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Authors
Jepps, Thomas
Abbott, Geoffrey

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Kcne4 deletion sex-dependently alters vascular reactivity

Geoffrey W. Abbott[1,*] and **Thomas A. Jepps**[2,*]

[1]Bioelectricity Laboratory, Department of Pharmacology and Department of Physiology and Biophysics, School of Medicine, University of California, Irvine, CA, USA

[2]Ion Channels Group, Department of Biomedical Sciences, University of Copenhagen, Denmark

Abstract

Voltage-gated potassium (Kv) channels formed by Kv7 (KCNQ) α-subunits are recognized as crucial for vascular smooth muscle function, in addition to their established roles in the heart (Kv7.1) and the brain (Kv7.2-5). *In vivo*, Kv7 α-subunits are often regulated by KCNE subfamily ancillary (β) subunits. We investigated the effects of targeted germline *Kcne4* deletion on mesenteric artery reactivity in adult male and female mice. *Kcne4* deletion increased mesenteric artery contractility in response to α-adrenoceptor agonist methoxamine, and decreased responses to Kv7.2-7.5 channel activator ML213, in male but not female mice. In contrast, *Kcne4* deletion markedly decreased vasorelaxation in response to isoprenaline in both male and female mice. *Kcne4* expression was two-fold lower in female versus male mouse mesenteric artery, and *Kcne4* deletion elicited only moderate changes of other *Kcne* transcripts, with no striking sex-specific differences. However, Kv7.4 protein expression in females was twice that in males, and reduced in both sexes by *Kcne4* deletion. Our findings confirm a crucial role for KCNE4 in the vasculature, and provide the first known molecular mechanism for sex-specificity of this modulation that has important implications on vascular reactivity and may underlie sex-differences in susceptibility to cardiovascular diseases.

Keywords

Kv7 channels; KCNE subunits; KCNE4; KCNQ; potassium channels; vascular physiology; smooth muscle

Introduction

KCNE4 is a member of the KCNE family (KCNE1-5) of single transmembrane-spanning β-subunits. Although these proteins do not form functional channels by themselves, they co-assemble with pore forming α-subunits of voltage-gated potassium (Kv) channels to regulate the trafficking, biophysical properties and pharmacology of the resultant heteromeric channel complexes (1). The KCNE β-subunits can dramatically affect the activity of different ion channels; however, our understanding of their functional impact in many native

*To whom correspondence should be addressed: Dr. Geoffrey W. Abbott, A-360 Medical Surge II, Department of Pharmacology, School of Medicine, University of California, Irvine, Irvine, CA 92697-4625, USA. abbottg@uci.edu; or Dr. Thomas A. Jepps, Department of Biomedical Sciences, University of Copenhagen, The Panum Institute, Blegdamsvej 3, 2200 Copenhagen N, Denmark. tjepps@sund.ku.dk.

systems is still limited. Recently, we showed that KCNE4 (also known as MiRP3) plays a fundamental role in regulating vascular tone (2). In mesenteric artery myocytes from adult male rats, KCNE4 co-assembled with the Kv7.4 and Kv7.5 α-subunits (encoded by KCNQ4 and KCNQ5, respectively), and knockdown of KCNE4 coincided with reduced Kv7.4 membrane expression and a more depolarized membrane potential (2).

Kv7.4 is a member of the Kv7 subfamily of Kv channel α-subunits, and has been widely reported to regulate vascular (and non-vascular) smooth muscle resting membrane potential (3-5). The Kv7 family is comprised of five members (Kv7.1-Kv7.5), of which Kv7.1, Kv7.4, and Kv7.5 are expressed in different rodent and human arteries (3, 5, 6). In vascular smooth muscle, Kv7.4 and Kv7.5 channels predominate functionally, and recent evidence suggests these isoforms co-assemble to form heteromultimeric channels (2, 7, 8). Pharmacological blockade of Kv7 channels produces vasoconstriction in several rodent and human blood vessels, and activation of Kv7 channels mediates relaxation in pre-contracted vessels (5, 6, 8-10). Moreover, Kv7 channel blockade, or Kv7.4 channel knockdown, impairs various receptor-mediated vasorelaxations in different arteries. Thus, the vascular Kv7 channels are regarded as functional mediators of different receptor-activated vasodilatations (7, 11-15).

To date, these studies have used mainly male animal models, even though there are many sex-dependent differences in vascular physiology (16, 17). Susceptibility to certain vascular diseases also differs between genders. Vascular diseases, such as hypertension, are typically more common in males and post-menopausal women than in pre-menopausal women (18-21). Sex-dependent differences have been associated with different circulating androgen levels; however, we still do not understand the molecular mechanisms responsible for these differences in the vasculature (17, 18, 22).

Recently, using a germline *Kcne4-deleted (Kcne4*$^{-/-}$*)* mouse line, we showed cardiac KCNE4 expression to be positively regulated by 5α-dihydrotestosterone (DHT), which corresponded with male mice displaying higher ventricular myocyte Kv current densities compared to female mice (23). Ventricular Kv current density in young sexually mature female mice was unaffected by *Kcne4* deletion and was similar to that of male *Kcne4*$^{-/-}$ mice. This difference was associated with KCNE4 upregulating Kv1.5 and Kv4.2 currents specifically in male *Kcne4*$^{+/+}$ mice due to *Kcne4* expression upregulation by DHT.

Given the newly identified role for KCNE4 in the vasculature, here we investigated the effects of *Kcne4* deletion on vascular function in mice, and found that *Kcne4* sex-specifically regulates mouse vascular reactivity.

Methods

Animals

The *Kcne4*$^{-/-}$ mouse line was generated by replacement of the sole coding exon of *Kcne4* with *neo* and *LacZ* genes in 129/Sv embryonic stem cells for embryonic injection followed by implantation into a C57 breeder. Progeny were genotyped by PCR and for final confirmation, Southern blot (performed by Lexicon, The Woodlands, TX; and Texas A&M Institute for Genomic Medicine, College Station, TX; data not shown) (23). Heterozygotes

(Kcne4$^{+/-}$*)* were then backcrossed for at least 10 generations into the C57BL/6 strain. We genotyped the colony by conventional PCR of genomic DNA isolated from tail tips, using primers of the following sequences: *Kcne4* forward 5′-CAACGACAGCAGTGAAGGC-3′, *Kcne4* reverse 5′-GCAGAGCAAAAGCAAAACCC-3′, Neo3a 5′-GCAGCGCATCGCCTTCTATC (23). Mice were housed in a pathogen-free facility; colony maintenance, genotyping, euthanasia and tissue isolations were approved by the Animal Care and Use Committees at University of California, Irvine, and were conducted in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Myography

Studies were performed on 6-8 month-old sexually mature male and female mice, after euthanasia by CO_2 asphyxiation. The intestines were immediately removed, placed in ice-cold physiological saline solution (PSS) and 2nd and 3rd order mesenteric arteries dissected. The arteries were cleaned of adherent tissue and segments (~2 mm) were mounted on 40 μm stainless steel wire in a myograph (Danish Myo Technology) for isometric tension recording. The myograph chambers were filled with PSS, maintained at 37 °C and aerated with 95 % O_2 / 5 % CO_2. The vessels were allowed to equilibrate at 37 °C before undergoing a passive force normalization procedure.

Increasing concentrations (0.1-30 μM) of the α-adrenoceptor agonist, methoxamine (Sigma), were applied. The vessels were then washed in fresh PSS before they were pre-contracted with 10 μM methoxamine and increasing concentrations of the Kv7.2-7.5 channel activator, ML213 (0.1-10 μM) (Tocris), or the β-adrenoceptor agonist, isoprenaline (0.1-3 μM) (Sigma), were applied. The isoprenaline concentration response curve was then repeated in the presence of the Kv7 channel blocker, linopirdine (10 μM) (Sigma).

Real-time quantitative PCR (qPCR)

We quantified relative expression of the *Kcne1-5* isoforms in the mesenteric arteries of male and female *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice by qPCR analysis, as we described previously (5). Briefly, RNA was extracted with the RNAEasy Micro Extraction Kit, including a DNase treatment, according to the manufacturer's instructions (Qiagen). The Nanoscript 2 kit (PrimerDesign Ltd., Southampton, U.K.) was used to reverse-transcribe the RNA. The cDNA samples (concentration of 1.5 ng/μl) were used for quantitative analysis of *Kcne* transcript expression using Precision PLUS-iC SYBR mastermix (PrimerDesign Ltd., Southampton, U.K.) in 20 μl samples containing 5 μl of cDNA and 300 nM primer. Experiments were run with the following cycling conditions: initial activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hertfordshire, U.K.), and data was collected during each cycling phase. Melt-curve analysis completed the protocol. RNA samples that were reverse transcribed without the nanoscript enzyme and no-template controls (NTCs) were run alongside all reactions to assess contamination. Quantification cycle (Cq) values were determined using Bio-Rad CFX96 Manager 3.0 software. The optimal reference genes were β-Actin and cytochrome c-1 as determined by geNorm analysis (Biogazelle). The expression levels of the *Kcne* isoform transcripts were calculated relative to these reference genes in

each artery to give a relative isoform expression profile using $2^{-\Delta Cq}$ (24). All primer assays were obtained from PrimerDesign Ltd. and the sequences for the *Kcne1-5* primers are given in Table 1.

Western blotting

We homogenized ~5 mm-long mouse mesenteric artery tissue samples (1 sample each from 4-5 different mice per sex per genotype) in 100 μl of PBS (pH 7.5) containing protease inhibitor cocktail (Thermo Fisher, Waltham, MA, USA) and 10% SDS (w/v) using a motorized Eppendorf homogenizer. Samples were then centrifuged for 10 minutes at 3 × g at room temperature (to avoid solidification arising from the high SDS content). The supernatants were resuspended in LDS gel loading buffer (Thermo Fisher) containing 25 mM tris(2-carboxyethyl)phosphine, heated for 10 minutes at 65 °C, vortexed, centrifuged for 3 minutes at 5 × g, and then separated by SDS-PAGE. Proteins were transferred to PVDF membranes and western blotted with 1/500 rabbit polyclonal anti-KCNQ4 (Santa Cruz Biotechnology, Dallas, TX, USA) and 1/1000 rabbit polyclonal anti-GAPDH (Abcam, Cambridge, UK) antibodies, with chemiluminescent detection via 1/5000 HRP-conjugated goat anti-rabbit IgG secondary antibodies (BioRad, Hercules, CA, USA). Band densities were quantified using ImageJ software (NIH, Bethesda, MA, USA) and Kv7.4 band density values each normalized to same-lane GAPDH band density.

Statistical Analysis

Results are shown as means ± s.e.m., unless otherwise stated. P-values <0.05 were taken as showing significant differences between means. Individual $LogEC_{50}$ values in figures 1C and 2C were calculated using a standard slope, equal to a Hill slope (or slope factor) of 1.0 for methoxamine and -1.0 for ML213. Differences between means were assessed for statistical significance with unpaired Student's t-tests. A two-way ANOVA followed by a Bonferroni posttest was used to determine the significance of linopirdine on the isoprenaline-concentration effect curves and to compare the effect of isoprenaline between *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ male and female mice. Unpaired Student's t-tests were used to compare changes in specific *KCNE* and *KCNQ* isoform transcript expression. ANOVA with post-hoc Tukey HSD test was used to compare Kv7.4 western blot band densities.

Results

Application of sequentially increasing concentrations of the α-adrenoceptor agonist, methoxamine, caused a similar concentration-dependent constriction of mesenteric artery segments from both female (n = 8) and male (n = 6) *Kcne4*$^{+/+}$ mice, with mean $LogEC_{50}$ values of -5.2 ± 0.05 M and -5.2 ± 0.05 M (P = 0.31, according to an unpaired t-test), respectively (Figure 1). In addition, the maximum force developed by the female and male artery segments did not differ significantly (4.3 ± 0.46 mN and 4.5 ± 0.53 mN, respectively; P = 0.75, according to an unpaired t-test). In the females, the response of mesenteric artery segments from the *Kcne4*$^{-/-}$ mice to methoxamine ($LogEC_{50}$ of -5.1 ± 0.01 M) was not significantly different from that of the *Kcne4*$^{+/+}$ females (P = 0.53, according to an unpaired t-test; Figure 1 B, C). In contrast, segments of mesenteric artery from male *Kcne4*$^{-/-}$ mice (n = 6) were significantly more sensitive to increasing concentrations of methoxamine

($LogEC_{50}$ of -5.6 ± 0.08 M; P = 0.006, according to an unpaired t-test) compared to the *Kcne4*$^{+/+}$ mesenteric arteries (Figure 1B, C); however, the maximum force developed at 30 μM methoxamine was not different in mesenteric artery segments between the male *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice (4.6 ± 0.89 mN; P = 0.97, according to an unpaired t-test).

Application of ML213 produced concentration-dependent relaxations that were not different in mesenteric arteries from the male and female *Kcne4*$^{+/+}$ mouse (P = 0.24 by unpaired t-test; Figure 2). In the females, there was no significant difference of vascular reactivity to ML213 in the *Kcne4*$^{-/-}$ mice (n = 6) compared to the *Kcne4*$^{+/+}$ mice (n = 5) (Figure 2 B,C). In contrast, *Kcne4* deletion in the mesenteric artery segments of male mice shifted the $LogEC_{50}$ for vascular response to ML213 from -6.01 ± 0.1 M (*Kcne4*$^{+/+}$, n = 5) to -5.7 ± 0.04 M (*Kcne4*$^{-/-}$, n = 6; P = 0.005 in unpaired t-test; Figure 2B,C).

As previously reported, isoprenaline-induced relaxations are mediated, in part, by Kv7 channels (11). Figure 3 shows isoprenaline caused robust, concentration-dependent relaxations in precontracted mesenteric artery segments from the *Kcne4*$^{+/+}$ male and female mice that were not statistically different between the sexes. In both male (Figure 3A) and female (Figure 3B) *Kcne4*$^{+/+}$ mouse mesenteric arteries, the isoprenaline relaxations were inhibited by linopirdine (10 μM) (n = 5 - 6).

We next determined the impact of *Kcne4* deletion on isoprenaline-mediated relaxations in mesenteric arteries from male and female mice. Isoprenaline was markedly less effective at relaxing the mesenteric arteries of *Kcne4*$^{-/-}$ male and female mice (>2-fold less relaxation at 1 μM and 3 μM isoprenaline compared to *Kcne4*$^{+/+}$; P < 0.05, n = 4-8) (Figure 4).

Finally, we employed qPCR to quantify the expression of *Kcne1-5* and *Kcnq1*-5 isoform transcripts using mesenteric artery cDNA from male and female *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice (Figure 5). Expression of *Kcne4* was >two-fold higher in male mouse mesenteric artery tissue compared to that of the female mice (n = 7; P = 0.0061 by unpaired t test). *Kcne1* was detectable in 5 out of 7 female mesenteric artery samples but not detectable in any male mouse mesenteric arteries (n = 7; P = 0.016 by unpaired t test). *Kcne3* displayed the highest levels of expression, and, as also observed for *Kcne2* and *Kcne5*, this expression level was sex-independent (Figure 5A). As expected, *Kcne4* transcript was not detectable in *Kcne4*$^{-/-}$ mesenteric arteries from either sex, confirming efficient germline deletion (Figure 5 B, C). *Kcne4* deletion was associated with ~50% increased expression of the already highly expressed *Kcne3* transcript in both male and female mice, which was significant in male (n = 6; P = 0.006) but not female mesenteric arteries (n = 6; P = 0.104) (Figure 5 B, C). *Kcne4* deletion was not associated with any other changes in other Kcne isoform expression in male or female mouse mesenteric arteries (Figure 5 B, C). We also analysed expression of *Kcnq1-5* transcripts in male and female *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice mesenteric arteries. There was no change in *Kcnq* transcript expression between *Kcne4*$^{+/+}$ male and female mesenteric arteries (n = 6; Figure 6 A), nor did we detect any change in *Kcnq* transcript expression in *Kcne4*$^{+/+}$ mice compared to *Kcne4*$^{-/-}$ in both male and female mice (n = 6; Figure 6 B, C).

In contrast, *Kcne4* deletion reduced Kv7.4 protein expression. Kv7.4 protein expression in mouse mesenteric artery tissue preparations from female mice was double of that observed in male mice ($P<0.05$), and was reduced in both sexes by Kcne4 deletion (Figure 7 A, B), with the comparison for female $Kcne4^{+/+}$ versus male or female $Kcne4^{-/-}$ mice achieving statistical significance by ANOVA after Tukey HSD test ($P = 0<0.01$; Figure 7 B).

Discussion

This is the first study to identify sex-dependent differences in the function and expression levels of an ion channel ancillary subunit in the vasculature. Our findings show that *Kcne4* expression is higher, and functional effects of its deletion more striking, in the mesenteric arteries of male mice compared to female mice. Using a $Kcne4^{-/-}$ mouse line we found that targeted germ line *Kcne4* deletion renders male, but not female, mouse mesenteric arteries more sensitive to α-adrenergic stimulation, and less responsive to the Kv7.2-7.5 activator ML213. These data suggest that males are more sensitive to changes in *Kcne4* expression in the vasculature than females. However, responses to the β-adrenoceptor agonist, isoprenaline, were inhibited in both male and female $Kcne4^{-/-}$ mice, suggesting Kcne4 is important in mediating the effects of this receptor in both sexes.

Kv7 channels, particularly Kv7.4 and Kv7.5, are important regulators of vascular tone (3, 6-15, 25, 26). These channels have been shown extensively to be expressed and functionally important in several human and rodent arteries. We previously reported that KCNE4 is expressed in several arteries, and that targeted KCNE4 knockdown in male rat mesenteric arteries using a morpholino strategy depolarized the smooth muscle resting membrane potential and reduced vasorelaxations in response to a Kv7.2-7.5 activator (S-1) (2). We found that KCNE4 co-assembles with Kv7.4 and Kv7.5 in mesenteric myocytes, left-shifting the voltage-dependence of Kv7.4 channel activation and increasing Kv7.4 membrane expression (2). Our findings in the current study, using male $Kcne4^{-/-}$ mice, confirm these previous findings and highlight the crucial regulatory role KCNE4 has on vascular Kv7 channel function in male mesenteric arteries. Importantly, this study highlights a novel role for the KCNE4 subunit in mediating β-adrenoceptor responses in the mesenteric artery. Kv7 channels in the vasculature contribute to different Gs-coupled receptor mediated relaxations, yet the exact signaling mechanisms are still unclear (7, 11, 12, 14, 15). Following Gs coupled-receptor activation, a cAMP-dependent mechanism (11) and the βγ-subunits (15) have both been shown to enhance Kv7.4/Kv7.5 channel activity. The current study confirms that isoprenaline effects in the mesenteric artery are sensitive to Kv7 blockade (linopirdine) and reveals an important role for KCNE4 in mediating these vasorelaxant effects in the mesenteric arteries of both males and females. Although Kv7 channel function was unaffected in the female $Kcne4^{-/-}$ mice, it is interesting that the responses to isoprenaline were equally impaired in the male and female $Kcne4^{-/-}$ mice. These data suggest the β-adrenoceptor intracellular signaling relies on KCNE4 to activate the vascular Kv7 channels. Interestingly, the KCNE4 protein contains a consensus cAMP phosphorylation site and a putative βγ-binding site (27) in close proximity to one another (amino acids 61-68; accession EAW70810.1) on the C-terminus; however, understanding the mechanisms by which the KCNE4 subunit interacts with Kv7.4 to facilitate the receptor-mediated vasorelaxant effects requires future attention.

Recently, we found that the KCNE4 β-subunit is positively regulated by DHT in the mouse cardiac myocytes (23). Male *Kcne4*$^{+/+}$ mice were shown to have a higher Kv current density compared with their age-matched, premenopausal female counterparts. The *Kcne4*$^{-/-}$ mice (males and females) had the same Kv current density as the *Kcne4*$^{+/+}$ females. *Kcne4* deletion did not alter ventricular repolarization in premenopausal female mice, but it delayed ventricular repolarization in postmenopausal female mice, which, unlike premenopausal female mice, showed equivalent ventricular *Kcne4* expression levels to those of adult male mice (23). Interestingly, in the present study we found no sex-dependent differences in the *Kcne4*$^{+/+}$ mouse mesenteric artery responses to methoxamine, isoprenaline or ML213, even though *Kcne4* mRNA expression in the male mouse mesenteric arteries was more than double that of the females. Although post-transcriptional modifications are possible, when taken together with the finding that the female *Kcne4*$^{-/-}$ mice showed similar responses to ML213 as their *Kcne4*$^{+/+}$ counterparts, these data suggest that female mice are less reliant on KCNE4 to regulate Kv7 channels in the vasculature, possibly relying on other *Kcne* isoforms. Aside from *Kcne4*, the only *Kcne* isoform to exhibit sex-dependent expression levels here was *Kcne1.* As reported previously (5, 28), *Kcne1* mRNA was not detected here in the mesenteric arteries of male mice; however, it was expressed in some of the female mesenteric arteries (Figure 5 A). KCNE1 reportedly increases Kv7.4 (5, 29) and Kv7.5 (30) currents *in vitro.* Based on these in vitro findings, one might expect KCNE1 to increase the function of the vascular Kv7.4/Kv7.5 channel, which could explain why the vascular responses to methoxamine and ML213 were normal in the female *Kcne4*$^{-/-}$ mice. The effect of KCNE1 on the Kv7.4/7.5 heteromeric channel that is likely to be found in the vascular smooth muscle is yet to be determined, however given the findings presented in this paper, the role of KCNE1 in the vasculature may be worth further exploration. Furthermore, the qPCR data in this study identified *Kcne3* mRNA as predominant in both male and female mesenteric arteries, and upregulated ~50% in males in response to *Kcne4* deletion. As the *Kcne3* expression levels in *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice were not sex-dependent, we consider it unlikely that Kcne3 underlies sex-specific differences in mouse mesenteric artery reactivity. However, we previously found that Kcne3 was upregulated in the gastric epithelium in response to germline *Kcne2* deletion and that this resulted in mistrafficking of Kcnq1 to the basolateral side of parietal cells, where it could not perform its normal function of recycling K^+ back into the stomach lumen (31). In addition, KCNE3 suppresses currents produced by Kv7.4 (29). It will, therefore, be interesting in the future to examine the possible functional effects of vascular *Kcne3* remodeling in response to *Kcne4* deletion.

Vascular diseases are in general more common in males than females, but the precise mechanisms responsible for these differences are incompletely understood. The effects of sex hormones in the cardiovascular system are thought be one important determinant of these differences (17, 18, 22). One factor that has been extensively researched in the vasculature is the male sex hormone, testosterone (22, 32). Together with higher levels of circulating testosterone, males have a higher incidence of vascular disease; however, reduced testosterone levels in aging men are known to increase the risk of developing cardiovascular diseases (33-35). Therefore, it is still unclear whether testosterone is a positive or negative factor in determining susceptibility to cardiovascular diseases.

The expression levels of *KCNE* transcripts and protein can be subject to regulatory influences, particularly by androgens such as oestrogen and testosterone, in at least some tissues. As mentioned, *Kcne4* transcript is positively regulated by DHT in mouse ventricular myocardium to the extent that it dictates sex-specific functional differences in ventricular repolarization currents (23). *Kcne2* is upregulated by oestrogen via a genomic mechanism (36), and may act as the female equivalent of *Kcne4* with respect to aspects of ventricular function, as both Kcne2 and Kcne4 regulate Kv4.2 and Kv1.5 channels in mouse ventricular myocytes (23, 37). Kcne3 protein expression in rat colonic crypts is reportedly reduced by oestrogen via a non-genomic action involving Kcne3 serine 82 (38), a PKC phosphorylation site (39). This activity may affect colonic Kv7.1-KCNE3 current properties and density during the oestrus cycle (38). In future work it will be important to define the different roles and interaction partners of all the KCNE subunits in the regulation of smooth muscle contractility, and investigate the possible role of sex hormones in regulating the vascular expression of both the KCNEs and their α-subunit partners, and the potentially dynamic effects of this on vascular reactivity. An important aspect of this will be to determine how the female mesenteric arteries compensate for loss of Kcne4 – is it simply less important in females and so compensation is easier, for example. Our Kv7.4 western blot data may provide some clues. These data suggest that Kv7.4 protein expression or stability in mouse mesenteric artery is reduced by *Kcne4* deletion, reminiscent of recent findings that transient KCNE4 knockdown in rat mesenteric artery impairs Kv7.4 surface expression (2). In addition, Kv7.4 protein was higher in mesenteric arteries isolated from female mice compared to those from males. This was despite the lack of a sex-dependent difference in *KCNQ4* transcript (which encodes Kv7.4) expression, an apparent discrepancy that could be explained by, for example, post-translational effects of hormones, as previously reported for KCNE3 in rat colon (39), described above. These findings raise the interesting possibility that female mouse mesenteric arteries maintain enough Kv7.4 protein and channels to ensure proper Kv7 function following the destabilizing effects of *Kcne4* deletion. Regardless, the current study emphasizes that in future experiments both sexes should be considered when determining the vascular roles of KCNE subunits.

Previously, Zhou et al., (40) showed that testosterone deprivation reduced Kv currents in rat aortic myocytes, which was associated with decreased Kv1.5 expression. We previously found that castration DHT-dependently reduced ventricular myocyte *Kcne4* expression, and that lower *Kcne4* expression was associated with lower Kv1.5 current (23). It is possible that *Kcne4* deletion could affect ion channels other than Kv7.4 in the mesenteric artery, including Kv1.5, however in this study we focused on its regulation of the Kv7 channels, as this interaction had been previously reported in the vasculature. It will be important in future experiments to determine other possible α-subunits with which KCNE4 might co-assemble, to fully understand the impact of DHT (or other androgen)-mediated changes in KCNE4 expression.

In summary, our findings highlight fundamental sex differences to the sensitivity of expression changes in KCNE4 in the vasculature that has important implications on vascular reactivity. These findings are in line with our previous studies that showed KCNE4 is regulated by DHT in mice, and that KCNE4 is an important regulator of Kv7.4 vascular function in male rats. We also provide novel evidence that KCNE4 is involved in the Kv7

channel recruitment following isoprenaline-induced activation of β-adrenoceptors. Given the important regulatory role of the KCNE family, not only with respect to Kv7 channels (41) but also many other Kv channel subtypes (42, 43), pacemaker channels (44) and even reportedly L-type Ca^{2+} channels (45), much more work needs to be done to elucidate the functional roles, and implications of sex-dependent differences in expression of the KCNE ancillary subunits in blood vessels.

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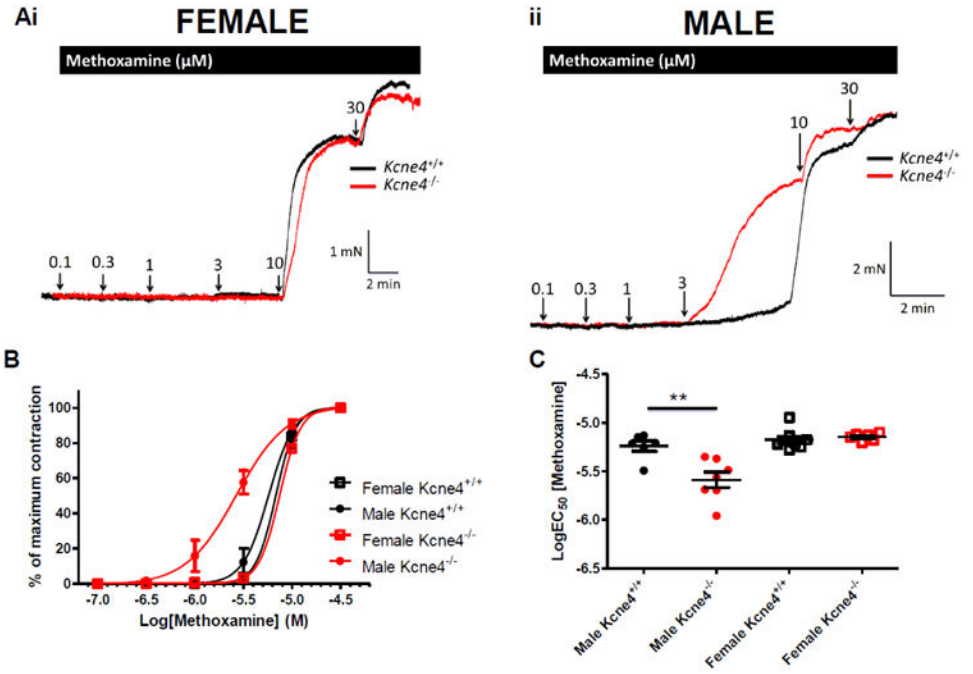

Figure 1.

Effect of methoxamine on male and female mesenteric arteries from *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice. (A) Representative isometric tension recordings from (i) female and (ii) male *Kcne4*$^{+/+}$ (black lines) and *Kcne4*$^{-/-}$ (red lines) to sequentially increasing concentrations of methoxamine. (B) Mean concentration-effect curves to methoxamine and (C) scatter plot of the EC_{50} values to methoxamine (n = 6-8). According to an unpaired t test, significance of P<0.001 is denoted ***.

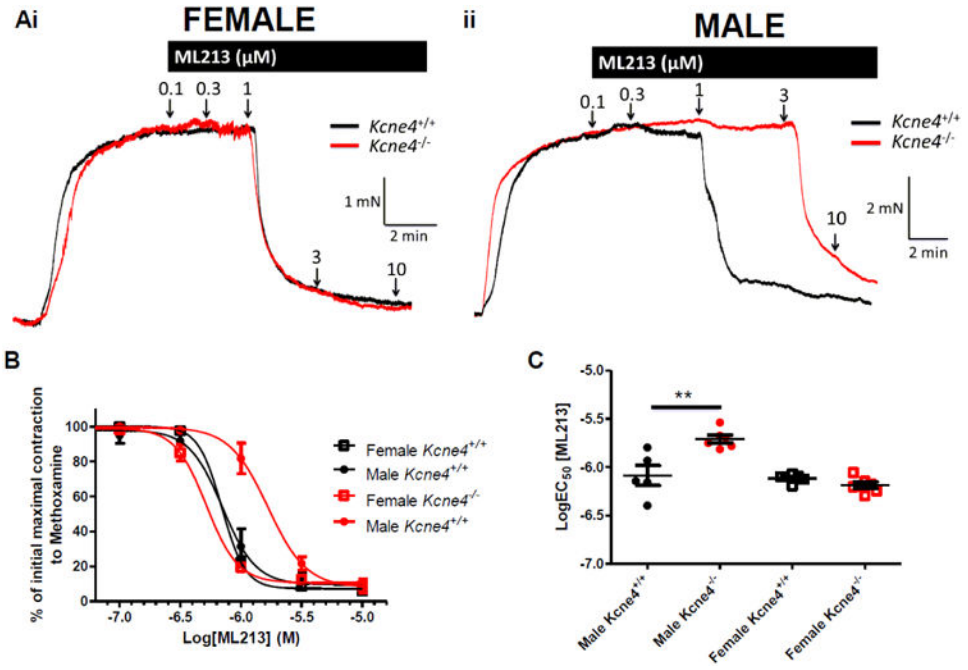

Figure 2.

Effect of ML213 on female and male mesenteric arteries from *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice precontracted with methoxamine. (A) Representative isometric tension recordings from (i) female and (ii) male *Kcne4*$^{+/+}$ (black lines) and *Kcne4*$^{-/-}$ (red lines) to sequentially increasing concentrations of ML213 (0.1 – 10 μM). (B) Mean concentration-effect curves to ML213 and (C) scatter plot of the EC_{50} values to ML213 (n = 5-6). According to an unpaired t test, significance of P<0.05 and P<0.001 are represented by * and ***, respectively.

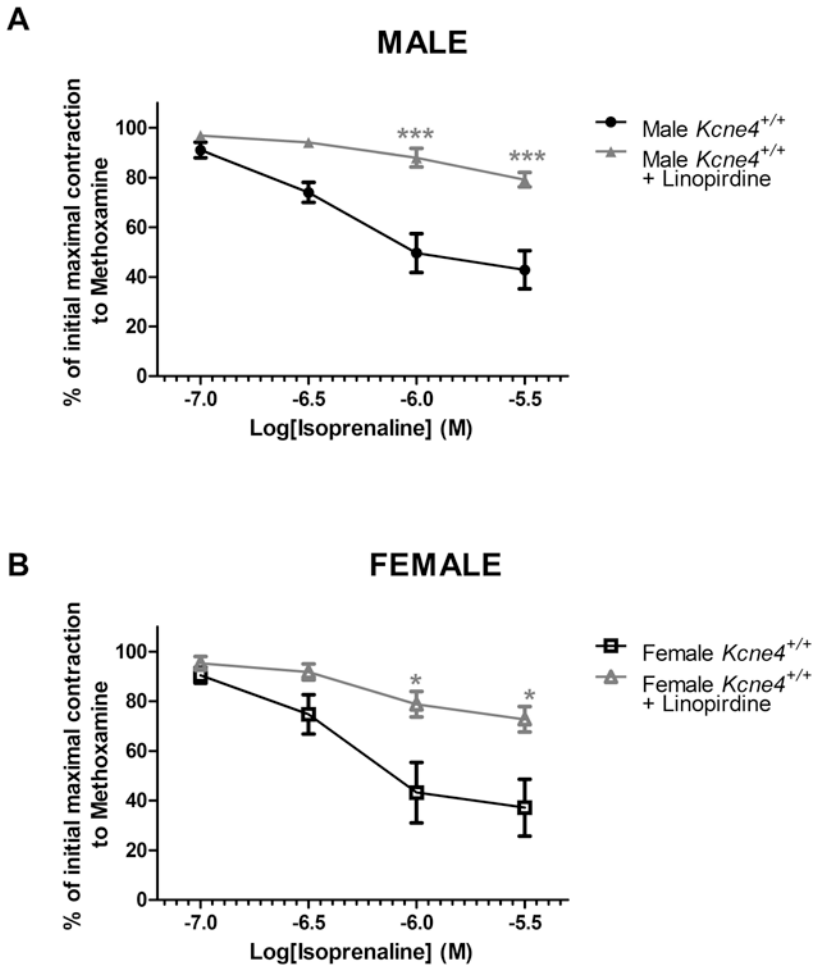

Figure 3.

Effect of the Kv7 channel blocker, linopirdine (grey), on the vasorelaxant effect of isoprenaline in *Kcne4*$^{+/+}$ (A) male (n = 6) and (B) female (n = 5) mesenteric arteries. A two-way ANOVA followed by a Bonferroni post-hoc test was performed and significance are denoted by * (P<0.05) and *** (P<0.001).

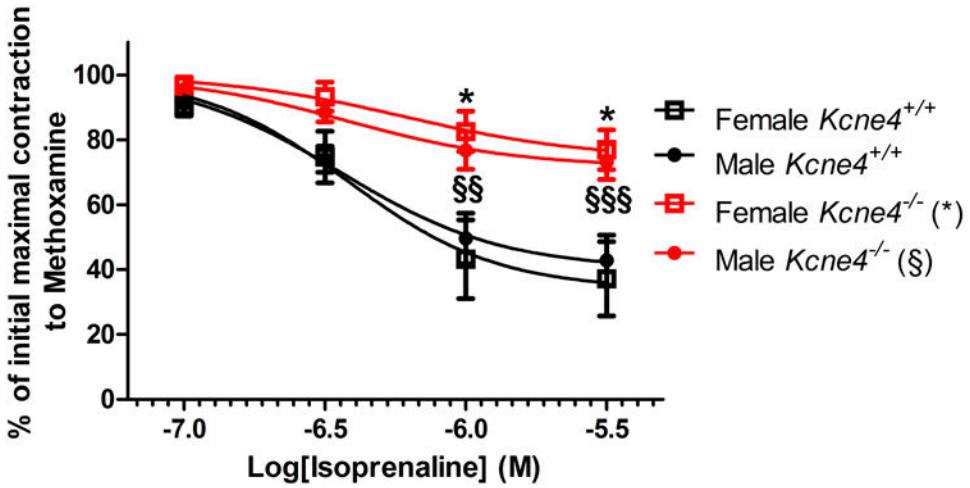

Figure 4.

Effect of isoprenaline on male and female mesenteric arteries from *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice. Mean concentration-effect curves to isoprenaline (n = 4-8). A two-way ANOVA followed by a Bonferroni posthoc test comparing the effect of isoprenaline in *Kcne4*$^{+/+}$ (black) with the *Kcne4*$^{-/-}$ (red) in both sexes. Significance is denoted by * for female and § for males.

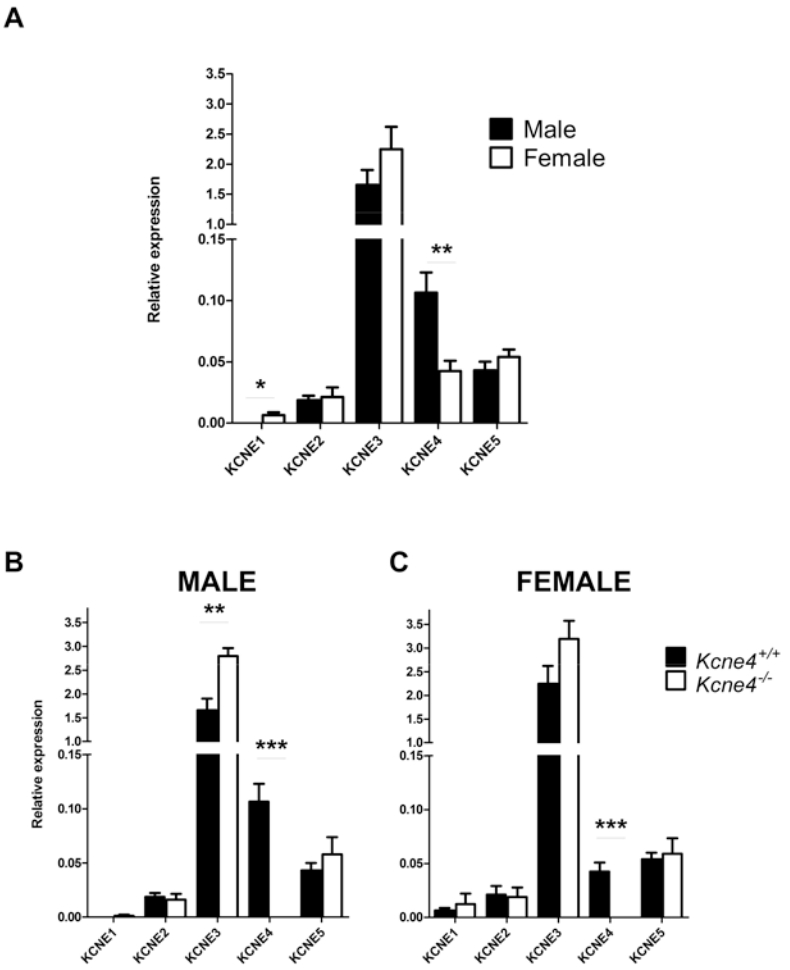

Figure 5.

Real-time quantitative PCR analysis comparing *Kcne1-5* mRNA expression in (A) male and female *Kcne4*$^{+/+}$ mice, and *Kcne4*$^{+/+}$ compared to *Kcne4*$^{-/-}$ in (B) male and (C) female mice (n = 5-6). Unpaired t tests were performed in A, B and C and significance are denoted by * (P<0.05), ** (P<0.01) and *** (P<0.001).

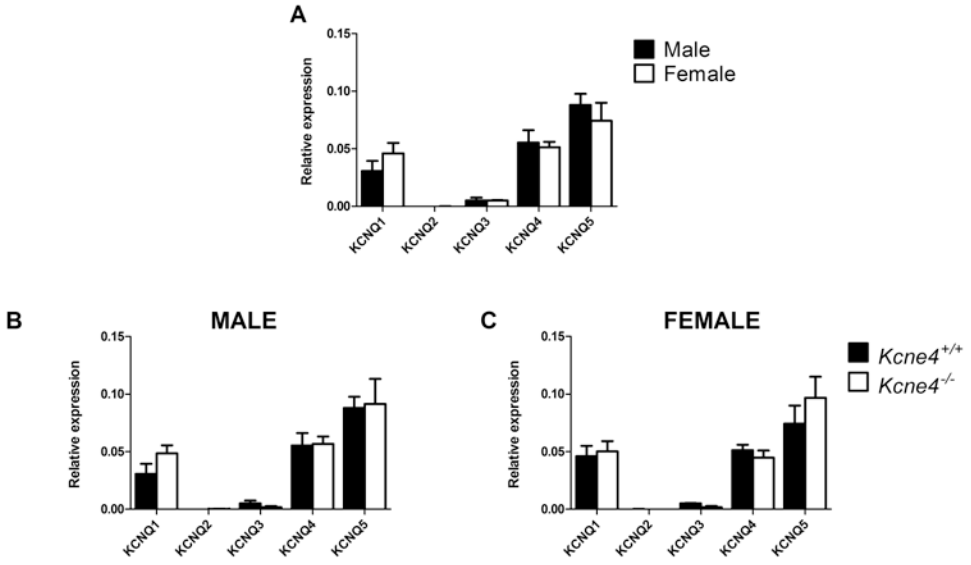

Figure 6.

Real-time quantitative PCR analysis comparing *Kcnq1-5* mRNA expression in (A) male and female *Kcne4*$^{+/+}$ mice, and *Kcne4*$^{+/+}$ compared to *Kcne4*$^{-/-}$ in (B) male and (C) female mice (n = 5-6). Unpaired t tests were performed in A, B and C and significance are denoted by * (P<0.05), ** (P<0.01) and *** (P<0.001).

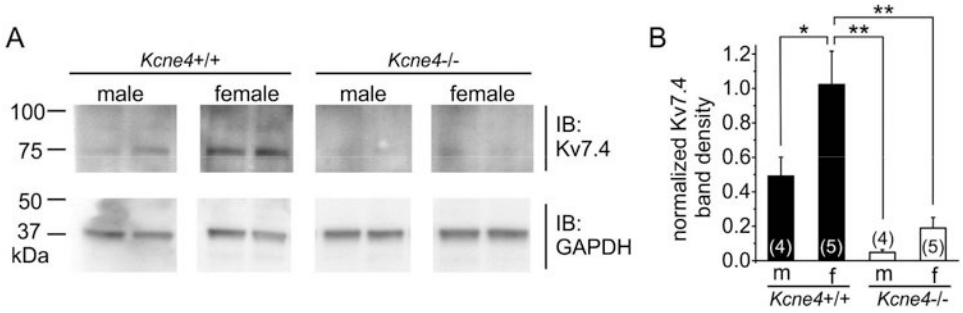

Figure 7.

Effects of sex and *Kcne4* deletion on mouse mesenteric artery Kv7.4 protein expression. (A) Exemplar western blots showing Kv7.4 (upper) and GAPDH (lower) expression in mouse mesenteric arteries. (B) Mean Kv7.4 band densities, normalized to same-lane GAPDH band densities, for blots as in panel A (n = 4-5 mice per group, as indicated in parentheses). * P < 0.05, **P<0.01, by ANOVA with Tukey's HSD test.

Table 1

Kcne and *Kcnq* primers used for real-time qPCR experiments.

Gene	Primer Sequence (+) sense, (-) antisense	GenBank Accession Number	Amplicon (bp)	Region Spanned
Kcne1	(+) 5′-ACTCGCACGACCCTTTCA-3′ (-) 5′-TTTCAATGACATAGCAAGCTCTG-3′	NM_012973	114	284-397
Kcne2	(+) 5′-TGTCATTTAAGTCCATTCCAATCAT-3′ (-) 5′-TGAGAAAGAAGGTTGAAAGATTTGT-3′	NM_133603	116	769-884
Kcne3	(+) 5′-TTATGATGTCTGAGGATTGTCTTCT-3′ (-) 5′ TGACCTAACTCTCTTACCAATTTCT-3′	NM_022235	114	449-562
Kcne4	(+) 5′-CCCTTGAGTCCCATGTGTCT-3′ (-) 5′-GTAGCCCAGCATGATTCCAAT-3′	NM_212526	113	135-247
Kcne5	(+) 5′-GTCAACGGCGTCCTGGAG-3′ (-) 5′-CAGCAGCAAGCGGTTCAA-3′	NM_00110100	96	27-122
Kcnq1	(+) 5′-CTCGGAGTCACACGCTTCT-3′ (-) 5′- GCTTGAACTTCTTCTTCTTTACCAT -3′	NM_008434	75	1301-1375
Kcnq2	(+) 5′-CCCTCATTGGTGTCTCGTTCT-3′ (-) 5′-GGTTCCGCCGTTTCTCAAAG-3′	NM_010611	111	1026-1136
Kcnq3	(+) 5′-GAAGAGGGGCAGAGGAGGA-3′ (-) 5′-CCTGTACTTGGCGTTGTTCC-3′	NM_152923	93	226-318
Kcnq4	(+) 5′-GTGGTCTTTGGCTTGGAGTATAT-3′ (-) 5′-CGATGACACAGAAGGGTTTCC-3′	NM_001081142	112	424-535
Kcnq5	(+) 5′-GTCAGATAAGAAGAGCCGAGAGA-3′ (-) 5′-CGATGGACTGGACCTGTTTCT-3′	NM_001160139	107	2164-2270