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RESEARCH ARTICLE

Communication Between the Plasma Membrane and Cytosolic Cardiac Pacemakers: A Role for Melatonin?

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ABSTRACT:

We review here briefly the essentials of the vertebrate cardiac pacemaking system consisting of the populations of ion channels that drive heartbeat. In addition to the sarcolemmal system, there is a second ion channel-based oscillator consisting centrally of a reciprocating Ca^{2+} flow between the sarcoplasmic reticulum and the cytosol regulated by release through ryanodine receptors and active return by the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase. We review the considerable similarity between vertebrate and the fly heart pacemakers. In our work with *Drosophila*, we have shown that melatonin is capable of increasing the rhythmicity of the fly heart to a remarkable level. This is true even in flies bearing mutations that severely damage ion channels central to the plasma membrane pacemaker. We review here evidence showing that mutations in the gene encoding the fly Calcium ATPase have severe effects on heart function. We present further evidence from our work that melatonin has only a modest effect in reversing these defects. We have hypothesized that melatonin acts by altering the relationship between the sarcolemmal and cytosolic oscillators and cite these and previous findings as provocative in this regard.

Introduction

Of considerable interest in cardiology is the question of regularity in heartbeat. Clearly, there are pathologies that result in highly arrhythmic heart function[1], but given that, normal heartbeat in both vertebrates and flies is not perfectly rhythmic and there is constitutive irregularity normally[2]. In fact, certain cardiac pathologies, including congestive heart failure, display extremely regular beat[3]. The nature of this has been under considerable study[3]. There are two theoretical sources. First, the irregularity is *1/f* "noise" in a deterministic system; the second regards the cardiac oscillator as fundamentally chaotic[4]. We can take as a given that a certain amount of irregularity is "normal" and look at it in the light of recent research on melatonin and its effect on the fly heart[5].

As the core of this discussion concerns evidence based on work with *Drosophila*, it is necessary to describe the fly model so its contributions may be seen clearly. Across the board, the fly has proven its relevance and value in cardiac research even to the point of the finding that a diet high in calories and fats can cause heart problems similar to those seen in humans[6,7]. The gene *ether-a-gogo* (*eag*) was initially discovered in *Drosophila*[8,9]. The cDNA from this gene was used to detect a closely related channel in mammals: human ether a go-go related gene (HERG)[10]. HERG is a homolog of the *Drosophila* gene *seizure*[11,12]. Mutations in HERG result in a cardiac arrhythmicity in humans, chromosome 7-linked long QT (LQT2) syndrome [13,14]. Individuals carrying mutations show delay in cardiac repolarization[14,15,16]. There are now 1682 human disease genes on record and nearly three quarters of them have homologs in *Drosophila;* roughly a third of these genes are functionally equivalent in flies and humans and There is an excellent and comprehensive summary of the scope of the fly cardiac model[17].

The Vertebrate Pacemaker System

The underlying ionic mechanism of the cardiac pacemaker has been a topic of intensive interest and research[18]. It is far beyond the scope of this paper to provide even a modest review of this work, but a concise description of the most widely accepted current model is in order as a basis. Pacemakers in the vertebrate heart are myogenic and consist of a population of ion channels interacting in an oscillatory fashion which triggers cyclic contractions in the myocardium[18]. In mammals, this primary oscillator is constrained to the Sinoatrial (SA) node, which is considered to be the "pacemaker" *per se*. This nodal tissue is histologically quite distinct from other cardiac tissue and retains is dominant[18]. Current evidence indicates that there are two interacting oscillators at work, one is found in the plasma (sarcolemmal) membrane, the other in the cytosol based on a cytoplasmic-based oscillator[19].

Unlike neurons, cardiac pacemakers, have no true "resting" potential[20]. By convention, the "resting potential" of true pacemaking cells is often taken to be the most negative potential the system reaches in its cycle[18]. It is possible to clamp a voltage in pacemaker cells at a level where little current is elicited, and this may be taken as a resting potential as well[18].

We first describe the sarcolemmal oscillator which was formerly considered to act alone. There are two major classes of ionic currents in SA nodal cells depending on time dependency[18]. Currents that are time-dependent are controlled by gated ion channels carrying Na^+ (I_{Na}), $Ca2^+$ ($I_{Ca,L}$ and $I_{Ca,T}$), K^+ (I_K and $I_{Ca,K}$), and a mixture (I_f or, variously, I_h)[21]. Ion pumps are responsible for the time constant currents. One exchanges three inward-bound Na^+ ions for one outgoing Ca^{2+} ions There is a net inward cation current (I_{NaCa}). There is also an exchanger that swaps two inward K^+ ions for three outward Na^+ ions (I_{NaK})[18].

Two separate Ca^{2+} currents modulated by distinct channels, constitute the cardiac action potential proper, starting with a sharp spike. The L type, so named because its current is Long-lived and Large ($I_{Ca,L}$) and the other Tiny and Transient ($I_{Ca,T}$)[21]. The interactive roles of these two Ca^{2+} channels involves $I_{Ca,T}$ being active transiently during early in the action potential, spiking the membrane sufficiently to activate $I_{Ca,L}$ which finishes the spike[22].

A K^+ current repolarizes the sarcolemma much as is seen in neurons [23]. In the heart, there are two major sub-categories of these channels based on activation. The first group utilizes voltage-activated gating. Above threshold, the channels open allowing outward flow of K^+ along its concentration gradient. The other consists of channels gated by cytoslic Ca^{2+} sensing. As the cardiac action potential is a calcium spike, the interaction is clear. These calcium-activated potassium channels (CaKs) play a central role in repolarization[23]. The current in early repolarization is usually termed the transient outward potassium current (I_{to})[23].

There is a crucial second role for K^+ in the sarcolemmal system- its participation in initiating the cardiac action potential. It does not act alone, as the channels responsible are permeable to both K^+ and Na^+ [24]. This is the "funny current," (I_f) also

called the hyperpolarization-activated current, or I_h. Its role has been somewhat controversial[24], but it is now the leading candidate to be the trigger for sarcolemmal depolarization[25].

The cytosol-based oscillator was not found until much later than the sarcolemmal. Starting with the work of Vinogradova et al., evidence for the cytosolic Ca^{2+}-based oscillator (local Calcium Release or LCR) has mounted[26]. A thorough description can be found in a paper by Lakatta and DiFrancesco which details both systems and compares and contrasts both ystems[27]. At its core the system involves release of Ca^{2+} from the sarcoplasmic reticulum of pacemaker cells via Ryanodine Receptors (RyR) and its reuptake by the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA)[28]. This oscillator works in consort with the sarcolemmal ion channel oscillator to effect heart pacemaking, and can even be seen to function when the plasma membrane is voltage-clamped[29]. The cooperation of the two systems is crucial to proper heart function[28]. One of the most consistently observed abnormalities in patients with ventricular arrhythmias is the impaired ability to handle intracellular calcium due to changes in ryanodine receptor (RyR) and/or sarco-endoplasmic reticulum Ca^{2+} - ATPase (SERCA)[30].

The Fly Heart

Our laboratory has approached uncovering heart function in an insect model. Using a combination of genetics and pharmacology, the essentials of the *Drosphila* pacemaker have been discovered which is remarkably similar to that found in vertebrates. Four genes encoding ion channels being necessary for normal heartbeat have been uncovered[31,32,33]. Three of the channels pass K^+ and the other Ca^{2+} [31,32,33]. These workers describe a sarcolemmal pacemaker model based on these genetic and pharmacological findings that is parallel in all respects to the vertebrate heart[33].

The role of melatonin in regulating cardiac rhythmicity

We have shown that the beating of the *Drosophila* early pupal heart becomes preternaturally rhythmic when treated with melatonin[5]. This is not hyperbole, as a quick perusal of our work will quickly attest. These results have major implications for pharmacological investigations in general, but in the context here, there are theoretical considerations about the very mechanism of heart pacemaking. We have postulated that the fact that the heart can beat with such precise regularity is evidence that the cardiac oscillator cannot be chaotic. Chaos would be a fundamental property of the underlying physics of the oscillator[34,35]. For example, the classic example of chaos, the double pendulum system, can never oscillate regularly unless it is fundamentally physically altered[36].

A further highly provocative finding was that the rhythmicity of hearts of flies bearing mutations that seriously damage genes encoding ion channels central to the oscillating mechanism beat as unnaturally rhythmically as those of wild-type flies after melatonin injection[5]. This finding almost literally demands that there be a fallback pacemaker configuration. We have formally proposed this solution[5,37]. A possibility for this exists. Which brings us to ground zero on understanding cardiac rhythmicity. In our work we have expanded on the mathematical analysis of heartbeat[38], but one very conventional way is to look at Inter Beat Interval, essentially a time series showing instantaneous rate essentially the length of each beat cycle[39]. Any irregularity in this interval can arise from only two sources, possibly from a combination of the two. The first is variation in the length of a given beat from its triggering to the next trigger, an alteration in the waveform. The other possibility is a variation in the trigger time, with the beat cycle duration remaining constant. This would necessitate a neutral "dead" state at the end of the cycle terminated by the trigger. This would fire irregularly.

Investigations into the cytosolic oscillator in *Drosophila*

Given the current consensus that the sarcolemmal and LCR oscillators operate cooperatively in tandem in vertebrates[25,26], it is essential to look at evidence in the fly. Ryanodine receptors and SERCA comprise the core of the cytoplasmic portion of the LCR system, and genetic probes have clearly shown that both are involved in the fly. Mutations in the gene encoding the RyR receptors cause serious cardiac malfunction[40]. Far more evidence comes from work with SERCA. Isolation of conditional mutations in the *Drosophila* SERCA gene, *Ca-P60A* provided considerable insight into the function of this Ca ATPase[41]. The two allelic mutants found were named *Kumbhakarna*^{170} and *Kumbhakarna*^{295} here, SERCA^{170} and SERCA^{295} [41]. They are recessive lethals and suffer paralysis at 40° in 3-5 minutes unlike wild-type, which is unaffected by this treatment[41]. These are loss-of-function mutations and calcium handling was shown to be the underlying issue as voltage-gated calcium currents are greatly reduced in heated SERCA^{170} individuals[41]. Critically, interference with muscle function was shown to be unrelated to any effects on SERCA in neurons[41]. Also, highly relevant here is

the observation that Ca^{2+}-gated K^+ currents are decreased in heated SERCA mutant flies. The gene encoding this channel in the fly is *slowpoke*, the most central of the K^+ channels mentioned above as components of the fly sarcolemmal pacemaker[31].

SERCA mutations have been thoroughly investigated with regard to fly cardiac function[42]. It has been shown that *Drosophila* SERCA protein is significantly enriched in the heart[42]. In addition, the temperature-sensitive mutations noted above, notably $SERCA^{170}$ and $SERCA^{295}$ have altered function. RI across strains was unaltered compared to wild-type, while Frequency (FR) was slightly, but significantly reduced[42]. However, after heat shock, both FR and RI were profoundly reduced. 41% of flies bearing the severe 170 allele showed no heartbeat at all and could not be included in the analysis[42]! The effect was localized to the heart using a muscle-specific molecular driver that caused expression of the mutant protein. Such flies showed mutant heart behavior that is not seen when the mutation was driven in nerves[42]. In another work, it should be noted that pinning the SERCA effect to the myocardium proved elusive[43]. Close analysis of the waveform of the heart systole showed that not only the interval between beats was lengthened, but the actual duration (i.e. waveform) of each beat was altered[42].

Effects of melatonin on flies bearing mutations in SERCA

The effect of melatonin on flies bearing the SERCA mutation have been investigated and we must consider these results in this light[44]. We present here work that appeared in the Van Kirk Doctoral thesis[44]. A brief specific materials and methods will follow, the general protocol can be found here[5].

Wild-type controls and $SERCA^{170}$ were collected at the third-instar larval stage just as they were entering the early pupal (P1) stage. At this point, they are translucent, making any heart movements transmitted to the surrounding organs easily visible. To monitor heart function, each pupa was placed on a glass slide in a drop of distilled water and set in the light beam of an Olympus binocular microscope. Temperature of the slide was maintained at 25 ° C. Recordings were done initially for 10 minutes for all subjects. Each pupa was allowed 1.5 minutes to equilibrate to 25 ° C after being placed under the microscope and before recording started. After injection, flies were immediately placed under the microscope and recording was started without the 1.5 minute equilibration time in order to observe the immediate effects of the injected substance. The protocols were as follows: groups of wild type and $SERCA^{170}$ subjects were left untreated between recordings to act as controls. A second group of experimentals and controls were injected with 46 nL of 1000µM melatonin (dissolved in 1% ethanol) after a preliminary 10 minute recording (details[5]). They were then exposed to a 2.5 minute heat shock by being housed in a glass vial and immersed in water at 41° C. They were then immediately returned to the recording apparatus and heartbeat was recorded for the requisite 10 minutes without any time to acclimate. A third protocol simply involved doing the melatonin injection after the heat shock, all else being identical. Control pupae were injected with *Drosophila* calcium-free ringers solution with 1% ethanol. Controls showed no significant change in heart rhythmicity with injection before or after 2.5 minute heat shock at 41 ° C ($p > 0.05$).

Data were analyzed by autocorrelation and Maximum Entropy Spectral Analysis (MESA)[38]. Estimations of heart rate frequency were based both on the spectral analysis and on inspection of the raw data plots and autocorrelation for verification. Regularity of the heartbeat was quantified from the autocorrelation analyses. Rhythmicity in the signal results in recurring peaks of positive and negative correlation and the decay envelope reflects regularity in the signal[38]. The height of the second peak (counting lag zero as the first peak) was expressed as a fraction of the height of the zeroth lag; we refer to this coefficient as the rhythmicity index[38] and it serves as our benchmark.

Results

For recordings where melatonin was injected before the 2.5 minute heat shock, minutes 1-10 of pre and post injection were compared and statistically analyzed. For recordings where melatonin was injected after the 2.5 minute heat shock, minutes 7-10 of pre and post injection were compared and statistically analyzed.

$SERCA^{170}$ pupae showed a significant decrease in heart rhythmicity with the 2.5 minute 41 ° C heat shock alone ($F(1,4) = 153.6$; $p < 0.001$ and $F(1,4) = 32.6$; $p < 0.001$). (Fig 1). Injections of melatonin before and after heat shock showed significant increase in heart rhythmicity in both wild-type and $SERCA^{170}$ strains (Melatonin injection before heat shock: $F(1,4) = 9.4$; $p < 0.05$ and $F(1,9) = 51.1$; $p < 0.001$; Melatonin injection after heat shock: $F(1,4) = 30.1$; $p < 0.001$ and $F(1,9) = 28.0$; $p < 0.001$). (Fig 1). When mutant pupae were injected with melatonin before heat

shock, they showed an increase in heart rhythmicity of 137%. When injected with melatonin after heat shock, they showed an increase in heart rhythmicity of 123%.

Comparison of wild-type to SERCA^{170} mutants showed a similar trend in which significant increase in heart rhythmicity occurred across the entire 10-minute recording with melatonin injection before heat shock, while significant increase in heart rhythmicity didn't begin until minute 7 with melatonin injections after heat shock, and continued to increase from that point (Fig 1).

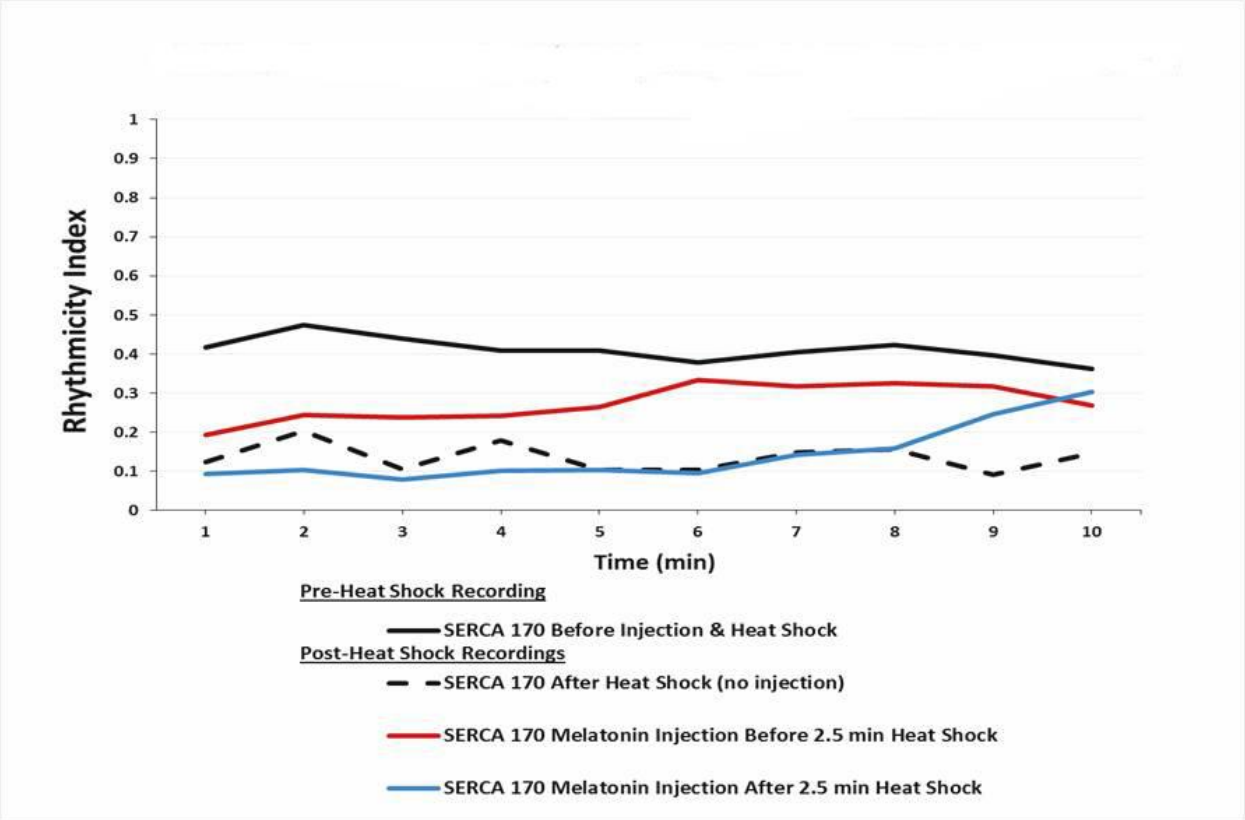

Figure 1. Response of SERCA^{170} pupae to heat shock and injection of melatonin. These mutant flies, as has been previously shown, do not have hearts as rhythmic as wild type even before heat shock, but this is not pronounced. After heat shock, as seen above, the rhythmicity declines precipitously. When melatonin is injected prior to the heat shock, there is a marked improvement over the untreated flies that increases throughout the duration of the recording. If the injection is postponed until after the heat shock, this delay is seen in the later recovery, but by the end of the recording, the hearts are clearly as robustly rhythmic as the other group and both groups are nearly as rhythmic as they were pre-shock.

Discussion

The mechanism whereby melatonin sharply increases the rhythmicity of the *Drosophila* heart demands an explanation. This is doubly crucial when contemplating the ability of this agent to rescue rhythmicity in hearts of flies bearing mutations affecting core elements of the pacemaker such as *slo*. As previously noted, if the sarcolemmal oscillator portion of the pacemaker is badly damaged, for example by a lesion in the *slo* gene (see above), it would be impossible for it to somehow become not just rhythmic, but more cleanly than untreated wild-type flies[5]. There seems to be no other option than hypothesizing a mechanism within the robust coupled system consisting of sarcolemmal and LCR components that demonstrably maintains pacemaking in the face of the failure of any of several individual components[25]. The extraordinary and abnormal regularity of melatonin-treated hearts is difficult to explain without drawing on the possibility of some fundamental alteration in this system.

The work referenced here begins to shed some light on this. While an active LCR oscillator has not been conclusively demonstrated in the fly, the work with RyR receptors and SERCA described above suggest it is highly likely. In the work we discuss above, we have an example of cardiac malfunction caused by SERCA being ameliorated by melatonin, and significantly so. But there is a difference that could be crucial. The hearts of heat-treated SERCA^{170} flies barely achieve the

same level of regularity as they had prior to heat shock, and that only after nearly 10 minutes post-shock. In addition, the kinetics of the change differ from those of wild-type. Wild-type fly hearts show an onset of high rhythmicity that occurs literally from one beat to the next and in the fly, this has been shown to be a likely result of receptor mediation[5]. The SERCA^{170} hearts improve only gradually after treatment.

Conclusions

We have hypothesized that melatonin is acting on the coupling mechanism between the sarcolemmal and cytosolic sub-oscillators to increase regularity. We have suggested that when the sarcolemmal pacemaker is badly damaged melatonin acts to switch full control over to the cytosolic system. The very different effects of melatonin on flies bearing SERCA^{170} mutations vs. those with damaged plasma membrane channels support this conclusion. It may further be postulated that this indicates the LCR system is actually more fundamental than the sarcolemmal, The inherent irregularity found in normal hearts may reside in the coupling between the two.

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