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The interaction of a new anti-tumour drug, KAR-2 with calmodulin

F. Orosz, B.G. Vértessy, *C. Salerno, *C. Crifo, *E. Capuozzo & [1]J. Ovádi

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, H-1518, P.O.B. 7, Hungary and *Department of Biochemical Sciences and Clinical Biochemistry Laboratory, University of Rome La Sapienza, and C.N.R. Center of Molecular Biology, 00185 Rome, Italy

1 KAR-2 (3″-(β-chloroethyl)-2″,4″-dioxo-3,5″-spiro-oxazolidino-4-deacetoxy-vinblastine) is a semisynthetic bis-indol derivative, with high anti-microtubular and anti-tumour activities but with low toxicity. KAR-2, in contrast to other biologically active bis-indols (e.g. vinblastine), did not show anti-calmodulin activity *in vitro* (enzyme kinetic, fluorescence anisotropy and immunological tests).

2 Direct binding studies (fluorescence resonance energy transfer, circular dichroism) provided evidence for the binding of KAR-2 to calmodulin. The binding affinity of KAR-2 to calmodulin (dissociation constant was about 5 μM) in the presence of Ca^{2+} was comparable to that of vinblastine.

3 KAR-2 was able to interact with apo-calmodulin as well; in the absence of Ca^{2+} the binding was of cooperative nature.

4 The effect of drugs on Ca^{2+} homeostasis in human neutrophil cells was investigated by means of a specific fluorescent probe. Trifluoperazine extensively inhibited the elevation of intracellular Ca^{2+} level, vinblastine did not appreciably affect it, KAR-2 stimulated the Ca^{2+} influx and after a transient enhancement the Ca^{2+} concentration reached a new steady-state level.

5 Comparison of the data obtained with KAR-2 and bis-indols used in chemotherapy suggests that the lack of anti-calmodulin potency resides on the spiro-oxazolidino portion of KAR-2. This character of KAR-2 manifested itself in various systems and might result in its low *in vivo* toxicity, established in an anti-tumour test.

Keywords: Vinca alkaloids; bis-indols; vinblastine; vincristine; calmodulin antagonism

Introduction

Numerous antimitotic drugs have been developed and therapeutically used as anti-cancer agents. They target the tubulin/microtubule (MT) network of the cytoskeleton fundamentally destroying its dynamic character and leading to cell death. The dynamic organization of MTs is essential in many cellular events including the formation of mitotic spindle required for chromosomal segregation (Bershadsky & Vasiliev, 1988). The assembly and function of MTs are regulated by MT associated proteins (MAPs) and a variety of other proteins including calmodulin (CaM). The rearrangement of MTs is due, in part, to changes in the centrosome, the principal MT organizing centre of the cell (McIntosh-Koonce, 1989) where CaM is also localized (De Brabander *et al*., 1980). Cellular CaM content can change during cell proliferation. Thus it has been suggested that CaM plays an important role in the initiation of DNA synthesis and in mitosis (Hidaka *et al*., 1981; Rasmussen & Means, 1989). Since Ca^{2+} is also implicated in the regulation or modulation of cell division and initiation of DNA synthesis, CaM is an extraordinarily versatile Ca^{2+}-binding protein, thus the Ca^{2+}-CaM complex could have a central role in many cellular events. It is also present in the soluble fraction and influences cell metabolism by activating/inhibiting regulatory enzymes (Migliaccio *et al*., 1984; Ovádi, 1989). Indeed, CaM can be found in most eukaryotic cells, its concentration varies in different tissues and the highest level has been found in the brain (500 mg kg^{-1} tissue) (Nairn & Perry, 1979).

In general, CaM antagonists compete directly with target enzymes for CaM binding since both the hydrophobic drugs and the enzymes interact with the hydrophobic binding pockets of CaM exposed by Ca^{2+}-binding. Pharmacological agents which selectively antagonize CaM function block cell-cycle progression both at the G_1/S boundary and during G_2/M and may cause cell cycle withdrawal and differentiation (Rasmussen & Means, 1989).

[1]Author for correspondence.

The bis-indol alkaloids (e.g. vinblastine, vincristine and navelbine (VBL, VCR and NAV, respectively), exhibit significant anti-microtubular activity and suspend the modulating effect of CaM (Gietzen *et al*., 1982; Watanabe & West, 1982; Molnár *et al*., 1995). The efficiencies of these drugs determined *in vitro* in anti-microtubular and anti-CaM tests were found to be comparable. KAR-2 (3″-(β-chloroethyl)-2″,4″-dioxo-3,5″-spiro-oxazolidino-4-deacetoxy-vinblastine) (see Figure 1), a new bis-indol derivative has been identified as a potent anti-mitotic agent (Orosz *et al*., 1997).

In the present work the CaM binding and anti-CaM activity of KAR-2 has been characterized. The effects of this drug detected in widely different systems were compared to that of other biologically active indol alkaloids as well as with that of trifluoperazine (TFP), a classical anti-CaM drug. The present data demonstrated that KAR-2 is of unique character concerning its interaction with CaM: it binds to the protein without significant anti-CaM activity.

Methods

Proteins

Phosphofructokinase (PFK) (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), aldolase and glycerol-3-phosphate dehydrogenase - triosephosphate isomerase purified from rabbit skeletal muscle were purchased in $(NH_4)_2SO_4$ suspensions from Sigma or Boehringer. The enzyme suspensions were centrifuged at 10,000 × *g* for 5 min. The pellets were suspended in standard buffer (50 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulphonic acid (HEPES), pH 7.0, containing 100 mM KCl, 5 mM $MgCl_2$ and 2 mM dithioerythritol), and gelfiltered or dialysed in the same buffer to remove $(NH_4)_2SO_4$.

Protein concentrations were determined spectrophotometrically, by use of molar absorption coefficients of

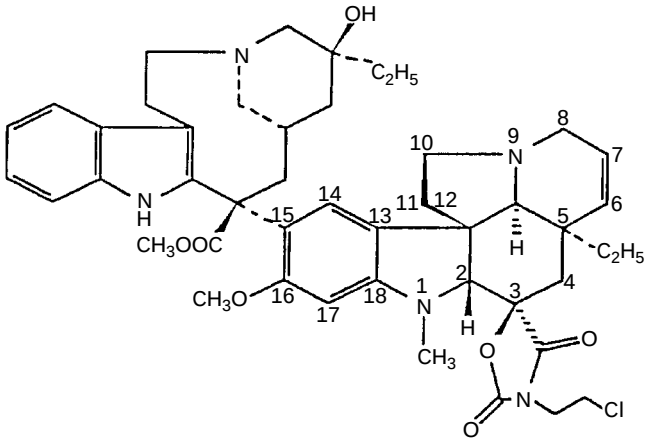

Figure 1 Structural formula of KAR-2

3.24×10^3 M^{-1} cm^{-1} at 276 nm for CaM (Watterson *et al.*, 1976), 8.88×10^4 M^{-1} cm^{-1} at 276 nm for PFK (Hesterberg & Lee, 1982) and 2.0×10^5 M^{-1} cm^{-1} at 280 nm for rabbit immunoglobulin G (Stevenson & Dorrington, 1970). Spectroscopic measurements were made on HP 8451A or JASCO V-550 spectrophotometers. Protein purity was determined by discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, as described by Laemmli (1970).

Purification of CaM and anti-CaM immunoglobulin G

CaM from bovine brain was purified to homogeneity by Phenyl-Sepharose (Pharmacia) chromatography (Gopalakrishna & Anderson, 1982). Its activity was checked by the phosphodiesterase test (Solti *et al.*, 1983), or PFK test (Orosz *et al.*, 1988a). Rabbits were immunized with dinitrobenzyl-derivatized CaM as described previously (Wallace & Cheung, 1979). For the isolation of the specific antibodies, a CaM affinity column was used (Liliom *et al.*, 1991). All measurements with CaM, unless indicated otherwise, were carried out in the presence of 3 mM $CaCl_2$.

Enzyme linked immunosorbent assay (ELISA) experiments

Standard ELISA protocols (Engwall, 1980) for indirect assays were used with some modifications. Ninety six-well microtiter plates with medium binding capacity, U shape (Greiner) were coated with CaM 2.5 μg ml^{-1} for 18 h at 4°C. Usually the experiments were performed in 100 μl volume in the wells. For the displacement experiments, the drug was added at different concentrations, and the antibody was used in constant subsaturating concentration (7.5 μg ml^{-1}). Incubation time was 2 h to ensure the equilibrium states of the binding processes. Incubation time was 2 h for the anti-rabbit immunoglobulin G peroxidase conjugate in 2000 fold dilution. Orto-phenylene diamine substrate was used at 3.7 mM and the absorbances were read on an Anthos II microplate reader at 492 nm after 30 min hydrolysis time, the reaction was stopped by addition of 1.5 M H_2SO_4. We used at least four parallel wells in a plate for each concentration and each experiment was repeated at least three times.

Circular dichroism measurements and calculation of difference spectra

Circular dichroism spectra were recorded with a JASCO J-720 spectropolarimeter. Measurements were performed at 25°C in thermostated cuvettes, in 10 mM piperazine-N,N′-*bis*(2-ethanesulphonic acid) pH 6.8 buffer, containing 150 mM KCl and 5 mM $CaCl_2$. For determination of the difference spectra of CaM-drug complexes the drugs were added to the protein and the circular dichroism spectra were recorded in the wavelength range 220–400 nm. Scanning was repeated twice and the spectra were averaged and corrected for the baseline of the instrument as described previously (Molnár *et al.*, 1995). Spectra were measured immediately after 30 μM CaM was mixed with various concentrations of the drugs and they were stable for at least 20 min under our experimental conditions. To look for complexation-induced circular dichroism signals, difference spectra were calculated by using the built-in JASCO software: the sum of the spectra of the protein and KAR-2 or VBL measured separately were subtracted from the spectra of the protein-drug mixture.

Fluorescence measurements

Fluorescence studies were conducted on a JASCO Model FP-777 spectrofluorometer, by use of excitation and emission slits of 3 nm. Anisotropy of dansyl-CaM was measured in standard buffer at an excitation wavelength of 370 nm and an emission wavelength of 500 nm, at 30°C. For each measurement, at least ten determinations of the vertically and horizontally polarized components of the fluorescent emission were made with a standard deviation of less than 5%. The anisotropy was calculated as previously described (Ovádi *et al.*, 1982). The binding of PFK and drugs to CaM was investigated in the presence of 1 mM $CaCl_2$.

Fluorescence excitation and emission spectra of KAR-2 and dansyl-CaM were measured in a buffer containing 10 mM 3-(N-morpholino) propanesulphonic acid, pH 7.0, 90 mM KCl, 2 mM ethylene glycol bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), in the absence and presence of 3 mM $CaCl_2$. The excitation wavelength were 295 nm and 340 nm for KAR-2 and dansyl-CaM, respectively. Fluorescence energy transfer between KAR-2 (donor) and dansyl-CaM (acceptor) was measured under the same conditions, with excitation at 295 nm.

PFK activity measurements

Drugs at 20 μM were preincubated with 3 μM CaM in the presence of 100 μM $CaCl_2$ for 60 min at 25°C in standard buffer. PFK (2 μM) was added to the CaM-drug mixture and incubated for an additional 20 min. The enzyme activity was assayed in 50 mM Tris, pH 8.0 containing 2 mM fructose-6-phosphate, 1 mM ATP, 3 mM $MgCl_2$, 0.2 mM NADH, 3 mM dithioerythritol, 0.1 mM EGTA, 2 u aldolase, 12 u triosephosphate isomerase, and 2 u glycerol-3-phosphate dehydrogenase at 30°C (Mayr, 1987; Lehotzky *et al.*, 1993). The specific activity of PFK was 185 u mg^{-1} measured at 0.8 μg ml^{-1} final concentration after dilution from 1 mg ml^{-1} stock solution into activity assay.

Neutrophil cultures and treatments

Human polymorphonuclear cells were purified by a one-step procedure (Ferrante & Thong, 1980) involving centrifugation of heparine-treated blood layered on Mono-Poly Resolving Medium (ICN Pharmaceuticals, Costa Mesa, CA). The cells were suspended in isotonic phosphate buffered saline (PBS (mM): NaCl 140, KCl 2.7, KH_2PO_4 1.5, Na_2HPO_4 12, $MgCl_2$ 0.49 and glucose 5; pH 7.4) and stored on ice. Each preparation produced erythrocyte-free populations containing 90–98% neutrophils that were more than 90% viable by trypan blue exclusion up to 6 h after purification.

Determination of intracellular Ca^{2+} level

Intracellular calcium was monitored by a Shimadzu spectrofluorophotometer in a thermostated (37°C) cell holder under continuous stirring by a magnetic flea by loading neutrophil cells with 5 μM of the cytosolic calcium probe, Indo-1 acetoxymethyl ester (Molecular Probes, Eugene, OR) as described

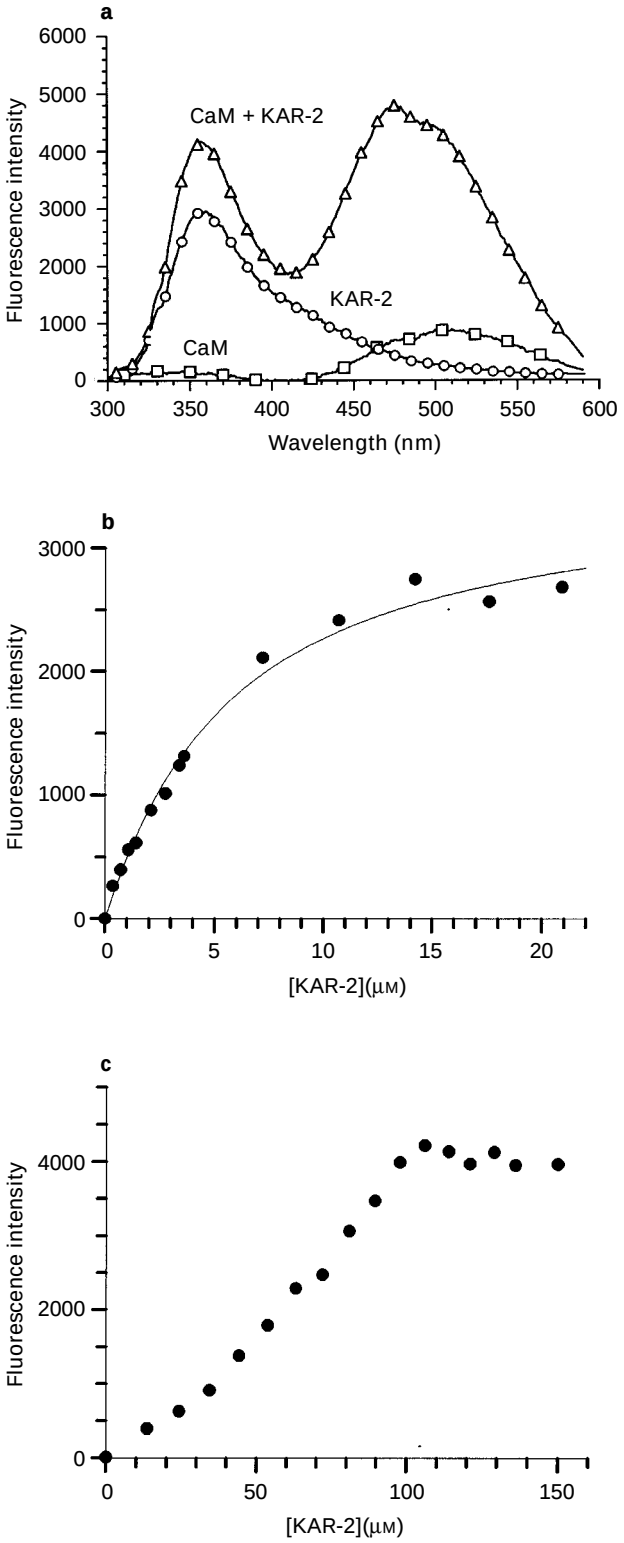

Figure 2 Fluorescence energy transfer between KAR-2 and dansyl-calmodulin (CaM). Fluorescence intensities were measured as described in Methods. Excitation was at 295 nm. (a) Fluorescence energy transfer in the presence of Ca^{2+}. Lines show emission spectra of 1.1 μM dansyl-CaM (□), 20 μM KAR-2 (○) and dansyl-CaM + KAR-2 (△). (b) Saturation of dansyl-CaM with KAR-2 in the presence of Ca^{2+}. Dansyl-CaM (1.1 μM) was titrated with KAR-2 in the presence of 3 mM $CaCl_2$. The fluorescence increase (ΔI) at 470 nm is shown as a function of added drug. Solid line shows theoretical curve calculated from the following equation:

$$\Delta I = \frac{\Delta I_{max} \times \{(K_d + [KAR-2]_T + [dCaM]) - \sqrt{[(K_d + [KAR-2]_T + [dCaM])^2 - 4[KAR-2]_T \times [dCaM]]}\}}{2[dCaM]}$$

where ΔI and ΔI_{max} are differences in the fluorescence intensities at 470 nm measured at a given and excess drug concentrations, respectively. $[KAR-2]_T$ and [dCaM] are the total concentrations of KAR-2 and dansyl-CaM, respectively. K_d is the dissociation constant of CaM-KAR-2 complex. The best fit was obtained with parameters $\Delta I_{max} = 3530 \pm 147$ and $K_d = 5.2 \pm 0.6$ μM. $\chi^2 = 10\ 510$. (c) Titration curve of dansyl-CaM with KAR-2 detected in the absence of Ca^{2+}. The fluorescence increase (ΔI) at 470 nm excited at 295 nm is shown as a function of added drug.

by Grynkiewicz *et al.* (1985). After 20 min incubation, neutrophils were washed twice and diluted to a final concentration of about 10^6 cells ml^{-1}. Excitation and emission wavelengths were 355 and 405 nm, respectively (5 nm bandwidths). The fluorescence signal was corrected for the contribution of the scattered light and the fluorescence of the drugs (if present). Cytosolic free calcium concentration was calculated from the relationship: $[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$; F was the measured fluorescence signal; F_{max} and F_{min} were obtained upon addition of 1.65 μM 4-bromo-A23187 (Molecular Probes, Eugene, OR) in the presence of 1 mM $CaCl_2$ or 10 mM EGTA, respectively; 75 μM digitonin was added to the EGTA-containing medium to facilitate the release of cytosolic calcium (Tsien *et al.*, 1982). K_d was taken to equal 250 nM (Grynkiewicz *et al.*, 1985). Control experiments showed that the drugs (2–50 μM) did not affect the fluorescence of the calcium probe (5 μM) in cell-free media containing 0–0.5 mM $CaCl_2$ and that no signal was obtained by adding the drugs to 10^6 cells ml^{-1} in the absence of Indo-1.

Chemicals

Indol-alkaloids - VBL, VCR, NAV, vindoline and catharantine as well as KAR-2 - were kindly provided by Chemical Works of Gedeon Richter Ltd., Budapest, Hungary. ATP, NADH and fructose-6-phosphate were purchased from Boehringer. Dansyl-CaM, TFP, digitonin and peroxidase conjugated anti-rabbit immunoglobulin G were purchased from Sigma. All other chemicals were reagent-grade commercial preparations.

Results

Binding of KAR-2 to CaM and the Ca^{2+}-sensitivity of the binding

Fluorescence studies The fluorescence energy transfer measurement, a direct and sensitive tool, was used to monitor and quantify the binding of KAR-2 to CaM. We used it previously to characterize the interaction of CaM with some vinca alkaloids (Molnár *et al.*, 1995). In the present work KAR-2 and dansyl-CaM were used as energy donor and acceptor, respectively. Since dansyl-CaM is excitable at 360 nm, where KAR-2 emits fluorescence, the KAR-2 – dansyl pair is expected to be suitable for fluorescence energy transfer studies. The drug was excited at 295 nm where, on the one hand, the direct excitation of dansyl-group is minimized, and on the other hand, there is practically no fluorescent emission of CaM since it does not have tryptophan (cf Figure 2a), which avoids an intramolecular energy transfer. However, significant fluorescence emission with a maximum at 470 nm was observed in a mixture of dansyl-CaM and KAR-2 (cf Figure 2a). This fluorescence energy transfer is indicative of the formation of the CaM – KAR-2 complex.

Quantitative characterization of the CaM – KAR-2 complex was obtained from the energy transfer measurements by titration of the dansyl-CaM with the drug. Figure 2b shows the saturation curve based on the increase of fluorescence intensity measured at 470 nm by addition of the drug. The dissociation constant calculated from this curve, assuming hyperbolic saturation of CaM with KAR-2 and a single binding site, was 5.2 ± 0.6 μM.

Binding of antagonists to CaM is highly dependent on Ca^{2+}-concentration; the classical antagonists and enzymes regulated by CaM do not interact with Ca^{2+}-free CaM. Recently, we found that the interaction of the vinca alkaloids, VBL, VCR and NAV, with CaM also requires Ca^{2+} (Molnár *et al*., 1995). In this study we examined the effect of Ca^{2+} on the formation of CaM - KAR-2 complex by energy transfer measurements. The addition of KAR-2 to dansyl-CaM caused a tremendous increase in the fluorescence intensity of dansyl-CaM with a maximum at 470 nm; this indicated the binding of KAR-2 to apo-CaM. The characteristics of the spectrum were similar to that measured in the presence of Ca^{2+}. The titration curve presented in Figure 2c shows that the binding of KAR-2 to the labelled apo-CaM is more complex than that measured in the presence of Ca^{2+}, and CaM is saturated with KAR-2 at a higher drug concentration. Nevertheless, the finding that CaM can be complexed with KAR-2 even in the absence of Ca^{2+} is novel and important.

Circular dichroism studies

The circular dichroism spectra of KAR-2 and VBL with and without CaM in the near u.v. range are shown in Figure 3a. With 30 μM VBL, a positive peak with maximum at 258 nm appeared, while KAR-2 at the same concentration negative signal in the 270–295 nm range was obtained. A broad positive peak in the 300–330 nm range was seen with both VBL and KAR-2. CaM 30 μM elicited negligible elipticity between 250 and 350 nm.

Figure 3b shows the difference spectra of circular dichroism of stoichiometric mixtures of CaM plus KAR-2 or CaM plus VBL in the near u.v. wavelength range. If complexation of CaM with drugs does not perturb the circular dichroism spectra then the difference spectrum has to be a flat zero line. However, we have found characteristic, concentration-dependent signals in the difference spectra: a relatively sharp negative peak with minimum at 300 nm with CaM plus KAR-2. In the case of the mixture of CaM and VBL, negative differential elipticity was observed at this wavelength. However, this signal had a much broader shape. Moreover, the addition of VBL to CaM induced the formation of a significant positive difference peak with a maximum at 260 nm. Saturation values of the difference signals were reached with both drugs at concentrations of approximately 60 μM (data not shown). These data indicate that the strength of the interactions between CaM and KAR-2 or CaM and VBL are comparable.

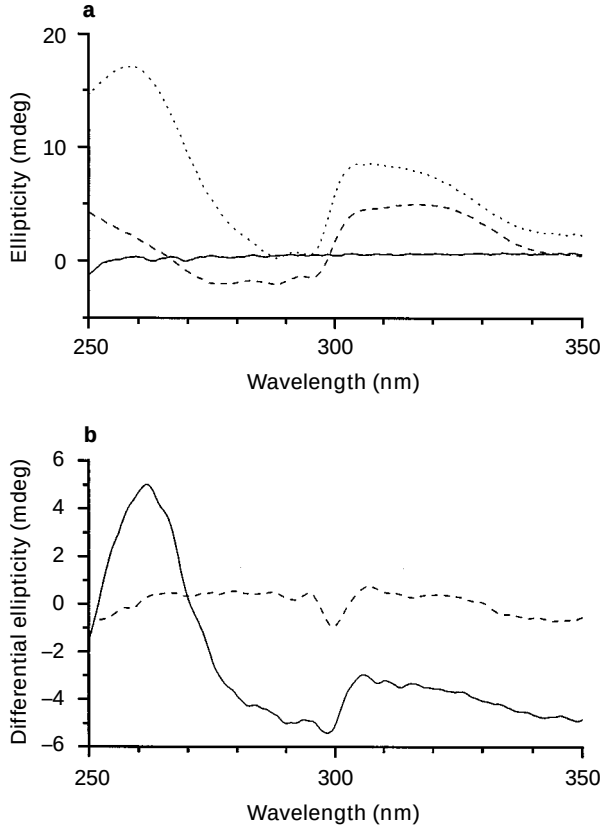

Figure 3 (a) Near u.v. circular dichroism spectra of calmodulin (CaM, solid line), KAR-2 (dashed line) and vinblastine (VBL, dotted line). (b) Near u.v. circular dichroism difference spectra of the CaM - KAR-2 (dashed line) or CaM - VBL (solid line) complexes. The concentrations of the drugs and CaM were equimolar (30 μM). Spectra were recorded and difference spectra were calculated as described in Methods.

Anti-CaM potency of KAR-2

Test for anti-CaM activity by ELISA

Previously, an indirect ELISA was developed to characterize the binding of drugs to CaM (Liliom *et al*., 1991; Orosz *et al*., 1992). Now we investigated how the binding of KAR-2 interferes with that of the monospecific anti-CaM antibody. Displacement ELISA experiments were carried out with KAR-2 and some reference dimeric (VBL, VCR and NAV) and monomeric (vindoline and catharantine) alkaloids. On the basis of the optimization experiments (Liliom *et al*., 1991) 2.5 μg CaM ml^{-1} and 7.5 μg antibody ml^{-1} were found to ensure the very sensitive detection of binding between antigen and antibody. Drugs at various concentrations were used in the indirect ELISA assay and the binding of antibody to coated CaM was detected. We have found that VBL but especially NAV inhibited extensively the immunocomplex formation. However, KAR-2 did not seem to perturb the binding of antibody to CaM up to concentrations of 100 μM (data not shown). The inhibitory effects of drugs (250 μM) including TFP on the antibody binding to CaM are summarized in Table 1. These data indicate a marked difference in the inhibitory potencies of the drugs although their binding affinities to CaM were comparable.

Fluorescence anisotropy studies As we demonstrated in previous papers, fluorescence anisotropy is a sensitive tool for investigation of the effect of drugs on the binding of enzymes to dansyl labelled CaM (Orosz *et al*., 1988a; 1990). The associations of PFK, phosphodiesterase and myosin light chain kinase to the dansyl labelled CaM were detected by the fluorescence anisotropy technique. The changes of anisotropy correlated to changes in molecular weights due to complex formation. In this study the CaM-PFK system was selected to probe the effect of KAR-2 on the binding of PFK to dansyl-

Table 1 Anti-calmodulin (CaM) effects of drugs

Compound	*ELISA Anti-CaM effect* (%)	*Fluorescence anisotropy Anti-CaM effect* (%)	*Enzyme activity Anti-CaM effect* (%)
KAR-2	18	3	7
VBL	34	39	61
VCR	43	NM	62
NAV	68	48	83
Vindoline	< 5	NM	0
Catharantine	< 5	NM	0
TFP	94	87	69
KHL-8430[a]	NM	0	100

Concentrations were in ELISA: CaM, 2.5 μg ml^{-1}; antibody, 7.5 μg ml^{-1}; drugs, 250 μM; in the anisotropy measurements: CaM, 1.5 μM; PFK, 2 μM; drugs, 20 μM; in the activity measurements: CaM, 3 μM; PFK, 2 μM; drugs, 20 μM. For experimental details see Methods. NM: means not measured. [a] Data taken from Orosz *et al*. (1990).

labelled CaM because the affinities of PFK and the drugs to CaM were comparable. As relatively low drug concentrations were sufficient to perturb the formation of the CaM-PFK complex, the nonspecific binding of drugs to the enzyme could be minimized. As shown in Table 1, the addition of 20 μM TFP, VBL or NAV reduced extensively the anisotropy value characteristic of the dansyl-CaM - PFK complex. However, KAR-2 was practically ineffective at the same concentration.

Enzymatic assay CaM can modulate the activity of numerous enzymes, which can be abolished by CaM antagonists. CaM can stimulate (e.g., phosphodiesterase) or inhibit (e.g., PFK) the activities of enzymes (Orosz *et al*., 1988b; Ovádi, 1989) and references therein). We found that the inhibitory potency of drugs to suppress the modulating effect of CaM manifested itself in both phosphodiesterase and PFK tests (Orosz *et al*., 1990).

The effect of KAR-2 on CaM-induced inactivation of PFK was measured and compared with that of other relevant drugs. As shown in Table 1, the inhibitory effect of 3 μM CaM was significantly attenuated by VBL, VCR, NAV and by TFP, but not by KAR-2. Control experiments indicated that the drugs themselves had no effect on PFK activity at concentrations up to 20 μM (data not shown). These results are in good agreement with those from the ELISA and fluorescence anisotropy experiments and demonstrate that the binding of the drugs to CaM does not necessarily result in functional effects.

Effect of KAR-2 on intracellular Ca^{2+} level

There are drugs that are potent CaM antagonists and influence a number of Ca^{2+}-dependent cellular processes including Ca^{2+} transport. The mechanisms for Ca^{2+} influx from external medium or from internal deposits may not be significantly different in different cell types. In human neutrophils the intracellular calcium level is influenced by transmembrane potential due to alterations in voltage-gate calcium channels (Majander & Wikström, 1989). The Ca^{2+} regulatory function of the plasma membrane depends on a CaM-dependent Ca^{2+}-ATPase and a Ca^{2+} channel (Lagast *et al*., 1984). It is known that TFP, a classic CaM antagonist, influences the cellular Ca^{2+} level by displacement of Ca^{2+} from membranes (Seeman, 1972), and blocking both passive (Landry *et al*., 1981) and active Ca^{2+} transport (Levin & Weiss, 1980; Raess & Vincenzi, 1980). Bis-indol alkaloids have been shown to be Ca^{2+}-uptake inhibitors (Yasin *et al*., 1973).

We studied the effect of some selected drugs on the calcium homeostasis in human neutrophils. The endogenous cytosolic Ca^{2+} concentration of the Indo-1 loaded neutrophils was 1.5–2 μM. The addition of 0.5 mM $CaCl_2$ increased transiently the intracellular calcium level, the maximum value was 0.4 μM as shown in Figure 4e. After 0.5–1 min the calcium concentration decreased again, so that only a small rise in the steady state value could be observed. The cytosolic calcium level was unaffected by the addition of 10 mM EGTA, suggesting stabilization of the intracellular concentration of the cation under our experimental conditions. By contrast, in the presence of a calcium ionophore, 4-bromo-A23187, the addition of 0.5 mM $CaCl_2$ caused dramatic (up to 1.2–1.4 μM) increase in the cytosolic calcium level (cf Figure 4a), which was followed by a subsequent decrease to a new steady-state value due to the stimulation of the Ca^{2+}-pump(s) in response to the increased Ca^{2+}-influx through the permeabilized membrane. The cytosolic calcium concentration fell to the initial value upon addition of 10 mM EGTA (Figure 4a).

We compared the effect of VBL, TFP and KAR-2 on the calcium homeostasis. Addition of the drugs (2–50 μM) did not cause changes in the endogenous cytosolic calcium level. However, at the addition of external Ca^{2+} both the amplitudes and the shapes of the calcium signal induced by 0.5 mM $CaCl_2$ varied depending on the nature of the drugs (Figure 4b, c and d). Figure 5 shows the maximal amplitude of the changes of Ca^{2+} level as a function of drug concentration. While VBL up

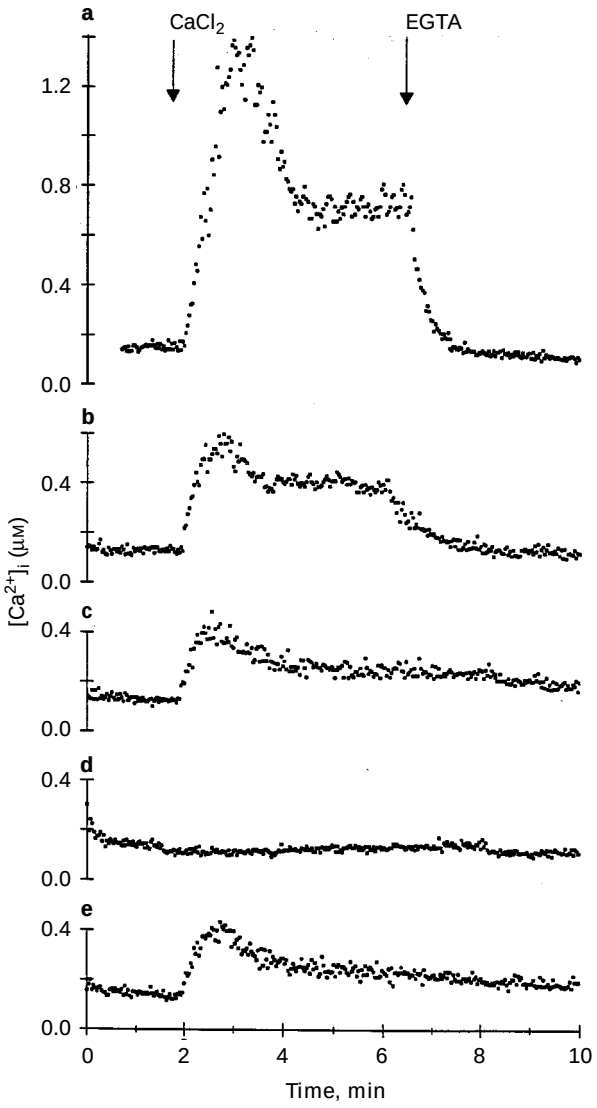

Figure 4 Time course of the changes in cytosolic free calcium concentration in human neutrophils triggered by adding 0.5 mM $CaCl_2$ and 10 mM EGTA. Neutrophils (10^6 cells ml^{-1}) were suspended in PBS buffer lacking in calcium and containing different drugs (a) 0.65 μM 4-bromo-A23187; (b) 25 μM KAR-2; (c) 25 μM vinblastine (VBL); (d) 25 μM trifluoperazine (TFP); (e) none. $CaCl_2$ and EGTA were added at the times indicated by the arrows.

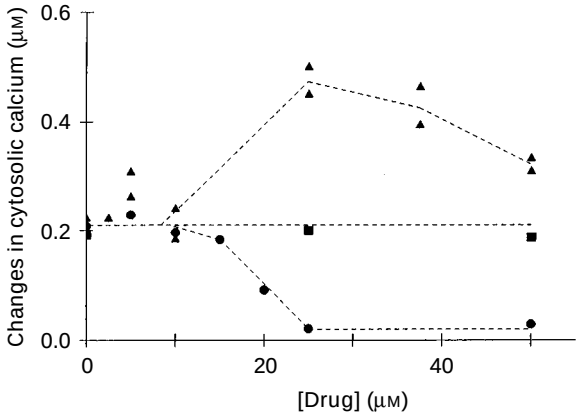

Figure 5 Amplitude of the transient increase in cytosolic Ca^{2+} in Indo-1 loaded neutrophils upon addition of 0.5 mM $CaCl_2$ as a function of the concentration of the drugs: vinblastine (VBL, ■), trifluoperazine (TFP, ●), KAR-2 (▲). Other conditions were as described in Figure 4.

to 100 μM did not appreciably affect Ca^{2+} incorporation, TFP extensively inhibited the elevation of the intracellular Ca^{2+} level, and in the presence of 20 μM TFP no Ca^{2+} accumulation could be detected within the cell. In contrast to these drugs, KAR-2 stimulated the Ca^{2+} efflux in a concentration-dependent manner; the maximal increase achieved was with approximately 20 μM KAR-2. This effect, as well as the time course of the changes in the intracellular Ca^{2+} concentration triggered by addition of $CaCl_2$ and EGTA, were similar to that observed on addition of 4-bromo-A23187. As shown in Figure 4a and b, in both cases after transient enhancement of the intracellular calcium concentration a new steady-state level was approached which fell to the initial level on addition of EGTA. These data show that KAR-2 does not behave like TFP or VBL, it affected the calcium homeostasis in neutrophils, this effect being significantly different from that produced by drugs with anti-CaM activity. This effect is probably related to Ca^{2+} entry in to the cells.

Discussion

Bis-indol derivatives, VBL, VCR and NAV, are among the most potent anti-mitotic agents. Recently, we identified a new bis-indol compound, KAR-2, a derivative of deacetoxy-VBL, which has similar or even higher anti-tubular and anti-tumour activity than other bis-indols, but with significantly lower toxicity (Orosz *et al*., 1997).

Vinca alkaloids display significant affinity towards CaM which is comparable to their affinities towards tubulin (Molnár *et al*., 1995). Direct binding studies (fluorescence and circular dichroism measurements) showed that KAR-2 does bind to Ca^{2+}-CaM and the value of the dissociation constant for CaM-KAR-2 complex (about 5 μM) was very close to that obtained for interactions of CaM with other active bis-indol derivatives (Molnár *et al*., 1995) and TFP (Orosz *et al*., 1990).

An additional important result of the direct binding experiments with KAR-2 was that Ca^{2+}-free CaM was able to bind the drug. This observation prompted us to attempt to crystallize apo-CaM - KAR-2 complex in Ca^{2+}-free form. Many efforts have been made in several laboratories to produce apo-CaM crystal (Kawasaki *et al*., 1985). Very recently, we were able to grow a single crystal of apo-CaM - KAR-2 complex which was the target of X-ray diffraction analysis (Vértessy *et al*., 1997). The recognition that a potent drug, KAR-2 - in contrast to many CaM antagonists - can bind to CaM in the absence of Ca^{2+} as well, is new and important and certainly has pharmacological relevance.

The immunological test, the fluorescence anisotropy and enzyme kinetic measurements rendered it possible to evaluate differences between the molecular mechanism for the actions of KAR-2 and other bis-indols on CaM-mediated processes. Whereas VBL, VCR and NAV antagonized the binding of target proteins (anti-CaM antibody or PFK) to CaM, KAR-2 was virtually ineffective. Therefore, although KAR-2 binds to CaM it does not display anti-CaM activity.

KAR-2 binds firmly to both tubulin and CaM, and exhibits potent anti-microtubular activity. However, it was virtually ineffective in inhibiting protein binding to CaM. The data from various types of experiments are consistent with a mechanism that assumes a ternary complex formation: KAR-2 - CaM - PFK in which CaM preserves its biological activity. We conclude, therefore, that KAR-2 is unique among bis-indol vinca alkaloids with regard to its behaviour in CaM-regulated processes. There are drugs of other CaM-antagonist families which act in a similar way. Newton *et al*. (1983) showed that a phenothiazine-CaM adduct (norchlorpromazine isothiocyanate - CaM) binds to the target enzymes, but it can no longer modulate the activity of the enzymes. Previously we found that an arylalkylamine derivative, KHL-8430, binds CaM complexed with PFK. However, it abolished the inhibitory effect of CaM within the ternary complex (Orosz *et al*., 1990) (see also Table 1). We suggested that this group of drugs should be termed 'functional antagonists'. It was concluded that KAR-2 is not a functional CaM-antagonist although it binds to the CaM-enzyme complex.

The bis-indol structure is necessary for tubulin binding, anti-microtubular activity and for anti-mitotic activity (Owellen *et al*., 1976). We have suggested that the binding of drugs to CaM also requires a dimeric (catharanthine plus vindoline moieties) structure and that the catharantine moiety is essential for the interaction with CaM (Molnár *et al*., 1995). NAV or 5′-nor-anhydro-VBL, a semisynthetic VBL-type alkaloid (Langlois *et al*., 1976), differs slightly in the catharantine part from VBL, which results in a lower inhibitory effect on tubulin polymerization (Fellous *et al*., 1989; Liliom *et al*., 1995), but higher inhibitory effect on CaM-modulated processes in comparison to other bis-indol derivatives (cf. Table 1).

However, with regard to the structure of KAR-2 and VBL, they are identical in the catharantine part, the vindoline moiety of KAR-2 is derived by a substituted oxazolidino ring, closed across the two C-3 substituents. This derivation does not significantly affect the ability of KAR-2 to be complexed with CaM, but it does affect the ability of KAR-2 to express anti-CaM activity. Thus it is postulated that the 'extra ring' of the vindoline moiety is responsible for the lack of anti-CaM activity; it probably makes KAR-2 unable to compete with the target enzyme for CaM binding.

A crucial problem in cancer chemotherapy is the neurotoxic side effect of drugs. Neurotoxicity means a temporary or permanent loss of neuronal function, which can appear as morphological changes, disturbed synthesis of transmitters, or the inhibition of their uptake and release. The role of CaM seems to be important in these processes (Asano *et al*., 1982). CaM acts as a go-between through which the action of Ca^{2+} on various enzymes is mediated. An increase in free intracellular Ca^{2+} concentration occurs in the neurones in response to a wide variety of agonists and it is perhaps the most important pathway by which cellular effects are expressed.

Experiments were designed to evaluate the effect of KAR-2 on the intracellular Ca^{2+} level. Our data on neutrophils suggest that KAR-2 affects the intracellular calcium homeostasis and this effect is quite different from that of other drugs possessing anti-CaM activities. Therefore KAR-2 appears to have a unique modulating effect on Ca^{2+} uptake. The effects of the different drugs may not be related directly to their anti-CaM potency, since the two CaM antagonists, TFP and VBL, affect the intracellular Ca^{2+} level differently.

Vinca alkaloids vary widely in their effectiveness against specific tumours and in their adverse effects (Watanabe & West, 1982). Himes *et al*. (1976) suggested that the different biological specificity of the drugs may be due to factors other than a direct interaction with tubulin. The antitumor activity of KAR-2 was demonstrated in the accompanying paper (Orosz *et al*., 1997). A cytotoxic effect was observed with low toxic side effects: no paralysis of bladder or lower extremities, an indication of neurotoxic effects, was observed with KAR-2 even during and after treatments with effective doses for several days. Our results suggest that the different actions of VBL and KAR-2 *in vivo* must be due to some biological processes other than the direct interaction with tubulin or MT. Since CaM exerts effects on important biological processes, such as proliferation, mitosis and migration, the investigation of drugs that binds to CaM with or without anti-CaM activity are crucial to the understanding of the relationship between toxic side effects and CaM antagonism and for the development of potent anti-cancer agents without undesirable side effects.

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References

ASANO, M., SUZUKI, Y. & HIDAKA, J. (1982). Effects of various calmodulin antagonists on contraction of rabbit aortic strips. *J. Pharmacol. Exp. Ther.*, **220,** 191 – 196.

BERSHADSKY, A.D. & VASILIEV, J.M. (1988). *Cytoskeleton*. New York: Plenum Press.

DE BRABANDER, M., NUYDENS, R., GEUENS, G., MOEREMANS, M., DE MEY, J. & HOPKINS, C. (1980). Nanovid ultramicroscopy: a new non-destructive approach providing new insights in subcellular motility. In *Microtubules and Microtubule Inhibitors*. ed. De Brabander, M. & De Mey, J. pp. 187 – 196. Amsterdam, New York: Elsevier.

ENGWALL, E. (1980). Enzyme Immunoassay ELISA and EMIT. *Methods Enzymol.*, **70,** 419 – 439.

FELLOUS, A., OHAYON, R., VACASSIN, T., BINET, S., LATASTE, H., KRIKORIAN, A., COUZINIER, J.C. & MEININGER, V. (1989). Biochemical effects of navelbine on tubulin and associated proteins. *Seminars in Oncology*, **16** (suppl. 4), 9 – 14.

FERRANTE, A. & THONG, Y.H. (1980). Optional conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human peripheral blood by the hypaque-ficoll method. *J. Immunol. Methods.*, **36,** 109 – 117.

GIETZEN, K., WÜTRICH, A. & BADER, H. (1982). Effects of microtubular inhibitors on plasma membrane calmodulin-dependent Ca^{2+}-transport ATPase. *Mol. Pharmacol.*, **22,** 413 – 420.

GOPALAKRISHNA, R. & ANDERSON, W.B. (1982). Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.*, **104,** 830 – 836.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260,** 3440 – 3450.

HESTERBERG, L.K. & LEE, J.C. (1982). Self-association of rabbit muscle phosphofructokinase: effects of ligands. *Biochemistry*, **21,** 216 – 222.

HIDAKA, H., SASAKI, Y., TANAKA, T., ENDO, T., OHNO, S., FUJII, Y. & NAGATA, T. (1981). N-(6-aminohexyl)-5-chloror-1-naphtalenesulfonamide, a calmodulin antagonist inhibitors cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.*, **78,** 4354 – 4357.

HIMES, R.H., KERSEY, R.N., HELLER-BETTINGER, I. & SAMSON, F.E. (1976). Action of the Vinca alkaloids vincristine, vinblastine, and desacetyl vinblastine amide on microtubules in vitro. *Cancer Res.*, **36,** 3798 – 3802.

KAWASAKI, H., ITOH, S., KASAI, H., MITSUI, Y., IITAKA, Y., NAKANASHI, M. & OKUYAMA, T. (1985). Crystallization of calcium-calmodulin-trifluoperazine complex and an attempt at crystallizing calcium-free calmodulin. *J. Biochem.* (*Tokyo*), **97,** 1815 – 1818.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, **227,** 680 – 688.

LAGAST, H., LEW, P.D. & WALDVOGEL, F.A. (1984). Adenosine triphosphate-dependent calcium pump in the plasma membrane of guinea pig and human neutrophils. *J. Clin. Invest.*, **73,** 107 – 115.

LANDRY, Y., AMELLAL, M. & RUCKSTUHL, M. (1981). Can calmodulin inhibitors be used to probe calmodulin effects? *Biochem. Pharmacol.*, **30,** 2031 – 2032.

LANGLOIS, N., GUERITTE, F. & LANGLOIS, Y. (1976). Application of a modification of the Polanowski reaction to the synthesis of Vinblastine-type alkaloid. *J. Am. Chem. Soc.*, **22,** 7017 – 7024.

LEHOTZKY, A., TELEGDI, M., LILIOM, K. & OVÁDI, J. (1993). Interaction of phosphofructokinase with tubulin and microtubule: quantitative evaluation of the mutual effects. *J. Biol. Chem.*, **268**, 10888 – 10894.

LEVIN, R.M. & WEISS, B. (1980). Inhibition by trifluoperazine of calmodulin-induced activation of ATPase activity of rat erythrocyte. *Neuropharmacology*, **19,** 169 – 174.

LILIOM, K., LEHOTZKY, A., MOLNÁR, A. & OVÁDI, J. (1995). Characterization of tubulin - drug interactions by ELISA. *Anal. Biochem.*, **228,** 18 – 27.

LILIOM, K., OROSZ, F., HORVÁTH, L. & OVÁDI, J. (1991). Quantitative evaluation of indirect ELISA. Effect of calmodulin antagonists on antibody binding to calmodulin. *J. Immunol. Methods*, **143,** 119 – 125.

MAJANDER, A. & WIKSTRÖM, M. (1989). The plasma membrane potential of human neutrophils. Role of ion channels and the sodium/potassium pump. *Biochim. Biophys. Acta*, **980,** 139 – 145.

MAYR, G.W. (1987). Interaction of calmodulin with phosphofructokinase: binding studies and evaluation of enzymatic and physicochemical changes. *Methods Enzymol.*, **139,** 745 – 763.

MCINTOSH-KOONCE, R. (1989). Mitosis. *Science (Wash, DC)*, **246,** 622 – 628.

MIGLIACCIO, A., ROTONDI, A. & AURICCHI, F. (1984). Calmodulin-stimulated phosphorylation of 17 beta-estradiol receptor in tyrosine. *Proc. Natl. Acad. Sci. U.S.A.*, **81,** 5921 – 5925.

MOLNÁR, A., LILIOM, K., OROSZ, F., VÉRTESSY, B.G. & OVÁDI, J. (1995). Anti-calmodulin potency of indol alkaloids in *in vitro* systems. *Eur. J. Pharmacol.*, **291,** 73 – 82.

NAIRN, A.C. & PERRY, S.V. (1979). Calmodulin and myosin light-chain kinase of rabbit fast skeletal muscle. *Biochem. J.*, **179,** 89 – 97.

NEWTON, D.L., BURKE, T.R., RICE, K.C. & KLEE, B. (1983). Calcium ion dependent covalent modification of calmodulin with norchlorpromazine isothiocyanate. *Biochemistry*, **22,** 5472 – 5476.

OROSZ, F., CHRISTOVA, T.Y. & OVÁDI, J. (1988a). Functional *in vitro* test of calmodulin antagonism: Effect of drugs on interaction between calmodulin and glycolytic enzymes. *Mol. Pharmacol.*, **33,** 678 – 682.

OROSZ, F., CHRISTOVA, T.Y. & OVÁDI, J. (1988b). Modulation of phosphofructokinase action by macromolecular interactions. Quantitative analysis of phosphofructokinase - aldolase - calmodulin interacting system. *Biochim. Biophys. Acta*, **957,** 293 – 300.

OROSZ, F., KOVÁCS, J., LÖW, P., VÉRTESSY, B.G., URBANYI, Z., ÁCS, T., KEVE, T. & OVADI, J. (1997). Interaction of a new bis-indole derivative, KAR-2 with tubulin and its antimitotic activity. *Br. J. Pharmacol.*, (MS 96 - 1560).

OROSZ, F., LILIOM, K., BARKHUDARYAN, N., HORVÁTH, L. & OVÁDI, J. (1992). Effects of calmodulin antagonists on antibody binding to calmodulin. Distinct conformers of calmodulin induced by the binding of drugs. *Biochem.*, **284,** 803 – 808.

OROSZ, F., TELEGDI, M., LILIOM, K., SOLTI, M., KORBONITS, D. & OVÁDI, J. (1990). Dissimilar mechanisms of action of anti-calmodulin drugs: quantitative analysis. *Mol. Pharmacol.*, **38,** 910 – 916.

OVÁDI, J. (1989). Effects of drugs on calmodulin-mediated enzymatic actions. A review. *Prog. Drug Res.*, **33,** 353 – 395.

OVÁDI, J., MOHAMED OSMAN, I.R. & BATKE, J. (1982). Local conformational changes induced by successive nicotinamide binding to dissociable tetrameric D-glyceraldehyde-3-phosphate dehydrogenase: quantitative analysis of a two step dissociation process. *Biochemistry*, **21,** 6375 – 6382.

OWELLEN, R.J., HARTKE, C.A., DICKERSON, R.M. & HAINES, F.O. (1976). Inhibition of tubulin-microtubule polymerization by drugs of the vinca alkaloid class. *Cancer Res.*, **36,** 1499 – 1502.

RAESS, B.U. & VINCENZI, F.F. (1980). Calmodulin activation of red blood cell (Ca^{2+} Mg^{2+})-ATPase and its antagonism by phenothiazines. *Molec. Pharmacol.*, **18,** 253 – 258.

RASMUSSEN, C.D. & MEANS, A.R. (1989). Calmodulin is required for cell-cycle progression during G_1 and mitosis. *EMBO J.*, **8,** 73 – 82.

SEEMAN, P. (1972). The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.*, **24,** 583 – 633.

SOLTI, M., DÉVAY, P., KISS, I., LONDESBOROUGH, J. & FRIEDRICH, P. (1983). Cyclic nucleotide phosphodiesterases in larval and brain of wild type and dunce mutant strains of *Drosophila melanogaster*: isozyme pattern and activation by Ca^{2+}/calmodulin. *Biochem. Biophys. Res. Commun.*, **111,** 652 – 658.

STEVENSON, G.T. & DORRINGTON, K.J. (1970). The recombination of dimers of immunoglobulin peptide chains. *Biochem. J.*, **118,** 703 – 712.

TSIEN, R.Y., POZZAN, T. & RINK, T.J. (1982). T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane in lymphocytes. *Nature*, **295,** 68 – 70.

VÉRTESSY, B.G., BÖCSKEI, Z., HARMATH, V., NÁRAY-SZABÓ, G. & OVÁDI, J. (1997). Crystallization and preliminary diffraction analysis of Ca^{2+}-calmodulin-drug and apocalmodulin-drug complexes. *PROTEINS: Structure, Function and Genetics*, (in press).

WALLACE, R.W. & CHEUNG, W.Y. (1979). Calmodulin. Production of an antibody in rabbit and development of a radioimmunoassay. *J. Biol. Chem.*, **254,** 6564 – 6571.

WATANABE, K. & WEST, W.L. (1982). Calmodulin, activated cyclic nucleotide phosphodiesterase, microtubules and vinca alkaloids. *Fedn. Proc.*, **41,** 2292 – 2299.

WATTERSON, D.M., HARRELSON, W.G., KELLER, P.M., SHARIEF, F. & VANAMAN, T.C. (1976). Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase and actomyosin ATPase. *J. Biol. Chem.*, **251,** 4501–4513.

YASIN, R., HUGHES, B.P. & PARKER, J.A. (1973). The effect of vincristine on the calcium transport and phospholipid composition of rat skeletal muscle microsomes. *Lab. Invest.*, **29,** 207–215.

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