Characterization of Multiple Ion Channels in Cultured Human Cardiac Fibroblasts

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Abstract

Background: Although fibroblast-to-myocyte electrical coupling is experimentally suggested, electrophysiology of cardiac fibroblasts is not as well established as contractile cardiac myocytes. The present study was therefore designed to characterize ion channels in cultured human cardiac fibroblasts.

Methods and Findings: A whole-cell patch voltage clamp technique and RT-PCR were employed to determine ion channels expression and their molecular identities. We found that multiple ion channels were heterogeneously expressed in human cardiac fibroblasts. These include a big conductance Ca2+-activated K+ current (BKCa) in most (88%) human cardiac fibroblasts, a delayed rectifier K+ current (IKdr) and a transient outward K+ current (INa) in a small population (15% and 14%, respectively) of cells, an inwardly-rectifying K+ current (IKir) in 24% of cells, and a chloride current (ICl) in 7% of cells under isotonic conditions. In addition, two types of voltage-gated Na+ currents (INa) with distinct properties were present in most (61%) human cardiac fibroblasts. One was a slowly inactivated current with a persistent component, sensitive to tetrodotoxin (TTX) inhibition (INa,TTR, IC50 = 7.8 nM), the other was a rapidly inactivated current, relatively resistant to TTX (INa,TTRY, IC50 = 1.8 μM). RT-PCR revealed the molecular identities (mRNAs) of these ion channels in human cardiac fibroblasts, including KCa.1.1 (responsible for BKCa), Kv1.5, Kv1.6 (responsible for IKr), NaVa.1.6, NaVa.1.7 (for INa,TTRY), and NaVa.1.5 (for INa,TTR).

Conclusions: These results provide the first information that multiple ion channels are present in cultured human cardiac fibroblasts, and suggest the potential contribution of these ion channels to fibroblast-myocytes electrical coupling.

Introduction

It is generally recognized that cardiac myocytes and fibroblasts form extensive networks in the heart, with numerous anatomical contacts between cells [1]. Cardiac fibroblasts play a central role in the maintenance of extra-cellular matrix in the normal heart and act as mediators of inflammatory and fibrotic myocardial remodeling in the injured heart, e.g. ischemic, hypertensive, hypertrophic, and dilated cardiomyopathies, and heart failure [2,3]. The cardiac myocyte network, coupled with gap junctions, is generally believed to be electrically isolated from fibroblasts in vivo. However, in the co-culture of cardiac myocytes and fibroblasts, the heterogeneous cell types form functional gap junctions, providing a substrate for electrical coupling of distant myocytes, interconnected by fibroblasts. In addition to the evidence of fibroblast-to-myocyte electrical coupling in the rabbit SA node [4], fibroblasts have been shown to be coupled electrotonically with myocytes in vitro [1,5–8]. Moreover, there is increasing evidence that implicates potential heterocellular electrical coupling in the diseased myocardium with arrhythmogenesis [9,10]; therefore, the cardiac fibroblasts are considered to be potential targets in managing cardiac disorders including hypertrophy, heart failure and arrhythmias [2,3,9,10].

Ion channels and their functions are well studied in cardiomyocytes; however, the ion channel expression and their physiological roles are not fully understood in cardiac fibroblasts. An inward rectifier K+ current (IKir), a delayed rectifier K+ current (IKr), and a non-selective cation channel current were recently reported in rat ventricular fibroblasts [11–13]. Although a Ga2+-activated big conductance K+ current (BKCa) was described in human cardiac fibroblasts [14], it is unknown whether other types of ion channel currents are present in human cardiac fibroblasts. The present study was designed to employ the approaches of whole-cell patch voltage clamp and RT-PCR to examine the functional ion channels in human cardiac fibroblasts. Using these techniques, we identified multiple ion channels expressed in cultured human cardiac fibroblasts.

Methods

Cell cultures

Human cardiac fibroblasts (adult ventricular, Catalog# 6310) were purchased from ScienCell Research Laboratory (San Diego,
CA). The cells were cultured as monolayers in completed DMEM containing 10% fetal bovine serum (Invitrogen, Hong Kong) and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin) at 37°C in a humidified atmosphere of 95% air, 5% CO2. No difference in cell growth and ion channel expression were observed with either our culture medium or the medium from ScienCell Research Laboratory. Cells used in this study were from the early passages 2 to 6 to limit the possible variations in functional ion channel currents and gene expression. The cells were harvested for electrophysiological recording and RT-PCR determination via trypsinization [15].

Solutions and reagents
Tyrode solution for electrophysiological study contained (mM): 140 NaCl, 5.0 KCl, 1.8 CaCl2, 10 glucose, and 10 HEPES; pH was adjusted to 7.3 with NaOH. The standard patch pipette solution contained (mM): 20 KCl, 110 K-aspartate, 1.0 MgCl2, 10 HEPES, 0.05 EGTA, 0.1 GTP, 5.0 Na2-phosphocreatine, and 5.0 Mg-ATP; pH was adjusted to 7.2 with KOH. When Na+ current was determined, K+ in pipette and bath solutions was replaced by equimolar Cs.

For volume sensitive chloride current (IC(Vol) recording, hypotonic 0.7T (~210 mosmol/L) Tyrode solution was made by reducing NaCl from 140 to 98 mM. When 1.0T (~300 mosmol/L) solution was prepared, 90 mM mannitol was added. The pipette solution for recording IC(Vol) contained (mM) 110 CsCl, 20 Cs-aspartate, 5 EGTA, 1.0 MgCl2, 10 HEPES, 0.1 GTP, 5.0 Na2-phosphocreatine, and 5.0 Mg-ATP (pH = 7.2 with CsOH).

The chloride channel blocker 5-nitro-1-(3-phenylpropylamino) benzoic acid (NPPB) was purchased from Tocris (Bristol, UK). All the other chemicals including DIDS (4,4’-disothiocyanostilbene-2,2’-disulfonic acid) were purchased from Sigma-Aldrich (St Louis, MO).

Electrophysiology
A small aliquot of the solution containing the cardiac fibroblasts was placed in an open perfusion chamber (1 ml) mounted on the stage of an inverted microscope. The cells were allowed to adhere to the bottom of the chamber for 10–20 min, and then superfused at 2–3 ml/min with Tyrode solution. The studies were conducted at room temperature (22–24°C).

The membrane ionic currents were recorded with a whole-cell patch-clamp technique as described previously [16]. Borosilicate glass electrodes (1.2 mm OD) were pulled with a Brown–Flaming puller (Model P-97, Sutter Instrument Co. Novato, CA), and had tip resistances of 2–3 MΩ when filled with pipette solution. The tip potentials were compensated before the pipette touched the cell. After a gigaohm-seal was obtained by negative pressure suction, the cell membrane was ruptured by a gentle suction to establish whole-cell configuration with a seal resistance >800 MΩ. The cell membrane capacitance (49.6±12.1 pF) was electrically compensated with the Pulse software. The series resistance (Rseries) 3–5 MΩ was compensated by 50–70% to minimize voltage errors. Membrane currents were elicited with voltage protocols as described in the following Results section for individual different current recording. Data were acquired with an EPC10 amplifier (Heka, Lambrecht, Germany). The membrane currents were low-pass filtered at 5 kHz and stored on the hard disk of an IBM compatible computer.

Messenger RNA determination
The messenger RNA was examined using RT-PCR technique using Table 1 primers as described previously [15]. Total RNA was extracted from human cardiac fibroblasts using Trizol reagent (Invitrogen), and further treated with DNase I (GE Healthcare, Hong Kong) for 30 min at 37°C, then heated to 75°C for 5 min and finally cooled to 4°C [17]. Reverse transcription was performed using a RT system (Promega, Madison, WI) in a 20 µl reaction mixture. A total of 2 µg RNA was used in the reaction and a random hexamer primer was used for the initiation of cDNA synthesis. After the RT procedure, the reaction mixture (cDNA) was used for PCR.

PCR was performed with thermal cycling conditions of 94°C for 2 min followed by 33 cycles at 94°C for 45 s, 55–58°C for 45 s, and 72°C for 1 min using a Promega PCR kit and oligonucleotide primers as shown in Table 1. This was followed by a final extension at 72°C (10 min) to ensure complete product extension. The PCR products were electrophoresed through 1.5% agarose gels and visualized under a UV transilluminator (BioRad, Hercules, CA) after staining with ethidium bromide.

Statistical analysis
Results are presented as means ± SEM. Paired and/or unpaired Student’s t-tests were used as appropriate to evaluate the statistical significance of differences between two group means, and analysis of variance was used for multiple groups. Values of P<0.05 were considered to indicate statistical significance.

Results
Families of membrane ionic currents in human cardiac fibroblasts
Figure 1 illustrates the families of membrane currents recorded in human cardiac fibroblasts using a standard pipette solution. Five types of membrane currents were observed in human cardiac fibroblasts (in a total of 265 cells). One current was activated at depolarization voltages between −70 and +60 from a holding potential of −80 mV (0.2 Hz), and showed an outward current with noisy oscillation between +20 and +60 mV (Fig. 1A). These features suggest that this current is likely a big conductance Ca2+-activated K+ current (BKCa) [15]. The noisy oscillatory BKCa was present with other currents in most (88%, 233 of 265) of fibroblasts. Another current activated by the same protocol was a transient outward current (Fig. 1B), and presented in 15% (40 of 265) of cells. Third current was an inward component activated by hyperpolarization voltage steps a holding potential of −2 mV (0.2 Hz), and co-existed with the noisy oscillatory current activated by depolarization voltage steps. This inward component exhibited the properties similar to inward rectifier K+ current (I Kir) (Fig. 1C). I Kir was observed in 24% (64 of 265) of cells. Fourth current was elicited by voltage steps between −120 and +60 from a holding potential of −40 mV, showing a very small inward component and a large outward current with outward rectification (Fig. 1D). This current was observed in 7% (19 of 265) of cells. Moreover, an inward current coexists with the oscillatory current in 61% (167 of 265) of human cardiac fibroblasts (Fig. 1E and 1F). Interestingly, the inward current exhibits either a fast inactivation (Fig. 1E) or a slow (Fig. 1F) inactivation.

Ca2+-activated noisy oscillatory current
Figure 2A displays the noisy oscillatory BKCa reversibly suppressed by the BKCa blocker paxilline (1 µM, 5 min exposure) in a representative fibroblast. Current-voltage (I-V) curves recorded with a 2-s voltage ramp (~80 to +80 mV from a holding potential of ~40 mV) in the absence of paxilline showed outward rectification (control) in another cell. The outwardly-rectifying current was remarkably reduced by paxilline (Fig. 2B). The current at +60 mV was reduced from 29.8±5.3 pA/pF of control...
to 3.9±0.2 pA/pF with 1 μM paxilline (n = 35, P<0.01 vs control).

We found that a paxilline-resistant current was present in a small population of human cardiac fibroblasts (14.2%, 5 of 35 cells). Figure 2G displays that paxilline (1 μM) partially suppressed the membrane current (+60 mV) to 8.9±1.6 pA/pF from 21.8±2.9, P<0.01); the remaining current was inhibited by 5 mM 4-aminopyridine (4-AP, to 2.1±0.8, P<0.01) (Fig. 2G). This suggests that a 4-AP sensitive delayed rectifier K⁺ channel (IKDR) is co-present with BKCa in these cells.

### Transient outward K⁺ current

The transient outward K⁺ current Ito was present in 15% of cardiac fibroblasts. Ito in human cardiac myocytes was sensitive to inhibition by 4-AP [18], therefore we determined whether Ito in human cardiac fibroblasts was sensitive to inhibition by 4-AP. Figure 3A shows the Ito traces recorded in a typical experiment in the absence and presence of 5 mM 4-AP. Ito (±60 mV) was substantially inhibited by 4-AP to 11.9±1.4 pA/pF from 36.5±2.6 pA/pF (n = 7, P<0.01).

Figure 3B illustrates the mean values of voltage-dependent activation (g/gmax) and inactivation (availability, I/Imax) of Ito. The g/gmax was determined from the I-V relationship of each cell as previously described [19]. The I/Imax was determined with the protocol as shown in the left inset (with 1-s conditioning pulses from voltages between −100 and −10 mV followed by a 300-ms test pulse to +60 mV). Data were fitted to a Boltzmann distribution to obtain the half activation or availability voltage (V0.5) and the slope factor (S). The V0.5 of activation and availability of Ito were 11.2±0.4 mV (n = 7) and −40.6±1.5 mV (n = 9), and the S was 11.1±1.1 and −8.4±1.3, respectively.

Fig. 3C shows the time course of the mean values of Ito recovery from inactivation, determined with a paired-pulse protocol as shown in the inset. Ito recovery was complete within 900 ms and fitted to a mono-exponential function with time constant (τ) of 257.4±5.9 ms (n = 7). These properties of Ito in human cardiac myocytes [19] and mesenchymal stem cells [15], though there are differences in the values of the recovery time constant and the V0.5 of voltage-dependent activation and availability.

### Inward rectifier K⁺ current

It is generally believed that inwardly-rectifying K⁺ channels are sensitive to inhibition by Ba²⁺ [20], therefore we determined the

| Gene name | Accession No. | Forward primer (5′-3′) | Reverse primer (5′-3′) | Product size (bp) |
|-----------|--------------|-----------------------|-----------------------|------------------|
| GAPDH     | J02642       | AACAGGCAACCACTCTCT     | GGAGGGAGATCAGTGTGT    | 258              |
| KCa1.1    | U11058       | ACAACATCCCCCCCCACCA   | TCACATCCTTTTCTCCTA    | 310              |
| KCa1.2    | NM_002250    | TGGAGGCGGAGAGACCAAGC  | GCAAGAAGAGGAGGAGGAG   | 187              |
| KCa2.1    | NM_170782    | GAAGTCCCTCAAGATCTCAA  | TCTTCCGTTTCTTTGCTT    | 498              |
| Kv1.4     | NM_002233    | CCAGAGGAAACCAGAGGAGTC | CCACAGATAGAGGGAGAG    | 426              |
| Kv1.5     | NM_002234    | CGATCCCCCAACACACCTCT  | CTGAACTGAGGAGTCTCT    | 410              |
| Kv1.6     | NM_002235    | CCGTTCTGGTTTCCCG      | ACCACATTGTTTCCACCA    | 456              |
| Kv2.1     | NM_004975    | GAGCAGATGAACCAGAGGAGC | ACAGGGAGATCAGTGTGAT   | 196              |
| Kv3.1     | NM_004976    | GAGACCGCTGAGAGGAGTC   | CAGAGAGTACAGTGTGAT    | 208              |
| Kir1.1    | NM_153765    | GGAGCTTGTGCATCTCT     | CCACATGGAATATCTCT     | 355              |
| Kir2.1    | NM_008891    | ACTCTCCTACCATGTC      | TCTTACTTCTCCAGGTCT    | 365              |
| Kir2.2    | BC027982     | CCAAGAAGCGGCAAGCAGA   | TGGGCCACACAGAAAGAT    | 243              |
| Kir2.3    | NM_152868    | CGGAGACCACAGAGACCA    | TGTCACTGGTGGCAGAGT    | 340              |
| Clcn2     | NM_004366    | AAGCGTGGCTTCTACATC    | ACCTCAGGTGGTCTCGT     | 368              |
| Clcn3     | NM_173872    | CATAGTCGACCACTCAGGTC  | TATTTCCGACCAACAGG     | 293              |
| Nav1.1    | NM_006920    | GAGAACAGCTCGAGAGTAGT  | CACCAACAGAGAGAAGA     | 208              |
| Nav1.2    | NM_021007    | CCCCCCTATCCATCATCT    | ACAGGGGTACAGTGCTCT    | 394              |
| Nav1.3    | NM_006922    | AAAAAAAGCTGAGCATAG    | ATCTTCACCATGGACAG     | 432              |
| Nav1.4    | NM_003334    | GTCATTGCCGACATTCA     | TCTCGGACTCAGACTGTT    | 454              |
| Nav1.5    | NM_198056    | ATGGACCCGTTTACTGACC   | CCACGTTGGACGGATG      | 367              |
| Nav1.6    | NM_014191    | TGCGGAAAGTACCACTATA   | AGAAGGAGCCGAGGATG     | 314              |
| Nav1.7    | NM_002977    | AAAAGGCTGTTGATCTCT    | GATCATTGGTGGTGTGTT    | 310              |
| Nav1.8    | NM_006514    | AACTCCTCCGCTGTTTACTCT| GAAGGTCATGCGGCTGCA    | 424              |
| Nav1.9    | NM_006514    | TGATGACTGACCGTTTGA    | ACAATGACCAAGGACCA     | 415              |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KCa, Ca²⁺-activated K⁺ channel; Kv, voltage-gated K⁺ channel; Kir, inward rectifier K⁺ channel; Clcn Cl⁻ channel; Nav, voltage-gated Na⁺ channel.
effect of Ba\(^{2+}\) on I\(_{Kr}\) in human cardiac fibroblasts. Figure 4 shows the current traces recorded in a representative cell with the voltage protocol as shown in the inset, suggesting that the I-V relationship of I\(_{Kr}\) was reversed by Ba\(^{2+}\). Figure 4B displays the increase of Ba\(^{2+}\) (0.5 mM) reversibly reduced I\(_{Kr}\). Figure 4C illustrates the I-V relationships of Ba\(^{2+}\)-sensitive I\(_{Kr}\) recorded in a representative cell with a 2-s ramp protocol (−120 to 0 mV from −40 mV) in solution containing 5 mM K\(^{+}\) (control) or 20 mM K\(^{+}\), and after application of 0.5 mM Ba\(^{2+}\) in bath solution. Ba\(^{2+}\) strongly inhibited I\(_{Kr}\). Ba\(^{2+}\)-sensitive current was obtained by digitally subtracting currents before and after application of Ba\(^{2+}\) (Fig. 4D). The I-V relationships of Ba\(^{2+}\)-sensitive I\(_{Kr}\) in 5 and 20 mM K\(^{+}\) exhibited a strong inward rectification, typical of an inwardly-rectifying K\(^{+}\) current. Similar results were obtained in 5 other cells.

Volume-sensitive chloride current in human cardiac fibroblasts

The current with outward rectification shown in Fig. 1D was insensitive to inhibition of K\(^{+}\) channel blockers including 5 mM tetraethylammonium (TEA), 5 mM 4-AP, or 0.5 mM Ba\(^{2+}\). Figure 2. BK\(_{Ca}\) and IK\(_{DR}\) in human cardiac fibroblasts. A. Voltage-dependent current was reversibly suppressed by the BK\(_{Ca}\) blocker paxilline (1 mM). Currents were elicited by the voltage protocol as shown in the inset. B. Current-voltage (I-V) relationships of membrane current were recorded by a 2-s ramp protocol (−80 to +80 mV from a holding potential −40 mV) in a representative cell in the absence and presence of 1 mM paxilline. C. Membrane currents recorded in a typical experiment with the same voltage protocol as in A were partially inhibited by 1 mM paxilline. The remaining current was suppressed by co-application of paxilline and 5 mM 4-AP. doi:10.1371/journal.pone.0007307.g002

Inward Na\(^{+}\) currents in human cardiac fibroblasts

The depolarization-elicted inward currents (Fig. 1E and 1F) were studied under K\(^{+}\)-free conditions. Figure 6 illustrates two types of
inward currents recorded in human cardiac fibroblasts with voltage steps (50 ms) to between −60 and +70 mV from −80 mV (inset) in 10-mV increments at 0.2 Hz. One of these currents exhibited an incomplete inactivation (or a persistent component) during 50 ms depolarization (control of Fig. 6A, 6B), similar to L-type Ca\textsuperscript{2+} current (ICa.L) in human cardiac myocytes [22]. However, this current was insensitive to inhibition by a high concentration of the ICa.L blocker nifedipine (10 \(\mu\)M), in contrast with human cardiac ICa.L, which is fully suppressed by nifedipine [22]. Interestingly, the current was abolished by replacing bath Na\textsuperscript{+} (Na\textsubscript{o}) with equimolar choline, and recovered upon restoration of Na\textsuperscript{+} (Fig. 6A, n = 6). In addition, this current is sensitive to inhibition by 10 and 100 nM tetrodotoxin (TTX), and the effect was reversed by washout (n = 6). These results suggest that this inward current is likely a TTX-sensitive INa (INa.TTX) with a persistent component.

Another inward current exhibited a complete inactivation (control of Fig. 6C & 6D). This current had no response to 10 nM TTX; however, nifedipine (10 \(\mu\)M) reversibly reduced the current (Fig. 6C, n = 7). Replacement of Na\textsuperscript{+} with equimolar choline reversibly abolished this inward current, and the current required a high concentration (10 \(\mu\)M) of TTX for a substantial suppression (Fig. 6D, n = 6). These results suggest that this inward current is likely a TTX-resistant Na\textsuperscript{+} current (INa.TTXR).

The concentration-dependent inhibitory effects of TTX on INa.TTX and INa.TTXR at 0 mV are illustrated in Fig. 6E. The IC\textsubscript{50} (50% inhibitory concentration) of TTX for inhibiting INa.TTX was 7.8 nM with a coefficient of 0.94, while the IC\textsubscript{50} of TTX for inhibiting INa.TTXR was 1.8 \(\mu\)M with a Hill coefficient of 0.58. The ICa.L blocker nifedipine had no significant inhibitory effect on INa.TTX, whereas it inhibited INa.TTXR with an IC\textsubscript{50} of 56.2 \(\mu\)M and a Hill coefficient of 0.59 (Fig. 6F).

The I-V relationships for the peak current of INa.TTX and INa.TTXR are illustrated in Fig. 7A. INa.TTX had a threshold potential of −240 mV and peaked at +10 mV, while INa.TTXR had a threshold potential of −50 mV and peaked at 0 mV. Inactivation of INa.TTX and INa.TTXR was fitted to a monoexponential function with time constant (\(\tau\)) as shown in the left panel of Fig. 7B. The inactivation process of INa.TTX was slower than that of INa.TTXR (Fig. 7B, n = 12, \(P<0.01\) at −20 to +60 mV).
respectively. While the \( V_{0.5}^{\text{so}} \) for \( g_{\text{Na}} \) and \( I_{\text{Na}}^{\text{max}} \) for \( \text{INa.TTX} \) and \( \text{INa.TTXR} \) were both more positive in \( \text{INa.TTX} \) than those in \( \text{INa.TTXR} \) (\( P \leq 0.01 \)). The arrows in the figure indicate the zero current level.

**Figure 5.** \( I_{\text{Cl}} \) in human cardiac fibroblasts. A. Voltage-dependent current was inhibited by the \( Cl^{-} \) channel blocker DIDS (150 \( \mu \)M). Current was elicited by the voltage steps as shown in the insert (0.2 Hz). B. \( I-V \) relation curve of DIDS-sensitive current obtained by subtracting currents before and after DIDS application in A. C. Voltage-dependent current recorded in a representative cells during control, after 20 min 0.7T exposure and application of 100 \( \mu \)M NPPB. D. \( I-V \) relationships for control current (1.0T), 0.7T and 0.7T with 100 \( \mu \)M NPPB. The 0.7T-induced current was significantly inhibited by NPPB at all test potentials (n = 5, \( P < 0.01 \)). The arrows in the figure indicate the zero current level.

Figure 7C illustrates the mean values of the steady-state voltage-dependent activation (\( g/g_{\text{max}} \)) and inactivation (availability, \( I/I_{\text{max}} \)) for both \( \text{INa.TTX} \) and \( \text{INa.TTXR} \). The \( g/g_{\text{max}} \) was determined from the \( I-V \) relationships of each cell in Fig. 7A as previously described [23]. The \( I/I_{\text{max}} \) of \( \text{INa.TTX} \) was determined with the protocol as shown in the left inset (with 1-s conditioning pulses from voltages between \(-120 \) and \(-10 \) mV then to a 50-ms test pulse to \( 0 \) mV). Data were fitted to a Boltzmann equation. The \( V_{0.5}^{g} \) of \( g/g_{\text{max}} \) and \( I/I_{\text{max}} \) for \( \text{INa.TTX} \) were \(-7.2 \pm 1.1 \) mV (n = 9) and \(-61.4 \pm 1.6 \) mV (n = 10), and the \( S \) was \(-8.7 \pm 1.2 \) and \(-10.8 \pm 1.2 \), respectively. While the \( V_{0.5}^{g} \) of \( g/g_{\text{max}} \) and \( I/I_{\text{max}} \) for \( \text{INa.TTXR} \) were \(-24.7 \pm 2.4 \) mV (n = 7) and \(-72.3 \pm 1.5 \) mV (n = 9), and the \( S \) was \(-8.5 \pm 1.3 \) and \(-8.5 \pm 1.3 \), respectively. The \( V_{0.5}^{g} \) of \( g/g_{\text{max}} \) and \( I/I_{\text{max}} \) were more positive in \( \text{INa.TTX} \) than those in \( \text{INa.TTXR} \) (\( P < 0.01 \)).

Figure 7E shows the time course of mean values of recovery of \( \text{INa.TTX} \) or \( \text{INa.TTXR} \) from inactivation, which was determined using a paired-pulse protocol shown in the inset as described previously [23]. The recovery of \( \text{INa.TTX} \) and \( \text{INa.TTXR} \) from inactivation was complete within 150 ms, and the curves were fitted to a mono-exponential function. The time constant (\( t \)) was 14.3 \pm 2.1 ms for \( \text{INa.TTX} \) (n = 11) and 21.4 \pm 2.9 ms for \( \text{INa.TTXR} \) (n = 9). The recovery of \( \text{INa.TTX} \) from inactivation was slower than that of \( \text{INa.TTXR} \) (\( P < 0.05 \)). These results indicate that two types of Na\(^{+}\) channels with distinct TTX-sensitivity and kinetics are present in human cardiac fibroblasts.

**Messenger RNAs of functional ion channels**

To explore the molecular identities of the functional ionic currents, we examined gene expression of various ionic channels in human cardiac fibroblasts with RT-PCR using the specific primers targeting human genes for KCa, Kv, Kir, Clcn, and Na\(^{+}\) channel families as shown in Table 1. Figure 8A displays the significant gene expression of KCa1.1 (responsible for BKCa), Kv1.5, Kv1.6 (responsible for IKDR), Kv4.2, Kv4.3 (responsible for IKur), Kir2.1, Kir2.3 (for IKur), Clcn3 (for ICl.vol), Na\(_{\text{v1.2}}, \text{Na}_{\text{v1.5}}, \text{Na}_{\text{v1.6}} \) and Na\(_{\text{v1.7}} \) (for \( \text{INa.TTX} \)) and Na\(_{\text{v1.5}} \) (for \( \text{INa.TTXR} \)) in human cardiac fibroblasts. In addition, Clcn2 was also significantly expressed in human cardiac fibroblasts. When RNA was directly amplified by PCR without reverse transcription, the bands for these positive genes disappeared (Fig. 8B), suggesting that the genes detected were not false-positive signals from genomic DNA contamination.

**Discussion**

In the present study, we have demonstrated that multiple ionic currents (BKCa, IKDR, IK1, ICl.vol, and \( \text{INa.TTX} \) and \( \text{INa.TTXR} \)) are present in human cardiac fibroblasts. BKCa was inhibited by paxilline, IKDR and IK1 were inhibited by 4-AP, IK1 was blocked by Ba\(^{2+}\) and ICl.vol was inhibited by DIDS or NPPB, while \( \text{INa.TTX} \) and \( \text{INa.TTXR} \) were suppressed by different concentrations of TTX. The channel genes corresponding to the functional currents (KCa1.1 for BKCa, Kv1.5/Kv1.6 for IKDR, Kv4.2/Kv4.3 for IKur, Kir2.1/Kir2.3 for IKur, Clcn3 for ICl.vol, Na\(_{\text{v1.2}}, \text{Na}_{\text{v1.5}}, \text{Na}_{\text{v1.6}} \) and Na\(_{\text{v1.7}} \) for \( \text{INa.TTX} \)) and Na\(_{\text{v1.5}} \) (for \( \text{INa.TTXR} \)) were confirmed by RT-PCR.

Recent studies demonstrated that an inward rectifier K\(^{+}\) current (IK1), a delayed rectifier K\(^{+}\) current (IKur), and a non-selective cation channel current were present in rat ventricular fibroblasts [11–13]. Only BKCa was described in human cardiac fibroblasts [14]. The present study provides novel information that multiple ionic channels are heterogeneously expressed in human cardiac fibroblasts. In addition to BKCa, as previously reported by Wang and colleagues [14], IKDR, IK1, IKur, ICl.vol, INa.TTX, and INa.TTXR were present with BKCa in different populations of human cardiac fibroblasts (Fig. 1). These currents have different distribution and properties compared to those in human cardiomyocytes [18,24–27].

Several K\(^{+}\) currents have been reported in myocytes from human hearts. They include 4-AP-sensitive IK1 (encoded by Kv1.4/Kv4.3) [28] in atrial and ventricular myocytes [18,26], 4-AP-sensitive ultra-rapid delayed rectifier K\(^{+}\) current (IKur, encoded by Kir2.1/Kir2.3) [24,28,30], and rapidly and slowly-activated delayed rectifier K\(^{+}\) currents (IKur and IKs) [25]. However, IK1 (likely encoded by Kv4.2/Kv4.3) and IKDR (likely encoded by Kv1.5/Kv1.6) were present only in a small population of human cardiac fibroblasts (15% and 14%, respectively) (Figs. 1–3). In addition, Ba\(^{2+}\)-sensitive inward rectifier K\(^{+}\) current (likely encoded by Kir2.1/Kir2.3) with a small amplitude was present in 24% human cardiac fibroblasts, not like in human cardiomyocytes where IK1 is detected in each cell [24,30]. It is interesting to note...
that BKCa was present in most (88%) human cardiac fibroblasts; however, this current has not been identified in human cardiomyocytes. The different distribution of K⁺ currents implies the various functions of these channels in these two types of heart cells.

Earlier studies have demonstrated that ICl.vol are present in human cardiac myocytes [31,32], and the current is only recorded when the hypotonic insult is applied [31,32]. Nonetheless, ICl.vol is recorded in a small population (7%) of human cardiac fibroblasts without hypotonic exposure (Fig. 1), and it is activated in almost all fibroblasts with hypotonic exposure (Fig. 5). ICl.vol is believed to play a role in arrhythmogenesis, myocardial injury, preconditioning, and apoptosis of myocytes [33]. Nonetheless, physiological function of ICl.vol in human cardiac fibroblasts remains to be studied in the future.

It is well recognized that INa channels expressed in cardiomyocytes (mainly encoded by NaV1.5) play an important role in controlling excitation-contraction and impulse conduction in the hearts. INa has been also found to participate in regulating sinus node pacemaker function [34]. In the present study, we found that INa was expressed in most (61%) human cardiac fibroblasts (Fig. 1). The INa.TTX in human cardiac fibroblasts (Figs. 6 & 7) shares some properties with neuronal

**Figure 6.** INa.TTX and INa.TTXR in human cardiac fibroblasts. A. An inward current with a persistent component (arrow) recorded in a representative cell under K⁺-free conditions using the voltage steps as shown in the inset. Nifedipine (10 μM) had no effect on the current, while the current disappeared when Na⁺o was replaced with equimolar choline, and recovered as restoration of Na⁺o. B. Similar inward current with persistent component (arrow) recorded in another cell was highly sensitive to inhibition by low concentrations of TTX. C. An inward current with fast inactivation recorded using the same voltage protocol as shown in the inset of A. The current was not affected by 10 nM TTX, but reversibly inhibited by 10 μM nifedipine. D. Similar current recorded in another cell disappeared with Na⁺o removal, and recovered as restoration of Na⁺o. The current was suppressed by a high concentration of TTX (10 μM). E. Concentration-dependent response of two types of inward currents to TTX. The data were fitted to the Hill equation: E = Emax(1+IC50/C)^b, where E is the percentage inhibition of current at concentration C, Emax is the maximum inhibition, IC50 is the concentration for a half inhibitory effect, and b is the Hill coefficient. The IC50 of TTX for inhibiting TTX-sensitive INa was 7.8 nM (n = 5–9 for each concentration), the Hill coefficient was 0.94. The IC50 of TTX for inhibiting TTX-resistant INa was 1.8 μM (n = 6–9 cell for each concentration), the Hill coefficient was 0.58. F. Concentration-dependent relationships of INa.TTX and INa.TTXR to nifedipine. The IC50 of nifedipine for inhibiting INa.TTX was 56.2 μM (n = 4–7 cells for each concentration) with a Hill coefficient of 0.59.

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INa, e.g. a transient inward current followed by a persistent component, sensitive to inhibition by nanomolar TTX, and likely encoded by NaV1.2, NaV1.3, NaV1.6, and NaV1.7 [35,36]. The INa.TTXR in human cardiac fibroblasts (Figs. 6 & 7) shares some features with INa in cardiomyocytes (e.g. inhibited by micromolar TTX and encoded by NaV1.5) [35,37]. Some properties of INa.TTXR in human cardiac fibroblasts are not identical to those of INa in human cardiomyocytes [27,37,38], e.g. more positive V0.5s of activation (−225 mV vs −239 mV) and availability (−72 mV vs −95 mV) and more positive peak current potential (0 mV vs −35 mV), compared to INa in human cardiomyocytes [27,37]. In addition, INa.TTXR in cardiac fibroblasts, as NaV1.5-encoded INa.TTXR in gastric epithelial cells [39], was inhibited by high concentrations of the I Ca.L blocker nifedipine (Fig. 6). Nonetheless, no report is available in the literature regarding the information whether INa of cardiomyocytes is sensitive to a high concentration of nifedipine. Moreover, it is unknown how INa.TTX and INa.TTXR participate in cellular function of cardiac fibroblasts.

It has been recognized that cardiac fibroblasts are electrically unexcitable, but they contribute to the electrophysiology of myocytes in various ways, such as electrical coupling of fibroblasts and myocytes [40]. The electrical coupling between fibroblasts and myocytes was observed at cellular and tissue level as well as in cell cultures [5,8,40–42]. Coupling between fibroblasts and myocytes was demonstrated to be via Cx43 gap junctions in sheep ventricles and Cx45 in rabbit sinoatrial node cells [4,6] and in sheep ventricular scars [43]. The cardiac fibroblasts are therefore believed to maintain electrical contact with myocytes. Our results of multiple ion channels in human cardiac fibroblasts likely provide a basis for understanding of the potential contribution of these ion channels to fibroblast-myocytes electrical coupling under physiological conditions, and also for future studies on the potential mechanism how cardiac fibroblasts participate in regulating cardiac electrophysiology.

In proliferative cells, ion channels play a role in cell cycle progression [44,45]. The activity of BKCa (i.e. KCa1.1) channels was regulated by the spontaneous Ca2+ oscillations, resulting in

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**Figure 7. Kinetics of INa.TTX and INa.TTXR.** A. Mean values of I–V relationships of INa.TTX and INa.TTXR. B. Left panel: inactivation time course of representative INa traces (at 0 mV) was fitted to a monoeponential function with time constant (τ) shown, 4.3 ms for INa.TTX and 1.82 ms for INa.TTXR. Right panel: mean values of voltage dependence of inactivation of INa.TTX (n = 8) and INa.TTXR (n = 10). P < 0.05 or P < 0.01 at −20 to +60 mV. C. Voltage-dependent availability (I/Imax) of INa was determined with the protocol as shown in the left inset (with 1-s conditioning pulses from voltages between −120 and −10 mV then a 50-ms test pulse to 0 mV). Curves of I/Imax and activation conductance (g/gmax) were fitted to a Boltzmann equation. E. Recovery curves of INa.TTX and INa.TTXR from inactivation were fitted to a monoeponential function.

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**Figure 8. RT-PCR for detecting ion channels expressed in human cardiac fibroblasts.** A. Images of RT-PCR products corresponding to significant gene expression of KCa1.1 (BKCa), Kv1.5 (IKNa), Kv4.3 (Ito), and Kir2.1 (IKir) and Clcn3 (ICl.vol), and NaV1.2, NaV1.3, NaV1.5, NaV1.6 and NaV1.7 in human cardiac fibroblasts. A weak expression of Kv4.2, Kir2.3, Clcn2 and NaV1.1 was also found in human cardiac fibroblasts. B. No significant bands were observed in the PCR experiment when RT product was replaced by total RNA.
fluctuations of membrane currents and potentials. BKCa was reported to play a role in regulating proliferation of human preadipocytes [46], endothelial cells [47], and breast cancer cells [48]. IKv was found to participate in regulating the proliferation of human hematopoietic progenitor cells [49]. Although the underlying mechanisms of ion channels in cell proliferation regulation remain elusive, the involvement of K+ channels in cell proliferation was well established [44,45,49]. Further exploration is required to find out whether these ion channels contribute to human cardiac fibroblast proliferation.

Clcn3 channel is regarded as one of the candidate channels for volume regulated anion channels and has been shown to play an important role in cell proliferation and apoptosis [45]. Blockade or disruption of Clcn3 channel resulted in arrest of cell cycle and prevention of cell proliferation in several cell types [21,50]. The present observation demonstrated that functional chloride current encoded by Clcn3, sensitive to cell volume, was observed in human cardiac fibroblasts (Fig. 5). Whether this ICl current would contribute to human cardiac fibroblast proliferation remains to be studied in the future.

One of limitations of the present study was that ion channels, BKCa, Ito, IKir and IClvol and INa, TTX, and Na, TTXR, were heterogeneously expressed within the same species of cultured human cardiac fibroblasts. This could result from heterogeneous cell population of the fibroblasts. An earlier study demonstrated that myofibroblast could differentiate from fibroblasts when plated at low density and could revert back to fibroblasts at higher density [51]. Consequently, a subpopulation of human cardiac fibroblasts may display different patterns of ion channel expression.

In summary, the present study provides the first information that multiple ion channel currents are present in cultured human cardiac fibroblasts, the patterns and properties of these ion channel currents differ from those observed in human cardiac myocytes.

The information obtained form the present study provides a basis for future study how ion channels participate in regulating cardiac electrophysiology.

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Author Contributions

Conceived and designed the experiments: GRL HFT CPL. Performed the experiments: HYS JBC YZ. Analyzed the data: GRL HYS. Wrote the paper: GRL.

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