The roles of different protein kinases and of calmodulin in the effects of Ca^{2+} mobilization on 3-hydroxy-3-methylglutaryl-CoA reductase activity in isolated rat hepatocytes

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The roles of protein kinase C, Ca^{2+} /calmodulin-dependent protein kinase and AMP-activated protein kinase in the phosphorylation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase induced by Ca^{2+} -mobilizing conditions in isolated hepatocytes were investigated. Only partial evidence for the involvement of AMP-activated kinase was found. Antagonism of calmodulin action prolonged the decrease in expressed/total activity ratio induced by vasopressin plus glucagon. Protease inhibitors active against Ca^{2+} -dependent cytosolic proteases or lysosomal proteolysis did not attenuate the loss of total HMG-CoA reductase induced by glucagon plus vasopressin, but calmodulin antagonists largely prevented this effect.

INTRODUCTION

We previously reported [1] that conditions that induce Ca^{2+} mobilization in isolated rat hepatocytes result in the rapid loss of total 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity. The effect appeared to be proportional to the intensity of the alteration of Ca^{2+} fluxes. Thus, glucagon enhanced the effects of vasopressin and phenylephrine; marked effects were observed with a maximally effective dose of the Ca^{2+} ionophore A23187 [1]. The loss of HMG-CoA reductase total activity was preceded by a transient increase in the degree of phosphorylation of the enzyme. However, there appeared to be no direct correlation between the degree of phosphorylation and the subsequent loss of total enzyme activity. For example, when cells were incubated with vasopressin, a 30% loss of total enzyme activity occurred within 40 min, in the absence of any detectable increase in the phosphorylation state of the enzyme.

These data raise the possibility that, although Ca^{2+} mobilization can trigger both increased phosphorylation and increased degradation and/or decreased synthesis of HMG-CoA reductase, the two processes are mediated by distinct mechanisms. In the present study we have sought to investigate the mechanisms involved in the two processes. Previous studies [2–4] had suggested a possible link between increased phosphorylation of HMG-CoA reductase and increased degradation of the enzyme protein by a Ca^{2+} -dependent protease (calpain II). We have therefore investigated the effects of inhibitors of the cytosolic Ca^{2+} -dependent proteases as well as of a lysosomally active protease inhibitor on the effect of Ca^{2+} mobilization on the total activity of HMG-CoA reductase in hepatocytes. Ca^{2+} may also be involved in the activation of two protein kinases for which reductase is a substrate, namely a Ca^{2+} /calmodulin-dependent kinase [5] and protein kinase C [6]. So we have tested the effects of calmodulin antagonists and protein kinase C activators on the phosphorylation state and total activity of HMG-CoA reductase, in the absence and presence of Ca^{2+} -mobilizing hormones. Finally, we have considered the possibility that changes in Ca^{2+} fluxes across the hepatocyte membranes may be accompanied by changes in AMP concentrations that could stimulate the activity of the AMP-activated kinase, for which HMG-CoA reductase is also a substrate [7,8].

MATERIALS AND METHODS

Animals

These were female Wistar rats (200–250 g) fed *ad libitum* on a standard laboratory chow diet. They were kept in a room with a 12 h-light/12 h-dark cycle with lights switched on between 20:00 h and 08:00 h. The animals were used at 11:00 h for preparation of hepatocytes, i.e. 3 h into the dark period.

Hepatocytes were prepared and incubated as described previously [1]. For the assay of HMG-CoA reductase activity the incubations were performed as in [1] and terminated by cold-quenching and sonication in either KCl- or KF-containing medium to obtain total and expressed activities, respectively. Expressed and total activities were determined as in [1]. For determination of cellular AMP concentrations, the entire cell-incubation mixtures were aspirated from the flasks by using wide-mouthed 5 ml plastic pipette tips and gently delivered into 12 ml plastic tubes which contained an oil layer (2.5 ml) [made up of a mixture of Dow Corning 702 fluid (60%, v/v) and Dow Corning 200/10 cs silicone fluid (40%, v/v)], which was in turn layered on top of 0.5 ml of 10% (w/v) HClO_4 . The cells were sedimented through the oil layer and into the acid by rapid centrifugation (3600 g for 20 s). The tubes were kept on ice until removal of denatured protein and subsequent neutralization of the HClO_4 extracts. These were frozen at -20°C before spectrophotometric assay of their AMP content [9].

Materials

The sources of most of the chemicals were as described previously [1], except that ionophore A23187 was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). In addition, {1-[bis-(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1*H*-imidazolium chloride (calmidazolium), adenylate kinase, pyruvate kinase and lactate dehydrogenase, trifluoperazine, methylamine hydrochloride, phorbol 12-myristate 13-acetate and 1-oleoyl-2-acetylgllycerol were also from Sigma. Dow Corning 200/10cs and 702 silicone fluids were obtained from Hopkin and Williams (Chadwell Heath, Essex, U.K.).

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

RESULTS AND DISCUSSION

Effects of activators of protein kinase C

In our previous experiments [1] we found that glucagon potentiated the effects of vasopressin and phenylephrine on the phosphorylation of reductase and the loss of total activity. As glucagon is known to enhance production of both $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol in response to these hormones, we tested the effects on expressed and total activities of HMG-CoA reductase of phorbol 12-myristate 13-acetate and a synthetic diacylglycerol (1-oleoyl-2-acetyl-glycerol; 0.1 mM), which are known to activate protein kinase C in rat hepatocytes [10,11]. Neither of these agents affected the phosphorylation state (i.e. expressed/total activity ratio) or the total activity of HMG-CoA reductase, nor did they augment the effects of vasopressin plus glucagon (results not shown). It is concluded that the effects of Ca^{2+} -mobilizing hormones on HMG-CoA reductase are not mediated via the activation of protein kinase C in rat hepatocytes, in spite of the fact that purified HMG-CoA reductase is a substrate *in vitro* for this protein kinase [5].

Effects of Ca^{2+} on cellular AMP concentrations

Another kinase for which HMG-CoA is a substrate is the AMP-activated protein kinase [7]. We examined the possibility that the effects of Ca^{2+} mobilization in increasing the degree of phosphorylation of HMG-CoA reductase in hepatocytes may be due indirectly to a simultaneous increase in cellular AMP concentration. As shown in Fig. 1, this occurred on incubation of cells with the Ca^{2+} ionophore A23187. The increase in AMP was fairly modest (about 2-fold) and reached a peak between 5 and 10 min after addition of the ionophore. In this respect, this time course was similar to that observed for increased phosphorylation (decreased expressed/total activity ratio) for reductase after addition of A23187 [1]. However, hepatocyte AMP

Fig. 1. Effect of ionophore A23187 on the AMP concentration in isolated rat liver cells

Rat hepatocytes were incubated in a total volume of 2.8 ml for 20 min, with continuous oxygenation (see the Materials and methods section) before addition of ionophore A23187 ($10 \mu\text{M}$) at zero time dissolved in $2.8 \mu\text{l}$ of dimethyl sulphoxide. The cells were harvested at the times shown by rapid centrifugation through oil (see the Materials and methods section), and AMP was measured in the HClO_4 extracts. Controls to which only dimethyl sulphoxide was added showed no difference from saline controls. ●, +A23187; ○, saline controls. The results are expressed as a percentage of the AMP concentration at zero time, to minimize the effects of variation in cellular AMP between different preparations. The absolute AMP content was $93.7 \pm 11.0 \text{ nmol/mg}$ of cells (wet wt.) for 8 preparations.

concentrations remained elevated even 35 min after addition of A23187 (Fig. 1), whereas the expressed/total activity ratio for reductase recovers its control value by this time point [1]. Consequently, the relationship between total cellular AMP concentration and reductase phosphorylation may not be a simple one. This was further demonstrated by the fact that we could not find any significant effects of vasopressin or phenylephrine (with or without glucagon) on hepatocyte AMP concentrations (results not shown). By contrast, addition of these hormone combinations results in substantial increases in the phosphorylation of HMG-CoA reductase (see Fig. 2 and [1]).

These observations provide only partial evidence for the involvement of elevated cellular AMP concentrations in the increased phosphorylation of HMG-CoA reductase elicited by Ca^{2+} mobilization in hepatocytes. It is possible that increases in AMP concentration are effectively localized to sites of increased ATP hydrolysis (owing to Ca-ATPase action), thus minimizing the experimentally determined increase in whole-cell AMP, but this is necessarily speculative at present. A second possibility is that the effects of changes in AMP concentration are amplified *in situ*, such that, although significant increases in AMP cannot be observed experimentally, their effects on HMG-CoA phosphorylation would still be evident. One way in which such amplification could occur would be if AMP stimulates the phosphorylation and consequent activation of reductase kinase (i.e. AMP-activated kinase [7,8]). It would be of interest to study the effects of Ca^{2+} mobilization in hepatocytes on reductase kinase [12] activity.

Effects of calmodulin antagonists

HMG-CoA reductase is also a substrate for a Ca^{2+} /calmodulin-dependent protein kinase *in vitro* [5], although it is not known whether this kinase is the Ca^{2+} /calmodulin protein kinase II, as the molecular mass cited in [5] is smaller than that established for this latter kinase (see [13]). We have tested the effects of two antagonists of calmodulin action (calmidazolium and trifluoperazine) on the action of vasopressin and glucagon on HMG-CoA reductase in hepatocytes. Cells were incubated with antagonists for 10–15 min before addition of glucagon, followed (after a further 2 min) by addition of vasopressin (Fig. 2). Neither calmidazolium (Fig. 2a) nor trifluoperazine (results not shown) affected the decrease in expressed/total activity ratio induced by the hormones, suggesting that increased phosphorylation of reductase is not a calmodulin-dependent process. However, in the presence of calmidazolium, there was a prolongation of the decrease in expressed/total activity ratio induced by hormone treatment, such that after 40 min of incubation HMG-CoA reductase appeared to be in a markedly more phosphorylated state in the presence of the calmodulin inhibitor. The simplest explanation for this observation is that a Ca^{2+} /calmodulin-dependent protein phosphatase is involved in the restoration of the phosphorylation state of reductase after challenge of hepatocytes with Ca^{2+} -mobilizing hormones or A23187 [1]. Such a Ca^{2+} /calmodulin-dependent enzyme is phosphatase 2B or calcineurin, which is known to occur in liver, albeit in low concentrations [14–16]. However, on the basis of its substrate specificity *in vitro*, this phosphatase has hitherto not been thought to act on the rate-limiting enzymes of metabolic pathways (see [17]).

Calmidazolium significantly diminished the effect of vasopressin plus glucagon on the loss of total HMG-CoA reductase activity (Fig. 2b). Qualitatively similar effects were obtained with trifluoperazine (results not shown). These observations suggest that calmodulin is involved in the mechanism through which Ca^{2+} mobilization results in a decreased rate of synthesis and/or an increased rate of degradation of HMG-CoA reductase in hepatocytes. The contrast between the effects of calmidazolium

Fig. 2. Effects of the calmodulin antagonist calmidazolium on the action of glucagon plus vasopressin on (a) the expressed/total activity ratio and (b) the total activity of HMG-CoA reductase in isolated rat liver cells

Hepatocytes were incubated for 10 min before addition of (○) calmidazolium (25 μM in dimethyl sulphoxide) or (●) carrier only. After a further 15 min, glucagon (10 nM) and vasopressin (0.1 μM) were added (zero time). At the specified times, cells were harvested by cold-quenching and sonicated in either chloride- or fluoride-containing media (see the Materials and methods section) to obtain total and expressed activities respectively. Microsomes were prepared and the activities assayed as described previously [1]. Values are means (± S.E.M.) for five separate cell preparations.

on the induction of increased phosphorylation and on the loss of total activity of HMG-CoA reductase reinforces the inference, drawn from our previous studies [1], that the two are not causally related in response to Ca^{2+} mobilization.

Effects of protease inhibitors

It has been shown [2] that phosphorylation of HMG-CoA reductase makes it more susceptible to proteolysis by the Ca^{2+} -dependent cytosolic protease calpain II *in vitro*. It is therefore possible that, although we did not observe a direct relationship between increased phosphorylation and loss of total activity of reductase [1], the increased cytosolic $[\text{Ca}^{2+}]$ induced by vasopressin and phenylephrine, especially in the presence of glucagon, could result in the activation of the calpain system *in situ* such

that enhanced degradation would be independently activated. Therefore we studied the effects of the incubation of hepatocytes with leupeptin (100 μg/ml) and antipain (50 μg/ml). These are known to be inhibitors of the cytosolic Ca^{2+} -dependent proteases [18–20], although they have also been used to inhibit intralysosomal proteases [21]. In addition, they have been shown to inhibit certain actions of Ca^{2+} mobilization in different cell types when included in incubation media (see [17]). We did not detect any effects of these protease inhibitors on the loss of total HMG-CoA reductase induced by vasopressin plus glucagon. The decrease in total reductase activity was $51.3 \pm 5.7\%$ ($n = 7$) and $48.0 \pm 4.8\%$ ($n = 3$) for control and protease-treated cells respectively. Methylamine, a lysosomotropic protease inhibitor, decreased the extent of loss of HMG-CoA reductase ($42.4 \pm 6.2\%$; $n = 4$) induced by the hormones by a small extent. However, this was probably related to the fact that lysosomally active protease inhibitors are known to inhibit the basal rate of HMG-CoA reductase degradation in hepatocytes [20]. Consequently, we found no evidence that the effect of Ca^{2+} mobilization on total HMG-CoA reductase can be counteracted by protease inhibitors active either on the Ca^{2+} -dependent calpain system in the cytosol or on lysosomal proteolysis. This may suggest either that other forms of degradation are involved (cf. the NH_4Cl -insensitive mevalonolactone-induced degradation of HMG-CoA reductase [21]) or that increased degradation of the protein is not part of the mechanism through which decreased HMG-CoA reductase content of hepatocytes is achieved after Ca^{2+} mobilization. Whatever the precise mechanism, it is clearly different from the mevalonate-induced degradation of HMG-CoA reductase, which is totally prevented by lysosomotropic protease inhibitors [2].

General conclusions

We have failed to find any evidence for the involvement of a Ca^{2+} /calmodulin-dependent protein kinase or of protein kinase C in the vasopressin-plus-glucagon-induced phosphorylation of HMG-CoA reductase in isolated rat hepatocytes. The increased AMP concentration that occurs after addition of A23187 to the cells (and which is accompanied by a decrease in expressed/total HMG-CoA reductase activity ratio from 0.8 to 0.2; see [1]) suggest that this kinase is involved, although direct evidence for this in the case of incubations with hormones was not obtained. Calmodulin antagonists partially prevent the effects of Ca^{2+} mobilization on total reductase activity and prolong the phosphorylation of the enzyme after a Ca^{2+} -mobilizing stimulus. These observations reinforce our previous conclusions [1] that the decrease in total reductase activity observed after Ca^{2+} mobilization is unrelated to the increased phosphorylation of the enzyme. Although it is not possible, from the present data, to ascertain whether the loss of total reductase activity is due to decreased synthesis and/or increased degradation of the enzyme, it is evident that neither the Ca^{2+} -dependent cytosolic proteinases nor accelerated lysosomal degradation appear to be involved.

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