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ALKALINE PHOSPHODIESTERASE-ACTIVE ANTIGENS IN PLASMA MEMBRANES OF RAT LIVER

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Summary

Enzyme activity of immunoprecipitated plasma membrane antigens was quantitatively investigated employing various phosphodiesterase substrates. The highest activity was found with thymidine 5'-monophospho-*p*-nitrophenyl ester at pH 9.5—10.0 thus characterizing the enzyme(s) as alkaline phosphodiesterase I (EC 3.1.4.1). Furthermore, inhibition studies of this activity with different nucleotide pyrophosphatase substrates demonstrated a dual specificity against phosphodiester and nucleotide pyrophosphate bonds.

Zymogram staining for alkaline phosphodiesterase activity of plasma membrane antigens separated in crossed immunoelectrophoresis revealed three different antigens posessing this activity. By inclusion of ATP, ADP or AMP into the staining medium, the alkaline phosphodiesterase activity of one of the antigens was completely inhibited while the staining of the two others was unaffected.

All three phosphodiesterase-active antigens were found to be identical with some plasma membrane antigens previously characterized as multienzyme complexes. Thus, all of these antigens also exhibited nucleoside di- and triphosphatase activity; two of them also showed an NADH-oxidizing function, and one was in addition arylamidase-active.

Affinity chromatography of plasma membrane extracts on columns of concanavalin A-Sepharose as well as analysis in fused rocket immunoelectrophoresis using concanavalin A-Sepharose containing intermediate gels revealed that all three alkaline phosphodiesterase-active antigens bound to the lectin, thus demonstrating the presence of carbohydrate in these antigens.

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Introduction

We have earlier reported on the polymorphism of ATPase-active antigens in plasma membranes from rat liver [1,2]. All of these antigens, which optimally can be resolved to ten, also exhibit an NADH-oxidizing function (NADH-neotetrazolium reductase) and two of them are acrylamidase-active as well [2]. Thus, several of these antigens, which we tentatively have considered as multienzyme complexes, contain similar enzyme activities, but they are individually distinct as judged by immunological criteria. Further characterization of these complex antigens revealed that in addition all of them contain phospholipid [2] and most of them also a carbohydrate moiety binding to the lectin concanavalin A [3]. Moreover, they possess the capacity specifically to bind epinephrine, although the physiological significance of that binding still is unclear [4].

The aim of the present work was to characterize further the enzyme active antigens in liver plasma membranes, by employing an additional zymogram staining for alkaline phosphodiesterase activity. Moreover, as this enzyme is known to be an intrinsic constituent of hepatocyte plasma membranes [5—12], it was of interest to see if the enzyme activity could be recovered in membrane extracts and to determine its antigenic nature in comparison with other rat liver plasma membrane components [1—4].

We have used an established histochemical staining method for phosphodiesterase activity [13] for zymogram staining. Concomitantly with such staining after crossed immunoelectrophoresis, we have assayed and characterized the phosphodiesterase activity of the total immunoprecipitate pattern also in test-tube assays, thus providing both qualitative and quantitative data of the activity present.

Materials and Methods

Plasma membrane extracts. Plasma membranes were isolated from starved, female Sprague-Dawley rats according to the method of Emmelot et al. [5]. After isolation the membranes were washed twice in 0.9% (w/v) NaCl and then resuspended in 0.9% NaCl containing 1% (w/v) sodium deoxycholate and 0.5% (w/v) Lubrol W (cetylpolyxyethylene condensate, I.C.I., Manchester, U.K.) for solubilization. This mixture was incubated in an ice-bath for 1 h and the extract was obtained by centrifugation at 105 000 × g for 1 h. Protein concentrations were determined according to the method of Lowry et al. [14], using bovine serum albumin as standard. The extracts were adjusted to 10 mg protein/ml.

Antisera against intact plasma membranes were prepared as previously described [2,15].

Rocket immunoelectrophoresis was performed as described by Laurell [16] using 1% (w/v) agarose gel (Behringwerke AG, Marburg Lahn, F.R.G.) and 0.05 M barbital buffer (pH 8.6). 40 μl of the membrane extract was applied in each antigen well. Electrophoresis was carried out at 4 V/cm for 22 h and the antigens precipitated with 27% (v/v) antiserum present in the gel during the run.

Fused rocket immunoelectrophoresis was performed with an intermediate gel containing either concanavalin A-Sepharose 4B or Sepharose 4B alone (Pharmacia Fine Chemicals, Uppsala, Sweden, 0.2 ml packed gel per ml of 1% (w/v) agarose gel) as described previously [3].

Crossed immunoelectrophoresis (two-dimensional immunoelectrophoresis) [17] was carried out under the conditions described for rocket immunoelectrophoresis, except that electrophoresis in the first dimension was run for 5 h and the antiserum concentration during the second run was 10% (v/v).

Quantitative enzyme assays. Pieces of agarose gel containing the immunoprecipitates obtained in rocket immunoelectrophoresis were cut out and dried on a plastic support, from which they were then easily collected as a thin film.

For determination of alkaline phosphodiesterase activity the dried pieces, containing the membrane antigens, were incubated in prewarmed media at 37°C for 15 min. The assay was performed according to the method of Ostrowski and Tsugita [18] using either 2.5 mM thymidine 5′-monophospho-*p*-nitrophenyl ester, thymidine 3′-monophospho-*p*-nitrophenyl ester or 10 mM bis-*p*-nitrophenyl phosphate as substrate. The incubation medium (0.4 ml) also contained 50 mM Tris buffer (pH 8.9) 10 mM $MgCl_2$ and 5 mM $CaCl_2$. The reaction was terminated by addition of 0.6 ml 0.5 M NaOH and the samples read at 400 nm.

Enzyme stainings. Plasma membrane immunoprecipitates in dried plates after crossed immunoelectrophoresis were stained for the following enzyme activities: nucleoside triphosphatase with ATP as substrate [1,19], NADH-neotetrazolium reductase [20], arylamidase using L-leucine 2-naphthylamide-HCl as substrate [21]. The incubation medium for alkaline phosphodiesterase activity consisted of 5 mg thymidine 5′-monophospho-1-naphthyl ester [13] or the 2-naphthyl phenylphosphonate ammonium salt (Regis Chem. Co., Morton Grove, IL, U.S.A.) [22] and 10 mg of diazonium salt Fast Blue B in 10 ml 50 mM Tris buffer (pH 8.9) containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$. The staining was developed in room temperature within one minute giving a distinct red colouring of the enzyme-active precipitates. Some weak nonspecific staining according to the criteria of Brogren and Bog-Hansen [23] was also encountered. The latter type of staining appeared more brown than the specific one and was developed more slowly. The nonspecific staining was probably mostly due to adsorption of precipitated diazonium salt to the immunoprecipitates.

Substrates and inhibitors were purchased from Sigma Chem. Co., St. Louis, MO, U.S.A. if not otherwise indicated.

Results

In order to characterize the phosphodiesterase activity of rat liver plasma membrane antigens, immunoprecipitates, obtained in rocket immunoelectrophoresis (see Materials and Methods) between detergent extracts of plasma membranes and anti-plasma membrane antiserum, were tested for their hydrolytic activity on different substrates. A marked activity was found with thymidine 5′-monophospho-*p*-nitrophenyl ester while almost no activity was detected using thymidine 3′-monophospho-*p*-nitrophenyl ester as substrate (2% of the activity with the former substrate). bis-*p*-Nitrophenyl phosphate was

also hydrolysed by the plasma membrane antigens although less efficiently (35% of the activity with thymidine 5′-monophospho-*p*-nitrophenyl ester). Thus, thymidine 5′-monophospho-*p*-nitrophenyl ester was chosen as the substrate for the following quantitative determinations of the phosphodiesterase activity.

The marginal inhibitory effect of 10^{-2} M theophylline on the phosphodiesterase activity (11% inhibition) indicated that the activity tested for did not involve any significant 3′ : 5′-cyclic AMP phosphodiesterase (EC 3.1.4.17) activity [24] but was a phosphodiesterase of type I (EC 3.1.4.1) [25].

Tests of the enzyme activity at different pH values showed an optimum at an alkaline pH in the range 9.5—10.0 with only minor activity at pH 5 or 6 (about 20% of the optimal activity). In order to characterize further the specificity of this phosphodiesterase activity different nucleotide pyrophosphate substrates were used for competition in the assay with the thymidine 5′-monophospho-*p*-nitrophenyl ester (Table I). As can be seen 10^{-2} M NADH or ATP markedly inhibited the activity, while 10^{-2} M PP_i gave essentially no effect. AMP was as efficient an inhibitor as ATP. Moreover, for maximal expression of the enzyme activity divalent cations were needed as indicated by the inhibition of the activity by EDTA (10^{-2} M).

Having thus established the occurrence of phosphodiesterase activity in the plasma membrane immunoprecipitates we were interested to investigate a possible localization of such activity to some of the plasma membrane antigens previously characterized in crossed immunoelectrophoresis [2,26]. The staining of such immunoprecipitates for phosphodiesterase activity, using thymidine 5′-monophospho-1-naphthyl ester or the 2-naphthyl phenylphosphonate as substrate, revealed three active antigens (Fig. 1b). Upon prolonged incubation a few other precipitates also appeared but were stained much weaker. It was, however, not possible to elucidate whether this latter staining was due to nonspecific background staining only.

When plates run in parallel were also stained for ATPase (Fig. 1a), NADH-neotetrazolium reductase and arylamidase activity in different combinations (selected results presented in Fig. 1a) it was evident that phosphodiesterase

TABLE I

THE EFFECT OF VARIOUS AGENTS ON ALKALINE PHOSPHODIESTERASE ACTIVITY OF IMMUNOPRECIPITATED PLASMA MEMBRANE ANTIGENS

Thymidine 5′-monophospho-*p*-nitrophenyl ester was used as substrate and the activity was determined as μmol nitrophenol formed/mg protein (referring to the total amount of protein in the sample applied for electrophoresis) per h. 100% relative activity represents a mean specific activity of 2.3 μmol/mg protein per h. Numbers within brackets indicate the number of tests performed.

Additive	Concentration	Relative activity (%)
None	—	100
NADH	10^{-2} M	49 (4)
ATP	10^{-2} M	40 (5)
AMP	10^{-2} M	37 (4)
PP_i	10^{-2} M	96 (3)
EDTA	10^{-2} M	50 (2)

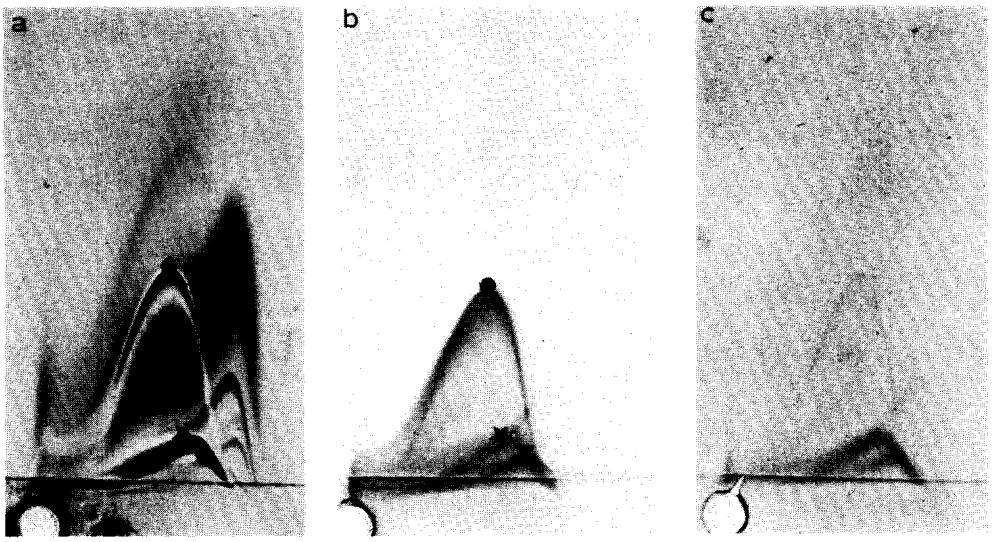

Fig. 1. Crossed immunoelectrophoresis of plasma membrane extract against anti-plasma membrane antiserum. The plates were stained for nucleoside di- and triphosphatase activity with ATP as substrate (a) and alkaline phosphodiesterase activity in the absence (b) or in the presence (c) of ATP in the staining medium. ▼, antigen staining for ATPase, NADH-neotetrazolium reductase, arylamidase and alkaline phosphodiesterase activity. ★, antigen exhibiting ATPase, NADH-neotetrazolium reductase and alkaline phosphodiesterase activity. ●, antigen staining for ATPase and alkaline phosphodiesterase activity. The weakly stained precipitates seen in (b) and (c) are due to background staining.

activity coincided with the other enzyme activities earlier seen in antigens referred to as multienzyme complexes [2,26]. One of these antigens (triangle, Fig. 1a, b) stained for all four enzyme activities mentioned above. Another antigen (dot, Fig. 1a, b) exhibited phosphodiesterase, ATPase and NADH-neotetrazolium reductase activity while the third antigen (star, Fig. 1a, b) showed only phosphodiesterase and ATPase activity. Interestingly, staining for phosphodiesterase activity after preincubation with and subsequently in the presence of ATP, ADP or AMP (10^{-2} M) in the staining mixture, showed that the staining of only one of the antigens was considerably inhibited, while the staining of the other two was unaffected (Fig. 1c). The precipitate with the inhibited activity appeared to be weakly brown as compared to the red colour of the ones not affected by the inhibitors. Therefore, the remaining staining of the inhibited precipitated was considered to be nonspecific.

When thymidine 5′-monophospho-*p*-nitrophenyl ester is used as substrate in zymogram staining for phosphodiesterase [10] a yellow product (*p*-nitrophenol) is formed at the site of enzyme reaction. This product is soluble, however, and thus diffuses during the course of the staining, causing a lower degree of resolution as compared to the procedure using the 1-naphthyl substrate. With thymidine 5′-monophospho-*p*-nitrophenyl ester one of the three phosphodiesterase active antigens stained particularly rapid (dot, Fig. 1b). Upon prolonged incubation the other two phosphodiesterase-active antigens were also stained. The same pattern of staining, but only after a considerably longer incubation time, was obtained using bis-*p*-nitrophenyl phosphate as substrate in the staining reaction. These results strongly indicate that the phos-

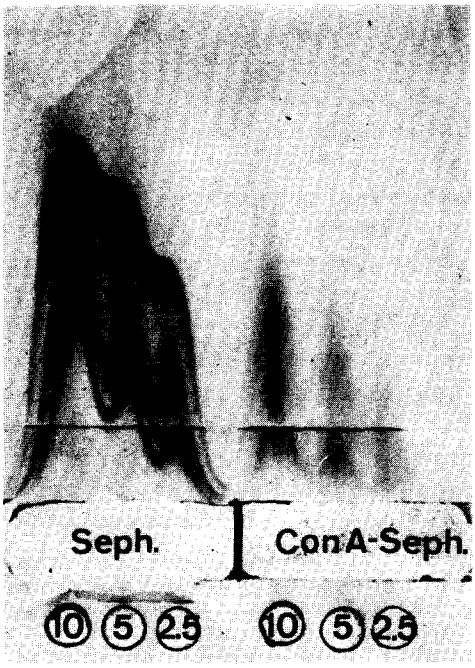

Fig. 2. Fused rocket immunoelectrophoresis with an intermediate gel containing concanavalin A-Sepharose (right) or Sepharose only (left). Plasma membrane extract, in three dilutions (10, 5 and 2.5 mg protein/ml, respectively), was reacted against anti-plasma membrane antiserum and the plate was stained for alkaline phosphodiesterase activity. All three of the active antigens were retained in the concanavalin A-containing gel as seen from the decreased heights of the precipitates as compared with the control. The control intermediate gel contained either Sepharose 4B only (results shown) or concanavalin A-Sepharose plus 0.3 M α-methyl-D-mannoside. Both types of controls gave comparable results.

phodiesterase activity seen in the test-tube assays and that detected by zymogram staining were due to the same enzyme entities.

We have shown earlier that the major part of the plasma membrane antigens in detergent extracts bind to concanavalin A, thus indicating that these antigens contain a carbohydrate moiety [3]. In the present studies when a plasma membrane extract was applied onto a concanavalin A-Sepharose 4B column all three phosphodiesterase active antigens bound to the column. The carbohydrate specificity of this binding was demonstrated as all three of these antigens were readily eluted with 0.3 M α-methyl-D-mannoside. Moreover, the carbohydrate content of the antigens was also shown by means of rocket immunoelectrophoresis empoying an intermediate gel containing concanavalin A-Sepharose 4B. In these experiments all three antigens were retained on the lectin in the intermediate gels (Fig. 2). When, instead, only Sepharose 4B or concanavalin A-Sepharose 4B plus 0.3 M α-methyl-D-mannoside were used in the intermediate gels the precipitate patterns were not affected.

Discussion

Alkaline phosphodiesterase I is a well established constituent of liver plasma membranes [5—12]. Enzymes of this type have been purified from such mem-

branes of mouse [10,11] and rat [9,12,27] and have been shown to be able to hydrolyse not only phosphodiester bonds, but also several pyrophosphate bonds. Accordingly, this enzyme has been referred to also as a nucleotide pyrophosphatase (EC 3.6.1.9) [9—12,27].

Our data on the inhibition of the phosphodiesterase activity of plasma membrane antigens with different nucleotide pyrophosphatase substrates strongly indicate that we are dealing with an overall enzyme activity similar to that of the enzyme described by Bischoff et al. [9,12]. Furthermore in our assays, despite the reaction with antibodies, these enzyme-active antigens basically retain their catalytic properties and their characteristics concerning pH optimum and ion requirement [9,12].

The nucleoside pyrophosphatase purified by Bischoff et al. [12] hydrolysed bis-*p*-nitrophenyl phosphate at a rate being 0.6% of that with thymidine 5′-monophospho-*p*-nitrophenyl ester, while it showed no reactivity towards thymidine 3′-monophospho-*p*-nitrophenyl ester. The relatively higher activity towards bis-*p*-nitrophenyl phosphate in our tests (35% of the activity with thymidine 5′-monophospho-*p*-nitrophenyl ester) might be explained by the presence of more than one phosphodiesterase among the plasma membrane antigens. The occurrence of several different alkaline phosphodiesterase in liver plasma membranes could also explain some of the conflicting data seen in the literature concerning ribonuclease activity and glycerophosphorylcholine hydrolysing activity in relationship to alkaline phosphodiesterase I [10,29—30]. In unpublished experiments we have shown that RNA at relatively high concentrations (10 mg/ml) inhibits the phosphodiesterase activity to about 40% of the optimal activity. We have, however, not yet elucidated if the RNA is hydrolysed in this reaction or if it, like AMP, only blocks the phosphodiesterase catalytic site [9,12,31].

Evans et al. [10] also employed crossed immunoelectrophoresis against anti-plasma membrane antiserum for analysis of a purified phosphodiesterase enzyme. They used thymidine 5′-monophospho-*p*-nitrophenyl ester as substrate for zymogram staining and obtained a very weak colour reaction in one immunoprecipitate. We chose, instead, the 1-naphthyl variant of the substrate [13] in combination with Fast Blue B salt as a coupling reagent in order to obtain an insoluble diazo dye at the site of enzyme reaction. By means of this zymogram technique we demonstrated the presence of three distinct antigens with alkaline phosphodiesterase activity in plasma membranes. Furthermore, the active antigens were shown to correspond to plasma membrane antigens earlier referred to as multienzyme complexes [2,26].

Interestingly, Bachorik and Dietrich [27] when solubilizing a nucleotide pyrophosphatase from a rat liver microsomal fraction with the non-ionic detergent Triton X-100, obtained the enzyme activity in large membrane fragments (mol. wt. approx. $1.5 \cdot 10^6$). By treatment with low concentration of sodium dodecyl sulfate, however, the membrane fragments were reduced in size and could be resolved into a number of protein components. The pyrophosphatase activity was then recovered in a component of an approximate molecular weight of $8 \cdot 10^5$. Although no assays on other enzyme activities were included in that study it is tempting to assume that the membrane fragments seen by these authors might correspond to the multienzyme complexes we have

detected in crossed immunoelectrophoresis.

In contrast, the nucleoside pyrophosphatases purified by Evans et al. [10] using the anionic detergent *N*-dodecyl sarcosinate and by Bischoff et al. [12] using Triton X-100 and enzyme digestions, both had a molecular weight of approx. 130 000. At this purified stage, however, no alkaline phosphatase or L-leucine 2-naphthylamidase activity was detected [10].

The three phosphodiesterase-active antigens described in this study employing crossed immunoelectrophoresis thus were distinguished from each other by their different associations with other enzyme activities. Furthermore, the phosphodiesterase activity of one of the antigens could be distinguished from the activity of the two others by its inhibited staining in the presence of ATP, ADP or AMP. This might indicate that several distinct peptides with this enzyme activity are present in the plasma membrane, but the particular peptides from each antigen would have to be isolated before such a conclusion can be safely drawn.

As judged from zymogram stainings in crossed immunoelectrophoresis the antigen inhibited by the various nucleoside phosphates mentioned above is able to hydrolyse ATP and ADP, but not AMP [1,2]. This property points to a correspondence of this particular antigen to the enzyme purified by Bischoff et al. [9,12]. These authors showed that their purified nucleotide pyrophosphatase hydrolysed ATP and ADP to give AMP and inorganic pyrophosphate or phosphate, respectively. The enzyme was inactive towards AMP, but was competitively inhibited by AMP and other nucleoside 5′-monophosphates [9,12].

Using the lectin concanavalin A we have been able to demonstrate that all the three phosphodiesterase-active antigens of rat liver plasma membranes contain a sugar moiety. However, we have not yet been able to determine if this carbohydrate is a constituent of the phosphodiesterase-active molecules themselves or of associated glycoproteins or glycolipids. By means of periodate-fuchsin staining of the purified nucleoside pyrophosphatase in polyacrylamide gels both Evans et al. [10,11] and Bischoff et al. [9,12] concluded that the enzyme was itself a glycoprotein.

In conclusion, we have demonstrated the presence of alkaline phosphodiesterase I activity in immunoprecipitates of detergent-solubilized liver plasma membranes. The activity appeared in three electrophoretically and immunologically distinct antigens, each of which also exhibited other enzyme activities. These results further strengthen the hypothesis that certain membrane enzymes are organized as individually distinct multienzyme complexes [2,26], although the functional reason for such organization remains obscure.

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