α1-adrenoceptors regulate only the caveolae-located subpopulation of cardiac Kv4 channels

Aintzane Alday, Janire Urrutia, Mónica Gallego and Oscar Casis*

Department of Physiology; School of Pharmacy; University of the Basque Country; Bilbao, Spain

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Introduction

The transient outward potassium current, I\textsubscript{to}, modulates the duration of the plateau phase of the cardiac action potential and therefore determines the amount of calcium that enters the myocyte. Thus, changes in the amplitude and/or kinetics of I\textsubscript{to} current have serious effects on both the strength and duration of cardiac contraction, as well as on the refractory period. We have previously described that the cardiac I\textsubscript{to} current is regulated by α1-adrenoceptors (α1-AR) through a mechanism involving a G\textsubscript{αs} protein, the subsequent activation of adenylate cyclase (AC) and finally the PKA dependent phosphorylation of the I\textsubscript{to} channel.\textsuperscript{1} This finding, however, raises the question of how does α1-AR stimulation activate PKA without affecting i.e., L-type calcium channels. Actually, PKA is a broad-specific kinase activated by cAMP. However, although a number of agonists increase cAMP and activate PKA, they have very different cellular effects (reviewed in refs. 2 and 3). It is possible that the α1-AR/PKA/I\textsubscript{to} pathway is compartmentalized and limited to specific membrane regions. In fact, the PKA dependent phosphorylation of the I\textsubscript{to} channels after α1-AR stimulation only occurs when both the sarcoplasmic membrane and the cytoskeleton integrity are maintained.\textsuperscript{3}

Recent works demonstrate the colocalization of different receptors with their intracellular signaling cascades in discrete membrane microdomains named membrane rafts.\textsuperscript{4} This preassembly of receptors and signaling molecules allows faster cellular responses as well as specific responses to specific stimuli. The membrane rafts are regions enriched in cholesterol and sphingolipids, whose lipidic composition gives them a rigid structure which floats in the membrane. One specific subtype of membrane raft is the caveolae. These are flask-shaped membrane invaginations characterized by the presence of the scaffolding protein caveolin.\textsuperscript{5} Whereas the presence of α1-AR in caveolae has been reported,\textsuperscript{6} the localization of the Kv4.2 and Kv4.3 proteins, molecular correlates of the I\textsubscript{to} channel, is controversial. Therefore, both groups of preassembled proteins are maintained in close proximity by caveolin-3. A different I\textsubscript{to} channel population localizes in non-caveolar membrane rafts and is not sensitive to α1-adrnergic regulation.

In ventricular myocytes, α1-AR stimulates G\textsubscript{αs} proteins and reduces the transient outward K\textsuperscript{+} current (I\textsubscript{to}) via a cAMP/PKA-mediated pathway and thus regulates cardiac contraction and excitability. This I\textsubscript{to} reduction is compartmentalized and limited to discrete membrane regions since PKA-dependent phosphorylation of the I\textsubscript{to} channels after α1-AR stimulation requires the integrity of both the sarcoplasmic membrane and the cytoskeleton. The aim of this work was to investigate the mechanisms involved in the compartmentalization of the PKA-dependent modulation of I\textsubscript{to} in response to α1-AR activation. I\textsubscript{to} current recordings were performed by the Patch-Clamp technique. Membrane rafts from isolated ventricular myocytes were extracted by centrifugation in a sucrose density gradient. The different proteins were visualized by western blot and protein-protein interactions determined by coimmunoprecipitation experiments. Localization of I\textsubscript{to} channel in caveolae, particular subtypes of membrane rafts, was achieved by electron microscopy. Patch-Clamp recordings show that a functional supramolecular complex, kept together by the A kinase anchoring protein AKAP100, exist in caveolae in living myocytes. Density gradients and immunoprecipitation experiments show that the components of the α1-AR/I\textsubscript{to} pathway localize in caveolae, forming two different groups of proteins. The Kv4.2/Kv4.3 channel forms a supramolecular complex with PKA through AKAP100 and is attached to caveolae by interacting with caveolin-3. On the other hand, α1-AR, G\textsubscript{αs} and adenylate cyclase gather in a second group also connected to caveolin-3. Therefore, both groups of preassembled proteins are maintained in close proximity by caveolin-3. A different I\textsubscript{to} channel population localizes in non-caveolar membrane rafts and is not sensitive to α1-adrnergic regulation.
Results

Our working model proposes that, in adult ventricular myocytes, the $\alpha_1$-AR/PKA/I$_{to}$ pathway is compartmentalized within discrete membrane regions, probably the caveolae. The first group of experiments was designed to demonstrate the functional existence of a supramolecular regulatory complex localized in caveolae. We performed patch-clamp experiments in freshly isolated cardiac myocytes and elicited I$_{to}$ current-voltage curves by applying depolarizing pulses ranging from -30 to +50 mV. As reported, 30 $\mu$M phenylephrine resulted in a reduction of the I$_{to}$ current of 32 $\pm$ 1.8% ($p < 0.001$). We then tested the inhibitory effect of phenylephrine on I$_{to}$ in the presence of the panAKAP blocker Ht31, a peptide that mimics the amphipathic helix of AKAPs that binds to the PKA, and has been widely used as a competitive inhibitor of PKA-AKAP interactions. We observed no difference in the I$_{to}$ current after the application of phenylephrine in cells pretreated with 20 $\mu$M Ht31, confirming the importance of AKAP in the maintenance of the functional complex. Additionally, the control peptide Ht31-P does not modify the $\alpha_1$-AR effect on I$_{to}$ (Fig. 1A and B).

Given their characteristic lipid composition, membrane rafts are very sensitive to cholesterol modifying agents. To confirm that these proteins locate in membrane rafts, we employed 2% MβCD, the most commonly used cholesterol modifying method. Membrane raft dilution after depletion of cellular cholesterol eliminates the $\alpha_1$-AR induced I$_{to}$ current reduction, suggesting that the regulatory complex must be raft associated in order to be functional. Cytoskeleton disruption internalizes caveolae and, therefore, microtubule disrupting agents are often used to discriminate between caveolae and non-caveolar membrane rafts. We found that microtubule disruption with colchicine also abolished the inhibitory effect of phenylephrine on the I$_{to}$ current. This result reinforces the idea of a supramolecular complex only functional when located in caveolae (Fig. 1C and D).

To further confirm this hypothesis we performed fractionation experiments to isolate membrane rafts from cardiomyocytes. Caveolae are a specific type of membrane raft and their major structural component, caveolin, is enriched in low density membrane fractions. Thus, in our experiments, we used caveolin-3, the myocyte-specific caveolin isoform, as a marker for membrane raft isolation. As a negative control we checked for the presence of the non-raft membrane protein Na+/K+ ATPase.

The detergent-based raft isolation method$^{12}$ isolates low density detergent-insoluble complexes from total cellular membranes. This method has been successfully employed in different cell types such as mouse L-cells, HEK293 cells or neurons.$^{3,14}$ We found however that the purity of the raft isolation was too low in ventricular myocytes. The membrane raft containing fraction appeared at the 35–40% sucrose interface, whereas the non-raft fraction was found in the 40% sucrose. We then switched to a detergent-free isolation method where caveolin-3 appears only at the top of the gradient (5–35% interface) and the Na+/K+ ATPase was confined to the bottom of the gradient (Fig. 2).

Once the quality of the isolation method was confirmed, we tested the presence in membrane rafts of the I$_{to}$ channel forming proteins K,4.2 and K,4.3, and of the proteins involved in the $\alpha_1$-AR regulation of the I$_{to}$ channel: $\alpha_1$-AR, G$\alpha$s, AC, PKA and AKAP100 (Fig. 2).

As previous work described, 4 we detected $\alpha_1$-AR only in membrane rafts. Interestingly, the downstream effectors G$\alpha$s protein and PKA were also found in the same fractions, mirroring the distribution of the receptor. The channel forming proteins K,4.2 and K,4.3 were also detected only in the membrane raft fractions. AC and the scaffolding protein AKAP100 appears both in low and high density gradient fractions (Fig. 2). These results indicate that the $\alpha_1$-AR/I$_{to}$ pathway localize in membrane rafts.

Next we incubated the myocytes with 2% MβCD for one hour and found that the cholesterol depleting agent dissociated the lipid rafts and altered the buoyancy of the raft associated proteins. Thus, all raft associated proteins, $\alpha_1$-AR, G$\alpha$s, AC, AKAP100, PKA, K,4.2 and K,4.3, were shifted toward the bottom of the gradient after cholesterol depletion (Fig. 3). These results confirm that the components of the $\alpha_1$-AR/I$_{to}$ regulatory pathway localize in membrane rafts, but do not distinguish whether these rafts are caveolae or not.

We then incubated the myocytes with 5 $\mu$M colchicine to alter caveolae structure before membrane extraction. We observed that in colchicine-treated myocytes $\alpha_1$-AR, G$\alpha$s, AC, AKAP100, PKA, K,4.2 and K,4.3 migrated toward higher density fractions, indicating their presence in caveolae (Fig. 3).

To further demonstrate the physical association between the I$_{to}$ channel molecules and the $\alpha_1$-Adrenergic pathway in caveolae, we performed immunoprecipitation experiments using an anti-Caveolin-3 antibody (Fig. 4). The presence of caveolin-3 in the precipitate, and its absence in the supernatant was confirmed by immunoblot. Caveolin-3 interacts with each of the $\alpha_1$-AR/I$_{to}$ pathway proteins, $\alpha_1$-AR, G$\alpha$s, AC, AKAP100, PKA, K,4.2 and K,4.3. These results support a model where all the components of the pathway remain attached to caveolar membrane rafts through the interaction with caveolin-3.

In ventricular myocytes, I$_{to}$ is reduced only ~35% by maximum $\alpha_1$-Adrenergic stimulation and we wondered why the current reduction never reaches the 100%. One possibility is that there are two different channel populations, one localized in caveolae and subjected to $\alpha_1$-AR regulation and another one localized outside the caveolae and $\alpha_1$-AR-independent. Supporting this hypothesis is the fact that K,4.2 and K,4.3 channel proteins also appear in the supernatant from caveolin-3 immunoprecipitation experiments. In all Caveolin-3 immunoprecipitation experiments the absence of Caveolin-3 in the supernatant was confirmed as control (Fig. 4).

Although both the I$_{to}$ channel and PKA locate in caveolae, interaction between them is possible only if they are in close proximity. Through anchoring and formation of multiprotein complexes, AKAPs attach PKA to specific targets. To test if this is the case, we immunoprecipitated AKAP100 from myocyte membranes and analyzed the presence of the components of the pathway by western blot. The presence of AKAP100 in the precipitate as well as its absence in the supernatant was confirmed as control. Our experiments showed that AKAP100 interacts with K,4.2, K,4.3 and PKA. Caveolin-3 was also found in the
precipitate, which is consistent with the presence of the complex in caveolae. Also as expected, AKAP100 did not immunoprecipitated with either α1-AR, Gαs or AC, proteins which were only detected in the supernatant of the immunoprecipitation experiment. These results suggest that binding to AKAP100 keeps PKA and I_{to} in a regulatory multiprotein complex which, in turn, interacts with caveolin-3 and thus remains attached to the caveolae (Fig. 5).
caveolae (Fig. 6C). Interestingly, when the channels localize in the caveolae they always appear in neck of the structure (Fig. 6B).

In summary (Fig. 7), the Kv4.2/Kv4.3-AKAP-PKA complex ensures the channel regulation by PKA. The presence of caveolin-3 in the complex maintains it in close proximity to the α1-AR-Gαs-AC signaling pathway, which is also concentrated in caveolae in cardiac myocytes. A second channel population localizes outside caveolae and is not α1-adrenergically regulated.

**Discussion**

In a previous work we reported that α1-AR stimulation couples the receptor to a Gαs protein and activates the cAMP/PKA signaling cascade, which leads to Kv4.2/Kv4.3 channel phosphorylation.
Protein kinase A regulates A-type potassium current in hippocampal neurons and the close-related transient outward current in ventricular myocytes. The Kv4.2 channel is phosphorylated by PKA at Thr38 and Ser552. However, PKA phosphorylation of the Kv4.2 is necessary but not sufficient for channel modulation. Channel modulation by PKA requires the formation of a supramolecular regulatory complex with an ancillary subunit, the K+ channel interacting protein (KChIP3). Therefore, PKA must of necessity act on a supramolecular complex of pore-forming α-subunits plus ancillary subunits to alter channel properties. A-Kinase Anchoring Proteins target PKA to its substrates and AKAP100 (or mAKAP, for muscle-specific AKAP) has been found in cardiac myocytes. Moreover, there is a putative AKAP binding motif in the C-terminal of the Kv4.2 amino-acid sequence, similar to that found in L-Type Ca++ channels or K₄.LQT1 K⁺ channels. In our experiments Kv4.2 and Kv4.3 and reduction of Iᵦ current. Membrane rafts, and particularly caveolae, have an important role in compartmentalization and modulation of a growing number of membrane-linked signal transduction pathways. Many GPCRs, G proteins and signaling molecules such as PKA, PKC or MAPK have been reported to be localized in membrane rafts (reviewed in ref. 15). The present results show that, in ventricular myocytes, the components of the α₁-AR/Iₒ signal transduction pathway that includes the receptor, Gₐₐₐ protein, AC, AKAP100, PKA, Kv4.2 and Kv4.3 are raft-associated and that these rafts are caveolae. This preassembly within membrane domains allows the quick regulation of the channel in response to α₁-adrenergic stimulation.

Figure 3. Protein buoyancy depends on cholesterol depletion and on cytoskeleton integrity. Sucrose density gradient fractions of detergent-free extracted membrane rafts, from rat ventricular myocytes incubated with 2% methyl-β-cyclodextrin (MβCD) or 5 µM colchicine (COLCH). All raft associated proteins, α₁-AR, Gₛₛ, AC, AKAP100, PKA, Kv4.2 and Kv4.3, migrate toward higher density fractions after cholesterol depletion with MβCD, further confirming their presence in cholesterol enriched membrane rafts. The microtubule disrupting agent colchicine also induces a protein displacement from low to high density gradient fractions, which indicates that these proteins are present in caveolae. Western blots shown are representative of 5 and 6 different gradients with MβCD and COLCH respectively.
results regarding the localization of the \( K_{v}4.2 \) proteins. \( K_{v}4.2 \) has been found in rafts in tsa201 cells but not in mouse 1 cells, and in hippocampal neurons there are contradictory results.\(^{13,14} \) Our results agree with those reporting the presence of \( K_{v}4.2 \) within membrane rafts.

As mentioned above, we first isolated membrane rafts from cardiac myocytes using a Triton-X-100 based method and then with a detergent-free one and obtained discrepant results. The isolation method must be therefore optimized for each cell type. However, our results in \( K_{v}4.2 \) channel differ from those of Martens et al.\(^{13} \) who employed the same method as we did. One possible explanation is that protein localization may be different in each cell type, probably due to association with different interacting proteins, as recently demonstrated for other channels. Thus, \( K_{v}1.5 \) channels do not appear in membrane rafts when expressed alone, but are directed to membrane rafts when coexpressed with \( K_{v}1.3 \) channels.\(^{25} \) Similarly, PSD95 increases the raft association of \( K_{v}1.4 \) channels.\(^{14} \) In this sense, the \( K_{v}4.3 \) channel protein has a consensus sequence for interaction with caveolin (\( \Phi_{4}X_{3}
\Phi_{3}X_{1}
\Phi_{3} \), aminoacids 165–173 of \( K_{v}4.3 \)) and \( K_{v}4.2 \) lacks this sequence. Therefore, \( K_{v}4.3 \) could be the scaffolding protein who directs \( K_{v}4.2 \) to caveolae in rat cardiac myocytes.

The localization of any ionic channel in a high resistance bottle-neck structure could impair the voltage gradient necessary for their correct function, although channels such as \( K_{v}1.5 \), Kir6.1, Na\(^{+}1.5 \), or Ca\(^{2+}1.2 \) have been described in caveolae.\(^{26,27} \) In our electron micrographs the \( K_{v}4.3 \) channel is always located at the neck of the caveola, avoiding this hypothetical shortcoming. Recent studies employing electron microscopy reveal that insulin receptors also associate predominantly with the neck, but not the bulb, of caveolae.\(^{28} \) This localization is also consistent with the higher concentration of caveolin at the neck connecting the caveola to the plasma membrane.\(^{29} \)

In our work, electron micrographs answer a second question regarding \( \alpha_{1}-\text{Adrenergic} \) regulation of \( I_{to} \). As mentioned, maximum \( \alpha_{1}-\text{AR} \) stimulation reduces \( I_{to} \) current amplitude up to a 35%. The presence of \( K_{v}4.3 \) channels outside of caveolae suggest that two different \( I_{to} \) channel populations exist. One caveola associated and sensitive to \( \alpha_{1}\)-adrenergic regulation and a second channel population localized in non-caveolar membrane rafts and PKA coimmunoprecipitate with AKAP100, consistent with the existence of a supramolecular regulatory complex. This complex also coimmunoprecipitates with caveolin-3, ensuring its concentration in caveolae.

The native \( I_{to} \) channel in rat ventricular myocytes is a heterotramer formed by the association of \( K_{v}4.2 \) and \( K_{v}4.3 \) proteins. As previously described for other voltage-gated \( K^{+} \) channels, such as \( K_{v}2.1 \) or \( K_{v}11.1 \),\(^{23,24} \) our two channel forming proteins appear in the same low density membrane fractions, suggesting their raft association. This is the first description of the raft association of the \( K_{v}4.3 \) channel protein. However, there are controversial
rafts and α1-adrenergic independent. This non-caveolar channel population could be still accessible to α1-adrenergic regulation, but to a significantly lower degree of efficacy.

Last, the physiological relevance of our work is the description of a mechanistic link between the sympathetic nervous system and the fine modulation of cardiac electrical activity. A growing body of evidence shows that ionic channels form supramolecular regulatory complexes with protein kinases, phosphatases and scaffolding proteins which provide a very fine regulation of cardiac activity. We propose that this fine regulation can be even more accurately tuned because of the existence of different channel populations, differentially modulated depending on their specific localization. This can also have pathophysiological and clinical implications. Although there are not electrophysiological studies on Caveolin-3 KO animals, these mice have a number of cardiac alterations including severe hypertrophy and hyperactivation of the MAPK pathway.30,31 In this regard, cardiac Ito becomes resistant to α1-AR stimulation in diabetic animals.32 The cause suggested years ago was the reduction of the α1-AR expression which occurs in diabetic animals.33 However, now we know that in the α1-AR/Gs/AC pathway the receptor is expressed in excess and is not the limiting step in cAMP production.1 It has been recently described that caveolin-3 expression is dramatically reduced in diabetic animals34 which could lead to the α1-AR/Ito pathway misslocalization and explain the formerly described Ito resistance to α1-AR regulation in diabetic animals.

**Materials and Methods**

The investigation fulfills to the Spaniard (RD 1201/2005) and European (D2003/65/CE and R2007/526/CE) rules for Care of Animals used for experimental and other research purposes, and has been approved by the Ethics Committee for Animal Care (CEBA) of the Universidad del País Vasco.
12 and taurine 20, bubbled with 95% O₂ and 5% CO₂, pH 7.4 at 37°C, followed by the same solution without Ca²⁺, and by the same nominally Ca²⁺-free solution containing collagenase Type I (0.5 mg ml⁻¹) and protease Type XIV (0.03 mg ml⁻¹). The hearts were finally perfused with a KB solution³⁵ (in mmol l⁻¹): taurine 10, glutamic acid 70, creatine 0.5, succinic acid 5, dextrose 10, KH₂PO₄ 10, KCl 20, HEPES-K⁺ 10, EGTA-K⁺ 0.2, adjusted to pH 7.4 with KOH. Single cells were obtained by mechanical agitation.

**Patch-clamp.**  Iᵣ current is rapidly activated and so, experiments were performed at room temperature (20–22°C) to isolate peak current from capacitive current. Ionic currents were recorded using the whole-cell variation of the Patch-Clamp technique³⁶ with an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc.). Recording pipettes were obtained from borosilicate tubes (Sutter Instruments), and had a tip resistance of 1–3 MΩ when filled with the internal solution (in mmol l⁻¹): L-aspartic acid (potassium salt) 80, KH₂PO₄ 10, MgSO₄ 1, KCl 50, HEPES-K⁺ 5, ATP-Na₂ 3, EGTA-K⁺ 10, adjusted to pH 7.2 with KOH.

Methyl-β-CD and Colchicine are the most commonly employed methods to ensure the localization of different proteins in membrane rafts and to discriminate between caveolae and non-caveolar membrane rafts, respectively.⁹,¹⁰,¹³,¹⁴,²³,²⁶ MβCD 2% and 5 µmol l⁻¹ Colchicine were added to the KB medium two hours before the start of the experiments. 20 µmol l⁻¹ Ht₃₁ was added to the pipette solution. After the patch rupture, currents were allowed to stabilize and control records were made. After recording control currents, cells were bathed with phenylephrine 30 µmol l⁻¹, dissolved with ascorbic acid to avoid oxidation and with propranolol 100 nmol l⁻¹ to block any possible β-AR mediated effect. As reported,¹³ MβCD treatment made the cells very difficult to patch clamp.

Following the patch rupture, whole cell membrane capacitances were measured from integration of the capacitive transients elicited by voltage steps from -50 to -60 mV, which did not activate any time dependent membrane current. Series resistances were compensated 80% in order to minimize voltage errors and were checked regularly throughout the experiment to ensure that there were no variations with time. The voltage-clamp experimental protocols were controlled with the “Clampex” program of the “pClamp” software (Axon Instruments Inc.). The bathing solution was (in mmol l⁻¹): NaCl 86, MgCl₂ 1, HEPES-Na⁺ 10, KCl 4, CaCl₂ 0.5, CoCl₂ 2, dextrose 12, TEA-Cl 50, adjusted to pH 7.4 with NaOH. Iᵣ was recorded applying depolarizing pulses ranging from -30 to +50 mV, starting from a holding potential of -60 mV. The TEA-resistant time-independent Iᵣ was digitally subtracted. Peak Iᵣ were normalized to cell capacitance, and expressed as pA pF⁻¹.

**Membrane isolation.** All procedures were performed at 4°C, in a homogenization buffer (HF) containing Tris-HCl 20 mmol l⁻¹ pH 7.4, EDTA 1 mmol l⁻¹, and 2.5 µl ml⁻¹ of the Sigma Protease inhibitor cocktail (Sigma Chemical Co.). Myocytes were homogenized 1 minute on ice. Nuclei and debris were pelleted by centrifugation at 500 g for 10 minutes. The supernatant was centrifuged at 25,000 g for 30 minutes. The pellet was resuspended in HF and centrifuged again at 25,000 g for 30 min. The final

**Cell isolation.** Sprague-Dawley rats (200–220 g) were anaesthetized with chloral hydrate (3 ml kg⁻¹, ip) and killed by cervical dislocation. The hearts were removed and perfused with a Tyrode solution containing (in mmol l⁻¹): NaCl 118, KCl 5.4, NaHCO₃ 24, MgCl₂ 1.02, CaCl₂ 1.8, NaH₂PO₄ 0.42, dextrose
pellet was stored at -80°C. The protein content was determined by using the Bradford method.37

**Raft isolation.** There are two main procedures to isolate membrane rafts, both of them based on the resistance of membrane rafts to either detergent- or detergent-free solubilisation and on the buoyancy of unsolubilised rafts.12,38 The detergent-free raft isolation were performed as follows. Cell membranes isolated from ventricular myocytes were resuspended into 2 ml of Mes-buffered saline (MBS; 25 mmol l⁻¹ Mes, pH 6.5, 150 mmol l⁻¹ NaCl; 2.5 µl ml⁻¹ of the Sigma Protease inhibitor cocktail; plus 250 mM sodium carbonate). Homogenization was carried out with three 10-s bursts on a Polytron tissue grinder (Sanyo Gallenkamp 23 Hz) and a 5 minutes burst on a sonicator (Ultrasons, Selecta). The homogenate was then adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above (3 ml of 5% sucrose/3 ml of 35% sucrose; both in MBS containing 250 mM sodium carbonate) and centrifuged at 200,000 g for 18 h in a TST41.14 Swinging-Bucket rotor (Kontron Instruments). The low density unsolubilised membrane raft containing fraction was confined to the 5–35% sucrose interface, whereas the soluble lysate was observed at the bottom of the density gradient. Gradient fractions were collected from the top of each gradient. The first 2 ml were collected together as they do not contain any detectable protein. After that, 1 ml gradient fractions were collected to yield a total of nine fractions, the last one containing the pellet. Proteins from each fraction were precipitated with trichloroacetic acid and resuspended in SDS sample buffer. Caveolin appears at the 5–35% sucrose interface, thus, Caveolin-3 was used as marker of the membrane raft isolation. The Na⁺/K⁺ ATPase appears always at the bottom of the tube, and it was used as non-raft marker.

**Coimmunoprecipitation.** Myocytic membranes were solubilized in RIPA buffer (Tris-HCl 50 mmol l⁻¹ pH 7.4, NaCl 150 mmol l⁻¹, EDTA 1 mmol l⁻¹, Igepal 1%, Sodium deoxycholate 1%, 2.5 µl ml⁻¹ of the Sigma Protease inhibitor cocktail and the crosslinker DTBP 0.5 mg ml⁻¹). 250 µg of membrane proteins were centrifuged at 25,000 g for 30 min. The pellet was resuspended in 150 µl of RIPA buffer and incubated for 1 hour at 4°C. Samples were cleared by centrifugation at 15,000 g for 25 minutes and supernatants were incubated for 3 hours at 4°C with 40 µl of protein G-sepharose. After that, samples were centrifuged 2 minutes at 4,000 g. 150 µl of the supernatant were obtained and incubated overnight with 2 µg of the anti-AKAP100 or anti-Caveolin-3 antibody (Santa Cruz Biotechnologies) in 140 µl of RIPA at 4°C. 100 µl of 50% protein G-sepharose were added and the mixture was incubated for 3 hours at 4°C. The beads were pelleted and washed three times in RIPA buffer at 4,000 g for 2 minutes. The bound proteins were eluted using 50 µl of SDS sample buffer. The pellet from each immunoprecipitation experiment was probed with Cav-3 as control, and with each one of the proteins of interest.

**Western blot.** Gradient fractions or immunoprecipitation samples were fractionated on 10% SDS-polyacrylamide gels and transferred to Nitrocellulose membranes (Amersham Biosciences). Nitrocellulose membranes were blocked in TTBS solution (Tris-HCl 50 mmol l⁻¹ pH 7.5, NaCl 150 mmol l⁻¹, Tween-20 0.05%) containing BSA 3%. Blots were incubated with primary antibodies: anti-Caveolin-3 (1:1,000), PKA (1:200), Na⁺-K⁺ ATPase (1:50), K⁺,4,2 (1:200), K⁺,4,3 (1:200), Gα₃ protein (1:200), AC (1:200) and AKAP100 (1:200) from Santa Cruz Biotechnology. α₁-AR (1:400) from Calbiochem. Secondary antibodies were: donkey anti-rabbit IgG (1:5,000, Amersham Biosciences), anti-mouse IgG (1:3,300, Sigma Chemical Co.) and anti-goat (1:5,000, Santa Cruz Biotechnology). Blots were developed using the West Pico chemiluminiscence reagent (Pierce).

**Electron microscopy.** Animals were transcardially perfused with PBS (0.1 M; pH 7.4) and then fixed by 500 ml of a fixative made up of 0.1% glutaraldehyde, 4% formaldehyde, and 0.2% picric acid in PBS. Perfusates were used at 4°C. Tissue blocks
were extensively rinsed in 0.1 M PBS, pH 7.4. 50 μm-thick heart vibrosions were collected in 0.1 M PBS, pH 7.4, at room temperature. Sections were preincubated in 10% blocking BSA prepared in PBS for 1 hour at room temperature. The localization of Kv4.3 channels was carried out by means of the preembedding silver-intensified immuno gold method. Following incubation with the primary monoclonal mouse antibody against amino acids 415–636 of Kv4.3 (1:1,000 in 1.5% BSA/PBS; NeuroMab, UC Davis, Ca, USA) for two days, heart sections were incubated with 1.4 nm gold-labeled rabbit anti-mouse IgG (Fab' fragment, 1:100, Nanoprobes Inc., Stony Brook, NY, USA) diluted in 1% BSA/PBS for four hours at room temperature. Tissue was subsequently postfixed in 1% glutaraldehyde for 10 min, rinsed extensively in double-distilled water, and gold particles were silver-intensified with an HQ Silver kit (Nanoprobes Inc.,) for about 8 min. Successfully stained sections were osmicated (1% OsO4 in 0.1 M PB, pH 7.4, 20 min), dehydrated in graded alcohols to propylene oxide and plastic-embedded flat in Epon 812. Ultrathin sections were collected on mesh nickel grids, stained with uranyl acetate and lead citrate. The preparations were finally examined in a Philips EM208S electron microscope at the Microscopy Core Facility of the University of the Basque Country.

Chemicals. Collagenase, Protease, Phenylenolhyamine, Ascorbic acid and Cyclodextrin were from Sigma Chemical Co., Propanolol-HCl was from Tocris, Colchicine was from Calbiochem, and InCELEcHt-31 was from Promega.

Data analysis. Current recordings were analyzed using the Clampfit program of pClamp 10.1 software (Axon Instruments Inc). Western blot bands were acquired with a Kodak Gel Logic 2200 and analyzed with the Kodak Molecular Imaging 4.0.4 software (Eastman Kodak Company). Immunohistochemical preparations were photographed by using standard electron microscopy negative plates. TheStudent’s paired t test was used for statistical analysis. The data are expressed as the mean ± S.E.M. A p value of less than 0.05 was considered to be statistically significant.

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References

1. Gallego M, Serion R, Puebla L, Boyano-Adanez MC, Artilla E, Casis O. alpha-Adrenoreceptors stimulate a G protein and reduce the transient outward K+ current via a cAMP/PKA-mediated pathway in the rat heart. Am J Physiol-Cell Physiol 2005; 228:577-85.
2. Steinberg SF, Brunton LL. Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. Annu Rev Pharmacol Toxicol 2001; 41:751-73.
3. Kapiloff MS. Contributions of protein kinase A anchoring proteins to compartmentation of cAMP signaling in the heart. Mol Pharmacol 2002; 62:193-9.
4. Pike LJ. Rafts defined: A report on the Keystone Symposium on lipid rafts and cell function. J Lipid Res 2006; 47:1557-88.
5. Frank PG, Hassan GS, Rodriguez-Feo JA, Lisanti MP. Caveolae and caveolae-1: Novel potential targets for the treatment of cardiovascular disease. Curr Pharm Des 2007; 13:1761-9.
6. Fujita T, Toyoda T, Inawashiro K, Tamura Y, Iwakura K, Onoda T, Kitamura K, Uemura S, et al. Accumulation of molecules involved in alpha-adrenergic signaling within caveolae: caveolin expression and the development of cardiac hypertrophy. Cardiovasc Res 2001; 51:709-16.
7. Carr DW, Haasen ZE, Fraser ID, Stoelho-Hahn RE, Scott JD. Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein. Cloning and characterization of the RII- binding domain. J Biol Chem 1992; 267:13376-82.
8. Fujita T, Toyoda T, Iwakura K, Onoda T, Kitamura K, Uemura S, et al. Accumulation of molecules involved in alpha-adrenergic signaling within caveolae: caveolin expression and the development of cardiac hypertrophy. Cardiovasc Res 2001; 51:709-16.
9. Carr DW, Haasen ZE, Fraser ID, Stoelho-Hahn RE, Scott JD. Association of the type II cAMP-dependent protein kinase with a human thyroid RI-anchoring protein. Cloning and characterization of the RII- binding domain. J Biol Chem 1992; 267:13376-82.
10. Inoue K, Andjel K, Kuzmek R, Kogyo H, Fujimori T, Fujita T, et al. Endothelial Ca2+ -wave propagation originates at specific loci in caveolin-rich cell edges. Proc Natl Acad Sci USA 1998; 95:5009-14.
11. Peisova Z, Novotny J, Cerny M, Mladek G, Sloboda P. Thyroid-stimulating hormone-induced depletion of α(1α)-caveolin from detergent-insensitive membrane domains. FEBS Lett 1999; 464:35-40.
12. Sargiacomo M, Sudul M, Tang Z, Lisanti MP. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol 1997; 135:789-97.
13. Martens JR, Kavan-Polanco R, Coppeko EA, Nishiyama A, Parkley L, Grohaski TD, et al. Differential targeting of Shaker-like potassium channels to lipid rafts. J Biol Chem 2000; 275:7443-6.
14. Wong WS, Schlichter LC. Differential recruitment of Kv1.4 and Kv4.2 to lipid rafts by PSD-95. J Biol Chem 2004; 279:444-52.
15. Patel HH, Murray E, Insel PA. Caveolae as organizers of pharmacologically relevant signal transduction molecules. Annu Rev Pharmacol Toxicol 2008; 48:359-91.
16. Hoffman DA, Johnston D. Downregulation of transient K+ channels in dendrites of hippocampal CA1 pyramidal neurons by activation of PKA and PKC. J Neurosci 1998; 18:3521-8.
17. Anderson AE, Adams JP, Qian Y, Cook RG, Paffinger PJ, Sweatt JD. K+2,4-phosphorylation by cyclic AMP-dependent protein kinase. J Biol Chem 2000; 75:5337-46.
18. Schrader LA, Anderson AE, Mayne A, Paffinger PJ, Sweatt JD. PKA modulation of K+2,4-encoded A-type potassium channels requires formation of a supramolecular complex. J Biol Chem 2007; 282:2501-12.
19. Yang J, Zdrahal JA, Ferguson DG, Bond M. A kinase anchoring protein 100 (AKAP100) is localized in multiple subcellular compartments in the adult rat heart. J Cell Biol 1998; 142:511-22.
20. Mccartney S, Little BM, Langeberg LK, Scott JD. Cloning and characterization of Akinase anchor protein 100 (AKAP100). A protein that targets A-kinase to the sarcoplasmic reticulum. J Biol Chem 1995; 270:9327-33.
21. Maia SO, Kurokawa J, Reiken S, Moteiko H, D’Alimontino J, Malek AR, et al. Requirement of a macromolecular signaling complex for β adrenergic receptor modulation of the KCNQ1-KCNQ1 potassium Channel. Science 2002; 295:496-9.
22. Holme JT, Ahn M, Haushka SD, Scheuer T, Catterall WA. A novel leucine zipper targets AKAP15 and cyclic AMP-dependent protein kinase to the C terminus of the skeletal muscle Ca2+ channel and modulates Its function. J Biol Chem 2002; 277:4079-87.
23. Baljepalli RC, Delsie BP, Baljepalli SY, Forel JD, Slind JK, Kamp TJ, et al. Kv1.1 (ERG1)-K+ channels localize in cholesterol and sphingolipid enriched membranes and are modulated by membrane cholesterol. Channels (Austin) 2007; 1:163-72.
24. Martens JR, Sakamoto N, Sullivan SA, Grohaski TD, Tamkun MM. Isoform-specific localization of voltage-gated K+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. J Biol Chem 2001; 276:8409-14.
25. Martinez-Marmol R, Villalonga N, Solé L, Vicente R, Tamkun MM, Soler C, et al. Multiple K+1.5 targeting to membrane surface microdomains. J Cell Physiol 2008; 217:667-73.
26. Magy A, Hebert TE, Natrel S. Involvement of lipid rafts and caveolae in cardiac ion channel function. Cardiovasc Res 2006; 69:798-809.
27. McEwen DP, Li Q, Jackson S, Jenkins PM, Martens JR. Caveolin regulates K+1.5 trafficking to cholesterol-rich membrane microdomains. Mol Pharmacol 2008; 73:678-85.
28. Fonti M, Porcheron G, Fournier M, Maeder C, Carpenter JL. The neck of caveolae is a distinct plasma membrane subdomain that concentrates insulin receptors in 3T3-L1 adipocytes. Proc Natl Acad Sci USA 2007; 104:1242-7.
29. Thorn H, Stenkula KG, Karlsson M, Ortegren U, Nystron FH, Gustavsson J, et al. Cell surface ori- fices of caveolae and localization of caveolin to the necks of caveolae in adipocytes. Mol Biol Cell 2003; 14:9867-76.
30. Park DS, Woodman SE, Schubert W, Cohen AW, Frank PG, Chandra M, et al. Caveolin-1/3 double-knockout mice are viable, but lack both muscle and non-muscle caveolae, and develop a severe cardiomyopathic phenotype. Am J Pathol 2002; 160:2207-17.

31. Hnasko R, Lisanti MP. The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. Mol Interact 2003; 3:445-64.

32. Gallego M, Casis O. Regulation of cardiac transient outward potassium current by norepinephrine in normal and diabetic rats. Diabetes Metab Res Rev 2001; 17:304-9.

33. Bitar MS, Koul M, Rapoport SI, Linnoila M. Adrenal catecholamine metabolism and myocardial adrenergic receptors in streptozotocin diabetic rats. Biochem Pharmacol 1987; 36:1011-6.

34. Penumathsa SV, Thirunavukkarasu M, Zhan L, Maulik G, Menon VP, Bagchi D, et al. Resveratrol enhances GLUT-4 translocation to the caveolar lipid raft fractions through AMPK/Akt/eNOS signaling pathway in diabetic myocardium. J Cell Mol Med 2008; 12:2350-61.

35. Ienborg G, Klockner U. Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium". Pflugers Arch 1982; 395:6-18.

36. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 1981; 391:85-100.

37. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54.

38. Song SK, Li S, Okamoto T, Quilliam LA, Sargiacomo M, Lisanti MP. Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. J Biol Chem 1996; 271:9690-7.