Angiotensin Receptor Type 1 Forms a Complex with the Transient Outward Potassium Channel Kv4.3 and Regulates Its Gating Properties and Intracellular Localization*

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We report a novel signal transduction complex of the angiotensin receptor type 1. In this complex the angiotensin receptor type 1 associates with the potassium channel α-subunit Kv4.3 and regulates its intracellular distribution and gating properties. Co-localization of Kv4.3 with angiotensin receptor type 1 and fluorescent resonance energy transfer between those two proteins labeled with cyan and yellow-green variants of green fluorescent protein revealed that Kv4.3 and angiotensin receptor type 1 are located in close proximity to each other in the cell. The angiotensin receptor type 1 also co-immunoprecipitates with Kv4.3 from canine ventricle or when co-expressed with Kv4.3 and its β-subunit KChIP2 in human embryonic kidney 293 cells. Treatment of the cells with angiotensin II results in the internalization of Kv4.3 in a complex with the angiotensin receptor type 1. When stimulated with angiotensin II, angiotensin receptors type 1 modulate gating properties of the remaining Kv4.3 channels on the cell surface by shifting their activation voltage threshold to more positive values. We hypothesize that the angiotensin receptor type 1 provides its internalization molecular scaffold to Kv4.3 and in this way regulates the cell surface representation of the ion channel.

Electrophysiological remodeling in hypertrophy and heart failure predisposes the heart to lethal arrhythmias, which account for half of the mortality (1, 2). Experimental evidence derived from large scale clinical trials shows that inhibition of angiotensin II synthesis by inhibitors of angiotensin-converting enzyme or direct blockade of angiotensin receptor type 1 (AT1 receptor) with the antagonist losartan protects the heart from hypertensive complications (3, 4). A substantial reduction in mortality has been attributed to a significant decrease in sudden cardiac deaths possibly because of fewer episodes of complex arrhythmias (5, 6). The positive influence of inhibition of angiotensin-converting enzyme has been linked to bradykinin-mediated effects (7, 8). However, the role of direct blockade of the AT1 receptor by losartan in episodes of sudden cardiac arrhythmias is still debated (9–11). Electrophysiologic remodeling affects the entire spectrum of cardiac ion channels including the transient outward potassium current (Ito) whose density is often decreased in heart failure (2, 12, 13).

The mechanism of Ito down-regulation in heart failure is not completely understood and is likely to have multiple etiologies. In part it can be explained by the inhibitory effects of angiotensin II. Experiments with spontaneously hypertensive rats suggest that the AT1 receptor might be directly involved in the regulation of Ito. In this animal model Ito is inhibited and can be recovered by treatment with the AT1 receptor specific antagonist losartan (14). Experiments with isolated cardiomyocytes show that stimulation of the AT1 receptor results in the inhibition of Ito in myocytes from rat or canine ventricle (15, 16). In large mammals such as dogs or humans with substantial ventricular wall thickness, Ito exhibits a transmural gradient that is vital for normal electrical activity (17, 18). Distortion of the Ito gradient leads to dispersion of repolarization across the ventricular wall, providing a substrate for ventricular arrhythmias (19). In both canine and human ventricle, Ito density is higher in epicardial and midmyocardial than in the endocardial cells (17, 18). The gradient of Ito inversely correlates with the density of angiotensinogen across the ventricular wall whose expression is more prominent in the subendocardial than in either midmyocardium or epicardium regions (20). Evidence exists that cardiomyocytes, Purkinje fibers, and cardiac fibroblasts produce angiotensin II (21–23). Therefore, locally produced angiotensin II could act in a paracrine and/or autocrine manner to regulate Ito. Experiments in vitro show that losartan stimulates Ito in canine cardiomyocytes isolated from endocardium and converts the configuration of the action potential of endocardial myocytes to that found in epicardium (16).

Ito is rapidly activated and inactivated in response to myocyte depolarization and the rapid repolarization phase (phase 1) of the cardiac action potential and gives rise to a notched appearance of the action potential in epicardial myocytes. The absence of a prominent notch in the endocardial action potential is a consequence of the much smaller Ito (18). The molecular correlates of Ito belong to the A-type family of ion channels that include several subfamilies of α-subunits: Shaker (Kv1.4), Shaw (Kv3.4), and Shal (Kv4.1, Kv4.2, and Kv4.3). Proteins of the Shal family, Kv4.2 and Kv4.3, are considered to be the dominant molecular correlates of Ito in cardiomyocytes (24–28). The molecular identity of Ito is species-specific. In ferret and rat hearts the molecular correlate of Ito is both Kv4.2 and Kv4.3 (24, 25), whereas in the human and canine hearts Kv4.3 is the dominant isoform (26, 27). Ion channels of the Shal family,
Kv4.2 and Kv4.3, are regulated by a variety of extracellular factors including inhibition via α1-adrenergic, muscarinic, endothelin, and angiotensin receptors (15, 16, 29–32). Insulin increases I\textsubscript{to} in the cardiac myocytes of diabetic rats (33, 34). A-type current density in myometric muscle cells, presumably mediated by Kv4.3, is dramatically decreased during pregnancy or after treatment with 17β-estradiol (35). Studies of I\textsubscript{to} regulation via α1-adrenergic, muscarinic, endothelin, and angiotensin receptors in isolated cardiomyocytes or in the Xenopus oocyte expression system reveal the important role of protein kinase C activation in I\textsubscript{to} inhibition (15, 29–32).

Investigations of ion channel regulation in the heart are hindered by the difficulties of acute myocyte isolation and cell culture. To conduct more detailed studies of the mechanism of I\textsubscript{to} regulation by angiotensin II, we co-expressed Kv4.3 with the AT1 receptor in HEK 293 cells. Recently, it was shown that ion transport by pore-forming α-subunits of the Kv4.x family depends on the presence of calcium sensing proteins, KChIPs, which form complexes with them and deliver the α-subunits to the cell surface (36, 37). For this reason we co-expressed Kv4.3 with its β-subunit KChIP2. In the studies presented below, we investigate how angiotensin II affects gating properties and the intracellular localization of the Kv4.3 channel.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat angiotensin receptor type 1 with an HA-tag at the N-terminal end (HA-AT1 receptor) in the pcDNA3.1 expression vector was generously provided by Dr. Caron (Duke University Medical Center Durham, NC). Rat cDNAs for Kv4.3 and KChIP2 were kindly provided by Dr. McKinnon (SUNY at Stony Brook, New York). Rabbit polyclonal anti-AT1 receptor (306) antibody, goat polyclonal anti-Kv4.3 (C-17) antibody, and mouse monoclonal anti-HA antibody (clone F7) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against Kv4.3 were purchased from Santa Cruz Biotechnology. Rabbit monoclonal anti-KChIP2 antibody was purchased from Invitrogen. Rabbit monoclonal anti-HA antibody (clone 3F10) covalently coupled to agarose beads was obtained from Roche Applied Science. Mouse monoclonal rhodamine-conjugated anti-HA antibody (clone F7, anti-HA-TRITC) and mouse monoclonal fluorescein isothiocyanate-conjugated anti-V5 antibody (anti-V5-FITC) were purchased from Santa Cruz Biotechnology and Invitrogen, respectively. Prestained molecular weight markers were purchased from New England Biolabs. All other reagents were purchased from Sigma.

**Expression Constructs**—Kv4.3 and KChIP2 were cloned into the pBudCE4.1 expression vector (Invitrogen). This expression vector allows expression of two recombinant proteins in mammalian cells from the EF-1α and cmv-megavirus promoters. Kv4.3 was cloned into the pBudCE4.1 expression vector after the EF-1α promoter between the NotI and BglII restriction sites and expressed as the C terminally V5-tagged derivative (Kv4.3-V5). KChIP2 was cloned into the pBudCE4.1 expression vector between the HindIII and XbaI restriction sites after the cytomegalovirus promoter and expressed as C terminally myc-tagged or untagged protein.

Green fluorescent protein-labeled derivatives of Kv4.3 and the AT1 receptor were obtained by cloning the proteins into the ECFP-C1 and EYFP-N1 expression vectors (Clontech), respectively. Kv4.3 was N-terminal-tagged by a cyan fluorescent variant of green fluorescent protein (ECPF-Kv4.3) after cloning into the ECFP-C1 expression vector at the BglII-XbaI restriction sites. The AT1 receptor, tagged at the C-terminal end with yellow-green fluorescent protein, AT1-EYFP, was obtained after AT1 receptor cloning into the EYFP-N1 expression vector at the HindIII-BamHI restriction sites.

**Cell Culture**—HEK 293 cells were maintained on DMEM supplemented with 5% fetal bovine serum, penicillin (60 μg/ml), streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. For the expression of recombinant proteins, cells were transiently transfected with expression vectors using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol.

**Assay of Kv4.3-dependent Ion Current**—HEK 293 cells on 35-mm plastic dishes were transiently transfected with the pbuCE4.1 expression vector encoding Kv4.3-V5 with (Kv4.3-V5-KChIP2-myc) and without KChIP2-myc. To study the effects of angiotensin II, the cells were co-transfected with Kv4.3-V5-KChIP2-myc and the HA-AT1 receptor. The EGFP-N1 expression vector (Clontech) was used to express the EGFP reporter gene (4 μg of total recombinant DNA, Kv4.3-V5-KChIP2-myc/HA-AT1/EGFP-N1 at a plasmid ratio of 1/2/1). Thirty hours after transfection, cells were serum-starved for 3 h in DMEM in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C and treated with or without 1 μM angiotensin II for 1 h.

Ion currents were recorded using the whole-cell patch clamp technique. Whole-cell patch clamp recording was performed using the Axopatch-1D amplifier coupled to the pCLAMP data acquisition and analysis software package (Axon Instruments, Inc.). Patch clamp electrodes contained 10 mM HEPES, pH 7.2, 50 mM KCl, 80 mM K-aspartate, 1 mM MgCl\textsubscript{2}, 3 mM magnesium-ATP, and 10 mM EGTA. Cells were maintained at 22°C on the microscope stage in a bath solution that contained 10 mM HEPES, pH 7.4, 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl\textsubscript{2}, 1.8 mM CaCl\textsubscript{2}, and 10 mM glucose. In the case of cells treated with angiotensin II, the bath solution contained 1 μM angiotensin II. Current density was assayed by depolarizing with a 400-mV step voltage from a sustained potential of −80 mV to a test potential of +50 mV. Recovery from inactivation was studied using a paired pulse protocol. A 200-ms test pulse from −80 mV to the test potential of +50 mV was followed by a variable recovery interval (10–300 ms) at −80 mV, then by a second test pulse to +50 mV. To study the dependence of I\textsubscript{to} on activation voltage, a current was elicited by a family of depolarizing voltage steps, in 10 mV increments, from a −80-mV holding potential to a maximal +80 mV test voltage. All current traces were processed with the ClampFit software package.

**Immunocytochemistry**—HEK 293 cells were plated on a Lab-Tek II chamber slide (4-well) (Nalge Nunc International), and transiently co-transfected with Kv4.3-V5-KChIP2-myc, and HA-AT1 receptor (0.4 μg of total DNA/well, with 4 μg of V5-KChIP2-myc/HA-AT1 plasmids at a ratio of 1/2). After 30 h, cells were washed in DMEM twice for 5 min, and serum-starved for 3 h in DMEM in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C, and treated with or without 1 μM angiotensin II in DMEM for 1 h. After that cells were fixed in 3.7% formaldehyde in phosphate-buffered saline for 10 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 5 min, blocked with 5% bovine serum albumin in phosphate-buffered saline for 30 min, and incubated for 2 h with anti-HA-TRITC (10 μg/ml) and anti-V5-FITC antibodies (5 μg/ml). After washing three times with phosphate-buffered saline, the nuclei were counterstained with 4,6-diamidino-2-phenylindole (Molecular Probes) according to the manufacturer’s protocol, and chamber slides were mounted on coverslips with VectaShield mounting media (Vector Laboratories).

Immunofluorescence was analyzed by deconvolution microscopy using the AxiosioVision 4.1 imaging software package coupled to an Axiovert 200M inverted fluorescence microscope (Carl Zeiss). Cross-sectional images were obtained with 250-nm Z-stack steps and processed using the AxiosioVision 4.1 constrained iterative algorithm.

**Fluorescent Resonance Energy Transfer (FRET)**—HEK 293 cells were plated on to poly-l-lysine coated 35-mm coverslip-bottom number 1 German glass cell culture dishes (BD Biosciences) and transiently co-transfected with Kv4.3-V5-KChIP2-myc and AT1/EYFP, with total DNA, with ECFP-Kv4.3/pBudCE4.1-KChIP2/AT1-EYFP plasmids in a ratio of 1/1/2). FRET experiments were performed 30 h after transfection. Cells were serum-starved in FRET incubation buffer containing 0.02 μM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2} for 3 h at 37°C, placed in the thermostatic chamber (37°C) of the Axiovert 200M fluorescence microscope, and treated with 1 μM angiotensin II in FRET buffer.

FRET in live cells was monitored using a donor (CFP), acceptor (YFP) and FRET filter set (Carl Zeiss). Acquired FRET images were processed with the AxioVision FRET software package using a donor-acceptor concentration-independent algorithm (38).

**Membrane Preparation from Heart Tissue**—Pieces of tissue from canine left epicardium or endocardium (1–2 g) were homogenized in ice cold 10-fold volume (weight/ml) of 10 mM NaHCO\textsubscript{3}, 10 mM histidine, 1.8 mM CaCl\textsubscript{2}, and 10 mM glucose. In the case of cells treated with angiotensin II, the bath solution contained 1 μM angiotensin II for 1 h.

**Protein Immunoprecipitation and Immunoblotting**—Kv4.3 was immunoprecipitated from the detergent extracts of heart membranes prepared as described above using rabbit polyclonal anti-Kv4.3 antibody. For this, membranes were treated with lysis buffer containing Tris-HCl, pH 7.4, 0.15 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, and a set of protease inhibitors and phosphatase inhibitors (cocktails type 1 and II) for 5 min at 22°C. The extract was cleared by
centrifugation at 15,000 × g and 4 °C for 30 min and used for protein immunoprecipitation. Kv4.3 was immunoprecipitated from 1 ml of lysate (1 mg of total protein) by incubation with 5 μg of primary antibodies for 4 h at 4 °C followed by adsorption of primary antibodies on protein A/G PLUS-agarose (Santa Cruz Biotechnology) at 4 °C overnight. Agarose beads were washed four times with 1 ml of wash buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100. Kv4.3 and the AT1 receptor were detected after the immunoblotting of SDS-PAGE-separated proteins with goat polyclonal anti-Kv4.3 antibody (C-17) and rabbit polyclonal anti-AT1 receptor antibody (306 antibody).

HA-AT1 receptor and Kv4.3-V5 expressed in HEK 293 cells were immunoprecipitated after treatment of the cells with the lysis buffer (described above) for 5 min at 22 °C. The lysate was cleared by centrifugation at 15,000 × g for 30 min at 4 °C and used for the HA-AT1 receptor and Kv4.3-V5 immunoprecipitation with rat monoclonal anti-HA high affinity antibody (clone 3F10) and anti-V5 antibody covalently coupled to protein A/G PLUS-agarose beads. The cleared cell lysate (1 ml, 1 mg of total protein) was incubated with antibodies for 4 h at 4 °C. The agarose beads were washed four times with wash buffer (described above). After protein separation on SDS-PAGE, HA-AT1 receptor and Kv4.3-V5 were detected by immunoblotting with the corresponding anti-HA and anti-V5 antibodies.

RESULTS

Effects of Angiotensin II on Kv4.3 Co-expressed with the Angiotensin Receptor Type 1—To simplify the immunochemical analysis Kv4.3, KChIP2, and the AT1 receptor were tagged with V5-, myc-, and HA-tags, respectively. The HA-tagged AT1 receptor is functional and routinely used to study angiotensin II signal transduction pathways (39, 40). The data presented in Fig. 1 show that labeling Kv4.3 with a V5-tag produces functional ion channels in HEK 293 cells. The co-expression of KChIP2-myc with Kv4.3-V5 resulted in a more rapid recovery from inactivation and translocation of the channel to the cell surface (Fig. 1) as was reported for the wild type proteins (36, 37). Kv4.3 is partially internalized in the absence of KChIP2 (Fig. 1B).

The co-expression of HA-AT1 receptor with Kv4.3-V5 and KChIP2-myc resulted in a 10% decrease in the current density (Fig. 2). The current density was decreased by 90% in cells expressing AT1 receptor and treated with angiotensin II (Fig. 2). The inactivation rate and the rate of recovery from inactivation are unchanged by co-expression with the AT1 receptor and treatment of cells with angiotensin II. Representative recordings are shown in Fig. 3. Treatment with angiotensin II leads to a significant positive shift in the voltage threshold for activation (Fig. 4). The threshold voltage estimated with and without the AT1 receptor co-expression was −35 mV. After treatment with 1 μM angiotensin II, the remaining current shows an activation voltage threshold of −20 mV (Fig. 4, F and G).

Effects of Angiotensin II on Intracellular Localization of Kv4.3—The AT1 receptor was located predominantly on the cell surface where it co-localized with Kv4.3 in the absence of angiotensin II (Fig. 5A). The addition of angiotensin II resulted in the internalization of the AT1 receptor (Fig. 5B). Remarkably, Kv4.3 was also removed from the cell surface by angiotensin II (Fig. 5B). The majority of the internalized AT1 receptor co-localizes with Kv4.3 in intracellular vesicles (Fig. 5B).

The prominent internalization of Kv4.3 correlates with the dramatic reduction in the peak current density in the presence of angiotensin II. The co-localization of the AT1 receptor with Kv4.3 in intracellular compartments suggests that Kv4.3 and the AT1 receptor might be targeted by angiotensin II into the same internalization complexes.

AT1 Receptor Association with Kv4.3—We used resonance energy transfer to test whether Kv4.3 and the AT1 receptor are located in close proximity to each other when co-expressed in HEK 293 cells. Previous investigators have shown that GFP-fused Kv4.3 produces a functional ion channel which is regulated by KChIP1 (41). A similar cloning strategy was used in the present study to produce N terminally tagged ECFP-Kv4.3. C terminally GFP-tagged AT1 receptor yields a functional receptor and is routinely used for receptor internalization assays (42). The data presented in Fig. 6 show a series of time lapse (0–55 min) FRET images of a living HEK 293 cell. The cells were co-transfected with AT1-EYFP, ECFP-Kv4.3, and untagged KChIP2. White and red colors in Fig. 6 represent the highest levels of resonance energy transfer, whereas blue and black correspond to low or absent resonance energy transfer.

The FRET signal is localized along the cytoplasmic membrane prior to the addition of angiotensin II. Cell treatment with angiotensin II leads to a gradual redistribution of the FRET signal from the cell surface into intracellular compartments. After 55 min in the presence of angiotensin II, the majority of the FRET signal is localized inside the cell (Fig. 6).

Data from the resonance energy transfer experiments suggest that the AT1 receptor and Kv4.3 are targeted into the same internalization complexes by angiotensin II and located in close proximity to each other. The expected distance between the AT1 receptor and Kv4.3 is less than 100 Å. This
serves as a model for the analysis of the functional consequences of receptor interactions in the presence of endogenous ligands and for the design of novel therapeutic strategies.

**Fig. 3.** Effects of AT1 receptor expression in HEK 293 cells and cells treatment with angiotensin II on the rates of Kv4.3 inactivation and recovery from inactivation. HEK 293 cells were transiently transfected with Kv4.3-V5-KChIP2-myc with or without co-transfection with HA-AT1 receptor. AT1 receptor-expressing cells were treated with or without 1 μM angiotensin II for 1 h. Expression of AT1 receptor or treatment of cells with angiotensin II does not change the rates of inactivation or recovery from inactivation. A, representative recordings of IF for cells transfected with Kv4.3-V5-KChIP2-myc in the absence of AT1 receptor. B, effects of HA-AT1 receptor co-expression with Kv4.3-V5-KChIP2-myc revealed by representative traces recorded from cells transfected with Kv4.3-V5-KChIP2-myc and HA-AT1 receptor, which were treated with angiotensin II.

**DISCUSSION**

The A-type voltage-gated ion channels of the Shal family, Kv4.2, and Kv4.3, play an important role in cardiac physiology by providing the outward current for repolarization during phase 0 of the cardiac action potential. Data obtained from both animal models of hypertension and randomized clinical trials argue that the beneficial effects of inhibition of the renin-angiotensin system are at least in part attributable to the prevention of episodes of sudden cardiac arrhythmias. The down-regulation of \( I_{K4.3} \), or of Kv4.3 in the myocardium, might be, at least in part, responsible for the arrhythmogenic potential of angiotensin II. Experiments with isolated cardiomyocytes demonstrated that angiotensin II inhibits \( I_{K4.3} \) in rat and canine myocytes (15, 16). As with other \( G_{\alpha} \)-coupled receptors (e.g., \( \alpha_{1D} \)-adrenergic, muscarinic, and endothelin receptors (16, 29–31)), the inhibitory effects of angiotensin II on \( I_{K4.3} \) are mediated by protein kinase C (15) and can be mimicked by the treatment of Xenopus oocytes expressing Kv4.2 or Kv4.3 with phorbol 12-myristate 13-acetate or diacylglycerol (29, 30).

One important difference between protein kinase C-dependent inhibition of \( I_{K4.3} \) in Xenopus oocytes and the angiotensin II-dependent inhibition of \( I_{K4.3} \) in canine cardiomyocytes (16) or HEK 293 cells is the change in the activation voltage threshold of Kv4.3. The activation voltage threshold of Kv4.3 was depolarized from −35 mV to −20 mV without changes in the inac-
tivation rate of the $I_{to}$ (Fig. 4). This means that Kv4.3 channels will open at more positive voltages and thus contribute less to the phase 1 repolarization of the myocyte action potential.

Modulation of the gating properties of Kv4.3 is important, but it is not the dominant mechanism of $I_{to}$ inhibition by angiotensin II. We observed a reduction in $I_{to}$ density by 90%
after treatment of cells with angiotensin II. Analysis of the intracellular distribution of Kv4.3 revealed that after exposure to angiotensin II most Kv4.3 was removed from the cell surface and co-localized with the AT1 receptor. It has been established that the cell surface delivery of Kv4.3/Kv4.2 depends on expression of the calcium sensing proteins KChIPs (36, 37). It was further suggested that a gradient in KChIP2 expression is responsible for the transmural gradient of \( I_{to} \) within the ventricular wall of large mammals (44). The rescue of endocardial \( I_{to} \) with losartan (14, 16) and the data presented above suggest that chronic exposure of cells to angiotensin II results in internalization of Kv4.3. Thus, the cardiac renin-angiotensin system might play a crucial role in establishing the gradient of \( I_{to} \). It is also possible that Kv4.3 internalization is responsible for the \( I_{to} \) inhibition during episodes of hypertension and heart failure.

In HEK 293 cells, internalized Kv4.3 co-localizes with AT1 receptor, suggesting that both the AT1 receptor and Kv4.3 are targeted to the same cellular compartments. To further investigate the mechanism of Kv4.3 internalization, we studied fluorescent resonance energy transfer between CFP-labeled Kv4.3 and the YFP-labeled AT1 receptor. FRET analysis showed that the distance between the AT1 receptor and Kv4.3 was less than 100 Å implying association of the AT1 receptor with Kv4.3 in a macromolecular complex. The co-immunoprecipitation of Kv4.3 with the AT1 receptor demonstrated that the ion channel and the AT1 receptor form a stable complex when co-expressed in HEK 293 cells or in native cardiac tissue. In this respect, the interaction of Kv4.3 with the AT1 receptor is similar to the association

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**Fig. 5.** Kv4.3 trafficking with the agonist activated AT1 receptor into endocytic vesicles. HEK 293 cells were transiently transfected with Kv4.3-V5-KChIP2-myc and the HA-AT1 receptor. The distribution of AT1 receptor and Kv4.3 was visualized after cell fixation with 3.7% formaldehyde using anti-HA-TRITC and anti-V5-FITC antibody before and after treatment with 1 µM angiotensin for 1 h. The distribution of Kv4.3 and AT1 receptor is shown before (A) and after treatment with angiotensin II (B). Nuclei (blue color) were counterstained with 4,6-diamidino-2-phenylindole.

**Fig. 6.** Fluorescent resonance energy transfer in live HEK 293 cells expressing ECFP-Kv4.3 and AT1-EYFP. HEK 293 cells were transiently transfected with ECFP-Kv4.3 (donor), KChIP2 and AT1-EYFP (acceptor) and treated with 1 µM angiotensin. The images show time a lapse series (0–55 min) of color-encoded FRET. White and red colors represent the highest levels of resonance energy transfer, and blue and black correspond to low or absent resonance energy transfer.

**Fig. 7.** Immunoprecipitation (IP) of AT1 receptor by Kv4.3 from detergent extract of cardiac membranes. AT1 receptor was co-immunoprecipitated by Kv4.3 from epicardial and endocardial membranes isolated from a canine left ventricle. AT1 receptor was detected as an 83-kDa band of mature glycosylated receptor (A). Kv4.3 was immunoprecipitated as a 75-kDa band (B). Similar results were obtained in three independent experiments. IB, immunoblot.
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and complex formation with
nalization occurs via the well established mechanism of AT1
ization complexes. It is reasonable to suggest that Kv4.3 inter-
and serves as a molecular scaffold for the assembly of internal-
independent complexes with Kir3 ion channels (45).

The addition of angiotensin does not have a dramatic effect on complex
receptor from cells co-transfected with HA-AT1 receptor and treated
out 1
5KChIP2 with or without co-transfection with HA-

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