Br. J. Pharmac. (1978), **62**, 79–89

PHOSPHOLIPASE A_2 ACTIVITY OF GUINEA-PIG ISOLATED PERFUSED LUNGS: STIMULATION, AND INHIBITION BY ANTI-INFLAMMATORY STEROIDS

G.J. BLACKWELL, R.J. FLOWER, F.P. NIJKAMP[1] & J.R. VANE

Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS

1 A simple double-isotope assay for phospholipase A_2 activity of perfused organs is described.

2 Guinea-pig lungs perfused through the pulmonary circulation exhibit a low background enzyme activity. This activity is blocked by dexamethasone, betamethasone and hydrocortisone, mepacrine, procaine or chlorpromazine. Aspirin and indomethacin are without effect.

3 Mechanical trauma, antigen challenge or injections of bradykinin, rabbit aorta contracting substance-releasing factor (RCS-RF) or histamine increase 'basal' phospholipase activity. The effect of these agents, except that of bradykinin, is blocked by dexamethasone or mepacrine.

4 The blocking effect of steroids is cumulative and dose-dependent. They do not work in cell-free systems. Inhibition by mepacrine is rapid and is effective in cell-free lung homogenates.

5 It is suggested that agents which liberate prostaglandin endoperoxides and thromboxane A_2 from perfused lungs do so by activating phospholipase A_2.

Introduction

A wide variety of stimuli release rabbit aorta contracting substance (RCS), now known to be a mixture of prostaglandin endoperoxides and thromboxane A_2 (TXA_2) (Hamberg, Svensson & Samuelsson, 1975) from isolated perfused lungs of the guinea-pig. The list includes histamine (Piper & Vane, 1969; Bakhle & Smith, 1972; Liebig, Bernhauer & Peskar, 1974), bradykinin (Piper & Vane, 1969; Vargaftig & Dao Hai, 1972), 5-hydroxytryptamine (Alabaster & Bakhle, 1970; 1976), and rabbit aorta contracting substance-releasing factor (RCS-RF) (Piper & Vane, 1969; Nijkamp, Flower, Moncada & Vane, 1976) as well as mechanical trauma or antigen challenge (Piper & Vane, 1969; 1971; Palmer, Piper & Vane, 1973; Liebig *et al.*, 1974). Injection or infusion of the precursor arachidonic acid into the preparation also leads to the generation of prostaglandin endoperoxides and TXA_2, (Vargaftig & Dao Hai, 1972; Palmer *et al.*, 1973; Hamberg & Samuelsson, 1974) and it has therefore been inferred that 'substrate availability' is an important rate-limiting step in this reaction, and that agents which release these fatty acid derivatives do so by liberating the substrate from some endogenous lipid pool. The arachidonate content of most cells is high but little arachidonic acid is present in the 'free' form (Kunze & Vogt, 1971; Samuelsson, 1972; Haye, Champion & Jacquemin, 1973). This 'lipid pool' must therefore comprise neutral lipid esters of arachidonic acid (mono- di- or tri-glycerides or cholesterol esters), or else the phospholipid store. It is on this last possibility that most attention has been focussed and the phosphatide pool has been implicated as the source of substrate for prostaglandin biosynthesis in several tissues (Haye, *et al.*, 1973; Flower & Blackwell, 1976; Blackwell, Duncombe, Flower, Parsons & Vane, 1977). Given that this idea is true, then the enzyme phospholipase would be implicated in the release mechanism. We have earlier shown that RCS-RF releases arachidonic acid from isolated perfused lungs of guinea-pig (thus leading to prostaglandin and TXA_2 release) and speculated that this could be due to a stimulation of lung phospholipase A_2 (Nijkamp *et al.*, 1976). We also showed that the release of prostaglandins and TXA_2 was blocked by anti-inflammatory steroids and suggested that their activity might be related to a suppression of phospholipase activity. We now present further evidence to support this concept and also show stimulation of phospholipase activity by several other substances.

[1] Present address: Rudolf Magnus Institute for Pharmacology, Vondellaan 6, Utrecht, The Netherlands.

Methods

Guinea-pig lung perfusion and superfusion bioassay

Lungs were removed from male guinea-pigs (200–300 g) and were perfused through the pulmonary artery

with Krebs solution (37°C) at 10 ml/min as described by Piper & Vane (1969). In some experiments lungs were taken from guinea-pigs that had been sensitized to ovalbumin (100 mg s.c. and 100 mg i.p.) 2–4 weeks earlier. Anaphylactic shock in these perfused lungs was induced by injection of 2 μg ovalbumin into the pulmonary artery.

For the bioassay of prostaglandin endoperoxides and thromboxanes, the effluent from the lungs was used to superfuse in cascade, strips of rabbit aorta, rat stomach and (on some occasions) guinea-pig trachea (Piper & Vane, 1969). The sensitivity and selectivity of the assay tissues was increased by an infusion into the lung effluent of a mixture of antagonists (Piper & Vane, 1969) which prevented the actions of histamine, acetylcholine, catecholamines and 5-hydroxytryptamine, together with indomethacin (1 μg/ml) to prevent endogenous synthesis of prostaglandins by the tissues (Eckenfels & Vane, 1972).

Preparation of specifically labelled 2-oleoyl phosphatidylcholine

This compound was prepared as described by Blackwell, *et al.* (1977), except that [9,10 (n)-^{3}H]- and not [1-^{14}C]-oleic acid was used as the fatty acid for esterification.

Liquid scintillation counting procedures

The radioactivity in the samples was estimated with a Beckman LS-150 liquid scintillation counter. The scintillation counting fluid used was Beckman 'Cocktail D' : 5 g PPO and 100 g naphthalene per litre dioxane. The double-isotope method depends on accurate counting so all samples were counted such that $2\sigma \not> 0.5\% \bar{x}$. All ct/min values were converted to d/min by the AES ratio method. The ^{14}C 'spillover' into the ^{3}H channel and appropriate correction to obtain the true ^{3}H d/min was calculated by a conventional formula with an Olivetti P602 computer.

Calculation of results

Each perfused lung acted as its own control. Because of this and the inherent variability between preparations it was rarely possible to obtain sufficient data for a meaningful statistical analysis. IC_{50} values calculated from partial dose-response curves are referred to as 'estimated IC_{50} values'.

Solubility of drugs

Steroids are difficult to dissolve in aqueous media and we used formulations of the soluble salts whenever possible. For most of these the vehicle was not available from the manufacturing company, but we have confirmed our data wherever possible by using the pure steroid, dissolved in ethanol and diluted 9:1 (v/v) with 0.9% w/v NaCl solution (saline).

Each steroid was infused into the lungs for 30 min before the experiment, for Nijkamp *et al.* (1976) demonstrated that these agents require some time to act. Non-steroid drugs were also infused for 30 min to obtain a comparison.

The following drugs and chemicals were used: dexamethasone sodium phosphate (Decadron), indomethacin and procaine hydrochloride (Merck), mepacrine hydrochloride and chlorpromazine (May and Baker), dexamethasone, hydrocortisone, oestradiol-17β and aldosterone (Sigma), betamethasone (Betsolan) and hydrocortisone sodium succinate (Efcortelan, Glaxo), [1-^{14}C]-oleic acid, 57 mCi/mmol, [9,10 (n)-^{3}H]-oleic acid 2.4 Ci/mmol, and phosphatidyl-[n-methyl-^{14}C]-choline, 60 mCi/mmol (Radiochemical Centre, Amersham), bradykinin (Sandoz), ovalbumin and pig pancreas phospholipase A_2 (Boehringer Manheim G.m.b.H), histamine acid phosphate and 5-hydroxytryptamine creatinine sulphate (BDH). All other reagents (buffer salts, chromatography solvents) were of 'Analar' grade or the highest purity obtainable. RCS-RF was prepared and purified as previously described (Nijkamp *et al.*, 1976). X-ray film for autoradiography was obtained from Kodak.

Results

Release from perfused lungs of biologically active substances, and inhibition of release by steroids

Figure 1 shows the release of RCS from isolated perfused lungs of guinea-pig induced by injections of arachidonic acid and RCS-RF. This RCS is a mixture of prostaglandin endoperoxides and TXA_2 (Hamberg *et al.*, 1975; Nijkamp *et al.*, 1976). Also shown in Figure 1 is the effect of infusion of two concentrations of dexamethasone (1.5 μg/ml and 3.0 μg/ml). This steroid caused a dose-related reduction in the output of RCS caused by RCS-RF, but not that generated directly from arachidonate. All other anti-inflammatory steroids tested (as well as oestradiol-17β) also produced this effect and in Table 1 their IC_{50} concentrations and their relative anti-inflammatory potencies are compared. This table is derived from our earlier paper (Nijkamp *et al.*, 1976). Dexamethasone did not inhibit the release of RCS by bradykinin (Figure 2). Figure 3 shows that dexamethasone also blocks the releasing activity of histamine and 5-hydroxytryptamine without effect on release by bradykinin or arachidonic acid.

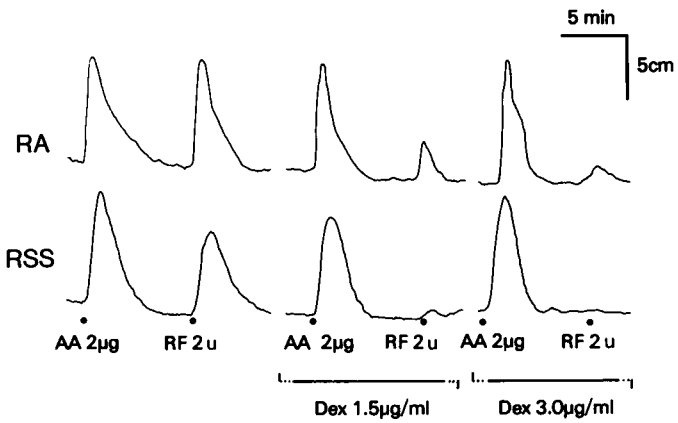

Figure 1 Release of rabbit aorta contracting substance (RCS) from guinea-pig perfused lung as indicated by a contraction of the rabbit aortic strip (RA) and rat stomach strip (RSS). Arachidonic acid (AA) or RCS-releasing factor (RF) injections were given alternately into the pulmonary artery. There was a dose-related reduction in responses to RCS-RF but not arachidonic acid observed during dexamethasone (Dex) infusions (which were begun 20–30 min before the responses).

Development of an assay for phospholipase A_2 activity

Elution and recovery of radioactivity from the perfused lung. An initial experiment was done to determine the salient characteristics of the assay system. The labelled phosphatide (0.1 μCi) was injected into the lung; 1 ml serial fractions of the effluent were collected and the radioactivity in an aliquot of each was measured. With a flow rate of 10 ml/min, approximately 50% of the injected radioactivity was recovered within 30 s, 85% within 1 min and the remainder within 2 minutes. For routine sample collection we therefore collected for periods longer than 2 min, usually for 5 minutes. Recovery of the radioactive phospholipids was good and fairly reproducible, in one lung it was carefully measured and found to be 96.4 ± 4.5% (mean ± s.e. mean; $n = 3$). Comparable recoveries were found in other experiments also. However, when a similar technique was used with oleic acid the amount recovered in the effluent varied significantly not only from one experiment to another, but also between serial injections into the same lung. This effect was probably partly due to the fatty acid becoming absorbed onto the glassware. This situation would clearly compromise the precision of the assay since oleic acid liberated from the phosphatides in the lung would be recovered in variable amounts in the effluent. To make the assay more precise we used a commercially prepared phosphatide, phosphatidyl-[^{14}C]-choline as an internal marker. Thus, hy-

Table 1* Effect of some anti-inflammatory steroids on the release of prostaglandin endoperoxides and thromboxane A_2 from perfused guinea-pig lungs in response to rabbit aorta contracting substance-releasing factor injections

Steroid	IC_{50}** *(μg/ml)*	*Relative potency‡*	*Relative anti-inflammatory potency‡‡*
Dexamethasone	1.4	31.2	25.0
Betamethasone	1.4	31.2	25.0
Triamcinolone	4.2	10.4	5.0
Fludrocortisone	5.1	8.5	10.0
Prednisolone	6.4	6.8	4.0
Corticosterone	33.6	1.3	0.4
Cortisone	43.5	1.0	0.8
Hydrocortisone	43.8	1.0	1.0
Ostradiol-17β	1.9	23.1	?

* Data calculated from results previously published (Nijkamp *et al.*, 1976); ** expressed as base; ‡ hydrocortisone = 1; ‡‡ data calculated from Sayers & Travis (1970), hydrocortisone = 1.

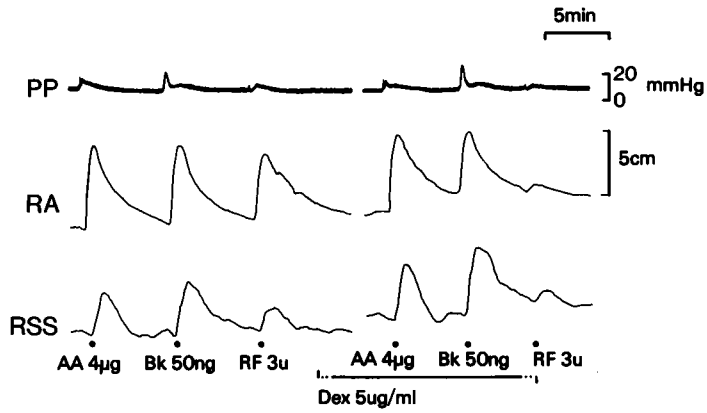

Figure 2 Release of rabbit aorta contracting substance (RCS) from guinea-pig perfused lungs by arachidonic acid (AA), bradykinin (BK) and RCS-releasing factor (RF) injections. The isolated tissues used were the rabbit aortic strip (RA) and rat stomach strip (RSS). The perfusion pressure (PP) of the lungs is also shown. The output of RCS induced by RCS-RF, but not by bradykinin or arachidonic acid, was almost completely abolished during the dexamethasone (Dex) infusion (which was begun 30 min before the responses).

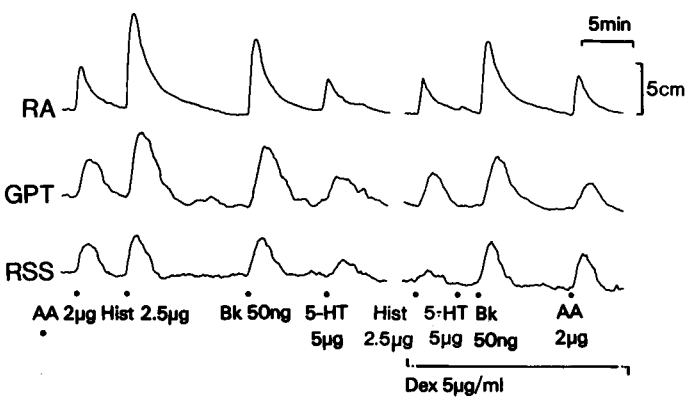

Figure 3 Release of rabbit aorta contracting substance (RCS) from guinea-pig perfused lungs by arachidonic acid (AA), histamine (Hist), bradykinin (Bk) and 5-hydroxytryptamine (5-HT). The isolated tissues used were the rabbit aortic strip (RA), guinea-pig trachea (GPT) and rat stomach strip (RSS). The output of contractile substances from the lung induced by histamine and 5-hydroxytryptamine, but not that by bradykinin or arachidonic acid, was almost completely abolished by the infusion of dexamethasone (Dex) (which was begun 30 min before the responses).

drolysis of the [^{3}H]-oleic acid from the phosphatide mixture would result in an increase in the $^{14}C/^3H$ ratio of the phospholipid/lysophospholipid fraction of the perfusate. We were still unable to obtain reproducible results with this technique apparently because the [^{14}C]-choline moiety was being hydrolysed from the remainder of the lecithin molecule. We therefore obtained quantitation by adding to the labelled phosphatide mixture a small amount of [^{14}C]-oleic acid. This acts as an internal marker to correct for losses of the liberated [^{3}H]-oleic acid in the preparation or on the glassware. It was clearly important to determine whether oleic acid was metabolized by the lung. In one experiment [^{14}C]-oleic acid (0.1 μCi) was injected through the lung, the effluent collected, acidified, and the fatty acids extracted into ether and methylated with ethereal diazomethane. The methyl esters of the radioactive products were resolved by t.l.c. on silica gel developed in ethyl acetate, *iso*-octane, water (50:100:100). Only one radioactive peak was present and that had the same mobility as authentic methyl oleate. Thus, there was no evidence for meta-

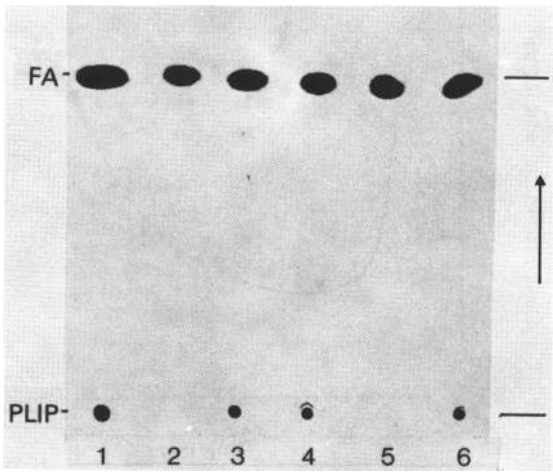

Figure 4 An autoradiogram of a t.l.c. plate developed in chloroform, methanol, acetic acid (90:10:2) showing the proportions of radioactive fatty acids (FA) and phosphatides (PLIP) extracted from a test sample of Krebs solution at pH 8 by various organic solvents. Lane 1 is a control zone showing the amounts of labelled phosphatides and fatty acids in the Krebs solution. Pentane and hexane (Lanes 2 and 5) selectively extracted the fatty acids with no phosphatide contamination. The other three solvents, ethyl acetate (Lane 3), diethyl ether (Lane 4) and chloroform (Lane 6) extracted both fatty acids and phosphatides. Extraction of the fatty acids is not quantitative, hexane gives approximately 63% and pentane, 78% extraction after 1 min vortex mixing. Because there is an internal marker present during the experiments this is not important. The arrow indicates the direction of development of the plate.

bolism of oleic acid during a single pass through the perfused lungs.

Choice of solvents for fatty acid extraction. Increased hydrolysis of the labelled phosphatide mixture is indicated by an increase in the $^3H/^{14}C$ ratio of the liberated fatty acids. Fatty acids can be selectively extracted from the crude effluent with the correct solvent. After some initial experimentation hexane was selected for this purpose (see Figure 4).

Validation of the assay and calculation of results. The procedure finally adopted for the phospholipase assay was based on the results obtained in the foregoing sections and is outlined in Figure 5. A mixture of the labelled phosphatide and the labelled fatty acid (0.1 μCi total) dissolved in tris buffer (pH 7.5), was injected into the Krebs solution entering the pulmonary artery. The effluent was collected for 5 min, 5 ml of tris buffer (pH 7.5; 100 mM) was added to stabilize the pH, and the labelled fatty acids were extracted into 5 ml hexane by 1 min vortex mixing. An aliquot of the hexane was transferred into scintillation vials and the solvent evaporated with a gentle stream of nitrogen. The $^3H/^{14}C$ ratio was estimated as described. The ratio of the two isotopes in a small aliquot of the aqueous solution was also determined. This gave a value for the 'total hydrolysis' and the actual hydrolysis was calculated from the following formula:

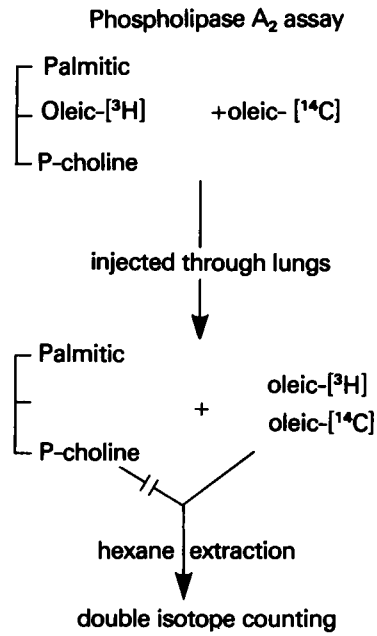

Figure 5 Schematic diagram of the phospholipase A_2 assay.

$$\text{units oleic acid liberated} = \frac{(\text{d/min } ^3\text{H in sample}) \times (\text{d/min } ^{14}\text{C in control})}{(\text{d/min } ^{14}\text{C in sample}) \times (\text{d/min } ^3\text{H in control})} \times S$$

where S is the number of units of oleic acid present in the phosphatide (i.e. pmol, μg etc.).

We tested the assay technique on an *in vitro* system using pig pancreas phospholipase A_2 as a source of enzyme. The conditions were those described by Blackwell *et al.* (1977). After incubation the labelled fatty acids were extracted with hexane and the radioactivity estimated. Figure 6 shows that a linear hy-

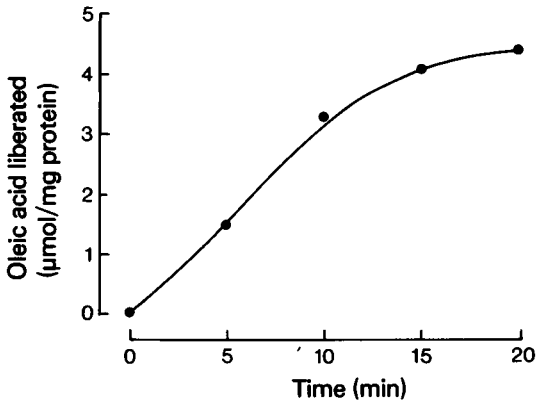

Figure 6 Graph showing the increase in [^{3}H]-oleic acid liberated from the substrate by pig pancreas phospholipase A_2 (abscissa scale) as a function of time (ordinate scale). This experiment was undertaken to validate the double isotope assay.

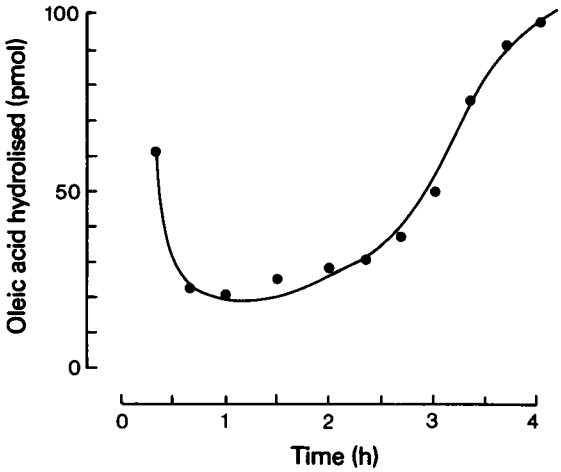

Figure 7 The hydrolysis of the labelled phosphatide (ordinate scale) as a function of the age of the preparation in hours (abscissa scale). Each point represents a single observation.

drolysis occurred for 0 to 10 min followed by near plateau conditions after 15 minutes.

A comparison of the accuracy and precision of the assay with a conventional t.l.c. assay is shown in Table 2. There was no significant difference between the two methods.

Measurement of 'basal hydrolysis' of phospholipids by the perfused lung preparation

In two experiments (one shown in Figure 7) the basal hydrolysis of the labelled phosphatides was measured over a period of time. An injection of the labelled phospholipid was made every 20 min and the hydrolysis estimated. Three distinct phases were seen: initially (20–40 min) an elevated hydrolysis; secondly, a period of relative stability (1 h to 2.5 h); finally a period of steadily increasing hydrolysis (3 h onward). The second experiment gave a comparable picture, we therefore used the 1 to 2.5 h 'stable period' of the preparation for our experiment.

Inhibition of basal phospholipase A_2 activity by drugs

Table 3 shows the results of experiments in which several substances were tested for their ability to inhibit basal phospholipid hydrolysis. Mepacrine, chlorpromazine and procaine have previously been reported to inhibit phospholipase, (Vargaftig & Dao Hai, 1972; Kunze, Nahas, Traynor & Wurl, 1976; Flower & Blackwell, 1976). In these experiments the decreasing order of activity was mepacrine > chlorpromazine > procaine. Aspirin and indomethacin were included as inhibitors of cyclo-oxygenase (Vane, 1971). They showed little activity against phospholipase A_2.

Amongst the five steroids, betamethasone and dexamethasone were approximately equipotent and hydrocortisone was active at higher doses. Aldosterone was inactive at the single dose used (80 μg/ml) but interestingly, oestradiol 17-β displayed substantial activity.

Stimulation of lung phospholipase activity

Table 4 shows the effects of various procedures, or substances known to stimulate the release of prostaglandin endoperoxides and TXA_2 from perfused lungs. All agents produced stimulation, the most potent being mechanical trauma which boosted phospholipase activity more than tenfold. The 'mechanical

Table 2 Comparison of double-isotope and conventional radiochromatographic assays of pig pancreas phospholipase A_2 activity

	Oleic acid liberated (μmol/mg protein)			
Assay	*Mean**	*s.d.*	*s.e. mean*	*Range*
Radiochromatographic	2.220	0.007	0.003	2.209–2.227
Double isotope	2.218	0.017	0.007	2.198–2.236

* $n = 5$.

trauma' used here was 15 s gentle mechanical vibration by means of an electrically driven vibrator held against the lung parenchyma.

Table 5 shows the results of experiments in which fixed concentrations of mepacrine or dexamethasone were used to block the stimulation of phospholipase activity. Both these agents produced a marked inhibition of all stimuli except bradykinin.

Time course of steroid block of phospholipase activity in lungs

Nijkamp *et al.*, 1976 noted that the inhibitory effect of infusions of steroids was a cumulative phenomenon, and estimated the time for maximal inhibition to be 20 minutes. We therefore used this standard time period for most of our current work. However, in two final experiments we found a 20 min period to be insufficient for full blockade. Figure 8 shows one of two experiments in which phospholipid hydrolysis was measured every 10 minutes. After a 20

Table 3 Inhibition of basal phospholipase A_2 activity in isolated perfused lungs of guinea-pigs

Drug	*Estimated IC_{50} (μg/ml)*
Non-steroids	
Mepacrine	20.0
Chlorpromazine	33.5
Procaine	73.4
Indomethacin	≃ 400.0
Aspirin	⩾ 500.0
Steroids	
Betamethasone	1.4
Dexamethasone	1.5
Hydrocortisone	48.0
Aldosterone	⩾ 80.0
Oestradiol-17β	1.6

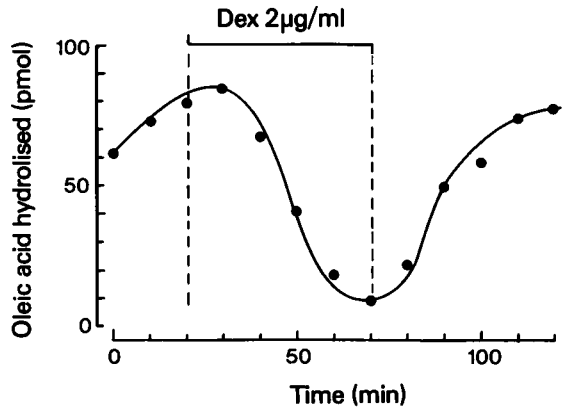

Figure 8 Inhibition of phospholipase A_2 activity in guinea-pig perfused lungs by an infusion of dexamethasone (Dex) at 2 μg/ml. Broken vertical lines represent the beginning and end of the infusion period. 'Time 0' corresponds to the beginning of the experiment, approximately 1 h after perfusion of the preparation started.

Table 4 Stimulation of phospholipase A_2 activity in isolated perfused lungs of guinea-pigs

Stimulus	*Oleic acid liberated (pmol)** *Control*	*Stimulated*	*△ Control %*
RCS-RF (5u)	584.6	865.9	+148.1
Bradykinin (1 μg)	328.2	756.6	+230.5
Antigen (ovalbumin; 2 μg)	282.8	718.1	+252.1
Histamine (2 μg)	625.3	1107.6	+177.1
Mechanical trauma	273.9	3194.5	+1166.3

* Each result is the mean of two observations.

Table 5 Inhibition by mepacrine and dexamethasone of phospholipase A_2 stimulation

Stimulus	*% inhibiton by* *Mepacrine (75 μg/ml)*	*Dexamethasone (3.9 μg/ml)*
Histamine (2 μg)	60.6	42.1
Bradykinin (1 μg)	14.7	14.1
RCS-RF (5u)	61.2	50.8
Antigen (ovalbumin; 2 μg)	83.1	58.7

* Each result is the mean of two observations

min control period an infusion of dexamethasone was begun, terminated 50 min later and the hydrolysis measured for a further hour. In this particular experiment an atypical rise in 'baseline' hydrolysis was observed but this was immediately reversed following the start of the dexamethasone infusion. Maximal inhibition did not occur after 20 min, but became progressively greater with time, leading to virtually 100% inhibition when the infusion was terminated. Thereafter, the hydrolysis returned to control levels after about 40 minutes. The other experiment gave a comparable result, except that the baseline hydrolysis was constant, and the steroid infusion was continued for only 30 minutes.

Inhibition by drugs of phospholipase activity in homogenates

Our results show that both mepacrine and steroid anti-inflammatory drugs suppress phospholipase activity in perfused lungs. We tested the same drugs in cell-free homogenates of lungs. Table 6 shows that although mepacrine still exerts an inhibitory effect on phospholipase A_2, the steroids were without effect, causing in fact a modest increase in hydrolysis.

Discussion

We have tested the concept that many agents that release prostaglandin endoperoxides and TXA_2 from guinea-pig perfused lungs do so by liberating the substrate from intracellular lipid stores (most probably phosphatides), through activation of phospholipase A_2, and furthermore that drugs that selectively block the release reaction (as opposed to cyclo-oxygenase inhibitors), of which the most notable examples are the anti-inflammatory steroids, do so by preventing activation of phospholipase A_2.

Much of the evidence for this hypothesis comes from earlier work. For example, when arachidonic acid is infused into guinea-pig isolated perfused lungs it is immediately converted to RCS (Vargaftig & Dao Hai, 1972; Palmer *et al.*, 1973); that this really is a conversion not a release is easily demonstrated with labelled arachidonate. This suggests that in this system at least, substrate availability is the rate limiting reaction. Nijkamp *et al.* (1976) demonstrated the release of arachidonic acid from lungs by the injection of RCS-RF and that this was blocked by anti-inflammatory steroids. We have now shown that the TXA_2 releasing activity of other stimuli (histamine, 5-hydroxytryptamine) is also blocked by infusion of steroids, although an interesting exception was bradykinin, whose activity was apparently steroid-insensitive.

In other tissues phospholipids seem to be a major source of substrate for prostaglandin biosynthesis. We therefore designed a simple assay for phospholipase A_2 activity in the lung to test whether there is any correlation between the release of prostaglandin endoperoxides or TXA_2 and the level of phospholipase activity. This is based on the injection of a specifically labelled phosphatide through the lung; we earlier used this technique to measure phospholipase A_2 activity of platelets during aggregation (Blackwell *et al.*, 1977). The perfused lung appears to exhibit a basal level of phospholipase A_2 activity which varies with the age of the preparation. When first perfused, the lung shows a high level of enzyme activity possibly due to the presence of blood components in the pulmonary circulation or to the trauma involved in the isolation procedure. This soon declines and then follows a phase (2 h) during which enzyme activity remains fairly stable. There is then a steady increase in the hydrolysis which is irreversible and probably represents cytolysis and the concomitant liberation of lysosomal enzymes.

Several types of drugs inhibited the basal phospholipase activity: amongst the non-steroids mepacrine and chlorpromazine were the most potent, procaine showing only weak activity. The two prostaglandin cyclo-oxygenase inhibitors aspirin and indomethacin (Vane, 1971) were virtually inactive. Perhaps more interesting was the effect of the anti-inflammatory steroids dexamethasone, betamethasone and hydrocortisone all of which reduced the basal phospholipase activity. Interestingly, this correlated with a phenomenon often observed when steroids were first infused into the lung during the bioassay experiments,

Table 6 Hydrolysis of phosphatidylcholine in guinea-pig lung homogenates: effects of mepacrine and steroids

Drug	*Oleic acid liberated* (nmol)*	*% inhibition*	P
None	1.76 ± 0.17	—	—
Dexamethasone (350 μg/ml)	1.99 ± 0.17	—	NS
Betamethasone (350 μg/ml)	2.17 ± 0.27	—	NS
Mepacrine (100 μg/ml)	1.17 ± 0.12	33.82	< 0.05

* Mean ± s.e. mean, $n = 5$.

a fall in tone of the smooth muscle organs, suggesting a decrease in the output of RCS-like activity (presumably prostaglandin endoperoxide and thromboxanes) from the lung.

All the stimulating agents we tested increased hydrolysis of the injected phosphatide suggesting an 'activation' of enzyme activity. In addition to blocking the basal phospholipase activity, mepacrine and dexamethasone (at least) prevent the stimulation of hydrolysis by histamine, RCS-RF and antigen; bradykinin stimulation was only weakly inhibited. The lack of effect of steroids on enzyme activity stimulated by bradykinin confirms the results obtained by bioassay. It is difficult to interpret the relative lack of effect of mepacrine which has previously been reported to block the effect of bradykinin in this sytem (Vargaftig & Dao Hai, 1972). It seems necessary to postulate the existence of a 'steroid-sensitive' and 'steroid-insensitive' population of receptors or phospholipase enzymes if we wish to retain the original hypothesis outlined at the beginning of this paper.

The results presented here do not enable us to reach any conclusion concerning the method of phospholipase 'activation'. Some types of phospholipase exist as pro-enzymes which are activated by the removal of a short peptide (Pickett, Jesse & Cohen, 1976; Drenth, Enzing, Kalk & Vessies, 1976), perhaps catalysed by a serine esterase. However, it is not known whether such a mechanism operates in this system but it is at least conceivable that some of the activating factors produce their effect by an action on such an esterase. Again, few conclusions can be drawn concerning the method of steroid inhibition of the enzyme activity. That steroids do not block phospholipase activity in cell-free homogenates but only in intact cells suggests that these drugs act on the enzyme indirectly, perhaps by modifying the microenvironment of the enzyme. There is evidently a difference in the mode of action of steroids and mepacrine, which works in both homogenates and intact cell situations.

From the results obtained concerning the time course of steroid block we can conclude that the blocking action of steroids is cumulative, increasing with the duration of the infusion and reversing when the infusion is stopped. It is of interest that the inhibitory action described here must be one of the very few effects of these drugs which occurs within minutes. Most of the reported actions of steroids have a much longer latency and are thought to involve synthesis of new enzyme protein or RNA. The cumulative nature of the inhibition illustrates that 'IC_{50} values' for steroids are meaningless unless accompanied by information concerning the duration for which these drugs were in contact with the preparation.

Most of the steroids used in this study are potent anti-inflammatory agents and it is possible that their ability to block phospholipase activity and therefore production of prostaglandins is one of underlying mechanisms of their anti-inflammatory effects. The discovery by Vane (1971) that the aspirin-like drugs directly block prostaglandin synthetase initiated a great deal of research and there is now considerable evidence that inhibition of synthesis of these prostaglandins is indeed the main mechanism of action of these drugs. An obvious question was whether the anti-inflammatory steroids also blocked prostaglandin biosynthesis. Vane demonstrated (1971) that hydrocortisone was inactive against prostaglandin biosynthesis in cell-free homogenates, and Flower, Gryglewski, Herbaczynska-Cedro & Vane (1972) demonstrated that several other potent anti-inflammatory steroids were also without effect on the cyclo-oxygenase. More recently, however, there has been increasing evidence that one steroid at least (hydrocortisone) interferes with prostaglandin biosynthesis in several whole cell or whole organ systems (Lewis & Piper, 1975; Gryglewski, Panczenco, Korbut, Grodzinska & Ocetkiewicz, 1975; Tashjina, Voelkel, McDonough & Levine, 1976; Kantrowitz, Robinson, McGuire & Levine, 1976). Nijkamp *et al.* (1976) investigated this problem further and found that anti-inflammatory steroids prevent the biosynthesis of TXA_2 by guinea-pig lungs by preventing the liberation of substrate from intra-cellular stores. We proposed that the mechanism involved was a phospholipase catalysed degradation of phosphatides. A similar hypothesis had already been advanced by Gryglewski and his colleagues (1975) to explain the blocking action of hydrocortisone in antigen challenged guinea-pig lungs, and more recently definitive evidence for such an effect has been provided by the work of Hong & Levine (1976). In contrast to the former authors we believe the enzyme involved to be a phospholipase A_2 located somewhere in the cell membrane or cytosol rather than in the lysosomes. An inhibition by steroids of phosphatide catabolism *in vivo* could well contribute to their anti-inflammatory effect since it would limit the supply of substrate to the cyclo-oxygenase as well as the lipoxygenase which generates HETE, shown by Turner, Tainer & Lynn (1975) to be strongly chemotactic for polymorphonuclear leucocytes. In addition to the formation of potentially toxic products of phosphatide metabolism such as lysolecithin and lipid peroxides would also be reduced.

Of some interest was the finding that oestradiol-17β also prevented phospholipase A_2 activity. Bodel, Dillard & Kaplan & Malawista, 1972, reported an anti-inflammatory activity of oestradiol-17β, but to our knowledge no one has investigated this phenomenon rigorously, or compared the activity with that of other steroids. The IC_{50} values, and relative activity of

other steroids in the phospholipase test compares well with their previously reported activity (Nijkamp *et al.*, 1976), and it would be of interest to compare the activity of oestradiol in experimental inflammation.

The current interest in prostaglandin and TXA_2 biosynthesis and metabolism has highlighted the role of phospholipase A_2, an enzyme that has never before been considered of significant pharmacological interest. We believe that this enzyme with its apparent capacity for 'activation' by miscellaneous agents and its sensitivity to anti-inflammatory steroids will prove to be of great importance as our knowledge concerning the functions of prostaglandins (and other lipid products) within the cell gradually accumulates.

F.P.N. was supported in part by a grant from the Royal Society (ZWO).

References

ALABASTER, V.A. & BAKHLE, Y.S. (1970). The release of biologically active substances from isolated lungs by 5-hydroxytryptamine and tryptamine. *Br. J. Pharmac.*, **40**, 582–583P.

BAKHLE, Y.S. & SMITH, T.W. (1972). Release of spasmogenic substances induced by vasoactive amines from isolated lungs. *Br. J. Pharmac.*, **46**, 543–544P.

BODEL, P., DILLARD, G.M., KAPLAN, S.S. & MALAWISTA, S.E. (1972). Anti-inflammatory effects of estradiol on human blood leukocytes. *J. Lab. clin. Med.* **80**, 373–384.

BLACKWELL, G.J., DUNCOMBE, W.G., FLOWER, R.J., PARSONS, M.F. & VANE, J.R. (1977). The distribution and metabolism of arachidonic acid in rabbit platelets during aggregation and its modification by drugs. *Br. J. Pharmac.*, **59**, 353–366.

DRENTH, J., ENZING, C.M., KALK, K.H. & VESSIES, J.C.A. (1976). Structure of porcine pancreatic prephospholipase A_2. *Nature, Lond.*, **264**, 373–377.

ECKENFELS, A. & VANE, J.R. (1972). Prostaglandins, oxygen tension and smooth muscle tone. *Br. J. Pharmac.*, **38**, 214–221.

FLOWER, R.J. & BLACKWELL, G.J. (1976). The importance of phospholipase A_2 in prostaglandin biosynthesis. *Biochem. Pharmac.*, **25**, 285–291.

FLOWER, R.J., GRYGLEWSKI, R., HERBACZYNSKA-CEDRO, K. & VANE, J.R. (1972). The effects of anti-inflammatory drugs on prostaglandin biosynthesis. *Nature, New Biol.*, **238**, 104–106.

GRYGLEWSKI, R.J., PANCZENKO, B., KORBUT, R., GRODZINSKA, L. & OCETKIEWICZ, A. (1975). Corticosteroids inhibit prostaglandin release from perfused mesenteric blood vessels of rabbit and from perfused lungs of sensitized guinea-pigs. *Prostaglandins*, **10**, 343–355.

HAMBERG, M. & SAMUELSSON, B. (1974). Prostaglandin endoperoxides VII. Novel transformations of arachidonic acid in guinea-pig lung. *Biochem. biophys. Res. Commun.* **16**, 942–949.

HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1975). Thromboxanes. A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. natn. Acad. Sci. U.S.A.*, **72**, 2994–2998.

HAYE, B., CHAMPION, S. & JACQUEMIN, C. (1973). Control by TSH of a phospholipase A_2 activity, a limiting factor in the biosynthesis of prostaglandins in the thyroid. *FEBS Letters*, **30**, 253–260.

HONG, S-C.L. & LEVINE, L. (1976). Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory steroids. *Proc. natn. Acad. Sci. U.S.A.*, **73**, 1730–1734.

KANTROWITZ, F., ROBINSON, D.W., McGUIRE, M.B. & LEVINE, L. (1976). Corticosteroids inhibit prostaglandin production by rheumatoid synovia. *Nature. Lond.*, **258**, 737–739.

KUNZE, H., NAHAS, N., TRAYNOR, J.R. & WURL, M. (1976). Effects of local anaesthetics on phospholipase. *Biochim. biophys. Acta*, **441**, 93–102.

KUNZE, H. & VOGT, W. (1971). Significance of phospholipase A for prostaglandins formation. *Ann. N.Y. Acad. Sci.* **180**, 123–125.

LEWIS, G.P. & PIPER, P.J. (1975). Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory steroids. *Nature, Lond.*, **254**, 308–311.

LIEBIG, R., BERNHAUER, W. & PESKAR, B.A. (1974). Release of prostaglandins, a prostaglandin metabolite, slow-reacting substance and histamine from anaphylactic lungs, and its modification by catecholamines. *Naunyn Schmiedebergs Arch. Pharmac.*, **284**, 279–293.

NIJKAMP, F.P., FLOWER, R.J., MONCADA, S. & VANE, J.R. (1976). Partial purification of rabbit aorta contracting substance—releasing factor and inhibition of its activity by anti-inflammatory steroids. *Nature, Lond.*, **263**, 479–482.

PALMER, M., PIPER, P.J. & VANE, J.R. (1973). Release of rabbit aorta contracting substance (RCS) and prostaglandins induced by chemical or mechanical stimulation of guinea-pig lungs. *Br. J. Pharmac.* **49**, 226–242.

PICKETT, W.C., JESSE, R.L. & COHEN, P. (1976). Trypsin-induced phospholipase activity in human platelets. *Biochem. J.*, **160**, 405–408.

PIPER, P.J. & VANE, J.R. (1969). Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature, Lond.*, **233**, 29–35.

PIPER, P.J. & VANE, J.R. (1971). The release of prostaglandins from lung and other tissues. *Ann. N.Y. Acad. Sci.*, **180**, 363–385.

SAMUELSSON, B. (1972). Biosynthesis of prostaglandins. *Fedn Proc.* **31**, 1442–1450.

SAYERS, G. & TRAVIS, R.H. (1970). *The Pharmacological Basis of Therapeutics*. fourth ed. ed. Goodman, L.S. & Gilman, A. pp. 1604–1642. New York: Macmillan.

TASHJIAN, A.H., VOELKEL, E.F., MCDONOUGH, J. & LEVINE, L. (1976). Hydrocortisone inhibits prostaglandin production by mouse fibrosarcoma cells. *Nature, Lond.*, **258**, 739–741.

TURNER, S.R., TAINER, J.A. & LYNN, W.S. (1975). Biogenesis of chemotactic molecules by the arachidonate lipoxygenase system of platelets. *Nature, Lond.*, **257**, 680–681.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature, New Biol.*, **231**, 232–235.

VARGAFTIG, B.B. & DAO HAI, N. (1972). Selective inhibition by mepacrine of the release of "rabbit aorta contracting substance" evoked by the administration of bradykinin. *J. Pharm. Pharmac.* **24**, 159–161.

(*Received May 16, 1977*)