Molecular mechanism for an inherited cardiac arrhythmia

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In the congenital long-QT syndrome, prolongation of the cardiac action potential occurs by an unknown mechanism[1,2] and predisposes individuals to syncope and sudden death as a result of ventricular arrhythmias[3]. Genetic heterogeneity has been demonstrated for autosomal dominant long-QT syndrome by the identification of multiple distinct loci[4,5], and associated mutations in two candidate genes have recently been reported[6,7]. One form of hereditary long QT (LQT3) has been linked to a mutation[7] in the gene encoding the human heart voltage-gated sodium-channel α-subunit (*SCN5A* on chromosome 3p21)[8]. Here we characterize this mutation using heterologous expression of recombinant human heart sodium channels. Mutant channels show a sustained inward current during membrane depolarization. Single-channel recordings indicate that mutant channels fluctuate between normal and non-inactivating gating modes. Persistent inward sodium current explains prolongation of cardiac action potentials, and provides a molecular mechanism for this form of congenital long-QT syndrome.

Figure 1 shows two-electrode voltage-clamp recording from *Xenopus laevis* oocytes expressing either wild-type (WT) human heart Na^+ channel (hH1) or the LQT3 mutation designated ΔKPQ (in-frame deletion of residues Lys 1,505, Pro 1,506, Gln 1,507 in *SCN5A*). Normalized tracings of both ΔKPQ and wild type demonstrate similar rapid macroscopic inactivation and each exhibit a similar biexponential time course. Remarkably, we found that time constants for current decay were decreased in the ΔKPQ mutant (WT-hH1: $\tau_{fast} = 1.47 \pm 0.11$ ms, $\tau_{slow} = 8.59 \pm 0.71$ ms, $n = 18$; ΔKPQ: $\tau_{fast} = 0.98 \pm 0.07$ ms, $\tau_{slow} = 5.40 \pm 0.55$ ms, $n = 13$; $P < 0.01$; data are means ± s.e.m.) not increased as was predicted[7] on the basis of deletion mutagenesis work performed with rat brain IIA Na^+ channels[9]. However, unlike WT-hH1, currents recorded in ΔKPQ-expressing cells did not decay completely within a 200-ms depolarization. This persistent current represented less than 5% of the peak inward current at −20 mV, and was blocked by 30 μM tetrodotoxin (TTX) (Fig. 1, insets). Another surprising feature of the ΔKPQ mutant was a significant shift in the membrane potential at which 50% of the current is inactivated (WT: -74.6 ± 0.9 mV, $n = 16$; ΔKPQ: -80.4 ± 1.1 mV, $n = 19$; $P < 0.002$). The slope factor for this relationship was not changed (WT: 4.35 ± 0.13 mV, $n = 16$; ΔKPQ: 4.22 ± 0.16, $n = 19$). In LQT cardiac myocytes, this shift in steady-state inactivation midpoint would decrease the availability of the mutant channel relative to WT-hH1 at the resting membrane potential. There was no observable difference in the kinetics of recovery from inactivation between WT and mutant Na^+ channels (data not shown). The same qualitative results were observed in one other independent mutant clone.

To understand fully the functional disturbance conferred by the ΔKPQ mutation, we performed single-channel analysis on both wild-type and mutant Na^+ channels. Figure 2 shows data from experiments using inside-out excised membrane patches. The WT-hH1 channels opened briefly (<1 ms) only once or a very few times during depolarization and these openings always occurred within the first 20 ms (Fig. 2*a*, *c*). Single-channel records obtained from oocytes expressing ΔKPQ channels demonstrated a similar frequency of initial openings, but mutant channels would occasionally exhibit multiple reopenings ('bursts') occurring much later than 20 ms (Fig. 2*b*, *d*). This intermittent reopening behaviour gave rise to a small sustained inward Na^+ current, as revealed by the ensemble average (Fig. 2*e*, *f*). In data obtained from three separate membrane patches (total 400 sweeps), bursting occurred in 3.5 ± 1.3% of sweeps recorded from ΔKPQ-expressing cells, but was not seen in WT-

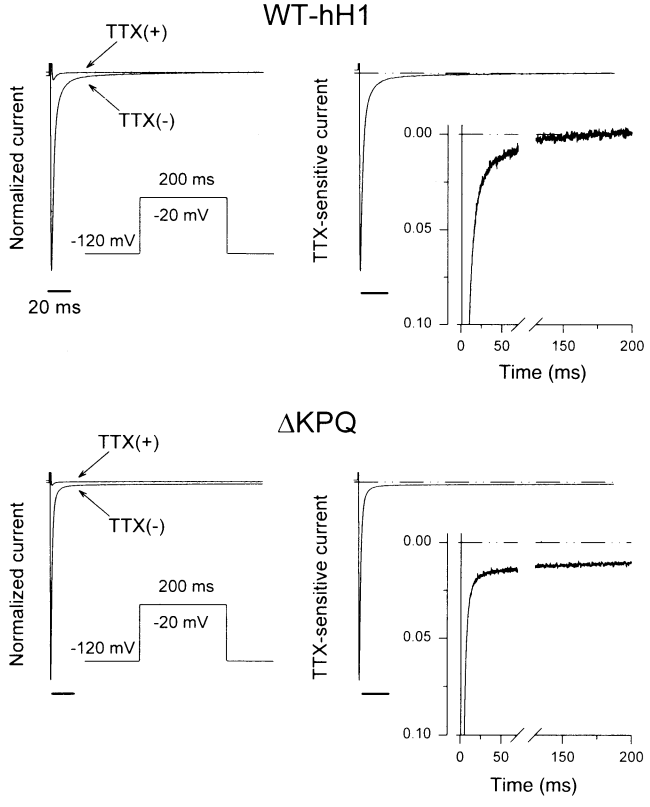

FIG. 1 Sodium currents recorded in *Xenopus* oocytes expressing either WT-hH1 (top) or ΔKPQ (bottom). Representative current traces obtained in the presence or absence of 30 μM TTX and recorded during a 200-ms test depolarization to −20 mV from a holding potential of −120 mV (left panels). Peak current amplitudes are normalized to that obtained in the absence of TTX. The result of subtraction of currents obtained in the presence from those in the absence of TTX are shown in the right panels. The same subtracted data are shown on an expanded scale in the insets. Sodium-current expression was similar in WT and ΔKPQ RNA-injected oocytes.

METHODS. Deletion of amino-acid residues 1,505–1,507 in the recombinant human heart Na^+-channel α-subunit (hH1) was accomplished by overlap extension polymerase chain reaction mutagenesis[19], and the final product was assembled into the plasmid pSP64T-hH1 (ref. 20). Multiple independent recombinants were selected, analysed by digestion with restriction enzymes, and the mutant inserts sequenced in their entirety. Clones were identified that were free of polymerase errors and used for electrophysiological studies. *In vitro* transcription reactions, microinjection of *Xenopus* oocytes, and two-electrode voltage-clamp recording were done as described[21]. Oocytes were injected with 20 ng RNA.

hH1 under the same experimental conditions. The single most striking feature of the ΔKPQ mutant channels was their propensity to reopen repeatedly at times much later than 20 ms, in contrast to WT-hH1. We specifically compared the late-opening behaviour of the mutant and wild-type channels occurring between 30–200 ms after the onset of the test depolarization. During this time interval, the probability of WT-hH1 channel openings was zero (data not shown). In contrast, mutant channels displayed a substantial open probability throughout the period (Fig. 3*a*). The abnormal gating behaviour displayed by the ΔKPQ channel was not associated with a change in the duration of single-channel openings (Fig. 3*b*, *c*).

We used computer simulations of Na^+-channel gating based upon multistate Markov gating models to test specific hypotheses put forth to explain the abnormal behaviour of the ΔKPQ channel (Fig. 4). Our data can be used to exclude the mechanisms shown in Fig. 4*b*–*d*. Therefore, we considered an alternative hypothesis: modal gating (Fig. 4*e*, *f*). Modal gating appears to be a general kinetic feature of mammalian Na^+ channels[10–13], but in contrast to the behaviour of normal cardiac Na^+ channels, which exhibit rare (<0.1%) transitions into a non-inactivating gating mode[14], the ΔKPQ mutant entered this mode in 3.5% of depolarizations. A simplified model for this hypothesis is shown in Fig. 4*e* and *f*. Here the channels gate normally most of the

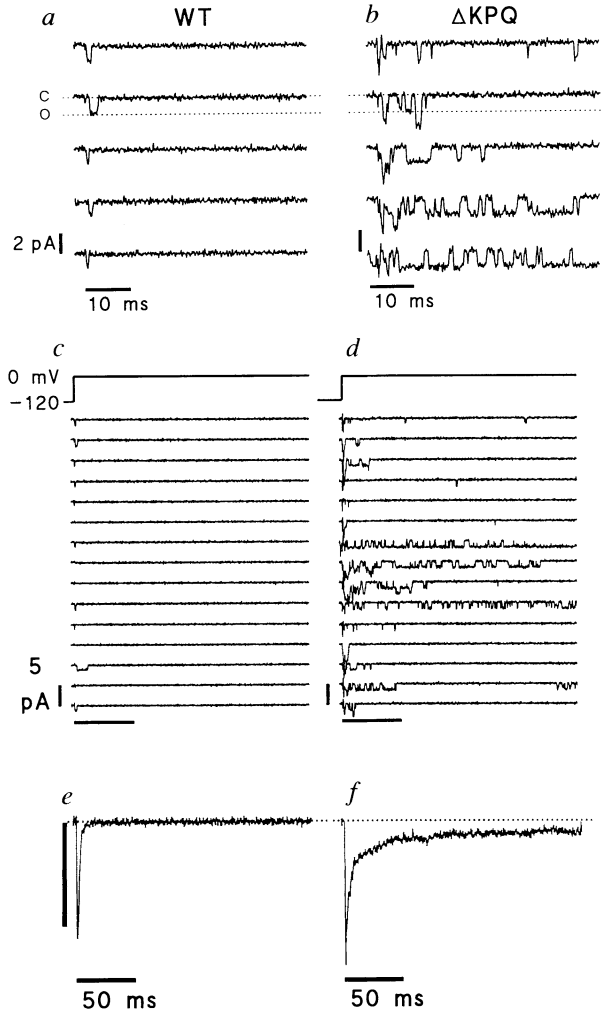

FIG. 2 Single-channel recordings from Na^+ channels in inside-out membrane patches excised from *Xenopus* oocytes during 200-ms duration voltage-clamp steps. *a*, Selected recordings of unitary WT-hH1 Na^+-channel current (data from the first 50 ms are shown). *b*, Selected recordings of unitary ΔKPQ mutant Na^+ channel current (data from the first 50 ms are shown). *c*, Records from WT-hH1 channels during 200-ms voltage-clamp steps. *d*, Records from ΔKPQ channels during 200-ms voltage-clamp steps. Time calibration bars in *c* and *d* represent 50 ms. *e*, Ensemble average of WT-hH1 Na^+ channel current. The vertical axis was scaled to open probability (P_{open}) by dividing the ensemble average current by the unitary current (i) and the number of channels in the patch (N). Vertical calibration bar represents P_{open} of 0.2. *f*, Ensemble average of ΔKPQ mutant Na^+-channel current.

METHODS. Oocyte vitelline membranes were removed by hypertonic stripping[22]. Single Na^+ channels were recorded in the inside-out configuration[23] and analysed as described[24]. Currents were recorded during voltage steps to 0 mV from a holding potential of −120 mV. Single Na^+-channel currents were filtered at 2 kHz and digitized at 10 kHz. Patch pipettes were pulled from borosilicate glass and filled with (in mM) 145 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.35. The bath solution ('intracellular') contained (in mM) 110 CsF, 10 NaF, 20 CsCl, 2 EGTA, 10 HEPES, pH 7.35.

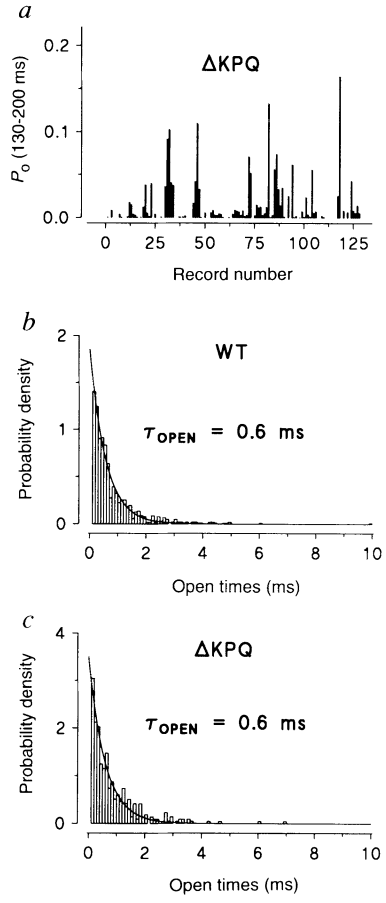

FIG. 3 Open-probability diaries and open-time distributions. *a*, Open probability in each record for ΔKPQ mutant channels 30–200 ms after a voltage step to −20 mV. No openings of WT-hH1 were observed during the interval 30–200 ms ($P_{open} = 0$). *b*, Distribution of open times for WT-hH1 recorded at −20 mV. *c*, Distribution of open times for ΔKPQ mutant recorded at −20 mV.

METHODS. Unitary current was estimated from all points amplitude histograms and open times were measured using a 50% threshold-crossing criterion as described[24]. Open-time distributions were made by sorting open durations into bins and plotting (or fitting) the number of openings per bin as a function of open time. Probability density was calculated by dividing the number of events in each bin by the total number of events and the bin width. Diaries were made by integrating the current ($N \times P_o \times i \times s$) in each record and dividing by the single channel current (i), the number of channels in the patch (N) and the duration of the record.

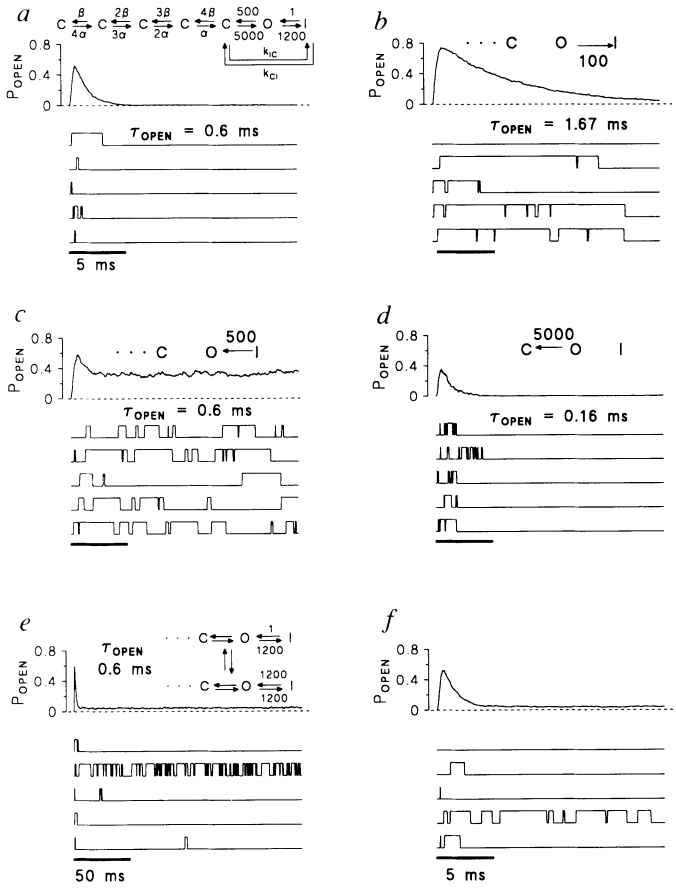

FIG. 4 Predicted behaviour of Na^+-channel gating based on multistate Markov gating models. For simplicity, we adopted a Na^+-channel model having a single open state[25]. *a*, WT gating model. In alternative models (*b–f*), only changes from the WT-model rate constants are shown. Predicted open times are shown in each panel. *b*, A 12-fold decrease of the rate constant entering the inactivated state causes slower macroscopic current decay and prolonged open times. *c*, A 500-fold increase of the rate constant leaving the inactivated state causes channels to return readily from the inactivated state. There is no effect on the open times, but the channels open and close continuously. Smaller increases in this rate constant did not cause a sufficient number of reopenings. *d*, An increase of the rate constant returning to the first closed state from the open state causes numerous open–closed transitions. However, this mechanism also shortens the open times. *e*, *f*, Modal gating model. The channel randomly enters an alternate gating mode with a probability of 0.05. In the alternate gating mode, the inactivation rate constant was unchanged. The rate constant for returning to the open state from the inactivated state was increased from 1 to 1,200 s^{-1}. All rate constant are given in units of s^{-1}.

METHODS. WT-hH1 was modelled as a series of closed states preceding a single open state followed by an inactivated state; τ_{open} is the mean open time. Typical single-channel openings and the ensemble open-channel probability are shown as functions of time. Forward rate constant α was 10,000 s^{-1}; reverse rate constant β was 100 s^{-1}; k_{IC} and k_{CI} are the rate constants going to or returning from the inactivated state to the first closed state, respectively; k_{IC} was set to 1,000 s^{-1} and k_{CI} was adjusted to enforce microscopic reversibility.

time, but occasionally enter an altered gating mode where the channel bursts for prolonged periods. Substantial evidence implicates the cytoplasmic region linking repeat domains 3 and 4 (ID3-4) in Na^+-channel inactivation[15,16]. It has been postulated that this region forms the inactivation gate of the Na^+ channel by forming a lid that closes over the open ion-conducting pore, causing inactivation. One possible molecular mechanism for the altered gating caused by the ΔKPQ deletion is that the flexibility of the ID3-4 is modified, which effectively increases the unbinding rate of the ID3-4 from its docking site.

Long-QT syndrome is the first recognized inherited myocardial ion-channel disease. Our demonstration of a disturbance in Na^+-channel function is necessary and sufficient proof that ΔKPQ can cause the LQT phenotype. In cardiac myocytes, the observed abnormal Na^+-channel gating will result in a small fraction of persistent inward Na^+ current flowing during the plateau phase of the cardiac action potential. The plateau is normally maintained by a delicate balance between inward and outward currents[17], and repolarization occurs as activating outward currents prevail over inactivating inward currents. Dysfunction of an outward potassium current is presumed to cause one form of congenital LQT (LQT2)[18]. In LQT3, the sustained inward current generated by ΔKPQ will prolong action potential duration by shifting this balance in favour of inward current, and cause lengthening of the Q–T interval. This sustained inward Na^+ current might also promote other inward currents (calcium current, electrogenic sodium–calcium exchange), thus amplifying the QT-prolonging effect of the mutation. □

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