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Intact Cell Binding Properties of Cells Expressing Altered β-Adrenergic Receptors

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SUMMARY

During the course of equilibrium competition binding assays with intact cells, agonists induce conversion of β-adrenergic receptors (BARs) from a native form with high affinity for agonists to a form with a markedly lower apparent affinity. The roles of receptor internalization, receptor-G_s coupling, and receptor phosphorylation in this agonist-induced conversion to the low affinity form were investigated. Agonist and antagonist competition for [^{125}I]iodopindolol binding to intact cells was measured in mouse L cells expressing wild-type BARs (C+I+), mutated BARs that do not couple to G_s but do internalize (C−I+), and mutated BARs that do not couple to G_s and do not internalize (C−I−). For C+I+ and C−I+ cells, most of the receptors exhibited apparent affinities for the agonist isoproterenol that were 500–900-fold lower in equilibrium assays with intact cells than in short-time assays with intact cells or in equilibrium assays with isolated membranes, similar to previous results with cells expressing native BARs. The extent of conversion to this lower affinity form for C−I− cells was markedly decreased. Binding properties for the antagonist metoprolol were similar for all three BARs in both short-time and equilibrium assays. Isoproterenol competition in short-time and equilibrium assays also was compared in Chinese hamster fibroblasts expressing wild-type BARs, mutated BARs that lack BAR kinase sites, mutated BARs that lack cAMP-dependent protein kinase sites, and mutated BARs that lack both types of phosphorylation sites. All three BAR phosphorylation mutants showed only small but significant decreases, relative to the wild-type BAR, in the extent of conversion to the low affinity form. These results provide additional evidence that receptor internalization is the major determinant for the conversion of intact cell BARs to the low affinity form. Receptor phosphorylation may play a minor role in conversion to the low affinity form, whereas receptor coupling to G_s is apparently not required.

In many receptor systems, prolonged agonist stimulation results in subsequent receptor desensitization. BARs are among the most well studied receptor systems in terms of the cellular and molecular mechanisms of agonist-induced desensitization. The current model for BAR desensitization includes a rapid uncoupling of receptors from G_s, followed by receptor sequestration and internalization and, finally, receptor down-regulation (1). Considerable evidence indicates that the uncoupling results from post-translational regulatory phosphorylation of BARs by PKA and BARK (2–4). However, the mechanisms and structural requirements for sequestration, internalization, and down-regulation remain unclear. Receptor phosphorylation appears not to be required for sequestration or internalization but may play some role in down-regulation (3).

In equilibrium competition binding assays, agonists exhibit markedly lower apparent affinity for BARs on intact cells than in isolated membrane preparations. This discrepancy is not observed for antagonists. The low affinity binding of agonists to intact cells results from agonist-induced conversion of BARs from a native high affinity form to the low apparent affinity form during the course of the equilibrium binding assay (5–7). This native high affinity form can be detected in short-time competition binding assays (5–7), in assays with hydrophilic radioligands (8), or in binding assays performed at reduced temperature (9, 10). Similar phenomena have also been shown to occur for α_1-adrenergic receptors (11), α_2-adrenergic receptors (12), and muscarinic acetylcholine receptors (13, 14).

We have postulated that the apparent low affinity observed for agonists in equilibrium competition binding assays with intact cells may be related to agonist-induced internalization of receptors and subsequent inaccessibility of the relatively hydrophilic agonists to the internalized receptors (11, 15, 16). An even lower affinity form of BARs that is observed in short-time competition binding assays with agonist-pretreated cells

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ABBREVIATIONS: BAR, β-adrenergic receptor; BARK, β-adrenergic receptor kinase; PKA, cAMP-dependent protein kinase; CHW, Chinese hamster fibroblast; ISO, isoproterenol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [^{125}I]IPIN, [^{125}I]iodopindolol; Gpp(NH)p, guanosine 5′-(βγ-imido)triphosphate.

almost certainly reflects limited accessibility and slow equilibration with internalized or sequestered receptors (15–17). The question addressed here is whether the low affinity binding observed even after prolonged incubation in equilibrium competition binding assays is also a reflection of internalization or sequestration or whether this phenomenon represents an additional agonist-induced change in these receptors. We have previously shown that permeabilization of cells with the detergents saponin or digitonin prevents both receptor internalization and conversion to low affinity binding for BARs on DDT_1 MF-2 hamster smooth muscle cells (18). Similarly, treatment of cells with antimycin A, which blocks receptor internalization in several cases, including BARs (19), also prevents the conversion of BARs in these cells to the low affinity form (20).

To further examine the roles of receptor internalization, receptor-G_s coupling, and receptor phosphorylation in the low affinity binding, we have characterized the binding properties of intact cells expressing normal BARs or mutated BARs with functional alterations in each of these processes (3, 21). Our results support a major role for receptor internalization in the conversion of BARs to the low affinity form in intact cells.

Experimental Procedures

Materials. Growth medium, serum, and G-418 were purchased from GIBCO. (−)-Pindolol and metoprolol were gifts from Sandoz Ltd. (Basel, Switzerland) and Ciba-Geigy (Summit, NJ), respectively. [^{125}I]-IPIN was synthesized using the chloramine-T method, as described previously (22). Other chemicals were purchased from Sigma Chemical Co.

BAR mutants and cell culture. The L cells expressing BARs defective in internalization and coupling were provided by Dr. Catherine Strader (Merck Sharp & Dohme, Rahway, NJ). The amino-terminal end of the third intracellular loop of the hamster β_2-adrenergic receptor was either deleted (D[222–229]BAR, referred to here as C−I−) or replaced by sequence from the human muscarinic acetylcholine receptor in the corresponding region (M_1[220–230]BAR, referred to here as C−I+), as described previously (21). The CHW cells expressing BARs defective in receptor phosphorylation were provided by Dr. Robert Lefkowitz (Duke University, Durham, NC). Serines and threonines in BARK and PKA phosphorylation sites were substituted with either alanine or glycine, as described previously (3). Mutant A in the previous study (3) is referred to here as the PKA mutant, mutant B in the previous study as the BARK mutant, and mutant AB in the previous study as the BARK/PKA double mutant. Both L cells and CHW cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and G-418 (700 μg/ml for L cells, 10 μg/ml for CHW cells), in a 5% CO_2 atmosphere, at 37°.

Membrane preparation and binding assays. Fresh membranes were prepared as described previously (21). Membrane binding was performed for 60 min at 37° in 75 mM Tris, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA, containing 100 pM [^{125}I]IPIN and the indicated concentrations of competing drugs. Nonspecific binding was defined as the binding occurring in the presence of 1 μM propranolol. Membranes were then filtered and washed with wash buffer (10 mM Tris, pH 7.5, 140 mM NaCl) over S&S no. 30 glass fiber filters, using a Brandel cell harvester. Radioactivity associated with the filters was quantitated by γ counting.

Intact cell binding assays. Cells were incubated at 37° in high-glucose Dulbecco's modified Eagle's medium buffered to pH 7.5 with 20 mM HEPES, containing 100 pM [^{125}I]IPIN and various concentrations of competing drugs, for either 1 min (short-time assays) or 60 min (equilibrium assays). Nonspecific binding was defined as the binding occurring in the presence of 1 μM propranolol. Cells were then washed with high-glucose Dulbecco's modified Eagle's medium/20 mM HEPES, pH 7.5, containing 100 μM propranolol and were dissolved in 0.2 N NaOH. Radioactivity was then quantitated by γ counting.

Receptor internalization assays. As described previously (23), cells incubated for 30 min at 37° in the absence or presence of 10 μM ISO were lysed at 4° with lysis buffer containing 20 mM Tris, pH 7.4, and 2 mM EDTA. Cells were then homogenized with a Tissumizer (Tekmar) and a 3.5-ml portion of the lysate was layered on top of a sucrose density gradient consisting of 3 ml of 55% (w/v), 3 ml of 32%, and 1.5 ml of 5% sucrose, in 12-ml tubes. Centrifugation was at 4° for 60 min at 35,000 rpm in a Beckman SW41 rotor. The membranes at the 5/32% interface were collected as the light vesicle fraction and those at the 32/55% interface were collected as the plasma membrane fraction. Aliquots of each fraction were assayed for BARs as described above for membrane assays.

Data analysis. Data (means ± standard deviations) from three independent competition binding experiments were fit to one- and two-site binding models using the computerized curve-fitting capacity of GraphPAD (San Diego, CA). The one-site fit was used when the two-site fit was not significantly ($p > 0.05$) better than the one-site fit. Only apparent affinities are given (expressed as IC_{50} values, the concentration of competing ligand that inhibits specific binding by 50%) because it is not clear that true "equilibrium" binding is achieved, due to accessibility barriers and the changing properties of the receptors (6, 16).

Results

Competition by the agonist ISO for [^{125}I]IPIN binding to BARs was compared between membrane preparations and intact cells for L cells transfected with the wild-type BAR (C+I+) (Fig. 1; Table 1). Binding to membranes in the presence of guanine nucleotides was better fit by the two-site model, with 76 ± 11% of the receptors exhibiting an IC_{50} of 1.3 ± 0.3 μM and the remaining 24% exhibiting an even higher affintity of 50 ± 30 nM. Binding to intact cells was obviously biphasic, with most of the receptors exhibiting markedly lower affinity than that observed in the membrane assays; 16 ± 1% of receptors exhibited the higher affinity (IC_{50} = 35 ± 9 nM) and the remaining 84 ± 1% of the receptors exhibited much lower affinity (IC_{50} = 100 ± 5 μM). These results with the wild-type

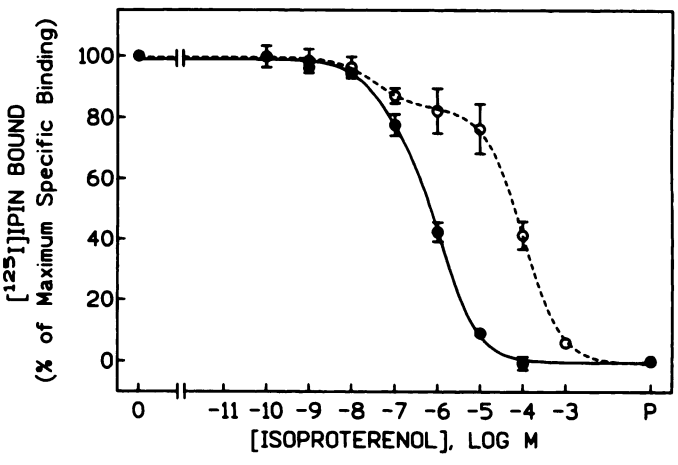


Fig. 1. Comparison of equilibrium binding of the agonist ISO to wild-type BARs of L cell membrane preparations and intact cells. Binding of [^{125}I]-IPIN to BARs in membrane preparations in the presence of 100 μM Gpp(NH)p (●) and on intact cells (○) was measured in the absence (*0*) or presence of the indicated concentrations of the BAR agonist ISO or 1 μM propranolol (*P*), for 60 min. Data are expressed as percentages of maximal specific binding and are means ± standard deviations for three experiments, each performed in duplicate.

TABLE 1

Intact cell binding properties of BAR-transfected L cells for the agonist ISO and the antagonist metoprolol

IC_{50} values and percentages of receptors in each fraction were obtained from data on the displacement of 100 pM [^{125}I]IPIN, as described in Experimental Procedures. Each value represents the mean ± standard deviation of three experiments performed in duplicate.

	I_H[a]	R_H	I_L	R_L
	nM	%	μM	%
C+I+				
ISO				
ST	200 ± 18	85 ± 2	1300 ± 520	15 ± 2
EQ	35 ± 9	16 ± 1	100 ± 5	84 ± 1
Metoprolol				
ST	500 ± 40	100[b]		
EQ	1800 ± 200	100[b]		
C−I+				
ISO				
ST	10 ± 1	69 ± 1	41 ± 8	31 ± 1
EQ	3 ± 1	24 ± 2	9 ± 1	76 ± 2
Metoprolol				
ST	100 ± 30	100[b]		
EQ	600 ± 100	100[b]		
C−I−				
ISO				
ST	12 ± 2	83 ± 3	27 ± 16	17 ± 3
EQ	13 ± 1	65 ± 2	5 ± 2	35 ± 2
Metoprolol				
ST	100 ± 10	100[b]		
EQ	500 ± 30	100[b]		

[a] I_H, high affinity IC_{50}; I_L low affinity IC_{50}; R_H, percentage of receptors in the high affinity form; R_L, percentage of receptors in the low affinity form; ST, intact cell short-time assay; EQ, intact cell equilibrium assay.

[b] Data were adequately fit by a one-site model.

BARs transfected in L cells are similar to those seen previously in cells expressing native BARs (5–7, 24).

The apparent low affinity binding to intact cells observed in equilibrium assays results from a change in the binding properties of the receptors during the course of the assay, as can be demonstrated by comparing short-time versus equilibrium assays with intact cells. Short-time and equilibrium assays of ISO competition for [^{125}I]IPIN binding to intact cells expressing wild-type BARs were compared (Fig. 2A; Table 1). Competition by ISO was better fit by the two-site model for both short-time and equilibrium assays. For short-time assays, most of the receptors (85 ± 2%) were in the higher affinity form, with an IC_{50} of 200 ± 18 nM. This is in marked contrast to the equilibrium assay, where most of the receptors (84 ± 1%) exhibited markedly lower apparent affinity, with an IC_{50} of 100 ± 5 μM. This change in binding properties is specific for agonists only. Fig. 3A shows the comparison of short-time and equilibrium competition binding assays for the antagonist metoprolol with intact cells expressing wild-type BARs. Both short-time and equilibrium assays were adequately fit by the one-site model. There was an approximately 4-fold change in IC_{50} values between the two assays, as expected based on the equation of Cheng and Prusoff (25), as detailed in previous studies (5–7).

To investigate the involvement of receptor-G_s coupling and receptor internalization in conversion to the low affinity form, L cells expressing mutated BARs that do not couple to G_s but internalize normally (C−I+) and mutated BARs that do not couple to G_s and do not internalize (C−I−) were chosen for a comparison of their binding properties in short-time and equilibrium assays (Fig. 2, B and C; Table 1). Competition by the agonist ISO was better fit by the two-site model for both BAR mutants in both assays. In short-time assays, both C−I+ and

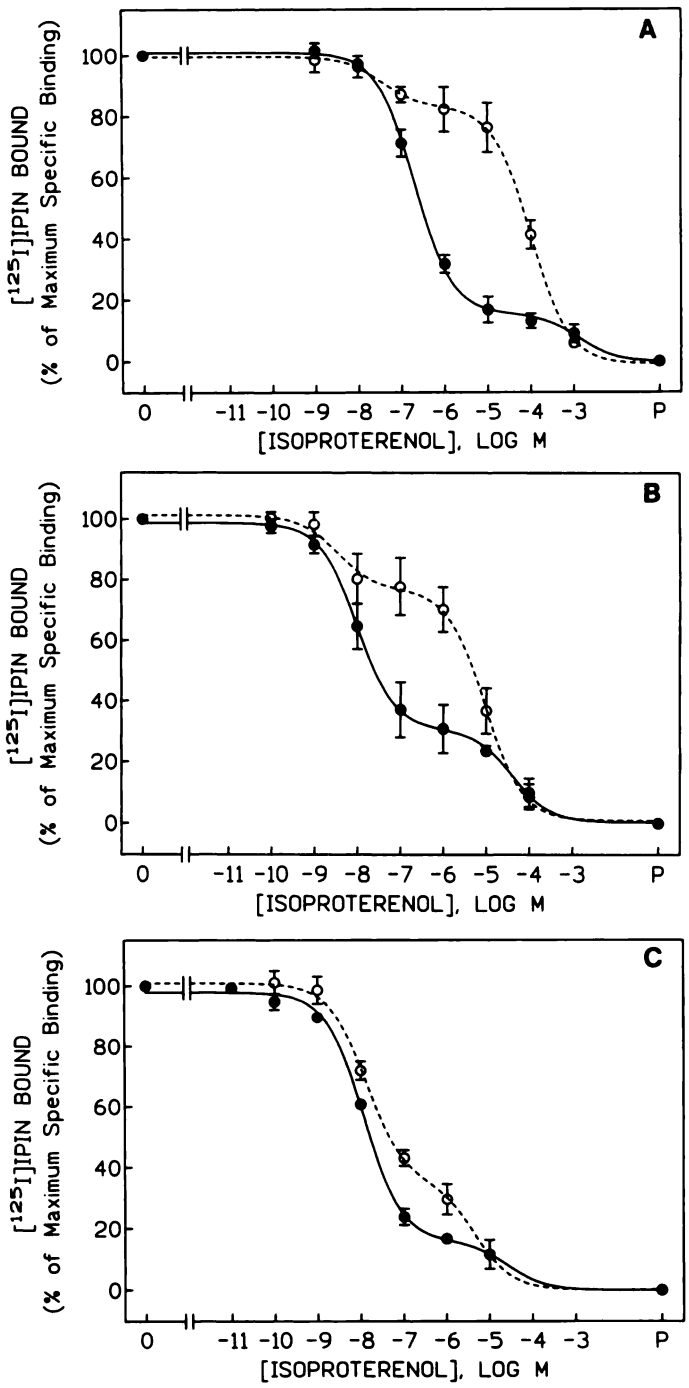


Fig. 2. Comparison of short-time and equilibrium binding of the agonist ISO to wild-type and mutated BARs of intact L cells. Binding of [^{125}I]IPIN to BARs in 1-min (●) and 60-min (○) assays was measured in the absence (*0*) or presence of the indicated concentrations of the BAR agonist ISO or 1 μM propranolol (*P*). Data are expressed as percentages of maximal specific binding and are means ± standard deviations for three experiments, each performed in duplicate. A, Wild-type C+I+; B, mutant C−I+; C, mutant C−I−. Typical values for specific binding in the absence of ISO (100% values) in 1-min assays (cpm/dish) were 7300 for C+I+, 1600 for C−I+, and 3400 for C−I−. The corresponding values in 60-min assays (cpm/dish) were 65,000 for C+I+, 12,000 for C−I+, and 28,000 for C−I−.

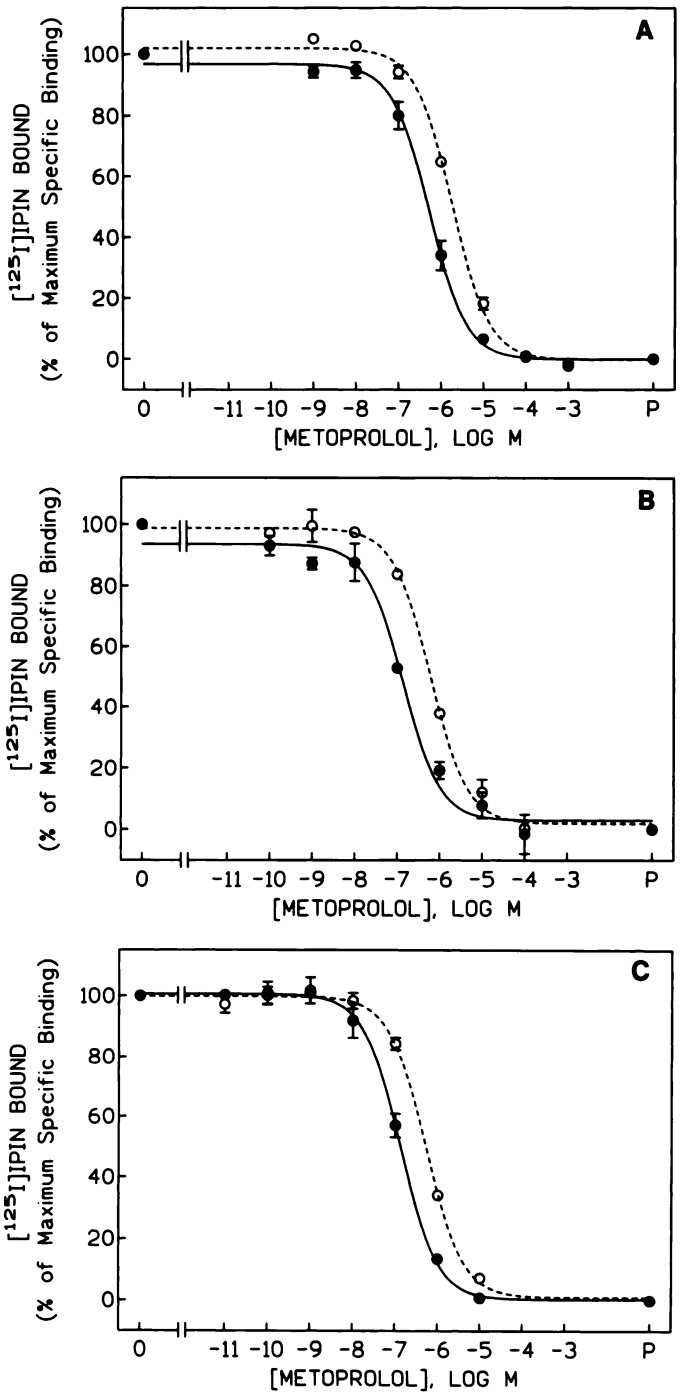


Fig. 3. Comparison of short-time and equilibrium binding of the antagonist metoprolol to wild-type and mutated BARs of intact L cells. Binding of [^{125}I]IPIN to BARs in 1-min (●) and 60-min (○) assays was measured in the absence (*0*) or presence of the indicated concentrations of the BAR antagonist metoprolol or 1 μM propranolol (*P*). Data are expressed as percentages of maximal specific binding and are means ± standard deviations for three experiments, each performed in duplicate. A, Wild-type C+I+; B, mutant C−I+; C, mutant C−I−.

C−I− cells exhibited predominantly high affinity binding (69 ± 1% and 83 ± 3%, respectively), similar to the wild-type BARs. In the equilibrium assays, 76 ± 2% of BARs in C−I+ cells showed markedly lower apparent affinity, similar to the wild-type C+I+ cells. In contrast, the C−I− cells had a much smaller proportion of the receptors in the lower affinity form (35 ± 2%). Although the two mutated BARs each had about 10–20-fold higher affinity for ISO at both the low and high affinity sites than did the wild-type BARs, the difference in IC_{50} values between the predominant high affinity form seen in short-time assays and the lower affinity form seen in equilibrium assays was similar for all three cell types (400–1000-fold).

Short-time and equilibrium assays of competition by the antagonist metoprolol also were compared for the two mutated BARs (Fig. 3, B and C; Table 1). Like the wild-type BAR, binding of metoprolol to the mutated BARs was adequately fit by the one-site model, and there was the expected 4–6-fold difference between IC_{50} values in short-time and equilibrium assays for both BARs. All three BARs showed similar affinities for the antagonist metoprolol.

The internalization properties of the mutated BARs were investigated. Agonist-induced sequestration of cell surface receptors of L cells expressing wild-type and mutated BARs was previously assessed by measuring binding of the hydrophilic antagonist [^{3}H]CGP-12,177 to intact cells on ice (21). Because of the possibility of different characteristics for sequestration and internalization (23), we used the sucrose density gradient centrifugation assay to further assess the property of internalization (Fig. 4). Exposure of cells to the agonist ISO induced a shift of receptors on sucrose density gradients from the plasma membrane fraction to the light vesicle fraction that is thought to represent receptors internalized in endocytotic vesicles. For wild-type C+I+ cells, only 24 ± 2% of the receptors were present in the light vesicle fraction in cells not exposed to ISO, whereas 59 ± 2% were present in the light vesicle fraction in cells exposed to 10 μM ISO for 30 min. A similar shift of receptors to the light vesicle fraction was observed for C−I+ cells (from 17 ± 3% to 49 ± 5%), whereas almost no change was detected for C−I− cells (from 24 ± 3% to 27 ± 2%). These results confirm the defect in internalization for the C−I− cells and are consistent with the previous sequestration assay results (21).

The G_s-coupling properties of the three cell lines reported

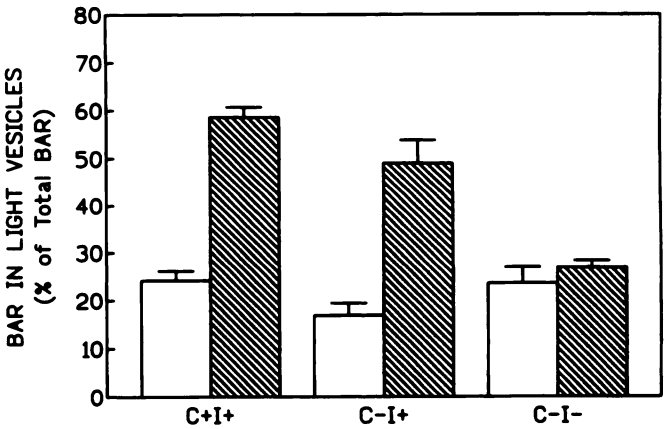
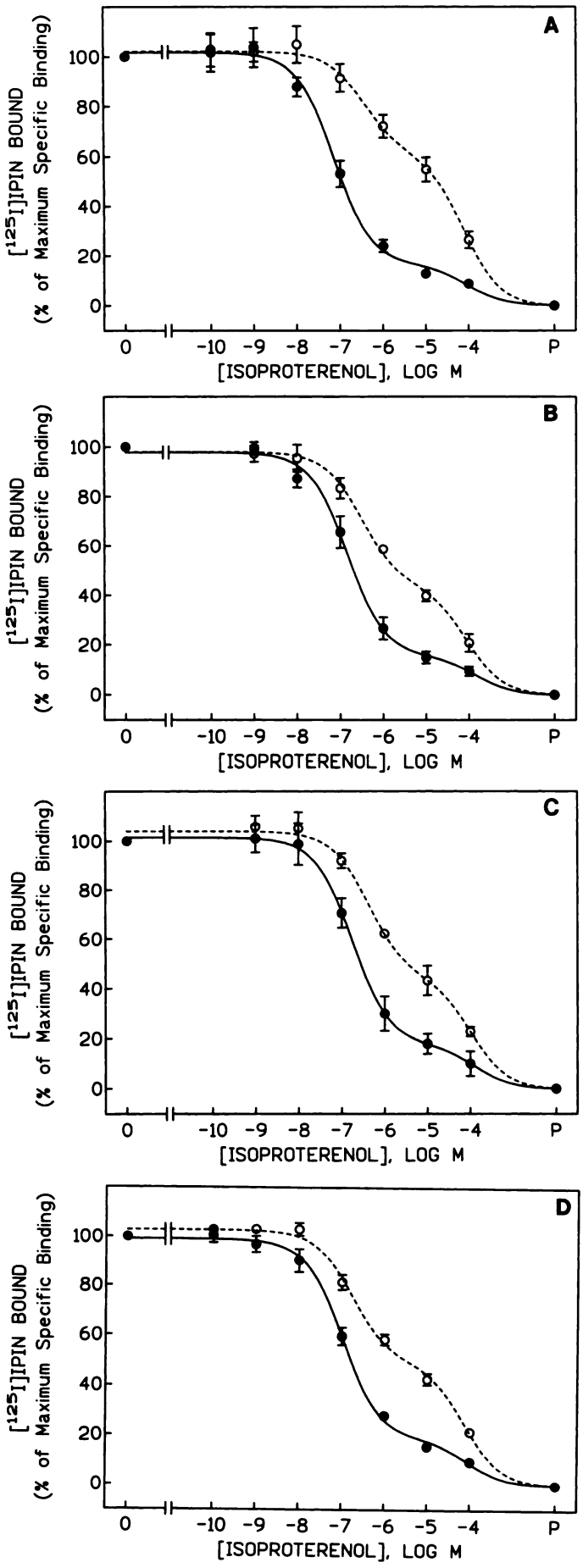


Fig. 4. Agonist-induced internalization of wild-type and mutated BARs of L cells. L cells expressing wild-type C+I+, mutant C−I+, or mutant C−I− BARs were incubated for 30 min in the absence (□) or presence (▨) of 10 μM ISO and were then lysed. The lysates were subjected to sucrose density gradient centrifugation. [^{125}I]IPIN binding to BARs in the light vesicle and plasma membrane fractions from the gradients was determined. Data are expressed as percentages of BARs in the light vesicle fraction and are means ± standard deviations from three independent experiments.



previously (21) were confirmed by measuring competition by ISO for binding to BARs in isolated membranes in the absence and presence of Gpp(NH)p (data not shown). Briefly, addition of Gpp(NH)p caused a marked rightward shift of the binding curve for wild-type BARs, an indication of normal receptor-G_s coupling (26). However, for both BAR mutants binding curves were essentially insensitive to Gpp(NH)p, confirming the lack of G_s coupling in the two mutants.

To investigate the possible role of BARK or PKA phosphorylation sites in the low affinity binding to intact cells, we performed limited experiments with intact CHW cells expressing wild-type BARs, mutated BARs that lack BARK sites, mutated BARs that lack PKA sites, and mutated BARs that lack both BARK and PKA sites. Short-time and equilibrium assays of competition by the agonist ISO for [^{125}I]IPIN binding were compared in each cell line (Fig. 5; Table 2). Most of the receptors were in the higher affinity form in short-time assays for all four BARs. In equilibrium assays, a larger fraction of the receptors were in the low affinity form for all four BARs. However, all four of the BAR-expressing CHW cell lines, including the wild-type line, showed smaller proportions of receptors in the low affinity form than did the L cells expressing the wild-type or C–I+ receptors (only 44–60% in CHW cells versus 76–84% in L cells). The three phosphorylation mutants each showed small but significant decreases (12–15%) in the fraction of receptors present in the low affinity form, compared with the wild-type receptor. However, this difference was much less dramatic than that for the L cell internalization mutant, which had about 50% fewer receptors in the low affinity form than did its corresponding wild-type line.

Discussion

In these studies, cells stably transfected with wild-type BARs showed the same agonist-induced change in intact cell binding properties that has been demonstrated in previous studies of a variety of cells expressing native BARs (5, 7, 24). This allowed us to use this system to investigate the structural features and molecular mechanisms involved in the change in binding properties induced by agonists, using site-directed mutagenesis.

Binding properties of intact L cells transfected with wild-type hamster BARs were similar to those observed in DDT_1 MF-2 hamster smooth muscle cells (11), 1321N1 human astrocytoma cells (6), and S49 mouse lymphoma cells (7, 27). L cells expressing BARs defective in receptor coupling to G_s but normal for internalization (C–I+) showed binding properties similar to those of wild-type BARs, indicating that receptor-G_s coupling is not required for conversion to the low affinity form.

Fig. 5. Comparison of short-time and equilibrium binding of the agonist ISO to wild-type and mutated BARs of intact CHW cells. Binding of [^{125}I]IPIN to BARs was measured in 1-min (●) and 60-min (○) assays in the absence (*0*) or presence of the indicated concentrations of the BAR agonist ISO or 1 μM propranolol (*P*). Data are expressed as percentages of maximal specific binding and are means ± standard deviations for three experiments, each performed in duplicate. A, Wild-type BAR; B, BARK site mutant; C, PKA site mutant; D, BARK/PKA site double mutant. Typical values for specific binding in the absence of ISO (100% values) in 1-min assays (cpm/dish) were 3600 for the wild-type BAR, 4900 for the BARK site mutant, 4100 for the PKA site mutant, and 4100 for the BARK/PKA double mutant. The corresponding values in 60-min assays (cpm/dish) were 38,000 for the wild-type BAR, 47,000 for the BARK site mutant, 41,000 for the PKA site mutant, and 66,000 for the BARK/PKA site double mutant.

TABLE 2

Intact cell binding properties of BAR-transfected CHW cells for the agonist ISO

IC_{50} values and percentages of receptors in each fraction were obtained from data on the displacement of 100 pM [^{125}I]IPIN, as described in Experimental Procedures. Each value represents the mean ± standard deviation of three experiments performed in duplicate.

	I_H*	R_H	I_L	R_L
	nM	%	μM	%
Wild-type				
ST	72 ± 12	84 ± 4	91 ± 58	16 ± 4
EQ	350 ± 110	40 ± 4	75 ± 16	60 ± 4
BARK site mutant				
ST	150 ± 77	84 ± 5	150 ± 110	16 ± 5
EQ	320 ± 180	55 ± 3	84 ± 51	46 ± 3
PKA site mutant				
ST	180 ± 43	82 ± 2	130 ± 98	18 ± 2
EQ	420 ± 270	56 ± 5	100 ± 74	44 ± 5
BARK/PKA site double mutant				
ST	100 ± 12	81 ± 3	87 ± 38	19 ± 3
EQ	170 ± 40	52 ± 3	74 ± 20	48 ± 3

* I_H, high affinity IC_{50}; I_L, low affinity IC_{50}; R_H, percentage of receptors in the high affinity form; R_L, percentage of receptors in the low affinity form; ST, intact cell short-time assay; EQ, intact cell equilibrium assay.

These results are consistent with previous studies showing normal conversion to the low affinity form (7, 27, 28) and normal internalization (29, 30) in S49 cyc$^-$ mutants, which lack functional G_s protein and coupling. In contrast, L cells expressing BARs defective in internalization (C−I−) showed markedly decreased conversion to the low affinity form, indicating that normal receptor internalization is required for conversion to the low apparent affinity form to occur. Together, these results support our hypothesis that this low affinity binding of agonists to BARs is a reflection of receptor internalization and the consequent relative inaccessibility of hydrophilic agonists to the internalized receptors (11, 15, 16).

Although the two BAR mutants and the wild-type BAR all had similar affinities for the antagonist metoprolol, both mutants showed about 10-fold higher affinity for the agonist ISO in assays with membrane preparations and in short-time assays with intact cells than did the wild-type BAR. This increase in agonist affinity in the mutated cells has been discussed previously (21). However, this difference in agonist affinity did not influence the extent of agonist-induced conversion of BARs to the form with low apparent affinity in equilibrium assays for C−I+ cells. This rules out the possibility that the decrease in conversion to the low apparent affinity form observed for the internalization mutant C−I− is due to its higher agonist binding affinity. The differences between the lower and higher affinity values in short-time and equilibrium assays in intact cells were similar for all three BARs; however, the fraction of receptors converted to low affinity was markedly lower in C−I− cells than in C+I+ or C−I+ cells. The cells with the highest (C+I+) and lowest (C−I+) levels of receptor expression showed similar marked conversion to the low affinity form, whereas the C−I− cells, which showed only minimal conversion to the low affinity form, had an intermediate level of receptor expression (see the legend to Fig. 2). Thus, the differences in conversion to the low affinity form are not likely to be related to differences in receptor expression levels among the three cell types.

Previous studies in our laboratory presented evidence that sequestration and internalization are separable steps for α_1-adrenergic receptors in DDT_1 MF-2 hamster smooth muscle cells and that sequestration alone might be sufficient to induce low affinity with α_1-adrenergic receptors on intact cells (11, 23, 31). A two-step internalization pathway may also occur for BARs (32–34). The previous studies of the L cell mutants used the hydrophilic antagonist [^{3}H]CGP-12,177 to label cell surface BARs, and the loss of [^{3}H]CGP-12,177 binding sites provided an assay for receptor sequestration (21). We used sucrose density gradient assays, which presumably monitor receptor internalization into endocytotic vesicles (34). Results in our internalization assays were similar to the previous sequestration assays, indicating that C−I+ is normal for both sequestration and internalization and that C−I− is defective in both processes.

Phosphorylation by multiple kinases has been shown to be involved in BAR desensitization (2–4). However, studies with kinase inhibitors and with mutations at BARK and PKA phosphorylation sites suggest that phosphorylation is not required for sequestration to occur (3, 4). In particular, previous studies with the CHW phosphorylation mutants used here showed normal receptor sequestration for all three mutants (3). Based on our hypothesis that the conversion to low affinity is due to agonist-induced receptor internalization, we therefore predicted that these phosphorylation mutants would show normal conversion to the low affinity form. All three phosphorylation mutants exhibited agonist-induced conversion to the low affinity form, although the extent of conversion was somewhat smaller than for the wild-type BAR. Thus, the possible involvement of phosphorylation cannot be excluded. However, the possibility that the phosphorylation modifications also altered other aspects of receptor function cannot be eliminated. In preliminary experiments using sucrose density gradient centrifugation to assess BAR internalization, rather than the CGP-12,177 competition binding assay used in previous studies to assess BAR sequestration (3), we observed somewhat lower internalization with the BARK/PKA site double mutant than with the wild-type BAR (data not shown). Two recent studies have presented evidence that more selective mutations of specific potential BARK phosphorylation sites resulted in dramatic inhibition of sequestration (35, 36). Thus, additional studies will be required to establish the roles of specific serine and threonine residues, either as structural determinants or as regulatory phosphorylation sites, in receptor sequestration and internalization and in conversion of receptors to the low affinity form.

These current results with mutated BARs are consistent with our previous studies of the involvement of receptor internalization in the low affinity binding phenomenon. Permeabilization of cells with detergents (18) or ATP depletion with antimycin A (20) prevented both receptor internalization and conversion to the low affinity form for BARs of DDT_1 MF-2 cells. ATP depletion with antimycin A also prevented both receptor internalization and conversion to the low affinity form for muscarinic acetylcholine receptors of 1321N1 human astrocytoma cells (13). Previous reports have also shown that high affinity binding of agonists to both BARs and muscarinic receptors of intact cells is observed if the radioligand used is very hydrophilic and thus able to label only cell surface receptors, whereas lower affinity is observed with lipophilic radioligands that presumably label both cell surface and sequestered or internalized receptors (8, 13, 14). Together, these studies provide considerable evidence in support of our hypothesis that

this low affinity is due to agonist-induced receptor internalization. Additional approaches will be required to fully explain the mechanism of the low affinity binding of agonists observed with intact cells. Further testing of additional mutants with selective defects in receptor sequestration, internalization, and phosphorylation will be useful. More detailed characterization of the possibly different nature of the "sequestration" and "internalization" steps, including identification of selective inhibitors of these two steps, may allow a fully quantitative explanation of the agonist-binding properties of these receptors measured on intact cells.

Acknowledgments

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