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α_2-Adrenergic Receptor Turnover in Adipose Tissue and Kidney: Irreversible Blockade of α_2-Adrenergic Receptors by Benextramine

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SUMMARY

The recovery of post- and extrasynaptic α_2-adrenergic receptor-binding sites was studied *in vivo* in male golden hamsters after treatment with an irreversible α-adrenoceptor antagonist benextramine, a tetramine disulfide that possesses a high affinity for α_2-binding sites. The kidney α_2-adrenergic receptor number was measured with [^{3}H]yohimbine, whereas [^{3}H]clonidine was used for fat cell and brain membrane α_2-binding site identification. Benextramine treatment of fat cell, kidney, and brain membranes reduced or completely supressed, in an irreversible manner, [^{3}H] clonidine and [^{3}H]yohimbine binding without modifying adenosine (A1-receptor) and β-adrenergic receptor sites. This irreversible binding was also found 1 and 2 hr after intraperitoneal administration of benextramine to the hamsters. Although it bound irreversibly to peripheral and central α_2-adrenergic receptors on isolated membranes, benextramine was unable to cross the blood-brain barrier of the hamster at the concentrations used (10–20 mg/kg). After the irreversible blockade, α_2-binding sites reappeared in kidney and adipose tissue following a monoexponential time course. Recovery of binding sites was more rapid in kidney than in adipose tissue; the half-lives of the receptor were 31 and 46 hr, respectively in the tissues. The rates of receptor production were 1.5 and 1.8 fmol/mg of protein/hr in kidney and adipose tissue. Reappearance of α_2-binding sites was associated with a rapid recovery of function (antilipolytic potencies of α_2-agonists) in fat cells inasmuch as occupancy of 15% of [^{3}H]clonidine-binding sites was sufficient to promote 40% inhibition of lipolysis. Benextramine is a useful tool to estimate turnover of α_2-adrenergic receptors under normal and pathological situations using the approach described in the present paper.

Changes in adrenergic receptor number, measured by radioligand binding studies, have been analyzed in various physiological and pathological situations. The observed changes may be the end result of various processes involving plasma membrane alterations or changes in the rate of synthesis or degradation of the receptor protein.

Two major processes of regulation of plasma membrane adrenergic receptors have been extensively investigated in various *in vitro* and *in vivo* biological systems. The first one concerns desensitization occurring after chronic exposure of the cell receptors to their agonist; it is generally but not exclusively associated with a decrease in receptor number (down-regulation) (1, 2). Down-regulation of adrenergic receptors *in vivo* is tissue and subtype selective, i.e., β_1- and α_1-adrenoceptors are down-regulated in certain tissues, whereas α_2-adrenergic receptors and mechanisms seem to be more resistant to desensitization in various models (3–7). In contrast, when the physiological agonist is lacking after denervation, presynaptic depletion of neurotransmitter, or adrenal medulla removal, the sensitivity of the biological systems is enhanced and the number of receptors is increased (up-regulation).

Variations in the adrenoceptor number imply that receptor synthesis or degradation is decreased or increased or that the receptor is present in a form which is unable to bind the radioligand. A combination of the different events cannot be excluded. It is important to obtain further information on the metabolism of adrenoreceptors in the different tissues. The dynamic turnover studies require the use of tools such as irreversible ligands able to bind tightly to the sites and to measure receptor reappearance after such an irreversible blockade. Concerning the adrenergic receptors, there are few reports concerning the study of α- and β-adrenergic receptor turnover. Some investigations were conducted on cell lines (8, 9), whereas studies have also been made with *in vivo* approaches for β_1-, β_2-, and α_1-adrenoceptors (10–15).

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ABBREVIATIONS: [^{3}H]YOH, [^{3}H]yohimbine; [^{3}H]CLO, [^{3}H]clonidine; [^{3}H]PIA, (−)-N6-*R*-[G-^{3}H]phenylisopropyladenosine; [^{3}H]DHA, 1-[4,6 propyl-^{3}H] dihydroalprenolol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ADA, adenosine deaminase; UK-14,304, 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.

α_2-Adrenergic receptor turnover has, as yet, retained little attention, probably due to the absence of reliable irreversible α_2-blocking agents (14). Because α_2-adrenergic receptors and functions have been well defined by us and some other groups in the adipose tissue of various species (16, 17), the study of the turnover of this family of receptors was carried out in an animal model possessing fat cell α_2-binding sites: the golden hamster (6, 7, 18).

In these studies, we first defined the effects of benextramine (*N,N"*-(dithiodi-2,1-ethanediyl)bis[*N'*-(2-methoxyphenylmethyl)-1,6-hexane-diamine]), a water-soluble, irreversible α_1/α_2-adrenergic receptor antagonist on isolated membranes and after intraperitoneal administration to the animals. The disappearance and the kinetics of the recovery of α_2-adrenergic receptor-binding sites and of the α_2-adrenergic responses initiated by the α_2-agonists were studied in the adipose tissue of hamsters, after a single administration of benextramine. Since α_2-adrenergic receptor distribution and function present some degree of variability according to age and fatness (18), we felt it important to compare the data obtained in adipose tissue with those of two other reference tissues (renal and brain cortex) known to possess α_2-binding sites (19–21). Thus, in addition to adipose tissue, disappearance and recovery of α_2-binding sites were also measured in membranes prepared from renal and brain cortex of the same animals to get a wider overview of α_2-adrenergic receptor turnover in this animal model.

Materials and Methods

Benextramine tetrahydrochloride monophosphate and benzamidine hydrochloride hydrate came from Aldrich Chemical Co. [^{3}H]YOH (90 Ci/mmol), [^{3}H]CLO (42 Ci/mmol), [^{3}H]DHA (90 Ci/mmol), and [^{3}H]PIA (46 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Bovine serum albumin (fraction V), phenylmethylsulfonyl fluoride, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). The following drugs were graciously given by the respective companies: clonidine hydrochloride, Boehringer Ingelheim (Reims, France); UK-14,304, Pfizer Inc. (Sandwich, UK); idazoxan hydrochloride [RX 781094, 2-(2-(1,4-benzodioxanyl))-2-imidazoline hydrochloride], Reckitt and Colman Corp. (Kingston-upon-Hull, UK); phentolamine mesylate, CIBA-Geigy Corp. (Basel, Switzerland). ADA, crude collagenase, phenylisopropyladenosine, and other enzymes came from Boehringer Mannheim Corp. (Mannheim, FRG). All other chemicals were reagent grade. Adult male golden hamsters (*Mesocricetus auratus*), weighing 130 ± 15 g, were housed in a temperature-controlled room (22°) and fed *ad libitum* with a standard diet (UAR Paris) with free access to drinking water.

Preparation of particulate fractions. Hamsters were killed by cervical dislocation. Epididymal adipose tissue, kidney, and brain were quickly removed and processed immediately for crude membrane preparation.

Fat cells. Isolation of fat cells and lipolysis measurements were made in Krebs-Ringer bicarbonate buffer containing 35 mg/ml of serum albumin and 6 μM glucose as recently described (7, 18). Crude membranes were obtained after osmotic lysis of the cells. The fat cells were washed four times at 8–10° in a lysing medium supplemented with proteinase inhibitors (2.5 mM $MgCl_2$; 1 mM $KHCO_3$; 2 mM Tris-HCl, 100 μM phenylmethylsulfonyl fluoride, 100 μM benzamidine, and 5 mM EGTA , pH 7.4). The fat cake was removed and the adipocyte ghosts were spun down (40,000 × *g*, 4°, 10 min). The pellet was washed twice with lysing medium and finally resuspended in the incubation buffer used for the binding studies (10 mM $MgCl_2$, 50 mM Tris-HCl; pH 7.5) at a final concentration of 2–2.5 mg of protein/ml and frozen immediately. The membrane preparation was stored at −80° and generally used within 2–3 days.

Kidney. After removal, the kidney was placed on an ice-cold glass support plate and treated, with minor modifications, as previously described (19). The renal cortex was dissected out from the medulla, cut into small pieces, and incubated in 25 ml of Krebs-Ringer buffer (pH 7.5) containing 5 mg of collagenase. Digestion was carried out under vigourous shaking at 37° for 30 min. After this initial treatment the digested slices were completely disrupted by repeated passage through a Pasteur pipette and the products of this treatment were passed through a nylon screen. The filtrate was pelleted, and the pellet was then resuspended and homogenized in 10 volumes of lysing medium containing proteinase inhibitors, using a Potter homogenizer with a tight-fitting Teflon pestle. This homogenate was pelleted (40,000 × *g*, 4°, 10 min) and washed three times to remove excess collagenase. The final pellet was frozen and kept at −80° until used.

Brain. Crude cerebrocortical membranes were prepared from fresh cortical tissue. After opening the skull, the bulbar region, olfactive lobes, and cerebellum were dissected out from the brain. The cerebral cortex was sliced and cut into small pieces on an ice-cold glass support plate and homogenized in a Potter apparatus in 50 mM Tris-HCl (pH 7.5). The crude particulate fraction was collected after centrifugation (40,000 × *g*, 4°, 10 min) and stored at −80°.

Blockade of α-adrenergic receptors with benextramine. Benextramine tetrahydrochloride was dissolved in sterile physiological saline (0.9% NaCl) at a concentration of 1 mg/ml. The drug was administered in the morning by the intraperitoneal route at a dose of 10 or 20 mg/kg, as specified in the experiments. The hamsters were kept for varying times with free access to food and water before their organs were removed. Injection of the drug at the concentrations above had a minor effect on eating and drinking behavior (a moderate increase in drinking was observed), but we did notice a reduced mobility of the animals only during the 2 hr following the injection of the drug; after this time they behaved normally. The treatments did not induce a single case of mortality.

Identification of α_2-binding sites. Binding assays were conducted according to the method reported previously (22) with minor modifications. [^{3}H]YOH (α_2-antagonist) was used for the identification of renal cortex α_2-binding sites. Since this ligand exhibited poor binding properties in fat cell and brain cortex membranes, [^{3}H]CLO was chosen for the identification of α_2-sites as previously shown (7, 18). In the hamster fat cell, α_2-binding sites are specifically and saturably labeled by the agonist [^{3}H]CLO but bind the antagonist [^{3}H]YOH poorly, as shown in the submandibular glands (23). Thawed frozen membranes were rehomogenized with four pestle strokes in a Potter apparatus immediately before use. Binding studies were conducted in a final volume of 400 μl, the incubation mixture consisted of 100 μl of aqueous radioligand solutions ranging in final concentration from 0.2 to 15 nM and 100 μl of membrane suspensions (200–250 μg of protein) made up to a final volume of 400 μl with 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$. Nonspecific binding was evaluated in the presence of 10 μM phentolamine. Incubations were carried out at 25° in a water bath for 20 min under constant shaking. At the end of the incubation, the suspensions were diluted in 4 ml of ice-cold buffer and filtered through Whatman GF/C glass fiber filters placed on a Millipore manifold. The filters were washed twice with 10-ml portions of ice-cold incubation buffer; they were placed in miniviales and counted in 4 ml of scintillation medium (Ready Solv M. E., Beckman Instrument Ltd.) in a Backard scintillation spectrometer at an efficiency of 35–40%. Specific binding was taken as the amount of radioactivity bound to the membranes (defined as total binding) minus binding in the presence of 10 μM phentolamine. The K_D and B_{max} values were calculated from a computer-assisted nonlinear regression of the data plotted as bound versus free ligand. Adenosine (A1-receptor) and β-adrenergic receptor sites were identified in binding analysis with [^{3}H]PIA and [^{3}H]DHA using 1 mM theophylline (or 0.1 μM PIA) and 10 μM isoproterenol, respectively, for definition of nonspecific binding. Protein was measured by

the method of Lowry *et al.* (24), using bovine serum albumin as standard.

Results

In binding experiments the specific binding of the ^{3}H-radioligands was saturable and Scatchard analysis gave straight lines indicating a single population of noninteracting binding sites (Table 1, Fig. 1). The specificity of binding of both ligands was, as previously shown (18, 19), typical of α_2-adrenergic receptor-binding sites (Table 2). The relative order of potency of the various antagonists in displacing the agonist [^{3}H]CLO from its binding site was found to be similar in fat cell and brain membranes with the following order: idazoxan > phentolamine > yohimbine ≫ prazosin. Idazoxan and phentolamine were more potent than yohimbine in displacing the [^{3}H]CLO from its site. In kidney, the relative order of potency of the antagonists in displacing [^{3}H]YOH from its binding site was found to be: phentolamine > idazoxan > prazosin. Although slightly different from that described for the agonist, such an order is in accordance with the definition of an α_2-site; in this rodent species, prazosin was more potent as compared to α_2-sites defined in human fat cells with the antagonist (22).

TABLE 1

Comparison of binding parameters of [^{3}H]CLO and [^{3}H]YOH to plasma membranes from adipocytes, kidney, and brain of golden hamsters

Assays were performed as described in Materials and Methods. Results are expressed as the mean ± standard error of at least five experiments performed in duplicate. Specific binding was found to be saturable in the three tissues. Scatchard plot analysis of the saturation curves yielded straight lines. K_D and B_{max} values were calculated by nonlinear curve fitting.

Radioligand	K_D	B_{max}
	nM	fmol/mg protein
[^{3}H]CLO		
Adipocyte	2.9 ± 0.1	116 ± 20
Brain	2.0 ± 0.2	78 ± 8
[^{3}H]YOH		
Kidney	8.1 ± 1.8	80 ± 7

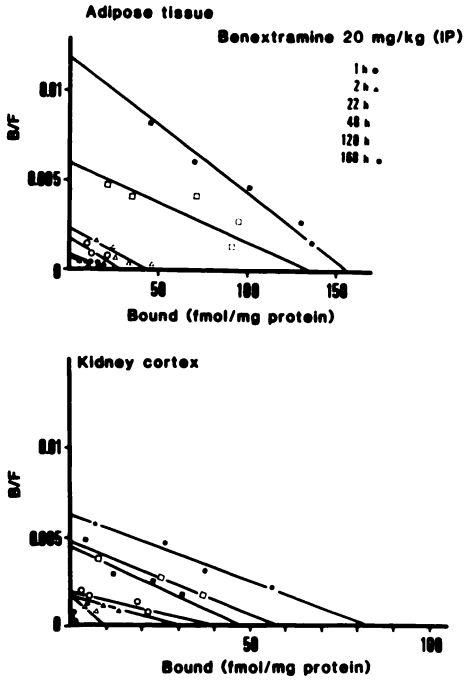

Fig. 1. Recovery of [^{3}H]YOH- and [^{3}H]CLO-binding sites after blockade with benextramine intraperitoneally (*IP*, 20 mg/kg) in kidney and adipose tissue. Results are from a typical experiment. The hamsters were killed after the varying time intervals indicated and particulate fractions were prepared; [^{3}H]YOH and [^{3}H]CLO binding were measured as described in Materials and Methods. A Scatchard plot of the data is shown where the ratio bound over free radioligand (*B/F*; fmol/mg of protein/nM) is plotted as a function of the [^{3}H]CLO and [^{3}H]YOH specifically bound. The *line* plotted by linear regression analysis has a slope of $-1/K_D$. The number of binding sites (B_{max}) is determined by the intercept of the line with the abscissa.

TABLE 2

Inhibition of [^{3}H]CLO and [^{3}H]YOH binding in various tissues by benextramine and various α-adrenergic receptor antagonists

EC_{50} values are means ± standard errors of (*n*) experiments (in parentheses). K_i values (mean K_i, in brackets) are means of (*n*) experiments. EC_{50} is the concentration of benextramine and antagonists producing 50% inhibition of specific binding of radioligands (determined by log probit analysis). Benextramine produces an irreversible inhibition of binding, thus it is not possible to calculate K_i values since the equation used to calculate them is based on a competitive model. The K_i (mean K_i) values were calculated from the equation $K_i = EC_{50}/(1 - [L]/K_D)$, where K_D is the dissociation constant for ^{3}H-ligands obtained in saturation analysis and [*L*] the concentration of ^{3}H-ligand used in the assay (6–7 nM for [^{3}H] CLO and 14–16 nM for [^{3}H]YOH).

	Fat cell [^{3}H]CLO (5)	Brain [^{3}H]CLO (5)	Kidney [^{3}H]YOH (4)
Benextramine	436 ± 160	2190 ± 880	26 ± 9
Idazoxan	24 ± 10	121 ± 40	400 ± 140
	[7]	[33]	[130]
Phentolamine	90 ± 16	280 ± 80	33 ± 17
	[22]	[80]	[10]
Yohimbine	945 ± 200	1030 ± 230	–
	[265]	[280]	[–]
Prazosin	≫ 100 000	≫ 100 000	620 ± 140
	[–]	[–]	[220]

The apparent affinity of benextramine for α_2-binding sites was tested in the tissues by comparison of its relative potency to displace the ^{3}H-radioligands from their corresponding α_2-binding sites. Results are given in Table 2. Benextramine had the highest affinity for α_2-sites in kidney, whereas the affinities were only 5-fold different for brain and fat cell α_2-adrenergic receptors. Although the effects of benextramine and its analogues have been thoroughly studied *in vitro*, but not *in vivo*, in several tissues from different species such as guinea pig atrium and ileum, rabbit aorta, and rat atrium or vas deferens, no data are available on the efficacy of benextramine as an irreversible α_2-blocker on fat cell or kidney. In order to define the properties of the compound more fully, for its use in the present studies we carried out *in vitro* investigations to assess its putative irreversible α_2-blocking potencies in the tissues.

Irreversible blockade of α_2-adrenergic receptors in membranes from different tissues. Two different experiments were conducted to assess the properties of benextramine. In the first set of experiments, membrane suspensions were incubated 15 min in binding buffer containing 10^{-7}–10^{-4} M benextramine. After this incubation period and centrifugation, the pellet was washed five times with 10-ml portions of binding buffer at intervals of 10 min between two washings. At the end of the washing procedure the membranes were resuspended in fresh binding buffer for saturation analysis with the corresponding radioligands. In some control experiments the washing procedure was carried out with binding buffer supplemented with 10^{-4} M cold clonidine or yohimbine to test a putative dissociation of benextramine in the presence of high concentrations of cold ligands. Under these conditions, this procedure

was immediately followed by a supplementary washing period to remove cold ligands before undergoing binding studies.

Two other series of control experiments were set up. First, membrane suspensions were incubated with cold clonidine and cold yohimbine (10^{-4} M) for 15 min and washed extensively as described for benextramine to test putative alterations of α_2-receptors during the incubation (with agonist and antagonist) and washing periods. Second, we tested the protection of the α_2-adrenergic receptor-binding site by the cold ligand: preincubation of membranes with 10^{-4} M clonidine (10 min) before addition of the irreversible antagonist benextramine (10^{-5} M, 15 min more) and extensive washing as described previously.

Results showing the irreversibility of α_2-adrenergic receptor blockade by benextramine are summarized in Table 3. Preincubation of membranes from the three tissues with 10^{-4} M benextramine (its effect is dose dependent within the duration of the experiment) promoted the complete loss of α_2-ligand binding. This effect was resistant to several washings with and without cold ligands. Preincubation of membranes with high concentrations (10^{-4} M) of cold clonidine and cold yohimbine was without any effect on the binding properties of the α_2-sites. Moreover, clonidine (10^{-4} M) protected (full protection, i.e., unchanged B_{max} values) α_2-sites against their inactivation by 10^{-5} M benextramine.

When freshly isolated fat cells were incubated in the same experimental conditions, the treatment with benextramine (30 min, 10^{-5} M or 10^{-4} M) promoted a complete loss of [^{3}H]CLO-binding sites and, concomitantly, of α_2-adrenergic responsiveness without alteration of the β-adrenergic-mediated lipolytic response or the phenylisopropyl-adenosine-dependent antilipolytic effect (not shown). Radioligand binding studies of adenosine sites ([^{3}H]PIA binding) and β-sites ([^{3}H]DHA binding) were carried out after benextramine treatment of the isolated fat cells. After cell lysis and extensive washing of the membranes, the number of [^{3}H]PIA sites (B_{max}) was 480 ± 35 fmol/mg of protein in controls and 420 ± 40 fmol/mg of protein in treated adipocytes (n = 5); β-receptors ([^{3}H]DHA sites) were unchanged after benextramine treatment (45 ± 6 in controls versus 37 ± 7 fmol/mg of protein in membranes of treated fat cells; n = 5).

In the fat cell and kidney, intraperitoneal injection of benextramine reduced the α_2-binding site number. Two hr after benextramine injection (20 mg/kg) the highest blockade of α_2-sites was determined in the kidney and fat cells (Table 4).

TABLE 3

Evidence for irreversible binding of benextramine on α_2-sites in membranes from adipocyte, renal cortex, and brain of golden hamsters

Membrane preparation and incubations were made as described in Materials and Methods. Values are means ± standard errors of three to four experiments performed in duplicate. Membranes were incubated with or without benextramine (10^{-4} M) and washed five times with 20 ml of Tris-$MgCl_2$ buffer as described in Materials and Methods. Control experiments (with cold ligand) were carried out with 10^{-4} M cold clonidine (fat cell, brain) or cold yohimbine (kidney).

	[^{3}H]YOH or [^{3}H]CLO binding			
	Without benextramine		With benextramine (10^{-4} M)	
	Controls	With cold ligand	Controls	With cold ligand
	fmol/mg of protein			
Fat cell	115 ± 20	113 ± 18	Und.	Und.
Kidney	80 ± 6	75 ± 2	Und.	Und.
Brain	78 ± 7	65 ± 4	Und.	Und.

[a] Und., undetectable.

TABLE 4

Blockade of α_2-adrenoceptors in different tissues following i.p. injections of benextramine

Different doses of benextramine (10 or 20 mg/kg) were injected intraperitoneally (IP) into golden hamsters. The animals were killed 1 or 2 hr later, particulate fractions were prepared, and ^{3}H-ligand binding ([^{3}H]CLO for fat cell and brain cortex or [^{3}H]YOH for kidney) were measured as described in Materials and Methods. B_{max} values (fmol/mg of protein) were determined by saturation binding assays made in duplicate. Values are means ± standard errors of four separate experiments for each treatment group.

	Controls	Benextramine administered IP	
		10 mg/kg	20 mg/kg
One hr			
Fat cell	120 ± 20		16 ± 4[a]
Kidney	80 ± 6		Und.[a,b]
Brain	78 ± 7		64 ± 6 (NS)[c]
Two hr			
Fat cell	120 ± 20	52 ± 7[d]	17 ± 2[d]
Kidney	80 ± 6	24 ± 6[d]	10 ± 2[a]
Brain	88 ± 7	64 ± 8 (NS)	106 ± 10 (NS)

[a] Significantly different from control ($p < 0.01$), Mann-Whitney U test.
[b] Und., undetectable.
[c] NS, not statistically significant.
[d] Significantly different from control ($p < 0.05$), Mann-Whitney U test.

Among all of the animals used, not a single death occurred after treatment. There was no noticeable reduction of maximum density of [^{3}H]CLO-binding sites in brain after administration of benextramine (Table 4).

Recovery of α_2-binding sites in fat cells and kidneys. Hamsters received a single injection of benextramine (20 mg/kg) and were killed after different periods of time. The number of α_2-binding sites and their dissociation constants were determined by Scatchard analysis. The time-dependent recovery of [^{3}H]CLO- and [^{3}H]YOH-binding activity after benextramine blockade is shown in Fig. 1. The K_d values for [^{3}H]CLO and [^{3}H]YOH were not strikingly modified during the receptor regeneration period. The affinities at each time point were very similar to that in control animals except for the earliest time points at which the affinity was slightly decreased (Fig. 1).

Repopulation kinetics, after irreversible blockade of a receptor, can be calculated as previously demonstrated *in vitro* (8, 9) and *in vivo* studies (10–14). On the hypothesis already confirmed for acetylcholine (25), insulin (26), α_1-adrenoceptors (10–14), and dopamine receptors (27)—that receptor production is constant during the period of reappearance and that the degradation of the receptors produced after blockade with benextramine is, at any time, proprotional to the concentration of these receptors in the cell, repopulation kinetics can be described according to the equation

$$[R_t] = r/k\ (1 - e^{-kt}) \qquad (1)$$

where $[R_t]$ = receptor concentration at time t, r = receptor production rate, and k = rate constant for receptor degradation. As the time of repopulation tends to infinity, $[R_t]$ approaches the value of the ratio r/k and therefore approaches the steady state receptor concentration $[Rss]$. The $[Rss]$ value did not vary noticeably from the beginning to the end of the experimental period in fat cells and kidney. The logarithmic transformation of Eq. 1 gives:

$$\ln [Rss]/[Rss] - [R_t] = kt \qquad (2)$$

Plots of receptor recovery data according to Eq. 2 are given in Fig. 2. The time course of reappearance was studied in adipose tissue and kidney (Fig. 2A). Although both types of

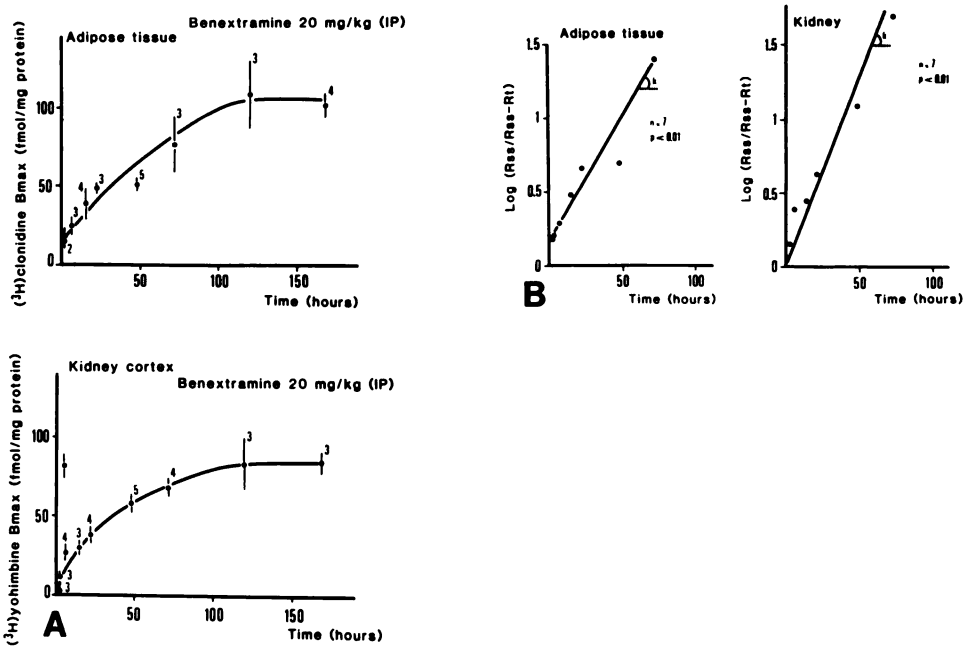

Fig. 2. Time course of α_2-adrenoceptor reappearance in kidney and adipose tissue after their irreversible blockade with benextramine. A. Maximal [^{3}H]CLO (adipocytes) and [^{3}H]YOH (kidney) binding. (R_t) values were determined by Scatchard analysis at different time intervals after intraperitoneal (*IP*) administration of benextramine. Values are means ± standard errors of separate experiments indicated by the *numbers*. B. Semilogarithmic plot of the time course of α_2-adrenoceptor recovery. [*Rss*] is the steady state receptor concentration. The slope of the line gives the rate constant for degradation of the receptor (k).

ligand were used (an agonist and an antagonist), the profile of the time-dependent recovery is very similar in both tissues. The logarithmic plot of the time course, represented by a straight line, confirms that the kinetics are, as hypothesized, a monoexponential process. The slopes of the plots give an estimate of the respective receptor degradation rate constants (k) in both tissues. Their values are $k = 0.015$ hr^{-1} in adipose tissue and $k = 0.022$ hr^{-1} in the kidney.

Substituting these values of k into Eq. 1, when t approaches infinite time and [Rt] tends to [Rss] allows the receptor production rate (r) to be calculated. It was 1.5 fmol/mg of protein/hr in adipose tissue and 1.8 fmol/mg of protein/hr in the kidney. The half-lives of the receptor were 46.2 and 31.2 hr, respectively in the tissues; these values were calculated by substitution of k into Eq. 2.

Evolution of the lipolytic responses during benextramine treatment and receptor recovery. Benextramine treatment promoted an important reduction of [^{3}H]CLO-binding sites in the fat cell. Concerning the lipolytic activity of fat cells obtained 1 or 2 hr after benextramine injections, there were no differences in the basal lipolytic rates (in the absence of lipolytic or antilipolytic agents). The slight differences observed during the recovery phase are probably linked to a circadian rhythm of cell activity (Table 5). Lipolytic responses were tested over a large range of isoproterenol concentrations (in the presence of 0.1 mM ascorbic acid). Benextramine treatment did not modify the effectiveness of isoproterenol in stimulating lipolysis during the recovery of the α_2-sites, and the response to isoproterenol was not modified; the stimulation over basal activity was equivalent in the different batches of hamsters (Table 5).

The efficiency of phenylisopropyladenosine to promote antilipolytic effects through A1-site stimulation was also unchanged. To test the antilipolytic effects, ADA (2 μg/ml) was included in the incubation flasks to inactivate the adenosine released in the incubation buffer; glycerol release rose in the presence of ADA (7). Because the study of receptor regulation is more meaningful if the changes in the binding parameters and the function of these receptors can be evaluated simultaneously, the capacity of clonidine and a new α_2-agonist (UK-14,304) (28) to elicit antilipolytic effects through α_2-adrenergic receptor stimulation was studied on isolated fat cells at various periods after benextramine administration. Clonidine and UK-14,304 exerted antilipolytic effects in fat cells incubated in the presence of ADA. Although there is some correlation between B_{max} values for α_2-sites and the extent of the antilipolytic effect initiated by the compounds (Fig. 3), a careful analysis of the time course of recovery of [^{3}H]CLO sites and of the responses revealed that the small number of sites being left (1 or 2 hr after benextramine treatment) is sufficient for clonidine to

TABLE 5

Lipolytic responsiveness of isolated fat cells during the recovery period after a single injection of benextramine (20 mg/kg)

Isolated fat cells from hamsters undergoing recovery of benextramine injection were incubated in Krebs-Ringer bicarbonate buffer as described in Materials and Methods. Basal lipolysis was determined from spontaneous glycerol release of isolated fat cells over a period of 90 min. Isoproterenol (10^{-6} M) (Iso.) was used for maximal stimulation of fat cell lipolysis. The lipolytic mixture ADA + Theoph., i.e., adenosine deaminase (2 μg/ml) associated with theophylline (0.5 mM), acts through adenosine removal, blockade of A1-adenosine receptors, and inhibition of cAMP-dependent phosphodiesterase. Benextramine treatment was without major incidence on the lipolytic responsiveness of the fat cells whatever the lipolytic procedure. Values are means ± standard errors.

	Glycerol release (μmol/100 mg lipid/90 min)				
	Controls	2 hr	22 hr	72 hr	120 hr
Basal	0.186 ± 0.02	0.110 ± 0.01	0.170 ± 0.01	0.147 ± 0.01	0.180 ± 0.01
Iso.	2.340 ± 0.28	1.800 ± 0.20	1.750 ± 0.12	2.800 ± 0.20	2.480 ± 0.10
ADA + Theoph.	1.950 ± 0.21	1.500 ± 0.16	1.790 ± 0.05	2.760 ± 0.26	2.300 ± 0.10

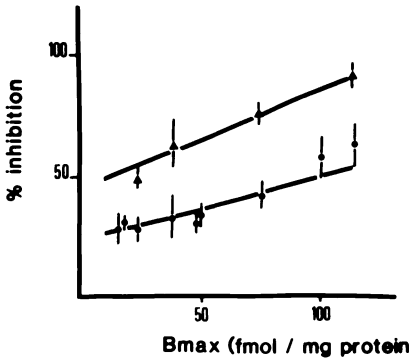

Fig. 3. Relationship between [^{3}H]CLO binding and the antilipolytic effects of clonidine (●) and UK-14,304 (▲). The relationship between total binding capacity (B_{max}) in membranes and maximal antilipolytic effects initiated by clonidine (10^{-5} M) or UK-14,304 (10^{-5} M) was analyzed in isolated fat cells. The antilipolytic effect of clonidine is expressed as the per cent inhibition of glycerol release measured in the presence of 2 μg/ml ADA associated with 0.5 mM theophylline.

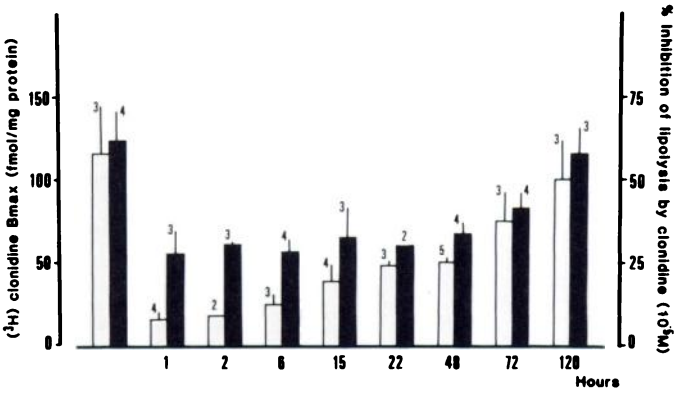

Fig. 4. Recovery of [^{3}H]CLO-binding sites and antilipolytic effect of clonidine after varying time intervals following the blockade of α_2-binding sites by benextramine (intraperitoneally, 20 mg/kg). The total binding capacity was determined by Scatchard analysis as shown in Fig. 1. The antilipolytic effect of clonidine is expressed as the per cent inhibition of stimulated lipolysis (2 μg/ml ADA + 0.5 mM theophylline). Values are means ± standard errors of separate experiments indicated by the *numbers*.

exert a significant antilipolytic action. Occupancy of this small fraction of α_2-sites promotes a significant inhibition of lipolysis. Thus, the correlation observed at a higher number of sites (Fig. 3) is not true for the lowest number of receptors (Fig. 4); occupancy of approximately 15% of the α_2-sites by clonidine elicits 40% inhibition of lipolysis.

Moreover, these experiments also reveal that UK-14,304 is a better agonist than clonidine in fat cells. Atlhough clonidine possesses noticeable α_2-agonist potencies on fat cells, it probably behaves as a partial agonist as described in various other tissues. To conclude, benextramine treatment did not strikingly modify the lipolytic capacities of fat cells or their hormonal responses, but the α_2-component of catecholamine action is specifically affected.

Discussion

The present study demonstrates that benextramine (15, 29, 30), which has a high affinity for the sites, is a valuable irreversible ligand for fat cell and kidney α_2-adrenergic receptors (Table 2). The rate of reappearance of α_2-sites was determined in both tissues after intraperitoneal benextramine administration (20 mg/kg, 1 or 2 hr) and the kinetic parameters of receptor turnover in kidney and adipose tissue were defined.

Radioligand-binding studies have allowed characterization of α_2-adrenergic receptors in the fat cells (17, 18, 22) and kidney of various species (19). Fat cell α_2-adrenergic receptor stimulation promotes inhibition of the lipolytic activity of the adipocytes (7, 16–18). In the kidney the receptors are involved in the control of renin release and in the control of electrolyte and fluid transport; some aspects of their action require further study before being fully established (31–34).

The α_2-site number presents large variations between tissues and could be altered in various physiological or pathological conditions (35–38). In the kidney, hypertensive rats have an increased number of α_2-sites (39); moreover, in rat strains sensitive to salt ingestion, the number of α_2-sites increases during a diet enriched in sodium (38–40). In adipose tissue, there is an increase in α_2-adrenergic responsiveness in large fat cells from obese or aging animals; also, α_2-sites are distributed in a heterogeneous manner in various fat deposits (17, 18). All of these data suggest a specific regulation of this family of receptors which seems to possess regulating processes different from those described for β- and α_1-sites as shown by ontogenesis or desensitization studies (3, 6, 7, 41). A better knowledge of their turnover rate in adipose tissue and kidney should bring valuable information about their characteristics in two tissues differing strikingly in function and innervation. The rat and hamster kidneys are densely innervated by sympathetic nerve endings, whereas the fat deposits are less innervated; 2–5% of white adipocytes are considered to be directly innervated in rat adipose tissue (42), but no data are available for hamster adipocytes.

The method selected for the study of the *in vivo* turnover of α_2-adrenergic receptors is based on the induction of an irreversible and rapid blockade of the sites followed by the measurement of their rate of reappearance. This strategy has been used by various investigators for the analysis of α_1-adrenoceptor turnover in cell cultures or *in vivo* (8–14). For this purpose, the authors used the haloalkylamine, phenoxybenzamine, which is a well known α_1-antagonist possessing some α_2-blocking properties. However, this compound presents some deserving properties including its poor water solubility associated with a high lipid solubility and various blocking properties toward 5-hydroxytryptamine, H1, or muscarinic receptors (15, 30). Moreover, it is known to easily cross the blood-brain barrier in rats (11, 13). Since this compound can be trapped in cell membranes or in adipose tissue, affecting its delivery after a single administration, we tested another recently described irreversible α-antagonist drug: benextramine. It is a water-soluble tetramine disulfide with poor lipid solubility. It possesses irreversible α_1/α_2-adrenoceptor antagonist properties and is able to act on α_1-adrenoceptors like phenoxybenzamine (15, 43). Recently, benextramine was shown to block vascular α_2-sites as well as brain α_2-binding sites after intracisternal administration in rabbits (44).

In the first part of our investigation we clearly demonstrated the affinity of benextramine for α_2-sites (Table 2) and that it is a strong irreversible ligand for α_2-sites on membranes from fat cells, kidney, and brain (Table 3) when it is added directly to membrane fractions *in vitro*. When administered intraperitoneally, benextramine promotes a strong reduction of α_2-binding sites in fat cells and kidney with only a minor impact

on brain α_2-sites (Fig. 1, Table 3). These data suggest that, under our working conditions and in this animal model, the compound, which has a lower affinity for brain α_2-sites (Table 2), does not cross the blood-brain barrier, differing from phenoxybenzamine which freely crosses the blood-brain barrier in the rat (11). It is an interesting property which might be remembered for further *in vivo* investigations.

Moreover, benextramine seems to be interesting for *in vivo* studies since it has no effect on β-adrenergic receptor or adenosine (A1-receptor) responsiveness in fat cells recovered 1 to 2 hr after benextramine injection (Table 5). Furthermore, its incubation *in vitro* for 30 min with freshly isolated fat cells promotes the complete disappearance of [^{3}H]CLO sites and of α_2-adrenergic responsiveness without affecting [^{3}H]DHA or [^{3}H]PIA binding or even β-adrenergic or adenosine responsiveness. From this part of our investigations benextramine seems to be a valuable irreversible antagonist for further studies on α_2-adrenergic receptor characteristics and metabolism. It seems to be more suitable than phenoxybenzamine (13) and EEDQ used previously in turnover studies of α_2-sites in vascular bed and brain, respectively (14).

The receptor recovery curves were analyzed according to the receptor repopulation model previously described for β- and α_1-adrenergic receptors (8–11). The various assumptions supporting this kind of repopulation kinetics model used here have not been verified for the tissues under consideration and for the α_2-adrenergic receptor as this is a very difficult task. A putative effect of benextramine on metabolism after penetration into the cells leading to modification of either the rates of synthesis or degradation cannot be fully excluded at present. Recent investigations in human erythroleukemia (HEL) cells have shown that benextramine can penetrate the cells only at very high concentrations (>mM).[1] Under our working conditions, the highest theorical plasma concentration of the compound reached after its i. p. administration did not exceed 4–6 μM.

The linear relationship found by analysis of recovery time (correlation coefficient-0.96 for fat cells and 0.99 for kidney) suggests that Eq. 1 represents a valid approximation of the α_2-repopulation kinetics in both tissues after benextramine blockade. Receptor recovery is faster in the kidney than in adipose tissue (Fig. 3). Several arguments can be considered to explain such an observation. Differential rates of clearance or metabolism, should they take place, might partly account for the differences in receptor recovery. Benextramine treatment certainly has vascular impacts (alteration of α_1- and α_2-adrenergic receptors of blood vessels) (43, 44), affecting tissue draining and benextramine bioavailibility in the two tissues. Moreover, the slower turnover rate of α_2-sites observed in the adipose tissue may reflect the existence of a lower degree of plasticity and protein synthesis in this tissue as compared with the kidney.

The half-life of α_2-adrenergic receptors is very close to that of α_1-adrenoceptors in submaxillary glands or BC_3H1 cells (33 and 23 hr, respectively) (8, 11) and faster than that defined during repopulation of α_2-adrenergic receptors in rat cerebral cortex after their blockade by EEDQ (99 hr) (14). We recently found that the half-life of α_2-adrenergic receptors is about 30 hr in the human colonic adenocarcinoma cell line HT29.[2] In the absence of more numerous investigations, it is still difficult to determine whether the variable half-lives of adrenergic receptors in different tissues are really linked to tissue variations in receptor metabolism or to some other problem involving the trapping or the liberation of the irreversible ligand used for the blockade.

Finally, concerning the relationships between receptor reappearance and recovery of biological responsiveness, these experiments indicate that a relationship exists between the density of [^{3}H]CLO-binding sites and the ability of fat cells to respond to α_2-agonists (Fig. 3). When all of the sites are permanently blocked after incubation of isolated fat cells with benextramine (10^{-5} or 10^{-4} M), the α_2-mediated antilipolysis initiated by clonidine is totally suppressed. This result fits with that obtained previously in very young hamsters, in which the absence of [^{3}H]CLO binding is well correlated with the absence of any α_2- effect (18). The present study focuses on the disproportion between the number of sites and the corresponding antilipolytic effects under conditions associated with the existence of a lower number of binding sites. The analysis of correlations between receptor number and antilipolysis in the early moments following benextramine treatment (Fig. 4) reveals that the remaining 15% of [^{3}H]CLO sites (1 or 2 hr after benextramine injection) is sufficient to elicit a 40% inhibition of lipolysis. However, at 15–22 hr, receptors have recovered to approximately 40% of their initial number, without great improvement of functional efficacy. Such a result suggests a more complex mechanism of receptor-effector coupling and the possible existence of a receptor reserve in fat cells as shown in human platelets (45). A more detailed delineation of receptor-effector coupling requires *in vitro* approaches which are considered to be out of the scope of the present paper. The idea of the existence of an α_2-adrenergic receptor reserve in fat cells also arose from desensitization studies showing that modifications of [^{3}H]CLO binding site number do not correlate with α_2-receptor-mediated responses in hamster fat cells (7).

To conclude, benextramine seems to be a useful tool for further investigations into α_2-adrenergic receptor turnover. In this field of research, an irreversible antagonist is still necessary since the technique, based on the appearance and disappearance of down-regulated sites (5) recently described for β-sites, is questionable for this family of receptors showing some resistance to desensitization (6, 7). This tool has permitted us to determine the turnover rate of α_2-sites in adipose tissue and kidney, two tissues where the existence and function of extrajunctional α_2-adrenergic receptor sites are not questioned, although their physiological function(s) have yet to be described in more detail.

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