Reciprocality Between Estrogen Biology and Calcium Signaling in the Cardiovascular System

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17β-Estradiol (E₂) is the main estrogenic hormone in the body and exerts many cardiovascular protective effects. Via three receptors known to date, including estrogen receptors α (ERα) and β (ERβ) and the G protein-coupled estrogen receptor 1 (GPER, aka GPR30), E₂ regulates numerous calcium-dependent activities in cardiovascular tissues. Nevertheless, effects of E₂ and its receptors on components of the calcium signaling machinery (CSM), the underlying mechanisms, and the linked functional impact are only beginning to be elucidated. A picture is emerging of the reciprocality between estrogen biology and Ca²⁺ signaling. Therein, E₂ and GPER, via both E₂-dependent and E₂-independent actions, moderate Ca²⁺-dependent activities; in turn, ERα and GPER are regulated by Ca²⁺ at the receptor level and downstream signaling via a feedforward loop. This article reviews current understanding of the effects of E₂ and its receptors on the cardiovascular CSM and vice versa with a focus on mechanisms and combined functional impact. An overview of the main CSM components in cardiovascular tissues will be first provided, followed by a brief review of estrogen receptors and their Ca²⁺-dependent regulation. The effects of estrogenic agonists to stimulate acute Ca²⁺ signals will then be reviewed. Subsequently, E₂-dependent and E₂-independent effects of GPER on components of the Ca²⁺ signals triggered by other stimuli will be discussed. Finally, a case study will illustrate how the many mechanisms are coordinated to moderate Ca²⁺-dependent activities in the cardiovascular system.

Keywords: estrogen, G protein—coupled estrogen receptor, calcium, calmodulin, calmodulin-binding proteins, cardiomyocytes, vascular smooth muscle, endothelium

MAIN COMPONENTS OF THE CALCIUM SIGNALING MACHINERY (CSM) IN CARDIOVASCULAR TISSUES

The CSM herein refers to proteins responsible for the generation or sequestration of intracellular Ca²⁺ signals and their transduction to target activities. In this section, key CSM components in cardiovascular tissues will be briefly described to facilitate review of the relevant effects and mechanisms of estrogenic agonists and receptors.

Intracellular Ca²⁺ Stores, Release, and Uptake Mechanisms

Organelles Functioning as Intracellular Ca²⁺ Stores

The sarcoplasmic/endoplasmic reticulum (SR/ER) is the main Ca²⁺ store in cardiomyocytes, vascular smooth muscle cells (VSMCs) (1, 2), and endothelial cells (ECs), where the ER stores ~75%
Ca$^{2+}$ and mitochondria house ~25% (3). The Golgi (4, 5) and lysosomes have more recently been recognized as Ca$^{2+}$ reservoirs (6, 7). Ca$^{2+}$ reaches 5 × 10$^{-4}$ M in the ER/SR and lysosomes and 1.3–2.5 × 10$^{-4}$ M between the trans-Golgi and cis-Golgi (5, 8). The medial Golgi also releases Ca$^{2+}$ in response to inositol-triphosphate receptor (IP$_3$R) and ryanodine receptor (RyR) stimulation (9). Crossstalk between the ER/SR and other organelles affects their Ca$^{2+}$ fluxes (10–14). In neonatal cardiomyocytes, beat-to-beat oscillations in mitochondrial and cytosolic Ca$^{2+}$ occur in parallel (15), and mitochondrial uptake reduces cytosolic Ca$^{2+}$ (16).

**Mechanisms of Ca$^{2+}$ Uptake Into Ca$^{2+}$ Stores**

SR/ER Ca$^{2+}$-ATPases (SERCAs) are the key Ca$^{2+}$ uptake mechanisms. For each ATP hydrolyzed, they pump 2 Ca$^{2+}$ ions into the ER/SR in exchange for less than four H$^+$ ions (17). SERCA2b is ubiquitously expressed. SERCA2a predominates in cardiomyocytes and is essential for cardiac development (18). SERCA3 is the predominant vascular isoform; its deletion causes smooth muscle relaxation abnormality (19, 20). SERCA3 has lower affinity for Ca$^{2+}$ and is only active at high Ca$^{2+}$ levels. Non-phosphorylated phospholamban interacts with SERCA1a, SERCA2a, and SERCA2b and reduces their Ca$^{2+}$ affinity. Phosphorylation at Ser16 and Thr17 removes phospholamban–SERCA interaction, promoting SERCA activity (21, 22). Sarcolipin also binds SERCAs and reduces their Ca$^{2+}$ affinity. Its deletion increases SR Ca$^{2+}$ uptake (23).

The secretory pathway Ca$^{2+}$ pump (SPCA) mediates Ca$^{2+}$ uptake into the Golgi with nanomolar affinity for Ca$^{2+}$. Unlike the SERCA, Ca$^{2+}$ transport by SPCA is not associated with counter transport of H$. In the medial Golgi, both SERCA and SPCA participate in Ca$^{2+}$ uptake (9).

Mitochondrial Ca$^{2+}$ uptake is mediated by the voltage-dependent anion channel (VDAC) and the mitochondrial Ca$^{2+}$ uniporter (MCU). VDACs are non-selective anion channels in the open state yet in the “closed” state permit influxes of cations such as K$^+$, Na$^+$, and Ca$^{2+}$ into the mitochondria (24). VDAC isoforms participate equally in transporting Ca$^{2+}$ triggered by IP$_3$-producing agonists; however, VDAC1 selectively transports apoptotic Ca$^{2+}$ signals (25). Myocardial VDAC2 regulates rhythmicity by influencing the spatial and temporal properties of cytoplasmic Ca$^{2+}$ signals (26). The MCU constitutes a low-affinity yet selective Ca$^{2+}$ channel pore as part of a mitochondrial Ca$^{2+}$ uptake protein complex (MICU) and the essential MCU regulator (27, 28).

**Mechanisms of Ca$^{2+}$ Release From Ca$^{2+}$ Stores**

In IP$_3$Rs, IP$_3$ activates RyR2 > IP$_3$R1 > IP$_3$R3 affinity order (29) and cooperatively switches IP$_3$R tetramers to an open conformation to form clusters and release Ca$^{2+}$ (30, 31). IP$_3$Rs regulate Ca$^{2+}$ release from the ER/SR, Golgi apparatus, and nucleus (32). ER/SR Ca$^{2+}$ release depletes ER Ca$^{2+}$ and triggers store-operated Ca$^{2+}$ entry (SOCE). IP$_3$R2 predominates in the cardiomyocytes (33). In failing hearts, IP$_3$-mediated Ca$^{2+}$ transients are enhanced, and mitochondrial Ca$^{2+}$ uptake is reduced, which facilitates contraction and spontaneous action potentials that increase arrhythmogenicity (34). In VSMCs, all IP$_3$Rs are expressed and are important for agonist-induced contraction (35). Endothelial IP$_3$R1 is predominant in the brain (36), whereas IP$_3$R2 and IP$_3$R3 are abundant in the aorta and pulmonary arteries (37, 38).

\[ \text{Rs} (\text{RyR1–RyR3}) \text{ are the main SR Ca}^{2+} \text{ release channels (39).} \]

\[ \text{IP}_3 \text{Rs, RyR2 predominates (40) and is closed, activated, and inhibited, respectively, at Ca}^{2+} < \text{10}^{-7} \text{M, } \text{10}^{-7} \text{–} \text{10}^{-5} \text{M, and } > \text{10}^{-3} \text{M (41).} \]

\[ \text{Entry via voltage-dependent Ca}^{2+} \text{ channels (VDCCs) stimulates Ca}^{2+} \text{-induced Ca}^{2+} \text{ release (CICR) via RyR2, contributing to myocardial contraction.} \]

\[ \text{In VSMCs, RyR2 predominates in the aorta and pulmonary and cerebral arteries, while RyR3 is the only isoform in basilar arteries (42–44). CICR also contributes to VSMC contraction, but not as critically as in cardiomyocytes; indeed, skinned smooth muscle fiber bundles can contract at Ca}^{2+} \text{ levels that do not activate RyRs (45).} \]

\[ \text{In ECS, RyR2 is on the ER and mitochondria (46); however, RyR agonists only cause a slow Ca}^{2+} \text{ release that corresponds to a reduction in the IP$_3$-sensitive Ca}^{2+} \text{ pool (47, 48).} \]

**SOCE** is a ubiquitous mechanism where Ca$^{2+}$ store depletion triggers Ca$^{2+}$ influx (52, 53). Proposed in the 1980s, SOCE was confirmed in the mid-2000s with the discoveries of the stromal interaction molecule 1 (STIM1) (54–56) and Orai Ca$^{2+}$ channels (57–59). STIM1 resides mainly on the ER/SR membrane and has a luminal EF hand that houses a Ca$^{2+}$-binding loop (60). In Ca$^{2+}$-full ER/SR, the loop is in a closed conformation. Upon ER/SR Ca$^{2+}$ depletion, Ca$^{2+}$ leaving the loop promotes STIM1 oligomerization to interact with Orai channels and

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**Abbreviations:** AF domain, transcriptional activation function domain; CaM, calmodulin; Ca$^{2+}$, Ca$^{2+}$-bound calmodulin; cAMP, cyclic adenosine monophosphate; CICR, Ca$^{2+}$-induced Ca$^{2+}$ release; CRAC, Ca$^{2+}$ release-activated channels; CSM, Ca$^{2+}$ signaling machinery; E$_{j}$, 17β-estradiol; EGS, endothelial cells; EGF, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; ERβ, estrogen receptor β; ERα, estrogen receptor α; ERK1/2, extracellular signal-related kinases 1 and 2; FRET, fluorescence resonance energy transfer; GPER, G protein-coupled estrogen receptor 1; GPR30, G protein-coupled estrogen receptor 1; HEK293 cells, human embryonic kidney 293 cells; J$_{Ca,l}$, L-type Ca$^{2+}$ channel current; IP$_3$Rs, inositol-triphosphate receptors; LTCC, L-type Ca$^{2+}$ channels; LV, left ventricle; MAPK, mitogen-activated protein kinases; mCRIC, mitochondrial Ca$^{2+}$ retention capacity; MCU, mitochondrial Ca$^{2+}$ uniporter; MEK1, MAP (mitogen-activated protein) kinase/ERK (extracellular signal-regulated kinase) kinase 1; mPTP, mitochondrial permeability transition pore; Ncx, Na$^+$/Ca$^{2+}$ exchanger; OXV, ovarioic tym; PDAZ, PSD-95/Dlg/ZO; PKC, protein kinase C; PLCβ, phospholipase C-β; PMCA, plasma membrane Ca$^{2+}$ ATPase; PSD-95, post-synaptic density protein 95; RMP, resting membrane potential; RyRs, ryanodine receptors; SCPR, secretory pathway Ca$^{2+}$ pump; SERCA, sarcoplasmic/endoplasmic reticulum Ca$^{2+}$ ATPase; SMD, submembrane domains of G protein-coupled receptors; SOCE, store-operated Ca$^{2+}$ entry; SOICR, store overload-induced Ca$^{2+}$ release; SR/ER, sarcoendoplasmic reticulum; STIM1, stromal interaction molecule 1; VDAC, voltage-dependent anion channel; VDCC, voltage-dependent Ca$^{2+}$ channels; VDC, voltage-dependent Ca$^{2+}$ entry; VSMCs, vascular smooth muscle cells.
trigger Ca$^{2+}$ entry (61–63). STIM1 also interacts with L-type Ca$^{2+}$ channels (LTCCs) (64), maintains ER/SR structure (65–67), and is upregulated in atherosclerosis and hypertension (68–71). Myocardial SOCE is normally not prominent; however, STIM1 and SOCE are increased in heart failure (67, 72–76). In VSMCs, SOCE contributes significantly to contraction; α1AR-mediated contraction is reduced ∼30% in SM-specific STIM1−/− animals (77). In ECs, SOCE is the major Ca$^{2+}$ entry and is required for many critical functions such as endothelial nitric oxide synthase (eNOS) activity and proliferation (78–82).

Voltage-Dependent Ca$^{2+}$ Entry (VDCE)

Functional voltage-dependent Ca$^{2+}$ channels (VDCCs) are the hallmark of tissue excitability and are present in cardiomyocytes and VSMCs, but not ECs. In cardiomyocytes, LTCCs are located mostly in transverse T tubules in apposition to RyR2s (83). Ca$^{2+}$ entry via LTCCs triggers CICR via RyR2. In VSMCs, LTCCs also play a critical role in Ca$^{2+}$ entry and contraction (84). The LTCC complex (85) consists of α1, α2δ, β, δ, and γ subunits. Four LTCC members are named according to their α1 pore-forming subunits: Ca.1.1, Ca.1.2, Ca.1.3, and Ca.1.4 (86). Ca.1.2 is predominant in cardiac and smooth muscles.

Ca$^{2+}$ Extrusion via the Plasma Membrane/Sarcolemma

The plasma membrane Ca$^{2+}$-ATPases (PMCA) prevail for Ca$^{2+}$ extrusion in non-excitable tissues while the Na$^+$-Ca$^{2+}$ exchanger (NCX) is more important in excitable cells. SERCA2a, NCX, and PMCA sequester, respectively, ∼70, 28, and 2% of cytosolic Ca$^{2+}$ in cardiomyocytes (83) and 25, 25, and 50% in ECs (87).

Plasma Membrane Ca$^{2+}$-ATPase

PMCA extrude one Ca$^{2+}$ ion for each ATP used and function as Ca$^{2+}$-H$^+$ exchangers (88–90). PMCA are regulated by a Ca$^{2+}$-dependent interaction with calmodulin (CaM). At low Ca$^{2+}$, a C-terminal autoinhibitory domain binds to two cytosolic loops and inhibits pump activity. Increased Ca$^{2+}$ promotes CaM–PMCA interaction, which removes inhibition and activates Ca$^{2+}$ efflux (91, 92). PSD-95 promotes expression and distribution of PMCA4b via PDZ binding (93). PMCA are inhibited by C-terminal tyrosine phosphorylation (94). Myocardial PMCA play a little role under physiological conditions. However, expressions of PMCA1 and PMCA4 are reduced by up to 70 and 50%, respectively, in end-stage heart failure (95), and cardiac-specific overexpression of PMCA4b improved myocardial functions in ischemia–reperfusion injury and heart failure (96). PMCA concentrate in the caveolae of VSMCs and ECs (97, 98). PMCA1 suppresses VSMC proliferation (99, 100), while PMCA4 mediates cell cycle (101, 102). In ECs, PMCA1b, and PMCA4b are predominant (87, 103, 104).

Na$^+$-Ca$^{2+}$ Exchanger

The NCX may function in two modes. In the forward mode, myocardial NCX1 balances LTCC-mediated Ca$^{2+}$ entry and RyR-mediated Ca$^{2+}$ release during cardiac excitation, extruding ∼25% of the Ca$^{2+}$ needed to activate myofilaments (105). NCX1 also predominates in VSMCs (106, 107). In ECs, NCX accounts for ∼25% of Ca$^{2+}$ removal (87). Endothelial NCX and PMCA dynamically adjust their Ca$^{2+}$ extrusion rates to maintain sufficient efflux (104). In the reverse mode, upon myocardial depolarization, Na$^+$ entry causes the NCX to transiently operate in this mode, promoting Ca$^{2+}$ entry. This is much less efficient in triggering SR Ca$^{2+}$ release compared to LTCC-mediated Ca$^{2+}$ entry (108, 109). However, it primes the dyad to increase LTCC-mediated CICR (110). In VSMCs, reverse-mode NCX1 facilitates Ca$^{2+}$ entry and mediates contraction, vascular tone, and blood pressure (111, 112). The reverse mode is not significant in ECs.

Sex Differences in Ca$^{2+}$ Signaling Proteins

Higher mRNA levels of Ca.1.2, RyR, and NCX, but not of phospholamban and SERCA2a, have been observed in female than in male rat hearts (113). However, caffeine-induced Ca$^{2+}$ release is lower in cardiomyocytes from female hearts (114). Ca.1.2 mRNA is higher in coronary smooth muscle from male than from female pigs (115). In smooth muscle cells (SMCs), expressions of ERα and ERβ, but not G protein-coupled estrogen receptor 1 (GPER), are higher in female than in male rats (116). These differences and the lower Ca.1.2 expression (115) may be responsible for less contraction of VSMCs from females (116). No studies have examined sex differences in Ca$^{2+}$ handling proteins in ECs.

Transduction of Ca$^{2+}$ Signals—The Essential Role of Calmodulin (CaM)

While some Ca$^{2+}$-dependent proteins are activated directly by Ca$^{2+}$, many are activated by a complex between Ca$^{2+}$ and CaM. CaM has two lobes linked by a flexible helix and can interact with ∼300 target proteins (117, 118). Ca$^{2+}$-free CaM binds or serves as structural subunits of ∼15 proteins (119). However, each CaM lobe has two Ca$^{2+}$-binding sites, and cooperative Ca$^{2+}$ binding induces conformations that allow CaM to interact with many proteins, aided by the flexibility of the central helix (120, 121). Thus, CaM is the ubiquitous Ca$^{2+}$ signal transducer. Activities of Ca$^{2+}$/CaM-binding proteins depend on the Ca$^{2+}$ signals, CaM availability, and properties of the interaction between Ca$^{2+}$/CaM and the target proteins. Many of these factors are subject to estrogenic moderation.

Despite being required for activation of many Ca$^{2+}$-dependent proteins, up to 50% of cellular CaM is engaged in inseparable interactions, leaving much less available for dynamic target binding (122). This generates an environment of limited CaM (123), as has been demonstrated in ECs (124), VSMCs (125), and cardiomyocytes (126). Consequently, competition for CaM generates a unique crosstalk among CaM-dependent proteins (124, 127), and factors that alter CaM level are predicted to have pervasive functional impact. It is noteworthy that virtually all CSM components interact with CaM and, in the context of reciprocity between estrogenic and Ca$^{2+}$ signaling pathways, that ERα and GPER are both regulated by direct interactions with Ca$^{2+}$/CaM.
ESTROGEN RECEPTORS AND THEIR CALCIUM-DEPENDENT REGULATION

Estrogen Receptor α (ERα)
ERα (128–130) is a nuclear receptor that, upon E2 binding (K_d ~ 10^{-10} M), assumes an active conformation to bind estrogen-responsive elements (EREs) in the promoters of target genes, modulating their transcription (131). Its N-terminus has a transcriptional activation function (AF-1) domain, a DNA-binding domain, and a hinge region; the C-terminus houses the ligand-binding domain and a second AF-2 domain. ERα is robustly expressed in the heart (132), VSMCs, and ECs (133–136).

ERα activities are strongly regulated by the Ca^{2+}-dependent interaction with CaM. ERα binds CaM in a Ca^{2+}-dependent fashion with a K_d of 1.6 × 10^{-10} M and an EC_{50}(Ca^{2+}) value of ~3 × 10^{-7} M (137). When ERαs from Wistar rats’ uteri is used, CaM decreases ERα-E2 binding but increases liganded ERα-ERE interaction (138, 139). A comparison of the CaM-bound/CaM-unbound ERα ratio in the cytosolic (unliganded) and nuclear (liganded) ERα pools isolated from MCF-7 cells suggests that E2 binding induces a conformation that favors ERα-CaM interaction (138). The CaM-binding domain was initially predicted to be a.a. 298–310 (137) but was later determined to be a.a. 298–317, with a.a. 248–317 required for maximal interaction (140). Further studies revealed that a.a. 287–311 is required to interact with both CaM lobes (141). CaM binding promotes ERα homodimerization that is critical for transcription activity (140, 142). With two lobes, each CaM binds two ERα molecules and thus stabilizes ERα dimerization (143). Notably, analogs of ERα17p (a.a. 295–311) that are unable to bind CaM downregulates ERα, stimulates ERα-dependent transcription, and enhances proliferation of MCF-7 cells, as does the wild-type ERα17p, indicating that this domain may also be involved in CaM-independent posttranslational regulation of ERα (144).

Estrogen Receptor β (ERβ)
ERβ has ~96% and 55–58% sequence homology with ERα in the DNA- and ligand-binding domains, respectively (145, 146). ERβ binds E2 with a K_d of ~4–6 × 10^{-10} M. ERβ forms homodimers but more preferentially forms heterodimers with ERα, which bind E2 with a K_d of ~2 × 10^{-9} M and are transcriptionally active (147). ERβ is abundantly expressed in the vasculature (133–136). However, its expression and direct actions in the heart are controversial; cardiac manifestations of ERβ−/− animals have been attributed to indirect effects from vascular changes (148). ERβ is not regulated by Ca^{2+} or CaM (149).

GPER
GPER (150), aka GPR30, was cloned from various tissues in the 1990s (151–156). GPR30 is required for estrogenic activation of extracellular signal-related kinase (ERK)1/2 via transactivation of the epidermal growth factor receptor (EGFR) and release of the heparan-bound epidermal growth factor (EGF) (157, 158). It was shown to bind E2 in 2005 (159, 160), and the designation GPER was adopted by the International Union of Basic and Clinical Pharmacology in 2007 (161). A host of steroidal and non-steroidal agents and specific GPER agonists can activate GPER (150). GPER couples with Goα, Goβ, or Goγ. Supporting Goα, coupling are data that (1) most membrane-bound [35S]GTPγ-S from cells overexpressing GPER and treated with E2 coimmunoprecipitate with Goα (159), (2) GPER is present in Goα, pull-down fraction from GPER-expressing cells, and (3) E2 promotes GPER-dependent cyclic adenosine monophosphate (cAMP) production (162). Supporting GPER-Goα interaction are results that pertussis toxin prevents (1) E2-induced, GPER-mediated ERK1/2 phosphorylation in cells transfected with GPER (134, 157); (2) upregulation of c-fos in ERα/ERβ-negative, GPER-positive SKBr3 cells (163); and (3) E2-induced Ca^{2+} signals in ECs (164). GPER is robustly expressed in cardiovascular tissues (133–136). In ECs, GPER mRNA is increased 8-fold by shear stress (154). GPER is localized on the ER/SR membrane (160) and responds to cell-permeable ligands (165). However, it also resides on the plasma membrane (166) and requires its C-terminal PDZ-binding motif to do so (167). The plasmalemmal GPER pool seems to constitutively undergo clathrin-dependent endocytosis and accumulate in the trans-Golgi network for ubiquitination in the proteasome without recycling to the plasma membrane, a process unaffected by agonist stimulation (168). Despite its predominant expression in the ER/SR, the sequence that drives GPER localization here has not been identified.

GPER is directly regulated by Ca^{2+}-CaM complexes. In VSMCs and ECs, GPER coimmunoprecipitates with CaM in a constitutive association that is promoted by treatment with E2, G-1, or receptor-independent stimulation of Ca^{2+} entry (169, 170). GPER is the first G protein-coupled receptor (GPCR) shown to possess four CaM-binding sites on its respective four submembrane domains (SMDs) (169). Fluorescence resonance energy transfer (FRET) biosensors based on SMDs of GPER bind CaM with K_d from 0.4 to 136 × 10^{-6} M and affinity ranging SMD2 > SMD4 > SMD3 > SMD1. These interactions are Ca^{2+} dependent, with an EC_{50} (Ca^{2+}) of 1.3 × 10^{-7}–5.5 × 10^{-6} M, values within the physiological Ca^{2+} range (169). Due to technical challenges with purifying full-length GPCRs, the K_{CaM} for GPER as a holoreceptor is not available. The presence of four CaM-binding sites makes this task even more challenging and, in some way, not useful functionally. Functionally, mutations that reduce CaM binding but that do not perturb GPER-Gαo preassociation drastically prevent GPER-mediated ERK1/2 phosphorylation (170).

STIMULATION OF CALCIUM SIGNALS BY ESTROGEN AND GPER AGONISTS

Observations
In rat hearts, E2 (10^{-12}–10^{-8} M) triggers {\textsuperscript{45}}Ca^{2+} uptake that is inhibited by LTCC antagonists (171). In VSMCs, GPER agonist G-1 triggers a slow-rising Ca^{2+} signal that is <2 × 10^{-7} M (172). In MCF-7 cells, E2 (10^{-7} M) induces Ca^{2+} store release and entry, yet only the former is required to activate mitogen-activated protein kinase (MAPK) (173). Interestingly, the ERα/ERβ antagonist ICI182,780 (10^{-6} M) also triggered
Ca\(^{2+}\) signals in these cells. In ECs, E\(_2\) (10\(^{-10}\)–10\(^{-9}\) M) triggers Ca\(^{2+}\) store release and entry, effects not affected by ER\(\alpha\)/ER\(\beta\) inhibitor tamoxifen (164, 174). The data with ICI182,780 and tamoxifen implicate a receptor other than ER\(\alpha\) or ER\(\beta\) in mediating the Ca\(^{2+}\) signal. Both reagents were later shown to be GPER agonists, triggering ERK1/2 phosphorylation only in cells expressing GPER (157, 159). Later studies confirmed Ca\(^{2+}\) signals stimulated by E\(_2\), GPER agonist G-1, and ICI182,780 in cells expressing GPER endogenously and absence of this effect in GPER\(^{-/-}\) cells (160, 175, 176).

**Mechanisms (Figure 1)**

**Direct E\(_2\)-Ca\(_{\alpha 1.2}\) Interaction**

E\(_2\) (10\(^{-11}\)–10\(^{-9}\) M) potentiates I\(_{Ca,L}\) in neurons and HEK293 cells overexpressing the \(\alpha 1C\) subunit; nifedipine displaces membrane E\(_2\) binding; and E\(_2\)'s effect is reduced by a dihydropyridine-insensitive LTCC mutant, indicating that E\(_2\) binds to the dihydropyridine-binding site (177). Intriguingly, E\(_2\) and the dihydropyridines exert opposite effects on I\(_{Ca,L}\).

**Direct, Membrane-Delimited Activation of Ca\(^{2+}\) Channels by Ga Subunits**

GPCR stimulation can trigger Ca\(^{2+}\) signals independently of the second messenger (178–180). GPER couples with Ga\(_i\) and Go\(_i\), which can interact with LTCC (178, 181, 182) and trigger Ca\(^{2+}\) entry.

**Release of G\(_{\beta\gamma}\) Subunit Upon GPER-Associated Ga\(_i\) Stimulation**

G\(_{\beta\gamma}\) stimulates PLC\(\beta\) (183–185) and activates IP\(_3\)R1 (186), both of which trigger Ca\(^{2+}\) store depletion and SOCE. Consistently, E\(_2\)-induced Ca\(^{2+}\) store release and entry in ECs are completely inhibited by pertussis toxin and PLC\(\beta\) inhibitor U73122 (164). Also, HEK293 cells only produce a Ca\(^{2+}\) response to E\(_2\) when expressing HA-tagged GPER (162). Since (1) Ca\(^{2+}\) entry channels are located on the membrane and (2) G\(_{\beta\gamma}\) activates IP\(_3\)Rs by interacting with the IP\(_3\)-binding sites (186) on IP\(_3\)Rs' cytosolic domains, both the membrane-delimited/Go-mediated and G\(_{\beta\gamma}\)-mediated mechanisms should only be operable by the plasmalemmal GPER pool. A distinguishing feature is that the former mechanism would not trigger SR/ER Ca\(^{2+}\) release in the absence of extracellular Ca\(^{2+}\), whereas the latter would. Based on this feature, data fitting the former are available from renal tubular cells (176); and data fitting the latter, from vascular ECs (164).

**Functional Impact**

Do Ca\(^{2+}\) signals stimulated by estrogenic agonists activate Ca\(^{2+}\)-dependent activities? When reported, the concentration of a Ca\(^{2+}\) signal allows for prediction of proteins that may or may not be affected by it. For example, E\(_2\) induces ER Ca\(^{2+}\) release signals of \(\sim 2 \times 10^{-7}\) M and activates MAPK (173), because this Ca\(^{2+}\) level is sufficient for MAPK activity (187); indeed, Ca\(^{2+}\) chelation...
abolishes E\textsubscript{2}'s effect (173). Considering that GPER mediates the effect of E\textsubscript{2} to trigger Ca\textsuperscript{2+} signals that activate MAPK, GPER activity can promote many downstream effects (163, 170, 188). In ECs, E\textsubscript{2} (10\textsuperscript{-9}–10\textsuperscript{-6} M) stimulates very small Ca\textsuperscript{2+} signals (<10\textsuperscript{-7} M) (174). One can predict that only proteins with very high Ca\textsuperscript{2+} sensitivity, for example, phosphorylated eNOS (170, 189, 190), would be activated by these signals. Whether a Ca\textsuperscript{2+} signal can produce a predicted effect also depends on other factors. For example, the Ca\textsuperscript{2+} signal of \sim 2 \times 10\textsuperscript{-7} M triggered by G-1 in VSMCs (172) would be sufficient to activate myosin light-chain kinase (MLCK) and cause vasoconstriction, based on MLCK's properties (191). However, G-1 causes vasodilatation (172, 192–194), likely by activating eNOS (170, 193, 195–198), inhibiting VSMC Ca\textsuperscript{2+} (199), and stimulating SMC K\textsuperscript{+} efflux (200).

**CALCIUM ENTRY INHIBITION BY ESTROGENIC AGONISTS AND ESTROGEN RECEPTORS**

To a large extent, estrogenic regulation of Ca\textsuperscript{2+} signaling involves effects of estrogenic agonists and receptors on the Ca\textsuperscript{2+} signals triggered by other stimuli, via both E\textsubscript{2}-dependent and E\textsubscript{2}-independent mechanisms.

**Store-Operated Ca\textsuperscript{2+} Entry (Figure 1)**

In VSMCs, E\textsubscript{2} (10\textsuperscript{-8}–10\textsuperscript{-5} M) inhibits norepinephrine- and phenylephrine-induced arterial constriction in the presence of extracellular Ca\textsuperscript{2+} but not that induced in Ca\textsuperscript{2+}-free medium (201, 202). These effects may be attributed to inhibition of both VDCE and SOCE, as α\textsubscript{1} adrenoceptor agonists can activate both (77). GPER-mediated inhibition of SOCE has been shown in ECs, where G-1 (10\textsuperscript{-8}–10\textsuperscript{-6} M) suppresses SOCE induced by thapsigargin or bradykinin (203). Interestingly, the observations that in the absence of any treatment with agonists, thapsigargin-induced SOCE is increased by 80% in GPER-knockdown ECs and is reduced by 40% in GPER-overexpressing HEK293 cells implicate E\textsubscript{2}-independent mechanisms (203).

How E\textsubscript{2}/GPER suppresses SOCE seems to involve STIM1. G-1 treatment prevents thapsigargin-induced STIM1 puncta, indicating inhibition of STIM1's association with the Ca\textsuperscript{2+} channel; and Ser575/608/621Ala mutations of STIM1 reduce the inhibitory effect of G-1 (203). Consistently, E\textsubscript{2} inhibits Ser575 STIM1 phosphorylation in bronchial epithelial cells, thus suppressing STIM1 mobility and SOCE (204). Our initial data also indicate that dynamic physical interaction between them contributes importantly to GPER's inhibition of SOCE (205).

**Voltage-Dependent Ca\textsuperscript{2+} Entry (Figure 1)**

Electrically induced Ca\textsuperscript{2+} signals are increased in cardiomyocytes from ovariectomized (OVX) animals (206–208). Many lines of evidence indicate that GPER mediates the inhibitory effect of E\textsubscript{2} on IC\textsubscript{aVL}. These include inhibitory effects of E\textsubscript{2} (1–3 \times 10\textsuperscript{-5} M) and combined ER\textalpha/ER\textbeta antagonists/GPER agonists (ICI182,780, tamoxifen, or raloxifene) on IC\textsubscript{aVL} in cardiomyocytes from both WT and ER\textalpha/-/ER\textbeta/- animals, as reviewed in (132). Similarly, in VSMCs, E\textsubscript{2} inhibits electrically induced IC\textsubscript{aVL} (209, 210), and ER\textalpha/ER\textbeta antagonists/GPER agonists tamoxifen and ICI164,384 inhibit high-K\textsuperscript{+}-induced contraction (202). GPER agonist G-1 (10\textsuperscript{-6} M) inhibits nifedipine-sensitive Ca\textsuperscript{2+} spikes in LTCC-expressing A7R5 SMCs, an effect prevented by GPER antagonist G-15 (10\textsuperscript{-6} M) (199); these concentrations are specific for GPER (175, 211). Consistently, ER\alpha knockout does not affect E\textsubscript{2}'s inhibition of KCl-induced 45Ca\textsuperscript{2+} uptake in VSMCs and vasorelaxation (212).

How E\textsubscript{2} inhibits electrically induced VDCE is still unknown. Hypothetically, at high levels, E\textsubscript{2} binding to the dihydropyridine-binding site on LTCC (177) may instead inhibit IC\textsubscript{aL}. As for prevention of β adrenoceptor (βAR)-mediated potentiation of VDCE, recent evidence suggests that GPER may be an intrinsic component of β1AR activation. Thus, G-1 inhibits isoproterenol-induced increases in left ventricle (LV) pressure, heart rate, ectopic contractions, IC\textsubscript{a,LTCC} LTCC phosphorylation, and total myocardial Ca\textsuperscript{2+} signal, while the GPER inhibitor G-36 promotes ISO-induced Ca\textsuperscript{2+} signal and LTCC phosphorylation (213). Speculatively, GPER may do so in part by interacting with β1AR or with A kinase-anchoring protein 5, thus inhibiting cAMP production (167). These may represent some E\textsubscript{2}-independent effects of GPER. Studies in GPER-knockout tissues are needed to further clarify the mechanisms.

**ESTROGENIC REGULATION OF CYTOPLASMIC CALCIUM REMOVAL MECHANISMS**

**SERCA Activity**

Few studies, mostly in cardiac tissues, have examined the effects of E\textsubscript{2} on SERCA activity, with somewhat conflicting results. E\textsubscript{2} (1–30 \times 10\textsuperscript{-6} M) does not affect the V\textsubscript{max} of SR vesicle Ca\textsuperscript{2+} uptake in canine LV tissue (214). However, ovariectomy reduces the V\textsubscript{max} but increases the Ca\textsuperscript{2+} sensitivity for SR Ca\textsuperscript{2+} uptake of rat LV homogenates or SR-enriched membrane fractions; *mechanistically*, these effects appear to be associated with reduced Thr17 phosphorylation of phospholamban and are restored by treatment with either E\textsubscript{2} or progesterone (215) (Figure 1). How E\textsubscript{2} and progesterone promote Thr17 phosphorylation of phospholamban is unknown, perhaps by inhibiting CaM kinase II (216), the enzyme that phosphorylates phospholamban (21). The effect of E\textsubscript{2} on SERCA activity in VSMCs has not been examined.

**NCX Activity**

As with SERCA activity, few studies have measured the effects of E\textsubscript{2} on NCX activity. Na\textsuperscript{+}-dependent 45Ca\textsuperscript{2+} uptake in rat LV myocytes is increased by \sim 3-fold after 60 days of ovariectomy, which is restored by replenishment with E\textsubscript{2} (1.5 mg/60 days) (208). During myocardial ischemia, intracellular Na\textsuperscript{+} concentration is higher in male than in female cardiomyocytes and is associated with increased Ca\textsuperscript{2+} concentration as a result of increased NCX activity (217). These studies are consistent with an inhibitory effect of E\textsubscript{2} on NCX activity in both the forward and reverse modes (Figure 1). However, the mechanisms of this inhibition are unclear.
Mitochondrial Ca\(^{2+}\) Uptake

In the heart, diethylstilbestrol (0.9–1.8 × 10^{-3} M) inhibits mitochondrial 45Ca\(^{2+}\) uptake (218). Mitochondrial Ca\(^{2+}\) retention capacity (mCRC), a combination of mitochondrial Ca\(^{2+}\) uptake, total mitochondrial Ca\(^{2+}\)-binding sites, and mitochondrial Ca\(^{2+}\) release mechanisms, is a determinant of the protective role of the mitochondria during cytoplasmic Ca\(^{2+}\) overload. E\(_2\) (4 × 10^{-8} M) increases myocardial mCRC following ischemia–reperfusion, an effect abolished by genetic deletion of GPER but not of ER\(_\alpha\) or ER\(_\beta\); mechanistically, this effect seems to involve PKC-dependent, MAPK-dependent phosphorylation of glycogen synthase kinase (GSK)-3\(\beta\), leading to inhibition of the mitochondrial permeability transition pore (219). Consistently, E\(_2\) (10^{-8} M) inhibits high Ca\(^{2+}\)-induced cytochrome c release from myocardial mitochondria (220). In ECs, 48-h E\(_2\) (10^{-8} M) treatment inhibits mitochondrial Ca\(^{2+}\) uptake, an effect abolished by the ER\(_\alpha\)/ER\(_\beta\) antagonist ICI182,780 (10^{-8} M) (221). The mechanisms whereby E\(_2\) inhibits mitochondrial Ca\(^{2+}\) uptake are still unknown (Figure 1).

PMCA Activity

Recent data show that GPER inhibits PMCA activity via both E\(_2\)-dependent and E\(_2\)-independent mechanisms (Figure 1). E\(_2\)-dependent mechanisms are evidenced by the effects of G-1 (10^{-8}–10^{-6} M) and E\(_2\) (1–5 × 10^{-9} M) to inhibit PMCA-mediated efflux in primary ECs without affecting PMCA expression levels and to promote PMCA phosphorylation at Tyr1176 (135, 170), which is known to inhibit pump activity (94). Notably, this phosphorylation masks the stimulatory effect of enhancing the PMCA–Ca\(^{2+}\) interaction produced by 48-h E\(_2\) treatment (170). E\(_2\)-independent mechanisms are indicated by the findings that (1) GPER constitutively interacts with PMCA4b via the anchoring action of PSD-95 at their C-terminal PDZ-binding motifs; (2) overexpression of GPER decreases PMCA activity; (3) GPER knockdown promotes PMCA activity; and (4) PSD-95 knockdown or truncation of the PDZ-binding motif on GPER releases GPER–PMCA association and promotes PMCA activity (135). Functionally, these mechanisms collectively prolong agonist-induced Ca\(^{2+}\) signal and enhance eNOS activity in ECs (135, 170, 203). Consistent with suppressed Ca\(^{2+}\) efflux, the Ca\(^{2+}\) signals stimulated by E\(_2\) and the GPER agonist G-1 in cells overexpressing GPER reported by various laboratories display much more prolonged plateau phases compared to Ca\(^{2+}\) signals in cells not overexpressing GPER or those stimulated by other agonists such as ATP or bradykinin (160, 162, 164, 175). GPER–PMCA4b interaction seems to be mutually influential, such that knockdown of PMCA decreases GPER-mediated ERK1/2 phosphorylation, while GPER knockdown does the opposite on PMCA activity (135).

ESTROGENIC MODERATION OF CALCIUM-DEPENDENT ACTIVITIES

How do the various mechanisms discussed so far come together in regulating cardiovascular functions? An immediate challenge is how to reconcile the effects of estrogenic agonists to both trigger acute Ca\(^{2+}\) signals by themselves and inhibit otherwise stimulated Ca\(^{2+}\) signals. The Ca\(^{2+}\) signals triggered by estrogenic agonists in primary cardiovascular cells are generally of very low amplitude. Furthermore, as in experiments testing their effects on Ca\(^{2+}\) signals otherwise triggered, estrogenic agonists are present in situ with other stimuli whose Ca\(^{2+}\) signals they inhibit. Thus, for mechanisms that generate cytoplasmic Ca\(^{2+}\) signals, E\(_2\) and GPER exert ultimate inhibitory effects. For cytoplasmic Ca\(^{2+}\) removal mechanisms, estrogenic agonists and GPER also are inhibitory. For Ca\(^{2+}\) signal transduction, E\(_2\), via a feedforward action at GPER, increases CaM expression and enhances linkage in the CaM-binding proteome.

All things considered, E\(_2\) and GPER, via both E\(_2\)-dependent and E\(_2\)-independent mechanisms, act to moderate...
Ca\(^{2+}\)-dependent activities in the cardiovascular system. They “clamp” cytoplasmic Ca\(^{2+}\) signals by lowering peaks (inhibition of signal generation) and raising troughs (inhibition of signal removal), collectively confining tissues in a narrower yet more sustained operating range of Ca\(^{2+}\). Also, GPER-mediated increases in CaM expression and CaM network linkage improve Ca\(^{2+}\) signal transduction efficiency. Considering the Ca\(^{2+}\) sensitivity of Ca\(^{2+}\)-dependent proteins in this context, one can predict that those with low Ca\(^{2+}\) sensitivity (requiring high Ca\(^{2+}\) for activation) are more likely to be affected by the inhibition of Ca\(^{2+}\) signal generation. On the other hand, proteins with high Ca\(^{2+}\) sensitivity (requiring low Ca\(^{2+}\) for activation) are more likely to be promoted by the inhibition of Ca\(^{2+}\) removal and less affected by the suppression of Ca\(^{2+}\) signal generation (Figure 2).

This notion has been demonstrated experimentally via the case of eNOS, a Ca\(^{2+}\)-dependent CaM-binding protein (222) with sub-nanomolar affinity for CaM (127). CaM interaction and subsequent activation of wild-type eNOS have high Ca\(^{2+}\) sensitivities, with respective EC\(_{50}\)(Ca\(^{2+}\)) values \(~1.8 \times 10^{-7}\) and \(~4 \times 10^{-7}\) M (190). eNOS is also regulated by multisite phosphorylation (223). Notably, its bi-phosphorylation at Ser617 and Ser1179 promotes NO production by increasing the Ca\(^{2+}\) sensitivity for both CaM binding and enzyme activation, reducing their respective EC\(_{50}\)(Ca\(^{2+}\)) values to \(~0.7 \times 10^{-7}\) and \(~1.3 \times 10^{-7}\) M, thus rendering the synthase active at resting cytoplasmic Ca\(^{2+}\) (189). E\(_2\) and GPER (1) prolong endothelial cytoplasmic Ca\(^{2+}\) signal by inhibiting Ca\(^{2+}\) efflux (135, 170), (2) promote eNOS phosphorylation at Ser617 and Ser1179 (170, 198), (3) increase CaM expression and eNOS–CaM interaction (170), and (4) suppress endothelial SOCE (203). When we incorporate these effects into a verified sequential “CaM binding eNOS activation” model (189, 190), eNOS activity and NO accumulation are shown to substantially increase across the time course of bradykinin-induced Ca\(^{2+}\) signal in ECs by treatment with G-1 (203). Importantly, major contributions to this outcome include the increases in CaM binding, phosphorylation, Ca\(^{2+}\) sensitivity, and duration of Ca\(^{2+}\) signals due to Ca\(^{2+}\) efflux inhibition, but little or no effect of the inhibition of SOCE (203), due obviously to the synthase’s high Ca\(^{2+}\) sensitivity (Figure 3). Thus, via multifaceted actions on components of the CSM, E\(_2\) and GPER moderate Ca\(^{2+}\)-dependent activities by differentially affecting the continuum of Ca\(^{2+}\)-dependent proteins based on their Ca\(^{2+}\) sensitivities for Ca\(^{2+}\) or Ca\(^{2+}\)–CaM complexes.

Considering the two Ca\(^{2+}\)-dependent estrogen receptors—ER\(_{\alpha}\) and GPER—how does the presence of one influence the effects of the other on Ca\(^{2+}\) signaling? A complex relationship is predicted to exist in which ER\(_{\alpha}\) transcriptional activities affect the expression of certain Ca\(^{2+}\) signaling proteins but are themselves influenced by the amplitudes and dynamics of Ca\(^{2+}\) signals limited by GPER activation and the availability of CaM that is promoted by GPER action (170). In turn, as CaM is limited in cells (122, 124, 126, 127), the high affinity binding of CaM by ER\(_{\alpha}\) and GPER further limits CaM availability and will influence CaM-dependent regulation of each other at the receptor level, a predictable outcome of the functional crosstalk via competition for limited CaM (124, 127). These relationships may represent but a small aspect of the reciprocity between estrogen and Ca\(^{2+}\) signaling.

**CONCLUSION AND FUTURE PERSPECTIVES**

Reciprocity between estrogen signaling and Ca\(^{2+}\)-dependent activities is becoming evident. Considering the impact of estrogen
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Estrogen Biology and Calcium Signaling

FIGURE 3 | Moderation of Ca2+-dependent eNOS activity by GPER activation. (A) Cytoplasmic Ca2+ clamping by GPER activation in ECs (203). The solid line represents Ca2+ signals produced in response to agonist stimulation in the absence of GPER activation. The sparsely dotted area represents the range of cytoplasmic Ca2+ signals, in which peak and trough are seen due to maximal effects of Ca2+ entry and Ca2+ efflux. The stippled blue line represents Ca2+ signals produced in the presence of GPER and its activation. These signals are clamped in a narrower range (the blue area) due to inhibitory effects on both SOCE [green stripes (135)] and PMCA4b-mediated Ca2+ efflux [red stripes (199, 210)]. (B) Average time courses of cytoplasmic Ca2+ signals measured in primary ECs treated with bradykinin in the absence of extracellular Ca2+ followed by treatment with vehicle or G-1; total Ca2+ signals were triggered by re-addition of extracellular Ca2+ [arrow (203)]. (C) Calculated eNOS point activity corresponding to each Ca2+ value in (B) considering only changes in Ca2+ due to GPER activation using a verified sequential eNOS–CaM binding eNOS activation model [equation, where (K1, K2) and (K3, K4) are derived products of the binding constants of Ca2+ at the Ca2+-binding sites on the N and C lobes of CaM in binding to CaM and interaction of Ca2+-CaM and eNOS (189, 190)]. (D) Calculated eNOS point activity corresponding to each Ca2+ value measured in (B), factoring in changes in Ca2+, CaM binding, and eNOS phosphorylation (170, 203). See details in text and (170, 203). Reproduced with permission from the author’s previous publication (203).

and its receptors on Ca2+ signaling, E2, and in many cases, GPER exert inhibitory effects on many components of the CSM in cardiovascular tissues, from Ca2+ store release and uptake (214, 215, 221) and Ca2+ entry (199, 201–210, 212, 213) to cytosolic Ca2+ removal mechanisms (135, 170, 208, 217–221). Considering the impact of Ca2+ signaling on estrogen biology, both ERα and GPER are strongly regulated by direct Ca2+-dependent interactions with CaM. These interactions serve to stabilize receptor dimerization and enhance subsequent transcriptional activities [the case of ERα (137, 138, 142, 143)] or promote receptor-mediated downstream signaling [the case of GPER (169, 170)]. Also, E2-induced MAPK activation has long been known to be dependent on the Ca2+ signal produced (173). Reciprocality between estrogen biology and Ca2+ signaling is further evidenced by the demonstration of a feedforward mechanism, in which E2, via GPER activation, upregulates total cellular CaM expression and free intracellular Ca2+-CaM concentration, which promotes functions of GPER and ERα and other classes of Ca2+-CaM-dependent proteins (170). The combination of these various actions is predicted to affect Ca2+-dependent functions depending on the affinity and Ca2+ sensitivities of the proteins involved, as exemplified by the case of eNOS (Figures 2, 3) (170, 203).

The moderating effects that estrogenic agonists and receptors exert on the CSM can explain many of their cardiovascular effects, such as preventing excessive cardiac contraction during sympathetic stress, limiting adverse outcomes related to Ca2+ overload, and reducing vascular tone. Nevertheless, the effects of E2 and estrogen receptors on many CSM components have not been examined. Additionally, many questions remain regarding
mechanisms of the observed effects that estrogen agonist receptors produce on the CSM. For example, how do E2 and GPER inhibit ICa,L? What are the mechanisms that position GPER as an intrinsic component of β1AR signaling in the myocardium? What are the mechanisms whereby E2 inhibits the activities of SERCA and NCX? What are the mechanisms whereby E2 inhibits mitochondrial Ca2+ uptake? Further studies are needed to answer these questions. Through many examples, however, it is clear that GPER produces both E2-dependent and E2-independent effects on the CSM. While the search is ongoing for approaches to apply specific estrogen receptor agonists to the prevention of cardiovascular disease, the therapeutic potential of E2-independent effects of GPER and other estrogen receptors is as yet an unexplored territory.

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**Conflict of Interest**: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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