0026-895X/86/050459-04$02.00/0

MOLECULAR PHARMACOLOGY, 30:459–462

Phenylarsine Oxide Inhibits Agonist-Induced Changes in Photolabeling But Not Agonist-Induced Desensitization of the β-Adrenergic Receptor

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Received May 19, 1986; Accepted August 22, 1986

SUMMARY

In the human lymphocyte, desensitization of the β-adrenergic receptor-adenylate cyclase complex is associated with sequestration of the receptor as well as a change in photolabeling of β-receptor proteins. Thus, desensitization of the lymphocyte β-adrenergic receptor-adenylate cyclase system is associated with a selective reduction in the photoaffinity labeling of an $M_r \simeq$ 55,000 β-adrenergic receptor-binding site as compared to an $M_r \simeq$ 68,000 β-adrenergic receptor-binding moiety. In order to examine the relationship between sequestration and reduction in labeling of the $M_r \simeq$ 55,000 peptide, we have studied the effect of phenylarsine oxide (an inhibitor of β-receptor sequestration in astrocytoma cells) on agonist-induced desensitization of the β-adrenergic receptor-adenylate cyclase system in circulating lymphocytes. Incubation of cells with phenylarsine oxide prior to exposure to agonists did not block the consequent reduction in isoproterenol-stimulated adenylate cyclase activity. However, sequestration of the receptor, as assessed by a decrease in accessibility of β-adrenergic receptors on intact cells to hydrophilic receptor ligands, is blocked by phenylarsine oxide. Thus, the agonist-induced reduction in binding of the hydrophilic β-adrenergic receptor ligand CGP-12177 was blocked by phenylarsine oxide (without phenylarsine oxide, 57 ± 6% of control, with phenylarsine oxide, 97 ± 3% of control). Photolabeling studies with [^{125}I]iodocyanopindolol diazirine revealed that phenylarsine oxide pretreatment also blocked the selective loss in labeling of the $M_r \simeq$ 55,000 β-adrenergic receptor protein. These data suggest that agonist-induced alterations in the photolabeling pattern of the lymphocyte β-adrenergic receptor that occur with desensitization closely parallel the apparent sequestration of β-adrenergic receptors but can be dissociated from the initial desensitization phenomenon.

Several mechanisms have been implicated in the process of desensitization of β-adrenergic receptor-adenylate cyclase complex. It has been noted in several systems that agonist-induced desensitization is characterized by a functional uncoupling of the receptor from the other components of the β-receptor-adenylate cyclase complex prior to any prominent loss in receptor density, i.e., prior to "down-regulation." It has been suggested that this uncoupling may be due to a physical sequestration of the receptor from other components of the adenylate cyclase system (1, 2). A number of laboratories have also demonstrated alterations in the structure of the β-receptor which parallel desensitization, namely, an apparent phosphorylation of the β-adrenergic receptor (3–6) which results, in some cases, in an altered migration of the photolabeled β-adrenergic receptor on SDS-PAGE (5–7).

R. D. F. is the recipient of the George Morris Piersol Research and Teaching Scholarship of the American College of Physicians. This study was supported by United States Public Health Service Grant HL 32501-1.

Desensitization of the human lymphocyte-β-receptor complex results in both sequestration of receptors and alterations in patterns of photoaffinity labeling. In recent studies from our laboratory the lymphocyte β-receptor has been characterized by two major polypeptide bands with $M_r \simeq$ 68,000 and $M_r \simeq$ 55,000 (8). Short-term desensitization studies (10 min of agonist exposure) have demonstrated a relative reduction in labeling of the $M_r \simeq$ 55,000 polypeptide and broadening of the $M_r \simeq$ 68,000 band (8). The lymphocyte-β-adrenergic receptor system also demonstrates apparent agonist-induced sequestration as assessed by the reduced accessibility of the hydrophilic ligand CGP-12177 following exposure to agonist (9–11).

The present studies were performed to determine whether there was a direct relationship between agonist-induced alterations in β-receptor-specific photolabeling patterns and in β-receptor sequestration, i.e., altered accessibility to hydrophilic ligands. We studied the effect of PAO on these two properties of the lymphocyte β-adrenergic receptor system be-

ABBREVIATIONS: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAO, phenylarsine oxide; EDTA, ethylenediaminetetraacetate; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute medium; FCS, fetal calf serum; DTT, dithiothreitol; IPIN, iodopindolol; ICYP, iodocyanopindolol.

fore and after agonist exposure since PAO has previously been demonstrated to block internalization of epidermal growth factor (12) and insulin (13) receptors and the internalization of β-adrenergic receptor triggered by agonist occupany of the receptor (14). We observe that PAO does not block functional desensitization of the β-adrenergic receptor complex but, interestingly, does block receptor sequestration. Therefore, under these conditions, receptor sequestration cannot be the mechanism by which receptor-stimulated adenylate cyclase activity is reduced. Similarly, PAO blocks agonist-induced changes in β-receptor photolabeling. Thus, agonist-induced alterations in β-receptor photoaffinity labeling appear to be directly related to β-receptor sequestration.

Materials and Methods

Preparation of cells. Whole blood was taken from healthy, normotensive volunteers aged 18–35 who had taken no medication for at least a week before the procedure. Lymphocytes were isolated from EDTA-anticoagulated blood according to the method of Boyum (15), as we have previously described (16). The isolated mononuclear cells were washed with ice-cold PBS (1.9 mM monobasic sodium phosphate, 8.1 mM dibasic sodium phosphate/0.84% NaCl/3 mM EDTA adjusted to pH 7.6 at room temperature) and centrifuged at 300 × *g* for 15 min at 4°. The supernatant was discarded, and the cells were resuspended in ice-cold PBS and centrifuged at 300 × *g* for 15 min at 4°. The supernatant was again discarded and the pellet was resuspended in RPMI/5% FCS.

Lymphocyte suspensions were incubated initially in the presence of dimethyl sulfoxide (0.1%) and in the presence or absence of PAO and/or other reagents for 15 min at 37° (see Table 1). Excess glutathione (1 mM) was also added (except as noted in Table 1) to block the nonspecific oxidizing effects of PAO (14). Following this, a second 10-min incubation in the presence or absence of isoproterenol (10 μM) was performed at 37°. All subsequent steps were performed at 4°. The cells were pelleted at 400 × *g* for 10 min and resuspended in an RPMI/5% FCS/5 mM DTT solution and pelleted at 400 × *g* for 10 min. The cells were then suspended and washed as above, first in RPMI/FCS, then in RPMI alone. In all experimental conditions, cell viability, as assessed by trypan blue exclusion, exceeded 85%. For radioligand binding studies, cells were then resuspended in PBS, washed as above, and resuspended in PBS at a final density of 4–6 × 10^6 cells/100 μl. For studies of adenylate cyclase activity, a broken cell preparation was used as previously described (16). Lysates for photoaffinity labeling were prepared as previously described (8). Preliminary studies demonstrated that the alterations in adenylate cyclase activity upon desensitization seen in the lymphocyte lysates used for photolabeling paralleled the changes seen in broken cell preparations.

Radioligand binding studies. β-Receptor binding studies were performed according to modifications of previously described methods, using the antagonist [^{125}I]IPIN as the monitor of β-receptor binding activity (2.2 Ci/μmol, New England Nuclear) (16). [^{125}I]IPIN and other drugs were prepared in 1.25 mM ascorbic acid as an antioxidant and with 10 μg/ml of bovine serum albumin. For saturation binding studies, concentrations of [^{125}I]IPIN ranging from 15 to 150 pM were used in each assay.

Binding assays were started by adding 100 μl of the intact cell preparation containing 4–6 × 10^6 cells into the incubation mixture containing the radioligand and competing drugs. The final volume of the incubation was 250 μl. The assays were performed in disposable polystyrene test tubes (Fisher Scientific). After incubation for 90 min at 25°, a duration sufficient to reach steady state for all concentrations of radioligand, the reactions were diluted to 10 ml with a 1/10 dilution of PBS at 25°. The assay tubes were incubated for an additional 3 min. Incubation was terminated by rapid vacuum filtration (Whatman GF/C filters). Each filter was washed with an additional 10 ml of diluted buffer (25°) and radioactivity was determined (Beckman Gamma 4000). Nonspecific binding was defined as that binding not blocked by 1 μM propranolol.

The proportion of specific binding blocked by the hydrophilic ligand CGP-12177 (30 nM) was used as a measure of surface-accessible (i.e., nonsequestered) β-receptor sites using a method similar to that of Hertel *et al.* (14).

Adenylate cyclase assays. Adenylate cyclase activity was determined by the conversion of [^{32}P]ATP to [^{32}P]cAMP as we have previously described (17). One hundred to 200 μg of protein suspended in 75 mM Tris-HCl and 25 mM $MgCl_2$ were added to a final incubation volume of 100 μl. To assess catalytic activity in the absence of a β-receptor agonist, activity was determined by the addition of NaF (3 mM) in the absence of GTP. β-Adrenergic receptor-stimulated activity was assayed with isoproterenol (100 μM) in the presence of GTP (100 μM).

Photoaffinity labeling studies. Covalent labeling of the β-receptor was performed using the radioligand [^{125}I]ICYP-diazirine according to modifications of our previous methods using [^{125}I]*p*-azidobenzylcarazolol (8). Washed lymphocyte lysates (at a β-receptor concentration of about 50 pM) were incubated in 75 mM Tris, 3 mM EDTA, 15 mM $MgCl_2$, and ICYP-diazirine (80–120 pM) for 60 min at 30° in the dark. The protocol was otherwise unchanged from that described above, with the following exceptions: with ICYP-diazirine, photolysis was performed for 20 min at 4° without transfer to a Petri dish. ICYP-diazirine specifically labeled two major polypeptides of $M_r \simeq 55{,}000$ and $M_r \simeq 68{,}000$, as previously described with *p*-azidobenzylcarazolol, and was blocked by co-incubation with unlabeled ligands with a potency and stereoselectivity characteristic of a β_2-adrenergic receptor (data not shown).

Results

Exposure of lymphocytes to isoproterenol (10 μM for 10 min) resulted in a greater than 50% reduction in isoproterenol-stimulated adenylate cyclase activity without reduction in NaF-stimulated activity (Table 1). Intact cell radioligand binding studies demonstrated that this protocol did not result in a

TABLE 1

Agonist-induced alterations in lymphocyte adenylate cyclase activity: Effects of PAO

All samples were incubated with dimethyl sulfoxide (0.1%) and glutathione (1 mM—except for the PAO/DTT samples). PAO and PAO + ISO samples were also initially incubated with PAO (65 μM). PAO/DTT + ISO samples were incubated with PAO (65 μM) and DTT (5 mM). ISO, PAO + ISO, and PAO/DTT + ISO samples were then incubated for 10 min with isoproterenol 10 μM. For the ISO + PAO sample, cells were *initially* incubated with isoproterenol (10 μM) for 10 min and PAO was *subsequently* added in a second 15-min incubation. The data represent the mean ± standard deviation from three experiments comparing all conditions and one experiment comparing Control and PAO only. The activities in these studies in the presence of GTP alone were (pmol of cAMP/min/mg): Control, 155 ± 37; PAO, 97 ± 39; ISO, 116 ± 36; PAO + ISO, 95 ± 34; PAO/DTT + ISO, 87 ± 41; ISO + PAO, 100 ± 48. Isoproterenol-stimulated activity is expressed as the increase in activity over GTP-stimulated levels. NaF-stimulated activity is expressed as the increase in activity over basal levels.

Condition	Lymphocyte exposure		Stimulation of adenylate cyclase activity by:	
	First incubation period	Second incubation period	ISO[a]	NaF
			% over GTP-stim.	% over basal
Control			77 ± 7	586 ± 49
PAO	PAO		78 ± 6	425 ± 29
ISO		ISO	24 ± 6	648 ± 38
PAO + ISO	PAO	ISO	36 ± 5	702 ± 204
PAO/DTT + ISO	PAO/DTT	ISO	28 ± 1	796 ± 129
ISO + PAO	ISO	PAO	33 ± 16	722 ± 24

[a] ISO, isoproterenol.

decrease in β-receptor density (control, 596 ± 30 sites/cell; desensitized, 637 ± 25) but was associated with a 40% reduction in binding which was accessible to the hydrophilic ligand CGP-12177 (control, 93 ± 1% of propranolol-competable binding; desensitized, 53 ± 3%). The data suggest that the receptors have not been "lost" (i.e., degraded) upon agonist exposure, since they all can be detected with the hydrophobic antagonist [^{125}I]IPIN. Nonetheless, agonist exposure has altered the cellular distribution of receptors since 40% of the receptors are no longer accessible to the hydrophilic ligand CGP-12177. [Previous studies from our laboratory have demonstrated that, under similar conditions of agonist-induced desensitization, there is no reduction in total β-receptor density detectable in the broken cell lysates used for photoaffinity labeling (8).]

Incubation of cells with PAO was associated with reductions in basal and stimulated adenylate cyclase activity as expressed in absolute terms (i.e., pmol of cAMP/min/mg of protein). However, similar to the data reported in astrocytoma cells by Hertel *et al.* (14), the degree of stimulation of adenylate cyclase activity to isoproterenol was not reduced by PAO (Table 1). Furthermore, PAO pretreatment did not alter the agonist-induced reduction in β-adrenergic-stimulated adenylate cyclase activation which is characteristic of desensitization (Table 1). In intact cell radioligand binding studies, PAO pretreatment of lymphocytes followed by extensive washing (cf. Materials and Methods) was not associated with any alterations in receptor density (control, 596 ± 30 sites/cell; PAO, 593 ± 73 sites/cell; $n = 4$) K_D for [^{125}I]IPIN (control, 25 ± 12 pM; PAO, 21 ± 7 pM) or in CGP-12177-accessible labeling (control, 93 ± 1% of propranolol-competable binding; PAO, 91 ± 2%). However, the agonist-induced reduction in binding accessible to competition with CGP-12177 was blocked by PAO pretreatment (Table 2), suggesting that PAO blocks agonist-provoked receptor redistribution. Co-incubation with the bifunctional thiol reagent DTT (but not the monofunctional reagent glutathione) reversed this effect consistent with the findings of Hertel *et al.* (14) in the astrocytoma β-receptor. Furthermore, incubation of lymphocytes with PAO *after* agonist pretreatment was not effective in blocking the agonist-induced reduction in CGP-12177-accessible binding (Table 2).

PAO pretreatment also blocked the pattern of agonist-induced alterations in β-receptor photolabeling (Fig. 1). With PAO exposure alone (Fig. 1, *lane 1*), there is β-receptor-specific labeling of two polypeptides ($M_r \simeq 55{,}000$ and $M_r \simeq 68{,}000$) as seen in previous control studies. As illustrated, and as we have previously demonstrated (8), exposure to isoproterenol results in a relative reduction in labeling of the $M_r \simeq 55{,}000$ polypeptide and broadening of the band at $M_r \simeq 68{,}000$ (Fig. 1, *lane 2*). PAO pretreatment of lymphocytes *prior to* isoproterenol exposure blocks these agonist-induced changes in β-receptor photolabeling (Fig. 1, *lane 3*). This did not appear to be a nonspecific effect of PAO on labeling of the $M_r \simeq 55{,}000$ moiety since PAO exposure *after* isoproterenol incubation did not block the agonist-induced alterations in β-receptor photolabeling (Fig. 1, *lane 5*). Thus, agonist-induced alterations in β-receptor photolabeling were only evident under those conditions where CGP-12177-accessible binding was also reduced (Fig. 1, *lanes 2, 4, and 5*).

TABLE 2

Agonist-induced alterations in [^{125}I]IPIN binding to intact lymphocytes: Effects of PAO

Conditions for lymphocyte exposure	Receptor density	K_D for IPIN	Percentage of receptors accessible to CGP-12177[a]
	sites/cell	*pM*	
PAO only	593 ± 73	21 ± 7	91 ± 2
ISO only[b]	637 ± 25	29 ± 14	53 ± 3
PAO followed by ISO	615 ± 51	27 ± 12	88 ± 3
PAO/DTT followed by ISO	596 ± 63	18 ± 5	56 ± 3
ISO followed by PAO	716 ± 50	32 ± 11	48 ± 2

[a] Percentage of receptors accessible to CGP-12177 was defined as the mean of:

$$\% \frac{\text{CGP-12177 competable}}{\text{Propranolol competable}} [^{125}\text{I}]\text{IPIN binding}$$

at each of four concentrations of [^{125}I]IPIN. The data represent the mean ± standard deviation from four experiments performed in triplicate.

[b] ISO, isoproterenol.

Discussion

In several models of the β-adrenergic receptor, exposure to agonist hormones leads to three alterations in receptor properties: (a) a reduction in β-adrenergic-mediated adenylate cyclase activity, (b) redistribution of the receptor, and (c) alterations in the photolabeling of β-receptor polypeptides (1–7). Whether these alterations in receptor redistribution and photolabeling represent the cause or the effect of functional desensitization is unclear. Furthermore, the relationship between receptor redistribution and alterations in photolabeling has not previously been explored.

Our data indicate that PAO pretreatment blocks agonist induced receptor redistribution and alterations in photolabeling without blocking agonist-induced desensitization. Thus, consistent with the findings of DeBlasi *et al.* (9), Hertel *et al.* (14), and Kassis *et al.* (18), our studies indicate that receptor redis-

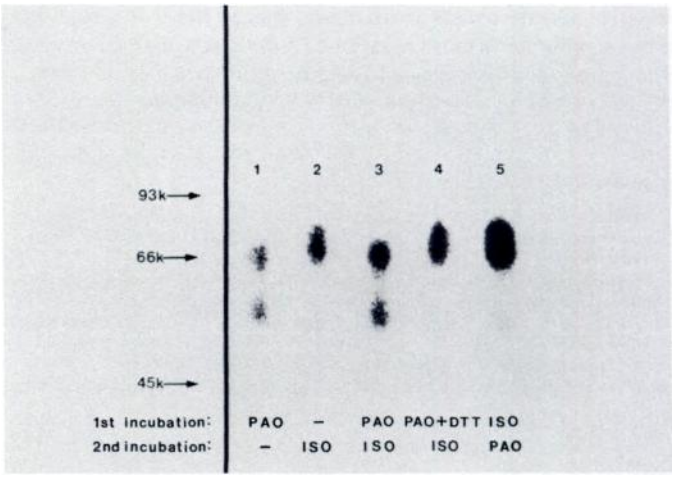

Fig. 1. Alterations in ICYP-diazirine photoaffinity labeling induced by preexposure to a β-adrenergic agonist: Effects of PAO (autoradiograph). Lymphocytes were exposed to PAO, isoproterenol (*ISO*), and DTT as noted. Lysates were prepared and photoaffinity labeling was performed as described under Materials and Methods. Proteins were separated by SDS-PAGE and β-receptor-associated polypeptides were identified by autoradiography ($M_r \simeq 68{,}000$ and $M_r \simeq 55{,}000$ as illustrated in *lane 1*). The *arrows* to the *left* indicate the relative mobility of known standards (Bio-Rad): phosphorylase *b*, $M_r = 93{,}000$; bovine serum albumin, $M_r = 66{,}000$; ovalbumin, $M_r = 45{,}000$. The experiment was repeated three times with the same results.

tribution cannot necessarily account for functional desensitization, as has been argued in the rat lung model system (19).

The novel finding in these studies is that PAO treatment also dissociates the changes in β-receptor photolabeling from the reduction in β-adrenergic-mediated adenylate cyclase activity. In the turkey erythrocyte, altered migration of photolabeled β-receptor polypeptides on SDS gels has been thought to be due to phosphorylation and that phosphorylation of the receptor leads to the functional desensitization of the complex (1). The altered migration of the 68,000-Da polypeptide of the lymphocyte β-receptor may represent an analogous event. If so, our studies suggest that agonist-induced changes in β-receptor photolabeling can be dissociated from the process of desensitization and might instead represent a signal or mechanistic event involved in receptor sequestration.

The significance of the two β-adrenergic receptor-associated polypeptides and the selective reduction of the $M_r \simeq 55{,}000$ polypeptide with desensitization is unclear. The finding of two major polypeptide bands has been described in many models of the β-receptors studied using photoaffinity labeling. In particular, the S49 lymphoma β-receptor demonstrates two polypeptide bands ($M_r \simeq 55{,}000$ and $\simeq 65{,}000$), and a similar reduction in photolabeling of the $M_r \simeq 55{,}000$ band has been described with desensitization of the receptor (6). Tryptic digests have suggested a precursor-product relationship between the $M_r \simeq 65{,}000$ and $\simeq 55{,}000$ polypeptides (20). A recent study by Moxham and Malbon (21), using a partially purified radioiodinated preparation of fat cell β_1-receptors, suggested an alternate explanation for the two major polypeptide bands, *viz.*, the lower molecular weight band represents the persistence of an oxidized form of the receptor and the higher molecular weight species represents the reduced form. If so, one might speculate that desensitization results in a reduction of certain receptor peptides and PAO acts by blocking this agonist-induced reduction. However, although the molecular explanation for the interconversion between the two molecular weight β-adrenergic receptor species awaits further evaluation, these studies nonetheless indicate a close relationship between agonist-induced β-receptor sequestration and alteration in labeling of β-receptor-associated polypeptides in the human lymphocyte-β-adrenergic receptor complex.

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