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Phosphodiesterase 4-dependent regulation of cyclic AMP levels and leukotriene B_4 biosynthesis in human polymorphonuclear leukocytes

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Abstract

Several selective phosphodiesterase 4 inhibitors were found to be potent inhibitors of the *N*-formyl-Met-Leu-Phe (fMLP)-induced leukotriene B_4 biosynthesis by human polymorphonuclear leukocytes with IC_{50}s in the nanomolar range (0.09–26 nM). The rank order of potency was 6-(4-pyridylmethyl)-8-(3-nitrophenyl)quinoline (RS-14203) > 3-benzyl-5-phenyl-3*H*-imidazo[4,5-*c*][1,8]naphthyridin-4(5*H*)-one (KF18280) > 8-aza-1-(3-nitrophenyl)-3-(4-pyridylmethyl)-2,4-quinazoline dione (RS-25344) > 3-cyclo-pentyloxy-*N*-[3,5-dichloro-4-pyridyl]-4-methoxybenzamide (RP-73401) > *R*-rolipram > *R*-4-[2-(3-cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl] pyridine (CDP840) > *S*-rolipram. Isoproterenol (IC_{50} = 350 nM) and prostaglandin E_2 (IC_{50} = 59 nM) also suppressed leukotriene B_4 biosynthesis. Inhibitors of the phosphodiesterase 1 (8-methoxymethyl-1-methyl-3-(2-methylpropyl)xanthine (8-MeOMe-IBMX)), phosphodiesterase 2 (*erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA)), phosphodiesterase 3 (quazinone and milrinone) and phosphodiesterase 5 (zaprinast and dipyridamole) had no inhibitory effects on the fMLP-induced leukotriene B_4 biosynthesis (IC_{50}s > 20 μM). All phosphodiesterase 4 inhibitors caused an accumulation of cellular cyclic AMP to 140–185% over the basal level of fMLP-treated control cells, comparable to that observed with high concentrations of isoproterenol and prostaglandin E_2. In contrast, the complete inhibition of leukotriene B_4 production by 5-lipoxygenase and 5-lipoxygenase-activating protein (FLAP) inhibitors had no effect on cyclic AMP levels. Phosphodiesterase 1, 2, 3 and 5 inhibitors had little effect on the level of cellular cyclic AMP (89–126% of the basal cyclic AMP level). Dose-dependencies for *R*-rolipram, RS-14203 and CDP840 indicated that the maximal accumulation of cyclic AMP occurred at concentrations of phosphodiesterase 4 inhibitors higher than those required for the inhibition of leukotriene B_4 production. The presence of a mixture of 8-MeOMe-IBMX, EHNA, milrinone and zaprinast to inhibit phosphodiesterase 1, 2, 3 and 5 had little effect on the dose-dependence of *R*-rolipram for the inhibition of leukotriene B_4 biosynthesis or cyclic AMP accumulation. These data demonstrate that selective phosphodiesterase 4 inhibitors can inhibit the fMLP-induced leukotriene B_4 biosynthesis in human polymorphonuclear leukocytes with a potency similar or greater than that of potent 5-lipoxygenase or FLAP inhibitors. This inhibition is accompanied by small variations in the levels of cellular cyclic AMP and appears to proceed independently of the other phosphodiesterases.

Keywords: Leukotriene biosynthesis; Phosphodiesterase; Phosphodiesterase inhibitor; Polymorphonuclear leukocyte; fMLP (*N*-formyl-Met-Leu-Phe)

1. Introduction

The elevation of intracellular cyclic AMP has been associated with the suppression of the functions of inflammatory cells. For example, agents that stimulate intracellular cyclic AMP levels have been shown to inhibit the chemotaxis and degranulation of eosinophils (Souness et al., 1995; Alves et al., 1996; Tenor et al., 1996), the release of TNF-α from macrophages (Lombardo, 1995; Moraes et al., 1998) and the release of lysosomal enzymes, platelet-activating factor (PAF), leukotrienes, nitric oxide and reactive oxygen species from neutrophils, eosinophils or macrophages (Moore and Willoughby, 1995; Tenor and Schudt, 1996). Since the phosphodiesterase 4 is the main enzyme responsible for the degradation of cyclic AMP in these cells (Torphy and Undem, 1991; Beavo et al., 1994), much attention has been devoted to the therapeutic potential of selective phosphodiesterase 4 inhibitors for the treatment of asthma and inflammation (Torphy et al., 1994; Hughes et al., 1997).

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The phosphodiesterase family is very complex as, to date, at least seven different isozymes and multiple spliced variants differing in subcellular localization have been identified (Bushnik and Conti, 1996; Houslay, 1996; Müller et al., 1996). The key role of phosphodiesterase 4 in the regulation of leukotriene biosynthesis has been demonstrated in numerous studies where the selective phosphodiesterase 4 inhibitor rolipram has been reported to inhibit the release of leukotriene C_4 from anti-immunoglobulin E-stimulated human basophils (Peachell et al., 1992), to inhibit the release of leukotriene and to raise cyclic AMP levels in *N*-formyl-Met-Leu-Phe (fMLP)/thimerosal-stimulated human polymorphonuclear leukocytes (Schudt et al., 1991), to inhibit PAF- and C5a-induced chemotaxis and leukotriene C_4 biosynthesis from human eosinophils (Tenor et al., 1996) and to inhibit the release of leukotriene B_4 from opsonized- and PAF-stimulated eosinophils (Banner et al., 1996). Studies on the effects of phosphodiesterase inhibitors on leukotriene release have focused mainly on the use of rolipram and non-specific inhibitors like theophylline and 3-isobutyl-1-methylxanthine (IBMX). These inhibitors have been reported to have variable effects on cyclic AMP accumulation depending on the cell system and the nature of the stimulus (Nielson et al., 1988; Schudt et al., 1991; Peachell et al., 1992). In the present study, we have extensively characterized the effect of numerous selective phosphodiesterase 4 inhibitors and compared their effects to those of inhibitors of phosphodiesterase 1, 2, 3 and 5, 5-lipoxygenase and 5-lipoxygenase activating protein (FLAP) on the biosynthesis of leukotriene B_4 and the modulation of cyclic AMP intracellular levels in fMLP-activated human polymorphonuclear leukocytes.

2. Materials and methods

2.1. Preparation of human polymorphonuclear leukocytes

Leukocytes were isolated from fresh citrated human blood by dextran sedimentation followed by differential centrifugation on Histopaque®-1077 according to previously described procedures (Gillard et al., 1989). The cells were washed twice in Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS) containing 10 mM HEPES (pH 7.4), resuspended in the same buffer at a concentration of 3×10^6 cells ml^{-1}, viability assessed by Trypan blue exclusion (routinely $> 95\%$) and kept at 37°C until used.

2.2. Stimulation of human polymorphonuclear leukocytes with fMLP

The assay was carried out in a 96-well format at a final cell concentration of 2.2×10^6 cells ml^{-1} and in a final volume of 400 μl. Inhibitors (5 μl in dimethylsulfoxide) were added to 300 μl of the cell suspension and preincubated for 1 or 10 min at 37°C. The cells were then stimulated (10 min, 37°C) by the addition of 100 μl of a solution of 0.8 or 1.6 μM fMLP or 8 μM A23187 in Ca^{2+}/Mg^{2+}-free HBSS/10 mM HEPES, pH 7.4, supplemented with 6.4 mM $CaCl_2$. The reaction was stopped by the addition of 100 μl of methanol and the samples were frozen in a mixture of solid CO_2/isopropanol and were kept frozen at −80°C until the time of the analysis. After thawing, samples were centrifuged to remove cell debris before the measurement of leukotriene B_4 and cyclic AMP by radioimmunoassay (RIA).

For the various donors used ($n = 16$), the stimulation of leukotriene B_4 production by fMLP was found to vary from four to 33 times that of unstimulated cells (no fMLP). IC_{50} values for the different inhibitors were determined using eight-point titrations performed in duplicates. Percentages of inhibition were calculated from the difference in leukotriene B_4 production between fMLP-stimulated and control cells. Cyclic AMP levels were expressed as percent of basal level of fMLP-stimulated cells in the absence of inhibitor.

2.3. Materials

Sodium citrate, dextran, Histopaque®-1077, HEPES, isoproterenol, zaprinast, pentoxifylline, IBMX, dipyridamole (2,6-bis(diethylamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine), calcium ionophore (A23187) and fMLP were purchased from Sigma (Oakville, Canada). HBSS (without calcium chloride, magnesium chloride and magnesium sulfate) was obtained from Life Technologies (Burlington, Canada). *erythro*-9-(2-Hydroxy-3-nonyl)adenine · HCl (EHNA), 8-methoxymethyl-1-methyl-3-(2-methylpropyl)xanthine (8-MeoMe-IBMX), milrinone and trequensin were purchased from Biomol (Plymouth Meeting, USA), theophylline from Aldrich (Milwaukee, USA) and quazinone from Calbiochem (La Jolla, USA). *R*-4-[2-(3-cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl] pyridine (CDP840) was obtained from Celltech Therapeutics (Slough, UK) (Hughes et al., 1996) and *R*-rolipram, *S*-rolipram, 8-aza-1-(3-nitrophenyl)-3-(4-pyridylmethyl)-2,4-quinazoline dione (RS-25344) (Alvarez et al., 1995), 6-(4-pyridylmethyl)-8-(3-nitrophenyl)quinoline (RS-14203) (Lombardo, 1995), 3-benzyl-5-phenyl-3*H*-imidazo[4,5-*c*] [1,8]naphthyridin-4(5*H*)-one (KF18280) (Suzuki et al., 1992), 3-cyclo-pentyloxy-*N*-[3,5-dichloro-4-pyridyl]-4-methoxybenzamide (RP-73401) (Souness et al., 1995), 2-cyano-4-(3-furyl)-7-[[6-[3-(3-ydroxy-6,8-dioxabicyclo [3.2.1] octanyl)]-2-pyridyl]methoy]naphthalene (L-739,010) (Hamel et al., 1997) and 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl] 2,2-dimethylpropanoic acid (MK-886) (Gillard et al., 1989) were synthetized at the Department of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research (Kirkland, Quebec). [^{3}H] leukotriene B_4 and [^{125}I] cyclic AMP RIA kits (non-acetylation protocol) were purchased from Amersham (Oakville, Canada).

3. Results

3.1. Production of leukotriene B_4 by fMLP-stimulated human polymorphonuclear leukocytes

Ca^{2+}-depleted human polymorphonuclear leukocytes have been shown to produce significant quantities of leukotriene B_4 in response to the simultaneous addition of Ca^{2+} and fMLP (Krump et al., 1995). This cellular model was used in the present study to examine the role of phosphodiesterase 4 in leukotriene B_4 biosynthesis in response to the soluble chemotactic peptide fMLP in the absence of any priming agent such as thimerosal or granulocyte and macrophage-colony stimulating factor (GM-CSF) (Krump and Borgeat, 1994; Villagrasa et al., 1996). As shown in Fig. 1a, fMLP dose-dependently stimulated the release of leukotriene B_4 by polymorphonuclear leukocytes by about 14-fold with half maximal production at a fMLP concentration of 0.05–0.1 μM. The synthesis of leukotriene B_4 was rapid, with a maximum being reached after 3 min (Fig. 1b). The production of leukotriene B_4 by fMLP-stimulated polymorphonuclear leukocytes (10 min incubation) obtained from various donors ($n = 16$) ranged from 45 to 400 pg/10^6 cells.

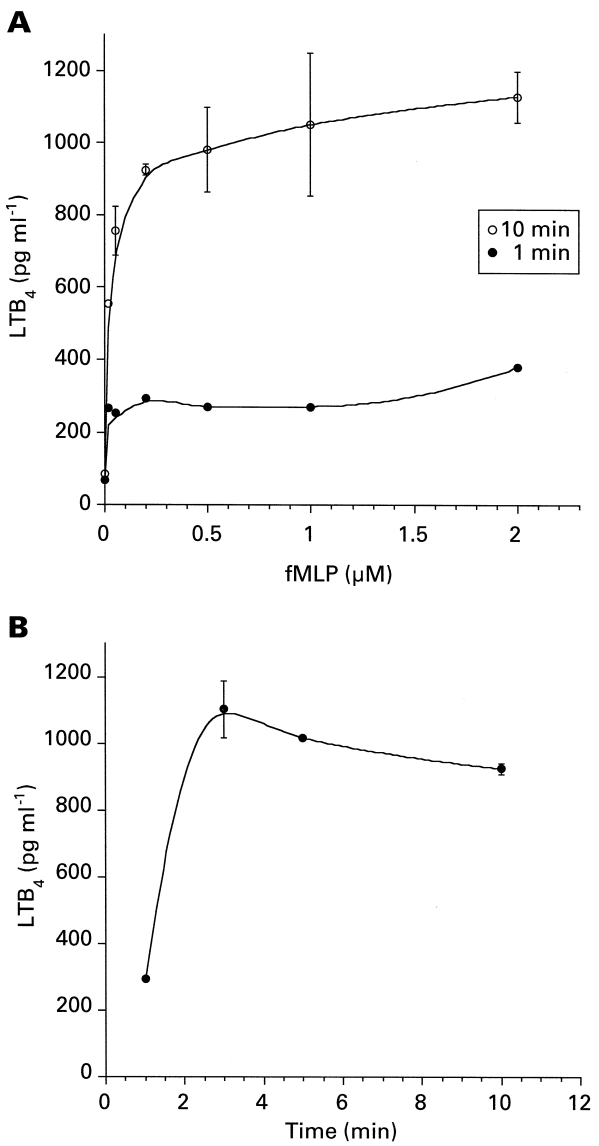

Fig. 1. Dependence of leukotriene B_4 biosynthesis by human polymorphonuclear leukocytes on fMLP concentration and incubation time. Human polymorphonuclear leukocytes were stimulated by the simultaneous addition of (A) Ca^{2+} and increasing concentrations of fMLP for 1 or 10 min, or (B) Ca^{2+} and 0.2 μM fMLP for the indicated incubation time. Values are from a single measurements or means ± S.E. of three determinations.

Table 1
IC_{50} values of various compounds for the inhibition of fMLP-induced leukotriene B_4 biosynthesis in human polymorphonuclear leukocytes

Compound	IC_{50} (nM) (n)	Class of inhibitor
L-739,010	2 ± 1 (7)	5-LO
MK-886	9 ± 1 (2)	FLAP
Isoproterenol	350 ± 220 (7)	
PGE_2	59 ± 21 (6)	
R-Rolipram	6 ± 2 (9)	PDE_4
S-Rolipram	26 ± 9 (4)	PDE_4
CDP840	14 ± 5 (4)	PDE_4
RS-25344	0.6 ± 0.2 (5)	PDE_4
RS-14203	0.09 ± 0.03 (9)	PDE_4
KF18280	0.4 ± 0.1 (3)	PDE_4
RP-73401	2.6 ± 0.7 (4)	PDE_4
Theophylline	> 100,000 (3)	Non-specific PDE
IBMX	> 100,000 (3)	Non-specific PDE
Pentoxifylline	> 20,000 (3)	Non-specific PDE
8-MeOMe-IBMX	> 20,000 (4)	PDE_1
EHNA	> 100,000 (3)	PDE_2
Quazinone	> 100,000 (3)	PDE_3
Trequensin	2400 ± 1100 (4)	$PDE_{3/4}$
Milrinone	> 20,000 (3)	PDE_3
Zaprinast	> 70,000 (2)	$PDE_{5/6}$
Dipyridamole	21,000 ± 6600 (5)	PDE_5

PDE: phosphodiesterase.

3.2. Inhibition of leukotriene B_4 biosynthesis by phosphodiesterase inhibitors

The effect of various modulators of cellular cyclic AMP levels and of leukotriene biosynthesis on the synthesis of leukotriene B_4 by fMLP-stimulated polymorphonuclear leukocytes was determined (Table 1). The selective 5-lipoxygenase inhibitor L-739,010 and the FLAP inhibitor MK-886 displayed a high inhibitory potency with IC_{50} values in the low nanomolar range. Adenylate cyclase activators, like isoproterenol and prostaglandin E_2, also inhibited leukotriene B_4 synthesis, confirming the role of cyclic AMP in the regulation of leukotriene biosynthesis in fMLP-activated polymorphonuclear leukocytes. Selective phosphodiesterase 4 inhibitors abolished fMLP-induced leukotriene B_4 synthesis with KF18280, RS-25344 and RS-14203 (IC_{50} of 0.1–0.6 nM) being more potent than *R*-rolipram and CDP840 (IC_{50} of 6 and 14 nM, respectively). Some stereoselectivity of inhibition was observed for rolipram as *R*-rolipram was about four-fold more

potent than the *S*-isomer. In contrast, the inhibitors of phosphodiesterase 1 (8-MeOMe-IBMX), phosphodiesterase 2 (EHNA), phosphodiesterase 3 (quazinone and milrinone) and phosphodiesterase 5 (zaprinast and dipyridamole) showed little or no inhibitory effects in this system (IC_{50}s > 10–100 μM). The non-specific phosphodiesterase inhibitors theophylline, IBMX and pentoxifylline were not potent inhibitors of fMLP-induced leukotriene B_4 synthesis (IC_{50}s > 20–100 μM). The inhibitory effect of the phosphodiesterase 3 inhibitor trequensin (IC_{50} of 2.4 μM) was probably due to its limited specificity since this compound has also been reported to inhibit phosphodiesterase 4 activity at these concentrations (IC_{50} of 0.4 μM) (Souness and Rao, 1997).

3.3. Variation in cellular cyclic AMP levels during inhibition of leukotriene B_4 biosynthesis

In order to further examine the relationship between the inhibition of leukotriene biosynthesis and the activity of phosphodiesterase 4 in human polymorphonuclear leukocytes, the levels of both leukotriene B_4 and cyclic AMP were measured from cells treated with *R*-rolipram, RS-14203, CDP840 (phosphodiesterase 4 inhibitors) and L-739,010 (5-lipoxygenase inhibitor). Each of these compounds was a potent inhibitor of fMLP-dependent leukotriene B_4 biosynthesis although a more complete inhibition (> 99%) was achieved with the 5-lipoxygenase inhibitor than with the phosphodiesterase 4 inhibitors (80–85% inhibition) (Fig. 2). A significant elevation of cyclic AMP was observed for each of the selective phosphodiesterase 4 inhibitors tested (Fig. 2a, b and c). The selectivity of this effect for phosphodiesterase 4 inhibition is further demonstrated by the fact that L-739,010, a 5-lipoxygenase inhibitor which is as potent as *R*-rolipram at inhibiting leukotriene B_4 production, had no effect on cyclic AMP levels (Fig. 2d). The maximal level of cyclic AMP in cells treated with phosphodiesterase 4 inhibitors reached 150–170% of that measured with fMLP-stimulated polymorphonuclear leukocytes and was detected at

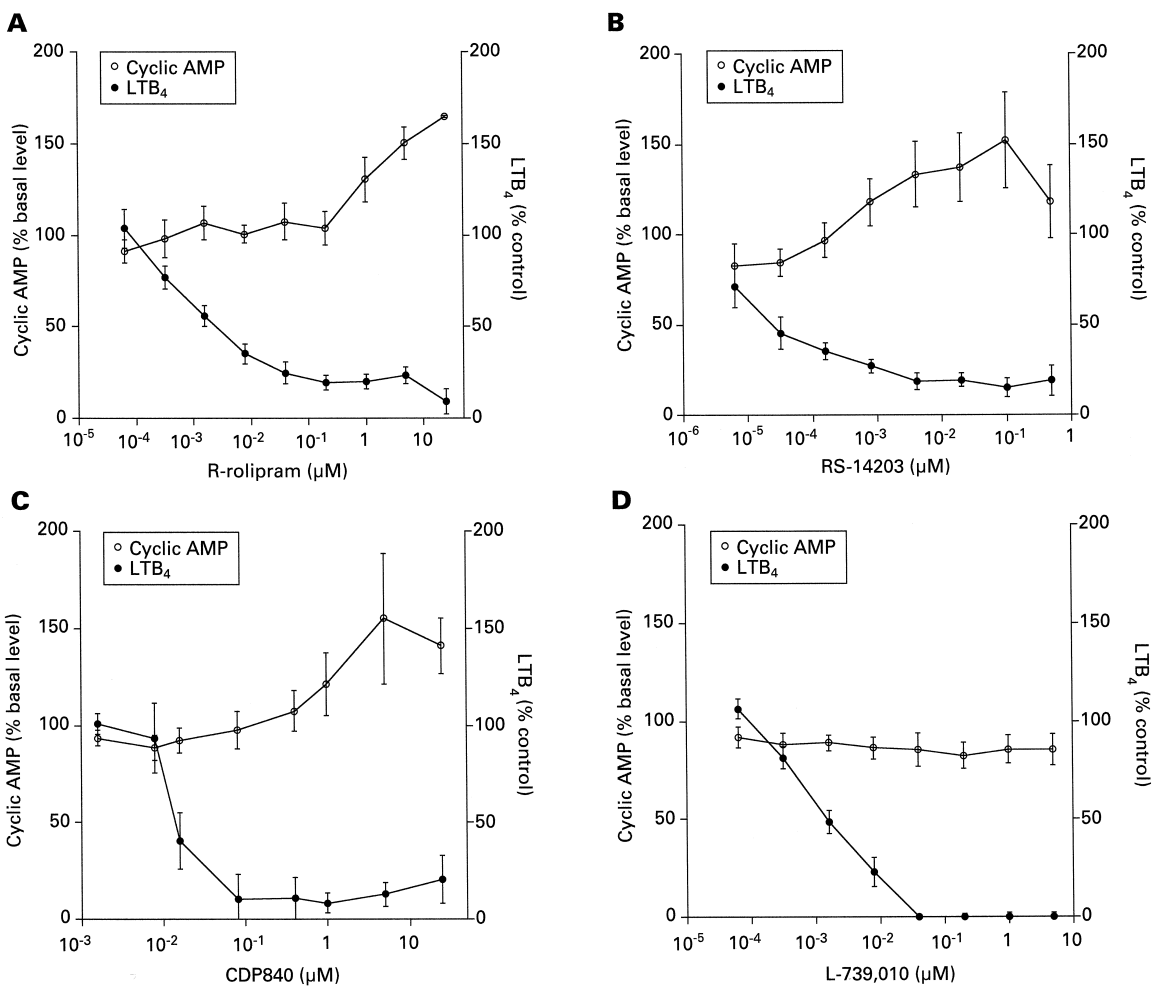

Fig. 2. Effect of phosphodiesterase 4 inhibitors on cellular cyclic AMP levels and leukotriene B_4 production of fMLP-stimulated human polymorphonuclear leukocytes. Cells were preincubated for 10 min with various concentrations of *R*-rolipram, RS-14203, CDP840 or of the 5-lipoxygenase inhibitor L-739,010 prior to stimulation with fMLP for 10 min. Samples were then analyzed for cyclic AMP and leukotriene B_4. Results are reported as the percentage of cyclic AMP and leukotriene B_4 measured in fMLP-stimulated cells incubated under the same conditions in absence of inhibitor. Values are means ± S.E. of three to eight determinations.

Table 2
Effect of various phosphodiesterase inhibitors on cyclic AMP levels in fMLP-stimulated human polymorphonuclear leukocytes

Compound	Concentration (μM)	Cyclic AMP (% basal level) (*n*)
L-739,010	5	85 ± 8 (7)
MK-886	5	104 ± 6 (2)
Isoproterenol	50	158 ± 11 (7)
PGE_2	10	165 ± 14 (6)
R-Rolipram	5	150 ± 9 (8)
S-Rolipram	25	136 ± 7 (2)
CDP840	25	141 ± 14 (2)
RS-25344	0.1	185 ± 37 (4)
RS-14023	0.1	152 ± 27 (8)
KF18280	0.1	142 ± 8 (2)
RP-73401	1	183 ± 45 (3)
Theophylline	100	105 ± 14 (3)
IBMX	100	127 ± 13 (3)
Pentoxifylline	100	119 ± 10 (3)
8-MeOMe-IBMX	20	89 ± 14 (4)
EHNA	20	112 ± 7 (3)
Quazinone	20	126 ± 9
Trequensin	4	122 ± 22
Milrinone	20	118 ± 3 (3)
Zaprinast	20	104 ± 1 (2)
Dipyridamole	20	98 ± 4 (5)

inhibitor concentrations that were in excess to those required for the inhibition of leukotriene B_4 production. Treatment by fMLP did not affect cyclic AMP levels of Ca^{2+}-repleted cells (1.0 ± 0.1 vs. 1.1 ± 0.1 pmol/10^6 cells, $n = 36$, for incubations without and with fMLP, respectively).

Similar to the results depicted for rolipram in Fig. 2, the phosphodiesterase 4 inhibitors RS-25344, KF18280 and RP-73401 caused an elevation of cyclic AMP levels which reached a maximum at concentrations higher than those for the IC_{50} values for the inhibition of leukotriene B_4 (data not shown). The magnitude of the maximal cellular increases in cyclic AMP caused by inhibitors of phosphodiesterase 4, as well as by other inhibitors of leukotriene B_4 production, are summarized in Table 2. The different phosphodiesterase 4 inhibitors caused an increase in cyclic AMP (150–180%) that was similar to that obtained by stimulation of adenylate cyclase with high doses of isoproterenol and prostaglandin E_2 (158% at 50 μM and 165% at 10 μM, respectively). In contrast, a complete inhibition of leukotriene B_4 production with high concentrations of either the 5-lipoxygenase inhibitor L-739,010 or the FLAP inhibitor MK-886 had little effect on the basal level of cyclic AMP (85 and 104% of the basal cyclic AMP levels, respectively, at inhibitor concentration of 5 μM). The non-specific phosphodiesterase inhibitors and the various selective inhibitors of phosphodiesterase 1, 2, 3 and 5, which had no effect on leukotriene B_4 biosynthesis (Table 1), also showed little effect on cyclic AMP levels at high doses (89–127% of basal levels). These results indicate that small, but significant changes in cellular cyclic AMP of human polymorphonuclear leukocytes are specifically associated with inhibition of leukotriene B_4 biosynthesis resulting from phosphodiesterase 4 inhibition.

3.4. Time course of the elevation of cyclic AMP following phosphodiesterase 4 inhibition

In order to determine whether the inhibition of phosphodiesterase 4 could have induced a rapid transient increase in cyclic AMP that would not have been detected after the 10 min incubation, the time course of the change in cyclic AMP following a short preincubation with phosphodiesterase 4 inhibitors was determined. Human polymorphonuclear leukocytes were preincubated for only 1 min with *R*-rolipram and RS-14203 and stimulated with fMLP for an additional 1.5, 3, 5 and 10 min before cyclic AMP measurement. The increase in cyclic AMP was found to increase in the 1st min following incubation with *R*-rolipram (Fig. 3a) or RS-14203 (Fig. 3b). The slightly

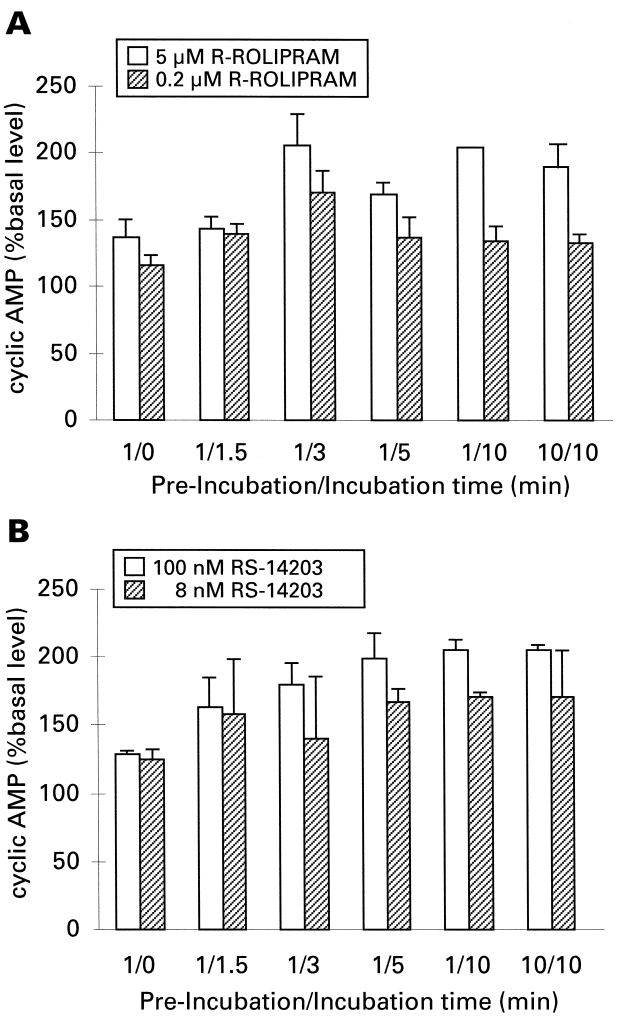

Fig. 3. Effect of incubation time on cyclic AMP accumulation induced by *R*-rolipram or RS-14203. Human polymorphonuclear leukocytes were preincubated with *R*-rolipram or RS-14203 for 1 or 10 min prior to fMLP stimulation for 0, 1.5, 3, 5 and 10 min as indicated. Values are means ± range of two independent determinations.

higher levels of cyclic AMP detected in this experiment as compared to the mean values reported in Table 2 appears to be due to the responsiveness of the donors used since the control values obtained for the regular 10 min assay were also higher than those reported in Table 2 (190% vs. 150% at 5 μM *R*-rolipram and 200% vs. 150% at 0.1 μM RS-14203). These results show that no major transient increase in cyclic AMP occurred during the first 10 min following inhibition of phosphodiesterase 4 and inhibition of leukotriene B_4 biosynthesis by selective phosphodiesterase 4 inhibitors.

3.5. Effect of phosphodiesterase 1, 2, 3 and 5 inhibitors on phosphodiesterase 4-dependent cyclic AMP accumulation

The possibility that the modest elevation of cyclic AMP detected following phosphodiesterase 4 inhibition could be due to cyclic AMP metabolism via other phosphodiesterases was investigated. Human polymorphonuclear leukocytes were preincubated for 10 min with increasing concentrations of *R*-rolipram in the presence or the absence of a mixture of phosphodiesterase inhibitors (8-MeOMe-IBMX, EHNA, milrinone and zaprinast for the inhibition of phosphodiesterase 1, 2, 3 and 5, respectively) at concentrations (10 μM) that had little or no effect on leukotriene B_4 biosynthesis or cyclic AMP accumulation (Tables 1 and 2). The cells were then stimulated with fMLP for 10 min and the levels of leukotriene B_4 and cyclic AMP were determined. A control experiment showed that the mixture of phosphodiesterase inhibitors caused only a slight increase of the levels of cyclic AMP (128%) and leukotriene B_4 (122%) in the absence of *R*-rolipram. Neither the extent of leukotriene B_4 biosynthesis inhibition or cyclic AMP accumulation by *R*-rolipram were strongly modified by the presence of the mixture of phosphodiesterase inhibitors (Fig. 4), although the presence of the mixture might have slightly diminished the accumulation of cyclic AMP at the highest doses of *R*-rolipram. Similar results were observed with RS-14203 (data not shown). The results of Fig. 4 and Tables 1 and 2 suggest that the phosphodiesterases other than phosphodiesterase 4 do not play significant roles in the regulation of leukotriene B_4 biosynthesis and cyclic AMP accumulation in this cellular system. These results support the conclusion that modest or compartmentalized phosphodiesterase 4-dependent changes in cellular cyclic AMP pools are sufficient to produce leukotriene B_4 inhibition in fMLP-stimulated human polymorphonuclear leukocytes.

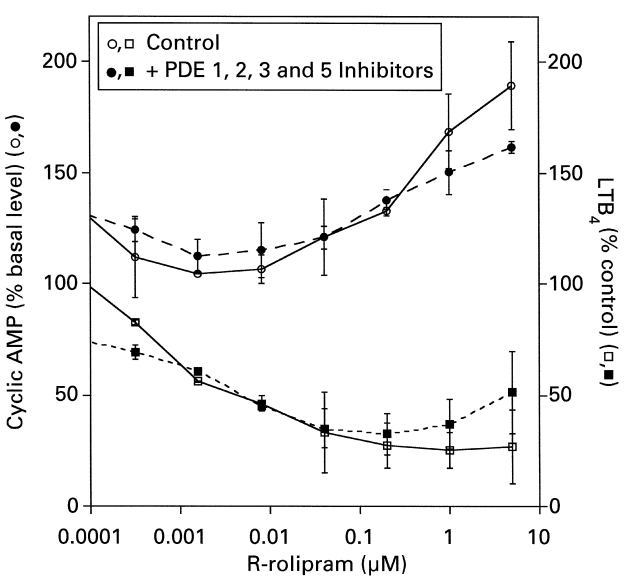

Fig. 4. Effect of a mixture of phosphodiesterase 1, 2, 3 and 5 inhibitors on leukotriene B_4 biosynthesis and cyclic AMP accumulation caused by *R*-rolipram treatment of fMLP-stimulated human polymorphonuclear leukocytes. Cells were pretreated for 10 min with increasing concentrations of *R*-rolipram with (closed symbols) or without (open symbols) a mixture of 8-MeOMe-IBMX, EHNA, milrinone and zaprinast at a concentration of 10 μM each. Leukotriene B_4 (squares) and cyclic AMP levels (circles) were determined after a 10-min stimulation with fMLP. Values are means ± range of two determinations.

4. Discussion

Many of the cellular systems reported for the study of leukotriene B_4 biosynthesis involve either the use of Ca^{2+} ionophore or a combination of fMLP and priming agents such as GM-CSF (Krump and Borgeat, 1994), thimerosal (Schudt et al., 1991) or dihydrocytochalasin B (Hughes et al., 1997). These agents, beside increasing agonist-induced leukotriene release, have diverse cellular actions such as causing a sustained elevation of intracellular calcium (Schudt et al., 1991) and the disruption of microtubules (Klotz and Jesaitis, 1994). In the present study, Ca^{2+}-depleted human polymorphonuclear leukocytes (Krump et al., 1995) were used to investigate the role of phosphodiesterase 4 in the release of leukotriene B_4 by polymorphonuclear leukocytes in response to the soluble agonist fMLP without potential interference from costimulators.

The present results confirm the role of phosphodiesterase 4 in the regulation of leukotriene B_4 biosynthesis in human polymorphonuclear leukocytes and indicate that several different selective phosphodiesterase 4 inhibitors are potent inhibitors of fMLP-induced leukotriene B_4 release. Both the inhibition of leukotriene B_4 release and the elevation of cyclic AMP in polymorphonuclear leukocytes showed selectivity for the inhibition of the phosphodiesterase 4 isozyme. The phosphodiesterase 4 inhibitors were considerably more potent at blocking leukotriene B_4 biosynthesis than the non-specific phosphodiesterase inhibitors, theophylline, IBMX and pentoxifylline, a result which is in agreement with previous reports on the low potency of theophylline (EC_{50} of 50–300 μM) in other cell assays (Nielson et al., 1988; Beavo, 1995; Tenor et al., 1996) and the low potency of IBMX and pentoxifylline on the purified enzyme (Beavo, 1995).

It is noteworthy that *R*-rolipram is a more potent inhibitor of leukotriene B_4 biosynthesis (IC_{50} of 6 nM) in calcium/fMLP-stimulated human polymorphonuclear leukocytes than in other assays such as the thimerosal/fMLP-induced leukotriene B_4 release from human neutrophils (IC_{50} of 40 nM, Schudt et al., 1991), the calcium ionophore- or the fMLP-induced respiratory burst in human polymorphonuclear leukocytes (IC_{50} of 1–10 and 0.2 μM, respectively, Nielson et al., 1990), the opsonized zymosan-stimulated superoxide anion generation from human eosinophils (IC_{50} of 40 μM, Dent et al., 1994) or the PAF/opsonized zymosan-stimulated leukotriene B_4 synthesis from guinea pig eosinophils (IC_{50} of 2.7 μM, Banner et al., 1996). Furthermore, *R*-rolipram and CDP840 were not potent inhibitors of calcium ionophore (A23187)-induced leukotriene B_4 release from human polymorphonuclear leukocytes ($IC_{50} > 5$ μM, unpublished data) unlike L-739,010 or MK-886 ($IC_{50} = 3.9 \pm 2.6$, 8.9 ± 0.9 nM, respectively, unpublished data). It thus appears that the nature of the stimulus is an important factor in the evaluation of the potency of phosphodiesterase 4 inhibitors as inhibitors of leukotriene biosynthesis. The availability of intracellular calcium, which can be reduced by phosphodiesterase inhibitors (Nielson et al., 1990; Schudt et al., 1991; Anderson et al., 1998), may be a major factor responsible for the difference in potency observed between the different systems.

In fMLP-stimulated human polymorphonuclear leukocytes, selective phosphodiesterase 4 inhibitors caused a modest elevation of cyclic AMP levels, but of the same order of magnitude as observed with adenylate cyclase activators (150–180%). The maximal accumulation of cyclic AMP was observed at doses of inhibitor higher than those required for the inhibition of leukotriene B_4 and was not detected following treatment with 5-lipoxygenase, FLAP, phosphodiesterase 1, 2, 3, 5 and 6 inhibitors. The lack of effect of 5-lipoxygenase and FLAP inhibitors rules out the possibility that the elevation of cyclic AMP caused by phosphodiesterase 4 inhibitors could be due to an effect of leukotriene B_4 or other metabolites of the 5-lipoxygenase pathway. However, more pronounced cyclic AMP rises (2–200-fold) have been reported for cell systems where the cyclic AMP accumulation was measured in the presence of adenylate cyclase activators including prostaglandin E_2 (Torphy et al., 1994; Alvarez et al., 1996), isoprenaline (Souness et al., 1995) or forskolin (Chini et al., 1997).

The present results differ from those obtained with thimerosal-primed and fMLP-stimulated polymorphonuclear leukocytes where a 10-fold stimulation of cyclic AMP by rolipram was observed (Schudt et al., 1991). In this assay, the IC_{50} for the inhibition of leukotriene B_4 biosynthesis by rolipram was about 50 nM and corresponded approximately to the concentration which caused a 50% increase in cyclic AMP levels. Similarly, a good correlation has been reported between the inhibition of superoxide generation and cyclic AMP accumulation in dihydrocytochalasin B/fMLP stimulated human neutrophils treated with rolipram (Hughes et al., 1997). In light of these results, we further investigated the potential reasons for the small changes in cyclic AMP detected in the present assay by examining the detailed time course of cyclic AMP levels and the possibility that the activity of other phosphodiesterases may have reduced cyclic AMP accumulation. The elevation of cyclic AMP was found to occur as early as after 1 min of exposure to the phosphodiesterase 4 inhibitor and to be stable for at least 10 min. In addition, the smaller accumulation of cyclic AMP in fMLP-stimulated polymorphonuclear leukocytes does not appear to be due to hydrolysis of cyclic AMP by other phosphodiesterases such as phosphodiesterase 3 and 5 since a mixture of inhibitors of these enzymes had little effect on cyclic AMP levels. The inhibition of leukotriene B_4 production in human polymorphonuclear leukocytes thus seems to be finely modulated by either a modest increase in total cellular cyclic AMP, by a change in cyclic AMP turnover or by a localized elevation in specific cyclic AMP pools. The concept of cyclic AMP pools has been previously discussed for rat mesangial cells where two different cyclic AMP pools, regulated by phosphodiesterase 4 and phosphodiesterase 3 activities, have different cellular targets, namely reactive oxygen metabolites generation and mesangial cells proliferation (Chini et al., 1997).

The present results demonstrate that inhibition of leukotriene B_4 biosynthesis can be achieved via selective phosphodiesterase 4 inhibition, with small changes in total cellular cyclic AMP levels. The unique role of phosphodiesterase 4 in the maintenance of cyclic AMP balance in inflammatory cells further stresses the benefit that could arise from specific and tolerable phosphodiesterase 4 inhibitors in the management of asthma and inflammatory diseases.

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