Calcium signaling in endothelial and vascular smooth muscle cells; sex differences and the influence of estrogens and androgens

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Abstract

Calcium signaling in vascular endothelial cells (ECs) and smooth muscle cells (VSMC) is essential for the regulation of vascular tone. However, the changes to intracellular Ca^{2+} concentrations are often influenced by sex differences. Furthermore, a large body of evidence shows that sex hormone imbalance leads to dysregulation of Ca^{2+} signaling and this is a key factor in the pathogenesis of cardiovascular diseases. In this review, the effects of estrogens and androgens on vascular calcium-handling proteins are discussed, with emphasis on the associated genomic or non-genomic molecular mechanisms. The experimental models from which data were collected were also considered. The review highlights: 1) in female ECs, TRPV4 and MCU enhances Ca^{2+}-dependent NO generation. In males, only TRPC3 plays a fundamental role in this effect. 2) Female VSMCs have lower cytosolic Ca^{2+} levels than males due to differences in the activity and expression of STIM1, Orai1, $Ca_V1.2$, NKCC1, and the Na^+/K^+-ATPase. 3) When compared with androgens, the influence of estrogens on Ca^{2+} homeostasis, vascular tone and incidence of vascular disease is better documented. 4) Many studies use supraphysiological concentrations of sex hormones, which may limit the physiological relevance of outcomes. 5) Sex-dependent differences in Ca^{2+} signaling mean both sexes ought to be included in experimental design.

KEY WORDS: sex hormones; calcium homeostasis; endothelium; vascular smooth muscle

New & Noteworthy

Around 50 years ago, researchers began studying the effects of sex hormones on Ca^{2+} handling in isolated vascular tissues, vascular smooth muscle cells, and endothelial cells. Since then, many researchers have made important discoveries to help us better understand how androgens and estrogens impact cellular Ca^{2+} homeostasis.

1. Introduction

The regulation of physiological blood pressures (BP) is essential for human health and could be influenced by factors such as age and sex (1). Many physiological processes contribute to the regulation of blood pressure, though the force required for the necessary changes to hemodynamics is usually generated by smooth muscle contraction. Smooth muscle contraction is dependent on excitation-contraction coupling for which Ca^{2+} signaling is essential (2). In vascular smooth muscle cells (VSMCs), cytosolic Ca^{2+} levels ($[Ca^{2+}]_{cyt}$) are finely tuned by many sarcolemma and intracellular channels, exchangers, and transporters (3) These processes can be influenced by many intrinsic and extrinsic factors to modulate smooth muscle contraction. However, dysregulation of these processes is a key contributor to vascular disease such as obesity, diabetes mellitus and hypertension and the progression of associated comorbidities such as cardiovascular, renal and ocular diseases (1).

Accumulating evidence suggests that estrogens, androgens and sex play vital roles in the homeostasis of Ca^{2+}, regulation of vascular tone and its cellular signaling through genomic and non-genomic mechanisms (4–8). This has led to an understanding of the difference in cardiovascular protection against hypertension between biological sex (9). Studies in hypertensive animals show that sex differences play an important role in BP regulation (10–12). Furthermore, ovariectomy (OVX) in spontaneously hypertensive rats (SHR) accelerates the development of hypertension which is then reversed by estrogen treatment (13). The presence or absence of gonads are also implicated in these responses (14). This raised the hypothesis that any sex differences in the mechanisms of Ca^{2+} mobilization in VSMC contraction would be more evident in hypertensive than in normotensive animals. Over 20 years ago, Murphy & Khalil showed that basal $[Ca^{2+}]_{cyt}$ levels in VSMC from female Wistar-Kyoto (WKY) rats were lower compared to males and that this same pattern was observed in the SHRs (15). In addition, OVX significantly increased contractility and basal $[Ca^{2+}]_{cyt}$ levels, an effect that was reversed when OVX rats were treated with 17β-estradiol ($E_2β$) (15). This sex-specific reduction in contractility and $[Ca^{2+}]_{cyt}$ levels in the VSMC of female rats was greater in SHR than in WKY rats (15) and provided insight into why Angiotensin II (Ang II) produces a greater $[Ca^{2+}]_{cyt}$ response in VSMC isolated from male SHRs compared with females (16).

The influence of sex on the management of Ca^{2+} at the vascular level has been a long-standing issue (17, 18), and there are more recent contributions such as that of Pabbidi et al. who reviewed

the role of $E_2\beta$ on underlying sex differences in vascular size, blood flow, shear stress, vascular compliance, myogenic properties of VSMCs, and the role of perivascular adipose tissue (19).

The purpose of this review is to inform, through an in-depth and updated analysis of the literature, the genomic and non-genomic influence of estrogens, and androgens, on the proteins that modulate Ca^{2+} homeostasis in the vasculature. We have detailed the associated molecular mechanism(s), the interactions with other signaling pathways, as well as the experimental models and species used in the studies **(Table S1 and S2, Supplementary material)**. Finally, we identified gaps and limitations in the existing knowledge, and suggested avenues through which understanding may be improved on for future studies.

2. Mechanisms of contraction in VSMC

Various physiological stimuli, including intravascular pressure and activation of membrane receptors, can initiate VSMC contraction leading to an increase vascular resistance (20). The Ca^{2+}-mediated canonical contraction mechanism begins with the activation of G-protein-coupled receptors (GPCRs) (20). GPCR activation leads to G_q/G_{11}-mediated phospholipase-C (PLC) stimulation, generating the secondary messenger inositol 3,4,5-triphosphate (IP_3) and diacylglycerol (DAG). (21, 22). IP_3 binds to its receptor (IP_3R) located on the sarcoplasmic reticulum (SR), and together with ryanodine receptors (RyR), generate Ca^{2+} release from internal stores. The subsequent increase of $[Ca^{2+}]_{cyt}$ is enhanced by sarcolemma Ca^{2+} influx via voltage-gated Ca^{2+} channels (VGCC), store-operated Ca^{2+} channels (SOCC), receptor-operated Ca^{2+} channels (ROCC), and potential transient receptors (TRP). Calcium diffuses into the contractile machinery and binds to calmodulin (CaM). The Ca^{2+}-CaM complex induces a conformational change in myosin light chain kinase (MLCK) activating it. Activated MLCK then induces myosin light chain (MLC) phosphorylation, thereby stimulating actin-myosin interaction, leading to force, and shortening, and thus contraction of the VSMCs (22, 23). Vascular contraction may also be enhanced by a Ca^{2+} sensitization mechanism. This signaling pathway involves the participation of a small GTP-binding protein RhoA, an increase in the activity of its kinase (ROCK), and the inhibition of MLC phosphatase (MLCP) that lead to increased MLC phosphorylation (24).

For VSMC relaxation, several mechanisms can work in concert to decrease $[Ca^{2+}]_{cyt}$. For instance, the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) resequesters Ca^{2+} into the SR while mitochondrial Ca^{2+} uniporter (MCU) complex facilitates mitochondrial Ca^{2+} uptake. Furthermore, the sodium-calcium exchanger (NCX) and plasma membrane Ca^{2+}-ATPase (PMCA) promote Ca^{2+} extrusion. (23).

3. The influence of sex hormones on mechanisms of Ca^{2+} mobilization in the sarcoplasmic/endoplasmic reticulum

3.1. Inositol 3,4,5-triphosphate receptor (IP_3R)

In VSMCs, IP_3 receptors (IP_3Rs) are expressed as 3 different isoforms (IP_3R1-3), with IP_3R1 being the predominant isoform in VSMC (25). IP_3Rs are essential for the generation of Ca^{2+} waves

produced by the release of Ca^{2+} from the SR (26, 27). A luminal excitatory Ca^{2+} binding site on IP_3R provides a direct explanation of how increases in $[Ca^{2+}]_{SR}$ activate IP_3R and continue the propagation of Ca^{2+} waves (25, 28).

To date, the effects of sex hormones on IP_3R are inconclusive. Early studies demonstrated that in rats, $E_2\beta$ reduces phenylephrine (PE) and KCl-induced contraction in the femoral artery and portal vein without endothelium (29). However, $E_2\beta$ did not alter the release of Ca^{2+} from the sarcoplasmic reticulum (SR) induced by agonists such as PE or IP_3, thus suggesting that the relaxing effect is not mediated by IP_3R inhibition (29). On the other hand, although it has been reported that progesterone is not capable of altering the expression of IP_3R in VSMC of human umbilical arteries (HUA) (30), other reports show that in a Ca^{2+}-free medium, both $E_2\beta$ and progesterone inhibit norepinephrine-induced contraction in rabbit aorta (31) and pulmonary arteries (32).

The activation of the G protein-coupled estrogen receptor (GPER), also known as GPR30, is reported to blunt the serotonin-induced vasoconstriction by reducing $[Ca^{2+}]_{cyt}$. In the aorta and carotid artery, the selective GPER agonist G-1 causes more potent dilation than $E_2\beta$ (33). Besides, G-1-mediated GPER activation produces a rapid and transient increase in $[Ca^{2+}]_{cyt}$, an event that reduces subsequent Ca^{2+} mobilization induced by ligands of other GPCRs such as serotonin (33). In GPER knockout mice, Meyer et al. showed that the vasoconstrictor response to endothelin-1 (ET-1) was significantly increased. However, a reduction in the ET-1-induced increase in $[Ca^{2+}]_{cyt}$ was observed, suggesting an increased myofilament sensitivity to $[Ca^{2+}]_{cyt}$ (34). Interestingly, acute activation of GPER has no effect on the Ca^{2+} response elicited by ET-1, indicating the differential effects of chronic basal activity and acute activation of GPER (34).

Although GPER is a receptor generally associated with female physiology, its expression in male vascular beds has also been described. In the vascular wall of carotid arteries, GPER is expressed in both vascular endothelial cells (ECs) and VSMCs of both sexes **(Figure 1)** and exhibits acute vasodilatory effects (35). Activation of estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and GPER in certain blood vessels initiates NO generation by activating endothelial nitric oxide synthase (eNOS), both in a Ca^{2+}-dependent **(Figure 1, A and B)** and independent manner (36). Downstream, the NO-cGMP-PKG signaling pathway takes place in VSMCs. NO activates soluble guanylate cyclase (sGC), an enzyme that catalyzes the reaction that forms cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G (PKG) (36). The activation of PKG could have a role in the inhibitory phosphorylation of IP_3R and the reduction in the release of Ca^{2+} from the SR (37). On the other hand, GPER activation favors an increase in the production of cyclic adenosine monophosphate (cAMP) and the subsequent activation of protein kinase A (PKA) (38), leading to the phosphorylation of IP_3R. Thus, PKA-PKG crosstalk could contribute to PKA-mediated inhibitory phosphorylation of IP_3R, but this requires further investigation (39). Finally, in aortic ECs within a Ca^{2+}-free medium, $E_2\beta$ induces an increase in $[Ca^{2+}]_{cyt}$ via ER-dependent pathways, but not via plasma membrane (PM) influx of Ca^{2+} (40). This supports the suggestion that the initial effects of $E_2\beta$ on $[Ca^{2+}]_{cyt}$ in the endothelium

could be due to the activation of IP_3R. Most studies on the influence of sex or the effects of sex hormones on IP_3R activity are non-specific, and do not appear to directly address the involvement of this Ca^{2+}-handling protein. Many effects can be explained by the participation of membrane proteins related to the influx of Ca^{2+}, as we will see later. Nonetheless, future studies should aim to elucidate whether IP_3R-dependent sex differences in Ca^{2+} handling are mediated by GPER at the vascular level.

3.2. Ryanodine receptor (RyR)

Ryanodine receptors (RyR1 and RyR2) contributes to at least two types of SR Ca^{2+} release in VSMCs; Ca^{2+} waves, related to IP_3R activation, and Ca^{2+} sparks, associated with the function of Ca^{2+}-activated K^+ channels (K_{Ca}) and Ca^{2+}-activated Cl^- channels (CaCC) (41, 42). RyR2 is predominant in different vascular beds (42), although both RyR1 and RyR2 are also involved with the generation of Ca^{2+} sparks (43). Ca^{2+} sparks are the result of rapid opening of a small RyR pool in the SR, leading to local and transient increases in submembranal Ca^{2+} concentrations (41, 42). Ca^{2+} sparks do not directly modify the net Ca^{2+} concentration, rather by activation of spontaneous transient outward currents (STOC). STOCs cause hyperpolarization of the membrane, decreasing global $[Ca^{2+}]_{cyt}$ and reducing vasoconstriction through the activation of K_{Ca} located in the PM (44).

Several studies show, that the non-genomic vasodilator effect of estrogens is independent of RyR (29, 45–47). However, Ca^{2+} sparks and the expressions of RyR are reported to increase during pregnancy **(Figure 1D)**. Uterine arteries incubated with $E_2\beta$ and progesterone mimics pregnancy-induced upregulation of RyR, increasing RyR-generated Ca^{2+} sparks and K_{Ca} activity, leading to vasodilation mediated by STOC (48) **(Figure 3B)**. Interestingly, the presence of RyR might not be essential for the generation of female hormone-induced STOCs through K_{Ca}. It has been proposed that in VSMCs, GPER activation substantially increases K_{Ca} channel activity (49). The participation of second messengers such as PKG and PKA downstream would partly explain the increase in K_{Ca} activity. However, although these effects have been elucidated in recent years, the contribution of GPER to K_{Ca}-mediated arterial relaxation is complex. This is because the expression of GPER and K_{Ca} occurs in both EC and VSMCs, and they are differentially coupled to downstream signaling and, therefore, may produce direct or indirect effects on VSMCs (36).

3.3. Sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA)

SERCA actively pumps Ca^{2+} into the sarco/endoplasmic reticulum, thereby regulating $[Ca^{2+}]_{cyt}$ and vascular tone (50). The activity of the pump is inhibited by dephosphorylated phospholamban (PLB). When PKA or Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) phosphorylate PLB, SERCA activity increases enhancing Ca^{2+} uptake into SR, leading to vasodilation (51). Mammals express three isoforms of SERCA (SERCA1-3). In the cardiovascular system, SERCA2 dominates (51). SERCA2 appears as two sub-isoforms, SERCA2a and SERCA2b, which have different functional and structural characteristics. SERCA2b shows higher affinity for Ca^{2+}, but

has a lower ATP hydrolysis turnover rate compared to SERCA2a, although both isoforms are inhibited by PLB and thapsigargin (51, 52).

Studies on the effects of sex hormones on SERCA activity have been carried out mainly in cardiac tissue (Reviewed in detail by (53–55)). In cardiomyocytes, estrogen deficiency suppresses SERCA2a expression and activity, in addition to a reduction in PLB phosphorylation levels. In OVX animals, estrogen supplementation improves SERCA2a activity and PLB phosphorylation levels, which favors an increase in the Ca^{2+} reuptake rate in the SR (53, 54).

In smooth muscle of female rat mesenteric arteries, GPER activates adenylyl cyclase resulting in an increase in cAMP, which can activate PKA and phosphorylate PLB thereby enhancing SERCA activity (56) **(Figure 2B)**. However, the effects on SERCA-dependent cAMP-mediated vasodilation remains unclear. Although sex differences of SERCA activity have not been measured in VSMCs of SHR, thapsigargin-sensitive Ca^{2+} stores are higher in female SHR aorta versus male SHR (16) **(Figure 1, C and D)**.

Hill & Muldrew demonstrated that $E_2\beta$ can induce a ~50% increase in the expression of SERCA2b, and the regulatory proteins PKA and PKG in the coronary arteries of mature female pigs, by a mechanism dependent on ERα and ERβ (57) **(Figure 3B)**. Another female hormone, progesterone can increase the expression of SERCA2 through the activation of the membrane progesterone receptor α (mPRα). Also, progesterone-induced mPRα activation increases PLB phosphorylation, thus enhanced SERCA function. These findings suggest that progesterone is capable of increasing Ca^{2+} sequestration in the SR ($[Ca^{2+}]_{SR}$), favoring VSMC relaxation through decreased $[Ca^{2+}]_{cyt}$. This signaling between mPRα and SERCA2/PLB would imply the participation of a Gi protein, MAP Kinase, and Akt/PI3K (Akt also known as protein kinase B; PKB), as well as a downregulation of RhoA/ROCK pathway (30) **(Figure 3B).**

To date, some questions that remain unanswered are whether sex hormones non-genomically modulate SERCA activity. An in silico study showed that selective estrogen receptor modulators (SERMs) did not directly interact with the transmembrane domain of the SERCA protein at the thapsigargin binding site (58).

3.4. Proteins that sense Ca^{2+} levels in the SR

The SR membrane of numerous cell types contains proteins known as Stromal Interaction Molecules (STIMs). STIMs are dynamic Ca^{2+} sensors and are activated when SR Ca^{2+} reserves are depleted, thus participate in store-operated Ca^{2+} entry (SOCE), a mechanism that sequesters Ca^{2+} in the SR (59). After STIMs activation, they translocate from SR to discrete PM-binding domains activating Ca^{2+} channels as Orai1 (59, 60). STIM dysregulation may contribute to the pathogenesis of hypertension and cardiovascular remodeling (61).

Giachini et al showed that STIM1 expression is greater in the aorta of male **(Figure 1C)** spontaneously hypertensive stroke-prone rats (SHRSP) than females (62) **(Figure 1D)**. On the other hand, the increase of GPER heterologous expression in ECs and its activation is capable

of inhibiting SOCE, and this is potentiated by G-1 treatment. In contrast, GPER silencing was associated with an increase in SOCE. A visible manifestation of STIM1 activation is its formation of puncta-like SR-PM junctions. 90% of thagpsigargin-treated cells showed STIM1 puncta, whereas only ~10% of G-1-treated cells exhibited this response. Taken together, these data suggest that in ECs, GPER activation prevents STIM1 oligomerization (63) **(Figure 2B)**. Co-immunoprecipitation and confocal microscopy studies showed that GPER interacts with STIM1 in different subcellular compartments. Furthermore, activation of GPER inhibits SOCE through alteration in the phosphorylation status of STIM1 (64) at Ser 575, 608 and 621 (63). A GPER sub membrane domain in the SR interacts with the Ca^{2+} binding loop in STIM1, although it appears that the Ca^{2+} and the GPER domain bind to different regions of the loop (65).

4. Mobilization of Ca^{2+} through the plasma membrane

In vascular smooth muscle and endothelial cells, intracellular Ca^{2+} handing involves the participation of several PM Ca^{2+} channels, such as Ca^{2+} voltage-dependent channels (VGCC), store-operated Ca^{2+} channels (SOCC), receptor-operated Ca^{2+} channels (ROCC), transient receptor potential channels (TRP), and mechanosensitive Ca^{2+} channels (Piezo). Also, Ca^{2+} transporters and Ca^{2+} related-exchangers such as PMCA, plasma membrane Na^{+}/Ca^{2+} exchanger (NCX), $Na^{+}/K^{+}-$ATPase pump, and $Na^{+}–K^{+}–2Cl^{-}$ symporter play a key role in Ca^{2+} homeostasis through the plasma membrane.

4.1. Voltage-gated Ca^{2+} channel (VGCC)

Voltage-gated Ca^{2+} channels (VGCC) open in response to changes in membrane potential, providing an important pathway for the regulated entry of Ca^{2+} ions. These channels include L, N, P/Q, and R-types (HVA, high-voltage-activated) and T-type channels (LVA, low-voltage-activated) (66). Here, we describe the L-type Ca^{2+} channel (LTCC) and T-type Ca^{2+} channel (TTCC) in VSMCs and ECs.

4.1.1. L-type Ca^{2+} channels (LTCC)

The LTCC presents four isoforms: $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ y $Ca_V1.4$ (67) The $Ca_V1.2$ is a key player in the hormonal regulation of blood pressure and the development of myogenic tone (68). Most sarcolemma Ca^{2+} influx that drives the contractile response in VSMCs is mediated by the $Ca_V1.2$ (68). The $Ca_V1.2$ channel consist of pore-forming α_{1C} subunits (voltage sensing domain) and auxiliary β, α2δ, and γ subunits that modulate channel function (67, 69, 70). $Ca_V1.2$ activation is prominent at more depolarized vascular smooth muscle membrane potentials (~ -45 to -36 mV) observed at higher intraluminal pressures, and single-channel conductance is high and may be in the range of 18-26 pS (70, 71). However, the channel is also activated by binding to PKA through of AKAP (A kinase anchoring protein), such as AKAP5 (68). In this way, AKAPs facilitate the regulation of $Ca_V1.2$ for kinases (PKA, PKC) and phosphatase (calcineurin) (72).

4.1.1.1. The influence of estrogens on LTCC

$Ca_V1.2$ channel plays an essential role in sex differences in the Ca^{2+} handling of VSMCs and vascular tone. In 1987, Stice et al. showed, for the first time, the role of sex hormones on Ca^{2+} influx through VGCC (73). By reducing Ca^{2+} influx through VGCC, estrogens increase uterine arterial blood flow (UBF) during the oestrus stage (74). This finding suggests that estrogen levels are negatively correlated with the uptake of extracellular Ca^{2+} and the contractile response in the uterine arteries during pregnancy (75). Addition of $E_2\beta$ and progesterone to the organ bath in vascular reactivity experiments significantly reduces the contraction induced by $CaCl_2$ in a Ca^{2+}-free medium in the aorta (31, 76), carotid artery (77), middle cerebral artery (78), pulmonary artery (32), coronary (79), mesenteric artery (80), and tail artery (80) of rabbit (31, 32, 77–79) and rat (76, 80). Even the active metabolites of $E_2\beta$, 2-hydroxyestradiol (2-HOE), and 2-methoxyestradiol (2-MeOH), attenuated KCl-induced contraction (~50%) in the coronary arteries of breeding sows (81) **(Table S2, supplementary material).**

In general, $E_2\beta$ produces rapid and reversible relaxation, independently of the blood vessel type or species (29, 82). In the aorta (46, 83), coronary artery (84), basilar artery (85) and internal carotid artery (85), estrogen causes vasodilation by blocking of Ca^{2+} influx through the LTCC, without involving ER and classical genomic pathway (86). On the other hand, the vasodilator effect of $E_2\beta$, but not progesterone, is different in hypertensive model. $E_2\beta$ vasodilation in the SHR aorta is partially mediated by opening ATP-sensitive K^+ channels (K_{ATP}), voltage-gated K^+ channels (K_V) and blocking VGCC. In contrast, progesterone-induced relaxation is primarily by VGCC blocking rather than K^+ channel activation in SHR rats (87). However, no significant differences were seen in the blocking effect of both sex hormones on VGCC between WKY and SHR (87).

Another study showed that $E_2\beta$ did not decrease the expression or activity of the LTCC in coronary arteries of hyperlipemic rabbits (88). Most studies implicate increases in the function and expression of $Ca_V1.2$ in disease models such as hypertension (68). However, with the limited evidence available, it is difficult to conclude whether VGCC blockade induced by female hormones does not change in models of hypertension and hyperlipidemia, requiring future studies.

In VSMC isolated from rat tail arteries (89) and human aorta (90), $E_2\beta$ inhibits the influx of extracellular Ca^{2+} through LTCC and prevents the KCl-induced $[Ca^{2+}]_{cyt}$ increase **(Figure 3B)**. In A7r5 cells (aortic thoracic smooth muscle of rat), $E_2\beta$ and progesterone reduce Ca^{2+} current through LTCC ($I_{Ca,L}$), while the reduction produced by 17α-estradiol ($E_2\alpha$) is significantly less (83, 91). However, another study showed that in the same cells, $E_2\beta$, but not progesterone, reduces $I_{Ca,L}$ in a reversible and concentration-dependent manner (92). Although the inhibition of the $Ca_V1.2$ channel by estrogens is mediated by a non-genomic mechanism through direct interaction with the LTCC channel (93), the involvement of a GTP-binding protein (G proteins sensitive to Pertussis toxin; PTX) was proposed (94). Incubation of VSMC isolated from the rat tail artery with progesterone inhibits the KCl-induced increase in $[Ca^{2+}]_{cyt}$ and suppresses $I_{Ca,L}$ (95) **(Figure 3B)**,

an effect that appears to be mediated by membrane progestin receptor α, through a mechanism that involve activation of a G_i protein and downregulation of cAMP-dependent signaling (96).

In the ECs, $E_2β$ increases $[Ca^{2+}]_{cyt}$ through a non-genomic mechanism to generate NO (40). In rat brain microvascular ECs (RBMVECs), GPER promotes Ca^{2+} entry by activating LTCC via PKA, leading to NO production and (97) **(Figure 2B).**

Sex hormones modulate the expression and activity of $Ca_V1.2$. In a culture of coronary arteries, $E_2β$ reduced $Ca_V1.2$ $α_{1C}$ subunit protein expression through an ER-dependent mechanism (98). In female rats, basal Ca^{2+} influx is significantly lower than in intact males or OVX females (99), while estrogen treatment reverses the increased influx of Ca^{2+} (100) and mRNA levels of the $α_{1C}$ subunit in female OVX rats (101) **(Figure 1, C and D)**. These results would explain why the chronic treatment with $E_2β$ decreases the mean arterial pressure in OVX rats (102). Estrogen treatment reduced the sensitivity to depolarization with KCl in coronary arteries of OVX female pigs, suggesting a decrease in LTCC activity. In this study, the mRNA expression of the $α_{1C}$ subunit of $Ca_V1.2$ did not decrease (103). Along the same lines, progesterone reduces the contractile response to KCl or Bay K8644 (LTCC agonist) in vascular smooth muscle of human umbilical vein without decreasing the expression of $Ca_V1.2$ or PR (47).

In various vascular beds of different species, $E_2β$ reduces Ca^{2+} influx by blocking LTCC in a concentration-dependent manner. However, some studies showed that estrogen-induced relaxation via non-genomic pathway in rat aorta, involved cAMP, but not LTCC or also known as dihydropyridine channel (104). This was confirmed when $E_2β$ did not interfere the relaxant response to LTCC blockers such as nifedipine, diltiazem, and verapamil in rat mesenteric artery (105). Although the lack of effect of $E_2β$ may be related to the type of vascular bed, the species, or the sex of the animal, this is unlikely given that a relaxing effect was previously demonstrated (29, 82). With the exception of studies using VSMCs, most studies using vascular tissues have mainly used high $E_2β$ concentrations in the micromolar range (**Table S2, Supplementary material**). Therefore, we cannot rule out that the blocking effect of $E_2β$ on LTCC is very subtle at low concentrations (105), and therefore, more selective molecules such as LTCC blockers (i.e nifedipine) mask the vasodilator effect of $E_2β$.

4.1.1.2. The influence of androgens on LTCC

Bowles et al. reported that LTCC activity is different in VSMC of male and females (106). They found an increase of the LTCC channel current ($I_{Ca,L}$) in coronary artery smooth muscle (CASM) of male pigs compared to females, probably due to higher $Ca_V1.2$ expression (106) **(Figure 1, C and D)**. In castrated animals, testosterone treatment restored the $Ca_V1.2$ expression and activity, demonstrating that endogenous testosterone is a primary regulator of $Ca_V1.2$ expression and activity in males (107) by a protein kinase C δ (PKCδ)-dependent mechanism (108) **(Figure 1C)**. On the other hand, the expression of $Ca_V1.2$ is directed by a promoter in the 2-kb 5'-flaking sequence of the $α_{1C}$-subunit in vascular smooth muscle. This consensus sequence presents a hormone response element (HRE) or cAMP response element (CRE) that could explain how

testosterone regulates $Ca_V1.2$ expression (109). Curiously, a study showed that 24-h treatment with DHT decreases α subunit ($Ca_V1.2$) expression in HUA, which could be explanained by the regulatory effect of androgens or their metabolites on $Ca_V1.2$ (110).

In human umbilical arteries (HUA), androgens, such as 5β-dihydrotestosterone (5β-DHT), are more potent vasodilators than progestins. At nanomolar concentrations, 5β-DHT and testosterone decrease the Ca^{2+} influx through LTCC (111, 112) **(Figure 3A)**, but at high concentrations (mM), only testosterone increases $[Ca^{2+}]_{cyt}$ (113).

In rat aorta, testosterone decreases KCl-induced contraction (99) by blocking Ca^{2+} influx through LTCC (114, 115). These effects appear to be independent of the androgen receptor (AR), conversion of testosterone to estrogen (115), release of NO or vasodilatory prostaglandins (116), K^+ channels, endothelium, aromatase enzyme, activity of 5α-reductase (117), or the production of cAMP/cGMP (118). In hypertensive animals, androgens cause greater vasodilation than in normotensive ones. Firstly, testosterone, 5α-dihydrotestosterone (5α-DHT), and 5β-DHT relaxed the thoracic aorta and mesenteric artery of WKY and SHR rats. Secondly, 5β-DHT produced the greatest vasorelaxant effect in SHR arteries pre-contracted with KCl and Bay K8644, suggesting an inhibitory effect on LTCC (119). Blood pressure decrease of testosterone and its metabolites were markedly greater in SHR than in WKY; 5β-DHT>testosterone>5α-DHT (120).

In A7r5 cells, testosterone directly blocks the LTCC channel over a physiological concentration range (121). In addition, testosterone and 5β-DHT reduce $[Ca^{2+}]_{cyt}$ increase in aortic VSMCs (122) and A7r5 cells (123). Interestingly, nifedipine (LTCC blocker), but not pimozide (TTCC blocker), blunted K^+-evoked rises of $[Ca^{2+}]_{cyt}$ (123), supporting the idea that the vasodilatory effect of testosterone involves the blockade of LTCC. Thus, Scragg et al demonstrated that a point mutation (T1007Y) in the α_{1C} subunit of $Ca_V1.2$, which drastically reduced affinity for organic Ca^{2+} channel antagonists, not only suppresses sensitivity to nifedipine, but also makes LTCC insensitive to testosterone. To date, evidence suggests that testosterone at physiological concentrations is capable of inhibiting $I_{Ca,L}$ in a voltage-independent manner. Perhaps, testosterone requires a direct molecular interaction with LTCC at the channel pore-forming α_{1C} subunit (124).

In general, an increase in the function and expression of $Ca_V1.2$ increase the myogenic tone in hypertension (125, 126), suggesting that the complex molecular mechanism of sex hormones on $Ca_V1.2$ have important implications under physiological and pathological conditions.

4.1.2. T-type Ca^{2+} channel (TTCC)

T-type Ca^{2+} channel (TTCC) together with LTCCs contribute to the vascular tone (72), and TTCC subunits $Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$ are found in several vascular beds (68). The activation of PKA and PKC inhibit TTCCs, decreasing extracellular Ca^{2+} entry.

Studies on the effect of sex hormones on TTCC are scarce, and most of them use non-physiological concentrations. $E_2β$ significantly reduces current through TTCC ($I_{Ca,T}$) **(Figure 3B)**,

while $E_2\alpha$ and tamoxifen cause significantly less reduction in A7r5 cells (91, 127). On the other hand, supraphysiologic concentrations of testosterone block TTCC human in A7r5 cells (121) **(Figure 3A)**.

4.2. Store-operated Ca^{2+} channels (SOCC)

After the initial release of Ca^{2+} from intracellular stores follows an event of sustained Ca^{2+} influx from the extracellular space. Depletion of the SR Ca^{2+} stores lead to SR refilling with external Ca^{2+} in a process known as store operated Ca^{2+} entry (SOCE) (128). STIM1 is a SR-localized Ca^{2+} sensor that activates the calcium release-activated channels (CRAC) consisting of tetramers of Orai1 protein (member of SOCC) in the PM. The result is to generate Ca^{2+} currents (I_{CRAC}) in the PM that replenish SR stores (72, 129).

Giachini et al. showed increased expression and activity of STIM1 and Orai1 proteins in males SHRSP compared to female SHRSP and WKY rats (62) **(Figure 1, C and D)**. Interestingly, aortas from OVX-SHRSP showed increased contraction during the Ca^{2+}-loading period and increased Orai1 expression, but no changes in the SR-buffering capacity or STIM1 expression (130). According to these findings, the authors hypothesize that stimulation of the STIM1/Orai-1 pathway might increase Ca^{2+} entry into VSMCs of hypertensive males compared to females. Consequently, hypertensive male rats exhibit higher $[Ca^{2+}]_{cyt}$ in their arteries, which may lead to greater vascular contraction (18). Although the non-genomic effects of $E_2\beta$ on SOCC remain to be elucidated, one study reported that $E_2\beta$ at supraphysiological concentrations can inhibit contractile responses to cyclopiazonic acid (CPA), a selective inhibitor of SERCA associated with SOCE to via non-L-type Ca^{2+} channels (46).

Testosterone reduces prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced Ca^{2+} influx through the SOCC in A7r5 cells, suggesting that testosterone-induced vasodilation involves a blocking effect on SOCC (131). In rat aorta, testosterone strongly inhibits CPA contractile responses, albeit at supraphysiological concentrations (115). The maximal testosterone-evoked dilation occurs after pre-constriction with agonist that activate VGCC, compared to the capacitive Ca^{2+} influx (via SOCC) induced by thapsigargin (116).

4.3. Transient receptor potential channels (TRP)

Transient receptor potential (TRP) channels are a superfamily of cation-conducting channels with 28 coding genes. Based on their sequence homology, TRP channels are grouped into six subfamilies; TRPM (melastatin), TRPC (canonical), TRPV (vanilloid), TRPP (polycystin), TRPA (ankyrin) and TRPML (mucolipin) (132).

4.3.1. TRPM channels

Although most TRP channels are permeable to Ca^{2+}, TRPM4 and TRPM5 are activated by Ca^{2+}, but are not permeable to Ca^{2+} or other divalent ions (133). The TRPM family's TRPM2, TRPM3, TRPM4, TRPM7, and TRPM8 are expressed in vascular tissue (132) and contribute to the development of vascular tone by regulating PM potential and contraction.

Sex hormones modulate the intracellular Ca^{2+} levels mediated by the TRPM channels activity. TRPM3 channels can be activated by sphingolipids, hypotonic shock, and the neurosteroid pregnenolone sulfate (134). In human saphenous vein vascular cells, pregnenolone sulfate stimulates TRPM3-dependent Ca^{2+} entry, while progesterone prevents pregnenolone sulfate-induced Ca^{2+} influx (135) **(Figure 3B).**

4.3.2. TRPC as functional channel of SOCC

In blood vessels, there exists evidence that TRPC channels (TRPC1, TRPC4, and/or TRPC5) can function as store-operated Ca^{2+} channels (SOCC) (128, 133). Thus, in response of the depletion of internal Ca^{2+} stores, TRPC channels can induce capacitive Ca^{2+} entry (128).

Studies on TRPC-related sex differences in vasculature are scarce and have not been studied in detail. Although $E_2\beta$ treatment in bovine aortic ECs significantly lowers TRPC4 gene expression (136), Park et al demonstrated that the acute activation of GPER in human ECs induces an increase in $[Ca^{2+}]_{cyt}$ through TRPC channels **(Figure 2B)**. This conclusion was based on the fact that pretreatment of ECs with the TRPC inhibitor SKF-96365 suppressed G-1-induced intracellular Ca^{2+}; however, pretreatment with HC-030031 (TRPA antagonist) showed no effect (137). In VSMC of human saphenous vein, progesterone inhibited Ca^{2+} entry through endogenous TRPC5-containing channels **(Figure 3B)**. This membrane-delimited effect was relatively rapid in onset and occurred in an independent way of the channel expression mechanism or intracellular organelles (138).

So far, evidence has not linked the signaling mechanisms of sex hormones with the SOCC-like activity of TRPC. The discovery that STIM1/Orai-1 complex is critically involved in SOCE casts doubt on the obligate role of TRP channels in this response. Even, it has been reported that SOCE can occur in the absence of TRPC1, TRPC4, and TRPC5 expression and that TRPC channels can function independently of STIM1 and Orai-1 (For review, see Ref. (139)). Therefore, the functional relationships between STIM1/Orai-1 and TRPC during SOCE remain to be investigated, and then to confirm whether sex hormones influence the activity of TRPC to mediate SOCE.

4.3.3. TRPC as functional channel of ROCC

Ca^{2+} entry via receptor-operated channels (ROCC) causes a sustained contractile response (plateau phase) called “receptor-operated Ca^{2+} entry” (ROCE) (133). Unlike SOCE, ROCE is activated without Ca^{2+} release from intracellular stores (140).
In blood vessels, TRPC channels can also function as receptor-operated channels (ROCC). For example, TRPC3, TRPC6, and TRPC7 channels belong to ROCC, and they are directly activated (i.e., independent of protein kinase C, PKC) by DAG in the vasculature (133, 140, 141).

Physiological concentrations of testosterone inhibit $PGF_{2\alpha}$-induced Ca^{2+} fluxes by a non-genomic mechanism, probably through ROCC in aortic VSMCs (142). In males, TRPC3 (ROCC) plays a key role in the vasodilator response induced by bradykinin in a NO- and EDHF-dependent manner

(Figure 1A), although the function of TRPC3 was sex dependent, the TRPC3 expression is not (143). Likewise, norgestimate (a synthetic estrogen) blocks TRPC-mediated cation currents induced by vasopressin in A7r5 cells. In addition, norgestimate causes vasorelaxation of the aorta through its inhibitory effects on DAG-sensitive TRPC3 and TRPC6 channels (144).

4.3.4. TRPV

The TRPV family's TRPV1, TRPV2, TRPV3 are expressed in vascular tissue, and contribute to the vascular tone increases by regulating PM potential (132). TRPV4 contributes to vasodilation involving the endothelial NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) pathways. TRPV4 activation can also directly cause hyperpolarization and vasodilation in VSMCs due to a functional coupling with K_{Ca} (145). On the other hand, the activation of TRPV4 can also cause constriction in some specific vascular beds or under pathological conditions (145).

Although there are no reported sex differences in the vascular TRPV4 expression, the function of TRPV4 channel seems different in male versus female. In female isolated pig coronary arteries, selective inhibition of TRPV4 by RN1734 significantly reduced vascular relaxation in NO-dependent bradykinin, but not coronary arteries from in male pigs (143) **(Figure 1B)**. These events are probably associated with the inhibitory effect of progesterone on the expression and activity of the TRPV4 channel through a genomic mechanism that involves the progesterone receptor (PR) (146) **(Figure 3B).** On the other hand, vasodilator effect of testosterone is mediated by the androgen receptor (AR) in ECs, which increases the production of hydrogen sulfide (H_2S), leading to H_2S-mediated vasodilatation, which involves Ca^{2+} influx through TRPV4 activation (147, 148) **(Figure 2A).**

4.4. Mechanosensitive Ca^{2+} channels (Piezo)

Piezo1 and Piezo2 mechanosensitive ion channels are nonselective ion channels permeable to Na^+, K^+, and Ca^{2+}, it also has a very low permeability to Cl^- (149, 150). Piezo1 acts as a blood flow sensor in the endothelium, exhibiting a slow current inactivation that causes a constant increase in the influx of intracellular Ca^{2+}, leading to NO generation (151).

As previously reported, estrogens enhance uterine blood flow (UBF) during pregnancy due to decreased contractile force of the uterine arteries (74, 75). When Piezo1 is stimulated by increasing intraluminal flow or by pharmacological treatment (Yoda1; Piezo1 agonist), it led to a NO vasodilation in the uterine artery, which is associated with significant increases in $[Ca^{2+}]_{cyt}$ in ECs. Piezo1 is mostly found in ECs of the uterine arteries of late pregnant rats, and its expression increases significantly during pregnancy (152) **(Figure 1B)**. Recently, Piezo1 was also discovered in uterine smooth muscle and the microvascular endothelium of pregnant human myometrium (153).

Arishe et al also found that Piezo1 activation increases drug dose-dependent vasodilation (Yoda1) in the uterine arteries of pregnant normotensive rats compared to virgin rats. However, this relaxation response in hypertensive rats was lower than in normotensive pregnant (controls), suggesting that the downregulation of the Piezo 1 channel contributes to vascular dysfunction in

hypertensive animals similar with preeclampsia (154). Although the activation of Piezo-1 in pregnant animals led to greater vasodilation in uterine arteries than the resistance mesenteric arteries, the Piezo1-mediated vasodilation in both vascular beds involves NO pathways. These findings would indicate that Piezo1-mediated vasodilation during pregnancy is dependent on the vascular bed (155).

5. Mechanisms of Ca^{2+} Removal

5.1. Plasma membrane Ca^{2+}-ATPase (PMCA)

Calcium homeostasis involves several distinct mechanisms, such as PMCA, a member of the P-type pump family. PMCA is essential for the removal of intracellular Ca^{2+} into the extracellular space. PMCA pump hydrolyzes 1 ATP molecule per Ca^{2+} ion transported (1:1 stoichiometry), and there are four isoforms defined as PMCA 1-4 (156). PMCA1 is the predominant isoform in VSMCs and ECs (157). In the vasculature, the activity of PMCA is stimulated by CaM (158), and also though activation via PKC- and PKG-induced phosphorylation (159, 160).

Tran et al, showed that GPER activation inhibited PMCA activity and blocked Ca^{2+} efflux **(Figure 2B)** mediated by tyrosine phosphorylation (Tyr1176 PMCA); this interaction between PMCA and GPER occurs when GPER C-terminal PDZ-binding motifs interact to inhibit PMCA in the presence of postsynaptic density protein PSD-95 (161). Secondy, $E_2\beta$ via GPER increased the expression of CaM which involved the epidermal growth factor receptor (EGFR), and downstream activation of the mitogen-activated protein kinase (MAPK) cascade. Thirdy, $E_2\beta$ enhanced the binding of CaM to GPER, ERα, eNOS, and particularly PMCA (162), although PMCA activation would be masked by the inhibitory effect of GPER-induced PMCA phosphorylation (161). In vascular ECs, $E_2\beta$ decreases the cytosolic Ca^{2+} by inhibition of PMCA activity and enhancing CaM levels, leading to increase of eNOS activity, contributing significantly to the decrease of vascular tone (162).

In coronary artery smooth muscles, $E_2\beta$ decreases $[Ca^{2+}]_{cyt}$ through PMCA stimulation. The inhibition of PMCA with lanthanum abolishes the decrease in $[Ca^{2+}]_{cyt}$ by $E_2\beta$ (163, 164).

5.2. Plasma membrane Na^+/Ca^{2+} exchanger (NCX)

Na^+/Ca^{2+} exchanger of the plasma membrane (NCX) is an important mechanism of intracellular Ca^{2+} removal. Because of its low affinity for Ca^{2+} but high Ca^{2+} transport capacity, NCX exchanges one Ca^{2+} for 3 Na^+ (165). The exchanger has two modes; forward which favors the exit of Ca^{2+} to extracellular space and reverse, which favors Ca^{2+} entry. (166). In vascular tissues, NCX1 is the dominant isoform, and NCX1.3 and NCX1.7 are the predominant splice variants (167). In human pulmonary VSMC, Zhang et al, showed that removal of extracellular Na^+, which switches the NCX exchanger from the forward mode to the reverse mode, significantly increases $[Ca^{2+}]_{cyt}$ (168). Apparently, the TRPC3-mediated Na^+ influx and membrane depolarization is able to shift the equilibrium of Na^+/Ca^{2+} exchange sufficiently to enable Ca^{2+} entry via reversed mode exchange, and therefore favoring $[Ca^{2+}]_{cyt}$ overload (169). This mechanism requires interaction between

NCX1 with the cytosolic C terminus of TRPC3, suggesting functional and physical interaction of nonselective TRPC cation channels with NCX proteins as a novel principle of TRPC-mediated Ca^{2+} signaling (170) (For review, see ref. (139))

To the best of our knowledge, to date there are no reports in which the influence of sex on the expression or activity of the NCX exchanger at the vasculature level has been clearly and conclusively described. However, in view of the expression patterns of NCX, particularly NCX1, the greatest amount and information is from cardiac tissues. Several studies found a higher expression and/or activity of NCX in female human, rat, and rabbit cardiomyocytes compared to their male counterparts (169). However, current evidence regarding the effect of estrogen deficiency on the activity and expression of NCX is not conclusive. Although NCX expression in cardiomyocytes from OVX animals is decreased and estrogen replacement is capable of reversing this effect, in other studies, no change in NCX expression was observed in both estrogen treatment and OVX animals. Some studies even show that long-term estrogen deprivation significantly increases NCX expression and activity (55, 169, 171).

A single study carried out in VSMC of rat aorta, found that treatment for 24 hours with sex hormones such as $E_2\beta$, progesterone and testosterone, had no effect on the activity of NCX, quantified as the reversed Na^+ gradient-dependent $^{45}Ca^{2+}$ influx (i.e. raising cell Na^+ and replacing external Na^+ with K^+) (172).

5.3. Na^+/K^+−ATPase pump

The Na^+/K^+−ATPase is an integral membrane protein that regulates cell membrane potential by maintaining Na^+ and K^+ ion gradients across the plasma membrane (173). The Na^+/K^+−ATPase is characterized by complex molecular heterogeneity resulting from differential expression and association of multiple isoforms of the α and β subunits. In mammals, up to four α isoforms (α_1-α_4) and three β isoforms (β_1-β_3) have been identified (174). The α subunit, responsible for the catalytic transport activities of the enzyme, contains binding sites for cations, ATP, and the inhibitor ouabain. The β subunit, is a highly glycosylated protein essential for normal enzyme activity (174). Although the α_1 isoform is homogeneously distributed in the smooth muscle cell membrane, the α_2 isoform of Na^+/K^+−ATPase is considered the most important for the regulation of vascular tone (175). This isoform localizes to spatially restricted areas of the plasma membrane of VSMCs very close to the SR, forming a microdomain that is capable of controlling the local Na^+ concentration near the NCX and thus influencing $[Ca^{2+}]_{cyt}$ (175).

The findings of Palacios et al indicated that acetylcholine (ACh) increases the Na^+/K^+−ATPase activity in female rat arteries through the action of estrogens and participation of NO (176). Female rats exhibited a higher proportion of α_2 isoform compared to males **(Figure 1, C and D)**, and this effect was reproduced by $E_2\beta$ administration in male arterial explants **(Figure 3B)**. In addition, ovariectomy completely abolished the stimulation of ACh-induced Na^+/K^+−ATPase activity, and $E_2\beta$ treatment reversed this effect (176). Interestingly, a study showed that $E_2\beta$ is also capable of increasing the α_1 subunit in rat aorta VSMC, by a mechanism dependent on multiple signaling

cascades, including phosphatidylinositol-3 kinase (PI3K), cytosolic phospholipase A_2 ($cPLA_2$), and mitogen-activated protein kinase ($p42/44^{MAPK}$) (177) **(Figure 3B)**.

Contrary to our findings above, a study by Dias et al. suggested that participation of Na^+/K^+−ATPase in the vascular relaxation is higher in male rats compared with females, and NO might have a greater influence on the Na^+/K^+−ATPase participation in the relaxation of the female rat aorta (178). Results from another study showed that ovariectomy for eight weeks increased free radical production, leading to decreased NO (179). However, ovariectomy did not decrease ACh or sodium nitroprusside-induced relaxation due to an increase in the activity of Na^+/K^+−ATPase (179).

5.4. Na^+–K^+–$2Cl^-$ symporter

NKCC co-transporters regulate the electroneutral movement of Na^+ and K^+ and are closely coupled to the movement of Cl^- across cell membranes (180, 181). NKCC1 is expressed on all cell types, including VSMCs and erythrocytes, while NKCC2 is exclusively expressed on the apical membranes of renal epithelial cells. Both isoforms are inhibited by high-ceiling diuretics (HCD), such as furosemide and bumetanide (182).

In general, NKCC1 participates in the elevation of BP through the increase of $[Cl^-]_i$ (180, 182). Vasoconstrictor agonists activate the Ca^{2+}-activated Cl^- channel (i.e., TMEM16A), leading to increase Cl^- efflux thence membrane depolarization. Simultaneous activation of LTCC channels increases intracellular Ca^{2+} producing vasoconstriction (183).

Substitution of Cl^- ions by thiocyanate ions in the extracellular medium of aortic rings causes a decrease of $[Ca^{2+}]_{cyt}$, and a reduction of the contractile response in female rats (184). Palacios et al. found that PE-stimulated NKCC1 activity was significantly higher in the aorta of male rats when compared with females **(Figure 1, C and D)**, and estradiol treatment prevented stimulation of NKCC1 activity in ovariectomized rats. Likewise, NKCC1 inhibition with bumetanide reduced the vascular contractile response to PE in male rats, but not in females (185). Further research is required to elucidate the role of NKCC1 in in vascular response sex differentials.

6. Other intracellular Ca^{2+} regulatory proteins

6.1. Mitochondrial Ca^{2+} uniporter (MCU)

The mitochondria also play an important role in Ca^{2+} homeostasis. In the heart, the regulation of Ca^{2+} ion channels and mitochondrial function is highly sensitive to estrogen deficiency. Compared with males, female mitochondria have lower Ca^{2+} uptake rates and can adequately maintain mitochondria membrane potential ($\Delta\psi_m$) under conditions of high $[Ca^{2+}]_{cyt}$ (186). Furthermore, reactive oxygen species (ROS) production rates are lower in the cardiac mitochondria of female rats compared to age-matched males. In $E_2\beta$-deficient OVX rats, there is reduced ATP production, which may contribute to an increase in cardiac injuries, such as ischemic reperfusion (I/R) injury. The impact on cellular energy homeostasis and reactive oxygen species (ROS) balance would

be explained due to the genomic and non-genomic actions of cellular and mitochondrial ER/GPER signaling initiated by $E_2\beta$ (Reviewed in detail by (169)).

On the other hand, in vascular tissue, the mitochondrial Ca^{2+} uniporter (MCU) complex transports Ca^{2+} into mitochondria and is responsible for the generation of rhythmic waves of $[Ca^{2+}]_{cyt}$ (187). Although mitochondrial morphology and function differ between men and women in various organs and cell types, studies on vascular tissue are scarce.

A recent study showed sex differences in the handling of mitochondrial Ca^{2+} of the vascular endothelium. Endothelial MCU inhibition reduced vasoconstriction in the mesenteric resistance arteries of female mice significantly more than in males (188) **(Figure 1, A and B).** In the same study, $E_2\beta$ treatment at physiological concentrations increased mitochondrial Ca^{2+} entry and Ca^{2+} storage capacity in human aortic endothelial cells (HAECs), reducing mitochondrial ROS generation. These changes appear to be caused by the existence of more mitochondria and higher mitochondrial membrane potential in female mice **(Figure 3B).** These effects were not produced by testosterone; there was even an increase in mitochondrial ROS levels (188) **(Figure 2A)**.

It has been hypothesized, that specific receptors on the mitochondrial membrane are involved in the mitochondrial Ca^{2+} uptake produced by estrogen agonists at pharmacological concentrations (189). It is not clear, whether the decrease of mitochondrial Ca^{2+} influx in ECs or VSMCs depends on the activation of sex hormone receptors.

6.2. Gap junctions

Gap junctions are essential for intercellular communication between ECs and between ECs and VSMCs in vascular tissue. Gap junction channels are formed by connexins (Cx); six connexins form a connexon or hemichannel and the docking of two connexons result in a full gap junction channel allowing for the exchange of ions and small metabolites between neighboring cell (190). There is a consensus that an increase in $[Ca^{2+}]_{cyt}$ and subsequent activation of K_{Ca} channels in the endothelium is a prerequisite for the generation of endothelium-derived hyperpolarization (EDH). K_{Ca} activation hyperpolarizes endothelial cells, and K^+ efflux through them can act as a diffusible EDHF by stimulating vascular smooth muscle Na^+/K^+-ATPase and K_{IR} (191). In parallel, hyperpolarizing current spreads from the endothelium to the VSMCs through myoendothelial gap junctions (MEGJs) located on endothelial projections, contributing to the intercellular transduction of vasodilator signals from ECs (190, 192).

Several studies demonstrate the impact of estrogen on EDHF-dependent vasodilation and the role of MEGJs in this response (193–195). In estrogen-deficient (196, 197) or hyperandrogenemic female rats (198), EDHF-mediated decrease of vasodilatation implicated reduced MEGJ (Cx40 or Cx43) expression (196–198), whereas $E_2\beta$ hormone replacement therapy recovered the vascular response (195–197, 199).

Moreover, estrogenic vasodilation induced by EDHF is greater in the reproductive vessels (uterine artery) than in the non-reproductive ones (mesenteric artery) (199). Connexins such as Cx37 and Cx43 are highly expressed in the endothelium of uterine arteries during the follicular phase, and even more so during pregnancy; physiological states associated with high estrogen levels (200) **(Figure 1B)**. The authors clarify that endothelial ATP-induced eNOS activation and NO production are mediated by Ca^{2+} and have a mandatory requirement for Cx43 (200). However, although this represents a great advancement in understanding the role of connexins on local vascular tone, it remains to be clear whether this applies to the control of systemic blood pressure, since it has previously been reported that neither ovariectomy nor hormone replacement had any effect on Cx43 expression in aortic VSMCs compared to control animals (201).

7. Priorities for further research

Women live longer than men, and this is partly due to a lower risk of cardiovascular diseases (CVDs). It is increasingly clear that Ca^{2+}-handling mechanisms in the cardiovascular system play an important role in the development and progression of CVDs such as arterial hypertension. Although there is abundant evidence that sex and sex hormones influence Ca^{2+} handling in cardiac muscle, this is a field that has not yet been widely explored in the vasculature.

Around 50 years ago, researchers began studying the effects of sex hormones on Ca^{2+} handling in isolated vascular tissues, VSMCs, and ECs. Since then, many researchers have made important discoveries to help us better understand how androgens and estrogens impact cellular Ca^{2+} homeostasis.

The knowledge we have about the influence of sex on vascular Ca^{2+} management mechanisms has been studied in only three species. In pigs, it has been shown that the activity/expression of TRPC3 (143), TRPV4 (143) and $Ca_V1.2$ (106, 107) differs between males and females. While, in rats, these differences involve STIM1 (62, 130), Orai-1 (62, 130), Na^+/K^+-ATPase (176, 178), and NCCK1(185). Likewise, the key role of MCU on vasorelaxation of the mesenteric arteries of females compared to males has been demonstrated only in a mouse model (188). Evidence of sex differences in humans are not available (**Table 1**), as such, more research is needed to either validate or confirm these effects. There remain gaps in knowledge regarding sex differences that should be studied in animal models before moving to humans. For example, a good starting point would be to identify whether there are also sex differences in the activity or expression of IP_3R, RyR, SERCA, TTCC, Piezo1, PMCA and NCX.

It is also the case that many of the studies that describe sex differences in vascular Ca^{2+} management have used conductive arteries - such as the aorta - for vascular reactivity assays (**Table 1**). However, since blood pressure is a product of cardiac output and total peripheral resistance, small arteries and resistance arterioles are key determinants of blood pressure because their contractile state defines vascular resistance. In contrast, conductive arteries have less direct influence on blood pressure and function to transport blood flow to resistance vessels which in turn control blood flow to downstream organs and tissues (202). Studies on basal NO

production and VGCC-mediated contractions, myogenic contractile tensions in smaller muscular arteries (femoral and mesenteric arteries) and the larger elastic conduit vessels (aorta and carotid artery) and associated sex differences should be a consideration for future research. This is because muscular arteries have a significantly higher sensitivity to the VGCC blockers, suggesting different populations of VGCC isoforms in these vessels (203).

In many countries, demographics reveal an aging population, therefore, elucidating age-related mechanisms of vascular dysfunction and integrating sex differences is essential if we are to better direct pharmacological interventions thus reduce/manage the severity of cardiovascular risk in both men and women. In addition to different morphological, mechanical, physiological and structural characteristics, large elastic arteries such as the aorta tend to develop greater arterial stiffness compared to small muscular arteries, and this mainly influenced by aging (205). While large arteries remodel to preserve Windkessel function (buffering effect), small blood vessels are reported to maintain greater longitudinal compliance (207).

Senescence induces arterial hyperreactivity mediated by α_1 adrenergic receptors through a mechanism related to an increase in Ca^{2+} influx and release of COX-derived vasoconstrictive prostanoids (209). Furthermore, Ca^{2+} signaling in the vascular endothelium decreases with age, further contributing to the impairment of endothelium-dependent vasodilation. With aging, there is a reduced level of calreticulin (Calr) - a multifunctional Ca^{2+} binding protein and ER chaperone - leading to decreased vasodilation. However, although advancing age reduces Calr protein expression in small resistance mesenteric arteries, this does not occur similarly in large arteries such as the aorta (210).

Interestingly, the controversy over the beneficial or harmful effects of estrogens in hormone replacement therapy (HRT) could also be resolved with studies linking sex, age and oxidative stress. So far it seems that HRT mainly favors young women when compared to postmenopausal women. This may be due to a mechanism by which NOS is uncoupled with advance in age, and instead of producing NO, it increases superoxide (O_2^-) levels (211). Likewise, ER stress could be associated with the lack of vasodilatory response due to calreticulin deficiency and Ca^{2+} events in the endothelium (210). Finally, given that the expression of STIM1 and Orai-1 is higher in males than in females (62, 130), it remains to be studied whether this sex difference also influences vascular inflammation. This hypothesis arises in view of the fact that CRAC channels (formed by STIM1 and Orai-1) are responsible for histamine-induced Ca^{2+} influx, thus increasing permeability and interleukin synthesis in lymphatic ECs (212).

Vascular Ca^{2+} homeostasis can also be studied through the use of experimental models with altered levels of sex hormones, Pregnancy (high estrogen levels) (48), ovariectomy (hypoestrogenism) (196, 197), orchiectomy (low androgen levels) (107), and the hyperandrogenemia model (198), could further elucidate how sex hormones impact the activity/expression of proteins that manage Ca^{2+} in the vasculature (**Table 1**).

Many studies that evaluated the effect of sex hormones or sex on the vasculature have used vasoactive chemicals, but did not measure myogenic differences in basal vascular tone. If sex-based differences affect basal vascular tone, then the exploration of the underlying mechanisms is crucial to better understand vascular physiology and pharmacology. In conclusion, to associate a mechanism with a particular physiological event, it is necessary that future studies make efforts to acquire data from different types of vascular beds (conducting and resistance arteries) in both sexes. Those studies ought to take into account age and metabolic status (oxidative stress and inflammation) of the specimen in both basal and pathological conditions. Furthermore, variables such as sex hormone levels, reproductive age, cycle phase, and other influencing factors should be considered and reported clearly in order to improve the rigor and reproducibility of the findings. (213).

Table 1. Advances in the field and gaps in knowledge pending study

Location	Protein	Sex-based studies was made in…		Type of artery		Are activity/ expression influenced by … ?			
		human	Animal	Large (conduit)	Small (resistance)	Pregnancy	Hypoestrogenism	Hyperandrogenemia	Hypoandrogenism
PM	IP_3R	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	RyR	N.D.	N.D.	N.D.	N.D.	Yes	N.D.	N.D.	N.D.
	SERCA	N.D.	Rat	Aorta	N.D.	N.D.	N.D.	N.D.	N.D.
	STIM1	N.D.	Rat	Aorta	N.D.	N.D.	No	N.D.	N.D.
PM (Ca^{2+} entry)	TRPC3	N.D.	Swine	N.D.	Coronary artery	N.D.	N.D.	N.D.	N.D.
	TRPV4	N.D.	Swine	N.D.	Coronary artery	N.D.	N.D.	N.D.	N.D.
	$Ca_V1.2$	N.D.	Swine / rat	Aorta	Coronary artery	N.D.	Yes	N.D.	Yes
	TTCC	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Orai-1	N.D.	Rat	Aorta	N.D.	N.D.	Yes	N.D.	N.D.
	Piezo1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PM (Ca^{2+} extrusion)	PMCA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	NCX	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	NKA	N.D.	Rat	Aorta	N.D.	N.D.	Yes	N.D.	N.D.
	NKCC1	N.D.	Rat	Aorta	N.D.	N.D.	Yes	N.D.	N.D.
Mitochondria	MCU	N.D.	Mice	N.D.	Mesenteric artery	N.D.	N.D.	N.D.	N.D.
MEGJs	Connexins	N.D.	Rat	N.D.	Mesenteric artery	Yes	Yes	Yes	N.D.

ND: No data, PM: Plasmatic membrane, MEGJs: myoendothelial gap junctions,

8. Summary of key findings

In this section we highlight the most important studies from which it is inferred that Ca^{2+} management in the vasculature is influenced by sex and sex hormones (**Table 2**). Many studies show that $[Ca^{2+}]_{cyt}$ in VSMCs is lower in females than in males (**Figure 1**). There exists

considerable evidence that sex influences the expression levels and activity of Ca^{2+}-handling proteins, and this is mainly regulated by the genomic and non-genomic actions of sex hormones (**Figure 2 and 3**). Our review demonstrates that in female VSMCs there is enhanced vasodilation due to a reduction in the expression or activity of proteins that promote Ca^{2+} influx such as $Ca_V1.2$ (106, 107), STIM1 (62, 130), Orai-1 (62, 130), and NKCC1 (185). Likewise, there is an increase in the activity/expression of the Na^+/K^+-ATPase (176), an enzyme that influences intracellular Na^+ levels thus Ca^{2+} extrusion via NCX (175). However, it is necessary to clarify the role of Na^+/K^+ ATPase in sex differences, since only one study showed greater activity of the enzyme in the aorta of males (178). In female ECs there is an increase in NO production favored by the entry of Ca^{2+} through TRPV4 (143) and the Ca^{2+} buffering capacity mediated by MCU (188). Some studies demonstrate that estrogen levels in females influence the expression of certain Ca^{2+} handling proteins. For instance, physiological elevations of estrogen such as those in pregnancy, upregulate RyR producing an increase in Ca^{2+} sparks (48). In turn, Ca^{2+} sparks activate STOCs producing membrane hyperpolarization through the activation of K_{Ca} (44). On the other hand, during states of hypoestrogenism, vasodilatory capacity is reduced due to lower expression of Cx43 (196, 197). Interestingly, when androgen levels are high, an increase in blood pressure occurs, likely due to reduced EDHF-mediated vasodilation and a reduction in the expression of Cx43 (198).

In males, TRPC3 plays an important role in NO-mediated vasodilation (143), however, the endothelial Ca^{2+} buffering capacity through MCU is reduced compared to females (188). Furthermore, $[Ca^{2+}]_{SR}$ in males is lower compared to females (16), this partly explains why the STIM1/Orai-1 pathway is upregulated (62, 130). This could mean that in males there is more cytosolic Ca^{2+} available to promote vascular contraction or that the mechanisms of Ca^{2+} reuptake into the sarcoplasmic reticulum are less effective compared to females (30).

Table 2 Summary of studies designed to investigate the influence of sex or sex hormones on Ca^{2+}-handling proteins in vasculature

Protein	Species	sex	Vascular tissue/ cell type	study design	Method/ technique	Hormone/ Molecule/ Intervention	Findings	Ref.
RyR	sheep	Female	Uterine artery (VSMC)	In vitro/ Ex vivo	qPCR + WB + CFM + Patch-clamp + Pressure-dependent vascular reactivity	Pregnant vs non-pregnant	Pregnancy effects: ↑ RyR1-3 expression ↑ Ca^{2+} sparks ↑ STOCs	(48)
RyR	sheep	Female	Uterine artery	In vitro	qPCR + WB	$E_2\beta$ + progesterone	↑ RyR1-3 expression in non-pregnant	(48)
SERCA	SHR	Male and female	Aorta (VSMC)	In vitro	Fluorescence microscopy	Thapsigargin	$[Ca^{2+}]_{SR}$ are higher in females than in males	(16)
SERCA	human	Female	Umbilical artery (VSMC)	In vitro/ Ex vivo	qPCR + WB + siRNA assay + fluorescence	Progesterone	↓ PLB expression	(30)

					microscopy + vascular reactivity		↑ SERCA activity and expression ↑ $[Ca^{2+}]_{SR}$ ↓ $[Ca^{2+}]_{Cyt}$	
STIM1/ Orai-1	SHRSP	Male and female	Aorta	Ex vivo	WB + vascular reactivity	Male vs female	STIM1/Orai-1 expression is higher in males compared to females.	(62, 130)
TRP	Swine	Male and female	Coronary artery	Ex vivo	WB + vascular reactivity	Male vs female	In females, TRPV4 plays a role in NO-mediated vasorelaxation. However, in men, TRPC3 plays a major role. Protein expression level of TRPV4 and TRPC3 were not influenced by sex	(143)
LTCC	Rat	Male and female	Thoracic aorta	Ex vivo	$[^{45}Ca^{2+}]$ influx assay + vascular reactivity	Male vs female	↓Ca^{2+} influx in females compared to males	(99)
LTCC	Rabbit	Male and female	Coronary artery	Ex vivo	Vascular reactivity	Progesterone	↓ $CaCl_2$ – induced contraction in free-Ca^{2+} medium	(79)
LTCC	human	Male	Aortic VSMC	In vitro	$[^{45}Ca^{2+}]$ influx assay	$E_2\beta$	↓ $^{45}Ca^{2+}$ influx in depolarized VSMC	(90)
LTCC	human	Pregnant female	Umbilical vein	In vitro/Ex vivo	qPCR + WB + vascular reactivity	Progesterone	↓ KCl– and Bay-k8644 and $CaCl_2$– induced contraction No changes in $Ca_V1.2$ expression	(47)
LTCC	human	pregnant female	Umbilical artery	In vitro	qPCR	DHT	↓ α_{1C} subunit mRNA expression	(110)
LTCC	Swine	Male and female	Coronary artery (VSMC)	In vitro	Whole cell voltage clamp	Male vs female	Higher I_{Ca} density in males than in females	(106)
LTCC	Swine	Male and female	Coronary artery (segments and VSMC)	In vitro	Whole cell voltage clamp + qPCR + WB +	Male vs female	- Higher $I_{Ca,L}$ density in males than in females due to higher	(107)

					immunohistochemistry		expression of $Ca_V1.2$	
Piezo-1	human	Female	Microvascular endothelium (ECs)	In vitro	Fluorescence microscopy	Yoda 1	Piezo-mediated Ca^{2+} influx	(153)
Piezo-1	Rat	Female	Main uterine artery	In vitro/Ex vivo	WB + immunohistochemistry + vascular reactivity	Pregnant vs non-pregnant	↑ Piezo-1 expression during late pregnancy Pregnancy does not affect Piezo-mediated Ca^{2+} entry	(152)
Na^+/K^+-ATPase	Rat	Male and female	Aorta	In vitro/Ex vivo	Semiquantitative PCR + WB + $^{86}Rb^+/K^+$ uptake assay + vascular reactivity	Male vs female	↑ Na^+/K^+–ATPase activity in females ↑Levels of α_2 isoform in females ↓ Na^+/K^+–ATPase activity in OVX rats	(176)
Na^+/K^+-ATPase	Rat	Male and female	Aorta	Ex vivo	Vascular reactivity	Male vs female	↑ Na^+/K^+–ATPase activity in males	(178)
NKCC1	Rat	Male and female	Aorta	Ex vivo	$^{86}Rb^+/K^+$ uptake assay + vascular reactivity	Male vs female	No differences in NKCC1 basal activity ↑ PE-stimulated NKCC1 activity in male compared with female	(185)
MCU	mice	Male and female	Mesenteric artery (endothelium)	Ex vivo	Transgenic MCU model + vascular reactivity	Male vs female	MCU ablation reduces ACh - induced vasodilatation to a greater extent in females than in males	(188)
Cx43	Rat	Female	Mesenteric artery	In vivo/Ex vivo/In vitro	Ovariectomy + $E_2\beta$ levels + electrophysiology + vascular reactivity + immunohistochemistry or WB + PCR	Hypoestrogenism model	↓ ACh - induced EDHF-mediated vasodilatation ↓ Cx43 expression	(196, 197)
Cx43	Rat	Female	Mesenteric artery	In vivo/Ex vivo/In vitro	Blood Pressure measurement + vascular reactivity +	Hyperandrogenism model	↑Blood pressure	(198)

					qPCR + WB + Immunofluorescence microscopy		↓ EDHF-mediated ACh relaxation ↓ Cx43 expression	

CFM: Confocal fluorescence microscopy, WB: Western blot, qPCR: quantitative polymerase chain reaction, PSHRSP: stroke prone spontaneously hypertensive rat, PE: phenylephrine, $E_2\beta$: 17β-estradiol, STOC: spontaneous transient outward current, SHR: spontaneously hypertensive rat, RyR : ryanodine receptor, siRNA: small interfering RNA, SERCA: Sarco/endoplasmic Ca^{2+}-ATPase, $[Ca^{2+}]_{cyt}$: Cytosolic Ca^{2+} levels, $[Ca^{2+}]_{SR}$: Ca^{2+} levels in the SR, PLB : Phospholamban, STIM1: Stromal Interaction Molecule 1, Orai-1: Ca^{2+} release-activated Ca^{2+} channel protein 1, TRP: transient receptor potential channel, TRPV: transient receptor potential vanilloid, TRPC: transient receptor potential canonical , $I_{Ca,L}$: LTCC current, OVX: ovariectomy, NKCC1: Na+–K+–2Cl−symporter type 1, MCU: mitochondrial calcium uniporter, ACh : acetylcholine.

Finally, this review highlights certain gaps in knowledge.

1) The role of IP_3R, RyR, SERCA, TTCC, Piezo1, PMCA and NCX in sex differences has not yet been clearly described in VSMCs and ECs. 2) Several studies suggest that during pregnancy - a physiological state associated with high levels of estrogen - there is an upregulation of Piezo1, RyR and MEGJs. Although this is an important advance in knowledge, it does not necessarily mean that these proteins play an important role in the sex differences in vascular Ca^{2+} homeostasis. 3) It is known that the physiological levels of sex hormones vary between males and females and across species. In addition, several studies used supraphysiological concentrations of sex hormones to better understand the Ca^{2+} homeostasis in blood vessels. Therefore, future research must use physiological concentrations of sex hormones and species must be considered.

To obtain more conclusive evidence of the participation of the genomic pathway in Ca^{2+} management, it will be necessary to use male and female sex hormones receptor knockout mice. Additionally, the tamoxifen-inducible Cre-LoxP-based genetic modification systems may be used to investigate specific genes of interest (For review, see (204)). However, a remaining concern relates to the toxic effects of tamoxifen on cardiomyocytes and vascular endothelial cells (206).

Another important area for future work is to identify whether protein kinases (such as PKA and PKG) are involved in sex differences, because several of their phosphorylation targets include the transporters, pumps, receptors and channels that regulate Ca^{2+} homeostasis in VSMCs and ECs. Furthermore, computational models and molecular docking techniques could shed light on the interaction between sex hormones and proteins that handle Ca^{2+}, thus contributing to the understanding of their non-genomic effects. These sex-based in silico models of VSMCs could indicate the fundamental ionic mechanisms underlying membrane potential and intracellular Ca^{2+} signaling during the development of myogenic tone (208).

9. Concluding remarks

The historical rationale for only using male animal models was to exclude the effects of changes in sex hormones during the estrous cycle in females. However, the evidence summarized here

demonstrates important fundamental sex differences in vascular Ca^{2+} handling thus blood vessel responses and regulation of tone. For a complete understanding of the field, and development of effective therapeutic strategies, investigators ought to consider these differences when interpreting previous work and designing future studies.

GLOSSARY

$[Ca^{2+}]_{cyt}$ Cytosolic Ca^{2+} levels

$[Ca^{2+}]_{SR}$ Sarcoplasmic Ca^{2+} levels

5-HT Serotonin

5α-DHT 5α-dihydrotestosterone

5β-DHT 5β-dihydrotestosterone

ACh acetylcholine

AKAP5 kinase-A anchor protein 5

Ang II Angiotensin II

AR Androgen receptor

ATP Adenosine triphosphate

BP Blood pressure

CaCC Ca^{2+}-activated Cl^- channels

CaM Calmodulin

CAMKII Ca^{2+}/CaM-dependent protein kinase II

cAMP Cyclic adenosine monophosphate

CASM Coronary artery smooth muscle

$Ca_V1.2$ voltage-gated Ca^{2+} channel subunit alpha 1C

cGMP Cyclic guanosine monophosphate

CPA Cyclopiazonic acid

$cPLA_2$ Cytosolic phospholipase A2

CRAC Ca^{2+} channel activated by Ca^{2+} release

CRE: cAMP response element

CVDs cardiovascular diseases

Cx Connexin

DAG Diacylglycerol

$E_2α$ 17α-estradiol

$E_2β$ 17β-estradiol

ECs Endothelial cell(s)

EDHF Endothelium-derived hyperpolarizing factor

EGFR Epidermal growth factor receptor

eNOS Endothelial nitric oxide synthase

ER Endoplasmic reticulum

ERα Estrogen receptor alpha

ERβ Estrogen receptor beta

ET-1 Endothelin-1

G-1 GPER/GPR30 agonist

GPCR G protein-coupled receptor

MCU Mitochondrial Ca^{2+} uniporter

MEGJs Myoendothelial gap junction(s)

MLC Myosin light chain

MLCK Myosin light chain kinase

MLCP Myosin light chain phosphatase

mPRα membrane progesterone receptor α

mRNA messenger RNA

NCX Plasma membrane Na^+/Ca^{2+} exchanger

NKCC Na^+–K^+–2Cl^-symporter type 1

NO Nitric oxide

Orai1 Ca^{2+} release-activated Ca^{2+} channel protein 1

OVX Ovariectomy

PE Phenylephrine

$PGF_{2α}$ Prostaglandin $F_{2α}$

PI3K Phosphatidylinositol-3 kinase

PKA Protein kinase A

PKC Protein kinase C

PKCδ Protein kinase C δ

PKG pProtein kinase G

PLB Phospholamban

PLC Phospholipase C

PM Plasma membrane

PMCA Plasma membrane Ca^{2+}-ATPase

PR Progesterone receptor

PSD-95 Postsynaptic density protein 95

PTX Pertussis toxin

RBMVECs rat brain microvascular ECs

RhoA Ras homolog family member A

ROCC Receptor-operated Ca^{2+} channel

ROCE receptor-operated Ca^{2+} entry

ROCK Rho-associated protein kinase

ROS reactive oxygen species

RyR Ryanodine receptor

SERCA Sarco/endoplasmic Ca^{2+}-ATPase

SERMs Selective estrogen receptor receptor(s)

sGC Soluble guanylate cyclase

GPER/GPR30 G protein-coupled estrogen receptor

GTP Guanosine-5'-triphosphate

HAECs Human aortic endothelial cell(s)

HRE hormone response element

HUA human umbilical arteries

HUVEC Human umbilical vascular endothelial cell

I/R ischemic reperfusion

$I_{Ca,L}$ LTCC current

$I_{Ca,T}$ TTCC current

I_{CRAC} CRAC current

IP_3 Inositol 3,4,5-triphosphate

IP_3R Inositol 3,4,5-triphosphate receptor

K_{ATP} ATP-sensitive K^+ channels

K_{Ca} Ca^{2+}-activated K^+ channels

KCl Potassium chloride

K_V Voltage-gated K^+ channels

LTCC L-type Ca^{2+} channel

MAPK Mitogen-activated protein kinase

SHR Spontaneously hypertensive rats

SHRSP Spontaneously hypertensive stroke-prone rats

SOCC Store-operated Ca^{2+} channel

SOCE Store-operated Ca^{2+} entry

SR Sarcoplasmic reticulum

STIM1 Stromal Interaction Molecule 1

STOC Spontaneous transient external currents

TRP Transient receptor potential channel

TRPC Transient receptor potential canonical

TRPM Transient receptor potential melastatin

TRPV Transient receptor potential vanilloid

TTCC T-type Ca^{2+} channel

UBF Uterine arterial blood flow

VGCC Voltage-gated Ca^{2+} channel

VSMCs Vascular Smooth muscle cell(s)

WKY Wistar-Kyoto

Δψm mitochondria membrane potential

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AUTHOR CONTRIBUTIONS

D.A.A. and J.P conceived and designed research; D.A.A. drafted the initial version of the manuscript and prepared figures; D.A.A., J.P., R.Y.J., R.C.S., C.R.N., F.C. and D.J.G. edited and revised manuscript; All authors approved final version of manuscript.

FIGURE LEGENDS

Figure 1. Sex differences in calcium-handling proteins in vascular smooth muscle cells (VSMCs) and vascular endothelial cells (ECs). (**A**) Activation of endothelial TRPC3 in males plays a key role in the NO- and EDHF-type vasorelaxation by increasing cytosolic Ca^{2+} levels ($[Ca^{2+}]_{cyt}$). (**B**) In female ECs, there is a greater capacity for uptake of Ca^{2+} through MCU (mitochondrial Ca^{2+} uniporter) complex favoring its buffering, and altogether with an increase in TRPV4 activity and Piezo1 expression lead to $[Ca^{2+}]_{cyt}$ increments. Downstream, $[Ca^{2+}]_{cyt}$ activate calmodulin (CaM), increase the activity of endothelial nitric oxide synthase (eNOS) and promote the production of nitric oxide (NO). $[Ca^{2+}]_{cyt}$ also opens K_{Ca} channels that produce endothelium-derived hyperpolarizing factor (EDHF), that spreads to VSMCs via myoendothelial gap junctions (MEGJs) mainly through the upregulated connexin-43 (Cx43). (**C and D**) In VSMCs, $[Ca^{2+}]_{cyt}$ are higher in men than in women due to multiple mechanisms that occur in the plasma membrane (PM) and sarcoplasmic reticulum (SR). In the SR of males, there is an upregulation of the STIM1/Orai1 pathway that favors store-operated Ca^{2+} entry (SOCE). Endogenous testosterone increases PKC□ levels and therefore favors Ca^{2+} entry through the PM by increasing the expression of the $Ca_V1.2$ channel. Furthermore, compared to females, males have greater activity of the Na^+–K^+–$2Cl^-$ cotransporter (NKCC). The increase in Cl^- efflux through the Ca^{2+}-activated Cl^- channel (CaCC) increases the membrane potential (V_m), favoring the activation of $Ca_V1.2$. On the other hand, in females there is a greater expression of the α_2 isoform of the Na^+/K^+-ATPase (NKA) pump, which may indirectly favor the release of Ca^{2+} due to its close relationship with the Na^+/Ca^{2+} exchanger (NCX) located in the PM. Finally, there is an upregulation of ryanodine receptors (RyR) in females, favoring the production of Ca^{2+} sparks that activate K_{Ca} channels that hyperpolarize the PM through spontaneous transient external currents (STOCs) leading $Ca_V1.2$ inhibition. The calcium-handling proteins in which sex differences have not yet been demonstrated are represented in gray scale. Sex differences associated with males and females are shown in red and blue, respectively. Dashed lines mean reduced activity. ER: endoplasmic reticulum, $[Ca^{2+}]_m$: mitochondrial Ca^{2+} levels, $[Ca^{2+}]_{SR}$: sarcoplasmic Ca^{2+} levels, IP_3R: Inositol triphosphate receptor, SERCA: Sarcoplasmic/endoplasmic Ca^{2+}-ATPase, PLB: phospholamban, NCX_m: mitochondrial Na^+/Ca^{2+} exchanger, GPER: G protein-coupled estrogen receptor, mPRα: membrane progesterone receptor α, STIM1: stromal interaction protein 1, Orai1: Ca^{2+} release-activated Ca^{2+} channel protein 1, TRPC3: transient receptor potential canonical 3, TRPV4: transient receptor potential vanilloid 4, TRPM3: transient receptor potential melastatin 3, K_{Ca}: Ca^{2+}-activated K^+ channels, PMCA: Plasma membrane Ca^{2+}-ATPase. Created with BioRender.com.

Figure 2. Sex hormones-based effects in calcium-handling proteins in vascular endothelial cells (ECs). (A) Testosterone (T) increases hydrogen sulfide (H_2S) levels by an androgen receptor (AR)-dependent mechanism, favoring the entry of Ca^{2+} through the TRPV4 channel. The increase in $[Ca^{2+}]_{cyt}$ enhances the CaM-eNOS-NO pathway, leading to vasodilation. Interestingly, T increases the levels of reactive oxygen species (ROS) in the mitochondria of ECs, which may further modulate vasoreactivity. (**B**) The female hormone 17β-estradiol ($E_2\beta$) promotes mitochondrial Ca^{2+} uptake through the MCU (mitochondrial Ca^{2+} uniporter) complex. Subsequently, high levels of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) contribute to Ca^{2+}-buffering capacity mediated by a dynamic interaction between NCX_m located in the mitochondria and SERCA and IP_3R in the endoplasmic reticulum (ER). This event is attributed to an increase in mitochondrial mass, the number of mitochondria, a greater mitochondrial membrane potential, and a reduction in ROS levels. On the other hand, the selective activation of the G protein-coupled estrogen receptor (GPER) induced by G-1 (selective GPER agonist) or $E_2\beta$ increases the influx of Ca^{2+} through the TRPC (transient receptor potential canonical) channel and the $Ca_V1.2$ (via cAMP-PKA). To reinforce this increase in $[Ca^{2+}]_{cyt}$, GPER inhibits the extrusion of Ca^{2+} through plasma membrane Ca^{2+}-ATPase (PMCA). Importantly, GPER activation clamps $[Ca^{2+}]_{cyt}$ signals by inhibiting STIM1 oligomerization, probably through a direct interaction in different subcellular compartments. Downstream, store-operated Ca^{2+} entry (SOCE) via Orai1 is suppressed. The calcium-handling proteins in which sex differences have not yet been demonstrated are represented in gray scale. The effect of male and female

hormones is shown in red and blue, respectively. TRPV4: transient receptor potential vanilloid 4, STIM1: stromal interaction protein 1, Orai1: Ca^{2+} release-activated Ca^{2+} channel protein 1, SERCA: sarcoplasmic/endoplasmic Ca^{2+}-ATPase, IP_3R: Inositol triphosphate receptor, K_{Ca}: Ca^{2+}-activated K^+ channels, EDHF: endothelium-derived hyperpolarizing factor, MEGJs: myoendothelial gap junctions, Cx43: connexin-43, CaM: calmodulin, eNOS: endothelial nitric oxide synthase, NO: nitric oxide. Created with BioRender.com.

Figure 3. Sex hormones-based effects on calcium-handling proteins in vascular smooth muscle cells (VSMCs). (**A**) Testosterone (T) and its metabolite 5β-dihydrotestosterone (5β-DHT) reduce Ca^{2+} influx by decreasing the activity and expression of the $Ca_V1.2$ channel. Furthermore, T can also reduce the activity of the T-type Ca^{2+} channel (TTCC). (**B**) Progesterone (P_4) is capable of increasing Ca^{2+} sequestration in the SR, favoring VSMC relaxation through decreased $[Ca^{2+}]_{cyt}$. First, P_4 reduces the influx of extracellular Ca^{2+} through the TRPC5, TRPM3 and TRPV4 channels, the latter by a progesterone receptor (PR)-dependent mechanism. Second, activation of the membrane progesterone receptor α (mPRα) favors the upregulation of SERCA2 and the downregulation of its inhibitory protein phospholamban (PLB). Third, progesterone-induced mPRα activation enhances SERCA2 function by facilitating PLB phosphorylation (not shown). This signaling mechanism between mPRα and SERCA2/PLB would involve the participation of a Gi protein, MAPK and Akt/PI3K, as well as a negative regulation of the RhoA/ROCK pathway. By a mechanism dependent on estrogen receptors α and β (ERα and ERβ), 17β-estradiol ($E_2β$) can induce an increase in SERCA2b expression. Furthermore, $E_2β$ also upregulates both isoforms ($α_1$ and $α_2$) of the Na^+/K^+-ATPase (NKA) pump by a mechanism that depends on multiple signaling cascades, including PI3K, $cPLA_2$ and $p42/44^{MAPK}$, thus being able to promote Ca^{2+} efflux due to its close relationship with the activity of the Na^+/Ca^{2+} exchanger (NCX) located in the plasma membrane (PM). Together, both $E_2β$ and P_4 upregulate ryanodine receptors (RyR) located in the SR, favoring the production of Ca^{2+} sparks that activate K_{Ca} channels that hyperpolarize PM through spontaneous transient external currents (STOCs). An increase in the frequency of STOCs can inhibit the activity of the $Ca_V1.2$ channel, however, both $E_2β$ and P_4 are capable of inhibiting $Ca_V1.2$ in a non-genomic manner. Like T, $E_2β$ is also capable of inhibiting Ca^{2+} influx through TTCC. The calcium-handling proteins in which sexl differences have not yet been demonstrated are represented in gray scale. The effect of male and female hormones is shown in red and blue, respectively. $[Ca^{2+}]_{cyt}$: cytosolic Ca^{2+} levels, $[Ca^{2+}]_{SR}$: sarcoplasmic Ca^{2+} levels, SERCA: sarcoplasmic/endoplasmic Ca^{2+}-ATPase, MAPK: mitogen-activated protein kinase, Akt: protein kinase B, PI3K: phosphatidylinositol-3 kinase, $cPLA_2$: cytosolic phospholipase A_2, $p42/44^{MAPK}$: p42/44 mitogen-activated protein kinase, RhoA: Ras homolog family member A, ROCK: Rho-associated protein kinase, K_{Ca}: Ca^{2+}-activated K^+ channels, GPER: G protein-coupled estrogen receptor, STIM1: stromal interaction protein 1, Orai1: Ca^{2+} release-activated Ca^{2+} channel protein 1, SOCE: store-operated Ca^{2+} entry, IP_3R: Inositol triphosphate receptor, K_{Ca}: Ca^{2+}-activated K^+ channels, Cx43: connexin-43, PMCA: plasma membrane Ca^{2+}-ATPase, TRPV4: transient receptor potential vanilloid 4, TRPC5: transient receptor potential canonical 5, TRPM3: transient receptor potential melastatin 3, Created with BioRender.com.

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