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Selective Blockade and Recovery of Cell Surface α_2-Adrenergic Receptors in Human Erythroleukemia (HEL) Cells. Studies with the Irreversible Antagonist Benextramine

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SUMMARY

α_2-Adrenergic receptors are present on human erythroleukemia (HEL) cells, both on the cell surface and in a sequestered compartment. In the current study we show that benextramine, a hydrophilic irreversible antagonist, can be used to investigate α_2-adrenergic receptor compartmentation in these cells. In membranes prepared from HEL cells, benextramine competed for all α_2-adrenergic receptors ([^{3}H]yohimbine sites). In intact cells, at 4°, benextramine exhibited a biphasic competition curve for α_2-adrenergic receptors, with EC_{50} values of ~10 μM and >1 mM for the high and low affinity components, respectively. We propose that the α_2-adrenergic receptors preferentially blocked by benextramine are those on the surface of the cell, whereas those with low affinity are sequestered receptors because: 1) only epinephrine-accessible sites [i.e., cell surface sites; McKernan *et al.*, *Mol. Pharmacol.* 32:258–265 (1987)] are removed by prior treatment of cells with benextramine, 2) a preparation enriched with surface membranes is also enriched in receptors with a high affinity for benextramine; and 3) after blockade of cell surface receptors (54 ± 6% of total sites, n = 7) by benextramine, the ability of the α_2-adrenergic agonists epinephrine and UK-14,304 to inhibit forskolin-stimulated cAMP accumulation is lost. The latter result implies that only cell surface and not sequestered receptors are functionally coupled to adenylate cyclase. The return of receptors from the sequestered compartment to the cell surface and the recovery of α_2-adrenergic receptor function were measured after HEL cells were treated with benextramine (50 μM for 1 hr at 4°). The recovery of receptor binding ($t_{1/2}$ = 25 min) was somewhat slower than the recovery of function ($t_{1/2}$ ~8 min). This is consistent with the existence of "spare receptors" and also suggests that the sequestered compartment of α_2-adrenergic receptors can rapidly exchange with those on the surface. When all α_2-adrenergic receptors were blocked by incubation of HEL cells with benextramine for 1 hr at 37°, repopulation of surface and sequestered receptors was much slower ($t_{1/2}$ = 9 hr for recovery of total receptors). Surface receptors recovered even more slowly than did total cellular receptors, consistent with the idea that α_2-adrenergic receptors must traverse through intracellular locations before insertion into the cell surface. Taken together, these data indicate the utility of a hydrophilic drug like benextramine as a novel approach for the study of compartmentation of receptors in intact cells. In addition, these results illustrate the dynamic nature of α_2-adrenergic receptors in HEL cells.

Understanding of the molecular properties of drug receptors has been greatly aided by the use of radioligand binding studies with target cell membranes (1–3). However, in the preparation of cell membrane fractions the normal distribution of receptors in subcellular compartments is lost. It is therefore desirable to carry out studies in intact cells in which the subcellular compartmentation of receptors is maintained. Use of intact cells can allow the examination of receptors under physiological conditions and can facilitate the study of receptor translocation from one compartment to another. A further advantage of using intact cells is that the plasma membrane presents a selective barrier to certain drugs; thus, intracellular and cell surface receptors should be available to lipophilic compounds, whereas very hydrophilic compounds should preferentially identify receptors on the cell surface (4, 5).

Studies of β-adrenergic and α_1-adrenergic receptors have provided evidence for compartmentation and agonist-mediated redistribution of adrenergic receptors (e.g., Refs. 4–10). Information about the cellular compartmentation of the other class of adrenergic receptors, α_2-adrenergic receptors, is much more limited.

We have recently described the use of HEL cells as a model for studying α_2-adrenergic receptors in intact cells (11). In our

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ABBREVIATIONS: HEL, human erythroleukemia; EDTA, ethylenediaminetetraacetic acid; UK-14,304, 5-bromo-6-[2-imidazolin-2-yl-amino]quinoxaline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RPMI, Roswell Park Memorial Institute (media); RPMIH, RPMI media + 25 mM HEPES, pH 7.3; EC_{50}, the concentration producing 50% of maximal effect.

initial studies we carried out experiments with assays conducted at 4°, and used agonists of different hydrophilicity to demonstrate that α_2-adrenergic receptors exist in both surface and intracellular compartments in these cells. We have now extended these findings and have tested a unique approach for the study of cell surface receptors. We reasoned that the drug benextramine might provide another means to investigate cell surface and sequestered compartments of α_2-adrenergic receptors. Benextramine is a hydrophilic and irreversible antagonist at α_2-adrenergic receptors (12, 13). It has been used as a tool to measure the rate of α_2-adrenergic receptor synthesis *in vitro* in HT-29 cells (14) and also *in vivo* in studies using hamster and rabbit tissues (15, 16). In experiments presented here we describe selective blockade of cell surface receptors by treatment of cells with benextramine at 4°, and the subsequent translocation of receptors from the sequestered compartment to the surface of the cell.

Experimental Procedures

Materials. HEL cells were obtained from T. Papayannopoulou, Department of Medicine, University of Washington (Seattle, WA). RPMI culture medium, glutamine, and fetal bovine serum were obtained from Gibco Laboratories (Grand Island, NY). [^{3}H]Yohimbine was from New England Nuclear (Boston, MA). Benextramine was purchased from Aldrich Chemical Co. Idazoxan and UK-14,304 were gifts from Prof. P. S. Sever, St. Mary's Hospital Medical School (London, England). Phentolamine was a gift from Ciba-Geigy Corp. (Summit, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Determination of lipophilicity. In order to have an indication of the relative distribution of the adrenergic drugs between the incubation buffer and the cell membrane, we determined the lipophilicity (octanol/buffer ratio) of all α_2-adrenergic agents used, under the conditions employed in our experiments. Drugs were dissolved in water and diluted in buffer (50 mM Tris, 1 mM EDTA, 120 mM NaCl, pH 7.5) to a final concentration of 100 μM. Two ml of the drug solution were mixed on a rotary wheel for 2 hr at 4° with 2 ml of water-saturated *n*-octanol. The absorbance of the aqueous phase before and after partitioning into octanol was measured at A_{280}. The buffer/octanol ratios of the adrenergic drugs used in these experiments are shown in Table 1. Benextramine and epinephrine are both very hydrophilic, having H_2O/octanol ratios of greater than 5, whereas yohimbine is the most lipophilic compound, with an H_2O/octanol ratio of 0.048.

Tissue culture of HEL cells. HEL cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal bovine serum as previously described (11). Cell viability was assessed by trypan blue exclusion at the end of each experiment and was always greater than 90%. In experiments using benextramine, cells were first removed from serum by centrifugation for 5 min at 170 × *g*, followed by one 50-ml wash in RPMI medium containing 25 mM HEPES buffer, pH 7.3. All subsequent procedures with intact cells (except radioligand binding) were carried out in this buffer. After incubation of intact HEL cells with benextramine, the cells were washed three times at 4° by centrifugation and then resuspended in 50 ml of RPMIH to remove unbound benextramine. Cells were finally resuspended in buffer (50 mM Tris, 1 mM EDTA, 120 mM NaCl, pH 7.5) prior to assay of radioligand binding or measurement of cAMP accumulation.

TABLE 1

Lipophilicity of adrenergic drugs used in this study

The lipophilicity index is calculated from the distribution of the drug between H_2O and *n*-octanol, as described under Experimental Procedures. Values represent the means of two separate determinations with less than 10% difference between them.

Compound	H_2O/octanol ratio
Yohimbine	0.048
Phentolamine	0.11
Idazoxan	0.55
UK-14,304	0.75
Benextramine	5.6
Epinephrine	6.5

Radioligand binding studies. Binding studies were carried out using intact HEL cells or HEL membranes. Membranes were prepared from cells washed twice with 50 mM Tris, 5 mM EDTA, 120 mM NaCl, pH 7.5, followed by homogenization in 50 mM Tris, 0.5 mM EDTA, pH 7.5 with three 5-sec bursts with an Ultra-Turrax homogenizer. Membranes were pelletted by centrifugation at 45,000 × *g* for 20 min, and were washed twice with 50 mM Tris-HCl, 5 mM EDTA, pH 7.5. All procedures were carried out at 4°. Binding was performed in a total volume of 200 μl at 25° for 1 hr. For studies with intact cells, cells were incubated with [^{3}H]yohimbine (0.4–25 nM) for 5 hr at 4° in a total volume of 200 μl. Nonspecific binding was defined as binding in the presence of phentolamine (10 μM). Cell surface receptor binding was defined as total binding minus binding at 4° in the presence of 1 mM epinephrine (11). Incubations were terminated by filtration through Whatman GF/C filters using a Brandel Cell Harvester. Filters were washed twice with 10 ml of ice-cold buffer (50 mM Tris, 1 mM EDTA, 120 mM NaCl, pH 7.5). Radioactivity retained on the filters was determined by liquid scintillation spectrometry. Proteins were assayed according to the method of Lowry *et al.* (17).

cAMP accumulation. HEL cells were washed twice and resuspended at a density of 10^6 cells/ml in RPMIH. cAMP formation was stimulated by the addition of 20 μM forskolin and α_2-adrenergic receptor-mediated inhibition by the addition of epinephrine or UK-14,304 as indicated. cAMP production was allowed to continue for 5 min. Incubations were terminated by centrifuging the cells, removing the media, adding 50 mM sodium acetate, 0.2 mM 3-isobutyl-1-methylxanthine (pH 4.0), and immediately boiling the material for 3–5 min. cAMP was assayed in aliquots of the boiled sample using a competitive binding assay (11). Results were expressed as pmol of cAMP generated/10^7 cells.

Data analysis. Data are presented as mean ± standard error. K_i values were calculated by nonlinear regression of competition binding curves, using the program GraphPAD (Institute for Scientific Information). Tests of significance were calculated by two-tailed paired *t* test.

Results

Use of benextramine to block α_2-adrenergic receptors in HEL cells. The ability of benextramine to compete for α_2-adrenergic receptors on intact HEL cells and on HEL cell membranes was investigated initially. When added directly to binding assays, benextramine competed for [^{3}H]yohimbine binding at α_2-adrenergic receptors (data not shown; see Refs. 11, 15, and 16). When HEL cells or HEL cell membranes were incubated with benextramine for 1 hr at 4° and washed three times by centrifugation and resuspension, the preincubation with benextramine prevented subsequent binding of [^{3}H]yohimbine to α_2-adrenergic receptors in a dose-dependent manner (Fig. 1). The EC_{50} for this effect in membranes was 2.4 ± 0.6 μM (n = 3). The extent of competition and the EC_{50} were the same independent of whether epinephrine (1 mM) or phentolamine (10 μM) was used to define specific binding. When benextramine was preincubated with intact cells for 1 hr at 4° in an analogous manner, the interaction with α_2-adrenergic receptors was more complex (Fig. 1). If specific binding was defined by 10 μM phentolamine, benextramine distinguished between two populations of sites, one with an EC_{50} of 9.2 ± 2 μM and the other with an EC_{50} of >1 mM.

This biphasic competition curve for benextramine and its

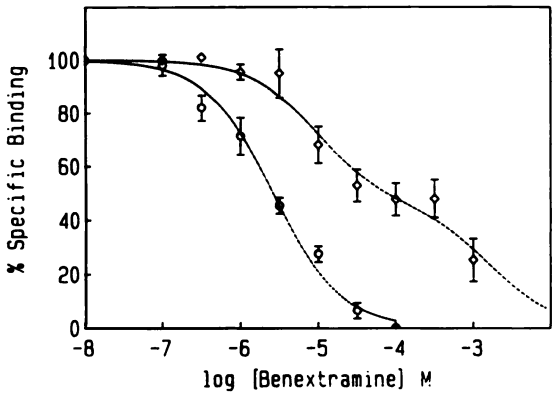

Fig. 1. Blockade of [^{3}H]yohimbine (8 nM) binding to intact cells and membranes by benextramine. Intact cells (◇) or membranes (○) were incubated with benextramine at various concentrations for 1 hr at 4°. Cells or membranes were then washed three times and binding of [^{3}H] yohimbine (8 nM) was carried out for 5 hr at 4°. Data represent the mean ± standard error of four separate experiments.

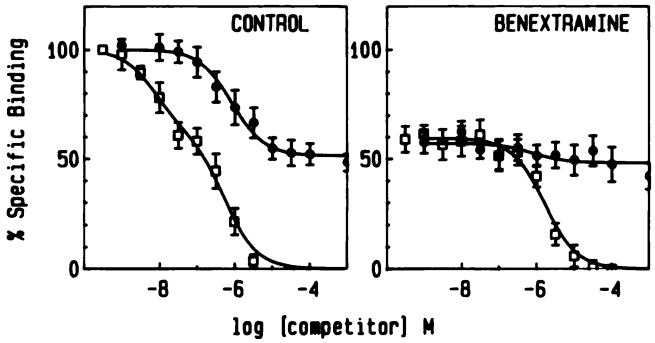

Fig. 2. Competition for [^{3}H]yohimbine binding after blockade of α_2-adrenergic receptors with 50 μM benextramine for 1 hr at 4°. Cells were washed three times before competition with phentolamine (□) and epinephrine (●) in intact control cells (A) or in cells that had been previously treated with benextramine (B). [^{3}H]Yohimbine binding was conducted for 5 hr at 4°, and the data shown represent the mean ± standard error of four experiments.

high hydrophilicity suggested that it might selectively block α_2-adrenergic receptors on the surface of HEL cells. To test this possibility, further experiments were carried out in which intact cells were treated at 4° for 1 hr with 50 μM benextramine, a concentration demonstrated in Fig. 1 to abolish selectively binding to the high affinity site, while leaving the low affinity site unaffected. We compared the ability of phentolamine and epinephrine to compete for [^{3}H]yohimbine binding in cells previously treated with benextramine at 4° (Fig. 2). Phentolamine, being relatively lipophilic (Table 1), is able to compete for all α_2-adrenergic receptors in HEL cells, whereas epinephrine, being more hydrophilic, would be expected to identify selectively only those sites on the cell surface (11). Therefore, as shown in Fig. 2A, approximately 48% of [^{3}H]yohimbine-binding sites were not competed for by epinephrine at 4°. We consider these to be receptors that are sequestered in a compartment away from the cell surface. After treatment of cells with benextramine at 4°, 52% of the receptors identified by competition with phenetolamine were not detectable, whereas 100% of those identified by epinephrine were removed. These results support the idea that benextramine (50 μM for 1 hr at 4°) selectively blocks α_2-adrenergic receptors on the surface of the HEL cells.

In intact HEL cells at both 4° and 37°, [^{3}H]yohimbine identifies a single population of sites (11), and after pretreatment of intact cells with 50 μM benextramine for 1 hr at 4°, the maximum number of binding sites was reduced by 54 ± 6% (n = 7) without a change in affinity for [^{3}H]yohimbine in either intact cells or membranes (Table 2, Fig. 3). By contrast, when intact cells were treated with 50 μM benextramine for 1 hr at 37°, no detectable α_2-adrenergic receptor-binding sites remained (Table 2). Thus, at 37°, treatment with benextramine appears to block all α_2-adrenergic receptors, whereas at 4°, benextramine appears to block preferentially α_2-adrenergic receptors on the surface of the cell. The similarity in K_d values, associated with the loss in B_{max}, indicates that benextramine is a noncompetitive antagonist of [^{3}H]yohimbine binding in these cells.

We investigated whether blockade of α_2-adrenergic receptor by benextramine was due to a direct interaction with the receptor-binding protein. If benextramine acted at, or near, the ligand-binding site of the receptor, then a reversible antagonist should protect the receptor from blockade by benextramine. As shown in Fig. 3, the α_2-adrenergic receptor antagonist phentolamine (100 μM) substantially prevented blockade of the α_2-adrenergic receptor sites by benextramine, consistent with an interaction of benextramine in the region of the receptor-binding site.

The effect of benextramine treatment on α_2-adrenergic receptors in HEL cell membranes. In several other cell types (e.g., Refs. 6, 7, 10, and 18–21), sequestered β- and α_1-adrenergic receptors have been identified in membrane fractions with properties distinct from those of the plasma mem-

TABLE 2

The effect of benextramine treatment of HEL cells on B_{max} and K_d of [^{3}H]yohimbine binding to intact cells

Treatment	B_{max}	K_d	Percentage of receptors blocked
Untreated	9380 ± 1060	4.2 ± 0.47	
Benextramine, 50 μM, 1 hr at 4°	4933 ± 909	4.4 ± 0.50	54 ± 6 (n = 7)
Benextramine, 50 μM 1 hr at 37°	No binding detectable		100 (n = 3)

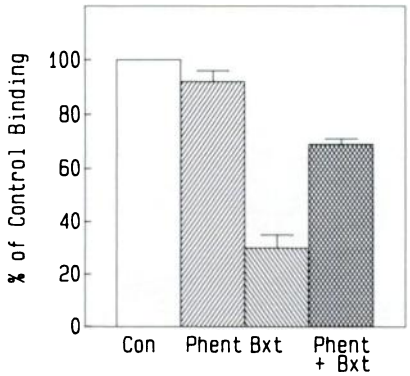

Fig. 3. α_2-Adrenergic receptors are partially protected from benextramine by prior incubation with phentolamine. Cells were incubated at room temperature for 30 min with phentolamine (100 μM), cooled to 4°, and incubated for a further 1 hr with benextramine (50 μM). Cells were washed eight times at room temperature to remove the drugs and [^{3}H] yohimbine binding was subsequently performed at 4°. Nonspecific binding was defined with 10 μM phentolamine. Data shown are for control cells (*Con*), cells treated with phentolamine (*Phent*) alone, benextramine (*Bxt*) alone, or phentolamine and benextramine (*Phent* + *Bxt*) and are the mean ± standard error of three experiments.

brane. We therefore examined whether the sites preferentially blocked by benextramine were associated with the surface membrane. We prepared a crude membrane fraction (described under Experimental Procedures), as is typically used in receptor binding studies, from untreated HEL cells, and from cells which had been previously incubated with benextramine. Approximately 60–70% of the total receptor population was recovered in the membrane preparation. Based on data from other cell types, e.g., DDT_1 smooth muscle cells (10), guinea pig heart (19) frog erythrocytes (18), 1321N1 astrocytoma cells (20), and S49 cells (21), receptors that were not recovered were presumably present in light membrane vesicles, perhaps in intracellular compartments that are not isolated in the preparation of crude membranes. After treatment of intact HEL cells at 4° with benextramine, 54 ± 6% ($n = 7$) of receptors were irreversibly blocked on intact cells (Table 2) whereas, when membranes were prepared from similarly treated cells, 80 ± 5% ($n = 4$) of the α_2-adrenergic receptors were blocked (Fig. 4, *inset*). These indirect data suggest that a preparation that is likely to be enriched for surface membranes is also enriched for α_2-adrenergic receptors which are sensitive to benextramine treatment at 4°.

Benextramine treatment and cAMP accumulation. We investigated whether the population of α_2-adrenergic receptors which was selectively blocked by benextramine was functionally coupled to adenylate cyclase (Fig. 5). The accumulation of cAMP in HEL cells can be inhibited by epinephrine and UK-14,304 with similar efficacy (11). We exploited the differences in lipophilicity of UK-14,304 and epinephrine to access (and, therefore, presumably stimulate) either all α_2-adrenergic receptors (stimulation by UK-14,304) or those on the cell surface only (stimulation by epinephrine, Ref. 11). Cells were preincubated in the presence or absence of benextramine for 1 hr at 4° and were then washed several times at 4° to remove unbound benextramine. cAMP production was stimulated by the addition of forskolin, and the inhibition of cAMP production was assessed by the addition of epinephrine or UK-14,304. In control cells, not treated with benextramine, both agonists inhibited cAMP production to a similar extent (42% inhibition for

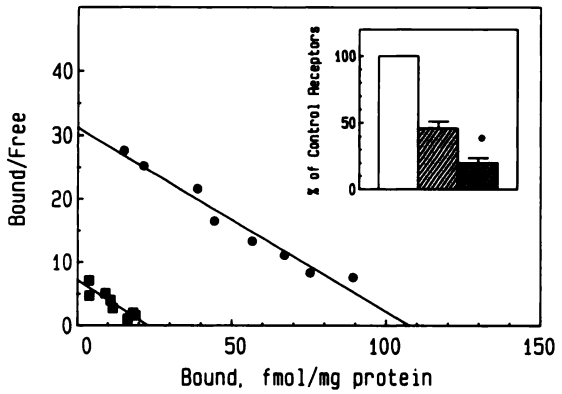

Fig. 4. Scatchard analysis of [^{3}H]yohimbine binding to membranes prepared from untreated (●) HEL cells and HEL cells which were treated with benextramine (50 μM) for 1 hr at 4° (■). Data shown are representative of four experiments performed in membranes. The *inset* shows a comparison of B_{max} determinations of [^{3}H]yohimbine binding in intact HEL cells (▨, $n = 7$) and binding in membranes prepared from intact cells which had been previously treated with benextramine for 1 hr at 4° (■, $n = 4$) compared with control binding in control cells or membranes (□).

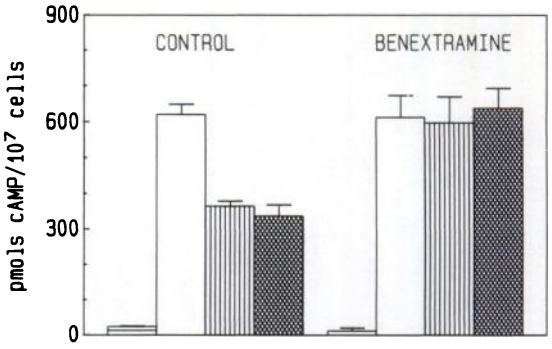

Fig. 5. The effect of pretreatment with benextramine on the ability of epinephrine and UK-14,304 to inhibit cAMP accumulation in HEL cells. Cells were incubated with 50 μM benextramine for 1 hr at 4°, then washed, and assayed for cAMP accumulation in response to a 5-min incubation with no addition (■), or in the presence of 20 μM forskolin (□), 20 μM forskolin and 1 μM epinephrine (■), or 20 μM forskolin and 1 μM UK-14,304 (■). Data shown are representative of three similar experiments.

epinephrine versus 46% for UK-14,304). In cells that were treated with benextramine, the stimulation elicited by forskolin was unchanged, but neither agonist was able to inhibit cAMP accumulation. Since UK-14,304 can bind to all α_2-adrenergic receptors in HEL cells assayed at 4° (11), we presume that during a 5-min incubation at 37° this hydrophobic agonist should be able to penetrate the cell and bind to sequestered receptors. We interpret the inability of UK-14,304 to inhibit cAMP accumulation in benextramine-treated cells as evidence that only surface receptors and not sequestered α_2-adrenergic receptors in HEL cells are functionally coupled to adenylate cyclase.

Translocation of α_2-adrenergic receptors between compartments. We next tested whether sequestered receptors could translocate to the surface of the cell, and whether recovery of binding after benextramine treatment would parallel recovery of the functional response at α_2-adrenergic receptors. Surface α_2-adrenergic receptors were blocked with benextramine; then, the cells were washed at 4° and then transferred into RPMIH medium at 37°. Cells were removed at various time intervals thereafter, and the number of receptors in the cell surface and sequestered compartments was measured by binding of [^{3}H]yohimbine at 4° in the presence of 1 mM epinephrine ("surface sites") or 10 μM phentolamine ("total sites"). The ability of the receptor to inhibit forskolin-stimulated cAMP accumulation was also assessed. The results of these experiments are shown in Fig. 6. After blockade of surface α_2-adrenergic receptors by benextramine, the receptors in the sequestered compartment were able to translocate to the surface of the cell. The number of "total sites" detectable with [^{3}H] yohimbine was unchanged throughout the experiment, indicating that the recovery of "surface sites" was not attributable to a release of benextramine from the receptors. Recovery of surface sites was maximal after 60 min. In these experiments the sequestered receptors constituted 52–63% of the total cellular population of α_2-adrenergic receptors. Thus, blockade of surface sites by benextramine would not be expected to allow the entire population of cellular receptors to recover over a short period of time. Restoration of surface α_2-adrenergic receptors occurred with a $t_{1/2}$ of 25 min. The recovery of α_2-adrenergic receptor function occurred more rapidly, with a $t_{1/2}$ of approximately 8 min, full response being restored within 15

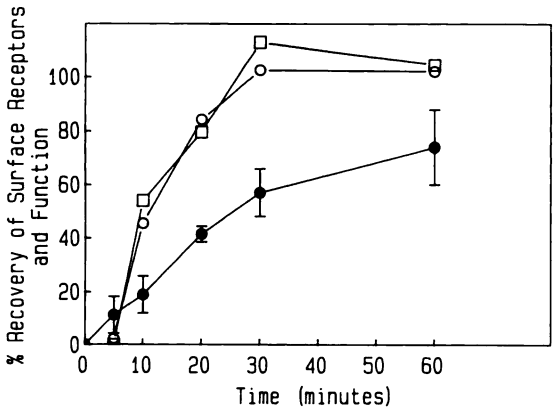

Fig. 6. Repopulation of surface α_2-adrenergic receptors and of α_2-adrenergic receptor function after benextramine treatment for 1 hr at 4°. Intact HEL cells were incubated with 50 μM benextramine at 4°, then washed three times at 4° and transferred to RPMIH at 37°. Aliquots were removed at various times, as indicated, and rapidly cooled to 4°. Total and surface (●) receptors were measured by assay with 8 nM [^{3}H] yohimbine (at 4° for 5 hr), and the inhibition of forskolin-stimulated cAMP accumulation by 1 μM epinephrine (□) or 1 μM UK-14,304 (○) for 5 min at 37° was determined at each time point. In parallel experiments, cells that had not been exposed to benextramine exhibited no difference in receptor distribution as a result of the experimental protocol. It was not possible to determine the inhibition of cAMP accumulation at t = 0 because the stimulation of cAMP continued for 5 min. Data shown for the inhibition of cAMP are the means of two experiments, differing by less than 8%. The receptor binding data are the mean ± standard error of three experiments where surface receptors were 2900–4100 receptors/cell.

min. This discrepancy between the rates of recovery of binding and function will be discussed below.

When HEL cells were treated with 50 μM benextramine at 37° for 1 hr, receptors in both cell surface and sequestered compartments were completely blocked, as shown in Table 2. We therefore also examined the rate of recovery from receptor blockade after benextramine treatment at 37°. We assessed the repopulation of both sequestered and surface receptor compartments by measuring recovery of binding in both sequestered and surface compartments, as shown in Fig. 7. The recovery of α_2-adrenergic receptors after benextramine treatment at 37° was much slower than was observed after treatment at 4°. The recovery of total cell receptors occurred with a half-time of approximately 9 hr, whereas recovery of the surface compartment occurred more slowly, with a half-time of ~13 hr. Recovery of receptors was prevented by the addition of the protein synthesis inhibitor cycloheximide (1 μM) 1 hr before receptor blockade with benextramine at 37° (data not shown).

Discussion

The data presented here confirm and extend our previous observations (11) that HEL cells possess α_2-adrenergic receptors in both surface and sequestered locations. In the current studies, we used the hydrophilic antagonist benextramine, a tetramine disulfide, to block selectively receptors on the cell surface. Benextramine is thought to block irreversibly α_2-adrenergic receptors by covalent bond formation with a protein thiol, perhaps through a disulfide-protein thiol interchange reaction (22). Such a mechanism seems likely for the α_2-adrenergic receptor since experiments with purified α_2-adrenergic receptors reveal the presence of an essential SH group at or near the ligand-binding site (23).

We have demonstrated that when cells are incubated at 4° with benextramine, α_2-adrenergic receptors in the cell surface compartment are selectively blocked. Several pieces of data support this conclusion. 1) Only those receptors that are accessible to epinephrine at 4° are blocked by benextramine (Fig. 2). 2) A crude membrane preparation is enriched for α_2-adrenergic receptors that are susceptible to blockade by benextramine at 4° (Fig. 4). 3) α_2-Adrenergic receptors that are not blocked by benextramine are also not functionally coupled to the inhibition of adenylate cyclase activity. 4) Surface sites and receptor function recover rapidly after benextramine treatment at 4°.

The nature of the surface and sequestered compartments cannot be precisely defined in anatomical terms. It seems likely that the surface receptors represent plasma membrane receptors accessible from the extracellular environment, since only the receptors in this compartment are able to inhibit the accumulation of cAMP. The sequestered pool of receptors might represent receptors in intracellular organelles (endosomes, Golgi, etc.) or in a plasma membrane domain less accessible to hydrophilic molecules.

It has been shown that the α_2-adrenergic receptor can be phosphorylated *in vitro* (24), and like the β- and α_1-adrenergic receptor (25, 26), this may be a signal for receptor sequestration in response to agonists or other treatments. The data presented here imply that receptor sequestration is, at least in part, a reversible process since, when surface α_2-adrenergic receptors are blocked with benextramine, the sequestered receptors can rapidly relocate to the cell surface. Moreover, since benextramine is unlikely to penetrate cells, its ability to block α_2-adrenergic receptors in both surface and sequestered compartments during a 1-hr incubation at 37° (Table 2) suggests that α_2-adrenergic receptors may be continually exchanging between the surface and sequestered compartments in the cell. If one assumes that blockade of surface receptors by benextramine does not interfere with the kinetics of receptor movement, then the rate of translocation of sequestered receptors to the surface of the cell ($t_{1/2}$ = 25 min) may represent an approximate rate of receptor movement between these two compartments. Whether internalization and recycling of α_2-adrenergic receptors in HEL cells might involve a phosphorylation/dephosphorylation cycle will require future studies.

Sequestered α_2-adrenergic receptors can most readily be detected when binding studies are carried out in intact cells, since, as demonstrated in the *inset* to Fig. 4, preparation of HEL cell membranes appears to result in the preferential isolation of cell surface receptors. It is interesting to note that the recovery of α_2-adrenergic receptors in intact HEL cells is somewhat quicker than in most other systems (27), even when benextramine has been used (14–16). In other studies with this drug, half-times observed for recovery of receptor binding were 27 hr in HT-29 cells (14) and 36–41 hr *in vivo* (15, 16). Although our estimate of a faster rate of recovery in HEL cells may merely relate to variations in receptor metabolism in different cell types, an alternative possibility is our use of an experimental protocol which distinguishes sequestered receptors from cell surface receptors. Previous workers who prepared crude membrane fractions would preferentially be assaying surface receptors (Fig. 4, *inset*), which have a slower rate of recovery (Fig. 7). This slower rate of recovery of surface sites is consistent with the idea that α_2-adrenergic receptors must first traverse

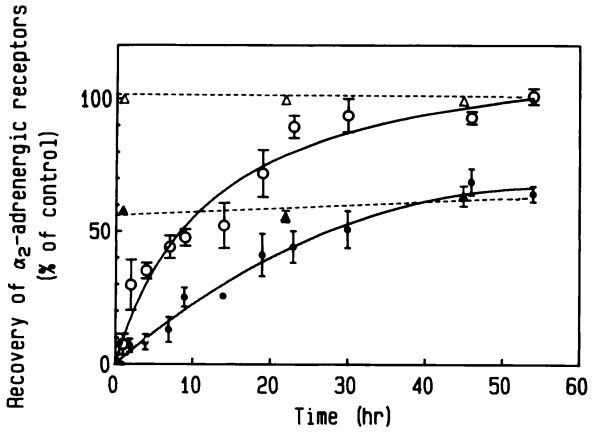

Fig. 7. Recovery of [^{3}H]yohimbine binding to total (○) and surface α_2-adrenergic receptors (●) after benextramine treatment at 37°. Intact cells were incubated for 1 hr with 50 μM benextramine; then, cells were washed three times with RPMI and then resuspended in RPMI with 10% fetal calf serum and the antibiotics penicillin (50 units/ml) and streptomycin (50 μg/ml) (which were without effect on cell growth or receptor number and distribution). Aliquots were removed at various times, as indicated, and rapidly cooled. Total and surface receptors were measured using 8 nM [^{3}H]yohimbine. Data shown are the mean ± standard error of three experiments. (Total receptor number = 6,930–10,200 receptors/cell, surface receptors = 3,060–5,440 receptors/cell.) In parallel experiments, in cells which had not been exposed to benextramine, the number of total receptors (△) and the number of surface receptors (▲) varied by less than 10% throughout the course of the experiment.

through intracellular locations before insertion into the plasma membrane.

In agreement with the studies of Paris *et al.* (14), we have observed that α_2-adrenergic receptor functional response, i.e., the inhibition of cAMP accumulation, recovers from treatment with benextramine before a full complement of receptors is restored. It was not possible for us to obtain an exact quantitation of the relationship between occupancy of α_2-adrenergic receptors and the functional response to inhibit cAMP accumulation in intact HEL cells because of the rapid relocation of sequestered receptors to the surface of the cell at 37°, and because we conducted functional assays over several minutes. Discrepancy between receptor number and receptor function has been observed for α_2-adrenergic receptor-mediated inhibition of adenylate cyclase (14, 28), inhibition of neurotransmitter release (29), and lipolysis (15). One possible explanation is that, since these functional responses occur distal to occupation of α_2-adrenergic receptors by agonist, amplification of the signal is possible, allowing for a nonlinear relationship between receptor occupancy and functional response. Since "spareness" is observed in the second messenger system (inhibition of adenylate cyclase), this suggests that the locus of amplification could be the inhibitory guanine nucleotide-regulatory (G_i) protein through which α_2-adrenergic receptors mediate cellular responses. Since data from reconstitution studies (30) and analysis of receptor-G_i stoichiometry (31, 32) indicate that one receptor molecule may interact with more than one G_i protein, amplification of the signal of receptor occupancy and a nonlinear relationship between receptor occupancy and inhibition of adenylate cyclase would be observed.

In summary, we have used benextramine as a novel tool for measuring the subcellular distribution of α_2-adrenergic receptors in intact HEL cells and for the assessment of receptor translocation between cellular compartments. Treatment of cells with a hydrophilic, irreversible antagonist appears to provide a simple, useful, and somewhat less perturbing means for probing cell surface receptors than are certain other methods, e.g., the removal of surface receptors by either brief acid exposure (8, 33) or limited trypsin digestion (34). Potentially, this technique can be extended to other receptor systems in which hydrophilic, irreversible antagonists are or will become available.

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