Biochem. J. (1991) 279, 377–383 (Printed in Great Britain)

Rapid decrease in the expression of 3-hydroxy-3-methylglutaryl-CoA reductase protein owing to inhibition of its rate of synthesis after Ca^{2+} mobilization in rat hepatocytes

Inability of taurolithocholate to mimic the effect

Victor A. ZAMMIT, Anna M. CALDWELL and Marian P. KOLODZIEJ
Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

The mechanisms through which Ca^{2+} mobilization in rat hepatocytes results in the loss of total activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [Zammit & Caldwell (1990) Biochem. J. **269**, 373–379] were investigated. The loss of total activity was shown to be paralleled by an equal loss of immunoreactive HMG-CoA reductase protein after exposure of hepatocytes to optimal concentrations of vasopressin plus glucagon for 40 min. This loss of enzyme protein was due to an inhibition of enzyme synthesis; the rate of degradation was unaffected. Other Ca^{2+}-mobilizing conditions (phenylephrine, glucagon, vasopressin added singly and A23187) also resulted in graded inhibition of synthesis of HMG-CoA reductase. These effects were accentuated by omission of Ca^{2+} from the cell incubation medium, suggesting that it is the depletion of an intracellular $InsP_3$-sensitive pool of Ca^{2+} to which synthesis of HMG-CoA reductase is sensitive. In agreement with this we found that t-butylhydroxybenzoquinone, which inhibits the activity of the Ca^{2+}-ATPase of the endoplasmic-reticular membrane, mimicked the action of Ca^{2+}-mobilizing hormones. However, taurolithocholate, which transiently mobilizes Ca^{2+} from the same pool, was ineffective. All these effects on HMG-CoA reductase were accompanied by parallel inhibition of ^{35}S incorporation from [^{35}S]methionine into total protein, suggesting that inhibition of reductase synthesis formed part of a generalized response of the hepatocyte to Ca^{2+} mobilization. Inhibition of the rate of synthesis of HMG-CoA reductase was, however, more responsive to Ca^{2+} mobilization in the absence of added Ca^{2+} from the extracellular medium. The concentrations of vasopressin required to elicit the inhibition of synthesis of HMG-CoA reductase were of the same order as those that elicited activation of glycogen phosphorylase in hepatocytes.

INTRODUCTION

The activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in isolated hepatocytes is decreased acutely when Ca^{2+} mobilization is induced by addition either of hormones such as vasopressin or of the Ca^{2+} ionophore A23187 [1]. This effect has two, apparently independent, components, namely a rapid increase in the phosphorylation of the enzyme, which is maximal within 5 min, and a more prolonged decrease in total activity of the enzyme, presumed to be due to loss of enzyme protein. Although some progress has been made in identifying the role of individual protein kinases and phosphatases in the first of these responses [1,2], the mechanism through which a rapid loss of enzyme protein could be achieved remains to be investigated. Previous studies on the possible connection between increased phosphorylation of the enzyme and loss of its activity have concentrated on the role of increaed protein degradation [3–6]. However, it is equally possible that Ca^{2+} mobilization acts via a decrease in the rate of synthesis of HMG-CoA reductase. Indeed, preliminary observations indicated that incubations of hepatocytes with inhibitors either of Ca^{2+}-dependent cytosolic proteinases or of lysosomal proteolysis were both ineffective in preventing the action of Ca^{2+} mobilization on the loss of HMG-CoA reductase in isolated hepatocytes [2]. A third possibility exists, namely that the decrease in total HMG-CoA reductase activity is unrelated to a decrease in the amount of protein, but results from masking of the activity. In the present study, therefore, we have measured the amount of immunoreactive HMG-CoA reductase protein and have measured the specific rates of synthesis and degradation of the protein in isolated rat hepatocytes exposed to conditions that alter the Ca^{2+} fluxes across the endoplasmic-reticulum (ER) and plasma membranes. The results indicate that decreases in the rate of synthesis of HMG-CoA reductase are exclusively responsible for the loss of enzyme protein in cells exposed to Ca^{2+}-mobilizing hormones.

MATERIALS AND METHODS

Animals

These were female Wistar rats (200–220 g) that were housed in a room at 23 °C in which the lights were on for 12 h per day (from 19:00 h to 07:00 h). They were fed on a standard laboratory-rat chow diet *ad libitum* (see [1]). Where indicated, 4 days before being used, the animals were fed on a diet supplemented with 2% (w/v) cholestyramine (Bristol–Myers, Uxbridge, U.K.) for 48 h and, additionally, with 0.2% simvastatin (Merck, Sharp & Dohme, Hoddesdon, Herts., U.K.) for the final 48 h before the experiments. They had access to water throughout.

Hepatocyte preparation and incubations

Rats were anaesthetized with pentobarbitone (60 mg/kg) 3.5 h into the dark period. Hepatocytes were prepared by collagenase digestion [7] and washed in Krebs–Henseleit buffer [8] supplemented with 25 mM-Hepes (pH 7.4) and containing 2% BSA, by centrifugation at 30 *g* for 90 s. For experiments in which cells were incubated in the absence or presence of external Ca^{2+} in the medium, the initial suspension of cells was divided into two: one half of the suspension was sedimented, washed and resuspended in Ca^{2+}-containing medium (2.5 mM-$CaCl_2$),

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; ER, endoplasmic reticulum; tBuBHQ, t-butylbenzohydroquinone; TLC, taurolithocholate; EC_{50}, concn. giving 50% inhibition.

whereas the other half was washed and resuspended in Ca^{2+}-free medium. Routinely, the cells were approx. 93% viable, as judged by Trypan Blue exclusion. They were incubated, with continuous shaking (180 oscillations/min) and oxygenation (O_2/CO_2, 19:1) at a density of $(1.0–1.6) \times 10^6$ cells/ml in 25 ml silicone-treated glass conical flasks. The volume of the total incubation mixture was 2.8 ml for non-radioactive incubations and 1.5 ml for experiments involving [^{35}S]methionine. Cells were preincubated for 20 min before addition of hormones and other effectors.

Measurement of rates of synthesis and degradation of HMG-CoA reductase

These were performed essentially as described in [9]. Hepatocytes were incubated under conditions identical with those described in [1] except for the addition of [^{35}S]methionine (1000 Ci/mmol; 50 μCi per flask). The rate of incorporation of [^{35}S]methionine into HMG-CoA reductase and total protein was experimentally determined to be linear for at least 20 min, and routinely incorporation of label 20 min after addition of [^{35}S]methionine to the cells was used to quantify the rate of synthesis. Incubations were terminated by rapid sedimentation of cells (7000 *g* for 1 s) in an Eppendorff 5412 bench centrifuge. They were resuspended and washed in ice-cold incubation medium containing 2 mM-methionine, and re-sedimented. They were finally solubilized by addition of 0.96 ml of medium A. This contained 100 mM-NaCl, 10 mM-potassium phosphate, 5 mM-EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01% NaN_3 and 1 μg of antipain, 1 μg of pepstatin and 4 μg of leupeptin per ml. The pH was 7.5 at room temperature. To obtain rates of degradation, the cells were labelled with ^{35}S for a period of 20 min in a bulk incubation (same cell density as above in 15–20 ml). They were then sedimented (30 *g* for 90 s), washed in incubation medium containing 2 mM-methionine, re-sedimented, resuspended in Krebs medium containing 2 mM-methionine and divided into 1.5 ml batches in flasks. Loss of ^{35}S radioactivity from HMG-CoA reductase and total protein was monitored at the times indicated. Incubations were terminated by rapid sedimentation and addition of 0.96 ml of medium A. In all instances, solubilization of the cells was aided by brief (10 s) sonication, after which the samples were incubated for 45 min at room temperature, followed by centrifugation at 70000 *g* for 30 min. The supernatant containing solubilized protein [9] was aspirated and, after addition of 5 mg of BSA, samples (20 μl) were taken and added to 1.0 ml of ice-cold 5% (w/v) trichloroacetic acid for measurement of ^{35}S label in total protein. The precipitated protein was collected by centrifugation and washed three times with ice-cold acetone/water (9:1, v/v). The final pellet was dissolved in 0.5 ml of Triton X-100 (10%, v/v) and added to scintillant for determination of associated radioactivity. For measurement of ^{35}S label in HMG-CoA reductase protein, the supernatant obtained after solubilization and centrifugation (as above) was treated with a portion (20–30 μl, depending on original cell density) of a suspension of agarose–Protein A. The suspension was incubated at room temperature for 45 min with occasional shaking. The agarose–Protein A was removed by centrifugation, and 20 μl of rabbit antiserum raised against purified rat liver HMG-CoA reductase (see under 'Materials') was added. The tubes were incubated for 16 h at 4 °C and further treated as described in [9]. After separation of the immunoprecipitated proteins by SDS/PAGE the gels were treated with salicylic acid [9], dried and exposed to Kodak X-OMAT XAR film. The bands corresponding to the 97 kDa polypeptide (HMG-CoA reductase) were localized, and the ^{35}S label associated with them was determined after solubilization of the dried gel strips in 1.0 ml of Soluene (60 °C for 4 h) and addition of Ultima Gold scintillant (Canberra Packard, Pangbourne, Berks., U.K.). We routinely observed the presence of a 180 kDa protein which co-precipitates with HMG-CoA reductase, as described in [10].

For immuno-quantification of HMG-CoA reductase protein, the hepatocytes were treated exactly as above, except that after SDS/PAGE the proteins were transblotted on to nitrocellulose. HMG-CoA reductase was detected quantitatively by incubation with anti-reductase antiserum, followed by ^{125}I-labelled Protein A. After autoradiography (as above) each band corresponding to reductase (97 kDa) was excised and the amount of ^{125}I radioactivity associated with it was measured with a γ-radiation counter.

Enzyme assays

HMG-CoA reductase and phosphorylase (*a* and *a*+*b*) activities were measured as described previously [1].

Materials

The source of most of these was as described previously [1,2]. In addition, Protein A and agarose–Protein A were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Na^{125}I and [^{35}S]methionine were obtained from Amersham International (Amersham, Bucks., U.K.). Iodo-Gen iodination reagent was from Pierce & Warriner (U.K.), Chester, Cheshire, U.K. ^{125}I-labelled Protein A was prepared as described in [11]. Polyclonal antibody against rat liver HMG-CoA reductase was raised in rabbits injected with the purified soluble 53 kDa fragment of reductase generated by proteolysis of the intact enzyme as described previously [12]. It was raised and donated by Dr. P. R. Clarke and Dr. D. G. Hardie (University of Dundee, U.K.).

RESULTS

Quantification of immunoreactive HMG-CoA reductase in hepatocytes

In order to verify that the previously observed decrease in total HMG-CoA reductase activity in hepatocytes treated with Ca^{2+}-mobilizing hormones was due to a loss of enzyme protein, we measured the amount of protein in control and hormone-treated cells. These experiments were performed on hepatocytes isolated from rats fed on a normal diet (see the Materials and methods section). We tested the effect of vasopressin (0.1 μM) addition 2 min after addition of glucagon (10 nM) to the cells, in order to optimize the Ca^{2+} mobilization induced by the hormone [1]. The cells were cold-quenched and sedimented as in [1], and HMG-CoA reductase was immunoprecipitated from the solubilized cell pellet. Triplicate incubations were performed. In three different cell preparations, the amount of immunoreactive protein was decreased by 39.8 ± 4.1% in cells treated for 40 min with vasopressin plus glucagon (300 ± 12 d.p.m./10^6 cells) as compared with saline-treated controls (508 ± 58 d.p.m./10^6 cells). This decrease was very similar to the decrease in total HMG-CoA reductase activity observed routinely (44.3 ± 3.2%; $n = 12$; see [1,2]).

Rate of HMG-CoA reductase degradation in hepatocytes

Preliminary experiments were performed on hepatocytes isolated from rats fed on a normal diet. In subsequent experiments rats were fed on cholestyramine and simvastatin to enhance HMG-CoA reductase expression. Throughout, the qualitative effects of hormones and other agents observed with cells from normal rats were the same as those obtained with cells in which HMG-CoA reductase was induced 10-fold by the special diet.

The rate of degradation of ^{35}S-labelled HMG-CoA reductase was very rapid; the half-life was approx. 70 min. Inclusion of vasopressin plus glucagon in the incubation medium did not increase the rate of degradation (Fig. 1*b*) during the period of

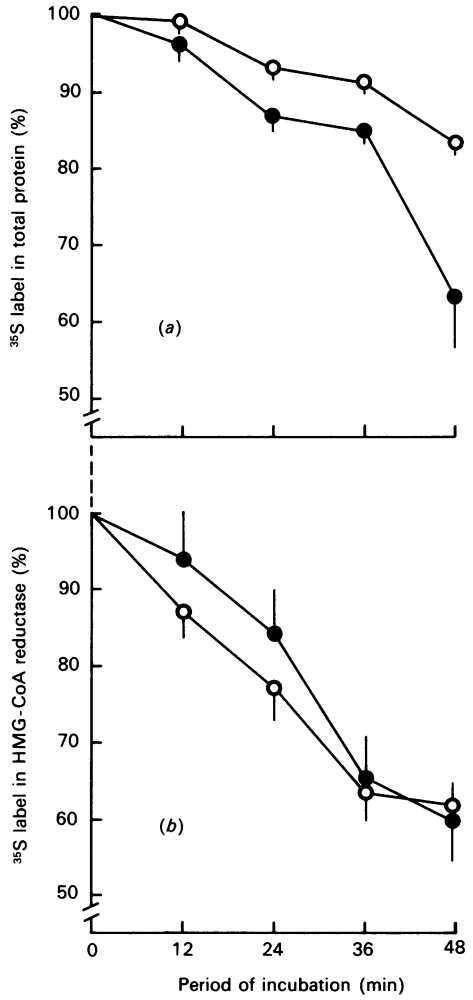

Fig. 1. Effects of vasopressin plus glucagon on the rate of degradation of (*a*) total protein and (*b*) HMG-CoA reductase in freshly isolated rat hepatocytes

Labelling of hepatocyte proteins with [^{35}S]methionine was performed as described in the Materials and methods section. The cells were washed and re-incubated in medium containing 2 mM-methionine (unlabelled), and the amount of ^{35}S associated specifically with HMG-CoA reductase immunoprecipitated by anti-reductase antiserum and with total protein was determined at the times indicated. Glucagon (10 nM) was added 2 min before addition of vasopressin (0.1 μM) at zero time. Values are means (±S.E.M.) for four separate cell preparations: ○, control; ●, glucagon plus vasopressin.

incubation examined (which corresponded to a period similar to that over which the experiments in [1] were conducted). By contrast, the inclusion of vasopressin and glucagon increased the rate of loss of ^{35}S from newly synthesized total protein by about 2-fold. As is apparent from Fig. 1(*a*), the rate of degradation of total protein was initially markedly slower than that of HMG-CoA reductase, although it appeared to accelerate over later time points, especially when the hormones were present.

Rate of HMG-CoA reductase synthesis in hepatocytes

As with experiments on the rate of degradation, it was ascertained that qualitatively similar results were obtained with either hepatocytes obtained from rats fed on a normal diet or hepatocytes from rats fed on cholestyramine plus simvastatin. Only data from experiments performed on cells prepared from rats fed on the special diet are given.

(i) Effects of vasopressin, glucagon and phenylephrine. In preliminary experiments, the rate of incorporation of [^{35}S]methionine into total and HMG-CoA reductase protein was shown to be linear from at least 20 min, and all subsequent experiments were performed over this time interval (see Table 1). When hepatocytes were incubated in the presence of normal concentrations of Ca^{2+} (Krebs–Henseleit medium containing 2.5 mM-$CaCl_2$ plus 2% BSA), vasopressin inhibited the rate of HMG-CoA reductase synthesis by approx. 25%. Phenylephrine had a much smaller effect, whereas glucagon had no significant effect. However, glucagon doubled the effect of vasopressin when added 2 min before the latter, such that the rate of HMG-CoA reductase synthesis was then inhibited by 51%. There was apparently close correlation between these hormonal effects on synthesis of reductase and on synthesis of total protein (Table 1).

(ii) Effects of A23187 and external Ca^{2+} concentration. Addition of A23187 to cells resulted in a very pronounced inhibition of the rates of synthesis of HMG-CoA reductase and total protein. As the ionophore permeabilizes both the plasma membrane and the membranes of intracellular Ca^{2+}-storing vesicles, we examined the effects of omission of extracellular Ca^{2+} from the medium in which the cells were incubated on the effects of the hormones described above (under these conditions the extracellular Ca^{2+} concentration is effectively diminished to about 100 μM, whereas the intracellular stores of Ca^{2+} are not depleted [13], as EGTA was not added to the medium). Determination of rates of ^{35}S incorporation in the presence or absence of Ca^{2+} were performed with the same batches of cells washed with or without Ca^{2+} respectively. The data were corrected for small differences in cell number and viability (routinely 91–94%) between the two sets of cells.

Incubation of cells without Ca^{2+} and with 0.5 mM-EGTA resulted in a large decrease in total protein and reductase synthesis (results not shown; see also [14]), whereas the sole absence of added extracellular Ca^{2+}, without addition of EGTA, had no effect on the rates of synthesis of either total protein or HMG-CoA reductase (Table 1). By contrast, omission of Ca^{2+} resulted in a very strong potentiation of the inhibition of HMG-CoA reductase and total protein synthesis induced by vasopressin, glucagon and phenylephrine (Table 1). This potentiation was more pronounced in the instances where the effect in the presence of added extracellular Ca^{2+} was marginal, e.g. when phenylephrine and glucagon were added separately. Interestingly, omission of extracellular Ca^{2+} resulted in a much greater subsequent effect of Ca^{2+} mobilization (by hormones and phenylephrine) on the rate of HMG-CoA reductase synthesis than on total protein synthesis. Thus, although the effects on both parameters were still proportional, inhibition of HMG-CoA reductase synthesis was markedly greater than that of total protein in the absence of extracellular Ca^{2+} (Fig. 2). This was particularly evident for glucagon and phenylephrine, which are both weak mobilizers of intracellular Ca^{2+} stores [15,16].

(iii) Effects of t-butylbenzohydroquinone (tBuBHQ) and taurolithocholate (TLC). The above results suggested that conditions that depleted Ca^{2+} from the ER (i.e. Ca^{2+} mobilization) and interfered with its repletion (e.g. absence of added extracellular Ca^{2+}) resulted in inhibition of synthesis of total protein and reductase. Therefore we investigated the effects of agents that are known to affect specifically the movement of Ca^{2+} across the ER membrane. As shown in Table 1, incubation of hepatocytes with tBuBHQ (which effectively depletes Ca^{2+} from the ER by inhibiting the Ca-ATPase of this membrane system, but does not result in Ca^{2+} influx across the plasma membrane [17,18]), resulted in a marked inhibition of synthesis of reductase and total protein. Monohydroxylated bile acids have also been reported to mobilize Ca^{2+} transiently from the ER [19] and to enhance Ca^{2+} efflux from the liver [20]. However, TLC (100 μM) showed no inhibition of reductase or total protein synthesis

Table 1. Relative rate of [^{35}S]methionine incorporation (%) into HMG-CoA reductase and total protein in control hepatocytes and cells incubated with various effectors in the absence and presence of added extracellular Ca^{2+}

Hepatocytes were washed and incubated in normal or Ca^{2+}-free medium (without addition of EGTA) as described in the Materials and methods section. The total extracellular Ca^{2+} concentrations were 2.5 mM and approx. 100 μM [13] respectively under these conditions. Incorporation of [^{35}S]methionine was measured over a period of 20 min after addition of glucagon (10 nM), vasopressin (0.1 μM), phenylephrine (1 μM) or A23187 (10 μM) and 5 min after addition of tBuBHQ (25 μM) or TLC (100 μM). Values are means (±S.E.M.) for the numbers of determinations on separate cell preparations shown in parentheses. At 100 μM external Ca^{2+}, the control rates of [^{35}S]methionine incorporation into HMG-CoA reductase and total protein were 108.7±3.5% ($n = 5$) and 105.8±5.7% ($n = 5$) respectively of those in the presence of 2.5 mM-Ca^{2+}. Statistically significant differences from controls ($P < 0.01$) are indicated by asterisks.

	2.5 mM-Ca^{2+}		100 μM-Ca^{2+}	
Addition	HMG-CoA reductase	Total protein	HMG-CoA reductase	Total protein
Control	100	100	100	100
Phenylephrine (3)	83.1±3.7*	85.9±3.2*	42.7±4.0*	52.7±1.2*
Glucagon (3)	87.6±6.0*	95.0±4.8	71.0±9.7*	88.1±1.9*
Vasopressin (3)	74.2±6.4*	77.1±6.2*	38.0±3.8*	46.3±3.3*
Vasopressin+glucagon (3)	48.8±1.5*	51.5±3.4*	30.6±3.6*	41.3±2.5*
A23187 (2)	17.5	18.9	13.8	27.9
tBuBHQ (3)	29.1±7.7*	35.9±6.2*	–	–
TLC (2)	96.8	97.7	–	–

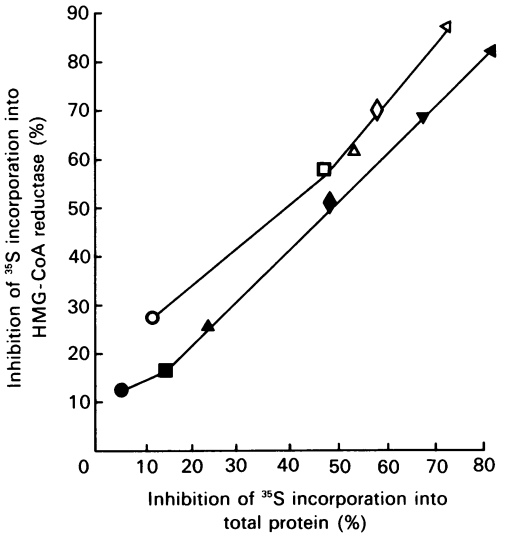

Fig. 2. Relationship between inhibiton of the synthesis of HMG-CoA reductase and of total protein in freshly prepared isolated rat hepatocytes incubated in the presence of 2.5 mM-Ca^{2+} (black symbols) and in the absence of added Ca^{2+} (approx. 100 μM; white symbols)

Data are abstracted from Table 1. Key: ●, ○, glucagon; ▲, △, vasopressin; ◆, ◇, vasopressin plus glucagon; ■, □, phenylephrine; ◁, ◀, A23187; ▼, tBuBHQ.

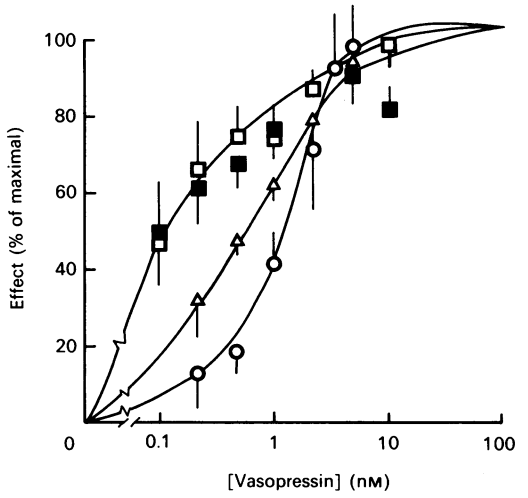

Fig. 3. Dose–response curve for the effects of vasopressin

Hepatocytes were preincubated for 20 min before addition of vasopressin (100 nM) at zero time. Incorporation of [^{35}S] methionine into HMG-CoA reductase and total protein was measured over the first 20 min after addition of hormone. Maximal inhibition was routinely obtained at 10 nM vasopressin, and was 39.2±3.5% ($n = 4$) for synthesis of HMG-CoA reductase and 38.1±4.3% ($n = 4$) for total protein. In separate cell preparations, the activities of phosphorylase (a and $a+b$) and of total HMG-CoA reductase were measured 4.5 min and 20 min respectively after addition of vasopressin. Basal phosphorylase a activity was 16.1±3.3% ($n = 3$) of maximally stimulated activity, whereas maximal loss of HMG-CoA reductase activity was 37.5±4.7% ($n = 3$) compared with the activity in control cells to which only saline was added and which were sampled simultaneously. Key: synthesis of total protein (□) and HMG-CoA reductase (■); activation of phosphorylase a (△); decrease in HMG-CoA reductase total activity (○).

(Table 1) and did not result in any decrease in total HMG-CoA reductase activity over a 40 min period of incubation. It is noteworthy that, whereas tBuBHQ produced a rapid 4-fold activation of phosphorylase a which was sustained throughout the rest of the incubation, the activation produced by TLC was very transient, such that by 5 min after addition phosphorylase a activity was back to basal levels (cf. [19]).

Dose–response curve for effects of vasopressin

All the above data were obtained with optimal concentrations of hormones and other effectors. In order to test whether the effects of Ca^{2+} mobilization by vasopressin are likely to be physiologically relevant, the dose–response curves for the effects of vasopressin on reductase and total protein synthesis, on loss of HMG-CoA reductase and on activation of glycogen phosphorylase were compared. The data in Fig. 3 show that the inhibition of [^{35}S]methionine incorporation into HMG-CoA reductase (and protein) was extremely sensitive to low

concentrations of vasopressin [EC_{50} (concn. giving 50% inhibition) approx. 0.1 nM]. Indeed, it was more sensitive than the activation of phosphorylase (EC_{50} approx. 0.6 nM). The dose-dependence, on vasopressin concentration, of the loss of expressed HMG-CoA reductase was much steeper and more similar to that of phosphorylase activation, but the EC_{50} was about an order of magnitude higher than that for protein labelling. This difference in sensitivity to vasopressin was presumed to result from a combination of the following: Ca^{2+} mobilization inhibits initiation, but not elongation, of polypeptide chains (see below), and measurement of HMG-CoA reductase activity includes mature protein that exists before addition of hormone, such that, during a relatively short incubation period, the contribution towards loss of functional reductase of the inhibition of initiation by sub-optimal concentrations of vasopressin would be minimized.

DISCUSSION

The present results demonstrate that the loss of total HMG-CoA reductase activity that we observed on incubation of isolated rat hepatocytes with Ca^{2+}-mobilizing hormones [1] was indeed due to a loss of enzyme protein. It is evident that this loss is almost entirely due to a rapid and marked decrease in the rate of synthesis of HMG-CoA reductase, rather than to accelerated degradation of existing protein. The net effect on the amount of reductase protein is so pronounced because the enzyme has a very short half-life. This was particularly so in our isolated hepatocyte preparation, in which $t_{\frac{1}{2}}$ was approx. 70 min (cf. approx. 120 min in [6]). The rate of degradation of HMG-CoA reductase was much more rapid than that of newly synthesized total cell protein. This was expected from the short half-life characteristic of reductase, even compared with the pool of newly synthesized protein that turns over rapidly. The rates of degradation of total protein observed in the present study were similar to those reported for newly synthesized protein in cultured hepatocytes [21].

Incubation with vasopressin plus glucagon doubled the rate of total protein degradation, suggesting that Ca^{2+} mobilization in hepatocytes enhances short-lived protein degradation. This observation agrees with the recent description of the acceleration of protein degradation, within the ER, by Ca^{2+} mobilization in CHO cells [22]. This contrasted with the total lack of effect of the hormones on HMG-CoA reductase degradation, which suggests that acceleration of protein degradation by Ca^{2+} mobilization may be more easily observed for proteins turning over more slowly than reductase, even though regulated reductase degradation may occur within the ER [23].

The effects of altered Ca^{2+} fluxes on the rate of synthesis of HMG-CoA reductase were proportional to those on total protein synthesis. This observation suggests that the effects of Ca^{2+} mobilization on HMG-CoA reductase are not specific to the enzyme, but occur as part of a generalized inhibition of protein synthesis on membrane-bound polysomes. The involvement of altered hepatocyte Ca^{2+} distribution in peptide-chain initiation in hepatocytes and other cell types has been recognized relatively recently [24–26]. The mechanism of the inhibition of protein synthesis by vasopressin in rat hepatocytes has been shown to result from inhibition of initiation and involves the increased phosphorylation of the α-subunit of initiation factor eIF2 [27], which is accompanied by decreased activity and recycling of eIF-2B.

Previous work [24–26,28] has suggested that it is not the elevation of cytosolic Ca^{2+} concentration in itself that mediates the inhibition of protein synthesis at initiation, but that it is the depletion of an intracellular Ca^{2+} store to which the protein-synthesis apparatus is sensitive. Consequently, it is the depletion of Ca^{2+} from the ER, which in the hepatocyte is extensive, that could affect the rate of protein synthesis. This theory [2,3] emerged from experiments in which hepatocytes were first treated with EGTA to deplete them of Ca^{2+} and then had Ca^{2+} added back to them in excess of the amount of EGTA present. We have sought to explore the mechanism further through the use of more gentle conditions, which did not alter the basal rate of protein synthesis and which did not result in Ca^{2+} depletion of the hepatocytes. When hepatocytes were washed and incubated in the absence of added Ca^{2+} (but without addition of EGTA; total external [Ca^{2+}] approx. 100 μM [13,29]), the rate of protein synthesis was unaffected. The most striking observation was that the effects of Ca^{2+} mobilization by hormones on protein synthesis were much more marked when hepatocytes were incubated in the absence of added Ca^{2+}. Because under these conditions Ca^{2+} efflux still occurs, but rises in cytosolic Ca^{2+} are small and very transient [15], the increased effect on protein synthesis must have resulted from the inability of the cells to replenish their $InsP_3$-sensitive Ca^{2+} pools under these conditions. Consequently, these results support the theory that depletion of the $InsP_3$-sensitive Ca^{2+} pool of hepatocytes is functionally linked to the inhibition of protein synthesis. In the studies of Brostrom *et al.* [14] the rate of synthesis of all populations of proteins (as judged by labelling of peptides separated on two-dimensional PAGE) appeared to be inhibited by Ca^{2+} depletion of hepatocytes with EGTA. Consequently, it was suggested that protein synthesis on both membrane-bound and free polysomes was affected by depletion of Ca^{2+} from the lumen of the ER. Our experiments on hepatocytes exposed to low extracellular Ca^{2+} but not depleted of Ca^{2+} before exposure to Ca^{2+}-mobilizing hormones indicate that protein synthesis on the rough ER may be more specifically affected. This is shown by the fact that the effects of omission of Ca^{2+} from the medium were much more pronounced on HMG-CoA reductase (which is exclusively synthesized on the ER [30]) than on total protein-synthesis rates.

The effects of phenylephrine and glucagon were of particular interest, as in our previous studies [1] we had found that the former gave a transient decrease in total HMG-CoA reductase activity, whereas glucagon left this parameter unaffected, within experimental error. Use of the much more sensitive techniques in the present study makes it is clear that, at least over the first 20 min of incubation, both phenylephrine and glucagon gave a small (10–15%) inhibition of HMG-CoA reductase synthesis. This glucagon effect would agree with the observations in [31] that glucagon inhibits the rate of synthesis of HMG-CoA reductase during a 2 h incubation period. In the studies by Menaya *et al.* [32], it was shown that phenylephrine was only effective as an inhibitor of protein synthesis when the cells were incubated in the absence of Ca^{2+}. Our results confirm this observation. However, we do not agree with the conclusion drawn by those authors, that this is due to the rise in cyclic AMP which phenylephrine induces in the absence of extracellular Ca^{2+} [33]. If elevation of cyclic AMP concentration were involved, then it would have been expected that glucagon when added to hepatocytes incubated with 2.5 mM-Ca^{2+} would have a similar effect. This was obviously not the case. As mentioned above, any minor effects of glucagon appear to be mediated through its ability to mobilize Ca^{2+} weakly from the ER [15].

Further evidence for the existence of an intracellular pool of sequestered Ca^{2+} to which protein synthesis is sensitive emerged from the data obtained with tBuBHQ. This compound is known to deplete the $InsP_3$-sensitive Ca^{2+} pool of hepatocytes by inhibiting the Ca^{2+}-ATPase activity of the ER membrane [17,18] without affecting Ca^{2+} fluxes across the plasma membrane. In

our experiments, tBuBHQ resulted in a very marked inhibition of HMG-CoA reductase synthesis, and this was accompanied by a 60% loss of total HMG-CoA reductase activity. Consequently, tBuBHQ mimicked the effects of Ca^{2+} mobilization induced by more physiological methods, as would be expected if the synthesis of HMG-CoA reductase was controlled by the level of repletion of the $InsP_3$-sensitive Ca^{2+} pool in hepatocytes.

The possible involvement of the inhibition of HMG-CoA reductase expression through the inhibition of its synthesis by bile-acid-induced depletion of Ca^{2+} from the ER was of obvious interest to our studies. Monohydroxylated bile acids are known to mediate the release of Ca^{2+} from hepatocyte ER, without raising intracellular $InsP_3$ concentrations [19,20]. This effect is independent of external Ca^{2+} [34] and results in net Ca^{2+} efflux from the liver [35]. Because of the general hepatotoxic and cholestatic effects of monohydroxylated bile acids, it was suggested that these effects were linked to their ability to mobilize Ca^{2+} from the ER [19,20,36]. Our data show that TLC, at concentrations (100 μM) which raise cytosolic [Ca^{2+}] [19], does not affect either total HMG-CoA reductase activity in hepatocytes or the rate of synthesis of the enzyme. This may be due to the very transient effect of TLC on cytosolic [Ca^{2+}], as evidenced by its effect on phosphorylase *a* (see also [34]). To the extent that bile acid secretion is dependent on the rate of cholesterol synthesis [37] (and therefore on HMG-CoA reductase activity), our conclusion agrees with that in [38], namely that release of Ca^{2+} from the ER may not be the mechanism for TLC-induced cholestasis in the liver.

We have attempted to address the question as to the physiological relevance of the observed effects of Ca^{2+} mobilization on total protein synthesis in general, and on HMG-CoA reductase synthesis in particular, by comparing the dose–response curves for vasopressin action on these parameters with that for its ability to activate glycogen phosphorylase. The data in Fig. 3 show that the inhibition of both total protein synthesis (cf. [27]), and specifically of HMG-CoA reductase synthesis, were extremely sensitive to vasopressin, and that the EC_{50} of the hormone was even lower than that observed for activation of phosphorylase. Consequently, it is concluded that the effects of Ca^{2+} mobilization on protein synthesis in the liver could be physiologically significant. This is particularly so for proteins with high rates of turnover, such as HMG-CoA reductase, because any inhibition of synthesis would rapidly result in a marked decrease in the expression of the enzyme. This effect is of particular interest because, at sub-nanomolar concentrations, vasopressin produces Ca^{2+} transients in individual hepatocytes [13,39]. Evidently, the inhibition of protein synthesis by vasopressin-induced Ca^{2+} mobilization does not necessitate a simplistic visualization of the effect in terms of a continuous depletion of the relevant Ca^{2+} pool for a prolonged period of time, but one that can be elicited by oscillatory depletion and repletion of this pool with Ca^{2+}. Nevertheless, it is important to emphasize that specialization of distinct ER vesicle populations for Ca^{2+} storage and protein synthesis exist in liver cells (see [40]). The greater sensitivity to vasopressin of protein synthesis than of activation of phosphorylase may be indicative of the much more localized effect that vasopressin would have on Ca^{2+} depletion from the responsive Ca^{2+} pool, as compared with the effects on cytosolic Ca^{2+}.

In conclusion, we have shown that the effects of Ca^{2+}-mobilizing hormones to decrease acutely the expression of HMG-CoA reductase is due to a marked inhibition of the synthesis of this protein, as part of a more generalized shut-down of protein synthesis and disruption of protein trafficking in hepatocytes. For a protein with such a short half-life as HMG-CoA reductase this has the effect of diminishing rapidly the amount of enzyme activity, especially when accompanied by an acute increase in the phosphorylation state of the enzyme [1,2].

This work was supported by the British Heart Foundation.

REFERENCES

1. Zammit, V. A. & Caldwell, A. M. (1990) Biochem. J. **269**, 373–379
2. Zammit, V. A. & Caldwell, A. M. (1991) Biochem. J. **273**, 485–488
3. Gibson, D. M., Parker, R. A., Stewart, C. S. & Evenson, K. J. (1982) Adv. Enzyme Regul. **20**, 263–283
4. Parker, R. A., Miller, S. J. & Gibson, D. M. (1984) Biochem. Biophys. Res. Commun. **125**, 629–635
5. Marrero, R. F., Haro, O. & Hegardt, F. G. (1986) FEBS Lett. **197**, 183–186
6. Parker, R. A., Miller, S. J. & Gibson, D. M. (1989) J. Biol. Chem. **264**, 4877–4887
7. Seglen, P. O. (1973) Methods Cell Biol. **13**, 30–83
8. Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. **210**, 33–66
9. Edwards, P. A., Lau, S.-F. & Fogelman, A. M. (1983) J. Biol. Chem. **258**, 10219–10222
10. Haro, D., Marrero, P. F., Ayte, J. & Hegardt, F. G. (1990) Eur. J. Biochem. **188**, 123–129
11. Fraker, P. J. & Speck, J. C., Jr. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857
12. Clarke, P. R. & Hardie, D. G. (1990) EMBO J. **9**, 2439–2446
13. Woods, N. M., Dixon, C. J., Cuthbertson, K. S. R. & Cobbold, P. H. (1990) Cell Calcium **11**, 353–360
14. Brostrom, C. O., Bocckino, S. B., Brostrom, M. A. & Galuska, E. M. (1986) Mol. Pharmacol. **29**, 104–111
15. Altin, J. G. & Bygrave, F. C. (1988) Biol. Rev. Cambridge Philos. Soc. **63**, 551–611
16. Williamson, J. R., Cooper, R. H., Joeseph, S. K. & Thomas, A. P. (1985) Am. J. Physiol. **248**, C203–C216
17. Moore, G. A., McConkey, D. J., Kass, G. E. N., O'Brien, P. J. & Orrenius, S. (1987) FEBS Lett. **224**, 331–336
18. Kass, G. E. N., Duddy, S. K., Moore, G. A. & Orrenius, S. (1989) J. Biol. Chem. **264**, 15192–15198
19. Combettes, L., Dumont, M., Berthon. B., Erlinger, S. & Claret, M. (1988) J. Biol. Chem. **263**, 2299–2303
20. Anwer, M. S., Engelking, L. R., Nolan, K., Sullivan, D., Zimniak, P. & Lester, R. (1988) Hepatology **8**, 887–891
21. Russell, J. L. & Khairallah, E. A. (1985) in Intracellular Protein Catabolism (Khairallah, E. A., Bond, J. S. & Bird, J. W. C., eds.), pp. 495–497, A. R. Liss, New York
22. Wileman, T., Kane, L. P., Carson, G. R. & Terhorst, C. (1991) J. Biol. Chem. **266**, 4500–4507
23. Chun, K. T., Bar-Nun, S. & Simoni, R. D. (1990) J. Biol. Chem. **265**, 22004–22010
24. Brostrom, C. D. & Brostrom, M. A. (1990) Annu. Rev. Physiol. **52**, 577–590
25. Chin, K.-V., Cade, C., Brostrom, C. O., Galuska, E. M. & Brostrom, M. A. (1987) J. Biol. Chem. **262**, 16509–16514
26. Kumar, R. V., Wolfman, A., Panniers, R. & Henshaw, E. C. (1989) J. Cell Biol. **108**, 2107–2115
27. Kimball, S. R. & Jefferson, L. S. (1990) J. Biol. Chem. **265**, 16794–16798
28. Chin, K.-V., Cade, C., Brostrom, M. A. & Brostrom, C. O. (1988) Int. J. Biochem. **20**, 1313–1319
29. Blackmore, P. F., Strickland, W. G., Bocckino, S. B. & Exton, J. H. (1986) Biochem. J. **237**, 235–242
30. Brown, D. H. & Simoni, R. D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 1674–1678
31. Edwards, P. A., Lau, S.-F. & Fogelman, A. M. (1986) J. Lipid Res. **27**, 398–403
32. Menaya, J., Parilla, R. & Ayuso, M. S. (1987) Biochem. J. **248**, 903–909
33. Chan, T. M. & Exton, J. H. (1977) J. Biol. Chem. **252**, 8645–8651
34. Combettes, L., Berthon, B., Douchet, E., Erlinger, S. & Claret, M. (1990) Eur. J. Biochem. **190**, 619–623
35. Anwer, M. S., Little, J. M. & Oelberg, D. G., Zimniak, P. & Lester, R. (1989) Proc. Soc. Exp. Biol. Med. **191**, 147–152
36. Combettes, L., Berthon, B., Douchet, E., Erlinger, S. & Claret, M. (1989) J. Biol. Chem. **264**, 157–167

37. Panolak, W. M., Heuman, D. M., Hylemon, P. B. & Vlahcevic, Z. R. (1990) J. Lipid Res. **31**, 79–90
38. Farrell, G. C., Duddy, S. K., Kass, G. E. N., Llopis, J., Gahm, A. & Orrenius, S. (1990) J. Clin. Invest. **85**, 1255–1259
39. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1987) Cell Calcium **8**, 79–100
40. Meldolesi, J., Madeddu, L. & Pozzan, T. (1990) Biochim. Biophys. Acta **1055**, 130–140

Received 28 February 1991/18 May 1991; accepted 21 May 1991