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Cyclic AMP Stimulates K^+ Channel Activity in Mesophyll Cells of *Vicia faba* L.[1]

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Whole-cell patch-clamp recordings from *Vicia faba* mesophyll protoplasts reveal that outward K^+ current is increased in a dose-dependent fashion by intracellular application of cAMP. The enhancement of the outward current by cAMP is specific and it cannot be mimicked by a series of nucleotides that includes AMP, cGMP, and GMP. The enhancement is evoked by micromolar concentrations of cAMP in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine. PKI or Walsh inhibitor, a specific peptide inhibitor of cAMP-dependent protein kinase (PKA), inhibits the outward K^+ current. Adenosine 3′,5′-phosphothioate, a competitive inhibitor of PKA, has a similar effect. Conversely, the catalytic subunit of PKA (cAMP independent) from bovine brain enhances the magnitude of the outward K^+ current in the absence of added cAMP. Our results indicate that cAMP modulates K^+ channel activity in mesophyll cells and suggest that this modulation occurs through a cAMP-regulated protein kinase.

In animal systems, cAMP has been well defined as a second messenger modulating many cellular functions, including the regulation of different types of ion channels (Trautwein and Hescheler, 1990; Hartzell et al., 1991; Wang and Giebisch, 1991). At least two mechanisms have been proposed for cAMP regulation of ion channels in animal systems. One is the membrane-delimited regulation of channels by cAMP. *Drosophila* larval muscle possesses such a K^+-selective channel that is activated directly and reversibly by cAMP (Delgado et al., 1991). The second and most common pathway involves a cytoplasmic signaling cascade in which cAMP serves as a second messenger to activate PKA. The activated PKA catalytic subunit then phosphorylates either ion channels or their associated proteins and modulates channel activities (Trautwein and Hescheler, 1990; Hartzell et al., 1991; Wang and Giebisch, 1991). To determine whether an ion channel is regulated by PKA, the typical experiments include intracellular application of PKA or PKA-specific inhibitors with micropipettes (Adams and Levitan, 1982; Alkon et al., 1983; Kume, 1989). For example, PKA holoenzyme increases open probability of Ca^{2+}-dependent K^+ channels of tracheal myocytes in the presence of cAMP, and similar results are obtained when the catalytic subunit of PKA is used (Kume, 1989). Intracellular perfusion of the specific PKA inhibitor, PKI, blocks the serotonin-induced increase of K^+ currents in squid neuron (Adams and Levitan, 1982). A cAMP antagonist, Rp-cAMP-S, which can bind to PKA holoenzyme and cause inactivation of PKA, has also been used in modulating cAMP-dependent pathways (DeWit et al., 1982; Botelho et al., 1988).

Previous studies of ion channel regulation have indicated several parallels in signal transduction pathways in plant and animal cells, such as G-proteins as transducer molecules and Ca^{2+} as a second messenger (Hepler and Wayne, 1985; Fairley-Grenot and Assmann, 1991; Li and Assmann, 1993). However, it is not certain whether a similar parallel can be drawn between plants and animals regarding cAMP-signaling pathways. The major questions are: Are cAMP and associated pathways of cAMP synthesis and degradation present in plant cells? Are there PKA-like protein kinases mediating cAMP functions in plants? What is the physiological role of cAMP in plant cells?

Studies from a number of laboratories have provided considerable evidence for the presence of cAMP in plants and a variety of proteins that interact with cAMP such as cyclic nucleotide phosphodiesterase, cAMP-binding proteins, and adenylate cyclase (Brown and Newton, 1981; Carricarte et al., 1988). A protein kinase activity similar to that of the PKA catalytic subunit has been detected in petunia (Polya et al., 1991). More recently, genes whose deduced amino acid sequences show a high degree of similarity to the conserved catalytic domains of PKA and protein kinase C have been cloned from *Pisum sativum, Phaseolus vulgaris, Oryza sativa,* and *Zea mays* (Lawton et al., 1989; Lin and Watson, 1992). However, PKA has not been purified from plants, nor has enzymatic activity been determined from the expression of cloned putative PKAs. To date, none of the proteins involved in the cAMP-signaling pathway has been functionally characterized from higher plants. Therefore, it is currently uncer-

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Abbreviations: IBMX, 3-isobutyl-1-methyl-xanthine; I_{K_o}, outward K^+ current; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKI, PKA inhibitor; *R*, access resistance; Rp-cAMP-S, Rp-cyclic-3′-5′-monophosphatethioate.

tain whether cAMP-signaling pathways exist in higher plants (Trewavas and Gilroy, 1991).

The physiological role of cAMP in higher plants has been controversial (Brown and Newton, 1981). The major limitations are methodology and the lack of a suitable experimental system with which to work. In previous studies, tissues, organs, or even whole plants were incubated with cAMP for hours or several days to detect the effects of cAMP on phenomena ranging from seed germination to plant growth rate. These procedures face two serious problems: (a) Is cAMP effectively taken up by the plant material? (b) Is cAMP metabolized during the lengthy assays?

To avoid these problems, we have used whole-cell patch-clamp techniques to study the possible role of cAMP in the regulation of K^+ channels. Regulators are directly introduced into the cell or added to the bath solution; therefore, changes of ion channel activities can be monitored instantaneously. Our previous studies have revealed an I_{K_o} in the plasma membrane from mesophyll cells of *Vicia faba* that is activated by depolarizing voltages (Li and Assmann, 1993). The activity of this K^+ channel is inhibited by elevation of cytoplasmic Ca^{2+} and by guanosine-5′-*O*-(3-thiotriphosphate), which activates GTP-binding proteins (Li and Assmann, 1993). By using protein phosphatase 1- and 2A-specific inhibitors, we find that dephosphorylation events inhibit I_{K_o} (Li et al., 1994). Here we report that cAMP dramatically stimulates K^+ channel activity in mesophyll cells of *V. faba*. We also present evidence that a PKA-like protein kinase may be involved in the activation of these outward K^+ channels.

MATERIALS AND METHODS

Plant Materials and Solutions for Whole-Cell Recordings

Plants of *Vicia faba* L. were grown in growth chambers as described previously (Li and Assmann, 1993). *V. faba* mesophyll protoplasts were prepared daily from individual leaves, and whole-cell recording was performed essentially as described by Li and Assmann (1993). Briefly, the standard patch pipette solution contained (in mM) 98 K-glutamate, 2 EGTA, 2 $MgCl_2$, 2 KCl, 10 Hepes, and 2 MgATP titrated to pH 7.2 with 3.6 KOH. The bath (external) solution consisted of 10 K-glutamate, 1 $CaCl_2$, 4 $MgCl_2$, and 10 Hepes titrated to pH 7.2 with 3 KOH. Osmolalities of pipette solution and bath solution were adjusted with D-mannitol to 700 mmol kg^{-1} and 628 mmol kg^{-1}, respectively. The calculated equilibrium potential for K^+ after correcting for ionic strength was −47 mV. During whole-cell recordings, the membrane potential was held at −47 mV except during voltage steps. Currents across the membrane were measured upon imposition of a series of 2-s voltage pulses from −55 to +85 mV with +20-mV increments. Liquid junction potential was measured and corrected as described by Li and Assmann (1993). Steady-state current as a function of membrane potential and leak current were measured as described by Li et al. (1994). Time-activated current at each voltage was calculated as the difference between steady-state currents and leak currents at different voltages. Immediately before each voltage family, seal resistance and whole-cell capacitance were measured from the current response to a 20-mV, 20-ms pulse, insufficient to activate channel activity. Seal resistances were in the range of 1 to 4 GΩ.

There is some variability between protoplasts, particularly protoplasts from different leaves or plants, and we took three measures to account for this. First, we always performed control and treatment measurements with the same preparation of protoplasts (from the same individual leaf) and repeated a given set of controls and treatments several times during a period of 3 to 4 weeks. Thus, each figure and the table has its own set of control measurements. Average control currents at +85 mV (see figures and table for *n*) were 703 pA (comparison with cAMP treatments), 743 pA (comparison with other nucleotide treatments), 849 pA (IBMX treatments), 667 pA (PKI treatments), and 664 pA (PKA treatments). Second, since cells vary in surface area, comparisons between controls and treatments are not made simply based on absolute current magnitude but, rather, on current magnitude normalized by a measure of cell surface area. Since protoplasts flatten as they come in contact with the dish, cell surface area cannot always be accurately calculated from measurements of cell diameter. As discussed by Pusch and Neher (1988; see also Li et al., 1994), membrane capacitance is therefore used as a more accurate measure of membrane area. Average whole-cell capacitances, measured with the compensation device of the patch-clamp amplifier (Bookman et al., 1991), were 23.1 pF (cAMP treatments), 23.1 pF (other nucleotide treatments), 26.7 pF (IBMX treatments), 22.0 pF (PKI treatments), and 21.3 pF (PKA treatments), yielding normalized control currents of 30.4 pA pF^{-1} (cAMP treatments), 32.2 pA pF^{-1} (other nucleotide treatments), 31.8 pA pF^{-1} (IBMX treatments), 30.3 pA pF^{-1} (PKI treatments), and 31.2 pA pF^{-1} (PKA treatments). Thus, it can be seen that control current density actually exhibited low variability during the entire experimental period. Third, statistical analyses were performed (see "Results") to verify the significance of results presented here. All data discussed here were statistically significant with $P \leq 0.05$ (*t* test) when control and experimental currents at +85 mV were compared.

Chemicals

Chemicals to be tested were included in the pipette solutions unless indicated in the text and figure legends. cAMP and other regulatory chemicals were dissolved in the pipette solution or bath solution on the day of recording. cAMP (Tris salt), 8-bromo-cAMP, dibutyryl-cAMP, AMP, cGMP, GMP, IBMX, and PKI were purchased from Sigma. PKA was from Promega (Madison, WI). Rp-cAMP-S was from Calbiochem (La Jolla, CA).

RESULTS

The I_{K_o} Is Enhanced by cAMP and Its Analogs

In the present study, we performed whole-cell patch-clamp experiments with cAMP included either in the bath solution or in the patch pipette solution. A typical whole-cell current time course in the absence of cAMP is shown in Figure 1A. For this cell and for other control cells, whole-cell current magnitude stabilized 0 to 10 min after formation of the whole-cell configuration and remained stable for up to 60

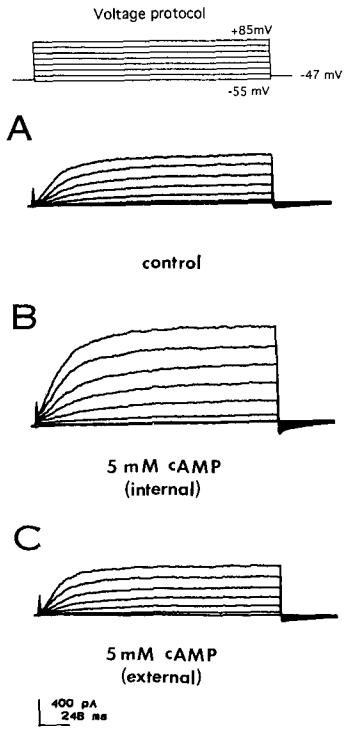

Figure 1. The stimulation of outward rectifying K^+ current by cAMP administered intracellularly to mesophyll protoplasts of *V. faba*. The voltage protocol used is described in "Materials and Methods." A, I_{Ko} in the absence of cAMP (control). *R* was 2.6 GΩ; whole-cell capacitance (*C*) was 21 pF. B, I_{Ko} when 5 mM cAMP Tris salt was perfused to the cell interior with patch pipette solution. R = 1.0 GΩ; C = 23 pF. C, I_{Ko} when 5 mM cAMP (Tris salt) was included in the bath solution. R = 2.3 GΩ; C = 21 pF.

min (data not shown). Addition of 5 mM cAMP to the cytoplasmic side of protoplasts stimulated voltage-dependent I_{Ko} (n = 11; Fig. 1B). The cAMP effect on I_{Ko} can be first observed within 5 to 15 min after the whole-cell formation. In other words, there was a delay of a few minutes before current enhancement by cAMP stabilized compared to the time required to observe the stabilized control currents. For both control and treatments, the current-voltage family data were collected at least 5 min after the current had stabilized. With the control solution loaded in the pipette, no effect on I_{Ko} was observed within 15 min after 5 mM cAMP (final concentration) was added to the bath solution (n = 13, Fig. 1C). Indeed, cAMP-binding assays showed that intact protoplasts did not bind cAMP, but protoplasts broken by hypoosmotic shock released cAMP-binding activity (S. Luan, unpublished data).

The cAMP concentration used in the experiment described in Figure 1 was higher than the physiological level, considering the cAMP levels measured previously from both animal (Kuo and Greenguard, 1969) and plant tissues (Brown and Newton, 1981; Francko, 1983). Therefore, we reduced the concentration of cAMP included in the pipette solution. As shown in Figure 2A, significant enhancement of I_{Ko} was observed only when 1 mM or higher concentrations of cAMP were delivered to the cells, whereas the physiological concentrations of cAMP are considered to be in the nanomolar to micromolar range (Francko, 1983). One of the possible explanations for this discrepancy is that plant cells have high levels of PDE activity (Brown and Newton, 1981; Francko, 1983) that may immediately hydrolyze the exogenous cAMP. To test this possibility, we included a PDE inhibitor, IBMX, in the pipette solution. As shown in Figure 2B, 1 mM IBMX alone had no effect on I_{Ko}. However, IBMX together with 5 μM cAMP dramatically increased the magnitude of I_{Ko}. In the presence of 1 mM IBMX, 50 nM cAMP nonsignificantly increased I_{Ko} (P = 0.066) for currents at +85 mV. The mean time-activated current at +85 mV was 31.2 ± 3.6 pA pF^{-1} in control cells (n = 8) and was 37.6 ± 5.7 pA pF^{-1} in the presence of 50 nM cAMP and 1 mM IBMX (n = 10). These results indicate that PDE activity in mesophyll cells is high and may partly explain why previous approaches have failed to unambigiously show cAMP function in higher plants.

To examine the specificity of cAMP function, active cAMP analogs, 8-bromo-cAMP and N^6,2′-*o*-dibutyryl-cAMP, and a number of other nucleotides, AMP, GMP, and cGMP, were introduced into mesophyll protoplasts at a concentration of 5 mM. As shown in Table I, 8-bromo-cAMP and N^6,2′-*o*-dibutyryl-cAMP had a similar stimulating effect on I_{Ko} as did cAMP, but none of the other nucleotides significantly af-

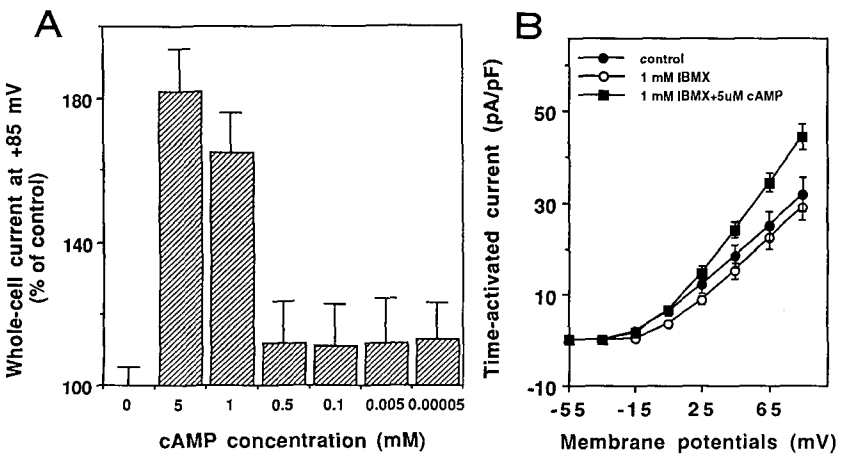

Figure 2. PDE inhibitor increases cAMP efficiency. A, Dosage effects of intracellularly perfused cAMP (Tris salt) on the time-activated I_{Ko}. Whole-cell current magnitude at +85 mV relative to control current at that voltage is shown. Only 1 mM or higher concentrations of cAMP significantly increased I_{Ko}. Control (0 mM cAMP, n = 8), 50 nM cAMP (n = 9), 5 μM cAMP (n = 8), 100 μM cAMP (n = 5), 500 μM cAMP (n = 6), 1 mM cAMP (n = 7), 5 mM cAMP (n = 11). B, PDE inhibitor IBMX potentiates the effect of cAMP on I_{Ko}. Control (n = 9), 1 mM IBMX alone (n = 5), and 1 mM IBMX plus 5 μM cAMP (n = 10). All results are means ± 1 SE.

Table I. *Effects of nucleotides (5 mM) and cAMP analogs (5 mM) on I_{Ko} in mesophyll cell protoplasts of V. faba L.*

Treatment (*n*)[a]	I_{Ko}[b]	P[c]
	pA pF^{-1}	
Control (11)	32.4 ± 2.7	
cAMP, Tris salt (11)	50.3 ± 3.4	≤0.01
8-Bromo-cAMP (7)	52.1 ± 5.1	≤0.01
N^6, 2′-o-dibutyryl-cAMP (7)	41.6 ± 3.5	≤0.01
AMP (8)	27.6 ± 4.1	>0.05
cGMP (5)	27.7 ± 3.6	>0.05
GMP (7)	28.7 ± 2.2	>0.05

[a] *n* is the number of protoplasts tested. [b] I_{Ko} is the time-activated outward current density measured at +85 mV. [c] Probability (*t* test) is calculated by comparing I_{Ko} (pA pF^{-1}) between control and different treatments.

fected I_{Ko}. These results indicate that cAMP is specifically involved in the regulation of the outward K^+ channel.

The I_{Ko} Is Regulated by PKA-Specific Inhibitors and the Catalytic Subunit of PKA

To investigate whether a cAMP-dependent kinase could be responsible for the cAMP effect observed here, we introduced two specific inhibitors of PKA into mesophyll protoplasts. One was PKI (or Walsh inhibitor), a heat-stable 10-kD protein that binds with high affinity to the catalytic subunit of PKA and inhibits its activity (Adams and Levitan, 1982; Kemp et al., 1988). As shown in Figure 3A, application of 10 μM PKI reduced the magnitude of I_{Ko} at +85 mV to 49% of the control level. A competitive inhibitor of PKA, Rp-cAMP-S, is an intracellular cAMP antagonist (Botelho et al., 1988). Intracellular delivery of 1 mM Rp-cAMP-S gave a similar inhibitory effect as PKI on I_{Ko}. These data indicate that a protein kinase similar to PKA controls the activities of outward K^+ channels in mesophyll cells.

Since a PKA homolog from plant sources is not available, we tested whether PKA from animal cells can modulate K^+ channel activity in plant cells. As shown in Figure 3B, when a catalytic subunit of PKA (cAMP independent) from bovine brain was applied at a concentration of 100 units mL^{-1} to the pipette solution dialyzing the interior of the mesophyll protoplasts, the I_{Ko} magnitude increased significantly in the absence of exogenous cAMP. This experiment indicates that elevation of the intracellular concentration of free catalytic PKA increases I_{Ko}.

DISCUSSION

As described earlier in this report, cAMP function in higher plant cells has been a controversial area of research. The present results address two major questions. First, we demonstrate, for the first time, cAMP regulation of an ion channel in a higher plant cell, indicating a physiological role for cAMP in ion transport across the plasma membrane. Second, our data provide strong evidence that cAMP functions at least in part by activating a PKA-like protein kinase in plant cells.

Because the patch-clamp technique affords access to both the cytosolic and the external side of the plasma membrane, we were also able to address the locus of cAMP action. cAMP functions from inside the mesophyll cell (Table I) as in most animal systems rather than through a cell surface receptor as in *Dictyostelium* (Gerisch, 1987). The function of cAMP is specific and cannot be substituted by several other nucleotides, such as AMP, GMP, or cGMP (Table I).

To test whether cAMP affects outward K^+ channels by activation of a PKA-like protein kinase in mesophyll cells, we used two types of PKA inhibitors as diagnostic tools for PKA involvement. These inhibitors, PKI and Rp-cAMP-S, are routinely used in the in vivo study of PKA function in animal cells (Botelho et al., 1988; Kemp et al., 1988). Both inhibitors have effects on I_{Ko} opposite to that of cAMP. Under the control conditions, these inhibitors reduce I_{Ko} by 50%, suggesting that a basal level of PKA-like protein kinase is operating and maintains the I_{Ko} at the control level. This basal activity could be due to a constitutively active form of the protein kinase. Addition of cAMP over a threshold concentration would further activate the kinase activity and, therefore, the magnitude of I_{Ko}. The effective cAMP concentration used in the present studies was 5 μM in the presence of the PDE inhibitor IBMX but is more than 1 mM in the absence of IBMX. This result implies that the threshold concentration of cAMP is in the millimolar range for the activation of a PKA-like kinase and that mesophyll cells contain a high level of PDE activity. The hypothesis of PKA involvement in I_{Ko} regulation is further supported by the observation (Fig. 3B) that intracellular perfusion of the catalytic subunit of PKA stimulates the activity of these outward K^+ channels. Recently, we reported the regulation of inward K^+ channels by a protein phosphatase 2B-like phosphatase in guard cells (Luan et al., 1993). Together with our observations that protein phosphatase 1 and/or 2A inhibit outward channel

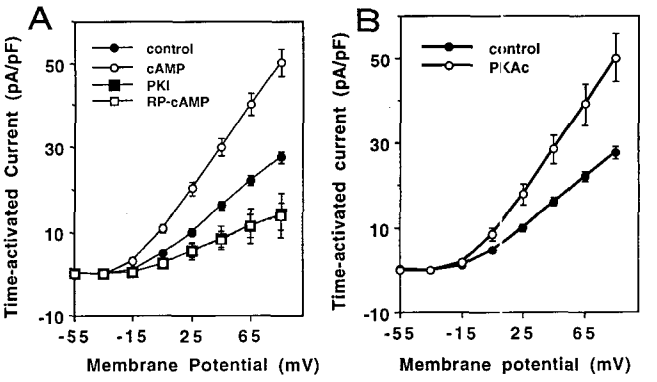

Figure 3. Evidence for PKA involvement in regulation of the outward K^+ channel activity in mesophyll cells of *V. faba*. A, PKA inhibitors PKI (n = 7) and Rp-cAMP-S (n = 6) inhibit K^+ channel activity. Concentrations of PKI and Rp-cAMP-S were 10 μM and 1 mM, respectively. Voltage-dependent outward currents in the absence (control, n = 3) and the presence of 5 mM cAMP (Tris salt, n = 11) are shown. B, The catalytic subunit of PKA (PKAc) increases K^+ current in the absence of added cAMP. O represents the average time-activated current in the presence of 100 units mL^{-1} PKA (n = 7); ● represents the control (n = 7) current. All results are means ± 1 SE.

activities in mesophyll cells (Li et al., 1994), we speculate that phosphorylation/dephosphorylation of K^+ channels or associated proteins modulates the activities of both outward and inward K^+ channel activities in plant cells.

Although our present experiments provide evidence that a PKA-like protein kinase regulates K^+ channel activity in the mesophyll cells of *V. faba*, the data shown here do not exclude the possibility that cAMP may alternately or additionally activate the K^+ channel by a direct gating mechanism. Recently, cDNAs for an inward K^+ channel were cloned from *Arabidopsis thaliana* (Anderson et al., 1992; Sentenac et al., 1992) and functionally expressed in *Xenopus* oocytes (Schachtman et al., 1992). Interestingly, a region of the deduced protein sequence of AKT1 is homologous to the cyclic nucleotide-binding domain of K^+ channels from animal cells (Sentenac et al., 1992). Functional analyses of this cDNA product in a model system, such as *Xenopus* oocytes, may help to further elucidate the regulation of K^+ channels by cAMP. Upon the recent recognition of electrical signals as a pathway for signal transmission by plant cells (Wildon et al., 1992), control of K^+ channel activity in plant cells by cAMP not only implies the conservation of ion channel regulation among higher eukaryotes but suggests a possible function of cAMP-modulated processes in the signaling networks of higher plant cells.

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