Basolateral localisation of KCNQ1 potassium channels in MDCK cells: molecular identification of an N-terminal targeting motif

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Summary
KCNQ1 potassium channels are expressed in many epithelial tissues as well as in the heart. In epithelia KCNQ1 channels play an important role in salt and water transport and the channel has been reported to be located apically in some cell types and basolaterally in others. Here we show that KCNQ1 channels are located basolaterally when expressed in polarised MDCK cells. The basolateral localisation of KCNQ1 is not affected by co-expression of any of the five KCNE β-subunits. We characterise two independent basolateral sorting signals present in the N-terminal tail of KCNQ1. Mutation of the tyrosine residue at position 51 resulted in a non-polarized steady-state distribution of the channel. The importance of tyrosine 51 in basolateral localisation was emphasized by the fact that a short peptide comprising this tyrosine was able to redirect the p75 neurotrophin receptor, an otherwise apically located protein, to the basolateral plasma membrane. Furthermore, a di-leucine-like motif at residues 38–40 (LEL) was found to affect the basolateral localisation of KCNQ1. Mutation of these two leucines resulted in a primarily intracellular localisation of the channel.

Key words: KCNQ1, KCNE, MDCK cells, Confocal microscopy, Subcellular distribution

Introduction
The voltage-gated potassium channel KCNQ1 plays a key role in different physiological functions. KCNQ1 channels are made up of four α-subunits, each containing six transmembrane segments, a pore loop constituting the central part of the pore holding the potassium selectivity filter and intracellular N- and C-termini. KCNQ1 channels associate with all five members of the KCNE β-subunit family, resulting in a β-subunit-specific change of the current characteristics (Angelo et al., 2002; Barhanin et al., 1996; Grunnet et al., 2002; Nicolas et al., 2001; Sanguinetti et al., 1996; Schroeder et al., 2000; Tinel et al., 2000; Warth et al., 2002). KCNQ1 channels have been characterised in several epithelial tissues, where they are involved in water and salt transport, as well as in cardiac myocytes, in which the KCNQ1 channel in complex with the KCNE1 β-subunit (minK) is partly responsible for terminating the cardiac action potential (Barhanin et al., 1996; Sanguinetti et al., 1996). The physiological importance of KCNQ1 channels is emphasized by studies of patients with mutations in KCNQ1 and by studies of KCNQ1 knockout mice, showing several functional defects (Lee et al., 2000; Wang et al., 1996). Cardiac arrhythmias and hearing loss in humans have been correlated with KCNQ1 and KCNE1 mutations, and KCNQ1 knockout mice show complete deafness, imbalance problems as well as gastrointestinal abnormalities. In addition, studies of knockout mice lacking KCNE1, which in several organs is associated with KCNQ1, reveal signs of chronic volume depletion associated with urinary and fecal salt wasting, lower plasma K+ concentration and an enhanced level of aldosterone, indicating that KCNQ1/KCNE1 channel complexes have important functions in the epithelial-rich tissues such as colon and kidney (Arrighi et al., 2001; Vallon et al., 2001; Warth and Barhanin, 2002).

Epithelial cells are polarised cells with an apical membrane facing the lumen, the mucosal side, and a basolateral membrane facing the interstitium, the serosal side. Tight junctions separate the two membrane domains. In epithelial cells many membrane-bound proteins are selectively located at either the apical or the basolateral membrane (Yeaman et al., 1999). The specific localisation to the basolateral side is obtained by the presence of basolateral sorting signals in the cytoplasmic tail(s). Most of the known basolateral sorting signals can be grouped into two classes (Heilker et al., 1999; Yeaman et al., 1999). The first class is characterised by an essential tyrosine either as a part of an NPXY-motif (where X is any amino acid) or as a part of a YXXΦ-motif (where Φ is a bulky hydrophobic residue), and the second class contains a di-leucine (LL) sequence. Both classes of sorting signals have in several cases been found to induce both endocytosis and basolateral sorting (Heilker et al., 1999).

Whether a given epithelial protein is located at the apical, the basolateral, or both surfaces is of crucial physiological
importance. Interestingly, KCNQ1 has been reported to be located apically in some epithelial cell types and basolaterally in others. KCNQ1 is located at the apical surface together with KCNE1 in stria vascularis in cochlea as well as in the proximal tubule of the kidney (Nicolas et al., 2001; Vallon et al., 2001). In the stomach KCNQ1 proteins are located in the apical plasma membrane of the parietal cells and at the basolateral membrane in the foveolar cells (Dedeck and Waldegger, 2001; Grahemmer et al., 2001a). Furthermore, KCNQ1 proteins have been found in the basolateral membrane in exocrine pancreas, in trachea epithelial cells and in the crypt cells of colon and small intestine (Dedeck and Waldegger, 2001; Grahemmer et al., 2001b; Warth et al., 2002).

Two different studies of the KCNE1 knockout mouse, in which vestibular dark cells and pancreatic acini were investigated, revealed an altered subcellular localisation of KCNQ1 (Nicolas et al., 2001; Warth et al., 2002). KCNQ β-subunits are in several epithelial tissues expressed together with KCNQ1 channels (Dedeck and Waldegger, 2001; Demolombe et al., 2001; Nicolas et al., 2001; Schroeder et al., 2000; Vallon et al., 2001). Based on the results from the KCNE1 knockout mice and the fact that KCNQ1 is located either apically or basolaterally depending on the cell type, it was suggested that KCNE β-subunits contain signals responsible for the subcellular localisation of KCNQ1/KCNE complexes (Warth et al., 2002). Such a function of the KCNE β-subunit would lead to a heterogenous subcellular localisation of KCNQ1 depending on which KCNE β-subunit is expressed in the cell.

In the present study, we have examined the subcellular localisation of KCNQ1 channels expressed in the MDCK cell line, which is widely used as a model for protein trafficking in polarised epithelial cells (Ikonen and Simons, 1998). We find that KCNQ1 channel proteins are located at the basolateral membrane of MDCK and that this localisation is not influenced by the co-expression of KCNE1-5. Furthermore, we have identified two sorting signals in the N-terminal part of the KCNQ1 protein important for basolateral localisation; an LXL-motif at position 38-40 and a tyrosine-based motif at position 51.

Materials and Methods
Molecular biology
Expression of human KCNQ1 (hKCNQ1) was obtained by the use of plasmid pIRES-KCNQ1 (Grunnet et al., 2002). hKCNQ1, hKCN2, mKCN3 and mKCN4 cDNA were inserted upstream of the internal ribosome entry site (IRES) element in pIRES-KCNQ1. By placing the two cDNAs in the same transcription unit, the mRNA for both genes will always be present in the transfected cells (pKCN1/2/3/4/5-IRES-KCNQ1). Translation of KCNQ1-4 is obtained by ordinary cap-scanning mechanism, whereas translation of KCNQ1 is achieved by placing an IRES from EMCV just upstream of the start codon (Morgan et al., 1992). For KCNQ1 and KCNQ5 co-expression experiments a co-transfection with human pIRES-KCNQ1 and human pHZOOM-KCNE5 was performed. The KCNQ1 deletion and substitution mutants (Δ20-KCNQ1, Δ20-40-KCNQ1, Δ40-KCNQ1, Δ47-KCNQ1, A80-KCNQ1, A95-KCNQ1, 2xL-A, Y51A, Y111A, and Y111C) were subcloned (BamHI/NotI) into pHZOOM, which provides efficient expression in both mammalian cells and Xenopus laevis (Jespersen et al., 2002). The carboxy-terminal deleted p75NTR (p75*, kind gift of Andre Le Bivic) was PCR amplified and subcloned into the BamHI and HindIII sites of pHZOOM. Immediately before the start-codon a Kozak sequence was introduced. The construct contains only five intracellular residues. The fragment of 17 aa including tyrosine 51 (wild-type or Y51A) was introduced onto the carboxy-terminal end of p75* by PCR (the sequence of the two peptides defined by oligonucleotides) and subcloned into the BamHI and HindIII sites of pHZOOM, thus generating p75*-Q1(Y51) and p75*-Q1(Y51A). All PCRs were performed with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) under buffer conditions as proposed by the supplier, supplemented with 4% betaine (Sigma), with initial denaturation at 97°C for 3 minutes, followed by 15 rounds of denaturation at 97°C, 30 seconds, annealing at 50°C, 30 seconds, and elongation at 68°C, 1 minute 20 seconds. The integrity of all constructs was confirmed by sequencing.

cRNA preparation and capping for oocyte injection were performed by in vitro transcription using the mCAP mRNA capping kit (Stratagene, La Jolla, CA, USA). mRNA was phenol/chloroform extracted, ethanol precipitated and dissolved in TE buffer to a concentration of approximately 1 µg/µl. The integrity of the transcripts was confirmed by agarose gel electrophoresis, and mRNA was stored at –80°C until injection.

Transient expression in MDCK cells and Xenopus oocytes
MDCK (strain II) cells were grown in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies) at 37°C, in 5% CO₂. Three hours prior to transfection, 3×10⁵ cells were plated in a cell culture T25 flask (Nunc). Cells were transfected with 2 µg of plasmid, using Lipofectamine and Plus Reagent (Life Technologies) according to the manufacturer’s instructions. Electrophysiological studies were performed 48-72 hours post-transfection on cells replated on glass coverslips (3.5 mm in diameter, VWR International, Albertslund, Denmark).

Xenopus laevis surgery and oocyte treatment were done as previously described (Grunnet et al., 2001). Xenopus oocytes were collected under anaesthesia. These experiments were approved by the Danish National Committee for Animal Studies. Oocytes were kept in Kulori medium consisting of (in mM) 90 NaCl, 1 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, pH 7.4, for 24 hours at 19°C before injection of 50 nl of mRNA (approximately 5 ng). All injections were performed using a Nanoject microinjector (Drummond Scientific, Broomall, PA). Oocytes were kept at 19°C in Kulori medium for 2-7 days before measurements were performed. The Kulori medium was changed once a day.

Electrophysiology
MDCK cells
Transfected cells were identified by co-expression of EGFP, which is included in the pIRES vector (Grunnet et al., 2002). Only single isolated cells were chosen. Experiments were performed in whole-cell patch-clamp configuration at room temperature with an EPC-9 amplifier (HEKA Electronics, Lambrcht, Germany). Pipettes were pulled from thin-walled borosilicate glass on a horizontal patch electrode puller (DMZ universal puller, Zeitz Instruments, München, Germany) and had a resistance of between 1.5 and 2.5 MΩ. A custom-made perfusion chamber (volume: 15 µl) with a fixed AgCl-Ag pellet electrode was mounted on the stage of an inverted microscope. Coverslips with MDCK cells were transferred to the perfusion chamber and superfused with physiological solutions consisting of (in mM) 150 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂ and 10 HEPES (pH 7.4 with NaOH). Pipettes were filled with solutions consisting of (in mM) 144 KCl, 10 EGTA and 10 HEPES (pH 7.2 with KOH). CaCl₂ and MgCl₂ were added in concentrations calculated (EqCal, BioSoft, Cambridge, UK) to give a free Mg²⁺ concentration of 1 mM and free Ca²⁺ concentrations of 100 nM. Cell capacitance and series resistance was updated before each pulse application. Series resistance values were between 2.5 and 10.0 MΩ, and only experiments in which the resistance remained constant during the experiments were analysed. Current signals were low-pass filtered at 3 kHz and acquired using Pulse software (HEKA).
All steps were performed at 4°C. Just prior to use, the membrane-cell surface biotinylation and chase fluorescent markers. expressed EGFP was quenched, allowing double-labelling with green (Molecular Probes). Under these experimental conditions the co-filter. After washing, the filters were mounted in Prolong Antifade for 1 hour in primary antibodies diluted in 0.05% saponin/0.125% gelatin/saponin/0.25% fish skin gelatin in PBS. The cells were then incubated with 0.05% paraformaldehyde in PBS for 30 minutes at room temperature. At this point the filters were cut out and placed in a humid chamber on parafilm.

Oocytes

Current through expressed KCNQ1 channels was recorded using a two-electrode voltage-clamp amplifier (Dagan CA-1B, Minneapolis, MN, USA). Electrodes were pulled from borosilicate glass capillaries on a horizontal patch electrode puller (DMZ universal puller, Zeitz Instruments) and had tip resistance between 0.3 and 2.0 MΩ when filled with 1 M KCl. During the experiments oocytes were placed in a small chamber (volume: 200 µl) connected to a continuous flow system (flow: 6 ml/min). KCNQ1 channels were activated by membrane depolarisation and channel activity was measured in Klori solution. All experiments were performed at room temperature. The condition of each single oocyte was controlled before measurements by recording membrane potentials. Only oocytes with membrane potentials below ~40 mV were used for current recordings. An exception was oocytes co-expressing Y111A-KCNQ1 and Y111C-KCNQ1 where a consistent change to slightly more depolarised membrane potentials was observed. For these experiments only oocytes with membrane potential below ~20 mV were used for current recordings. Measurements were performed 3-4 days after injection from at least two batches of oocytes.

Immunofluorescence

Transfected cells (3×10⁴) were plated on permeable membranes (0.4 µm pore size) using Transwell chambers (Costar Europe, Badhoevedorp, The Netherlands) 18-30 hours after transfection. Three days after plating, the MDCK cells were washed with PBS (composition in mM: 136 NaCl, 2.5 KCl, 1.5 KH₂PO₄, 6.5 NaHPO₄, pH 7.4) and then fixed with 3% paraformaldehyde in PBS for 30 minutes at room temperature. At this point the filters were cut out and placed in a humid chamber on parafilm. Quenching was performed by a 30-minute incubation with 0.05% saponin/0.25% fish skin gelatin in PBS. The cells were then incubated for 1 hour in primary antibodies diluted in 0.05% saponin/0.125% gelatin in PBS (wash buffer). Secondary antibodies and Alexa Fluor 488-conjugated streptavidin (Molecular Probes) were diluted in wash buffer and applied for 45 minutes. Antibodies were applied to both sides of the filter. After washing, the filters were mounted in Prolong Antifade (Molecular Probes). Under these experimental conditions the co-expressed EGFP was quenched, allowing double-labelling with green fluorescent markers.

Cell surface biotinylation and chase

All steps were performed at 4°C. Just prior to use, the membrane-impermeable sulfo-3-(biotinamido)ethyl-1,3-dithiopropionate (EZ-Link™ NHS-SS-biotin, Pierce) was dissolved in PBS++ (PBS containing 1 mM CaCl₂ and 1 mM MgCl₂) to a final concentration of 1 mg/ml. After washing twice in PBS++, transfected MDCK cells were incubated for 30 minutes in the biotin solution applied either basolaterally or apically. The cells were subsequently washed three times for five minutes in PBS++ containing 100 mM glycine to quench the residual biotin. A part of the cells was then subjected to a chase of 1 hour at 37°C in a 5% CO₂ atmosphere.

MesNa treatment

This procedure was applied to cleave off sulfo-NHS-biotin moieties present on the cell surface proteins. It was performed as previously described (Rasmussen et al., 2002) with slight modifications. All steps were performed on ice. After biotinylation and chase (see above) cells were washed twice in NT-buffer (20 mM N-(-3-(hydroxymethyl)-2-aminoethanesulfonic acid (TAPS), pH 8.6, 150 mM NaCl, 1 mM EDTA, 2 µg/ml BSA). Cells were subsequently incubated for 1 hour in freshly prepared 10 mM MesNa (2-mercaptoethanesulfonic acid) in NT-buffer. Freshly dissolved MesNa was added after 20 and 40 minutes of incubation. Finally, cells were washed three times in NT-buffer, followed by two washes in PBS before fixation in 3% paraformaldehyde in PBS.

Confocal microscopy and imaging

Laser scanning confocal microscopy was performed using the Leica TCS SP2 system equipped with argon and helium-neon lasers. The objective was 63×, NA 1.2. For double-labeling experiments sequential scanning was employed to allow the separation of signals from the two channels. Images were treated using Adobe Photoshop 5.5 and Adobe Illustrator 9.0.

Results

Wild-type KCNQ1 channels are primarily located at the basolateral plasma membrane of MDCK cells

In order to investigate the steady-state distribution of KCNQ1 channels, MDCK cells were transiently transfected with an expression vector carrying the KCNQ1 cDNA and grown to confluency. The subcellular localisation of the KCNQ1 protein was determined using a KCNQ1 specific antibody and examined by confocal microscopy (Fig. 1). KCNQ1 channels were observed almost exclusively in the basolateral membrane, co-localising with biotin surface labelling from the basolateral side. Biotin labelling of the basolateral plasma membranes was included for two reasons: first, to verify the polarisation of the MDCK cells, and second to visualise the membranes. To validate that the KCNQ1 channels were functional and present in the cell membrane when expressed in MDCK cells, whole-cell patch-clamp electrophysiology was performed (Fig. 1B). As expected, KCNQ1-expressing cells showed a slowly activating voltage-dependent current at potentials more positive than ~40 mV. The obtained current could be blocked by 100 µM XE991, a specific blocker of KCNQ currents (Zaczek et al., 1998). No KCNQ1 current (XE991-sensitive current) was detected in non-transfected cells (data not shown).

KCNE1-5 β-subunits do not alter the subcellular localisation of KCNQ1

Several reports suggest that KCNE β-subunits associate with KCNQ1 and affect the subcellular localisation of
KCNQ1/KCNE complexes in several epithelial tissues. This hypothesis was analysed using the polarised MDCK cells for co-expression of KCNQ1 and each of the five KCNE β-subunits (Fig. 2). To ensure co-expression of the KCNE proteins together with the KCNQ1 protein in all the transfected cells, bicistronic expression vectors were used, except for the co-expression of KCNQ1 and KCNE5, where a co-transfection of the two plasmids was performed (see Materials and Methods). The subcellular localisation of KCNQ1 was determined as described above. In MDCK cells expressing all combinations of KCNE1-5 and KCNQ1, basolateral staining of KCNQ1 was observed.

Expression of KCNE1-5 β-subunits and the KCNQ1 α-subunit in transfected MDCK cells was analysed by electrophysiology (Fig. 2B). Whole-cell patch-clamp analysis was done as described for KCNQ1 alone, except for KCNQ1/KCNE5 where the depolarising pulses were extended to +140 mV. All KCNE subunits modulate the KCNQ1 current in MDCK cells, thus verifying expression of both KCNQ1 and KCNE proteins in the same cell. The currents could all be blocked by 100 μM XE991, confirming that the currents were KCNQ-specific. KCNQ1/KCNE1-expressing MDCK cells showed a slowly activating voltage-dependent current, which was activated at potentials more positive than –20 mV (811 pA±437 pA at the end of the +60 mV pulse, n=4) in agreement with previous reports of the Ik current (Barhanin et al., 1996; Sanguinetti et al., 1996). Co-expression of KCNQ1 and KCNE2 resulted in channel complexes constitutively open at negative potentials and partly inactivating currents at positive potentials (269 pA±46 pA at +60 mV, n=5). These characteristics result in a linear I-V curve at negative potentials as previously reported in HEK293 and COS-7 cells (Chen et al., 2002; Dedek and Waldegger, 2001; Tinel et al., 2000). KCNQ3 provides the KCNQ1 channel with fast activation and deactivation kinetics and renders the I-V relationship very similar to that of KCNQ1/KCNE1 (1400 pA±495 pA at +60 mV, n=5). However, KCNQ1/KCNE3 currents have a very reduced tail current at –30 mV. The characteristics of the present KCNQ1/KCNE3 currents are different from those observed in Xenopus oocytes (Grunnet et al., 2002; Schroeder et al., 2000), but are in agreement with data from Chinese hamster ovary (CHO) cells (Mazhari et al., 2002). KCNE4 blocks the KCNQ1 current (61 pA±31 pA at +60 mV, n=3), as previously shown for both oocytes and HEK293 cells by our laboratory (Grunnet et al., 2002). KCNQ1/KCNE5 channels open at very positive potentials, and voltage steps up to 140 mV were therefore applied in the KCNQ1/KCNE5 experiments. A slowly activating current with a very right-shifted I-V curve was found in KCNQ1/KCNE5-expressing MDCK cells (83 pA±1 pA at +60 mV, 703 pA±140 pA at +140 mV, n=4), similar to previous observations in CHO cells (Angelo et al., 2002). Because expression of KCNE1-5 together with KCNQ1 in all cases modulated the KCNQ1 current, this strongly suggests direct interaction between KCNQ1 and each of the KCNE β-subunits.

Amino acids located between residues 20 and 40 in the N-terminal of KCNQ1 are important for the steady-state membrane localisation

To investigate whether basolateral sorting signals are present in the N-terminal portion of KCNQ1, five different truncation/deletion mutants and four transition mutants were constructed. Fig. 3 shows the N-terminal sequence of wild-type KCNQ1 until the first transmembrane segment, together with indications of the residues deleted/altered in the constructed mutants. The truncation mutants Δ20-KCNQ1, Δ40-KCNQ1, Δ47-KCNQ1, and Δ80-KCNQ1, as well as sKvLQT1 (Sanguinetti et al., 1996), were analysed in Xenopus laevis oocytes in order to verify correct folding and assembly of the proteins. Two-electrode voltage-clamp experiments showed specific, but reduced, KCNQ1 current, with kinetics resembling KCNQ1, of all the above-mentioned truncation mutants and sKvLQT1, strongly suggesting correct folding of
Basolateral localisation of KCNQ1

The proteins (data not shown). MDCK cells expressing Δ20-KCNQ1, Δ40-KCNQ1, Δ47-KCNQ1, and Δ80-KCNQ1 were subsequently analysed for the subcellular distribution of KCNQ1 as described above (Fig. 4A). The truncation mutant Δ20-KCNQ1 shows basolateral membrane staining similar to the one observed for wild-type (wt)-KCNQ1 channels, indicating that no sorting signals are located between residues 1-20. In contrast to this, Δ40-KCNQ1-transfected MDCK cells show a predominantly intracellular location of the protein, suggesting that signals important for the basolateral localisation are present between residues 20-40. MDCK cells transfected with Δ47-KCNQ1 and Δ80-KCNQ1 displayed an intracellular staining pattern similar to Δ40-KCNQ1 (data not shown). A construct in which the amino acids from residue 20 to residue 40 were deleted was also investigated. This construct gave rise to a protein with a similar localisation to Δ40-KCNQ1 (data not shown).

As the N-terminal part of the KCNQ1 protein is implicated in the subcellular distribution of KCNQ1, we were interested in investigating the cellular localisation of the KCNQ1 variant cloned by Sanguinetti and co-workers (Sanguinetti et al., 1996) and later named sKvLQT1 (Fig. 3) (Schmitt et al., 2000). The majority of sKvLQT1 staining in polarised MDCK cells is observed intracellularly with a staining pattern similar to Δ40-KCNQ1 (data not shown). To investigate whether the KCNE1 β-subunit could promote a more efficient membrane localisation, co-expression of sKvLQT1 and KCNE1 was performed (Fig. 4B, parts B1-2). However, KCNE1 expression did not alter the localisation of sKvLQT1 as the transfected cells also have the majority of sKvLQT1 staining located intracellularly.

A di-leucine-like motif is important for correct localisation of the KCNQ1 channel. Residues 38 to 40 in wt-KCNQ1 channels form a LEL (Leu-
Glu-Leu) sequence resembling a di-leucine signal reported to be important for basolateral targeting and internalisation (Yeaman et al., 1999). In order to investigate whether the LEL sequence affects the steady-state localisation of the KCNQ1 protein, the two leucines were mutated into alanines giving rise to a 2xL-A-KCNQ1 construct (Fig. 3). The 2xL-A-KCNQ1 protein had a mainly intracellular distribution similar to D40-KCNQ1, D47-KCNQ1, Δ80-KCNQ1, Δ20-40-KCNQ1, and sKvLQT1 (Fig. 5A, Fig. 5B, parts a,d). To analyse which intracellular compartment 2xL-A-KCNQ1 proteins are located in, double-labelling experiments with antibodies directed against PDI, a marker of the endoplasmic reticulum, and TGN-38, a marker of the trans-Golgi compartment, were performed (Fig. 5A). The mutant 2xL-A-KCNQ1 was not co-localised with either of these two proteins, indicating that 2xL-A-KCNQ1 is not retained in the exocytotic pathway.

To further analyse the subcellular localisation of 2xL-A-KCNQ1, a 1-hour chase of biotinylated plasma membrane proteins was performed (Fig. 5B). The basolateral membranes were biotinylated at time zero and the cells were cultured for another hour at 37°C. Before fixation, biotin residues present at the plasma membrane were cleaved off with MesNa (see Materials and Methods). After a 1-hour chase, 2xL-A-KCNQ1 proteins partly co-localise with endocytosed biotinylated proteins (Fig. 5B, part f), indicating that the proteins are located in endosomal compartments. Together, these observations strongly suggest that the majority of 2xL-A-KCNQ1 proteins are located in endosomal compartments.

The role of N-terminal tyrosine residues in basolateral targeting and protein folding

Having established that the di-leucine-like sequence is an important motif for subcellular localisation, we focused on the potential role of tyrosine residues in the N-terminal sequence for the steady-state localisation to the basolateral membrane. Two YXXΦ-motifs are present in the N-terminal tail of KCNQ1 (Fig. 3). The tyrosine residues at position 51 and 111 in these two potential basolateral targeting motifs were mutated into alanines and the localisation of the proteins analysed as described above. The tyrosine residue at position 111 was an interesting candidate, as a Y111C mutation has been described in a patient with long QT syndrome (Splawski et al., 2000). As illustrated, Y111A-KCNQ1 protein was located intracellularly when expressed in MDCK cells (Fig. 6a-c). KCNQ1 with a mutation similar to the one described in the human LQT patient (Y111C) had a similar subcellular distribution as Y111A-KCNQ1 (data not shown).

In contrast to Y111A/C-KCNQ1, Y51A-KCNQ1 is localised to the plasma membrane (Fig. 6d-f). However, the Y51A-KCNQ1 mutant displayed a non-polarized steady-state distribution and was detected in both the apical membrane (Fig. 6e) and in the basolateral plasma membrane (Fig. 6d). Hence, in contrast to wild-type KCNQ1 and all the other mutants tested, Y51A-KCNQ1 is present in the apical membrane. Thus, the tyrosine residue at position 51 plays an important role in the basolateral plasma membrane localisation of KCNQ1 channels.

The electrophysiological properties of the
Basolateral localisation of KCNQ1 transition mutants, 2xL-A-KCNQ1, Y51A-KCNQ1, Y111A-KCNQ1 and Y111C-KCNQ1, were analysed in both MDCK cells and Xenopus oocytes. The results obtained with oocytes were similar to the results obtained with MDCK cells and are therefore not shown. In MDCK cells, both Y51A-KCNQ1 and 2xL-A-KCNQ1 gave rise to current kinetics resembling the wt-KCNQ1 current, confirming correct folding of the proteins, and that the mutated amino acids do not play a role for KCNQ1-gating kinetics (Fig. 7A). The average current level for Y51A-KCNQ1 was not significantly different from wt-KCNQ1 channels, whereas 2xL-A-KCNQ1-expressing cells provided a third of the wild-type current level (Fig. 7B). No KCNQ1 current could be measured in Y111A-KCNQ1- and Y111C-KCNQ1-expressing MDCK cells.

A fragment of 17 residues comprising Y51 can function as a dominant basolateral targeting signal

After establishing that the Y51 residue plays an important role in basolateral targeting of KCNQ1, we analysed whether this residue, present in the context of the YXXΦ motif, is sufficient to redirect the otherwise apically located neurotrophin receptor p75NTR. The apical sorting of p75NTR has previously been shown to involve the O-glycosylated stalk domain, which is located in the extracellular domain of the protein (Yeaman et al., 1997). A truncated version of p75NTR, lacking almost the entire intracellular domain, has previously
tyrosine was replaced by an alanine [p75*-Q1(Y51A)], a preferential apical localisation, similar to the p75* localisation, was found. These results show that the fragment of 17 residues, comprising Y51, contains a basolateral targeting signal which is sufficient to redirect an otherwise apically located protein. Furthermore, the silencing of the basolateral localisation of the engrafted fragment by mutating tyrosine 51 into an alanine confirms that this residue plays a pivotal role in the basolateral targeting.

**Discussion**

KCNQ1 potassium channels are expressed in several epithelial tissues where they participate in transepithelial transport of salt and water. KCNQ1 channels are selectively located in the apical or the basolateral membrane of different epithelial cells (Dedek and Waldegger, 2001; Grahammer et al., 2001a; Grahammer et al., 2001b; Warth et al., 2002). We have used the polarised epithelial MDCK cell line to investigate molecular determinants involved in the subcellular localisation of KCNQ1 channels.

When KCNQ1 channels are expressed in polarised MDCK cells a strict basolateral localisation is observed. Only lateral staining was detected, an observation that can probably be attributed to the permeable membranes the cells were grown on as also the biotin staining from the basolateral side was restricted to the lateral membranes. The presence of functional channel complexes at the cell surface was confirmed by patch-clamp electrophysiology. It has previously been suggested that the KCNE β-subunits may carry the determinants for the subcellular localisation of the KCNQ1/KCNE complex (Warth et al., 2002). However, when either of the KCNE1-5 β-subunits were co-expressed with KCNQ1 in MDCK cells, a clear basolateral localisation, indistinguishable from that of KCNQ1 alone, was observed. This observation suggests that the KCNE β-subunits do not determine the subcellular localisation of KCNQ1. The currents obtained from the MDCK cells expressing KCNE1-5 together with KCNQ1 were similar to the currents reported in other mammalian cells. The present study thus confirms that KCNE β-subunits modulate the electrophysiological properties of the KCNQ1 channels, but it also demonstrates that the β-subunits are not involved in localisation of the channels to the apical or basolateral plasma membranes. Endogenous expression of KCNE proteins in MDCK cells has not been directly investigated in this study, but the electrophysiological data from the transfected cells indicate that endogenous expression of KCNE proteins is, at most, low. If an endogenous KCNE β-subunit had been expressed at significant levels, modulation of the KCNQ1 current in cells solely expressing KCNQ1 proteins would have been expected.

Having excluded the contribution of β-subunits in targeting of KCNQ1 channels, the focus was directed towards internal KCNQ1 motifs. The residues involved in membrane localisation and basolateral targeting of the KCNQ1 protein were analysed by N-terminal truncations and substitutions. The truncation mutants revealed that residues between amino acid 20 and 40 in the N-terminal tail of KCNQ1 are important for the steady-state localisation to the basolateral plasma membrane. A di-leucine-like motif is located within these 20 residues. In several cases di-leucine motifs have been shown...
to be signals for basolateral sorting and internalisation (Heilker et al., 1999). When altering the two leucines to alanines, the KCNQ1 protein forms functional channels in both oocytes and MDCK cells, suggesting correct folding of the protein. However, the current level of 2xL-A-KCNQ1 channels was decreased to approximately one-third of wild-type level. This observation corresponds well with the fact that 2xL-A-KCNQ1 was primarily located intracellularly. In order to establish the subcellular localisation of intracellular KCNQ1 proteins with a mutated di-leucine motif, stainings of intracellular compartments were performed. Co-stainings of 2xL-A-KCNQ1 and markers of the endoplasmic reticulum and Golgi apparatus revealed a very low degree of overlapping localisation, indicating that 2xL-A-KCNQ1 proteins do not accumulate in exocytotic compartments. In contrast, 2xL-A-KCNQ1 was found to co-localise with biotinylated proteins endocyotosed from the basolateral membrane, demonstrating that the 2xL-A-KCNQ1 proteins are present in endosomal compartments. The fact that 2xL-A-KCNQ1 proteins form functional channels, are present in the exocytotic pathway in a very low amount, and are found in endocytotic compartments indicates that the di-leucine motif is important for steady-state membrane localisation.

Another group of potential basolateral sorting signals are tyrosines as a part of a YXXΦ motif (Heilker et al., 1999). The tyrosine residue in position 51 in the N-terminal tail of KCNQ1 is potentially a part of such a YXXΦ motif and was therefore mutated into an alanine. The KCNQ1 proteins with a neutralised tyrosine (Y51A) are located at both the apical and basolateral plasma membranes. Electrophysiological measurements in MDCK cells and Xenopus oocytes expressing Y51A-KCNQ1 revealed a current amplitude and kinetics similar to wt-KCNQ1 channels. To confirm that tyrosine 51 is important for the steady-state localisation of KCNQ1 to the basolateral membrane, we engrafted a 17-residue peptide containing Y51 and the flanking residues into p75NTR. p75NTR is normally located at the apical membrane. However, the chimeric protein had a clear basolateral localisation, showing that this short peptide can function as a dominant basolateral localisation signal. Within the 17 amino acid peptide the only obvious localisation signal is the Y51XXIΦ, and indeed, when the tyrosine was mutated into an alanine a predominantly apical localisation was detected, confirming the crucial role of this residue. Thus, the Y51 residue is important in the steady-state localisation of KCNQ1 channels in the basolateral plasma membrane.

The basolateral sorting signals LXL and Y51 identified in this study are conserved in human, rat and mouse, indicating that their functions have been retained through the evolution of mammals.

Mutations in the KCNQ1 gene have been found to cause long QT syndrome (Barhanin et al., 1996; Sanguinetti et al., 1996). Long QT syndrome is characterised by a prolonged QT interval in the cardiac action potential. This prolongation is because of decreased outward potassium current as KCNQ1/KCNE1 is partly responsible for repolarising the cardiac myocytes. A mutation of tyrosine 111 into a cysteine was described in a long QT patient (Spawlski et al., 2000). Mutating this tyrosine into an alanine or a cysteine (Y111A-KCNQ1 and Y111C-KCNQ1) resulted in an intracellular localisation of these proteins. This observation is further supported by the fact that the Y111A and Y111C proteins produced no current in MDCK cells. The present investigation supports the idea that when analysing the clinical manifestations in patients suffering from long QT syndrome a wider perspective is needed (Attali, 2002). KCNQ1 channels are important delayed rectifier channels in the heart, but these channels do also have an important function in epithelia in controlling the potassium homeostasis. Hence, the cardiac arrhythmias observed in long QT patients are probably not only caused by a reduced function of KCNQ1 channels in the heart but also by a reduced function in epithelia.

In summary, we have found that KCNQ1 channels are located in the basolateral plasma membrane of MDCK cells. Co-expression of KCNE β-subunits together with KCNQ1 did not affect the steady-state localisation of KCNQ1. A tyrosine-containing motif at position 51 is crucial for the KCNQ1 targeting to the basolateral membrane, whereas a di-leucine-like motif at position 38-40 is important for the steady-state localisation of KCNQ1 in the surface membrane.

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