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$(Na^+ + K^+)$-ATPase

PHOSPHORYLATION-DEPENDENT CROSS-LINKING OF THE α-SUBUNITS IN THE PRESENCE OF Cu^{2+} AND *o*-PHENANTHROLINE

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In previous studies we had demonstrated that in the presence of 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline, cross-linking of the α-subunits of $(Na^+ + K^+)$-dependent adenosine triphosphatase was induced by the addition of Na^+ + ATP, and that the formation of the α,α-dimer was preceded by that of phosphoenzyme. The purpose of the present studies was the further evaluation of the role of phosphoenzyme in the process of cross-linking. Na^+ + UTP did not induce cross-linking unless Mg^{2+} was also added. In contrast, Na^+ + ATP-induced cross-linking did not require the addition of Mg^{2+}. The different effects of ATP and UTP in the absence of added Mg^{2+} could be accounted for by the presence in the enzyme preparation of bound Mg^{2+} which supported enzyme phosphorylation by ATP but not by UTP. When the enzyme was phosphorylated by P_i, in the presence of Mg^{2+} and ouabain, and then exposed to Cu^{2+} and *o*-phenanthroline, the α,α-dimer was obtained. Under these conditions, Na^+ blocked both phosphorylation and cross-linking. These results indicate that it is the formation of phosphoenzyme per se that leads to conformational transitions favorable to cross-linking. They also suggest that Cu^{2+} and *o*-phenanthroline participate in the cross-linking reaction, but not in the phosphorylation reactions. In the digitonin-treated enzyme, Na^+ and ATP induced the formation of phosphoenzyme, but not that of α,α-dimer. These findings indicate that in addition to phosphorylation, a proper orientation of α-subunits in an oligomer is also necessary for cross-linking.

Introduction

The active transports of Na^+ and K^+ across the plasma membranes of most eucaryotic cells are carried out by the $(Na^+ + K^+)$-dependent adenosinetriphosphatase $(Na^+ + K^+)$-ATPase, EC 3.6.1.3). That the ligand-induced conformational transitions of the enzyme are intimately related to its transport function is now well-established [1]; and, hence, the need for the detailed mapping of such transitions with various probes is apparent. In recent studies [2,3] we demonstrated the utility of chemical cross-linking experiments as novel probes of the conformational transitions of the enzyme. Among our findings were the observations [3] that under specified conditions the formation of a cross-linked dimer of the α-subunit of the enzyme in the presence of *o*-phenanthroline and Cu^{2+} seemed to be dependent on the phosphorylation of the enzyme by ATP. The continuation of these experiments presented here were done with two primary objectives: First, we wished to determine whether or not cross-linking occurs under conditions where the phosphoenzyme is obtained without the use of ATP. The results showing that cross-linking may also be induced by UTP and P_i indicate that it is the phosphoenzyme per se rather than the nature of the phosphorylating agent that is relevant to cross-

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linking. The second question was whether or not the formation of the phosphoenzyme is sufficient to induce cross-linking. Experiments with the digitonin-treated enzyme indicate that in addition of phosphorylation, the proper juxtaposition of the subunits are also required for the occurrence of cross-linking. In the course of these studies, some clarification of the roles of Cu^{2+} and *o*-phenanthroline in the process of phosphorylation-dependent cross-linking has also been achieved.

Methods

Highly purified enzyme from dog kidney outer medulla was prepared by the 'rapid' version of the procedure of Jorgensen [4]. The specific activities of the various preparations used in these studies were in the range of 800–1300 μmol P_i/mg per h.

All cross-linking experiments were performed with 0.25 mM $CuSO_4$, 1.25 mM *o*-phenanthroline, and 0.5 mg of enzyme protein/ml. Other details of the reaction conditions, procedures for SDS-polyacrylamide gel electrophoresis, and staining and scanning of the gels have been described before [3]. The formation and the measurement of the phosphoenzyme were also carried out by the previously described procedues [3]. The digitonin-treated enzyme was prepared according to Winter [5]. All experiments were carried out at 24°C.

[γ-^{32}P]ATP, [γ-^{32}P]UTP, and $^{32}P_i$ were purchased from New England Nuclear (Boston, MA). $^{32}P_i$ was purified according to Skou and Hilberg [6] immediately prior to use.

Results

Comparison of the effects of ATP and UTP on the formation of the cross-linked α,α-dimer

In our previous experiments [3] we showed that when the enzyme was incubated with 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline no cross-linking of the α-subunits occurred, and that the formation of a cross-linked α,α-dimer was induced when Na^+ and ATP, but not K^+ and ATP, were added along with Cu^{2+} and *o*-phenanthroline. We also showed that this induced cross-linking was preceded by the formation of the phosphoenzyme. Under the cross-linking conditions used, neither the formation of the phosphoenzyme nor that of the α,α-dimer required the addition of Mg^{2+}.

Because nucleoside triphosphates other than ATP are known to support Na^+-dependent phosphorylation of the enzyme, experiments were done to see if these nucleotides could replace ATP in the cross-linking reaction. CTP and ITP proved to be effective

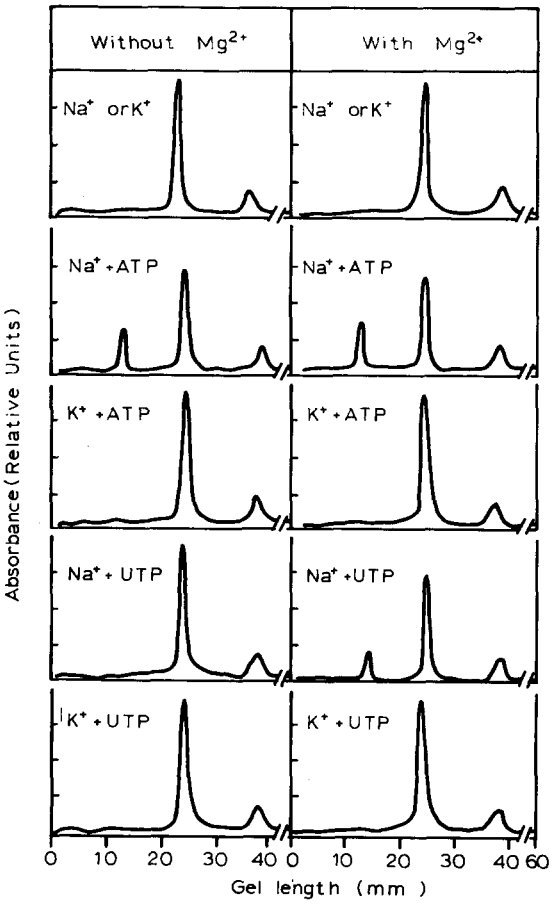

Fig. 1. Comparison between the effects of 1 mM ATP and 1 mM UTP, in the presence or absence of 2 mM Mg^{2+}, on the formation of the cross-linked α,α-dimer in the presence of Cu^{2+} and *o*-phenanthroline. Each crosslinking reaction was performed for a duration of 5 min. Na^+ and K^+ concentrations were 100 mM and 25 mM, respectively. Other reaction conditions and experimental procedures are indicated in Methods. Each panel is the photometric scan of the enzyme sample reacted under the indicated conditions, and then subjected to SDS-polyacrylamide gel electrophoresis. Migration was from left to right. The three major peaks (e.g. in the panel marked Na^+ + ATP), from left to right, are: α,α-dimer, α-monomer, and β-monomer.

[3]. UTP, however, did not induce cross-linking under the same conditions that ATP was effective (Fig. 1). This finding did not seem to support the notion that phosphorylation of the enzyme is responsible for the induction of cross-linking. Therefore, it prompted a more detailed examination of the UTP effect on cross-linking. Eventually, it was found that the addition of Mg^{2+} to the reaction mixture did, in fact, cause a Na^+ + UTP-induced formation of the cross-linked α,α-dimer. The comparison between the effects of ATP and UTP, in the presence or absence of Mg^+, on the formation of the cross-linked α,α-dimer is shown in Fig. 1.

The experiments whose results are summarized in the top portion of Table I show why UTP-induced cross-linking, but not ATP-induced cross-linking, requires the presence of Mg^{2+}. It is apparent that in the presence of Cu^{2+} and *o*-phenanthroline, when ATP is used Na^+-dependent phosphorylation occurs regardless of the presence or absence of Mg^{2+}; whereas with UTP Na^+-dependent phosphorylation does not occur unless Mg^{2+} is added. These data again indicate the necessity of enzyme phosphorylation as a step preliminary to the formation of the cross-linked α,α-dimer under the cross-linking conditions used in these experiments.

The above findings raised a new question: Without added Mg^{2+}, why did enzyme phosphorylation occur in the presence of ATP + Cu^{2+} + *o*-phenanthroline, but not in the presence of UTP + Cu^{2+} + *o*-phenanthroline? The experiments summarized in the bottom portion of Table I showed that Cu^{2+} and *o*-phenanthroline had nothing to do with the different behaviors of ATP and UTP in supporting phosphorylation. The data indicate that in the absence of Cu^{2+}, *o*-phenanthroline, and added Mg^{2+}, Na^+-dependent phos-

TABLE I

EFFECTS OF Mg^{2+}, Cu^{2+}, AND *o*-PHENANTHROLINE ON Na^+-DEPENDENT PHOSPHORYLATION OF THE ENZYME BY [γ-^{32}P]ATP AND [γ-^{32}P]UTP

For each reaction condition indicated below two sets of experiments were performed: One in the presence of 100 mM Na^+ and the other in the presence of 25 mM K^+. The difference between ^{32}P incorporation under these two conditions is expressed as the Na^+-dependent component. The indicated values are averages of three determinations. Reaction time was 15 s. The concentrations of other ligands used were: Mg^{2+}, 2 mM; Cu^{2+}, 0.25 mM; *o*-phenanthroline, 1.25 mM; ATP, 0.3 mM; UTP, 1 mM. Note that at the nucleotide concentrations used, maximal level of Na^+-dependent phosphorylation is expected to occur with ATP but not with UTP [9].

Reaction conditions		Na^+-dependent ^{32}P-incorporation (nmol/mg) in the presence of	
Cu^{2+} + *o*-phenanthroline	Mg^{2+}	ATP	UTP
+	–	3.4 ± 0.5	0.3 ± 0.2
+	+	3.6 ± 0.2	2.4 ± 0.5
–	–	2.7 ± 0.6	0
–	+	3.3 ± 0.3	2.1 ± 0.4

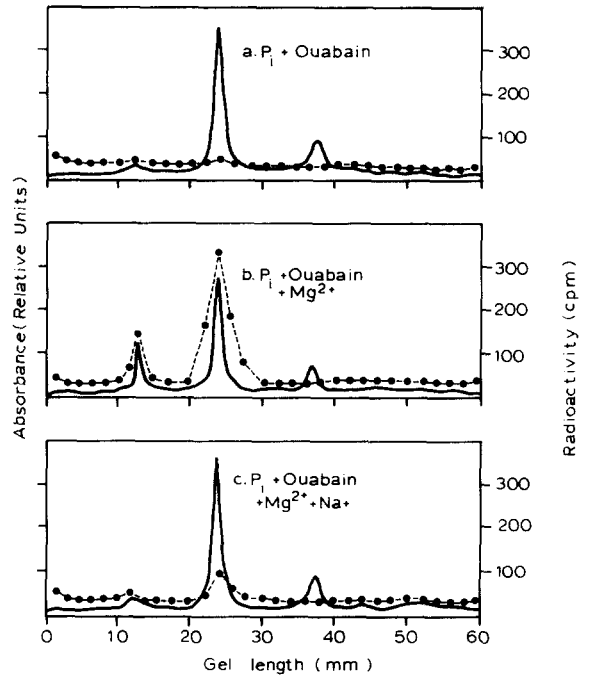

Fig. 2. P_i-induced phosphorylation and cross-linking of the α-subunits. The enzyme was preincubated with the indicated ligands, except P_i and the cross-linking reagent, for 5 min. $^{32}P_i$ (0.2 mM) was then added, and after 1 min Cu^{2+} and *o*-phenanthroline were introduced. After 5 min reactions were terminated, and samples were subjected to electrophoresis at pH 2.4 by the procedures indicated in Methods. The concentrations of ligands were: 2 mM Mg^{2+}, 100 mM Na^+, and 1 mM ouabain. Photometric scan is represented by the solid line, and radioactivity by the dashed line. The three major peaks, from left to right, are: α,α-dimer, α-monomer, and β-monomer.

phorylation of the enzymes occurs with ATP but not with UTP. In retrospect, the findings with ATP are not surprising. Previous work had indicated the existence of bound Mg^{2+} in several $(Na^+ + K^+)$-ATPase preparations [7,8]. Apparently, the preparation used here also contains such Mg^{2+}. What is not clear is why bound Mg^{2+} can support the reaction of ATP, but not that of UTP, with the enzyme. Perhaps because the apparent K_m of UTP is much larger than that of ATP [9,10], the small amount of bound Mg^{2+} permits the formation of sufficient MgATP, but not enough MgUTP, for interaction with the enzyme.

Effect of P_i on the formation of the cross-linked α,α-dimer

It is known that P_i, either in the presence or absence of ouabain, can phosphorylate the enzyme at the same site that phosphorylation from ATP occurs [11,12]. The data of Fig. 2 show that in the presence of Cu^{2+} and *o*-phenanthroline, P_i-induced phosphorylation and cross-linking are obtained. Several points concerning these experiments are worthy of note: (1) As in the experiments with UTP, here also the addition of Mg^{2+} is necessary for the occurrence of phosphorylation and cross-linking. (2) Preincubation of the enzyme with Na^+ inhibits the P_i-induced cross-linking, which is consistent with the known inhibitory effect of Na^+ on phosphorylation of the enzyme by P_i [11,12]. (3) The α,α-dimer obtained in the presence of $^{32}P_i$, like that obtained in the presence of [γ-^{32}P]ATP [3], contains the labeled phosphate.

Experiments with the digitonin-treated enzyme

Previous studies [5,13] had shown that when the digitonin-treated enzyme is exposed to Cu^{2+} and *o*-phenanthroline in the absence of the enzyme's physiological ligands, the cross-linked α,α-dimer is not obtained. Our findings that a phosphorylation-dependent formation of the cross-linked α,α-dimer may be induced in the native enzyme, raised the question as to whether such induced cross-linking may also be observed in the digitonin-treated enzyme. Although this preparation has little or no $(Na^+ + K^+)$-dependent ATPase activity, its ability to catalyze Na^+-dependent ADP-ATP exchange had already been demonstrated [5]. Hence, it was reasonable to expect that Na^+-dependent phosphorylation of the digitonin-treated enzyme by ATP may also occur. A portion of an enzyme preparation was treated with digitonin as indicated in Methods. ATP-induced phosphorylation of the treated and the untreated portions were attempted in experiments similar to those described in Table I. The level of Na^+-dependent ^{32}P-incorporation (average of three determinations) into the control sample was 2.8 ± 0.2 nmol/mg, and that into the digitonin-treated sample was 2.9 ± 0.4 nmol/mg. When the digitonin-treated enzyme was used in cross-linking experiments as described in Fig. 1, no Na^+ + ATP-induced formation of the cross-linked dimer could be detected.

Discussion

Our previous studies showing the $Na^+ + ATP$-stimulated formation of the cross-linked α,α-dimer upon exposure of the native enzyme to Cu^{2+} and *o*-phenanthroline, suggested that a conformational transition concomitant with phosphorylation of the α-subunits leads to the exposure of a set of sulfhydryl groups, and to the subsequent dimerization of the α-subunits through the oxidation of the exposed sulfhydryl groups [3]. It was not clear from these studies, however, whether the induced conformational change was because of phosphorylation per se, or due to the presence of ligands (i.e., ATP and Na^+) that induced both phosphorylation and cross-linking. The experiments presented here indicate that the relevant event is indeed the formation of the phosphoenzyme. It is apparent that as long as the phosphoenzyme is formed, regardless of whether it is obtained from ATP or UTP, or P_i, the cross-linked α,α-dimer is obtained. A special role of Na^+-binding to the enzyme as a requirement for cross-linking is also ruled out by the observations that Na^+ stimulates ATP-induced cross-linking but inhibits P_i-induced cross-linking. These effects of Na^+ again point to the primary role of the phosphoenzyme in the process of cross-linking.

The results of the experiments with UTP and P_i also lead to the clarification of the role of Cu^{2+} and *o*-phenanthroline in the process of phosphorylation-dependent cross-linking. Because in our previous studies [3] it was noted that phosphorylation-dependent cross-linking induced by $Na^+ + ATP$ did not require the addition of Mg^{2+}, we had suggested that perhaps the interaction of a ternary complex of ATP,

Cu^{2+}, and *o*-phenanthroline with the enzyme initiated the phosphorylation of the α-subunits and their subsequent cross-linking. The data presented here indicate that this is not the case. It is apparent that phosphorylation-dependent cross-linking in the presence of either UTP or P_i does require the addition of Mg^{2+}, and that the phosphorylation obtained in the presence of ATP, and absence of added Mg^{2+}, is due to traces of bound Mg^{2+} present in the enzyme preparation. These facts clearly suggest that Cu^{2+} and *o*-phenanthroline are not involved in the process of formation of the phosphoenzyme, and that their primary role is in the oxidation of sulfhydryl groups that are exposed due to phosphorylation-induced conformational transitions.

The theoretical difficulties in the interpretation of cross-linking studies of membrane proteins are well-known (e.g. Refs. 14 and 15). In the context of the present studies the following important questions may be posed: Is the phosphorylation-induced cross-linking an indication of the association of α-subunits in the native enzyme, or is it simply due to the increased reactivity of non-associated subunits towards cross-linking reagents within the membrane phase? Previous evidence favoring the former possibility has been discussed elsewhere [3,13]. The experiments with the digitonin-treated enzyme presented here lend further support to this alternative. The digitonin-treated enzyme is not a denatured preparation. Past studies [16] have shown that in this preparation the α,β-associations are retained, the α,α-associations are modified, and several partial reactions of the enzyme are detected. And our data demonstrate directly that the Na^+-dependent phosphorylation of the digitonin-treated enzyme, comparable to that of the native enzyme, also occurs. Therefore, the fact that under phosphorylating conditions cross-linking does not occur in the digitonin-treated enzyme strongly suggests that necessary conditions for cross-linking are not only the phosphorylation and the resulting conformational changes of the α-subunits, but also the associations and the appropriate juxtapositions of several α-subunits in an oligomer.

The findings with the digitonin-treated enzyme are also pertinent to the interpretation of certain previous experiments of our laboratory. We showed recently [17] that under optimal conditions for the phosphorylation of the native enzyme by ATP, a fraction of the phosphorylated α-subunits does not participate in the cross-linking reaction; and we interpreted this in the context of the enzyme's potential for half-of-the-sites reactivity. The complete lack of cross-linking reactivity of the phosphorylated α-subunit in the digitonin-treated enzyme raises the possibility that the incomplete cross-linking reactivity of the native enzyme may be due, at least in part, to the existence of a population of molecules within the native enzyme preparation that is similar to the digitonin-treated enzyme. The resolution of these questions must await the outcome of studies in progress.

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RUBESCENSLYSIN AND PHALLOLYSIN RELEASE MARKER MOLECULES FROM PHOSPHOLIPID CHOLESTEROL LIPOSOMES

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Multilamellar liposomes were prepared from phospholipid, cholesterol and dicetyl phosphate and marked with potassium chromate or with glucose. They were treated with 0.5–50 haemolytic units (HU)/ml of rubescenslysin from *Amanita rubescens* or of phallolysin from *Amanita phalloides*. Rubescenslysin caused a dose-dependent release of marker molecules from phosphatidylcholine and from sphingomyelin liposomes. A statistically significant marker leakage was caused by 0.5–1 HU/ml from liposomes prepared from sphingomyelin, phosphatidylcholine from egg yolk, and among the synthetic phosphatidylcholines from distearoyl > dipalmitoyl > dioleoyl phosphatidylcholine. With the lysin concentrations tested, at the best 50% of total markes were released from sphingomyelin liposomes and 75–95% from phosphatidylcholine liposomes. Also phallolysin released marker molecules from phosphatidylcholine liposomes; statistically significant amounts were released by 0.5 HU/ml from egg yolk phosphatidylcholine and from dipalmitoyl ≃ distearoyl > dioleoyl phosphatidylcholine liposomes; 60–90% of maximum release was achieved at the best. However, liposomes prepared from (bovine) sphingomyelin were resistant to phallolysin; even with 50 HU/ml no leakage of markers was observed. Heat-inactivated rubescenslysin, heat-inactivated phallolysin and human serum albumin failed to release markers. Our results lead to the conclusion that both, rubescenslysin and phallolysin, interact with membrane phospholipids, phallolysin showing a higher degree of specificity.

Introduction

Rubescenslysin from the Blusher *Amanita rubescens* (Pers. ex Fr.) Gray, and phallolysin from the Death-Cap *Amanita phalloides* (Vaill. ex Fr.) Secr. are cytolytically acting proteins (for review, see Ref. 1). Studies on the properties and the haemolytic effect of rubescenslysin indicated that this cytolysin possibly functions as a detergent: rubescenslysin is surface active; in aqueous solution it forms aggregates of varying sizes; only slight differences exist in the susceptibility of red cells of various animal species; moreover, rubescenslysin is expended on haemolysis and is inhibited by both, cholesterol and phospholipids [2]. On the other hand, the mode of action of phallolysin remains unclear: there was neither a basis for the assumption of a detergent effect, nor could phospholipase activity be detected. It became, however, evident from the high species specificity of this toxin, that it attacks cell membrane relatively specifically [3]. Even *N*-acetylglucosamide-containing lectin receptors were claimed to be the site of action of phallolysin [4]. The fact that bovine and sheep erythrocytes are resistant against phallolysin suggests that membrane phospholipids are probably involved in its cytolytic action: the membranes of these species lack phosphatidyl choline, but are abundant in sphingomyelin [5].

The present study of liposomes, already published in part [6], aimed to clarify to what extent membrane phospholipids could be the site of action of rubescenslysin and phallolysin.

Abbreviation: HU, haemolytic units.

Materials and Methods

The following substances were obtained from commercial sources; they were GR and purchased from Merck, unless otherwise stated: Chloroform, extra pure; cholesterol, from spinal cord (Schuchardt); dicetyl phosphate, research grade (Serva); D-glucose monohydrate, DAB 7; human albumin, purified (Behringwerke) di-potassium hydrogen phosphate 3-hydrate; L-α-phosphatidylcholine, from egg yolk (Sigma); L-α-phosphatidylcholine (dipalmitoyl), synthetic, research grade (Serva); L-α-phosphatidylcholine (distearoyl), synthetic, approx. 98% (Sigma); L-α-phosphatidylcholine (dioleoyl), chloroform sol., synth., approx. 98% (Sigma); potassium dihydrogen phosphate; sodium chloride; sphingomyelin, from bovine brain (Sigma).

Rubescenslysin and phallolysin were isolated from lyophilized fruit-bodies of *Amanita rubescens* and *Amanita phalloides,* respectively, as previously described [7,8]. The labile cytolysins were standardized in haemolytic units (HU) and retitered before use with washed blood cells. Protein was determined by the method of Lowry et al. [9]. 1 HU of rubescenslysin is defined as the activity, that completely clears 1 ml of a 1% suspension of washed preserved human erythrocytes at 37°C with 30 min; 1 HU of phallolysin as the activity that completely clears 1 ml of this cells suspension at 37°C within 1 h. With the preparations used 1 HU of rubescenslysin was equivalent to 1 μg of protein and 1 HU of phallolysin to 10–15 μg of protein.

Multilamellar liposomes were prepared essentially as described by Sessa et al. [10] and Richards and Gardner [11] from phospholipid, cholesterol and dicetyl phosphate. The molar ratio of these components was 7 : 2 : 1 in the case of phosphatidylcholine from egg yolk, synthetic dipalmitoyl and synthetic dioleoyl phosphatidylcholine and the liposomes were marked with potassium chromate; in the case of sphingomyelin and synthetic distearoyl phosphatidylcholine, the molar ratio was 7 : 4 : 1 and the liposomes were marked with glucose. The lipid film was sonicated (Branson Sonifier B 12) for 40 s at a frequency of 20 kHz in 0.145 M glucose or 0.145 M potassium chromate dissolved in 3.3 mM potassium phosphate buffer (pH 7.2) to yield a total lipid concentration of 6.4 mmol/l. Untrapped marker was removed from 3 ml aliquots on a Sepharose 6B (Pharmacia) column (300 × 27 mm) equilibrated and eluted with phosphate-buffered saline (6.7 mM potassium phosphate buffer (pH 7.2) in 0.9% sodium chloride) (room temp.; 0.3 ml/min; fractions of 1 ml). In the eluate liposomes were detected by turbidity measurement at 540 nm (Zeiss Spektralphotometer PMQ II). The liposomes-containing fractions were pooled, phosphate buffered saline being added to give a final volume of 20 ml. They were stored under a nitrogen atmosphere at 4–5°C for maximally five days. Those containing phosphatidylcholine from egg yolk or synthetic dioleoyl phosphatidylcholine were checked for autooxidation by calculation of the oxidation index (A_{233nm}/A_{215nm}) [12] and were used immediately after preparation. 2.5–3% of the C=C bonds of the paraffin chain were found to be oxidized.

The liposomes were incubated for 30 min at 37°C with an equal volume of cytolysin (rubescenslysin, phallolysin, or Triton X-100) in phosphate buffered saline pH 7.2 or with phosphate-buffered saline (controls), respectively. Then the samples were centrifuged at 45 000 × *g* for 90 min at 6–8°C (IEC centrifuge B-20 A). In the supernatant potassium chromate was determined by its absorbance at 370 nm; results were corrected for the absorbance of the added cytolysin. Glucose was determined by the hexokinase method, using commercially available test kits (Glucoquant®, Boehringer). To make sure that the observed effects were due to the cytolytic effect of rubescenslysin and phallolysin (and not to the presence of protein material), corresponding experiments were performed with human serum albumin and with heat-inactivated phallolysin and rubescenslysin. For statistical analysis the geometrical means of the % values of controls were compared by paired *t*-test.

Results

Rubescenslysin caused dose-dependent release of marker molecules from liposomes containing sphingomylein and from those containing phosphatidylcholine (Fig. 1). Its lytic potency against liposomes was comparable to some extent to its haemolytic potency: a statistically significant marker release from liposomes was obtained at 0.5 HU/ml or, in the

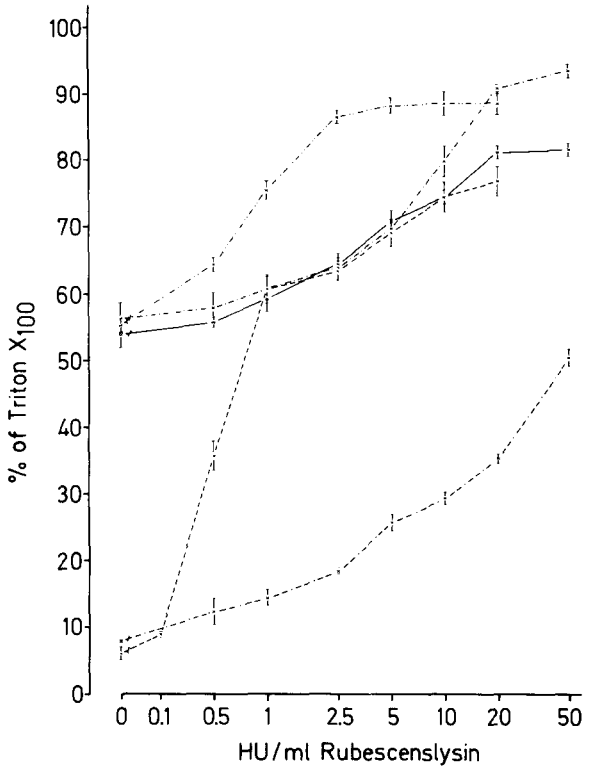

Fig. 1. Release of entrapped marker by rubescenslysin from liposomes prepared from different phospholipids, cholesterol and dicetyl phosphate. Phospholipid component: X– · – · –X, phosphatidyl choline from egg yolk; X– ·· – ·· –X, synthetic dipalmitoyl phosphatidyl choline; X- - - - - -X, synthetic distearoyl phosphatidyl choline; X———X, synthetic dioleoyl phosphatidyl choline; X- · - · - ·X, sphingomyelin from bovine brain. Data in % of maximum release by Triton X-100. $\bar{x}$ ± S.E.; n = 6–8.

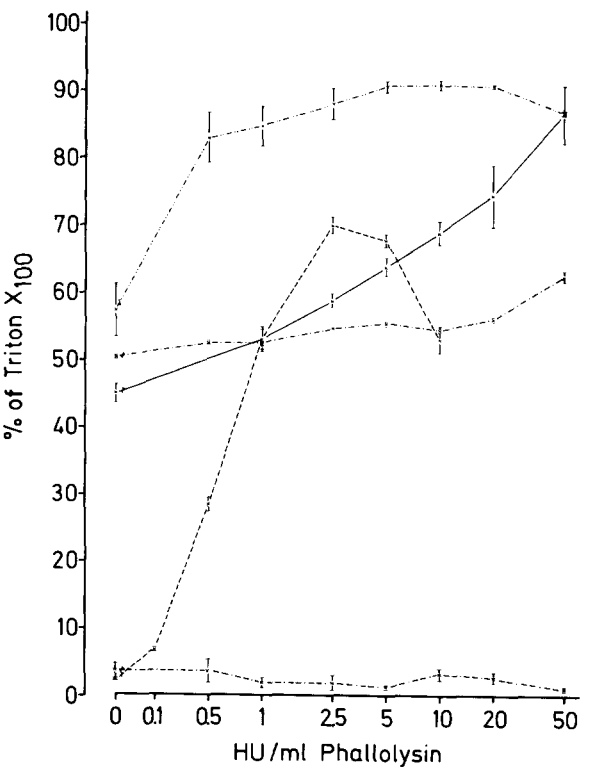

Fig. 2. Release of entrapped marker by phallolysin from liposomes prepared from different phospholipids, cholesterol and dicetyl phosphate. Phospholipid component: X– · – · –X, phosphatidyl choline from egg yolk; X– ·· – ·· –X, synthetic dipalmitoyl phosphatidyl choline; X- - - - -X, synthetic distearoyl phosphatidyl choline; X———X, synthetic dioleoyl phosphatidyl choline; X- · - · - ·X, sphingomyelin from bovine brain. Data in % of maximum release by 0.6% Triton X-100. $\bar{x}$ ± S.E.; n = 6–8.

case of sphingomyelin or dioleoyl phosphatidylcholine, at 1 HU/ml; the latter concentration completely lyzes a 1% suspension of red cells. With the concentrations tested, 50% of maximum release of markers was effected from sphingomyelin liposomes and 75–95% from phosphatidylcholine liposomes. As expected from inhibition experiments [2], the effect was influenced by the length and saturation of the hydrocarbon chain: liposomes prepared from dipalmitoyl or distearoyl phosphatidylcholine were particularly susceptible. Saturation phenomena became apparent here, resembling the consumption of rubescenslysin in haemolysis [2].

Phallolysin, too, released marker molecules from liposomes prepared with phosphatidylcholine (Fig. 1), and its effect was similar to the effect of rubescenslysin. Again 0.5 HU/ml caused statistically significant release of entrapped markers, and 60–90% of maximum release was at the best achieved with the concentrations tested. As observed with rubescenslysin, dipalmitoyl and distearoyl phosphatidylcholine liposomes were most sensitive, saturation phenomena here being pronounced. However, liposomes prepared from (bovine) sphingomyelin were resistant to phallolysin, even when applied in the 50-fold haemolytic concentration. Phallolysin itself was not inhibited by glucose, entrapped in these vesicles, even in concentrations far exeeding those obtainable in the medium surrounding the liposomes: the haemolytic activity of the cytolysin was unchanged whether it was deter-

mined in phosphate-buffered saline (controls) or in 0.008 M glucose made isotonic with phosphate-buffered saline.

Heat-inactivated rubescenslysin, heat-inactivated phallolysin, and human serum albumin in concentrations corresponding to the highest tested lysin concentration failed to release marker molecules from phosphatidylcholine (egg yolk) and from sphingomyelin liposomes.

Discussion

Our experiments indicate that rubescenslysin as well as phallolysin interact with membrane phospholipids. Marker release from liposomes is obtained with lysin concentrations comparable to the haemolytic concentrations of these cytolysins. Rubescenslysin releases markers from both, (long-chain-) phosphatidylcholine and sphingomyelin liposomes, though the latter are somewhat less susceptible. Cholesterol, which inhibits rubescenslysin haemolysis [2], might also be involved. This rather non-specific interaction of rubescenslysin with different membrane lipids corresponds to its low species specificity in haemolysis [2].

Phallolysin, on the other hand, interacts with phosphatidylcholines, but, in comparable concentrations, neither with (bovine) sphingomyelin nor with cholesterol in liposomes. The present results agree with findings that phallolysin stimulated cellular phospholipase A_2 in mouse fibroblasts, whereas sphingomyelinases were not significantly activated [13]. This phenomenon, termed activation of cellular phospholipases by cytolysins, might simply mean that cytolysins destroy the integrity of the cell membrane and consequently offer to those enzymes an increased amount of substrate. Interestingly enough, another fungal cytolysin, pleurotolysin, interacts only with (sheep) sphingomyelin, as revealed from inhibition experiments [14]. The significance of membrane phospholipids for the cytolytic action is stressed by the inverse correlation between the sensitivity of erythrocytes of various animal species to pleurotolysin and phallolysin.

Further membrane constituents, such as integral membrane proteins, may of course be involved in the action of rubescenslysin and phallolysin and could even be more susceptible to these compounds. It can at least be concluded from our results, that a cell membrane susceptible to reasonable concentrations of rubescenslysin and phallolysin needed not consist of anything else than phospholipid and cholesterol.

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