The role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis

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Abstract **This study assessed the effects of selective inhibitors of 3′,5′-cyclic nucleotide phosphodiesterases (PDEs) on adipocyte lipolysis. IC224, a selective inhibitor of type 1 phosphodiesterase (PDE1), suppressed lipolysis in murine 3T3-L1 adipocytes (69.6 ± 5.4% of vehicle control) but had no effect in human adipocytes. IC933, a selective inhibitor of PDE2, had no effect on lipolysis in either cultured murine 3T3-L1 adipocytes or human adipocytes. Inhibition of PDE3 with cilostamide moderately stimulated lipolysis in murine 3T3-L1 and rat adipocytes (397 ± 25% and 235 ± 26% of control, respectively) and markedly stimulated lipolysis in human adipocytes (932 ± 7.6% of control). Inhibition of PDE4 with rolipram moderately stimulated lipolysis in murine 3T3-L1 adipocytes (291 ± 13% of control) and weakly stimulated lipolysis in rat adipocytes (149 ± 7.0% of control) but had no effect on lipolysis in human adipocytes. Cultured adipocytes also responded differently to a combination of PDE3 and PDE4 inhibitors. Simultaneous exposure to cilostamide and rolipram had a synergistic effect on lipolysis in murine 3T3-L1 and rat adipocytes but not in human adipocytes. Hence, the relative importance of PDE3 and PDE4 in regulating lipolysis differed in cultured murine, rat, and human adipocytes.**—Snyder, P. B., J. M. Esselstyn, K. Loughney, S. L. Wolda, and V. A. Florio. **The role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis. *J. Lipid Res.* 2005.** 46: **494–503.**

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Adipose tissue functions as an energy storage organ in which excess calories are sequestered in the form of triglyceride (TG). TG contained in circulating chylomicrons and VLDL particles is hydrolyzed by extracellular lipoprotein lipase to yield glycerol and FFA. FFA is then taken up by adipocytes, converted to fatty acyl-CoA, and reesterified with glycerol-3-phosphate to form intracellular TG. The size of adipose TG stores is dynamically regulated by endocrine signals in response to energy intake and metabolic demands. Thus, anabolic hormones, such as insulin, stimulate adipocyte TG synthesis (lipogenesis), whereas catabolic hormones, such as epinephrine, glucagon, and corticotropin, stimulate hydrolysis of adipocyte TG to glycerol and FFA (lipolysis).

Cyclic AMP is an important second messenger in the signaling pathways that mobilize fat stores (1). Catecholamines (epinephrine and norepinephrine) stimulate adipocyte lipolysis by binding to β-adrenoceptors, which activate adenylyl cyclase (AC) via the stimulatory guanine nucleotide binding protein (G_s), leading to an increase in intracellular cAMP and activation of cAMP-dependent protein kinase (PKA). Initially, cAMP-mediated stimulation of lipolysis was thought to be attributable exclusively to PKA-dependent phosphorylation and activation of hormone-sensitive lipase (HSL), the primary neutral lipase in adipose tissue (2). However, adipocytes from HSL knockout mice retain considerable TG lipase activity, and lipolysis in these cells is partially responsive to the β-adrenoceptor agonist isoproterenol (ISO), suggesting that other lipases besides HSL play a role in lipolysis (3–5). Another layer of regulation of lipolysis is revealed by the observation that lipolytic stimuli cause translocation of HSL from the cytosol to the surface of lipid droplets, allowing HSL access to substrate (6). This redistribution of HSL is thought to be regulated by PKA-dependent phosphorylation of the lipid-associated protein perilipin A (7). In the absence of stimulation, hypophosphorylated perilipin A covers the surface of the TG droplet, protecting it from hydrolysis (8, 9). However, after phosphorylation by PKA, perilipin A stimulates lipolysis by facilitating the translocation of HSL, and possibly other uncharacterized lipases, to the surface of the lipid droplet (9–11). Recent studies have suggested that formation of a complex between PKA, perilipin A, and the scaffolding protein caveolin-1 is essential for PKA-dependent phosphorylation of perilipin A (12).

Abbreviations: AC, adenylyl cyclase; BMI, body mass index; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; G_s, stimulatory guanine nucleotide binding protein; HSL, hormone-sensitive lipase; IBMX, 3-isobutyl-1-methylxanthine; ISO, isoproterenol; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; PDEx, type x phosphodiesterase; PKA, cAMP-dependent protein kinase; RIIβ, cAMP-dependent protein kinase regulatory subunit IIβ; R_{max}, maximal response; TG, triglyceride.

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The importance of the cAMP/PKA pathway in the regulation of fat stores has been demonstrated in vivo by genetic inactivation of the PKA regulatory subunit IIβ (RIIβ). In RIIβ knockout mice, the catalytic subunit of PKA is constitutively active in tissues in which RIIβ is the predominant isoform of the regulatory subunit, including adipose tissue. This mutation, which mimics the condition of chronic cAMP increase, results in mice that are lean, despite normal food intake, and resistant to diet-induced obesity (13). In light of these findings, an agent that chronically and selectively increases cAMP in adipocytes may have use as an antiobesity therapeutic agent.

Intracellular levels of cAMP are determined by the rates of its synthesis by AC and its hydrolysis by 3′,5′-cyclic nucleotide phosphodiesterases (PDEs). The PDE enzymes are grouped into 11 families (PDE1 through PDE11) according to differences in their amino acid sequences, kinetic properties, modes of allosteric regulation, and sensitivity to chemical inhibitors (14). Some PDE families specifically hydrolyze cAMP (e.g., PDE4, PDE7, and PDE8), some specifically hydrolyze cGMP (e.g., PDE5, PDE6, and PDE9), and others can hydrolyze both substrates (e.g., PDE1, PDE2, PDE3, PDE10, and PDE11). Biochemical analysis of rat adipocyte extracts reveals the presence of at least four cAMP-hydrolyzing PDEs: PDE1 (Ca^{2+}/calmodulin-stimulated PDE), PDE2 (cGMP-stimulated PDE), PDE3 (cGMP-inhibited PDE), and PDE4 (cAMP-specific, rolipram-sensitive PDE) (15, 16). Most of the cAMP hydrolytic activity is accounted for by PDE3 and PDE4; however, these two isozymes are differentially localized within the rat adipocyte, with PDE3 predominantly recovered in the microsomal fraction and PDE4 predominantly recovered in the cytosolic fraction (15, 17). Two members of the PDE3 family have been identified (PDE3A and PDE3B); molecular analysis indicates that adipocytes express PDE3B (18). All four members of the PDE4 family (PDE4A, -B, -C, and -D) are expressed in the murine preadipocyte cell line 3T3-F442A (19); however, no information is currently available concerning the expression of PDE4 isoforms in differentiated adipocytes.

Just as lipolysis is stimulated by increasing cAMP levels, it is also inhibited by decreasing adipocyte cAMP. Binding of insulin to its receptor initiates a signaling cascade that leads to phosphorylation of PDE3B at Ser-279 and possibly at Ser-302, thereby increasing its activity 2- to 3-fold (20–22). The resultant increased rate of cAMP hydrolysis decreases intracellular cAMP levels and inhibits lipolysis. Selective inhibitors of PDE3 are able to overcome the antilipolytic action of insulin in vitro (22) and in vivo (23, 24).

Because the basal activity of PDE3B, in the absence of insulin, tonically inhibits lipolysis, PDE3 inhibitors also stimulate basal lipolysis. For example, the selective PDE3 inhibitor cilostamide induces lipolysis in cultured murine 3T3-L1 adipocytes (25). The selective PDE4 inhibitor Ro 20-1724 also stimulates lipolysis in this system, albeit less effectively than cilostamide. However, previous studies of the effects of selective PDE3 and PDE4 inhibitors on lipolysis in adipocytes have reported variable results in different adipocyte preparations. In primary rat epididymal adipocytes, the selective PDE3 inhibitors cilostamide and amrinone do not significantly affect basal lipolysis, although they potentiate β-adrenoceptor-stimulated lipolysis (15, 26). The PDE4 inhibitors rolipram (15) and Ro 20-1724 (27) weakly activate lipolysis in primary rat epididymal adipocytes; however, Ro 20-1724 does not stimulate lipolysis in primary human subcutaneous adipocytes (28). In human in vivo studies, intravenous administration of amrinone acutely activates lipolysis, as indicated by increased blood levels of glycerol and FFA (29, 30). Furthermore, in humans, delivery of amrinone to subcutaneous adipose tissue by microdialysis induces lipolysis locally (31). At present, little information is available about the role of other PDE isotypes in regulating adipocyte lipolysis.

In this report, we assess the ability of selective PDE inhibitors to induce lipolysis in three cultured adipocyte systems: murine 3T3-L1 adipocytes that were differentiated in vitro from the 3T3-L1 fibroblast cell line, human adipocytes that were differentiated in vitro from preadipocytes obtained from subcutaneous adipose tissue, and rat adipocytes that were differentiated in vitro from preadipocytes obtained from the epididymal fat pad. We found that selective inhibitors of PDE3 and PDE4, but not inhibitors of PDE1 and PDE2, significantly stimulated basal lipolysis in adipocytes. PDE3 inhibitors stimulated lipolysis in all three systems, whereas PDE4 inhibitors stimulated lipolysis only in murine and rat adipocytes. Furthermore, the combination of a PDE3 inhibitor and a PDE4 inhibitor had a synergistic effect on lipolysis in murine and rat adipocytes but not in human adipocytes. A partial account of this work has been reported previously in abstract form (32).

METHODS

Culture and differentiation of murine 3T3-L1 cells

Murine 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were grown on 24-well plates in a growth medium consisting of DMEM (Invitrogen-Gibco, Carlsbad, CA) supplemented with 25 mM glucose, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in an atmosphere containing 95% air and 5% CO_2. After reaching confluence, cells were differentiated (33) by incubating for 3 days in growth medium supplemented with 0.4 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine (IBMX), and 10 μg/ml bovine insulin (Sigma Chemical Co., St. Louis, MO), followed by 3 days in growth medium supplemented only with 10 μg/ml bovine insulin and an additional 3 days in growth medium alone. At this time, >90% of the 3T3-L1 cells had differentiated into adipocytes, as indicated by the accumulation of intracellular lipids. The cells were cultured for up to 10 days before use, with the medium changed every 2–3 days.

Culture of differentiated human adipocytes

In vitro differentiated human adipocytes (Zen Bio, Inc., Research Triangle Park, NC) were derived from stromal preadipocytes isolated from subcutaneous adipose tissue obtained from elective surgery of healthy females between 18 and 60 years old. Three preparations of adipocytes were used in the studies reported below. Prep 1 was derived from preadipocytes pooled from five donors with an average age of 38 years and an average

body mass index (BMI) of 24.7 kg/m^2. Prep 2 was derived from preadipocytes pooled from seven donors with an average age of 43 years and an average BMI of 23.1 kg/m^2. Prep 3 was derived from preadipocytes pooled from six donors with an average age of 36 years and an average BMI of 24.9 kg/m^2. Differentiated adipocytes were cultured on 96-well plates in maintenance medium [DMEM/Ham's F-10 medium (1:1), 3% FBS, 15 mM HEPES, pH 7.4, 33 μM biotin, 17 μM pantothenate, 100 nM human insulin, 1 μM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B] for up to 2 weeks, with the medium changed every 2–3 days. Greater than 75% of the cells exhibited an adipocyte phenotype (accumulation of intracellular lipids).

Culture and differentiation of rat adipocytes

Rat adipocytes were isolated, cultured, and differentiated according to published methods (34), modified as follows. Epididymal fat pads from male Sprague-Dawley rats were removed and placed in serum-free DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The tissue was minced and digested for 40 min in DMEM containing 3 mg/ml collagenase and 20 mg/ml BSA at 37°C with mild agitation. After trituration, the slurry was filtered through a 250 μm screen and the filtrate was centrifuged at 250 *g* for 8 min. The pellet containing the "vascular/stromal" cells was resuspended in DMEM supplemented with 8% FBS, filtered through a 25 μm screen, and centrifuged as before. The pellet was resuspended in a growth medium consisting of DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on 24-well plates at a density of 30,000 cells/cm^2. When the cells reached confluence, they were differentiated by incubating for 2 days in growth medium supplemented with 17 nM bovine insulin, 2 nM triiodothyronine (ICN Biomedicals, Inc., Aurora, OH), 200 μM IBMX, and 50 nM dexamethasone. Cell were subsequently maintained in growth medium supplemented with 17 nM insulin and 2 nM triiodothyronine, with the medium changed every 2–3 days. Cells were used 5–15 days after addition of differentiation medium, at which time >75% of the cells had differentiated into adipocytes, as indicated by the accumulation of intracellular lipids.

Lipolysis assays

PDE inhibitors were dissolved in 100% DMSO and working dilutions were made up in serum-free growth medium (as defined above for each cell type). The final concentration of DMSO was held constant in all dilutions, and growth medium containing DMSO alone was used as a vehicle control. For assays involving cultured human or rat adipocytes, the diluent also contained 4% BSA. The presence or absence of BSA in the diluent did not significantly affect the dose response of PDE inhibitors in 3T3-L1 adipocytes (data not shown). Confluent monolayers of adipocytes were washed with Dulbecco's PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and exposed to PDE inhibitors or vehicle for 6 h at 37°C in a 95% air/5% CO_2 atmosphere. Culture supernatants were collected and stored at −20°C until analyzed for glycerol, a breakdown product of TG.

For glycerol assays, 100 μl of culture supernatants and 100 μl of GPO-Trinder glycerol assay reagent (Sigma Chemical Co.) were mixed in a 96-well plate. After a 5 min incubation at room temperature, the absorbance at 540 nm was measured with a Spectramax 250 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The absorbance of a blank well (growth medium alone) was subtracted. Absolute glycerol concentrations were calculated from a standard curve. Means and SEM were calculated for each set of replicate wells, and these values are expressed as a percentage of the vehicle control. The values obtained were compared using a two-tailed Student's *t*-test for nonpaired samples; $P < 0.05$ was assumed to be statistically significant. EC_{50} and maximal response (R_{max}) values were derived from concentration-response curves fit to the experimental data by nonlinear regression using a three parameter logistic dose-response model according to the following equation:

$$R_I = 1 + \frac{R_{max}}{1 + \left[\frac{[I]}{EC_{50}}\right]^h} \quad \textit{(Eq. 1)}$$

where R_I is the response (expressed as a multiple of the vehicle control) at inhibitor concentration [I] and h is the Hill coefficient. The best fit values are reported ± the standard error of the fit. Table Curve 2D software (version 4.10; Systat Software, Inc., Richmond, CA) was used to obtain the fits.

RESULTS

Effect of selective PDE inhibitors on lipolysis in murine 3T3-L1 adipocytes

Murine 3T3-L1 cells are fibroblastic cells that differentiate into adipocytes in the presence of a cocktail of adipogenic factors (35). To investigate the role of PDEs in adipocyte lipolysis, we treated 3T3-L1 adipocytes with a panel of selective PDE inhibitors (**Table 1**) and monitored the lipolytic response by measuring the accumulation of glycerol in the culture medium. Murine 3T3-L1 adipocytes were exposed to selective PDE inhibitors at a final concentration of 1 or 10 μM (**Table 2**). The effects of PDE inhibitors were compared with ISO, which increases cAMP levels and stimulates lipolysis by activating G_s-coupled β-adrenoceptors.

Inhibition of PDE3 with cilostamide activated lipolysis in murine 3T3-L1 adipocytes (142 ± 15% of vehicle control at 1 μM, 289 ± 27% of control at 10 μM) (Table 2). Stimulation of lipolysis by the PDE4 inhibitor rolipram was similar (171 ± 7.4% of control at 1 μM, 204 ± 22% of control at 10 μM). In comparison, 1 μM ISO strongly activated lipolysis in 3T3-L1 adipocytes (1,162 ± 103% of control). This was a maximally effective concentration of ISO for this cell type (data not shown). Hence, the lipolytic response in 3T3-L1 cells induced by 10 μM cilostamide was 25% of the maximal ISO response, whereas the response

TABLE 1. Potency and selectivity of PDE inhibitors

Inhibitor	Target PDE	IC_{50}[a]	Selectivity[b]
		μM	
IC224	PDE1	0.08	127
IC933	PDE2	0.004	235
Cilostamide	PDE3	0.007	1,200
Milrinone	PDE3	0.23	68
Rolipram	PDE4	0.28	176
3-Isobutyl-1-methylxanthine	All but PDE8	1.6–59	Nonselective

PDE, 3′,5′-cyclic nucleotide phosphodiesterase; PDEx, type x phosphodiesterase.

[a] IC_{50} values were determined with recombinant human enzymes (our unpublished observations).

[b] Ratio of IC_{50} value for the next most sensitive PDE to IC_{50} value for the target PDE.

TABLE 2. Effect of selective PDE inhibitors on lipolysis in murine 3T3-L1 adipocytes

Inhibitor	Target PDE	Concentration	Lipolysis			
			No ISO		+1 nM ISO	
		μM	*% control*	*n*	*% control*	*n*
Vehicle	N.A.	N.A.	100 ± 3.3	18	165 ± 28[a]	9
IC224	PDE1	1	100 ± 8.1	6	160 ± 20	6
		10	69.6 ± 5.4[b]	6	117 ± 16	6
IC933	PDE2	1	97.5 ± 2.7	6	149 ± 25	6
		10	95.3 ± 2.9	6	167 ± 32	6
Cilostamide	PDE3	1	142 ± 15[b]	6	230 ± 54	6
		10	289 ± 27[c]	6	368 ± 22[d]	6
Rolipram	PDE4	1	171 ± 7.4[c]	6	227 ± 38	6
		10	204 ± 22[c]	6	281 ± 63	6
ISO	N.A.	1	1,162 ± 103[c]	18	N.A.	N.A.

Lipolysis induced by selective PDE inhibitors in the absence and presence of 1 nM isoproterenol (ISO). Values represent the glycerol concentration in the culture supernatant and are expressed as percentages of vehicle control (mean ± SEM for n replicate wells). The absolute glycerol concentration in the culture supernatant of vehicle-treated cells was 59.4 ± 9.0 μM. N.A., not applicable.

[a] $P < 0.01$ vs. vehicle control by two-tailed Student's *t*-test for independent samples.

[b] $P < 0.001$ vs. vehicle control by two-tailed Student's *t*-test for independent samples.

[c] $P < 0.0001$ vs. vehicle control by two-tailed Student's *t*-test for independent samples.

[d] $P < 0.001$ vs. 1 nM ISO alone by two-tailed Student's *t*-test for independent samples.

elicited by 10 μM rolipram was 18% of the maximal ISO response.

In contrast, neither PDE1 nor PDE2 inhibitors significantly stimulated lipolysis in murine 3T3-L1 adipocytes (Table 2). Unexpectedly, the PDE1 inhibitor IC224 suppressed basal lipolysis, although this effect was only apparent at 10 μM (69.6 ± 5.4% of control; $P < 0.001$).

In some instances, PDE inhibitors exert minimal effects on cell function in the absence of AC activation. Hence, we also measured the effects of PDE inhibitors on lipolysis in murine 3T3-L1 adipocytes after activation of AC with a submaximal concentration (1 nM) of ISO (Table 2). In the absence of PDE inhibitors, ISO at 1 nM stimulated lipolysis to 165 ± 28% of the vehicle control ($P < 0.01$). In the presence of 1 nM ISO, inhibition of PDE3 with cilostamide or PDE4 with rolipram stimulated lipolysis above the level seen in cells exposed to ISO alone; however, the increase was significant only for 10 μM cilostamide (368 ± 22% of control; $P < 0.001$ vs. 1 nM ISO alone). Neither PDE1 nor PDE2 inhibitors in combination with 1 nM ISO stimulated lipolysis above the level induced by 1 nM ISO alone. Inhibition of PDE1 tended to suppress ISO-stimulated lipolysis (117 ± 16% of control for 10 μM IC224 + 1 nM ISO vs. 165 ± 28% of control for 1 nM ISO alone); however, the difference was not statistically significant ($P = 0.22$).

Because inhibition of PDE3 or PDE4 stimulated lipolysis in 3T3-L1 adipocytes, further studies were carried out to determine the potency and efficacy of these agents. We examined the concentration-response relationship for cilostamide, rolipram, and the nonselective PDE inhibitor IBMX. Cilostamide induced lipolysis in murine 3T3-L1 adipocytes with an EC_{50} of 2.0 ± 1.4 μM and achieved an R_{max} of 397 ± 25% of vehicle control (**Fig. 1A**). Rolipram had an EC_{50} of 0.12 ± 0.07 μM and an R_{max} of 291 ± 13% of control. In contrast, the nonselective PDE inhibitor IBMX stimulated lipolysis in 3T3-L1 adipocytes with higher efficacy (752 ± 13% of control at 100 μM). The IBMX concentration-response curve did not reach a pla-

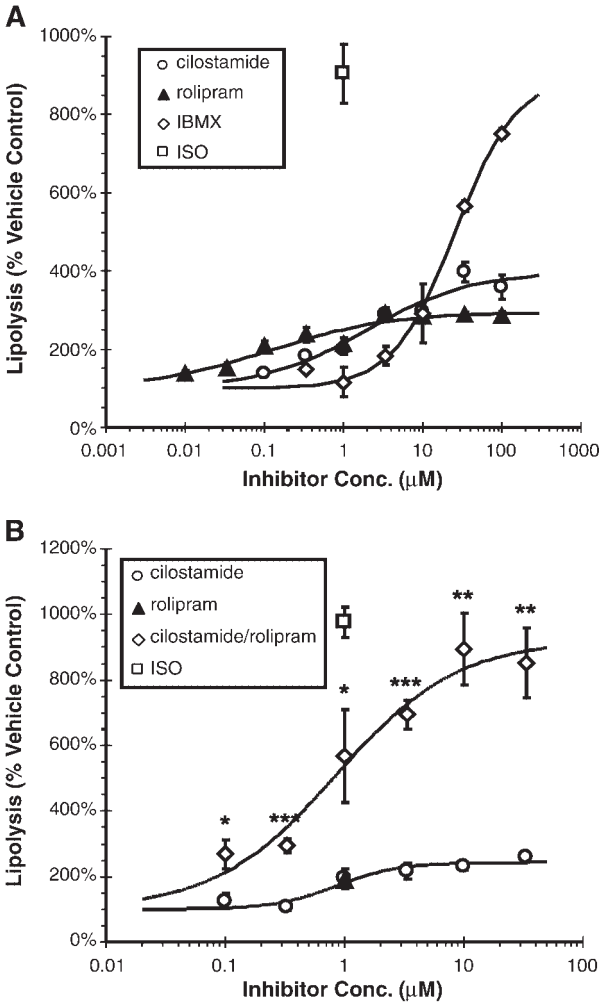

Fig. 1. Effects of 3′,5′-cyclic nucleotide phosphodiesterase (PDE) inhibitors on lipolysis in murine 3T3-L1 adipocytes. Lipolysis (glycerol release) is plotted as a function of inhibitor concentration. Data are normalized to the vehicle control and presented as means ± SEM for three replicates. Where error bars are not visible, the SEM was smaller than the symbol. Curves were fit to the experimental data using a three parameter logistic dose-response model. A: Concentration-response curves are shown for cilostamide [a type 3 phosphodiesterase (PDE3) inhibitor], rolipram (a PDE4 inhibitor), and 3-isobutyl-1-methylxanthine (IBMX; a nonselective PDE inhibitor). The glycerol concentration in the culture supernatant of the vehicle-treated wells was 46.7 ± 3.9 μM. B: Concentration-response curves are shown for cilostamide in the absence and presence of a fixed concentration of rolipram (1 μM). The effects of rolipram alone (1 μM) and isoproterenol (ISO; 1 μM) are shown for comparison. The glycerol concentration in the culture supernatant of the vehicle-treated wells was 109 ± 3.3 μM. Values for cilostamide in the presence of rolipram were compared with values for cilostamide alone by a one-tailed Student's *t*-test for independent samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

teau at the highest concentrations tested (100 μM). ISO (1 μM) also stimulated 3T3-L1 lipolysis to a high level (904 ± 76% of control). Hence, the R_{max} values of cilostamide and rolipram were only 44% and 32%, respectively, of ISO-induced lipolysis, whereas the R_{max} of IBMX was >83% of the maximal ISO-induced level.

IBMX, like other xanthines, is known to be an antagonist of adenosine A_1 receptors (36, 37). Because activation of A_1 receptors by secreted adenosine can inhibit lipolysis (36), the greater efficacy of IBMX could be attributable to a combination of PDE inhibition and A_1 receptor antagonism. To test this possibility, we treated 3T3-L1 adipocytes with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a potent and selective A_1 receptor antagonist that inhibits PDE1 through PDE4 with low affinity (37). At concentrations up to 1 μM, DPCPX did not stimulate lipolysis in 3T3-L1 cells [86 ± 4.6% of vehicle control at 1 μM (n = 3 replicates); not significantly different from control].

In light of this result, the higher effectiveness of the nonselective PDE inhibitor IBMX, relative to the selective inhibitors cilostamide and rolipram, may be attributable to simultaneous inhibition of multiple PDE isoforms. To test this hypothesis, we investigated whether a combination of a PDE3 inhibitor and a PDE4 inhibitor would be more effective than either inhibitor alone. 3T3-L1 cells were exposed to various concentrations of cilostamide in the absence or presence of a fixed concentration of rolipram (1 μM). Cilostamide alone displayed an R_{max} of 259 ± 12% of vehicle control and had an EC_{50} of 0.86 ± 0.32 μM (Fig. 1B). Exposure of cells to 1 μM rolipram alone stimulated lipolysis to 187 ± 12% of control. In the presence of 1 μM rolipram, cilostamide's EC_{50} did not change (0.86 ± 0.36 μM); however, its R_{max} increased to 894 ± 109% of control. Rolipram significantly potentiated the effects of cilostamide at all concentrations of cilostamide that were tested. Furthermore, the effects of cilostamide and rolipram were synergistic (i.e., greater than additive). The R_{max} of cilostamide in the absence of rolipram was 27% of the maximal ISO response (976 ± 45% of control), whereas in the presence of rolipram the R_{max} of cilostamide increased to 92% of the maximal ISO response.

Effect of selective PDE inhibitors on lipolysis in cultured human adipocytes

To determine if the various PDEs have a similar role in regulating lipolysis in human adipocytes, we carried out an analogous set of experiments. Human adipocytes were differentiated in vitro from preadipocytes isolated from subcutaneous adipose tissue. Cultured human adipocytes (Prep 1; see Methods) were exposed to selective PDE inhibitors at a final concentration of 1 or 10 μM (**Table 3**). Inhibition of PDE3 by cilostamide stimulated basal lipolysis in human adipocytes (165 ± 4.7% of control at 1 μM, 800 ± 81% of control at 10 μM). Cilostamide at a concentration of 10 μM stimulated lipolysis in human adipocytes by an amount comparable to the level of lipolysis induced by 1 μM ISO (780 ± 40% of control), a maximally effective concentration of ISO for this cell type (data not shown). In contrast, exposure of human adipocytes to inhibitors of PDE1, PDE2, or PDE4 did not significantly affect lipolysis (Table 3). The effects of inhibitors of PDE1, PDE2, PDE3, and PDE4 were similar in adipocytes from a second pool of donors (Prep 2; data not shown).

TABLE 3. Effect of selective PDE inhibitors on lipolysis in cultured human adipocytes

Inhibitor	Target PDE	Concentration	Lipolysis			
			No ISO		+1 nM ISO	
		μM	% control	n	% control	n
Vehicle	N.A.	N.A.	100 ± 3.3	12	246 ± 24[a]	6
IC224	PDE1	1	94.5 ± 8.2	3	266 ± 5.7	3
		10	91.3 ± 4.2	3	246 ± 27	3
IC933	PDE2	1	106 ± 4.2	3	301 ± 11	3
		10	96.1 ± 4.2	3	291 ± 11	3
Cilostamide	PDE3	1	165 ± 4.7[a]	3	817 ± 121[b]	3
		10	800 ± 81[c]	3	2,037 ± 221[d]	3
Rolipram	PDE4	1	108 ± 11	3	328 ± 23	3
		10	98.7 ± 4.0	3	266 ± 32	3
ISO	N.A.	1	780 ± 40[a]	6	N.A.	N.A.

Lipolysis induced by selective PDE inhibitors in the absence and presence of 1 nM ISO. Values represent the glycerol concentration in the culture supernatant and are expressed as percentages of vehicle control (mean ± SEM for n replicate wells). Absolute glycerol concentration in the culture supernatant of vehicle-treated cells was 8.3 ± 0.3 μM. N.A., not applicable.

[a] $P < 0.0001$ vs. vehicle control by two-tailed Student's *t*-test for independent samples.

[b] $P < 0.001$ vs. 1 nM ISO alone by two-tailed Student's *t*-test for independent samples.

[c] $P < 0.001$ vs. vehicle control by two-tailed Student's *t*-test for independent samples.

[d] $P < 0.0001$ vs. 1 nM ISO alone by two-tailed Student's *t*-test for independent samples.

We also measured the effect of selective PDE inhibitors on lipolysis in cultured human adipocytes stimulated with a submaximal concentration (1 nM) of ISO. Human adipocytes (Prep 1) were exposed to 1 nM ISO and selective PDE inhibitors at a final concentration of 1 or 10 μM (Table 3). In the absence of PDE inhibitors, ISO at 1 nM stimulated lipolysis by 246 ± 24% compared with vehicle controls ($P < 0.0001$). In the presence of 1 nM ISO, cilostamide activated lipolysis significantly above the level induced by ISO alone (817 ± 121% of control at 1 μM cilostamide, 2,037 ± 221% of control at 10 μM). The effect of the combination of cilostamide and ISO was synergistic (i.e., greater than additive). In addition, a combination of ISO and cilostamide induced a level of lipolysis significantly greater than the maximal ISO-induced lipolysis in the absence of cilostamide (780 ± 40% of control). In the presence of 1 nM ISO, inhibition of PDE1, PDE2, or PDE4 did not stimulate human adipocyte lipolysis above the level seen in cells exposed to ISO alone.

To further characterize the role of PDE3 and PDE4 in lipolysis in cultured human adipocytes, we determined the concentration-response relationship for cilostamide and rolipram. Human adipocytes (Prep 3) were exposed to various concentrations of cilostamide and rolipram (**Fig. 2**). Cilostamide had an EC_{50} for activation of lipolysis of 1.8 ± 0.14 μM and an R_{max} of 932 ± 7.6% of vehicle control. Rolipram did not significantly affect lipolysis in cultured human adipocytes at concentrations up to 33

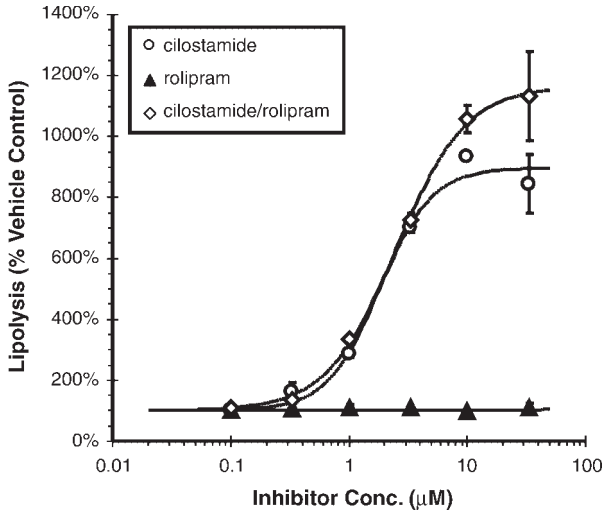

Fig. 2. Effects of selective PDE3 and PDE4 inhibitors on lipolysis in human adipocytes. Lipolysis (glycerol release) is plotted as a function of inhibitor concentration for cilostamide (a PDE3 inhibitor), rolipram (a PDE4 inhibitor), or a combination of the two inhibitors. For dual inhibition, cells were exposed to various concentrations of cilostamide in the presence of a fixed concentration of rolipram (1 μM). Data are normalized to the vehicle control and presented as means ± SEM for three replicates. Where error bars are not visible, the SEM was smaller than the symbol. Curves were fit to the experimental data using a three parameter logistic dose-response model. The glycerol concentration in the culture supernatant of the vehicle-treated wells was 29.5 ± 0.9 μM.

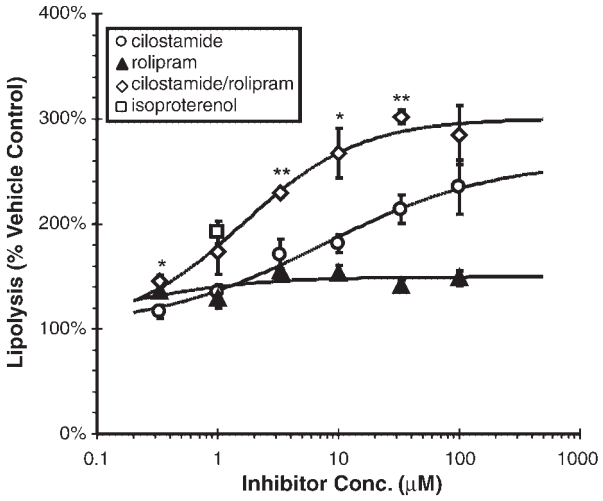

Fig. 3. Effects of selective PDE3 and PDE4 inhibitors on lipolysis in rat adipocytes. Lipolysis (glycerol release) is plotted as a function of inhibitor concentration for cilostamide (a PDE3 inhibitor), rolipram (a PDE4 inhibitor), or a combination of the two inhibitors. For dual inhibition, cells were exposed to various concentrations of cilostamide in the presence of a fixed concentration of rolipram (1 μM). The effect of 1 μM ISO is shown for comparison. Data are normalized to the vehicle control and presented as means ± SEM for three replicates. Curves were fit to the experimental data using a three parameter logistic dose-response model. The glycerol concentration in the culture supernatant of the vehicle-treated wells was 33.2 ± 9.6 μM. Values for cilostamide in the presence of rolipram were compared with values for cilostamide alone by a one-tailed Student's *t*-test for independent samples. * $P < 0.05$, ** $P < 0.01$.

μM. The response of cultured human adipocytes to cilostamide and rolipram was the same in adipocytes from a second pool of donors (Prep 2; data not shown). We also examined the effect of a combination of cilostamide and a fixed concentration of rolipram (1 μM) on lipolysis in human adipocytes (Prep 3). Under these conditions, cilostamide had an EC_{50} of 2.5 ± 0.31 μM and an R_{max} of 1,279 ± 25% of control. Potentiation of the cilostamide response by rolipram was slight and was only apparent at high cilostamide concentrations (≥10 μM). Lack of synergy between PDE3 and PDE4 inhibitors in stimulating lipolysis in cultured human adipocytes was confirmed with a different PDE3 inhibitor, milrinone (Table 1), using adipocytes from a second pool of donors (Prep 2; data not shown).

Effect of PDE3 and PDE4 inhibitors on lipolysis in cultured rat adipocytes

Because PDE3 and PDE4 appeared to have somewhat different roles in regulating lipolysis in cultured murine and human adipocytes, we also examined the effects of PDE3 and PDE4 inhibitors on lipolysis in cultured adipocytes from a third species. In rat adipocytes, differentiated in vitro from preadipocytes isolated from the epididymal fat pad, cilostamide had an EC_{50} of 7.4 ± 5.1 μM and stimulated lipolysis to 235 ± 26% of vehicle control at 100 μM (**Fig. 3**). The cilostamide concentration-response curve did not reach a plateau; however, the predicted maximum response based on fitting the experimental data to a three parameter logistic dose-response model was ~260% of control. Rolipram stimulated lipolysis in cultured rat adipocytes less strongly (R_{max} = 155 ± 2.4% of control; $P < 0.01$). In addition, activation of lipolysis by rolipram was only slightly dose-dependent over the concentration range tested (0.33–100 μM). ISO (1 μM) stimulated lipolysis in cultured rat adipocytes to 192 ± 11% of control. Maximal ISO-stimulated lipolysis in cultured rat adipocytes was 263 ± 4.6% of control at 3.3 μM ISO (data not shown). Hence, the predicted R_{max} for cilostamide was approximately equal to the R_{max} for ISO, whereas the R_{max} for rolipram was ~60% of the R_{max} for ISO.

Exposure of rat adipocytes to cilostamide in the presence of a fixed concentration of rolipram (1 μM) enhanced lipolysis over levels induced by either inhibitor alone (R_{max} = 302 ± 6.2% of vehicle control) (Fig. 3). Rolipram alone at 1 μM induced lipolysis by 129 ± 9.8% of control ($P < 0.05$). Therefore, the combined effects of cilostamide and rolipram appeared to be approximately additive with respect to maximal stimulation. However, the concentration-response curve for cilostamide was shifted to the left in the presence of 1 μM rolipram (EC_{50} = 1.6 ± 0.41 μM). As a result, 1 μM rolipram synergistically enhanced the cilostamide response at concentrations of cilostamide between 0.33 and 33 μM.

DISCUSSION

In this report, we describe the effects of selective inhibitors of PDE1, PDE2, PDE3, and PDE4 (Table 1) on lipoly-

sis in cultured murine, rat, and human adipocytes. The PDE3 inhibitor cilostamide was the most effective at stimulating lipolysis in cultured adipocytes of all three species (Figs. 1–3). Inhibition of PDE3 with cilostamide moderately stimulated lipolysis in murine 3T3-L1 adipocytes (259–397% of vehicle control in different experiments) and rat adipocytes (235 ± 26% of vehicle control) and markedly stimulated lipolysis in human adipocytes (932 ± 7.6% of control). In contrast, inhibition of PDE4 with rolipram moderately stimulated lipolysis in murine 3T3-L1 adipocytes (291 ± 13% of control) and weakly stimulated lipolysis in rat adipocytes (155 ± 2.4% of control) but did not affect lipolysis in human adipocytes. Inhibition of PDE1 and PDE2 did not stimulate lipolysis in murine, human, or rat adipocytes.

Our data are in agreement with a published report documenting a greater effect of PDE3 inhibition than PDE4 inhibition in stimulating murine 3T3-L1 adipocyte lipolysis (25) and are also consistent with reports of the effects of PDE4 inhibitors in human (28) and rat (15, 27) adipocytes. However, our observation that the PDE3 inhibitor cilostamide stimulated lipolysis in cultured rat adipocytes contrasts with studies that reported no activation of lipolysis in primary rat epididymal adipocytes by either cilostamide (15) or amrinone (26). In these studies, lipolysis was stimulated only when the PDE3 inhibitors were coadministered with a β-adrenoceptor agonist (noradrenaline or ISO). This may reflect a difference in the properties of primary rat adipocytes and the in vitro-differentiated rat adipocytes used in our studies.

It should be noted that the origin of the adipocytes used in our work differed among the three species. Murine 3T3-L1 adipocytes were differentiated from a fibroblast cell line; human adipocytes were differentiated from preadipocytes prepared from subcutaneous adipose tissue; and rat adipocytes were differentiated from preadipocytes prepared from the epididymal fat pad. Furthermore, the protocol used to differentiate each cell type was different. Hence, differences in the role of PDE3 and PDE4 in regulating lipolysis between the various adipocyte preparations may be attributable to these different methods of preparation. In this regard, it would be informative to compare the response of freshly isolated primary adipocytes from the three species in question.

We observed that maximal ISO-induced lipolysis was somewhat greater in murine 3T3-L1 adipocytes (904–1,162% of control in different experiments) than in cultured human adipocytes (780 ± 40% of control) and substantially greater than in cultured rat adipocytes (263 ± 4.6% of control). However, the maximal cilostamide-stimulated lipolysis, expressed as a percentage of maximal ISO-induced lipolysis, was markedly higher in cultured human (119%) and rat (99%) adipocytes than in murine 3T3-L1 adipocytes (27–44% in different experiments). Variation in the responsiveness to ISO may reflect different levels of expression of β-adrenoceptors in cultured adipocytes from the different species.

When the effects of selective PDE inhibitors on lipolysis stimulated by a low concentration of ISO were assessed in murine and human cultured adipocytes, there was no effect of inhibitors of PDE1, PDE2, or PDE4 (Tables 2, 3). In murine 3T3-L1 adipocytes, inhibition of PDE3 with cilostamide increased ISO-stimulated lipolysis, and the combined effects of the two agents were approximately additive. Hence, there was little synergy between β-adrenoceptor stimulation and PDE3 inhibition in these cells. In contrast, in cultured human adipocytes, ISO-stimulated lipolysis was strongly potentiated by cilostamide, and the effects of the two agents were synergistic (i.e., greater than additive). Because of their ability to mobilize adipocyte fat stores, selective β_3-adrenoceptor agonists have been considered as potential therapeutic agents for the treatment of obesity and metabolic syndrome in humans. To date, however, these agents have not produced weight loss in patients (38). Our findings that a β-adrenoceptor agonist and a PDE3 inhibitor exhibit synergy in the stimulation of lipolysis in cultured human adipocytes suggest that combination therapy with these two agents may have superior efficacy.

The EC_{50} value for rolipram in 3T3-L1 adipocytes (0.12 μM; Fig. 1A) was comparable to, and in fact somewhat lower than, its IC_{50} value against recombinant PDE4 (0.28 μM; Table 1). In contrast, EC_{50} values for cilostamide in 3T3-L1 adipocytes (2.0 ± 1.4 μM and 0.86 ± 0.32 μM in Fig. 1A, B, respectively) were more than 100-fold greater than its IC_{50} value for recombinant PDE3 (0.007 μM; Table 1). However, even at 2 μM, cilostamide was still 4-fold below its IC_{50} for the next closest PDE isotype. Thus, the effect of cilostamide in the cell-based assay was probably attributable to inhibition of PDE3 rather than other PDE families. Published EC_{50} values for cilostamide in cell-based assays, including inhibition of vascular smooth muscle cell proliferation (39) and stimulation of chloride secretion in colonic epithelial cells (40), are similar to the values we have obtained with cultured adipocytes. This suggests that the low relative potency of cilostamide in such assays may be to the result of poor penetration into cells.

Cultured murine, human, and rat adipocytes differed in their lipolytic responses to a combination of PDE3 and PDE4 inhibitors. In murine 3T3-L1 (Fig. 1B) and cultured rat adipocytes (Fig. 3), simultaneous exposure to cilostamide and a fixed concentration of rolipram had a synergistic effect on lipolysis. In contrast, in human adipocytes, the combination of a PDE3 and a PDE4 inhibitor was only slightly more effective than cilostamide alone in stimulating lipolysis (Fig. 2). Maximal stimulation of lipolysis in the presence of cilostamide and rolipram was 894 ± 109%, 302 ± 6.2%, and 1,279 ± 25% in murine, rat, and human adipocytes, respectively. Relative to the maximum lipolytic response to cilostamide alone, these values represent 3.5-, 1.3-, and 1.4-fold enhancements in murine, rat, and human adipocytes, respectively. Although the effect of rolipram on the maximal lipolytic rate was comparable in rat and human adipocytes, the potentiation of the cilostamide response by rolipram was greater in rat adipocytes, as indicated by the left shift of the concentration-response curve for cilostamide in the presence of rolipram (Fig. 3), an effect that was not observed with human adipocytes.

Synergistic effects of PDE3 and PDE4 inhibitors have

been documented previously in several cell- or tissue-based functional assays, including relaxation of smooth muscle strips (41–43), attenuation of vascular smooth muscle cell proliferation (39, 44), stimulation of vascular smooth muscle cell migration (45), induction of apoptosis in leukemic cells (44, 46), attenuation of T-lymphocyte proliferation (47, 48), and inhibition of T-lymphocyte interleukin-2 synthesis (47). In mice injected with T-cell mitogens, PDE3 and PDE4 inhibitors also act synergistically in vivo to attenuate systemic tumor necrosis factor-α release and protect against liver injury (49). However, to our knowledge, this report is the first to demonstrate synergism between PDE3 and PDE4 inhibitors in the stimulation of lipolysis in adipocytes.

The effects of a combination of cilostamide and Ro 20-1724 (a PDE4 inhibitor) on lipolysis in murine 3T3-L1 adipocytes have been reported previously (25). In that investigation, 0.3 μM cilostamide activated 3T3-L1 adipocyte lipolysis by 272 ± 18% relative to vehicle controls and 10 μM Ro 20-1724 activated lipolysis by 186 ± 12% of control; however, a combination of the two inhibitors was no more effective than was cilostamide alone. Because 10 μM Ro 20-1724 alone was reported to stimulate 3T3-L1 lipolysis to the same extent as 1 μM rolipram stimulated lipolysis in our experiments, the apparent absence of synergy between cilostamide and Ro 20-1724 was probably not attributable to insufficient inhibition of PDE4. Notably, the maximal ISO-stimulated lipolysis observed in the earlier experiments (in the presence of 1 μM ISO) was only 330% of vehicle controls; whereas we observed a stimulation of 904–1,162%. This difference in the maximal ISO-induced response may be attributable to the longer incubation time used in the present study (6 vs. 1 h). The greater dynamic range of the lipolysis assay under our experimental conditions, together with the comparison of a full concentration-response relationship for cilostamide in the absence and presence of rolipram, may explain why synergism between PDE3 and PDE4 inhibitors was detected in the present study but not in earlier investigations.

In murine 3T3-L1 adipocytes, we observed that maximal stimulation of lipolysis by the nonselective PDE inhibitor IBMX was considerably greater than by either cilostamide or rolipram alone (Fig. 1A). Our data suggest that the greater efficacy of IBMX is attributable to a synergistic effect of the inhibition of both PDE3 and PDE4. However, in addition to inhibiting PDEs, IBMX is also an adenosine A_1 receptor antagonist (36, 37). In adipocytes, A_1 receptors couple to the inhibitory guanine nucleotide-binding protein; activation of this receptor inhibits AC activity and suppresses lipolysis (36). Hence, IBMX could stimulate lipolysis by antagonizing the inhibitory effect of secreted adenosine. This activity could contribute to its greater efficacy relative to cilostamide and rolipram, which do not have affinity for A_1 receptors. However, we found that DPCPX, a xanthine that is a potent and selective adenosine A_1 receptor antagonist but inhibits PDE1 to PDE4 with low affinity (37), did not induce lipolysis in 3T3-L1 cells. Hence, we conclude that antagonism at adenosine A_1 receptors does not contribute significantly to the lipolytic activity of IBMX in 3T3-L1 adipocytes.

We found that a selective PDE1 inhibitor, IC224, did not stimulate lipolysis in either murine 3T3-L1 adipocytes or cultured human adipocytes (Tables 2, 3); however, IC224 suppressed 3T3-L1 lipolysis by ∼30% at a concentration of 10 μM (Table 2). A similar effect was not observed in cultured human adipocytes (Table 3). The antilipolytic effect of IC224 suggests the possibility that PDE1 tonically stimulates lipolysis in 3T3-L1 adipocytes. However, this effect is counter to that of PDE3 and PDE4, which inhibit lipolysis by decreasing intracellular levels of cAMP. We do not, at the present time, understand the mechanism by which IC224 inhibits lipolysis in 3T3-L1 adipocytes.

PDE2 has been detected in rat epididymal adipocytes (15), rat brown adipose tissue (16), 3T3-L1 adipocytes (our unpublished observations), and cultured human adipocytes (our unpublished observations). Hence, the inability of the PDE2 inhibitor IC933 to affect lipolysis in murine 3T3-L1 adipocytes and cultured human adipocytes suggests that PDE2 does not play a role in signaling pathways that regulate lipolysis.

Obesity is a major health concern in the developed world, affecting nearly one-third of the adult population (50) and an increasing percentage of children and adolescents (51). Behavioral therapies for obesity include diet and exercise; however, experience has shown that long-term weight loss is difficult to sustain by these methods alone. Safe and effective pharmacotherapy would be highly desirable as an adjunct to diet and exercise in the treatment of obesity. Drugs currently approved for the treatment of obesity primarily target energy intake by suppressing appetite or inhibiting nutrient absorption (52). Agents that induce mobilization of fat stores, such as β_3-adrenoceptor agonists (38) or selective inhibitors of adipocyte PDE3B (53), may provide additional benefit to obese patients. Our finding that PDE3 inhibitors are capable of inducing high levels of lipolysis in cultured human adipocytes lends support to this hypothesis. However, our observation that inhibition of both PDE3 and PDE4 is required for efficient stimulation of lipolysis in murine and rat adipocytes suggests that commonly used rodent models of obesity may not be appropriate for predicting the clinical effects of PDE3 inhibitors in humans.

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