Phytochemistry, Vol. 24, No. 11, pp. 2555–2558, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00
Pergamon Press Ltd.

DIBUCAINE, CHLORPROMAZINE, AND DETERGENTS MEDIATE MEMBRANE BREAKDOWN IN POTATO TUBER HOMOGENATES

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(*Received* 2 *January* 1985)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato tubers; calmodulin; calmodulin antagonists; dibucaine; lipolytic acyl hydrolase; membranes.

Abstract—Various strategies were evaluated for their ability to minimize the rate of breakdown of endogenous membrane lipids during cell fractionation studies with potato tubers. Buffering the homogenates at pH 7.5 to 8.0 resulted in significantly lower rates of phosphatidylcholine (PC) hydrolysis than were observed at lower pH values. Several potential inhibitors were added to homogenates to evaluate their ability to inhibit membrane lipid hydrolysis. The addition of bovine serum albumin (1% w/v) inhibited the rate of PC hydrolysis by 50%. The addition of low concentrations (25–100 μM) dibucaine (nupercaine) inhibited PC hydrolysis, but at higher concentrations (1–2 mM) it caused a 5- to 6-fold stimulation. Because dibucaine is a calmodulin antagonist, two other calmodulin antagonists (trifluoperazine and chlorpromazine) were tested and found to exhibit similar patterns of inhibition and stimulation. Similarly, the addition of low concentrations of deoxycholate also inhibited PC hydrolysis and high concentrations stimulated it. These results indicate that the hydrophobic properties of deoxycholate, dibucaine, and other calmodulin antagonists may explain their unusual effects on the rates of PC hydrolysis in potato tuber homogenates. Although the addition of exogenous calcium increased the rate of PC hydrolysis, the addition of calmodulin (bovine brain) had no effect. Other experiments revealed that the addition of 1% bovine serum albumin improved the yield and stability of mitochondrial and microsomal fractions from potato tubers. In constrast, the addition of 100 μM dibucaine caused deleterious effects.

INTRODUCTION

In 1970, Galliard [1] reported that there was a rapid breakdown of membrane lipids in homogenates of potato tubers. In order to control the breakdown of membrane lipids during cell fractionation studies, Galliard [2] recommended that the following precautions be taken: choose rapid isolation techniques, control temperature at 0–4°, buffer at pH 7.5 to 8.0, and add 0.1 to 1.0% bovine serum albumin to the homogenization buffer. Pun *et al.* [3] presented evidence which suggested that dibucaine (nupercaine) was an effective inhibitor of the lipolytic acyl hydrolase activity in potato tubers when added to the homogenization buffer. This observation was recently challenged by Bishop and Oertle [4] whose data suggested that dibucaine may only inhibit lipoxygenase activity in potato tubers and actually stimulate the lipolytic acyl hydrolase activity.

This study was undertaken in order to evaluate the effectiveness of buffers, dibucaine, bovine serum albumin, and other reagents as potential inhibitors of lipolytic acyl hydrolase in potato tuber homogenates. Because we measured the direct effect of potential inhibitors on the levels of endogenous phospholipids in the absence of detergents, this approach offers several advantages over the two previous studies [3, 4]. Since dibucaine is a calmodulin antagonist [5], we also investigated the possibility that calmodulin may be involved in this enzymatic reaction.

RESULTS

The first set of experiments (Fig. 1) was designed to determine whether the pH of the homogenization buffer significantly effected the rate of breakdown of endogenous phospholipids in filtered homogenates of potato tubers. At pH 5.5 and 4°, there was a very rapid breakdown of phospholipid as initially reported by Galliard [1]. However, at pH 6, 7 and 8, the rates of PC hydrolysis were reduced to 20, 9 and 4% per hr, respectively. Although we only chose to quantitate the levels of PC (because it is the most abundant phospholipid in potato tubers), comparable rates of hydrolysis of other phospholipids and galactolipids were observed in our TLC separations.

In order to evaluate the effectiveness of the various potential inhibitors, filtered tuber homogenates were buffered at pH 7.5 and incubated at 4° for 4 hr. The composition of the grinding buffer, the temperature, and the time period were comparable to those of most subcellular fractionation studies [6]. Under these conditions, 15% of the original level of PC was hydrolysed in 4 hr at 4° (Table 1). The addition of 1% bovine serum albumin (fat-free) inhibited the rate of hydrolysis by 50%. The addition of phenylmethylsulphonyl fluoride (1 mM), an inhibitor of many proteases and esterases [7], caused a slight stimulation in the rate of hydrolysis. However, when dibucaine (2 mM) was added, there was a 6-fold stimulation in hydrolysis. Because dibucaine caused such a dramatic stimulation, three other local anesthetics were also tested at the same concentrations. Tetracaine and procaine significantly stimulated the rates of hydrolysis, but lidocaine had no effect. Because dibucaine and tetracaine are calmodulin antagonists [5], we decided to test two other such compounds. The addition of either

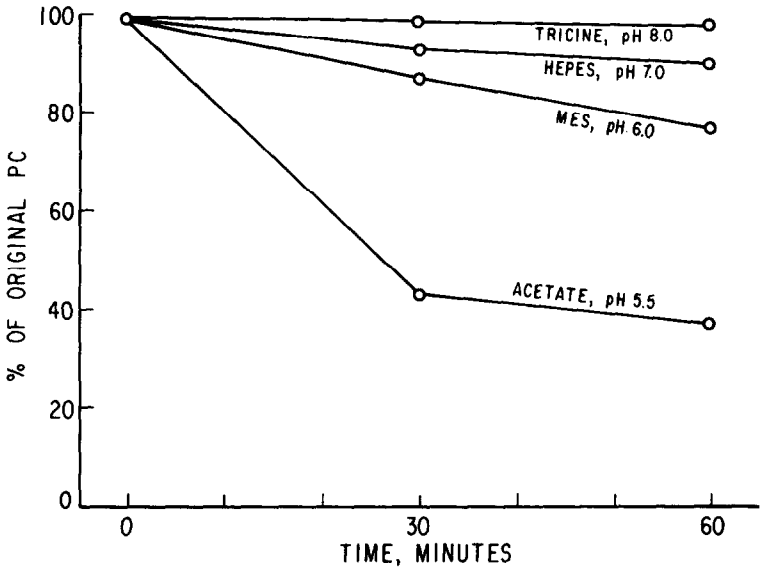

Fig. 1. Effect of the pH of the grinding buffer on the rate of PC hydrolysis in homogenates of potato tubers. After homogenization, the pHs of the homogenates were 7.81 for Tricine, 6.88 for HEPES, and 6.02 for MES. NaOH was the counter ion in each buffer system.

Table 1. Effect of various potential inhibitors on the rate of PC hydrolysis in potato tuber homogenates

Treatment	% of original PC hydrolysed during 4 hr at 4°
Control (2 mM EDTA)	15.0 ± 0.9
1 % bovine serum albumin	7.5 ± 0.5
1 mM phenylmethylsulfonyl fluoride	18.9 ± 1.1
2 mM dibucaine	89.3 ± 3.2
2 mM tetracaine	22.7 ± 1.3
2 mM lidocaine	15.2 ± 1.2
2 mM procaine	25.9 ± 0.8
100 μM trifluoperazine	52 ± 2.4
100 μM chlorpromazine	47 ± 2.7

Data represents the mean of triplicate samples ± standard deviation.

trifluoperazine (100 μM) or chlorpromazine (100 μM) more than tripled the rate of PC hydrolysis.

The effect of various concentrations of dibucaine, chlorpromazine and trifluoperazine on the rate of PC hydrolysis was tested (Fig. 2). Although high concentrations (1–2 mM) of dibucaine greatly stimulated the rate of PC hydrolysis (as was shown in Table 1), low concentrations (25–100 μM) actually inhibited the rate of hydrolysis by more than 50%. Both chlorpromazine and trifluoperazine produced concentration curves of the same shape as dibucaine, but shifted to much lower concentrations. Chlorpromazine and trifluoperazine were inhibitory at very low concentrations (1–5 μM) and dramatically stimulated PC hydrolysis at high concentrations (> 100 μM).

The unusual concentration-dependent effects (Fig. 2) caused by the three calmodulin antagonists indicated that either calmodulin is involved in regulating the rate of phospholipid hydrolysis or the effects are caused by the hydrophobic nature of the drugs [6, 8]. In order to investigate the former possibility, calcium and purified calmodulin were added to tuber homogenates (Table 2). Although the addition of calcium more than doubled the rate of PC hydrolysis, calmodulin (bovine brain) had no effect on the rate of PC hydrolysis. To investigate the latter possibility (that the effects caused by the calmodulin antagonists are due to their hydrophobic nature), we measured the effect of two other surfactant reagents (Table 2). Triton X-100 and deoxycholate both stimulated the rate of PC hydrolysis. When several other concentrations of deoxycholate were tested (Fig. 3), the resulting curve very much resembled those curves which were produced by the calmodulin antagonists (Fig. 2). Low concentrations (10–50 μM) of deoxycholate inhibited PC hydrolysis and high concentrations (0.5–1.0 mM) stimulated it. The similarities in shape and concentration-dependence caused by dibucaine (Fig. 2) and deoxycholate (Fig. 3) is striking. These results suggest that the hydrophobic nature of dibucaine may cause its unusual effects on the rate of PC hydrolysis in tuber homogenates.

Because BSA (Table 1) and low concentrations of dibucaine (Fig. 2) did significantly inhibit the rate of PC hydrolysis in potato tuber homogenates, an experiment was designed to test their effectiveness in preserving membrane phospholipids during subcellular fractionation (Table 3). The presence of 1 % BSA in the grinding buffer appeared to increase the amount of PC in freshly prepared (0 hr) mitochondria-enriched fractions. The presence of BSA in the resuspended particulate fractions also appeared to protect the membrane lipids in both fractions (especially the microsomes) from hydrolysis during storage (24 hr at 4°). In contrast, the incorporation of dibucaine (100 μM) in the grinding buffer resulted in a lower initial yield of membrane lipid and a higher rate of PC hydrolysis during storage.

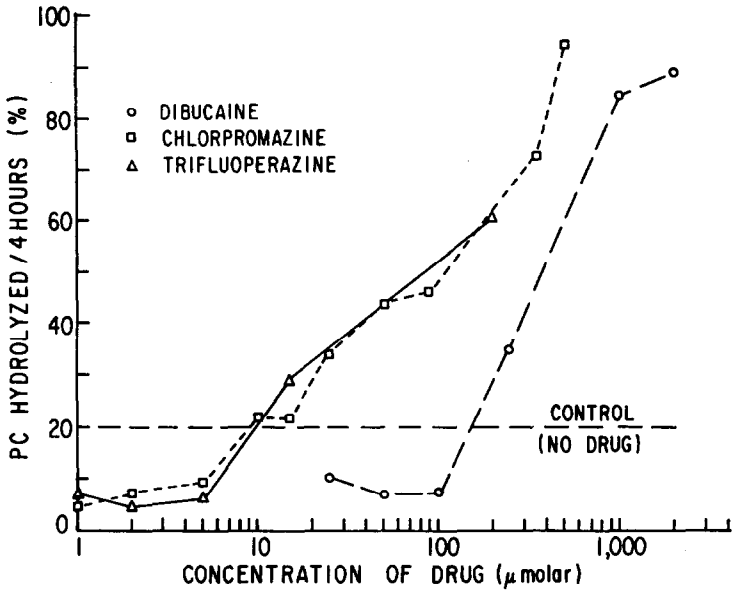

Fig. 2. Effect of various concentrations of calmodulin antagonists on the rate of PC hydrolysis in homogenates of potato tubers.

Table 2. Effect of calcium, calmodulin, and detergents on the rate of PC hydrolysis in potato tuber homogenates

Addition	% of original PC hydrolysed during 4 hr at 4°
Control (2 mM EDTA)	14.7 ± 1.4
5 mM $CaCl_2$	36.5 ± 1.9
5 mM $CaCl_2$ + 1 μM calmodulin	35.5 ± 0.8
0.1 % Triton X-100	95.9 ± 3.0
1 mM deoxycholate	56.3 ± 1.6

Data represents the mean of triplicate samples ± standard deviation.

DISCUSSION

This study revealed that dibucaine causes very unusual concentration-dependent effects on the lipolytic enzymes in potato tubers. These effects do not seem to be due to the local anesthetic or calmodulin antagonist properties of the

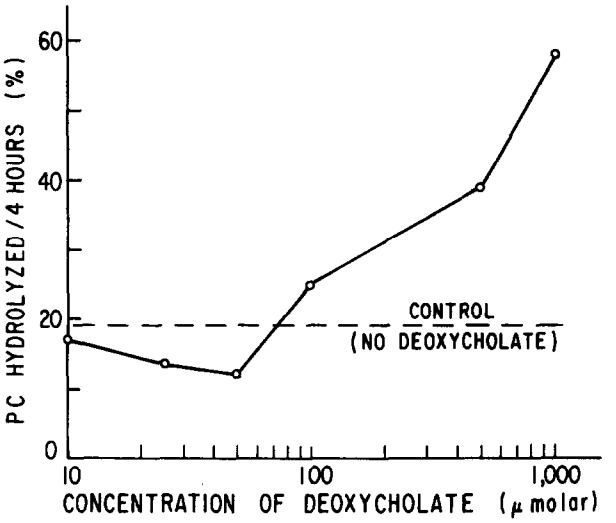

Fig. 3. Effect of various concentrations of sodium deoxycholate on the rate of PC hydrolysis in homogenates of potato tubers.

Table 3. Effect of the addition of 1 % BSA or 0.1 mM dibucaine to grinding buffer during the preparation of subcellular fractions from potato tubers

Composition of grinding buffer	nmol PC/fraction			
	Mitochondria-enriched fraction		Microsomal fraction	
	0 hr	24 hr	0 hr	24 hr
Control	769 ± 51	622 ± 40	2026 ± 91	1752 ± 9
1 % BSA	1161 ± 45	971 ± 41	2029 ± 31	2043 ± 11
0.1 mM dibucaine	741 ± 28	385 ± 4	1674 ± 60	1110 ± 52

Data represent the mean of triplicate samples ± standard deviation.

drug, since similar responses were observed using deoxycholate. The addition of exogenous calmodulin had no effect on the rate of membrane breakdown in homogenates of potato tuers, unlike our recent finding that calmodulin stimulated the rate of lipid breakdown in homogenates of potato leaves [9]. The results of this study may resolve the conflicting effects caused by dibucaine in the previous two studies with potato tubers [3,4]. Pun *et al.* [3], using 50 μM dibucaine, observed that it inhibited lipolysis in tuber preparations, and Bishop and Oertle [4], using 0.5 mM dibucaine, observed that it stimulated hydrolysis. Both of these reports are consistent with our results (Fig. 2).

Although the addition of dibucaine during homogenization may be beneficial with some types of plant tissue, our results (Table 3) indicate that dibucaine (100 μM) has deleterious effects on subcellular fractions from potato tubers. We do not know why 100 μM dibucaine should inhibit lipolysis in tuber homogenates (Fig. 2) and stimulate lipolysis in subcellular fractions obtained by differential centrifugation (Table 3). It is possible that the presence of lower concentrations of dibucaine may be beneficial during differential centrifugation. Perhaps the ratio of inhibitor molecules to membrane lipids is more important than the concentration of inhibitor. However, these results clearly indicate that this drug should only be used after its utility has been clearly established for each type of experimental conditions. In our study, the addition of BSA (1%) did seem to increase the yield and stability of subcellular fractions. Although dibucaine (150 μM) was shown to effectively inhibit phospholipase A_2 activity in isolated cauliflower mitochondria [10], there have been no other reports of its effect on other plant tissues. We are currently investigating the effect of dibucaine on the rate of membrane lipid degradation in homogenates of other plant tissues.

EXPERIMENTAL

Materials. Seed potato tubers (cv Kennebec) were stored at 10° and removed immediately before use. Trifluoperazine was a gift from Smith, Kline, and French (Philadelphia). Calmodulin (bovine brain) was obtained from Bio Rad. All other reagents were obtained from Sigma.

Lipid analysis of leaf homogenates. Homogenates were usually prepared by grinding 50 g peeled and diced tuber with 100 ml 0.3 M sucrose, 0.1 M HEPES buffer pH 7.5, 2 mM EDTA, 5 mM dithiothreitol, and 5 mM β-mercaptoethanol in a chilled Waring blender. The homogenate was filtered through two layers of cheesecloth and divided into 15 ml aliquots. For some studies, various potential inhibitors were added to some of the aliquots at this point. All aliquots were vortexed and incubated in a 4° water bath. Triplicate 4-ml samples were removed immediately (0 hr) and after the desired time of incubation. The reaction was stopped by adding 200 μl glacial acetic acid and the lipids were extracted with 7 ml 3:2 hexane–isopropanol and 5 ml 6.7% Na_2SO_4. Lipid samples were spotted on 250 μ silica gel G TLC plates, developed in $CHCl_3$–MeOH–HOAc–H_2O (85:15:10:3.5), and visualized with I_2. The spots which cochromatographed with authentic phosphatidylcholine were scraped from the plates and subjected to phosphorus analysis [11]. The rate of hydrolysis was expressed as the percent of the original (0 hr) PC which was hydrolysed in 4 hr at 4°.

Differential centrifugation study. Tubers were homogenized in grinding buffer containing either 1% BSA or 0.1 mM dibucaine. The filtered homogenates were centrifuged at 2000 *g* for 7 min to remove starch bodies and cell walls. The 2000 *g* supernatant was then centrifuged at 15 000 *g* for 15 min to obtain a mitochondria-enriched pellet. The 15 000 *g* supernatant was centrifuged at 100 000 *g* for 1 hr to obtain the microsomal fraction. The 15 000 *g* and 100 000 *g* pellets were each resuspended by adding 2 ml of the original grinding buffer (which included BSA or dibucaine, as noted). The resuspended pellets were incubated in a 4° water bath. Samples were removed at 0 hr and 24 hr and lipids were extracted, separated and analysed as described above.

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