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Divalent Metal Ions as Modulators of Rat Liver Microsomal Cholesterol Esterase

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The regulatory properties of the divalent metal ions Mg^{2+}, Ca^{2+} and Mn^{2+} on the activity and kinetic behaviour of rat liver microsomal cholesterol esterase were studied *in vitro*. Mg^{2+} and Ca^{2+} exhibited similar concentration and preincubation time-dependent increases in esterase activity, with maximal stimulation at a concentration of 2 mM. However, Mn^{2+} had no effect at this concentration but displayed a potent inhibitory effect at concentrations above 20 mM. Activation of cholesterol esterase by Mg^{2+} and Ca^{2+} was selective in relation to i) the changes that cations produced in the enzyme kinetic constants, and ii) the chelating agents that reversed the metal ion-induced activation. Hence, the maximum rate of cholesterol ester hydrolysis doubled in the presense of Mg^{2+} and activation was reversed by EDTA, whereas a significant decrease in the apparent Km for cholesterol oleate was found when Ca^{2+} was added and this effect was blocked by ATP and EGTA. Both cations were able to reactivate cholesterol ester hydrolase activity in metal–depleted microsomes.

Key words: Cholesterol esterase, Divalent cations, Liver microsomes.

It is well established that the liver is the major site of hydrolysis of long chain fatty acid esters of cholesterol in the rat. This is achieved by at least three cholesterol ester hydrolases (CEH) (EC 3.1.1.13) with specific biological functions and different subcellular localizations. The best characterized enzyme is the lysosomal acid cholesterol esterase which is involved in the hydrolysis of cholesteryl esters delivered to cells via receptor-mediated endo-

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cytosis of lipoproteins (3, 10, 19). Excess intracellular cholesterol is esterified with long chain fatty acids (1, 8, 21), and the resulting cholesteryl esters are either utilized or stored in the cytoplasm and in the membranes of the smooth endoplasmic reticulum (8, 14). Mobilization of these esters is due to the combined action of two neutral CEH located in cytosol and microsomes (6, 7, 9, 17, 19). Though soluble cholesterol esterase has been purified and rather well characterized, few studies have been reported on the precise function and regulation of the microsomal enzyme. It may be involved in preventing the accumulation of cholesteryl ester in the endoplasmic reticulum and in controlling the fate of the cell cholesterol ester pool between the biliary and the very low density lipoprotein cholesterol secretory pathways. It also hydrolyses esterified cholesterol stored in lipid droplets in the cytosol (19). Rat liver microsomal CEH has been partially solubilized (12). Seventy-five per-cent of its activity has been found on the luminal face of the rough microsomes (9) and it displays a diurnal rhythm unrelated to the feeding status (15). This activity has been found to be different from the hepatic lipase (5) and from the neutral rethinyl ester hydrolase activity also located in microsomes (11). Contradictory reports have appeared concerning the existence of compensatory changes in the esterase activity in response to altered dietary cholesterol fluxes in the liver (18, 20). Recently, using a cell-free system it has been found that cholesterol ester hydrolase from rat liver microsomes may be regulated by reversible phosphorylation with the phosphorylated form being active (16). Phosphorylation and concomitant activation was triggered by commercial cyclic AMP-dependent protein kinases and by Ca^{2+}/Calmodulin- and Mg^{2+}-activated kinases associated with the microsomal preparation (16). In order to examine further the regulatory role and the specificity of divalent cations on microsomal CEH, the effect of Mg^{2+}, Ca^{2+} and Mn^{2+} on CEH activity and its kinetic behaviour were studied *in vitro*. Here it is shown that cholesterol ester hydrolase from rat liver microsomes is consistently activated by Mg^{2+} and Ca^{2+}, and that they alter selectively the kinetic constants of the enzyme, whereas Mn^{2+} behaves in a different way. Moreover, the reversion and the rate of reversion of metal ion-induced activation by different chelators have also been investigated.

Materials and Methods

Materials. — Cholesterol oleate, dipalmitoyl phosphatidylcholine, sodium cholate, bovine serum albumin, $MgCl_2$, $CaCl_2$ and $MnCl_2$ were purchased from Sigma Chemical Co. Cholesterol [1-^{14}C] oleate and [^{3}H] oleic acid were from Amersham (UK). All other chemicals used were of reagent grade.

Preparation of rat liver microsomes. — Female Sprague-Dawley rats (180-200 g), housed in temperature and light controlled rooms and fed as described (15), were used at 2 hours of the light period. Animals were anaesthetized under light diethyl ether vapours and the livers were removed immediately, rinsed in ice cold saline and homogenized (15). The microsomal fraction was prepared by serial centrifugations (1000 × *g*, 15 min; 22000 × *g*, 20 min; 105000 × *g*, 60 min) at 4 °C, washed once and resuspended in 1 vol 20 mM Tris-HCl buffer, pH 7.4, with 250 mM sucrose. Microsomes could be stored at −20 °C under N_2 for up to 5 days without loss of CEH activity.

Assay of cholesterol esterase activity. — Enzyme activity was determined in terms of release of oleic acid from the cholesterol [1-^{14}C] oleate substrate. Routinely an assay mixture contained in a total volume of 0.4 ml: 12 nmol cholesterol oleate (sp. act.

6.25 Ci/mol) prepared as mixed micelles with dipalmitoyl phosphatidylcholine and sodium cholate in a 1:4:2 molar proportion (13), 0.5 mg microsomal protein, 75 mM Tris-HCl buffer, pH 7.4 containing 0.2 mg bovine serum albumin and the corresponding addition. In the assay system used there was no observable precipitation with any of the divalent metal ions studied. After incubation for 45 min, the reaction was stopped and the released labelled free fatty acids measured (15), the partitioning of free fatty acids being monitored by the addition of known amounts of [^{3}H] oleic acid to blank assays. Protein was determined as described by BRADFORD (2), with bovine serum albumin as standard. CEH activity was expressed as Units/mg microsomal protein, one Unit corresponding to the hydrolysis of one pmol cholesterol oleate per min at 37 °C.

Statistical analysis was done by the Student's *t* test unless otherwise stated.

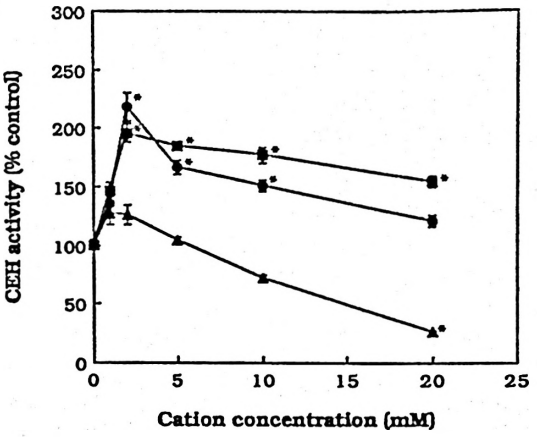

Fig. 1. *Effect of Mg^{2+}, Ca^{2+} and Mn^{2+} on microsomal CEH activity.*
Microsomes were incubated in the presence of increasing concentrations of $MgCl_2$ (■), $CaCl_2$ (●) or $MnCl_2$ (▲) and CEH assayed as described in Materials and Methods. The absolute value of 100 % CEH activity was 8.4 Units/mg protein. Points represent the means ± SE of four experiments in triplicate. * $P < 0.005$ versus incubates with no additions.

Results and Discussion

When microsomes isolated from fed female rats were incubated with increasing amounts of the metal chlorides $MgCl_2$, $CaCl_2$ and $MnCl_2$, a concentration-dependent profile was observed for cholesterol esterase (fig. 1). Concentrations of 2 mM Mg^{2+} or Ca^{2+} doubled CEH activity, but at higher concentration it declined slowly from this value. However, Mn^{2+} did not produce any significant effect at the concentration interval of 0.1-10 mM, but at 20 mM a drastic inactivation was seen. Similar results were obtained when cholesterol ester hydrolase activity was tested in the presense of Mn^{2+} levels up to 100 mM (data not shown). When microsomes were preincubated with 2 mM cations for up to 25 min (fig. 2) a continuous pattern of activation was attained with Mg^{2+} and Ca^{2+}, whereas no effect was found with Mn^{2+}.

In a recent study evidence supporting the idea that Mg^{2+} and Ca^{2+} indirectly increase microsomal CEH activity as a result of the stimulation of endogenous protein kinases has been obtained (16). On the other hand, it has not been established whether microsomal CEH and also liver esterases from other subcellular locations are metalloenzymes. Since protein phosphorylation is likely to have and important function in binding cations (4), these two regulatory mechanisms could be well interconnected. Irrespective of the mechanisms involved, the findings in this study reinforce the idea that the divalent metal ions Mg^{2+} and Ca^{2+} activate CEH. Mn^{2+}, in contrast, behaved as a potent inhibitor at high concentrations.

In order to ascertain whether Mg^{2+} and Ca^{2+} might affect the cholesterol esterase kinetic behaviour the microsomal fraction was incubated with each cation at 2 mM, the optimum activatory concentration, for

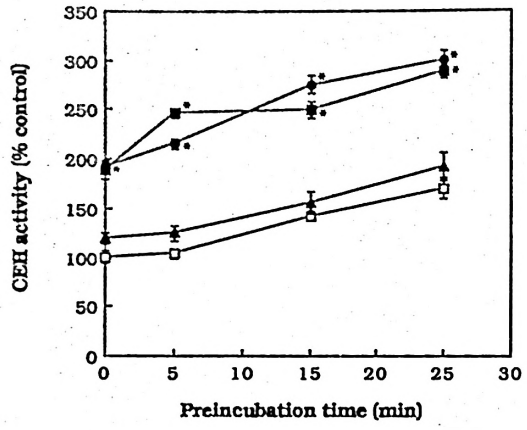

Fig. 2. *Time-course effect of Mg^{2+}, Ca^{2+} and Mn^{2+} on microsomal CEH activity.*
Microsomes were preincubated for up to 25 min without (□) or with 2 mM $MgCl_2$ (■), $CaCl_2$ (●) or $MnCl_2$ (▲) before assaying CEH activity as described in Materials and Methods. The absolute value of 100 % CEH activity was 6.9 Units/mg protein. Points represent the means ± SE of four experiments in triplicate. * $P < 0.005$ versus incubates with no additions at the same preincubation period.

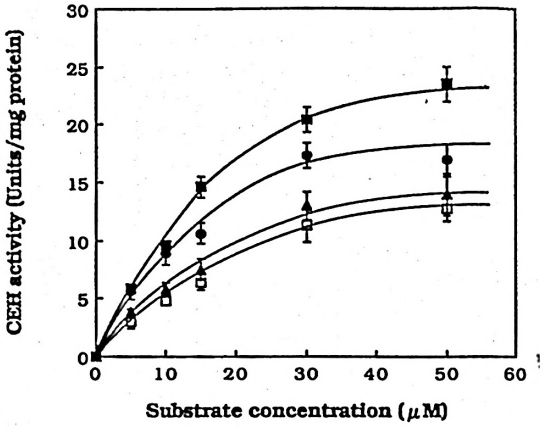

Fig. 3. *Effect of Mg^{2+}, Ca^{2+} and Mn^{2+} on the dependence of CEH activity towards the substrate cholesterol oleate.*
Microsomes were incubated without (□) or with 2 mM $MgCl_2$ (■), $CaCl_2$ (●) or $MnCl_2$ (▲) and cholesterol ester hydrolase assayed as described in Materials and Methods except that cholesterol oleate concentration ranged from 5 to 50 μM. Points represent the means ± SE from five experiments in triplicate. The probability of that difference of variances in double-reciprocal plots of values displayed for Mg^{2+} and Ca^{2+} respect to control profile is <0.01.

45 min at 37 °C with various substrate concentrations ranging from 5 to 50 μM. For comparison, Mn^{2+} was also tested under the same conditions. Results, directly plotted in fig. 3, indicate that the dependence of CEH activity on the substrate cholesterol oleate is selectively affected by the cations assayed. A computerized analysis of the double reciprocal plots of data in fig. 3 provided the CEH kinetic constants. Table I shows that Mg^{2+} doubled the maximum rate of hydrolysis whereas the apparent Km was not affected. In contrast, Ca^{2+} decreased the Km value significantly by around 45 % while the increase in the maximum velocity did not reach significance. As expected 2 mM Mn^{2+} did not modify the CEH kinetic pattern. It is therefore apparent that different mechanisms underlie CEH activation in rat liver microsomes by Mg^{2+} and Ca^{2+}.

As the metal ions tested in this study are substances capable of establishing a real link with either the cholesterol esterase or the endogenous protein kinases that in turn may activate CEH among other enzymes, the cation-induced activation was tested as to whether it could be reversibly counteracted by further incubation with chelating agents. For this purpose, aliquot portions of enzyme source were incubated with Mg^{2+} or Ca^{2+} for 5 min to allow activation to occur, and then ATP, EDTA or EGTA were added (table II). Firstly, it is noteworthy that chelators alone have no effect on CEH activity. This lack of effect was also found when other nucleotides such as ADP, GTP, GDP or CTP in concentrations 1-5 mM were tested (data not shown). In the presence of the activating metal ions, however, chelators had an in-

Table I. *Effect of 2 mM Mg^{2+}, Ca^{2+} and Mn^{2+} on the kinetic constants of microsomal CEH.*
Microsomes were treated as in legend to figure 3. The kinetic constants were calculated by linear regression analysis from values plotted in figure 3. Data are shown as the means ± SE from five experiments in triplicate. Statistical significance is given versus control with no additions.

Cation added	Km (μM)	Vmax (Units/mg)
none	27.6 ± 1.4	19.2 ± 1.2
Mg^{2+}	27.7 ± 1.6	38.3 ± 1.8[b]
Ca^{2+}	15.1 ± 0.9[a]	23.4 ± 0.8
Mn^{2+}	22.9 ± 1.4	18.5 ± 0.6

[a] $p < 0.025$; [b] $p < 0.01$.

Table II. *Effect of the chelators ATP, EDTA and EGTA on CEH activity in Mg^{2+} and Ca^{2+} treated microsomes.*
Microsomes were incubated without or with Mg^{2+} or Ca^{2+} for 5 min and then ATP, EDTA or EGTA were added and CEH activity measured as described. The absolute value of 100 % CEH activity was 14.9 Units/mg protein. Data are shown as the means ± SE from four experiments made in triplicate.

Addition	CEH activity (% control)
1. none	100 ± 4
2. 2 mM ATP	93 ± 5
3. 5 mM ATP	100 ± 2
4. 2 mM EDTA	98 ± 2
5. 5 mM EDTA	97 ± 3
6. 2 mM EGTA	96 ± 3
7. 5 mM EGTA	104 ± 4
8. 2 mM Mg^{2+}	188 ± 6*
9. + 2 mM ATP	174 ± 5
10. + 5 mM ATP	164 ± 8
11. + 10 mM ATP	152 ± 7**
12. + 2 mM EGTA	198 ± 4
13. + 5 mM EDTA	114 ± 9**
14. 2 mM Ca^{2+}	183 ± 6*
15. + 2 mM ATP	139 ± 5***
16. + 5 mM ATP	114 ± 3***
17. + 2 mM EGTA	105 ± 3***
18. + 5 mM EDTA	170 ± 9

* $P < 0.005$ versus line 1, ** at least $P < 0.025$ versus line 8 and *** at least $P < 0.01$ versus line 14.

activating effect which was dependent on the particular cation used. Data in table II indicate that EGTA was not able to reverse the Mg^{2+}-stimulation of CEH activity while EDTA decreased it markedly and ATP had a slight inhibitory effect. However, in Ca^{2+}-stimulated microsomes, EDTA had no effect, ATP caused a significant decrease in activity and EGTA abolished the stimulatory effect completely. Since the loss in activity in each case is time-dependent (fig. 4), it is unlikely that the inactivation is due to competition between binding of the chelators and substrate at the active site of the enzyme. The different rates of inactivation by EDTA and by EGTA or ATP suggest that different degrees of accessibility of chelators to the specific metal binding site may occur.

The data described above indicate that metal ions may be important for the activity or the structural integrity of microsomal cholesterol esterase. However, this tentative conclusion would be on much firmer ground if it were possible to add metal ions back to metal-depleted microsomes and observe regain of activity. To examine this, the effect of Mg^{2+} and Ca^{2+} on CEH activity was tested in microsomes isolated in buffers containing either 10 mM EDTA or 1 mM EGTA respectively. In both cases, cholesterol esterase activity declined to approximately 75 % of that of a control sample isolated in the absence of chelating agents (data not shown). Further addition of 2 mM Mg^{2+} led CEH activity to 40 % above the basal value (from 10.8 ± 0.9 to 15.4 ± 1.6 Units/mg protein). Two mM Ca^{2+} exerted a striking reactivatory effect, CEH activity being enhanced 120 % over that found in EGTA-isolated microsomes (from 11.3 ± 2.3 to 25.2 ± 4.1 Units/mg protein).

The situation with regard to the chelating agents is not fully clear. Though the results above mentioned support strongly that Mg^{2+} and Ca^{2+} are activators of the enzyme, the observation that the addition

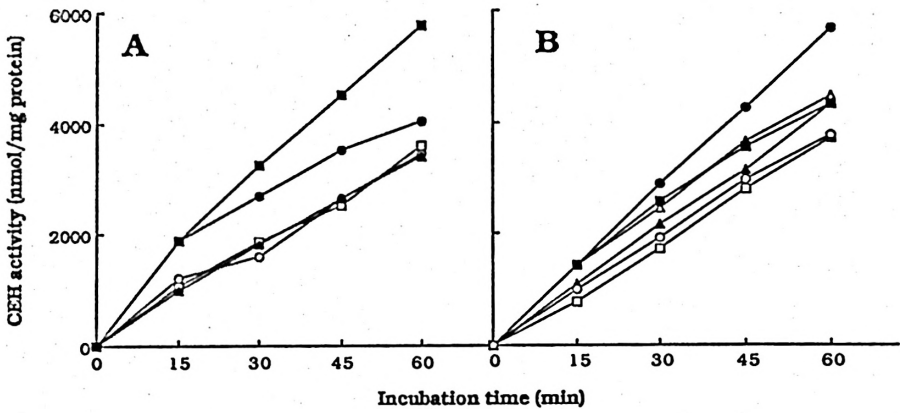

Fig. 4. *Time-reversal of Mg^{2+} and Ca^{2+}-induced microsomal CEH activity by EDTA, EGTA and ATP.* Points represent the mean values of two experiments in triplicate from microsomes incubated without (□) or with 2 mM Mg^{2+} or 2 mM Ca^{2+} for the indicated times. Chelators were added to the assay mixture either simultaneously with the cation (zero time) or after a 15 min incubation period run in the presence of the cation. Additions: Panel A; 2 mM $MgCl_2$ (■); 5 mM EDTA (○); 2 mM $MgCl_2$ and 5 mM EDTA added at zero time (▲) or after 15 min incubation (●). Panel B; 2 mM $CaCl_2$ (●); 2 mM $CaCl_2$ and 5 mM EGTA added at zero time (▲) or at 15 min (△); 2 mM $CaCl_2$ and 5 mM ATP added at zero time (□) or after 15 min incubation (■).

of chelators to control untreated microsomes does not affect CEH activity could question the need of cations in order for the reaction to take place.

The activatory effects of Mg^{2+} and Ca^{2+} on cholesterol esterase have been found to differ in two aspects: the changes promoted in the kinetic constants of the enzyme and the different degree of reversion induced by the metal-chelating agents ATP, EDTA and EGTA. This particular behaviour could arise from: i) the binding of the metal ions to specific catalytic or conformational sites of the cholesterol esterase or ii) that the activated kinases supposedly involved could be responsible for the union of phosphoryl groups to different sites of the enzyme molecule, or to one site that plays different roles in enzyme reaction. It is fairly common for proteins to be phosphorylated by multiple protein kinases at multiple sites and dephosphorylated by multiple protein phosphatases.

Nucleotides are well known as chelators for both Mg^{2+} and Ca^{2+}. Although a clear reversal of the Ca^{2+}-induced CEH activity by ATP, was found, surprisingly the Mg^{2+} effect was not abolished by this and other nucleotides. A strong binding of Mg^{2+} to the enzyme and/or a low accessibility of the chelator to the binding site could be responsible for this lack of reversal. Nucleotides could also exert a regulatory influence on CEH phosphorylation. Though we failed in our attempt to promote CEH phosphorylation by ATP *in vitro* (16), its participation in this process in the liver tissue cannot be excluded. Microsomal CEH phophorylation studies using liver cell suspensions as well as the purification of microsomal CEH are the subject of our current investigation.

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Resumen

Se estudia *in vitro* la regulación de la actividad y de la cinética de la colesterol esterasa de microsomas hepáticos por los iones metálicos divalentes Mg^{2+}, Ca^{2+} y Mn^{2+}. El Mg^{2+} y el Ca^{2+} producen análogos perfiles de activación del enzima tanto en función de la concentración como del tiempo de preincubación, obteniéndose el máximo efecto a 2 mM. Sin embargo, a esta concentración el Mn^{2+} no afecta la actividad enzimática, observándose una marcada inhibición a concentraciones superiores a 20 mM. La respuesta de la colesterol esterasa al Mg^{2+} y Ca^{2+} se puede considerar selectiva en lo que respecta a los cambios que dichos cationes producen en las constantes cinéticas del enzima, y a los quelantes que revierten la actividad inducida por el ion metálico. Así, el Mg^{2+} duplica la máxima velocidad hidrolítica y la activación es revertida por EDTA, mientras que el Ca^{2+} provoca un descenso sustancial de la Km aparente y la activación concomitante revierte por ATP y EGTA. Ambos iones metálicos reactivan la colesterol esterasa de microsomas tratados con quelantes.

Palabras clave: Colesterol esterasa, Cationes divalentes, Microsomas hepáticos.

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