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Expression of cyclic GMP-inhibited phosphodiesterases 3A and 3B (PDE3A and PDE3B) in rat tissues: Differential subcellular localization and regulated expression by cyclic AMP

[1]H. Liu & [1,2,3]D.H. Maurice

Departments of [1]Pharmacology & Toxicology and [2]Pathology, Queen's University, Kingston, Ontario, Canada, K7L 3N6

1 A combination of pharmacological, molecular biological and biochemical approaches were used to investigate the differential expression of two cyclic GMP-inhibited cyclic nucleotide phosphodiesterase genes (PDE3A and PDE3B) in the rat.

2 RT–PCR using PDE3A- or PDE3B-specific oligonucleotide primers allowed amplification of products encoding PDE3A (508 bp) or PDE3B (499 bp) sequences from several rat tissues (heart, aorta, liver, kidney and epididymal fat), from primary cultures of aortic vascular smooth muscle cells (VSMC) as well as from an SV40 large T-antigen immortalized aortic VSMC line.

3 Immunoblotting experiments with PDE3-selective antisera allowed the detection of both PDE3A and PDE3B immunoreactive proteins in several rat tissues, including tissues of the cardiovascular system, in primary cultures of aortic VSMC and in an SV40 large T-antigen immortalized aortic VSMC line. In all cases, PDE3A was expressed as a 120 kDa protein which was only detected in the cytosolic fraction. PDE3B was expressed as a 135 kDa protein and its expression was limited to the particulate fraction of all tissues and cells studied.

4 Prolonged incubation of cultured aortic VSMC with agents that increase VSMC cyclic AMP (forskolin or 8-bromo-cyclic AMP) produced marked time-dependent increases in PDE3 activity which correlated with increases in PDE3A and PDE3B RT–PCR signals and a marked increase in particulate PDE3 activity and PDE3B protein.

5 The physiological, pharmacological and biochemical implications of these findings are discussed based on previous reports of the effects of PDE3 inhibitors in the cardiovascular system and the relevance of our findings are presented in the context of the development of PDE3A and/or PDE3B-selective pharmacological agents.

Keywords: Cyclic AMP; cyclic nucleotide phosphodiesterase; phosphodiesterase 3; vascular smooth muscle

Introduction

Cyclic AMP and cyclic GMP are ubiquitous second messengers which regulate multiple functions in cells (Lincoln, 1989; Murray, 1990; Sunahara *et al*., 1996; Mcdonald & Murad, 1996). Recently, an important role for cyclic nucleotide phosphodiesterases (PDE), the enzymes that catalyse the hydrolysis of the 3′-5′ phosphodiester bond of cyclic nucleotides and terminate cyclic nucleotide-mediated signalling, has been elaborated for several tissues (Loughney & Ferguson, 1996; Polson & Strada, 1996). To date, there are no fewer than ten distinct PDE families, with each identified on the basis of molecular sequence, substrate selectivity, modes of regulation and sensitivity to specific inhibitors (Bolger *et al*., 1993; Beavo, 1995; Conti *et al*., 1995; Manganiello *et al*.,1995a, b; Loughney & Ferguson, 1996; Degerman *et al*., 1997; Fisher *et al*., 1998; Soderling *et al*., 1998). In addition, each PDE family comprises multiple genes that can each yield several distinct gene products through alternate splicing, or the use of alternate promoters. Most mammalian tissues express members of multiple PDE families, and selected tissues can express several variants of individual families (Bolger *et al*., 1993; Beavo, 1995; Conti *et al*., 1995; Manganiello *et al*., 1995a,b; Loughney & Ferguson, 1996; Degerman *et al*., 1997).

[3]Author for correspondence at: Departments of Pathology and Pharmacology & Toxicology, PMAC-HRF/MRC Career Research Scientist in Health Sciences, Botterell Hall, A221, Queen's University, Kingston, Ontario, Canada, K7L 3N6

Phosphodiesterase 3 (PDE3), also called the cyclic GMP-inhibited phosphodiesterase, has been purified from many sources including platelets (Grant & Colman, 1984), heart (Harrison *et al*., 1986), liver (Pyne *et al*., 1987), adipose tissue (Rahn *et al*., 1996) and vascular smooth muscle cells (VSMC) (Lindgren *et al*., 1991; Rascon *et al*., 1992), though usually as a proteolytic fragment of the intact enzyme. More recently, molecular cloning studies have revealed that two distinct genes encode PDE3 activity (Meacci *et al*., 1992; Taira *et al*., 1993; Kasuya *et al*., 1995). Cloning of human and rat cDNAs for both these genes, HPDE3A or RPDE3A, (PDE3A) and HPDE3B or RPDE3B (PDE3B), reveals that they encode proteins with identical predicted structural organizations and highly homologous, though not identical, catalytic domains. Though cDNAs for both PDE3A and PDE3B predict 120–125 kDa proteins (Meacci *et al*., 1992; Taira *et al*., 1993), enzymes with molecular masses of 130–135 kDa or 105–110 kDa are identified in particulate, and cytosolic fractions of tissues that express PDE3 activity (Grant & Colman, 1984; Harrison *et al*., 1986; Lindgren *et al*., 1991; Rascon *et al*., 1992; Smith *et al*., 1993; Rahn *et al*., 1996). Expressions of full length recombinant PDE3A, or PDE3B, in heterologous expression systems, yield enzymes with very similar kinetics, though PDE3A has been shown to be more sensitive to inhibition by cyclic GMP, than PDE3B (Leroy *et al*., 1996).

PDE3 activity represents a substantial percentage of total cyclic AMP PDE activity in tissues such as heart, blood vessels and platelets and pharmacological inhibitors of PDE3 activity

such as milrinone or cilostamide are potent positive inotropes, vasodilators and inhibitors of platelet aggregation (reviewed in Bolger *et al*., 1993; Beavo, 1995; Conti *et al*., 1995; Manganiello *et al*., 1995a,b; Loughney & Ferguson, 1996; Degerman *et al*., 1997). Since PDE3A was initially cloned from human heart this PDE3 is often referred to as the 'cardiac' PDE3 (Meacci *et al*., 1992). Similarly, since PDE3B was initially cloned from adipose tissue, this enzyme is referred to as the 'adipose' PDE3 (Taira *et al*., 1993). Although initial evidence from Northern blotting and *in situ* hybridization suggested that tissue selective expression of these two gene products might be a defining characteristic for these genes, more recent studies have cast some doubt on this (Maurice *et al*., 1995; Lobbert *et al*., 1996). Based on our earlier work (Maurice *et al*., 1995), and more recent evidence of the presence of mRNA for PDE3B in hepatocytic and T cell lines (Murata *et al*., 1996; Ekholm *et al*., 1997), we chose to investigate if both PDE3A and PDE3B were expressed in several rat tissues with a particular focus on cardiovascular tissues. Our approach involved using a combination of strategies including selective pharmacological inhibition of PDE3 activity, RT–PCR and immunoblotting with PDE3-specific antisera.

Methods

Cell culture

Primary cultures of rat aortic VSMC were a generous gift from Dr S. Pang (Department of Anatomy and Cell Biology, Queen's University) following isolation from rat aortae as described previously (Pang & Venance, 1992) and were cultured as described (Rose *et al*., 1997). Primary rat aortic VSMC immortalized with SV40 large T-antigen were obtained from Dr J.J. Castellot, Tufts University and maintained in culture as described previously (Caleb *et al*., 1996).

Treatment of cultured aortic VSMC with pharmacological agents

At confluence (3–4 days), culture media was removed and replaced with 1 or 5 ml of fresh culture media supplemented with either (i) forskolin (1–100 μM), (ii) 8-bromo-cyclic AMP (0.1–1 mM), or (iii) vehicle (0.1% dimethylsulphoxide, (DMSO)). After 4, 8 or 16 h, cells were harvested in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM benzamidine, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg ml^{-1} leupeptin and 1% Triton X-100. A $3000 \times g$ supernatant of the cell lysate was transferred to microtubes and stored at 4°C, or frozen at −70°C, until needed. In experiments in which cytosolic ($100{,}000 \times g$ supernatant) and particulate ($100{,}000 \times g$ pellet) fractions were prepared, Triton X-100 was omitted from the lysis buffer.

Assay of cyclic AMP phosphodiesterase activity

Cyclic nucleotide phosphodiesterase activity was assayed by a modification of the method of Davis & Daly (1979), as described previously (Rose *et al*., 1997), using 1 μM [^{3}H]-cyclic AMP as substrate. The product of the reaction ([^{3}H]-5′ AMP) was purified and quantified by liquid scintillation counting following correction for recovery of an internal standard (1200 d.p.m. of [^{14}C]-5′ AMP) and normalized to the total protein used in the assay. PDE3 activity was determined using 1 μM cilostamide, a concentration of this PDE3 inhibitor shown previously to specifically inhibit PDE3 activity in these tissues (Rose *et al*., 1997). Protein concentrations of samples were determined using the BCA Protein Assay system from Pierce, according to the manufacturer's methodology using bovine serum albumin as standard.

Reverse transcription-polymerase chain reaction

Rat tissues were harvested from sodium pentobarbital anaesthetized male Wistar rats (250–350 g body weight; Charles River) and pulverized in liquid nitrogen, while cultured rat aortic VSMC were processed directly from the tissue culture flasks. In both cases RNA was purified using the TRIzol Reagent. First strand cDNA was generated from 10 μg of total RNA using oligo(dT)$_{18}$ to prime reverse transcription (SuperScript Moloney murine leukaemia virus (MMLV) reverse transcriptase). Amplification was routinely performed using Taq DNA polymerase on 1/100th of the first strand reaction and 20 pmol each of selective PDE3A or PDE3B sense and antisense oligonucleotide primer pairs (Table 1). Conditions for PCR were 30 s at 95°C, 30 s at 58°C and 1 min at 72°C for 35 cycles (for non-quantitative amplification of either PDE3A or PDE3B), or for 18 cycles (PDE3A) or 28 cycles (PDE3B) for amplification within the linear range for each product. The number of PCR cycles which allowed linear amplification of PDE3A and PDE3B were determined empirically for each cDNA preparation. PCR products were separated by electrophoresis on 1.5% agarose gels, visualized with ethidium bromide under u.v. light and purified using Geneclean. Each analysis was repeated at least three times with RNA isolated from separate animals or cultures of VSMC. PCR products were sequenced as described previously (Maurice *et al*., 1995).

Immunoblotting

Two rabbit polyclonal PDE3 antisera were used in these studies. One of these antisera was raised against purified human platelet PDE3, a cell type which expresses a cytosolic PDE3 previously shown to be PDE3A. The second antisera was raised against a bacterially expressed fusion protein containing a carboxyl-terminal region of a recently cloned mouse PDE3B and the glutathione-binding region of glutathione-S-transferase (Zhao *et al*., 1997). For immunoblotting, samples (rat tissue homogenates ($3000 \times g$ supernatant), tissue homogenate subcellular fractions ($100{,}000 \times g$ cytosolic or particulate fractions), cultured rat aortic VSMC homogenates ($3000 \times g$ supernatant) or 3T3-L1 cell homogenates ($3000 \times g$ supernatant) were generated by homogenization in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM EGTA, 5 mM benzamidine, 1 μg ml^{-1} aprotinin and 1 μg ml^{-1} leupeptin and centrifugation. In some experiments, medial and adventitial cell layers of the rat aorta were isolated (Pang & Venance, 1992) and processed independently. Samples (5–40 μg) were subjected to SDS–PAGE 7.5–10% polyacrylamide gels at 100V. Following electrophoresis, proteins were transferred to nitrocellulose membranes and the membranes blocked by incubation with TBST (20 mM TrisHCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) supplemented with 5% powdered Nonfat milk, or 3% gelatin, overnight. Blots were incubated with an appropriate dilution of primary antibody (polyclonal anti-platelet PDE3, 1 : 1000; polyclonal anti-PDE3B-GST fusion protein, 1 : 5000) for 2 h and rinsed three times with TBST. Rinsed blots were incubated with horse radish peroxidase conjugated goat anti-rabbit IgG (1 : 3000

dilution) for 2 h, rinsed with TBST and immunoreactivity was detected by chemiluminescence as per manufacturers' recommendations. Immunoreactive proteins detected by immunoblot were quantitated by scanning densitometry using Corel Photo-Paint 7.0 Software as per manufacturers recommendations and the most abundant protein on each blot analysed was arbitrarily given a relative intensity score of 1.0.

3T3-L1 preadipocyte differentiation

The 3T3-L1 preadipocytic cell line was obtained from American Type Tissue Culture Collection (CCL92.1). Differentiation of 3T3-L1 preadipocytes to 3T3-L1-adipocytes was achieved as previously reported (Raptis *et al*., 1997). Briefly, approximately 1000 cells were seeded into each well of a 24-well Linbro plate in 10% FCS. At confluence, defined as high cell density with extensive cell-to-cell contacts, cultures were treated with differentiation medium containing insulin and dexamethasone. Lipid droplets which were indicative of differentiation appeared approximately 10 days later. Lipid droplets were visualized using Oil Red-O staining (Lu *et al*., 1990).

Statistical analysis

Cyclic nucleotide phosphodiesterase activity data are presented as mean ± standard error of the mean (s.e.mean) of at least three independent experiments. Within each experiment, values were means from at least three individual determinations for each experimental condition. The quantification of the abundance of immunodetected PDE3A or PDE3B is presented as relative optical densities (Rel. Int.) of representative immunoblots. All immunoblot analysis of rat tissue homogenates, or cells, were carried out at least three times on independent tissue extracts or cell homogenates. Statistical differences between cyclic AMP PDE activities were determined using the Student's *t*-test for either paired or unpaired samples with $P<0.05$ considered significant.

Materials

Tissue culture reagents (DMEM, calf serum, HEPES, penicillin/streptomycin, HBSS, trypsin-EDTA), SuperScript reverse transcriptase, TRIzol Reagent and Taq DNA polymerase were from GIBCO BRL, Ontario, Canada. Radioactive products were from NEN Life Science Products, Massachusetts, U.S.A., ([^{14}C]-cyclic AMP, [^{3}H]-cyclic AMP). Isobutyl methylxanthine (IBMX) was from the Aldrich Chemical Company, Ontario, Canada. Forskolin, 8-bromo-cyclic AMP and cilostamide were from Research Biochemicals International, Massachusetts, U.S.A., while Tris-HCl, benzamidine, EDTA, EGTA, DTT, PMSF, Triton X-100 and NaCl were from ICN Biomedicals Incorporated, Quebec, Canada. Leupeptin was from Boehringer Mannheim, Quebec, Canada. Affi-gel 601, column supports and nitrocellulose blotting paper were from BioRad, Ontario, Canada. The BCA protein assay and bovine serum albumin were from Pierce, Ontario, Canada. Renaissance Western Blot Chemiluminescence Reagent Plus was from NEN Life Sciences. All other chemicals were of reagent grade and purchased from Fisher Scientific, Ontario, Canada.

Results

Steady state levels of PDE3A and PDE3B mRNA in rat tissues and cultured cells

PDE3A and PDE3B expression was investigated by RT–PCR using PDE3A or PDE3B specific primers (Table 1), and oligo(dT) reverse-transcribed RNA isolated from several rat tissues, or from cultured rat aortic VSMC. Following 35 cycles of amplification, PCR products encoding PDE3A (508 bp) or PDE3B (499 bp) sequences were readily amplified from all rat tissues studied. As was the case with RNA isolated from rat tissues, RT–PCR with RNA isolated from primary rat aortic VSMC cultures (Figure 1c) or from an SV40 large T-antigen immortalized rat aortic VSMC cell line (TEX-18) (not shown) resulted in the amplification of both PDE3A and PDE3B PCR

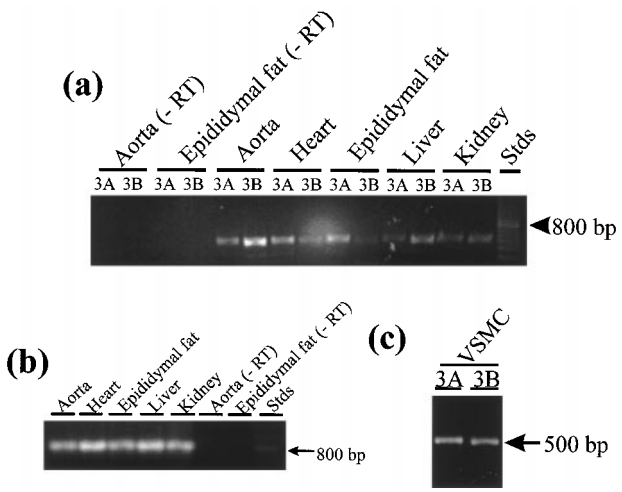


Figure 1 RT–PCR amplification of PDE3A and PDE3B in rat tissues and cultured aortic VSMC. PCR reactions were carried out as described in Methods. Briefly, PCR reactions with an aliquot (1 μl) of the first strand reactions of RNA isolated from selected rat tissues (a), or cultured rat aortic VSMC (c), were carried out using 20 pmol of both sense and antisense oligonucleotide primers (Table 1) for PDE3A or PDE3B (a, c) or GAPDH (b). PCR products from 100 μl reactions for PDE3A (15 μl), PDE3B (50 μl) or GAPDH (2 μl) were electrophoretically resolved on 2% agarose gels and ethidium bromide staining viewed using u.v. light.

Table 1 Primers used for RT–PCR of RNA from rat aortic VSMC and rat tissues

cDNA (GenBank accession number)	*Primer Pairs*[a]	*Nucleotides*
Rat PDE3A	5′-CCGAATTCCCTTATCATAACAGAATCCACGCCACT-3′	2248–2274
(U38179)	5′-GGGAATTCGTGTTTCTTCAGGTCAGTAGCC-3′	2718–2739
Rat PDE3B	5′-CCGAATTCTATCACAATCGTGTGCATGCCACAGA-3′	2216–2241
(Z22867)	5′-CCGAATTCTTTGAGATCTGTAGCAAGGATTGC-3′	2674–2698
Rat GADPH	5′-GTTGCCATCAACGACCCCTT-3′	115–134
(M17701)	5′-AGCATCAAAGGTGGAGGAATG-3′	895–915

[a]Underlined nucleotides are designed for introducing an *Eco*RI site in the PCR products.

products. Reactions carried out using RNA that had not been reverse transcribed yielded no products (Figure 1, (−RT)) and sequencing of the PDE3A and PDE3B RT–PCR generated products showed that they were identical to the previously published sequences for rat PDE3A and PDE3B (Table 2) (Meacci *et al*., 1992; Taira *et al*., 1993; Maurice *et al*., 1995). Results similar to those shown in Figure 1 were obtained using RNA isolated from tissues harvested from six separate rats and from five separate aortic VSMC cultures.

Steady state levels of PDE3A and PDE3B protein in rat tissues and cultured cells

The PDE3A and PDE3B RT–PCR data was consistent with a broad tissue distribution of both PDE3A and PDE3B in rat tissues and cultures of aortic VSMC. In order to further test this hypothesis, immunoblotting studies with PDE3 antisera were carried out. One of these, anti-PDE3A, was raised against purified human platelet PDE3, a cell type known to express a

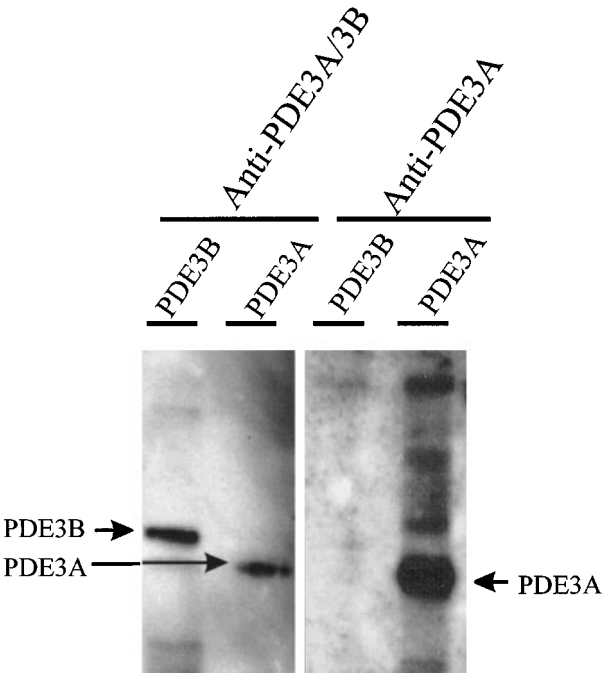
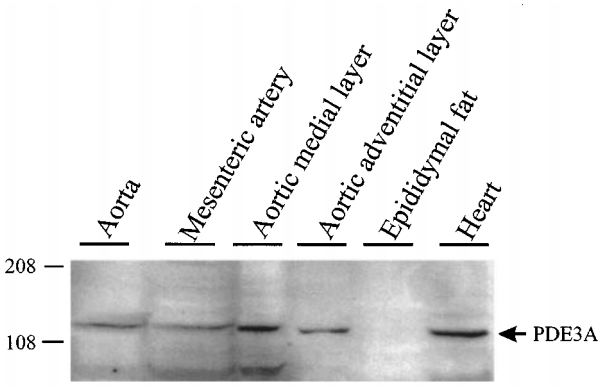


Figure 2 Reactivity of PDE3A and PDE3A/3B antisera with yeast-expressed human recombinant PDE3A and PDE3B. Lysates of yeast expressing human PDE3A (1 μg), or PDE3B (1 μg), were electrophoretically resolved, transfered to nitrocellulose membranes and blotted with either the anti-PDE3A antisera (1 : 1000) or the anti-PDE3A/3B antisera (1 : 5000) and goat anti-rabbit IgG. Immunoreactive proteins were detected by chemiluminescence (see Methods).



Homogenate (μg per lane)	10	10	10	10	10	10
cAMP PDE (pmol.min^{-1})	1.8	1.7	1.9	1.7	1.8	8.2
PDE3 (pmol.min^{-1})	0.8	0.8	1.0	0.8	2.0	4.1
PDE3A (Rel.Int.)	0.6	0.4	0.9	0.4	0	1.0

Figure 3 Immunoblot analysis of PDE3A in rat tissues. Homogenates (10 μg of protein) of various rat tissues were resolved electrophoretically, transfered to nitrocellulose and blotted using anti-PDE3A antisera at a 1 : 1000 dilution, goat anti-rabbit IgG, and immunoreactive proteins were detected by chemiluminescence (see Methods). Total cyclic AMP PDE activity was determined using 1 μM [^{3}H]-cyclic AMP as substrate and PDE3 activity taken as the amount of cyclic AMP PDE activity inhibited by 1 μM cilostamide (see Methods). Immunoblots were quantitated by scanning densitometry using Corel Photo-Paint 7.0 and the most prominant immunoreactive band designated a relative intensity score of 1.0 (see Methods).

Table 2 Sequences of PDE3A and PDE3B PCR-amplified products from rat aortic VSMC

Rat PDE3A

CCGAATTCCCTTATCATAACAGAATCCACGCCACTGATGTTTTGCACGCCGTGTGGTA
TCTCACAACACAGCCGATTCCTGGCCTCCCGAGTGTGATTGGTGATCACGGCTCGG
CAAGTGACTCTGATTCTGACAGTGGGTTTACACACGGACACATGGGATATGTGTTTT
CCAAAGCGTATCATGTGCCAGATGACAAATATGGATGCCTGTCTGGAAATATTCCA
GCCCTGGAGTTGATGGCCCTGTATGTTGCTGCAGCCATGCATGACTACGATCACCCA
GGAAGGACAAATGCTTTCCTGGTTGCCACTAGCGCCCCTCAGGCCGTGCTGTACAATG
ACCGTTCCGTTCTGGAGAACCATCACGCAGCTGCAGCCTGGAATCTCTTCATGTCCCG
GCCGGAGTATAACTTCTTAGTTAACCTGGACCATGTGGAATTTAAGCACTTCCGA
TTCCTAGTCATTGAAGCAATTCTGGCTACTGACCTGAAGAAACAC**GAATTCCC**

Rat PDE3B

CCGAATTCTATCACAATCGTGTGCATGCCACAGATGTCCTACATGCTGTTTGGTATT
TGACAACACGACCAATTCCTGGCTTACAGCAGCTCCATAATAACCATGAAACAGAAA
CCAAAGCAGATTCAGATGCTAGACTTAGTTCTGGACAGATTGCTTACCTTTCTTCGAA
GAGTTGCTGTATTCCAGATAAGAGTTATGGCTGCCTGTCTTCAAACATTCCTGCGTTA
GAACTGATGGCTTTATATGTGGCAGCTGCCATGCACGATTATGATCACCCAGGAAGA
ACAAATGCATTCCTAGTGGCTACAAATGCACCTCAGGCAGTTTTATACAATGACAGA
TCTGTTCTAGAAAATCATCATGCCGCATCAGCGTGGAATCTGTATCTTTCTCGCCCAG
AATACAACTTCCTCCTTAACCTTGATCACATGGAATTCAAGCGTTTTCGATTTTTAGT
CATAGAAGCAATCCTTGCTACAGATCTCAAA**GAATTCGG**

RT–PCR products were obtained from total RNA isolated from cultured rat aortic VSMC as described in Methods. Engineered *Eco*RI sites are underlined.

cytosolic PDE3A, while the other, anti-PDE3A/3B, was raised against a carboxyl-terminal portion of a recently cloned mouse PDE3B. The specificity of these antisera was tested by immunoblotting against lysates of yeast expressing either recombinant human PDE3A or PDE3B (Figure 2). In these experiments the anti-PDE3A antisera reacted strongly and specifically with the yeast expressed recombinant human PDE3A, but not with yeast-expressed recombinant PDE3B, while the second, anti-PDE3A/3B, reacted strongly with both yeast-expressed recombinant human PDE3A and PDE3B (Figure 2).

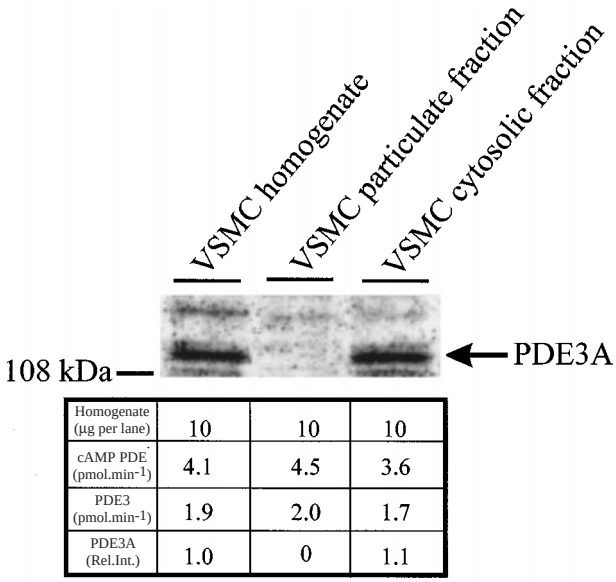
Immunoblots of 10 μg of various rat tissue homogenates with the anti-PDE3A-selective antisera allowed the identification of a 120 kDa PDE3A in several rat tissues including heart, aorta, aortic medial and adventitial layers, mesenteric artery as well as in cultured rat aortic VSMC, but not in epididymal fat (Figure 3). Although proteins smaller than the 120 kDa PDE3A were detected on some immunoblots, this antisera did not detect immunoreactive proteins larger than 120 kDa in any tissue or cell type studied. When equivalent amounts of homogenate from these tissues were blotted, densitometric analysis revealed that the 120 kDa PDE3A was most abundant in heart, with this tissue expressing twice the amount detected in aorta (Figure 3). This finding is consistent with the relative amount of PDE3 activity present in these two tissues (Table 3). Immunoblotting of subcellular fractions of cultured aortic VSMC homogenates demonstrated that the 120 kDa PDE3A was entirely cytosolic (Figure 4), with no anti-PDE3A immunoreactivity detected in the $100{,}000 \times g$ pellet (Figure 4). Similar results were obtained when cytosolic and particulate fractions of aorta or heart were immunoblotted with the anti-PDE3A antisera (not shown).

Immunoblots of rat tissues and of cultured VSMC with the anti-PDE3A/3B antisera allowed detection of two immunoreactive proteins with molecular weights of 120 kDa and 135 kDa, respectively (Figure 5). Binding of the anti-PDE3A/3B antibody with both proteins was efficiently competed by addition of the antigen, PDE3B-GST, but not by addition of an equivalent amount of another bacterially expressed GST fusion protein, cdc42-GST (Figure 6). Based on tissue expression, electrophoretic mobility and subcellular distribution, the 120 kDa protein detected with this antisera was indistinguishable from the 120 kDa PDE3A detected with the anti-PDE3A-selective antibody. Thus, the protein was expressed in heart, platelets, aorta and aortic VSMC (Figure 5) with roughly equivalent amounts detected in platelets and aorta. Subcellular fractionation of homogenates of aortic VSMC (Figure 5) or aorta (Figure 4) showed that both

Table 3 Total cyclic AMP PDE and PDE3 activity of rat aorta and heart

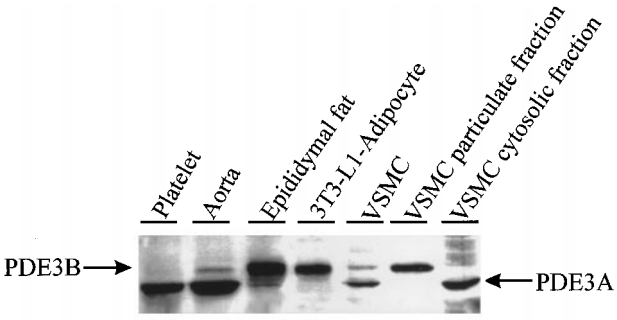
	cyclic AMP PDE activity (nmol min^{-1})	
Tissue	*No addition*	*1 μM cilostamide*
Aorta		
$3000 \times g$ supernatant	7.7±1.3	3.8±0.4
$100{,}000 \times g$ supernatant	6.3±0.7	3.1±0.5
$100{,}000 \times g$ pellet	0.6±0.0	0.2±0.0
Heart		
$3000 \times g$ supernatant	11.8±1.9	5.9±0.9
$100{,}000 \times g$ supernatant	11.4±2.7	6.2±1.0
$100{,}000 \times g$ pellet	0.3±0.0	0.1±0.0

Values are mean±s.d. of four experiments. Each was carried out in triplicate with identical amounts of fractional protein.



Homogenate (μg per lane)	10	10	10
cAMP PDE (pmol.min^{-1})	4.1	4.5	3.6
PDE3 (pmol.min-1)	1.9	2.0	1.7
PDE3A (Rel.Int.)	1.0	0	1.1

Figure 4 Subcellular expression of PDE3A in cultured rat aortic VSMC. Equal amounts (10 μg) of VSMC lysates ($3000 \times g$ supernatant), isolated cytosolic ($100{,}000 \times g$ supernatant), or particulate ($100{,}000 \times g$ pellet) fractions were electrophoretically resolved, transfered to nitrocellulose membranes and blotted with anti-PDE3A antisera (1 : 1000), goat-anti-rabbit IgG and immunoreactive proteins detected by chemiluminescence (see Methods). Total cyclic AMP PDE activity was determined using 1 μM [^{3}H]-cyclic AMP as substrate and PDE3 activity taken as the amount of cyclic AMP PDE activity inhibited by 1 μM cilostamide (see Methods). Immunoblots were quantitated by scanning densitometry using Corel Photo-Paint 7.0 and the most prominant immunoreactive band designated a relative intensity score of 1.0 (see Methods).



Homogenate (μg per lane)	25	25	25	25	18	40	40
cAMP PDE (pmol.min^{-1})	ND	17.4	11.8	8.3	1.8	6.7	5.5
PDE3 (pmol.min-1)	ND	9.4	7.2	5.8	0.4	1.6	1.5
PDE3A (Rel.Int.)	0.8	1.0	0.2	0	0.3	0	0.5
PDE3B (Rel.Int.)	0	0.1	1.0	0.6	0.1	0.5	0

Figure 5 Immunoblot analysis of PDE3A and PDE3B expression in rat tissues and cultured rat aortic VSMC. Homogenates ($3000 \times g$ supernatant) or resolved subcellular fractions of several rat tissues or of rat aortic VSMC were electrophoretically resolved, transfered to nitrocellulose, blotted sequentially with anti-PDE3A/3B (1 : 5000) and goat-anti-rabbit IgG and immunoreactive proteins detected by chemiluminescence as per manufacturer's recomendations (see Methods). Total cyclic AMP PDE activity was determined using 1 μM [^{3}H]-cyclic AMP as substrate and PDE3 activity taken as the amount of cyclic AMP PDE activity inhibited by 1 μM cilostamide (see Methods). Immunoblots were quantitated by scanning densitometry using Corel Photo-Paint 7.0 and the most prominant immunoreactive band for PDE3A, or PDE3B, designated a relative intensity score of 1.0 respectively (see Methods).

120 kDa proteins were exclusively cytosolic. Similarly, based on tissue expression, electrophoretic mobility and subcellular distribution, the 135 kDa immunoreactive protein was shown to be PDE3B. Thus, this protein was highly expressed in epididymal fat and differentiated 3T3-L1 adipocytes (Elks *et al*., 1983) (Figure 7a), but not in platelets or 3T3-L1 preadipocytes (Figure 5 and 7a), cell types known not to express PDE3B. In addition, immunoblotting of cytosolic and particulate fractions of epididymal fat (Figure 7b) or aortic VSMC homogenates (Figure 5) showed the 135 kDa protein to be exclusively particulate.

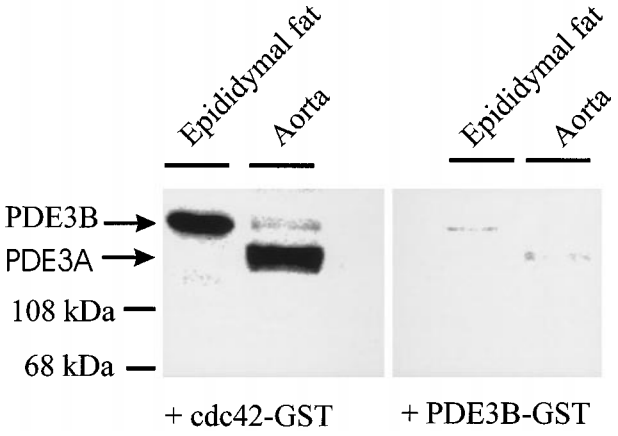


Figure 6 Specificity of PDE3A/3B antisera. Homogenates ($3000 \times g$ supernatant) of rat epididymal fat or of rat aorta were electrophoretically resolved, transfered to nitrocellulose. Prior to addition to the nitrocellulose membranes, anti-PDE3A/3B antisera (1 : 5000) was preincubated with 200 ng of either purified recombinant cdc42-GST, or purified recombinant PDE3B-GST. Following this pre-incubation step, anti-PDE3A/3B antisera was incubated with nitrocellulose membranes and immunoreactive proteins detected using goat-anti-rabbit IgG and chemiluminescence (see Methods).

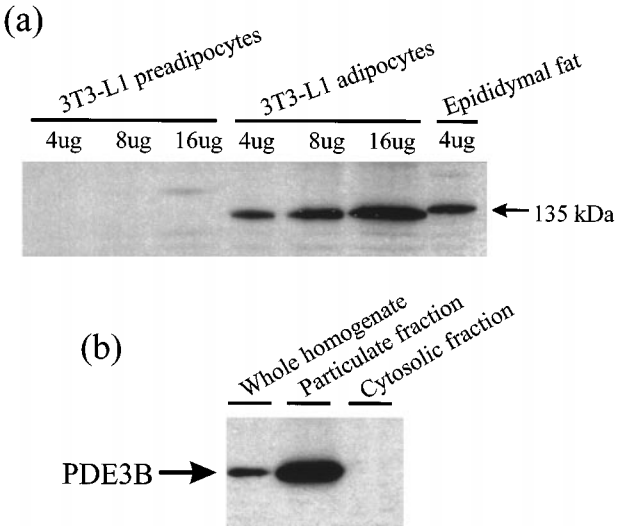


Figure 7 Up-regulation of PDE3B expressing during 3T3-L1 preadipocytic differentiation (a) and subcellular distribution of epididymal fat PDE3B. (a) Homogenates ($3000 \times g$ supernatant) of 3T3-L1 preadipocytes and 3T3-L1 adipocytes, or of epididymal fat, were electrophoretically resolved, transfered to nitrocellulose, blotted sequentially with anti-PDE3A/3B (1 : 5000) and goat-anti-rabbit IgG and immunoreactive proteins detected by chemiluminescence as per manufacturer's recommendations (see Methods). (b) An epididymal fat lysate was fractionated and the cytosolic ($100{,}000 \times g$ supernatant) and particulate ($100{,}000 \times g$ pellet) fractions individually resolved electrophoretically, blotted to nitrocellulose and probed as described in (a).

In tissues expressing both the 120 kDa PDE3A and the 135 kDa PDE3B (epididymal fat, aorta, cultured aortic VSMC, heart), marked difference in the relative amounts of these two proteins were observed (for example see Figure 5). Thus, densitometric analysis of seven immunoblots of rat tissues and cell lines demonstrated that whereas epididymal fat contained 12 times more of the particulate 135 kDa PDE3B than the cytosolic 120 kDa PDE3A, rat aorta expressed ten times more of the cytosolic 120 kDa PDE3A than the particulate 135 kDa PDE3B. Again, consistent with the identification of the cytosolic 120 kDa protein as PDE3A and the particulate 135 kDa protein as PDE3B, these ratios of cytosolic and particulate PDE3 expression are consistent with the relative abundance of PDE3 activities present in these fractions in rat aorta (Table 3) and epididymal fat (Degerman *et al*., 1997). The amount of particulate PDE3B expressed in aorta and in cultured VSMC were roughly equal.

Effects of forskolin or 8-bromo-cyclic AMP on PDE3 activity and expression in VSMC

In order to assess the potential relevance of the co-expression of both PDE3A and PDE3B in VSMC we studied the effects of prolonged incubations of cultured rat aortic VSMC with cyclic AMP-elevating agents, a treatment protocol which we have shown previously increases cyclic AMP PDE activity in these cells (Rose *et al*., 1997). In addition, since results of immunoblotting studies indicated that PDE3A was the cytosolic PDE3 and that PDE3B was the particulate PDE3 in VSMC, we further studied the effect of these treatments on PDE3 activity and expression in these two subcellular fractions. While prolonged incubations (4–16 h) of cultured rat aortic VSMC with forskolin, an activator of adenylyl cyclases, or with 8-bromo-cyclic AMP, a lipophilic analogue of cyclic AMP, increased total cyclic AMP PDE activity (Figure 8a) and total PDE3 activity (Figure 8b), this treatment had markedly different effects on cytosolic and particulate PDE3 activities (Figure 8b). Indeed, in these experiments cytosolic PDE3 decreased during the first 8 h of incubation and then increased during the subsequent 8 h of incubation (Figure 8), whereas particulate PDE3 activity increased linearly in a time-dependent fashion. Thus, in the five experiments in which it was measured, cytosolic PDE3 activity in cells treated with 8-bromo-cyclic AMP was shown to have decrease to $20 \pm 12\%$ of initial values after 8 h and then to increase to approximately $140 \pm 22\%$ of initial values after 16 h. In contrast to the biphasic effects observed when cytosolic PDE3 activity was measured, incubation of VSMC with either forskolin or 8-bromo-cyclic AMP caused a linear time-dependent increase in particulate PDE3 activity which was maximal at the 16 h time point. Thus, in the five experiments in which it was measured treatment of VSMC with 8-bromo-cyclic AMP for 16 h caused a $800 \pm 55\%$ increase in particulate PDE3 activity when compared to control untreated cells.

In order to determine if the changes in PDE3 activity observed in these two subcellular fractions resulted from differential effects of cyclic AMP on PDE3A and PDE3B, changes in the steady state levels of PDE3A or PDE3B were determined. Results of immunoblots of homogenates of control or treated VSMC showed that expression of PDE3A and PDE3B were differentially affected by increasing VSMC cyclic AMP. Thus, when homogenates of VSMC incubated with cyclic AMP-elevating agents for 8 h were probed with the PDE3A/3B antisera, PDE3A (120 kDa) was decreased in

VSMC, while PDE3B (135 kDa) was increased (Figure 9). At 16 h, the amount of PDE3A expressed in VSMC were seen to have returned to basal levels while those of PDE3B were still increased relative to basal (Figure 10). In order to unequivocally discriminate between the effects of cyclic AMP-elevating agents on PDE3A and PDE3B expression in VSMC, identical samples of control and treated VSMC were electrophoretically resolved on the same SDS–PAGE gel and subsequently probed with either the PDE3A-selective antibody or the PDE3A/3B antibody (Figure 10). Since the PDE3A antibody used reacted strongly with a single protein of 120 kDa in control and forskolin-treated VSMC homogenates, which after 16 h had not changed relative to basal, the 135 kDa protein identified in these samples when probed with the PDE3A/3B antisera, and which was elevated by cyclic AMP, cannot be attributed to a species of PDE3A expressed in these cells. In the five experiments in which it was measured, levels of PDE3A detected in immunoblots of VSMC treated for 8 h with 8-bromo-cyclic AMP were shown to be $67 \pm 20\%$ lower than those found in control untreated cells, while PDE3A levels after incubation for 16 h with this agent were not different from control untreated cells, $110 \pm 22\%$. In contrast, in the five experiments in which it was measured, levels of PDE3B immunoreactive protein were significantly elevated from basal values at all time points studied (for example Figure 9). Thus, incubation of VSMC with 10 μM forskolin for 16 h caused a $600 \pm 60\%$ increase in VSMC particulate PDE3B. Consistent with an increased expression of PDE3A and PDE3B underpinning the increased levels of these proteins at 16 h, $302 \pm 58\%$ and $280 \pm 39\%$ more PDE3A and PDE3B PCR products were obtained when RNA was isolated from forskolin, or 8-bromo-cyclic AMP, than from control. In addition, we have demonstrated previously that the effects of prolonged treatment with either forskolin or 8-bromo-cyclic AMP are inhibited by actinomycin D and cycloheximide (Rose *et al*., 1997). Similar results were obtained when 8-bromo-cyclic AMP was used to elevate cyclic AMP (Figure 11). Although PDE3A immunoreactivity detected in cells incubated with cyclic AMP-elevating agents was decreased at the 8 h time point in our studies, the amount of PDE3A PCR product obtained from cells treated for 8 h was slightly elevated ($50 \pm 20\%$) when compared to control.

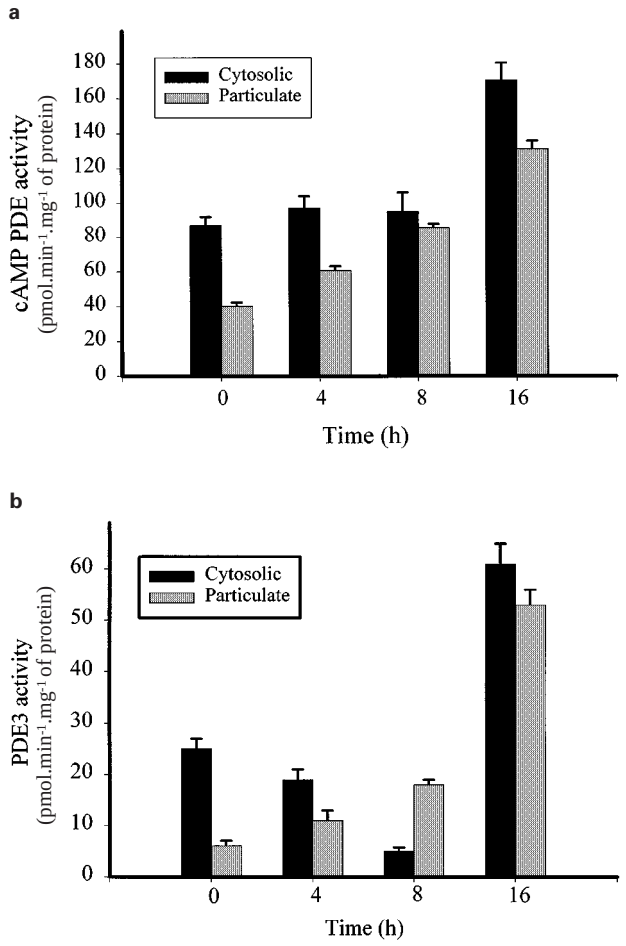
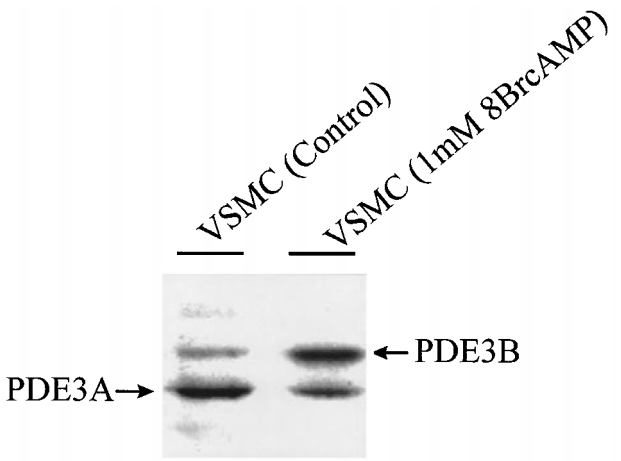


Figure 8 8-bromo-cyclic AMP-mediated increases in cyclic AMP PDE and PDE3 activity in cultured rat aortic VSMC. Confluent rat aortic VSMC were incubated with fresh culture media supplemented with 1 mM 8-bromo-cyclic AMP for 4, 8, or 16 h. At the end of this incubation, VSMC were rinsed with HBSS and subsequently lysed in ice cold lysis buffer supplemented with 1% Triton X-100 (see Methods). $3000 \times g$ VSMC supernatants of VSMC lysates were centrifuged for 1 h at $100{,}000 \times g$ and cytosolic (supernatant) and particulate (pellet) fractions were separated and cyclic AMP PDE activities measured using 1 μM [^{3}H]-cyclic AMP as substrate (see Methods). Values are total cyclic AMP PDE (a) or PDE3 (b) activities and PDE3 activity was determined using 1 μM cilostamide for PDE3 inhibition. Values represent mean $\pm$ s.e.mean of three experiments, each was carried out in triplicate.



Homogenate (μg per lane)	18	18
cAMP PDE (pmol.min^{-1})	1.8	4.3
PDE3 (pmol.min^{-1})	0.4	1.0
PDE3A (Rel.Int.)	0.3	0.1
PDE3B (Rel.Int.)	0.1	0.5

Figure 9 Different changes in PDE3A and PDE3B levels following treatment with forskolin for 8 h. Homogenates ($3000 \times g$) of cultured aortic VSMC incubated with saline (Control) or 1 mM 8-bromo-cyclic AMP for 8 h were electrophorectically resolved, transfered to a nitrocellulose membrane and blotted with anti-PDE3A/3B (1 : 5000), goat-anti-rabbit IgG and immunoreactive bands detected by chemiluminescence as per manufacturer's recommendations (see Methods). Total cyclic AMP PDE activity was determined using 1 μM [^{3}H]-cyclic AMP as substrate and PDE3 activity taken as the amount of cyclic AMP PDE activity inhibited by 1 μM cilostamide (see Methods). Immunoblots were quantitated by scanning densitometry using Corel Photo-Paint 7.0 and the most prominant immunoreactive band designated a relative intensity score of 1.0 (see Methods).

Discussion

Prior research has suggested that PDE3A is expressed in most tissues of the cardiovascular system, whereas PDE3B expression is limited to fat and perhaps certain hepatocytic and lymphoid cell lines (Murata *et al*., 1996; Ekholm *et al*., 1997; Degerman *et al*., 1997). In this study we have used a combination of molecular biological, biochemical and pharmacological approaches to examine PDE3 expression in several rat tissues with an emphasis on cardiovascular tissues. The data presented in this manuscript is consistent with the hypothesis that products of the PDE3A and the PDE3B genes are both expressed in several tissues of the rat including liver, kidney, epididymal fat, heart, aorta, and cultured aortic VSMC. In addition, our data demonstrates that the products of these two PDE3 genes are expressed in different subcellular fractions and that they are differentially expressed in response to increases in cellular cyclic AMP. First, PCR products for both PDE3A and PDE3B were readily amplified from RNA isolated from a host of rat tissues and from isolated rat aortic VSMC cultures. The formal possibility that the PDE3B PCR products obtained from rat tissues were derived from adipocytes present in the isolates was addressed using primary cultures of aortic VSMC as well as an extensively characterized SV40 large T-antigen immortalized rat aortic VSMC cell line (TEX-18) (Caleb *et al*., 1996). Since adipocytes are not present in cultures of rat aortic VSMC (Pang & Venance, 1992) and that the SV40 large T-antigen immortalized aortic cell line used was clonal, amplification of PDE3A and PDE3B RNA from these sources establishes that PDE3A and PDE3B mRNA is present in VSMC. Moreover, amplification of both PDE3A and PDE3B mRNA from the clonal VSMC cell line demonstrates that individual smooth muscle cells can express both these gene products. Consistent with previous reports, and the RT–PCR data, using a PDE3A-selective antisera a 120 kDa PDE3A was detected in homogenates of heart, platelet and blood vessels (aorta and mesenteric artery), with heart expressing significantly more of this enzyme than aorta, a result consistent with the amount of PDE3 activity detected in these two tissues. When the medial and adventitial layers of the aorta were assayed individually, PDE3A was detected in both, a result consistent with a broad level of expression of this protein in blood vessel-derived cell types (Maurice *et al*., 1995). Although the cDNA encoding PDE3A predicts a membrane associated protein, and overexpression of PDE3A in fibroblasts results in a predominantly particulate enzyme (Leroy *et al*., 1996), in our experiments PDE3A was only detected in the cytosolic fractions of all tissues studied. Consistent with our results, PDE3A purified from platelets, heart or VSMC were all obtained from the cytosolic fractions of these tissues (reviewed in Manganiello *et al*., 1996). The molecular basis for this difference in subcellular localization of 'endogenous' PDE3A and the expressed recombinant protein is at present unknown. In a previous study (Smith *et al*., 1993), a PDE3 antiserum was used to immunoprecipitate 116 kDa and 135 kDa proteins from the cytosolic and particulate fractions, respectively, of human, canine, rabbit and guinea-pig myocardial tissues. Although the authors of this earlier report suggested that these proteins represented differentially processed versions of PDE3A, based on our results we would suggest that the cytosolic protein identified was PDE3A while the 135 kDa particulate protein was more likely PDE3B. In our experiments immunoblot analysis of more than 20 μg of epididymal fat allowed the detection of small amounts of PDE3A in this tissue. Since no PDE3A was detected when pure cultures of 3T3-L1 adipocytes were blotted, this result was consistent with the presence of blood vessels in the epididymal fat used in our studies. A similar result has been reported previously (Taira *et al*., 1993). In our studies, PDE3B was detected in epididymal fat, heart, aorta, aortic VSMC and

Figure 10 Regulated expression of PDE3A and PDE3B by forskolin in rat cultured aortic VSMC. Cultured rat aortic VSMC were incubated with forskolin (10 μM) for 16 h. Following this treatment, homogenates ($3000 \times g$ supernatant) were prepared. Aliquot of control and forskolin-treated homogenates were loaded in duplicate lanes on the same gel, electrophoretically resolved and transferred to a nitrocellulose membrane. The membrane was cut into two parts after transfer such that each blot contained material from control and treated cells. One blot was incubated with anti-PDE3A/3B antibody and the other with anti-PDE3A antibody. Immunoreactive bands were detected by chemiluminescence as per manufacturers recommendations (see Methods).

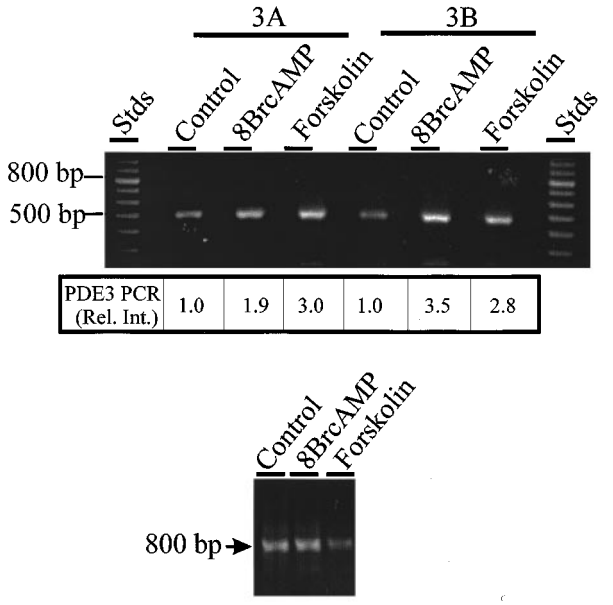


Figure 11 Effects of 8-bromo-cyclic AMP or forskolin on steady state mRNA levels of PDE3A and PDE3B in cultured rat aortic VSMC. Cultured rat aortic VSMC were incubated with 8-bromo-cyclic AMP (1 mM) or forskolin (10 μM) for 16 h and RT–PCR reactions for PDE3A, PDE3B (a) or GAPDH (b) were carried out as described in Methods. The number of PCR cycles which allowed for linear amplification of PDE3A (18 cycles) or PDE3B (28 cycles) with the primers described in Table 1 were determined empirically. PCR products for PDE3A (15 μl), PDE3B (50 μl) or GAPDH (1 μl) were separated on 2% agarose gels and ethidium bromide staining visualized under u.v. light. Relative intensity of PDE3A and PDE3B PCR products was determined by scanning densitometry (see Methods). Fold increase compared to control is indicated.

3T3-L1 adipocytes, but not in platelets or undifferentiated 3T3-L1 preadipocytes. Using several criteria, the PDE3B detected in heart, aorta and cultured aortic VSMC was indistinguishable from the protein expressed in adipocytes. Thus, both proteins had identical mobility on SDS–PAGE and each was expressed exclusively in the particulate fractions of every tissue examined. Also, the amount of PDE3B detected in each tissue was correlated with the amount of particulate PDE3 activity measured in these tissues.

Although the presence of PDE3 activities in both the cytosolic and particulate fractions of VSMC was consistent with previous reports (reviewed in Degerman *et al*., 1997), the presence of PDE3B in the particulate fraction of tissues of the cardiovascular system, and the more marked effect of prolonged increases in cyclic AMP on this PDE3, is entirely novel. Previous reports have documented that long-term incubations of Sertoli cells, or of cells involved in immune reactions (for example U937, Mono Mac6 or Jurkat cells) with activators of adenylyl cyclase, such as forskolin, or with lipophilic analogues of cyclic AMP, increases PDE4 activity (Verghese *et al*., 1995; Manning *et al*., 1996; Erdogan & Houslay, 1997). Recently, we and others reported that prolonged incubations of rat aortic VSMC or Jurkat T-cells with cyclic AMP elevating agents caused marked changes in both PDE3 and PDE4 activities (Erdogan & Houslay, 1997; Rose *et al*., 1997). Although changes in PDE3 activity accounted for more than half of the total increase of cyclic AMP PDE activity in Jurkat T cells, the PDE3 gene product(s) expressed in these cells, and the variants involved in the increased activity, were not reported in the studies of Erdogan (1997). Similarly, incubation of human erythroleukemia (HEL) cells with forskolin for 24 h has also been reported to increase PDE3 activity in these cells (Sheth *et al*., 1997). The cytosolic PDE3 expressed in HEL cells has been shown to be PDE3A, and presumably this gene product is that regulated by cyclic AMP, although this has not formally been tested. Results reported here show that the PDE3A and PDE3B expressed in VSMC are differentially regulated in response to prolonged increases in cyclic AMP. Studies to determine the molecular basis of these differences are underway in our laboratory. Although the mechanism which accounts for the differences are not yet clear, the fact that prolonged increases in cyclic AMP in VSMC had a more marked effect on PDE3B in these cells is clearly relevant to cyclic AMP-mediated functions of these cells. In this context, several previous reports, including our own (Rose *et al*., 1997), have shown that cyclic AMP-mediated up-regulation of cyclic AMP PDE in cells results in heterologous desensitization of these cells to activators of adenylyl cyclase (Torphy *et al*., 1995; Verghese *et al*., 1995; Manning *et al*., 1996). Previously, we showed that increased PDE3 activity played an important role in the cyclic AMP-mediated desensitization of VSMC to the cyclic AMP elevating effects of isoproterenol (Rose *et al*., 1997). Given that only modest changes in PDE3A expression occurred in VSMC under these conditions, it is perhaps reasonable to conclude that up-regulated PDE3B could be responsible for the PDE3 involvement in cyclic AMP-mediated desensitization. Since selective inhibitors of PDE3A, or PDE3B, are not available this assumption is not yet easily tested.

Although the generality of our findings concerning expression of PDE3B in cardiovascular tissues and the expression of this enzyme in the particulate fraction of these tissues, to other blood vessels or to other species will have to await further studies, some of the implications of our findings are clear. Thus, our data raises the interesting possibility that PDE3B, in addition to PDE3A, might be involved in mediating effects of PDE3 inhibitors in the cardiovascular system. An important fact in this context is the conclusion of earlier work in which a strong correlation between inhibition of particulate PDE3, and positive inotropic or vasorelaxant potential of PDE3 inhibitors, was shown (reviewed in Polson & Strada, 1996). Regrettably, the absence of specific PDE3A and PDE3B selective agents render this hypothesis untestable at the present time.

In conclusion, our data is consistent with expression of both PDE3A and PDE3B in several rat tissues. Moreover, our data shows that these two PDE3s are expressed in separate subcellular fractions with PDE3A accounting for all of the cytosolic PDE3 activity and PDE3B for all of the PDE3 activity present in the particulate fraction. The physiological, pharmacological and biochemical relevance of our findings is amplified by the fact that PDE3B, and to a lesser extent PDE3A, expressions were markedly increased following prolonged increases in VSMC cyclic AMP.

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