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Evidence for α_1-Adrenergic Receptor Internalization in DDT_1 MF-2 Cells Following Exposure to Agonists plus Protein Kinase C Activators

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SUMMARY

Agonist-induced sequestration and internalization of α_1-adrenergic receptors were examined in DDT_1 MF-2 cells. Pretreatment of cells with epinephrine or norepinephrine alone, but not with phorbol 12-myristate 13-acetate alone, resulted in a marked decrease in [^{3}H]prazosin binding to intact cells at 4°. These pretreatments resulted in little or no change in the fraction of α_1-adrenergic receptors exhibiting limited accessibility to the hydrophilic competing ligand epinephrine in short-time competition binding assays with intact cells and little or no change in the subcellular distribution of α_1-adrenergic receptors between the plasma membrane and light vesicle compartments as assessed by sucrose density gradient centrifugation assays. Pretreatment with a combination of agonist plus phorbol 12-myristate 13-acetate resulted in a greater decrease in [^{3}H]prazosin binding at 4° than was observed when cells were pretreated with agonist alone, induced the conversion of about half of cell surface α_1-adrenergic receptors to a form exhibiting limited accessibility to epinephrine in short-time assays, and induced a shift of about half of α_1-adrenergic receptors from the plasma membrane fraction to a light vesicle fraction on sucrose density gradients. A similar shift of α_1-adrenergic receptors was observed on sucrose density gradients after exposure to agonist plus either mezerein or β-phorbol didecanoate, but not with agonist plus α-phorbol didecanoate, indicating involvement of protein kinase C. These results suggest that pretreatment with agonist alone induces the sequestration of α_1-adrenergic receptors into a compartment that is inaccessible to [^{3}H]prazosin at 4° but that is accessible to hydrophilic ligands at 37° and remains associated with the plasma membrane. In contrast, α_1-adrenergic receptors are apparently internalized from the plasma membrane to a separate compartment, presumably an intracellular vesicle, when cells are pretreated simultaneously with a combination of agonist plus a protein kinase C activator.

Many receptor-coupled cellular responses become desensitized as the result of agonist-induced changes in the chemical and physical properties of their signal transduction pathways. These alterations limit the response to a subsequent challenge with agonist and often occur at the level of the receptor. In some systems, receptor sequestration or internalization appears to occur as a part of the overall process of agonist-induced desensitization (1).

Catecholamines and other agonists bind to AAR and elicit specific cellular responses that are mediated by the hydrolysis of membrane polyphosphoinositides and the formation of the second messengers inositol trisphosphate and diacylglycerol. Inositol trisphosphate acts to release Ca^{2+} from intracellular storage sites and diacylglycerol activates protein kinase C (2–4). Agonist binding to AAR has also been shown to induce desensitization of AAR-coupled phosphoinositide turnover (5–8) and to cause alterations in the agonist-binding properties of AAR on intact cells (8–13). Phorbol esters such as PMA, which are thought to mimic diacylglycerol and directly activate protein kinase C (4), inhibit agonist-stimulated phosphoinositide turnover in a variety of receptor systems, including AAR (11, 14–17). PMA also alters the agonist-binding properties of AAR (11, 14, 16). These findings suggest that protein kinase C may be involved in feedback inhibition of AAR function, inasmuch as these receptors are coupled to the formation of diacylglycerol.

The possible role of receptor sequestration or internalization in the regulation of AAR has not been extensively investigated and remains unclear. In contrast, sequestration and internalization of BAR are thought to occur as part of the overall process of agonist-induced desensitization of BAR-coupled adenylate cyclase (18–21). BAR sequestration and internalization have been well characterized in a variety of cell lines using several different methods.

First, sucrose density gradient centrifugation has been used to detect agonist-induced internalization of BAR (22, 23). In-

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ABBREVIATIONS: AAR, α_1-adrenergic receptors; DDT cells, DDT_1 MF-2 cells; BAR, β-adrenergic receptors; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2 ethane sulfonic acid.

ternalization is reflected as a shift of receptors from a high sucrose density fraction containing plasma membrane markers to a lower sucrose density fraction containing markers for intracellular vesicles (22, 24). Epidermal growth factor receptors, which are known to be internalized based on immunocytochemical electron microscopy studies (25), exhibit an agonist-induced shift to the light vesicle fraction on sucrose density gradients that is similar to that for BAR (1, 26, 27).

Second, BAR sequestration has been characterized using short-time assays of competition by hydrophilic ligands for [^{125}I]iodopindolol binding to intact cells (10, 11, 28). Competition curves generated using hydrophilic ligands are best fit to a two-site model in which a fraction of BAR exhibit very low apparent affinity. This very low apparent affinity form of BAR is thought to result from the relative inaccessibility and therefore slow equilibration of the hydrophilic competing ligand with sequestered BAR. The apparent low affinity observed in these assays does not reflect the true affinity of these BAR for the ligand. Most of the BAR exhibiting limited accessibility to hydrophilic ligands in short-time assays migrate with the light vesicle fraction on sucrose density gradients (23).

Third, [^{125}I]iodopindolol appears to selectively label cell surface BAR when binding to intact cells is assayed at 4° (29). Presumably, sequestered BAR are inaccessible to this radioligand under these conditions, due to a temperature-dependent decrease in the permeability of the plasma membrane to the radioligand. Again, most of the BAR exhibiting limited accessibility to [^{125}I]iodopindolol in assays at 4° also migrate in the light vesicle fraction on sucrose density gradients. Thus, in the case of BAR, the agreement among all three of these assays suggests that receptor internalization occurs during the course of agonist-induced desensitization of BAR-coupled adenylate cyclase.

In the case of AAR, in contrast, these assays have produced conflicting results with regard to the occurrence of receptor sequestration or internalization. We reported that pretreating suspended DDT cells with the AAR agonist epinephrine or with PMA resulted in desensitization of AAR-coupled phosphoinositide turnover but did not appear to induce AAR internalization as assessed by short-time competition binding assays and by sucrose density gradient centrifugation assays (11). Similarly, AAR internalization was not detected using these assays with monolayer rather than suspension cultures of DDT cells (10). In contrast, De Blasi and co-workers (8, 12, 13) recently reported that agonists can induce sequestration of AAR in DDT cells, as indicated by a decrease in the binding of [^{3}H]prazosin to intact cells assayed at 4°.

The present study examines the reasons for the difference in results obtained previously by comparing all three assays for AAR internalization in DDT cells after pretreatment with either agonist alone, PMA alone, or a combination of agonist plus PMA. These results are compared with those for agonist-induced changes in BAR in the same cell line. The results suggest that the apparent discrepancy described above reflects differences in the extent of receptor sequestration or internalization detected by the different assays. The data also demonstrate that agonist-induced regulation of AAR and BAR are distinctly different in DDT cells. Finally, evidence is provided to suggest that pretreatment of DDT cells with a combination of agonist plus a protein kinase C activator leads to translocation of cell surface AAR to an intracellular vesicle fraction as demonstrated by a shift of AAR on sucrose density gradients.

Methods

Materials. DDT cells were provided by Dr. J. S. Norris (University of Arkansas for Medical Sciences, Little Rock, AR). Concanavalin A was obtained from Calbiochem (La Jolla, CA). Phentolamine was a gift from Ciba Pharmaceutical Co. (Summit, NJ). [^{3}H]Prazosin was purchased from New England Nuclear (Boston, MA). Other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture. DDT cells, a transformed smooth muscle cell line isolated from hamster vas deferens (30), were maintained at 50,000 to 200,000 cells/ml in suspension culture in bicarbonate-buffered DMEM supplemented with 5% fetal bovine serum at 37° in an atmosphere of 5% CO_2/95% air.

Cell treatment. Cells were pelleted by centrifugation and resuspended at 3,000,000 cells/ml at 37° in DMEM buffered to pH 7.4 with 20 mM HEPES (DMEM-HEPES). Pretreatment drugs were dissolved in either 1 mM ascorbate or DMSO and added to cell suspensions to obtain the concentrations indicated. After incubation for 30 min at 37°, cells were washed twice with ice-cold DMEM-HEPES, pelleted, and prepared for the three different assays of AAR sequestration as described below.

[^{3}H]Prazosin binding to intact cells at 4°. Cells were resuspended to 5,000,000 cells/ml and kept on ice after the pretreatment protocol. Assays were begun by adding 100 μl of cell suspension (500,000 cells) to 900 μl of [^{3}H]prazosin (400 pM) in DMEM-HEPES at 4°. Nonspecific binding was defined by 10 μM phentolamine. Samples were then incubated with shaking for 6 hr at 4°, filtered rapidly over Schleicher and Schuell (Keene, NH) no. 30 glass fiber filter strips with a Brandel cell harvester, and washed with buffer containing 10 mM Tris (pH 7.4), 140 mM NaCl, and 100 μM of both propranolol and phentolamine. These drugs prevent further binding of the radioligand during the wash procedure and reduce nonspecific binding by unknown mechanisms. Radioactivity associated with the cells was then determined by counting filters in 10 ml of Budgetsolve (Research Products International, Mount Prospect, IL) in a scintillation counter.

Short-time agonist competition for [^{3}H]prazosin binding to intact cells at 37°. Short-time assays were performed as described previously (11). Intact cells were resuspended at 5,000,000 cells/ml and kept on ice. A total of 100 μl of cells was added to 900 μl of DMEM-HEPES containing [^{3}H]prazosin (400 pM) and 1 mM ascorbate in the absence or presence of the indicated concentrations of epinephrine and incubated for 1 min at 37°. In some experiments a single concentration of epinephrine (100 μM) was used to define the fractions of AAR exhibiting high and very low apparent affinity. This concentration of epinephrine blocks essentially all of the high affinity sites but does not block the sites with very low apparent affinity (10, 11). Nonspecific binding was defined by 10 μM phentolamine. Samples were then filtered, washed, and quantitated as described above.

Determination of AAR subcellular distribution by sucrose density gradient centrifugation. Cells were pelleted after pretreatment, resuspended to a density of 3,000,000 cells/ml in 5.0 ml of ice-cold DMEM-HEPES containing 0.5 mg/ml concanavalin A (22), and incubated for 20 min on ice. Samples were then pelleted, resuspended in 3.0 ml of ice-cold lysis buffer (1 mM Tris, 2 mM EDTA, pH 7.4), and incubated for 20 min on ice. Cells were then homogenized with a Tekmar (Cincinnati, OH) Tissuemizer (10 sec at full power) and 2.0 ml of lysate was layered onto either continuous (linear) or discontinuous (step) sucrose density gradients. Linear gradients contained 9.0 ml of 30–50% sucrose with a 70% sucrose pad (0.5 ml) and a 5% sucrose cap (0.5 ml). Step gradients contained 3.5 ml of 55% sucrose, 3.5 ml of 32% sucrose, and 3.0 ml of 5% sucrose. This was a modification of a step gradient procedure previously described (26). Subcellular membranes were separated by centrifugation at 4° for 60 min at 100,000 × *g* in an SW40Ti rotor and a Beckman ultracentrifuge. Fractions were collected from the top of the gradients and AAR were quantitated using

400 pM [^{3}H]prazosin (10). Samples were incubated for 60 min at 37°, filtered, and washed with 10 mM Tris (pH 7.4), 140 mM NaCl. Nonspecific binding was defined by 100 μM phentolamine. Radioactivity was determined as described above. The subcellular distribution of BAR was determined as described above for AAR using 100 pM [^{125}I]iodopindolol as radioligand and 100 μM isoproterenol to define nonspecific binding (10, 11). Radioactivity was quantitated using a γ-counter.

Data analysis. Nonlinear least squares curve fitting of the raw data from competition binding experiments to one-, two-, or three-site models was performed using BDATA (EMF Software, Knoxville, TN). Statistical comparison of the goodness of fit of these models was accomplished as previously described (31). Statistical comparisons among the different pretreatments used either a *t* test (Table 1) or ANOVA (Waller's LSD rule; Tables 2 and 3) (32).

Results

Comparison of different assays for agonist-induced sequestration and internalization of AAR. Three procedures were used to assess sequestration or internalization of AAR, namely [^{3}H]prazosin binding to intact cells assayed at 4°, short-time assays of competition by the hydrophilic ligand epinephrine for [^{3}H]prazosin binding to intact cells at 37°, and [^{3}H]prazosin binding to membrane fractions after sucrose density gradient centrifugation (Table 1). These assays were performed on cells pretreated either with 100 μM norepinephrine plus 1 μM propranolol in ascorbate-containing medium, conditions similar to those used previously by Fratelli and De Blasi (12) to demonstrate agonist-induced sequestration of AAR using [^{3}H]prazosin binding to intact cells at 4°, or with 10 μM epinephrine without propranolol in medium containing 1% DMSO, the conditions used in our previous study of AAR internalization using short-time competition binding assays and sucrose density gradient centrifugation assays (11).

Pretreatment of DDT cells with norepinephrine plus propranolol in ascorbate-containing medium resulted in a 32% decrease in [^{3}H]prazosin binding to intact cells at 4°. This change was similar to that previously reported (8, 12, 13) and is consistent with agonist-induced sequestration of AAR. However, results obtained from short-time competition binding assays and from sucrose density gradient centrifugation assays were in sharp contrast to the results obtained from binding assays at 4°. Pretreatment with norepinephrine plus propranolol resulted in a change of only 8% of AAR to a form exhibiting very low apparent affinity for epinephrine in short-time assays. Similarly, only a 5% shift of AAR from the plasma membrane fraction to a light vesicle fraction was detected on sucrose density gradients, indicating that pretreatment with norepinephrine plus propranolol did not markedly alter the subcellular distribution of AAR.

Similar to the results obtained when cells were pretreated with norepinephrine plus propranolol in ascorbate-containing medium, pretreatment with epinephrine in medium containing 1% DMSO but in the absence of propranolol resulted in a marked reduction in [^{3}H]prazosin binding to intact cells at 4° but did not induce significant changes in short-time assays or sucrose density gradient assays. The results obtained using the latter two assays are in agreement with our previous results (10, 11). Therefore, the lack of AAR internalization observed in our previous study was apparently not due to effects of DMSO, to the lack of propranolol, or to the use of 10 μM epinephrine instead of 100 μM norepinephrine in the pretreatments. The apparent discrepancy between our results and those of De Blasi and co-workers (8, 12, 13) thus appears to result from the different assays used rather than from differences in the pretreatment conditions or in the properties of DDT cells between the two laboratories.

Internalization of AAR induced by a combination of agonist plus protein kinase C activator. The three assays for sequestration or internalization of AAR described above were performed after pretreatment of DDT cells with either agonist alone, PMA alone, or agonist plus PMA simultaneously (Table 2). Propranolol was included during all pretreatments in order to block any BAR effects, and 10 μM norepinephrine was used instead of 100 μM to decrease possible artifacts due to retained pretreatment drug. Under these conditions, pretreatment with epinephrine or norepinephrine alone induced a reduction in [^{3}H]prazosin binding to about 75% of control with intact cells assayed at 4°. In contrast to the 25% reduction in AAR detected by this assay, only a 10% change was observed in short-time assays and no changes were detected in sucrose density gradient assays. Pretreatment with PMA had little or no effect on [^{3}H]prazosin binding at 4° or on sucrose density gradient assays but did induce some increase in receptors exhibiting low apparent affinity in short-time assays.

Results obtained when cells were pretreated with a combination of agonist plus PMA were in marked contrast to the results obtained when cells were pretreated with agonist alone or PMA alone (Table 2). Although PMA pretreatment alone did not alter the binding of [^{3}H]prazosin to intact cells assayed at 4°, PMA enhanced the decrease in binding elicited by agonist alone. Furthermore, pretreatment of cells with epinephrine or norepinephrine in the presence of PMA resulted in a pronounced quantitative conversion of about half of cell surface AAR from the native high affinity form to a form exhibiting very low apparent affinity for epinephrine in short-time assays. These pretreatments also induced a quantitative shift of about half of cell surface AAR from the plasma membrane fraction to the light vesicle fraction on sucrose density gradients.

TABLE 1

Comparison of different assays for agonist-induced sequestration or internalization of AAR

Cells were pretreated for 30 min at 37° in the absence or presence of 100 μM norepinephrine in DMEM-HEPES containing 1 mM ascorbate and 1 μM propranolol (A) or in the absence or presence of 10 μM epinephrine in DMEM-HEPES containing 1% DMSO (B). Cells were washed to remove pretreatment medium and assays were then performed as described in Methods using [^{3}H]prazosin to label AAR. RH and RL represent the percentages of AAR in the high and low affinity forms, respectively. RL represents [^{3}H]prazosin specific binding in the presence of 100 μM epinephrine to block RH. RH was calculated as total specific binding minus RL. PMF and LVF represent the percentages of AAR in the plasma membrane fraction and light vesicle fraction, respectively, on step gradients. Data represent means ± standard errors (3–17 determinations). Total and nonspecific binding to control cells were 2580 and 330 cpm for intact cell assays at 4° and 1150 and 210 cpm for short-time assays. The sum of the specific binding to PMF and LVF was 1180 cpm for sucrose density gradient assays. None of these values was markedly altered by any pretreatment.

Pretreatment	Intact cell assays at 4°	Short-time assays		Sucrose density gradient assays	
		RH	RL	PMF	LVF
	% of control	% of total			
A. Control	100	82 ± 2	18 ± 2	74 ± 3	26 ± 3
Norepinephrine	68 ± 3[a]	74 ± 1	26 ± 1	69 ± 3	31 ± 3
B. Control	100	82 ± 1	18 ± 1	72 ± 1	28 ± 1
Epinephrine	77 ± 2[a]	79 ± 2	21 ± 2	72 ± 2	28 ± 2

[a] Significantly different from control, $p < 0.01$.

TABLE 2

Internalization of AAR induced by a combination of agonist plus PMA

Cells were pretreated with the indicated drugs for 30 min at 37° in DMEM-HEPES containing 1% DMSO and 1 μM propranolol. Concentrations of norepinephrine, epinephrine, and PMA were 10, 10, and 1 μM, respectively. Cells were washed and assays were performed as described in Methods using [^{3}H]prazosin to label AAR. RH and RL represent the percentages of AAR in the high and low affinity forms, respectively. RL represents [^{3}H]prazosin specific binding in the presence of 100 μM epinephrine to block RH. RH was calculated as total specific binding minus RL. PMF and LVF represent the percentages of AAR in the plasma membrane fraction and light vesicle fraction, respectively, on step gradients. Data represent means ± standard errors (three to seven determinations).

Pretreatment	Intact cells assays at 4°	Short-time assays		Sucrose density gradient assays	
		RH	RL	PMF	LVF
	% of control	% of total			
Control	100	83 ± 1	17 ± 1	76 ± 1	24 ± 1
Epinephrine	75 ± 1[a]	72 ± 1	28 ± 1[a]	76 ± 2	24 ± 2
Norepinephrine	77 ± 2[a]	74 ± 3	26 ± 3[a]	73 ± 1	27 ± 1
PMA	97 ± 3	71 ± 1	29 ± 1[a]	72 ± 2	28 ± 2[a]
Epinephrine + PMA	60 ± 2[a,b]	49 ± 2	51 ± 2[a,b]	44 ± 3	56 ± 3[a,b]
Norepinephrine + PMA	66 ± 3[a,b]	46 ± 4	54 ± 4[a,b]	40 ± 2	60 ± 2[a,b]

[a] Significantly different from control, $p < 0.01$.
[b] Significantly different from agonist alone and PMA alone, $p < 0.01$.

In both control and pretreated cells, the distribution of AAR between the plasma membrane fraction and the light vesicle fraction on linear sucrose density gradients (Fig. 1) was similar to that observed using step gradients (Table 2). The shift in AAR distribution observed on linear gradients induced by a combination of agonist plus PMA appeared to be similar to the shift of BAR induced by agonists alone in DDT cells (10, 11) and in other cells (22–24, 33, 34).

In control cells, curves generated from short-time assays of epinephrine competition for [^{3}H]prazosin binding to intact cells were best fit to a two-site model, with about 80% of [^{3}H]prazosin binding sites exhibiting high affinity for epinephrine and 20% exhibiting very low apparent affinity (Fig. 2). Curves generated from cells pretreated with norepinephrine plus PMA were markedly better fit to a three-site model than to one- or two-site models. This pretreatment resulted in a marked increase in the fraction of AAR exhibiting very low apparent affinity and induced the appearance of a third form of AAR with intermediate affinity for epinephrine. The significance of the three affinity states remains to be determined, although it is interesting that another recent study using a different experimental approach also provided evidence for three forms of AAR in DDT cells (13).

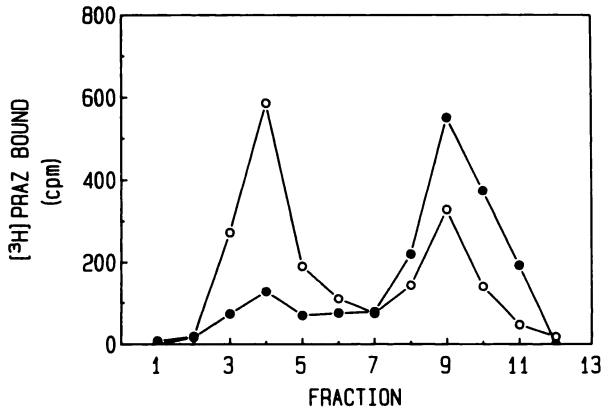

Fig. 1. Distribution of AAR on linear sucrose density gradients. Cells were pretreated for 30 min at 37° in the absence (●) or presence (○) of 10 μM norepinephrine plus 1 μM PMA in DMEM-HEPES containing 1% DMSO and 1 μM propranolol. Cells were then washed, treated with concanavalin A, and lysed. Lysates were subjected to sucrose density gradient centrifugation using linear gradients. Gradient fractions were then assayed for AAR using [^{3}H]prazosin (*[^{3}H]PRAZ*). Data shown are from a representative experiment.

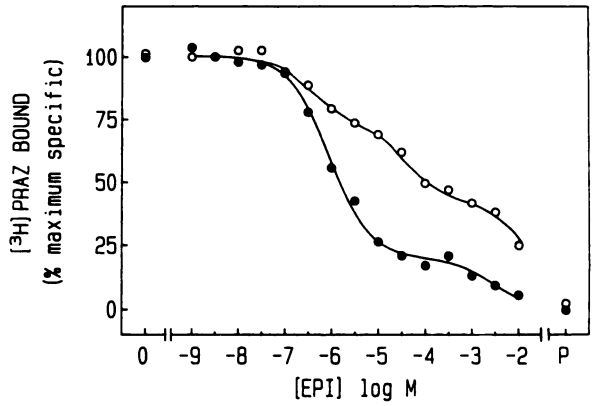

Fig. 2. Short-time assays of epinephrine competition for [^{3}H]prazosin binding to intact cells. Cells were pretreated for 30 min at 37° in the absence (●) or presence (○) of 10 μM norepinephrine plus 1 μM PMA in DMEM-HEPES containing 1% DMSO and 1 μM propranolol. After washing to remove pretreatment medium, the cells were further incubated for 1 min with [^{3}H]prazosin (*[^{3}H]PRAZ*) (400 pM) and the indicated concentrations of epinephrine (*EPI*). Nonspecific binding was determined by 10 μM phentolamine (*P*). The data *points* are averages from three separate experiments and the curves are those for computer-generated fits. Values for computerized fits were as follows: control, 81%, IC_{50} = 1 μM and 19%, IC_{50} >1 mM; norepinephrine plus PMA pretreated, 26%, IC_{50} = 0.3 μM, 30%, IC_{50} = 30 μM, and 44%, IC_{50} >1 mM.

Similar to results obtained when cells were pretreated with norepinephrine plus PMA, a shift of AAR on sucrose density gradients also was observed when cells were pretreated with a combination of norepinephrine plus 1 μM mezerein, a direct activator of protein kinase C structurally unrelated to PMA (Table 3). Pretreatment of cells with agonist plus the β-isomer of phorbol didecanoate, which activates protein kinase C, also induced a shift of AAR on sucrose density gradients. In contrast, pretreatment with agonist plus the inactive α-isomer of phorbol didecanoate did not induce a shift of AAR. These results implicate protein kinase C in the effect of agonist plus PMA.

The effect of agonists is apparently mediated through AAR (Table 3). Thus, the shift of AAR on sucrose density gradients induced by norepinephrine plus PMA was blocked by inclusion of the AAR-selective antagonist phentolamine. In contrast, the

TABLE 3

Specificity of protein kinase C activators and AAR ligands to induce redistribution of AAR on sucrose density gradients

Cells were pretreated for 30 min at 37° with the indicated drugs in DMEM-HEPES containing 1% DMSO and 1 μM propranolol. Cells were washed and assays were performed using step gradients as described in Methods. PMF and LVF represent the percentages of AAR in the plasma membrane fraction and light vesicle fraction, respectively. Data represent the means ± standard errors (three determinations). The concentration of mezerein and phorbol esters was 1 μM.

Pretreatment	AAR in PMF	AAR in LVF
	%	%
Control	80 ± 1	20 ± 1
10 μM Norepinephrine	73 ± 1	27 ± 1
10 μM Norepinephrine + PMA	42 ± 2	58 ± 2[a]
10 μM Norepinephrine + mezerein	44 ± 1	56 ± 1[a]
10 μM Norepinephrine + β-phorbol didecanoate	38 ± 2	62 ± 2[a]
10 μM Norepinephrine + α-phorbol didecanoate	74 ± 3	26 ± 3
PMA	77 ± 2	23 ± 2
PMA + 1 μM phentolamine	82 ± 1	18 ± 1
PMA + 1 μM norepinephrine	50 ± 3	50 ± 3[b]
PMA + 1 μM norepinephrine + 100 μM phentolamine	86 ± 2	14 ± 2
PMA + 100 μM phenylephrine	60 ± 2	40 ± 2[b]

[a] Significantly different from norepinephrine alone, $p < 0.01$.
[b] Significantly different from PMA alone, $p < 0.01$.

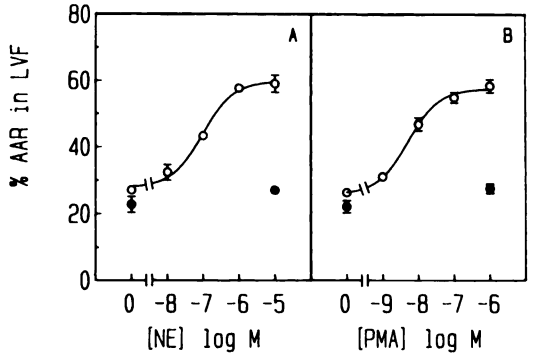

Fig. 3. Dose-response relationships for AAR internalization determined by sucrose density gradient centrifugation. Cells were pretreated for 30 min at 37° with the indicated concentrations of norepinephrine (*NE*) in the absence (●) or presence (○) of 1 μM PMA (A) or with the indicated concentrations of PMA in the absence (●) or presence (○) of 10 μM norepinephrine (B) in DMEM-HEPES containing 1% DMSO and 1 μM propranolol. Cells were then washed, treated with concanavalin A, and lysed. Lysates were subjected to sucrose density gradient centrifugation using step gradients. AAR in the plasma membrane fraction and light vesicle fraction (*LVF*) were quantitated using [^{3}H]prazosin (400 pM). Specific binding to LVF is expressed as a percentage of the sum of the specific binding to both fractions. Values are the means ± standard errors (three or four experiments).

shift was not blocked by the BAR-selective antagonist propranolol, which was included in all pretreatments. Furthermore, the relatively AAR-selective agonist phenylephrine in the presence of PMA induced a shift similar to that observed after pretreatment with norepinephrine plus PMA.

The effect of agonist plus PMA to shift AAR on sucrose density gradients was dose-dependent for both agonist in the presence of a fixed concentration of PMA (Fig. 3A) and for PMA in the presence of a fixed concentration of agonist (Fig. 3B). Half-maximal effects were observed at 100 nM for norepinephrine and 5 nM for PMA. In contrast to results obtained for AAR, pretreatment of DDT cells with agonist alone induced a dose-dependent shift of BAR on sucrose density gradients, and the inclusion of 1 μM PMA in the pretreatment did not alter the extent of BAR internalization at any agonist concentration (Fig. 4).

Discussion

We recently reported that pretreating DDT cells with epinephrine or with PMA resulted in desensitization of AAR-coupled phosphoinositide turnover but did not induce AAR sequestration or internalization as assessed by short-time competition binding assays and by sucrose density gradient centrifugation assays (11). In contrast, agonists have been reported to induce sequestration of AAR in DDT cells as determined by the binding of [^{3}H]prazosin to intact cells assayed at 4° (8, 12, 13). The present study was undertaken to examine the reasons for the different results previously obtained, by comparing all three of these assays within one study. This study also included an investigation of the effects of pretreating cells with a combination of agonist plus PMA on AAR sequestration and/or internalization in DDT cells.

Pretreatment of DDT cells with epinephrine or norepinephrine alone, but not with PMA alone, resulted in a decrease in [^{3}H]prazosin binding to intact cells at 4°, whereas these pretreatments resulted in only minimal changes in short-time competition binding assays or in sucrose density gradient assays. Without further characterization, assays at 4° and short-time assays on intact cells reflect receptor "sequestration" due to the limited accessibility of the radioligand and the competing ligand, respectively, to sequestered AAR. These assays do not provide information regarding the subcellular distribution of these receptors. On the other hand, sucrose density gradient centrifugation assays detect changes in the subcellular distribution of receptors and therefore presumably reflect true receptor "internalization" from the plasma membrane to an intracellular compartment (1, 22, 23, 26). Our results suggest that pretreatment with agonists alone may induce the sequestration

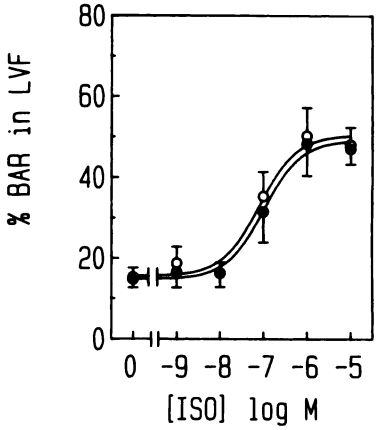

Fig. 4. Dose-response relationships for BAR internalization determined by sucrose density gradient centrifugation. Cells were pretreated for 30 min at 37° with the indicated concentrations of isoproterenol (*ISO*) in the absence (●) or presence (○) of 1 μM PMA in DMEM-HEPES containing 1% DMSO. Cells were then washed, treated with concanavalin A, and lysed. Lysates were subjected to sucrose density gradient centrifugation using step gradients. BAR in the plasma membrane fraction and light vesicle fraction (*LVF*) were quantitated using [^{125}I]iodopindolol (100 pM). Specific binding to LVF is expressed as a percentage of the sum of the specific binding to both fractions. Values are the means ± standard errors (three experiments). The sum of the specific binding to both fractions was 4810 cpm in control cells and was not markedly altered by any pretreatment.

of AAR into a compartment that is inaccessible to [^{3}H]prazosin at 4° but that remains associated with the plasma membrane and is accessible to hydrophilic ligands at 37°. However, neither agonists nor PMA, used separately, appear to induce AAR internalization as assessed by sucrose density gradient assays.

In contrast, pretreatment with a combination of agonist plus PMA resulted in a greater decrease in [^{3}H]prazosin binding at 4° than was observed when cells were pretreated with agonist alone, caused a marked increase in the fraction of AAR exhibiting limited accessibility to epinephrine in short-time assays, and induced a shift of about half of cell surface AAR from the plasma membrane fraction to a light vesicle fraction on sucrose density gradients. These effects most likely represent the internalization of AAR from the plasma membrane to an intracellular compartment. Regardless of the precise subcellular location of these apparently internalized receptors, it seems clear that the combination of agonist plus PMA induces a further change in AAR distribution that is not induced by agonist alone or by PMA alone.

The regulation of BAR by agonists has been studied extensively using the three assays described above in a variety of cell lines including 1321N1 human astrocytoma cells (22, 23, 28, 29), S49 mouse lymphoma cells (33), C6 rat glioma cells (34), and DDT cells (8, 10–13). DDT cells are an ideal cell line in which to compare the regulation of AAR and BAR because these cells have a similar number of both receptors (35, 36). Similar to results obtained previously in other cells, pretreatment of DDT cells with agonist alone appears to induce internalization of BAR as determined by all three assays (Fig. 5A). The changes observed in the three assays are in reasonable quantitative agreement and together provide strong evidence for agonist-induced internalization of BAR from the plasma membrane to an intracellular compartment.

In contrast to the results with BAR, pretreatment of DDT cells with agonist alone caused a substantial decrease in cell surface AAR as determined by [^{3}H]prazosin binding to intact cells at 4° (Fig. 5B) but resulted in much smaller changes (Fig. 5B) or no changes (10) in short-time assays and in sucrose density gradient assays. Thus, there appears to be a distinct difference between agonist-induced regulation of AAR and BAR in these cells. However, marked decreases in cell surface AAR were detected by all three assays when cells were pretreated with a combination of agonist plus PMA (Fig. 5C). These results suggest that the internalization of AAR induced by a combination of agonist plus PMA may be similar to that occurring for BAR during pretreatment with agonist alone.

Although the mechanisms responsible for the effect of PMA to facilitate agonist-induced internalization of AAR remain to be determined, it seems likely that protein kinase C is involved, based on the specificity of the PMA analogs tested. The receptor itself is a likely candidate for the protein kinase C substrate involved, inasmuch as both agonists (8) and PMA (14) have been shown to stimulate phosphorylation of AAR in DDT cells. The greater effect of agonist plus PMA on internalization of AAR could then result from enhanced phosphorylation of AAR, resulting from the combined effects of receptor occupancy by agonist and of marked activation of protein kinase C by PMA that has been demonstrated with purified components (37). In intact cells, agonist occupancy alone may not be sufficient to fully activate protein kinase C inasmuch as diacylglycerol is rapidly metabolized by diacylglycerol kinase and diacylglycerol lipase (2). Alternatively, the phosphorylation of AAR induced by agonist plus PMA may be qualitatively different from that induced by agonist alone or PMA alone. Finally, it is quite possible that phosphorylation of some component of the internalization machinery other than the receptor is involved. However, the observation that PMA did not alter the agonist-induced shift of BAR on sucrose density gradients suggests that this effect is selective for AAR or perhaps for those receptors coupled to phosphoinositide hydrolysis.

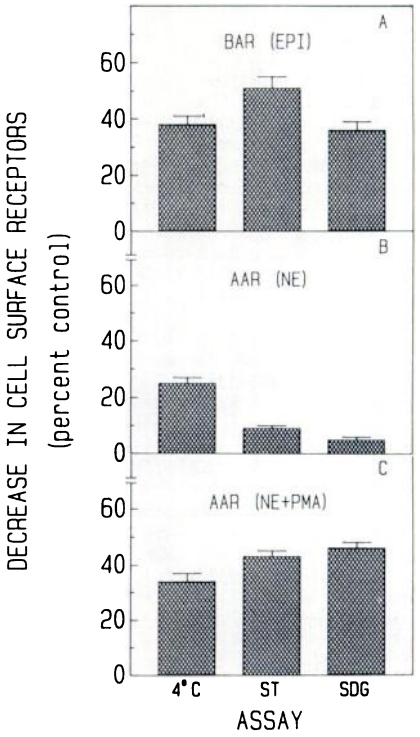

Fig. 5. Comparison of different assays for sequestration and/or internalization of BAR and AAR. A, For intact cell assays at 4°, cells were pretreated for 30 min at 37° in the absence (control) or presence of 10 μM epinephrine (*EPI*) in DMEM-HEPES containing 1% DMSO. Cells were then washed and assays for BAR were performed in DMEM-HEPES at 4° using [^{125}I]iodopindolol (100 pM) to label BAR. Nonspecific binding was defined by 1 μM propranolol. Data for short-time assays (*ST*) and sucrose density gradient assays (*SDG*) were recalculated from Ref. 11 for comparison. B and C, Data from Table 2 are presented for direct comparison with those for BAR. For ST and SDG, data have been recalculated as per cent decrease in cell surface receptors rather than as a percentage of total cellular receptors. (*NE*, norepinephrine).

In summary, our results suggest that exposure of DDT cells to agonist alone may lead to a form of AAR sequestration in which AAR are inaccessible to [^{3}H]prazosin at 4° but are accessible to hydrophilic ligands at 37° and remain associated with the plasma membrane. However, concomitant exposure to agonist plus protein kinase C activator leads to internalization of AAR into the presumably intracellular compartment, detected as the light vesicle fraction in sucrose density gradient assays and as the very low affinity form in short-time competition binding assays. Elucidation of the precise mechanisms involved in AAR internalization and the relationship of internalization to phosphorylation and other aspects of desensitization will require further investigation.

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References

1. Wakshull, E., C. Hertel, E. J. O'Keefe, and J. P. Perkins. Cellular redistribution of β-adrenergic receptors in a human astrocytoma cell line: a comparison with the epidermal growth factor receptor in murine fibroblasts. *J. Cell. Biochem.* **29**:127–141 (1985).
2. Berridge, M. J. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**:345–360 (1984).
3. Berridge, M. J., and R. F. Irvine. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.)* **312**:315–321 (1984).
4. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.)* **308**:693–698 (1984).
5. Bobik, A., J. H. Campbell, and P. J. Little. Desensitization of the *alpha*$_1$ adrenoceptor system in vascular smooth muscle. *Biochem. Pharmacol.* **33**:1143–1145 (1984).
6. Lurie, K. G., G. Tsujimoto, and B. B. Hoffman. Desensitization of *alpha*-1 adrenergic receptor-mediated vascular smooth muscle contraction. *J. Pharmacol. Exp. Ther.* **234**:147–152 (1985).
7. Hughes, R. J., and P. A. Insel. Agonist-mediated regulation of α_1- and β_2-adrenergic receptor metabolism in a muscle cell line, BC3H-1. *Mol. Pharmacol.* **29**:521–530 (1986).
8. Leeb-Lundberg, L. M. F., S. Cotecchia, A. De Blasi, M. G. Caron, and R. J. Lefkowitz. Regulation of adrenergic receptor function by phosphorylation. 1. Agonist-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT$_1$ MF-2 smooth muscle cells. *J. Biol. Chem.* **262**:3098–3105 (1987).
9. Schwarz, K. R., S. M. Lanier, E. A. Carter, R. M. Graham, and C. J. Homcy. Transient high affinity binding of agonists to *alpha*-1 adrenergic receptors of intact liver cells. *FEBS Lett.* **187**:205–210 (1987).
10. Toews, M. L. Comparison of agonist-induced changes in β- and α_1-adrenergic receptors of DDT$_1$ MF-2 cells. *Mol. Pharmacol.* **31**:58–68 (1986).
11. Cowlen, M. S., and M. L. Toews. Effects of agonist and phorbol ester on adrenergic receptors of DDT$_1$ MF-2 cells. *J. Pharmacol. Exp. Ther.* **243**:527–533 (1987).
12. Fratelli, M., and A. De Blasi. Agonist-induced α_1-adrenergic receptor changes: evidence for receptor sequestration. *FEBS Lett.* **212**:149–153 (1987).
13. Fratelli, M., O. Marasco, and A. De Blasi. Two subpopulations of α_1-adrenergic receptors with high and low affinity for agonist: short-term exposure to agonists reduced the high affinity sites. *Biochim. Biophys. Acta* **930**:87–96 (1987).
14. Leeb-Lundberg, L. M. F., S. Cotecchia, J. W. Lomasney, J. F. DeBernardis, R. J. Lefkowitz, and M. G. Caron. Phorbol esters promote α_1-adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc. Natl. Acad. Sci. USA* **82**:5651–5655 (1985).
15. Cotecchia, S., L. M. F. Leeb-Lundberg, P.-O. Hagen, R. J. Lefkowitz, and M. G. Caron. Phorbol ester effects on α_1-adrenoceptor binding and phosphatidylinositol metabolism in cultured vascular smooth muscle cells. *Life Sci.* **37**:2389–2398 (1985).
16. Corvera, S., K. R. Schwarz, R. M. Graham, and J. A. Garcia-Sainz. Phorbol esters inhibit α_1-adrenergic effects and decrease the affinity of liver cell α_1-adrenergic receptors for (−)-epinephrine. *J. Biol. Chem.* **261**:520–526 (1986).
17. McMillian, M., B. Chernow, and B. L. Roth. Phorbol esters inhibit *alpha*$_1$-adrenergic receptor-stimulated phosphoinositide hydrolysis and contraction in rat aorta: evidence for a link between vascular contraction and phosphoinositide turnover. *Biochem. Biophys. Res. Commun.* **134**:970–974 (1986).
18. Harden, T. K. Agonist-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase. *Pharmacol. Rev.* **35**:5–32 (1983).
19. Perkins, J. P., M. L. Toews, and T. K. Harden. Regulation of β-adrenergic receptors during exposure of astrocytoma cells to catecholamines. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**:37–46 (1984).
20. Hertel, C., and J. P. Perkins. Receptor-specific mechanisms of desensitization of β-adrenergic receptor function. *Mol. Cell. Endocrinol.* **37**:245–256 (1984).
21. Sibley, D. R., and R. J. Lefkowitz. Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature (Lond.)* . **317**:124–129 (1985).
22. Harden, T. K., C. U. Cotton, G. L. Waldo, J. K. Lutton, and J. P. Perkins. Catecholamine-induced alteration in sedimentation behavior of membrane bound β-adrenergic receptors. *Science (Wash. D. C.)* **210**:441–443 (1980).
23. Toews, M. L., G. L. Waldo, T. K. Harden, and J. P. Perkins. Relationship between an altered membrane form and a low affinity form of the β-adrenergic receptor occurring during catecholamine-induced desensitization: evidence for receptor internalization. *J. Biol. Chem.* **259**:11844–11850 (1984).
24. Waldo, G. L., J. K. Northup, J. P. Perkins, and T. K. Harden. Characterization of an altered membrane form of the β-adrenergic receptor produced during agonist-induced desensitization. *J. Biol. Chem.* **258**:13900–13908 (1983).
25. Beguinot, L., R. M. Lyall, M. C. Willingham, and I. Pastan. Down-regulation of the epidermal growth factor receptor in KB cells is due to receptor internalization and subsequent degradation in lysosomes. *Proc. Natl. Acad. Sci. USA* **81**:2384–2388 (1984).
26. Hertel, C., S. J. Coulter, and J. P. Perkins. A comparison of catecholamine-induced internalization of β-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells. *J. Biol. Chem.* **260**:12547–12553 (1985).
27. Hertel, C., and J. P. Perkins. Sequential appearance of epidermal growth factor in plasma membrane-associated and intracellular vesicles during endocytosis. *J. Biol. Chem.* **262**:11407–11409 (1987).
28. Toews, M. L., and J. P. Perkins. Agonist-induced changes in β-adrenergic receptors in intact cells. *J. Biol. Chem.* **259**:2227–2235 (1984).
29. Toews, M. L., G. L. Waldo, T. K. Harden, and J. P. Perkins. Comparison of binding of ^{125}I-iodopindolol to control and desensitized cells at 37° and on ice. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**:47–62 (1986).
30. Norris, J. S., J. Gorski, and P. O. Kohler. Androgen receptors in a syrian hamster ductus deferens tumor cell line. *Nature (Lond.)* **248**:422–424 (1974).
31. De Lean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
32. Snedecor, G. W., and W. G. Cochran. *Statistical Methods.* Iowa State University Press, Ames, IA (1980).
33. Clark, R. B., J. Friedman, N. Prashad, and A. E. Ruoho. Epinephrine-induced sequestration of the β-adrenergic receptor in cultured S49 WT and *cyc*$^-$ lymphoma cells. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **10**:97–119 (1985).
34. Kassis, S., T. Zaremba, J. Patel, and P. H. Fishman. Phorbol esters and β-adrenergic agonists mediate desensitization of adenylate cyclase in rat glioma C6 cells by distinct mechanisms. *J. Biol. Chem.* **260**:8911–8917 (1985).
35. Cornett, L. E., and J. S. Norris. Characterization of the *alpha*-1 adrenergic receptor subtype in a smooth muscle line. *J. Biol. Chem.* **257**:694–697 (1982).
36. Garmer, J., L. E. Cornett, and J. S. Norris. Adenylate cyclase coupled β_2 adrenergic receptors in a smooth muscle tumor cell line. *Fed. Proc.* **41**:1511 (1982).
37. Bouvier, M., L. M. F. Leeb-Lundberg, J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Regulation of adrenergic receptor function by phosphorylation. II. Effects of agonist occupancy on phosphorylation of α_1- and β_2-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **262**:3106–3113 (1987).

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