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CHARACTERIZATION OF A NOVEL POTENT AND SPECIFIC INHIBITOR OF TYPE V PHOSPHODIESTERASE

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Abstract—Guanosine cyclic 3′:5′-monophosphate (cGMP) plays a crucial role in regulating vascular smooth muscle contractile state. In rat aortic smooth muscle cells (RSMC) three isozymes of phosphodiesterase (PDE) may be involved in the degradation of cGMP, namely PDE I, PDE III, and PDE V. To study the effective contribution of PDE V to the control of intracellular cGMP levels, a specific and potent PDE V inhibitor 1,3-dimethyl-6-(2-propoxy-5-methanesulfonylamidophenyl)pyrazolo[3,4d]-pyrimidin-4-(5H)-one (DMPPO) was synthesized. DMPPO is a competitive inhibitor with respect to cGMP (K_i = 3 nM) and displayed high selectivity for PDE V as compared to other PDE isozymes. DMPPO strongly potentiated the cGMP response of atrial natriuretic peptide- or sodium nitroprusside-treated RSMC (EC_{50} = 0.5 μM). In addition, similar intracellular cGMP levels were obtained in the presence of a saturating concentration of DMPPO or 3-isobutyl-1-methylxanthine, a nonspecific PDE inhibitor, suggesting that cGMP is almost exclusively hydrolyzed by PDE V in RSMC. Stimulation of RSMC with atrial natriuretic factor resulted in accumulation of cGMP in the extracellular media. This egression was shown to be proportional to the intracellular level of cGMP and a first-order rate constant of 0.04 min^{-1} was determined for the egression process. DMPPO did not interfere with the efflux and allowed us to show that intracellular cGMP levels are mainly controlled by PDE V, rather than by egression in RSMC. DMPPO is, therefore, a useful tool for determining the role of PDE V in the control of cGMP levels in living cells and tissues.

Key words: phosphodiesterase; cGMP; smooth muscle cell; egression

Guanosine cyclic 3′:5′-monophosphate (cGMP) participates in signal transduction mechanisms in many eukaryotic cells [1]. The role of this cyclic nucleotide has been extensively studied in the cardiovascular system [2]. For instance, an increase in cGMP levels in vascular smooth muscle cells has been clearly demonstrated to induce vasorelaxation [3, 4].

The intracellular concentration of cGMP is controlled by 3 different mechanisms: (1) synthesis from guanosine triphosphate (GTP); (2) catabolism to 5′-guanosine monophosphate; and (3) egression from the cellular compartment. Synthesis of cGMP from GTP is catalyzed by guanylate cyclases (EC 4.6.1.2). Two forms of guanylate cyclases have been described [5]: a soluble guanylate cyclase activated by NO† [6–8] and a plasma membrane-associated guanylate cyclase activated by natriuretic peptides, such as ANF and CNP [9, 10]. The degradation of cyclic nucleotides by hydrolytic cleavage of the 3′-ribose-phosphate bond is catalyzed by cyclic nucleotide PDEs (EC 3.1.4.17) [11]. Most tissues contain multiple isozymes of PDE [12–14], the forms differing in their substrate preference (cAMP or cGMP) and sensitivity to effectors such as calmodulin, cGMP, or various inhibitors. These enzymes were classified by Sonnenburg and Beavo [15] into 7 families according to their primary protein and cDNA sequence, substrate specificity, and pharmacological information as follows: a calcium-calmodulin-dependent PDE (PDE I), a cGMP-stimulated PDE (PDE II), a cGMP-inhibited PDE (PDE III), a cAMP-specific PDE (PDE IV), a cGMP-specific PDE (PDE V), a photoreceptor cGMP-specific PDE (PDE VI), and a cAMP-specific rolipram-insensible PDE (PDE VII).

Egression of cyclic nucleotides has been described [16]. This phenomenon consists of secretion of either cAMP or cGMP into extracellular fluids [17, 18]. The egression is unidirectional [19] and energy dependent [20]. The role of efflux in the regulation of intracellular cAMP levels has not been clearly demonstrated [21, 22].

However, in the case of cGMP, the egression might be important in decreasing these cyclic nucleotide levels in cells [20, 23].

In rat aortic smooth muscle, 4 different PDE isoforms have been isolated: PDE I, III, IV, and V [24, 25]. PDE V has been postulated to regulate cGMP levels in vascular smooth muscle [26, 27]. This conclusion was based on the use of zaprinast (M&B 22,948), a moderately potent PDE I and PDE V inhibitor [27, and the present paper]. In addition, the contribution of cGMP efflux to the control of the cyclic nucleotide levels in vascular smooth muscle cells is still poorly understood.

In the present work, the synthesis of a potent and specific PDE V inhibitor (DMPPO) is described. The inhibitory activity of DMPPO in enzymatic PDE assays and in a cell system is characterized and compared with other PDE inhibitors. In addition, DMPPO is used as a tool to study the role of the efflux process in the control of cGMP levels in vascular smooth muscle cells. Finally, the synthesis and activities of a very potent and nonselective PDE inhibitor, IBMQ, are presented. This com-

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† Abbreviations: ANF, atrial natriuretic factor; CNP, C-type natriuretic peptide; DMPPO, 1,3-dimethyl-6-(2-propoxy-5-methanesulfonylamidophenyl)pyrazolo[3,4d]-pyrimidin-4-(5H)-one; IBMQ, 1-isobutyl-3-methylimidazo[1,5-a]quinoxalin-4-(5H)one; IBMX, 3-isobutyl-1-methylxanthine; NO, nitric oxide; PDE, phosphodiesterase; RSMC, rat aortic smooth muscle cells; SNP, sodium nitroprusside.

pound can be a powerful tool for enhancing cyclic nucleotide levels in cells.

MATERIALS AND METHODS

Chemical syntheses

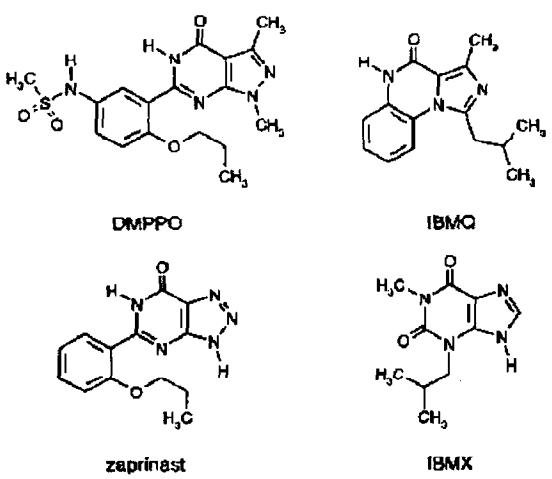
The structure of DMPPO, IBMQ, zaprinast, and IBMX is shown in Scheme 1. DMPPO, IBMQ, and zaprinast were synthesized as previously described [28–30]. IBMX was purchased from Sigma.

Materials for biochemical studies

Unless otherwise stated, all reagents were purchased from Sigma.

PDE preparations

PDE I, III, and V were purified from bovine aorta mainly as described by Lugnier *et al.* (1986) [24]. Briefly, fresh tissue was minced and homogenized using an Ultra Turrax in 5 volumes of buffer A (20 mM Tris-HCl pH 7.5, 2 mM Mg-acetate, 1 mM dithiothreitol, 5 mM EDTA, and 2 μg/mL aprotinin) per gramme of tissue. The extract was centrifuged at 105,000 g for 60 min and the supernatant applied to a DEAE-Trisacryl (Bio-Sepra, France) column (2.5 × 10 cm) equilibrated in buffer B (20 mM Tris-HCl pH 7.5, 2 mM Mg-acetate, 1 mM dithiothreitol). The column was washed with buffer B until no more absorbance at 280 nm was detected in the eluate. A linear NaCl gradient from 0 to 400 mM in 800 mL of buffer B was then applied. For loading, washing, and elution from the DEAE column, the flow rate was 30 mL/hr. Fractions containing mixed PDE I and PDE V activities on the one hand, and PDE III and PDE IV activities on the other, were pooled and glycerol added to yield a final concentration of 10% (v/v). The fractions were kept at –80°C. To separate PDE I from PDE V, the first pool containing both enzymatic activities (5 mL) was applied to a MONO Q HR5/5 column (Pharmacia-Biotech, France) in an FPLC system (Pharmacia-Biotech, France). The column was previously equilibrated with buffer B. The flow rate was 1 mL/min. At first, a linear NaCl gradient (0 to 175 mM) was applied for 35 min. After a 20-min step at 175 mM NaCl, a second NaCl linear gradient (from 175 to 500 mM) was applied for 20 min. The active fractions were pooled and glycerol added to yield a final concentration of 10% (v/v). The fractions were kept at –80°C. The specific activities obtained for each pool were 6.5 nmoles cAMP, 49.5 nmoles cAMP, and 4.8 nmoles cGMP hydrolyzed per min and per mg of protein for PDE I, PDE III, and PDE V, respectively (see below for assay conditions). Human recombinant PDE II and PDE IV were kindly provided by Mike Gadau, Lothar Uher, Dr. Tim Martins, and Dr. Vince Florio (ICOS Corporation, Bothell, WA, U.S.A.) [31].



Scheme 1. Structure of DMPPO, IBMQ, zaprinast, and IBMX.

PDE assay

The PDE assay, modified from Wells *et al.* [32], was based on the use of Multiscreen plates (Millipore, France) and a vacuum manifold (Millipore, France). In such plates, both the reaction and the subsequent separation between substrates and products can be achieved. The assay (100 μL) contained 50 mM Tris-HCl, 5 mM Mg acetate, 1 mM EGTA, and 250 μg/mL snake venom nucleotidase, pH 7.5. Fifty nM [8-^{3}H]-cAMP (26 Ci/mmol, Amersham, France) for the PDE I, III, and IV assays or 50 nM [8-^{3}H]-cGMP (15 Ci/mmol, Amersham, France) for the PDE II and V assays were added. For the PDE I assay, EGTA was replaced by 10 μM $CaCl_2$ and 20 nM calmodulin, which was purified from bovine testes and kindly supplied by Lothar Uher, Dr. Tim Martins, and Dr. Vince Florio (ICOS Corporation). For the PDE III assay, 10 μM of rolipram was added to completely inhibit the PDE IV activity present in the fraction. Reactions were started by the addition of 25 μL of the diluted enzyme preparations. The assays were incubated for 30 min at 30°C. Microcolumns were prepared by aliquoting of 300 μL per well of QAE Sephadex previously swollen for 2 hr in water (12 mL/g). At the end of the incubation, the total volume of each assay was loaded onto microcolumn plate by filtration. The elution of free radioactivity was effected by 200 μL of water from which 50 μL aliquots were analyzed by scintillation counting.

In this PDE assay, the substrate concentration never exceeded 30% of the K_m of the enzyme tested. Under such conditions, the IC_{50} obtained for any given compound closely corresponds to the K_i for such compound. In addition, all enzyme studies were performed under conditions of initial velocity (maximal substrate hydrolysis of 10% to 15%). For the determination of PDE V activity the enzyme concentration is estimated from gel electrophoresis to be about 15 pM in the assay. Stock solutions of PDE inhibitors were prepared in dimethylsulfoxide. The final solvent concentration in each assay was 2% (v/v).

Cell cultures

RSMC were prepared according to Chamley *et al.* (1977) [33]. Cells were cultured in Dulbecco's modified Eagle medium (Gibco, France) containing 10% fetal calf serum, 1% glutamine, and 1% penicillin-streptomycin at 37°C in a 95% air-5% CO_2 humidified atmosphere. The cells were used between the 5th and 15th passage.

Treatment of cells with PDE inhibitors and guanylate cyclase agonists

Cells were seeded in 24-well culture dishes at a density of 2 to 5.10^4 cells/well. Experiments were performed after 3 to 5 days in culture when cells reached

confluence. Medium were aspirated and replaced by 0.5 mL of PBS containing the PDE inhibitor. After 30 min at 37°C, soluble or particulate guanylate cyclase was stimulated by addition of SNP (0.5 μM) or ANF (0.1 μM), respectively, at 37°C. At the end of the incubation, the medium was removed and stored at −20°C for extracellular cyclic nucleotide determinations. Intracellular cyclic nucleotides were extracted by two ethanolic (65%) washes at 4°C for 5 min. The ethanolic extracts were pooled, evaporated to dryness using a Speed-Vac system, and stored at −20°C. cGMP and cAMP were measured by scintillation proximity immunoassay (Amersham, France).

In all cases, any given treatment with effectors was performed in duplicate or triplicate wells. Stock solutions of PDE inhibitors were made up in dimethylsulfoxide. In the assays, the final concentration of dimethylsulfoxide never exceeded 0.1% (v/v).

Estimation of the rate constant for cGMP efflux

As previously described by Barber and Butcher [21], assuming the efflux of cGMP is a saturable process, then:

$$\frac{dP}{dt} = \frac{V \cdot S}{Ke + S}$$

where P is the amount of extracellular cGMP, t is time, V represents the maximum velocity of efflux, S the intracellular cGMP concentration, and Ke the intracellular cGMP concentration at which efflux takes place at half its maximal rate. When S is much smaller than Ke,

$$Pt = \kappa \int_o^t Sdt \text{ with } \kappa = \frac{V}{Ke}$$

Where κ is the apparent first order rate constant for cGMP efflux. The time integral of intracellular cGMP is calculated as the area under the curve in the time course of intracellular cGMP accumulation between 0 and any given time t.

Incubation of RSMC in the presence of 1 μM cGMP resulted in no loss of extracellular cGMP over a period of 30 min, suggesting that there was no extracellular cGMP degradation. Therefore, the amount of cGMP found in the medium after stimulation was considered to represent the total amount secreted during incubation.

RESULTS

Comparison of the effect of DMPPO on the activity of different purified PDEs

Zaprinast is generally considered as a specific PDE V inhibitor [34]. However, as shown in Table 1, this compound inhibits PDE V only 15 times more potently than PDE I. In an attempt to obtain more potent and selective PDE V inhibitors, DMPPO was synthesized (Scheme 1). DMPPO is a very potent PDE V inhibitor with an IC_{50} of 3 nM, displaying a high selectivity towards PDE V compared to other PDEs (Table 1). This compound is 66-fold more potent than zaprinast on PDE V. In addition, the IC_{50} towards PDE V is 300-fold lower than that observed for PDE I. To characterize the properties of this new PDE V inhibitor, we determined its potency in the presence of different cGMP concentrations (Fig. 1). Inhibition of PDE V by DMPPO was highly dependent on the substrate concentration present in the assay. The Lineweaver-Burk plot (Fig. 1A) indicated that DMPPO is a competitive inhibitor versus cGMP. The plot of the apparent K_m *versus* different inhibitor concentrations (Fig. 1B) allowed us to determine an apparent K_i of 3 ± 0.7 nM (means ± SD of a representative experiment performed in triplicate and repeated twice) for DMPPO. Furthermore, inhibition of PDE V by DMPPO was reversible (data not shown).

Comparison of the effect of various PDE inhibitors on cGMP accumulation in ANF- or SNP-stimulated RSMC

In RSMC, cGMP levels can be increased either by stimulation of particulate guanylate cyclase with ANF or by stimulation of the soluble guanylate cyclase with SNP [35]. Using these two different stimuli, we compared the effect of various PDE inhibitors on intracellular cGMP levels (Table 2). Basal cGMP levels in unstimulated RSMC were 70 ± 28 fmoles/10^6 cells (mean ± SD, n = 3). When cells were incubated in the presence of ANF or SNP for 10 min, the levels of cGMP increased to 1540 ± 250 and 430 ± 80 fmoles/10^6 cells, respectively. Ten μM of DMPPO added 30 min before ANF or SNP strongly potentiated the cGMP response regardless of the stimulating agent used: in both ANF and SNP-treated cells, a 30-fold further stimulation in the cGMP levels was observed. A similar increase in cGMP levels was detected when DMPPO was added simultaneously with the guanylate cyclase-stimulating agent (data not shown). For comparison, accumulation of cGMP in cells treated with zaprinast (10 μM) and ANF or SNP was 2- to 3-fold lower than that obtained in DMPPO-treated cells. The PDE III inhibitor, cilostamide, and the PDE IV inhibitor, rolipram, were unable to produce an increase in cGMP levels in either SNP- or ANF-stimulated cells. However, when cilostamide or rolipram were added to

Table 1. Inhibition of different PDE isoforms by PDE inhibitors

Compounds	IC_{50} (μM)				
	PDE I	PDE II	PDE III	PDE IV	PDE V
DMPPO	1 ± 0.5	3 ± 0.2	10 ± 1.5	22 ± 3	0.003 ± 0.001
Zaprinast	3 ± 0.6	70 ± 5	>100	78 ± 7	0.2 ± 0.1
IBMQ	0.2 ± 0.07	0.3 ± 0.06	0.4 ± 0.1	1 ± 0.04	0.01 ± 0.005
IBMX	12 ± 3	20 ± 4	2 ± 0.4	5 ± 1.1	4 ± 1.7

PDE activities were determined as described in Materials and Methods. IC_{50} values represent the mean ± SD of at least 3 independent determinations in which dose-response curves were obtained using compound concentrations from 1 nM to 100 μM. PDE I results correspond to inhibition of the Ca^{2+} and calmodulin-activated enzyme. In the absence of Ca^{2+} and calmodulin, IC_{50}'s of compounds towards PDE I were comparable to those shown in the table.

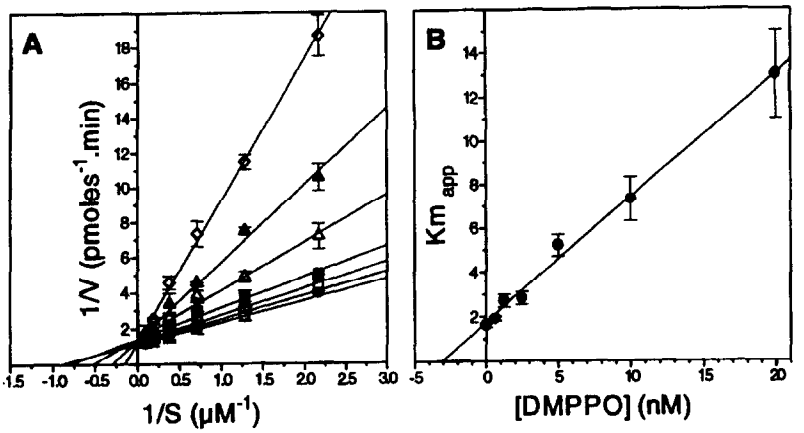


Fig. 1. Inhibition activity of DMPPO on bovine PDE V. PDE activity was measured as described in Materials and Methods. Panel (A) Lineweaver-Burk representation of typical results from saturation curves. DMPPO at the concentrations 0 nM (○), 0.062 nM (●), 0.125 nM (□), 2.5 nM (■), 5 nM (△), 10 nM (▲), and 20 nM (◇) were added to assay solution. Panel (B) Plot of the apparent K_m *versus* DMPPO concentrations. The data shown (mean ± SD) are of a representative experiment performed in triplicate wells and repeated twice.

forskolin- or isoproterenol-treated cells, intracellular cAMP levels were strongly enhanced (data not shown). These data show that PDE III and PDE IV hydrolyze cAMP without hydrolyzing cGMP in these cells. In the case of PDE IV, the result was expected because this enzyme displays high selectivity towards the hydrolysis of cAMP [11].

As shown in Table 2, the nonspecific PDE inhibitor IBMX at 10 μM produced a modest enhancement of cGMP levels in RSMC treated with ANF or SNP. This is probably due to the low efficacy of IBMX in inhibiting PDEs (see Table 1). A more potent nonspecific PDE inhibitor, IBMQ, has been synthesized in our laboratory. The structure of IBMQ is shown in Scheme 1 and its inhibitory activities against different PDEs are depicted in Table 1. Compared to IBMX, IBMQ is 50-, 33-, 5-, 5-, and 400-fold more potent against PDE I, II, III, IV, and V, respectively. When RSMC were stimulated with ANF or SNP in the presence of 10 μM IBMQ, accumulation of cGMP was similar to that obtained with DMPPO and the guanylate cyclase activators.

Table 2. Effect of various PDE inhibitors on SNP- or ANF-stimulation of intracellular cGMP levels in RSMC

	cGMP (fmoles/10^6 cells)	
Treatment	ANF	SNP
Control	1540 ± 250	430 ± 80
Cilostamide	1450 ± 90	397 ± 30
Rolipram	1550 ± 100	419 ± 20
Zaprinast	15,500 ± 1,400	4620 ± 510
DMPPO	45,100 ± 3,000	13,800 ± 1,700
IBMX	4890 ± 380	1540 ± 120
IBMQ	38,600 ± 3,600	13,500 ± 1,200

RSMC were incubated for 30 min with 10 μM of various PDE inhibitors as indicated. Cells were then stimulated either by ANF (0.1 μM) or by SNP (0.5 μM) for 10 min and intracellular cGMP levels determined as described in Materials and Methods. In unstimulated RSMC, the basal cGMP level was 70 ± 28 fmoles/10^6 cells. Data are presented as the mean ± SD, $n = 3$.

To determine the relative efficiency of the PDE inhibitors, dose responses of these compounds were performed on ANF-stimulated RSMC. As shown in Fig. 2, DMPPO, zaprinast, IBMQ, and IBMX dose-dependently increased intracellular cGMP levels. The extent of cGMP accumulation at a saturating concentration of the different PDE inhibitors was similar, suggesting that in RSMC, PDE V is the main PDE controlling the catabolism of cGMP. A correlation was observed between the efficacy of these compounds to promote cGMP accumulation in cells and their capacity to inhibit PDE V. DMPPO and IBMQ were the more active PDE inhibitors in cells (EC_{50} = 0.5 and 0.7 μM, respectively). IBMQ was 300-fold more potent than IBMX (EC_{50} = 200 μM) and DMPPO was 30-fold more potent than zaprinast (EC_{50} = 15 μM).

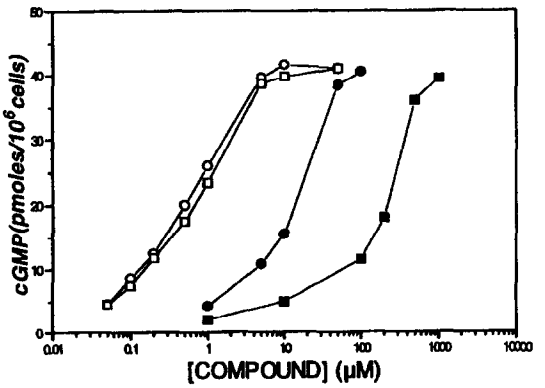


Fig. 2. Dose-response curves of DMPPO and nonspecific PDE inhibitors on ANF stimulated intracellular cGMP levels in RSMC. RSMC were incubated for 30 min with various concentrations of DMPPO (○), zaprinast (●), IBMQ (□), or IBMX (■). Cells were then stimulated with ANF (0.1 μM) for 10 min and intracellular cGMP levels determined as described in Materials and Methods. The data shown are of a representative experiment repeated 3 times and performed in duplicate wells.

in cells (EC_{50} = 0.5 and 0.7 μM, respectively). IBMQ was 300-fold more potent than IBMX (EC_{50} = 200 μM) and DMPPO was 30-fold more potent than zaprinast (EC_{50} = 15 μM).

Comparison between the effects of DMPPO on cGMP and cAMP levels in RSMC

The levels of cAMP and cGMP were compared in nonstimulated or ANF-stimulated RSMC in the presence or absence of DMPPO or IBMQ (Table 3). As previously reported [36], basal GMP levels were 13-fold lower than basal cAMP levels. Addition of 10 μM DMPPO increased basal cGMP levels by approximately 3-fold without any significant effect on basal cAMP levels, confirming the absence of effect of DMPPO on cAMP-hydrolyzing PDEs such as PDE III or PDE IV. In contrast, IBMQ, the nonspecific PDE inhibitor, induced at 10 μM a similar stimulation of both cGMP and cAMP basal levels.

When cyclic nucleotide levels were determined on ANF-stimulated RSMC, similar results were obtained. A 25-fold stimulation of cGMP levels was observed on ANF-stimulated RSMC in the presence of DMPPO without significant changes in cAMP levels. In contrast, IBMQ increased both cGMP and cAMP levels in ANF-treated cells.

As expected, forskolin was able to increase cAMP levels in these cells. A 6-fold stimulation in cAMP was achieved at 0.5 μM of forskolin without modification of the cGMP levels. IBMQ strongly potentiated the effect of forskolin on cAMP levels: a 10-fold enhancement of cAMP accumulation was observed in this case. When IBMQ-treated cells were stimulated with forskolin, cGMP levels were increased 3-fold. The effect on cGMP levels was similar to that observed on unstimulated cells. When forskolin-stimulated cells were preincubated with DMPPO, a 3-fold increase in cGMP was observed. The extent of cGMP accumulation was comparable to that found in cells treated with DMPPO alone. Unexpectedly, a modest but significant 1.5-fold increase in cAMP levels was detected in forskolin-stimulated cells incubated in the presence of DMPPO. However, this increase was considerably lower than the 10-fold enhancement obtained with IBMQ.

Effect of DMPPO on egression of cGMP from RSMC

Two main pathways have been described for decreasing intracellular cyclic nucleotide levels: one is the degradation of cyclic nucleotides by PDEs and the other the egression of nondegraded cyclic nucleotides outside the cell by an energy-dependent efflux mechanism [19, 21]. Reports in the literature describe the effect of PDE inhibitors on the egression of cyclic nucleotides [36, 37]. The possibility that DMPPO interferes with the efflux process has, thus, been tested.

Figure 3A shows the kinetics of intracellular and extracellular cGMP accumulation in response to 0.1 μM ANF. Intracellular cGMP levels followed an initial fast rise that peaked at approximately 2 min, and then declined rapidly. On the other hand, the accumulation of cGMP in the extracellular medium was gradual and reached a plateau value 15 min after addition of ANF. This time corresponded to the return of intracellular cGMP levels to basal values. A plot of extracellular cGMP *versus* the accumulation of intracellular cGMP during the time course (time integral, Fig. 3B) displayed a linear relationship between both parameters. From this plot, an apparent first-order rate constant κ for the efflux of cGMP could be calculated. κ was found to be 0.04 min^{-1}.

Addition of DMPPO dramatically modified not only the extent, but also the time course, of ANF-mediated accumulation of intracellular cGMP (Fig. 3C). In the presence of the PDE V inhibitor, intracellular cGMP was continuously accumulated up to 15 min after addition of ANF. After that time, a slow decrease in intracellular cGMP was observed. This phenomenon probably corresponds to the observed efflux of cGMP, as well as to a decrease in the rate of synthesis.

The apparent first-order rate constant of efflux was unaffected by DMPPO, suggesting that even at high levels of cGMP the efflux process is still proportional to intracellular cGMP levels and that DMPPO did not modify this phenomenon (Fig. 3D).

DISCUSSION

PDE V is a cGMP specific phosphodiesterase found in various tissues, including lungs, aorta, and platelets [12,

Table 3. Effect of DMPPO and IBMQ on intracellular cGMP and cAMP levels in RSMC

Treatment	Cyclic nucleotide (fmoles/10^6 cells)	
	cGMP	cAMP
None	60 ± 23	782 ± 110
IBMQ	180 ± 30*	3020 ± 130**
DMPPO	196 ± 28*	890 ± 130
ANF	1540 ± 250**	906 ± 150
ANF + IBMQ	38,700 ± 2400**	3230 ± 240**
ANF + DMPPO	36,800 ± 1800**	1070 ± 90
Forskolin	78 ± 15	4600 ± 520**
Forskolin + IBMQ	194 ± 40*	43,700 ± 4800**
Forskolin + DMPPO	218 ± 46*	6800 ± 980**

RSMC were incubated in the presence or absence of the different PDE inhibitors (10 μM) for 30 min at 37°C. Cells were then stimulated (or not) by addition of ANF (0.1 μM) or forskolin (0.5 μM) for 10 min and cyclic nucleotide levels determined as described in Materials and Methods. Data are presented as the mean ± SD, $n = 3$. Significant difference with respect to control: *$P < 0.05$ and **$P < 0.01$ (t-test).

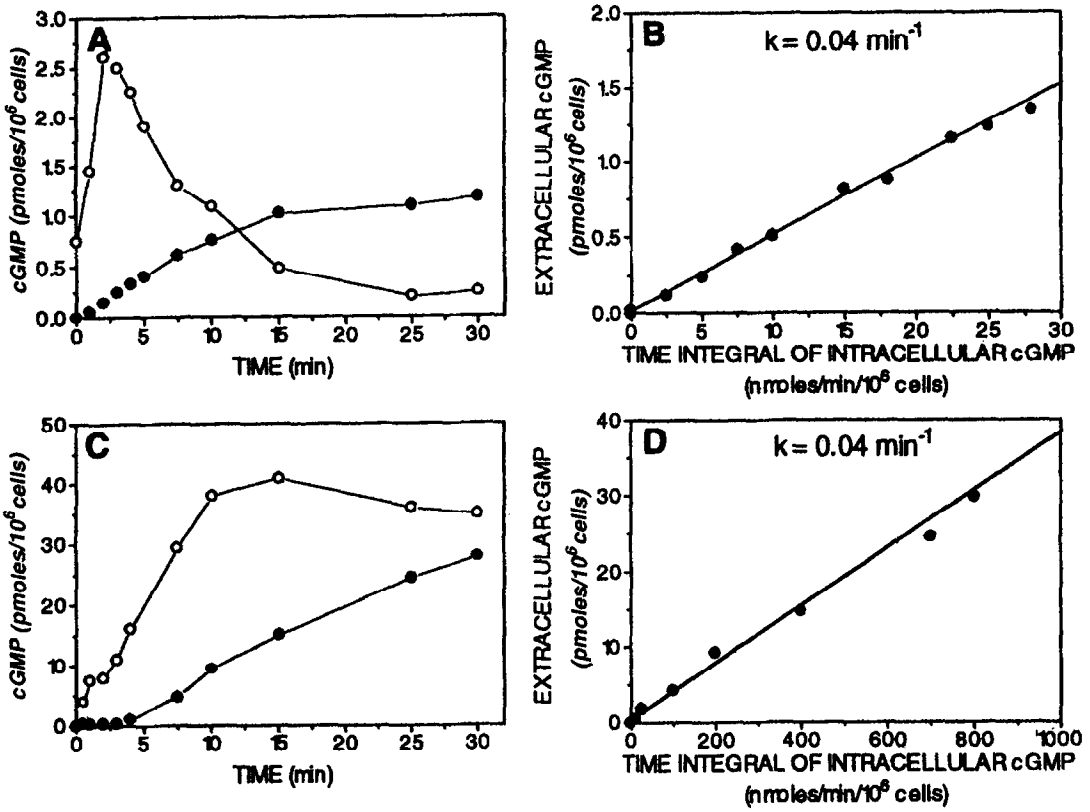


Fig. 3. Effect of DMPPO on ANF-dependent intra- and extracellular cGMP accumulation. RSMC were incubated for 30 min in the presence of DMPPO (10 μM) before stimulation by addition of ANF (0.1 μM). Intracellular cGMP levels (○) and extracellular cGMP levels (●) were determined at various times as described in Materials and Methods. Panels A and B, cells treated with ANF alone; panels C and D, cells treated with ANF in the presence of DMPPO. The data shown (mean ± SD) are of a representative experiment performed in triplicate wells and repeated twice.

24, 38, 39]. The actual contribution of PDE V to the regulation of intracellular cGMP levels in these tissues has not yet been elucidated. This is mainly due to the absence of specific and potent PDE V inhibitors. Zaprinast has been widely used as a reference compound to inhibit PDE V in cells and tissues [40–43]. However, as shown in Table 1, this compound is not very potent and, in addition, lacks specificity. In contrast, DMPPO has a K_i of 3 nM on PDE V and its IC_{50}s towards other PDEs range between 1 and 20 μM. Moreover, this compound is a reversible and competitive inhibitor towards cGMP (Fig. 1).

Natriuretic peptides and NO donors induce vasorelaxation by increasing cGMP levels in vascular smooth muscle cells [35]. In addition, PDE V has been involved in the degradation of cGMP in these cells. However, these conclusions were based upon the use of zaprinast. It was, therefore, of interest to look at the effect of DMPPO on cGMP levels in vascular smooth muscle cells. DMPPO was able to induce a 3-fold enhancement of the basal level of cGMP in RSMC (Table 3). However, when soluble or particulate guanylate cyclases were stimulated with SNP or ANF, respectively, a 30-fold increase in cGMP levels was observed (Table 2). The fact that DMPPO was more efficient in cells in which cGMP synthesis was stimulated as compared to unstimulated cells has also been observed for other inhibitors of PDEs [44, 45]. Furthermore, as shown in Table 3, the nonselective PDE inhibitor IBMQ was more effective in increasing cAMP levels in forskolin-treated cells compared to nonactivated cells. These findings can be probably explained by the K_m of a PDE toward its substrate: the higher the cyclic nucleotide concentration in a cell is, the higher its rate of hydrolysis by a PDE will be. Nevertheless, the possibility that cyclic nucleotide levels modify the activity of a PDE by mechanisms such a phosphorylation cannot be ruled out. Indeed, it has recently been described in the literature that cAMP-dependent protein kinase is able to phosphorylate PDE V leading to an increase in its catalytic activity [46].

The potency of DMPPO was approximately 170-fold higher in the enzymatic PDE V assay as compared to the cell model. DMPPO is a competitive inhibitor of PDE V towards cGMP. Therefore, because the cGMP concentration in cells (approximately 10 μM) is much higher than the cGMP concentration used in the enzymatic test (0.1 μM), a shift to the right in the dose-response curve to DMPPO in cells would be expected. Indeed, the same reasoning can be applied to explain the lower potency in the cellular model of the other PDE inhibitors used in this work. In addition, cell permeability or intracellular metabolism can also partially restrict the activity of DMPPO. However, the effect of DMPPO on cGMP levels was not affected by the time elicited between addi-

tion of the PDE V inhibitor and stimulation of guanylate cyclases (data not shown).

Similar intracellular cGMP levels were found in the presence of saturating concentrations of DMPPO or with the nonspecific PDE inhibitors IBMX and IBMQ (Fig. 2). These results suggest that, in RSMC, cGMP seems to be exclusively hydrolyzed by PDE V. However, the role of the calcium-calmodulin-dependent PDE (PDE I) in the control of cGMP levels in vascular smooth muscle cells *in vivo* cannot be totally ruled out. To test this hypothesis, calcium levels were enhanced in RSMC by treatment with 1 μM Arg-vasopressin, which activates phospholipase C in these cells via V1 receptors [47], or with the calcium ionophore A23187 (10 μM). Under these conditions, the levels of cGMP accumulated in response to ANF were identical to those obtained when intracellular calcium concentration was not artificially enhanced (data not shown). The results suggest that PDE I is not involved in the catabolism of cGMP in cultured RSMC.

cAMP levels in unstimulated or ANF-treated cells were not significantly affected by the presence of DMPPO (Table 3), confirming the specificity of this compound for the cGMP-specific PDE V. However, when adenylate cyclase was stimulated either by addition of forskolin (Table 3) or isoproterenol (data not shown) a small increase in cAMP levels was observed in presence of DMPPO. The 3-fold stimulation observed on the cGMP basal level in DMPPO-treated cells corresponds to an intracellular cGMP concentration of approximately 0.2 μM (assuming an intracellular volume of 1 μL per 10^6 cells). This concentration of cGMP has been described to produce approximately 50% inhibition of PDE III activity in vitro [48]. Therefore, the modest enhancement of cAMP levels by treatment of cells with DMPPO could be explained by the partial inhibition of PDE III by cGMP. Indeed, the specific PDE III inhibitor cilostamide induced accumulation of cAMP in RSMC (data not shown), confirming the presence of an active PDE III in these cells. The specificity of DMPPO towards PDE V in intact cells was, again, evidenced by comparison with the effects of a nonspecific PDE inhibitor: IBMQ was able to enhance cAMP levels in basal conditions or in ANF- or forskolin-treated cells.

Vascular smooth muscle, endothelial, and kidney epithelial cells secrete cGMP in response to ANF [18, 20]. The mechanism of cGMP secretion displays strong similarities with the egression of cAMP: both processes were shown to be unidirectional, to occur against a gradient of very high concentrations of extracellular cyclic nucleotide, and to be energy-dependent [19, 20]. In addition, efflux of cyclic nucleotides is inhibited by methylxanthines such as IBMX [36, 37]. In RSMC, we found that there was a linear relationship between the intracellular concentration of cGMP and the rate of egression of this cyclic nucleotide. This result strongly suggests that the efflux process is proportional to intracellular cGMP levels. An apparent first-rate efflux constant for cGMP of 0.04 min^{-1} was determined. Barber and Butcher [21] described a comparable rate of egression (from 0.02 min^{-1} to 0.08 min^{-1}) for cAMP in various cell lines. Addition of DMPPO at a concentration of 10 μM, at which maximal intracellular cGMP accumulation was achieved, did not modify the efflux rate constant. This result shows that, in contrast to IBMX, DMPPO can elicit cGMP accumulation through inhibition of PDE V without affecting the efflux of cGMP. On the other hand, the maximal intracellular cGMP concentrations obtained in the presence of DMPPO and ANF were 16-fold higher than the levels of cGMP obtained with ANF alone. Yet, the relationship between the amount of secreted cGMP and the cGMP accumulated in ANF + DMPPO-treated cells remained constant (Fig. 3D). These findings suggest that the cGMP transporter responsible for the efflux is not saturated even at high intracellular cGMP concentrations. Indeed, reports from the literature argue in favor of a high K_m for cAMP transporter [17, 21]. The fact that very high levels of intracellular cGMP are found in the presence of DMPPO and ANF (compare Fig. 3, panels A and C) also suggest that the efflux process does not significantly contribute to the regulation of cGMP concentration in cultured RSMC.

Another new molecule described in the present work is IBMQ. This compound displays low selectivity towards inhibition of different PDEs. However, compared to IBMX, IBMQ is far more potent. The difference in inhibitory potency between IBMX and IBMQ is more striking on PDE V: IBMQ is 400-fold more potent than IBMX against PDE V. This can explain the 300-fold higher efficacy of IBMQ on the stimulation of ANF-dependent cGMP accumulation in RSMC. As for DMPPO, IBMQ (10 μM) does not affect cGMP egression in ANF-stimulated RSMC (data not shown).

To summarize, our results demonstrate that DMPPO is a potent and specific PDE V inhibitor both *in vitro* and in intact cells. Using this new compound, we found that PDE V seems to be the only PDE hydrolyzing cGMP in cultured RSMC. Moreover, because DMPPO does not affect efflux of cGMP from cells, we determined that the efflux mechanism does not control cGMP levels in RSMC. We believe that DMPPO represents a useful experimental tool for determining the importance of PDE V in regulation of intracellular cGMP levels in various tissues. For example, it will be of particular interest to analyze the effect of DMPPO on the regulation of vascular tone by different contracting and relaxing agents.

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