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ADRENERGIC RECEPTOR CHARACTERISTICS OF CARDIAC MYOCYTES CULTURED IN SERUM-FREE MEDIUM: COMPARISON WITH SERUM-SUPPLEMENTED MEDIUM*

Joel S. Karliner, Paul C.Simpson, John E. Taylor, Norman Honbo and Wanda Woloszyn

Cardiology Section, Veterans Administration Medical Center, San Francisco, California

Department of Medicine and the Cardiovascular Research Institute, University of California, San Francisco, California

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Neonatal rat ventricular myocytes cultured in serum-free medium coexpress both α_1 and β_1 receptors as determined by radioligand binding studies. In cells exposed to serum for 48 hr surface area increased 3.69 fold, but the maximum number of binding sites ([^{125}I]-iodocynanopindolol) only increased 1.5 fold from 12956 ± 7579 to 19676 ± 5181 sites/cell (n=5, $p<.05$) yielding a value of 2.48 sites/um^2 for cells grown in serum-supplemented medium compared with 6.96 sites/um^2 for cells grown in serum-free medium. Thus serum-induced hypertrophy is associated with a decrease in β_1 receptor density relative to cell size; however, adenylate cyclase response is unaffected. This cell culture system constitutes an excellent model for studying interventions that may influence the regulation of cardiac myocyte hypertrophy by nonhemodynamic factors, particularly through the adrenergic receptor system.

Previous studies from this laboratory have indicated that serum-supplemented primary cultures from neonatal rat myocardial cells are well differentiated (1,2) and undergo hypertrophy over time in culture (2,3). As cells grown in serum-free medium constitute a potential ideal model system for the assessment of myocardial growth factors and other modulators of cardiac cell function, including hormones and neurotransmitters (2,3), we here report the β_1-adrenergic characteristics of primary cultures of highly purified neonatal rat ventricular myocytes cultured in serum-free medium and compare the information obtained to that from cells grown in serum-supplemented medium. Preliminary data regarding α_1-adrenergic receptors are also presented.

MATERIALS AND METHODS

Cell Culture and Size

Cultures were composed of single, isolated cells prepared from hearts of day-old rats as described previously (1-3). In summary, cells were obtained by brief, alternating cycles of room-temperature trypsinization and mechanical disaggregation. Cells were combined, washed, and preplated in the presence of 5% fetal bovine serum to reduce the number of contaminating

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mesenchymal non-myocardial cells. After 30 minutes the still-suspended myocardial cells were removed from the attached non-myocardial cells, counted (hemocytometer), and diluted to 260,000 viable (trypan blue-negative) cells per ml in culture medium with 5% fetal bovine serum. This cell suspension was distributed into 100mm culture dishes at a plating density of approximately 550 cells per mm^2. On culture day 1, after overnight attachment, those cultures designated to contain serum-free medium were given 8ml of a serum-free medium containing 10ug per ml insulin and 10ug per ml transferrin. Medium was routinely changed on day 4.

The standard serum-free culture medium was Gibco medium 199 (Hanks salts) supplemented with 1.5uM vitamin B_{12} and 50 U per ml penicillin. The medium through culture day 3 also contained 0.1 mM bromodeoxyuridine to prevent low-level non-myocardial cell proliferation, as previously described (1-3).

Cell yield was 3-5 million per heart, of which over 90% were viable. All cultures were kept at 37°C in humidified air with sufficient CO_2 (about 1%) to maintain pH 7.3. The cultures contained >90% myocardial cells and cell numbers were constant over time, as determined by counting cells in the dishes (1-3). Cell size was quantified by measurement of surface area in at least 200 myocardial cells (2), which was determined using a BioQuant (R&M Industries) image analysis system.

Radioligand Binding Studies

Radioligand binding studies were carried out 5 to 8 days after plating. For particulate preparations, cells were lysed in situ in 1 mM Tris buffer for 45 minutes at 4°C. Dish contents were centrifuged at 40,000 x g for 30 min. The pellet was resuspended in 50mM Tris-HCl buffer, pH 7.4, at a concentration of 0.5mg protein/ml after filtration through nylon gauze. Protein was determined by the method of Lowry et al. using bovine serum albumin as standard (4). Initially, the membrane preparations were incubated with [^{3}H]-dihydroalprenolol (DHA) as previously described (5). Additional radioligand binding studies were performed in whole cell preparations. For β_1-adrenergic receptor analysis [^{125}I]-iodocyanopindolol (ICYP) was used. For assessment of α_1-adrenergic receptors, the selective α_1-adrenergic antagonist [^{125}I]-I-2-[β-(4-hydroxyphenyl)ethylaminomethyl] tetralone (^{125}I-IBE-2254) was utilized, as described by Kupfer et al (6). All radioligand binding studies were carried out in triplicate. Analysis of saturation binding isotherms was performed according to the method of Scatchard (7). (-)-Isoproterenol competition curves were analyzed using the iterative curve fitting program "LIGAND" of Munson and Rodbard (8). Adenylate cyclase activity was determined by the method of Salomon et al (9).

RESULTS

Characterization of the β_1 Receptor

Initial studies in cells grown in serum-supplemented medium indicated that the β-adenergic receptor was characteristic of the β_1 subtype as previously reported (10). Agonist competition curves gave a rank order consistent with a β_1-adrenergic receptor(11); cells grown in serum-free medium exhibited a similar response (Fig 1A). In membranes prepared from cells grown both in serum-supplemented and in serum-free medium a rank order consistent with a β_1-adrenergic receptor subtype was also observed with (-)-isoproterenol (EC_{50}=5uM, n=3, data not shown) being more potent in stimulating adenylate cyclase activity than (-)-norepinephrine and (-)-epinephrine, which exhibited equivalent activity (EC_{50}=80uM, n=3). Stereoselectivity was demonstrated with (-)-propranolol (IC_{50}=4.58nM) being 124 times more potent than (+)-propranolol (IC_{50}=569 nM) in inhibiting [^{3}H]-DHA binding to these myocyte membranes (n=3, data not shown).

In 5 separate experiments in membranes from cells grown in serum-free medium, adenylate cyclase activity increased after a 30 min exposure to 1uM (-)-isoproterenol from 153±78 to 365±186 pmol/30min/mg protein ($p<.05$). This was 24% of maximal activity as determined by 10mM

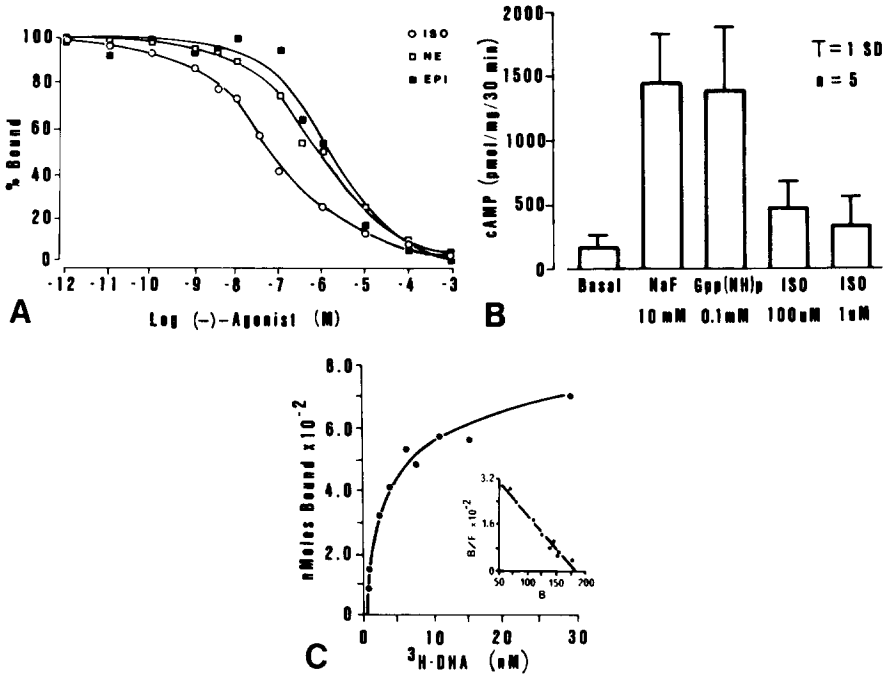


Figure 1A. Agonist competition curves showing a rank order consistent with a beta$_1$-adrenergic receptor. The data were obtained in membrane preparations prepared from neonatal rat ventricular myocytes grown in serum-free medium. Each point is the average of 3 separate experiments, each performed in triplicate.

Figure 1B. (-)-Isoproterenol stimulated adenylate cyclase actvity in membranes prepared from neonatal rat ventricular myocytes grown in serum-free medium. Isoproterenol produces a 2.5-3-fold stimulation of adenylate cyclase activity which is 24% of maximal activity stimulated by NaF. Results obtained in membranes prepared from cells grown in serum-supplemented medium were not different.

Figure 1C. A typical equilibrium binding isotherm using [^{3}H]-DHA as the radioligand. Membranes were prepared from neonatal rat ventricular myocytes. A plot of specific binding is shown. The insert depicts Scatchard analysis of these data (Kd=199nM; Bmax=182 fmol/mg protein).

NaF stimulated activity and 26% of activity stimulated by 0.1 mM Gpp(NH)p (Fig 1B). Similar results were obtained in membranes obtained from cells grown in serum-supplemented medium.

Competition curves utilizing [^{125}I]-ICYP and (-)-isoproterenol (range 1pM - 1mM) carried out in the presence and in the absence of 0.1mM guanosine triphosphate (GTP) in membranes prepared from cells grown in serum-free medium revealed an average Ki in control cells of 50 nM and a rightward shift after GTP exposure (294 nM $p<.05$, n=8). As expected, GTP increased the proportion of low affinity binding sites for [^{125}I]-ICYP determined by computer modeling (10) from 26±11 to 55±27% (n=8, $p<.025$)

β_1 Receptor Affinity and Density

In equilibrium binding experiments the Kd value of membranes prepared from cells grown in serum-supplemented medium was 1.62±0.5 nM (n=4). Kd values for membranes from cells grown in

serum-supplemented medium under relative hypoxic conditions (5% O_2) did not differ (Kd=1.29±0.56 nM, n=5). Kd values in membranes from cells grown in serum-free medium also were not different. A typical equilibrium binding experiment is shown in Fig 1C. For membranes prepared from cells grown in serum-supplemented medium the maximum number of binding sites (Bmax) using [^{3}H]-DHA as the radioligand was 260±71 fmol/mg protein (n=4) and for membranes prepared from cells grown in the presence of serum in 5% O_2 the value was 280±117 fmol/mg protein (n=5, p=NS). By contrast, the membranes prepared from cells grown in serum-free medium exhibited a significant decrease in Bmax (176±56 fmol/mg protein, $p<.05$ vs cells grown in serum-supplemented medium).

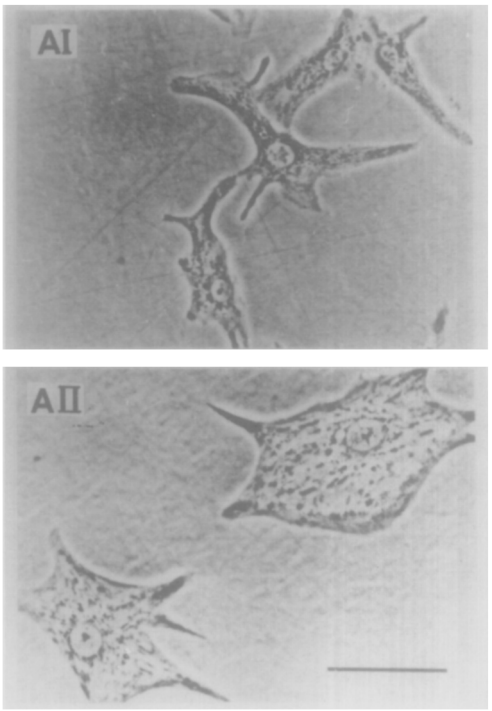
These data suggested that the increase in β_1-adrenergic receptor density associated with serum-induced growth might not be commensurate with the observed increase in cell size. To test this possibility directly, we compared cell surface area with the number of sites per cell using [^{125}I]-ICYP as the radioligand. Cell surface area was determined in the same cultures used for the radioligand binding studies. Serum induced a 3.69 fold increase in cell surface area from 2150± 294 to 7934 ± 848 um^2(n=4, $p<.0005$) (Fig 2A). However, the number of binding sites/cell only increased from 12956 ± 7579 to 19676 ± 5181 (n=5, $p<.05$) (Fig 2B), while the Kd values did not change (99 ± 45 vs 109 ± 35 pM (p=NS). Calculation of receptor density for cells grown in serum-free medium yielded a value of 6.96 sites/um^2, while for cells grown in serum this value was markedly reduced to 2.48 sites/um^2.

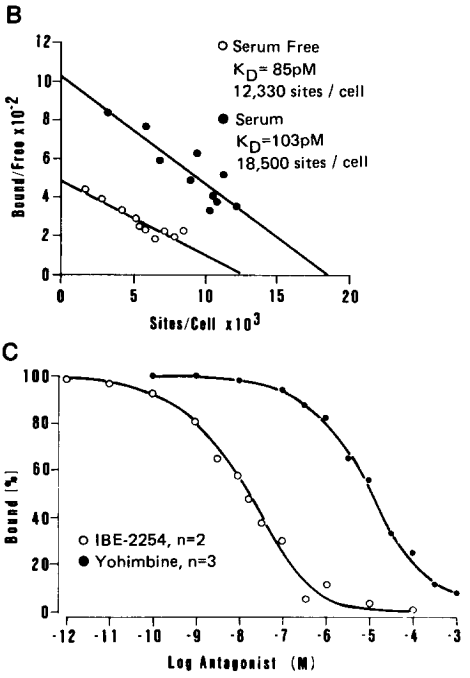
α_1 Adrenergic Receptors

Cells grown in serum-free medium also exhibited an α_1-adenergic binding site. Using [^{125}I]-IBE 2554 as the radioligand, non-iodinated IBE 2254 (α_1 selective) was markedly more potent than yohimbine (α_2 selective) in inhibiting binding to these cells (Fig 2C). These cells also exhibited a high capacity, low affinity site for α_1 receptors with a Kd of 567±188 pM and a Bmax of 27,514±9451 sites per cell (n=6).

DISCUSSION

Prior studies of the β-adenergic characteristics of rat neonatal ventricular cells have used serum-supplemented medium exclusively (10,12). Lau et al. performed studies using [^{125}I]-ICYP in serum-supplemented cells derived from neonatal rat ventricles (10). The Kd we observed was almost identical while the maximum number of binding sites in our serum-supplemented preparation was 2.59 fold higher. Moreover, we were able to confirm the β_1 specificity of our isolated myocardial cell preparation as noted both in neonatal (10) and in





Fugure 2AI. The upper panel depicts typical neonatal rat ventricular myocytes grown in serum-free medium after 7 days.

Figure 2AII shows typical myocytes that have been grown in 5% fetal bovine serum for the same time. The bar represents 50um.

Figure 2B. Typical Scatchard plots of equilibrium binding isotherms of cells grown in serum vs those grown in serum-free medium. For these studies the binding reaction was carried out in whole cells which had been frozen and thawed, as no difference in binding characteristics could be demonstrated when fresh cells were compared with preparations frozen in 0.25 M sucrose and 50mM Tris-HCl, pH 7.4, and lmM EDTA at -70°C for up to 4 months. The assay buffer contained in 50mM Tris-HCl pH 7.4: 10 mM MgCl; bovine serum albumin lmg/ml; and varying amounts of [^{125}I]-ICYP. Binding experiments were initiated by the addition of at least 10,000 cells and conducted at 37°C for 30 min. The reaction was terminated by adding 4ml of 50mM Tris-HCl at 25°C, followed by immediate rapid vacuum filtration through Whatman GF/C glass fiber filters. The test tubes and filters were washed with an additional 15ml of buffer and the radioactivity retained on the filter was determined in a gamma counter at 73% efficiency. Non-specific binding was defined as that component of total binding not inhibited by luM (-)-propranolol and did not exceed 20% of total binding.

Figure 2C. Antagonist competition curves of membranes prepared from neonatal rat ventricular myocytes grown in serum-free medium. The radioligand was [^{125}I]-IBE 2254; binding studies were carried out using the method of Kupfer et al. (6).

purified adult rat cardiac myocytes (13). Indeed, our Bmax value for serum-supplemented cells is almost identical to that recently reported by Buxton and Brunton in adult rat myocytes (13). However, our data differ from those of Porzig et al.(12) who were unable to utilize [^{3}H]-DHA in neonatal rat myocytes because of a large amount of nonspecific binding, whereas our nonspecific binding was in the range of 10 to 20%. In contrast to our observations, they reported that GTP

had no effect on the slope or IC_{50} values of isoproterenol competition curves. As indicated above, in our studies, [^{3}H]-DHA proved to be an excellent radioligand and is only limited in its utility by the fact that large numbers of cells are required.

Our data indicate a significant reduction in the maximum number of β_1 binding sites per cell in myocardial cells grown in serum-free medium. However, when this value was corrected for the difference in cell size between these myocardial cells and the cells grown in serum-supplemented medium, there was in fact a marked reduction in the number of sites per um^2 in the cells grown in serum-supplemented medium. Previous studies from this laboratory have indicated that exposure of myocardial cells to serum results in a three-fold increase in cell protein content (2), commensurate with the increase in cell surface area observed in the present study. Thus, the β_1-adrenergic receptor does not appear to share in the generalized increase of myocardial cell constituents accompanying the hypertrophic process. Whether this represents true down-regulation or merely selective inhibition of receptor protein synthesis is speculative, as is the potential protective effect against agonist stimulation that such a reduced receptor density might afford. Consistent with the latter interpretation is the fact that the adenylate cyclase response to (-)-isoproterenol was not different when membranes from cells grown in serum-free medium were compared with membranes from cells grown in serum-supplemented medium.

These data differ from reports indicating that when C6 glioma and S49 lymphoma cells grown in serum-containing medium are compared with cells grown in serum-free medium, there is an absolute decrease in β-adrenergic receptor number (14,15). The reasons for this decline remain unexplained; small molecules such as catecholamines are unlikely candidates for producing desensitization under these conditions, especially as the adenylate cyclase response to catecholamines is unimpaired (14). Others have shown that serum may influence membrane receptors. Thus no down-regulation by agonist of receptors for epidermal growth factor in Hela cells was observed following growth in serum-free medium by Wolfe et al (16). By contrast, a decreased production of prolactin in rat pituitary cells after they were transferred to a serum-free environment has been reported (17).

The data utilizing [^{125}I]-IBE 2254 are consistent with those recently reported by Kupfer et al. (6) and confirmed that this radioligand is highly selective for α_1 receptors based on competition curve data. Further, Kd and Bmax values in these two studies are very close

indicating that in whole cell preparations grown in serum-free medium the α_1-adenergic properties as measured by [^{125}I]-IBE2254 do not differ importantly from cells grown in serum-supplemented medium (6).

Thus, our data indicate that myocardial cells grown in serum-free medium co-express both α_1- and β_1-adenergic receptors. Furthermore, serum-induced cardiac myocyte cell hypertrophy is associated with a modest absolute increase in the total number of β_1-adrenergic receptors per cell, but a marked decreased in the density of these receptors on the cell surface; agonist coupling to adenylate cyclase is unaffected. Since cardiac myocyte cell size may be regulated through a variety of neurotransmitter and hormonal mechanisms (3, 18), this cell culture system constitutes an excellent model for the study of interventions that may influence the regulation of myocardial cell hypertrophy by nonhemodynamic factors, particularly through the adrenergic receptor system.

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