Synthesis and Properties of Fluorescent β-Adrenoceptor Ligands†

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ABSTRACT: We describe the synthesis of bordifluoropyrromethene (BODIPY), fluorescein, and related fluorescent derivatives of the β-adrenergic ligand CGP 12177. With these probes we screened insect (Sf9) cells stably transformed with the human β_2-adrenoceptor gene and expressing (2–3.5) × 10^5 human β_2-adrenoceptors per cell. Among these derivatives only BODIPY-CGP gave a receptor-specific signal sufficiently strong for measuring the on- and off-rate constants and the equilibrium dissociation constant of β-adrenoceptor-specific binding by spectrofluorometry or photon counting. Similar K_D values for BODIPY-CGP binding were obtained by kinetic measurements (approx. 250 pM) and under equilibrium conditions (400 ± 180 pM), and these were in the same range as those obtained with [^{3}H]CGP 12177 (200 ± 32 pM). The cell-bound fluorescence could be quenched specifically with nonfluorescent CGP 12177 to near background levels. The disposition of the β_2-adrenoceptors in BODIPY-CGP-stained Sf9 cells was mainly restricted to the cell surface at 4 and 30 °C. Hence, β-adrenoceptor-expressing cells can be stained specifically with BODIPY-CGP, and β-adrenoceptors on a single cell can be assessed by photon counting under the fluorescence microscope. Cells can also be scanned by fluorescence-activated flow cytometry.

Fluorescent probes have been used before in studies of receptors, such as nicotinic acetylcholine (Cheung et al., 1984; Covarrubias et al., 1984; Gonzalez-Ros et al., 1983; Herz et al., 1989; Prinz & Maelicke, 1983), dopamine (Ariano et al., 1989; Monsma et al., 1989), α-and β-adrenergic (Atlas & Levitzki, 1977; Correa et al., 1980; Heithier et al., 1988a; Hekman et al., 1984; Henis et al., 1982; Zemcik & Strader, 1988), benzodiazepine (McCabe et al., 1990), epidermal growth factor (Dickson et al., 1983), insulin (Sui et al., 1988), glucagon (Heithier et al., 1988b; Ward et al., 1988), vasopressin (Jans et al., 1989, 1990), opioid (Correa et al., 1980; Kolb et al., 1983; Mihara et al., 1985), adenosine (Jacobson et al., 1987), and steroid receptors (Bindal & Katzenellenbogen, 1985; Carlson et al., 1989; De Potter et al., 1985; Pomper et al., 1990) as well as L-type calcium channels (Knaus et al., 1992a,b). However, quantitation and visualization of specific ligand–receptor complexes in intact cells by fluorescence measurements have been compromised by a too weak signal, high levels of tissue autofluorescence, and a lack of fluorescent staining specificity (Barnes et al., 1980; Correa et al., 1980; Hess, 1979; Rademaker et al., 1985a,b). Thus, despite the obvious advantages of fluorescence over radioactivity measurements, fluorescently labeled ligands have until now not been able to replace radioligands for binding measurements. With the development of powerful cell-imaging systems, the interest in specific and highly fluorescent probes for cell surface receptors has been revived. CGP 12177[1] has been proved to be highly advantageous for studying β-adrenoceptors in intact cells (Affolter et al., 1985; Staehelin et al., 1983). Due to its hydrophilic nature, CGP 12177 binds almost exclusively to sites exposed on the cell surface, thereby minimizing nonspecific binding and intercalation in the membrane lipid bilayer (Affolter et al., 1985; Staehelin et al., 1983). We have previously synthesized a fluorescent derivative of CGP 12177 (Heithier et al., 1988a). Here, we describe the synthesis and use of a new fluorescent derivative of CGP 12177, BODIPY-CGP, characterize its binding properties, and compare these with other fluorescent derivatives.

BODIPY-CGP was tested using a stably transformed *Spodoptera frugiperda* (Sf9) cell line which expresses human β_2-adrenoceptors at a density of 2–3.5 × 10^5/cell. The human β_2-adrenoceptor gene is constitutively transcribed in Sf9 cells from the promotor of the IE_1 gene (an immediate early gene of the baculovirus *Autographa californica* nuclear polyhedrosis virus). We have used these stably transformed Sf9 cells because most of the properties of the human β_2-adrenergic receptor expressed in these cells, including ligand binding properties, coupling to endogenous G-proteins, and adenylyl

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[1] Abbreviations: CGP 12177, Ciba Geigy product no. 12177, a hydrophilic β-adrenergic antagonist, 4-[[3-[(4-amino-1,1,4-trimethylpentyl)amino]-2-hydroxypropyloxy]]-1,3-dihydro-1*H*-benzimidazol-2-one; BODIPY, "bordifluoropyrromethene", (N^1–*B*)-$N^{1'}$-(difluoroboryl)-3,5′-dimethyl-2,2′-pyrromethene-5-propionic acid, *N*-succinimidyl ester; BSA, bovine serum albumin; DTT, dithiothreitol; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; alprenolol-NBD, 7-(2-allylphenoxy)-2,2-dimethyl-6-hydroxy-1-(7-NBD)-1,4-diazaheptane; K_D, dissociation constant; B_{max}, maximal concentration of binding sites; PBS, phosphate-buffered saline.

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cyclase activation and desensitisation, have been described recently (Kleymann et al., 1993).

MATERIALS AND METHODS

Materials

BODIPY-succinate and propidium iodide were obtained from Molecular Probes, Eugene, OR. 2,5-Diamino-2,5-dimethylhexane was a product of Aldrich Chemicals (U.K.). Fluorescein isothiocyanate (isomer 1) and L-alprenolol were obtained from Fluka (Neu-Ulm, FRG). Nonlabeled CGP 12177 was a kind gift from Ciba-Geigy (Basel, Switzerland). [^{125}I]Iodocyanopindolol (2000 Ci/mmol), [^{3}H]CGP 12177 (43 Ci/mmol), and the RPA 509 radioimmunoassay kit for cAMP measurement were purchased from Amersham Buchler (Braunschweig, Germany). IPL 41 insect cell growth medium, pH 6.8 (GIBCO/BRL, Eggenstein, Germany), was supplemented with 5% fetal calf serum (Boehringer Mannheim, Germany). Geniticine G 418 was purchased from GIBCO/BRL. Preparative thin-layer chromatography plates (20 × 20 × 0.1 cm) and analytical KG 60 chromatography plates (Merck, Darmstadt, Germany) were activated at 110 °C for 2 h before use. Whatman GF/F glass fiber filters soaked in 0.3% polyethylenimine (Sigma, Deisenhofen, Germany) were used for ligand binding assays. All other chemicals and solvents were of analytical grade and were purchased from Merck or Sigma.

Methods

Synthesis of BODIPY-CGP. Reactions and separations were carried out under an argon atmosphere. BODIPY-succinate (3′,5′-dimethyl-2,2′-pyrromethene-1,1′ ((*N*^{1}–*B*)-*N*$^{1'}$-(difluoroboryl)-3′,5′-dimethyl-2,2′-pyrromethene-5-propionic acid, *N*-succinimidyl ester, 4.5 mg (1.2 × 10^{-5} mol)) and D,L-CGP 12177 (4-[[3-[(4-amino-1,1,4-trimethylpentyl)-amino]-2-hydroxypropyl]oxy]-1,3-dihydro-1*H*-benzimidazol-2-one, 3.6 mg (10^{-5} mol)) were dissolved in 2 mL of dimethylformamide/pyridine (1:1) and stirred for 48 h. The solvent was evaporated at 20 °C, and the resulting brown substance was chromatographed on preparative silica gel plates. The solvent used was chloroform:methanol:concentrated aqueous ammonia (90:10:1). After separation of the green-yellow fluorescent main zone, the product was eluted with a chloroform:methanol:concentrated ammonia:water (50:50:1:20) mixture. The solution was washed with water, dried over Na_2SO_4, and concentrated to dryness under reduced pressure. The dry substance was chromatographed on analytical silica gel plates with chloroform:methanol:concentrated aqueous ammonia (90:10:1). The structure of BODIPY-CGP shown in Figure 1 was confirmed by ^{1}H NMR, IR, and UV spectroscopy. The yield was 2.5 mg, or about 32% based on the concentration of the starting material. The final product was analyzed by thin-layer chromatography on analytical silica gel plates (the solvent was chloroform:methanol:concentrated ammonia (90:10:1)) and found to be essentially pure. The R_f values were as follows: BODIPY-succinate, 0.46; CGP 12177, 0.03; BODIPY-CGP, 0.24. H^1 NMR (ppm): 1.20–1.25 (4s, 12H), 1.48 (m, 4H), 2.24 (s, 3H), 2.56 (s, 3H), 2.90 (d, J = 4 Hz, 2H), 3.25 (t, J = 7 Hz, 2H), 3.54 (t, J = 7 Hz, 2H), 4.08–4.16 (m, 3H), 6.12 (s, 1H), 6.33 (d, J = 3.5 Hz, 1H), 6.62 (d, J = 9 Hz, 1H), 6.70 (d, J = 9 Hz, 1H), 6.86 (d, J = 3.5 Hz, 1H), 6.94 (m, 1H), 7.10 (s, 1H). BODIPY-CGP is a racemic mixture of two stereoisomers, due to the stereochemical properties of the starting material, 4-[[3-[(4-amino-1,1,4-trimethylpentyl)-amino]-2-hydroxypropyl]oxy]-1,3-dihydro-1*H*-benzimidazol-2-one, used for conjugation with BODIPY-succinate.

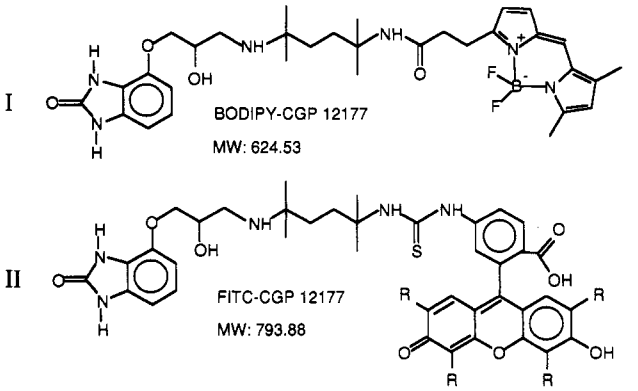

FIGURE 1: Structures of (I) BODIPY-CGP and (II) FITC-CGP 12177. In (II), R is H; R is Br in the eosin derivative; R is I in the erythrosin derivative.

Synthesis of Fluorescein-CGP 12177. 4-[(2,3-Epoxypropyl)oxy]-1,3-dihydro-1*H*-benzimidazol-2-one 0.412 g was dissolved in a solution of 2.9 g of 2,5-diamino-2,5-dimethylhexane in 20 mL of 2-propanol. The solution was heated to reflux for 5 h. The solvent was removed *in vacuo*, and the residue was dried at 60 °C and 3 × 10^{-2} mbar for 2 h. Crystallization of the dried substance yielded 0.403 g of colorless crystals of 4-[[3-(4-amino-1,1,4-trimethylpentyl)amino]-2-hydroxypropyl]oxy]-1,3-dihydro-1*H*-benzimidazol-2-one (**1**) ($C_{18}H_{30}N_4O_3$·(0.25)H_2O, MW 354.96). Thirty-five milligrams of **1** was dissolved in 1 mL of ethanol; a solution of 40 mg of fluorescein isothiocyanate (isomer 1) in 1 mL of dimethylformamide was added, and the mixture was kept at 60 °C for reaction for 1 h. After incubation overnight at room temperature, the solvents were removed under vacuum and the product was triturated with ethanol. We were able to obtain 61.1 mg of orange crystals of the FITC derivative shown in Figure 1 ($C_{39}H_{41}N_5H_{41}O_8S$·3$H_2O$, MW = 793.89). The eosin and erythrosin derivatives of CGP 12177 were prepared in an analogous manner. All these compounds were characterized and confirmed by ^{1}H NMR, IR, and mass spectra.

Cell Culture. Wild-type Sf9 cells (CRL 1711; American Type Culture Collection, Rockville, MD) and Sf9 cells stably transformed with the gene of the human β_2-adrenoceptor [for details, see Kleymann et al. (1993)] were grown at 27 °C in IPL 41 insect cell growth medium, pH 6.8, supplemented with 5% fetal calf serum in a moisturized atmosphere without additional CO_2. Additional oxygenation did not significantly increase cell growth. The transformed cell clone was reselected every 2 months by passaging in medium containing geniticine G418 (1 mg/mL). The transformed cells expressed 2–3.5 × 10^5 human β_2-adrenoceptors per cell. Receptor density remained constant with changes in cell density or time of culture (6 months or longer). Wild-type Sf9 cells do not express β-adrenoceptors (Reilander et al., 1991). Cell membranes were isolated by differential centrifugation after lysing the cells by nitrogen cavitation as described previously (Boege et al., 1988). Membranes containing 300–800 fmol of receptor per milligram of protein were stable for more than 2 months when stored with 20% glycerol at −70 °C.

Radioligand binding measurements on intact cells were carried out using [^{3}H]CGP 12177 under equilibrium conditions. Usually, 2 × 10^5 cells were suspended in serum-free medium at the temperatures and for the times specified in the caption to Figure 2a and Table 1. [^{3}H]CGP 12177 binding to cells was carried out as previously described (Boege et al., 1988; Kleymann et al., 1993; Reilander et al., 1991). In the case under consideration here, nonspecific binding was determined in the presence of 1 μM nonlabeled CGP 12177 or 10 μM L-isoproterenol (cf. Figure 2c). Bound and free

ligand were separated by rapid filtration using Whatman GF/F filters soaked in 0.3% polyethylenimine. Filter-bound radioactivity was determined by liquid scintillation counting. K_D and B_{max} values were calculated by computer-aided nonlinear regression analysis of the binding isotherms.

Fluorescent Ligand Binding. Membranes from transformed Sf9 cells were prepared as described (Kleymann et al., 1993) and incubated with BODIPY-CGP in PBS buffer, pH 7.4; see the caption to Figure 2b. Membrane-bound ligand was separated from free ligand by centrifugation [30 min, (2×10^4)g at 4 °C]. The membrane pellets were washed twice by resuspension in 1 mL of ice-cold PBS buffer, pH 7.4, and centrifugation [30 min, (2×10^4)g at 4 °C] and finally suspended in 100 μL of PBS buffer, pH 8.5, containing 0.8% cholate. After 45 min at room temperature, the lysates were centrifuged [30 min, (2.5×10^4)g at 20 °C] and 70 μL of the supernatant fluid was withdrawn, kept for 12 h at 20 °C in the dark for micellar equilibration, and then transferred to a quartz microcuvette for fluorescence measurements. Fluorescence emission was screened from 490 to 600 nm at 20 °C upon excitation at 470 nm using a Schoeffel RRS 1000 spectrometer. Autofluorescence was measured in the absence of BODIPY-CGP. Nonspecific binding of BODIPY-CGP was measured in the presence of nonfluorescent CGP 12177. At saturating concentrations of fluorescent ligand the intensity of autofluorescence and/or the fluorescence of nonspecifically bound ligand was 5–10% of the specifically bound fluorescence. The amount of fluorescent ligand bound to membranes or cells was estimated by linear regression analysis as a function of the background fluorescence intensity and the increase in fluorescence after addition of various concentrations of BODIPY-CGP. For purposes of standardization the relation of fluorescence intensity to ligand concentration was redetermined in each series of experiments to minimize experimental variability.

Fluorescence Microscopy and Photon Counting. Cells were seeded on glass coverslips placed in Petri dishes of 3.5-cm diameter at a density of 5×10^4 cells per coverslip and grown for 2 days under cell culture conditions, as described above. Coverslips were washed (3×) and incubated at the temperatures and the times given in the figure captions. Incubation was with serum-free medium containing BODIPY-CGP. For determination of nonspecific binding, nonfluorescent CGP 12177 was added together with the fluorescent ligand. Coverslips were then washed twice at room temperature with PBS buffer, pH 6.8, containing 0.4% glucose and 0.1% BSA. When cells were fixed, 2% paraformaldehyde was applied. A drop of the same buffer was added, and inspection was with a water-immersion lens (Zeiss, Ph2 Neofluar, 40×) of a Zeiss IM 35 inverted fluorescence light microscope. Optical images obtained by phase contrast and green fluorescence (460–600-nm emission) microscopy were documented using a Contax 139 quartz camera and Kodak TMAX p3200 color negative film. Measurements of single-cell fluorescence were carried out using an Ortec photon-counting system. B_{max} values were determined by means of measuring BODIPY-CGP binding to single cells by photon counting under the fluorescence microscope. The fluorescence was correlated and compared with B_{max} values determined by conventional [^{3}H]CGP 12177 binding under identical conditions. Based on this standard for calibration, the data are expressed as photon counts per second (cps) per cell.

Analysis of Binding Data. Binding isotherms and association/dissociation kinetics measured by fluorescence or radioactivity were analyzed by computer-aided nonlinear regression analysis, using a program collection by McPherson (McPherson, 1985; McPherson et al., 1983). With this program, the interaction of the ligands with one or more saturable specific binding sites and with nonsaturable nonspecific sites under equilibrium conditions was modeled according to

$$[B] = \sum_{i=1}^{i=n}\left[\frac{B_{imax}[L]}{K_{iD} + [L]}\right] + N[L]$$

where [B] is the concentration of bound ligand, K_{iD} and B_{imax} are the binding parameters, [L] is the free ligand concentration, and N is the ratio of bound over free ligand at infinite concentrations. Kinetic data were modeled using the equations

$$[B_{(t)}] = \sum_{i=1}^{i=n}[B_{i(t=0)}]e^{-k_{off}}$$

and

$$[B_{(t)}] = \sum_{i=1}^{i=n}[B_{iequ}](1 - e^{-k_{obs}t})$$

for dissociation and association, respectively. $B_{(t)}$, $B_{(t=0)}$, and B_{equ} are the concentrations of bound ligand at time t, at time $t = 0$, and at equilibrium, respectively; k_{off} is the dissociation rate constant; k_{obs} is the observed association rate constant; i is the number of binding sites; and [L] is the free concentration of ligand. The data points were fitted by the Marquardt–Levenberg modification of the Gauss–Newton method for least square regression curve fitting. The fit was routinely estimated from the sum of the squares, the least square value, and the degrees of freedom. Confidence limits of K_D, B_{max}, k_{obs}, or k_{off} were calculated from these values and did not exeed 15%. The data were tested to see whether the data points were randomly distributed to both sides of the regression curve. Usually, i was assumed to be 1, but in cases where the data could not be fitted on the basis of the above criteria, higher values for i were used and the best result was selected by the Fisher test.

Flow Cytometry. Transformed or wild-type Sf9 cells (2.5×10^5 cells) were incubated with 0.1 μM of BODIPY-CGP with or without nonfluorescent CGP 12177 (10 μM) in 50 μL of serum-free IPL 41 medium, pH 6.8, for 2 h on ice in the dark. Samples were then diluted 10-fold with ice-cold PBS buffer containing 0.4% glucose, 0.1% BSA, and 2 μg/mL propidium iodide and measured immediately using a FACScan flow cytometer (BectonDickinson, USA). Measurements were made in the pH 6.8–8.5 range.

To avoid artifacts arising from autofluorescence of dead cells and cellular debris, data acquisition was electronically gated (i) for large particles presumably representing intact cells by forward scatter (>600 arbitrary units) and (ii) for particles not stained by the polar fluorescent DNA-intercalating dye propidium iodide, which is excluded from vital cells (orange fluorescence < 40 arbitrary units). Since the wavelengths of excitation and emission of BODIPY-CGP are similar to those of fluorescein, we have used flow cytometer standard settings adjusted to routine double-staining techniques employing FITC and propidium iodide. Routinely, data from green fluorescence of 10^4 cells were subjected to histogram analysis.

RESULTS

We characterized recently the ligand binding properties of the human β_2-adrenoceptor stably expressed in transformed Sf9 cells by conventional radioactive methods (Kleymann et

Table 1: K_D Values of [^{3}H]CGP 12177 and Its Fluorescent Derivatives for Binding to Human β_2-Adrenoceptors Expressed in Sf9 Cells[a]

ligand	target	K_D ± SEM (n = 3)
		pM
[^{3}H]CGP	binding to intact cells[b]	200 ± 32
BODIPY-CGP	displacement of [^{3}H]CGP bound to cells[b]	290 ± 70
FITC-CGP	displacement of [^{3}H]CGP bound to cells[b]	2000 ± 250
BODIPY-CGP	binding to cell membranes[c]	115 ± 42
BODIPY-CGP	binding to single cells[c]	400 ± 180 (250)[d]
BODIPY-CGP	binding to single cells and displacement by nonfluorescent CGP[c]	173 ± 68

[a] Ligand binding was at pH 6.8. The cells expressed 2 × 10^5 human β_2-adrenoceptors per cell. [b] Radioactivity measurements. [c] Fluorescence measurements. For photon counting of single cells under the fluorescence microscope, the pH was raised to 8.5. For experimental details, see Materials and Methods and the captions to Figure 2b,c. [d] The value in parentheses has been calculated from the forward (k_{on}) and reverse (k_{off}) rate constants. See the caption to Figure 2d.

Table 2: Receptor Dependency of Cell-Bound Fluorescence[a]

		cps		
cells	fluorescent substances	total cell-bound fluorescence	receptor-independent fluorescence	autofluorescence
Sf9 WT	BODIPY-CGP	1082 ± 354	1074 ± 406	790 ± 224
Sf9 WT	BODIPY-succinate	869 ± 254	1032 ± 617	790 ± 224
Sf9 trans.	BODIPY-CGP	17 500 ± 3515	1095 ± 575	485 ± 210
Sf9 trans.	BODIPY-succinate	795 ± 140	865 ± 245	485 ± 210

[a] Sf9 wild-type cells (Sf9 WT) and the transformed clone expressing ~2 × 10^5 β-adrenoceptors/cell (Sf9 trans.) were used. The cells were grown in Petri dishes and after washing were incubated with 5 nM concentrations of the fluorescent substances in serum-free IPL 41 medium for 15 min at 30 °C. Receptor-independent fluorescence was determined in the presence of 1 μM nonfluorescent CGP 12177. Data are expressed as photon counts per second per cell (cps). Each data point represents the mean of 10 individual measurements ± standard error of the mean. The data are representative of measurements on two independent cell preparations. The experimental procedure is described in Materials and Methods.

al., 1993). In these experiments cell clones expressing 2–3.5 × 10^5 ligand binding sites/cell were studied. Comparable receptor numbers per cell were estimated on the basis of binding measurements using either the rather hydrophilic membrane-impermeable ligand [^{3}H]CGP 12177 or the hydrophobic ligand [^{125}I]iodocyanopindolol, known to cross membranes of intact cells (Hertel & Staehelin, 1983; Hertel et al., 1983; Portenier et al., 1984). The data summarized in Table 1 show that the K_D values for receptor binding of [^{3}H]CGP 12177 and BODIPY-CGP measured by radioactivity or fluorescence measurements in intact cells and cell membranes are comparable. Results from experiments carried out with intact Sf9 cells are shown in Figure 2a,c,d, and those from experiments carried out with membranes isolated from Sf9 cells are shown in Figure 2b. These experiments indicate that the human β_2-adrenoceptors expressed in these cells have uniform affinities for these ligands. The Hill plot in the inset to Figure 2a and the Scatchard plot in the inset to Figure 2b indicate that the binding of BODIPY-CGP to the human β_2-adrenoceptors expressed in Sf9 cells was best described by a model assuming a single class of equivalent and independent binding sites. Accordingly, on the basis of the Hill plot (Figure 2a, inset) one calculates a Hill coefficient near unity, e.g., n = 0.88. This model is also confirmed by data in Figure 2d, where it is shown that association followed pseudo-first-order and dissociation first-order kinetics: The k_{on} value was 2 × 10^7 M^{-1} min^{-1}, and the k_{off} value was 4.7 × 10^{-3} min^{-1}, for BODIPY-CGP binding to human β_2-adrenoceptors expressed in Sf9 cells. These data are in good aggreement with those previously measured for [^{3}H]CGP 12177 and in the same cells (Kleymann et al., 1993). Moreover, the K_D values calculated on the basis of the rate constants (see the caption to Figure 2d) and the $K_D$values measured under equilibrium conditions (Table 1) were in reasonably good agreement. Finally, the characteristics of CGP 12177 binding to recombinant human β_2-adrenoceptors expressed in Sf9 cells are similar to those reported from human cell lines constitutively expressing β_2-adrenoceptors (Boege et al., 1988; Heithier et al., 1988a; Staehelin et al., 1983). Comparing the K_D values obtained by displacement of radioactive [^{3}H]CGP 12177 bound to β-adrenoceptors of Sf9 cells by fluorescent BODIPY-CGP (Figure 2a) with those obtained by fluorometry of BODIPY-CGP bound to Sf9 cell membranes (Figure 2b) and with data obtained by direct photon counting of BODIPY-CGP bound to single intact cells (Figure 2c,d) shows that the favorable ligand binding properties of CGP 12177 are not compromised by the derivatization with BODIPY. The concentrations of BODIPY-CGP required to saturate the specific binding sites on membranes are about 10 nM (Figure 2b) but about 40 nM for cells grown on coverslips, where binding was measured by photon counting of single cells (Figure 2c). These differences may be exaggerated because a single representative experiment is shown in each of the figures. The differences noted in the K_D values in Table 1 are more realistic, although the scatter of the photon counting data is appreciable. In contrast, derivatization with FITC decreased the affinity of CGP 12177 10-fold (see Table 1 and Figure 2a). Data obtained with the corresponding eosin and erythrosin derivatives of CGP 12177 are not shown, because they were like those obtained with the FITC-CGP 12177 derivative. In each case the fluorescent signal was not strong enough to measure directly ligand binding to β-adrenoceptors on cells.

The UV and fluorescence spectra of BODIPY-CGP were identical to those of BODIPY-succinate. Therefore they are not shown. The emission upon excitation at 480 nm peaked at 510 nm. The specific fluorescence of BODIPY-CGP bound to intact Sf9 cells was about 17-fold higher than that of nonspecifically bound ligand (determined in the presence of 1 μM non-fluorescent CGP 12177), which exceeded the autofluorescence of the cells by about 1.5-fold (see Table 2). In contrast, the specific fluorescence signal of FITC-CGP 12177 under these conditions was (1.8 ± 0.4)-fold greater than the nonspecific fluorescence, which was similar to the autofluorescence of the cells. The high quantum yield (≈0.9) and the high binding specificity of the BODIPY derivative made it possible to directly visualize β-adrenoceptors on transformed insect cells, producing 2 × 10^5 receptors per cell. Figure 3 shows typical results: In each row the same cells are visualized by phase-contrast (left) and green fluorescence light

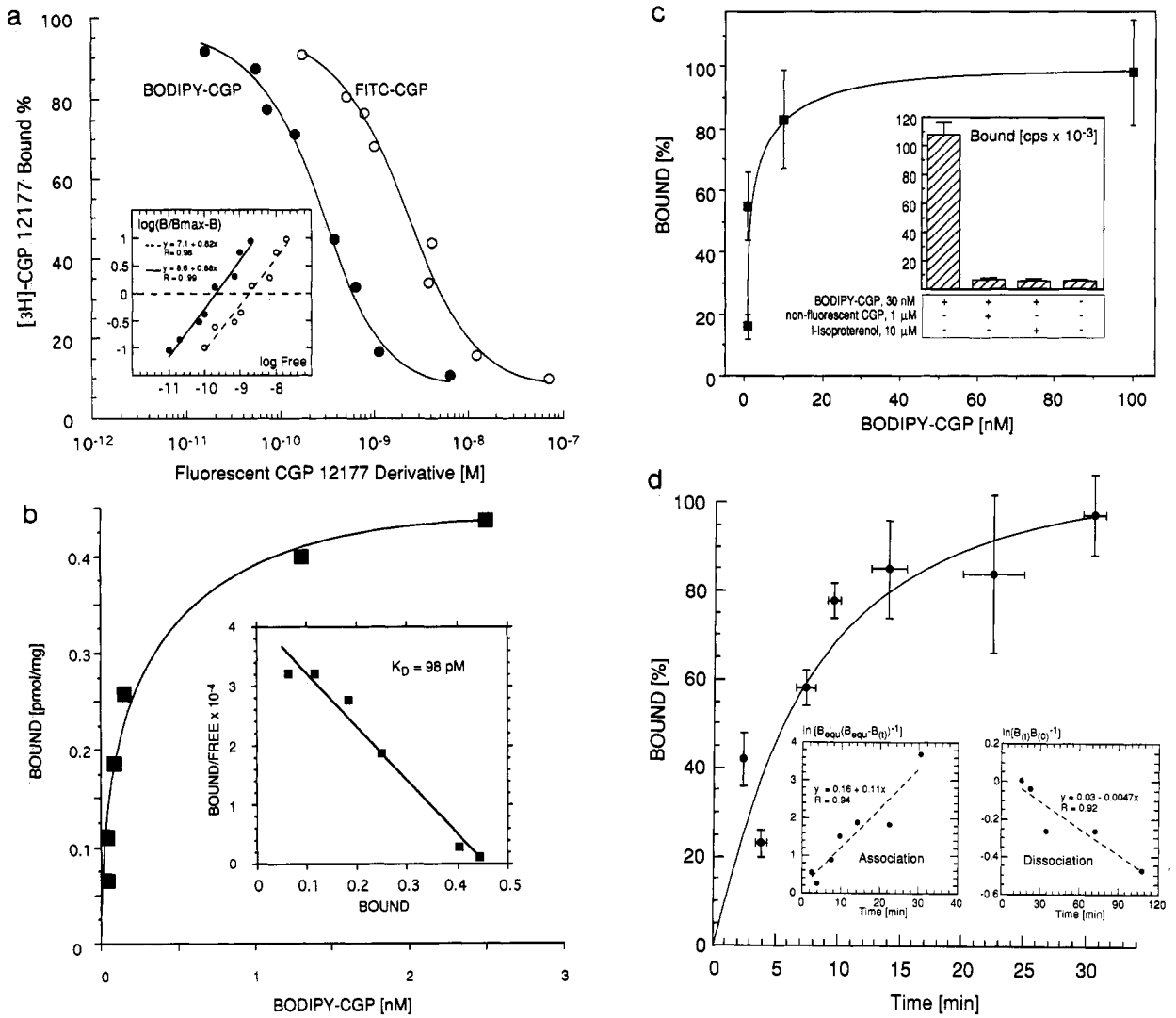

FIGURE 2: (a) Displacement of [^{3}H]CGP 12177 bound to human β_2-adrenoceptors expressed in transformed Sf9 cells by BODIPY-CGP (●) and FITC-CGP 12177 (○). Cells (2×10^5) were suspended in a final volume of 200 µL of serum-free medium, pH 6.8, and incubated with 200 pM [^{3}H]CGP 12177 and 2×10^{-11} to 2×10^{-9} M BODIPY-CGP or 10^{-10} to 10^{-7} M FITC-CGP for 30 min at 27 °C. Radioligand binding was measured as described in the Materials and Methods section. Solid lines were drawn by computer-aided nonlinear regression analysis of the data. The coefficients of variance of the residuals and the confidence intervals of the B_{max} and K_D values derived from these regressions were smaller than 15%. The inset shows a pseudo-Hill plot: Solid (—) and dashed (- - -) lines were drawn by linear regression analysis of the transformed data. The coefficients of the linear regression analysis are given. The Hill coefficients, derived from the slopes of the regression lines, were 0.88 and 0.82 for BODIPY-CGP and FITC-CGP, respectively. The IC_{50} values, derived from the x-values of the intercepts of the regression lines when $y = 0$, were 2×10^{-10} and 2×10^{-9} M for BODIPY-CGP and FITC-CGP, respectively. The K_i values were calculated from the IC_{50} values, the free ligand concentration [L], and the K_D value for [^{3}H]CGP 12177 (Table 1) according to $K_i = IC_{50}((1 + [L])(K_D^{-1}))^{-1}$; K_i was 100 pM and 1 nM for BODIPY-CGP and FITC-CGP, respectively. K_D values, derived from the nonlinear regression of the data, are given in Table 1. This is one of three experiments with similar results. (b) Binding of BODIPY-CGP to human β_2-adrenoceptors in membrane preparations of transformed Sf9 insect cells measured by fluorometry. Three hundred micrograms of membrane protein was incubated with 8×10^{-11} to 5×10^{-9} M BODIPY-CGP in 10 mL of PBS buffer, pH 7.4, for 3 h at 30 °C. Specific binding is thought to be represented by total cell-bound fluorescence minus the fluorescence that remained when membranes were treated with 1 µM nonfluorescent CGP 12177 added together with BODIPY-CGP. The experimental procedure is described in Materials and Methods. In the inset is a Scatchard plot: Solid lines are drawn by computer-aided nonlinear regression analysis of the data. This is one of three experiments with similar result. (c) Binding of BODIPY-CGP to single Sf9 cells expressing human β_2-adrenoceptors. Results were obtained by photon counting of single cells under the fluorescence microscope. Cells grown on coverslips were incubated with BODIPY-CGP at given concentrations in serum-free medium, pH 6.8, for 15 min at 27 °C. Each point represents the mean ± SEM of the data from five individual cells. Nonspecific binding was determined in the presence of 1 µM nonfluorescent CGP 12177 and subtracted. The data are normalized to maximal binding (100%). The inset shows actual photon counts per second obtained with unstained cells or with cells stained with 30 nM BODIPY-CGP alone or with 30 nM BODIPY-CGP and 1 µM nonfluorescent CGP 12177 or 10 µM L-isoproterenol. At these concentrations L-isoproterenol was equally effective as blocker. This is one of three independent experiments with similar results. Experimental details are in Materials and Methods. (d) Kinetics of association and dissociation of BODIPY-CGP with single Sf9 cells expressing human β_2-adrenoceptors. Cells grown on coverslips were incubated with 5 nM BODIPY-CGP in serum-free medium, pH 6.8, at 27 °C (see Materials and Methods) for the time intervals given (left inset) or were first labeled with 5 nM BODIPY-CGP for 20 min, washed exhaustively (5 times), and subsequently incubated with buffer alone for the times indicated (right inset). Single-cell fluorescence was corrected for nonspecific binding measured in the presence of 1 µM nonfluorescent CGP 12177. 100% binding represents maximal specific binding at equilibrium. Each point represents the mean of five independent measurements. The error bars indicate the SEM. The line was drawn by computer-aided nonlinear regression of the data (see Material and Methods). The two insets show semilog plots and linear regression analysis of the transformed data of association (left inset) and dissociation kinetics (right inset). K_{off} and the observed time constant for association, k_{obs}, were derived from the slopes of the respective regression lines in the insets. Similar values were obtained by nonlinear regression analysis of the nontransformed data. Since the off rate was very slow, that part of the dissociation curve for the specifically bound BODIPY-CGP resulting in up to 40% loss of bound ligand in about 110 min was considered for calculation of k_{off}. A true association constant, k_{on}, was calculated as 2×10^7 min^{-1} (mol/L)$^{-1}$ from k_{obs}, k_{off}, and the ligand concentration [L] by $k_{on} = (k_{obs} - k_{off})[L]^{-1}$. The K_D of BODIPY-CGP binding derived from the kinetics was 2.5×10^{-10} mol/L.

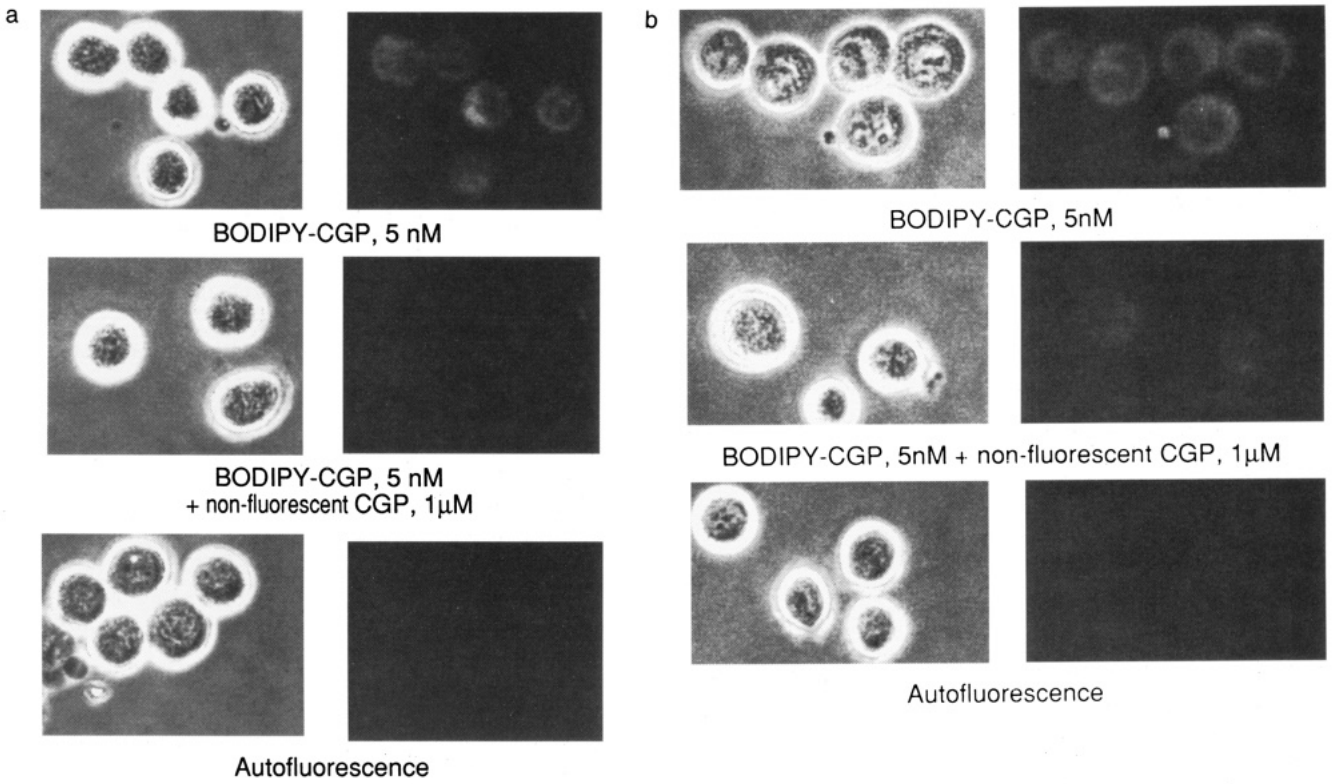

FIGURE 3: Fluorescence images of the β-adrenoceptors expressed in Sf9 cells and stained with BODIPY-CGP. Cells grown on coverslips were incubated with (top and middle rows) or without (bottom row) 5 nM BODIPY-CGP in PBS buffer, pH 6.8. Incubation was for 15 min at 30 °C (a) or for 20 min on ice (b). To cells shown in the middle row was added 1 μM nonfluorescent CGP 12177 together with BODIPY-CGP. After washing, cells were fixed with 2% paraformaldehyde for 10 min at 30 °C and visualized at pH 6.8 by phase-contrast (left column) and green fluorescence light microscopy (right column).

microscopy (right). The autofluorescence of cells incubated without fluorescent ligand can be seen in the bottom row. When the cells are stained with BODIPY-CGP, a bright fluorescent image of the cells is obtained (top row). This bright image is due to specific fluorescent β-adrenoceptor binding because it can be almost completely blocked by addition of nonfluorescent ligand during staining (middle row). Similar results were obtained when hydrophobic ligands such as L-alprenolol instead of more hydrophilic ligands such as nonfluorescent CGP 12177 or an agonist such as L-isoproterenol were used as blockers (compare Figure 2c). This made it possible to directly titrate cell-bound β-receptors by photon counting on single Sf9 cells. The experiments in Figure 3a were carried out at 30 °C, and those in Figure 3b, with cells kept on ice. Comparing the data indicates that binding of BODIPY-CGP to the receptors on transformed Sf9 cells was not much dependent on temperature, although somewhat less of the dye was bound on ice than at 30 °C. But in each case, the fluorescence was mainly on the cell surface, giving a half-moon pattern, typical for surface-restricted receptor expression. Internalization was not observed at the higher temperature. Moreover, when Sf9 cells were fixed first with 2% paraformaldehyde in order to immobilize receptors before binding of the fluorescent ligand at 30 °C, and receptor-specific fluorescence was compared by photon counting with that of nonfixed cells stained at 30 °C, only a small difference was observed (13 000 versus 16 000 cps, respectively). This is compatible with the assumption that the fluorescent β-receptor–ligand complex is not internalized by Sf9 cells. Wild-type Sf9 cells which do not have β-adrenoceptors did not bind or internalize BODIPY-CGP. Moreover, by exposing wild-type or transformed Sf9 cells to BODIPY derivatives not coupled to β-adrenergic ligands such as BODIPY-succinate, we could exclude receptor-independent cellular uptake of fluorescence or uptake of the dye component alone (Table 2). Similar results were also obtained with a tetramethyl-BODIPY derivative or fluorescamine (not shown). Thus, we can conclude that the cells were intact and were not leaky and that BODIPY-CGP not bound to β-adrenoceptors or fluorescence derived from it cannot gain access to the cell interior by receptor-independent means.

We have also studied the pH dependence of the fluorescence bound to transformed Sf9 cells, with results as to be expected: Whereas emission of BODIPY-CGP at 510 nm in aqueous solution increased by 30% when the pH was changed from 2.9 to 10.4, the intensity of the cell-bound fluorescence increased by about 50% in the pH range between 6.5 and 8.5. With the exceptions noted (see Table 1 and Figure 4) fluorescence bound to Sf9 cells was measured at room temperature and under the same pH conditions at which the cells were labeled.

A representative example of the analysis of Sf9 cells stained with BODIPY-CGP by fluorescence activated flow cytometry is presented in Figure 4, and the data are summarized in Table 3: Transformed Sf9 cells expressing $\sim 2 \times 10^5$ β-adrenoceptors/cell stained with BODIPY-CGP could be clearly distinguished (92% positive cells) either from cells which had not been stained (~2.5% positive cells) or from cells where specific staining of β-adrenoceptors had been blocked by an excess of nonfluorescent CGP 12177 (~3.3% positive). Sf9 wild-type cells, which do not possess β-adrenergic binding sites, were not stained (6.5% positive cells) by BODIPY-CGP, confirming specificity of the fluorescence staining which is nearly exclusively confined to the β-adrenoceptor. It becomes apparent, however, that the specific fluorescence emitted by the cells stained with BODIPY-CGP as related to the autofluorescence was much less in the FACS experiments than that of single ground-attached cells stained with BODIPY-CGP and measured by photon counting under the fluorescence microscope. Since the specific fluorescence did not increase much when the cells were copiously washed,

Table 3: FACS Analysis of Stably Transformed Sf9 Insect Cells Producing Human β_2-Adrenoceptor Stained with BODIPY-CGP[a]

cells	ligand	no. of cells screened			fluorescence	
		total	negative	positive	mean	cv, %[b]
Sf9 trans.	BODIPY-CGP	9850	798	9052	98	55
Sf9 trans.	BODIPY-CGP + nonfluorescent CGP	11 360	10 980	380	14	28
Sf9 trans.	no ligand, autofluorescence	9256	9021	235	11	29
Sf9 WT	BODIPY-CGP	9872	9223	649	19	36
Sf9 WT	BODIPY-CGP + nonfluorescent CGP	10 526	10 272	254	14	25
Sf9 WT	no ligand, autofluorescence	10 092	9824	268	12	21

[a] For conditions, see Materials and Methods and the caption to Figure 4. [b] Coefficient of variance: [(standard deviation)/(mean value)] × 100.

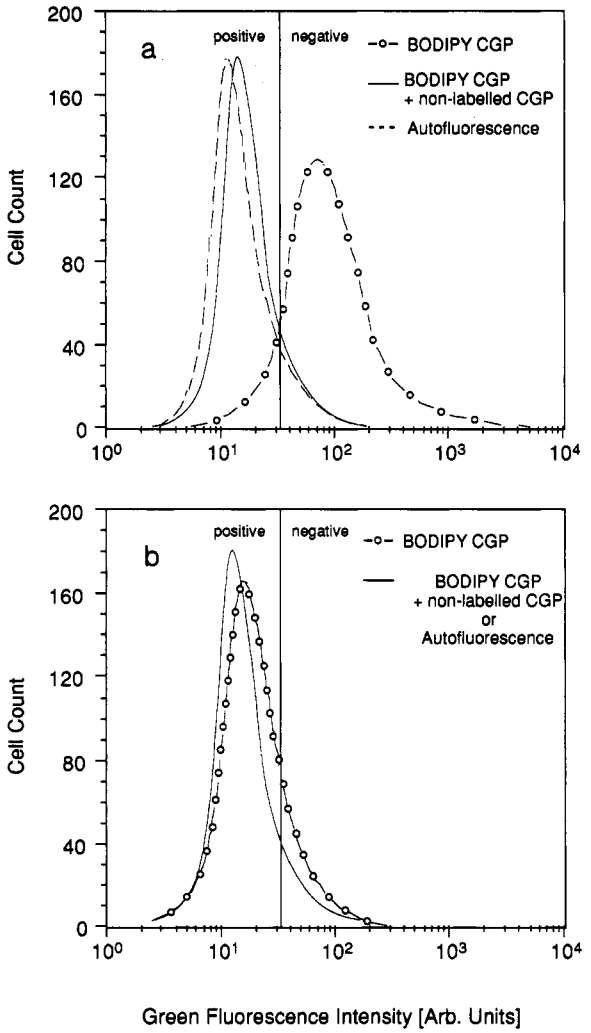

FIGURE 4: FACS-analysis of BODIPY-CGP binding to Sf9 cells: 2.5×10^5 transformed (a) or wild-type (b) Sf9 cells were incubated with 100 nM BODIPY-CGP alone (-O-) or with 100 nM BODIPY-CGP + 10 μM nonfluorescent CGP 12177 (—) as described in the Flow Cytometry section in Materials and Methods. Autofluorescence in panel a (- - -) was determined with cells incubated without ligand. After a 10-fold dilution, cells were stained with propidium iodide and measured with a FACScan. Data collection was electronically gated to intact cells not stained by propidium iodide. The solid vertical line indicates the discriminator selected for the statistical analysis (see Table 3).

and since the fluorescence due to nonspecific binding was comparable with the autofluorescence of the cells, we assume that this difference reflects the properties of adherent Sf9 cells as opposed to those of cell suspensions and the fact that under the microscope the cells could be selected for analysis by morphological criteria of viability (rounded shape, double contoured marginal refraction, etc.; compare Figure 3), while in the flow cytometry analysis a far less efficient viability selection (by the propidium exclusion test) had to be employed, resulting in a higher background of autofluorescence. Moreover, control experiments with [^{3}H]CGP 12177 indicated that Sf9 cells adherent on Petri dishes have about twice as many CGP 12177 binding sites ($4–5 \times 10^5$ sites per cell) as suspended cells ($2–3.5 \times 10^5$ sites per cell). Thus, the apparent lower specific staining of suspended cells might be due to an increase in autofluorescence and a decrease in receptor number.

DISCUSSION

We are interested in studying the disposition of β-adrenoceptors in intact cells (Hekman et al., 1984; Henis et al., 1982). For such studies highly fluorescent and specific receptor probes are required. One possibility is to use specific tight-binding antibodies that can be multiply tagged with fluorescent dyes (Bahouth et al., 1991; Chapot et al., 1989; Dunkel et al., 1989; Strosberg, 1989; von Zastrow & Kobilka, 1992; Wang et al., 1989). Another approach is to use suitable fluorescent ligands. The latter technique has the advantage that it allows one to distinguish on the basis of ligand binding properties between wild type and mutant and between properly and improperly folded β-adrenoceptors. For example, both would be expected to react with a common sequence-recognizing antibody, but only the former would be expected to bind the ligand with specificity and high affinity (von Zastrow & Kobilka, 1992). Therefore, we have made efforts to synthesize specific and tight-binding fluorescent β-adrenergic ligands (Heithier et al., 1988a; Henis et al., 1982). In previous work (Henis et al., 1982), NBD-alprenolol was used because CGP 12177 was not yet available. It is now well documented that more hydrophobic ligands such as alprenolol, in contrast to CGP 12177, can also label β-receptors, which are sequestered from the cell surface [for example, see Kurstjens et al. (1991)]. The chances of obtaining a suitable fluorescent probe for β-adrenergic receptors therefore increased considerably with the introduction of the hydrophilic ligand CGP 12177 and its favorable binding properties (Portenier et al., 1984; Staehelin et al., 1983). We have made use of CGP 12177 and have synthesized its NBD derivative (Heithier et al., 1988a). However, the fluorescence emission of this compound was extremely solvent sensitive, with the quantum yield increasing 23-fold upon transfer from H_2O to acetonitrile. Therefore, in order to measure by fluorometry CGP-NBD bound to β_2-adrenoceptors in $A_{431.E3}$ cells, it was necessary first to extract quantitatively the fluorescent ligand with chloroform from cells and membranes. Hence, compared with the NBD derivative of CGP 12177 synthesized by us in the past, the fluorescent BODIPY derivative of CGP 12177 described in this report appears to have more attractive properties, although the data obtained with NBD-CGP are not directly comparable with the data obtained with BODIPY-CGP. In order to compare the properties of more hydrophobic β-adrenergic ligands, such as NBD-alprenolol (Henis et al., 1982), or more hydrophilic ligands, such as NBD-CGP (Heithier et al., 1988a), with our BODIPY derivative, the same kind of cells should be used, which were studied before with the fluorescent NBD derivatives. These cells, Chang

liver cells (Henis et al., 1982), and $A_{431.E3}$ cells (Heithier et al., 1988a) contain at least 1 order of magnitude fewer β_2-adrenoceptors than the transformed Sf9 cells used in this work. We have initiated studies with $A_{431.E3}$ cells, which contain about 3×10^4 specific β_2-receptor binding sites. The results of these studies should allow us to decide wether the new BODIPY-CGP derivatives are better tools for biophysical studies than the NBD derivatives used in the past. However, the data reported here demonstrate impressively that BODIPY-CGP binds with high affinity and specificity to human β_2-adrenoceptors expressed in stably transformed *Spodoptera frugiperda* Sf9 cells. The level of nonspecific binding was low with up to 80% specific binding at 50 times K_D (Figure 2a,c), and the K_D values for nonderivatized CGP 12177 and BODIPY-CGP were in the same range (Table 1). Thus, the favorable ligand binding properties of CGP 12177 are largely preserved in its fluorescent analogue. The good fluorescence qualities made it also possible to directly visualize the heterologously produced human β_2-adrenoceptors in single transformed Sf9 cells (2×10^5 sites per cell) and to quantitate the receptor-bound fluorescence. This made it possible to estimate the number of β-receptors present on single cells simply by photon counting, although the scatter of the data is still too large (Figure 2c; Tables 1 and 2). In order to improve the data, a larger number of cells must be counted, perhaps with an automatic data acquisition device for practical applications. Moreover, BODIPY-CGP can be used to screen for β-adrenoceptor expression by FACS (Figure 4; Table 3).

On comparing panels a and b of Figure 3, it becomes apparent that in Sf9 cells kept on ice or at 30 °C the receptor-bound fluorescence remained mostly restricted to the cell surface. Thus, these cells do not take up receptor-bound fluorescence in a temperature-dependent manner. These data and a comparison of the number of binding sites titrated with [^{3}H]CGP 12177 at 4 and 30 °C suggested that most of the receptors on Sf9 cells are on the surface and accessible to either [^{3}H]CGP 12177 or BODIPY-CGP. Receptor-independent uptake of the fluorescent ligand into the cells can be excluded on the basis of the data shown in Table 2.

Thus BODIPY-CGP is a fluorescent β-adrenergic ligand, which allows specific and direct titration of receptor binding without the need to use radioactive ligands. Moreover, with this ligand we can measure binding sites present on single cells by photon counting under the fluorescence microscope. This allows us to assess differentially the expression of β-adrenoceptors in small samples of heterogeneous cell mixtures. Work is in progress to test the applicability of this new approach to visualize and quantify the number of β-receptor binding sites on single cells expressing only 10^4 or fewer receptors per cell.

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REFERENCES

Affolter, H., Hertel, C., Jaeggi, K., Portenier, M., & Staehelin, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 925.

Ariano, M. A., Monsma, F. J., Jr., Barton, A. C., Kang, H. C., Haugland, R. P., & Sibley, D. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8570.

Atlas, D., & Levitzki, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5290.

Bahouth, S. W., Wang, H. Y., & Malbon, C. C. (1991) *Trends Pharmacol. Sci. 12*, 338.

Barnes, P., Koppel, H., Lewis, P., Hutson, C., Blair, I., & Dollery, C. (1980) *Brain Res. 160*, 209.

Bindal, R. D., & Katzenellenbogen, J. A. (1985) *J. Steroid Biochem. 23*, 929.

Boege, F., Ward, M., Jurss, R., Hekman, M., & Helmreich, E. J. M. (1988) *J. Biol. Chem. 263*, 9040.

Carlson, K. E., Coppey, M., Magdelenat, H., & Katzenellenbogen, J. A. (1989) *J. Steroid Biochem. 32*, 345.

Chapot, M. P., Couraud, P. O., Schmutz, A., Strosberg, A. D., & Durieu, Trautmann, O. (1989) *Hybridoma 8*, 535.

Cheung, A. T., Johnson, D. A., & Taylor, P. (1984) *Biophys. J. 45*, 447.

Correa, F. M., Innis, R. B., Rouot, B., Pasternack, G. W., & Snyder, S. (1980) *Neurosci. Lett. 16*, 47.

Covarrubias, M., Prinz, H., & Maelicke, A. (1984) *FEBS Lett. 169*, 229.

De Potter, Cr., Eechaute, W., Roels, H., & Leusen, I. (1985) *J. Recept. Res. 5*, 245.

Dickson, R. B., Hanover, J. A., Willingham, M. C., & Pastan, I. (1983) *Biochemistry 22*, 5667.

Dunkel, F. G., Munch, G., Boege, F., Cantrill, R., & Kurstjens, N. P. (1989) *Biochem. Biophys. Res. Commun. 165*, 264.

Gonzalez-Ros, J. M., Farach, M. C., & Martinez-Carriou, M. (1983) *Biochemistry 22*, 3807.

Heithier, H., Jaeggi, K. A., Ward, L. D., Cantrill, R. C., & Helmreich, E. J. M. (1988a) *Biochimie 70*, 687.

Heithier, H., Ward, L. D., Cantrill, R. C., Klein, H. W., Im, M. J., Pollak, G., Freeman, B., Schiltz, E., Peters, R., & Helmreich, E. J. M. (1988b) *Biochi.m Biophys. Acta 971*, 298.

Hekman, M., Schiltz, E., Henis, Y. I., Elson, E. L., & Helmreich, E. J. M. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17*, 47.

Henis, Y. I., Hekman, M., Elson, E. L., & Helmreich, E. J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2907.

Hertel, C., & Staehelin, M. (1983) *J. Cell Biol. 97*, 1538.

Hertel, C., Staehelin, M., & Perkins, J. P. (1983) *J. Cyclic Nucleotide Protein Phosphorylation Res. 9*, 119.

Herz, J. M., Johnson, D. A., & Taylor, P. (1989) *J. Biol. Chem. 264*, 12439.

Hess, A. (1979) *Brain Res. 160*, 533.

Jacobson, K. A., Ukena, D., Padgett, W., Kirk, K. L., & Daly, J. W. (1987) *Biochem. Pharmacol. 36*, 1697.

Jans, D. A., Peters, R., Zsigo, J., & Fahrenholz, F. (1989) *EMBO J. 8*, 2481.

Jans, D. A., Peters, R., & Fahrenholz, F. (1990) *EMBO J. 9*, 2693.

Kleymann, G., Boege, F., Hahn, M., Hampe, W., Vasudevan, S., & Reiländer, H. (1993) *Eur. J. Biochem. 213*, 797.

Knaus, H. G., Moshammer, T., Friedrich, K., Kang, H. C., Haugland, R. P., & Glossman, H. (1992a) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3586.

Knaus, H. G., Moshammer, T., Kang, H. C., Haugland, R. P., & Glossmann, H. (1992b) *J. Biol. Chem. 267*, 2179.

Kolb, V. M., Koman, A., & Terenius, L. (1983) *Life Sci. 33*, 423.

Kurstjens, N. P., Fröhlich, M., Dees, C., Cantrill, R. C., Hekman, M., & Helmreich, E. J. M. (1991) *Eur. J. Biochem. 197*, 167.

McCabe, R. T., de Costa, B. R., Miller, R. L., Havunjian, R. H., Rice, K. C., & Skolnick, P. (1990) *FASEB J. 4*, 2934.

McPherson, G. A. (1985) *J. Pharmacol. Methods 14*, 213.

McPherson, G. A., Molenaar, P., Raper, C., & Malta, E. (1983) *J. Pharmacol. Methods 10*, 231.

Mihara, H., Lee, S., Shimohigashi, Y., Aoyagi, H., Kato, T., Izumiya, N., & Costa, T. (1985) *FEBS Lett. 193*, 35.

Monsma, F., Jr., Barton, A. C., Kang, H. C., Brassard, D. L., Haugland, R. P., & Sibley, D. R. (1989) *J. Neurochem. 52*, 1641.

Pomper, M. G., Pinney, K. G., Carlson, K. E., van Brocklin, H., Mathias, C. J., Welch, M. J., & Katzenellenbogen, J. A. (1990) *Int. J. Radiat. Appl. Instrum. 17*, 309.

Portenier, M., Hertel, C., Muller, P., & Staehelin, M. (1984) *J. Recept. Res. 4*, 103.
Prinz, H., & Maelicke, A. (1983) *J. Biol. Chem. 258*, 10263.
Rademaker, B., Kramer, K., Stroes, J. W., Vlug, J., Krielaart, M., & Zaagsma, J. (1985a) *Eur. J. Pharmacol. 111*, 31.
Rademaker, B., Kramer, K., van Ingen, H., Kranendonk, M., & Timmerman, H. (1985b) *J. Recept. Res. 5*, 121.
Reilander, H., Boege, F., Vasudevan, S., Maul, G., Hekman, M., Dees, C., Hampe, W., Helmreich, E. J. M., & Michel, H. (1991) *FEBS Lett. 282*, 441.
Staehelin, M., Simons, P., Jaeggi, K. A., & Wigger, N. (1983) *J. Biol. Chem. 258*, 3496.
Strosberg, A. D. (1989) *Methods Enzymol. 178*, 265.
Sui, S. F., Urumow, T., & Sackmann, E. (1988) *Biochemistry 27*, 7463.
von Zastrow, M., & Kobilka, B. K. (1992) *J. Biol. Chem. 267*, 3530.
Wang, H., Lipfert, L., Malbon, C. C., & Bahouth, S. (1989) *J. Biol. Chem. 264*, 14424.
Ward, L. D., Cantrill, R. C., Heithier, H., Peters, R., & Helmreich, E. J. M. (1988) *Biochim. Biophys. Acta 971*, 307.
Zemcik, B. A., & Strader, C. D. (1988) *Biochem. J. 251*, 333.