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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 μU (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 (Bt_2cAMP) (and also cholera enterotoxin) (5, 7, 8) or epinephrine (EP) (9), and the latter by administration of Bt_2cAMP 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 Ca^{2+} 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) Ca^{2+} 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 Bt_2cAMP markedly induced these enzyme activities regardless of the Ca^{2+} 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.

Abbreviations used: NCA, 0.07% Nikkol BO-10TX in 0.1 M citric acid; MEM, Eagle's minimum essential medium; αMEM, α-modified Eagle's minimum essential medium; Z 3-14, Zwittergent 3-14; Ins, insulin; Dex, dexamethasone; GH, growth hormone; EP, epinephrine; Glu, glucagon; Tch, taurocholate; ALP, alkaline phosphatase; 5′ND, 5′-nucleotidase; γGT, γ-glutamyltransferase.

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 $1.2–1.5 \times 10^7$ cells/ml in Williams' medium E were diluted to 6×10^5 cells/ml with Ca^{2+}-free α-modified Eagle's minimum essential medium (αMEM) containing amphotericin B (0.25 mg/l), gentamycin (60 mg/l) and penicillin G (10^5 units/l).

Ca^{2+}-free αMEM. This medium was prepared as reported (19), and its Ca^{2+} concentration was adjusted to 0.1 mM or 2 mM with 1 M $CaCl_2$.

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) Ca^{2+}-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% CO_2 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 Ca^{2+}. 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 (Bellco 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

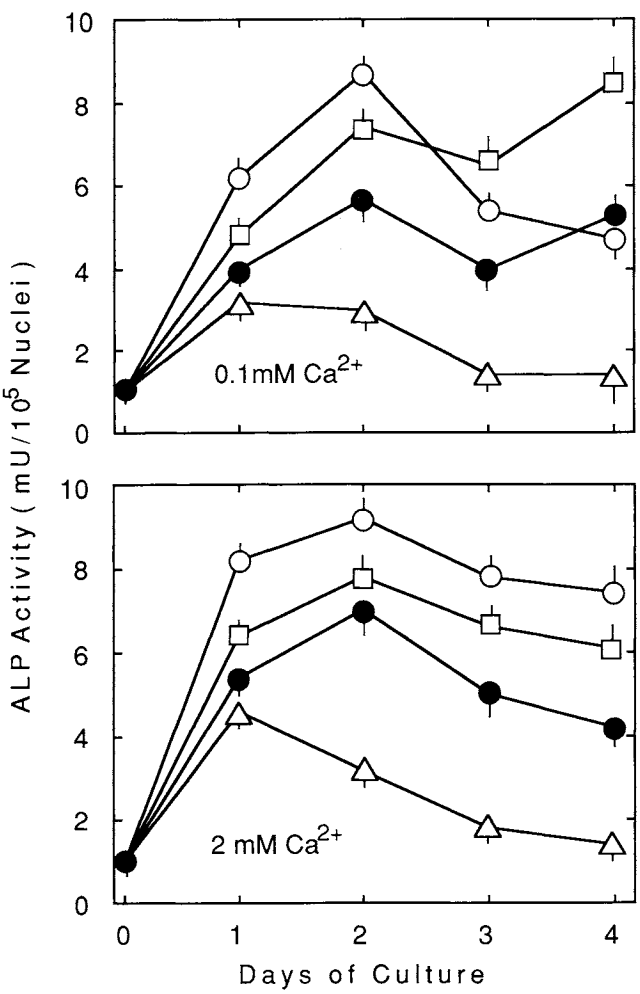


Fig. 1. Time courses of changes in ALP activity of cells cultured with Glu, EP or GH in 0.1 mM and 2 mM Ca^{2+} media.
Cells were cultured for the indicated days in media with or without hormones. Samples: no addition (●), with 0.1 μM Glu (○), with 10 μM EP (□), with 200 μU GH (△). Other experimental conditions were as described in the text.

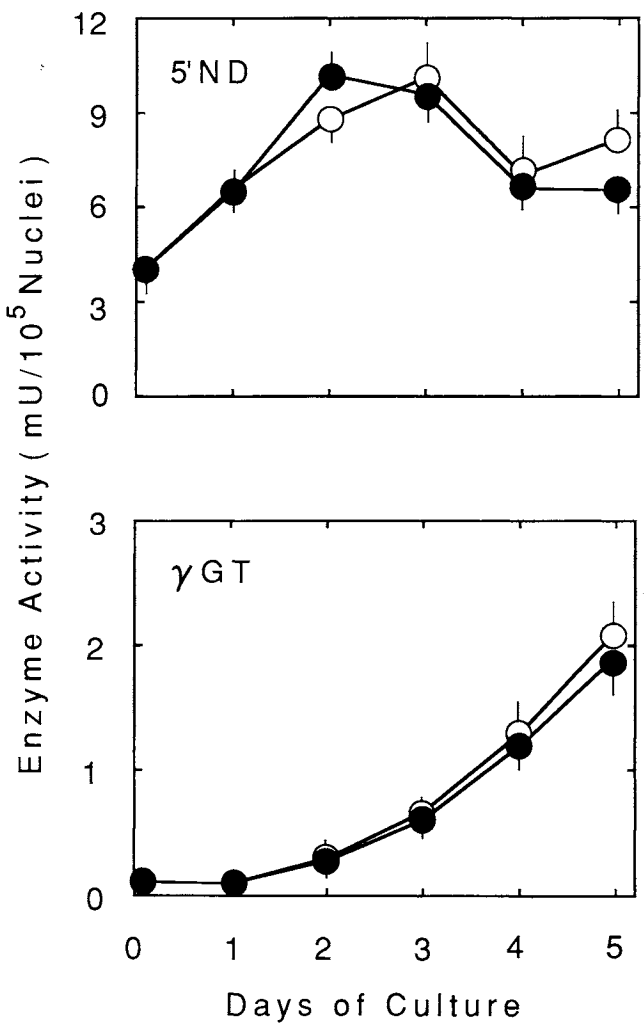


Fig. 2. Time courses of changes in 5'ND and γGT activities in cultures in 0.1 mM and 2 mM Ca^{2+} media.
Enzyme activities were assayed after the indicated days of culture in 0.1 mM (○) or 2 mM (●) Ca^{2+} media. Other experimental conditions were as described in the text.

with the digest. Cells were pelleted by centrifugation, washed with 0.15 M NaCl and lysed with 0.1% Zwittergent 3-14 detergent (Z 3-14) in 0.15 M NaCl (1 ml) as reported (19). The lysate 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 (=somatotropin from human pituitary, 4 I.U./mg), taurocholate (Tch), EP and Bt_2cAMP 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, Bt_2cAMP and GH on the activities of ALP, 5′ND and γGT in cultured hepatocytes. Hepatocytes on collagen gel remained viable for more than 4 days in serum-free culture medium containing 0.1 μM Ins, 0.1 μM Dex and 10 mM pyruvate in either low or high Ca^{2+} medium. After the culture period 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 activity in the cells cultured on the gel changed during the culture period of 4 days in low and high Ca^{2+} media containing Glu, EP or GH, as shown in Fig. 1. Glu (0.1 μM) markedly induced ALP activity in high Ca^{2+} medium throughout the 4-day period and in low Ca^{2+} medium for the first 2 days. EP (10 μM) induced ALP activity 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 hepatocytes and became appreciable after the culture period of 3 days, the changes in its activity in low and high Ca^{2+} media being similar (Fig. 2). We examined its activities in 4-day cultures in low and high Ca^{2+} media containing Bt_2cAMP or hormones that affected ALP activity (Fig. 3). EP markedly induced γGT activity in both media, but Glu and Bt_2cAMP induced γGT activity only in high Ca^{2+} 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, Bt_2cAMP, EP, and GH were examined in 4-day cultures only in the high Ca^{2+} medium (Figs. 4, 5). The time course of change in 5′ND activity in medium without added hormones was similar to that of ALP activity (Fig. 2). Glu had similar dose-dependent effects on the activities of ALP and 5′ND at concentrations of 1–10 nM, causing a maximum of 2-fold or more induction at 10 nM (Fig. 4). It also induced γGT activity, but at about ten times higher concentrations (10–100 nM) than those required for ALP or 5′ND. EP and Bt_2cAMP induced all three enzyme activities in similar dose ranges with maximal effects at 10 μM and 0.1 mM, respectively. The inductions by Bt_2cAMP at the highest concentrations tested were similar to, or slightly less

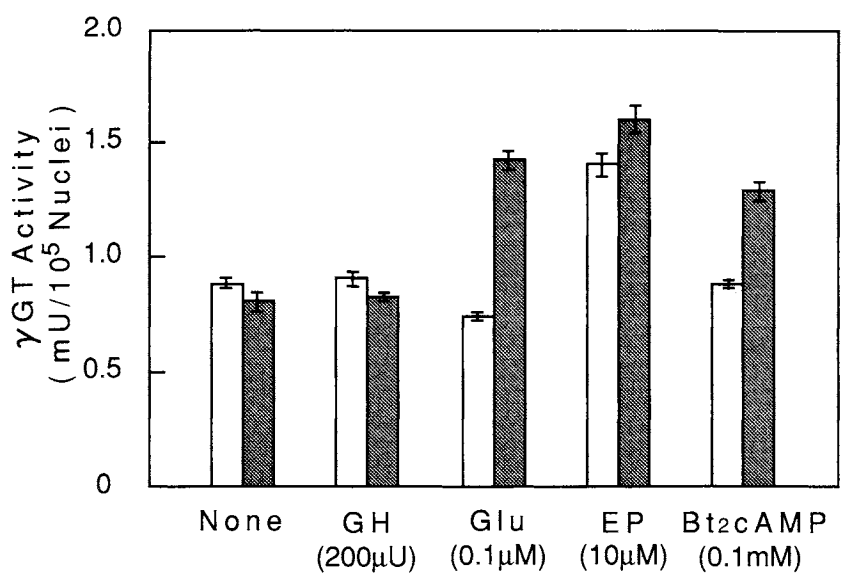


Fig. 3. γGT activities of cells cultured with Glu, EP, Bt_2cAMP or GH in 0.1 mM and 2 mM Ca^{2+} medium.
Cells were cultured for 4 days in 0.1 mM (□) or 2 mM (■) Ca^{2+} medium with or without Glu, EP, Bt_2cAMP or GH at the indicated concentrations. Other experimental conditions were as described in the text.

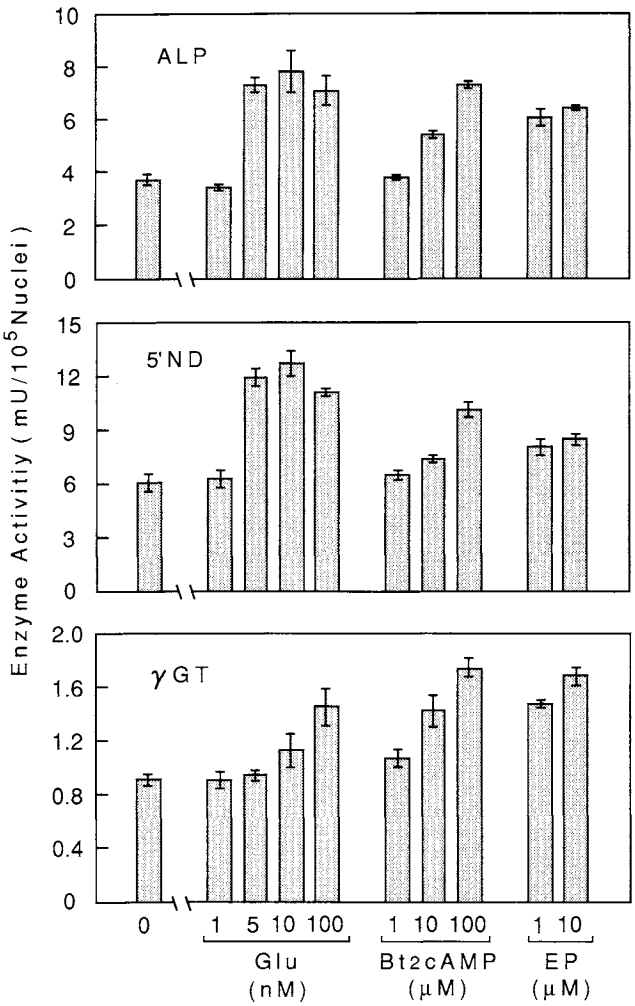


Fig. 4. Dose-dependent inductions of ALP, 5'ND and γGT activities by Glu, Bt_2cAMP or EP.
Cells were cultured for 4 days in 2 mM Ca^{2+} medium with or without Glu, Bt_2cAMP or EP at the indicated concentrations. Other experimental conditions were as described in the text.

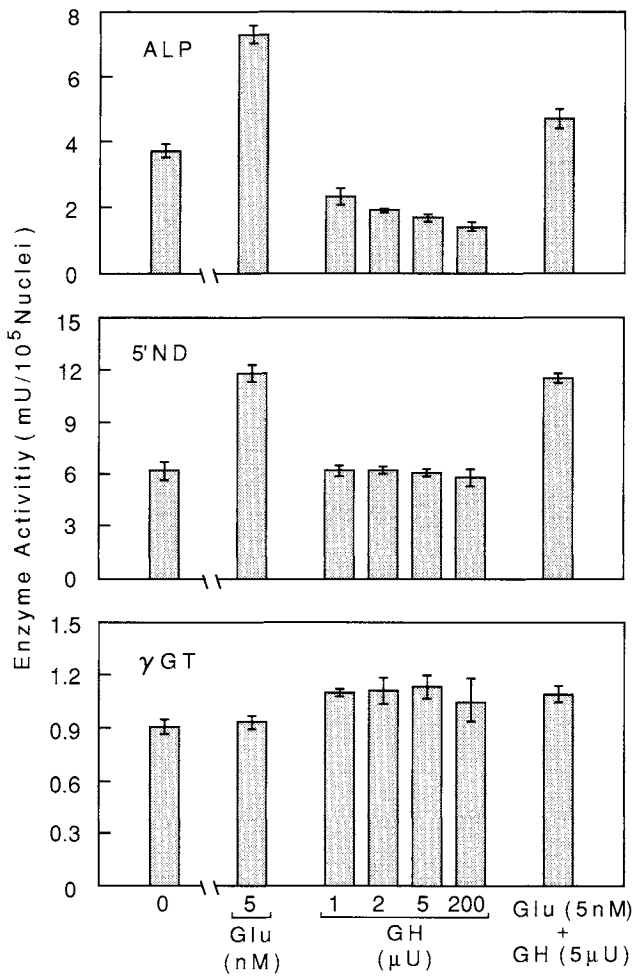


Fig. 5. Effect of GH concentration on ALP, 5'ND and γGT activities.
Cells were cultured for 4 days in 2 mM Ca^{2+} medium with or without Glu or GH at the indicated concentrations. Other experimental conditions were as described in the text.

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 nM 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

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

Reagent		Relative enzymatic activity (%)	
		ALP	γGT
None		100*±12	100**± 3
Taurocholate	1 mM	153 ±16	177 ±26
	2	146 ±16	147 ± 8

* 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.

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 I). 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 Bt_2cAMP 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, counteracting 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 Bt_2cAMP 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 endocrinal 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

1. Low, M.G. (1989). The glycosyl-phosphatidylinositol anchor of membrane proteins. *Biochim. Biophys. Acta*, **988**: 427–457.
2. Hughey, R.P., Coyle, P.J., and Curthoys, N.P. (1979). Comparison of the association and orientation of γ-glutamyltranspeptidase in lecithin vesicles and in native membranes. *J. Biol. Chem.*, **254**: 1124–1128.
3. Matsuda, Y., Tsuji, A., and Katunuma, N. (1983). Studies

on the structure of γ-glutamyltranspeptidase. III. Evidence that the amino terminus of the heavy subunit is the membrane binding segment. *J. Biochem.*, **93**: 1427–1433.
4. Laperche, Y., Bulle, F., Aissani, T., Chobert, M.-N., Aggerbeck, M., Hanoune, J., and Guellaen, G. (1986). Molecular cloning and nucleotide sequence of rat kidney γ-glutamyl transpeptidase cDNA. *Proc. Natl. Acad. Sci. USA*, **83**: 937–941.
5. Baker, A., Kaplan, M., and Kimberg, D.V. (1973). Alkaline phosphatase: Possible induction by cyclic AMP after cholera enterotoxin administration. *J. Clin. Invest.*, **52**: 2928–2934.
6. Puente, J., Varas, M.A., Beckhaus, G., and Sapag-Hagar, M. (1979). γ-Glutamyltranspeptidase activity and cyclic AMP levels in rat liver and mammary gland during the lactogenic cycle and in the oestradiol-progesterone pseudo-induced pregnancy. *FEBS Lett.*, **99**: 215–218.
7. Pekarthy, J.M., Short, J., Lansing, A.I., and Lieberman, I. (1972). Function and control of liver alkaline phosphatase. *J. Biol. Chem.*, **247**: 1767–1774.
8. Baker, A. and Kaplan, M. (1976). Effects of cholera enterotoxin, glucagon, and dibutyryl cyclic AMP on rat liver alkaline phosphatase, bile flow, and bile composition. *Gastroenterology*, **70**: 577–581.
9. Wilfred, G. and Rao, J.P. (1976). Induction of hepatic alkaline phosphatase: phosphodiesterase inhibitors and adrenaline. *Indian J. Biochem. Biophys.*, **13**: 331–334.
10. Higashi, T., Tateishi, N., Naruse, A., and Sakamoto, Y. (1976). Decrease of glutathione and induction of γ-glutamyltransferase by dibutyryl-3′,5′-cyclic AMP in rat liver. *Biochem. Biophys. Res. Commun.*, **68**: 1280–1286.
11. Tateishi, N., Higashi, T., Naruse, A., Nakashima, K., Shiozaki, H., and Sakamoto, Y. (1977). Rat liver glutathione: possible role as a reservoir of cysteine. *J. Nutr.*, **107**: 51–69.
12. Kato, S., Aoyama, K., Nakamura, T., and Ichihara, A. (1979). Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. *J. Biochem.*, **86**: 1419–1425.
13. Hatoff, D.E. and Hardison, W.G.M. (1979). Induced synthesis of alkaline phosphatase by bile acids in rat liver cell culture. *Gastroenterology*, **77**: 1062–1067.
14. Jung, W., Gebhardt, R., and Mecke, D. (1982). Alterations in activity and ultrastructural localization of several phosphatases on the surface of adult rat hepatocytes in primary monolayer culture. *Eur. J. Cell Biol.*, **27**: 230–241.
15. Sirica, A.E., Richards, W., Tsukada, Y., Sattler, C.A., and Pitot, H.C. (1979). Fetal phenotypic expression by adult rat hepatocytes on collagen gel/nylon meshes. *Proc. Natl. Acad. Sci. USA*, **76**: 283–287.
16. Edwards, A.M. (1982). Regulation of γ-glutamyltranspeptidase in rat hepatocyte monolayer cultures. *Cancer Res.*, **42**: 1107–1115.
17. Rosenberg, M.R., Strom, S.C., and Michalopoulos, G. (1982). Effect of hydrocortisone and nicotinamide on gamma glutamyltransferase in primary cultures of rat hepatocytes. *In Vitro*, **18**: 775–782.
18. Gomez-Lechon, M.J., Garcia, M.D., and Castell, J.V. (1983). Effect of glucocorticoids on the expression of γ-glutamyltransferase and tyrosine aminotransferase in serum-free-cultured hepatocytes. *Hoppe-Seyler's Z. Physiol. Chem.*, **364**: 501–508.
19. Horiuti, Y., Yano, K., and Shibuya, Y. (1992). Effect of calcium concentration on survival, proliferation and activities of alkaline phosphatase, 5′-nucleotidase, γ-glutamyltranspeptidase and lactate dehydrogenase of adult rat hepatocytes cultured in serum-free medium. *Cell Struct. Funct.*, **17**: 377–384.
20. Horiuti, Y., Ogishima, M., Yano, K., and Shibuya, Y. (1991). Quantification of cell nuclei isolated from hepatocytes by cell lysis with nonionic detergent in citric acid. *Cell Struct. Funct.*, **16**: 203–207.
21. Kaplan, M.M. and Righetti, A. (1969). Induction of liver alkaline phosphatase by bile duct ligation. *Biochim. Biophys. Acta*, **184**: 667–669.
22. Kaplan, M.M. and Righetti, A. (1970). Induction of rat liver alkaline phosphatase: the mechanism of the serum elevation in bile duct obstruction. *J. Clin. Invest.*, **49**: 508–516.
23. Kryszewski, A.J., Neale, G., Whitfield, J.B., and Moss, D.W. (1973). Enzyme changes in experimental biliary obstruction. *Clin. Chim. Acta*, **47**: 175–182.
24. Schlaeger, R. (1975). The mechanism of the increase in the activity of liver alkaline phosphatase in experimental cholestasis: measurement of an increased enzyme concentration by immunochemical titration. *Z. Klin. Chem. Klin. Biochem.*, **13**: 277–281.
25. Schulz, B.-O. and Schlaeger, R. (1979). Evidence for an enhanced *de novo* synthesis of alkaline phosphatase in cholestatic rat liver using immunotitration and precursor incorporation techniques. *Enzyme*, **24**: 173–180.
26. Kaplan, M.M., Ohkubo, A., Quaroni, E.G., and Sze-Tu, D. (1983). Increased synthesis of rat liver alkaline phosphatase by bile duct ligation. *Hepatology*, **3**: 368–376.
27. Kaplan, M.M., Kanel, G.C., and Singer, J.A. (1979). Enzyme changes and morphometric analysis of bile ducts in experimental bile duct obstruction. *Clin. Chim. Acta*, **99**: 113–119.
28. Zaphiropoulos, P.G., Mode, A., Norstedt, G., and Gustafsson, J.-A. (1989). Regulation of sexual differentiation in drug and steroid metabolism. *Trends Pharmacol. Sci.*, **10**: 149–153.
29. Norstedt, G., Enberg, B., Moller, C., and Mathews, L.S. (1990). Review Paper: Growth hormone regulation of gene expression. *Acta Paediatr. Scand* (Suppl), **366**: 79–83.
30. Tannenbaum, G.S. and Martin, J.B. (1976). Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology*, **98**: 562–570.
31. Willoughby, J.O., Martin, J.B., Renaud, L.P., and Brazeau, P. (1976). Pulsatile growth hormone release in the rat: failure to demonstrate a correlation with sleep phases. *Endocrinology*, **98**: 991–996.

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