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Anti-inflammatory activity of phosphodiesterase (PDE)-IV inhibitors in acute and chronic models of inflammation

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SUMMARY

Inhibitors of cyclic nucleotide phosphodiesterases are known to suppress lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) production *in vitro* in human monocytes. The most potent of these have selectivity for type IV PDEs, suggesting that this class of PDE is the major type involved in the regulation of human TNF-α production. Using compounds of two distinct chemical structural classes, a quinazolinedione (CP-77 059) and a 4 arylpyrrolidinone (rolipram), we show here that PDE-IV-specific inhibitors are also potent in suppressing LPS-induced TNF-α production *in vitro* in sodium periodate-elicited murine macrophages (IC_{50}s of 1 and 33, respectively). We then report the *in vivo* anti-inflammatory effect of PDE-IV inhibition in five murine models of inflammation: (i) elevation of serum TNF-α induced by a sublethal LPS injection; (ii) LPS-induced endotoxic shock; (iii) LPS/galactosamine-induced endotoxic shock; (iv) carrageenan-induced paw oedema; and (v) adjuvant arthritis. Following a sublethal (5 μg/mouse) injection of LPS, serum TNF-α levels in mice peaked sharply, reaching concentrations of 3–12 ng/ml 90 min after injection. In this sublethal LPS assay, CP-77 059 was about 30 times more potent than rolipram, with a minimum effective dose of 0·1 mg/kg *versus* 3 mg/kg for rolipram. This rank order is in keeping with the relative *in vitro* IC_{50}s for CP-77 059 and rolipram, as well as their relative Ki against the human PDE-IV enzyme (46 nM and 220 nM, respectively). In LPS-induced endotoxic shock, rolipram and CP-77 059 at relatively high doses of 30 and 10 mg/kg, respectively, significantly reduced serum TNF-α levels, and also inhibited mortality 66%. In the LPS/galactosamine shock model, in which mice are rendered exquisitely sensitive to LPS by co-injection with galactosamine, only 0·1 μg of LPS/mouse is necessary for serum TNF-α elevation and death. Both rolipram and the CP-77 059 caused dose-dependent reduction of serum TNF-α and lethality. In the carrageenan-induced paw oedema model, in which there is a pronounced local TNF-α response (without a serum TNF-α elevation), rolipram significantly inhibited paw swelling as well as localized TNF-α levels in the paw. In the adjuvant arthritis model, a chronic model of inflammation also possessing localized TNF-α elevation in the inflamed paw, rolipram and CP-77 059 suppressed ankle swelling and radiological evidence of joint damage. These data are consistent with a major role for PDE-IV in regulation of TNF-α production and inflammatory responses in murine systems. It suggests a potential therapeutic use for PDE-IV-specific inhibitors in inflammatory disease such as rheumatoid arthritis, septic shock and other inflammatory diseases where TNF-α has been postulated to be a contributing factor in the pathology of the disease.

Keywords arthritis *in vivo* PDE-IV inhibitors rolipram shock tumour necrosis factor-alpha

INTRODUCTION

The intracellular concentration of cAMP appears to play a major role in the response of inflammatory cells to a wide range of stimuli [1–4]. This is supported primarily by the similar effects on cell function of a variety of cyclic nucleotide-elevating agents including cAMP analogues such as dbcAMP, agents which stimulate adenylate cyclase activity such as prosta-

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glandin E_1 (PGE_1), forskolin and β2-agonists, and cyclic nucleotide phosphodiesterase inhibitors which slow the catabolism of cAMP and cGMP. One of the most striking examples is the suppressive effect of these agents on the production of tumour necrosis factor-alpha (TNF-α) monocytes and macrophages [5–10].

The relatively recent characterization of several PDE isoforms exhibiting differential regulation and tissue expression, and the availability of inhibitors selective for some of these isoforms, has spurred efforts to develop further PDE isoform-selective inhibitors as tools for investigating PDE function and as potential therapeutics. Based on the effect of prototypic PDE-IV selective inhibitors such as rolipram, and molecular cloning efforts, PDE-IV is thought to be a major phosphodiesterase isoform responsible for catabolism of cAMP and regulation of inflammatory function in many cells, including monocytes, lymphocytes, mast cells, basophils and neutrophils [1–4,11].

Like IL-1β, TNF-α is a pluripotent cytokine believed to be involved in the pathology of a variety of inflammatory diseases [12–14]. TNF-α bears many of the activities of IL-1, such as induction of bone resorption, activation of collagenase, stimulation of prostaglandin release and up-regulation of endothelial cells [14]. Again like IL-1β, TNF-α appears to play a key role in septic shock models, as demonstrated by Pfeffer *et al.* [15]. Presence of localized and systemic TNF-α in acute and chronic animal models of inflammation has been shown by Sekut *et al.* [16] and Smith-Oliver *et al.* [17].

In the present study, we investigate the effect of PDE-IV-selective inhibitors, representing two distinct chemical structural classes, on *in vitro* and *in vivo* murine TNF-α production, and on the inflammatory response in several murine inflammation models.

MATERIALS AND METHODS

Animals

Female inbred C57Bl/6, C3H/hen and C3H/HeJ mice (approximately 22 g each) were obtained from Charles River Laboratories Inc. (Raleigh, NC). Rats used in the carrageenan oedema studies were 250-g male, Lewis rats purchased from Charles River Labs. In the adjuvant arthritis model, male Lewis rats 160–170 g from Charles River Labs were free of pathogenic viruses as determined by a standard viral titre screen (Microbiological Associates, Bethesda, MD).

PDE inhibitors

The following PDE inhibitors have been previously described and were synthesized in house for these studies: PDE-IV-specific inhibitors rolipram and CP-77 059 [18]; PDE-III-specific inhibitor CI-930; PDE-V inhibitor zaprinast (Verghese *et al.*, J Pharm Exp Ther, accepted for publication, 1995).

TNF-α production by murine macrophages

C3H/HeJ female mice were injected intraperitoneally with 2 ml of 5 mM $NaIO_4$. Five days later, mice were euthanized and peritoneal exudate cells collected into cold PBS. Erythrocytes were lysed with ammonium chloride if necessary. Cells were plated at 5×10^5 cells/well in a 24-well tissue culture plate and allowed to adhere for 1·5 h. Non-adherent cells were removed and test PDE inhibitors were added in RPMI medium with 1% fetal bovine serum (FBS). Lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4; Sigma Chemical Co., St Louis, MO; 2·5 ng/ml final concentration) was then added and cultures were incubated for 24 h at 37°C. TNF-α protein was quantified in the supernatant fluids by a commercial murine TNF-α ELISA kit (Genzyme Diagnostics, Cambridge, MA).

Sublethal LPS injection

C3H/hen mice received an i.p. injection of 5 μg LPS in 0·5 ml PBS. Mice were bled from the abdominal vein 90 min following i.p. injection ($n = 4$). The blood was allowed to clot overnight at 4°C and then centrifuged for 15 min. Serum TNF-α levels were measured by ELISA kit.

The PDE-IV inhibitors were suspended in a 0·1% methyl cellulose solution and ground in a homogenizer (Eberbach) to ensure a uniform suspension. A 0·5-ml volume of compound was administered to each mouse by oral gavage 30 min before i.p. injection of LPS. Mice were fasted overnight before dosing.

Endotoxic shock models

Both endotoxic shock models were very similar. They differ mainly in the amount of LPS used and the additional inclusion of galactosamine. In the shock model with LPS alone, 500 μg/mouse of LPS were injected intraperitoneally into C3H/hen mice. In the LPS/galactosamine model of shock, C57/Bl mice received an i.p. injection of 0·5 ml of a mixture of LPS (0·1 μg/mouse) and galactosamine (600 mg/kg; Aldrich Chemical Co., Milwaukee, WI) as previously described [16]. Compounds were administered and serum was obtained and analysed as in the sublethal model ($n = 4$). The survival rate was assessed over a 24-h period ($n = 6$).

Carrageenan-induced paw oedema in rats

A 0·1-ml volume of a 1% solution of carrageenan was injected into the rat hind paw. Animals, previously fasted overnight, were dosed orally with compound in 0·1% methyl cellulose 1 h before carrageenan injection. Three hours after injection, animals were euthanized and paw swelling was measured with calipers. Each group contained eight animals.

For measurement of local TNF-α, paws were removed, weighed, snap-frozen in liquid N_2, pulverized and homogenized for 2 min in 10 ml saline at 4°C as previously described [16]. The suspension was centrifuged in a microcentrifuge to remove debris, and the supernatant assayed using the TNF-α ELISA kit.

Adjuvant arthritis in rats

Rats were injected subcutaneously in the base of the tail with 0·05 ml of Freund's complete adjuvant (FCA) containing 300 μg of *Mycobacterium tuberculosis* (Difco Labs, Detroit, MI) as described previously [19]. Compounds were suspended in a 0·1% solution of methyl cellulose, ground in a homogenizer, and administered by oral gavage once daily in a volume of 2 ml. Ankle diameter was measured throughout the time-course using calipers. At the end of the experiment, rats were euthanized, paws were removed and radiological analysis was performed as previously described [20]. Ankles were scored in a blinded fashion, and each of the five parameters were evaluated on a scale of 0–4. The features given a radiological score were (i) bone demineralization, (ii) bone erosion, (iii) periostitis, (iv) cartilage space reduction, and (v) soft tissue swelling. Thus the

Table 1. *In vitro* effect of phosphodiesterase inhibitors on lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF) production in murine peritoneal macrophages

	Per cent inhibition*			
Concentration (μM)	CP-77 059	Rolipram	CI930	Zaprinast
0·001	55	9	5	0
0·01	67	39	11	8
0·1	77	63	28	5
1	82	77	20	7
10	85	81	49	25

*Represents the mean of at least two experiments.

maximum total score could be 20 [20]. Each group contained eight animals.

Statistical analysis

Comparison of mean serum TNF inhibition and paw swelling was tested between groups using analysis of variance followed by Dunnett's test for multiple comparisons. Lethality rates were compared using Fisher's exact test. Group comparisons where multiple measurements were made on each animal were tested using a multivariate analysis of variance with repeated measures model. Alpha levels for all tests were set at 5%.

RESULTS

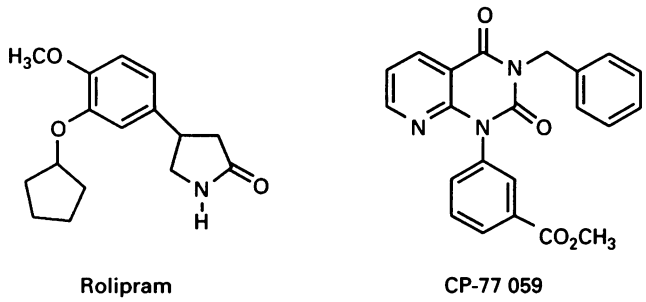
TNF-α production by murine macrophages in vitro

The PDE-IV selective inhibitors rolipram and CP-77 059 were potent suppressors of LPS-induced TNF-α production in Na periodate-elicited mouse peritoneal macrophages, with IC_{50}s of approximately 33 nM and 1 nM, respectively (Table 1). This rank order of potency is the same when the respective Ki against the human PDE-IV enzyme are calculated; with rolipram possessing a Ki of 220 nM ($n = 9$) and CP-77 059 exhibiting a Ki of 46 nM ($n = 4$; Feldman *et al.*, accepted for publication, J Med Chem, 1995).

By contrast, the PDE-III-selective inhibitor CI930 [21] was weakly active (approximately 10 μM), and zaprinast, a PDE-V inhibitor [22,23] had little or no effect ($\geqslant$ 10 μM).

Sublethal injection of LPS

As an extension of studies of TNF-α inhibition *in vitro*, PDE-IV inhibitors were tested *in vivo*. Mice were injected intraperitoneally with 5 μg LPS, a dose calibrated to elevate serum



Rolipram CP-77 059

Fig. 1. Structure of the PDE-IV inhibitors, rolipram and CP-77 059.

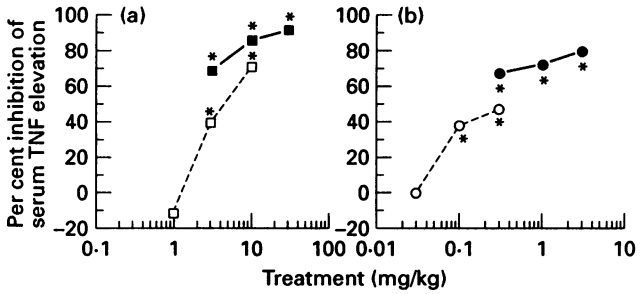


Fig. 2. The effect of PDE-IV inhibitors on serum tumour necrosis factor-alpha (TNF-α) levels in mice injected with a sublethal concentration of lipopolysaccharide (LPS). Fasted female C3H/hen mice were injected intraperitoneally with 5 μg LPS. Rolipram (a) or CP-77 059 (b) was given orally 30 min before LPS injection ($n = 4$). Blood was taken from the heart 90 min after LPS injection and serum TNF-α levels measured using a commercial available ELISA kit. *Significantly different ($P < 0·05$) from LPS control using Dunnett's multiple comparison. ■,●, Test 1; □,○, test 2.

TNF-α levels but not to cause death. Rolipram and CP-77 059 (Fig. 1) were given orally 30 min before LPS injection. In two experiments, rolipram was administered at doses ranging from 1 to 30 mg/kg (Fig. 2a). Rolipram had a minimum effective dose of 3 mg/kg, with maximum inhibition of 92% at 30 mg/kg and no effect at 1 mg/kg. Similar testing of CP-77 059 resulted in a minimum effective dose of 0·1 mg/kg (Fig. 2b). Thus CP-77 059 was about 30-fold more potent than rolipram. Background levels of serum TNF-α in normal mice were undetectable (< 50 pg/ml), while serum TNF-α levels in the LPS-injected mice ranged from 3 to 12 ng/ml.

LPS and LPS/galactosamine-induced endotoxic shock

Since PDE-IV inhibitors were active in their inhibition of low-dose LPS-induced serum TNF-α elevation, these same inhibitors were tested in models of endotoxic shock, thought to depend upon the presence of TNF-α. In the high-dose LPS shock model, serum TNF-α levels were evaluated over time and the 24-h mortality rate recorded (Fig. 3). Just as in the sublethal model, serum TNF-α levels peaked at 90 min post-injection. At high doses (10–30 mg/kg), CP-77 059 and rolipram reduced mortality rate 66% and serum TNF-α level 81–87%.

In the galactosamine (600 mg/kg) + LPS (0·1 μg/ml) model of endotoxic shock, galactosamine destroyed liver function so that normally non-lethal doses of LPS now caused death. In a dose response experiment, rolipram (1, 10 and 30 mg/kg) and CP-77 059 (1, 3 and 10 mg/kg) inhibited serum TNF-α between 84% and 94% (Fig. 4). Rolipram at the highest dose significantly inhibited mortality rate by 66%, while CP-77 059 at doses of 3 and 10 mg/kg also significantly inhibited mortality by 50–66%. However, both drugs failed to significantly block mortality at the lower doses.

Carrageenan oedema

Since elevated TNF-α levels have been observed in extracts of rat and mouse paws injected with carrageenan [16], rolipram was tested for anti-inflammatory activity in this model. At a dose of 3, 10 or 30 mg/kg, rolipram significantly inhibited carrageenan paw oedema by 21%, 43% and 45%, respectively (Fig. 5). It also significantly inhibited TNF-α levels in homogenates from the carrageenan-injected paws, with approxi-

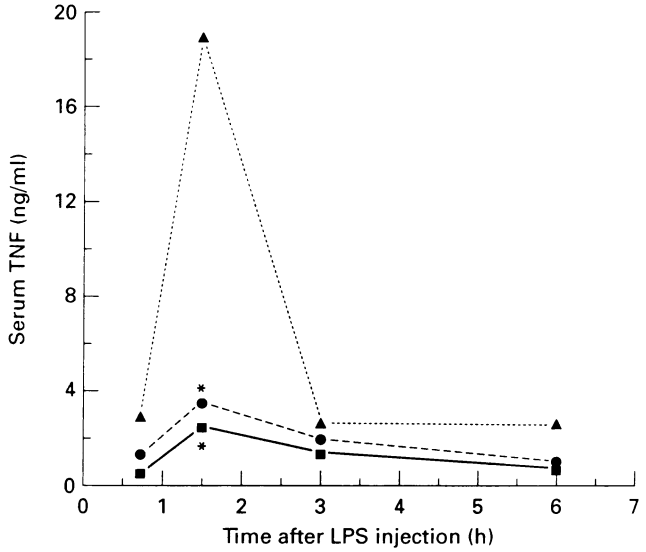


Fig. 3. Effect of PDE-IV inhibitors on serum tumour necrosis factor-alpha (TNF-α) levels and the lethality associated with the lipopolysaccharide (LPS) model of endotoxic shock. Fasted female C3H/hen mice were injected intraperitoneally with 500 μg LPS (n = 22). Four animals were bled at each time point. Six animals were observed for 24 h to establish mortality rate. For further details see Fig. 2 or Materials and Methods. *Significantly different (P < 0·05) from LPS control using Dunnett's multiple comparison. ▲, LPS control; ■, rolipram 30 mg/kg; ●, CP-77 059 10 mg/kg.

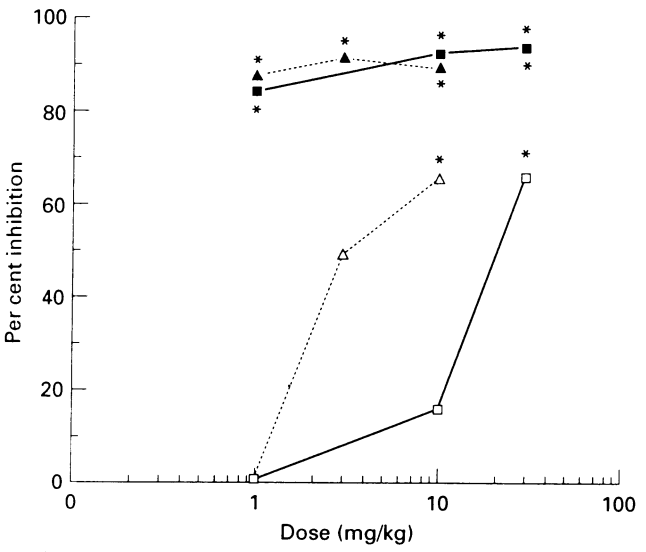


Fig. 4. Effect of PDE-IV inhibitors on serum tumour necrosis factor-alpha (TNF-α) levels and the lethality associated with the lipopolysaccharide (LPS)/galactosamine model of endotoxic shock. Fasted female C57/Bl mice were injected intraperitoneally with 0·1 μg LPS and 600 mg/kg galactosamine (n = 10). Four animals were bled 90 min after LPS injection. Six animals were observed for 24 h to establish mortality rate. For further details see Fig. 2 or Materials and Methods. *Significantly different (P < 0·05) from LPS control using Dunnett's multiple comparison. △, CP-77 059 inhibition of lethality; ▲, CP-77 059 inhibition of TNF; □, rolipram, inhibition of lethality; ■, rolipram, inhibition of TNF.

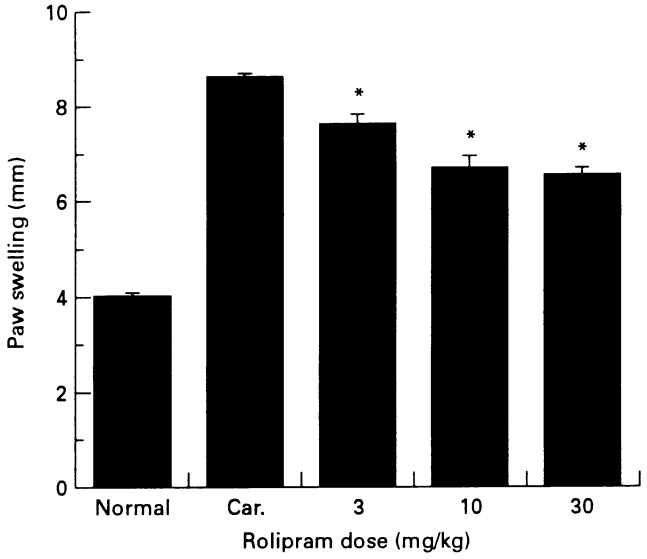


Fig. 5. Effect of rolipram on carrageenan paw oedema. Fasted male Lewis rates were injected in the footpad with 0·1 ml of a 1% solution of carrageenan. Rolipram was given orally 1 h before carrageenan injection (n = 8). Paw diameter was measured with calipers 3 h after carrageenan injection. *Significantly different (P < 0·05) from carrageenan control using Dunnett's multiple comparison.

mately 75% inhibition at the high dose (30 mg/kg). There was no detectable TNF-α in the serum of rats injected in the paw with TNF-α, nor was there any TNF-α in normal paw homogenates.

Adjuvant arthritis

Since PDE-IV inhibitors were successful in reducing serum TNF-α levels and soft tissue swelling, these drugs were tested in a chronic model of inflammation where TNF-α is also thought to play a role in soft tissue swelling [16]. In the 3-week adjuvant arthritic rat model, rats were dosed from day 1 with rolipram and CP-77 059 at 0·3, 1 and 3 mg/kg, po (Fig. 6). Both drugs inhibited ankle swelling in a dose response fashion. Rolipram was significantly inhibitory (32%) at 3 mg/kg, while CP-77 059 significantly inhibited ankle swelling by 40% and 42% at 1 and 3 mg/kg, respectively.

Ankles were also evaluated radiologically using five individually graded parameters (0–4). The features assessed were bone demineralization, bone erosion, periostitis, cartilage space reduction and soft tissue swelling as previously described [20]. As in inhibition of ankle swelling, rolipram and CP-77 059 exhibited a significant and dose-dependent effect on each individual parameter. Results depicting the total radiological score show that rolipram at 3 mg/kg significantly reduced radiological changes by 24%, while CP-77 059 at all doses significantly reduced radiological changes by 34–62% (Fig. 7).

In a second experiment, rolipram was dosed in a therapeutic fashion, from day 7 to day 14. Under this regimen, rolipram again exhibited inhibition of ankle swelling and radiological damage of approximately 60% (data not shown).

DISCUSSION

Agents that elevate the concentration of intracellular cAMP can inhibit inflammatory cell activities such as cytokine pro-

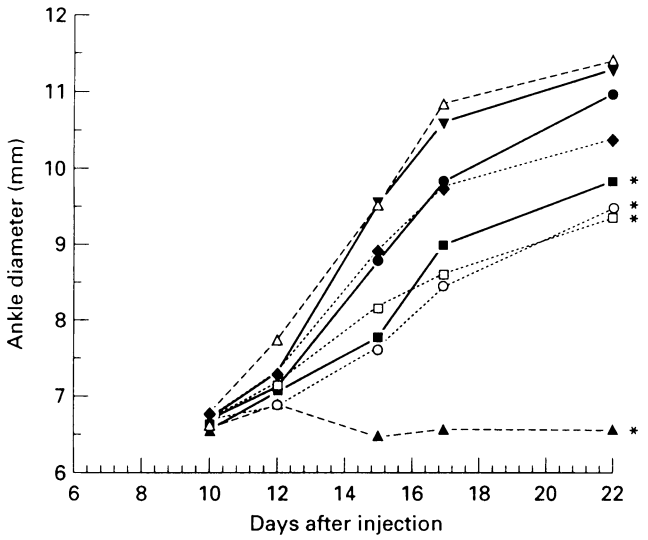


Fig. 6. Inhibition of ankle swelling in adjuvant arthritic rats treated with PDE-IV inhibitors. Male Lewis rats were injected subcutaneously in the tail with 0·05 ml Freund's complete adjuvant. Animals were dosed orally from day 1 to day 21 with rolipram or CP-77 059 ($n = 8$). Measurement of ankle swelling was taken throughout the time course using calipers. *Significantly different ($P < 0·05$) from arthritic control using Dunnett's multiple comparison. ▲, Normal; △, arthritic; ■, rolipram (3 mg/kg); ●, rolipram (1 mg/kg); ▼, rolipram (0·3 mg/kg); □, CP-77 059 (3 mg/kg); ○, CP-77 059 (1 mg/kg); ◆, CP-77 059 (0·3 mg/kg).

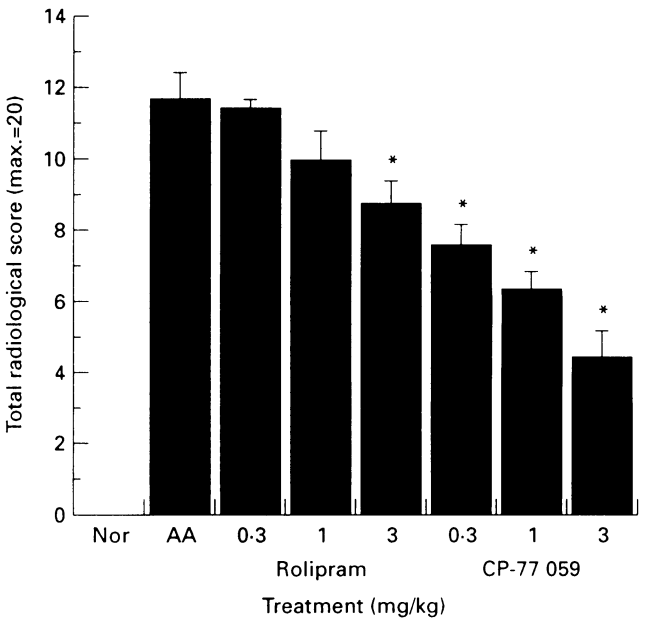


Fig. 7. Radiological score of adjuvant arthritic rats treated with PDE-IV inhibitors. Total radiological score was based on five parameters each scored from 0 (normal) to 4 (severe changes) as previously described [15]. Thus a maximum total score could be 20. For further details see Fig. 6 or Materials and Methods. *Significantly different ($P < 0·05$) from arthritic control using Dunnett's multiple comparison.

duction, chemotaxis, cytotoxicity and cell aggregation [1]. PDE type-IV inhibitors, by virtue of their ability to block phosphodiesterase activity in monocytic cells, thus elevating cAMP levels, may be a class of compounds from which useful anti-inflammatory drugs can be developed [1–4].

This study demonstrated the effectiveness of PDE type-IV inhibitors in various acute and chronic models of inflammation in which TNF-α may have a pathological role. PDE-IV inhibitors were potent suppressors of LPS-induced TNF-α production in murine macrophages, whereas inhibitors selective for PDE-III or -V were much less active. Similar findings have been reported for TNF-α production in human monocytes [5,7], supporting a major role for PDE-IV in the regulation of both human and murine TNF-α production.

The *in vivo* potency of PDE-IV inhibitors was assessed in a mouse model, where LPS was injected at a sublethal concentration to induce appearance of serum TNF-α. The PDE-IV inhibitors rolipram and CP-77 059 were orally active, dose-dependent inhibitors of serum TNF-α levels in this system. These PDE-IV inhibitors were tested in endotoxic shock models, where serum TNF-α levels as well as mortality rate could be measured. In the LPS/galactosamine model of endotoxic shock, rolipram and CP-77 059 not only effectively reduced serum TNF-α levels, but also showed protective effects in a dose-dependent fashion. However, reduction of serum TNF-α level did not guarantee complete protection against lethality, since animals suffered 100% mortality at doses which inhibited serum TNF-α levels over 80%. Since shock is accompanied by a potentially lethal fall in blood pressure, we considered the possibility that the hypotensive properties of the PDE-IV inhibitors were responsible for the difference in the relatively high protective dose *versus* the much lower TNF inhibitory dose. However, we have observed that non-hypotensive compounds such as glucocorticoids also manifested this discrepancy between the dose needed to protect against lethal shock and the dose needed to inhibit serum TNF levels. In addition, the PDE-IV inhibitors are active at high doses, where the hypotensive side-effects are most pronounced. If their haemodynamic profile contributed to their poor potency in the LPS lethality assay, then one would have predicted that the compounds would be active at the low dose (where TNF inhibition was high and hypotensive effect low) and inactive at the high dose (where the TNF inhibition would be countered by the pronounced hypotensive effect). While the haemodynamic effects of PDE-IV inhibitors may contribute to this lack of correlation between survival rate and reduction of serum TNF-α level, it is also possible that the difference may be due to the greater relevance of localized elevation of TNF-α in target tissues such as the lung, liver and paw as opposed to systemic levels in the blood [16]. Alternatively it may also be due in part to the pro-inflammatory activity of cytokines not affected by PDE-IV inhibitors.

Since we have previously demonstrated that elevation of TNF-α occurred in the paw but not in the serum of rats injected with carrageenan [16], we tested rolipram for its anti-inflammatory activity in this model of localized TNF-α elevation. Rolipram and CP-77 059 have previously been shown to suppress carrageenan paw oedema [18]. We have confirmed and extended that observation here by showing that rolipram significantly inhibited paw inflammation as well as local TNF-α production. It cannot be ruled out that the anti-oedema action of these agents may in part be the result of their induction of systemic hypotension. However, because of their profound

effect on neutrophils *in vitro* [21,22], it is likely that a substantial component of the anti-inflammatory activity seen in the carrageenan oedema model is due to inhibition of neutrophil activity *in vivo*.

Encouraged by the activity of these prototypic PDE type-IV inhibitors in models of acute inflammation, we next evaluated the compounds in a chronic model of inflammation. The rat adjuvant arthritis model mimics many aspects of human rheumatoid arthritis, including elevated levels of TNF-α in the arthritic joint [17,24]. In this model, rolipram and CP-77059 not only reduced tissue oedema but also inhibited radiological changes in the bone. In a previous paper we compared ankle swelling and joint TNF-α levels in arthritic rats treated with non-steroidal anti-inflammatory drugs or immunosuppressive compounds [17]. It will also be important to address the relationship between joint protection and joint TNF-α levels in arthritic rats treated with PDE-IV inhibitors.

The most suitable candidates of PDE inhibitors for evaluation in the clinic should possess the following properties: high specificity to a selected PDE-IV isozyme, high potency, low toxicity and good oral absorption. The recognition of multiple isoforms [11,25] and a non-random tissue distribution of PDE-IV [1–4,26] suggest the potential to develop more specific and less toxic PDE-IV inhibitors. Synergism of PDE inhibitors and β2-agonists should be evaluated. In the sublethal LPS model of serum TNF-α measurement, we have observed some mild synergistic effect between salmeterol and rolipram (unpublished data).

In conclusion, we have shown that PDE-IV inhibitors, in addition to being potent suppressors of TNF-α production, are also good anti-inflammatory agents in several models of acute and chronic inflammation. It is conceivable that the reported activities of these compounds are due to unknown reactivities, but the finding that compounds of two different chemical structural classes have similar activities supports the notion that PDE-IV inhibition is relevant. It should be emphasized that while the suppressive effect on TNF-α production is one of the most potent, consistent and complete inhibitory effects known for PDE inhibitors in relation to inflammatory cell function, the extent to which this contributes to anti-inflammatory activity in each of these models will not be clear until the role of TNF-α *in vivo* is better defined. For example, PDE-IV inhibition has been shown to have suppressive effects on several other potentially pro-inflammatory cellular activities, including IL-1β [7] and LTB4 [27] production by monocytes, neutrophil respiratory burst [28], histamine and LTB4 release from basophils [29], histamine and LTC4 from mast cells [27] and T lymphocyte blastogenesis [30]. In addition, there are several examples of cAMP-regulated inflammatory cell function which have not been fully explored with respect to PDE inhibition. Nevertheless, these data suggest that a specific PDE-IV inhibitor has potential therapeutic utility for acute and chronic inflammatory disease, and are consistent with the hypothesis that TNF-α is an important mediator in inflammatory disease.

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