Subclasses of Cyclic GMP–Specific Phosphodiesterase and Their Role in Regulating the Effects of Atrial Natriuretic Factor

Ronald E. Weishaar, Dianne C. Kobylarz-Singer, Joan Keiser, Stephen J. Haleen, Terry C. Major, Stephen Rapundalo, J.T. Peterson, and Robert Panek

Two subclasses of cyclic guanosine monophosphate (GMP)–specific phosphodiesterases were identified in vascular tissue from several beds. The activity of one subclass (phosphodiesterase IB) was stimulated severalfold by calmodulin and selectively inhibited by the phosphodiesterase inhibitor TCV-3B. The activity of the other subclass (phosphodiesterase IC) was not stimulated by calmodulin and was selectively inhibited by the phosphodiesterase inhibitor M&B 22,948. To assess the involvement of both subclasses in regulating cyclic GMP–dependent responses, the ability of TCV-3B and M&B 22,948 to potentiate the in vitro and in vivo responses to the endogenous guanylate cyclase stimulator atrial natriuretic factor (ANF) was evaluated. Both TCV-3B and M&B 22,948 relaxed isolated rabbit aortic and pulmonary artery rings and also potentiated the relaxant effect of ANF. In addition, both inhibitors produced small increases in urine flow and sodium excretion in anesthetized rats and potentiated the diuretic and natriuretic responses to exogenous ANF. M&B 22,948 (30 μg/kg/min) produced a threefold increase in the natriuretic response to simultaneously administered ANF, and TCV-3B (10 μg/kg/min) produced a twofold increase in the response to ANF. The results of the present experiments suggest that both the calmodulin-sensitive and calmodulin-insensitive subclasses of cyclic GMP–specific phosphodiesterase play a role in regulating the in vitro and in vivo response to ANF. (Hypertension 1990;15:528–540)

The role of atrial natriuretic factor (ANF) in regulating vascular muscle contractile function, diuresis and natriuresis, and secretion of renin and aldosterone is well known (for reviews see References 1 and 2). Murad and others have suggested that many of these actions are because of stimulation by ANF of a membrane-bound form of guanylate cyclase, leading to an increase in intracellular levels of the second messenger cyclic guanosine monophosphate (GMP).3,4 The link between cyclic GMP and ANF is supported by the observation that inhibitors of guanylate cyclase such as methylene blue block the vascular relaxant response to ANF5 and by the observation that changes in circulating and urinary levels of cyclic GMP correlate well with changes in circulating levels of ANF.6,7 Although considerable information is available regarding ANF-induced synthesis of cyclic GMP, relatively little is known about the factors regulating cyclic GMP degradation and termination of the response to ANF. A number of studies have demonstrated that ANF-responsive tissues and organs such as vascular smooth muscle and the kidney possess cyclic GMP phosphodiesterase (PDE).6–11 and Fiscus et al12 have shown that the nonselective PDE inhibitor isobutylmethylxanthine enhances ANF-stimulated cyclic GMP accumulation in cultured cells. However, the identification of multiple forms of cyclic nucleotide PDEs that vary with respect to substrate specificity, intracellular localization, and response to endogenous regulatory factors such as calmodulin,13,14 suggests that regulation of cyclic GMP degradation may be a complex process.

Weishaar et al15 previously reported that, in bovine coronary artery, cyclic GMP is hydrolyzed by a low Km PDE, which they labeled Type I PDE. Kukovetz...
et al. and Martin and coworkers have shown that the cyclic GMP PDE inhibitor M&B 22,948 can increase tissue levels of cyclic GMP in isolated vascular smooth muscle. M&B 22,948 also potentiates the vascular relaxant response to agents or interventions that stimulate guanylate cyclase activity (e.g., nitrovasodilators and endothelial cells). In the present investigation, the procedure previously used to isolate the low Km cyclic GMP PDE from smooth muscle was modified, revealing that this activity is actually composed of three different subclasses of the enzyme, two of which are sensitive to calmodulin (Types IA and IB) and one that is calmodulin-insensitive (Type IC). In addition, selective inhibitors of PDE IB and PDE IC were identified and used to assess the role these latter two subclasses play in regulating the vascular relaxant and renal responses to ANF.

**Methods**

Animal care and use in these studies conforms to the standards in the Guide for the Care and Use of Laboratory Animals (DHEW Publication NIH 78-12, revised 1978).

**Procedures for Isolating Phosphodiesterases**

PDEs were isolated from several vascular beds (rabbit aorta and pulmonary artery, bovine aorta) using the method previously described by Weishaar and coworkers, or using a modification of the procedure described by Lugnier et al. This latter method differed from previous isolation procedures in that the adsorption column contained diethylaminoethyl (DEAE)-Trisacryl M instead of DEAE-cellulose, and the proteins were eluted using different salt gradients to increase resolution. After connective tissue and adventitia were removed, 2-4 g vascular muscle from each bed was minced into 0.5 mm squares with a McIlwain tissue chopper (Brinkman Instrs., Westbury, New York), and suspended in 10 volumes (wt/vol) of buffer A (20 mM Tris-HCl, pH 7.5, containing 2 mM magnesium acetate, 1 mM dithiothreitol, and 5 mM Na$_2$EDTA). The proteinase inhibitors leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF) were also included in this buffer (final concentration of 100 nM each). The mince was homogenized with a Potter-Elvehjem homogenizer and centrifuged at 100,000g for 40 minutes. The supernatant was then removed and filtered through four layers of gauze. In preliminary experiments, the supernatant was applied to DEAE-cellulose and eluted with sodium acetate as described previously. In subsequent experiments, the supernatant was applied to a DEAE- Trisacryl M column. The column was washed with several bed volumes of buffer B (20 mM Tris-HCl, pH 7.5, containing 2 mM magnesium acetate, 1 mM dithiothreitol, and proteinase inhibitors) and eluted by two successive linear NaCl gradients (0.05-0.15 M, 300 ml total; 0.15-0.40 M, 200 ml total). Five milliliter fractions were collected and assayed for cyclic AMP and cyclic GMP PDE activity in the presence and absence of 0.3 µg calmodulin and 10 µM CaCl$_2$ (this amount of calmodulin is sufficient to maximally stimulate calmodulin-dependent PDE activity). Appropriate fractions were pooled and dialyzed overnight against 4 l buffer C (200 mM Tris-HCl, pH 7.5, containing 2 mM magnesium acetate and proteinase inhibitors). After complete separation, the PDEs were concentrated to 14% of the original volume. The protein was then diluted to 65% with ethylene glycol monooethyl ether and stored at –20° C for up to 6 weeks.

The pellet (containing the membrane-bound PDEs) was resuspended in 10 volumes buffer A and centrifuged as before. The resulting supernatant was discarded, and the pellet was washed once more. The final pellet was resuspended in 10 volumes (vol/vol) of buffer A containing 0.4 M NaCl, 1% Triton X-100, and 0.1% brij 30 (polyoxyethylene 4 lauryl ether) and was incubated overnight as described previously. The extracted proteins were then separated by centrifugation at 100,000g for 40 minutes. Using this latter procedure, Elks and Manganiello have reported that roughly 85-90% of the PDE activity in the pellet is recovered in the supernatant. The high salt, detergent-extracted proteins were then dialyzed for 5 hours against 4 l buffer C, applied to DEAE- Trisacryl M, and eluted as described above.

**Measuring Phosphodiesterase Activity**

PDE activity was measured as described by Thompson et al., in a reaction medium containing 40 mM Tris-HCl (pH 8.0), 5 mM MgCl$_2$, and 4 mM 2-mercaptoethanol. Unless otherwise indicated, the concentration of substrate ([3H]cyclic adenosine monophosphate (AMP) or [3H]cyclic GMP) was 1.0 µM. Selective and nonselective PDE inhibitors were dissolved in dimethyl sulfoxide (final concentration of 2.5%). This concentration of dimethyl sulfoxide inhibited enzyme activity by roughly 10%. The concentrations that inhibited substrate hydrolysis by 50% (IC$_{50}$) values for the various agents examined were determined from concentration–response curves in which concentrations typically ranged from 10 nM to 1 mM for the more potent inhibitors, and 10 µM to 1 mM for the less potent inhibitors (half-log increments). The substrate concentration at which half-maximal reaction velocity is observed (K$_{m}$) values were determined by using the method of Hofstee. For these studies, enzyme activity was measured at 14 substrate concentrations ranging from 0.1 to 100 µM in the presence of cyclic GMP (if cyclic GMP was the preferred substrate), or cyclic AMP (if cyclic AMP was the preferred substrate, or if there was no substrate specificity).

**Vascular Relaxation Studies**

Male New Zealand rabbits (Hazelton, Denver, Pennsylvania) weighing 2.5–3.0 kg were killed by cervical dislocation and exsanguination. A section of thoracic aorta and pulmonary artery 4–5 mm wide was rapidly removed and placed in oxygenated physiological salt solution (PSS) containing the following...
Hemodynamic Studies

Hemodynamic Studies were performed upon the isolated aortic rings of dogs, with the following modifications. Blood pressure was monitored with a Statham pressure transducer (Grass Instr. Co., Quincy, Massachusetts), which was connected to a Gould 2800 multichannel recorder (Cleveland, Ohio). After a 90-minute equilibration period, during which the rings were rinsed with fresh PSS, the rings were contracted with 1.0 μM norepinephrine. This concentration produced roughly 70% of the maximum response to norepinephrine. Fifteen minutes later, increasing concentrations of ANF, M&B 22,948, TCV-3B, or vehicle were added to the baths (at 30-minute intervals) and changes in tension development were noted.

In addition, norepinephrine-constricted rings were relaxed with increasing concentrations of ANF after previous application of M&B 22,948 or TCV-3B. For these latter studies, M&B 22,948 and TCV-3B were added to the baths at concentrations previously shown to produce roughly a 25% relaxant effect.

Measurement of Cyclic Guanosine Monophosphate and Cyclic Adenosine Monophosphate

For measurement of cyclic nucleotide levels, aortic rings were suspended in PSS under 4 g of tension at 37°C in water-jacketed organ baths. Equal concentrations (3 × 10−8 M) of M&B 22,948, TCV-3B, or imazodan were added to the baths, and the tissues were frozen in liquid nitrogen once a maximum relaxant response was achieved. Frozen tissues were homogenized in ice-cold 6% trichloroacetic acid, and the extracts were assayed for cyclic GMP and cyclic AMP with radioimmunoassay kits (New England Nuclear, Boston, Massachusetts) as described previously.18 Protein concentrations in the extracts were assayed with the BCA protein assay reagent (Pierce Chemical Co., Rockford, Illinois).

Renal Studies

Male Sprague-Dawley rats (CD Keepers, Charles River, Portage, Michigan) weighing 350–500 g were anesthetized with Inactin (100 mg/kg i.p., A. Lockwood Inc., Rochester, Minnesota). Cannuli were placed in the left carotid artery and jugular vein for recording arterial blood pressure and infusing drugs, respectively. Blood pressure was recorded with a P23ID Statham pressure transducer (Oxnard, California) and displayed on a Gould 2800 multichannel recorder. An infusion of lactated Ringer's solution (28 μl/min) was initiated through the venous cannula after placement. The infusion was maintained throughout the stabilization and experimental periods. Ureters were cannulated bilaterally with PE-10 tubing from a midline incision. Urine was collected into pretared tubes, and urine volume was measured gravimetrically. The sodium and potassium concentrations in urine were measured with ion-selective electrodes (Na-K Analyzer, model 1020, Orion Res. Inc., Cambridge, Massachusetts).

The experimental protocol consisted of a series of five 15-minute urine collections. The first collection period was designated control. During this time, the rats continued to receive lactated Ringer's at a rate of 28 μl/min. The second 15-minute collection period was for drug intervention. Rats received either ANF (30–1,000 ng/kg/min), M&B 22,948 (10–1,000 μg/kg/min), TCV-3B (10 and 30 μg/kg/min), or a combination of ANF and M&B 22,948 or TCV-3B. ANF was dissolved in lactated Ringer's and the PDE inhibitors in appropriate vehicles. Concentrations of drug were adjusted to achieve a fixed infusion rate of 28 μl/min. At the end of the second collection, all rats were switched back to lactated Ringer's infusions, and three additional 15-minute urine collections were performed to assess recovery.

Statistical Evaluation

For enzyme inhibitor studies IC50 values were calculated according to the method of Hubert,23 as described previously.21 For in vitro contractility studies, IC50 was calculated for each individual concentration–response curve by using a probit transformation of the data (normalized as percent of maximum relaxation) and then a linear regression analysis of this transformed data. An analysis of variance was then performed and differences in the
IC₅₀ mean values were assessed with Duncan's multiple range test.²⁴ A significant difference was considered at the p<0.05 level. The concentration of ANF required to achieve IC₅₀ was calculated for individual rings with probit analysis. Differences in the IC₅₀ values obtained in the absence and presence of drugs were assessed with a Student's t test, and p<0.05 was considered a statistically significant change. For in vivo hemodynamic studies, the responses to M&B 22,948 and TCV-3B were compared with vehicle control responses with a one-way analysis of variance. A sequential monotonic trend test was performed to determine the progressive effect of treatment. For the in vivo renal studies, statistical analysis of the maximal natriuretic or diuretic responses in each treatment group were compared with the vehicle control group with a one-way analysis of variance and a Student's t test with a weighted test (Bonferroni method) for multiple comparisons.²⁵

**Reagents**

All reagents used were of the highest obtainable commercial purity. Cyclic AMP labeled with [2,8-³H] (30–50 Ci/mmol) and [8-³H(N)]-labeled cyclic GMP (10–25 Ci/mmol) were obtained from New England Nuclear (Boston, Massachusetts). Calmodulin, Ophiophagus hannah snake venom, theophylline, cyclic AMP, cyclic GMP, pepstatin A, leupeptin, PMSF, Triton x-100 and brij 30 were obtained from Sigma Chemical Co. (St. Louis, Missouri). DEAE-Cellulose (product No. Cx583-1-9178) was obtained from Matheson, Coleman and Bell (East Rutherford, New Jersey). DEAE-Trisacryl M was obtained from IBF Techniques (Savage, Maryland). Inactin was obtained from A. Lockwood, Inc. (Rochester, Minnesota). Imazodan (hydrochloride salt), Ro 20-1724, TCV-3B, ICI 74,917, and M&B 22,948 were prepared by the Parke-Davis Chemistry Department. Rolipram was obtained from Scherring AG Pharmaceutical Co. (Berlin, GDR). Dipyridamole was obtained from CIBA-GEIGY (Summit, New Jersey). Isobutylmethylxanthine was obtained from the Aldrich Chemical Co. (Milwaukee, Wisconsin). ANF was obtained from Bachem, Inc. (Torrance, California). The purity of these reagents was more than 99%.

**Results**

**Isolation of Subclasses of Type I Phosphodiesterases**

By using standard isolation procedures with DEAE-cellulose chromatography, rabbit aortic smooth muscle was found to contain multiple forms of cyclic nucleotide PDE (Figure 1). These enzymes were labeled Type I, Type II, and Type III PDE, based on their order of elution. As shown in Figure 1, Type I PDE appeared to be composed of subclasses. Absorption on DEAE-Trisacryl M rather than DEAE-cellulose, combined with changes in the protein elution protocol, revealed that Type I PDE in rabbit aorta and pulmonary artery was actually composed of three distinct types of PDE enzymes. As seen in Figure 2, these three enzymes varied according to substrate specificity, sensitivity to calmodulin, and intracellular localization. Type IA PDE was the least abundant subclass of Type I PDE. This enzyme hydrolyzed both cyclic GMP and cyclic AMP and was present in both the soluble and particulate fractions. In addition, Type IA PDE activity could be stimulated roughly 20-fold by calmodulin. Type IB PDE preferentially hydrolyzed cyclic GMP and was present only in the soluble fraction. The activity of Type IB PDE was stimulated by calmodulin, but not to the degree observed for Type IA PDE. (The small amount of Type IA PDE that could be obtained precluded any detailed kinetic comparison of the differences in calmodulin responsiveness between the Type IA and IB subclasses). Like Type IB PDE, Type IC PDE preferentially hydrolyzed cyclic GMP and appeared to be primarily a soluble enzyme. In contrast to the Type IA and Type IB enzymes, the activity of the Type IC PDE was not stimulated by calmodulin.

The same three subclasses of the Type I PDE were also identified in rabbit pulmonary artery (Figure 3) and in bovine coronary artery (data not shown).

Kinetic studies revealed that all three subclasses of the Type I PDE from rabbit aorta were low Kₘ enzymes. The Kₘ for cyclic GMP hydrolysis was 1.1 μM for the Type IA PDE, 4.7 μM for the Type IB PDE, and 0.6 μM for the Type IC PDE (n=2–4 for each subclass). Because of the low basal activity of Type IA PDE, kinetic studies with this subclass were performed in the presence of saturating concentrations of CaCl₂ and calmodulin.
The Type II and Type III PDEs were also eluted from the DEAE-Trisacryl M. Both enzymes displayed the same substrate specificity as the enzymes previously eluted from DEAE-cellulose. Kinetic analysis revealed that the Type II PDE was a high \( K_m \) enzyme (\( K_m = 20 \) \( \mu \)M for cyclic AMP), whereas both high and low \( K_m \) sites for cyclic AMP hydrolysis were characterized for the Type III PDE (\( K_m = 1.4 \) and 9.4 \( \mu \)M). The Type III PDE appeared to be present in both the soluble and particulate fractions.

Response of Vascular Phosphodiesterases to Reference Inhibitors

To further characterize the differences between the subclasses of the Type I PDE, the inhibitory effects of several reference cyclic GMP PDE inhibi-

FIGURE 2. Plots showing separation of soluble (Panels A and B) and membrane-bound (Panels C and D) cyclic nucleotide phosphodiesterases isolated from rabbit aorta. Column fractions were eluted from diethylaminoethyl (DEAE)-Trisacryl M and assayed directly for enzyme activity in the presence of 1.0 \( \mu \)M cyclic guanosine monophosphate (GMP) (Panels A and C) or cyclic adenosine monophosphate (AMP) (Panels B and D), and in the presence (filled symbols) and absence (open symbols) of calmodulin and CaCl\(_2\). Procedure used to isolate the enzymes and elute them from DEAE-Trisacryl M is described in Methods section. Each panel is representative of three comparable isolations.

FIGURE 3. Plots showing separation of soluble (Panels A and B) and membrane-bound (Panels C and D) cyclic nucleotide phosphodiesterases isolated from rabbit pulmonary artery. Column fractions were eluted from diethylaminoethyl (DEAE)-Trisacryl M and assayed directly for enzyme activity in the presence of 1.0 \( \mu \)M cyclic guanosine monophosphate (GMP) (Panels A and C) or cyclic adenosine monophosphate (AMP) (Panels B and D), and in the presence (filled symbols) and absence (open symbols) of calmodulin and CaCl\(_2\). Procedure used to isolate enzymes and elute them from DEAE-Trisacryl M is described in Methods section. Each panel is representative of three comparable isolations.
In Vitro Effects of Selective Type I Phosphodiesterase Inhibitors

In addition to evaluating their inhibitory effect on the partially purified subclasses of cyclic GMP–specific PDE from vascular muscle, the ability of TCV-3B and M&B 22,948 to alter cyclic nucleotide levels in isolated aortic rings was also examined. For comparative purposes, the response to the selective cyclic AMP–specific PDE inhibitor, imazodan, was also evaluated. As shown in Figure 5, both TCV-3B and M&B 22,948 increased cyclic GMP levels in vascular rings sevenfold to 10-fold while having no effect on basal levels of cyclic AMP. In contrast, imazodan significantly increased cyclic AMP levels while having no effect on cyclic GMP (Figure 5).

Table 1. Effects of Reference Phosphodiesterase Inhibitors on Phosphodiesterases Isolated From Rabbit Aorta and Pulmonary Artery

<table>
<thead>
<tr>
<th>Agent</th>
<th>Tissue</th>
<th>Type IA PDE*</th>
<th>Type IB PDE</th>
<th>Type IC PDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI 74,917</td>
<td>Aorta</td>
<td>25(2)</td>
<td>23(3)</td>
<td>19(3)</td>
</tr>
<tr>
<td></td>
<td>Pulmonary artery</td>
<td>ND</td>
<td>30(3)</td>
<td>38(3)</td>
</tr>
<tr>
<td>TCV-3B</td>
<td>Aorta</td>
<td>&gt;100(2)</td>
<td>23(3)</td>
<td>120(3)</td>
</tr>
<tr>
<td></td>
<td>Pulmonary artery</td>
<td>ND</td>
<td>20(3)</td>
<td>&gt;100(3)</td>
</tr>
<tr>
<td>M&amp;B 22,948</td>
<td>Aorta</td>
<td>&gt;100(2)</td>
<td>20(3)</td>
<td>1.2(3)</td>
</tr>
<tr>
<td></td>
<td>Pulmonary artery</td>
<td>ND</td>
<td>20(3)</td>
<td>1.2(3)</td>
</tr>
</tbody>
</table>

The IC₅₀ (concentration that inhibits substrate hydrolysis by 50%) values were determined from concentration–response curves, in which concentrations ranged from 3 x 10⁻⁷ to 3 x 10⁻³ M for the more potent inhibitors, and from 10⁻⁵ to 10⁻³ M for the less potent inhibitors (half-log increments). Enzyme activity was measured as described in Methods section. The number of separate concentration–response curves generated for each agent is shown in parentheses. Typically, each concentration–response curve was generated using different enzyme preparations. Cyclic guanosine monophosphate was used as the substrate for inhibitor studies with the Types IA, IB, and IC phosphodiesterase (PDE). ND, not determined.

*Because of low basal activity, inhibitor studies with the Type IA PDE were conducted in the presence of saturating concentrations of calmodulin and CaCl₂.
To characterize the role that the Type IB and Type IC PDEs play in regulating in vitro vascular muscle contractile function, the relaxant response to the selective Type IB PDE inhibitor TCV-3B and the selective Type IC PDE inhibitor M&B 22,948 was evaluated. The inability to identify a selective Type IA PDE inhibitor precluded further studies regarding the involvement of this subclass in regulating in vitro vascular contractile function. In addition to examining the direct effects of both compounds, the ability of TCV-3B and M&B 22,948 to potentiate the relaxant response to the guanylate cyclase stimulator ANF was also evaluated.

**Direct effects.** As shown in Figure 6, both TCV-3B and M&B 22,948 relaxed isolated vascular rings previously contracted with 1.0 µM norepinephrine. TCV-3B and M&B 22,948 were equipotent in the rabbit pulmonary artery (IC50 = 22±4 µM for TCV-3B and 29±2 µM for M&B 22,948). In the rabbit aorta, TCV-3B was more potent than M&B 22,948 (IC50 = 41±17 µM for TCV-3B and >100 µM for M&B 22,948). Solubility problems precluded evaluating either compound at concentrations greater than 100 µM.

**Potentiation of the in vitro response to atrial natriuretic factor.** The vascular relaxant effect of ANF on isolated aortic rings was significantly (*p<0.05) potentiated by pretreatment with TCV-3B or M&B 22,948 (Figure 7A). For these experiments, rings were pretreated with equipotent concentrations of TCV-3B and M&B 22,948 before addition of increasing concentrations of ANF. TCV-3B and M&B 22,948 also significantly potentiated the relaxant response of ANF on isolated pulmonary artery rings (Figure 7B). These latter experiments were also conducted using equipotent concentrations of TCV-3B and M&B 22,948. The results of these experiments are summarized in Table 2.

In contrast to ANF, TCV-3B and M&B 22,948 did not potentiate the vascular relaxant response to the adenylate cyclase stimulator forskolin (Figure 8).

**In Vivo Effects of Selective Type I Phosphodiesterase Inhibitors**

Because TCV-3B and M&B 22,948 both exerted a relaxant effect on isolated vascular smooth muscle,
the direct effect of both compounds on blood pressure and total peripheral resistance was evaluated in the anesthetized dog. In addition, the ability of TCV-3B and M&B 22,948 to potentiate the renal effects of ANF in the anesthetized rat was also evaluated.

Direct effects. When administered intravenously to open-chest anesthetized dogs, M&B 22,948 produced a significant (p<0.05) dose-dependent reduction in blood pressure at doses ranging from 30 to 300 μg/kg (Table 3). This reduction was accompanied by a significant decrease in total peripheral resistance and a nonsignificant increase in cardiac output. Heart rate was not significantly altered at any dose tested. Infusion of TCV-3B also resulted in a significant dose-dependent reduction in blood pressure, total peripheral resistance, and in cardiac output at doses ranging from 100 to 3,000 μg/kg (Table 3). Infusion of TCV-3B also produced a slight decrease in heart rate that was not statistically significant (p<0.05).

Potentiation of the in vivo response to atrial natriuretic factor. To determine whether M&B 22,948 or TCV-3B would potentiate the in vivo effects of ANF, the ability of both compounds to influence the renal response to ANF was evaluated. When infused intravenously to anesthetized normotensive rats, both M&B 22,948 and TCV-3B significantly increased sodium excretion (Figures 9A and 9B). The maximal response to M&B 22,948 was a fivefold increase in sodium excretion at 300 μg/kg/min. TCV-3B produced a 2.5-fold increase in sodium excretion at 30 μg/kg/min. In contrast, ANF produced 30-fold increases in sodium excretion at 1,000 ng/kg/min (Figure 9C). The onset of the response to ANF was prompt and waned rapidly during the recovery periods. M&B 22,948 and TCV-3B both produced responses that were slower in onset and recovery.

**TABLE 2. In Vitro Relaxant Response to Atrial Natriuretic Factor in the Absence and Presence of TCV-3B or M&B 22,948**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Aorta</th>
<th>Pulmonary artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANF alone</td>
<td>4.9±1.1</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>ANF plus TCV-3B*</td>
<td>0.9±0.2†</td>
<td>0.4±0.1†</td>
</tr>
<tr>
<td>ANF plus M&amp;B 22,948*</td>
<td>0.6±0.1†</td>
<td>0.3±0.1†</td>
</tr>
</tbody>
</table>

*ANF, atrial natriuretic factor; IC50 concentration that inhibits substrate hydrolysis by 50%.
†Tissues were pretreated with a concentration of TCV-3B or M&B 22,948 that produced a 25% relaxation.
‡p<0.05 compared with vehicle control.

**TABLE 3. Hemodynamic Response to Increasing Doses of Selective Type 1 Phosphodiesterase Inhibitors**

<table>
<thead>
<tr>
<th>Dose (μg/kg)</th>
<th>Blood pressure (mm Hg)</th>
<th>Total peripheral resistance (mm Hg/min)</th>
<th>Cardiac output (l/min)</th>
<th>Heart rate (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>121±9</td>
<td>83±14</td>
<td>1.5±7</td>
<td>150±7</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0±2%</td>
<td>-5±0%</td>
<td>+3±3%</td>
<td>-4±2%</td>
</tr>
<tr>
<td>1 μg/kg</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>3 μg/kg</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>10 μg/kg</td>
<td>-1±1%</td>
<td>-1±3%</td>
<td>+1±1%</td>
<td>-2±1%</td>
</tr>
<tr>
<td>30 μg/kg</td>
<td>-2±1%</td>
<td>-5±1%</td>
<td>+6±2%</td>
<td>-2±1%</td>
</tr>
<tr>
<td>100 μg/kg</td>
<td>-4±1%*</td>
<td>-8±2%</td>
<td>+5±2%</td>
<td>-9±2%</td>
</tr>
<tr>
<td>300 μg/kg</td>
<td>-5±1%*</td>
<td>-13±3%*</td>
<td>+12±0%*</td>
<td>-8±2%</td>
</tr>
<tr>
<td>1,000 μg/kg</td>
<td>-19±4%*</td>
<td>-36±2%*</td>
<td>+18±2%*</td>
<td>-8±1%</td>
</tr>
<tr>
<td>3,000 μg/kg</td>
<td>-22±5%*</td>
<td>-46±8%*</td>
<td>+28±1%*</td>
<td>-10±2%</td>
</tr>
</tbody>
</table>

**TABLE 4. In Vitro Relaxant Response to Atrial Natriuretic Factor in the Absence and Presence of TCV-3B or M&B 22,948**

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<th>Treatment group</th>
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<tr>
<td>ANF plus M&amp;B 22,948*</td>
<td>0.6±0.1†</td>
<td>0.3±0.1†</td>
</tr>
</tbody>
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†Tissues were pretreated with a concentration of TCV-3B or M&B 22,948 that produced a 25% relaxation.
‡p<0.05 compared with vehicle control.

**FIGURE 8. Plots showing relaxant effect of increasing concentrations of forskolin on isolated rabbit aortic rings (Panel A) and pulmonary artery rings (Panel B) with absence (○) and presence of TCV-3B (△) or M&B 22,948 (●). Both aortic and pulmonary artery rings were precontracted with 1.0 μM norepinephrine. Responses at each concentration of forskolin are normalized as percent of maximum response to forskolin after equilibration with vehicle or one of phosphodiesterase inhibitors. Each value represents mean±SEM of four to six experiments. For potentiation experiments, rings were pretreated with a concentration of TCV-3B or M&B 22,948 that produced a 25% relaxation 30 minutes before adding forskolin to bath. For aortic rings, 8 μM TCV-3B and 8 μM M&B 22,948 were used. For pulmonary artery rings, 4 μM TCV-3B and 2 μM M&B 22,948 were used. There was no significant difference (p>0.05) in response to forskolin in presence of TCV-3B or M&B 22,948.
In addition to increasing sodium excretion, M&B 22,948 and TCV-3B also produced increases in urine volume and potassium excretion. The magnitude of these effects were less than those observed with ANF. No significant changes in mean arterial blood pressure were observed with TCV-3B. M&B 22,948 produced a reduction in blood pressure at the 1,000 μg/kg/min dose. These results are summarized in Table 4.

Administration of M&B 22,948 or TCV-3B also significantly potentiated the renal response to simultaneously administered ANF. For these experiments, TCV-3B or M&B 22,948 were administered at doses of 10 or 30 μg/kg/min, respectively. ANF was simultaneously infused at doses ranging from 30 to 300 ng/kg/min. As shown in Figure 10A, TCV-3B coadministered with ANF (100 ng/kg/min) increased sodium excretion by more than 4,000% of the basal level. This increase was more than double the response to ANF alone. A similar potentiation of the renal response was observed when M&B 22,948 and ANF were coadministered (Figure 10B).

M&B 22,948 and TCV-3B also significantly potentiated the effects of ANF on urine volume and potassium excretion. These results are summarized in Table 5.

Discussion

In 1971 Thompson and Appleman first demonstrated the presence of multiple molecular forms of PDE in the rat brain. Subsequent investigations in other laboratories demonstrated that multiple forms of PDE exist in a variety of organs and cells, including liver, heart, thyroid gland, adipocytes, and platelets. In addition, selective inhibitors of several forms of PDE have been identified and used to evaluate the role that these different enzymes play in regulating various physiological processes, including cardiac contractility, lipolysis, and the activity of the central nervous system. New isolation techniques have also revealed that some of the PDEs are actually composed of subclasses. In 1984, Yamamoto et al demonstrated that the cyclic AMP-specific PDE present in calf liver is actually composed of two subclasses, one that is inhibited by cyclic GMP and one that is insensitive to cyclic GMP. Similar results have been obtained in canine ventricular muscle. The involvement of the subclasses of cyclic AMP PDE in regulating ventricular contractile function was recently described by Weishaar et al. The present series of experiments was designed to characterize the subclasses of the Type I or cyclic GMP-specific PDE present in smooth muscle from different vascular beds. In addition, the role of two of these subclasses in modulating the response to the endogenous guanylate cyclase stimulator ANF was also evaluated.
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Table 4. Effect of M&B 22,948, TCV-3B, and Atrial Natriuretic Factor on Renal Function in Anesthetized Normotensive Rats

<table>
<thead>
<tr>
<th>Dose (ng/kg/min)</th>
<th>Change in sodium excretion (µmol/min)</th>
<th>Change in potassium excretion (µmol/min)</th>
<th>Change in urine output (µl/min)</th>
<th>Change in mean arterial blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+0.07±0.04(6)</td>
<td>+0.21±0.18(6)</td>
<td>+0.12±0.16(6)</td>
<td>-0.3±0.2(6)</td>
</tr>
<tr>
<td>30</td>
<td>+0.12±0.18(6)</td>
<td>+0.22±0.23(6)</td>
<td>+0.13±0.17(6)</td>
<td>-0.3±0.2(6)</td>
</tr>
<tr>
<td>100</td>
<td>+0.15±0.19(6)</td>
<td>+0.23±0.24(6)</td>
<td>+0.14±0.18(6)</td>
<td>-0.3±0.2(6)</td>
</tr>
<tr>
<td>300</td>
<td>+0.18±0.20(6)</td>
<td>+0.25±0.26(6)</td>
<td>+0.15±0.19(6)</td>
<td>-0.3±0.2(6)</td>
</tr>
<tr>
<td>1,000</td>
<td>+0.21±0.22(6)</td>
<td>+0.27±0.28(6)</td>
<td>+0.17±0.20(6)</td>
<td>-0.3±0.2(6)</td>
</tr>
</tbody>
</table>

Substitution of the anion-exchange resin DEAE-Trisacyl M for DEAE-cellulose in the PDE isolation procedure revealed that the Type I PDE present in rabbit and bovine vascular smooth muscle is actually composed of three distinct subclasses. The activity of two of these subclasses (Type IA and IB) is stimulated by calmodulin, whereas the third subclass (Type IC) is insensitive to calmodulin. In addition, although the Type IA PDE hydrolyzes both cyclic AMP and cyclic GMP, the Type IB and IC subclasses preferentially hydrolyze cyclic GMP. Sharma and Wang34 previously described two forms of calmodulin-stimulated PDE in bovine brain. These enzymes possess different molecular weights and are differentially regulated by calcium-protein kinases and cyclic AMP–protein kinases. In addition, the two enzymes are stimulated to different degrees by calmodulin; the activity of one form is stimulated roughly 30-fold, whereas the other form is stimulated only fivefold. Similar differences in the response of the Type IA and IB PDEs to calmodulin were noted in the present study, with the Type IA subclass being stimulated roughly 20-fold, whereas the Type IB PDE was stimulated only twofold to fourfold.

In addition to differences in substrate specificity and calmodulin sensitivity, the three subclasses of Type I PDE also differed in their response to the reference PDE inhibitors ICI 74,917, TCV-3B, and M&B 22,948. All three compounds have previously been characterized as selective inhibitors of the Type I or cyclic GMP PDE, although none have been previously evaluated for inhibitory effects on the subclasses of the Type I PDE. In the present studies, ICI 74,917 exerted a nonselective inhibitory effect on all three subclasses, whereas TCV-3B exerted a selective inhibitory effect on the Type IB PDE, and M&B 22,948 produced a greater inhibitory effect on the Type IC PDE. The inability to identify a selective inhibitor of the Type IA PDE precluded additional experiments to assess the physiological role of this subclass. Instead, subsequent studies focused on using the selective Type IB inhibitor TCV-3B and the selective Type IC inhibitor M&B 22,948 as pharmacological probes to investigate the involvement of these latter two subclasses in modulating vascular muscle contractile activity and renal function. In addition, because both the Type IB and IC PDEs preferentially hydrolyze cyclic GMP, the ability of TCV-3B and M&B 22,948 to influence the in vitro and in vivo response to the endogenous guanylate cyclase stimulator ANF was evaluated.

Both TCV-3B and M&B 22,948 have previously been shown to relax vascular smooth muscle in vitro, and Karpati et al36 have reported that administration of TCV-3B to anesthetized dogs resulted in an increase in cerebral blood flow. M&B 22,948 has also been shown to potentiate the increase in tissue levels of cyclic GMP produced by acetylcholine, presumably through its inhibitory effect on cyclic GMP PDE activity. In the present study, M&B 22,948 relaxed preconstricted rabbit aortic and
The role that the calmodulin-dependent and calmodulin-independent subclasses of cyclic GMP PDE play in regulating cyclic GMP-dependent metabolic events was further assessed by evaluation of the ability of TCV-3B and M&B 22,948 to potentiate in vivo vascular relaxant response to ANF. The physiological responses to ANF have been extensively documented and include relaxation of vascular smooth muscle, stimulation of sodium excretion and urine output, and inhibition of aldosterone secretion. Murad, Winquist and others have provided evidence to suggest that the response to ANF is due to activation of a particulate form of guanylate cyclase, resulting in an increase in tissue levels of cyclic GMP. This increase is associated with increased activity of cyclic GMP-dependent protein kinase.

In the present study, both TCV-3B and M&B 22,948 significantly potentiated the in vitro vascular relaxant response to ANF, suggesting that both the calmodulin-dependent and calmodulin-independent subclasses of the cyclic GMP-specific PDE play a role in modulating the effects of ANF on vascular smooth muscle. In contrast, neither TCV-3B nor M&B 22,948 potentiated the relaxant response to the adenylate cyclase stimulator forskolin, indicating that both inhibitors selectively influence cyclic GMP but not cyclic AMP–dependent responses. TCV-3B and M&B 22,948 also potentiated the in vivo effects of ANF on renal function (e.g., urine output and sodium excretion) and arterial blood pressure, suggesting that they did not represent an indirect response to changes in renal perfusion pressure.
sodium excretion). The response to coadministration of either inhibitor together with ANF was significantly greater than the response to either the inhibitors or ANF alone. These observations provide additional support to indicate that both the calmodulin-dependent and calmodulin-independent subclasses play a role in modulating the physiological response to ANF.

Although TCV-3B and M&B 22,948 produced effects on in vitro vascular muscle contractile and in vivo renal function that were qualitatively similar to those produced by ANF, high doses of both compounds were required to produce these effects. In addition, the maximum response to ANF was greater than the maximum response to either TCV-3B or M&B 22,948. These differences may indicate that under basal conditions (e.g., when circulating levels of ANF are low) tissue levels of cyclic GMP are reduced, thereby limiting the response to inhibition of cyclic GMP PDE activity. When circulating ANF levels are elevated by infusion of exogenous ANF or in response to pathological conditions such as hypertension or heart failure, the response to the two inhibitors should be increased. This latter possibility appears to be the case, as coadministration of TCV-3B or M&B 22,948 with submaximal doses of ANF markedly potentiated the renal response to ANF infusion alone. The renal response to TCV-3B and M&B 22,948 in hypertensive rats is currently under investigation.

In conclusion, the results of the present study demonstrate that vascular smooth muscle from two species (cow and rabbit) and three vascular beds (aorta, pulmonary artery, and coronary artery) possess subclasses of the Type I PDE. The three subclasses identified vary with respect to substrate specificity, sensitivity to calmodulin, and response to various PDE inhibitors. In addition, selective inhibitors of the calmodulin-dependent and calmodulin-independent forms of cyclic GMP-specific PDE were used to demonstrate that both subclasses are involved in mediating the in vitro and in vivo actions of ANF. These results provide new insight into the pathways regulating the cyclic GMP-dependent response to ANF and may provide new opportunities to develop therapeutic agents that mimic the actions of ANF.

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R E Weishaar, D C Kobylarz-Singer, J Keiser, S J Haleen, T C Major, S Rapundalo, J T Peterson and R Panek

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