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THE IMPORTANCE OF PHOSPHOLIPASE-A_2 IN PROSTAGLANDIN BIOSYNTHESIS

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Abstract—A model has been devised to examine the cellular biochemical events which culminate in prostaglandin (PG) biosynthesis and release from tissues. Slices of guinea-pig spleen incubated in buffer containing [1-^{14}C]arachidonic acid incorporate the label into cellular phospholipid and neutral lipid pools. The majority of incorporated radioactivity appeared in the lecithin fraction; smaller amounts were associated with neutral lipids (chiefly diglycerides) or remained unesterified. During incubation there was a small basal release of prostaglandins. When tissues were vibrated mechanically or shocked there was a loss of [1-^{14}C]arachidonic acid from the phospholipid pools, a corresponding rise in the free substrate levels, and an increase in the synthesis of ^{14}C-labelled PGE_2. The synthesis of prostaglandins was blocked by indomethacin, and the loss of arachidonate from the phospholipid fraction of the cells was blocked by the anti-malarial drug mepacrine. During mechanical vibration or immunological challenge the labelled arachidonic acid released as a substrate for prostaglandin biosynthesis originated solely from the phospholipid fraction. Phospholipase is therefore the key enzyme which mobilises free fatty acids for prostaglandin biosynthesis during these types of cell injury. Spleen slices were also vibrated in the presence of labelled arachidonic acid without prior incorporation. This procedure also increased prostaglandin biosynthesis several-fold, indicating that substrate availability is not the only requirement for stimulation of prostaglandin biosynthesis.

Mammalian cells release prostaglandins in response to physiological, pharmacological or pathological stimuli and since prostaglandins are not 'stored' within cells [1], biosynthesis must immediately precede release. The substrates for prostaglandin synthesis (C-20 unsaturated fatty acids) must be in non-esterified form [2, 3], but the intracellular levels of such precursors are extremely low [4, 5, 6]. The first step in the release of prostaglandins from any cell therefore, depends on the appearance of substrate at or near the microsomal synthetase complex.

These precursor acids could arise from a number of intracellular lipid pools; cholesterol esters, phosphatides, mono, di, or tri-glycerides might all contain sufficient substrate to support prostaglandin biosynthesis. Thus, several enzymes are potentially capable of mobilising fatty acid substrates. The suggestion which has received most attention is that the free substrate originates from the phospholipid fraction of the cell, under the influence of the hydrolytic enzyme phospholipase A_2. Although this idea has been widely canvassed there is little direct experimental evidence for it, although indirect evidence comes from the experiments of Vargaftig and Dao Hai [7]; Lands and Samuelsson [6], and Vonkeman and van Dorp [3].

We have attempted to answer the following questions:

(a) Does the substrate for prostaglandin synthesis arise from phospholipids or from some other lipid pool?
(b) What effect does an inhibitor of phospholipase have on the release of prostaglandins?
(c) Is the availability of substrate the rate limiting step in prostaglandin biosynthesis?

We have used slices of guinea-pig spleen because it is a soft and easily manipulated tissue; furthermore, spleen has previously been shown to release prostaglandins when vibrated [8]. We have initiated release of prostaglandins by two stimuli; anaphylaxis [9] and mechanical vibration [8]. The latter challenge was easier to quantitate, gave more-reproducible releases and hence was employed for most of our studies. Many tissues are known to take up and metabolise labelled fatty acids and we have used this technique to label different lipid pools within spleen cells.

MATERIALS

(a) *Unlabelled lipids.* Arachidonic acid (Grade I), sphingomyelin, L-α-lecithin, L-α-cephalin, cholesterol arachidonate and lipid standards for chromatography (containing mono, di and tri-glycerides, and mixtures of fatty acid methyl esters) were purchased from the Sigma Chemical Co. Prostaglandins E_2 and $F_{2\alpha}$ were purchased from Cambrian Chemicals Ltd.

(b) *Radioactive lipids.* [5,6,8,11,12,14,15-^{3}H]prostaglandin E_2 (batch 4, 180 Ci/m-mole) and [1-^{14}C]arachidonic acid (batch 3, 58 mCi/m-mole) were purchased from the Radiochemical Centre, Amersham. Both compounds were checked for radiochemical purity by t.l.c. before use and if necessary repurified (see Flower *et al.*) [10].

(c) *Chromatographic materials.* Plastic-backed t.l.c. plates (100 μm layer type, 6060 silica gel) were obtained from Eastman Kodak; preparative t.l.c. plates (2 mm layer, Kieselgel 60) from Merck, and glass-backed t.l.c. plates impregnated with 10% $AgNO_3$ (250 μm layer silica gel G) from Anachem Ltd. All chromatographic reagents were of 'Analar' grade, or the highest quality obtainable.

(d) *General.* Triton-X was obtained from the Sigma Chemical Company and pig pancreas phospholipase A_2 from Boehringer Mannheim G.m.b.H.

METHODS

(c) *Preparation of spleen slices.* Male guinea-pigs weighing 200–300 g were killed by cervical dislocation. The spleens were removed, trimmed free of connective tissue and placed pedicle uppermost in a tissue slicer designed to cut tissue slices (1·5 mm; *ca* 150–250 mg) from the (non-pedicle) surface of the spleen. The slices were transferred into small flasks containing Krebs' solution, continuously gassed with 95% O_2, 5% CO_2.

(b) *Pulse labelling of spleen phospholipids with [1-^{14}C]arachidonic acid.* In two initial experiments a time course for incorporation of [1-^{14}C]arachidonate into spleen phospholipids was measured. This indicated that a 30 min incubation was satisfactory (see Results). Thereafter 10–20 spleen slices were incubated for 30 min at 37° in a flask containing 10–15 ml Krebs' solution gassed with 95% O_2–5% CO_2 with the addition of 0·5–2·0 μCi [1-^{14}C]arachidonic acid dissolved as the Tris salt. After incubation the slices were removed, washed to remove excess label and allowed to stand in flasks containing 5 ml Krebs' solution (continuously gassed) for 45 min in a shaking water bath. In some experiments spleen slices were allowed to remain in contact with arachidonic acid for 10 min only and allowed to equilibrate for only 5 min. Under these conditions the incorporation of substrate into phospholipids or other lipid pools was negligible and virtually all the label within the tissue was unesterified.

(c) *Vibration of spleen slices.* Spleen slices were vibrated for 90 sec by holding the side of the flask against the rotating cup of a Vortex mixer. Since this procedure involved removing the flask from the water bath, the non-vibrated samples were also removed and allowed to remain on the bench for the same period.

(d) *Induction of anaphylactic shock in spleen slices.* In these experiments spleens were used from guinea-pigs sensitised 14 days earlier by injections (1 ml subcutaneously and 1 ml intraperitoneally) of a 10% (w/v) aqueous suspension of ovalbumin.

After the pre-incubations were completed, anaphylaxis was induced in the spleen slices by addition into each flask of 0·1 ml of the ovalbumin suspension. Control flasks received only saline.

(e) *Processing of spleen slices.* Immediately after vibration or anaphylaxis, spleen slices were removed from their flasks, drained briefly and frozen by transfer into a mixture of dry ice and methanol (5 ml). The incubation fluids were placed in the deep freeze to await analysis.

After the dry ice had evaporated, the spleen slices were homogenised and 2 vol of chloroform added (to bring the chloroform–methanol ratio to 2:1). After mixing, the spleen lipids were allowed to extract for 1 hr at 4°. After extraction the solution was filtered to remove precipitated protein and other debris, evaporated under reduced pressure and the residue redissolved in 500 μl chloroform–methanol (2:1). Aliquots of this were streaked (1·5 cm) onto preparative plates previously cut into 5 lanes (4-cm wide), and developed to a height of 16–18 cm in chloroform–methanol–acetic acid, (90:5:5). Authentic lipid markers were added to each lane. After development, the plates were dried and the lipid zones were visualised by exposure to iodine vapour. In this system, phospholipids remain at the origin. Typical R_f values (× 100) were 28·6 for mono-glycerides, 47·4 for di-glycerides, 69·2 for tri-glycerides, 42·1 for arachidonic acid and other fatty acids and 66·9 for cholesterol arachidonate.

After development the zones containing phospholipids were scraped off, the radioactivity eluted with 10 ml methanol and estimated by liquid scintillation counting (see below). The radioactive zones containing arachidonic acid, neutral lipids or other areas of the t.l.c. plate were eluted with 10 ml chloroform–methanol 2:1. Since chloroform is a powerful quenching agent the solvents were concentrated under reduced pressure prior to removal of a small aliquot for scintillation counting.

(f) *Processing of incubation fluids.* The incubation fluids were acidified to pH 3 with 1 N HCl and extracted into equal volumes of ethyl acetate. The solvent was evaporated under reduced pressure and the residue redissolved in 50 μl of chloroform–methanol (2:1) and spotted onto a plastic-backed silica gel t.l.c. plate. Arachidonic acid and PGE_2 and $F_{2\alpha}$ standards were also spotted on each lane. Plates were developed in iso octane–ethyl acetate–water–acetic acid (5:11:10:2), which gives good separation of prostaglandins and arachidonic acid. Typical R_f values (× 100) in this system are 18·2 for $PGF_{2\alpha}$, 28·5 for PGE_2, 52·5 for mono-glycerides, 66·7 for arachidonic acid, 77·2 for di-glycerides and 82·7 for tri-glycerides. Phosphatides remained at the origin. Zones corresponding to different lipids were detected by the exposure to iodine vapour, cut out with scissors and the radioactivity measured by liquid scintillation counting.

(g) *Measurement of organic phosphorus.* Organic phosphorus was measured by the direct colorimetric method of Raheja *et al.* [11] using lecithin as a standard.

(h) *Characterisation of phospholipids.* In two experiments portions of the phospholipid fraction from previous chromatography were subjected to further chromatography in chloroform–methanol–water (95:36:6) which resolves phospholipids into different classes. Authentic sphingomyelin, lecithin and cephalin were used as chromatography markers. After development, the plate was divided into 1-cm sections, the lipid material in each section eluted with methanol and the radioactivity and organic phosphorus in each section measured.

(i) *Enzymatic hydrolysis of lecithin fraction.* In order to determine whether the labelled fatty acid was esterified in the 1′ or 2′ position of lecithin, two experiments were carried out using a phospholipase A_2 from pig pancreas, which characteristically hydrolyses the 2-ester bond.

The radioactive lecithin fraction was divided into two portions and pipetted into small glass tubes. After evaporation of the organic solvent the lipids were resuspended in 0·5 ml 100 mM Tris buffer (pH 7·5); 10 μl of phospholipase A_2 (100 μg enzyme) was added to one tube together with 10 μl triton-X, the other tube received triton-X only. After mixing the tubes were incubated in a water bath (37°) for 2 hr, the labelled products were then extracted and separated by t.l.c. on a silica gel plate developed in chloroform–

methanol–acetic acid (90:5:5). In one experiment the radioactive fatty acid thus liberated was eluted from the plate, methylated with diazomethane, spotted onto a glass silica gel plate impregnated with 10% $AgNO_3$, and developed in hexane–diethyl ether (40:60) which separates methyl esters of fatty acids according to the degree of unsaturation as well as chain length. Authentic methyl arachidonate was run as a standard.

(j) *Measurement of 'accessibility' of prostaglandin synthetase.* In an attempt to measure the 'accessibility' of the enzyme to the substrate during mechanical damage to the tissue, we used an arachidonic acid analogue which is also an irreversible inhibitor of prostaglandin synthetase (5,8,11,14-eicosatetraynoic acid or TYA; see ref. [12]). We assumed that the access of substrate and analogue would be similar. Spleen slices were divided into three groups and were equilibrated for 5 min in individual flasks containing 5 ml Krebs' solution in a shaking water bath at 37°. TYA (final concn 1 μM) was added to the one set of flasks (Group A). The three sets of flasks were then vibrated for 60 sec after which the spleen slices were removed and transferred into new flasks all of which (except group C) contained 1 μM TYA in 5 ml Krebs' solution. After incubation for 30 min, labelled arachidonic acid (0·1 μCi) was added to each flask and the spleen slices were allowed to equilibrate for 5 min. After equilibration the spleen slices were again vibrated for 60 sec and the incubation fluids extracted, and the prostaglandin content measured.

The only difference between the three groups of spleen slices was that Group A had been pre-incubated and vibrated in the presence of TYA for 1 min longer than the others. Thus if mechanical damage induced by the vibration increases the access of the substrate to the enzyme (or in this case inhibitor to the enzyme) the prostaglandin biosynthesis by Group A should be much less than by the other groups.

(k) *Liquid scintillation counting procedures.* Strips of plastic-backed t.l.c. plate, or aliquots of the chloroform–methanol, or methanol eluate of silica gel scrapings from t.l.c. plates were added to plastic vials containing 10 ml Beckman liquid scintillation cocktail 'D' (5 g PPO and 100 g naphthalene/l dioxan). After mixing, samples were counted in a Beckman LS-150 liquid scintillation counter.

All samples were routinely counted to an accuracy such that 2 S.D. > 0·5% $\bar{x}$. All cpm values were converted to dis/min using the AES ratio method and a standard curve freshly constructed for each experiment.

(l) *Expression of results*

Because of the variability of uptake of the label by spleen slices, the results are expressed as percentages of the total pmoles of [1-^{14}C]arachidonate recovered from the chromatography plates after development, taking into account dilution factors. When the zones containing phospholipids, arachidonic acid or other lipids were removed from the t.l.c. plate, the remainder of the lane was scraped off and any remaining radioactivity eluted with chloroform–methanol (1:1) and an aliquot used for radioactivity determinations. Knowing the original specific activity of the arachidonic acid and the size of the aliquot analysed, the total number of pmoles chromatographed was calculated (this calculation does not allow for any isotope dilution by endogenous arachidonate). All subsequent results were expressed as percentages of this figure.

The figures relating to arachidonic acid, neutral lipids and phosphatides represent the sum of both intra and extra-cellular pools. We were never able to detect intracellular prostaglandins.

Student's *t*-test (independent) was used to analyse the results.

RESULTS

(a) *Prostaglandin biosynthesis by guinea-pig spleen homogenates.* To demonstrate the presence of prostaglandin synthetase activity in guinea-pig spleens, homogenates were incubated for 10 min in the presence of [1-^{14}C]arachidonic acid and 10 μg PGE_1 (to protect labelled prostaglandin products from inactivation).

Figure 1 shows that there was a considerable conversion to more polar compounds. The major product (which accounted for 80 per cent of the total arachidonate converted) co-chromatographed with the PGE_2 standard; a smaller amount of $PGF_{2\alpha}$ was also formed.

As Table 1 shows, the production of prostaglandins by spleen homogenates was blocked by indomethacin (10 μM) and to a lesser extent by mepacrine (1 mM).

(b) *Metabolism of PGE_2 by guinea-pig spleen homogenates.* When [^{3}H]PGE_2 was added to spleen homogenates, there was conversion to a less polar product which had an R_f value identical to authentic 15-keto PGE_2. However, the conversion was slow (≃ 30% in 10 min); no detectable metabolism occurred in 2 min.

(c) *Vibration of spleen slices in the presence of 1-^{14}C-labelled arachidonic acid.* As Table 2 shows, non-vibrated slices produced small amounts of prostaglandins; this production was increased 8-fold by vibration. Biosynthesis was blocked by indomethacin (50 μM) and to a lesser extent by mepacrine (1 mM).

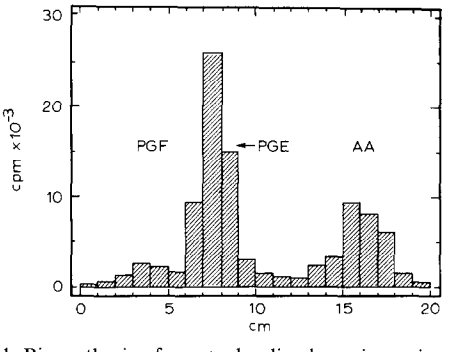

Fig. 1. Biosynthesis of prostaglandins by guinea-pig spleen homogenates. After incubation with labelled arachidonic acid the reaction products were extracted and processed as described in Methods and separated by t.l.c. on silica gel plates developed in iso-octane–ethyl acetate–water–acetic acid (5:11:10:2). When the developed plates were dry they were cut into 1-cm sections and the radioactivity in each zone estimated. 'PGF' 'PGE' and 'AA' refer to the positions of authentic prostaglandin $F_{2\alpha}$, E_2 and arachidonic acid markers.

Table 1. Synthesis of prostaglandins by guinea-pig spleen homogenates

Treatment	pmole PGE_2 (M ± S.E.M.) (n = 5)	% control value
None	1068·25 (±165·18)	—
+10 μM indomethacin	115·05 (±24·83)	10·8
+1 mM mepacrine	292·71 (±25·00)	27·4

Table 2. Synthesis of prostaglandins by guinea-pig spleen slices (no prior incorporation)

Treatment	pmole PGE_2 (M ± S.E.M.) (n = 5)	% 'vibrated' value
None	10·10 (±0·88)	11·44
Vibrated	88·26 (±1·79)	100·00
Vibrated +50 μM Indomethacin	4·28 (±0·79)	4·85
Vibrated +1 mM Mepacrine	21·44 (±1·13)	24·29

(d) *Uptake of [1-^{14}C]arachidonic acid into guinea-pig spleen slices.* Figure 2 shows the uptake of arachidonic acid into spleen phospholipids during a 90 min incubation. There was rapid incorporation in the first 30–45 min, during which 75% of the maximal uptake occurred. In all subsequent experiments, we used incubation periods of 30 min.

(e) *Analysis of labelled cell constituents after incubation with [1-^{14}C]arachidonate.* Figure 3 shows a radiochromatogram of the lipid extract of spleens incubated with [1-^{14}C]arachidonate for 30 min. The radioactivity was mainly incorporated into the phospholipid fraction, smaller amounts being present as neutral lipids (mainly di-glycerides) and non-esterified acid. There was some variation from day to day in the relative quantities incorporated into different lipid pools; Table 3 gives the ranges of values found in all experiments.

(f) *Characterisation of phospholipids.* Figure 4 shows the results of an experiment in which the phospholipids from labelled spleen slices were separated into individual classes. The majority of the organic phosphorus had a chromatographic mobility identical with the lecithin marker; much smaller amounts were associated with the sphingomyelin or cephalin markers. With the incubation times used, virtually all the radioactivity was associated with the lecithin fraction.

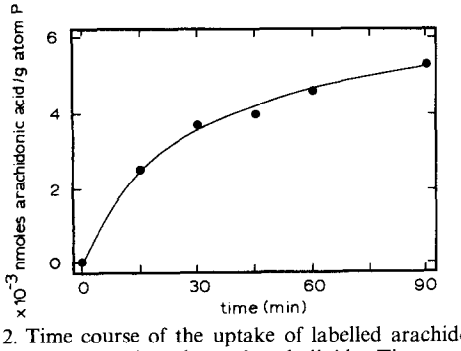

Fig. 2. Time course of the uptake of labelled arachidonic acid into guinea-pig spleen phospholipids. Tissues were extracted and processed as described in Methods. Organic phosphorus was measured by the method of Raheja *et al.* [11].

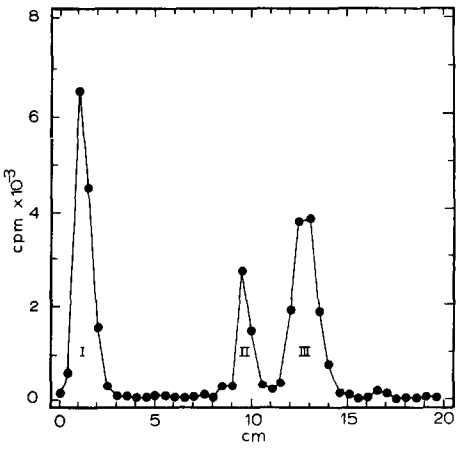

Fig. 3. Chromatographic analysis of labelled lipid fractions of guinea-pig spleen. After incubation with labelled arachidonic acid the spleen slices were processed and extracted as described in Methods. Lipid extracts were separated by t.l.c. on silica gel plates developed in chloroform–methanol–acetic acid (90:5:5). The developed plates were cut into 1-cm sections, three clear peaks of radioactivity were always observed and identified on the basis of their mobility as; peak I—phospholipids, peak II—arachidonic acid, peak III—di-glycerides.

(g) *Enzymatic hydrolysis of ^{14}C-labelled lecithin.* When the radioactive lecithin isolated by the above procedure was hydrolysed with phospholipase A_2 and the products rechromatographed, more than 70–90 per cent of all the radioactivity was released from the phospholipid fraction and appeared in the fatty acid zone. After methylation with diazomethane and further chromatography on silver nitrate plates this product co-chromatographed with the methyl arachidonate standard.

(h) *Effect of mechanical vibration on spleen lipid distribution.* Table 4 shows the relative distribution of radioactive lipids in spleen before and after 90 sec mechanical vibration (mean of 5 experiments). The

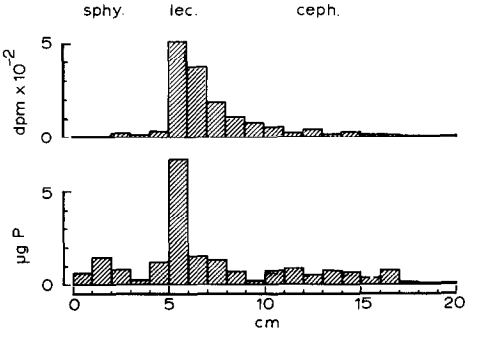

Fig. 4. Characterisation of labelled phospholipids from guinea-pig spleen. The phospholipid fraction of spleen homogenates was isolated by t.l.c. as shown in Fig. 3, and re-chromatographed on silica gel plates in chloroform–methanol–water (95:35:6). When developed the plates were dried and divided into 1-cm sections and parallel estimations of radioactivity, and organic phosphorus performed. 'Sphy', 'lec' and 'ceph' refer to authentic sphingomyelin, lecithin and cephalin marker positions.

Table 3. Distribution of label amongst lipid fractions

Fraction	% of total label incorporated
Phospholipids	20–95
Mono-glycerides	0–20
Di-glycerides	0–20
Tri-glycerides	0–16
Free arachidonate	5–60
Cholesterol esters	0–4
Prostaglandins (E or F)	undetectable

amount of substrate detected in the phospholipid fraction was always 10–20 per cent less after mechanical vibration. The release of fatty acids from the phospholipid pools was reflected by an increase in non-esterified label in the tissue slice. In the control, unvibrated spleen slices, the substrate levels were also high but prostaglandin synthesis was low. In the vibrated spleens the intra-cellular concentrations of free substrate only doubled, but prostaglandin biosynthesis increased 4-fold. When the isotope dilution effect is taken into account, the actual increase was probably greater.

(i) *Effect of inhibitors on the synthesis and release of prostaglandins induced by mechanical vibration.* Indomethacin is a potent prostaglandin synthetase inhibitor [13]. In spleen slices, a concentration of 50 μM was needed to give an 80 per cent block of biosynthesis, some 5-fold higher than that needed in homogenates. With these high concentrations there was also an effect on the uptake and incorporation of free fatty acids into phospholipids. In one series of experiments slices inhibited with indomethacin (50 μM) incorporated 31·1 (±4·3)% of the label into the phospholipids as against 52·3 (±0·9)% in control slices. With indomethacin, 57·4 (±8·0)% was incorporated into the neutral lipids as against 32·9 (±0·7)% in the controls. After vibration, the production of prostaglandins by the slices was reduced 41 per cent by the indomethacin. Thus in the high doses used, indomethacin apparently inhibited the enzyme system which esterifies fatty acids into 2′ positions of lysophospholipids.

The anti-malarial drug mepacrine also inhibits phospholipase [7]. When spleen slices were vibrated in the presence of 1 mM mepacrine (Table 5), there was no effect on the uptake of ^{14}C-fatty acids into phosphatides. The loss of substrate from phospholipids was much reduced (no significant differences from the control) and there was a smaller rise in intracellular substrate levels (significantly different from controls; $P < 0{\cdot}01$). The amount of prostaglandins produced was also reduced almost to control levels (no significant difference). The ^{14}C-fatty acid content of the neutral lipid pools was unchanged.

Table 4. Changes in the distribution of [1-^{14}C]arachidonic acid following mechanical vibration

Treatment	pmole % (M ± S.E.M.) Phospholipids	Arachidonic acid	Neutral lipids	PGs
Control (n = 5)	67·16 (±3·52)	25·36 (±3·35)	5·13 (±0·55)	2·06 (±0·13)
Vibrated (n = 5)	41·52 (±2·67)	44·98 (±3·04)	5·34 (±1·06)	8·02 (±0·84)
P 1 2	<0·001	<0·01	N.S.	<0·001

Table 5. Inhibition of phospholipid breakdown by mepacrine

Treatment	pmole % (M ± S.E.M.)‡ Phospholipids	Arachidonic acid	Neutral lipids	PGs
Control	77·72 (±5·27)	9·73 (±1·65)	10·51 (±3·58)	2·10 (±0·36)
Vibrated	62·35* (±1·09)	20·78§ (±0·66)	11·16 (±1·29)	5·73† (±0·11)
Vibrated +1 mM Mepacrine	73·63 (±1·78)	14·74† (±0·66)	9·79 (±1·19)	2·43 (±0·17)

* $P < 0{\cdot}05$ compared with controls
† $P < 0{\cdot}01$ compared with controls.
§ $P < 0{\cdot}001$ compared with controls.
‡ (n = 5).

Table 6. Changes in the distribution of [1-^{14}C]arachidonic acid following anaphylactic shock

Treatment	pmole % (M ± S.E.M.) Phospholipids	Arachidonic acid	Neutral lipids	PGs
Control (n = 5)	83·57 (±1·12)	6·38 (±1·84)	10·77 (±0·98)	0·86 (±0·26)
Anaphylaxis (n = 5)	78·20 (±1·12)	8·55 (±1·35)	10·39 (±0·46)	3·25 (±0·80)
P	<0·05	N.S.	N.S.	<0·02

(j) *Effect of homogenisation on phospholipase activity of spleen slices.* Homogenisation is a much more severe trauma to cells than vibration. In one experiment, spleen slices were homogenised and left standing at room temperature for 5 min. In the control (non-homogenised samples) 41·8 per cent (n = 3) of the total arachidonate was present in the phospholipids. After homogenisation, there was only 17·9 per cent (n = 3), and there was a corresponding rise in the substrate levels.

(k) *Effect of anaphylactic shock on the distribution of [1-^{14}C]arachidonic acid.* Although anaphylactic shock always induced a fall in phospholipid arachidonate and an increase in prostaglandin synthesis, the changes were smaller and less reproducible than those induced by mechanical vibration and sometimes were not statistically significant. In one set of experiments, however (Table 6), there was clear evidence that a similar situation obtains to that already described for mechanical vibration. Table 6 shows that 3 min after anaphylaxis there was a small but significant

Table 7. TYA experiment

Group A	Group B	Group C
1. Equilibrate slices for 5 min in Krebs' solution.	Equilibrate slices for 5 min in Krebs' solution.	Equilibrate slices for 5 min in Krebs' solution.
2. Vibrate for 1 min with 1 μM TYA.	Vibrate for 1 min in Krebs' solution only.	Vibrate for 1 min in Krebs' solution only.
3. Transfer into new flask containing 1 μM TYA. Incubate for 30 min.	Transfer into new flask containing 1 μM TYA. Incubate for 30 min.	Transfer into new flask. Incubate for 30 min.
4. Add label equilibrate for 5 min.	Add label equilibrate for 5 min.	Add label equilibrate for 5 min.
5. Vibrate for 1 min.	Vibrate for 1 min.	Vibrate for 1 min.
6. Measure PGE biosynthesis 151·27 (±6·66) pmole.	Measure PGE biosynthesis 249·09 (±8·26) pmole.	Measure PGE biosynthesis 314·86 (±9·53) pmole.

($P < 0.05$) fall in phospholipid arachidonate, a small (non-significant) rise in intracellular substrate levels and a 4-fold increase in prostaglandin levels ($P < 0.02$).

(l) *Effect of mechanical vibration on the access of a substrate analogue to prostaglandin synthetase.* The synthesis of prostaglandins by the spleen slices of Group B (shaken once with TYA) was some 21 per cent less than the controls with no TYA (Group C). Spleen slices vibrated twice with the substrate analogue (Group A) produced significantly ($P < 0.05$) less prostalgandins than Group B—only 48 per cent of control synthesis.

DISCUSSION

The evidence that phospholipase regulates prostaglandin formation may be summarised thus:

(1) Phospholipids could act as stores of precursor for prostaglandins [2, 3].

(2) Perfusion of guinea-pig lungs or frog intestine with phospholipase A leads to a rapid release of large quantities of prostaglandins [14, 15].

(3) Infusion of arachidonic acid through frog intestine [15] or lungs [7, 16] leads to an appearance of prostaglandins in the effluent.

(4) Infusion of arachidonic acid or bradykinin through guinea-pig lungs leads to an appearance of prostaglandins in the perfusate; mepacrine, a phospholipase inhibitor, blocks the releasing action of bradykinin but not of arachidonic acid [7]. These results are consistent with the idea that bradykinin releases prostaglandins by stimulating phospholipase, whereas arachidonic acid is converted to prostalgandins directly.

(5) Thyroid-stimulating hormone apparently increases the synthesis of prostaglandins in the thyroid by stimulating the activity of an endogenous phospholipase [5].

Although the foregoing evidence is strongly suggestive of the role of phospholipase in prostaglandin biosynthesis, it is not conclusive. We have now demonstrated that spleen slices incorporate [1-^{14}C]arachidonic acid chiefly into the 2′-position of lecithin, and that during cellular damage caused by homogenisation, mechanical stimulation or anaphylactic shock there is a loss of esterified labelled prostaglandin substrate from the phospholipid fraction, an increase in the free substrate levels and an increase in biosynthesis of prostaglandins. These experiments clearly show that phospholipids are an important source of substrate for prostaglandin biosynthesis and that this source can be mobilised within the time span and under the conditions in which prostaglandin synthesis and release occurs. Since the ^{14}C-fatty acid was esterified in the 2′-position of lecithin the enzyme involved in the mobilisation was presumably phospholipase A_2.

Our results confirm previous work [7] suggesting that mepacrine is an inhibitor of phospholipase. However, mepacrine also had some intrinsic activity against prostaglandin synthetase at the dose necessary for phospholipase blockade.

During mechanical or immunological damage, there was no release of [1-^{14}C]arachidonic acid from the neutral lipid pools, indicating that the phosphatides were the sole source of substrate for prostaglandin biosynthesis. However, our technique only detects changes in lipid pools which are labelled. If arachidonate was liberated from cholesterol esters, it would be difficult to detect, since this pool was not readily labelled (see Table 3).

It is possible that we underestimated prostaglandin biosynthesis in this work. The tracer quantities of [1-^{14}C]arachidonic acid released from phospholipids were presumably diluted by unlabelled substrate also released. This could decrease the specific activity of the substrate available for synthesis thereby leading to an underestimate of the prostaglandins formed. We did not estimate prostaglandin metabolite formation in these preparations since our experiments showed that metabolism of prostaglandin E_2 was negligible during the time of vibration.

One of the most interesting observations is that the presence of intracellular substrate is not in itself a sufficient condition for prostaglandin biosynthesis. Thus, whilst there is a small basal release of labelled prostaglandins in the presence of exogenous [1-^{14}C]arachidonic acid, this is increased more than 8-fold by vibration. When the label was previously incorporated, approximately 25 per cent was present as non-esterified fatty acids and the background production of prostaglandins was very low. During vibration the free substrate levels increased less than 2-fold, but prostaglandin biosynthesis increased by almost 4-fold. Gryglewski and Vane [8] reached the same conclusion from their experiments on vibrated dog spleen; repeated periods of vibration gave smaller and smaller outputs of prostaglandin. Addition of arachidonic acid restored the output during vibration.

The conclusion we draw from these results is that in spleen normal 'resting' cells do not synthesise prostaglandins (perhaps because the synthetase is compartmentalised) but need to undergo 'stimulation' first. This 'stimulation' can take the form of various sorts of damage, such as those investigated here, or it might be through the addition of vasoactive or other substances, many of which are known to release prostaglandins (see Flower [12]). What is the nature of this stimulation? We wish to speculate that many agents which release prostaglandins share the common property of interacting with the cell membrane in such a way that phospholipase A_2 is activated (perhaps by a change in calcium concentration), releasing fatty acid precursors and also promoting the access of fatty acids to prostaglandin synthetase. The experiments we report here with the inhibitor TYA support this idea. Exposure of the slices (Group B) to the substrate analogue for 30 min resulted in only small inhibition (21%) of prostaglandin biosynthesis—indicating that only low concentrations of the drug reached the enzyme. After vibration for 60 sec, however, there was a significant (52%; $P < 0.05$) increase in blockade of prostaglandin synthetase—indicating that during this brief period the inhibitor had access to the enzyme system. At first sight, the idea that the synthetase needs to be 'activated' in some way is not supported by other experimental evidence previously quoted—i.e. that arachidonic acid injected into isolated lungs or perfused gut results in a prostaglandin formation. However, by their very nature

such preparations are already 'damaged' and the amounts of prostaglandins produced by these preparations are well below the total synthetic capacity of these tissues, suggesting that only a small fraction of the enzyme is in operation.

Because the cellular membrane system to a large extent controls the integrity of the cell (prostaglandin synthetase is also membrane bound), its function as a storehouse of fatty acid substrates and as a regulator of permeability are intimately linked. Thus damage to a section of membrane resulting in a turnover of phosphatides could not only release fatty acids but also result in an increased diffusion of those precursors into the prostaglandin synthetase compartment. Audet *et al.* [17] investigated the activity of phospholipase A in three different strains of *E. coli.* Very little activity was found in two of the strains which had rigid cell envelopes and were thus relatively resistant to lysis. The other strain, however, had high phospholipase activity, was easily lysed, and during growth released lipid, protein and polysaccharide material into the medium. There was also production of free fatty acids. Other workers too, have noticed that the activity of this enzyme is greater in cells subjected at adverse conditions [18–20]. On the basis of this evidence, we propose that a primary lesion in cell damage is disruption of the cellular membrane system; this leads to release of fatty acids and to an increase in permeability which enables the liberated fatty acids to reach the prostaglandin synthetase enzyme, thus resulting in prostaglandin biosynthesis and release. The anti-malarial compound mepacrine blocks release of fatty acids and the aspirin-like drug indomethacin, the synthetase enzyme.

CONCLUSIONS

1. During the mechanical or immunological damage to cells, phospholipase A_2 is activated. This results in a loss of prostaglandin precursors from the 2′-position of lecithin and an increase in prostaglandin biosynthesis. Mepacrine inhibits both steps, but indomethacin only the latter.

2. During damage, there is an increase in 'permeability' of the cell which enables the substrate to diffuse into the prostaglandin synthetase compartment.

3. Our results confirm that phospholipase A_2 is an important enzyme in prostaglandin generation, but suggest that the availability of substrate is not the only condition for prostaglandin biosynthesis.

REFERENCES

1. P. J. Piper and J. R. Vane, *Ann. N.Y. Acad. Sci.* **180,** 363 (1971).
2. W. E. M. Lands and B. Samuelsson, *Biochim. biophys. Acta* **164,** 426 (1968).
3. H. Vonkeman and D. A. van Dorp, *Biochim. biophys. Acta* **164,** 430 (1968).
4. H. Kunze and W. Vogt, *Ann. N.Y. Acad. Sci.* **180,** 123 (1971).
5. B. Haye, S. Champion and C. Jacquemin, *FEBS Lett.* **30,** 253 (1973).
6. B. Samuelsson, *Fedn Proc. Fedn Am. Socs exp. Biol.* **31,** 1442 (1972).
7. B. B. Vargaftig and N. Dao Hai, *J. Pharm. Pharmac.* **24,** 159 (1972).
8. R. Gryglewski and J. R. Vane, *Br. J. Pharmac.* **45,** 37 (1972).
9. P. J. Piper and J. R. Vane, *Nature, Lond.* **233,** 29 (1969).
10. R. J. Flower, H. S. Cheung and D. W. Cushman, *Prostaglandins* **4,** 325 (1973).
11. R. K. Raheja, Charanjit Kaur, Ajit Singh and I. S. Bhatia, *J. Lipid Res.* **14,** 695 (1973).
12. R. J. Flower, *Pharmac. Rev.* **26,** 33 (1974).
13. J. R. Vane, *Nature, New Biol.* **231,** 232 (1971).
14. W. Vogt, U. Meyer, H. Kunze, E. Lufft and S. Babilli, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **262,** 124 (1969).
15. J. Bartels, H. Kunze, W. Vogt and G. Wille, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **266,** 199 (1970).
16. M. A. Palmer, P. J. Piper and J. R. Vane, *Br. J. Pharmac.* **49,** 226 (1973).
17. A. Audet, G. Nantel and P. Proulx, *Biochim. biophys. Acta* **348,** 334 (1974).
18. J. Scandella and A. Kornberg, *Biochemistry* **10,** 4447 (1971).
19. J. Bennet, J. Glavenovich, R. Liskay, D. L. Wullf and J. E. Cronan Jr., *Virology* **43,** 516 (1971).
20. P. Patriarca, S. Beckerdite and P. Elsbach, *Biochim. biophys. Acta* **260,** 593 (1972).