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EFFECTS OF VINPOCETINE ON CYCLIC NUCLEOTIDE METABOLISM IN VASCULAR SMOOTH MUSCLE

MASATOSHI HAGIWARA, TOYOSHI ENDO and HIROYOSHI HIDAKA*
Department of Pharmacology, Mie University School of Medicine, Tsu 514, Japan

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Abstract—A novel vasodilating agent, vinpocetine (14-ethoxycarbonyl-(3α,16α-ethyl)-14,15-eburnamenine) inhibits Ca^{2+}-dependent phosphodiesterase, selectively, among the three forms of cyclic nucleotide phosphodiesterase identified in the rabbit aorta. The concentration of vinpocetine producing 50% inhibition of Ca^{2+}-dependent phosphodiesterase activity was approximately 21 μM, both in the presence and absence of Ca^{2+}-calmodulin (CaM). Increasing the concentration of CaM in the presence of Ca^{2+} did not prevent vinpocetine-induced inhibition of Ca^{2+}-dependent phosphodiesterase, thereby indicating that vinpocetine inhibited the enzyme by interacting with the enzyme and not with CaM. To determine the influence of vinpocetine-induced inhibition of Ca^{2+}-dependent phosphodiesterase on cyclic nucleotide metabolism in vascular smooth muscle, cyclic nucleotide levels in isolated rabbit aortic strips were also investigated. Addition of vinpocetine produced dose-dependent increases in only the cyclic GMP levels and there was no significant effects on the cyclic AMP levels. These results provide pharmacological evidence that Ca^{2+}-dependent phosphodiesterase mainly hydrolyzes cyclic GMP in vascular smooth muscle. Vinpocetine may induce vascular relaxation by increasing cyclic GMP contents in vascular smooth muscle through selective inhibition of Ca^{2+}-dependent phosphodiesterase.

Recent studies on the role of cyclic nucleotides in vascular smooth muscle have suggested that cyclic nucleotides such as cyclic AMP or cyclic GMP play a regulatory role in vascular contraction [1–3]. These levels are probably controlled to some extent by cyclic nucleotide phosphodiesterase. We found that cyclic nucleotide phosphodiesterase exists in multiple forms in vascular smooth muscle, namely cyclic GMP phosphodiesterase (with high affinity for cyclic GMP), Ca^{2+}-dependent phosphodiesterase (with low affinity for cyclic AMP and cyclic GMP) and cyclic AMP phosphodiesterase (with high affinity for cyclic AMP) [4]. Among these three forms of the phosphodiesterase, vascular smooth muscle contains an enzyme highly dependent on Ca^{2+}, and *in vitro*, the enzyme has a lower K_m for cyclic GMP than for cyclic AMP [5]. Therefore, a selective inhibitor of the Ca^{2+}-dependent phosphodiesterase should provide a useful tool for elucidation of the role of Ca^{2+}-dependent phosphodiesterase in vascular smooth muscle. CaM-antagonists W-7 and phenothiazine derivatives also inhibit Ca^{2+}-dependent phosphodiesterase selectively, by interacting with CaM [6, 7]. However, CaM activates various enzymes including myosin light chain kinase from smooth muscle [8, 9]. Thus this complication necessarily limits the utility of CaM-antagonists as tools for elucidation of the role of Ca^{2+}-dependent phosphodiesterase in vascular smooth muscle.

We now report findings with vinpocetine, a novel vasodilating agent, which inhibits Ca^{2+}-dependent phosphodiesterase selectively by mechanisms which differ from those related to CaM-antagonists. Using this agent we obtained pharmacological evidence for the role of Ca^{2+}-dependent phosphodiesterase in vascular smooth muscle.

MATERIALS AND METHODS

Materials. Vinpocetine (Fig. 1) was kindly provided by Takeda Chemical Industries, Ltd. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was synthesized according to the method of Hidaka *et al.* [9]. Cyclic [^{3}H]adenosine 3′:5′-monophosphate, cyclic [^{3}H]guanosine 3′:5′-monophosphate, [^{3}H]adenosine triphosphate and [^{3}H]guanosine triphosphate were purchased from New England Nuclear Co. Unlabeled cyclic AMP, cyclic GMP and snake venom (Crotalus atrox) were purchased from Sigma Chemical Co. Cyclic AMP and cyclic GMP radioimmunoassay kits were purchased from Yamasa Co., Ltd. All other chemicals were of reagent grade or the best commercially available.

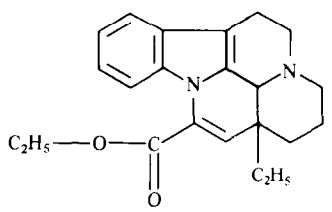

Fig. 1. Chemical structure of 14-ethoxycarbonyl-(3α,16α ethyl)-14,15-eburnamenine (vinpocetine).

* Author to whom all correspondence should be addressed.

† Abbreviations: vinpocetine, 14-ethoxycarbonyl-(3α,16α-ethyl)-14,15-eburnamenine; CaM, calmodulin; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

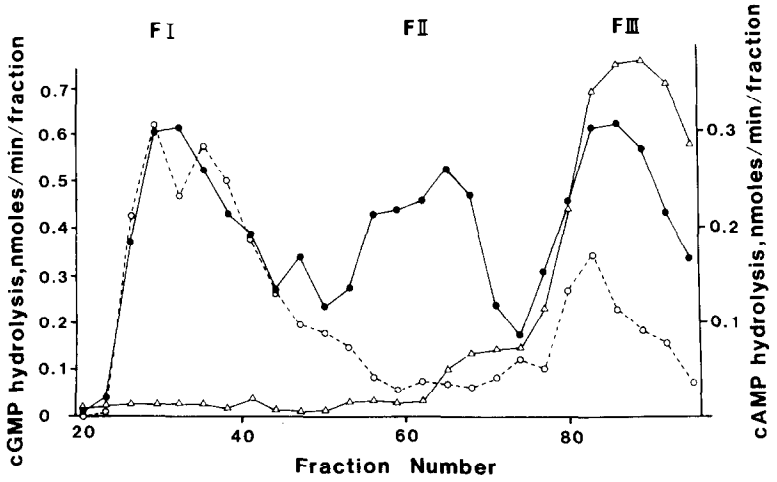

Fig. 2. Typical elution profile of DEAE-cellulose column chromatography. 105,000 *g* supernatant of rabbit aorta smooth muscle layer was applied to a DEAE-cellulose-column (1.5 × 20 cm) previously equilibrated with 50 mM Tris–acetate Buffer containing 1 mM $MgCl_2$ and 2.75 mM 2-mercaptoethanol (pH 6.0). The column was washed with approx. 5 volumes of equilibrating buffer followed by 300 ml of a linear gradient of sodium acetate from 0 to 0.5 M at a flow rate of 30 ml/hr. Aliquots, 0.01 ml for low substrate (4 μM) were assayed directly. (●—●), Cyclic GMP hydrolysis with Ca^{2+} and CaM; (○---○), cyclic GMP hydrolysis without Ca^{2+} and CaM; (△—△), cyclic AMP hydrolysis.

Preparation of cyclic nucleotide phosphodiesterase and CaM. Cyclic nucleotide phosphodiesterase was partially purified from rabbit aorta, as previously described [5]. The aorta obtained from 10 albino rabbits were homogenized with 5 volumes of 50 mM Tris–acetate buffer (pH 6.0) containing 3.75 mM 2-mercaptoethanol (Buffer A), using a glass tissue homogenizer. The homogenate was sonicated then centrifuged at 105,000 *g* for 60 min. The supernatant was applied to a DEAE-cellulose column (bed volume 35 ml) equilibrated with Buffer A. After washing the column with Buffer A, a linear gradient from 0 to 0.5 M sodium acetate was applied with a flow rate of 0.5 ml/min. As shown in Fig. 2, three cyclic nucleotide phosphodiesterase fractions, designated cyclic GMP phosphodiesterase (FI), Ca^{2+}-dependent phosphodiesterase (FII), cyclic AMP phosphodiesterase (FIII) were obtained. The specific activities of cyclic GMP phosphodiesterase, Ca^{2+}-dependent phosphodiesterase and cyclic AMP phosphodiesterase were 168, 3214 and 6561 pmol/min/mg protein, respectively. Bovine brain CaM was purified to homogeneity according to the methods previously described [10]. One unit of CaM is defined as the amount required to produce 50% maximal activation of Ca^{2+}-dependent phosphodiesterase, under standard conditions, and was equivalent to 10 ng of protein.

Phosphodiesterase assay. Phosphodiesterase activity was measured by the method previously reported [11]. The reaction mixture (0.5 ml) contained 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, cyclic [^{3}H]AMP or cyclic [^{3}H]GMP, various concentrations of vinpocetine and appropriate amounts of enzyme. Ca^{2+}-dependent phosphodiesterase was measured using cyclic GMP as a substrate. After 15 min incubation at 30°C, the reaction was terminated by boiling for 5 min, after which 50 μg of snake venom was added and the mixture was incubated for another 10 min. Three ml of water was added and the preparation was applied to a small cation exchange resin column (AG50-X4, 200–400 mesh, 0.7 × 1.5 cm). The product, [^{3}H]adenosine or [^{3}H]guanosine was eluted with 1.5 ml of 3 N ammonium hydroxide after the column had been washed with 15 ml of deionized water. The amount of product was determined in a liquid scintillation counter (Beckman, LS-7500).

Adenylate cyclase and guanylate cyclase assay. Adenylate and guanylate cyclases were prepared from human platelets as previously described [12]. In brief, platelets were homogenized in 50 mM Tris–HCl buffer (pH 7.4) with a tight Teflon pestle. The homogenate was centrifuged at 105,000 *g* for 60 min. The resultant supernatant was used as the crude soluble guanylate cyclase and the pellet as adenylate cyclase. The activities of the adenylate and guanylate cyclases were determined by the methods of Nakazawa *et al.* [13], and Asano and Hidaka [12], respectively. In brief, the standard assay mixture contained 2 mM [^{3}H]ATP (4 μCi/ml) or 1 mM [^{3}H]GTP (5 μCi/ml), 0.5 mM cyclic AMP or cyclic GMP, 15 mM creatine phosphate, 40 μg of creatine kinase, 10 mM $MgCl_2$ or 3 mM $MnCl_2$, 50 mM Tris–HCl buffer (pH 7.7), various concentrations of vinpocetine and an appropriate amount of the enzyme in a total volume of 0.25 or 0.2 ml. The reaction was started by the addition of the enzyme. After the incubation for 30 min at 30°, the reaction was terminated by boiling for 2 min, following the addition of 40 μl of 1 M HCl. The assay mixture was neutralized with 1 M Tris solution and 0.14 M sodium pyrophosphate then applied to a neutral aluminum oxide column (Bio-Rad Co., 0.5 g, 0.6 × 2.0 cm) and washed with 5 ml of 50 mM Tris–HCl buffer (pH 7.7). The washed solution containing the cyclic nucleotide passed directly to a AG1-X2 column (chloride form, 0.7 × 3 cm). The resin column was washed with 0.005 M HCl and cyclic AMP was eluted

from this column with 2 ml of 0.05 M HCl. Cyclic GMP was eluted with 2 ml of 0.5 M HCl, washing 0.05 M HCl.

Measurement of levels of cyclic AMP and cyclic GMP. Albino rabbits of both sexes, weighing 2.5–3.0 kg, were anesthetized with ether and exsanguinated from the common carotid arteries. The aorta was removed, cut helically and placed in bath medium consisting of 115 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM dextrose. Each strip (2.5 × 30 mm) was contracted by addition of an appropriate dose of KCl (25 ± 5 mM) to the bath and when contraction reached 50% of a maximum, vinpocetine was added to the bathing medium. Immediately after completion of relaxation response, tissue samples were frozen with a clamp cooled in dry-ice acetone. Frozen samples were homogenized in 5% trichloroacetic acid and precipitates were removed by centrifugation at 3000 *g* for 20 min. To remove the trichloroacetic acid, 5 ml of ether was added to 1 ml of the supernatant and the preparation centrifuged at 1000 *g* for 10 min. After discarding the ether, samples (each 1 ml) were freeze-dried and resuspended in 100 μl of distilled water. Cyclic AMP and cyclic GMP in the suspensions were measured using Yamasa cyclic AMP and cyclic GMP radioimmuno assay kits.

RESULTS

Inhibition of cyclic nucleotide phosphodiesterase. The effects of vinpocetine on rabbit aorta cyclic nucleotide phosphodiesterase, separated by DEAE-cellulose column chromatography, were examined. The three forms of cyclic nucleotide phosphodiesterase observed were cyclic GMP phosphodiesterase (FI), Ca^{2+}-dependent phosphodiesterase (FII) and cyclic AMP phosphodiesterase (FIII) (Fig. 2). Table 1 summarizes the effects of vinpocetine on these three forms of the enzymes. The greater potency of vinpocetine, as an inhibitor of Ca^{2+}-dependent phosphodiesterase is noteworthy and of particular interest as the concentrations of vinpocetine which produced 50% inhibition of the Ca^{2+}-dependent phosphodiesterase were about 21 μM, both in the presence and absence of Ca^{2+}-CaM. The kinetic analysis by Dixon plots [14] was linear and showed a non-competitive inhibition, with respect to cyclic GMP as substrate (Fig. 3). The

Table 1. Effect of vinpocetine on cyclic nucleotide phosphodiesterase from rabbit aorta

Phosphodiesterases (PDE)	Concentration required for 50% inhibition (μM)
Cyclic GMP-PDE (FI)	>500
Ca^{2+}-dependent PDE (FII)	
in the presence of Ca^{2+}-CaM	21
in the absence of Ca^{2+}-CaM	21
Cyclic AMP-PDE (FIII)	>500

* Four μM of nucleotides were used as substrates in these assays.

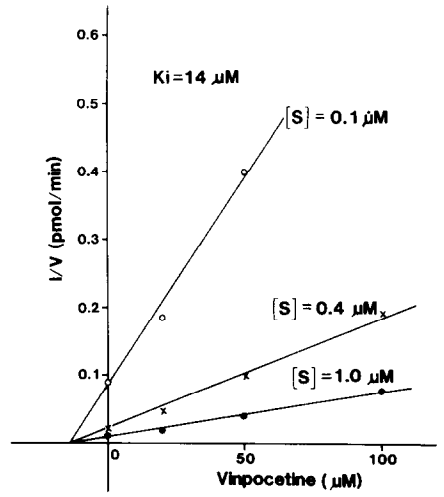

Fig. 3. Kinetic analysis of vinpocetine on Ca^{2+}-dependent phosphodiesterase (FII) by Dixon plots (Dixon, 1953). (S) = substrate concentration (cyclic GMP).

determined K_i value was 14 μM in the presence of Ca^{2+}-CaM.

Antagonism of CaM. The CaM-antagonists but not vinpocetine were all more potent against Ca^{2+}-dependent phosphodiesterase in its Ca^{2+}-CaM activated state than in its basal state. This suggested that

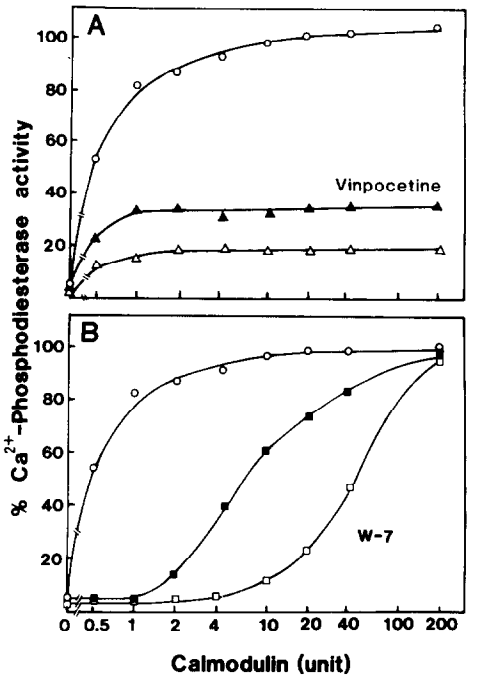

Fig. 4. Influence of CaM on vinpocetine-induced (A) or W-7-induced (B) inhibition of Ca^{2+}-dependent phosphodiesterase. One unit of CaM was equal to 10 ng of purified CaM. Each point represents the percentage of activation of Ca^{2+}-dependent phosphodiesterase activity by CaM. Vinpocetine at the concentrations of 0 (○), 20 (▲), and 40 (△) μM and W-7 at the concentrations of 0 (○), 20 (■), and 40 (□) μM were added to assay solutions.

vinpocetine may directly inhibit Ca^{2+}-dependent phosphodiesterase while the CaM antagonists antagonize the CaM stimulation of the enzyme. This was tested further by examining the ability of vinpocetine and W-7 to alter the activation of the phosphodiesterase by CaM. As shown in Fig. 4, vinpocetine-induced inhibition of the phosphodiesterase activity was not overcome by increasing the concentration of CaM and the extent of activation of CaM was greatly suppressed (Fig. 4(A)). On the other hand, the W-7-induced inhibition of the enzyme was overcome by increasing the concentration of CaM (Fig. 4(B)). These results indicate that vinpocetine inhibits Ca^{2+}- dependent phosphodiesterase by a mechanism differing from those related to CaM-antagonists. The possibility of interaction of vinpocetine with the enzyme has to be considered.

Cyclic AMP and cyclic GMP levels. To investigate the influence of the vinpocetine-induced inhibition of Ca^{2+}-dependent phosphodiesterase on cyclic nucleotide metabolism in vascular smooth muscle, cyclic AMP and cyclic GMP levels in isolated rabbit aortic strips were investigated.

The exposure of K^+-depolarized rabbit aorta to 1, 10 and 100 μM vinpocetine resulted in a decrease in tension of aortic strips, and as shown in Fig. 5, 10 and 100 μM of vinpocetine caused a significant increase in cyclic GMP levels. There was a tendency toward increase in cyclic GMP levels with 1 μM vinpocetine, albeit not statistically significant. There was no increase in cyclic AMP levels associated with relaxation, regardless of the concentration of vinpocetine.

In concentrations of vinpocetine ranging from 10 to 1000 μM, there was no increase in guanylate cyclase activity and vinpocetine also had no effect on adenylate cyclase (data not shown). Thus, the increase in cyclic GMP level may have been due to the inhibitory effect of vinpocetine on the Ca^{2+}-dependent phosphodiesterase activity.

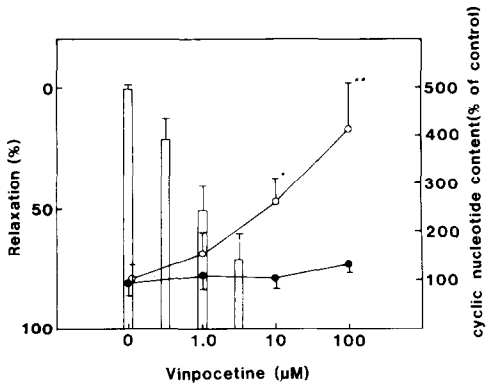

Fig. 5. Effect of vinpocetine on cyclic AMP and cyclic GMP content in rabbit aorta. Helically cut strips of rabbit aorta were contracted half maximally with KCl and cyclic AMP (—●—) and cyclic GMP (—○—) contents were assayed before and after the addition of various concentrations of vinpocetine by radioimmunoassay. Each value is the mean ± S.E. of five determinations. Results were compared using the Student's *t*-test. * Indicates $P < 0.05$, ** indicates $P < 0.005$. Basal cyclic AMP level (control) was 87.8 ± 45.7 pmol/g wet-wt tissue. Basal cyclic GMP level (control) was 9.36 ± 5.01 pmol/g wet-wt tissue. Vertical bars show the dose–relaxation response for vinpocetine in strips of rabbit aorta. The relaxation induced by 1×10^{-4} M of papaverine was taken as 100%.

DISCUSSION

A novel compound, vinpocetine had a highly selective inhibitory action toward Ca^{2+}-dependent phosphodiesterase. As reported earlier [9, 15], CaM-antagonists such as W-7 or phenothiazine derivatives also inhibit specific cyclic nucleotide phosphodiesterase, characterized by stimulation by Ca^{2+} and CaM. However, CaM is involved in the activation of enzymes other than Ca^{2+}-dependent phosphodiesterase such as myosin light chain kinase [8] and CaM appears to have multifunctional roles as a mediator of various Ca^{2+} regulatory signals, this complication necessarily limits the utility of CaM-antagonists as tools for investigating the role of Ca^{2+}-phosphodiesterase. On the other hand, vinpocetine inhibits Ca^{2+}-dependent phosphodiesterase not by interacting with CaM but by interacting with the enzyme. Accordingly, this opens the way for elucidation of the properties of Ca^{2+}-dependent phosphodiesterase and role of cyclic GMP in biological systems. Ca^{2+}-dependent phosphodiesterase reportedly [4] has a lower K_m for cyclic GMP than for cyclic AMP *in vitro*, our results provide pharmacological evidence that Ca^{2+}-dependent phosphodiesterase mainly hydrolyzes cyclic GMP, in vascular smooth muscle.

It has recently been suggested that cyclic GMP may be involved in processes related to smooth muscle relaxation [2, 3]. Vinpocetine increases cyclic GMP levels through inhibition of Ca^{2+}-dependent phosphodiesterase and seems to support the hypothesis of Schultz *et al.* [1] that relaxation of smooth muscle is accompanied by an increase in cyclic GMP. Kramer and Wells [16] reported a positive correlation between relaxation of pig coronary artery and inhibition of cyclic GMP hydrolysis. Gruetter *et al.* [17] also reported that cyclic GMP formation may be involved in nitric oxide-induced relaxation of bovine coronary arterial smooth muscle. However, further work will be necessary to clarify the role of cyclic GMP in vascular smooth muscle.

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