Biochemical Pharmacology, Vol. 51, pp. 949–956, 1996.
Copyright © 1996 Elsevier Science Inc.

ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00053-6

# Association of the Anti-inflammatory Activity of Phosphodiesterase 4 (PDE4) Inhibitors with Either Inhibition of PDE4 Catalytic Activity or Competition for [$^3$H]Rolipram Binding

*Mary S. Barnette,*† Joan O'Leary Bartus,‡ Miriam Burman,* Siegfried B. Christensen,§ Lenora B. Cieslinski,* Klaus M. Esser,‡ Uma S. Prabhakar,‖ Julia A. Rush* and Theodore J. Torphy*¶*

DEPARTMENTS OF *PULMONARY PHARMACOLOGY, ¶INFLAMMATION PHARMACOLOGY, ‡MOLECULAR VIROLOGY, §MEDICINAL CHEMISTRY, AND ‖CELLULAR BIOCHEMISTRY, SMITHKLINE BEECHAM PHARMACEUTICALS, KING OF PRUSSIA, PA 19406, U.S.A.

**ABSTRACT.** Phosphodiesterase 4 (PDE4) inhibitors are novel anti-inflammatory compounds. Unfortunately, the archetypal PDE4 inhibitor rolipram produces central nervous system and gastrointestinal side-effects. To exploit these agents, we need to identify PDE4 inhibitors that retain the anti-inflammatory activity with a reduced potential to elicit unwanted side-effects. PDE4 possesses both cyclic AMP catalytic activity that is inhibitable by rolipram and a high affinity binding site for rolipram. The function of this high affinity rolipram binding site is unclear; however, certain pharmacological effects of PDE4 inhibitors are associated with competition for this site. Since PDE4 inhibitors suppress both monocyte and neutrophil activation, the present experiments were carried out to establish a correlation between suppression of monocyte activation [tumor necrosis factor alpha (TNFα) formation] or suppression of neutrophil activation (degranulation) with inhibition of either PDE4 catalytic activity or [$^3$H]rolipram binding. Suppression of TNFα formation demonstrated a strong correlation with inhibition of PDE4 catalytic activity ($r = 0.87$; $P < 0.01$; Spearman's Rho = 0.79, $P < 0.05$), whereas there was no correlation with inhibition of [$^3$H]rolipram binding ($r = 0.21$, $P > 0.5$; Spearman's Rho = 0.16, $P > 0.5$). Suppression of neutrophil degranulation was not associated with inhibition of PDE4 catalytic activity ($r = 0.25$, $P > 0.4$; Spearman's Rho = 0.33, $P > 0.2$), but was associated with inhibition of [$^3$H]rolipram binding ($r = 0.68$, $P < 0.05$; Spearman's Rho = 0.6, $P = 0.06$). These results indicate that anti-inflammatory effects of PDE4 inhibitors can be associated with either inhibition of PDE4 catalytic activity or high affinity rolipram binding. BIOCHEM PHARMACOL 51;7:949–956, 1996.

**KEY WORDS.** phosphodiesterase; rolipram binding; neutrophil; monocyte; tumor necrosis factor alpha; myeloperoxidase

Previous studies with selective inhibitors of the low $K_m$ cAMP**-specific phosphodiesterase (PDE4) have demonstrated impressive *in vitro* anti-inflammatory actions [1–7]. The promise that these inhibitors may be useful anti-inflammatory agents is tempered by concern over their side-effects. In initial clinical trials, rolipram, a prototypical PDE4 inhibitor, and zardaverine, a mixed PDE3/4 inhibitor, produced gastrointestinal side-effects, i.e. pyrosis, nausea, and emesis, that were dose limiting [8, 9]. Furthermore, rolipram was being developed as an anti-depressant agent [10], indicating that it possesses psychotropic activity [11]. These pharmacological actions of rolipram are likely to limit its usefulness as an anti-inflammatory agent. The challenge for drug discovery is to identify the mechanism responsible for these effects and to design novel PDE4 inhibitors that retain the anti-inflammatory actions of rolipram but with a reduced potential to elicit unwanted side-effects.

Along with its catalytic activity, PDE4 also possesses a high affinity ($K_d = 1$ nM) binding site for rolipram [12, 13]. Surprisingly, the rank order potency of a variety of PDE4 inhibitors for this binding site differs from their rank order potency for inhibiting cAMP hydrolysis [12]. The functional role of the high affinity site is unknown, although it may represent an allosteric site [12, 14], or, alternatively, the catalytic site of one of two co-existing but conformationally distinct forms of PDE4 [12, 15]. Even though the exact function of the high affinity [$^3$H]rolipram-binding site relative to the actions of PDE4 inhibitors is unknown, cer-

† Corresponding author: Mary S. Barnette, Ph.D., Department of Pulmonary Pharmacology, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, 709 Swedeland Road, King of Prussia, PA 19406. Tel. (610) 270-6496; FAX (610) 270-5381.

** *Abbreviations:* cAMP, 3′,5′-adenosine monophosphate; DB-PBS, Dulbecco's phosphate-buffered saline; fMLP, formyl methionine leucine phenylalanine; LPS, lipopolysacchride; MPO, myeloperoxidase; PDE, phosphodiesterase; $PGE_2$, prostaglandin $E_2$; PMN, neutrophil; and TNFα, tumor necrosis factor alpha.

Received 30 January 1995; accepted 7 December 1995.

tain biological actions of selective PDE4 inhibitors seem to correlate with the ability of these compounds to displace [$^3$H]rolipram from this site and not with their ability to inhibit PDE4 catalytic activity. For example, the ability to reverse gastric glands [17] correlates with the ability of these compounds to displace rolipram from its high affinity site. In contrast, suppression of guinea pig mast cell activation by PDE4 inhibitors is better correlated with the ability of these compounds to inhibit PDE4 catalytic activity [18]. Thus, it appears that certain side-effects are associated with inhibition of rolipram binding and at least one potential therapeutic effect is correlated with inhibition of catalytic activity. These findings suggest that one approach to increasing the therapeutic index of PDE4 inhibitors is the design and synthesis of compounds with reduced ability to displace rolipram from its high affinity binding site. The success of this approach depends on defining which desirable pharmacological effects are associated with inhibition of PDE4 catalytic activity and which are associated with competition for rolipram binding. Since previous studies have shown that prototypical PDE4 inhibitors block the activation of both monocytes [19, 20] and PMNs [3, 5, 6, 20, 21], the present studies were carried out to fulfill two objectives: (1) to compare the ability of selective PDE4 inhibitors to suppress monocyte (measured by LPS-induced TNFα formation) or neutrophil (measured by MPO release) activation; and (2) to determine if an association exists between the ability of PDE4 inhibitors to suppress activation in these two cell types and their ability to inhibit PDE4 catalytic activity or [$^3$H]rolipram binding.

## MATERIALS AND METHODS

### *Materials*

RPMI 1640 medium was purchased from MA Bioproducts (Waldersville, MD) and fetal bovine serum was obtained from Hyclone Laboratories Inc. (Logan, UT). Bacterial LPS or endotoxin (*Escherichia coli* 055:B5), fMLP, $PGE_2$, *o*-dianisidine, cytochalasin B, Histopaque 1077, HEPES, gelatin and Earle's balanced salt solution were obtained from the Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase was purchased from Boehringer Mannheim (Mannheim West Germany). Hydrogen peroxide was obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ). Recombinant human TNFα was provided by Dr. J. Strickler (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Peroxide conjugated goat anti-rabbit antibodies were purchased from Pierce (Rockford, IL). The following compounds were synthesized by Dr. S. B. Christensen and colleagues (SmithKline Beecham Pharmaceuticals, King of Prussia): *R*-rolipram; *S*-rolipram; (A) 4 cyano-4-(3-cyclopentyloxy-4-methoxyphenyl) cyclohexanone; (B) *R* (+)-1-(4-aminobenzyl)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone; (C) *S* (−)-1-(4-aminobenzyl)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone; (D) *R* (+)-1-(4-acetamidobenzyl)-3-(3-cyclopentyloxy-4-methoxyphe-nyl)-2-pyrrolidinone; (E) *S* (−)-1-(4-acetamidobenzyl)-3-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone; (F) *S* (−)-1-(4-aminobenzyl)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-imidazolidinone; (G) *R* (+)-1-(4-aminobenzyl)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-imidazolidinone; (H) *R* (+)-1-(4-pyridinylbenzyl)-3-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone; and (I) *S* (−)-1-(4-pyridinylbenzyl)-3-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone.

### *Isolation and Culture of Peripheral Blood Monocytes*

Peripheral blood monocytes were purified from freshly obtained buffy coats or plasma-phoresis residues of blood obtained from normal donors according to the procedure of Collata *et al.* [22]. Purified mononuclear cells (>85% monocytes) were seeded at a density of $1 \times 10^6$ cells/mL in RPMI 1640 medium + 1% heat-inactivated fetal bovine serum, penicillin and streptomycin (10 U/mL) onto 24-well multidishes. The cells were allowed to adhere for 1 hr at 37°. After this incubation, non-adherent cells were removed by aspiration, and 1 mL of fresh medium was added to the enriched adherent monocytes. The cells were incubated for 45 min to 1 hr in the absence or presence of various concentrations of PDE4 inhibitors prior to the addition of LPS (0.1 μg/mL; final concentration). Culture supernatants were removed from the monocytes after 14–16 hr of incubation at 37°, 5% $CO_2$, and centrifuged at 1000 *g* to remove cell debris. TNFα levels of these samples either were determined immediately by ELISA or the culture supernatants were stored at −70° until assayed.

### *Isolation and Purification of PMNs*

PMNs were isolated from heparinized blood by gradient centrifugation using Ficoll (Histopaque 1077) followed by dextran sedimentation to remove the erythrocytes. Any remaining erythrocytes were lysed with water for 30 sec, and isotonicity was restored using 10x DB-PBS, without $Ca^{2+}$ or $Mg^{2+}$). PMNs were isolated by centrifugation and were washed one additional time with 1x DB-PBS prior to determining cell number and viability (trypan blue dye exclusion). Cell number was adjusted to $0.75–1.5 \times 10^6$ cells/mL depending on the individual donor.

### *Measurement of TNFα*

Levels of TNFα were measured using a sandwich ELISA [23] employing a murine monoclonal anti-human TNFα antibody as the capture antibody and a polyclonal rabbit anti-human TNFα as the second antibody. For detection, a peroxidase-conjugated goat anti-rabbit antibody was added followed by the addition of 1 mg/mL of *o*-phenylenediamine with 0.1% urea peroxide. TNFα levels in samples were calculated from a standard curve generated with recombinant human TNFα produced in *E. coli*. Monoclonal antibodies to human TNFα were prepared from spleens of BALB/c mice immunized with human TNFα by a modification of the method of Kohler and Milstein [24]. Poly-

clonal rabbit anti-human TNFα antibodies were prepared by repeated immunization of a New Zealand white rabbit with recombinant human TNFα emulsified in complete Freund's adjuvant.

### *Degranulation (Release of MPO)*

An aliquot (0.1 mL) of the above cell suspension was incubated in Earle's Balanced Salt Solution containing 20 mM HEPES buffer (pH 7.4) and 0.1% gelatin in the presence of 5 μg/mL of cytochalasin B for 5 min at 37° in a shaking water bath. Cells were pretreated for an additional 5 min with various concentrations of selective PDE4 inhibitors and $PGE_2$ (3–10 nM) prior to addition of fMLP (30 nM). fMLP was added, and the incubation was continued for an additional 30 min. The reaction was terminated by placement of the samples on ice followed by centrifugation. The supernatant fraction was removed and stored frozen (−30°) until assay for MPO activity.

MPO activity was determined using *o*-dianisidine as substrate and horseradish peroxidase as a standard. Briefly, an aliquot (50 μL) of supernatant was incubated with 100 μL of substrate (*o*-dianisidine, 0.53 mM; $H_2O_2$, 0.147 mM; final concentration) in 50 mM sodium phosphate buffer (pH 6.0). The reaction was terminated by the addition of 50 μL of 4 M $H_2SO_4$. Product formation was determined by measuring absorbance at 410 nm, and activity was determined by comparison to the standard curve using horseradish peroxidase. Data were expressed as percent of control (amount of MPO released in the presence of $PGE_2$ alone). Since the maximum inhibition produced by PDE4 inhibitors was approximately 30%, in $IC_{15}$ values were calculated by linear interpolation, using the responses obtained in 3–6 experiments.

### *PDE and [$^3$H]Rolipram Binding Assays*

PDE4 activity was determined by the method of Davis and Daly [25] using either partially purified PDE4 from monocytes or recombinant human PDE4A expressed in yeast and cAMP (1 μM) as the substrate [26]. [$^3$H]Rolipram binding to rat brain microsomes was measured as previously described [12].

### *Statistical Analyses*

To determine if an association existed between suppression of monocyte activation and inhibition of PDE4 activity or competition with high affinity [$^3$H]rolipram binding, we calculated the linear ($r$) or rank order correlation (Spearman's Rho) between the mean log ($IC_{50}$) values for suppression of TNFα formation and the log ($IC_{50}$) values for inhibition of PDE4 catalytic activity or displacement of the high affinity [$^3$H]rolipram binding. A correlation was considered significant if a $P$ value < 0.05 was obtained, using the STATVIEW II program written for the Macintosh computer. Similar comparisons were performed using the mean log ($IC_{15}$) values obtained for the neutrophils.

## RESULTS

Previous studies have demonstrated that PDE4 inhibitors suppress LPS-induced TNFα formation, as well as neutrophil degranulation or superoxide formation [3, 6, 7, 21, 27, 28]. This suppression was correlated with the ability of these compounds to elevate cAMP content in these cells [6, 19]. In this study, we compared the ability of PDE4 inhibitors to suppress TNFα formation or to inhibit fMLP-induced MPO release. Intriguingly, selective PDE4 inhibitors behaved differently in the two cell types. First, rolipram inhibited TNFα formation in monocytes without the addition of an exogenous activator of adenylyl cyclase, whereas in PMNs rolipram was without effect in the absence of a threshold concentration of $PGE_2$ (data not shown). Second, the enantiomers of rolipram, which display a marked difference in their ability to compete for [$^3$H]rolipram binding but little difference in their ability to inhibit PDE4 purified from human monocytes, differed in their relative potency against the activation of monocytes versus PMNs. In monocytes, *R*-rolipram ($IC_{50}$ = 0.065 μM) was about 15-fold more potent than *S*-rolipram ($IC_{50}$ = 0.95 μM) at inhibiting TNFα formation (Fig. 1A). This difference in potency is similar to the differences observed between these two enantiomers at displacing [$^3$H]rolipram binding (Table 1). In neutrophils, *R*-rolipram was about 3-fold more potent than *S*-rolipram ($IC_{15}$ = 0.037 μM, *R*-rolipram; $IC_{15}$ = 0.11 μM, *S*-rolipram), which is similar to the potency of these two enantiomers as inhibitors of PDE4 catalytic activity (Table 1). These initial results suggested that inhibition of TNFα formation was associated with displacement of rolipram binding and not inhibition of PDE4 catalytic activity, while the opposite was true for suppression of PMN activation.

To further characterize the relationship between suppression of cell activation and inhibition of PDE4 catalytic activity or displacement of [$^3$H]rolipram binding, we examined a number of structurally diverse PDE4 inhibitors for their ability to inhibit TNFα or MPO release. Within this series of compounds, there was no correlation between the ability to inhibit PDE4 catalytic activity and the ability to displace [$^3$H]rolipram binding ($r$ = 0.11, $P$ > 0.5; Spearman's Rho = −0.016, $P$ > 0.5) (Table 1). In contrast to the limited results with *R*- and *S*-rolipram, there was strong correlation between suppression of LPS-induced TNFα formation and inhibition of PDE4 catalytic activity ($r$ = 0.87, $P$ < 0.01; Spearman's Rho = 0.79, $P$ < 0.01; Fig. 2A). There was no statistically significant association between suppression of TNFα formation and competition for [$^3$H]rolipram binding ($r$ = 0.21, $P$ > 0.5; Spearman's Rho = 0.16, $P$ > 0.5; Fig. 3A). Thus, although the initial results with the enantiomers of rolipram suggested otherwise, results obtained with a broader panel of compounds indicate that suppression of TNFα formation is better correlated with inhibition

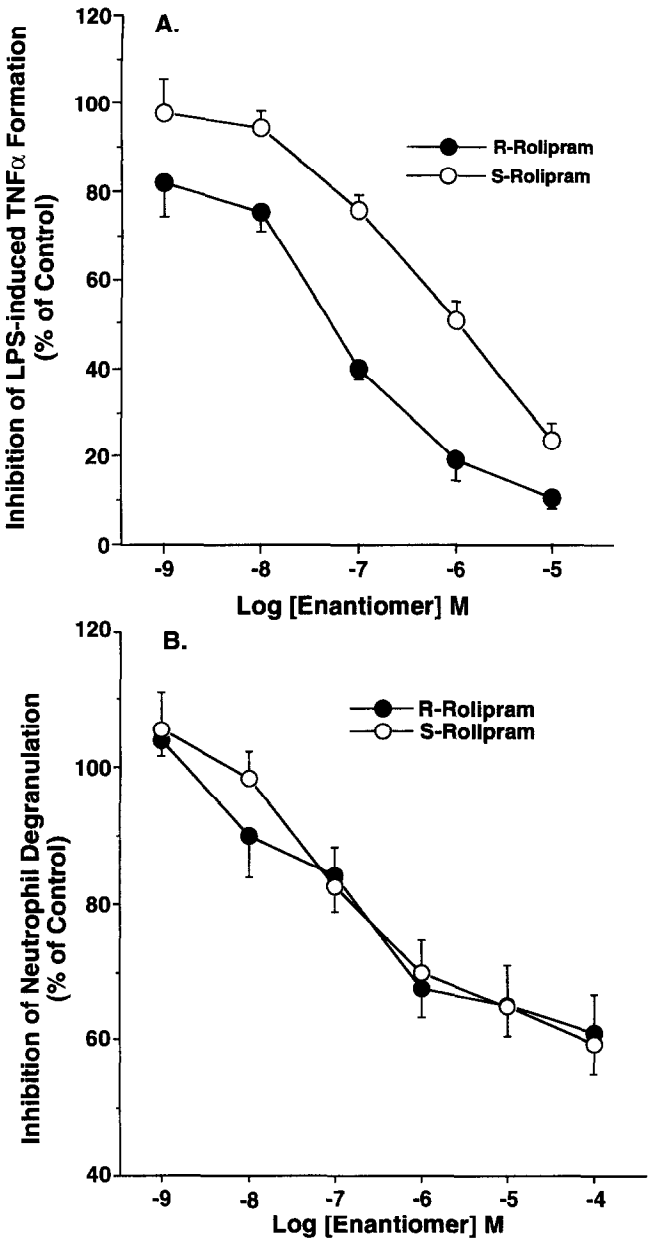


**FIG. 1. Effects of *R*-rolipram and *S*-rolipram on TNFα formation in monocytes (A) or fMLP-induced MPO release in PMNs (B). Data are expressed as a percent of the response to LPS (0.1 μg/mL) or fMLP (30 nM) in the absence of drug (control) and are the means ± SEM of 8–9 (*R*-rolipram) or 6–7 (*S*-rolipram) experiments. In these experiments, LPS-induced TNFα formation was 5 ± 1.25 ng/1 × $10^6$ cells, and fMLP increased the release of MPO from 1.9 ± 1.0 × $10^{-3}$ to 19 ± 2.6 × $10^{-3}$ U/mL.**

of PDE4 catalytic activity than with inhibition of [$^3$H]rolipram binding. The opposite association occurred with neutrophil degranulation. Inhibition of fMLP-induced MPO release did not correlate with inhibition of PDE4 catalytic activity ($r = 0.25$, $P > 0.4$; Spearman's Rho = 0.33, $P > 0.2$; Fig. 2B). In contrast, there was a significant linear correlation with inhibition of MPO release and competition for [$^3$H]rolipram binding ($r = 0.68$, $P < 0.05$; Spearman's Rho = 0.6, $P = 0.06$; Fig. 3B). Thus, our results indicate: (1) that the relative difference in potency for each enantiomer of rolipram is not always predictive of the association of that pharmacological effect with inhibition of PDE4 catalytic activity or competition for rolipram binding; and (2) that in a similar manner to certain undesirable actions of PDE4 inhibitors, some anti-inflammatory actions of PDE4 inhibitors may be associated with competition for rolipram binding.

## DISCUSSION

The enthusiasm surrounding the potential utility of PDE4 inhibitors as anti-inflammatory agents is dampened by the possibility of CNS and gastrointestinal side-effects [8, 9, 11]. A key question for drug discovery is whether the side-effects of these compounds can be separated from their anti-inflammatory effects. One of the more fascinating properties of PDE4 is the presence of a high affinity binding site for [$^3$H]rolipram on this protein [12, 29]. Selective PDE4 inhibitors not only inhibit catalytic activity of this enzyme but also compete for the high affinity rolipram binding. However, there does not appear to be a direct correlation between the potencies of compounds for these two effects [12]. Consequently, the functional role of the high affinity site is unknown, although it may represent an allosteric site [12, 14] or, alternately, the catalytic site of one of two co-existing but conformationally distinct forms of PDE4 [12, 15]. Although the exact function of this high affinity site is unknown, certain biological actions of selective PDE4 inhibitors seem to correlate with the ability of these compounds to displace [$^3$H]rolipram from this site. For example, the ability of PDE4 inhibitors to reverse reserpine-induced hypothermia [16] and to enhance acid secretion [17], both potential side-effects, is better correlated with the ability of these compounds to compete for [$^3$H]rolipram binding than with their capacity to inhibit PDE4 catalytic activity. In contrast, certain potential beneficial effects of PDE4 inhibitors, such as inhibition of fMLP-induced superoxide generation in guinea pig eosinophils [30] and suppression of antigen-induced bronchoconstriction in anesthetized guinea pigs [18], are better correlated with the ability of PDE4 inhibitors to inhibit the catalytic activity of PDE4 than with their ability to displace [$^3$H]rolipram binding.

In this report, we have evaluated the effects of a variety of PDE4 inhibitors on LPS-induced TNFα formation and fMLP-induced degranulation. As observed previously [7, 19], PDE4 inhibitors suppressed the production of TNFα. This activity correlates with their ability to elevate cAMP [19]. In our studies, *R*-rolipram was 15-fold more potent than *S*-rolipram at inhibiting LPS-induced TNFα formation. This finding is similar to that of Semmler *et al.* [7], who found that *R*-rolipram was approximately 5-fold more potent than the *S* enantiomer. At first glance, our findings suggest that suppression of TNFα is more closely associated with competition for rolipram binding than with inhibition of PDE4 catalytic activity. Indeed, these results are similar

**TABLE 1. Comparison of the ability of selective PDE4 inhibitors to suppress LPS-induced TNFα formation and neutrophil degranulation with their capacity to inhibit PDE4 catalytic activity or to compete for [$^3$H]rolipram binding**

| Compound | TNFα suppression Log ($IC_{50}$) M | Inhibition of MPO release Log ($IC_{15}$) M | Inhibition of human monocyte PDE4 activity Log ($IC_{50}$) M | Inhibition of [$^3$H]rolipram binding Log ($IC_{50}$) M |
|---|---|---|---|---|
| R-Rolipram | −71.9 ± 0.11 (9) | −7.43 ± 0.34 (8) | −5.86 | −8.39 |
| S-Rolipram | −6.02 ± 0.13 (6) | −6.95 ± 0.29 (7) | −6.06 | −7.43 |
| A | −7.5 (2) | −6.43 ± 0.12 (4) | −7.1 | −6.88 |
| B | −6.06 ± 0.19 (4) | −6.0 ± 0.28 (6) | −5.65 | −7.44 |
| C | −6.84 ± 0.07 (4) | −6.26 ± 0.2 (6) | −6.82 | 6.70 |
| D | −6.05 (2) | >−4.00 (6) | −5.65 | −5.89 |
| E | −7.85 (2) | −6.40 ± 0.19 (6) | −7.36 | −7.20 |
| F | −6.87 (2) | −6.93 ± 0.42 (4) | −6.00 | −6.10 |
| G | −5.76 (2) | −6.28 ± 0.36 (4) | −5.07 | −7.00 |
| H | 5.76 (2) | −5.54 ± 0.44 (3) | −5.13 | −6.17 |
| I | −6.79 (2) | −5.57 ± 0.06 (3) | −6.14 | −5.68 |

The log ($IC_{50}$) values obtained with the selective PDE4 inhibitors for suppression of LPS-induced TNFα formation are expressed as the means ± SEM (or the average where N = 2) of the number of experiments indicated in parentheses. The log ($IC_{15}$) values obtained for inhibition of MPO release are expressed as the means ± SEM of the number of experiments indicated in parentheses. The log ($IC_{50}$) values for inhibition of PDE4 catalytic activity or competition for [$^3$H]rolipram binding are the averages of 1–2 experiments. See Materials and Methods for chemical names of compounds.

to our findings with the enantiomers of rolipram on both acid secretion and superoxide production in eosinophils [17, 30]. However, when we examined additional PDE4 inhibitors we discovered that although the enantiomers of rolipram behaved comparably, the ability of PDE4 inhibitors to enhance acid secretion was related to their ability to displace high affinity rolipram binding [17], whereas the ability to inhibit superoxide formation [30] and TNFα formation (present study) was correlated more strongly with their ability to inhibit PDE4 catalytic activity. Thus, these results indicate that the relative potency difference between *R*- and *S*-rolipram is not uniformly predictive of the association of functional changes produced by PDE4 inhibitors with their biochemical actions, i.e. inhibition of catalytic activity or competition for [$^3$H]rolipram binding. In addition, it is important to emphasize that in these studies the comparison of functional effects to biochemical actions was made across species and cell types. However, it does not appear that the kinetic properties of PDE4 vary a great deal among species or cell types. Rolipram binding was determined using rat brain cytosols, since measurement of [$^3$H]rolipram binding sites in cellular extracts of monocytes met with little or no success, presumably because of the low abundance of these sites in these cells. Also, a small amount of specific binding was detected in neutrophils using heroic protein concentrations, which was not sufficient to run reliable competition studies. Since previous work has demonstrated similar binding affinities for compounds measured in rat brain versus rolipram binding to recombinant PDE4 protein [12], we believe this to be a valid comparison.

We also examined the ability of selective PDE4 inhibitors to reduce the activation of human neutrophils, as determined by the inhibition of fMLP-induced MPO release. Earlier studies demonstrated that activators of adenylyl cyclase or PDE inhibitors reduce the release of granular constituents from human neutrophils [1, 21]. As observed in our work, the maximal inhibition of release varied between 20 and 40% regardless of whether the release of primary (MPO or β-glucuronidase) or secondary (lysozyme) granules was determined (present study and Refs. 1 and 21). By comparison, when inhibition of the respiratory burst was used as a measure of neutrophil activation, a greater maximal effect was observed with PDE4 inhibitors. Regardless of the measures of activation, PDE4 inhibitors were still the most potent and effective class of isozyme-selective PDE inhibitors identified [3, 6].

In contrast to their action in human monocytes, guinea pig eosinophils, and rabbit gastric glands, the enantiomers of rolipram were approximately equipotent at inhibiting fMLP-induced degranulation of human neutrophils. Indeed, the pharmacological effects of a range of selective PDE4 inhibitors were associated with competition for [$^3$H]rolipram binding rather than with inhibition of PDE4 catalytic activity. These results are in contrast to our previous results with human monocytes (present study) and guinea pig eosinophils [30]. We should stress that within the series of compounds evaluated in this study, most were more potent at displacing [$^3$H]rolipram binding than inhibiting PDE4 catalytic activity, and none demonstrated selectivity for inhibition of PDE4 catalytic activity. Consequently, even though the functional effects of PDE4 inhibitors in monocytes and neutrophils were differentially associated with either inhibition of PDE4 catalytic activity (a low affinity form) or inhibition of high affinity rolipram binding (a high affinity form), we cannot eliminate the possibility that both forms of the enzyme are important. For example, it is possible that inhibition of the high affinity form is necessary but not sufficient, without the concomitant inhibition of the low affinity form of PDE4, to suppress monocyte activation. Nonetheless, our results do show that the functional effects of PDE4 inhibitors in inflammatory compounds can be associated with either inhibition of

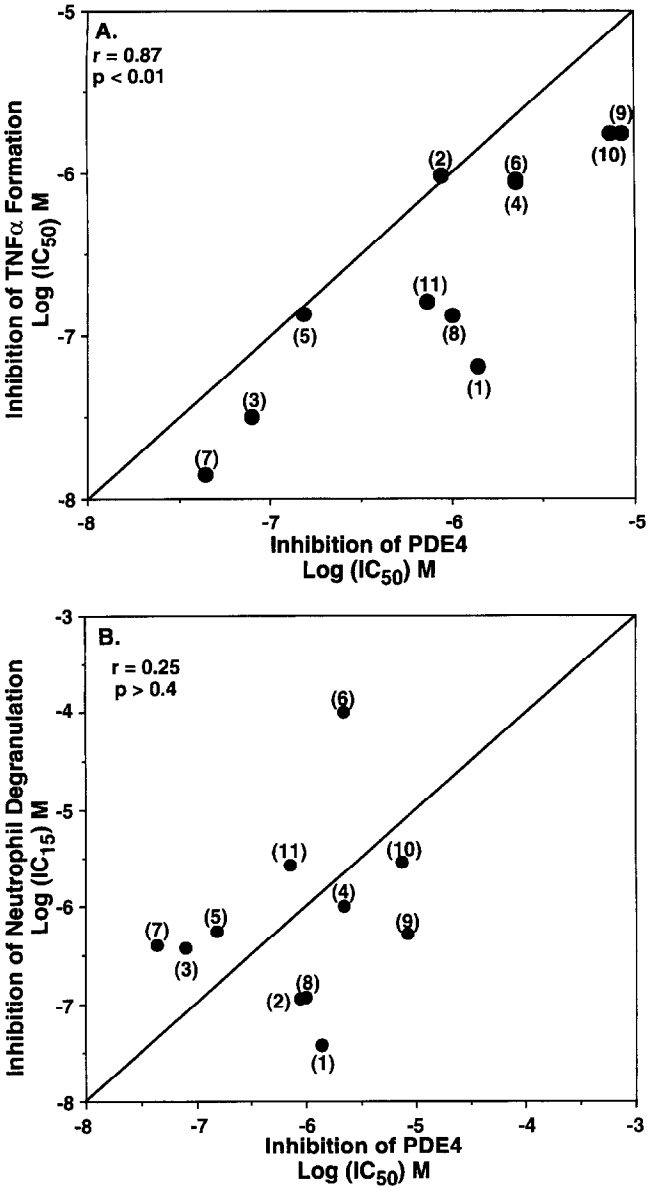


FIG. 2. Comparison between the ability of PDE4 inhibitors to inhibit PDE4 catalytic activity and their ability to suppress LPS-induced TNFα formation (A) or reduce fMLP-induced MPO release (B). The compounds used in these correlation are as follows: 1, R-rolipram; 2, S-rolipram; 3, A; 4, B; 5, C; 6, D; 7, E; 8, F; 9, G; 10, H; and 11, I. See Materials and Methods for chemical names of compounds.

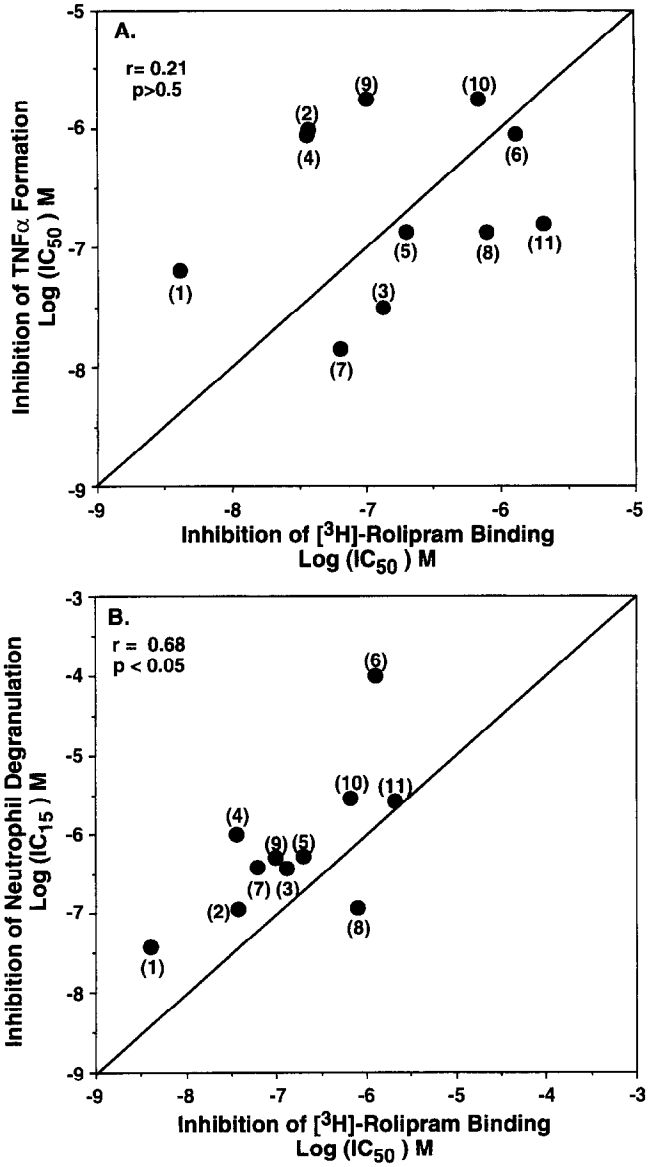


FIG. 3. Comparison between the ability of compounds to compete for [3H]rolipram binding and their ability to suppress LPS-induced TNFα formation (A) or reduce fMLP-induced MPO release (B). The compounds are as listed in the legend for Fig. 2.

PDE4 catalytic activity or rolipram binding. This represents, to our knowledge, the first reported comparison of PDE4 inhibitors among inflammatory cell types using the identical PDE4 inhibitors.

Perhaps a reason for the varying functional associations of PDE4 inhibitors among cell types and tissues is the presence of different subtypes of PDE4 in these cells. PDE4 exists as a family of enzymes derived from four distinct genes [31]. Since at least two of the major PDE4 subtypes demonstrate high affinity rolipram binding [12, 13] as well as catalytic activity, it is possible that these subtypes differ in the proportion of the enzyme found in the high affinity conformation as measured by rolipram binding. Thus, depending on the complement of PDE4 subtypes in a cell, the functional effects of PDE4 inhibitors may be associated with either inhibition of catalytic activity or competition for [3H]rolipram binding. Indeed, monocytes and PMNs appear to contain different subtypes [32, 33]. Monocytes contain PDE4A and PDE4B, whereas PMNs contain predominantly PDE4B [32, 33]. With the availability of recombinant proteins and the advent of subtype selective inhibitors, it should be possible to determine the role of high affinity rolipram binding in the functional actions of PDE4 inhibitors and to discern the functional role of the PDE4 subtypes.

Based on our findings, the approach to minimize side-effect potential of newer PDE4 inhibitors by identifying

compounds with decreased ability to displace [$^3$H]rolipram from its high affinity binding site may be limited, since certain therapeutic effects are also correlated with this biochemical action. In addition to inhibition of PMN degranulation, relaxation of histamine-induced bronchoconstriction in guinea pigs is associated with inhibition of the high affinity rolipram binding and not with inhibition of PDE4 catalytic activity [34].

In summary, we have confirmed earlier studies that PDE4 inhibitors are potent suppressors of monocyte and PMN activation and have demonstrated that these functional actions are associated with different biochemical effects of PDE4 inhibitors, namely inhibition of catalytic activity versus competition for rolipram binding.

*We gratefully acknowledge the careful and critical review of this manuscript by Drs. D. W. P. Hay and L. A. Marshall. We would like to thank Dr. P. Bender, C. Forster, A. Guider, and J. Karpinski for preparing the novel PDE4 inhibitors used in these studies.*

## References


1. Busse WW and Anderson CL, The granulocyte responses to the phosphodiesterase inhibitor RO 20-1724. *J Allergy Clin Immunol* **67:** 70–74, 1981.
2. Elliott KRF and Leonard EJ, Interaction of formylmethionyl-leucyl-phenylalanine, adenosine and phosphodiesterase inhibitors in human monocytes. *FEBS Lett* **254:** 94–98, 1989.
3. Nielson CP, Vestal RE, Sturm RJ and Heaslip R, Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. *J Allergy Clin Immunol* **86:** 801–808, 1990.
4. Peachell PT, Undem BJ, Schleimer RP, MacGlashan DW, Lichtenstein LM, Cieslinski LB and Torphy TJ, Preliminary identification and role of phosphodiesterase isozymes in human basophils. *J Immunol* **148:** 2503–2510, 1992.
5. Prabhakar U, Lipshutz D and Truneh A, Inhibition of CD44, CD45, and LFA-3 mediated cytokine release from human monocytes by SK&F 86002 and pentoxifylline. *Int J Immunopharmacol* **15:** 205–209, 1993.
6. Schudt C, Winder S, Forderkunz S, Hatzelmann A and Ullrich V, Influence of selective phosphodiesterase inhibitors on human neutrophil functions and levels of cAMP and $Ca_i$. *Naunyn Schmiedebergs Arch Pharmacol* **344:** 682–690, 1991.
7. Semmler J, Wachtel H and Endres S, The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor-α production by human mononuclear cells. *Int J Immunopharmacol* **15:** 409–413, 1993.
8. Horowski R and Sastre-Y-Hernandez M, Clinical effects of the neurotropic selective cAMP phosphodiesterase inhibitor rolipram in depressed patients: Global evaluation of the preliminary reports. *Curr Therap Res* **38:** 23–29, 1985.
9. Brunnee T, Engelstatter R, Steinijans VW and Kunkel G, Bronchodilatory effect of inhaled zardaverine, a phosphodiesterase III and IV inhibitor, in patients with asthma. *Eur Respir J* **5:** 982–985, 1992.
10. Wachtel H, Potential antidepressant activity of rolipram and other selective cyclic adenosine 3′,5′-monophosphate phosphodiesterase inhibitors. *Neuropharmacology* **22:** 267–272, 1983.
11. Wachtel H, Characteristic behavioral alteration in rats induced by rolipram and other selective adenosine cyclic 3′,5′-monophosphate phosphodiesterase inhibitors. *Psychopharmacology* **77:** 309–316, 1982.
12. Torphy TJ, Stadel JM, Burman M, Cieslinski LB, McLaughlin MM, White JR and Livi GP, Coexpression of human cAMP-specific phosphodiesterase activity and high affinity rolipram binding in yeast. *J Biol Chem* **267:** 1798–1804, 1992.
13. McLaughlin MM, Cieslinski LB, Burman M, Torphy TJ and Livi GP, A low $K_m$, rolipram-sensitive, cAMP-specific phosphodiesterase from human brain. *J Biol Chem* **268:** 6470–6476, 1993.
14. Souness JE, Malsen C and Scott LC, Effects of solubilization and vanadate/glutathione complex on inhibitor potencies against eosinophil cyclic AMP-specific phosphodiesterase. *FEBS Lett* **302:** 181–184, 1992.
15. Torphy TJ, DeWolf WE Jr, Green DW and Livi GP, Biochemical characteristics and cellular regulation of phosphodiesterase IV. *Agents Actions Suppl* **43:** 51–71, 1993.
16. Schmiechen R, Schneider HH and Wachtel H, Close correlation between behavioral response and binding *in vivo* for inhibitors of the rolipram-sensitive phosphodiesterase. *Psychopharmacology* **102:** 17–20, 1990.
17. Barnette MS, Grous M, Cieslinski LB, Burman M, Christensen SB and Torphy TJ, Inhibitors of phosphodiesterase IV (PDE IV) increase acid secretion in isolated rabbit gastric glands: Correlation between function and interaction with a high affinity rolipram-binding site. *J Pharmacol Exp Ther* **273:** 1396–1402, 1995.
18. Underwood DC, Osborn RR, Novak LB, Matthews JK, Newsholme SJ, Undem BJ, Hand JM and Torphy TJ, Inhibition of antigen-induced bronchoconstriction and eosinophil infiltration in the guinea pig by the cyclic AMP-specific phosphodiesterase inhibitor, rolipram. *J Pharmacol Exp Ther* **266:** 306–313, 1993.
19. Prabhakar U, Lipshutz D, O'Leary Bartus J, Slivjak MJ, Smith EF III and Esser KM, Characterization of cAMP-dependent inhibition of LPS-induced TNFα production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor. *Int J Immunopharmacol* **16:** 805–816, 1994.
20. Semmler J, Gerbert U, Eisenhut T, Moeller J, Schonharting MM, Allera A and Endress S, Xanthine derivatives: Comparison between suppression of tumour necrosis factor α production and inhibition of cAMP phosphodiesterase activity. *Immunology* **78:** 520–525, 1993.
21. Nourshargh S and Hoult JRS, Inhibition of human neutrophil degranulation by forskolin in the presence of phosphodiesterase inhibitors. *Eur J Pharmacol* **122:** 205–212, 1986.
22. Colotta F, Peri G, Villa A and Mantovani A, Rapid killing of actinomycin D-treated tumor cells by human mononuclear cells. *J Immunol* **132:** 936–944, 1984.
23. Winston SE, Fuller SA and Hurrel JGR, Enzyme linked immunosorbent assay (ELISA) for detection of antigens. In: *Current Protocols in Molecular Biology* (Eds. Ausubel FM, Brente R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K), P. 11.2.1. John Wiley, New York, 1987.
24. Köhler G and Milstein C, Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:** 495–497, 1975.
25. Davis CW and Daly JW, A simple direct assay of 3′,5′-cyclic nucleotide phosphodiesterase activity based on the use of polyacrylamide-boronate affinity gel chromatography. *J Cyclic Nucleotide Res* **5:** 65–74, 1979.
26. Livi GP, Kmetz P, McHale MM, Cieslinski LB, Sathe GM, Taylor DP, Davis RL, Torphy TJ and Balcarek JM, Cloning and expression of cDNA for a human low-$K_m$, rolipram-sensitive cyclic AMP phosphodiesterase. *Mol Cell Biol* **10:** 2678–2686, 1990.
27. Schade FU and Schudt C, The specific type III and IV phosphodiesterase inhibitor zardaverine suppresses formation of tumor necrosis factor by macrophages. *Eur J Pharmacol* **230:** 9–14, 1993.
28. Molnar-Kimber K, Yonno L, Heaslip R and Weichman B,

Modulation of TNFα and IL-1β from endotoxin-stimulated monocytes by selective PDE isozyme inhibitors. *Agents Actions* **39:** C77–C79, 1993.

29. Schneider HH, Schmiechen R, Brezinski M and Seidler J, Stereospecific binding of the antidepressant rolipram to brain protein structures. *Eur J Pharmacol* **127:** 105–115, 1986.
30. Barnette MS, Manning CD, Cieslinski LB, Burman M, Christensen SB and Torphy TJ, The ability of phosphodiesterase IV inhibitors to suppress superoxide production in guinea pig eosinophils is correlated with inhibition of phosphodiesterase catalytic activity. *J Pharmacol Exp Ther* **273:** 674–678, 1995.
31. Bolger G, Michaeli T, Martins T, St. John T, Steiner B, Rodgers L, Riggs M, Wigler M and Ferguson K, A family of human phosphodiesterases homologous to the *dunce* learning and memory gene product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol Cell Biol* **13:** 6558–6571, 1993.
32. Obernolte R, Bhakta S, Alvarez R, Bach C, Zuppan P, Mulkins M, Jarnagin K and Shelton ER, The cDNA of a human lymphocyte cyclic-AMP phosphodiesterase (PDE IV) reveals a multigene family. *Gene* **129:** 239–247, 1993.
33. Engels P, Fichtel K and Lubbert H, Expression and regulation of human rat phosphodiesterase type IV isogenes. *FEBS Lett* **350:** 291–295, 1994.
34. Harris AL, Connell MJ, Ferguson EW, Wallace AM, Gordon RJ, Pagani ED and Silver PJ, Role of low $K_m$ cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea pig. *J Pharmacol Exp Ther* **251:** 199–206, 1989.