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Differential type 4 cAMP-specific phosphodiesterase (PDE4) expression and functional sensitivity to PDE4 inhibitors among rats, monkeys and humans

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Abstract

It has been suggested that the rat is relatively more susceptible to toxicity induced by inhibitors for type 4 cAMP-specific phosphodiesterase (PDE4). In this study designed to elucidate possible biochemical basis for the higher susceptibility, we compared PDE4 expression levels and their functional relevance among rats, monkeys and humans. In several toxicologically relevant tissues and blood leukocytes, the mRNA expression levels of PDEs 4A, 4B, 4C and 4D were significantly higher in rats than in humans. We confirmed that higher PDE4 expression levels were correlated with a higher enzyme activity level in rat leukocytes. The PDE4 enzyme activity level of leukocytes in monkeys fell between that of rats and humans. Functionally, the potencies of the PDE4 inhibitors rolipram, SB 207499 and SCH 351591 in inhibiting tumor necrosis factor production from leukocytes were in the following order: rat > monkey > human. In addition, rolipram was about 10-fold more potent in rats than in humans in inhibiting phenylephrine-induced contraction of renal artery. These inhibitors were confirmed to be highly selective for PDE4 in comparison to all other PDE families, and to inhibit rat and human PDE4s with identical potencies. Taken together, these results suggest that the higher susceptibility of rats to PDE4 inhibitor-induced toxicity might be due to their higher expression levels of PDE4, and that PDE4 inhibitors may be safer in humans than in monkeys and, particularly, rats.

Keywords: Type 4 phosphodiesterase (PDE4); cAMP; mRNA; Tumor necrosis factor (TNF); Renal artery

1. Introduction

Recently, there has been significant interest in selective inhibitors for type 4 cAMP-specific phosphodiesterase (PDE4) as a potential novel therapy for asthma, chronic obstructive pulmonary disease and allergic rhinitis [1]. Clinically, PDE4 inhibitors are effective against these diseases and are safe, with emesis being the only significant side effect [2–4]. However, in several animal species a number of other toxic effects have been caused by PDE4 inhibitors. For instance, in rats, rolipram caused arteriopathy, myocardial degeneration and necrosis, endocardial fibrosis, epicarditis, mucosa necrosis, and hyperplasia and hyperkeratosis of stomach [5]. In particular, arteriopathy has been considered to be a 'class effect' of PDE4 inhibitors in rats, and was the dose-limiting toxic effect for RP 73401 [6]. CI-1018 also caused arteriopathy in rats [7]. SB 207499 caused adrenocortical hypertrophy, gastric erosion/epithelial hyperplasia and testis degeneration in addition to arteriopathy. Interestingly, these toxic effects were all rat-specific and were not observed in other species such as monkey (Stephen Newsholme, personal communication). Although SCH 351591 caused arteriopathy in both rats and monkeys [8], a much higher plasma exposure level of the inhibitor was required in the latter species (unpublished observations). Thus, rats appear to be relatively more susceptible to PDE4 inhibitor-induced toxicity than other species including monkeys.

To elucidate the possible biochemical basis for the differential susceptibility of different species to PDE4 inhibitor-induced toxicity, we compared PDE4 expression

Abbreviations: PDE, phosphodiesterase; TNF, tumor necrosis factor; PCR, polymerase chain reaction; PE, phenylephrine

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levels and their functional relevance among rats, monkeys and humans. The PDE4 family comprises four genes (subtypes) in both rats and humans: PDEs 4A, 4B, 4C and 4D [9], whereas monkey PDE4 cDNA sequences are currently unknown. From each PDE4 gene, multiple 5′-alternatively spliced variants are formed. We first quantified mRNA levels of all the PDE4 subtypes in various toxicologically relevant tissues in rats and humans. Then, we assessed correlation between PDE4 mRNA expression and total enzyme activity in blood leukocytes in rats, monkeys and humans. Finally, to evaluate functional relevance of PDE4 expression, effects of several PDE4 inhibitors on tumor necrosis factor (TNF) production from leukocytes and on renal artery contraction were examined.

2. Materials and methods

2.1. PDE4 inhibitors

Rolipram was purchased from Biomol, while SB 207499 and SCH 351591 were synthesized in this institute.

2.2. Isolation of rat, monkey and human total blood leukocytes

Rat (Sprague–Dawley, male), monkey (cynomolgus, male) (both from Charles River) or human (healthy male and female) venous blood was drawn and erythrocytes were removed as described [10,11]. The plasma was centrifuged at 350 × *g* for 10 min to isolate total leukocytes.

2.3. Quantification of rat and human PDE 4A, 4B, 4C and 4D mRNAs

Rat and human PDE 4A, 4B, 4C and 4D mRNAs in various tissues were quantified using real-time quantitative polymerase chain reaction (PCR) in ABI Prizm™ 7700 Sequence Detector by the TaqMan method (PE Biosystems). The positions and sequences of PDE4 subtype-specific primers and β-actin primers used, the sizes of their corresponding PCR products, and TaqMan probes used are shown in Table 1. Each primer set was able to detect all known variants derived from the particular PDE4 subtype gene [9]. All the primers and probes were synthesized by PE Biosystems. The specificity of each primer set was confirmed by a regular PCR (only one PCR product with the expected molecular size was detected). A standard curve for each PDE4 subtype or β-actin was generated in each PCR for quantification. To prepare standards, the primers shown in Table 1 were used to generate gene-specific PCR fragments using the following thermal cycle parameters: denaturation, 94 °C for 30 s; annealing, 60 °C for 30 s; and extension, 72 °C for 45 s. Each fragment was gel-purified then quantified by UV spectrometry. The real-time PCR in ABI Prizm™ 7700 Sequence Detector was performed using the standard thermal cycle parameters for this instrument. Results of PDE4 subtypes were normalized to those of β-actin.

For rat adrenal gland, aorta and stomach, total RNAs were obtained from Analytical Biological Services. From rat and human renal arteries (see Section 2.6) and total leukocytes, total RNAs were prepared using TRIzol Reagent (Invitrogen). From the total RNAs, first-strand cDNAs were synthesized using Advantage RT-for-PCR Kit (Clontech). For other tissues, cDNAs were obtained from Clontech.

2.4. Assay for total PDE4 activity in rat, monkey and human total blood leukocytes

Total leukocytes were homogenized in 45 mM Tris–HCl (pH 7.8) containing 5% glycerol, 0.1 mM sodium vanadate and Complete Protease Inhibitor Cocktail Tablet (Boehringer Mannheim) by sonication for 20 s in Branson's Sonifier 250 with output set at 4. Aliquots of the homogenates (1 μg protein/assay) were assayed for total cAMP-PDE activity as well as SB 207499-insensitive (total non-PDE4) PDE activity in the absence or presence of 10 μM SB 207499. Total PDE4 activity was determined as SB 207499-sensitive activity by subtracting the SB 207499-insensitive activity from the total cAMP-PDE activity. The PDE assays were performed using Amersham's cAMP-PDE SPA Assay kit at a final concentration of cAMP (Sigma) of 1 μM. Protein concentrations were determined by Bradford's method using BSA as standard [12].

2.5. Assay for TNF production in rat, monkey and human total blood leukocytes

Total leukocytes were suspended in RPMI 1640 supplemented with 1% penicillin–streptomycin (all from Invitrogen) and 2.5% FCS (Hyclone) at a density of 1×10^6 cells/ml. Aliquots (1 ml) of the cell suspension were transferred to 24-well plates, then TNF production was induced by 100 ng/ml [13] lipopolysaccharide (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2 for 18 h in the presence of various concentrations of each inhibitor. Inhibitors were dissolved in DMSO, and then diluted with PBS, with a final DMSO concentration of 0.1%. TNF amounts in the culture supernatants were determined using TNF ELISA kits (R&D Systems).

2.6. Assay for contraction of rat and human renal arteries

These experiments were performed in accordance with the NIH Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act in an AAALAC-accredited

Table 1
Positions and sequences of PCR primers used, and sizes of resultant PCR products in the quantification of PDE4 subtype mRNA levels in various tissues by real-time quantitative PCR

Gene	Primer sequences	Position	Access no.	Size (bp)	Probe sequence
rPDE4A	CATGGGCAGATCTCGTCCATGTCA GGCAGGGACGGAT	1279–1428	M26715	150	CCGGATGCCCAAGACATCCTGGA
rPDE4B	GGATTCTGAAGGACCGGAAAATAT GTGTCGATCAGAGACTTG	2528–2677	U95748	150	CAACTATTTCAGCAGCACAAAGACA CTTTGTGTG
rPDE4C	ATACATGCTGCCGACGTGGTGGTG CCAGCTCCGAGTTGGTGT	314–468	M25347	155	CGGACCTAGAAGTCTTGGCCGCCA
rPDE4D	ACTTTGGAGGACAATCGTGAGTT CAGTGTCTGACTCGCCATC	1577–1726	U09455	150	CCTGCACCTGATGACCAAGAGGACG
rβ-actin	CCCTGTATGCCTCTGGTCGTACAGTGT GGGTGACCCCGT	423–489	NM031144	67	CACTGGCATTGTGATGGACTCCGG
hPDE4A	TGGTGACATACATGCTGACGCGAGAA TCTCCAGGTCCGT	548–698	U97584	151	CTGACGTGCTGCAGTCCACCCAC
hPDE4B	TTAATGCATAGTTCAAGCCTAGGGT CTATTGTGAGAATATCC	1079–1228	L20966	150	TGAAGATCACCTGGCCAAGGAGCTG
hPDE4C	GGATGGTCATTGACATGGTGCTCTCCC TGCTGGAAGAACTCG	1581–1830	Z46632	250	TGGAGACCAAGAAGGTGACAAGCCTCG
hPDE4D	CCTGGAGGAGCTGGACTGGACAC TTGATTTCCAGACCGA	502–651	AF012074	150	TGGACCAGCTAGAGACCCTACAGA CCAGG

For each gene the upper and lower sequences are forward and reverse primers, respectively. All sequences are from 5′ to 3′. The position of each gene is according to the particular GenBank accession no. shown. r: rat; h: human. For human β-actin, commercially available primers and probe for the TaqMan method were used (PE Biosystems).

program. Rats were euthanized with CO_2 gas. Two renal arteries were isolated from each rat. Human renal artery was collected from male and female organ donors (49–74 years old) from Tissue Transformation Technologies or National Disease Research Interchange. Isometric force was measured as described previously [14] with modifications. Briefly, rat or human renal arteries were cut into 0.5 cm rings, attached to triangle hooks, and connected to force transducers (model FT-03, Grass) in Radnoti organ baths filled with Krebs' buffer. The tissues were continuously oxygenated (95% O_2/5% CO_2) at 37 °C. Isometric force was recorded using a physiograph (Astro–Med model K2G, Grass). Passive tension was set at 1 g and tissues were equilibrated for 45 min. Tissues were washed two to three times, 5 min apart before commencing the experiment.

Each tissue was incubated with rolipram (Sigma) or vehicle for 3 h. Isometric force in response to phenylephrine (PE, 0.001–100 μM, Sigma) was then recorded. Tissue dry weight was measured at the end of the experiment. The results were calibrated by tissue dry weight and expressed as g force/g tissue.

2.7. Cloning and expression of recombinant full-length PDEs

Human PDE4B2 [15], PDE7A1 [16], PDE8A1 [17] and PDE9A1 [18] were described previously. Human PDE1A3 (accession number U40370), PDE2A3 (U67733), PDE3A1 (M91667), PDE5A1 (AJ004865), PDE10A2 (AB026816) and PDE11A3 (AJ278682) and rat PDE4B2 (L27058) were cloned and expressed in SF9 insect cells by standard techniques as described previously [19].

2.8. Isolation of native human PDE4 from neutrophils

Blood neutrophils were isolated from healthy donors as described previously [13]. All the following procedures were performed at 4 °C. Neutrophils were homogenized with a glass–glass homogenizer in 20 mM Tris–HCl (pH 8.0) containing 2 mM EDTA, 1% Triton X-100, 0.5% deoxycholate and Complete Protease Inhibitor Cocktail Tablet (50 million cells per ml), and then the homogenate was stirred for 2 h. After centrifugation at 100,000 × *g* for 1 h, resultant supernatant was subjected to Mono Q column chromatography on a FPLC system (both from Amersham) using 20 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 and Complete Protease Inhibitor Cocktail Tablet. A NaCl concentration gradient was applied from 0 to 0.5 M over 30 min. PDE4 activity was eluted as a single peak at a NaCl concentration of 0.3 M.

2.9. Assays for recombinant PDEs

PDE4 assay was performed as described above. For PDE1, PDE2, PDE3, PDE7, PDE8, and PDE11 assays, the cAMP-PDE SPA Assay kit was used, with the following additions: 10 μM cAMP, 200 μM $CaCl_2$, 0.1 μM calmodulin (Calbiochem), 20 mM imidazole and 0.2 mg/ml BSA for PDE1 (at pH 8), 40 μM cAMP and 10 μM cGMP (Sigma) for PDE2, 0.4 μM cAMP for PDE3, 0.1 μM cAMP for PDE7, 0.05 μM cAMP for PDE8, and 0.0125 μM cAMP for PDE11. For PDE5, PDE9, and PDE10 assays, Amersham's cGMP-PDE SPA Assay kit was used with the following additions: 0.5 μM cGMP (PDE5), 0.15 μM cGMP and 100 μM $MnCl_2$ (PDE9), and 0.7 μM cGMP (PDE10).

3. Results

3.1. Comparison of mRNA expression of all PDE4 subtypes in toxicologically relevant tissues between rats and humans

To compare PDE4 expression among the various species, we first examined PDE4 mRNA expression levels in all toxicologically relevant tissues using quantitative real-time PCR by the TaqMan method (PE Biosystems). Since monkey PDE4 cDNA sequences are currently unknown, the mRNA expression profiling was performed only with rat and human tissues. All primers were designed so that they were able to specifically detect all known variants derived from each PDE4 gene, and hence the data represent total mRNAs derived from each gene (Table 2). Because the mRNA levels were determined from standard curves generated in every assay, they can be compared between tissues and between species. There were significant differences among different tissues in PDE4 subtype expression profile in rats and humans, and significant differences in most tissues between the two species. Nevertheless, in every tissue the total expression level of PDE4 in rats was significantly higher than that in humans.

Table 2
Comparison of mRNA expression of all PDE4 subtypes in toxicologically relevant tissues between rats and humans

Tissue	mRNA level (PDE4/β-actin, $\times 10^4$)			
	A[a]	B[a]	C[a]	D[a]
Adrenal gland				
Rat[b]	13.1 ± 3.5	6.7 ± 2.0	11.1 ± 0.3	18.8 ± 3.4
Human[b]	4.9 ± 1.4	2.0 ± 0.7	2.2 ± 0.1	0.4 ± 0.0
Aorta				
Rat[b]	92.0 ± 10.3	10.0 ± 2.7	3.8 ± 0.2	52.8 ± 7.7
Human[b]	1.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.0
Renal artery				
Rat[b]	12.5 ± 0.7	434.5 ± 202.4	0.0 ± 0.0	4.6 ± 0.8
Human[b]	3.2 ± 0.6	1.7 ± 1.4	5.4 ± 4.7	0.3 ± 0.1
Brain				
Rat[b]	217.0 ± 34.5	40.9 ± 9.1	5.2 ± 0.2	19.4 ± 6.4
Human[b]	29.4 ± 6.9	8.1 ± 0.4	19.4 ± 5.5	1.9 ± 0.1
Liver				
Rat[b]	5.2 ± 1.1	18.3 ± 5.1	9.4 ± 0.8	5.6 ± 1.4
Human[b]	4.0 ± 0.1	0.6 ± 0.1	1.3 ± 0.5	0.4 ± 0.0
Stomach				
Rat[b]	47.5 ± 7.6	4.0 ± 1.0	8.6 ± 0.4	15.6 ± 3.3
Human[b]	15.1 ± 1.6	2.2 ± 0.1	7.4 ± 0.6	4.3 ± 0.3
Testis				
Rat[b]	540.0 ± 56.6	11.1 ± 3.6	97.8 ± 3.7	9.4 ± 3.1
Human[b]	100.0 ± 2.9	3.6 ± 0.2	39.2 ± 11.6	1.5 ± 0.2

All determinations were performed in duplicate, and each set of experiments was performed at least three times. The data shown are from all experiments, representing mean ± S.E.M.
[a] PDE4s.
[b] Species.

Table 3
Comparison of PDE4 mRNA expression (A) and enzyme activity levels (B) and inhibition of TNF production by PDE4 inhibitors (C) in blood leukocytes among rats, monkeys and humans

	Species		
	Rat	Human	Monkey
(A) mRNA Level (PDE4/β-actin, $\times 10^4$)			
A[a]	5.7 ± 0.3	7.1 ± 0.6	–
B[a]	794.2 ± 42.8	31.2 ± 1.8	–
C[a]	2.1 ± 0.1	0.0 ± 0.0	–
D[a]	469.1 ± 11.0	2.6 ± 0.3	–
(B) Enzyme activity (pmole/min. mg protein)			
	8.70 ± 1.25	2.67 ± 0.14	6.15 ± 0.35
(C) TNF production inhibition (IC_{50}, nM)			
Rolipram	39 ± 24	200 ± 80	63 ± 16
SB 207499	48 ± 6	248 ± 78	118 ± 86
SCH 351591	42 ± 13	204 ± 63	145 ± 11

(A) and (B) All assays were performed in duplicate, and each set of experiments was performed at least three times in multiple donors. The data shown are from all experiments, representing mean ± S.E.M. (A) Monkey PDE4 mRNA levels could not be measured because monkey PDE4 cDNA sequences are currently unknown. (C) All experiments were performed in triplicate, and analysis of inhibitor dose–response data (Fig. 1) and calculation of IC_{50} values were performed using GraphPad Prism (GraphPad Software, San Diego, CA). The calculation of IC_{50} values was based on the percent inhibition data.
[a] PDE4s.

3.2. Comparison of PDE4 mRNA expression and enzyme activity levels in blood leukocytes among rats, monkeys and humans

Subsequently, to assess the correlation between mRNA expression and enzyme activity levels, PDE4 mRNA expression and total enzyme activity levels were examined in a single tissue, total blood leukocytes, in rats, monkeys and humans. Blood leukocytes were selected because they were readily available from all the three species. As shown in Table 3, the PDE4 mRNA expression levels in total leukocytes were much higher in rats than in humans as in all other tissues tested. Importantly, this higher mRNA expression level in rats was correlated with a significantly higher (more than three-fold higher than in human) PDE4 enzyme activity. With regard to total leukocyte PDE4 activity in monkeys, the level was between that in rats and humans. Monkey PDE4 mRNA levels could not be measured because monkey PDE4 cDNA sequences are currently unknown.

3.3. Comparison of functional sensitivity to PDE4 inhibitors among rats, monkeys and humans

To evaluate the functional relevance of the differential PDE4 expression among species, we first compared the effects of three PDE4 inhibitors, rolipram (an inhibitor with a complex mechanism of inhibition [20–23]), SB 207499 (a competitive inhibitor [24]) and SCH 351591 (a noncompetitive inhibitor [25]), on lipopolysaccharide-induced TNF production in total blood leukocytes from rats, monkeys, and humans. These inhibitors were selected due to their high

Table 4
PDE selectivity profiles of PDE4 inhibitors used

Inhibitor	IC_{50} (μM)									
	1[a]	2[a]	3[a]	4[a]	5[a]	7[a]	8[a]	9[a]	10[a]	11[a]
Rolipram	>100	>100	>100	0.070 ± 0.004	>100	>100	>100	>100	100	>100
SB 207499	>100	>100	>100	0.029 ± 0.002	>100	>100	31.3 ± 0.5	>100	>100	29.0 ± 0.7
SCH 351591	>100	>100	>100	0.051 ± 0.009	>100	>100	>100	>100	>100	>100

PDE4 was isolated from human neutrophils, whereas all other PDEs were recombinant enzymes. All assays were performed in duplicate, and each set of experiments was performed at least twice. Analysis of inhibitor dose–response data and calculation of IC_{50} values were performed using GraphPad Prism (GraphPad Software). The data shown are from all experiments, representing mean ± S.D. PDE6 was not tested because it is present only in photoreceptors.
[a] PDEs.

potencies for PDE4 (Table 4). In this assay, leukocytes from the various species were stimulated in the same culture medium to rule out the possibility of an effect by inhibitor-serum protein binding. Dose–response curves obtained in these TNF production inhibition experiments are shown in Fig. 1. IC_{50} values calculated from these experiments are shown in Table 3: all the three inhibitors exhibited the following order of potency: rat > monkey > human, with the potency in rats being about five-fold higher than that in humans.

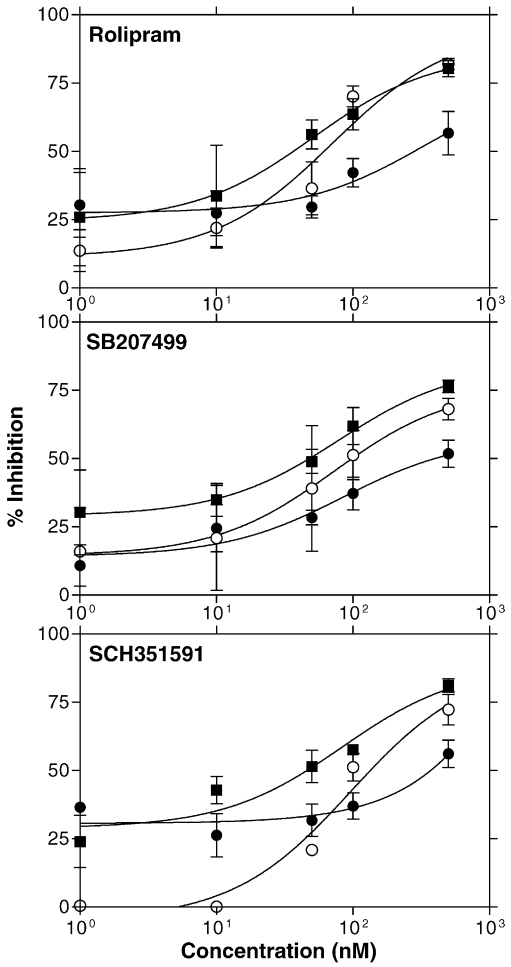

Fig. 1. Dose–response curves of inhibition of TNF production by PDE4 inhibitors in rat (■), monkey (○) and human (●) blood leukocytes. All experiments were performed in triplicate in multiple donors. The data analysis was performed using GraphPad Prism (GraphPad Software). The data shown are from all experiments, representing mean ± S.E.M.

In addition, the effects of rolipram on PE-induced contraction of rat and human renal arteries were compared. As shown in Fig. 2, rolipram inhibited PE-induced renal artery contraction in both rats and humans ($p < 0.05$). However, rolipram (0.3 μM) had a greater inhibitory effect in rat ($K_b = 4.0 \times 10^{-9}$ M) than in human ($K_b = 3.5 \times 10^{-8}$ M) arteries. Thus, rolipram was approximately 10-fold more potent in rats than in humans in inhibiting PE-induced contraction of renal artery.

3.4. PDE selectivity profile and comparative potencies of selected PDE4 inhibitors on rat and human PDE4s

Since high selectivity was critical to the evaluation of the correlation between PDE4 expression and functional sensitivity in this study, and since complete PDE selectivity

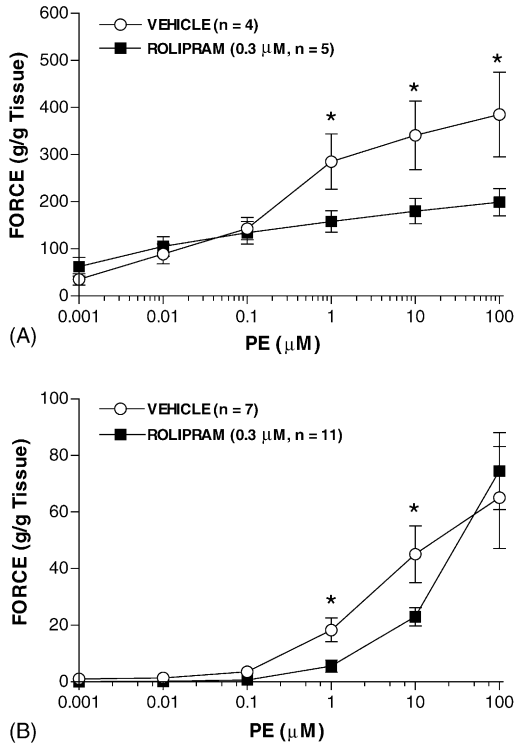

Fig. 2. Comparison of inhibition of PE-induced contraction of renal artery by rolipram between rats and humans. The effect of rolipram was examined in renal arteries obtained from rats (A) or humans (B). Values displayed represent the mean ± S.E.M. Statistical significance as indicated by (*) was achieved with $p < 0.05$ using an unpaired t-test.

Table 5
Comparison of potencies of PDE4 inhibitors used between rat and human PDE4B2s

Inhibitor	IC_{50} (nM)	
	Human	Rat
Rolipram	148 ± 27	143 ± 19
SB 207499	113 ± 3	117 ± 3
SCH 351591	105 ± 15	109 ± 9

Human and rat recombinant PDE4B2s were compared on the same 96-well plates. All assays were performed in duplicate, and each set of experiments was performed twice. Analysis of inhibitor dose–response data and calculation of IC_{50} values were performed using GraphPad Prism (GraphPad Software). The data shown representing mean ± S.D. Note that the different IC_{50} values for human PDE4s between Table 4 and in this table were presumably due to the different enzymes (native vs. recombinant).

data on these three inhibitors have not been available in the literature, we examined the PDE selectivity profile of these inhibitors against all PDE families except for PDE6 (i.e. PDEs 1–5 and 7–11 [26]). PDE6 was not tested since it is present only in photoreceptors. As shown in Table 4, all the three inhibitors were highly selective against any other PDEs.

Furthermore, to rule out the possibility that the relatively higher functional sensitivity to PDE4 inhibitors in rats was due to relatively higher potencies of the inhibitors on rat PDE4, we compared the potencies of these three inhibitors between recombinant human and rat PDE4B2s. The particular variant PDE4B2 was chosen because it is the predominant molecular species of PDE in neutrophils and monocytes [15], which are the principal producers of TNF in lipopolysaccharide-stimulated blood leukocytes [13]. Recombinant human and rat PDE4B2s had similar K_m values (5.9 ± 2.2 and 9.5 ± 2.9 μM, respectively). Their V_{max} values were several-fold different (15.5 ± 3.5 and 71.7 ± 7.3 nmol/min/mg, respectively), presumably due to a relatively higher expression level of the rat enzyme. As shown in Table 5, all the three inhibitors exhibited identical potencies between human and rat PDE4B2s. This result suggests that the relatively higher functional sensitivity of rats to PDE4 inhibitors might be due to the higher expression levels of PDE4, but not to high inhibitor potencies on rat PDE4.

4. Discussion

In the literature it has been suggested, based on sporadic observations using Northern blot analysis, RNase protection assay and/or qualitative PCR, that PDE4 subtypes are expressed differentially among tissues [9]. In this study, the use of quantitative real-time PCR to determine the absolute levels of mRNAs for all four PDE4 subtypes (covering all known splice variants derived from each gene) in various tissues represents the first quantitative and comprehensive investigation on tissue distribution of PDE4 in both rats and humans and the first quantitative comparison between the two species. Our results confirm the notion that all the four PDE4 subtypes are expressed differentially among various tissues (Table 2). The existence of some differences of PDE4 expression among species also has been suggested sporadically in the literature. For instance, PDE4C mRNA was barely detectable in rat brain [27] but readily detected in high abundance in human brain [28]. Our study clearly demonstrates that most rat and human tissues exhibit significant differences in PDE4 subtype expression (Table 2).

Importantly, in this study it was found that the PDE4 mRNA expression levels in all rat tissues tested were much higher than those in humans. Using one of the tissues, total blood leukocytes, it was subsequently confirmed that the higher mRNA expression levels in rats were correlated with a higher total PDE4 enzyme activity, suggesting that the higher enzyme activity might be due, at least in part, to higher gene expression (Table 3). However, despite the much higher mRNA levels (for example, 25-fold higher for PDE4B and 180-fold higher for PDE4D), the PDE4 enzyme activity in rat leukocytes was only about three-fold higher than that in human leukocytes. Therefore, it is possible that the relatively small difference in enzyme activity levels between rats and humans is related to protein levels rather than mRNA levels. There are several possible reasons for this hypothesized smaller difference in protein levels, such as higher translation efficiency of PDE4 mRNAs and/or higher stability of PDE4 proteins in humans. Unfortunately, a complete validated set of PDE4 antibodies is not currently available for measurement of various PDE4 subtypes and variants by Western blot analysis with similar efficiencies between rats and humans.

Functionally, the sensitivity of the various species to PDE4 inhibitors was compared using lipopolysaccharide-induced TNF production in total blood leukocytes, since the TNF production assay is highly sensitive to PDE4 inhibition [25,29,30]. In this assay the three PDE4 inhibitors tested, each with a different mechanism of inhibition [20–25], showed an order of potency identical to the order of PDE4 enzyme activity level (i.e. rat > monkey > human) (Table 3). In addition, a PE-induced renal artery contraction assay was used to further assess the sensitivity to PDE4 inhibitors between rats and humans, in light of the fact that arteriopathy has been considered to be a serious "class effect" of PDE4 inhibitors in rats [5–7]. In this functional vascular smooth muscle assay, rolipram was approximately 10-fold more potent as an inhibitor in rats than in humans (Fig. 2). Using a different model, Lindgren and Andersson [31] showed that rolipram was about two-fold more effective in relaxing noradrenaline-induced renal artery in rats than in humans. All these inhibitors were potent for PDE4 and were highly selective against all other PDE families (Table 4), and had identical potencies between rat and human PDE4 (Table 5). Moreover, these inhibitors were effective against all the PDE4 subtypes

[25,32–34]. Therefore, they were appropriate tools for the evaluation of the correlation between PDE4 expression levels and functional sensitivity to PDE4 inhibition. Taken together, these results strongly indicate that the sensitivity of the various species to PDE4 inhibitors is directly correlated with their PDE4 enzyme activity levels.

In the determination of inhibitor potencies on recombinant PDE4s (Table 5), when the amount of human PDE4 was increased three-fold to mimic the difference in leukocyte PDE4 enzyme activity levels between rats and humans (Table 3), the IC_{50} values remained the same (data not shown). Thus, it is conceivable that, at a particular concentration of a PDE4 inhibitor, the total PDE4 enzyme activity inhibited in rat leukocytes is three-fold higher than that in human leukocytes. The larger changes in absolute amount of PDE4 enzyme activity in rat leukocytes and, conceivably, many other tissues may explain the relatively higher functional sensitivity of rats to PDE4 inhibitors observed in this study as well as the reported higher susceptibility of rats to PDE4 inhibitor-induced toxicity. Of course, there may be other important contributing factors to the higher susceptibility of rats to PDE4 inhibitor-induced toxicity. For instance, it has been demonstrated that there is a hemodynamic basis for PDE3 inhibitor-induced arterial lesions [35]. Nevertheless, our study suggests that the higher susceptibility of rats to PDE4 inhibitor-induced toxicity may be due, at least in part, to the fact that rats express higher levels of PDE4 activity than the other species.

Monkey total blood leukocytes exhibited a total PDE4 enzyme activity level lower than that of rat leukocytes (Table 3). This result is consistent with a lower susceptibility to PDE4 inhibitor-induced toxicity in monkeys (see Section 1). Interestingly, the total PDE4 enzyme activity level of human total blood leukocytes was even lower than that of monkey leukocytes (Table 3). This result suggests that PDE4 inhibitors may be even safer in humans than in monkeys and, particularly, in rats. In fact, in human clinical studies thus far the only significant side effect of PDE4 inhibitors has been emesis [2–4].

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