mRNA expression and antilipolytic role of phosphodiesterase 4 in rat adipocytes in vitro

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Abstract **Adipocyte lipolysis is dependent on an increase in the intracellular concentration of cAMP. Intracellular phosphodiesterases (PDEs) hydrolyze cAMP and limit stimulation of lipolysis. In the present study, the mRNA expression of PDE4 subtypes and the antilipolytic role of PDE4 in rat adipocytes were investigated. Fragments encoding PDE4A (233 bp), PDE4B (786 bp), PDE4C (539 bp), and PDE4D (262 bp) sequences were amplified by RT-PCR. The mRNA expression of PDE4 subtypes (A, B, C, D) determined by real-time quantitative PCR was 7, 18.7, 18.9, and 7.2% relative to PDE3B. Inhibition of PDE4 by rolipram increased basal lipolysis and reversed in part prostaglandin E_2 antilipolysis. The combination of PDE3 and PDE4 inhibitors synergistically reversed both prostaglandin E_2 and phenylisopropyl adenosine antilipolysis. Stimulation of adipocytes with prostaglandin E_2 increased total PDE activity and PDE3 activity measured by hydrolysis of ^{3}[H]cAMP by the particulate fraction of adipocytes. The present study confirmed that mRNAs for all four PDE4 subtypes were expressed in rat adipocytes, with PDE4B and PDE4C predominant. Moreover, PDE4 not only limits the rate of basal lipolysis but also contributes to prostaglandin E_2 antilipolysis in rat adipocytes.**—Wang, H., and N. K. Edens. **mRNA expression and antilipolytic role of phosphodiesterase 4 in rat adipocytes in vitro.** ***J. Lipid Res.*** **2007.** 48: **1099–1107.**

Supplementary key words lipolysis • phosphodiesterase • prostaglandin E_2

Lipolysis, hydrolysis of stored triglyceride in adipose tissue, is catalyzed by hormone-sensitive lipase (HSL) and adipose tissue triglyceride lipase, yielding free fatty acids and glycerol (1). Activation of HSL is dependent on the increase in intracellular concentration of cyclic adenosine monophosphate (cAMP) and resultant activation of cAMP-dependent protein kinase A (2). cAMP is an important intracellular second messenger that is synthesized by adenylate cyclase and degraded by cyclic nucleotide phosphodiesterase (PDE) (3). Many hormones and factors regulate lipolysis by affecting the generation and degradation of cAMP. Activation of β-adrenergic receptors by catecholamines (epinephrine and norepinephrine) activates stimulatory G-protein and increases adenylate cyclase activity and, consequently, intracellular cAMP concentration (2). Conversely, insulin, an antilipolytic hormone, phosphorylates and activates PDE3B, which decreases cAMP concentration (4, 5). Prostaglandin E_2 (PGE_2) and adenosine, endogenous antilipolytic factors released by adipocytes, inhibit lipolysis by activating the inhibitory G-protein (Gi), decreasing adenylate cyclase activity and thus lowering intracellular cAMP concentration (6).

PDE4, encoded by four genes (A, B, C, D), consists of over 16 splice variants. PDE4 proteins are located in cytoplasm and membranes (3). PDE4 activity has been detected by biochemical and pharmacological methods in rat adipocytes (7, 8) and 3T3-L1 adipocytes (9, 10). PDE4A5, PDE4B2, PDE4C2, PDE4D3, and PDE4D5 are known to be expressed in 3T3-F442A preadipocytes (11). However, the expression and physiological role of PDE4 in differentiated rat adipocytes remain unclear. We previously reported that ginseng extract, but not insulin, inhibited lipolysis in part by activating PDE4 in rat adipocytes. The signaling pathway leading to PDE4 activation by ginseng was different from that activated by insulin (12). The purpose of the present study was to determine the gene expression of PDE4 subtypes and investigate whether PDE4 plays an antilipolytic role in rat adipocytes.

METHODS

Chemicals

Collagenase (type I) was purchased from Worthington Biomedical (Lakewood, NJ). Recombinant human insulin (Humulin-R) was from Eli Lilly (Indianapolis, IN). Bovine serum albumin (BSA, type V) and adenosine deaminase were purchased from Roche Biochemical (Indianapolis, IN). PGE_2, adenosine,

Abbreviations: ISO, isoproterenol; KRH medium, Krebs-Ringer's-HEPES medium; PDE, phosphodiesterase; PGE_2, prostaglandin E_2; PIA, phenylisopropyl adenosine; QPCR, real-time quantitative PCR.

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isoproterenol (ISO), phenylisopropyl adenosine (PIA), cilostamide, rolipram, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Animal and adipocyte isolation

The protocol for animal use was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. Young male Sprague-Dawley rats weighing 150 ± 10 g were purchased from Harlan (Indianapolis, IN) and housed in a temperature- and humidity-controlled environment (12 h light/dark cycle) with free access to tap water and a nonpurified commercial diet (Teklad Rodent Diet 8640; Harlan, Bartonville, IL). The rats were acclimated to the laboratory for at least 6 days before use. Fed rats were euthanized by decapitation for the lipolysis and PDE activity assays; rats were euthanized by carbon dioxide inhalation for RNA extraction. The epididymal and retroperitoneal fat pads of rats were removed and pooled for adipocyte isolation. The adipocytes were isolated by a modification (13) of a method described by Rodbell (14). Isolated adipocytes were washed three times and suspended at a 20% concentration (v/v) in Krebs-Ringer's-HEPES (KRH) medium containing 2.5% BSA, 200 nM adenosine, and 5 mM glucose.

Adipocyte RNA extraction

Total RNA was extracted from isolated rat adipocytes using TRIzol reagents (Invitrogen; Carlsbad, CA) according to a modification of the manufacturer's protocol. In brief, 400–500 μl packed adipocytes frozen in liquid nitrogen were thawed in 1 ml of TRIzol reagent and homogenized with 20 strokes using a 2 ml ground glass homogenizer (Wheaton; Millville, NJ). Homogenates were centrifuged at 12,000 *g* for 10 min at 4°C, and then the top lipid layer was discarded. Chloroform (0.2 ml) was added to extract RNA, and the mixture was centrifuged at 12,000 *g* for 15 min at 4°C. After centrifugation, the colorless upper aqueous phase was transferred to a fresh sterile tube, and 0.5 ml of isopropanol was added to precipitate RNA at −20°C overnight. RNA precipitate was obtained after centrifugation at 12,000 *g* for 30 min at 4°C. Quantity and purity of adipocyte RNA were determined by measuring absorbance at 260 nm and 280 nm by UV-Vis spectroscopy. Integrity of adipocyte RNA was determined by electrophoresis on 1% agarose gel for reverse-transcription PCR and analyzed using an Agilent 2100 Bioanalyzer and Eukaryote Total RNA Nano kit (Agilent Technologies; Palo Alto, CA) for real-time quantitative PCR (QPCR).

RT-PCR

RNA (3 μg) was reverse transcribed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA was amplified by PCR using *Taq* DNA Polymerase (Promega; Madison, WI). The published primers (**Table 1**) for PDE3B (15) and four PDE4 subtypes (16) were used. All oligonucleotides for primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Two microliters of the first-strand cDNA were used as template in 50 μl of reaction buffer. PCR was performed using protocols shown in Table 1. The PCR products were resolved by electrophoresis on 1% agarose gels in the presence of ethidium bromide and visualized by ultraviolet fluorescence. PCR products were purified by using QIAquick Gel Extraction kit (Qiagene; Santa Clarita, CA) and sequenced at the DNA Sequencing Facility of The Ohio State University Neurobiotechnology Center using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kits and an ABI 373XL Stretch DNA sequencer (Applied Biosystems; Foster City, CA).

QPCR

The primers and probes (**Table 2**) were designed by applying the Primer Express software program (Applied Biosystems) to a region of high similarity among PDE3B and PDE4 (A, B, C, D) sequences (**Table 3**). The TaqMan probes carried a 5′-FAM reporter and a 3′-TAMRA quencher. All oligonucleotides were synthesized by Integrated DNA Technologies. The assay was carried out on DNase I-treated samples using the Platinum Quantitative RT-PCR ThermoScript One-Step kit (Invitrogen). The fluorescence intensity of the reporter label was normalized to Texas Red, the passive reference label added to the buffer. All reactions were performed in an ABI-PRISM 7900HT Sequence Detection System (Applied Biosystems). Serially diluted samples of Universal Rat Reference RNA (Stratagene; La Jolla, CA) were used to generate a calibration curve for each gene. Each QPCR sample was amplified in triplicate. Relative expression levels were deter-

TABLE 1. PCR primers and PCR protocols

Gene (Genbank Accession Number)	Sequence	PCR Protocols	Amplicon (bp)
PDE3B (NM_017229)	F, CAGGAAGGATTCTCAGTCAG R, GTATTCTGGGCGAGAAAGAT	95°C/40 s 52°C/30 s 72°C/60 s 35 cycles	530
PDE4A (NM_013101)	F, GCGGGACCTACTGAAGAAATTCC R, CAGGGTGGTCCACATCGTGG	95°C/40 s 59°C/30 s 72°C/60 s 35 cycles	233
PDE4B (NM_017031)	F, CAGCTCATGACCCAGATAAGTGG R, GTCTGCACAATGTACCATGTTGCG	95°C/40 s 58°C/30 s 72°C/60 s 35 cycles	786
PDE4C (XM_214325)	F, ACTGAGTCTGCGCAGGATGG R, CCTCCTCTTCCTCTGTCTCCTC	95°C/40 s 58°C/30 s 72°C/60 s 35 cycles	539
PDE4D (NM_017032)	F, CCTCTGACTGTTATCATGCACACC R, GATCCACATCATGTATTGCACTGGC	95°C/40 s 59°C/30 s 72°C/60 s 35 cycles	262

F, forward primer; R, reverse primer.

mined by the Relative Standard Curve Method (Applied Biosystems). The level of 28s rat RNA was assayed as an endogenous control for each sample on every reaction plate.

Lipolysis assay

Lipolysis was assayed by measuring glycerol release into the incubation medium using the Trinder kit (Sigma-Aldrich). Adipocytes, at a final concentration of 2% (v/v), were incubated in 2 ml KRH medium containing 2.5% BSA, 0.8 U/ml adenosine deaminase, and 10 mM glucose. Incubations were carried out at 37°C for 1 h with shaking (80 rpm). Treatments (90 pM insulin, 10 nM PGE_2, 10 nM PIA) and PDE inhibitors (5 μM cilostamide and 10 μM rolipram) were added to incubations as indicated in Results. These concentrations of inhibitor were chosen based on previous experiments (12).

Preparation of subcellular fractions from isolated adipocytes

Isolated adipocytes (2 ml of 20% cell suspension in KRH-BSA) were washed twice with 5 ml of homogenization buffer containing 50 mM Tris (pH 7.4), 250 mM sucrose, 1 mM EDTA, 0.1 mM EGTA, 10 μg/ml antipain, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A. Subsequently, adipocytes were centrifuged at 125 *g* for 2 min at room temperature, resuspended in 0.8 ml of homogenization buffer with the addition of 100 nM calyculin A, homogenized at room temperature with 10 strokes in a 2 ml homogenizer, and then placed on ice immediately. Subcellular fractions were prepared as described by Manganiello and Vaughan (17). In brief, defatted homogenates (H fraction) were centrifuged at 10,000 *g* for 7 min at 4°C to produce the P1 fraction, and then the 10,000 × *g* supernatant was centrifuged at 100,000 *g* for 20 min at 4°C to produce the P2 fraction. The 100,000 *g* supernatant fraction was designated as the S fraction. The pellet fractions were resuspended in 200 μl of homogenization buffer. Protein was measured with a BCA kit (Pierce; Rockford, IL) using BSA as standard. For multiple incubations, isolated adipocytes (2 ml of 20% cell suspension) were incubated with 100 nM ISO, 90 nM insulin, 1 μM PGE_2, and 100 nM PIA for 15 min, respectively. The whole particulate fraction (P1+P2) produced by direct centrifugation of defatted homogenates at 100,000 *g* for 20 min at 4°C was subjected to the following PDE activity assay.

PDE activity assay

PDE activity was assayed as described by Ahmad et al. (18). Briefly, samples were incubated at 30°C for 10 min in a total volume of 300 μl containing 50 mM HEPES, pH 7.4, 0.1 mM EGTA, 8.3 mM $MgCl_2$, and 0.1 μM [^{3}H]cAMP (25–35,000 cpm) as substrate. To ensure that substrate supply was not limiting, samples were diluted before the incubation so that no more than 20% of the substrate was hydrolyzed. *Crotalus atrox* venom was added and incubation continued at 30°C for 30 min to dephosphorylate 5′-AMP to adenosine. Adenosine was separated from substrate using DEAE-Sephadex A-25 columns and quantified by scintillation counting. PDE activity is expressed as the amount of cAMP converted to AMP per min per mg of protein. The activity of each PDE isoform was calculated by the difference in activity in the absence and the presence of a specific isoform inhibitor. PDE3 activity and PDE4 activity were distinguished by the addition to the assay of the PDE3-specific inhibitor cilostamide (0.5 μM) and the PDE4-specific inhibitor rolipram (5 μM).

Statistical analysis

Data were presented as the means ± SEM. n = number of independent adipocyte preparations. For lipolysis assay with treat-

TABLE 2. Alignment of PDE3B and PDE4 (A, B, C, D) primers and probes for QPCR

PDE4A	.....	.....GGAC	AACTACTCTG	ACCGTATCCA	GGTCCTCAGG	AACATGGTGC	ACTGTGCAGA	CCTCAGCAAT
			<<<<<	<<<<<<<<<<				**********
PDE4B	GTGACGAGCT	CCGGTGTTCT	CCTCCTGGAC	ACCGGATACA	GGTTCTTCGC	AACATGGTAC	ATTGTGCAGA	CCTGAGCAAC
	<<<<<<<<<<	<<<<<<<<<<	**********	**********	**********			
PDE4C	.....	.....	.....	.....	.....	.....	.....	.....
PDE4D	.....	.....	.....	.....	.....	....CCTCCAG	AATATGGTGC	ACTGTGCAGA	CCTGAGCAAC
						<<<<<<<<<	<<<<<<<<<<	<<<<<	
PDE3B	.....	.....AGC	AGTGAAAACG	ATCGACTCTT	AGTCTGCCAG	GTGTGCATCA	AATTAGCAGA	CATCAACGGC
		<<<	<<<<<<<<<<	<<<<<<<<<<	*********	**********	**********	*
PDE4A	CCCACCAAGC	CCCTGGAGCT	GTACCGACAG	TGGACCGAC.	.....	.....	.....	.....	.....
	**********	****	>>>>>>>>>>	>>>>>>>>>					
PDE4B	CCTACCAAGT	CCTTGGAGTT	GTATCGGC..	.....	.....	.....	.....	.....	.....
	>>>>	>>>>>>>>>	>>>>>>>>>						
PDE4C	.....	.....	.....	.GGACGGAGC	GCATCATGGC	TGAGTTCTTC	CAGCAGGGTG	ACCGGGAACG	TGAGTCGGGC
				<<<<<<<<<	<<<<<<<<< *	**********	**********	****	>>>>>>>>>
PDE4D	CCCACAAAGC	CACTCCAGCT	CTACCGCCAG	TGGACGGACC	GGATAATGGA	GGAGTTCTTC	CGTCAGGGGG	ACCGGGAGCG	TGAGCGTGGC
				**********	**********	**			>>>>>>>>>
PDE3B	CCAGCAAAAG	ATCGGGATCT	TCATTTGAGA	TGGACAGAAG	GCA	.....	.....	.....	.....
			>>>>>>>>>>	>>>>>>>>>>	>>>				

QPCR, real-time quantitative PCR; <, forward primer; *, probe; >, reverse primer.

TABLE 3. A region of high similarity among PDE3B and PDE4 (A, B, C, D) sequences

NM_017229	PDE3B (2736)	————CCAATGATGTAAATAG——TAACGGTATAGAATGGAGCAGTGAAA
XM_214325	PDE4C (1833)	GGAGACCAAGAAAGTGACTAGCCTTGGCGTCCTGCTCTTGGACAACTACT
NM_013101	PDE4A (1727)	GGAGACGAAGAAAGTGACCAGCTCCGGAGTTCTCTTGCTGGACAACTACT
NM_017031	PDE4B (2065)	AGAAACCAAAAAGGTGACGAGCTCCGGTGTTCTCCTCCTGGACAACTATA
NM_017032	PDE4D (1359)	TGAAACGAAGAAGGTGACGAGCTCTGGCGTCCTCCTCCTTGATAACTATT
	Consensus	GA ACCAAGAA GTGAC AGCTCTGGCGTTCTCCTCCTGGACAACTA T
NM_017229	PDE3B (2778)	ACGATCGACTCTTAGTCTGCCAGGTGTGCATCAAATTAGCAGACATCAAC
XM_214325	PDE4C (1883)	CTGACCGCATCCAGGTCCTCCAGAGCCTGGTGCACTGCGCCGACCTCAGC
NM_013101	PDE4A (1777)	CTGACCGTATCCAGGTCCTCAGGAACATGGTGCACTGTGCAGACCTCAGC
NM_017031	PDE4B (2115)	CTGACCGGATACAGGTTCTTCGCAACATGGTACATTGTGCAGACCTGAGC
NM_017032	PDE4D (1409)	CTGACAGGATCCAGGTCCTCCAGAATATGGTGCACTGTGCAGACCTGAGC
	Consensus	CTGACCG ATCCAGGTCCTCCAGAACATGGTGCACTGTGCAGACCTCAGC
NM_017229	PDE3B (2828)	GGCCCAGCAAAAGATCGGGATCTTCATTTGAGATGGACAGAAGGCATTGT
XM_214325	PDE4C (1933)	AACCCTGCCAAGCCACTACCCCTCTACCGCCAGTGGACGGAGCGCATCAT
NM_013101	PDE4A (1827)	AATCCCACCAAGCCCCTGGAGCTGTACCGACAGTGGACCGACCGCATCAT
NM_017031	PDE4B (2165)	AACCCTACCAAGTCCTTGGAGTTGTATCGGCAATGGACTGATCGCATCAT
NM_017032	PDE4D (1459)	AACCCCACAAAGCCACTCCAGCTCTACCGCCAGTGGACGGACCGGATAAT
	Consensus	AACCC ACCAAGCC CTGGAGCT TACCG CAGTGGAC GA CGCATCAT

The boldface residues are identical among the five sequences at those positions. The parentheses indicate the position of residue.

ment and PDE inhibitor, data were analyzed by two-way ANOVA (SPSS 12.0; SPSS, Inc., Chicago, IL) to determine whether treatment and inhibitor had a significant effect, respectively. When a significant effect was found, a priori Bonferroni *t*-tests were used to compare eight pairs of interest. The α level was set at $0.05/8 = 0.006$. Student's *t*-test was used to evaluate the statistical significance for PDE activity assay, effects of PDE inhibitor on basal lipolysis, and the synergistic effect of combined PDE inhibitors; the α level was set at 0.05.

RESULTS

Determination of PDE4 subtype mRNA expression

The expression of PDE4A, PDE4B, PDE4C, and PDE4D was determined by RT-PCR using published generic PDE4 primers designed to amplify regions common to all known splice variants in a specific subtype of PDE4 (16). PCR products encoding PDE3B (530 bp), PDE4A (233 bp), PDE4B (786 bp), PDE4C (539 bp), and PDE4D (262 bp) sequences were amplified (**Fig. 1**). Amplification reactions performed using RNA that had not been reverse transcribed yielded no PCR products. Results similar to those shown in Fig. 1 were obtained using RNA extracted from three independent preparations of isolated rat adipocytes. Sequencing of the PDE3B and PDE4 (A, B, C, D) RT-PCR products showed that they were identical to the published sequences using NCBI BLAST (data not shown).

Quantitation of PDE4 subtype mRNA expression

The primer and probe sets for PDE3B and PDE4 (A, B, C, D) genes were designed according to a region of homology across the five genes to ensure equivalent reverse-transcription efficiency, which is a necessary requirement for gene-to-gene comparison in the absence of quantitative standards such as purified RNA transcripts. BLAST analysis of the amplicon sequences did not yield any significant cross-alignments (data not shown). The primer sets generated PCR amplification efficiencies of 66% for PDE4C, and 92% to 100% for the other PDE isomer transcripts (**Table 4**). Our QPCR results showed that PDE3B and PDE4 (A, B, C, D) genes were detected in rat adipocyte RNA samples (n = 5) and in a positive control rat heart

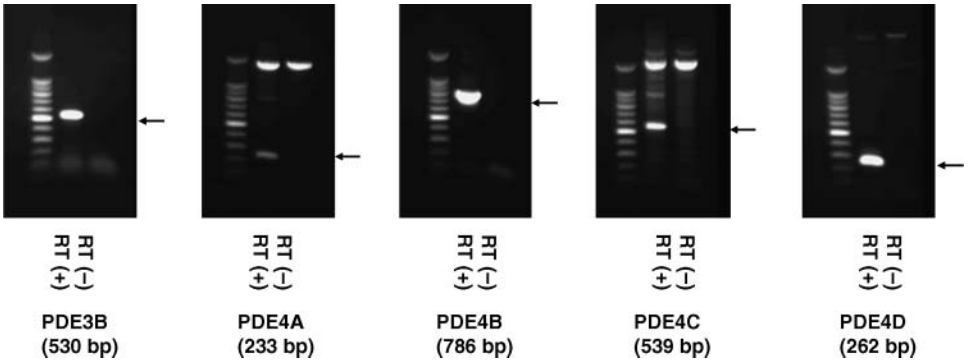

Fig. 1. Expression of PDE3B and PDE4 (A, B, C, D) mRNA in rat adipocytes. The predicted fragments for PDE3B (530 bp), PDE4A (233 bp), PDE4B (786 bp), PDE4C (539 bp), and PDE4D (262 bp) were amplified by PCR. Experiments were done in the presence (+) or absence (−) of reverse transcriptase (RT). Results shown are representative of three independent experiments. The first lane in each panel is DNA ladder.

TABLE 4. Properties of QPCR amplicons used for relative quantitation of PDE4 (A, B, C, D) gene expression

Gene (Genbank Accession Number)	Sequence	Amplicon (bp)	Standard Curve Equation (Correlation Coefficient)	Efficiency
PDE3B (NM_017229)	F, AGCAGTGAAAACGATCGACTCTT T, TGCCAGGTGTGCATCAAATTAGCAGAC R, TGCCTTCTGTCCATCTCAAATG	106	y = −3.47x + 32.31 (0.99)	94.18%
PDE4A (NM_013101)	F, GGACAACTACTCTGACCGTATCCA T, GTCGGTCCACTGTCGGTACA R, CTCAGCAATCCCACCAAGCCCCT	103	y = −3.29x + 28.81 (0.995)	101.18%
PDE4B (NM_017031)	F, GTGACGAGCTCCGGTGTTC T, TCCTGGACAACTATACTGACCGGATACAGGTT R, GCCGATACAACTCCAAGGACTT	118	y = −3.42x + 30.61 (0.992)	96.21%
PDE4C (XM_214325)	F, GGACGGAGCGCATCATG T, CTGAGTTCTTCCAGCAGGGTGACCG R, GATGTCCAAGCCCGACTCA	68	y = −4.52x + 33.10 (0.974)	66.42%
PDE4D (NM_017032)	F, CCTCCAGAATATGGTGCACTGT T, CAGTGGACGGACCGGATAATGGAGG R, TATCTCCATGCCACGCTCA	136	y = −3.55x + 30.21 (0.996)	91.46%

F, forward primer; R, reverse primer; T, TaqMan probe.

tissue RNA sample (n = 1). In rat adipocytes, the level of PDE3B, PDE4A, PDE4B, PDE4C, and PDE4D normalized to 28S rat RNA was 38.9 ± 6.6, 2.9 ± 0.9, 7.6 ± 2.4, 7.4 ± 1.8, and 2.5 ± 0.4, respectively. Relative to PDE3B, the levels of PDE4A, PDE4B, PDE4C, and PDE4D were 7%, 18.7%, 18.9%, and 7.2%, respectively (**Fig. 2A**). In rat heart tissue, the levels of PDE3B, PDE4A, PDE4B, PDE4C, and PDE4D normalized to 28S rat RNA were 1.4, 4.7, 1.8, 7.9, and 1.7, respectively (Fig. 2B).

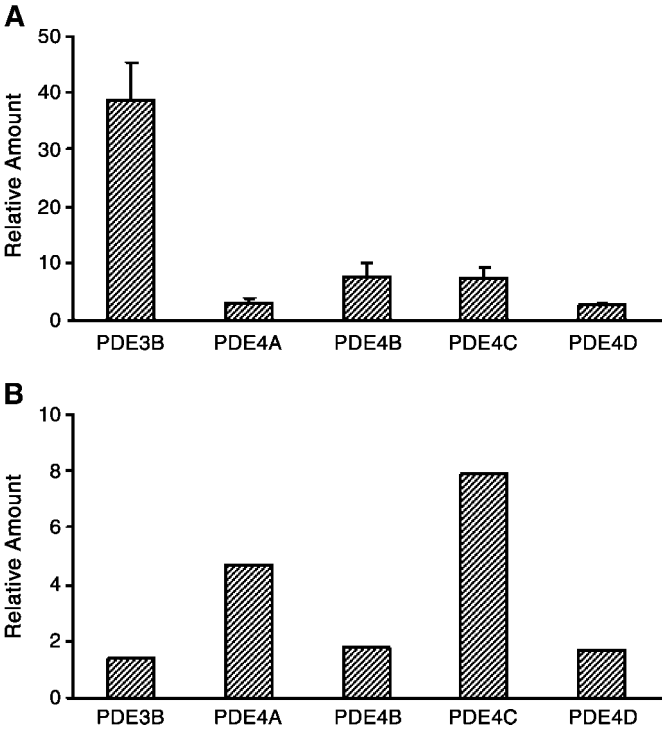

Fig. 2. Relative expression of PDE3B and PDE4 (A, B, C, D) mRNA normalized to 28s RNA in rat adipocytes (A) (n = 5) and rat heart tissue (B) (n = 1). Relative expression levels were determined by the Relative Standard Curve Method (Applied Biosystems). Data are mean ± SEM of 5 independent experiments.

Effects of PDE3 and PDE4 inhibitors on lipolysis

In the present study, the basal condition is defined as an adenosine-free system. Given that different adipocyte preparations may generate variations in lipolytic rates due to variations in endogenous adenosine release (19), adenosine deaminase (0.8 U/ml) was added to adipocyte incubations to remove variations in basal lipolysis and to keep adipocytes responsive to inhibition by insulin, PGE_2, and PIA. Eighteen independent experiments demonstrated that cilostamide and rolipram increased basal lipolysis by 28% and 33% in rat adipocytes, respectively ($P < 0.001$; **Fig. 3**). The combination of cilostamide and rolipram increased basal lipolysis by 51%, higher than cilostamide or rolipram alone ($P < 0.001$), such that the effect of the combined inhibitors was almost additive.

As previously reported (7), the PDE3-specific inhibitor cilostamide, but not the PDE4-specific inhibitor rolipram, completely reversed insulin antilipolysis (**Fig. 4A, B**).

PGE_2 inhibited lipolysis by 84% compared with basal (n = 5, $P < 0.006$; Fig. 4A). In the presence of cilostamide, PGE_2 inhibited lipolysis by 76.3% compared with basal

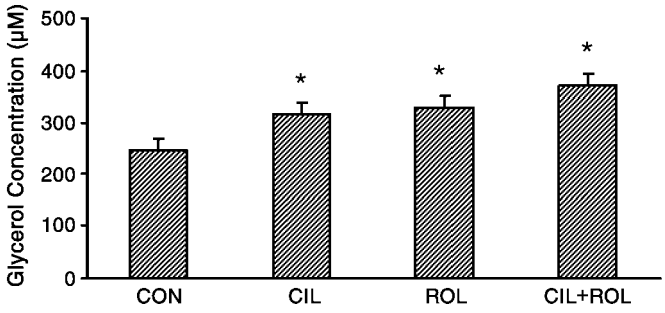

Fig. 3. Effects of the PDE3 and PDE4 inhibitors on basal lipolysis in rat adipocytes. Rat adipocytes were incubated with the PDE3 inhibitor cilostamide (CIL, 5 μM), the PDE4 inhibitor rolipram (ROL, 10 μM), and the combination of these two inhibitors. Control (CON) indicates that no PDE inhibitor was present. Data are presented as mean ± SEM of 18 independent experiments assayed in triplicate. * $P < 0.05$, compared with control.

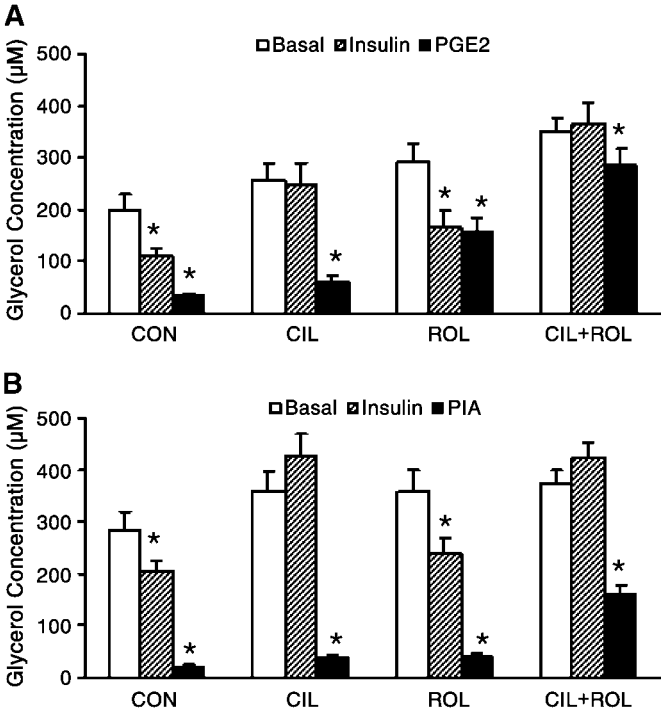

Fig. 4. Effects of the PDE3 and PDE4 inhibitors on insulin, prostaglandin E_2 (PGE_2), and phenylisopropyl adenosine (PIA) antilipolysis in rat adipocytes. A: Rat adipocytes were incubated without and with insulin (90 pM) and PGE_2 (10 nM) in the absence and presence of the PDE3 inhibitor cilostamide (CIL, 5 μM), the PDE4 inhibitor rolipram (ROL, 10 μM), and the combination of these two inhibitors (CIL+ ROL). B: Rat adipocytes were incubated without and with insulin (90 pM) and PIA (10 nM) in the absence and presence of the PDE3 inhibitor cilostamide (CIL, 5 μM), the PDE4 inhibitor rolipram (ROL, 10 μM), and the combination of these two inhibitors (CIL+ ROL). Basal indicates that no insulin, PGE_2, or PIA was present. Control (CON) indicates that no PDE inhibitor was present. Data are mean ± SEM of five independent experiments assayed in triplicate. Statistical significance compared with basal for each inhibitor is denoted with *, $P < 0.006$.

with cilostamide ($P < 0.006$). In the presence of rolipram, PGE_2 inhibited lipolysis by 46.6% compared with basal with rolipram ($P < 0.006$). In the presence of both rolipram and cilostamide, PGE_2 inhibited lipolysis by only 17.5% compared with basal with both inhibitors. The effect of combining cilostamide and rolipram was synergistic, defined as greater than the sum of the effects of each inhibitor alone ($P < 0.02$).

PIA inhibited lipolysis by 92.2% compared with basal (n = 5, $P < 0.006$; Fig. 4B). Neither cilostamide nor rolipram alone affected PIA antilipolysis significantly. However, in the presence of both cilostamide and rolipram, PIA inhibited lipolysis by only 56.8% compared with basal with both inhibitors; the effect of the combined inhibitors was synergistic ($P < 0.001$).

Subcellular distribution of PDE activity

The activity of PDE3 and PDE4 in whole homogenates (H) accounted for 65.7% and 19.2% of total PDE activity, respectively, leaving about 15% unaccounted for (**Fig. 5**; n = 5). PDE3 and PDE4 represented 79.3% and 13.6% of total PDE activity, respectively, in the 10,000 *g* pellet fraction (P1). PDE4 contributed only 10.1% of the total activity in the 100,000 *g* pellet fraction (P2), whereas PDE3 accounted for 90%. In contrast, PDE3 and PDE4 accounted for 41.1% and 31.9%, respectively, of total PDE activity in the 100,000 *g* supernatant fraction (S).

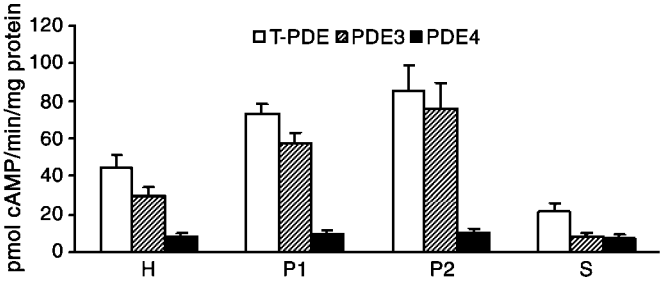

Fig. 5. Subcellular distribution of total PDE, PDE3B, and PDE4 activity in rat adipocytes assayed with 0.1 μM cAMP. Data are mean ± SEM of five independent experiments assayed in triplicate. H, homogenate; P1, 10,000 *g* pellet fraction; P2, 100,000 *g* pellet fraction; S, 100,000 *g* supernatant fraction.

Effects of ISO, insulin, PGE_2, and PIA on particulate PDE activity

ISO, insulin, and PGE_2 increased total PDE activity by 85.4% ($P < 0.01$), 26.2% ($P < 0.01$), and 25.7% ($P < 0.03$), respectively (n = 5; **Table 5**). ISO, insulin, and PGE_2 increased PDE3 activity by 86.3% ($P < 0.01$), 24.4% ($P < 0.03$), and 21.4% ($P < 0.01$), respectively. Also, ISO, insulin, and PGE_2 stimulated PDE4 activity by 108.4%, 23.6%, and 26.7%, respectively, although effects were not statistically significant because of variance among individual experiments. PIA decreased total PDE activity by 17.1% (data not shown; n = 5, $P < 0.01$). The effects of PIA on PDE3 and PDE4 were not determined.

DISCUSSION

The present study characterized PDE4 gene expression and its antilipolytic role in rat adipocytes. To our knowl-

TABLE 5. Effects of isoproterenol, insulin, and PGE_2 on total PDE, PDE3, and PDE4 activity in the particulate (P1+P2) fraction of rat adipocytes

	Total PDE	PDE3	PDE4
	pmol cAMP/min/mg protein		
Basal	59.8 ± 7.6	48.8 ± 5.6	12.1 ± 2.2
ISO (100 nM)	110.8 ± 13*	90.9 ± 11.8*	25.3 ± 4.5
Insulin (90 nM)	75.4 ± 7.8*	60.6 ± 5.6*	15.0 ± 2.7
PGE_2 (1 μM)	75.1 ± 10*	59.2 ± 6.5*	15.4 ± 3.0

ISO, isoproterenol; PDE, phosphodiesterase; PGE_2 prostaglandin E_2; P1, 10,000 *g* pellet fraction; P2, 100,000 *g* pellet fraction; basal indicates that no treatment was present; data are mean ± SEM of five independent experiments assayed in triplicate; * $P < 0.05$, compared with basal control.

edge, this is the first report to determine and quantitate the mRNA expression of PDE4 subtypes in rat adipocytes. The results showed that mRNA for all four PDE4 subtypes (A, B, C, D) were expressed in rat adipocytes, with PDE4B and PDE4C predominant. Subcellular fractionation of PDE4 activity showed that PDE4 activity accounted for 19% of total PDE activity in homogenates, and existed in both particulate and soluble fractions of rat adipocytes. Of note is that PDE4 not only played a role in inhibition of basal lipolysis, but also mediated in part the antilipolytic effect of PGE_2. Moreover, PGE_2 stimulated total PDE and PDE3 activity in rat adipocytes.

PDE4 subtype mRNA expression

Both RT-PCR and QPCR confirmed that all four PDE4 subtype genes were expressed in rat adipocytes. The mRNA level of PDE3B was about 14-fold higher than that of PDE4A and PDE4D, and about 5-fold higher than that of PDE4B and PDE4C in rat adipocytes. The expression of the PDE4 gene was around 50% that of the PDE3B gene in rat adipocytes. The significance of differential expression of PDE4 subtypes in a variety of organs, tissues, and cells remains unclear. A recent study has reported that the functions of PDE4B and PDE4D were complementary, rather than redundant, in neutrophils (20). It is possible that the four PDE4 subtypes have complementary roles in rat adipocytes as well.

In the present study, rat heart tissue mRNA was used as a positive control for QPCR. The observation that the mRNA level of PDE3B in heart tissue was much lower than in adipocytes is in accordance with the previous literature (21). Our data also showed that the mRNA expression of PDE4 subtypes (A, B, C, D) in heart tissue was comparable to that in adipocytes. Although PDE4C was not detected by RT-PCR in rat heart tissue in a previous study (16), it was detected by QPCR in the present study. This inconsistency may result from the use of different primers for amplification of PDE4C. In the present study, the efficiency for QPCR of PDE4C was lower than the other three PDE4 subtypes, suggesting possible difficulty in amplifying PDE4C.

Isoform and subcellular distribution of PDE activity

Eriksson et al. (22) reported that PDE4 activity accounted for 10% of total PDE activity in whole-rat adipocytes, as determined in the presence of the PDE4 inhibitor Ro 20-1724 (30 μM). The current study showed that PDE4 activity, as determined in the presence of rolipram (5 μM), accounted for 19% of total PDE activity in whole homogenates. The difference may be accounted for by differences in experimental technique.

PDE4 activity was found in both the particulate and soluble fractions of rat adipocytes. Although PDE4 activity was evenly distributed among three subcellular fractions, i.e., 10,000 *g* pellet, 100,000 *g* pellet, and 100,000 *g* supernatant, the proportion of PDE4 activity relative to total PDE activity was highest in the supernatant fraction. In contrast, PDE3 activity was predominant in 10,000 *g* pellet and 100,000 *g* pellet.

PDE activity and lipolysis

The role of PDE3 in lipolytic modulation by insulin and β-adrenergic agonists is well established. Consistent with previous reports (7, 9), insulin stimulated PDE3 activity in the particulate fraction of adipocytes, and insulin antilipolysis in intact adipocytes was reversed by the specific PDE3 inhibitor cilostamide. In contrast, insulin did not affect PDE4 activity in the particulate fraction of adipocytes, and the specific PDE4 inhibitor rolipram did not affect insulin antilipolysis in whole cells. As previously reported, ISO markedly stimulated PDE3 activity (5). This effect is thought to serve as a brake on ISO-stimulated lipolysis. ISO doubled PDE4 activity as well, but lack of statistical significance necessitates further investigation.

Basal lipolysis

Modulation of antilipolysis by PDE inhibitors must be interpreted in the context of their effects on basal lipolysis. As mentioned above, in the present report, in vitro basal lipolysis is defined as that occurring in the absence of endogenous adenosine. Previous studies have shown that inhibition of PDE4 increased the rate of basal lipolysis in 3T3-L1 adipocytes and rat adipocytes. Consistent with the previous literature (9, 23), the current study demonstrated that inhibition of PDE3 and PDE4 increased basal lipolysis by 28% and 33%, respectively. The combination of cilostamide and rolipram had an additive effect on basal lipolysis, while synergistic effects of PDE3 and PDE4 inhibitors on basal lipolysis have been observed in primary cultures of rat adipocytes (23). Additive or synergistic effects of PDE3 and PDE4 inhibitors suggest that both PDE3 and PDE4 attenuate basal lipolysis in rat adipocytes.

PIA-inhibited lipolysis

The nonhydrolyzable adenosine analog, PIA, significantly inhibited lipolysis and total particulate PDE activity. It has been reported that activation of the adenosine pathway stimulates PDE in 3T3-L1 adipocytes (24) but inhibits it in primary rat adipocytes (25). Adenosine may have discordant effects on PDE activity and lipolysis in primary rat adipocytes, as does isoproterenol.

PGE_2-inhibited lipolysis

PGE_2 antilipolysis was partially reversed by the PDE4 inhibitor rolipram but was not affected by the PDE3 inhibitor cilostamide. It is thought that PGE_2 exerts its antilipolytic effect by binding to EP3, activating Gi protein, and inhibiting adenylate cyclase (26), but reversal of antilipolysis by rolipram suggests that PDE4 may also be involved in PGE_2 action. The signaling pathway for PGE_2-mediated PDE4 activation in rat adipocytes deserves further investigation.

Although PGE_2 increased PDE3 activity in adipocyte membranes, the PDE3 inhibitor cilostamide did not re-

verse PGE_2 antilipolysis in the present study. This discrepancy may result from the different experimental systems used to assess PDE activity and lipolysis. The lipolysis assay was performed in intact adipocytes, whereas the PDE activity assay was performed using the P1+P2 particulate fraction of adipocytes with exogenous cAMP as substrate. cAMP is effectively compartmentalized in intact cells because endogenous PDE limits diffusion (27). A recent paper further proposed that PDE3B and PDE4 regulate different cAMP pools in adipocytes (28). It is possible that the cAMP pool hydrolyzed by PGE_2-stimulated PDE3 is not involved in regulation of lipolysis.

The combination of PDE3 and PDE4 inhibitors synergistically reversed both PGE_2 and PIA antilipolysis. It has been reported that the combination of PDE3 and PDE4 inhibitors has a synergistic effect on other cell-specific physiological functions, such as inhibition of vascular smooth muscle cell migration (29) and suppression of T-lymphocyte proliferation (30). The synergistic effect of combining PDE inhibitors may arise when adipocytes are treated with PIA or PGE_2, because adenylate cyclase is inhibited and the intracellular concentration of cAMP should be quite low. Under these circumstances, PDE activity may be in such excess of substrate availability that inhibition of one isoform with cilostamide or rolipram alone has no impact on the intracellular concentration of cAMP and the rate of lipolysis. In contrast, when both PDE isoforms are inhibited by the combination of cilostamide and rolipram, the intracellular concentration of cAMP may rise, reversing the antilipolytic effect of PIA or PGE_2.

The present study demonstrates that mRNAs for all four PDE4 subtypes are expressed in rat adipocytes. It was not feasible to demonstrate expression of PDE4 protein because available antibodies lacked sufficient specificity (data not shown). Although PDE4 does not mediate insulin antilipolysis, it restrains the rate of basal lipolysis even in the absence of adenosine. Moreover, PDE4 contributes to PGE_2 antilipolysis through an as yet unknown mechanism.

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