Hormonal Regulations of Alkaline Phosphatase, 5'-Nucleotidase and γ-Glutamyltransferase Activities in Adult Rat Hepatocytes Cultured in Serum-Free Medium on Collagen Gel

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ABSTRACT. Primary hepatocytes were cultured on collagen gel in serum-free, α-modified Eagle's minimum essential medium containing 0.1 μM insulin, 0.1 μM dexamethasone, 10 mM pyruvate and supplements such as glucagon, epinephrine or growth hormone. The activities of alkaline phosphatase, 5'-nucleotidase and γ-glutamyltransferase were assayed in cell extracts prepared from the cultures. All three enzyme activities were induced by glucagon, epinephrine or dibutyryl CAMP. The maximally effective concentration of glucagon was 5-10 nM for both alkaline phosphatase and 5'-nucleotidase and 100 nM for γ-glutamyltransferase. Only alkaline phosphatase activity was suppressed by growth hormone, which caused marked suppression at about 1 μg (0.25 ng)/ml. Taurocholate also induced both alkaline phosphatase and γ-glutamyltransferase activities at 1 mM.

The alkaline phosphatase (ALP), 5'-nucleotidase (5'ND) and γ-glutamyltransferase (γGT) activities of mature hepatocytes are mainly located in their plasma membrane. The two phosphatases ALP and 5'ND are anchored to the outer surface of the membrane through a covalent linkage to glycosyl phosphatidylinositol (1), while γGT is bound to the membrane through its hydrophobic peptide segment (2-4). The ALP and γGT activities in rat liver were reported to increase with increase in the amount of cAMP in the liver (5, 6). The former was induced by administration of glucagon (Glu), dibutyryl cAMP (Bt2cAMP) (and also cholera enterotoxin) (5, 7, 8) or epinephrine (EP) (9), and the latter by administration of Bt2cAMP or theophylline (10, 11). These inductions have not, however, been demonstrated in cultures of primary hepatocytes. Even in the absence of added hormone, the ALP and 5'ND activities in cultured hepatocytes increase spontaneously and rapidly in early stages (days 1-2) of culture (12-14), and γGT activity increases in later stages (days 3-5) of culture (15-17). The only hormone known to affect the three enzyme activities in primary hepatocytes is dexamethasone (Dex), which induces ALP (14) and γGT (16-18) activities, but suppresses 5'ND activity (14). In a previous study, we found that ALP and 5'ND activities in hepatocytes cultured on collagen-coated plastic are affected appreciably by the Ca2+ concentration of serum-free medium containing both insulin (Ins) and Dex, whereas the γGT activity is not (19). In this study, we examined whether hormones that generate cAMP in cultured hepatocytes and growth hormone (GH) affect these enzyme activities in the cells cultured in serum-free low (0.1 mM) and high (2 mM) Ca2+ media on collagen gel. Collagen gel was used because it enhances survival of the cells during culture for more than 4 days. Results showed that Glu, EP and Bt2cAMP markedly induced these enzyme activities regardless of the Ca2+ concentration of the medium, as observed in vivo, and that GH markedly suppressed ALP activity, but had no appreciable affect on 5'ND or γGT activity.

MATERIALS AND METHODS

Preparation of hepatocyte suspension. Hepatocytes were isolated from adult male Wistar rats weighing about 200 g, as described previously (19). Suspensions of 1.2-1.5 × 10⁷ cells/ml in Williams' medium E were diluted to 6 × 10⁶ cells/ml with Ca²⁺-free α-modified Eagle's minimum essential medium (αMEM) containing amphotericin B (0.25 mg/l), gentamycin (60 mg/l) and penicillin G (10⁸ units/l).
Ca²⁺-free αMEM. This medium was prepared as reported (19), and its Ca²⁺ concentration was adjusted to 0.1 mM or 2 mM with 1 M CaCl₂.

Culture of hepatocytes on collagen gel. Culture dishes (d: 35 mm) were coated with rat tail collagen gel as described (20), except that the stock collagen solution in 0.1% acetic acid was diluted to 0.7 mg/ml and mixed with one-fourth volume of conc. (× 5) Ca²⁺-free αMEM containing 50 mM Hepes just before gelation. The cell suspension (1 ml) was inoculated onto the gel and incubated for about 2 h in an incubator under 5% CO₂ in air at 37°C to allow cell attachment. The medium was then replaced by αMEM (1 ml) containing 0.1 μM Ins, 0.1 μM Dex, 10 mM pyruvate and 0.1 mM or 2 mM Ca²⁺. Test agents were added to the culture medium about 1 h after the first medium change. The medium was changed to fresh culture medium supplemented with test agents 24 h and 72 h after inoculation.

Measurement of number of cell nuclei. Cell nuclei were isolated from the cells after solubilizing the collagen gel with 0.07% Nikkol BO-10TX in 0.1 M citric acid (NCA) (1 ml) as reported (20). Nuclei were counted in a hemocytometer, and values are presented as means ± S.D. for triplicate dishes.

Assays of ALP, 5'ND and γGT activities of cultured cells. The culture medium was removed, and the collagen gel was digested with 0.1% collagenase solution containing soybean trypsin inhibitor (50 μg/ml) in Hank's solution (1 ml) with gentle shaking at 37°C for 30 min on a Low-Profile Rocker (Belco Glass, Vineland, N.J.). The digest was then transferred to a plastic test tube with a plastic pipette, the dish washed with 0.15 M NaCl (2 ml), and the washing fluid combined

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**Fig. 1.** Time courses of changes in ALP activity of cells cultured with Glu, EP or GH in 0.1 mM and 2 mM Ca²⁺ media.

**Fig. 2.** Time courses of changes in 5'ND and γGT activities in cultures in 0.1 mM and 2 mM Ca²⁺ media.

Cells were cultured for the indicated days in media with or without hormones. Samples: no addition (●), with 0.1 μM Glu (○), with 10 μM EP (▲), with 200 μU GH (●). Other experimental conditions were as described in the text.
with the digest. Cells were pelleted by centrifugation, washed with 0.15 M NaCl and lysed with 0.1% Zwittergent 3-14 deter-
gent (Z 3-14) in 0.15 M NaCl (1 ml) as reported (19). The ly-
sate was centrifuged, and the resulting supernatant was used for
assays of activities of ALP, 5'ND and γGT as described in
our previous paper (19).

Materials. Collagenase for cell isolation from the liver
(type 1), Ins, soybean trypsin inhibitor, Glu, GH (=somato-
tropin from human pituitary, 4 I.U./mg), taurocholate (Tch),
EP and Bt2cAMP were from Sigma Chemical Co., St. Louis.
Collagenase for cell detachment and Dex were from Wako
Pure Chemical Industries, Osaka. Williams' medium E was
from Flow Laboratories, Irvine. Z 3-14 was from Calbiochem
Co., La Jolla. Nikkol BO-10TX was from Nikko Chemicals,
Tokyo.

RESULTS

The effects of Glu, EP, Bt2cAMP and GH on the ac-
tivities of ALP, 5'ND and γGT in cultured hepato-
cytes. Hepatocytes on collagen gel remained viable for
more than 4 days in serum-free culture medium contain-
ing 0.1 μM Ins, 0.1 μM Dex and 10 mM pyruvate in
either low or high Ca²⁺ medium. After the culture peri-
od of 5 days, the number of nuclei of cells remaining on
the gel was about 70% of that at the time of inoculation
(data not shown), whereas that of cells inoculated onto
plastic was 25-50% of the initial number (19). ALP ac-
tivity in the cells cultured on the gel changed during the
culture period of 4 days in low and high Ca²⁺ media
containing Glu, EP or GH, as shown in Fig. 1. Glu (0.1
μM) markedly induced ALP activity in high Ca²⁺ medi-
um throughout the 4-day period and in low Ca²⁺ medi-
um for the first 2 days. EP (10 μM) induced ALP activi-
ty in both media throughout the 4-day period. GH (200
μU) suppressed the ALP activity in both media from
days 2 to 4.

γGT activity was very weak in freshly isolated hepato-
cytes and became appreciable after the culture period of
3 days, the changes in its activity in low and high Ca²⁺
media being similar (Fig. 2). We examined its activities
in 4-day cultures in low and high Ca²⁺ media contain-
ing Bt2cAMP or hormones that affected ALP activity
(Fig. 3). EP markedly induced γGT activity in both me-
dia, but Glu and Bt2cAMP induced γGT activity only in
high Ca²⁺ medium. GH did not affect the activity even
at a concentration of 200 μU (50 ng)/ml.

The dose-responses of the activities of ALP, γGT and
5'ND to Glu, Bt2cAMP, EP, and GH were examined in
4-day cultures only in the high Ca²⁺ medium (Figs. 4,
5). The time course of change in 5'ND activity in medi-

![Figure 3](image-url)

**Fig. 3.** γGT activities of cells cultured with Glu, EP, Bt2cAMP or GH in 0.1 mM and 2 mM Ca²⁺ medium.
Cells were cultured for 4 days in 0.1 mM (○) or 2 mM (■) Ca²⁺ medium with or without Glu, EP, Bt2cAMP or GH at the indicated concentra-
tions. Other experimental conditions were as described in the text.
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Fig. 4. Dose-dependent inductions of ALP, 5'ND and γGT activities by Glu, Bt2cAMP or EP.

Cells were cultured for 4 days in 2 mM Ca\(^{2+}\) medium with or without Glu, Bt2cAMP or EP at the indicated concentrations. Other experimental conditions were as described in the text.

Fig. 5. Effect of GH concentration on ALP, 5'ND and γGT activities.

Cells were cultured for 4 days in 2 mM Ca\(^{2+}\) medium with or without Glu or GH at the indicated concentrations. Other experimental conditions were as described in the text.

Table 1. Effect of Tch on the activities of ALP and γGT of cultured cells.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Relative enzymatic activity (%)</th>
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<tbody>
<tr>
<td></td>
<td>ALP</td>
</tr>
<tr>
<td>None</td>
<td>100*±12</td>
</tr>
<tr>
<td>Taurocholate 1 mM</td>
<td>153 ±16</td>
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<tr>
<td>2</td>
<td>146 ±16</td>
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* 5.64 mU/10^5 Nuclei, ** 1.12 mU/10^5 Nuclei

Cells were cultured for 4 days in 2 mM Ca\(^{2+}\) medium with or without Tch with the concentrations indicated. Other experimental conditions were as described in the text.

than those by the optimal concentration of Glu. These results support the idea that cAMP is an intracellular mediator of the effect of Glu. GH suppressed ALP activity only, decreasing its level to less than half the control level. It was effective at a concentration as low as 1 μU/ml (0.25 ng/ml) (Fig. 5). It also abolished the increase in the activity induced by Glu; 5 μU of GH suppressed the increase induced by 5 mM Glu to the control level.

The effect of Tch on the activities of ALP and γGT.

ALP activity in the liver increase in conditions of bile duct obstruction, such as cholestasis and experimental bile duct ligation (21, 22). This increase is associated with the induction of de novo synthesis of ALP protein in the liver (23–26). Hatoff and Hardison (13) examined
the effects of bile acids on ALP induction in primary cultures of hepatocytes, used as a model of liver with accumulated bile acids. They found that Tch induced ALP activity most effectively and that the induction was blocked by cycloheximide. Consistent with their results, we found that Tch caused marked induction of the enzyme activity, being maximally effective at 1 mM (Table 1). It also induced γGT activity. This result reproduces in an in vitro system the in vivo finding by Kaplan et al. (27) that bile duct ligation resulted in about a 2-fold increase in γGT activity in rat liver after 120 h.

**DISCUSSION**

In this study, we examined the inductions of ALP, 5'ND and γGT activities in hepatocytes cultured on collagen gel in serum-free αMEM containing Dex and Ins. We found that the ALP and 5'ND activities were induced by Glu and EP through an adenylate cyclase system and that the activity of only ALP was suppressed by GH. GH markedly suppressed both the basal level of ALP activity and its increased level induced by Glu, being effective at a concentration as low as 1 μU (0.25 ng/ml). Our results are consistent with those of in vivo experiments showing that Glu and Bt2cAMP induce ALP activity in the liver (5, 7, 8). GH was recently found to exert effects on the expressions of various hepatic proteins, including hormones, growth factor receptors, secretory proteins and enzymes (28, 29): e.g., insulinlike growth factor I (somatomedin C), EGF receptor, GH receptor, α2u-globulin, albumin, serine protease inhibitor and cytochrome P-450 family proteins. However, its effect on hepatic ALP activity has not been reported. Adult male rats (350–425 g) secrete GH periodically with peak values of >200 ng/ml and trough values of <1 ng (unmeasurable levels) at about 3.3 h intervals (30, 31). Therefore, the low effective concentration of GH suggests that ALP activity in the liver in vivo is suppressively controlled by this hormone. The ALP activity in freshly isolated hepatocyte culture is reported to increase within 2 days to a maximum of about 10 times that in whole liver or freshly isolated cells (12). The reason for this marked increase during culture has not previously been clarified. The present results show that GH in the culture medium reduced the increase in ALP activity to a value close to that in freshly isolated hepatocytes during culture for 4 days. This finding also suggests that GH regulates ALP activity in vivo, keeping its level low, as in freshly isolated hepatocytes or whole liver. Stress, induced by either disease or emotional or physiological conditions, activates the endocrine system, the main hormones involved being glucocorticoids, catecholamines, glucagon and GH. GH plays an essential role in mammals, not only in maintenance of postnatal body growth but also in a variety of stress-related responses. In man, GH secreted in response to exposure to stress seems to have a beneficial effect, countering the catabolic effects of stress by its protein anabolic actions. The ALP activity in human liver might be maintained homeostatically by a balance between cAMP-generating hormones and glucocorticoids that induce its activity and GH that suppresses its induction. On the other hand, in rats, exposure to stress suppresses GH secretion, abolishing the periodic peaks of its plasma levels, but stimulates the secretions of other stress hormones. Thus in rats, homeostatic control of ALP activity could not be compared to that in man. In rats, the induction of ALP activity may be suppressed continuously by GH to a low plasma level that is not decreased further by various stress stimuli, or it might be suppressed by some unknown humoral factor(s) other than GH that is released into the plasma in response to stress. Alternatively, its induction might be enhanced by stress. There are as yet no reports on the effect of stress on ALP activity in rat liver or in human liver. Liver ALP has been studied extensively for many years, but little is yet known about its precise physiological role(s). An understanding of hormonal interplay in the induction of ALP and the mechanism of action of each hormone(s) should provide clues to the physiological role(s) of this enzyme.

The 5'ND activity in primary cultures of hepatocytes also increases to a maximum during culture after 2 days, which is similar to the value in whole liver (12). This increase in its activity has been explained as due to repair of the plasma membranes of hepatocytes after their injury during cell isolation procedures.

In our experimental system, γGT activity was induced markedly by Bt2cAMP or EP, and moderately by Glu. These results are consistent with the conclusion from previous in vivo experiments that this enzyme activity is modulated by cAMP (6, 10, 11).

Thus, the present study confirmed and explained many endocrinical and pathological findings on changes in ALP, 5'ND and γGT activities in rat liver, and also demonstrated a regulatory effect of GH on ALP activity in the liver. Our culture system and methods for assays of the enzyme activities should be useful in further studies on hormonal regulations and pathological changes of the liver enzymes.

**REFERENCES**


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