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THE ACTION OF COBRA VENOM PHOSPHOLIPASE A_2 ISOENZYMES TOWARDS INTACT HUMAN ERYTHROCYTES

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Summary

1. Cobra venom phospholipase A_2 from three different sources has been fractionated into different isoenzymes by DEAE ion-exchange chromatography.
2. Treatment of intact human erythrocytes with the various isoenzymes revealed significant differences in the degree of phosphatidylcholine hydrolysis in those cells.
3. It is argued that the plateaus observed in dose-response curves for such treatments may be caused by an increase in lateral surface pressure within the outer half of the membrane due to the production of free fatty acids and lyso-compounds.

Phospholipases A_2 (EC 3.1.1.4) purified from snake venoms (in particular from *Naja naja* venom) have been widely used as tools to study the disposition of phospholipids in biological membranes (for a recent review see Ref. 1). These enzymes have been particularly useful in elucidating the localization of phospholipids in the erythrocyte membrane [2], and the results thus obtained for the localization of phosphatidylcholine in that membrane could be quantitatively confirmed by studies using phosphatidylcholine exchange protein [3, 4]. The reason why phospholipases could successfully be applied became apparent only recently, when analyses involving ^{31}P-NMR showed that even after extensive phospholipase treatments of the membrane, the residual phospholipids and products of hydrolysis remain in a bilayer configuration [5]. Indeed, fatty acids and lysophosphatidylcholine associate to form bilayers [6]. While the reliability of the results and the conclusions drawn from the above studies have been questioned by Martin et

al. [7], their experimental results could also be interpreted as actually supporting them [8]. Prior to lysis of the cells, the absence of which has been emphasized as one of the very first prerequisites in such studies [9, 10], exactly the same degradation ratios for each of the individual phospholipid classes were found, as had been reported in earlier studies [2, 11]. Nevertheless, as outlined below, there indeed may be a pitfall one should be aware of when using highly purified *N. naja* phospholipase A_2 towards intact human erythrocytes and, possibly, also other membrane systems. In our earlier work [2, 11] it has been reported that up to 68% of the phosphatidylcholine fraction in the membrane can be degraded when intact human red blood cells are incubated under isotonic conditions with an excess of phospholipase A_2 purified from *N. naja* venom supplied by Koch-Light Laboratories Ltd. (Colnbrook, U.K.). However, we failed recently to reproduce the result when using the enzyme purified in our laboratory from the very same source, and found a percentage for phosphatidylcholine degradation of only 55 or even lower. Increasing the amount of enzyme and/or time of incubation did not result in an appreciably higher degradation ratio.

The phospholipase A_2 activity found in crude *N. naja* venom, however, is known not to be due to the presence of one single compound, but rather to a complex mixture of various isoenzymes [12]. Since the molecular weights of those isoenzymes do not differ very much (if at all) from one another, they cannot be separated by ordinary gel filtration. There are, however, differences in their isoelectric points which enables a fractionation by ion-exchange chromatography.

N. naja venom obtained from Dr. N.E. Vad (Astik Farm and Laboratory, Bombay, India) was prepurified on Sephadex G-100 and the pooled fractions containing phospholipase A_2 activity were subsequently fractionated on DEAE-cellulose DE 52 (Whatman Ltd., U.K.). About 12 mg protein was loaded on a column of 0.8 × 15 cm, pre-equilibrated with 10 mM Tris-HCl, pH 7.8. The column was eluted with a continuous salt gradient (0.0 to 0.4 M NaCl) and fractions of 0.5 ml were collected. The elution pattern is shown in Fig. 1A. The peak fractions (I, II, III, IV) were each pooled, dialyzed, lyophilized and finally redissolved in a small volume of 50 mM Tris-HCl (pH 7.5)/1mM $CaCl_2$/50% of glycerol. Each of those fractions was assayed for phospholipase A_2 activity as described by Nieuwenhuizen et al. [13]. Activity was recovered in the void volume peak (V_0) — presumably a basic compound — and in four additional fractions (I–IV, Fig. 1A) which were eluted by the NaCl gradient. Each of those fractions was tested for its capacity to degrade phosphatidylcholine in intact human erythrocytes under the conditions previously described [2]. Haemolysis of the cells (determined as described in Ref. 14) in all experiments never exceeded 2%. As can be seen in Fig. 2A, only one (I) of the acidic isoenzymes was able to achieve the optimal degradation of phosphatidylcholine (assayed as described in Ref. 11), namely 68%, which is identical to previously published results [2, 11]. Also in agreement with those earlier observations is the fact that 68% degradation could not be enhanced by increasing the amount of enzyme. Dose-response curves for each of the other three isoenzymes appeared to level off at different values for the

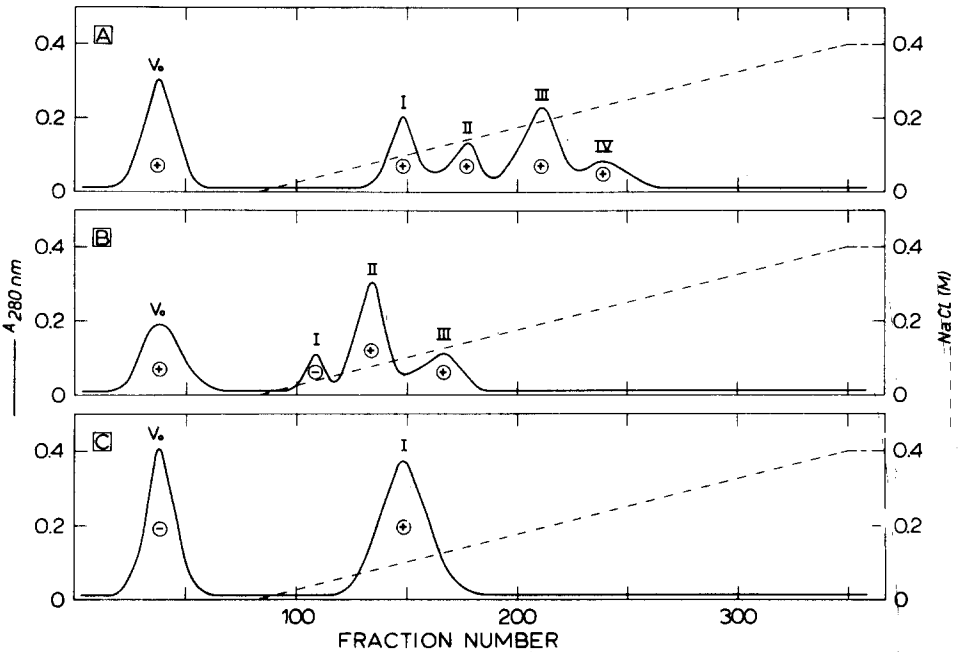

Fig. 1. Fractionation of cobra venom phospholipase A_2 on DEAE-cellulose. For experimental details see the text. A, *Naja naja naja* obtained from Dr. N.E. Vad (Astik Farm and Laboratory, Bombay, India) and prepurified by gel filtration; B, preparation prepurified by gel filtration of *N. naja* venom supplied by Koch-Light. C, *N naja* phospholipase A_2 purified by, and obtained from, Sigma ⊕ and ⊖ indicate respectively the presence and absence of phospholipase A_2 activity in the corresponding fraction.

degradation of phosphatidylcholine which are appreciably lower than 68% (Fig. 2A).

Using the same DEAE column under identical conditions, fractionation of phospholipase A_2, purified as described before [2] from *N. naja* venom supplied by Koch-Light (Batch No. 78887), resulted in four protein peaks (Fig. 1B). The fraction eluted in the void volume (V_0) and only two (II, III) of the three more acidic compounds appeared to contain phospholipase A_2 activity. As is shown by the dose-response curves (Fig. 2B), none of these three isoenzymes was able to degrade the phosphatidylcholine in intact human erythrocytes up to the limiting value of 68%. This agrees with our previous results as mentioned above, i.e. the inability to achieve 68% degradation of phosphatidylcholine, because we used a phospholipase A_2 preparation purified from *N. naja* venom (Koch-Light) by gel filtration [2] that contained a mixture of the three isoenzymes.

DEAE fractionation of phospholipase A_2 from *N. naja*, obtained in a purified form from Sigma Chemical Co. (St. Louis, MO, U.S.A., Lot No. 59C-9014), resulted in a void volume peak (V_0) in which no phospholipase A_2 activity could be detected, and the salt gradient eluted only one single fraction (I) which contained all of the enzymatic activity (Fig. 1C). This latter fraction (I) appeared to be very effective in degrading the phosphatidylcholine in the intact cells and its dose-response curve resembled rather closely that for compound I in Figure 1A.

An interesting question is, of course, why do the dose-response curves

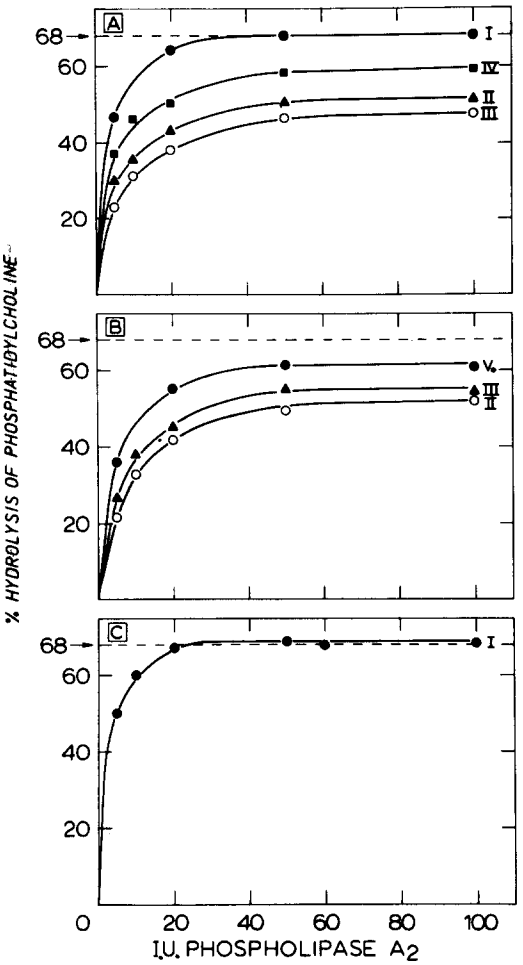

Fig. 2. Dose-response for the hydrolysis of phosphatidylcholine in intact human erythrocytes by treatment with different isoenzymes of *N. naja* phospholipase A_2. 5 ml of 5% suspensions of fresh cells were incubated for 1 h under isotonic conditions at 37° C with the indicated amounts of enzyme (I.U., international unit). For further experimental details see Ref. 2. A, isoenzymes obtained by DEAE fractionation shown in Fig. 1A; B, and C, idem. with respect to Figs. 2B and 2C, respectively 4. V_0 and I—IV refer to individual isoenzymes obtained by DEAE fractionation as shown in the corresponding sections of Fig. 1.

for the degradation of phosphatidylcholine by treatment of intact human erythrocytes with the various isoenzymes level off at different plateaus? It was thought before [15] that the apparent compression state of the phospholipids in the outer monolayer of the human erythrocyte membrane is rather close to the limit (34.8 dynes/cm) above which the *N. naja* phospholipase A_2 can no longer attack the membrane phospholipids. It is well known (see for instance Ref. 16) that the lipid content of erythrocytes decreases slightly with increasing cell age. This may imply that the lateral surface pressure in the membrane of young cells is higher than in older ones. Hence, the possibility was considered that young cells could be much more resistant to attack by the less potent isoenzymes. Subjecting separate samples of young and old cells (fractionated according to Murphy [17] to those phospholipase

A_2 treatments, however, revealed identical dose-response curves (results not shown) for a given isoenzyme.

It is also possible that the lateral pressure in the outer monolayer may increase slightly as the phosphatidylcholine molecules are converted into lyso-derivatives and free fatty acids, since it is well known that these digestion products remain in the membrane. On basis of the 'bilayer couple hypothesis' of Sheetz and Singer [18], Fujii and Tamura [19] recently suggested that the change of cell shape into echinocytes, caused by treatment of intact erythrocytes with *N. naja* phospholipase A_2, is due to an extension of the outer monolyer of the membrane relative to the inner half. Such a selective extension, of course, will imply also an increase in lateral surface pressure. If this pressure in the native membrane is already very close to the limit at which the less potent isoenzymes can still attack their substrates, it seems plausible that only a slight increase in pressure is needed to stop hydrolysis of phosphatidylcholine at a certain level. This reasoning in fact also implies that even the limit of 68% degradation of phosphatidylcholine achieved by the most potent isoenzyme may be the result of the same phenomenon. In contrast to the echinocyte formation caused by treatment of the cells with phospholipase A_2, treatment with sphingomyelinase C will lead to invaginations [19, 20] which are ascribed to the opposite event, namely a shrinkage of the outer monolayer [19] and, consequently, a decrease in lateral surface pressure in that layer. Consistent with this and the above theory is the observation that treatment of intact cells with *N. naja* phospholipase A_2, followed by sphingomyelinase C, results in an increase of the phosphatidylcholine degradation from 68% up to 76%, concomitant with 20% degradation of phosphatidylethanolamine [2, 11]. This view is further supported by the observation that exactly the same figures (76 and 20% hydrolysis for each of these two phospholipids, respectively) are obtained when the less potent *N. naja* isoenzymes are used in combination with sphingomyelinase C. This indeed indicates that the observed phenomena may be due to differences in sensitivity of the isoenzymes towards changes in lateral surface pressure, rather than to differences in product inhibition.

We feel that, in addition to the problems discussed earlier by Dennis and coworkers (see for instance Ref. 21), one should also be aware of the possible occurrence of the effects reported above. In particular when using *N. naja* phospholipase A_2 alone, the results obtained from phospholipid localization studies should be interpreted with some care as they may strongly depend upon the source of which the enzyme is purified. Indeed, it is known that the composition of snake venoms may be influenced by factors like origin and sex of the snake, its diet and the season and frequency of milking [22].

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