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Does cholinesterase participate in the intercellular interactions in pollen-pistil system?

L.V. KOVALEVA and V.V. ROSHCHINA*

Institute of Plant Physiology, Russian Academy of Sciences, 127276 Moscow, Russia
Institute of Cell Biophysics, Russian Academy of Sciences, 142292 Pushchino, Russia *

Abstract

Hydrolysis of acetylthiocholine and butyrylthiocholine has been observed in aqueous extracts from petunia pollen and pistils. The reproductive organs of self-compatible clone showed a higher rate of choline ester hydrolysis than those of self-incompatible clone. The highest rate of acetylthiocholine hydrolysis blocked by the cholinesterase inhibitors (physostigmine and neostigmine) was characteristic for the pollen of self-compatible clone. The incomplete (25 - 40 %) inhibition of hydrolysis in pistil extracts of self-compatible clone suggests the presence of unspecific esterases. The eight-fold lower hydrolysis was observed in the pistils of self-incompatible clone as compared to the pistils of compatible clone; neostigmine completely blocked this low hydrolytic activity. The treatment of flower buds with physostigmine and neostigmine (10^{-5} - 10^{-3} M) decreased the seed production by 10 - 20 % in compatible clone. When the surfaces of pistil stigmae were treated with physostigmine and neostigmine (10^{-5} - 10^{-3} M) before pollination, the seed formation was inhibited by 95 % after both self- and cross-pollination.

Additional key words: acetylthiocholine, butyrylthiocholine, *Petunia hybrida* L., self-compatible and self-incompatible clones, neostigmine, physostigmine

Introduction

Cholinesterase, a component of signalling cholinergic system in animals, was found also in plant cells (Roshchina and Semenova 1990, Roshchina 1991). The cholinesterase activity in pollen of *Vicia faba* was firstly evidenced by histochemical method (Bednarska 1992). Biochemically similar activity has been found in the homogenates from anthers of *Hippeastrum*, *Clivia* and *Gladiolus* (Semenova and Roshchina 1993) and in aqueous extracts from pollen of 13 plant species (Roshchina

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et al. 1994). Cholinesterase is localized in the exine of pollen grain. The enzyme is supposed to participate in fertilization (Roschina *et al.* 1994).

The aim of the present paper was to study cholinesterase activity in the reproductive organs of petunia during pollination and fertilization. To investigate the contribution of cholinesterase to pollination and fertilization, we measured cholinesterase activity itself and estimated the action of cholinesterase inhibitors on the seed formation after flower organs treatment.

Materials and methods

We studied the pollen and pistils in petunia (*Petunia hybrida* L.) plants cultivated in a greenhouse. Three clones differing in functional activity of male gametophyte were investigated. The seeds are produced by self-compatible clone after both self- and cross-pollination. In the self-incompatible clone, a male gametophyte inhibition occurs which results in seed production only after cross-pollination. In sterile clone (male sterility), the seeds are also formed only after cross-pollination.

The cholinesterase activity was measured in extracts from pollen or pistils (extracted for 1 h in 20 mM K-phosphate buffer (pH 7.4) at 0 °C and then filtered). Cholinesterase activity was measured as the rate of thiocholine formation after thiocholine ester hydrolysis by the colour reaction with Ellman reagent (DTNB - 5,5"-dithio-bis-nitrobenzoic acid). The procedure modified by Gorun *et al.* (1978) was described elsewhere (Roshchina *et al.* 1994). Acetylthiocholine and butyrylthiocholine were used as the substrates in the reaction (60 min at 25 °C). The reaction medium (0.2 cm^3) contained 0.05 cm^3 of protein extract, 0.149 cm^3 of 0.1 M K-phosphate or Na/K-phosphate buffer (pH 7.4) and 0.001 cm^3 of substrate. The reaction was blocked by the addition of 1.8 cm^3 of DTNB. The absorbance of yellow product was recorded at 412 nm with a spectrophotometer *Perkin-Elmer* or *Specord*

Table 1. The absorbance (A_{412}) before and after 1-h exposure of control 1 (protein extract + DTNB without any substrate) and experimental samples (protein extract + DTNB + acetylthiocholin 10^{-3}M) ($n = 5$).

Variant		Pollen A_{412}	ΔA_{412}	Pistils A_{412}	ΔA_{412}
Self-compatible clone (yellow)					
Control	0 min	0.435±0.002		0.023±0.001	
	60 min	0.440±0.001	+0.005	0.022±0.001	-0.001
Experimental	0 min	0.430±0.002		0.024±0.001	
sample	60 min	0.550±0.001	+0.120	0.081±0.001	+0.057
Self-incompatible clone					
Control	0 min	0.064±0.001		0.177±0.001	
	60 min	0.065±0.001	+0.001	0.176±0.001	-0.001
Experimental	0 min	0.062±0.002		0.178±0.001	
sample	60 min	0.018±0.001	+0.056	0.197±0.001	+0.019

M-40. There were two controls - control 1 (protein extract + DTNB without any substrate) and control 2 (a substrate + DTNB). During the exposure from 0 to 60 min the absorbance changes (against 0.1 M K-phosphate buffer as a standard) in control 1 (Table 1) and control 2 were about 0.001 that is out of the range of apparatus sensitivity, and it is insignificant for the enzyme activity calculation. Therefore, the absorbance associated with the substrate hydrolysis was suitable to measure in differential regime - experimental cuvette against control. The rate of the substrate hydrolysis was calculated on the bases of the measurement of the absorbance and expressed in μmol kg^{-1}(f.m.) s^{-1} according to Gorun *et al.* (1978).

The treatment of petunia flowers with the cholinesterase inhibitors physostigmine and neostigmine (10^{-3} - 10^{-6} M) was carried out as follows: the inhibitor solutions were either injected (2 - 3 times) into the flower buds before their opening or were loaded on the stigma surface of open flowers an hour prior to pollination (self- or cross-pollination). After a month, the seed production was estimated.

Results

The hydrolysis of acetylcholine was observed in the extracts from both pollen and pistils (Table 2). The participation of cholinesterase was evidenced from the significant decrease in rate of acetylthiocholine degradation by the inhibitors of animal cholinesterases, physostigmine and neostigmine (Table 2). In the extracts

Table 2. The maximal rate of acetylthiocholine hydrolysis (V_{max}) in water extracts from petunia pollen and pistils (concentrations of physostigmine and neostigmine 10^{-5} M; n = 5, S.E. = 2 - 3 %).

Plant material	V_{max} [μmol kg^{-1}(f.m.) s^{-1}]		
	control	+ physostigmine	+ neostigmine
Self-compatible clone (yellow pollen)			
Pollen	1400	93	0
Pistils	492	307	330
Self-compatible clone (blue pollen)			
Pollen	800	62	86
Pistils	412	310	320
Self-incompatible clone (yellow pollen)			
Pollen	330	280	175
Pistils	50	22	0

from petunia pollen, the rate of choline ester hydrolysis was not inhibited by high substrate concentrations (Fig.1). The reaction rate was 3 - 4 times higher with acetylcholine than with butyrylthiocholine. The Michaelis constants (Km) were 6×10^{-4} M with acetylthiocholine and 7×10^{-4} M with butyrylthiocholine, respectively, for the enzymatic reaction inhibited by heating at 70 °C.

High rate of acetylthiocholine hydrolysis in the pollen of two fertile clones was suppressed by 90 % with the inhibitors of cholinesterase (Table 2). In the self-

compatible clone, the pollen was characterized by a low rate of acetylthiocholine hydrolysis, and the inhibition by physostigmine and neostigmine was only 15 % and 47 %, respectively.

In the pistils, the choline ester was hydrolyzed not so effectively as in pollen. The lowest level was recorded in the pistils of self-incompatible clone; it was eight times lower than in the pistils of self-compatible clone. Physostigmine and neostigmine inhibited the activity of cholinesterase by 25 % and 40 %, respectively, in the pistils of self-compatible clone which indicated the presence of nonspecific esterases. In the similar extracts from self-incompatible clone, physostigmine inhibited the hydrolysis by 50 % and neostigmine suppressed it completely. Thus, the reproductive organs (pollen and pistils) of incompatible petunia clone were characterized by lower enzyme activity as compared to self-compatible clones.

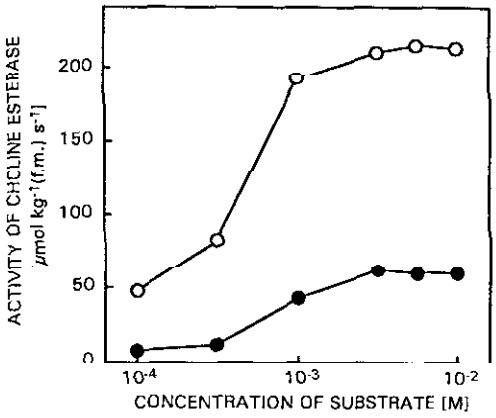

Fig. 1. Cholinesterase activity as affected by substrate concentration (*open circles* - acetylthiocholine, *closed circles* - butyrylthiocholine. Each point represents mean ± S.E. of 4 independent experiments. The standard error of the mean was less than 2 %.

From the dynamics of cholinesterase activity in the course of pollen storage (Table 3), a correlation between pollen viability and cholinesterase activity was evident. The enzyme activity declined to 25 % in 7 d and to 8 % in 20 d in the self-compatible clone while it dropped to zero by 20 d in self-incompatible clone.

Table 3. Cholinesterase activity during the pollen storage (n = 5, S.E. = 2 - 3 %).

Variant	Acetylthiocholine production [μmol kg^{-1}(f.m.) s^{-1}]		
	0 d	7 d	20 d
Pollen of self-compatible clone	1400	428	99
Pollen of self-compatible clone	800	134	110
Pollen of self-incompatible clone	330	207	0

To be sure in direct participation of cholinesterase in the fertilization, we treated the petunia reproductive organs with the inhibitors of cholinesterase, physostigmine and neostigmine.

The enzyme from fertile and sterile clones was insignificantly inhibited (by 10 - 20 %) by physostigmine at 10^{-5} - 10^{-4} M and by neostigmine at 10^{-3} - 10^{-4} M concentration (Fig. 2). The treatment of stigma surfaces just after flower opening with the inhibitors of cholinesterase resulted in a more prominent inhibition of seed productivity (Fig. 3). Seed production after self-pollination in the petunia flowers of self-compatible clone declined up to 10 % at 10^{-5} M physostigmine and 23 % at 10^{-5} M neostigmine. A similar tendency was observed with the pistils of sterile clone although the degree of inhibition was lower.

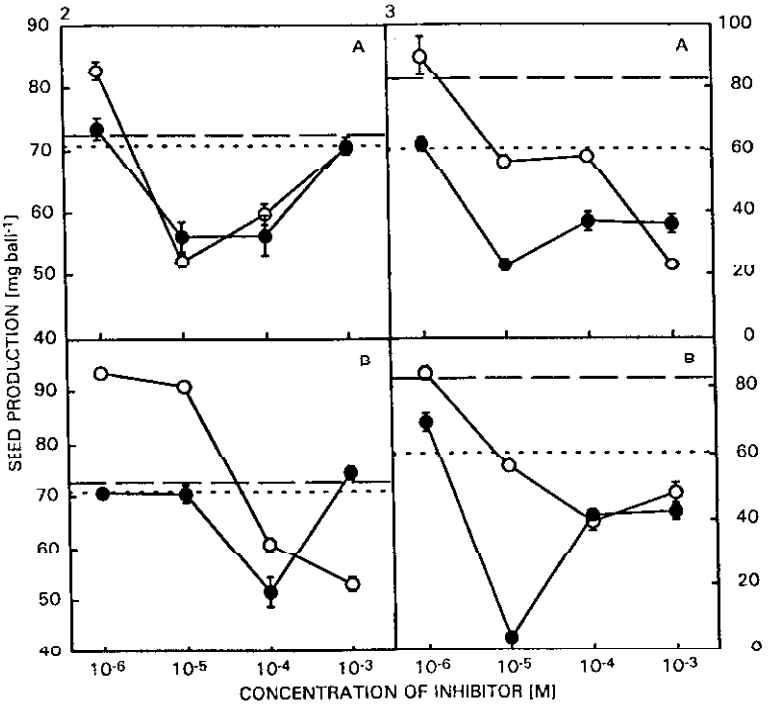

Fig. 2. Effects of bud treatment with cholinesterase inhibitors: (*A*) physostigmine and (*B*) neostigmine on seed production in petunia of self-compatible (*closed circles*) and sterile (*open circles*) clones. Dotted line represents control value for fertile plants (70.8±2.7) and dash line represents control value for sterile plants (72.5 ± 1.1). Each point represents the mean ± SE of 10 flowers. Control buds were injected with H_2O.

Fig. 3. Seed production in petunia of self-compatible (*closed circles*) and sterile (*open circles*) clones after treatment of stigmae with cholinesterase inhibitors 1 h prior to pollination: (*A*) with physostigmine, (*B*) with neostigmine. Dotted line represents control value for fertile plants (60.5 ± 2.6) and dash line represents control value for sterile plants (82.5 ± 3.7). Each point represents the mean ± SE of 10 flowers. Control stigmae were treated with H_2O.

The treatment of the pistils of self-incompatible clone with 10^{-3} - 10^{-6} M physostigmine and neostigmine (both in flower buds and before pollination) did not affect the seed production after self-pollination (data not presented).

Discussion

Cholinesterase, in combination with its natural substrate (acetylcholine) previously identified in the pollen (Marquardt and Vogt 1953) and acetylcholine receptor, constitutes a specific highly sensitive signal system in animals and plants (see Roshchina 1991). In this paper, the enzymatic hydrolysis of choline esters inhibited by neostigmine and physostigmine was found both in protein extracts from petunia pollen and pistils. K_m values calculated for the reaction rates were lower than K_m values estimated in mammalian tissues, although were close to the values obtained in the invertebrate tissues (Roshchina and Semenova 1990). Part of the visible hydrolysis of choline esters in generative organs was not inhibited by the cholinesterase inhibitors that shows also the contribution of non-specific esterases. The location of both cholinesterases and non-specific esterases has been demonstrated earlier by histochemical method in pollen grains of *Vicia faba* (Bednarska 1992) and on the surface of *Pharbitis nil* pistil stigma (Bednarska and Tretyn 1989). Apparently, cholinesterase is a most likely candidate for the specific recognition and signal transduction in the pollen-pistil system.

Previously, we have shown the participation of two enzymes in the reproductive process of petunia, *i.e.* of ribonuclease (Kovaleva *et al.* 1975) and peroxidase (Kovaleva *et al.* 1995). They are related to the operation of the barriers against self-pollination: ribonuclease takes part in the gametophytic self-incompatibility mechanism, whereas peroxidase contributes to the mechanism of male sterility. High activities of both enzymes were demonstrated in the reproductive organs of self-incompatible and sterile clones. On the contrary, high activity of cholinesterase was typical for reproductive organs of self-compatible clones, especially in the pollen. The pollen specific cholinesterases were destroyed early in a storage period, and the loss of enzyme activity resulted in the loss of pollen fertility. This enzyme is most active at the stage of flower bud opening and readiness to fertilization; it follows from the decline in seed production induced by physostigmine and neostigmine in self-compatible clone. No such effect was observed after the treatment of immature reproductive organs in the flower buds with the inhibitors.

Another evidence supporting the cholinesterase participation in the specific recognition in the pollen-pistil system comes from cholinesterase blockage by its inhibitors at low (10^{-6} - 10^{-5} M) concentrations. It is the pollen of self-compatible clone that is sensitive to physostigmine and neostigmine. Different sensitivity of the pollen of compatible clone to physostigmine and neostigmine is likely related to the differences in enzyme structure in the reproductive organs, as it was shown with invertebrate embryos (Roshchina and Semenova 1990), to the configuration of enzyme reactive center in particular. An easy diffusion of the enzyme from the pollen grain surface (Roshchina *et al.* 1994) indicates its superficial position that facilitates

its operation as a signal transducer. The blockage of the enzyme by its inhibitors results in acetylcholine accumulation and inactivation of the possible acetylcholine receptor (Roshchina 1991). It seems possible that it is this receptor that finally triggers the program of fertilization manifesting itself in the pollen tube growth to the ovule.

In conclusion, cholinesterase and other components of cholinergic signal system can take part in the operation of recognition mechanism in the pollen-pistil system.

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