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The Baculovirus/Insect Cell System as an Alternative to Xenopus Oocytes

FIRST CHARACTERIZATION OF THE AKT1 K⁺ CHANNEL FROM ARABIDOPSIS THALIANA*

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Two plant (Arabidopsis thaliana) K⁺ transport systems, KAT1 and AKT1, have been expressed in insect cells (Sf9 cell line) using recombinant baculoviruses. Microscopic observation after immunogold staining revealed that the expressed AKT1 and KAT1 polypeptides were mainly associated with internal membranes, but that a minute fraction was targeted to the cell membrane. AKT1 was known, from earlier electrophysiological characterization in Xenopus oocytes, to be an inwardly rectifying voltage-gated channel highly selective for K⁺, while similar experiments had failed to characterize AKT1. Insect cells expressing AKT1 displayed an exogenous inwardly rectifying K⁺ conductance reminiscent of that described previously in Xenopus oocytes expressing AKT1. Under similar conditions, cells expressing AKT1 showed a disturbed cell membrane electrical stability that precluded electrophysiological analysis. Use of a baculovirus transfer vector designed so as to decrease the expression level allowed the first electrophysiological characterization of AKT1. The baculovirus system can thus be used as an alternative method when expression in Xenopus oocytes is unsuccessful for electrophysiological characterization of the ion channel of interest. The plant AKT1 protein has been shown in this way to be an inwardly rectifying voltage-gated channel highly selective for K⁺ ions and sensitive to cGMP.

In plants, inwardly rectifying potassium channel activity is involved in long-term K⁺ uptake and in related functions at the cell or whole plant level, e.g. turgor regulation, stomatal guard cell movements, or cell expansion and plant growth (1–3). The first plant K⁺ channel characterized at the molecular level, AKT1 (4) and KAT1 (5) from Arabidopsis thaliana, were cloned by functional complementation of yeast strains defective in K⁺ transport. Several K⁺ channels have since been identified using probes from AKT1 or KAT1 cDNAs (Refs. 6–8 and sequences found in the EMBL Data Bank). These channels share strong homologies (~60% identity) and show structural and sequence homologies with K⁺ channels of the Shaker family found in insects and mammals (4, 5, 9). They display the characteristic hydrophobic domain consisting of six transmembrane segments, named S1 to S6, with a pore-forming region located between S5 and S6. A putative cyclic nucleotide-binding domain is present downstream of S6, as found in cyclic nucleotide-gated channels of the Shaker superfamily (4–8). Two subfamilies can be defined according to the presence or absence (channels of the AKT1 or KAT1 type, respectively) of an ankyrin domain in the polypeptidic chain downstream of the putative cyclic nucleotide-binding domain (3, 4).

Electrophysiological characterization by heterologous expression in Xenopus laevis oocytes or yeast indicated that AKT1 is an inwardly rectifying voltage-gated K⁺ channel highly selective for K⁺ (10–15). It is expressed in guard cells and thought to mediate long-term K⁺ influx leading to stomatal opening (16). Northern blot analysis indicated that AKT1 is expressed mainly in roots (17). Studies of its tissue-specific expression using the GUS reporter gene revealed that its promoter directs preferential expression in the peripheral cell layers of the mature region of roots (18), suggesting a role in K⁺ uptake from the soil solution. Injection of AKT1 cRNA in Xenopus oocytes did not, however, affect the membrane conductance. The K⁺ channel activity of the encoded polypeptide thus awaited characterization.

The insect cell line Sf9 can express high levels of foreign proteins when infected by a recombinant baculovirus. This expression system has been shown to be capable of performing most eukaryotic post-translational modifications (19, 20). It has been used, in particular, for expressing the Drosophila Shaker K⁺ channel in a functional form (21–23). In this study, functional expression of AKT1 and KAT1 polypeptides has been achieved using the baculovirus/Sf9 system.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies were raised against the ankyrin domain of AKT1 and the C-terminal region of KAT1 (see Fig. 1A). Nucleotide sequences coding for these domains were cloned into the pET-3c vector designed for expression in Escherichia coli (24). The required restriction sites (NdeI and BamHI) were introduced in AKT1 and KAT1 cDNAs as described below. The ankyrin domain of AKT1 was amplified by polymerase chain reaction using a 5′-primer (5′-TTTCTATATGGATCTTCCTC) introducing a NdeI site at position 1600 of AKT1 cDNA and a 3′-primer (5′-GGGACCGATCCCGGTTTAGATAGAG) introducing a TAA stop codon at position 2218, just before the unique BamHI site present in AKT1. The NdeI-BamHI fragment was sequenced on both strands. KAT1 cDNA was introduced into pBluescript® so that the BamHI polylinker site was present downstream of the KAT1 stop codon. The

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Ni61-BamHI fragment encoding the C-terminal part of KAT1 was cloned into the Sf9D184 vector (25), introducing a Ni61 site just upstream of the Ni51 site. The Ni61-BamHI fragment was thereafter introduced into the pET-3c vector.

The recombinant pET-3c vectors were introduced in *E. coli* strain BL21(DE3) (24). Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (final concentration of 0.4 mM) and lasted 3 h at 37 °C. Both expressed polypeptides formed inclusion bodies (26). They were purified by washing the inclusion bodies several times with a medium containing 50 mM Tris-HCl (pH 8), 2 mM EDTA, 1 mM NaCl, and 5% Triton X-100. The final pellet was suspended in 50 mM Tris-HCl (pH 8), 2 mM EDTA, 0.5 mM dithiothreitol, and 7 M urea. Approximately 40 µg of proteins were subjected to SDS-PAGE. 2 Acrylamide bands containing overexpressed polypeptides were crushed in 2 ml of PBS, and the resulting mixtures were used to immunize rabbits intradermally. Booster injections were given 2 months later.

**Insect Cell Culture**—The Sf9 cell line was maintained in monolayer culture at 28 °C in TC-100 medium (Life Technologies, Inc.) supplemented with 0.37 g/liter α-ketoglutaric acid, 0.4 g/liter β-fructose, 0.055 g/liter fumaric acid, 0.67 g/liter malic acid, 0.2 g/liter alanine, 2.7 g/liter sucrose, 0.2 g/liter choline chloride, 0.2 g/liter β-alanine, 0.35 g/liter NaHCO₃, 3.33 g/liter lactalbumin (Difco), 0.05 g/liter streptomycin sulfate, 0.125 g/liter penicillin, and 10% fetal calf serum. Cells were split every 4 days to maintain a density ranging from ~4 × 10⁸ to 7 × 10⁸ cells/ml.

**Recombinant Baculoviruses**—Two baculovirus transfer vectors (see Fig. 1B) were used, pGmAc34T (27) and pGmAc217. In the pGmAc34T vector, the initiator ATG codon of polyhedrin (corresponding to the first nucleotide of the polyhedrin initiator ATG codon), and residues +8 to +462 were deleted. In the pGmAc217 vector, residues −8 to +502 were deleted, and a BgIII site was introduced just downstream to position −8. Such a deletion in the promoter region has been shown to result in a decreased expression level (28).

Recombinant transfer vectors, named p34T-AKT1, p34T-KAT1, and p217Δ-AKT1, were obtained as described in the legend to Fig. 1B by cloning AKT1 and KAT1 cDNAs into pGmAc34T and AKT1 cDNA into pGmAc217, respectively. Sf9 cells were transfected with wild-type viral DNA and the recombinant transfer vector p34T-AKT1, p34T-KAT1, or p217Δ-AKT1 using N-[1-(2,3-dioleoyloxypropyl)-N,N,N-trimethylammonium methylsulfate (Boehringer Mannheim) as fusion agent according to Davrinche et al. (27). Recombinant baculoviruses were purified and named RB34T-AKT1, RB34T-KAT1, and RB217Δ-AKT1, respectively. They were amplified to 10⁷ plaque-forming units/ml and used for protein expression.

**Membrane Purification**—Sf9 cells in exponential phase were layered at a density of 5 × 10⁸ cells/ml and infected with recombinant baculoviruses at a multiplicity of infection of 10. After 2 days of incubation at 28 °C, cells were harvested and centrifuged at 500 × g for 5 min. The pellet was washed with ice-cold PBS. The cells were centrifuged for 5 min at 500 × g and resuspended at −10⁷ cells/ml in a grinding medium containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 250 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 2 mM phenylmethylsulfonfyl fluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 µg/ml antipain. The suspension was frozen in liquid nitrogen, quickly thawed at room temperature, and sonicated three times for 10 s with a probe sonicator. The homogenate (referred to as total extract) was centrifuged at 100,000 × g for 1 h. The crude membrane pellet was suspended in 2 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 mM phenylmethylsulfonfyl fluoride, and 10% glycerol and stored in liquid nitrogen. Proteins were assayed according to Schaffner and Weissman (29) using bovine serum albumin as a standard.

**Immunoblots**—Proteins were separated by SDS-PAGE according to Laemmli (30) and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell Corp.) at 100 V for 1 h in a medium containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. Blots were blocked in PBS containing 5% low fat milk for 30 min at room temperature. Primary antibody diluted in PBS containing 0.1% Tween 20 (PBS-T) was bound overnight at room temperature. After three 10-min washes in PBS-T, goat anti-rabbit IgG secondary antibody coupled to peroxidase (Sigma) diluted in PBS-T was added. Blots were incubated for 2 h at room temperature and washed as described above. Peroxidase activity was detected using a 0.5 mg/ml 4-chloronaphthol solution prepared in PBS, 20% hydrogen peroxide, and 0.01% H₂O₂.

**Immunogold Staining and Microscopy**—Sf9 cells were infected with recombinant baculoviruses as described above (see “Membrane Purification”) and harvested 2 days later by pelleting at 500 × g for 5 min. They were fixed for 1 h in 4% paraformaldehyde in PBS at 4 °C. Following dehydration (increasing ethanol concentration up to 100%), cells were embedded in LR white resin (Taab). Thin sections were made and immunostaining was performed as described (31). Observations were made with a Zeiss (Jena) EM 10C/CR electron microscope.

**Patch-clamp Experiments**—Sf9 cells were plated in 3-cm diameter cell culture dishes and infected as described above (see “Membrane Purification”). Prior to electrophysiological recordings, culture medium was replaced by a bath solution containing 10 mM KCl (or 100 mM KCl), 4 mM CaCl₂, 5 mM MgCl₂, 5 mM glucose, 10 mM MES-Tris (pH 6.3), and NaCl (to give an osmolality of 0.28). Standard voltage-clamp protocols (see “Results”) allowed macroscopic current recording in the whole cell configuration of patch-clamp (32).

**RESULTS**

**Expression of KAT1 and AKT1**—Sf9 cells were infected either with wild-type baculovirus or with viruses recombinant for *KAT1* or *AKT1* cDNA: RB34T-KAT1, RB34T-AKT1, and RB217Δ-AKT1 (see “Experimental Procedures” and Fig. 1). RB217Δ-AKT1 was obtained using a transfer vector designed (deletion in the polyhedrin promoter) (Fig. 1) so as to decrease the level of expression of the foreign sequence.

Infected Sf9 cells with wild-type baculovirus resulted in the appearance of many extra bands following SDS-PAGE of total cell extract (Fig. 2A). The major one, present at 31 kDa, corresponds to the virus polyhedrin gene product. When Sf9 cells were infected with viruses recombinant for *KAT1* or *AKT1* cDNA, the polyhedrin band was no longer present, while an extra major band was clearly visible. The relative molecular mass of this band was close to that expected for the corresponding plant channel: ~70 kDa for *KAT1* (predicted molecular mass of 78 kDa) and 95 kDa for *AKT1* (predicted molecular mass of 97 kDa) (Fig. 2A). Antibodies raised against *KAT1* or *AKT1* detected a 70-kDa band (Fig. 2B, lane 34T-KAT1) and a 95-kDa band (lanes 34T-AKT1 and 217Δ-AKT1), respectively. This confirmed that both plant channels were expressed in Sf9 cells. The level of expression of *AKT1* was lower in RB217Δ-AKT1-infected cells than in RB34T-AKT1-infected cells, as expected from the use of an 8 base pair-deleted polyhedrin promoter (28).

**Plant Channel Targeting in Sf9 Cells**—In a preliminary biochemical approach, the cellular localization of plant channels expressed in Sf9 cells was investigated by preparing soluble and membrane protein fractions and analyzing their polypeptide composition by SDS-PAGE. Polypeptide bands of the size expected for *AKT1* and *KAT1* were detected in the membrane protein fraction (Fig. 3). Western blotting performed with the corresponding antisera failed to detect the channels in the soluble protein fraction (Fig. 3B). A cellular distribution of *AKT1* in Sf9 cells was further investigated by immunogold staining. Infection with *AKT1* and *KAT1* recombinant viruses dramatically increased the amount of intracellular membrane (Fig. 4) when compared with that of

2 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MES, 4-morpholinethane-sulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
control cells (uninfected or infected with wild-type virus) (data not shown). Strong staining was observed in both RB34T-AKT1- and RB217D-AKT1-infected cells (Fig. 4, A–D), in contrast to control cells. Additionally, KAT1-expressing cells (RB34T-KAT1-infected) probed with the serum raised against the AKT1 ankyrin domain (Fig. 4E) also showed little staining, equivalent to that observed with the other controls. High magnification micrographs (Fig. 4, C–D) indicated that most AKT1 proteins had an intracellular localization in both cells and that only a minute fraction of the expressed polypeptide was targeted to the plasma membrane. The intracellular pool of AKT1 was associated with internal membranes. Counting of gold particles was performed on nine different cells of each type. While internal membrane staining in RB217D-AKT1-infected cells was 35% lower than in RB34T-AKT1-infected cells (24 and 37 particles/mm², respectively; S.D., 20% of mean values), plasma membrane staining was similar in both cell types (~2.7 ± 0.8 particles/μm² of plasma membrane) (data not shown).
Electrophysiological Evidence for Functional Channel Expression—From a holding potential of −10 mV, membrane potential was clamped for 800-ms periods to values ranging from 0 to −160 mV. Negligible currents were usually recorded in cells infected with wild-type virus as shown in Fig. 5A. In some cell batches, however, randomly activating currents could be recorded at membrane potential values negative to −120 mV (data not shown). Cell batches that exhibited this behavior were discarded.

Slowly activating inward currents could be recorded in cells infected with each of the recombinant viruses (Fig. 5, B–D). During double-pulse protocols, the above three cell types displayed tail currents that reversed at potential values close to the equilibrium potential for K⁺ in both cases). The homogenate was subjected to centrifugation (2 × 13,000 g for 20 min and 100,000 × g for 1 h). Soluble (supernatant) (lanes S) and microsomal (pellet) (lanes M) fractions were collected and loaded on a polyacrylamide gel. Coomassie Blue-stained gel. Each lane contained 60 μg of protein. B, Western blots. Lanes contained either 10 μg of microsomal protein or 30 μg of soluble protein. Blots were probed using a serum directed against the C-terminal part of KAT1 (left) or a serum directed against the ankyrin domain of AKT1 (right) (see Fig. 1).

AKT1 Channel Voltage Gating—Inward currents were recorded during hyperpolarizing pulses in cells bathed first in 10 mM K⁺ and thereafter in 100 mM K⁺. These currents activated slowly with a multiexponential time course (Fig. 7, A and B). Steady-state activation was virtually achieved within the 1100-ms hyperpolarizing pulses. Half-activation time was clearly voltage-dependent: 40 ms at −180 mV and 160 ms at −120 mV in the 10 mM K⁺ bath (36 and 140 ms, respectively, in 100 mM K⁺). Plotting steady-state current versus voltage (Fig. 7C) revealed a strong inward rectification. Due to the presence of a Shaker S4-like voltage sensor in AKT1 (4), we hypothesized that this rectification was due mainly to a voltage-dependent G/Gmax ratio and determined whether the steady-state IN’ curves shown in Fig. 7C could be fitted by a simplified voltage-gating model. As described previously for the KAT1 channel (12), the steady-state current was assumed to be predicted by the Goldman equation, multiplied by a voltage-dependent G/Gmax ratio changing from 0 to 1 upon hyperpolarization. The G/Gmax voltage dependence was described using a simple two-state Boltzmann equation, assuming it was independent of the K⁺ concentration in the bath (10 or 100 mM). The calculated steady-state IN’ curve was drawn in full line in Fig. 7C for each bath condition. The single Boltzmann curve corresponding to both fits is shown in Fig. 7D. The half-activa-
Effect of ATP and cGMP on AKT1 Activation—In most cases, a rapid decrease in AKT1 current was observed when the pipette solution contained no ATP (Fig. 8A). This decrease could generally be prevented by including 2 mM ATP in the pipette solution (Fig. 8B). The standard pipette solution thus contained 2 mM MgATP. In some cases, this ATP concentration caused an increase in the current recorded at a given potential. This was due to a slight positive shift in the activation potential (Fig. 8B). When the clamped cell was perfused with a bath solution supplemented with 0.1 mM 8-bromo-cGMP, the current decreased. This was due to a negative shift in the activation potential, as shown in Fig. 8C (example representative of six independent recordings). This shift in AKT1 activation potential was time-dependent; the maximal value was reached within 15 min and was in the -25 to -40 mV range.

DISCUSSION

Our current knowledge of the structure/function relationship of voltage-gated animal Shaker channels originates mainly from the literature reporting the functional characterization of wild-type and mutant channels expressed in Xenopus oocytes. This expression system is popular for electrophysiologists as it is readily amenable to current recordings. However, in some unpublished experiments aimed at characterizing new putative animal channels, no functional expression has been obtained from cRNA injection in oocytes. Similarly, although the KAT1 channel was expressed and characterized in Xenopus oocytes (10–14), similar attempts for AKT1 have failed up until now.

The baculovirus/insect cell system has often been used to express functional membrane proteins (20). The Drosophila Shaker K⁺ channel was shown to be expressed in Sf9 cells and targeted to the plasma membrane in a functional state (21). KAT1 and AKT1 are the first ion channels from the plant kingdom to be expressed using this system. Both plant chan-

![FIG. 5. Whole cell currents recorded in baculovirus-infected Sf9 cells. Cells were infected at a multiplicity of infection of 10 with wild-type baculovirus (A) or with RB34T-KAT1 (B), RB34T-AKT1 (C), or RB217a-AKT1 (D). Currents were recorded 2 days later. The bath contained 100 mM KCl, 4 mM CaCl₂, 5 mM MgCl₂, 5 mM glucose, 10 mM MES/Tris (pH 6.3), and NaCl (amount required to give an osmolality of 0.28). The pipette solution contained 60 mM KF, 50 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 2 mM MgATP, 10 mM MOPS/NaOH (pH 7.2), and NaCl (to give an osmolality of 0.30). From a holding potential of -10 mV, the membrane was clamped at values ranging from 0 to -160 mV, with a step of -20 mV during nine 800-ms-long successive pulses.](https://image)

![FIG. 6. Deactivating currents recorded in RB217a-AKT1-infected Sf9 cells and instantaneous current/voltage curve. The bath solution contained either 10 mM KCl (A) or 100 mM KCl (B). A and B, currents recorded during double-pulse protocols. Holding potential was 0 mV. The activating pulse at -150 mV lasted 800 ms. Tail currents were recorded in the -20 to -120 mV range in 10-mV step in 100 mM K⁺ bath solution (B). Leak and capacitive currents were mathematically subtracted after recording. C, instantaneous deactivating current plotted against voltage applied during the second pulse of the voltage-clamp protocol. The data are from A (○) and B (●).](https://image)

**TABLE I**

Reversal potential of AKT1 current compared with theoretical K⁺ equilibrium potential

| Bath/pipette K conc | 10/110 mM | 100/110 mM |
|---------------------|-----------|------------|
| Reversal potential of AKT1 current | -63 ± 9 (10) | -5 ± 4 (7) |
| Theoretical K⁺ equilibrium potential | -62 | -2 |

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5 O. Pongs, personal communication.
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Channels have been expressed in a functional form and partly targeted to the plasma membrane of the cells as revealed by whole cell current recording and immunogold staining (Figs. 4–8). This is the first report showing that the baculovirus expression system can be used in an alternative strategy for characterizing ion channels when attempts using Xenopus oocytes have failed. The reason why AKT1 is expressed in a functional state in the former system and not in the latter is still unknown.

The membrane of Sf9 cells was often unable to withstand hyperpolarizations beyond $-100$ mV. Due to this problem, Sf9 cells do not offer the ideal system for electrophysiological characterization of hyperpolarization-activated channels such as KAT1 and AKT1. Some cell batches, however, were able to withstand membrane potential as negative as $-160$ mV (Fig. 5A) or even $-180$ mV. It is worth noting that Sf9 cells infected with either BR34T-KAT1 or RB217$\Delta$-AKT1 recombinant baculovirus withstand large hyperpolarizations (see Fig. 6 for RB217$\Delta$-AKT1-infected cells) much more reproducibly than wild-type baculovirus-infected cells or RB34T-AKT1-infected cells.

AKT1 was highly expressed in cells infected with RB34T-AKT1 (Fig. 2); however, these cells showed membrane instability. Breakdowns occurred especially at potentials more negative than $-100$ to $-120$ mV, precluding any characterization of AKT1 channel activity using this construct (Fig. 5C). As expected, the expression level of AKT1 in cells infected with RB217$\Delta$-AKT1 was lower than in the former cells (Fig. 2). In both types of cells, the plant channel was present in the membrane fraction (Fig. 3), although the amount of AKT1 actually targeted to the plasma membrane was small and roughly the same in both cases (Fig. 4). Most expressed polypeptides remained associated with internal membranes; their functional competence is unknown. A similar phenomenon with the expression of the Drosophila Shaker channel in Sf9 cells was hypothesized from the discrepancy between the magnitude of the currents recorded in the infected cells and the intensity of the Shaker polypeptide band on a Coomassie Blue-stained protein gel (21). Similarly, a liver gap junction protein expressed in Sf9 cells has been shown to remain mainly associated with the endoplasmic reticulum, with only a small fraction reaching the cell surface (34). It seems that Sf9 cells are able to synthesize large amounts of membrane proteins, but the protein export machinery is overwhelmed by the high rates of synthesis (35).

By making use of the RB217$\Delta$-AKT1 virus instead of the RB34T-AKT1 virus, it was possible to obtain conditions allowing the functional characterization of AKT1. This work represents the first data on AKT1 channel activity. Our results demonstrated that the reversal potential for AKT1 current remained close to the equilibrium potential for K$^+$ ions when the external concentration of this ion was changed (Table I), indicating that this current is mainly carried by K$^+$ ions. This is in agreement with the presence in the putative selectivity filter-forming region of a GYGD motif (4) thought to be a hallmark of highly selective K$^+$ channels (36).

Like KAT1 current, AKT1 current activated slowly upon hyperpolarization and underwent no inactivation (Fig. 5). Comparison of traces in Fig. 5D to those in Fig. 5B reveals that AKT1 activation was slower than that of KAT1 and occurred from a more negative threshold potential. It should be noted, however, that KAT1 activation in Sf9 cells was faster (half-activation time of $-15$ ms at $-140$ mV) (Fig. 5B) than that observed in Xenopus oocytes (half-activation time of $-200$ ms at $-140$ mV) (11). Thus, the kinetic features of KAT1 are dependent on the expression system used. This might be due to

![Figure 7](image)

**Fig. 7.** Voltage gating is the major mechanism underlying inward rectification of AKT1 channels. A and B, whole cell currents recorded in RB217$\Delta$-AKT1-infected Sf9 cells. The bath contained 10 mM KCl (A) or 100 mM KCl (B). Holding potential was 0 mV. Voltage-clamp episodes lasted 1100 ms, and clamp potential ranged from 0 to $-180$ mV with a $-10$-mV step. C, steady-state current through AKT1 channels plotted against membrane potential. The steady-state current value was taken at the end of the voltage-clamp episode on current traces shown in A (□) and B (○). Full line curves represent the prediction of the voltage-gated channel theory (see “Results”). D, dependence of $G/G_{\text{max}}$ on membrane potential. Squares and circles represent $G/G_{\text{max}}$ values calculated from the data shown in C. The full line curve represents the voltage-dependent variation of $G/G_{\text{max}}$ predicted by the Boltzmann law (see ”Results”).
differences in expression levels in *Xenopus* oocytes and Sf9 cells since differences in the expression level of KAT1 within the same expression system (*Xenopus* oocytes) have been shown to result in changes in the kinetic features of this channel (11). Similarly, features of the *Drosophila* Shaker K⁺ channel have been shown to depend on the expression system used: the activation curves are qualitatively identical in Sf9 cells and *Xenopus* oocytes, but the curve obtained in the former system is shifted −15 mV to more depolarized potentials (21). Whether these kinds of differences are relevant or not to the regulation and the role of the channel in situ in the animal/plant is as yet unknown (11, 37).

As shown in steady-state IV curves (Fig. 7C), AKT1 displayed a strong inward rectification. This is likely to originate mainly from some voltage-dependent gating as shown by the Boltzmann fit in Fig. 7D. The presence of ATP in the pipette solution was required to obtain routinely a stable AKT1 activation upon repeated hyperpolarizations. AKT1 current decrease in the absence of ATP was variable between cells (in the example of Fig. 8A, the decrease was particularly fast). ATP (2 mmol/liter) generally shifted the IV curve positively along the voltage axis (Fig. 8B). A shift of this curve in the opposite direction was elicited by bathing the cell with 100 μM 8-bromo-cGMP solution (Fig. 8C). All but one of these observations are reminiscent of those recently reported regarding KAT1 expressed in oocytes: while the decrease in KAT1 current was mainly due to a negative shift in the IV curve along the voltage axis (13), that of AKT1 originated from a decrease in activable channels (Fig. 8A). Previous sequence analysis indicated that a putative cyclic nucleotide-binding site sharing sequence homologies with the cyclic nucleotide-binding domain of animal cyclic nucleotide-gated channels is present in both AKT1 and KAT1 polypeptides, downstream of the membrane-spanning region (3, 4). The effect of cGMP on AKT1 and KAT1 activity may thus indicate direct modulation by cGMP, i.e. resulting from cGMP binding to the channel. An indirect effect cannot, however, be ruled out since modulation by cyclic nucleotide-dependent protein kinases is a likely means of K⁺ channel regulation in *planta* (38). Also, the hypothesis of an indirect effect is supported by the time dependence of the shift in activation potential.

The highest similarities between AKT1 and KAT1 and the animal K⁺ channels of the Shaker superfamily are found with the *Drosophila* Eag gene product. The Eag channel has been shown to be both voltage-dependent and cAMP-modulated (39). The existence of a link between strictly voltage-gated K⁺ channels and cyclic nucleotide-gated ion channels has been proposed (40). Eag, KAT1, and AKT1 may be members of a class of channels representing such a link.

In conclusion, we have shown that the baculovirus system can be used as an alternative method when expression in *Xenopus* oocytes is unsuccessful for electrophysiological characterization of the ion channel of interest. The plant AKT1 protein has been shown in this way to be a K⁺-selective, voltage-gated, and probably cGMP-modulated channel.

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FIG. 8. AKT1 channel activation is ATP-dependent and cGMP-sensitive. Voltage ramps (from 0 to −180 mV within 10 s) were applied to the membrane of RB217 AKT1-infected Sf9 cells at time intervals after rupturing the patch. Pseudo steady-state current/voltage curves could be recorded. A, the absence of ATP in the pipette solution (same as given in the legend to Fig. 5, but without MgATP) caused a rapid decrease in outward current. Zero time was at rupturing the patch. The bath contained 100 mM K⁺ as given in the legend to Fig. 5, but without MgATP) caused a rapid decrease in outward current. Zero time was at rupturing the patch. B, a 2 mM MgATP pipette solution prevented the time-dependent decrease in current. Successive IV ramps shifted positively along the voltage axis. Zero time was at rupturing the patch. C, the addition of 100 μM 8-bromo-cGMP at zero time (upon recording the b ramp, 8 min after rupturing the patch) made the further IV ramps shift negatively along the voltage axis.
