# Flow cytometry analysis of $\alpha_1$-adrenoceptor subtypes

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**Abstract To characterize the $\alpha_1$-adrenoceptor subtypes, we developed a flow cytometry method using the fluorescent ligand BODIPY-FL prazosin and the anti-peptide antibody against the $\alpha_{1b}$-adrenoceptor amino terminus (designated 1B-N1-C) as probes. Three $\alpha_1$-adrenoceptors ($\alpha_{1a}$, $\alpha_{1b}$ and $\alpha_{1d}$) expressed in CHO cells were detected by BODIPY-FL prazosin; however, only $\alpha_{1b}$-adrenoceptor subtype was detected by the anti-peptide antibody 1B-N1-C. Furthermore, the flow cytometry analysis with 1B-N1-C specifically identified $\alpha_{1b}$-adrenoceptor in native cells of hamster DDT$_1$-MF2 cells, rat hepatocytes and1 cardiomyocytes.**

*Key words:* $\alpha_1$-Adrenoceptor; BODIPY-FL prazosin; Anti-peptide antibody; Flow cytometry; Hamster DDT$_1$-MF2 cell

## 1. Introduction

$\alpha_1$-adrenoceptors 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. Recently, it was found that $\alpha_1$-adrenoceptors comprise a heterogeneous family. Heterogeneity of $\alpha_1$-adrenoceptors ($\alpha_{1A}$ and $\alpha_{1B}$) was first suggested by pharmacological studies based on differential affinity of a variety of agents and differential sensitivity to the alkylating agent CEC, and differing requirements for extracellular calcium in signal transduction [1-5]. The cloning of three distinct cDNA encoding $\alpha_1$-adrenoceptor subtypes ($\alpha_{1a}$, $\alpha_{1b}$, $\alpha_{1d}$) has been reported [6-11]. The uncertain relationship between the cloned and native subtypes has been the source of much confusion; however, more recent studies provide evidence supporting the idea that the $\alpha_{1a}$-adrenoceptor cDNA encodes the pharmacological $\alpha_{1A}$-adrenoceptor subtype whereas the $\alpha_{1b}$-adrenoceptor cDNA clone appears to encode the natively expressed, pharmacologically defined $\alpha_{1B}$-adrenoceptor subtype [12,13]. However, the functional role of the native $\alpha_{1D}$-adrenoceptor still remains to be defined, and the $\alpha_{1D}$-adrenoceptor protein has not been identified yet.

Since neither $\alpha_1$-adrenoceptor subtype-selective ligand nor specific antibody is currently available, detection of each receptor transcript has been performed to characterize the tissue distribution of each $\alpha_1$-adrenoceptor expression; thus, the distribution of $\alpha_1$-adrenoceptor subtypes has been extensively characterized in rat, rabbit and human tissues by using Northern blot, in situ hybridization, RNase protection assay and RT-PCR techniques [6,7,9,10,14]. It is, however, suggested that the level of mRNA expression in a given tissue may not directly correlate with the level of receptor protein [15]. Ultimately, therefore, studies on both mRNA and receptor protein concentrations are required to understand the functional roles of adrenoceptors and mechanisms of how they are involved in physiology and pathology. Additionally, each native organ is composed of mixed cell populations expressing either a mixture or a unique population of $\alpha_1$-adrenoceptor subtypes. Thus, it is important to determine the expression profile of each receptor subtype protein not only at organ level but also at cellular level.

In this report, we examine whether the flow cytometry method with fluorescent probes could identify the cell surface $\alpha_1$-adrenoceptor of individual cells. We used the fluorescent ligand, BODIPY-FL prazosin, as a nonselective probe, and we also developed the anti-peptide antibody against $\alpha_{1b}$-adrenoceptor amino terminus (designated 1B-N1-C) as a subtype-selective probe. The antibody identified the $\alpha_{1b}$-adrenoceptor by Western blotting and immunoprecipitation. With the flow cytometry method, we characterized CHO and COS-7 cells that were transfected with each $\alpha_1$-adrenoceptor subtype cDNA. Furthermore, flow cytometry analysis with the antibody 1B-N1-C selectively identified $\alpha_{1b}$-adrenoceptor in native cells of DDT1-MF2, a hamster smooth muscle cell line [6], and hepatocytes and cardiomyocytes [1,4,15].

## 2. Materials and methods

### 2.1. Generation of anti-peptide antisera

Peptides were synthesized corresponding to amino acids 12–27 (Peptide: 1B-N1; (C)SAPAQWGELKDANFTG) of the published hamster $\alpha_{1b}$-adrenoceptor sequence [6], with the help of an ABI model 430A peptide synthesizer (Fig. 1A). Peptide 1B-N1 was conjugated through the extra amino-terminal cysteine to the activated carrier protein KLH (Boehringer Mannheim GmbH, Germany), according to the manufacturer's protocol. Synthesized peptide and conjugated KLH were purified by gel filtration on a G-25 column and stored at −20°C. The amino acid sequence of the peptide was verified by an ABI 477A protein sequencer. The conjugated peptide (500 μg) was emulsified in complete Freund's adjuvant and injected subcutaneously into Japanese white rabbits at 4–6 sites on the back. Rabbits were boosted with the same amount of the peptide emulsified in incomplete Freund's adjuvant and bled 14–21 days after the boost. Sera were stored at −20°C until use. Antisera were screened against peptides by using cross dot systems (Sebia, Moulineaux, France). On the pre-wetted 0.22-μm pore GVHP membrane (Nihon Millipore, Tokyo, Japan), slots were coated for 2 h at room temperature with peptide (0.01–10 μg/slot), washed three times with TTBS and blocked for 1 h at room temperature with 10% skimmed milk in TTBS; antisera were added at a dilution of 1/100 to 1/10000 for each slot and were allowed to

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Throughout this paper, we will use the standardized nomenclature system for $\alpha_1$-adrenoceptor subtypes recently recommended by the IUPHAR Committee on the Classification of Adrenoceptors. In this system, the cloned subtypes are designated in lower case letters as $\alpha_{1a}$, $\alpha_{1b}$, and $\alpha_{1d}$ which correspond to the clones previously defined as $\alpha_{1c}$, $\alpha_{1b}$, and $\alpha_{1a}$ (or $\alpha_{1a/d}$ and $\alpha_{1d}$), respectively. The corresponding pharmacological subtypes are designated with upper case letters and are defined as $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$, respectively.


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incubate for at least 12 h on a rotator at 4°C. The slots were washed three times with TTBS, and were visualized by ABC system (Vector Laboratories, Burlingame, CA) as described by the manufacturer.

*2.2. cDNA construction, cell culture and transfection*

Cell culture and transfection were performed as described previously [10,16]. For transient expression, human $\alpha_{1a}$-adrenoceptor [10], hamster $\alpha_{1b}$-adrenoceptor [9] and human $\alpha_{1d}$-adrenoceptor [17] cDNAs were ligated into the SR$\alpha$ promoter-based mammalian expression vector pME18S. The constructs were transfected into COS-7 cells by the DEAE-dextran method, and cells were assayed 48–72 h after transfection. For stable expression, human $\alpha_{1d}$-adrenoceptor cDNAs were ligated into the pSV-neo-R expression vector, which contains the neomycin resistance gene for selection of stable transformants. CHO cells were transfected with the above plasmids by LipofecTAMIN (Gibco/BRL, Life Technologies, Gaithersburg, MD, USA). The cells were maintained in F-12 medium (Gibco/BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and G418 (200 μg/ml). We also used a CHO cell line stably expressing $\alpha_{1b}$-adrenoceptor and $\alpha_{1a}$-adrenoceptor as reported previously [11,16]. $DDT_1MF2$ cells were maintained in Dulbecco's modified Eagle's medium containing glucose (4.5 g/l), streptomycin (100 μg/ml), penicillin ($10^5$ U/l) and supplemented with 10% fetal bovine serum. The medium was changed every 3 days, and the confluent cells were subcultured at a ratio of 1:10.

*2.3. Membrane preparation and radioligand binding assay*

Membranes of COS-7 and CHO cells were prepared as described previously [10,16]. Briefly, the cells were collected and disrupted by the sonicator (model SONIFIER 250, setting 5 for 8 s) in ice-cold buffer containing 5 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 10 mM EGTA, 250 mM sucrose, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 100 μM benzamidine). The mixture was then centrifuged at $3000\times g$ 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, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 100 μM benzamidine) and stored −80°C until use. Membrane aliquots (10 μg of protein) were incubated for 60 min at 25°C with approximately 80 pM of [$^{125}$I]HEAT, an $\alpha_1$-adrenoceptor antagonist radioligand, $^{125}$I-(2-β-(4-hydroxy phenyl)-ethylaminomethyl)-tetralone, 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 fiber filters with a Brandel cell harvester (Model-30, Gaithersburg, MD, USA). Specific [$^{125}$I]HEAT binding was experimentally determined from the difference between counts in the absence and presence of 10 μM phentolamine. $K_i$ values were generated by the iterative curve-fitting program LIGAND [18]. The protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA).

*2.4. Photoaffinity labeling*

Photoaffinity labeling was performed by a modification of the procedure of Terman et al. [19,20]. Briefly, 10 μg of membrane fraction was incubated at 37°C for 1 h in buffer containing 0.3 nM [$^{125}$I]aryl azidoprazosin, then photolyzed for 10 min under a EN160L/J UV mineral lamp (Spectronics, Westburg, NY, USA). The membranes in the reaction mixture were pelleted by centrifugation, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to a BAS 2000 system (Fuji, Tokyo, Japan) to visualize incorporated [$^{125}$I]aryl azidoprazosin.

*2.5. Immunoprecipitation*

After photoaffinity labeling, the labeled membrane (10 μg of protein) was resuspended in 450 μl of binding buffer, then 50 μl of 5% (v/v) Triton X-100 was added, and the mixture was vortexed before allowed to solubilize on ice for 1 h. Lysates were centrifuged in a microfuge for 30 min at $10\,000\times g$ two times, to sediment insoluble material. 400 μl of the supernatant was subsequently used for immunoprecipitation. Solubilized material was incubated with 4 μl of antiserum for at least 12 h on a rotator at 4°C. Nonspecific immunoprecipitation was assessed either by using nonimmune rabbit serum or by using the anti-peptide antiserum plus excess antigenic peptide. After 1 h incubation with 50 μl of 50% (w/v) protein A-Sepharose CL-4B (Pharmacia Biotech, Tokyo, Japan), the resulting complex was collected by centrifugation ($100\times g$, 30 s) and washed three times in 200 μl of buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 100 μM benzamidine. The pellets were solubilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

*2.6. Preparation of primary cells*

Rat isolated hepatocytes were prepared by the collagenase perfusion method as described previously [4]. The yield of cells from one liver averaged $1.5\times10^8$, with 88–95% viability as estimated by trypan blue exclusion. Light microscopic analysis showed that ~97% of the cells were hepatocytes.

Rat ventricular myocytes were enzymatically isolated using the method of Isenberg and Klockner [21]. Briefly, rats weighing 150–

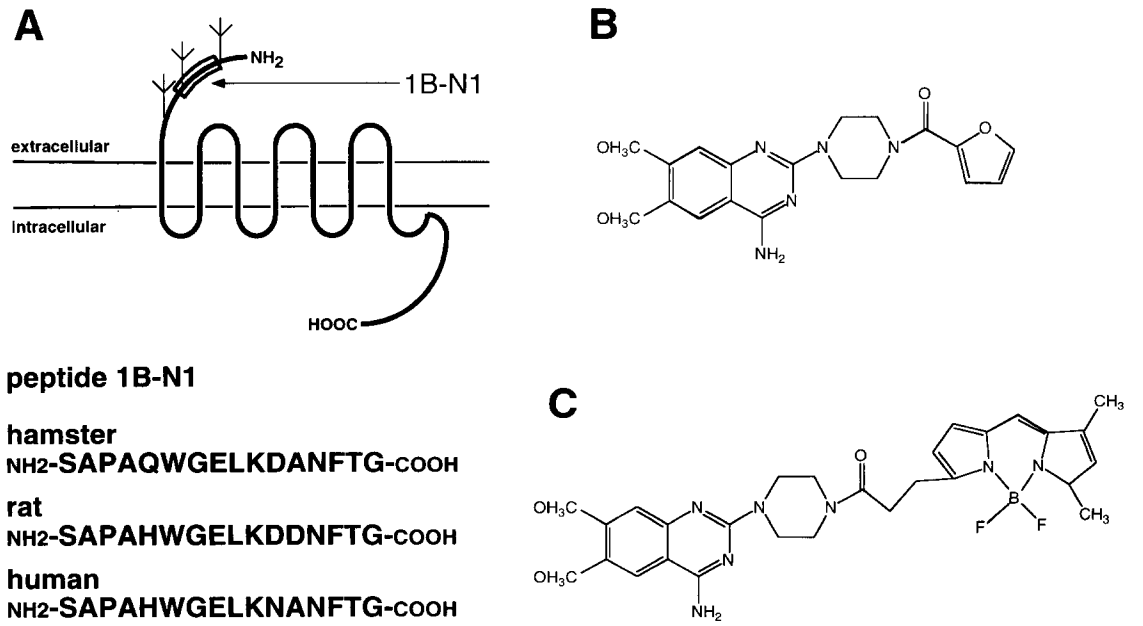


Fig. 1. Probes for flow cytometry. (A) Schematic diagram of the $\alpha_{1b}$-adrenoceptor. Following the generally accepted model of G-protein-coupled receptors, the seven highly homologous hydrophobic regions are shown as membrane-spanning domains. The amino acid sequences of the 1B-N1 (hamster), and the sequences of the same region for rat and human are also shown. (B) and (C) represent chemical structure of prazosin and BODIPY-FL prazosin, respectively.

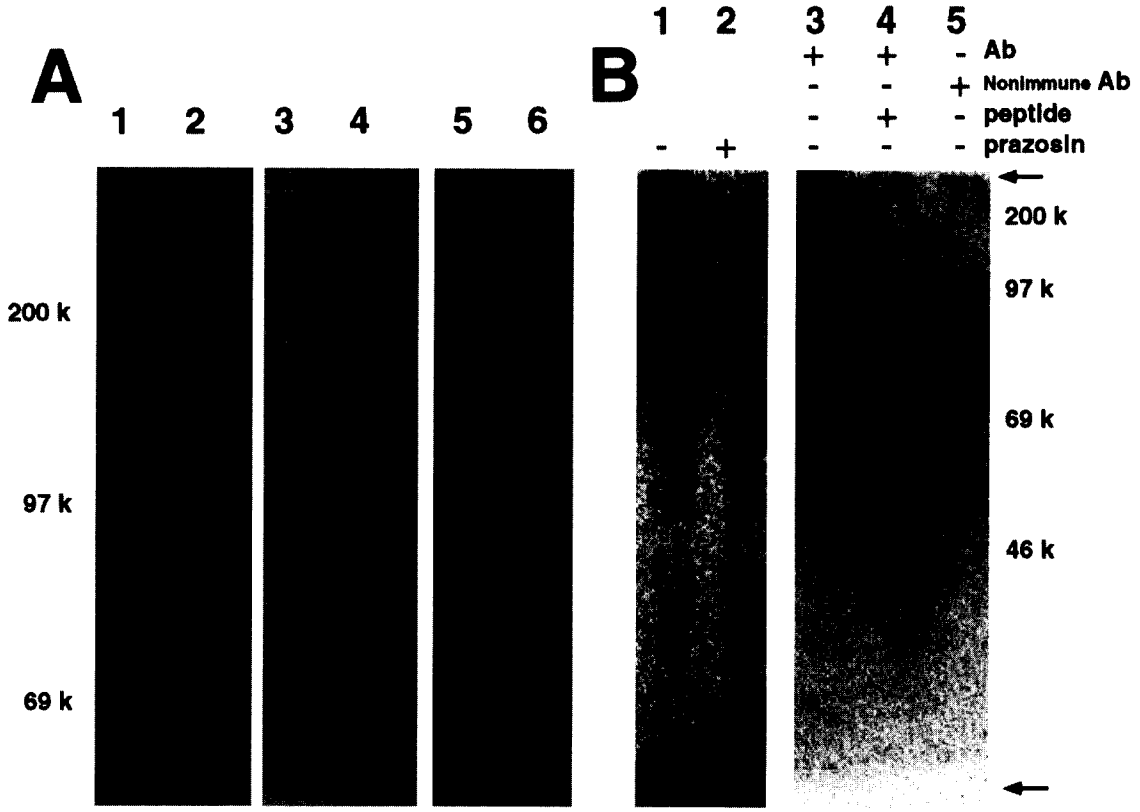


Fig. 2. Characterization of antibodies in $\alpha_{1b}$-adrenoceptor-transfected CHO cells. (A) Immunoblot against membrane proteins prepared from transfected and non-transfected CHO cells. Each sample (10 or 20 μg of protein) was applied on the 7.5% SDS-PAGE, transferred to GHPV membrane by Western blotting, and then probed with nonimmune, immune sera, or immune sera with immunogen peptide. CHO membrane proteins probed with the anti-peptide antibody (1:250 dilution) (lanes 1, 2), nonimmune antibody (lanes 3, 4) and the antibody with immunogen peptide (lanes 5, 6). Lanes 1, 3, 5: 10 μg of protein from CHO membrane-transfected $\alpha_{1b}$-adrenoceptor cDNA. Lanes 2, 4, 6: 20 μg of protein from wild-type CHO-K1 membrane. The numbers on the left show the relative positions of the molecular weight markers run simultaneously on the same gel. (B) Photoaffinity labeling with [$^{125}$I]aryl azidoprazosin and immunoprecipitation of $\alpha_{1b}$-adrenoceptor derived from transfected CHO cells. All procedures were performed as described in section 2. Photoaffinity labeling was performed with 300 pM [$^{125}$I]aryl azidoprazosin with (lane 2) or without (lane 1) prazosin. After photoaffinity labeling, membrane pellets were solubilized and immunoprecipitated. Lanes 3–5 represent the immunoprecipitates of antiserum (lane 3), pre-blocked by its corresponding peptide (lane 4), and pre-immune serum from the same rabbit (lane 5).

200 g were anesthetized by ether, and the hearts were quickly removed and rinsed in calcium-free Tyrode solutions [NaCl 135 mM, KCl 5.4 mM, $NaH_2PO_4$ 0.3 mM, HEPES 5.0 mM, $MgCl_2$ 0.5 mM, glucose 5.6 mM, pH 7.4]. Using a Langendorff technique, collagenase (Yakult, Tokyo) of 50 units/ml was perfused for 10–20 min. Ventricular muscles were minced by scissors and dispersed with gentle agitation, and filtered by a 200 μm nylon mesh. Cells were stored in KB medium (L-glutamic monopotassium 50 mM, taurine 10 mM, KCl 25 mM, $KH_2PO_4$ 10 mM, EGTA 0.5 mM, HEPES 10 mM, $MgCl_2$ 3 mM, glucose 10 mM, pH 7.4).

*2.7. Flow cytometry analysis*

For the analysis with BODIPY-FL prazosin, CHO and COS-7 cells were trypsinized and washed twice 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 up to 1 h.

For the analysis by anti-peptide antibodies, cells were trypsinized, washed twice with PBS, and fixed in 4% paraformaldehyde/PBS solution for 5 min at room temperature 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) or Cy3-conjugated goat anti-rabbit IgG (Chemicon International, Temecura, CA) for 60 min at 4°C, and again washed three times with PBS.

Analysis of the cells was performed using a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, CA, USA). To avoid artifacts arising from autofluorescence of dead cells and cellular debris, data acquisition was electrically gated for large particles presumably representing intact cells by forward and side scatter. Since the wavelengths of excitation and emission of BODIPY-FL prazosin are similar to those of fluorescein, we used a flow cytometry standard setting adjusted to routine double staining techniques employing FITC (FL-1) and Cy3 (FL-2) using the operating program LYSIS-II. Routinely, data from green fluorescence of $10^4$ cells were subjected to histogram and dot-plot analysis.

*2.8. Materials*

Complete Freund's adjuvant and incomplete Freund's adjuvant were purchased from Difco (Detroit, MI). [$^{125}$I]Aryl azidoprazosin and [$^{125}$I]HEAT (specific activity, 2200 Ci/mmol) were purchased from New England Nuclear. Acrylamide and other chemicals used for SDS-PAGE were from Bio-Rad (Richmond, CA). Reagents for peptide synthesis were from ABI-Japan (Tokyo, Japan); Prazosin HCl (Pfizer, Brooklyn, NY), BODIPY-FL prazosin (Molecular Probes, Eugene, OR) and phentolamine (Sigma, St. Louis, MO). All other reagents were of the highest analytical grade.

## 3. Results

*3.1. Transient and stable transformants*

COS-7 cells and CHO-K1 wild-type cells did not contain any detectable [$^{125}$I]HEAT binding sites. By contrast, when transfected with the plasmids containing $\alpha_1$-adrenoceptor cDNAs ($\alpha_{1a}$, $\alpha_{1b}$ and $\alpha_{1d}$-adrenoceptor), the COS-7 cells ex-

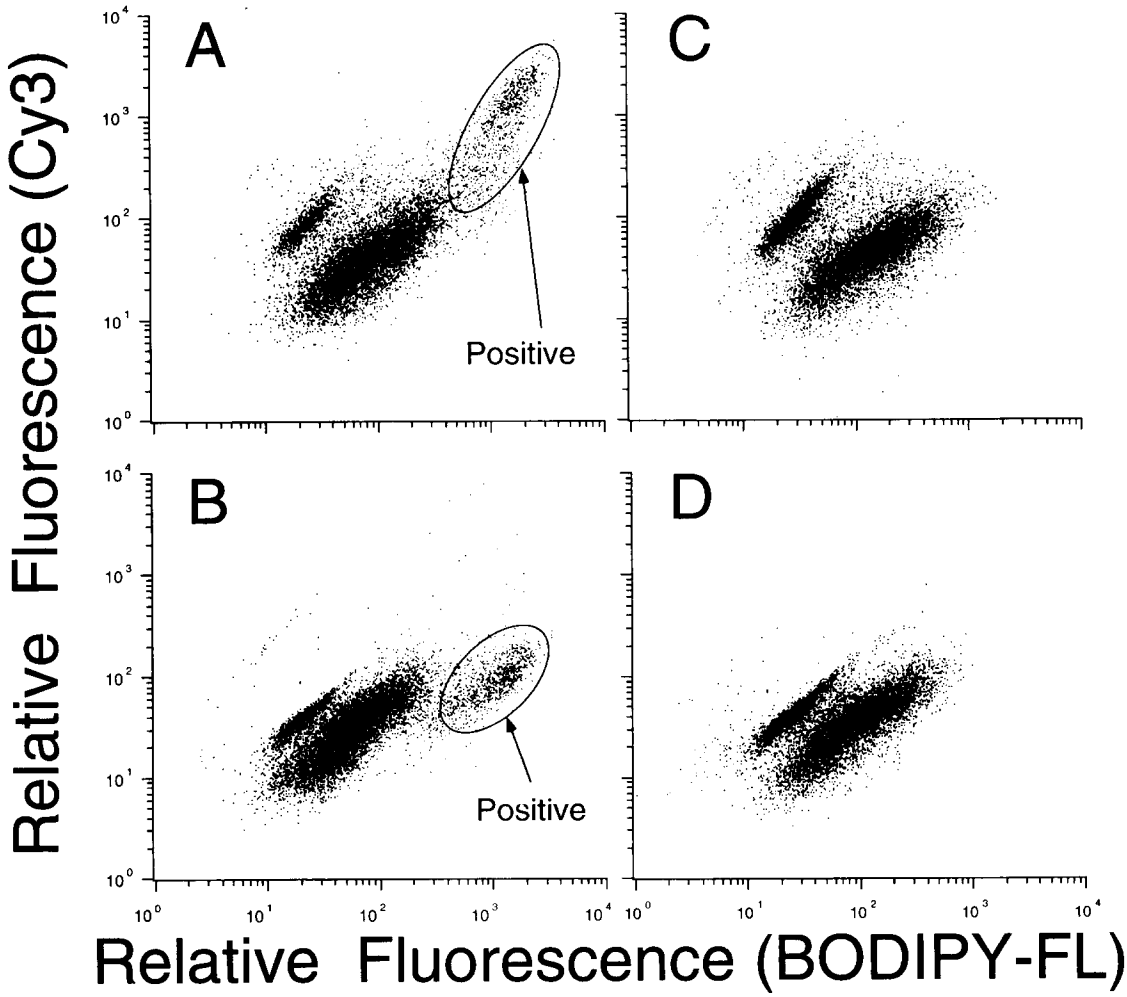


Fig. 3. Flow cytometry analysis of transfected COS-7 cells. COS-7 cells transiently transfected with hamster $\alpha_{1b}$-adrenoceptor (A and B) or expression vector pME18S only (C and D) were analyzed by the staining method using BODIPY-FL prazosin (A, B, C and D), with (A and C) and without anti-peptide antiserum with Cy3-labeled secondary antibody (B and D), respectively. The cells circled as 'Positive' are considered to be cells expressing hamster $\alpha_{1b}$-adrenoceptor (more details are discussed in the text). *X*-axis, relative fluorescence of BODIPY-FL; *Y*-axis, relative fluorescence of Cy3.

pressed [$^{125}$I]HEAT binding sites of only one affinity. The binding properties are characteristic of each $\alpha_1$-adrenoceptor subtype as previously reported [22]. We also examined the transfection efficiency by in situ β-gal assay [23], and 7–10% of the COS-7 cells were positively stained (not shown). For stable transformants, several G418 resistant clones for each receptor cDNA that express 1000–10000 fmol/mg protein binding sites were chosen and used for further studies. The $B_{max}$ and $K_d$ for each CHO cell line were 1300 ± 120 fmol/mg protein, 110 ± 17 pM ($\alpha_{1a}$, $n$ = 3), 5500 ± 80 fmol/mg protein, 60 ± 0.57 pM ($\alpha_{1b}$, $n$ = 3) and 1100 ± 51 fmol/mg protein, 300 ± 24 pM ($\alpha_{1d}$, $n$ = 6), respectively.

### 3.2. Characterization of the probes

Western blotting showed that the antiserum obtained (designated 1B-N1-C) specifically detected a broad band of ~90 kDa in membranes from $\alpha_{1b}$-transfected CHO cells (Fig. 2A, lane 1). On the other hand, however, the same antibody did not recognize any specific band in wild-type CHO-K1 cells (lane 2). The 90 kDa band was not detected by the nonimmune serum (lane 3, 4) or by the antiserum that was co-incubated with 1 μM of immunogen peptide (lanes 5, 6).

Photoaffinity labeling experiments showed a broad band with molecular weight of 90 kDa detected with [$^{125}$I]aryl azidoprazosin only in membranes from $\alpha_{1b}$-transfected CHO cells (Fig. 2B, lane 1), but no band labeled in membranes prepared from wild-type CHO-K1 cells (data not shown). The broad band was blocked by either the $\alpha_1$-adrenoceptor antagonist of prazosin (100 nM) (Fig. 1B, lane 2) or phentolamine (10 μM, data not shown), indicating that it represents $\alpha_{1B}$-adrenoceptor. This photoaffinity-labeled broad 90 kDa band is identical in its molecular size to that detected by Western blotting (Fig. 2A). Also, as shown in lane 3 of Fig. 2B, the antiserum immunoprecipitated the [$^{125}$I]aryl azidoprazosin-labeled 90 kDa protein in $\alpha_{1b}$-transfected CHO cell membranes. Pre-treatment with the corresponding antigen peptide (10 μM) inhibited the immunoprecipitation of the photoaffinity-labeled protein (Fig. 2B, lane 4). The nonimmune serum could not immunoprecipitate the photoaffinity-labeled protein (Fig. 2B, lane 5). Also, an unrelated peptide KSGLKTDKSD (corresponding to the $\alpha_{1a}$-adrenoceptor residues 219–228, 10 μM [10]) was unable to inhibit immunoprecipitation of the photoaffinity-labeled $\alpha_{1b}$-adrenoceptors in the same membrane (data not shown).

Utilizing the membrane preparations of $\alpha_{1a}$, $\alpha_{1b}$, $\alpha_{1d}$-adrenoceptor transformants, we also examined the ligand binding affinity of BODIPY-FL prazosin. The radioligand binding studies with [$^{125}$I]HEAT showed that the $K_d$ of BODIPY-FL prazosin is almost the same for all receptor subtypes; thus, 300 nM for $\alpha_{1a}$, 300 nM for $\alpha_{1b}$ and 350 nM for $\alpha_{1d}$, respectively ($n$ = 2 each).

### 3.3. Flow cytometry analysis

After transfection with the $\alpha_{1b}$-adrenoceptor cDNA, the COS-7 cells were double-stained by both BODIPY-FL prazosin and antibody 1B-N1C (Fig. 3A), or stained only by BODIPY-FL prazosin (Fig. 3B). When transfected, approximately 9–12% of COS-7 cells were positively stained with BODIPY-FL prazosin (circled as 'Positive' in Fig. 3B), which are also

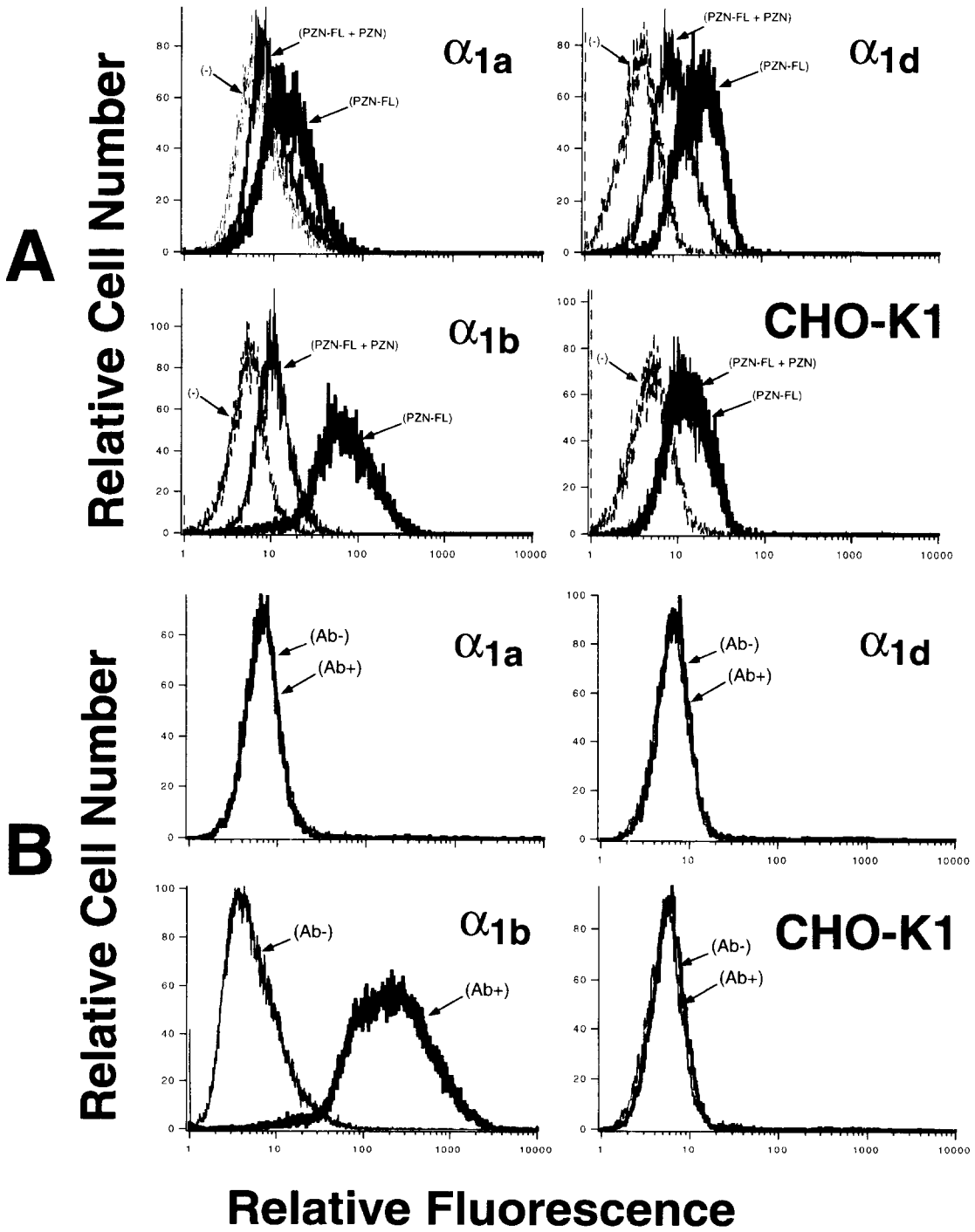


Fig. 4. Flow cytometry analysis of transfected CHO cells. CHO cells transfected with human $\alpha_{1a}$-adrenoceptor ($\alpha_{1a}$), hamster $\alpha_{1b}$-adrenoceptor ($\alpha_{1b}$), human $\alpha_{1d}$-adrenoceptor ($\alpha_{1d}$) and wild-type CHO-K1 cells (CHO-K1) were analyzed by FACScan flow cytometer. (A) Cells stained by 1 μM BODIPY-FL prazosin with (PZN-FL+PZN) or without (PZN-FL) 1 μM of cold prazosin, and no stain (−). (B) Cells stained with anti-peptide antiserum (Ab+) or nonimmune serum (Ab−) and FITC-labeled secondary antibody.

positively double-stained by the antibody 1B-N1C (circled as 'Positive' in Fig. 3A). Furthermore, the BODIPY-FL prazosin staining of these cells was inhibited by 1 μM of cold prazosin (data not shown), suggesting these cells (circled as 'Positive' in Fig. 3A and B) may be the COS-7 cells successfully expressing $\alpha_{1b}$-adrenoceptor. When the empty vector pME18s was transfected, no cell was positively stained by both BODIPY-FL prazosin and antibody 1B-N1C (Fig. 3C) or stained only by BODIPY-FL prazosin (Fig. 3D).

To characterize the subtype selectivity of these probes, we

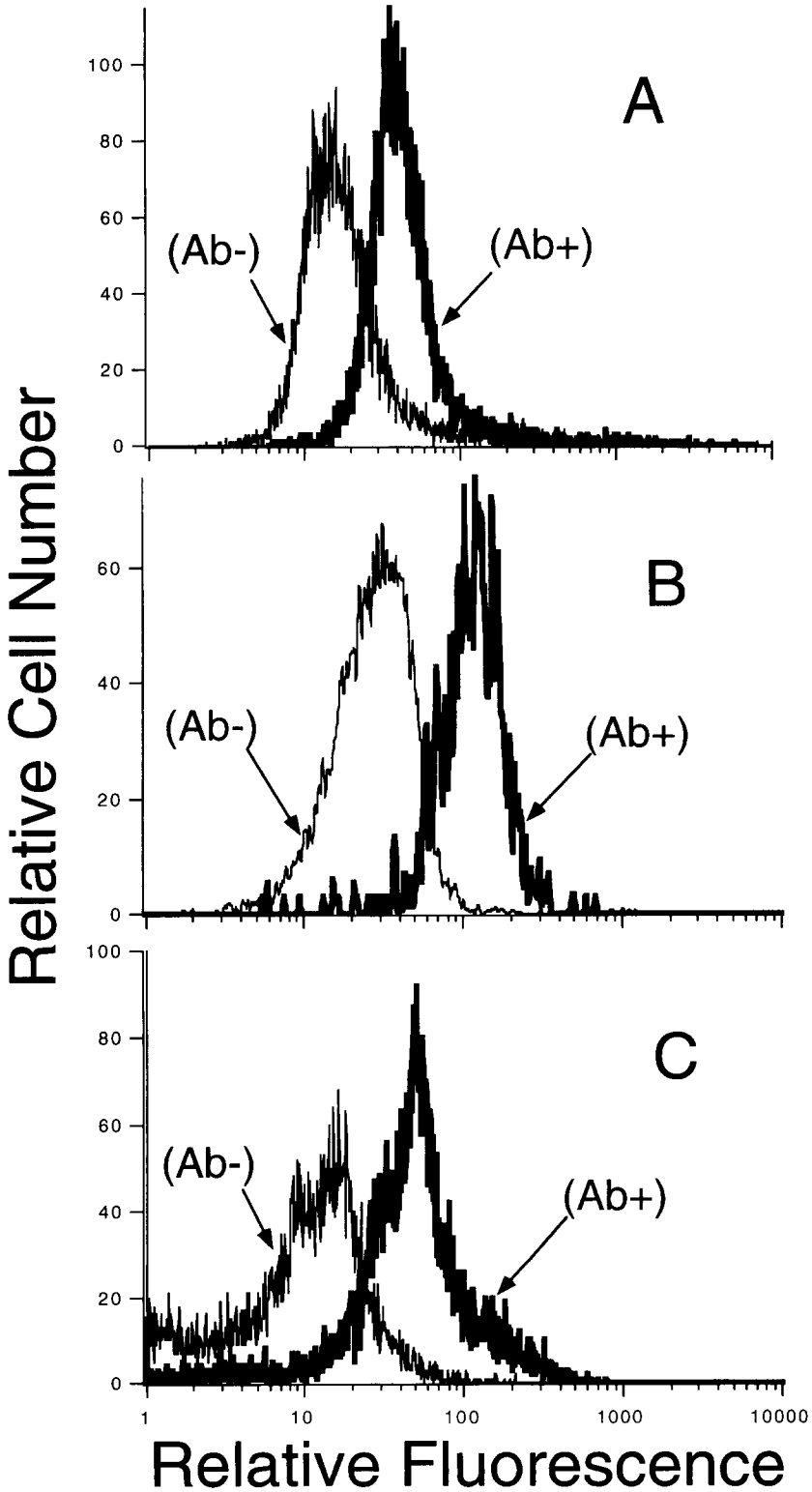


Fig. 5. Analysis of $DDT_1$-MF2 and freshly isolated native cells. $DDT_1$-MF2 cell line (A) and freshly isolated hepatocytes (B) and cardiomyocytes (C) were fixed, stained with anti-peptide antiserum (Ab+) or nonimmune serum (Ab−) and FITC-labeled secondary antibody and analyzed by FACScan flow cytometer.

further examined CHO cells stably expressing each $\alpha_1$-adrenoceptor subtype by either BODIPY-FL prazosin or the antibody 1B-N1C. As shown in Fig. 4, all the cell lines expressing $\alpha_1$-adrenoceptor subtype were stained by BODIPY-FL prazosin. This staining was inhibited by prazosin (1 μM), and no signal was detected in the wild-type CHO-K1 cells (Fig. 4 A). Interestingly, the intensity of the fluorescence was not equal for all the cell lines; thus, the fluorescence intensity of the $\alpha_{1a}$-adrenoceptor-expressing cells was weaker compared to the $\alpha_{1b}$- or $\alpha_{1d}$-adrenoceptor-expressing cells (Fig. 4A). These results showed that BODIPY-FL prazosin specifically stained the $\alpha_1$-adrenoceptor, but in a subtype-nonselective manner. In contrast to BODIPY-FL prazosin, the flow cytometry analysis with the antibody 1B-N1-C detected only the $\alpha_{1b}$-adrenoceptor-expressing CHO cells with little staining in the CHO cells expressing other receptor subtypes, indicating that the antibody 1B-N1C was an $\alpha_{1b}$-adrenoceptor-selective probe (Fig. 4B).

With the antibody 1B-N1-C, we further examined the several native cells which were previously shown to express pharmacologically defined $\alpha_{1B}$-adrenoceptor [24,25]. As shown in Fig. 5, $DDT_1MF2$ cells, hepatocytes and cardiomyocytes were specifically stained by the antibody 1B-N1-C.

## 4. Discussion

Fluorescence-activated flow cytometry has been widely adopted to identify specific antigens on the cell surface. However, few fluoroligands or antibodies have been developed and successfully used for G-protein coupled receptors [26-28], mainly because of the poor quantitation and visualization due to their weak signal, high level of tissue autofluorescence, and a lack of fluorescent staining specificity. In the present study, we showed that BODIPY-FL prazosin can be used as a specific fluorescent ligand for $\alpha_1$-adrenoceptor, although the $K_d$ value of BODIPY-FL prazosin is approximately 100 times larger than that of the original compound prazosin. All the cell lines expressing $\alpha_1$-adrenoceptor subtypes were stained by BODIPY-FL prazosin; however, the fluorescence intensity was not the same for all subtypes (Fig. 4). While it is not clear why only the $\alpha_{1a}$-adrenoceptor-expressing cells were weakly stained with BODIPY-FL prazosin compared to others, potential explanations could involve the different cellular distribution of the receptor protein; alternatively, the proportion of the receptor proteins localized on the cell surface could be different and smaller for $\alpha_{1a}$-adrenoceptor compared to other receptor subtypes, since the $K_d$ of BODIPY-FL prazosin and the density of receptor sites in membrane preparations monitored by radioligand binding assay were not much different among the subtypes.

The flow cytometry analysis provides us with important information, such as the amount of the receptor protein present on the cell surface. Also, the analysis can identify the cells expressing the specific receptor protein in a heterogenous group of cells. This information is often difficult to obtain for $\alpha_1$-adrenoceptor by conventional radioligand binding assay, since most radioligands used for identifying $\alpha_1$-adrenoceptor are highly lipophilic and easily taken up into the intact cells. Thus, it has been difficult to determine whether some specialized cells or all the cells in a tissue express $\alpha_{1b}$-adrenoceptor on the cell surface. With the antiserum 1B-N1-C that can specifically detect the extracellular N-terminus peptide of $\alpha_{1b}$-adrenoceptor, our flow cytometry analysis could show that most of the isolated hepatocytes and myocytes express $\alpha_{1b}$-adrenoceptors on the cell surface. This is the first demonstration that $\alpha_{1b}$-adrenoceptor protein can be identified in the pharmacologically well-characterized $\alpha_{1B}$-adrenoceptor-expressing tissues [1,4,6,15].

In summary, the flow cytometry analysis with BODIPY-FL prazosin can successfully identify $\alpha_1$-adrenoceptors, and with the specific probe for the $\alpha_{1b}$-adrenoceptor protein, the anti-peptide antibody 1B-N1-C, the flow cytometry analysis can selectively identify the $\alpha_{1b}$-adrenoceptor protein even in the native tissues. Flow cytometry analysis with specific fluoroligands or subtype-selective antibody would be a sensitive and useful method to pharmacologically characterize the cell surface receptor protein of individual cells. Also, the approach would be potentially useful to monitor the cellular regulation of the receptor protein, such as receptor internalization.

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