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Adventitious Bud Induction by Protein Kinase C Activators in *Torenia* Stem Segments

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In *Torenia* stem segments cultured in vitro, active meristematic divisions are induced in the epidermis by application with cytokinin, resulting in the formation of adventitious buds. Application of some activators for protein kinase C such as phorbol esters and diacylglycerol was found to induce meristematic divisions in the absence of cytokinin. The simultaneous addition of inhibitor for protein kinase C suppressed bud initiation induced by phorbol esters, diacylglycerol, calcium ionophore, dibutyryl cyclic AMP and cytokinin.

Introduction

In *Torenia* stem segments, active meristematic divisions in the epidermis occur prior to bud differentiation, and the number of meristematic zones (MZ) in the epidermis significantly increases by the application of cytokinin and is reduced by simultaneous addition of auxin[1]. Adventitious bud initiation is controlled by a balance between auxin and cytokinin applied to a culture medium in a large number of plant species[2].

In order to elucidate the mechanism of adventitious bud differentiation in higher plants, it seems important to develop different approaches which do not involve auxin-cytokinin mediated processes. We have demonstrated that application of Ca^{2+} ionophore A23187 promoted adventitious bud induction in *Torenia* stem segments and this process seemed to be mediated through a Ca^{2+}-binding mediator protein calmodulin[3]. The results indicate that cytokinin may not be directly involved in bud initiation in this material. Recently, we have reported that the addition of N^6, O^2-dibutyryl adenosine 3' : 5'-cyclic monophosphate (Bt_2-cAMP) also stimulated bud initiation and that adenosine 3' : 5'-cyclic monophosphate (cAMP)-dependent protein kinase was involved in this process[4].

In animal and microbial cells, cAMP[5] and Ca^{2+}-calmodulin[6] have been known to be the second messenger for gene expression. And a protein kinase C also controlled some physiological phenomena[7]. The activity of protein kinase C was stimulated by diacylglycerol or phorbol ester such as phorbol 12-myristate 13-acetate (PMA) and phorbol 12, 13-dibutyrate (PDBu)[8,9]. The 4α-derivatives of PMA could not stimulate the activity of protein kinase C[9]. We have previously reported that application of PMA, PDBu or diacylglycerol promoted bulblet initiation in lily bulb-scale segments[10].

In the present paper, we report promotive effects of some activators of protein kinase C, and involvement of protein kinase C in adventitious bud initiation in *Torenia* stem segments.

Materials and Methods

Plants of *Torenia fournieri* Lind. were grown in a growth chamber at a constant temperature of 25±2°C under a 16 h long day regime for 8 weeks. Explants (5 mm in length) were taken from apical stem internodes and cultured on a basal medium containing Murashige and Skoog's mineral salts and vitamines[11], 2% sucrose and 0.25% Gelrite (Merck) (MS medium). Benzyladenine (BA) was added to the MS medium at 0.5 μM. In another series of experiments, Ca^{2+} ionophore A23187 (Calbiochem-Behring), Bt_2-cAMP (Sigma), PMA, PDBu, 4-α-phorbol 12-myristate 13-acetate (4α-PMA) and diacylglycerol (Diolein) (all from LC services) were dissolved in dimethylsulfoxide and added to the medium. The final concentration of dimethylsulfoxide was adjusted to 0.3 % in all treatments of the experiments. To examine the possible involvement of protein kinase C, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) (LC services) was simultaneously added to the medium containing BA, A23187, Bt_2-cAMP, PMA, PDBu or Diolein.

Measurements of bud initiation were performed as follows. Meristematic divisions were induced in the epidermis of stem segments, and these MZ had a potency to develop buds[12]. The epidermal strips were peeled off from the 7 day-cultured stem segments, stained with aceto-carmine, and immediately observed under a microscope. The number of MZ in the epidermis were counted from more than 240 epidermal strips and the data presented show the average number of MZ formed per strip. The above experiments were repeated at least 3 times and the standard errors were then calculated.

Results and Discussion

When *Torenia* stem segments were cultured on the basal MS medium, the number of MZ formed in the epidermis was always less than 1. As shown in **Fig. 1**, the MZ number increased progressively by the addition of 0.1 to 1.0 μM PMA. Similar increases in the MZ formation were observed in the presence of PDBu or Diolein (**Fig. 1.**). The application of 0.1 μM PDBu induced the formation of 4.6 MZ, and 1 μM Diolein 3.8 MZ (**Fig. 1**). The most effective chemical was PMA, and 5.2 MZ per epidermal strip was obtained at 0.1 μM of PMA. The application of 4α-PMA did not affect MZ

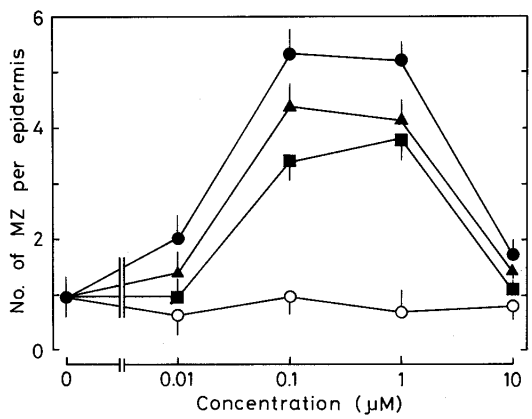

Fig. 1 Effects of phorbol esters and diacycerol on MZ formation in *Torenia* stem segments. Explants were cultured for 7 days on MS medium containing various concentrations of PMA (●), PDBu (▲), Diolein (■), or 4α-PMA (○). To calculate the average number of MZ, at least 240 epidermis for each treatment were examined. Experiments were repeated 3 times, and the standard errors were then calculated.

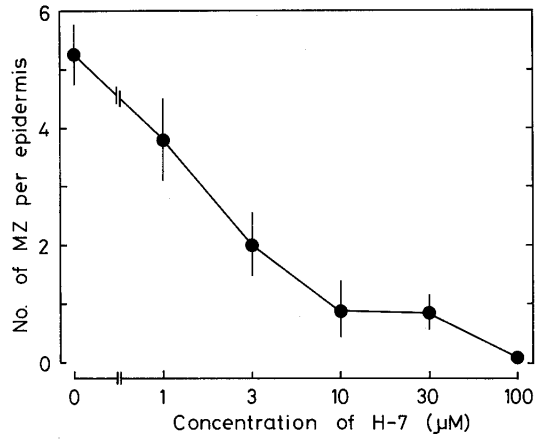

Fig. 2 Effects of H-7 on PMA-induced MZ formation in *Torenia* stem segments. Explants were cultured for 7 days on MS medium containing various concentrations of H-7 with 0.1 μM of PMA. To calculate the average number of MZ, at least 240 epidermis for each treatment were examined. Experiments were repeated 3 times, and the standard errors were then calculated.

formation (**Fig. 1**).

We have reported that application of Ca^{2+} ionophore A23187 promoted adventitious bud induction in *Torenia* stem segments and the process seemed to be mediated through a Ca^{2+}-binding mediator protein calmodulin[3]. Recently, we have demonstrated that addition of Bt_2-cAMP also stimulated bud initiation and that cAMP-dependent protein kinase was involved in this process[4]. In lily bulb -scale segments, A23187 and Bt_2-cAMP stimulated bulblet differentiation[13,14] and the differentiation was also promoted by PMA, PDBu or Diolein[10].

To elucidate the role of phorbol ester or diacylglycerol on bud initiation, the inhibitor of protein kinase C, H-7, was applied to the medium with 0.1 μM PMA. As shown in **Fig. 2**, H-7 suppressed PMA-induced bud initiation, and the MZ formation induced by PMA was completely inhibited by 10 μM H-7.

Table 1. Effect of H-7 on MZ formation induced by activators of protein kinase C, cyclic AMP (Bt_2 -cAMP), calcium ionophore (A23187) and cytokinin (BA).

Chemicals	Concentration (μM)	No. of MZ/epidermis	
		−H-7	+H-7
None	—	0.8±0.2	0.2±0.2
PMA	0.1	5.8±0.4	0.8±0.4
PDBu	0.1	5.2±0.2	0.6±0.4
Diolein	1	5.4±0.6	0.5±0.2
Bt_2-cAMP	1	8.2±0.8	0.8±0.6
A23187	10	7.6±0.8	0.9±0.6
BA	0.5	5.2±0.4	0.4±0.2

Explants were cultured on the medium containing PMA, PDBu, Diolein, Bt_2-cAMP, A23187 or BA with or without 10 μM H-7. To calculate the average number of MZ, at least 240 epidermis for each treatment were examined. Experiments were repeated 3 times, and the standard errors were then calculated.

In higher plants, the physiological role of protein kinase C has not been clearly understood. However, protein kinase C has been found in zucchini hypocotyl hooks and stems[15], wheat cells[16], *Amarnthus tricolor* seedlings and soybean callus[17].

Thus, we examined the effects of H-7 on MZ formation in the explants cultured on the medium together with substances stimulating the MZ formation. The application of H-7 strongly inhibited MZ formation induced by PMA, PDBu or Diolein (**Table 1**). Furthermore, H-7 inhibited A23187-, Bt_2-cAMP- and BA-induced MZ formation (**Table 1**).

Hidaka *et al.*[18] reported that H-7 was a more marked inhibitor for protein kinase C than for other kinases. The H-7 strongly inhibits MZ formation induced by all of the chemicals. These results suggest that adventitious bud initiation in *Torenia* stem segments may involve protein kinase C.

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《和文要約》

プロティンキナーゼC活性化剤によるトレニア茎切片からの不定芽誘導

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トレニア（*Torenia fournieri* Lind.）の茎切片をサイトカイニン存在下で培養すると，一部の表皮細胞で集中的に分裂が起こり不定芽原基が誘導される．サイトカイニンがなくとも，ホルボールエステルやジアシルグリセロールなどのプロテインキナーゼCの活性化剤の添加によって不定芽原基が分化した．プロティンキナーゼCの阻害剤を同時に添加するとそれらの活性化剤やカルシウムイオノフォア，サイクリックAMPさらにはサイトカイニンによる不定芽誘導は完全に抑制された．