Kinetic mechanism of activation by cardiolipin (diphosphatidylglycerol) of the rat liver multicatalytic proteinase

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The effect of phospholipids on the trypsin-like, chymotrypsin-like and peptidylglutamyl-peptide-hydrolysing activities of the so-called latent form of the rat liver multicatalytic proteinase was studied, assaying them with the following substrates: *N*-Cbz-ARR-4MNA (*N*-Cbz, *N*-benzyloxycarbonyl; 4MNA, 4-methoxy-β-naphthylamide), *N*-Suc-LLVY-MCA (*N*-Suc, *N*-succinyl; MCA, methylcoumarin) and *N*-Cbz-LLE-β-NA (β-NA, β-naphthylamide) respectively (amino acids are shown as their one-letter symbol). For the most part neither lysophospholipids nor phospholipids at 20 μg/ml have any effect on the activity of the enzyme (assayed at 50 μM peptide), except for phosphatidylserine, which activates 2-fold the hydrolysis of *N*-Suc-LLVY-MCA, and phosphatidylinositol, which inhibits by 20% the hydrolysis of *N*-Cbz-LLE-β-NA. By contrast, cardiolipin (diphosphatidylglycerol) is a strong activator of the hydrolysis of *N*-Suc-LLVY-MCA (60-fold) and *N*-Cbz-LLE-β-NA (30-fold), with half-maximal activation at concentrations of 0.15 μg/ml and 1.5 μg/ml respectively. The activation of *N*-Suc-LLVY-MCA hydrolysis is due to an increase of the affinity of the enzyme for the peptide and to an increase in the $V_{max.}$ (30-fold). The activation of *N*-Cbz-LLE-β-NA hydrolysis is explained by suppressing the co-operativity for this substrate, producing hyperbolic kinetics with a K_m of 60 μM and a 15-fold increase in the $V_{max.}$ of the enzyme. This activation by cardiolipin was completely suppressed by micromolar concentrations of fluophenazine, a drug known to inhibit other phospholipid-regulated process. Cardiolipin activation and the known activation by SDS are additive, either at suboptimal or optimal concentrations of both activators. Cardiolipin also activates the *in vitro* degradation of some proteins from metabolically labelled total cellular extracts by the latent multicatalytic proteinase. These results clearly show that cardiolipin is a natural positive modulator of the peptidase and proteolytic activities of the multicatalytic proteinase, probably acting through a binding site different from that of SDS.

INTRODUCTION

The multicatalytic proteinase (MCP) complex (also known as the proteasome) is a high-molecular-mass proteinase composed of 13–15 low-molecular-mass (22–34 kDa) non-identical subunits (for reviews, see [1,2]). The MCP is found in all eukaryotes, from yeast to man [3], and a simpler form, composed of only two subunits, has been found in archaebacteria [4]. The enzyme from eukaryotes has three distinct peptidase activities, cleaving bonds on the carboxy side of basic, hydrophobic and acidic amino acids. On the basis of the structure of the amino acid residue in the P1 position of the substrate these activities were designated 'trypsin-like', 'chymotrypsin-like' and 'peptidylglutamyl-peptide-hydrolysing' activities respectively.

The peptidylglutamyl-peptide-hydrolysing and the chymotrypsin-like activities of the MCP (as well as its proteinase activity) are strongly activated by low concentrations of SDS and fatty acids, a property of most MCPs isolated from variety of mammalian and other sources [5–15], indicating that the purified enzyme is in a so-called 'latent' form. In a search for other possible natural modulators that, like fatty acids, may mimic the effect of SDS, we have conducted a detailed study of the effect of different phospho- and lysophospho-lipids on the peptidase activities of the MCP from rat liver using three peptide substrates: *N*-Cbz-Ala-Arg-Arg-4MNA, *N*-Suc-Leu-Leu-Val-Tyr-MCA, *N*-Cbz-Leu-Leu-Glu-2-NA, for assaying the trypsin, chymotrypsin and peptidyl-glutamyl-peptide-hydrolysing activities respectively (*N*-Cbz- is *N*-benzyloxycarbonyl-, *N*-Suc- is *N*-succinyl-, 4-MNA is 4-methoxy-β-naphthylamide, MCA is methylcoumarin and β-NA is β-naphthylamide). The results presented show, for the first time, that a naturally occurring phospholipid, cardiolipin (diphosphatidylglycerol), is a very potent activator of the chymotrypsin and peptidyl-glutamyl-peptide-hydrolysing activities of the MCP. The kinetic mechanism of activation by cardiolipin, although similar in some respects to the SDS mechanism [15], is not identical with it, and in fact the activation observed in the presence of both SDS and cardiolipin equals the sum of the activation observed with each of these compounds added separately, suggesting that they act through different binding sites.

MATERIALS AND METHODS

Reagents

Fluorescent peptides (*N*-Cbz-Ala-Arg-Arg-4MNA, *N*-Suc-Leu-Leu-Val-Tyr-MCA, *N*-Cbz-Leu-Leu-Glu-β-NA) for peptidase assay and fluophenazine were obtained from Sigma. The sources of the phospholipids used in this study (all from Sigma) were as follows: dioleoyl phosphatidic acid; oleoyl lysophosphatidic acid; lysophosphatidylethanolamine and phosphatidylethanolamine from bovine liver; lysophosphatidylcholine and phosphatidylcholine from bovine liver; phosphatidylinositol from bovine liver and lysophosphatidylinositol from soybean; lysophosphatidylserine and phosphatidylserine from bovine brain; cardiolipin from bovine heart, and dioleoyl phosphatidylglycerol prepared

Abbreviations used: *N*-Cbz-, *N*-benzyloxycarbonyl-; *N*-Suc-, *N*-succinyl-; 4-MNA, 4-methoxy-β-naphthylamide; MCA, methylcoumarin; β-NA, β-naphthylamide; PMSF, phenylmethanesulphonyl fluoride; MCP, multicatalytic proteinase (proteasome); cardiolipin, diphosphatidylglycerol; DMEM, Dulbecco's modified Eagle medium; NRK, normal rat kidney.

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from egg yolk. Thin-layer Silica-Gel-60 plates without fluorescent indicator were obtained from Merck.

Purification of MCP from rat liver

The rat liver MCP was purified as described [15], adding one further step after the glycerol/1 M urea gradient sedimentation. A pool of the glycerol gradient fractions was applied to an h.p.l.c. (System Gold; Beckman) DEAE-5PW column equilibrated with 50 mM Tris/HCl (pH 8.0)/25 mM KCl/10% glycerol/1 mM $MgCl_2$ and the MCP was eluted with a linear gradient from 25 to 500 mM KCl in the same buffer (buffer B); the protein and activity peak was eluted at 70% buffer B (0.38 M KCl).

Peptidase assay

The enzymic proteinase activity was assayed by continuous recording of the enzymically released fluorochrome; initial rates were calculated from the slope of the corresponding recordings (absorbance versus time) when less than 10% of the substrate has been consumed. In all assays these plots were linear for at least 10 min, indicating pseudo-first-order reaction conditions. The reaction mixture contained, in a final volume of 0.5 ml: 50 mM Tris/HCl, pH 7.4, 1 mM DTT, different concentrations of the fluorogenic peptide, as indicated, and 25 μl of the purified rat liver (2 μg). Peptides used and wavelengths of excitation and emission were, respectively: 335–410 nm for *N*-Cbz-Ala-Arg-Arg-4MNA (hereafter, for simplicity, called 'ARR') and *N*-Cbz-Leu-Leu-Glu-β-NA (hereafter called 'LLE'); 380–460 nm for *N*-Suc-Leu-Leu-Val-Tyr-MCA (hereafter called 'LLVY'). The different lipids were evaporated to dryness with nitrogen from their solution in chloroform and resuspended in the reaction buffer by sonication in a water bath (2 min) immediately before being used or dissolved in 100% methanol (cardiolipin) at high concentration (5–10 mg/ml). Controls, run in parallel, showed that the amount of methanol does not significantly affect the peptidase activity of the proteinase towards any of the three peptides. To rule out a possible effect of glycerol (0.5% final concn. in the routine assay; see above), an aliquot of the purified enzyme was buffer-exchanged by dilution and concentration in a Centrikon ultrafilter (30 kDa molecular-mass-cut-off) in a buffer containing 50 mM Tris/HCl (pH 7.5)/1 mM DTT (five times; 20-fold dilution each time); with this enzyme preparation (virtually free of glycerol, as measured in a coupled assay with glycerol kinase and glycerol-3-phosphate dehydrogenase) glycerol up to a 5% final concentration in the assay had no effect on the enzyme activity on any of the three peptide substrates not on activation by cardiolipin or SDS of the hydrolysis of LLVY and LLE peptides.

Metabolic labelling of cells and *in vitro* protein degradation

Normal rat kidney (NRK) cells (A.T.C.C. catalogue no. CRL 1570) were grown in Dulbecco's modified Eagles medium (DMEM) with 10% fetal-calf serum. For labelling, cells grown at 80% confluence in 90 mm-diameter plastic Petri dishes (Costar) were washed three times with DMEM without methionine and incubated for 3 h at 37 °C with 4 ml of DMEM without methionine in the presence of 100 μCi/ml of [^{35}S]methionine/cysteine (Translabel; ICN). After the labelling period cells were placed on ice, washed three times with cold TBS [50 mM Tris/HCl (pH 7.4)/150 mM NaCl] and solubilized in TBS plus 0.5% Nonidet P40 and 0.1 mM phenylmethanesulphonyl fluoride (PMSF) (1 ml/90 mm dish); after incubation for 10 min at 4 °C, a cell-free extract was obtained by centrifugation of the crude homogenate at 100000 *g* for 30 min. The *in vitro* proteolytic assay of the MCP contained, in a final volume of 20 μl: 50 mM Tris/HCl, pH 8.0, 1 mM DTT, 2 μl of the ^{35}S-labelled cell-free extract (200000 c.p.m.) and different additions, as indicated: 1 μg of purified rat liver MCP, 5 μg/ml cardiolipin or 0.01% SDS. The reaction was allowed to proceed at 37 °C for the times indicated and stopped by boiling after the addition of 5 μl of concentrated SDS sample buffer [16]. Proteins were resolved on 13%-SDS/polyacrylamide gels [16]; the gels were fixed and stained in 20% methanol/5% acetic acid/0.25% Coomassie Blue, destained in 10% ethanol/5% acetic acid, then dried and exposed to X-ray film for a maximum of 6 h at −70 °C. Quantification of the degradation of the labelled proteins was done by scanning densitometry of the autoradiograms with a SilverScan instrument connected to a Macintosh IICi computer, using the NIH image 1.42 software; peak area quantification was done in pixels after equalizing the acquired image. The quantitation of the degradation of the 22 kDa protein is expressed as a percentage of total arbitrary densitometric units and is obtained as the quotient of the values obtained for this protein band divided by the total densitometric units obtained per lane in the 20.1–29 kDa molecular-mass range.

RESULTS

Cardiolipin is a potent activator of LLVY and LLE hydrolysis by the MCP

The possible effect of different phospholipids and lysophospholipids on the MCP activity was initially assayed at a fixed concentration (50 μM) of the peptide substrates (ARR, LLE and LLVY) and at both 1 and 20 μg/ml concentrations of the different lipids. Phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine and their corresponding lyso derivatives were without effect on the hydrolysis of LLVY and LLE by the MCP. Phosphatidylserine at 20 μg/ml, but not lysophosphatidylserine, activated LLVY hydrolysis 2-fold, but had no effect on LLE hydrolysis. Phosphatidylinositol, but not lysophosphatidylinositol, slightly inhibited (20% inhibition at 20 μg/ml) LLE hydrolysis, but did not affect LLVY hydrolysis. The trypsin-like activity of MCP assayed with ARR peptide was not affected by any of the above-mentioned phospholipids.

In contrast with this situation, cardiolipin was able to activate the hydrolysis by the enzyme of LLE and LLVY (assayed at 50 μM peptide concentration), as shown in Figure 1, but has no effect on the trypsin-like activity of the MCP assayed with ARR peptide. The maximal activation of LLVY hydrolysis by cardiolipin was about 50-fold, with an EC_{50} of 0.15 μg/ml. The activation of LLE hydrolysis was about 30-fold with an EC_{50} of 1.5 μg/ml. This activation by cardiolipin was completely reversible by simple dilution and did not affect the native molecular mass of the MCP complex, as judged by gel-filtration chromatography of the enzyme in the presence of cardiolipin (results not shown). The effect of cardiolipin, like that of SDS [15], is not due to an alteration of the peptide solubility or structure (it is unlikely that the peptides used have secondary structure), as cardiolipin in concentrations up to 50 μg/ml was without effect on the hydrolysis of LLVY by chymotrypsin, ARR by trypsin or LLE by staphylococcal V8 proteinase.

To establish that cardiolipin was the actual activator and not an unrelated substance contaminating the commercial lipid, cardiolipin was subjected to silica-gel t.l.c. and developed in chloroform/methanol/water (65:25:4, by vol.). A major

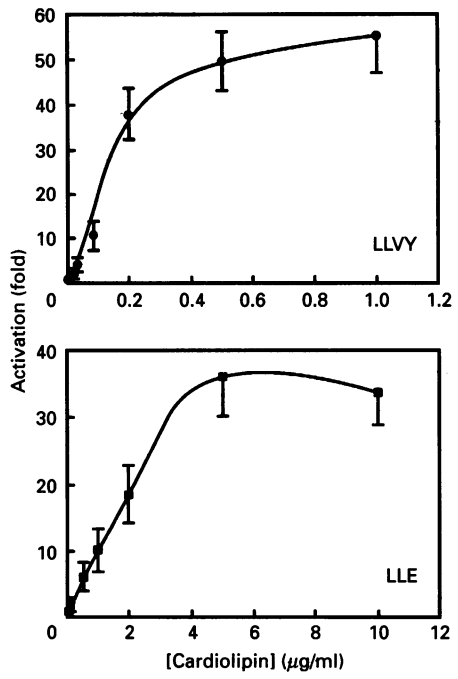

Figure 1 Effect of different amounts of cardiolipin on the hydrolysis of LLVY and LLE by rat liver MCP

The activity observed at a peptide concentration of 50 μM in the absence of cardiolipin, corresponding to 0.15 ± 0.02 nmol/min per μg for LLVY and 0.065 ± 0.007 nmol/min per μg for LLE hydrolysis, was taken as 1. The values presented (mean ± SD from three different experiments) are (activity observed at the different cardiolipin concentrations)/(activity obtained in its absence). The half-maximal dose for activation, K_a, was obtained from double-reciprocal plots of the results presented.

phospholipid stained with iodine was visible with an R_F of 0.7, identical with the one reported for cardiolipin in this system [17], both the lipid present in that spot and in the front of the chromatogram (where free fatty acids migrate) were extracted and assayed for possible activation of the MCP activity. The lipid extracted from the spot migrating with the R_F of cardiolipin was the only one able to activate the MCP hydrolysis of LLVY and LLE peptides (results not shown). These results show that cardiolipin is the actual activator of the MCP; furthermore, both phosphatidyl moieties of cardiolipin seem to be required for the activation, as phosphatidylglycerol, a precursor of cardiolipin, at concentrations up to 100 μg/ml, was without effect on the hydrolysis of LLE and LLVY by the enzyme.

Kinetic mechanism of the activation of the MCP by cardiolipin

The above results prompted us to study the effect of cardiolipin on the saturation curve of the MCP for LLVY and LLE peptides. Results of the kinetic parameters obtained for both peptides in the absence or in the presence of cardiolipin are presented in Table 1. Cardiolipin activation of LLVY hydrolysis is due to an increase in the $V_{max.}$ of the enzyme (30-fold) and a decrease in the apparent K_m (from 130 μM in the control to 40 μM in the presence of cardiolipin). The kinetics of LLE hydrolysis in the absence of cardiolipin are complex [15,18,19]; with the purified enzyme obtained by ourselves we observed two components, one co-operative (see also Table 1) with lower $s_{0.5}$ [60 μM; h (Hill coefficient) = 1.6] and $V_{max.}$ (0.12 ± 0.02 nmol/min per μg) and a second non-co-operative component with a higher K_m (320 μM) and $V_{max.}$ (0.32 ± 0.03 nmol/min per μg). In the presence of cardiolipin the saturation curve of LLE hydrolysis becomes hyperbolic (h = 1) with an apparent K_m of 60 μM and a 15-fold increase in the $V_{max.}$ of the enzyme.

Effect of cardiolipin and SDS on the activity of the MCP

SDS at low concentrations (0.01 %) is a strong activator of MCP hydrolysis of LLVY and LLE. SDS activation of LLVY hydrolysis is due to a 10-fold increase in the $V_{max.}$ and slight decrease in the K_m of the proteinase for this peptide; the activation of LLE hydrolysis by SDS (see also Table 1) is kinetically explained by the disappearance of co-operativity for this substrate, which decreases the apparent K_m with a minor effect on the $V_{max.}$ of the enzyme [15]. From these results and those presented above it can be concluded that SDS and cardiolipin activation of the hydrolysis of LLVY share a similar kinetic mechanism (increasing the $V_{max.}$ and decreasing the K_m), and the activation of LLE hydrolysis is due, in the case of both compounds, to the suppression of the co-operativity of the MCP for LLE; however, they differ in that cardiolipin strongly increases the apparent $V_{max.}$ of the enzyme.

As both activators show some analogy in their kinetic mechanism of action, it was decided to study the effect of SDS and cardiolipin, when added together, on the activity of MCP; results are presented in Table 2. When the hydrolysis of LLVY and LLE was studied at a fixed peptide concentration (50 μM), the presence of optimal concentrations of cardiolipin (5 μg/ml) and SDS (0.01 %) gave a total activation that equals the sum of the

Table 1 Summary of the kinetic parameters of the MCP in the absence and in the presence of cardiolipin

Kinetic parameters (mean ± SD) are derived from the corresponding double-reciprocal plots by linear least-squares fitting from three different experiments. [a] $V_{max.}$ and K_m values of the second non-co-operative component of the saturation curve for LLE peptide. [b] $V_{max.}$, $s_{0.5}$ and h of the first component of the saturation curve for LLE peptide. The data with no additions (repeated for the present work, confirming our previously reported parameters) and in the presence of 0.01 % SDS are taken from our own published data [15].

	LLVY		LLE			
Addition	K_m (μM)	$V_{max.}$ (nmol/min per μg)	K_m (μM)	$s_{0.5}$	h	$V_{max.}$ (nmol/min per μg)
None	130 ± 20	0.56 ± 0.06	320 ± 20[a]	60 ± 5[b]	1.6[b]	0.32 ± 0.03[a]; 0.12 ± 0.02[b]
Cardiolipin (5 μg/ml)	40 ± 3	16.8 ± 0.6	60 ± 4		1	4.5 ± 0.2
SDS (0.01 %)	90 ± 8	6.2 ± 0.4	30 ± 4		1	0.50 ± 0.04

Table 2 Additive effect of cardiolipin and SDS on the hydrolysis of LLVY and LLE by rat liver MCP

Enzyme activity was determined at a concentration of 50 μM for each peptide; the activity in the absence of both activators, corresponding to 0.15 ± 0.02 nmol/min per μg for LLVY and 0.065 ± 0.007 nmol/min per μg for LLE hydrolysis, was taken as 1. The values presented (mean ± SD from three different experiments) are the quotient of (activity observed in the presence of the different additions)/(activity obtained with no additions). Abbreviation: n.d., not determined

Additions (final concns.)			Activation (fold)	
Cardiolipin (μg/ml)	SDS (%)	Substrate...	LLVY	LLE
—	—		1	1
0.1	—		n.d.	28 ± 2
1.0	—		10 ± 2	n.d.
5.0	—		30 ± 3	60 ± 5
—	0.001		2.0 ± 0.2	5.0 ± 0.5
—	0.01		10 ± 2	15 ± 1
1.0	0.001		11 ± 1	n.d.
1.0	0.01		21 ± 2	n.d.
0.2	0.001		n.d.	34 ± 3
0.1	0.01		n.d.	42 ± 3
5.0	—		34 ± 3	64 ± 5
5.0	—		41 ± 3	78 ± 6

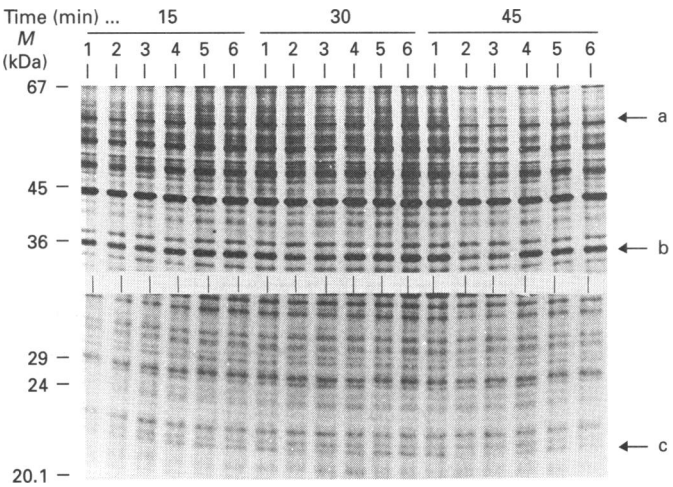

Figure 2 Cardiolipin activates the proteolytic activity of the MCP

The *in vitro* proteolytic activity of the purified latent MCP was assayed as described in the Materials and methods section. The reaction mixtures contained [^{35}S]methionine-labelled cell-free extract incubated for the different periods indicated on the upper part of the Figure, with the following additions: Lanes 1, MCP (1 μg); lanes 2, MCP (1 μg) + cardiolipin (5 μg/ml); lanes 3, MCP (1 μg) + SDS (0.01 %); lanes 4, no additions (controls); lanes 5, cardiolipin (5 μg/ml) and lanes 6, SDS (0.01 %). The Figure shows the corresponding autoradiogram of the SDS/13 % polyacrylamide gel used to analyse the protein degradation under the different reaction conditions, in which proteins labelled a (57 kDa), b (38 kDa) and c (22 kDa) are only degraded in reactions where the MCP with either cardiolipin (lanes 2) or SDS (lanes 3) was added. Positions of the molecular-mass (*M*) markers are shown.

respective activations observed with each compound alone. Similar additive results were obtained (Table 2) when either suboptimal concentrations of cardiolipin (1 μg/ml for LLE hydrolysis and 0.1 μg/ml for LLVY) were added together to the assay mixture with optimal (0.01 %) concentrations of SDS or suboptimal concentrations of SDS (0.001 %) are added in the presence of optimal concentrations of cardiolipin (5 μg/ml). As a consequence, the activation of the hydrolysis of LLVY and LLE by SDS and cardiolipin is additive. These results clearly suggest that the binding sites through which cardiolipin and SDS activate MCP are independent and do not seem to compete or show synergism.

The activation effect of cardiolipin can be completely blocked by fluophenazine

The effect of phospholipids on several enzyme activities can be counteracted by the presence of psychotropic drugs [20,21], indicating that disruption of lipid structure prevents its action. The effect of different amounts of the tranquilizer fluophenazine on the activation of MCP by cardiolipin (at a concentration of 5 μg/ml), both on the LLVY and LLE hydrolysis, shows that fluophenazine was able to inhibit completely the activation by cardiolipin, with half-maximal inhibition being obtained at a concentration of 2 μM. Fluophenazine, even at 100 μM, has no effect in the absence of cardiolipin or in the activation of LLE and LLVY hydrolysis by SDS (0.01 %).

Effect of cardiolipin on the proteolytic activity of MCP

The protocol followed for the purification of the MCP from rat liver used here (always in the presence of 10 % glycerol [15]) leads one to obtain the so-called 'latent' form of the enzyme. This form is known to exhibit poor proteolytic activity towards native natural proteins like casein and is activated by low concentrations of SDS [9,22–24]. We have tested the effect of cardiolipin on the degradation of casein; cardiolipin effectively activates casein degradation by the latent enzyme, but it requires higher concentrations of cardiolipin (100 μg/ml) than those needed for the activation of the peptidase activities of the MCP (see above). As a consequence, we conducted experiments like the one shown in Figure 2, in which a crude extract of total cellular proteins labelled with [^{35}S]methionine was incubated in the presence or absence of the MCP, cardiolipin and SDS, added independently or in combinations, as described in the legend to Figure 2. Under the conditions used to prepare the labelled cell extract (see the Materials and methods section) no significant endogenous protein degradation was observed when the extract was incubated alone for the time periods indicated (Figure 2, lanes 4). The addition to the incubation mixture of either 5 μg/ml cardiolipin (Figure 2, lanes 5), 0.01 % SDS (Figure 2, lanes 6) or 1 μg of the MCP (Figure 2, lanes 1) also did not produce any significant degradation. Only when 1 μg of the MCP was added together with either 5 μg/ml cardiolipin (Figure 2, lanes 2) or 0.01 % SDS (Figure 2, lanes 3), was degradation of some proteins, with molecular masses of 57 (labelled a), 38 (labelled b) and 22 kDa (labelled c), observed during the incubation period. Most proteins still remained undegraded during the course of the incubation, which is consistent with the fact that no significant change occurred in the total trichloroacetic acid-precipitatable ^{35}S-labelled proteins during the incubation time under the different reaction conditions, less than 1 % of the total input of radioactivity being liberated as acid-soluble material. The densitometric quantification of the amount of the 22 kDa protein during the time of incubation under the different experimental conditions shows that is degraded in a time-dependent manner when the MCP is added together with 5 μg/ml cardiolipin (lanes 2) or

0.01 % SDS (lanes 3), reaching 70–90 % degradation, with respect to the controls, after 45 min of incubation. This activating effect of cardiolipin (but not that of SDS) can be suppressed by the presence of 20 μM fluophenazine (data not shown).

DISCUSSION

The results clearly show that cardiolipin behaves like an allosteric positive modulator of the chymotrypsin and peptidylglutamyl-peptide-hydrolysing activities of the MCP, acting through a binding site that is different from the SDS site. This conclusion is based mainly on three observations: (1) cardiolipin has no structural similarity to the substrates of the enzyme, and its activation, like that of SDS, is reversible, indicating that the effect is due to a conformational change of the enzyme in the presence of cardiolipin; (2) cardiolipin and SDS activation are additive, with different kinetic mechanisms of activation; and (3) activation by cardiolipin, but not by SDS, is suppressed by fluophenazine. We have also found that this activation by cardiolipin of MCP activity is observed with the enzyme purified from *Xenopus laevis* ovaries and from human erythrocytes [25], indicating that cardiolipin activation is a property of the MCP from different eukaryotic sources.

The MCP was reported initially as a latent proteinase whose activity on casein as substrate was stimulated by the presence of low concentrations of SDS and fatty acids [7,9,22–24]. This form of the enzyme is the one used in the present studies and is obtained by purification of the enzyme in the presence of buffers containing glycerol [15]. To assay its proteolytic activity we have used, as substrate, total [^{35}S]methionine/cysteine-labelled cell proteins, showing that cardiolipin activates the degradation of some cellular proteins by the latent form of MCP at a concentration similar to the one used for activation of the peptidase activities of the enzyme, higher concentrations of cardiolipin (100 μg/ml) being required for the activation of casein degradation by the enzyme. Part of the activation of degradation by cardiolipin could be due to binding of cardiolipin to the proteins that are degraded in the presence of the MCP, making them more susceptible to degradation. This possibility is difficult to completely exclude, but it seems to us unlikely, because the same proteins are degraded by the so-called 'activated' form of the MCP (obtained by purification of the rat liver MCP in buffers containing no glycerol), in a way independent of the presence of cardiolipin or SDS. In this *in vitro* proteolytic assay, two main precautions are necessary: (1) the [^{35}S]methionine-labelled cell-free extract has to be freshly prepared or, if stored in aliquots at -70 °C, 0.1 mM PMSF (this concentration of PMSF does not affect any of the peptidase activities or the proteolytic activity of the enzyme) has to be added to the defrosted extract at 4 °C to prevent non-specific degradation; (2) the latent form of the MCP has to be either freshly obtained or stored at -70 °C and defrosted at 4 °C only once, prolonged storage at -70 °C or repeated freezing and thawing led to a preparation of MCP whose proteolytic activity is not longer modulated either by cardiolipin or by SDS.

The reported activation by cardiolipin opens the question of the possible role of cardiolipin as a possible allosteric regulator of the activity of the MCP *in vivo*. Cardiolipin biosynthesis is entirely confined to the mitochondria, these subcellular organelles being the location within the cell where the lipid is most abundant [26]. The subcellular location (see [27] for a recent review) of the MCP is not fully determined, the enzyme having been described as being located both in the nucleus [28–30] and in the cytoplasm [9,30,31]; this might be due to there being different MCP subpopulations within the cell. Preliminary experiments of subcellular fractions prepared from rat liver and immunoblotting with specific antibodies against the MCP show that the MCP is mainly present in the soluble and microsomal fraction of rat liver (J. Arribas, G. Egea and J. G. Castaño, unpublished work), but it is not present in association with the mitochondrial fraction, in agreement with the reported ultrastructural localization [30]. As discussed with the reported activation of the MCP by fatty acids [7], further work will be required to clarify the possible role of cardiolipin in the physiological regulation of MCP *in vivo*.

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