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Comparison of Agonist-Induced Changes in β- and α_1-Adrenergic Receptors of DDT_1 MF-2 Cells

MYRON L. TOEWS

Department of Pharmacology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

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SUMMARY

Agonist-induced changes in β- and α_1-adrenergic receptors (BARs and AARs) were compared in the DDT_1 MF-2 smooth muscle cell line. During equilibrium competition binding assays with intact cells at 37°, agonists induced conversion of both BARs and AARs from a native form with high affinity for agonists to a form with much lower affinity for agonists. The native high affinity form of both receptors could be detected either in short-time competition binding assays at 37° or in equilibrium competition binding assays on ice. Conversion to the low affinity form was nearly complete for BARs, but only about half of the AARs were converted to the low affinity form. For BARs, the high affinity form of the receptor observed in short-time assays with intact cells was similar to that observed in membrane preparations, whereas for AARs this form exhibited much higher affinity than was seen in membrane assays. None of these changes were observed during competition binding assays with antagonists. Both short-time competition binding assays with hydrophilic competing ligands and sucrose density gradient centrifugation assays were consistent with the occurrence of agonist-induced internalization of BARs. These same assays for AARs were consistent with the presence of some AARs in an intracellular compartment in the native state, but no agonist-induced increases in intracellular AARs were detected. During more prolonged exposure (13 hr) to agonists, about 80% down-regulation of BARs occurred, whereas only about 20% down-regulation of AARs was detected. These results may indicate that internalization and down-regulation are not involved in conversion of these receptors to the low affinity form observed in intact cell binding assays.

Exposure of mammalian cells containing adrenergic receptors to catecholamines or other adrenergic agonists can lead to a variety of changes in the properties of these receptors and in the responses they mediate (1–3). These changes have been most extensively documented and studied in the case of BARs (1, 2). In one well studied system, 1321N1 human astrocytoma cells in culture, a series of agonist-induced changes in BARs has been shown to occur (reviewed in Ref. 4). A rapid uncoupling of cell surface BARs from stimulation of the enzyme adenylate cyclase (5, 6) is followed by changes in the physical (7, 8) and pharmacological (9, 10) properties of the receptor that have been interpreted in terms of internalization of BARs, or at least sequestration of BARs away from the cell surface. These changes are followed by a much slower down-regulation of BARs (decrease of total receptor number as measured in radioligand binding assays) (6, 11, 12). Similar changes have been shown to occur for BARs in a variety of other mammalian cells, although the relative rates of the various steps may be different (13–18).

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An additional change in BAR-binding properties can be observed in binding studies with intact cells. Agonists exhibit apparent binding affinities for BARs of intact cells that are markedly lower than their affinities for BARs in isolated membranes and their potencies for stimulation of cAMP production in intact cells (19–22). This lower apparent affinity results from agonist-induced conversion of BARs during the course of the assay from a native state of predominantly high affinity for agonists to the low affinity state observed in equilibrium assays (21–26). The native high affinity state of the receptor can be observed either in short-time competition binding assays carried out at 37° (21–25) or in equilibrium competition binding assays carried out on ice (22, 26). The possible roles of receptor uncoupling, internalization, and down-regulation in the conversion of BARs to the low affinity form observed in equilibrium competition binding assays with agonists remain unclear.

Agonist-induced changes in AARs of intact cells have been much less extensively characterized than those in BARs. Sladeczek *et al.* (27) reported that agonists exhibited shallow competition curves of unexpectedly low affinity in equilibrium assays with intact BC3H1 smooth muscle cells at 37°, but not in assays on ice. Schwarz *et al.* (28) obtained similar results in

ABBREVIATIONS: BAR, β-adrenergic receptor; AAR, α_1-adrenergic receptor; PRAZ, ^{3}H-prazosin; HEAT, ^{125}I-BE-2254; IPIN, ^{125}I-iodopindolol; DDT, the DDT_1 MF-2 smooth muscle cell line; EDTA, ethylenediaminetetraacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

equilibrium competition binding assays with AARs of intact isolated hepatocytes. In addition, they were able to detect a native state of the AARs exhibiting transient high affinity for agonists in short-time competition binding assays at 37°. We have performed similar experiments with AARs of intact DDT_1 MF-2 smooth muscle cells in culture. Since these cells contain comparable numbers of both BARs (β_2 subtype) and AARs (29, 30), we have compared the agonist-induced changes in intact cell-binding properties for these two receptors in the same cells. The extent of occurrence of apparent receptor internalization and receptor down-regulation following exposure of cells to agonists also was compared for these two receptor systems. We show that the agonist-induced change in intact cell-binding properties occurs together with apparent internalization and down-regulation in the case of BARs, but in the absence of detectable internalization or down-regulation in the case of AARs.

Materials and Methods

Chemicals. The following drugs were gifts: metoprolol and phentolamine from Ciba-Geigy (Summit, NJ), sotalol from Bristol-Myers (Evansville, IN), and (−)-pindolol from Sandoz (Basel, Switzerland). Other drugs were from Sigma Chemical Co. PRAZ and HEAT were from Amersham. IPIN was prepared as previously described (31).

Cell culture. DDT_1 MF-2 smooth muscle (DDT) cells (32), originally isolated from a leiosarcoma of hamster vas deferens, were obtained from Dr. J. S. Norris. Cells were maintained in monolayer culture at 37° in a humidified incubator under an atmosphere containing 8% CO_2. Growth medium was high glucose Dulbecco's modified Eagle's medium (Gibco) containing 5% fetal calf serum (Gibco). Cells were removed from dishes with 0.05% trypsin and plated for experiments at about 20,000 cells/cm^2 on tissue culture dishes (Falcon), and used in experiments 3 days later. Pretreatment of cells in the absence or presence of agonists was in growth medium containing 1 mM sodium ascorbate.

Intact cell binding assays. Assays of BARs and AARs on intact cells were performed in a manner similar to that previously described for BARs of intact 1321N1 cells (9, 22). Cells (naive or pretreated as indicated) on 35-mm dishes were washed once or twice at 37° with 2 ml of Eagle's medium buffered to pH 7.4 with 20 mM Hepes (Eagle's-Hepes). Cells were then further incubated for the indicated times at 37° or on ice in Eagle's-Hepes medium containing 1 mM sodium ascorbate, either IPIN or PRAZ, and various competing drugs as indicated. Following incubation, the cell sheets were rinsed twice at 37° with Eagle's-Hepes medium containing 100 μM propranolol (BAR assays) or 100 μM propranolol plus 100 μM phentolamine (AAR assays). These drugs were included during the wash step to prevent any further binding of radioligand during the wash procedure and because they markedly reduced nonspecific binding (by unknown mechanisms). Radioactivity associated with the cells was then determined following removal from the dishes with 1 ml of 0.2 N NaOH. ^{125}I was determined in a gamma counter; ^{3}H was determined by liquid scintillation counting in 10 ml of Budget Solve (Research Products International Corp., Mt. Prospect, IL). Nonspecific binding was defined as that occurring in the presence of 1 μM propranolol (BAR assays) or 10 μM phentolamine (AAR assays).

Sucrose density gradient centrifugation of cell lysates. Sucrose density gradient centrifugation studies of receptor distribution were performed essentially as described previously (7, 8). Cells grown on 100-mm dishes were preincubated at 37° in the absence or presence of agonists. This medium was removed and the cell sheets were incubated on ice for 20 min with Eagle's-Hepes medium containing 0.5 mg/ml concanavalin A. Following two washes with 5 ml of ice-cold lysis buffer (1 mM Tris, 2 mM EDTA, pH 7.4), cells were scraped from the dish with a rubber policeman. Cells (about 1 ml of cell suspension/dish) were then homogenized on ice with a Tekmar Tissumizer (5–10 sec at full power).

A 9.5-ml linear sucrose density gradient from 30–45% sucrose was formed with a Buchler gradient former. A 0.5-ml pad of 5% sucrose was layered on top of the gradient, and lysates (2–2.5 ml) were layered on top of this pad. Centrifugation was carried out at 4° for 60 min at 35,000 rpm in a Beckman SW40Ti rotor in a Beckman L5-65 refrigerated ultracentrifuge. Gradient fractions of 0.8 ml each were then collected at 4° from the top of the gradient using an ISCO gradient fractionator.

Down-regulation experiments. Cells grown on 100-mm dishes were incubated at 37° for the indicated times in the presence of 1 mM ascorbate or 1 mM ascorbate plus 10 μM epinephrine in the original growth medium. For the 21-hr time point, epinephrine and/or ascorbate were re-added at 13 hr. Cell sheets were then washed twice with 5 ml of ice-cold lysis buffer and lysed as described above. (Cells were not treated with concanavalin A in these experiments.) The lysates were centrifuged at 18,000 rpm for 20 min in an SM24 rotor and a Sorvall RC2B centrifuge at 4°. The membrane pellets from each dish were resuspended in 0.5 ml of 50 mM Tris buffer (pH 7.5) containing 250 mM sucrose and 5 mM $MgCl_2$ and stored frozen overnight prior to assay.

Binding assays on broken cell preparations. Gradient fractions or membrane suspensions were diluted to about 1.5 ml with 20 mM Tris buffer (pH 7.4) containing 2 mM $MgCl_2$ and 140 mM NaCl. Binding assays (60 min) were performed as previously described for BARs (12), using IPIN (100 pM) to label BARs and either PRAZ (400 pM) or HEAT (100 pM) to label AARs. Tissue (180 μl) was added to polypropylene tubes containing 50 μl of radioligand in the above buffer and 20 μl of competing drug in 1 mM HCl. Nonspecific binding was defined as that occurring in the presence of 100 μM isoproterenol (BAR assays) or 100 μM phentolamine (AAR assays). Following incubation for 60 min at 37°, assays were terminated by dilution with 20 mM Tris buffer (pH 7.4) containing 140 mM NaCl followed by rapid filtration over Whatman GF/B or Schleicher and Schuell No. 30 glass fiber filters. Filtration was either with a Millipore filtration manifold or with a Brandel cell harvester modified for receptor binding assays. The filters were washed an additional time with the same buffer and then transferred to tubes for gamma counting. Competition binding assays with membrane suspensions were performed in a similar manner, using IPIN to label BARs and PRAZ to label AARs. Both BAR and AAR assays contained 100 μM guanosine triphosphate.

Computerized curve fitting. Nonlinear least squares curve fitting of the raw data from competition curves to one- or two-site models was performed using PROC NLIN of SAS as previously described (9, 22) or using the CDATA program (EMF Software, Knoxville, TN) on an IBM PC-XT. Statistical comparison of the goodness of fit of the one- and two-site models was as previously described (33), with significance being taken as $p < 0.05$. Data from competition binding experiments are expressed as the percentage of maximal specific binding measured in the absence of competing ligand. The IC_{50} is defined as the concentration of competing ligand which reduces the amount of radioligand specifically bound to one-half of the amount specifically bound in the absence of competing ligand. As described previously (9), the model used is an empirical one based on equations for equilibrium binding to either one or two classes of specific sites. Since this model is technically correct only in those cases where the competing ligand is at equilibrium with both sites, IC_{50} values are generated rather than K_D (equilibrium dissociation constants) values. Where appropriate, K_D values have been calculated from IC_{50} values using the Cheng-Prusoff equation (34). Values in the text are expressed ± standard error for curves analyzed using CDATA and as the 95% confidence limits for curves analyzed using SAS. Saturation analyses of radioligand binding to determine K_D and B_{max} values for the radioligands used were according to the method of Rosenthal (35). All values reported are the averages of at least three separate determinations.

Results

IPIN binding to BARs of intact cells. IPIN was used to label BARs on intact DDT cells grown in monolayer culture as described in Materials and Methods. Binding of IPIN to intact cells was inhibited by adrenergic ligands in a stereoselective manner and with the rank order of potency expected for BARs (isoproterenol > epinephrine; propranolol ≫ phentolamine). Saturation analysis indicated that IPIN labeled a single class of binding sites, with a K_D of 52 ± 7 pM and B_{max} of 32 ± 2 fmol/35-mm dish or approximately 6000 receptors/cell.

Previous studies have documented that for rapidly equilibrating competing ligands, the IC_{50} obtained in short-time competition binding assays can be taken as the true K_D for the competing ligand (22–25). Competition by antagonists and agonists for IPIN binding to intact cells was measured in both short-time (30 sec) and equilibrium (60 min) competition binding assays. Curves obtained with the antagonist metoprolol were consistent with interaction with a single class of sites in both short-time and equilibrium assays (Fig. 1, Table 1). The K_D value (0.56 μM) calculated from the equilibrium IC_{50} value (1.78 μM) using the Cheng-Prusoff equation (34) was in good agreement with the IC_{50} value (0.51 μM) obtained directly in short-time assays. Thus, the affinity of BARs for metoprolol is not altered during 60 min exposure of intact cells to this antagonist.

The curves obtained with the agonists isoproterenol and epinephrine in both short-time and equilibrium assays (Fig. 1, Table 1) were significantly better fit by the two-component model. In short-time assays with both agonists, about 80% of IPIN binding was inhibited with high affinity and the remaining 20% was inhibited with an IC_{50} greater than 1 mM. In equilibrium assays, both of the agonists inhibited about 20% of IPIN binding with very high apparent affinity, whereas the remaining 80% was inhibited with a much lower IC_{50}. For both of these agonists, the affinity of the predominant form of the BARs observed in short-time assays (0.26 μM for isoproterenol, 1.68 μM for epinephrine) is 200–300-fold higher than that calculated (51 μM for isoproterenol, 520 μM for epinephrine) from the IC_{50} of the predominant low affinity form of the receptor observed in equilibrium assays. As described previously for BARs in several other cell lines (21–24), these results are consistent with an agonist-induced conversion of intact cell BARs from a native form with high affinity for agonists to the predominantly low affinity form observed following prolonged incubation in the presence of agonists during equilibrium competition binding assays.

Binding properties of AARs of intact cells. PRAZ was used to label AARs of intact DDT cells. Binding of PRAZ to intact cells was inhibited by adrenergic ligands in a stereoselective manner and with the rank order of potency expected for AARs (prazosin > phentolamine > yohimbine). Saturation analysis indicated interaction of PRAZ with a single class of sites with K_D of 114 ± 11 pM and B_{max} of 59 ± 2 fmol/35-mm dish or about 11,000 receptors/cell.

Competition by antagonists and agonists for PRAZ binding to intact cells was compared in both short-time (1 min) and equilibrium (60 min) assays (Fig. 2, Table 1). The α_2-adrenergic receptor-selective antagonist yohimbine was chosen for most of these experiments since it has an affinity for AARs that is similar to that of epinephrine in short-time assays. Competition curves obtained with yohimbine in both short-time and equilibrium assays were consistent with interaction of this antagonist with a single class of binding sites. The K_D value (0.45 μM) calculated from the equilibrium IC_{50} value (2.4 μM) was in good agreement with the IC_{50} value (0.30 μM) obtained directly in short-time assays. The higher affinity antagonist phentolamine also exhibited single-site competition curves in both short-time and equilibrium assay (Table 1), and the short-time IC_{50} (0.06

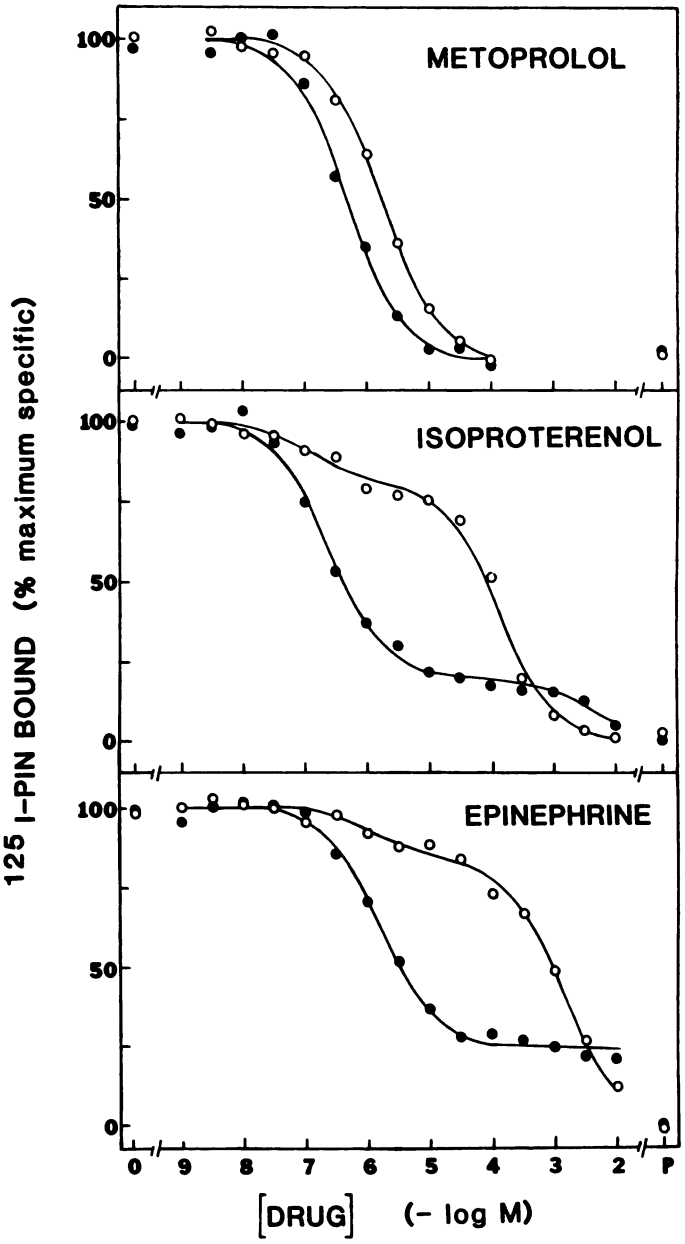

Fig. 1. Competition by metoprolol, isoproterenol, and epinephrine for IPIN binding to intact cells in 30-sec and 60-min assays. Intact cells were incubated with IPIN (100 pM) and the indicated concentrations of metoprolol (*upper panel*), isoproterenol (*middle panel*), or epinephrine (*lower panel*) for either 30 sec (●) or 60 min (○). Specific binding was then determined as described in Materials and Methods. In 30-sec assays, specific binding was approximately 4,500 cpm and nonspecific binding was approximately 1,300 cpm; in 60-min assays, specific binding was approximately 50,000 cpm and nonspecific binding was approximately 2,000 cpm. The data points shown are the averages of three experiments with each point determined in duplicate; the curves are those for the computerized fits of the data. Numerical values for the computerized fits of the data are presented in Table 1.

TABLE 1

Agonist and antagonist competition for radioligand binding to BARs and AARs

Assays were performed as described in Materials and Methods and Figs. 1 and 2, in all cases using IPIN to label BARs and PRAZ to label AARs. Data are means ± standard errors for computer-generated fits from three experiments. R_H and R_L represent the high and low affinity forms of the receptor, respectively. Where appropriate, K_D values have been calculated from equilibrium IC_{50} values using the Cheng-Prusoff equation (34). K_D and IC_{50} values are expressed as μM except where indicated otherwise.

Ligand	Tissue and assay condition						
	Intact cells, short-time assays (37°)		Intact cells, equilibrium assays (37°)		Intact cells, equilibrium assays (ice)		Membranes, equilibrium assays (37°)
	R_H	R_L	R_H	R_L	R_H	R_L	R_H
Competition for IPIN at BAR							
Metoprolol							
Percentage	100		100		100		100
IC_{50}	0.51 ± 0.10		1.78 ± 0.22		2.63 ± 0.74		2.98 ± 0.23
K_D			0.56		1.19		1.28
Isoproterenol							
Percentage	80 ± 3	20	19 ± 2	81	84 ± 2	16	100
IC_{50}	0.26 ± 0.06	>1 mM	0.12 ± 0.07	145 ± 22	0.61 ± 0.08	>1 mM	2.01 ± 0.87
K_D			0.04	51	0.24		0.88
Epinephrine							
Percentage	76 ± 4	24	17 ± 3	83			100
IC_{50}	1.68 ± 0.48	>1 mM	1.45 ± 1.27	1400 ± 300			12.8 ± 0.1
K_D			0.54	520			5.4
Competition for PRAZ at AAR							
Yohimbine							
Percentage	100		100		100		100
IC_{50}	0.30 ± 0.05		2.40 ± 0.38		2.24 ± 0.49		4.58 ± 1.58
K_D			0.45		0.44		1.40
Phentolamine							
Percentage	100		100				
IC_{50}	0.06 ± 0.01		0.20 ± 0.02				
K_D			0.07				
Epinephrine							
Percentage	80 ± 4	20	42 ± 5	58	86 ± 2	14	100
IC_{50}	0.16 ± 0.04	1 mM	2.26 ± 1.06	491 ± 185	0.05 ± 0.01	490 ± 190	27.8 ± 3.6
K_D			0.50	109	0.01		7.1
Norepinephrine							
Percentage	70 ± 4	30	48 ± 7	52			
IC_{50}	0.40 ± 0.14	>1 mM	5.08 ± 2.45	785 ± 409			
K_D			1.32	204			

μM) was in good agreement with the calculated equilibrium K_D (0.07 μM). Thus antagonist-binding properties of AARs are not changed during the course of equilibrium assays with intact cells.

Competition curves obtained in both short-time and equilibrium assays with the agonists epinephrine and norepinephrine (Fig. 2, Table 1) were significantly better fit by the two-component binding model. In short-time assays with both agonists, about 75% of PRAZ binding was inhibited with high affinity and the remaining 25% was only slightly inhibited even at concentrations greater than 1 mM. In equilibrium assays, both agonists inhibited about 45% of PRAZ binding with high affinity, whereas the remaining 55% was inhibited with lower affinity. For both of these agonists, the high affinity form of the AARs observed in short-time assays exhibited an affinity (0.16 μM for epinephrine, 0.40 μM for norepinephrine) about 500-fold higher than that calculated (109 μM for epinephrine, 204 μM for norepinephrine) from the IC_{50} for the low affinity form observed in equilibrium assays. These data are consistent with an agonist-induced conversion of AARs during equilibrium assays from a native form of predominantly high affinity for agonists to the lower affinity form observed in equilibrium assays, in a manner similar to that for BARs in these (Fig. 1) and other cells (21–24) and for AARs in intact hepatocytes (28). Similarities and differences in these phenomena for BARs and AARs are addressed in the Discussion.

Binding properties of BARs and AARs of intact cells on ice. Competition by antagonists and agonists for IPIN binding to intact cells also was measured in 4-hr (equilibrium) competition binding assays on ice (Table 1). IPIN labeled a single class of sites with K_D of 95 ± 12 pM and B_{max} of 32 ± 2 fmol/dish, similar to that observed at 37°. Competition curves obtained with the antagonist metoprolol were consistent with interaction with a single class of sites, and the affinity observed in equilibrium assays on ice was similar to that in equilibrium assays at 37°. Competition curves obtained with the agonist isoproterenol were better fit by the two-component model. However, isoproterenol exhibited predominantly high affinity interaction with IPIN-binding sites on ice, in marked contrast to the predominantly low apparent affinity for isoproterenol observed in equilibrium assays at 37°. The results obtained with intact cells on ice were thus very similar to those obtained in short-time assays at 37°.

Similar results were obtained in studies of PRAZ binding to

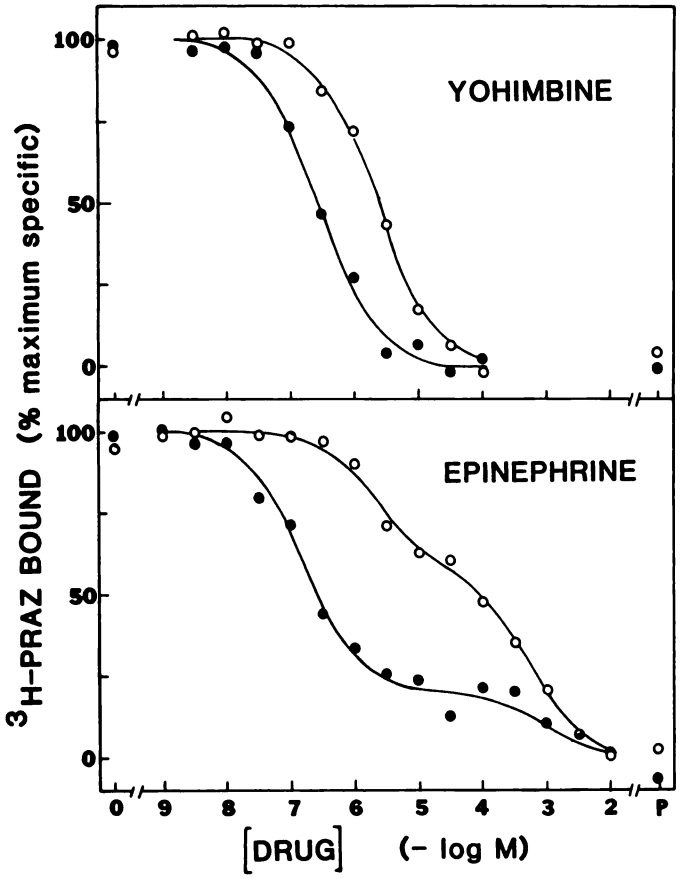

Fig. 2. Competition by yohimbine and epinephrine for PRAZ binding to intact cells in 1-min and 60-min assays. Intact cells were incubated with PRAZ (400 pM) and the indicated concentrations of yohimbine (*upper panel*) or epinephrine (*lower panel*) for either 1 min (●) or 60 min (○). Specific binding was then determined as described in Materials and Methods. In 1-min assays, specific binding was approximately 550 cpm and nonspecific binding was approximately 200 cpm; in 60-min assays, specific binding was approximately 3000 cpm and nonspecific binding was approximately 300 cpm. The data points shown are the averages of three experiments with each point determined in duplicate; the curves are those for the computerized fits of the data. Numerical values for the computerized fits of the data are presented in Table 1.

AARs of intact cells on ice (Table 1). PRAZ labeled a single class of sites with K_D of 135 ± 11 pM and B_{max} of 51 ± 2 fmol/dish. The antagonist yohimbine exhibited single-site competition curves with an affinity in assays on ice that was similar to that observed at 37°. The agonist epinephrine exhibited two-component competition curves with predominantly high affinity binding in assays on ice, in contrast to the predominantly low affinity observed at 37°. However, the affinity of epinephrine for the predominant site in assays on ice was much higher than that observed even in short-time assays with intact cells at 37°. These results indicate that the agonist-induced conversion of both BARs and AARs to the low affinity form observed at 37° does not occur in cells on ice, even following prolonged incubation in the presence of agonist during equilibrium competition binding assays.

Binding properties of BARs and AARs in membrane preparations. The interactions of antagonists and agonists with BARs and AARs in isolated membrane preparations were studied in 60-min equilibrium assays at 37° (Table 1). The K_D values in membrane assays were 92 ± 22 pM for IPIN and 190 ± 38 pM for PRAZ. These assays for both BARs and AARs were carried out in the presence of guanosine triphosphate, since this is presumably the relevant condition for comparison with the intact cell studies. In studies of IPIN binding to BARs in membranes, both the antagonist metoprolol and the agonists isoproterenol and epinephrine exhibited single-site competition curves. The affinities of all three ligands in membrane assays were similar to or slightly lower than those for the high affinity forms observed in intact cell assays at 37° and on ice. Similarly, in studies of PRAZ binding to AARs in membranes, both the antagonist yohimbine and the agonist epinephrine exhibited single-site competition curves. The affinity of yohimbine in membrane assays was only slightly lower than that observed with intact cells at 37° and on ice. The affinity of epinephrine in membrane assays, however, was markedly lower than the high affinity observed with intact cells in short-time assays at 37° or in equilibrium assays on ice.

Intact cell assays for receptor internalization (sequestration). Agonist-induced changes in the apparent affinity of intact cell BARs for hydrophilic ligands in short-time competition binding assays have been shown to correlate with an apparent internalization or sequestration of BARs away from the cell surface (9, 10). Short-time competition binding assays with various ligands were compared for both BARs and AARs of intact cells pretreated in the absence or presence of agonist (Fig. 3, Table 2). For BAR assays, intact cells were incubated for 20 min at 37° in the absence or presence of 10 μM epinephrine (Fig. 3) or 1 μM isoproterenol (Table 2) and washed; then, competition by various ligands for IPIN binding to intact cells was measured in short-time competition binding assays. Epinephrine competition curves obtained with control cells preincubated in the absence of epinephrine (Fig. 3) were similar to those obtained with naive cells (Fig. 1, Table 1), with about 25% of IPIN-binding sites exhibiting very low apparent affinity for epinephrine. Cells pretreated with 10 μM epinephrine showed a dramatic increase (to about 75%) in the fraction of IPIN-binding sites exhibiting low apparent affinity for epinephrine, with no change in IPIN bound in the absence of competing ligand. A similar change in binding of the hydrophilic agonists epinephrine and isoproterenol was observed in cells pretreated with 1 μM isoproterenol (Table 2). Pretreatment with isoproterenol led to a similar change in the binding properties of the hydrophilic antagonist sotalol (Table 2). In contrast, no changes were observed in the binding of the lipophilic antagonist metoprolol (Table 2). Thus, pretreatment of DDT cells with agonists appears to lead to a decrease in the accessibility of hydrophilic ligands, but not lipophilic ligands, to a portion of BARs in short-time binding assays.

To determine whether a similar change occurs for AARs, cells were pretreated at 37° for 20 min in the absence or presence of 10 μM epinephrine and washed, then, competition by various ligands for PRAZ binding to intact cells was measured in short-time competition binding assays (Fig. 3, Table 2). The epinephrine competition curves obtained with control cells pretreated without epinephrine were similar to those obtained with naive cells (Fig. 2, Table 1). However, the proportion of PRAZ-binding sites exhibiting the lower affinity for epinephrine was consistently higher in ascorbate-pretreated cells (Table 2) than in naive cells (Table 1). Curves obtained with cells pretreated with epinephrine were essentially identical to those with control cells. No changes in the affinity of AARs for the antagonist yohimbine were observed in epinephrine-

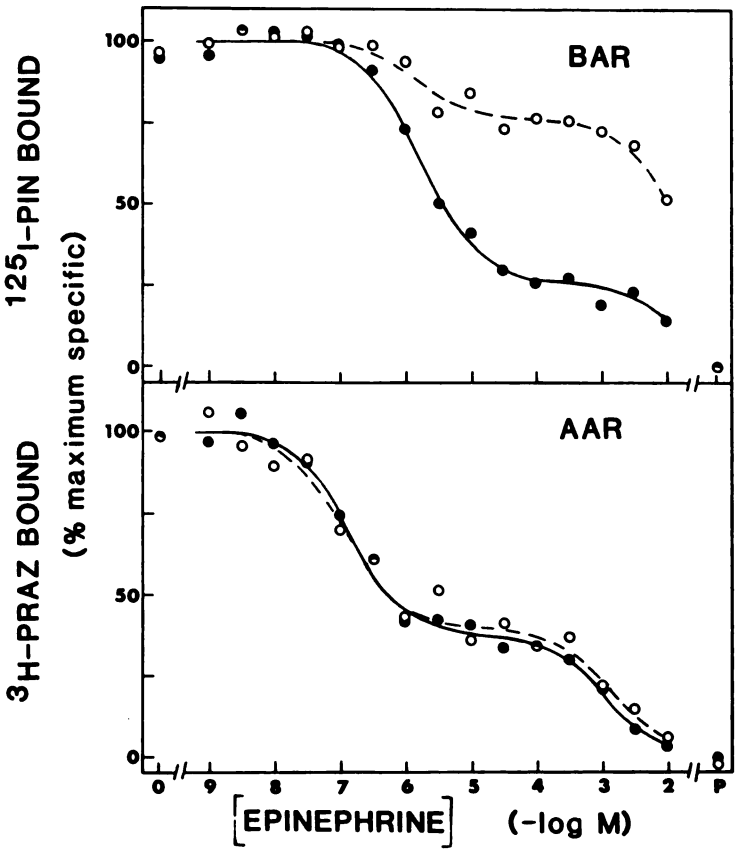

Fig. 3. Short-time assays of epinephrine competition for binding to control and epinephrine-pretreated intact cells. Cells were incubated for 20 min in the absence (●) or presence (○) of 10 μM epinephrine. After washing to remove pretreatment medium, the cells were further incubated for 1 min with IPIN (100 pM, *upper panel*) or PRAZ (260 pM, *lower panel*) and the indicated concentrations of epinephrine. Specific binding was then determined as described in Materials and Methods. Specific binding of IPIN was 7130 cpm for control and 7915 cpm for isoproterenol-pretreated cells; PRAZ binding was 445 cpm for control and 450 cpm for epinephrine-pretreated cells. The data points shown are averages from three to four separate experiments; the curves are those for the computerized fits of the data. Computerized fits for IPIN binding were as follows: control, 74 ± 5%, IC_{50} = 1.9 ± 0.7 μM and 26%, IC_{50} > 10 mM; epinephrine-pretreated, 24 ± 7%, IC_{50} = 1.7 ± 2.7 μM and 76%, IC_{50} > 10 mM. Values for PRAZ binding are presented in Table 2.

TABLE 2

Short-time competition binding assays on control and agonist-pretreated intact cells

Intact cells were incubated for 20 min at 37° in the absence or presence of 1 μM isoproterenol (BAR assays) or 10 μM epinephrine (AAR assays). Cell sheets were washed and then short-time competition binding assays were performed (30-sec assays for IPIN, 1-min assays for PRAZ). Data are means ± standard errors for computer-generated fits from three experiments. R_H and R_L represent the high and low affinity forms of the receptor, respectively. IC_{50} values are expressed as μM.

	Control cells		Agonist-pretreated cells	
	R_H	R_L	R_H	R_L
Competition for IPIN at BAR				
Metoprolol				
Percentage	100		100	
IC_{50}	0.36 ± 0.15		0.35 ± 0.15	
Sotalol				
Percentage	76 ± 3	24	30 ± 4	70
IC_{50}	0.12 ± 0.04	130 ± 40	0.12 ± 0.04	220 ± 100
Isoproterenol				
Percentage	75 ± 2	25	30 ± 2	70
IC_{50}	0.16 ± 0.02	7700 ± 3700	0.16 ± 0.04	4700 ± 1700
Competition for PRAZ at AAR				
Yohimbine				
Percentage	100		100	
IC_{50}	0.40 ± 0.11		0.51 ± 0.14	
Epinephrine				
Percentage	62 ± 5	38	60 ± 8	40
IC_{50}	0.15 ± 0.05	1200 ± 600	0.11 ± 0.08	1600 ± 1700

pretreated cells either (Table 2). There was no significant difference in the amount of PRAZ bound to control or epinephrine-pretreated cells in the absence of competing ligand. Thus, agonist pretreatment does not lead to changes in accessibility of AARs to either lipophilic or hydrophilic ligands in short-time binding assays.

Sucrose density gradient centrifugation assays for receptor internalization. As an additional means to monitor receptor internalization, the subcellular distribution of BARs and AARs was determined following sucrose density gradient centrifugation of lysates from cells pretreated in the absence or presence of agonist. Cells were incubated at 37° for 20 min in the absence or presence of 10 μM epinephrine, washed, and treated with concanavalin A.[1] Cells were then lysed, and the lysates were subjected to sucrose density gradient centrifuga-

[1] As in previous studies (7, 15), concanavalin A treatment led to greatly improved resolution of the plasma membrane fraction but was not necessary to detect the shift in BAR distribution.

tion. Fractions from these gradients were assayed for BAR distribution using IPIN and for AAR distribution using PRAZ (Fig. 4).

For BARs, the results obtained were similar to those previously obtained with several other cell lines (7, 8, 14–17). BARs of control cells were predominantly localized in "heavy peak" fractions together with the plasma membrane marker adenylate cyclase (not shown). A smaller portion of BARs was found in a "light vesicle" fraction near the top of the gradient. Gradients from cells pretreated with epinephrine revealed a dramatic shift of BARs from the heavy peak fraction to the light vesicle fraction, with little or no change in total binding of IPIN. Adenylate cyclase activity was not shifted to the light vesicle fractions along with the BARs (not shown).

AARs from control cells exhibited a distribution on sucrose density gradients similar to that for BARs, with most of the AARs in the heavy peak plasma membrane fractions and the remainder in the light vesicle fraction. However, in contrast to results obtained with BARs, no redistribution of AARs to the light vesicle fractions occurred following agonist pretreatment. Little or no change in total binding of PRAZ was observed either. Similar results were obtained using HEAT as the radioligand (K_D = 74 ± 5 pM). No evidence for internalization of AARs was observed under any of the following conditions (using HEAT as radioligand, not shown): longer incubation (up to 21 hr) with 10 μM epinephrine, incubation with 100 μM epinephrine or 100 μM norepinephrine, or selective activation of AARs either with the AAR-selective agonist oxymetazoline or with epinephrine plus propranolol to block activation and internalization of BARs.

Studies of receptor down-regulation. To determine whether down-regulation of BARs and AARs occurred during exposure to agonist, cells were incubated for various times at 37° in the absence or presence of 10 μM epinephrine. BARs and AARs were then determined in membrane fractions prepared from these cells (Fig. 5). Down-regulation of BARs occurred rapidly, with a $t_{1/2}$ of about 3 hr, and proceeded to 80–85% loss of receptors by 13 hr. Saturation analysis was performed on membranes from cells incubated for 13 hr in the absence and presence of 10 μM epinephrine (not shown). The results indicated that the decrease in IPIN binding resulted from a decrease in B_{max} with no significant change in the K_D value for IPIN.

The same membrane fractions used in the BAR assays above also were analyzed for AARs using both PRAZ and HEAT as radioligands. Essentially no loss of AARs was observed up to 2 hr of exposure to epinephrine, and only about 20% reduction in AARs was observed following twenty-one hr exposure to epinephrine. No further down-regulation of AARs was observed following prolonged exposure (12–24 hr) to a higher concentration (100 μM) of either epinephrine or norepinephrine (not shown).

Discussion

Two recent studies have used PRAZ to investigate the binding properties of AARs of intact cells, namely the BC3H1 smooth muscle cell line (27) and isolated hepatocytes (28). In both studies, antagonists bound to AARs of intact cells in a manner similar to their interaction with AARs in isolated membranes. In contrast, agonists exhibited shallow competition curves and unexpectedly low apparent binding affinities in assays with intact cells but not in isolated membranes. Binding to AARs of intact cells on ice gave steeper competition

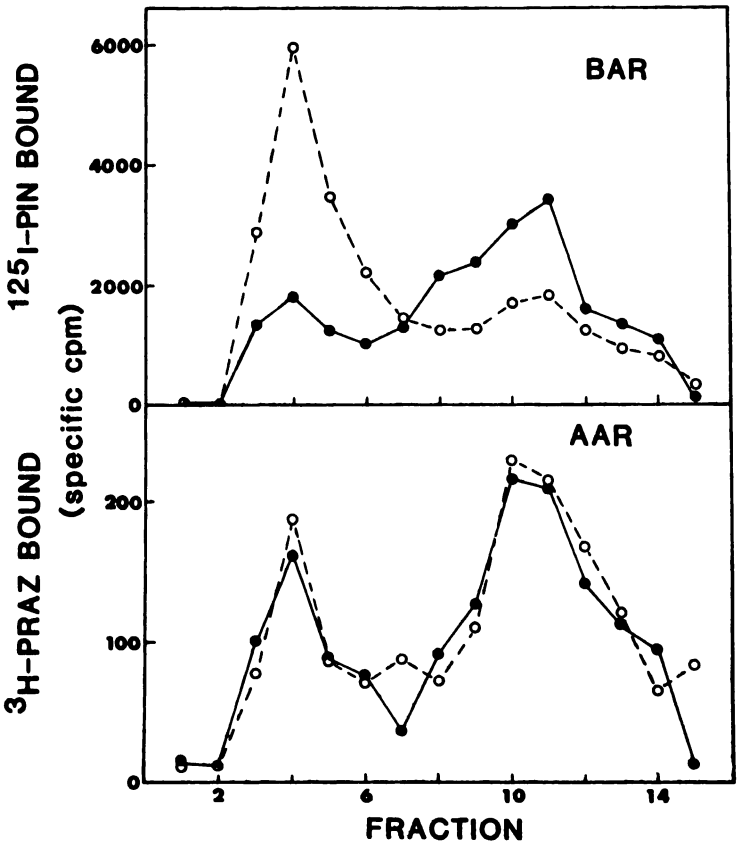

Fig. 4. Distribution of BARs and AARs on sucrose density gradients from control and epinephrine-pretreated cells. Cells were incubated for 20 min in the absence (●) or presence (○) of 10 μM epinephrine, washed, treated with concanavalin A, and then lysed. The lysates were subjected to sucrose density gradient centrifugation. Gradient fractions were then assayed for BARs using IPIN (*upper panel*) and for AARs using PRAZ (*lower panel*). The results shown are from one of four experiments which gave similar results. The sum of specific binding to all gradient fractions was 22,270 for BARs in the control gradient, 25,675 for BARs in the epinephrine-pretreated gradient, 1,500 for AARs in the control gradient, and 1,600 for AARs in the epinephrine-pretreated gradient.

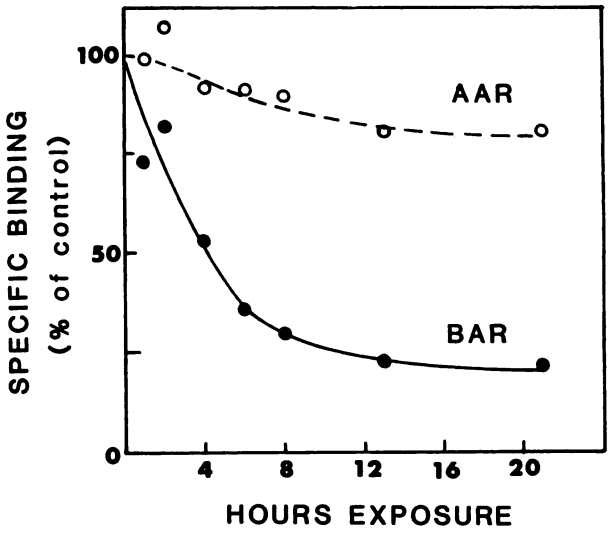

Fig. 5. Down-regulation of BARs and AARs during exposure of cells to epinephrine. Cells were incubated for the indicated times in the absence or presence of 10 μM epinephrine and lysed, and a membrane fraction was isolated. BARs (●) and AARs (○) in these membranes were then determined using IPIN and PRAZ and/or HEAT, respectively. Similar results were obtained using either radioligand for measuring AARs. The specific binding in membranes from cells pretreated with epinephrine is expressed as a percentage of specific binding in membranes from cells pretreated for the same time in the absence of epinephrine. Specific binding to membranes from control cells did not change as a function of time of exposure. The results shown are the averages of six experiments.

curves and much higher binding affinities. These results are similar to those reported previously for BARs of intact cells (19–24). In the case of BARs, the low affinity for agonists observed in equilibrium competition binding assays (typically 60-min assays) has been shown to result from agonist-induced conversion of BARs during the course of the assay from a native state of predominantly high affinity for agonists to the low affinity state observed in equilibrium assays (21–24). The native high affinity state of the receptor can be detected either in short-time competition binding assays at 37° (21–25) or in equilibrium competition binding assays carried out on ice to prevent the change in receptor-binding properties (22, 26).

We chose to investigate possible similarities in agonist-induced changes in binding properties of intact cell BARs and AARs in DDT$_1$ MF-2 smooth muscle cells. These cells contain comparable numbers of both BARs and AARs and can be grown conveniently either in monolayer or suspension culture (29, 30). We chose to carry out these studies on cells in monolayer culture since Pittman and Molinoff (36) had reported that the agonist-induced conversion of BARs to the form with low apparent affinity for agonists occurred in L6 muscle cells in monolayer culture but not in suspension. However, our subsequent studies have found that agonist-induced changes in BARs occur in a similar manner in DDT cells in both monolayer and suspension culture.[2]

Agonist-induced changes in the binding properties of BARs on intact DDT cells were essentially identical to those previously observed by us in 1321N1 human astrocytoma cells and by others in a variety of cultured cells (9, 21–24). Antagonists inhibited IPIN binding to intact cell BARs in a manner consistent with interaction with a single class of binding sites. The IC_{50} observed in short-time competition binding assays, which is equivalent to the true K_D in the case of a rapidly equilibrating competing ligand (22, 25), was similar to the K_D calculated from the IC_{50} obtained in equilibrium competition binding assays using the Cheng-Prusoff equation (34). This result indicates that no changes in the antagonist-binding properties of intact cell BARs are occurring during equilibrium competition binding assays. Competition curves obtained in equilibrium competition binding assays with agonists indicated apparent interaction with two classes of binding sites, with the predominant site being of low affinity. In short-time competition binding assays, two-component competition curves again were obtained, but in this case predominantly high binding affinity was observed. Predominantly high affinity binding of agonists to intact cell BARs also was observed even following prolonged incubation in the presence of agonist during equilibrium competition binding assays carried out on ice. These results together indicate that BARs of DDT cells undergo an agonist-induced, temperature-dependent conversion from a native form with high affinity for agonists to the low affinity form observed in equilibrium competition binding assays at 37°.

Internalization of BARs also has been postulated to occur during exposure of intact cells to BAR agonists. Evidence for internalization includes a shift of receptors from the plasma membrane fraction to a light vesicle fraction observed following sucrose density gradient centrifugation (7, 8, 14–17), a decrease in the accessibility of BARs to hydrophilic ligands (9, 10, 37, 38), and a decrease in the accessibility of BARs even to relatively lipophilic ligands in assays on ice (16, 39, 40). Whether these changes reflect internalization of BARs within endocytotic vesicles as in the case of several peptide receptor systems (41) or sequestration of receptors into a subdomain of the plasma membrane having these properties (42, 43) remains to be determined. Regardless of the morphological location of the compartment containing these receptors, our results show that this same change occurs for BARs of DDT cells. Agonist pretreatment induced conversion of approximately half of cellular BARs from the high affinity form observed in short-time assays with intact cells to the form exhibiting very low apparent affinity for hydrophilic ligands (isoproterenol, epinephrine, and sotalol) in short-time assays, with no changes in the binding properties of more lipophilic ligands (metoprolol and IPIN). Agonist pretreatment also induced a shift of approximately half of DDT cell BARs from plasma membrane fractions to light vesicle fractions on sucrose density gradients.

Down-regulation of BARs also occurred following more prolonged exposure of cells to BAR agonist. Our results are similar to those recently reported by Scarpace *et al.* (44) for down-regulation of DDT cell BARs. The time course for this agonist-induced loss of BARs was much slower than that for the apparent internalization, occurring over the course of several hours rather than in a few minutes as for internalization. Thus, a series of agonist-induced changes in BARs occurs in DDT cells that is quite similar to that which has been studied in various other cells.

Agonist-induced changes in the binding properties of AARs of intact DDT cells were investigated using PRAZ as the radioligand. PRAZ gave much better ratios of specific to nonspecific binding, particularly in short-time assays, than did HEAT, which was used in another recent study of intact DDT cell AARs (45). Antagonist competition for PRAZ binding to

[2] R. Hoover and M. Toews, manuscript in preparation.

intact cells was consistent with interaction with a single class of binding sites in both short-time and equilibrium assays. The shift observed between the curves obtained in short-time and equilibrium assays was similar to that predicted by the Cheng-Prusoff equation (34), indicating that antagonist-binding properties are not changed during equilibrium assays. In equilibrium competition binding assays, agonists exhibited two-component competition curves, with the majority of sites in a low apparent affinity form. Short-time competition curves also indicated interaction of agonists with two classes of PRAZ-binding sites, with most of the sites exhibiting high affinity. Predominantly high affinity binding of agonists to intact DDT cell AARs also was observed in equilibrium competition binding assays carried out on ice. However, in contrast to the results obtained for BARs, the higher affinity form of AARs observed in assays on ice exhibited markedly higher affinity than that observed in short-time assays at 37°. The reason for this difference in the temperature dependence of agonist binding to these two receptors in intact cells remains to be determined.

Our results indicate that the native state of AARs of intact DDT cells is one with predominantly high affinity for agonists and that an agonist-induced, temperature-dependent conversion of AARs to a form of low apparent affinity for agonists occurs during equilibrium competition binding assays at 37°. Similar results were obtained by Schwarz *et al.* (28) in studies of AARs of intact isolated hepatocytes. Their results, together with ours and those of Sladeczek *et al.* (27), indicate that this change in agonist binding properties of AARs may be a general feature of AAR regulation. It should be noted, however, that two previous studies of agonist competition for binding of ^{3}H-dihydroergocryptine (29, 45) or HEAT (46) to AARs of intact DDT cells found no major discrepancy between agonist binding to intact cells and isolated membranes. Whether the difference between their results and ours results from the different radioligands used or from actual differences in DDT cells in different laboratories has not been investigated.

Our studies in DDT cells allow a direct assessment of the similarity of the change in intact cell binding properties of AARs to that which occurs for BARs in these cells, which is similar to that which occurs for BARs in other cells. The change which occurs in AARs appears at least qualitatively similar to that for BARs in the same cells; however, several differences should be pointed out. In the case of BARs, the high affinity state observed in short-time assays with intact cells is only about 3-fold higher than that observed in isolated membranes. A similar 3-fold difference was observed with the antagonist metoprolol. In the case of AARs, the high affinity state of the receptor observed in short-time assays with intact cells exhibits nearly 50-fold higher affinity than that observed in membranes for the agonist epinephrine, whereas only a 4-fold difference was observed for the antagonist yohimbine. The extent to which these phenomena may relate to differential coupling of AARs to guanine nucleotide-regulatory proteins in these various preparations remains unknown. Although evidence exists for coupling of AARs to such regulatory proteins in several other systems (47, 48), studies to date with DDT cells, from our laboratory (unpublished) and others (45), have failed to provide evidence for such coupling in DDT cells. Alternatively, the low affinity for epinephrine in equilibrium assays with membranes may result from agonist-induced conversion of AARs to the low affinity form even in broken cell preparations as recently reported by Schwarz *et al.* (49) for AARs of isolated hepatocytes. Experiments to investigate this phenomenon for both BARs and AARs in DDT cell membranes are in progress.

A second difference between these phenomena for BARs and AARs is in the high affinity form of the receptor observed following prolonged incubation with agonists in equilibrium assays at 37°. In the case of BARs in these and other cells (22–24), this form of the receptor represents only 10–20% of IPIN binding and is of equal or higher affinity than the high affinity form observed in short-time assays at 37°. This apparent high affinity form of the receptor has been attributed to agonist-induced loss of IPIN-binding sites (down-regulation) during the assay rather than to agonist competition for IPIN-binding sites remaining on the cells in a high affinity form (22). Thus, it is proposed that, for cells incubated for 60 min with concentrations of agonist higher than about 10 μM, only 75% of the original number of receptors remains able to bind IPIN and that all of these receptors are in the low affinity form. The results in Fig. 5 indicate that, in fact, approximately 20–25% down-regulation of BARs is observed following 60-min exposure to agonist, consistent with this explanation.

In contrast, in the case of AARs, the high affinity form of the receptor observed in equilibrium competition binding assays represents about 45% of total PRAZ-binding sites. The affinity of these sites is only slightly lower than that of the high affinity sites observed in short-time assays. Furthermore, the apparent lack of agonist-induced down-regulation of AAR by 60 min in these cells indicates that down-regulation cannot explain the residual high affinity binding in equilibrium assays with AARs as it can for BARs. Thus, in the case of AARs, the high affinity sites observed in equilibrium competition binding assays most likely represent receptors remaining in the native high affinity form even after prolonged exposure to agonist. This is in contrast to the apparently complete conversion of all receptors remaining in these cells to the low affinity form in the case of BARs. An understanding of these phenomena at the molecular level may be required to determine whether the agonist-induced changes in agonist-binding properties of intact cell BARs and AARs result from similar or different processes.

We also investigated whether changes in AARs occur that are similar to those thought to reflect receptor internalization in the case of BARs. Thus, short-time assays of competition for PRAZ binding by the hydrophilic agonist epinephrine were compared for control cells and for cells preincubated with epinephrine. No differences in binding were observed, indicating that agonist-induced changes in receptor affinity for, or accessibility to, epinephrine under these conditions do not occur. No changes in the binding of the more lipophilic ligands PRAZ and yohimbine were observed either. Sucrose density gradient centrifugation studies led to the same conclusion, since no agonist-induced changes in the distribution of AARs were observed. It should be emphasized that these assays of AAR distribution were done on the same gradient fractions from the same experiment in which redistribution of BAR was observed. Although these negative findings cannot be taken as proof that AAR internalization does not occur, the concurrent studies with BARs in the same experiments strongly support the conclusion that agonist-induced sequestration or internalization of AARs does not occur in these cells to the same extent or in the same manner as it does for BARs.

In the case of BARs in 1321N1 cells, the form of the receptor

exhibiting low affinity for hydrophilic ligands in short-time assays has been shown to correlate with those receptors migrating in the light vesicle fraction on sucrose density gradients (10). These results are taken as evidence for the existence of internalized BARs. In the case of AARs of DDT cells, about 35% of receptors were observed in the low affinity form in short-time competition binding assays with control cells; a similar fraction of AARs was observed in the light vesicle fraction on sucrose density gradients from control cells. These distributions are similar to those observed for BARs in these cells, and they suggest that a portion of both BARs and AARs exists in an intracellular or sequestered compartment, even in the absence of agonist exposure. Exposure of cells to agonist leads to redistribution of additional BARs to this compartment but to no change in the distribution of AARs.

We observed only a slight down-regulation of AARs even following prolonged exposure of cells to the agonist epinephrine. These AAR assays were carried out on the same membrane preparations in which marked down-regulation of BARs by epinephrine was observed. In other studies where the two processes have been directly compared, AAR down-regulation also was found to occur more slowly than BAR down-regulation (50, 51). However, AAR down-regulation in several other cultured cells proceeded to a much greater extent than what we have observed for DDT cells (50–54). Studies of agonist-induced AAR internalization have not been previously reported. Whether DDT cells will be unique for their lack of AAR internalization and minimal down-regulation remains to be determined.

The relationship of the apparent internalization of BARs to the low affinity form of the receptor observed in equilibrium agonist competition binding assays with intact cells has not been established. Although limited accessibility and therefore slow equilibration of hydrophilic competing ligands with internalized receptors can explain the low apparent affinity for these ligands observed in short-time competition binding assays with agonist-pretreated cells, it is not clear how this could explain the low apparent affinity observed in equilibrium assays, which should allow sufficient time for full equilibration of these ligands even with intracellular receptors. Our studies of this phenomenon in DDT cells may shed new light on this question, since conversion to the low affinity form during equilibrium assays occurs together with apparent internalization in the case of BARs but in the absence of internalization in the case of AARs. Clearly, in the case of AARs, agonist-induced internalization cannot explain the conversion of receptors to the low affinity form for agonists observed in equilibrium competition binding assays. To the extent that the low affinity forms observed for BARs and AARs represent the same modifications of these two receptors, these results indicate that receptor internalization may not explain the low affinity form of BARs observed in equilibrium competition binding assays either. Thus, further studies will be required to fully understand this change in the agonist-binding properties of BARs and AARs of intact cells.

In summary, we find that a time-dependent change in agonist-binding properties occurs for AARs of intact DDT cells which appears to be similar to that which occurs for BARs in the same cells. Although we observe apparent internalization and down-regulation of BARs in these cells, there is no evidence for agonist-induced internalization of AARs and only a small down-regulation of AARs under the same conditions. These observations may lead to important new insights on the relationships among these changes in adrenergic receptors which occur during exposure of cells to agonists.

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Send reprint requests to: Dr. Myron L. Toews, Department of Pharmacology, School of Medicine, University of Missouri-Columbia, Columbia, MO 65212.