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In Vitro Study of α2-Adrenoceptor Turnover and Metabolism Using the Adenocarcinoma Cell Line HT29

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SUMMARY

We have utilized the adenocarcinoma cell line HT29 as an *in vitro* model to investigate the turnover and the metabolism of the α2-adrenoceptor. The biosynthesis rate of the receptor was studied in postconfluent HT29 cells, when its density expressed as fmol/mg of cell membrane protein is constant, by following the recovery of the receptor binding capacity after blockade with the nonreversible α-adrenergic antagonist benextramine. Study of the inhibition of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding showed that benextramine was a more potent antagonist at α2-adrenoceptor than phenoxybenzamine. The incubation of intact HT29 cells for 30 min in the presence of 10^{-5} M benextramine irreversibly blocked more than 95% of the α2-adrenoceptors and totally suppressed the inhibitory effect of UK-14,304 on cyclic AMP production. The blockade appeared specific, since benextramine effects were prevented by α2-adrenergic agents. Moreover, neither vasoactive intestinal polypeptide responsiveness nor other tested aspects of the regulation of the adenylate cyclase was altered by the treatment. Study of the time course of receptor recovery after irreversible blockade indicated that α2-adrenoceptors reappeared in the cells with a monoexponential kinetic. The linearization of the repopulation curve obtained with the labeled antagonist [^{3}H]yohimbine allowed the determination of the rate constant for receptor degradation (k = 0.0268 ± 0.0025 hr^{-1}) and the rate of receptor synthesis (6.91 ± 0.64 fmol/mg of cell membrane protein/hr) corresponding to the synthesis of about 500 receptors/cell/hr. The α2-adrenoceptor half-life was 26 ± 3 hr. Measurement of the biological effects associated to the α-adrenoceptor stimulation during the course of receptor recovery indicated a relationship between the number of cell receptors and the percentage of inhibition of the cyclic AMP accumulation induced by forskolin. The receptor reappearance was totally inhibited by either actinomycin or cycloheximide or tunicamycin, showing that the recovery corresponded to *de novo* synthesized receptor and giving indirect evidence for the glycoproteic nature of the α2-adrenoceptor. Deprivation for glucose or glutamine also impeded the recovery process; by contrast, addition of UK-14,304 or clonidine did not interfere, indicating that the expression of the α2-adrenoceptor is not subject to homologous regulation in the HT29 cell.

It is now well established that change in membrane receptivity is an important means by which target cells alter their responsiveness to hormones. During the last decade a great deal of interest has been devoted to the study of this phenomenon, and a large amount of information has been accumulated on the cellular events and the biochemical mechanisms involved in this process (1–3).

For adrenergic receptors mediating the responses to catecholamines, numerous *in vitro* and *in vivo* studies have documented the regulation of the β-adrenoceptor which is linked to adenylate cyclase activation (4). By contrast, even if many reports described changes in α2-adrenoceptor expression under various physiological or pathological situations (5–7), much limited data are available on the mechanisms responsible for the regulation of this receptor which is negatively associated to adenylate cyclase.

Changes in membrane receptor number measured by the radioligand binding technique can be the end result of a number of cellular events including the rate of receptor synthesis and degradation as well as cell membrane modifications allowing internalization or reinsertion of existing receptors. Until now, the respective role of these processes in the regulation of α2-adrenergic receptivity has still been obscure, and precious information may come from dynamic studies of the receptor turnover.

Among the different methods which have been used for examining the turnover of membrane receptor, one is to study the recovery of the binding capacity and/or the restoration of the biological activity of the receptor after its blockade with an irreversible ligand. A number of such chemical tools have been

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ABBREVIATIONS: UK-14,304, 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline; benextramine, *N,N″*-dithiodi-2,1-ethanediyl) bis[*N′*-(2-methoxyphenylmethyl)-1,6-hexane-diamine; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GTP, guanosine 5′-triphosphate; GppNHp, guanosine-5′-(imido)-triphosphate; IBMX, 3-isobutyl-1-methylxanthine; VIP, vasoactive intestinal peptide; DMEM, Dulbecco's modified Eagle's medium.

employed for *in vivo* and *in vitro* studies of α1- and β-adrenoceptor metabolism (8–11). Even if phenoxybenzamine (12, 13) and EEDQ (14) have been used for *in vivo* studies of α2-adrenoceptor turnover, research in this area has been handicapped, so far, by the absence of sensitive and specific irreversible markers of the receptor as well as by the lack of a simple *in vitro* model for such investigations.

Benextramine is a tetramine disulfide which is considered to alkylate α-adrenoceptors by a disulfide interchange reaction with sulfhydryl groups of the receptor polypeptide (15, 16). This compound was employed as an irreversible adrenoceptor antagonist to study α-adrenergic mediated vasoconstriction in pithed rats (17) and to investigate *in vivo* the turnover of the α2-adrenoceptor in rabbit spleen (18). Very recently, using benextramine on golden hamster, we reported that the α2-adrenoceptor has a half-life of 31 hr in kidney and 46 hr in adipose tissue (19). Since these values were obtained *in vivo* and may have been overestimated, it was of importance to compare them with those we could get from an *in vitro* study. In the present work, we investigate the turnover and the metabolism of the α2-adrenoceptor on the cultured human adenocarcinoma cell line, HT29, which possesses a well characterized α2-adrenoceptor (20, 21). The repopulation kinetics of α2-adrenergic binding sites and the recovery of the α2-mediated inhibition of forskolin-induced cyclic AMP accumulation were analyzed after irreversible blockade of the receptor with benextramine.

Materials and Methods

Cell culture. The HT29 cell line has been established in culture from a cancer from human colon by Dr. J. Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY) (22). Cell culture conditions were identical to those described previously (21). Cells were seeded at a density of 5×10^4 cells/cm^2 in plastic Petri dishes 65 mm in diameter (Falcon, Becton Dickinson, France) and grown at 37° under a controlled atmosphere (95% air/5% CO_2) in 5 ml of DMEM supplemented with 10% fetal calf serum. During the exponential phase of growth, medium was renewed every 48 hr. Under these conditions, cell confluency was reached at day 9 of the culture. Thereafter, medium was changed every 24 hr in order to avoid nutrient exhaustion.

Receptor blockade with benextramine. We showed in a previous study (23) that α2-adrenoceptor number on HT29 cell membrane is a function of cell density within the dish. In order to avoid any interference between the growth-related receptor maturation process and the receptor reappearance following irreversible blockade, all experiments were started at day 13 of the culture with postconfluent cells (stationary phase of growth). For receptor blockade the cell layers were incubated at 37° in 5 ml of serum-free DMEM containing the appropriate concentration of benextramine. At the end of the incubation period, the medium was sucked out and the cells were washed four times with 5 ml of serum-free DMEM. The cells were finally placed in 5 ml of DMEM supplemented with 10% calf fetal serum and further incubated in the same conditions as above. During the whole time of receptor reappearance the culture medium was changed every 12 hr. At the indicated times, dishes were collected for the determination of receptor number and the measurement of the intensity of the biological effect associated to their stimulation.

Measurements of intracellular cyclic AMP levels. Measurements of intracellular levels of cyclic AMP were performed as previously described (23), but on isolated HT29 cells rather than on attached ones. The HT29 cells were isolated by a 5-min incubation of the cell layer in a phosphate buffer solution containing 0.25% trypsin and 0.5 mM EDTA. The detached cells were pelleted by gentle centrifugation (800 × *g*, 5 min) and finally resuspended in 5 ml of DMEM buffered with 50 mM Hepes. Incubations were carried out as follows: 100-μl samples of the HT29 cell suspension (0.2–0.3 mg of cell protein) were mixed with 100 μl of Hepes-buffered DMEM containing 0.5 mM IBMX and the appropriate concentration of forskolin and α2-adrenergic agonist. After a 20-min incubation period at 37°, 1.8 ml of an ice-cold 95% methanol, 5% formic acid solution was added to stop the reaction. The suspension was sonicated and centrifuged at 2500 × *g* for 15 min, and 100-μl aliquots of the supernatant were evaporated. For analysis, the dried samples were rediluted in sodium acetate buffer (0.2 M, pH 6.5) and cyclic AMP was measured by a radioimmunological method (24).

Preparation of the crude membrane fraction. The dishes used for binding studies or adenylate cyclase measurements were collected as follows: after medium removal, the cell layer was rapidly rinsed twice with 5 ml of ice-cold 0.9% NaCl solution, quickly frozen, and stored at −80°. All the steps of the membrane preparation were carried out at 4°. The frozen cells were scraped in 2 ml of ice-cold Tris-Mg^{2+} buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) and further disrupted using a Dounce homogenizer. The cell lysate was diluted up to 50 ml in Tris-Mg^{2+} buffer and centrifuged for 10 min at 25,000 × *g*. The obtained pellet was resuspended in 50 ml of homogenization buffer and recentrifuged under the same conditions. The final crude membrane fraction was resuspended in Tris-Mg^{2+} buffer at a protein concentration of 1.5–3 mg/ml and immediately used for studies of binding capacity or adenylate cyclase activity.

In some experiments, the membranes from cells which were exposed to adrenergic agonists (*i.e.* clonidine and UK-14,304) were prepared in a way which differed in two points: 1) the Tris-Mg^{2+} buffer used to homogenize the cells was added with 50 mM EDTA, and 2) the suspended membranes were incubated twice for 20 min at room temperature before each centrifugation step. Direct studies of [^{3}H]clonidine and [^{3}H]UK-14,304 binding indicated that this procedure allowed total dissociation of tightly bound agonist and did not interfere with the receptor quantification in control cells.[1]

Binding assays. Binding assays were performed as previously described (23). Specific binding was defined as the difference between the total and nonspecific binding. The total binding was determined by incubating 100 μl of a HT29 cell membrane suspension with ^{3}H-ligand in a 400-μl final volume of 50 mM Tris-HCl buffer containing 0.5 mM $MgCl_2$; whereas the nonspecific binding was measured in parallel assay but in the presence of 10 μM phentolamine. After 20 min of incubation at 25°, 4 ml of ice-cold buffer were added into each incubation tube and the diluted assays immediately filtered through a fiberglass filter (Whatman GF/C) using a Millipore Manifold Sampling unit. The filters were rapidly washed twice with 10 ml of ice-cold buffer and placed into scintillation minivials. The retained radioactivity was determined in presence of 4 ml of scintillation mixture (Beckman Ready Solv) using a scintillation counter (Packard) with an efficiency of 30–35%.

For saturation studies, the final concentrations of [^{3}H]yohimbine and [^{3}H]UK-14,304 ranged respectively from 0.1 to 20 nM and from 0.05 to 8 nM. In control assays, nonspecific binding was 40–45% at 20 nM [^{3}H]yohimbine and 15–20% at 8 nM [^{3}H]UK-14,304.

For competition studies, the desired concentrations of competing drug were added into the incubation mixture prior addition of the membrane preparation. All experiments were carried out at radioligand concentration corresponding to more than twice the k_d. According to the Hill coefficient values not different from 1, data were analyzed using INHIBITION, a computerized curve-fitting program to a one-site inhibition model (25). The EC_{50} value which is the concentration of drug displacing 50% of bound radioligand was calculated by Hill plot analysis using 10 different concentrations of each competing agent.

Adenylate cyclase assays. Adenylate cyclase activity was measured in crude membrane fraction according to the method described by Salomon *et al.* (26). Briefly, 30–50 μg of membrane protein were incubated with 0.2 mM [α-^{32}P]ATP in 50 μl of Tris-HCl buffer (40 mM,

[1] H. Paris, M. Taouis, and J. Galitzky, unpublished data.

pH 7.5) containing 2 mM $MgCl_2$, 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, 1 mM cyclic AMP, 0.5 mM IBMX, 100 μM EGTA, and 0.2% bovine serum albumin. After 20 min at 30° the reaction was arrested by addition of 100 μl of a solution containing 2% sodium dodecyl sulfate, 40 mM ATP, 1.4 mM cyclic AMP, and 12 nCi of ^{3}H-cyclic AMP as an internal standard. Cyclic AMP was separated from ATP by successive chromatography on Dowex and alumina columns. Activities are expressed as picomoles of cyclic AMP produced/mg of protein/min.

Cell viability and protein content determination. Cell viability was assessed by exclusion of trypan blue dye. Protein concentrations were determined by the Coomassie Brilliant Blue method (27) using bovine serum albumin as standard.

Analysis of binding data and statistics. The results from binding studies were treated with an Apple IIe computer using the Biodata Handling programs written by Barlow (25). The saturation curves were analyzed with HYPMIC. According to Hill coefficient values not different from 1, the maximum number of binding sites (B_{max}) and their dissociation constant (K_d) were determined by linear least-squares fit of the Scatchard plot.

The data from the kinetic of receptor recovery were fitted to the equation $[R_t] = V/K\ (1-e^{-Kt})$, where R_t is expressed as fmol/mg of cell membrane protein and represents the receptor number at discrete time t, V is the appearance rate constant of the receptor expressed as fmol/mg of cell membrane protein/hr, and K is the disappearance rate constant of the receptor in unit hr^{-1}.

All reported values are means ± SE from n determinations.

Drugs and chemicals. [^{3}H]UK-14,304 (specific radioactivity, 77–87.6 Ci/mmol), [^{3}H]clonidine (specific radioactivity, 45.2 Ci/mmol), [α-^{32}P]ATP (specific radioactivity, 30–35 Ci/mmol), and the ^{125}I-cyclic AMP radioimmunoassay kit were from New England Nuclear (Boston, MA). ^{3}H-Cyclic AMP (specific radioactivity, 41.7 Ci/mmol) and [^{3}H]yohimbine (specific radioactivity, 90 Ci/mmol) were from Amersham (Les Ulis, France). Phentolamine and UK-14,304 were kindly provided by CIBA-Geigy (Basel, Switzerland) and Pfizer (Sandwich, UK), respectively. Phenoxybenzamine hydrochloride was obtained from Smith Kline and French Laboratories (Philadelphia, PA); benextramine from Aldrich Chemical Co.; and forskolin from Behring Diagnostics. Yohimbine hydrochloride, EDTA, EGTA, and IBMX were purchased from Sigma. Coomassie Brilliant Blue was from Serva (Heidelberg, F. R. G.). Fetal calf serum, bovine serum albumin (fraction V), GTP, GppNHp, cycloheximide, actinomycin, and tunicamycin were from Boehringer (Mannheim, F. R. G.). Dulbecco's modified Eagle's medium was from Eurobio (France). VIP (vasoactive intestinal peptide) was a generous gift from Dr. Laburthe (INSERM, Villejuif, France).

Results

Affinity of benextramine for the α2-adrenoceptor. The apparent affinity of benextramine for α2-adrenoceptor was assessed on HT29 cell membrane preparations by determining its potency to inhibit [^{3}H]yohimbine and [^{3}H]UK-14,304 binding. The results from these experiments as well as those obtained with phenoxybenzamine and other well known α-adrenergic antagonists (yohimbine and prazosin) are summarized in Table 1.

In all cases, the Hill coefficients were near unity and the inhibition curves were treated according to a one-site inhibition model. The inhibition constants of yohimbine and prazosin confirmed the α2 characteristics of the labeled receptor. The selective α2-adrenergic antagonist yohimbine was more than 1000-fold more potent than the selective α1-adrenergic antagonist, prazosin.

Analysis of the inhibition curves obtained with the irreversible antagonists showed clearly that against both radioligands, benextramine was significantly more potent than phenoxybenzamine. In addition to its weaker affinity, it must be pointed out that phenoxybenzamine had the disadvantageous property to hugely increase the nonspecific binding of [^{3}H]yohimbine.

Irreversible blockade of α2-adrenoceptors by benextramine on intact HT29 cells. HT29 cell layers were incubated for 30 min at 37° in presence of increasing concentrations of benextramine (10^{-8}, 10^{-7}, 10^{-6}, 5×10^{-6}, and 10^{-5} M). As indicated under Materials and Methods, the cell layers were then extensively washed in DMEM, rinsed twice with cold saline solution, and frozen. The crude membrane preparations from the benextramine-treated HT29 cells were tested for their capacity to bind [^{3}H]UK-14,304. The results from this study, which are reported in Table 2, indicated that benextramine produced a dose-dependent reduction of the number of binding sites with no significant modification of the affinity of the remaining sites for the radioligand. The decrease in [^{3}H]UK-14,304 site number was about 25% after treatment with 10^{-7} M benextramine and reached 80% with 10^{-6} M benextramine. At 5×10^{-6} M concentration, more than 90% of the binding sites were blocked and no Scatchard plot could be drawn when the cells were treated with a 10^{-5} M concentration. The blocking action of benextramine 10^{-5} M was prevented by the prior addition of 2×10^{-5} M yohimbine into the incubation medium. Clonidine or UK-14,304 also protected the α2-adrenoceptor against benextramine blockade (not shown); by contrast, prazosin and propranolol were ineffective.

The kinetic of benextramine action (Fig. 1A) indicated that the irreversible blockade was very rapid and was completed within 10 min at 37°. Under the same conditions, treatment of HT29 cells with 10^{-5} M yohimbine for as long as 30 min did not result in any significant modification of either [^{3}H]UK-14,304 binding site number or affinity of the receptor for the ligand. Moreover, the results reported Fig. 1B indicated clearly that the blockade of α2-adrenoceptors by benextramine was accomplished by a total loss of their associated biological effect. Exposure of HT29 cells to 10^{-5} M forskolin provoked a huge accumulation of intracellular cyclic AMP (about 500-fold the basal value). Concomitant addition of UK-14,304 (10^{-5} M) resulted in a 90% inhibition of the forskolin-induced cyclic AMP accumulation in control HT29 cells. This inhibitory effect of UK-14,304 was not affected by prior treatment of the cells for 30 min with yohimbine (10^{-5} M), but was totally abolished in the cells treated for 30 min with benextramine (10^{-5} M).

The experiments reported in Table 3 were performed in order to verify that other aspects of the regulation of adenylate cyclase were not affected by benextramine exposure. On both membrane preparations from control and benextramine-treated cells, GppNHp had the same efficiency to inhibit the cyclic AMP production elicited by forskolin, suggesting that benextramine had no effect on the inhibitory capacity of the guanine nucleotide-binding protein, N_i. The results showed, moreover, that the adenylate cyclase from benextramine-treated cells was still responsive to VIP stimulation. The sensitivity to the polypeptide and the extent of activation were not altered, indicating that the VIP-ergic receptor and its positive coupling to the adenylate cyclase were not altered. Besides, the results obtained on intact cells were confirmed by the adenylate cyclase assays. In membrane from control cells, UK-14,304 (10^{-6} M) provoked a significant inhibition of the activity of adenylate cyclase stimulated by 10^{-8} M VIP (34% inhibition).

TABLE 1

Inhibition of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding on HT29 cell membrane by benextramine and various α-adrenergic antagonists

Membrane preparation and inhibition studies were performed as described under Materials and Methods. The membranes were incubated with either [^{3}H]yohimbine or [^{3}H]UK-14,304 in the presence of a range of concentrations of the indicated adrenergic antagonists. According to Hill coefficients (*nH*) not different from 1, the EC_{50} values were determined using the computer program *INHIBITION*. Inhibition constants (K_i) were calculated from the equation $K_i = EC_{50}/(1 + [RL]/K_d)$, where $[RL]$ is the concentration of radioligand and K_d the dissociation constant of the radioligand. The concentrations of radioligands used were 10 nM [^{3}H]yohimbine and 4 nM [^{3}H]UK-14,304. Under our binding conditions the mean values of the K_d for [^{3}H]yohimbine and [^{3}H]UK-14,304 were, respectively, 3.5 and 0.7 nM. EC_{50} and K_i values are expressed in nanomolars and are means ± SE of *n* determinations.

Antagonist	Radioligand							
	[^{3}H]Yohimbine				[^{3}H]UK-14,304			
	EC_{50}	K_i	*nH*	*n*	EC_{50}	K_i	*nH*	*n*
Yohimbine	32 ± 10	10 ± 3	1.05 ± 0.10	3	89 ± 6	13 ± 1	0.95 ± 0.05	4
Benextramine	300 ± 38		0.86 ± 0.11	3	630 ± 60		0.91 ± 0.09	5
Phenoxybenzamine	938 ± 150		0.93 ± 0.15	3	2537 ± 480		0.92 ± 0.10	5
Prazosin	45,000 ± 3,900	14,330 ± 1,240	0.95 ± 0.13	3	80,300 ± 11,000	12,050 ± 1,650	0.85 ± 0.15	3

TABLE 2

α2-adrenoceptor blockade: study of the dose-dependent effect of benextramine and of the protecting action of yohimbine

The HT29 cell layers were incubated for 30 min at 37° in the presence of the indicated concentrations of benextramine and/or yohimbine, propranolol, and prazosin. At the end of the treatment, the cells were extensively washed and the membrane fractions were prepared as indicated under Materials and Methods. The number (B_{max}) and the dissociation constant (K_d) of [^{3}H]UK-14,304 binding sites were determined from Scatchard analysis using six different radioligand concentrations

Treatment	[^{3}H]UK-14,304 binding parameters	
	K_d	B_{max}
	nM	*fmol/mg protein*
None	0.6 ± 0.1	190 ± 17
Benextramine (10^{-8} M)	0.8 ± 0.3	184 ± 13
Benextramine (10^{-7} M)	1.0 ± 0.2	136 ± 10
Benextramine (10^{-6} M)	0.8 ± 0.3	42 ± 5
Benextramine (5×10^{-6} M)	1.6 ± 0.8	14 ± 4
Benextramine (10^{-5} M)	–[a]	<2
Yohimbine (2×10^{-5} M)	1.4 ± 0.3	156 ± 10
Yohimbine (2×10^{-5} M) + benextramine (10^{-5} M)	1.2 ± 0.2	163 ± 12
Propranolol (2×10^{-5} M)	0.6 ± 0.1	172 ± 9
Propranolol (2×10^{-5} M) + benextramine (10^{-5} M)	–	<8
Prazosin (2×10^{-5} M)	0.8 ± 0.2	210 ± 10
Prazosin (2×10^{-5} M) + benextramine (10^{-5} M)	–	<10

[a] Indeterminable.

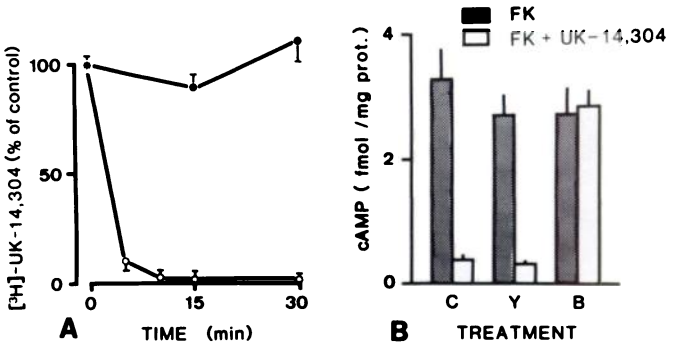

Fig. 1. Effect of benextramine treatment on α2-adrenergic receptivity of HT29 cells. A, kinetic study of the irreversible blockade of α2-adrenoceptors by benextramine. Postconfluent HT29 cells were treated with either 10 μM benextramine (○) or 10 μM yohimbine (●). At discrete time, the cells were extensively washed and membrane fractions were prepared as indicated under Materials and Methods. The number of [^{3}H]UK-14,304 binding sites was determined by Scatchard analysis using six different radioligand concentrations. Results are expressed as percent of control (untreated cells). B, influence of benextramine and yohimbine treatment on α2-agonist-induced biological effect. Postconfluent HT29 cells were incubated with either 10 μM benextramine or 10 μM yohimbine. After 30 min of treatment, the cells were washed and detached using a trypsin-EDTA solution. The cells were then suspended in Hepes-buffer DMEM and assayed for the capacity of UK-14,304 (10^{-5} M) to inhibit the intracellular accumulation of cyclic AMP induced by forskolin (10^{-5} M). *C*, *Y*, and *B* refer respectively to control, yohimbine-treated, and benextramine-treated cells; *FK*, forskolin. Results expressed as femtomoles of cyclic AMP/mg of protein are mean ± SE of five determinations.

This effect was totally abolished in membranes from benextramine-treated cells.

Recovery of α_2-adrenoceptors after benextramine treatment. HT29 cell layers were treated with 10^{-5} M benextramine for 30 min at 37°. The medium containing benextramine was sucked out, and the cell layers were washed four times with 5 ml of serum-free DMEM and finally replaced in standard DMEM. The cells were allowed to recover for up to 5 days.

During the entire time course of the experiment, the total number of cells and the total protein content per dish did not vary significantly. Furthermore, the number of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding sites in cells which did not receive benextramine treatment remained fairly constant. From the six determinations which were done at zero time and 24, 48, 72, 96, and 120 hr after the beginning of the experiment, the mean values of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding site were, respectively, 258 ± 19 and 174 ± 10 fmol/mg of cell membrane protein. These values were taken as binding site concentration at steady state (R_{ss}).

Benextramine-treated dishes were collected after different periods of time and the binding parameters of [^{3}H]yohimbine and [^{3}H]UK-14,304 were determined. The entire time course of receptor reappearance was studied. Results from a typical experiment are presented Fig. 2. During the first hours following the blockade, no reliable Scatchard plots could be drawn with either [^{3}H]yohimbine or [^{3}H]UK-14,304. At 6 hr, a significant [^{3}H]UK-14,304 binding was detected but no reliable data could be obtained using [^{3}H]yohimbine because of its high level of nonspecific binding. The [^{3}H]UK-14,304 binding site which reappeared at 6 hr had the same affinity as in control cells (K_d = 0.68 ± 0.3 nM) and its density was 12 ± 2 fmol/mg of membrane protein. [^{3}H]UK-14,304 as well as [^{3}H]yohimbine binding were determined at 12, 24, 36, 48, 72, 96, and 120 hr. At any time, the ratio [^{3}H]UK-14,304/[^{3}H]yohimbine remained fairly constant (0.65–0.75) and was similar to that found in

TABLE 3

Adenylate cyclase activity in membrane preparations from control and benextramine-treated HT29 cells

Membrane fractions were prepared and adenylate cyclase assays were performed as described under Materials and Methods. Adenylate cyclase activity is expressed as picomoles of cyclic AMP produced/mg of protein/min. Reported values are means ± SE of *n* determinations in parentheses.

Addition	Adenylate cyclase activity	
	Control	Benextramine-treated
	pmol cyclic AMP produced/mg protein/min	
None	1.84 ± 0.10 (6)	1.74 ± 0.15 (6)
NaCl (0.1 M)	3.02 ± 0.65 (6)	2.26 ± 0.27 (6)
NaCl (0.1 M) + FK_a (10^{-5} M)	65.5 ± 6.8 (8)	53.5 ± 5.5 (8)
NaCl (0.1 M) + FK (10^{-5} M) + GppNHp (10^{-7} M)	52.8 ± 4.6 (8)	45.3 ± 4.5 (8)
NaCl (0.1 M) + FK (10^{-5} M) + GppNHp (10^{-6} M)	30.2 ± 2.9 (8)	25.7 ± 3.5 (8)
NaCl (0.1 M) + FK (10^{-5} M) + GppNHp (10^{-5} M)	24.5 ± 2.1 (8)	21.9 ± 2.3 (8)
GTP (10^{-7} M)	2.06 ± 0.08 (4)	3.50 ± 0.20 (4)
GTP (10^{-7} M) + VIP (10^{-10} M)	5.59 ± 0.68 (4)	7.17 ± 0.28 (4)
GTP (10^{-7} M) + VIP (10^{-9} M)	10.7 ± 0.2 (4)	13.2 ± 1.3 (4)
GTP (10^{-7} M) + VIP (10^{-8} M)	19.4 ± 2.3 (4)	22.2 ± 2.1 (4)
GTP (10^{-7} M) + VIP (10^{-8} M) + UK-14,304 (10^{-6} M)	12.8 ± 0.3 (4)	18.9 ± 1.2 (4)

FK, forskolin.

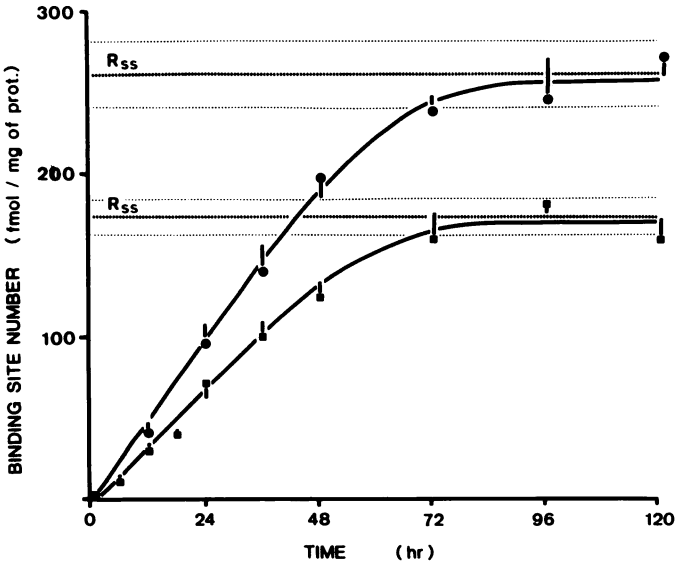

Fig. 2. Repopulation of α2-adrenoceptors on HT29 cells after irreversible blockade with benextramine. Postconfluent HT29 cells were incubated for 30 min with 10 μM benextramine. Cell layers were then washed four times with serum-free DMEM and finally replaced in standard culture conditions. At the indicated times, the dishes were collected and membrane fractions were prepared as described under Materials and Methods. The density of α2-adrenoceptors at the discrete times (R_t) was determined by Scatchard analysis of [^{3}H]yohimbine (●) and [^{3}H]UK-14,304 (■) saturation isotherms. Maximum number of binding sites is expressed as femtomoles/mg of cell membrane protein. Throughout the experiment, the number of binding sites for both radioligands was found constant in control cells. The mean values from these control dishes were taken as the number of binding sites at steady state (R_{ss}) and were respectively 258 ± 19 fmol/mg of protein for [^{3}H]yohimbine sites and 174 ± 10 fmol/mg of protein for [^{3}H]UK-14,304 sites.

control cells (0.67). With both radioligands, more than 50% of the respective binding sites were recovered at 36 hr and site densities not significantly different from control were found at 72 hr.

The repopulation kinetics of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding sites were fitted to a model in which 1) the rate of receptor synthesis and insertion into the membrane is constant during the entire period of the recovery process, and 2) the rate of degradation of the produced receptor is proportional to its concentration on the cells. According to this model, which has already been used to study the turnover of α- and β-adrenergic receptors (8, 11), the rate of receptor recovery can be described by Eq. 1: $[R_t] = V/K\ (1-e^{-Kt})$, where R_t expressed as fmol/mg of protein represents the receptor number at discrete time t, V is the appearance rate constant of the receptor expressed as fmol/mg of protein/hr, and K is the disappearance rate constant of the receptor in unit hr^{-1}. When applied to our data, the logarithmic transformation of Eq. 1 (which is Eq. 2: $\ln([R_{ss}]/[R_{ss}]-[R_t]) = Kt$) gave straight lines (Fig. 3), indicating that the recoveries of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding sites were monoexponential processes. From linear regression analysis of the plots obtained in two different experiments, it was possible to calculate a mean value of the rate constant of receptor degradation (K = 0.0268 ± 0.0025 hr^{-1}) which yielded to a value of the receptor half-life of 26 ± 3 hr.

Recovery of the α2-adrenoceptor-mediated inhibition of cyclic AMP production after benextramine treatment. This part of the study was carried out to determine whether the α2-adrenoceptor repopulation was accompanied by the restoration of the UK-14,304-elicited biological effect. For this purpose, HT29 cells were treated with benextramine (10^{-5} M) and were allowed to recover as described above. At the indicated time the cells were detached with a solution of EDTA-trypsin and suspended in Hepes-buffered DMEM. Aliquots of the cell suspension were taken to study the effect of UK-14,304 (10^{-5} M) on the accumulation of cyclic AMP induced by forskolin (5×10^{-6} M); whereas the remaining cells were pelleted and used for membrane preparation and [^{3}H]UK-14,304 binding assay.

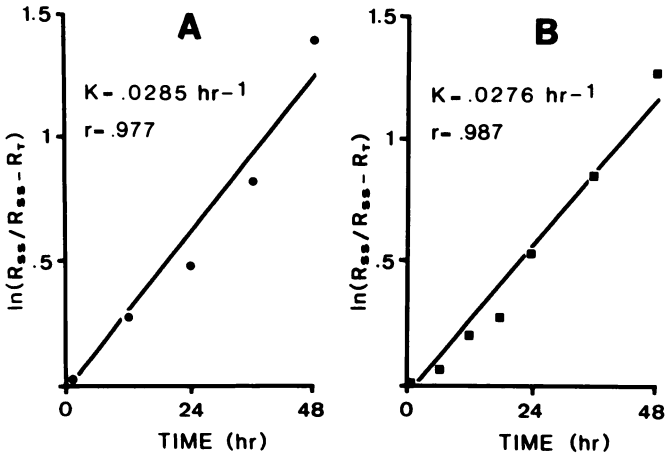

Fig. 3. Semilogarithmic plot of the time course of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding site recovery. The results from the time course study of the recovery of [^{3}H]yohimbine (A) and [^{3}H]UK-14,304 (B) binding sites were plotted according to Eq 2: $\ln(R_{ss}/R_{ss} > R_t) = Kt$, where R_{ss} is the steady-state binding site number and R_t the binding site number at any time after benextramine blockade. The slopes of the plots were determined by linear regression analysis and represented the rate constant of receptor degradation (K).

Fig. 4 shows the recovery with time of the UK-14,304-mediated inhibition of forskolin-induced cyclic AMP accumulation. It can be seen that there was a rapid restoration of the α2-agonist effect since a 100% recovery of the control response was observed at 48 hr. Although functional activity rather than receptor was measured, the assumption was made that the recovery of UK-14,304 effect was also a monoexponential process. Indeed, analysis of the data by Eq. 2 ($\ln [R_{ss}/R_{ss}-R_t] = Kt$, *where* R_{ss} was the maximum response in control cells and R_t is the response at time t after benextramine treatment) produced a linear plot with a correlation coefficient r of 0.977. Calculated in this way, the half-time ($t_{1/2}$) for recovery of UK-14,304-mediated inhibition was 14 hr. It must be mentioned that, in this specific experiment, the half-time for [^{3}H]UK-14,304 binding sites recovery was found to be 30 ± 2 hr, which was not different from that reported in the previous paragraph.

Effects of antibiotics, nutrient deprivation, and agonist exposure on α2-adrenoceptor recovery. HT29 cells were treated with benextramine (10^{-5} M) for 30 min. After extensive washing with serum-free culture medium, the cell layers were replaced in either standard DMEM (control) or in DMEM containing the antibiotic or the α-adrenergic agonist to be tested or in DMEM deprived for glucose or glutamine. The cells were allowed to recover for 32 hr and then collected for [^{3}H]UK-14,304 binding study. Results are reported in Table 4.

As judged by trypan blue dye exclusion, the percentage of viable cells in treated on nutrient-deprived cells was not significantly different from that found in control ones (more than 95%).

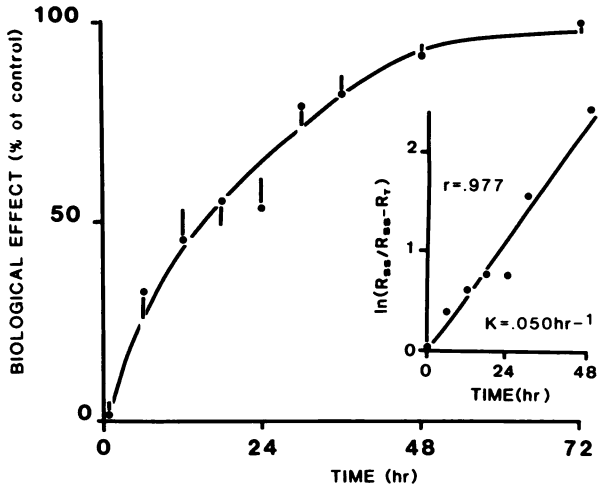

Fig. 4. Time course of α2-agonist-induced biological effect recovery in HT29 cells after benextramine treatment. Postconfluent HT29 culture dishes were incubated for 30 min with 10 μM benextramine. Cell layers were then washed four times with serum-free DMEM and finally replaced in standard culture conditions. At the indicated times, the dishes were collected and the cells detached by treatment with a trypsin-EDTA solution. The cells were then suspended in Hepes-buffer DMEM and assayed for the capacity of UK-14,304 (10^{-5} M) to inhibit the intracellular accumulation of cyclic AMP induced by forskolin (5×10^{-6} M). The intensity of the biological effect of UK-14,304 is expressed as percent of the response elicited by UK-14,304 in control cells (untreated). *Inset* is the semilogarithmic plot of the time course of UK-14,304 biological effect recovery according to Eq. 2: $\ln (R_{ss}/R_{ss} - R_t) = Kt$, where R_{ss} and R_t are respectively the intensity of response to UK-14,304 in control cells and in benextramine-treated cells at time t. The slope of the plot (K) was determined by linear regression analysis.

TABLE 4

Effects of antibiotics, agonist exposure, and nutrient deprivation on the recovery of HT29 cell α2-adrenoceptor

Postconfluent HT29 cells were incubated for 30 min with 10 μM benextramine. Cell layers were then washed four times with 5 ml of serum-free DMEM and further cultured for 32 hr under different conditions. The effects of the various additions as well as the influence of glucose or glutamine deprivation were tested. The number of [^{3}H]UK-14,304 binding sites was determined from Scatchard analysis using six different radioligand concentrations. The level of receptor recovery in standard culture conditions was taken as control and the results were expressed as percent of control.

Treatment	[^{3}H]UK-14,304 binding site recovery at 32 hr
	%
None	100
Actinomycin (0.2 μg/ml)	3 ± 2
Cycloheximide (1 μg/ml)	5 ± 1
Tunicamycin (5 μg/ml)	11 ± 4
Clonidine (10^{-5} M)	92 ± 5[a]
UK-14,304 (10^{-5} M)	107 ± 12[a]
Glucose deprivation	<2
Glutamine deprivation	<2

[a] As specified under Materials and Methods, the membranes from agonist-treated cells were prepared using a homogenization buffer containing 50 mM EDTA.

Actinomycin (0.2 μg/ml), cycloheximide (1 μg/ml), and tunicamycin (5 μg/ml) strongly inhibited the reappearance of [^{3}H]UK-14,304 binding sites. Exposure of the cells to the α2-adrenergic partial agonist clonidine (10^{-5} M) or to the α2-adrenergic full agonist UK-14,304 (10^{-5} M) did not modify the rate of [^{3}H]UK-14,304 binding site recovery. As indicated under Materials and Methods, it must be noticed, however, that the membranes from these agonist-exposed cells were prepared in a homogenization buffer containing 50 mM EDTA. When membranes were prepared in standard conditions, we observed a significant decrease (60%) of the receptor recovery in UK-14,304-treated cells, but not in clonidine-treated ones. Finally, deprivation of the cells for glucose or glutamine totally abolished the recovery process.

Conclusion

Up to now, only scanty information has been available concerning the metabolism and the regulation of the α2-adrenoceptor (for review, see Ref. 28). By using [^{3}H]yohimbine, we described the presence of α2-adrenoceptors on the adenocarcinoma cell line, HT29 (20). Further studies showed that this receptor was negatively coupled to the adenylate cyclase and could be identified by either [^{3}H]clonidine (21) or [^{3}H]UK-14,304 (29), indicating that the HT29 cell line might be a good model for studies of the α2-adrenoceptor regulation. Benextramine is a water-soluble tetramine possessing irreversible α-adrenergic antagonist properties (15, 16), and was recently used *in vivo* to investigate the turnover of α2-adrenoceptor in spleen, kidney, and adipose tissue (18, 19). In the present work we used the HT29 cell line in combination with benextramine to investigate the α2-adrenoceptor biosynthesis and metabolism in an *in vitro* model.

The data obtained on HT29 cells indicated that benextramine bound irreversibly to the α2-adrenoceptor and had a better affinity than phenoxybenzamine for this receptor. This covalent binding led to a dose-related reduction in [^{3}H]UK-14,304 binding site number and to a disappearance of the effect of UK-14,304 on the forskolin-induced cyclic AMP accumulation. The blocking effect was prevented by yohimbine, clonidine, or UK-14,304 but not by propranolol or prazosin. Other

aspects of the regulation of adenylate cyclase activity and sensitivity to VIP were not affected by the treatment. Moreover, neither cell viability nor cellular metabolism (appreciated respectively by trypan blue exclusion and glucose consumption) was altered. All together, these observations indicated that benextramine was rather specific for the α2-adrenoceptor and might be a more convenient tool for investigation of the α2-adrenoceptor turnover in HT29 cells than previously used phenoxybenzamine (12, 13) and EEDQ (14), which were found to present deserving properties. Indeed, in addition to its effect toward α-adrenoceptor, phenoxybenzamine was found to inactivate 5-hydroxytryptamine, H1, and muscarinic receptors (15, 16). EEDQ is similarly active and blocks β-adrenoceptors (11) and dopaminergic (30) receptors.

In control HT29 cells, [^{3}H]yohimbine labeled a number of sites (R_{ss} = 258 ± 19 fmol/mg of membrane protein) which was significantly higher than with [^{3}H]UK-14,304 (R_{ss} = 174 ± 10 fmol/mg of membrane protein). In agreement with that found in human platelets (31), study of the inhibition of [^{3}H]yohimbine binding by nonradioactive UK-14,304 (not shown) showed that this difference was obviously due to the fact that, under our binding conditions (Tris-Mg^{2+} buffer), [^{3}H]UK-14,304 labeled only the high affinity class of the receptor, whereas [^{3}H]yohimbine labeled the whole α2-adrenoceptor population. Study of the recovery of both [^{3}H]yohimbine and [^{3}H]UK-14,304 binding sites in HT29 cells treated with 10^{-5} M benextramine indicated that the cells recuperated up to 100% of the steady-state binding site density within 72 hr. Such a level of recovery suggested that neither the rate constant of receptor synthesis (V) nor that for receptor degradation (K) was affected by benextramine treatment. This point of view was further confirmed by the transformation of our data following Eq. 2. Analysis of the time course of [^{3}H]yohimbine and [^{3}H]UK-14,304 binding site recovery following this equation gave straight lines which confirmed that Eq. 1 correctly described the repopulation as a monoexponential process and that V and K did not change significantly during the recovery period. The ratio (number of [^{3}H]UK-14,304 sites/number of [^{3}H]yohimbine sites) did not vary significantly during the whole recovery period, indicating that the percentage of high affinity state receptor remained fairly constant. As a consequence, the values of K found with [^{3}H]yohimbine or [^{3}H]UK-14,304 were very similar. From two different experiments, where recovery of [^{3}H]yohimbine or [^{3}H]UK-14,304 binding were followed in parallel, it was calculated that the mean value of the rate constant for receptor degradation was 0.0268 ± 0.0025 hr^{-1}. Therefore, the half-life of α2-adrenoceptors in HT29 cells was 26 ± 3 hr. Such a value is close to that obtained in kidney and adipose tissue (31 and 46 hr, respectively) using benextramine (19) and in spleen (39 hr) using phenoxybenzamine (13). By contrast, it differs considerably from the half-life of 99 hr reported in brain (14). The reason for this discrepancy is still unclear. It may be due to a difference between the metabolism of central and peripheral α2-adrenoceptors, but it may also be related to the use of a different alkylating agent. The use of EEDQ on HT29 cells could bring an answer to this last hypothesis. It is remarkable, in this respect, that the use of EEDQ to study dopamine receptor turnover led to a significantly higher half-life value than phenoxybenzamine (32).

Assuming that [^{3}H]yohimbine labeled the whole receptor population and taking 258 ± 10 fmol/mg of membrane protein as α2-adrenoceptor density at steady state, it can be calculated that the rate of synthesis of the α2-adrenoceptor was 6.91 ± 0.64 fmol/mg of membrane protein/hr. Since cell membrane proteins were regularly found to represent 34–38% of the total cell proteins and as 1 mg of total protein corresponded to 3 × 10^6 HT29 cells, one can extrapolate that the rate of synthesis of α2-adrenoceptor was about 450–550 receptors/cell/hr.

The receptor recovery was accompanied by the restoration of the biological effect of α2-agonist. Interestingly, the $t_{1/2}$ for recovery of the UK-14,304-mediated response (i.e., inhibition of the forskolin-induced cyclic AMP accumulation) was significantly shorter than the $t_{1/2}$ for recovery of the receptor. A similar observation was done in rat brain; indeed, after EEDQ treatment, the UK-14,304-mediated inhibition of noradrenaline release recovered much more rapidly than [^{3}H]RX-781094 binding sites in this tissue (14). To better understand the relationship between UK-14,304 biological effect and binding site number after benextramine treatment, the intensity of UK-14,304-mediated inhibition of cyclic AMP production was plotted as a function of [^{3}H]UK-14,304 binding site number in Fig. 5. It appeared clear that the two recovery processes were linked. However, the plot was not linear but rather hyperbolic, indicating that there is no direct proportionality between α2-agonist efficacy and α2-adrenoceptor number. A similar observation was made when studying the evolution of α2-adrenergic receptivity during HT29 cell growth (22). These results suggest a complex receptor-effector coupling and the existence of spare receptors. In this respect, the HT29 cell resembles other cell types, since the presence of a α2-adrenoceptor reserve was also demonstrated in platelets (33) and adipocytes (19).

The effects of actinomycin and cycloheximide indicated clearly that the repopulation process was only imputable to *de novo* synthetized receptor. By contrast, the inhibition of protein synthesis alone by cycloheximide did not modify the density of α2-adrenoceptor in control cells (not shown), making it impossible to measure directly the rate of receptor degradation. Such a phenomenon was also reported in other systems. According to conclusions from the studies of insulin receptor (34) and α1-adrenoceptor (9), this could be due to the necessity for α2-adrenoceptor degradation of a protein component having a very short half-life. The marked reduction in receptor recovery in the presence of tunicamycin suggested that, like many other

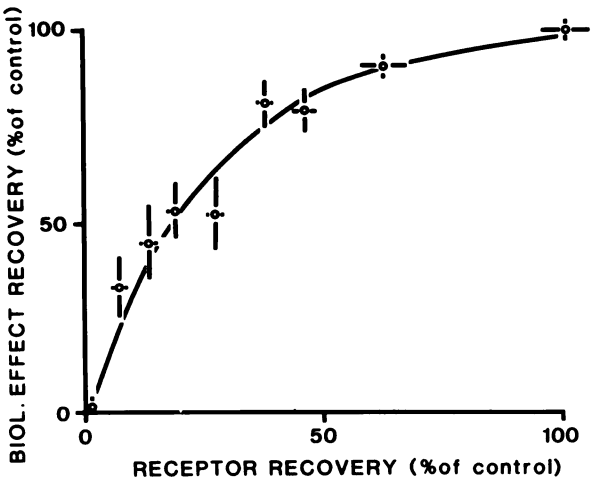

Fig. 5. Evolution of the intensity of UK-14,304 biological effects as a function of [^{3}H]UK-14,304 binding site number on HT29 cell membrane.

ectoproteins of the plasma membrane, the α2-adrenoceptor is a glycoprotein. It is now well established that the expression of numerous membrane receptors can be altered by inhibition of the dolichol pyrophosphate-mediated protein glycosylation (34, 35); to our knowledge, it is, however, the first time such an effect is reported for α2-adrenoceptor. The effect of tunicamycin might be explained by two most likely consequences of the inhibition of glycosylation: 1) complete translation of the α2-adrenoceptor polypeptide cannot occur because glycosylation is a co-requisite event and 2) complete translation occurs, but the aglycosylated α2-adrenoceptor either is not processed and sorted to the membrane or is rapidly degraded or is nonfunctional. To date, it is still impossible to differentiate between these different possibilities, and further knowledge of the intracellular pathways of α2-adrenoceptor processing will require immunological techniques allowing recognition of the immature forms of the receptor, which are not available yet.

Possible down-regulation of α2-adrenoceptor is still subject to intensive discussion. Under our working conditions (i.e., preparing the membrane in a Tris-Mg^{2+} buffer containing 50 mM EDTA), the rate of α2-adrenoceptor recovery was not modified by the addition of clonidine (partial agonist) or UK-14,304 (full agonist) into the incubation medium. The present observation fits with previous data showing that the density of α2-adrenoceptor on HT29 cells was not lowered after a 2-day or even a 10-day exposure to clonidine (36) and confirms that the receptor is not down-regulated by agonists. Such a conclusion also agrees with that reported in other tissues (37–39), but strikingly differs from that found in neuroblastoma x glioma hybrid cells NG 108–15, since UK-14,304 was reported to down-regulate the α2-adrenoceptor (40). We have no definite explanation to account for this discrepancy. However, it must be emphasized that when membrane fractions from agonist-treated cells were prepared in Tris-Mg^{2+} buffer without EDTA, we also found a significant decrease in receptor recovery in UK-14,304-treated cells, but not in clonidine-treated ones. From direct studies of the dissociation kinetics of [^{3}H]UK-14,304 and [^{3}H]clonidine, it appeared clear that the full agonist, but not the partial one, was able to induce the formation of an agonist-receptor complex of super high affinity which dissociated very slowly in the absence of EDTA.[2] Therefore, as already observed in platelets (41), the down-regulation found with UK-14,304 when working in the absence of a chelating agent was only apparent and due to tightly bound UK-14,304 retained on the HT29 cell membrane preparation.

To conclude, all together, our results indicate that the turnover rate and the metabolism of the α2-adrenoceptor in HT29 cells are very similar to those described in intact animals. This reinforces our previous idea that this human cell line, even if cancerous, is a very good model to study the modulation of α2-adrenoceptivity under different hormonal, pharmacological, or nutritional conditions.

Acknowledgments

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