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EFFECTS OF VINCA ALKALOIDS ON CALCIUM–CALMODULIN REGULATED CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE ACTIVITY FROM BRAIN*

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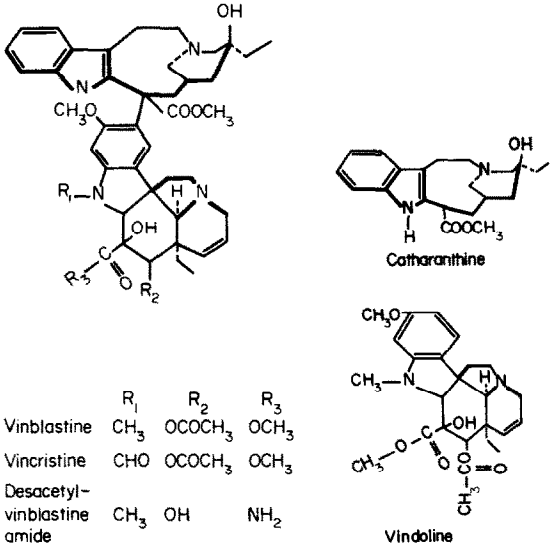
Abstract—The effects of a series of vinca alkaloids on calcium–calmodulin regulated brain cyclic adenosine 3′,5′-monophosphate phosphodiesterase (PDE) activity were examined. The alkaloids tested included the dimeric indole alkaloids, vinblastine, vincristine, and desacetylvinblastine amide, and the monomeric alkaloids, catharanthine and vindoline. The order of magnitude of the inhibitory effects on the calcium–calmodulin stimulated activity of phosphodiesterase was vinblastine > desacetylvinblastine amide > vincristine = catharanthine > vindoline. In contrast, both catharanthine and vindoline were more potent inhibitors than the dimeric vinca alkaloids of the basal unstimulated phosphodiesterase activity. Vinblastine and vincristine inhibition of calcium–calmodulin activated partially purified PDE was non-competitive with substrate. In contrast, the inhibitory actions of vinblastine, desacetylvinblastine amide, vincristine and catharanthine, but not of vindoline, were competitive with calcium–calmodulin. Colchicine inhibited both activated and basal phosphodiesterase activity. The inhibitory effect of colchicine was not reversed by the addition of either calmodulin or calcium. These results suggest that the calcium–calmodulin dependent inhibition of PDE activity by the dimeric vinca alkaloids had the greater specificity, and that this inhibitory action required the catharanthine moiety. In view of these results, dimeric vinca alkaloids may provide a useful tool for the elucidation of the physiological role of calcium–calmodulin.

Among vinca alkaloids, vinblastine (VB) and vincristine (VC) have been used, either alone or in combination with other drugs, for the treatment of neoplasms (cf. Ref. 1). Experimental evidence suggests that these compounds have similar effects, such as inhibition of mitosis and of polymerization of microtubules, and formation of highly regulated tubulin crystals [2–6]. Based on a study of structure–activity relationships, Owellen *et al.* [5] suggested that the dimeric indole moiety may be necessary for the inhibition of microtubule polymerization. Wilson *et al.* [3], on the basis of both inhibition of mitosis and the stabilization of colchicine binding activity to tubulin, postulated that the biological activity of vinca alkaloids resides in the catharanthine (CR) moiety, a compound related structurally to the upper half of the dimeric indole alkaloid molecule (see Fig. 1). The lower half of the dimeric compound, vindoline (VD), is inactive for both inhibition of mitosis and microtubule polymerization [3, 4]. Desacetylvinblastine amide (DVB), a newer vinca alkaloid, has effects similar to those of VB and VC on microtubule polymerization and mitosis [4–7]; desacetylvinblastine is a metabolite of VB [8]. Although the biological effects of these alkaloids (VB, VC, and DVB) are similar, both their clinical efficacies in the treatment of neoplastic disease and their adverse effects are dissimilar [1, 7]. Hence, it has been suggested that the different clinical actions of these alkaloids may be based on biological processes



	R_1	R_2	R_3
Vinblastine	CH_3	$OCOCH_3$	OCH_3
Vincristine	CHO	$OCOCH_3$	OCH_3
Desacetyl-vinblastine amide	CH_3	OH	NH_2

Fig. 1. Chemical structures of vinblastine, vincristine and desacetylvinblastine amide, (left panel) and catharanthine and vindoline (right panel).

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other than the reaction with tubulin or with microtubules *per se* [6].

We have reported previously that a fraction of brain supernatant cyclic nucleotide phosphodiesterase (PDE) activity was associated with microtubule proteins prepared by various methods of purification [9, 10]. The brain supernatant proteins precipitated by VB had a 2- to 3-fold higher PDE activity than the proteins precipitated with VC [10]. Since VB specifically inhibited the Ca^{2+}–calmodulin activated brain PDE activity [11], we now report the effects of VB, VC, DVB, CR and VD on the Ca^{2+}–calmodulin regulated PDE activity. The effect of colchicine on this PDE activity was also compared.

METHODS

Crude brain supernatant PDE activity. Rat brain was homogenized with 2 vol. of 10 mM imidazole buffer, pH 6.9, containing 1.5 mM $MgCl_2$ and, after centrifugation at 78,000 *g* for 30 min, the supernatant fraction was used as a source of enzyme activity [11].

Calcium–calmodulin dependent PDE activity. Calcium–calmodulin dependent PDE was prepared by the method of Klee and Krinks [12] with a modification, using 50 mM 2(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.7, containing 0.5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol.

Purification of calmodulin. Calmodulin was purified from bovine brain by modifications of the method of Kanamori *et al.* [13]. Brain was homogenized with ice-cold 50 mM MES buffer, pH 6.7, containing 0.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 0.2 mM ethylene glycol bis (β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA). The supernatant fraction obtained by centrifugation for 30 min at 78,000 *g* was heated for 5 min at 100°. The solution was centrifuged for 30 min and the clear supernatant fluid was brought to 50% saturation of $(NH_4)_2SO_4$ at 4°. The pellet obtained after centrifugation for 20 min at 16,000 *g* was discarded and the supernatant fraction was brought to 100% saturation of $(NH_4)_2SO_4$ at pH 6.0. The pellet obtained after centrifugation at 16,000 *g* for 20 min was dissolved in the buffer and dialyzed against the same buffer overnight. The solution was applied to a DEAE-Sephadex column pre-equilibrated with the buffer. The column was washed with 0.22 M NaCl in the buffer, and the calmodulin was eluted with a linear gradient of 0.22 to 0.8 M NaCl. The fraction showing the peak activity of calmodulin was collected. This protein either showed as a single band following electrophoresis in 12.5% acrylamide gel containing 0.1% sodium dodecylsulfate (SDS) or was further purified by Sephadex G-100 column chromatography. Protein concentration was measured by the method of Bradford [14] using bovine serum albumin as a standard. SDS-gel electrophoresis was performed by the method of Sandoval and Cuatrecasas [15].

PDE assay. PDE activity was measured by the method by described previously [11]. The incubation mixture contained 1 μM [U-^{3}H]cyclic AMP (sp. act. 27 Ci/mmole), 2 mM $MgCl_2$, 0.02% albumin and 50 mM Tris–HCl buffer, pH 7.4, in a final volume of 250 μl. Calmodulin and drugs were incubated at 30° for 5 min in the presence of 0.1 mM calcium. Five minutes after the addition of enzyme, the reaction was initiated by the addition of cyclic AMP. The reaction was stopped by immersing the tube in boiling water for 2 min.

MATERIALS

The vinca alkaloids, VB, DVB, VC, CR and VD, were gifts from Eli Lilly & Co., Indianapolis, IN (Drs. R. J. Hosley and K. Gorezon). AG 1 × 2 resin was purchased from Bio-Rad Laboratories, Richmond, CA. Cyclic AMP, Sephadex G-100, DEAE-Sephadex (A-50), Trizma (Tris–HCl buffer, pH 7.4), albumin, colchicine, and EGTA were purchased from the Sigma Chemical Co., St. Louis, MO [U-^{3}H]Cyclic AMP was obtained from the Amersham/Searle Co., Arlington Heights, IL.

RESULTS

Vinca alkaloids and the calcium-activated crude brain supernatant PDE activity. The crude supernatant PDE activity of rat brain was activated 330 per cent by the addition of 0.125 mM Ca^{2+} in the presence of 0.1 mM EGTA above the activity measured in the presence of EGTA alone. The concentrations of alkaloids required for 50 per cent inhibition of the Ca^{2+}-activated PDE activity were: VB, 7×10^{-5} M; DVB, 1.6×10^{-4} M; CR, 2.1×10^{-4} M; VC, 3.6×10^{-4} M; and VD, 4.8×10^{-4} M (Fig. 2).

Vinca alkaloids and the basal, unstimulated crude brain supernatant PDE activity. Inhibitory potencies of various vinca alkaloids toward the basal, unstimulated crude brain supernatant PDE activity were measured in the presence of 0.1 mM EGTA. Of the

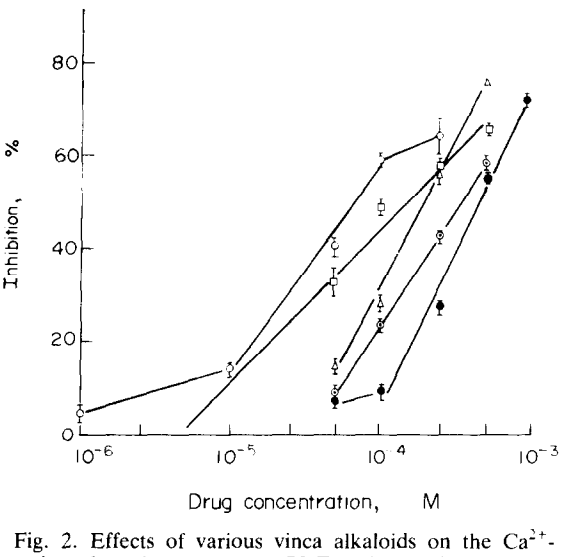

Fig. 2. Effects of various vinca alkaloids on the Ca^{2+}-activated crude supernatant PDE activity. The rat brain crude supernatant fraction obtained by centrifugation for 30 min at 78,000 *g* was used as the source of the PDE activity. The supernatant PDE was activated by the addition of 0.125 mM Ca^{2+} in the presence of 0.1 mM EGTA; 100% was 770 pmoles·(mg protein)$^{-1}$·min^{-1}. Key: vinblastine (○), desacetylvinblastine amide (□), catharanthine (△), vincristine (⊙), and vindoline ●. The value of each point is the mean ± S.E.M. of eight to eighteen determinations.

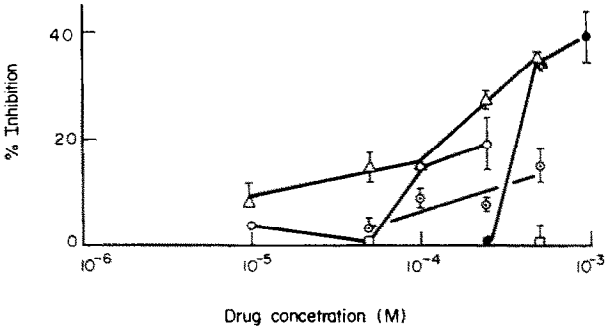

Fig. 3. Effects of various vinca alkaloids on the basal, unstimulated crude supernatant PDE activity. All experimental conditions are the same as in Fig. 2, except that the basal, unstimulated PDE activity was measured in the presence of 0.1 mM EGTA; 100% was 230 pmoles·(mg protein)$^{-1}$·min^{-1}. Key: Vinblastine (○), desacetylvinblastine amide (□), vincristine (⊙), catharanthine (△), and vindoline (●). The value of each point is the mean ± S.E.M. of three to seven determinations.

compounds tested, none was able to reduce PDE activity by 50 per cent in the doses used (Fig. 3). When the per cent inhibition of basal PDE activity obtained in the presence of EGTA alone was compared to the doses causing 50 per cent inhibition of the Ca^{2+}-activated PDE activity and expressed as ratios, the values of VB, DVB, and VC were 3.79 or more; on the other hand, those of CR and VD were 2.04 and 1.79 respectively.

Vinca alkaloids and Ca^{2+}-calmodulin activated partially purified PDE activity. The half-maximum activation of bovine brain Ca^{2+}–calmodulin dependent PDE activity was obtained by addition of 0.01 to 0.02 μg calmodulin in the presence of 0.1 mM Ca^{2+}. The maximum activation (about 5-fold), obtained by addition of 0.21 μg calmodulin, was more than any activation caused by Ca^{2+} alone. The degree of inhibiting of Ca^{2+}–calmodulin regulated PDE activity by various vinca alkaloids was determined in the presence of 0.1 μg calmodulin and 0.1 mM Ca^{2+}. As seen in Fig. 4, the doses required for 50 per cent inhibition were: VB, 1.6×10^{-5} M; DVB, 4.6×10^{-5} M; VC, 1.14×10^{-4} M; CR, 1.14×10^{-4} M; and VD, 3.1×10^{-4} M. In the absence of Ca^{2+}, dimeric vinca alkaloids (VB and VC) had either no effect or slightly stimulated the partially purified PDE activity, whereas the effects of CR and VD were still inhibitory (data not shown).

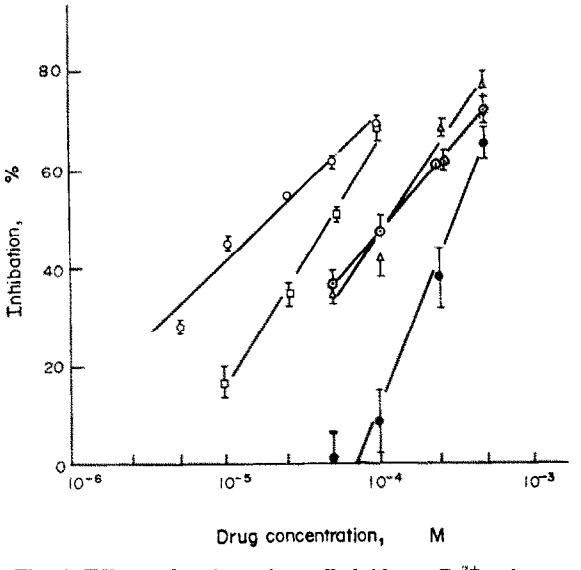

Fig. 4. Effects of various vinca alkaloids on Ca^{2+}–calmodulin activated partially purified bovine brain PDE activity. The calmodulin-free PDE activity was partially purified from bovine brain. Calmodulin was also purified from bovine brain as shown in Methods. The PDE activity was stimulated by the addition of 0.1 μg calmodulin and 0.1 mM Ca^{2+}; 100% was 590 pmoles·(mg protein)$^{-1}$·min^{-1}. Key: vinblastine (○), desacetylvinblastine amide (□), vincristine (⊙), catharanthine (△), and vindoline (●). The value of each point is the mean ±S.E.M. of six to fifteen determinations.

Effects of various amounts of calmodulin on the inhibitory effects of vinca alkaloids. As was observed earlier in this laboratory, the inhibitory effect of VB on the Ca^{2+}–calmodulin regulated PDE activity was abolished with large amounts of calmodulin [11]. To determine the mechanisms of inhibition with other vinca alkaloids, the effects of various concentrations of calmodulin in the presence of 0.1 mM Ca^{2+} were measured and expressed by Hofstee plots [16]. As shown in Fig. 5, the inhibitory effects of VB, DVB, VC, and CR, but not VD, were found to be competitive with Ca^{2+}–calmodulin.

Types of inhibition by VB and VC with substrate. Both VB and VC inhibited the Ca^{2+}–calmodulin activated partially purified PDE activity non-competitively with substrate (Fig. 6) as is indicated by a change in maximum velocity and no change in K_m.

Effect of colchicine. Colchicine was examined for its effect on partially purified PDE activity. The maximumly activated PDE activity in the presence of Ca^{2+}–calmodulin was inhibited by colchicine slightly more than was unstimulated (EGTA) or submaximumly activated PDE activity (calmodulin concentration, 0.038 to 0.19 μg). The inhibition by colchicine was not reversed by large amounts of calmodulin (Fig. 7) or Ca^{2+} (data not shown).

DISCUSSION

These data demonstrate that all of the vinca alkaloids examined inhibited the Ca^{2+}–calmodulin regu-

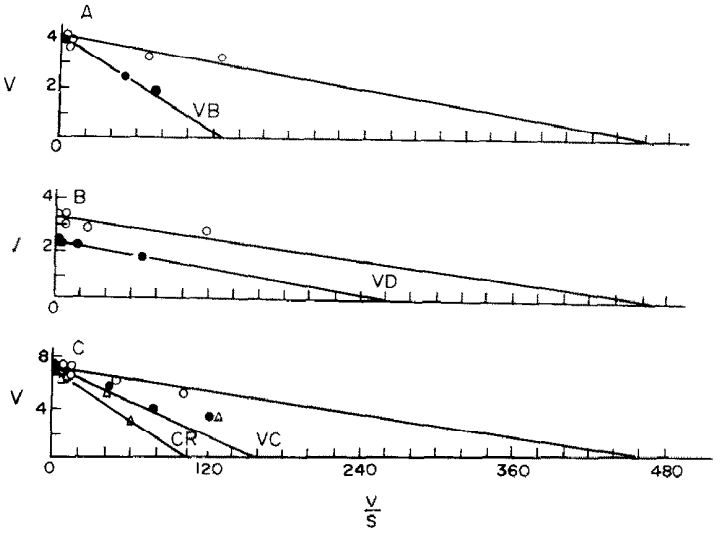

Fig. 5. Hofstee plots of the effect of increasing amounts of calmodulin on the inhibitory actions of various vinca alkaloids on the Ca^{2+}–calmodulin activated PDE activity. A partially purified bovine brain PDE preparation and purified calmodulin were used. The effects of various vinca alkaloids were examined at a fixed concentration of Ca^{2+} (0.1 mM) and with various amounts of calmodulin. Initial velocity, v, is plotted versus the ratio v/S (Hofstee plots [16]), where S represents the various concentrations of calmodulin. The concentrations of alkaloids used in this study were: vinblastine, 1.25×10^{-5}M (A); vindoline, 2.5×10^{-4}M (B); vincristine, 1.25×10^{-4}M and catharanthine, 1.25×10^{-4}M (C). The plot for desacetylvinblastine amide (5×10^{-5}M) was similar to that of catharanthine (C) (data not shown).

lated PDE activity, although their potencies appear to differ. The order of inhibitory potency toward the Ca^{2+}-activated crude brain supernatant PDE activity was VB > DVB > CR > VC > VD. However, CR and VD, monomeric vinca alkaloids, were stronger inhibitors of the basal unstimulated enzyme activity than the other compounds. Although the dose required for 50 per cent inhibition of Ca^{2+}-activated crude brain supernatant PDE by VC was larger than that of CR, VC, as well as the other dimeric vinca alkaloids, appears to have been a more selective inhibitor of the Ca^{2+}-activated PDE activity. The use of partially purified PDE and purified calmodulin resulted in an increase in the effects of the dimeric alkaloids, with the potency of VB > DVB > VC = CR > VD. The effect of CR (a monomeric alkaloid) also was abolished with increasing

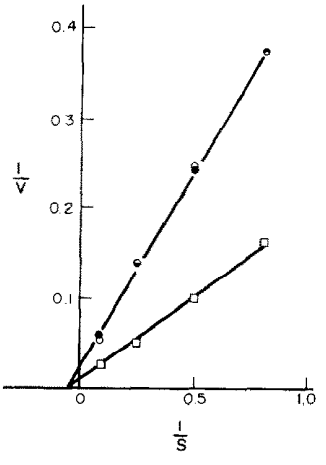

Fig. 6. Inhibition by vinblastine and vincristine of partially purified bovine brain PDE activity activated by Ca^{2+} and calmodulin. The PDE activity was measured as described in Fig. 4 with various concentrations of substrate (cyclic AMP). The concentration of vinblastine (●) was 1.25×10^{-5} M and that of vincristine (○) was 1.25×10^{-4} M; (□) is without inhibitor. Data were plotted reciprocally.

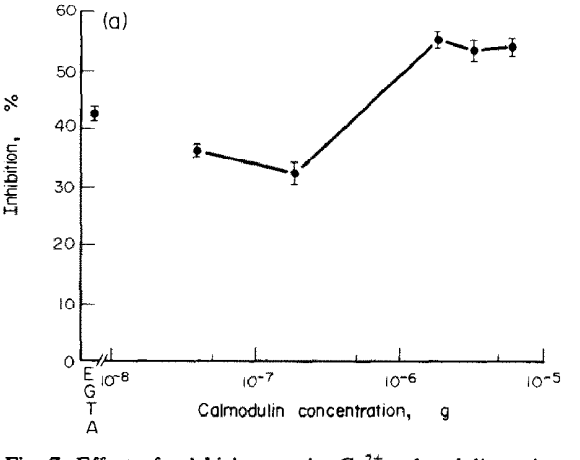

Fig. 7. Effect of colchicine on the Ca^{2+}–calmodulin activated and basal PDE activity. The effects of various concentrations of calmodulin at a fixed concentration of Ca^{2+} (0.1 mM) on the inhibitory effect of colchicine (0.4 mM) were determined. The value of each point is the mean ± S.E.M. of three to twenty-seven determinations. The single point at the left represents the effects of colchicine on basal PDE activity in EGTA without calmodulin; 100% equaled 73 pmol·(mg protein)$^{-1}$·min^{-1}.

amounts of calmodulin in the presence of Ca^{2+}, whereas the effect of VD was not. Overall, these results suggest that the Ca^{2+}–calmodulin dependent inhibitory effect of vinca alkaloids on PDE activity is competitive with Ca^{2+}–calmodulin, but not with substrate, and that the cause of the effect resides in the CR moiety. The dimeric indole structure is necessary for even more potent inhibition of the PDE activity. The results with colchicine suggest that the effect of Ca^{2+}–calmodulin is less specific for colchicine than for dimeric vinca alkaloids, which is consistent with the previous finding that the binding sites on tubulin for colchicine are different from those of the vinca alkaloids [17].

Among the various compounds showing inhibition of PDE activity [18], the dimeric vinca alkaloid [11] and phenothiazines [19] are known as selective inhibitors of Ca^{2+}–calmodulin activated PDE activity. These agents are also known to inhibit microtubule polymerization or to cause specific aggregation of microtubules [1–6, 20, 21]. The effects of these agents on PDE and microtubules may not be related to each other. However, we have recently shown that Ca^{2+}–calmodulin activated PDE activity was associated with microtubules prepared by various methods of isolation including VB- and VC-induced precipitates of brain supernatant proteins [9, 10]. Runge *et al.* [22] confirmed our observation, and both have shown that a higher specific activity of PDE is located in microtubule associated protein fraction [22–24]. Microtubule associated proteins are necessary for the induction of a stable spiral structure by VC [25]. Since higher specific activity of PDE was found in the microtubule associated protein fraction [22–24], and the VC-induced precipitate contains PDE activity [10] as well, the interaction between VC and Ca^{2+}–calmodulin activated PDE activity shown in the present study suggests that one of the microtubule associated proteins that is responsible for the induction of a spiral structure of microtubule by VC may be PDE or a PDE–Ca^{2+}–calmodulin complex. In addition, a possible inhibitory role of calmodulin in microtubule polymerization has been reported, and calmodulin-binding proteins in microtubule preparation have been found [22–24, 26].

The concentration of VB (1.6×10^{-5} M) required for 50 per cent inhibition of Ca^{2+}–calmodulin activated PDE activity from bovine brain is the same as that of VB for 50 per cent binding of bovine brain tubulin [27]. This dose of VB is the same as the dose of VB causing 50 per cent binding to low affinity VB binding sites of rat brain tubulin [28]. Wilson *et al.* [3] have suggested that the biological activity of vinca alkaloids resides in the CR portion of the molecule. The same CR moiety appears to be essential for the inhibition of calmodulin regulated PDE activity. VD, inactive as an antimitotic [3], was also found to be the weakest compound for the suppression of the PDE activity. On the other hand, VC and DVB at 10^{-6} to 10^{-8} M were as potent as VB in producing these previously reported effects [3–6]. The question arises whether those effects of vinca alkaloids on the Ca^{2+}–calmodulin regulated system are related to previously reported actions on microtubules [29]. Since it is not known whether the presence of both Ca^{2+} and calmodulin affects the binding constants or the efficacy of the drugs for the inhibition of mitosis or microtubule polymerization, further studies are indicated.

The different vinca alkaloids, VB and VC, vary widely in their effectiveness against specific tumors. They also differ in adverse effects [1, 7]. DVB has mixed types of adverse effects similar to those of both VB and VC [7, 30]. Himes *et al.* [4] suggest that the differential biological specificities of the drugs may be due to factors other than direct interaction with tubulin. Our results with PDE activity, showing great differences among the vinca alkaloids in their potencies, suggest that Ca^{2+}–calmodulin or Ca^{2+}–calmodulin regulated PDE may be one of the factors which Himes *et al.* [4] suggested. Increased levels of calmodulin have been reported in certain Morris hepatomas and RNA virus transformed cells, but not in DNA virus transformed cells [31–33]. Experiments are in progress to determine whether the levels of calmodulin in various tumors may be used as a determinant for the selection of vinca alkaloid therapy.

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