Cyclic AMP and prostaglandin E in perfusates of rat hind paws during the development of adjuvant arthritis

M. J. PARNHAM, I. L. BONTA, AND M. J. P. ADOLFS

From the Department of Pharmacology, Medical Faculty, Erasmus University Rotterdam, PO Box 1738, Rotterdam, The Netherlands

SUMMARY The contralateral un.injected hind paws of rats which had been injected with Freund's complete (FCA) or incomplete (FIA) adjuvant 10, 14, 18, or 22 days previously were perfused under urethane anaesthesia using a stainless steel coaxial catheter. In one series of experiments cyclic AMP (cAMP) levels were determined after a 2-hour perfusion. cAMP levels were also determined in rats treated on days 16–22 with either prostaglandin E$_1$ (PGE$_1$) (500 μg/kg per day subcutaneously) or saline. When compared with control rats injected with FIA, after 22 days, cAMP levels in FCA-injected rats fell between days 10 and 18 and then rose between days 18 and 22. After PGE, treatment, paw volume on day 22 increased in rats injected with FCA, but cAMP levels were not significantly altered when compared with day 22 FCA-injected controls treated with saline.

In a second series of experiments, using a slightly modified perfusion method, PG levels in extracts of 30-minute perfusates were determined after chromatography and bioassay. Protein levels and paw volume were also measured. No PGF was detectable in any perfusate. Changes in PGE levels in FCA-injected animals paralleled changes in paw volume, increasing from day 14 and reaching a maximum on day 22. Both parameters remained unchanged in FIA-injected rats. Protein levels in perfusates from FCA-injected animals were significantly greater than FIA-injected controls only on day 22.

We suggest that the early changes in cAMP reflect the infiltration of activated leucocytes into the inflamed joint and that the increase in PGE is attributable to lysosomal enzyme activity. Later increases in cAMP are attributable both to the increasing PGE levels and to other changes in cellular activation. We suggest that high levels of PGE contribute to tissue damage which is reflected in raised protein levels.

Prostaglandins (PGs), particularly those of the E series, are released locally during various inflammatory responses, both in man and in laboratory animals, and exert both pro- and anti-inflammatory actions (see Zurier, 1974; Vane, 1976). PGs have thus been ascribed a dual role as modulators of inflammation (Willoughby and Dieppe, 1976).

The anti-inflammatory property of pharmacological doses of PGE was first demonstrated in adjuvant arthritis in rats (Aspinall and Cammarata, 1969; Zurier and Quagliata, 1971). The inhibitory effects of PGE on leucocyte function in vitro are related to increases in intracellular cyclic AMP (cAMP) (Bourne et al., 1974), and theophylline, an inhibitor of cAMP phosphodiesterase, was found in vivo to potentiate the PGE$_{1}$-induced suppression of the arthritic syndrome (Bonta et al., 1977b, 1978).

Adjuvant arthritis in the rat is similar in several respects to rheumatoid arthritis (RA) (Pearson, 1964) and we have suggested that the interactions between PGE and cAMP in the two diseases may also be similar (Parnham et al., 1977). However, although PGE production has been shown to increase in synovial joints of RA patients, with associated changes in cAMP (Higgs et al., 1974; Robinson and Levine, 1974; Robinson et al., 1975a), no such changes have been reported in the inflamed joints of rats with adjuvant arthritis. We describe here the changes which occur in the levels of cAMP and PGE.
in perfusates of arthritic rat joints obtained at different stages during the development of chronic inflammation. Preliminary reports of some of these data have been published (Adolfs et al., 1977; Parnham et al., 1977).

**Materials and methods**

**Animals and treatment**

Male rats of an inbred Lewis strain (Central Animal Breeding Institute, Hanover, W. Germany), weighing 180–250 g, were maintained on standard laboratory diet and drinking water *ad libitum*. Arthritis was induced by injecting 0.1 ml Freund’s complete adjuvant (FCA) (5 mg/ml *Mycobacterium butyricum* in liquid paraffin; Difco, Detroit) into the left hind paw. Control animals received 0.1 ml incomplete adjuvant (FIA) (liquid paraffin only). FCA injection was accompanied by an acute inflammatory response, and subsequent swelling in the uninjected right hind paw about 10 days after FCA injection was taken to indicate the chronic arthritic response.

In the first series of experiments, for cAMP determination, arthritic animals were anaesthetised with urethane (1.65 g/kg) 10, 14, 18, or 22 days after FCA injection. Because of the small number of animals available, control animals were anaesthetised only on day 22 after FIA injection. A group of FCA-injected rats were also treated with either saline (1 ml/kg per day subcutaneously, 5 animals) or PGE₁ (Unilever, Vlaardingen) (0.5 mg/kg per day subcutaneously, 5 animals) on days 16–22 inclusive, before being anaesthetised on day 22. Uninjected hind paw volumes were also determined in this group on day 22, using a differential volume meter (Ugo Basile, Milan). All animals were weighed before anaesthesia.

In the second series of experiments, for PG determination, both FCA- and FIA-injected animals were anaesthetised with urethane 10, 14, 18, or 22 days after injection. Uninjected hind paw volumes and body weights of all rats were measured before adjuvant injection and before anaesthesia.

**Perfusion**

Two modifications of the technique of Rocha e Silva and Antonio (1960) were used, the method of determining PG levels being an improvement of the technique initially used in determining cAMP levels. Both modifications are shown in Fig. 1 *A* and *B*.

For measurement of cAMP levels in paw perfusates, an incision was made, after anaesthesia, in the skin covering the thigh of the uninjected hind leg of

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**Fig. 1** Stainless-steel, coaxial catheters used to perfuse uninjected hind paws of rats. (*A*) Method used in determining cAMP levels. The outer efferent tube (inner diameter 2 mm) was straight, and the inner afferent tube (inner diameter 0.8 mm) was bent and exited through the side of the outer tube. The needle-shaped tip covered the tibiotalar joint. (*B*) Method used in determining PGE and protein levels. The inner tube was straightened to receive a thin wire before the perfusion. The needle-shaped tip was pushed into the joint space between tibia and calcaneus.
the rat and a stainless steel coaxial catheter inserted (Fig. 1A). The catheter was pushed subdermally down the outer side of the leg until the tip covered the tibiotalar joint. The catheter was then secured by a thread which also closed the incision. Sterile 6% dextran/saline (Dextran Povit 70, Povit Products, Amsterdam) was pumped for 2 hours, at 0·2 ml/min (using a Braun-Melsungen Unita II pump), down the small inner tube and the perfusate was collected on ice from the larger outer tube. To test for possible interference of the dextran, 0·1 ml of the 6% dextran/saline solution was injected into the hind paws of 4 unanaesthetised normal rats and 4 normal rats anaesthetised of paws over maintained solution of 5% dextran (using a Braun-Melsungen tube). Furthermore, the catheter was pushed subdermally down the back (instead of the side) of the uninject ed leg so that the tip lay in the space between the tibia and the calcaneus. As in the earlier perfusion experiments, the catheter was secured round the incision with thread. Although the perfusion rate was maintained at 0·2 ml/min, the perfusion time was limited to 30 minutes, and since the perfusate was collected over ice, breakdown of the unstable PGs was reduced. Initial experiments, using this modified technique, were carried out with 6% dextran/saline as the perfusion fluid. However, recovery of labelled PGs, after extraction from the perfusate, was low, presumably due to binding of PGs to the dextran. Thus our results on PG levels in the perfusates were obtained using Krebs solution (composition g/1: NaCl 6·9, KCl 0·35, CaCl2 0·27, KH2PO4 0·16, MgSO4·7H2O 0·29, NaHCO3 2·1, glucose 1·0) as the perfusion fluid.

**CYCLIC AMP ASSAY**

Four ml of the 2-hour perfusate were shaken with 10 ml chloroform: methanol (2:1), to remove total lipids. The dextran was then emulsified by the addition of 2 × 10 ml absolute ethanol and the mixture centrifuged at 2500 rpm for 5 minutes. The supernatant was evaporated to dryness under vacuum on a rotary evaporator (Büchi, Flawil, Switzerland) and resuspended in 350 μl distilled water. Each perfusate extract was then assayed for cAMP, in duplicate, by the protein-binding method of Gilman (1970), using a commercially-available kit (Boehringer-Mannheim). The mean recovery of 3H-cAMP (7500 cpm), added to the perfusate, through the extraction procedure was 82·3%. The mean difference between duplicate samples was 29·7%. cAMP binding was undetectable after incubation of the samples with phosphodiesterase (Sigma, London) for 1 hour at 37°C.

Results were expressed as pmol cAMP/2 hour, i.e. the total amount of cAMP present in the perfusate after a 2-hour perfusion (approx 25 ml). All values were corrected for recovery.

**PG EXTRACTION, SEPARATION, AND BIOASSAY**

1 ml of the 30-minute perfusate was removed for protein determination. The remainder was acidified to pH 3 with 1 M HCl and the PGs extracted with 3 × 1 volume ethyl acetate. The acetate layers were removed with a Pasteur pipette and evaporated to dryness on a rotary evaporator. After storage overnight at −21°C, the dried extracts were resuspended in 1 ml redistilled chloroform and subjected to chromatography on silicic acid minicolumns using increasing concentrations of methanol in chloroform as successive eluants (Parnham and Sneddon, 1975). The fractions containing the PGEs and PGFs were each resuspended in 0·5 ml saline for bioassay. Mean recoveries of 3H-PGE2 (17 000 cpm, Radiochemical Centre, Amersham) and 3H-PGF2α (15 000 cpm, Radiochemical Centre) through extraction and chromatography were 65·8% and 66·3% respectively.

PGE column fractions were assayed against authentic PGE2 (Upjohn, Kalamazoo) on the isolated rat stomach strip (Vane, 1957) and PGF fractions were assayed against authentic PGF2α (Upjohn) on the isolated rat colon (Regoli and Vane, 1964). All assays were carried out by bracketing. The isolated tissues were suspended in an organ bath containing liquid paraffin at 37°C (Ferreira and De Souza Costa, 1976) and superfused at 0·2 ml/min with Krebs solution containing a mixture of antagonists (Gilmore et al., 1968) plus indomethacin (Merck, Sharpe and Dohme) (2 μg/ml). Samples and standards were injected directly over the tissues in constant volumes of 50 or 100 μl (Bult et al., 1977). Results were expressed as total PGE (ng) present in the 30-minute perfusate (approx 6 ml).

**PROTEIN DETERMINATION**

One ml samples of perfusates used for PG assay were
assayed for protein by the method of Lowry et al. (1951) using egg albumin as a standard. Results were expressed as total protein (mg) present in the 30-minute perfusate (approx 6 ml).

**Statistical Analysis**
The significance of differences between FIA- and FCA-injected animals was determined by the one-tailed Mann-Whitney U test. For cAMP levels, the significance of differences from the FIA-injected control group, taken on day 22, was determined for all FCA-injected groups, irrespective of the length of time from FCA injection. With the remaining data, the significance of differences was determined between FIA-injected and FCA-injected groups perfused on the same day after injection.

**Results**

**Cyclic AMP Levels**
The mean cAMP levels in perfusates from paws taken at different stages during the development of the chronic arthritic response are shown in Fig. 2. cAMP levels in perfusates from day 10 FCA-injected rats were not significantly different from day 22 FIA-injected controls, although by day 14 cAMP levels were markedly reduced when compared with controls. This persisted in day 18 perfusates, but by day 22, although still significantly reduced, cAMP levels had increased towards control levels once again.

![Cyclic AMP levels after PGE1 treatment](image)

**Fig. 3** Mean PGE levels and protein levels in perfusates and mean changes in volume of uninjected hind paws of adjuvant-injected rats. (a) Mean PGE levels; (b) mean changes in paw volume. Values were calculated by subtracting paw volume/100 g body weight on the day of adjuvant injection from paw volume/100 g weight on the day of perfusion. (c) Mean protein levels. • FIA-injected animals, ○ FCA-injected animals. The numbers indicate the number of perfusions per mean value. Significance of differences (FIA vs FCA): * P<0.05, ** P<0.01.
0.02 ml, 5 rats). However, mean cAMP levels in 2-hour perfusates from these same paws were not significantly different (P > 0.05), though the mean levels in perfusates of PGE-treated paws (902.2 ± 311.8 pmol/2 h) were somewhat higher than those in perfusates of saline-treated paws (593.2 ± 168.2 pmol/2 h).

**PG and Protein Levels**

The changes in mean PGE and protein levels and in mean paw volumes during the development of the arthritic response are shown in Fig. 3a, b, c. PGF levels in perfusates from both FIA- and FCA-injected animals were below the detection limit of our bioassay (500 pg PGF/100 µl, i.e. 2.5 ng PGF/30-min perfusate). The detection limit for the PGE bioassay was 100 pg PGE/100 µl, i.e. 500 pg PGE/30-min perfusate. Mean PGE levels in perfusates from FCA-injected animals, taken 10 and 14 days after adjuvant injection, were not significantly different from those in perfusates from FIA-injected controls (Fig. 3a). However, PGE levels in FCA-injected rats had increased significantly by day 18 and further by day 22.

This time pattern was reflected in the increase in the mean volume of the uninjected hind paws of the FCA-injected animals when compared with FIA-injected controls (Fig. 3b), confirming earlier paw volume measurements in similarly-treated Lewis rats (Bonta et al., 1977a, b, 1978). However, the increase seen on day 18 was not significant, as one of the 4 FCA-treated rats perfused on this day exhibited no marked arthritic symptoms.

Protein levels in perfusates from FCA-injected rats also increased between days 14 and 22 (Fig. 3c), achieving a significant difference from FIA-injected controls on day 22 only. Similar PGE and protein levels were also obtained in initial experiments using 6% dextran/saline as the perfusing fluid.

**Discussion**

Our results indicate that while PGE and protein release from chronic arthritic rat paws increases with paw swelling, cAMP release falls initially and then rises again as the paw swelling reaches a peak. Random sampling of perfusates, obtained for both cAMP and PG assay, showed that the fluid was cell-free at all stages of the arthritic response. It is possible that cells adhered to both the tissue and the inside of the steel catheter used for the perfusions. cAMP release into the culture medium has been reported with several isolated cell types, including rat macrophages (Gemsa et al., 1975) and human fibroblasts (Franklin and Foster, 1973), and in both cases this release was stimulated by PGE. Thus, the cAMP detected in the perfusates in our study was probably released into the extracellular fluid by cells in and around the tibiotarsal joint.

There are doubts as to which leucocytes are the first to migrate into the joints of uninjected limbs of FCA-injected rats (Jones and Ward, 1963; Burnstein and Waksman, 1964; Perper et al., 1975). However, pharmacological inhibition in vitro of the function of all leucocytes detectable in arthritic joints is associated with an increase in intracellular cAMP (Bourne et al., 1974). It is likely then that activation in vivo is associated with a decrease in cAMP levels.

This decrease, observed between days 10 and 18 in our experiments, probably reflects the infiltration into the joint of activated leucocytes.

Although cAMP levels in perfusates of day 22 arthritic joints were still significantly lower than in control perfusates, they were higher than in day 18 perfusates, probably reflecting the changes in intracellular cAMP which occur in different cell types in established chronic joint disease (see Parnham et al., 1977). Our results also show that by day 22 PGE levels in arthritic paws are markedly higher, an increase which is also seen in the synovia of RA patients (Higgs et al., 1974; Robinson and Levine, 1974). Robinson et al. (1975a) have shown that the increased production of PGE by cultured RA synovial cells is associated with increased cAMP production. Thus, a large component of the increase in cAMP towards control levels, seen in our day 22 arthritic rat paw perfusates, may have been due to stimulation of synovial cell adenylate cyclase by the increased amounts of PGE.

The complex nature of the changes in cAMP occurring in arthritic joint synovia is indicated by the results of the short series of experiments in which rats were treated with PGE, on days 16–22. Surprisingly, at the relatively low dose used, PGE increased paw volume as measured on day 22. This is in contrast to previous results (Aspinall and Cammarata, 1969; Zurier and Quagliata, 1971; Bonta et al., 1977b, 1978). However, there is some indication that low doses of PGE increase lysosomal enzyme release from granulocytes in vitro (Weissmann, 1972) and this may have accounted for the increase in paw volume in our study. cAMP levels in perfusates from the PGE-treated animals showed large variations, suggesting that the PGE may have affected several independent cAMP-mediated processes in the arthritic joints, such as fibroblast, leucocyte, and osteoclast activity (Parnham et al., 1977). Consequently, cAMP levels in PGE-treated animals were not significantly different from saline-treated controls.

The gradual increase in PGE levels in arthritic rat paw perfusates, occurring between days 14 and 22, closely paralleled the increase in paw volume.
Since relatively low doses of PGE potentiate the oedema-producing activity of other mediators, such as histamine and bradykinin (see Vane, 1976), this sensitising activity of PGE is probably involved in the production of the early inflammatory symptoms in adjuvant arthritis. It has been suggested that phospholipase A, released by lysosomes, is the main trigger to the release of PGs in inflammation (Kunze and Vogt, 1971; Anderson et al., 1971). Certainly, lysosomal enzyme activity in uninjected hind paws of FCA-injected animals starts to increase about 8 to 9 days after FCA injection (Anderson 1970; Ignarro and Slywka, 1972). Since we were unable to determine the efficiency of our perfusion in extracting PGs from the joints, the true increase in PGE production in the inflamed paws may have begun earlier than we were able to detect.

Thus, it is highly probable that release of lysosomal phospholipase A was responsible for the increased PGE release. PGF2\alpha may also be involved in inflammatory processes (see Willoughby and Dieppe, 1976), but we detected no PGF in our rat paw perfusates. Recently, it has been suggested that PGG2, an unstable intermediate in PG biosynthesis, may be more important than the classical PGs in inflammatory processes (Kuehl et al., 1977). Thus, although we measured PGE-like activity in perfusates of arthritic rat paws, with regard to pro-inflammatory actions this may simply be a more stable metabolite of the more active PGG2.

The increase in PGE levels was followed by a significant increase in protein release into paw perfusates between 18 and 22 days after FCA injection. This was taken to indicate the necrotic changes occurring in the joint at this time. Since PGE released by rheumatoid synovial tissue has been shown to induce bone resorption in vitro (Robinson et al., 1975b) and intra-articular PGE1 causes marked cartilage damage in the rabbit in vivo (Teitz and Chrisman, 1975), it is likely that the increased PGE released by adjuvant arthritic paws also contributes to bone and cartilage necrosis.

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