intervene during the reaction by facilitating enzyme-substrate interaction as the lysis proceeds. The mechanism by which SDS could intervene in elastinolysis is ambiguous. In the first place, it depends upon the nature of the enzyme, since SDS enhances elastinolysis only with macrophage elastase. Then, a main point is to distinguish between SDS action on the substrate (before the reaction) and on the enzyme (during the reaction). Nevertheless, some investigators have suggested that the conformation of elastin could be modified by SDS and so the substrate would be more accessible to degradation[15]. Others[14,15] suggest that SDS could increase the binding of the enzyme to the substrate. In our hands, addition of SDS during the reaction inactivates the enzyme. If following its preincubation with elastin, SDS was removed completely by thorough washing no effect could be discerned.

The reported observations emphasize the role of Mg^{++} salts in the elastinolysis mechanism. A direct effect on the enzymatic reaction is indicated by the greater increase of elastinolysis when Mg^{++} was incorporated in the reaction buffer rather than applied to the substrate as part of the washing process[5]. As pointed out by several authors[4,8] the biological effects of Mg^{++} are due to its ability to form weak chelates. Enzymes activated by Mg^{++} demonstrate a bell-shaped p Mg^{++} dependency similar to that found in this study. As regards the effects of anions it has been reported[3] that iodine may be more effective than chloride in enhancing elastinolysis; however, our findings indicate that anions do not affect the elastinolysis process. A major problem in biological studies is the lack of sufficiently large amounts of material for adequate tests. The 2.6-fold enhancement of elastinolysis may therefore be of great importance. Furthermore, if destruction of elastin in the arterial wall is the first event in the etiology of atherogenesis, the dependence of the elastinolysis rate on Mg^{++} concentration which is highest under physiological conditions will have a significant effect.

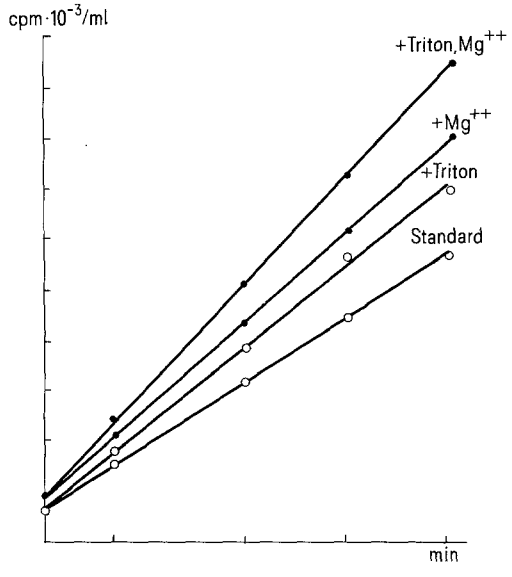

Figure 4. Effect of Triton on elastinolysis with and without added Mg^{++}. O–O, Elastinolysis as previously described with and without pre-incubation with 0.5% (w/v) Triton during the washing of elastin; ●–●, elastinolysis in the presence of 10^{-3} M $MgCl_2$ added at the start of the assay and with and without Triton pre-incubation. Each point is the mean of five experiments.

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Role of phospholipids in the binding activity of vasoactive intestinal peptide receptors[1]

A. Sarrieau, N. Boige and M. Laburthe

Equipe de Recherches sur le Mécanisme d'Action des Hormones et Neuropeptides Digestifs, Unité de Recherches de Diabétologie et d'Etudes Radio-Immunologiques des Hormones Protéiques (INSERM U.55), Hôpital Saint-Antoine, 184, rue du Faubourg Saint-Antoine, F-75012 Paris (France), 30 March 1984

Summary. Phospholipase digestion of rat intestinal epithelial cell membranes was performed in order to study the influence of membrane phospholipids on the binding activity of VIP receptors. Phospholipases A_2 and C strongly ($ED_{50} \simeq 4 \times 10^{-2}$ and 4×10^{-1} µg/ml, respectively) and rapidly reduced ^{125}I-VIP binding to membranes whereas phospholipase D was ineffective. This suggests an important role of both hydrophobic and hydrophilic groups of phospholipids on VIP receptor binding activity.
Key words. Intestinal epithelium, rat; VIP receptors; phospholipids.

The binding of vasoactive intestinal peptide (VIP) to specific receptors on the exterior surface of target cells initiates cellular responses[2]. The functional properties of VIP receptors[2] as well as their protein nature and molecular size[3,4] have been characterized in the intestinal epithelium. Phospholipids, a major component of membrane lipids, interact with membrane proteins, in particular hormone receptors, and often modulate their functional activity[5]. The influence of phospholipids on

VIP receptor activity has nevertheless not been documented. The present study describes the effect of phospholipase attack of intestinal membranes on their ability to bind VIP specifically. Because of their well-defined properties three phospholipases, A_2, C and D have been employed in modifying membrane phospholipids.

Material and methods. Purified porcine VIP was obtained from Prof. V. Mutt (Karolinska Institute, Stockholm, Sweden). Phospholipases A_2 (from *Crotalus durissus*) and C (from *Bacillus cereus*, grade II) were from Boehringer Mannheim and phospholipase D (from peanut, type III) from Sigma. Other chemicals were of reagent grade purity. VIP was iodinated at a specific activity of 250 Ci/g[6]. Rat intestinal epithelial cell membranes were prepared as described[7]. Enzymatic digestion of membranes was performed as reported previously[3]. Intestinal membranes (about 600 µg protein) were incubated at 37°C alone or in the presence of enzyme for various periods as indicated in the legends to the figures. The incubation medium (500 µl) was 50 mM Tris-HCl buffer (pH 7.5) for phospholipases C and D. It was supplemented with 10 mM $CaCl_2$ in the case of phospholipase A_2[8]. The reaction was stopped by adding 1 ml of ice-cold 50 mM Tris-HCl buffer. The incubation medium was then centrifuged at 20,000 × g for 20 min at 4°C. The resulting membrane pellet was then resuspended in 25 mM Tris-HCl and used in further studies. The binding assay of ^{125}I-VIP was conducted as described[9]. The incubation medium contained in a 250 µl final volume, 25 mM Tris-HCl (pH 7.5), 2 mg/ml bovine serum albumin, 1 mg/ml bacitracin and ^{125}I-VIP at 5×10^{-11} M. Reactions were started with the addition of enzyme-treated or control membrane preparations (about 10 µg proteins in 50 µl). After 30 min at 30°C, the incubation was stopped and membrane-bound ^{125}I-VIP determined as described[9]. Results are expressed as specific binding; this is obtained by substracting from the total bound that amount of ^{125}I-VIP which remained bound in the presence of a large excess of native VIP (10^{-6} M). Inactivation of ^{125}I-VIP by membranes was tested by radioreceptor assay as previously described[2]. Membrane proteins were determined according to Bradford[10] using bovine serum albumin as standard.

Results. Figure 1 shows that digestion of intestinal membranes with phospholipases A_2 and C induced a time-dependent loss of their ability to bind specifically ^{125}I-VIP. In sharp contrast, identical digestion with phospholipase D was without effect on VIP binding. At the concentration tested (0.3 µg/ml), the half maximum inactivations of VIP binding were observed at about 2 min and 6 min for phospholipases A_2 and C, respectively. In accordance with the Ca^{++} requirement of phospholipase A_2[8], the absence of Ca^{++} in the buffer during the digestion of membranes completely abolished the effect of phospholipase A_2 on ^{125}I-VIP binding (not shown). The loss of the ability of intestinal membranes to bind ^{125}I-VIP after enzyme treatment is attributable to phospholipase-induced loss of specific receptors since: a) nonspecific binding of ^{125}I-VIP is not altered by enzyme treatment; b) the degradation of ^{125}I-VIP by membranes is similar in enzyme-treated and control membranes; c) in the absence of enzymes, membranes retained almost completely their ability to bind ^{125}I-VIP. Figure 2 shows the dose-effect of phospholipases in inducing the loss of ^{125}I-VIP binding to intestinal membranes. Phospholipase A_2 was 10 times more potent than phospholipase C, the ED_{50} being 4×10^{-2} and 4×10^{-1} µg/ml, respectively. At very high concentrations (40 µg/ml), phospholipases A_2 and C almost completely abolished the binding capacity of membranes whereas phospholipase D remained inactive.

Discussion. Each phospholipase alters a specific portion of a phospholipid[11], permitting us to draw some conclusions about the phospholipid requirement for VIP binding to membrane receptors. The present results indicate that the hydrolysis of hydrophilic groups of the membrane phospholipids by phospholipase C has a pronounced effect on the membrane components involved in the VIP binding activity. In sharp contrast, phospholipase D, which also acts on the polar head groups of phospholipids but does not remove phosphate, has no effect; this supports a major role of the negatively charged phosphoric acid moiety in the ability of intestinal membranes to bind VIP. Hydrolysis of ester bonds of phospholipids by phospholipase A_2, which removes a fatty acid at position 2, also results in a dramatic loss of VIP receptor activity. These data suggest that both hydrophobic and hydrophilic groups of phospholipids may be involved in the VIP binding activity of intestinal membranes. Such results present similarities with the effect of phospholipases on gonadotropin receptors in plasma membranes of bovine corpus luteum[12].

The importance of hydrophobic interaction between VIP receptor protein and fatty acid chains for binding activity, suggested here by the effect of phospholipase A_2, is further supported by the extreme sensitivity of VIP binding activity to various detergents that modify lipid-protein interactions[13]. Lipids may even be involved in the binding of VIP to receptors, as suggested previously for the interaction of other peptide hormones with membrane receptor sites[14]. The ability of VIP to bind to phospholipids has been observed previously[15] and is consistent with the fact that the peptide can form an amphiphatic helix[14,16], at least in some portions of the mole-

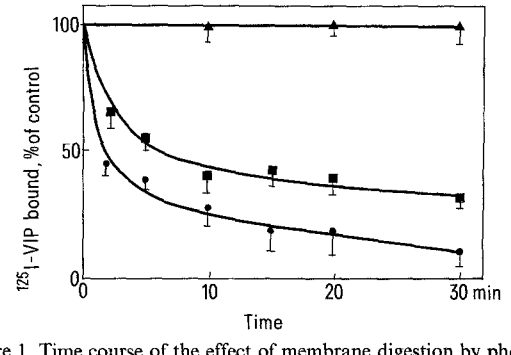

Figure 1. Time course of the effect of membrane digestion by phospholipases on the binding of ^{125}I-VIP. Intestinal membranes were incubated with 0.3 µg/ml of phospholipases A_2, C or D at 37°C for indicated times. The subsequent specific binding was then measured as described under 'methods' and expressed as a percentage of ^{125}I-VIP binding at time zero of enzyme digestion. Each point is the mean ± SEM of three separate experiments. Phospholipase A_2, ●–●; phospholipase C, ■–■; phospholipase D, ▲–▲.

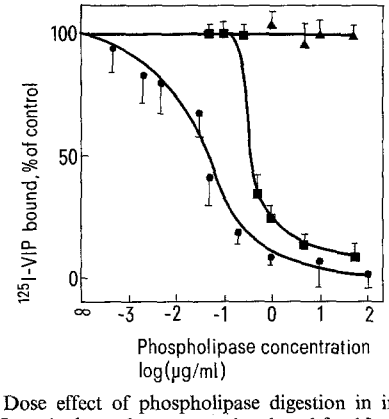

Figure 2. Dose effect of phospholipase digestion in inactivating VIP receptors. Intestinal membranes were incubated for 15 min with increasing phospholipase levels as described under 'methods'. The subsequent specific binding was then determined and expressed as a percentage of the initial ^{125}I-VIP binding (incubation without phospholipase). Each point is the mean ± SEM of four separate experiments. Phospholipase A_2, ●–●; phospholipase C, ■–■; phospholipase D, ▲–▲.

cule. Electrostatic interaction between VIP, a strongly basic protein[17], and acidic groups of phospholipids may also be involved and could explain the differential effect of phospholipases C and D in altering VIP binding to membranes. In conclusion, the observed effect of phospholipases on VIP binding clearly indicate the important role of phospholipids in the process of VIP binding to its membrane receptors.

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Changes of the level of proteinase inhibitors in rat plasma during turpentine-induced inflammation[1]

M. Warwas and J. Osada

Department of Biochemistry, Faculty of Pharmacy, Medical Academy, Szewska 38/39, PL-50-139 Wrocław (Poland), 3 April 1984

Summary. The levels of rat plasma α-macroglobulins, α-cysteine proteinase inhibitor, haptoglobin and antipapain activity were studied during the acute-phase reaction after an injection of α-pinen. An increase in concentration of all the compounds examined was observed.
Key words. α-Cysteine proteinase inhibitor; haptoglobin, α-macroglobulins; inflammation; rat plasma.

Three high mol.wt cysteine proteinase inhibitors have been described in serum: α_2-macroglobulin (α_2-M), haptoglobin (Hp) and α-cysteine proteinase inhibitor (α-CPI) which appears in different molecular forms[14,21]. Recently, cysteine proteinase inhibitors of low mol.wt have been identified in human, rat and bovine serum[4,5,7]. The biological function of the α-CPI and the low mol.wt inhibitor are still unknown. However, it has been suggested that these inhibitors might play a role in the control of cysteine proteinases participating in the inflammatory response[14,16], and these which are involved in myofibrillar protein degradation[19].

The aim of this study was to investigate changes in the rat plasma concentration of α-CPI in comparison with other known acute-phase reactants such as haptoglobin and α-macroglobulins during the course of turpentine inflammation.

Materials and methods. Experiments were carried out with 35 healthy male rats of the Buffalo strain. Inflammation was induced by c.s. injection of α-pinen (0.2 ml/100 g b.wt) in the scapular region. After one, two, three, five and seven days of the experiment the groups of five rats were anesthetized with ether and bled by intracardiac puncture into a syringe containing heparin. The control group (10 animals) received a similar volume of saline and were bled 24 h after injection. The amount of functional α-macroglobulin was determined by Ganrot's method[2]. The sum of the normally present α_1-macroglobulin and the acute-phase α_2-macroglobulin is determined by this method[3]. The concentration of haptoglobin was measured by the peroxidase method of Jayle[8]. Antipapain capacity (APC) of rat plasma was determined in the presence of 6 mmoles/l cysteine according to Sasaki et al.[17] using casein as a substrate. The inhibitory activity of α-CPI against papain was determined after methylamine inactivation of α-macroglobulins according to Minakata et al.[12]. One unit of α-CPI or antipapain capacity was expressed as the amount inhibiting 1 mg of papain (up to 50% inhibition).

Results and discussion. As can be seen in the figure, during the inflammatory reaction increased levels of haptoglobin, α-macroglobulins, α-CPI and antipapain capacity were observed.

The plasma concentration of Hp increased significantly 24 h after turpentine injection and reached a maximum level of seven times the normal value after 48 h. It was observed that α-CPI activity increased twice after 24 h and five times after 48 h. α-macroglobulins and antipapain capacity did not increase so markedly, and reached the maximum value of about 2.5 times normal after 72 h. Calculated correlation coefficients 'r' between α-CPI and Hp, α-M, and antipapain capacity are: 0.52, 0.72 and 0.94 respectively.

Inflammation induced by various agents is reflected in the production of modulatory proteins by the liver. These proteins, referred to as 'acute phase reactants'[9], circulate in the blood. The precise functions of these proteins have not been elucidated yet. The observed changes in Hp and α-macroglobulin concentrations during the course of inflammation are in good agreement with the previous findings[3,6,10,11,18]. The correlation between α-CPI and antipapain capacity is rather low. In the human serum the inhibitor activity of α-CPI against ficin was found to be twice higher then that of α_2-macroglobulin. In our experiments we used papain as a cysteine proteinase and we found that the inhibitory activity of α-CPI in the rat plasma comprised ony about 10% of the antipapain capacity. From the work of Sasaki et al.[17] it is known that the inhibitory power of α-CPI towards the hydrolytic activity decreases in the following order: ficin, papain, cathepsin B and bromelain. We choose papain as a cysteine enzyme because of its general resemblance to cathepsins B and H[20]. Furthermore, Valeri et al.[22] demonstrated that plasma antithrombin III is also capable of