Cyclic Adenosine Monophosphate Regulates Calcium Channels in the Plasma Membrane of Arabidopsis Leaf Guard and Mesophyll Cells*

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The effect of cAMP on Ca\(^{2+}\)-permeable channels from Arabidopsis thaliana leaf guard cell and mesophyll cell protoplasts was studied using the patch clamp technique. In the whole cell configuration, dibutyril cAMP was found to increase a hyperpolarization-activated Ba\(^{2+}\) conductance \(I_{Na}\). The increase of \(I_{Na}\) was blocked by the addition of GdCl\(_3\). In excised outside-out patches, the addition of dibutyril cAMP consistently activated a channel with particularly fast gating kinetics. Current/voltage analyses indicated a single channel conductance of ~13 picosiemens. In patches where we measured some channel activity prior to cAMP application, the data suggest that cAMP enhances channel activity without affecting the single channel conductance. The cAMP activation of these channels was reversible upon washout. The results obtained with excised patches indicate that the cAMP-activated \(I_{Na}\) seen in the whole cell configuration could be explained by a direct effect of cAMP on the Ca\(^{2+}\) channel itself or a close entity to the channel. This work represents the first demonstration using patch clamp analysis of the presence in plant cell membranes of an ion channel directly activated by cAMP.

The Arabidopsis thaliana genome encodes 20 putative members of the cyclic nucleotide-gated channel (CNGC) family (1–4). Using electrophysiological techniques in combination with heterologous expression systems, prior work from this laboratory (5, 6) demonstrated that the translation products of cloned plant CNGC cDNAs, like their animal counterparts found in rod and olfactory cells, are activated by the binding of cyclic nucleotide monophosphate (cNMP; cAMP and/or cGMP), a defining attribute of this channel family. Animal CNGCs (six genes are present in humans) are nonsensitive cation (i.e. conducting Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\)) channels whose opening produces membrane depolarization and/or cytosolic Ca\(^{2+}\) rise, important components of signal transduction pathways in the animal cell (7).

CNGC proteins are expressed in a number of plant tissues (4); however, the presence and role of the CNGC-activating ligand cAMP in plants is still controversial. Early reviews (8) cite work that questioned the presence in plants of cAMP at physiologically relevant levels. However, more recent studies using a cyclic nucleotide fluorosensor (9) injected into pollen tubes have reported cAMP levels as high as ~150 nM. Use of tandem mass spectrometry (10) for cyclic nucleotide quantification has confirmed the presence of cAMP (as well as cGMP) in plant cells. Furthermore, a partial clone encoding a protein with homology to fungal adenylate cyclase has been identified in corn pollen; importantly, its translation product was shown to have adenylate cyclase activity (9). Recent work has identified adenylate cyclase activity in (tobacco) chloroplasts (11), suggesting its ubiquitous presence in photosynthetic tissue. cAMP has been associated with cation uptake and associated cell growth/expansion in pollen tubes (9, 12), as well as cell cycle progression and hormone signaling in plants (see Refs. 4 and 12 for reviews). cGMP may be involved in ion uptake into guard cells and stomatal opening, although the effect of cGMP on ion transport is probably indirect and mediated through a signal cascade (13, 14). Conflicting studies have associated cGMP increases in the guard cell cytosol with stomatal closure and presumably loss of ions from guard cells, however (15, 16).

Examination of mutagenized plants has identified a role for the Arabidopsis CNGCs AtCNGC2 (17) and AtCNGC4 (18) in plant hypersensitive response to pathogen infection. Interestingly, an early step in plant response to pathogen infection is Ca\(^{2+}\) influx into cells (19, 20). The molecular mechanisms facilitating this inward Ca\(^{2+}\) current are not known. AtCNGC2 and AtCNGC4 also play a role in normal plant growth and development (i.e. in the absence of pathogen infection); Arabidopsis plants lacking functional copies of these genes display reduced growth as compared with wild type plants (17, 18, 21). AtCNGC2 and AtCNGC1 are probably involved in cation transport in plant cells; translational arrest of those genes affects plant sensitivity to cations in the growth medium (21, 22). In both of these studies, the authors speculated that these plant CNGCs might play a role in Ca\(^{2+}\) movement into or within the plant. Support for the hypothesis that CNGCs may be involved in Ca\(^{2+}\) fluxes across the plant cell membrane can be found in the work of Volotovsky et al. (23). Using protoplasts isolated from tobacco plants expressing recombinant apoaequorin (a cytosolic Ca\(^{2+}\) sensor), they demonstrated that physiological responses such as protoplast swelling and change in cell Ca\(^{2+}\) homeostasis occurred in response to exposure of protoplasts to cyclic nucleotides. Having said that, the authors also show that intracellular stores of Ca\(^{2+}\) could be activated by cyclic nucleotides, since increase of [Ca\(^{2+}\)]\(_{cyt}\) can occur in external Ca\(^{2+}\)-free medium (23).

Prior work from this laboratory has demonstrated that Arabidopsis CNGCs are inward rectified, nonactivating channels that can conduct Ca\(^{2+}\) across the cell membrane (5, 6). Cyclic...
nucleotides are present in plant cells and play critical roles in numerous signal transduction pathways (4, 12). Recent studies (9, 24) have identified proteins with adenylyl (as discussed above) and guanylyl cyclase activities in plants. Thus, the cytosolic machinery necessary for the generation of the activating ligand (cNMP) of CNGCs is present in plant cells. However, no study to date has demonstrated the presence of a cyclic nucleotide-gated inward rectifying ion channel in plant cell membranes. It was the objective of the work described in this report to apply voltage clamp methods to plant cell protoplasts to probe for the presence of this current in plants.

EXPERIMENTAL PROCEDURES

Protoplast Isolation—*A. thaliana* (Columbia) seeds were grown on standard potting mix in a controlled environment growth chamber at 18 °C on a 16:8-h light/dark cycle. Guard cell protoplasts and mesophyll cell protoplasts were isolated from 6- to 8-week-old *Arabidopsis* plants. Guard cell protoplasts were isolated from abaxial epidermal strips as described previously (25). Briefly, epidermal strips were floated on medium containing 1.8–2.5% (w/v) Cellulase Onozuka RS (Yacult Honsha, Tokyo, Japan), 1.7–2% (w/v) Cellulysin (Calbiochem, Behring Diagnostics, La Jolla, CA), 0.026% (w/v) Pectolyase Y-23 (Yacult Honsha), 0.26% (w/v) bovine serum albumin, and 1 mM CaCl₂ (pH 5.6) with osmolality adjusted to 360 mosm/kg with mannitol. After 2–3 h of incubation at 28 °C with gentle shaking, released protoplasts were passed through a 25-μm mesh and kept on ice for 2–3 min before centrifugation (100 × g for 4 min at room temperature). For mesophyll cell protoplasts, we used peeled leaves (mesophyll layer exposed to the light) to collect guard cell protoplasts. Guard cell protoplasts (or membrane patches) were perfused for a few minutes prior to and following the breakage of the junction potential as described (36). Nernst potentials were calculated without correction for ionic activities. Current-voltage relationships for *Iₜ₅₆* were plotted as steady-state currents versus test potentials.

RESULTS

**cAMP Regulates IₐCa in Plant Cells**

**cAMP Modulates an Inward Rectifying Ca²⁺ Current (IₐCa) across the Plasma Membrane of Guard Cell Protoplasts—*Arabidopsis* guard cell protoplasts were patch-clamped (whole cell configuration) in order to assess the effect of cAMP on the hyperpolarization-activated Ba²⁺ current (*IₐBa*). Ba²⁺ was preferred as the charge carrier instead of Ca²⁺, since the use of Ba²⁺ has the advantages of (a) blocking inward and outward K⁺ currents through K⁺-selective channels and therefore unmasking other less dominant conductances, and (b) Ba²⁺ permeates Ca²⁺ channels much better than Ca²⁺ itself (37).

In the whole cell configuration, the addition of Bt₂cAMP to the perfusion bath resulted in an enhancement of *IₐCa* in seven of nine guard cell protoplasts assayed. In the presence of Bt₂cAMP, *IₐBa* measured at −140 mV increased by an average of 3.4 ± 0.28-fold (*n = 7*). A typical experiment demonstrating such an effect (i.e., increase in whole cell current upon the addition of activating ligand) is shown in Fig. 1. In this case, the IV relationships of *IₐBa* measured from one *Arabidopsis* guard cell prior to and 8 min following the application of 1 mM Bt₂cAMP are shown. In the absence of Bt₂cAMP, only a small background conductance is noticeable (~13 pA at −110 mV), but in the presence of Bt₂cAMP, a much larger conductance (~47 pA at −110 mV) was measured, a ~3.5-fold increase (Fig. 1A). The Bt₂cAMP-induced *IₐBa* current, obtained by subtracting the control IV curve (−dB-cAMP) from the test IV curve (+d-b-cAMP), is plotted in Fig. 1B. This analysis shows an inward rectification of this conductance in the whole cell configuration, with a voltage threshold for activation approaching −30 mV. Results presented in Fig. 1C show the whole range of the Bt₂cAMP-activated *IₐBa*-V plot, as we extended the range of the hyperpolarizing voltage ramp to values near −190 mV. At these negative voltages, approximately −195 pA current could be measured. The addition of GdCl₃ at 50 μM, a concentration at which only Ca²⁺ channels are affected (see Ref. 38), and keeping the same concentration of Bt₂cAMP in the bath, led to a dramatic and swift block of the Bt₂cAMP-activated *IₐBa* (Fig. 1C).
A precedent for a direct (or at least membrane-delimited) cAMP activation of native plant plasma membrane ion channels has not been reported in the literature. However, Maathuis and Sanders (39) described a direct effect of cAMP-induced Ca\(^{2+}\)-conducting channels that were silent in the absence of added ligand or dramatically potentiated channels that were opening even before the addition of exogenous Bt\(_2\)cAMP. A representative set of data is shown in Fig. 2 from one Arabidopsis guard cell plasma membrane patch. Note the virtual absence of channel activity before Bt\(_2\)cAMP application in control current traces (at all but one voltage; \(-132\) mV) and compare it with the strong activation that followed the application of Bt\(_2\)cAMP (Fig. 2A). Plotting the I-V relationship of this current prior to and after Bt\(_2\)cAMP application (Fig. 2B) reveals that both curves change sign virtually at the same reversal potential (\(+35\) mV), just slightly positive of \(E_{\text{Ba}} (+29\) mV) but far away from \(E_{\text{Ca}} (-31\) mV) and/or \(E_{\text{K}} (-75\) mV). In this particular example, it can be seen from the current traces shown in Fig. 2A (right panel) that the probability of opening of Ca\(^{2+}\)-conducting channels depends not only on the presence of the ligand Bt\(_2\)cAMP, but also on the membrane voltage. In Fig. 2C, the Bt\(_2\)cAMP-induced \(I_{\text{Ba}}\) (obtained by subtracting the control background current curve from the +Bt\(_2\)cAMP curve) is shown. Although it is difficult to distinguish channel open and closed states when channel gating is as fast as is the case here with these cAMP-activated \(I_{\text{Ba}}\) channels, it can be estimated that the \(I_{\text{Ba}}\) curve plotted in Fig. 2C shows up to five open states. Each of the dashed lines was fitted to a linear equation with a basic slope of 13 pS for one channel being open: \(O_1\) (26 pS for \(O_2\), 39 pS for \(O_3\), etc.). Also, notice the reversal potential just slightly positive of the calculated \(E_{\text{Ba}}\) pointing to a predominant Ba\(^{2+}\) conductance in the presence of Bt\(_2\)cAMP.

Some channel activity could be clearly detected even before Bt\(_2\)cAMP perfusion, as is evident in the recording from the membrane patch shown in Fig. 3. However, in this case, a clear effect of cyclic nucleotide can still be observed. Prior to perfusion with Bt\(_2\)cAMP, the channel seems to be predominantly in a slow kinetic mode of opening and closing (with some intermittent fast channel flickering; see Fig. 3A, left panel). Channel activity appears markedly different after application of Bt\(_2\)cAMP (Fig. 3A, right panel). The channel is now predominately in a fast flickering kinetic mode (with some relatively long periods of silence, as is the case at \(-21.8\) mV; see boxed region of recording in Fig. 3A, right panel).

The activating effect of ligand addition (i.e. an increase in the
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The $I_{Na}$-$V$ relationship of this membrane patch is shown in Fig. 3C. The data points were fitted to a linear relation with a slope factor of 12.2 pS. In this experiment, the apparent reversal potential (Fig. 3C) of +20 mV is slightly less positive than the calculated $E_{Na}$, but is still far removed from both $E_{Na}$ and/or $E_{Cl}$; this result is in good agreement with the primarily Ba$^{2+}$ nature of this conductance. A portion (i.e. the region within the box bordered by broken lines) of the recording made at −31.8 mV command potential from this membrane patch in the presence of Bt$_2$CAMP as shown in Fig. 3A (right panel) is presented in Fig. 4D with an expanded time scale. In this manner, discreet channel opening/closing events can be more easily discerned.

cAMP-activated $I_{Ca}$ in Mesophyll Cell Membranes—Many of the 20 specific Arabidopsis CNGC isoforms are expressed in leaf mesophyll cells (4, 44). We therefore extended our studies to determine whether cyclic nucleotide could activate a Ca$^{2+}$-dependent conductance in the plasma membrane of Arabidopsis leaf mesophyll protoplasts. Of the nine mesophyll cell protoplasts tested, seven (three of three measured in the whole cell configuration, two of two in the outside-out configuration, two of four in the inside-out configuration) demonstrated increased current upon application of exogenous Bt$_2$CAMP (cAMP in case of excised inside-out patches) to the cytosolic portion of the channel. Fig. 4 shows the results of three experiments that were performed on these cells. Recordings from outside-out membrane patches are shown in Fig. 4, A and B. Results from an inside-out patch are shown in Fig. 4, C and D. This figure highlights several important results: (a) cAMP effects on $I_{Ca}$ are not restricted to the guard cell type but could also be seen in mesophyll cells; (b) the effect of Bt$_2$CAMP is fully reversible (Fig. 4B). After activation of the current by the addition of Bt$_2$CAMP to the perfusion bath, removal of the ligand from the bath (i.e. “washout”) resulted in a reversal of channel activation; washout was observed in two of four membrane patches tested. c, activation could also be observed with inside-out membrane patches, using cAMP instead of Bt$_2$CAMP as an activating ligand. Results from a third cell are shown in Fig. 4, C and D. In this experiment, ligand activation was demonstrated using an inside-out patch pulled from a mesophyll cell. Ramp recordings were obtained from this patch prior to and then 8 min after the addition of 1 mM cAMP to the perfusion bath (Fig. 4C). Again, we note an increase in current upon application of CAMP. The cAMP-induced current is shown in Fig. 4D. The reversal potential of the cAMP-dependent current is at $E_{Ba}$ in this experiment.

DISCUSSION

Data presented in this report show that cAMP, a well established cytosolic second messenger in animals, activates a Ca$^{2+}$-dependent conductance channel located in the plasma membrane of plant cells. In whole cell experiments designed to measure $I_{Ca}$ activated by hyperpolarization (e.g. 37, 45–48), Arabidopsis guard cell protoplasts increased their $I_{Ca}$, in response to a lipophilic analog of cAMP (Bt$_2$CAMP) by an average of 3–4-fold (Fig. 1A). The cAMP-induced activation of $I_{Ca}$ measured in guard cell protoplasts in the whole cell configuration, showed a marked inward rectification with no apparent current flow in the outward direction at voltages up to +100 mV (Fig. 1B). It should be noted that prior studies have identified a hyperpolarization-activated $I_{Ca}$ current in the guard cell plasma membrane that is modulated by abscisic acid and hydrogen peroxide (47, 48). The fast gating kinetics and short dwell time (i.e.

**Fig. 2.** Bt$_2$CAMP (db-cAMP) increases single channel activity of $I_{Na}$ in excised outside-out patches. Current traces and $I$-$V$ relationships from one A. thaliana guard cell protoplast (bath and pipette media 1) are shown. A, six current traces (membrane voltages in mV are indicated to the right) of single channel activity present in the membrane patch prior to and 10 min after perfusing with bath solution containing 2 mM Bt$_2$CAMP. B, $I$-$V$ relationship before (○) and after (●) the addition of 2 mM Bt$_2$CAMP to the perfusion bath. Currents were induced by voltage ramps going from +48 to −160 mV in −2 s. C, $I$-$V$ relationship of the cAMP-induced single channel current activity obtained by subtracting the control curve (○) from the test curve (●) shown in B. Respective positions of the calculated values of $E_{Na}$, $E_{Cl}$, and $E_{K}$ are indicated in B and C by arrows along the voltage axis. Five open channel levels are shown and are indicated by $O_1$, $O_2$, etc.; each of the five corresponding dashed lines is a fit of a linear equation (see “Results” for details). The conductance values used to generate the lines corresponding to these five open states were obtained from the analysis (Fig. 3C) of the membrane patch shown in Fig. 3.

“flickery” nature of the channel upon the addition of Bt$_2$CAMP (as observed by comparing the currents shown in the left and right panels of Fig. 3A) is more clearly portrayed in the analysis shown in Fig. 3B. In this case, the dwell time (i.e. the interval of uninterrupted time spent by the channel(s) in a closed and/or any open state; analysis was limited to only three or four open states) is plotted as a function of single channel current amplitude. Fig. 3B highlights two notable effects of ligand application. a, there is an increase in the number of channels in the open state. This is consistent with a recruitment of channels that were silent prior to the addition of cAMP. b, there is a decrease in the channel dwell time at any single command voltage shown in Fig. 3A. A decrease in channel dwell time is consistent with an increase in the flickery nature of the channel in the presence of cAMP.

The $I_{Na}$-$V$ relationship of this membrane patch is shown in Fig. 3C. The data points were fitted to a linear relation with a slope factor of 12.2 pS. In this experiment, the apparent reversal potential (Fig. 3C) of +20 mV is slightly less positive than the calculated $E_{Na}$, but is still far removed from both $E_{Na}$ and/or $E_{Cl}$; this result is in good agreement with the primarily Ba$^{2+}$ nature of this conductance. A portion (i.e. the region within the box bordered by broken lines) of the recording made at −31.8 mV command potential from this membrane patch in the presence of Bt$_2$CAMP as shown in Fig. 3A (right panel) is presented in Fig. 4D with an expanded time scale. In this manner, discreet channel opening/closing events can be more easily discerned.

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flickery nature) of the cAMP-activated inward current we observe in our studies is similar to that of the guard cell hyperpolarization-activated Ca^{2+} current reported by Hamilton et al. (47). We also note with interest that the single channel conductance reported by Hamilton et al. (47) (13 pS) is similar to that reported here for the guard cell cAMP-activated Ca^{2+} channel; documentation that the same channel is responding to all three of these messenger molecules (i.e. cAMP, hydrogen peroxide, and abscisic acid) awaits further study, however.

![Image](http://www.jbc.org/)
Our results indicate that Arabidopsis mesophyll cells also contain an inward rectified Ca\(^{2+}\) channel directly activated by cAMP. In other studies (data not shown), we have also identified a cAMP-activated inward rectified Ca\(^{2+}\) channel in the plasma membrane of guard cell protoplasts isolated from Vicia faba. The cyclic nucleotide-activated channel in Vicia appears to have similar properties as that of the Arabidopsis guard cell channel we have characterized in the work presented here.

The Ca\(^{2+}\) channel blocker Gd\(^{3+}\) (38, 47, 49) completely blocked the cAMP-activated current. These results provide the first electrophysiological evidence supporting previous speculations that increase of cytosolic [Ca\(^{2+}\)] triggered by cNMP occurs mainly through a plasma membrane-localized Ca\(^{2+}\)-permeable channel (23, 50).

The effect of Bt\(_2\)cAMP in the whole cell configuration could be interpreted as either indirect (i.e. mediated through cAMP-activated protein kinases (which could in turn activate \(I_{Ca}\) by phosphorylation; see Ref. 51)) or a direct effect of the ligand on the \(I_{Ca}\) channel itself. Our results with excised patches indicate that cAMP was able to stimulate channel activation directly. Thus, cAMP facilitated the opening of single \(I_{Ca}\) channels upon hyperpolarization in excised patch modes. In other cases, single channel activity could be resolved in cAMP-free patches containing only a few channels. The cAMP-induced \(I_{Ca}\) current activation occurred without apparent modification of the single channel conductance (Fig. 3C). The reversal potential obtained from the single channel \(I-V\) relationship (+20 mV) indicated that these channels were preferentially carrying Ba\(^{2+}\) despite the presence of other ions (K\(^{+}\) and Cl\(^{-}\)) in the recording medium. Prior work from this laboratory (5, 6) has shown that cloned plant CNGCs expressed in heterologous systems conduct Ca\(^{2+}\) (as well as K\(^{+}\)) and, further, that exogenous Ca\(^{2+}\) partially blocks K\(^{+}\) conductance by these channels. We note that in some experiments, cAMP-activated current shows a reversal potential very close to \(E_{Ba}\) (Figs. 2B and 4D), whereas in others (Figs. 3C and 4B) the reversal potential is near (relative to \(E_K\) and \(E_{Cl}\)) but not at \(E_{Ba}\). This result could be explained by the cAMP-activated channel predominantly conducting Ca\(^{2+}\) (Ba\(^{2+}\)) but also allowing, to some extent, K\(^{+}\) to permeate the channel. These results are consistent with the conductance properties of cloned plant CNGCs as reported in earlier work from this laboratory (5, 6).

As discussed above, the use of the excised patch configurations allowed us to identify cAMP as a direct activator of Ca\(^{2+}\) channels. To our knowledge, this is the first report showing a direct stimulatory action of cAMP on any ion channel present in a native plant membrane. We conclude that the work presented in this report showing cAMP-dependent activation of Ca\(^{2+}\) permeation in these plant cell membranes is consistent with the presence of functional CNGCs in these cells. It should be noted that in all cases reported to date, CNGCs have been found to be heterotetramers composed of subunits encoded by different CNGC genes (7). The translation products of at least 12 of the 20 different CNGC genes in Arabidopsis are expressed in leaves (4). Thus, we cannot know if the cyclic nucleotide-activated current we recorded from the leaf cell membrane in our experiments can be attributed to one or several different CNGC channel protein complexes or if the presumed CNGC channel protein(s) facilitating the currents we recorded is composed of one or several CNGC gene translation products.

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