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Effect of Calcium Concentration on Survival, Proliferation and Activities of Alkaline Phosphatase, 5′-Nucleotidase, γ-Glutamyltransferase and Lactate Dehydrogenase of Adult Rat Hepatocytes Cultured in Serum-free Medium

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ABSTRACT. **Mature rat hepatocytes were cultured on collagen coated dishes in serum-free α-modified Eagle's minimum essential medium containing 0.1 μM insulin, 0.1 μM dexamethasone, 10 mM pyruvate and Ca^{2+} at concentrations of 0–2 mM. Survival of nondivided cells was best in medium containing 2 mM Ca^{2+}. Proliferation during 5-day culture was greatest with 0.4 mM Ca^{2+}, but DNA synthesis was scarcely affected by the concentration of Ca^{2+}. Both the activities of alkaline phosphatase, 5′-nucleotidase, γ-glutamyltransferase and lactate dehydrogenase and the number of cell nuclei of cultures in 0.1 mM and 2 mM Ca^{2+} media were assayed over a 5-day period, and their activities were calculated as enzyme activities per unit number of cell nuclei. Alkaline phosphatase activity increased rapidly during the first day of culture in both media, and its activity in 0.1 mM medium was higher than that in 2 mM medium after culture for 3 days. The activity of 5′-nucleotidase became higher in 0.1 mM medium than in 2 mM medium from day 2 and was maximal on day 3 in both media. γ-Glutamyltransferase activity increased and lactate dehydrogenase activity decreased with time in culture, both activities showing no appreciable difference in the two media.**

The Ca^{2+} concentration ($[Ca^{2+}]$) of culture media markedly affects the proliferation and differentiation of epithelial cells. For example, proliferation of rat urinary bladder epithelial cells in organ culture was inhibited at 1.8 mM Ca^{2+} of medium but induced at 0.1–0.9 mM Ca^{2+} (1). Slimilarly, mouse epidermal cells proliferate more rapidly at lower $[Ca^{2+}]$ and show greater terminal differentiation at higher $[Ca^{2+}]$ (2). As hepatocytes are epithelial cells, their ability to proliferate and express differentiated functions may also be affected by the $[Ca^{2+}]$ of the culture medium. There are a few reports on the effects of the $[Ca^{2+}]$ on DNA synthesis (3) and cell division (4, 5) of mature hepatocytes in culture, but no report on the effects on the survival and enzymatic activities of hepatocytes during culture. Hasegawa *et al.* (4) could observed the cell divisions in culture medium containing no growth factor, when $[Ca^{2+}]$ of culture medium was reduced to below 0.4 mM, but they scarcely observed them at above 1 mM. They explained the observation as the result of loosing cell-to-cell contact caused by the lower $[Ca^{2+}]$ of medium. In addition, Eckl *et al.* (5) observed that the percentage of second cell division supported by epidermal growth factor (EGF) occurred 2-to 4-times more in media of $[Ca^{2+}]$s below 0.8 mM than in the medium of 1.8 mM. Thus proliferation of mature hepatocytes increases at lower $[Ca^{2+}]$ of culture medium. In the present study, we examined the effect of $[Ca^{2+}]$ on the survival, DNA synthesis, proliferation and activities of three plasma membrane enzymes, alkaline phosphatase (ALP), 5′-nucleotidase (5'ND) and γ-glutamyltransferase (γGT), and one cytosolic enzyme, lactate dehydrogenase (LDH), of cultured mature hepatocytes. The three enzyme activities were examined because the activities and proliferation are regulated reciprocally by cell density. The activities per mg cell protein increased (6, 7) and proliferation of the cells decreased (7, 8) as the density of inoculated cells was increased. Therefore, we supposed that, if the activities and proliferation are also regulated reciprocally by $[Ca^{2+}]$ of medium, the activities would be decreased at lower $[Ca^{2+}]$ where proliferation is stimulated. Contrary to expectation, we obtained the results

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Abbreviations used: NCA, 0.07% Nikkol BO-10TX in 0.1 M citric acid; αMEM, α-modified Eagle's minimum essential medium; PBS, phosphate buffered saline; Z 3-14, Zwittergent 3-14; Ins, insulin; Dex, dexamethasone; EGF, epidermal growth factor; I-UdR, 5-iodo-2-deoxyuridine; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; 5'ND, 5′-nucleotidase; γGT, γ-glutamyltransferase; $[Ca^{2+}]$, Ca^{2+} concentration.

that the ALP and 5'ND activities were higher in low (0.1 mM) Ca^{2+} medium than in high (2 mM) Ca^{2+} medium after culture for 3 days, whereas the γGT and LDH activities were not appreciably different in the two media during culture.

MATERIALS AND METHODS

Preparation of hepatocyte suspension. Hepatocytes were isolated from adult male Wistar rats weighing about 200 g by perfusion of the liver *in situ* with collagenase solution essentially as described by Seglen (9). The cells fractionated and washed by centrifugation in Eagle's minimum essential medium (MEM) were finally suspended in Williams' medium E at $1.2–1.5 \times 10^7$ cells/ml and diluted to various cell concentrations with Ca^{2+}-free α-modified Eagle's minimum essential medium (αMEM). All media were supplemented with amphotericin B (0.25 mg/l), gentamycin (60 mg/l) and penicillin G (10^5 units/l).

Preparation of Ca^{2+}-free αMEM. This medium (1 liter) was prepared by mixing MEM containing amino acids and vitamins alone (commercial product of Nissui Pharmaceutical Co., 0.88 g), L-alanine (25 mg), L-asparagine H_2O (50 mg), L-aspartic acid (30 mg), L-cysteine $HCl \cdot H_2O$ (82 mg), L-cystine 2 HCl (10 mg), L-glutamic acid (75 mg), L-proline (40 mg), L-serine (25 mg), glycine (50 mg), biotin (0.1 mg), vitamin B_{12} (1.4 mg), lipoic acid (0.2 mg), L-ascorbic acid (50 mg), sodium pyruvate (110 mg), glucose (1 g), phenol red (10 mg) and inorganic salts without $CaCl_2$ in the same amounts as in αMEM in distilled, deionized water. The resulting solution was supplemented with the three antibiotics and adjusted to about pH 7.4 with NaOH solution.

Culture of hepatocytes on collagen-coated plastic. Culture dishes (d: 35 mm) were coated with rat tail collagen as described previously (10). The cell suspension (2 ml) was inoculated onto the dishes and incubated for about 2 h under 5% CO_2 in air at 37°C to allow cell attachment. The medium was then replaced by αMEM (2 ml) containing 0.1 μM insulin (Ins), 0.1 μM dexamethasone (Dex), 10 mM pyruvate and Ca^{2+} at various concentrations. Culture in the CO_2-incubator was continued with change to fresh medium 24 h and 72 h after cell inoculation.

Measurement of number of cell nuclei. The cells were detached from the plastic surface by incubation with 0.07% Nikkol BO-10TX in 0.1 M citric acid (NCA) (2 ml), and their nuclei were isolated from them (10). Nuclei were counted in a hemocytometer, and values are presented as means or means ±S.D. for triplicate dishes.

Assay of DNA synthesis. Cell suspensions in Ca^{2+}-free αMEM (0.8×10^5 cells in 1 ml) were inoculated into wells (d: 20 mm) coated with rat tail collagen (0.5 ml) as described previously (10) and incubated for 2 h. The medium was then changed to αMEM (1 ml) containing 0.1 μM Ins, 0.1 μM Dex, 10 mM pyruvate, EGF (10 ng) and Ca^{2+} at various concentrations, and was renewed 24 h after cell inoculation. After 44 h the medium was supplemented with 0.25 μCi 5-[^{125}I]iodo-2-deoxyuridine ([^{125}I]IUdR) solution (20 μl). The [^{125}I]IUdR solution was prepared by diluting 200 μCi [^{125}I]IUdR in saline (0.25 ml) with 6 μM 5-iodo-2-deoxyuridine (IUdR) in PBS (16 ml) containing 20 mg bovine serum albumin. DNA synthesis was assayed by measuring incorporation of [^{125}I]IUdR into DNA in 3 h with or without addition of aphidicolin (5 μg) in 50% dimethylsulfoxide-PBS (10 μl) (11). After the incubation, the medium was aspirated, and the cells on the well washed with ice-cold PBS (1 ml) and detached with NCA (1 ml) by incubation with shaking for 10 min at 37°C. The resulting suspension was transferred to a plastic test tube with a plastic pipette. The well was washed with cold NCA (1.4 ml) and the washing solution was combined with the suspension. Unbroken cells and cell nuclei liberated during the detachment process were pelleted by centrifugation at 500 × g for 5 min and washed with cold NCA (3 ml) by centrifugation. The resulting pellet was washed again with cold NCA (3 ml) by centrifugation, and its radioactivity was measured in a gamma counter. The radioactivity incorporated into the pellet was similar to that in a pellet of cell nuclei only, which was prepared from the pellet of unbroken cells and cell nuclei by further incubation with NCA according to the procedure described under "measurement of number of cell nuclei". Activity for DNA syntesis was calculated from the mean ±S.D. for the radioactivity and the mean for the number of cell nuclei in triplicate wells, respectively. The numbers of nuclei were determined by counting the numbers in wells without added [^{125}I]IUdR.

Preparation of cell extracts. The culture medium was removed, and the cultured cells were detached with 0.1% collagenase in Hanks' solution (1 ml) containing soybean trypsin inhibitor (50 mg/l) by incubation at 37°C for 10 min with shaking. The resulting cell suspension was supplemented with 0.15 M NaCl (1 ml) and transferred to a plastic test tube with a plastic pipette. The dish was washed with 0.15 M NaCl (2 ml), and the washing fluid was combined with the suspension. Cells were pelleted by centrifugation at 500 × g for 5 min, washed by addition of 0.15 M NaCl (3 ml) without shaking, collected by centrifugation and suspended in 0.1% Zwittergent 3-14 (Z 3-14) in 0.15 M NaCl (1 ml) with the aid of a vibrator. The suspension was then incubated at 37°C for 10 min with continuous shaking. The cell lysate was centrifuged at 1,000 × g for 10 min at 25°C, and the resulting supernatant was kept in ice-water until assays of enzyme activities.

Assays of enzyme activities. Enzyme activities are presented as activiies per unit number (10^5) of cell nuclei calculated from the mean numbers of cell nuclei and means ±S.D. of the enzyme activities in triplicate dishes. ALP activity was measured by the King-King method as modified by Watanabe *et al.* (12). The reaction mixture (1 ml) was composed of 0.1 ml of cell lysate, 0.2 ml of 0.25 M sodium carbonate-sodium bicarbonate buffer (pH 10.2), 0.1 ml of 75 mM disodium phenyl phosphate, 0.5 ml of 15 mM 4-aminoantipyrine, 0.025 ml of 0.1 M $MgCl_2$ and 0.075 ml of distilled water. The reaction

was carried out at 37°C for 20 min and stopped by adding 0.5 ml of 36 mM potassium ferricyanide-0.21 M boric acid mixture. The absorbance of the resulting solution was measured at 500 nm. Phenol solution instead of the cell lysate was used as a standard. One unit of enzyme activity was defined as the amount releasing 1 μmol of phenol per min.

γGT activity was measured by the method of Persijn and van der Slik (13) with some modifications. The reaction mixture (1 ml) was composed of 0.3 ml of cell lysate, 0.2 ml of 0.5 M of Tris-HCl buffer (pH 8.2), 0.2 ml of 0.5 M glycylglycine-NaOH (pH 8.2), 0.2 ml of 15 mM L-glutamic acid-(3-carboxy-4-nitroanilide), and 0.1 ml of distilled water. The reaction was carried out at 37°C for 30 min and stopped by adding 0.1 ml of 10% SDS. The absorbance of the resulting solution was measured at 405 nm. The amount of 5-amino-2-nitrobenzoic acid liberated was calculated taking the millimolar extinction coefficient of the acid as 9.49. One unit of enzyme activity was defined as the amount liberating 1 μmol of the acid per min.

5′ND activity was measured by a modification of the methods of others (14–16). The reaction mixture (0.8 ml) was composed of 0.3 ml of cell lysate, 0.16 ml of 0.5 M Tris-HCl buffer (pH 8), 0.08 ml of 20 mM AMP (pH 8), 0.08 ml of 10 mM $MnCl_2$, 0.08 ml of 6.6% sodium β-glycerophosphate n-hydrate (pH 8) and 0.1 ml of distilled water. The reaction was started by adding the lysate, continued for 40 min at 37°C and stopped by adding 0.2 ml of 1 M trichloroacetic acid. The resulting mixture was stood for 30 min in ice-water and then centrifuged at 1,200 × g for 10 min at 5°C. Pi in the supernatant was then measured with a kit for inorganic phosphorus determination, Phospha-B-Test (Wako). A suitable volume of the supernatant was mixed with 1.7 ml of the reagent solution in the kit and 0.15 M NaCl in a final volume of 2 ml. The amount of the supernatant used (0.1–0.3 ml) was sufficiently small to avoid precipitation of insoluble chromogen by binding of proteins in the lysate to the blue phosphomolybdenum complex. One unit of enzyme activity was defined as the amount liberating 1 μmol of inorganic phosphorus per min. LDH activity was assayed as described previously (10) except that the lysate prepared with 0.1% Z 3-14 was used as an enzyme source.

Materials. Ins, trypsin inhibitor from soybean, collagenase for cell isolation (type 1), aphidicolin, and L-glutamic acid-(3-carboxy-4-nitroanilide) were from Sigma Chemical Co, St Louis. Collagenase used for cell detachment, Dex, IUdR, sodium β-glycerophosphate n-hydrate and Phospha-B-Test (Wako) were from Wako Pure Chemical Industries, Osaka. Modified MEM containing only amino acids and vitamins was from Nissui Pharmaceutical Co., Tokyo. Williams' medium E was from Flow Laboratories, Irvine. EGF was from Toyobo Co., Osaka. Detergent Z 3-14 was from Calbiochem Co., La Jolla. Nikkol BO-10TX was from Nikko Chemicals Co., Tokyo. [^{125}I]IUdR was from Amersham Japan Co., Tokyo.

RESULTS

Effect of [Ca^{2+}] on survival and proliferation of hepatocytes in culture media with and without EGF. Survival of hepatocytes in culture was measured in a 5-day period by scoring numbers of cell nuclei in cultures on collagen coated dishes in serum-free αMEM containing 0.1 μM Ins, 0.1 μM Dex, 10 mM pyruvate and Ca^{2+} at various concentrations. The two hormones and pyruvate, the concentration of which was a quater that in Koga's medium L for serum-free culture (4), were added because they enhanced cell viability (10). When cells were inoculated at a subconfluent cell density (5.8

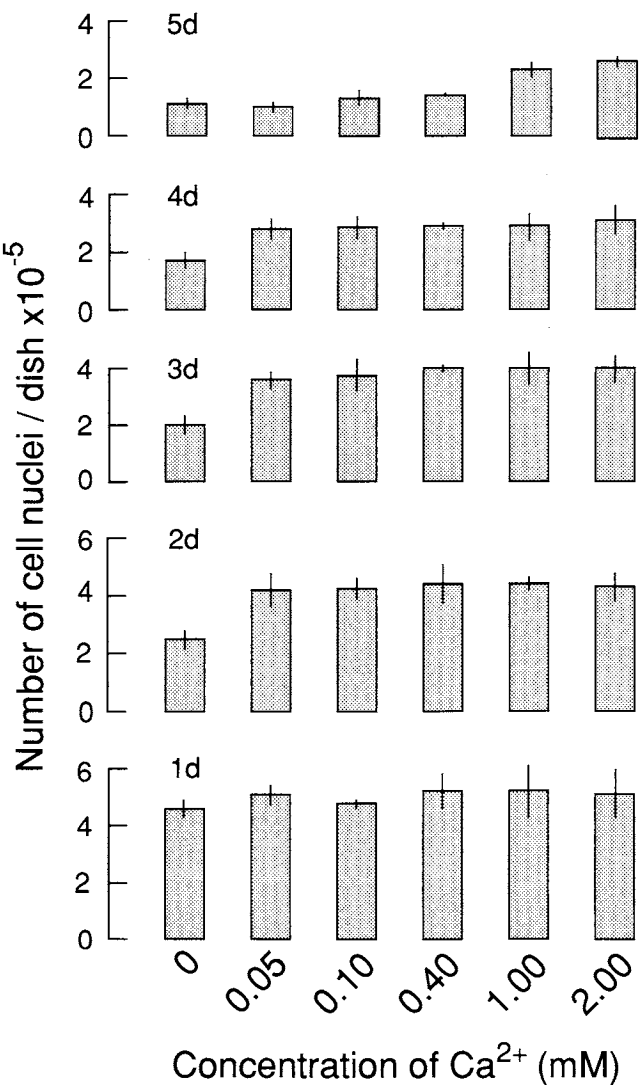

Fig. 1. Effect of [Ca^{2+}] on survival of nonproliferative hepatocytes. Cells were seeded at 5.8×10^5 nuclei per dish, and cultured in media with the indicated Ca^{2+} concentrations. Other experimental conditions were as described in the text. Values are means ± S.D. for triplicate dishes.

$\times 10^5$ cell nuclei/dish), the number of cell nuclei decreased similarly in 0.05–2 mM Ca^{2+} media during the first 4 days, but then the decrease was greatest in 0–0.4 mM Ca^{2+} media. On day 5, the numbers of nuclei in 0.1 mM and 2 mM Ca^{2+} media were less than one third and half that at seeding, respectively (Fig. 1). Thus nonproliferating hepatocytes survived best in medium containing about 2 mM Ca^{2+}. Hepatocytes in culture are induced to proliferation by EGF when the cell density is below about half that for confluence (8). Hepatocytes inoculated at lower cell density (1.5×10^5 cell nuclei/dish) and cultivated in the presence of EGF increased their number of cell nuclei by about 20% in 0.05–2 mM Ca^{2+} media in 2 days and by about 50% in 0.4–2 mM Ca^{2+} media in 3 days (Fig. 2). During further culture, however, the numbers increased in only 0.4 mM Ca^{2+} medium, attaining twice the inoculated number. Thus during 5-day culture hepatocytes proliferated best in 0.4 mM Ca^{2+} medium. This is consistent with a report (5) that the percentage of second division metaphases caused by EGF was highest in 0.4 mM Ca^{2+} medium. Under the present conditions, the first cell division occurred about 48 h after the start of culture with EGF and the second cell division after about 72 h (data not shown). Therefore, the number of nuclei on day 5 represents the increase by cell proliferation in at least two cell divisions minus the decrease due to cell death. With 0.4 mM Ca^{2+} dividing and newly divided cells in cell division cycles seemed to survive and enter another round of cell division without marked loss of viable cells.

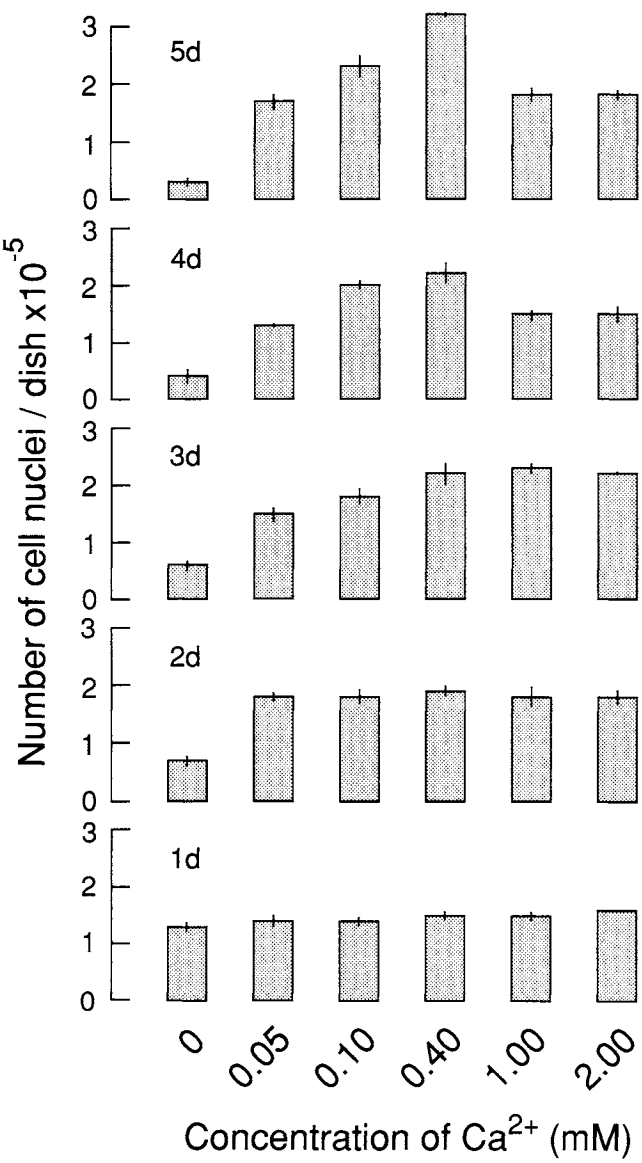

Fig. 2. Effect of $[Ca^{2+}]$ on survival of hepatocytes induced to proliferate by EGF. Cells were seeded at 1.6×10^5 nuclei per dish, and cultured in media containing the indicated concentrations of Ca^{2+}. EGF (10 ng/ml) was added to the medium on medium changes at 2 h, 24 h and 72 h. Other experimental conditions were as described in the text. Values are means±S.D. for triplicate dishes.

We obtained similar results on survival and proliferation of hepatocytes in culture media with and without EGF when the cell density inoculated was set at about 3×10^5 cell nuclei/dish (data not shown). At the cell density, however, the optimal concentration of Ca^{2+} (0.4 mM) for proliferation was less conspicuous because proliferation was terminated after the first cell division cycle probably due to the increase in cell density.

Effect of $[Ca^{2+}]$ on DNA synthesis. DNA synthesis induced by EGF reached a peak about 45 h after addition of EGF at the first medium change (data not shown). The peak activity of DNA synthesis was hardly affected by Ca^{2+} at concentrations of more than 0.05 mM (Table I). These results are consistent with a report (3) that serum-free media with a wide range of $[Ca^{2+}]$s (0.4–1.8 mM) yielded maximal DNA synthesis in cultures. The dose-dependencies of induction of DNA synthesis by EGF were similar in media containing 0.1, 0.4 and 2 mM Ca^{2+} (Fig. 3).

Cell shapes in low (0.1 mM) and high (2 mM) Ca^{2+} media in the absence and presence of EGF. Phase contrast photomicrographs of cells cultured in low and

Table I. DNA SYNTHESIS ACTIVITIES OF CELLS CULTURED AT VARIOUS Ca^{2+} CONCENTRATIONS.

Ca^{2+} (mM)	DNA synthesis activity (cpm/10^5 nuclei $\times 10^{-3}$)
0	9.8±0.8
0.05	10.6±0.8
0.10	10.4±0.4
0.40	10.2±0.6
2.00	10.4±1.0

Experimental conditions were as described in the text, except that cells were cultured in medium containing the indicated concentrations of Ca^{2+}.

high Ca^{2+} media are shown in Fig. 4. On day 2, in the absence of EGF (A, B) the cells in low Ca^{2+} medium (A) were more flattened and formed less compact cell islands than those in high Ca^{2+} medium (B). The low Ca^{2+} medium appeared to cause looser intercellular contact. In the presence of EGF (C, D) the cells in low Ca^{2+} medium (C) were more elongated and disordered than those in high Ca^{2+} medium (D).

Extractions of ALP, 5'ND, γGT and LDH from hepatocytes cultured in low (0.1 mM) and high (2 mM) Ca^{2+} media for assays of their activities. The ALP, 5'ND and γGT of cultured hepatocytes are mainly bound to the plasma membranes, and so their activities could be affected by cell-cell interaction and the $[Ca^{2+}]$ in medium. For assaying their activities in cultured cells, we examined the method for their extraction from the cells in comparison with the extraction of a typical cytosolic enzyme, LDH, as a control. Bailyes *et al.* (17) reported that 5'ND is solubilized from rat liver plasma membranes more efficiently with a zwitterionic detergent Z 3-14 (=Sulphobetaine 14) than with other detergents, such as sodium deoxycholate, Lubrol 12A9, sodium dodecyl sulfate and Triton X-100. Solubilization with the detergent Z 3-14 gave the best recoveries of all four enzyme activities when compared with solubilizations with Triton X-100 or by sonication (Fig. 5). From these results, we used this detergent at a concentration of 0.1% (w/v) in 0.15 NaCl for extractions of all four

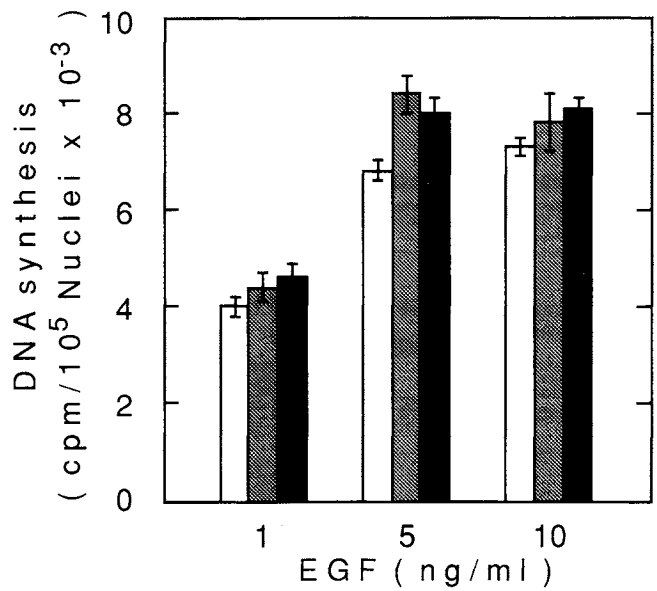

Fig. 3. Dependency of DNA synthesis on the concentration of EGF in culture media containing 0.1 mM, 0.4 mM and 2 mM Ca^{2+}. Cells were inoculated at 0.8×10^5 nuclei per well. The culture medium contained 0.1 mM (□), 0.4 mM (■) or 2 mM (■) Ca^{2+}. EGF was added to the medium in the amounts indicated. Other experimental conditions were as described in the text. Values are means±S.D. for triplicate wells.

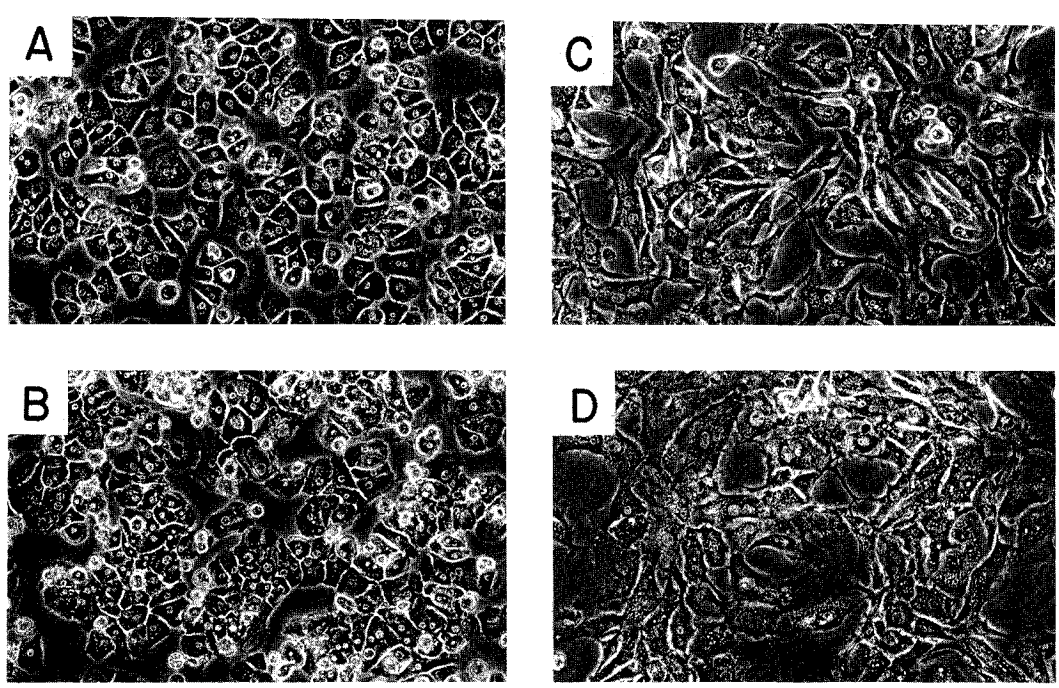

Fig. 4. Phase-contrast micrograph of cultured hepatocytes in culture media containing 0.1 mM and 2 mM Ca^{2+} with and without EGF. Cells at 5.6×10^5 nuclei (A, B) and 1.8×10^5 nuclei (C, D) were cultured for 2 days in 0.1 mM (A, C) and 2 mM (B, D) Ca^{2+} medium, and with (C, D) and without (A, B) EGF. Magnification. ×100

activities.

Changes in the activities of ALP, 5'ND, γGT and LDH with time in culture in low (0.1 mM) and high (2 mM) Ca^{2+} media. The number of nuclei of cells in culture decreased almost linearly with time in both media, although the decrease was much more in low Ca^{2+} medium after 4 days (Fig. 1). In both media, LDH activity decreased linearly for 3 days and then remaind constant during further culture (Fig. 6). In high Ca^{2+} medium, the activity of ALP increased rapidly in the first 1 day, reached a maximum on day 2 and then decreased (Fig. 6). This time-course of change was similar to that observed by others in ordinary culture media (6, 18, 19). On the other hand, in low Ca^{2+} medium, the activity increased to a plateau on days 2–3, and then gradually increased. Thus changes in the activity in the two media were similar until days 2–3, but became different during further culture. The activities of 5'ND in low and high

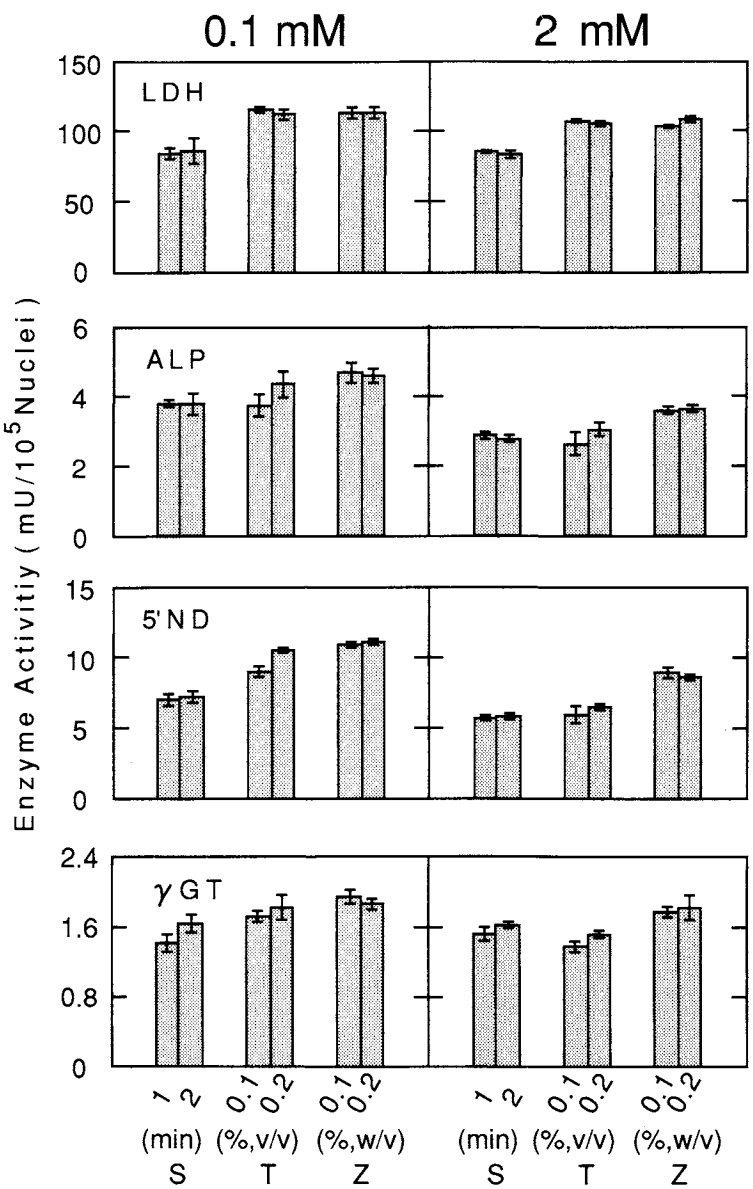

Fig. 5. Comparison of activities of ALP, 5'ND, γGT and LDH in cell extracts prepared by sonication (S) or solubilization with Triton X-100 (T) or Z 3-14 (Z).

Cells were seeded at 6.3×10^5 nuclei per dish and cultured in 0.1 mM or 2 mM Ca^{2+} medium for 4 days. They were then detached, collected and washed as described in the text. Washed cells were resuspended in ice-cold 0.15 M NaCl (1 ml) and disrupted by sonication for 1 or 2 min or lysed with 0.1% or 0.2% Triton X-100 or Z 3-14 in 0.15 M NaCl (1 ml). Other experimental conditions were as described in the text.

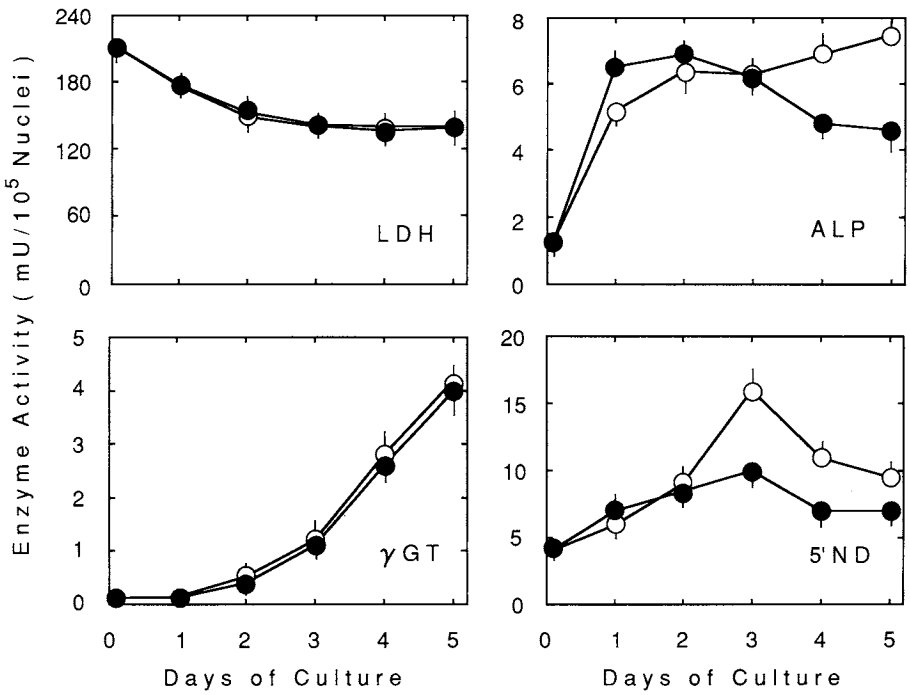

Fig. 6. Time-courses of change in activities of LDH, ALP, 5'ND and γGT of cells cultured in 0.1 mM and 2 mM Ca^{2+} media. Numbers of cell nuclei and enzyme activities were assayed after cell culture for the indicated days. Cells were seeded at 5.8×10^5 cell nuclei per dish and cultured in 0.1 mM (○) or 2 mM (●) Ca^{2+} medium. Other experimental conditions were as described in the text.

Ca^{2+} media increased similarly until day 2, reaching maxima on day 3. But on days 3–5, the activities in low Ca^{2+} medium were higher than those in high Ca^{2+} medium like those of ALP. γGT activity in hepatocytes from adult rat liver increases during primary culture to the level of that from fetal or immediate-post natal rat liver (20–22). Similarly, the activity in the cells in present medium increased rapidly after a lag period of 2 days. The time-courses of change in the activity were not appreciably different in low and high Ca^{2+} media. Thus differences in $[Ca^{2+}]$ in the culture medium did not cause appreciable differences in the activities of γGT and LDH. The increase in γGT activity appeared to be inversely related with that of decrease in the number of cell nuclei, as if reflecting deterioration of cultured cells.

DISCUSSION

Our results that ALP and 5'ND activities of mature hepatocytes increased during culture for 2–3 days in low and high Ca^{2+} media are in good accordance with other reports using ordinary culture media (6, 18, 19). But, although ALP activity in low Ca^{2+} medium rapidly increased to and reached a plateau, it increased again in further culture, showing no distinct peak. On the other hand, ALP activity in high Ca^{2+} medium and 5'ND activities in both Ca^{2+} media reached a maximum on day 2 and 3, respectively, and then they decreased in further culture, showing a broad peak. In a previous study (6) both ALP and 5'ND activities in hepatocytes were found to increase with increase in the density of inoculated cells during the initial 2–3 days in culture. In cultures at confluent or subconfluent density, the cells tend to reaggregate in a few days and reestablish intimate intercellular contacts including structures similar to bile canaliculi (19). Therefore, the intercellular contacts in cultures may enhance induction of activities of the two plasma membrane enzymes ALP and 5'ND by altering the cell surface structure. If this is so, the decreases in their activities after day 3 might be explained by loss of plasma membrane contact, causing suppression, deletion or denaturation of the enzymes. But there are two problems in this explanation. One is that the levels of these enzyme activities in low Ca^{2+} medium became higher than those in high Ca^{2+} medium after day 3, whereas intercellular contact seem to be less in low Ca^{2+} medium than in high Ca^{2+} medium (Fig. 4). The other is that ALP activity increased in later stages of culture in low Ca^{2+} medium, whereas LDH activity ($mU/10^5$ cell nuclei) decreased gradually during culture, indicating that the cells were gradually deteriorating, losing intercellular contact and died (Fig. 6). Therefore, we surmise that the inductions of these enzyme activities might be governed by a more complex and still unknown mechanism(s) that involves factor(s) dependent on plasma membrane interactions and other additional

factor (s). In a subsequent paper, we show that the activities of ALP, 5'ND and γGT are also under humoral regulaion by various hormones.

REFERENCES

1. Reese, D.H., and Friedman, R.D. (1978). Suppression of dysplasia and hyperplasia by calcium in organ-cultured urinary bladder epithelium. *Cancer Res.,* **38**: 586–592.
2. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S.H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell,* **19**: 245–254.
3. Sand, T-E., Gladhaug, I.P., Refsnes, M., and Christoffersen, T. (1989). DNA synthesis in cultured adult rat hepatocytes: effect of serum and calcium. *Liver,* **9**: 20–26.
4. Hasegawa, K., Watanabe, K., and Koga, M. (1982). Induction of mitosis in primary cultures of adult rat hepatocytes under serum-free conditions. *Biochem. Biophys. Res. Commun.,* **104**: 259–265.
5. Eckl, P.M., Whitcomb, W.R., Michalopoulos, G., and Jirtle, R.L. (1987). Effect of EGF and calcium on adult parenchymal hepatocyte proliferation. *J. Cell. Physiol.,* **132**: 363–366.
6. 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.
7. Edwards, A.M., Lucas, C.M., and Baddams, H.M. (1987). Modulation of γ-glutamyltranspeptidase in normal rat hepatocytes in culture by cell density, epidermal growth factor and agents which alter cell differentiation. *Carcinogenesis,* **8**: 1837–1842.
8. Nakamura, T., Tomita, Y., and Ichihara, A. (1983). Density-dependent growth control of adult rat hepatocytes in primary culture. *J. Biochem.,* **94**: 1029–1035.
9. Seglen, P.O. (1976). Preparation of isolated rat liver cells. In *Methods in Cell Biology* (D. Prescott, Ed.), Vol. 13, Academic Press, New York, pp 29–83.
10. 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.
11. Nakamura, T., Arakaki, R., and Ichihara, A. (1988). Interleukin-1 β is a potent growth inhibitor of adult rat hepatocytes in primary culture. *Exp. Cell Res.,* **179**: 488–497.
12. Watanabe, K., Tsuda, T., and Kitamura, M. (1967). A method for the determination of serum alkaline phosphatase. *The Japanese Journal of Clinical Pathology,* **15**: 708–712.
13. Persijn, J.P., and van der Slik, W. (1976). A new method for the determination of γ-glutamyltransferase in serum. *J. Clin. Chem. Biochem.,* **14**: 421–427.
14. Gerlach, U., and Hiby, W. (1974). 5'-Nucleotidase. In *Methods of Enzymatic Analysis* (H.U. Bergmeyer, Ed.), Vol. 2, Academic Press, New York, pp. 871–875.
15. Newby, A.C., Luzio, J.P., and Hales, C.N. (1975). The properties and extracellular location of 5'-nucleotidase of the rat fat-cell plasma membrane. *Biochem. J.,* **146**: 625–633.
16. Stanley, K.K., Edwards, M.R., and Luzio, J.P. (1980). Subcellular distribution and movement of 5'-nucleotidase in rat cells. *Biochem. J.,* **186**: 59–69.
17. Bailyes, E.M., Newby, A.C., Siddle, K., and Luzio, J.P. (1982). Solubilization and purification of rat liver 5'-nucleotidase by use of a zwitterionic detergent and a monoclonal-antibody immunoadsorbent. *Biochem. J.,* **203**: 245–251.
18. 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.
19. 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.
20. 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.
21. Edwards, A.M. (1982). Regulation of γ-glutamyltranspeptidase in rat hepatocyte monolayer cultures. *Cancer Res.,* **42**: 1107–1115.
22. 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.

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