

AUTOLYSIS OF MEMBRANE LIPIDS IN POTATO LEAF HOMOGENATES: EFFECTS OF CALMODULIN AND CALMODULIN ANTAGONISTS

Potato leaves contain high levels of lipolytic acyl hydrolase activity which degrades phospholipids and galactolipids during homogenization and organelle isolation. Four calmodulin antagonists (dibucaine, tetracaine, trifluoperazine and chlorpromazine) were found to inhibit the rate of hydrolysis of endogenous membrane lipids in homogenates of potato leaves. In contrast, the addition of calcium and purified calmodulin stimulated the rate of hydrolysis. These results indicate that a lipolytic acyl hydrolase activity in potato leaves appears to be mediated either directly or indirectly by calcium and calmodulin.

Key words: phospholipase; membranes; calmodulin; potato; *Solanum tuberosum*

Introduction

During the homogenization of potato leaves at 0–4°C, there is a rapid breakdown of endogenous membrane lipids which is catalyzed by lipolytic acyl hydrolase [1,2]. This enzymatic activity exhibits both phospholipase activity and galactolipase activity [2,3]. The local anesthetic, dibucaine, has been used to inhibit phospholipase activity in purified cauliflower mitochondria [4] and potato tuber organelle fractions [5]. Our preliminary studies revealed that dibucaine also inhibited phospholipase activity in potato leaf homogenates. Because dibucaine is also a calmodulin antagonist, the following experiments were designed to investigate the hypothesis that calmodulin may regulate the activity of a lipolytic acyl hydrolase in potato leaves. Prior to this report, only five calmodulin-dependent plant enzymes have been identified [6].

Abbreviations: BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PC, phosphatidylcholine; TLC, thin-layer chromatography.

Materials and methods

Materials

Seed potato tubers (cv. Kennebec) were planted in 6-inch clay pots in commercial potting soil. Plants were grown in a greenhouse with supplemental light from four 34-W fluorescent lamps. Calmodulin (bovine brain) was obtained from BioRad. All other reagents were obtained from Sigma.

Lipid analysis of leaf homogenates

Homogenates were prepared by grinding 5 g of leaves (3–4 cm in length) with 20 ml of a solution containing 0.3 M sucrose, 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 2 mM EDTA, 5 mM dithiothreitol, and 5 mM β -mercaptoethanol in a chilled mortar and pestle. The homogenate was filtered through two layers of cheese cloth and divided into 5-ml aliquots. For some studies the various potential inhibitors were added to some of the aliquots. All aliquots were vortexed and incubated in a 4°C water bath. Triplicate 1-ml samples were removed immediately

(0 h) and after 4 h. The reactions were stopped by adding 50 μ l of glacial acetic acid. Lipids were extracted with 7 ml of 3:2 hexane/isopropanol (v/v) and 5 ml of 6.7% (w/v) Na_2SO_4 , spotted on 250- μ Silica gel G TLC plates, developed in 85:15:10:3.5 (by vol) chloroform, methanol, acetic acid, water and visualized with I_2 . The spots which co-chromatographed with phosphatidylcholine (PC) standards were scraped from the thin-layer chromatography (TLC) plates and subjected to analysis for total phosphorous [7]. The rate of hydrolysis was expressed as the percent of the total PC (average = 0.8 to 1.1 μ mol/g fresh wt.) which was hydrolyzed in 4 h at 4°C.

Results

When potato leaves were homogenized at pH 7.5 and the filtered homogenates incubated at 4°C for 4 h, 22.5% of the original

PC was hydrolyzed (Table I). This rate is consistent with the published results of Rodionov et al. [1] and ourselves [2]. Although we only chose to quantitate the levels of PC (because it is the most abundant phospholipid in potato leaves), comparable rates of hydrolysis of other phospholipids and galactolipids were observed in our thin-layer chromatographic separations. Since no accumulation of phosphatidic acid or lysophospholipids was observed with any of our treatments, a lipolytic acyl hydrolase activity [2,3] was probably responsible for the PC hydrolysis. Because we were interested in identifying a chemical inhibitor which could be routinely used to inhibit membrane lipid breakdown, four compounds which have been reported to inhibit similar enzymes (bovine serum albumin (BSA), bromophenacyl bromide, phenyl methyl sulfonyl fluoride (PMSF), and dibucaine) [4,5,8–10] were tested (Table I). Among these four com-

Table I. Effect of various potential inhibitors on the rate of PC hydrolysis in potato leaf homogenates

Treatment	% of original PC hydrolyzed during 4 h at 4°C
Control (buffer contained	
2 mM EDTA)	22.5 \pm 2.1 ^a
1% BSA	22.3 \pm 1.3
1 mM phenylmethylsulfonyl fluoride ^b	27.8 \pm 0.9
100 μ M bromophenacyl bromide ^b	24.1 \pm 1.6
2 mM dibucaine	11.0 \pm 0.8
2 mM tetracaine	18.6 \pm 2.2
2 mM lidocaine	27.2 \pm 1.3
2 mM procaine	28.0 \pm 2.4
100 μ M trifluoperazine	15.1 \pm 2.0
100 μ M chlorpromazine	16.5 \pm 1.7

^aData reported are the means of triplicate analyses \pm S.D.

^bThese compounds were added in ethanol to a final concentration of 1% (v/v) ethanol; controls showed that this concentration of ethanol was without effect.

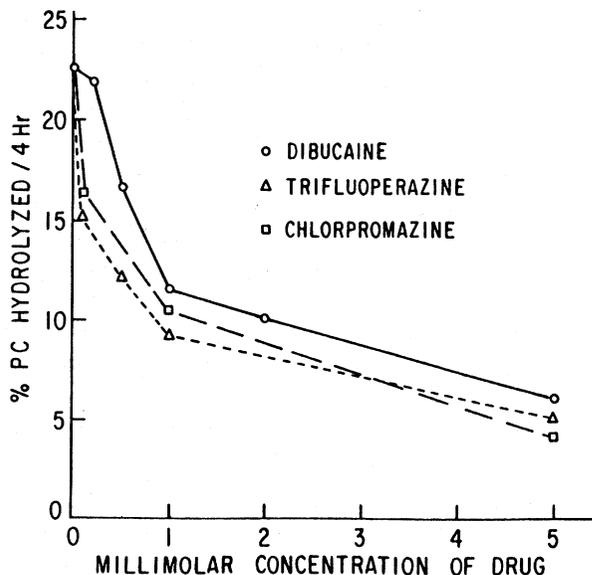


Fig. 1. Effect of various concentrations of calmodulin antagonists on the rate of hydrolysis of endogenous PC in homogenates of potato leaves.

pounds, only dibucaine inhibited (by about 50%) the rate of hydrolysis of PC in leaf homogenates. When three other local anesthetics were tested (tetracaine, lidocaine, and procaine) only tetracaine was inhibitory. Because both dibucaine and tetracaine are calmodulin antagonists [11] we chose to test two other common calmodulin antagonists (trifluoperazine and chlorpromazine) which are usually inhibitory at much lower concentrations. At 100 μ M, chlorpromazine and trifluoperazine inhibited PC hydrolysis by 27 and 33%, respectively. Various concentrations of dibucaine, trifluoperazine, and chlorpromazine were then compared for their ability to inhibit PC hydrolysis in potato tuber homogenates (Fig. 1). At low concentrations (100–200 μ M) trifluoperazine and chlorpromazine were more effective inhibitors than dibucaine, but at high concentrations (1–5 mM), all three were almost equally effective.

In order to investigate whether the inhibition which we observed with the calmodulin

antagonists actually involved calmodulin or was due to the general hydrophobic properties of these drugs [11], the direct effects of calcium and exogenous calmodulin were measured (Table II). Comparable results were obtained when leaf homogenates were incubated at 4°C for 4 h or 25°C for 1/2 h. The addition of 5 mM CaCl₂ to leaf homogenates caused a 46% stimulation of the rate of hydrolysis. The combination of calcium and 1 μ M calmodulin (bovine brain) caused an 80% stimulation of the rate of PC hydrolysis. The calcium and calmodulin stimulation was completely overcome by adding 100 μ M chlorpromazine. A slight inhibition (7%) was observed when calcium ionophore (A23187) was added. The deletion of EDTA from the homogenization buffer (control No. 2) had little or no effect on the rate of autolytic hydrolysis of PC. The subsequent addition of calmodulin (in the absence of exogenous calcium) stimulated PC hydrolysis by more than 50%. This experiment indicates that the degree of calmodulin stimulation was the same in the presence of endogenous levels of Ca²⁺ or 5 mM exogenous Ca²⁺ (actually about 3 mM when the presence of EDTA is considered).

Table II. Effect of calcium and calmodulin on the rate of PC hydrolysis in potato leaf homogenates.

Treatment	% of original PC hydrolyzed during	
	4 h at 4°C	1/2 h at 25°C
Control 1 (buffer contained 2 mM EDTA)	18.3 ± 2.3 ^a	14.6 ± 1.3
+5 mM CaCl ₂	26.7 ± 1.7	24.1 ± 1.1
+5 mM CaCl ₂ + 1 μ M calmodulin	32.8 ± 2.2	32.4 ± 1.8
+5 mM CaCl ₂ + 1 μ M calmodulin + 100 μ M chlorpromazine	18.1 ± 0.8	14.3 ± 0.9
+100 μ M Ca ionophore A23187 ^b	17.0 ± 1.3	13.8 ± 1.5
Control no. 2 (buffer contained no EDTA)	18.7 ± 1.9	14.3 ± 0.9
+1 μ M calmodulin	29.0 ± 0.4	22.4 ± 1.1

^aData reported are the means of triplicate analyses ± S.D.

^bSee footnote b of Table I.

Discussion

This report has provided the first evidence that the rate of autolysis of membrane lipids in potato leaves is apparently stimulated by calmodulin and inhibited by calmodulin antagonists. We are currently conducting experiments to determine whether calmodulin binds directly to the lipolytic enzyme, or stimulates indirectly. An indirect mechanism of calmodulin regulation, via phosphorylation by a protein kinase was recently exhibited by another plant enzyme, Quinate: NAD⁺ oxidoreductase [6]. Since the exogenous calmodulin used in this study was obtained from bovine brain, it is conceivable that the degree of stimulation may be different if potato calmodulin is used. Even though calmodulin has been structurally conserved

and functionally preserved throughout the plant and animal kingdoms, small differences in the level of enzymatic stimulation have been reported using calmodulin from different species [6].

Further experimentation is also required in order to learn whether the leaves of other plant species contain comparable lipolytic enzymes which are also regulated by calmodulin. Leshem et al. [12] recently showed that the application of calcium ionophore (A23187) to pea leaves promoted membrane deterioration and treatment of the tissue with fluo-phenazine delayed membrane deterioration. Based on these observations they predicted that calmodulin mediates the action of phospholipase A₂ on membranes during the senescence of pea foliage.

Although this is the first report of a lipolytic enzyme in plants which is mediated by calmodulin, phospholipase A₂ activity in human platelets was previously shown to be stimulated by calmodulin and inhibited by calmodulin antagonists [13]. That preliminary report has not yet been confirmed [14]. In contrast, pancreatic phospholipase A₂ was also shown to be inhibited by calmodulin antagonists, but the addition of exogenous calmodulin had no effect [15].

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