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# Characterization and Effects of Methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline Carboxylate Sulfate (T-1032), a Novel Potent Inhibitor of cGMP-Binding cGMP-Specific Phosphodiesterase (PDE5)

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**ABSTRACT.** An isoquinolone derivative, methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate sulfate (T-1032), was found to be a novel potent inhibitor of cyclic GMP (cGMP)-binding cGMP-specific phosphodiesterase (PDE5). We investigated the inhibitory effects of T-1032 on six PDE isozymes isolated from canine tissues. T-1032 specifically inhibited the hydrolysis of cGMP by PDE5 partially purified from canine lung, at a low concentration ($IC_{50}$ = 1.0 nM, $K_i$ = 1.2 nM), in a competitive manner. In contrast, the $IC_{50}$ values of T-1032 for PDE1, PDE2, PDE3, and PDE4 were more than 1 μM. T-1032 also inhibited PDE6 from canine retina with an $IC_{50}$ of 28 nM, which is of the same order of magnitude as the $IC_{50}$ of sildenafil. cGMP hydrolytic activities of two alternative splice variants of canine PDE5 expressed in COS-7 cells were inhibited by this compound to a similar extent. T-1032 increased the intracellular concentration of cGMP in cultured rat vascular smooth muscle cells in the presence and absence of C-type natriuretic peptide, an activator of membrane-bound guanylate cyclase, whereas the compound did not change cyclic AMP levels. These data indicated that T-1032, which belongs to a new structural class of PDE5 inhibitors, is a potent and selective PDE5 inhibitor. This compound may be useful in pharmacological studies to examine the role of a cGMP/PDE5 pathway in tissues. BIOCHEM PHARMACOL **60**;9:1333–1341, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** cGMP; phosphodiesterase; isozyme; canine; inhibitor; vascular smooth muscle cells

cGMP§ acts as a second messenger for vasodilation [1–3] and a synaptic signaling agent in the central nervous system [4–7]. It has been reported that cGMP plays roles in the physiological functions in other tissues such as the intestine, the pancreas, and adipocytes [8–10]. Two major pathways for cGMP production have been reported. One is mediated by NO stimulation, which activates soluble guanylate cyclases [11, 12], and another is mediated by natriuretic peptides and guanylin/uroguanylin via membrane-bound receptors: guanylate cyclase-A, guanylate cyclase-B, and guanylate cyclase-C [13, 14]. cGMP degradation is controlled by cGMP hydrolytic PDEs. Based on amino acid sequence analysis and biochemical properties, eleven PDE families have been recognized [15–18]. PDE1, PDE2, PDE5, PDE6, PDE9, PDE10, and PDE11 are categorized as cGMP hydrolytic PDEs [16–18], and each PDE isozyme shows a specific tissue expression pattern. In the vascular system, NO released from endothelial cells and natriuretic peptides in blood or from endothelial cells lead to vasodilation via cGMP generation in VSMCs. PDEs associated with cGMP degradation in VSMCs have been reported to be PDE1, PDE2, PDE3, and PDE5 [19–21].

PDE5 activity has been found in the lung, vascular and tracheal smooth muscle cells, the spleen, and platelets [22–24]. Recently, we cloned rat, canine, and human PDE5 cDNAs and investigated the tissue distributions of the transcripts in these species [25–27]. High levels of PDE5 transcripts were observed in many tissues, such as the cerebellum and intestine, in addition to tissues containing blood vessels. More recently, immunohistochemical analysis using an anti-PDE5 antibody has demonstrated that

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§ *Abbreviations*: cGMP, cyclic GMP; cAMP, cyclic AMP; NO, nitric oxide; PDE(s), cyclic nucleotide phosphodiesterase(s); PDE5, cGMP-binding cGMP-specific PDE; VSMCs, vascular smooth muscle cells; CNP, C-type natriuretic peptide; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-adenine; and IBMX, 3-isobutyl-1-methylxanthine.

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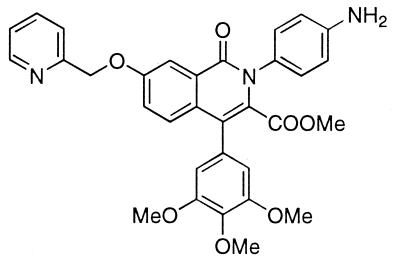
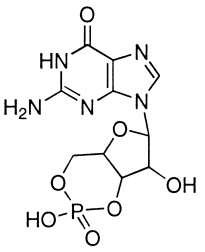
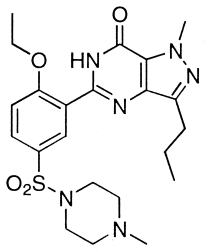



T-1032

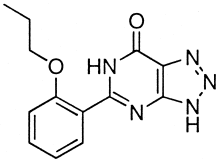



cGMP

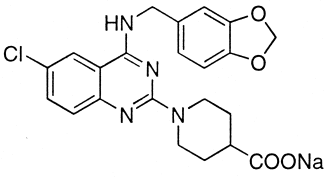
**FIG. 1. Chemical structures of PDE5 inhibitors and cGMP.**




Sildenafil




Zaprinast




E4021

PDE5 is also present in proximal renal tubules, collecting renal ducts, and epithelial cells of pancreatic ducts in rats [28]. We found two alternative splice variants (PDE5A1 and PDE5A2) possessing distinct N-terminal sequences in humans, canines, and rats, and two variants showed similar kinetic properties including $K_{m,}$ $V_{max,}$ and inhibition by a PDE5 inhibitor, zaprinast [27]. In humans, different expression patterns of the two different spliced transcripts of human PDE5As in certain adult and fetal tissues have been observed [29]. In our previous study, the levels of the PDE5A transcripts, especially the PDE5A2 transcripts, have been revealed to be increased by cAMP in cultured rat VSMCs, indicating that the PDE5A2 is an inducible variant of PDE5A in rats [29].

Zaprinast (Fig. 1) is effective in enhancing cGMP accumulation in VSMCs [19, 30] and promotes induction of cerebellar long-term depression in rat cerebellar slices [31]. Sodium 1-[6-chloro-4-(3,4-methylenedioxybenzyl)-aminoquinazolin-2-yl]piperidine-4-carboxylate sesquihydrate (E4021; Fig. 1), a selective PDE5-specific inhibitor, was reported to increase cGMP levels in VSMCs and reduce hypoxic vasoconstriction, pulmonary artery pressure, and total pulmonary resistance with no significant effect on cardiac output, systemic pressure, and resistance [32, 33]. A potent PDE5-specific inhibitor, sildenafil (Fig. 1), is effective in treating male erectile dysfunction via cGMP increase in corpus cavernosum smooth muscle [34]. More recently, another PDE5 inhibitor, 4-(3-chloro-4-methoxybenzyl)amino-1-(4-hydroxypiperidino)-6-phthalazinecarbonitrile monohydrochloride (E4010), has been reported to attenuate hypoxic or monocrotaline-induced pulmonary hypertension in rats [35]. In the present study, we isolated six canine PDE isozymes, which were used to characterize a novel PDE5 inhibitor, methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate sulfate (T-1032; Fig. 1). We also examined the effects of T-1032 on the intracellular accumulation of cGMP and cAMP in cultured rat VSMCs.

## MATERIALS AND METHODS

### *Materials*

T-1032, sildenafil, milrinone, and rolipram were synthesized at the Discovery Research Laboratory of the Tanabe Seiyaku Co., Ltd. With regard to T-1032 and sildenafil, we confirmed by HPLC that the purity of these compounds used in this work was more than 99%. [$^3$H]cGMP, [$^3$H]cAMP, a cGMP EIA System, and a cAMP EIA System were from Amersham Pharmacia Biotech. cGMP, cAMP, Dowex (1 × 8–400), EHNA, IBMX, and calmodulin were purchased from the Sigma Chemical Co. CNP was obtained from the Peptide Institute. *Crotalus atrox* snake venom was purchased from Nakarai Tesque. Male canines were purchased from Oriental Yeast. Male Sprague–Dawley rats were obtained from Japan SLC. All experimental protocols for animal studies were approved by the Animal Care and Use Committee of the Tanabe Seiyaku Co., Ltd.

### *Preparation of PDE Isozymes from Canine Tissues*

Male canines at 48 weeks of age were anesthetized with sodium pentobarbital before various tissues were excised and stored in liquid nitrogen until used. PDE1, PDE4, and PDE5 were partially purified from canine lung. One gram of canine lung was homogenized in 10 mL of ice-cold homogenization buffer A (20 mM Tris–HCl, pH 7.8, 2 mM Mg

acetate, 0.3 mM $CaCl_2$, 1 mM dithiothreitol, 1.3 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride) in a Polytron homogenizer for 1 min at medium speed. The homogenate was centrifuged at 6000 *g* for 20 min, and then the supernatant was recentrifuged at 100,000 *g* for 60 min. The cytosolic fraction was applied to a DEAE–Sepharose FF column (Amersham Pharmacia Biotech) equilibrated in elution buffer A (20 mM Tris–HCl, pH 7.8, 1 mM dithiothreitol, 1 mM $CaCl_2$, and 5 mM benzamidine). The column was washed with 60 mL of elution buffer A, and proteins were then eluted from the column by running a linear NaCl gradient (0 to 0.35 M, 180 mL) in elution buffer A. Fractions (2 mL each) were collected on ice and assayed for cGMP and cAMP hydrolytic activities.

Partial purification of PDE3 and PDE2 from canine heart and canine adrenal gland was done as follows. One gram of canine heart or 1 g of canine adrenal gland was homogenized in 5 mL of ice-cold homogenization buffer B (25 mM Tris–HCl, pH 7.5, 4 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM EGTA, 4 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) and then centrifuged as described above. The cytosolic fractions were applied to a DEAE–Sepharose FF column equilibrated in elution buffer B (25 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EGTA, and 5 mM benzamidine). The column was washed with 60 mL of elution buffer B, and proteins were then eluted from the column by running a linear NaCl gradient (0 to 0.55 M for heart or 0 to 0.45 M for adrenal gland, 180 mL) in elution buffer B. Fractions (2 mL each) were collected on ice and assayed for cGMP and cAMP hydrolytic PDE activities.

Purification of PDE6 from canine retina was performed by a modified version of the methods of Yamazaki *et al.* [36]. In brief, after twenty canine retinas (approximately 2 g) were excised in room light, rod outer segments were prepared by a 3-layer stepwise sucrose gradient composed of 1.10, 1.11, 1.13, and 1.15 g/mL concentrations of sucrose according to the reported method. Crude PDE6 fractions were eluted from the rod outer segments by hypotonic buffer (5 mM Tris–HCl, pH 7.5, 5 mM dithiothreitol, 0.5 mM $MgSO_4$, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin, and 10 μM leupeptin). The eluate containing PDE6 was applied to a Bio-Gel 1.5-m Gel column (Bio-Rad Laboratories) equilibrated with elution buffer C (50 mM Tris–HCl, pH 7.5, 2 mM dithiothreitol, and 0.15 M NaCl). Fractions (2 mL each) were collected on ice and assayed for cGMP hydrolytic PDE activities. We checked the purity of the isolated PDE6 fractions by SDS–PAGE and silver staining. This analysis showed only two bands derived from PDE6α and PDE6β. Moreover, the identity of PDE6 isolated from retinas was also confirmed through its activation by trypsin.

### *PDE Assay*

The PDE assay was performed by the radiolabeled nucleotide method [37]. Assay buffer contained 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 0.33 mg/mL of BSA (Sigma), 0.1 to 30 μL of enzyme solution, unlabeled cGMP or cAMP, and 12.5 nM [$^3$H]cGMP or 4.88 nM [$^3$H]cAMP. The reaction was started by mixing the substrate into 500 μL of the assay buffer, and tubes were incubated at 37° for 30 min. After boiling for 1.5 min, the mixtures were added to 100 μL of a 1 mg/mL solution of *Crotalus atrox* snake venom and incubated at 37° for 30 min. The reaction was stopped by the addition of 500 μL methanol, and the resultant solutions were applied to a Dowex (1 × 8–400) column (volume 0.25 mL). Aqueous scintillation fluid was added to each eluate, and the radioactivity was measured.

T-1032 and sildenafil were dissolved in DMSO. For studies of inhibition of PDE activities, inhibitors in DMSO (final concentration 1%, v/v) were added to the assay buffer containing enzyme and preincubated for 5 min before reactions were initiated by the addition of substrate.

### *Expression of Two Variants of Canine PDE5 in COS-7 Cells*

Two types of canine PDE5 proteins (PDE5A1 and PDE5A2) were prepared as described previously [27]. In brief, the expression plasmids, pSVL-CB and pSVL-CR, were transfected into COS-7 cells with the LipofectAMINE Reagent (Gibco) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed with ice-cold PBS, and scraped in the ice-cold homogenization buffer A described above. After cells were disrupted by a sonicator (TOMY Seiko), lysates were centrifuged at 100,000 *g* for 60 min, and cytosolic extracts containing canine PDE5A1 and PDE5A2 were obtained.

### *Cell Culture and the Effects of Inhibitors on Cyclic Nucleotide Elevation in Cultured Rat VSMCs*

Cultured rat VSMCs were obtained from the thoracic aortae of 8-week-old male Sprague–Dawley rats by the explant method [38]. Cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37° in a 95% air-5% $CO_2$ humidified atmosphere, and were serially passaged before reaching confluence. Passage 7 cultures were used in this experiment.

With regard to the effects of inhibitors on cGMP and cAMP production, subconfluent cells were treated with fresh medium containing inhibitors or CNP. After a 15-min incubation with inhibitors or a 5-min incubation with CNP, the medium was aspirated, and 500 μL of ice-cold ethanol was added to the cells quickly to terminate the reaction. cGMP and cAMP in the extracts were measured according to the manufacturer's instructions for the cGMP EIA System and the cAMP EIA System, respectively. With regard to the effects of inhibitors on the intracellular cGMP elevation in response to CNP stimulation, subconfluent

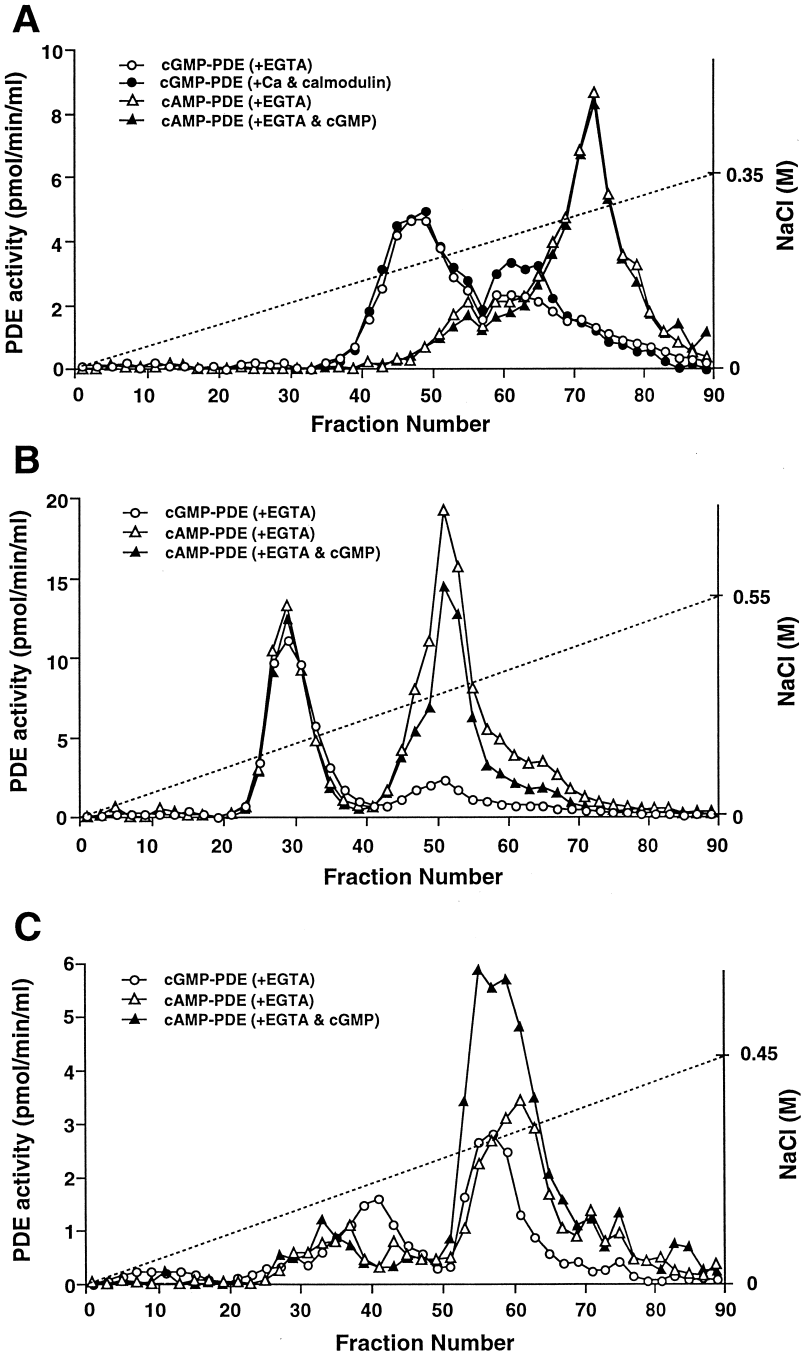



**FIG. 2. Elution profiles after DEAE–Sepharose FF chromatography of cytosolic extracts from canine tissues. Chromatography of cytosolic extracts of canine lung (A), canine heart (B), and canine adrenal gland (C) is shown. A linear gradient of NaCl from 0 to 0.35 M (A), from 0 to 0.55 M (B), or from 0 to 0.45 M (C) was applied. The cGMP hydrolytic activities were measured using 1 μM cGMP with EGTA (1 mM) (open circles) or with 2 mM $CaCl_2$ and 20 U calmodulin (closed circles). The cAMP hydrolytic activities were determined using 1 μM cAMP with (closed triangles) or without (open triangles) 1 μM cGMP.**

cells were treated with fresh medium containing inhibitors. After a 10-min incubation, CNP was added to the medium, and cells were incubated for 5 min. To terminate the reactions, medium was aspirated and ice-cold ethanol was added quickly to the cells. cGMP was quantified as described above.

### *Statistical Analysis*

Data were analyzed using one-way ANOVA followed by Dunnett's two-tailed test. The statistical analysis of the difference between two values was done by Student's *t*-test. Values were considered to be statistically significant at $P < 0.05$.

## RESULTS

### *Isolation and Characterization of PDE Isozymes from Canine Tissues*

DEAE–Sepharose FF column chromatography was carried out to isolate five PDE isozymes from canine lung, heart, and adrenal gland. First, PDE1, PDE4, and PDE5 isozymes were isolated from canine lung using chromatography in

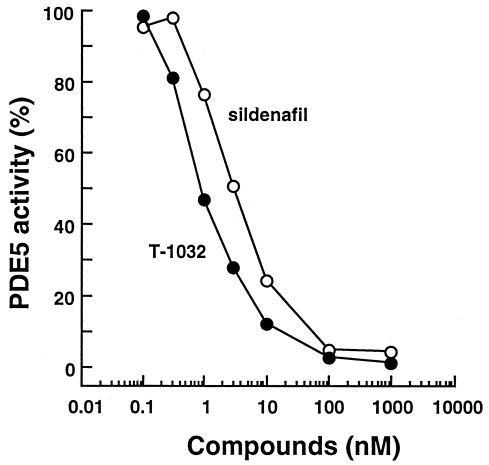



**FIG. 3. Inhibition of PDE5 partially purified from canine lung by various concentrations of T-1032 and sildenafil. cGMP hydrolytic activity (4.5 pmol/min/mL) in the absence of the inhibitors was taken as 100%. Each point represents the mean value from the results of three or four assays.**

the presence of $Ca^{2+}$. Figure 2A shows the elution profile using cytosolic extracts from canine lung. There was one major and one minor peak of cGMP hydrolytic activity and one major peak of cAMP hydrolytic activity. With regard to cGMP hydrolytic PDE activity, fractions 39–47 (the first half of the major peak) were insensitive to $Ca^{2+}$ and calmodulin and were inhibited by a PDE5-specific inhibitor, sildenafil (Fig. 3), indicating that the major peak of cGMP hydrolytic activity contained PDE5. The cGMP hydrolytic activity of the minor peak (fractions 59–65) was increased approximately 1.5-fold by the addition of $Ca^{2+}$ and calmodulin, compared with the activity in the presence of EGTA, indicating that these fractions contained $Ca^{2+}$/calmodulin-dependent PDE (PDE1). These fractions also showed cAMP hydrolytic activity. The major peak of cAMP hydrolytic activity (fractions 67–76) eluted at a concentration of approximately 0.3 M NaCl. The cAMP hydrolytic activity in these fractions was not inhibited by the addition of 1 μM cGMP, and it was sensitive to rolipram ($IC_{50}$ = 0.86 μM). No cGMP hydrolytic activity was observed in these fractions. These findings demonstrated that the isozyme in this peak was rolipram-sensitive PDE (PDE4) and that these fractions did not contain PDE3.

PDE3 isozyme was detected in cytosolic fractions of canine heart using DEAE chromatography in the presence of EGTA (Fig. 2B). Two major peaks showing cAMP hydrolytic activity were observed. The first peak (fractions 23–37) also exhibited cGMP hydrolytic activity, and $Ca^{2+}$ and calmodulin stimulated this activity (data not shown). The cAMP hydrolytic activity in the first half of the second peak (fractions 53–55) was decreased slightly in the presence of 1 μM cGMP or 10 μM milrinone, a PDE3-specific inhibitor. The activity in these fractions was also inhibited by 10 μM rolipram (data not shown). These fractions appeared to contain both PDE3 and PDE4. The cAMP hydrolytic activity in the latter half of the peak (fractions 57–67) was inhibited strongly by 1 μM cGMP or milrinone ($IC_{50}$ = 2.6 μM), indicating that the activity contained cGMP-inhibited PDE (PDE3). In addition, a high concentration (100 μM) of rolipram showed weak inhibition of the cAMP hydrolytic PDE in fractions 57–67, suggesting that these fractions did not contain PDE4.

Levels of cGMP-stimulated PDE (PDE2) in DEAE fractions from canine lung and heart were too low to use for characterization by PDE inhibitors, and therefore PDE2 was isolated from canine adrenal gland (Fig. 2C). There was one major peak of cAMP hydrolytic activity. The cAMP hydrolysis in fractions 53–61 was stimulated by 1 μM cGMP, and the cGMP hydrolytic activity in the first half of the major peak (fraction 53–57) was inhibited by a PDE2-specific inhibitor, EHNA ($IC_{50}$ = 10 μM). Because this activity was not increased by the addition of $Ca^{2+}$ and calmodulin and was not inhibited by rolipram and milrinone, these fractions did not appear to contain PDEs other than PDE2.

PDE1 in fractions 59–65 from the lung, PDE2 in fractions 53–57 from the adrenal gland, PDE3 in fractions 57–67 from the heart, PDE4 in fractions 67–76 from the lung, and PDE5 in fractions 39–47 from the lung were used for analysis of the inhibition of PDE isozymes by T-1032 and sildenafil.

### *Inhibition by T-1032 of Various PDE Isozymes Isolated from Canine Tissues and of Alternative Splice Variants of Canine PDE5* in Vitro

The inhibitory potency of T-1032 against six PDE isozymes was investigated using the partially purified enzymes described above. Figure 3 shows concentration-dependent inhibition of cGMP hydrolytic activity of PDE5 by T-1032 and sildenafil. T-1032 exhibited strong inhibition of the cGMP hydrolytic activity of PDE5, with an $IC_{50}$ of 1.0 nM (Fig. 3 and Table 1). Sildenafil was a potent PDE5 inhibitor with an $IC_{50}$ of 3.6 nM. Thus, T-1032 was more potent as a PDE5 inhibitor than was sildenafil. As shown in Table 1, T-1032 and sildenafil also inhibited PDE6 isolated from canine retinas with $IC_{50}$ values of 28 and 29 nM, respectively, which were 30- and 8-fold higher than those for inhibition of PDE5. In contrast, T-1032 did not inhibit the cAMP hydrolytic activity of PDE3 even at a concentration of 100 μM, and the cGMP hydrolytic activity of PDE1 and PDE2 and the cAMP hydrolytic activity of PDE4 were inhibited weakly by T-1032, with $IC_{50}$ values of 3.0, 9.7, and 3.3 μM, respectively. Sildenafil moderately inhibited the cGMP hydrolysis of PDE1 ($IC_{50}$ value = 0.27 μM). Very weak inhibition by sildenafil was observed using the other PDE isozymes except for PDE5 and PDE6 (Table 1).

We examined the inhibitory effects of T-1032 and sildenafil on two variants of recombinant canine PDE5. T-1032 inhibited the cGMP hydrolytic activities of both PDE5A1 and PDE5A2 with similar $IC_{50}$ values of 2.7 and 2.0 nM, respectively (Table 1). Sildenafil also inhibited both PDE5A1 and PDE5A2 with similar $IC_{50}$ values of 4.9 and 4.5 nM, respectively.

**TABLE 1. Inhibitory effects of T-1032 and sildenafil in the six PDE isozymes from canine tissue and recombinant PDE5A splice variants**

| | $IC_{50}$ (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Compounds | PDE5 | rPDE5A1 | rPDE5A2 | PDE1 | PDE2 | PDE3 | PDE4 | PDE6 |
| T-1032 | 0.0010 ± 0.00012 (N = 7) | 0.0027 (N = 2) | 0.0020 (N = 2) | 3.0 ± 0.37 (N = 3) | 9.7 ± 0.74 (N = 3 | >100 (N = 3) | 3.3 ± 0.37 (N = 3) | 0.028 ± 0.00047 (N = 4) |
| Sildenafil | 0.0036 ± 0.00026 (N = 9) | 0.0049 (N = 2) | 0.0045 (N = 2) | 0.27 ± 0.038 (N = 3) | 43 ± 1.5 (N = 3) | >100 (N = 3) | 11 ± 3.1 (N = 4) | 0.029 ± 0.00093 (N = 4) |

PDE1, PDE4, and PDE5 were isolated from canine lung, and PDE3 was isolated from canine heart. PDE2 and PDE6 were prepared from canine adrenal gland and canine retinas, respectively. Recombinant canine PDE5A1 (rPDE5A1) and recombinant canine PDE5A2 (rPDE5A2) were obtained from transfected COS-7 cells. PDE1, PDE2, and PDE5 activities were assayed with 1 μM cGMP as a substrate, and PDE3 and PDE4 activities were assayed with 1 μM cAMP. PDE6 activity was measured with 10 μM cGMP. In each experiment, PDE activity was 2–33 pmol/min/ml. The $IC_{50}$ values were determined by linear regression. The number of experiments (N) is shown in parentheses. Values represent means ± SEM for three to nine experiments or the mean for two experiments.

### *Kinetic Analysis of the Effects of T-1032 on PDE5 from Canine Lung*

To elucidate the mechanism of inhibition by T-1032, Lineweaver–Burk plots and Dixon plots were constructed at concentrations of 0.27 to 4.02 μM cGMP substrate. The PDE5 partially purified from canine lung had a cGMP $K_m$ value of 2.0 μM, and T-1032 inhibited the PDE5 activity in a competitive manner with respect to cGMP hydrolysis (Fig. 4). The inhibition constant ($K_i$ value) of T-1032 for PDE5 was 1.2 ± 0.1 nM according to the Dixon plots.

### *Effects of T-1032 on cGMP and cAMP Accumulation in Cultured Rat VSMCs*

We investigated alterations in the intracellular concentrations of cGMP and cAMP in response to T-1032 and a nonselective PDE inhibitor, IBMX, in the presence and absence of CNP. In cultured rat VSMCs, CNP (10 nM) stimulation for 5 min without a PDE inhibitor elevated the cGMP level from 100 to 350 fmol/$10^6$ cells. T-1032 slightly increased basal cGMP levels in the absence of CNP stimulation (Fig. 5A). IBMX (10 μM) also elevated the intracellular cGMP levels without CNP. Treatment of rat VSMCs with T-1032 in the presence of 10 nM CNP resulted in an up to 4-fold increase in cGMP concentrations over 10 nM CNP treatment alone in a concentration-dependent manner (Fig. 5B). IBMX also increased the intracellular cGMP levels by 1.6-fold in the presence of CNP. As expected, T-1032 did not alter basal cAMP levels, whereas IBMX increased cAMP levels (Fig. 5C).

## DISCUSSION

In this study, we have characterized a novel PDE5 inhibitor, T-1032. The inhibitory effects of T-1032 on six canine PDE isozymes were compared with those of sildenafil. The $IC_{50}$ value of T-1032 for PDE5 was of the same order of magnitude as that of sildenafil. T-1032 inhibited not only canine PDE5 but also human platelet PDE5 to a similar extent (data not shown). Sequence analysis has demonstrated previously that there is only one amino acid difference between the catalytic domains of human and canine PDE5 [26, 27]. This observation is in accord with the similarity of the inhibitory potency of T-1032 against canine and human PDE5. The $IC_{50}$ values of T-1032 for other PDE isozymes except for PDE6 were more than 1 μM.

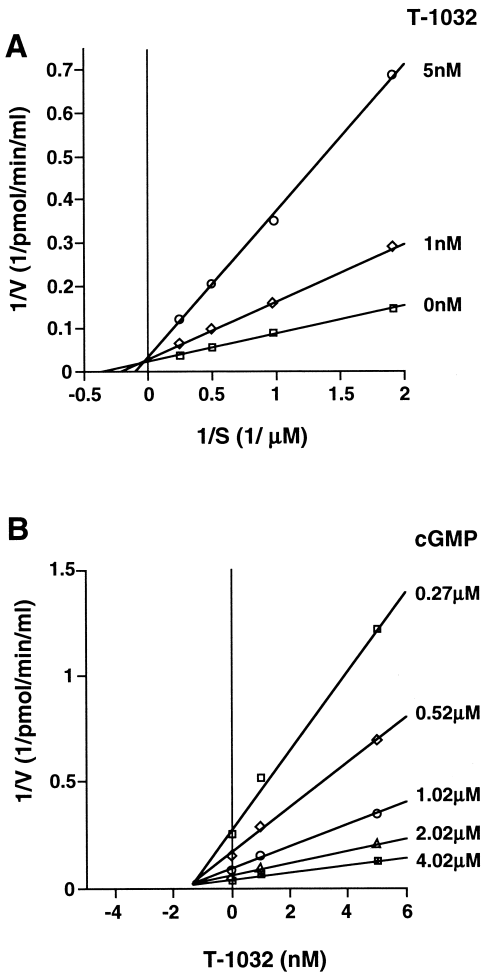
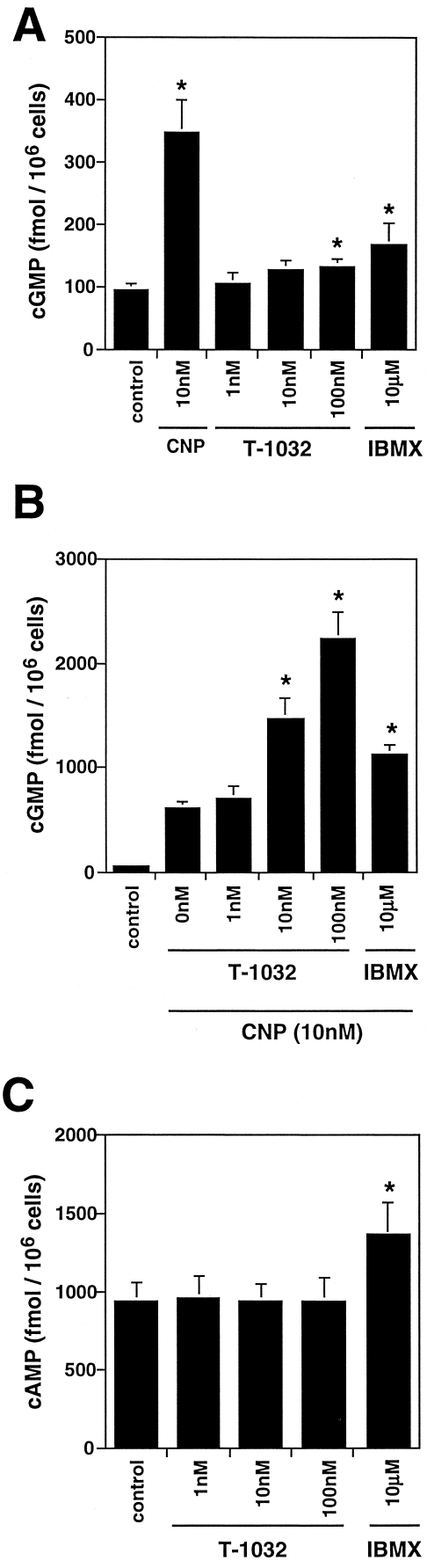



**FIG. 4. Kinetic analysis of the inhibition of T-1032 on PDE5 partially purified from canine lung. Lineweaver–Burk plots at concentrations of 0.27 to 4.02 μM cGMP are shown in panel A. Dixon plots for T-1032 at concentrations of 1 and 5 nM are shown in panel B. In each experiment, cGMP hydrolytic activity was 15 pmol/min/mL. All assays were performed in triplicate. A plot typical of three independent experiments is shown.**




The $IC_{50}$ of T-1032 for PDE1 was 10-fold higher than that of sildenafil. Therefore, with regard to PDE1 and PDE5 isolated from canine lung, T-1032 was more PDE5-selective than sildenafil.

The structure of T-1032 is quite different from that of cGMP, whereas sildenafil and zaprinast are structurally related to cGMP. It is likely that the selectivity of T-1032 for PDE1 and PDE5 inhibition is due to the structural difference between T-1032 and cGMP. T-1032 binds to the catalytic site of PDE5 and inhibits cGMP hydrolysis. In contrast, a high concentration (1 μM) of T-1032 stimulated cGMP binding to the allosteric sites, indicating that T-1032 does not interact with the allosteric sites (data not shown). Sildenafil and zaprinast also do not bind to the cGMP binding allosteric sites, in spite of the structural similarity to cGMP [39]. These observations support the proposal [40] that the conformation of the cGMP-binding pockets of the allosteric sites and the catalytic domain is quite different.

Zaprinast, a classical PDE5 inhibitor with a $K_i$ of 160 nM [19], has been used in pharmacological studies to investigate the roles of PDE5 and cGMP in various tissues. However, this compound also inhibits PDE1 at high concentrations ($K_i$ = 9.9 μM for PDE1 isolated from rat aorta) [19]. Zaprinast has approximately 60-fold selectivity for PDE5 when compared with PDE1. Therefore, zaprinast is not specific enough to allow assignments of physiological roles for PDE5; administration of zaprinast at high concentrations may affect cellular functions via PDE1 inhibition. More selective PDE5 inhibitors such as T-1032 should be more useful in pharmacological studies to elucidate the contribution of the cGMP/PDE5 pathway in tissues.

Characterization of PDE isozymes in canine lung has not been reported in detail previously. We have demonstrated DEAE elution profiles of PDE isozymes in canines. PDE1, PDE4, and PDE5 were identified in the canine lung using chromatography in the presence of $Ca^{2+}$. Although PDE3 has been identified as a milrinone-sensitive PDE in the canine aorta [41], PDE3 activity, compared with PDE4, was barely detectable in the canine lung, where VSMCs are abundant. Many previous studies indicated that PDE5 is a major enzyme for cGMP hydrolysis in rat and bovine lung

**FIG. 5. Effect of T-1032 on the intracellular levels of cGMP in cultured rat VSMCs. (A) Cells were incubated with CNP for 5 min or with T-1032 or IBMX for 15 min at 37°, and then the medium was aspirated. Five hundred microliters of ice-cold ethanol was added to the cells, and the amounts of cGMP in the extracts were quantified as described in Materials and Methods. (B) Cells were treated with T-1032 or IBMX for 10 min prior to the addition of CNP. After the cell culture was incubated with CNP for 5 min at 37°, cGMP was extracted and measured. (C) cAMP in the extracts under the same conditions as for panel A was measured as described in Materials and Methods. Each data point represents the mean ± SEM from three to four separate determinations. Key: (*) significantly different from control (A and C) or CNP alone (B) ($P < 0.05$).**

[42, 43]. However, complete separation of PDE5 from PDE1 in the lung has not been done by chromatography in the presence of $Ca^{2+}$. Here, we have demonstrated by chromatography that the activity of PDE5 was the major contributor to the cGMP hydrolytic activity in canine lung in both the presence and absence of $Ca^{2+}$ and calmodulin. In human and pig aortae, PDE1 activity in the presence of $Ca^{2+}$ and calmodulin is more abundant than PDE5 activity [20, 21], whereas PDE5 activity is dominant in the presence of EGTA. It also has been reported that in the canine aorta PDE1 activity appears to be more abundant than that of PDE5 [41]. In contrast, human pulmonary arteries contain a high level of PDE5 activity, compared with PDE1 activity. In conscious, pulmonary-hypertensive rats, E4021, which is a more selective PDE5 inhibitor than is sildenafil, effectively reduces pulmonary artery pressure and total pulmonary resistance strongly compared with systemic arterial pressures [32]. Thus, the major contributor to cGMP hydrolysis in the rat pulmonary artery may be PDE5. It has been reported that a major isozyme of cGMP hydrolytic activity in the human corpus cavernosum is PDE5 and a minor isozyme is PDE2, but PDE1 activity has not been detected [34, 44]. Sildenafil enhances relaxation of corpus cavernosal smooth muscle and is effective in treating male erectile dysfunction [34]. These observations suggest that the relative contributions of PDE1 and PDE5 to cGMP hydrolysis in tissues are more important for the pharmacological effect of the selective PDE5 inhibitors.

cGMP elevation by natriuretic peptides such as CNP has been revealed to inhibit the proliferation of cultured rat VSMCs, which show a proliferative phenotype [45]. In addition to PDE1, PDE5 is expressed in cultured human VSMCs from atherosclerotic lesions [46]. We have examined the effects of T-1032 on cGMP accumulation in cultured rat VSMCs. T-1032 increased the intracellular concentrations of cGMP with and without CNP in cultured rat VSMCs. This result indicated that T-1032 is a membrane-permeable drug in intact cells and may be effective *in vivo*.

In conclusion, our results demonstrated that an isoquinolone derivative, T-1032, is a potent and selective PDE5 inhibitor *in vitro* and in intact cells. Because PDE5 is present not only in blood vessels and the central nervous system but also in many other tissues, T-1032 may be useful to study cGMP functions via PDE5 in these tissues.

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