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9-Phenanthrol and Flufenamic Acid Inhibit Calcium Oscillations in HL-1 Mouse Cardiomyocytes

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Abstract

It is well established that intracellular calcium ($[Ca^{2+}]_i$) controls the inotropic state of the myocardium, and evidence mounts that a “ Ca^{2+} clock” controls the chronotropic state of the heart. Recent findings describe a calcium-activated nonselective cation channel (NSC_{Ca}) in various cardiac preparations sharing hallmark characteristics of the Transient Receptor Potential Melastatin 4, (TRPM4). TRPM4 is functionally expressed throughout the heart and has been implicated as a NSC_{Ca} that mediates membrane depolarization. However, the functional significance of TRPM4 in regards to Ca^{2+} signaling and its effects on cellular excitability and pacemaker function remains inconclusive. Here, we show by Fura2 Ca-imaging that pharmacological inhibition of TRPM4 in HL-1 mouse cardiac myocytes by 9-phenanthrol (10 μ M) and flufenamic acid (10 & 100 μ M) decreases Ca^{2+} oscillations followed by an overall increase in $[Ca^{2+}]_i$.

The latter occurs also in HL-1 cells in Ca^{2+} -free solution and after depletion of sarcoplasmic reticulum Ca^{2+} with thapsigargin (10 μ M). These pharmacologic agents also depolarize HL-1 cell mitochondrial membrane potential. Furthermore, by on-cell voltage clamp we show that 9-phenanthrol reversibly inhibits membrane current; by fluorescence immunohistochemistry we demonstrate that HL-1 cells display punctate surface labeling with TRPM4 antibody; and by immunoblotting using this antibody we show these cells express a 130-150 kD protein, as expected for TRPM4. We conclude that 9-phenanthrol inhibits TRPM4 ion channels in HL-1 cells, which in turn decreases Ca^{2+} oscillations followed by a compensatory increase in $[Ca^{2+}]_i$ from an intracellular store other than the sarcoplasmic reticulum. We speculate that the most likely source is the mitochondrion.

Keywords

HL-1 Cardiomyocytes; TRPM4; $[Ca^{2+}]_i$

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1. Introduction

Calcium-activated non-selective cation channels (NSC_{Ca}) in myocardium have long been documented [1], but their physiologic significance in heart function remains unclear. The transient receptor potential melastatin 4 (TRPM4), along with the closely related TRPM5, is such a non-selective cation channel that unlike most TRPs is impermeable to Ca²⁺ [2, 3]. Yet, intracellular Ca²⁺ and depolarization of the transmembrane voltage activate TRPM4 [4]. The topology of the TRPM4 channel subunit indicates that the Ca²⁺ sensitivity for gating is regulated by ATP, PKC-dependent phosphorylation and calmodulin binding at the peptide C terminus [5]. The mechanism of its voltage activation is less clear, but structural evaluations indicate the peptide C terminus is a locus for voltage dependence [4].

Ongoing biophysical characterization of TRPM4 notwithstanding, there is mounting evidence that this channel plays an important physiologic role throughout the cardiovascular system. TRPM4 is important for myogenic constriction of cerebral arteries [6], and its regulation depends on Ca²⁺ released from the inositol 1,4,5-trisphosphate receptor on the sarcoplasmic reticulum [7]. Guinamard and colleagues first characterized a Ca²⁺-activated non-selective cation channel in dedifferentiated cultured rat ventricular cardiomyocytes [8]. They showed later that human atrial cardiomyocytes expressed a similar channel with characteristics of TRPM4 [9]. Mouse sinoatrial node cells also express TRPM4, and these channels may well contribute to generation of heart rhythm or cardiac arrhythmias [10]. In this regard, 9-phenanthrol selectively inhibits TRPM4 compared with TRPM5 [11, 12], and it abolishes mouse ventricular arrhythmias created by hypoxia and re-oxygenation [13].

Oscillations of [Ca²⁺]_i govern the strength of myocyte contraction and the cardiac contractile reserve [14]. The frequency and intensity of these oscillations are essential in maintaining cardiac contractility as well as numerous other cellular functions, including various transcription programs [15]. In sinoatrial node (SAN) cells, a local increase in [Ca²⁺]_i via the 'Ca²⁺ clock' [16] generates an inward current that involves complex interactions of multiple inwardly and outwardly directed ion currents. These changes increase the slope of diastolic depolarization and, thereby, modulate the SAN action potential and ultimately the heart rate [14, 17]. On the other hand, I_f has been implicated as the principle pacemaker current [18]; however, despite numerous studies conducted in an array of tissue preparations, the contribution of I_f to pacemaker activity is inconclusive [17].

HL-1 immortalized mouse cardiomyocytes retain various phenotypes and functions of cardiac muscle cells [19, 20]. As such they are an important cardiomyocyte surrogate free of in situ neurohumoral modulators. About 30-40% of HL-1 cells display [Ca²⁺]_i oscillations that result in part from spontaneous action potentials, which are generated by the depolarizing I_f attributed to hyperpolarization-activated cyclic nucleotide-gate (HCN) ion channels [21, 22]. We have shown that lipopolysaccharides directly inhibit HL-1 cell [Ca²⁺]_i oscillations as well as I_f [22] and extend duration of the action potential [23]. We also have shown that activation of I_f in HL-1 cells occurs typically at voltages more negative than the resting membrane potential of -60 mV [22]. This has led us to ask if other mechanisms are involved. We report here that 9-phenanthrol and flufenamic acid, inhibitors of TRPM4 [11, 12], inhibit [Ca²⁺]_i oscillations in HL-1 cells followed by an elevation of [Ca²⁺]_i. This increase resulted from an HL-1 intracellular Ca²⁺ store other than the sarcoplasmic/endoplasmic reticulum store-operated Ca²⁺ entry [24].

2. Materials and Methods

2.1 HL-1 Cell Culture

HL-1 atrial cardiomyocytes were a gift of Dr. William Claycomb (Louisiana State University Medical Center, New Orleans, LA). Cells were grown in 5% CO₂ at 37°C in Claycomb media (Sigma) and supplemented with lot-specific 10% FBS (Sigma), 100 U/ml: 100 µg/ml Penicillin/Streptomycin (Invitrogen), 0.1 mM norepinephrine (Sigma) and 2 mM L-glutamine (Invitrogen) [19, 20]. Prior to culturing cells, flasks were treated for a 12 hour period with 0.02% Bacto gelatin (Fisher Scientific); 0.5% Fibronectin (Invitrogen). Glass cover slips (12-mm diameter) were flamed briefly to enhance coating and transferred to a 35-mm culture dish where they were treated overnight with gelatin/fibronectin. Cells were plated at a density of 3×10^5 cells/35-mm culture dish.

2.2 Intracellular Calcium Measurements

Experiments were performed after 2-3 days in culture when small islands of confluent HL-1 cells had formed. Cells were loaded with Fura-2 AM (TefLabs, Austin, TX, USA) by treating them with a standard salt solution containing 2-µM Fura-2 AM for 30 minutes at room temperature (22-23°C). Cells were then washed with the standard external salt solution and incubated at 37°C in 5% CO₂ for 30 minutes in the Claycomb media. The cells were transferred to an acrylic chamber (Warner Instruments, Hamden, CT) on the microscope stage and washed with the standard external salt solution for 2-3 minutes prior to data collection. Temperature throughout experiments was maintained at 37°C by a stage/inline controller (Warner Instruments). Fluorescence was measured with an imaging system composed of a filter wheel and a Basler A311F VGA camera connected to an Olympus IX71 inverted microscope. InCyt2 software (version 5.29; Intracellular Imaging, Cincinnati, OH) controlled the data collection and filter wheel. $[Ca^{2+}]_i$ was determined by interpolation from a standard curve generated by a Ca²⁺ calibration buffer kit #2 (Molecular Probes) and Fura-2/K5-salt. Background fluorescence was corrected, and the ratio of the fluorescence at excitation wavelengths (F340/F380) was monitored in 30-40 cells. Cells were then superfused constantly in solutions as indicated in the results.

2.3 Mitochondrial Membrane Potential (ψ_{mito}) Measurements

Tetramethylrhodamine, methyl ester (TMRM) is a lipophilic potentiometric dye that partitions between the mitochondria and cytosol by virtue of its positive charge. HL-1 cells were loaded with TMRM (500 nM for 30 minutes at 37°C), and washed 2X in phosphate buffered saline. TMRM-loaded cells were imaged in standard external salt solution containing TMRM (50 nM) at room temperature (~22 °C). TMRM was excited using a Zeiss Observer Z1 inverted microscope equipped with a Colibri LED at a wavelength of 554 nm and emission was recorded at wavelength 566 nm. Relative fluorescent intensity (RFI) was recorded from pixel regions containing mitochondria.

2.4 Whole-Cell Voltage Clamp Measurements

Glass cover slips containing the cells were transferred to an acrylic chamber (Warner; New Haven, CT, USA) on the stage of an inverted microscope (Olympus IMT-2) equipped with Hoffman modulation contrast optics. Cells were superfused at 22-23° C with a standard external salt solution. Patch pipets (3-6 MΩ in the bath solution) were fabricated from glass capillaries (1.1-1.2 mm ID, 0.2mm wall thickness, non-heparinized micro-hemacrit capillary tubes; Fisher Scientific) with a Brown-Flaming horizontal micropipette puller (P-97, Sutter Instruments; Novato, CA, USA). A micromanipulator (Scientifica, East Sussex, England) was used to position pipettes. On-cell patch configurations were obtained by standard patch clamp technique [25]. Voltage-clamp currents were measured using a patch clamp amplifier

(Axopatch 200B, Axon Instruments; Foster City, CA, USA) with the lowpass, Bessel filtering (-3bD) set at 5kHz. Signals from the patch clamp amplifier were fed into a computer via a digital interface (Digidata 1322A) and processed by Clampex 8 software (Axon Instruments).

2.5 Immunocytochemistry and Immunoblotting

Cells mounted on glass cover slips were immunostained at 22-23°C. Cells were fixed for 1 hour in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 8°C. Cells were then brought to room temperature and rinsed 6×10 minutes with 0.1M PBS (pH 7.3). Following rinses, cells were either permeabilized with 0.4% Triton X-100 in PBS or left intact and then blocked with 5% normal donkey serum (Jackson ImmunoResearch, 017-000-121) for 2 hours. Permeabilized cells were then incubated in rabbit anti-TRPM4 antibody (1:2000; Abcam, ab63080) diluted with 0.4% Triton X-100 in PBS, while unpermeabilized cells were incubated in antibody diluted with PBS overnight at 22-23°C. Cells were then washed with PBS and incubated for 2 hours in Alexa Fluor 555 donkey anti-rabbit IgG (1:200; Invitrogen, A31572). Hoechst counterstain was applied to visualize nuclei. Cover-slips were mounted on glass slides with Citifluor mounting medium (Ted Pella, Redding, CA, USA) and edges of coverslips were sealed with clear nail polish. The cells were viewed and photographed using a Zeiss Axio Observer.Z1 with an AxioCam MRm camera.

Confluent HL-1 cell cultures were lysed, homogenized and fractionated by differential centrifugation. Protein concentration was determined by BCA assay (Pierce). Equal protein samples were fractionated on 5-15% gradient SDS-PAGE gels and electroblotted to PVDF (Millipore). Blots were blocked, reacted with the rabbit anti-TRPM4 antibody (1:200; Abcam ab63080), and developed by chemiluminescence (Pierce).

2.6 Solutions and Chemicals

Standard external salt solution contained (mM): NaCl 150, KCl 6, MgCl₂ 1, CaCl₂ 1.5, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, glucose 10 (pH adjusted to 7.4 with NaOH; 315 mosmol kg⁻¹). In some instances Ca²⁺ was removed from the standard solution when cells were subjected to a Ca²⁺ free extracellular environment (pH adjusted to 7.4 with NaOH; 312 mosmol·KgH₂O⁻¹). N-methyl-D-glucamine (NMDG⁺; as a Na⁺ substitute) solution contained in (mM): NMDG 140, NaCl 10, KCl 6, MgCl₂ 1, CaCl₂ 1.5, HEPES 10, glucose 10 (pH adjusted to 7.4 with HCl; 309 mosmol·KgH₂O⁻¹). Solution osmolality was measured by freezing point depression (Micro-osmette, Precision System, Inc., Natick, MA). Pipette solution contained (in mM): CsCl 20, Cs aspartate 100, MgCl₂ 1, Na₂ATP 4, HEPES 10, ethylene glycol-bis(-aminoethylether) N, N, N',N'-tetraacetic acid (EGTA) 5, CaCl₂ 7.085 (free Ca²⁺ = 100 μM; computed by WCaBuf, G. Droogmans, KU Leuven, Leuven, Belgium), pH 7.2 (adjusted with CsOH). Fura-2 (AM) was acquired from TEFLabs, Austin, TX, and TMRM was obtained from Molecular Probes/Invitrogen, Grand Island, NY Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was obtained from Tocris, R&D Systems, Minneapolis. Dimethylsulphoxide (DMSO), thapsigargin, flufenamic acid, and 9-Phenanthrol were obtained from Sigma-Aldrich (St. Louis, MO, USA). 9-Phenanthrol and flufenamic acid were reconstituted in DMSO to 10- and 100-mM stock solutions and diluted to 10 μM and 100 μM with standard external salt solution (0.1% DMSO final).

3 Results

3.1 9-Phenanthrol inhibits [Ca²⁺]_i oscillations

Consistent with our previous report [22] [Ca²⁺]_i oscillations were evident in approximately 30-40% of HL-1 cells in sub-confluent culture for 2-3 days, Fig. 1A. 9-Phenanthrol (10 μM)

abolished these $[Ca^{2+}]_i$ oscillations, which resulted in substantial increases in $[Ca^{2+}]_i$ that decreased steadily over 6-7 minutes, Fig. 1A. These increases in $[Ca^{2+}]_i$ by 9-phenanthrol treatment occurred in all HL-1 cells regardless of whether-or-not they displayed Ca^{2+} oscillations, Fig. 1B. These effects of 9-phenanthrol reversed on washout. DMSO alone, as vehicle control, did not affect HL-1 $[Ca^{2+}]_i$ [26].

3.2 9-Phenanthrol and flufenamic acid increase $[Ca^{2+}]_i$ from an intracellular pool

To explore further the mechanism by which 9-phenanthrol affects $[Ca^{2+}]_i$ we repeated the treatment in cells that were perfused first with Ca^{2+} -free external solution. The $[Ca^{2+}]_i$ oscillations disappeared with depletion of extracellular Ca^{2+} , Fig. 2. None the less, 9-phenanthrol (10 μ M) still resulted in a 80-nM increase in $[Ca^{2+}]_i$ in the absence of external Ca^{2+} , Fig. 2. 9-Phenanthrol auto-fluoresces at 340 nm, and at 10 μ M this accounted for only 6.0 nM $[Ca^{2+}]_i$ (*unpublished observations*). This artifact increased markedly at higher drug concentrations, which obviated its utility beyond 10 μ M. This concentration, however, agreed with $IC_{50} = 10$ -20 μ M for 9-phenanthrol's inhibition of TRPM4 reported by others [11, 27]. None the less, we utilized an additional inhibitor of TRPM4 to evaluate concentration dependence.

Flufenamic acid inhibits TRPM4, albeit its specificity is less than that of 9-phenanthrol [11, 12]. It also inhibited $[Ca^{2+}]_i$ oscillations at 10 μ M in single HL-1 cells followed by ~40-nM increase in $[Ca^{2+}]_i$, which increased by ~80 nM at 100 μ M, Fig. 3A, i & ii. In some cells the $[Ca^{2+}]_i$ oscillations returned on washout of 100 μ M flufenamic acid, Fig. 3A, ii. The mean $\pm$ SE of the effect of flufenamic acid on $[Ca^{2+}]_i$ in 40 cells is shown in Fig. 3A, iii. Increases in $[Ca^{2+}]_i$ by flufenamic acid also occurred in Ca^{2+} -free external solution, Fig. 3B, i, and the $[Ca^{2+}]_i$ oscillations returned in some cells after washout of flufenamic acid and restoration of external Ca^{2+} , Fig. 3B, ii. The mean $\pm$ SE of the effect of flufenamic acid in Ca^{2+} -free external solution on $[Ca^{2+}]_i$ in 35 cells is shown in Fig. 3B, iii.

3.3 9-Phenanthrol increases $[Ca^{2+}]_i$ from an intracellular pool other than the SR/ER

9-Phenanthrol is a putative inhibitor of TRPM4 [11] and also hyperpolarizes smooth muscle [27]. Thus, we postulated that increases in $[Ca^{2+}]_i$ by added 9-phenanthrol resulted from reduced Na^+ influx through the TRPM4 monovalent cation channel; this in turn effected a compensatory increase in $[Ca^{2+}]_i$ since TRPM4 is activated in part by Ca^{2+} [4]. To test this, we substituted isosmotically all but 10 mM extracellular Na^+ with the impermeable quaternary ammonium cation, NMDG⁺. This change resulted in further increase in $[Ca^{2+}]_i$, Fig. 2. This increase was transient, however, compared with the sustained increase in $[Ca^{2+}]_i$ resulting from added 9-phenanthrol, Fig. 2. Thus, we conducted additional experiments to determine if these increases in $[Ca^{2+}]_i$, either by added 9-phenanthrol or by external Na^+ substitution, arose from an identical intracellular Ca^{2+} pool.

Under conditions of Ca-free extracellular solution we first added thapsigargin (10 μ M) to inhibit the SERCA pump and deplete the sarcoplasmic/endoplasmic reticulum of Ca^{2+} , Fig. 4A. Subsequent addition of 9-phenanthrol (10 μ M) resulted again in a 80-nM sustained increase in $[Ca^{2+}]_i$, which reversed on washout, Fig. 4A. Therefore, the increase in $[Ca^{2+}]_i$ by added 9-phenanthrol came from an intracellular source other than the sarcoplasmic/endoplasmic reticulum. We conducted additional experiments to validate this conclusion and to determine whether this unexpected increase in $[Ca^{2+}]_i$ by 9-phenanthrol resulted from reduced influx of Na^+ through TRPM4. Here, sarcoplasmic/endoplasmic reticulum was again depleted of Ca^{2+} by addition of thapsigargin. Ca-free extracellular solution then was superfused over the cells followed by a Ca^{2+} -free solution in which all but 10 mM of Na^+ was substituted with NMDG⁺. This resulted in no increase in $[Ca^{2+}]_i$, Fig. 4B., which

indicated that the increase in $[Ca^{2+}]_i$ by 9-phenanthrol did not result from reduced influx of Na^+ .

3.4 Substitution of external Na^+ with NMDG $^+$ increases $[Ca^{2+}]_i$

Notwithstanding this testing to account for the effect of 9-phenanthrol on HL-1 $[Ca^{2+}]_i$, reduced external Na^+ alone did increase in $[Ca^{2+}]_i$, Fig. 5. Firstly, substitution of all but 10 mM external Na^+ alone with NMDG $^+$ resulted in a substantial increase in $[Ca^{2+}]_i$, Fig. 5A. This increase in $[Ca^{2+}]_i$ comprised two components: 1. $[Ca^{2+}]_i$ increased rapidly to 150 nM, rising from 200 nM to 350 nM; 2. $[Ca^{2+}]_i$ then decreased by 50 mM over 5 minutes to a sustained, elevated $[Ca^{2+}]_i$ of approximately 300 nM, Fig. 5A. To test whether a component of this increase in $[Ca^{2+}]_i$ resulted from reversal of the Na-Ca exchange, the NMDG $^+$ substitution was repeated in cells that were superfused first with Ca^{2+} -free external solution, Fig. 5B. Here, the NMDG $^+$ substitution again resulted in a rapid, ~150-nM rise in $[Ca^{2+}]_i$, but this increase was transient only and decreased rapidly to the control level of the Ca^{2+} -free solution and less. The $[Ca^{2+}]_i$ increased rapidly on restoration of external Na^+ and Ca^{2+} , Fig. 5B.

3.5 HL-1 cells express membrane TRPM4 ion channels

Since 9-phenanthrol is known to inhibit TRPM4, we evaluated the expression of TRPM4 ion channels in HL-1 cells by immunofluorescent detection of the anti-TRPM4 antibody. Punctate fluorescent labeling was evident in cells treated without and with Triton X-100, Fig. 6 (A, B & C). This indicates that HL-1 cells express TRPM4 ion channel protein primarily at plasma membrane sites. In contrast, control cells without Triton X-100 treatment and without anti-TRPM4 antibody showed no fluorescent labeling, Fig. 6D. TRPM4 labeling was not uniform in all cells. Some cells showed greater density of punctate fluorescence compared with others while many had none, Fig. 6A-C. The anti-TRPM4 antibody also was utilized to generate immunoblots of various HL-1 cell fractions. A faint band corresponding to the 130-150 kDa range was detected in the HL-1 cell membrane fraction, Fig. 7. This range of molecular weight is consistent with that reported for the TRPM4b splice variant [2]. We cannot state with certainty that this band is a doublet, but it is known that murine TRPM4a and TRPM4b splice variants co-localize [28].

3.6 9-Phenanthrol and flufenamic acid depolarize HL-1 cell mitochondria membrane potential

As an initial step to determine whether mitochondria may be the source of increased cytoplasmic Ca^{2+} in response to 9-phenanthrol, we measured the effect of 9-phenanthrol and flufenamic acid on mitochondrial membrane potential. HL-1 cells loaded with TMRM displayed punctate, mitochondrial fluorescence recorded at 566 nm, Fig. 8A. Fluorescence intensity did not diminish with added vehicle (0.1% DMSO), Fig. 8A & 8B. Addition of 9-phenanthrol (10 μ M) resulted in rapid, significant decreases in fluorescence intensity, which plateaued over 20 minutes, but it did not decrease to zero, Fig. 8A & 8B. Flufenamic acid (10 μ M) also significantly depolarized HL-1 cell mitochondria with small additional depolarization at 100 μ M, Fig. 8C. It is noteworthy that neither 9-phenanthrol (10 μ M) nor flufenamic acid (10 & 100 μ M) collapsed mitochondrial membrane potential compared with the complete collapse by added FCCP (1 μ M), Fig. 8A

3.7 9-Phenanthrol inhibits membrane ion channel current

To evaluate functional effects of 9-phenanthrol on membrane ion channel activity, we obtained patch-clamp on-cell recordings at 0 mV pipette potential. Inward and outward transient channel openings and closings were observed at baseline current. 9-Phenanthrol (10 μ M) completely inhibited these events, and this inhibition reversed on washout, Fig. 9A

and 9B. The predominant transmembrane cation gradients at zero pipette potential (assuming maximum diastolic potential of -65 mV [4, 21, 22, 29]) are attributed to Cs^+ (120 mM) and free- Ca^{2+} (100 μM) in the on-cell pipette solution, and either or both of these likely constitute the inward currents in Fig. 9A and 9B. The outward currents evident in Fig. 9B(*inset*) are of the magnitude we reported previously and attributed to the repolarizing K^+ currents of HL-1 cell action potentials [22].

4 Discussion

These findings demonstrate that 9-phenanthrol and flufenamic acid added to HL-1 cardiomyocytes abolish the Ca^{2+} oscillations that coincide with firing of spontaneous action potentials [21, 22]. This implies that TRPM4 plays a role in the automaticity of HL-1 cells, which serve as an important mouse cardiomyocyte model [19, 20]. It is noteworthy that the mechanism of cardiac pacemaking remains unresolved [30, 31]; nor is the role of Ca^{2+} -activated nonselective cationic channels fully understood in this process [1]. Yet, TRPM4 is functionally expressed in mouse sinoatrial node cells [10] as well as HL-1 cells. Therefore, the present findings shed some light on this matter: Ca^{2+} oscillations depend upon influx of extracellular Ca^{2+} through voltage activation of both L- and T-type membrane Ca^{2+} channels, which are found in HL-1 cells [29, 32]. This Ca^{2+} entry induces Ca^{2+} release from the sarcoplasmic reticulum [33]. We propose that the rise in $[\text{Ca}^{2+}]_i$ also activates TRPM4, which enhances depolarization of the membrane potential leading to threshold. This, then, constitutes a positive feedback that sustains depolarization, which alone might deactivate the hyperpolarization-activated “funny current,” I_f , and prevent further depolarization. Thus, I_f and the Ca^{2+} -activated nonselective cationic current of TRPM4 may work together to achieve depolarization throughout the range of diastolic membrane potential.

The unexpected finding here is that the abolition of HL-1 cell Ca^{2+} oscillations by 9-phenanthrol and flufenamic acid results in an increase in $[\text{Ca}^{2+}]_i$ from an intracellular store. Others have reported a similar effect of 9-phenanthrol on $[\text{Ca}^{2+}]_i$ from bladder smooth muscle [34]. Moreover, this intracellular store is not the sarcoplasmic/endoplasmic reticulum, which leaves mitochondria as the remaining source of this increase in $[\text{Ca}^{2+}]_i$. We conclude that depolarization of mito by both 9-phenanthrol and flufenamic acid elicits an equilibrium redistribution of mitochondrial Ca^{2+} to the cytoplasm. We cannot infer a mechanism by which inhibition of TRPM4 releases mitochondrial Ca^{2+} ; however, the fact that neither agent completely depolarizes the mitochondria indicates that this effect does not result from complete uncoupling of oxidation as seen with added FCCP. To the best of our knowledge, there have been no reports of TRPM4 localized in mitochondria. None the less, our findings imply that a cellular, compensatory mechanism involves redistribution of mitochondrial Ca^{2+} stores to upregulate plasma membrane TRPM4 current, whose activation by Ca^{2+} and voltage is well established [4]. Molecular crosstalk between mitochondria and sarcoplasmic reticulum Ca^{2+} cycling has been reported recently to modulate cardiac pacemaker cell automaticity [35]. In this regard, both ryanodine and thapsigargin slow automaticity in HL-1 cells [29]. Accordingly, mitochondrial Ca^{2+} may play a significant role in the TRPM4 pacemaker current generated by a “calcium clock” [16]. Furthermore, release of mitochondrial Ca^{2+} secondary to metabolic dysfunction such as hypoxia/reperfusion injury [13, 36] may in turn activate TRPM4 as a consequence of $[\text{Ca}^{2+}]_i$ overload and lead to development of oscillatory depolarizations and corresponding arrhythmia that may accompany these disorders [13].

9-Phenanthrol not only inhibited spontaneous Ca^{2+} oscillations in HL-1 cells, but it also inhibited inward and outward on-cell membrane currents in the same time course. This further implicates a role for TRPM4 in rhythmic excitability of HL-1 cells. The latter has long been attributed to the “funny current,” I_f , and associated HCN channels in HL-1 cells

[21] as well as isolated sinoatrial node cells [37]. However, our prior study of HL-1 cells [22] demonstrated I_f to be activated primarily at membrane potentials more negative than the -60 to -65 mV found to be the maximal diastolic potential in the HL-1 cells [22, 23]. TRPM4 on the other hand is activated by depolarization [4] and is expressed in mouse sinoatrial node cells [10]. We do not exclude the importance of I_f in spontaneous activation of HL-1 cells; rather, our findings indicate that TRPM4 may act in concert with the well-established I_f to determine the rate of firing of HL-1 cells.

These results also show a role for intracellular Na^+ in the regulation of $[\text{Ca}^{2+}]_i$ in HL-1 cells. Substitution of all but 10 mM of extracellular Na^+ resulted in an increase in $[\text{Ca}^{2+}]_i$ comprising phasic and static components. The static component was eliminated in nominal extracellular Ca^{2+} -free solution, suggesting that it results from reversal of the Na-Ca exchange when NMDG⁺ is substituted for external Na^+ . The phasic component, on the other hand, was eliminated by prior treatment with thapsigargin and superfusing with Ca^{2+} -free solution, which indicates the sarcoplasmic/endoplasmic reticulum of HL-1 cells is the source of this phasic rise in $[\text{Ca}^{2+}]_i$. Here, the reversal of the Na-Ca exchange with corresponding rise in $[\text{Ca}^{2+}]_i$ triggers Ca^{2+} release from the sarcoplasmic reticulum [38]. Our findings indicate, however, that such release of Ca^{2+} by the sarcoplasmic/endoplasmic reticulum occurs by Na^+ substitution even in the absence of extracellular Ca^{2+} , which suggests a more complex regulation. Mitochondrial extrusion of Ca^{2+} was postulated to depend on Na^+ [39], which was later established as the Na-Ca exchanger [40]. However, we have no ready explanation as to how the dynamics of this mitochondrial Na-Ca exchange might be activated by TRPM4 inhibition nor whether it might be stimulated by 9-phenanthrol or flufenamic acid.

Immunoblots show that HL-1 cells express the TRPM4 protein whose molecular weight is consistent with that previously reported [2]. The punctate fluorescent labelling of TRPM4 in HL-1 cells suggests they localize in clusters [41]. Similar labeling in cells permeabilized with TritonX-100 suggests that most TRPM4 is localized to cell membranes, although we cannot exclude the possibility that mitochondria contain TRPM4 channels. None the less, it is tempting to postulate that activation of TRPM4 at an intracellular locus may contribute to oscillations of $[\text{Ca}^{2+}]_i$. It also is interesting to note that not all HL-1 cells express the punctate TRPM4 labeling and that only 30-40% of the cells oscillate $[\text{Ca}^{2+}]_i$. We cannot, however, imply causality with this correlation. Nor can we fully explain why 9-phenanthrol increased $[\text{Ca}^{2+}]_i$ in all cells; whereas, only a fraction express plasma membrane TRPM4. It is significant that HL-1 cells are coupled by gap junctions [19, 20]. Thus, it is likely that $[\text{Ca}^{2+}]_i$ released in some cells diffuses rapidly to juxtaposed cells.

In summary, we have shown that 9-phenanthrol, an established specific inhibitor of TRPM4 ion channels, reversibly blocks on-cell recorded inward membrane currents in HL-1 cells. 9-phenanthrol inhibits HL-1 cell $[\text{Ca}^{2+}]_i$ oscillations resulting in an increase in $[\text{Ca}^{2+}]_i$. This increase derives from an intracellular Ca^{2+} store other than the sarcoplasmic/endoplasmic reticulum, most likely the mitochondria. A rise in $[\text{Ca}^{2+}]_i$ from mitochondria on inhibition of TRPM4 by 9-phenanthrol implicates a regulatory feed-back mechanism between the intracellular Ca^{2+} store and regulation of TRPM4, particularly since its activation by Ca^{2+} is well established. Release of mitochondrial Ca^{2+} secondary to metabolic dysfunction such as hypoxia/reperfusion injury or sepsis may in turn activate TRPM4 as a consequence of $[\text{Ca}^{2+}]_i$ overload and lead to development of oscillatory depolarizations and corresponding arrhythmia that may accompany these disorders.

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Fig. 1. Effect of 9-phenanthrol (10 μM) on [Ca²⁺]_i oscillations recorded by Fura2 fluorescence imaging of HL-1 mouse cardiomyocytes. *A.* [Ca²⁺]_i versus time in three independently oscillating cells. *B.* [Ca²⁺]_i versus time. Mean ± SE (n = 40 cells)

Fig. 2. Sequential effects of 9-phenanthrol (10 μ M) and NMDG⁺ substitution for all but 10 mM external Na⁺ on $[Ca^{2+}]_i$ in a HL-1 cardiomyocyte superfused with Ca²⁺-free external salt solution

Fig. 3. Effect of flufenamic acid (10 & 100 μ M) on $[Ca^{2+}]_i$ oscillations recorded by Fura2 fluorescence imaging of HL-1 mouse cardiomyocytes. *A. i & ii* Effect of flufenamic acid on $[Ca^{2+}]_i$ in single cells; *iii*. $[Ca^{2+}]_i$ versus time. Mean $\pm$ SE (n = 40 cells). *B. i & ii* Effect of flufenamic acid on $[Ca^{2+}]_i$ in single cells in Ca^{2+} -free external solution. *iii*. $[Ca^{2+}]_i$ versus time in Ca^{2+} -free solution. Mean $\pm$ SE (n = 35 cells).

Fig. 4.

Sequential effects of store depletion of SR/ER Ca^{2+} via SERCA pump inhibition by thapsigargin (TG, 10 μ M). A. Effect of Ca^{2+} -free solution and 9-phenanthrol (10 μ M) on $[Ca^{2+}]_i$ in mouse HL-1 cardiomyocytes after store depletion. B. Effect of Ca^{2+} -free solution and NMDG⁺ substitution on $[Ca^{2+}]_i$ in mouse HL-1 cardiomyocytes after store depletion. Mean $\pm$ SE (n = 40 cells)

Fig. 5.

Effect of NMDG⁺ substitution for all but 10 mM of external Na⁺ on $[Ca^{2+}]_i$ in HL-1 cardiomyocytes. *A.* HL-1 cells superfused with standard external salt solution before and after NMDG⁺ substitution. Mean $\pm$ SE (n = 40 cells). *B.* HL-1 cells superfused with Ca²⁺-free external salt solution prior to NMDG⁺ substitution. Mean $\pm$ SE (n = 40 cells)

Fig. 6. Fluorescent immunohistochemistry labeling of TRPM4 protein with anti-TRPM4 antibody in HL-1 cardiomyocytes. *A.* Punctate labeling for TRPM4 was associated with the plasma membrane of some HL-1 cells, which were stained without Triton X-100 treatment. *B.* As in *A*, but also showing a cell exhibiting higher density punctate labeling. *C.* As in *A* & *B*, but cells permeabilized with TritonX-100. *D.* Control cells without anti-TRPM4 antibody. Nuclei were counterstained with Hoechst. Scale bar = 25 μ m

Fig 7.

Immuno-identification of TRPM4 expression in HL-1 cells. Confluent HL-1 cell cultures were homogenized and fractionated by centrifugation. Equal protein samples (100 μ g/lane) of each fraction were separated by SDS-PAGE and blotted to PVDF. The blot was reacted with anti-TRPM4 antibody. Lane: 1) protein marker; 2) Whole-cell lysate; 3) Low-speed supernatant (10,000g); 4) Nuclei and mitochondria lysate; 5) High-speed supernatant (45,000g); 6) Membrane protein.

Fig. 8.

Mitochondrial membrane potential (Ψ_{mito}) measured in HL-1 cells using the potentiometric dye TMRM. **A.** Representative images of HL-1 cells loaded with TMRM and treated with: Control, vehicle control (0.1% DMSO, 9-Phenanthrol (10 μ M) or FCCP (1 μ M). **B.** Inhibition by 9-phenanthrol ($p < 0.05$) of TMRM relative fluorescent intensity (RFI) of mitochondrial regions. Mean $\pm$ SE ($n = 4$). **C.** Inhibition by flufenamic acid ($p < 0.05$ at 10 & 100 μ M) of TMRM relative fluorescent intensity (RFI) of mitochondrial regions. Mean $\pm$ SE ($n = 4$)

Fig. 9.

Effect of 9-phenanthrol (10 μM) on membrane ion channel activity recorded in HL-1 cells from patch-clamp electrophysiology in the on-cell recording mode. *A.* On-cell recording showing ion channels with predominant inward current. *B.* On-cell recording showing ion channels with both inward and outward currents. *Inset.* Displays expanded record of ion channel outward currents. Current and time base applies to A & B.