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Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A

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Cantharidin, a natural toxicant of blister beetles, is a strong inhibitor of protein phosphatases types 1(PP1) and 2A (PP2A). Like okadaic acid, cantharidin inhibits the activity of the purified catalytic subunit of PP2A (IC_{50} = 0.16 μM) at a lower concentration than that of PP1 (IC_{50} = 1.7 μM) and only inhibits the activity of protein phosphatase type 2B (PP2B) at high concentrations. Dose–inhibition studies conducted with whole cell homogenates indicate that cantharidin also inhibits the native forms of these enzymes. Thus, cantharidin, which is economical and readily available, may be useful as an additional probe for studying the functions of serine/threonine protein phosphatases.

Cantharidin; Serine/threonine, Protein; Phosphatase; Inhibitor; Dephosphorylation; Toxin

1. INTRODUCTION

Mylabris, the dried body of the Chinese blister beetle (*Mylabris phalerata* or *M. cichorii*), can be traced back more than 2,000 years as a traditional medicine in China and is still used as a folk medicine in Asia today [1]. The active constituent of mylabris, cantharidin (exo, exo-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride) (Fig. 1), is produced by as many as 1500 different species of blister beetles, with the Spanish fly *Cantharis vesicatoria* probably being the best known source [1–3]. Cantharidin also has a history of medical usage in Europe where it was first isolated in the early 1800's as the active principle of a blister beetle extract [3]. Although occasionally utilized as a topical vesicant for the removal of warts [4], cantharidin was considered as obsolete and too toxic for use as an internal medicine by the early 1900's. Nonetheless, folklore surrounding the purported properties of 'Spanish fly' as an aphrodisiac and abortifacient continues to result in human poisonings today, with 26–50 mg estimated as a lethal dose for humans [5]. Livestock toxicosis due to the consumption of feed containing blister beetles also continues to present a problem for ranchers [6].

The toxic properties of cantharidin are well characterized. Dermal contact produces blisters characterized by a distinctive vesiculobullous eruption [7,8]. Oral ingestion leads to severe irritation and ulceration of the gastrointestinal and urinary tract epithelial linings [3,9], and intraperitineal injection causes severe congestion and edema of the liver, with a $LD_{50} \cong 1$ mg/kg [10,11].

The cellular mechanisms underlying the toxicity of cantharidin have been under investigation for several years. Early studies indicated that cantharidin interfered with mitochondrial respiration [12,13] or induced the activation of an acantholytic factor [8]. Cantharidin was then shown to bind with high affinity to a specific and saturable binding site in a cytosolic fraction produced from mouse liver [14,15]. This high-affinity cantharidin binding site was subsequently identified in several other tissues including: heart, kidney, lung, pancreas, skin, spleen, brain, blood and stomach [15]. Recently, Li and Casida [16] purified the cantharidin-binding protein (CBP) to homogeneity, identifying it as protein phosphatase type 2A (i.e. the amino acid sequences of seven peptides from CBP were identical with deduced amino acid sequences for $PP2A_\alpha$. CPB had phosphorylase a phosphatase activity which was inhibited by both okadaic acid and cantharidic acid, and okadaic acid inhibited the binding of [^{3}H]cantharidin to CBP).

The present report characterizes the inhibitory activity of cantharidin on the activity of the purified catalytic subunits of serine/threonine protein phosphatases types 1 (PP1), 2A (PP2A) and 2B (PP2B) and in whole cell homogenates of PC12 cells. These studies indicate that, like okadaic acid, cantharidin is a strong inhibitor of PP1 and PP2A and a weak inhibitor of PP2B.

2. MATERIALS AND METHODS

2.1. *Materials*

Phosphorylase kinase (EC 2.7.1.38) and protein kinase A (3′,5′-cyclic AMP-dependent protein kinase) from rabbit muscle, histone (type 2AS), PP2B (calcineurin) and calmodulin (phosphodiesterase 3′:5′-cyclic nucleotide activator) from bovine brain, and *p*-nitrophenyl phosphate (PNPP) were obtained from Sigma Company and utilized without further purification. Cantharidin was purchased from Sigma

chemical company and exhibited >98% purity by GC/MS analysis. Okadaic acid was generously provided by Dr. R. Dickey.

2.2. *Determination of protein phosphatase activity*

The catalytic subunit of PP1 and PP2A were purified to apparent homogeneity, demonstrating a single band upon SDS-PAGE and silver staining, as described by Honkanen et al. [17–19]. Inhibition assays conducted with purified PP1 and PP2A contained 125 pM PP1 or 25 pM PP2A having phosphohistone phosphatase activity of 265 ± 15 and 1250 ± 62 nmol/min/mg of protein, respectively. Phosphohistone, specific activity 4.5×10^6 dpm/nmol of incorporated phosphate, was prepared by the phosphorylation of histone (type 2AS from Sigma) with cAMP-dependent protein kinase (protein kinase A) in the presence of [γ-^{32}P]ATP (150 μM ATP) as described in detail previously [18]. Protein phosphatase activity against phosphohistone was determined by the liberation of ^{32}P using either the purified catalytic subunits of the indicated protein phosphatases (Figs. 2 and 3) or a dilute whole cell homogenate of PC12 cells (Fig. 4). The dephosphorylation of substrate was < 10% of the total, and reactions were linear with respect to enzyme concentration and time [18]. ^{32}P-phosphate liberated by the enzymes was extracted as a phosphomolybdate complex and measured according to the methods of Killilea et al [20]. Inhibition of phosphatase activity was determined by the addition of toxins to the assay mixture 10 min before initiation of the reaction with the addition of substrate [18]. Okadaic acid and cantharidin were dissolved in dimethyl formamide (DMF), which was added in equal amounts to controls. PP2B activity was measured using PNPP as a substrate in the presence of calmodulin and Ca^{2+}, as described previously [18].

2.3. *Estimation of K_i values*

Previous experiments have indicated that for compounds that behave as 'tightly binding inhibitors' the determination of K_i by fitting dose–inhibition relationships to the theoretical function by an ordinary nonlinear least-squares methods does not provide reliable confidence limits [21]. However, as demonstrated for okadaic acid, the dose–inhibition of 'tightly binding inhibitors' is well described by the following equation, derived with steady-state assumptions:

$$x = [(E_t - I_t - K_i) + \sqrt{(E_t - I_t - K_i)^2 + 4\,E_t \cdot K_i}]\,/2E_t \quad (1)$$

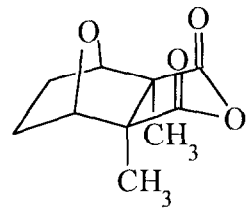
where x stands for the fractional activity v_i/v_o, K_i is the dissociation constant, and E_t and I_t are the total concentration of the enzyme and inhibitor, respectively [21] For the set of observations (I_t, x) and (I'_t, x'), rearrangement of equation 1 gives.

$$x(1-x)E_t + (1-x)K_i = x \cdot I_t \text{ and } x'(1-x')E_t + (1-x')K_i = x' \cdot I_t'$$

K_i values were estimated as means of the solutions obtained by solving the simultaneous equations for all possible sets of observations [21]. This procedure is the direct-linear plot method for estimation of enzyme-kinetic constants [22] as modified by Takai et al. [21]. Theoretical limits of this application have been discussed previously [21,23].

3. RESULTS

The dephosphorylation of phosphohistone by the purified catalytic subunits of both PP1 and PP2A is inhibited by cantharidin, which also inhibits the activity of PP2B at higher concentrations (Fig. 2, Table I). Cantharidin inhibits the activity of PP1 and PP2A in a dose-dependent manner, inhibiting PP2A (IC_{50} = 0.16 ± 0.06 μM; mean ± S.D., n = 4) at a 10.6- fold lower concentration than PP1(IC_{50} = 1.70 ± 0.5 μM; mean ± S.D., n = 4); PP2B is inhibited by cantharidin



Cantharidin

Fig. 1 Structure of cantharidin.

at a >500-fold higher concentration than that which inhibits PP1 (Fig. 2). Under identical assay conditions okadaic acid inhibits the activity of PP2A (IC_{50}~0.04 nM) at a >1,000-fold lower concentration than cantharidin (Fig. 3).

To determine whether cantharidin inhibits the activity of the native forms of PP1 and PP2A at a comparable concentration to that observed with the respective purified catalytic subunits, we examined the effects of cantharidin in whole cell homogenates of PC12 cells. Fig. 4 shows the inhibitory dose–response of cantharidin and okadaic acid in a dilute whole cell homogenate of PC12 cells (60 μg protein/ml). Both toxins inhibited the divalent cation-independent protein phosphatase activity (predominately PP1 and PP2A) in the whole-cell homogenate, with 0.1 μM okadaic acid and 100 μM cantharidin causing 100% inhibition. Nonlinear regression analyses of the dose–inhibition curves obtained with both okadaic acid and cantharidin indicate that the divalent cation-independent phosphatase activity contained in the PC12 cell homogenate best fits a

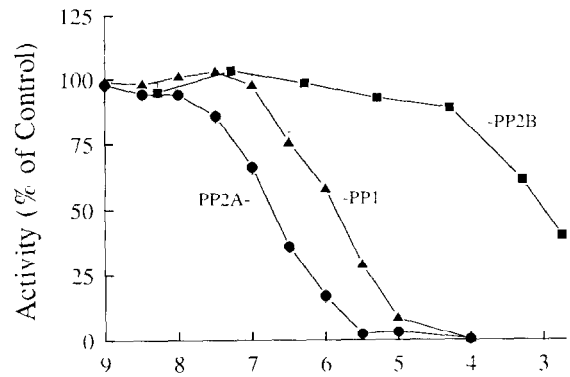


Fig. 2. Inhibition of protein phosphatases by cantharidin. The catalytic subunits of PP1 and PP2A were assayed using ^{32}P-labeled histone as a substrate and PP2B using PNPP as a substrate as described in Section 2. The data is expressed as % of control, with control activity against phosphohistone being 265 ± 15 and 1,250 ± 62 nmol/min/mg protein for PP1 and PP2A, respectively. Cantharidin was mixed with the enzymes for 10 min at 23°C prior to the initiation of the reaction with the addition of substrate. The data represents the mean ± S.D. of all data obtained from at least 4 separate experiments employing two different preparations of each enzyme tested.

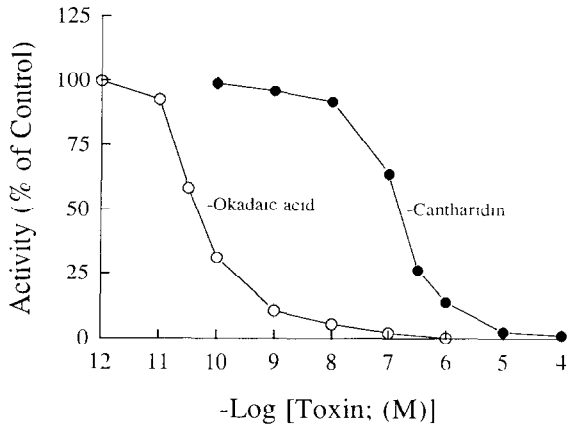


Fig. 3. Comparison of the inhibitory effect of okadaic acid and cantharidin on the activity of the purified catalytic subunit of PP2A from muscle. Assays were conducted as described in Figure 2 using phosphohistone as a substrate and with enzymes having comparable initial activity. Both toxins were mixed with the enzyme 10 min at 23°C prior to the addition of substrate. Each point represents the mean ± S.D. ($n = 4$).

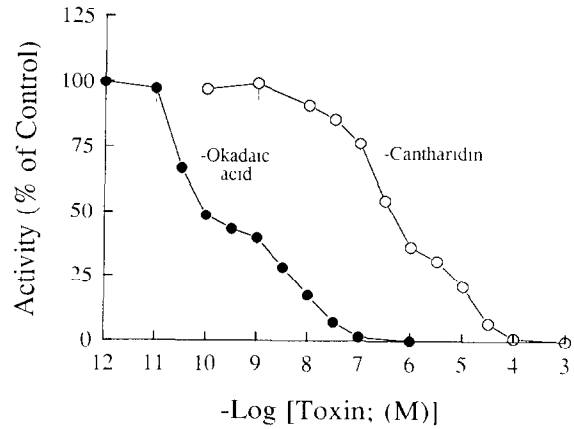


Fig. 4. Effect of okadaic acid and cantharidin on protein phosphatase activity contained in a homogenate of PC12 cells. Cells from a single ~50% confluent 100-mm dish were rinsed with 4°C phosphate-buffered saline, scraped and sonicated in 1 ml of Tris-buffer (20 mM Tris-HCl, 1 mM EDTA and 2 mM dithiothreitol, pH 7.4). The homogenate was then diluted below the titration end point (the concentration after which further dilution no longer affects the IC_{50} [18,19], ~60 μg protein/ml) and assayed for phosphohistone phosphatase activity as described in Fig. 2 Each point represents the mean ± S D. of three experiments conducted in triplicate.

two-site model, which is consistent with that predicted for an assay containing a mixture of both PP1 and PP2A [18,19].

4. DISCUSSION

This study shows that cantharidin, a natural toxicant of several species of blister beetles [1,3] is a potent inhibitor of serine/threonine protein phosphatases types 1(PP1) and 2A (PP2A) and a weak inhibitor of the Ca^{2+}/calmodulin-dependent phosphatase, PP2B. Thus, cantharidin may be added to a growing list of natural toxins that strongly inhibit the activity of both PP1 and PP2A which include: okadaic acid, a polyether carboxylic acid produced by several species of marine dinoflagelates [24,25]; the microcystins, a family of cyclic heptapeptides produced by certain strains of cyanobacteria [19,26,27]; nodularin, a cyclic pentapeptide produced by *Nodularia spumigena* [18]; calyculin A, a spiro ketal obtained from a marine sponge [28] and tautomycin, a dialkylmaleic anhydride produced by a strain of *Streptomyces spiroverticillatus* [29–31].

Like okadaic acid and the cyanobacterial toxins (microcystins and nodularin), the inhibitory effect of cantharidin differs substantially among the cantharidin-sensitive phosphatases. The purified catalytic subunit of PP2A from muscle is inhibited by cantharidin at a concentration that is 5–10-times lower than that required to inhibit the activity of purified PP1 (Table I, Fig. 2). With okadaic acid, PP2A is inhibited at a 10–100-fold lower concentration than PP1 [18,24,25], while both microcystin-LR [19,26] and nodularin [18] are 4–60-fold more potent inhibitors of PP2A than PP1. However, due the extremely high apparent affinity of microcystin-LR and nodularin for both PP1 and PP2A, the difference in the relative potency of these compounds can only be observed in very dilute or high volume assays employing radiolabeled substrates with very high spe-

Table I

Inhibition of PP1 and PP2A by cantharidin

		PP2A		PP1		
Inhibitor	Substrate	K_i (μM)	n	K_i (μM)	n	PP1/PP2A
Cantharidin	Phosphohistone	0.194 (0.037)	84	1.11 (0.27)	35	5.72

The dissociation constants (K_i) estimated by the dose-inhibition analysis are listed for PP1 and PP2A. The K_i values are expressed in μM with 95% confidence (in parentheses); n denotes the total number of determinations utilized in deriving K_i estimates, and the PP1/PP2A is the ratio of K_i values for PP1 to that for PP2A using phosphohistone as a substrate.

cific activity [18,19]. In contrast, calyculin A inhibits the activity of both PP1 and PP2A at a similar concentration [28], and tautomycin, which is a relatively unstable compound [31], has been reported to inhibit PP1 at a slightly lower concentration than PP2A [29,30].

Because of the inhibitory effects it has on PP1 and PP2A, okadaic acid has proven to be a valuable research tool for determining the physiological and pathological roles of serine/threonine protein phosphatases [24,33]. Cantharidin, which is readily available and economical, may also be useful as a research tool. In dilute whole cell homogenates, PP1 and PP2A make up the majority of the divalent cation independent protein phosphatase activity contained in most mammalian tissues [32,33]. Like okadaic acid, cantharidin completely inhibits the divalent cation-independent protein phosphatase activity contained in a crude PC12 cell homogenate, with essentially complete inhibition occurring at a concentration <100 μM (Fig. 4). Therefore, cantharidin also appears to be an effective inhibitor of the 'native forms' of PP1 and PP2A. Furthermore, like okadaic acid, the dose–inhibition relationship produced with cantharidin is biphasic, with characteristics similar to that predicted from a solution containing both PP1 and PP2A [18,19]. Nonetheless, additional experiments addressing the specificity, stability and metabolism of cantharidin will be required to fully assess the value of this toxin as a research tool for the detection and analysis of protein phosphatases.

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