Nucleotides and Mg\(^{2+}\) Ions Differentially Regulate K\(^{+}\) Channels and Non-Selective Cation Channels Present in Cells Forming the Stomatal Complex

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Voltage-dependent inward-rectifying (\(K_{in}\)) and outward-rectifying (\(K_{out}\)) K\(^{+}\) channels are capable of mediating K\(^{+}\) fluxes across the plasma membrane. Previous studies on guard cells or heterologously expressed K\(^{+}\) channels provided evidence for the requirement of ATP to maintain K\(^{+}\) channel activity. Here, the nucleotide and Mg\(^{2+}\) dependencies of time-dependent \(K_{in}\) and \(K_{out}\) channels from maize subsidiary cells were examined, showing that MgATP as well as MgADP function as channel activators. In addition to \(K_{in}\) channels, these studies revealed the presence of another outward-rectifying channel type (MgC) in the plasma membrane that however gates in a nucleotide-independent manner. MgC represents a new channel type distinguished from \(K_{out}\) channels by fast activation kinetics, inhibition by elevated intracellular Mg\(^{2+}\) concentration, permeability for K\(^{+}\) as well as for Na\(^{+}\) and insensitivity towards TEA\(^{+}\). Similar observations made for guard cells from *Zea mays* and *Vicia faba* suggest a conserved regulation of channel-mediated K\(^{+}\) and Na\(^{+}\) transport in both cell types and species.

Keywords: Guard cell — K\(^{+}\) channel — Mg\(^{2+}\) — non-selective cation channel — Nucleotides — Subsidiary cell.

Introduction

Ion channel activities are fine-tuned by a variety of regulation mechanisms. Depending on the channel type and its physiological role within the cell and tissue, different sensitivities towards extra- and intracellular regulators evolved to control channel gating. Among them, nucleotides and Mg\(^{2+}\) ions have been identified as important regulators of ion channels in different animal and plant cell types. For instance, the animal ATP-sensitive potassium channels (\(K_{ATP}\)) are inhibited by ATP and stimulated by ADP (Campbell et al. 2003). A series of animal cation channels are regulated by intracellular Mg\(^{2+}\) ions i.e. glutamate-activated channels and inward-rectifying K\(^{+}\) channels (Matsuda et al. 1987, Nowak et al. 1984).

In plants, most studies concerning the nucleotide- and Mg\(^{2+}\)-dependent regulation were performed on ion channels from guard cells. Thereby, it was shown that the activity of the cloned guard cell K\(^{+}\) channels KST1 and KAT1 in heterologous expression systems is maintained in the presence of intracellular ATP (Hoshi 1995, Müller-Röber et al. 1995). Removal of ATP results in rundown of channel activity. Likewise, ATP is required for activation of guard cell K\(^{+}\) channels in vivo (Schroeder 1988, Wu and Assmann 1995, Goh et al. 2002). In comparison little is known about Mg\(^{2+}\)-dependent ion channels in plants. The slow(SV)- and fast(FV)-activating vacuolar channels are stimulated and inhibited by Mg\(^{2+}\) ions, respectively (Carpaneto et al. 2001, Pei et al. 1999). Inward rectification of plant K\(^{+}\) uptake channels in the plasma membrane, however, is not mediated via Mg\(^{2+}\)-dependent block but represents an intrinsic voltage-dependent feature (Hedrich et al. 1995, Schroeder 1995).

In contrast, information about the nucleotide or Mg\(^{2+}\) sensitivity of ion channels from subsidiary cells was not yet available. Subsidiary cells are flanking the pair of guard cells in grasses to form with them the stomatal complex. During stomatal movement a shuttle transport of K\(^{+}\) ions appears to take place between subsidiary cells and guard cells (Raschke and Fellows 1971, Penny and Bowling 1974, Willmer and Pallas 1973) probably enabled by K\(^{+}\)-permeable channels in the plasma membrane of these cell types. So far two populations of K\(^{+}\)-selective ion channels have been identified in subsidiary cells (Majore et al. 2002). They give rise to time-dependent inward-rectifying and outward-rectifying K\(^{+}\) currents, respectively, similar to those found in guard cells from maize and other species (Schroeder 1988, Fairley-Grenot and Assmann 1992, Ichida et al. 1997, Dietrich et al. 1998, Ache et al. 2000). Thus these K\(^{+}\) channel types may mediate K\(^{+}\) uptake and K\(^{+}\) release during stomatal movement underlying the shuttle K\(^{+}\) transport between subsidiary cells and guard cells (Raschke and Fellows 1971, Willmer and Pallas 1973). In the present work we examined the effect of several nucleotides and Mg\(^{2+}\) ions on the different types of K\(^{+}\)-permeable channels expressed in maize subsidiary cells. Thereby we found that MgATP or MgADP were required for K\(^{+}\) channel activation. In addition, a new outward-rectifying cation channel type permeable to K\(^{+}\) and Na\(^{+}\) was identified. In contrast to the slow-activating outward-rectifying K\(^{+}\)-selective channels, these fast-activating non-selective cation channels were not stimulated by nucleotides but inhibited by intracellular Mg\(^{2+}\) ions. Since these three channel types were found in guard cells from

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maize and broad bean, too, they appear to represent the general transport pathways for K$^+$ uptake as well as for K$^+$ and Na$^+$ release during stomatal movement.

**Results**

Recently maize subsidiary cells were shown to possess at least two voltage-dependent K$^+$ channel types that mediate either time-dependent outward- or inward-rectifying K$^+$ currents across the plasma membrane at depolarization and hyperpolarization, respectively (Majore et al. 2002). To gain new insights into the nucleotide-dependent regulation of cells forming the stomatal complex, with the patch-clamp-technique we recorded macroscopic outward-rectifying K$^+$ currents from subsidiary cell protoplasts in the presence of 5 mM ADP in the Mg$^{2+}$-containing standard pipette solution.

To reveal whether time-dependent and instantaneous currents are mediated by different channel types or reflect different modes of the same channel, in the following we examined their nucleotide-dependent regulation, selectivity and pharmacology.

**Nucleotide dependency of slowly activating outward-rectifying K$^+$ channels**

In the presence of cytosolic GTP and GDP (Fig. 2a, e, g) or in the absence of nucleotides (Fig. 2b, g) the time-depend-
ent $K_{\text{out}}$ currents completely vanished in subsidiary cells soon after whole cell access (Fig. 2e). However, ADP and ATP led to activation of time-dependent $K_{\text{out}}$ currents (Fig. 1a, 2a, e, g) (Majore et al. 2002) which were stable between the recording time of 4 and 30 min (Fig. 2e). Reduction in the intracellular ATP concentration resulted in a dose-dependent decrease of the time-dependent $K_{\text{out}}$ current amplitudes (Fig. 2b). To test whether the stimulatory effect of ATP on $K_{\text{out}}$ channels is attributed to the action of protein kinases, K252a, an inhibitor of serine/threonine-dependent protein kinases, was applied to 5-mM-ATP-containing standard pipette solution. However, K252a did not affect the time-dependent $K_{\text{out}}$ currents (at +66 mV; control: 26.2 ± 9.0 pA/pF; 500 nM K252a: 26.0 ± 9.8 pA/pF), indicating that serine/threonine-dependent protein kinases are not involved in ATP-dependent $K_{\text{out}}$ channel activation.

Since mitochondria provide a potential source of ATP for $K^+$ channel activation in rice mesophyll cells (Goh et al., 2003), we further examined whether mitochondria synthesize ATP from ADP added to the patch pipette solution. When ADP and the adenylate kinase inhibitor ApA, which prevents the conversion of two ADP to AMP and ATP, were simultaneously applied via the patch pipette, the time-dependent $K_{\text{out}}$ currents could not be distinguished from those evoked in ADP-containing solutions. Likewise, oligomycin, a blocker of the mitochondrial ATP synthase, did not affect the time-dependent $K_{\text{out}}$ currents in ADP (at +66 mV; control: 68.2 ± 29.5 pA/pF; n = 9; 100 µM ApA: 83.5 ± 68.8 pA/pF, n = 5; oligomycin (5 µg/ml): 95.3 ± 46.9 pA/pF, n = 6). Thus, ATP itself seems to activate the time-dependent $K_{\text{out}}$ currents of maize subsidiary cells in an as yet unknown manner. Similar to subsidiary cells, activation of time-dependent $K_{\text{out}}$ currents from Zea mays and Vicia faba guard cells was not just observed in the presence of ADP (Fig. 1b–d) but also with ATP in the Mg$^{2+}$-containing standard pipette solution (Fig. 3a, c, d, f). $K^+$ currents disappeared in the absence of ATP or ADP and lacked activation upon GTP application (Fig. 3c, f). The time-dependent $K_{\text{out}}$ currents from Vicia faba were similar after 4 min and 30 min in the whole cell configuration showing that channel activity did not significantly rundown during this period (4 min in ATP: $I_{\text{t}}/C_m = 21.8±11.6$ pA/pF; in ADP: $I_{\text{t}}/C_m = 42.8±8.8$ pA/pF; 4 min in GTP: $I_{\text{t}}/C_m = 8.0±6.9$ pA/pF).

![Fig. 3](https://academic.oup.com/pcp/article-abstract/46/10/1682/1904267)

**Fig. 3** Effect of different nucleotides on outward-rectifying currents of Zea mays (a-c) and Vicia faba guard cells (d-f). a, b, d, e Time-dependent and instantaneous current densities ($I_{\text{t}}/C_m$, $I_{\text{inst}}/C_m$) are given as a function of membrane voltage. SC and GC indicate protoplasts of subsidiary cells and guard cells, respectively, a, b Z. mays GC: 5 mM ATP (filled triangle) (n = 4); 5 mM ADP (triangle) (n = 4), 3 mM GTP (filled rhombus) (n = 3), d, e V. faba GC: 5 mM ATP (filled triangle) (n = 6); 5 mM ADP (triangle) (n = 9), 3 mM GTP (filled rhombus) (n = 4). c, f Representative traces of outward-rectifying currents elicited upon voltage pulses in the range of –44 and +76 mV in 20-mV-steps are shown. Except for those current traces which were recorded 5 min after whole cell access from Z. mays guard cell protoplasts in the absence of nucleotides (0 mM NTP), all other current traces were monitored 30 min after establishment of the whole cell configuration. a-f The Mg$^{2+}$-containing standard pipette solution was used in the absence or presence of nucleotides.

![Fig. 4](https://academic.oup.com/pcp/article-abstract/46/10/1682/1904267)

**Fig. 4** Effect of intracellular Mg$^{2+}$ on the instantaneous outward-rectifying currents of subsidiary cells. a, b Representative traces of $I_{\text{inst}}$ currents were evoked by voltage pulses in the range of –14 and +86 mV in 20-mV-steps in the presence/absence of nucleotides in nominal Mg$^{2+}$-free pipette solution. c Instantaneous current densities ($I_{\text{inst}}/C_m$) were determined in the absence (triangle, n = 3–4) and presence of 7 mM total Mg$^{2+}$ (filled square, n = 13) in 5 mM ATP-containing standard pipette solution and plotted as a function of membrane voltage. d $I_{\text{inst}}/C_m$ (V) curves determined in the absence and presence of nucleotides and/or Mg$^{2+}$ are presented (triangle 5 mM ATP, 0 mM Mg$^{2+}$, n = 3–4; filled circle 5 mM ADP, 0 mM Mg$^{2+}$, n = 4; square 0 mM NTP, 0 mM Mg$^{2+}$, n = 3; asterisk 0 mM NTP, 2 mM Mg$^{2+}$, n = 8).
**Table 1** Nucleotide-containing and -free standard pipette solutions used in Fig. 2 are characterized by different free Mg$^{2+}$ concentrations

<table>
<thead>
<tr>
<th>Solution</th>
<th>$\log K$ in M$^{-1}$</th>
<th>total [Mg$^{2+}$] in µM</th>
<th>free [Mg$^{2+}$] in µM</th>
<th>$I_{\text{inst}}$/C$_m$ $\pm$SE in [pA/pF]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM NTP</td>
<td>–</td>
<td>2,000</td>
<td>2,000</td>
<td>2.3±0.6 (8)</td>
</tr>
<tr>
<td>5 mM ATP</td>
<td>4.70</td>
<td>7,000</td>
<td>1,400</td>
<td>5.2±2.2 (13)</td>
</tr>
<tr>
<td>5 mM GDP</td>
<td>3.52</td>
<td>2,000</td>
<td>&gt;370 $^b$</td>
<td>27.3±1.6 (6)</td>
</tr>
<tr>
<td>5 mM ADP</td>
<td>4.11</td>
<td>2,000</td>
<td>370</td>
<td>31.3±5.0 (9)</td>
</tr>
</tbody>
</table>
| 5 mM GTP | 4.45                  | 2,000                   | 93                    | 32.5±2.5 (5)                             

$^a$ $K$ = equilibrium constant for the reaction of a nucleotide species and Mg$^{2+}$ to give the complex (Pecoraro et al. 1984, Stumber et al. 2002).

$^b$ not known, but considering $K$ and the same total Mg$^{2+}$ concentration present in ADP it should be higher than 370 µM.

$^c$ Instantaneous current densities ($I_{\text{inst}}$/C$_m$) were determined at +66 mV from experiments shown in Fig. 2c and d. Parenthesis gives the number of experiments (see Fig. 2c, d).

30 min in ATP: $I_{\text{m}}/C_m = 16.8±11.3$ pA/pF and in ADP: $I_{\text{m}}/C_m = 36.9±10.0$ pA/pF.

Nucleotide insensitivity and Mg$^{2+}$ block of fast-activating outward-rectifying cation channels

In comparison to ADP (Fig. 1a, 2c) the instantaneous outward-rectifying currents ($I_{\text{inst}}$) in subsidiary cells were similar in the presence of GTP and GDP (Fig. 2d, e), but appeared largely reduced in ATP-containing standard solution (Fig. 2c, g). This reduction was already accomplished 4 min after whole cell access pointing to a fast channel regulation (Fig. 2f). These results may further indicate that $I_{\text{inst}}$ is inhibited by ATP or the elevated free Mg$^{2+}$ concentration accompanying the treatment with ATP. In ATP-containing standard solutions the free Mg$^{2+}$ concentration was calculated to be about 1.4 mM while it was only 0.4 mM in ADP-containing standard solutions (Table 1). To study the effect of Mg$^{2+}$ and nucleotides independently, ATP was applied in the absence of Mg$^{2+}$. Under this condition $I_{\text{inst}}$ increased while time-dependent $I_{\text{out}}$ currents disappeared (Fig. 4a, c). Thus, the absence of $I_{\text{inst}}$ in the presence of ATP (Fig. 2c, 4c, Table 1) very likely results from an inhibition by an excess of free Mg$^{2+}$ ions. In line with the features of a Mg$^{2+}$-sensitive channel, in the presence or absence of nucleotides similar $I_{\text{inst}}$ amplitudes could be elicited under Mg$^{2+}$-free conditions (Fig. 4d). On the other hand, $I_{\text{inst}}$ could be blocked by Mg$^{2+}$ even under nucleotide-free conditions (Fig. 4d). In contrast, activation of time-dependent $I_{\text{out}}$ currents required Mg$^{2+}$ in the presence of either ATP or ADP suggesting that the MgADP or MgATP complex rather than the free nucleotides function as channel activators. Taken together, these results show that time-dependent $I_{\text{out}}$ currents are stimulated by nucleotides in a Mg$^{2+}$-dependent manner while $I_{\text{inst}}$ is insensitive towards nucleotides and blocked with increasing intracellular Mg$^{2+}$ concentration. In guard cells from Zea mays (Fig. 1b, e, 3b, c) and Vicia faba (Fig. 1c, e, 3e, f) similar fast-activating outward-rectifying currents were induced at low intracellular Mg$^{2+}$ concentration with e.g. ADP or GTP in the standard pipette solution and they appeared largely reduced at high intracellular Mg$^{2+}$ concentration as well.

Pharmacology and selectivity of fast-activating outward-rectifying cation channels

To gain further insights into the nature of the channel type mediating the instantaneous currents, the corresponding cation channel

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**Fig. 5** Different effect of extracellular TEA$^+$ and intracellular Na$^+$ on time-dependent and instantaneous outward-rectifying currents of subsidiary cells. a Representative current traces recorded at +56 mV in the presence of 5 mM ATP (upper traces) or 5 mM GTP (lower traces) before (–TEA$^+$, left column) and about 5 min after external addition of 30 mM TEA$^+$ (right column). Dashed lines represent zero current level. Note the TEA$^+$ insensitivity of the instantaneous currents. b, c Time-dependent ($I_{\text{t}}$) and instantaneous ($I_{\text{inst}}$) current-voltage curves were determined in the presence of intracellular K$^+$ (circle) or Na$^+$ (filled circle) by using the Mg$^{2+}$-containing standard pipette medium or sodium pipette solution. $I_{\text{t}}$ and $I_{\text{inst}}$ were induced upon addition of (b) 5 mM Mg-ATP or (c) 5 mM Tris-GTP to the pipette solutions, respectively. The number of experiments in (b) was filled circle $n = 3$, circle $n = 13$ and in (c) filled circle $n = 6$, circle $n = 5$. 

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permeability and sensitivity towards the $K_{\text{out}}$ channel blocker TEA$^+$ were studied. Time-dependent $K_{\text{out}}$ currents almost completely diminished upon addition of 30 mM TEA$^+$ (Fig. 5a, upper traces) (Majore et al. 2002). No time-dependent $K_{\text{out}}$ currents were observed when intracellular K$^+$ was replaced by Na$^+$ (Fig. 5b). This discrimination between K$^+$ and Na$^+$ is well in agreement with previous studies on time-dependent $K_{\text{out}}$ channels in subsidiary cells (Majore et al. 2002). In contrast to these $K^+$-selective channels, $I_{\text{in}}$ remained unaffected by external TEA$^+$ treatment or replacement of intracellular K$^+$ by Na$^+$ (Fig. 5a, lower traces, and 5c). The latter finding indicates that both K$^+$ and Na$^+$ can permeate this channel type. The difference in the susceptibility towards TEA$^+$, Mg$^{2+}$, Na$^+$ and nucleotides demonstrates that the time-dependent and the instantaneous outward-rectifying currents in subsidiary cells result from different depolarization-dependent channel types such as $K_{\text{out}}$ channels and Mg$^{2+}$-sensitive non-selective cation channels (MgC).

Effect of nucleotides and Mg$^{2+}$ on inward-rectifying $K^+$ channels

As in the case of the $K_{\text{out}}$ channels (Fig. 2a), ATP in Mg$^{2+}$-containing standard pipette solution was similarly efficient as ADP in the stimulation of inward-rectifying K$^+$ ($K_{\text{in}}$) currents from subsidiary cells (Fig. 6a, b). GTP and GDP, however, were unable to activate $K_{\text{in}}$ channels from subsidiary cells (Fig. 6a, d). $K_{\text{in}}$ currents also vanished in AMP-containing or nucleotide-free standard pipette solution (Fig. 6a, c, d). The stimulatory ATP effect on the $K_{\text{in}}$ currents from subsidiary cells was concentration-dependent (Fig. 6a, c). When the intracellular ATP concentration was lowered from 5 to 1 mM, $K_{\text{in}}$ currents were reduced by approximately 73% at $-164$ mV (Fig. 6c). In contrast to $K_{\text{out}}$ and MgC channels (Fig. 2e, f), the $K_{\text{in}}$ channels, however, were characterized by rundown behavior between the recording time of 12 and 37 min. For instance the $K_{\text{in}}$ current amplitude at $-164$ mV was $-111.5 \pm 15.8$ pA/pF in ATP ($n = 10$) and $-93.0 \pm 11.6$ pA/pF in ADP ($n = 9$) after 12 min but $-59.1 \pm 10.3$ pA/pF in ATP ($n = 10$) and $-59.4 \pm 10.4$ pA/pF ($n = 9$) in ADP after 37 min in the whole cell configuration. The application of the protein kinase inhibitor K252a in the presence of 5 mM ATP in the standard pipette medium did not significantly alter the $K_{\text{in}}$ current amplitudes (e.g. at $-184$ mV; control: $-184.2 \pm 20.3$ pA/pF, $n = 10$; 500 nM K252a: $-136.6 \pm 20.3$ pA/pF, $n = 10$). Thus threonine/serine-dependent protein kinases are probably not involved in maintaining $K_{\text{in}}$ channel function. However, $K_{\text{in}}$ currents disappeared in ATP- or ADP-containing pipette solutions under nominal Mg$^{2+}$-free conditions (data not shown) suggesting that the MgATP or MgADP complex rather than the free nucleotides are required to activate the $K_{\text{in}}$ channels. In the presence of 5 mM ADP, the adenylyl kinase inhibitor Ap$_5$A did not affect the $K_{\text{in}}$ currents from subsidiary cells while the ATP synthase inhibitor oligomycin reduced them by about 30% at $-184$ mV (control: $-90.7 \pm 14.0$ pA/pF; $n = 9$; Ap$_5$A (100 nM): $-84.7 \pm 20.1$ pA/pF, $n = 5$; oligomycin (5 µg/ml): $-63.3 \pm 11.1$ pA/pF, $n = 6$). Thus, it can be assumed that this resting activity of $K_{\text{in}}$ channels is most likely due to action of ADP and not to ATP generated by mitochondria. The potential of ADP and ATP to maintain the $K_{\text{in}}$ channel activity seems to represent a general rather than a cell-type specific feature since these nucleotides prevented its rundown in guard cells from Zea mays as well as Vicia faba as well (Fig. 7c, d).

Discussion

Here, we identified a new fast-activating non-selective cation channel type (MgC). This channel is active upon depolarization in Zea mays subsidiary cells and guard cells as well as in Vicia faba guard cells. Open MgC channels give rise to
The physiological role and molecular basis of MgC currently remain unknown. Thus a similar situation exists as with non-selective cation channels as well as with stretch-, cold- and elicitor-activated channels in planta. However, one obvious function of MgC in planta would be to guarantee a pathway for the release of K\(^+\), parallel to K\(_{\text{out}}\) channels, providing a redundant system for K\(^+\) transport alike that reported for K\(_{\text{out}}\) channels (Szyroki et al. 2001). Even more interesting, considering its permeability not only for K\(^+\) but also for Na\(^+\), MgC is also a suitable element for preventing Na\(^+\) accumulation under salt stress. Na\(^+\) can enter the cell via non-selective cation channels (Amtmann et al. 1997, Roberts and Tester 1997, Tyerman et al. 1997). However, high levels of Na\(^+\) in the cytosol are toxic to plants, thus maintaining a low cytosolic Na\(^+\) level is essential for the viability of the plant. Under salt stress, stomatal opening will result in an increase in cytosolic and vacuolar Na\(^+\) levels in guard cells. The Na\(^+\) level will also rise within the neighboring subsidiary cells during stomatal closure. If under salt stress conditions only K\(_{\text{out}}\) channels would operate, Na\(^+\) accumulation in the cytosol of guard cells and subsidiary cells would accompany stomatal closure and stomatal opening, respectively. This problem could be circumvented by Na\(^+\)-permeable channels like MgC.

While MgC is regulated in a nucleotide-independent manner, the K\(^+\)-selective channels (K\(_{\text{in}}\), K\(_{\text{out}}\)) were found to require MgATP or MgADP for channel activation (Fig. 1–3, 6, 7). In the absence of these nucleotides we observed K\(^+\) channel rundown within about 30 min (see also Goh et al. 2002). In contrast to this ATP or ADP dependency, in earlier studies on Vicia faba guard cells stable whole cell K\(^+\) currents were recorded even in the absence of nucleotides in the pipette solution, probably because of a reduced diffusional loss of the cytoplasmic content and incomplete equilibration with the pipette solution (Wu and Assmann 1995). Here oligomycin, an inhibitor of the mitochondrial ATP synthase, caused a decrease in the K\(_{\text{in}}\) currents in the presence of ADP suggesting that K\(_{\text{in}}\) channel activation is not only attributed to ADP but partially also to ATP generated by mitochondria. Thus in contrast to the animal K\(_{\text{ATP}}\) channels (Campbell et al. 2003) both ATP and ADP appear to stimulate the K\(_{\text{in}}\) channels in subsidiary cells. An inhibitory effect of oligomycin or the adenylate kinase inhibitor Ap5A on the K\(_{\text{out}}\) channels in subsidiary cells, however, was not monitored in the presence of ADP. These results suggest that ADP can also activate K\(_{\text{out}}\) channels while the mitochondria-produced ATP level, however, seems to be not sufficient for their stimulation. Accordingly it remains unclear whether K\(_{\text{in}}\) channel activation observed with ATP-containing pipette solution is correlated to ATP or to ADP present or produced to a minor extent in this solution. The involvement of ATP in channel regulation, however, is supported by previous works on guard cell K\(_{\text{in}}\) and K\(_{\text{out}}\) channels from Vicia faba and Arabidopsis thaliana showing that phosphorylation/dephosphorylation processes participate in K\(^+\) channel regulation (Luan et al. 1993, Li et al. 1994, Thiel and Blatt 1994, Li et al. 1998, Mori et al. 2000).
Though the present study shows that the $K_{m}$ and $K_{out}$ channels from subsidiary cells were insensitive to K252a, it still does not exclude the possibility of the involvement of phosphatases or K252a-insensitive protein kinases in channel regulation. More interestingly, we could identify a major role of ADP in $K_{out}$ channels. 

The observation that ADP was not able to exclude the possibility of the involvement of phosphatases or K252a-insensitive protein kinases in channel regulation. More interestingly, we could identify a major role of ADP in $K_{out}$ channels. 

**Materials and Methods**

**Plant material and protoplast isolation**

Maize plants (Z. mays L. var. Caráibe, Saaten Union, Hannover, Germany) were grown for 8 days in a climate chamber at 70% humidity, at 26°C/16°C (day/night) with a 12.5 h photoperiod and a photon flux density of about 130 µmol m$^{-2}$ s$^{-1}$. Vicia faba L. cv. Französische Weissknige plants (Benary, Hahn-Münden, Germany) were cultivated for 2 weeks in a climate chamber at 50–70% humidity, at 22°C/16°C (day/night) with a 12 h photoperiod and a photon flux density of about 80 µmol m$^{-2}$ s$^{-1}$.

For isolation of subsidiary cell protoplasts, maize epidermal peels were incubated at 30°C for 110 min in an enzyme solution composed of 0.6% (w/v) cellulase Onozuka-RS (Serva, Heidelberg, Germany), 0.064% (w/v) pectolyase Y-23 (Seishin Corp., Tokyo, Japan), 0.4% macerozyme R-10 (Serva, Heidelberg, Germany), 0.8% (w/v) bovine serum albumine, 0.4% (w/v) polyvinyl pyrrolidone (PVP-40), 0.8 mM CaCl$_2$, pH 6.5/KOH, $\pi = 530$ mosmol kg$^{-1}$ (D-mannititol). For isolation of guard cell protoplasts of Zea mays or Vicia faba, epidermal peels were gently shaken at 30°C for 3–3.5 h or 2 h, respectively, in the subsidiary-cell enzyme solution (see above) which was mixed in a 1 : 1 ratio with an enzyme solution composed of 2.5% (w/v) cellulase Onozuka-RS (Yakult, Tokyo, Japan), 2% (w/v) macerozyme Onozuka-R10 (Yakult, Tokyo, Japan), 0.026% (w/v) pectolyase Y-23 (Sigma-Aldrich, Deisenhofen, Germany), 0.26% bovine serum albumine, 1 mM CaCl$_2$, 10 mM HEPES/Tris pH 6.5, $\pi = 310$ mosmol kg$^{-1}$ (D-sorbitol). Following enzyme treatment, the suspension containing subsidiary cell or guard cell protoplasts from maize was filtered through a 200 and 20 µm nylon mesh and rinsed with wash solution. The suspension containing Vicia faba protoplasts was filtered through a 400 and 50 µm mesh. The wash solution for subsidiary cell protoplasts was composed of 1 mM CaCl$_2$, 5 mM MES, pH 6.5/KOH, $\pi = 530$ mosmol kg$^{-1}$/D-mannititol while the wash solution for guard cell protoplasts contained 1 mM CaCl$_2$, $\pi = 400$ mosmol kg$^{-1}$/D-mannititol, pH 5.6. After centrifugation at 122×g (4°C) for 12 min and subsequent removal of the supernatant, the protoplasts were stored on ice.

**Electrophysiological measurements**

Whole-cell patch-clamp experiments were performed as previously described (Hamill et al. 1981, Dietrich et al. 1998, Majore et al. 2002). Patch pipettes with a pipette resistance around 3 MΩ in standard patch solutions were pulled from Kimax-51 glass capillaries (Kimble products, Vineland, NY, USA) and heat-polished. Voltage pulses in the range of –184 to +96 mV were applied in 10-mV-steps from a holding voltage of –64 mV. The clamped voltages were corrected off-line for the liquid junction potential (Neher 1992). Instantaneous currents ($I_{inst}$) were determined at the beginning of the voltage pulses immediately after decay of the capacitance transient while the steady-state currents ($I_{ss}$) were measured at the end of the voltage pulses. Currents ($I_{inst}$, $I_{ss}$) were corrected for background currents which were determined in a voltage range of high membrane resistance usually between –64 and –44 mV. Time-dependent outward-rectifying currents ($I_{out}$) were quantified by subtracting $I_{inst}$ from $I_{ss}$. To compare the current amplitude of different protoplasts, the current densities ($I_{out}/C_{pip}$, $I_{out}/C_{mem}$ or $I_{out}/C_{cell}$) were determined upon dividing the macroscopic currents by the whole cell membrane capacitance $C_{cell}$ of the individual protoplasts and were plotted against the membrane voltages. If not otherwise mentioned, the outward- and inward-rectifying currents were recorded 30 and 37 min, respectively, after whole-cell access and complete equilibration of the cytosol with the pipette solution. Data values are given as mean ± standard error of the mean (SE), n denotes the number of independent experiments.

**Patch clamp solutions**

For the measurements on subsidiary cell protoplasts the osmolarity of the pipette (intracellular) and bath (extracellular) solutions were adapted to 560 and 540 mosmol kg$^{-1}$ with D-mannititol, respectively. The pipette and bath solutions used for the experiments on guard cell protoplasts were adjusted to 480 and 450 mosmol kg$^{-1}$ with D-mannititol, respectively. The standard bath solution contained (in mM) 10 K-glucolate, 10 Ca-glucolate$_2$, 10 MES pH 5.6/Tris. The control pipette medium was composed of (in mM) 150 K-glucolate, 2 MgCl$_2$, 10 EGTA, ± nucleotides (NTP, at given concentration), 10 HEPES pH 7.4/Tris. The free Mg$^{2+}$ concentration of the control solutions was calculated by using the program “calcium.exe“ v2.0 and 2.1 developed by K.J. Foehr and written by W. Warchol. For measurements under nominal Mg$^{2+}$-free conditions (Fig. 4), pipette solutions were used containing (in mM) 136 K-glucolate, 10 EGTA, 10 EDTA, 5 Mg-ATP or (5 K-ADP plus 5 K-glucolate) or (0 NTP plus 10 K-glucolate), 4 KCl, 10 HEPES pH 7.4/Tris. The Na$^+$ permeability of the outward-rectifying currents (Fig. 5b, c) was examined by using a sodium pipette solution composed of 150 Na-glucolate, 5 K-glucolate$_2$, 2 MgCl$_2$, 10 EGTA, 5 Mg-ATP or 5 Tris-GTP, 10 HEPES pH 7.4/Tris.

**Chemicals**

TEA-Cl (tetra-ethylammonium chloride), ATP (Mg salt, application in standard pipette solution and in sodium pipette medium), ATP (K salt, application only in Mg$^{2+}$-free pipette solution), ADP (K salt), GTP (Tris salt), bovine serum albumine, oligomycin, ApA (P$^\beta$P$^\gamma$) D(adenosine-5') pentaphosphate, Li salt) and polyvinylpyrrolidone (PVP-40) were from Sigma-Aldrich (Deisenhofen, Germany). GDP (Tris salt) and AMP (Na salt) were purchased from ICN Biomedicals (Asse-Relegem, Belgium) and K252a, an inhibitor of serine/threonine-dependent protein kinases, from Alexis Biochemicals (Grünberg, Germany).

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**References**

