Molecular Mechanisms of Cardiostimulatory Effects of Sildenafil

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ABSTRACT—To better understand the molecular mechanisms of the previously described cardiostimulatory action of the phosphodiesterase type-5 (PDE5) inhibitor sildenafil, we first evaluated its effects on cyclic AMP level in the canine ventricular membrane preparation. Sildenafil (10 μmol/L) significantly increased the net cyclic AMP production rate, the potency of which was similar to that of 3-isobutyl-1-methylxanthine (IBMX). Next, we assessed the inhibitory effect of sildenafil on PDE of bovine heart. Sildenafil (1–10 μmol/L) as well as IBMX significantly decreased the cyclic AMP hydrolyzing speed of PDE. These results suggest that a supra-therapeutic concentration of sildenafil may directly inhibit cyclic AMP hydrolyzing PDEs in the heart, although indirect inhibition of PDE3 via the “cross-talk” pathway cannot be totally excluded.

Keywords: Phosphodiesterase, cyclic AMP, Sildenafil

Sildenafil (Viagra™) is a relatively selective inhibitor of cyclic GMP-specific phosphodiesterase type-5 (PDE5) which is predominantly found in the corpus cavernosum (1). In our recent study using the canine in vivo model (2), a supra-therapeutic concentration of sildenafil (>10×) exerted positive chronotropic and inotropic effects together with a reduction of total peripheral resistance. Those cardiostimulatory actions can be largely attributed to the sympathetically mediated reflex resulting from the vasodilator effect of sildenafil, while it is also possible that non-specific PDE inhibition occurs at these higher sildenafil concentrations. Wallis et al. showed that sildenafil had no direct effect on the contraction force of the canine isolated trabeculae carnea (1), which supports the former hypothesis. Meanwhile, Stief et al. reported that sildenafil increased cyclic AMP concentration in human cardiac tissue (3), which indicates the possibility of the latter. To better understand the mechanism of cardiostimulatory actions of sildenafil observed in the in situ heart, we first evaluated its effects on the net cyclic AMP production rate in the canine ventricular membrane preparation, which contains active PDE as well as adenylate cyclase. Next, we assessed the inhibitory effect of sildenafil on the commercially available PDE from bovine heart. An enzymatic fluorometric assay technique was adopted for precisely estimating the concentration of cyclic AMP (4, 5). A non-specific inhibitor of PDE, 3-isobutyl-1-methylxanthine (IBMX), was used as a reference compound.

All experimentation was performed in accordance with the rules and regulation of the Committee for Research at the Yamanashi Medical University. Ventricular membranes were prepared from six female beagle dogs weighing approximately 10 kg, as previously described (4, 6). Briefly, the dog was anesthetized with pentobarbital sodium (30 mg/kg, i.v.), given heparin calcium (500 U/kg), and exsanguinated. The hearts were excised and immediately placed in ice-cold SET buffer (0.25 mol/L sucrose; 0.1 mmol/L EDTA; 5.0 mmol/L Tris-acetate, pH 7.4). Portions (3–5 g) from the left ventricular apex were minced and then homogenized in SET buffer in 1/10 W/V. The homogenate was filtered (Nitex filter; Tetko, Los Angeles, CA, USA) and centrifuged for 20 min at 1,500 x g. The pellet was resuspended and centrifuged three more times. The final pellet was resuspended in SET buffer. Protein concentration was measured using a commercially available protein assay reagent (Pierce, Rockford, IL, USA). The membrane suspension was diluted with SET buffer to a concentration of 3–5 mg protein/ml, and it was stored at –80°C until enzyme activity was measured.

The net cyclic AMP production rate of the membrane preparation was measured with an enzymatic fluorometric assay technique for adenylate cyclase activity, which was essentially the same as that previously described (4). Fifty microliters of reaction mix (100 mmol/L Tris-acetate, pH 7.4; 20 mmol/L KCl; 10 mmol/L MgCl2; 20 mmol/L
phosphoenolpyruvate; 2 mmol/L ATP; 2 mmol/L GTP; 2 mmol/L dithiothreitol; 0.4 mg/L bovine serum albumin; 100 μg/ml pyruvate kinase) was added to each microcentrifugation tube in duplicate with or without either of sildenafil (2 and 20 μmol/L) or IBMX (20 and 200 μmol/L). Next, the membrane suspension in a volume of 50 μL was added to each tube. The reaction mixture and membrane suspension, both before and after being mixed, were maintained at 4°C to ensure the same starting time for all assay tubes. The reaction was initiated by placing the tubes in a water bath maintained at 37°C. After 30 min, the reaction was terminated by heating at 90°C for 3 min. The mixture was vortexed 3 times and centrifuged at 10,000 × g for 5 min. A volume of 5 μL of the supernatant was transferred to a 10 × 75 mm disposable assay tube (Iwaki Lab Ware, Tokyo) in triplicate. For the cyclic AMP standard, 5 μL of a known amount of cyclic AMP was added to the tubes. The cyclic AMP concentration was assayed using the enzymatic fluorometric method as previously described (5).

Next, the effects of sildenafil on the hydrolyzing speed of cyclic AMP was determined using the commercially available PDE from bovine heart. Fifty microliters of reaction mix (100 mmol/L Tris-acetate, pH 7.4; 10 mmol/L MgCl₂; 0.4 mg/L bovine serum albumin; 20 μmol/L cyclic AMP) was added to each microcentrifugation tube in triplicate with or without either of sildenafil (2 and 20 μmol/L cyclic AMP) or IBMX (2, 20 and 200 μmol/L). Next, 50 μL of PDE solution (40 μg/mL) suspended in SET buffer was added to each tube on ice. The reaction was initiated by placing the tubes in a water bath maintained at 37°C. After 20 min, the reaction was terminated by heating at 95°C for 5 min. The mixture was vortexed 3 times and centrifuged at 10,000 × g for 5 min. Twenty-five microliters of the supernatant was diluted with 100 μL of distilled water (5 times dilution). A volume of 5 μL of the diluted supernatant was transferred to the assay tube in triplicate and the cyclic AMP concentration was assayed using the enzymatic fluorometric method (5).

Sildenafil citrate (MW = 666.71) was generously provided by Pfizer Pharmaceuticals, Inc. (Tokyo). The following drugs were purchased: pentobarbital sodium (Tokyo-Kasei, Tokyo) and heparin calcium (Mitsui, Tokyo). All other enzymes and substrates were obtained from Sigma Chemical Company (St. Louis, MO, USA). The data are presented as the mean ± S.E.M. The statistical comparisons of mean values were carried out using one-way repeated-measures ANOVA followed by Contrast. A P-value less than 0.05 was considered significant.

The effects of sildenafil and IBMX on the net cyclic AMP production rate in the membrane preparation obtained from left ventricle are summarized in Fig. 1 (n = 6). Sildenafil as well as IBMX increased the net cyclic AMP production rate in a dose-related manner. Significant increases were induced by 10 μmol/L of sildenafil and 10–100 μmol/L of IBMX. The in vitro effect of 10 μmol/L of sildenafil on the net cyclic AMP production rate was almost equivalent to that of the same concentration of IBMX.

The effects of sildenafil on the cyclic AMP hydrolyzing speed of PDE are summarized in Fig. 2 (n = 3). Basal
hydrolyzing speed of 20 μg/mL of PDE was 16.1 ± 0.5 nmol·min⁻¹·ng protein⁻¹. Sildenafil (≥1 μmol/L) as well as IBMX decreased the cyclic AMP hydrolyzing speed of PDE in a dose-related manner.

There are two new findings in this study: One is that sildenafil significantly increased the net cyclic AMP production rate in the canine ventricular membrane preparation with a similar potency to IBMX. The other is that sildenafil as well as IBMX decreased the cyclic AMP hydrolyzing speed of PDE. These results are essentially in accordance with the previous report (3) that sildenafil increased cyclic AMP levels in the human cardiac auricle with a similar potency to the typical PDE3 inhibitor milrinone in the range of 0.1 to 1.0 μmol/L. Thus, the current result may provide alternative mechanisms for some cardiovascular side effects that have been reported with sildenafil use (7, 8). On the other hand, the present result and our recent in vivo finding (2) may not explain the observation of the previous report (1) that sildenafil was devoid of direct inotropic effects on the canine isolated trabeculae carneae at concentrations of 0.01 to 10 μmol/L. Further study should be performed to bridge the gap between the different manifestation of the drug action using other experimental designs like the canine isolated, blood-perfused heart preparations (9).

Two potential mechanisms have been proposed to explain the increase of cyclic AMP level in the heart (3). Intracellular levels of cyclic AMP as well as cyclic GMP are controlled by a balance between their formation and degradation by the respective enzymes (1, 4, 8). PDE1 and PDE2 hydrolyze both cyclic AMP and cyclic GMP, whereas PDE3 and 4 are specific for cyclic AMP, and PDE5 and PDE6 are specific for cyclic GMP (1, 7, 8). It should be noted that cardiac tissue does not have significant PDE5 activity (1, 7, 8). It has been shown that sildenafil potent inhibits PDE5 expressed in the corpus cavernosum, which is 80 to 19,000-fold more effective than for PDE1 to 4 (1, 7, 8). Since PDE1 to 4 have been detected in the heart, each of which possesses cyclic AMP hydrolyzing activity (1, 8, 10), a supra-therapeutic concentration of sildenafil might directly inhibit these PDEs, leading to an increase of cyclic AMP concentration. The current results at least in part support this hypothesis. An alternative explanation is a “cross-talk” mechanism between cyclic GMP and cyclic AMP-dependent signal transduction pathways (3). Since PDE1 and PDE2 possess cyclic GMP hydrolyzing activity (1, 7, 8), an increase of cyclic GMP via an inhibition of these PDEs by the high concentration of sildenafil might exert an inhibitory effect on PDE3 (1, 7, 8), resulting in an elevation of cyclic AMP. The present results cannot totally exclude this mechanism. Experiments are now on-going to further analyze the indirect inhibition of PDE3 via the “cross-talk” pathway, using cultured cardiomycocytes with more complex experimental design.

In summary, a supra-therapeutic concentration of sildenafil can increase the cyclic AMP level in the heart possibly through the direct inhibition of cyclic AMP hydrolyzing PDEs. The data shown in this study will provide convenient guidelines for comparing the potency of cyclic AMP hydrolyzing PDEs inhibitors. Moreover, the present study suggest that care must be taken on patients with ischemic heart disease, obstructive hypertrophic cardiomyopathy and/or ventricular arhythmias during the sildenafil therapy, since high concentration of sildenafil would directly exert cardiotonic action.

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