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Functional reconstitution of bacterially expressed human potassium channels in proteoliposomes: membrane potential measurements with JC-1 to assay ion channel activity

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Abstract

Structure-function studies on ion channels have been greatly facilitated by the cloning of cDNAs from a variety of sources. However, obtaining detailed structural information on these proteins requires overexpression, purification and reconstitution in a functionally competent form. In this communication, we report on the functional reconstitution of a human potassium channel, Kv1.4, overexpressed in bacteria. We have assessed the activity of these channels using a spectroscopic assay with a potential-sensitive dye, JC-1. The presence of ion channels renders proteoliposomes selectively permeable to potassium ions as monitored by measurements of transmembrane electrical potential. We have optimised conditions wherein a 12% change in the fluorescence signal of the carbocyanine dye JC-1 per 10 mV change in membrane potential is obtained. Using this assay, we find that the reconstituted protein is potassium selective and its activity is blocked by 4-aminopyridine, a known potassium channel blocker.

Keywords: Potassium channel; Reconstitution; JC-1; Membrane potential; Fluorescence assay

1. Introduction

The report of the first detailed crystal structure of a potassium selective channel from *Streptomyces lividans* demonstrates a paradigm shift towards structural understanding of the mechanisms of membrane transport which was initiated by reconstitution of several membrane proteins [1–3]. Subunits in the tetrameric *S. lividans* channel have pore regions flanked by one transmembrane segment on each side. Shaker type potassium channels are also tetramers with six transmembrane segments and a highly conserved pore region in each subunit. These latter channels are voltage gated and are involved in a variety of cellular functions most spectacularly the action potential in excitable tissue [4].

In principle, monitoring of channel proteins site-specifically labelled with spectroscopic probes can yield real time information on the structural changes these proteins undergo [5]. The absence of a reliable overexpression system has hampered progress in this area. Several members of the Shaker family have been expressed in heterologous eukaryotic overexpression systems. However, expression systems using

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mammalian or insect cell culture are prohibitively expensive methods for the generation of large quantities of functional protein. Unfortunately, bacterial systems, which are the method of choice for many soluble proteins, have not been adequately explored as an expression system for voltage-gated ion channels from eukaryotes. Generation of non-functional channels either due to non-glycosylation or formation of inclusion bodies has been a major deterrent.

The human potassium channel hKv1.4 (HuKII) [6] is a member of the Shaker family. We overexpressed ΔN50hKv1.4, a truncated version of hKv1.4 in bacteria, using a T7 polymerase based system. Although the protein was directed into inclusion bodies, we were able to solubilise and reconstitute it into artificial vesicles. Functionality of the protein was assayed by monitoring transmembrane potentials in the reconstituted proteoliposomes. The presence of an ion selective conductance should lead to Nernstian potentials which we have assayed using the potential-sensitive probe, JC-1. This dye has been widely used to monitor mitochondrial membrane potential changes [7]. The potential sensitivity of the dye arises from the ability of the dye to form highly fluorescent aggregates with spectral properties distinct from the monomer. We have monitored an aggregate of the dye which responds with a 12% change in fluorescence per 10 mV change in membrane potential. The reconstituted protein has been shown to be selective for K^+ over Na^+ and its conductance demonstrated to be sensitive to 4-aminopyridine, a blocker of Shaker type potassium channels.

2. Materials and methods

5′,5′,6,6′-Tetrachloro-1,1,3,3′-tetraethylbenzimadazolylcarbocyanine iodide (JC-1) was obtained from Molecular Probes. All the other fine chemicals were obtained from Sigma.

2.1. Expression and partial purification of human Kv1.4

The ΔN50hKv1.4 cDNA was generated by deleting about 900 base pairs from the 5′ end of the human Kv1.4 cDNA leaving about 50 amino acid residues upstream from the first hydrophobic segment [6]. ΔN50hKv1.4 was subcloned into pET 20b (Novagen, USA) under a T7 promoter with the start site in frame with a 22 residue *pel B* leader sequence. Capped RNA was transcribed in vitro from this construct using the mMessage mMachine kit (Ambion, USA) and injected into *Xenopus* oocytes. Currents were measured with a two-electrode voltage-clamp amplifier, OC-725 (Warner Instruments, USA).

Ten independent single colonies were picked from a plate containing *Escherichia coli* BL21(*DE3*)*pLysS* transformed with ΔN50hKv1.4. Each colony was individually inoculated into 1 l Rich medium and allowed to grow at 37°C till the optical density at 600 nm reached 0.5–0.6. Expression was then induced with 1 mM IPTG for 3 h. Cells were harvested and aliquots monitored by SDS-PAGE for expression of an induced band at approx. 43 kDa. Cell pellets from expressing cultures were frozen at −20°C till further processed.

The pellet corresponding to 500 ml of culture was resuspended in 10 ml Buffer I (20 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 1 mM dithiothreitol (DTT). RNase A (10 μg/ml), DNase I (10 μg/ml), 10 mM EGTA, $MgCl_2$ (2 mM) and 1 mM phenylmethylsulphonyl fluoride (PMSF) were added, cells disrupted in a French Press and incubated on ice for 15 min. DNase I was inhibited by adding EDTA to a final concentration of 5 mM and centrifuged at $27\,000 \times g$ for 15 min at 4°C. The pellet was resuspended in 1 ml Buffer I and fractionated by centrifugation at $131\,000 \times g$ for 4.5 h at 4°C, through a step gradient made of 4.0 ml of 40%, 5.0 ml of 53% and 2 ml of 70% (w/v) solutions of sucrose dissolved in Buffer I. The inclusion body fraction formed a band at the interface between the 53% and 70% sucrose solutions. The banded material was washed twice with 10 ml each of Buffer II (0.2 mg/ml lysozyme, 1 mM EDTA and 1 mg/ml deoxycholate and 0.2 M phosphate buffer, pH 7.4) and centrifuged at $12\,000 \times g$ for 15 min at 4°C.

The resulting pellet was dissolved in 20 ml Buffer III (8 M urea, 1% CHAPS and 0.1 M sodium phosphate, pH 7.4) using Polytron (Kinematica, Switzerland) followed by rotary mixing in a cell mixer for 1 h. The redissolved fraction was spun at $100\,000 \times g$ for 1 h and the supernatant concentrated to 1 ml using Centricon (Amicon, USA) with 30 kDa cut off. The buffer was exchanged to 3 ml of Buffer IV

(1% CHAPS, 2 mg/ml of azolecithin, 1 mM DTT, 5 mM HEPES, pH 8.0 and 150 mM KCl) by gel filtration. Protein concentration was determined by a modified Lowry method. 300 μl of the protein sample (150 μg/ml) was added to 3 ml of reconstitution buffer (1.5% CHAPS, 35 mg/ml of soy lecithin, 5 mM HEPES, pH 8.0, 150 mM KCl and 1 mM EDTA) and dialysed against 400 ml of 150 mM KCl, 5 mM HEPES, pH 8.0. for 48 h with six changes of dialysis buffer at 4°C. Inclusion body fractions from non-expressing cultures had vanishingly small quantities of protein. A mock reconstitution was set up containing all the contents of the reconstitution buffer except protein. The reconstituted protein samples were aliquotted, flash frozen in liquid nitrogen and stored at −80°C for later use.

2.2. Calibration of JC-1 response

Small unilamellar vesicles (SUV) were prepared in Buffer V (143 mM KCl, 7 mM NaCl, 5 mM HEPES, pH 8.0) following the procedure described earlier [8]. The lipid concentration was estimated as described by New [9]. Liposomes were resuspended in Buffer VI (143 mM NaCl, 7 mM KCl and 5 mM HEPES, pH 8.0) and 20 nM valinomycin. JC-1 was added to a final concentration of 8 μM and allowed to equilibrate. The presence of valinomycin clamps the transmembrane potential to −60 mV. Thus, by varying $[K^+]_{ext}$ using KCl the liposomes can be clamped at different membrane potentials [10].

2.3. Measurement of membrane potential to assay ion channel activity

Frozen proteoliposomes were thawed and added to SUVs and the suspension subjected to three freeze thaw cycles. The lipid-protein mixture thus generated was sonicated and centrifuged at 100 000×*g* for 1 h. The top one-third fraction which is rich in small unilamellar vesicles was used for the assay. JC-1 was added to proteoliposomes and allowed to equilibrate against a 10× gradient of potassium (150 mM internal:15 mM external). Dye responses were recorded in response to changes in $[K^+]_{ext}$ achieved by adding KCl to the buffer. 4-Aminopyridine was included in the reconstitution mixture and in the resuspending medium at 1 mM when used.

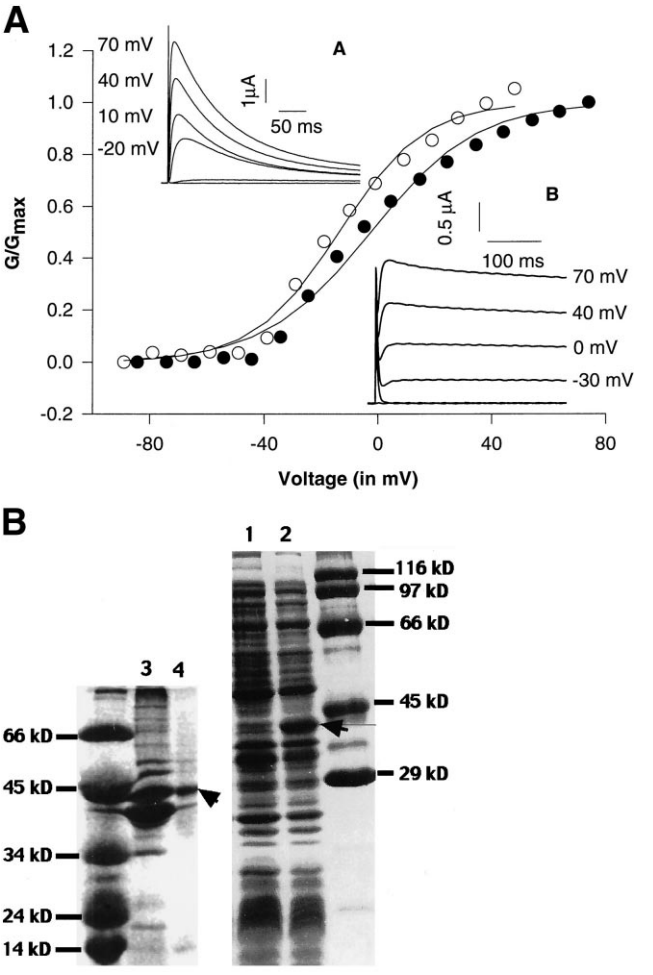

Fig. 1. Expression of human K^+ channel (Kv1.4) in *Xenopus* oocytes and bacteria. (A) Voltage dependence of conductance. Representative plots of full length hKv1.4 (filled circles) and ΔN50hKv1.4 (open circles). Relative conductances were obtained by dividing the conductance at the indicated potentials by the maximum observed conductance. The conductance was determined from the equation $G = I/(V - V_K)$, assuming a V_K of −80 mV. The insets show the macroscopic outward currents of hKv1.4 (inset A) and ΔN50hKv1.4 (inset B). The membrane potential was held at −80 mV followed by a 1 s hyperpolarising prepulse to −120 mV, and then stepped to test potentials as indicated against the traces. (B) Expression and partial purification of ΔN50hKv1.4 monitored by SDS-PAGE (12%). Total cell protein before (lane 1) and after induction (lane 2) with 1 mM IPTG for 3 h. The cell pellets were lysed by boiling at 100°C for 2 min after resuspending in 1×SDS-PAGE gel loading buffer. The washed inclusion body pellet was resolubilised in buffer containing 8 M urea and 1% CHAPS. Lane 3, TCA precipitate of 1/5th of the insoluble fraction. Lane 4, TCA precipitate of 1/200th of the total solubilised fraction. The arrowhead indicates the induced band. The gel was stained with Coomassie brilliant blue R-250.

3. Results

3.1. Expression and reconstitution of human Kv1.4 channels

A construct lacking the first 254 residues from the N terminus of human Kv1.4 (ΔN50hKv1.4) was generated using PCR based mutagenesis. The mutant channels lack fast inactivation but have the same voltage sensitivity as the wild type channels (Fig. 1A and inset).

We had previously made unsuccessful attempts to overexpress the full length channel. The deletion of 254 residues resulted in adequate expression possibly due to a reduction in size of the expressed protein. A further benefit of the deletion is the elimination of inactivation which is essential for the membrane potential based assay we have adopted (see Section 4).

ΔN50hKv1.4 was cloned into the T7 expression vector pET 20b and introduced into the *BL21(DE3)-pLysS* strain of *E. coli*. Inducing expression with IPTG results in the appearance of a 43 kDa band on SDS-PAGE (Fig. 1B, lane 1). Often, only one flask out of ten inoculated would have strongly expressing cultures. We estimate that the induced band amounts to 5–20 mg per litre of an expressing culture. Though the expression system is very unstable, the yields obtained are more than the maximum reported in any other system [2,11]. The protein is, however, localised in inclusion bodies and is absent from the membrane or soluble fractions (data not shown). The protein could be solubilised in 8 M urea containing 1% CHAPS in phosphate buffer in relatively pure form (Fig. 1B, lane 3). We estimate that the protein accounts for at least 80% of the inclusion body extract. Urea was removed by gel filtration through a column equilibrated with detergent and soy lecithin. The solubilised protein was reconstituted into soy lecithin liposomes by dialysis of a suspension of protein in 1.5% CHAPS and 35 mg/ml soy lecithin against a 150 mM KCl and 15 mM HEPES buffer over a period of 48 h. Faster dialysis or rapid removal of detergent by gel filtration resulted in loss of viable protein. The reconstituted protein was assayed by a novel method based on detecting membrane potential changes.

Table 1
The spectroscopic properties of JC-1

Mode	Peak I (nm)	Peak II (nm)	CJC (μM)
Absorption[a]			
A	520	–	0.3
B	500	590	
Emission[a]			
A	525	–	0.6
B	525 (0.003)	590 (−0.2)	
Synchronous[a]			
A	530	–	0.4
B	530	590 (0.012)	
Absorption[b]			
A	520	–	–
B	520	590	
Emission[b]			
A	535	–	0.8
B	535	596	
Synchronous[b]			
A	540	–	1.0
B	535	595	

Peak I at low concentration, below CJC (A), is mainly monomers and a combination of H-aggregates and monomers at high concentration above CJC (B). Peak II represents the J-aggregate peak. Emission spectra are collected in the range of 500–600 nm with excitation at 490 nm. Synchronous scan spectra are collected from 500 to 600 nm with 15 nm difference in the emission and excitation monochromater. [a] represents data collected in aqueous buffer and [b] represents data collected in the presence of lipids. The numbers in parentheses indicate the anisotropy values.

3.2. Membrane potential changes probed with JC-1

Cyanine dyes have been reported to respond to changes in membrane potential by redistributing across the membrane. At high concentrations, they form two types of aggregates: the H- and J-aggregates whose spectroscopic characteristics have been well studied [12,13]. We have reinvestigated absorption and emission characteristics of JC-1 as function of concentration and have summarised the results in Table 1. Our results on the J-aggregate are broadly in agreement with those of Reers et al. but they do not mention the presence of H-aggregates. The high quantum yield of J-aggregates has been used to report on mitochondrial membrane potential in cells and in isolated mitochondria [14,15].

J-Aggregates absorb at a lower energy than the molecular solutions of cyanine dyes and they exhibit resonance fluorescence (very little Stokes shift) [13]. The mitochondrial membrane potential measure-

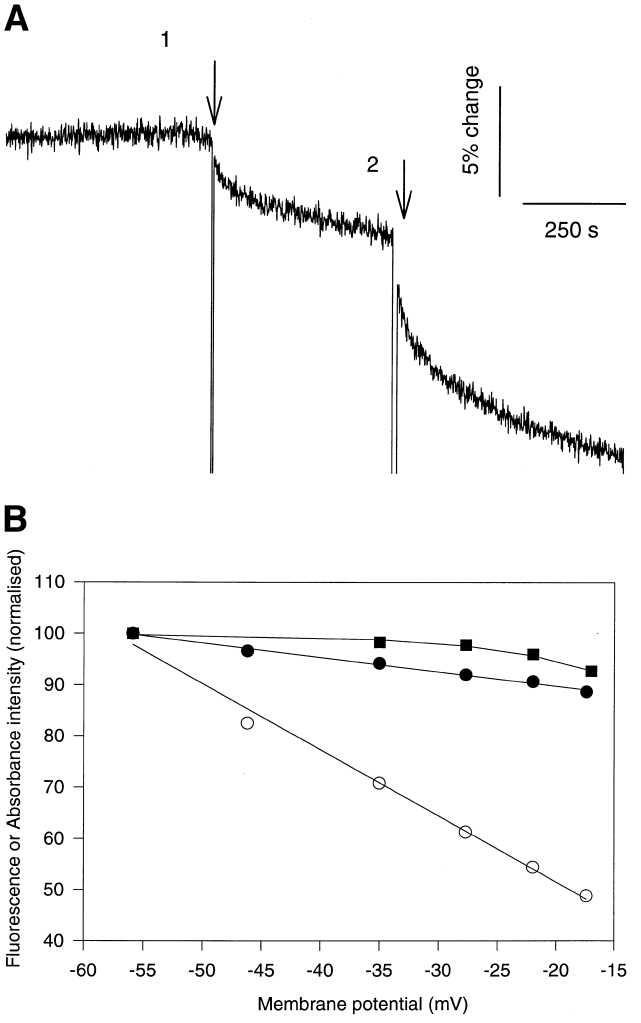

Fig. 2. JC-1 as a membrane potential indicator in liposomes. (A) Dependence of the J-aggregate on the membrane potential. The intravesicular buffer composition was 143 mM KCl, 7 mM NaCl, 5 mM HEPES, pH 8.0. The medium buffer composition was 14 mM KCl, 143.5 mM NaCl, 5 mM HEPES, pH 8.0. The total lipid concentration in the cuvette was 10 μg/ml and the dye concentration was 6 μM. Valinomycin was present to 20 nM. JC-1 fluorescence intensity (λ_{ex} = 580 nm and λ_{em} = 595 nm) decreases as the membrane potential is depolarised. The arrow indicates addition of KCl which changes the membrane potential to (1) −55 mV and (2) −49 mV (a baseline drift was subtracted from the traces). (B) Variation of the normalised fluorescence intensity of the J-aggregate, λ_{em} = 595 nm, with membrane potential. (a) λ_{ex} = 580 nm (filled circles), (b) λ_{ex} = 490 nm (open circles), and (c) absorbance at 590 nm (filled squares) normalised to maximum absorbance.

ments monitor the 590 nm J-aggregate peak excited at 500 nm. We have made anisotropy measurements to understand the nature of this chromophore. A large negative anisotropy value of −0.2 of the J-aggregate peak excited at 500 nm is diagnostic of a right angle between the excitation and the emission dipole [16]. This rules out energy transfer to the J-aggregate from any other chromophore as that would have led to randomisation of orientation and hence depolarisation of fluorescence. In the presence of lipid, the CJC shifts to higher concentrations indicative of monomers partitioning into bilayers.

Transmembrane electrical potentials established in liposomes using valinomycin and a 10-fold gradient of K^+ can be collapsed by adding K^+ to the external medium. Fig. 2A shows the variation in J-aggregate fluorescence on adding KCl. No response was seen on adding KCl to liposomes in the absence of valinomycin nor did valinomycin elicit a response on addition to liposomes with a symmetrical K^+ distribution (data not shown). Fig. 2B presents the emission and absorption responses of the J-aggregate as a function of membrane potential. The 500 nm excitation exhibits a 3% change in intensity per 10 mV change in potential while the resonance fluorescence intensity changes approx. 12% per 10 mV.

3.3. Ion channel activity monitored with JC-1

Liposomes containing functional K^+ channels should be selectively permeable to K^+. Changes in gradients of permeant ions should result in changes in membrane potential which should be reflected in changes in dye fluorescence. Liposomes were prepared in buffer containing 150 mM KCl and resuspended in buffer containing 15 mM KCl. K^+ permeable liposomes should have a membrane potential of about −60 mV at this point. Addition of KCl to the medium collapses the gradient, reducing the transmembrane electrical potential. Fig. 3A shows that the fluorescence of J-aggregates in proteoliposomes reconstituted with ΔN50hKv1.4 decreased on addition of external K^+ as expected while control liposomes not containing the protein did not respond to such additions. If the liposomes were selectively permeable to K^+, the membrane potential should be predicted by the Nernst equation. The Nernst equation specifies a linear relationship between $\Delta\Psi$ and $\log[K^+]_{ext}$. $\Delta\Psi$ varies linearly with $\Delta F/F$ (Fig. 2B). A plot of $\Delta F/F$ against $\log[K^+]_{ext}$ is linear (Fig. 3B) suggesting that the liposomes are selectively permeable to potassium.

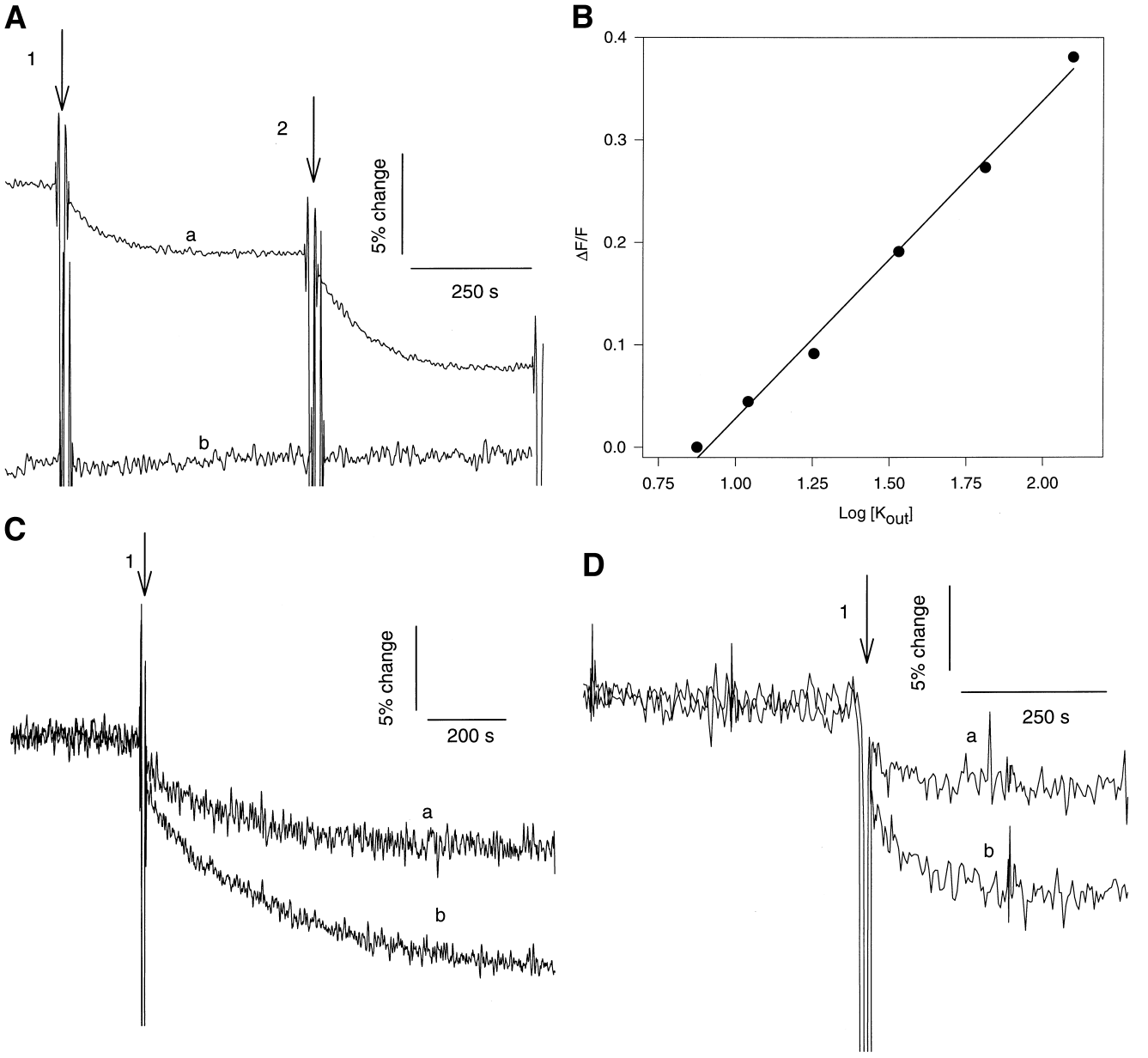

Fig. 3. Assay of ΔN50hKv1.4 reconstituted into soy lecithin liposomes monitored with JC-1 aggregate fluorescence $\lambda_{ex} = 580$ nm; $\lambda_{em} = 595$ nm. (A) Fluorescence intensity changes in (a) reconstituted and (b) control vesicles in response to KCl additions. KCl addition at arrow 1 to medium $[K^+]_{ext} = 27$ mM and arrow 2 to 67 mM. The intravesicular concentration is 150 mM. (B) Variation in normalised fluorescence intensity with external K^+ concentration. (C) Effect of 4-aminopyridine. Fluorescence intensity changes in vesicles (a) containing 1 mM 4-AP or (b) absence of 4-AP. KCl was added at the arrow to raise $[K^+]_{ext}$ to 47 mM. (D) Ion specificity of fluorescence response. (a) NaCl added to 40 mM outside; (b) KCl added to 47 mM outside.

Selectivity of liposomes for K^+ was tested by varying the external Na^+ while keeping $[K^+]_{ext}$ constant. Fig. 3C shows that while variation in $[K^+]_{ext}$ resulted in large fluorescence changes, variation in $[Na^+]_{ext}$ had a much smaller effect on dye fluorescence. Addition of 40 mM $[K^+]$ should lower $\Delta\Psi$ from -60 mV to -30 mV and results in a 10% reduction in JC-1 fluorescence. Addition of 40 mM Na^+ to the external medium should result in very large change in membrane potential if the vesicles had a significant permeability for this ion. The observed fluorescence change is only 5% indicating that the permeability

for Na^+ is much less than for K^+. It may be noted that voltage-gated K^+ channels have a 30:1 selectivity for K^+ over Na^+.

4-Aminopyridine (4-AP) blocks hKv1.4 with an IC_{50} of 0.8 mM [6] and ΔN50hKv1.4 is also sensitive to the blocker in electrophysiological experiments (data not shown). Addition of 1 mM 4-AP to the assay mixture reduced the dye response by 50% as seen in Fig. 3D. This implies that 50% of the liposomes that were previously permeable to K^+ are no longer permeable. Had there been more than one functional channel in a liposome this concentration of 4-AP would block only half those channels leaving the liposome still selectively permeable to K^+. The observed efficacy of the blocker therefore requires that most liposomes contain no more than one functional channel.

4. Discussion

In order to carry out structural studies, it is essential that large amounts of functionally active proteins be reconstituted into artificial membranes free of contaminating proteins. Since purification from tissue has resulted in low yields of a mixture of potassium channels, heterologous overexpression in systems lacking such proteins is the approach of choice [17]. Bacterial overexpression has the advantages of low cost and ease of making mutant proteins. As in many other instances, overexpression of ΔN50hKv1.4 in bacteria results in protein being directed to inclusion bodies. The washed inclusion body could be resolubilised using 8 M urea and 1% CHAPS and the protein reconstituted in a functionally active form. To the best of our knowledge, the only previous report of a eukaryotic membrane transport protein being functionally reconstituted from inclusion bodies is that of the bovine mitochondrial oxoglutarate carrier [18].

Aggregation of the carbocyanine dye JC-1 has been used to monitor mitochondrial membrane potentials with higher signal-to-noise ratios than was possible using the monomer alone [15]. The J-aggregate peak can be excited either at 590 nm or 490 nm. The former is ascribed to the resonance fluorescence and the latter has been used by Reers et al. to monitor membrane potential changes. The anisotropy of −0.2 of the 490 nm excitation peak requires that excitation and emission dipoles be orthogonal to each other and is thus inconsistent with an energy transfer mechanism. The $S_2 \leftarrow S_0$ transition dipole is known to be orthogonal to the $S_0 \leftarrow S_1$ transition dipole in systems such as the indole chromophore of tryptophan [16]. We propose that excitation at 490 nm results in excitation to S_2 followed by internal conversion to S_1 and emission therefrom. Absorption would then be at higher energy than the $S_1 \leftarrow S_0$ transition of the J-aggregate, while the short lifetime and the long correlation time of the aggregate would ensure that there was no rotation of the chromophore before emission. Orthogonality of the transition dipoles would then ensure that the observed fluorescence anisotropy was −0.2.

Valinomycin imposed, negative-inside membrane potentials result in concentration of JC-1 in the intravesicular space as the dye is positively charged and membrane permeant. At the bulk concentrations used, the dye will aggregate in the intravesicular space to form intensely fluorescent J-aggregates whose concentration then reflects the magnitude of the imposed potential. Changes in the intensity of J-aggregate fluorescence would then be limited not by the rate of change in membrane potential but rather by dye transport across the membrane and subsequent aggregation-disaggregation reactions. The actual time course may depend to a large extent on the composition of the membrane as differences are seen between lipid-only membranes (valinomycin experiments, Fig. 2A) and proteoliposomes while mitochondria respond somewhat faster than either [15].

Monitoring membrane potentials as an assay for ion transport requires that the transporter be functional at the potentials used. At the time of reconstitution, intravesicular and extravesicular solutions are the same (150 mM K^+) and no transmembrane electrical potentials arise. On reducing external K^+ to 15 mM, a potential can be generated only if the transporter is active at 0 mV. ΔN50hKv1.4 is open at 0 mV but closed at −60 mV (Fig. 1A). It is likely that there are as many inside out channels as right side out channels and the former would experience a potential corresponding to +60 mV in this situation. An additional complication would be introduced by channel inactivation. ΔN50hKv1.4 does not, however, undergo rapid inactivation of the type observed

in hKv1.4. It should be noted that the protein would not be glycosylated in *E. coli* whereas the native Shaker type channels have been shown to be glycosylated. These channels have, however, been shown to be functional in the absence of glycosylation [19]. The demonstration of K^+ selective permeability in ΔN50hKv1.4 reconstituted liposomes establishes that these channels are in the open state at 0 mV. Similar reconstitutions carried out with mutant channels that are normally closed at 0 mV should allow characterisation of the channels in the closed state.

Functional reconstitution of ion channels into liposomal systems is an essential step in the process of characterising these proteins spectroscopically. Once reconstitution has been carried out it is necessary to establish that the proteins are selectively permeable and that they retain voltage sensitivity. At reconstitution stoichiometries of at least one functional channel per liposome, membrane potential measurements can be used to estimate selectivity of the reconstituted protein. We have demonstrated that variations in transmembrane K^+ gradients affect the electrical potentials far more than equivalent variations in Na^+ gradients reflecting the selectivity of the reconstituted protein. Had every liposome in the sample contained at least one functional channel the measured membrane potential should have been predicted by the Nernst equation for the permeant ion.

The membrane potential of the liposomes is established very rapidly when the channels are open because movement of a few ions will be sufficient to charge up the membrane capacitance. But when these channels are non-conducting the membrane potential should decay back to zero since there is no concentration gradient. As a result, even in case of channels which open and close over the course of an experiment, dye response will settle to a steady state depending both on the mean open time of the channels and the membrane potential. Hence, reversible blockers like 4-AP should decrease their mean open time thus setting a new steady state with a lower amplitude for the dye response. The mean open time is potential dependent. However, for symmetric reconstitutions of channels with conductance voltage profiles like ΔN50hKv1.4, decreases in mean open time of one population of channels (say, right side out) will be compensated for by increases in the other population (inside out).

Most investigations of reconstituted proteins will involve an average signal from the entire sample population. It is thus essential to estimate the fraction of the reconstituted protein that is functionally active. We have demonstrated that the K^+ selective permeability can be blocked by 4-AP, a known blocker of the Kv1 family of channels. Titration with such blockers should yield information on the number of conducting channels in the population provided three of at least one functional channel per liposome could be achieved. With our reconstitution stoichiometry of one monomer per five liposomes, the fraction of liposomes containing at least four monomers is less than 5%. At concentrations of 4-AP comparable to IC_{50} reported with hKv1.4, we observe a 50% decrease in JC-1 response indicating that nearly all liposomes that exhibit K^+ permeability can be blocked by 4-AP.

Ligand binding assays which have been used for quantitating functional reconstitution rely on the assumption that the presence of a ligand binding site necessarily correlates with channel function [1,20,21]. On the other hand, direct measurements of ion flux using radioactive tracers are cumbersome and inconvenient to use for establishing selectivity [22]. Fusion of proteoliposomes to planar membranes allows electrophysiological characterisation of the protein [2]. This combined with 'Nystatin spike analysis' to monitor vesicle fusion would be a good method for estimating the fraction of vesicles containing functional channels [23]. Electrophysiological characterisation by patch-clamping giant liposomes is a possibility that has been explored [24]. This approach has been used to study single channels in patches excised from giant liposomes. However, technical limitations have precluded the widespread use of either of these approaches to characterising reconstituted ion channels. In the meantime, the use of JC-1 under the conditions described here joins the arsenal of assays available to practitioners of the art of channel reconstitution.

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