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# DISTINCT INHIBITION OF MEMBRANE-BOUND AND LYSOSOMAL PHOSPHOLIPASE $A_2$ BY GLUCOCORTICOID-INDUCED PROTEINS

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**Abstract**—Anti-inflammatory steroids induce the release *in vivo* of antiphospholipase proteins (APP) into the peritoneal cavities of rats. APP were partially purified by ion-exhange chromatography. The main anti-phospholipase activity was recovered in two zones of the elution gradient named APP I and APP II; their molecular weight (mol. wt) was determined with molecular sieve chromatography. Two phospholipase $A_2$ ($PLA_2$) activities were identified from rat peritoneal leucocytes, one with a pH optimum at 4.5 (a lysosomal enzyme) and one with pH optimum at 8.5 (a membrane-bound enzyme); the selective secretion of the former was observed when leucocytes were stimulated by phagocytosis. The effect of APP on both enzyme activities was studied on enzyme preparations from resting leucocytes. APP were also added to leucocytes incubated with or without phagocytozable material. After incubation, $PLA_2$ activities were determined both inside the cells and in the culture medium. APP I revealed a mol. wt of 200 k with a small fragment of 15 k and inhibited membrane-bound $PLA_2$; APP II revealed a mol. wt of 40 k and inhibited lysosomal $PLA_2$.

The importance of $PLA_2$‡ in the generation of proinflammatory eicosanoids has been well demonstrated, and it is now a common belief that many physiological, pharmacological and pathological stimuli that cause prostaglandin and leukotriene biosynthesis share the common property of activating phospholipase [1, 2]. Anti-inflammatory steroids inhibit eicosanoid biosynthesis by preventing arachidonic acid release from phospholipids [3–5]. This action depends on occupation of the receptors and on *de novo* mRNA and protein synthesis [6–9]. We have shown that the $PLA_2$ inhibition by steroids is mediated by a proteinaceous second messenger named "Macrocortin" [10]. We have also reported that dexamethasone and hydrocortisone induce the release of antiphospholipase proteins into the peritoneal cavity of rats, and that these proteins have anti-inflammatory properties in rat carrageenin pleurisy [11] as well as in rat carrageenin hind-paw oedema§. Recently two $PLA_2$ pools have been described in mouse peritoneal macrophages, one in the lysosomes which is active at acid pH and $Ca^{2+}$-independent and one in the plasma membrane which is active at alkaline pH and showing $Ca^{2+}$-requirement for its activity [12].

The present study was undertaken to investigate $PLA_2$ activities in rat peritoneal leucocytes and the effect of APP on these enzymes. We report that rat leucocytes also have two $PLA_2$ activites, one $Ca^{2+}$-independent active at pH 4.5 (a lysomal enzyme) and one $Ca^{2+}$-dependent active at pH 8.5 (a membrane-bound enzyme). The selective secretion of the lysosomal enzyme during phagocytosis is also described. APP are shown to be a mixture of the two major components with a mol. wt of 40 and 200 k respectively. The effect of APP on $PLA_2$ activities is discussed.

## MATERIALS AND METHODS

### *Collection and purification of APP*

Male Wistar rats were injected subcutaneously with dexamethasone (1 mg/kg). Sixty minutes later the animals were killed and the peritoneal cavity washed with 10 ml saline containing 50 mM potassium phosphate buffer, 2 u/ml heparin and 50 $\mu$M phenylmethylsulphonylfluoride to retard proteolysis. The fluid was aspirated and cells removed by gentle centrifugation. The fluid containing crude APP was then dialyzed to equilibrium against 2 × 100 vol of 25 mM pH 8.0 Tris buffer and applied to pre-equilibrated 10 × 1 cm column of Whatman DE 52 DEAE cellulose. After washing the column with 5 column volumes buffer, a linear gradient (0–1 M NaCl) was then run at 0.5 ml/min and 10 min fractions were collected and assessed for anti-$PLA_2$ activity on porcine pancreatic phospholipase $A_2$ as previously des-

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‡ Abbreviations: APP, antiphospholipase proteins; $PLA_2$, phospholipase $A_2$; CELL, intracellular enzymes; EXT, extracellular enzymes; $PGE_2$, prostaglandin $E_2$; $LTB_4$, leukotriene $B_4$; S.D., standard deviation; S.E.M., standard error of the means.

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cribed [11]. Anti-phospholipase chromatographic fractions ex-DEAE were dialyzed to equilibrium against 2 × 100 vol of 0.01 M ammonium carbonate buffer pH 8.0 and lyophylized. After redissolving in 2 ml of the same buffer, the samples (1 ml) were applied to a 70 × 1 cm column of Sephacryl S-300 equilibrated in the ammonium carbonate buffer. The flow rate was 0.5 ml/min and 2 min fractions were collected and tested for anti-$PLA_2$ activity as above [11].

*Identification and assay of $PLA_2$ activity*

*Resting leucocytes.* The technique was modified from Wightman and coworkers' method [12]. Male Wistar rats (250–300 g) were killed through exposure to ether and bled. The peritoneal cavity was washed with 10 ml of 0.9% w/v NaCl solution (saline) containing 2 μm/ml heparin. The cell-rich fluid was harvested and centrifuged (50 *g*), the cells from 6 to 8 animals were pooled and suspended to a final concentration of $10^7$ cells/ml (about 80% mononuclear and 20% polymorphonuclear cells) in heparinized saline. The suspension was placed in a 15 ml glass tube and pulse-sonicated for 5 min while suspended in an ice bath. The homogenate was then centrifuged for 10 min (1000 *g*), and the supernatant was retained as the enzyme source. The protein content was determined with the method of Lowry [13]. Enzyme activities were assayed in Britton and Robinson Universal buffer composed as follows: citric acid 20 mM, potassium biphosphate 20 mM, boric acid 30 mM, diethylbarbituric acid 8 mM and $CaCl_2$ 2 mM; pH values were obtained adding aliquots of 0.2 M NaOH.

Some preliminary experiments were performed in order to determine the optimal conditions for the assay of $PLA_2$ activity. We found that this was linearly dependent on the protein concentration and on the time of incubation for up to 24 hr (data not shown). Since all experiments were performed within these linear ranges for both time and protein, the assay gives the true initial rate for $PLA_2$ activity. The reaction set contained 15 μg of 1000 *g* supernatant proteins (isolated enzymes), 40 nmoles of α′-stearoyl, β-(1-$^{14}$C) arachidonyl, L-3-phosphatidylcholine in a total volume of 200 μl. The reaction was performed at 37° and stopped after 16 hr with 50 μl of 1 M trichloroacetic acid. $^{14}$C-Arachidonic acid was separated from unhydrolysed phosphatide using small columns of Florisil (0.5 × 4 cm) eluted with 2 ml absolute methanol. The radioactivity was then estimated by conventional liquid scintillation techniques. Enzyme activities were corrected for non enzymic hydrolysis and expressed as dpm of $^{14}$C-arachidonic acid. In the experiments where the effect of APP was examined (Fig. 4), the reaction set contained 15 μg of isolated enzymes and 15 μg of APP in a total volume of 150 μl. This was incubated for 60 min at 4°; in some previous (unpublished) exeriments we observed that this "pre-incubation" was necessary to observe an effect of APP on $PLA_2$. Thereafter, 40 nmoles of the labeled phospholipid in 50 μl were added and the reaction was performed at 37° as above.

*Incubated leucocytes.* Rat peritoneal leucocytes were collected with heparinized Krebs as described previously [14]. Groups of $10^8$ cells in 10 ml were incubated for 120 min at 37° in a metabolic shaker with or without killed bodies of *Bordetella pertussis* at a ratio of 1000 bacteria per cell. At the end of the incubation, the cell suspension was centrifuged (50 *g*) and the supernatant stored at 4°; the cells were resuspended in 10 ml of fresh heparinized Krebs and rapidly disrupted by sonication and the homogenate was centrifuged (1000 *g*). The phospholipase activities of 50 *g*-supernatants (EXT) and of 1000 *g*-supernatants (CELL) were then determined in pH 4.5 and 8.5 buffers as described in the previous paragraph. In the experiments where the effect of APP was examined (Fig. 5), at the beginning of incubation either crude APP or anti-$PLA_2$ fractions ex-DEAE suspended in saline (100 μg/ml) were added, while the control incubations received equivalent volumes of saline.

*Materials*

The following drugs and chemicals were used: Dexamethasone sodium phosphate (Decadron Merck, Sharp and Dohme); phenylmethylsulphonylfluoride, porcine pancreatic phospholipase $A_2$ (Sigma); DE52 ion-exchange resin (Whatman); Sephacryl S-300 superfine and molecular weight calibration kits (Pharmacia); α′-stearoyl, β-1-$^{14}$C-arachidonyl, L-3-phosphatidylcholine (The Radiochemical Centre, Amersham); Florisil, 60-100 U.S. Mesh (BDH); *Bordetella pertussis* (Wellcome Laboratories). All other reagents were of "AnalaR" grade.

*Statistics*

The statistical analysis was performed on the absolute values using the *t*-test for non-paired samples. The differences between the values were considered statistically significant only when $P < 0.01$.

## RESULTS

*Purification of APP.* We have reported previously that peritoneal washings from saline-treated animals often contained some inhibitory activity (probably released from the cells by the normal steroid background) but that this was increased several fold by the administration of dexamethasone [11]. Ion exchange chromatography of the fluid from dexamethasone-treated rats, containing crude APP, gave rise to three separate peaks of anti-$PLA_2$ activity eluting at about 0.1–0.5–0.8 M NaCl respectively. The first two peaks (purified APP I and II) were always observed, whilst the third peak was not present in every preparation. For this reason, attention was focused on APP I and II, which underwent molecular sieve chromatography. Figure 1 shows that the anti-$PLA_2$ activity of APP I co-chromatographed with a mol. wt of 200 k with a smaller peak at about 15 k, probably a product of proteolytic cleavage. The activity of APP II was recovered in the 40 k zone.

*Characterization of two $PLA_2$ activities.* Initial experiments were undertaken to determine pH optima for $PLA_2$ activity from resting leucocytes. The isolated enzymes were assayed over a range of increasing pH from 3 to 9 and two peaks of activity were

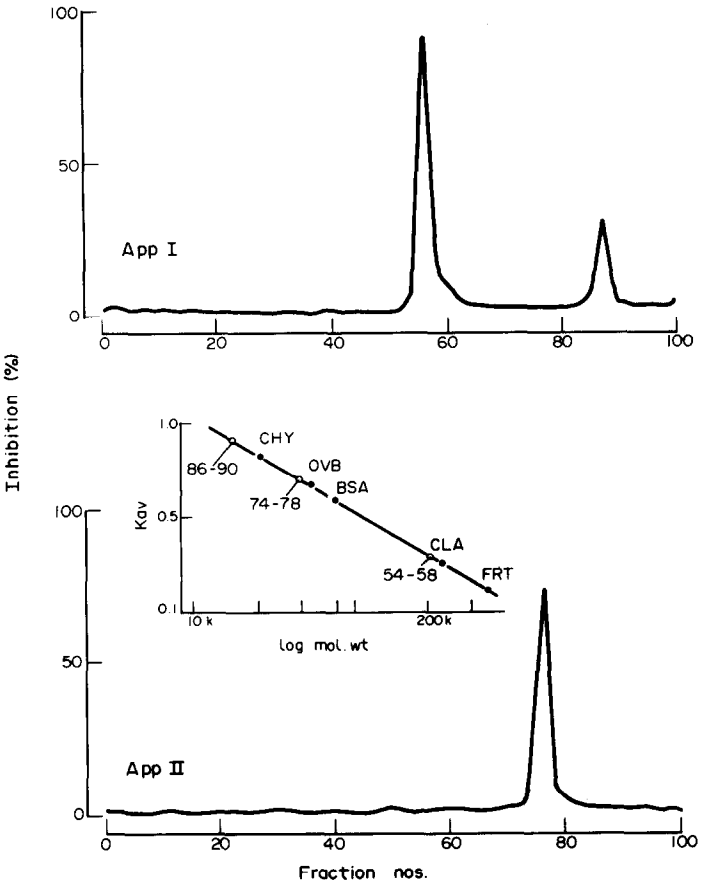


Fig. 1. Estimation of the mol. wt of the anti-$PLA_2$ fractions ex-DEAE, APP I and APP II, with Sephacryl S-300 chromatography. See inset for calibration curve (CHY = Chymotrypsin; OVB = Ovalbumin; BSA = Bovine Serum Albumin; CLA = Catalase; FRT = Ferritin). The fractions were applied to a 70 × 1 cm column of Sephacryl S-300 equilibrated in 0.01 M ammonium carbonate bufffer pH 8.0. The flow rate was 0.5 ml/min and 2 min fractions were collected and assessed for anti-$PLA_2$ activity. The results are expressed as percent inhibition of porcine pancreas $PLA_2$ (see Ref. 11 for analytical procedure of the assay).

observed, in pH 4.5 and 8.5 buffers (Fig. 2). In the subsequent experiments the phospholipase activity was therefore tested only in pH 4.5 and 8.5 buffers. The $Ca^{2+}$ requirement for the two $PLA_2$ activities was estimated by incubation of isolated enzymes in the presence of either 2 mM $CaCl_2$ or 1 mM EDTA and it was noted that only the activity with pH optimum 8.5 required $Ca^{2+}$ ions in the medium of incubation (data not shown). In subsequent experiments, the two $PLA_2$ activities were measured both inside and outside leucocytes incubated with or without phagocytozable material. Figure 3 shows that the pH 4.5 $PLA_2$ was present to a greater extent inside control cells and it was secreted into the culture medium when the cells were stimulated by phagocytosis. The pH 8.5 $PLA_2$ activity remained virtually unchanged in control and phagocytosing leucocytes.

*Effect of crude and purified APP on $PLA_2$ activities from resting leucocytes.* Figure 4 shows that pH 4.5 and 8.5 $PLA_2$ activities were inhibited to a similar extent by crude APP. On the other hand a distinct inhibitory action on the two $PLA_2$ by purified APP was observed; in fact APP I inhibited pH 8.5 $PLA_2$ alone, while APP I inhibited only pH 4.5 $PLA_2$.

*Effect of crude and purified APP on $PLA_2$ activities from incubated leucocytes.* Figure 5 shows that the pH 4.5 $PLA_2$ was inhibited by crude APP and APP II inside the control cells and in the supernatant of phagocytosing cells; thus, bearing in mind that phagocytosis stimulates the release of lysosomal $PLA_2$ (see Fig. 3), it is evident that pH 4.5 $PLA_2$ was chiefly inhibited where present in greater amounts. APP I was not effective on pH 4.5 $PLA_2$ from either control or phagocytosing cells.

pH 8.5 $PLA_2$, which is not secreted during phagocytosis (see Fig. 3), was inhibited by crude APP and APP I in all preparations, APP I being more potent than crude APP. The inhibitory effect of APP I was always more evident inside the cells.

## DISCUSSION

Eicosanoids are not stored within the cells and biosynthesis immediately precedes release. This

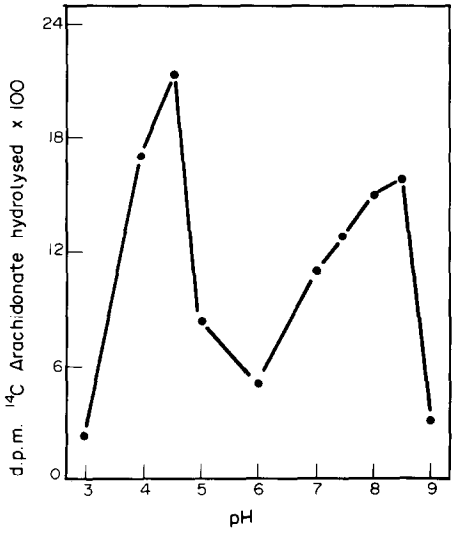


Fig. 2. pH Optima for the hydrolysis of $^{14}$C-arachidonate labelled phospholipid by isolated $PLA_2$ from resting leucocytes (see Methods). Fifteen micrograms of the isolated enzymes were incubated in 200 $\mu$l of Britton and Robinson universal buffers with 40 nmoles of $\alpha'$-stearoyl, $\beta$-(1-$^{14}$C) arachidonyl, L-3-phosphatidylcholine. Incubations were carried out for 16 hr at 37° and stopped with 50 $\mu$l of 1 M trichloroacetic acid. The hydrolysed $^{14}$C-arachidonic acid was separated from unhydrolysed phosphatide by small columns of Florisil (0.5 × 4 cm) eluted with 2 ml of absolute methanol. Enzyme activities were corrected for non enzymic hydrolysis and expressed as dpm of $^{14}$C-arachidonic acid. Each point is the mean of triplicate determinations with S.D. less than 10%.

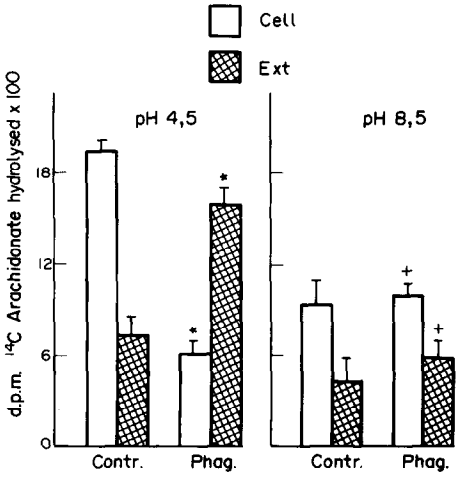


Fig. 3. Pattern of pH 4.5 and 8.5 $PLA_2$ activities from leucocytes incubated alone (Contr) or with killed bodies of *Bordetella pertussis* (Phag) (see Methods). After the incubation, intracellular (CELL) and extracellular (EXT) enzyme activities were tested. Incubations and assessment of $^{14}$C-arachidonic acid hydrolysis were carried out as in Fig. 2. Results are the mean ± S.E.M. of 8 experiments. Key: (*) significantly different ($P < 0.001$) from corresponding control incubations; (†) not significantly different from corresponding control incubations.

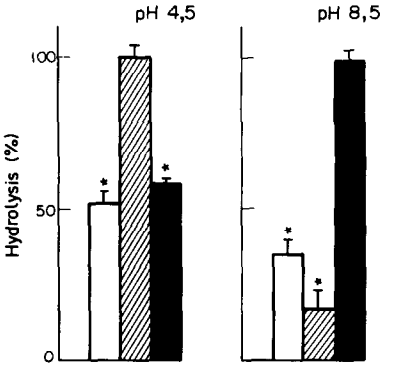


Fig. 4. Effect of crude APP (open columns), APP I (hatched columns) and APP II (filled columns) on pH 4.5 and 8.5 $PLA_2$ activities from resting leucocytes. Fifteen micrograms of APP were incubated with 15 $\mu$g of isolated enzymes in pH 4.5 and 8.5 buffers (total volume = 150 $\mu$l) for 60 min at 4°. Then 40 nmoles/50 $\mu$l of $\alpha'$-stearoyl, $\beta$-(1-$^{14}$C)-arachidonyl, L-3-phosphatidylcholine were added. Thereafter the incubation for the assessment of enzymic activities and the hydrolysis of $^{14}$C-arachidonic acid was carried out at 37° for 16 hr as in Fig. 2. The results are the mean ± S.E.M. of 6 experiments and are expressed as percent of the hydrolysis shown in Fig. 2. Key: (*) significantly lower ($P < 0.001$) than the hydrolysis shown in Fig. 2.

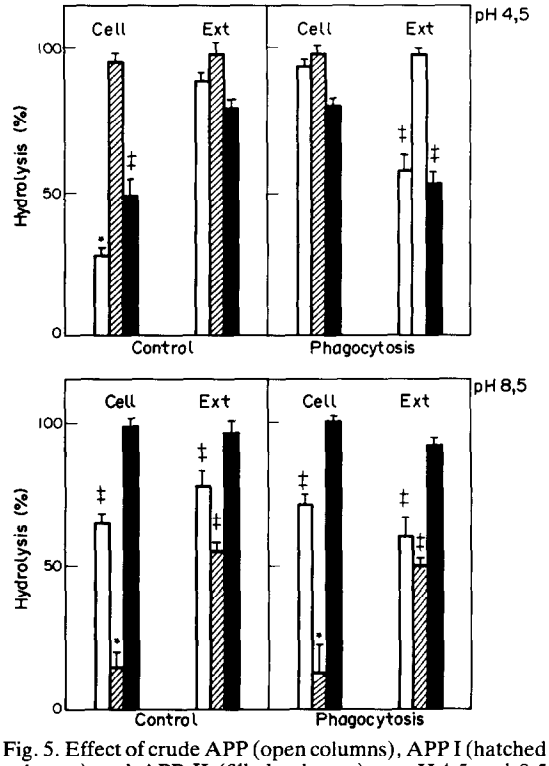


Fig. 5. Effect of crude APP (open columns), APP I (hatched columns) and APP II (filled columns) on pH 4.5 and 8.5 activities from leucocytes incubated alone (Control) or with killed bodies of *Bordetella pertussis* (Phagocytosis) (see Methods). At the beginning of incubation APP (100 $\mu$g/ml) were added to the cells. At the end of incubation, CELL and EXT $PLA_2$ activities were assessed (see legends of Figs. 2 and 3). The results are the mean ± S.E.M. of 6 experiments and are expressed as percent of the hydrolysis shown in Fig. 3. Key: significantly (‡) ($P < 0.01$) and (*) ($P < 0.001$) lower than the hydrolysis shown in Fig. 3.

biosynthesis is limited by the availability of C-20 unsaturated fatty acids, such as arachidonic acid as free precursor. The precursor acids can arise from the phospholipid fraction of the cells through the action of the hydrolytic enzyme phospholipase $A_2$ [1, 2] or phospholipase C [15], depending on the cell system examined.

Hsueh *et al.* [16] have shown that in rabbit alveolar macrophages there are two phospholipases, one in the lysosomes and one elsewhere. Two $PLA_2$ pools have also been described in mouse peritoneal macrophages, a lysosomal enzyme which is active at acid pH and $Ca^{2+}$-independent, and a membrane-bound enzyme which is active at alkaline pH and showing $Ca^{2+}$ requirement for its activity [12]. The results reported here show that also in rat peritoneal leucocytes (mainly mononuclear cells), two distinct pools of $PLA_2$ are found.

Due to the different characteristics and localization of these two $PLA_2$ activities, a different mechanism of their activation is conceivable.

Changes in intracellular $Ca^{2+}$ concentration is frequently assumed as a major (if not the major) controlling factor in the activation of membrane-bound $PLA_2$ [17]. Phospholipid methylation [18] and cyclic nucleotides [19] have recently been proposed as other regulators of this $PLA_2$.

The mechanism through which lysosomal $PLA_2$ is controlled is at present practically unknown. Nevertheless, an important role for lysosomes in eicosanoid biosynthesis has been found during phagocytosis and interaction with immunocomplexes [20, 21].

Macrophages that are directly involved in the inflammatory process can thus respond to phlogistic stimuli by activating both $PLA_2$ activities, which in turn can trigger the synthesis of pro-inflammatory eicosanoids or mediate directly vascular alterations occurring during inflammation, such as hyperaemia [22].

Anti-inflammatory steroids inhibit eicosanoid biosynthesis by preventing arachidonic acid release from phospholipids [3–5]. This action depends on receptor occupation and on *de novo* mRNA and protein synthesis [6–9]. We and others have shown that the effect of steroids is mediated by proteinaceous second messengers which inhibit directly $PLA_2$ [10, 23, 24]. It has been observed that these proteins also inhibit phospholipase C and D [25]. We have also reported that these proteins have anti-inflammatory effects in rat carrageenin pleurisy [11] as well as in rat carrageenin hind-paw oedema.*

In the present study we report that crude APP is a mixture of two major components, one called APP I which has a mol. wt of about 200 k, with a smaller fragment of 15 k (probably a product of proteolytic cleavage) and one, called APP II, which has a mol. wt of about 40 k. APP I selectively inhibits the membrane-bound $PLA_2$, APP II selectively inhibits the lysosomal $PLA_2$. Crude APP reduced both pH 4.5 and 8.5 $PLA_2$ activities, as can be expected, it being a mixture of both components.

The relationship existing between APP I and APP II is very uncertain at the present time. Although we used a protease inhibitor, we cannot exclude that APP II is a proteolytic fragment of APP I or, more likely, that APP I and II are two fragments of a larger protein.

The results reported here give greater emphasis to the anti-inflammatory properties of glucocorticoid-induced proteins; as a matter of fact, we have demonstrated here that two components of APP exert selective inhibition of both membrane-bound $PLA_2$, sensitive to soluble inflammatory stimuli and lysosomal $PLA_2$ which is activated by phagocytozable material.

It has been reported that soluble membrane-mediated inflammatory stimuli can trigger the formation of only $PGE_2$, while phagocytozable inflammatory mediators stimulate the synthesis of both cycloxygenase and lipoxygenase products. Thus the membrane-bound $PLA_2$ could be in the vicinity only of cycloxygenase whilst the lysosomal $PLA_2$ may release arachidonate in the vicinity of both 5-lipoxygenase and cycloxygenase [26]. We have recently shown that 40 k APP at the same concentration used in the present paper inhibits the synthesis of both $PGE_2$ and $LTB_4$ to a similar extent.* These results are in agreement with the selective inhibition of lysosomal $PLA_2$ by APP II.

As APP exert a direct inhibitory effect on isolated enzymes, some questions arise about the mechanism by which these proteins could reach the two described $PLA_2$ in a whole-cell system.

When leucocytes were incubated with APP I, only pH 8.5 $PLA_2$ activity was inhibited and this action was always more evident inside the cells, regardless of phagocytosis, i.e. the inhibition was greater in the presence of higher enzyme concentrations. The inhibitory interaction with a membrane-bound enzyme can be explained by two mechanisms: (i) APP I does not enter the cell but interacts with the enzyme situated on the outer cell membrane surface; (ii) APP I enters the cell through a specific carrier.

When leucocytes were incubated with APP II, only pH 4.5 $PLA_2$ activity was inhibited and again the inhibition was greater where the enzyme was present in considerable amounts, i.e. it was more evident inside the control cells and outside the phagocytosing cells. In the latter case it is conceivable that APP II interacts with the secreted lysosomal enzyme. With regard to the inhibition inside the control cells, there are two possible mechanisms by which the enzyme and the inhibitor can come into contact: (i) APP II is taken up by the cell through a mechanism of pinocytosis, then the pinocytotic vesicle merges into the lysosome; (ii) APP II enters the cells through a specific carrier and interacts with the lysosomal membrane. The first hypothesis is supported by the work of Brown *et al.* [27] and Steinman *et al.* [28] who described a similar pinocytotic mechanism for other proteins.

Whatever the mechanism that makes these interactions possible, it is evident from this study that glucocorticoid-induced proteins are a family of very specific phospholipase inhibitors, that can represent an endogenous mechanism for the control of inflammatory responses.

* L. Parente, M. Di Rosa, R. J. Flower, P. Ghiara, R. Meli, P. Persico, J. A. Salmon and J. N. Wood, *Eur. J. Pharmac.* submitted for publication.

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