Electrophysiological characterization of murine HL-5 atrial cardiomyocytes

Yong-Fu Xiao,1 Erica M. TenBroek,2 Joshua J. Wilhelm,1 Paul A. Iaizzo,3 and Daniel C. Sigg1

1Cardiac Rhythm Disease Management and 2Corporate Science and Technology, Medtronic Incorporated, Minneapolis, and 3Departments of Surgery and Physiology, University of Minnesota, Minneapolis, Minnesota

Submitted 18 January 2006; accepted in final form 27 March 2006

Xiao, Yong-Fu, Erica M. TenBroek, Joshua J. Wilhelm, Paul A. Iaizzo, and Daniel C. Sigg. Electrophysiological characterization of murine HL-5 atrial cardiomyocytes. Am J Physiol Cell Physiol 291: C407–C416, 2006.—HL-5 cells are cultured murine atrial cardiomyocytes and have been used in studies to address important cellular and molecular questions. However, electrophysiological features of HL-5 cells have not been characterized. In this study, we examined such properties using whole cell patch-clamp techniques. Membrane capacitance of the HL-5 cells was from 8 to 62 pF. The resting membrane potential was −57.8 ± 1.4 mV (n = 51). Intracellular injection of depolarizing currents evoked action potentials (APs) with variable morphologies in 71% of the patched cells. Interestingly, the incidence of successful, current-induced APs positively correlated with the hyperpolarizing degrees of resting membrane potentials (r = 0.99, P < 0.001). Only a few of the patched cells (4 of 51, 7.8%) exhibited spontaneous APs. The muscarinic agonist carbachol activated the acetylcholine-activated K+ current and significantly shortened the duration of APs. Immunostaining confirmed the presence of the muscarinic receptor type 2 in HL-5 cells. The hyperpolarization-activated cation current (Ih) was detected in 39% of the patched cells. The voltage to activate 50% of Ih channels was −73.4 ± 1.2 mV (n = 12). Voltage-gated Na+, Ca2+, and K+ currents were observed in the HL-5 cells with variable incidences. Compared with the adult mouse cardiomyocytes, the HL-5 cells had prolonged APs and small outward K+ currents. Our data indicate that HL-5 cells display significant electrophysiological heterogeneity of morphological appearance of APs and expression of functional ion channels. Compared with adult murine cardiomyocytes, HL-5 cells show an immature phenotype of cardiac AP morphology.

action potential; ion channel; muscarinic receptor

TO GENERATE A CARDIAC CELL LINE, Field (8) in an early study used transgenic mice that carry fusions between the transcriptional regulatory sequences of atrial natriuretic factor and those encoding SV40 T antigen to derive AT-1 cells. These cells can be maintained as a subcutaneous tumor lineage in syngeneic mice. To have a cardiac cell line that can be serially passaged after culture while maintaining a differentiated phenotype and also be recovered from frozen stocks, Claycomb et al. (5) derived the HL-1 cell line from the AT-1 cell line. HL-1 cells have been used in a variety of experiments to address important questions on cellular and molecular cardiac biology (33). Several studies have described the biological features of HL-1 cells. HL-1 cells express a time-dependent outward current similar to the delayed rectifier K+ current (5). HL-1 cells were used to study mutations in the human ether-a ´-go-go-related gene (HERG) (2) and the effect of KCR1, a novel HERG channel binding protein, on the sensitivity of HERG channels to antiarrhythmic agents (16). Recently, the sarcolemmal ATP-sensitive K+ channel was identified in HL-1 cells (9). In addition, Sartiani et al. (26) carried out an extensive charac-
terization of HL-1 cells by studying calcium cycling, action potential (AP), and the hyperpolarization-activated cyclic nucleotide-gated (HCN) “pacemaking” channel. Functional expression of the voltage-gated L- and T-type Ca2+ channels also has been reported in HL-1 cells (37). The expression of Ca2+ channels in HL-1 cells also was observed in other studies (25, 43). Finally, rapid stimulation of HL-1 cells for 24 h significantly shortened the duration of APs and induced a downregulation of L- and T-type Ca2+ channels (43). Taken together, the data from these studies suggest that the electrophysiological properties of HL-1 cells are similar to those of primary cardiomyocytes. HL-1 cells have been used as a model cardiac cell to study their utility as a portable, cell-based biosensor system to monitor the effects of chemical and biological agents on cardiac function (6, 10).

The HL-5 cell line also was derived from the AT-1 cell line (33). Wu et al. (36) evaluated the intracellular process of atrial natriuretic peptide in HL-5 cells by using the RNA interference technology. In addition, HL-5 cells have been used to investigate the signaling mechanism of cardiomyocyte apoptosis induced by ischemia-reperfusion in vitro (4). Nevertheless, there is a lack of information regarding the electrophysiological features of HL-5 cells. Furthermore, HL-1 cells resemble embryonic atrial cardiac muscle cells ultrastructurally (5) but have electrophysiological properties that are very similar to those of primary adult cardiomyocytes (33). Therefore, a goal of the present study was to characterize the basic electrophysiological properties of HL-5 cells. We also attempted to evaluate their relative homogeneity based on their electrophysiological features and maturity via a comparison of our observed results with electrophysiological properties of isolated adult mouse cardiomyocytes.

MATERIALS AND METHODS

Culture of HL-5 cells. HL-5 cells at passage 70 purchased from Dr. W. C. Claycomb (Louisiana State University Health Science Center, New Orleans, LA) were cultured on gelatin/fibronectin-coated glass coverslips placed in 35-mm culture dishes. Cells were maintained in Claycomb medium (JRH Biosciences, Lenexa, KS) (5), supplemented with 10% fetal bovine serum (Life Technologies, Rockville, MD), 4 mM I-glutamine (Life Technologies), and 10 μM norepinephrine (Sigma Aldrich, St. Louis, MO). The medium was changed every 24 h. HL-5 cells at different passages (from passages 74 to 78) were split when they reached a state of confluence by adding trypsin-EDEA (Life Technologies) to the culture dishes. Digestion was stopped by the addition of culture medium. Dissociated cells were either replated for a new passage or used for patch-clamp experiments. HL-5 cells were cultured at 37°C under an atmosphere of 5% CO2 and 95% air with ~95% humidity.

Address for reprint requests and other correspondence: Y.-F. Xiao, Cardiac Rhythm Disease Management, Medtronic Inc., 7000 Central Ave. NE, B252, Minneapolis, MN 55432-3576 (e-mail: yong-fu.xiao@medtronic.com).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Isolation of adult mouse cardiomyocyte. Single left ventricular cardiomyocytes were enzymatically isolated from adult mouse hearts by using methods described previously (41). Briefly, hearts were rapidly excised, cannulated via the aorta, and connected to a modified Langendorff apparatus. Hearts were initially perfused for 4 min with oxygenated 37°C normal Tyrode solution. Hearts were then perfused with Ca²⁺-free Tyrode solution for 5–6 min, recirculated with Ca²⁺-free Tyrode solution containing 0.7 mg/ml collagenase (type I) and 0.02 mg/ml protease (type XIV) (Sigma Aldrich) for 10–15 min, and finally perfused with Tyrode solution containing 200 μM CaCl₂ for 5 min. Several pieces of myocardium were then removed from the left ventricle, placed into a petri dish with Tyrode solution containing 200 μM CaCl₂, minced, and gently agitated to separate the cells at room temperature. Quiescent, rod-shaped ventricular myocytes with clear striations were patched for electrophysiology studies.

Electrophysiological recordings. After dissociation of HL-5 cells from a culture dish, the cells (~10,000 cells/cm²) were replated on gelatin/fibronectin-coated coverslips for patch-clamp experiments. One day after reculture, the cells plated on a coverslip were transported to a chamber mounted on the stage of a Nikon microscope (Tokyo, Japan). The chamber was continuously superfused (~0.5 ml/min) with the Tyrode solution. The whole cell configuration of the patch-clamp technique (12) was applied. Briefly, glass electrodes (World Precision Instruments, Sarasota, FL) with 1- to 3-MΩ resistance were connected via an Ag-AgCl wire to an Axopatch 200A amplifier interfaced with a DigiData-1320 acquisition system (Axon Instruments, Foster City, CA). After a conventional “gigahm” seal was formed, electrode capacitance was compensated. Additional suction ruptured the patched membrane and formed the whole cell configuration. Cell membrane capacitance (Cm) was measured in each patched cell with the pCLAMP program (version 9.2; Axon Instruments). The average value of Cm was 22.2 ± 1.1 pF for the HL-5 cells (n = 120).

During recording of APs, HL-5 or adult cardiomyocytes were superfused with the normal Tyrode solution. APs were measured under the current-clamp condition. Before initiation of an AP, the membrane potential of a patched cell was held at approximately −76 mV (~76.1 ± 0.61 mV, n = 57) via intracellular injection of a constant-hyperpolarized current. Electrically stimulated APs were elicited at a rate of 0.1 Hz by 5-ms square current pulses and sampled at 5 kHz. Spontaneous APs were recorded under the zero-current clamp condition. The outward (Iₒ) and inward (Iₖ) K⁺ currents were evoked using protocols similar to those described previously (40). The protocol used to elicit acetylcholine-activated K⁺ current (I_KACHCl) was similar to the one described in a previous report by our group (38). The K⁺ currents were recorded under bath perfusion with the normal Tyrode solution plus 5 μM verapamil to block Ca²⁺ and 20 μM tetrodotoxin to block Na⁺ channels. The methods for recording the voltage-gated Na⁺ (I_Na) and Ca²⁺ (I_Ca) currents were similar to those of our group described previously (39, 41). The hyperpolarization-activated cyclic nucleotide-gated (HCN) inward current (I_h) was measured with the modified Tyrode bath solution. I_h was evoked by 2- to 6-s hyperpolarizing steps to potentials ranging from −50 to −130 mV from a holding potential of −40 mV. The reversal potential of I_h was measured using tail currents recorded by 1.2-s “tail” steps to membrane potentials ranging from −80 to 0 mV in 10-mV increments following a 2-s conditioning potential step to −120 mV for 10 s. The holding potential was set at −40 mV. The activation of I_h was elicited by 3-s tail pulses to −120 mV following 5-s conditioning pulses from 0 to −130 mV in 10-mV increments. The membrane holding potential was −40 mV, and the pulse rate was once every 30 s.

Immunohistochemistry. HL-5 cells were replated to poly-d-lysine/ laminin-coated coverslips (BD Biosciences, Bedford, MA) at a low cell density (~10,000 cells/cm²) to visualize colonies and individual cells, and they were grown for 24 h. Cells were then fixed with 4% paraformaldehyde (Polysciences, Warrington, PA) for 10 min at room temperature, permeabilized with 0.15% Triton X-100 (Sigma Aldrich) in complete medium, and blocked overnight with 10% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA). Coverslips were washed three times with Dulbecco’s phosphate-buffered saline before being incubated with primary antibody to muscarinic acetylcholine receptor M₂ (Santa Cruz Biotechnology) at a 1:50 dilution for 1 h at room temperature. After coverslips were washed three times, incubation was done with goat anti-rabbit-FITC secondary antibody (Santa Cruz) at 1:50 and wheat germ agglutinin conjugated to Texas red (Molecular Probes, Eugene, OR) at 1:100 dilution. Coverslips were then washed extensively before being mounted on glass microscope slides with Ultra-Cruz mounting medium (Santa Cruz Biotechnology), containing 4,6-diamidino-2-phenylindole counterstain for visualization of nuclei. Confocal microscopy was performed on a Bio-Rad 1024 Multi-Photon system with a Titanium sapphire tunable solid-state laser (Life Science Research Group, Hercules, CA). Colorization was determined from overlaying colorized images using the Adobe Photoshop technique.

Solutions and chemicals. The normal Tyrode solution for recordings of APs contained (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 d-glucose, and 10 HEPES (pH adjusted to 7.4 with NaOH). For the recording of K⁺ currents, the bath solution contained (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 0.005 verapamil, 0.02 tetrodotoxin, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The modified Tyrode solution for the recordings of I_Ca contained the normal Tyrode solution as described above with supplement of 2 mM NiCl₂ and 0.2 mM CdCl₂ to block the Ca²⁺ current and Ca²⁺-activated current, 2 mM BaCl₂ to block the inward rectifier K⁺ current (I_K1), and 1 mM 4-aminopyridine (4-AP) to block the transient outward K⁺ current (I_o). The concentration of KCl was increased to 25 mM to amplify I_Ca. The pipette solution for recording APs and I_Ca contained (in mM) 130 K-glutamate, 15 KCl, 5 NaCl, 5 Mg-ATP, 1 MgCl₂, 5 EGTA, 1 CaCl₂, and 10 HEPES (pH adjusted to 7.2 with KOH).

For the recording of Ca²⁺ currents, the bath solution contained (in mM) 120 N-methyl-d-glucamine, 5 CsCl, 1 MgCl₂, 1.8 CaCl₂, 10 glucose, and 10 HEPES (pH 7.4 with HCl) plus 20 μM tetrodotoxin. The bath solution for the recording of Na⁺ currents contained (in mM) 60 NaCl, 60 N-methyl-d-glucamine, 10 CsCl, 1 MgCl₂, 1.8 CaCl₂, 10 glucose, and 10 HEPES (pH 7.4 with HCl) plus 5 μM verapamil. The pipette solution was the same for the recording of both Ca²⁺ and Na⁺ currents and contained (in mM) 100 CsCl, 40 CsOH, 1 MgCl₂, 1 CaCl₂, 11 EGTA, 5 Mg-ATP, and 10 HEPES (pH 7.3 with CsOH). Carbachol, isoproterenol, ATP, and other chemicals used in this study were obtained from Sigma Aldrich. A perfusion system (Warner Instruments, Hamden, CT) was used to change the extracellular solution. Data were collected using pCLAMP software. Experiments were conducted at room temperature (~22°C).

Statistical analysis. The parameters of APs were analyzed similarly to our group’s previous report (13). Peak currents were measured for I_Na, I_Ca, and I_o. I_K1, I_h, and I_I were evaluated at a point near the end of each test pulse unless stated otherwise. The current amplitudes were normalized with respect to the corresponding values of Cm to minimize the current difference due to cell size. Some data were fitted by a Boltzmann equation \(1/[1 + \exp (V_{1/2} - V)/k]\), where \(V_{1/2}\) is the half-inactivation potential, \(V\) is the voltage potential, and \(k\) is the slope factor (in mV/e-fold change in current). The best-fit procedure was performed with a commercial software program (Origin 7.5; Microcal Software, Northampton, MA). All data are presented as means ± SE unless otherwise stated. Paired or unpaired Student’s t-test was applied for statistical analysis as appropriate. Differences were considered significant if \(P < 0.05\).

RESULTS

APs in HL-5 cells. To characterize APs of HL-5 cells, we patched single cells under whole cell current-clamp conditions. Both electrically stimulated and spontaneous APs were ob-

AJP-Cell Physiol • VOL 291 • SEPTEMBER 2006 • www.ajpcell.org

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.4 on November 28, 2017
served in HL-5 cells. However, the morphology of APs varied greatly from cell to cell. Figure 1, A, B, and C, show the representative APs recorded from three individual HL-5 cells. These APs were elicited by intracellular injection of depolarizing currents. The amplitude of APs was 96.5 ± 3.1 mV with an overshoot of 19.2 ± 2.7 mV (n = 32). Action potential duration (APD) measured at 50 and 90% repolarization was 41.5 ± 3.0 and 120.0 ± 7.1 ms (n = 32), respectively. The spontaneous APs in Fig. 1D were recorded without intracellular injection of depolarizing currents. However, in some cells there were neither spontaneous APs nor electrically stimulated APs (Fig. 1C). The expanded recording at right in Fig. 1D indicated by the arrow clearly displays a spontaneous diastolic depolarization phase.

To determine the resting membrane potential of HL-5 cells, we applied the zero-current clamp mode under the whole cell configuration. The value was recorded when the negative membrane potential of a cell reached the maximum following dialysis with the internal solution. Most of the patched cells had resting potentials in the range of −40 to −69 mV (42 of 51 patched cells; Fig. 2, A and B). On average, the HL-5 cells had a resting potential of −57.8 ± 1.4 mV (n = 51). Only five cells had resting membrane potentials at −70 mV or more negative. The most negative measured resting membrane potential was −78 mV. We hypothesized that a more negative resting potential might be associated with a greater likelihood of AP induction. Therefore, we correlated the incidence of electrically stimulated APs with the resting membrane potential (Fig. 2B). Interestingly, the incidence of stimulated APs was 100% if the cells had resting membrane potentials of −70 mV or more negative. In contrast, the incidence of stimulated APs was only 25% if the cells had resting membrane potentials between −30 and −39 mV. Some of the HL-5 cells exhibited spontaneous APs; however, the incidence was only ~8% (4 of 51 cells). Electric stimuli with various strengths failed to elicit APs in ~30% of the patched HL-5 cells (Figs. 1C and 2C).

Both mammalian atrial and ventricular myocytes, including mouse heart cells, express muscarinic receptors (11, 14, 24, 27, 29, 38). Thus, to determine whether the murine atrial HL-5 cell line expressed muscarinic receptors and what the effects would be when the receptors were activated, we administered the muscarinic receptor agonist carbachol to the HL-5 cells. Figure 3A shows that bath perfusion of 5 μM carbachol reduced both the amplitude and duration of APs. This reduction probably resulted from the activation of I_{K(ACh)} (Fig. 3B). Table 1 summarizes the effects of bath perfusion of 5 μM carbachol on the properties of APs in these HL-5 cells. Specifically, in the presence of 5 μM carbachol, the amplitude, overshoot, and duration of 90% repolarization of APs were significantly decreased. In addition, immunostaining showed the presence of muscarinic M2 receptors in the sarcolemmal membrane of the HL-5 cells (Fig. 3C). These results indicate that HL-5 cells express muscarinic receptors and that the agonist carbachol is able to modify their influence on APs.

The β-adrenergic receptor plays important roles in positive chronotropic and inotropic effects of heart cells. Therefore, we also tested the effects of the β-adrenergic agonist isoproterenol on the properties of APs in HL-5 cells. However, extracellular application of 2 μM isoproterenol caused variable and very small effects on the duration of APs. Actually, the duration of APs increased in two cells and decreased in two others (P > 0.05; data not shown). This probably resulted from the continuous exposure to adrenergic stimulation of the HL-5 cells, because the culture medium contained norepinephrine.

Voltage-gated Na+ and Ca2+ channels in HL-5 cells. In our cultured HL-5 cells, I_{Na} with fast activation and fast inactivation kinetics was evoked by depolarizing pulses from −80 to 40 mV with 10-mV increments. Figure 4A shows the current-
activation was $-40.6 \pm 0.5$ mV with a $k$ (slope) value of $5.8 \pm 0.4$ ($n = 10$, Fig. 4B). The steady-state inactivation of $I_{Na}$ in these HL-5 cells had a mean value of $-96.3 \pm 0.6$ mV ($n = 6$) at the $V_{1/2}$ point with a $k$ value of $10.7 \pm 0.7$ (Fig. 4B). This $V_{1/2}$ value of inactivation in these cells was more hyperpolarized compared with that of $I_{Na}$ in adult mouse ventricular cardiomyocytes. The $V_{1/2}$ of $I_{Na}$ was $-87.7 \pm 0.2$ mV in the adult cardiomyocytes ($n = 15$), and the peak current density was $-59 \pm 4$ pA/pF elicited by test pulses from $-120$ to $-30$ mV. In addition, the window current between the activation and inactivation curves of $I_{Na}$ was very small in the HL-5 cells (Fig. 4B). Compared with that in adult cardiomyocytes, the small $I_{Na}$ density and the negative shift of the $V_{1/2}$ in HL-5 cells may reduce the amplitude, overshoot, and maximal velocity of the upstroke of APs.

The whole cell L-type $I_{Ca}$ of HL-5 cells was obtained by applying a group of test pulses from $-60$ to $40$ mV (see inset in Fig. 4C). The current-voltage curve was bell-shaped with the maximal current at $0$ mV. The peak current density of $I_{Ca}$ at $0$ mV in these HL-5 cells was $-4.5 \pm 0.8$ pA/pF ($n = 10$, Fig. 4F); this is significantly lower than that ($-9.7 \pm 0.6$ pA/pF, $n = 60$) in the adult mouse ventricular cardiomyocytes (41). The $V_{1/2}$ of $I_{Ca}$ activation in the HL-5 cells was $-15.2 \pm 0.8$ mV with a $k$ value of $8.1 \pm 1.3$ ($n = 10$, Fig. 4F). The steady-state inactivation of $I_{Ca}$ in the HL-5 cells had a mean value of $-29.5 \pm 1.0$ mV ($n = 10$, Fig. 4F) at the $V_{1/2}$ with a $k$ value of $6.8 \pm 1.2$ (Fig. 4D). The $V_{1/2}$ of the steady-state inactivation of $I_{Ca}$ in the HL-5 cells was very similar to that ($-30.2 \pm 0.9$ mV, $n = 17$) in the adult mouse ventricular cardiomyocytes (41). The normalized activation and inactivation curves of $I_{Ca}$ show that the HL-5 cells displayed a large window Ca$^{2+}$ current (Fig. 4D). Studies have shown that the L-type Ca$^{2+}$ channel window current plays a major role in the initiation of early afterdepolarizations (22) and that reduction of the window Ca$^{2+}$ current may reduce the likelihood of early afterdepolarizations (34), but the significance of the large window Ca$^{2+}$ current in cultured HL-5 cells is unknown.

Voltage-activated K$^+$ currents in HL-5 cells. Two major voltage-activated outward K$^+$ currents, $I_{K}$ and the delayed outward rectifier K$^+$ current ($I_{K}$), have been broadly classified in cardiomyocytes. However, multiple components of $I_{K}$ and $I_{K}$ have been used for analysis of the outward K$^+$ currents in different studies (23, 30, 42). In the present study, we analyzed the peak amplitude ($I_{peak}$; Fig. 5A) and the sustained component ($I_{ss}$; Fig. 5A) of such outward K$^+$ currents in HL-5 cells. $I_{peak}$ and $I_{ss}$ were measured at the point of the maximal peak and at the point near the end of outward currents elicited by 5-s depolarization pulses (see inset in Fig. 5A), respectively. $I_{to}$ was calculated by subtraction of $I_{ss}$ from the corresponding $I_{peak}$. Figure 5A shows the current-voltage relationships of $I_{peak}$, $I_{ss}$, and $I_{to}$ that were elicited by the 5-s test pulses from $-90$ to $60$ mV. The current densities measured at the test pulse of $60$ mV ($n = 18$) were $17.0 \pm 1.9$, $13.5 \pm 1.6$, and $3.7 \pm 0.3$ pA/pF for $I_{peak}$, $I_{ss}$, and $I_{to}$, respectively. We also examined the main inward K$^+$ current, $I_{K}$, in HL-5 cells. $I_{K}$ was evoked by 2-s hyperpolarizing pulses from the holding potential of $-40$ mV down to $-160$ mV with $-10$-mV increments every 20 s. The resultant current-voltage relationship is shown in Fig. 5B, and the averaged current density measured at $-160$ mV was $-7.3 \pm 2.7$ pA/pF ($n = 10$, Fig. 5B). These results strongly
suggest that cultured HL-5 atrial cardiomyocytes express these three main $K^+$ channels.

**Hyperpolarization-activated cation currents in HL-5 cells.**

To determine the expression of the HCN inward current ($I_f$) in cultured HL-5 cells, we applied different voltage protocols during the experiments (see insets in Fig. 6). Such applied hyperpolarizing pulses elicited the time-dependent and non-inactivating inward current, $I_f$. The amplitudes of $I_f$ were increased at more negative test potentials (Fig. 6). Representative $I_f$ traces recorded in a single HL-5 cell are shown in Fig. 6A. The current densities were measured at the time point indicated in Fig. 6A. The mean values from 13 cells are plotted in Fig. 6B as a function of membrane potentials. The density of $I_f$ at $-130$ mV was $-0.5 \pm 1.3$ pA/pF. Time constants ($\tau$) of activating currents were determined by single-exponential fitting of individual current traces. As the voltage became more negative, the activation of the current became faster. The mean $\tau$ values of $I_f$ were $1.3 \pm 0.2$ s at $-80$ mV and $0.42 \pm 0.04$ s at $-130$ mV ($n = 12$). Extracellular perfusion of 4 mM Cs$^+$ almost completely blocked $I_f$ in the HL-5 cells, and the blocking effect was reversible after washout of Cs$^+$ (data not shown).

---

Fig. 3. Effect of carbachol on action potential duration (APD) in HL-5 cells. A: APs in the absence (control) and presence of 5 $\mu$M carbachol in an HL-5 cell. B: mean $K^+$ currents ($n=6$) in the absence (control) and presence of 5 $\mu$M carbachol in the bath perfusion solution. The differences (carbachol − control) of the currents represents the acetylcholine-activated $K^+$ current [$I_{K(ACh)}$]. The currents were elicited by 250-ms pulses from a holding potential of $-40$ mV down to $-130$ mV and up to $-30$ mV with 10-mV increments every 10 s. C: immunohistochemistry with confocal microscopy confirms the presence of muscarinic M$_2$ receptors in HL-5 cells ($\times$40 magnification). Brightfield images show morphology of the single HL-5 cells or a cell mass. UV images visualize nuclei stained by 4,6-diamidino-2-phenylindole. WGA-Texas red images were obtained using wheat germ agglutinin (WGA) conjugated to Texas red to stain cell membranes by binding to N-acetyleneuraminic acid and N-acetylglycosamine residues (24). Anti-mAChR M$_2$ images were stained with the antibody against the muscarinic receptor M$_2$. Colocalization is indicated by merging WGA-Texas red with anti-mAChR M$_2$ images.
The reversal potentials of $I_f$ in HL-5 cells were determined using the measurement of tail currents. The tail-current traces recorded from a HL-5 cell were elicited by depolarizing steps ranging from −80 to 30 mV in 10-mV increments following a hyperpolarizing pulse to −120 mV, which was sufficient to maximally activate $I_f$ in HL-5 cells (Fig. 6F). Tail-current amplitudes were measured at the time point indicated in Fig. 6C. The current-voltage relationship shown in Fig. 6D was obtained by plotting the means ($n = 13$) of current densities vs. their associated membrane potentials. These obtained data were well fitted by a linear regression function, which intersected with the $x$-axis at −16 mV, reflecting the average reversal potential of $I_f$ of the HL-5 cells. HCN channels are permeable to both Na$^+$ and K$^+$ with a permeability ratio $P_{Na}/P_K$ of 0.3 (28). Our modified Tyrode solution contained 25 mM K$^+$ to enhance the amplitude of $I_f$ and several blockers (see MATERIALS AND METHODS) to minimize possible interference from Ca$^{2+}$ and K$^+$ conductances. The reversal potential (−16 mV) obtained in this study is very similar to that of $I_f$ in HL-1 cells (−20.8 ± 1.5 mV) and HCN3 channels (−20.5 ± 4 mV) (26, 28).

To measure the activation conductance of $I_f$ in cultured HL-5 cells, we used an experimental protocol composed of 5-s test pulses with various voltages from −130 to 0 mV, followed by 2-s tail pulses of −120 mV (see inset in Fig. 6E). Figure 6E shows representative current traces of $I_f$ elicited using the protocol in a single HL-5 cell. The resultant activation conductance was calculated using the tail currents measured at the time point indicated and was normalized with respect to the maximal conductance value for each cell. Figure 6F shows the mean normalized activation conductance of $I_f$ obtained from 12 cells. The activation curve was obtained from data fitted with a Boltzmann function. The threshold of $I_f$ activation in the HL-5 cells was −50 mV, and the $V_{1/2}$ of activation was −73.4 ± 1.2 mV with a $k$ value of 10.5 ± 0.7.

Our results show that $I_f$ was observed in 39% of the patched HL-5 cells (21 of 54 cells). Only 62% of the patched cells exhibited an obvious $I_{Na}$. However, most of the patched HL-5 cells elicited $I_{Na}$ (85%), $I_{Ca}$ (79%), $I_k$ (93%), and $I_{K1}$ (86%) currents. The incidences of obtaining these main currents in the HL-5 cells are correlated relatively well with the chance (71%) to successfully induce APs after electrical stimulation (Fig. 2C).

**Comparison of APs between HL-5 and adult mouse cardiomyocytes.** Developmental changes in the morphology of APs occur in both atrial and ventricular cardiomyocytes in mice (30, 32). Both HL-1 and HL-5 cell lines were derived from the AT-1 adult atrial myocyte line (33), but the ultrastructural

---

**Table 1. Effects of carbachol on action potentials in HL-5 cardiomyocytes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>MP, mV</th>
<th>AAP, mV</th>
<th>OS, mV</th>
<th>$V_{max}$, V/s</th>
<th>APD$_{50}$, ms</th>
<th>APD$_{90}$, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−72.7 ± 2.4</td>
<td>84.9 ± 4.7</td>
<td>12.1 ± 3.1</td>
<td>13.4 ± 3.5</td>
<td>45.5 ± 7.0</td>
<td>118.1 ± 15.9</td>
</tr>
<tr>
<td>Carbachol</td>
<td>−72.4 ± 2.0</td>
<td>78.9 ± 4.9†</td>
<td>6.4 ± 3.7†</td>
<td>12.2 ± 3.4</td>
<td>36.4 ± 5.6</td>
<td>85.3 ± 11.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 9$). MP, membrane potential measured and maintained by intracellular injection of hyperpolarizing currents before elicitation of action potentials; AAP, amplitude of action potential; OS, overshoot; $V_{max}$, maximum upstroke velocity of action potential; APD, action potential duration measured at 50% (APD$_{50}$) and 90% (APD$_{90}$) of repolarization. *$P < 0.05$; †$P < 0.01$ vs. corresponding control with paired Student’s $t$-test.
characteristics of HL-1 cells are typical of embryonic atrial cardiac muscle cells (5). In contrast, molecular analyses have confirmed a pattern of gene expression similar to that of adult atrial myocytes (5). Therefore, it was of interest to compare the characteristics of APs of HL-5 cells with those of isolated adult mouse cardiomyocytes. To do so, we examined the APs in HL-5 cells (Fig. 7A) and adult mouse ventricular cardiomyocytes (Fig. 7B). Compared with the HL-5 cells, the adult mouse ventricular cardiomyocytes displayed significantly higher AP amplitudes, larger overshoots, and greater maximum upstroke velocities. The duration of APs was significantly shortened in the adult mouse ventricular cardiomyocytes compared with the HL-5 cells (Table 2).

Voltage-gated outward K⁺ channels play an important role in the amplitude and duration of APs in cardiomyocytes. Based primarily on time- and voltage-dependent properties, there are two broad classes of outward K⁺ currents, Iₒ and I𝐾 (23, 42). Shortening of the duration of APs in adult mouse cardiomyocytes is considered to be caused by increased outward K⁺ currents (23, 42). We thus examined whole cell outward K⁺ currents in HL-5 cells and adult mouse ventricular cardiomyocytes. Depolarization pulses elicited superimposed outward
currents in both the HL-5 cell (Fig. 7C) and the adult cardiomyocyte (Fig. 7D). Yet, the adult mouse ventricular cardiomyocyte had large $I_{\text{to}}$ ($I_{\text{to}} = I_{\text{peak}} - I_{\text{ss}}$) that rose rapidly to the peak and then decayed (Fig. 7D). The values of $I_{\text{peak}}$, $I_{\text{ss}}$, and $I_{\text{to}}$ were $11.4 \pm 2.4$, $7.6 \pm 1.5$, and $3.8 \pm 1.0$ pA/pF for the HL-5 cells ($n = 11$) and $62.4 \pm 3.2$, $23.8 \pm 1.6$, and $38.6 \pm 2.8$ pA/pF for the adult cardiomyocytes ($n = 22$), respectively. Compared with that in adult mouse cardiomyocytes, $I_{\text{to}}$ was significantly decreased in the HL-5 cells (Fig. 7E). The ratios $I_{\text{to}}/I_{\text{peak}}$ were $31.4 \pm 3.4$ and $61.9 \pm 3.8\%$ for the HL-5 cells and adult mouse ventricular cardiomyocytes, respectively. Importantly, $I_{\text{to}}/I_{\text{peak}}$ was almost twofold greater in the adult mouse ventricular cardiomyocytes than in the HL-5 cells (Fig. 7F). In contrast, $I_{\text{to}}/I_{\text{peak}}$ in the adult cardiomyocytes is much smaller than in the HL-5 cells. These results suggest that compared with the adult mouse cardiomyocytes, prolongation of the APD in the HL-5 cells is primarily caused by a relatively smaller outward K$^+$ current.

**DISCUSSION**

In the present study, we characterized the electrophysiological features of the HL-5 atrial myocyte line. Our data show that these immortalized HL-5 cells had variable resting membrane potentials and that most of them fell in the range of $-50$ to $-69$ mV. Whereas the frequency of spontaneous APs was only 8% in these patch HL-5 cells, electric stimuli could induce APs in most of them (71%). However, the morphology of APs in our cells varied considerably (Fig. 1). It is interesting that in the HL-5 cells with more hyperpolarized resting membrane potentials, electrically induced APs were readily induced; the inverse was observed in relatively more depolarized cells (Fig. 2). More specifically, APs were inducible in all of the cells (100%) when their resting membrane potentials were $-70$ mV or more negative (Fig. 2). The incidence of electrically stimulated APs was significantly decreased in the cells with more depolarized potentials. Our data indicate that HL-5 cells do not have uniform electrophysiological characteristics. These results suggest that HL-5 cells with more negative resting potentials may be healthier than those with depolarized resting membrane potentials. The underlying cause of these variabilities in membrane physiological properties in HL-5 cells is unknown. One speculation is that HL-5 cells in culture are at various differentiating stages or are probably damaged and dying. Another possibility is that after multiple passages in culture, HL-5 cells may contain some non-atrial muscle cells that are then no longer a phenotype of cardiomyocytes. Investigation of these possibilities was

**Table 2. Characteristics of action potentials in murine HL-5 and adult ventricular cells**

<table>
<thead>
<tr>
<th>Myocyte</th>
<th>$n$</th>
<th>MP, mV</th>
<th>AAP, mV</th>
<th>OS, mV</th>
<th>$V_{\text{max}}$, $V_{\text{ss}}$</th>
<th>APD$_{50}$, ms</th>
<th>APD$_{90}$, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-5</td>
<td>32</td>
<td>$-77 \pm 1.3$</td>
<td>$96.5 \pm 3.1$</td>
<td>$19.2 \pm 2.7$</td>
<td>$20.2 \pm 4.2$</td>
<td>$41.5 \pm 3.0$</td>
<td>$120.0 \pm 7.1$</td>
</tr>
<tr>
<td>Adult</td>
<td>25</td>
<td>$-80 \pm 2.3$</td>
<td>$112.7 \pm 2.5^*$</td>
<td>$32.4 \pm 2.5^*$</td>
<td>$54.2 \pm 4.5^*$</td>
<td>$8.9 \pm 0.7^*$</td>
<td>$28.3 \pm 2.8^*$</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE; $n$, no. of cells. *$P < 0.05$; †$P < 0.01$ vs. corresponding values of HL-5 cells.
outside the scope of the present study, but we plan to investigate such possibilities in the future.

Importantly, the extracellular application of the muscarinic receptor agonist carbachol significantly reduced the duration of APs in HL-5 cells (Fig. 3A and Table 1). This reduction probably resulted from the activation of $\mathit{I}_{\mathit{K(ACh)}}$ (Fig. 3B). Previous work by our group (38) and a report from Boyett et al. (3) showed that stimulation of muscarinic receptors significantly decreases the duration of APs in ferret ventricular cardiomycocytes because of activation of $\mathit{I}_{\mathit{K(ACh)}}$. The presence of muscarinic receptors in HL-5 cells was further confirmed by immunocytochemistry staining. We found that the muscarinic M$_2$ receptor was well expressed in the sarcolemmal cell membrane of HL-5 cells (Fig. 3C). Hence, this result is consistent with the previous findings that both mammalian atrial and ventricular myocytes isolated from several different species, including humans, contain muscarinic receptors (15, 17, 18, 19, 38). Therefore, our data in this study suggest that even after prolonged culture periods and multiple replications, HL-5 cells maintain their abilities to express functional muscarinic receptors.

Our present data demonstrate the presence of $\mathit{I}_{\mathit{f}}$ currents in immortalized HL-5 atrial cardiomycocytes. The electrophysiological characteristics of $\mathit{I}_{\mathit{f}}$ in HL-5 cells are similar to those found in cardiac pacemaker cells (7) and HL-1 atrial myocytes (26). In the HL-5 cells, $\mathit{I}_{\mathit{f}}$ was activated when the membrane potential was hyperpolarized to $-50$ mV and was increased as the potential become more negative. Under the 25 mM K$^+$/H$\mathit{1}$002 external solution, the current-voltage relationship of $\mathit{I}_{\mathit{f}}$ in HL-5 cells was reversed around $-15$ mV, which is close to the reversal potential of $-21$ mV in HL-1 cells (26). $\mathit{I}_{\mathit{f}}$ in the HL-5 cells was also blocked by Cs$^+$. However, $\mathit{I}_{\mathit{f}}$ was observed in only 39% of the HL-5 cells, even under the condition of 25 mM extracellular K$^+$ (which amplifies the $\mathit{I}_{\mathit{f}}$ current significantly). This percentage is slightly higher than the 30% reported by Sartiani et al. (26) for HL-1 atrial cardiomyocytes. We also observed that the passage number of the cell culture did not affect the presence of $\mathit{I}_{\mathit{f}}$ in HL-5 cells, which is consistent with the finding in HL-1 cells (26).

Compared with the number of $\mathit{I}_{\mathit{f}}$-positive cells (39%), the number of the HL-5 cells with spontaneous APs was much lower (only 8%). The possible relationship between $\mathit{I}_{\mathit{f}}$ and automaticity of HL-5 cells was beyond the scope of this study. We speculate that $\mathit{I}_{\mathit{f}}$ may not correlate with automaticity in these cells and that other ion currents may contribute to the initiation of APs in spontaneously beating myocytes. For example, proliferating immature or early mouse myocytes express functional $\mathit{I}_{\mathit{f}}$ channels (1), but electrical activity can be initiated by spontaneous Ca$^{2+}$ release from the sarcoplasmic reticulum (31). In addition, Miike et al. (20) showed that the use of viral gene transfer of the dominant negative Kir2.1AAA to inhibit the inward rectifier current ($\mathit{I}_{\mathit{k}}$) converted quiescent heart muscle cells of the left ventricle into pacemaker cells. These cells successfully generated spontaneous and rhythmic cardiac activities in guinea pigs (20, 21). Therefore, the mismatch between the number of cells having $\mathit{I}_{\mathit{f}}$ currents and the number of cells actually showing spontaneous APs may imply that the presence of $\mathit{I}_{\mathit{f}}$ in a cardiomyocyte is not sufficient to generate spontaneous contractile activity for the cells. We should point out that in the present study, single HL-5 cells, not confluent monolayer cultures, were studied using the patch-clamp technique.

Previous studies showed that the duration and configuration of APs dramatically changed during postnatal development. With this in mind, in the present study we compared the morphologies of the APs of HL-5 cells with the APs of adult mouse cardiomycocytes. The APD of the HL-5 cells was longer than that of the mouse adult ventricular myocytes (Fig. 7 and Table 2). Postnatal development of the APD and voltage-dependent K$^+$ currents has been examined in mouse atrial myocytes (30). The APDs were found to be significantly longer in day 1 neonatal mouse atrial myocytes (APD 50%, $\sim50$ ms; APD 90%, $\sim103$ ms) compared with the values obtained in other, older age groups (30). However, outward K$^+$ currents, including $\mathit{I}_{\mathit{o}}$, underwent significant upregulation during postnatal life in mouse atrium, resulting in a dramatic shortening of the APD (30). Similar developmental changes in duration and morphology of APs have been observed in mouse ventricular cardiomyocytes (32). As in the present study, the shortened APDs of adult mouse ventricular cells were considered mainly the consequence of increased expression of $\mathit{I}_{\mathit{o}}$ channels (32). Consistent with this notion, the elimination of the transient outward current in transgenic mouse atrial myocytes was shown to markedly increase the APDs (42). In addition, pharmacological blockade of the $\mathit{I}_{\mathit{o}}$ channel by 4-AP significantly prolongs APDs in adult atrial and ventricular myocytes (30, 32). Our data indicate that compared with adult mouse ventricular cardiomyocytes, HL-5 cells have much lower levels of $\mathit{I}_{\mathit{o}}$, and this may underlie the relative prolongation of APDs compared with adult cardiomyocytes. Adult mouse ventricular cardiomyocytes do share close similarities in APs and outward K$^+$ currents as parallel and similar between murine atrial and ventricular cardiomyocytes (30, 32). Because the duration of the APs in the HL-5 cells (Table 1) is longer than that in the day 1 neonatal mouse atrial myocytes (30), we could speculate that the HL-5 cell line is probably an earlier, more immature cardiac cell line, more comparable to a fetal or embryonic phenotype. This is consistent with findings that HL-1 cells have shown typical ultrastructural features of embryonic atrial cardiac muscle cells (5).

The electrophysiology in HL-1 cells, including APs and $\mathit{I}_{\mathit{f}}$, Na$^+$, Ca$^{2+}$, and K$^+$ channels, has been reported in several studies (2, 5, 6, 10, 16, 26, 37). Our present study filled in the lack of electrophysiological information in HL-5 cells. Major cardiac ion channel currents and APs could be elicited in the majority of our cultured HL-5 cells. Yet, only in a very small portion of these immortalized HL-5 cells was spontaneous activity recorded. Furthermore, a significant electrophysiological heterogeneity was observed in both the individual type of ion channels and the morphology of APs. Compared with those of adult cardiomyocytes, the APDs of HL-5 cells were prolonged, and this probably resulted from decreases in outward K$^+$ currents. From an electrophysiological view, HL-5 cells appear to be relatively immature and more comparable to a prenatal phenotype. HL-5, HL-1, and other similar cardiac cell lines are valuable cell sources for in vitro studies of cardiomyocyte biology at the cellular and molecular levels (33). Nevertheless, caution should be taken when interpreting data obtained from commercial cardiac cell lines, because our observations show significant electrophysiological heterogeneity and relative immaturity of HL-5 cells.
REFERENCES


