Altered Phosphodiesterase 3–Mediated cAMP Hydrolysis Contributes to a Hypermotile Phenotype in Obese JCR:LA-cp Rat Aortic Vascular Smooth Muscle Cells

Implications for Diabetes-Associated Cardiovascular Disease

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Cardiovascular diseases represent a significant cause of morbidity and mortality in diabetes. Of the many animal models used in the study of non–insulin-dependent (type 2) diabetes, the JCR:LA-cp rat is unique in that it develops insulin resistance in the presence of obesity and manifests both peripheral and coronary vasculopathies. In this animal model, arterial vascular smooth muscle cells (VSMCs) from homozygous obese (*cp/cp*) rats, but not from age-matched healthy (+/+ or +/*cp*, collectively defined +/?) littermates, display an "activated" phenotype in vitro and in vivo and have an elevated level of cAMP phosphodiesterase (PDE) activity. In this report, we confirm that *cp/cp* rat aortic VSMCs have an elevated level of PDE3 activity and show that only particulate PDE3 (PDE3B) activity is elevated. In marked contrast to results obtained in +/? VSMCs, simultaneous activation of adenylyl cyclase and inhibition of PDE3 activity in *cp/cp* VSMCs synergistically increased cAMP. Although PDE3 inhibition did not potentiate the antimigratory effects of forskolin on +/? VSMCs, PDE3 inhibition did markedly potentiate the forskolin-induced inhibition of migration of *cp/cp*-derived VSMCs. Although PDE3 activity was elevated in *cp/cp* rat aortic VSMCs, levels of expression of cytosolic PDE3 (PDE3A) and PDE3B in +/? and *cp/cp* VSMCs, as well as activation of these enzymes following activation of the cAMP–protein kinase A signaling cascade, were not different. Our data are consistent with an increased role for PDE3 in regulating cAMP-dependent signaling in *cp/cp* VSMCs and identify PDE3 as a cellular activity potentially responsible for the phenotype of *cp/cp* VSMCs. *Diabetes* 51:1194–1200, 2002

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MAPK, mitogen-activated protein kinase; PDE, phosphodiesterase; PDE3A, cytosolic PDE3; PDE3B, particulate PDE3; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PMA, phorbol myristate acetate; RIMAC, radial invasion of matrix by aggregated cells; VSMC, vascular smooth muscle cell.

Atherosclerosis and other cardiovascular diseases are much more prevalent in people with diabetes than in the population at large and represent a significant cause of morbidity and early mortality in diabetes (1–5). In this context, epidemiological evidence consistent with an increased risk of atherosclerosis in diabetes is mounting, with a possible role for *1*) insulin resistance and its associated hyperinsulinemia, *2*) poor glycemic control leading to elevated levels of advanced glycosylated end products, or *3*) elevated circulating leptin levels associated with increased adipose mass (1–5). Atherosclerotic lesions occur in the context of endothelial cell dysfunction and involve activation, migration, and proliferation of vascular smooth muscle cells (VSMCs). A link between an "activated" phenotype of VSMC in diabetes and atherosclerosis has been documented in animal models and humans (6).

Endothelium-derived relaxing factors, such as nitric oxide or prostacyclin, relax blood vessels and inhibit the proliferation and migration of VSMCs by increasing the synthesis of the cyclic nucleotides cAMP or cGMP (7–10). A large family of enzymes named cyclic nucleotide phosphodiesterases (PDEs) inactivate cAMP and cGMP through their hydrolysis (11–14). VSMC cAMP hydrolysis is catalyzed by members of the phosphodiesterase 3 (PDE3) and PDE4 families (12), whereas VSMC cGMP is inactivated by PDE1 or PDE5 enzymes (15).

Hydrolysis of cAMP by PDE3 is potently inhibited by cGMP, as well as by a large number of pharmacological agents capable of stimulating myocardial contractility, inhibiting platelet aggregation, and relaxing vascular and airway smooth muscle (e.g., cilostamide, enoximone, lixazinone) (11,13,16–19). Two different genes encoding

PDE3 activity have been identified, *PDE3A* and *PDE3B*, and each is expressed in rat and human VSMCs (20,21). Forskolin, β-adrenergic agonists, or glucagon increase cAMP and activate PDE3 through a cAMP-dependent protein kinase A (PKA)-mediated phosphorylation. Similarly, insulin, IGF-1, or leptin activates PDE3 by a protein kinase B (PKB)-mediated, phosphatidylinositol 3-kinase (PI3K)-dependent phosphorylation reaction. Indeed, activation of particulate PDE3 (PDE3B) by insulin in adipocytes, leptin in hepatocytes, or IGF-1 in pancreatic β-cells has been shown to be essential for the metabolic effects of these hormones in these tissues (22–24). Four PDE4 genes have been identified, *PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D* (25). Perhaps owing to the weak vasorelaxation caused by PDE4 inhibition (12), expression of these enzymes in VSMCs has not been extensively studied. Recently, we demonstrated that two PDE4D variants (PDE4D3 and PDE4D5) are expressed in both rat and human VSMCs (26,27). As with PDE3, PKA-dependent phosphorylation of some PDE4s, including PDE4D3, stimulates these enzymes (28,29). Activation of the mitogen-activated protein kinase (MAPK) cascade can activate, or inhibit, PDE4 perhaps in a cell type–dependent manner (26,30).

Of the many animal models used in the study of non–insulin-dependent (type 2) diabetes, the JCR:LA-cp rat is unique in that it develops insulin resistance in the presence of obesity and manifests both peripheral and coronary vasculopathies (6,31). Although the homozygous (+/+) and heterozygous (+/*cp*) animals (collectively defined +/?) are metabolically normal, homozygotes for the autosomal recessive corpulent gene (*cp/cp*) are insulin resistant, hyperinsulinemic, obese, glucose intolerant, and hyperlipidemic and show signs of pancreatic β-cell hyperplasia (31). In previous reports, *cp/cp* VSMCs have been shown to display an activated phenotype, compared with +/? VSMCs, both in vivo and after culture (6,32,33). Regrettably, little is known concerning the molecular basis of this activated phenotype. In one previous report, *cp/cp* rat aortae were shown to express increased PDE3 and PDE4 activities compared with aortae from age-matched +/? littermates (34). In the work described in this report, we have confirmed that *cp/cp* rat aortic VSMCs have elevated PDE3 activity and that this activity plays an important role in cAMP catabolism in *cp/cp* VSMCs. Our data are discussed in the context of the hypothesis that aberrant regulation of PDE3 activity in *cp/cp* VSMCs may alter the capacity of these cells to regulate cAMP-mediated effects.

RESEARCH DESIGN AND METHODS

Aortic tissues and cultured aortic cells. Care provided for the rats and the treatments used in experiments conformed to the guidelines of the Canadian Council on Animal Care and were subject to prior institutional approval. JCR:LA-cp rats were bred at the University of Alberta as described previously (35). Rat aortic tissues were collected from anesthetized animals (4% halothane). JCR:LA-cp rat aortic VSMCs were grown from explanted segments of aorta isolated from 3-week-old or 6-month-old rats and subcultured as described previously (33,36). Cells between passages 4 and 10 were used in these studies; no passage-dependent differences were observed.

Measurement of cAMP PDE activity. Total cAMP PDE, PDE3, and PDE4 activities in lysates of aortic tissues or of cultured VSMCs were measured as described previously (35), with isobutyl-methylxanthine (500 μmol/l), cilostamide (1 μmol/l), or Ro,201724 (10 μmol/l) used to determine total, PDE3, and PDE4 cAMP PDE activities, respectively. Concentrations of cilostamide and Ro,201724 used in these experiments were chosen from concentration-response curve analyses with each VSMC cell type. Because combinations of these two agents with concentrations of cilostamide >1 μmol/l and Ro,201724 >10 μmol/l were subadditive, and hence nonselective, those maximally effective concentrations were used. When PDE3 and PDE4 activities were measured in isolated cytosolic and particulate fractions of aortic tissue or of VSMCs, subcellular fractions were isolated by ultracentrifugation as described previously (20).

TABLE 1
cAMP PDE activities ($pmol \cdot min^{-1} \cdot mg^{-1}$) in fractionated +/? and *cp/cp* JCR:LA-cp rat aorta

	+/? rat aorta		*cp/cp* rat aorta	
	Cytosol	Membrane	Cytosol	Membrane
Additions				
None	252 ± 5	148 ± 9	253 ± 4	206 ± 3*
Cilostamide-inhibited	139 ± 5	97 ± 8	135 ± 5	133 ± 3*
Ro,201724-inhibited	90 ± 9	66 ± 3	99 ± 3	65 ± 4
IBMX-inhibited	242 ± 1	148 ± 0	240 ± 4	198 ± 5

Cilostamide (1 μmol/l), Ro,201724 (10 μmol/l), and IBMX (500 μmol/l) were used to determine PDE3, PDE4, and total cAMP PDE activities, respectively. Data are means ± SE from three independent determinations. Similar results were obtained in five independent experiments, each conducted in triplicate. *Significant difference compared with particulate PDE3 activity in +/? aortic tissue, $P < 0.05$.

Immunoblot analysis. PDE3 expression was verified and quantitated by immunoblot analysis using procedures described previously (20,21). Three separate antisera were used: a rabbit polyclonal antibody generated against the carboxy-terminal region of a murine PDE3B as antigen (provided by Dr. J.A. Beavo, University of Washington, Seattle, WA) and two polyclonal antisera raised to amino- and carboxy-terminal regions of human PDE3 (Santa-Cruz Biotech, Santa Cruz, CA).

Determination of VSMC cAMP levels. Drug-induced changes in VSMC cAMP were measured using a prelabeling method described previously (37). Briefly, VSMC ATP was metabolically labeled by incubation of cells with 2 μmol/l [^{3}H]hypoxanthine (10 μCi/ml for 16 h). Prelabeled VSMCs were incubated with pharmacological agents, and incubations were terminated by addition of trichloroacetic acid (5% vol/vol final concentration). [^{3}H]cAMP was isolated and purified by sequential chromatography on alumina/Dowex and quantitated by liquid scintillation (37).

Migration assays. Two separate migration assays were used in these studies. The modified Boyden chamber assay was conducted as previously described by us (38). The radial invasion of matrix by aggregated cells (RIMAC) assay was carried out as described previously (39), except that VSMCs were used rather than bovine aortic endothelial cells.

RESULTS

PDE3 and PDE4 activities in JCR:LA-cp rat aortae. Aortic cAMP PDE activity of the JCR:LA-cp rat strain was predominantly made up of PDE3 and PDE4 activities (Table 1). Consistent with a previous report (34), PDE3 activity of aortae derived from 6-month-old *cp/cp* rats was elevated significantly (18 ± 3%, $n = 9$) compared with that of age-matched controls (+/?). In contrast, no differences in PDE4 activity in aortic tissues isolated from *cp/cp* and +/? aortae were noted (Table 1). Analysis of cytosolic and particulate cAMP PDE activity identified an increase in particulate PDE3 activity in *cp/cp* rat aortic tissue compared with +/? aortae. There were no differences in cytosolic PDE3 activity between +/? and *cp/cp* aortae (Table 1).

PDE3 and PDE4 activities and expression in JCR:LA-cp rat aortic VSMCs. To more fully assess the potential significance of differences in PDE3 activity in *cp/cp* aortae, we determined the PDE3 and PDE4 activities

TABLE 2
cAMP PDE activities (pmol · min^{-1} · mg^{-1}) in fractionated +/? and *cp/cp* JCR:LA-cp rat aortic cultured VSMCs

	+/? rat aortic VSMCs		*cp/cp* rat aortic VSMCs	
	Cytosol	Membrane	Cytosol	Membrane
Additions				
None	78 ± 2	69 ± 3	75 ± 3	76 ± 4
Cilostamide-inhibited	5 ± 1	3 ± 1	7 ± 0	22 ± 3*
Ro,201724-inhibited	70 ± 4	28 ± 3	84 ± 7	22 ± 6
IBMX-inhibited	65 ± 3	58 ± 3	68 ± 2	62 ± 7

Cilostamide (1 μmol/l), Ro,201724 (10 μmol/l), and IBMX (500 μmol/l) were used to determine PDE3, PDE4, and total cAMP PDE activities, respectively. Data are means ± SE from three independent determinations. Similar results were obtained in seven independent experiments conducted in triplicate. *Significant difference compared with particulate PDE3 activity in cultured +/? aortic VSMCs; $P < 0.05$.

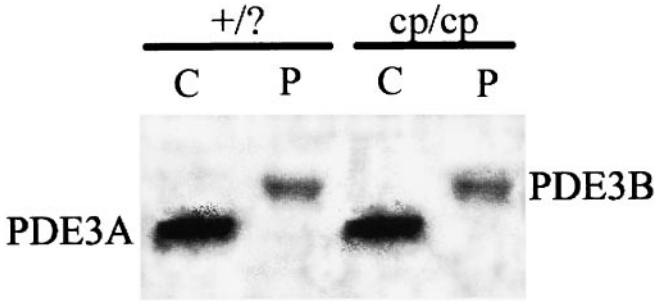

FIG. 1. Immunoblot analysis of PDE3A and PDE3B expression in cytosolic (C) and particulate (P) fractions of +/? and *cp/cp* aortic VSMCs. Cytosolic and particulate fractions of cultured rat aortic VSMC cultures (+/?, *cp/cp*) were generated by differential centrifugation at 100,000*g*. Aliquots of each fraction isolated from +/? or *cp/cp* VSMCs (5 μg protein) were subjected to SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes, and the membranes were blocked by incubation with Tris-buffered saline with Tween (TBST) (20 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 0.1% Tween-20) supplemented with 5% powdered nonfat milk for 1 h. Blots were incubated with a diluted rabbit polyclonal antibody raised against murine PDE3B (1:4,000) for 1–2 h and rinsed three times with TBST. The rinsed membrane was incubated with horseradish peroxidase–conjugated goat anti–rabbit IgG for 1 h and rinsed, and immunoreactivity was detected by chemiluminescence following the manufacturer's recommendations.

of VSMCs cultured from +/? and *cp/cp* aortae. Our data indicated that homogenates of cultured *cp/cp*-derived VSMCs had higher PDE3 activity than cultured VSMCs derived from age-matched +/? aortae (Table 2). PDE4 activity was not different between the +/?- and *cp/cp*-derived VSMCs. Subcellular fractionation revealed that *cp/cp* VSMC particulate PDE3 activity was increased relative to +/?-derived VSMCs. Cytosolic PDE3 activity was similar in +/? and *cp/cp* VSMCs (Table 2). Consistent with previous experiments in our laboratory, total PDE3 activity was lower in cultured VSMCs than in aortae. This difference is related to changes in expression of PDE3A in VSMCs upon culturing (H.A.D., D.H.M.: Altered expression of cyclic nucleotide phosphodiesterases in cultured and intimal vascular smooth muscle cells. Presented at the Joint Annual Meeting of ASPET, the American Society for Biochemistry and Molecular Biology, the Pharmacological Society of Canada, and French Pharmacological Society, Boston, MA, June 4–8, 2000).

We have reported previously that PDE3A and PDE3B were both expressed in rat and human aortic VSMCs, and that these two enzymes were selectively expressed in cytosolic (PDE3A) and particulate (PDE3B) fractions of rat tissues, respectively. Because particulate PDE3 activity was selectively elevated in *cp/cp* aortae and cultured aortic VSMCs (Tables 1 and 2), we hypothesized that levels of PDE3B would be elevated in *cp/cp* tissues relative to +/? tissues. Immunoblot analysis using several PDE3A and PDE3B antisera revealed the expression of a 105-kDa cytosolic PDE3A and a 135-kDa particulate PDE3B in both +/? and *cp/cp* VSMCs. In contrast to our prediction, however, of the four separate VSMC samples of each (+/? and *cp/cp*) that were immunoblotted using five separate antisera for PDE3A or PDE3B, there were no significant differences detected in the levels of PDE3A or PDE3B expressed (representative immunoblot, Fig. 1). Consistent with the activity data, levels of PDE4D expression were similar between +/? and *cp/cp* VSMCs (not shown).

Effects of forskolin, phorbol myristate acetate (PMA), or IGF-1 on +/? or *cp/cp* VSMC PDE3 activity. As described above, activation of either the cAMP-PKA or the PI3K-PKB signaling cascades increases PDE3 activity in some cell types. To assess the possibility that differences in the regulation of PDE3 activity were involved in the altered PDE3 activity in *cp/cp* VSMCs, we measured PDE3 activity in +/? or *cp/cp* VSMCs after incubation with agents that would activate PKA (50 μmol/l forskolin for 20 min) (26–27) or PI3K-PKB (100 nmol/l PMA for 20 min or 100 ng/ml IGF-1 for 5 min) (22–24). Our data were not consistent with the idea that differences in PKA or PI3K-PKB activation of PDE3 played a major role in the observed differences in particulate PDE3 activity between +/? and *cp/cp* VSMCs. Thus, incubation of VSMCs with forskolin increased particulate PDE3 activity by 20 ± 2% and 23 ± 3% in +/? and *cp/cp* VSMCs, respectively. The magnitude of this effect was similar to that previously reported by us for VSMCs cultured from Wistar rat aortae (27,36). Similarly, whereas incubation of +/? or *cp/cp* VSMCs with PMA or IGF-1 stimulated PDE4D3 activity by a MAPK-dependent mechanism (which we have reported previously [26–27]) and led to the accumulation of phosphorylated PKB (as determined using a phospho-PKB selective antisera [not shown]), neither of these agents altered cytosolic or particulate PDE3 activity in either VSMC population.

Selective inhibition of PDE3 activity in *cp/cp* VSMCs and adenylyl cyclase activation–mediated increases in cAMP. In previous work, we reported that increases in VSMC cAMP resulting from activation of adenylyl cyclase could be potentiated by simultaneous inhibition of VSMC PDE activity (26,27,36). Moreover, we showed that the level of potentiation achieved correlated to the cellular level of cAMP PDE activity. Using the same approach, we undertook to determine if the slight elevation of PDE3 activity detected in *cp/cp* VSMCs could alter the impact of PDE3 inhibition on cAMP responses in these cells. Our data identify a marked increase in the contribution of PDE3, relative to PDE4, to cAMP hydrolysis in *cp/cp* VSMCs compared with +/? VSMCs (Fig. 2*A–D*).

Whereas cilostamide had no effect on forskolin-induced

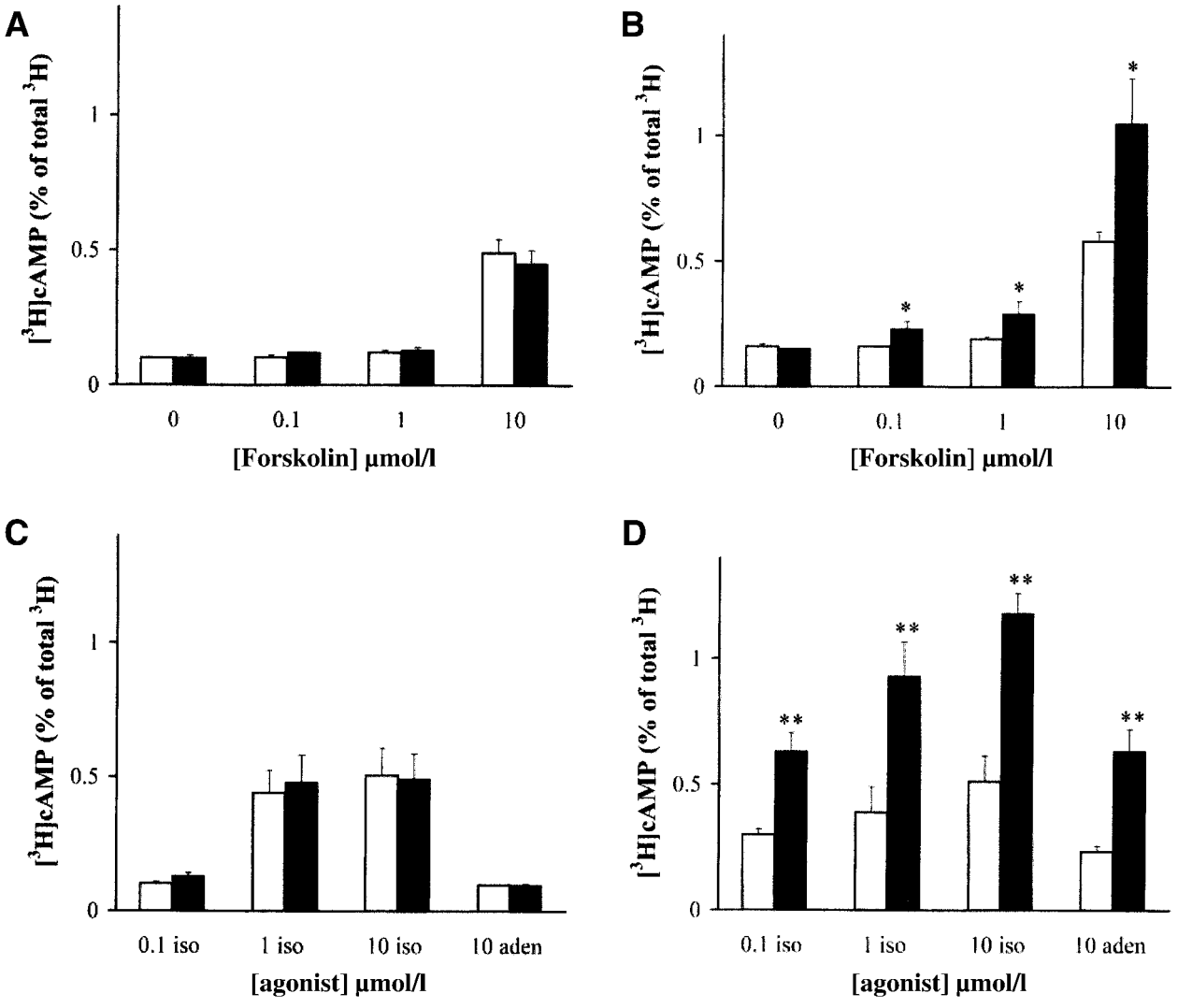

FIG. 2. Differential impact of PDE3 inhibition on [^{3}H]cAMP in +/? and *cp/cp* VSMCs. Changes in [^{3}H]cAMP in +/? VSMCs (*A*) or *cp/cp* VSMCs (*B*) prelabeled with [^{3}H]hypoxanthine (RESEARCH DESIGN AND METHODS) were measured after incubation with forskolin (0.1–10 μmol/l) (□) or a combination of forskolin (0.1–10 μmol/l) and cilostamide (1 μmol/l) (■) for 5 min. Similarly, cAMP levels in prelabeled +/? VSMCs (*C*) or *cp/cp* VSMCs (*D*) were determined after incubation with isoproterenol (0.1–10 μmol/l) (iso) or adenosine (10 μmol/l) (aden) alone or in combination with cilostamide (1 μmol/l) for 5 min. [^{3}H]cAMP was expressed as a percentage of the total [^{3}H] in each well. Individual treatments in experiments were assayed in triplicate, and the means ± SD of three independent experiments are shown. **P* < 0.05 compared with [^{3}H]cAMP in cells incubated with forskolin alone; ***P* < 0.05 compared with [^{3}H]cAMP in cells incubated with either isoproterenol or adenosine alone.

increases in cAMP in +/? VSMCs, this PDE3 inhibitor markedly potentiated the forskolin-induced increases in cAMP in *cp/cp* VSMCs (Fig. 2*A* and *B*). The inability of cilostamide to increase cAMP in +/? VSMC cAMP was consistent with previous work from our laboratory using VSMCs derived from other rat strains (36,38). Similar results were obtained when VSMC adenylyl cyclase was stimulated using isoproterenol or adenosine. Thus, whereas cilostamide did not increase the amount of cAMP that accumulated in response to isoproterenol or adenosine in +/? VSMCs, this agent markedly increased their effects in *cp/cp* VSMCs (Fig. 2*C* and *D*). Because 6-month-old rats would have established insulin resistance, similar experiments were carried out using VSMCs derived from 3-week-old rats, an age at which both +/? and *cp/cp* rats have normal insulin sensitivity and are essentially normoinsulinemic. In all respects, data obtained with VSMCs derived from the 3-week-old animals mirrored those obtained with the VSMCs derived from the older animals (Table 3).

Hypermotile phenotype of VSMCs derived from *cp/cp* rats. In a previous report, *cp/cp* aortic VSMCs were shown to exhibit a hyperproliferative phenotype relative to age-matched +/?-derived aortic VSMCs (33). Because VSMC migration is critical to development of diabetes-

TABLE 3
PDE3-mediated potentiation (% increase from basal [^{3}H]cAMP) of forskolin-induced increases in cAMP in VSMCs derived from 3-week-old +/? and *cp/cp* rat aorta

Additions	+/? VSMC [^{3}H]cAMP	*cp/cp* VSMC [^{3}H]cAMP
Forskolin	618 ± 36	207 ± 36
Cilostamide	73 ± 38	89 ± 37
Forskolin + cilostamide	764 ± 42*	439 ± 32*

Data are means ± SE from three independent determinations. *Significant difference compared with incubation with forskolin alone, $P < 0.05$.

TABLE 4
PDGF- or IGF-1–stimulated migration (number of cells migrated) of +/?- or *cp/cp*-derived aortic VSMCs

	+/? VSMCs	*cp/cp* VSMCs
Control	24 ± 3	50 ± 8†
PDGF (ng/ml)		
10	53 ± 7	126 ± 13*
30	86 ± 9*	127 ± 7*†
100	104 ± 7*	286 ± 12*†
IGF-1 (ng/ml)		
30	33 ± 4	86 ± 12†
100	29 ± 6	105 ± 12*†
300	35 ± 6	91 ± 10*†

Data are means ± SD from quadruplicate determinations. *Significant difference compared with migration of +/? or *cp/cp* VSMCs without addition of chemotactic factor, $P < 0.05$. †Significant difference compared with +/? VSMCs under the same experimental conditions, $P < 0.05$.

associated atherosclerosis, we determined if *cp/cp* VSMCs displayed a hypermotile phenotype relative to +/? VSMCs. Using both two-dimensional (modified Boyden's assay [38]) and three-dimensional (RIMAC [39]) migration assays, *cp/cp*-derived aortic VSMCs were shown to be hypermotile relative to age-matched +/? aorta–derived VSMCs. Indeed, marked differences in basal and stimulated migration were observed between these cells in each assay (Table 4; Fig. 3).

Effects of cilostamide on migration of *cp/cp* and +/? VSMCs. Because we had previously shown that forskolin inhibited platelet-derived growth factor (PDGF)-induced migration and that PDE inhibition could markedly potentiate this effect (38), we next determined if a functional link existed between the increased role of PDE3 in cAMP signaling in *cp/cp* VSMCs and the hypermotile phenotype of these cells. Consistent with our previous work, cilostamide did not inhibit migration of +/? VSMCs, nor did it increase the inhibition of migration caused by forskolin. In contrast, cilostamide caused a slight inhibition of PDGF-induced migration in *cp/cp* VSMCs and markedly potentiated the inhibitory effect of forskolin in these VSMCs (Table 5). In fact, the inhibition caused by simultaneous addition of forskolin and cilostamide in *cp/cp* VSMCs was larger than that caused by the combined actions of forskolin and Ro,201724 in these cells. This effect of cilostamide correlated positively with the effect of this PDE3 inhibitor on forskolin-, isoproterenol-, or adenosine-induced increases in cAMP in *cp/cp* VSMCs (Fig. 2), which supports the concept that even relatively minor differences in PDE3 activity between different VSMCs can have marked effects on cAMP-dependent VSMC function such as migration.

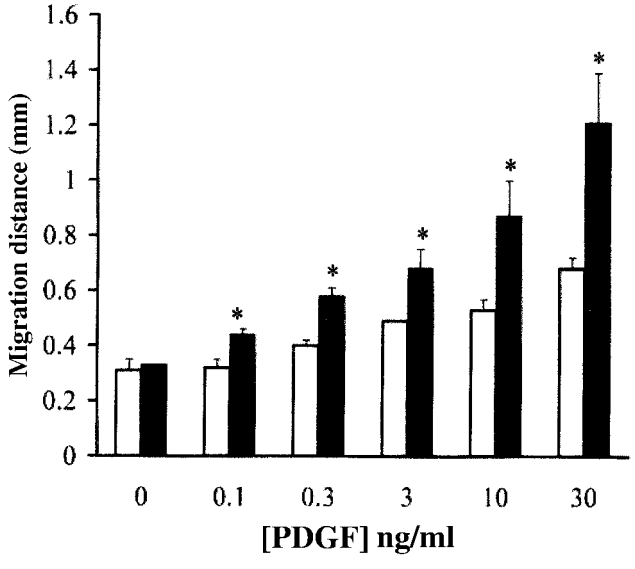

FIG. 3. PDGF-induced migration of +/? and *cp/cp* VSMCs. RIMAC assays were carried out using aggregates containing 10,000 VSMCs (+/? or *cp/cp*). Collagen-embedded VSMC aggregates were incubated in 100 μl Dulbecco's modified Eagle's medium supplemented with PDGF (0.1–30 ng/ml) in 96-well tissue culture plates. VSMCs were allowed to migrate for 18 h, after which they were fixed with 1% paraformaldehyde and stained with crystal violet. Stained cells were visualized by microscopy and digitally photographed, and the images were transferred from the camera to an IBM ThinkPad and converted to TIFF format with ScionImage software (Scioncorp, Frederick, MD). Migration was quantified by taking an average of the maximum migration of VSMCs along 64 radii superimposed on the digital image in ScionImage. The effect of each condition was tested in quadruplicate, and values obtained from the replicates were averaged to yield means and SD. □, +/? VSMCs; ■, *cp/cp* VSMCs.

DISCUSSION

Because an activated VSMC phenotype in obese *cp/cp* JCR:LA-cp rats predisposes these animals to the development of cardiovascular disease, our aim in this study was to determine if differences in arterial VSMC cAMP PDE activity between lean and obese JCR:LA-cp rats were involved in this activated phenotype. Overall, our findings are consistent with an increased role for PDE3 in the inactivation of cAMP in obese *cp/cp* rat arterial VSMCs and define PDE3 as a potential cellular activity responsible for this activated phenotype.

In this study, we confirmed previous work of others (34) indicating that at 6 months, the *cp/cp* rat aorta has higher PDE3 activity than age-matched lean +/? rat aorta. Although our findings were in general agreement with those reported previously, some differences were observed. Whereas Nagaoka et al. (34) described a twofold higher PDE3 activity in *cp/cp* aorta than in +/? aorta, a much more modest difference (~18%) was observed in our studies. Similarly, whereas Nagaoka et al., reported increased PDE4 activity in aorta of *cp/cp* animals, no such difference was observed in our experiments. Although the basis for these differences has not been fully elucidated, we suggest that the differences in total cAMP PDE activity determined for aorta isolated from 3-week-old and 6-month-old animals in the earlier report may have been a contributing factor. In this context, no age dependence of cAMP-PDE activity was seen in our experiments. Whereas the increase in *cp/cp* aortic PDE3 activity reported by Nagaoka et al. (34) correlated positively with an increased amount of PDE3A mRNA, no differences in PDE3A or PDE3B expression were observed in our experiments. Although PDE3A and PDE3B mRNA levels were not measured in our studies, based on our finding that similar amounts of these proteins were expressed in +/? and *cp/cp* aorta using five distinct PDE3 antibodies, we conclude that PDE3A and PDE3B expression is not different between these +/? and *cp/cp* tissues. Interestingly, we show that the difference in PDE3 activity in the aorta was maintained when aortic VSMCs were cultured from these

TABLE 5
Inhibition of PDGF-induced migration (mm) of +/?- and *cp/cp*-derived VSMCs by forskolin and impact of PDE3 inhibition

	+/? VSMCs Mean ± SD	% of effect of PDGF	*cp/cp* VSMCs Means ± SD	% of effect of PDGF
No stimulus	0.39 ± 0.02	—	0.40 ± 0.03	—
PDGF	0.60 ± 0.03	100	0.87 ± 0.04	100
PDGF + cilostamide	0.60 ± 0.03	100	0.80 ± 0.04	91
PDGF + Ro,201724	0.59 ± 0.02	98	0.79 ± 0.05	91
PDGF, cilostamide, + Ro,201724	0.56 ± 0.04	93	0.80 ± 0.03	91
PDGF + forskolin (1 μmol/l)	0.61 ± 0.02	102	0.88 ± 0.07	101
PDGF, forskolin (1 μmol/l), + cilostamide	0.58 ± 0.06	97	0.67 ± 0.04*	77†
PDGF, forskolin (1 μmol/l), + Ro,201724	0.54 ± 0.02	90	0.81 ± 0.02	93
PDGF, forskolin (1 μmol/l), cilostamide + Ro,201724	0.56 ± 0.03	93	0.59 ± 0.01*	68†
PDGF + forskolin (10 μmol/l)	0.59 ± 0.02	98	0.70 ± 0.04*	80†
PDGF, forskolin (10 μmol/l) + cilostamide	0.56 ± 0.05	93	0.41 ± 0.05*	47†
PDGF, forskolin (10 μmol/l) + Ro,201724	0.46 ± 0.04*	77†	0.52 ± 0.02*	60†
PDGF, forskolin (10 μmol/l), cilostamide, + Ro,201724	0.48 ± 0.02*	80†	0.36 ± 0.03*	41†

Data are means ± SD from nine individual determinations. The amount of PDGF added was 10 ng/ml; cilostamide, 1 μmol/l; and Ro,201724, 10 μmol/l. *Significant difference compared with migration caused by PDGF, $P < 0.05$. †Significant difference compared with effect of forskolin alone, $P < 0.05$.

animals. This fact lends credence to the hypothesis that the difference observed in the aorta is related to differences in aortic VSMCs, and that the differences in PDE3 activity detected between +/? and *cp/cp* VSMCs were intrinsic to these cells (40) and not due to the influence of blood-borne factors that could potentially influence PDE3 activity.

The molecular basis for the increase in particulate PDE3 activity in *cp/cp* VSMCs, in the absence of elevated expression of PDE3B in these cells, has not yet been fully elucidated, although we have tested several potential mechanisms. Based on our studies, it is unlikely that differences in PKA or PI3K-PKB signaling between these cells could account for the increase in particulate PDE3 detected in *cp/cp* VSMCs. Indeed, incubation of VSMCs with agents shown to activate PKA or PI3K-PKB, in both +/? and *cp/cp* VSMCs, had the expected impact on VSMC PDE4D activity and identical effects on PDE3 activity. Further studies, perhaps using cell types expressing significantly larger levels of PDE3, will be required to determine the molecular basis for the differences observed in this study. For example, if the increased particulate PDE3 activity seen in *cp/cp* VSMCs was due to aberrant targeting of this enzyme between the endoplasmic reticulum (41) or plasma membrane (42,43), levels of PDE3B in the cells used in this study would likely prohibit analysis of this event.

A novel, intriguing, and potentially important finding of our work is that the modest increase in particulate PDE3 activity detected in *cp/cp* VSMCs was associated with marked changes in the role of this enzyme in these cells. Whereas selective pharmacological inhibition of PDE3 had no effect on forskolin-, isoproterenol-, or adenosine-induced increases in cAMP in +/? VSMCs (results consistent with data reported previously using Wistar aortic VSMCs [36]), similar incubation of *cp/cp* VSMCs caused markedly different responses. Indeed, selective pharmacological inhibition of PDE3 in *cp/cp* VSMCs resulted in a large potentiation of the increase in cAMP caused by each of these agents. These results are all the more intriguing if one considers that PDE3 activity in VSMCs represents <30% of the total cAMP PDE activity in each of these populations of VSMCs. In addition, we found that the increase in PDE3 activity was limited to the subcellular fraction expressing only PDE3B, and not the more dominant PDE3A, expanding on earlier findings of Chini et al. (44) showing that individual cellular functions could be regulated in cAMP PDE-dependent fashions. Indeed, our work expands this finding to include differential effects of isozymes of the same cAMP PDE family.

Previous work has been reported indicating that *cp/cp* VSMCs exhibit an activated behavior in vivo characterized by an irregular shape and a propensity to migrate from the medial layer and invade the intimal layer (6,32). In addition, cultured *cp/cp* VSMCs display a hyperproliferative phenotype in response to several mitogens (33). We report here that *cp/cp* VSMCs are hypermotile in response to two chemotactic agents, IGF-1 and PDGF. The exaggerated response of *cp/cp* VSMCs to these factors is consistent with the earlier reports and adds motility and invasion of extracellular matrix to the list of exaggerated responses of these phenotypically activated cells. Although the molecular basis for the hypermotility of the *cp/cp* VSMCs has not been determined, from our studies a possible role for PDE3 is evident. Because pharmacological inhibition of PDE3 activity in *cp/cp* VSMCs markedly increases the antimigratory and invasion effects of forskolin in *cp/cp* VSMCs, but not +/? VSMCs, our data are consistent with a role for PDE3 in the hypermotile and invasive potential of these cells. Although the effectiveness of the PDE3 inhibitor was perhaps unexpected given the modest fraction of the total cAMP PDE activity that this agent inhibited, its impact was entirely consistent with its impact on VSMC cAMP levels.

In conclusion, our study provides a comprehensive analysis of cAMP PDE activity expressed in aortic VSMCs from lean and obese JCR:LA cp rats and describes a marked difference in the contribution of PDE3 to cAMP hydrolysis in aortic VSMCs isolated from the obese and lean animals. Moreover, we show that the *cp/cp* VSMCs display an exaggerated motility and invasiveness compared with +/? VSMCs that is sensitive to PDE3 inhibition. Because the JCR:LA-cp strain develops insulin resistance and hyperinsulinemia, is both glucose intolerant and hyperlipidemic, and has been an extensively studied model

of type 2 diabetes that develops cardiovascular disease, we believe our findings should stimulate further interest in PDE3 as a therapeutic target in continuing efforts to reduce diabetes-associated cardiovascular disease.

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