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ACCELERATED COMMUNICATION

Subtype-Specific Differences in Subcellular Localization of α_1-Adrenoceptors: Chlorethylclonidine Preferentially Alkylates the Accessible Cell Surface α_1-Adrenoceptors Irrespective of the Subtype

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SUMMARY

Selective inactivation of α_{1B}-adrenoceptor (AR) by the site-directed alkylating agent chlorethylclonidine (CEC) has been used as one of major pharmacological criteria to subclassify α_1-AR; however, the mechanism for the differential CEC sensitivity of the two subtypes is uncertain, and the extent of CEC inactivation varies depending on the treatment employed. In this study, we examined the correlation between the subcellular localization of α_1-AR subtypes (α_{1A} and α_{1B}) and CEC sensitivity. Constructing α_1-AR tagged with the FLAG epitope at the amino terminus and/or green fluorescent protein (GFP) at the carboxyl terminus, we examined the subcellular distribution of α_1-ARs expressed in COS-7 cells. Flow cytometry analysis showed that most populations of GFP-expressing α_{1B}-AR cells, but very few GFP-expressing α_{1A}-AR cells, were detected by the anti-amino terminus antibodies. The immunocytochemical and GFP-fluorescence confocal micrographs showed that α_{1A}-ARs predominantly localize intracellularly, whereas α_{1B}-ARs localize on the cell surface. Furthermore, CEC (10 μM) treatment of intact cells resulted in an inactivation of approximately 42% of α_{1A}-ARs and 93% of α_{1B}-ARs, whereas treatment of the membrane preparations resulted in an inactivation of approximately 83% of α_{1A}-ARs and 88% of α_{1B}-ARs, respectively. Together, the results showed that a hydrophilic alkylating agent CEC preferentially inactivates α_1-AR on the cell surface irrespective of its subtype, and that the subtype-specific subcellular localization rather than the receptor structure is a major determinant for CEC inactivation of α_1-AR. Subtype-specific subcellular localization suggests an additional class of functional properties that provide new insight into drug action.

α_1-ARs play critical roles in the regulation of a variety of physiological processes, such as smooth muscle contraction, myocardial inotropy and chronotropy, and hepatic glucose metabolism (1, 2). Increasing evidence suggests that these physiological responses are regulated by multiple receptor subtypes that are structurally homologous (3, 4). Heterogeneity of α_1-ARs (α_{1A} and α_{1B}) was first suggested by pharmacological studies based on differential affinity of a variety of agonist and antagonists, and differential sensitivity to the alkylating agent CEC (5–11). Molecular cloning identified three distinct cDNA encoding α_1-AR subtypes (α_{1a}, α_{1b}, and α_{1d}) (12–17). More recent studies provide evidence supporting the idea that the α_{1a}-AR (formerly α_{1c}-AR) cDNA encodes the pharmacological α_{1A}-AR subtype, whereas the α_{1b}-AR cDNA clone appears to encode the natively expressed, pharmacologically defined α_{1B}-AR subtype (18–20).

A selective inactivation of α_{1B}-AR by CEC has been used as one of major criteria to subclassify the α_1-AR binding sites and functional responses; however, the mechanism for the preferential inactivation of α_{1B}-AR by CEC is uncertain (4).

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ABBREVIATIONS: AR, adrenoceptor; CEC, chlorethylclonidine; HEAT, (2-β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone; PBS, phosphate-buffered saline; GFP, green fluorescent protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Also, the extent of CEC inactivation has been shown to be varied depending on the CEC treatment employed (intact cell or membrane preparation), and the relative proportion of each subtype in native tissues determined by CEC inactivation does not correlate well with that delineated by subtype-selective competitive antagonists (21, 22). With these problems, it is difficult at present to elucidate the functional role of each receptor subtype. Possible mechanisms that could explain these CEC-related problems include the subtype-specific difference in cellular localization, because CEC is known to bind equally well to both α_{1A}- and α_{1B}-ARs and is highly hydrophilic (23), thereby alkylating only the α_1-AR that can be accessed. In support of the hypothesis, our recent flow cytometry analysis of α_1-AR subtypes with fluorescent ligand BODIPY-FL prazosin indicated that α_{1A}- and α_{1B}-ARs may distribute differently at steady state in stably expressing Chinese hamster ovary cells (24). In the present study, therefore, we examined the subcellular localization of α_{1A}- and α_{1B}-ARs, and compared it with their CEC sensitivity of each subtype. Flow cytometry analysis and immunocytochemical and GFP-fluorescent localization analysis by confocal microscopy show that α_{1A}-ARs predominantly localize intracellularly, whereas most of α_{1B}-ARs localize on the cell surface, and that CEC preferentially inactivates the cell surface α_1-AR irrespective of the subtype. This study indicates that subtype-specific subcellular localization constitutes a new class of properties, in addition to ligand binding and G protein-coupling selectivity, which may pharmacologically distinguish structurally homologous subtypes of α_1-AR (as well as other G protein-coupled receptors).

Experimental Procedures

cDNA Construction

Expression constructs used in the present study are shown in Fig. 1. For tagging FLAG and HisX6 epitope to the amino terminus of α_{1A}-AR, primer 1 (gtactatggactacaaggacgacgatgacaagcatcaccatcaccatcacgc) and 2 (acgtcatgatacctgatgttcctgctgctactgttcgtagtggtagtggtagtgcggtac) were annealed and ligated into the *Nco*I- and *Pst*I-digested pME-α1A expression vector [previously pME-α1c (16)], which designated pME-α_{1A}-FH. The amino acid sequence coded by the primers are also shown in Fig. 1.

For tagging GFP to carboxyl terminus of α_1-ARs, the coding region of GFP mutant S65T (kind gift from Dr. H. Takahashi, Mitsubishi Kasei Inst. Life Sciences, Tokyo, Japan) was amplified by primer 3 (aaagggcccatgagtaaaggagaagaacttttc) and primer 4 (aaaactagttttgtatagttcatccatggc), which produce the 5′-*Apa*I site for ligation. All α_{1A}- and α_{1B}-AR expression vectors [pME-α_{1A}, pME-α_{1B} (25), and pME-α_{1A}-FH] were digested by *Apa*I and *Xba*I, to which the enzyme-digested GFP-polymerase chain reaction products ligated, and resulting constructs were designated pME-α_{1A}-GFP, pME-α_{1B}-GFP, and pME-α_{1A}-FH-GFP, respectively (Fig. 1). Modified regions of these constructs were confirmed by sequencing with an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

We chose the *Apa*I site in the carboxyl terminus for the GFP to be integrated because the region distal to the *Apa*I site varies in α_{1A}-AR splice variants with similar pharmacological properties (17), and deletion of the region in α_{1B}-AR was shown not to affect binding and signal transduction properties (26). All experiments were performed with wild-type receptors in parallel whenever possible to confirm that the observation of subcellular localization is not an artifact of the epitope tagging and/or GFP fusion technique (i.e., that the expressed receptors fold and sort properly).

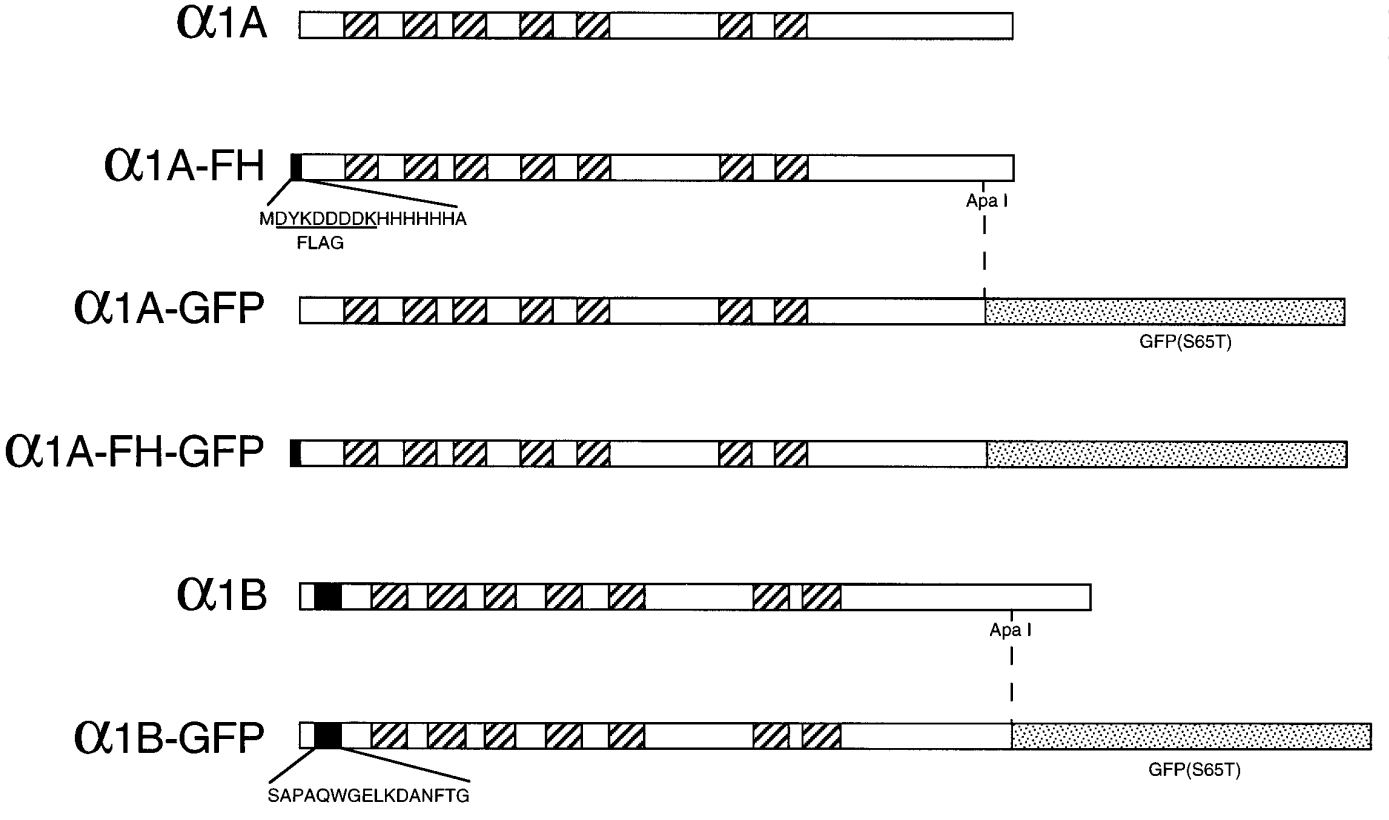

Fig. 1. Constructs for epitope-tagged and GFP-fused α_1-ARs. As described in Experimental Procedures, we constructed epitope-tagged and GFP-fused α_{1A}- and α_{1B}-ARs. ▨, putative membrane spanning domains; ■, epitope regions for anti-peptide antibodies or FLAG-tagged region. Epitope sequences are also shown.

Cell Culture and Transfection

COS-7 cells, obtained from the American Type Culture Collection (Rockville, MD), were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The constructs were transfected into COS-7 cells by the electroporation method using Cell-Porator (BRL Life Technologies, Bethesda, MD) according to the manufacturer's instructions, and cells were assayed 48–72 hr after transfection.

Antibody Preparation

Generation of an anti-peptide antibody (designated as 1B-N1-C) was described previously (23). Briefly, peptide was synthesized corresponding to amino acids 12–27 [peptide: 1B-N1; (C)SAPAQWGELKDANFTG] of the published hamster α_{1B}-AR sequence (12), conjugated to the carrier protein keyhole limpet hemocyanin and injected to rabbits. Antisera were screened against the peptides by using cross-dot systems (Sebia, Moulineaux, France) and visualized by ABC system (Vector Laboratories, Burlingame, CA). By immunoblotting and immunoprecipitation studies, we confirmed that the antibody detect the α_{1B}-AR (24).

Antiserum was purified on 1 ml of a protein A-Sepharose CL-4B column (Pharmacia Biotech, Tokyo, Japan) equilibrated with 20 mM phosphate buffer, pH 7.5, and eluted with glycine-HCl 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 microconcentrator (Amicon, Danvers, MA) and stored at −20°.

Flow Cytometry Analysis

For the flow cytometry analysis with specific antibodies, attached cells were trypsinized and washed twice with PBS. Cells were then incubated for 30 min at 4° with the primary antiserum (1B-N1-C, diluted 1/100) or anti-FLAG monoclonal antibody (10 μg/ml; Eastman Kodak, New Haven, CT), washed three times with PBS, and incubated with Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG (Chemicon International, Temecura, CA) for 60 min at 4°.

Analysis of the cells was performed by using FACScan flow cytometer (Becton Dickinson, Mountain View, CA). To avoid artifacts arising from auto-fluorescence of dead cells and cellular debris, data acquisition was electrically gated for large particles presumably representing intact cells by forward and side scatter. We have used flow cytometry standard setting adjusted to routine double staining techniques employing GFP-associated fluorescence (FL-1) and Cy3 (FL-2) using operating program LYSIS-II. Routinely, data from green fluorescence of 10^4 cells were subjected to histogram and dot-plot analysis, and FL-2 fluorescence of GFP-positive cells were compared with the control.

Laser Scanning Microscope Analysis

Immunofluorescence detection. Transfected and untransfected COS-7 cells were seeded at 1×10^5 per well of the 8-well Lab tek chamber slide (Nunc, Naperville, CT) in 0.5 ml of medium. Fixation was performed in 80% acetone for 5 min. Cells were then incubated with 0.05% Triton X-100 in PBS. The primary antibodies, 10 μg/ml of anti-FLAG monoclonal antibody (Eastman Kodak) and 5 μg/ml affinity-purified anti-α_{1B}-AR antibody (1B-N1-C) (24), were brought in PBS containing 10% goat serum and 0.05% Triton X-100, and applied to cells, which were subsequently kept in a humidified chamber for 1 hr at room temperature. Fluorescein isothiocyanate-conjugated goat anti-mouse or anti-rabbit IgG (Chemicon International, Temecura, CA) was diluted 1/200 in PBS containing 2% bovine serum albumin and 0.05% Triton X-100, and applied to cells for 1 hr at room temperature. Cells were then washed twice with PBS, and coverslips were applied using Gel/Mount (Biomeda, Foster City, CA). After immunocytochemical staining, cells were examined by using LSM-GB200 laser scanning microscope (Olympus, Tokyo, Japan) with argon-ion laser set at 488 nm for excitation of fluorescein isothiocyanate.

GFP detection. Transfected and untransfected COS-7 cells were seeded at 1×10^5 per well of the cover-glass bottom culture dish (MatTek, Ashland, MA) in 2.0 ml of medium. Two days after transfection, cells were washed with PBS and examined using LSM-GB200 within 30 min at room temperature.

^{125}I-HEAT-Binding Assay

Membrane preparation. Membrane preparation of the cells was performed as described previously (16, 17, 27). Briefly, the cells were collected and disrupted by the sonicator (model SONIFIER 250, setting 5 for 8 sec) in ice-cold buffer A (250 mM sucrose, 5 mM Tris·HCl, 1 mM $MgCl_2$, pH 7.4) and centrifuged at $3,000 \times g$ at 4° for 10 min to remove nuclei. The supernatant fraction was centrifuged at $35,000 \times g$ for 20 min at 4°. The resulting pellet was resuspended in binding buffer (50 mM Tris·HCl, 12.5 mM $MgCl_2$, 10 mM EGTA, pH 7.4), and was frozen at −80° until assay. All buffers contain protein inhibitors of 1 mM phenylmethylsulfonyl fluoride, 100 μM benzamidine, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin.

^{125}I-HEAT binding. ^{125}I-HEAT-binding assay was performed as described previously (16, 17, 27). Briefly, membrane aliquots (~10 μg of protein) were incubated with ^{125}I-HEAT in a final volume of 250 μl of binding buffer for 60 min at 25°. The incubation was terminated by adding the ice-cold buffer B and immediately filtering through Whatmann GF/C glass-fiber filters with a Brandel cell harvester (model-30; Brandel, Gaithersburg, MD). Each filter was collected, and the radioactivity was measured. Binding assays were always performed in duplicate, and specific ^{125}I-HEAT binding was determined experimentally 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, using computer-assisted iterative nonlinear regression analysis. The protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL). Values are expressed as the mean ± standard deviation.

CEC treatment. Intact cell treatment: $5–10 \times 10^6$ cells suspended in 1 ml of the buffered salt solution (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1 mM $NaHPO_4$, 5 mM HEPES, 11 mM glucose and 0.1% bovine serum albumin, pH 7.4) was incubated with or without CEC (10 μM) at 37° for 30 min. After incubation, cells were washed three times with the buffered salt solution and used for the binding assay.

Hypoosmotic membrane treatment: Membranes prepared from cells were incubated in 1 ml volume of hypotonic buffer (5 mM Tris·HCl, 5 mM EDTA, pH 7.6) with or without CEC (10 μM) at 37° for 30 min (8, 9). The reactions were stopped by adding 16 ml of ice-cold buffer, and the solution was centrifuged at $35,000 \times g$ for 20 min at 4°. The membranes were washed and resuspended in buffer B, and residual ^{125}I-HEAT binding was assessed.

Materials

Materials were obtained from the following sources: ^{125}I-HEAT (specific activity, 2200 Ci/mmol; New England Nuclear, Boston, MA); prazosin HCl (Pfizer, Brooklyn, NY); phentolamine mesylate (Ciba-Geigy, Summit, NJ); fetal bovine serum and goat serum (Gibco, Gaithersburg, MD); and Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan). All other reagents were of the highest analytical grade.

Results and Discussion

We first compared ligand binding of the α_1-AR conjugates we constructed with their wild-type α_1-AR subtype. All constructs were expressed transiently in COS-7 cells to characterize their binding properties to the antagonist ^{125}I-HEAT. The antagonist-binding isotherms for α_{1A}-FH, α_{1A}-GFP, α_{1A}-

TABLE 1

Comparison of K_d and B_{max} of wild-type, epitope-tagged, and GFP-fused α_1-ARs.

Affinity for ^{125}I-HEAT binding was determined in membrane preparations from cultured COS-7 cells transiently transfected with constructs for epitope-tagged and GFP-fused α_1-ARs. Each value is the mean of two to three different experiments.

Constructs	K_d	B_{max}
	nM	*pmol/mg*
α_{1A}	81.3	3.94
α_{1A}-FH	72.2	1.96
α_{1A}-GFP	80.5	0.88
α_{1A}-FH-GFP	48.3	1.58
α_{1B}	86.6	2.13
α_{1B}-GFP	74.5	4.10

FH-GFP and α_{1B}-GFP conjugates were nearly identical to the wild-type α_{1A}-AR or α_{1B}-AR, respectively (Table 1), confirming that epitope tagging and/or GFP fusion in this manner does not perturb normal ligand binding. Receptor subtypes displayed similar transfection efficiencies (assessed by the percentage of transfected cells expressing GFP-associated fluorescence; Fig. 2). Both wild-type and conjugated receptors expressed in COS-7 cells were found to be functional, as a norepinephrine (10^{-7} M)-induced intracellular free Ca^{2+} concentration response was observed by using Indo-1 (28) (data not shown). Although all the receptor conjugates showed similar ligand binding to ^{125}I-HEAT, the tagging technique may have an artifactual effect on receptor folding and sorting. Hence, all experiments below were performed with wild-type receptors in parallel whenever possible.

Using the epitope- and/or GFP-tagged receptors, we characterized the subcellular localization of α_1-AR subtypes. We used flow cytometry analysis and confocal microscopy analysis of immunolocalization and GFP fluorescence, which enable us to detect the cell surface receptor and also to visualize the subcellular distribution of α_1-ARs, respectively. First, the cells transfected with α_{1A}-FH-GFP and α_{1B}-GFP conjugates were examined by flow cytometry. When transfected, approximately 10–20% of COS-7 cells were positively detected as GFP-associated fluorescence for α_{1A}-FH-GFP and α_{1B}-GFP receptors (Fig. 2, A and C). The mean value of fluorescence intensity of α_{1B}-GFP receptor was noted to be stronger than that of α_{1A}-FH-GFP or α_{1A}-GFP (*x*-axis; Fig. 2, A versus C). Then, the cells transfected with α_{1A}-FH-GFP and α_{1B}-GFP conjugates were stained by the anti-FLAG monoclonal antibody and by the antibody 1B-N1-C, respectively. Interestingly, as shown in Fig. 2, B and D, most populations of the cells expressing GFP were further detected (an upward shift) by 1B-N1-C (Fig. 2D), whereas very few cells expressing GFP

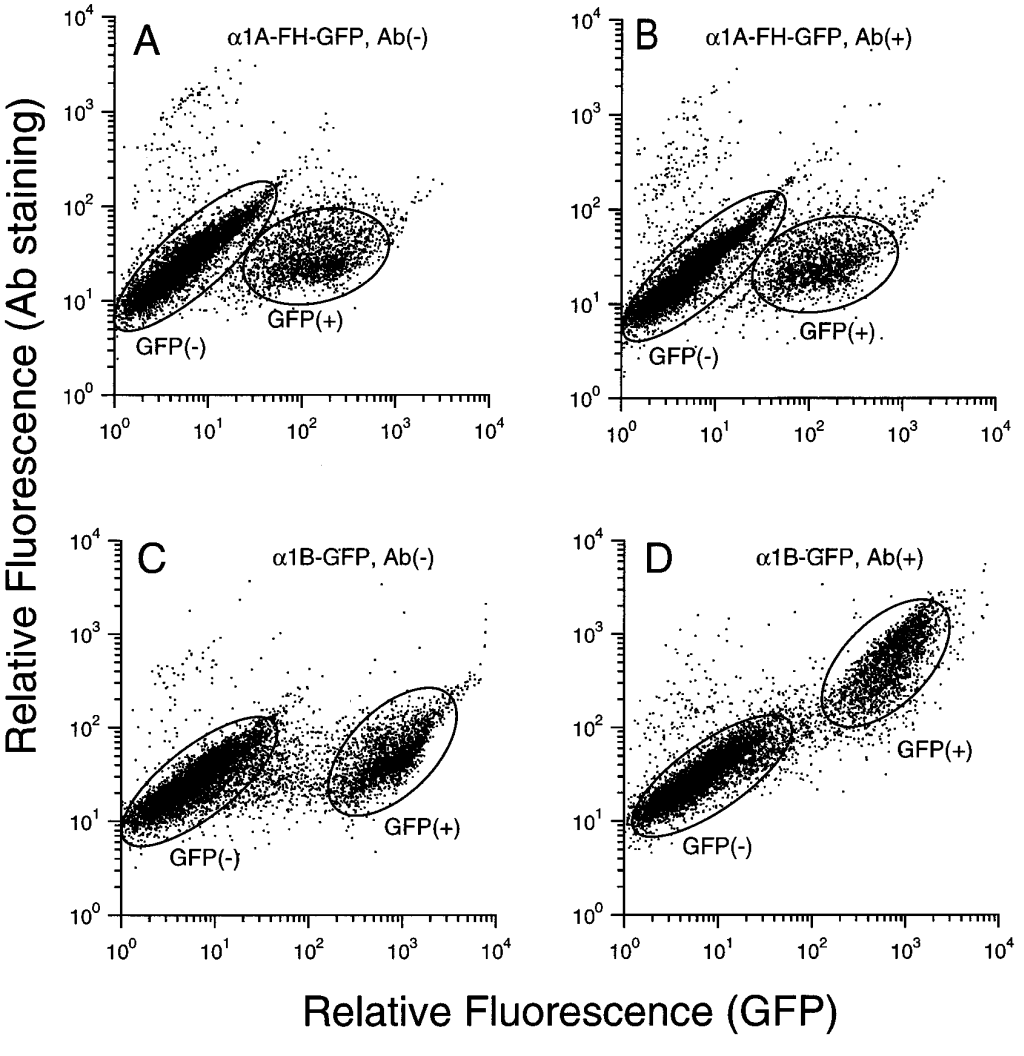

Fig. 2. Flow cytometry analysis for α_1-ARs. COS-7 cells transfected with pME-α1A-FH-GFP (A and B) and pME-α_{1B}-GFP (C and D) were analyzed by fluorescence-activated cell sorter flow cytometer. The cells were stained with nonimmune serum (A and C), anti-FLAG antibody (B), or anti-peptide antiserum against the α_{1B}-amino terminus (D). Results are showed by two-dimensional dot-plot using GFP fluorescence (FL-1: *horizontal*) and Cy3 labeled secondary antibody (FL-2: *vertical*). *GFP+*, cells expressing GFP-fused receptor; *GFP−*, cells that do not express GFP-fused receptor. The *x*-axis is relative fluorescence of GFP; *y*-axis is relative fluorescence of Cy3.

were detected by anti-FLAG antibody (no upward shift) (Fig. 2B). The failure for α_{1A}-GFP-expressing cells to be detected by anti-FLAG monoclonal antibody is not due to the construct or the weak affinity of antibody, because α_{1A}-FH and α_{1A}-FH-GFP can be immunostained by the same antibody (as shown below). Additionally, when the empty vector pME18s was transfected, no cell was positively detected as GFP-associated fluorescence or stained by antibodies. The results show that amino terminus of the α1B-AR can be, but that of α_{1A}-AR cannot be, detected by the antibody.

We further visually examined the cellular distribution of receptors using fluorescent antibody (α_{1A}-FH and α_{1B}-AR) and also the endogenous receptor fluorescence (α_{1A}-GFP and α_{1B}-GFP) by fluorescent confocal microscopy. As seen in Fig. 3 (*left*), immunocytochemical analysis showed that the fluorescence distribution of α_{1A}-AR is characteristic of the cytoplasmic distribution (enhanced perinuclear fluorescence), whereas that of α_{1B}-AR is typical of a plasma membrane-labeling pattern. Furthermore, corresponding well with immunocytochemical localization, the fluorescence micrograph of GFP shows that a strong green fluorescence densely localizes in a perinuclear region for α_{1A}-GFP, whereas fluorescence is diffuse in plasma membranes for α_{1B}-GFP (Fig. 3, *right*). Additionally, a similar cytoplasmic distribution was observed for α_{1A}-FH-GFP by using fluorescent antibody and also endogenous receptor fluorescence, and no fluorescence signal was detected in untransfected cells (data not shown). Together with the results of flow cytometry analysis, these confocal microscopy analyses show that α_{1A}-AR predominantly localizes intracellularly, whereas most α_{1B}-AR localizes on the cell surface.

We showed previously that the transfected α_{1A}-ARs could be inactivated easily by CEC when treated in membrane preparation, whereas they were relatively resistant to CEC inactivation compared with α_{1B}-AR when treated in intact cells (17, 29). To examine the correlation between the subcellular localization and the extent of CEC inactivation, we further compared the extent of CEC inactivation when treated in intact cells with that when treated in membrane preparation. As summarized in Fig. 4, CEC (10 μM 30 min) treatment of intact cells reduced the number of ^{125}I-HEAT binding sites by 41.8 $\pm$ 12.3%, 27.1 $\pm$ 18.1%, 37.7 $\pm$ 17.7%, and 43.7 $\pm$ 17.1% (n = 3 each) in the wild-type α_{1A}-AR, α_{1A}-FH, α_{1A}-GFP, and α_{1A}-FH-GFP, respectively; on the other hand, by 86.0 $\pm$ 4.5% and by 92.6 $\pm$ 1.9% (n = 3 each) in the wild-type α_{1B}-AR and α_{1B}-GFP, respectively. In contrast to the intact cell treatment, membrane preparation treatment resulted in a marked reduction of ^{125}I-HEAT binding sites in α_{1A}-AR (by 83.3 $\pm$ 2.5%, n = 5); however, it did not cause any further decrease of ^{125}I-HEAT binding sites in α_{1B}-AR (by 88.2 $\pm$ 4.5%, n = 4). In all cells, the K_d values to ^{125}I-HEAT obtained after CEC treatment were not much different from those obtained without CEC treatment (see Fig. 4 legend). Together with the observation that the two α_1-AR subtypes differentially localize, the results may suggest that a highly hydrophilic alkylating agent CEC inactivates only α_1-AR on the cell surface irrespective of its subtype; thus, the subtype-specific cellular distribution rather

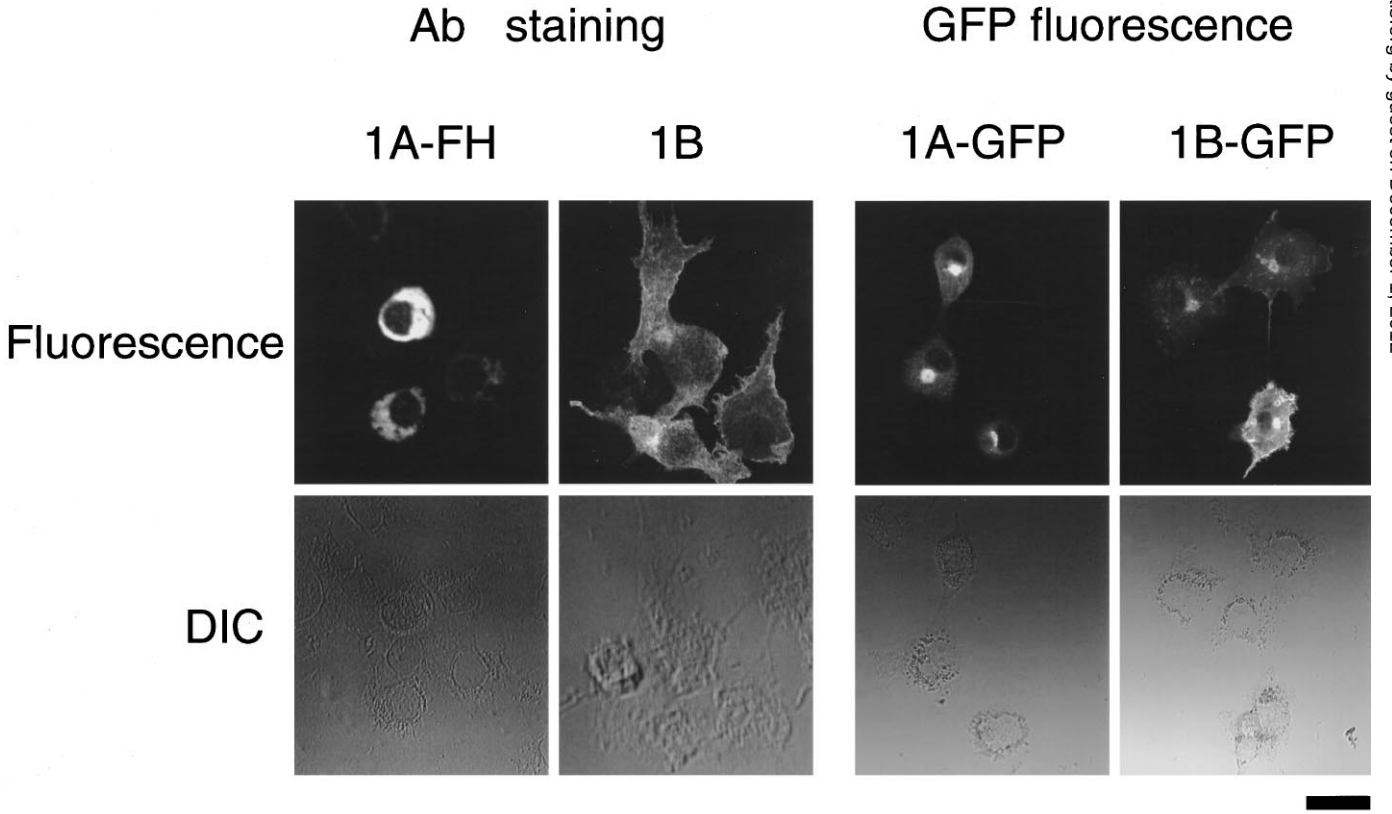

Fig. 3. The immunocytochemical and GFP fluorescence confocal micrographs of the α_{1A}-AR and α_{1B}-AR in COS-7 cells. Immunocytostaining with anti-peptide antiserum (1B-N1-C) and anti-FLAG antibody were performed in the pME-α_{1A}-FH (*left*)- or pME-α_{1B} (*right*)-transfected COS-7 cells. GFP fluorescence was also observed in the pME-α_{1A}-GFP (*left*)- or pME-α_{1B}-GFP (*right*)-transfected COS-7 cells in intact condition. *Bottom,* cells were also observed by electrical pseudo-differential interference contrast (*DIC*) image. *Scale bar*, 10 μm.

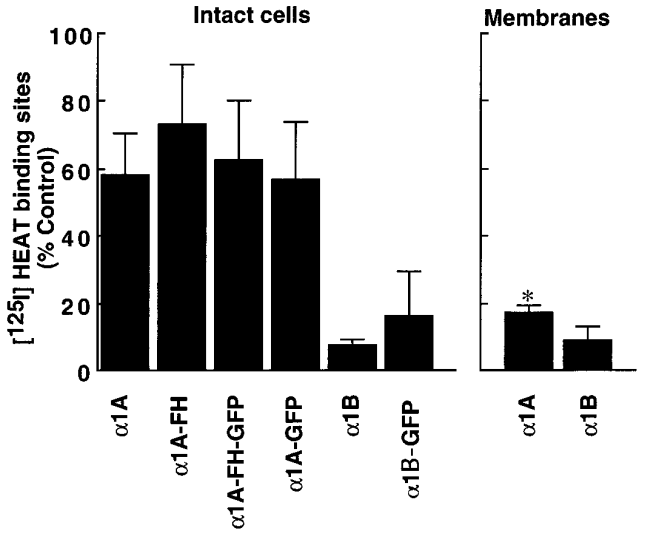

Fig. 4. Comparison of CEC inactivation by intact cell treatment and by membrane preparation treatment. For the intact cell treatment, COS-7 cells incubated with or without CEC (10 μM) at 37° for 30 min. After incubation, cells were washed three times with the buffered salt solution and used for the binding assay. In the membrane preparation treatment, membranes prepared from cells were incubated in hypotonic buffer (5 mM Tris·HCl, 5 mM EDTA, pH 7.6) with CEC (10 μM) at 37° for 30 min and residual ^{125}I-HEAT binding was assessed. The mean K_d values obtained in cells after CEC treatment were 48.5, 142, 33.0, 37.0, 28.8, and 67.0 nM, n = 2–3, in the wild-type α_{1A}-AR, α_{1A}-FH, α_{1A}-GFP, α_{1A}-FH-GFP, wild-type α_{1B}-AR, and α_{1B}-GFP, respectively. The results are expressed as the decrease in percentage of the control ^{125}I-HEAT binding. The values correspond to the mean ± standard deviation of at least three independent experiments. *, CEC inactivation was significantly different from that obtained in the intact cell treatment (unpaired t test, $p < 0.05$).

than the receptor structure is a major determinant for CEC inactivation of α_1-AR.

As indicated in the introductory material, three α_1-AR cDNA clones have been isolated, but their relationship to the pharmacologically defined α_{1A}- and α_{1B}-AR subtypes has been controversial. More recently, based on the comparison of the K_i values of native α_1-ARs from tissues with those of cloned α_1-ARs, and on the comparison of the tissue distribution of native α_1-ARs assessed by using subtype-selective drugs with that of mRNA expression of cloned receptors (18, 19), the IUPHAR Committee on the Classification of Adrenoceptor recommended that the α_{1a}-AR (formerly α_{1c}-AR) cDNA encodes the pharmacological α_{1A}-AR subtype, whereas the α_{1b}-AR cDNA clone appears to encode the natively expressed, pharmacologically defined α_{1B}-AR subtype (20). In this report, we demonstrated that α_{1A}-AR predominantly localizes intracellularly, whereas most of α_{1B}-ARs localize on the cell surface, and further indicate that the subtype-specific difference in receptor distribution appears to be reflected by inactivation of the highly hydrophilic alkylating agent CEC. The current results emphasize that one must be cautious in interpreting the data obtained by using CEC inactivation to study the functional role of each subtype.

Differential distribution of receptor subtypes appears to be generally observed for G protein-coupled receptors. von Zastrow *et al.* (30) and Saunders *et al.* (31) showed that subtype-specific different localization of α_2-AR subtypes. Thus, subtype-specific sorting comprises another important parameter, in addition to ligand binding affinity and G protein-coupling specificity, which may functionally differentiate receptor subtypes, thereby increase signal diversity. It is postulated that subtype-specific sorting might segregate individual receptors, G proteins, and effectors into functionally specialized microdomains (compartmentalization) and specify the signal transduction (30). Subtype-specific sorting may play an important role not only in increasing the signal diversity but also in the regulatory properties of receptor. Subtype-specific differences in susceptibility to agonist-promoted desensitization (and receptor phosphorylation) appeared to be well correlated with the cellular localization of receptor subtype; thus, α_2-C10-ARs and α_{1B}-ARs, which localize in the plasma membrane, are more easily desensitized compared with α_2-C4-ARs and α_{1A}-ARs, which predominantly localize intracellularly (32, 33). It is also suggested that intracellular receptors could serve as a reserve pool for delivery to the plasma membrane (30). In support of this view, we had previously shown a sizable pool of spare receptor for α_{1A}-AR and an absence of a receptor reserve for α_{1B}-AR by using the phenoxybenzamine-inactivation method (9, 10). Such internal stores could contribute to the rapid up-regulation of G protein-coupled receptor observed in physiological and pathophysiological conditions (34, 35). Further studies are clearly required to clarify the functional role of the subtype-specific subcellular localization.

In conclusion, we demonstrate for the first time that α_1-AR subtypes have differential subcellular localization, which appears to be a major determinant for CEC inactivation of α_1-AR. Thus, receptor subtype-specific localization suggests an additional functional properties that may explain the signal and functional diversity, and moreover it is of importance in assessing drug action. With the availability of experimental system as exemplified in the present study, elucidation of the molecular mechanism and function of the subtype-specific subcellular distribution may provide new insight into signal transduction as well as drug action.

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