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EFFECTS OF CON A AND OTHER LECTINS ON PURE 5'NUCLEOTIDASE ISOLATED FROM LYMPHOCYTE PLASMA MEMBRANES ⁂

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SUMMARY Inhibition of purified or membrane-bound 5'nucleotidase by various lectins was studied in lymphocytes from pig mesenteric lymph nodes. Con A or *Lens culinaris* lectin LcH inhibited (75 %) purified 5'nucleotidase by a non-competitive process without cooperativity. Inhibition by these lectins of 5' nucleotidase activity in whole lymphocytes, plasma membranes (untreated or solubilized) and LcH-receptor fraction displayed high positive cooperativity, reached higher level (90 %) and was of mixed type. An interaction between lectin receptors and 5'nucleotidase accounted for these differences. Wheat germ agglutinin (WGA) and divalent Con A which are not mitogenic for T lymphocytes had no effect on 5'nucleotidase; pokeweed mitogen (PWM), mitogen of T and B cells, was not inhibitor. When membrane proteins were cross-linked by glutaraldehyde, Con A inhibition of whole lymphocyte 5'nucleotidase presented the same properties as the purified enzyme. Possible correlation between 5'nucleotidase inhibition and lymphocyte stimulation is discussed.

INTRODUCTION The *Canavalia ensiformis* lectin, concanavalin A (Con A), binds sugars with D-arabinose configuration - like D-mannose or D-glucose - and membrane glycoproteins containing such sugars. Con A binding on plasma membranes from various cell types induces changes in their biophysical or biochemical properties and several membrane enzymes are affected. Some modifications come from direct interaction of the lectin with membrane receptors, others are due to secondary effects on cell metabolism. With isolated plasma membranes of different tissues direct effects of Con A can be evidenced, as on (Mg^{2+}) ATPase (1,2), (Ca^{2+}) ATPase (3), (Na^{+} + K^{+}) ATPase (3,4,5) and 5'nucleotidase (5,6,7,8) activities. With plasma membranes of lymphocytes from pig mesenteric lymph nodes we showed that Con A stimulated (Ca^{2+}) or (Mg^{2+}) ATPase activity (3,9) and more recently that Con A inhibited 5'nucleotidase activity (10). This inhibition displayed high positive cooperativity in untreated membranes or when membrane phospholipid matrix was solubilized with detergent (10). The hypothesis that 5'nucleotidase inhibition resulted from a direct interaction of Con A with the enzyme molecule was postula-

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ted. However inhibition by the lectin of 5'nucleotidase in solubilized membranes was not sufficient to support this hypothesis; in the presence of detergent molecules, the enzyme could remain associated with Con A binding proteins and the observed effect of Con A on 5'nucleotidase could result from binding of the lectin on such a complex. It was necessary to purify 5'nucleotidase in order to know if Con A binds to this enzyme and if this binding results in inhibition of its activity. We obtained pure 5'nucleotidase by two successive affinity chromatographies (11) : the first one on *Lens culinaris* lectin (LcH) immobilized on Sepharose 4B yielded 12 glycoproteins which bound LcH (and Con A which has the same sugar specificity); the second one on 5'AMP-Sepharose 4B gave pure 5'nucleotidase. This enzyme displays only one band on polyacrylamide-SDS electrophoresis (glycoprotein with a molecular weight of about 130,000 Daltons) and its specific activity is very high (2,500-3,000 µmoles Pi/h per mg protein). The purification level of our method is about the same as that obtained for rat liver 5'nucleotidase with a completely different process (12) but its yield (60 per cent) and its speediness are notably better. We used this pure enzyme to study the effects of Con A and some other lectins which are mitogenic or not for lymphocytes.

MATERIALS AND METHODS Preparation of lymphocytes and plasma membranes from pig mesenteric lymph nodes was reported previously (3) as well as membrane characterization by electron microscopy and enzymatic markers. Purification of 5'nucleotidase from membranes solubilized in 0.5 % sodium deoxycholate was published elsewhere (11).

5'nucleotidase activity was determined by measuring the inorganic phosphate (Pi) amount released by 5'AMP hydrolysis, under conditions reported earlier (10-11). Protein concentrations were measured either by Lowry's method (13) or by Böhlen's fluorimetric method (14).

Among studied lectins, Con A and WGA (wheat germ agglutinin) were from Pharmacia, PWM (pokeweed mitogen) from Difco. Divalent Con A was obtained by maleic anhydride treatment of Con A and purified following Greene (15). *Lens culinaris* lectin (LcH) was isolated from common lentils following Howard's method (16) as modified by Hayman and Crumpton (17).

Treatment of whole lymphocytes by 1 % glutaraldehyde was performed as described by Uusitalo and Karnovsky (18).

RESULTS 5'nucleotidase from whole lymphocytes, purified plasma membranes or deoxycholate-solubilized plasma membranes is inhibited by Con A (10). This inhibition results from a specific interaction of the lectin with some sugars and can be prevented or reversed by Methyl-α-D-mannopyranoside (αMM) which binds specifically Con A. With very low lectin concentrations (10 µg/ml) 90 % 5'nucleotidase inhibition is obtained with a membrane fraction (25 µg protein/ml) the specific activity of which is 12 µmoles Pi/h per mg protein. The high positive cooperativity of this inhibition (Hill number n_H varies from 1.8 for solubilized membranes to 2.4 for membrane enzyme) suggests an interaction between at least two lectin receptors.

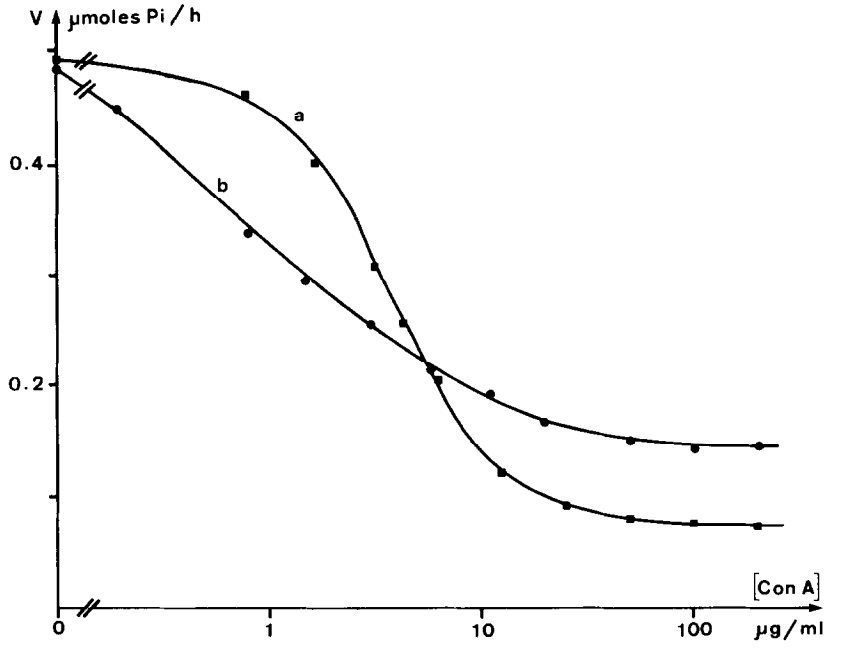

FIGURE 1 : Effects of increasing Con A concentrations on 5'AMP hydrolysis rate (V) with either 5 µg LcH-receptor fraction (*a*-curve) or 0.15 µg purified 5'nucleotidase (*b*-curve), at 37°C and pH 7.5.

This inhibition is of mixed type with both V_m decrease and K_m increase.

The effects of increasing concentrations of Con A on 5'nucleotidase specific activity are presented in figure 1, for LcH-receptor fraction (eluted from LcH-Sepharose 4B by αMM and dialysed) - *a*-curve - and for purified enzyme - *b*-curve - ; enzyme amounts are choosen in order to hydrolyse 5'AMP to the same extent in both cases. Inhibition of 5'nucleotidase in LcH-receptors displays a sigmoid shape, with high positive cooperativity (n_H = 2.0) and the maximum inhibition level reaches 90 %. Purified 5'nucleotidase is still inhibited by Con A but the characteristics of this inhibition are entirely different. At low lectin concentrations, the inhibition level is higher than for membrane-bound enzyme; the presence of other lectin receptors appears to reduce inhibition, by interfering with either the binding of Con A to the enzyme or the mechanism leading to inhibition. The shape of *b*-curve is no longer sigmoid and cooperativity is lost (n_H = 0.8). Moreover the maximum inhibition level is never higher than 75 % (over 7 different preparations of purified 5'nucleotidase).

Lineweaver-Burk plots (19) of 5'nucleotidase activity in the presence or in the absence of lectin show that inhibition of purified enzyme is non-competitive (figure 2), although it was of mixed type for membrane-bound 5'nucleotida-

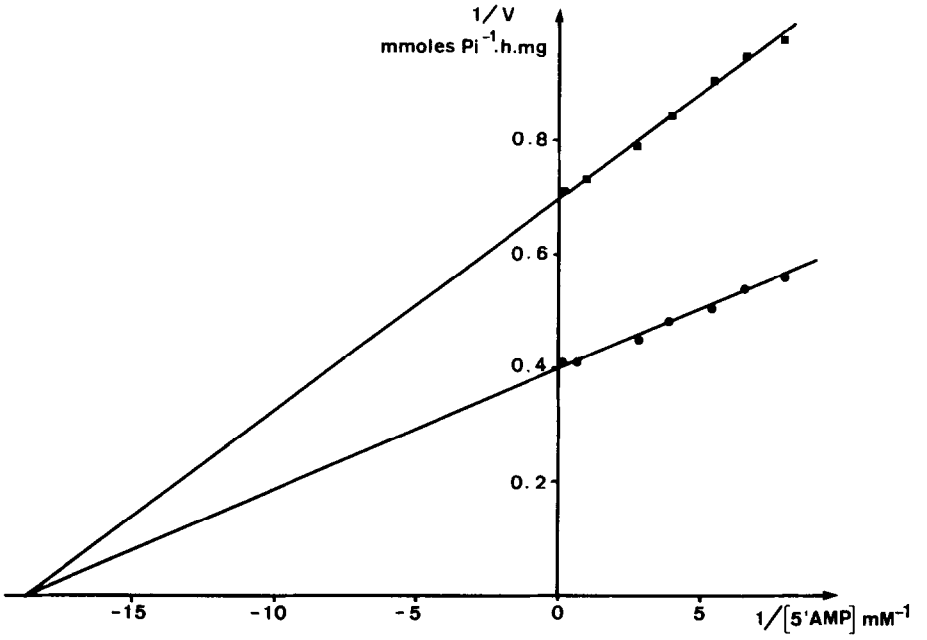

FIGURE 2 : Lineweaver-Burk plots of specific activity (V) of 5'nucleotidase as a function of 5'AMP concentration in the absence (●---●) or in the presence (■---■) of 12 µg/ml Con A.

se (10). The Michaelis constant for 5'AMP (K_m = 55 µM) is not modified by Con A.

Another significant difference in 5'nucleotidase properties between purified and membrane-bound enzyme concerns its pH dependence and was reported elsewhere (11). We showed that pH curve was narrow, with a sharp maximum at pH 7.5 in the case of purified 5'nucleotidase and very broad with maximum activity over a wide pH area (7.5-8.3) for membrane-bound and LcH-receptor 5'nucleotidase. This result indicates that one (or several) lectin-binding protein(s) protect(s) some aminoacids which are ionized at basic pH and are involved in some way in 5'AMP hydrolysis.

The properties of purified 5'nucleotidase and of membrane or LcH-receptor 5'nucleotidase are completely different at least in two points, pH dependence and inhibition by Con A. These differences seem the result of interactions of the enzyme molecule with lectin receptor(s). Indeed the behavior of 5'nucleotidase in LcH-receptors is exactly the same as in whole membranes although LcH-receptors are freed from phospholipids (10,11); so the differences of this behavior with that of purified enzyme are not induced by changes in membrane properties (such as phospholipid viscosity). Moreover these differences do not arise from interactions of 5'nucleotidase with proteins which do not bind Con A. If we assume that

loss of positive cooperativity in purified enzyme inhibition is linked to the absence of LcH receptor(s), we should be able to restore this cooperativity by reconstituting a complex of purified 5'nucleotidase with the fraction of LcH-receptors unretained on 5'AMP-Sepharose. We carried out experiments of this kind with mixture enzyme-receptors concentrated on Diaflow MP-10 membranes in order to obtain the same volume as the initial fraction before affinity chromatography. Under all conditions tested we were unable to restore the properties of the membrane-bound enzyme : 5'nucleotidase activity of this mixture displays a pH dependence identical with that of purified enzyme and its inhibition by Con A is not cooperative and is limited to 75 %.

Effects of other lectins on lymphocyte 5'nucleotidase were also studied. LcH has the same sugar specificity as Con A with 50-fold lower affinity (16) and was used in 5'nucleotidase purification (11) as we showed it inhibited this enzyme exactly as Con A with the exception of its higher concentration for half maximum inhibition (170 µg LcH per mg membrane protein). With LcH we find the same differences between the inhibition of purified enzyme and that of membrane-bound enzyme. Wheat germ agglutinin does not inhibit lymphocyte 5'nucleotidase in contrast with the results of Carraway for plasma membranes of mammary glands (20) and Slavik for rat liver (12). Pokeweed mitogen also has no effects on enzyme activity. Phytohemagglutinin from *Phaseolus vulgaris* (PHA) was not tested as we were unable to eliminate its high phosphatase activity which hydrolysed notably 5'AMP and could be responsible of the so-called ATPase activity reported by Nochumson *et al* (21).

Divalent Con A, obtained by maleic anhydride treatment of native Con A, does not inhibit either purified or membrane-bound 5'nucleotidase. An identical result was found with rat liver membranes (12). However it appears that divalent Con A binds the enzyme as it prevents its inhibition by native Con A.

Schmidt-Ullrich and Wallach showed recently (22) that Con A binding on whole cells was cooperative and this positive cooperativity was lost in glutaraldehyde pretreated cells, probably because receptor cross-linking prevented their mobility during lectin binding. The effects of Con A on 5'nucleotidase activity in whole lymphocytes pretreated or not with glutaraldehyde are shown in figure 3. *b*-curve corresponding to cells treated with the cross-linking reagent is identical to that obtained for purified enzyme (figure 1, *b*-curve). Here again we find loss of cooperativity and decrease in inhibition maximum.

DISCUSSION Con A was shown to have a biphasic effect on membrane 5'nucleotidase activity of rat liver (6) and murine plasmocytoma cells (5) : weak stimulation at low concentration (0.1 µM) and inhibition at higher level. This biphasic effect was lost in purified 5'nucleotidase from rat liver (12) which was only in-

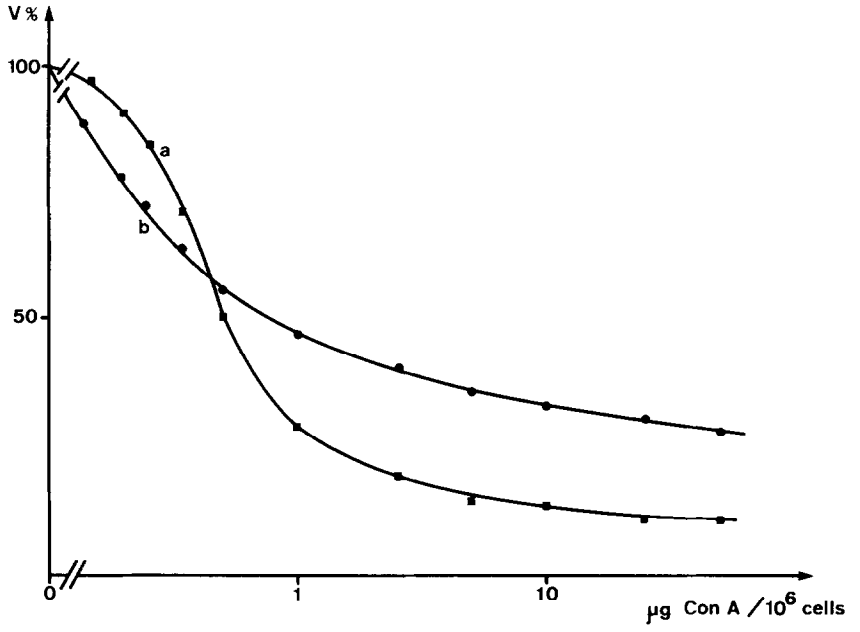

FIGURE 3 : Effects of increasing Con A concentrations on 5'AMP hydrolysis rate (V) by whole lymphocytes either untreated (*a*-curve) or pretreated with 1 % glutaraldehyde (*b*-curve).

hibited by Con A, and in solubilized 5'nucleotidase from plasmocytoma cells which was only stimulated by Con A (8). We have previously reported that Con A inhibits lymphocyte 5'nucleotidase even when small doses of the lectin are used, in the case of whole lymphocytes or plasma membranes, and that 5-fold lower doses of Con A are required to obtain in lymphocytes the same inhibition level as in rat liver (10). The same result is shown here for solubilized and pure 5'nucleotidase. This lack of biphasic effect of Con A was reported for membrane 5'nucleotidase of mammary glands (7,19,23), C_6 glioma cells (24) and rat liver (25).

Con A inhibition of 5'nucleotidase from lymphocyte plasma membranes is an early phenomenon which could play a role in lymphocyte stimulation by this lectin. In order to determine if this inhibition results from direct interaction of Con A with the enzyme, we prepared pure 5'nucleotidase which was found homogenous on polyacrylamide-SDS electrophoresis (11) and independent towards phospholipids. This ecto-enzyme is a glycoprotein acting as membrane receptor of Con A : it binds on Sepharose 4B conjugated with Con A or LcH (10,11,17) and is eluted by αMM, a specific sugar of these lectins; moreover Con A inhibition is obtained with purified enzyme in the absence of any other Con A binding protein. As for membrane-bound 5'nucleotidase, Con A inhibition of purified enzyme is prevented or reversed

by αMM, indicating specific interaction of the lectin with one or several saccharidic group(s) of the enzyme. Inhibition characteristics are completely different for purified enzyme and for lectin receptors which have the same behavior as whole membranes (figure 1). These differences concern maximum inhibition level, lower for purified enzyme (75 instead of 90 %), positive cooperativity, found only in membrane-bound enzyme, and inhibition type, mixed or non-competitive in membrane-bound or purified 5'nucleotidase, respectively. These differences suggest that other Con A receptors are involved in the inhibition when all receptors are present together. The interaction enzyme-receptors must be strong enough to remain after affinity chromatography on LcH-Sepharose. After dissociation this hypothetical enzyme-receptor complex cannot be reconstituted and we cannot restore positive cooperativity or increase inhibition level by mixing purified 5'nucleotidase with the other lectin-binding proteins separated by chromatography on 5'AMP-Sepharose. Glutaraldehyde treatment of whole lymphocytes prevent interaction between Con A receptors and under these conditions 5'nucleotidase inhibition has the same properties as that of pure enzyme.

The absence of inhibitory effects of divalent Con A is more difficult to explain and at least two hypothesis can be suggested : either Con A modification by maleic anhydride affects a site involved in the inhibition mechanism (and apparently not in its binding) or the tetravalent nature of Con A is required for inhibition; these two sites could be on either the same or different enzyme molecules (or sub-units); the interaction with another Con A receptor is necessary to obtain cooperativity (it should be pointed out that an interaction enzyme-receptor exists in the absence of lectin as evidenced by pH dependence changes).

Concerning the possible role of 5'nucleotidase inhibition in lymphocyte stimulation, studies with various lectins give interesting results. Con A and LcH which are T cell mitogens strongly inhibit the enzyme, as already stated. WGA and divalent Con A which have no mitogenic properties have no effect on enzyme activity. However this correlation is not found with PWM which not inhibitor of 5'nucleotidase although it stimulates T lymphocytes; nevertheless it is possible that PWM stimulation mechanism is different from that of Con A as this mitogen stimulates also B lymphocytes. In a recent paper (26) Decker *et al* showed that a lymphocyte membrane glycoprotein with a molecular weight of about 150,000 Daltons is a receptor of both Con A and antilymphocyte immunoglobulin. This glycoprotein could be 5'nucleotidase as Gurd and Evans (27) showed that this enzyme is inhibited by anti-lymphocyte membrane immunoglobulin; inhibition of 5'nucleotidase by antibodies might occur by the same way as Con A inhibition. Finally we must point out that 5'nucleotidase seems necessary for lymphocyte normal function; this enzyme is absent or has very low activity in lymphocytes of patients with chronic lymphocytic leukemia (28 and ourselves, unpublished results), infectious mononucleo-

sis (29) or hypogammaglobulinemia (30). The important role of 5'nucleotidase could be related with transport through plasma membranes of adenosine or other nucleosides obtained by hydrolysis of their monophosphates which are unable to cross the lymphocyte plasma membrane (31).

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