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Electron Microscopic Localization of the β-Adrenergic Receptor Using a Ferritin-Alprenolol Probe

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SUMMARY

This report describes electron microscopic localization of the β-adrenergic receptor using a β-adrenergic receptor antagonist conjugated to ferritin. The conjugate was synthesized by reacting a carboxylic acid derivative of alprenolol with ferritin. The ferritin-alprenolol compound was shown to be effective in displacing specific [^{3}H]dihydroalprenolol binding from rat erythrocyte membranes with a dissociation constant (K_d) of 25 nM. Rat erythrocyte ghosts were incubated with the compound and quantitative electron micrographic analysis yielded total binding of 1367 ± 129 ferritin particles and nonspecific binding of 688 ± 111 (six experiments). Thus, specific binding was 680 ± 60 ferritin particles per red cell profile. Qualitative observations suggested that the particles were distributed randomly on the surface of the erythrocyte, although an occasional cluster was seen. A compound from another synthesis was shown be to effective in displacing specific [^{125}I]iodocyanopindolol binding from neonatal rat cardiac myocyte membranes, with a dissociation constant of 13.8 nM, whereas native alprenolol had a dissociation constant of 1.3 nM. Neonatal rat cardiac myocytes were incubated with the compound and processed for electron microscopy. Total binding along the sarcolemmal membrane was 504 ± 38 ferritin particles/100 μm of membrane and nonspecific binding was 301 ± 26 ferritin particles/100 μm of membrane (seven experiments), yielding specific binding of 203 ferritin particles/100 μm of membrane. In additional studies, specific binding was inhibited 95% with 10^{-5} M *l*-isoproterenol and 29% with *d*-isoproterenol, indicating stereoselectivity (seven experiments). The probe was distributed randomly along the sarcolemma with no preferential localization to coated pits or other membrane specializations. From measurements of the surface area of the average cardiac myocyte (732 μm^2), the specific binding of ferritin-alprenolol per 100 μm of membrane (203), and section thickness (0.08 μm), we calculated that cardiac myocytes had 18,575 β-adrenergic membrane receptor sites. Thus, we have described a method for synthesizing and applying an electron-dense probe for electron microscopic localization of β-adrenergic receptors. In these studies we determined the distribution of these receptors on rat erythrocyte ghosts and neonatal rat cardiac myocytes.

The β-adrenergic receptor is an important receptor that exerts numerous effects on the functions of several cell types by stimulating adenylate cyclase and thereby increasing cyclic AMP (1, 2). Therefore, the localization of β-adrenergic receptors is important for understanding the effects of adrenergic stimulation. The distribution of β-adrenergic receptors in different tissue components of various organs has been determined at the light microscopic level using autoradiography (3–5). This technique does not have the resolution to determine the localization of the β-receptor at the level of single cells. The subcellular localization of the β-receptor has been studied by some investigators using radioligand binding techniques in combination with differential centrifugation (6) or sucrose density gradients (7). In addition, the development of hydrophilic ligands such as CPG-12177, which bind only to receptors on the plasma membrane, has allowed investigators to study internal and external receptors (8, 9). Other investigators have studied the subcellular distribution of β-receptors using hydrophilic ligand binding to whole cells at either 4°, to label the plasma membrane receptors, or at 37°, to label the entire population of receptors (10). However, few attempts have been made to study the receptors at the cellular level using morphological techniques. Henis *et al.* (11) in 1982, using a fluorescent labeled probe, suggested that β-receptors were clustered and that binding with alprenolol caused the receptors to then be distributed randomly on the membrane surface. Strader *et al.* (12), using an antibody directed against the β-receptor and horseradish peroxidase as a label, localized the β-receptor on postsynaptic

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ABBREVIATIONS: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DHA, dihydroalprenolol; ICYP, iodocyanopindolol; LDL, low density lipoproteins.

membranes in the rat and frog cerebellum. However, horseradish peroxidase techniques do not have the resolution to determine the precise localization of the receptor along the membrane. The distribution and quantitative localization of β-adrenergic receptors on the plasma membrane have not been confirmed in cardiac myocytes, a cell in which β-receptor function is important for modulating contractile function.

The purposes of this study were to synthesize a ferritin-labeled probe that could bind to the β-adrenergic receptor and to use this probe to visualize the β-adrenergic receptor at the electron microscopic level in erythrocyte ghosts, as well as neonatal rat cardiac myocytes.

Methods

Synthesis

The procedures used in the preparation of a carboxylic acid derivative of alprenolol and its conjugation to ferritin by the scheme depicted in Fig. 1 are outlined below.

Dihydroalprenolol methylthioacetate (I). L-Alprenolol tartrate (Sigma Chemical Company, St. Louis, MO) was allowed to react with a 10% molar excess of methylthioglycolate (Aldrich Chemical Company, Milwaukee, WI) in the presence of a catalytic amount of potassium persulfate using 10% ethanol in water as solvent. The reaction mixture was gently refluxed under a nitrogen atmosphere for about 5 hr at 90°. The reaction mixture was made basic by the addition of 10% aqueous sodium carbonate. The basic mixture was extracted with ethyl acetate. The organic phase was washed with water, then brine, dried on $MgSO_4$ and filtered, and the solvent was removed *in vacuo* to furnish crude I as a thick oil. The product was used without purification.

Dihydroalprenolol thioacetic acid (II). The title compound was prepared by saponification of I using a slight excess of 2 N NaOH in a 2:1 mixture of dioxane/water. The reaction required several hours at room temperature, after which the dioxane was removed by rotary evaporation and the resulting aqueous residue was acidified to pH 3 with 1 N HCl. The acidic aqueous phase was extracted three times with methylene chloride. The combined organic phase was washed with water, then brine, and dried on $MgSO_4$. Filtration, removal of solvent, and drying *in vacuo* furnished II as an amorphous solid.

Dihydroalprenolol *N*-succinimidyl thioacetate (III). Compound II (400 mg of crude material) was dissolved in methylene chloride and a 1.1 molar equivalent amount of *N*-hydroxysuccinimide (Aldrich) was added. The mixture was cooled to 4° in an ice bath and a 1.5 molar equivalent amount of dicyclohexyl carbodiimide (Fluka, Hauppauge, New York) added as a solid. After 2 hr of stirring, the ice bath was removed, and the reaction mixture was allowed to come to room temperature for 2 hr. Solid dicyclohexylurea by-product was removed by filtration (Whatman no. 3) and the solvent was evaporated to give III as an oily residue, which was dissolved directly in dry dimethylformamide and used immediately for coupling to ferritin.

Ferritin conjugation. Compound III dissolved in dimethylformamide (200 μl) was reacted overnight at 8° with ferritin (EM grade; Polysciences, Warrington, PA) dissolved in 10 mM potassium phosphate, pH 7.0, in various molar ratios, respectively. The reaction mixture was filtered through a 0.45-μm syringe filter and dialyzed exhaustively against 10 mM potassium phosphate, pH 7.0. The conjugate was then subjected to gel filtration chromatography (Ultragel-AcA 34; LKB, Gaithersburg, MD). In the first preparation of the compound (preparation I), the substitution was approximately 1 mol of alprenolol/1 mol of ferritin. In the second preparation (preparation II), the substitution was 11 mol of alprenolol/mol of ferritin as determined by radioimmunoassay analysis of the coupled alprenolol conjugate (13).

Validation of Binding

Preparation I was used in studying the β-receptors in the rat erythrocyte membranes. The rat erythrocyte was chosen because properties of β-receptor binding have been well characterized in this cell (14) and because this cell type could be easily manipulated for electron microscopy. β-Receptors were analyzed on rat erythrocyte membranes according to the methods of Fleming and Ross (14), who characterized [^{3}H]DHA binding in this model. Male Sprague Dawley rats were exsanguinated using cardiac puncture and the blood was placed in an equal volume of a buffer consisting of 20 mM Tris·HCl, 10 mM EDTA, 2 mM EGTA, and 140 mM NaCl (pH 7.5). Erythrocytes were sedimented in a table top centrifuge and washed one time with the buffer. The packed erythrocytes were lysed by suspension in 20 mM Na·HEPES, 2 mM EDTA, 0.1 mM PMSF. The PMSF concentration was then adjusted to 0.5 mM by the addition of 0.5 M PMSF in dimethylsulfoxide and the membranes were washed six times with the HEPES buffer. Experiments were done to determine whether the ferritin-alprenolol compound could displace [^{3}H]DHA binding. The membranes were incubated in 20 mM Tris·HCl (pH 7.5), 0.1 mM ascorbic acid, 1 mM EDTA, and 11 mM $MgCl_2$ in the presence of several concentrations of

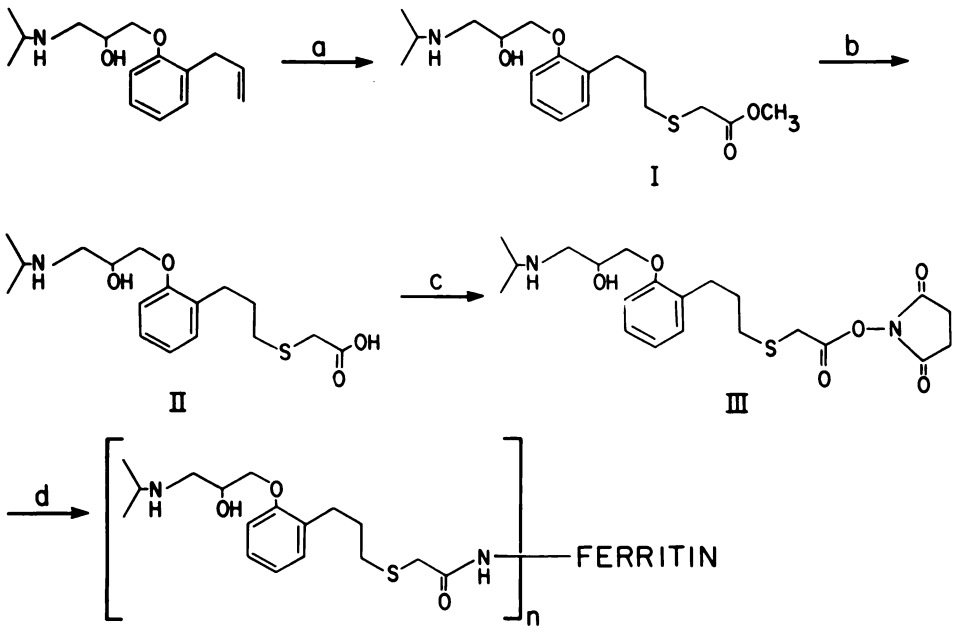

Fig. 1. This figure outlines the synthesis of the ferritin-alprenolol compound, as described in more detail in Methods.

alprenolol or ferritin-alprenolol. Saturation experiments determined that the dissociation constant (K_d) of [^{3}H]DHA was approximately 1 nM. The membranes were incubated in approximately 1 nM [^{3}H]DHA in the presence of several concentrations of the ferritin-alprenolol compound (preparation I) to determine whether the compound could displace specific [^{3}H]DHA binding.

Additional studies were performed in cardiac myocytes, a cell type in which β-adrenergic receptor stimulation modulates contractile function. Neonatal rat cardiac myocytes were prepared according to a modification of the techniques of Harary and Farley (15) as previously described (16). Briefly, the hearts of 2- to 3-day-old rats were removed and the ventricles were digested using a combination of collagenase and pancreatin. A 3-hr differential attachment period was used to separate the myocytes from the fibroblasts. The unattached cells were then plated in tissue culture dishes at a concentration of 1.5×10^6 cells/60 mm plate for the electron microscopic studies or at a concentration of 4×10^6 cells/10 cm plate for the biochemical studies. The cells were grown in 68% Dulbecco's modified Eagles medium with 18 mM glucose, 17% medium 199 with Earle's salts, 10% horse serum, 5% fetal calf serum, and antibiotics (penicillin, 100,000 Units/ml; streptomycin 10,000 μg/ml).

Initial experiments were done to determine whether the ferritin-labeled compounds could displace [^{125}I]ICYP (New England Nuclear, Boston, MA) in membranes prepared from the cardiac myocytes. For these studies preparation II of the ferritin-alprenolol was used. ICYP, an iodinated compound with a higher specific activity than dihydroalprenolol, was used because of the small amount of protein available from the cell homogenates. The binding of the [^{125}I]ICYP to the rat cardiac membranes has been previously characterized in our laboratory (16).

The cells were washed and membranes were prepared according to our previously described methods (16). Membranes were incubated in the presence of approximately 40 pM [^{125}I]cyanopindolol with four concentrations of the ferritin-alprenolol preparation. The dissociation constant of the ferritin-alprenolol preparations was calculated using the Cheng and Prusoff equation (17), using a K_d of 30 pM for [^{125}I] ICYP as determined by Scatchard analysis.

Electron Microscopic Localization

For the localization studies on the rat erythrocyte membranes, blood was removed as described for the membrane preparation and washed four times in the 20 mM Tris·HCl, 10 mM EDTA, 2 mM EGTA, 140 mM NaCl (pH 7.5). At all times the blood was kept at 2°. After the final centrifugation, the red cells were suspended in this buffer to make approximately a 50% suspension of red cells. The cells were then lysed on distilled water using the methods of Nicolson and Singer (18), in which some of the lysed cells spread out on the air-water interface. The red cell ghosts were then picked up on Parlodion-coated nickel grids that had been coated with a thin layer of carbon. The ghosts were then incubated in preparation I of the ferritin-alprenolol (25 nM ferritin-alprenolol) without (total binding) and with (nonspecific binding) 10^{-6} M *dl*-propranolol (Sigma) in 20 mM Tris (pH 7.5), 11 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ascorbic acid for 1 hr at room temperature. The grids were rinsed six times in cold buffer and then were rinsed quickly in cold deionized water and dried. The grids were examined in a 100C JEOL electron microscope using an accelerating voltage of 80 kV. Only cells that appeared intact and round were selected and placed in the center of the screen. At this magnification no ferritin particles could be seen, so the observer was not biased as to site selection for photography. The micrograph was taken at 5000× for determination of the area of the red cell profile. The magnification was then increased to 26,000× and a micrograph was taken of the ferritin particles. A calibration grid was included with each group of negatives taken. For each experiment, 10–20 ghosts were analyzed per treatment group. The negatives were processed and placed on a light box with a Nikon Macrolens, which was attached to a Nikon Magiscan image analysis system. From the negatives of the red cell ghosts, the area of the cell profile was measured, and in the higher magnification pictures the ferritin particles were counted on the entire negative. The number of particles per red cell profile was then calculated.

For the localization studies in the neonatal rat cardiac myocytes, myocytes were prepared as described above and cultured in 60-mm dishes plated at a density of 1.5×10^6 cells per plate. The cells were allowed to grow for 3 days in an incubator and then rinsed two times with 3 ml of ice-cold HEPES-buffered balanced salt solution (pH 7.4). Cold buffer was used to prevent the receptors from moving laterally in the membrane (19). The dishes were then incubated in 34 nM preparation II of the ferritin-alprenolol compound in the HEPES buffer in the absence of (total binding) and in the presence of (nonspecific) of 10^{-5} M *l*-alprenolol. Some dishes were incubated in ferritin not labeled with alprenolol at the same concentration of ferritin as was used in the incubations with the ferritin-alprenolol compound (17 μg/ml). This was done to determine the amount of nonspecific binding of ferritin alone. The incubation was done at 4° for 6 hr. After the incubation, the plates were rinsed three times with 3 ml of cold HEPES-buffered salt solution. The monolayer of cells was then fixed with 3 ml of cold 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). In addition to fixing the tissue, this step should cross-link the ferritin to the membrane, to prevent loss of the label during processing for electron microscopy. Glutaraldehyde cross-links proteins, and both intra- and intermolecular cross-links are formed. For a review of the biochemistry of the reaction of glutaraldehyde with proteins, see Hayat (20). The cells were fixed for at least 14 hr in the cold and then rinsed with phosphate buffer and post-fixed in 1% osmium in 0.1 M phosphate buffer (pH 7.3) for 30 min. The cells were then dehydrated with ethanol and embedded in Epon 812.

After the plastic hardened, the thin layer of plastic containing the cells was pulled off the plate and strips of cells were re-embedded in plastic and sectioned at approximately 80 nm in thickness. The sections were cut so that the cells could be viewed on edge. The sections were placed on copper grids and stained for 3 min with 2% uranyl acetate. Lead citrate was not used because this obscured the ferritin particles. The cells were viewed on a JEOL 100C electron microscope at 26,000× magnification, and negatives were taken along the entire length of the monolayer of cells present in the section. Only the top layer of the membrane was analyzed, and only cells that had myofibrils and the large mitochondria associated with cardiac myocytes were analyzed. Each time a series of negatives of the cells was taken, an additional negative was taken of a calibration grid (57,600 lines per inch; Ernest Fullum, Latham, NY) at the same magnification. The negatives were then placed on a light box and the image was projected to a Nikon Magiscan Image Analyzer with a Macrolens. The length of the membrane was measured with the image analyzer and the number of ferritin particles was counted. Calibration of the system was done with the negative of the calibration grid. Two blocks were analyzed from each culture dish and the results were pooled. In addition to the ferritin particles, coated pits were quantified along the entire length of the membrane analyzed. Only areas that were indented with a thickened plasma membrane were counted as coated pits (21).

In additional experiments, cells were incubated with the ferritin-alprenolol in the absence or in the presence of 10^{-5} M *l*-isoproterenol bitartrate, 10^{-5} M *d*-isoproterenol bitartrate, or 10^{-5} M alprenolol tartrate ($n = 7$ plates in each group). These experiments were done exactly like the previous experiments except that 10^{-5} M ascorbic acid was included to prevent oxidation of the isoproterenol.

Determination of the Surface Area of the Cardiac Myocytes

To calculate the number of ferritin-alprenolol binding sites per cell, it was necessary to determine the surface area of the cardiac myocytes. For these experiments, myocytes were grown in culture dishes as previously described and were washed with balanced salt solution. A trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA in balanced salt solution) was placed on the cells, and the culture dish was placed in the incubator for 5 min. The plates were rocked gently for an additional

5 min and the cells were gently pipetted several times through a Pasteur pipet. The detached cell suspension was added to 4 ml of ice-cold neonatal calf serum to stop the action of the trypsin on the cells, and the cells were spun down and resuspended in balanced salt solution. A drop of cells was placed on a siliconized slide and a siliconized coverslip was placed on top. The slide was immediately placed on a Nikon microscope and the area of the cells was determined using an image analyzer. Only cells that appeared round and were completely isolated were analyzed. From the area, the average radius of the cells was determined, and the surface area of the cells was determined using the formula for the surface area of a sphere (surface area = 12.57 r^2).

Statistical Analyses

To determine whether there was significant specific binding of the ferritin-alprenolol, total binding was compared with nonspecific binding using Student's t test. To determine stereoselectivity, the ferritin counts obtained from the cells incubated with the ferritin-alprenolol in the presence of 10^{-5} M 1-isoproterenol were compared with the ferritin counts obtained from cultures incubated with 10^{-5} M d-isoproterenol, using Student's t test. A p value of less than 0.05 was considered to be significant. Data are expressed as the mean ± standard error.

Results

Synthesis. A carboxylic acid derivative of alprenolol was synthesized by the scheme depicted in Fig. 1. The key step involved free radical-initiated addition of the thiol group of methylthioglycolate to the double bond of the allyl substituent of alprenolol. A similar procedure has been used to attach alprenolol to a thiolated derivative of Sepharose (22). After saponification of the resulting methyl ester derivative (**I**), the free acid (**II**) was converted to the N-hydroxysuccinimide mediated condensation. The active ester (**III**), in excess, was reacted with "available" amino groups (presumably from lysine residues) of ferritin to give the desired conjugates. The substitution achieved for the conjugates depended on the molar excess of N-hydroxysuccinimide ester to ferritin used in the reaction.

Validation of binding. In competition experiments, binding of the ferritin-alprenolol (preparation I) to rat erythrocyte membranes demonstrated a K_d of 25 nM (Fig. 2). Unlabeled alprenolol had a K_d of 0.93 nM, as seen in this figure. Thus, the ferritin-alprenolol was approximately 25 times less effective than unlabeled alprenolol at displacing specific [^{3}H]DHA binding from erythrocyte membranes. For the erythrocyte ghost electron microscopic experiments, a concentration of 25 nM alprenolol of the ferritin-alprenolol was used. In the studies using the rat neonatal cardiac myocytes, the dissociation constant of the ferritin-alprenolol (preparation II) was 13.8 nM (n = 3). This was approximately 10 times less effective than free alprenolol, which had a dissociation constant of 1.3 nM. For the electron microscopic studies with the myocytes, a concentration of 34 nM ferritin-alprenolol was used, with the goal of obtaining occupancy of most of the available receptors.

Electron microscopic localization. Fig. 3 is a low magnification of the profile of an erythrocyte ghost. The cells used for the analysis were round, smooth, and flat. Cells that appeared torn or that were in clumps were not used in the analysis. Table 1 demonstrates the results obtained from six experiments done with the erythrocyte ghosts. As seen from the quantification, there were 1367 ± 129 particles of total binding per red cell profile and 688 ± 111 particles of nonspecific binding per red cell profile, with 680 ± 60 ferritin particles of specific binding per red cell profile. There was a significant difference between total and nonspecific binding ($p < 0.001$).

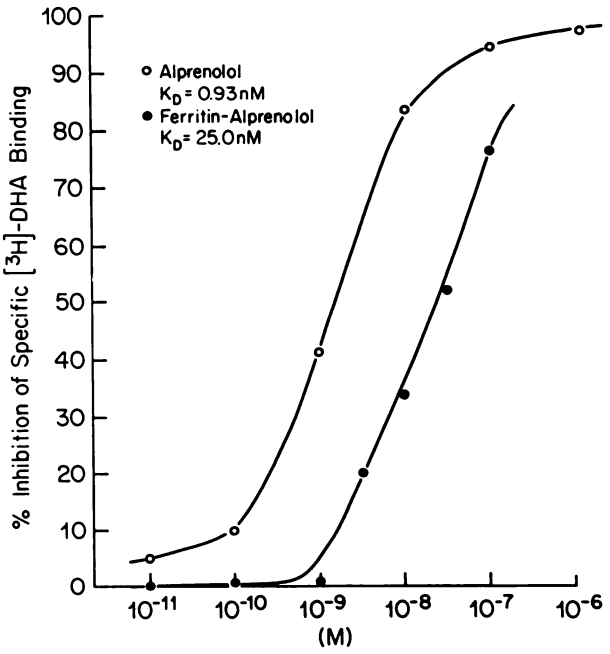

Fig. 2. This figure demonstrates the per cent inhibition of specific [^{3}H]DHA binding to rat erythrocyte membranes by alprenolol and ferritin-alprenolol. The concentration of [^{3}H]DHA used in the assay was approximately 1 nM and nonspecific binding was determined using 10^{-6} M *dl*-propranolol. Each *point* is the average of two or three separate experiments, each done in duplicate.

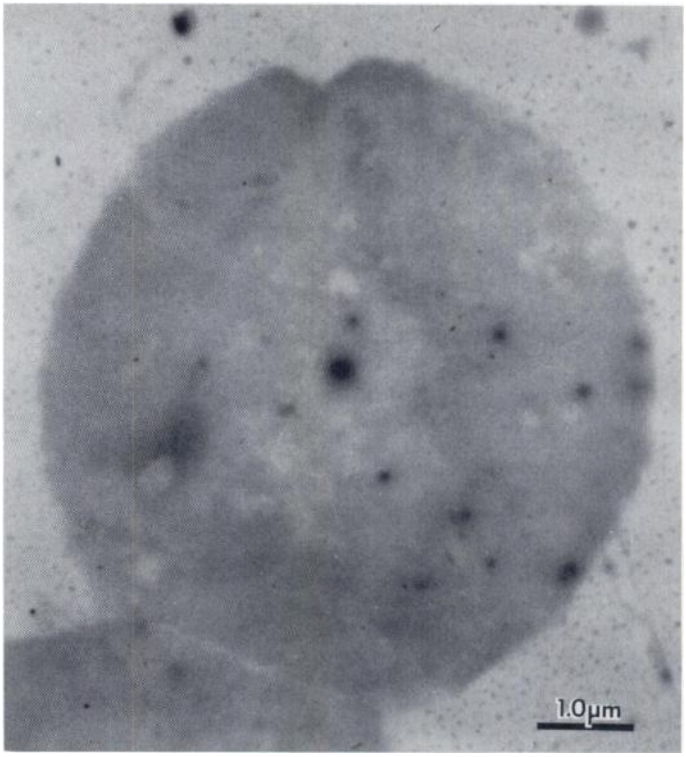

Fig. 3. This is a low magnification of the red cell ghosts used in the electron microscopic analyses of ferritin-alprenolol binding. Only ghosts that were round and not in clumps were used for the analysis.

TABLE 1

Ferritin particles on rat erythrocyte ghosts

Data are expressed as mean grains per red blood cell profile ± standard error. The numbers in parentheses are the total number of red blood cell profiles analyzed in that treatment group.

Exp	Total	Nonspecific	Specific
1	850 ± 102 (14)	342 ± 39 (17)	508
2	1306 ± 182 (21)	462 ± 84 (17)	844
3	1277 ± 186 (14)	629 ± 84 (17)	648
4	1804 ± 472 (18)	1042 ± 176 (12)	762
5	1505 ± 160 (8)	698 ± 82 (10)	807
6	1462 ± 246 (10)	954 ± 95 (14)	508
Mean	1367 ± 129[a]	688 ± 60	680 ± 60

[a] Significantly higher than nonspecific binding ($p < 0.001$) by Student's t test.

Qualitative observations revealed that there did not seem to be any difference in the distribution of the ferritin particles when comparing total binding with nonspecific binding (Fig. 4). Although occasional clusters were seen, these were also observed in the nonspecific binding group.

In the rat cardiac myocyte cultures that were used for the ferritin analysis, cardiac myocytes were readily identified by the presence of myofibrils and mitochondria characteristic of these cells (Fig. 5). These cultures are approximately 80% myocytes (16). The fibroblasts are very easy to distinguish from the myocytes, in that they have no organized myofibrillar structure, and they have abundant endoplasmic reticulum and large nuclei. Non-myocytes were not included in any of the analyses.

The ferritin particles were distributed along the sarcolemma of the cardiac myocytes usually as single particles, and occasionally in small clusters of two to six particles (Fig. 6). The occasional small clusters of ferritin were present in both the total and nonspecific binding preparations and probably represented slight clustering of the ferritin particles in the compound. The ferritin-alprenolol was not localized in coated pits or other membrane specializations, as seen in Fig. 6. There were no large clusters of the ferritin particles along the membrane.

To determine whether there was significant specific binding to the sarcolemma, quantification was done as described in Methods. Fourteen plates from three separate cultures were analyzed, 7 plates for total binding and 7 plates for nonspecific binding. The data are summarized in Table 2. Approximately the same length of membrane was analyzed in both the total binding and nonspecific binding categories. As seen in Table 2, there was a significant difference ($p < 0.001$) between the total (504 ± 38 particles per 100 μm of membrane) and nonspecific binding (301 ± 26 particles per 100 μm of membrane). Specific binding, calculated as total minus nonspecific binding, was 203 ferritin-alprenolol particles per 100 μm of membrane. Two plates were incubated with unlabeled ferritin at the same concentration of ferritin used in the studies in which cultures were incubated with ferritin-alprenolol. Ferritin could be seen along the membrane with occasional small clusters, as seen with the ferritin-alprenolol. In these studies, 54.68 μm of membrane was analyzed and quantification determined that there were 209 ferritin particles/100 μm of membrane in the plates incubated

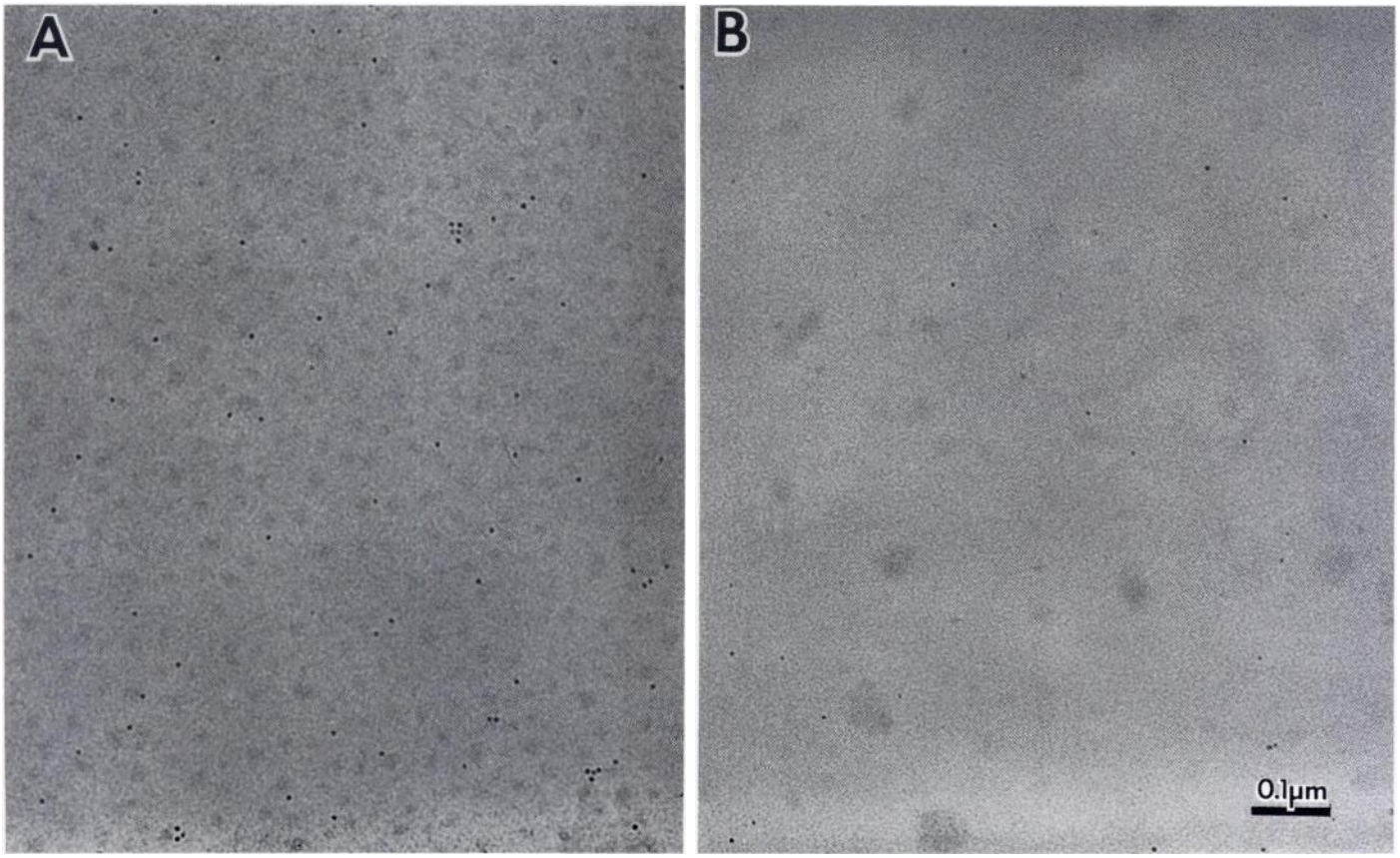

Fig. 4. This is a high magnification of the red cell ghosts, demonstrating the ferritin particles binding to the surface of the cell. A, Total binding of the compound to the erythrocyte ghosts. Although an occasional small cluster was seen, these were also seen in the nonspecific binding. B, Nonspecific binding, as defined by 10^{-6} M propranolol.

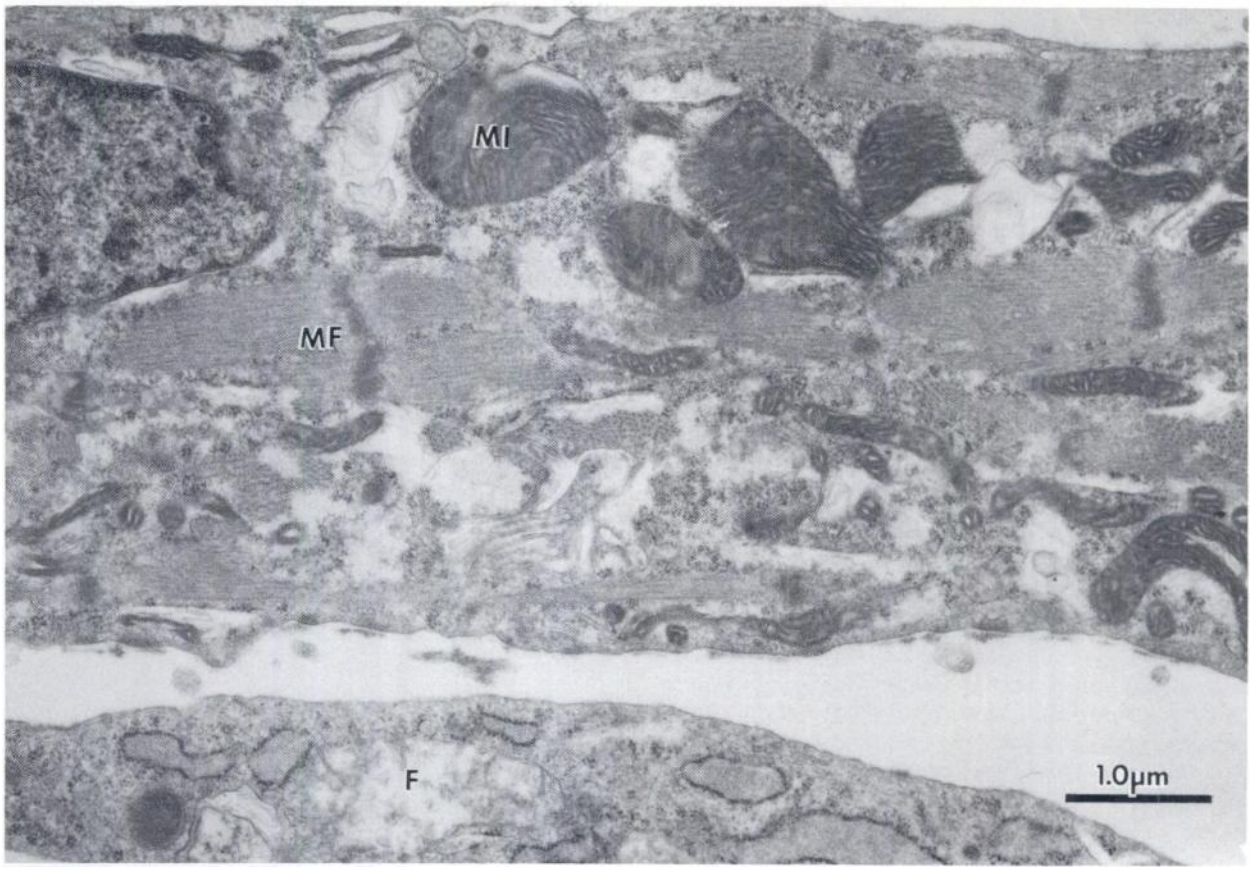

Fig. 5. This is an electron micrograph of the sections of the cardiac myocyte cultures that were used for the ferritin analysis. At this magnification, the ferritin cannot be seen. The cells used in the analysis had myofibrils (*MF*) and the large mitochondria (*MI*) characteristic of neonatal rat cardiac myocytes. The cultures are approximately 80% myocytes, and fibroblasts (*F*), which are easily recognizable by lack of myofibrils, prominent endoplasmic reticulum, and large nuclei, were excluded from the analysis.

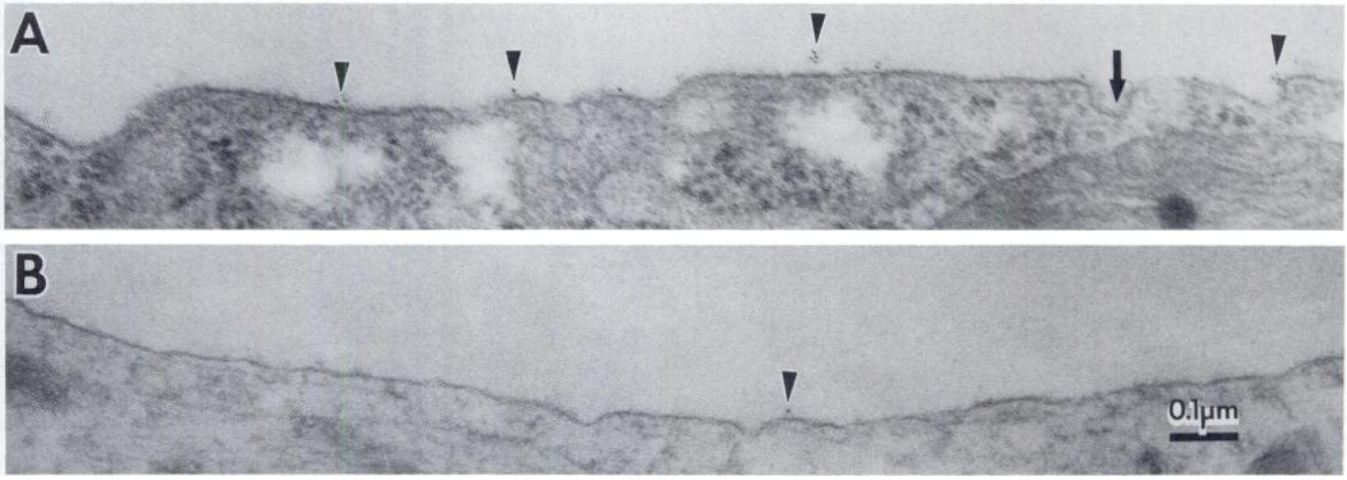

Fig. 6. This figure shows electron micrographs of myocardial cells incubated in ferritin-alprenolol and processed for electron microscopy as described in Methods. A, Myocardial cells incubated in preparation II of the ferritin-alprenolol. Notice the presence of electron-dense particles along the membrane (*pointers*). There is no particular concentration of particles to membrane specializations (*arrows*). The particles appear as single ferritin particles or as small clusters. B, Myocardial cells incubated with the ferritin-alprenolol in the presence of 10^{-5} M alprenolol (nonspecific binding).

with ferritin alone. When this binding was subtracted, the ferritin-alprenolol compound had a per cent specific binding of 81%.

In the experiments designed to determine whether the binding was stereoselective, Student's *t* tests determined that there was a significant difference between the ferritin particles counted in the presence of 10^{-5} M *l*-isoproterenol and *d*-isoproterenol ($p < 0.01$). The overall per cent inhibition with the *l*-isomer was 95 ± 4% whereas the *d*-isomer inhibited specific binding 29 ± 12%. Thus, stereoselectivity was demonstrated in these experiments (seven experiments).

To calculate the number of sites per cell, the surface area of an average cardiac myocyte had to be determined. Cells that had been in culture for 3 days were detached from the plates

TABLE 2
Binding of ferritin-alprenolol to neonatal rat cardiac myocytes

Binding	Total membrane analyzed	Total no. of ferritin particles	Average membrane analyzed/plate	No. of ferritin particles/100 μm of membrane
	μm			
Total	370.86	1813	53 ± 5	504 ± 38[a]
Nonspecific	333.60	995	48 ± 4	301 ± 26

[a] Mean ± standard error. Total binding was significantly different from nonspecific binding ($p < 0.001$) by Student's t test. The calculated specific binding was 203 ferritin-alprenolol particles per 100 μm of membrane. $n = 7$ plates/group.

as described in Methods and the area of the cells was quantified on the image analyzer. Most of the cells were round, but there were some that were oddly shaped. Only the round cells were measured. In addition, groups of cells were seen in which the trypsin had not completely dispersed the cells. These cells were not analyzed because often the entire cell could not be seen. Fifty cells that met the above criteria were analyzed. The cells were quite variable in size, ranging in area from 35.5 μm^2 to 412 μm^2; the average area of the 50 cells was 182.9 + 7.2 μm^2. The average area per cell (182.9 μm^2) converted to an average radius of 7.63 μm. Using the formula for the surface area of a sphere, it was determined that the surface area of an average cardiac myocyte was 732.2 μm^2 and the volume of an average cardiac myocyte was 1861 μm^3. Because the cardiac myocytes had 206 specifically bound ferritin particles per 100 μm of membrane and the sections were approximately 0.08 μm in thickness (as estimated by interference colors) the myocytes had 203 particles per 8 μm^2 of membrane. Thus, the calculated number of specifically bound ferritin particles per cell is 18,575.

Quantification of coated pits. The myocardial cells had numerous coated pits. In this study, we counted 36 coated pits on 225.7 μm of membrane in the group of cells used for total binding measurements, or 16 coated pits per 100 μm of cell membrane. In the group of cells used for nonspecific binding, we counted 39 coated pits on 209.64 μm of membrane, or 19 coated pits per 100 μm of membrane. With an average of 18 coated pits per μm of membrane, and a section thickness of 0.08 μm, the cardiac myocytes have a total of 1647 coated pits per cell.

Discussion

This study provides a demonstration of the electron microscopic localization of the β-receptor in two model systems, the rat erythrocyte membrane and the cardiac myocyte plasma membrane. For this purpose we used a carboxylic acid derivative of alprenolol conjugated to the ferritin particle. Although several other investigators have used ferritin-coupled ligands to study cell surface receptors, the ligands used in these other studies were much larger than the ligands that bind to the β-receptor (21, 23). Thus, this method of coupling a small ligand to the large ferritin molecule and retaining binding activity may be useful in studying other receptors that bind small ligands. The ferritin-alprenolol compound displaced specifically bound [^{3}H]DHA in rat erythrocyte membranes, with a dissociation constant of 25 nM, and displaced specifically bound [^{125}I]ICYP in myocyte membranes with a dissociation constant of 13.8 nM. The difference in the dissociation constant between the two preparations may be related to the fact that in the preparation used with the erythrocytes the molar ratio of alprenolol to ferritin was 1:1, whereas in the preparation used with the myocytes the ratio of alprenolol to ferritin was 11:1.

Specific binding was found to rat erythrocytes membranes. No significant clustering of the particles was apparent in this model. It has been shown that, as rat reticulocytes mature to erythrocytes, there is a loss of coupling of the β-receptor to adenylate cyclase (24). Because we did not study reticulocytes, it is unclear whether the distribution of the receptor is different in the reticulocyte, but these probes could provide an answer to this question.

In studying the localization of the receptor along the plasma membrane of cardiac myocytes, it was evident that, in contrast to the LDL receptor (21), the β-adrenergic receptor was not localized to coated pits or other membrane specializations such as caveolae. It is possible that the large ferritin-labeled ligand was not accessible to coated pits, but Anderson et al. (21), in their original work on LDL receptors, did localize ferritin-LDL to coated pits using similar techniques. Although small clusters of ferritin were seen in the total binding preparations, these were also seen in the nonspecific binding preparations, as well as in the cells incubated with ferritin alone. These clusters probably represented microaggregation of the ferritin-alprenolol compound during storage. No large clusters of particles were seen along the membrane.

Cell surface receptors, at least receptors for polypeptides, can be divided into two types (25). Activation of the first type of receptor leads to changes in cell behavior or metabolism. These changes result from the interaction between the ligand and the receptor and, although internalization may occur, it is not necessary for hormone action. The second type of receptor mediates ligand internalization and an example of this type of receptor would be the LDL receptor (26). Thus, it would not be surprising that the β-receptor, which logically would fit into the type I category, would not be distributed along the membrane like the LDL receptor, a type II receptor. Studies of type I receptors for molecules such as epidermal growth factor and insulin (27, 28), as well as luteinizing hormone (23), have determined that these receptors are distributed randomly on the membrane, and with ligand binding they aggregate and then are internalized through coated pits. In contrast, Henis *et al.* (11), using the binding of a fluorescent β-receptor antagonist to whole cells, reported that β-receptors are clustered and that binding with isoproterenol causes the receptors to then be distributed randomly along the membrane. It is possible that, with our electron microscopic techniques, we do not see enough of the membrane to detect large clusters and that light microscopic studies in whole cells may provide additional information on the distribution of the β-receptor. However, it is also possible that our electron microscopic studies have defined the distribution of the β-receptor more accurately.

The nonspecific binding seen in this study was predominately due to a nonspecific binding of ferritin to the cardiac myocyte membrane. The binding of ferritin alone accounted for 70% of the nonspecific binding to the membrane. It is unclear why the ferritin bound to the cardiac myocyte membrane, as others have not reported ferritin binding in fibroblasts or in luteal cells (21, 23), but this may be related to the thick glycocalyx present in the cardiac myocyte. Recently, we have done experiments using bovine serum albumin to reduce nonspecific binding of ferritin. These studies suggest that a significant component of this nonspecific binding can be eliminated.

Strong evidence that the ferritin-alprenolol conjugates defined the β-receptor rather than nonspecific sites was obtained from several lines of evidence. First, the ferritin-alprenolol compounds displaced specific [^{3}H]DHA binding from rat erythrocyte membranes and [^{125}I]ICYP binding from neonatal rat cardiac myocytes, and this displacement was dose dependent. In addition, there was a significant difference between total and nonspecific binding as analyzed by Student's *t* test in both the rat erythrocyte ghosts and the neonatal rat cardiac myocytes. When the sites per cell were calculated in the cardiac myocytes by estimating the area of the myocardial cells, there was reasonable agreement between the number obtained morphologically (18,575) and the numbers obtained biochemically (16) (approximately 13,000–14,000 sites per cell). Stereoselectivity studies, which determined that *l*-isoproterenol was more effective in displacing the label than was *d*-isoproterenol, provided additional evidence that this probe is indeed binding to the β-adrenergic receptor. Thus, we have synthesized an agent that can be used to localize the β-adrenergic receptor in rat neonatal cardiac myocytes with very high resolution. The experiments in these studies were designed to examine the localization of external receptors and not the localization of internal receptors. The probe could possibly be used in conjunction with ultrathin frozen sections to examine the location of internal pools of receptors. In addition, the β-receptor probe should prove useful in analyzing the distribution of the β-receptor under various physiological and pathological states.

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