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Rapid Reciprocal Changes in Adrenergic Receptors in Intact Isolated Hepatocytes during Primary Cell Culture

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SUMMARY

In hepatocytes freshly isolated from adult female rat livers, catecholamine-stimulated glycogenolysis is mediated predominantly by α_1-adrenergic receptors, and to only a minimal extent by β_2 receptors. Primary cell culture of these hepatocytes results in a switch in the adrenergic control of glycogenolysis from an α_1 to a predominant β_2 type of response. To investigate whether this switch is due to an alteration in the plasma membrane receptor composition, we characterized α_1 and β_2-adrenergic receptors in both freshly isolated and cultured hepatocytes, using radioligand-binding techniques. Binding of the selective α_1-adrenergic antagonist [^{3}H]prazosin and the β-adrenergic antagonist [^{125}I]pindolol to intact freshly isolated hepatocytes was of high affinity, saturable, and of appropriate specificity for an α_1- and β_2-adrenergic receptor, respectively. Equilibrium binding studies evaluated by a computer-assisted curve-fitting procedure indicated interaction with a single class of high affinity sites for radiolabeled prazosin (K_D = 126 ± 10 pM; B_{max} = 93,000 ± 5,500 sites/cell) and pindolol (K_D = 66 ± 6 pM; B_{max} = 2,000 ± 700 sites/cell). In intact hepatocytes and in membranes prepared from these hepatocytes, competitive inhibition curves revealed the coexistence of two different sites with high and low affinities for agonists at both α_1- and β_2-adrenergic receptors. When isolated hepatocytes were kept in monolayer cell culture for up to 72 hr, the switch in adrenergic control of glycogenolysis (phosphorylase *a* activation) from an α to a β pathway was confirmed and was associated with a progressive decrease in the number of α_1 receptors and an increase in β_2-adrenergic receptor density, without marked change in the affinity of agonists or antagonists. To investigate the mechanism(s) of this reciprocal change, a number of perturbations were examined including alterations in the composition of the culture medium and the influence of various hormones and inhibitors of cellular function. *De novo* protein synthesis is implicated in both receptor alterations as the inhibitors cycloheximide and actinomycin D prevented the increase in β- and attenuated the decrease in α-adrenergic sites. The other perturbations were without effect. Thus, these studies provide evidence for a coupling of the functional alteration in glycogenolysis to changes at the receptor level per se. The mechanism underlying the reciprocal changes in hepatocyte adrenergic receptors during culture remains undefined. As similar reciprocal changes also occur in a variety of pathophysiologic states *in vivo*, the cultured hepatocytes is a useful model for the study of this phenomenon *in vitro*.

INTRODUCTION

In most tissues, α- and β-adrenergic receptors mediate opposing effects (1). In the rat liver, however, catecholamine-induced activation of glycogenolysis can be mediated potentially by both receptor subtypes (2–5). While these adrenergic receptors use distinct pathways for signal transduction, evidence is accumulating that some as yet unidentified links may exist between these two systems (6–8).[1] For example, under a variety of conditions, including primary cell culture of isolated hepatocytes (9), a reciprocal change in the balance between α and β receptor mediated adrenergic stimulation of glycogenol-

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[1] C. J. Lynch, R. Charest, P. F. Blackmore, and J. H. Exton, submitted for publication.

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ysis has been observed (10, 11). To investigate the role of adrenergic receptors in this functional change, we developed techniques to characterize adrenergic receptors in intact rat hepatocytes, immediately after isolation and also after primary cell culture for up to 72 hr.

MATERIALS AND METHODS

Chemicals. [^{3}H]Prazosin (26 Ci/mmol) was purchased from Amersham. (±)Pindolol was a gift from Sandoz Corp. and iodinated as described by Israelson *et al.* (12). The following were gifts: prazosin (Pfizer, Groton, CT), rauwolscine (Roth, Karlsruhe, West Germany), (+)epinephrine (Sterling Winthrop, Rensselaer, NY), alprenolol (Hassle, Molndal, Sweden), phenoxybenzamine (Smith, Kline and French, Philadelphia, PA), and phentolamine (Ciba-Geigy, Summit, NJ). All other chemicals were purchased from Sigma Corp. (St. Louis, MO).

Preparation of hepatocytes: Female Sprague-Dawley rats (100 to 180 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and maintained on Purina Laboratory Chow. Hepatocytes were isolated by collagenase digestion of the perfused rat liver using a modification of previously published methods (13, 14). Briefly, the animal was anesthetized with intraperitoneal sodium methohexital (0.2 mg/g in weight). Following cannulation of the portal vein and inferior vena cava above the diaphragm, the liver was perfused with Ca^{2+}-free Krebs-Ringer bicarbonate buffer (pH 7.4, 37°) containing 5.5 mM glucose at 50–60 ml/min in a temperature-controlled jacketed hood. Oxygenation of the perfusate with 95% O_2/5% CO_2 was performed with a membrane oxygenator. After perfusion with 500 ml of buffer, the liver was perfused *in situ* in a recirculating system for 10 min with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5 mM Ca^{2+}, 5.5 mM glucose, and collagenase (0.5 mg/ml) (Worthington Biochemical Co., Freehold, NJ). As collagenase preparations contain varying amounts of proteases, cells prepared with several different batches of collagenase were initially tested for their effects on specific [^{3}H]prazosin binding. All subsequent experiments were performed using the same batch of collagenase (Lot 42B217). The liver was removed to a Petri dish containing ice-cold Dulbecco's minimal essential medium (M. A. Bioproducts, Walkersville, MD) and gently teased apart. The digest was poured through a silkscreen and the filtrate was collected in 15-ml polystyrene tubes. Cells were harvested by centrifugation (50 × *g* × 2 min), resuspended in ice-cold Dulbecco's minimal essential medium, washed twice, and maintained in the same medium at a concentration of 1–2 × 10^7 cells/ml at 37° prior to use or subsequent plating. All equipment and solutions were sterilized by autoclaving prior to use; perfusion solutions contained penicillin (10,000 units/liter) and streptomycin (100 μg/ml).

Cells were counted in a hemocytometer; viability as judged by trypan blue exclusion was routinely above 90%. Prior to the binding experiments, cells were diluted to a concentration of 2 × 10^6 cells/ml and washed two times with ice-cold PBS[2] (10 mM, Dulbecco's formula, pH 7.4) at 50 × *g* × 4 min.

Cell culture. Isolated hepatocytes were cultured as monolayers in Dulbecco's minimal essential medium supplemented with glucose (4.5 g/ml), penicillin (10,000 units/ml), hydrocortisone (10 μg/ml), and 10% fetal calf serum on 240 × 240 mm bioassay dishes (Nunc, Algade, Denmark) previously coated with collagen (Vitrogen 100, Flow Laboratories, McLean, VA) at a concentration of 250,000 cell/ml (total volume, 100 ml). In a series of experiments, cells were plated in serum-free Williams E medium (GIBCO, Grand Island, NY) or in the absence of hydrocortisone. The medium was changed after 24 hr. When indicated, medium was supplemented with (−)norepinephrine (10 μM), isoproterenol (10 μM), triiodothyronine (15 nM), colchicine (100 μM), cytochalazine B (10 μM), chloroquine (100 μM), methylamine (100 μM), cycloheximide (10 or 75 μM), or actinomycin D (1 μg/ml). Protein synthesis was determined by incorporation of [^{3}H]leucine as described in Ref. 15. Prior to the binding studies, cell sheets were rinsed with 3 × 40 ml of PBS. Cells were harvested with a large bladed rubber policeman (Bellco, Vineland, NJ), centrifuged at 50 × *g*, and passed gently through a single layer of silkscreen. This resulted in a suspension of >90% single cells with a viability >95% as judged by trypan blue exclusion.

Preparation of a crude membrane fraction from isolated hepatocytes. Cells (10^8) were homogenized on ice with a tight fitting Dounce tissue homogenizer and centrifuged at 4,000 × *g* for 5 min. The supernatant was saved and the pellet was resuspended, rehomogenized, and recentrifuged at the same speed. The supernatants from both spins were pooled, and the membranes pelleted at 36,000 × *g* for 20 min, and then washed twice with ice-cold buffer containing 100 mM Tris, 5 mM EDTA, and 1 mM $MgCl_2$ (pH 7.4). Protein concentration was determined according to Lowry *et al.* (16).

Binding studies. A typical binding experiment employed 200,000 cells, [^{3}H]prazosin (0.02–5.0 nM) with or without phentolamine mesylate (10^{-5} M) or [^{125}I]pindolol (0.01–5 nM) with or without alprenolol (10^{-5} M) in a total volume of 150 μl. In assays with particulate fractions, a protein concentration of 100 μg/test tube was used. Test tubes were incubated in a shaking water bath at 25° for 30 min. Inhibition curves for agonists were performed in the presence of pyrogallol (0.5 mM), pargyline (0.5 mM), pyrocatechol (3 mM), and ascorbic acid (0.1 mM). The reaction was terminated by washing tubes with 4 × 4 ml aliquots of ice-cold PBS onto 24-mm glass fiber filters and unbound ligand was separated by vacuum filtration. Filters were placed in 10 ml of scintillation fluid (Hydrofluor) and counted in a liquid scintillation spectrometer (Beckman) with a counting efficiency of 56% (tritium) or by direct counting in a gamma spectrometer (Micromedic) at 80% efficiency (^{125}I).

Measurement of phosphorylase a activity. In both fresh and cultured hepatocytes, phosphorylase *a* activity was determined by the method of Stalmans and Hers (17). Briefly, hepatocytes (4 × 10^6 cells/ml) were incubated in Krebs-Henseleit bicarbonate buffer (containing in millimolar: NaCl, 118.2; KCl, 4.7; KH_2PO_4,1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; glucose, 5.5) at 37° with continuous oxygen (95% O_2/5% CO_2). After a 30-min stabilization period, a 0.45-ml aliquot of cells was transferred to a plastic vial containing 50 μl of agonist or vehicle and the incubation was terminated after 2 min by immersing the tubes in liquid nitrogen. Antagonists or their vehicle were added to cells 15 min prior to agonist exposure. Care was taken to handle cells as gently as possible to maintain maximum viability (>95% trypan blue exclusion).

Cell homogenates were prepared according to Hutson *et al.* (18). Briefly, 1.3 ml of homogenization buffer (containing in millimolar: β-glycerol phosphate, 138; NaF, 138; EDTA, 28; sucrose, 550; cysteine HCl, 28; pH 6.0) were added to the frozen aliquot of cells (0.5 ml). After thawing on ice, the cells were disrupted using a Brinkmann Polytron (10 sec, setting 7) and 50-μl aliquots were then incubated for 15 min at 30° with 50 μl of assay buffer containing 2% (w/v) repurified oyster glycogen, 1 mM caffeine, 100 mM [^{14}C]glucose 1-phosphate (specific activity, 294 mCi/mmol, 50,000 cpm/tube). The reaction was stopped by spotting 50 μl of the incubation medium onto Whatman 3-mm-thick filter paper discs that were then immersed in 66% (v/v) ethanol to precipitate glycogen, and washed and counted for radioactivity according to Gilboe *et al.* (19). Phosphorylase *a* activity is expressed as nanomoles of [^{14}C]glucose incorporated into glycogen/min/mg of cell protein.

Data analysis. Data derived from radioligand studies were analyzed by LIGAND, a nonlinear computer-assisted iterative weighted least squares curve-fitting procedure (20). Briefly, the untransformed experimental data (i.e., total bound ligand) were fitted to possible curves derived from the law of mass action by least squares regression analysis using Marquardt's algorithm. The data were fitted in different subsequent calculation steps for several possible models of ligand-receptor interaction (one site, two or more sites). "Goodness of fit" for each possible model of interaction was then compared using the *F* test to define the underlying model of ligand-receptor interaction for the given data.

[2] The abbreviations used are: PBS, phosphate-buffered saline; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetracetic acid.

RESULTS

Studies in freshly isolated hepatocytes. [^{3}H]Prazosin bound to isolated hepatocytes in a saturable (Fig. 1) and reversible (Fig. 2) manner. Equilibrium was reached within 10 min at 25° and the binding remained constant for up to 2 hr. Analysis of equilibrium binding studies, using either LIGAND or conventional Scatchard plots (Fig. 1), suggested that the binding of the radioligand occurred to a single class of high affinity sites with a K_D of 125 ± 10 pM and a concentration of 93,000 ± 5,500 sites/cell. With both methods of analysis, specific binding typically represented >85% of total binding at a radioligand concentration up to six times the K_D. Binding in crude membranes prepared from the isolated hepatocytes (K_D = 133 ± 13 pM, B_{max} = 114 ± 15 fmol/mg protein, n = 8) was virtually identical to that in intact cells, although at higher ligand concentrations nonspecific binding was less than in intact cells. The K_D determined from the kinetic studies, 150 pM, was in agreement with that determined from equilibrium binding studies (see above).

Competitive inhibition studies with various adrenergic agents showed an order of potency typical for the α_1-adrenergic receptor (Fig. 3). Thus, unlabeled prazosin inhibited [^{3}H]prazosin binding more potently than the nonselective α antagonist phentolamine. The β-adrenergic ligand alprenolol and the α_2-selective antagonist rauwolscine were almost 10,000-fold weaker in their ability

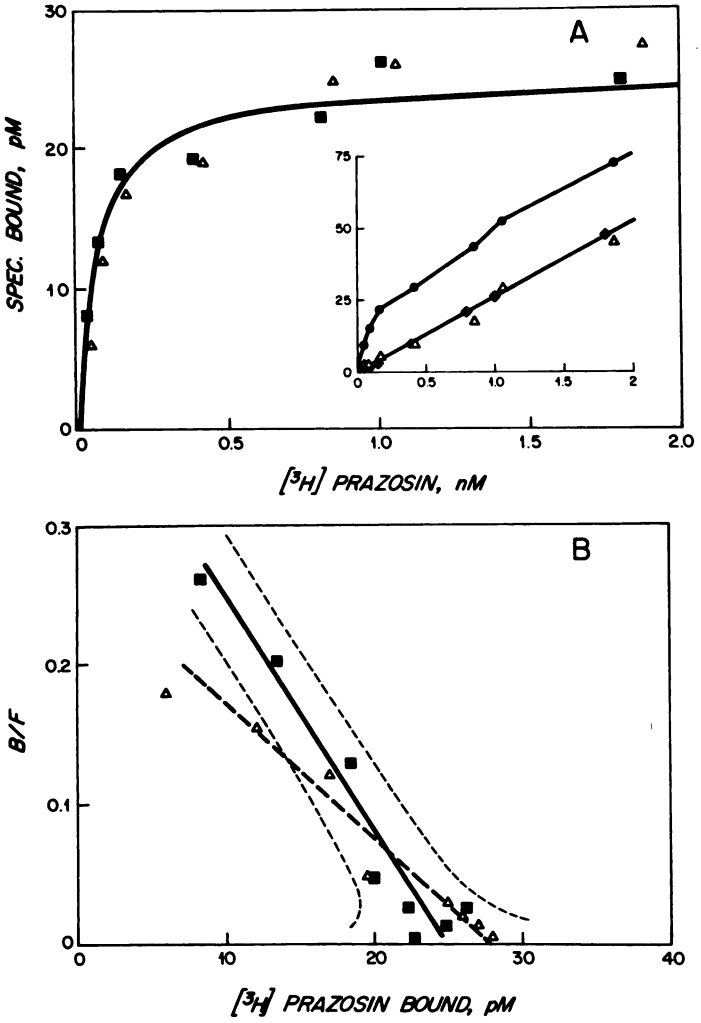

FIG. 1. *Equilibrium binding studies performed with the radioligand [^{3}H]prazosin and freshly isolated hepatocytes*

A, binding isotherm of specific [^{3}H]prazosin binding determined from computer analysis (■) of total binding data using LIGAND (20), which treats "nonspecific" binding as a fitted parameter, or by conventional Scatchard analysis (Δ) in which specific binding is determined by subtracting the binding in the presence of phentolamine (10^{-5} M) from total binding. Inset, comparison of LIGAND (♦) and conventionally determined nonspecific binding (Δ). The total binding isotherm (●) is also shown. In these experiments, [^{3}H]prazosin (0.02–5.0 nM, final concentration) was incubated with isolated hepatocytes (180,000 cell/ml) in a total volume of 1 ml in the presence or absence of phentolamine (10^{-5} M), as described in Materials and Methods. Values shown are the means of triplicate determinations. B, Scatchard plot of the equilibrium binding data shown in A determined with LIGAND (■——■) or by conventional linear regression analysis (Δ- - -Δ). Parallel broken lines represent 95% confidence limits. Receptor concentration (B_{max}) was determined from the x intercept and the affinity from the negative reciprocal of the slope of the line relating bound/free (B/F) radioligand to the concentration of specifically bound [^{3}H]prazosin. Using LIGAND, a K_D of 58 pM and a B_{max} of 83,000 ± 5,800 sites/cell were determined, compared to 120 pM and 93,000 sites calculated by linear regression analysis (r = 0.97).

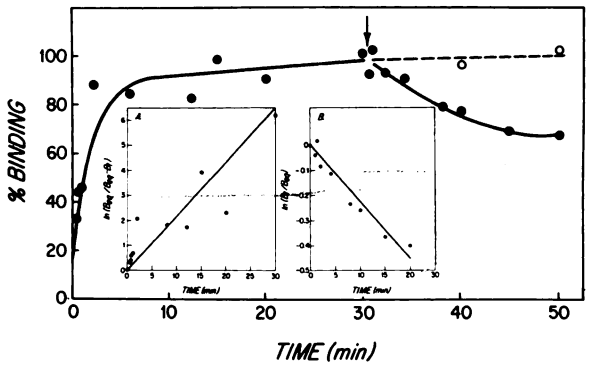

FIG. 2. *Kinetics of [^{3}H]prazosin binding to freshly isolated hepatocytes*

In the association reaction, hepatocytes (200,000 cells in 100 μl of PBS buffer) were incubated with 25 μl of [^{3}H]prazosin (0.3 nM, final concentration) and 25 μl of either 10^{-5} M phentolamine or buffer at 25°. At the times indicated, the incubation was terminated by vacuum filtration as described in Materials and Methods. In the dissociation reaction, after 30-min preincubation, the reaction mixture in some tubes was diluted (arrow) to 4–5 ml with buffer and the incubation continued at 25° for the times indicated. O, per cent binding in control tubes incubated for 40 and 50 min; stability of the binding is shown over these times. Association and dissociation rate constants were calculated as previously described (24). The apparent rate constant (k_{app}) for the pseudo-first order association reaction was calculated from the slope of the line (inset A) relating ln $[B_{eq}/(B_{eq} - B_t)]$ and time (determined by linear regression analysis, r = 0.91), where B_{eq} is the amount of [^{3}H]prazosin bound at equilibrium and B_t is the amount bound at each time t. The second order rate constant (k_1) was calculated according to the equation $k_1 = (k_{ap} - k_2)/[[^3H]prazosin]$, where k_2 is the dissociation rate constant. This latter value was determined from the dissociation reaction, where k_2 is the slope of the line (inset B) relating ln (B_t/B_{eq}) and time (determined by linear regression analysis, r = −0.95), where B_t is the amount of specific binding at each time after dilution and B_{eq} is the amount of binding at equilibrium. A k_1 = 143.4 × 10^6 M^{-1} min^{-1} and a k_2 = 0.022 min^{-1} were calculated from these studies giving a $K_D = k_2/k_1$ of 150 pM.

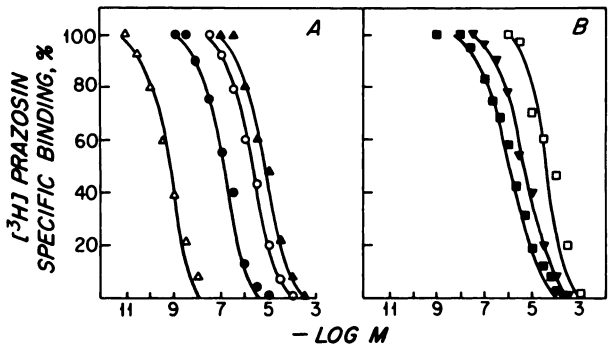

FIG. 3. *Inhibition of [^{3}H]prazosin (0.5–1 nM) binding to freshly isolated hepatocytes by various adrenergic agents*

A, antagonists: △, prazosin; ●, phentolamine; ○, rauwolscine; ▲, alprenolol. B, agonists: ■, (−)epinephrine; ▼, (−)norepinephrine; □, (+)epinephrine. Values shown are the means of triplicate determinations.

to inhibit [^{3}H]prazosin binding. Stereospecificity of binding was also observed; the (−) isomer of epinephrine was at least 10-fold more potent than its (+) isomer.

In equilibrium binding studies with freshly isolated hepatocytes, [^{125}I]pindolol interacted with a single class of binding sites with a K_D of 66 ± 6 pM (n = 12) (Fig. 4). The mean binding site concentration per cell was calculated as 2,000 ± 700. Due to the low binding site concentrations, specific binding represented only 30–40% of the total binding at the K_D. Goodness of fit was best for a one-site model for the ligand-receptor interaction. Identical dissociation constants were obtained in membrane preparations from isolated hepatocytes. Nonspecific binding of the nonselective β antagonist [^{3}H]alprenolol was significantly higher than that observed with [^{125}I]-pindolol, to the extent that computer-assisted curve-fitting could not be performed to give meaningful estimates of the K_D and binding site concentrations. Therefore, subsequent studies were performed with the radioiodinated ligand. Binding studies in intact hepatocytes performed with the hydrophilic β-adrenergic antagonist [^{3}H]CGP 12177 yielded binding site concentrations similar to those observed with [^{125}I]pindolol. The binding of this radioligand was investigated as it has previously been shown that [^{125}I]pindolol, a liphophilic ligand, may detect binding sites that are not confined to the cell surface when assays are performed at 25°, as was the case in this study.

Competitive inhibition studies with the isolated hepatocytes showed the expected order of potency for the β_2-adrenergic receptor (Fig. 5). (−)Norepinephrine was 5-fold less potent than (−)epinephrine in its ability to inhibit [^{125}I]pindolol binding. Stereospecificity of binding was confirmed by the finding that the (−)isomer of propranolol was almost 2 log-fold more potent than its (+)isomer.

While computer-assisted analysis showed that all competing antagonists bound to a single class of sites, agonist inhibition curves for both the α_1- and β_2-adrenergic sites were best described by a two-site model, in which approximately 30% of all the receptors were in a high affinity state (Table 1). A similar proportion of high and low affinity receptors was observed in membrane prepa-

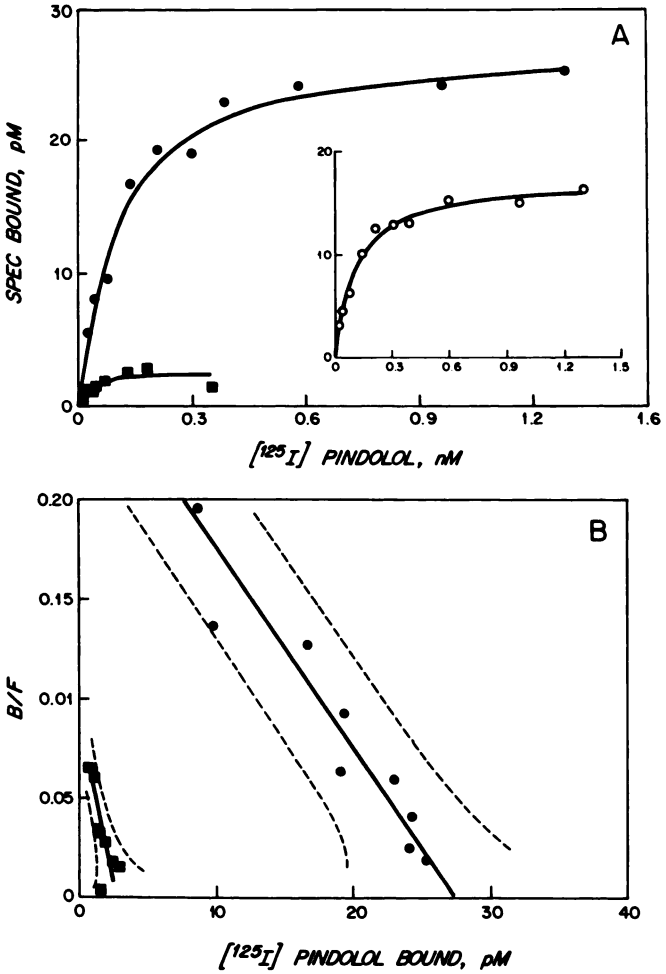

FIG. 4. *Equilibrium binding studies performed with the radioligand [^{125}I]pindolol and freshly isolated hepatocytes (■), hepatocytes maintained in monolayer cell culture for 24 hr (●), and in membranes prepared from the cultured cells (○)*

A, binding isotherm. B, Scatchard plot of the data for freshly isolated and cultured hepatocytes shown in A. Studies were performed as described in Materials and Methods. Values shown are the means of triplicate determinations. Parallel broken lines indicate 95%, confidence limits.

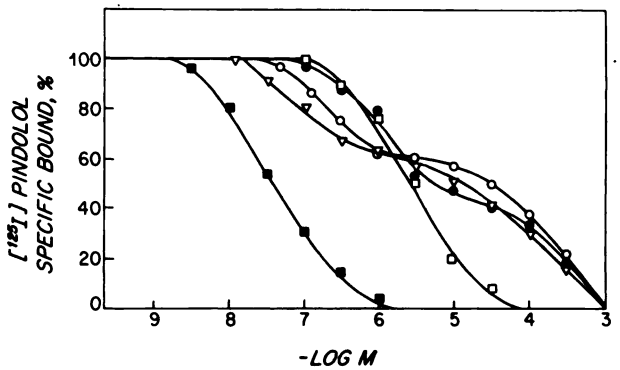

FIG. 5. *Inhibition of [^{125}I]pindolol (0.1 nM) binding to freshly isolated hepatocytes by various adrenergic agents*

■, (−)propranolol; □, (+)propranolol; ▽,(−)isoproterenol; ○, (−)epinephrine; ●, (−)norepinephrine. Values shown are the means of triplicate determinations.

TABLE 1

Apparent dissociation constants (K_D ± the standard error in nanomolar) of various adrenergic agents determined in intact isolated hepatocytes

	α_1 receptor[a]		β_2 receptor[a]	
	K_H	K_L	K_H	K_L
Agonists[b]				
(−)Epinephrine	20 ± 4	3,800 ± 500	48 ± 7	72,000 ± 16,000
(+)Epinephrine		26,000 ± 1,800	-	-
(−)Norepinephrine	212 ± 24	30,500 ± 900	244 ± 26	63,000 ± 18,000
(−)Isoproterenol			23 ± 4	57,000 ± 20,000
Antagonists		K_D		K_D
Prazosin		0.15 ± 0.06		
Phentolamine		34 ± 4		
Rauwolscine		9,000 ± 400		
(−)Alprenolol		5,900 ± 70		
(−)Propranolol				4.0 ± 0.5
(+)Propranolol				530 ± 60

[a] K_D values were determined by computer analysis (LIGAND) of competitive inhibition curves using the radioligands [^{3}H]prazosin or [^{125}I]pindolol for α_1- and β_2-adrenergic receptors, respectively as described in Materials and Methods. Values shown are derived from three to six separate studies per drug.

[b] In most cases, the competitive inhibition curves for agonists could best be fitted to a two-site model. The K_D values for the computed high (K_H) and low (K_L) affinity sites are shown.

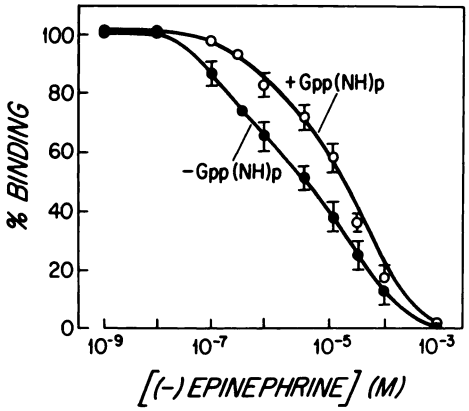

FIG. 6. *Competition by (−)epinephrine of [^{3}H]prazosin binding to membranes prepared from freshly isolated hepatocytes*

Studies were performed in the presence or absence of the nonhydrolyzable GTP analog Gpp(NH)p (0.1 mM). Values shown are the means ± standard error (bars) of four separate studies performed in triplicate.

rations from isolated hepatocytes. The dissociation constants for the high affinity sites were similar in intact cells and in membranes.

In competition studies using [^{3}H]prazosin as the radioligand and membranes prepared from isolated hepatocytes, Gpp(NH)p, a nonhydrolyzable analog of GTP, shifted the (−)epinephrine curve to the right, and computer analysis of the binding data now revealed only a single class of low affinity sites (Fig. 6). To demonstrate this Gpp(NH)p shift, it was necessary to prepare the membranes in the presence of 5 mM EGTA as reported by Lynch *et al.*[1] A similar Gpp(NH)p-induced shift of the (−)epinephrine competitive inhibition curve to a single class of binding sites was also observed for membrane β_2-adrenergic receptors.

To investigate the functional significance of the binding data, the adrenergic control of a receptor-coupled response, glycogenolysis, was evaluated in hepatocytes freshly isolated, as for the binding studies. In these experiments, the activity of phosphorylase *a*, an enzyme involved in the glycogenolytic pathway, was quantitated. Activity of this enzyme has previously been demonstrated to be mediated by both α_1- and β_2-adrenergic receptors, with the α_1 response predominating (2). These findings were confirmed in the present study. As shown in Fig. 7, the nonspecific agonist (−)epinephrine caused a dose-related increase in phosphorylase *a* activity. (−)Epinephrine induced a maximal increase of 140% ($p < 0.001$) over control (15.1 nmol min^{-1} per mg of protein) at a concentration of 10^{-5} M. Enzyme activation was also observed with the β-adrenergic receptor-specific agonist isoproterenol (60%, $p < 0.05$) and the α_1-selective agonist phenylephrine (110%, $p < 0.01$). Additionally, the α_1-selective antagonist prazosin (10^{-6} M) inhibited the activation of phosphorylase *a*, induced by maximal doses of (−)epinephrine (10^{-5} M), by 85% ($p < 0.001$), whereas the β-antagonist propranolol (10^{-6} M) was without effect (Fig. 10).

Studies in cultured hepatocytes. To investigate the effect of cell culture on the adrenergic receptor composition of isolated hepatocytes, the cells were maintained in monolayer culture for defined intervals and then resuspended for use in radioligand binding assays. Under these conditions, the cells remain nondividing as evidenced by the finding that DNA synthesis was almost absent (<5% [^{3}H]thymidine incorporated into nuclei as determined by autoradiography). Viability of the resuspended cells was >90% as defined by trypan blue exclusion.

After 3 hr in cell culture, the number of α_1-adrenergic receptors was unaltered (93,000 ± 5,400 sites/cell, control; 90,000 ± 6,200 cultured cells). Subsequently, the number of α_1 receptors decreased with a half-life of 18 hr to 15,000 ± 2,100 ($p < 0.001$) at 72 hr (Fig. 8). In cells cultured for 24 hr, there was a slight but insignificant increase in the affinity of the radioligand [^{3}H]prazosin for the α_1-binding sites from a K_D of 127 to 114 pM. In contrast to the α_1 receptor response, the number of β-

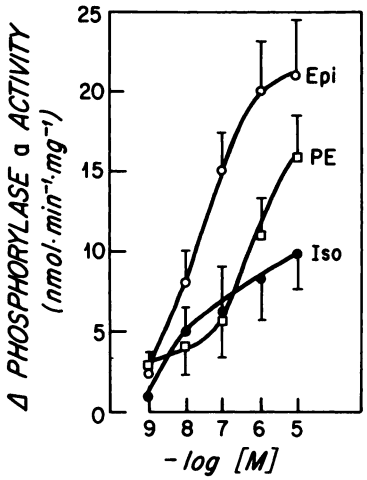

FIG. 7. *Effect of epinephrine (Epi), phenylephrine (PE), and isoproterenol (Iso) on phosphorylase a activity in freshly isolated hepatocytes*

Basal phosphorylase *a* activity was 15.1 ± 2.5 nmol min^{-1} mg^{-1} protein. Values shown are the means ± standard error (bars) of 3–6 experiments per drug.

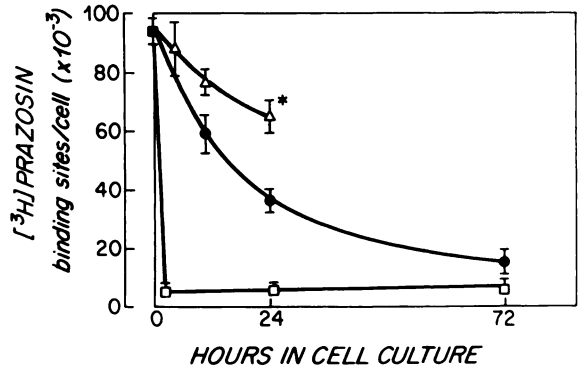

FIG. 8. *Effect of cell culture on α_1-adrenergic receptor density in untreated hepatocytes (●), cells cultured in the presence of cycloheximide (△), and cells pretreated with phenoxybenzamine (10 μM) (□) as described in Materials and Methods*

Values were determined from Scatchard plots of equilibrium binding studies and are the means ± standard error (bars) of 4–12 different experiments at each time. The asterisk indicates that these studies were not performed for longer than 24 hr, as the rate of cell death increased markedly after this time.

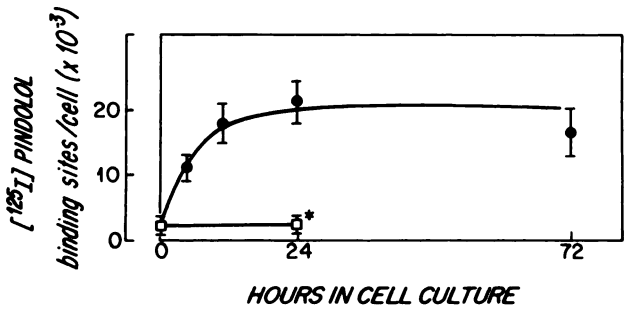

FIG. 9. *Effect of cell culture on β_2-adrenergic receptor density in untreated hepatocyte (●) and cells cultured in the presence of cycloheximide (□)*

Studies were performed as described in Materials and Methods. Values shown are the means ± standard error (bars) of 7–12 different experiments at each time. See legend to Fig. 8 for significance of asterisk.

adrenergic receptors increased 10-fold during primary cell culture from 2,000 ± 700 to 21,500 ± 2,100 ($p < 0.001$) at 72 hr (Fig. 9). Indeed, a 10-fold increase in binding site concentration was already observed after 12 hr. At 24 hr, the K_D of [^{125}I]pindolol increased from 66 to 112 pM ($p < 0.05$).

Similar changes in binding site concentrations were observed in membranes prepared from the cultured hepatocytes. The number of α_1-adrenergic receptors decreased from 116 ± 11 to 46 ± 4.0 fmol/mg of protein ($p < 0.05$), and the number of β-receptors increased from 4.9 ± 0.5 to 23.6 ± 4.3 fmol/mg of protein ($p < 0.01$) after 24 hr of culture. These culture-induced changes in adrenergic receptors were associated with a switch in the adrenergic control of glycogenolysis. Thus, in contrast to the response in freshly isolated hepatocytes, after 24 hr in culture, the β antagonist propranolol (10^{-6} M) now inhibited (−)epinephrine (10^{5} M)-induced activation of phosphorylase *a* by 90% (Fig. 10).

To investigate the mechanism(s) of the culture-induced changes in hepatocyte adrenergic receptors, a variety of perturbations were examined. As shown in Table

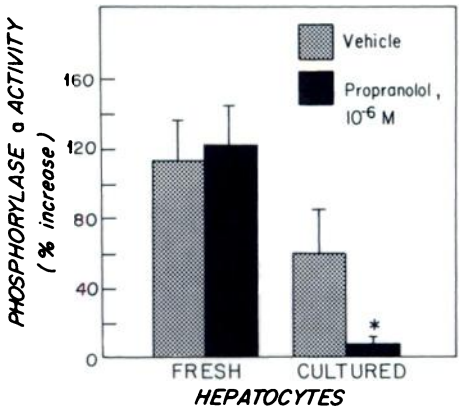

FIG. 10. *Effect of propranolol on epinephrine (10^{-5} M)-induced activation of phosphorylase a activity determined in freshly isolated hepatocytes and cells maintained in culture for 24 hr*

Values shown are the means ± standard error (bars). The asterisk indicates significant difference ($p < 0.01$) from the vehicle value. Basal phosphorylase *a* activity in fresh and cultured hepatocytes was 18.0 ± 2.5 and 14.8 ± 2.0 nmol min^{-1} mg^{-1} protein.

2 and Fig. 9, the translational and transcriptional protein synthesis inhibitors cycloheximide and actinomycin D prevented the increase in β_2-adrenergic receptors and attenuated the decrease in α_1 receptor-binding sites, respectively, (Fig. 8). However, omission from the culture medium of hydrocortisone, which was routinely included to improve plating efficiency and longevity (21), or culture in serum-free medium was without effect. Similarly, alterations in the hormonal composition of the culture medium by the addition of triiodothyronine, the α agonist (−)norepinephrine, or the β antagonist (−)isoproterenol did not prevent the reciprocal changes in adrenergic receptors. The effect of colchicine, an agent which disrupts the microtubular system, cytochalazine B, an inhibitor of microfilament formation, and the lysosome-stabilizing agents chloroquine and methylamine on the culture-induced decrease in α_1 receptors was also examined. Again these perturbants did not prevent the decrease in number of α_1-adrenergic receptors.

The ability of the irreversible α-adrenergic antagonist phenoxybenzamine to inactivate α_1-adrenergic receptors was also investigated. In these studies, freshly isolated hepatocytes were incubated with various concentrations of phenoxybenzamine for 20 min and the cells then were washed extensively. This procedure led to a dose-dependent decrease in the number of [^{3}H]prazosin-binding sites (Fig. 11). Phenoxybenzamine (1 μM, $n = 3$) resulted in a 51 ± 3% loss of binding sites, whereas a concentration of 10 μM decreased the number of binding sites by 90 ± 6% ($n = 5$). At this dose (and at higher doses, which inactivated more than 95% of the binding sites), the K_D of [^{3}H]prazosin was also markedly higher. This increase in K_D may be artifactual due to the low binding site concentrations, which did not permit the precise estimation of the computed binding parameters. This is evidenced by the broad 95% confidence limits observed in these experiments (Fig. 11).

When freshly isolated hepatocytes were plated after treatment with phenoxybenzamine (10 μM), there was no change in the number of the remaining α_1-adrenergic

TABLE 2

Influence of various perturbations on the effect of cell culture (24 hr) on hepatocyte α_1- and β_2-adrenergic receptor concentrations (B_{max}, sites/cell) and affinity (K_D in nanomolar for the radioligands [^{3}H]prazosin and [^{125}I]pindolol

Condition[a]	[^{3}H]Prazosin		[^{125}I]Pindolol	
	B_{max} ($\times 10^3$)	K_D	B_{max} ($\times 10^3$)	K_D
Freshly isolated hepatocytes	93.0 ± 5.4[b]	0.127 ± 0.01	2.0 ± 0.7[b]	0.066 ± 0.007
Cultured hepatocytes				
Control	36.0 ± 3.5	0.116 ± 0.013	21.5 ± 2.1	0.116 ± 0.014
−Hydrocortisone	32.3 ± 2.9	0.111 ± 0.020	16.8 ± 3.0	0.094 ± 0.025
Serum-free medium	29.4 ± 2.1	0.115 ± 0.011	17.4 ± 3.1	0.086 ± 0.030
Triiodothyronine (15 nM)	37.0 ± 2.0	0.129 ± 0.015	22.5 ± 4.1	0.129 ± 0.039
(−)Norepinephrine (10 μM)[c]	39.2 ± 1.9	0.015 ± 0.019	19.8 ± 2.9	0.123 ± 0.032
(−)Isoproterenol (10 μM)[c]	36.1 ± 2.0	0.016 ± 0.013	19.2 ± 3.5	0.140 ± 0.029
Colchicine (10 μM)	39.2 ± 3.0	0.117 ± 0.016		
Cytochalasine B (10 μM)	41.0 ± 2.9	0.119 ± 0.016		
Cycloheximide (75 μM)	66.5 ± 4.1[d]	0.121 ± 0.015	2.0 ± 0.19[b]	0.055 ± 0.010
Actinomycin D (1 μg/ml)	64.2 ± 5.6[d]	0.122 ± 0.018	1.9 ± 0.21[b]	0.046 ± 0.012
Chloroquine (0.1 mM)	39.4 ± 4.1	0.115 ± 0.013		
Methylamine (0.1 mM)	36.5 ± 3.6	0.125 ± 0.020		

[a] Studies were performed as detailed in Materials and Methods. Results shown are the means ± standard error for 3–18 separate studies per condition.

[b] $p < 0.001$ vs. control.

[c] $p < 0.01$ vs. control.

[d] In these studies, hepatocytes were incubated with medium containing the adrenergic agonists at the doses indicated. After resuspension of the cultured cells they were washed extensively (4 × 50 ml of PBS) to remove the agonists before assay for adrenergic receptors.

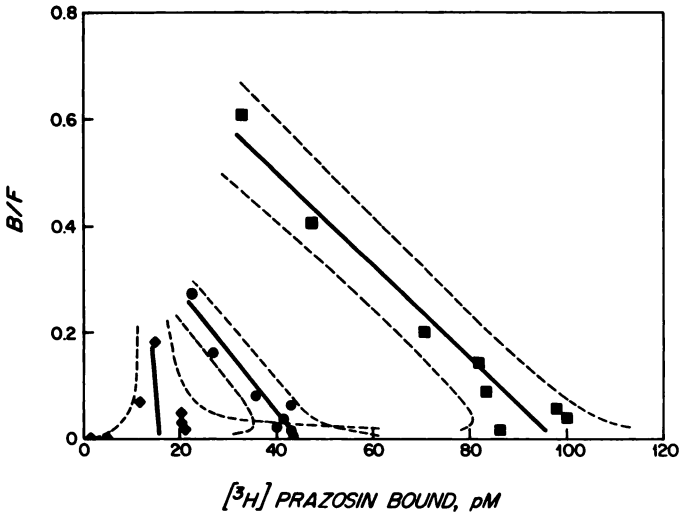

FIG. 11. *Representative Scatchard plots of equilibrium binding studies performed with untreated hepatocytes (■) and cells pretreated with phenoxybenzamine*

●, 1 μM; ♦, 10 μM. Cells were incubated with buffer or phenoxybenzamine for 30 min at 32° and then washed with 4 × 50 ml aliquots of PBS before assay. Parallel broken lines indicate 95% confidence limits.

receptors over a period of 72 hr (Fig. 8). The increase in the number of β-adrenergic receptors was not affected by preincubation of hepatocytes with phenoxybenzamine. This finding indicates per se that phenoxybenzamine, an alkylating agent, did not interfere with protein synthesis.

DISCUSSION

Isolated rat hepatocytes are widely used as a model system to study the effects of hormones *in vitro* (22). While rat liver has been a useful tissue source for the pharmacological and molecular characterization of adrenergic receptors (23–26), as well as of their signal transduction mechanisms (27, 28) and effector-coupled responses (29), few studies have evaluated the binding characteristics of adrenergic receptors in intact isolated hepatocytes. Attempts to identify adrenergic receptors in these cells have met with limited success (30, 31). As the viability and functional integrity of hepatocytes isolated by collagenase perfusion techniques have been established, and as radioligand binding in intact cells more closely approximates the *in vivo* hormone-receptor interaction, we initiated the present study in an effort to characterize hepatocyte adrenergic receptors from whole cell-binding experiments. We then applied these techniques to investigate the role of adrenergic receptors in the switch in the adrenergic control of glycogenolysis from an α to β pathway that has been observed with culture of hepatocytes (9), as well as a variety of pathophysiological states such as precarcinogenesis (32, 33), partial hepatectomy (34), bile duct ligation (35–37), adrenalectomy (38, 39), and thyroidectomy (40). To substantiate our findings from whole cell-binding experiments, radioligand-binding studies were also performed in membranes prepared from both freshly isolated and cultured hepatocytes, while the functional relevance of the binding data was evaluated via studies on the adrenergic control of phosphorylase *a* activity.

As demonstrated in Figs. 1–5, binding of the highly selective α_1-adrenergic antagonist [^{3}H]prazosin was of high affinity, saturable, kinetic, and specific. Similar binding characteristics were observed in membranes prepared from the isolated hepatocytes. Binding sites with a typical β_2-adrenergic receptor specificity were identi-

fied in studies with the β-adrenergic antagonist [^{125}I] pindolol performed with either isolated whole cells or membrane preparations. In the intact hepatocytes, both radioligands [^{3}H]prazosin and [^{125}I]pindolol interacted with an apparent single class of sites. However, analysis of equilibrium binding data indicated markedly different receptor concentrations for α_1- and β_2-adrenergic sites (93,000 and 2,000 sites/cell, respectively). Thus, the dominance of the α_1-adrenergic pathway in the control of glycogenolysis, demonstrated in previous studies (29) and confirmed in the present investigations (Fig. 7), is associated with a 46-fold excess of α_1- over β_2-adrenergic receptors.

In both freshly isolated hepatocytes and in membranes prepared from these cells, competition curves with antagonists at both α_1- and β_2-adrenergic receptors were typically "steep" and monophasic. By contrast, curves for agonists were "shallow" and the binding was best modeled to two sites with high and low affinity (Table 1). In the presence of Gpp(NH)p, only a single class of low affinity sites could be detected in membranes. With β-adrenergic receptors, this conversion by Gpp(NH)p is postulated to result from a dissociation of the ternary complex between agonist, receptor, and G-protein (41). Although a mechanistic role of a guanine nucleotide-binding protein has not been clearly defined for the signal transduction pathwy of α_1-adrenergic receptors, it appears that under certain circumstances α_1 receptors can mediate an increase in cAMP formation (4). A coupling between this receptor subtype, and the pathway normally used for the β-adrenergic system may, therefore be operative in the hepatocyte. Indeed, in keeping with the findings of Goodhardt *et al.* (42) and Lynch *et al.*[1] a Gpp(NH)p-induced conversion of α_1-adrenergic receptors to a state of low affinity for agonists could be demonstrated in membranes prepared from freshly isolated hepatocytes.

Whole cell-binding techniques were then applied to isolated hepatocytes maintained in monolayer cell culture under nondividing conditions. The ability to successfully perform radioligand-binding assays in cultured hepatocytes was dependent upon a number of factors. First, cells had to be plated onto collagen-coated culture dishes. This allowed the formation of monolayer cell cultures, resuspension by gentle mechanical means not involving trypsinization and dispersion of the hepatocytes to form single cell suspensions. Second, as plasma membrane markers change during isolation by collagenase perfusion and with monolayer cell culture (43), binding site concentrations were expressed in terms of a reliable denominator, cell number. Third, by assaying cells obtained from the same tissue culture plates, the precision of direct comparisons between α_1- and β_2-adrenergic receptor subtypes was enhanced.

After 24 h in cell culture, the number of α_1-adrenergic receptors per cell decreased by 60% from 93,000 to 36,000 and the number of β_2-adrenergic receptors increased 10-fold from 2,000 to 21,000 sites/cell. These changes were rapid in onset with reciprocal alterations in receptor densities already apparent after 8 hr. It is unlikely that plating itself was responsible for these changes in receptor density, as 3 hr after plating, at a time when the cells had already attached to the culture dishes, no marked alterations were observed in the receptor densities over those found in the freshly isolated hepatocytes. A differential dying off of some cells also seems unlikely, as there was only a slight decline in total DNA content over 24 hr in culture. Similarly, the changes cannot be explained by overgrowth of one cell type as <5% of all nuclei showed [^{3}H]thymidine uptake.

Associated with these changes in receptor density, the adrenergic control of phosphorylase *a* activity was found to switch from a predominantly α_1 to a β_2 type. In a previously reported study (44), in which only membrane β_2-adrenergic receptor concentrations were investigated, a 7-fold increase in the number of membrane-binding sites was observed at 7 hr of cell culture. It is of interest that in this study hepatocytes from adult male rats were employed. These cells, in contrast to those used in the present study, which were obtained from adult female rat livers, lack a β-adrenergic component to the control of glycogenolysis (9, 44). Nevertheless, culture of adult male rat hepatocytes results in the emergence of functionally coupled membrane β-adrenergic receptors as well as a decreased sensitivity of glycogen-phosphorylase to α_1-adrenergic stimulation (9).

With the identification of the culture-induced changes in adrenergic receptors, studies were performed to investigate potential mechanisms. Involvement of a serum-derived factor was excluded by culture in serum-free medium. As adrenalectomy leads to a change in the number of β-adrenergic receptors and may modulate agonist affinity at α_1-adrenergic sites (42), studies were performed in which hydrocortisone was omitted from the culture medium. The changes in adrenergic receptor densities were unaltered by this perturbation. Thyroidectomy leads to a decreased α-adrenergic sensitivity of glycogenolysis in isolated hepatocytes (40), which can be reversed by application of triiodothyronine. However, culture in the presence of this hormone did not influence the change in receptor densities. To determine if the presence of α and β agonists influences the changes in adrenergic receptors, hepatocytes were cultured in the presence of (−)isoproterenol and (−)norepinephrine, which may be expected to promote down-regulation of the receptors. No further decreases in the number of α_1 receptors were observed under these conditions, and the increase in β_1-adrenergic receptors could not be prevented.

The increase in the number of β-adrenergic receptors could be prevented by inhibition of protein synthesis with the translational inhibitor cycloheximide, as previously reported by Nakamura *et al.* (44). Similar results were obtained with actinomycin D, which acts at the transcriptional level. These findings suggest that the culture-induced increase in β-adrenergic sites is due to *de novo* synthesis rather than to other mechanisms such as unmasking of existing receptor sites. However, the trigger for this *de novo* synthesis of receptor protein remains unclear.

In contrast to these results with β receptors, no *de novo* synthesis of α_1-adrenergic receptors seems to occur

with culture, as neither cycloheximide nor actinomycin D accelerates the loss of α_1 receptors. Rather, these agents attenuated the decline in the number of α_1 receptor sites. Similar attenuation of protein degradation by protein synthesis inhibitors has been observed in other systems (45). For example, cycloheximide leads to an accumulation of surface receptors for insulin (46) and human chorionic gonadotropin (47) as well as to an increase in the half-life of epidermal growth factor receptors (48). The nature of this process is unclear. As constant recycling, initiated by endocytosis, has been demonstrated for these surface receptors, it has been suggested that a protein with a short half-life is involved in the initial step of this process, as the reappearance of internalized receptors is not affected by cycloheximide (49).

Further evidence that the degradation of α_1-adrenergic receptors is accelerated in culture, comes from finding that receptor concentration decreased with a half-life of 18 hr. This is considerably faster than the 42 hr reported by Lynch *et al.* (50) from *in vivo* studies in rat liver. In addition, studies with the irreversible α-adrenergic antagonist phenoxybenzamine provide additional evidence for a lack of *de novo* α_1 receptor synthesis in culture. Thus, when freshly isolated hepatocytes, pretreated with high concentrations of phenoxybenzamine, were cultured, no increase in α_1-adrenergic receptors was observed. In the same cells, however, β-adrenergic receptors increased 10-fold. This latter observation excludes a direct effect of phenoxybenzamine on protein synthesis.

Finally, changes in the cellular cytoskeleton probably do not contribute to the loss of α_1-adrenergic receptors, nor is it likely that lysosomes are involved as neither cytocholazine B and colchicine nor chloroquine and methylamine prevented the loss of receptors.

In conclusion, these studies provide evidence that reciprocal changes in the number of adrenergic receptors are responsible for the switch in the adrenergic control of glycogenolysis from an α_1 to a β_2 type observed with culture of hepatocytes. The exact trigger mechanism for these reciprocal changes in adrenergic receptors remains unclear, although *de novo* synthesis of β_2-adrenergic receptors and accelerated degradation of α_1-adrenergic receptors are clearly involved. Additionally, as the changes in receptor density are not affected by measures that profoundly influence receptor expression *in vivo*, they may be part of a "fetalization" phenomenon as suggested by Okajima and Ui (9). Evidence for such a process is the loss of liver-specific functions and the appearance of fetal enzymes such as α-fetoprotein in cultured hepatocytes (51). Altered cellular contacts during primary cell culture may initiate this process as it can be reversed by membrane proteins isolated from adult rat livers (52, 53). This possibility, which is currently under investigation, suggests a direct influence of cell surface components on plasma membrane adrenergic receptor composition.

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