Subclassification of β-Adrenergic Receptors in Cultured Rat Cardiac Myoblasts and Fibroblasts

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SUMMARY β-Adrenergic receptors in primary cultures of neonatal rat cardiac cells were identified with the radioligand [125I]iodohydroxybenzylpindolol ([125I]IHYP). At the time of cell plating, a differential attachment procedure was employed to separate myocardial (M) cells from fibroblast-like (F) cells. After 3–4 days, the cultures enriched in M cells were still more than 90% pure and the cultures enriched in F cells were more than 95% pure. For binding studies, confluent cell layers were nonenzymatically detached as single cell suspensions. Both M and F cells contained a limited number of β-adrenergic receptors (M, 7600 ± 2100 sites/cell; F, 9000 ± 2400 sites/cell) which had very high affinity (M, Kd = 88 ± 33 pm; F, Kd = 71 ± 23 pm) for [125I]IHYP. For each cell type, the binding sites were stereoselective for the L-isomers of agonists and antagonists. Further binding studies on the relative potency of β-agonists showed that the β-receptors in M cells could be subclassified as β1 (isoproterenol > epinephrine = norepinephrine), whereas the β-receptors in F cells were more typical of β2 (isoproterenol > epinephrine > norepinephrine). The order of potency of these catecholamines in stimulating the adenylate cyclase activity in M and F cells was consistent with the order of potency observed in binding studies. Practolol, a β1 inhibitor, was 30 times more effective as a competitor of [125I]IHYP binding in M cells than in F cells. It thus appears that, whereas M and F cells share a similar number of receptors per cell and similar affinities for [125I]IHYP, the receptors in the two cell types nevertheless cannot be distinguished on the basis of their subclassification. These results also emphasize the importance of obtaining a homogeneous cell population for studies of the β-adrenergic receptor in cultured cardiac tissue. Circ Res 47: 41–48, 1980

β-ADRENERGIC receptors with high affinity, low capacity, and stereoselectivity have been identified in cardiac microsomal preparations. Some (U’Prichard et al., 1978), but not all (Harden et al., 1976; Alexander et al., 1975), of these analyses have supported physiological observations that the heart can be subclassified as a β1 system (Lands et al., 1967), since epinephrine and norepinephrine are equipotent in binding to the receptor. In addition, direct binding studies have shown that there are changes in the number of β-adrenergic receptors in certain disorders associated with altered adrenergic tone (Ciaraldi and Marinetti, 1977; Williams et al., 1977; Limas and Limas, 1978).

A different approach to the study of β-adrenergic receptors and their regulation is through cardiac cells in primary tissue culture. This provides an opportunity for one to examine β-adrenergic receptors under conditions in which the external environment can be controlled precisely and perturbed readily. Cultures of cardiac cells already have been shown to respond to adrenergic agonists (Lane et al., 1977; Hermsmeyer and Robinson, 1977) and, recently, the effects of thyroid hormone on the β-adrenergic receptors of cultured rat myocardial cells also have been reported (Tsai and Chen, 1978). However, analysis of the β-adrenergic response in cultured cardiac cells at the receptor level must take into account the potential presence of other cell types which also could contain β-receptors. For example, fibroblast-like cells (F) are difficult to exclude when cardiac cells are plated. These F cells may present a problem in the interpretation of data, especially if they possess β-adrenergic receptors and have the capacity to respond to the β-adrenergic catecholamines. To address this possibility, we have modified the differential attachment procedure of Blondel et al. (1971) to produce cultures of myocardial (M) and F cells that are 80% and 95% pure (respectively) after 3–4 days. This paper reports the results of studies of the characteristics of the β-adrenergic receptor in these purified M and F cultures.
Methods

Materials

[125I]Iodohydroxybenzylpindolol (specific activity, 2200 Ci/mm) and [α-32P]ATP were obtained from New England Nuclear; l- and d-isoproterenol bitartrate, l-epinephrine bitartrate, l-norepinephrine bitartrate, and gentamycin all were obtained from Sigma Chemicals. The l- and d-Propranolol and dl-practolol were gifts of Ayerst Pharmaceuticals. Gelman A/E glass fiber filters were obtained through A.H. Thomas.

Preparation of Single Cells for Culture

Under aseptic conditions, hearts from eight to 25 Wistar rats (2–4 days old) were removed and placed in a sterile solution containing 130 mM NaCl, 4.7 mM KCl, 16.5 mM dextrose, 5 µg/ml phenol red, and 20 mM Na-HEPES at pH 7.2. The atria were removed and the ventricles were minced into about 10 pieces each. The minced cardiac tissue was then subjected to 12 successive periods of trypsin digestion at 37°C. The trypsin medium, 10–15 ml each time, was identical to that described above except for the presence of trypsin (0.37 mg/ml). The first two digests were carried out for 30 minutes each, and each of the subsequent digestions was for 15 minutes. The supernatant fractions from the first two digestions were discarded; all subsequent supernatant extracts were diluted 10% with cold horse serum and pooled in chilled centrifuge tubes.

The cell suspension obtained from the trypsin digests was centrifuged at 200 g for 5 minutes and resuspended in a medium containing MEM modified by increasing the NaHCO3 concentration to 16 mM and adding 10% (non-heat inactivated) horse serum, hypoxanthine (2 µg/ml) and gentamycin (20 µg/ml) (growth medium). The final concentration of suspended cells was approximately 5 × 10^6 cells/ml.

Differential Cell Plating

To obtain relatively pure M and F cultures, the differential plating procedure of Blondel et al. (1971) was adapted, with slight modifications. The suspended cells were plated in large petri dishes (Falcon #3003, 10 cm in diameter), at about 5 × 10^6 cells/dish. The dishes were placed in a water-saturated incubator with 5% CO2 at 37°C for 120–150 minutes. This period was sufficient for most of the F cells to become attached to the petri dishes but was inadequate for most of the M cells, which remained nonattached. At the end of the incubation period, the dishes were swirled gently to resuspend any remaining nonattached cells. The cell suspension, containing mostly M cells, then was transferred rapidly to new petri dishes. These dishes became the source of M cells. The dishes containing the adherent F cells were rinsed with 5 ml of growth medium and refilled with 10 ml of the same medium. All the dishes then were returned to the incubator. The media were changed after about 20 hours of incubation to remove nonattached or nonviable cells (approximately 20% of initial plating). Confluent monolayers of M and F cells usually were obtained after 2–3 days in culture. Myocardial cells produced in this manner are spontaneously active and exhibit a high degree of ultrastructural and electrophysiological differentiation. Maximum diastolic potential is −69.1 ± 1.5 mV and mean maximal upstroke velocity of phase 0 of the transmembrane action potential is 90.1 ± 4.7 V/sec (SEM). As indicated by the small standard errors, these electrical characteristics are uniform among cultures (Robinson and Legato, 1980).

Determination of Cell Types in Culture

For each preparation, dishes were set aside for determination of the number of M and F cells at the time of [125I]IHYP assays. The dishes were rinsed twice with a solution containing 130 mM NaCl, 5 mM KCl, 5 mM MgCl2, and 10 mM sucrose. The cells then were fixed in Bouin's fixative for 30 minutes, and, after extensive washing with water, were exposed to Masson's trichrome stain (Masson, 1929), which distinguishes between muscle and nonmuscle cells in cardiac cultures (Marvin et al., 1979). The stained cells were observed and photographed by means of phase contrast microscopy. At least 1000 cells were counted from random locations to determine the percentage of M and F cells in each culture. Agreement between counts from two dishes of the same culture was within 5%.

Harvest of Cells

The procedure used to detach adherent M or F cells was identical. All dishes first were rinsed with 10 ml of cell-detaching medium which contained 130 mM NaCl, 16 mM NaHCO3, 3 mM KCl, 0.5 mM NaH2PO4, 10 mM sucrose, and 1 mM EDTA (EDTA stock solution adjusted to pH 7.3 with Tris buffer). All dishes were refilled with 4 ml of cell-detaching medium and incubated at 37°C for 10 minutes. The cells were resuspended by gently pipetting, collected in centrifuge tubes, and diluted to three times their volume with a similar medium containing MgCl2 (2 mM) instead of EDTA. The cells were pelleted by centrifugation at 700 g for 5 minutes and resuspended in the Mg2+-containing medium at about 3.5 × 10^6 cells/ml. The exact yield of cells was determined by counting in a hemocytometer.

Detection of β-Adrenergic Receptors: Binding of [125I]Hydroxybenzylpindolol

The binding assay was performed in 12 × 75-mm glass test tubes with the following components in a total volume of 0.5 ml: [125I]IHYP, M or F cells (2–
4 x 10^6 cells) and the assay medium which contained 150 mM NaCl, 10 mM KCl, 10 mM dextrose, bovine serum albumin (1 g/liter), and 10 mM Tris, at pH 7.4. β-adrenergic agonists and antagonists used as competitors of [125I]IYP binding are described in individual experiments. For these experiments, the concentration of [125I]IYP was 34–60 pm. For Scatchard analysis, the concentration range of [125I]IYP was 4.8–960 pm. When catecholamines were employed, 1 mM ascorbic acid—which did not interfere with the binding assay—was included in the assay medium as an antioxidant. The samples were incubated at 37°C for 40–50 minutes and then filtered over Gelman A/E glass filters and washed twice with 15 ml of 10 mM Tris-HCl (pH 7.4) at room temperature. The [125I]IYP bound to cells was retained on the filters and detected in a Packard Autogamma spectrometer. For every concentration of radioligand used, parallel tubes were assayed in the presence of l-propranolol (0.1 μM) to account for nonspecific binding. The Kd for l-propranolol in M and F cells was less than 0.5 nM. Specific binding of [125I]IYP was defined as the difference between [125I]IYP bound in the absence and presence of propranolol. This amount constituted approximately 60% of total binding when the ligand concentration was 48 pm. The assay blank (radioligand trapped on the filter in the absence of cells) was approximately 1–2% of the radioactivity filtered. All data points are the result of triplicate determinations.

Adenylate Cyclase Assay

Adenylate cyclase activity in M and F cells, lysed by freezing and thawing, was assayed according to the method of Salomon et al. (1974). This method monitors the conversion of [α-32P]ATP to [32P]cyclic AMP and employs sequential Dowex and alumina chromatography to isolate the product. Approximately 25 μg of cellular protein were used in the assay. The protein content of all lysates was determined by the method of Lowry et al. (1951). The assay blank was approximately 50 counts/min (cpm)/10^6 cpm of labeled substrate. All determinations were performed in triplicate, for which the coefficient of variation was 10%.

Statistical Analyses

The binding capacity and affinity of M and F cultures were computed from the Scatchard plots by least squares linear regression (Snedecor and Cochran, 1967). The average M and F values of these parameters from all experiments were compared by Student’s t-test. In Figure 3 the concentrations of isoproterenol required for half-maximal inhibition of binding in M and F cultures were computed by weighted probit transformation of the log dose-response curves, and their 95% confidence intervals were compared. All error estimates in the text are ± standard deviation unless otherwise stated.

Results

Morphology and Purity of M and F Cultures

The M and F cells in rat cardiac cell culture are readily distinguishable from each other, particularly after staining (Marvin et al., 1979; Blondel et al., 1971). Figure 1, A and B, illustrates trichrome-stained M and F cultures, respectively. The M cells often have small, uninucleolate nuclei and rather coarse, granular cytoplasm. Many of these cells have a stellate configuration. Due to the presence of myofilaments, the M cells are stained deep red and pink. In contrast, F cells usually have a larger nucleus with several nucleoli. The F cells spread out flatly and their cytoplasm is almost agranular. Due to a lack of myofilaments, F cells are stained pale blue instead of red.

Based on these morphological differences, the percentage of M cells in the F cell cultures and, conversely, the percentage of F cells in M cell cultures were determined, as described in Methods. M cell cultures produced using the differential attachment procedure (see Methods) remained more than 80% pure after 3–4 days in culture. The average purity of the M cell cultures from eight separate preparations was 85.8 ± 6.2%. When the differential attachment procedure was not employed, the cardiac cell culture usually was infiltrated by more than 60% F cells after 3–4 days. Similarly, F cell cultures more than 95% pure were obtained after 3–4 days in culture. Based on eight separate preparations, the average purity of F cells was 96 ± 0.6%. In the experiments to be described, only cell cultures so enriched were used.

Binding of [125I]IYP to M and F cells

After M or F cells had been harvested separately and resuspended as single cells, the presence of β-adrenergic receptors was detected using [125I]IYP as the radioligand (see Methods). Specific binding was clearly saturated by 480 pm for both cell types. Scatchard analysis of these data from two sets of experiments for M and F cells is shown in Figure 2. The data points are best fit to a straight line, suggesting in each case a single class of binding sites. The average binding capacity for the M cells (five cultures) is 1.26 ± 0.34 x 10^-10 mol/cell or 7600 ± 2100 sites/cell; the average binding capacity for F cells (four cultures) is 1.49 ± 0.36 x 10^-10 mol/cell or 9000 ± 2400 sites/cell. Further information from Scatchard analyses of these data indicate that the equilibrium dissociation constant Kd is 88 ± 33 pm for M cells and 71 ± 23 pm for F cells. Figure 3 shows that the β-adrenergic agonist l-isoproterenol inhibited the specific binding of [125I]IYP to M cells half-maximally at approximately 0.2 μM (95% confidence interval = 0.02–0.7 μM). For F cells, half-
FIGURE 1 Phase-contrast photomicrographs of M- and F-enriched cultures, 3 days after initial plating, fixed and stained as described in Methods. Calibration bar = 100 μm. A: M-enriched culture, with two contaminating F cells marked. B: F-enriched culture.
maximal inhibition of $^{[125\text{I}]}$HYP binding by isoproterenol occurred at 0.5 $\mu$M (95% confidence interval 0.1-1.0 $\mu$M). None of the aforementioned differences between M and F cells was statistically significant ($P > 0.05$). Both M and F cells demonstrated, in addition, stereoselectivity of binding with marked preference for the levorotatory isomers of propranolol and isoproterenol (Fig. 4).

In view of the similarities of binding capacity in two culture systems, each containing a preponderance of a different cell type, it seemed most unlikely that only one type of cell contained $\beta$-adrenergic receptors. However, the binding experiments reported so far do not provide any basis for distinguishing between the $\beta$-adrenergic receptors of M cells and those of F cells. The next series of experiments was performed to determine whether the subclassification of $\beta$-adrenergic receptors might be different among M and F cells. Accordingly, the respective potencies of the three $\beta$-adrenergic agonists, l-isoproterenol, l-epinephrine, and l-norepinephrine, in inhibiting $^{[125\text{I}]}$HYP binding were studied. The results are summarized in Figure 5. For both cell types, isoproterenol is clearly the most potent inhibitor. However, the order of potency for M cells (isoproterenol > epinephrine > norepinephrine) suggests a $\beta_1$ subclassification, whereas the order of potency for F cells (isoproterenol > epinephrine > norepinephrine) suggests a $\beta_2$ subclassification. In one experiment, a complete range of agonist concentrations was employed. In M cells, the $K_a$'s for epinephrine and norepinephrine were approximately the same (1.0 $\mu$M vs. 0.9 $\mu$M) whereas, in F cells, epinephrine was much more effective than norepinephrine (1.5 $\mu$M vs. 23 $\mu$M). An experiment was performed with practolol, a selective $\beta_1$
The relative potencies of β-adrenergic agonists in inhibiting the specific binding of $[^{125}I]$HYP to M and F cells. l-Isoproterenol (Iso), l-epinephrine (Epi) and l-norepinephrine (NE), each 1 μM or 10 μM, were tested as inhibitors of maximal specific $[^{125}I]$HYP binding to F and M cells. The results shown are the mean ± SD of three experiments for which data points were obtained in triplicate. At each concentration, the differences between inhibition by epinephrine and norepinephrine were significant for F cells ($P < 0.05$) but not for M cells ($P > 0.05$).

FIGURE 5

### Adenylate Cyclase Activity of M and F Cells

The $[^{125}I]$HYP binding data suggest that despite the similarity of many binding characteristics, the receptors of M and F cells are distinguishable on the basis of their adrenergic subtypes. If this is the case, the classification of adrenergic subtype might be reflected as well in the differential ability of certain β-adrenergic agonists to stimulate adenylate cyclase activities in lysates of these cells.

The relative potencies of the β-adrenergic agonists in stimulating adenylate cyclase are illustrated in Figure 7. Isoproterenol is the most potent activator of adenylate cyclase for both M and F cells. The order of potency for M cells (isoproterenol > epinephrine > norepinephrine) suggests a β1 subclassification similar to data obtained in the binding studies. Likewise, the order of potencies for F cells (isoproterenol > epinephrine > norepinephrine) suggests a β2 subclassification which agrees with the binding data. Thus, the effectiveness of each β-adrenergic agonist to stimulate adenylate cyclase activity directly parallels its potency in inhibiting $[^{125}I]$HYP binding for both M and F cells.

### Discussion

Our results using $[^{125}I]$HYP to identify β-adrenergic receptors in myocardial and fibroblast-like cultures agree well with those published by other laboratories using $[^{125}I]$HYP in other cell systems (Maguire et al., 1977; Wolfe et al., 1977; Insel et al., 1976; Bilezikian et al., 1977; Brown et al., 1976; Schonberg et al., 1978). The only other report of $[^{125}I]$HYP binding to cardiac tissue presents results qualitatively similar to those reported in this study (Harden et al., 1976), although the apparent $K_d$ for IYP was extremely high. The very high protein concentration used in that study could be responsible for a $K_d$ higher than that shown in our study. This possibility is supported by the data of Chang et al. (1975), who have shown that the protein...
concentration of membrane preparations bears a direct relationship to the experimentally derived $K_d$ for binding.

Specific binding to the $\beta$-adrenergic receptor in cultured rat myoblasts has been reported, using $[^3H]$dihydroalprenolol, but it is difficult to make direct comparisons because little information was presented in that study on direct characteristics such as the $K_d$ or the binding capacity (Tsai and Chen, 1978). In this study we report additional binding characteristics such as stereoselectivity for $\alpha$-antipodes of $\beta$-adrenergic agonists and antagonists and linkage to adenylate cyclase activity which help to establish the likelihood that the M and F cells studied in this report contain bona fide receptors.

With respect to the binding affinity and binding capacity, $\beta$-adrenergic receptors in M and F cells are indistinguishable from each other. These observations cannot be attributed to significant contamination of F cells in the M cultures or M cells in F cultures combined with a significant difference in binding capacities. If this were the case, the binding capacities of the M and F cultures would reflect the relative ratios of M and F cells in the two cultures. In fact, if one recalculates the number of $[^3H]$IHYP binding sites in M and F cells, taking into account the percentage of contaminated cells in each culture, values are not significantly different from the data presented.

The existence of $\beta$-adrenergic receptors in non-muscle cells in culture is not completely unexpected and was suggested by the studies of Walker (1978), who worked with a purified preparation of non-muscle cells similar to our F cells from neonatal rat hearts. These non-muscle cells are of mesenchymal origin and probably consist of both fibroblasts from the connective tissue of the heart and endothelial cells of the coronary vasculature (Blondel et al., 1971; Mark and Strasser, 1966). The present study does not provide any data on precisely which of these two cells is responsible for the $[^3H]$IHYP binding sites in our F cultures. Other studies have provided direct evidence for the presence of $\beta$-adrenergic receptors in at least one fibroblast cell type, with a $K_d$ comparable to that found for F cells in our study (Maguire et al., 1976). The other non-muscle cell type in our F cultures, the endothelial cell, although responsive to several hormones such as angiotension II (Richardson and Beaulnes, 1971) and histamine (Majno et al., 1969), has not been reported to contain $\beta$-adrenergic receptors. In preliminary studies (Bilezikian and Jaffe, unpublished observations), cultured human umbilical endothelial cells could not be shown to contain $\beta$-adrenergic receptors. Thus it is likely that the cells containing $\beta$-adrenergic receptors in our F cultures are fibroblasts.

It is of great interest that, despite the apparent similarities of $\beta$-adrenergic receptors between M and F cells, we were able to distinguish between the two receptor systems on the basis of their subclassification. M cells display an order of potency for $\beta$-adrenergic agonist binding and adenylate cyclase activation that is $\beta_1$, according to conventional criteria (Lands et al., 1966; 1967), whereas the same analysis for F cells shows them to be $\beta_2$. Furthermore, practolol, a $\beta_1$ inhibitor, was much more effective in competing for $\beta$ receptors in M cells than in F cells. Previous studies have shown that $\beta$ receptors from cardiac tissue are $\beta_1$ (Lands et al., 1967; U’Prichard et al., 1978) and, from more indirect evidence, fibroblasts appear to be $\beta_2$ (Rao et al., 1971; Franklin et al., 1975).

These data suggest that there are other cell types derived from cardiac tissue besides myocardial cells that contain $\beta$-adrenergic receptors. Two points are evident: (1) in studying $\beta$-adrenergic receptors in cardiac culture, one should take measures to ensure that the culture is enriched in M cells and independently assess the effect of any experimental intervention on purified F cell cultures; (2) in binding studies employing the ventricular myocardium, it should be appreciated that non-myocardial elements, which may be present in significant quantities, could be erroneously included in the assessment and evaluation of cardiac $\beta$-adrenergic receptors.

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