Studies on Bulblet Differentiation in Bulb-scale Segments of *Lilium longiflorum*
VI. Promotive Effects of Cyclic AMP

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Received October 31, 1991

Summary

In lily bulb-scale segments cultured *in vitro*, adventitious bulblets could be induced by application of dibutyryl cyclic AMP in the absence of phytohormones. Similar promotive effects on bulblet differentiation were observed when various promoting reagents to accumulate endogenous cyclic AMP were added, such as activator for adenylate cyclase, forskolin, and inhibitors of cyclic nucleotide phosphodiesterase, theophylline and 3-isobutyl-1-methyl xanthine. Endogenous content of cyclic AMP in lily bulb-scale segments were increased by the application of the above chemicals or calcium ionophore A23187. These results suggested that endogenous concentrations of cyclic AMP were involved in adventitious bulbet differentiation of lily bulb-scale segments. Furthermore, some inhibitors of protein kinases suppressed bulblet initiation induced by cyclic AMP-accumulating reagents.

Key words: bulblet differentiation, cyclic AMP, *Lilium longiflorum*

Introduction

Adventitious bud initiation in *Torenia* stem segments is induced by cytokinin[22, 23, 25] and stimulated by wounding[21], application of traumatic acid[26], calcium ionophore A23187[27] and anaerobic treatment[24]. Bulblet differentiation in lily bulb-scale segments was also induced by application of phytohormones (auxin and cytokinin), and promoted by wounding, application of traumatic acid[10], some phospholipids, phorbol ester[12] and calcium ionophore A23187[28], and anaerobic treatment[11]. Therefore, bud initiation in *Torenia* and bulblet initiation in lily seemed to be regulated by similar mechanism.

Although cyclic AMP has been known to be a second messenger for gene expression in microbial and animal cells[18], physiological roles of cyclic AMP in higher plants have not been clear[3]. Cyclic AMP promotes auxin-induced root formation in carrot callus[7], and stimulates betacyanin synthesis in *Amaranthus*[6, 19]. Furthermore, cyclic AMP also slightly promoted bud initiation in *Torenia* stem segments[26]. Entering of cyclic AMP into cells is thought to be slow, while dibutyryl cyclic AMP is rather readilly taken up[19]. In *Torenia* stem segments, bud initiation was promoted by dibutyryl cyclic AMP[9]. Accordingly, we tried to examine the effects of dibutyryl cyclic AMP on the bulblet differentiation.

The control of endogenous levels of cyclic AMP are mediated by adenylate cyclase and

cyclic nucleotide phosphodiesterase (PDE). The activity of adenylate cyclase is stimulated by forskolin, and the activity of PDE is suppressed by theophylline or 3-isobutyl-1-methyl xanthine (IBMX)[1, 5, 14, 20]. To elucidate the exact role of cyclic AMP, we examined the effects of these reagents, which may influence the endogenous concentrations of cyclic AMP, on the adventitious bulblet differentiation in lily bulb-scale segments.

Cyclic AMP is thought to act through cyclic AMP-dependent protein kinase, and the action of some protein kinases is inhibited by 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), N-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide (H-8), and N-(2-aminoethyl)-5-isoquinoline sulfonamide (H-9)[8]. Thus, we tried to examine the effects of these inhibitors on cyclic AMP-induced bulblet initiation.

Materials and Methods

Plantlets of *Lilium longiflorum* Thunb. were grown *in vitro* as reported previously[10], the bulbs (about 14 mm in diameter) formed in the basal part of plantlets were harvested and the outer 2 bulb-scales were used. The bulb-scales were transversally cut to 6 segments and the segments were used as explants. The explants were cultured on the basal medium containing Murashige and Skoog's mineral salts[16], 4% sucrose and 0.25% Gelrite (Merck) (hereafter referred to as MS medium) with 0.1 μM naphthaleneacetic acid (NAA) and 1 μM benzyladenine (BA). Dibutyryl cyclic AMP, forskolin, theophylline, IBMX (all from Sigma), calcium ionophore A23187 (Calbiochem-Behring) and 12-O-tetradecanoyl phorbol-13-acetate (TPA, Funakoshi) were dissolved in the MS medium. To examine the possible involvement of protein kinase, H-7, H-8, or H-9 (all from LC Services) was simultaneously added to the culture medium containing NAA, BA, A23187 or other chemicals related to the control of endogenous concentrations of cyclic AMP.

The cultures were maintained under 16 hr long-day photoperiod (6,000 lux) and constant temperature of 25±2°C. After 3 weeks culture, bulblet differentiation in the cultured explants and the number of bulblets formed in the explants were observed.

Cyclic AMP accumulation in the cultured explants was determined as follows. Lily bulb-scale segments were cultured in the medium containing various chemicals for 2 weeks. The segments were homogenized in ice-cold 0.3 M perchloric acid and the extract was centrifuged. The resultant supernatant was neutralized with 3 M $KHCO_3$, and cyclic AMP was measured by a radioimmunoassay kit (Yamasa, Japan). [^{125}I] Succinyl-cyclic AMP and corresponding antisera (1 : 25,000) were incubated with standards and samples for 16 h. To confirm assayed samples as cyclic AMP, bovine PDE (from Sigma) was added to the mixture. Sheep anti-rabbit serum (100 μl diluted 1 : 1 with buffer) and 1 ml of 7.5% polyethylene glycol (mol wt 8,000) were added to each tube, and the tube were centrifuged at 2,500×g for 30 min. Radioactivity in the pellet was determined. The concentration of cyclic AMP was expressed as pmol per g fresh weight of segments.

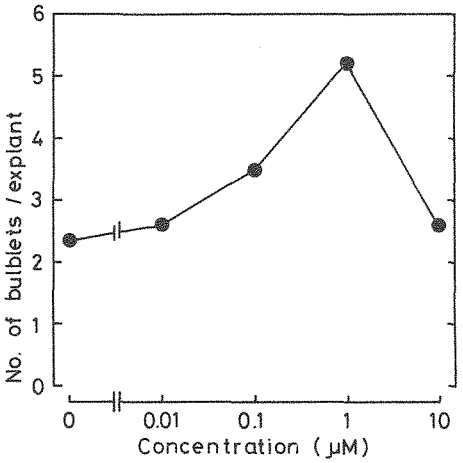

Fig. 1 Effects of dibutyryl cyclic AMP on bulblet differentiation in lily bulb-scale segments.

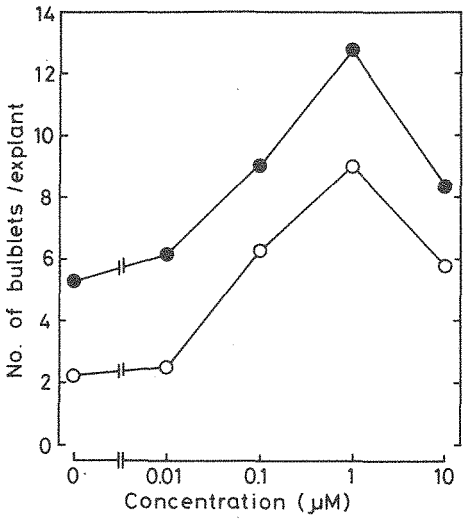

Fig. 2 Effects of forskolin on bulblet differentiation in lily bulb-scale segments. Explants were cultured on the medium containing various concentrations of forskolin with (●) or whthout (○) 1 μM of dibutyryl cyclic AMP.

Results

When lily bulb-scale segments were cultured on the basal MS medium contained NAA and BA without cyclic AMP-related chemicals, the number of bulblets formed in the segments was always less than 3. The number increased progressively by the addition of increasing concentrations of dibutyryl cyclic AMP (Fig.). The largest number of bulblets, 5.2 per explant, was obtained at 1 μM of dibutyryl cyclic AMP.

Similar increases in the formation of bulblets were observed in the presence of forskolin (Fig. 2). The application of 1 μM forskolin induced the formation of 9 bulblets and the bulblet initiation was further stimulated by simultaneous addition of dibutyryl cyclic AMP (Fig. 2).

Adventitious bulblet differentiation were also promoted by the application of PDE inhibitors, theophylline or IBMX (Fig. 3). The most effective chemical was IBMX, and about 9.5 bulblets were formed in the segment with 10 μM of IBMX present (Fig. 3). The

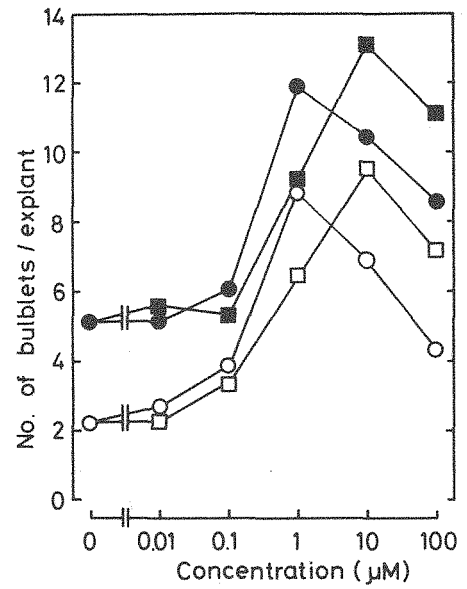

Fig. 3 Effects of theophylline and IBMX on bulblet differentiation in lily bulb-scale segments. Explants were cultured on the medium containing various concentrations of theophylline (○, ●) or IBMX (□, ■) with (●, ■) or without (○, □) 1 μM of dibutyryl cyclic AMP.

Table 1 Cyclic AMP accumulation in bulb-scale segments cultured on the medium containing cyclic AMP-related chemicals, calcium ionophore (A23187) and phorbol ester (TPA).

Chemicals	Concentration (μM)	Cyclic AMP content (pmol/g fr wt)	
		− PDE	+PDE*
None	—	23.8	9.8
Forskolin	1	99.8	9.9
Theophylline	1	110.6	10.9
IBMX	10	124.8	12.2
A23187	1	120.4	11.8
TPA	0.1	122.6	12.0

Bulb-scale segments cultured on the medium containing various chemicals for 2 weeks were homogenized, and cyclic AMP was measured by a radioimmunoassay kit. *To confirm assaed samples as cyclic AMP, bovine PDE was added to the mixture.

simultaneous addition of dibutyryl cyclic AMP further promoted the bulblet differentiation (Fig. 3).

The endogenous cyclic AMP content was increased in lily bulb-scale segments cultured on the medium containing the above substances (Table 1). The segments cultured on the medium without cyclic AMP-related substances contained 24 pmol cyclic AMP per g fresh weight, whereas the cyclic AMP content was 4-5 times higher when explants were cultured on medium containing forskolin, theophylline or IBMX. A similar accumulation of endogenous cyclic AMP was also observed in explants cultured on medium containing A23187 or TPA (Table 1). When PDE was added to sample solution from the cultured explants, the solutions contained about 10 to 12 pmol cyclic AMP per g fresh weight (Table 1).

To elucidate the role of cyclic AMP on bulblet differentiation, the inhibitors for protein kinases, H-7, H-8 and H-9 were applied to the medium with 10 μM of IBMX. As shown in Fig. 1, all of inhibitors were suppressed IBMX-induced bulblet initiation. The most effective inhibitor was H-8, and the bulblet formation induced by IBMX was completely inhibited by 10 μM H-8 (Fig. 4). The simultaneous application of H-8 strongly inhibited bulblet formation induced by dibutyryl cyclic AMP, forskolin, theophylline, or IBMX (Table2). Furthermore, H-8 inhibited TPA-and A23187-induced bulblet

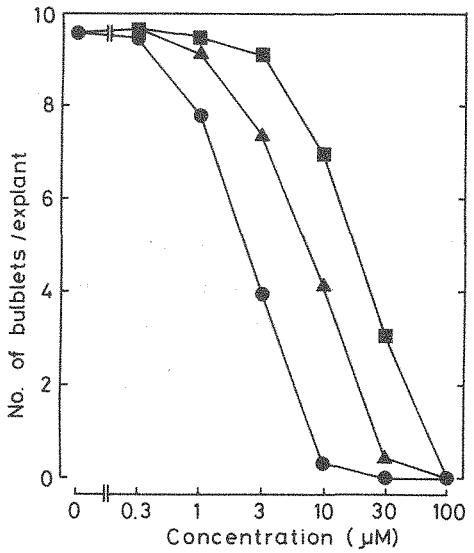

Fig. 4 Effects of isoquinolinesulfonamides (H-7, H-8 and H-9) on IBMX-induced bulblet differentiation. Explants were cultured on the medium containing various concentrations of H-7 (▲), H-8 (●), or H-9 (■) with 10 μM of IBMX.

Table 2 Effects of H-8 on bulblet differentiation induced by cyclic AMP-related chemicals, calcium ionophore (A23187) and phorbol ester (TPA).

Chemicals	Concentration (μM)	No. of bulblet/explant	
		− H-8	+ H-8
None	—	1.8	0
Forskolin	1	8.8	0.2
Theophylline	1	9.0	0.2
IBMX	10	9.6	0.4
A23187	1	6.6	0
TPA	0.1	9.6	0.4

The explants were cultured on medium containing forskolin, theophylline, IBMX, A23187 or TPA with or without 10 μM H-8.

formation (Table 2).

Discussion

In higher plants, the physiological role of cyclic AMP has not been clearly understood. However, cyclic AMP affects some phytohormone-regulated events[6, 7, 19]. As we have already reported, addition of cyclic AMP to culture medium slightly promotes adventitious bud initiation[26] and dibutyryl cyclic AMP strongly stimulated bud initiation[9] in *Torenia* stem segments. In lily bulb-scale segments, adventitious bulblet differentiation also promoted by dibutyryl cyclic AMP (Fig. 1).

Endogenous cyclic AMP is synthesized by adenylate cyclase and this enzyme is stimulated by forskolin. The activity of the cyclic AMP-degradating enzyme PDE inhibited by theophylline and IBMX[1, 5, 14, 20]. Therefore, application of the above chemicals increases the endogenous contents of cyclic AMP in animal systems. Theophylline and IBMX are reported as poor inhibitors of plant PDE[2], however, the increases in cyclic AMP accumulation are observed in *Torenia* stem segments[9] and lily bulb-scle segments (Table 1) cultured on the medium with theophylline, IBMX or forskolin. These cyclic AMP-accumulating reagents also stimulate bulblet differentiation (Fig. 2 and 3).

Application of the calcium ionophore A23187 or phorbol ester TPA also caused an increase in endogenous cyclic AMP conecentration (Table 1). Carricarte et al.[4] demonstrated that adenylate cyclase in alfalfa roots was active with calcium ions and calmodulin activated the activity of this enzyme. Another calcium-regulating system is protein kinase C, whose activity is stimulated by phospholipids and phorbol esters. Both chemicals stimulate cyclic AMP accumulation in animal cells[17, 20]. The application of phorbol esters[12] or calcium ionophore[28] promoted bulblet differentiation in lily bulb-scale cultures, and calmodulin was present in lily bulbs[29]. These observations and our results suggest that an increase in intracellular concentration of calcium ions enhance adenylate cyclase activity and induce cyclic AMP accumulation.

The physiological action of cyclic AMP in fungi and animals is expressed through

cyclic AMP-dependent protein kinase[3], and cyclic AMP promoted protein phosphorylation in higher plants[13, 15]. The activities of some protein kinases including protein kinase C and cyclic nucleotide-dependent protein kinase are inhibited by isoquinolinesulfonamides such as H-7, H-8 and H-9[8]. The simultaneous application of isoquinolinesulfonamides strongly inhibits bud initiation induced by theophylline in *Torenia* stem segments[9] and bulblet formation induced by IBMX (Fig. 4). The most effective inhibitor was H-8, 10 μM of H-8 completely inhibited bulblet differentiation (Fig. 4). Hidaka et al.[8] reported that H-8 was more marked inhibitor for cyclic AMP-dependent protein kinase than that for other kinases. The H-8 strongly inhibits bulblet differentiation induced by all of cyclic AMP-accumulating reagents (Table 2). These results suggest that adventitious bulblet differentiation in lily bulb-scale segments may involve cyclic AMP and cyclic AMP-dependent protein kinase.

Efforts are directed to obtain more information regarding the mechanism of action of cyclic AMP and protein kinases during adventitious bulblet differentiation in lily bulb-scale segments.

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鉄砲ユリの鱗片切片培養における球根分化に関する研究
VI. Cyclic AMP の分化促進効果

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摘　　要

鉄砲ユリの鱗片切片培養において，dibutyryl cyclic AMP の添加によって球根分化が誘導された．同様の促進効果は内生 cyclic AMP 濃度を上昇させる物質，すなわち adenylate cyclase の活性化剤あるいは phosphodiesterase の阻害剤の添加によっても認められた．さらに細胞内の cyclic AMP 濃度はそれらの物質やカルシウムイオノフォアの添加によって著しく上昇した．さらに protein kinase の阻害剤は球根分化を阻害した．以上の結果から鉄砲ユリの球根分化には内生 cyclic AMP 濃度が重要であり，さらに cyclic AMP は protein kinase を活性化することを経由して分化を制御している可能性が示された．