Evidence for the Involvement of Phospholipase C in the Anaerobic Signal Transduction

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Pre-treatment of rice roots for 2 h in aerobic conditions with two phospholipase C (PLC) antagonists, neomycin and compound 48/80 (C48/80), inhibited accumulation of γ-aminobutyric acid and increased the loss of K+ in the medium during 3 h of anoxia. The presence of Ca2+ and A23187 (Ca2+ ionophore) nullified the effect of PLC inhibitors. Pre-treatment of rice roots with neomycin and C48/80 abolished the anaerobic increase in the concentration of the PLC product inositol 1,4,5-triphosphate. Stimulation of the anaerobic signal transduction pathway with aluminum fluoride (G protein activator) was attenuated by PLC inhibitors. These findings are consistent with the participation of PLC in the anaerobic response.

Keywords: γ-Aminobutyric acid — Anoxia — Inositol 1,4,5-triphosphate — Oryza sativa — Phospholipase C — Signal transduction.

Abbreviations: AlF4−, aluminium fluoride; CaM, calmodulin; C48/80, compound 48/80; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; IP3, inositol 1,4,5-triphosphate; PLC, phospholipase C; RR, ruthenium red; TEA, tetraethylammonium chloride; TFP, trifluoperazine.

Introduction

In nature, higher plants often experience stress conditions. Flooding is considered as the primary cause of anaerobic stress in plants. After an oxygen shortage is sensed, the signal is transduced inside the cell through the production of second messengers. In anoxia, Ca2+ mobilization toward cytoplasm from intracellular stores is necessary for mRNA transcription of anaerobic proteins such as alcohol dehydrogenase and sucrose synthetase (Subbaiah et al. 1994a). Ca2+ is also involved in the selective translation of cytoplasmic mRNAs through phosphorylation of the cap-binding protein eIF4E during oxygen deprivation (Manjunath et al. 1999). Moreover, Ca2+/calmodulin (CaM) complex has been shown to control anaerobic protein degradation and solute release (Aurisano et al. 1995a).

A target of the anaerobic signal transduction is the CaM-dependent glutamic acid decarboxylase (GAD). This enzyme converts glutamate to γ-aminobutyric acid (GABA) in a reaction of α-decarboxylation which consumes protons (Bown and Shelp 1989). The metabolic consumption of protons has been suggested to be an adaptive response of plants to the stress-induced cellular acidosis (Aurisano et al. 1995b). GABA is accumulated at high levels in response to anoxia (Reggiani et al. 1988). Anaerobic GABA accumulation was inhibited by ruthenium red (RR, Ca2+ channel blocker), trifluoperazine (TFP) and W-7 (CaM antagonists) in rice roots (Aurisano et al. 1995a). The accumulation of this amino acid was restored after the addition of Ca2+ and A23187 (Ca2+ ionophore), indicating a Ca2+ dependence of this response.

An early event in the anaerobic signal transduction may be the activation of heterotrimeric G proteins. The level of GABA was increased by stimulation of GTP-binding proteins by GTPγS or aluminium fluoride (AlF4−) in rice roots, while the opposite was observed by inhibiting G proteins with GDP or GDPβS (Aurisano et al. 1996). Stimulation of GABA synthesis by AlF4− was completely inhibited by 400 μM RR, indicating that activated G proteins opened the same Ca2+ channels involved in the anaerobic response. In higher plants, a possible target of activated GTP-binding proteins is phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-biphosphate and generates two second messengers, inositol 1,4,5-triphosphate (IP3, inducer of Ca2+ release) and 1,2-diacylglycerol (activator of protein kinase C; Berridge and Irvine 1989). Activation of G proteins by mastorapan stimulated phosphoinositide-dependent PLC and the release of IP3 in Chlamidomonas moewusii and carrot cells (Drøbak and Watkins 1994, Cho et al. 1995, Van Himbergen et al. 1999). Moreover, evidence for IP3-dependent Ca2+ release in plants has been reported (Brosnan and Sanders 1993, Allen et al. 1995).

In the present study, we investigated the role of PLC in mediating the process of anaerobic GABA accumulation and K+ release in rice roots. In this tissue, the involvement of G proteins and Ca2+/CaM complex was previously shown (Aurisano et al. 1995a, Aurisano et al. 1996).

Materials and Methods

Chemicals

A23187, compound 48/80 (C48/80), neomycin, RR, tetraethylammonium chloride (TEA) and TFP were purchased from Sigma-Aldrich (Milano, Italy).

Plant material and treatment

Dehulled seeds of rice (Oryza sativa L. cv. Arborio) were steri-
lized and germinated in Petri dishes at 30°C in the dark for 3 d. Six seedlings were aerobically incubated for 2 h in 20 ml of 1 mM MES-Tris (pH 6.0) supplemented with the compounds described in figures. The aerobic pre-treatment with various agents has been shown to be a good method to study the anaerobic stress response (Subbaiah et al. 1994a, Aurisano et al. 1995a). After 2 h, the seedlings were subjected to anaerobic conditions in an anaerobic jar (Merek, Darmstadt, Germany) in which anoxia was generated by BBL GasPack Plus (Becton Dickinson, Cockeysville, MD, U.S.A.).

**GABA extraction and assay**

After the treatment, the roots were excised and ground in a mortar with 0.6 M HClO₄ (100 mg FW ml⁻¹) and the homogenate was centrifuged at 13,000 g for 15 min. GABA was assayed on the supernatant as previously described (Aurisano et al. 1993).

**IP₃ determination**

Short-term anaerobic experiments were carried out as previously described (Reggiani 1997). Three-days-old rice seedlings were then fully immersed in 10 ml of 1 mM MES-Tris (pH 6.0) in closed jars with inlet/outlet ways for gas. The jars were made anaerobic by flushing nitrogen gas (99.99% N₂) through the medium. Since in a short-term experiment the time required to reach complete anaerobiosis is critical, the N₂ gas flux was maintained elevated throughout the experimental period. The anaerobic treatment was applied for different lengths of time (0, 2.5, 5, 7.5 and 10 min). At the end of the treatment, the roots were disrupted by grinding with 0.6 M HClO₄ and the homogenate was cleared by centrifugation at 13,000 g for 15 min. The supernatant was neutralized by titrating with ice-cold 1.5 M KOH containing 60 mM HEPES and bromocresol purple as indicator. KClO₄ was then sedimented by centrifugation at 2,000 g for 15 min at 4°C. On the supernatant, IP₃ was determined by radioreceptor assay using D-myo-inositol-1,4,5-triphosphate assay system (Amersham Pharmacia Biotech, Cologno Monzese, Italy). The assay was performed according to the manufacturer’s protocol.

**Determination of K⁺**

At the end of the anaerobic treatment, the amount of K⁺ was estimated in the incubation buffer by an ion-selective electrode using the manufacturer’s protocol (Ingold, Urdorf, Switzerland). Values were obtained by comparison with standard solutions containing different K⁺ concentrations.

**Results**

**Effect of PLC inhibitors on GABA accumulation**

Anaerobic accumulation of GABA and its dependence from second messengers can be studied by 3-h anaerobiosis experiments (Aurisano et al. 1995a, Aurisano et al. 1996). In the present study, we examined the effects of two PLC antagonists, neomycin and C48/80, on the accumulation of GABA. In rice roots, the level of GABA was 1.10 μmol (g FW)⁻¹ in air and became 3.09 μmol (g FW)⁻¹ after 3 h of anoxia (Fig. 1). Neomycin inhibited anaerobic GABA accumulation at concentrations ≥ 2 mM and with complete inhibition at 10 mM. C48/80 inhibited GABA accumulation at lower concentrations than neomycin and it abolished amino acid accumulation at 1 mM. For both PLC inhibitors, the addition of 2 mM CaCl₂ and 10 μM A23187 restored GABA accumulation (Fig. 1), suggesting that the inhibition was upstream of the Ca⁺ release.

**Effect of PLC inhibitors on AlF₄⁻-stimulated anaerobic GABA accumulation**

The seedlings aerobically pre-treated with AlF₄⁻ (G protein activator) showed a higher anaerobic GABA concentration in comparison with untreated roots (+43%, Table 1). This finding confirmed previous observations on the effect of AlF₄⁻ in anaerobic rice roots (Aurisano et al. 1996). GABA accumulation under anoxia was strongly inhibited by neomycin or C48/80 in AlF₄⁻-treated roots, indicating that both compounds inhibited downstream to G protein stimulation.

**Anaerobic levels of IP₃**

The increase of IP₃ in plants after stimulation by stress or
Phospholipase C and anaerobic signal transduction

G protein activators has been reported to occur in the first few min of treatment (Drøbak and Watkins 1994, Beno-Moualem et al. 1995, Cho et al. 1995). Figure 2A illustrates the time-course of IP3 levels in the first 10 min of anaerobic stress. The IP3 level in aerobic rice roots was 33.7 pmol (g FW)-1. The concentration of IP3 increased within 5 min of anoxia becoming 3.5-fold the level in air. Thereafter, the level of second messenger decreased, though it remained 64% higher than in air (55.3 pmol (g FW)-1). IP3 concentration was then evaluated at 5 min of anaerobiosis after pre-treatment with neomycin, C48/80, AlF4- and Ca2+ (Fig. 2B). The PLC antagonists, neomycin and C48/80, both abolished the anaerobic increase in IP3 concentration. Stimulation of GTP-binding proteins with AlF4- strongly increased the IP3 level (207.9 pmol (g FW)-1). Neomycin and C48/80, both completely abolished the effect of AlF4-, suggesting that G proteins stimulated a PLC-mediated signal transduction pathway. The presence of Ca2+ reduced the IP3 concentration in anaerobic and AlF4- -stimulated roots to the aerobic level. The effect of Ca2+ might indicate a negative feedback of this ion on the anaerobic signal transduction pathway.

Release of K+ during anaerobic treatments

The loss of K+ in the root-incubation medium was previously shown to be controlled by Ca2+/CaM (Aurisano et al. 1995a). As shown in Fig. 3, a 3-h anaerobic stress increased the release of K+ from rice roots (26 pmol (g FW)-1 against 11.6 pmol (g FW)-1 in the aerobic control). The inhibition of the Ca2+ signal by RR or TFP induced a release of K+ greater than in untreated anaerobic roots (about 2.7 and 2.9 times, respectively). A similar effect was observed in roots pre-treated with neomycin or C48/80, the effect being nullified by Ca2+ ions. AlF4- slightly reduced the loss of K+ from anaerobic rice roots. Both PLC inhibitors increased the release of K+ in AlF4- -treated roots. It is interesting to note that TEA, an inhibitor of K+ channels, caused a loss of cations similar to that observed with RR, TFP and PLC antagonists. Thus, a possible target during anoxia of the Ca2+/CaM complex is the activation of K+ reassimilation.

Discussion

In rice roots, a 3-h anoxic stress induced GABA accumulation and K+ release. These responses are controlled by the Ca2+/CaM complex (Aurisano et al. 1995b). The role of Ca2+ in the anaerobic response was previously established in seedlings and cells of maize, Arabidopsis seedlings and rice roots (Subbaiah et al. 1994a, Subbaiah et al. 1994b, Subbaiah et al. 1998, Aurisano et al. 1995a, Sedbrook et al. 1996). Here, we present evidence for the involvement of PLC in the anaerobic signal transduction. The PLC antagonists, neomycin and C48/80, inhibited GABA accumulation and stimulated the loss of K+ during anaerobic treatments.
Phospholipase C and anaerobic signal transduction

of K⁺ in the medium (Fig. 1, 3). This effect was nullified by addition of 2 mM Ca²⁺ and 10 μM A23187, suggesting that inhibition of PLC affected the release of Ca²⁺.

PLC is known to induce Ca²⁺ release by the second messenger IP₃ (Berridge and Irvine 1989). Anaerobiosis induced a peak of IP₃ concentration after 5 min of stress and then the level decreased, though remaining higher than in air (Fig. 2A). A transient increase in 5 min of IP₃ level has also been observed after exposure of red beet slices to salt stress (Beno-Moualem et al. 1995). The concentration of IP₃ in rice roots was negatively affected by PLC inhibitors (Fig. 2B), indicating that these inhibitors acted on PLC as in animal systems. This may be explained by the homology found between plant PLCs and animal δ-PLCs (Hirayama et al. 1995, Shi et al. 1995).

The activation of GTP-binding proteins by AlF₄⁻ was previously shown to stimulate the anaerobic GABA accumulation through the release of Ca²⁺ from RR-sensitive channels (Aurisano et al. 1996). Here, we showed that AlF₄⁻, besides stimulating GABA accumulation (Table 1), increased the IP₃ concentration and reduced the anaerobic loss of K⁺ (Fig. 2B, 3). These effects were completely abolished by the addition of PLC antagonists to AlF₄⁻-treated roots. Therefore, in the anaerobic signaling cascade, G proteins appear to act upstream than PLC. Considering the results of the present study and the known signal transduction pathways in animals, we can draw a hypothetical pathway for the induction of GABA accumulation (Fig. 4). In this model, the perception of oxygen deprivation activates heterotrimeric GTP-binding proteins. The cytoplasmic IP₃ level would be increased by the stimulation of PLC activity by activated G proteins. A Gα subunit of heterotrimeric GTP-binding proteins has been cloned in rice (Seo et al. 1995). Further, IP₃ can induce Ca²⁺ release from intracellular stores through IP₃-gated Ca²⁺ channels. The Ca²⁺/CaM complex is associated with GAD, increasing enzyme activity and GABA synthesis. As shown in Fig. 4, Ca²⁺ is probably involved in a negative feedback that would attenuate the IP₃ signal. This might be a mechanism to avoid toxicity for Ca²⁺ overloading of the cytosol (Subbaiah et al. 1994b). A negative effect of Ca²⁺ on the level of IP₃ was observed also in rice aleurone layers (Kashem et al. 2000). Other targets of the anaerobic signal transduction pathway could be plasmalemma channels. The effects of RR, TFP and TEA on the loss of K⁺ in the medium suggest a role of Ca²⁺/CaM complex in activating K⁺ reassimilation.

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**Fig. 3** Release of K⁺ in the incubation medium during 3 h of anaerobiosis. Neomycin (10 mM), C48/80 (1 mM), AlF₄⁻ (100 μM), RR (400 μM), TFP (100 μM) and TEA (20 mM) were supplied during 2 h of aerobic pre-treatment. Calcium (2 mM CaCl₂ + 10 μM A23187) was added to reverse the effect of PLC antagonists. Aerobic control represents the release from roots after 3 h in air. Each bar is the mean of at least three independent experiments and the SE are indicated.

**Fig. 4** Proposed anaerobic signal transduction pathway for the process of GABA accumulation and K⁺ reassimilation. Gα and IP₃R represent activated-Gα subunit of heterotrimeric G proteins and IP₃ receptor, respectively.
PLC was recently found to be involved in ABA-induced free Ca\(^{2+}\) oscillation in guard cells (Staxén et al. 1999). Since ABA was previously shown to induce GABA accumulation and K\(^+\) release in wheat seedlings (Aurisano et al. 1993, Reggiani et al. 1993), this opens the question of the role of ABA in the anaerobic signal transduction pathway. Further research on this point is necessary.

References


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