Studies on Responsiveness of Hepatoma Cells to Catecholamines

I. Lack of \( \beta \)-Adrenergic Responsiveness in Rat Ascites Hepatoma AH13 Cells

Takayuki MATSUNAGA, Norito TAKEMOTO, Kenichi MIYAMOTO
and Ryozo KOSHIURA

Department of Pharmacology, Hokuriku University School of Pharmacy,
Ho-3, Kanagawa-machi, Kanazawa 920-11, Japan

Accepted September 6, 1984

Abstract—The \( \beta \)-adrenoceptor density and the activities of adenylate cyclase and cyclic AMP phosphodiesterase were examined to compare AH13 cells having lower \( \beta \)-adrenergic responsiveness with other rat ascites hepatoma cells and normal rat liver cells. Normal rat liver cells used were cultured for 24 hr after the collagenase digestion of liver. The density of binding sites of \( ^{3} \text{H} \)-dihydroalprenolol in AH13 cell plasma membrane was very similar to the density in AH44 and normal liver cell membrane, but that in AH130 cell plasma membrane was about 10-fold greater than those in the other three cell lines. The activity of cyclic AMP phosphodiesterase was about 2.5- to 7-fold higher in hepatoma cells than in rat liver cells, but this enzyme activity of AH13 cells was not especially high among the hepatoma cells examined. The basal adenylate cyclase activity was lower in AH44 cells, but was higher in AH13 and AH130 cells than in rat liver cells. However, adenylate cyclase of AH13 cells was hardly activated by isoproterenol, while the enzyme of the other cells was activated 3- to 5-fold. On the other hand, adenylate cyclase of each cell line including AH13 was activated 4- to 14-fold by NaF. From these results, it is suggested that AH13 cells can hardly produce cyclic AMP by the \( \beta \)-adrenergic stimulation because of the disordered interaction of \( \beta \)-adrenoceptors with adenylate cyclase.

Cyclic AMP is known to play an important role in controlling cell growth (1). It is also shown that this nucleotide acts as an intracellular second messenger by the stimulation of several hormones and bioactive substances and acts to control a large number of metabolic reactions (2). On the other hand, many investigators have pointed out that several tumor cell lines and transformed cells possessed lower cyclic AMP level (3) and lower responsiveness to some hormones than the corresponding normal cells and/or the original cells (4). We have recently reported that isoproterenol, a potent \( \beta \)-adrenergic stimulant, synergistically enhanced the cytotoxicity of alkylating agents in HeLa cells and some rat ascites hepatoma cells (5–7). However, the synergism was not observed in AH13 cells in which the intracellular level of cyclic AMP was hardly elevated by isoproterenol (6).

So, in order to clarify the mechanism for the lack of the responsiveness of AH13 cells to isoproterenol, we investigated the cyclic AMP production and degradation systems of AH13 cells in comparison with those of other hepatoma cells and normal rat liver cells.

Materials and Methods

Drugs used: \( (2,8-{^{3}} \text{H}) \)-Cyclic AMP (34.5 Ci/mmol) and \( 1-(\text{propyl}-1,2,3-{^{3}} \text{H}) \)-dihydroalprenolol \( (^{9} \text{H}-\text{DHA}, 102.7 \text{ Ci/mmol}) \) (New England Nuclear), dl-isoproterenol hydrochloride (Nakarai Chemicals), sodium fluoride (NaF) and adenosine triphosphate
disodium salt (ATP) (Wako Pure Chemical), dl-propranolol hydrochloride (Sigma Chemicals) and cyclic AMP (Kohjin) were commercially purchased.

Tumor cells and preparation of normal rat liver cells: Rat ascites hepatoma AH13, AH44 and AH130 cells were maintained serially by the intraperitoneal passage at weekly intervals in female Donryu rats (weighing 100-150 g, Shizuoka Agricultural Cooperative Association for Laboratory Animals). Hepatoma cells in the respective logarithmic growing phase were obtained from the abdominal cavity of each tumor bearing rat. Normal rat liver cells were isolated from female Donryu rat (5 to 6 weeks-old) by the collagenase digestion in situ according to a modification (8) of the method of Berry and Friend (9). In brief, after a rat was anesthetized, the abdomen was opened and the portal vein was cannulated. The liver was cleared of blood by washing with the pre-perfusion buffer and then was perfused with the collagenase solution. After 15 to 30 min of perfusion, the digested liver was cut away from the abdomen with a scissor and was placed on a petri dish. The liver cells were dispersed from the liver and were purified by a low speed centrifugation after filtration. The cell viability estimated by the Trypan Blue dye exclusion test was about 90%. The liver cells were cultured for 24 hr in Eagle’s minimum essential medium (MEM) containing 10% fetal calf serum. Hormones such as glucocorticoids and insulin were not added to the culture medium. After the culture, cells were collected, suspended in 5 mM Tris-HCl buffer (pH 7.4), homogenized in a Teflon-glass homogenizer and used for the enzyme assay after dilution with each assay buffer.

Preparation of plasma membrane from rat ascites hepatoma and cultured normal rat liver cells: The plasma membrane of cultured liver cells was isolated according to a slight modification of the method of Prpic et al. (10). Cultured cells were suspended in 1 mM Na2CO3 solution containing 2 mM CaCl2 to a concentration of 2 to 4x107 cells/ml, allowed to stand at 0°C for 15 min to swell and homogenized with a Dounce homogenizer (pestle A). After dilution, the homogenate was centrifuged at 1,450xg for 10 min, and the resulting pellet was resuspended in 0.25 M sucrose-10 mM Tris-HCl buffer (pH 7.5) containing 2 mM ethyleneglycol-bis (β-aminoethylether)-N,N’-tetraacetic acid (EGTA) (STE solution). A volume (10.4 ml) of this suspension was mixed with 1.4 ml of Percoll (Pharmacia) and centrifuged at 33,000xg for 30 min. The second layer from the top was collected and washed twice with 42 mM Tris-HCl buffer (pH 7.5). The pellet was resuspended in the same buffer and stored at -80°C until use for the binding assay.

The plasma membrane of rat ascites hepatoma cells was isolated according to a slight modification of the method of Nakamura et al. (11). Rat ascites hepatoma cells were suspended in 1 mM Na2CO3 solution containing 2 mM CaCl2 to a concentration of 2 to 4x107 cells/ml, allowed to stand at 0°C for 15 min to swell and homogenized with a Dounce homogenizer (pestle A). After dilution, the homogenate was centrifuged at 300xg for 10 min, and the supernatant was centrifuged at 5,000xg for 10 min. The resulting pellet was resuspended in STE solution and 0.4 ml of the suspension was mixed in 8 ml of isoosmotic Percoll solution composed of Percoll : 2M sucrose-80 mM Tris-HCl buffer (pH 7.5) containing 8 mM EGTA : STE solution (7:1:32). The mixture was centrifuged at 11,000xg for 15 min. The floating layer of membrane was collected and washed twice with 50 mM Tris-HCl buffer (pH 7.5). The pellet was resuspended in the same buffer and stored at -80°C until use for the binding assay.

By these procedures, activities of Na+-K+ ATPase, as a marker enzyme (12), of plasma membranes used were increased about 4- to 5-fold in each cell line from the respective cell homogenate.

3H-DHA binding assay: The cell membrane was incubated with varying concentrations of 3H-DHA in a total volume of 0.25 ml in 42 mM Tris-HCl buffer (pH 7.4) containing 15 mM MgCl2 for 10 min at 37°C (13). The incubation was terminated by the dilution with 5 ml of ice-cold incubation buffer followed by rapid vacuum filtration through a Whatman GF/C glass fiber filter.
The filter was washed, dried and placed into a Triton/toluene-based scintillation fluid, and the radioactivity was counted by a Beckman LS-230 liquid scintillation counter. The specific binding was defined as total bindings minus bindings in the presence of 10 μM dl-propranolol. The amount of 3H-DHA bound and the dissociation constant (Kd) of the binding of DHA to the cell membrane were determined by Scatchard analysis.

**Cyclic AMP phosphodiesterase assay:** Cyclic AMP phosphodiesterase activity was measured by the 2-step assay system proposed by Thompson and Appleman (14). The reaction mixture (0.4 ml) composed of 40 mM Tris-HCl buffer (pH 8.0) containing 0.2 μCi 3H-cyclic AMP, 5 mM MgCl2, 4 mM 2-mercaptoethanol and the cell homogenate was incubated for 10 min at 30°C and boiled to stop the reaction. Then, the 5'-AMP produced was converted to adenosine by Crotalus atrox snake venom (Sigma Chemicals). The converting reaction was stopped by the addition of 1.0 ml of a slurry (1:3) of Bio-Rad 1-X2 resin (200-400 mesh). After mixing well, the sample was centrifuged and the radioactivity in the supernatant was measured by a liquid scintillation counter. The value of the blank obtained by carrying out the same assay using the heat-inactivated enzyme was subtracted from the experimental value. The apparent Km value and the apparent Vmax value were determined by Hofstee plots of activity values obtained.

**Adenylate cyclase assay:** It has been reported for liver and hepatoma cells that adrenergic responsiveness in the plasma membrane was lower than that in the cell homogenate (15, 16). Therefore, the cell homogenates of these cell lines were used in this assay. The cell homogenate was incubated with or without a stimulant in 30 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl2, 1 mM ethylenediaminetetraacetate, 10 mM theophylline, 10 μM guanosine triphosphate (GTP), 10 mM phosphoenolpyruvate, 25 μg/ml pyruvate kinase and 2.5 mM ATP (17). After the incubation for 10 min at 37°C, the sample was boiled for 2–3 min to stop the enzyme reaction and centrifuged for 5 min at 3,000 rpm, and the cyclic AMP contents in the supernatant were measured by using a cyclic AMP assay kit (Yamasa Shoyu) according to the method of Honma et al. (18).

**Assay of protein contents:** Protein contents were estimated by the method of Lowry et al. (19) with bovine serum albumin as the standard.

**Results**

**Binding of 3H-DHA:** The binding of 3H-DHA to the cell membrane of each cell line was saturable, and the binding sites exhibited no cooperative interactions and no heterogeneity to DHA as assessed by Hill plots of the saturation curves (20). The Hill coefficients were 0.95, 0.98, 1.01 and 0.96 for AH13, AH44, AH130 and cultured normal rat liver cells, respectively. Furthermore, the binding was clearly stereoselective as (-) form isoproterenol inhibited the DHA binding about 100-fold more than (+)-form isoproterenol did in each cell line (data not shown). The density of 3H-DHA binding sites and the affinity of 3H-DHA in each cell membrane were estimated by a Scatchard plot (Fig. 1), and the binding parameters are indicated in Table 1. The Kd value for normal liver cells was similar to the Kd value for AH130 cells, but was lower than those for AH13 and AH44 cells. The Bmax of 3H-DHA was about 10-fold greater in AH130 cells than in the other three cell lines, of which the values were similar to one another.

**Cyclic AMP phosphodiesterase activity:** The rate of hydrolysis of cyclic AMP by the enzyme of these four cell lines is expressed by means of a Hofstee plot in Fig. 2. In this plot, the slope is the negative value of the apparent Km value, where the intercept on the ordinate gives the value of the apparent Vmax. These cells appeared to contain two enzyme activities, a high affinity form and a low affinity form. The maximum activity of the enzyme with each Km value was higher in hepatoma cells than in cultured rat liver cells, but in AH13 cells, the activity was not especially high in comparison with other hepatoma cells. The high Km value was similar in the four cell lines, but the low Km value was larger in hepatoma cells than in rat liver cells.
Fig. 1. Scatchard analysis of specific DHA binding in the membrane of cultured normal rat liver cells (●), AH13 cells (○), AH44 cells (■) and AH130 cells (▲). The amount of specifically bound DHA was determined at five concentrations of DHA (0.1–100 nM).

Table 1. $K_d$ value and number of $^3$H-DHA bound to the membrane of cultured normal liver and ascites hepatoma cells

|                | Liver | AH13 | AH44 | AH130 |
|----------------|-------|------|------|-------|
| Specific binding of $^3$H-DHA (f mole/mg protein) | 24.2±1.0 | 23.4±3.4 | 20.8±4.5 | 254.9±46.1 |
| $K_d$ value (nM) | 4.7±0.4 | 10.3±1.3 | 10.2±1.9 | 3.9±2.1 |

Values are the mean±S.E. for triplicate measurements in 3 experiments.

Table 2. Basal and NaF-stimulated activity of adenylate cyclase in cultured normal liver and ascites hepatoma cells

| Cell   | Basal activity (pmole/10 min/mg protein) | NaF-stimulated activity (pmole/10 min/mg protein) | Activation ratio |
|--------|------------------------------------------|--------------------------------------------------|-----------------|
| Liver  | 15.1±1.4                                 | 104.2±15.3                                       | 6.9             |
| AH13   | 67.9±9.3                                 | 487.8±26.1                                       | 7.2             |
| AH44   | 6.4±0.7                                  | 90.2±7.3                                         | 14.1            |
| AH130  | 48.9±4.2                                 | 195.3±32.8                                       | 4.0             |

The concentration of NaF used was $1 \times 10^{-2} \text{ M}$ which induced the maximum activity of adenylate cyclase. The right column indicates the activation ratio of NaF-stimulated activity against basal activity. Values are the mean±S.E. for triplicate measurements in 10 experiments for cultured normal rat liver cells (liver) and AH130 cells or 3 experiments for AH13 and AH44 cells.

Adenylate cyclase activity: The basal activity of adenylate cyclase and the activity stimulated with 10 mM NaF are shown in Table 2. The adenylate cyclase of AH13 cells showed sufficient activity for the formation of cyclic AMP from ATP and was activated to the same extent in cultured normal rat liver cells by NaF. The basal activity of the enzyme of AH44 cells was the lowest among these cell lines, but the activity showed the highest
increase after the stimulation by NaF. The effect of isoproterenol on the adenylate cyclase activity in hepatoma cells and cultured rat liver cells is shown in Fig. 3. This figure also indicates that isoproterenol activates the enzyme via $\beta$-adrenoceptors because the activation curves are shifted in parallel to the right by propranolol ($1 \times 10^{-7}$ M). The adenylate cyclase activity of AH44, AH130 and rat liver cells was increased to a similar extent by the $\beta$-stimulant, but the enzyme of AH13 cells was hardly activated by the treatment with any concentration of isoproterenol or other $\beta$-adrenergic agents such as epinephrine, norepinephrine and salbutamol (data not shown).

**Discussion**

Our previous paper (6) showed that the responsiveness to isoproterenol assessed by the extent of increase of intracellular cyclic AMP level varied among six cell lines of rat ascites hepatoma, and isoproterenol, a potent $\beta$-adrenergic stimulant, potentiated the effect of mitomycin C in high responsive hepatoma cells, but did not in AH13 cells in which cyclic AMP contents were not increased by the stimulant.
In the present study, we investigated the lack of \( \beta \)-adrenergic responsiveness of AH13 cells by means of the determination of the density of \( \beta \)-adrenoceptor, the activity of adenylate cyclase and the activity of cyclic AMP phosphodiesterase in comparison with those of AH44, AH130 and cultured normal rat liver cells. The density of binding sites of \(^3\)H-DHA, a selective \( \beta \)-adrenergic antagonist, regarded as \( \beta \)-adrenoceptors in the AH13 cell membrane was very similar to the density in AH44 and cultured rat liver cell membrane, but the density in AH130 cell membrane was about 10-fold more than those in the other three cell membranes (Table 1). Here, the adrenergic responsiveness of AH130 cells was not especially high in comparison with AH44 and normal rat liver cells (Ref. 6 and Fig. 3). This seems to be due to AH130 cells having many spare receptors which cannot interact with adenylate cyclase (21, 22). The basal activity of the adenylate cyclase of AH13 cells was the highest among those of these cell lines examined (Table 2). For cyclic AMP phosphodiesterase, there were a low affinity form and a high affinity form in each cell line as reported for other cells (23, 24). Both activities of cyclic AMP phosphodiesterase were higher in hepatoma cells than in rat liver cells (Fig. 2). Thus, as AH13 cells possessed \( \beta \)-adrenoceptors and adenylate cyclase which provided a sufficient activity to produce cyclic AMP and the activity of the cyclic AMP degrading enzyme was not especially higher than that of other hepatoma cells which accumulated cyclic AMP by the stimulation of isoproterenol, it is difficult from these results to elucidate the low responsiveness of AH13 cells to isoproterenol. The most significant difference between these hepatoma cells was observed when the adenylate cyclase activity was assayed in the presence of isoproterenol. While the adenylate cyclases of AH44, AH130 and rat liver cells were all activated 3- to 5-fold by isoproterenol, the enzyme of AH13 cells was scarcely activated (Fig. 3). It was reported that the adenylate cyclase system consisted of at least three main components: the receptor, the GTP-regulatory protein and the
catalytic unit (25). In this study, NaF known to act on the GTP-regulatory protein and to stimulate adenylate cyclase, markedly activated adenylate cyclase in AH13 cells as much as in normal rat liver cells (Table 2). These findings seem to be similar to the observations that were reported in the UNC mutant of S49 lymphoma (26) or the cells treated with polyene antibiotics (27) or phospholipase (28). Haga et al. (26) reported in the UNC mutant that the adenylate cyclase was activated only by NaF or cholera toxin, but was not activated by β-adrenergic agonists or prostaglandin E1; and the down-regulation of β-adrenoceptor by β-agonist and the effect of GTP on the binding of β-agonist to the receptor was not observed in the mutant. From these results, they concluded that the interaction of β-adrenoceptor with GTP-regulatory protein was deficient in the mutant.

From these results and evidences, it seems that AH13 cells may be a mutant which developed a defect in the interaction of β-adrenoceptor with GTP-regulatory protein and adenylate cyclase in the process of the carcinogenesis by dimethylaminoazobenzene or by the passage for a long term.

References

1 Pastan, I.H. and Johnson, G.S.: Cyclic AMP and transformation of fibroblast. Adv. Cancer Res. 19, 303–329 (1974)
2 Jost, J.P. and Rickenberg, H.V.: Cyclic AMP. Annu. Rev. Biochem. 40, 741–774 (1971)
3 Sheppard, J.R.: Difference in the cyclic adenosine 3',5'-monophosphate levels in normal and transformed cells. Nature New Biol. 236, 14–16 (1972)
4 Okamura, N. and Terayama, H.: Comparison of the epinephrine-mediated activation of adenylate cyclase in plasma membranes from liver and ascites hepatomas of rats. Biochim. Biophys. Acta 456, 297–314 (1976)
5 Miyamoto, K., Sanae, F., Iwasaki, M. and Koshiura, R.: Effect of β-adrenergic stimulants on cytotoxicity of mitomycin C in HeLa cells. Japan. J. Pharmacol. 32, 1019–1026 (1982)
6 Matsunaga, T., Iwasaki, M., Miyamoto, K. and Koshiura, R.: Relationship between enhancement of cytotoxic effect of mitomycin C and increase of intracellular cyclic adenosine 3',5'-monophosphate by isoproterenol in rat ascites hepatoma cells. J. Pharmacobiodyn. 7, 25–32 (1983)
7 Sanae, F., Miyamoto, K. and Koshiura, R.: Combined cytotoxic effect of isoproterenol with alkylating agents in HeLa cells. Japan. J. Pharmacol. 34, 359–362 (1984)
8 Tanaka, K., Sato, M., Tomita, Y. and Ichihara, A.: Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. J. Biochem. 84, 937–946 (1978)
9 Berry, M.N. and Friend, D.S.: High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structure study. J. Cell Biol. 43, 506–520 (1969)
10 Prpić, V., Green, K.C., Blackmore, P.F. and Exton, J.H.: Vasopressin-, angiotensin II-, and α1-adrenergic-induced inhibition of Ca2+ transport by rat liver plasma membrane vesicles. J. Biol. Chem. 259, 1382–1385 (1984)
11 Nakamura, T., Tonomura, A., Noda, C., Shinogi, M. and Ichihara, A.: Acquisition of a β-adrenergic response by adult rat hepatocytes during primary culture. J. Biol. Chem. 258, 9283–9289 (1983)
12 Shimizu, S.: The cell membrane of rat ascites hepatoma. In The Method of Cell Fractionation. Edited by Satoh, R., p. 330–339. Iwanami Shoten, Tokyo (1972) (in Japanese)
13 Lefkowitz, R.J., Mullikin, D. and Caron, M.G.: Regulation of β-adrenergic receptors by guanylyl-5'-yl imidodiphosphate and other purine nucleotides. J. Biol. Chem. 251, 4686–4692 (1976)
14 Thompson, W.J. and Appleman, M.M.: Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochemistry 10, 311–316 (1971)
15 Christoffensen, T., Refsens, M., Bronstad, G.O., Ostby, E., Huse, J., Haffner, F., Sand, T.E., Hunt, N.H. and Sonne, O.: Changes in hormone responsiveness and cyclic AMP metabolism in rat hepatocytes during primary culture and effects of supplementing the medium with insulin and dexamethasone. Eur. J. Biochem. 138, 217–226 (1984)
16 Hickie, R.A., Jan, S.H. and Datta, A.: Comparative adenylate cyclase activities in homogenate and plasma membrane fractions of Morris hepatoma 512C(n). Cancer Res. 35, 596–600 (1975)
17 Oka, H., Kaneko, T., Yamashita, K., Suzuki, S. and Oda, T.: The glucagon and fluoride sensitive adenylate cyclase in plasma membrane of rat liver. Endocrinol. Japon. 20, 263–270 (1973)
18 Honma, M., Satoh, T., Takazawa, J. and Ui, M.: An ultrasensitive method for the simultaneous determination of cyclic AMP and cyclic GMP
in small volume samples from blood and tissue. Biochem. Med. 18, 257–273 (1977)

19 Lowry, O.H., Rosebrough, N.T., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)

20 Weiland, G.A. and Molinoff, P.B.: Quantitative analysis of drug-receptor interactions: I. Determination of kinetic and equilibrium properties. Life Sci. 29, 313–330 (1981)

21 Takayanagi, I., Yoshioka, M., Takagi, K. and Tamura, Z.: Photoaffinity labeling of the β-adrenergic receptors and receptor reserve for isoprenaline. Eur. J. Pharmacol. 35, 121–125 (1976)

22 Terasaki, W.L., Linden, J. and Brooken, G.: Quantitative relationship between β-adrenergic receptor number and physiologic responses as studied with a long-lasting β-adrenergic antagonist. Proc. Natl. Acad. Sci. U.S.A. 76, 6401–6405 (1979)

23 Cohen, L.A., Straka, D. and Chan, Po-C.: Cyclic nucleotide phosphodiesterase activity in normal and neoplastic rat mammary cells grown in monolayer culture. Cancer Res. 36, 2007–2012 (1976)

24 Cho-Chung, Y.S. and Newcomer, S.F.: Adenylate cyclase, cyclic adenosine 3':5'-monophosphate phosphodiesterase, and regression of Walker 256 mammary carcinoma. Cancer Res. 37, 4493–4499 (1977)

25 Ross, E.M. and Gilman, A.G.: Reconstitution of catecholamine-sensitive adenylate cyclase activity: Interaction of solubilized components with receptor-replete membranes. Proc. Natl. Acad. Sci. U.S.A. 74, 3715–3719 (1977)

26 Haga, T., Ross, E.M., Anderson, H.J. and Gilman, A.G.: Adenylate cyclase permanently uncoupled from hormone receptors in a novel variant of S49 mouse lymphoma cells. Proc. Natl. Acad. Sci. U.S.A. 74, 2016–2020 (1977)

27 Puchwein, G., Pfeuffer, T. and Helmreich, E.J.M.: Uncoupling of catecholamine activation of pigeon erythrocyte membrane adenylate cyclase by filipin. J. Biol. Chem. 249, 3232–3240 (1974)

28 Rubalcava, B. and Rodbell, M.: The role of acidic phospholipids in glucagon action on rat liver adenylate cyclase. J. Biol. Chem. 248, 3831–3837 (1973)