Functional Assembly of Kv7.1/Kv7.5 Channels With Emerging Properties on Vascular Muscle Physiology

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Objective—Voltage-dependent K^+ (Kv) channels from the Kv7 family are expressed in blood vessels and contribute to cardiovascular physiology. Although Kv7 channel blockers trigger muscle contractions, Kv7 activators act as vasorelaxants. Kv7.1 and Kv7.5 are expressed in many vessels. Kv7.1 is under intense investigation because Kv7.1 blockers fail to modulate smooth muscle reactivity. In this study, we analyzed whether Kv7.1 and Kv7.5 may form functional heterotetrameric channels increasing the channel diversity in vascular smooth muscles.

Approach and Results—Kv7.1 and Kv7.5 currents elicited in arterial myocytes, oocyte, and mammalian expression systems suggest the formation of heterotetrameric complexes. Kv7.1/Kv7.5 heteromers, exhibiting different pharmacological characteristics, participate in the arterial tone. Kv7.1/Kv7.5 associations were confirmed by coimmunoprecipitation, fluorescence resonance energy transfer, and fluorescence recovery after photobleaching experiments. Kv7.1/Kv7.5 heterotetramers were highly retained at the endoplasmic reticulum. Studies in HEK-293 cells, heart, brain, and smooth and skeletal muscles demonstrated that the predominant presence of Kv7.5 stimulates release of Kv7.1/Kv7.5 oligomers out of lipid raft microdomains. Electrophysiological studies supported that KCNE1 and KCNE3 regulatory subunits further increased the channel diversity. Finally, the analysis of rat isolated myocytes and human blood vessels demonstrated that Kv7.1 and Kv7.5 exhibited a differential expression, which may lead to channel diversity.

Conclusions—Kv7.1 and Kv7.5 form heterotetrameric channels increasing the diversity of structures which fine-tune blood vessel reactivity. Because the lipid raft localization of ion channels is crucial for cardiovascular physiology, Kv7.1/Kv7.5 heteromers provide efficient spatial and temporal regulation of smooth muscle function. Our results shed light on the debate about the contribution of Kv7 channels to vasoconstriction and hypertension. **(*Arterioscler Thromb Vasc Biol*. 2014;34:1522-1530.)**

Key Words: blood vessels ■ KCNQ1 potassium channel ■ muscle, smooth

Voltage-dependent potassium (Kv) channels modulate action potentials in nerve and muscle.[1] The Kv7 channels control important neuronal K^+ currents produced by homomeric and heteromeric assemblies of related isoforms.[2] The Kv7 family consists of 5 members (Kv7.1–Kv7.5), encoded by single genes (*KCNQ1–5*) that generate slowly activating K^+ channels.[2,3] Mutations in 4 of the 5 members (Kv7.1–Kv7.4) are associated with different human disorders, establishing the physiological relevance of this family.[4]

Kv7 channels control vascular tone.[5,6] The Kv7 biophysical properties resemble currents underlying K^+ conductance of arterial and venous musculature. However, a relationship between functional currents and specific genes has not been established.[6] Heterotetramerization of Kv7 isoforms shapes K^+ currents, and this complexity is further increased by KCNE regulatory subunits.[7,8] Cardiovascular musculature mostly expresses Kv7.1, Kv7.4, and Kv7.5.[9,10] Accordingly, Kv7 blockers linopirdine and XE991 generate contractions that may vary in magnitude depending on the vascular bed.[11,12] Therefore, the existence of channel combinations makes it difficult to decipher pharmacological implications. For this reason, determining the entities that control smooth muscle tone is a topic of intense investigation.

Heteromeric channels maintain resting membrane potential and fine-tune physiological responses. Kv7 channels tetramerize via coil–coil domains at the C terminus.[13] Although all Kv7 channels contain tetramerization coil–coil domains, limited associations have been demonstrated.[8] Early reports indicated that Kv7.5 only interacted with Kv7.3.[14–16] However, the presence of Kv7.1 and Kv7.4 in vascular musculature

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Nonstandard Abbreviations and Acronyms	
ER	endoplasmic reticulum
Kv	voltage-dependent K^+

and the confirmation of Kv7.4/Kv7.5 heteromers pave the way for novel interactions that could shed light on pharmacological results in vascular musculature.[5,6,10,17–19] The role of Kv7.1 in cardiovascular smooth muscle is of particular interest.[6] Chromanol 293B, which blocks Kv7.1, has limited effect on human arterial tone, suggesting that Kv7.1 homotetramers have no function in vascular myocytes.[5] However, Kv7.1 and Kv7.5 are coexpressed in smooth and skeletal muscle cells.[2,6,20,21] To date, there are no reports on the association of Kv7.1 and Kv7.5, which could have important influences in vascular reactivity.

Finally, many cardiovascular channels are located in lipid rafts, which is important for action potentials.[22,23] Lipid rafts are membrane microdomains where many signaling proteins interface with their targets.[24] Kv7.1 and Kv7.5 differentially localize into rafts.[25,26] Although Kv7.1 targets to rafts, Kv7.5 is mostly absent of these domains. Lipid rafts, especially caveolae, are important in vascular musculature, where Ca^{2+} triggers important signals that contribute to vascular tone.[27] We demonstrate that Kv7.1 and Kv7.5 form functional channels. Unlike Kv7.5, heterotetrameric channels showed high endoplasmic reticulum (ER) retention and, in contrast to Kv7.1, are localized outside of lipid raft microdomains. Our results are of physiological interest because Kv7.1/Kv7.5 channels have a putative role in vascular musculature reactivity.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Expression of Kv7.1 and Kv7.5 in Cardiovascular Myocytes

Kv7.1 and Kv7.5 are present in blood vessels. However, their association, putatively relevant for the vascular muscle tone, has not been investigated. Kv7.1 and Kv7.5 are differentially coexpressed in the brain, skeletal muscle, heart, aorta, and cava from rats (Figure 1A). Kv7.1 was more abundant in the heart, whereas Kv7.5 was predominant in the brain, aorta, and cava. Similar levels of Kv7.1 and Kv7.5 were observed in quadriceps. Figure 1B demonstrated that Kv7.1, Kv7.5, and KCNE1 coimmunoprecipitated in rat aorta myocytes. Because previous studies are controversial,[10,12,17,28,29] we analyzed the pharmacology of K^+ currents in isolated rat coronary myocytes (Figure 1C–1E). Myocytes expressed Kv7.1 and Kv7.5 (Figure 1C), and concomitantly, K^+ currents were similarly blocked by linopirdine and chromanol 293B. Interestingly, the double addition of linopirdine plus chromanol 293B did not trigger any further blockade (Figure 1D and 1E). We next studied relaxant responses induced by retigabine, a Kv7.2 to Kv7.5 activator, in endothelium-intact rat coronary arteries stimulated with 5-HT (serotonin; Figure 1F and 1G). Retigabine induced a relaxant response that was differentially inhibited by the presence of chromanol 293B and linopirdine. We further confirmed the presence of both channels in rat isolated aorta and cava myocytes (Figure 1H and 1I). Histological analysis of human veins and arteries further supported Kv7.1 and Kv7.5 coexpression in blood vessels. Kv7.5 was more abundant than Kv7.1 in cardiovascular smooth muscle structures identified by α-actin expression (Figure I in the online-only Data Supplement). In the carotid artery, Kv7.1 notably stained the subendothelial layer, whereas Kv7.5 labeled the endothelium of the tunica intima. Similar Kv7.5 pattern was observed in the subclavian vein. In general, tunica media and externa layers homogeneously expressed Kv7.1 and Kv7.5. Kv7.1 and Kv7.5 staining was specific because controls with preimmune serum and Kv7.1 antigen peptide were negative (Figure II in the online-only Data Supplement). Our results indicate that Kv7.1 and Kv7.5 are present in blood vessels, control the arterial tone, and suggest that could form heterotetrameric channels with a differential pharmacological profile.

Kv7.1/Kv7.5 Heterotetramers Exhibit Differentiated Biophysical and Pharmacological Properties

Although work in native myocytes is crucial, conclusions should be carefully taken because of a complex scenario. Therefore, we sought evidences for Kv7.1/7.5 association in expression systems. Figure 2 illustrates representative K^+ currents and normalized peak current intensities from individually Kv7.1-, Kv7.5-, and doubly Kv7.1/Kv7.5-injected *Xenopus* oocytes. No currents were detected in water-injected oocytes (not shown). Kv7.1-injected oocytes yielded currents that required several seconds to fully activate on depolarization (Figure 2A). Similar patterns were observed with Kv7.5- and Kv7.1/Kv7.5-injected oocytes (Figure 2B and 2C, respectively); all groups displayed an inward rectification at potentials >0 mV (Figure 2D). Activation of Kv7.1 ($V_{0.5}$, −29.3±2.6 mV; *s*, 12.1±0.8 mV) was less hyperpolarized than that of Kv7.5 ($V_{0.5}$, −46.7±1.6 mV; *s*, 12.8±0.9 mV). The half-maximal activation of Kv7.1/Kv7.5 was intermediate ($V_{0.5}$, −40.6±2.6 mV; *s*, 11.8±0.9 mV) and significantly different from Kv7.1 and Kv7.5 (Figure 2F).

Figure 2G through 2L shows combinations of Kv7.1 and Kv7.5 in the presence of nonconducting Kv7.1(G314S) and Kv7.2(I340E) dominant-negative mutants.[29–31] To discard saturated responses, we first coinjected 10 ng of Kv7.1 plus 10 ng of Kv.7.5 cRNA. Double-injected oocytes elicited twice the K^+ current amplitude of those injected with only 10 ng of Kv7.1 (Figure 2G). Hyperpolarizing test pulses in Kv7.1/Kv7.1(G314S)-injected oocytes (10 ng of each) showed that the presence of Kv7.1(G314S) results in nonfunctional channels (Figure 2H). Conversely, coinjection of Kv7.5 (10 ng) and Kv7.2(I340E) (10 ng) did not support any interaction (Figure 2I), an expected result because Kv7.2 and Kv7.5 do not associate with each other.[15,16] Finally, oocytes coinjected with 10 ng each of Kv7.5 and Kv7.1(G314S) elicited currents that were ≈50% lower than K^+ current amplitudes with 10 ng of Kv7.5 (Figure 2J). Figure 2L demonstrates that Kv7.1(G314S) abolished Kv7.1 currents and inhibited Kv7.5 currents.

We further studied Kv7.1 and Kv7.5 interactions in COS7 cells (Figure 3). Figure 3A through 3C shows representative

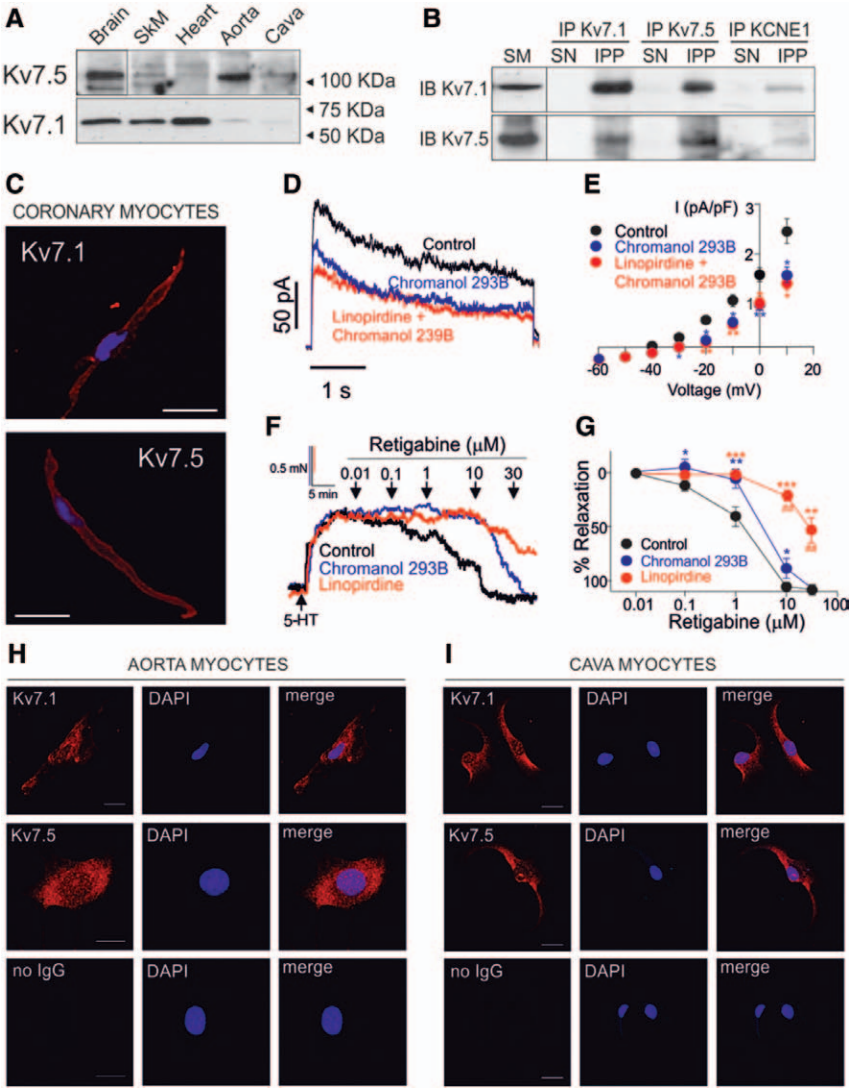

Figure 1. Kv7.1 and Kv7.5 in rat blood vessel myocytes. **A**, Kv7.1 and Kv7.5 abundance in enriched plasma membrane preparations of brain, quadriceps (skeletal muscle [SkM]), heart, aorta artery, and cava vein. **B**, Kv7.1 coimmunoprecipitates with Kv7.5 and KCNE1 in isolated aorta myocytes. Membrane extracts were immunoprecipitated (IP) with anti-Kv7.1, -Kv7.5 and -KCNE1 antibodies. **Top** (IB Kv7.1), Immunoblot (IB) demonstrating that Kv7.1 is detected in immunoprecipitates (IPP). **Bottom** (IB Kv7.5), Western blot against Kv7.5 demonstrating that Kv7.5 coimmunoprecipitates. SM indicates starting material; and SN, immunoprecipitated supernatant. **C**, Kv7.1 and Kv7.5 in isolated coronary myocytes. Myocytes were stained with anti-Kv7.1 (**top**) and anti-Kv7.5 (**bottom**). Bars, 20 μm. **D** and **E**, Voltage-dependent K^+ currents in isolated coronary myocytes were recorded in the absence or the presence of linopirdine and chromanol 293B. **D**, Representative current traces of 4-second +10 mV depolarization pulses from a holding potential of −60 mV before (control) and after the addition of 10 μmol/L chromanol 293B in the presence or the absence of 10 μmol/L linopirdine. **E**, Current–voltage relationship of n=6 to 8 independent cells. Black traces and circles indicate control; blue traces and circles, chromanol 293B; and red traces and circles, chromanol 293B+linopirdine. Representative traces (**F**) and average values (**G**) showing the attenuated vasodilation induced by retigabine in coronary arteries incubated with chromanol 293B and linopirdine as compared with those incubated with vehicle (control) for 20 minutes before the addition of the vasoconstrictor 1 μmol/L serotonin (5-HT). Results are means±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001 vs control and ##*P*<0.01 linopirdine vs chromanol 293B (1-way ANOVA, Tukey post hoc test). Black traces indicate control; blue traces, chromanol 293B; and red traces, linopirdine. **H**, Kv7.1 and Kv7.5 in isolated aorta myocytes. **Top**, Kv7.1 (**left**), 4,6-diamidino-2-phenylindole (DAPI) nuclei staining (**center**), and merge (**right**). Bar, 20 μm. **Center**, Kv7.5 (**left**), DAPI nuclei staining (**center**), and merge (**right**). Bar, 10 μm. **Bottom**, Negative controls demonstrating antibody-specific staining: no primary antibody (no IgG; **left**), DAPI nuclei staining (**center**), and merge (**right**). Bar, 10 μm. **I**, Kv7.1 and Kv7.5 expression in isolated cava myocytes. **Top**, Kv7.1 (**left**), DAPI nuclei staining (**center**), and merge (**right**). Bar, 20 μm. **Center**, Kv7.5 (**left**), DAPI nuclei staining (**center**), and merge (**right**). Bar, 20 μm. **Bottom**, Negative control demonstrating antibody-specific staining: no primary antibody (no IgG; **left**), DAPI nuclei staining (**center**), and merge (**right**). Bars, 20 μm.

K^+ currents from Kv7.1, Kv7.5, and Kv7.1/Kv7.5 channels, respectively. No important differences in current intensities were observed (Figure 3D and 3E). However, activation of Kv7.1 ($V_{0.5}$, −18±2 mV and *s*, 10±1 mV) was less hyperpolarized than that of Kv7.5 and Kv7.1/Kv7.5 ($V_{0.5}$, −46±3 mV and *s*, 15±1 mV and $V_{0.5}$, −41.0±3 mV and *s*, 7±3 mV, respectively; Figure 3F). In addition, although retigabine did not affect Kv7.1 (Figure 3G), Kv7.5 and Kv7.1/Kv/7.5 currents were activated >2-fold (Figure 3H–3J).

KCNE1 and KCNE3 regulate Kv7.5,[20] we therefore analyzed Kv7.1/Kv7.5 currents in the presence of KCNE1 and KCNE3. KCNE1 raised the current amplitude of the Kv7.1/

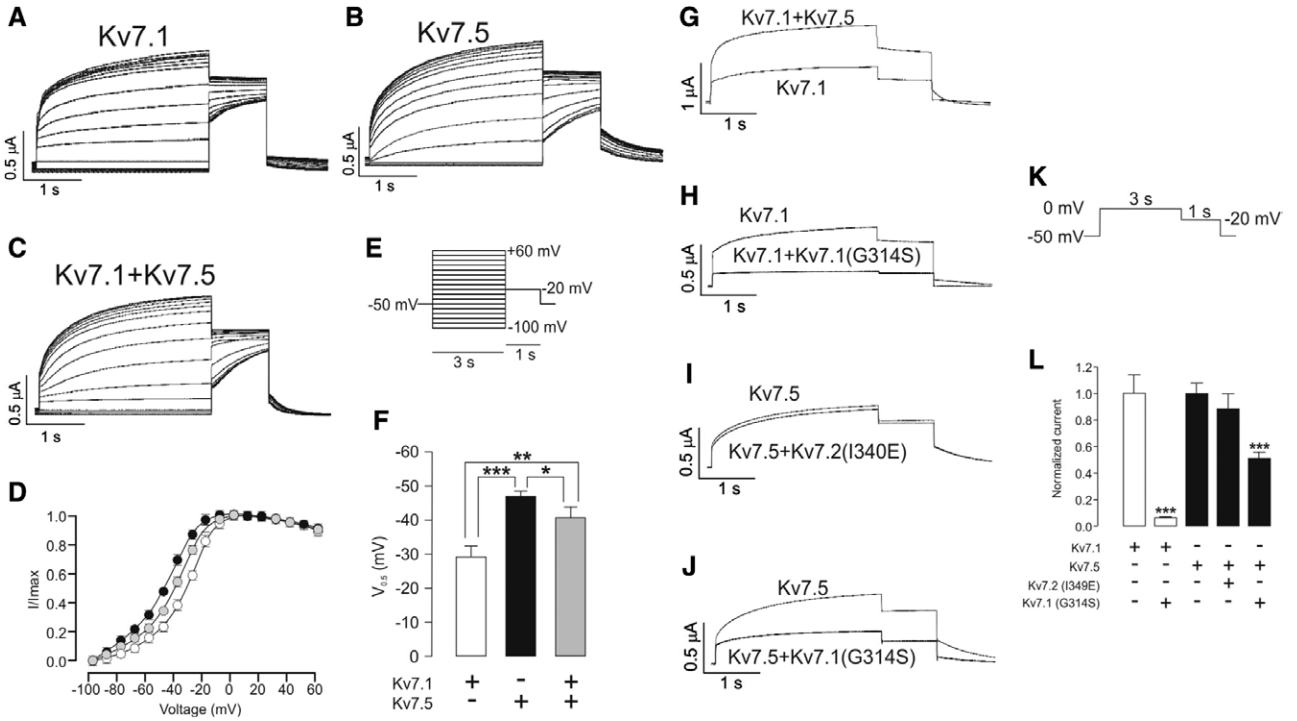

Figure 2. Voltage-dependent K^+ currents in Kv7.1-, Kv7.5-, and Kv7.1/Kv7.5-injected *Xenopus* oocytes. Kv7.1 (**A**), Kv7.5 (**B**), and Kv7.1/Kv7.5 (**C**) oocytes were injected with 10 ng of cRNA for individual Kv7.1 and Kv7.5 or coinjected with 10 ng total cRNA for Kv7.1/Kv7.5 (1:1). The current activated at different voltage test pulses was estimated by measuring the amplitude of corresponding tail currents at −20 mV. **D**, Kv7.1, Kv7.5, and Kv7.1/Kv7.5 current–voltage relationships. Current amplitudes, recorded in n>12 oocytes, were normalized to the maximum value. White circles indicate Kv7.1; black circles, Kv7.5; and gray circles, Kv7.1/Kv7.5. **E**, Pulse protocol. **F**, Half-activation voltages of Kv7.1, Kv7.5, and Kv7.1/Kv7.5. White columns indicate Kv7.1; black columns, Kv7.5; and gray columns, Kv7.1/Kv7.5. + indicates injection of cRNA; and –, absence of cRNA. Values are the mean±SEM (n>12). **P*<0.05; ***P*<0.01; ****P*<0.001 by 1-way ANOVA, Tukey post hoc test. **G** through **L**, Kv7.1 and Kv7.5 currents in the presence of Kv7.1(G314S) and Kv7.2(I340E) mutants. Oocytes were injected with cRNA of Kv7.1, Kv7.5, Kv7.1(G314S), and Kv7.2(I340E) ranging from 10 to 20 ng. Representative traces from oocytes injected with (**G**) 10 ng of Kv7.1 and 20 ng of Kv7.1/Kv7.5 (1:1), (**H**) 10 ng of Kv7.1 and 20 ng of Kv7.1/ Kv7.1(G314S) (1:1), (**I**) 10 ng of Kv7.5 and 20 ng of Kv7.5/Kv7.2(I340E) (1:1), and (**J**) 10 ng of Kv7.5 and 20 ng of Kv7.5/ Kv7.1(G314S) (1:1). **K**, Pulse protocol. **L**, Normalized peak current values at −20 mV of oocytes injected with Kv7.1, Kv7.5, Kv7.1(G314S), and Kv7.2(I340E) cRNAs. + indicates injection of cRNA; and –, absence of cRNA. White columns represent Kv7.1-injected oocytes; ****P*<0.001 vs Kv7.1 alone by Student *t* test. Black columns represent Kv7.5-injected oocytes; ****P*<0.001 vs Kv7.5 alone 1-way ANOVA, Tukey post hoc test. Values are the mean±SEM (n>6).

Kv7.5 heterotetramers (Figure IIIA and IIIC in the online-only Data Supplement), and conversely, KCNE3 reduced K^+ currents (Figure IIIB and IIIC in the online-only Data Supplement). In addition, although KCNE1 triggered a depolarizing shift of $V_{0.5}$ (49±3 mV), KCNE3 left-shifted the $V_{0.5}$ to highly negative values (−50±3 mV; Figure IIID in the online-only Data Supplement). Therefore, Kv7.1/Kv7.5/KCNE3 currents were greater than those elicited by Kv7.1/Kv7.5/KCNE1 at physiologically relevant potentials (0 mV). These data indicated that KCNE peptides, which assemble Kv7.5, also modulate Kv7.1/Kv7.5 heteromeric channels.

A physical association between Kv7.1 and Kv7.5 was unequivocally demonstrated by coimmunoprecipitation and fluorescence resonance energy transfer experiments (Figure 4). The left panel of Figure 4A shows that Kv7.1 is detected in the immunoprecipitate collected during a pull-down assay of Kv7.5YFP in the presence of anti–green fluorescent protein (+AB) but not in its absence (−AB). The right panel of Figure 4A demonstrates that Kv7.5YFP was immunoprecipitated in the presence of anti–green fluorescent protein (+AB). Reciprocal Kv7.1YFP pull-down assays yielded similar results (not shown). In addition, fluorescence resonance energy transfer analysis demonstrated that Kv7.1 and Kv7.5 are in close molecular proximity (Figure 4B–4E). Homotetrameric association between Kv7.1YFP and Kv7.1CFP resulted in 14.7±0.6% positive fluorescence resonance energy transfer. Kv7.1CFP/Kv7.5YFP experiments yielded a similar value of 10.1±0.9%. Fluorescence resonance energy transfer analysis of Kv7.1 and Kv1.5, which share intracellular colocalization (Figure 4F–4H), and Kv7.5 and Kv1.3, both with better surface expression (Figure 5I–5K), gave negative results. Therefore, our results demonstrated that Kv7.1 forms heterotetramers with Kv7.5 with specific biophysical and pharmacological consequences.

Intracellular and Lipid Raft Localization of Kv7.1/Kv7.5 Heterotetrameric Channels

Oligomeric associations influence channel trafficking triggering functional consequences.[30,31] Therefore, we studied the trafficking of Kv7.1, Kv7.5, and Kv7.1/Kv7.5 channels in HEK-293 cells. Kv7.1 showed deficient (7±1%) plasma membrane targeting (Figure 5A–5D) and was mostly retained (84±5%) at the ER (Figure 5E–5H). Kv7.5 exhibited better membrane targeting than Kv7.1 (Figure 5I–5L), although the ER signal is noticeable (Figure 5M–5P). The percentage of colocalization was 14±8% and 46±4% for membrane and ER, respectively.

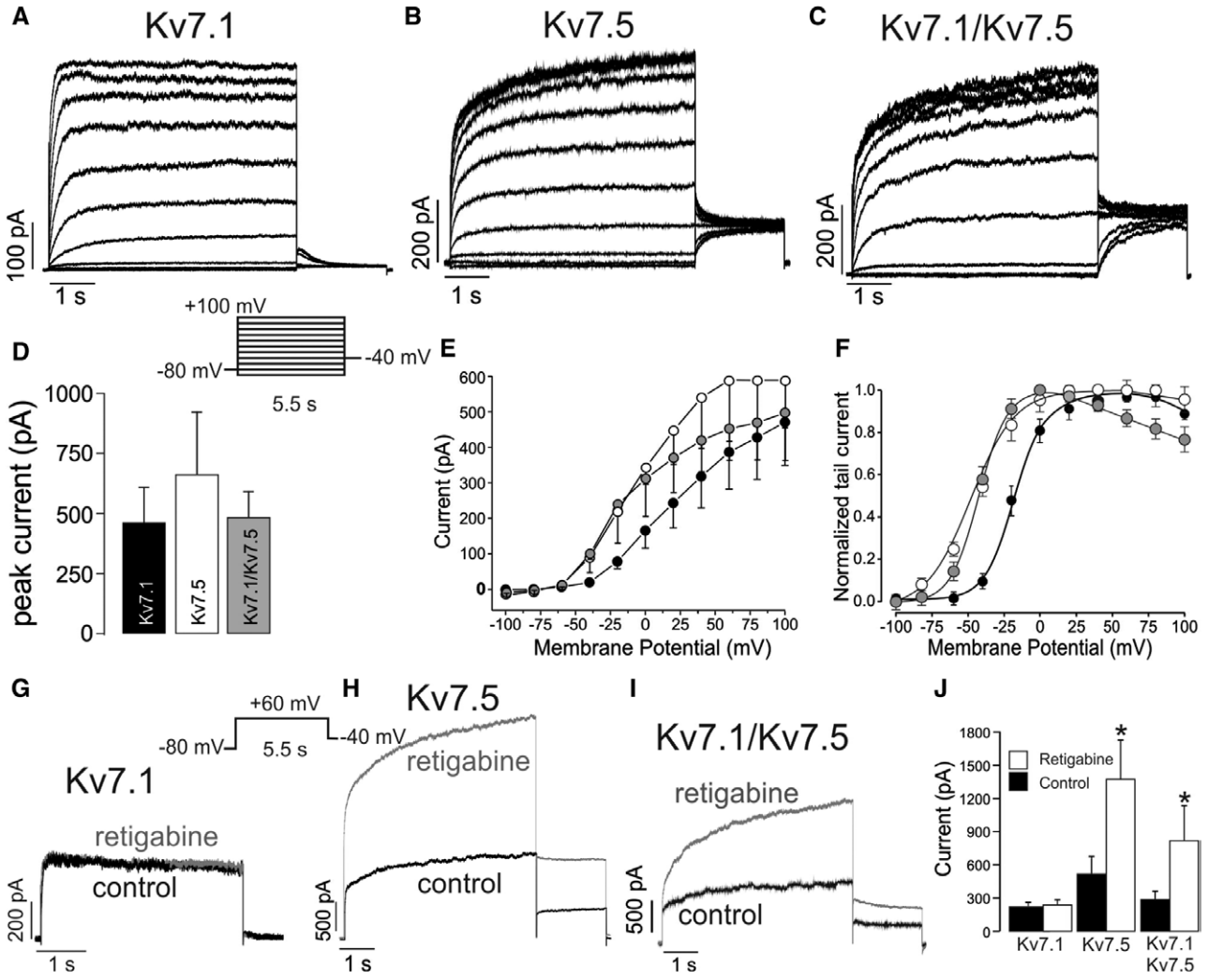

Figure 3. Voltage-dependent K^+ currents in COS7 cells expressing Kv7.1, Kv7.5, and Kv7.1/Kv7.5 channels. **A** to **C**, Representative currents from COS7 cells transfected with Kv7.1 (**A**), Kv7.5 (**B**), and Kv7.1/Kv7.5 (**C**). Currents were evoked by series of 5.5-second pulses from −100 to +100 mV in 20 mV steps from a holding potential of −80 mV and returned to −40 mV. **D**, Peak current intensities at +100 mV. **E**, Current–voltage relationship measured at the end of the pulse for Kv7.1, Kv7.5, and Kv7.1/Kv7.5 channels. **F**, Voltage dependence of activation. Activation curves were obtained by plotting the normalized tail currents vs the membrane potential. The fit with a Boltzmann equation is shown with a solid line. **D** through **F**, Black bar and circles indicate Kv7.1; white bar and circles, Kv7.5; and gray bar and circles, Kv7.1/Kv7.5. Results show the mean±SEM of 4 to 6 cells per group. **G** to **I**, Representative currents at + 60 mV from COS7 cells transfected with Kv7.1 (**G**), Kv7.5 (**H**), and Kv7.1/Kv7.5 (**I**) in the absence or the presence of 10 μmol/L retigabine. Currents were evoked by a 5.5-second pulse from a holding potential of −80 to +60 mV. Black traces indicate control (absence of retigabine); and gray traces, retigabine. **J**, Peak current intensities at +60 mV. Results show the mean±SEM of 4 to 6 cells per group. *P<0.05 retigabine (white bars) vs control (black bars) for Kv7.1, Kv7.5, and Kv7.1/Kv7.5 channels (Student t test).

Kv7.1 and Kv7.5 colocalized (65±4%) and their association increased Kv7.5 retention at the ER (75±5%; Figure 5Q–5T). Conversely, Kv7.1/Kv7.5 surface targeting was low (11±5%; Figure 5U–5X). These data indicate that the presence of Kv7.1 impairs the Kv7.5 traffic to the membrane and increases 2-fold the amount of channel retained in the ER. Although KCNE1 affected the Kv7.1/Kv7.5 biophysical characteristics (Figure III in the online-only Data Supplement) and facilitated the Kv7.1 surface expression,[25] it did not apparently alter the Kv7.1/Kv7.5 intracellular retention. KCNE2 was used as negative control.[26] Thus, a pixel-by-pixel analysis demonstrated that Kv7.1, Kv7.5, and Kv7.1/Kv7.5 in the presence of KCNE1 targeted 20±2%, 23±2% and 18±3%/24±3% to the membrane surface, respectively. KCNE1 mostly colocalized intracellularly with Kv7.1, Kv7.5, and Kv7.1/Kv7.5 (Figure IV in the online-only Data Supplement).

The spatial localization of cardiovascular ion channels in lipid raft microdomains is physiologically relevant.[22–24] Furthermore, vascular smooth muscle caveolar rafts are important for muscular reactivity.[32] We next analyzed whether Kv7.1/Kv7.5 heterotetrameric channels targeted to lipid rafts. Kv7.1 was enriched in rafts (44±8%; Figure 6A), whereas Kv7.5 was almost completely confined to clathrin fractions (90±3%; Figure 6B). When Kv7.1 was cotransfected with Kv7.5, Kv7.1 shifted out of raft domains (96±7%; Figure 6C and 6D). Therefore, the association of Kv7.5 with Kv7.1 contributes to the spatial regulation of Kv7.1/Kv7.5 channels.

Lipid rafts are more ordered and tightly packed than nonraft domains.[27] In this context, we studied channel dynamics by

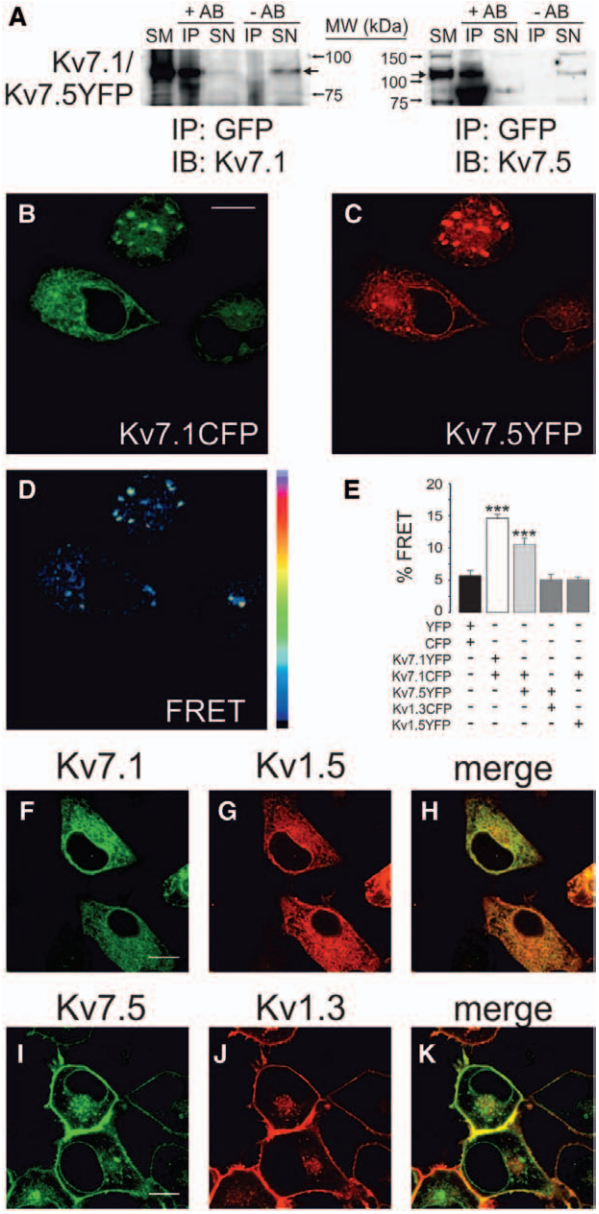

Figure 4. Kv7.1 associates with Kv7.5. **A**, Kv7.1 coimmunoprecipitates with Kv7.5. HEK-293 cells were transfected with Kv7.1 and Kv7.5YFP. Cells were lysed, and total cell extracts were immunoprecipitated (IP) with anti–green fluorescent protein (GFP) antibody. **Left**, Immunoblot (IB) demonstrating that Kv7.1 coimmunoprecipitates with Kv7.5YFP (arrow). **Right**, Western blot against GFP (IB) demonstrating that Kv7.5 immunoprecipitates (arrow). +AB indicates immunoprecipitation in the presence of antibody; –AB, immunoprecipitation in the absence of antibody; SM, starting material; IP, immunoprecipitated; and SN, immunoprecipitated supernatant. **B** through **E**, Representative fluorescence resonance energy transfer (FRET) experiment. Fluorescence signals of Kv7.1CFP and Kv7.5YFP are shown. Kv7.1 (**B**), Kv7.5 (**C**), FRET channel (**D**). **D**, Yellow fluorescent protein (YFP) fluorescence intensity after ciano fluorescent protein (CFP) excitation. **E**, Histogram showing FRET efficiency of different protein combinations. Negative control experiments were performed with cells expressing CFP and YFP alone (black column), Kv7.1 and Kv1.5 and Kv7.5 and Kv1.3 (dark gray columns). Positive control experiments were performed using cells expressing Kv7.1CFP and Kv7.1YFP (white column). ***P<0.001 vs CFP/YFP (n=10, Student t test). **F** through **H**, Representative FRET experiment between Kv7.1 and Kv1.5: (**F**) Kv7.1, (**G**) Kv1.5, and (**H**) merge in yellow. **I** to **K**, Representative FRET experiment between Kv7.5 and Kv1.3: (**I**) Kv7.5, (**J**) Kv1.3, and (**K**) merge in yellow. Bars, 10 μm.

fluorescence recovery after photobleaching. Because Kv7.5 altered Kv7.1 location, we analyzed Kv7.1YFP in the presence or the absence of Kv7.5 (Figure V in the online-only Data Supplement). Figure VA and VB in the online-only Data Supplement shows representative fluorescence recovery after photobleaching experiments, and Figure VC to VE in the online-only Data Supplement depicts the results collected in n>12 independent cells. Fluorescence recovery was monitored until a steady state was achieved (Figure VC in the online-only Data Supplement). Mobile fractions were similar (69±4% and 62±5% for Kv7.1 and Kv7.1/Kv7.5, respectively; Figure VD in the online-only Data Supplement), but Kv7.1/Kv7.5 heterotetramers exhibited higher lateral mobility (16±1 and 12±1 seconds for Kv7.1 and Kv7.1/Kv7.5, respectively; P<0.05; Figure VE in the online-only Data Supplement). These results further indicated that the association of Kv7.5 with Kv7.1 targets the Kv7.1-containing channels to different domains with higher mobility.

Because heterologous results were striking, we explore their raft distribution in the heart, brain, quadriceps, aorta, and cava (see Figure 1A for expression). Figure 6E shows that Kv7.1 targeted to lipid rafts in the heart. However, Kv7.1 localization in the rest of tissues, where Kv7.5 expression is notable, resembled that observed in HEK-293 cells (Figure 6F–6I).

Discussion

We demonstrate that Kv7.1 and Kv7.5 form functional heteromeric channels with novel properties. Although the effect on macroscopic currents was small, this association dramatically affects subunit trafficking and localization. On assembly with Kv7.5, Kv7.1 subunits are excluded from lipid rafts. In addition, the presence of Kv7.1 results in intracellular retention of the Kv7.1/Kv7.5 heterotetramer.

Kv channels are crucial in cardiovascular muscle contraction, and Kv7.1, Kv7.4, and Kv7.5 are important for appropriate smooth muscle tone.[6] Kv7.5 was previously thought to form heterotetramers with Kv7.3 exclusively.[8,15,16] However, Kv7.4 and Kv7.5 associate forming heteromers that are responsible for differential responses to diclofenac in smooth muscle.[18,33] In contrast, it was thought that Kv7.1 does not associate with any other Kv7 subunit.[2,3,13,18] However, Kv7.1 coimmunoprecipitates with Kv11.1 in the heart.[34] Lerche et al[15] indicated that currents in oocytes fail to reveal interactions between Kv7.1 and Kv7.5. Our results are in line with those of Lerche et al[15] because the currents were indeed similar. Although the lack of functional evidence for interactions between Kv7.1 and Kv7.5 reported by Lerche et al[15] has become a consolidated argument against the existence of Kv7.1 and Kv7.5 associations,[15] our results provide functional evidence that such interactions do indeed take place.

Pharmacological paradigms, used to decipher the role of Kv7 channels in blood vessels, generate an important debate. In vascular myocytes, Kv7 current suppression triggers Ca2+ influx and vasoconstriction, whereas Kv7 channel activation is typically associated with vasodilatation.[6] Vessels constrict after application of Kv7 channel blockers XE991 and linopirdine but not chromanol 293B.[5,11,12,35] These results point to Kv7.4 and Kv7.5 but not Kv7.1 as major determinants of resting membrane potential in vascular smooth muscle cells.[36]

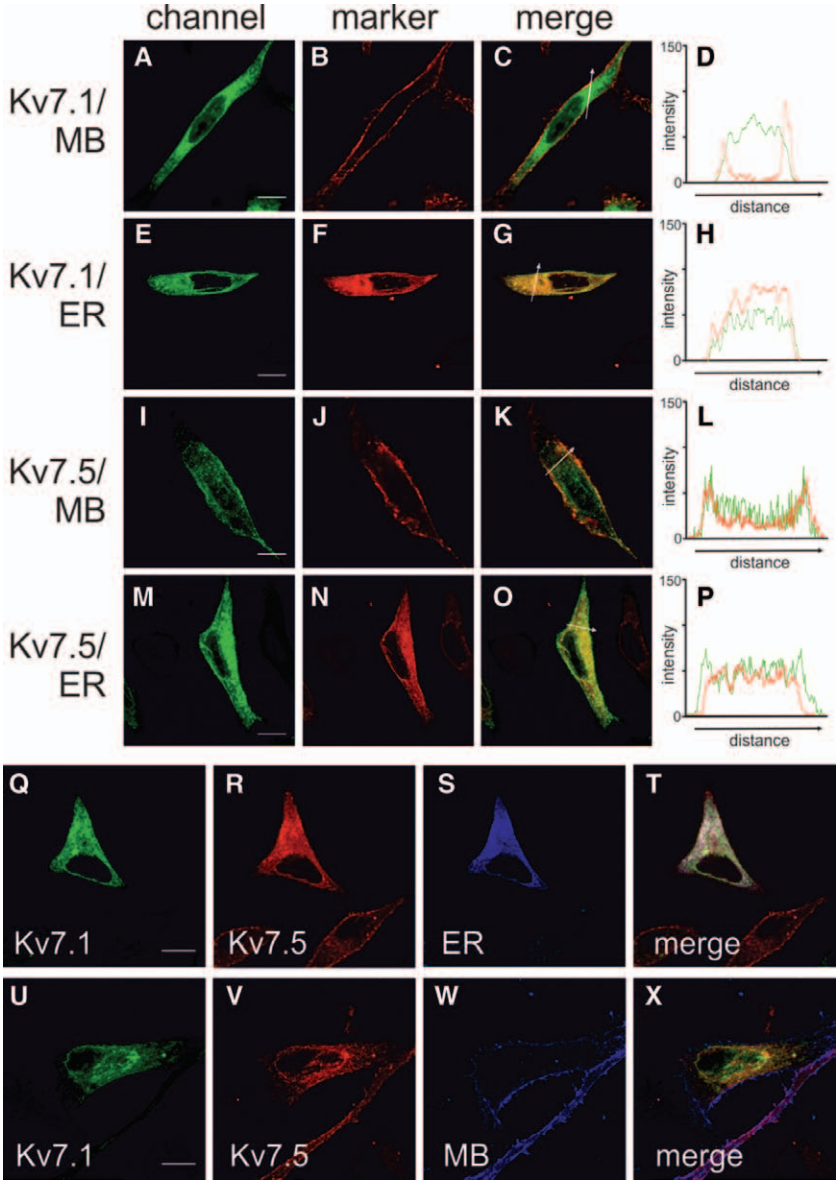

Figure 5. Kv7.1 impairs Kv7.5 cell surface expression. HEK-293 cells were transfected with Kv7.1YFP, Kv7.5YFP, and Akt-PH-pDsRed as membrane (MB) marker and DsRed-ER as endoplasmic reticulum (ER) marker. **A** through **D**, Kv7.1 MB surface expression. **E** through **H**, Kv7.1 endoplasmic reticulum retention. **I** through **L**, Kv7.5 MB surface expression. **M** through **P**, Kv7.5 and the endoplasmic reticulum. **A**, **E**, **I**, and **M**, Channels in green. **B**, **F**, **J**, and **N**, Markers in red. **C**, **G**, **K**, and **O**, Merge in yellow. **D**, **H**, **L**, and **P**, Histogram of the pixel-by-pixel analysis of the arrow section in merge panels. **Q** through **T**, Coexpression of Kv7.1, Kv7.5, and DsRed-ER marker. **U** through **X**, Coexpression of Kv7.1, Kv7.5, and Akt-PH-pDsRed MB marker. Kv7.1 (**Q** and **U**), Kv7.5 (**R** and **V**), markers (**S** and **W**), and merge (**T** and **X**). Yellow and white represent double and triple colocalization, respectively. Bars, 10 μm.

However, KCNE1 and KCNE3 are present in myoblasts[10,20] and alter the sensitivity of Kv7.1 to chromanol 293B[5]; KCNE3 also modulates Kv7.1 and Kv7.5 currents.[20,26] For that reason, pharmacological tools are unreliable in evaluating Kv7 contribution. Therefore, although Kv7.1 is highly expressed in several blood vessels,[5,6,10,17] its role remains uncertain. However, Greenwood and coworkers demonstrate that in contrast to previous assumptions, Kv7.1 channels expressed in arterial myocytes are functional and although do not seem to contribute to resting vascular tone,[36] Kv7.1 activators are effective vasorelaxants.[29] The formation of heterotetrameric Kv7.1/Kv7.5 and Kv7.4/Kv7.5 channels, in addition to the association of KCNE subunits, may help decipher ambiguous pharmacological results.[36] In this context, our Kv7.1(G314S)/Kv7.5 results unequivocally support Kv7.1/Kv7.5 heterotetramer formation. The role of KCNE in the Kv7.1/Kv7.5 hybrid complex warrants further research.

Kv7.5 targeted to the cell membrane more efficiently than Kv7.1, but its association with Kv7.1 shifted Kv7.5 to the ER. These results further suggest physical interactions that perturb the trafficking of Kv7.1/Kv7.5 heteromers to the surface. Many Kv heteromeric associations behave similarly. For instance, Kv1.5 associates with Kv1.3, and its expression results in dose-dependent ER retention of Kv1.3.[31] Furthermore, localization in membrane microdomains is also affected. Kv7.1/Kv7.5 association impairs Kv7.1 localization to cholesterol-rich rafts. This spatial regulation has important consequences on physiological responses. Many cardiovascular channels are concentrated in caveolar and noncaveolar rafts to interface with signaling molecules.[22,23] For instance, displacement of heteromeric Kv7.2/Kv7.3 M-channels from rafts prevents muscarinic-mediated suppression of the M-current in sympathetic neurons.[37]

The physiological consequences of Kv7.1/Kv7.5 heteromeric channel spatial regulation require several considerations: (1) the putative association of Kv7 channels to KCNE peptides, (2) calmodulin-mediated Ca^{2+} sensitivity of Kv7 channels, and (3) the physiological events initiated in cardiovascular smooth muscle rafts. First, KCNE1 and KCNE3,

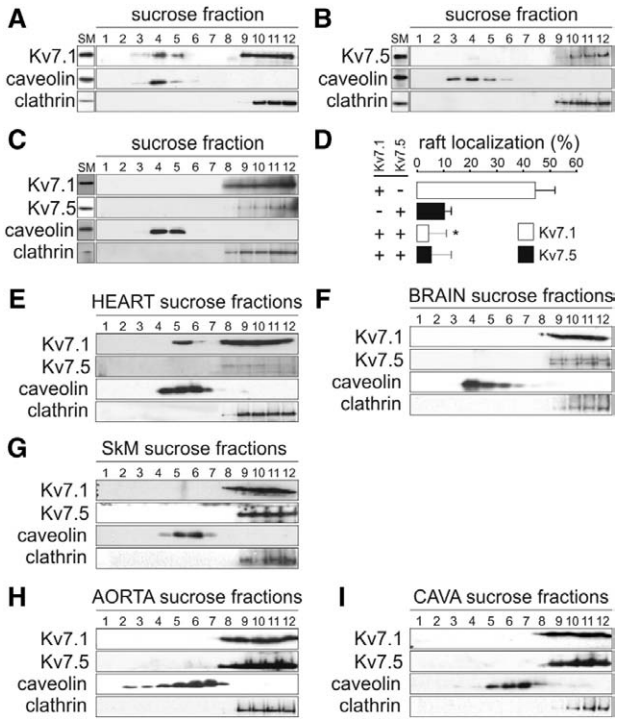

Figure 6. Kv7.1 and Kv7.5 channels differentially target to lipid rafts in HEK-293 cells and in rat tissues. Unlike Kv7.5 and Kv7.1/Kv7.5 heterotetramers, Kv7.1 channels target to lipid rafts. **A** through **C**, Sucrose density gradient of extracts from cells transfected with Kv7.1 (**A**), Kv7.5 (**B**), or cotransfected with Kv7.1 and Kv7.5 (**C**). Although caveolin indicates low-buoyancy rafts, clathrin distributes in nonfloating fractions. Numbers denote different fractions from the top (1) to the bottom (12) of the sucrose density gradient. **D**, Percentage of Kv7.1 (white columns) and Kv7.5 (black columns) localized in rafts in the absence (–) or the presence (+) of Kv7.1 and Kv7.5. Values are the mean±SEM (n=3). *P<0.05 vs Kv7.1 in the absence of Kv7.5 (Student t test). **E** through **I**, Representative sucrose density gradient fractions of the heart (**E**), brain (**F**), skeletal muscle (SkM; **G**), aorta (**H**), and cava (**I**).

which associate with Kv7.1 and Kv7.5 and modulate channel trafficking and gating, are highly expressed in blood vessels.[10] Although KCNE1 increases Kv7.1 and Kv7.5 currents,[20,38,39] the association of KCNE3 with Kv7.1 and Kv7.5 confines channels to nonraft domains.[25,26] It is intriguing that KCNE1 and KCNE3 affect both channels similarly. Second, Kv7 channels have differential calmodulin-mediated Ca^{2+} sensitivity. Ca^{2+} has dramatic effects on Kv7 channels.[40] Ca^{2+}-calmodulin regulates the trafficking of Kv7 channels and strongly reduces Kv7.5 currents but not Kv7.1 or Kv7.1/KCNE1.[40,41] Calmodulin is also involved in KCNE4 modulation of Kv7.1[42]; and KCNE4, but not KCNE1, impairs Kv7.1 trafficking to rafts.[25] Taken together, these results suggest that heteromeric associations of cardiovascular Kv7 channels influence their localization to microdomains, and their location fine-tunes the blood vessel vasoconstriction response to diverse effectors. Finally, membrane rafts and caveolae are crucial in cardiovascular signaling.[22,23] These microdomains are important in regulating vascular smooth muscle cells by initiating Ca^{2+} signal transduction pathways, thereby providing spatially and temporally efficient regulation.[32] Rafts concentrate channels and pumps that are important for Ca^{2+} homeostasis.[43] For example, Ca^{2+} extrusion by plasma membrane Ca^{2+} pumps is impaired in caveolin-1 knockout mice and in chemically disrupted rafts.[44] A rise in intracellular Ca^{2+} facilitates Kv7 interaction with calmodulin, activating smooth muscle cell contraction in blood vessel walls and leading to vasoconstriction. Vasoconstrictor mediators trigger vascular smooth muscle cell contraction by increasing cytosolic Ca^{2+} concentration, and aberrant Ca^{2+} signaling is characteristic of pulmonary arterial hypertension disease.[11] Physiological concentrations of arginine vasopressin suppress Kv7.5 currents and activate Ca^{2+} channels, thereby stimulating action potentials and triggering blood vessel constriction via protein kinase C, which is concentrated in lipid rafts.[43,45] Interestingly, the anticonvulsant retigabine, a Kv7 activator, causes hypotension[28] and effectively activates Kv7.1/Kv7.5 heterotetrameric channels. Until now, the role of Kv7.1 has been uncertain. Our results have clinical significance because Kv7 channels control smooth muscle contractility, which makes them excellent targets for the development of pharmaceutical therapies. Although pharmacology suggests a complex scenario, Kv7.1, Kv7.4, and Kv7.5 exhibit preferential cardiovascular expression which opens the treatment of smooth muscle disorders with few hazardous neurological consequences.

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Disclosures

None.

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Significance

Voltage-dependent K^+ (Kv) channels from the Kv7 family modulate vascular smooth muscle tone. Kv7.1, Kv7.4, and Kv7.5 are expressed in blood vessels and contribute to cardiovascular physiology. Kv7.1 is highly expressed in many vessels, but Kv7.1 blockers fail to modulate smooth muscle reactivity. We describe that Kv7.1 and Kv7.5 can form functional heterotetrameric channels. We include electrophysiology studies in isolated arterial myocytes, mammalian, and oocyte expression systems, fluorescence resonance energy transfer experiments, lipid rafts in native tissues (heart, brain, skeletal muscle, and blood vessels), and protein studies with rat and human tissue samples. Assembly of Kv7.1/Kv7.5 channels triggers emerging properties on vascular muscle physiology. Lipid raft localization of ion channels is crucial for cardiovascular physiology, and we propose that the formation of Kv7.1/Kv7.5 heteromeric channels provides efficient spatial and temporal regulation of smooth muscle cell function. Our results shed light on the debate about the relative contribution of Kv7 channels to vasoconstriction and hypertension.