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Microtubules and angiotensin II receptors contribute to modulation of repolarization induced by ventricular pacing

Nazira Özgen, MD, PhD^{*}, Zhongju Lu, MD, PhD[‡], Gerard J.J. Boink, MSc^{*}, David H. Lau, MD, PhD[#], Iryna N. Shlapakova, MD^{*}, Yevgeniy Bobkov, BS^{*}, Peter Danilo Jr., PhD^{*}, Ira S. Cohen, MD, PhD[‡], and Michael R. Rosen, MD, FHR^{*,†,‡}

^{*}Department of Pharmacology, Columbia University, New York, NY

[†]Department of Pediatrics, Columbia University, New York, NY

[#]Department of Medicine, Columbia University, New York, NY

[‡]Department of Physiology & Biophysics, Stony Brook University[†], Stony Brook, NY

Abstract

Background—Left ventricular pacing (LVP) in canine heart alters ventricular activation leading to reduced transient outward potassium current, I_{to} , loss of the epicardial action potential notch, and T wave vector displacement (TVD). These repolarization changes, referred to as cardiac memory, are initiated by locally increased angiotensin II (AngII) levels. In HEK293 cells in which Kv4.3 and KChIP2, the channel subunits contributing to I_{to} , are overexpressed with the AngII receptor 1 (AT1R), AngII induces a decrease in I_{to} as the result of internalization of a Kv4.3/KChIP2/AT1R macromolecular complex.

Objective—We tested the hypothesis that in canine heart in situ, 2h LVP-induced decreases in membrane KChIP2, AT1R and I_{to} are prevented by blocking subunit trafficking.

Methods—We used standard electrophysiologic, biophysical and biochemical methods to study 4 groups of dogs: 1) Sham, 2) 2h LVP, 3) LVP+colchicine (microtubule disrupting agent) and 4) LVP+losartan (AT1R blocker).

Results—TVD was significantly greater in LVP than Shams and was inhibited by colchicine or losartan. Epicardial biopsies showed significant decreases in membrane KChIP2 and AT1R protein after LVP but not after sham treatment, and these decreases were prevented by colchicine or losartan. Colchicine but not losartan significantly reduced microtubular polymerization. In

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Corresponding author: Michael R. Rosen, M.D., Gustavus A. Pfeiffer Professor of Pharmacology, Professor of Pediatrics, College of Physicians and Surgeons of Columbia University, Department of Pharmacology, 630 West 168 Street, PH 7West-321, New York, NY 10032, Telephone: 212-305-8754, Fax: 212-305-8780 (Pharmacology Dept.).

Gerard J.J. Boink has returned to the Heart Failure Research Center, Academic Medical Center, University of Amsterdam, in the Netherlands to complete his doctorate. Iryna N. Shlapakova is the Operating Room RN at Meadowlands Hospital Medical Center in Secaucus, NJ.

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isolated ventricular myocytes AngII-induced I_{to} reduction and loss of action potential notch were blocked by colchicine.

Conclusions—LVP-induced reduction of KChIP2 in plasma light membranes depends on an AngII-mediated pathway and intact microtubular status. Loss of I_{to} and the action potential notch appear to derive from AngII-initiated trafficking of channel subunits.

Keywords

Ventricular pacing; Angiotensin II; Angiotensin receptor; Ion channel; Microtubule

1. Introduction

Ventricular pacing-induced T wave vector displacement is a manifestation of cardiac memory and results from the altered myocardial stretch that is produced by altered ventricular activation.¹ We have shown that the T wave vector displacement induced by 20 min – 2 hours of left ventricular pacing is prevented by agents which block the transient outward current, I_{to} ,² the angiotensin II receptor 1 (AT1R)^{3, 4} or the L-type Ca channel.⁵ Whereas long term pacing (which results in long-term cardiac memory) induces changes in gene transcription for the pore-forming and accessory subunits of the channel determining I_{to} ,^{4, 6} the mechanism for the T wave vector displacement following short-term pacing (and seen as short-term memory) has been more elusive.⁷ Recently Doronin *et al* demonstrated that the Kv4.3/KChIP2 channel subunits responsible for I_{to} form a macromolecular complex with the AT1R.⁸ When transfected into a cell line, this complex produces a typical I_{to} , which decreases to near 0 following angiotensin II addition to the superfusate.⁸ This results from internalization of the macromolecular complex following angiotensin II binding to the AT1R, and suggests that internalization of the channel complex explains the loss of I_{to} .⁸ These observations have been validated in single ventricular myocytes.⁸

Microtubules are a major component of the cardiac myocyte cytoskeleton, and play a central role in the trafficking of channel subunits to and from the plasma membrane.^{9, 10} Microtubular network disruption induced by treating cells with depolymerizing agents decreases internalization and increases cell surface expression of channel subunits.^{11–13} The result is increased outward potassium current and/or shortened action potential duration in rat ventricular myocytes and/or in cells stably expressing Kv1.5, Kv4.2, Kv2.1 or Kv3.1. Microtubules also are critical to membrane receptor regulation in cardiac myocytes. For example, G protein-coupled receptor desensitization resulting from agonist binding-induced receptor internalization is inhibited by disrupting the microtubular network.^{14, 15}

Whether microtubular-mediated trafficking is responsible for the changes in repolarization that occur soon after onset of ventricular pacing in situ has been hypothesized⁷ but not tested. Therefore, we used a 2-hour pacing protocol that induces cardiac memory¹⁶ to test the hypothesis that left ventricular pacing-induced decreases in KChIP2 and AT1R protein in plasma membranes of the intact canine heart are prevented by the microtubule disrupting agent, colchicine. Sham instrumented animals and those treated with the AT1R blocker, losartan, provided control groups. Because we previously have shown in both a cell line and in cardiac myocytes that KChIP2 and Kv4.3 form a macromolecular complex with the

AT1R in the setting of angiotensin II treatment,⁸ in the present studies we considered only the receptor and KChIP2. Concurrent studies in isolated canine ventricular myocytes were performed to determine whether the pharmacological intervention does in fact impact on I_{to} and the transmembrane action potential.

2. Methods

Experiments were performed using protocols approved by Columbia University's and Stony Brook University's Institutional Animal Care and Use Committees and conform to the Guide for Care and Use of Laboratory Animals (NIH Publication NO. 85-23, revised 1996). All the chemicals, except those specified, are from Sigma-Aldrich, St. Louis, MO, USA.

2.1 Pacing Protocol

The pacing protocol was modified after one previously described.¹⁶ In brief, 2–3 year old adult male mongrel dogs weighing 22–25 kg (Chestnut Grove Kennels, Shippensburg, PA, USA) were anesthetized using propofol (10 mg/kg IV, APP Pharmaceuticals Inc., Schaumburg, IL, USA), intubated, and ventilated with isoflurane (2%, Baxter International, Deerfield, IL, USA). Depth of anesthesia was monitored by a veterinary anesthesia technician throughout all surgical procedures. Systemic arterial blood pressure and a body surface electrocardiogram were continuously monitored intra-operatively. Increases in heart rate and blood pressure greater than 20% of initial values were used to indicate the need for increases in isoflurane levels. In addition, palpebral or corneal reflexes were monitored. Using sterile technique a left thoracotomy was performed and the heart was suspended in a pericardial cradle. Epicardial bipolar pacing electrodes were sewn to the left atrium, and the anterior wall or posterior left ventricular wall.

Figure 1 shows the experimental protocol. The sham group was atrially paced throughout the experiment (n=9). The other groups were left ventricular pacing (anterior wall, n=7; posterior wall, n=5), left ventricular pacing + colchicine (anterior wall, n=5; posterior wall, n=9) and left ventricular pacing + losartan (anterior wall, n=2; posterior wall, n=6). Colchicine was infused as a 1 mg/Kg IV bolus 30 min before left ventricular pacing and infused at 16 µg/kg/min throughout left ventricular pacing. For AT1R blockade, losartan was injected as a 1 mg/Kg IV bolus 30 min before left ventricular pacing and infused at 30 µg/kg/min throughout the remaining protocol. At the start and end of each protocol an epicardial biopsy, 6 mm in diameter and 2 mm deep was taken 1–2 cm away from the left ventricular pacing electrode and quick frozen in liquid nitrogen for biochemical analysis. Switching the pacing site did not have any influence on the outcome of our biochemical studies. Due to the small size of the biopsies, there was insufficient material in any one biopsy to permit all the biochemical studies to be conducted on each sample. Thus, correlations that relate biochemical results with T wave vector displacement include only the electrophysiological results obtained from the dogs that actually provided samples for the appropriate biochemical measurement.

2.2 Preparation of whole cell lysates and extraction of light membrane fractions from biopsies

Whole cell lysates of cardiac biopsies were prepared with a lysate buffer containing 1x phosphate buffer saline, 1% triton X-100, 0.5% NaDoc, 0.1% Tween-20 and protease inhibitors (Roche, Mannheim, Germany). After biopsies were sonicated on ice they were rotated for 15 min at 4°C, and then centrifuged for 10 min at 3000 rpm. The supernatants were used as whole cell lysates.

Preparation of light membrane fractions was modified from previous descriptions.¹⁷ Biopsies were rinsed with cold MT buffer (pH 7.4, 10 mM of Tris-MOPS, 1 mM EGTA, 250 mM sucrose and various protease inhibitors) and subjected to 20 strokes in a glass Dounce homogenizer (10 strokes with loose pestle and 10 strokes with tight pestle, Wheaton, Millville, NJ, USA). Debris which mainly includes connective tissue and non-broken cells was removed by a centrifugation at 2900 rpm for 5 min. The heavy membrane fraction, mainly including organelles, was removed by 10 min centrifugation at 9300 rpm. After additional high speed centrifugation at 53000 rpm for 1h, the pellet was used as the light membrane fraction and the supernatant was considered as the cytosolic fraction. To confirm the quality of the light membrane fractions, all fractions were subjected to Western blot for anti-adenylyl cyclase 6 antibody, which is used as a membrane marker (Figure 2A).¹⁸ We confirmed the specificity of the KChIP2 and AT1R antibodies by Western blot using whole cell lysates of left ventricular free wall epicardium, midmyocardium and endocardium. Consistent with the literature,¹⁹ KChIP2 protein was highly expressed in epicardium and midmyocardium and absent from endocardium. In contrast, AT1R protein (Figure 2B) and message (Supplementary Figure 1) were equivalent transmurally. Because prior research has shown the most prominent left ventricular pacing-induced changes in KChIP2 occur in epicardium,⁶ we focused on epicardial tissues in this study.

2.3 Extraction of total, non-polymerized and polymerized β -tubulin fractions from biopsies

Beta-tubulin fractions were extracted from biopsies using a method modified from Putnam et al.²⁰ Briefly, biopsies were washed in microtubule stabilization buffer (MTSB, 0.1 M piperazine-N,N'-bis (2-ethanesulfonic acid) (Pipes, pH 6.75), 1 mM EGTA, 1 mM MgSO₄, 2M glycerol, and protease inhibitor tablet (Roche, Mannheim, Germany) minced in Kontes Duall tissue grinder glass containing 1 ml of MTSB with 50 strokes (Kimble Kontes, Rochester, NY, USA). The lysates were centrifuged at 2900 rpm for 5 min to remove connective tissue and unbroken cells. The supernatant was centrifuged at 63,000 rpm for 45 min at room temperature and the supernatant was used as the non-polymerized tubulin fraction. After sonication in lysate buffer (25 mM Tris-HCl, pH 7.4, 0.4 M NaCl, 0.5% sodium dodecyl sulfate and protease inhibit tablets) for 30 sec the pellet were incubated on ice for 30 min and used as polymerized tubulin fraction.

2.4 Western Blotting

Western blotting was performed as described previously²¹ using anti-KChIP2 (UC Davis/NIH NeuroMab Facility, Davis, CA, USA), anti-AT1R (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-adenylyl cyclase 6 (Santa Cruz Biotechnology, Santa Cruz,

CA, USA) and anti- β -tubulin (Sigma-Aldrich St. Louis, MO, USA.) antibodies. Each image in each figure represents at least 4 independent experiments.

2.5 Patch clamp recording of calcium-insensitive transient outward potassium current

Myocytes were isolated from canine ventricular epicardium as previously described²² and incubated with AngII (2 μ M) in the presence or absence of colchicine (150 μ M) for 2 hours at room temperature and then stored at 4°C in angiotensin II or angiotensin II + colchicine prior to use (5–8 hours). Using a previously described patch clamp protocol²¹ cells were held at –65 mV, then briefly depolarized (5 ms) to –30 mV to inactivate I_{Na} and then depolarized to voltages from –30 to +50 mV for 300–500 ms. Currents were normalized to cell capacitance.

To record action potentials in these myocytes, the pipette solution contained 115 mM potassium aspartate, 35 mM KOH, 3 mM MgCl₂, 3 mM MgATP, 10 mM HEPES, 11 mM EGTA and 5 mM glucose. The pH of the pipette solution was adjusted to 7.4 with KOH. The external solution contained 3 mM KCl, 140 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES and 10 mM glucose. The pH of the external solution was adjusted to 7.4 with NaOH.

2.6 Statistical analysis

Results are expressed as means $\pm$ S.E.M. Comparisons among group means were made by one way ANOVA. $p < 0.05$ was considered significant.

3.1 Results

The T wave vector displacement induced by left ventricular pacing was significantly reduced by colchicine or losartan

The T wave vector displacement, at the end of 2 hours pacing, was significantly higher in the left ventricular paced group than the sham group (Figure 3). In the colchicine and losartan groups the magnitude of the left ventricular pacing-induced T wave vector displacement was significantly reduced (Figure 3). Infusion of colchicine in the absence of ventricular pacing did not affect the T wave vector (0.091 $\pm$ 0.027 mV for control group (n=6) and 0.088 $\pm$ 0.033 mV for colchicine (n=3), $p > 0.05$). We previously have reported there is no effect of AT1 receptor blockade or ACE inhibition on the T wave vector in the absence of ventricular pacing.³

3.2 Left ventricular pacing resulted in loss of KChIP2 and AT1R from canine ventricular membrane fractions. This was blocked by colchicine or losartan

We previously showed that 3 weeks of ventricular pacing induced a significant decrease in the total expression level of KChIP2 in canine ventricular epicardium whole cell lysates.⁶ In contrast, 2 hours left of ventricular pacing induced no changes in total protein levels or mRNA levels of KChIP2 and AT1R (Figure 4A). This indicates that total KChIP2 and AT1R expression in the cell are constant at 2 hours. However, as shown in Figure 4B and C, KChIP2 and AT1R protein in the light membrane fraction were significantly decreased by left ventricular pacing but not by sham treatment, and these decreases were prevented by

colchicine or losartan ($p < 0.05$). To summarize, the total KChIP2 and AT1R protein in the cell (whole cell lysate) remain unchanged during pacing; both subunits are lost from the cell membrane during pacing; and this loss is prevented by colchicine or losartan.

We also found a significant correlation between the reduction in KChIP2 or AT1R in the light membrane fractions and the increase in T wave vector displacement (Figure 4D). These data strongly suggest that left ventricular pacing-induced loss of the I_{to} channel constituents by trafficking from plasma membrane in vivo plays a significant role in the left ventricular pacing-induced changes in T wave vector displacement.

3.3 Colchicine but not losartan significantly reduces microtubular polymerization

To investigate the mechanism of the colchicine effect on left ventricular pacing-induced T wave vector displacement, we studied microtubule dynamics in the biopsies. The microtubular disrupting effect of colchicine in canine cardiomyocytes has been previously shown by others using immunofluorescence confocal microscopy^{23, 24}. We employed β -tubulin fractionation and Western blot technique to determine the β -tubulin level in each fraction in each group. Figure 5A and B shows that with left ventricular pacing alone (left) or left ventricular pacing + losartan (right) there were no significant changes in total, non-polymerized or polymerized β -tubulin levels. In contrast, with left ventricular pacing + colchicine (center), the total and polymerized β -tubulin were significantly decreased and non-polymerized β -tubulin was significantly increased. Figure 5C shows that the dogs with the greatest reduction in polymerized tubulin and greatest increase in non-polymerized tubulin had the least T wave vector displacement. In contrast, the dogs with the least changes in polymerized and non-polymerized tubulin had the greatest T wave vector displacement. These results suggest that an intact microtubular network is critical for the left ventricular pacing-induced internalization of channel subunits and the induction of T wave vector displacement.

3.4 Colchicine reverses angiotensin II-induced reduction of I_{to} in isolated canine LV epicardial myocytes

We previously showed that 2 hours of left ventricular pacing significantly increase angiotensin II content in canine myocardium¹⁶ and that treating isolated canine ventricular epicardial myocytes with angiotensin II reduces I_{to} .²² The patch clamp experiments in Figure 6 confirm that angiotensin II reduces I_{to} . Importantly, Figure 6 also shows that colchicine alone has no effect on I_{to} , but it completely prevents the effect of angiotensin II to reduce the current. This outcome suggests that following microtubular disruption angiotensin II cannot internalize the I_{to} channel constituents, and the result is maintenance of the current. The impact of this on the action potential is shown in figure 7. As demonstrated by us previously²² angiotensin II significantly reduces the phase 1 notch and prolongs action potential duration. Note that the effects on the notch and on duration are prevented by colchicine.

4. Discussion

The molecular mechanisms of ventricular pacing-induced cardiac memory have been studied extensively.^{1, 4–6, 16} Moreover, cardiac memory provides a useful framework for understanding the evolution of remodeling because the pacing used to induce memory initiates two different yet complimentary processes. While long-term cardiac memory is the result of transcriptional changes of ion channels and Cx43,^{4, 5, 25, 26} short-term memory has been proposed to result from ion channel trafficking initiated by altered myocardial stretch-induced synthesis and release of angiotensin II.^{7,8} In this study, we demonstrated in intact animals that the internalization of I_{to} channel subunits initiated by the binding of angiotensin II to the AT1R previously shown in single cell studies contributes to the induction of short-term cardiac memory and this internalization process uses a microtubule-mediated pathway.

Surface expression of ion channels is regulated by many factors including gene expression, post translational modification, and channel trafficking and insertion into the plasma membrane.⁹ Channels expressed at the cell surface are regularly internalized for recycling or degradation. Interference with recycling and degradation can alter the expression of functional ion channels in plasma membranes, and thereby, alter the electrical activity of cardiomyocytes.

We previously reported that I_{to} is critically involved in the induction of cardiac memory,^{2, 4, 6, 27, 28} and now have studied the molecular mechanism for rapid change following 2 hours of ventricular pacing. In this study, pacing did not alter the total protein level of KChIP2 or the AT1R in whole cell lysates (Figure 4A). Moreover, there is no change in mRNA for KChIP2 or the AT1R (Figure 4A). This excludes the occurrence of pacing-induced transcriptional remodeling contributing to the physiological outcomes at 2 hour. However, the KChIP2 and AT1R protein in the membrane fraction are significantly decreased after left ventricular pacing (Figure 4B and C), and the loss of protein from the membrane correlates with the magnitude of the T wave vector displacement change. That is, those dogs having greater decreases in KChIP2 and AT1R had greater T wave vector displacements (Figure 4D). In addition, the AT1R blocker, losartan, not only inhibited the induction of T wave vector displacement but it also prevented the loss of KChIP2 and AT1R from the membrane fractions. Together these findings strongly support our hypothesis that left ventricular pacing-induced T wave vector displacement is at least in part the result of the reduction of KChIP2 in plasma membranes through an internalization process which is mediated by an angiotensin II-AT1R-pathway.

There are multiple routes via which ion channels might be internalized and eventually recycled or degraded.²⁹ In this study, disruption of the microtubular network by colchicine not only inhibited pacing-induced T wave vector displacement (Figure 3), it also prevented the loss of KChIP2 and AT1R from the membrane fraction (Figures 4B and C), and prevented the effect of angiotensin II to reduce I_{to} or the action potential notch (Figures 6,7). This suggests the microtubular network is important to the pacing-induced ion channel internalization. Although losartan inhibited the induction of T wave vector displacement and also prevented the loss of KChIP2 and AT1R from the membrane fraction, it did not affect microtubular dynamics. These results suggest that colchicine and losartan inhibit pacing-

induced T wave vector displacement by completely different mechanisms: colchicine disrupts the microtubular network, thereby preventing internalization of the Kv4.3/KChIP2/AT1R macromolecular complex, while losartan inhibits the action of angiotensin II by blocking the AT1R. This is consistent with our previous in vitro experiments in which a stable Kv4.3/KChIP2/AT1R macromolecular complex in a cell line or myocyte delivers a robust I_{to} and angiotensin II binding to the AT1R in the macromolecular complex is essential for its internalization.⁸

The results in Figure 5A and B show that exposure to colchicine not only disrupted the microtubular network, but it induced a significant decrease in the total β -tubulin protein level. This finding is consistent with studies showing that in mammalian cells the production of tubulin mRNA is controlled by the concentration of non-polymerized tubulin, via autoregulation of tubulin synthesis.^{30, 31} Disruption of microtubules by colchicine and the concomitant increase in non-polymerized tubulin concentrations result in cessation of tubulin mRNA production. Tubulin mRNA has a half-life of only 2 hours and colchicine-induced microtubulin disruption has been shown to result in a decrease in total tubulin protein levels,³⁰ a phenomenon we observed as well in our study.

In conclusion, our results suggest that left ventricular pacing-induced reduction of KChIP2 in plasma membranes depends on an angiotensin II-AT1R-mediated pathway and intact microtubular status. By implication, loss of I_{to} and the action potential notch in heart in situ appear to derive from angiotensin II -AT1R-induced trafficking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary of abbreviations

AT1R	angiotensin II receptor 1
KChIP2	K ⁺ channel interacting protein 2

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VCG, vectorcardiogram; AP, atrial pacing; AV, atrioventricular; LA, left atrium; LV, left ventricle

Figure 1.

Pacing and drug administration protocol. Atrial pacing was performed for 45 min. During the first 15 min, a reference biopsy was taken 1–2 cm away from the LV electrode. For dogs receiving drugs, colchicine or losartan bolus and infusion were started 30 min before LV pacing began and continued throughout the experiment. ECG and VCG were recorded at the times indicated. At the end of 2 h of pacing, another biopsy was taken 1–2 cm from the LV pacing electrode.

Figure 2.

Panel A: Light membrane fraction (LM), heavy membrane fraction (HM) and cytosolic fraction (Cyto) of biopsies taken before (Ref) and after 2 h of left ventricular pacing (2h) were Western blotted for adenylyl cyclase 6 (AC6) antibody. Note the presence of AC6 signal in the LM fraction but not in the HM or cytosolic fractions. Panel B: Whole cell lysates of epicardial (EPI), midmyocardial (MID) and endocardial (ENDO) tissues from control canine left ventricular free wall, Western blotted for KChIP2 and angiotensin II receptor 1 (AT1R) antibodies. KChIP2 is highly expressed in EPI and MID and absent from ENDO, whereas AT1R is equally expressed throughout the left ventricular free wall.

Figure 3.

Summary data of T wave vector displacement (TVD). The TVD is significantly higher following 2h left ventricular pacing (LVP, n=5) compared to Sham (n=9, $p<0.05$).

Colchicine (LVP + Colch, n=9) or losartan (LVP + Losar, n=6) infusion significantly reduced TVD compared to LVP ($p<0.05$). Because of the variability in T wave vector changes with left ventricular anterior and posterior wall pacing, we include here the analysis of posterior wall pacing only. *, $P<0.05$ vs Sham; +, $P<0.05$ vs LVP; ‡, $P<0.05$ vs Sham.

Figure 4.

Panel A (top): Representative Western blot images showing whole cell lysate expression of KChIP2 and AT1R in reference (Ref) and 2 hours of left ventricular pacing (LVP, 2h) biopsies. Panel A (bottom): Summary data of KChIP2 and AT1R protein level in whole cell lysate (n=6) and mRNA level (n=5). There are no significant changes in protein level or mRNA level after 2 hours of LVP.

Panel B: Representative Western blots of KChIP2 and AT1R in light membrane fractions. A non-specific band (around 80 kDa) from AT1R Western blot was used as a loading control. Panel C: Summary data for all experiments on light membranes (Sham, n=5; LVP, n=9 for KChIP2 and n=6 for AT1R; LVP + Colch, n=6 for KChIP2 and n=4 for AT1R; LVP + Losar, n=4; *, p<0.05 vs Ref).

Panel D: Correlation between the decrease in KChIP2 or AT1R in light membrane fractions and T wave vector displacement (TVD). Because in sham animals the T vector varied by up to 0.15 mV, we excluded all LVP animals that had a T vector displacement of less than 0.15 mV from the analysis in Panel D.

Figure 5.

Panel A: Whole cell lysate, non-polymerized and polymerized tubulin fractions of biopsies taken before (Ref) and after pacing (2h) were Western blotted for β -tubulin antibody. LVP, left ventricular pacing; Colch, colchicine; Losar, losartan. Panel B: Summary data for all experiments (*, $p<0.05$ vs LVP; LVP, $n=5$; LVP+Colch, $n=8$; LVP+Losar, $n=4$).

Panel C: In the presence of colchicine ($n=8$; why aren't all 9 animals included? Because the tubulin fractionation was done only for 8 animals), the magnitude of T wave vector displacement (TVD) is strongly correlated to the decrease in polymerized tubulin and the increase in non-polymerized tubulin.

Figure 6.

Colchicine reverses the reduction of I_{to} induced by angiotensin II (AngII) in isolated canine ventricular myocytes. Top: Experimental traces of I_{to} in cardiomyocytes isolated from ventricular epicardium in the absence (Control) or presence of 2 μ M angiotensin II (AngII), 150 μ M colchicine, or AngII + colchicine (Colch). Bottom: Summary of I_{to} density in the conditions indicated. (**, $P < 0.01$ vs Control, ANOVA).

Figure 7.

Colchicine reverses the action of angiotensin II to reduce the subepicardial action potential (AP) notch and prolong repolarization. Top: Representative AP in Control (left panel), Ang II (2uM, Middle panel) and Ang II with Colchicine (150uM, right panel), respectively. Cells were injected with a 180pA current for 10ms. APs were recorded under steady state conditions in current clamp. Bottom: Averaged AP durations in angiotensin II with or without colchicine. Note a significantly prolonged APD in the presence of angiotensin II which was reversed in the presence of angiotensin II and colchicine ($P < 0.01$ vs. Control, ANOVA). A comparable result was seen for the phase 1 notch: this was 12.0 ± 1.5 mV in control, -11.1 ± 1.4 mV in angiotensin II plus colchicine and 5.5 ± 1.1 mV in angiotensin II ($P < 0.01$ vs. Control and angiotensin II + colchicine).