KCNE Beta Subunits Determine pH Sensitivity of KCNQ1 Potassium Channels

Dirk Heitzmann*, Viktoria Koren*, Michael Wagner#, Christina Sterner, Markus Reichold, Ines Tegtmeier, Tilmann Volk# and Richard Warth

Institute of Physiology, University of Regensburg, # Institute of Cellular and Molecular Physiology, University of Erlangen-Nürnberg, *These authors contributed equally

Key Words

pH Sensitivity • Cardiac action potential • KvLQT1 • IsK • Kv7.1

Abstract

Background/Aims: Heteromeric KCNE_x/KCNQ1 (=KvLQT1, Kv7.1) K⁺ channels are important for repolarization of cardiac myocytes, endolymph secretion in the inner ear, gastric acid secretion, and transport across epithelia. They are modulated by pH in a complex way: homomeric KCNQ1 is inhibited by external acidification (low pH_e); KCNE2/KCNQ1 is activated; and for KCNE1/KCNQ1, variable effects have been reported. **Methods:** The role of KCNE subunits for the effect of pH_e on KCNQ1 was analyzed in transfected COS cells and cardiac myocytes by the patch-clamp technique. **Results:** In outside-out patches of transfected cells, hKCNE2/hKCNQ1 current was increased by acidification down to pH 4.5. Chimeras with the acid-insensitive hKCNE3 revealed that the extracellular N-terminus and at least part of the transmembrane domain of hKCNE2 are needed for activation by low pH_e. hKCNE1/hKCNQ1 heteromeric channels exhibited marked changes of

biophysical properties at low pH_e: The slowly activating hKCNE1/hKCNQ1 channels were converted into constitutively open, non-deactivating channels. Experiments on guinea pig and mouse cardiac myocytes pointed to an important role of KCNQ1 during acidosis implicating a significant contribution to cardiac repolarization under acidic conditions. **Conclusion:** External pH can modify current amplitude and biophysical properties of KCNQ1. KCNE subunits work as molecular switches by modulating the pH sensitivity of human KCNQ1.

Copyright © 2007 S. Karger AG, Basel

Introduction

The Shaker-type K⁺ channel KCNQ1 is strongly expressed in various epithelia and in cardiac muscle. To form native channels, the pore-forming alpha-subunit KCNQ1 assembles with the beta-subunits KCNE1-5 in a tissue specific manner. The precise stoichiometry of the channels subunits and whether or not KCNQ1 can

assemble with various KCNE proteins to form one channel is still a matter of debate [1, 2]. The small KCNE beta-subunits determine biophysical and pharmacological properties, they improve surface localization of the heteromeric KCNQ1 channel complex and they modify functional properties and channel regulation [3-5].

Assembly of KCNQ1 with KCNE1 leads to a voltage-dependent heteromeric channel with slow activation and deactivation kinetics [6, 7]. In cardiac myocytes, heteromeric KCNE1/KCNQ1 channels underlie the slow component of the delayed rectifier K⁺ current (I_{Ks}) which plays an important role for repolarization of the cardiac action potential (AP). The physiological significance of KCNE1/KCNQ1 for normal repolarization and frequency-dependent shortening of cardiac APs, especially in the ventricles, is exemplified by mutations of both human genes that lead to a marked prolongation of the AP and a high risk for the occurrence of life-threatening ventricular arrhythmias (long QT syndromes type 1 and 5, OMIM 192500 and 176261) [8-10]. Moreover, KCNE1/KCNQ1 channels are important for endolymph secretion in the inner ear and homozygous mutations of both genes cause congenital deafness (cardioauditory syndrome of "Jervell and Lange-Nielsen", OMIM 220400). Interestingly, heterologously expressed KCNQ1 has been shown to be affected by acidic extracellular and intracellular pH in a complex and KCNE1-dependent manner: Homomeric KCNQ1 channels are strongly inhibited by acidic extracellular and intracellular pH [11-13]; heteromeric KCNE1/KCNQ1 channels exhibit changes of activation and deactivation kinetics and variable effects on current amplitude upon low extracellular pH (pH_e) [11, 12]. Moreover, the glycosylation status of rat KCNE1 possibly affects regulation of rKCNE1/hKCNQ1 by low extracellular pH [11]. Intracellular acidification, however, activates KCNE1/KCNQ1 channels [13]. These properties of KCNE1/KCNQ1 may be of important pathophysiological relevance during conditions such as ischemia which leads to acidification of cardiac muscle tissue (pH 5.5-6.5) and consecutive changes in electrical properties thereby promoting cardiac arrhythmias.

In addition to KCNE1, KCNE2-5 are also expressed in cardiac myocytes and modulate KCNQ1 function in a complex way [14-16]: e.g. KCNE4 and KCNE5 reduce the KCNQ1 current in the range of physiological membrane voltages and induce marked shifts in voltage-dependence to much more depolarized values [6, 7, 15, 17, 18]. Very recently, evidence has been provided that the functional diversity of KCNQ1 channels can be

broadened by heteromeric assembly with more than one type of KCNE beta subunit [19].

Assembly with KCNE1, KCNE4, and KCNE5 results in voltage-dependent KCNQ1 channels. KCNE2 and KCNE3, however, change channel kinetics from slow activation and deactivation towards time-independent activity [3, 20]. In intestinal crypt cells and airway epithelial cells KCNQ1 associates with KCNE3 to form a cAMP-stimulated basolateral K⁺ conductance which is of physiological and pathophysiological importance to drive electrogenic Cl⁻ secretion [21, 22]. It is still a matter of debate whether KCNE3 mutations underlie "periodic paralysis" and "thyrotoxic hypokalemic periodic paralysis" (OMIM 604433) [23, 24].

KCNE2 has been originally described as a gene mutated in long QT syndrome (LQT-6, OMIM 603796); and mutations of KCNE2 can cause atrial fibrillation (OMIM 607554) [25]. In stomach mucosa, KCNE2/KCNQ1 channels play a pivotal role for K⁺ recycling across the luminal membrane of gastric parietal cells [26-30]. KCNE2/KCNQ1 channels are activated by second messengers stimulating acid secretion (i.e. cAMP, L- α -phosphatidylinositol-4,5-bisphosphate). In addition, acid secretion itself appears to activate KCNE2/KCNQ1 in the luminal membrane because this channel complex is activated by external acidic pH via a yet unidentified mechanism [31].

Thus, pH can exert multifaceted effects on the different heteromeric KCNQ1 channels. This study has been focused on pH-induced biophysical changes of KCNQ1 assembled with KCNE1, KCNE2, and KCNE3. Specifically, we determined the domains of KCNE2 needed for activation by acidic extracellular pH using a chimera approach; and we investigated the effect of acidic extracellular pH on KCNE1/KCNQ1 expressed in mammalian cells and cardiac myocytes of mouse, rat, and guinea pig.

Materials and Methods

Patch-clamp experiments

Human KCNE1 (hKCNE1; acc. No.: NM_000219), human KCNE2 (hKCNE2; acc. No.: NM_172201), human mutant KCNE2_{R27C} (hKCNE2_{R27C}) and human KCNE3 (hKCNE3; acc. No.: NM_005472) have been cloned into the pIRES-CD8 mammalian expression vector (plasmids were kind gifts of Dr. J. Barhanin, CNRS, Sophia-Antipolis, France [32]). COS and CHO cells were transiently co-transfected with pCI-hKCNQ1 (human KCNQ1 GenBank accession number AF00571) and either KCNE1 or KCNE2 or KCNE3 (each 0.5 μ g per dish) using the FuGENE transfection reagent (Roche, Mannheim, Germany).

Whole cell recordings were performed on transfected COS and CHO cells, as visualized by CD8 expression (Dynabeads M-450 CD8, Dynal GmbH, Hamburg, Germany). The patch pipette solution was (in mM): 95 K-gluconate, 30 KCl, 4.8 Na₂HPO₄, 1.2 NaH₂PO₄, 5 Glucose, 2.38 MgCl₂, 0.726 CaCl₂, 1 EGTA, 3 ATP, pH 7.2. For experiments with KCNE2/KCNQ1 co-expressing COS cells, cAMP 100 μ M has been added to the pipette solution. The standard extracellular solution for experiments with COS and CHO cells was (in mM): 145 NaCl, 0.4 KH₂PO₄, 1.6 K₂HPO₄, 5 Glucose, 1 MgCl₂, 1.3 CaCl₂, 5 HEPES, pH 7.4. For the acetate-containing solution, 20 mM NaCl were replaced by 20 mM Na-acetate. Whole cell currents were normalized to cell capacitance (pA/pF and pS/pF, respectively) as a measure of cell surface area. Excised outside-out patches were generated by very slow excision from the whole cell configuration. The reference electrode was of the flowing KCl (1 M) type in order to minimize liquid junction voltages. The pipette capacitance was cancelled by the compensation circuit of the amplifier (EPC-7-like amplifier made by U. Fröbe and R. Busche, Institute of Physiology, Freiburg, Germany) with the help of sine wave command voltage. Current signals were filtered at 300 Hz and digitized at 1 kHz. Analysis was performed using patch-clamp software written by U. Fröbe (Institute of Physiology, Freiburg, Germany). Leak subtraction was not performed. All experiments were performed at room temperature.

Mutagenesis

KCNE2/3 chimeras (in pIRES-CD8) were produced by PCR-based site-directed mutagenesis using mutagenic primers and Turbo-Pfu DNA polymerase (Stratagene). Each mutant was verified by DNA sequencing.

Preparation of cardiac myocytes

Guinea pigs and rats of 150–200 g were used for this study. The experimental protocols were approved by the local councils for animal care and were conducted according to the German law for the care. After intra-peritoneal application of ketamine (200 μ g/bw) and xylazine (1 μ g/g bw), ventricular myocytes were isolated in a similar way as described previously [33, 34]. The heart was removed, put into ice-cold cardioplegic solution (in mM: NaCl 15, KCl 9, MgCl₂ 4, NaH₂PO₄ 0.33, CaCl₂ 0.015, glucose 10, mannitol 238, pH 7.4). Then the aorta was cannulated and the heart was perfused for 3 min with 37°C warm Tyrode-like Ca²⁺-free solution (NaCl 138, KCl 4, NaH₂PO₄ 0.33, MgCl₂ 1, EGTA 1, HEPES 10, glucose 10, pH 7.3). The perfusion was continued for 15 min at 37°C with Ca²⁺-free solution containing collagenase (type CLS II, 200 U/ml, Biochrom AG, Berlin, Germany) and protease (type XIV, 0.7 U/ml, Sigma, Deisenhofen, Germany). All solutions were equilibrated with 100% oxygen. After the perfusion, the heart was quickly excised and placed into a modified Tyrode solution containing 100 nM Ca²⁺. The left ventricular muscle was cut into slices which were gently shaken for 5 minutes. Cardiac myocytes were stored at room temperature in modified Tyrode solution containing 100 nM Ca²⁺. For the experiments, cardiac myocytes were transferred into Ca²⁺-containing solution (NaCl 138, KCl 4, NaH₂PO₄ 0.33, MgCl₂ 1, CaCl₂ 2, HEPES 10, glucose 10, pH 7.4). Only single rod-shaped cells with clear cross-

striations and no spontaneous contractions were used for experiments. For primary cultures of cardiac myocytes, hearts of guinea pig embryos (embryonic day 40) or of newborn mice (day 1–3) were digested in the enzyme solution for 15 min and seeded on glass cover slips and cultured for 2–5 days in 5% CO₂ atmosphere at 37°C in cell culture medium consisting of DMEM (Gibco 41965) plus 10% FCS, penicillin 50 IE/ml, streptomycin 50 μ g/ml, L-glutamine 2 mM, and Na-pyruvate 1 mM. Spontaneously beating primary cultured myocytes were used to study the effect of KCNQ1 inhibition on action potentials.

Cytosolic pH measurements

Intracellular pH was measured with a filter wheel based imaging system (Universal Imaging Corporation, Downingtown, PA, USA) mounted on an inverted microscope (Axiovert 200, Zeiss, Jena, Germany) using the pH dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF, Molecular Probes; excitation at 440 $\pm$ 5 nm and 492 $\pm$ 5 nm, emission 522–550 nm). COS cells were loaded with BCECF-AM (1 μ mol/l) for 30 min. Mean fluorescence ratios were calculated for single cells using the Metafluor software (Universal Imaging, West Chester, PA, USA). For pH_i, the fluorescence emission (492 nm/440 nm) ratio was calculated. The BCECF fluorescence ratio was calibrated using nigericin (30 μ M). All experiments were performed at room temperature.

Chemicals

Standard chemicals and substances were purchased from Sigma and Aldrich (Deisenhofen, Germany); HMR1556 and 293B (kind gifts from Aventis Pharma GmbH, Frankfurt a. M., Germany) were dissolved in DMSO (stock concentration 0.1 M) and diluted to its final concentration in the experimental solutions.

Statistics

Data are shown as mean values $\pm$ SEM from *n* observations. Paired as well as unpaired Student's *t*-test was used as appropriate. A *P* value of <0.05 was accepted to indicate statistical significance.

Results

The parietal cell channel complex KCNE2/KCNQ1 is regulated by extracellular and intracellular pH

Previously we and others have shown that KCNE2/KCNQ1 channels are activated during gastric acid secretion. The heteromeric channel complex is activated by extracellular acidic pH and allows K⁺ recycling across the luminal membrane which is required for ongoing acid secretion [29–31]. Here, we performed an extended analysis of the pH regulation in COS cells expressing human KCNE2 (hKCNE2) and human KCNQ1 (hKCNQ1). In whole cell experiments, hKCNE2/

Fig. 1. Effect of pH on KCNE2/KCNQ1 channels. Whole cell currents of hKCNE2/hKCNQ1-coexpressing COS cells (normalized to cell capacitance (I/C_m)) were activated by acidic bath pH (A), inhibited by alkaline bath pH (B) and by intracellular acidification due to addition of 20 mM Na-acetate (C) to the bath. (D) Outside-out patch formed by slow excision from whole cell configuration. Stepwise acidification of bath led to increases in K^+ outward current which could be blocked by the KCNQ1 blocker 293B (10 μ M). (E) Summary of outside-out experiments as shown in (D). The “gain-of-function” mutation KCNE_{R27C} exhibits acid-induced stimulation of KCNQ1 current: (F) Typical trace of a whole cell experiment; (G) normalized whole cell current of experiments as shown in (F).

hKCNQ1 channels were inhibited by alkaline and activated by acidic pH_e . To test the effect of cytosolic acidification, a solution containing Na-acetate (20 mM) was added to the bath. In BCECF fluorescence measurements, cellular uptake of acetic acid led to acidification of the cytosol by 0.30 ± 0.06 pH units ($n=12$). In contrast to extracellular acidification, cytosolic acidification reduced the hKCNE2/hKCNQ1 current (Figure 1, A-C). To exclude indirect effects of pH changes on hKCNE2/hKCNQ1 in whole cell experiments, we performed outside-out patch clamp experiments using the inhibitor 293B as a pharmacological tool for identifying

KCNQ1-specific currents. Only patches showing clear 293B-sensitive outward currents have been included in this series. Acidification of the extracellular solution resulted in activation of the K^+ current with a maximum at pH 4.5. Further acidification was accompanied by slight reduction of the current (Figure 1, D and E). Filtered at 300 Hz, the current trace was “noisy” and we could not discriminate single channel events indicating that apparently single channel conductance is below 5 pS.

Recently, a hKCNE2 N-terminal “gain-of-function” mutation (arginine-to-cysteine mutation at position 27; R27C) has been reported to cause atrial fibrillation by

Fig. 2. Assembly of KCNE3 and KCNQ1 results in an acid-insensitive channel complex. Whole cell currents (normalized to membrane capacitance (I/C_m)) of KCNE3/KCNQ1-coexpressing COS cells were not changed by bath acidification (A), by alkalinization (B) and by intracellular acidification via Na-acetate (20 mM) application to the bath (C).

increasing the cardiac hKCNE2/hKCNQ1 current [25]. Here we examined whether the mechanism underlying the “gain-of-function” of hKCNE2_{R27C} mutant is a shift of the acid-induced activation into the range of physiological pH. Interestingly, activation of the hKCNE2_{R27C}/hKCNQ1 current by pH 5.5 was not reduced compared to wildtype hKCNE2/hKCNQ1 (Figure 1, F and G).

N-terminus and transmembrane domain of hKCNE2 are needed to mediate acid activation

Assembly of KCNQ1 with its beta subunit of intestinal and airway epithelia, KCNE3 [3], led to a completely acid-insensitive channel: hKCNE3/hKCNQ1 activity was affected neither by extracellular nor by intracellular pH changes (Figure 2, A-C). This is in contrast to acid-activated hKCNE2/hKCNQ1 channels (Figure 1) and - as previously described by others [11, 12] - in contrast to homomeric hKCNQ1 channels which are inhibited by low extracellular pH (Figure 3A). To identify domains of hKCNE2 responsible for pH sensitivity, we designed three chimeras between acid-sensitive hKCNE2 and acid-insensitive hKCNE3. All chimeras assembled with hKCNQ1 as indicated by the almost linear I/V curve and the hyperpolarized membrane voltage (-75 to -85 mV) of chimera/hKCNQ1-co-expressing cells compared to the slight voltage dependence and the more depolarized membrane voltage (-55 mV) of homomeric hKCNQ1-expressing cells (Figure 3, A-D). Moreover, the slow activation kinetics of hKCNQ1 was changed to a mainly instantaneous current (right panels of Figure 3, A-D). Replacement of the extracellular N-terminus of hKCNE2 by the N-terminus of hKCNE3 converted activation by external pH 5.5 into inhibition indicating that the hKCNE2 N-terminus is a prerequisite for acid

activation of the channel complex (Figure 3B). However, the opposite chimera consisting of hKCNE2 N-terminus and hKCNE3 transmembrane region and C-terminus did not lead to activation of hKCNQ1 by acidic pH (Figure 3C) indicating that the N-terminus of hKCNE2 alone is necessary but does not suffice to mediate acid activation. Next, we examined the effect of a chimera consisting of hKCNE2 N-terminus and transmembrane region fused to the intracellular C-terminus of hKCNE3. Interestingly, co-expression of this chimera and hKCNQ1 fully reconstituted acid activation (Figure 3D). Taken together, these data suggest that acid activation of heteromeric hKCNE2/hKCNQ1 channels requires the extracellular N-terminus and at least part of the transmembrane region of hKCNE2.

Acidic pH modifies the biophysical properties of hKCNE1/hKCNQ1 and increases its current amplitude

Previously, acidic extracellular pH has been reported to slow down activation and deactivation kinetics of KCNE1/KCNQ1 and to reduce current amplitudes in CHO cells and *Xenopus laevis* oocytes [11, 12]. To further analyze the effect of external pH on heteromeric hKCNE1/hKCNQ1 channels, we performed whole cell and excised outside-out patch-clamp experiments on transfected COS cells (without “leak subtraction”). In contrast to the above mentioned previous reports, we observed an increase in maximal whole cell current by pH 5.5 and a slight but significant reduction by pH 8.5. The increase in maximal hKCNE1/hKCNQ1-current during low external pH was paralleled by hyperpolarization of the cell membrane. Moreover, intracellular acidification increased the maximal current slightly as reported earlier [13] (Figure 4, A-C). To exclude

Fig. 3. Effect of pH on KCNQ1 and KCNE2/KCNE3 chimera. Whole cell currents of COS cells expressing homomeric or heteromeric KCNQ1 are shown as typical traces (right panel) and as summaries (currents at the end of the depolarizing pulses were normalized to membrane capacitance (I/C_m)). Leak subtraction was not performed to make sure that possible chimera-induced changes in ion selectivity are detectable. (A) Currents of homomeric KCNQ1 channels are depicted for comparison. They were slightly voltage-dependent and inhibited by external pH 5.5. To measure the tail current, membrane voltage was clamped to -55 mV after the depolarizing step of +25 mV. (B-C) Effect of external pH 5.5 on cells co-expressing hKCNQ1 and hKCNE2/hKCNE3 chimeras. Please note that all three chimeras changed the slow activation kinetics of homomeric KCNQ1 channels into instantaneous currents. (B) Replacement of the extracellular N-terminus of

KCNE2 (amino acids 1-48 of KCNE2) by the N-terminus of human KCNE3 (amino acids 1-60 of KCNE3) led to inhibition of whole cell current by external pH 5.5. (C) The chimera consisting of KCNE2 N-terminus (amino acid 1-48 of KCNE2) and KCNE3 transmembrane region and C-terminus (amino acids 61-103 of KCNE3) led to an acid-insensitive channel complex. (D) The chimera consisting of KCNE2 N-terminus plus transmembrane domain (amino acids 1-72 of KCNE2) and KCNE3 C-terminus (amino acids 81-103 of KCNE3) showed high current densities and was activated by pH 5.5.

cell type specific difference as cause for the differing results, we performed similar experiments in CHO cells: Again, low extracellular pH increased maximal current density. The effect of stepwise decrease in pH on a hKCNE1/hKCNQ1-co-expressing CHO cell is depicted in Figure 4D. Apparently, protons not only increased maximal current density but in a concentration-dependent manner they converted the slowly activating and deactivating hKCNE1/hKCNQ1 channel into a constitutively open channel with an almost linear I/V

relationship (Figure 4, D and E). Supported by the use of the KCNQ1-specific inhibitor HMR1556 (1 μ M), which blocked the current almost completely, a substantial contribution of non-KCNQ1 K^+ channels to the observed effect was unlikely (Figure 4D). Additionally, non-transfected or solely KCNQ1-transfected cells did not show such effects at low pH (data not shown). Further analysis of the effect of acidic pH on hKCNE1/hKCNQ1 current revealed that the instantaneous current component (current at the very beginning (after 20 ms)

Fig. 4. Effect of pH on KCNE1/KCNQ1 channels. Whole cell currents (normalized to membrane capacitance (I/C_m)) of KCNE1/KCNQ1-coexpressing COS cells were stimulated by bath acidification (A), inhibited by bath alkalinization (B) and slightly activated by intracellular acidification via Na-acetate application to the bath (C). (D) Increasing acidification of bath pH led to increase in whole cell K^+ current and changed the biophysical properties. Dependent on external H^+ concentration, the slowly activating and deactivating current was converted into an instantaneous, very slowly deactivating current, which could be blocked by 1 μM HMR1556 (HMR). To measure the tail current, membrane voltage was clamped to -55 mV after the depolarizing step of +25 mV. (E) I/V curves of maximal current of the experiment shown in (D). (F) Summary of 9 similar experiments, depicted are currents at $V_c = +25$ mV. Please note that the slowly activating current was decreased by acidification, but the instantaneous current component was increased (stars indicate differences from the value at pH 7.4). (G) Outside-out patch of a KCNE1/KCNQ1-co-expressing COS cell. The “noisy” K^+ current was activated by acidic pH and inhibited by application of HMR1556 ($V_c = -10$ mV).

of the voltage step from -95 to +25 mV) increased pH-dependently; the slowly activating component (maximal current at the end of the depolarizing voltage step minus instantaneous current), however, decreased (Figure 4F). The conversion of the deactivating hKCNE1/hKCNQ1 current into a non-deactivating current hyperpolarized the membrane voltage (as measured during 20 s in the “current clamp zero” mode between two voltage clamp periods;

Figure 4F, lower panel). So far, such fundamental changes in biophysical properties of hKCNQ1 channel have only been attributed to different beta subunits of hKCNQ1, e.g. hKCNE1 versus hKCNE2 or hKCNE3 [3, 20]. The acidic pH-induced increase in hKCNE1/hKCNQ1 current could also be observed in excised outside-out patches. Similar to outside-out patches of hKCNE2/hKCNQ1-expressing cells, single channel events could not be

Fig. 5. Effect of extracellular acidification on cardiac myocytes. (A) Action potential of a spontaneously beating primary cultured guinea pig cardiac myocyte (solid line); an action potential of the same cell in the presence of HMR1556 is superimposed for comparison (dotted line). Application of HMR1556 (1 μ M) led to inhibition of I_{Ks} and prolongation of the action potential. At bath pH 5.5 (right panel), repolarization of the action potential was prolonged. Under these conditions, the prolongation by HMR1556 was even larger compared to the effect at bath pH 7.4. (B) Effect of external pH on the HMR1556 (1 μ M) sensitive current in guinea pig cardiac myocytes (measured at +25 mV; current normalized to cell capacity; $n=6$). (C) Summary of measurements of the HMR1556 (1 μ M)-sensitive whole cell currents at the end of depolarizing pulses in primary cultured mouse cardiac myocytes. The HMR1556-sensitive current component was augmented by acidification of the bath solution.

resolved suggesting a single channel amplitude of less than 5 pS (Figure 3G).

Effect of acidic pH on I_{Ks} of cardiac myocytes

Under pathophysiological conditions, the pH of ischemic cardiac tissue can be as low as 6.2 after 15 min of stop flow [35]. In order to test the possible pathophysiological significance of the KCNE1/KCNQ1 current during acidosis, we performed experiments on spontaneous beating and non-beating primary cultured ventricular cardiac myocytes from guinea pig. The KCNQ1 inhibitor HMR1556 was used to evaluate the impact of KCNE1/KCNQ1 (I_{Ks}) on the shape of the AP under control conditions and during acidic extracellular pH (without hypoxia). The membrane potential was measured in whole cell patch-clamp experiments (current-clamp-zero mode). At pH 7.4, HMR1556 (1 μ M) prolonged the AP duration at 90% repolarization (APD_{90}) by some 10-40%. Reduction of the bath pH from 7.4 to 5.5 changed AP shape and delayed the repolarization. Under these acidic conditions, HMR1556 (1 μ M) further lengthened the repolarization phase indicating that I_{Ks} plays an important role for repolarization especially under acidic conditions (Figure 5A). The shape of the cardiac AP is dependent on many different ion conductances and, therefore, changes in shape and duration of the AP don't allow for definite conclusions of pH-induced changes of the I_{Ks} current. For that reason, we performed another set of whole cell experiments on primary cultured and on

freshly isolated ventricular guinea pig cardiac myocytes. However, reduction of extracellular pH from 7.4 to 6.5 and 5.5 did neither change current shape nor current amplitude ($n=6$, Figure 5B) of the HMR1556-sensitive current component. To test, whether cardiac I_{Ks} is acid-insensitive in general, we performed additional experiments on rat and mouse cardiac myocytes. In freshly isolated ventricular rat cardiac myocytes, no detectable HMR1556-sensitive current was observed at external pH 7.4 and pH 5.5 ($n=8$). In primary cultured mouse cardiac myocytes, a very small I_{Ks} current (as measured as HMR1556 (1 μ M) sensitive current component) was detected. In contrast to guinea pig, however, the HMR1556-sensitive current was significantly increased by external acidosis (Figure 5C) suggesting that the acid insensitivity of I_{Ks} might be guinea pig specific.

Discussion

Heteromeric KCNE x /KCNQ1 potassium channels play an important role for hyperpolarization of the plasma membrane of cardiac myocytes and epithelial cells. The relevance of these channels has been highlighted by diseases which are linked to mutations of KCNQ1 or KCNE x , e.g. long QT syndromes 1, 5, and 6 [9, 16, 36], atrial fibrillation [25, 37, 38], and AMME contiguous gene syndrome (Alport syndrome, mental retardation, midface

hypoplasia, and elliptocytosis) [39]. To adapt KCNQ1 channels to the variable biological needs, the function of the pore-forming KCNQ1 alpha subunit is modified by small beta subunits (KCNE1-5) which regulate biophysical properties, pharmacology, and regulation of the channel complex [3, 6, 7, 18, 20, 31, 40]. KCNE α /KCNQ1 channels can be influenced by a variety of cellular factors and pathways, among them [41] intracellular pH (pH_i) and extracellular pH (pH_e) [11-13, 29, 31]. Apparently, the regulation of KCNQ1 channels by pH is rather complex and dependent on the subunit-composition. The aim of the present study was to analyze the mechanism by which extracellular pH regulates hKCNQ1 channels associated with hKCNE1, hKCNE2, and hKCNE3.

pH regulation of hKCNE2/hKCNQ1 channels

Homomeric hKCNQ1 channels have been shown to be inhibited by acidic extracellular pH [11, 12]. In contrast to homomeric channels, assembly of hKCNQ1 with hKCNE2 leads to a channel complex which is activated by acidic extracellular pH [29, 31]. Here, we performed a profound analysis of the H^+ concentration-dependent activation of hKCNE2/hKCNQ1 channels expressed in COS cells (Figure 1). In whole cell experiments, we confirmed the activation by acidic pH_e and we observed an inhibition by alkaline pH_e . Intracellular acidification, however, inhibited hKCNE2/hKCNQ1. In gastric parietal cells, this mechanism leads to stimulation of the channel during acid secretion via extracellular acidification and it reduces KCNE2/KCNQ1-dependent luminal K^+ export when the cytosol acidifies, e.g. due to pathological back-leakage of gastric acid into the intracellular compartment. In excised outside-out patches of transfected COS cells, the maximal channel current was observed at pH 4.5; and further acidification slightly reduced the current suggesting that hKCNE2/hKCNQ1 channels generate highest currents in the initial phase of acid secretion. We were not able to test more acidic pH because the COS cell membrane suffered from such drastic conditions. Interestingly, the hKCNE2_{R27C} “gain of function” mutant, which is related to atrial fibrillation [25], showed normal activation at pH 5.5 suggesting that the “gain-of-function” is not caused by mutation-induced mimicking of the “low pH confirmation” of wildtype hKCNE2. In contrast to acid sensitivity of hKCNE2/hKCNQ1, assembly of hKCNQ1 with hKCNE3 led to a completely pH-insensitive channel which underlines the importance of the type of KCNE subunit assembled with KCNQ1 for the modulation by pH.

Next, we were interested in identifying protein

domains of hKCNE2 responsible for the stimulation by acidic extracellular pH. For this purpose, we generated chimeras of the pH-insensitive hKCNE3 and acid-activated hKCNE2 (Figure 2 and 3). Although the pH effect differed between the various chimeras, all chimeras changed the slowly activating and deactivating homomeric hKCNQ1 channels into constitutively open channels indicating that they all were able to assemble with hKCNQ1. Deduced from the membrane topology of KCNE proteins, it was likely that the extracellular N-terminus of hKCNE2 is involved in mediating the acid activation. In fact, analysis of the respective chimera coexpressed with hKCNQ1 indicated, that the extracellular N-terminus plus at least part of the transmembrane domain is sufficient for extracellular pH sensitivity. Although desirable, a more precise identification of the molecular nature of the pH sensor was beyond the scope of the present work.

pH-induced conversion of biophysical properties of hKCNE1/hKCNQ1

Intracellular acidification has been shown to activate heteromeric KCNE1/KCNQ1 channels [13]. For extracellular acidification, the effects described in the literature are less homogenous and more complex: In one study, only little effects on current amplitude by extracellular acidification but a slow-down of activation and deactivation kinetics of KCNE1/KCNQ1 have been observed [12]. Another group has described strong inhibitory action of acidic pH in rat KCNE1/human KCNQ1 co-expressing CHO cells and dependence of the pH effect on the glycosylation status of KCNE1 [11]. In the present study, we have performed similar experiments in COS and CHO cells expressing hKCNE1/hKCNQ1. In contrast to the previous reports, we observed an increase in current amplitude and a H^+ concentration-dependent progressive change of activation and deactivation kinetics, finally leading to constantly open channels with a linear I/V relationship at pH 4.0. Although the kinetics of KCNE1/KCNQ1 at pH 4.0 did not resemble “conventional” hKCNE1/hKCNQ1 kinetics, use of the KCNQ1 blocker HMR1556 [42] indicated that hKCNQ1 underlies the instantaneous current at pH 4.0 (Figure 4D). Loss of voltage dependence and deactivation increasingly hyperpolarized the cells with a significant effect already at pH 7.0 (Figure 4) speaking in favor of the physiological importance of pH-induced conversion of kinetics. What are possible explanations for the differences between the data presented in previous reports [11, 12] and our results? Technical and methodological

variations (e.g. cell types and culture conditions, pipette and bath solutions, differences in expression levels for KCNE's and KCNQ1) might account for different results. Specifically, in the two other studies "leak subtraction" has been performed, which was not used for our experiments. Since acidic pH leads to a progressive conversion of the voltage-dependent slowly activating KCNE1/KCNQ1 current into a "leak-like" instantaneous current, part of the KCNE1/KCNQ1 current could have been considered as "leak current" and could have been subtracted (tributary on the way, "leak subtraction" has been performed). We decided to avoid leak subtraction in order to make sure that possible pH- or chimera-induced changes of ion selectivity were detectable. To exclude indirect effects of pH being responsible for the changes observed, acidic pH was also tested on outside/out patches of hKCNE1/hKCNQ1-co-expressing cells. Under these experimental conditions, pH 5.5 led to an increase of a "noisy" K^+ current which could be almost completely blocked by the KCNQ1 blocker HMR1556. At 300 Hz filtering frequency, we could not resolve single channel events suggesting that single channel amplitude of hKCNE1/hKCNQ1 was below 5 pS and/or very flickery, which is in agreement with previous reports [43-46]. Next we addressed the question whether the pH-mediated regulation, which we observed in expression systems, applies for cardiac myocytes where KCNE1/KCNQ1 underlies the slow component (I_{Ks}) of the delayed rectifier current.

Effects of acidic pH in guinea pig and mouse cardiac myocytes

To assess the role of KCNE1/KCNQ1 in native cells, we first used guinea pig cardiac myocytes which are known to express a relatively large I_{Ks} current component. Application of HMR1556 substantially prolonged the AP of spontaneously beating primary cultured guinea pig cardiac myocytes. During extracellular acidosis (pH 5.5), the prolongation of the AP by HMR1556 was more pronounced pointing to the important role of I_{Ks} for the repolarization of the AP under acidic conditions. Interestingly, in whole cell voltage-clamp experiments on primary cultured and freshly isolated guinea pig cardiac myocytes, we did not observe changes of current amplitude or activation/deactivation kinetics of the HMR1556 sensitive I_{Ks} current. What might be the cause of the discrepancy between acidic pH effects on hKCNE1/hKCNQ1 in COS or CHO cells and the absence of such effects on I_{Ks} of guinea pig cardiac myocytes? Possible explanations are: (i) other factors present in

guinea pig cardiac myocytes modify the pH sensitivity of KCNE1/KCNQ1 channels. Such factors of cardiac myocytes can be other members of the KCNE family (as shown for human heart [15]), i.e. KCNE3 (which is pH insensitive), KCNE4, and KCNE5 or accessory and regulatory factors, which are differentially expressed in guinea pig cardiac myocytes versus COS and CHO cells. (ii) Species differences between guinea pig KCNE1/KCNQ1 and human KCNE1/KCNQ1. Such species-dependent differences have been reported for the effect of protein kinase C on KCNE1 from mouse, rat, and guinea pig [47]. To test whether or not activation of KCNQ1-dependent current by acidic extracellular pH is absent in cardiac cells in general, we performed two sets of experiments on rat and mouse cardiac myocytes. The HMR1556-sensitive current was virtually absent in freshly isolated rat ventricular cardiac myocytes and it couldn't be activated by external acidosis. In primary cultured murine cardiac myocytes, the HMR1556-sensitive component was very small compared to guinea pig. However, the HMR1556-sensitive current was increased by acidic extracellular pH suggesting that acidic extracellular pH can increase KCNQ1 currents also in cardiac cells. Apparently, the size of the I_{Ks} current and the pH regulation of KCNE1/KCNQ1 in cardiac myocytes show species-dependent variability. The nature of these species differences is not yet clear and it could involve a variety of mechanisms, e.g. differences in KCNE1/KCNQ1 proteins themselves, co-assembly with other pH-insensitive KCNE x proteins and/or additional factors. Further studies will be needed for the investigation of the diversity of the pH effect in different species and its relevance in human cardiac myocytes.

Physiological significance of the pH regulation of heteromeric KCNQ1 channels

For KCNE2, the regulation by pH evidently makes sense: In gastric parietal cells, KCNE2/KCNQ1 is activated by external acidic pH and inhibited by intracellular acidification. By this mechanism, low pH within the gland lumen can act as positive feedback mechanism - at least if the pH is not too low. Inhibition of KCNE2/KCNQ1 by acidic intracellular pH could serve as a self-protection mechanism: It would reduce acid secretion of parietal cells which are unable to keep the intracellular pH stable, e.g. due to back-leakage of gastric acid through a damaged luminal membrane. KCNE3-containing KCNQ1 channels were acid-insensitive leading to a basolateral K^+ conductance in intestine and airways which is unaltered by basolateral pH changes. Regulation

of KCNE1/KCNQ1 by pH appears to be much more variable and complex: In our hands, extracellular and intracellular acidosis activated human KCNE1/KCNQ1 in COS and CHO cells. Extracellular acidosis, in particular, led to dramatic changes in channel kinetics similar to those observed by assembly of KCNQ1 with KCNE3. In cardiac myocytes, I_{Ks} could be of importance for repolarizing the action potential during extracellular acidosis - even in species which don't exhibit changes of channel amplitude or kinetics - because other repolarizing channels like HERG are inhibited by extracellular acidosis [48]. This would hold true especially for cardiac myocytes exposed to acidic environment but not to levels of hypoxia sufficient to activate K_{ATP} channels [49]. The acid-induced conversion of KCNE1/KCNQ1 during acidosis could mimic "gain-of-function" of KCNE2_{R27C}/KCNQ1 which

is related to atrial fibrillation [25].

In conclusion, KCNE subunit-dependent pH regulation appears to be an important physiological and pathophysiological mechanism for modifying critical functional and biophysical features of heteromeric KCNQ1 K^+ channel complexes.

Acknowledgements

The authors thank Dr. J. Barhanin, CNRS, Sophia-Antipolis, France, for kindly providing plasmids and for fruitful discussions. The support by the Deutsche Forschungsgemeinschaft (Wa1275/4-1 and SFB699) is gratefully acknowledged.

References

- Wang W, Xia J, Kass RS: MinK-KvLQT1 fusion proteins evidence for multiple stoichiometries of the assembled IsK channel. *J Biol Chem* 1998;273:34069-34074.
- Chen H, Kim LA, Rajan S, Xu S, Goldstein SA: Charybdotoxin binding in the I(Ks) pore demonstrates two MinK subunits in each channel complex. *Neuron* 2003;40:15-23.
- Schroeder BC, Waldegger S, Fehr S, Bleich M, Warth R, Greger R, Jentsch TJ: A constitutively open potassium channel formed by KCNQ1 and KCNE3. *Nature* 2000;403:196-199.
- Warth R, Barhanin J: The multifaceted phenotype of the knockout mouse for the KCNE1 potassium channel gene. *Am J Physiol* 2002;282:R639-R648.
- McCrossan ZA, Abbott GW: The MinK-related peptides. *Neuropharmacol* 2004;47:787-821.
- Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M, Romey G: K_v LQT1 and IsK (minK) proteins associate to form the I_{Ks} cardiac potassium current. *Nature* 1996;384:78-80.
- Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, Keating MT: Coassembly of K_v LQT1 and minK (IsK) proteins to form cardiac I_{Ks} potassium channel. *Nature* 1996;384:80-83.
- Tyson J, Tranebjaerg L, Bellman S, Wren C, Taylor JF, Bathen J, Aslaksen B, Sorland SJ, Lund O, Malcolm S, Pembrey M, Bhattacharya S, Bitner-Glindzicz M: IsK and KvLQT1 mutation in either of the two subunits of the slow component of the delayed rectifier potassium channel can cause Jervell and Lange-Nielsen syndrome. *Hum Mol Genet* 1997;6:2179-2185.
- Wang Q, Curran ME, Splawski I, Burn TC, Millholland JM, VanRaay TJ, Shen J, Timothy KW, Vincent GM, de Jager T, Schwartz PJ, Toubin JA, Moss AJ, Atkinson DL, Landes GM, Connors TD, Keating MT: Positional cloning of a novel potassium channel gene KVLQT1 mutations cause cardiac arrhythmias. *Nature Genet* 1996;12:17-23.
- Keating MT, Sanguinetti MC: Molecular and cellular mechanisms of cardiac arrhythmias. *Cell* 2001;104:569-580.
- Freeman LC, Lippold JJ, Mitchell KE: Glycosylation influences gating and pH sensitivity of $I(sK)$. *J Membr Biol* 2000;177:65-79.
- Peretz A, Schottelndreier H, Aharon-Shamgar LB, Attali B: Modulation of homomeric and heteromeric KCNQ1 channels by external acidification. *J Physiol* 2002;545:751-766.
- Unsold B, Kerst G, Brousos H, Hubner M, Schreiber R, Nitschke R, Greger R, Bleich M: KCNE1 reverses the response of the human K^+ channel KCNQ1 to cytosolic pH changes and alters its pharmacology and sensitivity to temperature. *Pflugers Arch* 2000;441:368-378.
- Lundquist AL, Manderfield LJ, Vanoye CG, Rogers CS, Donahue BS, Chang PA, Drinkwater DC, Murray KT, George AL, Jr: Expression of multiple KCNE genes in human heart may enable variable modulation of I(Ks). *J Mol Cell Cardiol* 2005;38:277-287.
- Bendahhou S, Marionneau C, Haurogne K, Larroque MM, Derand R, Szuts V, Escande D, Demolombe S, Barhanin J: In vitro molecular interactions and distribution of KCNE family with KCNQ1 in the human heart. *Cardiovasc Res* 2005;67:529-538.
- Abbott GW, Sesti F, Splawski I, Buck ME, Lehmann MH, Timothy KW, Keating MT, Goldstein SA: MiRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmia. *Cell* 1999;97:175-187.
- Grunnet M, Olesen SP, Klaerke DA, Jespersen T: hKCNE4 inhibits the hKCNQ1 potassium current without affecting the activation kinetics. *Biochem Biophys Res Commun* 2005;328:1146-1153.

- 18 Angelo K, Jespersen T, Grunnet M, Nielsen MS, Klaerke DA, Olesen SP: KCNE5 induces time- and voltage-dependent modulation of the KCNQ1 current. *Biophys J* 2002;83:1997-2006.
- 19 Toyoda F, Ueyama H, Ding WG, Matsuura H: Modulation of functional properties of KCNQ1 channel by association of KCNE1 and KCNE2. *Biochem Biophys Res Commun* 2006;344:814-820.
- 20 Tinel N, Diocot S, Borsotto M, Lazdunski M, Barhanin J: KCNE2 confers background current characteristics to the cardiac KCNQ1 potassium channel. *EMBO J* 2000;19:6326-6330.
- 21 Bleich M, Warth R: The very small conductance K⁺ channel K_vLQT1 and epithelial function. *Pflügers Arch* 2000;440:202-206.
- 22 Kunzelmann K, Mall M: Electrolyte transport in the mammalian colon mechanisms and implications for disease. *Physiol Rev* 2002;82:245-289.
- 23 Abbott GW, Butler MH, Bendahhou S, Dalakas MC, Ptacek LJ, Goldstein SA: MiRP2 forms potassium channels in skeletal muscle with Kv3.4 and is associated with periodic paralysis. *Cell* 2001;104:217-231.
- 24 Dias Da Silva MR, Cerutti JM, Arnaldi LA, Maciel RM: A mutation in the KCNE3 potassium channel gene is associated with susceptibility to thyrotoxic hypokalemic periodic paralysis. *J Clin Endocrinol Metab* 2002;87:4881-4884.
- 25 Yang Y, Xia M, Jin Q, Bendahhou S, Shi J, Chen Y, Liang B, Lin J, Liu Y, Liu B, Zhou Q, Zhang D, Wang R, Ma N, Su X, Niu K, Pei Y, Xu W, Chen Z, Wan H, Cui J, Barhanin J, Chen Y: Identification of a KCNE2 gain-of-function mutation in patients with familial atrial fibrillation. *Am J Hum Genet* 2004;75:899-905.
- 26 Dedek K, Waldegger S: Colocalization of KCNQ1/KCNE channel subunits in the mouse gastrointestinal tract. *Pflügers Arch* 2001;442:896-902.
- 27 Roepke TK, Anantharam A, Kirchhoff P, Busque SM, Young JB, Geibel JP, Lerner DJ, Abbott GW: The KCNE2 potassium channel ancillary subunit is essential for gastric acid secretion. *J Biol Chem* 2006;18:281:23740-7.
- 28 Lee MP, Ravenel JD, Hu RJ, Lustig LR, Tomaselli G, Berger RD, Brandenburg SA, Litzi TJ, Bunton TE, Limb C, Francis H, Gorelikow M, Gu H, Washington K, Argani P, Goldenring JR, Coffey RJ, Feinberg AP: Targeted disruption of the Kvlqt1 gene causes deafness and gastric hyperplasia in mice. *J Clin Invest* 2000;106:1447-1455.
- 29 Grahammer F, Herling AW, Lang HJ, Schmitt-Gräff A, Wittekindt OH, Nitschke R, Bleich M, Barhanin J, Warth R: The cardiac K⁺ channel KCNQ1 is essential for gastric acid secretion. *Gastroenterology* 2001;120:1363-1371.
- 30 Lambrecht NW, Yakubov I, Scott D, Sachs G: Identification of the K efflux channel coupled to the gastric H,K ATPase during acid secretion. *Physiol Genomics* 2005;21:81-91.
- 31 Heitzmann D, Grahammer F, von Hahn T, Schmitt-Gräff A, Romeo E, Nitschke R, Gerlach U, Lang HJ, Verrey F, Barhanin J, Warth R: Heteromeric KCNE2/KCNQ1 potassium channels in the luminal membrane of gastric parietal cells. *J Physiol* 2004;561:547-557.
- 32 Fink M, Lesage F, Duprat F, Heurteaux C, Reyes R, Fosset M, Lazdunski M: A neuronal two P domain K⁺ channel stimulated by arachidonic acid and polyunsaturated fatty acids. *EMBO J* 1998;17:3297-3308.
- 33 Isenberg G, Klockner U: Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium". *Pflügers Arch* 1982;395:6-18.
- 34 Volk T, Ehmke H: Conservation of L-type Ca²⁺ current characteristics in endo- and epicardial myocytes from rat left ventricle with pressure-induced hypertrophy. *Pflügers Arch* 2002;443:399-404.
- 35 Marzouk SA, Ufer S, Buck RP, Johnson TA, Dunlap LA, Cascio WE: Electrodeposited iridium oxide pH electrode for measurement of extracellular myocardial acidosis during acute ischemia. *Anal Chem* 1998;70:5054-5061.
- 36 Schulze-Bahr E, Wang Q, Wedekind H, Haverkamp W, Chen Q, Sun Y, Rubie C, Hordt M, Towbin JA, Borggreve M, Assmann G, Qu X, Somberg JC, Breithardt G, Oberti C, Funke H: KCNE1 mutations cause jervell and Lange-Nielsen syndrome. *Nature Genet* 1997;17:267-268.
- 37 Chen YH, Xu SJ, Bendahhou S, Wang XL, Wang Y, Xu WY, Jin HW, Sun H, Su XY, Zhuang QN, Yang YQ, Li YB, Liu Y, Xu HJ, Li XF, Ma N, Mou CP, Chen Z, Barhanin J, Huang W: KCNQ1 gain-of-function mutation in familial atrial fibrillation. *Science* 2003;299:251-254.
- 38 Ravn LS, Hofman-Bang J, Dixon U, Larsen SO, Jensen G, Haunso S, Svendsen JH, Christiansen M: Relation of 97T polymorphism in KCNE5 to risk of atrial fibrillation. *Am J Cardiol* 2005;96:405-407.
- 39 Piccini M, Vitelli F, Seri M, Galletta LJ, Moran O, Bulfone A, Banfi S, Pober B, Renieri A: KCNE1-like gene is deleted in AMME contiguous gene syndrome identification and characterization of the human and mouse homologs. *Genomics* 1999;60:251-257.
- 40 Grunnet M, Jespersen T, Rasmussen HB, Ljungstrom T, Jorgensen NK, Olesen SP, Klaerke DA: KCNE4 is an inhibitory subunit to the KCNQ1 channel. *J Physiol* 2002;542:119-130.
- 41 Shamgar L, Ma L, Schmitt N, Haitin Y, Peretz A, Wiener R, Hirsch J, Pongs O, Attali B: Calmodulin is essential for cardiac IKS channel gating and assembly impaired function in long-QT mutations. *Circ Res* 2006;98:1055-1063.
- 42 Gogelein H, Brüggemann A, Gerlach U, Brendel J, Busch AE: Inhibition of IKs channels by HMR 1556. *Naunyn Schmiedeberg's Arch Pharmacol* 2000;362:480-488.
- 43 Pusch M, Bertorello L, Conti F: Gating and Flickery Block Differentially Affected by Rubidium in Homomeric KCNQ1 and Heteromeric KCNQ1/KCNE1 Potassium Channels. *Biophys J* 2000;78:211-226.
- 44 Sesti F, Goldstein SA: Single-channel characteristics of wild-type IKs channels and channels formed with two minK mutants that cause long QT syndrome. *J Gen Physiol* 1998;112:651-663.
- 45 Romey G, Attali B, Chouabe C, Abitbol I, Guillemare E, Barhanin J, Lazdunski M: Molecular mechanism and functional significance of the minK control of the KvLQT channel activity. *J Biol Chem* 1997;272:16713-16716.
- 46 Yang Y, Sigworth FJ: Single-channel properties of IKs potassium channels. [published erratum appears in *J Gen Physiol* 1999 Mar;113(3):505] *J Gen Physiol* 1998;112:665-678.
- 47 Lo CF, Numann R: PKC modulation of minK current involves multiple phosphorylation sites. *Ann N Y Acad Sci* 1999;868:431-433.
- 48 Vereecke J, Carmeliet E: The effect of external pH on the delayed rectifying K⁺ current in cardiac ventricular myocytes. *Pflügers Arch* 2000;439:739-751.
- 49 Kane GC, Liu XK, Yamada S, Olson TM, Terzic A: Cardiac KATP channels in health and disease. *J Mol Cell Cardiol* 2005;38:937-943.