Studies on Responsiveness of Hepatoma Cells to Catecholamines. V.[1] Loss of Adrenergic Response of Glycogen Phosphorylase in Rat Ascites Hepatoma AH130 Cells

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The β-adrenoceptor-cyclic adenosine monophosphate (AMP) dependent glycogenolytic cascade was examined in normal rat hepatocytes and rat ascites hepatoma AH130 cells. The cyclic AMP content in AH130 cells was half of that in normal hepatocytes, and the cyclic AMP levels in both kinds of cells were clearly increased by isoproterenol (IPN). Cyclic AMP-dependent protein kinase activity was higher in AH130 cells than in normal hepatocytes. Phosphorylase kinase activities in 10000 × *g* supernatant of normal hepatocytes and AH130 cells were also increased in the presence of cyclic AMP. Phosphorylase *a* activities in the supernatant of both kinds of cells gradually decreased during incubation with 40 mM glucose at 37 °C, and the enzyme activity of normal hepatocytes was completely restored by the addition of Mg^{2+}-adenosine triphosphate (ATP), but in the case of the hepatoma cells the recovery was small. The decreased phosphorylase *a* activity in the hepatoma cells was increased by additional glycogen but did not exceed the level before the incubation. In the case of normal hepatocytes it was not affected by glycogen. This indicates that glycogen contained in the cells influences the activation of phosphorylase; the glycogen content in AH130 cells was far less than in normal hepatocytes. On the other hand, when intact cells were incubated with a high concentration of glucose, phosphorylase *a* activity in the homogenate of normal hepatocytes was decreased and could be restored by IPN and dibutyryl cyclic AMP, but the enzyme activity in the homogenate of AH130 cells was very low and hardly changed after the incubation and treatment with these agents. Phosphorylase phosphatase activity was lower in AH130 cells than in normal hepatocytes. Only phosphorylase *b* converted from the *a* form by the phosphatase may be able to act as the substrate of phosphorylase kinase. The present study indicates that the key enzymes in the glycogenolytic cascade of AH130 cells functionally act as well as normal hepatocytes, but glycogen phosphorylase is unresponsive to stimulation through cyclic AMP. A part of the lack of response of the phosphorylase in AH130 cells may be due to its low ability to interconvert between the active form and the inactive form in the tumor cells owing to their low glycogen content and low phosphatase activity.

Keywords glycogenolysis; adrenergic control; cyclic AMP; glycogen; normal rat hepatocyte; rat ascites hepatoma; AH130 cell; glycogenolytic cascade; enzyme

Catecholamine-induced glycogenolysis is thought to be mediated by both α- and β-adrenoceptors in rat liver.[2] Glucagon also regulates the glycogen metabolism in the liver. The β-adrenoceptors and glucagon receptors are coupled to adenylate cyclase, and cyclic adenosine monophosphate (AMP) directs the glucose production.[3] α_1-Adrenergic stimulation results in glycogenolysis in a cyclic AMP-independent manner.[4] We have previously indicated that adenylate cyclase in several rat ascites hepatoma cells was activated by β-adrenergic stimulation[5] but glycogen phosphorylase in these cells was insensitive to catecholamines and glucagon.[1] We have further shown in the AH130 cell line, a rat ascites hepatoma cell line, changes of α-adrenoceptors from the normal α_1-type to the α_2-type and of β-adrenoceptors from the β_2-type to the β_1-type.[6] The loss of α-adrenergic response can be understood in terms of the lack of α_1-adrenoceptors in AH130 cell membrane.[6b] However, the lack of response of phosphorylase to β-adrenergic stimulation can not be explained by the switch of receptor subtypes in the hepatoma cells.

We investigated here the β-adrenergic stimuli- and cyclic AMP-dependent cascade of glycogenolysis in AH130 cells compared with that in normal rat hepatocytes.

Materials and Methods

Cells Rat ascites hepatoma AH130 cells were propagated in the abdominal cavities of female Donryu rats (5—7 weeks old, Shizuoka Laboratory Animal Center). Hepatocytes were isolated from female Donryu rats by *in situ* collagenase digestion as described elsewhere.[7]

Assay of Glycogen Content Cells were digested with 30% KOH, and the glycogen was treated with anthrone reagent and measured colorimetrically as glucose.[8]

Assay of Cyclic AMP Content The intracellular cyclic AMP was extracted by homogenizing in 6% trichloroacetic acid and assayed using a cyclic AMP assay kit (Yamasa Shoyu) following the procedure developed by Honma *et al.*[9]

Cyclic AMP-Dependent Protein Kinase Activity[10] Cells were homogenized with an ultrasonic homogenizer in 25 mM Tris–HCl (pH 7.5) containing 2 mM ethylenediaminetetracetic acid (EDTA), 0.25 M sucrose, 50 mM 2-mercaptoethanol and 0.001% leupeptin and centrifuged at 10000 × *g* for 20 min. The resultant supernatant fraction (20—50 μg protein) was incubated in 25 mM Tris–HCl (pH 7.0) buffer containing 10 mM $MgCl_2$, 10 μM adenosine triphosphate (ATP), 1×10^5 cpm [γ-^{32}P]ATP (278 TBq/mmol, Amersham), 10 μM cyclic AMP and 40 μg of histone (calf thymus; type II-AS, Sigma) at 37 °C for 3 min. The reaction was stopped by addition of 3 ml of 20% trichloroacetic acid, then 100 μg of bovine serum albumin was added and the mixture was centrifuged at 3000 rpm for 10 min. The resultant pellet was washed twice with 3 ml of 5% trichloroacetic acid and resolved with 1 N NaOH. A blank experiment was conducted without cyclic AMP. Radioactivity was measured in a Triton-toluene scintillation cocktail (33% Triton X-100, 0.4% DPO, 0.01% POPOP).

Phosphorylase Kinase Activity[11] Twenty microliters of the 10000 × *g* supernatant was combined with 80 μl of 20 mM Tris–HCl buffer (pH 6.8) containing 25 mM sodium-β-glycerophosphate, 0.625 mM magnesium acetate, 0.625 mM ATP, 1×10^6 cpm [γ-^{32}P]ATP and 100 μg of phosphorylase *b* (rabbit skeletal muscle, Sigma) and the mixture was incubated at 30 °C for 10 min. A blank experiment was conducted without phosporylase *b*. Radioactivity was measured as described above.

Phosphorylase Activity[12] Cells were treated with agents in 10 mM Hepes buffer (pH 7.4) containing 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$ and 40 mM glucose. The cells were homogenized following the addition of an equal volume of 50 mM glycyl-glycine buffer (pH 7.4) containing 20 mM EDTA, 100 mM NaF, 1% glycogen, 5 mM 2-mercaptoethanol and 0.001% leupeptin. Fifty microliters of the homogenate or its 10000 × *g* supernatant fluid was combined with 100 μl of 75 mM glycyl-glycine buffer (pH 6.1) contain-

ing 15 mM glucose-1-phosphate, 2×10^4 cpm [^{14}C]glucose-1-phosphate (11.91 GBq/mmol, New England Nuclear) and 0.15 M NaF. The glycogen concentration in the reaction mixture was 1%, unless otherwise described. In the case of the assay of phosphorylase *a*, 0.5 mM caffeine was added to the medium, and in the case of the assay of the total phosphorylase activity, 0.5 M Na_2SO_4 and 1 mM AMP were added for hepatocytes and 1 mM AMP alone was added for AH130 cells. The reaction mixture was incubated for 20 min at 30 °C, and then a portion was adsorbed onto Whatman 31ET filter paper. The filter paper was washed 3 times in 67% ethanol and dried, and the radioactivity was measured in a toluene scintillation cocktail (0.4% DPO, 0.01% POPOP). Phosphorylase activity was expressed in units; one unit was defined as the activity which converts 1 μmol of glucose per min to glycogen.

Phosphorylase Phosphatase Activity[13] Cells were sonicated in ice-cold 50 mM imidazole chloride buffer (pH 7.4) containing 5 mM EDTA and 0.5 mM dithiothreitol. The homogenate was combined with an equal volume of 50 mM imidazole chloride reaction medium (pH 7.2) containing 0.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM theophylline and 0.4 mg/ml phosphorylase *a* (rabbit skeletal muscle, Boehringer Mannheim), and the mixture was incubated at 30 °C for 5 min. The reaction was stopped by the addition of an equal volume of 200 mM imidazole chloride buffer (pH 6.5) containing 0.2 M NaF, 5 mM EDTA, 0.5 mM dithiothreitol and 2 mg/ml bovine serum albumin. The remaining phosphorylase *a* activity was measured as described above. Phosphorylase phosphatase activity was expressed by a unit that is defined as the amount converting 1 unit of phosphorylase a per min.

Protein was measured by the Lowry method.[14]

Agents 1-Isoproterenol (IPN), dibutyryl cyclic AMP and glycogen were purchased from Sigma Chemical Co. Other agents were also obtained from commercial sources; cyclic AMP (Kojin Co.) and *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8, Seikagaku Kogyo Co.).

Results

Cyclic AMP and Glycogen Contents The cyclic AMP content in AH130 cells (0.81 ± 0.01 pmol/10^6 cells) was half of that in normal hepatocytes (2.00 ± 0.02 pmol/10^6 cells), and the levels in both kinds of cells were increased about 1.7-fold over the untreated levels by treatment with 10 μM IPN for 2 min. Glycogen content in AH130 cells (1.7 ± 0.5 μg/10^7 cells) was far less than in normal hepatocytes (3.4 ± 0.1 mg/10^7 cells).

Cyclic AMP-Dependent Protein Kinase Activity The activity for phosphorylation of calf thymus histone was much higher in the $10000\times g$ supernatant of AH130 cells than in normal hepatocytes. These activities were further increased in both cells by cyclic AMP, and the increased activity was inhibited by H-8, an inhibitor of cyclic AMP-dependent protein kinase,[15] in a dose-dependent manner (Fig. 1). The cyclic AMP-dependent protein kinase activities in normal hepatocytes and AH130 cells were estimated to be 2.80 ± 0.06 and 10.11 ± 0.27 nmol Pi/100 mg protein, respectively.

Glycogen Phosphorylase Kinase Activity As shown in Fig. 2, there was no significant difference between the basal activities of the enzyme in the $10000\times g$ supernatant of normal hepatocytes and AH130 cells. The kinase activity in hepatocytes was gradually increased by increasing the concentration of cyclic AMP. The activity in AH130 cells was also increased by cyclic AMP, and the maximum activation was given by 1 μM cyclic AMP. The response to cyclic AMP of the enzyme in AH130 cells was higher than that in normal hepatocytes; the enzyme activity in normal hepatocytes and AH130 cells was increased 0.13 ± 0.1 and 0.18 ± 0.2 nmol Pi/min/100 mg protein, respectively, by cyclic AMP. These results indicate that in the $10000\times g$ supernatant of the cells glycogen phosphorylase kinase can be activated by cyclic AMP through activation of cyclic AMP-dependent protein kinase.

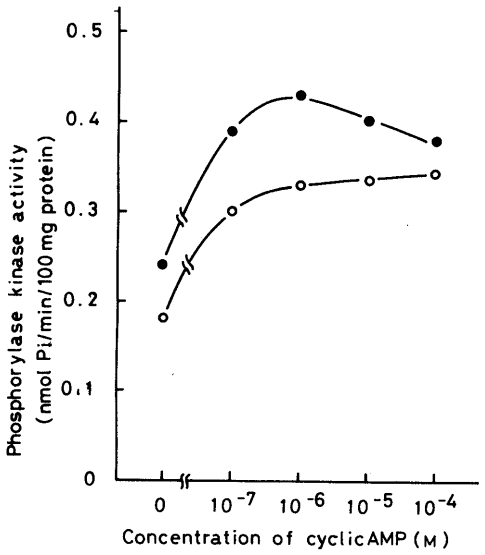

Fig. 2. Effect of Cyclic AMP on Phosphorylase Kinase Activity in $10000\times g$ Supernatant of Normal Rat Hepatocytes and AH130 Cells

Each supernatant was incubated with [γ-^{32}P]ATP and various concentrations of cyclic AMP for 10 min at 30 °C. Normal rat hepatocytes (○); AH130 cells (●).

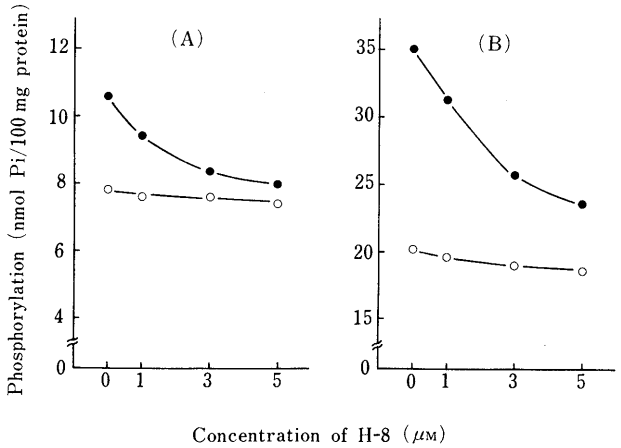

Fig. 1. Cyclic AMP-Dependent Protein Kinase Activity in $10000\times g$ Supernatant of Normal Rat Hepatocytes (A) and AH130 Cells (B)

Each supernatant was incubated with [γ-^{32}P]ATP and various concentrations of H-8 in the presence (●) or absence (○) of 10 μM cyclic AMP for 3 min at 30 °C.

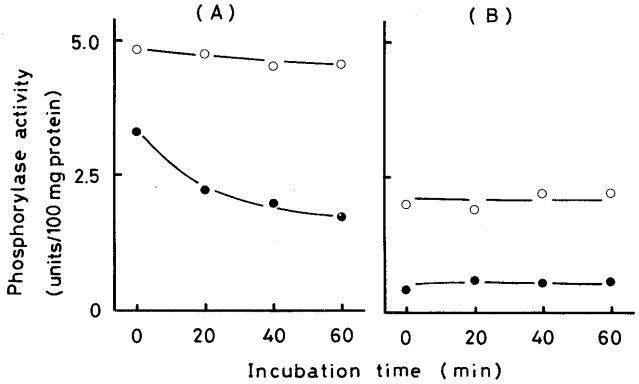

Fig. 3. Comparison of Glycogen Phosphorylase Activities between Normal Rat Hepatocytes (A) and AH130 Cells (B)

Cells were incubated in 40 mM glucose at 37 °C, and the total phosphorylase (○) and phosphorylase *a* (●) activities in the homogenates were measured at the indicated times.

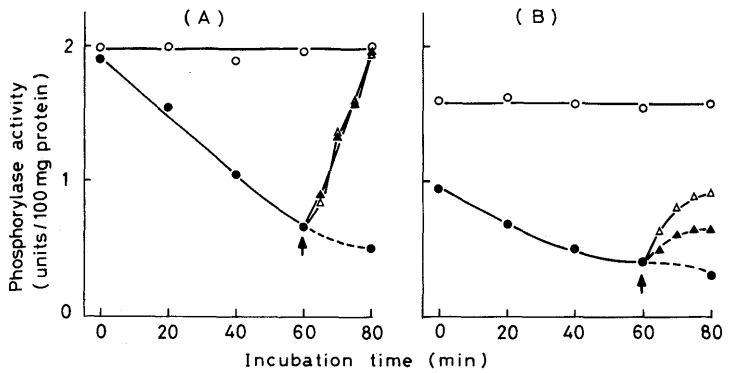

Fig. 4. Interconversion of Glycogen Phosphorylase Activities in 10000 × *g* Supernatant of Normal Rat Hepatocytes (A) and AH130 Cells (B)

The supernatant was incubated in 40 mM glucose at 37 °C, and at the times indicated by arrows 0.5 mM Mg^{2+}-ATP (▲) or 0.5 mM Mg^{2+}-ATP and 10 mg/ml glycogen (△) were added to the medium and incubated. The total phosphorylase activity (○) and phosphorylase *a* activity (●, ▲, △) are shown.

Table I. Effects of IPN and Dibutyryl Cyclic AMP on Phosphorylase *a* Activity in Normal Rat Hepatocytes and AH130 Cells

Treatment	Hepatocytes (units/100 mg protein)	AH130 cells (units/100 mg protein)
Before incubation	3.27±0.16	0.80±0.14
Control	1.95±0.03	0.91±0.03
IPN 10 μM	3.07±0.10[a)]	0.94±0.05
Dibutyryl cyclic AMP 10 μM	3.51±0.30[a)]	0.88±0.07

Cells were incubated in 40 mM glucose for 30 min at 37 °C and treated with agents for 2 min. After the treatment, phosphorylase *a* activity in the cell homogenate was measured. Each value represents the mean±S.E. *a)* $p<0.05$, control *vs.* treated.

Table II. Effect of Glycogen on Phosphorylase *a* Activity in 10000 × *g* Supernatant of Normal Rat Hepatocytes and AH130 Cells

Concentration (mg/ml)	Hepatocytes (units/100 mg protein)	AH130 cells (units/100 mg protein)
0	1.35±0.05	0.53±0.02
0.1	1.41±0.08	0.60±0.02[a)]
1.0	1.33±0.04	0.66±0.05[a)]
10	1.52±0.07[a)]	0.70±0.02[b)]

After preincubation for 60 min, the 10000 × *g* supernatant was further incubated with the indicated concentration of glycogen in the presence of 0.5 mM Mg^{2+}-ATP for 10 min at 37 °C. *a)* $p<0.05$. *b)* $p<0.01$.

Glycogen Phosphorylase Activity The total phosphorylase activity in both kinds of cells was higher in the homogenates than in the supernatants, while there was a small difference in phosphorylase *a* activity between the homogenates and the supernatants (Figs. 3 and 4). The total phosphorylase activity in the homogenate of AH130 cells was about half that of normal rat hepatocytes. Phosphorylase *a* activity in the homogenate of AH130 cells represented only about one-fifth of the total phosphorylase activity, but in normal hepatocytes it was about two-thirds of the total activity (Fig. 3). Phosphorylase *a* activity in the supernatant of hepatocytes accounted for most of the total activity, whereas in AH130 cells it was about half of the total activity (Fig. 4).

When cells were incubated at 37 °C in 40 mM glucose, phosphorylase *a* activity in normal hepatocytes gradually declined during the incubation, but the activity in AH130 cells did not change. The total activities in both kinds of cells were maintained at constant levels during the incubation (Fig. 3). The decreased activity in normal hepatocytes after the incubation for 30 min was restored by treatment with IPN and dibutyryl cyclic AMP for 2 min, but in AH130 cells the activity was not influenced by these agents (Table I). Thus, the β-adrenergic and cyclic AMP-dependent response of phosphorylase *a* was not observed in intact AH130 cells. When the 10000 × *g* supernatant of the cells was incubated in the presence of a high concentration of glucose, phosphorylase *a* activities in both kinds of cells declined. The decreased activity in normal hepatocytes was completely restored by addition of Mg^{2+}-ATP to the incubation media, but in AH130 cells the restoration by Mg^{2+}-ATP alone was partial and became complete with additional glycogen (Fig. 4). The effects of glycogen on the activation were dose-dependent in AH130 cells, while in normal hepatocytes glycogen did not affect the enzyme activity (Table II).

The conversion of glycogen phosphorylase *a* to *b* is caused by dephosphorylation of the *a* form by phosphorylase phosphatase. The phosphatase activity in the homogenates was 61.2±6.6 units/100 mg protein in normal hepatocytes and 39.0±7.0 units/100 mg protein in AH130 cells.

Discussion

We have found that a rat ascites hepatoma AH130 cell line did not respond to IPN in glycogen phosphorylase activation, although the cyclic AMP level in the cells was significantly increased by the β-adrenergic stimulation. Cyclic AMP regulates various functions of cells through the action of cyclic AMP-dependent protein kinase. Glycogenolysis in rat liver is one of them, and it is promoted by the activation of phosphorylase kinase.[16)]

It has been indicated that the isozyme pattern of cyclic AMP-dependent protein kinase and its intracellular localization in tumor cells differ from those of normal cells.[17)] As regards hepatoma cells, the activity of cyclic AMP-dependent protein kinase was lower than that in rat liver cells.[17b,18)] However, this study showed that cyclic AMP-dependent protein kinase activity in AH130 cells is higher than that in normal rat hepatocytes. This study also indicated that cytosolic phosphorylase kinase in AH130 cells is activated by cyclic AMP. The activation seems to be caused through cyclic AMP-dependent protein kinase. When these cell supernatants were incubated in a high concentration of glucose, the conversion of phosphorylase

a to *b* occured, and the decreased phosphorylase *a* activity in the supernatant of AH130 cells did not recover to the original activity after the addition of ATP alone but did do so completely with additional glycogen, while normal hepatocytes fully recovered with ATP alone. Phosphorylase kinase catalyzes its own activation dependent on Mg^{2+} and Ca^{2+}.[19] Glycogen is known as an activator of phosphorylase kinase,[20] but the content in AH130 cells was far less than in normal hepatocytes, as reported for other hepatomas.[21] A part of the unresponsiveness of AH130 cells to β-adrenergic stimuli may be attributed to their very low glycogen content.

The phosphorylase activity in AH130 cells after addition of ATP and glycogen never exceeded the initial *a* activity. This may indicate that only phosphorylase *b* converted from the *a* form by phosphorylase phosphatase may be able to be the substrate of phosphorylase kinase. Thus, it has been clarified that the key enzymes in the glycogenolytic cascade act functionally in AH130 cells. However, the interconversion of phosphorylase was not observed in intact tumor cells. A possible reason for the difference between the reaction in intact tumor cells and their supernatant fraction may be the low phosphorylase phosphatase activity in the cells. Some inhibitors of the phosphatase may be present in the particulate fraction of the hepatoma cells. On the other hand, the total phosphorylase activity was higher in the cell homogenates than in the cell supernatants, while there was a small difference in the activity of the active form between the homogenates and supernatants. This may suggest the localization of the inactive form in the particulate fraction of the cells. Furthermore, phosphorylase *a* in the supernatant of AH130 cells accounted for about half of the total activity, while in normal hepatocytes it accounted for most of the total activity. Sato *et al.*[22] have shown the existence of a fetal type isozyme of phosphorylase in AH130 cells. It is not known whether this isozyme is involved in the interconversion. It has been reported that the interconversion of glycogen synthase is closely associated with that of the phosphorylase.[23] The synthase activity in AH130 cells was very low, as reported,[12a,21] and the interconversion and the adrenergic response were not observed (data not shown). The cause of the unresponsiveness of phosphorylase in the hepatoma cells may also be related to the loss of regulation of synthase.

These results indicate that glycogenolysis in AH130 cells is hardly caused by β-adrenoceptor stimulation, although the hepatoma cells have enzymes of the cyclic AMP-dependent cascade. The hepatoma cells lack another type of adrenoceptor, α_1.[6b] Consequently, it seems that glycogen metabolism in the hepatoma cells is not regulated by catecholamines.

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