Phosphoprotein Synthesis in Epidermis During Acantholysis[1] (34391)

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Cantharidin, the active principle in the venom of cantharides, induces the formation of an intra-epidermal blister when it is applied to mammalian skin either *in vivo* or *in vitro*. The blister appears histologically to result from the loss of adherence of the individual prickle cells (Fig. 1), and it resembles the acantholysis seen in several skin diseases, particularly pemphigus vulgaris.

Previous studies indicated that endogenous ATP is required for the induction of acantholysis by cantharidin (1–4), and the recent finding of increased amounts of phosphoprotein in acantholytic epidermis suggests that the ATP may be used in the phosphorylation of epidermal proteins (5).

The present experiments were concerned with the biochemical requirements for synthesis of phosphoprotein in the epidermis and with the relationship of this synthesis to cantharidin-induced acantholysis. It was shown that protein phosphorylation is an energy-dependent reaction which is independent of the synthesis of proteins or of nucleic acids. The increased phosphorylation of protein is not a general response to cell damage but seems to be unique to acantholysis and to precede it.

Materials and Methods. All of the studies were made with epidermis from beef snout. Snouts were obtained on the morning of slaughter, and slices were taken with a keratotome set at 0.3 mm. The snout was shaved twice, and the first slice (consisting of stratum corneum and stratum granulosum) was discarded. The second slice, representing mainly stratum spinosum, was cut into pieces

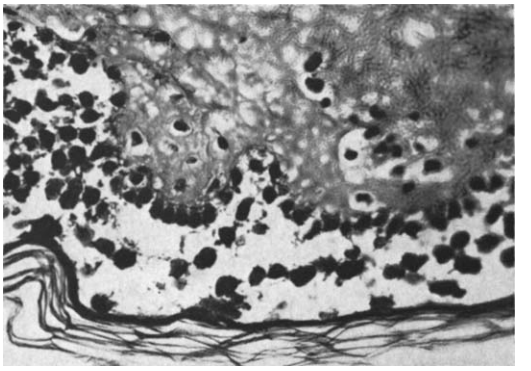

FIG. 1. Acantholytic epidermis; normal human skin (1 cm^2) was incubated for 1 hr in isotonic saline and cantharidin, 5×10^{-4} *M* (hematoxylin and eosin; × 260).

weighing approximately 50 mg and placed in cold isotonic saline until incubation. Some specimens were frozen with Fluoroethyl for 30 sec or were heated in saline at 55° for 90 sec prior to incubation.

The weighed specimens were incubated in open (6) 25-ml Erlenmeyer vessels in 2 ml of buffered medium which contained the following: 1.5 ml of Krebs–Ringer phosphate buffer, pH 7.3; 0.15 ml of 0.15 *M* Tris, pH 7.3; 0.15 ml of 0.1 *M* glucose; 0.1 ml chloramphenicol (500 μg/ml); and 0.1 ml of H_2O containing either 1 μCi of $H_3{}^{32}PO_4$ (carrier-free), 0.5 μCi of L-alanine-1-^{14}C (7.5 mCi/mmole) or 0.5 μCi of adenine-8-^{14}C (3.8 mCi/mmole). Other components were added to some vessels in 0.1 ml of H_2O: disodium cantharidin (2 μmoles), trypsin (1.5 mg), pronase (0.7 mg), citrate at pH 6.5 (100 μmoles), glutathione (10 μmoles), urea (1 mmole), cyanide (2 μmoles), fluoride (2 μmoles), Amytal (2 μmoles), azide (10 μmoles), cycloheximide (100 μg), puromycin (10 μg), and actinomycin D (20 μg). Vessels were shaken at 37° for 90 min unless indi-

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TABLE I. Distribution of Protein-Bound ^{32}P and Protein in Bovine Epidermal Slices.[a]

	Normal	Cantharidin treated	Ratio (acantholysis/control)
^{32}P in soluble protein (cpm)	1616	42,988	26.7
Soluble protein (mg)	1.7	5.2	3.1
Sp act (cpm/mg of protein)	952	8270	8.7
^{32}P in residual protein (cpm)	11,028	28,504	2.6
Residual protein (mg)	23.0	16.8	0.7
Sp act (cpm/mg of protein)	480	1700	3.5

[a] Each specimen weighed 100 mg, wet wt, before incubation.

cated otherwise. In studies in which histologic examination was made, an additional specimen (3 mm^2) was added to the incubation medium along with the weighed specimen and was removed at the appropriate time to be fixed in cold formalin. It was sectioned and stained with hematoxylin and eosin.

The weighed specimens were homogenized in 2 ml of 0.05 *M* Tris, pH 7.3. For the separation of phosphoproteins into soluble and insoluble fractions, the homogenates were centrifuged (9000*g*, 10 min) and the supernates were isolated before the addition of perchloric acid to each fraction. Otherwise, 3 ml of 1 *M* perchloric acid was added to the entire homogenates, and the residues, which contained all the protein as well as nucleic acids and phospholipids, were sedimented by centrifugation. The residues were washed twice with 2 ml of 0.5 *M* perchloric acid, and the lipids were extracted with 2 ml of chloroform–methanol (2:1).

Incorporation of isotope from alanine-1-^{14}C or from adenine-8-^{14}C into this residue was used as a measure of protein or nucleic acid synthesis, respectively. Residues were suspended in 1 ml of Hyamine and were counted in "diotol" (7) in a liquid scintillation spectrometer. Phosphoprotein ^{32}P was determined as described previously (5). Briefly, 1 ml of 1 *N* sodium hydroxide was added to the residue and the mixture was heated in a boiling water bath for 15 min. The soluble proteins and DNA were removed with 0.3 ml of 0.1 *M* silicotungstic acid after the solution was acidified with perchloric acid. Aliquots of the supernate were counted in diotol, and the counts were corrected for ^{32}P decay.

Results. When an epidermal slice is incubated for 90 min in buffered medium containing ^{32}P-labeled phosphate, normally about 3% of the cellular ^{32}P is covalently bound to protein; when cantharidin is included in the incubation mixture so that acantholysis occurs, the ^{32}P content of the protein fraction is increased to as high as 30% of the total cellular ^{32}P (5). The increase in protein–phosphate during acantholysis is particularly prominent in the soluble protein fraction, which shows a 27-fold increase in ^{32}P content (Table I). Since protein is also solubilized during acantholysis, the net increase in specific activity, about ninefold, is not as great.

Energy dependence of protein phosphorylation. The uptake of ^{32}P into epidermal proteins is energy dependent; inhibitors of respiration reduced phosphorylation by 55–75%, while fluoride, which blocks glycolysis, inhibited by 79% (Table II). The simultaneous inhibition of glycolysis and respiration completely abolished phosphoprotein synthesis.

Synthesis of proteins during protein phos-

TABLE II. Influence of Metabolic Inhibitors on Cantharidin-Induced Phosphoprotein Synthesis.[a]

Inhibitor	Inhibition (%)
Cyanide	63
Azide	57
Amytal	75
Fluoride	79
Cycloheximide	0
Puromycin	0
Actinomycin D	0

[a] All reactions contained disodium cantharidin, 5×10^{-4} *M*.

TABLE III. Effect of Metabolic Inhibitors on Incorporation of Alanine-1-^{14}C into Proteins by Bovine Epidermal Slices.

Inhibitor	Incorporation (cpm/100 mg of tissue, wet wt)	Inhibition (%)
Control	19,270	—
Cantharidin	1140	94.0
Cycloheximide	6500	66.5
Puromycin	5350	72.3

phorylation. Phosphoprotein synthesis is not dependent on the synthesis of new protein. Under conditions in which cycloheximide and puromycin effectively inhibited the incorporation of alanine-^{14}C into epidermal proteins (Table III), the synthesis of phosphoprotein was not affected (Table II). In fact, cantharidin itself reduced the uptake of alanine-^{14}C by 94% even while it was inducing phosphoprotein synthesis. The enzymatic reaction during acantholysis must involve the phosphorylation of a previously existing protein.

Synthesis of nucleic acids during protein phosphorylation. Epidermal slices incorporated adenine-^{14}C into the macromolecular fraction which was precipitated with perchloric acid. That this was RNA was indicated by its solubility in 1 *N* sodium hydroxide after 16 hr at 37° and by its solubility in hot 5% trichloroacetic acid. The presence of cantharidin during incubation doubled the rate of incorporation of adenine-^{14}C into this residual fraction (Table IV). However, the new synthesis of RNA is apparently unnecessary for protein phosphorylation; actinomycin D inhibited the synthesis of RNA in both controls and in cantharidin-treated slices, but it had no effect on protein phosphorylation (Table II) or on acantholysis.

TABLE IV. Effect of Inhibitors on Incorporation of Adenine-8-^{14}C into Nucleic Acids by Bovine Epidermal Slices.

Inhibitor	Incorporation (cpm/100 mg of tissue, wet wt)	Stimulation (+) or inhibition (—) (%)
Control	1765	—
Cantharidin	3465	+96.5
Actinomycin D	850	—52.0
+ cantharidin	530	—69.6

Conditions which induce phosphoprotein synthesis. Attempts were made to induce the synthesis of phosphoproteins by other agents which cause cell separation (8) or by conditions which produce mild damage to epidermal cells. While none of these enzymes or conditions produce a histologic change identical to that produced by cantharidin, they serve to show whether increased protein phosphorylation is related to cell damage in general rather than specifically to the cantharidin-induced reaction. Cantharidin was the only agent which stimulated protein phosphorylation during acantholysis (Table V).

TABLE V. Influence of Other Acantholytic Conditions on Phosphoprotein Synthesis in Epidermal Slices.

Added	Phosphoprotein (% of control)
Glutathione	72
Citrate	14
Urea	54
Pronase	37
Trypsin	51
Heated for 90 sec at 55°	83
Frozen and thawed 3 times	30
Cantharidin	317

Comparison of rates of protein phosphorylation and development of acantholysis. An increase in the rate of phosphoprotein synthesis can be observed as early as 10 min after the addition of cantharidin. Figure 2 shows that, within 30 min, slices which were incubated with cantharidin contained over twice the protein-bound ^{32}P as controls but they displayed no evidence of acantholysis at that time; phosphoprotein synthesis clearly preceded the histologic appearance of acantholysis. After this preacantholytic period, the extent of the acantholysis paralleled the degree of protein phosphorylation.

Turnover of phosphoprotein in slices. Once formed, the protein–phosphate complex is metabolically stable as shown by a "cold chase" experiment. Slices were incubated with buffered ^{32}P and 5×10^{-4} *M* cantharidin in the usual manner for 60 min. The

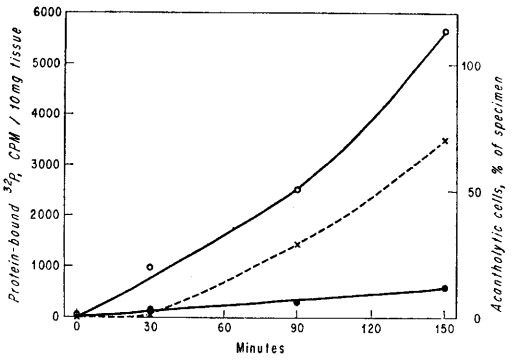

FIG. 2. Rates of development of acantholysis (- - -) and of protein phosphorylation in bovine epidermis (protein-bound ^{32}P in control (●); protein-bound ^{32}P in specimens treated with cantharidin (O).

protein-bound ^{32}P was determined in four specimens, and eight other specimens were rinsed and transferred to a buffered medium containing ^{31}P, with or without cyanide and iodoacetate, and were incubated for an additional 120 min before determination of protein-bound ^{32}P. Epidermal proteins retained ^{32}P-labeled phosphorus despite the presence of unlabeled phosphorus for 2 hr (1680 vs. 1900 cpm/10 mg of tissue). The presence of inhibitors of energy metabolism decreased the radioactivity of the protein fraction (1160 cpm/10 mg of tissue).

Discussion. The protein–phosphate complex produced during acantholysis was previously shown to be a covalent complex of phosphate and serine moieties of keratin-like proteins of the epidermis (5). The present observations make it clear that the formation of the complex is an enzyme-catalyzed reaction, the stimulation of which is unique to the type of acantholysis induced by cantharidin. Phosphoprotein formation is clearly an energy-dependent reaction; both glycolysis and respiration can supply this energy, and it is probable that ATP is the intermediate in this synthesis. It was also shown previously that the increase in the amount of ^{32}P complexed with protein in acantholytic epidermis is the result of a net increase in total phosphoprotein and not the result of an increased rate of exchange of ^{32}P with phosphate of preexisting phosphoprotein. The formation of new phosphoprotein during acantholysis, however, does not involve the synthesis of new protein, since inhibitors of protein synthesis had no effect on the incorporation of ^{32}P into protein. In fact, of all the inhibitors tested, cantharidin itself most effectively blocked the incorporation of alanine-^{14}C into epidermal protein. It does this under conditions which result in the formation of phosphoprotein and in acantholysis. This inhibition is probably not related causally to acantholysis because other inhibitors of protein synthesis do not induce acantholysis.

The data show that there is an increase in the synthesis of RNA during acantholysis and phosphoprotein formation, but this, too, is not obligatory for acantholysis or for protein phosphorylation. This conclusion is based on the fact that ^{32}P uptake into protein is unaltered during inhibition of RNA synthesis. Rather, it is likely that the increased synthesis of RNA is secondary to acantholysis, perhaps as a result of the phosphorylation of a protein repressor (9).

From these facts, the most likely reaction in the formation of these phosphoproteins is one catalyzed by a protein kinase. Other studies (unpublished data) have shown that this enzyme is quite active in mammalian epidermis. The hypothesis for a primary relationship between increased protein phosphorylation and cantharidin-induced acantholysis is based on three conditions: (i) both events require energy, probably via ATP; (ii) the appearance of increased phosphoprotein in the epidermis has so far been unique to this type of acantholysis; and (iii) this increase occurs concomitantly with or preceding the development of acantholysis.

Wilgram (10) and Wolff and associates (11) recently examined the histologic characteristics of acantholytic cells with the electron microscope and found that there is a disappearance of the desmosome–tonofilament complex and eventually a solubilization of various cellular cytoplasmic components. In view of this, it is interesting to note that during acantholysis the amount of soluble protein is tripled and the major portion of the phosphoprotein is in the water-soluble fraction. It seems probable that phosphorylation of proteins increases their solubility, and

perhaps it is this event which leads to the disintegration of the structures which characteristically disappear during acantholysis.

Whether acantholysis which is induced by cantharidin has any more in common with acantholytic diseases than a similar histologic picture remains unknown. The association between phosphoprotein content and the degree of experimental acantholysis which we have reported here offers a chemical parameter that may be used to compare experimental acantholysis with lesions which are produced in such diseases.

Summary. The effect of the acantholytic agent, cantharidin, on several biosynthetic systems was examined with epidermal tissue slices. Cantharidin is an effective inhibitor of protein synthesis, it stimulates RNA synthesis, and it leads to extensive phosphorylation of proteins. The inhibition of protein synthesis and the stimulation of RNA synthesis are probably reactions secondary to those which lead to acantholysis. The phosphorylation of proteins, on the other hand, appears to have a causal role in acantholysis. This reaction requires ATP, and its increased rate was induced only by cantharidin during acantholysis. Acantholysis leads to a threefold increase in soluble protein, and most of the phosphoprotein is in this fraction.

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