Characterization of calcineurin from *Hymenolepis microstoma* and *H. diminuta* and its interaction with cyclosporin A

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(*Received 17 June 1996; revised 3 August 1996; accepted 3 August 1996*)

SUMMARY

The drug cyclosporin A (CsA) exerts its immunosuppressive action by binding to the cytosolic protein, cyclophilin (CyP) and, as a complex, binding to and inhibiting the calcium/calmodulin-dependent serine threonine phosphatase, calcineurin. It is unknown whether a similar mode of action occurs during the drug's antiparasite activity. Calmodulin-binding proteins from the helminth parasites *Hymenolepis microstoma* and *H. diminuta* were purified by affinity chromatography, yielding single polypeptide bands of 60000 M_r, according to SDS–PAGE. These proteins were tested for calcineurin activity by the dephosphorylation of the R_{II} peptide (part of the catalytic subunit of cAMP-dependent protein kinase). Both proteins were calcium- and calmodulin-dependent and were inhibited by mammalian cyclophilin complexed with cyclosporin A (IC_{50} values of 0·75 μg CyP for *H. microstoma* and 0·90 μg CyP for *H. diminuta*). However, neither of the parasite calcineurins was inhibited by *H. microstoma* cyclophilin/CsA. These data suggest the anthelmintic mode of action of CsA in these helminth models does not involve the inhibition of a signal transduction pathway requiring interaction with calcineurin.

Key words: calcineurin, parasites, *Hymenolepis* spp., cyclosporin, cyclophilin.

INTRODUCTION

Cyclosporin A (CsA) is a clinically important immunomodulatory drug that exerts its immunosuppressive action by acting as a pro-drug and binding to the intracellular receptor, cyclophilin (CyP). This complex blocks the signal transduction pathway in the T cell, at a step between T cell receptor stimulation and cytokine gene transcription. The target molecule for this is calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase (Type 2B) (Wiederrecht *et al*. 1993). Inhibition of calcineurin blocks nuclear translocation of the transcription factor NF-AT which is necessary for activation of cytokine genes during T-cell activation.

CsA also possesses potent but selective anti-parasitic activities (Chappell & Wastling, 1992), but its mode of anti-parasite activity remains unexplained. Against certain model parasitic infections it is demonstrably effective (e.g. *Trypanosoma cruzi*, *Schistosoma mansoni*), whereas with others it is immunomodulatory, exacerbating infections (e.g. *T. brucei*). We have employed the closely related tapeworms *Hymenolepis microstoma* and *H. diminuta* as models to examine the mode of anti-parasite activity of CsA. The former species is killed both *in vivo* and *in vitro* by CsA, while the latter is unaffected (Chappell & Wastling, 1992). Cyclophilin is present in *H. microstoma* and binds CsA, although with lower affinity than mammalian cyclophilin (Roberts *et al*. 1995), while cyclophilin of *H. diminuta* is either not present or has such low affinity for CsA that it cannot be purified by conventional methods (Roberts *et al*. 1995). In this paper, we examine the inhibition of calcineurin in these tapeworms by CsA/CyP complexes to determine whether this is an important factor in the anthelmintic activity as has been described in immunosuppression (Wiederrecht *et al*. 1993).

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MATERIALS AND METHODS

Parasites

Hymenolepis microstoma and *H. diminuta* were maintained as cysticercoids in flour beetles (*Tribolium confusum*) and as adults in male MF1 mice or male Sprague–Dawley rats, respectively, as described by Roberts *et al*. (1995).

Calcineurin assay

Bovine brain calcineurin was purchased from Sigma (Poole, UK) and used without further purification.

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cAMP-dependent protein kinase (Sigma) was used for phosphorylation of the peptide substrate. DLDVPIPGRFDRRVSVAAE, a partial sequence corresponding to the R_{II} subunit of cAMP-dependent protein kinase, was purchased from Bachem Feinchemikalian (Bubendorf, Switzerland). All other reagents used were analytical grade and purchased from Sigma.

An HPLC assay, based on the method of Enz *et al.* (1994), was used to determine calcineurin activity. The R_{II} peptide was phosphorylated as described by Enz *et al.* (1994). Briefly, peptide (1 mg) was dissolved in 1 ml (total volume) of 20 mM Tris buffer, pH 7·4, containing 0·1 mM EGTA, 0·5 mM DTT, 10 mM $MgCl_2$, 0·5 mM $CaCl_2$ and 1 mM ATP. Phosphorylation was initiated by addition of cAMP-dependent protein kinase (3 μg/ml) and 1 mM cAMP, and the mixture incubated for 30 min at 30 °C. After phosphorylation of the peptide (as determined by HPLC), the mixture was heated (95 °C, 15 min in a water bath) and centrifuged (15 min, 13 500 ***g*** at 4 °C). The supernatant fraction containing the phosphopeptide (500 μM) was frozen in 100 μl aliquots and stored at −70 °C until use.

The phosphatase activity of calcineurin was determined at 30 °C in a total volume of 100 μl of 50 mM Tris, pH 7·0, containing (final concentrations) 0·5 mM $CaCl_2$, 0·5 mM DTT, 0·1 μM calmodulin. In inhibition experiments, reaction mixtures included appropriate amounts of parasite or mammalian cyclophilin and cyclosporin A, as indicated.

Phosphorylated peptide was separated from the peptide by reverse-phase chromatography on a Spherisorb C18 column (25 cm length, particle size 5 μm, Fisons, UK). The mobile phase, 10 mM phosphate buffer (pH 6)/16 % acetonitrile, was run under isocratic conditions at 60 °C, with a flow rate of 1 ml/min, a run time of 8 min and wavelength for detection at 205 nm. Under these conditions, dephosphorylated and phosphorylated peptides exhibited symmetrical peaks with retention times of 3 min and 1·8 min, respectively. The phosphopeptide eluted reproducibly under the chromatographic conditions used, and this was quantitated by peak area integration in order to determine kinetic parameters.

Increasing the concentration of acetonitrile resulted in shorter retention times and merging of the 2 peaks. Standard curves of substrate concentration were linear and the limit of detection was approximately 0·1 nmol.

Preparation of cyclophilin

Mammalian and parasite cyclophilins were prepared as described previously (Roberts *et al.* 1995) using affinity chromatography. Briefly, parasite and mouse kidney tissue homogenates were prepared in the presence of protease inhibitors iodoacetamide (1 mg/ml), PMSF (1 mM), EDTA (1 mM), pepstatin A (0·1 μM) and *O*-phenanthroline (1 mM) at 4 °C, and centrifuged at 100 000 ***g*** for 60 min at 4 °C. The supernatant fraction was removed and applied to an affinity chromatography column of cyclosporin-Affigel (Roberts *et al.* 1995). Unbound proteins were removed with 50 mM (pH 7) phosphate buffer, bound proteins were then eluted with 50 mM (pH 3) phosphate buffer, and the pH of these eluted proteins adjusted to pH 7. The purity of the resulting cyclophilin was confirmed by SDS–PAGE on 10–20 % gradient gels (Laemmli, 1970).

Preparation of calcineurin

Parasite calcineurins were purified according to a modified method of Stewart & Cohen (1988). Parasite tissues were prepared by homogenizing 1 g in the presence of protease inhibitors (PMSF, 1 mM; iodoacetamide, 1 mg/ml; *O*-phenanthroline, 1 mM; pepstatin A, 0·1 μM; EDTA, 1 mM) and 25 mM β-mercaptoethanol in 50 mM Tris buffer (pH 7·4) at 24 000 rpm (Ultra-Turrax, Labortechnik). The homogenate was centrifuged for 60 min at 100 000 ***g*** at 4 °C, and the supernatant fraction was removed and applied to an affinity chromatography column consisting of calmodulin–agarose equilibrated in 40 mM Tris (pH 7·5), 50 mM NaCl, 0·3 mM $CaCl_2$, 3·0 mM magnesium acetate containing 1·0 mg/ml BSA. The column was washed in equilibrating buffer containing 200 mM NaCl under gravity (flow rate of approximately 0·5 ml/min). Calcineurin was eluted with 40 mM Tris, pH 7·5, 2·0 mM EGTA, 1·0 mM magnesium acetate and 200 mM NaCl and protein-containing fractions analysed by SDS–PAGE on 10–20 % acrylamide gels (Laemmli, 1970).

RESULTS

Purification of calmodulin-binding proteins

Tapeworm homogenates, prepared as described above, were subjected to calmodulin–agarose affinity chromatography under conditions in which calmodulin-binding proteins are retained (Stewart & Cohen, 1988). These were eluted with 200 mM NaCl and the eluate fractions assayed for protein by the method of Bradford (1976). Fractions containing protein were analysed by SDS–PAGE. In both *H. microstoma* and *H. diminuta*, only 1 major polypeptide was recovered (Fig. 1, lanes 4 and 5, respectively) of M_r 60 000. This was similar to the molecular weight of bovine calcineurin (shown in lane 2). A mouse brain preparation was also made, and again gave a single band of 60 000 M_r (lane 3). The lower molecular mass (18 000) subunit of commercial calcineurin can also be seen in lane 1, but there was insufficient protein loaded from the other samples for this subunit to be detected.

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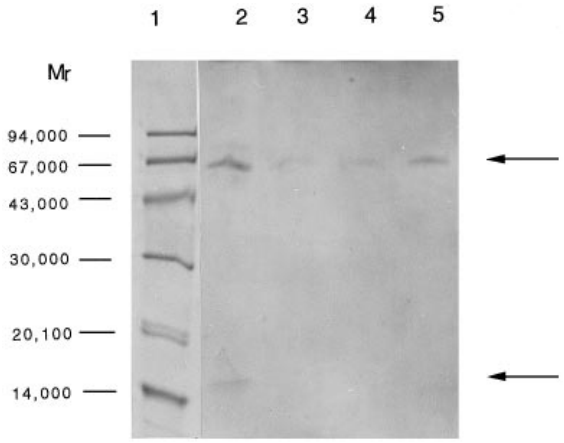

Fig. 1. SDS–PAGE of calmodulin-binding proteins eluted from an affinity column. Lane 1 molecular weight markers; Lane 2 bovine (commercial) calcineurin; Lane 3 mouse brain extract; Lane 4 *Hymenolepis microstoma* extracts; lane 5 *H. diminuta* extracts.

Table 1. Calcineurin activity of tapeworm and murine brain calmodulin binding fraction

(Results are expressed as nmol phosphorylated substrate/min/μg protein and are the means of 3 experiments ($\pm$S.D.) with 3 replicate determinations/experiment.)

Species	Activity with 0·5 mM Ca^{2+} and 0·1 μM calmodulin	No calmodulin	No calcium
Bovine	57·12$\pm$6·41	4·82$\pm$2·58	13·16$\pm$4·97
Murine	18·83$\pm$0·97	2·33$\pm$1·54	1·51$\pm$0·5
H. microstoma	5·72$\pm$1·35	1·38$\pm$0·68	0·98$\pm$0·25
H. diminuta	8·85$\pm$0·98	0·86$\pm$0·23	1·28$\pm$0·57

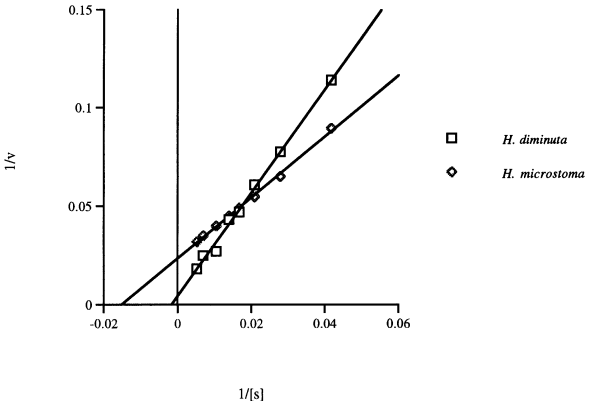

Fig. 2. Lineweaver-Burke plots of *Hymenolepis microstoma* and *H. diminuta* calcineurin. Y-axis intercept yields $1/V_{max}$, X-axis intercept $-1/K_m$. Data represent means of 3 independent tissue preparations. [s] = phosphopeptide concentration (μM); v = rate of dephosphorylation (μmol/min/mg protein). Lines were fitted by linear regression.

Table 2. Inhibition of parasite and mammalian calcineurin by cyclophilin

(Activities are expressed as nmol substrate/min/μg protein and are the means of 3 experiments. Concentration of CsA: 5 μg/ml.)

Calcineurin	Additions*	Activity $\pm$S.D. (mean % inhibition)
H. microstoma	—	5·72$\pm$1·35
	CsA	5·57$\pm$0·56 (2·62)
	CyP^m	5·73$\pm$1·84 (0)
	CsA/CyP^m	0·95$\pm$0·09 (83·4)
	CyP^p	5·05$\pm$0·54 (11·7)
	CsA/CyP^p	4·91$\pm$1·20 (14·2)
H. diminuta	—	8·85$\pm$0·98
	CsA	8·31$\pm$1·12 (6·1)
	CyP^m	6·73$\pm$1·24 (23·9)
	CsA/CyP^m	1·27$\pm$1·06 (85·6)
	CyP^p	9·00$\pm$0·74 (0)
	CsA/CyP^p	9·56$\pm$0·65 (0)
Bovine (commercial)	—	57·12$\pm$6·41
	CsA	58·15$\pm$4·57 (0)
	CyP^m	64·58$\pm$3·37 (0)
	CsA/CyP^m	12·48$\pm$6·12 (78·2)
	CyP^p	61·37$\pm$8·33 (0)
	CsA/CyP^p	55·47$\pm$1·57 (2·9)

* CyP^m = Mammalian cyclophilin; CyP^p = parasite (*H. microstoma*) cyclophilin; CaN = calcineurin.

Calcineurin activity of tapeworm calmodulin-binding fraction

Calmodulin-binding proteins from both *H. microstoma* and *H. diminuta* were examined for their ability to catalyse the dephosphorylation of the peptide substrate. Both were able to do so, although at rates lower than bovine calcineurin. In each case, the phosphatase activity was abrogated on removal of calmodulin or calcium from the assay buffer (Table 1).

Kinetic analyses of the parasite calcineurin activity indicated a K_m of 0·63 μM and V_{max} of 0·014 μmol/min/μg protein for *H. microstoma* calcineurin and a K_m of 13·76 μM and V_{max} of 0·174 μmol/min/μg protein for *H. diminuta* calcineurin (Fig. 2).

Inhibition of calcineurin activity

In order to test the hypothesis that the anti-parasitic activity of CsA is mediated via the inhibition of calcineurin by the CsA/CyP complex, we examined the inhibition of calcineurin using combinations of CsA and CyP. Table 2 shows the inhibition characteristics of *H. microstoma* calcineurin. Whilst the activity is strongly (83 %) inhibited by CsA in combination with murine CyP, only a slight inhibition was observed when CsA and parasite CyP were added in combination. There was also slight inhibition with parasite CyP alone. These amounts

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(14·2 and 11·7 %) were too small to be considered as responsible for the anti-parasitic effects of CsA. A similar result was obtained for *H. diminuta* calcineurin (Table 2) where murine CyP in combination with CsA led to 85 % inhibition of calcineurin activity, but parasite CyP/CsA had no significant effect. Table 2 also shows that with bovine calcineurin, the parasite CyP was non-inhibitory in the presence of CsA, while murine CyP/CsA gave 78·8 % inhibition. CsA alone and parasite or mammalian CyP alone were not significantly inhibitory. IC_{50} values for the inhibition of *H. microstoma*, *H. diminuta* and murine calcineurin by mammalian CyP/CsA were similar (0·90, 0·75 and 0·40 μM respectively).

DISCUSSION

In this study, affinity chromatography was used to purify calmodulin-binding proteins that exhibit calcineurin activity. The inhibition of parasite calcineurins by the mammalian CyP/CsA complex confirmed their similarity to mammalian calcineurin. Although kinetic data show that the specific activities of the calcineurins from both *H. microstoma* and *H. diminuta* are lower than that of bovine calcineurin, this may be due to impurities in the preparations. The IC_{50} data suggest that all 3 enzymes have similar affinities for mammalian CyP/CsA complex. The lack of inhibition by parasite CyP of parasite calcineurin was mirrored by the lack of inhibition of mammalian calcineurin, and suggests this is a feature of parasite CyP rather than of calcineurin.

Calcineurin has been demonstrated in many species, including humans, rodents, bovids, *Saccharomyces cerevisiae*, *Naegleria gruberi*, and *Caenorhabditis briggsae* (Klee & Cohen, 1988; Chaudhuri, Hammerle & Furst, 1995; Remillard *et al*. 1995). In all cases, calcineurin acts as a functional phosphatase involved in diverse cellular functions including signal transduction and cation transport (Breuder *et al*. 1994). It also binds the immunosuppressants CsA and FK506, thus interfering with T cell-interactions, as well as neutrophil chemotaxis, stomatal aperture in plants and recovery of yeast from pheromone cell cycle arrest (Kunz & Hall, 1993). Calcineurin consists of 2 subunits, calcineurin A (61000 M_r) containing the active catalytic site, which is also an autoinhibitory domain and the binding site for both calmodulin and calcineurin B (19000 M_r), which contains both the regulatory site and the calcium-binding site (Parsons *et al*. 1994; Sikkink *et al*. 1994). This is the first demonstration of parasite calcineurins, although calmodulin and calmodulin-binding proteins have previously been identified in *H. diminuta* (Eastlake, Branford-White & Whish, 1994).

Several suggestions for the mode of action of cyclosporin in its anti-parasite capacity have been made, including a role for cyclophilin and calcineurin leading to the modulation of protein phosphorylation (Bell, Wernli & Franklin, 1994; Page *et al*. 1995; Bell, Roberts & Chappell, 1996). Since mammalian CyP binds parasite calcineurin, but parasite CyP does not, this suggests that parasite calcineurin is functionally the same as mammalian calcineurin, but parasite CyP is different from its mammalian counterpart. Our data also suggest that CyP binding to calcineurin, when the former is complexed with CsA, may not be the primary mode of anthelmintic action. CsA and CyP bind to the A subunit only in the presence of the B subunit via CsA residues (Parsons *et al*. 1994), although the topological location and conformation of the calcineurin binding site and the exact mechanism for inhibition are not fully understood. It has not been confirmed that cyclophilins play a significant role in mediating the anti-parasite action of CsA, but the data presented here strongly suggest that calcineurin is not involved. Moreover, no clear relationship between the binding of CsA (and derivatives) and anti-parasite activity has been identified (Bell *et al*. 1994; Roberts *et al*., unpublished observations). Thus an alternative mode of action for CsA needs to be explored. This might include competition for cyclophilin binding by alternative proteins, inhibition of P-glycoproteins, interference with parasite development through lipid binding or inhibition of energy metabolism and mitochondrial function. All these postulated mechanisms have precedents (Gaveriaux *et al*. 1991; Furlong, Thibault & Rogers, 1992; Ke *et al*. 1993; Pastorino *et al*. 1993; Steinkasserer *et al*. 1995).

This work was supported by funding from the AFRC (BBSRC; grant number AG1/591) to J. M. S. and L. H. C. The authors would like to thank Professor J. F. Borel (Sandoz, Basle, Switzerland) and Sandoz, UK Ltd for providing the CsA and for their comments on the manuscript and their continued support.

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