

High lung PDE5: A strong basis for treating pulmonary hypertension with PDE5 inhibitors ☆,☆☆

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Abstract

[³H]Vardenafil (Levitra) or [³H]tadalafil (Cialis) binding was used to quantify PDE5 in rat lung and heart tissue. Each radioligand bound to purified recombinant phosphodiesterase-5 (PDE5) or to PDE5 in crude extracts with strong affinity, high specificity, slow dissociation, and good stoichiometry. PDE5, the only ³H inhibitor-binding protein detected in extracts, was 15 times higher in lung than in heart extracts, and the level measured by PDE5 catalytic activity agreed with that determined by ³H inhibitor binding. High level of PDE5 in lung approximated that in penile corpus cavernosum, the tissue targeted by PDE5 inhibitors. PDE5 was the predominant cGMP-PDE in lung, and on a molar basis was five times higher than cGMP-dependent protein kinase (PKG), which phosphorylates PDE5 in vivo. The PDE5 level was one-half that of PKG in heart. Thus, abundance of PDE5 in lung vascular smooth muscle provides a strong molecular basis for PDE5 inhibitor treatment of pulmonary hypertension.

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The level of phosphodiesterase-5 (PDE5) was found to be very high in penile corpus cavernosum, and it was suggested that this could account in part for the relative targeting of PDE5 inhibitors for penile erection in men [1]. It is hypothesized that the presence of a high level of PDE5 in a particular tissue in vitro should predict the effect of a PDE5 inhibitor in vivo. Numerous reports have indicated early success with the use of PDE5 inhibitors for pulmonary hypertension [2–6], although quan-

tification of PDE5 in lung tissue has not been performed. The original discovery, cloning, and characterization of PDE5 were done in this laboratory using lung tissue as the source of the enzyme [7–10]. Some enzymes of the cGMP signaling pathway have a relatively narrow tissue distribution as compared with those of the cAMP pathway [11,12]. Both PDE5 and cGMP-dependent protein kinase (PKG) are believed to be distributed strongly in smooth muscle cells such as those in the vasculature. Since PDE5 is an important substrate for PKG in mediating negative feedback regulation of cGMP levels [1,13,14], it seems logical that these enzymes would have similar tissue distributions. Very few quantitative analyses of PKG and PDE5 have been conducted, and most have relied on Western immunoblot analyses without proper standards. Using this qualitative approach, PDE5 has been shown to be localized in vascular smooth muscle of lung tissue [15], and the level of this enzyme increases in pulmonary

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☆☆ **Abbreviations:** PDE, 3',5'-cyclic nucleotide phosphodiesterase; PDE5, cGMP-binding cGMP-specific PDE; KPM, 10 mM potassium phosphate, pH 6.8, and 25 mM β-mercaptoethanol; IBMX, 3-isobutyl-1-methylxanthine; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; 8-CPT-cGMP, 8-chlorophenylthio-cGMP; PKI, protein kinase inhibitor peptide 5–24.

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hypertension [16]. Biochemical quantification has been done for PKG in pig coronary artery [17] and for PKG and PDE5 in rabbit penile corpus cavernosum [1]. The advent of potent and specific commercialized PDE5 inhibitors used to treat erectile dysfunction, such as sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis), has provided new probes to investigate PDE5. We have recently produced these PDE5 inhibitors in tritium-labeled form, and have demonstrated strong specificity, potency, and stoichiometry for binding of the inhibitors to purified PDE5 [14,18]. We have now used [^3H]vardenafil and [^3H]tadalafil to measure PDE5 levels in both crude supernatant and particulate fractions of lung and heart tissue extracts. This is the first study to address the tissue concentration of PDE5 and associated proteins as a consideration for PDE5 inhibitor therapy.

Materials and methods

Materials. [^3H]cGMP and [^3H]cAMP, were purchased from Amersham Biosciences (Piscataway, NJ). Sephadex G-25, and DEAE-Sephacel were from Pfizer. Histone type II-AS, histone type VIII-S, 3-isobutyl-1-methylxanthine (IBMX), 8-chlorophenylthio-cGMP (8-CPT-cGMP), *Crotalus atrox* snake venom, cilostamide, rolipram, cAMP, and cGMP were from Sigma Chemical (St. Louis, MO). PKG-selective heptapeptide (RKRSRAE), PKA-selective heptapeptide (LRRASLG, Kemptide), and PKI (protein kinase inhibitor peptide, PKI 5–24) were purchased from Peninsula Laboratories (Belmont, CA). [γ - ^{32}P]ATP was obtained from PerkinElmer (Boston, MA). His-tagged, full-length recombinant bovine PDE5 was isolated from infected Sf9 cells using nickel/nitrilotriacetic acid agarose (Qiagen, Valencia, CA) as described previously [14]. Sildenafil was purified from Viagra (Pfizer) tablets as described earlier [14,19]. Tadalafil was synthesized and submitted to Amersham Biosciences for tritiation [18]. The specific radioactivity of [^3H]tadalafil was 34,000 CPM/pmol. During latter stages of this work, unlabeled tadalafil was also provided by ICOS (Bothell, WA). Vardenafil and [^3H]vardenafil (specific radioactivity = 60,000 CPM/pmol) were provided by Bayer AG (Wuppertal, Germany).

Preparation of tissue extracts. Fresh rat lung or heart (~5 g) was cut into small pieces and homogenized in 80 ml KPM (10 mmol/L potassium phosphate, pH 6.8, plus 15 mmol/L β -mercaptoethanol) containing 1 mmol/L CaCl_2 with a Cuisinart apparatus (high speed) using three bursts of 15 s each at 4 °C. The supernatant fraction was obtained by centrifugation in a Beckman JA-20 rotor for 30 min at 10,000g. This supernatant was used for assays and for DEAE chromatography. The resulting pellet was re-suspended in the same volume as the original and re-centrifuged as before to obtain pellet 2, which was re-suspended in the same volume of the buffer for assays.

^3H inhibitor membrane filtration protein binding assay. Unless indicated otherwise, 400 μl sample (extract or DEAE fraction) was added to 1.8 ml of 10 mmol/L KPM containing 0.2 mg/ml histone IIA-S and 10 $\mu\text{mol/L}$ cGMP. For lung supernatant fraction, 80 μl sample was used since ^3H inhibitor binding was not linear with concentration of the sample at >200 μl sample. The 0.8 mol/L NaCl-eluted fraction from DEAE was diluted 10-fold before assay since the high NaCl carry-over was inhibitory for ^3H inhibitor binding. The [^3H]vardenafil or [^3H]tadalafil was added to the KPM–histone mixture before starting the reaction with sample. Reaction mixtures were incubated in an ice-water bath for 20 min. Two hundred microliters of 25% Triton X-100 (2.2% final concentration) was added to each sample and reactions were rapidly filtered under house vacuum through Mil-

lipore HAWP nitrocellulose membranes (0.45 μm) that had been pre-wetted with 1.5 ml of cold 10 mmol/L potassium phosphate, pH 6.8 (KP), containing 0.1% Triton X-100. Tubes were each rinsed with 3 ml of the same buffer and filtered. The filter membranes were removed, dried, and counted. ^3H inhibitor binding values were corrected for 25% loss of PDE5 through the filter [18].

PDE, PKG, and PKA activities. PDE activity was determined by a modified assay [20] as described earlier [1] using 0.4 $\mu\text{mol/L}$ [^3H]cGMP or [^3H]cAMP as substrate. PKG activity was determined using a synthetic PKG-selective heptapeptide substrate in the presence and absence of 2 $\mu\text{mol/L}$ cGMP and in the presence of 1 $\mu\text{mol/L}$ PKI (Kemptide) as described [1], and PKA activity was determined using a synthetic PKA-selective heptapeptide (Kemptide) substrate in the presence and absence of 2 $\mu\text{mol/L}$ cAMP [21].

PDE5-specific [^3H]cGMP-binding. The procedure was modified slightly from that described previously [1]. Twenty microliters of DEAE fraction was added to 50 μl of a mixture containing 20 mmol/L sodium phosphate (pH 6.8), 0.2 $\mu\text{mol/L}$ [^3H]cGMP, 1 mmol/L EDTA, 25 mmol/L 2-mercaptoethanol, 0.5 mg/ml Type VIII-S histone (Sigma), 0.1 mmol/L IBMX, 0.5 $\mu\text{mol/L}$ cAMP, and 0.25 $\mu\text{mol/L}$ 8-CPT-cGMP. The presence of cAMP and 8-CPT-cGMP blocks binding of [^3H]cGMP to PKA and PKG, respectively. After 60 min at 0 °C, 1.8 ml cold KP was added and the sample was filtered onto a Millipore HAWP nitrocellulose membrane (pore size 0.45 μm) that had been pre-moistened with the same buffer. Filters were then rinsed two times with the cold phosphate buffer, dried, and counted. PDE5-specific [^3H]cGMP binding represents binding to the regulatory domain of PDE5, which contains cGMP-binding GAF subdomains [10,11]. For calculation of intracellular PDE5 based on [^3H]cGMP binding, it was assumed that PDE5 bound 0.104 mol cGMP/subunit under the binding assay conditions used, which was the value found using purified recombinant bovine PDE5. Other assumptions were the same as described under Results for calculating intracellular concentration of PDE5 using [^3H]vardenafil or [^3H]tadalafil binding.

Results

^3H inhibitor binding in crude extracts of rat lung and heart

[^3H]Vardenafil and [^3H]tadalafil were shown to bind with high affinity, stoichiometry (nearly 1 mol inhibitor per PDE5 subunit), and specificity for the catalytic domain of purified, recombinant PDE5 [18]. Thus, it seemed logical that these ^3H inhibitor-binding assays could be used to estimate the levels of PDE5 in crude fractions of tissue extracts. Using the supernatant from rat heart, the time course and volume-dependence of the sample for binding of [^3H]vardenafil at 4 °C are shown in Fig. 1. [^3H]Vardenafil binding equilibration occurred within 20 min, which was similar to the finding using purified recombinant PDE5 [18], and binding was linear up to 400 μl supernatant fraction. Similar results were obtained using lung supernatant and particulate fractions of heart or lung except that ^3H inhibitor binding was not linear with concentration of sample using >200 μl lung supernatant fraction (not shown). Four hundred microliter sample and 20-min incubation were chosen for subsequent experiments except when using lung supernatant (80 μl sample).

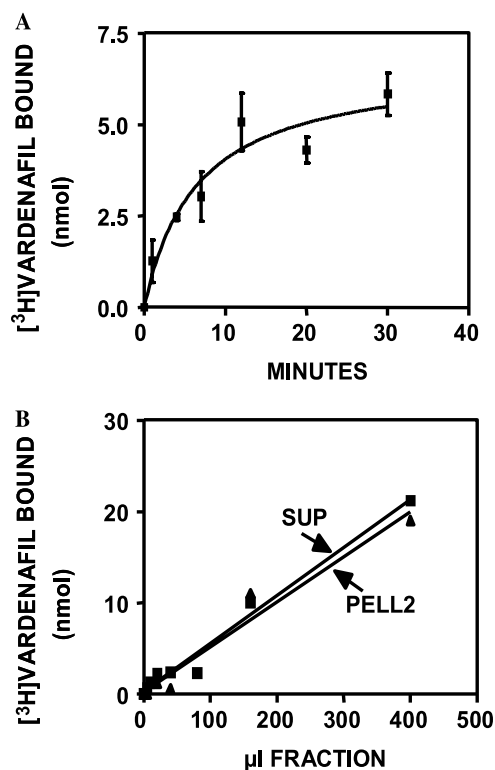


Fig. 1. Extract concentration- and time-dependence of $[^3\text{H}]$ vardenafil binding in rat heart supernatant and pellet 2 fractions. Rat heart was used for the experiment, which was performed using 0.4 nmol/L $[^3\text{H}]$ vardenafil as described under Materials and methods. Values in (A) are the means of duplicates in a single typical experiment using the supernatant fraction, and the experiment was performed three times. SEM in (B) represents triplicates in a single typical experiment, and the experiment was performed three times. SUP, supernatant fraction; PELL2, pellet 2 fraction.

^3H inhibitor binding exhibited specificity suggesting that the binding activity measured in the supernatant or pellet 2 fractions of lung was that of PDE5. Binding of 0.5 nmol/L $[^3\text{H}]$ vardenafil was inhibited by 100 nmol/L of cold vardenafil by $94\% \pm 1.8\%$ ($n = 6$), and by 100 nmol/L of cold tadalafil by $86\% \pm 1\%$ ($n = 3$). Cold rolipram (100 nmol/L), a PDE4 inhibitor, did not significantly affect binding ($119\% \pm 16\%$, $n = 3$), and 100 nmol/L cold cilostamide, a PDE3 inhibitor, did not significantly affect binding ($114\% \pm 8.5\%$, $n = 3$). The K_D values for binding of $[^3\text{H}]$ vardenafil to supernatant and pellet 2 fractions of lung (Fig. 2, upper panel) were 1.4 ± 1.6 nmol/L ($n = 4$) and 2.0 ± 0.41 nmol/L ($n = 5$), respectively. These values were similar to that (0.59 ± 0.02 nmol/L) obtained at 4°C using purified recombinant bovine PDE5 [18]. The supernatant fraction of heart exhibited a K_D for $[^3\text{H}]$ vardenafil (Fig. 2, lower panel) of 0.93 ± 0.25 nmol/L, which was also similar to the K_D values of lung supernatant and pellet 2 fractions, and to that of the recombinant PDE5. However, $[^3\text{H}]$ vardenafil binding to the pellet 2 fraction of heart was not saturable, suggesting that most of the $[^3\text{H}]$ var-

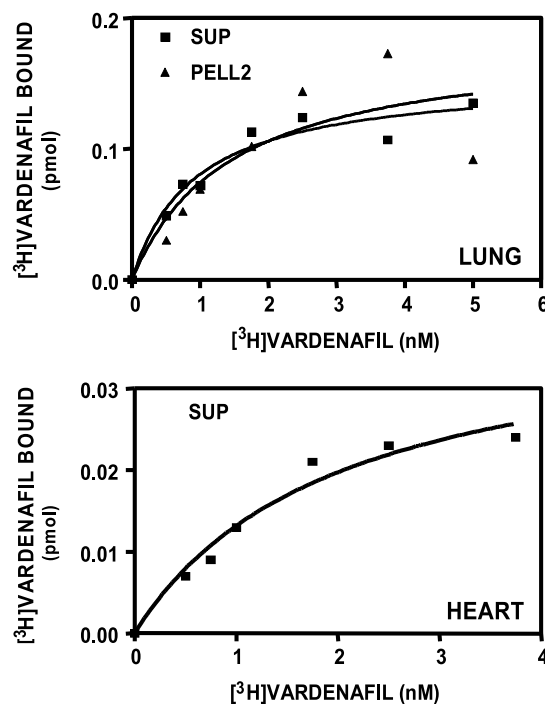


Fig. 2. Concentration-dependence of $[^3\text{H}]$ vardenafil binding in the supernatant and pellet fractions of lung, and of the supernatant fraction of heart. Rat lung and heart were used for the experiment, which was performed using variable concentrations of $[^3\text{H}]$ vardenafil as described under Materials and methods. Values in the upper panel are the means of duplicates in a single typical experiment using rat lung supernatant or pellet 2 fractions, and the experiment was performed four or five times, respectively. Values in the lower panel are the means of duplicates in a single typical experiment using rat heart supernatant fraction, and the experiment was performed four times. SUP, supernatant fraction; PELL2, pellet 2 fraction.

denafil binding measured in this fraction was not PDE5-specific (not shown). This conclusion was also supported by the finding that 0.5 nmol/L $[^3\text{H}]$ vardenafil binding in heart pellet 2 was blocked $<30\%$ by 100 nmol/L unlabeled vardenafil (not shown). The level of PDE5 determined by $[^3\text{H}]$ vardenafil binding in the lung supernatant fraction was very much similar to that determined by cGMP-PDE catalytic activity (Fig. 3). The specificity of ^3H inhibitor binding was also assessed by first saturating PDE5 with $[^3\text{H}]$ tadalafil and then measuring the disappearance of bound $[^3\text{H}]$ tadalafil in the presence of excess tadalafil, which blocked rebinding of the dissociated $[^3\text{H}]$ tadalafil. It can be seen in Fig. 4 that $[^3\text{H}]$ tadalafil dissociation using the crude rat lung supernatant fraction displayed a curvilinear pattern, which was not significantly different from that obtained using the purified recombinant PDE5, providing convincing proof that $[^3\text{H}]$ tadalafil was binding specifically to PDE5 in the crude rat lung extract. Collectively, the results were strongly indicative that, except for the pellet 2 fraction of heart, ^3H inhibitor binding was specific for PDE5 in crude extracts of lung, and that the biphasic

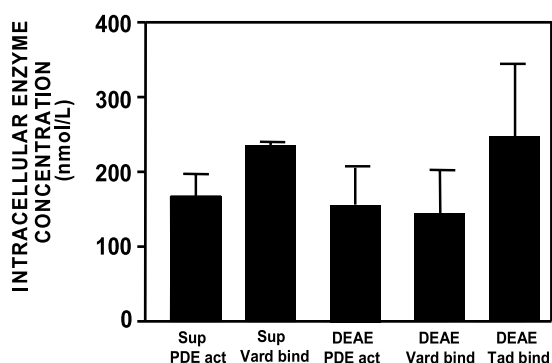


Fig. 3. Measurement of the level of PDE5 in lung using several methods. Values are presented as cellular enzyme subunit concentrations, which were calculated as described under Materials and methods. Concentrations were calculated from PDE5 catalytic activity, [^3H]varidenafil binding (0.5 nmol/L), and [^3H]tadafil binding (5 nmol/L) in supernatant fractions (sup) and in DEAE fractions (DEAE) as described under Results. Act, activity; bind, binding; Var, varidenafil; Tad, tadafil.

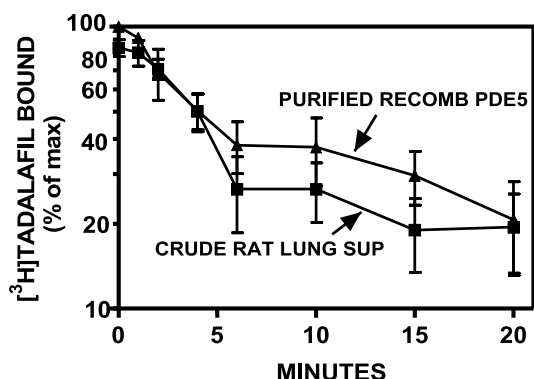


Fig. 4. Exchange-dissociation of [^3H]tadafil using crude rat lung supernatant fraction: comparison with that using purified recombinant PDE5. Recombinant bovine PDE5 (0.35 nmol/L final concentration in assay) prepared as described [18] or 800 μL crude rat lung supernatant was added to 14.5 ml of binding reaction mixture containing [^3H]tadafil (8 nmol/L final concentration). To determine the zero time point, a 1.8 ml aliquot of this mixture was filtered as described under Materials and methods. Next, 13 μL of 11 mmol/L unlabeled tadafil was added to the remaining binding reaction mixture at 4 $^{\circ}\text{C}$. Aliquots were removed and filtered by the same procedure at the indicated time points after incubation at 4 $^{\circ}\text{C}$. Values ($\pm\text{SEM}$) for recombinant PDE5 represent three experiments and data for crude rat lung supernatant represent 11 experiments.

exchange dissociation kinetics are due to inherent characteristics of PDE5.

DEAE-Sephacel chromatography of supernatant fractions of lung and heart extracts

In order to confirm identities and levels of PDE5, PKG, and PKA in rat lung and heart, supernatant fractions were chromatographed on DEAE-Sephacel. Fractions (1.8 ml) were collected and analyzed (substrate = 0.4 $\mu\text{mol/L}$ [^3H]cGMP) for PDE activity $\pm$ 5 nmol/L varidenafil in order to calculate PDE5-specific

PDE activity. For lung, it can be seen in Fig. 5A that cGMP-PDE catalytic activity was resolved into two peaks. The elution position of the first peak ($\sim$ 0.15 mol/L NaCl) was consistent with the elution of the purified PDE5 on DEAE-Sephacel, and the activity in this peak was strongly inhibited by 5 nmol/L varidenafil. This concentration of varidenafil is sufficient to fully inhibit the catalytic activity of the purified PDE5 [18]. Based on these results, obtained by subtracting cGMP-PDE activity (+5 nmol/L varidenafil) from cGMP-PDE activity ($-$ varidenafil), the majority of the PDE catalytic activity in the first peak was assumed to be that of PDE5. A second peak of cGMP PDE activity ($\sim$ 0.25 mol/L NaCl) eluted just after PDE5, and this activity, which was not inhibited by varidenafil, accounted for about 35% of the total cGMP PDE activity in the profile. The elution position of this peak and the finding that the cAMP-PDE activity of this peak was stimulated $\sim$ 2-fold by the addition of 1 $\mu\text{mol/L}$ cGMP (not shown) suggested that most of this activity was due to PDE2. Both [^3H]varidenafil and [^3H]tadafil binding activity co-eluted with the PDE5 catalytic activity peak (Figs. 5B and C), indicating that the binding activity in each case represented that of PDE5. This was confirmed by co-elution of [^3H]tadafil binding activity and PDE5-specific [^3H]cGMP-binding activity (Fig. 5D), which represents the regulatory domain of PDE5. It can be seen that PDE5 was the only significant peak of ^3H inhibitor binding that was resolved. Following completion of the initial gradient, the column was eluted with a bolus (20 ml) of 0.8 mol/L NaCl and no ^3H inhibitor binding was detected in this elution (not shown). The combined data suggested that the ^3H inhibitors are highly selective for PDE5 in the crude mixtures of proteins, and that these tissues do not contain other [^3H]varidenafil- or [^3H]tadafil-binding proteins as determined by the experimental conditions used.

DEAE-Sephacel chromatography was also performed for heart extract supernatant fraction using a similar procedure as that described above for lung supernatant fraction (Fig. 6). Two peaks of cGMP-PDE activity (Fig. 6A) eluted in similar positions as found using lung tissue (Fig. 5A), however, in this case the second peak was larger than the first peak. Furthermore, the total cGMP-PDE catalytic activity eluted using heart as the tissue source was lower as compared with lung (compare Fig. 6A with Fig. 5A). Again, as revealed by varidenafil inhibition of catalytic activity, the first peak primarily represented PDE5. [^3H]varidenafil binding activity co-eluted with the corrected PDE5 activity (Fig. 6B), indicating that even when very low levels of PDE5 are present (8% of that in lung based on catalytic activity), the enzyme can be detected using ^3H inhibitor binding assays. It can be seen in Fig. 6C that PKG activity eluted slightly later than did corrected PDE5 activity, a position which suggested that most of

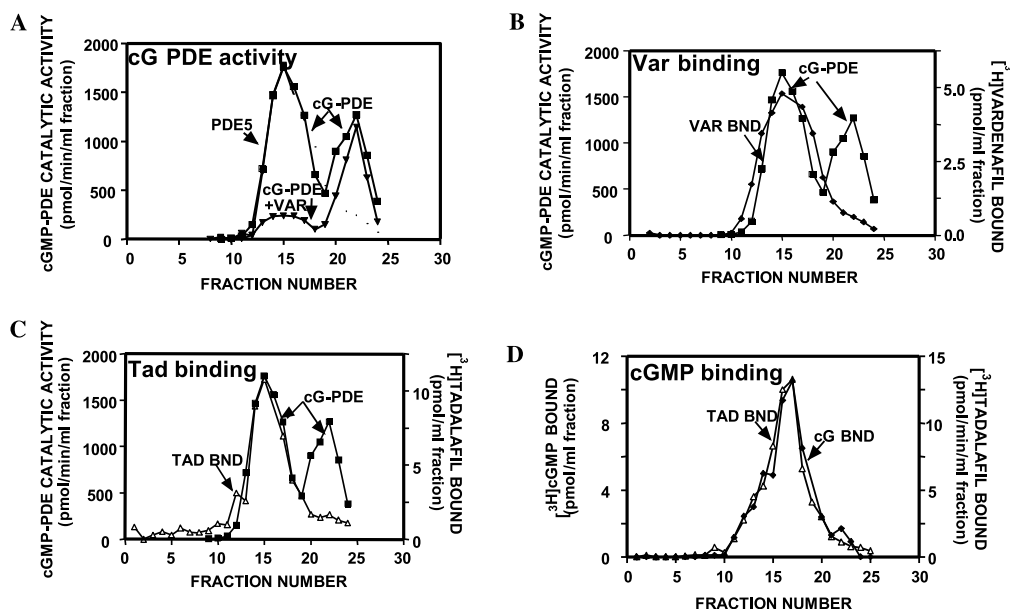


Fig. 5. DEAE-Sephacel chromatography of rat lung supernatant fraction. Rat lung (60–80 ml) supernatant fraction, which was prepared as described under Materials and methods, was applied to a DEAE-Sephacel column (0.9 × 10 cm) equilibrated in KPM and the column was washed with 20 ml of KPM containing 20 mmol/L NaCl and 1 mmol/L CaCl₂. The column was developed with a linear (20–300 mmol/L) NaCl gradient in KPM containing 1 mmol/L CaCl₂. [³H]Vardenafil (0.5 nmol/L) or [³H]tadalafil (5 nmol/L) binding activities were measured in Figs. 3B, C, and D using 400 μ l aliquots as described under Materials and methods. PDE5-specific [³H]cGMP-binding activity shown in Fig. 3B was determined as described under Materials and methods. TAD BND, tadalafil binding; VAR BND, vardenafil binding; cG-PDE, cGMP PDE catalytic activity; cG BND, cGMP binding activity.

the PKG in rat heart is PKGI α , although there may be a slight amount of PKGI β as suggested by the presence of a trailing edge of this peak.

Comparison of tissue concentrations of PDE5 in lung and heart

From the results of Figs. 1, 2, 5, and 6, estimations of tissue concentrations of PDE5 were made. PDE5 catalytic activity was converted to molar amount by using a specific enzyme activity of 0.42 μ mol/min/mg using 0.4 μ mol/L [³H]cGMP as substrate and assuming subunit M_r = 100,000 [1]. Total mole enzyme subunit (based on catalytic activity) in extracts or in fractions eluting from the DEAE column was converted to mole subunit/g tissue by dividing that total activity by the weight of tissue used to obtain the supernatant fraction applied to the column. Values were expressed as intracellular subunit concentrations. For this calculation and all subsequent calculations, it was assumed that tissues contained 0.5 ml intracellular water per gram tissue, enzymes were distributed equally in all cell types in the tissue, and recovery from the DEAE column was quantitative. This same intracellular water concentration was assumed earlier for calculating protein and cyclic nucleotide levels in pig coronary artery smooth muscle [17]. Estimations of PDE5 subunit were also made using [³H]vardenafil- or [³H]tadalafil-binding activity. The same general procedure was used as described for cata-

lytic activity except that the binding stoichiometry of each inhibitor was assumed to be 0.19 mol/subunit at the concentration of ³H inhibitor used, which was the value found in each case using purified recombinant bovine PDE5 [18].

No significant difference in values (150–300 nM intracellular subunit) for lung tissue concentrations of PDE5 were found using either crude extract supernatant fractions or DEAE-purified values obtained by either PDE catalytic activity or ³H inhibitor ([³H]vardenafil or [³H]tadalafil) binding activity (Fig. 3). In addition to these five methods, we also employed [³H]cGMP binding activity of PDE5, which targets a different domain of PDE5, namely, the regulatory domain instead of the catalytic domain. The value obtained for the intracellular concentration of PDE5 based on this method was 330 $\pm$ 60 nmol/L, which was not significantly different from that obtained by two of the five methods as shown in Fig. 3. These combined results verified the use of ³H inhibitor-binding assays to estimate PDE5 levels in crude fractions.

Based on [³H]vardenafil binding in supernatant fractions, much lower levels (~15-fold) of PDE5 were measured in heart compared with lung (Fig. 7A). As measured by PDE5 catalytic activity in fractions after DEAE chromatography, the calculated intracellular PDE5 concentration in heart was 13 $\pm$ 1.5 nmol/L (n = 3), which was ~12-fold lower than that measured the same way in lung [158 $\pm$ 50 nmol/L (n = 3)]. As

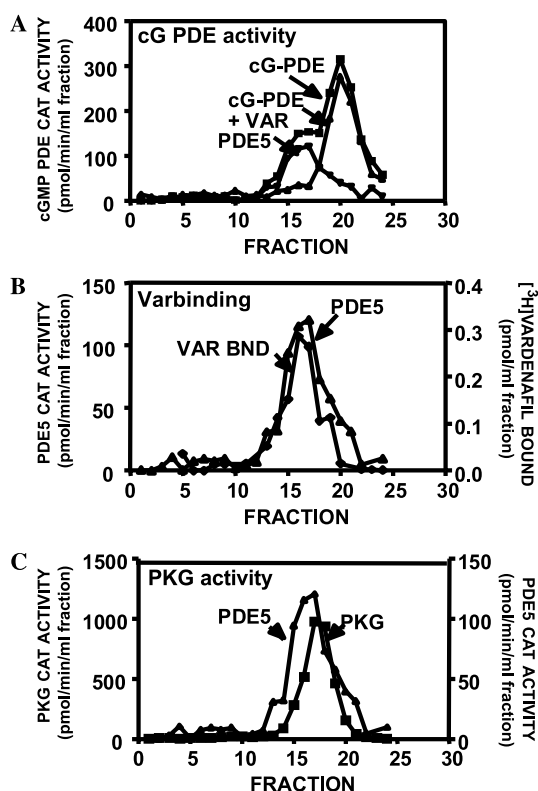


Fig. 6. DEAE-Sephacel chromatography of rat heart supernatant fraction. The experiment was performed exactly as described in Fig. 3 except that rat heart supernatant fraction was used. PDE5 catalytic activity was determined by subtracting cGMP-PDE catalytic activity determined in the presence of 5 nmol/L vardenafil from that in the absence of vardenafil. PKG activity was determined as described under Materials and methods. Labels are as in Fig. 3.

measured by [³H]vardenafil binding in fractions after DEAE chromatography, the calculated intracellular PDE5 concentration in heart was 3.9 ± 1.8 nmol/L ($n = 3$), which was ~48-fold lower than that measured the same way in lung [145 ± 58 nmol/L ($n = 3$)].

Soluble and particulate PDE5

Even though catalytic activity provides a more sensitive assay as compared with ³H inhibitor binding, the presence of high levels of PDEs other than PDE5 in heart produced very low signal/noise using the catalytic activity determination in crude supernatant or particulate fraction. It can be seen in Fig. 7A that, based on [³H]vardenafil binding, the particulate fraction (pellet 2) of lung tissue contained only 15% as much PDE5 as did the supernatant fraction of this tissue. When physiological ionic strength was included in the rinse buffer to obtain pellet 2, the particulate fraction contained only 7% of that found in the supernatant fraction (not shown). The supernatant fraction of heart tissue contained a low, albeit detectable, level of PDE5. It appeared that the particulate fraction of heart tissue contained a significant level of PDE5, but, as noted above, this binding

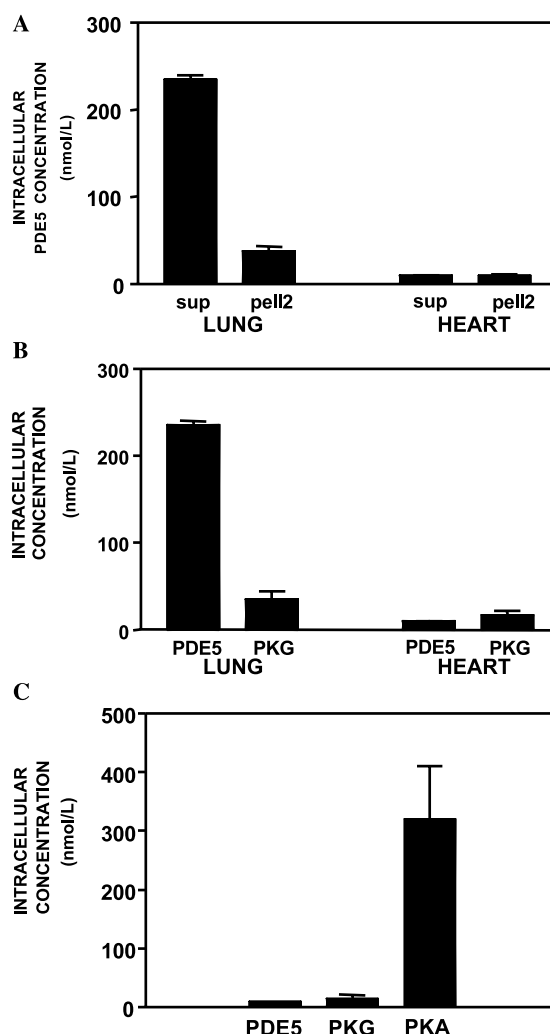


Fig. 7. (A) Tissue levels of PDE5 in supernatant and particulate (pellet 2) fractions of rat lung and heart. Values represent PDE5 subunit concentrations. [³H]Vardenafil binding activity was used to calculate values. SEM represents three experiments, each of which was performed in triplicate. Description of procedures and calculations are under Materials and methods and under Results. Sup, supernatant fraction; pell2, pellet 2 fraction. (B) Comparison of PDE5 and PKG levels in rat lung and heart. PDE5 subunit levels were calculated from [³H]vardenafil binding in supernatant fractions, and PKG subunit levels were calculated from PKG kinase activity in DEAE fractions as described in the text. SEM represents three experiments. (C) Comparison of PDE5, PKG, and PKA levels in rat heart. PDE5 and PKG subunit values are from Fig. 6. PKA subunit levels were calculated from PKA kinase activity in DEAE fractions as described in the text. SEM represents three experiments.

activity was not saturable with [³H]vardenafil and was blocked by <30% with excess unlabeled vardenafil, suggesting that most of it was artifactual.

Comparison of tissue concentrations of PDE5, PKG, and PKA in lung and heart

PKG is considered to be the main catalyst for phosphorylation of serine-102 (human PDE5), although a

role for PKA cannot be excluded as a kinase that phosphorylates this residue in certain instances [10,22]. This phosphorylation reaction increases enzyme catalytic activity as well as cGMP binding to the regulatory GAF domains of the enzyme [13,23,24]. Among other possible consequences, these effects have been suggested to result in negative feedback regulation of the cGMP pathway in cells [13]. Based on activities in DEAE fractions, the level of PKG in lung was 13% of the level of PDE5 (calculated from [^3H]vardenafil binding) in this tissue (Fig. 7B). However, the level of PKG in heart was 600% of the level of PDE5 in this tissue. Consistent with its key role in regulating heart functions, the PKA level in heart was 2200% of the level of PKG in this tissue (Fig. 7C).

Discussion

The traditional approach of using PDE catalytic activity to quantify a particular PDE is oftentimes inadequate. The contribution of other PDEs to the observed PDE activity can be substantial even after optimization of assay conditions (substrate, $\pm$ PDE inhibitors, etc.). This is much less of a concern using ^3H inhibitor-binding activity, which has not yet revealed the presence of detectable ^3H inhibitor binding proteins other than PDE5 in a limited number of tissue extracts. The binding assay has the added advantage of being stoichiometric rather than catalytic, therefore providing direct measurement of enzyme molar levels, and the sensitivity of the assay approaches 1 nmol/L cellular binding sites. Except for heart particulate fraction, the ligand specificity, K_D of [^3H]vardenafil or [^3H]tadalafil binding, [^3H]tadalafil dissociation pattern in extracts of rat lung or heart, co-elution of ^3H inhibitor-binding activity with PDE5 catalytic activity, and similar calculated PDE5 level using binding or catalytic activity validate the ^3H inhibitor-binding approach. Similarity of values obtained using the two independent methods provides high confidence in their accuracy. Furthermore, kinetic properties of vardenafil or tadalafil binding to PDE5 in crude tissue extracts are similar to those of purified, recombinant PDE5 expressed in insect cells. The advent of the ^3H inhibitor-binding assay for PDE5 provides a new and valuable tool to quantify and characterize PDE5.

The amount of PDE5 in cells of whole lung tissue (~ 200 nmol/L) is quite high and comparable to that found in corpus cavernosum based on PDE5 catalytic activity [1]. This suggests that early successes in PDE5 inhibitor therapy for pulmonary hypertension are due to direct effects of these inhibitors on PDE5 itself. Since PDE5 is present primarily in the smooth muscle of the vasculature of the adult rat lung [15], the actual concentration of PDE5 in these cells would be far greater than

200 nmol/L since the calculations assume equal distribution of PDE5 in all lung cells. Therefore, the targeting of PDE5 inhibitor therapy for penile erection is not due solely to relative abundance of PDE5 in corpus cavernosum. Perhaps targeting for penile erection is also partly due to selective release of nitric oxide in penis during sexual arousal.

The presence of >200 nmol/L PDE5 subunit in corpus cavernosal or lung smooth muscle raises questions about the mechanism of action of PDE5 inhibitors in treatment of erectile dysfunction or pulmonary hypertension. At maximum recommended doses in men, the concentration of these PDE5 inhibitors in plasma is about 400 nmol/L (sildenafil and tadalafil) or 20 nmol/L (vardenafil) [25]. If binding of the PDE5 inhibitors to plasma albumin reduces the plasma free concentration of each drug by 95% [25], the effective drug plasma concentration would be 20 nmol/L (sildenafil and tadalafil) or 1 nmol/L (vardenafil). Assuming equilibration, the smooth muscle cell concentration in corpus cavernosum or lung would be the same as the plasma concentration. Since binding of each inhibitor to PDE5 is stoichiometric, and intracellular PDE5 is likely to be far greater than 200 nmol/L, then 20 nmol/L intracellular sildenafil or tadalafil could inhibit at most 10% of PDE5 activity, and vardenafil could inhibit at most 0.5% of PDE5 activity. Even if all plasma PDE5 inhibitor is active, the vardenafil plasma concentration is still only 20 nmol/L, which is at most just 10% of the intracellular concentration of PDE5, and thus capable of inhibiting only 10% of intracellular PDE5 activity. It is possible that 10% inhibition of PDE5 is sufficient to elevate cGMP to levels that produce penile erection or lower pulmonary artery pressure in most patients. Another possibility is that because of its high affinity PDE5 could act as a pump or an intracellular sink to increase the cellular concentration of PDE5 inhibitor, which might eventually approach the intracellular concentration of PDE5 and therefore inhibit the enzyme by nearly 100%. Slow reversal of this process could increase the duration of action of the inhibitors well past that predicted from the plasma half-life of each inhibitor depending on the PDE5 affinity of the particular inhibitor. Moreover, elevation of cGMP in cells should increase binding affinity of PDE5 inhibitors to PDE5 both by allosteric activation and by phosphorylation [13,14,18,26], which would be predicted to further concentrate the intracellular PDE5 inhibitors and increase their duration of action.

As measured by either ^3H inhibitor binding or PDE5 catalytic activity, heart tissue contains a relatively low level of PDE5. Most of the PDE5 detected in heart extracts could be derived from the smooth muscles of the coronary vessels of this tissue rather than from the myocardial contractile cells of the whole tissue extract, although presence of PDE5 in the latter cells has been

reported [27]. We did not detect any effect of up to 1 $\mu\text{mol/L}$ sildenafil on contractile force of heart tissue slices [28], which is consistent with the presence of low levels or absence of PDE5 in myocardial contractile cells. The combined results suggest absence of direct effects of sildenafil, vardenafil, and tadalafil on the heart in men taking these drugs. However, in a rat model of aortic stenosis, administration of sildenafil blocked or reversed cardiac hypertrophy [29], suggesting a functional effect of a PDE5 inhibitor in cardiomyocytes.

The ^3H inhibitor-binding assay offers distinct advantages over Western immunoblot analysis or the PDE catalytic assay in potentially revealing the presence of proteins other than PDE5 that bind either of the inhibitors. Such proteins would escape detection by Western analysis unless they are evolutionarily very closely related. Search for potential cross-reactants using the catalytic assay is biased in the sense that it assumes that such a reactant would be a PDE, which ignores thousands of proteins, while the binding assay should detect any high-affinity ^3H inhibitor-binding protein that is present at detectable levels. No such protein(s) were found in heart or lung, thus reducing concern about side effects or safety issues of the drugs.

To our knowledge, this is the first report of tissue quantification of a kinase and its substrate for a physiological phosphorylation reaction. It is of interest that PKG is present at 20% the level of PDE5 in the lung, and PDE5 is a substrate for phosphorylation by PKG. We propose that phosphorylation and activation of PDE5 by PKG in lung is one of several mechanisms to engender negative feedback control of the cGMP pathway in the lung vasculature, which would accentuate effects of PDE5 inhibitors. The presence of PKG in excess of PDE5 in heart tissue suggests that PKG is not solely coupled with PDE5 for negative feedback regulation of the cGMP pathway in this tissue.

We previously did careful studies comparing PKG and PKA for phosphorylation of PDE5 [30]. Using purified enzymes, the rate of phosphorylation was ten times faster using PKG. Furthermore, we found that PDE5 cannot be phosphorylated at a significant rate by either kinase unless cGMP is bound to the PDE5 itself. We have referred to this phenomenon as “substrate-directed regulation of phosphorylation of PDE5”. In other words, cGMP needs to be elevated in tissues before phosphorylation of PDE5 can take place. Activation of the kinase alone (either PKG or PKA) is not sufficient. Elevation of cAMP alone, which activates PKA, is not sufficient since this nucleotide does not bind to PDE5. In fact, using bovine aorta, we showed [22] that elevation of cAMP alone does not cause phosphorylation of PDE5. Thus, it is unlikely that PKA phosphorylates PDE5 in many tissues, but a suggestion derived from the present report is that PKA could phosphorylate PDE5 in heart cells consid-

ering the very high level of PKA (but low level of PKG) in heart cells; this would also apply to other cells containing PDE5 and a high PKA to PKG ratio. For heart tissue, this likelihood would be precluded if PKA is localized mainly to myocardial contractile cells and PDE5 is localized mainly to coronary smooth muscle cells. Moreover, cGMP would have to be elevated and bound to PDE5 in order for the PDE5 phosphorylation site to be accessible to PKA.

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