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Differences between Agonist and Antagonist Binding to *Alpha*$_1$-Adrenergic Receptors of Intact and Broken-Cell Preparations

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SUMMARY

Alpha$_1$-Adrenergic receptors of a nonfusing muscle cell line (BC_3H_1) have been identified on intact and broken-cell preparations by using the high-affinity antagonist [^{3}H]prazosin. In intact cells, both equilibrium and kinetic studies gave K_D values in the range of 0.07–0.12 nM. The maximal number of binding sites determined by Scatchard analysis was about 85,000 ± 9,000 sites/cell. Antagonists bound to the [^{3}H]prazosin binding sites with Michaelis-Menten characteristics, and their specificity was typical of *alpha*$_1$-adrenergic receptors. No significant modification of antagonist binding was observed either after cell disruption or by lowering incubation temperature to 4°. In contrast, in intact cells at 37°, agonist competition curves were shallow with Hill coefficients of less than 1. The heterogeneity of [^{3}H]prazosin binding sites toward (−)-norepinephrine also appeared in [^{3}H]prazosin saturation experiments carried out in the absence and in the presence of this agonist. After cell disruption, the EC_{50} values of agonist competition curves decreased and Hill coefficients were close to 1. When the temperature was lowered from 37° to 4°, the affinity for (−)-norepinephrine in intact cells increased dramatically by 10,000 times and the Hill coefficient of the competition curve was equal to unity. This affinity shift induced by temperature was not so important in broken-cell preparations (50 times).

INTRODUCTION

The development of highly labeled antagonists and agonists has allowed the analysis of the biochemical and pharmacological properties of *alpha*- and *beta*-adrenergic receptors at the molecular level (for a review see ref. 1). Most of these studies were conducted with crude or purified membranes prepared from target tissues. The main criticism that can be made of this approach is that cell disruption could induce changes in receptor properties and regulation. Detailed analyses of the properties of *beta*-adrenergic receptors in intact cells have been reported (2–10). The main finding was that the properties of agonist binding to the *beta*-adrenergic receptor of intact cells were different from those found in membrane preparations. At equilibrium the affinities of agonists for the *beta*-adrenergic receptors were much weaker in intact cells than in membranes. This was not the case for antagonist binding.

Only two reports have been issued on the characteristics of *alpha*-adrenergic receptors in intact cells using the nonselective radioligand [^{3}H]dihydroergocryptine (11, 12). We recently studied the properties and the turnover of α_1-adrenergic receptors in a nonfusing muscle cell line (BC_3H_1) by binding studies of the *alpha*$_1$-selective radioligand [^{3}H]prazosin on particulate fractions (13). In this report we describe the binding characteristics of [^{3}H]prazosin on intact cells and compare them with results on broken-cell preparations of the same system.

MATERIALS AND METHODS

Tissues. The BC_3H_1 muscle cell line was a gift from Dr. J. Patrick (the Salk Institute, San Diego, Calif.). The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin G (50 IU/ml), and streptomycin sulfate (50 μl/ml) at 37° in an atmosphere of 5% CO_2 and 95% air. The line was maintained in the exponentiel growth phase by passage every 4 days. Cells used for experimentation were plated in 35-mm or 90-mm diameter plastic tissue culture dishes at a density of 10^3 cells/cm^2. They were used between 13 and 15 days after seeding, and the medium was changed on days 7 and 11. Cell number per plate was determined by resuspending the cells and counting them in a hemocytometer.

Preparation of particulate fractions. Particulate fractions were prepared as previously described (13) except that the final pellet was resuspended in BSS[3] having the following composition (millimolar):

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[3] The abbreviations used are: BSS, balanced salt solution; PBS, phosphate-buffered saline.

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NaCl, 130; KCl, 5.4; $MgSO_4$, 0.8; $CaCl_2$, 1.8; NaH_2PO_4, 0.9; glucose, 5.5; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, 20/8.5 (pH 7.3).

Measurement of [^{3}H]prazosin on intact cells. [^{3}H]prazosin-binding experiments on intact cells were performed in 35-mm tissue culture dishes in BSS at 37° or 4°. A series of sister cultures were removed from the incubator, and cells were washed with 2 ml of BSS and allowed to equilibrate for 15 min at 37° or 4° before the assay was initiated by addition of the radioligand and the indicated drugs. The [^{3}H]prazosin concentration was varied from 0.02 to 2 nM in a total volume of 2 ml. The assays were performed during 45 min at 37° or 7 hr at 4°. The incubation was terminated by aspiration of the incubation medium followed by thrice washing the culture dishes with 3 ml of PBS. Cells were collected from the dishes by the addition of 1 ml of 0.5% Triton X-100/0.2 M NaOH, transferred in a counting vial, and counted in scintillation fluid. Figures 1–4 show specific binding defined as the binding which was inhibited by 10 μM phentolamine. Computer analysis of competition experiments was obtained by a nonlinear least-squares curve-fitting procedure as in ref. 14. Using the extra sum of the squares principle as applied in ref. 15, the good fit was evaluated, assuming that the receptor had either one or two affinity states.

Measurement of [^{3}H]prazosin binding on particulate fractions. Particulate fractions (100–200 μg) were incubated for 45 min at 37° in BSS containing a 0.02–2 nM concentration of [^{3}H]prazosin in a total volume of 1 ml. Incubation was terminated by rapid filtration of the entire mixture through Whatman GF/B glass-fiber filters and by washing three times with 5 ml of PBS. The filters were dried and counted in scintillation fluid. Figures 3 and 4 show specific binding, defined as binding which was inhibited by 10 μM phentolamine.

Materials. [^{3}H]Prazosin was purchased from the Radiochemical Centre, Amersham. (−)-Epinephrine bitartrate, (−)-norepinephrine hydrochloride, (−)-phenylephrine hydrochloride, yohimbine hydrochloride, (−)-alprenolol tartrate, and (−)-isoproterenol bitartrate were obtained from Sigma Chemical Company; (+)-epinephrine bitartrate from Sterling Winthrop Research Institute; prazosin hydrochloride from Pfizer; and phentolamine hydrochloride from Ciba-Geigy Corporation.

RESULTS

Binding characteristics of [^{3}H]prazosin to BC_3H_1 cells at 37°. Kinetic and equilibrium experiments showed that [^{3}H]prazosin bound to a single category of binding sites on intact cells (data not shown). The dissociation constant (K_D = 0.07 nM) determined by kinetic studies was close to the value found by Scatchard analysis (K_D = 0.115 ± 0.07 nM; n = 5). The nonspecific binding determined in the presence of 10 μM phentolamine was about 25% of total binding for a [^{3}H]prazosin concentration close to the K_D value. The total number of sites was 137 ± 11 fmoles/dish (n = 5), corresponding to 85,000 ± 9,000 $alpha_1$-adrenergic receptor sites per cell. Under the same conditions, we found on particulate fractions a K_D of 0.07 nM; from the B_{max} of 142 fmoles/mg of protein it could be calculated that one cell contained 64,000 sites. This figure correlated well with the number of sites measured directly on intact cells.

Interaction of adrenergic drugs with [^{3}H]prazosin-binding sites on intact cells at 37°. The potency order of adrenergic antagonists to inhibit [^{3}H]prazosin binding on intact cells, presented in Table 1, is characteristic of *alpha*-adrenergic receptors. As indicated by the Hill coefficients, these competition curves are homogeneous. Similar results were found in particulate fractions of BC_3H_1 cells in a previous study (13).

The competition curves of adrenergic agonists displayed *alpha*-adrenergic specificity (Fig. 1A). The Hofstee plots of epinephrine, norepinephrine, and phenylephrine inhibition curves showed a nonhomogeneous competition pattern (Fig. 1B–D), and Hill coefficients of these curves were below 1 (Table 2).

TABLE 1

Inhibition constants and Hill coefficients for adrenergic antagonists in intact BC_3H_1 cells at 37°

Competition experiments were performed as described under Materials and Methods. K_I values were calculated from the formula $K_I = EC_{50}/(1 + [\text{prazosin}]/K_D)$ (23) and are reported as either the mean (n = 2) or the mean ± standard error (n = 3).

Competitor	K_I	n	n_H
	nM		
Prazosin	0.07	2	0.99
Phentolamine	6.2 ± 1.9	3	0.97 ± 0.07
Yohimbine	220	2	1.01
(−)-Alprenolol	2500	2	0.96

To verify that these heterogeneous competition curves were not due to a dose-dependent degradation of agonists, we performed the following experiment. Solutions of (−)-epinephrine in BSS medium were first incubated for 45 min at various concentrations with intact cells and then used to compete for [^{3}H]prazosin from $alpha_1$-adrenergic receptors in particulate fractions. The competition potency of preincubated (−)-epinephrine solutions was equal to that of freshly prepared ones (data not shown). Furthermore, when intact cells were incubated in the presence of ascorbic acid (0.5 mM), pargyline (10 μM), and pyrocatechol (3 mM) to inhibit the eventual (−)-epinephrine degradation, we obtained the same competition pattern as in BSS medium (data not shown).

One possible explanation for the nonlinear Hofstee plots shown in Fig. 1B–D is the presence of two affinity states of the receptor for these agonists. Table 3 shows the Fischer coefficients (F) and the probability of errors (p) for a model of two independent agonist sites compared with a one-site model. Because only 4 of 12 experiments gave $p < 0.05$ for the two-site model, this model does not produce a satisfactory explanation for the nonclassical competition curves observed.

We performed saturation experiments in the presence of 10 and 100 μM norepinephrine (Fig. 2). If the agonist had a classical competitive behavior for the [^{3}H]prazosin binding sites, increasing concentrations of norepinephrine should decrease the apparent affinity of [^{3}H]prazosin and the B_{max} value should remain constant. Figure 2 shows that 10 μM norepinephrine did not interact in the classical manner with the [^{3}H]prazosin binding sites. For this agonist concentration, we found an apparent loss of 27% (mean value 23.8 ± 1.7%; n = 4) of the [^{3}H]prazosin binding sites and no significant increase in the apparent K_D value [0.115 ± 0.007 nM (n = 5) for control and 0.111 ± 0.008 nM (n = 3)]. Only a 10-times higher concentration of norepinephrine seemed to interact competitively with the remaining [^{3}H]prazosin binding sites [$K_{D_{app}}$ = 0.23 ± 0.006 nM (n = 4). From the apparent dissociation constant, $K_{D_{app}}$, for [^{3}H]prazosin as determined by Scatchard analysis in the presence of 100 μM norepinephrine, we were able to calculate the norepinephrine dissociation constant for this fraction of sites by the following classical

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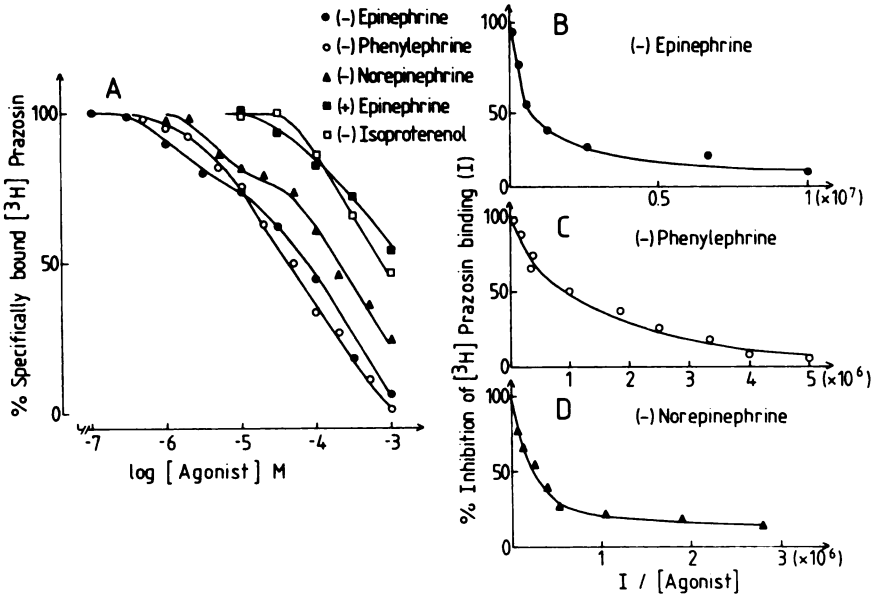

FIG. 1. *Comparison of relative potencies of various adrenergic agonists competing with [^{3}H]prazosin binding sites on intact BC_3H_1 cells*

A. Inhibition of specific [^{3}H]prazosin binding by different concentrations of adrenergic agonists. Cells were incubated at 37° in the presence of 0.14 nM [^{3}H]prazosin.

B–D. Hofstee plots for the inhibition of specific [^{3}H]prazosin binding by agonists. Experimental data are taken from A. This experiment is typical of two to five others.

relationship:

$$K_D^* = \frac{K_D[NE]}{K_{D_{app}} - K_D}$$

where K_D is the dissociation constant for [^{3}H]prazosin in the absence of norepinephrine. The value obtained was $K_D^* = 160 \pm 40$ μM (n = 5).

Influence of temperature and cell disruption on the properties of [^{3}H]prazosin binding sites. A decrease in the incubation temperature did not greatly influence the binding of [^{3}H]prazosin. At 4° the [^{3}H]prazosin dissociation constant was $K_D = 0.08$ nM and the maximal binding capacity was $B_{max} = 109$ fmoles/dish (65,000 sites/cell) (data not shown). These values are close to those found at 37° on intact cells and on particulate fractions. Similarly, the affinity of another *alpha*-adrenergic antagonist, phentolamine, was not significantly changed by cell disruption and temperature variations (Fig. 3; Table 2).

On the other hand, both treatments modified the heterogeneous agonist competition curves observed under physiological conditions (37°, intact cells). Hofstee plots became linear, and Hill coefficients increased to unity (Table 2) at 4° in intact cells and at 37° as well as at 4° in membranes (Fig. 4B, C, and E). Furthermore, a decrease in temperature from 37° to 4° in intact cells resulted in a dramatic decrease in the apparent affinity of (−)-norepinephrine for *alpha*$_1$-adrenergic receptors (EC_{50} decreased about 10,000 times from 220,000 nM to 25 nM (Fig. 4A; Table 2)]. In membranes, this temperature-induced shift in norepinephrine affinity was not so important (K_D decreased 52 times from 12 μM to 230 nM).

DISCUSSION

To study pharmacological and regulatory properties of receptors under physiological conditions it is necessary to perform binding experiments of specific radioligands on intact cells. Various reasons account for the low number of studies carried out on intact cells as compared

TABLE 2

Effects of temperature and cell disruption on drug competition constants

EC_{50} values were taken from representative experiments in which similar [^{3}H]prazosin concentrations were used (about 0.1 nM). Hill coefficients are mean values of the number of experiments indicated in parentheses.

Competitor	Intact cells				Particulate fractions			
	EC_{50}		Hill coefficient		EC_{50}		Hill coefficient	
	37°	4°	37°	4°	37°	4°	37°	4°
	nM				nM			
(−)-Norepinephrine	260,000	25	0.62 ± 0.04 (6)	0.90 ± 0.05 (6)	12,000	230	0.94 ± 0.02 (3)	0.97 ± 0.06 (3)
(−)-Epinephrine	76,000	—	0.75 ± 0.06 (4)	—[a]	5,600	—	0.96 ± 0.05 (3)	—
(−)-Phenylephrine	44,000	—	0.78 ± 0.03 (3)	—	11,000	—	0.97 ± 0.07 (3)	—
(−)-Phentolamine	80	30	0.97 ± 0.07 (3)	0.96 ± 0.03 (3)	80	80	0.94 ± 0.03 (3)	0.95 ± 0.04 (3)

[a] —, Value not determined.

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TABLE 3

Statistical analysis of agonist competition curves

Competition experiments were performed as described under Materials and Methods. The competition curves obtained were fitted by a model of two independent binding sites. The Fischer coefficients for this model and the error probabilities obtained by the comparison of this model with a classical one-site model were calculated as previously described (14).

Competitor	Fischer coefficient (F)	Error probability (p)
Norepinephrine	10.0	<0.01
Norepinephrine	1.9	>0.05
Norepinephrine	1.6	>0.05
Norepinephrine	0.9	>0.05
Norepinephrine	3.9	<0.05
Norepinephrine	2.0	>0.05
Epinephrine	5.0	<0.01
Epinephrine	2.0	>0.05
Epinephrine	4.2	<0.05
Epinephrine	3.4	≥0.05
Phenylephrine	2.0	>0.05
Phenylephrine	2.4	>0.05

with those on membrane preparations. Among them are (a) the difficulty of finding a homogeneous cell population with receptors easily accessible to the radioligand. Cell culture systems have been shown to fulfill these conditions (4–8, 10). (b) The degradation of ligands, which could be more pronounced on intact cells than on membrane preparations. In our experiments with BC_3H_1 cells we verified that, during a 45-min incubation period, [^{3}H]prazosin and epinephrine were not degraded (data not shown). (c) The uptake of radioligands into the cell. This has been observed in studies of binding of [^{3}H] dihydroalprenolol and [^{3}H]quinuclidinyl benzilate to human polymorphonuclear leukocytes (9, 16, 17) and to C_6 glioma cells.[4] It is likely that these radioligands accumulated in lysosomes, since this uptake could be eliminated by permeable amines such as phentolamine, chloroquine, or methylamine (7, 9, 16). This pitfall did not occur in our system, since methylamine had no effect on [^{3}H]prazosin binding (data not shown) and since similar numbers of binding sites were detected at 37° and 4° (data not shown).

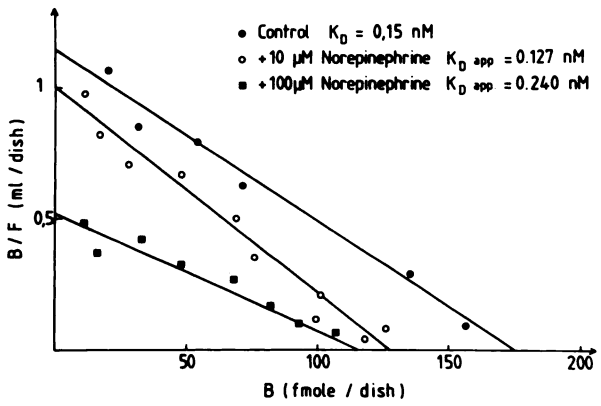

FIG. 2. *Scatchard analysis of [^{3}H]prazosin binding to BC_3H_1 cells in the presence of different doses of norepinephrine*

BC_3H_1 cells were incubated with [^{3}H]prazosin (0.02–1.7 nM), as described under Materials and Methods, without norepinephrine or with 100 µM or 10 µM norepinephrine. Norepinephrine was added in the incubation medium at the same time as [^{3}H]prazosin. This experiment is typical of three to four others.

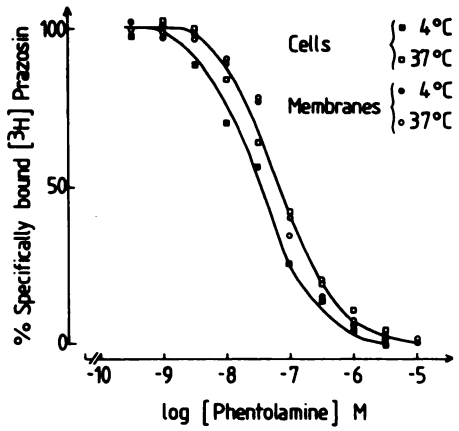

FIG. 3. *Effects of temperature and cell disruption on antagonist binding characteristics*

Inhibition of [^{3}H]prazosin binding by phentolamine. Cells or particulate fractions (175 µg of proteins) were incubated at 4° or 37° with 0.13 nM [^{3}H]prazosin and various concentrations of phentolamine. This experiment is typical of two others.

Both kinetic and equilibrium analyses indicated that [^{3}H]prazosin interacts with intact BC_3H_1 cells via one category of independent binding sites having a high affinity for [^{3}H]prazosin (K_D = 0.115 ± 0.007 nM; n = 5). These binding sites are homogeneous toward other adrenergic antagonists, as indicated by the Hill coefficients of their competition curves shown in Table 1. The specificity of these binding sites was typical of *alpha*$_1$-adrenergic receptors (prazosin > phentolamine > yohimbine > (−)-alprenolol).

The main finding of this study was that the interaction of the agonists (−)-epinephrine, (−)-norepinephrine, and (−)-phenylephrine with the *alpha*$_1$-adrenergic receptors had different characteristics on membranes and intact cells. On membranes, the agonist competition curves were of the Michaelis-Menten type with a linear Hofstee plot and a Hill coefficient close to 1 (Table 2), indicating a single affinity state of *alpha*$_1$-adrenergic receptors. In contrast, on intact cells at 37°, these agonists exhibited shallow competition curves with Hill coefficients less than 1 (Fig. 1; Table 2).

We have tried to explain these findings by a model of two independent agonist binding sites. The results presented in Table 3 show that this model did not explain in a satisfactory manner the nonclassical behavior of the agonists epinephrine, norepinephrine, and phenylephrine for the [^{3}H]prazosin binding sites.

The results found in [^{3}H]prazosin saturation experiments in the presence of 10 µM and 100 µM norepinephrine indicated that this agonist interacted with only about

[4] V. Homburger, unpublished results.

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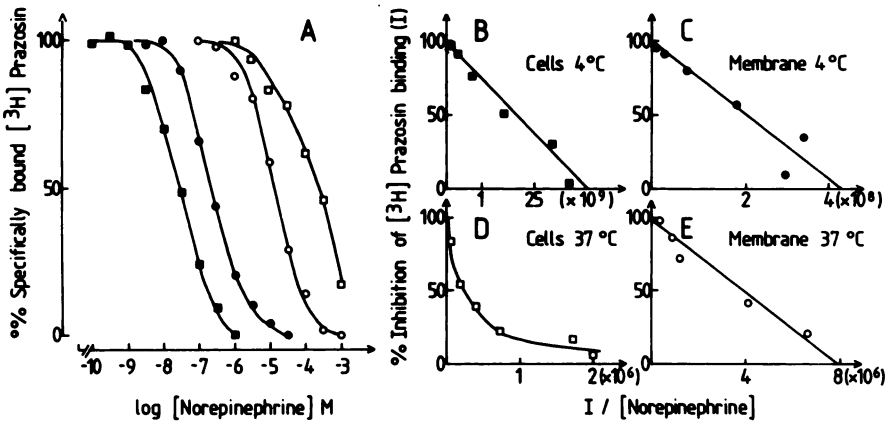

FIG. 4. *Effects of temperature and cell disruption on inhibition of specific [^{3}H]prazosin binding by norepinephrine*

A. Cells or particulate fractions (180 μg of protein) were incubated at 4° or 37° with 0.14 nM [^{3}H]prazosin and different concentrations of norepinephrine.

B–E. Hofstee plots from the inhibition of specific [^{3}H]prazosin binding by norepinephrine on intact or broken-cell preparations at 37° or 4°. Data are taken from A. This experiment is typical of two to five others.

70% of the [^{3}H]prazosin binding sites in a competitive manner. A recently described allosteric model (18) might explain our results. Further studies are required to clarify this point.

Another interesting observation made in this study was that the EC_{50} values of the agonist competition curves were 4 [(−)-phenylephrine], 14 [(−)-epinephrine], or 22 [(−)-norepinephrine] times greater for intact cells than for particulate fractions. This has already been reported for *beta*-adrenergic systems (5, 8–10). Dulis and Wilson (9) also observed a Hill coefficient below 1 for agonist binding on the *beta*-adrenergic receptors of intact cells.

The findings of two recent papers (19, 20) can explain these observations. The authors found this difference in the agonist competition properties between intact cells and particulate fractions only when they used lipophilic radioligands. By using a hydrophilic radioligand they found that isoproterenol had nearly the same affinity on intact cells as on broken-cell preparations. Porzig *et al.* (19) explained their observations by the hypothesis that the lipophilic radioligands could transform the *beta*-receptors of intact cells into a low agonist-affinity state. Staehelin *et al.* (20) proposed that lipophilic substances could penetrate endocytic vesicles formed by agonist-induced endocytosis during the competition experiments and in this way could bind to the receptors contained in these vesicles. Hydrophilic substances could bind only to receptors on the cell surface. For this reason, the hydrophilic agonist isoproterenol could easily inhibit the binding of hydrophilic but not of lipophilic radioligands (20). As prazosin is a highly lipophilic substance, a similar phenomenon could explain the agonist-affinity shifts and the nonclassical competition curves observed by us in intact cells. As we do not possess a hydrophilic *alpha*$_1$-adrenergic radioligand, we cannot test these hypotheses at the moment.

The most striking finding of this study was that lowering the temperature to 4° had a dramatic effect on the (−)-norepinephrine affinity for *alpha*$_1$-adrenergic receptors on intact cells (the EC_{50} was reduced by a factor of 10,000). Furthermore, at low temperature, (−)-norepinephrine exhibited (as observed on particulate fractions) homogeneous competition curves. This temperature effect was much less pronounced in membrane preparations (the K_D values were reduced 52 times). Such a moderate affinity shift has also been reported for *beta*-receptors, in both intact and broken-cell preparations (21, 22).

The fact that drugs which are known to have no properties stimulatory to the *alpha*$_1$-receptor system did not show shallow competition curves nor a significant temperature dependence of their affinity for the [^{3}H] prazosin binding sites might be a sign that the binding characteristics observed for the agonists in intact cells could be linked with the physiological actions of these drugs. The observations made in this study might open the way to a new understanding of the *alpha*$_1$-adrenergic receptor system.

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