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A prokaryotic potassium ion channel with two predicted transmembrane segments from *Streptomyces lividans*

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We report the identification, functional expression, purification, reconstitution and electrophysiological characterization of an up to now unique prokaryotic potassium ion channel (KcsA). It has a rectifying current–voltage relationship and displays subconductance states, the largest of which amounts to $\Lambda \cong 90$ pS. The channel is blocked by Cs^+ ions and gating requires the presence of Mg^{2+} ions. The *kcsA* gene has been identified in the gram-positive soil bacterium *Streptomyces lividans*. It encodes a predicted 17.6 kDa protein with two potential membrane-spanning helices linked by a central domain which shares a high degree of similarity with the H5 segment conserved among eukaryotic ion channels. Multiple alignments of deduced amino acids suggest that the novel channel has the closest kinship to the S5, H5 and S6 regions of voltage-gated K^+ channel families, mainly to the subfamily represented by the *Shaker* protein from *Drosophila melanogaster*. Moreover, KcsA is most distantly related to eukaryotic inwardly rectifying channels with two putative predicted transmembrane segments.

Keywords: bilayer/H5 region/patch clamp/potassium ion channels/*Streptomyces*

Introduction

Ion channels which are prevalent in most, if not all, eukaryotic cells, including animals, plants, yeast and protozoa, are involved in many physiological processes, such as secretion, regulation of membrane potential, signal transduction and osmoregulation. The large superfamily of eukaryotic ion channels includes Ca^{2+}, Na^+ and K^+ channel proteins; they sense changes in membrane potential and respond by enhancing the flux of ions extremely rapidly. For Na^+ and Ca^{2+} channels, a single gene encodes four similar repeats, each corresponding to the single subunit of a K^+ channel with six putative membrane helices (S1–S6) and the H5 segment which links the C-terminal helices S5 and S6 (for a review, see Hille, 1992). These canonical segments were initially found within four subfamilies (Shaker, Shal, Shab and Shaw) of K^+ channels from *Drosophila melanogaster*, and they have been discovered conserved in mice and humans (for a review, see Salkoff *et al.*, 1992; Pongs, 1993) as well as in the plant *Arabidopsis thaliana* (Schachtman *et al.*, 1992; Sentenac *et al.*, 1992). Recently, inwardly rectifying K^+ channels lacking the canonical motif of six membrane-spanning helices were identified in kidney cells of rats (ROMK1; Ho *et al.*, 1993), in rabbit renal cortical collecting tubule cells (RACTK1; Suzuki *et al.*, 1994) as well as in a mouse macrophage cell line (IRK1; Kubo *et al.*, 1993). These predicted proteins include only two putative membrane-spanning helices. Based on electrophysiological properties and sequence similarities, it has been proposed that intragenic duplications of an ancestral single-domain channel gave rise to a broad diversity of channel genes (Strong *et al.*, 1993).

Streptomycetes are Gram-positive soil bacteria which grow as substrate hyphae and differentiate to aerial mycelia as well as spores, if nutrients are depleted (for a review, see Kutzner, 1981). They synthesize a wide range of chemically different antibiotics, cytostatics and fungicides, and degrade numerous macromolecules. Here we report the cloning and functional expression of a so far unique prokaryotic gene encoding a potassium ion channel (KcsA) from *Streptomyces lividans*. In addition, we describe the purification and reconstitution of the channel protein, as well as its electrophysiological characterization.

Results

Identification of the S.lividans kcsA gene

In the course of an analysis of various genes from the *S.lividans* genome, we characterized several overlapping hybrid phages from a gene library (in Charon 35) of this strain (Kessler *et al.*, 1989; Dittrich *et al.*, 1991). We found an open reading frame (*kcsA*) of 483 bp with a high G + C content of 70.6% (typical of *Streptomyces* DNA) and a coding capacity for a 17.6 kDa protein (Figure 1, top). Various stretches of the deduced amino acids were compared with those derived from known predicted proteins (Figure 1, bottom). It is interesting that, within an overlap of 116 amino acids (aa), 32.8–31% of them are identical with deduced K^+ channels, such as ShC1 (*Shaker*) from *D.melanogaster* (Schwarz *et al.*, 1988), Kv3.2c from rats (Luneau *et al.* 1991) as well as HBK2 from humans (Grupe *et al.*, 1990) and various others. A total of 66.7% of identical amino acids are present in an overlap of 24 aa which corresponds to the H5 domain (25 aa) of the above quoted and several other K^+ channel proteins. The deduced novel protein possesses two potential transmembrane segments flanking the H5-like domain (Figure 2). The hydrophobicity profile (Kyte and Doolittle, 1982) of the deduced KcsA protein from *S.lividans* shares similarities with each of the two C-terminal helices S5 and S6 of the deduced ShC1 (see above), the KAT1 (*A.thaliana*) and others, as well as with inwardly rectifying

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GCATGCTGGCTCCTTTGGGATCGATCCGTCCGGTTCTTCTCCGGCCGGCCACCTCTCGAAGGTGACGCTGTCGCCGACGAGCCACCGACATCCGACCGACAGCCCCCGACAGCGCTCCTA  120
 Sph I
CGCGGTGCCGACATGACACCGACACCGCAGGTCGGACGACGGGGGCTCAGGCGCGACGGGCGCGGATCACGACGGCCGTACCGCCGCGACGGCGAGCACCGCCGCGCCGCCGAGGAGTGG  240
                                                                                                  M  P  P  M  L  S  G  L  L  A
CCGAAGGAGTGAAGATCGGTTACGGACCGTAAAGGAGTACCTGGCGCACCGGCGCGTTGTCGCATCGTCGTCCCGGCCGGTGGCGGAGCATGCCACCCATGCTGTCCGGTCTTCTGGCCA  360

R  L  V  K  L  L  L  G  R  H  G  S  A  L  H  W  R  A  A  G  A  A  T  V  L  L  V  I  V  L  L  A  G  S  Y  L  A  V  L  A
GATTGGTCAAACTGCTGCTCGGGCGCCACGGCAGTGCGCTGCACTGGAGGGCCGCGGGTGCCGCGACGGTCCTCCTGGTGATCGTCCTCCTCGCGGGCTCGTACTTGGCCGTCCTGGCTG  480

E  R  G  A  P  G  A  Q  L  I  T  Y  P  R  A  L  W  W  S  V  E  T  A  T  T  V  G  Y  G  D  L  Y  P  V  T  L  W  G  R  L
AGCGCGGCGCACCGGGCGCGCAGCTGATCACGTATCCGCGGGCGCTGTGGTGGTCCGTGGAGACCGCGACGACCGTCGGCTACGGCGACCTGTACCCCGTGACTCTGTGGGGCCGGCTCG  600

V  A  V  V  V  M  V  A  G  I  T  S  F  G  L  V  T  A  A  L  A  T  W  F  V  G  R  E  Q  E  R  R  G  H  F  V  R  H  S  E
TGGCCGTGGTGGTGATGGTCGCCGGGATCACCTCCTTCGGTCTGGTGACCGCCGCGCTGGCCACCTGGTTCGTCGGCCGGGAACAAGAGCGCCGGGGCCACTTCGTGCGCCACTCCGAGA  720

K  A  A  E  E  A  Y  T  R  T  T  R  A  L  H  E  R  F  D  R  L  E  R  M  L  D  D  N  R  R  *
AGGCCGCCGAGGAGGCGTACACGCGGACGACCCGGGCGCTGCACGAGCGTTTCGACCGTTTGGAGCGAATGCTCGACGACAACCGCCGGTGACTCCGCCGGTGACCGCCCGAGCGAGGCC  840

GCACCGATGAGTCTGCGGCGGTTGTGCGGTCTACCCGTCGACGAAGGGAGCGCACCATGCGCAAGATCATCATTTGCACGTTCCTGACGCTGGACGGCGTCATGCAGGCGCCGGGCGGCC  960

CGGACGAGGACGCCGAGAGCGGCTTCGAACACGGCGGCTGGCAGAAGCCGGTGGACGACGACGAGGTCGGCACGGCCATCGCCGGCTGGTACGAGGACTCCGACGCCATGCTCCTCGGCC 1080

GCAAGACCTACGACATCTTCGCGTCGTACTGGCCGACCGCCGACCCCGACAACCCGTTCACCCATCGGATGAACAGCATGC
                                                                                  Sph I
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                    H5
ShC1   IPDAFWWAVVTMTTVGYGDMTPVGFWGK   - 404
KcsA   YPRALWWSVETATTVGYGDLYPVTLWGR   -  89
Kv3.2  IPIGFWWAVVTMTTLGYGDMYPQTWSGM   - 451
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Fig. 1. Top: The nucleotide sequence of the *S.lividans kcsA* gene and the deduced amino acid sequence. The start and stop codons are underlined. The sequence was analysed with the help of the GENMON program (GBF, Braunschweig, Germany) and reading frames were determined using the GC-WIND program (D.Shields, Dublin, Ireland). The sequence appears in the EMBL GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number Z37969. Bottom: Amino acid comparisons of the deduced part of the KcsA protein sharing a high number of identical amino acids with the H5 region of the deduced ShC1 (Schwarz *et al.*, 1988) and Kv3.2c (Luneau *et al.*, 1991) proteins.

K^+ channels featuring only the two transmembrane segments M1 and M2 (Ho *et al.*, 1993; Kubo *et al.*, 1993; Suzuki *et al.*, 1994).

Cloning of the kcsA gene in an S.lividans mutant

The genome of *S.lividans* was cleaved by various enzymes and hybridized with the *kcsA* gene and neighbouring DNA. The arrangement within the genome corresponded to the insert of the hybrid phage from the gene library. To find a suitable homologous cloning host, we screened our collection of mutants and found that the previously identified spontaneous mutant *S.lividans* N1 (Kessler *et al.*, 1989; Dittrich *et al.*, 1991) carried a deletion of the *kcsA* gene (Figure 3).

In order to test the function of the novel gene, it was cloned together with its upstream region (as a partial 1.15 kb *Sph*I–*Sph*I–*Sph*I fragment; Figure 3) in the multicopy *Streptomyces* vector pIJ702 (Hopwood *et al.*, 1985). The resulting hybrid plasmid (pKCS1) was transformed into the spontaneous *S.lividans* mutant N1. Transformants which contained the correct construct pKCS1 were analysed in comparison with the mutants N1 and N1 carrying the vector pIJ702 only (controls). The transformants grew more rapidly than the controls, and the myceliar densities were ~3 times higher at KCl concentrations of 1–5 mM.

Electrophysiological characterization of the novel K^+ channel

To test for possible K^+ channel activities, protoplasts were prepared from *Streptomyces* mycelia (see Materials and methods). The size of the diameters usually ranged from 2 to 3 μm, some of them were larger (5–10 μm). However, all attempts to obtain high-resistance seals between the outer membrane of the large protoplasts and the patch pipette (GΩ seal) failed. Therefore, protoplasts were fused with small unilamellar liposomes which were subsequently enlarged to giant vesicles (Criado and Keller, 1987; Enz *et al.*, 1993; and Materials and methods). Using these preparations, seals with resistances between 10 and 50 GΩ were reproducibly obtained in the course of patch-clamp measurements.

Giant liposome–protoplast vesicles derived from *S.lividans* N1 (pKCS1) contained ion channel activity (Figure 4). Current recordings at a holding potential of V_h = +40 mV yielded deflections with different amplitudes (see the histogram in Figure 4). Similar data were obtained at all positive pipette potentials, whereas at negative values almost no channel activity could be observed. The *I/V* relationship in the lower part of Figure 5 reveals that the current data may be grouped into three different classes of open channel amplitudes with the following slope conductance values: $\Lambda \cong 20 \pm 7$ pS (n = 5), $\Lambda \cong 40 \pm 8$ pS (n = 11), $\Lambda \cong 90 \pm 18$ pS (n = 6). The channel chord conductance values given in Figure 4 for the different peaks in the amplitude histogram were $\Lambda \cong 16$ pS, $\Lambda \cong 48$ pS and $\Lambda \cong 72$ pS, whereas the reversal potential was $E_{rev.} = -30$ mV in this particular experiment. The arrows in Figure 4 mark transitions indicative of subconductance states. The shown sequence of channel gatings could only be explained by the presence of four simultaneously active channels, two of them approaching a conductance of $\Lambda \cong 20$ pS. The conductances of the others would have to be $\Lambda \cong 100$ pS and $\Lambda \cong 60$ pS, respectively. However, if more than one active channel protein were present in the patch, these channels would have to open simultaneously within <1 ms, which is statistically extremely unlikely (Patlak, 1988). Moreover,

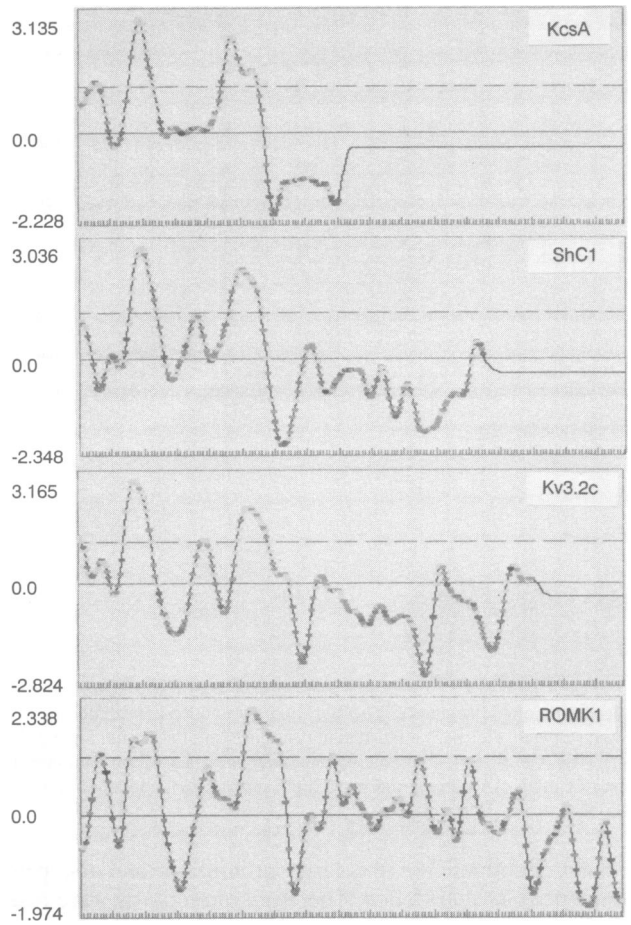

Fig. 2. Hydrophobicity profiles of the complete deduced KcsA (aa 1–160), the S4, S5, H5 and S6 regions, and C-terminal parts (left to right) of the deduced ShC1 (aa 315–564; Pongs *et al.*, 1988) and KAT (aa 186–510, the extension to 677 aa is not shown; Anderson *et al.* 1992), as well as of ROMK1 (aa 67–391; Ho *et al.*, 1993) including its M1, H5, M2 segments and the C-terminus.

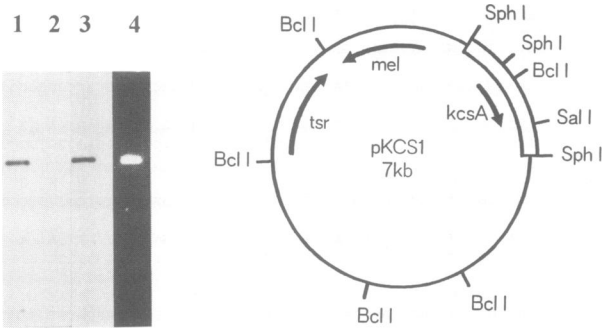

Fig. 3. Left: hybridization of the *ksc*A gene (internal *Sph*I–*Sal*I fragment) with *Sph*I-cleaved total DNA from *S.lividans* wild type (lane 1), the *kcsA* deletion mutant N1 (lane 2), the N1 (pKCS1) transformant (lane 3) and control (lane 4: *Sph*I–*Sph*I fragment carrying the *kcsA* gene without upstream region, see right). Right: the plasmid construct pKcsA was constructed by inserting the *kcsA* gene and its upstream region (*Sph*I–*Sph*I–*Sph*I 1.15 kb fragment) into the single *Sph*I site of the *Streptomyces* vector pIJ702.

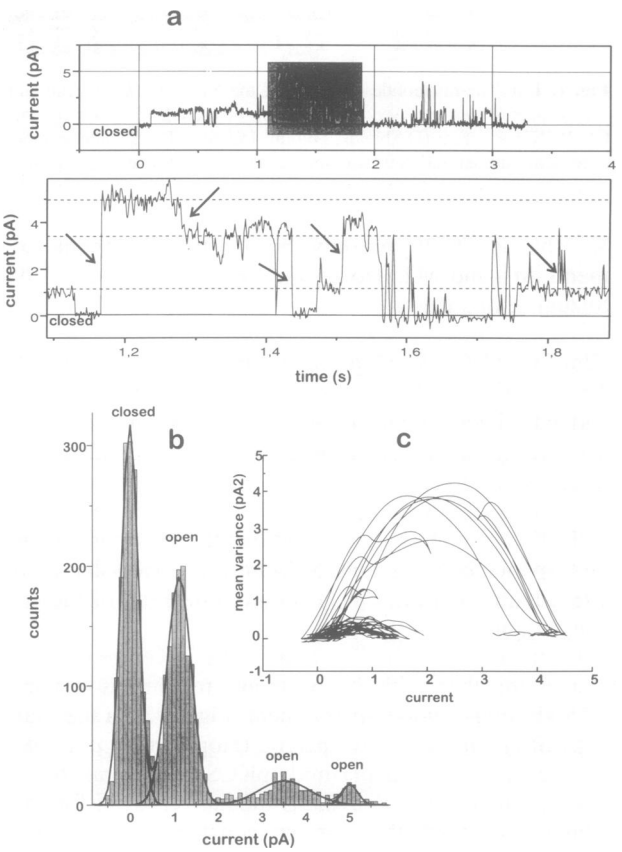

Fig. 4. Single-channel current recordings of giant liposome–protoplast vesicles from *S.lividans* N1 (pKCS1). (**a**) Top lane: measurements (holding potential V_m = +30 mV) in the excised (inside-out) patch configuration with asymmetrical buffers on both sides of the membrane [bath: 0 mM HEPES/Tris (pH 7.2), 40 mM KCl; pipette: 10 mM HEPES/Tris (pH 7.2), 200 mM KCl]. The second lane represents the shadowed part of lane 1 on an expanded scale. The arrows mark transitions indicative of subconductant states. After closing the 20 pS state, the channel fully opens to the 90 pS state (first arrow). From this state, partial closure to the 40 pS state occurs (second arrow), which is followed by a longer lasting complete closure (third arrow). Then the channel switches to the 20 pS open state, before opening from the closed to the intermediate 40 pS state (fourth arrow). (**b**) All-point amplitude histogram; the line indicates its Gaus fit. (**c**) Mean-variance plot of the data from the top lane.

in the course of many experiments revealing different current amplitudes, we have never observed sequential openings and closures of two or more active channels. Therefore, the temporal sequence of the current data (Figures 4–6) indicates that the different amplitudes are due to subconductance states rather than to the simultaneous activities of two or more channels. The reversal potential for the different channel transitions was identical with a value of $E_{rev.}$ = –33 mV (Figure 5), near to E_{K^+} = –40.6 mV. The comparative current voltage curves show that the channel activity described above (Figures 4, 5a and b) is absent in the control strain (Figure 5c).

In summary, the data demonstrate that the *kcsA* gene encodes a K^+-selective ion channel. Its maximal conductance of $\Lambda \cong 90$ pS is considerably less often observed than its smaller subconductance states of 20 and 40 pS. All conductance states have a high selectivity for potassium ions. When K^+ was partially substituted by Na^+ (bath: 200 mM K^+ + 100 mM Na^+; pipette: 40 mM K^+), the reversal potential changed from $E_{rev.} \cong -33$ mV to $E_{rev.}$ = –37 mV. Assuming that the K^+ permeability did not alter after the addition of Na^+, the permeability ratio P_{K^+}/P_{Na^+} can be calculated as $P_{K^+}/P_{Na^+} \cong 2.87$. In 30 independent experiments, Mg^{2+} ions (1 mM, absolutely necessary only

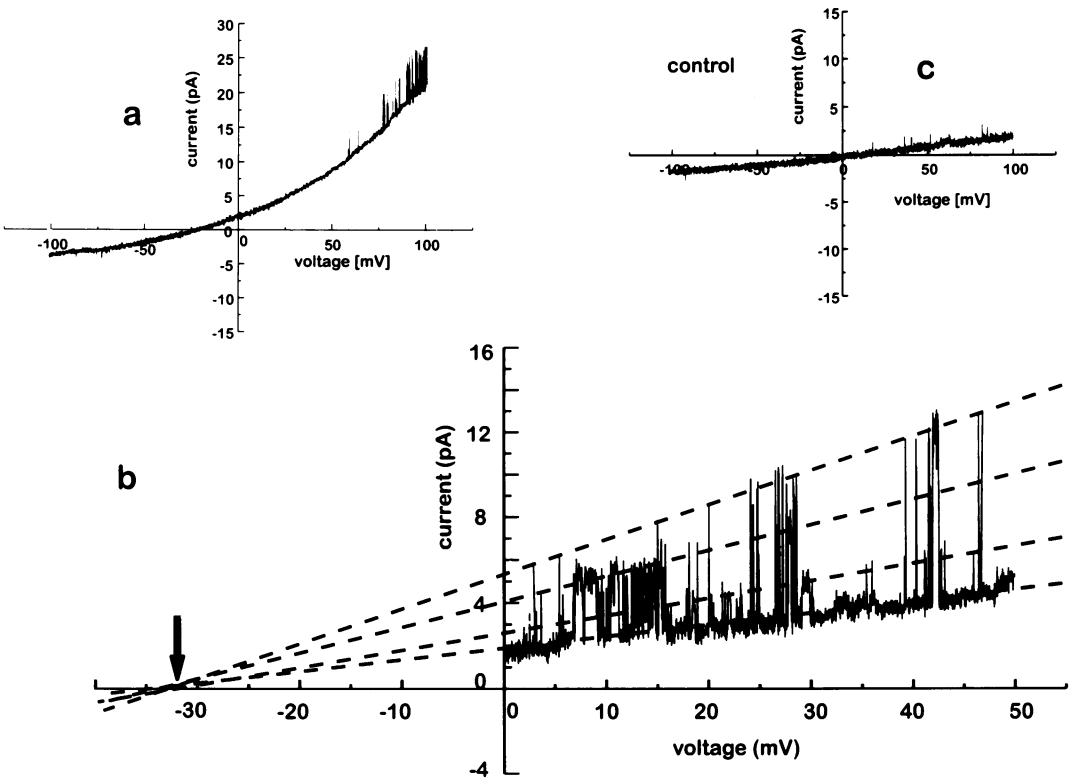

Fig. 5. Current recordings of giant liposome–protoplast vesicles from *S.lividans* N1 (pKCS1) [excised patch, bathed in asymmetric (bath: 10 mM HEPES/Tris pH 7.2, 40 mM KCl; pipette: 10 mM HEPES/Tris pH 7.2, 200 mM KCl) buffer], in response to a voltage sweep between 0 and +50 mV. (**a**) and (**c**) Current voltage curves with a pipette potential ranging from V_h = +100 mV to V_h = −100 mV were obtained using vesicles from *S.lividans* N1 containing the plasmid pKCS1 (a) and the control *S.lividans* N1 with vector only (c). In all experiments (two preparations, five attempts each), only small leak currents were observed at the varying holding potentials. The seal resistance (all channels closed) amounted to ~50 GΩ and the *I/V* relationship (all channels closed) was comparable for *S.lividans* N1 (pKCS1; a) and the control strain (c), indicating that the positive current offset at V_h = 0 mV is due to a second channel in the course of the voltage sweep. (**b**) The conductance values were calculated from the slope of the curves.

in the bath, not within the pipette) were required for channel activity. No channel activity was recorded in the course of five independent measurements when the $MgCl_2$ concentration was <1 mM. These data clearly demonstrate that Mg^{2+} ions activate the KcsA channel. Channel gating was only ascertained at mainly positive pipette potentials (Figure 5a), suggesting that the KcsA channels were unidirectionally incorporated into the membranes of the protoplast–liposome vesicles. Since the probability of channel openings was close to zero at negative pipette potentials, currents must be rectified. It was previously observed that channel orientation did not change during the fusion of native membranes with liposomes (Keller *et al.*, 1988; Enz *et al.*, 1993). Assuming that this is also the case in our study, the bacterial membranes will be oriented right-side out in the liposome–protoplast vesicles. Consequently, the observed potentials of the pipette and the bath refer to the outside and the cytoplasmic side, respectively. It must thus be concluded that the KcsA channel is more open at negative membrane potentials, and currents are inwardly rectified. In this case, the suspected Mg^{2+} binding site will be located in the cytosol. However, it is worth noting that, in contrast to other inward rectifiers like ROMK1 and IRK1, rectification is not due to a cytosolic Mg^{2+} block (Ho *et al.*, 1993; Kubo *et al.*, 1993). As reported for other ion channels (Kubo *et al.*, 1993), the activity of KcsA decreased slowly ($\tau/2 \geq 1$ min) with varying half-times (channel run-down). In the presence of CsCl, the overall open probability ($P_{open} \cong 0.52$) was drastically reduced ($P_{open} \cong 0.1$), and only the 40 pS open amplitude as well as flickering of currents were observed (Figure 6).

Overproduction and purification of the His-tag KcsA protein

The 545 bp *Sph*I–*Sal*I fragment containing the *kcsA* gene (without upstream region and start codon; Figures 1 and 3) was cloned into the polylinker of the *Escherichia coli* pQE-32 vector. This vector includes a ribosomal binding site, a start codon and six codons encoding histidine (Hochuli *et al.*, 1987). A transformant of *E.coli* XL-1 which carried the correct construct (verified by sequencing) was further tested for overexpression of the K^+ channel protein containing the His-affinity tag. The expected protein could be identified, after having optimized cultivation time, temperature and induction conditions for the strain (see Materials and methods), in the membrane fraction and not as inclusion bodies. After affinity chromatography, ~3 μg of the pure fusion protein could be obtained from a 1 ml culture.

Electrophysiological characterization of the reconstituted protein

The purified fusion protein was reconstituted into small unilamellar liposomes using the dialysis technique. After fusion of the proteoliposomes into planar bilayers (Mueller

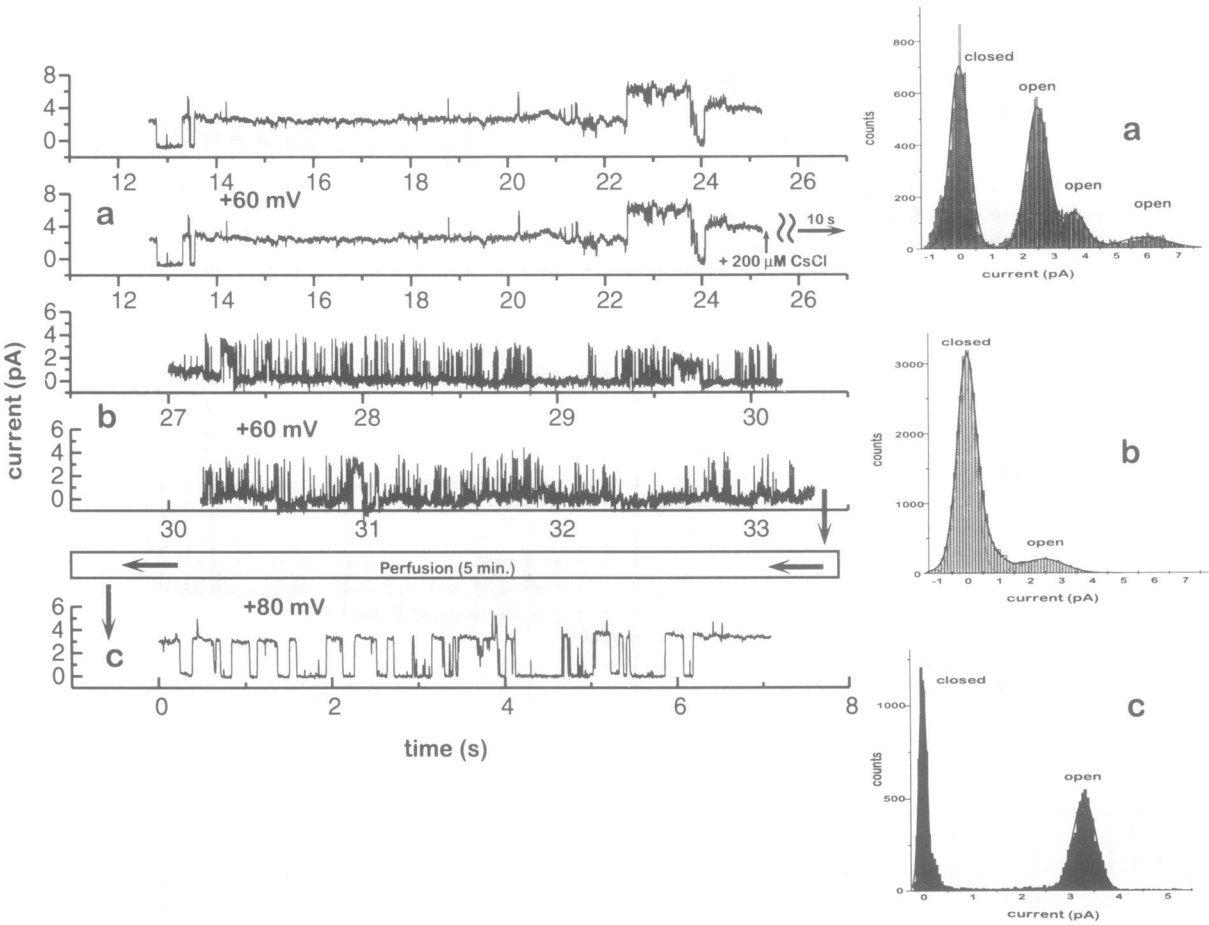

Fig. 6. Time sequence of single-channel current recordings with giant liposome–protoplast vesicles from *S.lividans* N1 (pKCS1) prior to and after the addition of 200 μM CsCl. (**a**, left) Measurements (two lanes, holding potential V_m = +60 mV) in the excised (inside-out) patch configuration with asymmetrical buffers on both sides of the membrane [bath: 10 mM HEPES/Tris (pH 7.2), 40 mM KCl; pipette: 10 mM HEPES/Tris (pH 7.2), 200 mM KCl]. (**b**, left) The current recordings (two lanes, gap 10 s) following the addition (arrow in second lane) of CsCl (under stirring) are presented in a five times expanded time scale. (**c**, left) Current recordings after bath perfusion for 5 min (buffer see above) at V_h = +80 mV. (**a**, right) All-point amplitude histogram in the absence of CsCl. (**b**, right) All-point amplitude histogram after addition of CsCl. The lines show the Gaus fit of the histogram. Considering $E_{rev.} \cong 30$ mV, the peak at 2.5 pA corresponds to a chord conductance of $\Lambda \cong 26$ pS. The overall open probability was calculated from the histogram peak areas of the closed state and those of the open states according to: P = a closed/E_{open} + a closed. (**c**, right) All-point amplitude histogram after perfusion.

et al., 1962), voltage-dependent single-channel activity was observed (Figure 7). The current–voltage relationship of the reconstituted channel was linear and its reversal potential of $E_{rev.} \cong +59$ mV was close to the Nernst potential for potassium ($E_{K^+} \cong +64$ mV). For protoplast–liposome vesicles, we mainly observed a conductance of $\Lambda \cong 42 \pm 6$ pS, whereas the larger conductance of $\Lambda \cong 90 \pm 12$ pS was less frequently recorded (Figure 7). The reconstituted channel showed no rectifying currents, and its gating was not affected by either Mg^{2+} or by Cs^+. These data suggest that the conformation of the reconstituted channel differs from that expressed within the liposome–protoplast vesicles, as the bilayer may lack essential lipids, proteins and components originally located within the cytoplasmic membrane of *S.lividans*.

Discussion

Using a newly established protoplast–liposome vesicle system, we succeeded in identifying a so far unique K^+ ion channel (KcsA) and its gene in a bacterium. The channel has a rectifying voltage relationship as well as several subconductance states, with the main open state of $\Lambda \cong 40$ pS. Channel gating requires Mg^{2+} ions and the channel is blocked by Cs^+ ions. In addition, we were able to overexpress and purify a His-tag KcsA protein. After its reconstitution in liposomes, followed by subsequent fusion into bilayers, we also succeeded in assigning K^+ ion channel activity to the purified protein. The results of both systems unambiguously proved that we have identified an up to now unique bacterial channel. Our data also demonstrate the first successful reconstitution of a bacterial K^+ ion channel protein. Furthermore, we have revealed that the reconstituted channel is no longer rectifying, and not affected by Mg^{2+} or Cs^+. However, this result is not surprising, as the artificial composition of the bilayer is different from that of the protoplasts (generated from the mycelia of the *Streptomyces* strain), and the bilayer also lacks any other component present in the protoplasts. This interpretation is in agreement with a recent study showing that the composition of the lipid membrane plays an important part in the action of channel effectors (Rehberg *et al.*, 1995). Other data have demonstrated that the addition of fatty acids or charged lipids can affect ion channel activity

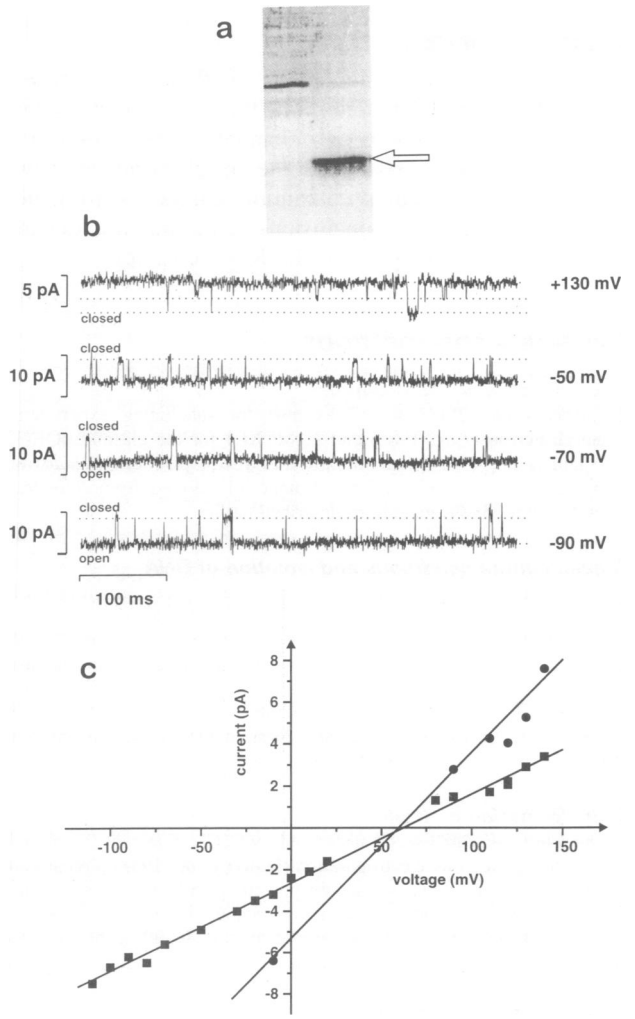

Fig. 7. Current recordings with the purified fusion protein. The His-tag–KcsA protein had been isolated from an *E.coli* XL-1 transformant grown to the mid-logarithmic phase and induced with IPTG (0.5 mM) for 90–100 min. The protein was purified to near homogeneity by affinity chromatography with Ni-NTA resin (see Materials and methods) and analysed by SDS–PAGE. (**a**) Left lane: eluate of proteins without channel activity, right lane: eluted (⇐) KcsA fusion protein. (**b**) (Four lanes): bilayer containing the reconstituted purified His-tag–KcsA (a, right) in 10 mM HEPES/Tris (pH 7.0), 20 mM KCl (*cis* side) and 10 mM HEPES/Tris (pH 7.0), 10 mM $CaCl_2$, 280 mM KCl (*trans* side). (**c**) Individual current amplitudes were taken from amplitude histograms of episodes of ~20 s duration (digitized at 0.2 ms, bandwith 2 kHz).

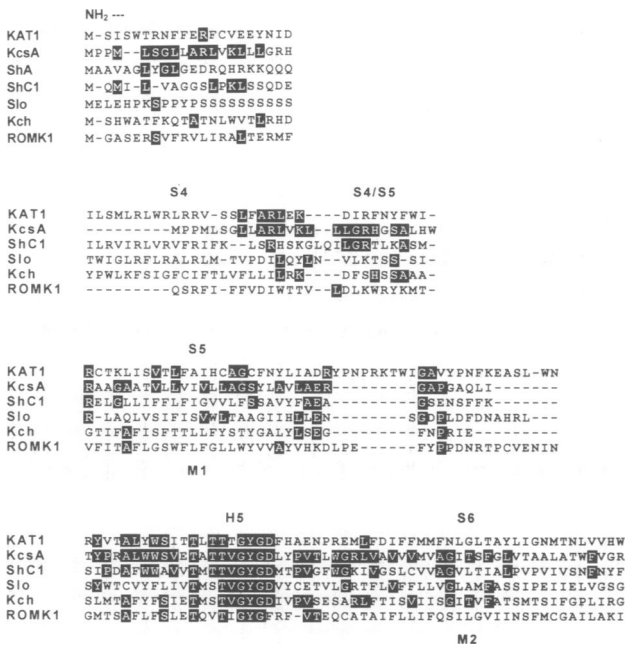

Fig. 8. Multiple alignments of deduced amino acids of the S4, S4/5 linker, S5, H5 and S6 regions, as well as of the N-terminal regions of the deduced KAT1 (Anderson *et al.*, 1992), KSC1, ShC1 (Pongs *et al.*, 1988), Slo (Atkinson *et al.*, 1991), Kch (Milkman, 1994) and ROMK1 (Ho *et al.*, 1993) proteins.

in muscle cells (Petrou *et al.*, 1995). Various K^+ ion channels from different eukaryotes have been analysed after the translation of the corresponding mRNA within oocytes (for a review, see Hille, 1992). Our preliminary experiments using polyadenylated mRNA from the *kcsA* gene did not lead to measurable K^+ channel activity in oocytes (D.Becker, R.Kümmerlen, R.Hedrich and H.Schrempf, unpublished). With antibodies raised against *kcsA*, we will test whether the mRNA was correctly translated.

The hydrophobicity profile of the small deduced protein allows the prediction of two transmembrane domains (Figure 2) with similarity to the segments S5 and S6. The latter were found within eukaryotic voltage-dependent K^+ channels containing six predicted transmembrane segments. The S5 and S6 segments were shown to play an important part in the determination of the lifetime of the activated and inactivated channel states (for a review, see Hille, 1992; Salkoff *et al.*, 1992; Schroeder *et al.*, 1994). The prokaryotic KcsA protein contains a segment, the amino acid composition of which is most closely related to the H5 region of voltage-gated eukaryotic K^+ channels. The H5 segment appears to be the major determinant of pore function. In addition to the S4/S5 linker region, the S6 segment as well as a short stretch beyond S6 contribute to the formation of the inner part of the pore (Jan and Jan, 1994). It has been widely assumed that the S4 segment in voltage-gated eukaryotic channels is a main component of the voltage sensor (for a review, see Hille, 1992; Brown, 1993; Pongs, 1993; Schroeder *et al.*, 1994). This region includes cationic arginine or lysine residues at every third position, interspersed with non-polar and hydrophobic residues. The identified bacterial KcsA protein lacks a stretch of amino acids corresponding to the complete S4 motif. However, the N-terminal part of KcsA contains seven leucine and two arginine residues, some of which can be aligned (with gaps) to a small part of the S4 region (Figure 8), and whose hydrophobicity profile resembles S4 (Figure 2). Moreover, several leucine residues as well as a few non-polar amino acids (corresponding to those from the S4–S5 linker region) are also present within the deduced N-terminal part of the KcsA protein. The C-terminal part of the deduced KcsA protein is relatively short, compared with deduced eukaryotic K^+ channel proteins (Figure 2). It is very rich in arginine as well as in glutamic and aspartic acid residues (Figures 1 and 8), and shares only a few amino acids with the region following the S6 segment in ShC1 (Schwarz *et al.*, 1988) and Kv3.2c (Luneau *et al.*, 1991), respectively.

The deduced eukaryotic inwardly rectifying K^+ channel proteins (IRKs), which are about three times longer than KcsA, possess two predicted transmembrane segments

Identity (%) for H5-segments

KAT1	100					
KcsA	44	100				
ShC1	36	64	100			
Slo	40	40	40	100		
Kch	44	48	48	52	100	
ROMK1	28	40	32	28	48	100
	KAT1	KcsA	ShC1	Slo	Kch	ROMK1

Identity (%) for S5-segments

KAT1	100					
KcsA	23	100				
ShC1	18	23	100			
Slo	0	14	9	100		
Kch	0	9	18	14	100	
ROMK1	4	14	14	4	27	100
	KAT1	KcsA	ShC1	Slo	Kch	ROMK1

Fig. 9. Identities (%) of deduced amino acids from representatives of the various channel proteins also compared in Figure 8.

(M1 and M2; Figure 2), the amino acid composition of which (Figure 8) differs considerably from that of the putative transmembrane helices of KcsA. Only the H5 domains of IRKs share ~40% of amino acids identical with those of the KcsA protein (Figure 8). The C-terminus, which apparently plays a major part in the specification of the properties of the pore within IRKs (Taglialatela *et al.*, 1994), has no amino acids in common with KcsA.

Interesting conclusions can be drawn from multiple alignments of deduced amino acids from one member of each of the major K^+ channel families (Figures 8 and 9). (i) Most remarkably, the two transmembrane helices of KcsA share the highest number of identical amino acids within the S5 (i. e. ShC1 > KAT1 > Slo) and S6 (ShC1 > Slo) segments of voltage-gated channel families and are most distantly related to the M1 and M2 segments from inwardly rectifying channels (i.e. ROMK1). (ii) The H5 segment of KcsA has the closest kinship to members of the voltage-gated *Shaker* subfamily. (iii) The S4/5 linkers of deduced *Shaker* proteins and their N-termini (ShA > ShC1) share several identical amino acids with KcsA. The variable N-terminus of the *Shaker* protein is known to form an inactivation ball (Choi *et al.*, 1991).

Recently, an open reading frame (*kch*) was found in the bacterium *E.coli* whose predicted protein is similar to six transmembrane segments of large eukaryotic K^+ channels. So far it is not known whether this gene is functional (Milkman, 1994). The alignment of its deduced amino acids reveals that its deduced H5 segment is most closely related to those within IRKs (Figures 8 and 9), whereas the transmembrane segments (M1 and M2, S5 and S6) from various eukaryotic channels, as well as KcsA from *S.lividans*, share only a few identical amino acids or none at all with Kch from *E.coli*.

As we succeeded in establishing an efficient overexpression system, we plan to determine the structural characteristics of the so far unique K^+ channel protein. Additional studies of the protoplast–liposome vesicles obtained from *S.lividans* transformants containing mutated *kcsA* genes should allow detailed conclusions to be drawn about the functions of various parts of the KcsA channel.

Materials and methods

Bacterial strains and plasmids

Escherichia coli JM83 served as host for subcloning experiments (Yanisch-Perron *et al.*, 1985) and *E.coli* XL1 for the plasmid pQE-32 (Hochuli *et al.*, 1987). The *Streptomyces* vector pIJ702, *S.lividans* 66 (Hopwood *et al.*, 1985) and the mutant N1 derived from *S.lividans* (Kessler *et al.*, 1989) have been described earlier.

Media, culture conditions and isolation of DNA

For the isolation of plasmid DNA, *E.coli* strains were grown in Luria broth (LB) containing 100 μg of ampicillin/ml to the logarithmic phase, and plasmid DNA was isolated as described previously (Sambrook *et al.*, 1989). *Streptomyces lividans* strains were grown either in minimal (Hopwood *et al.*, 1985) or in complete medium (Pigac *et al.*, 1988) if mycelia was needed to generate protoplasts for transformation or if DNA was to be isolated. Total and plasmid DNAs were isolated as depicted previously (Hopwood *et al.*, 1985).

Transformation of strains

Escherichia coli JM83 was transformed by $CaCl_2$ treatment (Sambrook *et al.*, 1989), *E.coli* by electroporation (Dower *et al.*, 1988). Preparation of *Streptomyces* protoplasts and their polyethylene glycol-assisted transformation were performed according to the standard procedure (Hopwood *et al.*, 1985). An overlay of 0.4% agarose containing 500 μg thiostrepton/ml was utilized to select transformants.

General DNA techniques

Cleavage of DNA by restriction enzymes, ligation of DNA fragments, separation of DNA fragments by agarose gel electrophoresis and DNA–DNA hybridizations were performed using standard procedures (Sambrook *et al.*, 1989). DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) with double-standard DNA templates and T7 polymerase kit (Pharmacia sequencing kit). Oligonucleotides were individually synthesized or corresponded to primers of the *lacZ* system (Sambrook *et al.*, 1989).

Nucleotide sequence accession number

The nucleotide sequence of the *kcsA* gene appears in the EMBL Gene Bank DDBJ Nucleotide Sequence Data Libraries under the accession number Z37969.

Generation of the fusion protein and its purification

The 545 bp *SphI–SalI* fragment containing the *kcsA* gene (Figures 1 and 3) without upstream region was fused in frame with six histidine codons in the *E.coli* vector pQE-32 (Quiagen, Germany). Having verified the construction by sequencing, the induction parameters for a transformant of *E.coli* XL1 carrying the hybrid plasmid pQE-32-KSC1 were optimized. It was best to grow the strain to the mid-logarithmic phase on a rotary shaker and to induce it with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 90 min. The cells (from a 200 ml culture) were harvested by low-speed centrifugation and washed twice. They were then suspended in 1.5 ml PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and disrupted by sonification. The pellet was obtained by high-speed centrifugation (10 min, 100 000 *g*) and extracted with buffer [PBS with 40 mM Mega 9 (Calbiochem)]. Proteins were bound to Ni-NTA according to the supplier's instructions (Quiagen, Germany), based on the studies of Hochuli *et al.* (1987). They were released by gradual elution with increasing concentrations of imidazol. Fractions were analysed by SDS–PAGE performed with 10% acrylamide gels in the presence of 0.1% SDS (Laemmli, 1970). The novel protein was eluted in the presence of 300 mM imidazol.

Preparation of protoplast–liposome vesicles

Streptomyces mycelia grown in complete medium (Kessler *et al.*, 1989) were treated with lysozyme in a buffer containing 15% sucrose as well as 10 mM $MgCl_2$ and 25 mM $CaCl_2$ for 5–6 h at 30°C. After microscopic inspection, protoplasts were washed three times in the same buffer. In order to expand their diameters, they were shifted to a buffer containing 10% sucrose. Suspensions included ~5×10^8 to 1×10^9 protoplasts/ml. KCl (30 mM) was added prior to fusion experiments.

Azolectin (50–100 mg/ml, Sigma type IV-S) was solubilized in the protoplast buffer by sonification for 20 s in a Branson sonifier equipped with a microtip (30% line voltage, 50% duty cycle) and liposomes were formed by the freeze–thaw technique (Kasahara and Hinkle, 1977). Protoplasts from *S.lividans* and liposomes were mixed 1:1 (v/v) by sonification (see above) and again subjected to two freeze–thaw cycles. Giant vesicles were obtained by a modified dehydration–rehydration procedure (Criado and Keller, 1987). After thawing, 5–7 µl of the protoplast–liposome mixture were spread on a glass slide and dehydrated at 4°C for 45–60 min in a 500 ml dessiccator filled to 1/3 volume with dry $CaCl_2$. Rehydration was performed in a Petri dish, the bottom of which was covered with water-saturated paper. Ten microlitres of the electrolyte solution to be used in the patch-clamp measurements were added to the partially dried sample on the slide. About 1 h later, giant liposomes with incorporated protoplasts could be observed. These giant liposome–protoplast vesicles (diameters ranging from 20 to 50 µm) were placed in a tissue bath (0.5 ml final volume), mounted on an Olympus IMT-2 inverted microscope and viewed with phase-contrast optics. Using the patch-clamp technique (Hamill *et al.*, 1981), single-channel current recordings were performed. The bath (agar electrode) was grounded and membrane voltages across excised inside-out patches referred to the pipette. Seals ranging from 10 to 50 GΩ were achieved by slight suction, once the pipette tip had been brought into contact with the giant liposome–protoplast vesicles. Current recordings were digitized at 36 kHz sampling rate using a VR 10 (Instrumental Corp.) digitizer and recorded on VHS video tapes.

Bilayer studies

For reconstitution, the purified solubilized fusion protein (~3–5 µg/ml) and small unilamellar liposomes were mixed 1:1 (v/v) by brief sonification and the mixture was kept for 30 min at 4°C. Liposomes were either formed by passing the mixture twice through NAP5™ columns (Pharmacia) equilibrated with a buffer containing 10 mM HEPES–KOH (pH 7), 1 mM $CaCl_2$ and 50 mM KCl, or by dialysis in this buffer (2 l, 4°C, overnight).

Planar lipid bilayers were produced by the painting technique (Mueller *et al.*, 1962). A solution of azolectin (Sigma type IV-S) 50 mg/ml in *n*-decan (analytical grade, Merck) was applied in the aperture (100–300 µm diameter) of a Teflon septum separating the two bath chambers. Formation of the bilayer was monitored optically as well as by capacitance measurement. The resulting bilayers revealed after 30 min a typical capacitance of ~0.5 µF/cm^2 with a peak to peak noise of ~1 pA at 1 kHz bandwidth. The *cis* and *trans* compartments (total volume ~3 ml) were equipped with magnetic stirrers and both Ag/AgCl electrodes were connected by an agar bridge containing 3 M KCl. After a stable bilayer had formed in symmetrical solutions [10 mM HEPES/Tris (pH 7.0), 20 mM KCl], 10 mM $CaCl_2$ and 280 mM KCl (final concentrations) were added to the *cis* compartment. Under vigorous stirring, 5–20 µl of the liposomes were passed through the tip of a microloader so that they slowly moved across the bilayer from the top to the bottom. The procedure was controlled microscopically and, when required, the fusion process was stopped by addition of EDTA. The electrode of the *trans* compartment was directly mounted on the headstage (CV-5B-100G) of a current amplifier (GeneClamp 500, Axon Instruments) and amplified currents were digitized at 48 kHz on a modified DAT recorder (Panasonic SV 3700) and recorded on DAT tapes.

For analysis, current recordings were low-pass filtered with an 8-pole bessel filter, usually at 1–2 kHz, digitized at sampling intervals of 0.2–5 ms, fed into an Axolab 1100 A/D converter (Axon Instruments) and stored in a personal computer. For analysis, a Windows™-based analysis software using the Borland Pascal™ language was developed. This program (SCIP, single-channel investigation program) is based on the mean-variance analysis (Patlak, 1988) and is available on request.

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References

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.

Atkinson,N.S., Robertson,G.A. and Ganetzky,B. (1991) A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science*, **253**, 551–555.

Brown,A.M. (1993) Functional bases for interpreting amino acid sequences of voltage-dependent K^+ channels. *Annu. Rev. Biophys. Biomol. Struct.*, **22**, 173–198.

Choi,K.L., Aldrich,R.W. and Yellen,G. (1991) Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K^+ channels. *Proc. Natl Acad. Sci. USA*, **88**, 5092–5095.

Criado,M. and Keller,B.U. (1987) A membrane fusion strategy for single channel recordings of membranes usually non-accessible to patch-clamp pipette electrodes. *FEBS Lett.*, **224**, 172–176.

Dittrich,W., Betzler,M. and Schrempf,H. (1991) An amplifiable and deletable chloramphenicol-resistance determinant of *Streptomyces lividans* 1326 encodes a putative transmembrane protein. *Mol. Microbiol.*, **5**, 2789–2797.

Dower,W.J., Miller,J.F. and Ragsdale,C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.*, **16**, 6127–6145.

Enz,C., Steinkamp,T. and Wagner,R. (1993) Ion channels in the thylakoid membrane (a patch clamp study). *Biochim. Biophys. Acta*, **1143**, 67–76.

Grupe,A., Schroeter,K.H., Ruppersberg,J.P., Stocker,M., Drewes,T., Beckh,S. and Pongs,O. (1990) Cloning and expression of a human voltage-gated potassium channel. A novel member of the RCK potassium channel family. *EMBO J.*, **9**, 1749–1756.

Hamill,O.P., Marty,A., Neher,E., Sakmann,B. and Sigworth,F.J. (1981) Improved patch clamp technique for high-resolution current recording from cells and cell free patches. *Pfluegers Arch.*, **391**, 85–100.

Hille,B. (1992) *Ionic Channels of Excitable Membranes*. Sinauer Associates Inc., Sunderland, MA.

Ho,K., Nichols,C.G., Lederer,W.J., Lytton,J., Vassilev,P.M., Kanazirska, M.V. and Hebert,S.C. (1993) Cloning and expression of an inwardly rectifying ATP regulated potassium channel. *Nature*, **362**, 31–38.

Hochuli,E., Döbeli,H. and Schacher,A. (1987) New metal chelate adsorbents selective for proteins and peptide containing neighbouring histidine residues. *J. Chromatogr.*, **411**, 177–184.

Hopwood,D.A., Bibb,M.J., Chater,K.F., Kieser,T., Bruton,C.J., Kieser,H.M., Lydiate,D.J., Smith,C.P., Ward,J.M. and Schrempf,H. (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual*. John Innes Foundation, Norwich, UK.

Jan,L.Y. and Jan,Y.N. (1994) Potassium channels and their evolving gates. *Nature*, **371**, 119–122.

Kasahara,M. and Hinkle,P.C. (1977) Reconstitution and purification of the D-glucose transporter from human erythrocytes. *J. Biol. Chem.*, **257**, 7384–7390.

Keller,B.U., Hedrich,R., Vaz,W.L. and Criado,M. (1988) Single channel recordings of reconstituted ion channel proteins: an improved technique. *Pfluegers Arch.*, **411**, 94–100.

Kessler,A., Dittrich,W., Betzler,M. and Schrempf,H. (1989) Cloning and analysis of a deletable tetracycline-resistance determinant of *Streptomyces lividans* 1326. *Mol. Microbiol.*, **3**, 1103–1109.

Kubo,Y., Reuveny,E., Slesinger,P.A., Jan,Y.N. and Jan,L.Y. (1993) Primary structure and functional expression of a rat g-protein-coupled muscarinic potassium channel. *Nature*, **364**, 802–806.

Kutzner,H.J. (1981). The family Streptomycetaceae. In Starr,M.P., Stolp,H., Trüper,H.G., Balows,A. and Schlegel,H. (eds), *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria*. Springer-Verlag, Berlin, pp. 2028–2090.

Kyte,J. and Doolittle,R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Luneau,C., Wiedmann,R., Smith,J.S. and Williams,J.B. (1991) *Shaw*-like rat brain potassium channel cDNA's with divergent 3′ ends. *FEBS Lett.*, **288**, 163–167.

Milkman,R. (1994) An *Escherichia coli* homologue of eucaryotic potassium channel proteins. *Proc. Natl Acad. Sci. USA*, **91**, 3510–3514.

Mueller,P., Rudin,D.O., Tien,H. and Wescott,W.C. (1962) Reconstitution of cell membrane structure *in vitro* and its transformation into an excitable system. *Nature (London)*, **194**, 979–980.

Patlak,B.J. (1988) Sodium channel subconductance levels measured with a new variance-mean analysis. *J. Gen. Physiol.*, **98**, 413–430.

Petrou,S., Ordway,R.W., Kirber,M.T., Dopico,A.M., Hamilton,J.A., Walsh,J.V. and Singer,J.J. (1995) Direct effects of fatty acids and other charged lipids on ion channel activity in smooth muscle cells. *Prostagland. Leuk. Essent. Fatty Acids*, **52**, 173–178.

Pigac,J., Vujaklija,D., Toman,Z., Gamulin,V. and Schrempf,H. (1988) Structural instability of a bifunctional plasmid pZG1 and single-stranded DNA formation in *Streptomyces*. *Plasmid*, **19**, 222–230.

Pongs,O. (1993) Structure-function studies on the pore of potassium channels. *J. Membr. Biol.*, **136**, 1–8.

Pongs,O., Kecskemethy,N., Mueller,R., Krah-Jentgens,I., Baumann,A., Kiltz,H.H., Canal,I., Llamazares,S. and Ferrus,A. (1988) *Shaker* encodes a family of putative potassium channel proteins in the nervous system of *Drosophila*. *EMBO J.*, **7**, 1087–1096.

Rehberg,B., Urban,B.W. and Duch,D.S. (1995) The membrane lipid cholesterol modulates anesthetic actions on a human brain ion channel. *Anesthesiology*, **82**, 749–758.

Salkoff,L., Baker,K., Butler,A., Covarrubias,M., Pak,M.D. and Wei,A. (1992) An essential 'set' of K^+ channels conserved in flies, mice and humans. *Trends Neurosci.*, **15**, 161–166.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

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.

Schroeder,J.I., Ward,J.M. and Gassmann,W. (1994) Perspectives on the physiology and structure of inward-rectifying K^+ channels in higher plants: Biophysical implications for K^+ uptake. *Annu. Rev. Biophys. Biomol. Struct.*, **23**, 441–471.

Schwarz,T.L., Tempel,B.L., Papazian,D.M., Jan,Y.N. and Jan,L.Y. (1988) Multiple potassium channel components are produced by alternative splicing at the *Shaker* locus in *Drosophila*. *Nature*, **331**, 137–142.

Sentenac,H., Bonneaud,N., Minet,M., Lacroute,F., Salmon,J.-M., Gaymard,F. and Grignon,C. (1992) Cloning and expression in yeast of a plant potassium ion transport system. *Science*, **256**, 663–665.

Strong,M., Chandy,K.G. and Gutman,G.A. (1993) Molecular evolution of voltage-sensitive ion channel genes—on the origins of electrical excitability. *Mol. Biol. Evol.*, **10**, 221–242.

Suzuki,M., Takahashi,K., Ikeda,M., Hayakawa,H., Ogawa,A., Kawaguchi,Y. and Sakai,O. (1994) Cloning of a pH-sensitive K^+ channel possessing two trans-membrane segments. *Nature*, **367**, 642–645.

Taglialatela,M., Wible,B.A., Caporaso,R. and Brown,A.M. (1995) Specification of pore properties by the carboxyl terminus of inwardly rectifying K^+ channels. *Science*, **264**, 844–847.

Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.

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