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GLUCOCORTICOIDS INDUCE THE FORMATION AND RELEASE OF ANTI-INFLAMMATORY AND ANTI-PHOSPHOLIPASE PROTEINS INTO THE PERITONEAL CAVITY OF THE RAT

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1 Dexamethasone and hydrocortisone induce the release of anti-phospholipase proteins into the peritoneal cavities of rats.

2 Adrenocorticotrophic hormone (ACTH) also releases these proteins in normal but not in adrenalectomized rats.

3 Peritoneal lavage proteins were separated by ion-exchange and size exclusion chromatography. The anti-phospholipase activity occurred in four separate fractions with the major component having an apparent mol.wt. of 40 k.

4 Column fractions containing these anti-phospholipase proteins had anti-inflammatory effects in the rat carrageenin pleurisy model whereas other fractions were inactive.

5 The proteins appear to be identical to macrocortin and lipomodulin, the 'second messengers' of glucocorticoid hormone action on the arachidonate system.

Introduction

By inhibiting phospholipase A_2, certain steroids prevent the biosynthesis by cells of pro-inflammatory mediators such as the prostaglandins, hydroxy-acids and leukotrienes (Gryglewski, Panczenko, Korbut, Grodzinska & Ocetkiewicz, 1975; Hong & Levine, 1976; Nijkamp, Flower, Moncada & Vane, 1976; Tam, Hong & Levine, 1977; Blackwell, Flower, Nijkamp & Vane, 1978; Flower & Blackwell, 1979; Di Rosa & Persico, 1979; Carnuccio, Di Rosa & Persico, 1980; Hirata, Schiffman, Venkatasubramanian, Salomon & Axelrod, 1980; Blackwell, Carnuccio, Di Rosa, Flower, Parente & Persico, 1980; Hirata, Del Carmine, Nelson, Axelrod, Schiffman, Warabi, De Blas, Nirenberg, Manganiello, Vaughan, Kumagai, Green, Decker & Steinberg, 1981; Carnuccio, Di Rosa, Flower & Pinto, 1981). This effect is specific for the glucocorticoids (Hong & Levine, 1976; Nijkamp *et al.*, 1976; Tam *et al.*, 1977; Blackwell *et al.*, 1978; Flower & Blackwell, 1979; Di Rosa & Persico, 1979; Carnuccio *et al.*, 1980; Hirata *et al.*, 1980; Blackwell *et al.*, 1980; Hirata *et al.*, 1981), and depends upon receptor occupation as well as transcriptional and translational events within the target cells (Danon & Assouline, 1978; Russo-Marie, Paing & Duval, 1979; Flower & Blackwell, 1979; Di Rosa & Persico, 1979; Carnuccio *et al.*, 1980; Hirata *et al.*, 1980; Blackwell *et al.*, 1980; Hirata *et al.*, 1981). The anti-inflammatory effect of glucocorticoids in the rat is also dependent on such a chain of events (Tsurufuji, Sugio & Takemasa, 1979).

We and others (Flower & Blackwell, 1979; Di Rosa & Persico, 1979; Carnuccio *et al.*, 1980; Blackwell *et al.*, 1980; Hirata *et al.*, 1980; 1981) have demonstrated that the anti-phospholipase effect is exerted by a 'second messenger' synthesized by and released from cells in the presence of the steroid. We have isolated this substance from rat peritoneal leucocytes (where it is apparently stored) and shown it to be a polypeptide of 15k mol.wt.—'Macrocortin' (Blackwell *et al.*, 1980); a protein of 40k mol.wt. ('Lipomodulin') having many similar properties has also been isolated from steroid-stimulated rabbit polymorphonuclear leucocytes by Hirata *et al.* (1980, 1981).

We now describe experiments demonstrating that both these proteins are released into the pleural and peritoneal cavities following systemic glucocorticoid or adrenocorticotrophic hormone (ACTH) administration. Moreover, crude extracts or chromatography fractions containing these proteins possess anti-inflammatory effects in a model of acute inflammation of the pleural cavity.

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Methods

Collection of anti-phospholipase A_2 proteins in vivo

In a previous study (Blackwell *et al.*, 1980) we demonstrated that an anti-phospholipase protein was released by glucocorticoids from suspensions of rat peritoneal leucocytes *in vitro*. We argued that a similar event should occur *in vivo* following the systemic administration of steroids, and that the anti-phospholipase protein thus released should produce a local anti-inflammatory effect. The pleural and peritoneal cavities of rats are easily accessible and contain a large number of 'resident' leucocytes, and are thus convenient both for the collection of secreted cellular proteins and the determination of inflammatory responses. Male Wistar rats (200–250 g) were injected subcutaneously with steroid or ACTH dissolved in 0.1 ml 0.9% w/v NaCl solution (saline) (or the equivalent volume of saline alone) and killed after various times with an intracardiac injection of 0.2 ml Euthasate and the peritoneal or pleural cavity washed with 10 ml saline containing 50 mM potassium phosphate buffer, 2 u/ml heparin and 50 μM phenylmethylsulphonylfluoride to retard proteolysis. Fluid was aspirated, the cells removed by centrifugation and the protein content of the sample estimated spectrophotometrically at 280 nM (A_{280}). Minor adjustments in sample volume were made to ensure that all protein concentrations were equivalent. This is important in crude extracts because large amounts of proteins interfere with the phospholipase assays in a non-specific manner (albumin seems to be the major inhibitor, IC_{50} approx. 5 mg/ml). To obtain reproducible results it was essential that the control animals also received an injection (i.e. of saline) at the same time as the dexamethasone-treated animals. Only rats that have been 'acclimatized' to the laboratory atmosphere for 24 h were used.

Phospholipase A_2 assay

Reaction sets for the phospholipase assay comprised the following: 0.1 ml 0.5 M Tris buffer (pH 8.0) containing 50 mM $CaCl_2$ (optimal concentration) and 200 ng porcine pancreatic phospholipase A_2; 0.3 ml peritoneal protein (or other sample or buffer blank); 0.1 ml of a 200 nCi/ml solution of α'-palmitoyl, β-,1-^{14}C-oleoyl, Lα-phosphatidyl choline (final concentration 0.15 μg per 0.5 ml) dispersed in buffer. Samples were incubated for 1 h at 4°C before the addition of the substrate. The reaction is too rapid at 37°C to allow accurate rate measurements but a 2 min incubation at room temperature permitted measurements over a linear range. At the end of the incubation the reaction was quenched with 1 ml ice-cold methanol. After the further addition of 2 ml chloroform the samples were extracted by vortex mixing for 15 s and centrifuged. The top (aqueous) phase was discarded and an aliquot of the lower phase taken to dryness under N_2. Labelled oleic acid was separated from unhydrolysed phosphatide by t.l.c. on silica gel, using chloroform, methanol, acetic acid (90:10:1) as a developing solvent. The radioactivity in the fatty acid zone and the phosphatide zone was estimated by conventional liquid scintillation counting techniques. A plot of % hydrolysis vs log [phospholipase A_2] enabled us to estimate how much enzyme was 'inactivated' by the inhibitor. We defined 1 unit as that amount which 'inactivated' 1 pmol of enzyme. Assuming a 1:1 relationship between inhibitor and enzyme and a mol.wt. of 40k (see below), this corresponds to approximately 40 ng protein. Dexamethasone itself produced less than 5% inhibition at the highest concentration tested (100 μg/ml).

Isolation of anti-phospholipase proteins

Hirata *et al.* (1980, 1981) found that their anti-phospholipase protein (lipomodulin) could be purified using a combination of DEAE cellulose and G-75 Sephadex chromatography. We have also found that these techniques are effective for the isolation of the anti-phospholipase protein fractions from the crude peritoneal proteins. Typically, we started with the accumulated peritoneal proteins taken by lavage from 5 rats (about 150 mg protein in 35 ml saline).

(a) *DEAE cellulose chromatography* After collection the proteins were dialysed to equilibrium against 2 × 100 vol of 25 mM pH 8.0 Tris buffer and applied to a pre-equilibrated 10 × 1 cm column of Whatman DE52 DEAE cellulose (DEAE Sephadex was also effective) and washed though with 5 column vol buffer. A linear gradient (0–1 M NaCl in the same Tris buffer) was then run at 0.5 ml/min and 10 min fractions were collected and assessed for anti-phospholipase activity. On some occasions fractions were assayed for anti-inflammatory activity (see below). All analytical procedures were performed at 4°C.

(b) *CM cellulose chromatography* After collection the proteins were dialysed to equilibrium against 2 × 100 vol of 25 mM phosphate buffer pH 6.5, and applied to a pre-equilibrated 10 × 1 cm column of Whatman CM52 CM cellulose and washed through with 5 column vol buffer. A linear gradient (0–1 M NaCl in the same phosphate buffer) was then run at 0.5 ml/min and 10 min fractions were collected and assessed for antiphospholipase activity. On some occasions fractions were assayed for anti-

inflammatory activity. All analytical procedures were performed at 4°C.

(c) *Sephadex G-75 chromatography* After collection the proteins were dialysed to equilibrium against 2 × 100 vol of 0.02 M ammonium bicarbonate buffer pH 8.0, and lyophilized. After redissolving the protein in 2 ml of the same buffer, the sample was applied to a 100 × 1 cm column of Sephadex G-75 (medium) equilibrated in the ammonium carbonate buffer. The flow rate was 0.38 ml/min and 10 min fractions were collected and assayed for anti-phospholipase activity as described. All analytical procedures were performed at 4°C.

(d) *h.p.l.c. gel exclusion chromatography* of partially purified anti-phospholipase protein was performed at room temperature using a Waters h.p.l.c. equipped with a TSK-2000 SW column eluting at 0.5 ml/min with 0.2 M ammonium carbonate (pH 8.0). After DEAE chromatography the partially purified active fractions (peptide II) were pooled, dialysed to equilibrium against 2 × 100 vol of 0.02 M ammonium bicarbonate (pH 8.0) and lyophilized. The residue was redissolved in 100 μl column buffer and injected onto the column. The column was precalibrated with a mixture of proteins of known molecular weight (blue dextran, ribonuclease (RNUC), dihydrofolate reductase (DHFR), chymotrypsinogen A(CHYMO), ovalbumin (OVALB), bovine serum albumin (BSA) and aldolase (ALD)) dissolved in the same buffer and a graph of the Kav (Kav = (Ve − Vo)/(Vt − Vo); where Ve is the elution volume of the protein; Vo the void volume, and Vt the total gel volume of the column) versus log mol.wt. plotted. When the sample was run, 2 min fractions were collected and each aliquot assayed for anti-phospholipase activity as described.

Anti-inflammatory testing

Peritoneal proteins were collected from saline or dexamethasone-treated rats as described and dialysed to equilibrium against 2 × 100 vol of 0.02 M ammonium carbonate buffer pH 8.0. After lyophilization the proteins were either used immediately for anti-inflammatory studies or were subjected to further chromatographic isolation as described. In the latter case, fractions were screened for anti-phospholipase activity, pooled together in small groups then dialysed against 2 × 100 vol of the ammonium carbonate buffer and lyophilized. Shortly before testing, the crude proteins or fractions were resuspended in saline. Anti-inflammatory activity was assessed using the conventional carrageenin pleurisy technique as follows. Rats (male, Wistar, 200–250 g) were lightly anaesthetized with ether and pleurisy was initiated by injection into the pleural cavity of 0.2 ml of a 1% w/v carrageenin suspension prepared either in saline alone or reconstituted samples of peritoneal proteins or column fractions. After 4 h, the rats were killed by exposure to ether, the exudates collected, and the volume and numbers of leucocytes present were recorded.

Materials

The following drugs and chemicals were used. Euthasate solution for euthanasia (Willows Francis); dexamethasone sodium phosphate (Decadron Merck, Sharpe and Dohme); hydrocortisone sodium succinate, ACTH (porcine), phenylmethylsulphonylfluoride, actinomycin D, cycloheximide, and puromycin dihydrochloride (Sigma); CM52 and DE52 ion-exchange resin (Whatman); Sephadex G-75 medium, and molecular weight calibration kits (Pharmacia); specifically labelled α′-palmitoyl, β-, 1-^{14}C-oleoyl, L-α-phosphatidylcholine, sp.act. 56 mCi/mmol, was purchased from Amersham International; and the porcine pancreatic phospholipase A_2 from Boehringer, and carrageenin (lot 590214) from Marine Colloids, Inc. USA. The TSK-2000 SW h.p.l.c. column was obtained from Anachem. All other reagents were of 'Analar' grade or the highest purity available. Adrenalectomized rats and sham operated controls were obtained from Charles River.

Statistical analyses were performed using the unpaired Student's *t* test.

Results

Anti-phospholipase activity of peritoneal lavage fluid

In initial studies we compared the ability of crude pleural or peritoneal lavage fluid obtained from saline or dexamethasone-treated rats to inhibit phospholipase A_2-catalysed hydrolysis of a specifically labelled phosphatidycholine. We found that there was often some inhibitory activity already present in pleural or peritoneal lavage fluid from saline-treated rats but that this was increased several fold by the administration of dexamethasone (1 mg/kg s.c.) 1 h before collection (see below). Peritoneal fluid was used for most of our studies because it generally contained larger amounts of inhibitory activity and was less contaminated with blood components than pleural fluid. The average amount of crude peritoneal proteins recovered from control rats was 35.5 ± 3.0 mg/rat (mean ± s.e.mean, $n = 12$). There was no significant change after preinjection with 1 mg/kg dexamethasone (31.4 ± 1.6 mg/rat, $n = 10$). Likewise there was no significant difference between

the total protein recovered from adrenalectomized rats, whether or not they were treated with dexamethasone, or from the sham operated controls (34.6 ± 4.4 mg/rat, $n = 7$, compared with 33.6 ± 1.8 mg/rat, $n = 7$).

Figure 1 shows that the amount of anti-phospholipase activity released into the peritoneal cavity after 1 h was approximately proportional to the log dose of steroid but the dose-response relationship was shallow; however, the relationship is more complex than this figure implies since the dose of dexamethasone affects the latency as well as the magnitude of the response with lower doses of the drug having a longer latency and *vice versa*. Although clear effects were seen at the lowest dose of steroid tested (20 μg/kg) we chose 1 mg/kg for our studies.

We observed a great variation in the generation of anti-phospholipase proteins both after dexamethasone pretreatment (1 h) (range 113-255 u) and in control saline-injected animals (15U-108 u). The reasons for this variability are not known but might include the fluctuating endocrine state of the animal, variations in the amount of proteolytic enzymes, and slight alterations in technique etc.

Figure 2 shows the time course of the appearance of phospholipase inhibitory protein following dexamethasone treatment. Substantial amounts were present 0.5 h after injection of steroid, with the peak being at 1 h. After 3–4 h the elevated levels had declined, in some cases below control levels. All measurements referred to hereafter were therefore made at 1 h.

Table 1 shows the effect of steroid and ACTH

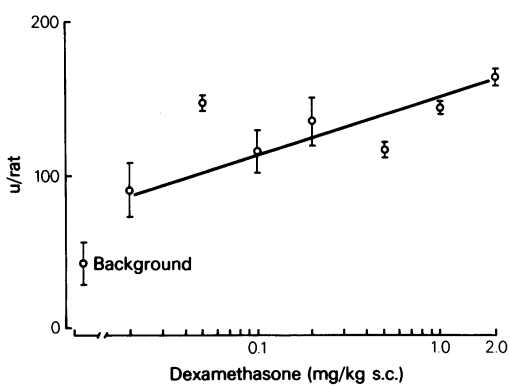

Figure 1 The relationship between dose of dexamethasone given subcutaneously in the back of the neck and the appearance 1 h later of anti-phospholipase activity in rat peritoneal fluid. Each point is the mean of triplicate estimations on the pooled proteins from 2 rats and bars show s.e.mean.

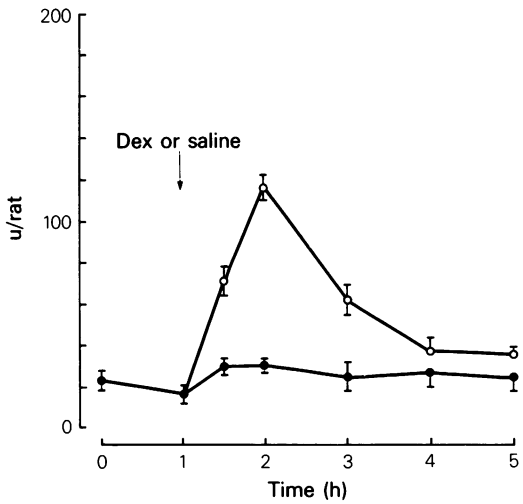

Figure 2 Time-course of dexamethasone-induced appearance of anti-phospholipase proteins in the rat. The graph shows one of two experiments and each point is the mean (with s.e.mean) of triplicate estimations on the pooled peritoneal proteins from 2 rats. The rats received either steroid (1 mg/kg s.c., ○) or the equivalent volume of saline (●). At various intervals after the injection, rats in the control and treated groups were killed and the peritoneal proteins collected, processed and assayed as described in Methods.

Table 1 Effect of steroids and ACTH on the content of anti-phospholipase proteins in rat peritoneal fluid

	Anti-phospholipase activity				
Type of rat	*Background*	*Dexamethasone (1 mg/kg)*	*Hydrocortisone (10 mg/kg)*	*ACTH (300 μg/kg)*	*Net increase induced by Dex (1 mg/kg)*
Normal (or sham operated)	81.02 ± 5.30 (11)	193.37** ± 10.02 (25)	144.4** ± 1.09 (2)	153.86** ± 1.3 (2)	112.35 ± 10.0 (25)
Adrenalectomized	29.92* ± 11.34 (6)	129.27** ± 14.98 (6)	68.77 ± 0.48 (2)	15.52 ± 0.9 (2)	99.35 ± 14.98 (6)

** Significantly greater than background levels ($P < 0.001$); * significantly different from sham operated controls ($P < 0.05$)

Results show mean values ± s.e.mean, (n) = number of experiments

treatments on the secretion of the anti-phospholipase proteins into peritoneal fluid of both normal and adrenalectomized rats. As noted above, lavage fluid from control saline-treated animals always contained some inhibitory activity: in part this was an artefact probably due to the presence of large amounts of protein which interfere with the assay but some of which may be genuine inhibitory protein released from cells by the normal steroid background. This is supported by the finding that the 'background' in adrenalectomized animals was much lower than normal or 'sham operated' controls, even though they responded normally to steroid pretreatment. Table 1 also shows that hydrocortisone as well as dexamethasone released the protein into lavage fluid. ACTH was also effective but not in adrenalectomized rats.

Table 2 illustrates the effects of inhibitors of protein and RNA synthesis on dexamethasone-induced release of antiphospholipase proteins as determined in two experiments. Actinomycin D at 1 and 5 mg/kg (given simultaneously with the steroid) produced 65.9 and 94.3% inhibition of production. Cycloheximide at 5 and 10 mg/kg produced 74.2 and 79.2% inhibition. Puromycin was almost inactive at 10 mg/kg, but at 50 mg/kg reduced levels below the background, again suggesting that there is a constant 'steroid drive' causing the release of this protein in control animals.

The anti-phospholipase activity of the crude peritoneal protein mixture was destroyed or severely attenuated when it was boiled for 5 min, but practically unaffected by heating to 70°C for 5 min. This property is very reminiscent of the activity of macrocortin (Blackwell *et al.*, 1980). We also confirmed the ability of the crude protein extracts to block prostaglandin generation by phagocytosing rat peritoneal leucocytes, as does macrocortin (Blackwell *et al.*, 1980): using our previously described technique the generation by control cells was 10.9 ± 1.2 ng PGE_2 equivalents/10^7 cells. Incubation of the cells with 1 μg/ml dexamethasone reduced this to 5.0 ± 0.4 ng (54.8% inhibition). Incubation of the cells in the presence of 3 μl/ml of crude peritoneal proteins from saline-treated animals inhibited production by 19.1% (8.9 ± 0.8 ng E_2) but incubation with proteins from steroid-treated rats produced a 55% inhibition (4.9 ± 0.9 ng E_2). In each case the inhibition by the peritoneal proteins was reversed by the addition of 1 μg/ml sodium arachidonate, indicating that the leucocyte cyclo-oxygenase itself was not inhibited. This experiment reinforces the idea that the proteins elicited *in vivo* by the steroid have very similar biological properties to those released from suspensions of leucocytes *in vitro*, and also underlines the concept that there is a certain amount of the protein present in control rats.

Partial purification of anti-phospholipase proteins (Figure 3)

Figure 3 is a composite diagram showing the chromatographic profiles of anti-phospholipase inhibitory activity from rats treated with either saline, dexamethasone, or dexamethasone plus puromycin. Up to four separate zones of antiphospholipase activity were seen; peptide II (eluting at about 0.4 M NaCl) was the major product and was always observed, but peptides I, III, and IV were not present in every preparation and are possibly complexes with, or proteolytic cleavage products of II (see below).

A highly active fraction could also be separated from the crude proteins using CM cellulose (Figure

Table 2 Effect of inhibitors of protein and RNA synthesis on the content of anti-phospholipase (Anti-P-lipase) proteins in rat peritoneal fluid

Expt.		*Inhibitor*	*Dose (mg/kg)*	*Anti-P-lipase proteins (u)*	*% mean inhibition*
1	Saline	None	–	38.3 ±0.66	–
	Dex 1 mg/kg	None	–	156.98 ± 0.48	Nil
	,,	Actinomycin D	2.0	78.73 ± 1.10	65.9
	,,	Cycloheximide	5.0	68.88 ± 0.81	74.2
	,,	Puromycin	10.0	143.91 ± 0.79	11.0
2	Saline	None	–	70.54 ± 0.60	–
	Dex 1 mg/kg	None	–	215.33 ± 0.67	Nil
	,,	Actinomycin D	5.0	78.79 ± 0.43	94.3
	,,	Cycloheximide	10.0	100.63 ± 0.65	79.2
	,,	Puromycin	50.0	19.61 ± 0.7	> 100

Each figure is the mean ± s.e.mean of triplicate assays on the pooled peritoneal proteins from 2 rats. Dex = dexamethasone, injected 1 h before peritoneal lavage.

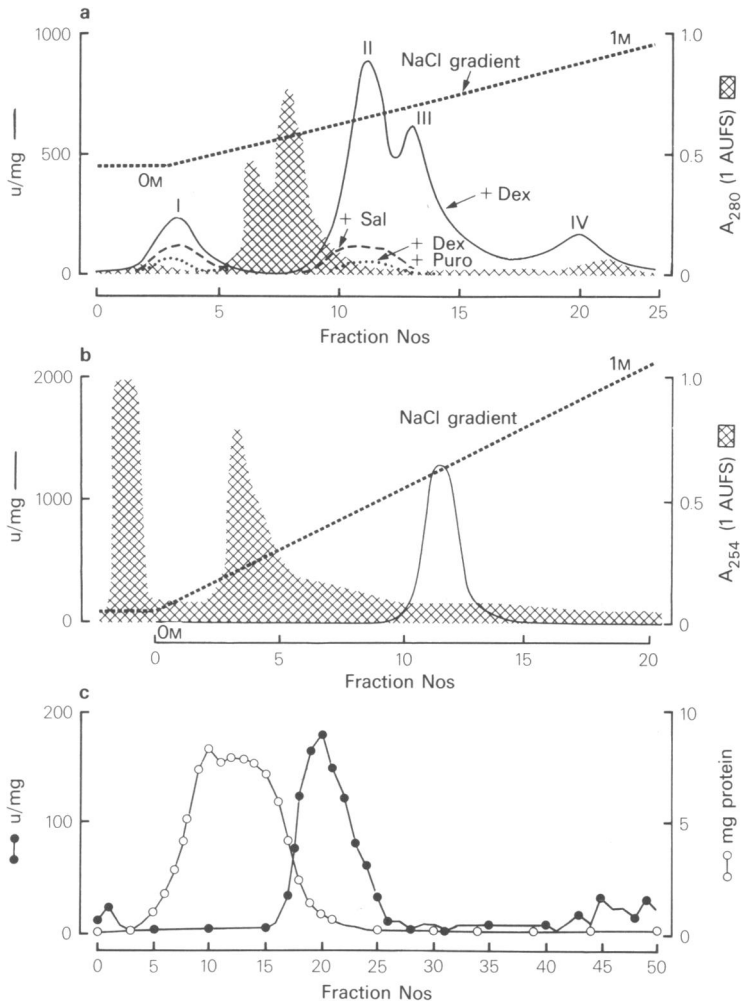

Figure 3 Partial purification of anti-phospholipase proteins by column chromatography using DEAE cellulose (a), CM cellulose (b) and Sephadex G-75 (c), as described in Methods. Rats receiving saline only (Sal), or dexamethasone plus puromycin (Dex + Puro) had substantially less anti-phospholipase activity than the dexamethasone-treated animals. The chromatograms shown here represent the peritoneal proteins from 5 rats. AUFS–absorbance units full scale.

3b). With this technique the majority of the crude proteins passed through the column, and the NaCl gradient eluted the absorbed material including one major zone of anti-phospholipase activity which appeared at about 0.6 M NaCl. This material was either peptide II itself, or a biologically active fragment, since partially purified (DEAE) preparations containing this peptide also eluted at this position on CM cellulose.

When the crude peritoneal proteins were subjected to G75 Sephadex chromatography (Figure 3c) the anti-phospholipase activity was only partially resolved from the other proteins. The position of elution was approximately the same as ovalbumin (mol.wt. 43 k) but small zones of activity were also seen close to the void volume of the column (> 70 k, possibly an artefact) and in the low mol.wt. zone (Figure 3c). When these active fractions (from the G75) were pooled and applied to the DEAE column, anti-phospholipase activity eluted in the same fractions as peptide II.

To estimate the mol.wt. more closely, partially purified fractions of peptide II (ex DEAE) were concentrated and chromatographed on a h.p.l.c. gel exclusion column which had previously been calibrated with marker proteins (Figure 4). The major

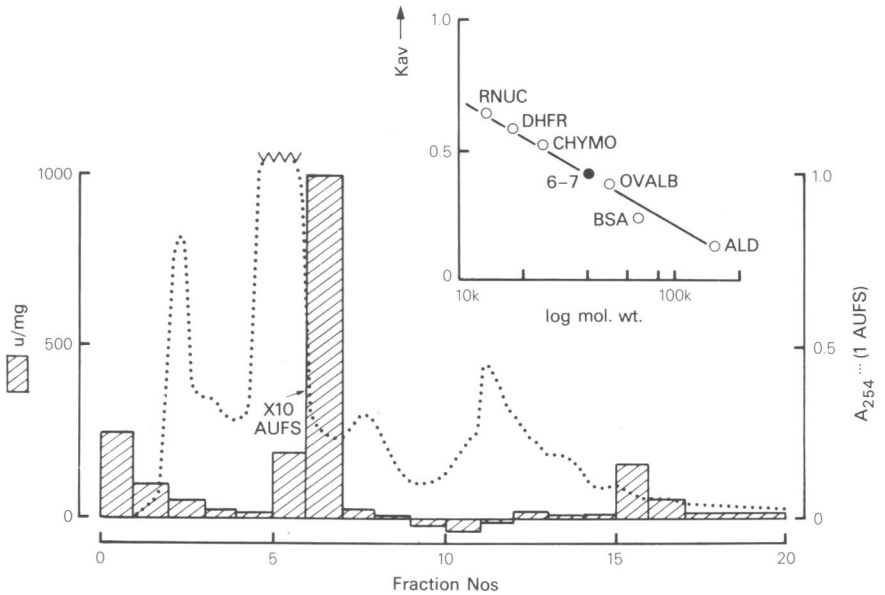

Figure 4 Estimation of the mol.wt. of the partially purified anti-phospholipase protein (peptide II) by h.p.l.c. gel exclusion chromatography (see Methods). The bulk of the activity eluted in fraction 7, corresponding to an apparent mol.wt. of 39–40 k (see inset for calibration curve). AUFS-absorbance units full scale.

biologically active fractions eluted corresponding to an apparent mol.wt. of 39–40 k, but other active zones were sometimes seen including one at > 200 k, 15 k as well as 4.2 k, 3.4 k, and 1.6 k. It is possible that the low mol.wt. peaks are proteolytic fragments of the 40 k peptide, and it is significant that a peak at 15 k was sometimes observed, since this corresponds to the estimated molecular weight of the phospholipase inhibitory peptide released from perfused lungs and leucocytes (Blackwell *et al.*, 1980). That this protein was not invariably seen could be because it eluted in the same fractions as phospholipase itself (mol.wt. approx. 13–15 k). We frequently encountered this enzyme in our extracts and its presence sometimes complicated the interpretation of chromatographic data.

Hirata and his colleagues (1980, 1981) provided evidence that 'lipomodulin' is a glycoprotein. In one experiment we also observed that anti-phospholipase activity on partially purified preparations of peptide II was removed after passage through Con-A Sepharose and could be thereafter eluted, enriched approximately twofold, with 0.5 M α-methyl-D-mannoside.

Boiling the column fractions containing peptide II for 5 min or more led to substantial (> 80%) or complete inactivation of the anti-enzyme activity.

Anti-inflammatory activity

Anti-phospholipase proteins should be anti-inflammatory because they would prevent the formation by cells of prostaglandins, hydroxy-acids, leukotrienes, and other lipid mediators. To test this hypothesis we first did experiments where preparations of crude peritoneal proteins from dexamethasone-treated rats were dialysed (to remove salts, steroid etc.), then concentrated and injected together with carrageenin into the pleural cavity of rats. The progress of this 'carrageenin pleurisy' was assessed after 4 h by measuring the exudate volumes and the leucocyte infiltration, which were compared to those in rats treated with carrageenin only, or carrageenin mixed with peritoneal proteins from saline-injected animals. Table 3 shows the results. Highly significant reductions in both exudate volume and leucocyte number were seen in rats injected with the peritoneal proteins from dexamethasone-treated rats, but extracts from saline-treated rats were not significantly different from controls (i.e. carrageenin alone), although a slight elevation was sometimes seen (see expt. 1, Table 3). Boiling the peritoneal proteins almost completely abolished the activity (expt. 2, Table 3), and pretreatment of rats with 2 mg/kg actinomycin D also substantially reduced the anti-inflammatory properties of their peritoneal proteins (see expt. 3, Table 3). Allowing for about 10–20% loss of active material during sample preparation, the peritoneal proteins prepared from 1 rat were sufficient to produce a highly significant anti-inflammatory effect in 1 test rat (but, perhaps because of proteolysis, this

Table 3 Anti-inflammatory effect of anti-phospholipase proteins from dexamethasone (Dex)-treated rats in rat carrageenin pleurisy test

Expt.	*Treatment*	*Exudate vol.* (ml/rat)	*Leucocytes* (10^6/rat)
1	Saline	0.84 ± 0.08 (11)	90.8 ± 9.1 (11)
	Proteins from saline-treated rats	0.89 ± 0.13 (14)	95.7 ± 12.5 (15)
	Proteins from Dex-treated rats	0.41 ± 0.09 (14)**	53.6 ± 6.9 (15)***
2	Saline	0.59 ± 0.02 (3)	35.4 ± 3.7 (3)
	Proteins from Dex-treated rats	0.30 ± 0.13 (3) **	10.6 ± 3.3 (3)***
	Boiled proteins from Dex-treated rats	0.62 ± 0.07 (3)	28.7 ± 2.6 (3)
3	Proteins from saline-treated rats	0.84 ± 0.06 (4)	55.2 ± 8.7 (4)
	Proteins from Dex-treated rats	0.27 ± 0.08 (3)*	32.5 ± 5.1 (4)*
	Proteins from Dex + AcD-treated rats	0.77 ± 0.04 (4)	50.1 ± 1.8 (4)
4	Saline	0.83 ± 0.10 (6)	64.0 ± 7.0 (6)
	Anti-phospholipase fractions from DEAE cellulose (see Figure 3a)	0.62 ± 0.07 (6)	38.6 ± 4.1 (6)*
	All other fractions‡	0.97 ± 0.04 (15)	55.4 ± 3.6 (15)
5	Saline	0.50 ± 0.18 (3)	66.9 ± 5.4 (3)
	Anti-phospholipase fractions from CM cellulose (see Figure 3b)	0.27 ± 0.12 (3)	38.8 ± 7.1 (3)*
	All other fractions‡	0.42 ± 0.06 (8)	59.6 ± 2.8 (8)

Values are mean ± s.e.mean (n)
Significance of difference shown with respect to control (first entry for each experiment): *$P<0.05$; **$P<0.01$; ***$P<0.001$
‡ These were individually assessed in small groups but are combined here to save space.
AcD = actinomycin D, 2 mg/kg

stoichiometry did not always hold for purified fractions, see below).

These experiments demonstrate that soluble, heat-labile, non-dialysable, anti-inflammatory factors are present in the peritoneal lavage fluid of rats which have received steroids but not in saline-treated rats. However, they do not prove that these factors are the anti-phospholipase proteins. Evidence that there is an association between the two is furnished by the other data in Table 3 in which the peritoneal proteins were fractionated and the location of the anti-inflammatory proteins ascertained. Expt. 4 demonstrates that the DEAE cellulose fractions which contain the anti-phospholipase protein peptide II also possess anti-inflammatory properties. Little or no activity was seen in other fractions indicating either that peptides I, III and IV were absent from these preparations, or that although active against the soluble phospholipase *in vitro* these proteins (proteolytic fragments of II?) have little activity against the cellular enzyme *in vivo*. Expt. 5 (Table 3) demonstrates that a similar situation obtains when the peritoneal proteins are chromatographed on CM cellulose. The anti-phospholipase fractions also show anti-inflammatory activity, whereas the other fractions did not.

Because of the semiquantitative nature of the carrageenin pleurisy test it is not possible to say what proportion of the total anti-inflammatory activity of the crude proteins is due to anti-phospholipase peptides.

Discussion

In 1979 we proposed that steroids blocked prostaglandin synthesis in guinea-pig perfused lung by causing target cells to synthesize and release a polypeptide with anti-phospholipase properties (Flower & Blackwell, 1979). We later demonstrated that this inhibitor (macrocortin) was present preformed in rat leucocytes and was released in the presence of glucocorticoids (di Rosa & Persico, 1979; Carnuccio *et al.*, 1980; Blackwell *et al.*, 1980). The inhibitory action of steroids on these cells was correlated with and absolutely dependent upon their macrocortin content: when the preformed stores were exhausted the cells became refractory to steroids and only regained sensitivity when the peptide was resynthesized (Carnuccio *et al.*, 1981).

The mol.wt. of macrocortin was estimated as 15 k by gel chromatography (Blackwell *et al.*, 1980).

Hirata and his colleagues have reported that rabbit polymorphs release a protein of mol.wt. 40 k with anti-phospholipase activities (lipomodulin). Both macrocortin and lipomodulin have substantially similar properties and several lines of evidence suggest they are related moieties. We propose that macrocortin (15 k) is a (biologically active) fragment of the 40 k lipomodulin, perhaps produced by proteases in the crude extract. The evidence supporting this contention is that: (a) Hirata and his colleagues found that in the absence of protease inhibitors, lipomodulin decomposed to various fragments including a 16 k and 24 k peptide (Hirata *et al.*, 1980; 1981) and 16 k is close to the estimated mol.wt. of macrocortin; (b) in experiments described here we detect active peptides at 39–40 k, 15 k as well as some low mol.wt. fragments: the mol.wt. of peptide II is identical to that reported for lipomodulin by Hirata *et al.* (1980, 1981); (c) in a recent unpublished collaborative study between our two groups it was found that samples containing macrocortin exhibited a high degree of cross-reactivity with antibodies to lipomodulin. It seems plausible that all the peptides with anti-phospholipase activity found here are proteolytic breakdown products of one 40 k peptide. Our earlier experiments, in which we described the 15 k protein (Blackwell *et al.*, 1980), were done in perfused organs and simple cell suspensions, conditions where one might anticipate a high proteolysis. In these experiments we have routinely employed protease inhibitors.

The most likely source of the anti-phospholipase proteins found in these experiments is the resident peritoneal leucocytes (mainly macrophages). These cells release preformed anti-phospholipase proteins *in vitro* when stimulated with steroids and we have previously found that the cells are depleted of the proteins if the host animal is treated 1 h beforehand with steroids (Blackwell *et al.*, 1980). This agrees well with the current finding that the content of peritoneal anti-phospholipase proteins is maximum at 1 h after pretreatment with 1 mg/kg dexamethasone.

Although not yet purified to homogeneity, it seems that the anti-inflammatory activity in the crude peritoneal proteins co-chromatographs with the anti-phospholipase activity (peptide II) in at least two different systems. This strongly suggests that the two effects are mediated by the same agent, an idea reinforced by the similarities in the way in which both the anti-phospholipase and anti-inflammatory activities of the peritoneal proteins are increased by steroids, blocked by actinomycin D and destroyed by boiling. We already know from our previous work (as well as that of Hirata and colleagues) that these proteins can prevent the generation of pro-inflammatory lipid mediators by cells and reduce cell chemotaxis, and the idea that these peptides are important in the control of inflammation is further supported by work recently described by the same group, who found that many patients with chronic inflammatory disease have auto-antibodies to lipomodulin in their plasma (Hirata *et al.*, 1981).

Clearly the effect of steroids described here is a rapid one, but it is a matter of practical laboratory experience that the anti-inflammatory effect of dexamethasone is clearly manifested when given 30 min before the phlogogenic agent in experimental models of inflammation.

In addition to mediating some of the effects of exogenous steroids, these proteins must be released when leucocytes are stimulated by endogenous steroids from the adrenal cortex. This idea is supported by the finding that ACTH leads to the liberation of anti-phospholipase proteins from leucocytes. It is a well documented fact that rats exposed to new environmental situations respond with an elevation of plasma corticosteroids secondary to ACTH release (e.g. see Hennessy, Heybach, Vernikos & Levine, 1979). It is therefore plausible that the release of anti-phospholipase proteins constitutes an endogenous mechanism for the control of the inflammatory response, in the same way that endorphins act to limit pain, and interferon and lysozyme, viral and bacterial infections. This mechanism can be activated by the pituitary-adrenal axis in response to environmental situations (perhaps explaining certain aspects of the enigmatic 'counter irritant effect'), as well as by the administration of exogenous steroids. One consequence of this would be that adrenalectomized animals would be less able to terminate inflammatory reactions: there is already some evidence that this may be so (Sendelbeck & Yates, 1970).

Steroids have such diverse and widespread action throughout the organism, and it is evident that it must be the target cells themselves which translate their general biochemical message into specific and appropriate cellular responses. Most of these effects seem to be mediated by the induction or repression of enzymes. The release of anti-phospholipase proteins from leucocytes (and probably other cells) is one way in which the steroids can exert their anti-inflammatory action; it is not the only way, neither is it the only way in which they could affect the prostaglandin system (Saeed, McDonald-Gibson, Cuthbert, Copas, Schneider, Gardiner, Butt & Collier, 1977; Moore & Hoult, 1980; Araki, Peck, Lefer & Smith, 1981). Nevertheless it is a mechanism which could account for many hitherto unexplained features of steroid action, and which therefore deserves careful scrutiny.

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