KCNQ4 channels expressed in mammalian cells: functional characteristics and pharmacology

RIKKE SØGAARD,1* TRINE LJUNGSTRÖM,1* KAMILLA ANGELO PEDERSEN,1 SØREN-PETER OLESEN,1,2 AND BO SKAANING JENSEN1,2

1Division of Cellular and Molecular Physiology, Department of Medical Physiology, University of Copenhagen, DK-2200 Copenhagen N; and 2NeuroSearch A/S, DK-2750 Ballerup, Denmark

Received 29 February 2000; accepted in final form 12 October 2000

KCNQ4 channels constitute a family of voltage-gated K+ channels with ~40% identity at the amino acid level. Besides the homology at the protein level, these K+ channels share biophysical characteristics, i.e., they activate and deactivate slowly, albeit with subtype differences. Furthermore, they all conduct a significant noninactivating current at negative membrane potentials. KCNQ1 channels are partly responsible for the repolarization of cardiac myocytes and are also involved in secretion of endolymph in the inner ear (18) as well as epithelial transport (7, 22, 29). KCNQ2 and KCNQ3 can form heteromeric channels, and KCNQ2+3 channels have been suggested to be the molecular constituent of the classic M current recorded in native cells because they show similar biophysical and pharmacological characteristics (28). The M current is an important regulator of neuronal excitability because it tunes the membrane potential about threshold level (9, 15, 16).

The KCNQ4 channel is expressed at high levels in the outer hair cells of the cochlea and in a number of nuclei in the brain stem (10), and mutations in the gene encoding KCNQ4 are known to underlie a form of nonsyndromic dominant deafness (8, 12). KCNQ4 is also expressed at significant levels in brain, heart, and skeletal muscle as determined by Northern analysis (12). KCNQ4 channels share some of the characteristics of KCNQ2+3 channels and of the native M current, but recordings of KCNQ4 channels expressed in Xenopus oocytes suggest that there may also be incongruities such as a sensitivity to M current blockers that is too low and an activation threshold that is too positive (12). However, M currents recorded in native cells also show variable characteristics. For this reason some of them have been called M-like currents, and it is likely that these currents reflect several different molecular entities (17, 22, 27). Because the role of M currents and M-like currents in excitable cells is well-characterized, and the functional effects of blockers of these currents are well-known, it is important to establish whether KCNQ4 qualifies as a candidate for an M-like current.

In the present study we found that KCNQ4 channels expressed in mammalian cells show characteristics that are significantly more M-like than when expressed in Xenopus oocytes, i.e., sensitivity to XE-991 and linopirdine in the low micromolar range and an activation threshold of about −60 mV, with the latter being important for its function.
MATERIALS AND METHODS

Stable expression of KCNQ4 channels in HEK-293 cells. KCNQ4 was the kind gift of Dr. Thomas Jentsch in the Xenopus oocyte expression vector pTLN. KCNQ4 was excised from pTLN using HindIII and XhoI and subcloned into the mammalian expression vector pNSIn (NeuroSearch), a custom-designed derivative of pcDNA3neo (Invitrogen). HEK-293 cells (American Type Culture Collection) were grown in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies) at 37°C in 5% CO₂. One day before transfection, 10⁶ cells were plated in a cell culture T25 flask (Nunc). Cells were transfected with 2.5 μg of the plasmid pNSIn_KCNQ4 by using Lipofectamine (Life Technologies) according to the manufacturer’s instructions. Cells transfected with pNSIn_KCNQ4 were selected in media supplemented with 0.5 mg/ml genetin (G418; Life Technologies). Single clones were picked and propagated in selection media for five passages, after which they were considered stable. Subsequently, the cells were cultured in regular medium without selection agent. Expression of functional KCNQ4 channels was verified by patch-clamp measurements.

Electrophysiology. Transfected cells were cultured on glass coverslips (diameter 3 mm), which were transferred to a small recording chamber. The chamber was perfused with extracellular solutions at a rate of 1 ml/min, giving rise to full exchange of the chamber volume (15 μl) each second. Whole cell currents were recorded with the use of a HEKA EPC-9 amplifier and borosilicate glass micropipettes with tip resistances of 1.5–3 MΩ. The fast and slow capacitances were automatically compensated by using the standard procedures of the HEKA amplifier (trains of 5-mV square wave pulses, averaging of the resulting current, and fitting of an exponential to deduce the compensation values required to cancel the capacitative currents). The slow capacitance and the series resistance were determined before each sweep. If the cancellation failed or the series resistance rose above 10 MΩ, the experiment was discarded. The series resistance was compensated by 70%. The currents were not leak-subtracted. The sampling interval was 400 μs, and the signals were low-pass filtered with a cut-off frequency of 1,000 Hz. The sampling interval was 400 μs, and the signals were low-pass filtered with a cut-off frequency of 1,000 Hz. The KCNQ4 current exhibited some rundown during the first 10–30 ms after addition of the physiological solution, and then stabilized at a steady current level that was always obtained before electrophysiological analysis or drug application. All experiments were performed at room temperature (21–25°C). Igor software (WaveMetrics, Lake Oswego, OR) was used for the analysis.

Calculations. Steady-state activation curves were fitted to Boltzmann functions as follows

$$I_{\text{tail}}(V_m) = I_{\text{tail}}(\infty)/(1 + \exp[-(V_m - V_{1/2})/S])$$

where $V_m$ is the membrane potential, $V_{1/2}$ is the half-activation potential, and $S$ is the slope factor ($kT/e$, where $e$ is the equivalent charge). $I_{\text{tail}}(\infty)$ is the maximal tail current. Current and membrane potential levels are stated as means ± SE.

Solutions and chemicals. One intracellular pipette solution and two extracellular solutions were used. The composition of the intracellular solution was (in mM) 110 KCl, 10 EGTA, 30 KOH, 5.1 CaCl₂, 2.8 MgCl₂, 1.6 ATP, and 10 HEPES (pH 7.2). The free Ca²⁺, Mg²⁺, and ATP concentrations were calculated to be 100 nM, 1 mM, and 200 μM, respectively (Eqcalc; Biosoft, Cambridge, UK). The composition of the extracellular physiological solution was (in mM) 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4). The composition of the extracellular high-K⁺ solution was (in mM) 144 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4). Linopirdine and clofilium tosylate were purchased from RBI, and bepridil was from Sigma. XE-991 was synthesized at NeuroSearch.

RESULTS

Biophysical characteristics of the KCNQ4 channel. Functional expression of KCNQ4 channels in the HEK-293 cells was studied by whole cell patch-clamp measurements. The cells were voltage clamped at −80 mV and stepped to potentials ranging from −100 to +80 mV, which gave rise to slowly activating currents as shown in Fig. 1A, left. After the 2-s duration command pulse, the cells were clamped at −30 mV, at which potential these voltage-activated channels conducted a significant outward current. Native HEK-293 cells express endogenous K⁺ currents (31), and a voltage-activated current was activated by the protocol used. This current is shown in Fig. 1A, right, together with the leak current. The endogenous current was inactivating in contrast to that of KCNQ4, and it activated at
more positive potentials (threshold −20 mV). The current-voltage (I-V) curves of the endogenous current and KCNQ4 current are shown in Fig. 1B. The current density of the KCNQ4 current measured at +140 mV in the extracellular physiological solution was 246 ± 17 pA/pF (n = 5), whereas that of the endogenous current was 6 ± 1 pA/pF (n = 13), i.e., the endogenous current constituted <2.5% of the measured current and could thus be neglected.

In symmetrical high-K⁺ solutions, a similar slowly activating KCNQ4 current was seen with a significant inward current at potentials more positive than +60 mV (Fig. 2A). The steady-state I-V curve was linear at potentials positive to +20 mV. In contrast, the instantaneous I-V curve shown in Fig. 2B reveals a significant inward rectification with a slope conductance at +80 mV of 222 nS compared with 38 nS at +180 mV, i.e., a 5.8-fold larger inward conductance. The following voltage-clamp experiments were all conducted in symmetrical high-K⁺-containing solutions.

The steady-state activation curve of KCNQ4 was determined by stepping from a holding potential of −80 mV to a number of potentials from −110 mV to +90 mV for 2 s, followed by a step to −120 mV, at which potential the peak tail current was measured (Fig. 3A). The peak tail current was plotted as a function of the activation potential and normalized to the maximal tail current. Figure 3A shows that the KCNQ4 channels conducted a significant steady-state current at a potential of −50 mV, and the half-activation potential was determined to be −32 mV. By fitting the curves to a single Boltzmann function, the steepness of the activation curve S = 17.4 mV was also determined, which corresponds to a gating charge of 1.4 elementary charges, similar to the finding by Kubisch et al. (12). It is worth noting that the equivalent gating charge is far less than the 13 charges that have been determined for the Shaker type of voltage-gated K⁺ channels (2), but it is quite close to the 1.2 charges determined for the voltage- and Ca²⁺-gated large-conductance K⁺ (BK) channels (23).

The KCNQ4 channels activated relatively slowly (Figs. 1A, 2A, and 3A). The activation kinetics showed two clear time constants, which for steps from −80 to +40 mV were 45 ± 7 and 531 ± 57 ms (n = 4) and for steps from −80 to +80 mV were 45 ± 5 and 636 ± 74 ms (n = 3), respectively (means ± SE, symmetrical K⁺, Fig. 2A). No inactivation was determined with the use of a protocol with 1-s steps to voltages ranging from −100 to +60 mV, followed by a 2-s step to +60 mV (Fig. 3B, n = 5). In a series of experiments in which we used 500-ms prepulses ranging from −80 to −160 mV, we did not find any indication that the KCNQ4 channel could be partly inactivated at the holding potential of −80 mV (data not shown).

Pharmacology of KCNQ4. The effects on KCNQ4 of XE-991 and linopirdine (KCNQ2/3 channel blockers) and of bepridil and clofilium (KCNQ1 channel blockers) were studied in whole cell configuration. XE-991 was administered to the bath at increasing concentra-
tions from 0.1 to 100 μM, leading to a concentration-dependent block with an IC$_{50}$ of 5.5 μM and a Hill coefficient of 0.76 (Fig. 4, $n = 3–6$ at each concentration). The degree of block was independent of whether it was determined from the outward plateau current at +40 mV or from the inward tail current at −120 mV. No significant voltage dependency of the block was observed by using a tail-current protocol similar to the one shown in Fig. 3A (data not shown). Linopirdine, the parent compound of XE-991, was studied for blocking effect at five concentrations ranging from 0.2 to 200 μM ($n = 2–11$ at each concentration), and the fit to the blocking curve gave an IC$_{50}$ value of 14 μM and a Hill coefficient of 0.92 (Fig. 5). The KCNQ4 currents showed initial rundown, as shown in Figs. 4A and 5A, and although the drug application was not started before this rundown had leveled out, it may have led to a slight overestimation of the drug affinity.

Bepridil (1–100 μM) inhibited the KCNQ4 current in a concentration-dependent manner (Fig. 6A), and the IC$_{50}$ value was estimated at 9.4 μM ($n = 5–8$ at each concentration) with a Hill coefficient of 0.96. The time course of the inhibition experiments, as shown in Fig. 6A, was interrupted for short periods at each concentration of bepridil to run a full plateau- and tail-current analysis (Fig. 6B). Clofilium showed a weak affinity for the KCNQ4 channel with an IC$_{50}$ of 100 μM ($n = 6$).

**Effects of KCNQ4 currents on membrane potential.** The membrane potential of the HEK-293 cells was measured in current-clamp mode in physiological solutions. KCNQ4-transfected HEK-293 cells had an average resting membrane potential of −56 ± 2 mV ($n = 25$) in contrast to native HEK-293 cells, which had a resting membrane potential of −12 ± 1 mV ($n = 6$; Fig. 7A). Administration of the channel blockers (100 μM XE-991, 100 μM bepridil, or 200 μM linopirdine) had a depolarizing effect on the membrane potential, verifying that the KCNQ4 channels contribute markedly to the setting of the membrane potential of HEK-KCNQ4 cells. The effect of bepridil on the resting membrane potential showed an immediate onset and was readily reversible (Fig. 7B).

**DISCUSSION**

Expression of the human KCNQ4 channels in HEK-293 cells gave rise to voltage-gated currents that activated and deactivated slowly. The channels showed no inactivation during 2-s pulses, a pronounced inward rectification, and high sensitivity to the channel blockers XE-991, linopirdine, and bepridil. Expression of KCNQ4 channels in the HEK-293 cells mediated a hyperpolarization of the cells, which was counteracted by the channel blockers.

The KCNQ4 channels expressed in HEK-293 cells activated at potentials positive to −60 mV (Figs. 1–3), and full activation was seen at +30 mV. Fitting the activation curve to a single Boltzmann function showed that half-activation was obtained at −32 mV. The characteristics of these channels indicate that the KCNQ4 channels expressed in HEK-293 cells activate...
channels in native tissue, this finding suggests that the function of the KCNQ4 is not to set the resting membrane potential but, rather, to contribute to the regulation of excitability by conducting a significant current near the threshold for firing of action potentials.

The instantaneous *I-V* relationship of KCNQ4 showed, surprisingly, that the current rectified about sixfold stronger in the inward than in the outward direction. Slight rectification has been shown for KCNQ4 as well as KCNQ2 at very positive potentials (3, 12).

The activation kinetics of KCNQ4 clearly showed two components, one with a time constant of 20–45 ms and another with a time constant of 200–700 ms. The two components may be related to the fast and slow gating mode, as has been described for single M channels (14).

The functional hallmarks of KCNQ channels are the negative activation threshold and the relatively slow kinetics. Pharmacologically, they are characterized by a high sensitivity to the compounds XE-991 and linopirdine.
nopirdine. The sensitivities of KCNQ1, KCNQ2, and KCNQ2+3 to XE-991 range from 0.6 to 0.8 μM, and those to linopirdine range from 4 to 9 μM (28). We found that the two compounds were slightly less potent as blockers of KCNQ4, with IC50 values of 5.5 and 14 μM for XE-991 and linopirdine, respectively. It is remarkable that linopirdine has been found to be significantly weaker when studied on channels expressed in Xenopus oocytes, which showed 30% block of KCNQ4 and 75% block of KCNQ3+4 by 200 μM linopirdine (12). Differences of up to 100-fold in pharmacological potencies of channel blockers have likewise been observed for human ether-a-go-go-related gene (HERG) and small-conductance Ca2+-activated K+ (SK) channels when expressed in mammalian cells vs. Xenopus oocytes (11, 24, 26, 33).

Linopirdine and XE-991 are cognition enhancers (32) that probably exert their effect by blocking the K+ conductance in neuronal cells that is called M current (1, 28). The M current is a slowly activating and deactivating current that regulates neuronal excitability and responsiveness to synaptic input by conducting a significant hyperpolarizing current at subthreshold potentials (4, 15). The native M currents resemble the KCNQ currents, and in particular, it has been shown that the characteristics of the native M current in rat sympathetic ganglia resemble those of the heteromeric KCNQ2+3 channels (28). In NG108-15 neuroblastoma cells and in mouse superior cervical ganglion cells, the M current may be a composite current conducted by

---

**Fig. 6.** Effect of bepridil on KCNQ4 current. **A**: time course of an experiment in which the cell was superfused with 1, 5, 10, 50, and 100 μM bepridil, as indicated by bars. Pulse protocol and tail current measurements are as described in Fig. 4A. **B**: in the presence of each concentration of bepridil, the experiment was interrupted to characterize plateau currents at voltages from –110 to 70 mV (left) and the associated tail currents elicited at –120 mV (right).

**Fig. 7.** Influence of KCNQ4 on resting membrane potential. Current-clamp experiments were performed on nontransfected (A) and KCNQ4-transfected (B) HEK-293 cells. The cells were superfused with high-K+ extracellular solution (exK) at the beginning and end of the experiment. The resting membrane potential of the cells was recorded during superfusion with the physiological Ringer solution (exNa). In B, 100 μM bepridil was added to the extracellular solution for 1.5 min and washed out for the following 2.5 min.
KCNQ2+3 as well as by erg K+ channels (17, 22). The concept is that M currents encompass a number of M current-like conductances, which vary significantly in kinetics and pharmacology, and are present in a number of tissues including brain, sympathetic ganglia, retina, and muscle (5, 13, 27). The molecular channels underlying these currents are probably quite heterogeneous and may be composed of different subunits from the KCNQ and erg families at least. A relevant question would be whether KCNQ4 alone or in conjunction with other subunits could underlie an M-like current. The activation and deactivation kinetics of KCNQ4 are similar to those of M-like currents, and the currents activate at similar voltages (V_{1/2} for M current: ~35 to ~44 mV). The M current and the KCNQ4 current both show significant rundown. The pharmacology of M currents and M-like currents display significant variation, as shown by the sensitivities to linopirdine, which vary with IC_{50} from 1.2 \mu M in NG108-15 neuroblastoma cells to 3.5 \mu M in mouse superior cervical ganglia (22), 7.0 \mu M in rat superior cervical ganglia (28), 25 \mu M in NGPM1-27 cells, and 36 \mu M in rat cortical pyramidal neurons (19). The sensitivity of KCNQ4 to linopirdine falls well within this range. We suggest that KCNQ4 serves the function of an M-like current in the nervous system and may constitute part of this composite current.

The function of KCNQ4 channels could be slightly different in other tissues. The present KCNQ4 channel has been cloned from retina (12), in which the photoreceptors express M-like currents (I_{Kc}), probably contributing to the setting of the resting membrane potential and accelerating the response to dim light (13). In the inner ear and cochlea, where the KCNQ channels are heavily expressed in the outer and type I hair cells, KCNQ4 has been suggested to represent the K+ currents I_{Kc} and g_{K,L}, which are already open at resting membrane potential (8, 10, 12). In the heart, the KCNQ4 channel would probably not influence the threshold potential but, rather, would contribute to the repolarization in a manner similar to KCNQ1, where it shares bepridil, but not clofilium, sensitivity.

Expert technical support from Inge Kjeldsen and Pernille O. Hulgaard is gratefully acknowledged.

This work was supported by the Danish Medical Research Council (9701799), The Vera and Carl Johan Michaelsen Foundation, and the Danish Heart Association (S.-P. Olesen). R. Søgaard holds a fellowship from the Danish Natural Sciences Research Council. Hulgaard is gratefully acknowledged.

REFERENCES


