Br. J. Pharmac. (1972), **46**, 449–457.

The generation from arachidonic acid of rabbit aorta contracting substance (RCS) by a microsomal enzyme preparation which also generates prostaglandins

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Summary

1. Sodium arachidonate was incubated with a crude prostaglandin synthetase preparation made from dog spleen. The incubation was made in a dynamic system so that the products could be delivered to strips of rabbit aorta and rat stomach.

2. A rabbit aorta contracting substance (RCS) and a prostaglandin-like substance were formed. The RCS had similar properties to that released in anaphylaxis. It was unstable and as the RCS activity declined, so the prostaglandin-like activity increased. Its formation was prevented by prostaglandin synthetase inhibitors such as indomethacin.

3. A different rabbit aorta contracting substance was formed by incubation of arachidonate with lipoxygenase, which generates peroxides. This substance was stable and did not lead to prostaglandin production.

4. We conclude that RCS may be an intermediate in prostaglandin production, such as the postulated cyclic endoperoxide.

Introduction

Rabbit aorta contracting substance (RCS) is an unstable principle released from guinea-pig isolated lungs by anaphylaxis (Piper & Vane, 1969, 1971), by mechanical stimulation (Palmer, Piper & Vane, 1970a) or by infusion into the perfused lungs of bradykinin, slow reacting substance released in anaphylaxis (SRS-A) or from egg yolk (SRS-C), arachidonic acid or a colloidal suspension of particles (Palmer, Piper & Vane, 1970b ; Piper & Vane, 1971 ; Vargaftig & Dao, 1971). RCS is also generated by vibrated or stirred slices of rabbit spleen (Gryglewski & Vane, 1972). The appearance of RCS in the effluent from the challenged tissues is accompanied by an output of prostaglandins. The release of both RCS and prostaglandins is blocked by non-steroidal anti-inflammatory drugs (Piper & Vane, 1969, 1971 ; Gryglewski & Vane, 1972), which are potent inhibitors of prostaglandin biosynthesis (Vane, 1971). Because these results suggest that RCS may be related to the prostaglandins, we have looked for RCS formation by a prostaglandin synthetase system. Some of this work was communicated to the British Pharmacological Society (Gryglewski & Vane, 1971).

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Methods

Prostaglandin synthetase system

The enzyme preparation was prepared as described previously (Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972). Dog spleen was cut with scissors into pieces approximately 1 cm^3, washed repeatedly with ice-cold Krebs solution, blotted and weighed. The spleen was then macerated for 2 min in 4 times its weight of ice-cold 100 mM phosphate buffer (pH 7·4) using a Waring-blender at full speed. After centrifugation at 100 *g* for 10 min the precipitate was discarded and the supernatant recentrifuged at 5,000 *g* for 10 min to give a pellet and a supernatant which was further centrifuged at 80,000 *g* for 60 minutes. This gave a further pellet which, after resuspension in phosphate buffer, was used as the source of enzyme activity. The amount of spleen protein preserved in the microsomal enzyme preparation was 7–10 mg/g wet weight of spleen. The particulate fraction was stored below −20° C and used within two weeks of preparation. It contained 20 mg protein/ml as estimated by the biuret method.

Enzyme activity was estimated by incubating in 2 ml of 50 mM phosphate buffer (pH 7·4): enzyme (10 mg protein), sodium arachidonate (20 μg), reduced glutathione (100 μg) and hydroquinone (10 μg). The mixture was incubated aerobically with shaking at 37° C for 20 min and the reaction was then stopped by heating the sample in a boiling water bath to coagulate the protein. The sample was centrifuged, the supernatant was diluted 10–50 times with saline and assayed for prostaglandin content (in terms of prostaglandin E_2) on the rat stomach strip (Vane, 1957). Zero time samples were similarly assayed. The assay tissues were superfused at 10 ml/min with Krebs solution containing a mixture of antagonists (see later) to make the assay more specific and indomethacin (1 μg/ml) which, by preventing prostaglandin production in the rat stomach strip, reduced the spontaneous tone of the tissue and somewhat increased its sensitivity to prostaglandin E_2 (Eckenfels & Vane, 1972).

The zero time content of prostaglandin-like activity in four different enzyme preparations was 127 ng/mg (range 90–170 ng/mg). Although the enzyme preparations had different activities, each one gave consistent results during the two week period for which it was used.

Continuous detection of generation of RCS and prostaglandins

Samples of the enzyme incubation mixture did not contract strips of rabbit aorta, but RCS is evanescent with a half life of less than 2 min (Piper & Vane, unpublished). To test more immediately for RCS generation, a bioassay system was used in which arachidonate and enzyme were incubated not in test tubes but in a silicone rubber coil, of 30 ml volume, immersed in a water bath at 37° C. Krebs solution was continuously pumped (5 ml/min) through this incubation coil and then it superfused a strip of thoracic rabbit aorta and a strip of rat stomach. Prostaglandins E_2 or $F_{2\alpha}$, the enzyme, arachidonate or inhibitors of the enzyme (indomethacin or meclofenamate) were infused into the coil through a needle pushed through its wall. By changing the site of infusion, the substances could be incubated together in the coil for 0·5–6 min before they reached the assay tissues. This incubation method is referred to as the single bank assay system.

For some experiments, a double assay system was devised. Two banks of tissues each containing a rabbit aorta and rat stomach strip, were superfused at 5 ml/min with Krebs solution containing the antagonists as well as sodium arachidonate (5 μg/ml). The Krebs solution passed through a 10 ml incubation coil (maintained at 37° C) before superfusing the first rabbit aorta and rat stomach strip. After re-oxygenation, the Krebs solution then passed through a second coil (20 ml volume, 37° C) before superfusing the second rabbit aorta and rat stomach strip. Indomethacin (1 μg/ml) or sodium meclofenamate (1 μg/ml) were infused at the end of the second incubation coil, so that the second bank of assay tissues was continuously exposed to a prostaglandin synthetase inhibitor. This will be referred to as the double bank (series) assay system. The ED50 against the spleen enzyme preparation for indomethacin was 0·06 μg/ml and for meclofenamate was 0·03 μg/ml (Flower *et al.*, 1972). Since the antagonists were infused at 1 μg/ml, the enzyme should have been maximally inhibited.

Changes in length of the assay tissues were detected by Harvard smooth muscle transducers fixed with a pendulum lever (Paton, 1957) to give an initial load on the tissues of 2 g (stomach strips) and 4 g (aortas). The output from the transducers was suitably amplified and displayed on a multi-channel pen-recorder (Watanabe).

The Krebs solution was composed of the following in g/l. (mM): NaCl, 6·9 (118); KCl, 0·35 (4·7); $CaCl_2$ $6H_2O$, 0·55 (2·5); KH_2PO_4, 0·16 (1·2); $MgSO_4.7H_2O$, 0·29 (1·17); glucose, 1·0 (5·6); $NaHCO_3$, 2·1 (25·0). It was pre-gassed with 5% CO_2 in oxygen and also contained the following antagonists to make the reactions of the assay tissues more specific for RCS and prostaglandins (Piper & Vane, 1969): hyoscine (100 ng/ml), phenoxybenzamine (100 ng/ml), propranolol (2 μg/ml), methysergide (200 ng/ml), mepyramine (100 ng/ml).

Drugs

Drugs used were arachidonic acid (Sigma), hyoscine hydrobromide (British Drug Houses), indomethacin (Merck, Sharp & Dohme), mepyramine maleate (May & Baker Ltd.), methysergide maleate (Sandoz), phenoxybenzamine hydrochloride (Smith, Kline & French), (±)-propranolol hydrochloride (ICI), prostaglandins E_2 and $F_{2\alpha}$ (Upjohn), sodium meclofenamate (Parke, Davis & Co.), soya bean lipoxygenase (Sigma). Indomethacin was diluted from a fresh solution made up by dissolving 10–25 mg in ethanol (0·2–0·5 ml) and then diluted with Krebs solution to give a solution of 1 mg/ml. Arachidonic acid was dissolved with sodium carbonate solution (0·2 mg/ml) to give 1 mg/ml.

Results

Generation of RCS and prostaglandins from arachidonate

Prostaglandins E_2 and $F_{2\alpha}$ (10–160 ng/ml) contracted the rat stomach strip in proportion to the dose, although concentrations higher than 100 ng/ml gave near-maximal effects. The rabbit aorta was relatively insensitive to prostaglandins E_2 and $F_{2\alpha}$, only the higher concentrations sometimes inducing small contractions.

Sodium arachidonate contracted both the rat stomach strip and rabbit aorta. The contraction of the rat stomach strip produced by 5 μg/ml was equivalent to prostaglandin E_2 at 10–20 ng/ml.

The enzyme preparations alone (0·1–0·4 mg protein/ml) contracted the rat

stomach strip and the height of this contraction roughly corresponded to the amount of prostaglandin-like activity in terms of prostaglandin E_2 detected in the zero time samples. The enzyme alone had little effect on the rabbit aorta, except in higher concentrations when a small contraction was observed.

In the single bank assay system, the simultaneous infusion of arachidonate (5 μg/ml) and the enzyme (0·1–0·4 mg protein/ml) into the incubation coil to give a 2–3 min incubation resulted in a strong contraction of the rabbit aorta. The height of this contraction far exceeded the heights of contractions (if any) produced by infusion of the enzyme or arachidonate separately (Fig. 1). The rabbit aorta relaxed when either enzyme or arachidonate were withdrawn and contracted again when both enzyme and arachidonate were present (Fig. 1). The contraction of the

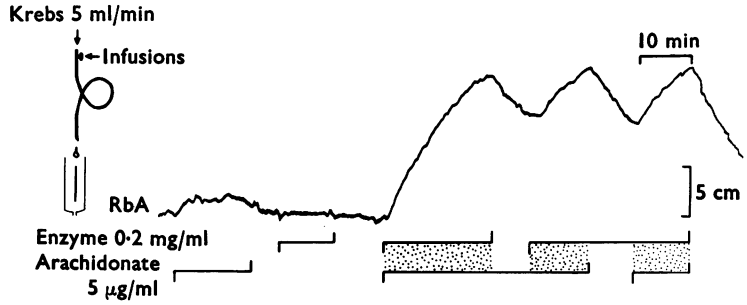

FIG. 1. A strip of rabbit aorta (RbA) was superfused with Krebs solution delivered at 5 ml/min from an incubating coil at 37° C. Arachidonate (5 μg/ml) or enzyme preparation (0·2 mg protein/ml) were infused into the coil to give a 2 min incubation. Simultaneous infusion of both substances (dotted area) caused a strong contraction of the rabbit aorta; withdrawal of either one or both caused relaxation. Time: 10 min; vertical scale 5 cm.

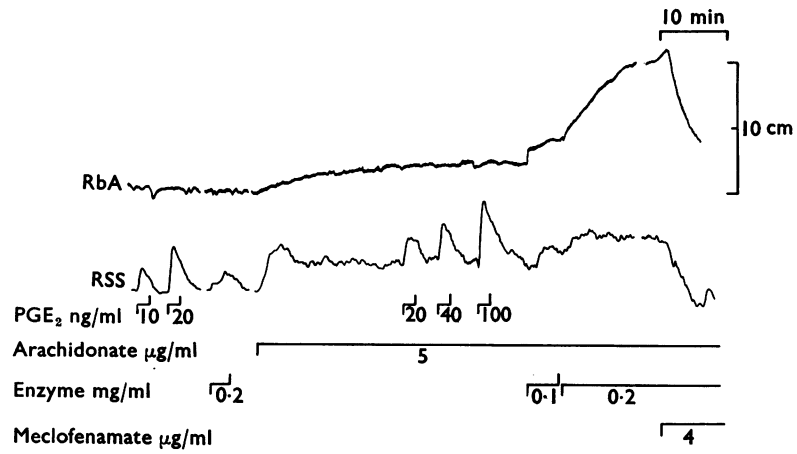

FIG. 2. A rabbit aorta (RbA) and rat stomach strip (RSS) were superfused with Krebs solution delivered at 5 ml/min from an incubating coil at 37° C. Prostaglandin E_2 (PGE_2 10–100 ng/ml), arachidonate (5 μg/ml), spleen enzyme (0·1–0·2 mg protein/ml) or meclofenamate (4 μg/ml) were infused to give a 2 min incubation in the coil. Enzyme alone contracted RSS but not RbA: arachidonate contracted both tissues. During an arachidonate infusion the sensitivity of the RSS to PGE_2 was reduced. During the arachidonate infusion, enzyme caused a strong contraction of RbA and RSS: these were reversed by meclofenamate. Time 10 min; vertical scale 10 cm.

rabbit aorta was accompanied by a contraction of the rat stomach strip, the magnitude of which indicated a doubling of the prostaglandin-like activity associated with infusion of the enzyme alone. Infusion of indomethacin or meclofenamate (1–5 μg/ml) into the coil was followed by relaxation of both tissues (Fig. 2), indicating that the generation of RCS and of prostaglandin from arachidonate was inhibited.

This suggestion was confirmed by using the double bank (series) assay system, with sodium arachidonate (5 μg/ml) added to the Krebs solution. Infusions of prostaglandins E_2 and $F_{2\alpha}$ (20–160 ng/ml) contracted the rat stomach strips in both banks of tissues but had little effect on the rabbit aortas (Fig. 3). When spleen

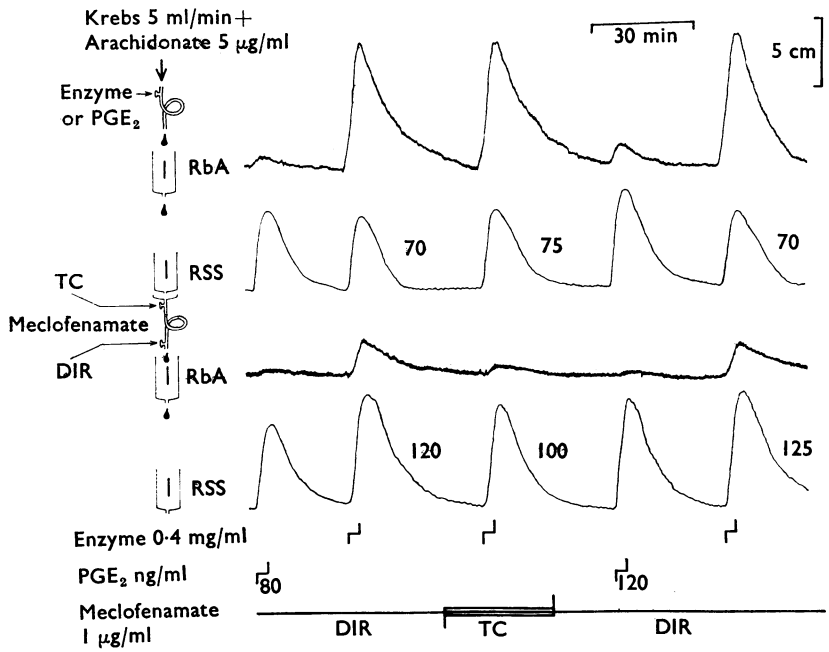

FIG. 3. A double bank (series) assay system was used with a rabbit aorta (RbA) and rat stomach strip (RSS) in both banks. Meclofenamate (1 μg/ml) was infused directly to the second bank of assay tissues. Prostaglandin E_2 (PGE_2: 80–120 ng/ml) into the first coil contracted RSS in both banks but had little effect on RbA. Enzyme (0·4 mg protein/ml) into the first coil generated PGE_2-like activity equivalent to 70 ng/ml on the first RSS and 120 ng/ml on the second. RCS was also generated and more was present after the first coil than after the second. When meclofenamate was infused into the second coil (TC), the contraction of the second RbA due to enzyme infusion was almost absent. PG-like activity on the second RSS was also reduced. The effects returned when meclofenamate was withdrawn from the coil. Time 30 min; vertical scale 5 cm.

enzyme (0·4 mg protein/ml) was infused into the first coil, contractions of both stomach strips showed the presence of a prostaglandin-like substance in far higher concentration (70 ng/ml at first strip) than could be accounted for by the amount present in the enzyme at zero time (60 ng/mg). The second stomach strip contracted even more, showing that in the second coil there had been a further increase in prostaglandin-like activity (to 120 ng/ml). Both rabbit aortas also contracted, showing the presence of an RCS-like substance. However, in all experiments, the contractions of the second rabbit aorta were consistently smaller than those of the first, even though the strips were from the same rabbit and the amplifications were adjusted to be the same.

An enzyme inhibitor (meclofenamate, 1 μg/ml) was then infused through the second coil and the infusion of enzyme to both banks repeated. There was a similar generation of RCS-like and prostaglandin-like activity in the first coil, as shown by the contractions of the first rabbit aorta and rat stomach strip, but the contractions of the second bank of assay tissues were reduced. However, there was still some increase in the prostaglandin-like activity (from 75–100 ng/ml) but the RCS-like activity detected by the second rabbit aorta had almost disappeared. Increasing the concentration of meclofenamate or indomethacin to 5 μg/ml (five experiments) still did not completely suppress the small additional generation of prostaglandin-like activity in the second coil.

Instability of RCS generated from arachidonate

The smaller contraction of the second rabbit aorta during enzyme infusion in the above experiments might have been due to a rapid formation of RCS in the first incubation coil with a decline in concentration (due to instability) before reaching the second rabbit aorta. To test this possibility, a single bank assay system was used, with arachidonate (5 μg/ml) added to the Krebs solution. Sodium meclofenamate (1 μg/ml) was infused at the end of the incubation coil so that the assay tissues were constantly exposed to the inhibitor. Enzyme (0·1 mg protein/ml) was infused for 2 min periods so that it incubated with the arachidonate for 0, 30 and 60 s before reaching the rabbit aorta (Fig. 4). RCS was produced in the greatest amount during the 30 s incubation. Since meclofenamate was infused 30 s away from the assay tissue and the enzyme infusion was repeated at 60 s, the arachidonate and enzyme could react freely for 30 s, after which time the enzyme was mixed with inhibitor for the next 30 seconds. If the substance which contracted the rabbit

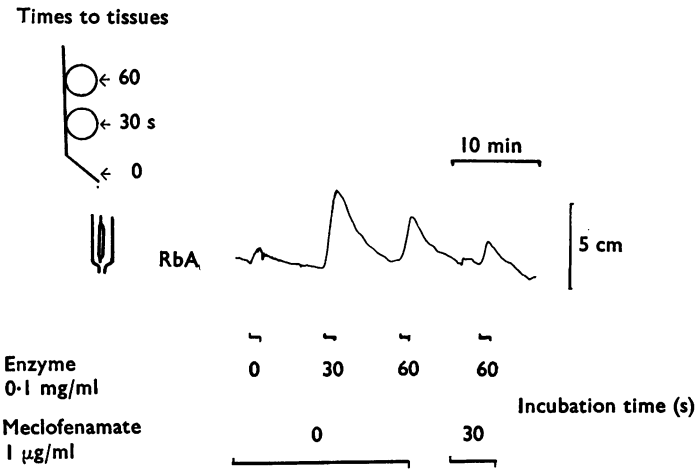

FIG. 4. A rabbit aorta (RbA) was superfused with Krebs solution (containing arachidonate, 5 μg/ml) delivered at 5 ml/min from an incubating coil at 37° C. Enzyme (0·1 mg protein/ml) was infused to incubate in the coil for 0, 30 and 60 seconds. Meclofenamate (1 μg/ml) was infused directly to the RbA. Enzyme infusion at 0 s produced a little RCS; there was maximum RCS production at 30 seconds. Meclofenamate was then infused at 30 s and enzyme at 60 s, so that the enzyme was incubated with arachidonate for 30 s before suppression of activity by meclofenamate. The contraction of RbA was small, indicating that the RCS produced in the first 30 s had declined in activity during the second 30 seconds. Time 10 min; vertical scale 5 cm.

aorta was stable, the size of the contraction should at least match that produced by the previous 30 s incubation of enzyme with arachidonate. However, as shown in Fig. 4 the contraction was much smaller, showing that the RCS-like activity had declined. Similar results were obtained in two other experiments.

Formation of an RCS from arachidonate by lipoxygenase

Soya bean lipoxygenase, like prostaglandin synthetase, removes 13-L hydrogen from arachidonate and forms a peroxide, although the oxygen molecule is inserted at a different position with these two enzymatic systems (Hamberg & Samuelsson, 1967). The activity of lipoxygenase was compared with that of spleen enzyme.

Infusion of lipoxygenase alone (2–20 μg/ml ; Fig. 5) had little or no effect on

FIG. 5. A rabbit aorta (RbA) and rat stomach strip (RSS) were superfused with Krebs solution delivered at 5 ml/min from an incubating coil at 37° C. Lipoxygenase (2 and 20 μg/ml) had little effect in the tissues. Arachidonate (5 μg/ml) was then infused ; this caused contraction of both tissues. In the presence of arachidonate PGE_2 was less active on the RSS but lipoxygenase (2 μg/ml) now contracted both RbA and RSS, as did the crude prostaglandin synthetase preparation from dog spleen. Time 20 min ; vertical scale 5 cm.

the assay tissues. However, an infusion of lipoxygenase (2 μg/ml) in the presence of arachidonate (5 μg/ml) resulted in a contraction of the rabbit aorta. The contraction was similar in height to that produced by an infusion of spleen enzyme at a concentration 0·4 mg protein/ml in the presence of arachidonate. The lipoxygenase-induced contractions of the rat stomach strips in the presence of arachidonate were much smaller than the contractions induced by spleen enzyme (Fig. 5). Further incubation in the double bank (series) assay system showed that the activity of the RCS formed by lipoxygenase did not change within 3 minutes. There was also no increase in prostaglandin-like activity in the second coil.

Discussion

We have previously shown (Gryglewski & Vane, 1972), using a superfused preparation of rabbit chopped spleen, that vibrating or stirring the tissue released RCS- and prostaglandin E_2-like activity and that further incubation of the superfusate

caused the concentration of RCS to decline and that of prostaglandin E_2 to increase. This result was interpreted to mean that the process of prostaglandin biosynthesis includes RCS as an intermediate and that, once formed, RCS can spontaneously give rise to a prostaglandin.

The present results reinforce this conclusion, for it has now been shown that a crude enzyme system which generates prostaglandin E_2 and $F_{2\alpha}$ from arachidonate (Flower *et al.*, 1972) can also generate RCS. Because of the instability of RCS, a dynamic incubation system was used. This allowed the products of the incubation of arachidonic acid with the microsomal enzyme preparation from dog spleen to be delivered to bioassay tissues chosen to detect RCS- and prostaglandin-like activity.

The RCS formed during the enzyme/arachidonate interaction had similar characteristics to that released from guinea-pig lungs (Piper & Vane, 1969) in that it was unstable, losing activity in a few minutes and its formation was inhibited by anti-inflammatory drugs such as indomethacin or meclofenamate. Thus, when two incubation coils were used in series, infusion of enzyme and arachidonic acid caused formation of RCS and prostaglandin-like activity in both. Whereas the prostaglandin activity was greater in the second coil, the RCS activity was, if anything, less. Furthermore, even when indomethacin or meclofenamate was infused into the second coil some increase in prostaglandin-like activity was seen. This result suggests that RCS formation in the second coil had been inhibited by the synthetase inhibitors, but that the RCS, which was already present due to the first coil incubation, continued to lead to formation of prostaglandins.

Incubation of soya bean lipoxygenase with arachidonate also produced a substance which contracted the rabbit aorta and weakly contracted the rat stomach strip. It is reasonable to suppose that this substance is a peroxide of arachidonic acid, which is isomeric with the peroxide formed in the first stage of the enzymic formation of prostaglandins (Hamberg & Samuelsson, 1967). Thus the rabbit aorta, although relatively insensitive to prostaglandins, may be highly sensitive to peroxides of arachidonic acid (or to peroxides of unsaturated fatty acids in general), while the rat stomach strip is more sensitive to prostaglandins than to peroxides. This conclusion is supported by the present results, if RCS is a peroxide.

All these results, coupled with the previous observations (Piper & Vane, 1969, 1971; Gryglewski & Vane, 1972) suggest that RCS is a peroxide, or the cyclic endoperoxide of arachidonic acid postulated as the unstable intermediate in biosynthesis of prostaglandins (Nugteren, Beerthuis & van Dorp, 1967; Samuelsson, Granstrom & Hamberg, 1967). If it is either, anti-inflammatory drugs inhibit biosynthesis of prostaglandins at the stage before formation of RCS.

Our results can also be interpreted in another way. The enzyme preparation was relatively crude and could have contained, as well as the prostaglandin synthetase system, another enzyme capable of utilizing arachidonate as a substrate. If this were so, then RCS could be generated separately from, and in parallel to, the production of prostaglandins, without participating as an intermediate. Certainly we cannot at present exclude this possibility but we think it unlikely because, in all situations so far tested, RCS release is accompanied by prostaglandin biosynthesis and release. Such a close relationship implies more than the fortuitous occurrence of a second unidentified enzyme, able to use the same substrate, appearing in the same preparations, stimulated into activity by the same processes and inhibited by the same substances as the prostaglandin synthetase system.

We are grateful to Mr. M. Peck, Mr. N. Marley, and Miss S. Blennerhasset for technical assistance and to the Wellcome Trust for a grant.

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(*Received May* 2, 1972)