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# Lateral motion of β receptors in membranes of cultured liver cells

(β-adrenergic receptors/photobleaching recovery/fluorescence image intensification)

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**ABSTRACT** **We have studied the lateral mobility and distribution of β receptors on Chang human liver cells by fluorescence photobleaching recovery and video intensification microscopy. The β receptors were labeled with the fluorescent antagonist 7-(2-allylphenoxy)-2,2-dimethyl-6-hydroxy-1-(4-nitrobenzo-2-oxa-1,3-diazolyl)-1,4-diazaheptane (Alp-NBD). Sixty to 75% of the staining was specific (displaceable by unlabeled antagonists). Most of the antagonist-occupied β receptors were immobile, because only 15–25% of their fluorescence recovered on the experimental time scale at 23°C. This immobility correlates with the clustered distribution of Alp-NBD–β-receptor complexes at 4°C and 37°C. The β receptors appear to be aggregated prior to antagonist binding, because visible patches were observed immediately after labeling for 30 sec at 4°C. Preincubation at 37°C with (−)-isoproterenol, a β agonist, prior to Alp-NBD labeling induced a time-dependent release of the β receptors to a more homogeneous distribution and increased the mobile fraction to 70–80% (lateral diffusion coefficient = $1.4 \times 10^{-9}$ cm²/sec at 23°C). This is not due to an effect on membrane fluidity, because the diffusion coefficient of a lipid probe was not altered. The time course of agonist-induced β-receptor mobilization correlates with receptor loss and adenylate cyclase desensitization but is much slower than adenylate cyclase activation. This indicates that adenylate cyclase activation by β receptors does not require macroscopic lateral mobility of the majority of the β receptors.**

Recent models of adenylate cyclase activation by β receptors stress the role of diffusional encounters between hormone–receptor complexes in the plasma membrane and other components of the cyclase system (1). The hybridization experiments of Schramm *et al.* (2) and the kinetic analyses of Levitzki *et al.* (3) are usually cited to support these models based on the floating receptor hypothesis (4). Lateral mobility of hormone–receptor complexes relative to the other cyclase components is supposed to be necessary for their effective interaction, although the receptor complexes themselves need not be mobile if the other components are. Recent studies of reconstituted systems (5) have, however, shown that the encounter of the hormone–receptor complex with the guanyl nucleotide binding protein (G protein) (6, 7) is much faster than the rate of cyclase activation and so cannot be rate-limiting in the activation process. This result must still be tested in native membranes. Further consideration of the kinetic role of receptor–G protein encounters requires direct measurement of the lateral mobility and distribution of both the receptors and the G proteins in living cells. Here we report measurements of the distribution and lateral diffusion coefficients of β receptors labeled with a fluorescent antagonist on Chang human liver cells, in which the influence of membrane structure on β-adrenergic stimulation of adenylate cyclase has previously been studied (8). The experiments used fluorescence photobleaching recovery (FPR) (9, 10) and video intensification microscopy (VIM) (11). We find that the majority of β receptors occupied by a fluorescent antagonist are clustered and immobile on Chang cell surfaces, that the receptors seem to be in this clustered, immobile state prior to binding the antagonist, and that preincubation of the cells with β agonist releases the receptors from this state.

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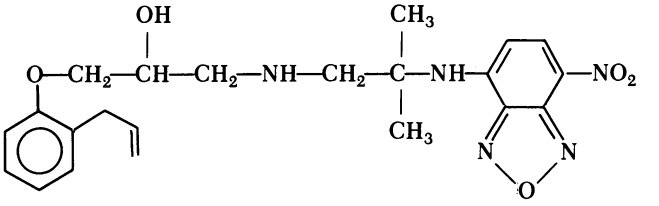
## MATERIALS AND METHODS

**Reagents.** 4-Nitrobenzo-2-oxa-1,3-diazolyl-phosphatidylethanolamine (NBD-PtdEtn) was from Avanti, propranolol was from Ayerst Laboratories (New York), and isoproterenol was from Sterling (New York). 1-(Isopropylamino)-3-indolyloxy-2-propanol [(−)- and (+)-pindolol] was a gift from Gary Brooker (University of Virginia Medical School, Charlottesville, VA).

**Synthesis of a Fluorescent β Antagonist.** The β-receptor antagonist 7-(2-allylphenoxy)-2,2-dimethyl-6-hydroxy-1-(7-NBD)-1,4-diazaheptane (Alp-NBD)



was synthesized as follows (ref. 12; unpublished data): *o*-Allylphenol was dissolved in an equimolar KOH solution. Epichlorohydrin was added with stirring. The mixture was extracted with ether, and the ether phase was washed and dried over $Na_2SO_4$. The alprenolol epoxide was distilled under reduced pressure and allowed to react with 1,2-diamino-2-methylpropane (13) in dioxane at 80°C to yield alprenolol-$NH_2$, which was precipitated with HCl/acetone, 1:1 (vol/vol), at 4°C. Alprenolol-$NH_2$·HCl was dissolved with NBD-Cl in ethanol and potassium acetate was added. After reaction at room temperature for 36 hr, the product was purified by chromatography on silica gel (30 × 2 cm) with $CHCl_3$/MeOH, 30:1 (vol/vol) as solvent. The product was precipitated with concentrated HCl/acetone, 1:1 (vol/vol). The yield was 1.4%. Purity was ascertained by IR,

Abbreviations: G protein, guanylnucleotide binding protein; FPR, fluorescence photobleaching recovery, VIM, video intensification microscopy; NBD, 4-nitrobenzo-2-oxa-1,3-diazolyl; NBD-PtdEtn, NBD-phosphatidylethanolamine; propranolol, 1-(isopropylamino)-3-naphthyloxy-2-propanol; isoproterenol, 1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol; Alp-NBD, 7-(2-allylphenoxy)-2,2-dimethyl-6-hydroxy-1-(7-NBD)-1,4-diazaheptane; carazolol, 4-(2-hydroxy-3-isopropylaminopropoxy)carbazole; *D*, lateral diffusion coefficient; $R_f$, mobile fraction.

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$^1$H NMR, and UV spectroscopy and elementary analysis. $K_d$ was 30 nM as determined in competition–binding assays with [$^{125}$I]iodohydroxybenzylpindolol on Chang liver cells (12).

**Cells and Cell Labeling.** Chang liver cells were obtained from the American Type Culture Collection. Monolayers were grown in Eagle's minimal essential medium with Earle's salts containing 10% fetal calf serum, penicillin at 100 units/liter, and streptomycin at 100 μg/liter. Cells were treated with trypsin and replated every 3–4 days. For FPR and VIM experiments, 3–5 × $10^4$ cells were plated 2 days earlier on glass coverslips placed in 35-mm Petri dishes. Each dish contained 1–2 × $10^5$ cells on the day of the experiment. Prior to labeling with Alp-NBD, cells were washed twice with 145 mM NaCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/5 mM KCl/25 mM glucose/20 mM Hepes, pH 7.4 (Hepes buffer). The coverslip was placed (cells facing downward) over a buffer-filled serological slide (Scientific Products M 6229-2), and was secured with vacuum grease. For experiments at 4°C or 37°C, a thermostatted holder was used.

Cells in Hepes buffer were labeled at 4°C or 23°C for 30 sec or 30 min with 0.04 or 2 μM Alp-NBD in Hepes buffer containing 1% (vol/vol) ethanol, followed by washing three times with buffer. To determine specificity cells were preincubated with 0.01 or 1 mM propranolol in Hepes buffer for 30 min at 23°C and then incubated in the presence of the competitor with 0.04 or 2 μM Alp-NBD, respectively. Specificity was also verified by displacement with 10 μM carazolol or with 0.05 μM (+)- and (−)-pindolol. Labeling with NBD-PtdEtn was achieved by a 1:100 dilution of NBD-PtdEtn at 2 mg/ml in ethanol into the Hepes buffer, followed by 15-min incubation at 23°C and washing twice with the buffer.

**FPR.** Diffusion coefficients ($D$) and mobile fractions ($R_f$) were measured by FPR (9, 10), using the apparatus described (14). The monitoring laser beam ($\lambda$ = 476.5 nm, 0.02 μW) from an argon ion laser was focused on the cell membrane through a Zeiss Universal microscope to a spot of 2.25-μm radius, using a ×100 oil-immersion lens. Fifty to 70% of the fluorescence in this region was irreversibly bleached by a 40-msec pulse of 0.2-mW intensity. The $D$ and $R_f$ values of the fluorescent probe were determined by using the attenuated beam to monitor the rate of fluorescence recovery in the bleached spot due to entry of unbleached fluorophores (10). Incomplete fluorescence recovery was interpreted as indicating probe molecules immobile on the experimental time scale ($D \leq 5 \times 10^{-12}$ cm$^2$/sec).

**VIM.** Cell-surface Alp-NBD fluorescence was visualized by an RCA 1005/N01 low-bloom television camera, coupled to the microscope through a Ni-Tec NVC-100 image intensifier. Phase and fluorescent images recorded on videotape (Panasonic VTR NV-8030) were photographed from the television monitor (Panasonic WV 5310) with an Olympus OM-2N camera, using 400 ASA film. All photographs were taken under identical settings of the TV equipment and the film camera, to enable direct comparison of the fluorescence micrographs.

## RESULTS

**Labeling of Chang Liver Cells with Alp-NBD.** Chang liver cells have (2 ± 1) × $10^4$ β receptors per cell, determined by [$^{125}$I]iodohydroxybenzylpindolol binding. This is a comparatively large complement (15, 16). The specificity of the fluorescent staining by Alp-NBD was determined by photon counting using the FPR system (Table 1). Competition by 10 μM propranolol ($K_d$ = 5.7 nM) or carazolol ($K_d$ = 0.4 nM) displaced 60% and 75% of the fluorescence from cells labeled with 40 nM Alp-NBD for 30 min or 30 sec, respectively (Table 1). Similar results were obtained whether the competitors were present during labeling with Alp-NBD or were added after 30-min incubation at 37°C with Alp-NBD, indicating little or no internalization of the receptors during that period. Specificity was also checked by preincubating cells with 50 nm (+)- or (−)-pindolol (90 min, 23°C) and then labeling with 40 nM Alp-NBD (30 sec, 23°C) in Hepes buffer. The (−) isomer, which binds tightly ($K_d$ = 0.03 nM) to β receptors (17), displaced 60% of the fluorescence, whereas (+)-pindolol displaced less than 10%. Moreover, 20–30% of the nonspecific fluorescence is due to cellular autofluorescence (Table 1). Thus, we conclude from measurements of fluorescence that the specificity of Alp-NBD labeling is 50–60% and 60–75% for labeling periods of 30 min and 30 sec, respectively. We have assumed that unlabeled antagonists displace only specifically bound Alp-NBD. The similarity of results using propranolol at high (10 μM) and (−)-pindolol at much lower (50 nM) concentration supports this assumption. The Alp-NBD nonspecific fluorescence has two components: a patchy nonhomogeneous component and a diffuse component with lipid probe characteristics that is selectively diminished with shorter (30-sec) incubations. That labeling with a higher Alp-NBD concentration (2 μM) yielded intenser and more homogenous fluorescence (Table 1) that was not displaced by propranolol and diffused like a lipid probe (Table 2) suggests that the diffuse component is due to Alp-NBD dissolved nonspecifically in the membrane lipid bilayer.

Fixation of the cells [3% (wt/vol) paraformaldehyde, 23°C, 1 hr] prevented subsequent labeling by 40 nM Alp-NBD, but not the diffuse labeling at 2,000 nM Alp-NBD (Table 1).

**Distribution of β-Adrenergic Receptors on Chang Liver Cells.** Alp-NBD complexes observed by VIM (11) on Chang cells labeled with 40 nM Alp-NBD at 4°C were mainly in aggregates (Fig. 1*A*) and remained patchy after 1 hr at 37°C or 23°C (Fig. 1*C*). The patches represent mostly specific labeling, because the nonspecific staining was significantly weaker (Fig. 1*B*; Table 1). Observation of patches after brief labeling (30 sec) at 4°C suggests that the β receptors on Chang liver cells are aggregated prior to Alp-NBD binding (see *Discussion*). Similar results were obtained when labeling was at 23°C or 37°C, or for 30 min. Therefore the patched distribution does not seem to

Table 1. Labeling of Chang liver cells with Alp-NBD

| Conditions | Alp-NBD, nM | Fluorescence intensity, arbitrary units: Without competitor | With competitor |
|---|---|---|---|
| (*a*) 30 sec | 40 | 5,560 ± 800 | 1,360 ± 190 |
| (*b*) 30 min | 40 | 2,800 ± 500 | 1,110 ± 140 |
| (*c*) 30 min | 2,000 | 27,800 ± 2,000 | 28,900 ± 1,700 |
| (*d*) No labeling | — | 300 ± 40 | 290 ± 50 |
| (*e*) Fixed; 30 min | 40 | 590 ± 100 | 750 ± 140 |
| (*f*) Fixed; 30 min | 2,000 | 19,000 ± 1,200 | 19,700 ± 1,200 |
| (*g*) Fixed; unlabeled | — | 670 ± 100 | 70 ± 120 |

Cells were incubated at 23°C with Alp-NBD for 30 sec or 30 min. Similar results were obtained at 4°C. Fluorescence was measured from 3.15-μm radius areas, illuminated by the laser (476.5 nm, 0.2 μW). Twenty to 30 cells were scored for each entry. Data are given as mean ± SEM. Measurements were performed after Alp-NBD labeling (*a–c*), without labeling (*d, g*), and after fixation with 3% paraformaldehyde (23°C, 1 hr) followed by Alp-NBD labeling (*e, f*). The fraction of specific fluorescence, $\rho^{(s)}$, was determined by competition with propranolol or carazolol. $\rho^{(s)}$ values were 0.76 ± 0.07 and 0.60 ± 0.12 for *a* and *b*, respectively. $\rho^{(s)}$ was calculated as 1 − (with competitor)/(without competitor). For 30-sec labeling with Alp-NBD, fluorescence was measured only on patched regions, because no staining was detected outside the patches. For direct comparison, the nonspecific staining (with competitor) was also measured at patchy regions. For 30-min labeling, some measurements were done outside of the patched regions, thus yielding lower average fluorescence.

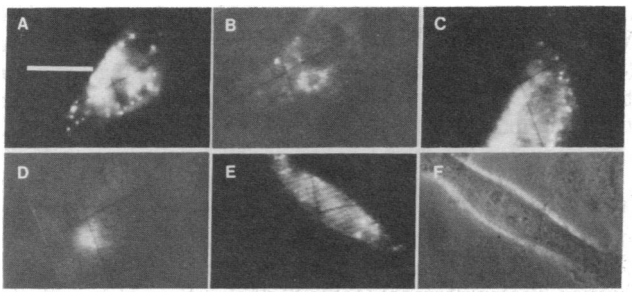


FIG. 1. Distribution of Alp-NBD on Chang liver cells. Cells were labeled with 40 nM Alp-NBD for 30 sec at 4°C. Labeling for 30 min at 4°C or at 23°C gave similar results. Photographs were taken from the television screen after image intensification, except *F*, which was taken directly through the microscope. Bar, 20 μm. (*A*) Fluorescence of Alp-NBD at 4°C. (*B*) Nonspecific Alp-NBD fluorescence labeling was performed in the presence of 10 μM propranolol introduced 30 min earlier. (*C*) Fluorescence of Alp-NBD after 1 hr at 37°C. (*D*) Alp-NBD fluorescence on cells fixed with 3% paraformaldehyde prior to labeling. (*E*) Alp-NBD fluorescence after 30-min preincubation with 16 μM (−)-isoproterenol at 37°C and labeling for 30 sec at 4°C with 40 nM Alp-NBD. (*F*) Phase-contrast micrograph of *E*.

result from exposure of the cells to cold or from failure to reach ligand binding equilibrium. Paraformaldehyde fixation prior to labeling prevented Alp-NBD binding, presumably due to receptor modification (Fig. 1*D*).

**Lateral Mobility of the β-Receptor Complexes with Alp-NBD.** The $D$ and $R_f$ values of Alp-NBD on Chang liver cells were measured at 23°C. $R_f$ was evaluated from the fraction of fluorescence recovery after the first bleach, and the $D$ values were obtained from the average of the subsequent four bleaches on the same spot. Representative FPR curves are in Fig. 2.

The patchy fluorescence suggests that the β-receptor Alp-NBD complexes should be largely immobile. This expectation is correct. The fraction of the specifically bound Alp-NBD that is mobile can be derived from eq. 1:

$$R_f^{(t)} = \rho^{(s)}R_f^{(s)} + \rho^{(n)}R_f^{(n)}. \quad [1]$$

Here $R_f^{(t)}$, $R_f^{(s)}$, and $R_f^{(n)}$ are the mobile fractions of the total, specific, and nonspecific fluorescence, respectively. $\rho^{(s)}$ and $\rho^{(n)}$ are the fractions of total fluorescence that are specific and nonspecific. Identical fluorescence and photobleaching properties of all detected fluorophores were assumed. Values of $\rho^{(s)}$ ($\rho^{(s)} = 1 - p^{(n)}$) are listed in the legend to Table 1. Values of $R_f^{(t)}$, $R_f^{(n)}$, and $R_f^{(s)}$ are listed in Table 2. The value of $R_f^{(s)}$ for cells labeled for 30 sec indicates that over 80% of the specifically bound

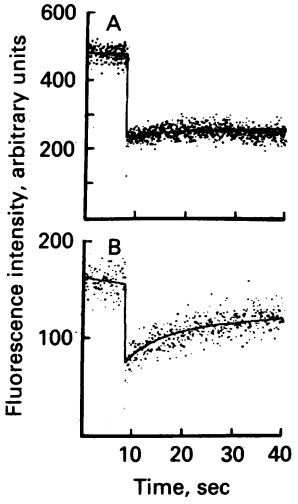


FIG. 2. Representative FPR curves of Alp-NBD on Chang liver cells. Cells were labeled with 40 nM Alp-NBD for 30 sec at 23°C before (*A*) and after (*B*) 30-min preincubation at 37°C with 16 μM (−)-isoproterenol. FPR measurements were performed at 23°C. The points represent the photons counted per 40-msec dwell time. Solid lines are computer-generated nonlinear regression best fit fluorescence recovery curves, obtained for a lateral diffusion process with a single diffusion coefficient $D$ (10). Only curves of a first bleach are shown. The computer fit yields $R_f = 0.15$ and 0.73 for *A* and *B*.

Table 2. Diffusion coefficients ($D$) and mobile fractions ($R_f$) of Alp-NBD on Chang liver cells

| Conditions | $D$, (cm²/sec) × 10⁹ | $R_f^{(t)}$ | $R_f^{(s)}$ |
|---|---|---|---|
| (*a*) 30 sec | 1.4 ± 0.1 | 0.15 ± 0.04 | 0.03 ± 0.14 |
| (*b*) 30 sec + propranolol | 3.5 ± 0.5 | 0.51 ± 0.05 | |
| (*c*) 30 min | 5.1 ± 0.4 | 0.51 ± 0.04 | 0.35 ± 0.37 |
| (*d*) 30 min + propranolol | 5.8 ± 0.5 | 0.74 ± 0.02 | |
| (*e*) 30 min | 8.9 ± 1 | 0.87 ± 0.03 | |
| (*f*) 30 min + propranolol | 10.7 ± 1 | 0.90 ± 0.03 | |

Cells were treated as described for Table 1, and FPR measurements were performed at 23°C. Incubation with Alp-NBD was for times listed at 40 nM (*a–d*) or 2,000 nM (*e*, *f*) and propranolol concentration was 10 μM (*b*, *d*) and 1 mM (*f*). Data are given as mean ± SEM. Each entry represents measurements on 20–30 cells. $R_f^{(s)}$ was calculated from Eq. 1, taking the $R_f^{(n)}$ values in rows *a* and *c* as those of $R_f^{(t)}$ in rows *b* and *d*, respectively. Propagation of errors in the calculation of $R_f^{(s)}$ was obtained by standard procedures (18).

Alp-NBD is immobile and is consistent with the possibility that all of these molecules are immobile (Table 2). Both the value of $R_f^{(s)}$ and its limits of error are larger for measurements on cells labeled for 30 min (Table 2). Nevertheless these results are also consistent specifically with the possibility that all of the bound Alp-NBD is immobile.

Interpretation of the measured diffusion coefficients is complicated by contributions from nonspecifically bound Alp-NBD. On cells labeled with Alp-NBD at high concentrations (2,000 nM), binding is mostly nonspecific and not diminished by propranolol (Table 2, rows *e* and *f*). The values of $R_f = 0.87$ and $D = 8.9 \times 10^{-9}$ cm²/sec suggest that this nonspecifically bound Alp-NBD is behaving like a lipid incorporated into the bilayer (19). Nonspecific Alp-NBD labeling at low concentration (40 nM) and for a short time (30 sec) yields lower mobility; $R_f = 0.51$ and $D = 3.5 \times 10^{-9}$ cm²/sec (Table 2, row *b*). Intermediate values of $R_f$ and $D$ are observed for nonspecific labeling at 40 nM for 30 min (Table 2, row *d*). Thus there are two types of nonspecific binding: one, which predominates at high labeling concentrations, is lipid-like, and the other, most clearly seen after labeling at low concentrations for short periods, has lower $R_f$ and $D$.

Under conditions that maximize the fraction of specifically bound Alp-NBD (Table 2, row *a*) $D = 1.4 \times 10^{-9}$ cm²/sec. This value is lower than the value for the nonspecific fluorescence (Table 2, row *b*) and might suggest that up to 20% of the β receptors move with $D \approx 10^{-9}$ cm²/sec. The values of $D$ and $R_f$ increase when cells are labeled for 30 min instead of 30 sec with 40 nM Alp-NBD, presumably due to greater nonspecific lipid-like labeling. In summary, most, if not all, of the Alp-NBD receptor complexes on Chang liver cells are laterally immobile over micrometer distances during time periods of at least 10 min.

**Effect of Isoproterenol on β-Receptor Mobility.** Functional differences exist between β receptors occupied by agonists and by antagonists (1, 20, 21). It was therefore of interest to study the effect of an agonist on the lateral mobility and distribution of β receptors.

Chang liver cells were incubated in the Hepes buffer at 37°C with 16 μM (−)-isoproterenol for 5 or 30 min, washed with Hepes buffer, and labeled with 40 nM Alp-NBD for 30 sec or 30 min at 4°C or at 23°C. Regardless of the Alp-NBD labeling temperature, preincubation with isoproterenol had two effects: it decreased the patchy appearance (Fig. 1*E*) and it increased the $R_f$ of the β-receptor–Alp-NBD complexes (Fig. 2, Table 3). $R_f^{(t)}$ of the β receptors occupied with Alp-NBD increased to 0.75 for 30-sec and to 0.91 for 30-min Alp-NBD labeling (Table 3).

Table 3. Effect of isoproterenol on the diffusion coefficient ($D$) and mobile fraction ($R_f$) of Alp-NBD on Chang liver cells

| Conditions | $D$, ($cm^2/sec$) × $10^9$ | $R_f^{(t)}$ | Fluorescence intensity, arbitrary units |
|---|---|---|---|
| (*a*) 30 sec | 1.5 ± 0.1 | 0.75 ± 0.05 | 1,390 ± 190 |
| (*b*) 30 sec + propranolol | 3.0 ± 1.0 | 0.6 ± 0.2 | 510 ± 210 |
| (*c*) 30 min | 3.6 ± 0.3 | 0.91 ± 0.03 | 1,870 ± 240 |
| (*d*) 30 min + propranolol | 5.9 ± 0.5 | 0.86 ± 0.03 | 810 ± 190 |
| (*e*) 30 sec; 0–15 min at 23°C | 1.7 ± 0.6 | 0.20 ± 0.05 | ND |
| (*f*) 30 sec; 15–30 min at 23°C | 1.4 ± 0.2 | 0.64 ± 0.06 | ND |
| (*g*) 30 min; 0–15 min at 23°C | 4.9 ± 0.7 | 0.46 ± 0.07 | ND |
| (*h*) 30 min; 15–30 min at 23°C | 4.0 ± 1.0 | 0.79 ± 0.05 | ND |

Cells were incubated at 37°C with 16 μM (−)-isoproterenol for 30 min (*a–d*) or 5 min followed by 0–30 min at 23°C (*e–h*). After washing three times with Hepes buffer, the cells were labeled with 40 nM Alp-NBD for 30 sec at 23°C (*a, b, e, f*) or for 30 min at 4°C (*c, d, g, h*). The values in rows *e–h* were determined on samples incubated 0–15 min at 23°C (*e, g*) and 15–30 min at 23°C (*f, h*) after preincubation with isoproterenol for 5 min at 37°C. The $D$ and $R_f$ (specific + nonspecific) values were determined by FPR at 23°C. The conditions for fluorescence intensity measurements were as for Table 1. Data are given as mean ± SEM. Each entry represents measurements on 10–20 cells. ND, not determined.

The higher $R_f$ of the 30-min labeling probably reflects the higher lipid-like fluorescence under these conditions.

Isoproterenol did not alter the specificity of Alp-NBD labeling (Table 3), excluding the possibility that isoproterenol increased the proportion of Alp-NBD incorporated into the membrane bilayer. The labeling seems to be lower (Tables 1 and 3), possibly due to redistribution of staining towards a more diffuse pattern and not necessarily due to a reduction in the binding capacity.

The similar results for Alp-NBD labeling at 4°C and at 23°C indicate that cold exposure after isoproterenol treatment is not required for the effect. Moreover, cold exposure alone (30 min, 4°C) did not affect the lateral mobility or distribution of Alp-NBD on the cell surface.

The time dependence of the isoproterenol effect was investigated by using FPR. Five-minute preincubation with isoproterenol (16 μM, 37°C) did not affect the mobility or the distribution of Alp-NBD on the cells (data not shown). No change was observed for an additional 15 min at 23°C (Table 3). However, after 15–30 min at 23°C, the fluorescence became more diffuse, and the $R_f$ increased gradually (Table 3).

The lateral mobility of the lipid probe NBD-PtdEtn was measured to test whether isoproterenol affects membrane fluidity. The mean ± SEM values at 23°C of the $R_f$ (0.87 ± 0.08 and 0.86 ± 0.05) or $D$ (5.7 ± 1.2 × $10^{-9}$ and 5.3 ± 1.0 × $10^{-9}$ $cm^2/sec$) before and after preincubation with 16 μM (−)-isoproterenol (30 min, 37°C) were similar. This rules out increased lateral lipid mobility (detectable by NBD-PtdEtn) as the mechanism of the isoproterenol effect.

## DISCUSSION

The majority of the β receptors occupied by a fluorescent antagonist on Chang liver cells are immobile on the experimental time scale (Fig. 2, Table 2). This is consistent with the patchy distribution of antagonist–receptor complexes demonstrated by VIM (Fig. 1). The observation of patches after brief labeling (30 sec) at 4°C suggests that the β receptors are aggregated *prior* to antagonist binding. This suggestion is based on analogy with the inhibition of clustering of several membrane proteins at 4°C (22–24) and on the short labeling period.

Pretreatment (30 min, 37°C) with (−)-isoproterenol prior to Alp-NBD labeling induced a more homogeneous distribution of β receptors and increased the fraction of mobile receptors (Figs. 1 and 2; Table 3). The time course of β-receptor mobilization by isoproterenol (Table 3) does not correlate with the activation of adenylate cyclase via the β receptors. The latter is maximal in Chang cells at 37°C 2–3 min after addition of the β agonist; no effect on the mobility and distribution of Alp-NBD-labeled β receptors is seen in that time (Table 3). This indicates that macroscopic lateral mobility of the majority of the β receptors is not required for adenylate cyclase activation. Dispersal of receptors may, however, be related to desensitization, which follows a slower time course similar to that of the isoproterenol effect on β-receptor mobility. The effect of isoproterenol on β-receptor distribution is in accord with a previous report (25) that pretreatment of frog erythrocytes with isoproterenol alters the distribution of β-adrenergic receptors on subsequently prepared membrane fractions. Our results also agree largely with a recent study of the rotational mobility of propranolol bound to frog erythrocytes (26). The dispersal and mobilization of Alp-NBD fluorescence by isoproterenol supports our contention that a substantial fraction of Alp-NBD is bound specifically to immobile β receptors in the absence of agonist, because otherwise isoproterenol would have to mobilize Alp-NBD bound to aggregated immobile structures unrelated to β receptors. Because a small fraction of the β receptors can activate the entire pool of G proteins and catalytic units (3, 5, 27), and because the catalytic unit, the G protein, and the β receptors are physically separate entities (2, 6, 28), the activation of adenylate cyclase through the β receptor has been proposed to involve lateral diffusion of the receptors relative to the other components of the system (3, 6, 27). In view of the lateral immobility of the major portion of the β receptors, several possibilities should be considered: (*i*) The G protein may be freely mobile in the membrane, and thus can meet and be activated by the agonist-occupied β receptor. (*ii*) The G protein and the catalytic units may be sequestered in the same local regions on the cell surface as the β receptors. Then, lateral or rotational diffusion of receptors over submicrometer distances (not measured by FPR) may be required for activation, but not lateral diffusion over micrometer distances. Measurements of the mobilities of lectin receptors (29) and the apparent discrepancies between lateral and rotational mobilities of erythrocyte proteins (30) have been interpreted in similar terms. If this explanation is correct, mobilization of the β receptors, the G protein, or the catalytic units by treatment with Sendai virus or other fusing agents is required to explain the heterokaryon fusion experiments of Orly and Schramm (2). It is also possible that a small fraction of the receptors is laterally mobile and that this fraction is responsible for adenylate cyclase activation. This possibility seems less plausible, because then the majority of the receptors would never be biologically active.

In future work it will be important to characterize the mobility and distribution of G proteins and catalytic units as well as the causes of clustering and immobilization of the β receptors in the absence of agonist. Experiments with other cell types are required to show that the clustering and immobility detected in this study are not peculiar to Chang liver cells. The reports that antagonist-labeled β receptors on L84 myoblasts are in patches (31) and arguments against random distribution of β

receptors in vesicles prepared from S49 cell membranes (32) suggest that this phenomenon is not specific to Chang cells.

Our results point out an interesting contrast between $\beta$ receptors and the receptors for insulin, epidermal growth factor, and nerve growth factor. Whereas the $\beta$ receptors are preaggregated and slowly released by agonist, the latter are initially mobile, and their specific ligands induce their aggregation and internalization (33–35). Whether this contrast reflects fundamental differences in the mechanism of these hormones remains an open question.

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