. Pharmacol.* **21**:36–43 (1982).
4. Kahn, D. J., J. C. Mitrius, and D. C. U'Prichard. Alpha$_2$-adrenergic receptors in neuroblastoma × glioma hybrid cells: Characterization with agonist and antagonist radioligands and relationship to adenylate cyclase. *Mol. Pharmacol.* **21**:17–26 (1982).
5. Bylund, D. B. Heterogeneity of alpha-2 adrenergic receptors. *Pharmacol. Biochem. Behav.* **22**:835–843 (1985).
6. Fogh, J., and G. Trempe. New human tumor cell lines. in *Human Tumor Cells In Vitro* (J. Fogh, ed.). Plenum Press, New York, 115–159 (1975).
7. Carpene, C., H. Paris, C. Cortinovis, V. Viallard, and J. C. Murat. Characterization of α_2-adrenergic receptors in the human colon adenocarcinoma cell line HT29 in culture by [^{3}H]yohimbine binding. *Gen. Pharmacol.* **14**:701–703 (1983).
8. Bouscarel, B., C. Cortinovis, C. Carpene, J. C. Murat, and H. Paris. α_2-Adrenoceptors in the HT29 human colon adenocarcinoma cell line: characterization with [^{3}H]clonidine; effects on cyclic AMP accumulation. *Eur. J. Pharmacol.* **107**:223–231 (1985).
9. Zweibaum, A., M. Rousset, M. Pinto, G. Chevalier, E. Dussaulx, and J.-L. Brun. Mucus glycoprotein differentiation by serum deprivation of the human colon carcinoma cell line HT-29 in culture. *Biol. Cell* **45**:91 (1982).
10. Laburthe, M., M. Rousset, G. Chevalier, C. Boissard, C. Dupont, A. Zweibaum, and G. Rosselin. Vasoactive intestinal peptide control of cyclic adenosine 3′:5′-monophosphate levels in seven human colorectal adenocarcinoma cell lines in culture. *Cancer Res.* **40**:2529–2533 (1980).
11. Rousset, M., M. Laburthe, G. Chevalier, C. Boissard, G. Rosselin, and A. Zweibaum. Vasoactive intestinal peptide (VIP) control of glycogenolysis in the human colon carcinoma cell line HT-29 in culture. *FEBS Lett.* **126**:38–40 (1981).
12. Grant, J. A., and M. C. Scrutton. Interaction of selective α-adrenoceptor agonists and antagonists with human and rabbit blood platelets. *Br. J. Pharmacol.* **71**:121–134 (1980).
13. Loftus, D. J., J. M. Stolk, and D. C. U'Prichard. Binding of the imidazoline UK-14,304, a putative full α_2-adrenoceptor agonist, to rat cerebral cortex membranes. *Life Sci.* **35**:61–69 (1984a).
14. Loftus, D., R. Guchhait, G. Vantini, J. Stolk, and D. C. U'Prichard. Characterization of binding to rat cortical membranes by a full α_2-adrenoceptor agonist. *Soc. Neurosci. Abstr.* **10**:279 (1984b).
15. 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).
16. Bylund, D. B., J. R. Martinez, and D. L. Pierce. Regulation of autonomic receptors in rat submandibular gland. *Mol. Pharmacol.* **21**:27–35 (1982).
17. De Lean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
18. Bennett, J. P., Jr., and H. I. Yamamura. Neurotransmitter, hormone, or drug receptor binding methods, in *Neurotransmitter Receptor Binding*, Ed 2 (H. I. Yamamura, S. J. Enna, and M. J. Kuhar, eds.). Raven Press, New York, 84–87, 1985.
19. Sedman, A. J., and J. G. Wagner. CSTRIP, a Fortran IV computer program for obtaining initial polyexponential parameter estimates. *J. Clin. Pharm. Sci.* **65**:1006–1010 (1976).
20. Aranyi, P. Kinetics of the hormone-receptor interaction. Competition experiments with slowly equilibrating ligands. *Biochim. Biophys. Acta* **628**:220–227 (1980).
21. Nickols, G. A., D. L. Carnes, C. S. Anast, and L. R. Forte. Parathyroid hormone-mediated refractoriness of rat kidney cyclic AMP system. *Am. J. Physiol.* **236**:E401–E409 (1979).
22. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
23. Goldstein, A. *Biostatistics: An Introductory Text*. Macmillan, New York, 71–73 (1964).
24. Laburthe, M., B. Amiranoff, and C. Boissard. α-Adrenergic inhibition of cyclic AMP accumulation in epithelial cells isolated from rat small intestine. *Biochim. Biophys. Acta* **721**:101–108 (1982).
25. Nakaki, T., T. Nakadate, S. Yamamoto and R. Kato. Alpha$_2$-adrenergic receptor in intestinal epithelial cells. Identification by [^{3}H]yohimbine and failure to inhibit cyclic AMP accumulation. *Mol. Pharmacol.* **23**:228–234 (1983).
26. Hoffman, B. B., and R. J. Lefkowitz. Radioligand binding studies of adrenergic receptors;: new insights into molecular and physiological regulation. *Annu. Rev. Pharmacol. Toxicol.* **20**:581–608 (1980).
27. Feller, D. J., and D. B. Bylund. Comparison of alpha-2 adrenergic receptors and their regulation in rodent and porcine species. *J. Pharmacol. Exp. Ther.* **228**:275–282 (1984).
28. Limbird, L. E., J. L. Speck, and S. K. Smith. Sodium ion modulates agonist and antagonist interactions with the human platelet alpha$_2$-adrenergic receptor in membrane and solubilized preparations. *Mol. Pharmacol.* **21**:609–617 (1982).
29. Hoffman, B. B., T. Michel, T. B. Brenneman, and R. J. Lefkowitz. Interaction of agonists with platelet α_2-adrenergic receptors. *Endocrinology* **110**:926–932 (1982).
30. Turner, J. T., and D. B. Bylund. Alpha-2 adrenergic receptors and inhibition of VIP stimulated adenylate cyclase in HT29 cells. *Fed. Proc.* **44**:509 (1985).

Send reprint requests to: Dr. John T. Turner, Department of Pharmacology, University of Missouri, M-517B Medical Sciences Building, Columbia, MO 65212.