Synapse-associated protein-97 (SAP97) is a membrane-associated guanylate kinase scaffolding protein expressed in cardiomyocytes. SAP97 has been shown to associate and modulate voltage-gated potassium (Kv) channel function. In contrast to Kv channels, little information is available on interactions involving SAP97 and inward rectifier potassium (Kir2.x) channels that underlie the classical inward rectifier current, I_{K1}. To investigate the functional effects of silencing SAP97 on I_{K1} in adult rat ventricular myocytes, SAP97 was silenced using an adenoviral short hairpin RNA vector. Western blot analysis showed that SAP97 was silenced by ~85% on day 3 post-infection. Immunostaining showed that Kir2.1 and Kir2.2 co-localize with SAP97. Co-immunoprecipitation (co-IP) results demonstrated that Kir2.x channels associate with SAP97. Voltage clamp experiments showed that silencing SAP97 reduced I_{K1} whole cell density by ~55%. I_{K1} density at -100 mV was -1.45 ± 0.15 pA/picofarads (n = 6) in SAP97-silenced cells as compared with -3.03 ± 0.37 pA/picofarads (n = 5) in control cells. Unitary conductance properties of I_{K1} were unaffected by SAP97 silencing. The major mechanism for the reduction of I_{K1} density appears to be a decrease in Kir2.x channel abundance. Furthermore, SAP97 silencing impaired I_{K1} regulation by β_{1}-adrenergic receptor (β1-AR) stimulation. In control, isoproterenol reduced I_{K1} amplitude by ~75%, an effect that was blunted following SAP97 silencing. Our co-IP data show that β1-AR associates with SAP97 and Kir2.1 and also that Kir2.1 co-IPs with protein kinase A and β1-AR. SAP97 immunolocalizes with protein kinase A and β1-AR in the cardiac myocytes. Our results suggest that in cardiac myocytes SAP97 regulates surface expression of channels underlying I_{K1}, as well as assembling a signaling complex involved in β1-AR regulation of I_{K1}.

In the heart, inward rectifier potassium current (I_{K1}) plays a key role in stabilizing the resting membrane potential, determining the excitation threshold, and initiating the final repolarization phase of the action potential. Inward rectifier potassium channel subfamily 2 members (Kir2.1, Kir2.2, and Kir2.3 (Kir2.x)) are the molecular correlates of I_{K1} in the heart (1, 2). In contrast to other cardiac ion channels, relatively little information is available on protein-protein interactions involving Kir2.x channels that are important for their function. We have shown recently that Kir2.3 is regulated by synapse-associated protein 97 (SAP97) in a heterologous expression system, but it is not known if cardiac Kir2.x channels are regulated in a similar manner in situ (3).

SAP97 belongs to the membrane-associated guanylate kinase family of proteins and is ubiquitously expressed in the heart (4). The membrane-associated guanylate kinase family of proteins are involved in the trafficking and assembly of proteins into macromolecular signaling complexes (5). They have protein-protein interaction domains such as PDZ (PSD-95, disc-large, ZO-1) domains, guanylate kinase homology domains, Src homology domain 3 and domains found on LIN2 and LIN7 (L27). The PDZ domains of SAP97 can bind to the C terminus of ion channels that have the sequence (S/T)X(I/L/V) (where X is any amino acid) as the last three amino acids on the C terminus (5). Leonoudakis et al. (6, 7) reported that SAP97 interacts with the last three residues (SXI) on the C terminus of Kir2.x channels. The functional effects of the potential associations between Kir2.x channels and SAP97 in cardiac myocytes are currently unknown. Furthermore, it is interesting that the SAP97-binding site on the C terminus of Kir2.x channels overlaps a putative protein kinase A (PKA) phosphorylation site (RRXS) at the extreme C terminus of Kir2.x channels. Leonoudakis et al. (7) have shown that if this site is phosphorylated, SAP97 fails to bind to the Kir2.x channel protein. Moreover, SAP97, with its Src homology domain 3 and guanylate kinase domains, can interact with A kinase anchoring protein (AKAP) and help assemble kinases and phosphatases in macromolecular signaling complexes (8). It was recently shown that SAP97 associates and modulates the function and regulation of transient outward potassium current by associating with Kv4.2/Kv4.3 and Ca^{2+}/calmodulin-dependent protein kinase (9).

The abbreviations used are: Kir2.x, inward rectifier potassium channel subfamily 2 (Kir2.1–Kir2.3); ARVM, adult rat ventricular myocytes; shRNA, short hairpin RNA; shSAP97, SAP97 silencing construct; shCTR, control silencing construct; PKA, protein kinase A; β_{1}-AR, β_{1}-adrenergic receptor; CaM, calcium kinase; Ca^{2+}/calmodulin-dependent protein kinase; pF, picofarad; AKAP, A kinase anchoring protein; PDZ, PSD-95, disc-large, ZO-1; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
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Given that SAP97 with its modular structure can assemble macromolecular signaling complexes and that Kir2.x channels are regulated by PKA on $\beta_1$-adrenergic receptor ($\beta_1$-AR) stimulation, we have also investigated the functional effects of silencing SAP97 on $I_{K1}$ in adult rat ventricular myocytes (ARVMs) to test the hypothesis that SAP97 plays a critical role in assembling a macromolecular complex involving Kir2.x channels, $\beta_1$-AR and PKA.

EXPERIMENTAL PROCEDURES

Culturing Adult Rat Ventricular Myocytes—Sprague-Dawley rats (200–250 g) were heparinized and anesthetized (Nembutal, 45 g/kg), and the hearts were removed following thoracotomy. The hearts were cannulated on a Langendorff apparatus and perfused for 5 min with modified Krebs-Henseleit buffer (KHB-A) (in mM) containing NaCl (140), KCl (5.4), HEPES (25), MgSO$_4$ (1.25), glucose (10), CaCl$_2$ (1), pH 7.40, with NaOH. Hearts were perfused with KHB-A until the blood cleared followed by KHB lacking Ca$^{2+}$ (KHB-B) for another 5 min or until the heart stopped beating. The KHB-B solution containing collagenase type II (200 units/ml), hyaluronidase (33.3 units/ml), and blebbistatin (33.3 μM) was then recirculated for 15 min. The enzymatically digested ventricles were isolated, minced, and gently shaken in digestion solution. Undigested ventricular tissue was removed using a 250-μm mesh sieve. The cell suspension from the previous step was centrifuged (800 g), and the Ca$^{2+}$ concentration was increased to 1 mM after resuspending the cell pellet in KHB-A containing 2% bovine serum albumin and blebbistatin (50 μM). The cell suspension was centrifuged again and resuspended in culture media (Medium 119, 45 g/kg) and serum (modified culture media with fetal bovine serum). Rod-shaped ventricular myocytes were counted with a hemocytometer, and ~40,000 cells were plated on each laminin (40 μg/ml)-coated tissue culture dishes/coverslips. Media were changed to modified culture media without fetal bovine serum after 2 h, and cells were cultured for up 4 days.

Adenovirus-mediated Silencing—We used viral transfer technology to knock down the expression of SAP97 in cultured ARVMs. Cells in culture were infected with adenovirus containing short hairpin RNA (shRNA) for SAP97 (shSAP97) (10). The sequences previously described by Nakagawa et al. (11) sense, 5′-GATATCCAGGAGCATAATG-3′, and antisense, 5′-TTTTATGCTTCTGGAT-3′, were used in designing the shRNA-SAP97 hairpin. For negative controls, cells were treated with an alternative adenoviral construct sense, 5′-GAGGTTAGCTGAAGTGAATG-3′, and antisense, 5′-GACCTACTTGCTGACCT-3′, containing a shRNA that has been previously used as a negative control (10). Experiments were conducted at multiplicities of infection of 100, and samples were collected from days 2 to 4 after infection. Silencing of SAP97 expression was confirmed by Western blot analysis on whole cell lysate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control for these experiments.

Preparation of Membrane Protein—All procedures were performed at 2–4 °C. Rat ventricular tissue or isolated cardiomyocyte samples were homogenized with 10 volumes of buffer A containing (in mM) sucrose (320), EDTA (5), EGTA (5), PMSF (1), protease inhibitor mixture (1) (Roche Applied Science), HEPES (20), pH 7.5. The homogenate was then centrifuged at 800 × g for 10 min to remove nuclei and DNA. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. Membrane pellets were then solubilized in HNNS buffer containing (in mM) IGEPAL CA-630 (1%), SDS (0.1%), NaCl (150), HEPES (20), and protease inhibitor mixture (1), pH 7.5. Protein concentrations were determined using a modified Lowry assay (Pierce). Bovine serum albumin of known concentration was used to calculate the standard concentration curve. Ionic detergent compatibility reagent (Pierce) was used to counteract the effect of ionic detergent on the protein assay. All samples were analyzed in triplicate at three different dilutions for accuracy and then averaged to determine the protein concentration. Membrane preparations of known concentration were then used for co-immunoprecipitation experiments (see below) and analyzed by SDS-PAGE and immunoblotting.

Co-immunoprecipitation (Co-IP)—For each co-IP reaction, ~4 μg of total membrane protein was used. Membrane proteins solubilized in HNNS buffer were pre-cleared with the addition of recombinant protein A/G-Sepharose beads (Pierce or Santa Cruz Biotechnology) for 1 h at 4 °C. After a brief spin, the pre-cleared supernatant was incubated with primary antibodies (4–15 μg/reaction) overnight at 4 °C, and protein-antibody complexes were recovered using recombinant protein A/G-Sepharose beads (Pierce or Santa Cruz Biotechnology). Samples were separated by SDS-PAGE and immunoblotted as described below. Primary antibodies for Kir2.1 were from Santa Cruz Biotechnology and/or Antibodies Inc.; Kir2.2 was from Millipore and/or Antibodies Inc.; Kir2.3 was from Alomone; β1AR was from Abcam and/or Santa Cruz Biotechnology; PKA and SAP97 were from Assay Designs Inc. Horseradish peroxydase-conjugated secondary antibodies were purchased from Jackson Immunoresearch. Antibody dilutions were used as specified by the manufacturer.

SDS-PAGE and Immunoblotting—4–20% pre-cast polyacrylamide gels in Tris-glycine buffer were used to separate proteins. Proteins separated on SDS-PAGE were transferred to nitrocellulose (Bio-Rad, 0.45 μm pore size). Nonspecific binding sites were blocked by incubation with 5% nonfat dry milk, in PBS, 0.05% Tween 20 (PBS-T). Membranes were then incubated with specific primary antibodies (0.5–1 μg/ml) diluted in 5% nonfat dry milk overnight. Following four 5-min washes in PBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Antigen complexes were visualized on x-ray film using enhanced chemiluminescence (Pierce). Protein bands were quantified by digital densitometry with a Bio-Rad Fluor-S imager and Quantity One software (Bio-Rad). For experiments in which whole cell lysate was used (Fig. 2A), GAPDH was used as a loading control. Equal protein loading was done for experiments involving membrane preparations.

Immunohisto(cyto)chemistry—Heart tissue was harvested from adult rats, cryoprotected in 30% sucrose/PBS, and frozen in Tissue-TEK OCT compound (Torrance, CA); and 7–10-μm sections were cut on a cryostat. The sections were fixed in 4% paraformaldehyde in PBS (10 min), washed in PBS, and perme-
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abilized with 0.1% Triton X-100 in PBS (5 min). Isolated cells cultured on laminin-coated coverslips were fixed with 4% paraformaldehyde in PBS (5 min), washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS (5 min). After blocking with 5% normal donkey serum in PBS, heart sections or cells were incubated with specific primary antibodies overnight at 4 °C. After washing with PBS-T, donkey secondary antibodies conjugated to DyLight-488 or DyLight-549 (Jackson Immunoresearch) were added for 60 min to heart sections or isolated cells. Samples were then washed in PBS-T and mounted onto slides using ProLong Gold anti-fade mounting kit (Invitrogen). Fluorescence was analyzed, and images were acquired on an Olympus or Zeiss confocal microscope. High resolution images were converted to 8-bit TIFF files, and then ImageJ software was used to investigate co-localization as discussed previously (12). Co-localization was determined by measuring Pearson’s correlation coefficient.

Electrophysiology—Whole cell patch clamp experiments were carried out at 35–37 °C, and cell-attached patch clamp experiments were done at room temperature. The Axopatch 200B (voltage clamp) and Multiclamp 700B (current clamp) amplifiers (MDS Analytical Technologies) were used for patch clamp experiments. 3–4 megohms (whole cell) and 10–11 megohms (cell attached) patch pipettes used in our experiments were pulled from borosilicate glass using a pipette puller (model P97, Sutter Instruments). The bath solution for whole cell recordings contained (mM) NaCl 148, KCl 5.4, MgCl$_2$ 1.0, CaCl$_2$ 1.8, Na$_2$HPO$_4$ 0.4, glucose 5.5, HEPES 15, pH 7.4 (NaOH). For $I_{K1}$ measurement, 5 μM nifedipine was added to block Ca$^{2+}$ current and the Ca$^{2+}$-sensitive chloride current. BaCl$_2$ (1 mM) was used to isolate $I_{K1}$ from other background currents. 1 μM isoproterenol was added to the bath solution to activate β-adrenergic receptors (1, 13). The pipette filling solution contained (mM) KCl 148, MgCl$_2$ 1, EGTA 5, HEPES 5, creatine 2, ATP 5, phosphocreatine 5, pH 7.2 (KOH). The solution for recording single channel unitary events for the bath solution contained (mM) KCl 140, CaCl$_2$ 1.8, HEPES 5, Na$_2$HPO$_4$ 0.33, pH 7.4 (KOH). The pipette filling solution for recording single channel unitary events contained (mM) KCl 140, CaCl$_2$ 1, HEPES 5; pH 7.4 (KOH).

Whole cell $I_{K1}$ was recorded using the step protocol with a holding at −50 mV to inactivate sodium channels and steps from a voltage range of −100 to +20 mV in 10-mV increments with a duration of 500 ms. Nifedipine (5 μM) was used to block the $I_{Ca-L}$ (14). For single channel unitary current recordings, we used a step protocol with a holding potential at 0 mV and stepped to −120 mV to record single channel events in cell-attached configuration. The bath solution contained 140 mM [K$^+$] to maintain the cell at 0 mV (15).

Measurement of Unitary Current Amplitude ($\gamma$), Open Time ($\tau_o$), and Closed Time ($\tau_c$)—The pCLAMP10 suite of programs was used to analyze single channel amplitudes and their open and close duration as a function of voltage (16, 17). Single channel current amplitudes were measured directly from and to base line and were only accepted as valid events if their open durations were more than 15 ms (15). An amplitude histogram was then constructed using 300–400 events (bin size = 0.5) and fitted with a Gaussian function to determine the most frequently occurring single channel conductance. Dwell time histograms were plotted similarly and fitted with first order exponential decay functions to calculate the $\tau_o$ and $\tau_c$.

Statistical Analysis—Statistical comparisons were carried out using Student’s paired and unpaired $t$ test or analysis of variance using the built-in programs in OriginLab or Microsoft Excel software (Northampton, MA). All data are presented as mean ± S.E. of the mean.

RESULTS

SAP97 Co-localizes and Co-immunoprecipitates with Kir2.x Channels in the Myocardium—Unitary conductance measurements of $I_{K1}$ in rat ventricular cells are reminiscent of heterologously expressed Kir2.1 and Kir2.2 channels, (18, 19) suggesting the predominance of these isoforms in this cell type. Therefore, we investigated possible interactions between SAP97 and Kir2.1 and Kir2.2 channel proteins in the myocardium. Immunolocalization was performed in both cardiac tissue and isolated cardiac myocytes, results of which are shown in Fig. 1A. SAP97 localizes at both the T-tubules (Fig. 1A, double arrows; panels c4, f2, i4, and i2) and intercalated discs (asterisks; panels c2 and i2). Kir2.1 co-localized with SAP97 at the intercalated discs (Fig. 1A, asterisk; panel c1) and T-tubules (double arrows; panel c3 and f1), although Kir2.2 and SAP97 co-localized only at the T-tubules (double arrows; panels i3 and i1). A population of nonmyocytes was positive for Kir2.2 consistent with the report of Kir2.2 expression in vascular endothelial cells (20). This population of myocytes is identified by arrowheads in Fig. 1A, panel i. Quantification of co-localization showed significant overlap between the fluorescent staining patterns and subcellular localization of Kir2.1/Kir2.2 and SAP97. The average Pearson’s correlation coefficient was 0.83 ± 0.10 (n = 36) for Kir2.1 and SAP97 and 0.94 ± 0.08 (n = 46) for Kir2.2 and SAP97. These results suggest that SAP97 and Kir2.1/Kir2.2 co-localize to the same discrete subcellular regions.

Co-IP was used to investigate whether Kir2.x channels and SAP97 associate (directly or indirectly) with each other. Immunoprecipitation of SAP97 pulled down Kir2.1 and Kir2.2 (Fig. 1B). Kir2.1 and Kir2.2 failed to co-immunoprecipitate when a nonimmune antibody was used as a negative control. In the immunoblot for detecting Kir2.1, the membrane input has two bands. The lower band in Fig. 1B is a cross-reacting protein that is seen when this antibody is used on cardiac membrane preparations or tissue homogenate. When antibodies recognizing Kir2.1 or Kir2.2 were used for the immunoprecipitation, SAP97 co-immunoprecipitated with Kir2.1 and Kir2.2 but failed to immunoprecipitate with purified rabbit IgG (negative control) (Fig. 1C). The protein band corresponding to SAP97 in the anti-Kir2.2 antibody immunoprecipitation lane is faint; hence a digitally enhanced image (Fig. 1C, #) is shown below the original blot for clarity. Taken together, these results suggest that Kir2.x channels are in a complex with SAP97 in cardiac myocytes.

Silencing SAP97 Expression Decreases $I_{K1}$ Density—We used viral transfer technology to knock down the expression of SAP97 in cultured ARVMs. Cells were infected with adenovirus containing shRNA silencing construct for SAP97 (shSAP97) or a control shRNA that has been previously used as a negative control (shCTR) (10). The efficiency of adenoviral infection in
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cultured myocytes using an adenoviral green fluorescent protein vector at a multiplicity of infection of 5 yielded $\sim 95\%$ infected cells (supplemental Fig. I). For all our experiments, adenoviral shRNA vectors were used at multiplicity of infection of 100. Fig. 2A (left) is a representative Western blot probed for SAP97 (top) and GAPDH (bottom) on whole cell lysates. The right panel of Fig. 2A is a histogram of averaged results from three preparations. In a separate set of experiments, the mRNA levels of SAP97 relative to GAPDH were also measured using quantitative real time PCR.Comparable with our results with relative protein expression levels of SAP97, mRNA were reduced by $\sim 60\%$ as compared with shCTR infected control (data not shown) on day 2. The resting membrane potential of SAP97-infected cells was significantly depolarized as compared with control cells ($p = 0.00004$) as follows: $-69.54 \pm 0.55 \text{ mV}$ ($n = 5$) and $-66.02 \pm 0.64 \text{ mV}$ ($n = 5$) in control and SAP97-silenced cells, respectively. Given that our Western blot data showed that SAP97 expression was silenced by day 3, $I_{K1}$ was recorded on day 3 or 4 in all subsequent experiments. Fig. 2B shows representative traces from uninfected (left, top) and SAP97-silenced (left, bottom) cells. Fig. 2B (right) is the average barium-sensitive $I_{K1}$ density plot from cells in control and following SAP97 silencing. Silencing of SAP97 decreased $I_{K1}$ density by $\sim 55\%$ as compared with controls. The average current density at $-100 \text{ mV}$ (peak inward) and $-60 \text{ mV}$ (peak outward) for SAP97-silenced cells, respectively, were $-1.45 \pm 0.15 \text{ pA/pF}$ and $0.14 \pm 0.03 \text{ pA/pF}$ ($n = 6, n = 2$). These values were significantly reduced as compared with shCTR ($-3.03 \pm 0.37 \text{ pA/pF}$ and $0.27 \pm 0.05 \text{ pA/pF}$ ($n = 8, n = 2$) ($p < 0.0001$). The inset in Fig. 2B is a magnification of the current density-voltage ($I-V$) plot at depolarized voltages ($-80 \text{ to } -40 \text{ mV}$).

To investigate if the decrease in $I_{K1}$ density was due to the silencing of SAP97 and not a result of morphological dedifferentiation and/or changes in cell size and/or down-regulation of ion channels, we investigated these properties in myocytes under our culture conditions in both uninfected and shCTR-infected ARVMs. Our results show that there was no morphological dedifferentiation of ARVM in culture because under our culture conditions ARVMs maintained a rod-shaped morphology for up to 4 days (Fig. 3A) for both uninfected and shCTR-infected cells (21). The cell capacitance recorded from control ($78.15 \pm 5.51 \text{ pF}$ ($n = 16$)) and SAP97-silenced ($78.77 \pm 4.6 \text{ pF}$, $n = 27$) cells was not significantly different ($p =$ not significant) after 3 days in culture. It has been reported previously that $I_{K1}$ density may change in culture (22). To investigate this possibility during 5 days of culture, we recorded $I_{K1}$ density using a step protocol (Fig. 3B, left, top). Representative traces recorded on day 1 and 5 are shown in Fig. 3B, left, middle and bottom panels, respectively. Fig. 3B, right, shows an average barium-sensitive $I_{K1}$ $I-V$ plot. The peak inward current ($-100 \text{ mV}$) on day 1 was $-3 \pm 0.4 \text{ pA/pF}$ ($n = 5, n = 2$) and was $-3 \pm 0.4 \text{ pA/pF}$ ($n = 5, n = 2$) on day 5. The peak outward current ($-60 \text{ mV}$) on day 1 was $0.16 \pm 0.09 \text{ pA/pF}$ ($n = 5, n = 2$) and was $0.25 \pm 0.13 \text{ pA/pF}$ ($n = 5, n = 2$) on day 5. These values were not significantly different ($p =$ not significant). Taken together, the data from Fig. 3 suggest that $I_{K1}$ density is not altered by our culture conditions.

To investigate the possible mechanism(s) for the decrease in whole cell $I_{K1}$ density, we studied the unitary conductance ($\gamma$)
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**Figure 2.** Silencing SAP97 reduces $I_{K1}$ density. A, left panel, an immunoblot demonstrating the effect of shSAP97 adenoviral treatment on SAP97 expression in adult rat ventricular myocytes in culture. GAPDH was used as a loading control. Right panel, histogram of densitometric analysis of SAP97 expression normalized to GAPDH in uninfected, shCTR, and shSAP97-infected cells. Values represent mean results from three experiments harvested on day 2 and day 3 after infection. SAP97 expression was effectively knocked down by ∼80% on day 3. B, representative traces of $I_{K1}$ from uninfected cells (left, top panel) and from shSAP97-infected cells (left, bottom panel) recorded after 3 days in culture. Average barium-sensitive I-V plot (right panel).

**Figure 3.** ARVMs do not show signs of dedifferentiation in culture. A, photomicrograph of uninfected and shCTR-infected ARVMs in culture on day 0–3. Scale bar denotes 20 μm. B, representative traces of $I_{K1}$, measured using the step protocol (left, top panel) on day 1 (left, middle panel) and day 5 (left, bottom panel) in culture. Average barium sensitive $I_{K1}$ density versus voltage (L-V) plot (right panel).

**Figure 4.** Effects of silencing SAP97 on unitary conductance properties of $I_{K1}$ in ARVMs. A, average percentage of events/cell conductance histogram recorded at −120 mV in control (shCTR) and following SAP97 silencing (shSAP97). Inset representative cell-attached patch recording with transitions of ∼44 pS. Solid line represents channels resting state. B, open (left) and closed (right) time histograms of unitary amplitude events.

Properties in cells infected with shCTR or shSAP97 (Fig. 4). Representative cell-attached patch recordings with transitions of ∼44 pS from controls and from SAP97-silenced cells are shown in Fig. 4A (inset). Given conductance variability in each patch, the average number of events at each conductance value was normalized to the total number of events and then plotted against unitary conductance for each condition (shCTR ($n = 338$, $n = 5$) and shSAP97 ($n = 293$, $n = 6$)) (20). Fig. 4A is a histogram of the average unitary conductance events. A wide range of $\gamma$ values was observed, with three prominent peaks between 15 and 20-, 20 and 30-, and 30 and 50-pS conductances. Statistical analysis showed that there was no difference between the two conditions. Note that the larger conductances have a broad distribution. Interestingly, there is a peak at ∼18 pS in shCTR and shSAP97 (although more prominent in control), which is reminiscent of the $\gamma$ of Kir2.3 isoform, and might therefore suggest the presence of this isoform in ARVMs. This issue is further addressed under “Discussion” and in the supplemental material. Fig. 4B depicts single order exponential fits to the mean open ($\tau_o$) (left) and closed ($\tau_c$) times (right) of the unitary events. The values of $\tau_o$ and $\tau_c$ were 32 ± 8.0 and 30.1 ± 5.1 ms for control ($n = 4$ and 2) and 29 ± 2.3 and 22.1 ± 3.1 ms in shSAP97-infected cells ($n = 4$ and 2), respectively. These $\tau_o$ and $\tau_c$ values were not significantly different between shCTR and shSAP97. Further analysis of the $\tau_o$ and $\tau_c$ for
Silencing of SAP97 Reduces Expression of Kir2.x Channels—Given that the unitary conductance properties of $I_{K_1}$ are not affected by silencing SAP97 expression, we investigated whether SAP97 silencing might impact the expression of Kir2.x channels (Fig. 5). Expression of Kir2 channels was assessed by quantitative Western blot analysis (Fig. 5A) and immunostaining of myocytes infected with shCTR or shSAP97 (Fig. 5, B and C). Fig. 5A is an immunoblot of membrane proteins harvested from ARVMs in culture on day 4 after infection with shCTR or shSAP97. A total of 20 μg of membrane protein was loaded in Fig. 5A, 1st and 2nd lanes, although 10 μg of total membrane protein was loaded in the 2nd and 3rd lanes, as indicated. Fig. 5A left, top panel shows that SAP97 expression was reduced on day 4 (left, upper panel, 2nd and 4th lanes) as compared with control (1st and 3rd lanes). Relative protein levels of Kir2.1 and Kir2.2 were also decreased in cells infected with shSAP97 as compared with controls (Fig. 5A, middle and lower panels). Similar results were also observed for Kir2.3 expression (see supplemental Fig. II, panel C). Fig. 5A (right) is a histogram of the average results from three different experiments; protein expression was normalized to control. We also investigated the subcellular localization of Kir2.x channels post SAP97 silencing. A striated pattern (double arrows) was detected for Kir2.1 (Fig. 5B, panels b1 and b2) and Kir2.2 (Fig. 5C, panels c1 and c2) on day 4 in cells infected with shCTR. In contrast, a punctate expression pattern (arrowheads) was apparent for Kir2.1 (Fig. 5B, panels b3 and b4) and Kir2.2 (Fig. 5C, panels c3 and c4) in cells where SAP97 was silenced.

Regulation of $I_{K_1}$ by β1-AR Stimulation Is Lost in SAP97-silenced Myocytes—To test the hypothesis that SAP97 plays a role in β1-AR-mediated regulation of $I_{K_1}$ in cardiac myocytes, we investigated the effects of SAP97 silencing on the response of $I_{K_1}$ to β1-AR stimulation by isoproterenol (Fig. 6). $I_{K_1}$ was inhibited in the presence of isoproterenol (1 μM) (Fig. 6A, left). At −100 mV, isoproterenol decreased $I_{K_1}$ by ~75% (Fig. 6A, right, n = 4). However, this effect of isoproterenol was ablated with SAP97 silencing (Fig. 6B). Representative traces and normalized current values of $I_{K_1}$ at −100 mV from cells infected with shSAP97 are shown in Fig. 6B, left and right, respectively. These results suggest that SAP97 plays an important role in assembling a macromolecular complex involved in the β1-AR-mediated regulation of $I_{K_1}$. In addition to SAP97, key components of such a complex are thought to involve a channel, a kinase, a phosphatase, and adaptor proteins (9, 23, 24). Therefore, we probed myocytes with anti-SAP97 and either anti-PKA (Fig. 6C, left) or anti-β1-AR (Fig. 6C, right) antibodies. A striated pattern reminiscent of T-tubular staining was identified as double arrows in Fig. 6C, panels c1 and c2 and f1 and f2. Pearsons correlation coefficient was significant for co-localization of SAP97 with PKA (0.83 ± 0.04, n = 3) and with β1-AR (0.80 ± 0.03, n = 7), suggesting that SAP97 and PKA/β1-AR are found at the same discrete subcellular locations. We then investigated whether SAP97, Kir2.1, PKA, and β1-AR associate with each other. Co-immunoprecipitation results (Fig. 6D) demonstrate that SAP97 and Kir2.1 immunoprecipitate with β1-AR. Additionally, SAP97, PKA, and β1-AR immunoprecipitated with Kir2.1

**FIGURE 5.** SAP97 affects Kir2 channel cell surface expression in ARVMs. A, left, representative Western blot of membrane preparations from ARVMs infected with shCTR and shSAP97. 20 μg (lanes 1 and 2) or 10 μg (lanes 3 and 4) of total membrane protein were loaded on the gel for samples collected in both conditions. Right, densitometric analysis of Western blot data comparing relative protein levels of Kir2.1 and Kir2.2 in ARVM treated with shCTR or shSAP97 indicates that after 4 days post-infection, SAP97, Kir2.1, and Kir2.2 expression is decreased in cells infected with shSAP97 as compared with control. Results are expressed as protein (Kir2.1 or Kir2.2) expression normalized to control levels. This histogram represents data from three preparations. B and C, confocal micrographs showing mislocalization of Kir2.1 (panel b3) and Kir2.2 (panel c3) channels when SAP97 is silenced in ARVMs as compared with controls (Kir2.1 (panel b1) and Kir2.2 (panel c1)). The areas identified in boxes are shown at higher magnification on the right, Kir2.1 (panels b1 and b3) and Kir2.2 (panels c1 and c3). Double arrows indicate the sarcomeric localization of Kir2.1 (panel b2) and Kir2.2 (panel c2) in shCTR-infected ARVMs. Arrowheads identify the punctate pattern of Kir2.1 (panel b4) and Kir2.2 (panel c4) in ARVMs infected with shSAP97. Scale bar denotes 20 μm.
Importantly, neither SAP97 nor Kir2.1 immunoprecipitated when nonimmune antibodies were used as a negative control. These results therefore suggest that Kir2.1, SAP97, PKA, and β1-AR are all in a macromolecular complex.

**DISCUSSION**

In this study, we have investigated the functional effects of associations between SAP97 and Kir2.x channels in native cardiac cells. We report that silencing SAP97 reduces the whole cell density of IK1 in adult rat ventricular myocytes. We present evidence that the major mechanism by which SAP97 modulates the whole cell current density of IK1 is by reducing the number of Kir2.x channels. Furthermore, silencing SAP97 blunted the β1-AR-mediated regulation of IK1. These results suggest that SAP97 plays a role in modulation of Kir2.x channel surface expression and further suggest that this scaffolding protein is important for assembling a macromolecular signaling complex. To our knowledge, this is the first demonstration that the scaffolding protein SAP97 plays an important role in the regulation of the classical inward rectifier current in the heart.

**SAP97 Regulates the Classical Inward Rectifier Current in the Heart**—Previous studies have demonstrated that Kir2.x channels interact with SAP97 at the extreme C-terminal residues of the Kir2.x channels and that the PDZ2 and/or PDZ3 domain(s) of SAP97 are important for the association (3, 7). Our experiments show that Kir2.x channel proteins and SAP97 co-immunolocalize and co-immunoprecipitate in ARVMs and that the PDZ2 and/or PDZ3 domain(s) of SAP97 are important for the association (3, 7). Our experiments show that Kir2.x channel proteins and SAP97 co-immunolocalize and co-immunoprecipitate in ARVMs and that they interact (directly or indirectly) in native cardiomyocytes, presumably to modify channel function.

To study the functional role of SAP97 in the modulation of IK1, we silenced SAP97 expression in cardiac myocytes. Our results show that there was a significant depolarization of the resting membrane potential in SAP97-silenced cells as
compared with control cells. This change in resting membrane potential is consistent with the reduction of \( I_{K1} \) density (25) after SAP97 silencing. The decrease in the whole cell \( I_{K1} \) density following SAP97 silencing can be explained by one or more of three possible mechanisms: 1) a decrease in the unitary conductance properties of \( I_{K1} \); 2) a decrease in the open probability of the unitary conductance of channels; and/or 3) a decrease in the number of functional channels in the membrane. The unitary conductance properties, i.e. unitary current amplitude, \( \tau_o \) and \( \tau_r \) were not significantly different between control and SAP97-silenced cells. This outcome therefore suggests that these are not the probable mechanisms for the decrease observed in whole cell density of \( I_{K1} \) following SAP97 silencing.

A noteworthy finding was the presence of a peak at \( \sim 18 \) pS in the unitary events histogram, suggesting the expression of Kir2.3 in the myocytes. To date, there have been no studies to identify directly the molecular correlate(s) of \( I_{K1} \), i.e. which isoform(s) of the Kir2.x family is (are) expressed in rat ventricular myocytes. However, a previous study of the unitary \( I_{K1} \) properties in cultured ARVMs reported small conductance values consistent with the presence of the Kir2.3 isoform in the cells (26). Given these findings, Western blot analysis was used in our study to investigate the presence of the Kir2.3 isoform in rat ventricular membrane preparations in a separate set of experiments (supplemental Fig. II, panel A). Ovine right and left ventricular membrane preparations were used as positive controls (1).

Our results show that Kir2.3 co-immunoprecipitates with SAP97 in the rat membrane preparations (supplemental Fig. II, panel B).

Taken together, our results demonstrate that the main mechanism for the decrease in \( I_{K1} \) density following SAP97 silencing is likely a decrease in the number of Kir2.x channels, as illustrated by quantitative Western blot and immunostaining. Our results show an \( \sim 55\% \) decrease in \( I_{K1} \) density, although we report an \( \sim 70\% \) decrease in Kir2.x protein levels. It is noteworthy that we have previously demonstrated (3) that overexpression of SAP97 in a heterologous expression system increases the whole cell current density of Kir2.3; and this was achieved by multiple mechanisms, one of which was an increase in the number of functional channels in the membrane. The other mechanism that was seen in the HEK cell expression system was an increase in the unitary conductance amplitude when SAP97 was overexpressed (3). However, our results in adult rat ventricular myocytes do not show any change in unitary conductance amplitude. Given that Kir2.x channels in the heart inherently have different unitary conductance amplitudes with Kir2.1 (20–35 pS), Kir2.2 (35–55 pS), and Kir2.3 (10–20 pS) (27), we can speculate that the change could have been well masked because of the presence of multiple Kir2.x subfamily members in the native cell system, as compared with the expression of only one isoform (Kir2.3) in the HEK293 cells used in the Vikstrom et al. study (3). Moreover, proteins of the SAP97 family have been reported to regulate the properties of Kv channels by modulating the channel surface expression (9, 28–31). This is in accordance with our results in ARVMs.

The activation of PKA reduces \( I_{K1} \) density in cardiac myocytes because of a change in the channel open probability (32). Terminal residues at the Kir2.x C terminus contain a PKA consensus sequence, RR\( \times \)S that overlaps the PDZ domain binding sequence. It is possible that silencing SAP97 could unmask this site and alter channel phosphorylation levels at steady state. However, our results show no significant change in the open probability of the channel when SAP97 was knocked down.

This argument further strengthens the hypothesis that the major mechanism for the decrease in \( I_{K1} \) density following SAP97 silencing is a change in Kir2.x channel abundance. In addition, \( I_{K1} \) no longer decreased following isoproterenol stimulation in SAP97-silenced cells. The loss of \( I_{K1} \) responsiveness to \( \beta1\)-AR stimulation may reflect the inability of the SAP97-silenced cardiac myocytes to form the appropriate signaling complex to transduce this response. However, we must also...
consider the possibility that the absence of SAP97 blocks phosphorylation of Kir2.x more directly. It has been suggested that the extreme C terminus of Kir2.1 is disordered (33, 34) and that this disordered structure becomes stabilized when the extreme C terminus associates with PDZ domains (35). We can speculate that in the chronic absence of SAP97, this region of the C terminus forms a destabilized structure that is resistant to PKA phosphorylation (36, 37).

It is our hypothesis that SAP97 is critical in the assembly of a macromolecular complex involving Kir2.x channels and β1-AR. Our results show that SAP97 associates with Kir2.x channels PKA and β1-AR in the heart, providing compelling evidence for the role of SAP97 in assembling such a macromolecular complex. This complex is functional, as shown in the present study and by others (13, 24, 38). However, there is always a possibility of complex subpopulations with different combinations of interacting proteins SAP97 and β1-AR, SAP97 and Kir2.1, and SAP97, Kir2.1, and β1-AR.

Based on the results of our study, we propose a model to show the possible interactions of a "channelosome" composed of, at a minimum, SAP97, Kir2.1, and β1AR (Fig. 7). Central to this model is the modular structure of SAP97 with an N-terminal L27 domain that can dimerize with an L27 domain of another SAP97 protein molecule. This dimerization is important for increasing the number of proteins available for protein-protein interactions, which thereby increases clustering of membrane proteins (11). The PDZ domain exhibits preference for proteins with a specific C-terminal motif (S/T)X(V/L/I); and in our model, the PDZ domains interact with both Kir2.x channels and β1-AR. Kir2.x channels can bind either the PDZ2 or PDZ3 domain of SAP97 (7), whereas β1-AR can associate with any one of the three PDZ domains. SAP97 can bind AKAPs with its Src homology domain 3 and/or guanylate kinase domains (8). The C terminus of Kir2.1 can also directly associate with an AKAP (23). Moreover, the AKAP family of proteins binds to phosphatases such as protein phosphate 2B and kinases (i.e., PKA, both protein kinase A-regulatory subunit and protein kinase A-catalytic subunit) and protein kinase C (39). The associations between Kir2.x, β1-AR, and AKAP are possible due to the modular nature of SAP97, which is important in assembling such a macromolecular signaling complex.

**Physiological Implications**—The physiological role of \( I_{K1} \) in the heart would suggest that perturbations altering this current could be arrhythmogenic. In fact, overexpression of Kir2.1 in a transgenic mouse model leads to very stable episodes of ventricular fibrillation (40), and gain of function mutations in Kir2.1 have been identified for short QT syndrome 3 (41) and familial atrial fibrillation (42). Targeted deletion of the gene KCNJ2 underlying Kir2.1 channel protein in the murine model, or a loss of function of the same gene in humans has been shown to have major pathophysiological consequences. One example is the Andersen syndrome (43). Furthermore, these scaffolding proteins and adaptor proteins play an important role in ion channel function. These observations clearly justify the need to better understand how individual components of the Kir2.x macromolecular complex contribute to ion channel function. In conclusion, our results suggest that in cardiac myocytes SAP97 is important for surface expression of channels underlying \( I_{K1} \), as well as for assembling a signaling complex involved in β1-AR regulation of the current.

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**REFERENCES**

1. Dhamoon, A. S., Pandit, S. V., Sarmast, F., Parisian, K. R., Guha, P., Li, Y., Bagwe, S., Taffet, S. M., and Anumonwo, J. M. (2004) Circ. Res. 94, 1332–1339
2. Anumonwo, J. M., and Lopatin, A. N. (2010) J. Mol. Cell. Cardiol. 48, 45–54
3. Vikstrom, K. L., Vaidyanathan, R., Levinsohn, S., O’Connell, R. P., Qian, Y., Crye, M., Mills, J. H., and Anumonwo, J. M. (2009) Am. J. Physiol. Heart Circ. Physiol. 297, H1387–H1397
4. Godreau, D., Vranckx, R., Maguy, A., Rücker-Martin, C., Goyenvalle, C., Abdelhafa, S., Tessier, S., Coutellet, J. P., and Hatem, S. N. (2002) Cardiovasc. Res. 56, 433–442
5. Sheng, M., and Sala, C. (2001) Annu. Rev. Neurosci. 24, 1–29
6. Leonoudakis, D., Conti, L. R., Radeke, C. M., McGuire, L. M., and Vandenberg, C. A. (2004) J. Biol. Chem. 279, 19051–19063
7. Leonoudakis, D., Mailliard, W., Wingerd, K., Clegg, D., and Vandenberg, C. (2001) J. Cell Sci. 114, 987–998
8. Colledge, M., Dean, R. A., Scott, G. K., Langeberg, L. K., Huganir, R. L., and Scott, J. D. (2000) Neuron 27, 107–119
9. El-Haou, S., Balse, E., Neyroud, N., Dilanian, G., Gavillet, B., Abriel, H., Coulombe, A., Jeromin, A., and Hatem, S. N. (2002) Circ. Res. 104, 758–769
10. Oxford, E. M., Musa, H., Maass, K., Coombs, W., Taffet, S. M., and Delmar, M. (2007) Circ. Res. 101, 703–711
11. Nakagawa, T., Futai, K., Lashuel, H. A., Lo, I., Okamoto, K., Walz, T., Hayashi, Y., and Sheng, M. (2004) Neuron 44, 453–467
12. Costes, S. V., Daelemans, D., Cho, E. H., Dobkin, Z., Pavlakis, G., and Lockett, S. (2004) Biophys. J. 86, 4003–1357–1364
13. Koumi, S., Wasserstrom, J. A., and Ten Eick, R. E. (1995) J. Physiol. 486, 647–659
14. Dhamoon, A. S., and Jalife, J. (2005) Heart Rhythm 2, 316–324
15. Muñoz, V., Vaidyanathan, R., Tolkacheva, E. G., Dhamoon, A. S., Taffet, S. M., and Anumonwo, J. M. (2007) Heart Rhythm 4, 487–496
16. Liu, G. X., Zhou, J., Nattel, S., and Koren, G. (2004) J. Physiol. 556, 401–413
17. Luu, T., Gage, P. W., and Tierney, M. L. (2006) J. Biol. Chem. 281, 35699–35708
18. Josephson, I. R. (1988) Mol. Cell. Biochem. 80, 21–26
19. Wahler, G. M. (1992) Am. J. Physiol. 262, C1266–C1272
20. Fang, Y., Schram, G., Romanenko, V. G., Shi, C., Conti, L., Veldkamp, C. A., Davies, P. F., Nattel, S., and Levitan, I. (2005) Am. J. Physiol. Cell Physiol. 289, C1134–C1144
21. Horackova, M., and Byczko, Z. (1997) Exp. Cell Res. 237, 158–175
22. Veldkamp, M. W., de Jonge, B., and van Ginneken, A. C. (1999) Cardiovasc. Res. 42, 424–433
23. Dart, C., and Leyland, M. L. (2001) J. Biol. Chem. 276, 20499–20505
24. Gardner, L. A., Naren, A. P., and Bahouw, S. W. (2007) J. Biol. Chem. 282, 5085–5099
25. Rinne, A., Littwitz, C., Kienitz, M. C., Gmerek, A., Bösche, L. I., Pott, L., and Bender, K. (2006) J. Muscle Res. Cell Motil. 27, 413–421
26. Josephson, I. R., and Brown, A. M. (1986) J. Membr. Biol. 94, 19–35
27. Liu, G. X., Derst, C., Schlüchtörlich, G., Heinen, S., Seebohm, G., Brüggemann, A., Kummer, W., Veh, R. W., Daut, I., and Preisig-Müller, R. (2001) J. Physiol. 532, 115–126
28. Neuringer, R. B., Wischmeyer, E., Döring, F., Veh, R. W., Sheng, M., and Karsch, A. (2000) J. Neurosci. 20, 156–162
29. Jegloff, D. G., Khanna, R., Schlüchtörlich, L. C., and Jones, O. T. (2000) J. Biol. Chem. 275, 1357–1364
30. Tiffany, A. M., Manganas, L. N., Kim, E., Hsueh, Y. P., Sheng, M., and
Trimmer, J. S. (2000) J. Cell Biol. 148, 147–158
31. Abi-Char, J., El-Haou, S., Balse, E., Neyroud, N., Vranckx, R., Coulombe, A., and Hatem, S. N. (2008) Am. J. Physiol. Heart Circ. Physiol. 294, H1851–H1861
32. Koumi, S., Backer, C. L., Arentzen, C. E., and Sato, R. (1995) J. Clin. Invest. 96, 2870–2881
33. Pegan, S., Arrabit, C., Zhou, W., Kwiatkowski, W., Collins, A., Slesinger, P. A., and Choe, S. (2005) Nat. Neurosci. 8, 279–287
34. Tao, X., Avalos, J. L., Chen, J., and MacKinnon, R. (2009) Science 326, 1668–1674
35. Goult, B. T., Rapley, J. D., Dart, C., Kitmitto, A., Grossmann, J. G., Leyland, M. L., and Lian, L. Y. (2007) Biochemistry 46, 14117–14128
36. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) J. Biol. Chem. 276, 41310–41317
37. Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J., and Hall, R. A. (2000) J. Biol. Chem. 275, 38659–38666
38. Koumi, S., Wasserstrom, J. A., and Ten Eick, R. E. (1995) J. Physiol. 486, 661–678
39. Michel, J. J., and Scott, J. D. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 235–257
40. Noujaim, S. F., Pandit, S. V., Berenfeld, O., Vikstrom, K., Cerrone, M., Mironov, S., Zugermayr, M., Lopatin, A. N., and Jalife, J. (2007) J. Physiol. 578, 315–326
41. Priori, S. G., Pandit, S. V., Rivolta, I., Berenfeld, O., Ronchetti, E., Dhamoon, A., Napolitano, C., Anumonwo, J., di Barletta, M. R., Gudapakkam, S., Bosi, G., Stramba-Badiale, M., and Jalife, J. (2005) Circ. Res. 96, 800–807
42. Xia, M., Jin, Q., Bendahhou, S., He, Y., Larroque, M. M., Chen, Y., Zhou, Q., Yang, Y., Liu, Y., Liu, B., Zhu, Q., Zhou, Y., Lin, J., Liang, B., Li, L., Dong, X., Pan, Z., Wang, R., Wan, H., Qiu, W., Xu, W., Eurlings, P., Barhanin, J., and Chen, Y. (2005) Biochem. Biophys. Res. Commun. 332, 1012–1019
43. Plaster, N. M., Tawil, R., Tristani-Firouzi, M., Canin, S., Bendahhou, S., Tsunoda, A., Donaldson, M. R., Iannaccone, S. T., Brunt, E., Barohn, R., Clark, J., Deymeer, F., George, A. L., Jr., Fish, F. A., Hahn, A., Nitu, A., Ozdemir, C., Serdaroglu, P., Subramony, S. H., Wolfe, G., Fu, Y. H., and Ptacek, L. J. (2001) Cell 105, 511–519