Conditions that result in the mobilization and influx of Ca^{2+} into rat hepatocytes induce the rapid loss of 3-hydroxy-3-methylglutaryl-CoA reductase activity that is not reversed by phosphatase treatment

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We investigated the effects of conditions that induce Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx into hepatocytes on the expressed and total (fully dephosphorylated) activities of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Vasopressin and phenylephrine when added alone had small or negligible effects on the phosphorylation state of the enzyme, as judged from the expressed/total activity ratio. However, when added in combination with glucagon, they elicited appreciable increases in the phosphorylation of the enzyme. Glucagon on its own had no effect either on phosphorylation state or on total HMG-CoA reductase activity during 40 min of incubation. Under conditions of sustained Ca^{2+} influx (i.e. vasopressin or phenylephrine plus glucagon), there was a marked loss of total HMG-CoA reductase activity. This effect was more pronounced when vasopressin was used; 50 % of the enzyme activity was lost within 40 min. The involvement of Ca^{2+} in these effects was verified directly by the use of ionophore A23187. Its addition to hepatocytes resulted both in a very pronounced increase in the phosphorylation state of the enzyme and in the loss of 50 % of the total activity within 30 min. There was no correlation between the ability of any set of conditions to increase the phosphorylation of the enzyme and the subsequent loss of total HMG-CoA reductase activity. The latter parameter appeared to be directly related, however, to the maintenance of prolonged Ca^{2+} influx, as indicated by the continued activation of glycogen phosphorylase, measured in the same cells. The lack of a causal relationship between increased phosphorylation and loss of total activity was demonstrated directly by studies in which okadaic acid was used to induce phosphorylation of HMG-CoA reductase in hepatocytes by inhibition of phosphatase 1 and 2A activities. This was not accompanied by any loss of total enzyme activity. Neither did okadaic acid enhance the loss of reductase induced by A23187 when the two agents were added together. It is concluded that altered Ca^{2+} fluxes in hepatocytes *in vivo*, under conditions of acute or chronic stress (such as may be associated with trauma or diabetes respectively), may be involved in the regulation of the expression of HMG-CoA reductase activity through alteration of enzyme concentration in the liver.

INTRODUCTION

The expression of HMG-CoA reductase activity in the liver is controlled at the transcriptional and translational levels [1], by modulation of its rate of synthesis and/or degradation [2] and by reversible phosphorylation [3]. Various hormones (e.g. insulin, thyroxine) and metabolites (e.g. mevalonate) are known to modulate the rates of synthesis and/or degradation of the enzyme. For example, exposure of hepatocytes to glucagon results in a several-fold decrease in the rate of synthesis [4], whereas oxysterols both decrease the synthesis rate and increase the degradation rate [5]. Similarly, modulation of the phosphorylation state of the liver enzyme by various hormones has been studied both *in vivo* and in isolated hepatocytes, although there is disagreement about some of these effects. Thus early reports that glucagon results in increased phosphorylation of the enzyme *in vivo* (see [6]) and in isolated hepatocytes [7] have not been confirmed for normal rats [4,8].

More recently it has been suggested that there is a causal relationship between the phosphorylation state of HMG-CoA reductase and the rate of degradation of the enzyme [9–11]. Conditions that elicit increased phosphorylation of the enzyme in isolated hepatocytes (e.g. incubation with mevalonate) are associated with the accelerated loss of total HMG-CoA reductase

activity. In addition, reductase either in microsomes isolated from mevalonate-incubated cells or in microsomes incubated with ATP-Mg *in vitro* is more susceptible to proteolysis by calpain II [9]. Consequently, it has been proposed by these authors that, by extrapolation to the situation in the intact cell, increased phosphorylation of the enzyme contributes towards an increase in degradation rate.

HMG-CoA reductase is a substrate for several protein kinases and phosphatases *in vitro*. The kinases include a Ca^{2+} -calmodulin-dependent protein kinase, protein kinase C (see [6]) and the AMP-stimulated kinase [12], which may be identical with the 'reductase kinase' previously described [13]. By contrast, reductase is not a substrate for cyclic AMP-dependent protein kinase (nor is the AMP-stimulated kinase, which is itself activated by phosphorylation [14]). These observations have raised important questions as to how glucagon could promote the phosphorylation of HMG-CoA reductase [3,15]. By contrast, all common protein phosphatases (type 1, 2A and 2C) are capable of dephosphorylating reductase, although phosphatase 2C is the most potent in crude liver homogenates [16]. It is not known, however, which of the protein kinases or phosphatases effective *in vitro* are involved in the modulation of HMG-CoA reductase phosphorylation state in intact cells.

It is therefore apparent that several possibly related aspects of

HMG-CoA reductase regulation need elucidation: (i) is activation of Ca^{2+} -dependent protein kinases in intact cells, through Ca^{2+} mobilization/influx, associated with increased phosphorylation of HMG-CoA reductase; (ii) does phosphorylation of reductase necessarily presage loss of total activity of the enzyme?; and (iii) can the effects of glucagon observed under some conditions *in vivo* (e.g. diabetes), but not others, be related to its capacity to increase the effectiveness of Ca^{2+} -mobilizing hormones? In providing answers to these questions, we have found that the sustained elevation of Ca^{2+} concentrations in hepatocytes under conditions that promote Ca^{2+} influx results in a profound and rapid loss of total HMG-CoA reductase activity that is unrelated to the magnitude of any increase in the phosphorylation state of the enzyme.

MATERIALS AND METHODS

Animals

These were female Wistar rats (180–200 g) maintained on a standard laboratory rat chow fed *ad libitum*. They were kept under a 12 h-light: 12 h-dark cycle and used 2.5 h into the dark period.

Preparation and incubation of hepatocytes

Liver cells were isolated by collagenase digestion as described in [17]. Routinely, they were approx. 90% viable as judged by Trypan Blue exclusion. They were finally resuspended in Krebs–Henseleit buffer [18] (2.5 mM- CaCl_2) containing 2% (w/v) defatted BSA and 12 mM-glucose. Unless otherwise indicated, 0.5 ml samples of the cell suspension were added to 2.3 ml of the same medium contained in 25 ml silicone-treated glass conical flasks such that a density of $(0.8\text{--}1.2) \times 10^6$ cells per flask was obtained. They were incubated at 37 °C with shaking (180 strokes/min) and continuous oxygenation (O_2/CO_2 , 19:1) for 15–20 min before making additions (at zero time). The incubations were then continued for the times indicated. Hormones were added as concentrated stock solutions in Krebs–Henseleit buffer; ionophore A23187 and okadaic acid were added in dimethyl sulphoxide (final volume 0.1% of total). An equivalent amount of dimethyl sulphoxide was added to control flasks.

Termination of incubations and cell extraction

Routinely, incubations were terminated by the rapid dilution of the contents of the flasks into 40 ml of Krebs–Henseleit buffer at 0 °C, followed by immediate centrifugation (20s) at 350 g performed at 0 °C. The supernatant was decanted, and 0.5 ml of ice-cold medium containing 150 mM sucrose, 5 mM-EDTA, 5 mM-EGTA, 10 mM-Tris (pH 7.3 at 0 °C), 1 mM-phenylmethane sulphonyl fluoride, 1 µg each of leupeptin, antipain and pepstatin/ml, and either 100 mM-KCl or 100 mM-KF was added to the cell pellet. This addition also resuspended the cells, and they were immediately sonicated (2×10 s), while cooled on ice, by using a Kontes sonicator operating at 70 W. Sonicated preparations were subsequently centrifuged at 10 000 g for 10 min. Samples (0.2 ml) of the resultant supernatants of sonicated preparations in F^- medium were frozen directly by squirting into liquid N_2 and were stored at –20 °C before determination of phosphorylase activities. Microsomes were prepared from both types of 10 000 g supernatants (F^- and Cl^-) by centrifugation of 240 µl samples for 10 min at 100 000 g [138 kPa (20 lb/in²) in a Beckman Airfuge]. Microsomes from F^- extracts were resuspended in F^- -containing medium (see above), washed and resedimented. Microsomes from Cl^- -extracted cells were resuspended in 100 µl of Cl^- medium and incubated at 30 °C for 5 min with another 100 µl of Cl^- medium containing

1–2 units of partially purified rat liver protein phosphatase (see [19]). These conditions were found to give maximal activation of HMG-CoA reductase by dephosphorylation, in pilot experiments. All pellets were finally resuspended in 100–200 µl of fluoride medium to yield a protein concentration of approx. 2.5 mg/ml. This ensured that, although two sets of microsomes (F^- and Cl^-) were prepared, all assays were performed under identical conditions. The protein content of the final microsomal suspensions were assayed by the method of Lowry *et al.* [20], with BSA as standard.

Assay of HMG-CoA reductase and phosphorylase activities

HMG-CoA reductase was assayed in the freshly prepared final microsomal suspensions at 30 °C as described previously [19]. Phosphorylase *a* and (*a* + *b*) activities were assayed in extracts of frozen samples (see above) prepared in ice-cold medium containing 100 mM-glycylglycine, 20 mM-EDTA, 100 mM-NaF, 0.5% glycogen and 1 mM-dithiothreitol (pH 7.5 at 0 °C) by using a Polytron (OD 10) tissue disintegrator. Phosphorylase *a* and (*a* + *b*) activities were assayed as described in [21]. Phosphorylase *b* was activated by inclusion of 1.25 mM-AMP and 1.2 M-1,2-methoxyethane in the assay medium.

Materials

Defatted BSA was from Calbiochem (Cambridge, U.K.). [¹⁴C]HMG-CoA was synthesized from [¹⁴C]HMG by the method of Williamson & Rodwell [22]. [³H]Mevalonolactone, [¹⁴C]HMG and [¹⁴C]glucose 1-phosphate were from Amersham International (Aylesbury, Bucks, U.K.). [Arginine]vasopressin, glucagon and phenylephrine were from Sigma Chemical Co. (Poole, Dorset, U.K.). Ionophore A23187 was from Ely Lilly and Co. (Indianapolis, IN, U.S.A.). Antipain, pepstatin and leupeptin were from Peptide Institute Inc. (Scientific Marketing, London N.1, U.K.). Okadaic acid was obtained from Dr. Y. Tsukitani (Fujisawa Chemical Co., Tokyo, Japan) through a generous gift from Professor P. Cohen (Department of Biochemistry, University of Dundee, U.K.).

RESULTS

In initial experiments on HMG-CoA reductase activity, in which we terminated cell incubations by simple centrifugation of incubating mixtures at room temperature (effective temperature of medium approx. 30 °C), we obtained erratic and spuriously low expressed/total activity ratios. These were accompanied by spuriously high activities of phosphorylase *a* (results not shown). That these results were due to artefacts arising during centrifugation was demonstrated in experiments in which, for each set of hepatocytes, we determined phosphorylase activity either in cells directly frozen into liquid N_2 [21] or in cells frozen after centrifugation at room temperature. Spuriously high phosphorylation was only observed in centrifuged cells. Moreover, incubation of cells with glucagon appeared to exacerbate this effect (results not shown). As freezing of entire incubation mixtures could not be adopted for HMG-CoA reductase assays because of the low activity of the enzyme, we had to devise a method in which a cell sedimentation step was retained but artefacts were avoided. This we achieved by centrifugation at 0 °C, as described in the Materials and methods section. Using this protocol we obtained identical time courses for activation of phosphorylase by hormones and other effectors, when compared with those obtained by direct freezing of samples of the same incubations (Fig. 1). They were also strictly comparable with those obtained by other workers on directly frozen cells [21,23–25]. It therefore appears plausible that this protocol also retained the phosphorylation state of reductase as it existed in

Fig. 1. Time courses of the changes in the phosphorylase $a/(a+b)$ activity ratio in hepatocytes exposed to conditions that elevate cytosolic Ca^{2+} and/or cyclic AMP concentrations

Hepatocytes were incubated as described in the Materials and methods section for 15–20 min before addition of (a) phenylephrine (1 μM , $\square$), glucagon (10 nM, $\bullet$) or vasopressin (0.1 μM , ∇) and (b) ionophore A23187 ($\blacktriangle$) (30 μM), phenylephrine plus glucagon ($\blacksquare$) or vasopressin plus glucagon ($\blacktriangledown$). In (b), phenylephrine or vasopressin was added (at zero time) 2 min after the addition of glucagon, all at the same concentrations as in (a). Control values, obtained in incubations using the same preparations as above and to which only an equivalent volume of Krebs–Henseleit buffer was added, are denoted by ($\circ$). Incubations were cold-quenched as described in the Materials and methods section; the resulting cell pellets were sonicated in F^- -containing medium and centrifuged for 10 min at 10000 g . Samples (0.2 ml) of the supernatants (which were also used for the preparation of microsomes; see Figs. 2–4) were frozen in liquid N_2 before determination of phosphorylase activities. Values are means ($\pm$ S.E.M.) of 3–5 determinations on separate cell incubations.

the cells at the time of sampling by dilution into ice-cold medium (see the Materials and methods section).

The time courses shown in Fig. 1 were obtained with the same cell incubations used for reductase determinations, in order to ascertain the effectiveness of action of the agents added. In

addition, they enabled us to monitor indirectly the changes in cytosolic Ca^{2+} induced by Ca^{2+} -mobilizing effectors (as described elsewhere [24,26]) in the same incubations used for experiments on reductase. In agreement with previous observations, we found that the activation of phosphorylase produced by phenylephrine was much less pronounced and more transient than that given by vasopressin (both at maximally effective concentrations). The more transient nature of the phenylephrine effect was also evident in the presence of glucagon, and reflects the more rapid decline in the rate of Ca^{2+} influx under these conditions compared with that observed in the presence of vasopressin plus glucagon [27]. With respect to the effects of these treatments on HMG-CoA reductase, it is important to note that vasopressin effects were much longer lasting than those of phenylephrine, in both the presence and the absence of glucagon, reflecting the larger Ca^{2+} influx that has been described by others (see, e.g., [27]). When ionophore A23187 was added to hepatocytes, the time course of activation of phosphorylase also reflected the prolonged elevation of cytosolic Ca^{2+} concentrations (Fig. 1b).

Effects of glucagon, phenylephrine and vasopressin on HMG-CoA reductase

Glucagon, when added alone, had no effect on HMG-CoA reductase activity over the 40 min incubation periods studied. It altered neither the total activity (obtained in microsomes extracted in Cl^- medium, followed by total dephosphorylation *in vitro*), nor the fraction of the enzyme in the active form (F^- -extracted/ Cl^- -extracted activity ratio). Changes in this ratio (referred to below as expressed/total) have been shown to be directly correlated with the degree of phosphorylation of the enzyme as determined by ^{32}P incorporation from [^{32}P]ATP *in vitro* [11]. Therefore these data are in agreement with those of Edwards *et al.* [4], who found that glucagon did not increase the incorporation of ^{32}P into reductase protein immunoprecipitated from hepatocytes equilibrated with [^{32}P]P $_i$. However, they are in contrast with those in refs. [7] and [15], which indicated that, after much more prolonged incubations with glucagon, there was a decrease in the expressed/total activity ratio. However, because of the length of incubation necessary to demonstrate these effects, these previous observations are unlikely to have arisen from the normal action of glucagon on hepatocytes.

Because of one report [28] which claimed that glucagon may act via inositol 1,4,5-trisphosphate formation at lower concentrations (1 nM) and via cyclic AMP at higher concentrations (10 nM), the experiments were done at both concentrations. Identical results were obtained at both concentrations, and only the results obtained with 10 nM-glucagon are shown.

Addition of phenylephrine (1 μM) to the cells resulted in a small decrease in the expressed/total activity ratio of HMG-CoA reductase at 5 min (Fig. 2a), which reached a moderate degree of significance ($0.1 < P < 0.05$). This indicated that there was a transient increase in the degree of phosphorylation of the enzyme that accompanied the rise in cytosolic Ca^{2+} elicited by the α_1 -agonist. Phenylephrine also elicited a transient decrease in total reductase activity, which was evident up to 20 min but was reversed thereafter. Vasopressin (0.1 μM) had no detectable effect on the expressed/total activity ratio (Fig. 2b). However, the hormone induced a marked and stable loss of total HMG-CoA reductase activity, such that approx. 25% of the activity was lost after 40 min (Fig. 3b). When vasopressin was added to hepatocytes 2 min after the addition of glucagon, the loss of total activity of reductase was much more pronounced (50%; Fig. 3e). Vasopressin plus glucagon also resulted in a significant decrease in the expressed/total activity ratio, which appeared to be sustained after 5 min. Glucagon also potentiated the increase in

Fig. 2. Effects of conditions that elevate the concentrations of Ca^{2+} and/or cyclic AMP in the cytosol of hepatocytes on the phosphorylation state of HMG-CoA reductase as reflected in the expressed/total activity ratio

Hepatocytes were incubated as described in the legend to Fig. 1 with the same concentrations of effectors: (a) phenylephrine, (b) vasopressin, (c) glucagon, (d) phenylephrine plus glucagon, (e) vasopressin plus glucagon, and (f) ionophore A23187. In (d) and (e) glucagon was added 2 min before the addition of phenylephrine or vasopressin respectively. Control values ($\circ$) were obtained in each experiment using the same cell preparations. Incubations were cold-quenched as described in the Materials and methods section and sonicated in either Cl^- or F^- -containing media. HMG-CoA reductase activity was measured in microsomes that were either maintained in F^- throughout or that were obtained in Cl^- , incubated with exogenous phosphatase and, finally, resuspended in F^- -containing medium (see the Materials and methods section). Values are means ($\pm$ S.E.M.) of 3–5 determinations on separate cell preparations.

phosphorylation induced by phenylephrine (Fig. 2d). Between 5 and 30 min of incubation, the phosphorylation induced by phenylephrine plus glucagon was greater or equal to that achieved by vasopressin plus glucagon. However, phenylephrine in the presence of glucagon resulted only in a small decrease in total activity during the 40 min period of incubation (Fig. 3d). In synthesis, therefore, whereas phenylephrine had greater effects on phosphorylation of reductase, vasopressin had more marked effects on the total activity of reductase. This difference was also evident when the respective effects were accentuated by pre-addition of glucagon.

Effects of ionophore A23187

Both phenylephrine and vasopressin mobilize Ca^{2+} from intracellular stores and result in Ca^{2+} influx. The latter effect is more pronounced for vasopressin and is accentuated by previous addition of glucagon (see [27,29]). Consequently, the observations described above indicated that an increase in cytosolic Ca^{2+} , and especially the prolongation of Ca^{2+} influx in the presence of glucagon, resulted in both increased phosphorylation of reductase and extensive loss of total activity. These inferences were tested directly in experiments in which the Ca^{2+} ionophore A23187 was added to hepatocyte incubations. This resulted in a profound increase in the phosphorylation state of reductase (Fig.

2f), which at its maximum (at 5 min) decreased the expressed/total activity ratio from 0.80 to about 0.35. Thereafter it recovered only very slowly and was still approx. 0.45 after 20 min, but was back to normal values after 40 min. These changes were accompanied by the loss of total HMG-CoA reductase activity of a similar magnitude (approx. 50% after 40 min) to that observed in the presence of vasopressin plus glucagon.

Effects of okadaic acid

The data presented in Figs. 2 and 3 suggest that there was no quantitative relationship between the degree of transient phosphorylation of reductase that accompanied the elevation of cytosolic Ca^{2+} and the subsequent loss of total reductase activity. We tested this lack of correlation between the two parameters by eliciting an increase in phosphorylation of reductase in a Ca^{2+} -independent manner. Hepatocytes were incubated with okadaic acid ($1 \mu\text{M}$), which is a potent inhibitor of type 1 protein phosphatases and of phosphatase 2A [30]. As shown in Fig. 4(a), this resulted in a rapid decrease in the expressed/total ratio from 0.8 to less than 0.2, within 5 min. This increased phosphorylation of HMG-CoA reductase was maintained throughout the incubation period, as would be expected from the mode of action of okadaic acid. The degree of phosphorylation induced by okadaic acid was greater than that observed with A23187. In

Fig. 3. Effects of conditions that elevate Ca^{2+} and/or cyclic AMP concentrations in the cytosol of hepatocytes on the total activity of HMG-CoA reductase

The microsomes in which total activity was measured were obtained from Cl^- -extracted hepatocytes (see the Materials and methods section) and were incubated with exogenous partially purified protein phosphatase in order to obtain complete dephosphorylation of HMG-CoA reductase. After re-sedimentation at $100\,000\text{ g}$ for 10 min, they were finally resuspended in F^- -containing medium before assay. Values (means $\pm$ S.E.M.) are presented as a percentage of the activity at zero time, to eliminate interference from variations in absolute activities between different hepatocyte preparations. The mean value for the absolute activity of totally dephosphorylated HMG-CoA reductase at zero time was 0.60 ± 0.05 nmol/min per mg of protein at 37°C ($n = 31$, including preparations used for experiments shown in Fig. 4). The same notation is used as in Fig. 2.

spite of this, okadaic acid did not induce any change in the total activity of HMG-CoA reductase (Fig. 4b).

In order to test whether the lack of effect of okadaic acid on total activity was due to interference with the (unknown) mechanism(s) responsible for such loss, the phosphatase inhibitor was added together with A23187. As shown in Fig. 4(b), okadaic acid did not diminish the extent of the loss of total activity induced by the A23187, whereas, as expected, the full phosphorylation effect of okadaic acid (plus A23187) was observed (Fig. 4a).

DISCUSSION

The present data show that Ca^{2+} mobilization and influx into hepatocytes are associated with an increase in the degree of phosphorylation of HMG-CoA reductase, and also result in the rapid loss of a substantial proportion of the total reductase activity. The latter phenomenon was the major effect observed when hormonal conditions known to result in sustained Ca^{2+} influx into hepatocytes (e.g. incubation with vasopressin plus glucagon) were used. The magnitude and rapidity of the loss of reductase were of the same order as those observed previously in response to effectors such as oxysterols and mevalonate [5,31], and are likely to form the basis for an important mechanism of control of reductase expression in the liver *in vivo*.

The decreases in expressed/total activity ratio of HMG-CoA reductase induced by changes in cytosolic Ca^{2+} mediated by phenylephrine or vasopressin added singly were either undetectable or very small, respectively. This was surprising as, *in vitro*, HMG-CoA reductase is a substrate for at least two protein kinases that would be expected to be activated under these conditions: protein kinase C and a Ca^{2+} -calmodulin-dependent protein kinase (see the Introduction). Another noteworthy observation was that, whereas phenylephrine was more effective than vasopressin in mediating phosphorylation of HMG-CoA reductase, the opposite was true for activation of phosphorylase. It therefore appears that α_1 -agonists and vasopressin have selective effects on the phosphorylation of individual hepatic enzymes (see also [32,33]). The observation that, in the presence of glucagon, the maximal phosphorylation of reductase induced by either phenylephrine or vasopressin was much larger suggests that only at the higher concentrations of cytosolic Ca^{2+} achieved when Ca^{2+} mobilization and influx are potentiated [34,35] are the protein kinase(s) involved in phosphorylating reductase able to overcome significantly the opposing effects of phosphatase activity. Similarly, it was only on incubation of cells with A23187 that major phosphorylation of reductase occurred. Even then, it was significantly less than the maximal phosphorylation observed in the presence of okadaic acid. The possibility cannot be excluded, of course, that con-

Fig. 4. Effects of okadaic acid in the presence or absence of A23187 on the total activity and phosphorylation state of HMG-CoA reductase in rat hepatocytes

(a) Expressed/total activity ratio; (b) total activity. Hepatocytes were incubated as described in the legend to Fig. 1, except that the total volume was 1.0 ml. After 15–20 min equilibration, additions were made of okadaic acid (1 μ M, ●), or A23187 (30 μ M, △), or okadaic acid plus A23187 (▲) (all in 1 μ l of dimethyl sulphoxide). Controls received 1 μ l of carrier. Incubations were terminated, and microsomes were prepared, as described in the legend to Fig. 1. Values are means ($\pm$ S.E.M.) for 2 or 3 determinations.

ditions that result in Ca^{2+} influx into hepatocytes also result in the elevation of other potential effectors of the phosphorylation state of reductase, e.g. an increase in AMP concentrations that would activate the AMP-stimulated kinase; see the Introduction.

These observations indicate that the phosphatase(s) involved in HMG-CoA reductase dephosphorylation (and possibly of a kinase for which it is a substrate; see the Introduction) are very abundant and/or active. In this respect, the data obtained with okadaic acid are important in demonstrating that, in intact cells, the phosphatases responsible for modulation of HMG-CoA reductase activity are of the type 1 and/or 2A classes. This is because, at the concentration used, okadaic acid only inhibits these phosphatases [30]. Interestingly, studies using liver homogenates had indicated that, although these two

phosphatases could act on reductase, the enzyme was a better substrate for phosphatase 2C [16]. This difference between results obtained with intact cells and with homogenates suggests that factors exist *in vivo* that enable type 1 and 2A phosphatases to act preferentially on reductase itself and/or on reductase kinase.

Several lines of evidence suggested that phosphorylation of HMG-CoA reductase and subsequent loss of total activity were not causally related, although they could both be induced by conditions that resulted in elevation of cytosolic Ca^{2+} concentration. First, there was no relation between the degree of phosphorylation of the enzyme, induced by the various hormonal conditions, and the loss of total reductase activity. Indeed, when vasopressin was added alone, it elicited a 25% loss in total activity in the absence of any net increase in phosphorylation. Second, whereas the loss in total activity was the same (approx. 50%) in the presence of either A23187 or vasopressin plus glucagon, the maximal degree of phosphorylation induced by the former was several-fold greater. Third, when phosphorylation was induced by inhibition of phosphatase activity with okadaic acid, there was no loss of total reductase activity; nor was the loss of reductase induced by A23187 enhanced by the simultaneous presence of okadaic acid, in spite of the increased phosphorylation. The simplest explanation for these findings is that, in intact hepatocytes, both phosphorylation and turnover of HMG-CoA reductase are under control by cytosolic Ca^{2+} concentrations, but that the two processes are mediated independently.

The parameter that appeared to be most closely correlated with the ability of any set of conditions to induce a sustained loss of total reductase activity was the induction and maintenance of an increase in cytosolic Ca^{2+} concentration, e.g. in the presence of vasopressin (in either the presence or the absence of glucagon) or on addition of A23187. The present data do not allow a distinction between whether this effect is mediated through alteration of the rates of synthesis and/or degradation of HMG-CoA reductase. However, it was evident from the data obtained with phenylephrine alone (Fig. 3a) that the system is very dynamic, in that the initial loss in total activity which occurred in response to the Ca^{2+} mobilization induced by phenylephrine was rapidly reversed when cytosolic Ca^{2+} returned to basal levels (as indicated from the response of phosphorylase α ; Fig. 1a).

These data provide no evidence in favour of the postulate that increased phosphorylation of reductase necessarily leads to increased degradation [9–11]. The results with okadaic acid show that phosphorylation of the regulatory site is not sufficient to trigger degradation (although it may still be necessary). It is also possible that phosphorylation of some other site, which is dephosphorylated by an okadaic acid-insensitive phosphatase (e.g. 2C) could be responsible. In this respect it is worth noting that, although examples of changes in HMG-CoA reductase phosphorylation state and enzyme concentration in the liver *in vivo* have been used previously [9] in support of such a postulate, there is little evidence to suggest that this apparent relationship holds *in vivo*. Thus the decline in total enzyme activity that follows the increased state of phosphorylation during the dark phase in the diurnal cycle [36] is achieved exclusively through cessation of enzyme synthesis (see [37]) rather than through increased degradation. Similarly, at least one physiological condition is known (late pregnancy in the rat) in which the phosphorylation state of the enzyme becomes markedly increased for 2–3 days around parturition without any change in total activity (4 h into dark period) [38].

The effects of Ca^{2+} mobilization and influx on hepatic HMG-CoA reductase observed in this study may be of potentially major physiological importance. For example, the loss of cholesterol-synthetic capacity that would be expected to accompany such large losses of reductase activity may explain the

decrease in hepatic very-low-density lipoprotein secretion that occurs in acute trauma [39], when the secretion of stress hormones would be increased [40]. Similarly, under conditions that may involve a more chronic elevation of these hormones (e.g. diabetes [41]), there may be a more sustained activation of Ca^{2+} -dependent processes in liver cytosol, such that the maintenance of a lower concentration of hepatic HMG-CoA reductase is favoured. Thus the basal cytosolic free Ca^{2+} concentration is known to be significantly increased in hepatocytes isolated from acute and chronically diabetic rats [42]. The possibility of a sustained increase in the activation of Ca^{2+} -dependent processes in diabetes has also been suggested to account for the loss of G_i function in this pathological state [43].

The data may also explain the otherwise paradoxical effects of glucagon. As discussed above (see the Introduction), glucagon does not appear to be able to affect the activity of reductase directly, because the kinase/phosphatase cascade that controls the activity of the enzyme is not affected by cyclic AMP-dependent protein kinase. Although glucagon raises cytosolic Ca^{2+} concentration in hepatocytes, the extent to which it does this is smaller than that caused by other Ca^{2+} -mobilizing agonists [29,34]. Consequently, the lack of effect on hepatic reductase phosphorylation state observed *in vivo* after glucagon treatment in normal rats [8] is to be expected. However, glucagon injection does effect increased phosphorylation of HMG-CoA reductase *in vivo* in diabetic rats [8]. The present data suggest that this selective effect may arise through the ability of glucagon to optimize the effects of such hormones as vasopressin and noradrenaline on Ca^{2+} mobilization and influx into hepatocytes (see above). Thus glucagon may play a role in the regulation of reductase phosphorylation and concentration in the liver under conditions characterized by elevated concentrations of stress hormones. Of course, in conditions such as diabetes, other factors will also act to increase the phosphorylation of HMG-CoA reductase and decrease its total activity [44].

It would be of interest to investigate whether changes in Ca^{2+} fluxes are also involved in the action of other effectors of total HMG-CoA reductase activity in liver cells, as well as in other cell types. Perhaps the most intriguing of these possibilities arises from the observations that binding of low-density lipoprotein to arterial smooth-muscle cells [45,46] and platelets [47] results in rises in cytosolic Ca^{2+} in these cell types. The possibility is raised (see also [48]) that this early effect of LDL binding may be transmitted to HMG-CoA reductase via the mechanism(s) responsible for the effects described in the present study, thus triggering an early phase in the suppression of reductase activity, followed by a later phase involving the effects of such products as oxysterols.

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