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The renin-angiotensin system regulates transmural electrical remodeling in response to mechanical load

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I) Introduction: The history of the T-wave and transmural heterogeneity

Initial studies first documenting electrical activity in the heart defined two electrical events, which we now know correlate with excitation and repolarization (Burdon-Sanderson and Page, 1880, 1883). If excitation occurs at a particular site and repolarization also starts at the same site, then given the opposite polarity of the currents flowing, the wave of excitation (the QRS complex) should evince an opposite polarity to that of repolarization (the T-wave). Indeed in these initial studies of Burden Sanderson and Page using the frog heart, the QRS complex was of opposite polarity to the T-wave. However with the recording of the initial large animal and human EKGs (Waller, 1887; Mines, 1913, Bayliss and Starling, 1892; Einthoven, 1913), the QRS complex had the same polarity as the T-wave, implying that the spread of depolarization was in the opposite direction to the spread of repolarization. This could only occur if the action potential was shorter where repolarization was initiated than where excitation began. This conclusion is further supported by the additional conduction time from the excitation point to that where repolarization began. Thus, more than half a century prior to the first microelectrode recordings (Ling and Gerard, 1949), it was clear that there was a difference in the electrical activity of the endocardial base where the action potential was initiated in the ventricle and the epicardial apex where the wave of repolarization began. The questions became 1) how was this electrical “heterogeneity” generated, and 2) for what purpose?

II) Multiple types of heterogeneity exist within the ventricular wall

Following the discovery of the microelectrode (Ling and Gerard, 1949), it became possible to record action potentials from a single cell within a section of cardiac tissue. The first detailed characterization of all these different results was presented in the text by Hoffman and Cranefield (1960). However, recording from cardiac myocytes within a tissue did not allow separation of differences in ion currents due to differing electrical properties from those due to differences in anatomy (like restricted extracellular spaces where ion concentrations could change during electrical activity). Indeed one explanation of the differences in transmural electrical activity proposed differences in extracellular anatomy in

the EPI and ENDO allowing for different changes in driving force. The authors' argument was that the differences in APD disappeared with a sufficient period of quiescence (Cohen *et al*, 1976). However with the advent of the patch clamp technique (Hamill *et al*, 1981) and dissociation protocols to permit the study of individual mammalian cardiac myocytes (Isenberg and Klockner, 1982), it was possible to record action potentials from individual heart cells. From these studies it was clear that some of the EPI, ENDO differences were due to the gating and ion transport properties of individual ion currents. There seemed to be two types of heterogeneity. One that existed transmurally and could be responsible for the surprising polarity of the T-wave (Noble and Cohen, 1978), and others that differed specifically in the midmyocardium, whose isolated myocytes had longer action potential durations (APDs) than those close to either surface (Isenberg and Klockner, 1982). The first current discovered to be distributed transmurally was the transient outward potassium current I_{to} , which was larger in EPI than in ENDO (Furukawa *et al*, 1990; Liu *et al*, 1993; Clark *et al*, 1993; Nabauer *et al*, 1996; Brahmajothi *et al*, 1999). Since i_{to} is an outward current, which contributes to repolarization, the larger it is, the shorter the expected APD (Dong *et al*, 2010). Since it is larger in EPI than ENDO, it alone should make the EPI APD shorter than that in ENDO. Other currents like the persistent Na current I_{NaP} are largest in the midmyocardium (Isenberg and Klockner, 1982). Since I_{NaP} is an inward current, it should contribute to making the APD of midmyocardial (M) cells longer than either EPI or ENDO. The focus of this review is on the transmural gradient in ion channel properties and its regulation by A2; only those currents that differ transmurally will be considered further.

III) Multiple currents contribute to transmural heterogeneity

It has long been known that I_{to} is larger in EPI than ENDO (Furukawa *et al*, 1990; Liu *et al*, 1993; Clark *et al*, 1993; Nabauer *et al*, 1996; Brahmajothi *et al*, 1999). One example from Furukawa *et al*. (Furukawa *et al*, 1990) is provided in Figure 1 from the feline heart. This ENDO, EPI difference is seen in humans, other large mammals and small mammals like rat and mouse. Multiple alternative hypotheses have been developed to explain this gradient. These alternatives are discussed by Hilgemann (2014).

Interestingly I_{to} is not unique in being expressed to a larger degree in EPI than in ENDO. The Na/K pump current I_p also presents this gradient. Gao *et al* (2005) divided the left ventricle of the canine heart into three layers. EPI, MID and ENDO. I_p was defined as the difference between currents recorded in the absence and presence of the I_p blocker dihydroouabain (DHO). As can be seen in Figure 2 from their studies, the measured current is larger in EPI than ENDO and that MID possessed an intermediate I_p amplitude. Their experiments further demonstrated that this difference was not due to a difference in the K^+ affinity, Na^+ affinity or voltage dependence. Not all currents that have a transmural gradient are larger in EPI than ENDO. Wang and Cohen (2003) demonstrated that the L-type Ca^{2+} current, I_{CaL} , and the T-type calcium current I_{CaT} were larger in ENDO than EPI (This transmural gradient in I_{CaL} is controversial as it was not seen in some studies (Cordeiro *et al*, 2004; Li *et al*, 2002) but confirmed in others (Gao *et al*, 2014). One example is provided in Figure 3.

IV) The combination of this transmural current distribution makes contraction larger in the ENDO than EPI

There are at least three currents regulated transmurally. I_{CaL} , I_{to} and I_p . Contraction is dependent on the level of calcium entry and so a larger I_{CaL} in ENDO would be expected to be associated with a larger $[Ca^{2+}]_i$ and contractile strength than EPI. I_{to} is smaller in ENDO than EPI. The effects of this change were studied by using the dynamic clamp to insert differing amounts of I_{to} and measuring APD, $[Ca^{2+}]_i$ and cell shortening (Dong *et al*, 2010). Their results, provided in Figure 4, show that increasing I_{to} reduces plateau height, resulting in less calcium entry. Thus the lower I_{to} density in ENDO contributes to in a greater contractile shortening in ENDO than that found in EPI. Finally the sodium pump current I_p is smaller in ENDO than in EPI (see Figure 2 above), while the Inward background current is largely constant across the wall (see Figure 5). With a constant Na/Ca exchanger current across the wall as well (Figure 6), the lower pump current density and equal I_{inb} leads to a larger $[Na^+]_i$ in ENDO than in EPI (see Figure 7). This leads to an increase in both systolic and diastolic $[Ca^{2+}]_i$ (Figure 8). Under physiologic conditions, this coherent transmural regulation of I_{CaL} , I_{to} and I_p is just what would be required to produce the observed greater shortening seen in ENDO than in EPI (Dong *et al*, 2010) (Figure 4 above).

V) Regulation of all 3 currents by Angiotensin II

A2 is known to regulate the heart's response to long term imbalances in load versus contractility by increasing synthesis and expression of contractile proteins via hypertrophy. It might also regulate the three currents identified above to increase contractility in response to moderate changes in loads of minutes in duration. An increase in load could induce the myocardium to transmurally modify the three currents identified above to make them similar to ENDO. A2 activation of AT_1 Rs initiates several distinct signaling cascades, which have acute and chronic effects on ventricular myocytes (Wang *et al*, 2010; Kass and Blair, 1981; Kim *et al*, 2015). The activated receptor is coupled through G proteins to activation of several kinases, which cause the acute effects. After about 30 minutes, the G protein signaling ends and the receptors are internalized to a sub plasma membrane pool of endosomal vesicles where they act as a scaffold for a number of signaling proteins. In this section we will focus on the effects which follow internalization and require at least 30 minutes exposure to produce. If there is to be a coherent response to increase contractility across the ventricular wall, A2 must reduce I_{to} , and I_p while increasing I_{CaL} .

I_{to} was the first of these three currents to be studied after chronic exposure to A2 (Yu *et al*, 2000). In the absence of exogenous A2, EPI myocytes produce a larger I_{to} with a more negative inactivation versus voltage curve and a more rapid recovery from inactivation (Figure 9). EPI myocytes exposed to saturating [A2] for 2 or more hours exhibited multiple changes including: 1) a decrease in the current's amplitude (Figure 10), 2) a more positive voltage dependence of inactivation (Figure 11), and 3) a dramatically slowed speed of recovery from inactivation (Figure 11). Interestingly, block of the AT_1 R by Losartan caused exactly the opposite effects in ENDO: 1) increasing the current amplitude (Figure 12), 2) shifting inactivation more negative (Figure 13), and 3) slowing its time course of recovery

from inactivation (Figure 13). Controls showed no effect of A2 on I_{to} in ENDO nor Losartan on I_{to} in EPI. The action potential in EPI has a prominent notch due to the large size of I_{to} while ENDO lacks the large I_{to} and the notch it induces. Incubation of EPI with A2 produces an action potential lacking a notch, while incubating ENDO with losartan induces the opposite effect (figure 14)

More recently, similar experiments (Gao *et al*, 2014) were performed on I_P and I_{CaL} while also investigating the transmural effects on I_{to} in more detail. The canine ventricular myocardium was divided into 5 transmural segments and myocytes were isolated from each. In control conditions, the magnitude of I_{to} decreased monotonically across the wall from EPI to ENDO (Figure 15). Incubation with a saturating [A2] reduced the magnitude of I_{to} to the greatest extent in EPI, to a lesser extent in MID and not at all in ENDO myocytes (Figure 15), resulting in a nearly uniform transmural amplitude. Multiple concentrations of A2 were employed for incubation of EPI myocytes. Their effects on the magnitude of the basal I_{to} and I_P current allowed calculation of the K_D for the functional effect of A2 on these 2 currents. The K_D was estimated to be roughly 170 nM for either current (Figure 16). Using the percentage of maximal inhibition of I_{to} , in the myocytes from the 5 positions across the ventricular wall, it was possible to estimate the ambient [A2] at each position. The results in Figure 16 demonstrate a continuous decline from more than 1.4 μ M in ENDO to 0 in EPI.

A larger I_{CaL} was observed in ENDO versus EPI myocytes. Further, acute exposure to A2 decreased reduced I_{CaL} in EPI myocytes while chronic (2 hour) exposure to A2 increased I_{CaL} in the same tissue (Figure 3). Thus, taken as a whole, there is coherent regulation of cardiac membrane currents by A2 to produce greater contractility in ENDO. The estimated ambient [A2] continuously declines as one proceeds transmurally from ENDO to EPI

VI) A2 regulation is endogenous, depends on stretch and involves the T-tubular system

At this stage of investigation, the major questions that remained were: (1) What is the signal for this transmural heterogeneity in A2? (2) Is there an autocrine A2 system in the myocytes? (3) How are the differences in concentration maintained in each cardiac region? And 4) what is the molecular mechanism that regulates functional ion channel expression/gating properties?

1. Stretch of rat neonatal myocytes in culture induces the release of A2 into the bathing medium (26). The canine left ventricle has been reported to have a transmural gradient in both systolic contraction and diastolic stretch. Both are highest in ENDO. These two results suggest that there is a transmural mechanical signal that is highest in ENDO. Thus it is not surprising that there is a physiologic gradient suggesting higher A2 levels in the region of maximal stretch (ENDO) and lower A2 levels in EPI as the myocytes experience lower systolic contraction and diastolic stretch (LeWinter *et al*, 1975; Streeter *et al*, 1970; Waldman *et al*, 1985).
2. Evidence for the existence of an autocrine RAS has existed since 1998 in a study of pigs (van Kats *et al*, 1998) and we have reported similar evidence

for its existence in canine ventricular myocytes (Pan *et al*, 2004). Thus the machinery necessary for A2 synthesis is present in canine mammalian myocytes.

3. Gao *et al* (2014) reported a K_D of roughly 170 nM for the angiotensin receptors that regulate this transmural gradient. Even with an endogenous RAS discussed above, there still is the question of how such a high concentration of A2 can be generated. Clearly if the A2 is secreted into the bulk extracellular space, it would not be possible. Thus secretion into a lower volume space is required, a space which has a significant restriction to diffusion into the bulk medium. The authors (Gao *et al*, 2014) tested the hypothesis that this restricted extracellular space is the T-tubular system (Figure 17). For this hypothesis to be plausible the flux of A2 into the T-tubular system must allow this restricted space to reach an average concentration of up to 1.4 μ M. Their calculations suggest, that given the volume of the T-system and the expected A2 diffusion coefficient, the myocyte would have to secrete A2 at the rate of 10^{-17} moles/sec into the T-system lumen. Another requirement of this hypothesis is that most if not all of the channels and transporters regulated by A2 reside in the T-tubular membrane. They studied the effects of detubulation on I_p in ENDO and EPI canine myocytes. Detubulation reduced the measured capacitance (demonstrating the effectiveness of the detubulation protocol). It also dramatically reduced I_p in EPI with essentially no effect on I_p in ENDO. These results were consistent with the I_p density in the T-tubular membrane being twice as large as that in the surface membrane in EPI, while the T-tubular I_p density was less than 20% of that in the surface in ENDO (Figure 18). Additional evidence supporting the T-tubular system as the restricted extracellular space was obtained from experiments examining the effects of incubation of canine EPI myocytes with a saturating [A2]. This incubation produced no change in the measured capacitance in the absence or presence of detubulation while it dramatically reduced I_p in control conditions and had no effect on detubulated myocytes (figure 19). In ENDO and EPI, the effect of detubulation on I_p was essentially the same as the effect of saturating exogenous [A2], suggesting A2 could inhibit 100% of the pumps in the T-system membranes.
4. Given that the density of I_p and I_{to} are reduced in the T-tubular system of ENDO, how might this reduction be achieved? Doronin *et al* (2004) investigated this question by overexpressing the alpha subunit of I_{to} , Kv4.3 (V5 tagged), the beta subunit of I_{to} , KChIP2 (myc tagged) and the AT_1R (HA-tagged) in HEK293 cells. Patch-clamping of these transfected HEK293 cells demonstrated that A2 (1 μ M) incubation for 1 hour reduced the current by 90%. This reduction in current was accompanied by internalization of both Kv4.3 and AT_1R proteins from the surface membrane to the intracellular compartments where they colocalized

(Figure 20). Further immunoprecipitation studies in canine ENDO and EPI myocytes demonstrated that the AT_1R , and Kv4.3 protein coimmunoprecipitated each other, suggesting they form a complex (figure 21). In summary, these studies suggest that internalization of the channel with the AT_1R occurs upon exposure to A2, reducing surface expression and corresponding I_{to} density. Similar coimmunoprecipitation of the Na/K pump with the AT_1R (Figure 22) has also been shown (Gao *et al*, 2005).

VII) Conclusion and remaining questions

The previous sections suggest some conclusions:

1. There are a coterie of at least 3 ion currents that differ transmurally and which by themselves contribute to a larger contraction in ENDO than EPI.
2. All three of these currents are regulated by increases in A2 in a manner to cause increases in contractility.
3. These same currents achieve this physiologic transmural distribution because of a difference in endogenous A2 levels.
4. The endogenous A2 levels are achieved by secretion into the restricted extracellular space of the T-tubular lumen.
5. The regulation by A2 is due to the higher density of the three regulated channels/transporters in the T-tubular system membranes.
6. Increasing stress within the ventricular wall is likely to increase endogenous secretion of A2 and change the transmural density of these three currents in the T-tubular system membranes, thus increasing contraction. The load-induced increase in A2 within the wall coherently regulates these three currents to help match contractile strength to applied load. Figure 23 is a block diagram from Gao *et al* (2014), suggesting how a feedback system, which includes A2's actions, might match the ventricular wall's contractile strength to a specific load.
7. This group of adaptations suggest that A2 induced remodeling of the ventricle presents an important step between the beat to beat response to changes in load described by Starlings law, and the long term anatomic remodeling required to respond to excessive long term imbalances between load and maximum contractile strength. Thus we hypothesize that the process between these two extremes is A2 induced electrical remodeling.
8. The transmural distribution of A2 is sufficient to explain the transmural gradients in I_{to} , I_P and I_{Ca} . As referenced above (Hilgemann, 2014), alternative explanations exist. These alternative explanations for the transmural gradient in I_{to} must be able to explain the observed actions of A2 which can begin to be seen with a delay of only 30 minutes and must

further include the observation that the transmural differences are removed by detubulation.

9. A surprising result in the study by Gao et al, (2014) is that the K_D for A2 of the AT_1R 's in the T-system is 170 nM. This is almost 2 orders of magnitude lower affinity than the systemic AT_1R s. On the one hand this observation makes it easy to understand how this cardiac regulation can be largely independent of the low systemic A2 concentrations. On the other hand, we have no understanding of how this lower affinity is achieved.

VIII. References

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Figure 1.
Comparison of transient outward current in feline ventricular ENDO and EPI myocytes
(Furukawa *et al*, 1990).

Figure 2.

Measurement of I_p in canine EPI and ENDO myocytes.

A) Method of measurement.

B) I_p in EPI. C) I_p in ENDO. D) Summary of all data including MID (Gao *et al*, 2005).

Figure 3.

A and B) I_{CaL} is larger in ENDO than in EPI canine ventricular myocytes. C) Upon acute exposure to A2 there is a reduction in I_{CaL} . D) Upon prolonged exposure to A2 there is an increase in I_{CaL} (Gao *et al*, 2014).

Figure 4.

A comparison of EPI and ENDO action potentials. A) The action potentials themselves. The magnitude of B) I_{to} , C) shortening and D) the calcium transient (Dong *et al*, 2010).

Figure 5.

A) Magnitude of the I_p as a function of pipette $[Na]$. B) Estimate of I_{inb} in ENDO And EPI as a function of $[Na]_i$ C) I_p as a function of pipette sodium in ENDO and EPI (Gao *et al*, 2005).

Figure 6.

A comparison of the sodium calcium exchanger ($I_{\text{Na/Ca}}$) in canine EPI, MID and ENDO. A) The protocol. B) The protocol executed at multiple $[\text{Ca}^{2+}]_o$. C) The reversal potential dependence on $[\text{Ca}^{2+}]_o$. D) Histogram of results (Wang *et al*, 2010).

Figure 7. Intracellular [Na]⁺I in A) quiescent EPI, MID and ENDO myocytes, B) a MID cell in quiescence and paced at 1Hz and 2Hz, and C) [Na]⁺I in all three regions (Wang *et al*, 2010).

Figure 8.

$[Ca]_i$ measurements in myocytes from EPI and ENDO stimulated at 1 Hz. The measurements are normalized to each cell's resting $[Ca]_i$, which is below the range of calcium sensitivity for Fluo3. The quiescent value was higher in ENDO (76 nM) than in EPI (38 nM). The systolic and diastolic values are also higher in ENDO than EPI. The decay in ENDO is slower (Wang, 2006).

Figure 9.

Differences in I_{to} between canine EPI and ENDO. A) Inactivation curve protocol. B) Inactivation curves in EPI and ENDO. C) Raw data for EPI in recovery from inactivation D) A graph to determine the EPI time constant. E) Raw data for ENDO in recovery from inactivation. F) A graph to determine the ENDO time constant (Yu *et al*, 2000).

Figure 10.

Effect of A2 on the amplitude and voltage dependence of activation of I_{to} in EPI. A) Control currents. B) Currents from EPI myocytes treated with A2. C) Graphs of current amplitudes vs. voltage (Yu *et al*, 2000).

Figure 11.

Effects of A2 on I_{to} in EPI myocytes incubated for at least 2 hours. A) The voltage dependence of inactivation. B) Raw data showing recovery from Inactivation in a two pulse protocol. C) A graph of data to generate the time constant for recovery from inactivation (Yu *et al*, 2000).

Figure 12. Effects of losartan on the amplitude and voltage dependence of I_{to} in ENDO A) Control. B) With losartan. C) Graphs of average raw data as a function of voltage for conditions of A and B (Yu *et al*, 2000).

Figure 13.

Effects of ENDO incubation with losartan. A) The voltage dependence of Inactivation. B) Raw data illustrating rapid recovery during a 2 pulse protocol and C) A graph of raw data to indicate the time constant of recovery from inactivation (Yu *et al*, 2000).

Figure 14. Effects of A2 on EPI and the AT₁R blocker losartan on ENDO. A) EPI control. B) ENDO control. C) EPI + A2. D) ENDO + losartan (Yu *et al.*, 2000).

Figure 15.

I_{to} density transmurally. A) Raw data from 5 regions. B) A graph of peak I_{to} density through the wall. C) Peak amplitude relative to EPI, as a function of voltage. D) The ratio of $I_{to}(EPI)/I_{to}(EPI+A2)$ is the same at each test potential. (Gao *et al*, 2014).

Figure 16.

Estimate of endogenous transmural A2 concentrations. A) Dose response curve on I_{t0} and I_p
 B) I_{t0} density as a function of position in control and after incubation with A2 (5 micromolar for at least 2 hours). C) Percentage of maximal inhibition by A2. D) Estimated Endogenous A2 as a function of position based on A-C (Gao *et al*, 2014).

Figure 17.

Cartoon indicating the differential effects of endogenous versus exogenous A2. Note the lack of uniformity in concentration in the endogenous situation as the A2 is secreted into the Restricted space of the T-system and then diffuses out creating a concentration gradient

Figure 18.

Effects of detubulation on the pump current I_p in EPI and ENDO myocytes. Capacitance in: A) EPI, and B) ENDO. I_p amplitude in control myocyte, detubulated myocyte and T-Tubules in: C) EPI, D) and ENDO. I_p density for the same three preparations in: E) EPI, and F) ENDO (Gao *et al*, 2014).

Figure 19.
Effects of detubulation on the effects of A2 in control and detubulated myocytes A)
Capacitance. B) Amplitude of I_p (Gao *et al*, 2014).

Figure 20.

The AT1R (red) and the Kv4.3 protein (green) are coexpressed in HEK293 cells A) In the absence of A2 they both have membrane surface localization. B) In the presence of A2 both are internalized and colocalized (Doronin *et al*, 2004).

Figure 21.

Western Blots indicating: A) that the alpha subunit protein of I_{to} , Kv4.3 coimmunoprecipitates with the AT1R in canine ENDO and EPI, and B) It has the same molecular weight when directly Immunoprecipitated with the Kv4.3 antibody (Doronin *et al*, 2004).

Figure 22.
Western blot coimmunoprecipitating the $\alpha 1$ subunit of Na/K pump protein after Immunoprecipitation with an antibody to the AT₁R.

Figure 23.

Systems diagram indicating the known and unknown steps in the feedback Loops that regulate electrical and anatomical remodeling (Gao *et al*, 2014)