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9-Phenanthrol and flufenamic acid inhibit calcium oscillations in HL-1 mouse cardiomyocytes

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Electrical potentials exist across the membranes of nearly every cell type in the body. In addition, excitable cells, such as neurons, myocytes and even some endocrine cells elicit electrochemical fluctuations, action potentials (AP), in the cell membrane to initiate cell-to-cell communication or intracellular processes. The basis for the electrical potential is rooted within an array of complex interactions between monovalent ions and their associated membrane channels and transporters that regulate the flux of these charged species across the hydrophobic bilayer. Here, an expansion of our recently published work [1] will serve to explore the modern concepts regarding the origin of the AP as well as to examine the mechanisms by which intracellular calcium ($[Ca^{2+}]_i$) is regulated within the HL-1 mouse cardiac myocyte.

Basics of the Cardiac Action Potential in Sino-atrial Node Cells (SAN)

In nerve and skeletal muscle namely two ionic channels are crucial for the production of an AP; these are the voltage-gated Na^+ channels (VGSC) and the voltage-gated K^+ channels (VGPC). However, in cardiac myocytes, an ensemble of ionic channels contributes to the various characteristics of the AP.

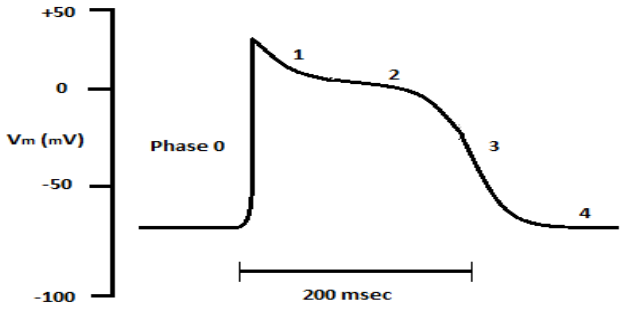

Fig1. (Above) Typical AP in ventricular and atrial cardiomyocytes.

APs in ventricular and atrial myocytes can be defined in five distinct phases: phase 0 (depolarization), phase 1 (partial repolarization), phase 2 (plateau), phase 3 (repolarization), and phase 4 (resting potential). The AP observed in SAN myocytes is markedly different from APs generated in other cardiac myocytes. Automaticity or spontaneous depolarization is a hallmark characteristic of the SAN cells of the heart's right atrium and these cells produce repetitive and rhythmic firing of APs that propagate to the working myocardium to elicit contraction and ultimately propel blood from the heart [6].

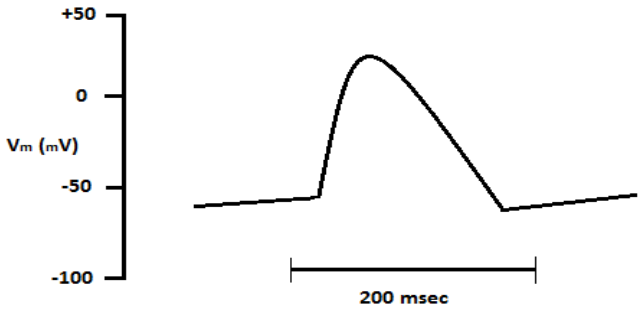

Fig 2. (Above) AP displayed by SAN cells.

SAN cells are differentiated from other cardiomyocytes inasmuch as they lack phases 1 and 2 of the AP while rapid depolarization in phase 0 is is attributable to voltage-gated

Ca^{2+} channels (VGCCs) and followed by a more gradual repolarization as the cell returns to its resting membrane potential (RMP). Phase 3 or membrane repolarization, is initiated by the inactivation of VGCCs and by the gradual increase in the cell's permeability to K^+ ions. Prior to reaching the RMP in phase 4, SAN pacemaker channels activate and begin to generate inward ionic flux. In actuality, there is no true RMP for SAN cells given that pacemaker channels are activated by repolarization and thus oppose the increasing polar state. As a consequence, SAN cells display a less negative RMP, approximately -65 mV while neurons and skeletal muscle reside in the -90 mV range. While functioning synchronously and rhythmically, pacemaker channels (see below: *Sarcolemmal Currents in Pacemaking*) work in unison to drive the cell membrane to a threshold potential by which inducing the activation of VGSCs and contributing to the rapid upstroke observed in phase 0 of the AP. Hence, these ionic channels mediate spontaneous depolarization and are of utmost importance to researches and clinicians alike.

HL-1 Cardiac Myocytes

While the use of primary cardiac myocytes is ideal, the practical aspects of the sacrificial harvesting of cardiac cells for experimental use can be complicated and time consuming. It is well established that long-term cultures of primary cells tend to exhibit a progressive loss in phenotype and become overgrown by non-myocytes after only a few days in culture [2, 3]. Nonetheless, isolated embryonic and neonatal primary cardiomyocytes have been widely used as a model system to study cardiac muscle structure and function in vitro [4]. However, their use is somewhat limited as they lack

many adult cardiomyocyte characteristics. Given this, a growing demand for a stable cardiac cell line gave rise to the development of the HL-1 cell.

The HL-1 cell line was established from AT-1 cells, which are myocytes obtained from an atrial tumor in transgenic mice expressing the Simian virus 40 (SV40) where a large T antigen was targeted to the atrial natriuretic factor (ANF) promoter [5]. The AT-1 cell line was an initial attempt to provide a suitable surrogate model for primary cardiomyocytes; however, the cell's continued passage in culture is finite and therefore could not be recovered from frozen stocks [4, 5]. Nonetheless, the cells did maintain a differentiated cardiac phenotype and provided the template for to develop the HL-1. Cancerous AT-1 tissue was excised from syngeneic mice, trimmed of connective tissues, minced, and incubated under 0.125% trypsin. Cells were obtained by 0.1% collagenase digestions and plated (15×10^6 cell/5ml). A proprietary growth medium developed by Claycomb et al., induced cell growth and division and bore the HL-1 cell line. Currently, the HL-1 is the only cell line available that continuously divides and spontaneously contracts while maintaining a differentiated adult cardiac phenotype through indefinite passages in culture [4, 5]. With increasing validity as a suitable cell culture shown through microscopic, immunohistochemical, electrophysiological, and pharmacological methods, the HL-1 cardiac myocyte has gained increasing popularity amongst researchers to assess normal cardiac function as well as pathophysiological conditions at the cellular and molecular levels. About 30% of confluent HL-1 cells generate spontaneous action potentials and display oscillations of intracellular calcium ($[Ca^{2+}]_i$), however, evidence about the electrophysiological properties of these cells is

limited. Therefore, our aim, in part, was to further elucidate the molecular components that initiate and propagate excitability within the HL-1 cell.

Sarcolemmal Currents in Pacemaking

The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels have been postulated to provide protection from the bradycardic effect of hyperpolarized myocardium following repolarization [10], but also may be key molecular players in the perpetuation of spontaneous AP rhythmicity. The current derived from these channels is “funny” inasmuch as typical currents in cardiac tissue are activated by hyperpolarization rather than by depolarization. In fact, this funny current, I_f, is a net inward current comprising a mixed inward Na^+ and outward K^+ current that is activated at membrane potentials between -50 to -60 mV in HL-1 cells [9]. Early hypotheses suggested I_f as the principle “pacemaker current” given its activation in the early stages of diastolic depolarization; however, this designation has been challenged as blockade of I_f seems to only extend cycle length, but does not cease pacemaking completely [8, 11]. Interestingly, some cardiac preparations displaying automaticity do not express the biophysical properties of HCN channel presence (e.g., the bullfrog) [8, 12]. Nonetheless, Sartiani et al., report: (1) not only the presence of I_f in the HL-1 cell, but also a strong correlate between I_f and spontaneous depolarization, noting that approximately 30% of the cultured cells display presence of the HCN 1 and HCN 2 isoforms; and (2) I_f activation in the HL-1 cell occurs at membrane potentials less negative than the resting membrane potential. This data suggests that HCN channels serve a functional role in the generation of the AP, but do not comprise pacemaking in its entirety.

Of the ions involved in the working myocardium, Ca^{2+} serves a ubiquitous role. Carrying a +2 formal charge, Ca^{2+} ions rapidly alter the membrane potential while serving as direct activators of myofilaments in the cardiac contractile apparatus by binding to troponin receptors. The notable plateau (phase 2) in the AP of ventricular cardiomyocytes reflects the extended contraction in the muscle fiber [15]. Given $[Ca^{2+}]_i$ coincides with the AP and determines myocyte contractility, growing interest surrounds the mechanisms by which Ca^{2+} handling is orchestrated in cells as it relates to pacemaking.

Voltage-gated Ca^{2+} channels (VGCCs) belong to the superfamily of membrane channels including the voltage-gated Na^+ and K^+ channels. Following activation, VGCCs become selectively permeable to Ca^{2+} whilst constituting the greatest contribution of Ca^{2+} ions to the inner-membrane pool. There are two major types of VGCCs; T-type and L-type channels with stimulation in the HL-1 cell occurring at -60mV and -40mV, respectively [13, 14]. L-type channel currents ($I_{Ca,L}$) activate with slow kinetics at voltages slightly more negative than the threshold potential and are attributed to the upstroke of the action potential, whereas T-type currents ($I_{Ca,T}$), which are predominately expressed in SAN cells, presumably are involved in early depolarization [13].

Pacemaking further involves depolarizing, inward currents of the electrogenic Na^+-Ca^{2+} exchange protein (NCX) [7, 8]. Initially, the inward current generated by NCX (I_{NCX}) was largely overlooked as an integral factor in pacemaking due to it being an exchange activity instead of a channel mediated current [8]. The presence of $[Ca^{2+}]_I$ instantaneously activates NCX, which serves to extrude from the cell a single Ca^{2+} for

three Na^+ ions. However, due to the fact that this exchanger is electrogenic in nature, the direction of transport can be reversed during the membrane potential nadir. Nonetheless, typical forward motion initiates a depolarizing current across the membrane and unlike many voltage-gated ion channels, NCX functions throughout the cardiac cycle [8].

Store Operated Ca^{2+} Entry (SOCE)

The sarcoplasmic reticulum (SR) constitutes the largest intracellular store of Ca^{2+} ions and its role in pacemaker function was initially noted from observations that blocking Ca^{2+} release via inhibition of the ryanodine receptors (RyRs) led to a progressive decrease in the rate of late diastolic depolarization [16]. RyRs function as "leaky pumps" that spontaneously and rhythmically generate local releases of Ca^{2+} (LCR's) from the SR into the cytosol with peak concentrations of LCRs occurring just prior to threshold potential. The presence of $[Ca^{2+}]_i$ released from the SR functions to not only alter membrane potential, but also stimulates the RyRs to release more Ca^{2+} ions into the cytosol through a positive-feedback mechanism dubbed, Ca^{2+} induced Ca^{2+} release (CICR).

Following the AP upstroke, Ca^{2+} ions entering from VGCCs elicit a larger CICR from the SR in which remaining Ca^{2+} content is dumped into the cytosol. This allows for a synchronization, albeit a depleted one, of SR Ca^{2+} content across SAN cells and prevents SR Ca^{2+} overload [8]. Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps mediate the re-uptake of SR Ca^{2+} and in time, RyRs will begin to produce LCRs once more. Interestingly, LCRs generated by the RyRs are unimpeded in cell

membranes that have been permeabilized, thus suggesting its perpetuation is independent of membrane-bound ion channels [8].

Mounting evidence suggests the mitochondria play a fundamental role in Ca^{2+} buffering that modulates SR Ca^{2+} load and thus indirectly influences SOCE in SAN cells. The mitochondrial Ca^{2+} uniporter (MCU) is predominately responsible for Ca^{2+} uptake, however, influx of Ca^{2+} ions into the mitochondrial lumen is still present in cells that have had MCU down-regulated suggesting that other mechanisms of Ca^{2+} entry are present [24]. In the mitochondria of rat liver and brain cells, Feng et al., describe the presence of a transient receptor potential (TRP) protein channel that functions to regulate mitochondrial Ca^{2+} (Ca^{2+}_m) homeostasis [25].

TRPM4 as a pacemaker component

The existence of TRP channels was first noted in the photoreceptors of *Drosophilia* where TRP gene mutations displayed voltage response to light [17]. TRP channel properties are not fully described; however, other than channel architecture and overall amino acid sequence homology, no distinct feature defining the TRP family has yet been defined [18]. Mammalian TRP channels comprise six related protein sub-families: TRPA, TRPC, TRPML, TRPM, TRPV, and TRPP and their importance in sensory transduction at the cellular and multicellular level is irrefutable across organismal hierarchy. TRP channels in yeast are integral in the perception of and response to hypertonic environments [19]. Nematode TRP channels are utilized as chemosensory receptors for the detection of the chemical quality of a substance [20]. Mice utilize a pheromone-sensing TRP channel in sex discrimination [21]. In the taste receptors of humans, TRP channels allow for the distinction of spicy, sweet, sour, and

umami flavors [22]. Moreover, these channels allow for the discrimination of hot and cold substances [23].

Single-channel electrophysiological recordings describe a Ca^{2+}-activated non-selective cation channel (NSC_{Ca}) in a variety of mammalian SAN cells that is voltage dependent, impermeable to Ca^{2+} ions, and has a channel conductance between 20 and 30 pS [26]. Several studies have postulated the TRP sub-family member, melastatin 4 (TRPM4) as being such a channel and its presence has been noted in mouse SAN cells [27]. TRPM4, designated TRPM4b (the active splice variant), appears to serve a functional role in allowing cells to depolarize in a Ca^{2+} dependent manner. We report the novel expression of TRPM4 in the HL-1 mouse cardiac myocyte and postulate its role in pacemaker function from an intracellular location, possibly the mitochondria.

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