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PROTEIN PHOSPHATASE 2A AND ITS [^{3}H]CANTHARIDIN/[^{3}H]ENDOTHALL THIOANHYDRIDE BINDING SITE

INHIBITOR SPECIFICITY OF CANTHARIDIN AND ATP ANALOGUES

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Abstract—The target site for cantharidin (CA) and its analogues was isolated recently from mouse liver and identified as protein phosphatase 2A (PP2A) in the heterodimeric form known as $PP2A_2$. The most toxic CA analogue, endothall thioanhydride (ETA) (mouse i.p. LD_{50} 0.3 mg/kg), appears to have the same binding site in mouse liver and brain based on studies comparing [^{3}H]ETA and [^{3}H]CA. ATP and its nonhydrolyzable analogues and pyrophosphate and related compounds including phosphonoformic acid inhibited both [^{3}H]CA and [^{3}H]ETA binding with IC_{50} values ranging from 2 to 81 μM. As with CA itself, the most potent inhibitors have two negatively charged groups in close proximity to each other. Inhibition of [^{3}H]CA binding by 5,5′-dithiobis(2-nitrobenzoic acid) and stimulation by *N*-ethylmaleimide indicated the involvement of a thiol site in the CA-binding domain. CA and three analogues (cantharidic acid, palasonin and endothall) inhibited PP2A and protein phosphatase 1 (PP1) but not PP2B or PP2C. The catalytic subunit of PP2A was 5- to 12-fold more sensitive to these CA analogues than the catalytic subunit of PP1. CA and the herbicide endothall also inhibited spinach leaf PP1 and PP2A and, at 50 μM, decreased the PP2A-mediated light-induced activation of nitrate reductase in intact spinach leaves by 62 and 56%, respectively. This is consistent with PP2A as their site of action in plants, and indicates the potential use of CA analogues as pharmacological probes to investigate cellular processes that are regulated by reversible protein phosphorylation *in vivo*.

Cantharidin (CA)‖ and its analogues have a remarkable range of biological activities. They are acantholytic and antitumor agents, pharmaceuticals and pesticides, and purported aphrodisiacs [1–3 and references cited therein]. These diverse actions may result from a single biochemical block. The specific binding site for CA in liver is associated with its acute toxicity [4, 5]. The CA-binding protein (CBP) was isolated and identified as protein phosphatase 2A (PP2A) (EC 3.1.3.16) which in the heterodimeric form is known as $PP2A_2$ [6]. PP2A is a regulatory enzyme involved in the control of many cellular processes in eukaryotic cells [7]. Therefore, it seems probable that all of the biological effects of CA and its analogues could be due to changes in intracellular protein phosphorylation caused by inhibition of protein dephosphorylation by PP2A, and possibly other protein phosphatases related to PP2A. This proposal is tested further in the present study which examines the specificity and structural aspects of interactions between CA and its analogues and PP2A and related enzymes.

First, the toxicity of CA analogues is generally correlated with their potency as inhibitors of [^{3}H]-CA binding to CBP [4, 5] with the possible exception of endothall thioanhydride (ETA) which is the most toxic analogue [8] and yet is a relatively poor inhibitor of [^{3}H]CA and [^{3}H]ETA binding (see Table 1) [5, 9]. Further, severe hepatohemia is a prominent feature of poisoning with CA but not ETA [9]. These anomalies indicate that CA and ETA may act at different target sites or with different binding kinetics at the same target, hypotheses tested here.

Second, ATP and pyrophosphate (PP_i) and their analogues inhibit both [^{3}H]CA binding to CBP [5] and PP2A activity [10, 11]. The structure–activity relationships of these inhibitors are therefore of interest for the [^{3}H]CA and [^{3}H]ETA binding sites.

Finally, the studies to date on CA and its analogues as inhibitors of protein phosphatase activity are restricted to CBP, i.e. $PP2A_2$ [6]. It is important to define the specificity of these inhibitors for other protein phosphatases, i.e. PP2A catalytic subunit and the structurally related enzymes, PP1 and PP2B, and PP2C which belongs to a different gene family from PP1, PP2A and PP2B [7, 12]. The suggestion that the herbicidal activity of endothall may be due to blocking PP2A or another protein phosphatase

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‖ Abbreviations: AMP-CPP, α,β-methylene-ATP; AMP-PCP, β,γ-methylene-ATP; CA, cantharidin; CBP, cantharidin-binding protein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; ETA, endothall thioanhydride; FPLC, fast protein liquid chromatography; NEM, *N*-ethylmaleimide; NSB non-specific binding; PP_i, pyrophosphate; and TB, total binding.

[6] was also examined by considering endothall and CA as possible inhibitors of PP1 and PP2A and the PP2A-mediated light-induced activation of nitrate reductase in spinach.

MATERIALS AND METHODS

Chemicals

Our previous studies describe the preparation of the CA analogues [8, 9] and the radioligands [^{3}H]CA and [^{3}H]ETA [4, 5, 9].

[^{3}H]*CA and* [^{3}H]*ETA binding assays*

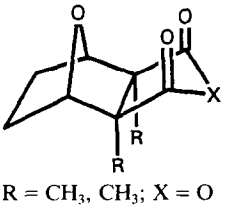
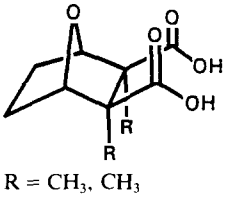
The procedures used were those of Graziano *et al.* [5] for [^{3}H]CA and of Kawamura *et al.* [9] for [^{3}H]ETA. Mouse liver and brain were homogenized in 50 mM Tris-maleate, pH 7.4, or 50 mM imidazole-HCl, pH 7.4. The homogenate was centrifuged at 15,000 *g* for 15 min and the supernatant thereof at 105,000 *g* for 60 min. All preparations were carried out at 4°, and the final supernatant (cytosol) and pellet (membrane) were stored at −70°. Protein was determined by the method of Bradford [13].

For binding assays, the standard incubation mixtures consisted of 0.5 mg cytosolic protein in 1 mL volume with 5 nM [^{3}H]CA in 50 mM Tris-maleate or imidazole-HCl, pH 7.4, or 3 nM [^{3}H]-ETA in 50 mM imidazole-HCl pH 6.0. Following incubation for 90 min at 37° with gentle shaking, the samples were simultaneously filtered under vacuum through Whatman GF/B glass fiber filters [presoaked with 0.03% poly(ethylenimine) for at least 1 hr] and rapidly rinsed with cold buffer (3 × 5 mL) using a Brandell Cell Harvester (model M-24R; Brandell Instruments, Gaithersburg, MD). Radioactivity retained on the filters was quantitated by liquid scintillation counting. Specific binding is defined as the difference between total binding (TB) (with [^{3}H]CA or [^{3}H]ETA only) and non-specific binding (NSB) (with 10 μM unlabeled CA or ETA, respectively).

For saturation assays the receptor was incubated with increasing amounts of unlabeled ligand under a set concentration of radioligand and non-specific binding was determined at 10 μM unlabeled ligand. The pH profile was determined with 50 mM imidazole-HCl, measuring the final pH value of the buffered solutions with protein present directly on a similar preparation. The rate of association of [^{3}H]ETA with its binding site was determined by filtration of the mixture at various time intervals during incubation. The binding parameters were analyzed by the computer program "LIGAND" [14].

In vitro inhibition was determined by adding the test compounds as solutions in acetone (10 μL) or buffer (10 μL) to the assay buffer, then the radioligand and protein were added in order to the incubation mixtures under the standard conditions. *In vivo* inhibition studies involved intraperitoneal dosing with the test compounds in dimethyl sulfoxide:methoxytriglycol (1:1). The mice were killed at predetermined

Table 1. Potency of cantharidin and its analogues as toxicants to mice and inhibitors of [^{3}H]CA and [^{3}H]ETA binding to mouse liver cytosol

Compound		Mouse i.p. LD_{50}* (mg/kg)	Binding site IC_{50} (nM) [^{3}H]CA†	[^{3}H]ETA*
Anhydrides				
Cantharidin (CA)	R = CH_3, CH_3; X = O	1.0	30	8.4
Palasonin	R = CH_3, H, X = O	3.1		12
Endothall anhydride	R = H, H; X = O	4.0	1800‡	26
Endothall thioanhydride (ETA)	R = H, H ; X = S	0.31	1500‡	32
Dicarboxylic acids				
Cantharidic acid	R = CH_3, CH_3	1.8	29	6.7
Endothall	R = H, H	14	930	26

* Kawamura *et al.* [9].
† Graziano *et al.* [5].
‡ This study.

Table 2. *In vivo* inhibition of [^{3}H]CA binding to liver cytosol from mice 60 min after i.p. injection of ETA and endothall anhydride

Compound	Dose mg/kg	Dose Rel. to LD_{50}	Inhibition*
ETA	0.3	1x	5
	1.2	4x	16
Endothall anhydride	4	1x	27
	16	4x	56

* Average of two experiments with three mice at each dose.

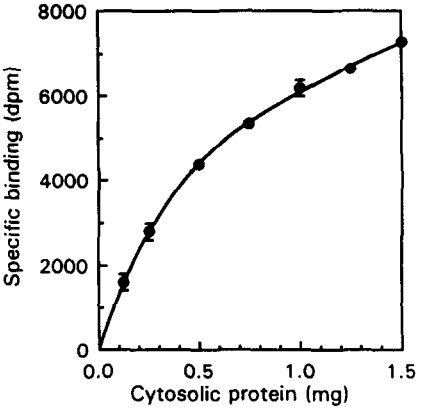


Fig. 2. Relation of [^{3}H]ETA binding to amount of mouse liver cytosolic protein. Each point is the mean ± SEM of three experiments.

times after treatment, and the cytosol fraction of liver and brain was prepared and assayed under the standard conditions described above.

Chromatographic properties of [^{3}H]CA and [^{3}H]ETA binding sites of mouse liver and brain cytosol

Liver cytosol was subjected to fast protein liquid chromatography (FPLC) involving anion exchange (mono Q), cation exchange (mono S), hydrophobic interaction (Phenyl Superose) and gel filtration (Superose 6) by the general procedure of Li and Casida [6]. Each fraction was concentrated and desalted by Centricon®-10 (Amicon, Beverly, MA) before binding assays. In an alternative approach, the [^{3}H]CA–CBP or [^{3}H]ETA–CBP complex was subjected to gel filtration with Superose 6 to determine free and bound ligand.

Protein phosphatase assays

The catalytic subunits of PP1 and PP2A [15] were purified to homogeneity by Dr. Don Schelling in the MRC Protein Phosphorylation Unit at Dundee. PP1 and PP2A were assayed at 0.2 mU/mL in the absence of divalent cations using 10 μM ^{32}P-labeled glycogen phosphorylase by the method of Cohen *et al.* [15]. PP2B from bovine brain (calcineurin) was assayed at 0.01 mU/mL as described by Blumenthal *et al.* [16] using a ^{32}P-labeled peptide (DLDVPIPGRFDRRVSVAAE), which corresponds to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase. PP2C in extracts of spinach leaves and rabbit liver was measured as the okadaic acid-insensitive casein phosphatase activity determined in the presence of 20 mM magnesium acetate using 6 μM [^{32}P]casein [17]. PP2C activity was 0.015 to 0.027 mU/mL in the assays. One unit of protein phosphatase was that amount which catalyzed the dephosphorylation of 1 μmol of substrate in 1 min.

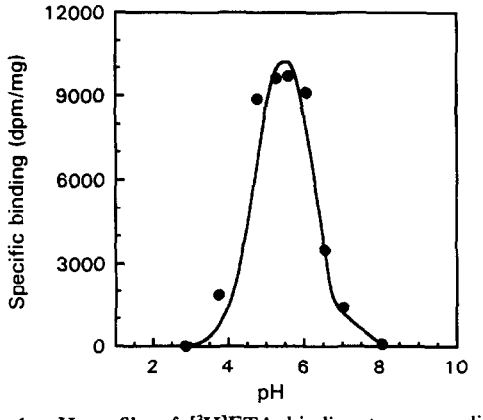


Fig. 1. pH profile of [^{3}H]ETA binding to mouse liver cytosol. Each point is the average of two experiments.

Light-induced activation of nitrate reductase in intact spinach leaves

Nitrate reductase is a physiological substrate of PP2A in plants and is dephosphorylated and activated by PP2A upon illumination of the leaves [18]. Okadaic acid and microcystin prevent the rapid light-induced activation of nitrate reductase in spinach leaves by interaction with PP2A [18]. Accordingly, the effect of CA and endothall on light-activation of nitrate reductase was examined to determine whether these herbicidal compounds act as protein phosphatase inhibitors in intact spinach leaves. This involved illumination for 30 min and subsequent assay of nitrate reductase activity in the presence of 5 mM $MgCl_2$. These results were compared to leaves (2 g) whose petioles had been immersed in CA and endothall solutions for 2 hr before illumination.

RESULTS

Comparison of [^{3}H]ETA and [^{3}H]CA binding sites

Potency of ETA as an inhibitor of [^{3}H]CA binding sites in mouse liver and brain cytosol. ETA is more than 10-fold more toxic than endothall anhydride [8]. It was therefore surprising to find that they were almost identical in potency as *in vitro* inhibitors of [^{3}H]CA binding with IC_{50} values of 1500 and 1800 nM,

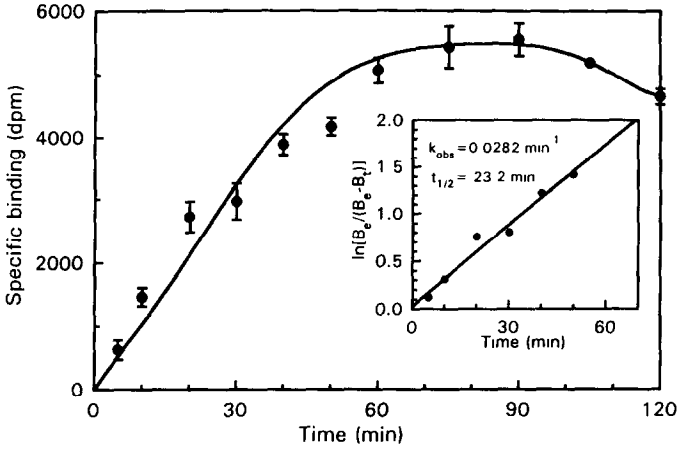


Fig. 3. Kinetics of association of [^{3}H]ETA binding to mouse liver cytosol. Each point is the mean ± SEM of three experiments. Inset: pseudo first-order plot.

respectively, in liver cytosol (Table 1) and 1100 and 1100 nm in brain cytosol.

The unusually high toxicity of ETA was not paralleled by high potency as an *in vivo* inhibitor of [^{3}H]CA binding (Table 2). Thus, in liver cytosol, ETA gave 6-fold less inhibition than endothall anhydride at the LD_{50} dose and 3.5-fold less at 4 times the LD_{50}. In brain cytosol there was no inhibition of [^{3}H]CA binding by ETA at 0.3 and 3 mg/kg with analyses at 1, 2 or 3 hr after treatments in which the mice were severely poisoned or dead. These findings are in contrast to studies with CA-poisoned mice (1, 3 and 10 mg/kg) showing *in vivo* inhibition of [^{3}H]CA binding in liver cytosol correlating with the toxicity [5]. They suggest that ETA and CA may differ in their distribution, metabolism, or site of action. The latter possibility was considered by the preparation of [^{3}H]ETA [9] and direct examination of its binding site.

Properties of [^{3}H]*ETA binding site in mouse liver cytosol.* Specific binding was optimal at pH 5.0 to 6.0 with little binding below pH 5.0 or above pH 6.5 using 50 mM imidazole-HCl buffer (Fig. 1). Binding of [^{3}H]ETA was almost linear with protein concentration up to 0.5 mg/mL (Fig. 2). [^{3}H]ETA associated with its binding site under the standard assay conditions with a $T_1/_2$ of 23.2 min and k_{obs} of 0.0282 min^{-1} (Fig. 3). Equilibrium was achieved at 75 min, and the binding complex was unstable after 90 min. The dissociation rate was therefore not determined. The binding of [^{3}H]ETA was not greatly affected by thiols (data not shown). Thus, upon removal of endogenous small molecules by Sephadex G25, cytosol and Sephadexed-cytosol had the same specific binding for

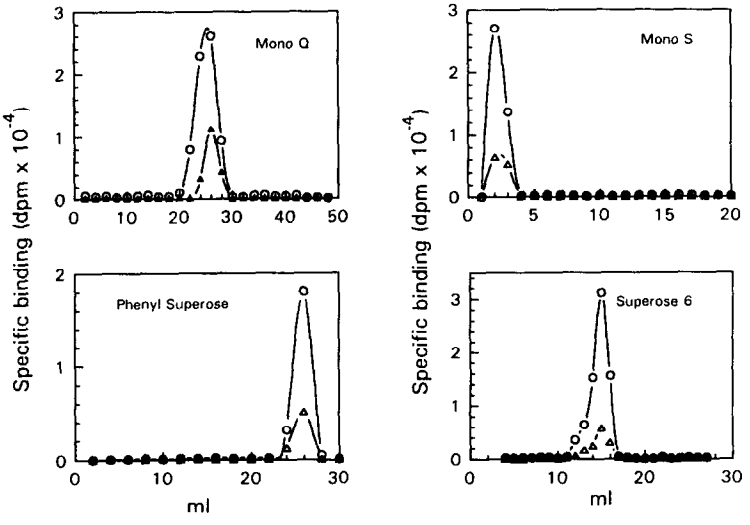


Fig. 4. FPLC on four columns of varied type of [^{3}H]CA- and [^{3}H]ETA-binding protein(s) (○ and △, respectively) from mouse liver cytosol.

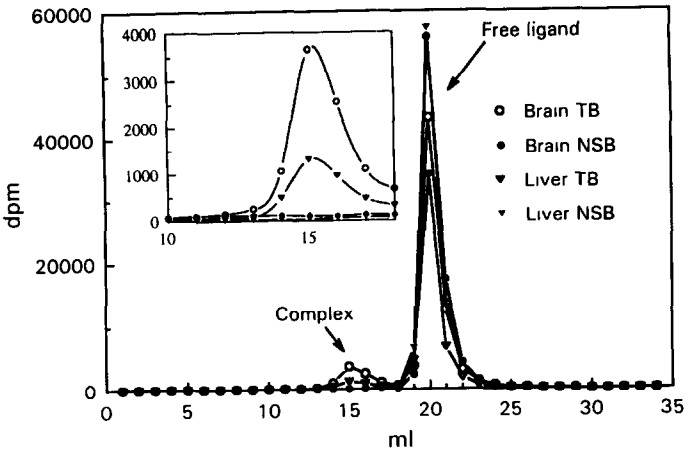


Fig. 5. Gel filtration (FPLC Superose 6) of [^{3}H]CA–CBP complex of mouse liver and brain cytosol. Inset: fractions 10–18 mL. Abbreviations: NSB, non-specific binding; and TB, total binding.

[^{3}H]ETA and there was no effect on [^{3}H]ETA binding upon addition of dithiothreitol (DTT) and/or glutathione at 1 mM to the Sephadexed cytosol.

Identity of [^{3}H]*ETA and* [^{3}H]*CA binding sites in mouse liver and brain cytosol.* The binding sites for [^{3}H]ETA and [^{3}H]CA were compared by several criteria and in each case were found to be identical. The mouse liver cytosol binding site for [^{3}H]ETA was not separable from that for [^{3}H]CA on four FPLC columns including anion exchange, cation exchange, hydrophobic interaction and gel filtration (Fig. 4). [^{3}H]CA also bound to the same protein fraction of mouse brain cytosol (as with liver) following Superose 6 chromatography (data not shown). Alternatively, after binding of [^{3}H]CA to the cytosolic proteins, the binding mixture was introduced into a Superose 6 column (gel filtration) and the radioactivity was monitored. A peak was detected for the [^{3}H]CA–CBP complex from brain cytosol, as with liver cytosol, followed by one for the free ligand (Fig. 5). This complex from mouse brain was similar in molecular size to that from liver. The radioactivity of the complex was absent under the non-specific binding condition. The [^{3}H]ETA binding site complex was unstable upon gel filtration (data not shown). The [^{3}H]CA binding site was also found in the membrane fraction of mouse brain but not appreciably in liver; the nature of this membrane CBP was not examined further.

In competition studies assayed in 50 mM imidazole-HCl at pH 6.0, the B_{max} values for [^{3}H]CA binding were the same (3.5 to 3.6 pmol/mg protein) in the absence or presence of unlabeled ETA, and the K_d values were 2-fold different (Fig. 6), indicating that ETA is a competitive inhibitor of [^{3}H]CA binding. CA was also a competitive inhibitor for [^{3}H]ETA binding (Fig. 6).

Inhibition of [^{3}H]*ETA binding to mouse liver cytosol by ATP and pyrophosphate analogues (Table 3)*

The nucleotide triphosphates ATP, UTP, and GTP inhibited [^{3}H]ETA binding to mouse liver cytosol with IC_{50} values of 4–6 μM. ATP did not appear to inhibit the binding site by initiating a phosphorylation reaction since hydrolysis-resistant ATP analogues such as α,β-methylene-ATP (AMP-CPP) and β,γ-methylene-ATP (AMP-PCP) were also effective. The diphosphates ADP, UDP, and CDP were somewhat less active (IC_{50} values 12–21 μM), whereas analogues with a single phosphate substituent (CDP-choline, AMP and cAMP) were much less effective (IC_{50} values 103 to >1000 μM). PP_i and phosphonoformate were equal to or more effective than ATP. Other active compounds have two negatively charged groups with free phosphono, phosphoro, and/or carboxyl groups at close proximity.

The inhibitors were more effective with [^{3}H]ETA in imidazole-HCl, pH 6.0, than with [^{3}H]CA in Tris-maleate, pH 7.4, perhaps due more to the change in buffer than in pH or radioligand since ETA inhibition of [^{3}H]CA binding to mouse liver cytosol was much higher in 50 mM imidazole-HCl than in 50 mM Tris-maleate, each at pH 7.4. These findings suggest that maleate at this high level may itself interact with the [^{3}H]ETA- and [^{3}H]CA-binding sites.

Inhibition of [^{3}H]*CA binding to mouse liver cytosol by sulfhydryl blocking reagents*

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) partially inhibited and *N*-ethylmaleimide (NEM) stimulated [^{3}H]CA binding to mouse liver cytosol in 50 mM imidazole-HCl, pH 7.4 (Fig. 7). DTT at 1 mM and β-mercaptoethanol at 10 mM had no effect on [^{3}H]CA binding. However, DTT (1 mM) reduced or prevented the reactivity of DTNB and NEM.

Specificity of CA analogues as protein phosphatase inhibitors

The purified catalytic subunits of PP1 and PP2A were inhibited by the anhydrides, CA and palasonin, and the dicarboxylic acids, cantharidic acid and endothall (Table 4). Inhibition curves for CA with

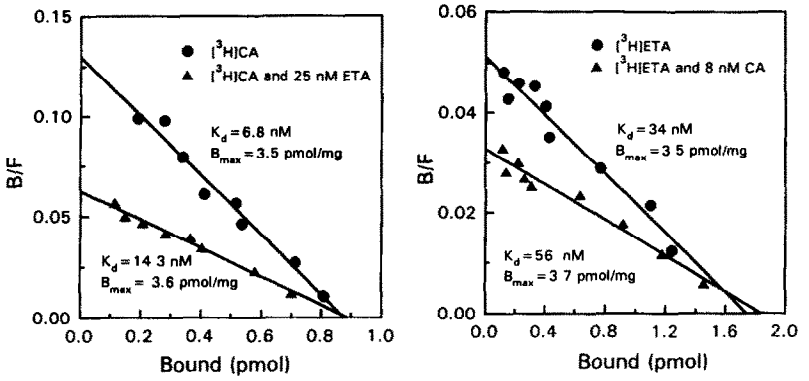


Fig. 6. Scatchard analysis of ETA as an inhibitor of [^{3}H]CA binding and CA as an inhibitor of [^{3}H]-ETA binding in mouse liver cytosol. The results represent one of two experiments, both of which showed the same relationship.

Table 3. ATP and pyrophosphate analogues as inhibitors of [^{3}H]ETA and [^{3}H]CA binding site(s) of mouse liver cytosol

Compound	IC_{50} (μM) [^{3}H]ETA*	[^{3}H]CA†
Nucleotides		
Triphosphates		
ATP	6.4	81
UTP	4.0	75
GTP	4.7	52
AMP-CPP	9.1	
AMP-PCP	7.1	
Diphosphates		
ADP	12	170
UDP	21	1000
GDP		240
CDP	19	
CDP-choline	>1000	
Monophosphates		
AMP	103	>1000
cAMP	>1000	>1000
Pyrophosphate analogues		
Phosphonoformate	2.4	
PP_i	3.6	27
Methylenediphosphonate	12	
Phosphonoacetate	40	
Carbamyl phosphate	66	
Imidodiphosphate	88	

* Average of two or three assays in imidazole-HCl, pH 6.0. The IC_{50} values (μM) for non-phosphorus analogues were 44 and >500 for oxalate and malonate, respectively.
† Assayed in Tris-maleate, pH 7.4 [5].

PP1 and PP2A are shown in Fig. 8. The anhydrides and dicarboxylic acids, i.e. CA and cantharidic acid, were equipotent in these assays. PP2A was more sensitive than PP1 by 12-fold for CA and its dicarboxylic acid and 5-fold for palasonin and endothall. PP2B and PP2C were insensitive to the CA analogues.

The active high-molecular mass forms of PP1 and

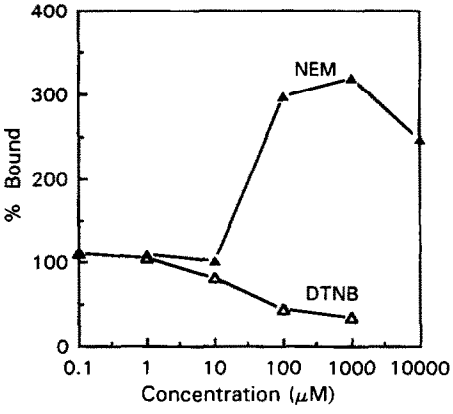


Fig. 7. Effects of DTNB and NEM on [^{3}H]CA binding to mouse liver cytosol in 50 mM imidazole-HCl, pH 7.4. Each point is the average of two experiments.

Table 4. Specificity of cantharidin analogues as inhibitors of protein phosphatases

Compound	IC_{50}* (nM) PP1†	PP2A†	PP2B	PP2C
Cantharidin	473	40	>30,000‡	$>10^6$
Palasonin	656‡	120‡	ND§	$>10^6$‡
Cantharidic acid	562	53	ND§	$>10^6$
Endothall	5000	970	>60,000‡	$>10^6$

* Enzyme was preincubated with inhibitor for 20 min at 30° before the assay was started by addition of substrate. Average of two experiments unless indicated otherwise.
† Purified catalytic subunits at approximately 0.18 and 1.5 nM for PP1 and PP2A, respectively.
‡ Single determination.
§ ND, not determined.

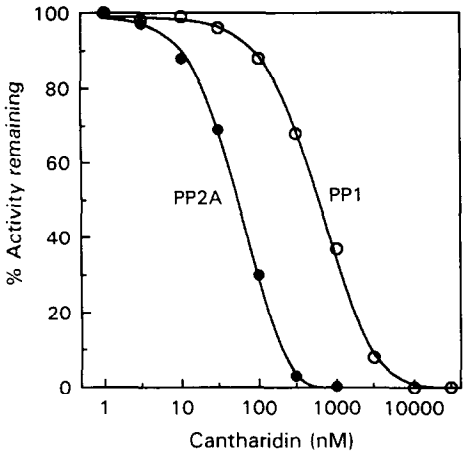


Fig. 8. CA inhibition of the protein phosphatase activities of the purified catalytic subunits of PP2A and PP1. The results are averages of duplicate determinations, which varied by less than 5%.

PP2A present in dilute extracts of rat liver or spinach leaves were also inhibited by CA and endothall with IC_{50} values similar to those observed with the free catalytic subunits (data not shown).

When used at 1 mM neither CA nor endothall has any effect on the activities of cyclic AMP-dependent protein kinase, phosphorylase kinase, the AMP-activated protein kinase [19] and MAP kinase.

Specificity of CA and endothall as inhibitors of the rapid light-induced activation of nitrate reductase in intact spinach leaves

When spinach leaves were illuminated for 30 min, subsequent assay in the presence of 5 mM $MgCl_2$ revealed that nitrate reductase activity was increased 8-fold compared with leaves that remained in the dark. In leaves whose petioles were incubated for 2 hr in 50 μM CA or endothall before illumination, activation was prevented with CA and endothall causing decreases of 62 ± 7 and $56 \pm 5\%$ (6 determinations), respectively, relative to controls.

DISCUSSION

The unusually high toxicity of ETA compared with other CA analogues might be due to differences in distribution, biotransformation or site of action. Possible preferential action of the more lipophilic ETA in the brain was examined in relation to the binding sites for both [^{3}H]CA and [^{3}H]ETA. The characteristics of the specific binding site in brain are very similar to those established in liver with respect to K_d, B_{max}, association/dissociation kinetics, and sensitivity to inhibitors [5]. The present study shows the similarity in size of CBP and the CBP–CA complex from liver and brain. Endothall anhydride and ETA had similar inhibitory potencies *in vitro* for the [^{3}H]CA binding sites of mouse liver and brain cytosol. Further evidence against brain as the target of ETA action was the failure to observe inhibition of the brain [^{3}H]CA binding site *in vivo* 3 hr after high ETA doses. [^{3}H]ETA underwent high affinity binding to a site in mouse liver and brain cytosol fractions with binding parameters (K_d, B_{max} and association kinetics) similar to those of [^{3}H]CA binding. The interaction of ETA with its binding site was more easily dissociated than that of the CA-binding site system because the ETA complex was unstable on passing through the gel filtration column. These findings suggest that the high toxicity of ETA does not result from the formation of a stable or covalently bound complex, such as a disulfide. Direct evidence of protein properties and competition studies demonstrate that [^{3}H]CA and [^{3}H]ETA probably bind to the same protein and the same site in mouse liver cytosol. It is reasonable to assume that this also happens in other tissues. These findings indicate that the high toxicity of ETA is not due solely to its high affinity for the binding site, but also to its action in a tissue other than liver or brain where the disruption leads more directly to lethality.

The CA and ETA binding sites interacted with ATP and PP_i and their analogues. This interaction did not appear to involve a nucleotide site since AMP, cAMP and CDP-choline were less active and there was no nucleotide specificity. The structural requirements for inhibition of [^{3}H]ETA binding indicate that the PP_i moiety may be important for the interaction. The concentration of ATP effective in blocking [^{3}H]CA and [^{3}H]ETA binding was lower than the physiological level (1–3 mM). The physiological relevance of the interaction with ATP *in vivo* remains to be established. The relative effects of ATP, ADP, AMP and PP_i on [^{3}H]ETA binding were very similar to those on [^{3}H]CA binding [5]. However, the [^{3}H]ETA site was about 10-fold more sensitive than the [^{3}H]CA site, perhaps due to a change in the buffer and pH involved. A similar ATP/PP_i site has also been described for PP2A [10, 11], DNA polymerases [20], glucocorticoid receptors [21–23] and the aryl hydrocarbon receptor [24, 25].

CBP isolated on the basis of the [^{3}H]CA binding assay is $PP2A_2$ (heterodimeric form) only without other protein phosphatases [6]. It is surprising that no $PP2A_1$ (heterotrimeric form) is evident since CA can certainly inhibit $PP2A_1$ as well as $PP2A_2$ and they are well separated on mono Q [26]; perhaps the β-subunit was lost by proteolysis during preparation of the extract. The failure to isolate PP1 from mouse liver cytosol may result from two factors. First, the active forms of PP1 are largely particulate; in liver, brain or kidney homogenates, up to 70% of PP1 activity is sedimented at 100,000 *g* [7]. Second, the [^{3}H]CA filtration assay is not sensitive enough to detect the low affinity interaction of CA and PP1 [27]. No appreciable specific [^{3}H]CA binding is detected in the 105,000 *g* pellet of mouse liver homogenate using this method [4]. PP2A therefore appears to be a major target for CA analogues and PP1 may also interact *in vivo*. Inhibition of dephosphorylation catalyzed primarily by PP2A, and perhaps secondarily by PP1, is probably responsible for the toxicity of CA analogues.

The catalytic subunits of PP1 and PP2A are complexed to regulatory and/or targeting subunits

[7]. The complexes are similar in sensitivity to the catalytic subunits alone, indicating that the CA binding site is not greatly altered on complex formation. CA and cantharidic acid showed the same potency to block PP1 and PP2A. This agrees with the earlier finding that oxabicycloheptanedicarboxylic acid anhydrides and the corresponding dicarboxylic acids have the same affinity for the [^{3}H]CA and [^{3}H]ETA binding sites in mouse liver cytosol [5, 9]. Endothall is not only less potent but is also less selective than dimethyl-endothall (cantharidic acid) in blocking PP2A compared with PP1.

The herbicidal action of endothall and CA may be due to PP2A inhibition leading to alterations in activity of several cytosolic enzymes undergoing reversible phosphorylation, e.g. nitrate reductase and sucrose phosphate synthase. By analogy with other eukaryotic cells it is likely that many processes in plants are regulated by enzymes related to PP2A. In the present study CA and endothall were equally potent in inhibiting activation/dephosphorylation of nitrate reductase in intact spinach leaves, with about one-fiftieth of the activity of okadaic acid and microcystin (IC_{50} values of $\sim 1\ \mu M$) [18]. CA was much more potent than endothall in the PP1 and PP2A enzyme assays with spinach cytosol, yet the two compounds were equally potent when fed to leaves in inhibiting activation (dephosphorylation) of nitrate reductase. Endothall and its anhydride are more potent than cantharidic acid and its anhydride (CA) as inhibitors of root growth of barnyard grass and wild mustard [8]. It is not known whether these differences are due to uptake and distribution or to specificity of the relevant PP2As involved.

CA and endothall are convenient inhibitors of phosphorylation/dephosphorylation events mediated by PP2A and PP1, especially in large-scale experiments to inhibit PP1 and PP2A during purification of phosphorylated proteins. First, they are less expensive and more abundant than okadaic acid, microcystin and tautomycin. Second, the anhydride or dicarboxylic acid form of CA analogues can be applied in either hydrophobic or hydrophilic condition. Third, the alternative inhibitors sodium PP_i and NaF are sometimes unsuitable, e.g. NaF inhibits many enzymes including some protein kinases. Finally, CA and its analogues are structurally simple probes to study the structures of PP2A and PP1 molecules.

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