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Isolation of a potassium-selective ion channel from the plasma membrane of the broad bean *Vicia faba* L.

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Abstract

Potassium ions are essential for plant nutrition and play an important role in osmoregulation and plant movement. Recent patch clamp investigations and molecular analysis have identified voltage-dependent potassium channels responsible for translocation of this ion across plant membranes. In order to characterize these transporters on the biochemical level, a potassium channel protein was isolated from *Vicia faba* under preservation of its functional integrity. Potassium channel activity was monitored by tracer flux and black lipid bilayer measurements. The capability for K^+ transport copurified with a 67 kDa protein from *V. faba* plasma membranes. A monoclonal antibody directed against the 67 kDa protein blocked channel activity. Single channels with a unit conductance of 40 pS were highly selective for K^+ over Na^+.

Key words: Plasma membrane; $^{86}Rb^+$ transport (K^+ channel); Protein isolation; Bilayer technique; *Vicia faba*

1. Introduction

Voltage-regulated potassium channels are key factors in the control of growth, development, osmoregulation, or stomatal movement. The characterization of potassium channels in the plasma membrane of guard cell protoplasts with the patch clamp technique revealed differences in voltage dependence, kinetics and pharmacology of two K^+ channel types: (a) an inward-rectifying K^+ channel mediating K^+ uptake, and (b) an outward-rectifyer allowing K^+ release [1,2].

Similar activities were also found in other plant cells such as motor cells, mesophyll cells, endosperm cells and root cells [3]. Two plant K^+ channels from *Arabidopsis thaliana* were cloned by complementation of a yeast mutant defective in K^+ uptake [4,5]. These plant genes (KAT1 and AKT1) show sequence similarities to members of the *Shaker*-related potassium channel family identified in *Drosophila* mutants [6,7]. After expression of the KAT1 cDNA in *Xenopus laevis* oocytes, an inward-rectifying potassium current was observed similar to the K^+ channels in higher plant cells [8]. The expression analysis of maize cDNA in *Xenopus laevis* oocytes revealed outward-rectifying potassium currents [9].

High affinity ligand binding and functional expression are successful tools to isolate animal Ca^{2+}, Na^+, or K^+ channels [10,11]. Toxins for plant ion channels, with similar high binding specificities as those for vertebrate ion channels are, however, not yet available. In this paper, functional reconstitution of the TEA-sensitive transport activity was used for the isolation of a mesophyll plasma membrane K^+ channel.

2. Materials and methods

2.1. Plasma membrane isolation

Plasma membrane vesicles were prepared by aqueous two-phase partitioning [12,13] from 2- to 3-week-old *V. faba* leaves using 5 mM KH_2PO_4/K_2HPO_4 (pH 7.8), 6.3% dextran, 6.3% PEG, 4 mM KCl and 330 mM sucrose. Plasma membrane vesicles were washed and resuspended in 10 mM K-HEPES (pH 7.2), 50 mM KCl and 330 mM sucrose. Protein was determined as described by Lowry et al. [14].

2.2. Solubilization and reconstitution of membrane proteins

Membrane proteins (1.0–1.5 mg·ml^{-1}) were solubilized for 5 min at 10°C in detergent buffer (10 mM K-HEPES (pH 7.2), 60 mM Mega-9, 5 mM KCl, 1 mM DTT, 0.01 mM *o*-phenanthroline, 0.01 mM PMSF and 0.1% asolectin. The detergent extract was centrifuged (150,000 × *g*, 20 min, 8°C) and the supernatant was dialysed for 24 h against 2 × 250 volumes of detergent-free buffer (10 mM K-HEPES (pH 7.2), 50 mM KCl and 1 mM $CaCl_2$). After addition of CHAPS (10 mM) and 10 mg·ml^{-1} asolectin, dialysis of the supernatant was continued for 36 h.

2.3. $^{86}Rb^+$ flux experiments

Plasma membrane vesicles and reconstituted vesicles containing about 50 μg protein (unless stated otherwise) were used for Rb^+-flux measurements. Vesicles were incubated for 30 min on ice with $^{86}Rb^+$ (3.7×10^4 Bq) in tracer buffer (10 mM K-HEPES (pH 7.2), 50 mM KCl, and 1 mM $CaCl_2$); plasma membrane suspensions contained in addition 330 mM sucrose.

To quantify uptake of $^{86}Rb^+$ into vesicles, external tracer was removed by filtration (filter type ME24, Schleicher and Schuell, Dassel, Germany) followed by 3 × 1 ml washes with chilled wash buffer (10 mM K-HEPES (pH 7.2), 200 mM K^+ and 1 mM $CaCl_2$). Uptake was further corrected for the background (1.7–2.3 pmol $^{86}Rb^+$) which was estimated from measurements without vesicles.

In order to demonstrate the sensitivity of the $^{86}Rb^+$ uptake towards the K^+ channel blocker TEA, vesicles were preincubated for 30 min with TEA (5 mM, unless stated otherwise) prior the addition of $^{86}Rb^+$.

2.4. Purification of the $^{86}Rb^+$-transporter

Plasma membrane-rich vesicles (10–15 mg of protein) were solubilized with 10 mM K-HEPES (pH 7.2), 60 mM Mega-9 (detergent to protein ratio = 20:1), 1 mM DTT, 0.01 mM *o*-phenanthroline,

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PMSF, phenylmethylsulfonylfluoride; Mega-9, nonanoyl-*N*-methylglucamide; TEA, tetraethylammoniumchloride; PEG, polyethylene glycol.

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2 μg·ml^{-1} leupeptin, 0.01 mM PMSF and 0.01% asolectin. After centrifugation of the detergent extract (150,000 × g, 20 min, 8°C), the supernatant was charged onto a Q-Sepharose column (Pharmacia, 0.75 × 20 cm) equilibrated with 10 mM K-HEPES (pH 7.2) and 10 mM CHAPS. Proteins were eluted with salt concentrations of 0.4 and 1 M KCl. The high salt eluate (1 M KCl) was subsequently diluted to a final concentration of 0.4 M KCl and was applied onto a Mono Q column (Pharmacia, Mono Q HR5/5). Proteins were eluted by a linear salt gradient (0.5 to 1 M KCl). The protein fraction irrigated by 680 mM KCl were incubated for 30 min at 4°C with affinity matrix Fractogel TSK AF-Red (Merck). The gel was flushed successively with buffer containing 10 mM K-HEPES (pH 7.2), 10 mM CHAPS, 0.75 M and 1.5 M KCl. All eluted probes were collected in 2 ml fractions. Aliquots (1.5 ml) were reconstituted into liposomes for Rb^+ uptake measurements.

2.5. Generation of monoclonal antibody

The monoclonal antibody (mab) G39–8-C1 was generated by immunizing BALB/c mice with 0.8–1.0 μg purified pm67. The generation of the mab and characterization of its reactivity according to Key and Weiler [15] was performed with both plasma membranes and purified pm67 for the selection of positive clones.

2.6. Electrophoretic and immunological analysis

Purified membrane proteins were separated electrophoretically on 12.5% SDS-polyacrylamide gels as described by Laemmli [16] followed by silver staining [17].

Immunological analysis was performed with polypeptides which were electroblotted at 5 mA · cm^{-2} onto nitrocellulose membranes (Sartorius, Göttingen, Germany) using a semi-dry apparatus (Biometra, Göttingen, Germany). Immunodetection of blotted proteins was performed using the monoclonal antibody (G39–8-C1), which was decorated by a second antibody (anti IgM) coupled to alkaline phosphatase according to Blake et al. [18]. The AKT1-antibody was a gift from Dr. H. Sentenac (Montpellier, France).

2.7. Single channel recordings

Ion channel properties of the purified membrane properties were investigated by fusing proteoliposomes into lipid bilayers formed from 20 mg asolectin in 1 ml *n*-decan [19]. The single channel recordings were performed under symmetrical solute compositions (10 mM K-HEPES (pH 7.2), 200 mM KCl and 1 mM $CaCl_2$; except studies on ion selectivity). The selectivity of the transport system was studied under biionic conditions by replacing 200 mM K^+ against 200 mM Na^+ on the *trans*-

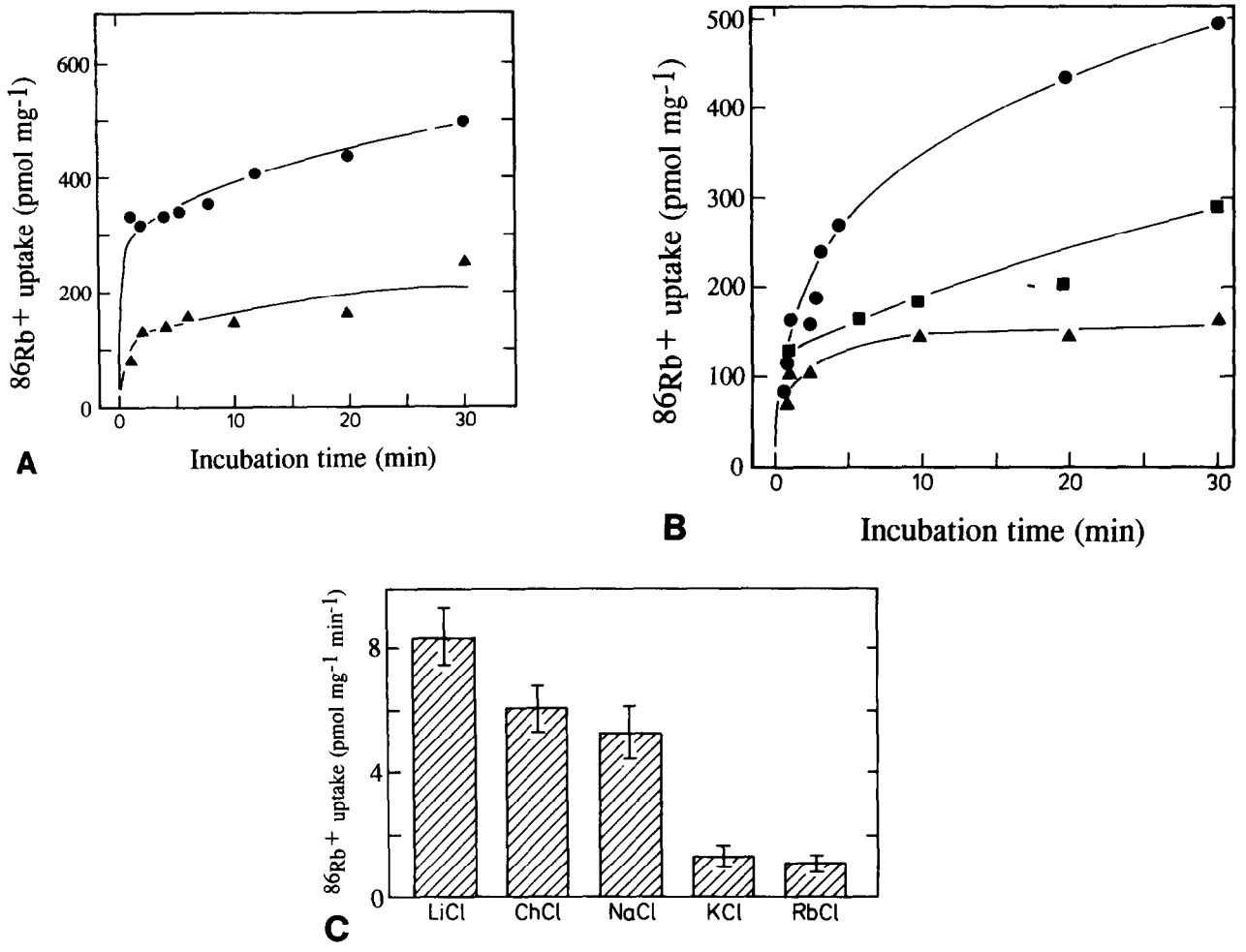

Fig. 1. Rb^+-uptake into plasma membrane vesicles and liposomes fused with plasma membrane proteins of *V. faba* L.. (A) Plasma membrane vesicles (50 μg protein, 0.4 ml assay volume) were pretreated with (▲) or without (●) 5 mM TEA (30 min) followed by incubation with $^{86}Rb^+$. (B) $^{86}Rb^+$-influx of control liposomes (▲) and reconstituted proteoliposomes in the absence (●) and presence (■) of 5 mM TEA. The reconstituted vesicles were prepared from a Mega-9 extract of enriched plasma membranes at a protein to lipid ratio of 0.1 (w/w). Liposomes were prepared by the detergent dialysis technique. Aliquots (0.2 ml) of the reconstituted vesicles or control liposomes were then incubated with tracer (assay volume: 0.21 ml). For details see section 2. (C) Effect of different extravesicular cations on the $^{86}Rb^+$-uptake into reconstituted vesicles. A Mega-9 extract of enriched plasma membranes was reconstituted in the presence of the detergent CHAPS at a protein to lipid ratio of 0.14 (w/w) with 50 mM KCl in the dialysis buffer. The reconstituted vesicles were centrifuged for 30 min at 150,000 × g and resuspended in dialysis buffer without potassium chloride. Reconstituted vesicles were incubated (30 min) with tracer and in the presence of 100 mM salt (ChCl = choline chloride, KCl, NaCl, LiCl, RbCl). The bars represent the standard deviation of three different preparations.

side of the chamber. The *trans*-side of the cuvette was held at 'virtual' ground and the voltage was applied to the *cis*-side to record the activity of single K^+ channels [19]. The current fluctuations were filtered at 1 kHz, stored on FM tape (Racal store 4FM) and analysed off-line on IBM computers at a sample rate of 2 kHz using software from RC-electronics.

3. Results and discussion

In order to study the potassium transport of 'crude' plasma membrane vesicles $^{86}Rb^+$ was used as tracer for K^+. Rb^+ fluxes were optimized with respect to the amount of protein and salt concentration. $^{86}Rb^+$ uptake of plasma membrane vesicles increased proportional with the protein concentration of the vesicle population ($\leq$ 75 μg per assay, data not shown).

Upon raising the extravesicular potassium concentration above the intravesicular level (50 mM), the transport rates of the membrane vesicles decreased whereas a reversed gradient (3-fold, data not shown) increased transport capacity. Uptake rates exhibited bi-phasic kinetics, with the fast component being responsible for the majority of the K^+ flux (Fig. 1A). When TEA, a blocker of potassium channels [20], was added to the membrane vesicles, $^{86}Rb^+$ uptake was inhibited in a dose-dependent manner (IC_{50} = 5 mM).

To identify the TEA-sensitive transporter, conditions for solubilization and reconstitution of plasma membrane proteins into lipid vesicles were analyzed (Fig. 1B).

Table 1
Purification of a Rb^+ transporter from plasma membranes of *Vicia faba*

Step	Protein (mg)	Activity (pmol·mg^{-1}·min^{-1})	Purification (fold)
Plasma membrane	10	15.5	1
Q-Sepharose	0.4	166	10.7
Mono Q	0.02	4162	268
Fractogel TSK AF-Red	0.002	6696	432

Mega-9 was the most effective detergent. Therefore, the detergent was applied at a concentration of 60 mM and at a detergent to protein ratio of 20:1 (w/w) throughout protein solubilization. The TEA-sensitive transport capacity was restored when Mega-9 was replaced by CHAPS for reconstitution into liposomes.

Rb^+ uptake into reconstituted proteoliposomes showed similar characteristics to plasma membrane vesicles with respect to its sensitivity towards TEA (Fig. 1B). The increase in transport with the protein concentration indicated the protein nature of the K^+ transport in the presence of a low $^{86}Rb^+$ binding capacity of lipid vesicles.

Reconstituted vesicles were permeable for Rb^+ and K^+ since these ions were able to compete the tracer for transport (Fig. 1C). Other cations such as Na^+, Li^+ or choline$^+$ were less permeable. Transport studies performed in the

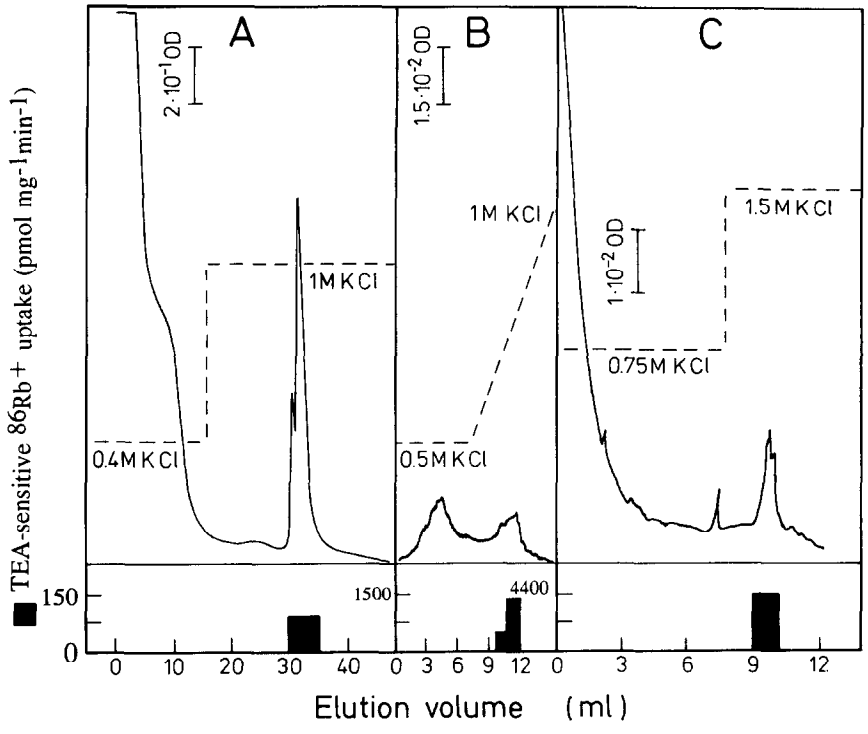

Fig. 2. Purification of a plasma membrane Rb^+ transporter by chromatography on Q-Sepharose column (A), Mono Q (HR 5/5, 1 ml gel bed) (B) and Fractogel TSK AF-Red (5 ml gel bed) (C). Protein was monitored at 280 nm. $^{86}Rb^+$ uptake of the reconstituted fractions was measured in absence or presence of TEA (5 mM).

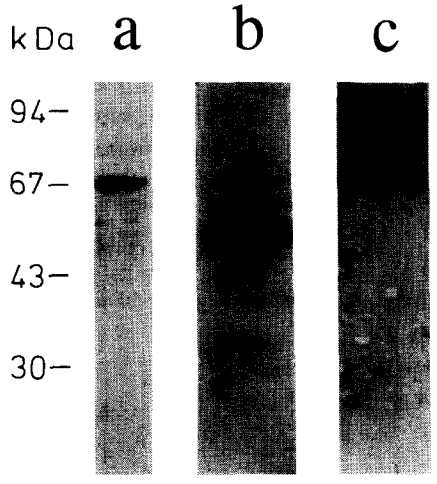

Fig. 3. Identification of pm67 by immunological techniques. Lane a, SDS-PAGE/ silver staining of the high salt fraction obtained from the Fractogel TSK AF-Red column (purified pm67). Lane b, immunblot analysis of plasma membranes by the monoclonal antibody G39–8-C1. Plasma membranes (25 μg) were separated by SDS-PAGE (12.5%) and transferred to nitrocellulose membranes. After preincubation with buffer (10 mM Tris (pH 7.8), 1% BSA, 100 mM NaCl and 1 mM $MgCl_2$) immunodetection was performed with cell a culture supernatant from the monoclonal antibody producing cell line (dilution with blocking buffer 1:200, monoclonal antibody was incubated with blot for 2 h). Lane c, G39–8-C1 directed against the purified K^+ transport protein cross-react with the purified pm67 (2 μg).

presence and absence of 1 mM Ca^{2+} revealed identical K^+ flux characteristics. This is in contrast to K_{in} channels in guard cells, but in line with observations on ion channels in xylem parenchyma cells from barley roots [21].

To isolate the TEA-sensitive transporter from a detergent protein extract, anion exchange and affinity chromatography was used. The purification was monitored by functional reconstitution of the TEA-sensitive Rb^+ transport activity in the lipid vesicles. The solubilized proteins were bound to Q-Sepharose and eluted with 0.4 M KCl and 1 M KCl buffers (Fig. 2A). The TEA-sensitive transporter appeared in the 1 M KCl fraction indicating its strong interaction with the anion exchanger.

The high-salt fraction (containing at least 7 different polypeptides) was then diluted to 0.4 M KCl and applied to a Mono Q column. Following high salt elution, $^{86}Rb^+$-transport activity was associated with the 680–750 mM KCl fraction (Fig. 2B). This sample was further purified by affinity chromatography using Fractogel TSK AF-Red. Fractions showing K^+ transport activity were eluted at 1.5 M KCl (Fig. 2C). The overall purification was 430-fold and resulted in the isolation of a single major protein band with an apparent molecular mass of 67 kDa (Fig. 3, lane a; Table 1).

The presence of the 67 kDa protein (pm67) within plasma membranes was analyzed with the monoclonal antibody G39–8-C1 generated against the 67 kDa protein. Western blot analysis showed that this antibody recognized a 67 and 49 kDa polypeptide in 'crude' plasma membranes and cross-reacted with purified pm67 (Fig. 3, lanes b,c). This antibody had the capability to inhibit the Rb^+ uptake (Fig. 4), whereas antibodies directed against total plasma membranes of *V. faba* had no effect. No cross-reaction was observed with an antibody directed against the plant K^+ channel, AKT1.

In order to demonstrate that pm67 represents a K^+-selective ion channel, pm67 containing liposomes were incorporated into black lipid films. In 200 mM KCl, open-to-closed transitions of single ion channels were resolved (Fig. 5A). At high voltages single channel amplitudes approached a steady-state indicating possible saturation of the ion conductance [1,22]. From the linear portion of the current–voltage curve, a slope conductance of 43 pS ± 1.26 (n = 13) was deduced (Fig. 5B). Similar unit conductances were observed for RbCl and KCl, but in NaCl it dropped to 8 pS (data not shown). In line with a K^+-selective channel being ten times more permeable to K^+ than to Na^+, the reversal potential shifted by −59 mV when K^+ was replaced by Na^+ (data not shown).

In the absence of any membrane potential across the vesicle membrane, K^+ transport was observed suggesting that the transport was mediated by a Rb^+/K^+ channel with a significant open probability at 0 mV. This transporter thus differs from the K_{in} and K_{out} channels of guard cells which activate at membrane potentials negative to −90 and positive to −20 mV, respectively [2,23].

The antibody detect pm67 in the native plasma membrane without any cross-reactivity with proteins from microsomes and other plant plasma membranes. Recognition of another protein (pm49; Fig. 3, lane b) in meso-

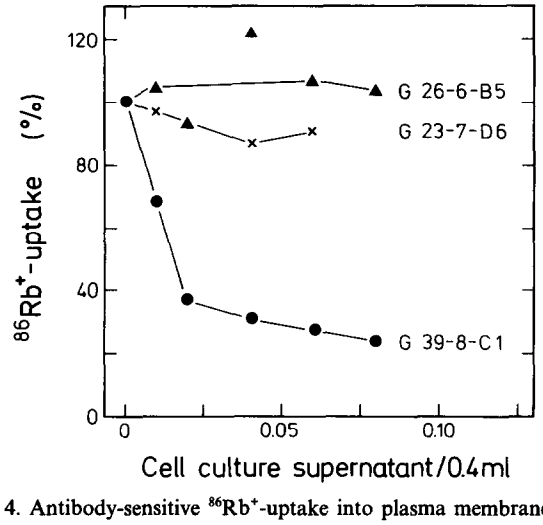

Fig. 4. Antibody-sensitive $^{86}Rb^+$-uptake into plasma membrane vesicles. After preincubation in cell culture supernatant obtained from antibody-producing cell lines, plasma membranes (50 μg) of *Vicia faba* were treated with 37 kBq $^{86}Rb^+$ for 30 min. G23–7-D6, G26–6-B5 and G39–8-C1 indicate different monoclonal antibodies. The 100% value corresponds to 16 pmol·mg^{-1}·min^{-1}.

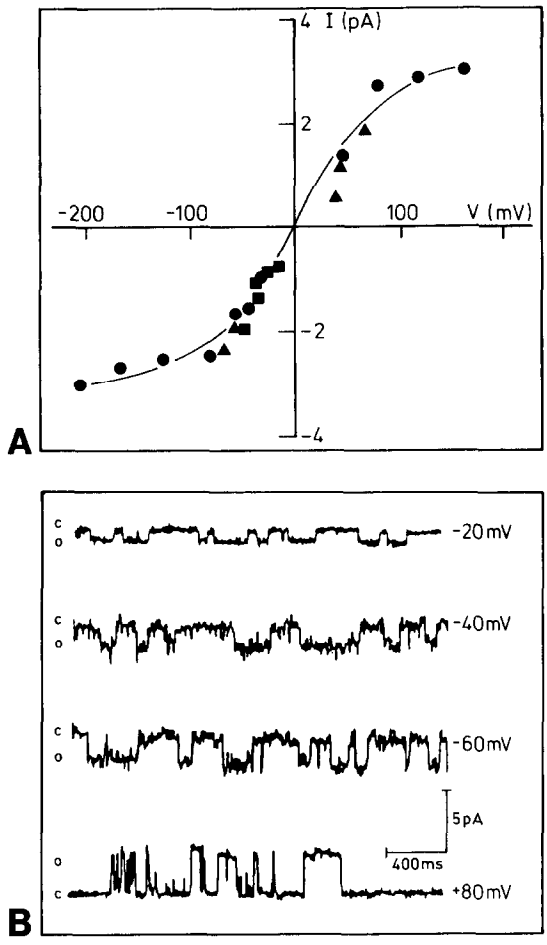

Fig. 5. Single channel activity related to purified pm67 after fusion into lipid bilayers. (A) Single-channel fluctuations as a function of voltage. A reconstituted pm67 preparation (1 μg protein · ml^{-1} lipid) was added to the *cis*-side of the cuvette. Measurements were performed in symmetrical KCl (200 mM), c = closed state and o = open state of the K^+ channel. (B) Current–voltage relation of the open K^+ channel in the range of +160 and −220 mV. Each symbol (●, ■, ▲) represents different pm67 preparations.

phyll plasma membranes might result from proteolytic degradation of pm67, since reconstitution of pm67 was sufficient to generate K^+ transport.

Unit conductances of single K^+ channels in the plant plasma membrane have been reported to be 20–50 pS for guard cell protoplasts [23–25], 12–18 pS for aleuron cell protoplasts [26], 22 pS for motor cells [27], 44 and 66 pS for *Arabidopsis* mesophyll cells [28] and 28.5 pS for KAT1 [8]. The reconstituted mesophyll channel exhibited a Rb^+ competition sequence of monovalent cations (Fig. 1C): $Rb^+ = K^+ > Na^+ > choline^+ > Li^+$and a permeability ratio of 10:1 between K^+ and Na^+. This permeability sequence as well as the single channel conductance and the saturation behaviour at high voltages are in line with the properties of K^+ channels identified in the plasma membrane of other plant cell types [2,29].

Despite the differences in molecular masses found between AKT1 (94 kDa), KAT1 (75 kDa) and pm67, the unique properties of pm67, i.e. the lack of pronounced voltage-dependence, Ca^{2+}-insensitivity and the lack of cross-reactivity with the AKT1-antibody, suggest that pm67 represents a new K^+ channel type from the mesophyll plasma membrane of *Vicia faba*.

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