Biochemical and Biophysical Research Communications **274,** 130–135 (2000)
doi:10.1006/bbrc.2000.3095, available online at http://www.idealibrary.com on IDEAL®

Temperature-Dependent Functional Expression of a Plant K^+ Channel in Mammalian Cells

Ildikò Szabò,*[,1,2] Alessandro Negro,†[,1] Patrick Mark Downey,* Mario Zoratti,‡ Fiorella Lo Schiavo,*[,†] and Giorgio Mario Giacometti*

**Department of Biology, †CRIBI, and ‡CNR Unit for Biomembranes, University of Padua, Via G. Colombo 3, 35121 Padua, Italy*

Received June 16, 2000

The *Arabidopsis thaliana* potassium channel KAT1 was expressed and characterized in Chinese hamster ovary cells. KAT1-GFP fusion protein was successfully targeted to the plasma membrane and electrophysiological analysis revealed functional expression of KAT1 only in cells cultured at 30°C. The main biophysical characteristics of KAT1 are similar to those described for the channel expressed in other systems. CHO cells represent an advantageous expression system and may be the system of choice to study the expression, assembly, function, and regulation of plant potassium channels in general.

Key Words: **heterologous expression system; potassium channel; plant 1.**

Potassium channels in plants are involved in a number of processes, including the uptake of K^+ from the soil by roots, the maintenance of membrane potential (1), turgor regulation in stomatal guard cells (2), and rapid leaf movement (3).

The study of plant ion channels in their native environment is technically rather difficult and whole-cell currents often arise from the simultaneous activity of more than one type of pore. The expression of isolated gene products in heterologous systems has greatly contributed to our understanding of these proteins (4, 5).

The most studied plant K^+ channel is the product of the *kat1* cDNA originally cloned from *Arabidopsis thaliana* (6). KAT1 encodes a *shaker*-type channel and produces an inward current in all expression system used so far. KAT1-like channels are expressed in guard cells (7, 8).

Functional expression of plant channels, including KAT1, has been achieved using various heterologous cells. *Xenopus* oocytes have so far been the system of choice (e.g., 9–11), but it fails for some (e.g., AKT1, SKT1) (12). Several K^+ channels, including AKT1 (12), KAT1 (13), and SKT1 (14) have been successfully expressed in the insect cell line Sf9. In one study of KAT1 advantage has been taken of the Trk1Δ Trk2Δ strain of *Saccharomyces cerevisiae* (15). Recently, KAT1 has been functionally expressed and characterized in tobacco mesophyll cells as well (16).

Chinese hamster ovary (CHO) cells have been widely used to study cloned animal K^+ channels since they lack endogenous voltage-dependent potassium channels. The present work reports the successful functional expression and characterization of KAT1 in CHO cells.

MATERIALS AND METHODS

DNA constructs and cell culture. To fuse the gene encoding KAT1 with the GFP protein, restriction sites were generated by PCR using modified primers. A 2059 bp DNA fragment was amplified and cloned directly in the plasmid pEGFP (Clontech) in order to give plasmid pGFP-KAT1. *Kat1* was amplified by PCR and cloned as a blunt ended fragment into pCDNA3. CHO-KI cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 on glass coverslips in Dulbecco's essential medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. 40% confluent culture was transfected using the Lipofectamine reagent. The transfected cells were cultured for 76 h at 30 or 37°C.

Fluorescence microscopy. GFP fluorescence was examined after fixation using an inverted fluorescence microscope (Olympus TM20). The fluorescence filters used were: excitation HQ480/40, dichroic Q480/40 and emission HQ510LP (Chroma Technology Corp.).

Electrophysiology. Patch clamp experiments were performed in the whole-cell and outside-out excised patch configurations on control or transfected CHO cells grown on glass coverslips. Bath solution: 150 mM K gluconate, 20 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM Hepes, pH 6.8 or 7.2. Pipet solution: 114 mM K gluconate, 20 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM Hepes pH 7.35 and 4 mM ATP. Modified bath solutions containing 20 mM K gluconate and 150 mM NaCl or 50 mM K gluconate and 120 mM NaCl were used for Fig. 3B. The latter solution was used also for the determination of selectivity (pipette solution:164 mM K^+ and 134 mM Cl^-). Liquid junction potentials were measured and corrected for in all experiments (17). Leak current was not subtracted. Currents were monitored using an EPC-7 amplifier (HEKA-List). Capacitive currents were canceled manually and series resistance was compensated. Pulse protocols were applied and single channel analysis was performed using the Pclamp6 program set (Axon). Sampling frequency: 2 kHz; filtering: 1 kHz. All data are presented as mean ± SD.

[1] The first two authors contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: 00-39-049-8276344. E-mail: ildi@civ.bio.unipd.it.

0006-291X/00 $35.00

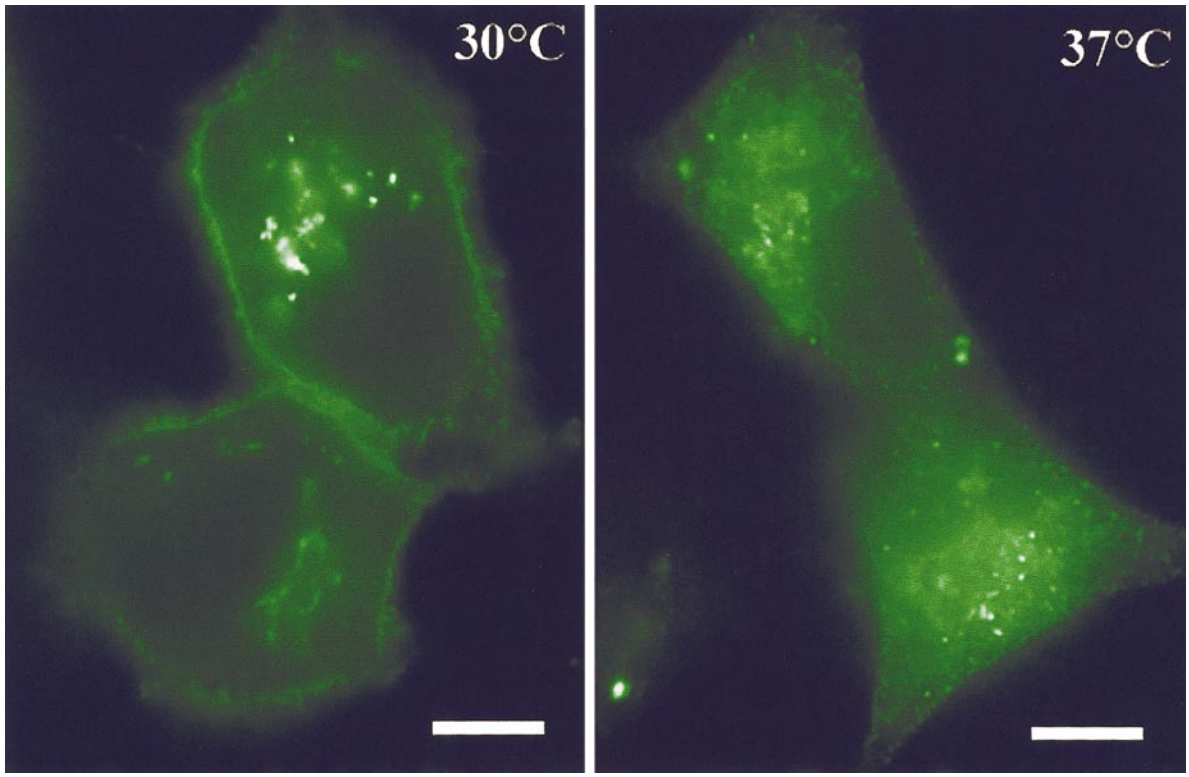

FIG. 1. KAT1-GFP expression in CHO cells at 30 and 37°C. Fluorescence microscopy images of KAT1-GFP expressing CHO cells cultured at 30°C (left) and at 37°C (right). Bar: 10 μm.

RESULTS

Since KAT1 has not been previously been expressed in mammalian cells, we created a KAT1-GFP (green fluorescent protein) fusion protein and examined its localization in transfected CHO cells by fluorescence microscopy. In cells incubated at 37°C (Fig. 1, right panel) the fusion protein is highly expressed, but seems to be retained in the endoplasmic reticulum and possibly in aggresomes which have been proposed to form in the presence of misfolded proteins (18). Analysis of several images similar to the one presented in the left panel of Fig. 1. revealed that if the transfected cells were incubated at 30°C, instead, at least a part of the fusion protein reproducibly reached the plasma membrane. The efficiency of transfection, determined as the percentage of fluorescent cells in the whole population, was 30 ± 10%. Transfection of HeLa cells with KAT1-GFP gave similar results (data not shown) indicating that the correctness of the folding was dependent on the incubation temperature rather than on the cell line used.

To test whether the plasma membrane-localized KAT1-GFP fusion protein was functional as a channel and the expression/activity correlation, we performed whole-cell patch clamp experiments on transfected CHO cells. Figure 2 shows representative current traces recorded from KAT1 transfected (n = 32) (Fig. 2A) and KAT1-GFP transfected (n = 3) (Fig. 2B) CHO cells incubated at 30°C. In both cases transient expression of the protein resulted in the appearance of large, voltage-dependent currents at hyperpolarizing voltages 2 to 4 days after transfection, altogether in 36% of the trials (35 out of 96). This % is in agreement with the transfection efficiency. Control non-transfected (n = 14) and pCDNA3 transfected (Fig. 2C) (n = 3) cells, cultured at 30°C, did not display any such activity. Importantly, transient expression of KAT1 in cells cultured at 37°C failed to result in channel activity (n = 11) (not shown). Thus the plasma membrane localization correlates with the functional expression of the protein.

Studies in various expression systems show that KAT1 behaves in a slightly different manner depending on the system used. Therefore we performed a detailed characterization of this plant protein expressed in animal cells. Since channel activity in cells transfected with KAT1 alone (Fig. 2A) showed a strong similarity to KAT1 expressed in oocytes, we used cells transfected with KAT1 (not with KAT1-GFP) for the rest of the study. In the absence of ATP in the pipet solution a rapid rundown (approx. 70%) was observed within 5–10 min. The currents elicited at various potentials decreased only slightly (by 6% within 15 and

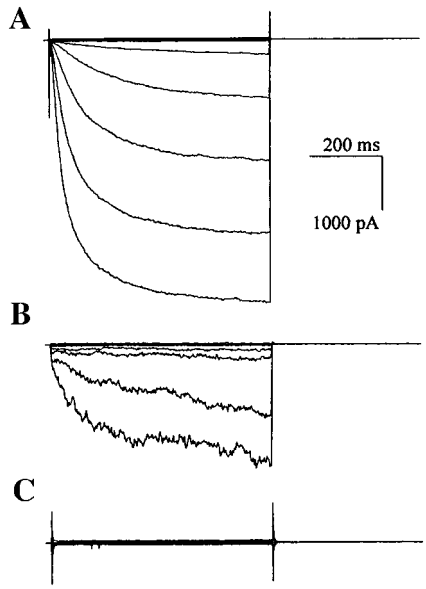

FIG. 2. Functional expression of KAT1 in CHO cells. (A) Whole-cell currents recorded in a KAT-transfected CHO cell. Inward rectifying currents were elicited by applying pulses of 600 ms duration ranging from −180 to +80 mV in 20 mV steps, at 45-s intervals. The same pulse protocol was used in B and C. (B) Representative whole-cell currents recorded in a KAT1-GFP transfected CHO cell (voltages from −160 to +80 mV in 40 mV steps). (C) Whole-cell currents recorded in a control, GFP-transfected CHO cell. In all figures cells were cultured at 30°C.

by 15% within 50 min; at −180 mV) with ATP in the pipet (Fig. 3A). Figure 3B shows the current–voltage relationship of KAT1-induced currents in the presence of 20 (n = 5), 50 (n = 4) and 170 (n = 11) mM external K^+. In accordance with previous results (15), almost complete saturation was achieved at 50 mM K^+. The normalized currents are reported in Fig. 3C showing that the intrinsic voltage-dependence and the activation potential of the channel did not change significantly with varying the potassium concentration. The threshold potential for activation was approximately −100 mV (n = 25). At pH 7.2 activation was half-maximal at −158 ± 12 mV (n = 11) as determined from the Boltzmann fit of G/G_{max} as a function of the membrane potential (not shown). Figure 3D reports the time constants of activation measured at various applied potentials showing that increasing hyperpolarization caused a faster activation of KAT1.

To determine the selectivity of the channel expressed in CHO, relaxation tail currents (Fig. 4A, inset) were recorded under asymmetric ionic conditions. The theoretical E_K^+ was −30 mV. The current reversal potential was −28.5 ± 5 mV (Fig. 4A) (n = 5) indicating a high selectivity of KAT1 for K^+ over Na^+ (P_{Na}^+/P_K^+ = 0.025). KAT1 was shown to be permeable to NH_4^+ when expressed in oocytes (19) and in mesophyll cells (16) but not in yeast (15) and in Sf9 cells (13). In CHO cells, exchanging the K^+-containing bath solution (20 mM) (Fig. 4B, upper part) with a NH_4^+-containing one (20 mM) during whole-cell experiments resulted in a 81 ± 5% decrease of the current recorded at −180 mV (Fig. 4B, lower part) (n = 3). Figure 4C shows traces recorded from an experiment with the NH_4^+ bath solution (a) and after addition of 10 mM KCl (b). KAT1 in CHO cells, like plant inward rectifying potassium channels in general, are inhibited by TEA^+ (72% inhibition by 10 mM TEA^+ at −180 mV in CHO cells; n = 3) and Cs^+ (60% inhibition by 3 mM Cs^+ at −180 mV; n = 3).

The single channel conductance of KAT1 was determined in outside-out excised patches. Figure 5A shows a representative current trace recorded upon application of a voltage step to −140 mV. The activation kinetics typical for KAT1 can be observed. Figure 5B shows single channel activity at steady-state hyperpolarizing voltage (−140 mV). The conductance, as determined from the current–voltage relationship, is in the range of 7–10 pS and the open probability of the channel increased with increasing hyperpolarization (not shown).

DISCUSSION

The results reported here demonstrate that CHO cells are a valid system for the study of KAT1. This system has various advantages. Patch clamp experiments can be easily performed for the study of both whole-cell currents and single channel activity, without any pretreatment of the cells, in contrast to yeast, mesophyll cells and oocytes. Importantly, CHO cells tolerate large hyperpolarizing voltages, in contrast to Sf9 cells. KAT1 is expressed in CHO cells at high levels within a short time, giving reproducible currents, in contrast with the somewhat unreliable performance of the oocyte expression system. In this work we induced transient expression, but, a stable cell line expressing another plant K_{in}^+ has been obtained (Lo Schiavo *et al.*, unpublished result), suggesting the possibility to create a stable cell line expressing KAT1 as well. CHO cells expressing plant ion channels could become an ideal tool also for biochemical and regulation studies of these channels, since large number of cells can be obtained within a short time, and the cell membrane is directly accessible. Importantly, CHO cells do not express other voltage-gated or inward rectifying potassium channels which could interfere with plant K_{in}^+ activity recording. However, the appropriate conditions must be chosen in order to avoid activation of the swelling-activated chloride channels (20, 21) and of the calcium-activated chloride (22) and potassium channels, which are present in both HeLa (23) and CHO cells (Szabò *et al.*, unpublished).

The possibility to follow the fate of ion channel subunits fused to GFP in CHO cells by fluorescence microscopy while at the same time testing for functional activity might open new perspectives for the study of

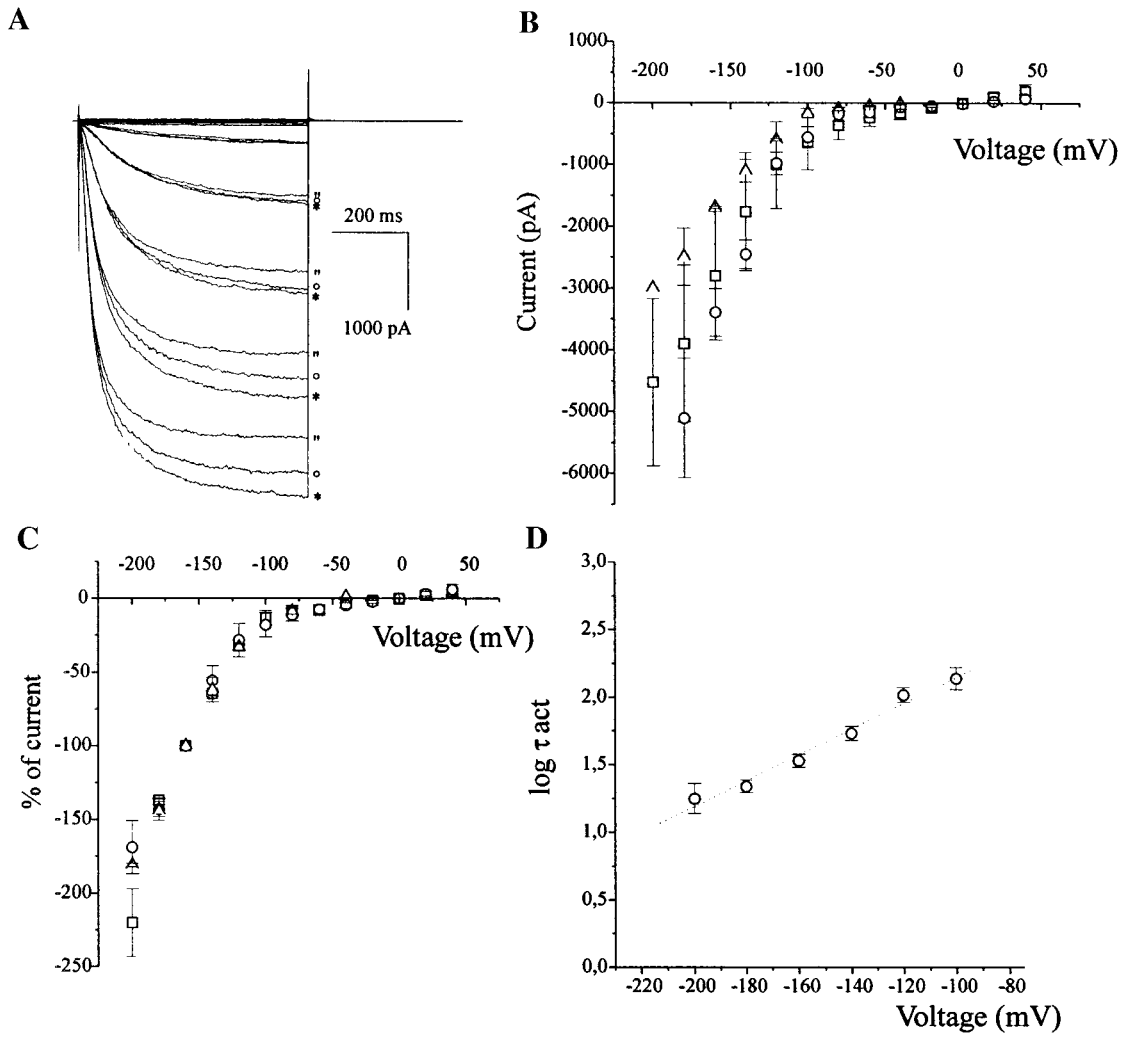

FIG. 3. Characterization of KAT1 expressed in CHO cells. (A) Representative KAT1 current traces recorded as in Fig. 2A: 3 min (*), 15 min (°) and 50 min (“) following the establishment of the whole-cell configuration with ATP in the pipet. (B) Current–voltage relationships in the presence of 20 mM (△), 50 mM (□), and 170 mM (○) bath potassium concentrations. Whole-cell currents were recorded from different cells for each point displayed on the figure (see Results); variability of cell size and of KAT1 expression from cell to cell resulted in relatively large standard deviation. (C) Intrinsic voltage-dependence of currents recorded as in Fig. 2B. Whole-cell currents recorded at various test potentials were expressed as % of the current measured at −160 mV for each experiment. The obtained values were averaged and reported. (D) Whole-cell currents recorded with 170 mM external potassium at various potentials were fitted with two exponentials. The logarithm values of the time constants of the fast component are reported on the figure as function of the applied voltage.

channel expression and subunit assembly. In this work for example, GFP was fused to the C-terminal part of the subunit, which is thought to play an important role in the association of plant K^+_{in} channel subunits (24, 25). Probably as a consequence, currents recorded in the presence of GFP at the C-terminus were low and “noisy” (Fig. 2B). C-terminus fusion nevertheless allowed us to verify that the *KAT1*-encoded protein was effectively translated.

The observed strong influence of temperature on KAT1 targeting might seem surprising. An analogous observation was reported for CFTR (26). Furthermore, the formation of aggresomes, due to the accumulation of incorrectly folded proteins in the cytosol, has been shown to decrease at 30°C with respect to culturing at 37°C (27). All expression systems used so far for the study of plant ion channels require the culturing of the various cell types at temperatures ≤30°C.

Various similarities and differences can be observed in the properties of KAT1 by comparing results obtained in different expression systems. Concerning the dependence of the KAT1-induced inward current magnitude on the external potassium concentration and the voltage at which the currents were activated (−100 mV), the behavior of KAT1 in CHO cells was comparable to that observed in other systems (9, 10, 15, 16). The half-activation voltage in CHO cells was more similar to that found in the yeast expression system

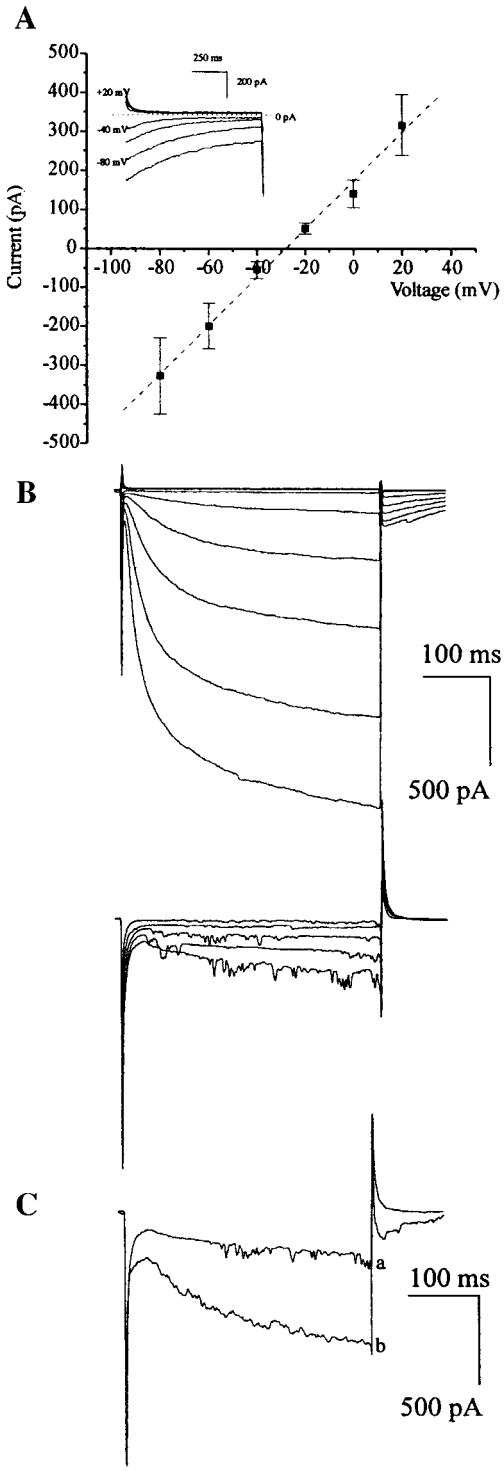

FIG. 4. Selectivity of KAT1 expressed in mammalian cells. (A) Tail currents recorded by stepping the voltage form the holding potential (−70 mV) to −180 mV, and subsequently to voltages ranging from −120 to +40 mV (bath: 50 mM K^+; pipet: 164 mM K^+ solution). A set of representative relaxation tail currents are shown in the inset. The averages of the tail currents are reported as a function of the voltage. (B) Upper part: Whole-cell traces recorded as in Fig. 1A with 20 mM K^+ bath solution. Lower part: currents recorded under the same conditions, following exchange of the bath solution with a 20 mM NH_4^+, 0 mM K^+ containing solution. (C) Current trace recorded at −180 mV in the presence of 20 mM NH_4^+ (a) (see B) and following the addition of 10 mM K^+ to the bath (b).

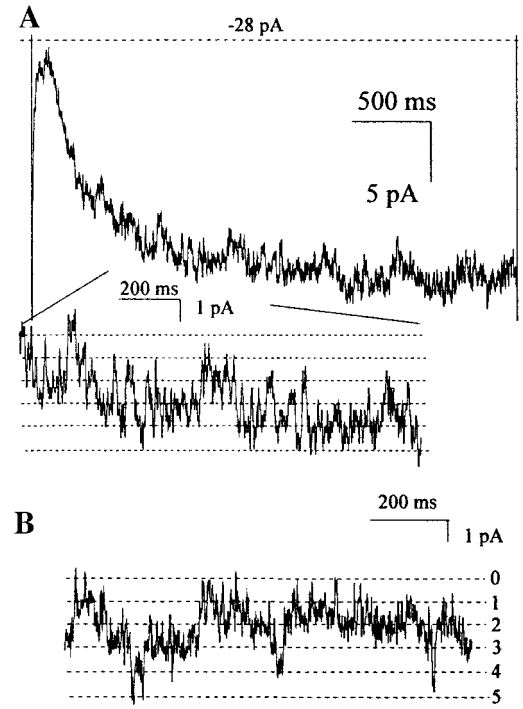

FIG. 5. Single channels in KAT1-transfected cells. (A) Current elicited by application of a voltage pulse (−140 mV) of 3 s duration in outside-out configuration (bath, 20 mM K^+). Lower part: expanded time and current scale. (B) KAT1 activity in an outside-out patch, at −140 mV (steady-state) (bath, 50 mM K^+; pipet, 164 mM K^+). (A and B) Sampling frequency, 5 kHz; filter, 500 Hz. Dashed lines correspond to the current level of one open channel.

(−160 −180 mV) than to that recorded in oocytes (−120 −130 mV). The half-activation time was similar to that found in oocytes (200 ms at −140 mV) (30) (also, Fig. 3D). The permeability of KAT1 expressed in CHO for NH_4^+ is comparable to that observed in mesophyll cells (16) and in oocytes (9, 29). 10 mM TEA^+, a typical potassium channel blocker, caused an 70–81% inhibition in all systems studied, including CHO cells. 10 mM Cs^+ almost completely blocked KAT1 expressed in mesophyll cells (16). In our case 3 mM Cs^+ caused a 60% inhibition of the current. The single channel conductance of KAT1 expressed in CHO cells is consistent with other reports (11, 16, 28).

In conclusion, the data reported here demonstrate that CHO cells represent a valid and easily accessible heterologous expression system with important advantages with respect to other systems used. Expression in CHO cells could be used for the characterization of several putative plant ion channels which do not express in other systems.

ACKNOWLEDGMENTS

The authors are grateful to Dr. M. Massimiliano for help with cell transfection and to Professor R. Hedrich and Professor F. Gambale for help and discussions. This work was supported by "PRIN99" and "Biotecnologie Vegetali" MIPA to F.L.S.

REFERENCES

1. Brüggemann, L., Dietrich, P., Becker, D., Dreyer, I., Palme, K., and Hedrich, R. (1999) Channel-mediated high-affinity K^+ uptake into guard cells from *Arabidopsis. Proc. Natl. Acad. Sci. USA* **96,** 3298–3302.
2. Schroeder, J. I., Ward, J. M., and Gassmann, W. (1994) Perspectives on the physiology and structure of inward-rectifying K^+ channels in higher plants. *Annu. Rev. Biophys. Biomol. Struct.* **23,** 441–471.
3. Moran, N., Ehrenstein, G., Iwasa, K., Mischke, C., Bare, C., and Satter, R. L. (1988) Potassium channels in motor cells of *Samanea saman. Plant Physiol.* **88,** 643–648.
4. Maathuis, F. J. M., Ichida, A. M., Sanders, D., and Schroeder, J. I. (1997) Roles of higher plant K^+ channels. *Plant Physiol.* **114,** 1141–1149.
5. Hedrich, R. (1995) Technical approaches to studying specific properties of ion channels in plants. *In* Single Channel Recording (Sakmann, B., and Neher, E., Eds.), pp. 277–305, Plenum Press, New York.
6. Anderson, J. A., Huprikar, S. S., Kochian, L. V., Lucas, W. J., and Gaber, R. F. (1992) Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **89,** 3736–3740.
7. Nakamura, R. L., Mckendree, W. L., Hirsch, R. E., Sedbrook, J. C., Gaber, R. F., and Sussman, M. R. (1995) Expression of an *Arabidopsis* potassium channel gene in guard cells. *Plant Physiol.* **109,** 371–374.
8. Müller-Rober, B., Ellenberg, J., Provart, N., Willmitzer, L., Busch, H., Becker, D., Dietrich, P., Hoth, S., and Hedrich, R. (1995) Cloning and electrophysiological analysis of KST1, an inward rectifying K^+ channel expressed in potato guard cells. *EMBO J.* **14,** 2409–2416.
9. Schachtman, D. P., Schroeder, J. I., Lucas, W. J., Anderson, J. A., and Gaber, R. F. (1992) Expression of an inward rectifying potassium channel by the *Arabidopsis* KAT1 cDNA. *Science* **258,** 1654–1658.
10. Very, A. A., Gaymard, F., Bosseux, C., Sentenac, H., and Thibaud, J. B. (1995) Expression of a cloned plant K^+ channel in *Xenopus* oocytes: Analysis of macroscopic current. *Plant J.* **7,** 321–332.
11. Hoshi, T. (1995) Regulation of voltage dependence of the KAT1 channel by intracellular factors. *J. Gen. Physiol.* **105,** 309–328
12. Gaymard, F., Cerutti, M., Horeau, C., Lemaillet, G., Urbach, S., Ravallec, M., Devauchelle, G., Sentenac, H., and Thibaud, J. B. (1996) The baculovirus/insect cell system as an alternative to *Xenopus* oocytes. *J. Biol. Chem.* **271,** 22863–22870.
13. Marten, I., Gaymard, F., Lemaillet, G., Thibaud, J. B., Sentenac, H., and Hedrich, R. (1996) Functional expression of the plant K^+ channel KAT1 in insect cells. *FEBS Lett.* **380,** 229–232.
14. Zimmermann, S., Talke, I., Ehrhardt, T., Nast, G., and Muller-Rober, B. (1998) Characterization of SKT1, an inwardly rectifying potassium channel from potato, by heterologous expression in insect cells. *Plant Physiol.* **116,** 879–890.
15. Bertl, A., Anderson, J., Slayman, C. L., and Gaber, R. (1995) Use of *Saccharomyces cerevisiae* for patch clamp analysis of heterologous membrane proteins: Characterization of Kat1, an inward-rectifying K^+ channel from *Arabidopsis thaliana,* and comparison with endogenous yeast channels and carriers. *Proc. Natl. Acad. Sci. USA* **92,** 2701–2705.
16. Bei, Q., and Luan, S. (1998) Functional expression and characterization of a plant K^+ channel gene in a plant cell model. *Plant J.* **13,** 857–865.
17. Neher, E. (1992) Correction for liquid junction potentials in patch clamp experiments. *Methods Enzymol.* **207,** 123–131.
18. Johnston, J. A., Ward, C. L., and Kopito, R. R. (1998) Aggresomes: A cellular response to misfolded proteins. *J. Cell Biol.* **143,** 1883–1898.
19. Hedrich, R., Moran, O., Conti, F., Busch, H., Becker, D., Gambale, F., Dreyer, I., Kuech, A., Neuwinger, K., and Palme, K. (1995) Inward rectifier potassium channels in plants differ from their animal counterparts in response to voltage and channel modulators. *Eur. J. Biophys.* **24,** 107–115.
20. Sorota, S. (1992) Swelling-induced chloride-sensitive current in canine arterial cells revealed by whole-cell patch clamp method. *Circ. Res.* **70,** 679–687.
21. Doroshenko, P., and Neher, E. (1992) Volume-sensitive chloride conductance in bovine chromaffin cell membrane. *J. Physiol.* **449,** 197–218.
22. Nilius, B., Prenen, J., Szucs, G., Wei, L., Tanzi, F., Voets, T., and Droogmans, G. (1997) Calcium-activated chloride channels in bovine pulmonary artery endothelial cells. *J. Physiol.* **498,** 381–396.
23. Szabò, I., Brutsche, S., Tombola, F., Moschioni, M., Satin, B., Telford, J. L., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999) Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity. *EMBO J.* **18,** 5517–5527.
24. Daram, P., Urbach, S., Gaymard, F., Sentenac, H., and Cherel, I. (1997) Tetramerization of the AKT1 plant potassium channel involves its C-terminal cytoplasmic domain. *EMBO J.* **16,** 3455–3463.
25. Erhardt, T., Zimmermann, S., and Muller-Rober, B. (1997) Association of plant K^+ in channels is mediated by conserved C-termini and does not affect channel assembly. *FEBS Lett.* **409,** 166–170.
26. Lukacs, G. L., Chang, X. B., Bear, C., Kartner, N., Mohamed, A., Riordan, J. R., and Grinstein, S. (1993) The delta F508 mutation decreases the stability of cystic fibrosis transmembrane conductance regulator in the plasma membrane. Determination of functional half-lives on transfected cells. *J. Biol. Chem.* **268,** 21592–21598.
27. Mata, R. G., Bebok, Zs., Sorscher, E. J., and Sztul, E. (1999) Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J. Cell Biol.* **146,** 1239–1254.
28. Becker, D., Dreyer, I., Hoth, S., Reid, J. D., Busch, H., Lehnen, M., Palme, K., and Hedrich, R. (1996) Changes in voltage activation, Cs^+ sensitivity, and ion permeability in H5 mutants of the plant K^+ channel, KAT1. *Proc. Natl. Acad. Sci. USA* **93,** 8123–8128.
29. Moroni, A., Bardella, L., and Thiel, G. (1998) The impermeant ion methyl ammonium blocks K^+ and $NH4^+$ currents through KAT1 channel differently: Evidence for ion interaction in channel permeation. *J. Membr. Biol.* **163,** 25–35.
30. Very, A. A, Bosseux, C., Gaymard, F., Sentenac, H., and Thibaud, J. B. (1994) Level of expression in *Xenopus* oocytes affects some characteristics of a plant inward-rectifying voltage-gated K^+ channel. *Eur. J. Physiol. Pflugers-Arch.* **428,** 422–424.