A NON-PENETRATING THIOL REAGENT MIMICS CYTOCHALASIN A INHIBITION OF SECRETORY PROTEIN SYNTHESIS.

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SUMMARY

Mimicry of selective cytochalasin A (CA) inhibition of cellulase synthesis in the water mold Achlya by the non-penetrating thiol reagent, p-chloromercuribenzene sulphonate suggests tnat CA may act as a non-permeant sulphydryl reagent, and that inhibition of cellulase synthesis may be exerted via relatively superficial thiol groups in the plasma membrane.

Although Carter has pointed out that the rapidity and rapid reversibility of cell responses to the cytochalasin family of fungal metabolites are consistent with action on the plasma membrane (1), evidence on the location of responses is lacking. In contrast, evidence that cytochalasins act, in certain systems, in the manner of thiol reagents (2, 3) provides clues to a mode of action for these compounds. In a previous note (4) we reported that cytochalasin A (CA) selectively inhibits synthesis of a secretory protein, cellulase, in the water mold Achlya, without affecting general protein synthesis. We now report that CA acts as a thiol reagent in Achlya, and that the non-penetrating thiol reagent, p-chloromercuribenzene sulphonate (pCMBS) mimics CA in its effects.

MATERIALS AND METHODS

Achlya ambisexualis (male strain E 87) mycelia were grown for 4 days in peptone yeast extract glucose (PYG) medium (peptone 1.2 grams; yeast extract 1.2 grams; D-glucose 3.2 grams/1 liter distilled water) aerated with bubbling. Mycelia were harvested by filtration at the time of the experiment.

Cellulase Induction and Assay. A detailed description of cellulase induction and assay is found elsewhere (4). Briefly, after filtration, 0.5

gram quantities of mycelium were incubated in replicates in 5 ml of growth medium. Cellulase induction was achieved by shaking the mycelia in growth medium at room temperature on a reciprocating shaker at 120 cycles/minute. After a 3 hour shake induction, 1 ml samples of medium were assayed viscometrically for cellulase using carboxymethyl cellulose as substrate. One unit of cellulase was defined as reported previously (4).

(^{203}Hg)pCMBS Binding by Mycelia. Binding of p-CMBS by Achlya mycelia was measured using isotopically labeled pCMBS. Fresh mycelia in 0.2 gram quantities were incubated for varying periods of time at room temperature in 5 ml of fresh growth medium containing 0.08 μCi/ml (^{203}Hg)pCMBS (specific activity, 1 mCi/50 mg). Unless otherwise indicated, at the end of the incubation period the mycelia were washed 3 times on fiber glass filters (Whatman) with fresh growth medium. Washing was completed within 3 minutes. Mycelia were homogenized in 3 ml 0.1 N NaOH and a 0.2 ml aliquant of the homogenate was sampled for liquid scintillation counting.

The radioisotopes used were obtained from Amersham/Searle. CA (Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in dimethyl sulphoxide (DMSO) to give a 1% (w/v) stock solution; controls where appropriate, contained DMSO at a concentration of 2% (v/v). All experiments have been repeated one or more times.

RESULTS AND DISCUSSION

Thiol Reagents, Thiols and Cellulase. Thiol inhibition of the CA response, inhibition of cellulase synthesis and secretion, was obtained not only with cysteine (data not shown), but with the relatively non-penetrating thiol (5) glutathione (GSH) (Table 1). In testing thiol reagents for CA-like effects on protein synthesis in Achlya we found that both the relatively penetrating (6) agent N-ethyl maleimide (NEM) (1×10^{-5}M to 5×10^{-3}M) and the relatively non-permeant (7) thiol reagent pCMBS (2.4×10^{-5}M to 4.8×10^{-4}M) inhibited cellulase synthesis and secretion. At 1×10^{-4}M, cellulase secretion in the presence of NEM or pCMBS was 25% and 65% of the control values respectively. Although glutathione eliminated pCMBS inhibition (Table 1), thiol failed to inhibit the NEM response. In contrast to the overall inhibition of protein synthesis elicited by NEM (Table 2), comparable to inhibition by cycloheximide, pCMBS failed to affect overall protein synthesis, as determined by labeled leucine incorporation into mycelial protein. Thus, pCMBS resembles CA in selectively inhibiting cellulase without affecting general protein synthesis.

Localization of pCMBS in Achlya. Incubation of mycelia in fresh growth

TABLE 1

Glutathione (GSH) inhibition of cytochalasin A (CA) and p-chloromercuribenzene sulphonate (pCMBS) responses in Achlya.

Experiment	Treatments	Cellulase Activity Units
	Control	27.4 ± 3.55 [a]
I	CA (4×10^{-5}M)	7.5 ± 0.45
	CA (4×10^{-5}M) + GSH (3.25×10^{-4}M)	30.0 ± 2.05
	Control	18.2 ± 0.70
II	pCMBS (1.92×10^{-4}M)	13.1 ± 0.35
	pCMBS (1.92×10^{-4}M) + GSH (3.25×10^{-4}M)	19.4 ± 0.65

a - mean values $\pm$ standard deviation

All treatments were in duplicate and contained 0.5 gram fresh weight of mycelium in 5 ml PYG medium. Glutathione (GSH) and CA or pCMBS were added to the growth medium simultaneously at the start of the experiment. CA and pCMBS produced cellulase levels different from controls ($P < 0.01$). This difference was eliminated in the presence of added GSH. GSH alone had no effect on cellulase.

medium containing (^{203}Hg)pCMBS resulted in rapid binding of radioactivity which attained a maximum level within 1 hour (Fig. 1). Further incubation, up to 3 hours, was not accompanied by any substantial increase in bound radioactivity. Labeled mycelium, washed and subsequently incubated in fresh growth medium shows no loss of bound radioactivity (Fig. 2). A similar treatment with a normally inhibitory concentration of pCMBS, followed by a wash and replacement in fresh medium has no significant inhibitory effect on

TABLE 2

Effects of a penetrating (NEM) and a non-penetrating (pCMBS) thiol reagent, and cycloheximide on (^{14}C)leucine incorporation into mycelial protein.

Experiment	Treatments	Mycelial Protein c.p.m./mg after 3 Hours
I	Control	2604 ± 133 [a]
	pCMBS (2.4×10^{-4}M)	2660 ± 15 NS
	Cycloheximide (7×10^{-5}M)	720 ± 68
II	Control	755 ± 50
	NEM (4×10^{-4}M)	447 ± 22 *
	Cycloheximide (7×10^{-5}M)	407 ± 19

a - mean values $\pm$ standard deviation

* - $P < 0.05$

NS - no significant difference

All treatments were in duplicate, and contained 0.5 gram fresh weight of mycelium in 5 ml of PYG medium containing 0.5 mM leucine. The specific activities of the L-U(^{14}C)leucine used in these experiments were 8.7×10^3 c.p.m./u mole and 1.3×10^3 c.p.m./u mole respectively. Radioactivity was determined by liquid scintillation counting as reported previously (4). In Achlya, cycloheximide is a known inhibitor of protein, including cellulase, synthesis (8).

cellulase induction and secretion (results not shown), indicating that pCMBS inhibition of cellulase responses is not mediated by tightly bound reagent. From Fig. 2 it is clear that about 70 percent of the bound radioactivity is lost within 5 minutes of treatment with the thiol dithiothreitol (DTT) or with unlabeled pCMBS, indicating that pCMBS acts in Achlya as in other systems (7), as a relatively non-penetrating reagent.

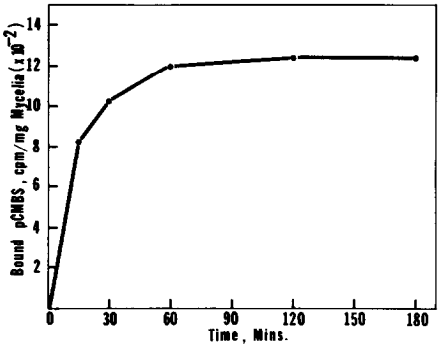

Fig. 1. Binding of pCMBS by Achlya mycelia. Fresh mycelia in 0.2 gram quantities were incubated in duplicates for varying periods of time at room temperature in 5 ml of fresh growth medium containing 0.08 uCi/ml (^{203}Hg)pCMBS (1 mCi/50 mg). After incubation, the mycelia were washed 3 times with fresh growth medium and the associated radioactivity was determined as described under Materials and Methods.

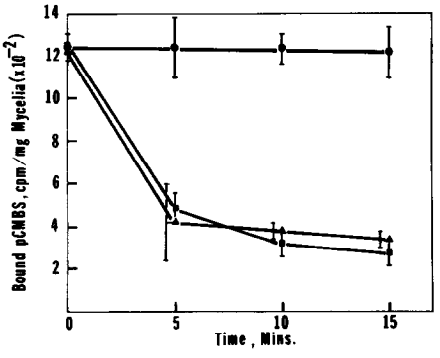

Fig. 2. Elution of pCMBS from Achlya mycelia. Fresh mycelia in 0.2 gram quantities were pre-incubated in fresh growth medium containing 0.08 uCi/ml (^{203}Hg)pCMBS (1 mCi/50 mg) at room temperature for 1 hour and then washed 3 times with fresh medium to remove unbound pCMBS. The washed mycelia were transferred to fresh growth medium (●——●), or fresh growth medium with 1 mM unlabeled pCMBS (■——■), or with 1 mM dithiothreitol (DTT)(▲——▲). After incubation periods of varying times, mycelia were harvested by filtration and the associated radioactivity was determined as described in Materials and Methods. Each point represents the mean value of duplicate treatments; vertical bars denote 2 x standard deviation.

CONCLUSIONS

Together the facts that the CA response in Achlya is eliminated by glutathione, a non-penetrating thiol, that CA fails to inhibit a thiol reagent sensitive system such as overall mycelial protein synthesis, and

that the relatively non-penetrating thiol reagent pCMBS mimics the CA response, selectively inhibiting secretory protein synthesis without affecting general protein synthesis, indicate that CA may act in Achlya as a non-penetrating thiol reagent, and that inhibition of cellulase synthesis may be exerted via relatively superficial thiol groups in the plasma membrane.

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