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Differential mechanism for the cell surface sorting and agonist-promoted internalization of the α_{1B}-adrenoceptor

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1 α_{1B}-adrenoceptors are localized at a steady state in the plasma membrane in untreated cells, and internalize to intracellular vesicles when exposed to agonist. Flow cytometry analysis with an anti-N-terminus-antibody (1B-N1-C, (Hirasawa *et al*., 1996)) facilitated the quantification of cell surface α_{1B}-adrenoceptor. Also, the cellular distribution of α_{1B}-adrenoceptors was visually monitored by immunocytochemical confocal microscopy.

2 Utilizing this combined approach, we have examined the molecular mechanism for cellular trafficking of α_{1B}-adrenoceptors, including the process of sorting of the synthesized receptor protein to the cell surface, and the agonist-induced internalization. The two processes were separately examined by using α_{1B}-adrenoceptor inducible DDT_1MF-2 cells for the sorting process and CHO cells stably expressing α_{1B}-adrenoceptors for the agonist-promoted internalization.

3 We examined the effects of cytochalasin D and mycalolide B (actin depolymerization agents), demecolcine (a microtubule disrupting agent), brefeldin A (an inhibitor of vesicular transport and Golgi function), bafilomycin A1 (a specific inhibitor of the vacuolar proton pump) or hyperosmotic sucrose treatment (that may inhibit clathrin-mediated endocytosis) on these processes.

4 We found that the agonist-promoted internalization was blocked by cytochalasin D and mycalolide B, while the cell surface sorting process was specifically blocked by brefeldin A, indicating that the two processes involve different components of the cellular endocytic machinery.

5 The experimental approach as exemplified in this study would provide a valuable system to study further the molecular mechanism(s) of cellular trafficking of G protein-coupled receptors.

Keywords: α_{1B}-Adrenoceptor; BODIPY-FL prazosin; anti-peptide antibody; flow cytometry; hamster DDT_1-MF2 cell; laser scanning confocal microscopy

Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of plasma membrane proteins and play important roles as cell surface sensor molecules, by which many extracellular stimuli can transduce signals via an intracellular signalling cascade. The receptors and their signal transduction systems are also dynamically regulated by desensitization, internalization and down-regulation (Lefkowitz *et al*., 1990; Hein & Kobilka, 1995). Several biochemical results have shown that the phosphorylation, palmitoylation and ubiqutination of these receptors are also dynamically changed by agonist stimulation (Lefkowitz *et al*., 1990; Milligan *et al*., 1995; Geli & Riezman, 1996; Hicke & Riezman, 1996) and are supposed to be correlated to their regulation.

The search for the mechanism of internalization has occurred at a rapid pace because of the importance of internalization in receptor function and regulation. Most GPCRs are internalized, and therefore the process of internalization might be expected to involve ill-defined components of the cellular endocytic machinery. A large number of domains have been implicated in the internalization of specific GPCRs (Haga & Haga, 1990; Hausdorff *et al*., 1991; Rodriguez *et al*., 1992; Nussenzveig *et al*., 1993; Barak *et al*., 1994; Hunyady *et al*., 1994). However, thus far, generalized domains and mechanisms for receptor internalization have been difficult to ascertain. Several complications have hindered the search for common structural features related to receptor internalization. Methodologies for investigating receptor internalization vary among receptor classes and are not strictly comparable; receptor internalization can vary depending on the cell types and multiple mechanisms are involved in internalization. Similarly, biosynthetic trafficking pathway of GPCRs has been poorly characterized, even though the sorting mechanisms for other molecules including viral proteins, secretory proteins and membrane proteins have been well examined (Bauerfeind & Huttner, 1993), mainly because of a lack of appropriate experimental approaches.

Recently, we showed the utility of several probes, N-terminus anti-peptide antibodies and fluorescent conjugated ligand, for the study of the subcellular localization of α_1-adrenoceptor (Hirasawa *et al*., 1996; 1997). These probes also provide a unique means of identifying α_1-adrenoceptor subtypes at the protein level. In the present study, we further showed that flow cytometry analysis with these probes can effectively quantify cell surface α_1-adrenoceptor levels. In addition, the cellular distribution of α_{1B}-adrenoceptors can be visually monitored by immunocytochemical confocal microscopy. Combining these approaches, we examined the effects of several pharmacological tools on receptor internalization and the cell surface sorting process. The results show that the two processes involve different components of the cellular endocytic machinery.

Methods

Cell culture, transfection and the α_{1B}-adrenoceptor-inducible DDT_1MF-2 cells

α_{1B}-Adrenoceptor-inducible hamster DDT_1MF-2 cells (Esbenshade *et al*., 1995b) were used to study the mechanism of cell

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surface sorting, in which the LacSwitch-inducible expression system was used to control α_{1B}-adrenoceptor expression. Briefly, one vector (p3'SS) constitutively expresses the lac repressor protein in this system, inhibiting expression of the operator vector (pOPRSV) containing the α_{1B}-adrenoceptor cDNA preceded by idealized lac operon sequences superimposed on a strong RSV promoter. The inducer isopropyl-β-D-thiogalactopyranoside (IPTG) which has no known effects on mammalian cells, prevents binding of the lac repressor to the lac operator sequences allowing gene expression. DDT_1MF-2 cells were cultured in a 7% CO_2 incubator in Dulbecco's Modified Eagle's medium (DMEM) containing glucose (4.5 g l^{-1}), streptomycin (100 mg l^{-1}) and penicillin (10^5 u l^{-1}) and supplemented with 2.5% foetal bovine serum, 2.5% horse serum, 400 μg ml^{-1} of G418 and 200 μg ml^{-1} of hygromycin B. In experiments involving IPTG induction, DDT_1MF-2 cells were maintained at lower (1/10) antibiotic concentrations as shown previously (Esbenshade *et al*., 1995a,b).

COS-7 cells, obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were maintained in DMEM supplemented with 10% foetal bovine serum. α_{1B}-Adrenoceptor expression constructs (Foglar *et al*., 1995) were transfected into COS-7 cells by the electroporation method by use of a Cell-Porator (BRL Life Technologies, Bethesda, MD, U.S.A.) according to instructions, and cells were assayed 48–72 h after transfection.

In order to examine the agonist-induced internalization, we used a Chinese hamster ovary (CHO) cell line stably expressing α1B-adrenoceptor (CHO-α1B), the pharmacological properties of which were previously described (Horie *et al*., 1994). CHO cells were cultured in 5% CO_2 at 37 °C in F12 medium (GIBCO) supplemented with 10% foetal bovine serum, 200 μg ml^{-1} of G418, penicillin (10^5 u l^{-1}) and streptomycin (100 mg l^{-1}).

Antibody preparation

Generation of an anti-peptide antibody (designated as 1B-N1-C) was as described previously. Briefly, peptide was synthesized corresponding to amino acids 12–27 (Peptide: 1B-N1; (C)SAPAQWGELKDANFTG) of the published hamster α_{1B}-adrenoceptor sequence (Cotecchia *et al*., 1988), conjugated to the carrier protein keyhole limpet haemocyanin and injected into two rabbits. Antisera were screened against the peptides by using cross dot systems (Sebia, Moulineaux, France) and visualized by the ABC system (Vector Laboratories, Burlingame, CA). We also performed immunoblotting and immunoprecipitation of α_{1B}-adrenoceptors and confirmed that the antibody specifically detects this receptor (Hirasawa *et al*., 1996).

Antiserum was purified on 1 ml of protein A-sepharose CL-4B column (Pharmacia Biotech, Tokyo, Japan) equilibrated with 20 mM phosphate buffer, pH 7.5, and eluted with glycine-HC1 buffer (100 mM, pH 2.2), into 1 ml fractions, which were immediately neutralized with 1 M Tris-HCl buffer, pH 8.5. The resulting antibody fractions were concentrated by Centricon 30 micro concentrator (Amicon, Danvers, MA, U.S.A.) and stored at −20 °C.

Membrane preparation and [^{125}I]-HEAT-binding assay

Membrane preparation of the cells and [^{125}I]-(2-β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone ([^{125}I]-HEAT)-binding assay was performed as described previously (Hirasawa *et al*., 1993; 1995; Shibata *et al*., 1996). Briefly, the cells were collected and disrupted by a sonicator (model SONIFIER 250, setting 5 for 8 s) (Branson, Danburg, CT, U.S.A.) in ice-cold buffer containing 5 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 10 mM EGTA, 250 mM sucrose. The mixture was then centrifuged at $3{,}000 \times g$ for 10 min. The supernatant fraction was centrifuged at $35{,}000 \times g$ for 20 min. The resulting pellet was resuspended in binding buffer (50 mM Tris-HCl pH 7.4, 12.5 mM $MgCl_2$, 10 mM EGTA). All buffers contained proteinase inhibitors; 1 mM PMSF, 100 μM benzamidine, 1 μg ml^{-1} pepstatin A and 1 μg ml^{-1} leupeptin. Membrane aliquots (10 μg of protein) were incubated for 60 min at 25 °C with [^{125}I]-HEAT in a final volume of 250 μl of binding buffer. After dilution with ice-cold buffer, samples were immediately filtered through Whatmann GF/C glass fibre filters with a Brandel cell harvester (Model-30, Gaithersburg, MD, U.S.A.). Specific [^{125}I]-HEAT binding was experimentally determined from the difference between counts in the absence and presence of 10 μM phentolamine. B_{max} and K_d values were obtained by fitting rectangular hyperbolic functions to the experimental data, by use of computer-assisted iterative nonlinear regression analysis. The protein concentration was measured with the BCA protein assay kit (PIERCE, Rockford, IL, U.S.A.).

Flow cytometry

Cells expressing α_{1B}-adrenoceptors were analysed by FACScan (Becton Dickinson & Co., Mountain View, CA, U.S.A.) with either BODIPY-FL prazosin or the anti-peptide antibody (1B-N1-C). For analysis with BODIPY-FL prazosin, DDT_1MF-2 cells were detached by shaking and washed once with PBS. Cells were incubated for 30 min at room temperature in 100 μl of BODIPY-FL prazosin buffer containing 1% BSA, 0.01% Pluoronic F127, 1 μM BODIPY-FL prazosin in PBS. Cells were diluted to 500 μl with PBS and stored for up to 1 h. For analysis with the anti-peptide antibodies, CHO-α_{1B} cells were trypsinized and washed twice with PBS. In some cases, cells were fixed in 4% paraformaldehyde/PBS solution for 5 min at room temperature and stored for up to 24 h at 4°C then washed with PBS. Cells were incubated for 30 min at 4°C with the primary antiserum (diluted 1/100), washed three times, incubated with FITC-conjugated sheep anti-rabbit IgG (Organon teknika, Durham, NC, U.S.A.) or Cy3-conjugated goat anti-rabbit IgG (Chemicon international, Temecura, CA, U.S.A.) for 60 min at 4°C, and again washed three times with PBS. To avoid artifacts arising from autofluorescence of dead cells and cellular debris, data acquisition were electrically gated for large particles presumably representing intact cells by forward and side scatter. Since the wavelength of excitation and emission of BODIPY-FL prazosin are similar to those of FITC, we used standard settings adjusted to routine double staining techniques employing FITC (FL-1) and Cy3 (FL-2) using the operating program LYSIS-II. Routinely, data from the green fluorescence of 10^4 cells were subjected to histogram and dot-plot analysis.

Immunocytochemical confocal microscopy analysis

α_{1B}-Adrenoceptor cDNA-transfected (CHO-α_{1B}) and non-transfected CHO cells were seeded at 1×10^5 per well in an 8 well Lab tek chamber slide (Nunc, Napervile, IL, U.S.A.) in 0.5 ml of medium. For DDT_1MF-2 cells, the chambers were coated with rat tail collagen. After drug treatment, medium was removed and cells were washed twice with PBS. Fixation was performed in 80% acetone for 5 min. Cells were then incubated with 0.5% Triton X-100 in PBS for permeabilization. The primary antiserum was brought to a final dilution of 1/100 in PBS containing 10% goat serum and was applied to the cells, which were subsequently kept in a humidified

chamber for 1 h at room temperature. FITC-conjugated goat anti-rabbit IgG (MBL, Nagoya, Japan) was diluted 1/200 in PBS containing 10% goat serum, and applied to cells for 1 h at room temperature. Cells were then washed twice with PBS and coverslips were applied using Gel/Mount (Biomeda, Foster City, CA, U.S.A.). After immunohistochemical staining, cells

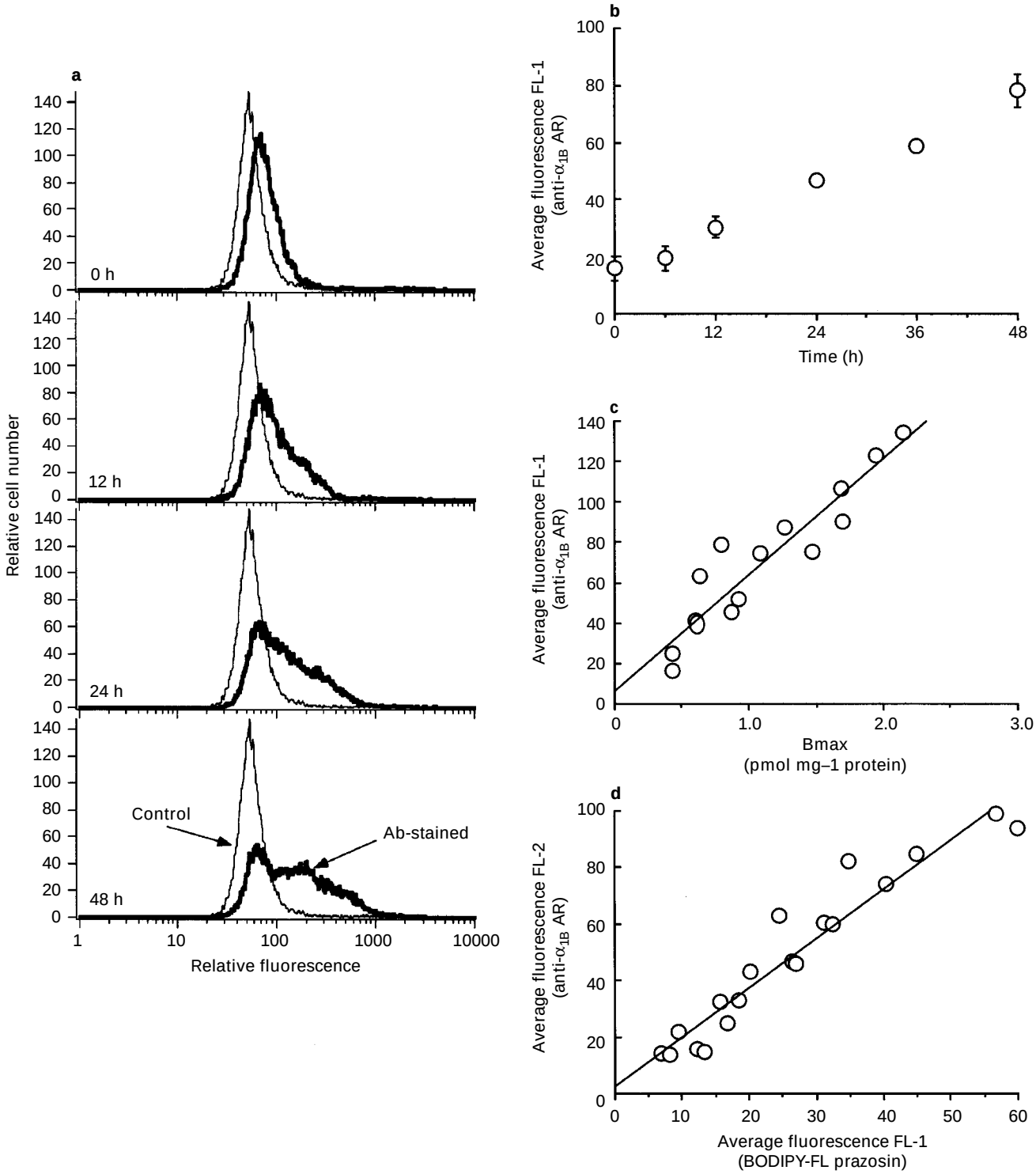

Figure 1 Flow cytometry analysis of α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells. (a and b) Time course for the induction of α_{1B}-adrenoceptor (AR) expression by 1 mM IPTG in DDT$_1$MF-2 cells. DDT$_1$MF-2 cells co-transfected with inducible hamster α_{1B}-adrenoceptor vector (pOP α1B) and repressor vector (p3'SS) were stained with or without anti-peptide antiserum (1B-N1-C), then stained with FITC-labelled secondary antibody and subjected to flow cytometry. (a) Histograms in FL-1 fluorescence were shown 0, 12, 24 and 48 h after IPTG induction. (b) The average fluorescence was measured by flow cytometry analysis 0, 6, 12, 24 and 48 h after IPTG induction. The values correspond to the mean of at least four independent experiments, vertical lines show s.d. (c) Correlation between B_{max} obtained by radioligand binding assay and the average fluorescence measured by flow cytometry analysis. The α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells were treated by various IPTG induction conditions (concentration: 0.1–10 mM, induction time: 0, 6, 12, 24, 48 h). Utilizing the same batch of cells, the flow cytometry analyses and radioligand binding studies were performed as described in Methods. (d) Correlation between the average fluorescence with antibody staining and BODIPY-FL prazosin staining measured by flow cytometry analysis. The α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells were treated in various IPTG induction conditions (concentration: 0.1–10 mM, induction time: 0, 6, 12, 24, 48 h). The collected cells were stained simultaneously with anti-peptide antibody and BODIPY-FL prazosin, and subjected to flow cytometry analysis.

were viewed with an LSM-GB200 laser scanning microscope (Olympus, Tokyo, Japan) with an argon-ion laser set at 488 nm for excitation of FITC (Awaji *et al*., 1996).

Pharmacological characterization of cell surface sorting and internalization

The inducible DDT$_1$MF-2 cells at 80% confluence were used to analyse cell surface sorting. IPTG (1 mM) was added to the medium, then drugs were added at 12 h after induction. At 48 h after drug application, the effect of the drugs on the expression of cell surface receptor was examined. For each drug, five independent experiments were performed. Data were expressed as % of the control IPTG-induction that had no drug treatment.

For the internalization experiments, we used CHO-α_{1B} cells that stably express α_{1B}-adrenoceptor (Horie *et al*., 1994). We did not examine the inducible DDT$_1$MF-2 cells to study the internalization process, because they were found to contain heterogeneous cell populations in the α_{1B}-adrenoceptor induction levels as described below. Drugs were applied to CHO-α_{1B} cells at 80% confluence, the α_1-adrenoceptor agonist phenylephrine (10 μM) was added 30 min later, and cells were then incubated for 2 h. For the immunocytochemical examination, cells were then trypsinized, fixed by paraformaldehyde, washed with PBS and examined for immunostaining. In preliminary experiments, we observed that drugs (brefeldin A, cytochalasin D, mycalolide B, bafilomycin A1, and demecolcine) and hyperosmotic sucrose treatment used in the present study had no significant effect on the basal expression level of α_{1B}-adrenoceptors, as measured by flow cytometry analysis. In all experiments, cells without any drug treatment, cells with phenylephrine (10 μM) alone and cells with phenylephrine (10 μM) plus prazosin (100 nM) were examined in parallel.

Each drug treatment was performed by use of previously described *in vitro* effective concentrations; brefeldin A 10 μg ml^{-1} (Schonhorn & Wessling, 1994), cytochalasin D 100 nM (Ozawa *et al*., 1996), mycalolide B 1 μM (Saito *et al*., 1994), bafilomycin A1 2 μM (Gekle *et al*., 1995) and demecolcine 100 nM (Schrader *et al*., 1996).

Materials

[^{125}I]-HEAT (specific activity, 2,200 Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA), brefeldin A, bafilomycin A1 and demecolcine from Wako (Osaka, Japan), prazosin HCl from Pfizer (Brooklyn, NY, U.S.A.). BODIPY-FL prazosin from Molecular Probes (Eugene, OR, U.S.A.), cytochalasin D and phenylephrine were from Sigma (St. Louis, MO, U.S.A.), and all other reagents were of the highest analytical grade.

Results

Quantification of cell surface α_{1B}-adrenoceptors by flow cytometry

We have previously shown that flow cytometry with the anti-N-terminus anti-peptide antibody (1B-N1-C) is sensitive and specific enough to detect α_{1B}-adrenoceptors on the cell surface (Hirasawa *et al*., 1996). In order to examine whether the flow cytometry analysis with 1B-N1-C can quantify cell surface α_{1B}-adrenoceptor levels, we compared the results of flow cytometry analysis with results from radioligand binding assay by using cells with different levels of expression of α_{1B}-adrenoceptors. To obtain the cells with different expression levels of α_{1B}-adrenoceptors, we used inducible DDT$_1$MF-2 cells treated with different concentrations and duration of IPTG induction (Esbenshade *et al*., 1995b).

First, we examined the time course of the inducible expression of α_{1B}-adrenoceptors IPTG treatment. As shown in Figure 1a and b, following the application of 1 mM IPTG, 1B-N1-C fluorescence in the DDT$_1$MF-2 cells increased in a time-dependent manner. However, the fluorescence intensity was found not to be unipolar (Figure 1a), indicating that the cultures contained populations of cells that were heterogeneous for the inducible α_{1B}-adrenoceptor expression (Esbenshade *et al*., 1995b). We subsequently compared the average fluorescence intensity obtained in flow cytometry analysis with B_{max} by radioligand binding assay, which also reflects the average value (Figure 1a). Cells were treated with IPTG with

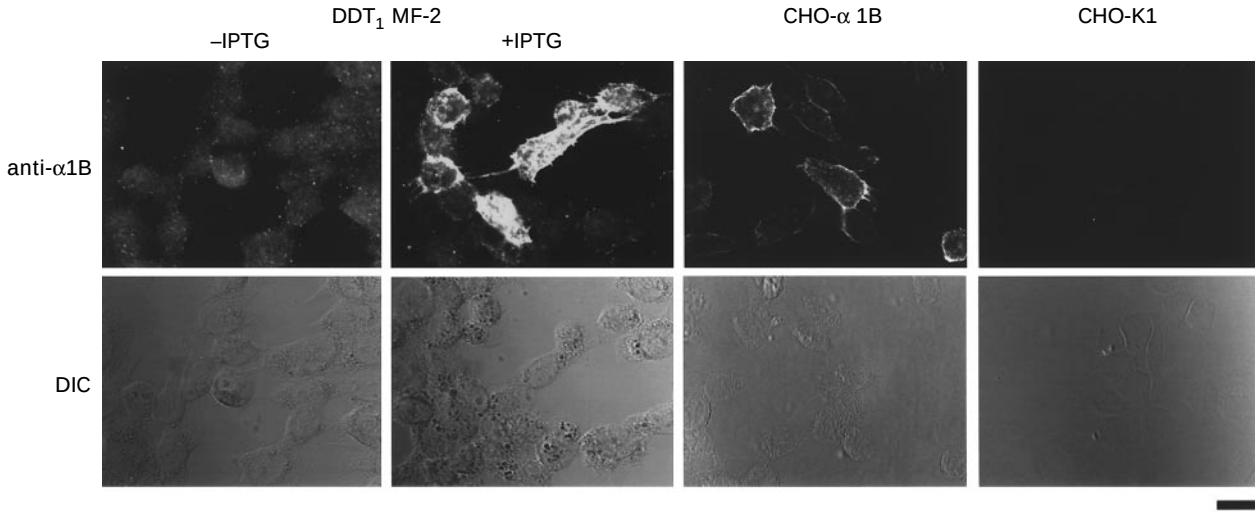

Figure 2 Immunocytochemistry of the α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells, CHO-α_{1B} cells and CHO-K1 cells. Immunocytostaining with anti-peptide antiserum (1B-N1-C) was performed in the α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells (−IPTG: without IPTG treatment; +IPTG: 48 h, 1 mM IPTG treatment), CHO-α_{1B} cells and CHO-K1 cells. Cells were reacted with the affinity purified anti-peptide antibody as described in Methods (upper panel). Cells were also observed by electrical pseudo differential interference contrast (DIC) image (lower panel). Scale bar, 10 μm.

different times of induction (0, 6, 12, 24, 48 h) and different concentrations (0.1 mM – 1.0 mM of IPTG). By using a radioligand binding assay with [^{125}I]-HEAT, we confirmed an increase in B_{max} as previously found by Esbenshade *et al.* (1995a,b); 48 h exposure to 1 mM IPTG caused a 5 fold increase in expression level. As shown in Figure 1c, there was a good correlation between the average fluorescence of antibody (FL-1) and B_{max}. Furthermore, cells exposed to IPTG were double stained by the antibody in FL-2 as well as by BODIPY-FL prazosin in FL-1, and the average fluorescent intensities of FL-1 and FL-2 were compared. As shown in Figure 1d, the average fluorescent intensity of antibody binding (FL-2) and fluorescent ligand binding (FL-1) were well correlated even in individual cells (data not shown). These results suggest that only α_{1B}-adrenoceptors localized on the cell surface can be quantitatively monitored by flow cytometry with 1B-N1-C, since the antibody is a hydrophilic macromolecule and is unlikely therefore to penetrate the cells during the staining treatment (4°C, 30 min).

Immunocytochemical localization of α_{1B}-adrenoceptors

Although flow cytometry analysis was shown to be useful for quantifying the cell surface α_{1B}-adrenoceptors, this analysis does not allow visualization of the intracellular localization of α_{1B}-adrenoceptors. We therefore determined whether the antibody could also be used to study the distribution of α_{1B}-adrenoceptors by immunocytochemical confocal microscopy.

The CHO-α_{1B} and inducible DDT$_1$MF-2 cells were examined. As shown in Figure 2, the pattern of immunostaining obtained with this antibody was diffuse and uniformly distributed over the cell surface. The specificity of the immunostaining was demonstrated by the following criteria (data not shown). First, preabsorption of the antiserum with the corresponding peptide abolished the specific staining. Second, no positive immunofluorescence was observed when the anti-peptide antiserum was substituted with preimmune serum. Third, no immunofluorescence was observed in untransfected CHO cells (Figure 2, CHO-K1).

The inducible DDT$_1$MF-2 cells were examined before or after 24 h exposure to IPTG (1 mM). In untreated cells (Figure 2, −IPTG), there was a weak fluorescent signal distributed over the cell, while with the IPTG-treated DDT$_1$MF-2 cells (Figure 2, +IPTG) a much stronger fluorescent signal was detected on the cell surface. In addition, the fluorescence intensity was found to differ between cells, supporting the observation by flow cytometry that the induced DDT$_1$MF-2 cell populations are not homogeneous for the inducible α_{1B}-adrenoceptor levels of expression.

These results suggest that the anti-peptide antibody is also sufficiently specific to characterize the localization of α_{1B}-adrenoceptors by immunocytochemical confocal microscopy. By combining the quantitative flow cytometry analysis with visualization of cellular distribution by the immunocytochemical confocal microscopy, we further examined the effects of several pharmacological tools on the processes of receptor internalization and cell surface sorting.

Pharmacological characterization of cell surface sorting

Taking advantage of the inducible expression system of α_{1B}-adrenoceptors in DDT$_1$MF-2 cells, we first examined the biosynthetic trafficking pathway of α_{1B}-adrenoceptors. As summarized in Figure 3a, flow cytometry showed that brefeldin A completely blocked the IPTG-induced expression of α_{1B}-adrenoceptors in DDT$_1$MF-2 cells, whereas the other drugs had no significant effect. The cell surface sorting was found to be rapidly restored upon brefeldin A removal (data not shown). Immunocytochemical confocal microscopy showed that IPTG induction resulted in a strong fluorescent signal predominantly distributed over the cell surface in control DDT$_1$MF-2 cells, while in the brefeldin A-treated cells no strong fluorescent signal was observed over the cell surface, and a much weaker fluorescent signal was detected intracellularly. The inhibitory effect of brefeldin A was also observed on the transient expression of α_{1B}-adrenoceptors in COS-7 cells (data not shown), suggesting that brefeldin A affects the cell surface sorting step of the receptor irrespective of cell type.

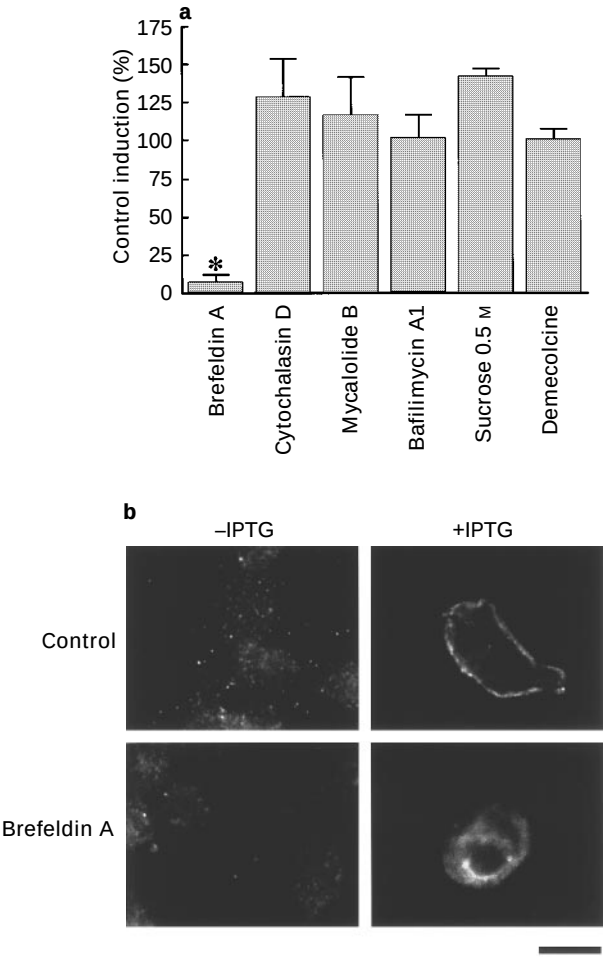

Figure 3 Effects of various pharmacological agents and hyperosmotic sucrose treatment on the cell surface sorting of α_{1B}-adrenoceptors. (a) At 12 h after treatment of the α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells with 1 mM IPTG, drugs were added, and after 36 h treatment effects of drugs on the IPTG-induced expression of the cell surface receptor were determined by flow cytometry. Concentrations of drugs were as follows; brefeldin A 10 μg ml^{-1}, cytochalasin D 100 nM, mycalolide B 1 μM, bafilomycin A1 2 μM and demecolcine 100 nM. The cells were fixed, washed by PBS, and labelled with anti-peptide antiserum (1B-N1-C) and subjected to the FACScan. The results are expressed as the percentage of controls. The values correspond to the mean ± s.d. of at least four independent experiments. *$P<0.05$ compared to control. (b) Effect of brefeldin A on the sorting of α_{1B}-adrenoceptors. The effect of brefeldin A treatment (10 μg ml^{-1}, 36 h) was examined in α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells either treated with 1 mM IPTG (+IPTG) or untreated (−IPTG). At 12 h after treatment of the α_{1B}-adrenoceptor inducible DDT$_1$MF-2 cells with 1 mM IPTG, brefeldin A was added, and a further 36 h treatment effect of brefeldin A on the subcellular localization of α_{1B}-adrenoceptors was determined by immunocytochemical confocal microscopy. Scale bar, 10 μm.

Pharmacological characterization of agonist-promoted internalization

The flow cytometry analysis showed that the exposure of CHO-α_{1B} cells to 10 μM phenylephrine for 2 h resulted in a decrease in fluorescent intensity of $29.4 \pm 2.4\%$ ($n=7$) compared to the control (Figure 4a). Higher concentrations of phenylephrine (up to 100 μM) or a longer incubation (up to 24 h) did not cause any further decrease. The phenylephrine-induced decrease of fluorescent intensity was α_{1B}-adrenoceptor-mediated, since 1 μM prazosin completely blocked the decrease, and it also recovered to control levels within 30 min after washout of the phenylephrine (Figure 4a).

We next examined the effects of drugs and hyperosmotic sucrose treatment on this phenylephrine-induced α_{1B}-adrenoceptor internalization. As summarized in Figure 4b, the agonist-induced α_{1B}-adrenoceptor internalization was specifically blocked by the actin depolymerization drugs, cytochalasin D and mycalolide B (Hori *et al*., 1993; Saito *et al*., 1994). Under the same conditions, immunocytochemical confocal microscopy showed that phenylephrine-treated cells had non-uniform and bright patches of immunofluorescence localized internally in the perinuclear region. Fluorescence of

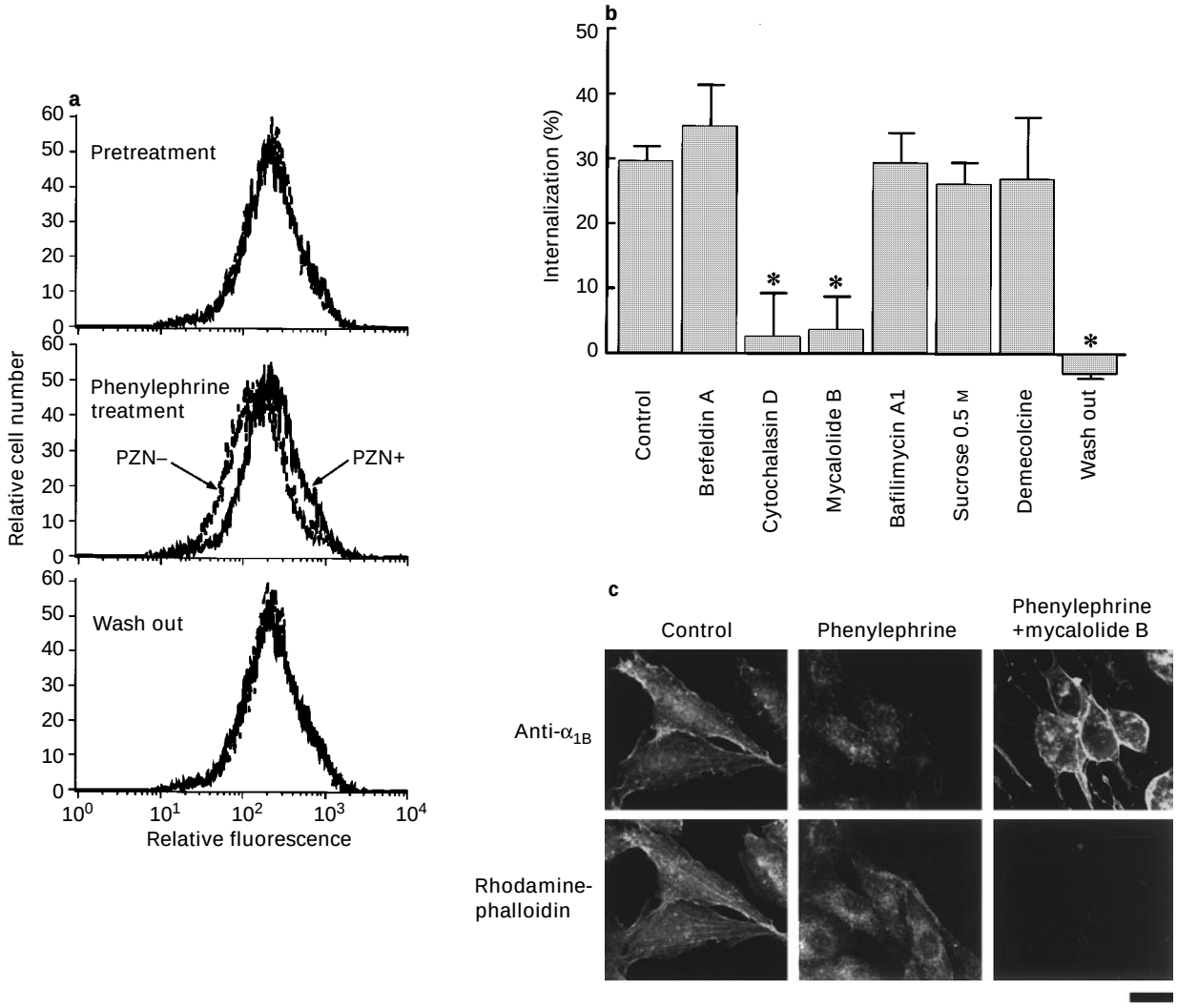

Figure 4 Effects of various pharmacological agents and hyperosmotic sucrose treatment on the internalization of α_{1B}-adrenoceptors. (a) Flow cytometry analysis of agonist-induced internalization and recycling of α_{1B}-adrenoceptors. The cell surface α_{1B}-adrenoceptors were quantitatively monitored in CHO-α_{1B} cells by flow cytometry. The cells were fixed, washed and incubated with antiserum and FITC-labelled anti-rabbit IgG and subjected to FACScan. CHO-α_{1B} cells (upper panel; pretreatment) were treated with 10 μM phenylephrine for 2 h with (middle panel, PZN+) or without (middle panel, PZN−) 100 nM prazosin, then harvested and processed for flow cytometry. Thirty minutes after wash-out of phenylephrine, CHO-α_{1B} cells were examined (bottom panel). (b) Effects of various pharmacological agents and hyperosmotic sucrose treatment on the internalization process of α_{1B}-adrenoceptors. CHO-α_{1B} cells were pretreated with drugs for 30 min, then 10 μM phenylephrine was applied. Following 2 h incubation, the cell surface α_{1B}-adrenoceptor was quantified by flow cytometry. Thirty minutes after wash-out of phenylephrine, cells were also examined (Wash out). The results are expressed as the decrease in percentage of the control cell surface fluorescence. Concentrations of drugs were as follows; brefeldin A 10 μg ml^{-1}, cytochalasin D 100 nM, mycalolide B 1 μM, bafilomycin A1 2 μM, demecolcine 100 nM. The values correspond to the mean$\pm$s.d. of at least four independent experiments. *$P<0.05$ compared to control. (c) Effect of phenylephrine alone and with mycalolide B on the subcellular localization of α_{1B}-adrenoceptors observed by immunocytochemical confocal microscopy. Confocal microscopy images are shown of: control cells, phenylephrine 10 μM-treated cells, or phenylephrine (10 μM) and mycalolide B (1 μM)-treated cells. Phenylephrine treatment interval was 2 h. In CHO-α_{1B} cells treated with phenylephrine and mycalolide B, cells were pretreated with mycalolide B for 30 min, then 10 μM phenylephrine was applied and incubated for 2 h. Cells were processed for immunocytochemical confocal microscopy by use of the anti-peptide antibody (upper panels) and also by rhodamine-phalloidin (lower panels). Scale bar, 10 μm.

rhodamine-phalloidin were also changed after phenylephrine treatment. Together with the flow cytometry results, these images clearly demonstrate that the α_1-adrenoceptor agonist elicits a profound redistribution of receptor; with a significant fraction of receptors translocating from the cell surface to an intracellular location. Furthermore, in accordance with the flow cytometry analysis, phenylephrine-promoted subcellular redistribution of α_{1B}-adrenoceptors was blocked by mycalolide B treatment (Figure 4c). In addition, rhodamine-phalloidin staining showed that F-actin was hardly detected in mycalolide B-treated cells (Figure 4b), confirming that mycalolide B completely disassembled actin filaments.

Discussion

The combination of quantitative flow cytometry analysis and immunocytochemical confocal microscopy analysis has enabled us to quantify effectively the cell surface α_{1B}-adrenoceptor, and to visualize the subcellular localization of this receptor. By using the α_{1B}-adrenoceptor inducible DDT_1MF-2 cells, we could examine the two processes of cell surface sorting and agonist-induced internalization of α_{1B}-adrenoceptors separately. Pharmacological evidence is presented suggesting the involvement of actin polymerization in the agonist-promoted receptor internalization, but not in the cell surface sorting process. As exemplified in this study, this experimental approach provides a valuable system for the study of the molecular mechanism(s) involved in the cellular trafficking of GPCR.

The present study has shown the utility of the quantitative flow cytometry analysis for the study of GPCR. Flow cytometry analysis of cell surface antigens is widely used in immunological and haematological studies, but it has not been widely applied to study GPCR. Flow cytometry has several clear advantages over the radioligand binding assay, since it can provide two different kinds of information; the amount of cell surface receptor can be quantified, and the specific cells expressing the certain receptor can be identified, even in mixed cell populations. In transiently transfected cells, the α_{1B}-adrenoceptor-expressing cells could be clearly distinguished from untransfected ones (Hirasawa *et al*., 1996). Also, as observed in the present study with the inducible DDT_1MF-2 cells, the levels of receptor expression can be assessed, enabling the efficient and easy collection of a highly expressing clone (or highly inducible cells) (Figure 1a). Practically, the sensitivity of detection with a fluorescent probe is now almost as sensitive as a radiolabelled assay, and the hardware system is popular and easy to handle. Quantitative monitoring of cell surface receptors is a particular advantage of flow cytometry. However, the precise subcellular localization of a receptor cannot be ascertained by this method. Immunocytochemical confocal microscopy is more applicable for the determination of subcellular localization of receptors although results may not be very quantitative. Hence, the two approaches compliment each other well for the analysis of receptor regulation.

Evidence is presented suggesting that the cell surface sorting process and the agonist-promoted internalization process involve different components of the cellular endocytic machinery. The cell surface sorting process was not affected by either cytochalasin D or mycalolide B, and was specifically blocked by brefeldin A. The fungal antibiotic brefeldin A is considered to cause the morphological disassembly of Golgi apparatus, and to block transport of proteins into post-Golgi compartments in the cell and redistribute Golgi-resident proteins back into the endoplasmic reticulum (Lippincott-Schwartz *et al*., 1990). Our observations showed that GPCRs are sorted to the cell surface following synthesis through a brefeldin A-sensitive and actin-independent pathway. In contrast to the cell surface sorting, the agonist-promoted internalization process is specifically blocked by actin depolymerization drugs, confirming that the agonist-promoted α_{1B}-adrenoceptor internalization is through actin-dependent endocytic pathways (Munn *et al*., 1995; Geli & Riezman, 1996).

Furthermore, our observation that the agonist-promoted α_{1B}-adrenoceptor internalization process is sensitive to cytochalasin D, but insensitive to hypertonic treatment, suggested that the α_{1B}-adrenoceptor endocytic process is via a non-coated pathway including caveolae, based on the proposal of Lamaze and Schmid (1995). However, this finding may not hold for GPCRs in general, since recent studies showed the involvement of caveolae in GPCR internalization, while other recent studies of β_2-adrenoceptors indicated a role for the coated vesicle (clathrin) pathway (Goodman *et al*., 1996). Furthermore, Fonseca *et al*. (1995) suggested that α_{1B}-adrenoceptor internalization process is dependent on coated vesicles, because internalization can be blocked by hyperosmotic treatment. These discrepant observations suggest that the two mechanisms can vary depending on the type of cell and GPCR, and also show the limitation of the pharmacological tools.

The present study characterized the pharmacology of internalization and cell surface sorting of α_{1B}-adrenoceptors. The results clearly showed that internalization and cell surface sorting involve different components of the cellular endocytic machinery. The experimental approach as exemplified in this study would provide a valuable system to study further the molecular mechanism(s) of cellular trafficking of GPCR.

Abbreviations
$[^{125}I]$-HEAT, $[^{125}I]$-(2-β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone; CHO cells, Chinese hamster ovary cells; PBS, phosphate-buffered saline; G protein, guanine nucleotide-binding protein; FITC, fluorescein isothiocyanate; GPCR, G protein coupled receptor; IPTG, isopropyl-β-D-thiogalactopyranoside

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