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ORIGINAL ARTICLE

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KCNE1 reverses the response of the human K^+ channel KCNQ1 to cytosolic pH changes and alters its pharmacology and sensitivity to temperature

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Abstract Previous studies have shown that heteromultimeric KCNQ1/KCNE1 (KvLQT1/minK) channels and homomultimeric KCNQ1 (KvLQT1) channels exhibit different current properties, e.g. distinct kinetics and different sensitivities to drugs. In this study we report on the divergent responses to internal pH changes and further characterize some of the current properties of the human isoforms of KCNQ1 and KCNE1 expressed in Chinese hamster ovary (CHO) cells or *Xenopus laevis* oocytes. Decreasing the bath temperature from 37 °C to 20 °C increased the half-activation time by a factor of 5 for KCNQ1/KCNE1 currents (I_{Ks}) but by only twofold (not significant) for KCNQ1 currents (I_K) in CHO cells. Acidification of cytosolic pH (pH_i) increased I_{Ks} but decreased I_K whereas intracellular alkalinization decreased I_{Ks} but increased I_K. pH_i-induced changes in intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) did not correlate with the current responses. At 20 °C mefenamic acid (0.1 mM) significantly augmented I_{Ks} but slightly decreased I_K. It changed the slow activation kinetics of I_{Ks} to an instantaneous onset. The form of the current/voltage (*I*/*V*) curve changed from sigmoidal to almost linear. In contrast, at 37 °C, mefenamic acid also increased I_{Ks} but slowed the activation kinetics and shifted the voltage activation to more hyperpolarized values without markedly affecting the sigmoidal shape of the *I*/*V* curve. The potassium channel blockers clotrimazole and tetrapentylammonium (TPeA) inhibited I_{Ks} with a lower potency than I_K. These results show that coexpression of KCNE1 reversed pH regulation of KCNQ1 from inhibition to activation by acidic pH_i. In addition, KCNE1 altered the pharmacological properties and sensitivity to temperature of KCNQ1. The pH-dependence of I_{Ks} might be of clinical and pathophysiological relevance in the pathogenesis of ischaemic cardiac arrhythmias.

Keywords KvLQT1 · minK · Temperature · Clotrimazole · Tetrapentylammonium · Mefenamic acid

Introduction

The K^+ channel KCNQ1 can either form homomeric channels or coassemble with the regulatory proteins KCNE1 or KCNE3 to form heteromeric channel complexes [1, 33, 34]. These K^+ channels are crucial for the function of many organs [37]. They play an important role in epithelia [4], providing the driving force for Cl^- secretion. In the colon, KCNQ1 associates with KCNE3, in the pancreas and inner ear it interacts with KCNE1 [23, 28, 29, 34]. KCNQ1/KCNE1 channels underlie the cardiac I_{Ks} current [39] which is responsible for repolarization of cardiac myocytes during the action potential. Mutations in either KCNQ1 or KCNE1 cause one type of the inherited long QT syndrome [14, 16, 36, 38, 41].

Previous studies have shown that KCNQ1 and KCNQ1/KCNE1 channels exhibit distinct properties. Both channels produce a voltage-dependent K^+ current that is activated by depolarization. The current through KCNQ1/KCNE1 channels, however, activates much more slowly than that through KCNQ1 homomers and more depolarized clamp voltages are required for half-maximal current activation. KCNQ1/KCNE1 channels, moreover, are more sensitive than KCNQ1 channels to the blockers 293B, azimilide, 17-β-oestradiol and to the channel activator mefenamic acid [13]. The aim of this study was to characterize further these two channels. We examined the effects of temperature, pH_i, clotrimazole, tetrapentylammonium (TPeA) and mefenamic acid.

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Materials and methods

Cell culture and stable transfection of CHO-K1 cells

CHO-K1 cells (American Type Culture Collection, Washington, D.C., USA) were cultured in minimum essential medium (MEM) alpha supplemented with 80 g/l fetal calf serum and penicillin/streptomycin (10 g/l) at 5% CO_2 and 95% air at 37 °C. CHO-K1 cells were transfected with pcDNA3.1-hKCNQ1 using *N*-(1-[2,3-dioleoyloxy]propyl)-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) according to the instructions of the distributor (Boehringer Mannheim, Mannheim, Germany). Cells were grown on 60-mm tissue culture dishes and incubated for 24 h with a mixture of the plasmid and DOTAP. After the incubation period the incubation mixture was aspirated and replaced by a medium containing 250 µg/ml Geneticin (G418, Life Technologies, Karlsruhe, Germany). CHO-K1 cells stably expressing hKCNQ1 and hKCNE1 (pcDNA3.1-hKCNQ1 plus pcDNA3.1zeo-hKCNE1) as well as the plasmid pcDNA3.1-hKCNQ1 were kindly provided by Dr. H.W. Jansen (Aventis, Frankfurt, Germany). The plasmids used for transfection encoded the full-length sequences of human KCNQ1, isoform 1 [44] and hKCNE1. Cells were grown to confluency, trypsinized and seeded as single cells on 60-mm culture dishes. Clones were selected 5–7 days after seeding and were shown to contain the respective messenger ribonucleic acids (mRNAs) by reverse-transcription polymerase chain reaction (RT-PCR) and electrophysiological analysis. Antibiotic selection [250 µg/ml Geneticin or 250 µg/ml Geneticin plus 250 µg/ml Zeocin (Invitrogen, San Diego, Calif., USA)] was continued till the day of the experiment.

Patch-clamp recordings from CHO cells

The standard patch-clamp techniques with the modifications implemented by ourselves have been described in detail [19]. The pipette capacitance, series resistance and cell capacitance were cancelled by the compensation circuit of the amplifier (U. Fröbe and R. Busche, this institute) with the help of a sine wave command voltage [22]. Only experiments with constant series resistance values were accepted for data analysis. A flowing (1 M) KCl electrode served as a bath reference to minimize liquid junction potentials. The tip resistance of the patch pipettes used was 2–3 MΩ, seal resistance ≥1 GΩ. Experiments were performed at 20 °C or 37 °C, as indicated in the Results. Two voltage-clamp protocols were used. To obtain current/voltage (*I*/*V*) relationships cells were clamped to a holding potential of –80 mV, depolarized with 10-mV increments and hyperpolarized to –80 mV after each depolarizing step. The pulse duration was 2 s. For the alternative clamp protocol the membrane potential was changed between –80 mV and 0 mV every 2 s. For the experiments at 20 °C the duration of the voltage-clamp pulses was prolonged to 4 s. The data were low-pass filtered at 10 kHz and stored on DAT tape (digitalisation rate 48 kHz). Analysis was performed from the online record or with patch-clamp software written by U. Fröbe (this institute).

The patch pipettes were filled with the following solution (mM): K-gluconate 95; KCl 30; NaH_2PO_4 1.2; Na_2HPO_4 4.8; Ca-gluconate 0.73; $MgCl_2$ 2.4; 1,2-bis(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 1; D-glucose 5; adenosine 5′-triphosphate (ATP) 3; pH 7.2. The standard bath solution, called control solution from hereon, contained (in mM): NaCl 145; K_2HPO_4 1.6; KH_2PO_4 0.4; Ca-gluconate 1.3; $MgCl_2$ 1; D-glucose 5. In the solutions containing buffer systems, 20 mM NaCl was replaced by 20 mM NH_4Cl, 20 mM Na-acetate or 20 mM trimethylamine hydrochloride (TriMA) to keep the osmolarity constant. In the solution containing bicarbonate, 25 mM NaCl was replaced by $NaHCO_3$. It was kept at 37 °C and gassed with 95% O_2 and 5% CO_2. The pH of all solutions was adjusted to 7.4. All chemicals used were of highest grade of purity and purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). *Trans*-6-cyano-4-(*N*-ethylsulphonyl-*N*-methylamino)-3-hydroxy-2,2-dimethylchromane (293B) was the kind gift of Aventis.

Fluorescence measurements of pH_i and $[Ca^{2+}]_i$ in CHO cells

The experiments were performed as described in detail elsewhere [31, 32]. In brief, CHO cells were loaded at room temperature for 40–60 min with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethylester (BCECF/AM, 30 µM) or 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranoyloxy]-2-(2-amino 5-methylphenoxy) ethane-N,N,N′N′-tetraacetic acid, autoxy methylester (fura-2/AM, 20 µM). Pluronic F127 (80 µM) was added to improve dye loading. During the experiments cells were superfused continuously with control solution at 37 °C at a bath solution exchange rate of 1 Hz. The emission signal was collected from an average of between six and eight cells at a time resolution of 10 Hz. BCECF was excited at 436 nm and 488 nm. Emission was recorded between 515 and 565 nm and the 488 nm/436 nm ratio calculated. The ratio signal was calibrated in pH units using nigericin (40 µM) and high-K^+ buffer solutions (145 mM) of defined pH (6.5; 7.0; 7.5; 8.0). Fura-2 was excited at 340, 360 and 380 nm. The emission was recorded at 500–530 nm and the emission ratio 340 nm/380 nm served as a measure of $[Ca^{2+}]_i$. Calibration of the ratio (*R*) as described in [18, 31] was not successful because no reliable maximum (R_{max}) values could be obtained in CHO cells. For qualitative comparison, peak ratios induced by the store depleting agonist ATP (0.1 mM) are given in Table 1.

Expression of hKCNE1 and hKCNQ1 in *Xenopus laevis* oocytes

The methods for isolation, microinjection and double voltage clamp of *Xenopus* oocytes have been described previously [20, 27]. In brief, the oocytes were isolated from adult female *Xenopus laevis* frogs (H. Kähler, Bedarf für Entwicklungsbiologie, Hamburg, Germany) and dispersed and defolliculated by a 0.5-h treatment with collagenase type A (Boehringer Mannheim). Afterwards the oocytes were rinsed and kept at 18 °C in an ND96-buffer containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 5, Na-pyruvate 2.5. The solution was adjusted to pH 7.55 and supplemented with theophylline (0.5 mM) and gentamycin (5 mg/l). For complementary RNA (cRNA) synthesis the plasmid pSP64-hKCNQ1 (kindly provided by M. Keating and M. Sanguinetti, University of Utah, USA) was linearised with EcoRI and transcribed in vitro using Sp6 polymerase and a 5′-cap (mCAP mRNA capping kit, Stratagene, La Jolla, Calif., USA). *NotI*, T7 polymerase and a 5′-cap were used for linearisation and in vitro transcription of pBS-hKCNE1 (kindly provided by A.E. Busch, Aventis). Using a pneumatic pico-pump (PV830, WPI, Germany), oocytes of identical batches were injected each with 10 ng of hKCNQ1-cRNA alone or together with 10 ng hKCNE1-cRNA dissolved in 50 nl H_2O. Experiments were performed 3–4 days after cRNA injection. The bath solution contained (in mM): NaCl 96; KCl 2; $CaCl_2$ 1.8; $MgCl_2$ 1; HEPES 5; Na-pyruvate 2.5 and the pH was 7.55. For the butyrate solution 10 mM NaCl was replaced by 10 mM Na-butyrate and pH was readjusted to 7.55. The oocytes were clamped to a holding potential of –80 mV and depolarized to 0 mV for 5 s every 10 s. Experiments in *Xenopus* oocytes were performed at room temperature.

Data analysis

Currents were analysed as absolute values (total current) or after subtraction of the non-specific baseline current, as indicated in the Results. Currents induced by KCNQ1 expression are referred to as I_K, currents induced by the expression of KCNQ1/KCNE1 are referred to as I_{Ks}. Time dependence of the currents was determined using a Levenberg-Marquardt algorithm (Origin 5.0, Microcal Software, Northampton, Mass., USA). The voltage dependence of current activation was determined by fitting the normalized *I*/*V* relationship by a Boltzmann function: $I = I_{max} + (I_{min} - I_{max})/1 + e^{(V_m - V_{1/2})/k}$, where V_m is the membrane voltage, $V_{1/2}$ the voltage needed for half-maximal activation of the current and *k* the slope factor. Concen-

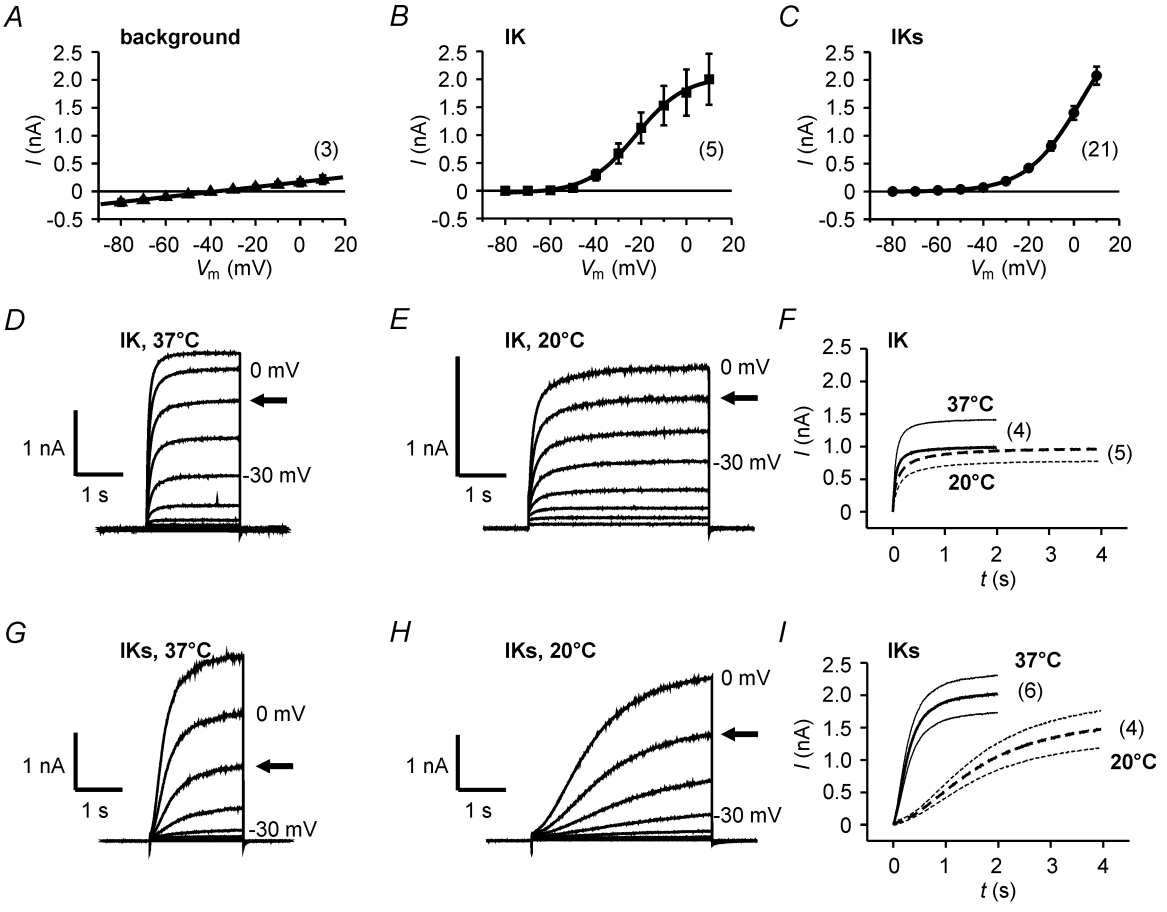

Fig. 1A–I Temperature dependence of K^+ channel currents I_K and I_{Ks}. **A–C** Current/voltage (*I*/*V*) relationship of total currents recorded in non-transfected Chinese hamster ovary (CHO) cells (**A**) and in cells transfected with KCNQ1 (**B**) or KCNQ1/KCNE1 (**C**) channel proteins (V_m membrane voltage). **D–I** Temperature dependence of total currents of CHO cells expressing KCNQ1 (**D–F**, unpaired experiments) and KCNQ1/KCNE1 (**G–I**, unpaired experiments) at 37 °C (**D**, **G**) and 20 °C (**E**, **H**), respectively. Currents were driven by voltage clamp pulses from –80 mV in +10-mV increments. *Arrows* indicate the currents at a V_m of –10 mV used for the calculation of mean currents (±SEM, *thin lines*) at 37 °C (*continuous line*) and at 20 °C (*broken line*), (**F**, **I**). The *numbers* in *parentheses* indicate the numbers of experiments

tration/response curves were described by a Hill function: $I_{max}x^n/(IC_{50}^n+x^n)$ where IC_{50} is the concentration eliciting half-maximal current inhibition and *n* the Hill coefficient. The data are presented as original recordings and as means ±SEM for *n* experiments. Paired and unpaired Student's *t*-tests were used for analysis. $P<0.05$ was considered significant.

Results

Characterization and temperature dependence of I_K and I_{Ks}

Figure 1A–C shows the *I*/*V* curves of total currents of non-transfected (A) and KCNQ1- (B) or KCNQ1/KCNE1- (C) expressing CHO cells. The *I*/*V* relationship of non-transfected CHO cells was linear. The zero current potential of around –40 mV corresponds to the combination of a background K^+ conductance and non-selective cation conductances [21]. KCNQ1-transfected CHO cells showed a sigmoidal *I*/*V* curve with a $V_{1/2}$ of –22.1±2.5 mV and a slope factor *k* of 10.0±0.7 mV (*n*=5). KCNQ1/KCNE1 transfected CHO cells showed an almost exponential *I*/*V* curve, it had a comparable *k* (11.8±0.7 mV) but the current activated at a more depolarized voltage with a $V_{1/2}$ of 3.9±1.5 mV. The hyperpolarized zero current voltage of –64.2±1.7 mV (*n*=40) and –57.3±3.5 mV (*n*=5) of KCNQ1- and KCNQ1/KCNE1-expressing CHO cells, respectively, is in accordance with a dominating, but voltage-gated, K^+ conductance.

The activation time course was characteristic for each of the described currents and is depicted in Fig. 1D and G. Non-transfected cells showed a current of instantaneous onset (not shown), while KCNQ1-transfected cells showed a time-dependent outward current after membrane depolarization to potentials above –60 mV at 37 °C (Fig. 1D). The activation time course in KCNQ1/KCNE1 transfected cells was slower and had a slightly sigmoidal shape, as shown in Fig. 1G.

I_{Ks} in *Xenopus laevis* oocytes is strongly temperature-dependent [7]. Since the interaction of KCNE1 with KCNQ1 was not known at that time, the temperature dependence of KCNQ1 homomers has not been investigated so far. We therefore investigated this effect in CHO

Table 1 Changes in cytosolic pH (ΔpH_i) and their effects on cytosolic [Ca^{2+}] ($\Delta[Ca^{2+}]_i$) in Chinese hamster ovary (CHO) cells (*TriMA* trimethylamine, *ATP* adenosine 5′-triphosphate)

Experiment (addition of)	ΔpH_i	*n*	$\Delta[Ca^{2+}]_i$ (340 nm/380 nm)	*n*
Acidification				
20 mM acetic acid/acetate	–0.25±0.03*	9	+0.06±0.02*	6
25 mM CO_2/HCO_3^-	–0.17±0.03*	5	+0.05±0.02*	7
Alkalinization				
20 mM NH_3/NH_4^+	+0.59±0.05*	11	+0.79±0.10*	6
20 mM TriMA/$TriMA^+$	+0.61±0.03*	9	+0.71±0.19*	7
Reference signal				
0.1 mM ATP			+2.44±0.28*	10

*P<0.05 vs. 0

cells which lack endogenous expression of KCNE1 or KCNQ1 [33]. In contrast to, and significantly different from I_{Ks}, temperature only slightly affected the properties of I_K (Fig. 1D–I). While the time necessary for half-maximal current activation ($t_{1/2}$) of I_K at –10 mV increased only twofold (not significant) from 0.05±0.01 s (n=4) at 37 °C to 0.10±0.02 s (n=5) at 20 °C, $t_{1/2}$ for I_{Ks} increased fivefold (P<0.05) from 0.27±0.02 s (n=6) to 1.31±0.10 s (n=4). It is also worth noting that the sigmoidal shape of the I_{Ks}, which is characteristic for this current in oocytes at room temperature, is much less pronounced at 37 °C. In addition, in three paired experiments, I_{Ks} amplitude decreased by about 40 % when the temperature was lowered from 37 °C to 20 °C during the experiment.

Divergent responses of I_K and I_{Ks} to changes in pH_i

It is unknown whether, and if so, to what extent, I_K and I_{Ks} are sensitive to pH_i changes. We addressed this issue by altering pH_i by the use of diffusible buffer systems, acetic acid/acetate, CO_2/bicarbonate, NH_3/NH_4^+, TriMA/$TriMA^+$ and butyric acid/butyrate, in the bath solution. Table 1 shows the changes in pH_i obtained by their addition. Figure 2A shows two original experiments with

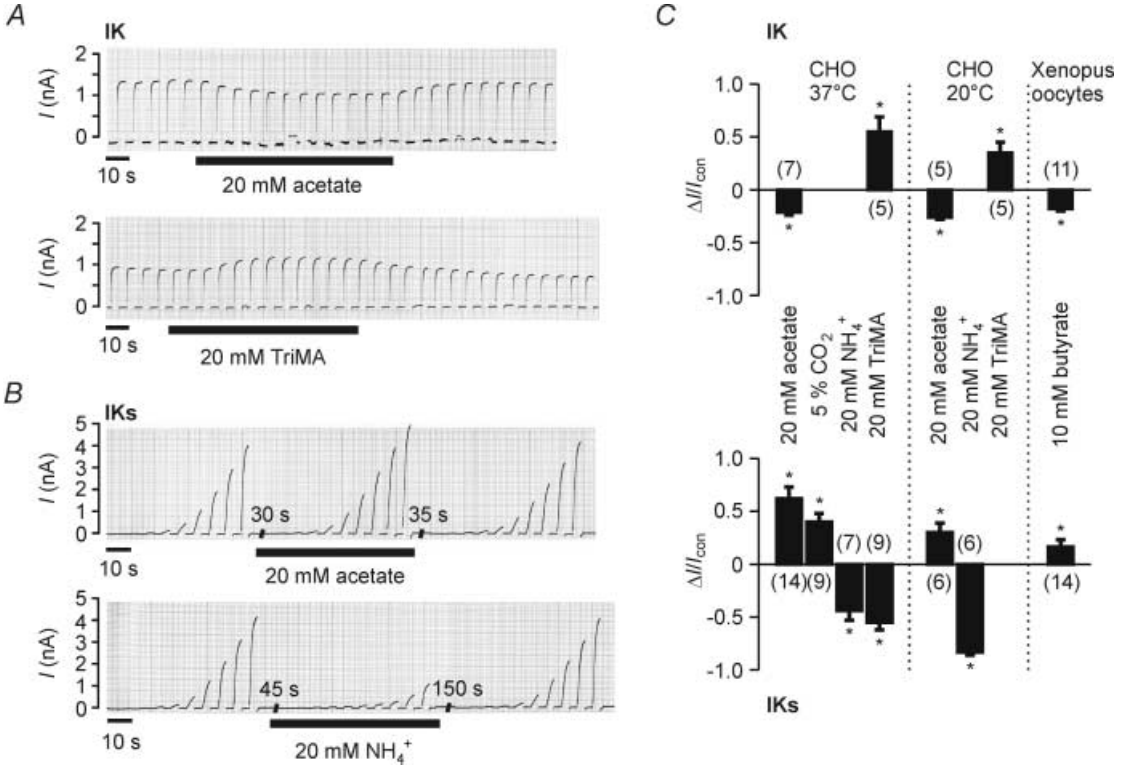

Fig. 2A–C Effect of cytosolic pH (pH_i) on I_K and I_{Ks}. **A** Original recordings of I_K from KCNQ1-expressing CHO cells at a bath temperature of 20 °C. The currents were elicited by 4 s pulses from –80 mV to 0 mV. *Upper panel*: exposure to 20 mM acetate; *lower panel*: exposure to 20 mM trimethylamine (*TriMA*). **B** Original experiments in CHO cells coexpressing KCNQ1/KCNE1 performed at a bath temperature of 20 °C. The currents were elicited by 4-s pulses from –70 mV to +10 mV. *Upper panel*: exposure to 20 mM acetate; *lower panel*: exposure to 20 mM NH_4^+. **C** Effects of cytosolic acidification and alkalinization on I_K and I_{Ks} in CHO cells and *Xenopus laevis* oocytes. Current amplitudes were measured at 0 mV. *P<0.05 vs. 0

KCNQ1-expressing cells at 20 °C. Acidification by addition of acetate decreased I_K whereas alkalinization by addition of TriMA had the opposite effect. Compared with I_K, I_{Ks} showed a reversed response to pH_i changes. This is illustrated in Fig. 2B by two typical recordings from KCNQ1/KCNE1-expressing CHO cells. At corresponding voltages addition of acetate led to a current increase and addition of NH_4^+ (alkalinization) to a current decrease. Figure 2C summarizes the effects of changes in pH_i by various buffer systems on I_K and I_{Ks} in CHO cells and *Xenopus laevis* oocytes. Unfortunately it is not possible to investigate the effect of cytosolic alkalinization in oocytes since, for unknown reasons, primary, secondary and tertiary amines are not able to alkalinize the cytosol in this expression system [6].

To investigate further the pH_i-induced effects on I_{Ks} we plotted normalized *I*/*V* curves of six paired experiments in CHO cells under control conditions, during acidification by acetate and during alkalinization by NH_4^+ at 20 °C (Fig. 3A). Under control conditions $V_{1/2}$ was –3.7±0.8 mV (*n*=22). During cytosolic acidification $V_{1/2}$ moved significantly leftwards to –7.2±1.9 mV (*n*=6). Cytosolic alkalinization significantly changed $V_{1/2}$ to 12.5±1.9 mV (*n*=5). These results signify that pH_i modulates the voltage-dependent activation of this current. To investigate if the activation kinetics were affected as well we calculated normalized mean currents recorded at –10 mV at 20 °C (Fig. 3B). The current under control conditions showed the characteristic sigmoidal shape and the typical slow activation kinetics with a $t_{1/2}$ of 1.36±0.06 s (*n*=9). Cytosolic acidification by 20 mM acetate did not change the current kinetics significantly ($t_{1/2}$ 1.38±0.14 s, *n*=4). In contrast, after cytosolic alkalinization the current lost its sigmoidal shape and the activation $t_{1/2}$ decreased significantly compared with control to 1.04±0.04 s (*n*=5).

These findings show that pH_i affects the amplitude, the voltage-dependent activation and the activation kinetics of I_{Ks}. These effects were investigated such that pH_i was changed during repetitive activation of the current. Hence it seemed reasonable to investigate whether stimulation of I_{Ks} by acidification was also present at steady-state activation. Cytosolic acidification by acetate enhanced the fully activated I_{Ks} to 202±26 % (*n*=3). One experiment is shown in Fig. 3C.

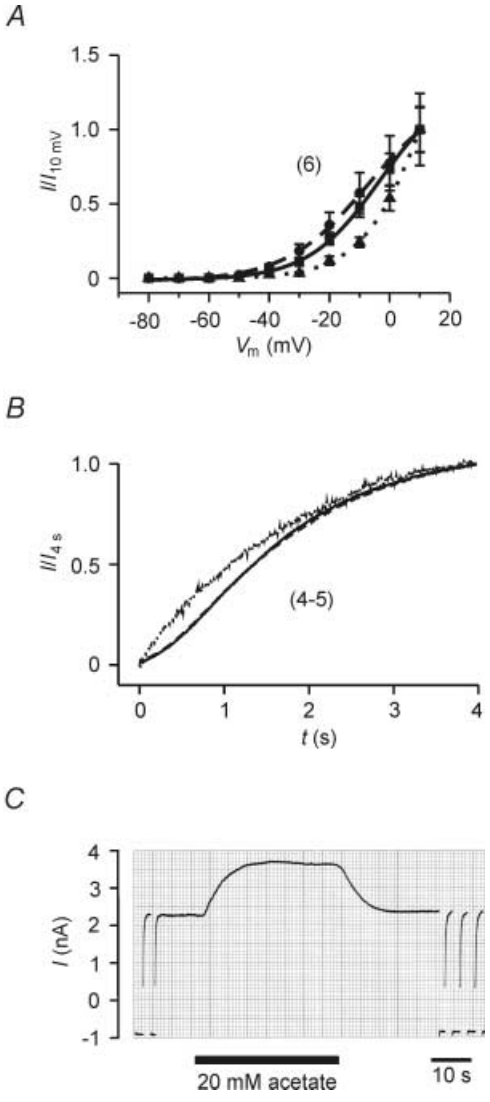

Fig. 3A–C Effects of acetate and NH_4^+ on I_{Ks} activation. **A** Normalized *I*/*V* relationships of I_{Ks} in CHO cells. Acidification by 20 mM acetate (*broken line*, *circles*) shifted the *I*/*V* curve to hyperpolarized values compared with control (*continuous line*, *squares*). Alkalinization by 20 mM NH_4^+ (*dotted line*, *triangles*) shifted the curve to depolarized voltages. **B** Activation time course of I_{Ks} under control conditions (*continuous line*), during acidification by acetate (*broken line*) and alkalinization by NH_4^+ (*dotted line*). Currents were sampled during a 4-s depolarizing step from –80 mV to –10 mV and are given as normalized mean values. Experiments shown in **A** and **B** were performed at 20 °C. **C** Original recording showing the increase of steady state current by 20 mM acetate in KCNQ1/KCNE1 expressing CHO cells (V_m 0 mV, 37 °C)

Effects of diffusible buffers on pH_i and $[Ca^{2+}]_i$

To quantify pH_i changes in CHO cells by the used buffer systems (c.f. above) we performed pH_i measurements with the fluorescent dye BCECF. Since changes in pH_i are paralleled frequently by changes in $[Ca^{2+}]_i$ [32] we also measured $[Ca^{2+}]_i$ with the fluorescent dye fura-2. The results are summarized in Table 1. Since the calibration of $[Ca^{2+}]_i$ was not possible in CHO cells, the mean peak of the ATP elicited $[Ca^{2+}]_i$ increase is given as a reference value. Figure 4 shows typical experiments. Addition of 20 mM acetate to the extracellular solution decreased pH_i by 0.25±0.03 units (*n*=9). The maximal acidification was reached some 30 s after the application of acetate and was followed by a moderate recovery of pH_i in the presence of acetate. When the acetate buffer was replaced by control solution, pH_i alkalinized before it returned to control values. As shown in Fig. 4A $[Ca^{2+}]_i$ did not change during the initial phase of acetate application. Only after some minutes could a very weak increase in $[Ca^{2+}]_i$ be detected. After the washout of acetate, how-

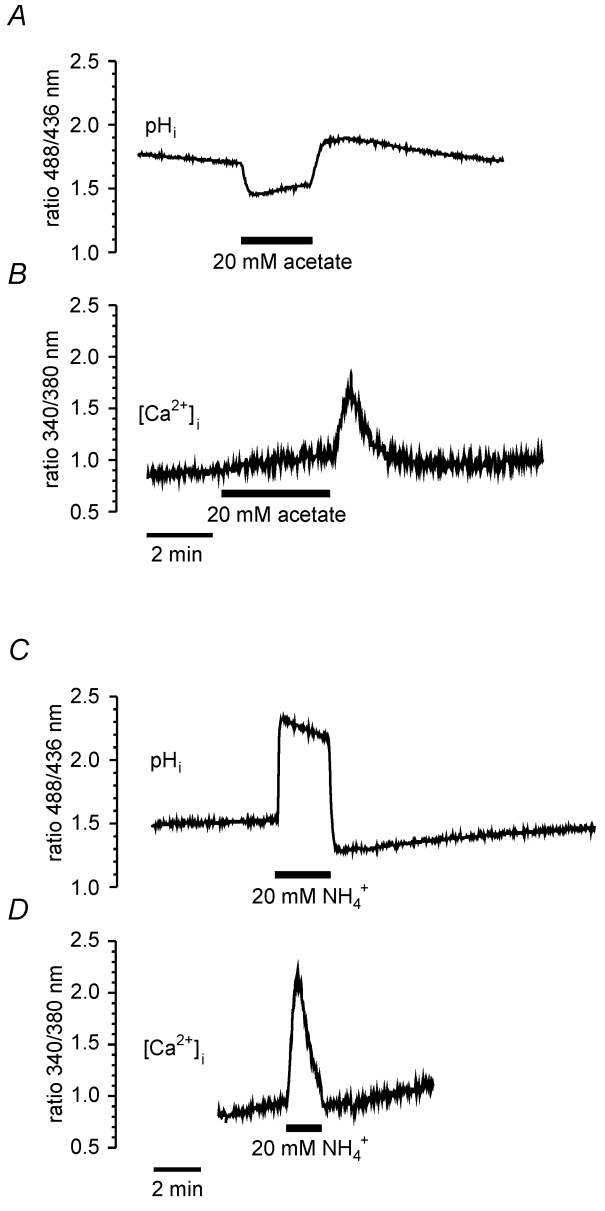

Fig. 4A–D Effects of acetate and NH_4^+ on pH_i and cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$). **A** A representative experiment in BCECF-loaded CHO cells showing the decrease of the fluorescence ratio (decrease in pH_i) following addition of acetate. **B** Trace showing the weak change in $[Ca^{2+}]_i$ in fura-2-AM-loaded cells following acidification by acetate and the Ca^{2+} transient on alkalinization by removal. **C** A BCECF experiment showing the increased fluorescence ratio (increased pH_i) following exposure to NH_4^+. **D** A fura-2 experiment corresponding to **C**, showing the pronounced increase in $[Ca^{2+}]_i$ following alkalinization by NH_4^+

ever, a very marked increase in $[Ca^{2+}]_i$ was observed (n=6). This correlated with the alkalinization of the cells. The application of 20 mM NH_4^+ increased pH_i by 0.54±0.05 units (n=11), Fig. 4B. After an initial peak, pH_i recovered slightly. Correspondingly, washout of NH_4^+ acidified the cells before pH_i normalized to the resting value. Again, and in parallel to the increase in pH_i, $[Ca^{2+}]_i$ showed a transient peak shortly after the application of NH_4^+. On removal of NH_4^+ $[Ca^{2+}]_i$ returned to control values (Fig. 4B). These data show that a rise in pH_i is accompanied by a rise in $[Ca^{2+}]_i$ in CHO cells whereas a fall in pH_i does not influence $[Ca^{2+}]_i$ markedly.

Effect of mefenamic acid, 293B, TPeA and clotrimazole on I_K and I_{Ks}

Activation of I_{Ks} has been described for fenamates, like mefenamic acid, which are thought to stabilize the channels in an open state [9, 12]. Here we examined the temperature dependence of the effects of 0.1 mM mefenamic acid on I_K and I_{Ks}. Figure 5 shows typical experiments. In KCNQ1-expressing cells at 20 °C, I_K was inhibited slightly but significantly to 84±6 % (n=6) of the control value (Fig. 5A). Addition of 30 µM 293B further decreased I_K to 38±1 % (n=6) of the current in the presence of mefenamic acid. The inhibitory effect of 293B in the presence of 0.1 mM mefenamic acid was significantly stronger than under control conditions where 49±3 % of the current remained after the application of 30 µM 293B. At the same bath temperature KCNQ1/KCNE1-coexpressing cells showed a completely different response (Fig. 5B). Application of 0.1 mM mefenamic acid increased I_{Ks} to 163±11 % (n=8) of control. During this increase the activation kinetics of this current changed markedly. The initial phase accelerated to an almost instantaneous upstroke and only a small delayed component was left. Addition of 30 µM 293B blocked I_{Ks} almost completely and only 10±0.5 % (n=4) of the current remained in the presence of mefenamic acid. This inhibition was again significantly more potent than without the previous application of mefenamic acid where 35±5 % (n=4) of the current remained after the addition of 30 µM 293B. At a bath temperature of 37 °C the effect of 0.1 mM mefenamic acid on I_K was very similar to that at 20 °C (data not shown). In contrast I_{Ks} showed a distinct response to mefenamic acid at the higher temperature (Fig. 5C). The current was also increased to 198±17 % (n=12), but the initial phase of the current was not accelerated. Figure 5D summarizes the results in stacked bars for the instantaneous part (white) and the delayed part (black) of the respective currents.

As shown in Fig. 6, the effects of mefenamic acid on I_{Ks} were studied in more detail. At 37 °C the activation of I_{Ks} was shifted to more hyperpolarized values ($V_{1/2}$ –16.0±4.8 mV vs. control 5.9±5.2 mV, Fig. 6A) keeping its typical shape, whereas at 20 °C the I/V curve in addition became almost linear (Fig. 6C). Figure 6B and D illustrates the striking temperature dependence of the mefenamic acid effects on the activation kinetics of I_{Ks}. At 37 °C mefenamic acid slowed the initial phase of the current activation (from a $t_{1/2}$ of 0.16±0.03 to 0.26±0.04 s; n=6). The current trace became slightly sigmoidal in the presence of mefenamic acid. In contrast, at 20 °C mefenamic acid markedly accelerated the initial phase and removed the sigmoidal shape.

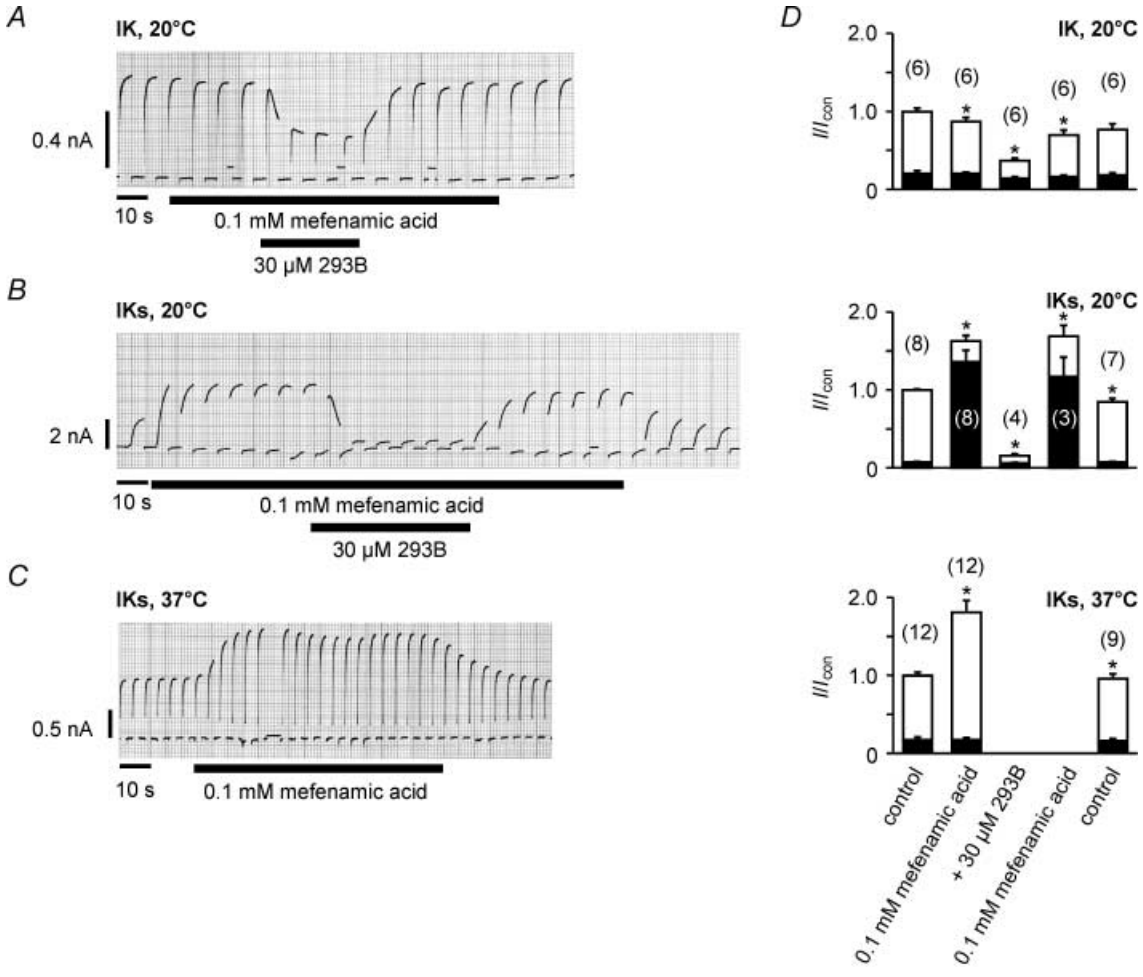

Fig. 5A–D Effect of mefenamic acid on I_K and I_{Ks} in CHO cells. **A** An original recording of I_K at 20 °C. Addition of 0.1 mM mefenamic acid and 293B (30 µM) is shown by the *solid bars* below the trace. Mefenamic acid slightly inhibited I_K. 293B led to a further and marked decrease of this current. **B** Effect of mefenemic acid and 293B (addition shown by *solid bars* below the trace) on I_{Ks} from KCNQ1/KCNE1-coexpressing CHO cells. Mefenamic acid markeldy increased the total current and caused the current to activate instantaneously. 293B inhibited this current almost completely indicating that these effects were due to an increase of I_{Ks}. **C** An experiment similar to that in **B**, but at 37 °C. Mefenamic acid also increased I_{Ks} but did not accelerate the activation time course. **D** Summary of experiments performed. The *solid bars* represent the instantaneous component of the current, the *open bars* the delayed component. The currents were elicited by pulses from –80 mV to 0 mV. Analysis was performed on the delayed current except for I_{Ks} at 20° where total currents were analysed. **P*<0.05 vs. the preceding bar

As a weak acid at pH 7.4 mefenamic acid partly permeates the cell membrane in its uncharged form, thereby changing pH_i. We therefore measured the influence of the concentration used on pH_i and $[Ca^{2+}]_i$. Mefenamic acid (0.1 mM) acidified pH_i significantly by 0.09±0.01 units (n=8). $[Ca^{2+}]_i$ was not affected significantly (n=6). The effect of mefenamic acid on pH_i is worth noting since it might explain in part the divergent responses of I_K and I_{Ks}. The temperature-dependent effect of mefenamic acid on the activation kinetics and on the slope of the *I*/*V* curve of I_{Ks} were, however, not related to pH_i.

293B is a selective blocker of KCNQ1 in native tissues as well as in expression systems [5, 10, 23, 24, 25]. In *Xenopus laevis* oocytes coexpression of KCNQ1 with KCNE1 leads to a higher sensitivity of the current to the chromanol 293B than for KCNQ1 alone [13], whereas in COS-7 cells the sensitivities of I_K and I_{Ks} to 293B are not different [26]. In our experiments with CHO cells, 30 µM 293B inhibited 55±2 % (n=8) of the current in KCNQ1-expressing cells and 68±2 % (n=6) in KCNQ1/KCNE1-coexpressing cells. This difference was significant.

In previous studies, clotrimazole, recently shown to be a selective blocker of intermediate-conductance, Ca^{2+}-activated K^+ channels [15], blocked both homomeric sKCNQ1 channels cloned from *Squalus acanthias* [3] and heteromeric KCNQ1/KCNE1 channels in pancreatic acinar cells [23]. In the present study, clotrimazole blocked I_K with a lower IC_{50} (4.6 µM, n=4–8) than I_{Ks} (IC_{50} 11.2 µM, n=6), Fig. 7A.

TPeA, an unspecific K^+ channel blocker, inhibits short-circuit currents in human colonic epithelium [30]. It therefore seemed reasonable to investigate whether TPeA blocks I_K, a channel which is involved in NaCl se-

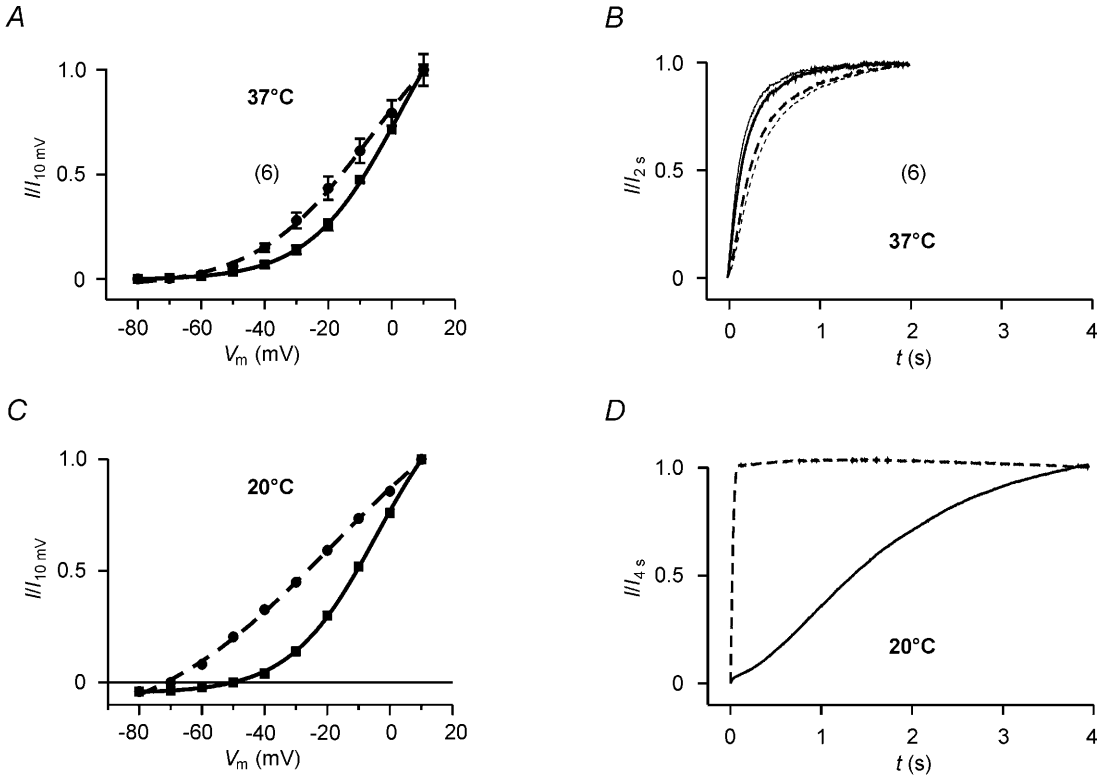

Fig. 6A–D Effect of mefenamic acid on voltage dependence and activation time course of I_{Ks} in CHO cells. **A** Normalized *I*/*V* relationships of I_{Ks} in CHO cells at 37 °C. Control (*continuous line*, *squares*) and 0.1 mM mefenamic acid (*broken line*, *circles*). Mefenamic acid shifted the curve to the left. **B** Activation time course of normalized mean I_{Ks} (±SEM, *thin lines*) recorded at 37 °C at 0 mV under control conditions (*continuous lines*) and during exposure to 0.1 mM mefenamic acid (*broken lines*). Mefenamic acid led to a slower current activation and to a sigmoidal slope of the activation time course compared to control conditions. **C** *I*/*V* relationship of I_{Ks} in a single CHO cell at 20 °C under control conditions (*continuous line*, *squares*) and during exposure to 0.1 mM mefenamic acid (*broken line*, *circles*). The *I*/*V* relation in the presence of mefenamic acid in almost linear at this temperature. **D** Activation time course of I_{Ks} of a single CHO cell under control conditions (*continuous line*) and in the presence of 0.1 mM mefenamic acid (*broken line*) at 20 °C (V_m=0 mV). In contrast to the effect at 37°C, mefenamic acid strongly accelerated the initial phase of the activation time course

cretion in this tissue. Like clotrimazole and unlike 293B, TPeA blocked I_K (IC_{50} 12.3 µM, *n*=8), Fig. 7B, with a higher potency than I_{Ks} (IC_{50} 78.9 µM, *n*=4–7). The effect of TPeA on I_{Ks} was delayed.

Discussion

After the discovery that KCNQ1 and KCNE1 or KCNE3 coassemble to form heteromeric channels [1, 33, 34] there was much excitement about the fundamental changes of KCNQ1 channel properties brought about by these subunits. In this study we confirmed some of the known divergent properties of KCNQ1 and KCNQ1/KCNE1 channels. In addition, the following

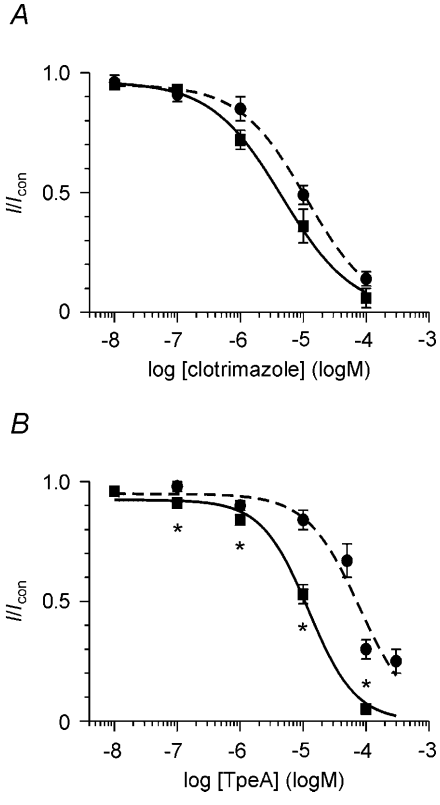

Fig. 7A, B Concentration response curves of clotrimazole and tetrapentylammonium (*TpeA*) on I_K and I_{Ks} in CHO cells. **A** Concentration/response curves for the effect of clotrimazole on I_K (*continuous line*, *squares*) and I_{Ks} (*broken line*, *circles*) in CHO cells (V_m=0 mV). **B** Concentration/response curves for the effect of TpeA on I_K (*continuous line*, *squares*) and I_{Ks} (*broken line*, *circles*) in CHO cells (V_m=0 mV). **P*<0.05 vs. I_{Ks}

new aspects came to light. First, KCNQ1/KCNE1 channels showed a higher temperature dependence than KCNQ1 channels. Second, both KCNQ1 and KCNQ1/KCNE1 channels were sensitive to pH_i changes, although in opposite directions. Third, mefenamic acid activated KCNQ1/KCNE1 channels but inhibited KCNQ1. Its effect on KCNQ1/KCNE1 was temperature dependent. Finally, TPeA and clotrimazole blocked KCNQ1 and KCNQ1/KCNE1 channels with different potencies.

Temperature dependence of I_K and I_{Ks}

The temperature effect on I_K is in the range observed for voltage-gated neuronal and muscular Na^+ and K^+ currents [2, 17] whereas the effect on I_{Ks} is much stronger. This strong temperature dependence and the sigmoidal activation time course of I_{Ks} probably reflect the complex gating mechanism of the corresponding channel complex. Since a conformational change within a preformed channel molecule is not expected to be that temperature sensitive [7] it has been proposed that KCNQ1 and KCNE1 subunits coassemble during a depolarising voltage step. This hypothesis could explain the slower activation kinetics at a lower temperature by the higher rigidity of the membrane lipids and proteins at this temperature. In line with this hypothesis is the observation that destruction of cytoskeletal structures by cytochalasin D alters activation kinetics [11] and that chemical cross-linkers accelerate the activation kinetics of I_{Ks} [40].

Divergent pH_i responses of I_K and I_{Ks}

To date, only the effect of extracellular pH on I_{Ks} has been investigated: these heteromeric channels are moderately inhibited by an acidification [43]. In the present study we showed that I_K and I_{Ks} were much more sensitive to pH_i changes and that they respond differently. Irrespective of the buffer system and bath temperature used, all manoeuvres acidifying the cell inhibited I_K and activated I_{Ks}. The opposite was observed for alkalinization. This renders unspecific or species-dependent effects rather unlikely. KCNQ1/KCNE1 channels show a permeability for NH_4^+ that is 8 times smaller than that for K^+ [35]. The addition of 20 mM NH_4^+ to the extracellular solution could therefore reduce the driving force for K^+ by 15%. Whilst this effect could contribute to the strong inhibition of I_{Ks} following the addition of NH_4^+, it cannot account for the full effect. Inhibition of I_{Ks} was also seen after alkalinization by TriMA which, because of its size, should not be able to permeate the channels, and in some experiments after removal of acetate or CO_2 when the cells were transiently alkaline before returning to the resting pH (data not shown). For CHO cells pH_i changes were quantified by BCECF fluorescence measurements showing that the experimental pH_i changes were within a range which can be expected under pathophysiological conditions. Since changes in pH_i can affect $[Ca^{2+}]_i$ [32], which then increases I_{Ks} [7, 8, 23, 24], it is important to consider $[Ca^{2+}]_i$ in the discussion of pH effects on I_K and I_{Ks}. Acidification by acetate or CO_2 did not affect $[Ca^{2+}]_i$ initially, whereas the current responses to cytosolic acidification were immediate. In contrast, alkalinization immediately caused a steep but transient rise in $[Ca^{2+}]_i$. In most experiments $[Ca^{2+}]_i$ reached the baseline value while cytosolic pH was still alkaline. The current responses, however, sustained. Moreover, I_{Ks} was inhibited by a rise in pH_i although $[Ca^{2+}]_i$ increased. It therefore seems reasonable to conclude that pH_i is the signal responsible for the divergent current responses. It still remains unclear whether pH_i itself or an unknown, pH_i-controlled mechanism mediates the effects on I_K and I_{Ks}.

There is some evidence that I_{Ks} is up-regulated by acidic pH_i also in native tissue. In vestibular dark cells a rise in electrogenic transepithelial transport is observed after cytosolic acidification by 20 mM propionate [42] and KCNQ1/KCNE1 channels carry I_{Ks} in these cells. It is tempting to speculate that activation of I_{Ks} by acidification also plays a role in other tissues. In the heart this effect might contribute to the pathogenesis of ischaemic cardiac arrhythmias.

How KCNE1 alters the pH response of KCNQ1 channels remains open. From the experiments performed it can not be deduced whether titratable amino acids, an interaction of amino acids, an altered pore or an altered coassembly are responsible for the divergent pH responses.

Effects of mefenamic acid on I_K and I_{Ks}

Mefenamic acid, other fenamates and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) activate I_{Ks}. In *Xenopus* oocytes expressing human KCNE1 (together with endogenous KCNQ1), application of 0.1 mM mefenamic acid increases I_{Ks} and the current loses its delayed activation kinetics and becomes almost instantaneous, reflecting persistent current activation. I_K is slightly inhibited by mefenamic acid [12, 13]. In our experiments at 20 °C we observed the same effects. Mefenamic acid had a small inhibitory effect on I_K whereas it increased I_{Ks} substantially. The current became instantaneous and showed an almost linear *I*/*V* relationship, indicating permanent activation of I_{Ks} similar to that observed after the application of chemical cross-linkers [40]. In the presence of mefenamic acid the current could be blocked very potently by 293B, strongly suggesting that the current increase was caused by the activation of I_{Ks} and not by the activation of a background conductance. However, when the experimental temperature was 37 °C I_{Ks} kept its voltage dependence and showed a slowed activation time course. We can only speculate about the mechanism for temperature dependence of the action of mefenamic acid. One possible explanation could be that binding of mefenamic acid to the channel proteins is stronger

at lower temperatures and that binding at 37 °C is too weak to arrest the channels in an open state. Our fluorescence measurements show that pH_i was slightly decreased in the presence of mefenamic acid. This acidification might contribute partially to the inhibitory effect observed on I_K and to the activation of I_{Ks}. Quantitatively, acidification by mefenamic acid was not sufficient to explain activation of I_{Ks} and, in addition, the changes in current kinetics caused by mefenamic acid could not be detected during acidification. As shown by fura-2 measurements, $[Ca^{2+}]_i$ was not significantly changed by mefenamic acid and therefore can be excluded as a mediator of its effects.

In summary, we showed that KCNE1 reverses the response of KCNQ1 to pH_i and alters its sensitivity to temperature and drugs. It will be interesting to elucidate the underlying mechanism further and to investigate the pathophysiological relevance of cytosolic pH changes for I_{Ks} in native tissues.

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