Switching from α_1- to β-subtypes in adrenergic response during primary culture of adult-rat hepatocytes as affected by the cell-to-cell interaction through plasma membranes

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The α_1-adrenergic response was predominant over the β-adrenergic one in adult rat hepatocytes, when the responses were measured as the agonist-induced generations of Ins(1,4,5)P_3 and cyclic AMP, respectively. During primary culture of the adult rat hepatocytes, the β-adrenergic response developed rapidly, whereas the α_1-response decreased gradually. Such receptor-subtype switching did not occur unless the cells were cultured under conditions favourable for cell growth, i.e. at low cell density (10^4 cells/cm^2). The switching was prevented progressively as the cell culture density was increased up to 20-fold or the low-density culture was achieved by addition of increasing amounts of liver plasma membranes. The gradual decrease in α_1-response was accounted for by a concurrent decrease in the receptor site density, whereas rapid development of the β-response definitely preceded the increase in β-ligand binding sites during the culture. This rapid development of the β-response reflected enhanced coupling of the receptor to G-protein during the early stage of culture, as evidenced by the progressively developed ability of GTP to lower the affinity of β-agonist binding to membranes prepared from these short-time-cultured hepatocytes.

INTRODUCTION

Adrenergic responses of adult male rat hepatocytes reflect α_1-adrenergic-receptor functions almost exclusively. This forms a sharp contrast with the overwhelming predominance of β_2-receptor-mediated responses over α_1-responses in livers of fetal or newborn rats (Blair et al., 1979; Morgan et al., 1983; Katz et al., 1985; Noguchi et al., 1985; Schleifer et al., 1989) or in regenerating livers of mature rats after surgical (Huerta-Bahena et al., 1983a,b; Aggerbeck et al., 1983; Goodhardt et al., 1984; Okajima and Ui, 1984) or chemical (García-Sáinz and Nájera-Alvarado, 1986) injury. It would therefore be reasonable to assume that development of β-adrenergic responses would be an event somehow related to hepatocyte proliferation. Maintenance of hepatocytes at the growth-arrested quiescent stage appears to provide conditions favourable for α_1-receptor-mediated functions to take the place of β-receptor-mediated ones.

The similar transition of adrenergic responses from α_1- to β-type occurred *in vitro* during primary culture of adult-rat hepatocytes (Okajima and Ui, 1982; Nakamura et al., 1983a), which proved to be a good system for the study of underlying mechanisms such as alteration of the receptor site density on the cell surface and modification of intracellular signals arising from the receptor stimulation (Nakamura et al., 1984a; Itoh et al., 1984; Kunos et al., 1984; Schwarz et al., 1985a; Tsujimoto et al., 1986; Ishac and Kunos, 1986).

Development of various hepatic functions, including cell proliferation and differentiation (e.g. expression of genes coding for the hepatic enzymes), is strictly dependent on the density of hepatocytes during culture (Nakamura et al., 1983b,c). The effect of increasing the cell density on the development was mimicked by the addition of plasma membranes to the low-cell-density culture (Nakamura et al., 1984b). The purpose of the present paper is to show 'α_1-to-β' subtype transition, as well as inhibition of the transition, of receptor-mediated signalling during culture of hepatocytes as a function of the culture time, cell density or the amount of plasma membranes added to the low-cell-density culture. These culture conditions exerted profound influences not only on α- and β-receptor densities on the cell surface but also on the efficiency of β-receptor coupling to the signal-transducer G-protein.

EXPERIMENTAL

Isolation of hepatocytes

Hepatocytes were isolated from male rats of the Wistar-derived Donryu strain, aged 8–12 weeks, by the collagenase perfusion method (Berry and Friend, 1969), mostly by the procedure described by Dunn et al. (1989). Briefly, the liver was perfused with Ca^{2+}-free Hank's solution (pH 7.4), containing 0.3 mM EGTA, 5.5 mM glucose and 20 mM Hepes, at a rate of 20 ml/min for 5 min in the 'flow-through' manner. The perfusate was maintained at 37 °C and equilibrated with O_2/CO_2 (19:1). The liver was subsequently perfused with 0.05% collagenase (type IV, Wako) solution supplemented with 5 mM $CaCl_2$, 5 mM sodium pyruvate and 100 units/ml aprotinin for 10 min in a recirculating circuit, and finally made free of collagenase by 'flow-through' perfusion with 50 ml of ice-cold Hepes-buffered solution. Hepatocytes were then dispersed in Leibovitz L-15 medium (ICN Flow) to obtain the cell pellet by repeated filtration

Abbreviations used cAMP, cyclic AMP; GTP[S], guanosine 5'-[γ-thio]triphosphate; G_s, heterotrimeric GTP-binding protein involved in adenylate cyclase stimulation; G_q, heterotrimeric GTP-binding protein involved in phospholipase C activation.

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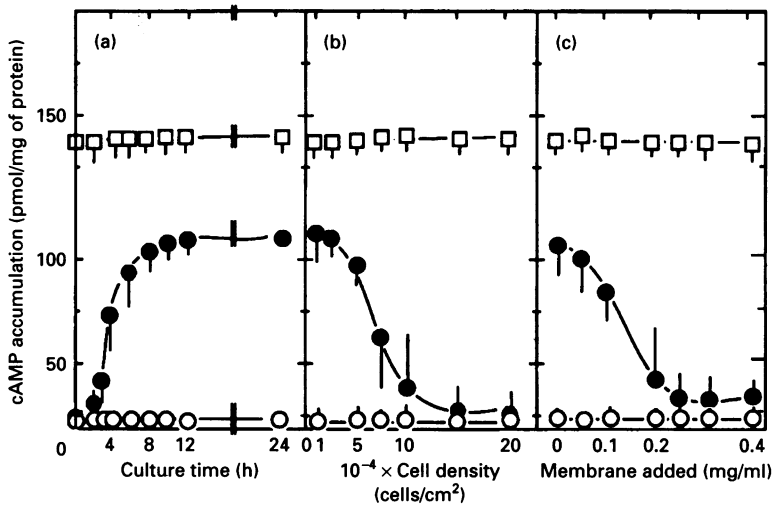

Figure 1 Progressive increases in β-adrenergic responses during hepatocyte culture, and its prevention by increasing the cell density or by adding liver plasma membranes

Adult-rat hepatocytes were cultured at a density of 10^4 cells/cm^2 for the indicated periods of time (**a**), at the indicated cell densities for 24 h (**b**), or at a density of 10^4 cells/cm^2 for 24 h with addition of the indicated amounts (mg of protein/ml of culture medium) of partially purified liver plasma membranes (**c**). The membranes had been purified from adult-rat liver as described in the Experimental section and added at 1 h after the beginning of culture. The cell monolayers thus cultured and washed were incubated with 100 μM isoprenaline (●) or 1 μM glucagon (□) to measure cAMP generation as described in the Experimental section. Data obtained without agonist are also shown (○). Each point represents the mean ± S.E.M. from triplicates within a typical experiment which was repeated more than three times with similar results.

through two nylon meshes with grid sizes of 250 and 52 μm, each time followed by centrifugation at 50 *g* for 1.5 min at 4 °C. To minimize (< 1%) contamination with non-parenchymal cells, the cell pellet was resuspended to 50 ml, and 12.5 ml of the cell suspension was added to 10.8 ml of Percoll (Pharmacia, $\rho = 1.13$ g/ml) and 1.2 ml of 10-times-concentrated Williams' E (WE) medium (ICN Flow). The mixture was centrifuged at 500 *g* for 5 min, and the pellet was washed twice with WE medium.

Hepatocyte culture

The isolated parenchymal hepatocytes were cultured as monolayers on 35 mm-diameter plastic dishes previously coated with collagen at cell densities indicated in the text at 37 °C under a 5%-CO_2 atmosphere. Collagen was prepared from rat tail tendon as described by Dunn et al. (1989). The culture medium (0.75 ml/dish) used was WE medium (unless otherwise noted below) containing 5% (v/v) fetal-calf serum (Gibco), 10 units/ml aprotinin, 1 nM insulin (Sigma), 1 nM dexamethasone (Sigma), 100 units/ml pencillin G (Banyu Seiyaku), 0.1 mg/ml streptomycin (Meiji Seika) and 10 units/ml mycostatin (Sigma). Cell attachment to dishes was complete within 1–1.5 h. After 3 h, the medium was changed to the same medium not supplemented with serum, in which cells were maintained viable with parenchymal-cell-specific appearance for 4–5 days if the serum-free medium was changed every 24 h. The first change of medium was made after three washings with the new medium to eliminate serum completely. Where indicated, the plasma-membrane preparation from adult-rat liver (see below) was added to the medium at 1 h. At the end of culture, the cell monolayers were washed several times with appropriate medium before being applied to the subsequent assays.

Purification of rat plasma-membrane preparation

The general procedure was as follows. The liver was excised from a male Donryu-strain rat (body wt. 350–400 g), made free of blood by washing in ice-cold saline, and then subjected to the subcellular-fractionation scheme at 0–4 °C as described by Seyfred and Wells (1984). Briefly, the liver was homogenized with 5 ml of homogenization buffer (HB: 0.25 M sucrose containing 1 mM EDTA, 75 μg/ml phenylmethanesulphonyl fluoride, 10 mM Tricine and 25 units/ml aprotinin) for 2 min in a Waring blender and then for 5 min in a Potter–Elvehjem homogenizer with a Teflon pestle at the rate of 4 strokes/min. The pellet obtained by centrifugation at 1500 *g* for 5 min was washed once with 4 ml of HB by mixing in the Potter homogenizer and centrifugation under the same conditions before being subjected to gradient centrifugation (10000 *g* for 20 min) as a mixture of the suspension (14 ml in HB) and 45% Percoll ($\rho = 1.05$ g/ml, 11 ml). The 3 ml upper phase was diluted in 10 ml of HB and centrifuged at 1500 *g* for 5 min to obtain the plasma-membrane-rich pellet, which was washed with HB three times and stored at −80 °C until use.

Addition of plasma-membrane preparations to hepatocyte culture was made at 1 h of culture time in all experiments, in which most of the added plasma membranes were removed by washing at 3 h when the culture medium was changed to the fresh serum-free medium (see above). Thus, hepatocytes were exposed to the amount of plasma membranes indicated in the Figures for only 2 h. The effects of plasma membranes recorded in the text should therefore be 'irreversible' effects of these amounts of membranes exerted during the first 2 h, or, alternatively, the prolonged effects of much smaller amounts of the membranes surviving the process of washing at 3 h. The protein content of the crude membranes prepared from the plasma-membrane-supplemented monolayers (for assay of adenylate cyclase or radioligand binding) was usually 100–102% of the protein content of the preparation from control monolayers (not supplemented with plasma membranes). There was no significant difference in the assay data based on the protein content between these two preparations.

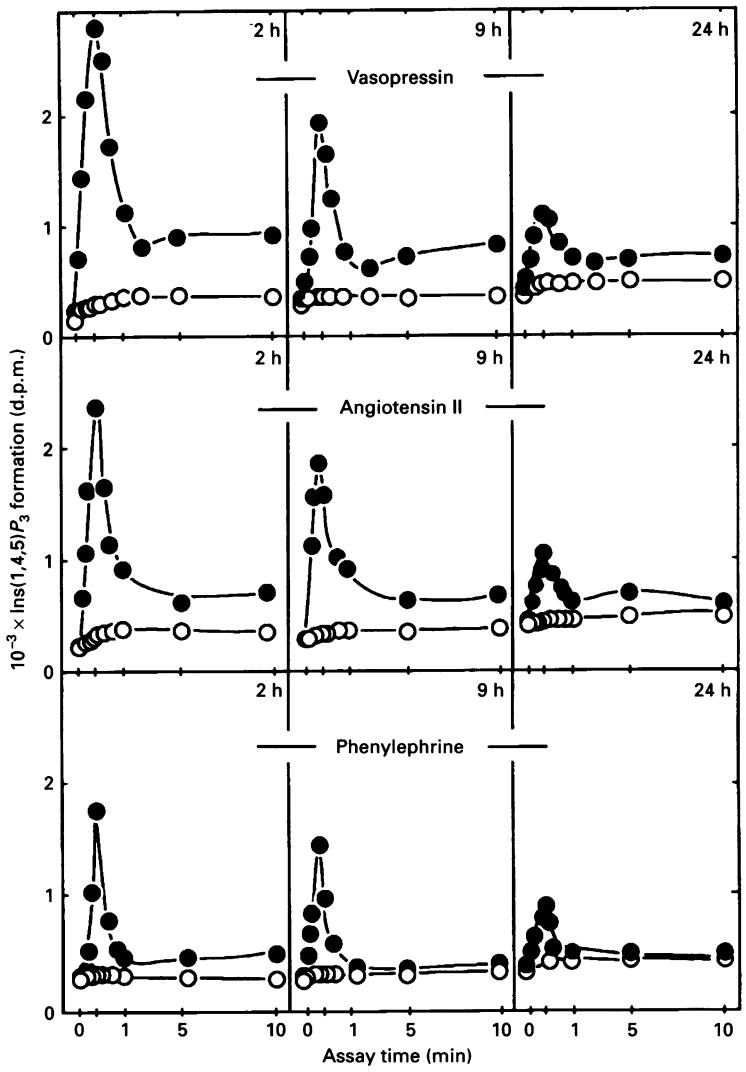

Figure 2 Transient increases in Ins(1,4,5)P_3 on addition of Ca^{2+}-mobilizing-receptor agonists to cultured hepatocytes

Hepatocyte monolayers that had been cultured at low cell density (10^4 cells/cm^2) for 2, 9 or 24 h (as indicated in the panels) were labelled with *myo*-[2-^{3}H]inositol for the last 90 min period of culture, and then assayed for InsP_3 generation in the presence of 0.1 μM vasopressin (top panels), 0.1 μM angiotensin II (middle panels) or 100 μM phenylephrine (bottom panels) as described in the Experimental section. The cell contents of radioactive InsP_3 (○, without addition; ●, with agonist) are plotted as a function of incubation time for assay. The data are representative ones from experiments performed more than three times with similar results.

Cyclic AMP (cAMP)-generating response of hepatocytes

At the end of culture, the culture medium was discarded and the cell monolayers were washed several times with Ca^{2+}-free Krebs–Ringer–Hepes (10 mM) medium (pH 7.4; Okajima and Ui, 1982). The monolayers were then incubated at 37 °C in the same medium (usually 0.8 ml/dish) supplemented with 2.5 mM $CaCl_2$, 0.2 mM 3-isobutyl-1-methylxanthine (Sigma) and 0.1 % BSA (Sigma). After incubation for 3 min, receptor agonists were added as indicated in the text, and the incubation was terminated at 10 min by addition of HCl to 0.2 M final concn., followed by immersing the dishes in a boiling-water bath for 3 min. Cellular cAMP quantitatively transferred to the supernatant was determined radioimmunochemically (Honma et al., 1977). Generation of cAMP progressed rapidly, and levelled off at 5–7 min of incubation under these conditions. For fresh (uncultured) hepatocytes, the cell suspension at a density of (1–2) × 10^5 cells/ml was incubated under the same conditions.

InsP_3-generating response of hepatocytes

Labelling of hepatocytes with *myo*-[2-^{3}H]inositol (NEN; 100 μCi/ml) was carried out at 37 °C for 90 min in TCM199 (Nissui Seiyaku, Tokyo, Japan) medium (containing 0.1 % BSA) for cultured cells or in Krebs–Ringer–Hepes medium for fresh cells. The incorporation of ^{3}H into phospholipid fractions was essentially the same for cells that had been cultured for different times. These ^{3}H-labelled cells were washed three times with Krebs–Ringer–Hepes medium and incubated with 15 mM LiCl for 15 min before the start of incubation with receptor agonists at 37 °C in the same medium. The reaction was terminated by adding 5 % trichloroacetic acid, which was then eliminated by shaking with water-saturated ether. The supernatant was submitted to batch-wise separation of inositol phosphates through a column of Dowex 1-X8 and counted for [^{3}H]InsP_3 radioactivity as described previously (Murayama and Ui, 1987). To increase the ^{3}H content of the InsP_3 fraction, we used 0.1 %-BSA-

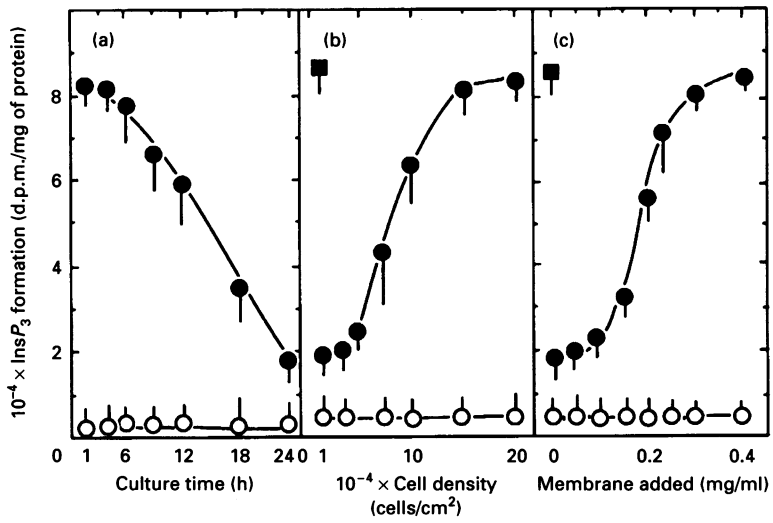

Figure 3 Progressive decreases in α_1-adrenergic-receptor-mediated InsP_3 generation during hepatocyte culture, and its prevention by increasing cell densities or adding liver plasma membranes

Hepatocytes were cultured in panels (**a**), (**b**) and (**c**) as described in Fig. 1. The cell monolayer thus cultured was then assayed for InsP_3 generation with 100 μM phenylephrine as in Figure 2. The peak value of InsP_3 with (●) or without (○) phenylephrine is plotted as a function of time in the low-cell-density culture (**a**), of cell density (**b**), or of the amounts of membranes added to the low-cell-density culture (**c**). The culture time was 24 h for (**b**) and (**c**). The initial value before the start of culture is shown in panels (**b**) and (**c**) (■). Each point represents the mean ± S.E.M. from triplicates within a typical experiment which was repeated more than three times with similar results.

supplemented TCM199 medium, instead of WE medium, which contains inositol, for culture of hepatocytes in this series of experiments. It was ascertained that hepatocytes cultured in the TCM199 medium up to 24 h displayed the same viability and alteration of adrenergic responses as did those cultured in WE medium.

Radioligand binding to α_1- and β-adrenergic receptors in hepatocytes

Most of the ligand-binding experiments were performed with crude membranes prepared by homogenizing, centrifuging (1500 *g* for 15 min) and washing cultured (or fresh) hepatocytes in Hepes-buffered medium. The membrane preparation (50 μg) was incubated for 30 min at 25 °C in 50 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$/1 mM EGTA/1 μM propranolol/0.8 mM ascorbic acid in a total volume of 200 μl for binding of [^{3}H]prazosin (0.01–5 nM; NEN) to α_1-receptors (Lynch et al., 1985b). Hepes buffer (50 mM, pH 7.4) and 2.5 μM 5-hydroxytryptamine were used in place of Tris/HCl and propranolol, respectively, for binding of [^{3}H]CGP-12,177 (0.01–4 nM; NEN) to β-receptors (Cervantes-Olivier et al., 1988). In some experiments a 500 μl incubation volume was employed to decrease the ratio of bound to free radioligands at their low concentrations. Prolongation of incubation time to 60 min gave similar results. After incubation, samples were diluted to 3 ml with ice-cold buffer (50 mM Hepes/10 mM $MgCl_2$) and rapidly filtered, under vacuum, through 24 mm Whatman GF/C glass-fibre filters. The filters were washed with a further 8 ml of the dilution buffer, and transferred to scintillation vials to be counted for radioactivity of bound ^{3}H. Non-specific binding was determined by including 10 μM phentolamine or propranolol for α_1- and β-receptor binding, respectively, in the assay mixture, and specific binding was defined as the difference between total and non-specific binding. Since Scatchard plots were linear, the slope and the intersection with abscissa were estimated for each plot to give the K_d (apparent dissociation constant) and the maximal binding capacity (the number of receptor) respectively.

Inhibition of the binding of [^{3}H]prazosin (1 nM) or [^{3}H]CGP-12,177 (0.2 nM) by various concentrations of noradrenaline or isoprenaline, respectively, was performed mostly as described above. The incubation medium was supplemented with 100 μM GTP where indicated. The concentration-dependent displacement curves of the radioligands, as shown in Figures 5 and 6, were analysed by non-linear curve-fitting according to the two-site model, $B_T = [B_H/(1+IC_{50H}/F)]+[B_L/(1+IC_{50L}/F)]$, where B and F represent the molar concentrations of specifically bound and free isoprenaline, and B_T is the maximal binding capacity and IC_{50} is the molar concentration of isoprenaline required for half-maximal inhibition of [^{3}H]CGP-12,177 binding. The subscripts H and L stand for the high- and low-affinity sites respectively. The K_d values (for high- and low-affinity sites for isoprenaline binding) were calculated from IC_{50} by using the equation $K_d = IC_{50}/(1+L/K_d')$, where L is the concentration (0.2 nM) of radioligand ([^{3}H]CGP-12,177) and K_d' is the K_d value for the radioligand binding.

The number of cell-surface receptors was determined for cultured cell monolayers, without cell disruption, in experiments shown in Figure 4. Hepatocytes were cultured on a 30 mm-diameter cover-glass placed on the bottom of a dish. After culture, the monolayer on the cover-glass was washed three times with Hanks' solution (pH 7.4) before being applied to the radioligand-binding assay as described above for hepatocyte membranes. The incubation time was shortened to 3 min, to minimize internalization of radioligand into cells. The binding to hepatocytes cultured for 0–1 h was determined by filtration through glass-fibre filters as for membranes. Essentially the same sets of linear Scatchard plots were obtained for [^{3}H]prazosin (Schwarz et al., 1985a,b) or [^{3}H]CGP-12,177 binding with intact cells as with membrane preparations under the experimental conditions employed in Figure 4.

Adenylate cyclase assay

Crude membranes (40–60 μg of protein) were incubated for 10 min at 30 °C in 0.2 ml of 10 mM Hepes (pH 7.5) containing 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 mM ATP, 5 mM phosphocreatine, 30 units/ml creatine kinase and 0.1% BSA. Guanosine 5'-[γ-thio]triphosphate (GTP[S]) (100 μM) with or without 100 μM isoprenaline was also added to some assay tubes. The enzyme reaction was terminated by adding HCl (final concn. 0.1 M), and the supernatant after centrifugation at 1500 *g* for 5 min was subjected to radioimmunoassay (see above) of cAMP formed to obtain the cyclase activity as pmol of cAMP/10 min per mg of membrane protein.

Sources of reagents

Sources of reagents, other than those described above, were as follows. Glucagon, isoprenaline, noradrenaline, vasopressin, angiotensin II and propranolol were purchased from Sigma. Phentolamine was obtained from Ciba Geigy, Japan. Aprotinin and reagents of radioimmunoassay of cAMP were gifts from Hoechst, Japan, and Yamasa Shoyu Co., respectively. Other reagents from commercial sources were of analytical grade.

RESULTS

Rapid development of β-adrenergic response during low-cell-density culture of hepatocytes and its inhibition by increasing the cell density or adding the plasma-membrane fraction

Hepatocytes that had been cultured for various times were incubated with isoprenaline or glucagon for 7 min at 37 °C to observe increases in cellular cAMP in response to these receptor agonists (Figure 1). There was essentially no cAMP response to isoprenaline in fresh non-cultured cells, but rapid and large increases in the cAMP response were observed when the low-cell-density (10^4 cells/cm^2) culture was conducted for a period from 3 to 24 h (Figure 1a). The cell culture density was altered in Figure 1(b). The β-response of hepatocytes observable at 24 h of culture decreased progressively as the cell density was increased from 10^4 to 2×10^5 cells/cm^2; essentially no β-response developed when the cell density was kept higher than 1.5×10^5 cells/cm^2. The development of β-response during 24 h of culture was prevented by adding plasma membranes to the low-cell-density culture as well (Figure 1c). The cell-to-cell contact through plasma membranes appears to be responsible for the inhibition of culture-induced development of the β-response. In sharp contrast, the cAMP-generating response of hepatocytes to glucagon remained unchanged under all culture conditions employed in Figures 1(a), 1(b) and 1(c).

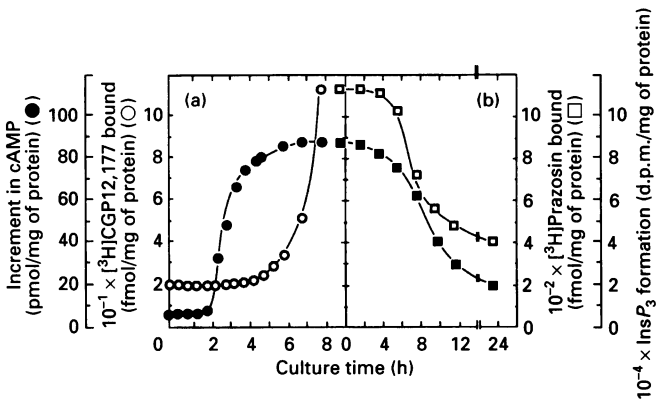

Figure 4 Progressive changes in β-adrenergic and α_1-adrenergic responses during hepatocyte culture as compared with concurrent changes in receptor numbers

Hepatocytes were cultured at low cell density (10^4 cells/cm^2) for the indicated periods of time. A fraction of monolayers in dishes was then assayed for cAMP with 100 μM isoprenaline as in Figure 1 (**a**; ●) or for $InsP_3$ with 100 μM phenylephrine as in Figure 3 (**b**; ■). The other fraction of dishes was subjected to the ligand-binding assay with [^{3}H]CGP-12,177 (**a**; ○) or with [^{3}H]prazosin (**b**; □) for determination of the number of β- or α_1-receptors, respectively, as the maximum binding capacity measured by Scatchard analysis as described in the Experimental section. The data represent means of duplicates within a typical experiment which was repeated more than three times with similar results.

Isoprenaline-induced increases in membrane adenylate cyclase activity displayed similar changes when membranes were prepared from hepatocytes that had been cultured under conditions similar to those for Figure 1, although there was essentially no change in the cyclase activity when assay was performed without isoprenaline (results not shown). The percentage increases caused by addition of isoprenaline to the assay mixture containing GTP[S], a non-hydrolysable GTP analogue, were 10, 69 and 130 for membranes prepared from 0 h-, 3 h- and 6 h-cultured cells, respectively, at the low cell density (10^4 cells/cm^2) in a typical experiment. These values were 9, 24 and 40 for membranes from the medium-cell-density (10^5 cells/cm^2) cultured cells, 10, 19 and 24 for those from the dense (2×10^5 cells/cm^2) cultured cells and 9, 10 and 11 for those from cells cultured at the low cell density but supplemented with plasma membranes (0.2 mg of protein/ml). Thus dependence of the hepatic β-adrenergic response development on culture conditions was observed whether the response was measured on the basis of intact-cell cAMP production or membrane adenylate cyclase activity.

Gradual decreases in hepatocyte responses to α_1-adrenergic and other Ca^{2+}-mobilizing receptor stimulation during culture under the same conditions as those causing development of the β-adrenergic response

Generation of $InsP_3$, a product of receptor-coupled phospholipase C, was monitored at short intervals to measure the peak value during incubation of hepatocytes that has been cultured at a low cell density (10^4 cells/cm^2) for various lengths of time (Figure 2). The peak $InsP_3$-generating responses to phenylephrine-induced stimulation of α_1-receptors tended to decrease gradually as the time of prior culture was prolonged from 2 to 24 h. Such was the case with vasopressin or angiotensin II, which also activated phospholipase C.

Peak values of $InsP_3$-generating responses to phenylephrine obtained as in Figure 2 were then plotted in Figure 3, where the response was maintained at its highest level for the initial 3 h period of culture time, but declined progressively during the subsequent low-cell-density culture up to 24 h (Figure 3a). Such a decrease in the α_1-response was prevented by increasing cell density (Figure 3b) or adding plasma membranes to the low-cell-density culture (Figure 3c). All the trends in Figure 3 were reproduced when phenylephrine was replaced by vasopressin or angiotensin II (result not shown), although the rank order of their efficacy was vasopressin > angiotensin II > phenylephrine (see Figure 2).

Changes in the number of functional α_1- and β-adrenergic receptors as correlated or not correlated with alterations of the respective adrenergic responses during culture

The specific binding of [^{3}H]CGP-12,177, a hydrophilic antagonist which binds selectively to cell-surface β-receptors

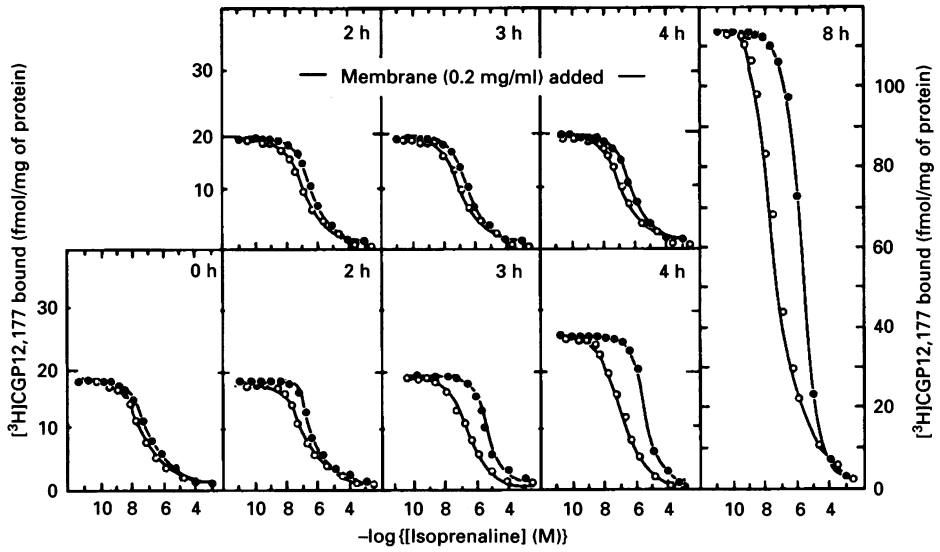

Figure 5 β-Adrenergic-agonist binding to membranes from cultured hepatocytes

Hepatocytes were cultured at low cell density (10^4 cells/cm^2) for periods of time up to 8 h as indicated in the panels. Partially purified liver plasma membranes (0.2 mg of protein/ml) were added to the culture medium for the top three panels. Membranes prepared from the cultured cells were submitted to the β-agonist-binding assay as described in the Experimental section. Each plot represents the amount of [^{3}H]CGP-12,177 bound in the presence of the indicated concentrations of isoprenaline with (●) or without (○) 100 μM GTP in the assay mixture. Representative data are shown from experiments repeated twice with similar results.

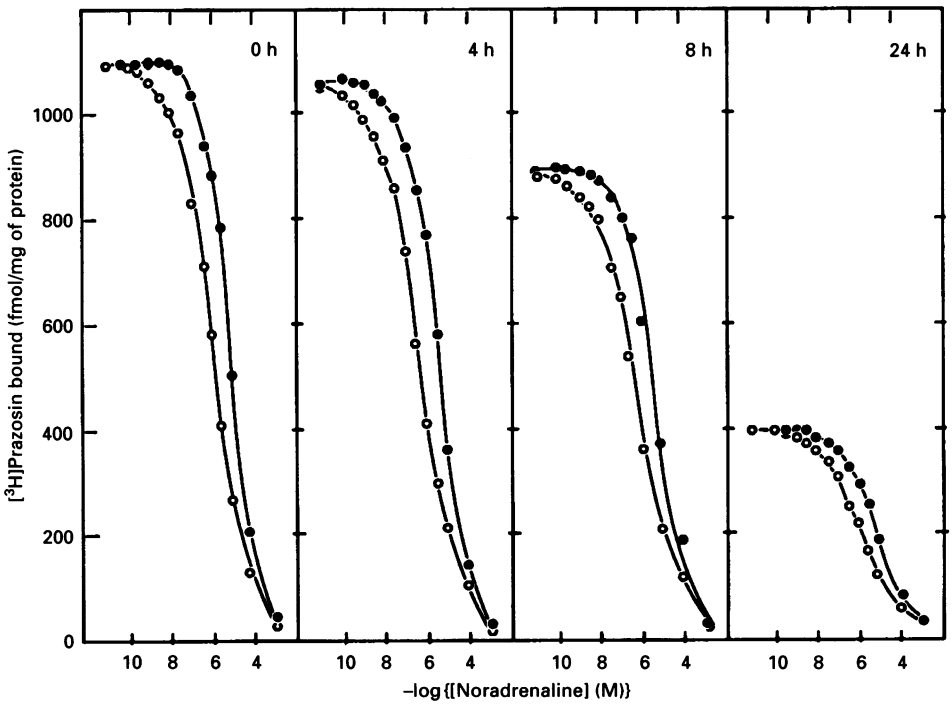

Figure 6 α-Agonist binding to membranes from cultured hepatocytes

Membranes were prepared from hepatocytes that had been cultured at low cell density (10^4 cells/cm^2) for indicated periods of time, and submitted to the α-agonist binding assay as described in the Experimental section. Each plot represents the amount of [^{3}H]prazosin in the presence of indicated concentrations of noradrenaline with (●) or without (○) 100 μM GTP in the assay mixture. Typical data are taken from experiments repeated twice with similar results.

(Cervantes-Olivier et al., 1988), or [^{3}H]prazosin, an α_1-antagonist, to membranes was subjected to Scatchard analysis. A linear Scatchard plot was obtained for each case as an indication of a single case of α_1- or β-adrenergic receptors on fresh (not cultured) hepatocytes (results not shown). Membranes of hepatocytes that had been submitted to the low-cell-density culture for various

lengths of time gave sets of the Scatchard plots each with the same slope but with different values of the intersection with abscissa (results not shown). The low-cell-density culture of hepatocytes was considered to cause changes in the numbers of α_1- and β-adrenergic receptors without significant changes in their affinities for the antagonists ($K_d = 0.20$ and 0.29 nM for α_1- and β-receptors respectively).

The numbers of α_1 and β-receptors on hepatocytes thus measured are plotted as a function of time in the low-cell-density culture in Figure 4. Intact-cell preparations were used, instead of crude membrane preparations, for radioactive-ligand binding in a series of experiments shown in Figure 4, for technical reasons, as to the simultaneous determination of cellular cAMP or InsP_3 in the same preparations. The progressive changes in these receptor numbers were totally inhibited by increasing cell densities 20-fold during culture or by addition of plasma membranes (0.2 mg of protein/ml) to the low-cell-density culture (results not shown).

As shown in Figure 4(b), the number of α_1-receptors started to decrease at around 6 h of the low-cell-density culture and reached the lowest level (about one-third of the initial value) at the end (24 h) of the culture. There was no further decrease when the culture time was prolonged beyond 24 h (results not shown). The α_1-response, measured as InsP_3 generation, diminished to one-fifth of the original value during the 24 h culture, with a time course in parallel with the progressive decrease in the α_1-receptor number. The culture-induced diminution of the α_1-receptor-mediated response is likely to be accounted for by the decrease in the density of receptor molecules on the cell surface.

The β-receptor number started to increase at roughly the same time, 6 h of culture, as the onset of the diminution of α_1-receptor number (Figure 4a). The rate of increase in β-receptor number was, however, much higher than the rate of decrease in α_1-receptor number; there was a 5–6-fold increase in the β-receptor number up to 120 fmol/mg of protein within a further 2–3 h of culture. Whereas decreases in InsP_3 generation and in α_1-receptor density were temporally coincident throughout the entire period of low-cell-density culture, there was no parallelism between changes in the cell-surface β-receptor number and cAMP-generating response to the receptor stimulation under the same conditions; the β-response developed promptly from virtually zero to almost the maximum, despite there being no real receptor-number increase, during the initial period of culture up to 5–6 h. Thus changes in the cell-surface receptor density are not responsible for rapid development of the β-response immediately after the onset of hepatocyte culture.

Uncoupling of G_s from β-receptors in fresh hepatocytes and rapid re-coupling during the initial stage of the low-cell-density culture as revealed by GTP-induced decrease in the affinity for agonist binding

The affinity of β-receptors for a typical agonist, isoprenaline, was determined by measuring its ability to compete with the radiolabelled antagonist in the absence or presence of GTP. Two competition curves (with or without GTP) are plotted in each panel in Figure 5. The left-hand end of each plot represents the number of β-receptors on the cells.

As shown in the '0 h culture' panel at the bottom of Figure 5, there was essentially no difference between the two competition curves obtained with and without GTP when binding was studied for membranes from fresh (not cultured) hepatocytes. After 2 h of the low-cell-density culture, the curve tended to be steepened and shifted to the right in the presence of GTP, despite there being just the same number of β-receptors at this culture time as before culture. This effect of GTP became more striking as the culture time was increased from 2 to 8 h. In sharp contrast, GTP was mostly without effect at 2–4 h of culture when the low-cell-density culture had been performed in the presence of plasma membranes (top panels in Figure 5), or when the cell density during culture was increased (results not shown).

The distribution of β-adrenergic receptors between the high-affinity and low-affinity states was then calculated by applying the two-state-model analysis to the data obtained as in Figure 5. Values calculated for K_d were 0.39 and 23 μM respectively for the high- and low-affinity sites. Essentially all the fraction (92–98%) of β-receptor was in the low-affinity state when the binding experiments were done in the presence of GTP, regardless of whether membranes were prepared from cells either not cultured or cultured under various conditions. Before culture, only a small fraction (15%) of β-receptor in adult-rat hepatocytes was in the agonist high-affinity binding state that reflects the receptor coupling to GDP-bound G-proteins or that is capable of being converted into the low-affinity state upon addition of GTP. The residual large fraction (85%) was in the low-affinity binding and GTP-non-susceptible state reflecting uncoupling from G proteins. At the early stage (2–4 h) of the low cell-density culture, these uncoupled β-receptors became rapidly coupled to G_s, as revealed by increases in the proportion of the GTP-susceptible high-affinity state from 15 to 84%. The increase was abolished by addition of plasma membranes to the low-cell-density culture.

Invariable coupling of α_1-receptors to G_q in hepatocytes either before or after culture for 24 h

G_q is known to couple Ca^{2+}-mobilizing receptors, including α_1-receptors, to phospholipase C activation in hepatocytes (Taylor et al., 1990; Wange et al., 1991; Blank et al., 1991). The coupling was evidenced by a GTP-induced decrease in the affinity for agonist (noradrenaline) binding to membranes from non-cultured cells ('0 h' panel in Figure 6). The effect of GTP was invariably observed in membranes prepared from 4 h-, 8 h- and 24 h-cultured cells (other panels in Figure 6). Application of the two-site model analysis to these data revealed that 58–65% of the total binding sites were in the high-affinity state in the absence of GTP in membranes from 0 h- to 24 h-cultured cells. The coupling of α_1-receptors to G_q does not appear to be markedly altered by hepatocyte culture.

The density of α_1-receptors on hepatocyte membranes as shown by the left-hand end of plots in each panel in Figure 6 showed a progressive decrease by a time course not significantly different from the time-dependent decrease observed for intact-cell preparations in Figure 4(b).

DISCUSSION

Previous reports (see references cited in the Introduction) have shown opposite-directional progressive changes in α_1- and β_2-adrenergic responses during primary culture of adult-rat hepatocytes, taking advantage of α_1-receptor-agonist-induced (or α_1-antagonist-suppressive) activation of glycogen phosphorylase as an index of the receptor-mediated response. The present paper is thus the first to show that similar changes occurred in α_1-receptor-mediated generation of InsP_3 and β-receptor-mediated cAMP production during primary culture of the hepatocytes under various conditions. InsP_3 and cAMP are the two second messengers immediately arising from receptor stimulation, i.e. the products of the effector enzymes phospholipase C_β and adenylate cyclase, that are directly activated by α_1-receptor-

coupled G_q and β-receptor-coupled G_s respectively. Conceivably, the generation of the second messengers reflects the receptor functions very closely in hepatocytes. Significant observations thus obtained here are as follows.

Rapid development of β-adrenergic response due to coupling of the receptor to G_s during low-cell-density culture of hepatocytes

There was essentially no, if any, cAMP production when adult-rat hepatocytes, before culture, were incubated with isoprenaline, although the uncultured cells possessed detectable amounts (20 fmol/mg of protein) of cell-surface receptors that are capable of binding β-receptor-selective agonists and antagonists. The cAMP-generating response to isoprenaline developed promptly, attaining almost the maximal level, although the number of β-receptors capable of binding their own ligands did not increase significantly during the early (2–6 h) period of low-cell-density culture (Figure 4a). The results confirm our previous conclusion (Okajima and Ui, 1982) that rapid development of β-receptor-mediated functions during hepatocyte culture is not solely accounted for by the increase in receptor density on the cell surface. The increase in the β-receptor density lagged definitely behind the β-response development and started at the final stage (5–6 h) of the response development (Figure 4a). The increase in the β-receptor number was again very rapid and of great magnitude (attaining roughly 6–8 times the pre-culture value), probably as a result of receptor-gene expression, and almost completed at 8 h of culture.

The rapid β-response development during culture did not result from increases in the adenylate cyclase catalytic subunit or G_s as well as in the receptor protein, because the membrane adenylate cyclase activity determined in either the presence or absence of GTP[S] never increased during culture, unless the assay mixture was further supplemented with a β-stimulant. Itoh et al. (1984) have reported that membrane G_s, detected by cholera-toxin-catalysed ADP-ribosylation, was maintained as its constant level during 24 h of culture. Instead, modification of β-receptor function has been clearly shown in the present paper. G_s was mostly uncoupled from β-receptors in membranes of the hepatocytes that had been cultured at a low density for a period of time shorter than 2 h (Figure 5). Afterwards, G_s became coupled to β-receptors very promptly, as evidenced by GTP-sensitive shallower binding curves of β-receptor agonists, in parallel with the development of the β-response during the low-cell-density culture. Thus the state of coupling between the receptor and G_s is an essential factor to determine development of the β-response during hepatocyte culture. The site of the functional modification induced by the low-cell-density culture would be likely to be on the β-receptor protein, rather than G_s, because the cAMP response to glucagon via a common G_s was not altered at all during hepatocyte culture under the same conditions (Figure 1). The molecular mechanism for the modification will be the subject for further investigation.

Gradual decreases in InsP_3-generating response due to decreases in the coupled receptor density on the surface of cultured hepatocytes

The α_1-adrenergic receptor is one of what are called Ca^{2+}-mobilizing receptors, which include vasopressin (V_1) and angiotensin II receptors in rat hepatocytes. Stimulation of these three types of receptors gave rise to rapid generation of InsP_3, via a common G_q, with the rank order of efficacy being vasopressin > angiotensin II > an α_1-agonist (Figure 2), in good agreement with previous reports (Creba et al., 1983; Charest et al., 1985). The different efficacies would reflect different densities of these receptors on the hepatocyte surface (Lynch et al., 1985a). In sharp contrast with the development of β-response without any change in the same G_s-mediated cAMP response to glucagon during culture, the culture-induced diminution of α_1-response was accompanied consistently by the same-directional changes in responses to vasopressin and angiotensin II (Figure 2). This is not surprising, however, in view of the previous reports that hepatocyte responses via these three receptors changed in parallel during the process of liver regeneration (Huerta-Bahena and García-Saínz, 1983) or as the age of the rat changed (Butlen et al., 1980; Freemark and Handwerger, 1984; Noguchi et al., 1985).

An additional characteristic of the α_1-receptor-mediated InsP_3 response that strikingly differs from that of the β-receptor-mediated cAMP response was that the culture-induced decrease in the former was in parallel with the decrease in the cellular density of the coupled receptors specifically binding a radioligand (Figure 4b). In fact, the coupling between α_1-receptor and G_q was not altered significantly by submitting hepatocytes to 24 h in culture (Figure 6). The decrease in the InsP_3-releasing response via α_1-receptors is therefore very likely to have resulted from the decrease in the α_1-receptor number during culture. Our results *in vitro* might be at variance with previous observations *in vivo* that there was marked difference between young and old rats in the guanine nucleotide effect on α_1-agonist binding to hepatocyte membranes (Lynch et al., 1985b, 1986).

The decrease in the InsP_3 production via α_1-receptors, which occurred in parallel with the decrease in the receptor number, lagged, by 4–5 h, behind the increase in β-receptor-mediated cAMP response in a low-cell-density culture of hepatocytes. This appeared to be at variance with our previous report (Okajima and Ui, 1982) that the final response to α_1-receptor stimulation, i.e. α_1-antagonist-susceptible conversion of phosphorylase *b* into *a*, decreased significantly as early as within 4 h, attaining the minimum level at 8 h, of hepatocyte culture. It is therefore highly probable that the action, rather than the production, of InsP_3 leading to the eventual α_1-response of phosphorylase activation was impaired during the early stage of 8 h in culture.

In rat hepatocytes, the concentration-dependence curves for Ca^{2+}-mobilizing-receptor-coupled release of InsP_3 (or diacylglycerol) were consistently, by one or two orders of magnitude, to the right of the curves for the same receptor-dependent activation of phosphorylase (Creba et al., 1983; Thomas et al., 1984; Charest et al., 1985; Lynch et al., 1985a; Bocckino et al., 1985). In some of these reports, concentration–response curves for intracellular Ca^{2+} mobilization and phosphorylase activation were shown to be superimposable after α_1-receptor stimulation (Charest et al., 1985; Lynch et al., 1985a). The data indicated that a very small and sub-maximal increase in InsP_3 was sufficient to mobilize intracellular Ca^{2+} maximally and to activate phosphorylase, presumably due to the presence of spare receptors for InsP_3 on the endoplasmic reticulum. It is very tempting to speculate that diminution of the spare receptor availability, if it might occur during the early stage of the hepatocyte culture, would lower the Ca^{2+}-mobilizing and phosphorylase-activating responses to the release of the same amount of InsP_3 under these conditions. The validity of this speculation is the subject of further investigations in our laboratory.

Altered receptor-mediated responses as inhibited by cell-to-cell interactions through plasma membranes

All the alterations of receptors and receptor functions described above were events occurring during the primary culture of

hepatocytes at a low cell density. They did not occur when cell-to-cell contacts through plasma membranes were favoured during the culture by increasing the cell density 20-fold or by achieving a low-cell-density culture by addition of liver plasma-membrane preparations (Figures 1 and 3). Such cell-to-cell interactions were reported to be an important factor in inhibition of cell growth in favour of cell differentiation or expression of liver-specific characteristics (Nakamura et al., 1983b, 1984b,c). Since the β-response development, at the expense of the α_1-response, is observed *in vivo* under such growth-favouring conditions as after partial hepatectomy, bile-duct ligation and chemical hepatocyte injury (see references in the Introduction), the β-receptor-mediated response is one of the growth-related liver functions, whereas the α_1-receptor-mediated response should be included in the differentiated liver functions, according to Ichihara's (1991) classification although stimulation of α_1-adrenergic receptors enhanced, unexpectedly, hepatocyte proliferation during primary culture (Takai et al., 1988; Michalopoulos, 1990).

Further extensive studies would be indispensable before elucidation of the molecular mechanisms by which functions of β_2- and α_1-adrenergic and other Ca^{2+}-mobilizing receptors, together with expression of the receptor molecule on the cell surface, are dependent on hepatocyte proliferation or differentiation.

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