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Age-dependent differences in the inhibition of HCN2 current in rat ventricular myocytes by the tyrosine kinase inhibitor erbstatin

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Abstract

Previously, we have shown that murine HCN2 channels over-expressed in newborn and adult cardiac myocytes produce currents with different biophysical characteristics. To investigate the role of tyrosine kinase modulation in these age-dependent differences, we employed the broad spectrum tyrosine kinase inhibitor erbstatin. Our results demonstrated distinct and separable effects of erbstatin on channel gating and current amplitude and a marked age dependence to these effects. In newborn myocytes, erbstatin decreased current amplitude, shifted the activation relation negative, and slowed activation kinetics. The effect on activation voltage but not that on amplitude was absent when expressing a cAMP-insensitive mutant (HCN2R/E), while a C-terminal truncated form of HCN2 (HCN2ΔCx) exhibited only the voltage dependent but not the amplitude effect of erbstatin. Thus, the action of erbstatin on the activation relation and current amplitude are distinct and separable in newborn myocytes, and the effect on activation voltage depends on the cAMP status of HCN2 channels. In contrast to newborn myocytes, erbstatin had no effect on HCN2 under control conditions in adult myocytes but induced a negative shift with no change in amplitude when saturated cAMP was added to the pipette solution. We conclude that erbstatin's effects on HCN2 current magnitude and voltage dependence are distinct and separable, and there are fundamental developmental differences in the heart that affect channel function and its modulation by the tyrosine kinase inhibitor erbstatin.

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Keywords

Hyperpolarization-activated channels; Cardiac myocytes; Ion channel modulation; Tyrosine kinase; Development

Introduction

The HCN gene family represents the molecular correlate of the pacemaker current I_f , which contributes to diastolic depolarization in cells of the sinoatrial node (SAN) and therefore to the regulation of cardiac rhythm. There are four known isoforms, three of which (HCN1, HCN2, and HCN4) are expressed in the heart [18,30], with HCN2 being the major isoform in the ventricle [11,13,30].

Biophysical properties of I_f vary dramatically with cardiac region, age, and disease [6,27]. The current activates at -40 mV in sinoatrial node myocytes [10], at -90 mV in Purkinje myocytes [36], and at voltages negative to -100 mV in adult ventricular cells [27,36]. Moreover, in rat ventricular myocytes, the voltage dependence of activation changes with age [27]. In newborn ventricular myocytes, the threshold is about -70 mV and becomes more negative when the animal matures. Some of these changes may relate to expression of different HCN subunits. In this regard, studies on expressed channels can provide insights beyond what is achieved from studies of native current. Qu et al. [25] have reported that murine HCN2 channels over-expressed in cardiac myocytes exhibited developmental differences in current characteristics similar to those of native I_f . HCN2 current also varies significantly when it is expressed in different cell types [24]. Taken together, these data identify an important role of cell environment in HCN channel function. These differences can arise as a result of a range of mechanisms, including differential processing and/or regulation of the channel, or the presence of different associated proteins as a function of cell type or age [12,15,22,26,29].

The conventional model of I_f regulation focuses on the role of cAMP, which binds to the cyclic nucleotide binding domain (CNBD) [4,8]. Recent data strongly suggest an important role of phosphoinositides (including PIP2) in regulation of different HCN isoforms [22,39]. However, I_f has also emerged as a target for regulatory phosphorylations that are controlled by various cellular kinases and phosphatases and lead to important changes in pacemaker current and SAN rate. Importantly, this regulation may be isoform and tissue specific. In canine Purkinje fibers, the protein phosphatase inhibitor calyculin A induces a positive shift of I_f activation on the voltage axis [36], whereas the non-specific protein kinase inhibitor H-7 induces a shift in the opposite direction [7]. Calyculin A also influences channel properties in rabbit sinoatrial node myocytes. Here, the effect of calyculin A is to increase channel conductance without changing voltage dependence [1].

There is a growing body of evidence identifying I_f channels as targets for phosphorylation by protein tyrosine kinases (PTK). Early studies identified an effect of epidermal growth factor (EGF) to accelerate heart rate [19]. The tyrosine kinase inhibitors herbimycin A and genistein (but not its inactive analog daidzein) were subsequently reported to decrease current amplitude without affecting voltage dependence in rabbit SAN [34]. EGF-dependent modulation of I_f in a manner opposite to that described for PTK inhibitors (and blocked by genistein) also has been reported [35]. Two recent studies have provided a more detailed analysis of PTK regulation of heterologously expressed channels. Yu et al. have identified an effect of genistein to suppress HCN2 (but not HCN1) current in *Xenopus* oocytes via a mechanism that involves a reduction in current magnitude and a negative shift on the voltage axis [37]. Both HCN2 and HCN4 interact with Src in HEK293 cells. The functional relevance of this interaction is a matter of controversy. Zong et al. demonstrated that Src inhibitors (PP2 or genistein) slow the kinetics

of activation and deactivation of both isoforms without altering channel voltage dependence or current amplitude [40]. On the other hand, two other studies showed that Src regulation of HCN4 involves changes in kinetics, voltage dependence, and current density [3,16]. Of note, while PP2 is a relatively selective PTK inhibitor (with selectivity for Src family kinases, Abl, the stem cell factor receptor c-Kit, and PDGF receptors [33]), the role of tyrosine kinases in the cellular actions of genistein have not been unambiguously established since genistein is reported to directly block I_f [2].

Thus, tyrosine phosphorylation has emerged as a mechanism to modulate HCN channels in an isoform and, at least in the case of HCN2, tissue-specific manner. Efforts to develop methods to use HCN channels as biological pacemakers in the heart [28] provide a need to study HCN channel regulation in cardiac myocytes to obtain a detailed understanding of how channel function may be regulated (for good or bad) in the relevant cell type within the intact organism.

Materials and methods

Cell culture

Cultures of newborn and adult rat ventricular myocytes were prepared as previously described [23]. Briefly, 1–2-day-old Wistar rats were killed according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University, hearts were quickly removed, and ventricles were dissociated by a standard trypsinization procedure. After preplating for 1 h to minimize fibroblast contamination, myocytes were harvested and plated in protamine-sulfate-coated 35- or 100-mm dishes. For the first 24 h, cells were incubated in MEM (Sigma) containing 10% fetal bovine serum and then switched to serum-free medium (SFM; [17]). On the day of the experiment, the cell monolayer was resuspended by brief exposure to 0.1% trypsin, and the cells were then re-plated onto fibronectin-coated coverslips.

Langendorff perfusion of collagenase was employed to dissociate adult rat ventricular myocytes as previously described [25]. Cells were plated onto laminin-coated coverslips, cultured in ACCIT medium, and incubated at 37°C, 5%CO₂ for at least 3 h prior to adenovirus infection (see protocol below).

Adenoviral vectors and infection

Adenoviruses were employed to over-express the mouse HCN2 channel. AdmHCN2 was prepared as previously described [25]. A cAMP-insensitive mutant of mHCN2 (HCN2R/E; substitution of arginine 591 with glutamate) and truncated channel (HCN2ΔCx; L665 stop) were taken from their original vectors (kindly provided by Dr. Steven Siegelbaum, Columbia University) and subcloned in the pDC516.IRES.hrGFP shuttle vector between *EcoRI* and *BamHI* restriction sites. Each shuttle vector was co-transfected with pBHGfrtΔE1,3FLP plasmid (AdMax™) into E1-complimenting HEK293 cells. Successful recombination was observed by CPE in passages 1–2. Resulting AdmHCN2R/E and AdmHCN2ΔCx were plaque-purified and amplified in HEK293 cells. Final titers were 1×10^9 – 5×10^9 fluorescence forming units/ml.

Infection of newborn ventricular myocytes was performed on monolayer cultures 4 days after initial plating. Cells were exposed to a virus-containing mix (m.o.i. 20, in 250 μl SFM for 35-mm dishes and 700 μl for 100-mm dishes) for 2 h, rinsed twice, and incubated in SFM at 37°C, 5% CO₂ for 24–48 h until use for experiments. Adult myocytes were infected 2–3 h after plating at m.o.i. 50–60 and used for experiments 48 h later. Since we had previously found greater than 90% expression of HCN2 in both newborn and adult myocytes infected this way [25], we did not select cells for study based on GFP fluorescence.

Electrophysiology

The whole-cell patch clamp technique was employed to record the pacemaker current I_f from myocytes. Experiments were carried out on cells superfused at 35°C. Extracellular solution contained (millimolar): NaCl, 140; NaOH, 2.3; MgCl₂, 1; KCl, 10; CaCl₂, 1; HEPES, 5; glucose, 10; MnCl₂ (2 mM) and BaCl₂ (4 mM) to eliminate I_{Ca} and I_{K1} ; pH 7.4. The pipette solution included (millimolar): aspartic acid, 130; KOH, 146; NaCl, 10; CaCl₂, 2; EGTA-KOH, 5; Mg-ATP, 2; HEPES-KOH, 10; pH 7.2. The pipette resistance was typically 3–5 MΩ. An Axopatch-200B amplifier and pClamp8 software (Axon Instruments) were used for data acquisition and analysis. HCN current was defined as the time-dependent component taken at the end of hyperpolarizing test voltage steps from –35 to –145 mV, with increments of 10–15 mV from the holding potential –35 mV. The test pulses were followed by a voltage step to a negative voltage (–100 to –115 mV), where the tail currents were recorded. The specific voltage range employed varied with HCN2 construct to obtain the full I – V curve as efficiently as possible. In order to approximate steady-state activation and get a more accurate measurement of the biophysical properties of the current, the test pulse length was 5 s for hyperpolarizing test steps and 6 s for the tail measurement. After that, a pulse to –5 mV for 0.5 s was applied between sweeps to deactivate the HCN channels. The tail current, after being plotted in relation to the test voltages, gave the maximum channel conductance (maximal tail current). The normalized plot of tail current vs. test voltages was fitted with a Boltzmann function, and then the voltage of half maximal activation and slope factor were defined from the fitting. Time constants of activation were obtained by fitting the early time course of current traces with a monoexponential function; the initial delay and slow activation phase were ignored. Kinetics of deactivation were determined by a single exponential fit of the time course of the current trace at the test voltage (from –95 to –35 mV; increment –10 mV) after activation by a prepulse to –110 mV.

Since ventricular myocytes have native I_f current (although it is small), only cells that exhibited current greater than 200 pA were included in the analysis. For more accurate measurements and to avoid potential errors from current rundown, all drug effects were compared with vehicle experiments over the same time course in parallel cells from the same preparation.

Immunoprecipitation and immunoblotting

Forty-eight hours after infection, cardiomyocytes were scraped into homogenization buffer [20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 0.1 mM sodium orthovanadate, 0.042% β-mercaptoethanol, 0.05 mg/ml aprotinin, 0.05 mg/ml benzamidin, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.005 mM pepstatin A and 0.1 mM sodium fluoride; 0.5 ml per 100-mm dish], sonicated, and centrifuged at 185,000×*g* for 1 h at 4°C. The pellet, representing the particulate fraction, was resuspended in solubilization buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 0.1 mM sodium orthovanadate and 0.1 mM sodium fluoride] and cleared of insoluble materials by sedimentation at 10,000×*g* for 30 min at 4°C. HCN2 was immunoprecipitated from 700 μg of particulate fraction, with 4 μg of anti-HCN2 antibodies (Alomone Labs, Israel) according to manufacturer's instructions. Proteins were separated by SDS-PAGE (8% acrylamide, acrylamide/bis-acrylamide ratio 49:1) and transferred to nitrocellulose for immunoblotting with anti-HCN2 or anti-pTyr (Upstate Biotechnology) antibodies. Proteins were visualized by enhanced chemiluminescence, with each panel in each figure derived from a single gel exposed for a uniform duration. Scanning densitometry was used for quantification of bands. For each experiment, the band intensity in drug-treated group was normalized to corresponding control. All results were replicated in at least three separate experiments on separate culture preparations.

Statistics

All data are presented as mean $\pm$ SEM. Statistical significance was examined by *t* test. Values of $p<0.05$ were determined to be significant.

Results

Effect of a broad spectrum PTK inhibitor erbstatin on HCN2 current in newborn rat myocytes

To study possible tyrosine phosphorylation-dependent regulation of the HCN2 subunit of the pacemaker channel within myocytes, we used the PTK inhibitor erbstatin, which is a potent inhibitor of both receptor and non-receptor tyrosine kinases [5,14]. Representative current recordings in newborn myocytes for both vehicle and erbstatin are shown in Fig. 1a and b. Superfusion of newborn rat myocytes with 25 μ M erbstatin resulted in strong inhibition of expressed HCN2 current. This inhibition resulted from a decrease of maximal current amplitude (by $58.5\pm3.8\%$ vs. $18.1\pm4.6\%$ in vehicle over the same time; $p<0.001$) and a negative shift in the midpoint of activation (change in $V_{1/2}$: -17.9 ± 1.8 mV vs. -5.2 ± 1.8 mV in vehicle; $p<0.001$; Fig. 1c and d). Erbstatin also resulted in a comparable negative shift of about 15-mV shift in the time constants of both activation and deactivation (Fig. 1e). To be certain that rundown during the time course of the experiment was not affecting the result, we also performed a separate population study (supplementary Fig. 1).

Since cAMP binding to HCN channels results in a positive shift in activation voltage, we considered the possibility that the effect of erbstatin on $V_{1/2}$ might be secondary to a decreased cellular cAMP concentration at the channel. To evaluate the role of cAMP-dependent mechanisms in the erbstatin-induced effects, we used a cAMP-insensitive form of the channel. Substitution of a single arginine in position 591 with glutamate (HCN2R/E) does not affect current density of heterologously expressed HCN2 channels but decreases the affinity of the CNBD for cAMP by more than $2,000\times$ [8]. Such a mutant channel, when expressed in newborn myocytes, produced a current with midpoint of activation 12 mV more negative than wild type (WT; -83.5 ± 1.1 mV vs. -71.9 ± 1.3 mV in WT; $p<0.001$; corresponding slopes factors were 9.46 ± 0.60 mV and 8.37 ± 0.60 mV; $n=22$), consistent with a degree of basal activation of expressed HCN2 channels by cAMP in newborn myocytes. In contrast to the earlier oocyte study [8], in newborn ventricular myocytes, the activation kinetics of HCN2R/E were faster than for WT at membrane potentials between -70 and -90 mV and were not different at more negative voltages (supplementary Fig. 2). Superfusion of HCN2R/E expressing myocytes with erbstatin resulted in significant inhibition of maximal current amplitude (Fig. 2a, upper traces; supplementary Fig. 3, upper traces). The decrease of amplitude was $41.1\pm4.7\%$ ($p<0.001$; vs. $17.0\pm4.4\%$ in corresponding control, $n=11$). There was also a slowing of activation kinetics, observed only at a physiologically relevant voltage (-90 mV) and not at more negative voltages (Fig. 2b, upper panel). However, unlike the situation with WT channels, no significant shift in voltage dependence was detected (change in $V_{1/2}$: -8.56 ± 1.87 mV vs. -7.61 ± 1.5 mV in vehicle, $p>0.05$). These results indicate that the effect of erbstatin on HCN2 amplitude and activation voltage might involve separate mechanisms and suggest that the effect on activation voltage in some way depends on cAMP.

Separable mechanisms of erbstatin effects were further confirmed in experiments using a truncated form of HCN2 (HCN2 Δ Cx) that terminates at amino acid L665 and therefore lacks the distal part of the C-terminus, including one putative PTK phosphorylation consensus motif. Deletion of this last portion of the HCN2 C-terminus did not affect current density and only slightly shifted activation to more negative voltage (-77.0 ± 1.0 mV vs. -71.9 ± 1.3 mV in WT, $p<0.005$; $n=22$; supplementary Fig. 3, lower panel). Erbstatin (25 μ M) induced a $33\pm7.1\%$ decrease of current amplitude, which was not significantly different from vehicle ($23.9\pm4.1\%$) but was statistically different from the effect in WT ($p<0.05$; Fig. 2a and c; supplementary Fig.

3, lower panel). No differences in activation kinetics were found compared to vehicle (Fig. 2b, lower panel). In contrast, there was a significant negative shift induced by erbstatin (change in $V_{1/2}$: -13.3 ± 1.8 mV vs. -7.1 ± 1.1 in vehicle, $n=10$, $p < 0.05$; Fig. 2d). These results further support the hypothesis that effects of erbstatin on amplitude and voltage dependence of HCN2 in newborn rat ventricular myocytes are separable. They indicate that the amplitude effect requires the distal C-terminus of HCN2, while the effect on activation voltage does not require the distal C-terminus but in some way depends on cAMP.

Next, we further investigated the cAMP dependence of the erbstatin effect on voltage dependence. One possible explanation for the data with HCN2R/E is that erbstatin lowers intracellular cAMP levels, thereby accounting for the negative shift in voltage dependence. There are some reports that PTK inhibitors may change activity of other enzymes (including adenylyl cyclase and phosphodiesterase) in a PTK-dependent or non-specific manner [20,32]. To test this possibility, we repeated the experiments on the effect of erbstatin on expressed HCN2 current but now included a saturating concentration of cAMP in the pipette, thereby eliminating any contribution from changes in intracellular cAMP. Figure 2c and d provides summary data for all experiments described so far, including those with cAMP. It shows that when $5 \mu\text{M}$ cAMP was added to the pipette solution, erbstatin still induced both a decrease of current amplitude and a negative shift in activation, comparable to the effect observed without cAMP. Erbstatin also significantly slowed activation kinetics at voltages positive to -100 mV in the presence of cAMP (data not shown). Thus, the electrophysiological data suggest that the broad spectrum PTK inhibitor erbstatin suppresses expressed HCN2 current in newborn rat ventricular myocytes via both a decrease of current amplitude and a negative shift in voltage dependence and that these two effects involve distinct and separable mechanisms. While neither effect is secondary to alterations in cAMP, the shift in voltage dependence appears to require the cAMP-bound state of the channel.

Effect of erbstatin in adult rat ventricular myocytes

Previously, we have shown that when the HCN2 isoform of the channel is expressed in newborn and adult cardiac myocytes, it produces currents with differing voltage dependence [25]. At the same time, saturating cAMP concentrations induce a similar positive shift in activation in both newborn and adult myocytes, suggesting that the basal difference in voltage dependence is likely not fully accounted for by age-dependent differences in cAMP level or responsiveness [25]. However, studies with the cAMP-insensitive mutant HCN2R/E identify subtle differences in cAMP responsiveness as a function of age in ventricular myocytes (Fig. 3). In contrast to the negative shift of the activation curve in newborn cultures described earlier and illustrated in Fig. 3, the midpoint of activation of HCN2R/E was not significantly shifted compared to HCN2 in adult myocytes (-91.8 ± 1.4 mV vs. -87.6 ± 1.9 mV in WT, $n=25$, $p > 0.05$), suggesting the absence of significant basal activation of HCN2 by cAMP in adult ventricle cells in culture. These results also confirm a cAMP-independent difference in voltage dependence as a function of age, based on the observation that HCN2R/E activates at more positive potentials in newborn than in adult myocytes (midpoint values of -83.5 ± 1.1 mV vs. -91.8 ± 1.4 mV; $p < 0.001$). We hypothesized that the remaining difference in voltage dependence might be due at least in part to differences in basal tyrosine kinase phosphorylation of the channel.

Superfusion of adult myocytes with erbstatin did not affect current amplitude, voltage dependence, or activation kinetics (Figs. 4 and 5; supplementary Fig. 4). These results suggest a lack of basal tyrosine phosphorylation of HCN2 in adult myocytes. Since our earlier experiments suggested that to develop the erbstatin-induced effect on activation voltage the channel needed to be in the cAMP-bound form and since we find no evidence of significant basal cAMP activation of expressed HCN2 channels in adult myocytes (see Fig. 3), we repeated

these experiments with 10 μ M cAMP added to the pipette solution (Fig. 4b). Under these conditions, we were able to observe an erbstatin-induced shift in voltage dependence (Fig. 5), but not a decrease in the current density or activation kinetics. These data again demonstrate that erbstatin alters voltage dependence and amplitude through distinct mechanisms.

Figure 5 summarizes data on erbstatin effects on voltage dependence of activation in newborn and adult cardiac myocytes under the various experimental conditions. It clearly shows that erbstatin has an effect on the midpoint of activation of HCN2 in both newborn and adult myocytes, but only when the channel is in the cAMP-bound form.

Basal tyrosine phosphorylation of HCN2 and its regulation by erbstatin

To confirm that erbstatin was affecting the degree of tyrosine phosphorylation of these channels, HCN2 was immunoprecipitated from newborn myocytes and then probed with an antiphosphotyrosine antibody to detect channel tyrosine phosphorylation. Figure 6 shows that anti-HCN2 antibodies pull down three epitope-specific bands that migrate as ~93–116-kDa proteins. All three bands are recognized by antiphosphotyrosine antibodies in resting cardiomyocytes. HCN2 tyrosine phosphorylation is reduced in cardiomyocytes treated with erbstatin (band intensity is 0.68 ± 0.11 relative to control, $p < 0.05$, one-tail t test) and increased in cardiomyocytes treated with the tyrosine phosphatase inhibitor hydrogen peroxide (H_2O_2 ; 3.38 ± 1.36 relative to control, $p < 0.05$, one-tail t test; Fig. 6b).

Discussion

In the present study, we show that the broad spectrum PTK inhibitor erbstatin inhibits HCN2 current in newborn rat ventricular myocytes via both a decrease in current amplitude and a negative shift in voltage dependence, in association with a modest decrease in basal tyrosine phosphorylation of the channel protein. The functional effects appear to be mediated by distinctive mechanisms involving different channel domains. Moreover, the effect of erbstatin to inhibit current in newborn myocytes was not detected in studies on adult myocytes under normal recording conditions.

Several reports suggest that tyrosine phosphorylation might regulate pacemaker current [34, 35]. A broad spectrum PTK inhibitor genistein (but not its inactive analog diadzein) inhibited I_f in rabbit SAN via decrease of current amplitude [34]. EGF increased current, and this effect could be blocked by genistein [35]. Another group also reported effects of several PTK inhibitors on pacemaker current in porcine SAN, but concluded that they are not related to tyrosine phosphorylation [31]. Incubation of rabbit SAN myocytes with PP2 or genistein slowed activation kinetics of I_f without any effect on current amplitude and voltage dependence [40].

Several studies focused on possible PTK regulation of heterologously expressed HCN channels. In *Xenopus* oocytes, genistein's effect appears to be isoform specific [37]. In particular, it has no effect on HCN1, decreases current amplitude of HCN4, and suppresses HCN2 current via both a decrease of amplitude and a negative shift [37]. One group found that in HEK293 cells, both genistein and PP2 slowed kinetics of activation and inactivation of mHCN2 and hHCN4 but did not affect current density and voltage dependence [40]. Recent work by Li et al. demonstrated that in addition to kinetic effects, regulation of hHCN4 by Src also involves changes in current density and voltage dependence [16]. The data suggest the presence of at least two different sites of channel phosphorylation with Tyr531 being responsible for most of Src's action and Tyr554 being associated only with kinetic changes [16]. Interestingly, substitution of these residues with phenylalanine does not eliminate channel phosphorylation, suggesting that there are multiple phosphorylation sites. Our data further support the idea of multiple, functionally distinct, phosphorylation targets since different

HCN2 domains seems to be responsible for erbstatin effects on voltage dependence and current amplitude.

In the study by Zong et al. [40], the presence of PTK inhibitors did not alter cAMP responsiveness, suggesting that regulation of HCN2 channels by cAMP and tyrosine phosphorylation are independent. In our study, erbstatin effects on amplitude were independent of cAMP. On the other hand, we observed a clear correlation between cAMP regulation and erbstatin effects on the midpoint of activation. In newborn rat myocytes expressing the cAMP-insensitive channel HCN2R/E, erbstatin decreased current amplitude but had no effect on activation voltages. Moreover, when a saturating concentration of cAMP was added to the pipette solution, erbstatin still induced a significant negative shift of WT-HCN2, suggesting that its effect on voltage dependence of HCN2 is not due to intracellular changes in cAMP. Thus, the negative shift in the $V_{1/2}$ of activation in response to erbstatin appears to be consistent with one of the following two possibilities: (1) Erbstatin caused a marked reduction in the cAMP affinity of the channel, effectively converting the channel to a cAMP-insensitive form regardless of the amount of cAMP dialyzed into the cell; (2) erbstatin requires that the channel be in the cAMP-bound form, but does not necessarily displace the cAMP from the channel. It also is interesting that erbstatin is able to slow activation kinetics under conditions where there is no effect on voltage dependence (e.g., in the case of HCN2R/E) since in the wild-type channel, these two parameters shift together. This is similar to what Zong et al. [40] observed for WT-HCN2 expressed in HEK293 cells and would be consistent with an interpretation of low basal activation of the channel by cAMP in HEK293 cells.

In our prior study, we demonstrated that voltage dependence of HCN2 current activation in newborn and adult rat myocytes differed dramatically, and a positive shift induced by cAMP was similar in both groups [25]. Therefore, in this study, we over-expressed a mutant channel, HCN2R/E, which is insensitive to cAMP. In newborn myocytes HCN2R/E, current was 12 mV more negative than WT, but in adult HCN2R/E and WT, currents were not significantly different, indicating that there are age-dependent differences in the cAMP status of HCN2. This could arise from either different intracellular cAMP concentrations in newborn and adult ventricular myocytes or different sensitivity of the channel to cAMP as a function of age, modulated by other mechanisms.

In this regard, the absence of any effect of erbstatin on voltage dependence in adult myocytes, where we found no significant basal activation of HCN2 by cAMP, is understandable. Adding cAMP into the pipette solution allowed rescue of the erbstatin-induced negative shift, consistent with the proposal that erbstatin only acts on the cAMP-bound form of the channel (and not consistent with the alternative hypothesis that erbstatin reduces the cAMP affinity of the channel). Moreover, the persistent absence of an erbstatin effect on amplitude and kinetics in adult myocytes, regardless of the presence or absence of cAMP, also suggests some fundamental difference between adult and newborn myocytes, which affects erbstatin's action on the channel. One potential mechanism could involve age-dependent differences in tyrosine kinase expression patterns. For example, cAbl has been detected in isolated newborn myocytes, but not adult myocytes [9].

It should be noted that inhibition of I_f in adult ventricular myocytes by genistein has been previously reported by Yu et al. [37], which differs from the insensitivity observed in the present study. Experimental differences (native heteromeric channels vs. expressed HCN2 channels, genistein vs. erbstatin as the inhibitor; acutely isolated cells vs. short term culture) probably contribute to this apparent inconsistency.

To date, the evidence that pacemaker channels are regulated through tyrosine phosphorylation in cardiac cells is based largely on pharmacological studies with inhibitors with known non-

specific actions and variable efficacy between cell types. Currently, the direct biochemical evidence that HCN channels are phosphorylated on tyrosine residues is limited to two recent studies examining HCN2 and HCN4 tyrosine phosphorylation in HEK293 cells and mouse brain [3,16,40]. Given the marked differences in HCN channel function between cell types, the relevance of these studies to HCN2 action in the heart is uncertain. In this context, our studies provide important direct biochemical evidence that HCN2 can be regulated through tyrosine phosphorylation in cardiac myocytes.

This study shows that there is basal tyrosine phosphorylation of HCN2 in newborn myocytes. The tyrosine kinase inhibitor erbstatin decreases but does not eliminate this phosphorylation, and it remains to be demonstrated that this reduction in phosphorylation accounts for the functional effects of erbstatin. While phosphorylation-independent effects of erbstatin on K⁺ channels [38] and other PTK inhibitors on pacemaker (I_f) current in rabbit SAN myocytes [2] have been identified, our observation that erbstatin does not inhibit HCN2 when it is expressed in adult myocytes argues against a non-specific effect of erbstatin on HCN2 function. Erbstatin also is not likely to alter HCN channel function indirectly by inhibiting the catalytic activity of one or more serine/threonine kinases since any effects of erbstatin to inhibit PKC or PKA are detected only at high concentrations, in excess of those used in this study [5]. However, we cannot rule out a possible additional functionally significant tyrosine phosphorylation on a protein that associates with HCN2, in addition to the phosphorylation of the HCN2 channel itself. Such a possibility could explain the observation that very modest changes in channel phosphorylation in response to erbstatin are accompanied by profound changes in current. Such an auxiliary protein is unlikely to be the HCN beta subunit MiRP1 since tyrosine residues in MiRP1 do not conform to TK-consensus phosphorylation motifs and MiRP1 overexpression in *Xenopus* oocytes does not influence HCN regulation by another broad spectrum inhibitor of tyrosine kinases [37]. However, the list of proteins that can act as HCN binding partners has grown in recent years [12,15,29], suggesting that tyrosine phosphorylation of an associated protein deserves further consideration in future studies.

The erbstatin effect on current density could result from displacement of channel subunits from the membrane or a change in their membrane sublocalization. It is well known that phosphorylation of many membrane proteins, including ion channels, can be important for their subcellular localization and subsequent function [21]. Dephosphorylation of these proteins by a phosphatase can lead to internalization and targeting for degradation. Removal of HCN channels from the membrane could also be related to the previously reported ability of erbstatin to disrupt protein-protein interaction [38] since the importance of such interaction for HCN proper trafficking and localization has been established [12,15]. However, preliminary data with a GFP-tagged HCN2 construct do not support the idea that erbstatin-induced reduction in current is largely due to displacement of channel subunits from the membrane (see supplementary Fig. 5).

Regardless of molecular mechanisms, this study exposes distinct and separable effects of the tyrosine kinase inhibitor erbstatin on pacemaker current magnitude and voltage dependence as well as developmental differences in HCN2 channel modulation by erbstatin, which suggest that cellular microenvironment may play a fundamental role to control pacemaker function in the heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Effect of vehicle (a) or 25 μM erbstatin (b) on HCN2 current in newborn myocytes. Current traces (in nA) of HCN2 were generated by applying hyperpolarizing steps to -40, -50, -60, -70, -90, -110, -120, and -130 mV from holding potential of -35 mV. The test pulses were followed by a voltage step to -100 mV, where the tail current was recorded. c Maximal channel conductance in control (open circles; representing time shortly after start of whole-cell recording), vehicle (filled circles), and after 25 μM erbstatin (filled squares) was obtained after plotting tail current in relation to the test voltage. Tail currents in vehicle and erbstatin group were normalized to corresponding controls. d Fractional activation of HCN2 channels. The mean value for midpoint of activation and slope factor are: in control -71.9 ± 1.3 mV and 9.46 ± 0.60 mV; in vehicle -73.4 ± 1.8 mV and 8.2 ± 1.4 mV; in erbstatin -89.3 ± 2.7 mV and 11.3 ± 1.1 mV. The solid lines in c and d are the fits to the Boltzmann function. e Time constant of activation (circles) and deactivation (squares) of HCN2 after vehicle (open symbols) or erbstatin (filled symbols)

Fig. 2.

Effect of erbstatin on current amplitude, midpoint of activation, and kinetics of HCN current in newborn myocytes. **a** Representative tail currents of HCN2R/E and HCN2ΔCx before (left) and after (right) erbstatin. Tail currents were recorded at voltage step to -110 (R/E) or -100 mV (ΔCx) after applying a test voltage (as described in the “Materials and methods”). Arrows indicate -90 mV test step. **b** Kinetics of activation of HCN2R/E (upper panel) and HCN2ΔCx (lower panel). Filled circles: Vehicle, filled squares: erbstatin. **c, d** Average data demonstrating the effect of erbstatin on current amplitude (**c**) and midpoint (**d**) of mHCN2 activation. Maximal channel conductance was obtained after plotting tail current in relation to the test voltage. Results are presented as a percentage decrease of maximal tail current relative to control. In **d**, shift in $V_{1/2}$ was analyzed from each individual cell as the difference between midpoints of activation in control and after erbstatin (or vehicle). White bars: Vehicle experiments, gray bars: effect of 25 μM erbstatin. * $p < 0.05$; ** $p < 0.001$.

Fig. 3. Activation relation of HCN2 (*open circles*) and HCN2R/E (*filled circles*) in newborn and adult myocytes. The normalized plot of tail current vs. test voltages was fitted with the Boltzmann function

Fig. 4.

Effect of erbstatin on HCN2 current in adult ventricular myocytes. **a** Maximal channel conductance was obtained after plotting tail current in relation to the test voltage. Tail currents in vehicle and erbstatin group were normalized to corresponding controls. The *solid lines* are the fits to the Boltzmann function. **b** Effect of erbstatin on maximal tail current with and without cAMP in pipette solution. *White bars* Vehicle, *gray bars* erbstatin, *far left bar* corresponding effect of erbstatin in newborn (NB) myocytes. **c** Effect of erbstatin on activation kinetics of HCN2. *Open circles* Vehicle, *filled circles* erbstatin

Fig. 5. Summary plot of effect of erbstatin on midpoint of activation of mHCN2 in rat ventricular myocytes. *Open circles* Vehicle, *filled circles* erbstatin. * $p < 0.05$; ** $p < 0.001$

Fig. 6.

Tyrosine phosphorylation of HCN2 in newborn rat ventricular myocytes. **a** Cardiomyocyte cultures were treated with erbstatin (25 μ M) for 5 min or H_2O_2 (5 mM) for 10 min and then harvested in homogenization buffer as described in the “Materials and methods”. Protein (0.7 mg) was immunoprecipitated with anti-HCN2 antibodies and then visualized with anti-HCN2 antibodies (using protein immunoprecipitated from 100 μ g starting cell membrane preparation, *top row*) and antiphosphotyrosine antibodies (using protein immunoprecipitated from 600 μ g starting cell membrane preparation, *lower row*). **b** Average data from densitometry from three separate experiments. Summary density of all three epitope-specific bands from erbstatin-treated myocytes was significantly less than in control. * $p < 0.05$. Statistical significance was defined in one-tail t test