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Interactions of Full and Partial Agonists with HT29 Cell α_2-Adrenoceptor: Comparative Study of [^{3}H]UK-14,304 and [^{3}H] Clonidine Binding

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SUMMARY

The HT29 cell line expresses α_2-adrenoceptors that are negatively coupled to the adenylate cyclase system and is, in this respect, a valuable model for *in vitro* study of α_2-adrenergic receptivity in a tissue from human origin. In these cancerous cells, UK-14,304 is a full agonist of the α_2-adrenergic-mediated inhibition of the vasoactive intestinal peptide-induced cyclic AMP accumulation, whereas clonidine acts only as a partial agonist. In the present report, we used [^{3}H]UK-14,304 as radioligand and compared its binding characteristics with those of [^{3}H]clonidine in order to better understand the difference between full and partial agonism on the basis of agonist/receptor interactions. [^{3}H]UK-14,304 labeled with high affinity (K_D = 0.39 ± 0.05 nM) a single class of sites having the pharmacological specificity of an α_2-adrenoceptor. Comparison of [^{3}H]UK-14,304, [^{3}H]clonidine, and [^{3}H]yohimbine B_{max} proved that both ^{3}H-agonists labeled the same number of sites (172 ± 14 versus 179 ± 21 fmol/mg of protein), whereas the ^{3}H-antagonist recognized more sites (246 ± 22 fmol/mg of protein). Inhibition of [^{3}H]yohimbine by the two agonists was consistent with the existence of an heterogeneous population of receptors and analysis of the data according a two-site inhibition model showed 1) that the K_{i_1}/K_{i_H} ratio was higher for UK-14,304 than for clonidine and 2) that the percentages of high affinity state receptor recognized by both agonists were identical (56 ± 4% with UK-14,304 and 59 ± 5% with clonidine). Kinetics of [^{3}H]UK-14,304 and [^{3}H]clonidine binding indicated more complex agonist-receptor interactions than equilibrium data did. Association as well as dissociation of both radioligands appeared to be biphasic, suggesting a relative heterogeneity of ^{3}H-agonist binding sites. Kinetic behavior of [^{3}H] UK-14,304 only differed from that of [^{3}H]clonidine by a much slower dissociation rate and by its ability to induce the formation of a tightly bound component, which corresponded to the formation of a very stable full agonist/receptor/G_i complex. Effects of guanosine-5′-(imido)-triphosphate and GTP on [^{3}H]UK-14,304 and [^{3}H]clonidine binding also proved that the agonists were not similarly sensitive to guanine nucleotides.

Although it has been sometimes overestimated (1), the radioligand binding technique proved to be extremely useful and was the basis of major advances in our knowledge of the molecular pharmacology of adrenoceptors. Indeed, the utilization of this method allowed direct identification and quantification of the different adrenoceptor subtypes in a wide number of tissues (2, 3); the availability of labeled agonists and antagonists gave rise to the concept of high and low affinity state receptors and pointed out the regulatory role of guanine nucleotides (4). In this respect, [^{3}H]clonidine and [^{3}H] *p*-aminoclonidine on the one hand (5, 6) and [^{3}H]yohimbine, [^{3}H] rauwolscine, and [^{3}H]-RX 781094 on the other hand (7–9) were very successfully used for α_2-adrenoceptor investigations.

Although recent reports indicated that some of the α_2-adrenergic-elicited responses are mediated via a pathway involving Na^+/H^+ exchange (10, 11), the most well characterized effect of α_2-adrenergic agonists is an inhibition of the adenylate cyclase system resulting in a decrease in the intracellular cyclic AMP level. Resolution of the molecular mechanisms responsible for this inhibition demonstrated that three separate macromolecules are involved in the signal transduction, the α_2-adrenoceptor itself, the GTP-binding protein (G_i), and the catalytic subunit of the adenylate cyclase. Comparative binding of ^{3}H-antagonist and ^{3}H-agonist also revealed that, similarly to β-adrenoceptors, α_2-adrenoceptors exist in two conformational states displaying either high or low affinity toward agonists. Many studies focused on the chronology of the protein-protein interactions taking place during receptor activation, but it is not clear yet whether the high affinity receptor is a preexisting state corresponding to precoupled receptor or whether it represents a proportion of the whole receptor population that is able to undergo coupling with G_i after agonist-receptor interaction (12, 13).

Another still open question concerns the notion of full and

ABBREVIATIONS: VIP: vasoactive intestinal peptide. UK-14,304, 5-bromo-6(2-imidazoline-2-ylamino)-quinoxaline; benextramine: *N,N″*-(dithiodi-2,1-ethanediyl) bis [*N′*-(2-methoxyphenylmethyl)-1,6-hexane-diamine]; HEPES, *N*-2-hydroxyethylpiperazine-2-ethoxy-1,2-dihydroquinoline; Gpp(NH)p, guanosine-5′-(imido)-triphosphate; DMEM, Dulbecco's modified Eagle medium; IBMX, 3-isobutyl-1-methylxanthine.

partial agonism. It has been widely documented that α_2-adrenergic compounds display different intrinsic activities to inhibit adenylate cyclase and behave either as full or partial agonists of the receptor. From the various studies that were carried out to understand this difference on the basis of agonist/receptor interactions, two possible interpretations were given. First, in brain (14) and in neuroblastoma × glioma hybrid cells (15), full agonists were proposed to label more high affinity state receptors than partial ones. Second, in human platelets, Hoffman *et al.* (16) reported that full and partial agonists recognized the same proportion of high affinity receptors but they observed a good correlation between the agonist efficacy and the ratio of the K_i values for the low and the high affinity state receptors (K_{i_L}/K_{i_H}). The mechanisms leading to full or partial activation of the receptor remain, therefore, poorly understood in terms of binding parameters. This is certainly due, in part, to the lack of a cellular model allowing accurate measurement of antagonist and agonist binding under very standardized conditions and, overall, to the lack of a suitable labeled adrenergic agonist exhibiting high α_2-selectivity and full intrinsic activity.

Using [^{3}H]yohimbine (17) and [^{3}H]clonidine (18), we previously showed that the colonic adenocarcinoma cell-line HT29 exhibits a homogeneous population of α_2-adrenoceptors that, according to the classification proposed by Bylund (19), is of the A subtype only. This cell line, which is established in permanent culture, is in this respect an excellent tool for studying α_2-adrenoceptors in a cellular model from human origin. Measurement of the inhibition of the VIP-induced cyclic AMP accumulation indicated, moreover, that compared with (−)-adrenaline or (−)-noradrenaline, clonidine was only a partial agonist on HT29 α_2-adrenoceptors (20), whereas UK-14,304 displayed full agonist activity, similar to catecholamines (21).

In the present report, UK-14,304 was confirmed to be a full agonist in HT29 cells, provoking a 90–95% inhibition of the intracellular accumulation of cyclic AMP induced by VIP. A detailed study of [^{3}H]UK-14,304 binding was performed on membrane preparations and the binding characteristics of the full agonist were compared with those displayed by the partial agonist [^{3}H]clonidine and the antagonist [^{3}H]yohimbine. We found that [^{3}H]UK-14,304 and [^{3}H]clonidine labeled the same number of receptors, which, from study of the inhibition of [^{3}H]yohimbine binding, approximately corresponded to α_2-adrenergic receptors in the high affinity conformation. In apparent contradiction with equilibrium data, kinetic studies demonstrated a relative heterogeneity of the ^{3}H-agonist binding sites. Time course of [^{3}H]UK-14,304 dissociation also revealed that the full agonist has the ability to tightly bind to the receptor. Such a tight agonist binding is not observed with [^{3}H]clonidine.

Materials and Methods

Cell culture. The HT29 cell line was established in permanent culture by Dr. J. Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY) (22) and cultured as described previously (18). Routinely, seeding was done from a post-confluent dish as follows: cells were detached in the presence of a phosphate buffer solution containing 0.25% trypsin and 0.5 mM EDTA; they were then collected by gentle centrifugation (800 × *g* for 5 min), resuspended in fresh culture medium, and seeded at a density of 5 × 10^4 cells/cm^2 in plastic Petri dishes 65 mm in diameter (Falcon, Becton Dickinson, France). The cells were grown at 37° under a controlled atmosphere (95% air/5% CO_2) in DMEM (Eurobio, France) supplemented with 10% fetal calf serum (Boehringer, Mannheim, FRG).

During the first days of growth (exponential phase), the culture medium was changed every 48 hr. Under such conditions, cell confluency was reached at day 9 of the culture. Then, medium was renewed daily in order to avoid nutrient exhaustion. α_2-Adrenoceptor number on HT29 cell membranes being a function of cell density within the dish (20), all experiments were done with post-confluent cells (day 13 of the culture).

Measurements of intracellular cyclic AMP levels. Measurements of VIP-induced cyclic AMP accumulation and its attenuation by α_2-adrenergic agonists were done as described by Paris *et al.* (23). Briefly, 4 hr before the experiment, the standard culture medium was replaced by 4 ml of serum-free DMEM. The cells were detached by trypsin-EDTA treatment, washed three times in serum-free DMEM to eliminate trypsin, and finally resuspended in DMEM containing 50 mM HEPES. Isolated cells (corresponding to about 0.4 mg of protein) were incubated in 200 μl of HEPES-buffered DMEM containing 0.5 mM IBMX, 0.2% bacitracin, and the appropriate concentration of drug to be tested. After 15 min at 37°, the incubation was stopped by addition of 1.8 ml of a 95% methanol-formic acid 5% solution. The suspension was sonicated briefly and centrifuged at 2500 × *g* for 15 min and aliquots (100 μl) of the supernatant were evaporated. Dry samples were resuspended in sodium acetate buffer (0.2 M, pH 7.5) and cyclic AMP was measured by the method of Steiner *et al.* (24) using the radioimmunoassay kit from New England Nuclear (Boston, MA).

Membrane preparation and binding assays. The dishes used for binding studies were collected 18 hr after the last medium change. After medium removal, cell layers were rinsed twice with 5 ml of ice-cold phosphate-buffered saline, quickly frozen, and stored at −80° until analysis.

All steps of membrane preparation were carried out at 4°. The frozen cell layers were scraped in 2 ml of an ice-cold hypotonic buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5), further disrupted with a Dounce homogenizer, and centrifuged at 25,000 × *g* for 10 min. The pellet obtained was suspended in Tris-Mg^{2+} buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) and recentrifuged in the same conditions as above. The final crude membrane fraction was taken up in the appropriate volume of Tris-Mg^{2+} buffer for immediate use.

Unless otherwise specified, all binding experiments were performed at 25°, as described by Paris *et al.* (20). In standard assay conditions, total binding was measured by incubating 100 μl of cell membrane suspension (150–300 μg of protein) with the ^{3}H-ligand in a total volume of 400 μl of Tris-Mg^{2+} buffer. After 30 min of incubation, 4 ml of ice-cold washing buffer (10 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) were added to each tube and assays were immediately filtered through fiberglass filters (Whatman GF/C) using a Millipore Manifold Sampling unit. The filters were then rapidly washed twice with 10 ml of washing buffer and placed into scintillation minivials for counting. Specific binding was defined as the difference between total and nonspecific binding determined in parallel assays but in the presence of an excess of phentolamine (10 μM).

For saturation studies, the final concentrations of radioligand ranged from 0.04 to 6 nM for [^{3}H]UK-14,304, from 0.1 to 8 nM for [^{3}H]clonidine and from 1 to 20 nM for [^{3}H]yohimbine. In all experiments reported herein, nonspecific binding was less than 10% at 6 nM [^{3}H]UK-14,304, less than 20% at 8 nM [^{3}H]clonidine, and 35–40% at 20 nM [^{3}H]yohimbine.

Unless otherwise specified, inhibition studies were carried out as follows. The indicated concentrations of unlabeled agonist or antagonist were added to the incubation mixture before addition of the membrane suspension. All experiments were carried out at radioligand concentrations corresponding to more than twice the K_D value (2–3 nM for [^{3}H]UK-14,304 and 8–10 nM for [^{3}H]yohimbine). Membrane-bound radioactivity was measured after 30 min of incubation, as indicated above. When catecholamines were tested, 0.5 mM ascorbic acid and 0.1 mM pyrocatechol were added into the binding medium in order to

prevent oxidation and degradation. Addition of this preservation cocktail did not affect the binding parameters of [^{3}H]UK-14,304 or [^{3}H] adrenaline on HT29 cell membrane.[1]

For kinetic studies of the association reaction, 1 volume of HT29 cell membrane preparation was incubated with 3 volumes of Tris-Mg^{2+} buffer containing either [^{3}H]UK-14,304 (2–3 nM) or [^{3}H]clonidine (4–5 nM). At the indicated times, triplicate 0.4-ml aliquots of the incubation mixture were taken, diluted in 4 ml of washing buffer, and filtered as indicated above. The pseudo-first order kinetic plot of the association reaction was drawn by plotting In $(B_e/B_e - B)$, where B_e is the specific binding at steady state and B is the specific binding at discrete times, versus time.

The reversibility of both [^{3}H]UK-14,304 and [^{3}H]clonidine binding was assessed by preincubating the membrane preparation for 30 min in the presence of radioligand. At 0 time, an excess of competing drug (10 μM phentolamine) was added and specific binding was determined in triplicate at indicated times. The first order kinetic plot was drawn by plotting In (B/B_e), where B_e was the specific binding at steady state and B the specific binding at indicated times, versus time.

The effects of Gpp(NH)p and sodium chloride were tested as follows: HT29 cell membranes were incubated in a 300 μl final volume of Tris-Mg^{2+} buffer containing either 2–3 nM [^{3}H]UK-14,304 or 4–6 nM [^{3}H] clonidine; after 30 min of incubation, 100 μl of either Gpp(NH)p (final concentration, 10^{-7} to 10^{-2} M) or NaCl (final concentration, 0.1 mM to 1 M) were added and specific radioligand binding was determined after an additional 30-min period.

Protein content determination, analysis of binding data, and statistics. Protein concentrations were determined by the brilliant blue method (25) using bovine serum albumin as standard.

Results from binding experiments were analyzed with the computer programs written by Barlow (26) and McPherson (27). Association and dissociation data were modeled with KINETIC, a program specifically designed for analysis of multiple exponential processes. Saturation isotherms were fitted with EBDA-LIGAND. These two program use a weighted nonlinear curve-fitting technique coupled with a partial F test to determine the most appropriate model (27). According to whether Hill coefficient values were different or not from unity, competitive binding curves were analyzed using TWOSITEINHIB or INHIBITION (26). In all analysis procedures the nonspecific binding was determined experimentally and was not allowed to float.

Reported values are means ± standard error from n determinations.

Drugs and chemicals. The radioligands were from the following sources. [^{3}H]UK-14,304 (specific radioactivity, 82.7–93.9 Ci/mmol) and [^{3}H]clonidine (specific radioactivity, 42–45.2 Ci/mmol) were from New England Nuclear. [^{3}H]Yohimbine (specific radioactivity, 88–90 Ci/mmol) was from Amersham (Amersham, England).

UK-14,304 tartrate and prazosin hydrochloride were generously provided by Pfizer, England and Pfizer, Belgium, respectively. (+)-Epinephrine bitartrate and (+)-norepinephrine bitartrate were kindly donated by Sterling-Winthrop Research Institute (Rensselaer, NY). VIP was a generous gift from Dr. M. Laburthe (Paris, France).

Phentolamine was obtained from CIBA-Geigy, Switzerland; propranolol from ICI, England; amidephrine mesylate from Bristol Myers (Evansville, IN); clonidine hydrochloride from Boehringer-Ingelheim, FRG; RX-781094 (Idazoxan) from Reckitt and Colman, England, and benextramine from Aldrich-Chimie, France.

Yohimbine hydrochloride, phenylephrine hydrochloride, dopamine, (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, ascorbic acid, pargyline, pyrocatechol, iproniazid phosphate, N-ethylmaleimide, and IBMX were purchased from Sigma Chemical Company (St. Louis, MO). Bacitracin and Coomassie blue were from Serva (Heidelberg, FRG). Gpp(NH)p and bovine serum albumin (fraction V) were from Boehringer (Mannheim, FRG).

All other chemicals were reagent grade of the best quality available.

[1] J. M. Sénard, P. Mauriège, D. Daviaud, and H. Paris, manuscript in preparation.

Results

Inhibition of VIP-Induced Cyclic AMP Accumulation

Under our experimental conditions, the basal level of cyclic AMP in isolated HT29 cells was 12 ± 3 pmol/mg of cell protein (n = 4), which was not different from that found previously in attached cells (20). The isolated cells were also very sensitive to VIP exposure and responded to 10 nM VIP by a huge accumulation of intracellular cyclic AMP (1037 ± 12 pmol/mg of cell protein, n = 8).

Inhibitory effects of (−)-epinephrine, UK-14,304, and clonidine on VIP-induced cyclic AMP accumulation are depicted in Fig. 1. The maximum rate of inhibition reached 92.5 ± 1% (n = 4) with 10 μM UK-14,304 and was very similar to that observed with (−)-epinephrine (91 ± 2%, n = 4). Under identical conditions, clonidine provoked a 66 ± 1% inhibition only (n = 4), which represented 70–73% of the effect produced by the two other agonists. This agrees with earlier data (18, 20, 21) and clearly confirms that UK-14,304 and (−)-epinephrine are full agonists of HT29 α_2-adrenoceptor, whereas clonidine is only a partial agonist.

Examination of the dose-response curves showed also that the concentration of α_2-agonist producing half-maximal inhibition of cyclic AMP accumulation was 14 ± 2 nM for (−)-epinephrine, 6.5 ± 1.5 nM for UK-14,304, and 41 ± 8 nM for clonidine. The effects of 1 μM UK-14,304, (−)-epinephrine, or clonidine were totally abolished by the selective α_2-antagonists yohimbine or idazoxan, but not by prazosin up to a 100 μM concentration. The concentration of yohimbine suppressing 50% of the α_2-adrenergic-mediated effect was 0.3 μM for 1 μM (−)-epinephrine and 0.9 μM for 1 μM UK-14,304. Finally, none

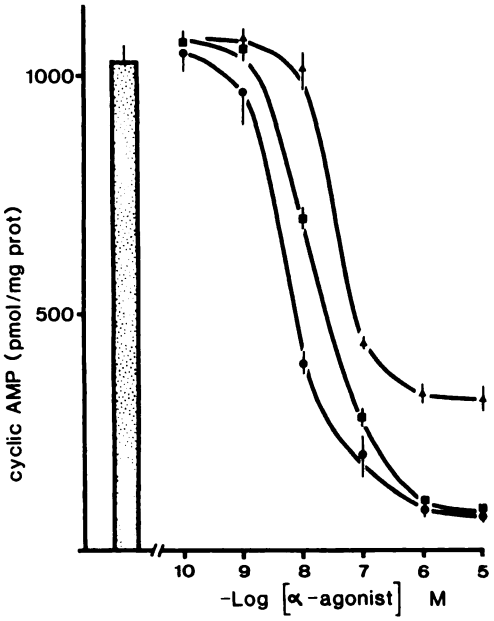

Fig. 1. Effects of UK-14,304, epinephrine, and clonidine on the VIP-induced cyclic AMP accumulation. Isolated HT29 cells were incubated as indicated in Materials and Methods, in the presence of 10 nM VIP alone (■) or in combination with the indicated concentrations of either UK-14,304 (●), clonidine (▲), or epinephrine plus 10^{-6} M propranolol (■). The experiments were performed in the presence of 0.5 mM IBMX and 0.02% bacitracin. After a 15-min incubation at 37°, the cyclic AMP was extracted and determined using a radioimmunoassay technique. The results are expressed as picomole of cyclic AMP per mg of cell protein. The reported values are means ± standard error of three experiments.

of the three agonists, even at very high concentration, was able to lower the cyclic AMP level in nonstimulated cells (not shown).

Binding Studies

Kinetic study of [^{3}H]UK-14304 and [^{3}H]clonidine binding. Typical examples of [^{3}H]UK-14,304 association and dissociation are presented in Fig. 2. The association time-course (Fig. 2A) indicated that [^{3}H]UK-14,304 binding was very rapid, with steady state being reached within 20 min. Then, specific binding remained stable for at least 4 hr. Semilogarithmic transformation of the plot showed a tendency to curvilinearity and data were analyzed using the KINETIC program (27). According to the F test, the fit by a biexponential model was significantly better than by a monoexponential ($p < 0.001$) and allowed two kinetically different components to be distinguished. From four independent experiments, the mean values of the two rate constants of the pseudo-first order association reaction (K_{ob}) were 2.41 ± 0.08 min^{-1} and 0.197 ± 0.044 min^{-1}. It was also calculated that the fast ($t_{½}$ = 0.29 min) and the slow ($t_{½}$ = 3.9 min) components accounted for 56 ± 8% and 44 ± 9% of the reaction amplitude, respectively.

Dissociation of [^{3}H]UK-14,304 (Fig. 2B) was also complex and showed a biphasic pattern. Rather rapid during the first minutes after phentolamine addition, the dissociation reaction then slackened in such a way that approximately 25% of the initial amount of bound [^{3}H]UK-14,304 was still tightly associated with HT29 membrane after 2 hr. The curve presented in Fig. 2B was obtained using phentolamine (10 μM) as competing agent; it must be emphasized, however, that identical kinetics were observed when clonidine, yohimbine, (−)-epinephrine, or UK-14,304 were used. The residual binding still observed 2 hr after the addition of phentolamine was not imputable to an irreversible reaction, because it can be very rapidly abolished by the addition of EDTA (10 mM) or *N*-ethylmaleimide (10 mM) (data not shown). Study of the dissociation process in Tris buffer containing 10 mM Mg^{2+} indicated, in contrast, that a higher concentration of the divalent cation significantly delayed the dissociation reaction.

Dissociation data, when plotted as $\ln(B/B_o)$ versus time, were obviously curvilinear and the KINETIC program showed the results fit better with a biexponential model. From four experiments, constants for the backward reaction (K_{-1}) were estimated to be 0.115 ± 0.018 min^{-1} and 0.0095 ± 0.0007 min^{-1}. The rapidly dissociated fraction ($t_{½}$ = 6.02 min) accounted for 48 ± 6% of the reaction amplitude, whereas the slow fraction ($t_{½}$ = 73 min) corresponded to 52 ± 6%.

A parallel study of the kinetics of [^{3}H]clonidine binding was performed under strictly identical conditions (Fig. 3). The [^{3}H]clonidine association reaction resembled very much that of [^{3}H]UK-14,304. The binding was very rapid, steady state being attained within 15 min at 25°. Analysis of three different experiments proved, moreover, that [^{3}H]clonidine association was also significantly better fitted by a biexponential model at $p < 0.001$. The estimates of the pseudo-first order rate constants (K_{ob}) were 4.56 ± 0.52 and 0.24 ± 0.04 min^{-1} (n = 3). The fast component ($t_{½}$ = 0.15 min) accounted for 34 ± 2% of the reaction amplitude, whereas the slow one ($t_{½}$ = 2.9 min) represented 66 ± 3%.

Dissociation of [^{3}H]clonidine appeared different from that of [^{3}H]UK-14,304, in that it was fully accomplished within 30 min after phentolamine addition. Analysis of the data according to

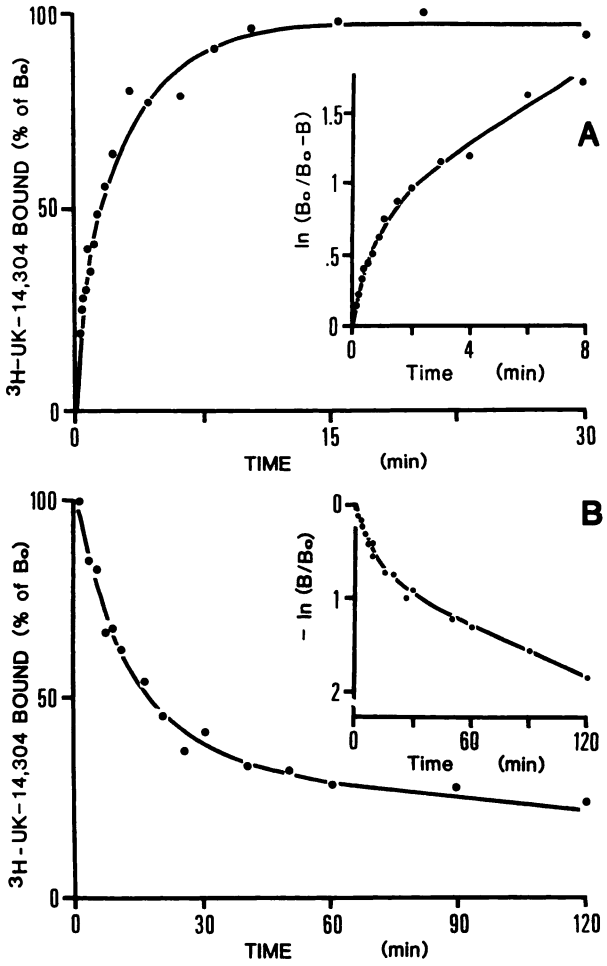

Fig. 2. Kinetic study of [^{3}H]UK-14,304 binding to HT29 cell membrane preparation. A, Study of the association reaction. A cell membrane preparation was incubated at 25° with 2–3 nM [^{3}H]UK-14,304 and the specific binding was determined in triplicate at the indicated times. The data from this typical experiment are representative of that obtained in three others. Analysis of the plot using the computer program KINETIC indicated that the association process of [^{3}H]UK-14,304 was better fitted by a biexponential model at $p < 0.001$. Pseudo-first order association rate constants (K_{ob}) of the two components of the reaction were 3.16 ± 0.62 min^{-1} and 0.23 ± 0.11 min^{-1} (mean ± standard error). The fast component accounted for 54 ± 9%. The *inset* is the pseudo-first order kinetic plot drawn from the mean values calculated from four experiments. In this representation, B_o is the amount of [^{3}H]UK-14,304 bound at steady state and B the amount of [^{3}H]UK-14,304 bound at indicated times. The slopes represent the pseudo-first order association rate constants (K_{ob}) of the two components. B, Study of the dissociation reaction. A membrane preparation was preincubated for 30 min at 25° in the presence of 2–3 nM [^{3}H]UK-14,304. At 0 time, an excess of phentolamine (10^{-5} M) was added and the specific binding was determined in triplicate at the indicated times. The data from this specific experiment are representative of those obtained in three others. Analysis of this plot using the computer program KINETIC indicated that the reaction was better fitted by a biexponential model at $p < 0.001$. Dissociation rate constants (K_{-1}) of the two components of the reaction were in this case 0.08 ± 0.02 min^{-1} and 0.005 ± 0.002 min^{-1} (mean ± standard error); the fast component accounted for 62 ± 7%. The *inset* is the semilogarithmic plot drawn from the mean values calculated from four experiments. In this representation, B_o is the amount of [^{3}H]UK-14,304 bound at steady state and B corresponds to the binding of [^{3}H]UK-14,304 at the indicated times. The slopes represent the dissociation rate constants (K_{-1}) of the two components.

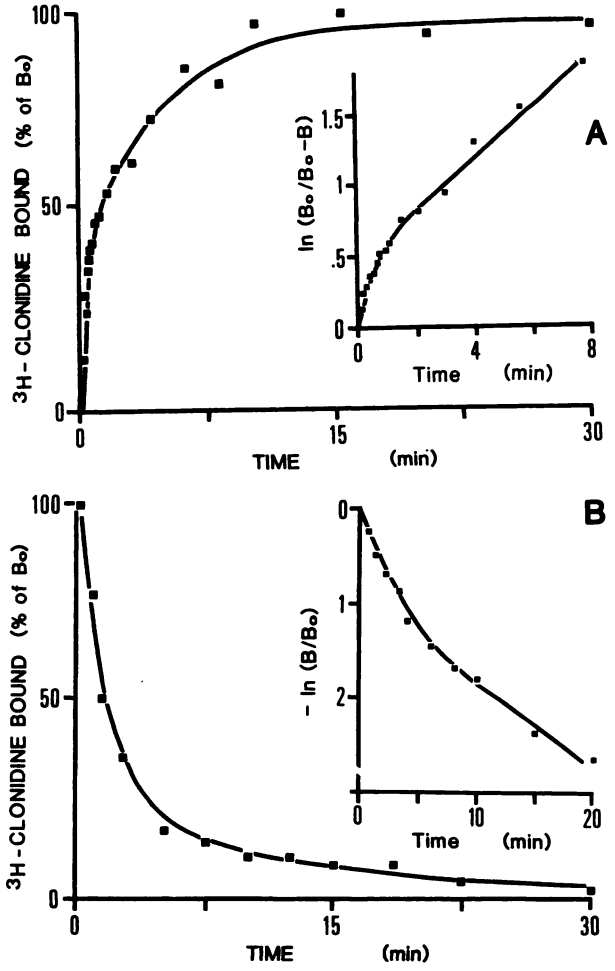

Fig. 3. Kinetic study of [^{3}H]clonidine binding to HT29 cell membrane preparations. The experiment and the analysis of the data were carried out exactly as described in Fig. 2, but in the presence of 4–5 nM [^{3}H] clonidine. A, Study of the association reaction. The presented data are representative of those obtained in three independent experiments. Analysis of the plot using the computer program KINETIC indicated that the association process of [^{3}H]clonidine was better fitted by a biexponential model at $p < 0.001$. Pseudo-first order association rate constants (K_{ob}) of the two components of the reaction were 4.16 ± 0.93 and 0.22 ± 0.08 min^{-1} (mean ± standard error). The fast component accounted for 36 ± 6%. The *inset* is pseudo-first order kinetic plot drawn from the means ($n = 3$), where B_∞ is the amount of [^{3}H]clonidine bound at steady state and B the amount of [^{3}H]clonidine bound at the indicated times. The slopes represent the pseudo-first order association rate constants (K_{ob}) of the two components. B, Study of the dissociation reaction. The data from this specific experiment are representative of those obtained in four others. Analysis of the plot using the computer program KINETIC according to a monoexponential model gave a K_{-1} value of 0.179 ± 0.021 min^{-1}. Fitting to a biexponential model was significantly better only at $p < 0.22$. From this second type of analysis, estimations of the dissociation constants (K_{-1}) of the two components were 1.0 ± 0.8 and 0.12 ± 0.03 min^{-1} (mean ± standard error). The rapidly dissociated fraction represented 71 ± 44%. The *inset* is the semilogarithmic plot of the mean values obtained from five dissociation experiments. In this representation, B_∞ is the amount of [^{3}H]clonidine bound at steady state and B corresponds to the binding of [^{3}H]clonidine at the indicated times.

a monoexponential model led to an estimated K_{-1} value of 0.113 ± 0.035 min^{-1} ($n = 5$). For all experiments, fitting of the data to a biexponential model was also possible. Although the significance may appear marginal ($0.262 > p > 0.107$), this second method of analysis was really better, in view of a lower sum of squares and better estimation of the initial amount of bound [^{3}H]clonidine. From the five experiments, K_{-1} values of the two components were calculated to be 0.79 ± 0.31 and 0.08 ± 0.02 min^{-1}. Moreover, the rapidly dissociated fraction of the reaction ($t_{1/2}$ = 0.88 min) accounted for 63 ± 15%

Specificity of [^{3}H]UK-14304 binding. Specificity of [^{3}H] UK-14,304 was assessed by comparing the ability of a series of adrenergic agonists and antagonists to inhibit its specific binding. Results from this study are reported in Table 1. All inhibition curves displayed a Hill coefficient value not different from unit and were, therefore, better fitted by a one-site inhibition model. The relative order of potency for agonists (UK-14,304 ≥ clonidine > (−)-epinephrine > (−)-norepinephrine > phenylephrine ≫ amidephrine) and for antagonists (yohimbine = RX 781094 > phentolamine ≫ prazosine) is as typically expected for drugs acting upon α_2-adrenoceptors.

The stereospecificity of [^{3}H]UK-14,304 binding was demonstrated by the use of catecholamine enantiomers, (+)-epinephrine and (+)-norepinephrine being, respectively, 10 and 40 times less potent in displacing [^{3}H]UK-14,304 than their corresponding (−)-enantiomers.

Fig. 4 shows the results obtained from the study of inhibition by phentolamine of [^{3}H]clonidine and [^{3}H]UK-14,304 binding under different conditions. These experiments were carried out either under "competition" conditions (i.e., phentolamine and the radioligand were simultaneously added to the membranes and binding was measured after a 30-min incubation) or under "displacement" conditions (i.e., membranes were incubated in the presence of radioligand until steady state was reached, then phentolamine was added and binding was measured after an additional 30-min period). As expected from the kinetic data, inhibition curve profiles differed according to the experimental protocol used and the radioligand tested. Both experimental conditions gave similar results when [^{3}H]clonidine was used; thus, maximum extent of inhibition and estimations of phentolamine (EC_{50} (48 ± 10 nM versus 66 ± 9 nM) were not

TABLE 1

Inhibition of [^{3}H]UK-14,304 binding by α-adrenergic agents and catecholamines

The inhibition of [^{3}H]UK-14,304 binding to HT29 cell membranes was investigated as described in Materials and Methods. The concentration of [^{3}H]UK-14,304 was 2–3 nM. Because Hill coefficients not different from unity, competitive curves were analyzed using INHIBITION, a computer program for curve fitting to a one-site inhibition model (26). Inhibition constants were calculated from the equation $K_i = EC_{50}/(1 + [^3H]UK\text{-}14,304/K_D)$, where EC_{50} is the concentration of drug displacing 50% of bound radioligand. Reported values are means ± standard error of n determinations.

Compound	n	K_i	N_H
		nM	
Agonists			
UK-14,304	5	0.53 ± 0.04	0.994 ± 0.063
Clonidine	5	1.18 ± 0.03	0.948 ± 0.054
(−)-Epinephrine	5	3.1 ± 0.5	0.888 ± 0.028
(−)-Norepinephrine	6	6.8 ± 0.8	0.979 ± 0.018
Phenylephrine	3	25.5 ± 0.5	0.881 ± 0.040
Dopamine	6	37 ± 5	0.851 ± 0.037
(+)-Epinephrine	4	43 ± 5	0.937 ± 0.066
(+)-Norepinephrine	4	290 ± 32	0.885 ± 0.092
Amidephrine	3	3,008 ± 170	1.125 ± 0.069
Antagonists			
Yohimbine	4	6.7 ± 0.5	0.928 ± 0.036
RX781094	4	7.9 ± 1.1	1.028 ± 0.005
Phentolamine	4	21 ± 6	0.961 ± 0.019
Prazosin	2	>15,000	ND[a]

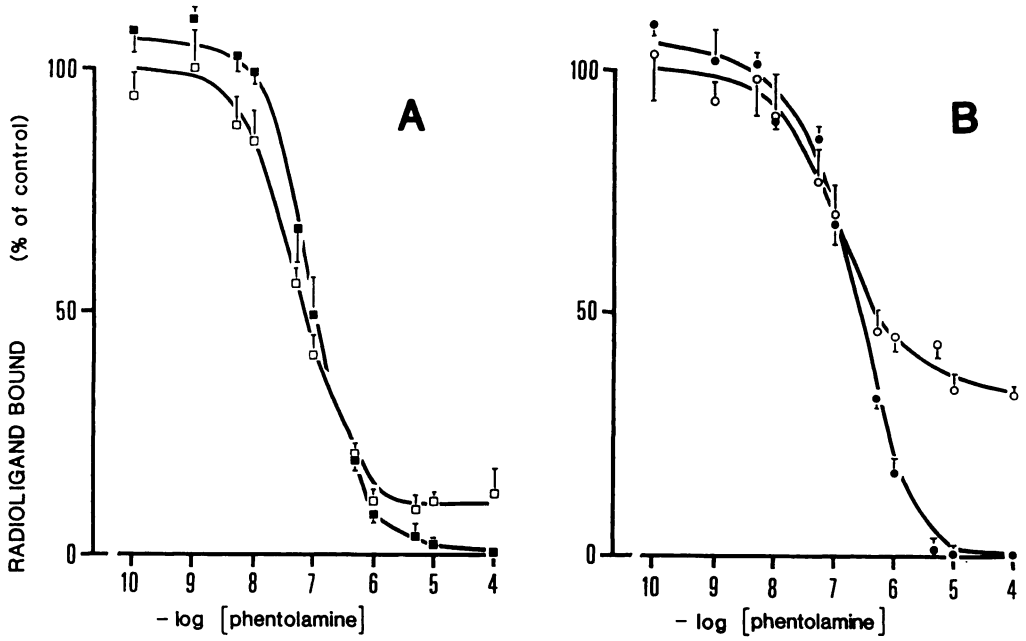

Fig. 4. Inhibition of [^{3}H]clonidine (A) and [^{3}H]UK-14,304 (B) binding under competition or displacement conditions. Inhibition of specifically bound [^{3}H]clonidine or [^{3}H]UK-14,304 by phentolamine was studied as described in Materials and Methods, but phentolamine was either added concomitantly with the radioligand (competition experiments, *filled symbols*) or after membranes were previously incubated for 30 min with the radioligand (displacement experiments, *open symbols*). Results are expressed as percent of control, which corresponded to specific binding measured in the absence of phentolamine. Each *point* is the mean ± standard error from three experiments performed in duplicate.

significantly different. Inhibition of [^{3}H]UK-14,304 binding under displacement conditions was only partial and a rather large fraction of bound [^{3}H]UK-14,304 was not displaced (30–35% of initial amount). Interestingly, there was no shift in the affinity of the displaced sites, inasmuch as phentolamine EC_{50} was 149 ± 32 nM under competition and 114 ± 55 nM under displacement conditions.

Saturation Experiments

The evolution of [^{3}H]UK-14,304 binding as a function of radioligand concentration is shown in Fig. 5. Binding was saturable and of high affinity. Analysis of the saturation isotherm using the EBDA-LIGAND program (27) indicated that the data were not better fitted by the two-site model; thus, the one-site model was used. From 20 independent experiments, B_{max} and K_D values were, respectively, estimated as 189 ± 12 fmol/mg of cell membrane protein and 0.39 ± 0.05 nM (n_H = 1.012 ± 0.013). Furthermore, incubation for much longer periods (2 or 4 hr) did not cause any modification of the B_{max} value; K_D was slightly reduced (0.22 ± 0.05 nM), but Scatchard plots were still linear.

In order to compare our results with the observations reported by Turner *et al.* (28), [^{3}H]UK-14,304 saturation isotherms were examined in 25 mM glycylglycine (pH 7.6) for five different membrane preparations. In four of the five experiments, there was curvature of the Scatchard plot and nonlinear least squares analysis of the data indicated the existence of two different classes of binding sites with K_D of 0.19 ± 0.03 nM and 16.3 ± 8 nM. The high and low affinity binding sites corresponded, respectively, to 53 and 47% of the total binding sites.

Experiments reported in Fig. 6 were designed to verify that the discrepancy between equilibrium and kinetic data was not due to technical limitations that would not permit evidence for the heterogeneity of UK-14,304 binding sites to be obtained in Tris-Mg^{2+} buffer. In these experiments, a HT29 membrane preparation was incubated for 30 min with 10 nM unlabeled UK-14,304, then an excess (10 μM) of the nonreversible α_2-adrenergic antagonist benextramine was added. After an additional 30-min incubation, UK-14,304/benextramine-treated

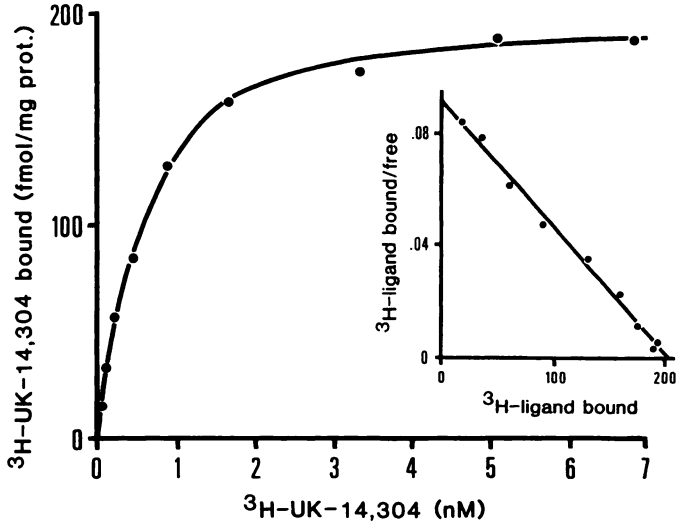

Fig. 5. Specific binding of [^{3}H]UK-14,304 to HT29 cell membrane preparations as a function of [^{3}H]UK-14,304 concentration. HT29 cell membrane fractions were incubated in Tris-Mg^{2+} buffer with the indicated concentrations of [^{3}H]UK-14,304 (0.04 to 8 nM). After 30 min at 25°, the amount of specifically bound [^{3}H]UK-14,304 was measured as described in Materials and Methods. The *inset* is the Scatchard plot, where the ratio of bound to free [^{3}H]UK-14,304 is plotted as a function of bound [^{3}H]UK14,304. Computer-assisted analysis using the EBDA-LIGAND program indicated that the results were consistent with the existence of a single class of binding sites. Nonlinear regression analysis of the Scatchard plot from this specific experiment gave a K_D of 0.49 ± 0.04 nM and a B_{max} of 204 ± 16 fmol of [^{3}H]UK-14,304 binding sites/mg of cell membrane protein. Such values are representative of 20 independent experiments.

membrane were diluted in a large volume of Tris buffer containing 0.5 mM EDTA, pelleted by centrifugation, and washed twice in Tris-Mg^{2+} buffer. [^{3}H]UK-14,304 binding capacity of treated membrane was finally compared with that of the corresponding control (untreated membrane). Considering 1) the time-course of [^{3}H]UK-14,304 dissociation when using benextramine as competitor (Fig. 6A) and 2) the irreversible binding properties of benextramine at α_2-adrenoceptors (23), one can

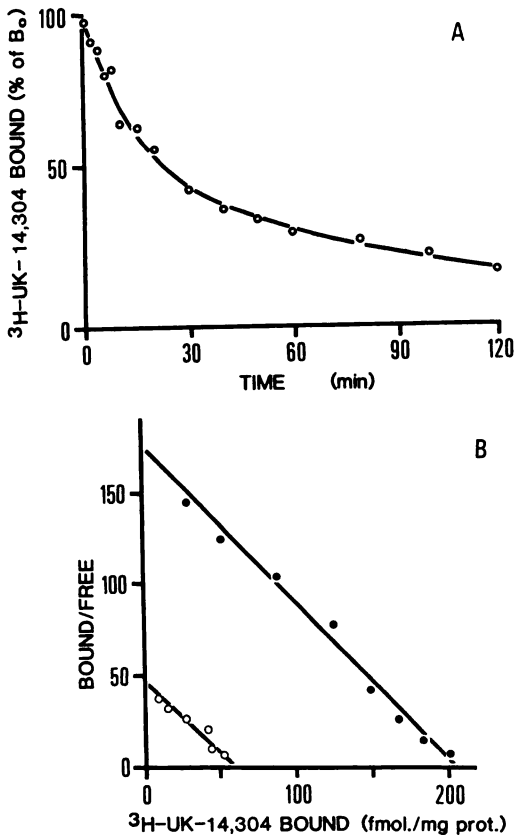

Fig. 6. Characteristics of the UK-14,304-protected α_2-adrenoceptors after partial irreversible blockade with benextramine. A, Study of the dissociation reaction of [^{3}H]UK-14,304 with benextramine. A membrane preparation was preincubated for 30 min at 25° in the presence of 3 nM [^{3}H]UK-14,304. At 0 time, an excess of benextramine (10^{-5} M) was added and the specific binding was determined in triplicate at the indicated times. The data from this specific experiment are representative of those obtained in two others. Kinetic parameters obtained after computerized treatment were not significantly different from those described in Fig. 2. B, Binding characteristics of the α_2-adrenoceptors protected by tightly bound UK-14-304 from irreversible blockade with benextramine. A membrane preparation was preincubated for 30 min at 25° in the presence of 5 mM unlabeled UK-14,304. At 0 time, an excess of benextramine (10^{-5} M) was added. After 30 min of dissociation, the reaction was stopped by dilution in a large volume of Tris-EDTA (5 mM), which, moreover, allowed dissociation of tightly bound UK-14,304. After centrifugation, the membrane pellet was washed twice with Tris-Mg^{2+} buffer, before being used for saturation experiments with [^{3}H]UK-14,304, as described above. The Scatchard plot obtained from UK-14,304/benextramine-pretreated membranes (○) is compared with that obtained in the corresponding untreated control (●).

logically expect that membranes prepared as indicated above should contain almost exclusively the slowly dissociating site fraction, which was protected from benextramine blockade by UK-14,304 and then released from tightly bound UK-14,304 by subsequent EDTA treatment. As predicted, the treated membranes displayed fewer [^{3}H]UK-14,304 binding sites than did the control ones; however, the K_D for the remaining sites was surprisingly unaffected (Fig. 6B).

Comparative Study of [^{3}H]UK-14,304, [^{3}H]Clonidine, and [^{3}H] Yohimbine Binding Parameters

A comparative determination of [^{3}H]UK-14,304, [^{3}H]clonidine, and [^{3}H]yohimbine binding parameters was performed. Results from this study, which was carried out at the same time on eight different membrane preparations, are summarized in Table 2. Binding of the three radioligands was consistent with the existence of a single class of sites. The K_D value was 3.57 ± 0.23 nM for [^{3}H]yohimbine, 0.91 ± 0.06 nM for [^{3}H]clonidine, and 0.44 ± 0.06 nM for [^{3}H]UK-14,304. Comparison of B_{max} revealed 1) that [^{3}H]clonidine labeled as many sites as [^{3}H]UK-14,304 (179 ± 21 versus 172 ± 14 fmol/mg of cell membrane protein) and 2) that the number of sites recognized by [^{3}H]yohimbine (246 ± 22 fmol/mg of cell membrane protein) was significantly higher than with ^{3}H-agonists.

Study of [^{3}H]yohimbine binding inhibition was also undertaken and the values of inhibition constants obtained from the computerized analysis are summarized in Table 3. As expected, the pattern of the curves was very different according to the competing drug tested. Inhibition of [^{3}H]yohimbine binding by itself was consistent with a one-site model and gave a K_i of 4.6 ± 0.8 nM, which is very similar to that obtained from equilibrium experiments. Results with the two agonists were, by contrast, better fitted by a two-site model and use of the TWOSITEINHIB computer program (26) allowed a high (R_H) and a low (R_L) affinity state receptor to be clearly distinguished. For clonidine, the value of K_i at the high affinity state receptor (K_{i_H}) was 1.1 ± 0.2 nM, whereas the one at the low affinity state receptor (K_{i_L}) was 121 ± 59 nM. The values obtained with UK-14,304 were 0.34 ± 0.3 nM for K_{i_H} and 203 ± 72 nM for K_{i_L}. In addition, both partial and full agonists recognized the same fraction of high affinity receptor, the percentage of R_H being 59 ± 5 for clonidine and 56 ± 4 for UK-14,304.

TABLE 2

Comparative study of the binding parameters of the full agonist [^{3}H]UK-14,304, the partial agonist [^{3}H]clonidine, and the antagonist [^{3}H]yohimbine.

Membrane preparations were incubated in the presence of increasing concentrations of [^{3}H]UK-14,304 (0.04–6 nM), [^{3}H]clonidine (0.1–8 nM), or [^{3}H]yohimbine (1–20 nM) and specific binding was determined as described in Materials and Methods. Computer analysis of the binding data indicated that the three radioligands bind to a single class of sites. The maximum number of binding sites (B_{max}) and the dissociation constant values (K_D) were calculated by nonlinear regression analysis of the data. Reported values are means ± standard error from eight different experiments.

Radioligand	B_{max}	K_D	n_H
	fmol/mg of protein	*nM*	
[^{3}H]UK-14,304	172 ± 14	0.44 ± 0.06	0.99 ± 0.03
[^{3}H]Clonidine	179 ± 21	0.91 ± 0.06	0.99 ± 0.03
[^{3}H]Yohimbine	246 ± 22	3.57 ± 0.23	1.05 ± 0.02

TABLE 3

Inhibition of [^{3}H]yohimbine binding to HT29 membrane preparations by yohimbine, clonidine, and UK-14,304

Membrane preparation and inhibition studies were performed as described in Materials and Methods. Data were analyzed by probit transformation and the Hill coefficients were determined. Inhibition by yohimbine itself fitted better with a one-site inhibition model and was analyzed using INHIBITION (26). Inhibition by clonidine and UK-14,304 were, by contrast, better fitted by two-site inhibition models and values were obtained using TWOSITEINHIB (26). K_H and K_L, which represent, respectively, the inhibition constants at the high and low affinity state receptor, were calculated from the equation: $K_i = EC_{50}/(1 + [^3H]yohimbine/K_D)$, where EC_{50} is the concentration of drug displacing 50% of bound radioligand. The proportion of receptor under the high affinity state (R_H) is given in percent. Reported values are means ± standard error of six determinations.

Competitor	K_H	K_L	RH	n_H
			%	
Yohimbine	4.6 ± 0.8			1.10 ± 0.05
Clonidine	1.1 ± 0.2	121 ± 59	59 ± 5	0.51 ± 0.04
UK-14,304	0.34 ± 0.3	203 ± 72	56 ± 4	0.42 ± 0.04

Effects of Sodium and Guanine Nucleotide

The effects of NaCl and Gpp(NH)p on [^{3}H]UK-14,304 and [^{3}H]clonidine binding are depicted in Fig. 7.

As previously observed with [^{3}H]clonidine (29), addition of sodium or Gpp(NH)p strongly inhibited the binding of both ^{3}H-agonists. Inhibition was dose dependent and binding was totally eliminated at 0.5 M NaCl or 10 mM Gpp(NH)p. The inhibition curves with NaCl were very steep and analysis of the data from three experiments run in parallel gave IC_{50} values of 58 ± 13 mM and of 66 ± 8 mM for [^{3}H]clonidine and [^{3}H]UK-14,304 binding sites, respectively.

Unlike sodium, Gpp(NH)p yielded very shallow inhibition curves and displayed different IC_{50} values according to the ^{3}H-agonist tested. The concentration of Gpp(NH)p inhibiting 50% of the initial amount of bound radioligand was 46.9 ± 15.5 μM (n = 7) for [^{3}H]UK-14,304 whereas it was 1.64 ± 0.57 μM (n = 7) for [^{3}H]clonidine. Comparison of these EC_{50} values using Student's t test showed a significant difference at $p < 0.005$. A difference in IC_{50} was also observed with GTP (1.9 ± 0.6 μM versus 23 ± 6 μM), suggesting that [^{3}H]UK-14,304 and [^{3}H] clonidine binding were not similarly sensitive to guanine nucleotide.

Discussion

In earlier direct binding studies using [^{3}H]yohimbine (17) or [^{3}H]clonidine (18), we showed that the human cancer cell line HT29, which is derived from human colon, possesses α_2-adrenoceptors. In addition, measurement of the biological effects associated with stimulation of these receptors indicated that, when compared with (−)-epinephrine, clonidine was an agonist with only partial intrinsic activity on HT29 α_2-adrenoceptors (20). In spite of being cancerous, HT29 cells behave, in this respect, as do the normal corresponding intestinal cells, inasmuch as clonidine was reported to be a very poor agonist on human colocytes (30) and isolated cells from rat small intestine (31).

The imidazole derivative UK-14,304 has long been described as a highly selective α_2-adrenergic agent having full intrinsic activity not only in the central nervous system but also in peripheral tissues, like blood platelets, where clonidine is a partial agonist or not an agonist at all (for review see Ref. 32). When [^{3}H]UK-14,304 became available, this observation prompted many investigators to use this radioligand. Loftus and co-workers (33, 34) were the first and reported that the molecule fulfilled the pharmacological characteristics of a selective α_2-adrenergic agent in rat cortex membrane preparations. Complementary information came from studies performed in human platelets (35–37), calf retina (38), and HT29 cells (28).

In the present work, study of the effects of UK-14,304 on the intracellular accumulation of cyclic AMP induced by VIP clearly showed that this compound is a full agonist at HT29 α_2-adrenergic receptors. Analysis of the dose-response curves of clonidine, UK-14,304, and (−)-epinephrine revealed that UK-14,304 counteracted up to 90–95% of the effect of VIP and displayed a higher affinity than (−)-epinephrine at HT29 α_2-adrenoceptors. Compared with (−)-epinephrine or UK-14,304, the agonist intrinsic activity of clonidine was only 0.7, confirming the previous observations on attached HT29 cells in exponential (18) or post-confluent (20, 21) phase of growth. In this respect, our results also agree with those reported by others in HT29 membrane preparations (28), UK-14,304 and (−)-epinephrine having been found to be similarly efficient for inhibiting forskolin-stimulated adenylate cyclase activity.

In order to better understand the difference between clonidine and UK-14,304 efficacy, a study of the binding parameters of the labeled full agonist was undertaken and results were compared with those found for [^{3}H]clonidine.

Inhibition of [^{3}H]UK-14,304 binding indicated that the radioligand fulfilled the pharmacological specificity of a highly selective α_2-adrenergic agent. The rank order of potency of antagonists and agonists to compete at [^{3}H]UK-14,304 binding sites is strictly identical to that previously observed with [^{3}H] yohimbine (17) and [^{3}H]clonidine (18) and, moreover, agrees with the pharmacological definition of a α_{2A}-adrenoceptor subtype (19). A striking point concerns dopamine, which exhibited

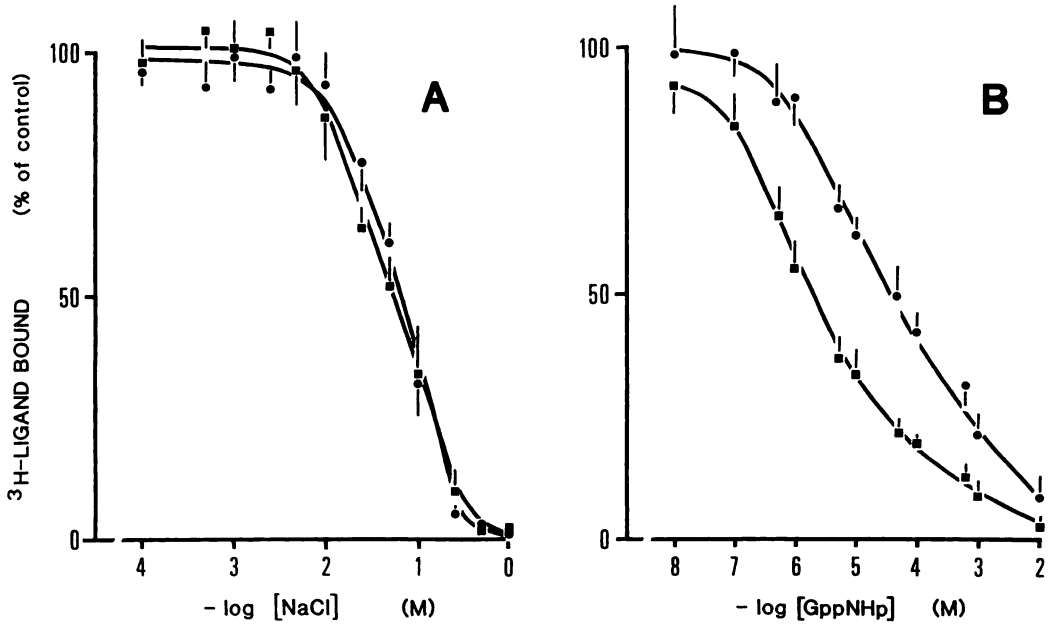

Fig. 7. Effect of sodium (NaCl) and guanine nucleotide (GppNHp) on the specific binding of [^{3}H]UK-14,304 (●) and [^{3}H]clonidine (■). HT29 cell membrane fractions were incubated in Tris-Mg^{2+} buffer with 2–3 nM [^{3}H]UK-14304 or 4–6 nM [^{3}H]clonidine. After 30 min, NaCl (A) or GppNHp (B) were added at the indicated concentrations and specific binding was determined after an additional 30-min period. Results are expressed as percent of control, which corresponded to specific binding measured in the absence of inhibitor. Each *point* is the mean ± standard error from four to seven experiments performed in duplicate.

a K_i of 37 ± 5 nM. Similar values being obtained irrespective of the radioligand used ([^{3}H]clonidine or [^{3}H]yohimbine),[2] it appears that dopamine has a rather high affinity for HT29 α_2-adrenoceptors. This differs somewhat from that observed in rat cortex membrane preparations (33, 34) and might reflect differences due to α_2-adrenoceptor heterogeneity.

Analysis of the saturation isotherms revealed that [^{3}H]UK-14,304 binds with high affinity to a single class of sites and all attempts to get significative evidence for Scatchard curvilinearity remained unsuccessful. Using incubation conditions very close to ours (i.e., Tris buffer containing either 10 mM Mg^{2+} or 0.1 mM Mn^{2+}), Neubig *et al.* (35) and Gibson *et al.* (36) reported the existence of only one quantifiable class of high affinity [^{3}H] UK-14,304 binding sites in human platelet membranes. Turner *et al.* (28) also observed linear Scatchard plots when working with HT29 cell membranes in the presence of 1 mM Mg^{2+}. In agreement with data reported by these latter authors, we also observed that saturation isotherms performed in glycylglycine buffer yielded curvilinear Scatchard plots, which reflected [^{3}H] UK-14,304 binding at high (R_H) and low (R_L) affinity state receptors (28). Thus, replacement of Tris-Mg^{2+} by glycylglycine provoked Scatchard plot curvature due to an increase in [^{3}H] UK-14,304 affinity for the low affinity state α_2-adrenoceptor. Among the ^{3}H-agonists tested so far on HT29 α_2-adrenoceptors (i.e., [^{3}H]clonidine, [^{3}H]UK-14,304, [^{3}H]*p*-amino-clonidine, and [^{3}H]adrenaline), [^{3}H]UK-14,304 is the one that displayed the higher affinity and gave the lower percentage of nonspecific binding.[2] Together with its full agonist activity, such binding properties make this radioligand the most convenient tool for studying agonist binding at α_2-adrenoceptors.

Direct comparison of [^{3}H]UK-14,304, [^{3}H]clonidine, and [^{3}H] yohimbine binding parameters indicated that the two ^{3}H-agonists labeled the same number of sites. [^{3}H]Yohimbine labeled more sites and exhibited a K_D value of 3.57 ± 0.23 nM which is slightly lower than previously reported (17). This increase of [^{3}H]yohimbine affinity is due to the use of lower Mg^{2+} concentrations in the present work (i.e., 0.5 mM instead of 10 mM).

Inhibition of [^{3}H]yohimbine binding by UK-14,304 and clonidine was consistent with the existence of an heterogeneous population of sites and allowed us to distinguish two classes of sites having either high (R_H) or low (R_L) affinity towards agonists. The dissociation constants of UK-14,304 and clonidine at the high affinity state receptor (K_{i_H}) are in good agreement with the K_D calculated from [^{3}H]UK-14,304 and [^{3}H] clonidine saturation isotherms. In contrast to observations by Turner *et al.* (28) with another partial agonist, *p*-amino-clonidine, we found that both UK-14,304 and clonidine interacted at the low affinity state receptor. Determination of K_{i_L} values revealed, moreover, that the ratio K_{i_L}/K_{i_H} was 600 for the full agonist whereas it was only 110 for the partial one. By contrast, identical percentages of high affinity state receptors were recognized by the two agonists. Such results agree with the observations in human platelets (16) and support the existence of a positive correlation between the α_2-agonist intrinsic activity and the ratio of its dissociation constants for the low and high affinity state of the receptor.

Kinetic studies provided valuable information on the mechanism of agonist binding and strongly suggested an heterogeneity of high affinity binding sites that is not discernible at equilibrium. Analysis of the data clearly indicated 1) that the two radioligands behave very similarly and 2) that the interactions of the agonists with the α_2-adrenoceptor are complex, inasmuch as both association and dissociation processes were better fitted according to biexponential models, which is somewhat discrepant with the linearity of the Scatchard plots. It must, however, be emphasized that similar observations were obtained by Convents *et al.* (38) in calf retina. From agonist inhibition of [^{3}H]yohimbine binding, it is obvious that at the radioligand concentrations that are used in kinetic experiments, [^{3}H]UK-14,304 or [^{3}H]clonidine bind almost exclusively to the high affinity state receptor. Thus, biexponential kinetics are not due to interaction of the agonist with the high and low affinity state receptor. In a recent report on [^{3}H]UK-14,304 binding to human platelet membranes, Neubig *et al.* (39) also observed complex kinetics that were not consistent with an apparent bimolecular reaction. Accurate study of the dependence of the forward kinetic constants on radioligand concentration and temperature allowed these authors to propose a new model better accounting for the association kinetics of agonist and agreeing with the present data. According to this model, the whole α_2-adrenoceptor population is composed of three kinds of receptors, 1) the "precoupled" ones, which are associated with G_i even without agonist-receptor interaction and are responsible for the fast phase of agonist association, 2) the "uncoupled/couplable" ones, which undergo coupling only after agonist occupancy and represent the slow phase of agonist association, and 3) the "uncoupled/uncouplable" ones, which cannot undergo coupling and correspond to the low affinity state (R_L). Taking this model and our data from equilibrium and association experiments, one can calculate that the relative amounts of these three kinds of α_2-adrenoceptors are about 30/30/40 in HT29 cells. A fairly similar repartition was found in human platelets (39).

Dissociation of the two ^{3}H-agonists appeared biphasic, but there was obvious difference between [^{3}H]UK-14,304 and [^{3}H] clonidine. The full agonist dissociated much more slowly than the partial one and induced tight binding. Even if the functional consequences of this phenomenon are still unclear, it is important to emphasize that the existence of this tightly bound fraction may have major consequences when the α_2-adrenoceptor response or density after UK-14,304 exposition is studied.

The tightly bound [^{3}H]UK-14,304 fraction being sensitive to EDTA, it represents a very stable form of the agonist-receptor-G_i complex. Such a point of view is further supported by the effects of guanyl nucleotides. Tight binding not being observed with [^{3}H]clonidine, it would be of interest to know whether the activity of full agonists may be related to their ability to induce this very stable ternary complex. Major questions remain to be resolved to strengthen this hypothesis. The first is whether our observations can be extended to other tissues in which UK-14,304 and clonidine act as full and partial agonists, respectively. The second is whether tight binding is a peculiar property of [^{3}H]UK-14,304 or whether it is a characteristic shared by other full agonists. Recent investigations indicate, in this respect, 1) that [^{3}H]UK-14,304 but not [^{3}H]clonidine is able to induce tight agonist binding in human adipocytes[3] and 2) that the kinetic behavior of [^{3}H]adrenaline is strictly identical to that of [^{3}H]UK-14,304 in HT29 cells.[1]

[2] H. Paris, J. Galitzky, and J. M. Senard, unpublished observations.

[3] J. Galitzky, M. Lafontan, H. Paris, and M. Berlan manuscript in preparation.

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