The anchoring protein SAP97 retains Kv1.5 channels in the plasma membrane of cardiac myocytes

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Membrane-associated guanylate kinase (MAGUK) proteins are important for the organization of ion channels and receptors at neuronal synapses and tight junctions (10). MAGUKs are expressed in cardiac myocytes, and in particular, the synapse-associated protein 97 (SAP97) is found at the level of intercalated disks in the atrial (18) and ventricular myocardium (32), where it colocalizes with Kv1.5 channels (18, 32). Moreover, cardiac SAP97 and Kv1.5 subunits have been coprecipitated, suggesting a direct interaction between the two proteins (18).

Recently, a strong interaction between SAP97 and Kv1.5 was demonstrated using protein microarrays and quantitative fluorescence polarization to characterize the binding selectivity of PDZ domains (42). However, indirect interactions through the NH2-terminal part of the channel or involving second messengers are also possible (14, 28). Whatever the nature of the interaction between the two proteins, all studies agree that the coexpression of SAP97 and Kv1.5 subunits results in an increased outward potassium current in heterologous systems (14, 17, 18, 32). However, the exact role of SAP97 in the membrane expression of endogenous Kv1.5 channel and cardiac excitability has not been reported.

Important breakthroughs on the regulation of membrane expression of ion channels in the plasma membrane have been obtained with genetically engineered mutant proteins labeled with green fluorescent protein (GFP) and physical chemistry techniques including fluorescence recovery after photobleaching (FRAP) (3, 13, 37). These studies have shown that ion channels are mobile proteins and that channel mobility is regulated by various factors, including protein concentration and lipid-protein and protein-protein interactions (4, 34, 35, 43, 47, 53). For instance, the neuronal MAGUK PSD95 protein immobilizes Kv1.4 channels and prevents their internalization (5, 23, 38). However, nothing is known about the effect of SAP97 on channel mobility. The reduced mobility of channels may favor their recruitment into large protein complexes where they can interact with regulatory partners, as shown for the regulation of inward rectifier K+ channels by the Gq protein in atrial myocytes (7).

We investigated the effect of SAP97 on potassium channel localization and current properties in cardiac myocytes. We examined how the anchoring protein regulates the surface expression of Kv1.5 channels in cardiac myocytes. We used neonatal rat myocytes in culture, which have been demonstrated to be a valuable model for studying the establishment of
Materials and Methods

Myocyte isolation and culture. Animal handling was performed in accordance with guidelines of our institution (BEA INSERM, agreement no. 751308) and was performed by an experienced investigator. Wistar rats were anesthetized by intraperitoneal injection of a mixture of heparin (0.1 ml/100 mg body wt) and pentobarbital sodium (0.15 ml/100 g body wt). For neonatal cardiomyocyte culture, hearts of 1-day-old Wistar rats were rapidly excised with sharp scissors and washed to remove blood and debris in preoxygenated Tyrode solution containing (in mM) 135 NaCl, 4 KCl, 2 MgCl2, 10 HEPES, 1 NaH2PO4, 20 glucose, and 2.5 Na-pyruvate, adjusted to pH 7.4 with NaOH. Hearts were carefully minced and dissociated into single cells by digestion with proteolytic enzymes in Tyrode solution containing 0.1 mg/ml collagenase A (Roche) and 1% bovine serum albumin (BSA). Tissue pieces were subjected to repeated digestion with gentle continuous stirring and aeration with 100% O2 at 37°C for 10 min until complete dissociation. Cells were pelleted from these suspensions by centrifugation at 800 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in growth medium containing 10% fetal bovine serum (FBS) to deactivate proteolytic enzymes. This step was repeated six times, and resuspended pellets were pooled after each digestion. After 1 h of preplating to purify the myocyte population, the cells were counted, adjusted to the desired density (1x10^6/h), and grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in standard conditions (37°C, 5% CO2) (1).

Adult rat atrial myocytes were isolated as previously described (1). Atria were dissected out and chopped into small pieces in Tyrode-bicarbonate solution containing (in mM) 120 NaCl, 5.6 KCL, 0.6 NaH2PO4, 1.1 MgCl2, 11.1 glucose, and 24 NaHCO3, with pH adjusted to 7.6 by bubbling with carbogen (95% O2-5% CO2) and supplemented with 30 mM 2,3-butanedione monoxime (BDM), 10 mM taurine, and 0.5 mM EGTA. Tissue fragments were then transferred into a cell-cir and predigested for 15 min with a mixture of type XXIV protease (20 U/ml; Sigma) and type V collagenase (10 U/ml; Sigma) diluted in calcium-free Krebs solution containing (in mM) 10 HEPES, 35 NaCl, 10 glucose, 134 sucrose, 16 Na2HPO4, 25 NaHCO3, 4.75 KCl, 1.2 KH2PO4, and 1% BSA. The supernatant was discarded, and tissue pieces were then subjected to four or five successive baths of 10 min each with type V collagenase (685 U/ml). Each supernatant was centrifuged to remove the enzyme, and the pellets were resuspended in the calcium-free Tyrode solution used for neonatal cardiomyocytes and supplemented with 10 mM BDM and 1% BSA. At the end of the digestion, resuspended pellets were pooled, centrifuged, and resuspended in 3 ml of Tyrode solution containing 0.2 mM calcium for 5 min. To gently increase the calcium concentration, the same volume of culture medium was added and the cells were incubated for an additional 5 min. After a final centrifugation, the pellet was resuspended in M199 medium (GIBCO) with 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 1% FBS (GIBCO) and 1:1000 insulin-transferrin-sodium selenite (Sigma). Isolated cells were cultured in standard conditions (37°C, 5% CO2) until patch-clamp recordings were made.

Chinese hamster ovarian (CHO) cells were cultured in HAM-F12 medium (GIBCO Nutrient Mixture) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (GIBCO BRL) in standard conditions (37°C, 5% CO2) (17).

Recombinant proteins, adenovirus, and transfection. Human (h) Kv1.5 channel and recombinant (r) SAP97 cDNAs were generated by RT-PCR and inserted into expression vectors pcDNA3 and pRSV2-DSRed, respectively. The GFP sequence was inserted in-frame into hKv1.5 at the BamHI restriction site downstream from the S6 segment sequence to generate an internally tagged channel protein, to maintain the COOH- and NH2-terminal ends free for interactions (17, 18). The cardiac isoform SAP97 containing the I3 domain but not the II domain, tagged or not with the GFP protein, was inserted into pShuttle-CMV, and SAP97 adenovirus was generated by homologous recombination of pShuttle-CMV-SAP97 and pAdEasy-1 in Escherichia coli (21). Myocytes were transduced by a 1-h incubation with a culture medium containing 7.3 x 10^5 infectious particles of GFP-adenovirus or SAP97-GFP adenovirus in 1.8-cm2 LabTek wells (~125,000 cardiomyocytes/well).

Amounts of adenoviruses were used to transfect SAP97 and Kv1.5 subunit in rat neonatal cardiac myocytes or CHO cells. Cells were transfected with 0.5 μg of GFP-Kv1.5 cDNA alone or with 1.5 μg of DsRed-SAP97 cDNA with 3 μl of FuGENE6. For the cardiac myocytes, we used a solution of FuGENE plus OptiMEM (GIBCO). Transfection procedures resulted in ~10 and ~30% of myocytes and CHO cells, respectively, being transfected.

For single-channel recordings, adult rat cardiomyocytes cultured in 35-mm Nunc petri dishes coated with 5 μg/ml laminin (Roche) were incubated for 1 h with GFP alone or SAP97-GFP adenoviruses (5 x 10^7 infectious particles/ml) the day of the isolation and studied between day 2 and day 3.

Immunohistochemistry. Indirect immunofluorescence was performed on cultured neonatal ventricular myocytes fixed with 4% formaldehyde at room temperature for 10 min, washed in phosphate-buffered saline (PBS) containing 5% BSA and 0.2% Triton X-100, and then incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: rabbit polyclonal antibodies directed against the N-cadherin (1:100; Sigma), the connexin43 (Cx43; 1:100; Zymed Laboratories), or the Kv1.5 subunit (1:20; Alomone Labs) and mouse monoclonal antibodies directed against sarcomeric α-actinin (1:200; Sigma) or PSD-95 family (20 μg/ml; Upstate Biotechnology). The following secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit IgG (1:300; Molecular Probes), Alexa Fluor 594 goat anti-rabbit IgG (1:300; Molecular Probes), or Alexa Fluor 594 goat anti-mouse IgG (1:300; Molecular Probes). For control experiments, the primary antibody was omitted.

Western blot analysis. Cultured rat neonatal cardiomyocytes were harvested at 4°C in lysis buffer containing (in mM) 5 Tris/HCl (pH 6.8), 0.1 EDTA, 250 sucrose, and 1× protease inhibitor cocktail (Roche). They were then homogenized with a potter and centrifuged at 3,500 g for 10 min at 4°C. The supernatant was collected and centrifuged at 40,000 g for 60 min at 4°C, and the microsomal pellet, i.e., the total membrane fraction, was resuspended in lysis buffer.

The protein concentration was determined using the Bradford assay (Bio-Rad). Samples were electrophoresed on 10% SDS-PAGE (equal amounts of membrane protein in each lane), transferred to polyvinylidene difluoride membranes, and probed with the following primary antibodies: anti-Kv1.5 (1:100), anti-PSD-95 family (20 μg/ml), and anti-sarco(endo)plasmic reticulum Ca2+ ATPase 2a (SERCA2a) (1:500; provided by Dr. F. Wuytack, University of Leuven, Belgium). The specificity of the anti-PSD95 family antibody to recognize the ~140-kDa SAP97 product has been established previously in both cardiac myocytes and a heterologous system (17, 18). For quantification, experiments were repeated three times.

Confocal microscopy and FRAP experiments. FRAP experiments were performed with a Zeiss LSM 510 META confocal microscope. All experiments were performed at 37°C in the external solution also
used for patch-clamp recordings in the whole cell configuration (see below). A ×63, 1.4 NA oil-immersion objective was used for imaging, with the pinhole aperture set for 1 airy unit. Regions of interest (ROI) were randomly selected from 115 cells transfected with either GFP alone, GFP-Kv1.5, or GFP-Kv1.5/DsRed-SAP97. Pre- and post-bleach images were acquired by laser beam scanning once every 5 s with the 488-nm line of an Argon2 set to 2% transmission. For the photobleaching of ROIs, the laser was set to 100% transmission. The fluorescence intensity was measured in the bleached area as a function of time t, and mean background values were determined outside the ROI and subtracted from all intensity data points before further analysis to provide correction for overall photobleaching. The average fluorescence intensity within bleached ROIs was normalized to prebleach intensity for each time point, and the recovery half time (t1/2) was calculated as t1/2 = ln 2/k (3). To study the effect of SAP97 overexpression on Kv1.5 distribution, we analyzed the fluorescence intensity in regions of cell-cell contact in fixed-cell cultures. Fluorescence signals of Kv1.5 within these ROIs were measured in control and GFP-SAP-transduced cells using ImageJ software.

Current measurements. Whole cell patch-clamp currents were recorded with borosilicate glass pipettes (resistance 1.5-2 MΩ) connected to the input stage of a patch-clamp amplifier (Axoclamp 200B; Axon Instrument). Resistance in series was compensated to obtain the fastest capacity transient current, but the capacitive and leakage currents were not compensated. Currents were filtered at 10 kHz, digitized with a DigiData 1200 (Axon Instrument), and stored on a computer. Data were acquired and analyzed with Acquis-1 software (provided by G. Sadoc, Centre National de la Recherche Scientifique, Gif/Yvette, France).

The cell-attached configuration was used to record single-channel currents. Pipettes with resistances of 10–15 MΩ were filled with the pipette solution and connected to an Axoclamp 200B amplifier (Axon Instrument). Junction potentials were zeroed before formation of the seal, and suction was applied to the patch until the cell-attached configuration was reached (~10 GΩ). Leak currents were partially compensated. Depolarizing 750-ms pulses were applied to the patch from a holding potential of ~80 to 80 mV at a rate of 0.2 Hz. Data were low-pass filtered at 2 kHz and digitized at 15 kHz with a DigiData 1200 (Axon Instrument).

Solutions and drugs. For whole cell recording experiments, cells were bathed in an external solution containing (in mM) 135 NaCl, 4 KCl, 2.5 Na-pyruvate, 1 NaH2PO4, 2 CaCl2, 2 MgCl2, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH. Patch pipettes were filled with an internal solution containing (in mM) 115 K-aspartate, 1 MgCl2, 4 MgATP, 3 MgCl2, 5 EGTA, and 10 HEPES, adjusted to pH 7.2 with KOH. All the experiments were carried out at room temperature. For single-channel recording experiments, the solutions were prepared according to the protocol described elsewhere (52). Cells were bathed in a solution containing (in mM) 135 K-aspartate, 1 MgCl2, 10 HEPES, 1 CaCl2, 0.33 NaH2PO4, and 10 glucose, with pH adjusted to 7.4 with N-methyl-D-glucamine (NMDG)-OH. Recording pipettes were filled with the following solution (in mM): 130 NMDG-aspartate, 5.4 K-aspartate, 1 MgCl2, 10 HEPES, 1 CaCl2, 0.33 NaH2PO4, and 10 glucose, with pH adjusted to 7.4 with NMDG-OH. Cd2+ (200 μM), atropine (200 nM), and E-4031 (5 μM) were added to both bath and pipette solutions to prevent contamination from calcium (Ica2+).

ACh-activated inwardly rectifier (Ikr) currents, respectively. When appropriate, 4-aminopyridine (4-AP; Sigma-Aldrich) was diluted in the perfusion solution (same as the bath) at a final concentration of 100 μM and delivered to the cell.

Statistical analysis. Data were means ± SE. Student’s paired or unpaired t-test and ANOVA were used to determine the significance of differences. P values <0.05 were considered significant.

RESULTS

SAP97 clusters Kv1.5 subunits in the plasma membrane of cardiac myocytes. To examine whether SAP97 can modulate membrane expression of cardiac Kv1.5 α-subunits, we overexpressed the anchoring protein in rat neonatal myocytes using adenoviral vectors. Myocytes were studied between day 3 and day 5 of culture when they began to reestablish contacts, as revealed by the well-organized patterns of N-cadherin and Cx43 staining (Fig. 1). In control myocytes, staining revealed endogenous Kv1.5 subunits as faint stringlike patterns at the membrane periphery and a patchy and diffuse distribution in the rest of the cell (Fig. 2A). In sharp contrast, in myocytes overexpressing GFP-SAP97, endogenous Kv1.5 channels were clustered at the membrane periphery in myocyte contact zones and were not detected elsewhere in the cells (Fig. 2B). Kv1.5 fluorescent signal intensity at cell-cell contacts was compared between control and GFP-SAP97-transduced cells (Fig. 2, C and D). Endogenous Kv1.5 fluorescence intensity in regions of cell-cell contacts in SAP-transduced cells was higher than that in nontransduced cells (n = 39 and 35, respectively; P < 0.001). GFP-SAP97 directly visualized as green fluorescence was also organized in large dense spots at myocyte-myocyte contacts (Fig. 3). Moreover, the red fluorescence of the secondary antibody and the green fluorescence of the GFP-SAP97 overlapped substantially (Pearson’s coefficient = 0.81 ± 0.07; n = 21), indicating that the two proteins colocalized (Fig. 3, right). For control experiments, myocytes were transduced with GFP-recombinant adenovirus: this had no detectable ef-

Fig. 1. Immunohistochemical characterization of cell-cell contacts in cultured rat neonatal cardiomyocytes. Between day 3 and day 5, cultured neonatal myocytes (stained in red with the sarcomeric α-actinin antibody) reestablish adherens junctions and tight contacts as indicated by N-cadherin (A) and connexin43 (Cx43; B) staining in green. The Nomarski image (right) shows that Cx43 is concentrated at myocyte-myocyte contacts. Bars: A, 20 μm; B, 10 μm.
fect on the diffuse distribution of endogenous Kv1.5 channels (data not shown).

To determine whether SAP97 overexpression increased the amount of Kv1.5 subunits, we performed Western blot experiments with total membrane fractions from cardiomyocytes transduced either with SAP97 adenovirus or GFP adenovirus as a control (Fig. 4). Two days after transduction, when myocytes were at ~60% of confluence (i.e., myocytes spread until establishing maximal contacts and covering the entire dish), there was a fourfold increase in the amount of SAP97 but no significant change in the amount of Kv1.5 subunits (n = 3). After 5 days of culture, when myocytes had reached conflu-
ence, the expression level of both SAP97 and Kv1.5 subunits in the membrane protein fraction was increased (Fig. 4). These results indicate that SAP97 overexpression favors the aggregation of Kv1.5 channels in the plasma membrane at myocyte-myocyte contacts.

SAP97 overexpression increases Kv1.5-encoded potassium current in neonatal myocytes. We then examined whether the effects of SAP97 on the abundance of Kv1.5 α-subunit membrane expression were associated with changes in potassium current properties. Currents were recorded on day 3 of culture, when myocytes were at ~60% of confluence; thus some myocytes remained isolated, allowing patch-clamp recording. A 20-ms pulse from −80 to −40 mV was used to inactivate the fast sodium current and was followed by incremental depolarization with 750-ms test pulses from −40 to +80 mV. In both control and infected myocytes, the outward potassium current activated during membrane depolarization was characterized by a transient current component (I_to) and a small sustained current component (I_kur) measured at the end of the 750-ms test pulse. In SAP97-transduced myocytes, the outward potassium current was increased compared with controls, an effect more pronounced on the sustained component than the transient component (at +60 mV: I_to was 4.63 ± 0.42 vs. 3.29 ± 0.51 pA/pF, P = 0.065; I_kur was 5.64 ± 0.57 vs. 3.23 ± 0.43 pA/pF, P < 0.05; n = 7) (Fig. 5A). The increase in I_kur was in the range of potentials at which the current activated, and there was no difference in the activation-voltage relationship parameters between SAP97-transduced myocytes and controls (Fig. 5B). No change in the activation-voltage relationship of I_to was observed (data not shown). A low concentration of 4-AP (100 μM) inhibited the sustained component of both control and SAP97 by 21 ± 3.5% (n = 5) and 35 ± 2.6% (n = 5), respectively (P < 0.01; Fig. 5, C and D), suggesting that the Kv1.5 channel-encoded current was involved in the SAP97 effect on outward current.

To study further the effects of SAP97 on surface expression of the channels, we performed single-channel recordings in adult atrial myocytes, which express Kv1.5 channels at a high level. Figure 6A shows typical recording segments of unitary currents obtained in the cell-attached patch configuration, in a cell transduced with the control GFP vector (GFP alone), during 750-ms test pulses from −80 to 80 mV. The channel activity (top) was blocked by 100 μM 4-AP (bottom) as previously reported by others (6, 52). In SAP97-transduced cells, channel activity (Fig. 6B) was clearly increasing, showing up to five to six unitary equidistant current levels, whereas there were only two to one two unitary current levels in control cells (Fig. 6, C and D). SAP97-increased activity was blocked by 100 μM 4-AP. Mean relative patch currents (9) were computed from 8 different membrane patches for both conditions: they were 0.15 ± 0.07 for GFP alone and 0.67 ± 0.1 for SAP97-transduced myocytes (n = 11; P < 0.001; Fig. 6, C and D). These results indicate that SAP97 overexpression increases the 4-AP-sensitive maintained I_kur component of the outward current.

SAP97 immobilizes Kv1.5 channels in cardiac myocytes. One mechanism by which MAGUK proteins could regulate ion channel expression is by anchoring and immobilizing proteins in the plasma membrane (5). We used the FRAP technique to investigate this possibility for SAP97 in live cardiac myocytes expressing GFP-tagged Kv1.5 subunits. In live myocytes, GFP-Kv1.5 were organized in clusters mostly at the basal membrane of the cell, i.e., that is in contact with the culture dish (cluster sizes varied between 0.2 and 1.5 μm^2, n = 360) (Fig. 7A). We have shown previously that a large outward potassium current can be recorded in transduced myocytes, indicating that most of these GFP-Kv1.5 subunits are functional (1). In FRAP experiments, randomly selected GFP-Kv1.5 clusters in 44 myocytes were photobleached, and fluorescence recovery within the ROI was monitored by laser beam scanning once every 5 s until a steady state was reached (Fig. 7, A–C). Around 90 s after the photobleaching, 70.6 ± 6.1% (n = 23) of the fluorescence had recovered (i.e., mobile fraction, M_f), with a t_1/2 of 45.3 ± 7.6 s (Fig. 7). GFP alone totally recovered within the first 5-s scan-time increment with a t_1/2 of 0.20 ± 0.18 s (data not shown). To examine the effect of SAP97 on Kv1.5 channel mobility, we cotransfected myocytes with GFP-Kv1.5 and DsRed-SAP97 plasmids (we checked that the DsRed-protein that forms aggregates had no effect on Kv1.5 distribution). Under this condition, clusters of channels were mostly localized at the membrane of the cell periphery (Fig. 7B) and tagged channels were poorly mobile, as indicated by the slow and incomplete fluorescence recovery: M_f = 33.2 ± 3.4% and t_1/2 = 70.5 ± 14.9 s (n = 21, P < 0.001; Fig. 7, D and E). The number and size of clusters were not significantly modified by SAP97 overexpression (data not shown). These results indicate that SAP97 reduced by almost threefold the mobility of Kv1.5 subunits in cardiac myocytes.
SAP97 immobilizes Kv1.5 subunits in plaquelike clusters in CHO cells. The mobility of Kv1.5 channels was also studied in CHO cells known to be lacking specialized membrane contact domains. In these cells, SAP97 has been shown to favor Kv1.5 channel organization into plaquelike clusters (17). In CHO cells expressing only the GFP-Kv1.5 channels, the fluorescence was homogeneously distributed throughout the cell body but not in the nucleus (Fig. 8A). There was no clear membrane localization, although a large Kv1.5-encoded current could be recorded in these cells as previously observed (17). Whatever the localization of the ROI, GFP-Kv1.5 subunits were highly mobile, as indicated by the fast (100 s) and almost complete recovery of the fluorescence within the ROI 5 min after photobleaching: \( M_f = 87.4 \pm 6.8\% \) and \( t_{1/2} = 20.6 \pm 3.8\ s \) (\( n = 18; \) Fig. 8). GFP proteins alone showed an almost instantaneous recovery of the fluorescence following photobleaching (data not shown). In CHO cells coexpressing Kv1.5 channels and SAP97, the green fluorescence was organized into dense spots in the plasma membrane (Fig. 8B), resembling the plaquelike clusters described for other channels (24). A few aggresomes were also observed in the cytosol (17, 46). In cotransfected cells, only 47.1 \( \pm \) 4.1\% (\( n = 29 \)) of the fluorescence recovered after photobleaching of plaquelike clusters with a \( t_{1/2} \) of 67.5 \( \pm \) 8.2 s (Fig. 8E). These results provide further evidence that SAP97 anchors Kv1.5 subunits in the plasma membrane.

DISCUSSION

Previous studies have shown that SAP97 and Kv1.5 subunits can interact, directly or indirectly, both in the heart and in heterologous systems (14, 17, 18, 24, 32). Recently, these findings have been strengthened by proteomic studies, which demonstrated a strong interaction between the two proteins (42). In the present study, we provide the first evidence that SAP97 can regulate the surface expression of Kv1.5 potassium channels.
channels of cardiac myocytes. Our study indicates that the retention of Kv1.5 channels in the plasma membrane by an anchoring protein is an important determinant for the density of functional channels and current properties in cardiac myocytes.

In control myocytes after few days of culture, endogenous Kv1.5 channels show a diffuse and patchy distribution, with the greatest density at the bottom of the cell and some staining at the membrane of the cell periphery. In sharp contrast, most Kv1.5 channels in myocytes overexpressing SAP97 are localized at the edge of the cells, where the plasma membrane reestablishes contacts with neighboring cells. SAP97 overexpression is also associated with an increase in both the maintained component of the outward potassium current $I_{Kur}$ and the activity of 4-AP-sensitive voltage-dependent potassium channels (15, 49). Notably, the patch-clamp experiments can be performed only in isolated myocytes with probably few or not well-organized clusters of channels. It is possible that with the increase in channel clustering at the myocyte-myocyte contacts, even more channels are functional and/or their coupling with regulatory partners is improved. It is likely that other Kv channels are involved in the SAP97 stimulatory effects on the cardiac outward current, including Shal type Kv channels (16, 33, 50). However, it is difficult to dissect precisely the molecular determinants of the distinct components of the outward potassium current in cardiac myocytes. Western blot experiments also showed an increase in Kv1.5 subunits in the membrane protein fraction in SAP97-transduced cells; this increase was dependent on the time in culture and was maximal when cultured myocytes reached confluence and formed contacts. However, given the procedure used to extract membrane protein, it is possible that part of the increase in Kv1.5 protein level in total membrane fraction was also due to the accumulation of the channel in the sarcolemma. Together, these findings indicate that increasing the expression of SAP97 promotes the expression of functional Kv1.5 channels in the plasma membrane of cardiac myocytes. This conclusion is in agreement with the well-known anchoring properties of MAGUK proteins reported for several ion channels (24), N-methyl-d-aspartate receptors (NMDA), and adenomatous polyposis coli (APC) proteins (10). MAGUK clusterize proteins at the plasma membrane because of their ability to multimerize and organize proteins into large networks at sites of contact with the cytoskeleton (24, 27, 30, 38). For instance, the cardiac SAP97 isoform containing the I1A domain, which prevents protein oligomerization, failed both to increase Kv1.5 subunit-encoded current and to organize channels into plaque-like clusters (17). In control myocytes, only part of endogenous Kv1.5 channels are localized at the level of cell-cell contacts despite the presence of endogenous SAP97. This observation suggests that the other actors involved in the normal channel targeting, such as cytoskeleton or microtubules, are altered in cultured myocytes as the result of the lack of mechanical coupling, two-dimensional environment and that myocyte-myocyte contacts in vitro do not fully reproduce the ultrastructure of a normal myocardial intercalated disk.

In live myocytes, GFP-tagged Kv1.5 channels were also organized into clusters of large size. Clustering appears to be a general feature of ion channels and has been observed for calcium channels in H9C2 cells (22), Kv2.1 channels in dendrites of spinal motoneurons (31), and also for Kv1.5 channels in atrial myocardium (12). In human embryonic kidney (HEK-293) cells and cultured hippocampal neurons, clusters of Kv2.1 channels are well-delineated membrane domains to which channels are delivered via trafficking vesicles and trapped by several retention proteins within the perimeter of the clusters (34, 44). Hence, the larger cluster size of GFP-Kv1.5 channels compared with that of endogenous channels may be due to an excessive accumulation at the sites of delivery of newly synthesized subunits in transduced myocytes (34). The discrepancy of the size of clusters between control and transduced myocytes could also be due to the difference between immunostaining and direct visualization in live myocytes of GFP.

Fig. 6. Increase in the number of Kv1.5 channels in myocytes overexpressing SAP97. Sample recordings are shown from cell-attached membrane patches of adult rat atrial myocytes during 750-ms pulses from $-80$ to $+80$ mV. Upward channel openings of a GFP-transduced myocyte (A, top) and following application (to the cell) of 4-AP (same patch; A, bottom) and of a SAP97-transduced myocyte before (B, top) and after 4-AP application (same patch; B, bottom). Arrows indicate the closed state. C and D: amplitude histograms determined from the corresponding current traces indicated. C, closed state; L1–L6, levels corresponding to the successive measurable current levels that are the indication of the number of active channels present in the membrane patch. Gaussian fitted curves corresponding to each current level are also shown.
tagged channels. Our FRAP experiments unraveled the dynamic behavior of Kv1.5 channels in cardiac myocytes. After photobleaching, bleached clusters recovered within 5 min very close to their initial intensity and appearance. Moreover, since clusters constituted isolated fluorescent areas, the recovery of fluorescence and restitution of their original shape and size could not account for lateral diffusion from surrounding fluorescent regions. Therefore, these observations suggest the recruitment of newly inserted channels, rather than the lateral diffusion of GFP-proteins from unbleached neighboring membrane areas or from adjacent clusters. These data are in good agreement with previous studies using single-particle tracking of cell surface channels showing that Kv2.1 channels move directly into clusters via trafficking vesicles (35, 44). Interestingly, SAP97 overexpression reduced the mobility of GFP-Kv1.5 subunits in both cardiac myocytes and CHO cells. In CHO cells, Kv1.5 subunits are diffusely distributed and freely mobile throughout the cell body probably because of the lack of specific membrane domains for Kv channel targeting. In these cells, the marked effect of SAP97 on the clustering and the mobility of GFP-Kv1.5, which is associated with the increase in the corresponding potassium current (17, 18), is further strong evidence of the crucial role played by SAP97 for the anchoring and the retention of Kv channels in the plasma membrane. The neuronal MAGUK protein PSD95 also reduces the mobility of GFP-Kv1.4 channels expressed in HEK-293 cells (5), probably through the interaction of MAGUK with elements of the cytoskeleton (26, 38). MAGUK proteins are probably important actors for the retention of channels in distinct domains of the plasma membrane, as described for the retention of Kv4.2 channels by SAP97 in dendritic spines of hippocampal neurons (16) or for the effect of PSD95 protein on Kv1.4 channels (23). It has been already shown that Kv channels can be mobile in cardiac myocytes and that their mobility differs between atrial and ventricular myocytes and depends on the Kv isoforms (36). These findings suggest that there are various distinct anchoring and targeting mechanisms for channels in the plasma membrane of cardiac myocytes. Further studies are now required to establish the relation between the mobility and the functional properties of Kv channels.

Although it is not possible to determine whether endogenous Kv1.5 channels are also mobile, several arguments indicate that the membrane expression of Kv1.5 channels is a dynamic...
process in cardiac myocytes. For instance, the inhibition of the dynein motor that regulates retrograde trafficking is associated with a relatively fast increase in the outward Kv1.5-encoded current in cardiac myocytes; this is consistent with continuous trafficking of newly formed channels (8).

The reduced mobility of Kv subunits by anchoring proteins might be an important determinant of the proper assemblage of multiprotein complexes and the normal activation and regulation of potassium currents. Indeed, a relationship between the mobility and the functional properties of proteins has been shown for phosphatidylinositol 4,5-bisphosphate, the immobilization of which results in the modulation of the Kir channel by Gq proteins in atrial myocytes (7). In neurons, the concentration and the immobilization of Kv channels at the synapse is crucial to control the amplitude and time course of depolarization at synaptic sites (31). In neurons, distinct membrane pools of channels or receptors differ by their mobility, and this phenomenon appears to play an important role in synaptic transmission by facilitating the replacement and recruitment of channels in this specialized domain in response to various stimuli (11, 31, 45).

In cardiac myocytes, we found that SAP97 regulates the formation of channel complexes by retaining and anchoring Kv subunits in the plasma membrane. This regulatory mechanism may contribute to the targeting of newly addressed channels to their appropriate membrane microdomains such as intercalated disks. This is also suggested by our observation that Kv1.5 channels accumulate at the adherens junction of myocytes overexpressing SAP97. Proteins associated with the cytoskeleton or cell junctions, such as MAGUK, are involved in the precise delivery of ion channels. For instance, in cardiac myocytes, Cx43 is directly targeted to cell-cell junctions through microtubules and dynein-dynactin complexes that tether microtubules to adherens junctions (41). During atrial fibrillation or dilation, the density of \( I_{\text{Kur}} \) has been reported to be unaltered or slightly decreased (25, 48, 51) despite the reduction in Kv1.5 channel content of the atrial myocardium (48). It might be that mechanisms regulating channel targeting and retention at the plasma membrane, including that involving SAP97, compensate for the downregulation of Kv1.5 channels. The disorganization of channels, such as that associated with connexins observed during myocardial remodeling, may also involve alterations of mechanisms that anchor channels to cytoskeleton (2, 39). Clearly, a better understanding of how channels are targeted and retained in the specialized domains of the plasma membrane should open a new field of research in the pathophysiology of cardiac diseases and, notably, arrhythmias.

Fig. 8. SAP97 aggregates GFP-Kv1.5 \( \alpha \)-subunits in plaquelike clusters and reduces their mobility in Chinese hamster ovary (CHO) cells. A: GFP-Kv1.5 channels showed a diffuse distribution and were highly mobile. B: in CHO cells coexpressing GFP-Kv1.5 channels and SAP97, the channels were organized in plaquelike clusters at the membrane and were poorly mobile. C: kinetics of fluorescence recovery of GFP-Kv1.5 clusters in CHO cells transfected either with Kv1.5 plasmid or with both Kv1.5 and SAP97 plasmids. The fluorescence recovery intensity was normalized to the prebleach fluorescence intensity. Data are means of at least 18–29 experiments. Bar graphs showing the percentage of \( M_t \) (D) and the recovery half time (E) in CHO cells expressing GFP-Kv1.5 alone or GFP-Kv1.5/SAP97. Bars, 5 \( \mu \)m. *** \( P < 0.001 \).
SAP97 PROTEIN ANCHORS CARDIAC POTASSIUM Kv1.5 CHANNELS

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