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Contribution of Phosphodiesterase Isoenzymes and Cyclic Nucleotide Efflux to the Regulation of Cyclic GMP Levels in Aortic Smooth Muscle Cells

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ABSTRACT. Involvement of phosphodiesterase isoenzymes (PDEs) in guanosine-3′,5′-cyclic monophosphate (cGMP) hydrolysis was analyzed in aortic smooth muscle cells. Four families of PDEs were separated from pig aorta: PDE1 (calcium–calmodulin-activated), PDE3 (cGMP-inhibited), PDE4 (adenosine 3′,5′-cyclic monophosphate [cAMP]-specific), and PDE5 (cGMP-specific). Within this PDE complement, PDE1 and PDE5 mostly contributed to the hydrolysis of cGMP both in the presence and absence of calcium–calmodulin. The role of these isoenzymes in cGMP degradation was analyzed in primary cultures of porcine aortic smooth muscle cells after stimulation with sodium nitroprusside (SNP) or atrial natriuretic factor (ANF). Pretreatment with 10 μM zaprinast, a concentration that selectively inhibits PDE5, did not potentiate the SNP- or ANF-induced rise of cGMP, questioning the widespread opinion that only PDE5 accounts for cGMP hydrolysis in this tissue. Further evidence came from experiments assessing the effect of zaprinast or 3-isobutyl-1-methylxanthine at concentrations inhibiting both type 1 and type 5 isoenzymes, in which this potentiation was clearly seen. Contribution of cGMP egression to the control of intracellular cGMP levels after SNP or ANF stimulation was also investigated. Shortly after guanylate cyclase activation, extracellular cGMP levels surpassed intracellular levels. However, comparison of the amounts of cGMP extruded to the extracellular medium with those degraded by PDEs leads to the conclusion that efflux is of relatively minor importance in regulating intracellular cGMP levels. In cells made tolerant to SNP, selective PDE5 inhibition synergistically increased intra- and extracellular cGMP amounts after SNP stimulation. These results indicate a previously undescribed greater relevance of PDE5 after tolerance development in aortic smooth muscle cells. BIOCHEM PHARMACOL **58**;10:1675–1683, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cyclic GMP; tolerance; aorta; zaprinast; cGMP phosphodiesterase; vascular smooth muscle cells

Agents that stimulate GC† induce vasorelaxation in vascular smooth muscle by elevating cGMP concentration [1–3]. This increase activates cGMP-dependent protein kinase, leading to the lowering of cytosolic calcium levels [4]. VSMC of the arterial wall are rich in cGMP-dependent protein kinase [5], which is activated in response to a variety of endogenous vasodilators, such as nitric oxide released from endothelial cells [6] or ANF [2, 7]. The intensity and duration of the relaxing effect of cGMP depend on the cytosolic concentration of this nucleotide, which is thought to be determined by the dynamic balance between the activities of GCs and PDEs, which synthesize and hydrolyze cGMP, respectively. An active transport of cGMP through cellular membrane following cGMP synthesis activation has also been described in several tissues including VSMC [8], but its relative importance in lowering intracellular cGMP content in this tissue remains unknown.

cGMP is also implicated in the therapeutic effect of organic nitrates, such as nitroglycerin and SNP [1, 3], widely used as vasorelaxants in the treatment of congestive heart failure and coronary vascular diseases. These nitric oxide-generating compounds activate the soluble form of GC, leading to cGMP accumulation in VSMC. However, the clinical use of nitrovasodilators is severely limited by the fast development of tolerance to these compounds [9]. Tolerance is associated with diminished synthesis of cGMP in VSMC in response to subsequent nitrate exposure [10]. Thus, elevating or preserving intracellular cGMP levels via alternative mechanisms may reverse the effects of tolerance, providing a rationale for the combination of cGMP phosphodiesterase inhibitors with nitrocompounds [11].

PDEs comprise several isoenzymes which by virtue of their varying regulatory properties and cellular localization allow a tissue-specific control of cyclic nucleotide levels

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† *Abbreviations:* GC, guanylate cyclase; VSMC, vascular smooth muscle cells; cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; PDE, cyclic nucleotide phosphodiesterase; ANF, atrial natriuretic factor; Ca/CaM, Ca^{2+}/calmodulin; DEAE, diethylaminoethyl; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; and SNP, sodium nitroprusside.

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[12]. PDE1, 2, 3, 4, and 5 have been described in VSMC of mammal species [13–16]. Since PDE5 is the only cGMP-selective PDE in the VSMC, inhibition of this isoenzyme has been regarded as a way to increase cGMP cellular content without affecting cAMP levels [14, 17–21]. Indeed, it has been proposed that cGMP is almost exclusively hydrolyzed by PDE5 in rat VSMC [14]. However, the preponderant role assigned to PDE5 is uncertain, since the potentiation of the effect of GC agonists obtained in pharmacological studies requires inhibitor concentrations markedly higher than those needed to inhibit PDE5 [10, 14, 17, 18, 21]. In addition, experimental results seem to be dependent on the animal model used [14, 16, 20–23]. In this respect, similarities between human and pig models, with regard to kinetic properties of PDEs present in this tissue and their sensitivities to inhibitors, have been pointed out [15].

The aim of the present work was to determine the role of PDE isoenzymes and cGMP expulsion in the regulation of intracellular cGMP levels in cells tolerant and non-tolerant to sodium nitroprusside. For this purpose, PDE isoenzymes from pig aorta were separated by anion exchange chromatography. To analyze the role of these isoenzymes in cGMP degradation, the effect caused by PDE inhibition on intra- and extracellular cGMP accumulation after stimulation of GC was measured in porcine VSMC. Results obtained contradict the idea of selective PDE5 inhibition as a way to synergistically increase the effect of GC agonists. However, data reported in the present study provide evidence that cGMP degradation becomes more dependent on this isoenzyme after development of tolerance to SNP. Implications of these results to the combination of nitrocompounds with phosphodiesterase inhibitors are discussed.

MATERIALS AND METHODS

Materials

DEAE-Sepharose CL-6B was from Pharmacia. Aprotinin, leupeptine, pepstatin, and bestatin were obtained from Boehringer Mannheim. FBS was from Gibco and the other tissue culture reagents were from BioWhittaker. Benzamidine, phenylmethylsulfonyl fluoride, cAMP, cGMP, calmodulin, nicardipine, IBMX, dipyridamole, collagenase III, elastase I, soybean trypsin inhibitor, SNP, and human ANF were from Sigma. [8-^{3}H]cAMP and [8-^{3}H]cGMP were obtained from Amersham. Zaprinast and rolipram were kindly supplied by Rhône-Poulenc and Schering Spain, respectively. Porcine aortas used throughout this study were obtained from a local slaughterhouse within a few minutes of the death of the animal, and transported on ice to the laboratory.

Isolation of PDE Isoenzymes

PDE extraction was performed as described by Saeki and Saito [13] with modifications. Porcine aorta was excised and adhering fat, adventitia, and endothelium scraped off by mechanical procedures. Medial tissue layer (15 g) was minced into small fragments and homogenized using a Polytron in 100 mL of ice-cold homogenization buffer (20 mM Tris–HCl, 0.1 mM $CaCl_2$, 2 mM magnesium acetate, 1 mM dithiothreitol, pH 7.4) containing 10 mg/L aprotinin, 10 mM benzamidine, 10 mg/L bestatin, 1 mM phenylmethylsulfonyl fluoride, 10 mg/L pepstatin, and 10 mg/L leupeptine. The homogenate was centrifuged at 15,000 *g* for 50 min at 4° and the supernatant applied to a DEAE-Sepharose CL-6B column pre-equilibrated with homogenization buffer. The column was washed with the same buffer and eluted with 200 mL of a linear gradient of 0.15–0.5 M NaCl. Forty-four fractions of 4.5 mL each were collected in tubes containing 100 μL of 35 mM EGTA, and PDE activities were assayed using 1 μM [8-^{3}H]cAMP or [8-^{3}H]cGMP as substrate, with or without Ca/CaM. Fractions containing each PDE isoenzyme were pooled and ethylene glycol was added to a final concentration of 30% (v/v) prior to their storage at −20°.

PDE Activity Assay

The assay was started by the addition of the enzyme preparation to an incubation mixture containing 40 mM Tris–HCl (pH 8), 4 mM β-mercaptoethanol, 0.1 mg/mL bovine albumin, 5 mM $MgCl_2$, 0.2 mM EGTA, and 1 μM [8-^{3}H]cAMP (0.1 μCi/tube) or 1 μM [8-^{3}H]cGMP (0.1 μCi/tube) as substrate, in a total volume of 200 μL. Kinetics were done in autotubes, allowing the use of a multichannel pipette to dispose the reagents, as described [24]. For assays performed in the presence of CaM, EGTA was replaced by 30 U/mL CaM and 1 mM $CaCl_2$. Following a 10-min incubation at 30°, reactions were terminated by placing the tubes in boiling water for 45 sec. After addition of 50 μL of snake venom 5′-nucleotidase solution (1 mg/mL) per tube, they were incubated further for 10 min at 30°. Isolation of the products (nucleosides) was achieved by removal of the substrates (nucleotides) with 0.5 mL of a cationic AG1X-2 resin (Bio-Rad). The suspension was shaken and centrifuged at 9200 *g* for 4 min. Finally, radioactivity in 0.2 mL of the supernatant was measured in a liquid scintillation counter. PDE inhibitors were tested at appropriate concentrations ranging from 0.1 to 250 μM. All PDE inhibitors were dissolved in DMSO, and the final concentration of DMSO in the incubation medium never exceeded 1% (v/v). Enzyme stocks were diluted to maintain substrate consumption always below 15%.

cGMP Measurement on Porcine Aortic Smooth Muscle Cell Cultures

Porcine aorta free of fat and adhering tissue was cut into pieces around 1 cm^2 and incubated at 37° for 10 min in a medium containing 200 U/mL collagenase, 15 U/mL elastase, and 0.5 mg/mL soybean trypsin inhibitor. The media layer was then stripped from adventitia and endothelium using sterile tweezers. The smooth muscle layer was excised

into 2–4 mm^2 pieces and around 10–15 explants were placed on each culture flask with Dulbecco's modified Eagle's medium previously supplemented with 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. Once cellular confluence was around 20–30%, explants were removed and FBS concentration reduced to 5%. Cell passages were performed only with confluent cells showing the characteristic "hill and valley" pattern, by two consecutive washes with Hank's Balanced Salt Solution and incubation with trypsin–EDTA for 3–4 min at 37°. Following trypsin inhibition with FBS, cells were centrifuged and resuspended in a suitable volume of culture medium. Cultured cells were used between the first and third passages in order to avoid changes in protein expression.

For assays, cells were cultured in 12-well plates for 3–4 days until confluence was reached. Medium was then replaced by PBS containing the appropriate PDE inhibitor and plates were incubated at 37° for 30 min before adding the GC activator. At the end of the incubation, the medium was removed and stored at −20° for extracellular nucleotide determinations. Intracellular cGMP was extracted with a cold ethanolic solution (70%) and extracts were evaporated to dryness. cGMP was measured by a commercial cGMP-[^{125}I] scintillation proximity assay (Amersham). PDE inhibitors were dissolved in DMSO and stored at −20° at a concentration 500 times higher than needed for assays. When indicated, tolerance to SNP was induced by incubating porcine aortic SMC for 1 hr with 0.5 mM SNP. Then, cells were rinsed with 3 washes of 10 min of PBS. Each data point represents the average of four samples of a representative experiment repeated at least twice.

Kinetics of cGMP Expulsion

Assuming that efflux of cGMP is a saturable process, then

$$\frac{d[\mathrm{cGMP}]_e}{dt} = \frac{V \times [\mathrm{cGMP}]_i}{K_{1/2} + [\mathrm{cGMP}]_i}$$

where V is the maximum velocity of efflux, $[\mathrm{cGMP}]_e$ and $[\mathrm{cGMP}]_i$ are the extra- and intracellular cGMP concentrations, t is time, and $K_{1/2}$ is the intracellular cGMP amount when the efflux rate equals $V/2$ [14]. When $[\mathrm{cGMP}]_i$ is much smaller than $K_{1/2}$,

$$[\mathrm{cGMP}]_e = k \int_0^t [\mathrm{cGMP}]_i \, dt$$

where k is the apparent first-order rate constant for cGMP expulsion ($V/K_{1/2}$), and the time integral of $[\mathrm{cGMP}]_i$ is the area under the curve in the time–course of intracellular cGMP from time 0 to t. $[\mathrm{cGMP}]_e$ was obtained directly by measuring the amount of cGMP accumulated in the medium after a certain time (t) of GC activation. The time integral of $[\mathrm{cGMP}]_i$ was calculated by adding all the partial areas between consecutive time points from time 0 to t. Each area was estimated by averaging the intracellular cGMP content between two successive points and multiplying by the interval of time.

RESULTS

Isolation of Porcine Aorta PDEs

Fractions eluted from a DEAE-Sepharose CL-6B column were assayed for cGMP and cAMP hydrolysis either in the presence or absence of Ca/CaM and selective PDE inhibitors (Fig. 1). Four PDE activities were identified, corresponding in order of elution to the cGMP-binding, cGMP-specific PDE (PDE5), the Ca/CaM-sensitive PDE (PDE1), the cGMP-inhibited PDE (PDE3), and the cAMP-specific PDE (PDE4). PDE1 and 5 were the major contributors to cGMP hydrolysis when physiological concentrations of substrate were used, both in the presence and absence of Ca/CaM. PDE5 was inhibited by the selective inhibitor zaprinast (IC_{50} = 0.23 μM) and by dipyridamole (IC_{50} = 0.33 μM), an adenosine uptake blocker. cGMP phosphodiesterase activity of PDE1 was much less sensitive to zaprinast and almost not inhibited by dipyridamole (34% of inhibition at 250 μM), but was effectively inhibited by the calcium channel blocker nicardipine (IC_{50} = 3.7 μM). In order to evaluate the contribution of PDE isoenzymes to cGMP hydrolysis in cultured VSMC, 10 μM zaprinast was used as a selective PDE5 inhibitor and 100 μM IBMX as a non-selective PDE inhibitor. Dose–response curves for zaprinast and IBMX on the four isolated PDEs are plotted in Fig. 2. Appropriate inhibitors for PDE1 isoenzymes have not yet been developed.

cGMP Synthesis and Egression in ANF- or SNP-Stimulated VSMC

Synthesis of cGMP in cultured VSMC was achieved either by stimulation of soluble GC by SNP or of particulate GC by ANF (Fig. 3). After SNP or ANF stimulation, considerable amounts of cGMP were secreted to the extracellular medium. Interestingly, the efflux was greater when the soluble GC was stimulated. To quantitatively assess the role of the expulsion system in regulating intracellular cGMP levels, the kinetics of egression was analyzed (Fig. 3, inset). The egression rate of cGMP was proportional to the intracellular cGMP concentration, but the values of the kinetic constant greatly depended on the agent used, being 0.02 min^{-1} for ANF and 0.1 min^{-1} for SNP. Rate constants were independent of intracellular cGMP concentration and thus, when cells were stimulated with 1, 10, or 100 μM SNP, the slope obtained was the same (0.1 min^{-1}), whereas cGMP levels reached inside cells were 6-, 10-, and 15-fold, respectively that of non-stimulated cells (0.2 ± 0.04 pmol/10^6 cells). This observation shows that the cGMP transport system is not saturable by the intracellular concentrations reached by this cyclic nucleotide.

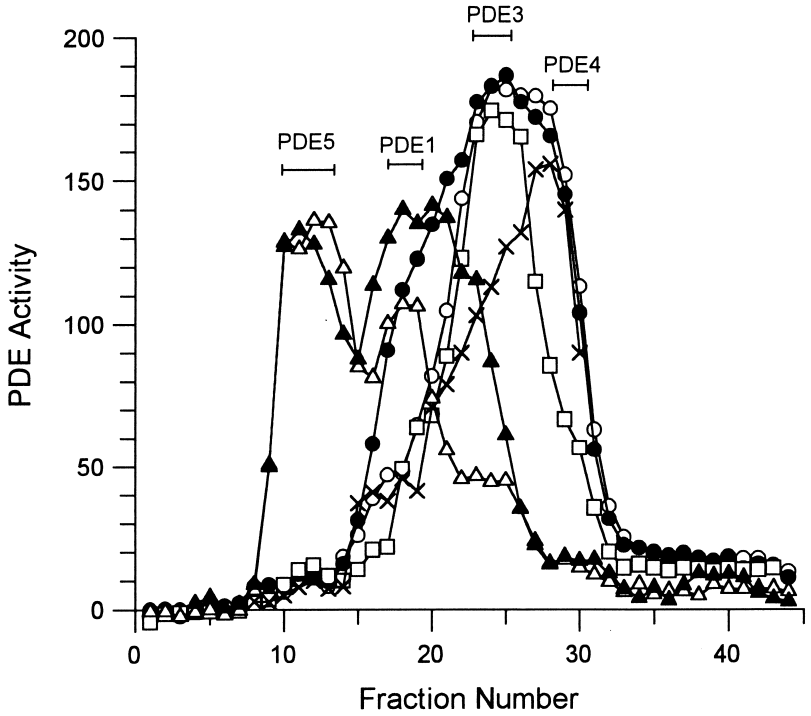

FIG. 1. PDE extraction from smooth muscle layer of porcine aorta. PDE elution profile from DEAE-Sepharose CL-6B chromatography of porcine smooth muscle aorta, obtained in the presence of Ca^{2+}. Cyclic nucleotide hydrolysis rates were determined using as substrate either [^{3}H]cAMP (circles) or [^{3}H]cGMP (triangles) with (closed symbols) or without (open symbols) Ca/CaM. Measurements of phosphodiesterase activity with 1 μM cAMP as substrate in the presence of 4 μM rolipram or 2 μM cGMP are depicted as squares and crosses, respectively. PDE activity is expressed as pmol of nucleotide hydrolized × min^{-1} × mL^{-1} by 100 μL of enzymatic extract.

Effect of PDE Inhibitors on ANF- and SNP-Stimulated cGMP Content

To analyze the role of PDE5 in cGMP degradation, VSMC preincubated with PDE inhibitors were stimulated with ANF or SNP. Zaprinast at 10 μM, a concentration that selectively inhibits PDE5 (Fig. 2), was unable to increase the cGMP levels reached with 0.5 μM ANF alone (Fig. 4A). In contrast, the ANF-induced intracellular cGMP rise was clearly enhanced by 100 μM IBMX, a non-selective PDE inhibitor. Similar results were obtained when soluble GC was activated: 10 μM zaprinast did not potentiate the SNP effect (Fig. 4B), while the intracellular cGMP content in VSMC preincubated with 100 μM IBMX increased from 0.76 to 14 pmol cGMP/10^6 cells within 2–10 min of cGMP synthesis activation with 10 μM SNP. These observations suggest that cGMP hydrolysis is a very active process and that most of the cGMP synthesized after GC stimulation is not detected in time–course assays, since it is quickly hydrolyzed by intracellular PDEs. These results also demonstrate that cGMP hydrolysis by intracellular PDEs is far more relevant than outward transport to the control of intracellular cGMP: non-selective inhibition of PDE activity led to a 15-fold enhancement of the intracellular cGMP peak in response to SNP, while extracellular accumulation of cGMP elicited by SNP alone was similar to that present inside the cells (Fig. 3). The PDE5-selective inhibitor zaprinast at 100 μM enhanced the effect of SNP (Fig. 4B), but at this concentration zaprinast inhibited both PDE1 and PDE5 (Fig. 2), suggesting that both isoenzymes contributed to cGMP hydrolysis.

In order to determine if the lack of synergy between SNP and zaprinast (at concentrations inhibiting only PDE5) was due to the concentration of SNP added, VSMC pretreated for 30 min with 10 μM zaprinast were stimulated with lower concentrations of SNP. Intracellular and extracellular cGMP was measured after 2 and 20 min of SNP treatment, respectively. Zaprinast was unable to increase the intracellular content of cGMP after GC stimulation, irrespective of the concentration of SNP added (Fig. 5). In these experiments, extracellular cGMP levels in cells pretreated with 10 μM zaprinast were also similar to those obtained with non-pretreated cells (Fig. 5 legend).

Effect of Zaprinast on cGMP Accumulation in SNP-Tolerant Cells

In contrast to non-tolerant cells, selective PDE5 inhibition by zaprinast was effective in potentiating the SNP-induced cGMP rise in SNP-tolerant VSMC (Fig. 6). Tolerance to SNP was induced by incubating VSMC with 500 μM SNP for 1 hr as described in Methods. After the cells were washed, their intracellular cGMP content (0.1 ± 0.02 pmol/10^6 cells) was similar to that present in non-tolerant cells, indicating that the procedure effectively removed the SNP used to induce tolerance. After a subsequent SNP addition, tolerant cells quickly increased their cGMP content in a dose–response manner, although cGMP values inside SNP-tolerant cells were markedly lower than in non-tolerant cells (Fig. 7). In spite of the fact that less cGMP was synthesized in tolerant cells, preincubation with 10 μM zaprinast enhanced accumulation of cGMP within the cells treated with SNP, and this effect was observed for all SNP concentrations tested (Fig. 7). This potentiation

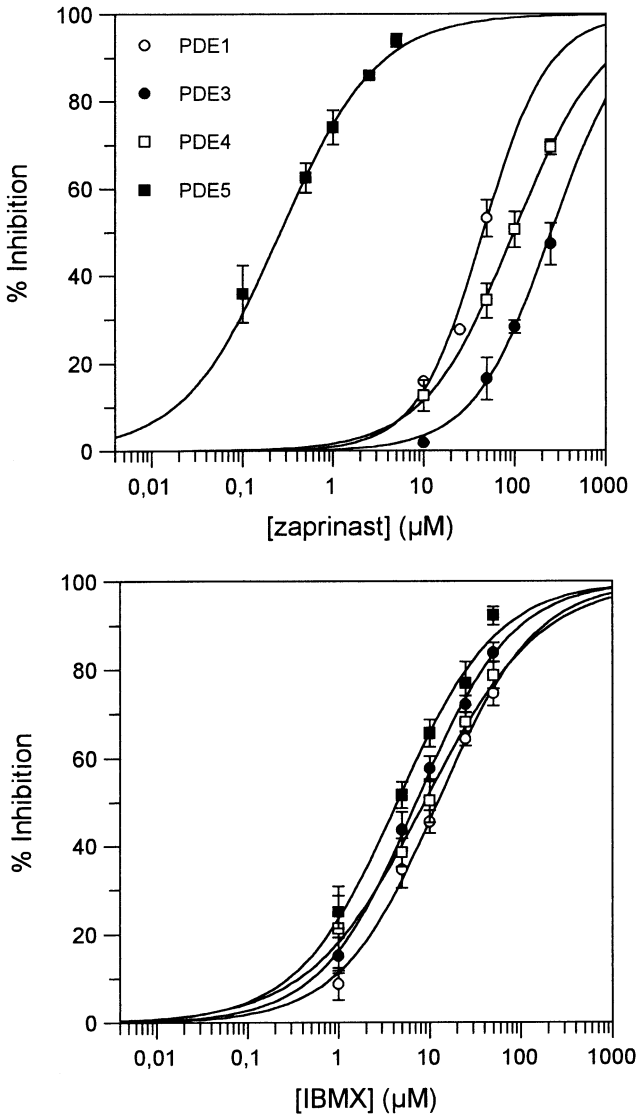

FIG. 2. Inhibition by zaprinast and IBMX of PDE isoenzymes isolated from smooth muscle porcine aorta. IC_{50} values were calculated by fitting experimental values by unweighted non-linear regression using Grafit (Erithacus software) as described [25]. IC_{50} values of zaprinast for PDE1, 3, 4, and 5 were 47, 250, 100, and 0.23 μM, respectively. For IBMX, IC_{50} values were 12, 7, 9, and 4 μM, respectively. Data represent the means ± SD of four different assays.

was also reflected in the amount of cGMP accumulated in the extracellular medium (Fig. 7).

DISCUSSION

The chromatogram obtained when separating PDEs from aortic smooth muscle showed that both PDE1 and PDE5 contributed to degradation of cGMP; physiological concentrations of substrate were used to establish the PDE profile since the intracellular cGMP content in non-stimulated cells was estimated to be roughly 0.2 μM (assuming a cell volume of 1 μL [14]). We looked for the presence of PDE2 in the profile by measuring the stimulation of cAMP hydrolysis by 2 μM cGMP. Using this assay, we were able to detect small quantities of this specific PDE in dog heart and in bovine aortic muscle, but not in porcine aortic muscle. The lack of PDE2 activity in this tissue has been reported in most studies [16], though Saeki and Saito [13] reported the presence of small amounts of PDE2 activity in soluble extracts of porcine aortic smooth muscle, and Miyahara *et al.* [15] observed that this activity was also present in low amounts in human aorta. We did not pursue this isoenzyme further for two main reasons. First, the K_m of PDE2 for cGMP (about 15 μM) is nearly two orders of magnitude above cellular concentrations of this cyclic nucleotide, and non-physiological cGMP increases would be needed to allow this isoenzyme to metabolize significant amounts of cGMP. On the other hand, cGMP at 10^{-7} M, within the physiological range, can stimulate the hydrolysis of physiological concentrations of cAMP, in the micromolar range, by PDE2. Thus cGMP probably behaves *in vivo* as an allosteric regulator of cAMP degradation for this isoenzyme. In any case, the lack of specific inhibitors for PDE2 prevented us from testing if this isoenzyme had any effect on cGMP degradation.

Intracellular hydrolysis of cGMP by PDEs in porcine VSMC after GC stimulation is a very active process, as confirmed by the strong enhancing effect of the non-selective PDE inhibitor IBMX on intracellular cGMP levels. In spite of the fact that most of the cGMP synthesized is quickly hydrolyzed by intracellular PDEs, inhibition of PDE5 does not seem to be a good approach to elevate intracellular cGMP levels after treatment with GC activators. The commonly used PDE5 inhibitor zaprinast, at concentrations that selectively inhibit PDE5, fails to potentiate the effect of GC activators. Our data suggest that this is not due to a lack of potency or bioavailability of zaprinast; instead, potentiation appears to be prevented by the presence of PDE1. The actual contribution of PDE1 to this process has not yet been determined, mainly because of the lack of specific and selective inhibitors. We tried to determine the role of PDE1 by measuring the effect of the selective PDE1 inhibitor nicardipine, at a concentration of 1 to 100 μM, on the cGMP content of SNP-stimulated VSMC. However, cGMP levels reached were always markedly lower when nicardipine was present (results not shown), probably due to a direct inhibitory effect on cGMP synthesis, as suggested for other dihydropyridines [26].

The relevance of cGMP egression to the control of intracellular cGMP levels in this tissue appears to be less than what has been suggested for other cell types such as platelets [27]. Compared to the amount of cGMP hydrolyzed by intracellular PDEs, cGMP expulsion is quantitatively of minor importance, though relevant physiological roles for the extruded cGMP should not be ruled out. The importance of the expulsion system in modulating intracellular cGMP levels may be easily overestimated if the amount of cGMP outside and inside the cells is compared in time–course assays. This is due to the progressive accumulation of extracellular cGMP not subject to degra-

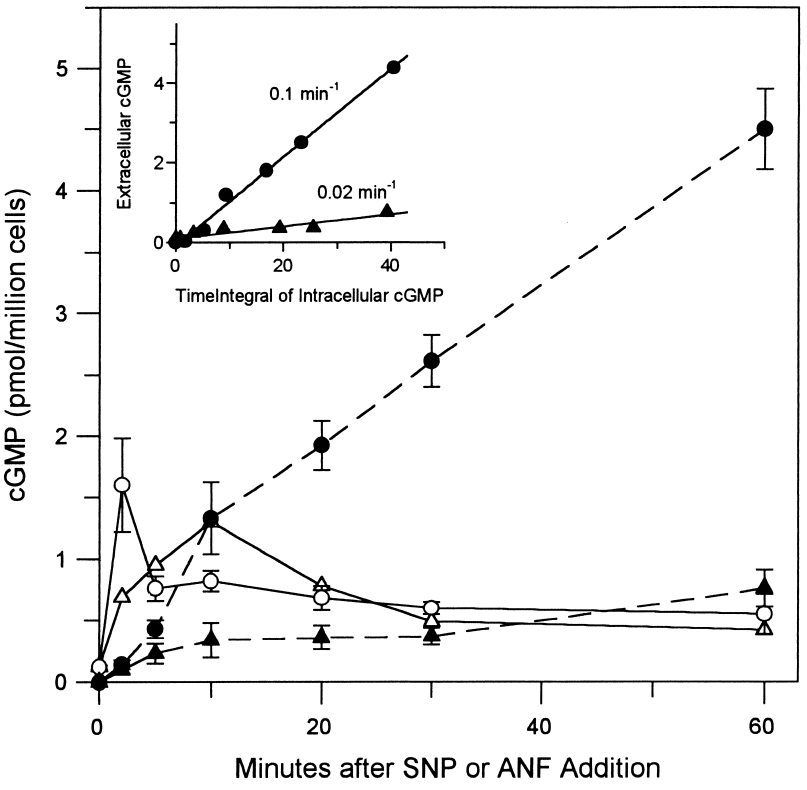

FIG. 3. Kinetics of cGMP egression in response to ANF or SNP. VSMC were treated with 10 μM SNP (circles) or 0.5 μM ANF (triangles) and the time–course of intra- (open symbols) and extracellular (closed symbols) cGMP levels was followed. The kinetics of the cGMP efflux in each case was determined as indicated in Methods. Data are the means ± SD of four wells of a representative experiment. The basal intracellular concentration of cGMP before treatments was 0.18 ± 0.04 pmol/10^6 cells.

dation, while most of the cGMP synthesized after GC stimulation is quickly hydrolyzed by intracellular PDEs and therefore not detected, as shown in experiments with IBMX. There was a linear relationship between the efflux rate and intracellular concentration of cGMP. It is of interest to note that the rate constant for cGMP egression was 5-fold greater when soluble GC as compared to particulate GC was stimulated, in spite of the fact that similar intracellular cGMP levels were obtained with SNP or ANF. This result can be interpreted in at least two ways. One explanation could be that cGMP synthesized by particulate or soluble GCs was compartmentalized in some way; this seems to be an important characteristic of the cyclic nucleotide signaling system [28, 29]. In this way, a discrete cGMP spatial distribution would be conditioning accessibility to the expulsion system. Alternatively, cGMP efflux might be regulated by a mechanism independent of the intracellular cGMP level. In fact, ANF receptors with GC activity only represent 10% of total ANF receptors [30], and some effects of nitric oxide are independent of GC activation [31]. A regulation of this type has been described for cAMP in pig aortic smooth muscle, where the apparent first-order rate constant for the efflux of cAMP depends on the extracellular adenosine concentration [32].

The role of PDE isoenzymes in the hydrolysis of cGMP has been analyzed by other authors. In these pharmacological and biochemical studies, PDE5 is frequently considered to be responsible for cGMP degradation in VSMC [10, 14, 17, 18]. Nevertheless, experimental support for this hypothesis comes mainly from data obtained with rat and rabbit aorta, where it has been reported that pretreatment with 30 or 100 μM zaprinast effectively potentiates the vasorelaxant effect of SNP [17, 18, 21]. It has also been found that pretreatment with 10 μM zaprinast enhances the intracellular amount of cGMP obtained after SNP or ANF stimulation in rat aortic smooth muscle cells [14]. However, the interpretation of this effect as being caused by PDE5 inhibition alone is questionable, since the sensitivity of PDE isoenzymes to inhibitors varies greatly from one mammalian species to another [13–18, 21, 33]. PDE5 isolated from porcine aorta is inhibited by zaprinast with an IC_{50} of 0.23 μM, similar to values obtained in all types of mammalian arteries [13–16]. In contrast, IC_{50} values of zaprinast on PDE1 of pig (47 μM) and human aortic smooth muscle cells (26 μM) [15] are far from those reported for this isoenzyme in rat or rabbit (3–8 μM) [14, 16–18, 33]. Thus, while 10 μM zaprinast behaves as a selective inhibitor of human and porcine PDE5, it inhibits both PDE5 and PDE1 in rat and rabbit aorta. Interestingly, the vasorelaxant effect of nitroglycerin in rat aorta is potentiated by 10, but not by 0.3 μM zaprinast [10]. This species-dependent susceptibility to zaprinast is probably due to the differential expression of kinetically different PDE1 isoforms [12]. In addition to zaprinast, more potent PDE5 inhibitors have been synthesized. Although they inhibit this isoenzyme in the nanomolar range, the concentrations of these compounds needed to potentiate the ANF- or SNP-induced intracellular cGMP rise are 1–2 orders of magnitude greater than those required to inhibit PDE5 [14, 21, 34]. Taken together, these data suggest that, as found in this work, inhibition of PDE1 and PDE5 isoenzymes is also required in rat and rabbit aortic smooth muscle cells to

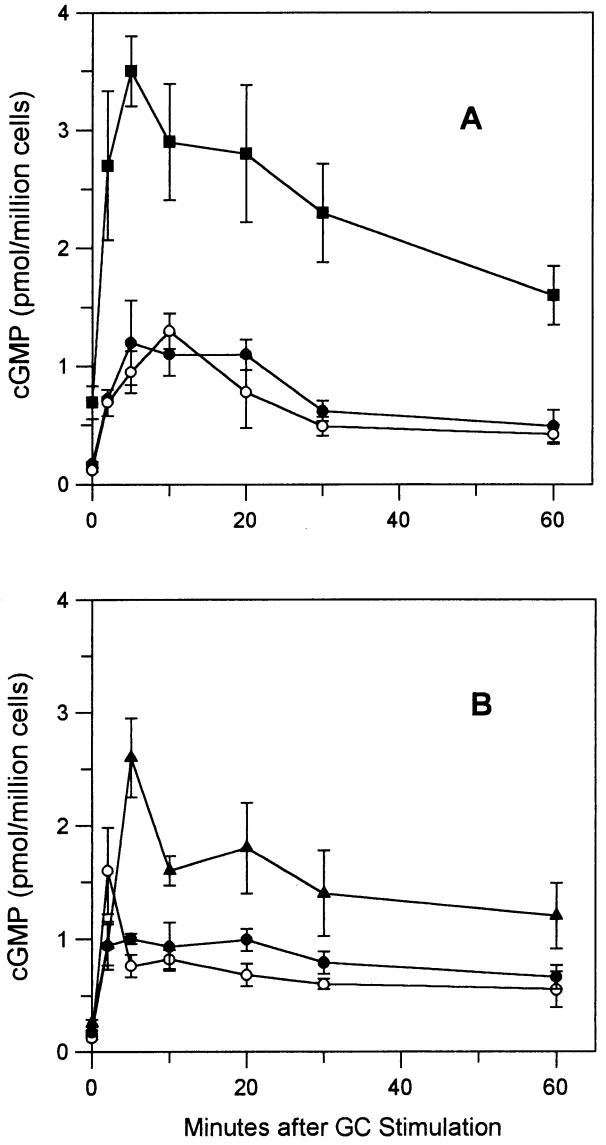

FIG. 4. Effect of PDE inhibitors on intracellular cGMP content of ANF- or SNP-stimulated VSMC. (A) Porcine VSMC were preincubated for 30 min with PBS (○), PBS with 10 μM zaprinast (●), or PBS with 100 μM IBMX (■), and then stimulated with 0.5 μM ANF. (B) Cells were preincubated for 30 min with PBS (○), PBS carrying 10 μM (●), or 100 μM zaprinast (▲), and then stimulated with 10 μM SNP. Data are the means ± SD of four wells of a representative experiment. In the absence of GC stimulation, pretreatment with 100 μM IBMX increased the basal intracellular content of cGMP 3- to 4-fold, while zaprinast had no effect.

enhance the intracellular cGMP levels obtained in response to GC stimulation.

Results obtained with cells tolerant to SNP indicate that cGMP degradation becomes more dependent on PDE5 when acute cGMP elevations have previously been elicited. In contrast to non-tolerant cells, selective inhibition of PDE5 by zaprinast synergistically increases the SNP-induced cGMP rise in SNP-tolerant aortic smooth muscle cells. The amount of zaprinast needed to obtain this potentiation is close to that needed to inhibit PDE5

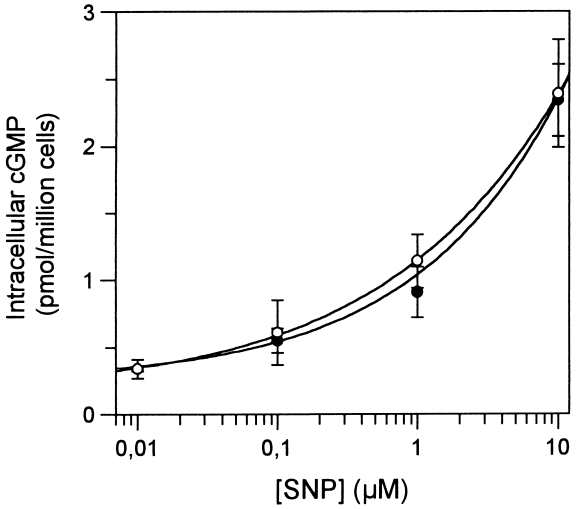

FIG. 5. Effect of selective PDE5 inhibition on intracellular cGMP levels after SNP stimulation in porcine VSMC. Non-treated VSMC (○) or VSMC pretreated for 30 min with 10 μM zaprinast (●) were stimulated with SNP. Intracellular levels of cGMP were measured after 2 min of SNP stimulation. Data are the means ± SD of four wells of a representative experiment. Extracellular levels after 20 min of stimulation with 0.01, 0.1, 1, and 10 μM SNP were, in pmol/million cells, 1.1 ± 0.3, 0.7 ± 0.3, 1.7 ± 0.7, and 4.3 ± 0.8, respectively for non-treated cells, and 1.3 ± 0.4, 1.0 ± 0.2, 2.0 ± 0.3, and 4.1 ± 0.7 for zaprinast-treated cells.

activity, suggesting that the membrane permeability for zaprinast is good, and that the lack of synergy found in non-tolerant cells is not due to a low bioavailability of the

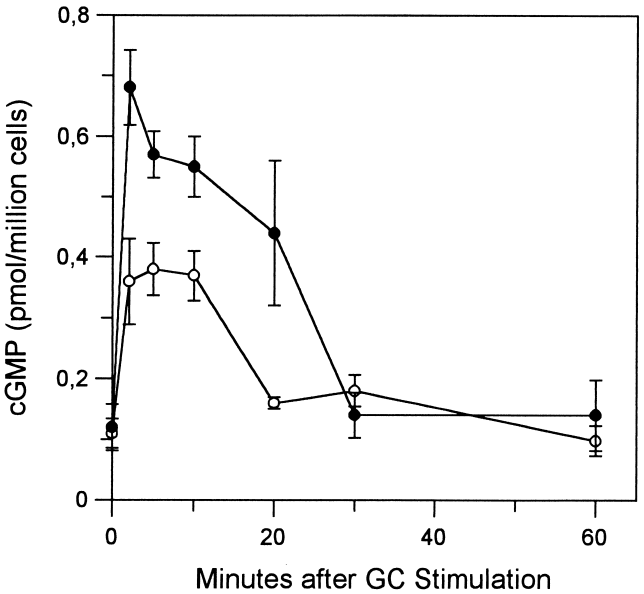

FIG. 6. Effect of 10 μM zaprinast on the cGMP time–course in response to SNP in tolerant cells. SNP (1 μM) was added to SNP-tolerant VSMC pretreated for 30 min with PBS (○) or PBS with 10 μM zaprinast (●). Intracellular cGMP was measured at the indicated times following SNP addition. Data are the means ± SD of four wells of a representative experiment. Zaprinast, when used alone, did not alter the basal amount of cGMP (0.1 pmol/10^6 cells).

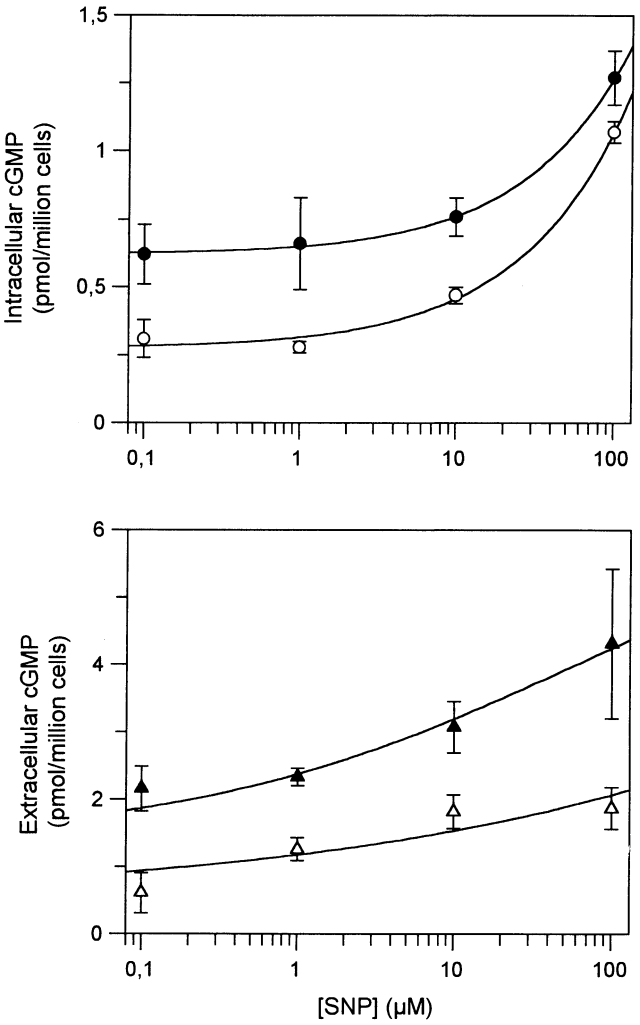

FIG. 7. Potentiation of SNP effect by zaprinast in SNP-tolerant porcine VSMC. Tolerance to SNP was induced by incubating cultured VSMC for 1 hr with 0.5 mM SNP, as described in Methods. Cells were then washed 3 times, incubated for 30 min with PBS (open symbols) or PBS containing 10 μM zaprinast (closed symbols) and then stimulated with SNP. Graphs show the intracellular (circles) and extracellular (triangles) cGMP content after 2 or 20 min of SNP addition, respectively. Data are the means ± SD of four wells of a representative experiment.

compound. The enhanced contribution of PDE5 in tolerant cells suggests that in the development of tolerance to nitrocompounds, in addition to the well-known decrease in cGMP synthesis, a regulation of the relative activity of the cGMP-hydrolyzing PDEs takes place. This does not mean that this isoenzyme is responsible for all cGMP hydrolysis in tolerant cells, since in the presence of IBMX the amount of cGMP accumulated is higher than with zaprinast. We suspect that the changes observed are related to the regulation by phosphorylation of these PDE isoenzymes. *In vitro*, PDE1 and 5 are substrates for calcium- or cyclic nucleotide-dependent protein kinases, but physiological roles for the phosphorylated PDEs remain unknown [12, 35–37]. Some PDE1 isoforms are substrates for the cAMP protein kinase and, when phosphorylated, show reduced affinity for CaM [38]. PDE5 can also be phosphorylated by the cAMP-dependent protein kinase [35] and becomes a good substrate for cGMP protein kinase when cGMP is bound to a high-affinity non-catalytic site [37]. Recently, it has been demonstrated that PDE5 is phosphorylated in intact cells following an ANF-induced cGMP elevation, leading to an increased PDE5 activity [36].

These findings also have potential practical applications. The rapid development of tolerance to nitrovasodilators severely limits their clinical usefulness [9]. Tolerance is associated with reduced cGMP synthesis in response to subsequent exposition to the compound. Previous studies measuring blood pressure or relaxation of whole arteries have shown that tolerance can be partially reversed by some PDE5 inhibitors, such as zaprinast [10, 11, 39]. These results can now be explained by the synergistic interaction between PDE5 inhibitors and nitrovasodilators in tolerant aortic smooth muscle cells, resulting in increased cGMP levels. However, pretreatment with these inhibitors does not prevent the development of tolerance toward the nitrovasodilator [11], so the main problem, the reduced synthesis of cGMP by tolerant cells, persists. Since tolerance is a gradual phenomenon and the degree of tolerance of the muscle depends on the concentration of nitrovasodilator to which it had been exposed, a reasonable therapeutic approach could be to combine a molecule with ability to inhibit both PDE1 and PDE5 with a nitrocompound. In this way, the potentiation of the effect of the nitrovasodilator would help to reduce the dose needed to reach some specific effect, reducing at the same time the risk of tolerance development.

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