Closing report

Tamas Banyasz

The role of calmodulin kinase in heart rate adaptation and cardiac arrhythmias

OTKA 101196

Summary

The central goal of the project was to determine how CaMKII modulates the function of cardiac ion channels, and how these modulations influence the frequency response and beat to beat variation of the action potential. In our proposal we planned to involve four ionic currents ($I_{Ca,L}$, I_{Kr}, I_{Ks}, $I_{Na/Ca}$) in the study, but later, because of its high clinical importance we decided to add $I_{Na,L}$.

The following changes were made to the plan during the accomplishment of the project.

1. Cocaine stimulation of CaMKII was replaced by pathologic model: According to the proposal, cocaine was planned to be used as activator of CaMKII to study the consequences of increased CaMKII activity on ionic channels. We decided to change the plan, because we had access to pressure and volume overloaded heart failure rabbit model at UC Davis via our collaborating team at Department of Pharmacology. Since CaMKII is known to be elevated in heart failure it was plausible to change to switch to this pathologic model.
2. Camui measurements were cancelled. Originally, we planned to use Camui to monitor the CaMKII activity in isolated cardiac myocytes. We had two reasons to change the plan, and drop Camui measurements
    a. Use of and established pathologic model with elevated CaMKII activity instead of pharmacological stimulation of CaMKII reduces the importance of CaMKII activity measurements.
    b. The dynamic range of Camui in cell model is less than 50% of the basal activity. When CaMKII is activated, by cardiac agonists (Isoproterenol, Phenylephrine or Angiotensine II) the increase is the signal is less than 40% of the control value with 8-10% standard error of mean [see Erickson et al in Circ Res 109:729-738). This offers only a limited resolution and assessment for activity measurement. Further disadvantage of the use of Camui is the 36 hours long incubation time in the expression phase. During this incubation period isolated cardiomyocytes start to dedifferentiate. When the grant proposal was submitted, these weaknesses were already known, but Camui was still the best tool to confirm increased CaMKII activity following cocaine stimulation. At that time we did not expect that we will have access to a well-established pathologic animal model with increased CaMKII activity. With the use of heart failure model, the value of Camui measurement has significantly reduced in the procejt. Thus, we decided to cancel this part.

Summary of the productivity of the project
Number of research papers published in peer reviewed journals: 20
Number of published papers with our partner team at UC Davis: 5
Cumulative Impact Factor: 62.728
Highest & Lowest Impact Factor: 5.148 & 0.892
Impact factor to FTI ratio: 3.3

Summary of the cooperation with UC Davis
The project has benefited significantly from our cooperation with the Department of Pharmacology at UC Davis. First of all, the heart failure rabbit model was produced in Davit California, thus we had access to this unique pathologic model. The experiments with HF rabbit were carried out in Davis. We published 5 papers together in peer reviewed journals and presented four co-authored lectures at the annual biophysical meetings. Our Debrecen team members spent 18 months in Davis working on the mutual project. To continue this cooperation, we formed a consortium from our two teams (Davis & Debrecen) submitted two NIH grant proposals.

Results of the Research

In this chapter the scientific results are presented.

Impact of heart failure (increased CaMKII activity) on cardiac ion currents

We aimed to perform a comprehensive electrophysiological investigation on the calcium-dependence of the main ion currents shaping AP in rabbit failing ventricular myocytes. APs were recorded and the main cardiac ion currents were measured using AP-clamp Sequential Dissection technique both with and without buffering the cytosolic calcium. AP lengthening happened only at 1 Hz but not at higher pacing rates with preserved calcium cycling in HF, however, when calcium was buffered AP lengthening occurred also at high pacing rates. Late sodium current was significantly increased in HF, which was blunted by calcium buffering. L-type calcium current decreased in HF, but net nifedipine-sensitive inward current was unchanged suggesting the upregulation of NCX. Both rapid and slow delayed rectifier potassium currents were significantly upregulated in HF during the AP, but only when measured without cytosolic calcium buffering. Inward rectifier potassium current was decreased in HF regardless of calcium buffering. Our data indicate that calcium-dependent upregulation of IKr and IKs counterbalance the increased depolarization drive in HF under physiological calcium cycling, especially at higher heart rates. It implies an important compensatory mechanism against cardiac arrhythmias in HF.

Coordination of ionic currents during ß-adrenergic/CaMKII stimulation

1. **Change in the dominance pattern of potassium currents following ß-adrenergic/CaMKII stimulation:** Adrenergic stimuli is known to be mediated via CaMKII pathway and differentially modulates different K^+ channels fine-tuning cardiac action potential (AP) repolarization. We used our innovative AP-clamp sequential dissection technique to directly record the dynamic I_{Ks}, I_{Kr}, and I_{K1} currents during the AP in Guinea pig ventricular myocytes to explore, how the ß-adrenergic/CaMKII modulation changed the contribution of individual potassium currents to the

repolarization. Our data show that isoproterenol treatment facilitates I_{K1} during the AP plateau phase, significantly increases the magnitude of I_{Ks}, but has little effect on I_{Kr}. Consequently, isoproterenol increases the contribution of I_{Ks} but decreases the contribution of I_{Kr} to the total repolarization reserve, leading to a reversal of the dominance of I_{Ks} versus I_{Kr} in repolarizing the AP. Therefore, the dominant K^+ current switches from I_{Kr} under the control condition to I_{Ks} under β-adrenergic/CaMKII stimulation. Such a reversal of dominance pattern has a significant implication for using specific K^+ channel blockers, to treat cardiac arrhythmias.

2. **Asynchronous activation of inward and outward currents by ß-adrenergic/CaMKII stimulation:** Our data indicate that inward currents activate faster than outward currents following ß-adrenergic/CaMKII stimulation. This may explain the temporal differences observed in the development of the isoproterenol induced plateau elevation and action potential shortening. The faster activation of $I_{Ca,L}$ comparing to I_{Ks} is useful when the heart must respond immediately to the demand of increased contractility. However, it may carry serious proarrhythmic risk as well, since the delay in the concomitant activation of K^+ currents increases the propensity of early afterdepolarizations, which are known to be associated with adrenergic activation.
3. **Exploring the impact of ß-adrenergic/CaMKII stimulation on calcium activated chloride current ($I_{Cl(Ca)}$):** Previous observations indicated that $I_{Ca,L}$ plays critical role in in activation of $I_{Cl(Ca)}$. We explored how ß-adrenergic/CaMKII stimulation modulates the relationship of these currents. $I_{Ca,L}$ was facilitated by the β-adrenergic agonist isoproterenol (ISO) applied in a concentration of 10 nM. In another set of experiments direct pharmacological stimulation of $I_{Ca,L}$ was achieved by 20 nM of Bay K8644. As it was confirmed with the recording of calcium transients, both interventions resulted in an increased systolic calcium concentration, although ISO was more potent in this regard. The two drugs acted similarly on action potential configuration, the plateau potential was increased and phase-1 amplitude was reduced by both interventions. $I_{Cl(Ca)}$ was recorded with action potential clamp method as 9-antracene sensitive current. Both inward and outward peaks of $I_{9\text{-}AC}$ were significantly larger after pre-treatment with either 10 nM isoproterenol or 20 nM Bay K8644 as compared to untreated controls. In addition, its profile, its amplitude and the total charge carried by $I_{9\text{-}AC}$ were similar after these pharmacological interventions, irrespective of the way of $I_{Ca,L}$ enhancement. Enhancement of $I_{Ca,L}$ led to a 2-fold increase in both the outward and the inward components of $I_{9\text{-}AC}$. These results support the view that Ca^{2+}-entry through the L-type calcium channels directly controls $I_{Cl(Ca)}$ without involving CaMKII activation.

Pharmacological observations

1. **Tetrodotoxin (TTX) blocks cardiac L-type calcium channels**. TTX is a popular drug to inhibit voltage activated sodium current. Because of its high affinity to sodium channels, it is regarded to be a highly selective tool among electrophysiologists. In cardiac tissues, however, the Kd is higher, thus micromolar concentrations of TTX are required to suppress sodium current effectively. TTX sensitive calcium currents have been identified in rodents but not in big mammals, like dog or men. We tested the TTX sensitivity of canine cardiac L-type calcium channels over the range of micromolar TTX concentration. We found that, TTX can specifically block cardiac $I_{Ca,L}$ with and IC_{50} of 55±2 μM (Hill coefficient is 1.0 ± 0.04). We also observed that the inhibitory potential of TTX on $I_{Ca,L}$ is modulated by pH and redox state. Alkaline or strong oxidant milieu could reduce the inhibitory effect of TTX by 40-60%. These observations led us to two important conclusions:

a. When $I_{Ca,L}$ is recorded in the presence of TTX inhibited sodium currents, the magnitude of calcium current could be underestimated because of the partially blocked calcium channels.
b. When $I_{Na,L}$ is recorded with TTX under action potential clamp protocol, the magnitude of $I_{Na,L}$ could be overestimated because of the contribution of inhibited $I_{Ca,L}$ to $I_{Na,L}$. Therefore, the maximum TTX concentration used in these experiments should not exceed 10 μM.
c. Redox and pH sensitivity of TTX must be taken into consideration when it is used in milieu with changing pH or redox state.

2. **KN-93 inhibits rapid component of the delayed rectifier potassium current (I_{Kr}).** KN-93 is widely used to inhibit CaMKII on the field. However, besides its primary effect, KN-93 has been found to have off-target effects including an open-channel blockade of some voltage-gated potassium channels. We explored the inhibitory effect of KN-93 on the rapid component of delayed rectifier potassium current (I_{Kr}) in freshly isolated mammalian ventricular myocytes. Our data indicate that KN-93 exerts direct inhibitory effect on I_{Kr} that is not mediated via CaMKII. The IC_{50} value for KN93 inhibition of I_{Kr} determined in our experiments (102.57 ± 9.28 nM) is significantly lower than the previously reported IC_{50} value of KN-93 (>300 nM) for inhibiting CaMKII. This off-target effect of KN93 should be taken into account when interpreting the data from using KN93 to investigate the role of CaMKII in cardiac function.

L-type calcium current ($I_{Ca,L}$)

1. **Impact of ß-adrenergic/CaMKII stimulation on the profile of $I_{Ca,L}$:** The isoproterenol induced facilitation of the current was statistically significant after 30 seconds and saturation was reached within 2 minutes. Blockade of β_1-adrenoceptors by 300 nM CGP-20712A reduced the facilitation of $I_{Ca,L}$ but interestingly shortened the build-up of the isoproterenol effect. These data indicated that $I_{Ca,L}$ is modulated by both $ß_1$ & $ß_2$ adrenergic receptors.
2. **Impact of calcium buffering on the profile of $I_{Ca,L}$:** The calcium sensitivity of $I_{Ca,L}$ was studied with action potential clamp method. $I_{Ca,L}$ was recorded as nisoldipine-sensitive current (I_{NISO}). Cytosolic calcium was buffered with two exogenous calcium chelator with low (EGTA) and high (BAPTA) calcium affinity. Using BAPTA-containing pipette solution resulted in a massive prolongation of APs accompanied by a pronounced positive shift in the plateau potential. Phase-1 amplitude was decreased by both BAPTA and EGTA, this effect was greater in the case of BAPTA than EGTA. In contrast to BAPTA, EGTA failed to increase the duration of APs. Similarly, the profile of nisoldipine-sensitive current was strongly different in the presence of the two Ca2+ buffers. BAPTA increased both the amplitude and integral of I_{NISO} significantly, while EGTA had no such effect on these parameters. On the other hand, I_{NISO} displayed a pronounced early inactivation allowing a second inward peak to rise under Ca^{2+} buffer-free conditions in cells possessing a spike and dome type of AP. This second peak disappeared in the presence of the calcium buffers.

Sodium/calcium exchange current (I_{NCX})

The influence of the Na+-Ca2+ exchanger (NCX) on beat-to-beat variability was studied after inhibition of I_{NCX} by SEA0400. This agent is thought to be a selective blocker of NCX, when applied at a sufficiently low concentration of 300 nM. Exposure of the cells to 300 nM SEA0400 for 25 min increased beat-to-beat variability and decreased APD significantly. I_{NCX} is believed to be the main mechanism of Ca^{2+}

extrusion from the intracellular space in cardiac cells, thus playing key role in downregulation of CaMKII. Suppression of I_{NCX} of Ca^{2+} elimination is expected to increase $[Ca^{2+}]_i$ beneath the sarcolemma, which effect was well documented in the literature in rat myocytes. Although no significant increase in $[Ca^{2+}]_i$ could be detected in canine cells on exposure to I_{NCX} inhibitor SEA0400 when measuring $[Ca^{2+}]_i$ in the bulk phase of the cytosol, it is likely that suppression of I_{NCX} results in an elevated $[Ca^{2+}]_i$ in the submembrane compartment of canine myocytes as well. Indeed, selective suppression of I_{NCX} by SEA0400 increased beat-to-beat variability while APD was reduced. Both changes were likely consequences of the elevated submembrane Ca^{2+} concentration.

Rapid component of the delayed rectifier potassium current (I_{Kr})

1. **Impact of ß-adrenergic/CaMKII stimulation on the profile of I_{Kr}:** The effects of β-adrenergic stimulation on I_{Kr} have been controversial in literature. Some reported facilitation, others reported inhibition and we can find publications where ß-adrenergic stimulation had no effect on I_{Kr}. The I_{Kr} profile we recorded is largely consistent with the previous model simulations of the current under the control condition, although some quantitative differences exist. Our data confirm that isoproterenol did not significantly alter I_{Kr} in the concentrations below 30 nM and caused only moderate reduction of I_{Kr} at 30 nM. Our data provide the first experimental measures on the isoproterenol dose–response of I_{Kr} in the presence of cytosolic Ca^{2+} transient; these data can be used to fine-tune the quantitative models.

Slow component of the delayed rectifier potassium current (I_{Ks})

1. **Impact of ß-adrenergic/CaMKII stimulation on the profile of I_{Ks}:** The profile of I_{Ks} displays a small and flat current throughout the AP under the control condition. The I_{Ks} was yero during diastole, built up slowly during the AP phases 1 and 2, reached a peak value at the end of phase 2 (near 0 mV membrane potential), and then declined rapidly during phase 3 in correspondence to AP repolarization. Isoproterenol treatment caused significant changes in I_{Ks} throughout the AP. The magnitude of I_{Ks} was augmented by isoproterenol in a dose-dependent manner, with a slight increase at 3 nM isoproterenol, and a substantial increase at 30 nM isoproterenol. The peak of the current shifted from mid plateau to negative membrane potential values similar to that of I_{Kr}, but the magnitude of I_{Ks} even surpassed that of I_{Kr}. Blockade of $ß_1$-adrenoceptors by 300 nM CGP-20712A prevented the ISO-induced enhancement of I_{Ks}.
2. **Time-dependency of ß-adrenergic stimulation:** I_{Ks} activation developed with a time lag. Statistically significant facilitation of the current required 1 minutes following the isoproterenol application but development of full effect exceeded 5 minutes (delay caused by the dead space of the perfusion system subtracted).

Sodium Current (I_{Na})

1. **Determining the submembrane sodium concentration with electrophysiological method:** Knowing the exact Na^+ concentration in the cytosol is important for the estimation of transmembrane sodium transport during cardiac cycle. Standard fluorescent methods to determine Na^+ concentration report bulk cytosol values and carry big error originating from the optical detection. Electrophysiological methods report are more accurate with less error per se. Thus, we developed a voltage-clamp technique to determine the upper and lower bounds of the

submembrane Na^+ concentration in isolated cardiac myocytes at different pacing rates. Our data showed that at low pacing rate (0.5 Hz) the upper and lower bounds converge at 9 mM, resulting a firm submembrane Na^+ concentration. When stimulation rate was increased to 2 Hz, the lower and upper boundaries were found to be 9 and 11.5 mM respectively. By our best knowledge, these are the first data reporting local Na^+ concentration during electric cycle.

2. **Dynamics of the late Na^+ current during cardiac action potential:** Using our sequential action potential clamp technique we (a) determined the trajectory of late sodium current ($I_{Na,L}$) during action potential and (b) studied the impact of CaMKII on the profile with unbuffered cytosolic calcium.
    a. $I_{Na,L}$ recorded under sAP-clamp displays a strikingly different profile from the small and monotonically decaying current seen under rectangular pulse voltage-clamp; the late Na^+ current is clearly separated from the fast (transient) Na^+ current. $I_{Na,L}$ was a small current at AP phase-1, increased gradually during phase-2 and -3, reached a peak at APD50, and then declined rapidly. Earlier studies reported monotonically declining current profile during the AP. However, all of them used AP waveforms recorded from other cells or reconstructed from model and therefore caused the measured current to deviate from the original current naturally flowing under the cell's own AP. Moreover, the earlier AP-clamp experiments also used ion substitution (replacing Na^+ with Cs^+ or K^+ with TEA) and exogenous Ca2+ buffer which, albeit useful for dissecting the biophysical properties of the channel, also deviate from physiological conditions and introduce some artifacts. The present sAP-clamp study was designed to directly record the $I_{Na,L}$ following under the cell's own AP during excitation–contraction coupling under physiological conditions.
    b. Ca^{2+} was proposed to modulate $I_{Na,L}$ by direct and indirect mechanisms and CaMKII is known to facilitate Na^+ current. In our experiments when BAPTA was used to buffer cytosolic Ca^{2+}, The APD and plateau height were significantly increased, which give rise to substantially increased $I_{Na,L}$ during the AP. We found positive correlation between the triangulation factor of AP and the peak amplitude of $I_{Na,L}$ that further supports the conclusion that $I_{Na,L}$ is more strongly influenced by the voltage profile during the AP rather than by Ca^{2+}. CaMKII inhibition using KN-93 reduced $I_{Na,L}$ magnitude throughout the time course of AP and also in the I–V relationship. Meanwhile, increasing Ca^{2+} load did not further increase $I_{Na,L}$, indicating that the Ca^{2+}-CaMKII modulation of $I_{Na,L}$ is already saturated at the control condition. The voltage dependence of the $I_{Na,L}$ recorded under sAP-clamp did not change under all the conditions tested, seen as no horizontal shift in the instant I–V relationship albeit changes in the amplitude of the current density (vertical shift in I–V). Our data provide, for the first time, a visualization of the impact of Ca^{2+} chelation and CaMKII inhibition on the dynamic profile of $I_{Na,L}$ during the AP.