Defective “pacemaker” current ($I_h$) in a zebrafish mutant with a slow heart rate

KEITH BAKER†‡, KERRI S. WARREN§§, GARY YELLEN*, AND MARK C. FISHMAN¶¶

Departments of *Neurobiology and †Anesthesia and the ‡Cardiovascular Research Center, Harvard Medical School and Massachusetts General Hospital, Boston, MA 02114

Communicated by Alexander Leaf, Massachusetts General Hospital, Charlestown, MA, February 21, 1997 (received for review November 8, 1996)

ABSTRACT At a cellular level, cardiac pacemaking, which sets the rate and rhythm of the heartbeat, is produced by the slow membrane depolarization that occurs between action potentials. Several ionic currents could account for this pacemaker potential, but their relative prominence is controversial, and it is not known which ones actually play a pacemaking role in vivo. To correlate currents in individual heart cells with the rhythmic properties of the intact heart, we have examined slow mo (smo), a recessive mutation we discovered in the zebrafish Danio rerio. This mutation causes a reduced heart rate in the embryo, which we can quantitate because the embryo is transparent. We developed methods for culture of cardiocytes from zebrafish embryos and found that, even in culture, cells from smo continue to beat relatively slowly. By patch-clamp analysis, we discovered that a large repertoire of cardiac currents noted in other species are present in these cultured cells, including sodium, T-type, and L-type calcium and several potassium currents, all of which appear normal in the mutant. The only abnormality appears to be in a hyperpolarization-activated inward current with the properties of $I_h$, a current described previously in the nervous system, pacemaker, and other cardiac tissue. smo cardiomyocytes have a reduction in $I_h$ that appears to result from severe diminution of one kinetic component of the $I_h$ current. This provides strong evidence that $I_h$ is an important contributor to the pacemaking behavior of the intact heart.

During the relaxation phase of the heart (diastole), cells of the normal pacemaker region exhibit a slow depolarization that brings the membrane potential up to the threshold for the next action potential and hence has been termed the “pacemaker potential.” Several currents contribute to the pacemaker potential, but there is debate about their relative importance for pacemaking (1–3). One proposal is that the pacemaker potential is due principally to the decay of the potassium-delayed rectifier current ($I_{Kd}$) in combination with a time-independent inward background current (possibly supplied by the Na–Ca2+ exchanger) (4). Another view is that the slow depolarization is due to the $I_h$ current, a nonselective Na+/K+ conductance activated upon hyperpolarization in a time-dependent fashion (5, 6). This current is also referred to as $I_τ$ (7), or as the pacemaker current, because it was first described in cells of the pacemaker region (8, 9). In fact, $I_h$ is present in cells throughout the Purkinje system (10–12) and even in ventricular myocytes, where it is activated at more hyperpolarized voltages than in the sino-atrial node (13, 14). $I_h$ also is found in some neurons. For instance, in the thalamus, it helps to generate sleep-regulated oscillatory rhythms (15).

To understand the roles of single genes in the development of organ function, we undertook a large-scale screen for mutations that affect the heart in the embryo of the zebrafish Danio rerio (16). The embryos of this vertebrate are transparent, so cardiac function can be analyzed in real time. The chamber design and function of the 2-day postfertilization embryonic fish heart is quite similar to that of the 3-week gestation human embryo heart. Unlike the human, the fish embryo does not depend for survival upon its circulation at the early stages because diffusion suffices (16–18). Therefore, mutants can survive without any functioning heart beat for days and can be studied specifically with regard to cardiac effects without complications due to nutritional deficiency or secondary deterioration. As part of our screen, we discovered 57 recessive mutations that affect the heart or vessels (16).

Of these zebrafish mutations, one, slow mo, affects the heart rate. From the onset of normal contractions, the mutant heart rate is slower than wild type, a condition termed “bradycardia.” This appears to be due to the properties of individual heart cells because they beat slowly compared with wild type even after dissociation into cell culture. Of the several potential pacemaking currents, only $I_h$ is perturbed in the mutants. There is a severe reduction in the fast kinetic component whereas a slow component of $I_h$ remains intact. Thus, selective diminution of one component of $I_h$, as assayed in individual cells, appears to cause bradycardia but otherwise does not perturb cardiac development.

MATERIALS AND METHODS

Cardiomyocyte Culture. Care and breeding of zebrafish (D. rerio) was as described (19). Developmental staging was carried out using standard morphological features (20) of fish raised at 28.5°C. The zebrafish embryo is transparent, so phenotypic characterization and all heart rate data were obtained by direct visual inspection under a dissecting microscope. Throughout this paper, “smo” refers to fish or cells that are homozygous for the smo mutation. The hearts from tricaine-anesthetized day 3 embryos were removed and placed in L15 media, rinsed in Hanks’ balanced salt solution, and incubated in collagenase type II (250 units/ml) and protease type XIV (1.0 unit/ml) with trituration to isolate the cardiomyocytes. Cells were plated on polylysine-coated coverslips and incubated in L15 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 28.5°C. For dissociated beat rate analysis, cell clusters were cultured for 1–4 h. For patch-clamp recording, all cells were used 18–36 h after plating to allow for single cell attachment onto coverslips. Only healthy cultures with beating cells were used for analysis.

Patch-Clamp Recording. Standard whole cell recording techniques (21) were applied to single cells. All data were

†To whom reprint requests should be addressed at: Cardiovascular Research Center, Massachusetts General Hospital-East, 149 13th Street, 4th Floor, Charlestown, MA 02129; email: fishman@cvcrc.mgh.harvard.edu.

§K.B. and K.S.W. contributed equally to this paper.
acquired and analyzed blind to the genotype. For \(I_{\text{Kr}}\) and \(I_{\text{K}}\), bath solution contained (in mM): NaCl 135, KCl 20, MgCl\(_2\) 1, CaCl\(_2\) 2, Hepes 10, and glucose 10 (pH 7.4 with NaOH). Pipette solution contained (in mM): KF 70, KCl 70, MgCl\(_2\) 0.5, Hepes 10, and EGTA 10 (pH 7.4 with NaOH). For \(I_{\text{Ca,L}}\), \(I_{\text{T}}\) bath solution contained (in mM): CaCl\(_2\) 15, CsCl 5, Hepes 10, glucose 10, and N-methyl-D-glucamine 125 (pH 7.4 with HCl). Pipette solution contained (in mM): tetraethylammonium-chloride 140, MgCl\(_2\) 0.5, Hepes 1, and EGTA 1 (pH 7.4 with CsOH). Pipettes were coated with Sylgard (Dow Corning, Midland, MI) and fire polished to resistances of 2–5 MΩ. Data acquisition and analysis were carried out using pClamp6 software (Axon Instruments, Foster City, CA). All recordings were at room temperature (21–23°C). For comparison of \(I_{\text{h}}\) and \(I_{\text{Kr}}\) from the same cell, the amplitude of the \(I_{\text{h}}\) current was determined from the amplitude of the Boltzmann fit, and the amplitude of the \(I_{\text{Kr}}\) current was determined in an analogous fashion. The leak current and nonlinear open channel properties were minimized for both the \(I_{\text{h}}\) and \(I_{\text{Kr}}\) records by taking all of the current amplitudes at a constant voltage.

The kinetics of activation for \(I_{\text{h}}\) were determined at −130 mV. Data traces were filtered at 1 kHz and fitted with either a single or double exponential curve. All traces were initially fit with a single exponential. The need for a second component was determined by a trial fit with two components. If the fitting algorithm converged and gave a nontrivial second component (i.e., time constant and amplitude > 0), then two components were assumed. The fitting and number of components was determined blind to the genotype. In most cases, the number of components was easily confirmed by visual inspection.

RESULTS

**slow mo** Mutants Have a Reduced Heart Rate. The \(smo\) mutation is evident as a slow heartbeat in 25% of embryos from a \(smo\)××\(smo\) cross, a recessive inheritance pattern that has been evident over seven generations. The heart and the embryo appear otherwise normal, and there is no evidence for diminished viability of \(smo−/−\) embryos compared with wild type at 28.5°C. Homozygotes have been raised to adulthood and can breed successfully. No other alleles of this mutation were isolated among the combined 6647 mutations generated in the two recent, large-scale screens (16, 22).

In the wild-type zebrafish embryo, the single heart tube is silent at the time it is generated by fusion of the bilateral tubes. Isolated twitches begin diffusely in the myocardium at 21 h postfertilization (hpf), followed by irregular peristaltic motions, which flow in a wave from the venous to the arterial end. By 24 h postfertilization, there are regular peristaltic contractions, which establish blood flow through the circulation, and by 32 h postfertilization, there are sequential contractions of the atrium followed by the ventricle.

\(smo\) mutants have a reduced heart rate from the onset of regular contractions in the heart tube. The wild-type heart rate increases in the first days postfertilization, as it does in other species (17), and even the early embryo responds to warming with an increased rate and to cooling with a decreased rate (Fig. 1A). The heart rate of \(smo\) mutants is significantly slower at all stages of development and at all temperatures (Fig. 1B), and the temperature response is abnormal (warming diminishes the rate).

The intrinsic heart rate in \(smo\) embryos is slow before innervation, suggesting that the \(smo\) defect is not directly neural in nature. In fact, when embryonic hearts are explanted into culture, the difference between wild type and \(smo\) beat rates is still apparent. Day 3 \(smo\) extracted hearts beat at 46.9 ± 3.7 beats per minute (mean ± SD; n = 48) whereas wild-type hearts beat at 78.7 ± 6.2 beats per minute (mean ± SD; n = 50; significant, \(P < 0.0005\)). To rule out selective failure of specialized pacemaker or conduction tissue within the intact heart, we dissociated hearts into small clusters of cells and found that \(smo\) cardiomyocyte clusters beat slower than wild type clusters [wild type 39.6 ± 11.8 beats per minute (mean ± SD; n = 30); \(smo\) 20.8 ± 4.6 beats per minute (mean ± SD; n = 30; significant, \(P < 0.0005\)]. Because the \(smo\) phenotype present in the mutant fish persists even when \(smo\) hearts are dispersed in culture, we reasoned that the defect may be present in single cells. We therefore developed methods for isolated cardiomyocyte culture and electrophysiological analysis of individual cells from wild type or \(smo\) hearts.

**Embryonic Cardiocytes Have a Hyperpolarization-Activated Inward Current.** We examined channel activity in individual dissociated cardiomyocytes by voltage clamp. Embryonic zebrafish cardiocytes have, as described in embryonic hearts of other species, voltage-gated sodium currents (\(I_{\text{Na}}\)), L-type calcium currents (\(I_{\text{Ca,L}}\)), and T-type calcium currents (\(I_{\text{Ca,T}}\)), as well as potassium currents with the properties of an ultrarapid delayed rectifier (\(I_{\text{Kur}}\)), transient outward (\(I_{\text{A}}\)), and a rapid delayed rectifier with inward rectification (\(I_{\text{Kr}}\)) (unpublished work). These did not appear different between wild-type and \(smo\) mutant fish (data not shown).

We also identified a hyperpolarization-activated current with properties that correspond to \(I_{\text{h}}\) noted previously (11). As shown in Fig. 2, this current meets the criteria for \(I_{\text{h}}\) because it is an inward current, carried by both Na\(^+\) and K\(^+\). In the cardiocytes, it is evident as a slowly activating current that appears when the membrane voltage is stepped from a holding voltage of −40 mV to more negative voltages (Fig. 2A). This inward current is due to an increase in conductance, measured by the instantaneous change in current upon stepping to a different voltage after activation (Fig. 2B). The conductance continues to increase with more negative activating voltages, reaching a half-maximal value by −95 mV. The conductance has a reversal potential of −14 mV, compared with the potassium equilibrium potential of −49 mV (Fig. 2B, bottom). This is compatible with the known properties of \(I_{\text{h}}\) in other systems (11). The current is blocked completely by 2 mM extracellular Cs\(^+\), as is \(I_{\text{h}}\) elsewhere (23, 24) (Fig. 2C).

**smo Mutants Have Diminished \(I_{\text{h}}\).** For quantification, we focused on currents potentially involved in diastolic depolarization, including \(I_{\text{Ca,T}}\), the delayed rectifier, \(I_{\text{Kr}}\), and \(I_{\text{h}}\). We found a substantial and specific reduction of \(I_{\text{h}}\) in cardiocytes of \(smo\) mutants compared with wild type over the entire
Fig. 2. A hyperpolarization-activated $I_h$ current is present in zebrafish cardiac myocytes. (A) Activation of $I_h$ current by hyperpolarization. An individual dissociated cardiomyocyte was voltage-clamped from a holding potential of $-40$ mV to various hyperpolarizing voltages ($-70$ to $-130$ mV in 10-mV increments) once every 15 s. Each hyperpolarization was followed by a step to $-130$ mV to determine the current at a constant voltage. The current amplitude immediately after the voltage step to $-130$ mV was then plotted as a function of the activation voltage. The data were fitted with a Boltzmann function. The amplitude of the $I_h$ current was given by the net amplitude of the Boltzmann function. The leak current was determined by the baseline of the fit and was not part of the $I_h$ determination. Current traces were filtered at 1 kHz. (B) Tail current analysis of the $I_h$ reversal potential. After $I_h$ was activated for 1 s by a step from 0 mV to $-130$ mV, another step was applied to a new voltage (from $-50$ to $+30$ mV in 10-mV increments). The tail current amplitude immediately after the voltage clamp settled is plotted as a function of the tail current voltage. The tail currents reversed at $-14$ mV, as determined by the line fitted to the data points. A perfectly potassium-selective channel would have a reversal potential of $-49$ mV in the solutions used. The dihydropyridine nifedipine (1 μM) was included to block the calcium currents present in these cells. If the cell was held at 0 mV and not hyperpolarized to activate the $I_h$ current, no tail currents were observed (data not shown). Current traces were filtered at 2 kHz. The voltage protocol was delivered every 4 s. (C) Inhibition of $I_h$ by cesium. $I_h$ currents with and without 2 mM CsCl applied extracellularly, in response to a step from $-40$ mV to $-130$ mV. Current traces were filtered at 1 kHz.

The voltage range of activation, from $-65$ to $-130$ mV, Fig. 3A shows representative recordings of $I_h$ from a wild-type and a smo mutant cell, and 3B shows the quantitation for many cells measured at the test voltage of $-130$ mV. The amplitude of the $I_h$ current at this test voltage is reduced by $\sim 85\%$ in smo. As a control for conditions and cell viability, we assayed $I_{Kr}$ in the same cells as $I_h$. We also measured $I_{Ca,T}$ because blockade of this current with Ni$^{2+}$ is reported to slow the heart rate (1). There was no significant change in $I_{Kr}$ or in $I_{Ca,T}$ (Fig. 3A and B). Therefore, of the several currents potentially involved in pacemaking, only $I_h$ appears to be affected by the mutation.

We noted that $I_h$ in smo is diminished but not absent. There are several possibilities for this. One is that the mutation changes the properties of the $I_h$ channel rather than eliminating its function. To examine this, we more closely studied its kinetic properties. In wild-type $I_h$, the kinetics of opening are well described by the sum of two exponentials, as shown in Fig. 4A. One component is larger and more rapidly activating and is referred to as “fast”; the other is smaller and more slowly activating and is referred to as “slow.” The smaller $I_h$ current in the smo mutant cells appears to consist mainly of the slow component (Fig. 4A). The fast component in smo cells is severely reduced, and in many cells it is undetectable (Fig. 4B). Although it is possible that smo causes interconversion of fast and slow components, this seems unlikely given that the slow component is unchanged in the mutant, both in its amplitude and kinetics. Rather, it appears that there may be two separate types of $I_h$ channel corresponding to the currents with fast and slow kinetics and that the smo gene product is required, directly or indirectly, to generate a normal fast component.

DISCUSSION

We describe here a recessive mutation that causes a marked reduction in heart rate and is reflected at the cellular level by specific and selective perturbation of a single current, the hyperpolarization-activated cation current referred to as $I_h$. This provides strong genetic evidence that $I_h$ helps control heart rate. The selective reduction of the fast kinetic component, with the maintenance of an intact slow component, suggests that there may be two channels normally involved in $I_h$ and that this mutation severely affects the fast one.

There are two principal models for how pacemaking is achieved. These are not mutually exclusive, and, in fact, either could predominate in different pacemaker regions or at different stages of development. One places emphasis upon the delayed rectifier potassium current $I_{Kr}$, the current that underlies repolarization of the action potential. Pacemaking, by this model, is due to the decay of $I_{Kr}$ combined with a time-independent depolarizing tendency due to background currents or to the Na$^{+}$-Ca$^{2+}$ exchanger (4). We found that $I_{Kr}$ and its decay at negative voltages are normal in the smo mutant (Fig. 3A).

The other model for pacemaking depends upon $I_h$. $I_h$ is an inward Na$^{+}$ and K$^{+}$ current activated by hyperpolarization. Therefore, it is inactive until after the action potential, when
**FIG. 3.** $I_h$ is reduced in smo cardiac myocytes. (A) Representative $I_h$, $I_{Kr}$, and $I_{Ca,T}$ currents from wild-type and smo cells. $I_h$ was elicited from a holding voltage of $-50$ mV by steps to various voltages ($-65$ to $-130$ mV in $5$-mV increments). A test voltage of $-130$ mV was applied at the end of each hyperpolarizing step. $I_{Kr}$ was activated by a $1$-s prepulse to various voltages ($+60$ to $-70$ mV in $10$-mV increments); the inwardly rectifying current was then measured at a constant test voltage of $-120$ mV. $I_{Ca,T}$ was elicited from a holding potential of $-100$ mV to voltages ranging from $-90$ to $+50$ mV once every $2$ s. Current traces were filtered at $1$ kHz ($I_h$) or $5$ kHz ($I_{Kr}$ and $I_{Ca,T}$). (B) Amplitude of $I_h$, $I_{Kr}$, and $I_{Ca,T}$ currents from many wild-type and smo cells. The data are displayed as box plots. The box delimits the central $50\%$ of the data values. The median is shown as a solid line tagged with a solid box, and the mean is shown as a solid circle. The $99\%$ confidence interval for a robust median is shown by the gray shaded zone. The confidence interval is based on the biweight locator and estimate of scale (25). Boxplots of the $I_h$ current amplitudes are shown for wild-type ($n = 47$) and smo ($n = 48$) cells. Boxplots of the $I_{Kr}$ current amplitudes also are shown for wild-type ($n = 31$) and smo ($n = 23$) cells and for $I_{Ca,T}$, wild-type ($n = 20$), and smo ($n = 20$) cells. The size of whole cell $I_h$ currents in smo cells was very small (average $12.0$ pA), making it difficult to fit some of the data to a Boltzmann function. The amplitude comparison between wild-type and smo cells was thus repeated but with recording conditions that were chosen to enhance the amplitude of the $I_h$ currents. The bath solution was as described in Materials and Methods except that KCl was increased to $140$ mM, $2$ mM BaCl$_2$ was added, and NaCl was omitted. The pipette solution was supplemented with $100$ µM cAMP. The $I_h$ current amplitude was indeed enhanced $2.5$-fold by the changes in recording solutions, and the effects of the smo mutation on the $I_h$ current were the same. For the $I_h$ current, the confidence interval was $110$–$260$ pA in wild-type cells ($n = 19$) compared with $5$–$44$ pA in smo cells ($n = 20$). $I_{Kr}$ current was again unchanged by the mutation: $99\%$ confidence interval was $67$–$358$ pA for wild-type ($n = 20$) and $109$–$261$ pA for smo cells ($n = 19$). The reduction in $I_h$ current in smo cells was not due to a difference in cell size between wild-type and smo because the cell capacitances were the same for the two genotypes; the $99\%$ confidence interval on the robust median for wild-type ($n = 34$) was $2.3$–$3.2$ pF and for smo ($n = 28$) was $2.4$–$3.1$ pF. Data were obtained for both $I_h$ and $I_{Kr}$ from each cell whenever possible. The amplitude of the $I_h$ current was determined as shown in Fig. 24 from the amplitude of the Boltzmann fit, and the amplitude of the $I_{Kr}$ current was determined in an analogous fashion. The current amplitude for $I_{Ca,T}$ was determined at $-20$ mV after leak subtraction. The leak current and nonlinear open channel properties were minimized for both the $I_h$ and $I_{Kr}$ records by taking all of the current amplitudes at a constant voltage. For each value in the legend, we report a range of numbers giving the $99\%$ confidence interval for the robust median. WT, wild-type.
and can do so without reliance on pharmacological intervention.

Most cardiac cells have latent pacemaking properties, but in the intact heart, those at the sino-atrial node beat faster and thus override the other pacemakers. Consistent with this regional difference in pacemaking properties, \( I_h \) has been shown in other species to activate at less negative potentials in nodal cells than in ventricular cardiomyocytes (4, 6, 13). Although we acknowledge that cell behavior in culture may not directly reflect pacing in the intact heart, we did observe a range of activation voltages for \( I_h \) in a mixed population of zebrafish cardiac cells (data not shown), and it may be that primary pacemaking by \( I_h \) normally occurs only in those cells in which \( I_h \) activates at less negative voltages. It is also important to note that the difference between \( smo I_h \) and wild type \( I_h \) is dramatic throughout the entire range of \( I_h \) activation and is even larger with activation at less negative voltages (data not shown).

If \( I_h \) is important for pacemaking, why does nearly complete elimination of the fast component produce only a 2-fold change in the heart rate? Presumably there are several redundant or backup pacemaking mechanisms, as one might expect for a function so critical to survival. One of these might be the remaining slow component of \( I_h \). Another could be the decay or a turnoff of outward K\(^+\) current. In any case, it is interesting that disruption of a large component of \( I_h \) leaves rhythm and conduction sufficiently intact to permit development to proceed normally. This observation makes it reasonable to target subtypes of \( I_h \) for therapeutic purposes of slowing the heart, an important treatment for ischemic heart disease.

One candidate for the \( smo \) gene product is the channel protein that carries the \( I_h \) current or, more precisely, the fast component of \( I_h \). However, it could also be another protein necessary for full \( I_h \) function. We have no evidence to distinguish the two, which ultimately rests upon the cloning and expression of the gene. \( I_h \) has not yet been cloned from any system or species. Therefore, because the molecular basis of pacemaking is so poorly understood, it will be important to achieve molecular definition of the \( smo \) gene, whether it is the channel or a regulator. A complete infrastructure for positional cloning is being assembled for the zebrafish. Framework maps have been generated, using microsatellites (30) and randomly amplified polymorphic DNA (31), and yeast artificial chromosome libraries are under construction (T. Zhong, E. Lander, and M.C.F., unpublished work). Clearly, \( smo \) will be a high priority as positional cloning in the zebrafish becomes more routine over the next few years.

We thank Margaret H. Boulus for her excellent technical assistance. This work was supported by: National Institutes of Health Grants R01 HL49579 (M.C.F.), R01 RO18888 (M.C.F.), T32 HL07208 (M.C.F.), and R01 NS26903 (G.Y.); a McKnight Investigator Award (G.Y.); a Research Starter Grant from the Foundation for Anesthesia Education and Research and the Society for Cardiovascular Anesthesiologists (K.B.); and a sponsored research agreement with Bristol Myers–Squibb (M.C.F.).