Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 486-494

EVIDENCE FOR INTRINSIC PROTEOLYTIC ACTIVITY IN RAT LIVER PLASMA MEMBRANES

Lucienne Guenet, Geneviève Leray, Jean-Pierre Codet, André Le Treut and Jean-Yves Le Gall

Laboratoire de Biochimie Médicale B and INSERM Unité de Recherche U 49 Hôpital Pontchaillou 35011 Rennes Cédex (France).

Received June 18, 1982

Electrophoresis on 10 % acrylamide-SDS gels showed that incubation of rat liver plasma membranes with certain detergents induces a proteolysis. The results obtained using different detergents, temperatures and pH levels, as well as the action of some protease inhibitors are in favor of an enzymatically-induced proteolysis. Since the proteolytic activity remains unchanged, even after the peripheral proteins have been released, it is proposed that this activity may reflect one of the integral proteins functions.

INTRODUCTION

Plasma membrane bound proteolytic activities have been recently reported in a variety of cell types : intestinal (1, 2) and renal (3) brush border cells, adipocytes (4), anterior pituitary cells (5), brain cells (6), leucocytes (7), erythrocytes (8-10), spermatozoa (11) and lymphocytes (12). The roles of these membrane proteases have only been partially elucidated. Among them are regulation of the AMP cyclic dependent protein kinase activity (13), generation of active neuropeptides from β endorphin (6) and participation in membrane fusion (14). These proteases may be involved in the liberation of a peptidic "second messager" which stimulates mitochondrial pyruvate deshydrogenase after binding of insulin to adipocyte membrane receptors (4, 15-17) ; such a peptidic "second messager" activates non-histone nuclear protein phosphorylation and induces the proliferation and differentiation of B cells, in response to the binding of anti-immunoglobulins to the surface of B lymphocytes (12).

In this paper we show that liver plasma membranes also possess proteases whose activity can be induced by certain detergents.

0006-291X/82/180486-09$01.00/0

MATERIAL AND METHODS

Preparation of plasma membranes : Plasma membranes were prepared from the livers of female Wistar rats (100 to 120 g body weight), fed "ad libidum". Neville's procedure (18) was used up to step 11. The purified membrane preparations were washed twice in 1 mM $NaHCO_3$, resuspended in the same buffer and stored in liquid nitrogen.

Assays for marker enzymes : In order to assess the purification, the following enzyme activities were used as markers and measured in the whole homogenate and in the membrane preparation : Cytochrome C oxydase (19, 20), Acid-β-D-galactosidase (21), Glucose-6-phosphatase (20), Lactate deshydrogenase (22), Catalase (23) and 5'Nucleotidase (24).

Incubations : Plasma membranes (5 mg/ml, final concentration) were incubated at 20° C, with or without detergents, in 0,1 M sodium phosphate buffer, pH 7,5 (except when otherwise indicated) over a 20 H period. The following detergents were tested : Cetyltrimethylammonium bromide (CTAB), Sodium deoxycholate (DOC), Sodium dodecyl sulfate (SDS), N-lauroyl sarcosine (SARK), Triton X-100 (TRI). The concentrations employed were 5 mM to 96 mM depending on the detergent used. Aliquots were taken at different intervals, frozen and stored in liquid nitrogen.

Some incubations were performed at different temperatures (4° C, 20° C and 37° C) and others were carried out at 20° C after boiling the membranes for 5 min.

In other experiments, before incubation, the proteins of the plasma membranes were separated into peripheral proteins, those binding to the membrane by predominantly electrostatic interactions, and integral proteins, those binding to the membrane through extensive hydrophobic interactions, according to Marchmont's method (25).

Effect of various protease inhibitors : before incubation with detergent, membranes were preincubated with protease inhibitors at ambient temperature for 1 hour. The following inhibitors were tested : 1 mM phenylmethylsulfonyl fluoride (PMSF), 8 mM ethylenediaminetetracetic acid (EDTA), 5mM iodoacetamide, 100 µg/ml pepstatin, 1 mM L.1.tosylamide-2-phenylethyl chloromethyl ketone (TPCK), 200 µg/ml leupeptin, 1000 KIU/ml aprotinin and 1 mg/ml soybean trypsin inhibitor (STI) (final concentration).

SDS electrophoresis : The samples were mixed with the "sample buffer", which contained (final concentrations) 62.5 mM Tris HCl pH 6.8, 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol. Then the proteins were completely dissolved by boiling for 5 min. The running sample volume, which contained 100 µg of protein, was 50 µl. Electrophoresis was carried out on 10 % acrylamide-sodium dodecyl sulfate gels as described by Laemmli (25). The gels were stained with 0.05 % Coomassie blue in ethanol, acetic acid, water (8:1:7, v/v) and washed with methanol, acetic acid, glycerol, water (8:2:1:29, v/v). Gels were scanned using a Cellosystem scanner (Sebia).

RESULTS

Enzymatic control of the membrane preparation

The results of the marker enzymes activities are summarized in table 1.

Table 1 : Enzymatic control of the membrane preparation. Relative specific activity is the ratio of the specific activity of the membranes to that of the whole homogenate. Results are given as the mean with the standard deviation (the number of experiments are indicated in parenthesis).

	Yield in %	Relative Specific Activity
5' Nucleotidase (3)	25 ± 10	21 ± 4,2
Glucose 6 Phosphatase (2)	0,5 ± 0,2	0,4 ± 0,1
Acid β D Galactosidase (3)	0,1 ± 0,2	0,2 ± 0,2
Lacticodeshydrogenase (3)	1,2 ± 0,2	0,9 ± 0,3
Catalase (3)	0,4 ± 0,4	0,7 ± 0,1
Cytochrome C Oxydase (1)	0,9	0,6

Effects of various detergents

In the conditions described above, plasma membrane electrophoresis, yielded about 60 bands, of which seven were consistently prominent. We have classified them as follows : 1 (mw : 100 000), 2 (mw : 43 000), 3 (mw : 40 000), 4 (mw : 36 000), 5 (mw : 30 000), 6 and 7 (mw < 30 000). Membrane proteins incubated over a 20 H period, at 20° C, in 0.1 M sodium phosphate buffer pH 7.5, displayed a remarkable stability with regard to the number and the color of the bands.

Detergents added to the incubation medium modified the electrophoretic pattern after 4 H or 20 H of incubation. The most important characteristics of these modifications were the reduction, then the disappearance of bands 2 and 6, the disappearance of many of less prominent bands and the appearance of a few, new,low molecular weight bands. To obtain this proteolysis, at least 6 mM CTAB, at least 24 mM DOC or at least 12 mM SARK was required ; these modifications became more pronounced with the increase in concentration. On the other hand, neither 24 mM SDS nor 96 mM TRI had any effect, although protein solubilization was complete at these concentrations (fig. 1). Moreover, the addition of 24 mM SDS with 9 mM CTAB prevented the proteolysis induced by CTAB alone.

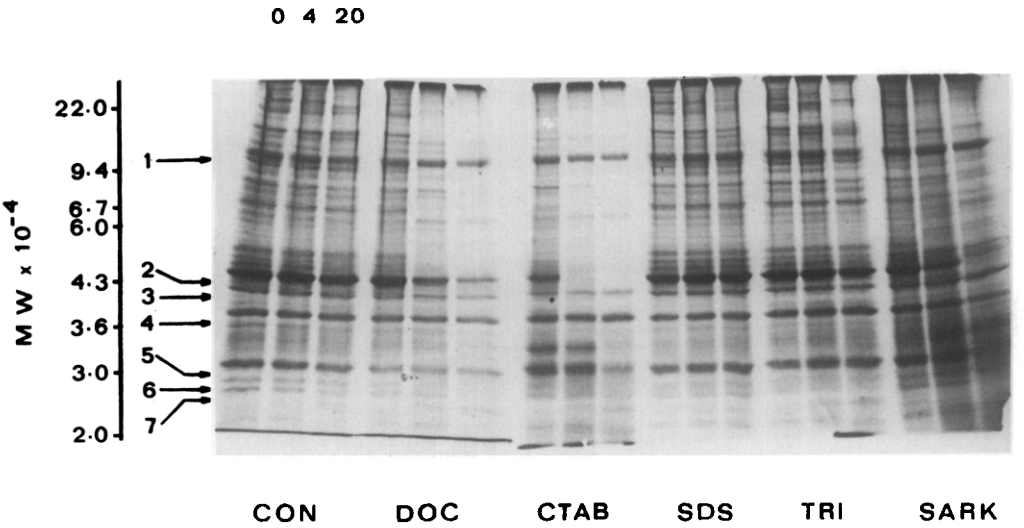

Figure 1 : Effect of detergents on liver plasma membranes. Membranes (5 mg/ml) were incubated in 0.1 M sodium phosphate buffer pH 7.5 for 0, 4, or 20 hours (control : CON). The following detergents were added : 24 mM sodium deoxycholate (DOC), 9 mM Cethyltrimethylammonium bromide (CTAB), 24 mM sodium dodecyl sulfate (SDS), 96 mM Triton X 100 (TRI) or 24 mM N-lauroyl sarcosine (SARK).

Replacement of 0.1 M sodium phosphate buffer pH 7.5 by 0.1 M TRIS HCl buffer at the same pH did not change the results.

Effect of pH

The influence of pH on the rate of proteolysis was studied in 0.1 M sodium citrate buffer (pH 2 to 4), in 0.1 M sodium acetate buffer (pH 5 and 6) and in 0.1 M sodium phosphate buffer (pH 7 to 9). The incubations were performed over a 4 H or 20 H period with 6 mM or 24 mM CTAB. Fig. 2 shows the pH dependance of the induced protease activity. The curve is biphasic with two maxima, one around pH 5 and the other one near pH 8 with the minima at pH 6.

Influence of temperature

Incubations with 6 mM or 9 mM CTAB, at 4° C, 20° C or 37° C showed (fig. 3) that the rate of proteolysis depended not only on the detergent concentration but also on incubation temperature.

Previous boiling of the membrane fraction prior to incubation abolished the proteolysis.

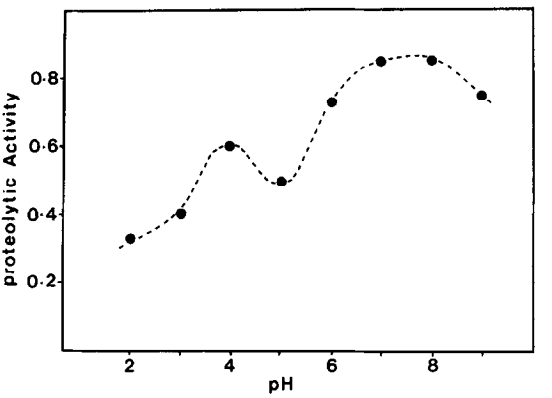

Figure 2 : Effect of pH on the rate of CTAB-induced proteolysis. Incubation medium composition was : from pH 2 to pH 4 : 0.1 M sodium citrate buffer, for pH 5 and pH 6 : 0.1 M sodium acetate buffer, and from pH 7 to pH 9 : 0.1 M sodium phosphate buffer. In each case, 9 mM CTAB was used. Proteolytic activity was given, after gel scanning, as the relative decrease of band 2 after a 4 hour incubation.

Separation of proteins into peripheral proteins and integral proteins

Marchmont's method, when used on our membrane preparation, gave a supernatant which contained the peripheral proteins and a pellet of integral proteins. Most of bands 3, 4 and 5 were recovered in the superna-

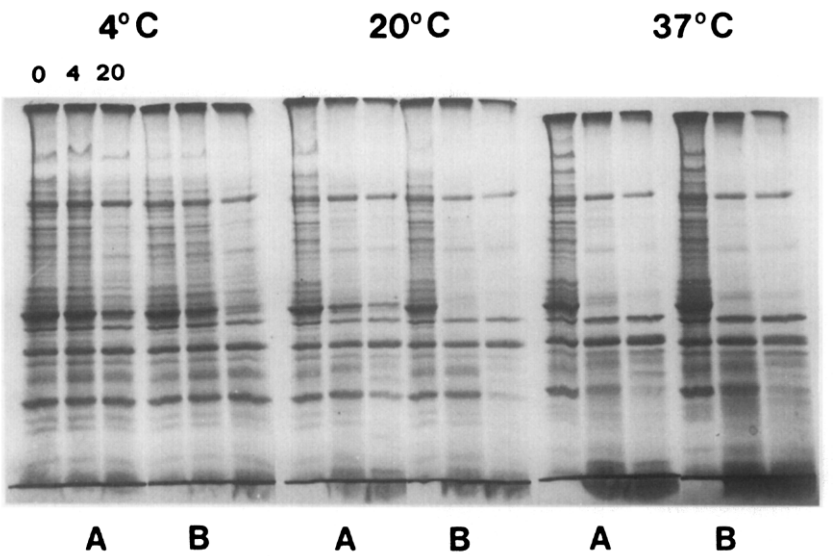

Figure 3 : Effect of temperature on the rate of CTAB induced proteolysis. Membranes (5 mg/ml) were incubated in 0.1 M sodium phosphate buffer pH 7.5 at 4°, 20° or 37° C with 6 mM CTAB (A) or 9 mM CTAB (B).

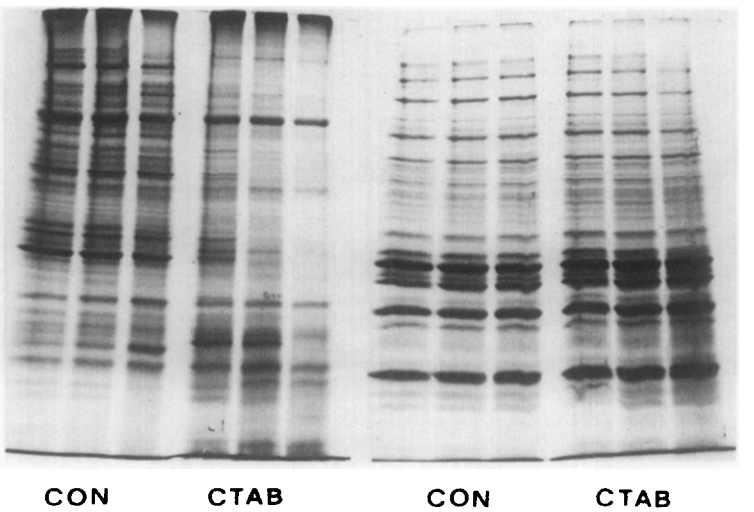

Figure 4 : Effect of CTAB on integral proteins and peripheral proteins obtained from liver plasma membranes according to the Marchmont's method. Incubations were performed in 0.1 M sodium phosphate buffer pH 7.5 without (CON) or with 9 mM CTAB, for 0, 4 or 20 hours.

tant ; bands 2, 6 and 7 were distributed in the same proportion between supernatant and pellet ; band 1 remained in totality in the pellet. CTAB (9 mM in 0.1 M sodium phosphate buffer pH 7.5) induced a marked proteolysis when added to the pellet but was without effect on the supernatant proteins (fig. 4).

Effect of various protease inhibitors

1 mM PMSF, 5 mM iodoacetamide, 8 mM EDTA, 100 µg/ml pepstatin, 1 mM TPCK, as well as the combination of PMSF, iodoacetamide and pepstatin, had little or no effect on 9 mM CTAB induced proteolysis. However, 200 µg/ml leupeptin or 1000 KIU/ml aprotinin completely inhibited the proteolysis ; 1 mg/ml STI yielded only a partial inhibition (fig. 5).

DISCUSSION

There are several arguments in favour of an enzymatic origin of the proteolysis observed on membrane proteins after solubilization by certain detergents, including the increased rate of proteolysis with tem-

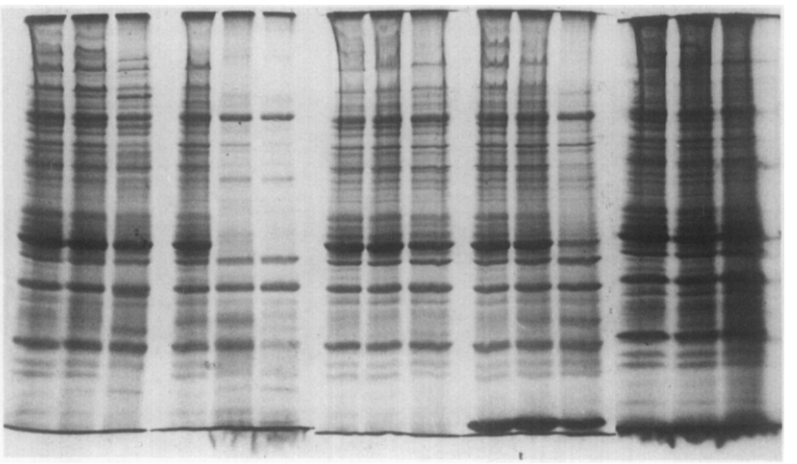

Figure 5 : Inhibition of CTAB induced proteolysis. Membranes (5 mg/ml) were preincubated in 0.1 M sodium phosphate buffer pH 7.5, for 1 hour, at room temperature, with 200 µg/ml leupeptin (LEU), 1 mg/ml soybean trypsin inhibitor (STI), or 1000 KIU/ml aprotinin (APRO). Then 9 mM CTAB was added and incubations were carried out at 20° C for 0, 4 or 20 hours.

perature, and the biphasic curve of proteolytic activity. The lack of action of some detergents (SDS, TRI) indicates that the induction of proteolysis is not the result of the effect of solubilization alone. Finally, the loss of proteolytic effects after thermodenaturation of the membrane proteins, and the action of protease inhibitors (leupeptin, aprotinin, STI) are fully consistent with enzyme involvment in this process.

The observed proteolytic actions might be the result of contamination by non-membrane enzymes, such as cytoplasmic proteases or lysosomal cathepsins. The existence of intramembranous proteases responsible for the observations is supported by the enzymatic markers used as controls during the fractionation (table 1). This point of view is buttressed by arguments from several other sources. Using Marchmont's technique we showed that CTAB could not induce proteolytic activity in the supernatant of peripheral proteins. In contrast, CTAB did induce proteolytic activity when exposed to pellet proteins. It seems that the intrinsic membrane proteins may be responsible for the observed proteolytic activity.

A detergent's action results in part from the solubilization of membrane components, thus permitting enzyme and substrate interaction (2). But our results show that solubilization is not the only effect of the detergents studied. Depending on the chemical properties of the detergent, there was activation, non-activation, or inhibition of the enzyme. The absence of proteolysis after the stimultaneous addition of CTAB and SDS is consistent with an inhibitory effect. Furthermore, it cannot be excluded that detergent-substrate interaction might play a role in the inhibition or activation of proteolysis.

Our results demonstrate the existence of an intrinsic proteolytic activity in liver plasma membranes. The nature and the characteristics of these proteases remain unclear as do their mechanism of action, the regulation of their activity and their biological functions.

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