Hetero-oligomeric Complex between the G Protein-coupled Estrogen Receptor 1 and the Plasma Membrane Ca\(^{2+}\)-ATPase 4b

Quang-Kim Tran\(^1\), Mark VerMeer\(^1\), Michelle A. Burgard, Ali B. Hassan\(^3\), and Jennifer Giles

From the Department of Physiology and Pharmacology, College of Osteopathic Medicine, Des Moines University, Des Moines, Iowa 50312

**Background:** GPER/GPR30’s actions are unclear.

**Results:** GPER/GPR30 and PMCA4b constitutively interact via PDZ-binding motifs. This inhibits PMCA but enhances GPER/GPR30 activity. GPER/GPR30 activation further supresses PMCA activity via tyrosine phosphorylation.

**Conclusion:** GPER/GPR30 and PMCA4b form a physical and functional complex.

**Significance:** GPER/GPR30-PMCA4b interactions mediate cross-talk between GPER/GPR30 and Ca\(^{2+}\) signaling.

The new G protein-coupled estrogen receptor 1 (GPER/GPR30) plays important roles in many organ systems. The plasma membrane Ca\(^{2+}\)-ATPase (PMCA) is essential for removal of cytoplasmic Ca\(^{2+}\) and for shaping the time courses of Ca\(^{2+}\)-dependent activities. Here, we show that PMCA and GPER/GPR30 physically interact and functionally influence each other. In primary endothelial cells, GPER/GPR30 agonist G-1 decreases PMCA-mediated Ca\(^{2+}\) extrusion by promoting PMCA tyrosine phosphorylation. GPER/GPR30 overexpression decreases PMCA activity, and G-1 further potentiates this effect. GPER/GPR30 knockdown increases PMCA activity, whereas PMCA knockdown substantially reduces GPER/GPR30-mediated phosphorylation of the extracellular signal-related kinase (ERK1/2). GPER/GPR30 co-immunoprecipitates with PMCA with or without treatment with 17\(\beta\)-estradiol, thapsigargin, or G-1. Heterologously expressed GPER/GPR30 in HEK 293 cells co-localizes with PMCA4b, the main endothelial PMCA isoform. Endothelial cells robustly express the PDZ post-synaptic density protein (PSD)-95, whose knockdown reduces the association between GPER/GPR30 and PMCA. Additionally, the association between PMCA4b and GPER/GPR30 is substantially reduced by truncation of either or both of their C-terminal PDZ-binding motifs. Functionally, inhibition of PMCA activity is significantly reduced by truncation of GPER/GPR30’s C-terminal PDZ-binding motif. These data strongly indicate that GPER/GPR30 and PMCA4b form a hetero-oligomeric complex in part via the anchoring action of PSD-95, in which they constitutively affect each other’s function. Activation of GPER/GPR30 further inhibits PMCA activity through tyrosine phosphorylation of the pump. These interactions represent cross-talk between Ca\(^{2+}\) signaling and GPER/GPR30-mediated activities.

The G protein-coupled estrogen receptor 1 (GPER/GPR30) is a newly found seven-pass transmembrane receptor that is sensitive to estrogen (1–3). GPER/GPR30 has been implicated in many physiological processes (4), such as cholinergeric neuron responses and cognitive function (5), autoimmune diseases and inflammation (6, 7), and myocardial contraction and left ventricular functions (8, 9), to name a few. GPER/GPR30 also appears to mediate different physiological effects in males and females, indicating its involvement in sex-independent processes (9). At the cellular level, GPER/GPR30 has been reported to participate in many functions, including Ca\(^{2+}\) mobilization (2, 3), cAMP production (2, 10), activation of protein kinases (1, 3), and activation of transcription (11–14). Despite the many actions described for GPER/GPR30, the underlying mechanisms are still unclear. For example, although GPER/GPR30 has been shown to associate with Go\(_{\alpha}\) (2), neither 17\(\beta\)-estradiol nor GPER/GPR30 agonist G-1 triggers cAMP production in cells overexpressing this receptor; on the contrary, expression of GPER/GPR30 decreases adenyl cyclase-mediated cAMP production through interactions with members of the membrane-associated guanylate cyclase (MAGUK) family and AKAP5 (15). In addition, although GPER/GPR30 stimulation with 17\(\beta\)-estradiol or GPR30 agonist G-1 has frequently been shown to induce intracellular Ca\(^{2+}\) signals (2, 3, 16), the effects of GPER/GPR30 activation on the activity of the key components of Ca\(^{2+}\) homeostasis and the underlying mechanisms remain unclear.

---

\(^{1}\) This work was supported, in whole or in part, by National Institutes of Health Grant HL12184 (to Q.-K. T.).

\(^{2}\) Both authors contributed equally to this work.

\(^{3}\) Supported by the Mentored Research Program at Des Moines University.

---

The abbreviations used are: GPER/GPR30, G protein-coupled estrogen receptor 1 or GPR30; PMCA, plasma membrane Ca\(^{2+}\)-transporting ATPase isoform 4b; PSD-95, postsynaptic density protein 95; PDZ domain, postsynaptic density protein, Drosophila disc large tumor suppressor (Dlg1) and Zonula occludens-1 protein domain; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; PAEC, porcine aortic endothelial cell; VSMC, vascular smooth muscle cell; oligo, oligonucleotide; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced YFP; GPCR, G protein-coupled receptor; eNOS, endothelial.
Hetero-oligomeric Complex between GPER/GPR30 and PMCA4b

Removal of cytoplasmic Ca\(^{2+}\) following agonist-induced Ca\(^{2+}\) signals is important for cell functions by controlling the dynamics of Ca\(^{2+}\) signals. For example, in vascular smooth muscle cells, the enhanced rate of cytoplasmic Ca\(^{2+}\) removal causes faster relaxation, allowing for a more dynamic control of vascular tone (17). In addition, cytoplasmic Ca\(^{2+}\) removal prevents Ca\(^{2+}\) overload and its consequences. In the opposite direction, reductions in the rate of cytoplasmic Ca\(^{2+}\) removal prolong the time courses of Ca\(^{2+}\)-dependent processes (18, 19). The plasma membrane Ca\(^{2+}\)-ATPase (PMCA) is a major Ca\(^{2+}\) extrusion mechanism in many cell types. In vascular endothelial cells, PMCA has been suggested to be responsible for up to 50% of the removal of cytoplasmic Ca\(^{2+}\) (20). PMCA4b is the predominant isoform in vascular endothelial cells, smooth muscle cells, and human embryonic kidney (HEK) 293 cells (21). PMCA activity is regulated by its interactions with many other proteins. Calmodulin (CaM) tightly regulates the pump’s activity through high affinity interaction with the PMCas (22, 23). A significant number of other proteins interact with PMCA via its PDZ-binding domain in the C terminus, including members of the MAGUKs, such as PSD-95/SAP90, SAP97/hDlg, SAP102, and PSD-93/chapsyn-110, and neuronal nitric-oxide synthase (24–28). Interaction with PSD-95 facilitates plasma membrane targeting and function of PMCA4b (27). These interactions are important for anchoring and targeting of PMCA to the plasma membrane and regulate its roles in signaling.

It is completely unknown whether GPER/GPR30 affects cellular Ca\(^{2+}\) homeostasis via modulation of PMCA activity. We have begun to test the idea that significant cross-talk exists between GPER/GPR30 signaling and Ca\(^{2+}\) signaling via interactions between this receptor and components of the Ca\(^{2+}\) signaling machinery. In this study, we describe physical interactions and mutual functional influences between GPER/GPR30 and PMCA4b in the vascular endothelium. Multiple experimental paradigms performed under basal conditions, receptor agonism, and gene silencing in primary porcine aortic endothelial cells (PAECs) or with overexpression of wild type and modified versions of GPER/GPR30 and PMCA4b in HEK 293 cells demonstrated the following: 1) GPER/GPR30 activation inhibits PMCA activity by promoting tyrosine phosphorylation of the pump; 2) GPER/GPR30 and PMCA4b constitutively and physically interact; 3) this interaction inhibits PMCA activity independently of phosphorylation but promotes GPER/GPR30 activity; and 4) interaction occurs via their C-terminal PDZ-binding domains and their association with PSD-95. The implications of these results on Ca\(^{2+}\)-dependent activities and GPER/GPR30 signaling are discussed.

Experimental Procedures

Cell Isolation and Culture—PAECs were obtained as described previously (29). Briefly, the intima of freshly isolated porcine thoracic aortas was gently scraped and resuspended in M-199 medium (Caisson Laboratories, Logan, UT) containing 10% newborn calf serum (Fisher) and 1% penicillin/streptomycin (MP Biomedicals, Solon, OH). Primary vascular smooth muscle cells (VSMCs) were subsequently obtained from the same vessels as described previously (30). Human embryonic kidney (HEK) 293 cells (ATCC) were cultured in DMEM containing 5–10% fetal bovine serum. Cells were cultured in a 37 °C incubator containing 5% CO\(_2\) humidified air. The medium was frequently renewed.

Molecular Biology—Total mRNA was obtained from primary PAECs, VSMCs, HEK 293 cells, and primary human umbilical vein endothelial cells (ATCC) using Promega’s ImProm-II reverse transcription system (Promega). cDNAs of human PMCA4b and GPER/GPR30 were then reverse-transcribed from HEK 293 cells’ mRNA. A BamHI and an XbaI restriction site was introduced into the N- and C-terminal ends, respectively, of human PMCA4b and GPER/GPR30 by PCR amplification. These sequences were then incorporated in a pcDNA3.1 mammalian expression vector. C-terminal fusions PMCA4b-DsRed2 and GPER/GPR30-DsRed2 were then constructed by inserting DsRed2 (Clontech) via a linker (IDYDVLDDYG) to the C-terminal end of PMCA4b and GPER/GPR30. The GPER/GPR30-ECFP fusion was constructed by replacing the EYFP moiety and the CaM-binding linker sequence of a previously published GPER/GPR30 biosensor (BSGPER(330–351)) (30) with the entire GPER/GPR30 sequence. The N-terminal fusions DsRed2-GPER/GPR30 and ECFP-PMCA4b were generated by inserting DsRed2 or ECFP between the HindIII and BamHI restriction sites upstream of PMCA4b or GPER/GPR30 in the pcDNA3.1 plasmids.

FLAG (MDYKDDDDK) and HA (MYPYDVPDYA) tag sequences were PCR-generated using forward and reversed primers designed to span the entire tag length, with a KpnI and a BamHI restriction site added to the N- and C-terminal ends of the tag sequences, respectively. These FLAG and HA tags were then inserted upstream of the N terminus of GPER/GPR30 or PMCA4b, respectively, in the pcDNA3.1 vector. The FLAG-tagged GPER/GPR30 and HA-tagged PMCA4b mutants with deletions of the four C-terminal residues (SSAV for GPER/GPR30) were generated using standard molecular biology techniques. All constructs were verified by DNA sequencing (University of Missouri-Columbia DNA Core Facility).

Transfection—Oligonucleotides and plasmids encoding various wild-type or mutant constructs were transfected into primary PAECs, HEK 293 cells using the Transit-2020 or Transit-Oligo transfection kits (Mirus Bio LLC) as per the manufacturer’s instructions.

Gene Silencing of GPER/GPR30, PMCA, and PSD-95—GPER/GPR30 was knocked down in primary PAECs using an antisense oligonucleotide directed against the porcine GPER/GPR30 sequence. For identification of transfected cells during PMCA activity measurement, GPER/GPR30 antisense and scrambled oligonucleotides were both conjugated with TAMS, a red fluorescent marker. PSD-95 antisense and scrambled sequences were based partly on previously published data, with some adjustment to match porcine sequences (31). All oligomer nucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). The antisense and scrambled oligo sequences for these targets are shown in Table 1.

For experiments measuring PMCA activity in GPER/GPR30 knockdown cells, transfection was performed on PAECs plated on 60-mm dishes containing number 1.5 coverslips. Two days
after transfection, the coverslips were processed for imaging to measure PMCA activity, and the remaining cells on the dishes were collected for Western blotting to verify effective knockdown of GPER/GPR30. Cells on coverslips were first loaded with fura-2/AM. Following loading, cells expressing GPER/GPR30 antisense or scrambled oligo were detected and marked using TAMS fluorescence emission at $\lambda 610$ nm in response to excitation at $\lambda 570$ nm. Imaging cube was then switched for measurement of PMCA activity using fura-2 fluorescence on pre-marked TAMS (+) cells. After the imaging experiments, the same coverslips were washed and fixed with 0.4% Triton X-100 for 10 min, followed by incubation overnight with a rabbit anti-GPR30 antibody (N-15, SC-48525, Santa Cruz Biotechnology). Cell Imaging Workbench 6.0 software (INDEC Biosystems). The dye was then removed, and cells were equilibrated in Ca$^{2+}$-free medium until subconfluence with or without treatments prior to experiment. Cells were incubated with 4 $\mu$M fura-2/AM (Invitrogen) in culture medium for 30 min at 37°C. The dye was then removed, and cells were equilibrated in Ca$^{2+}$-containing modified Tyrode’s buffer (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 10 HEPES, 1 CaCl$_2$, pH 7.4) for 15 min at room temperature. Excitation of fura-2 alternated between A340 nm and $\lambda 380$ nm for 50 ms each per 500-msec cycle from an ultra-high speed wavelength switcher (Lambda DG-4, Sutters Instruments). The excitation switching lapse between the two wavelengths was 1 ms. Emission light was collected at A510 nm through an ultra-rapid filter wheel (Lambda-10B, Sutters Instruments) and processed by an electron-multiplying charge-coupled digital camera (DU-885, Andor Technology). Pairs of images were analyzed using the Imaging Workbench 6.0 software (INDEC Biosystems). The ratio of emission fluorescence intensities collected following the excitation at A340 nm and A380 nm was used as a measure of intracellular Ca$^{2+}$ concentration. For some experimental paradigms, free intracellular Ca$^{2+}$ concentrations were calculated using Equation 1,

$$Ca^{2+}(nm) = K_d \times \frac{S_f}{S_b} \times \frac{R - R_{min}}{R_{max} - R} \quad (Eq. 1)$$

where the $K_d$ value is 224 nm for fura-2; $R$ is the observed ratio fluorescent signal during the experiment. $S_f$ and $S_b$ represent the emission intensities collected at A510 nm corresponding to the Ca$^{2+}$-free and Ca$^{2+}$-bound states of fura-2. We avoided potential errors associated with using fixed values for $R_{min}$ and $R_{max}$ by determining these values in individual cells in each experiment. We first determined the average relationship between $R_{min}$, $R_{max}$ and the basal $R_{basal}$ value, namely the $R$ value obtained in Ca$^{2+}$-free medium from unstimulated cells. Following dye loading, $R_{basal}$ values were obtained. The same cells were then incubated on stage with 20 $\mu$M BAPTA/AM (Tocris Bioscience, Ellisville, MO) for 30 min to obtain $R_{min}$ values. $R_{max}$ values from the same cells were next obtained by adding 5 $\mu$M ionomycin and 10 mM CaCl$_2$. Comparing the average ($n = 100$) absolute $R_{min}$ values with the observed fura-2 ratios in the same cells in Ca$^{2+}$-free medium prior to addition of BAPTA/AM ($R_{basal}$) and the average $R_{max}$ value, Equation 2 was obtained.

$$R_{min} = R_{basal} - 0.06 \times (R_{max} - R_{basal}) \quad (Eq. 2)$$

Similarly, Equation 3 was obtained between $S_f$ and $S_{basal}$, which is the fluorescence emission intensity at A510 nm at the beginning of the experiment with cells in Ca$^{2+}$-free medium in response to excitation at A380 nm.

$$S_f = S_{basal} + 0.85 \times (S_{basal} - S_b) \quad (Eq. 3)$$

No statistical differences ($n = 100$ cells from five separate pilot experiments) were observed between the measured $R_{min}$ and $S_f$ values versus the calculated $R_{min}$ and $S_f$ values using Equations 2 and 3. Because $R_{basal}$ and $S_{basal}$ values were readily measured at the beginning of every experiment, and corresponding $R_{max}$ and $S_b$ values were also easily obtained by the end of each imaging time course by adding high concentrations of ionomycin and Ca$^{2+}$, free Ca$^{2+}$ concentrations in individual cells could be calculated from Equations 1–3 with relatively high reliability.

Measurement of PMCA Activity in Living Cells—Cells were prepared as described above in the Ca$^{2+}$ imaging section. Thapsigargin (1 $\mu$M) was added to nominally Ca$^{2+}$-free buffer to deplete the endoplasmic reticulum of Ca$^{2+}$. Ca$^{2+}$ influx was initiated by the addition of 1.5 mM CaCl$_2$ with or without specified concentrations of G-1. When peak influx was reached, the extracellular medium was replaced by one containing 5 mM BAPTA and 150 mM N-methylglucamine in place of NaCl, to block the Na$^+/Ca^{2+}$ exchanger and abolish Ca$^{2+}$ entry. The rate of decay in intracellular Ca$^{2+}$ concentration now only reflected the rate of Ca$^{2+}$ extrusion via the PMCA, and it was determined by fitting the time course of the apparent free Ca$^{2+}$ concentration to the mono-exponential Equation 4.

$$y = y_0 + Ae^{-xt} \quad (Eq. 4)$$
Hetero-oligomeric Complex between GPER/GPR30 and PMCA4b

In previous pilot studies, a number of fitting approaches were tested, including linear, mono-, bi-, and tri-exponential equations; the mono-exponential equation provided the most reliable data with the best residuals (18, 19). The extrusion rates of Ca$^{2+}$ are presented as relaxation times (τ). Because the activity of PMCA is intrinsically Ca$^{2+}$-dependent, comparisons were made only among cells in which the free Ca$^{2+}$ concentrations at the beginning of the Ca$^{2+}$ extrusion time course were in the same ranges (18, 19). Absolute Ca$^{2+}$ values in individual cells were calculated as described above. Relative PMCA activity was expressed as the inverse of the relaxation time.

Confocal Microscopy—Confocal microscopy was performed using a Leica TCS SP8 confocal microscopy system (University of Iowa). HEK 293 cells plated on number 1.5 coverglass were transiently transfected with wild-type or mutant GPER and PMCA4b fused at their N terminus with DsRed2 or ECFP, respectively, followed by mounting with Prolong Gold Antifade Reagent (Fisher) 24 h prior to imaging. RGB images were analyzed using the ImageJ software (rsb.info.nih.gov) for visualization of separately labeled proteins.

Western Blotting—Cells were lysed in a buffer containing (in mM) 25 Tris-HCl, 148 NaCl, 97.6 NaF, 27.8 Na$_4$P$_2$O$_7$, 271.8 Na$_3$VO$_4$, Triton X-100 (1% v/v), trypsin inhibitor (45 μM), 1:200 protease inhibitor mixture (Sigma), and 2 μg/ml PMSF. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Following SDS-PAGE, proteins were transferred to a PVDF membrane (Thermo Scientific, Rockford, IL). The membranes were then blocked with 5% BSA overnight at 4 °C. Primary antibodies were applied in Tris-buffered saline containing Tween 20 (TBST) for 1 h at room temperature. Membranes were subsequently incubated with an appropriate secondary antibody in TBST. Enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) was used for film development and visualization of protein bands. Films were then scanned using Chemidoc XRS Imager (Bio-Rad), and densitometric values of bands were quantified using Image Lab 5.0 software (Bio-Rad). Densitometric values of the proteins of interest were corrected for corresponding values of the simultaneously probed loading control protein.

Co-immunoprecipitation—Co-immunoprecipitation was performed using protein A/G (Thermo Scientific) as per the manufacturer’s instructions. Cell lysis was performed on monolayer cells without trypsinization to avoid complications having to do with shear during the process. Cells were lysed on ice for 15 min, and the lysate was centrifuged at 21,000 × g for 5 min at 4 °C. Following pre-clearing, the protein content of the eluant of the preys using the ImageLab 5.0 software (rectangular volume tool) for analysis.

Statistical Analysis—Data are expressed as means ± S.E. Statistical analysis was performed using Student’s t test, assuming unequal variances between control and treated groups. Statistical significance was determined as p < 0.05.

Results

GPER/GPR30 Activation Inhibits PMCA Activity—We first verified the expression of GPER/GPR30 in a number of vascular cells and cell lines, including primary PAECs and VSMCs, primary human umbilical vein endothelial cells and HEK 293 cells. Total mRNAs were isolated from these cells, followed by RT-PCR to amplify a segment of the submembrane domain 4 of GPER/GPR30 (amino acids 330–375). Total lysate from these cells were probed for GPER/GPR30 using the N-15 antibody. Fig. 1A shows both mRNA (upper panel) and protein expression levels (lower panel) of GPER/GPR30 in these cell types.

To examine the potential role of GPER/GPR30 on Ca$^{2+}$ efflux, we first tested the effects of the GPER/GPR30 agonist G-1 (16) on PMCA activity in primary vascular endothelial cells. PMCA is known to be the key component of cytoplasmic Ca$^{2+}$ removal in these cells (20), which play an indisputable role in nitric oxide production and other vascular functions. We and others have successfully developed a protocol to measure PMCA activity in these cells (18–20). This protocol involves initial depletion of intracellular Ca$^{2+}$ stores with the irreversible sarco/endoplasmic reticulum Ca$^{2+}$/ATPase pump inhibitor thapsigargin in Ca$^{2+}$-free medium, followed by activation of Ca$^{2+}$ entry by addition of extracellular Ca$^{2+}$. PMCA activity is subsequently assessed as extracellular Ca$^{2+}$ is removed and Na$^+$-Ca$^{2+}$ exchanger prevented by substituting Na$^+$ with N-methyl-d-glucamine. A typical experiment time course is shown in Fig. 1B. Because PMCA activity is intrinsically Ca$^{2+}$-dependent, namely the higher starting Ca$^{2+}$ values are associated with higher PMCA activity, Ca$^{2+}$ values at the beginning of the extrusion time courses were binned, and comparisons were made among cells with values in the same range. $R_{\text{min}}$ and $R_{\text{max}}$ values for individual cells were determined in each experiment so that the calculated Ca$^{2+}$ values reflect more precisely the free intracellular Ca$^{2+}$ concentrations (see “Experimental Procedures”). This binning approach helps minimize erroneous interpretation of effects on PMCA due to differences in total cytoplasmic Ca$^{2+}$ signals that might be due to other factors. To initially test the effect of GPER/GPR30 activation on PMCA activity, different doses of G-1 were added together with thapsigargin in this protocol. When PAECs with similar free Ca$^{2+}$ levels at the beginning of Ca$^{2+}$ extrusion, following removal of extracellular Ca$^{2+}$, were binned together and rates of extrusion compared, G-1 clearly demonstrated a dose-dependent inhibitory effect on PMCA activity, as evidenced by increases in the Ca$^{2+}$ extrusion times. Fig. 1C summarizes the dose-dependent effect of G-1 on PMCA activity. Maximal effects were about 45% reduction in PMCA activity. Fig. 1D shows fits of average time courses from cells with free cytoplasmic Ca$^{2+}$ at the start of the Ca$^{2+}$ extrusion binned around 1000 nm from cells treated with a number of G-1 doses. Fig. 1, E and F, showed representative residuals (goodness of fits) of the mono-exponential fits for PMCA activity from control cells and cells treated with 1 μg G-1. All other fits yielded equally good residuals (data not shown).
G-1 Inhibits Ca\(^{2+}\) Efflux via Tyrosine Phosphorylation of PMCA—A number of factors have been shown to inhibit PMCA activity. These include calpain-mediated cleavage (32), interaction with POST (partner of stromal interaction molecule 1) (33), or Src-dependent phosphorylation at Tyr-1176 in the C terminus of PMCA4b (34, 35). Estrogen has been shown to activate Src-related tyrosine kinase activity and tyrosine phosphorylation of Shc adapter protein (1). As PMCA4b is the major PMCA isoform in endothelial cells (36), we considered the possibility that G-1 also inhibits PMCA activity by promoting tyrosine phosphorylation of the pump. To test this possibility, we first verified that G-1 can promote tyrosine phosphorylation of the PMCA. Primary PAECs were treated with vehicle or G-1, with or without pretreatment with the Src kinase inhibitor PP2, which has been shown to effectively inhibit tyrosine phosphorylation of PMCA4b (34). Following lysis, PMCA was immunoprecipitated using the PMCA antibody 5F10, followed by probing of the pulldown fractions with anti-phosphotyrosine antibody PY20 (Thermo Scientific). Fig. 2A clearly shows that G-1 stimulated robust tyrosine phosphorylation of the PMCA.
and that inhibition of Src activity using PP2 prevented this effect. We next examined the effects of these treatments on PMCA activity. G-1 (0.5 μM) produced the observed inhibitory effect as in Fig. 1, whereas pretreatment with Src kinase inhibitor PP2 prevented this effect (Fig. 2B). Fig. 2C shows average time courses of the Ca^{2+} extrusion phases in these experiments. These data indicate that G-1 inhibits PMCA activity by promoting tyrosine phosphorylation of PMCA.

**Heterologous Expression of GPER/GPR30 Decreases PMCA Activity**—To further confirm that GPER/GPR30 inhibits PMCA activity, we heterologously expressed human GPER/GPR30 in HEK 293 cells. As shown in Fig. 1A, both PAECs and HEK 293 cells express GPER/GPR30 at the mRNA and protein levels. However, these overexpression experiments were performed in HEK 293 cells for relative ease of transfection of large plasmids as compared with primary endothelial cells. To confirm imaging was performed in cells expressing GPER/GPR30, the receptor was fused with the fluorescent protein DsRed2. As a first control, only the DsRed2 moiety was expressed, followed by loading with fura-2/AM. As shown in Fig. 3, A and B, shows fluorescence images of DsRed2 and fura-2 in the same cell population containing both DsRed2-transfected and nontransfected cells. Merged image (Fig. 3C) allows easy distinction of these two populations. PMCA activity was then performed as described in Fig. 1B and was compared between DsRed2-expressing and nonexpressing cells in the same microscopic field. There was no difference in Ca^{2+} extrusion rates between these two groups, indicating that the expression of DsRed2 alone did not interfere with PMCA activity (data not shown). The same approach was then performed for cells expressing GPER/GPR30-DsRed2 fusion (Fig. 3, D–F). Fig. 3G shows fits of Ca^{2+} extrusion time courses between and mock-transfected cells and cells transfected with GPER/GPR30-DsRed2 with or without treatment of 0.5 μM G-1 prior to Ca^{2+} extrusion. Interestingly, overexpression of GPER/GPR30 inhibited PMCA activity by 40% in the absence of agonist treatment, and this effect is significantly further pronounced by treatment with G-1 (Fig. 3H).

**Knockdown of GPER/GPR30 in Endothelial Cells Increases PMCA Activity**—To further assess the effect of GPER/GPR30 on PMCA activity, we designed antisense oligonucleotides directed against porcine GPER/GPR30, and we compared PMCA activity in primary PAECs transfected with antisense or scrambled nucleotides. Both the antisense and scrambled oligonucleotides were conjugated with TAMS, a red fluorescent tag that would help identify cells transfected with the respective oligo prior to measuring PMCA activity using fura-2 fluorescence in the same field of cells. Transfection with GPER/GPR30 antisense resulted in a 70% reduction in PMCA activity, we heterologously expressed human GPER/GPR30 in HEK 293 cells. As shown in Fig. 1A, both PAECs and HEK 293 cells express GPER/GPR30 at the mRNA and protein levels. However, these overexpression experiments were performed in HEK 293 cells for relative ease of transfection of large plasmids as compared with primary endothelial cells. To confirm imaging was performed in cells expressing GPER/GPR30, the receptor was fused with the fluorescent protein DsRed2. As a first control, only the DsRed2 moiety was expressed, followed by loading with fura-2/AM. As shown in Fig. 3, A and B, shows fluorescence images of DsRed2 and fura-2 in the same cell population containing both DsRed2-transfected and nontransfected cells. Merged image (Fig. 3C) allows easy distinction of these two populations. PMCA activity was then performed as described in Fig. 1B and was compared between DsRed2-expressing and nonexpressing cells in the same microscopic field. There was no difference in Ca^{2+} extrusion rates between these two groups, indicating that the expression of DsRed2 alone did not interfere with PMCA activity (data not shown). The same approach was then performed for cells expressing GPER/GPR30-DsRed2 fusion (Fig. 3, D–F). Fig. 3G shows fits of Ca^{2+} extrusion time courses between and mock-transfected cells and cells transfected with GPER/GPR30-DsRed2 with or without treatment of 0.5 μM G-1 prior to Ca^{2+} extrusion. Interestingly, overexpression of GPER/GPR30 inhibited PMCA activity by 40% in the absence of agonist treatment, and this effect is significantly further pronounced by treatment with G-1 (Fig. 3H).
and it further confirms that PMCA activity was compared between cells with different levels of GPER/GPR30 expression. Fig. 4C shows the average time courses of Ca$^{2+}$ extrusion in cells transfected with GPER/GPR30 antisense or scrambled oligo. Knockdown of GPER/GPR30 apparently was associated with a 2-fold increase in the rate of Ca$^{2+}$ extrusion via the PMCA. This result is quite consistent with the pharmacological data in Fig. 1 and overexpression data in Fig. 3.

**PMCA Affects Function of GPER/GPR30**—The effect of GPER/GPR30 to inhibit PMCA activity raised the question whether PMCA reciprocally affects GPER/GPR30’s function. Our initial task was to verify a parameter for GPER/GPR30 activity. Despite evidence that GPER/GPR30 was a G$\alpha_{i}$-associated GPCR (1), it has been shown that 17$\beta$-estradiol failed to stimulate cAMP production in cells endogenously expressing GPER/GPR30 or heterologously overexpressing the receptor (37, 38). More recently, it was shown that neither 17$\beta$-estradiol nor GPER/GPR30 agonist G-1 stimulated cAMP production in cells overexpressing GPER/GPR30 and that GPER/GPR30 constitutively inhibits adenylyl cyclase-mediated cAMP production by interacting with the MAGUKs and AKAP5 (15). However, G-1 has been shown previously to increase ERK1/2 phosphorylation in many publications (1, 39–41). We therefore first verified in our cell systems whether G-1-induced ERK1/2 phosphorylation could be utilized as a measure of GPER/GPR30 activity. In primary PAECs, G-1 shows a clear dose-dependent effect to stimulate ERK1/2 phosphorylation (Fig. 5A). It was noted that this effect is reduced in later passages of endothelial cells (data not shown). To further confirm the specificity of this effect, G-1-induced ERK1/2 phosphorylation was compared in primary PAECs transfected with GPER/GPR30 antisense or scrambled oligo as described in Fig. 4A. Fig. 5B clearly shows that G-1-induced ERK1/2 phosphorylation was substantially reduced in GPER/GPR30 knockdown cells. To further confirm this possibility, we turned to overexpression experiments in HEK 293 cells. As noted in Fig. 1A, we observed that HEK 293 cells express GPER/GPR30 both at the mRNA and protein levels. Treatment of mock-transfected HEK 293 cells with G-1 triggered ERK1/2 phosphorylation; however, overexpression of GPER/GPR30 in these cells (Fig. 5C, upper...
panels) substantially increased G-1-induced ERK1/2 phosphorylation (Fig. 5C, lower panels). These data confirm that G-1-induced ERK1/2 phosphorylation can be used as a parameter for GPER/GPR30 activity. Additionally, the middle panel in Fig. 5C, showing increased GPER/GPR30 in cells transfected with GPER/GPR30, together with data in Fig. 4A, showing that GPER/GPR30 expression is reduced in cells transfected with GPER/GPR30 antisense, confirmed the specificity of the GPER/GPR30 antibody used.

Having confirmed a functional assay for GPER/GPR30 activity, we next tested whether PMCA knockdown would affect GPER/GPR30 function. An antisense sequence was designed to target all isoforms of PMCA in porcine endothelial cells (Table 1). Fig. 6A shows an ~60% reduction in total PMCA expression in primary PAECs transfected with the PMCA antisense. The effect of PMCA knockdown was now examined on G-1-induced ERK1/2 phosphorylation as an indicator of GPER/GPR30 activity. G-1 robustly stimulated ERK1/2 phosphorylation in primary PAECs (Fig. 6B, left panel), consistent with the data in Fig. 5. PMCA knockdown markedly reduced this effect (Fig. 6B, right panel). To verify that PMCA knockdown is associated with reduction in PMCA functions, we compared Ca\(^{2+}\) extrusion time courses in cells transfected with the PMCA antisense or scrambled oligo (Fig. 6C). There was a clear reduction in PMCA activity in cells transfected with the PMCA antisense (Fig. 6D), indicating the functional efficiency of PMCA knockdown.

GPER/GPR30 Co-immunoprecipitates with PMCA in Vascular Endothelial Cells—The mutual functional effects between GPER/GPR30 and PMCA, and the fact that overexpression of GPER/GPR30 inhibits PMCA independently of agonist stimulation, raised the possibility that these proteins might physically interact in a hetero-oligomeric complex. We first tested this idea by performing reciprocal co-immunoprecipitation in primary endothelial cells under nonstimulated conditions or acute treatment with thapsigargin, endogenous GPER/GPR30 ligand
E₂, or GPER/GPR30 agonist G-1 in the presence of 1 mM extracellular Ca²⁺. Immunoblotting using anti-PMCA antibody (clone 5F10, Thermo Scientific) in GPER/GPR30 pulldown fractions shows clear PMCA bands from all samples (Fig. 7A). Likewise, GPER/GPR30 was clearly detected in all PMCA pull-down fractions from all samples (Fig. 7B). The upper and lower immunoblots of PMCA or GPER/GPR30 in Fig. 7A and 7B, were two fragments of the same SDS-PAGE membranes, guarantee-
ing that the prey protein levels detected truly corresponded to the “input” levels of the bait proteins shown. These data clearly show that PMCA and GPER/GPR30 are in the same complex in cells under different scenarios, including basal condition. We did not see a significant difference in the association between submembrane domain of GPER/GPR30. These fusions were expressed in HEK 293 cells, followed by a series of Western blotting analyses. As seen in the left panel in Fig. 7E, probing with anti-GFP antibody showed bands compatible with the size of EYFP and the submembrane domain of GPER/GPR30 (~27 kDa). Following stripping and reprobing with the N-15 antibody, the lower bands at the level of GPER/GPR30 in the left panel in Fig. 7E were no longer recognized, because this antibody should not recognize any of the submembrane domains of GPER/GPR30. In contrast, this panel in Fig. 7E shows clear bands at the level of ~38 kDa, the size of the endogenous nascent form of GPER/GPR30, consistent with data in Fig. 1A showing both mRNA and protein expression of GPER/GPR30 in HEK 293 cells. After another stripping and reprobing of the membrane with the H-300 antibody, both the endogenous

FIGURE 7. Reciprocal co-immunoprecipitation between PMCA and GPER/GPR30. A and B, reciprocal co-immunoprecipitation between PMCA and GPER/GPR30. Primary PAECs were treated with the specified concentrations of G-1, E2, and thapsigargin (TGN) for 3 min in the presence of 1 μM extracellular Ca2+. Following immunoprecipitation (IP) and SDS-PAGE, the membranes were cut between predicted PMCA and GPER/GPR30 levels, and fragments were simultaneously probed for PMCA or GPER/GPR30. C and D, specificity of antibodies used. Total lysate from the same samples as represented in A and B was first probed for nonimmune IgGs (upper immunoblots) species-matched with the GPER/GPR30 antibody (C) or PMCA (D) antibody used in A and B. Following stripping, the membranes were reprobed for GPER/GPR30 or PMCA using the antibodies used in A and B, respectively. E, confirmation of the epitope specificities of the GPER/GPR30 antibodies. Lysate samples of HEK 293 cells expressing fusions between EYFP and the specified submembrane domain (SMO) of GPER/GPR30 were first probed for GFP (left immunoblot). Following stripping, the membrane was reprobed for GPER/GPR30 using the N-15 antibody (middle immunoblot). The membrane was stripped again, followed by probing for GPER/GPR30 using the H-300 antibody (right immunoblot). See text for explanation.

validity of the co-immunoprecipitation data in Fig. 7A and 7B. In our studies, two anti-GPER/GPR30 antibodies were used, each with a different epitope. The N-15 antibody recognizes the N-terminal fragment of GPER/GPR30, although the H-300 antibody (SC-134576, Santa Cruz Biotechnology) recognizes amino acids 75–375 of the GPER/GPR30 sequence. To further confirm the specificity of these antibodies for their respective epitopes, we generated fusion proteins between EYFP and each submembrane domain of GPER/GPR30. These fusions were expressed in HEK 293 cells, followed by a series of Western blotting analyses. As seen in the left panel in Fig. 7E, probing with anti-GFP antibody showed bands compatible with the size of EYFP and the submembrane domains of GPER/GPR30 (~27 kDa). Following stripping and reprobing with the N-15 antibody, the lower bands at the level of GPER/GPR30 in the left panel in Fig. 7E were no longer recognized, because this antibody should not recognize any of the submembrane domains of GPER/GPR30. In contrast, this panel in Fig. 7E shows clear bands at the level of ~38 kDa, the size of the endogenous nascent form of GPER/GPR30, consistent with data in Fig. 1A showing both mRNA and protein expression of GPER/GPR30 in HEK 293 cells. After another stripping and reprobing of the membrane with the H-300 antibody, both the endogenous

FIGURE 6. Effect of PMCA gene silencing on GPER/GPR30 function. A, physical efficiency of PMCA knockdown. Primary PAECs transfected as specified were probed for PMCA (5F10 clone). Following immunoblotting for PMCA, the same membrane was stripped and reprobed for vinculin. B, effect of PMCA gene silencing on G-1-induced ERK1/2 phosphorylation. Left panel, nontransfected PAECs were treated with or without 100 nM G-1 for 15 min prior to cell lysis. Right panel, PAECs transfected with PMCA antisense or scrambled oligo were treated with 100 nM G-1 for 15 min prior to lysis. Immunoblotting and densitometric analysis were performed as above. C, functional efficiency of PMCA knockdown. Primary PAECs were transfected with antisense against PMCA (closed circles) or scrambled oligo (open circles). These data clearly represent average ratio between PMCA/vinculin. D, nontransfected and PAECs transfected with PMCA antisense or scrambled oligo were treated with 100 nM G-1 for 15 min prior to cell lysis. Immunoblotting and densitometric analysis were performed as above. These data clearly show both mRNA and protein expression of GPER/GPR30 in HEK 293 cells. After another stripping and reprobing of the membrane with the H-300 antibody, both the endogenous

A PMCA → B Vinculin → C p-ERK1/2 → D ERK1/2 →

\[ \text{Scrambled PMCA Oligo Antisense} \]

1. **A** PMCA → Vinculin → B p-ERK1/2 → C ERK1/2 →
2. **A** IP GPER GPER GPER GPER GPER GPER GPER GPER
3. **A** IB PMCA → IB GPER → G-1 (100 nM) E2 (5 nM) TGN (1 μM)
4. **B** IB PMCA → IB GPER → G-1 (100 nM) E2 (5 nM) TGN (1 μM)
5. **C** Nonimmune rabbit IgG Rabbit anti-GPER Ab
6. **D** Nonimmune mouse IgG Mouse anti-PMCA Ab
7. **E** 38 kDa → Anti-GFP 27 kDa → Anti-GFP
8. **F** 27 kDa → Anti-GFP N-15 Ab EYFP→SMO
GPER/GPR30 and the heterologously expressed fragments that contain only the submembrane domains were now evident. Together with data in Figs. 4A and 5C, these data strongly validate the specificity of the antibodies used in our co-immunoprecipitation experiments and indicate that GPER/GPR30 and PMCA are constitutively in the same complex in primary PAECs.

**Co-localization of PMCA4b and GPER/GPR30**—The data presented so far suggest a possibility of direct interaction between PMCA and GPER/GPR30. PMCA4b is the predominant isoform in endothelial cells, vascular smooth muscle, and HEK 293 cells (36). To further strengthen the possibility that PMCA4b and GPER/GPR30 physically interact, ECFP and DsRed2 were fused to the N termini of PMCA4b and GPER/GPR30, respectively. The two fusions were then co-expressed in HEK 293 cells. Confocal microscopy scanning clearly showed co-localization of ECFP-PMCA4b and DsRed2-GPER/GPR30 (arrows, Fig. 8, A–C).

**PMCA4b and GPER/GPR30 Interact via PSD-95**—Our data so far clearly demonstrate mutual inhibitory effects between PMCA4b and GPER/GPR30. It has been demonstrated that both PMCA4b and GPER/GPR30 interact with the PSD-95 via their C-terminal PDZ-binding motifs (15, 26, 27, 42). For both proteins, the interaction with PSD-95 was shown to facilitate plasma membrane targeting. Given this background, we hypothesized that the physical and functional interactions between PMCA4b and GPER/GPR30 are mediated in part by the anchoring effect of PSD-95. To test this idea, we first examined the effect of silencing PSD-95 in primary PAECs on the interaction between PMCA and GPER/GPR30. Fig. 9A shows expression of PSD-95 in primary vascular endothelial cells and the effect of PSD-95 gene silencing. A 50% reduction in PSD-95 expression was achieved. PMCA–GPER/GPR30 interaction was then examined in cells transfected with PSD-95 antisense or scrambled oligo using co-immunoprecipitation. An ~45% reduction was observed in the association between PMCA and GPER/GPR30 in cells in which PSD-95 was knocked down (Fig. 9B), indicating a role of PSD-95 in mediating the interaction between GPER/GPR30 and PMCA4b.

**PMCA4b and GPER/GPR30 Interact through their C-terminal PDZ-binding Motifs**—Both PMCA4b and GPER/GPR30 contain in their C terminus a type I PDZ-binding motif, which mediates their respective interaction with PSD-95. To test the idea that PMCA4b and GPER/GPR30 interact in part through these PDZ-binding motifs, we examined the effect of truncation of PMCA4b’s PDZ-binding motif (ETSV) or GPER/GPR30’s (SSAV) on their association. HA-tagged wild-type PMCA4b or HA-tagged PMCA4bΔETSV was co-transfected with FLAG-tagged wild-type GPER/GPR30 or truncated GPER/GPR30ΔSSAV into HEK 293 cells. The transfected PMCA4b was immunoprecipitated using anti-HA antibody, followed by probing of the respective membrane fragments for the HA tag (heterologous PMCA4b) or GPER/GPR30 using anti-FLAG antibody. Immunoblotting of equal amounts of total cell lysate shows that all samples expressed both HA-PMCA variants and FLAG-GPER/GPR30 variants as specified (Fig. 9C). Co-immunoprecipitation from the same samples clearly shows that truncation of the PDZ-binding motifs in GPER/GPR30 and/or PMCA4b significantly reduced their association in cells (Fig. 9, D and E). Interestingly, removal of GPER/GPR30’s PDZ-binding motif apparently has a much more profound effect, virtually abolishing the interaction between PMCA4b and GPER/GPR30 (Fig. 9, D and E).

To examine the functional impact of the interaction between GPER/GPR30 and PMCA4b via their C-terminal PDZ-binding domains, we compared PMCA activity in cells transfected with either the FLAG-GPER/GPR30 or FLAG-GPER/GPR30ΔSSAV. Fig. 10A shows average Ca²⁺ extrusion time courses in mock-transfected cells, cells expressing FLAG-GPER/GPR30, and cells expressing FLAG-GPERΔSSAV. Again, only cells with similar free Ca²⁺ values at the beginning of the extrusion time courses were binned for comparison (“Experimental Procedures”). PMCA activity was significantly reduced by ~40% in cells overexpressing wild-type GPER/GPR30 and PMCA4b.
GPR30. Overexpression of the truncated version FLAG-GPER/GPR30/H9004SSAV significantly reduced the inhibitory effect on PMCA activity compared with the wild-type FLAG-GPER/GPR30 (Fig. 10B), despite a small residual inhibitory effect. These functional data are consistent with the finding that truncation of the C-terminal PDZ-binding motif in GPER/GPR30 substantially reduced the interaction between GPER/GPR30 and PMCA4b (Fig. 9, C–E).

Discussion

In this study, we demonstrate physical and functional interactions between GPER/GPR30 and PMCA4b, a nonreceptor transmembrane protein with well established roles in the control of Ca\(^{2+}\) homeostasis. Our data indicate that GPER/GPR30 can inhibit Ca\(^{2+}\) efflux in the vascular endothelium via the PMCA by two independent mechanisms as follows: 1) PMCA tyrosine phosphorylation that occurs upon GPER/GPR30 activation, and 2) constitutive physical interaction between GPER/GPR30 and PMCA4b. The link between agonist G-1 and GPER/GPR30 was supported by two lines of evidence. First, G-1 promotes ERK1/2 phosphorylation in primary PAECs, and knockdown of GPER/GPR30 drastically reduced this effect (Fig. 4, A and B). Second, G-1 promotes ERK1/2 phosphorylation in HEK 293 cells, which also express GPER/GPR30 mRNA and protein, and overexpression of GPER/GPR30 in these cells substantially increases G-1-induced ERK1/2 phosphorylation (Fig. 4C). These results are consistent with previous studies showing that estrogen induced ERK1/2 phosphorylation via GPER/GPR30 (1, 43).

The observed effect of G-1 to inhibit PMCA activity via tyrosine phosphorylation of the pump is quite consistent with previous studies demonstrating a clear inhibitory role of phosphorylation of Tyr-1176 on PMCA4b activity (34, 44, 45). Activation...
Hetero-oligomeric Complex between GPER/GPR30 and PMCA4b

Our data also provide several lines of evidence to show that GPER/GPR30 and PMCA4b physically interact via their PDZ-binding motifs at the C terminus and a significant role of PSD-95 in facilitating this interaction. These include co-immunoprecipitation in primary PAECs, confocal microscopic evidence for the co-localization of GPER/GPR30 and PMCA4b, and co-immunoprecipitation in HEK 293 cells overexpressing epitope-tagged wild-type or PDZ-binding domain-truncated versions of the two proteins. We also provide functional data to demonstrate that these interactions affect both partners’ activities. Interestingly, the inhibitory effect on PMCA activity due to this physical interaction appears to be constitutive and independent of GPER/GPR30 activation. Thus GPER/GPR30 can clearly affect PMCA activity by two distinct, additive mechanisms.

Knockdown of PMCA resulted in a substantial reduction in GPER/GPR30-mediated ERK1/2 phosphorylation (Fig. 6, A and B), indicating that PMCA-GPER/GPR30 interaction promotes GPER/GPR30 activity. It is noted, however, that the effect of PMCA knockdown on PMCA activity per se was not as pronounced (Fig. 6D). We currently do not have experimental data to explain this observation. It is unlikely for the increased cytoplasmic Ca\textsuperscript{2+} as a result of PMCA knockdown to decrease G-1-induced ERK1/2 phosphorylation, as MAPK functions in most cases are promoted rather than inhibited by cytoplasmic Ca\textsuperscript{2+} signals. Nevertheless, it is not uncommon for trans-membrane proteins and GPCRs to be distributed at different subcellular locales, and speculatively, a small difference in the pools of PMCA participating in Ca\textsuperscript{2+} extrusion and in interaction with GPER/GPR30 might explain this discrepancy. Further studies will be necessary to fully understand this observation.

Our data on the role of PDZ-binding motifs to promote interactions between GPER/GPR30 and PMCA4b are supported by a recent study demonstrating GPER/GPR30 interaction through its C-terminal PDZ-binding motif (SSAV) with the tandem PDZ domains 1 and 2 of PSD-95 in hippocampal tissue (42); this interaction promotes membrane targeting of GPER/GPR30 and increases the possibility of interactions between GPER/GPR30 and other receptors present in the hippocampus. In a different direction, GPER/GPR30 recently was nicely demonstrated to constitutively inhibit adenylyl cyclase-mediated production of cAMP via PDZ domain-mediated interactions with AKAP5 (15). Interactions between GPCRs and nonreceptor transmembrane proteins through hetero-oligomeric complexes are increasingly recognized as an important regulatory input in receptor function (46). The most striking example of this type of interaction is the case of receptor activity-modifying proteins, which are single-pass trans-membrane proteins that interact with and regulate the functions of a number of GPCRs. For GPER/GPR30, RAMP3 was the first nonreceptor membrane protein shown to interact with it and to regulate its subcellular localization and cardioprotective effects (47). The role of PDZ domains in promoting homodimeric or heterodimeric interactions between GPCRs has also been shown, with the example of the endothelin receptors ETA and ETB (48). Our data demonstrate mutual functional impact between GPER/GPR30 and PMCA4b and highlight the importance of C-terminal PDZ-binding motifs in forming such complexes. Through these examples, the roles of C-terminal PDZ-binding motifs in GPER/GPR30 and other GPCRs are being further demonstrated, and it is important to assess the multidirectional functional impact of such associations. This is particularly true considering that PDZ proteins such as PSD-95 contain multiple PDZ domains that could facilitate linkage among multiple partners in the same macromolecular complex.

Implications on Ca\textsuperscript{2+}-dependent Activities—Our data demonstrate a novel mechanism whereby Ca\textsuperscript{2+} efflux via the PMCA can be controlled: direct, constitutive interaction with a GPCR. Recently, a 10-pass transmembrane protein (partner of stromal interaction molecule 1- POST) was identified in complex with Stim1 and shuttles between the endoplasmic reticulum and plasma membrane to interact with the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase and the PMCA; similarly to GPER/GPR30, this interaction also decreases PMCA activity (33). Differently from GPER/GPR30, the interaction between POST and PMCA appears to be dynamic, although GPER/GPR30 seems to constitutively interact with PMCA4b. Clearly, inhibition of PMCA activity by GPER/GPR30 prolongs the time course of a particular Ca\textsuperscript{2+} signal. When considering the potential impact of this effect on the activity of Ca\textsuperscript{2+}-dependent proteins, the Ca\textsuperscript{2+} sensitivity of their activation plays an important role. For example, we have previously reported EC\textsubscript{50}(Ca\textsuperscript{2+}) values of 422 ± 23 and 130 ± 5 nm for the activation of wild-type and doubly phosphorylated eNOS, respectively, in the presence of saturating CaM availability (49). If we take the example of the experiment in Fig. 1D and assume sufficient CaM to saturate eNOS in these cells, when GPER/GPR30 is activated, wild-type and doubly phosphorylated eNOS would still remain 50 and 100% active at the end of the measured time course. However, all else being equal, based on our previous data (49), the same eNOS species would only be <5% and ~20% active, respectively, at the same time point without GPER/GPR30’s inhibitory effect on Ca\textsuperscript{2+} efflux. This inhibitory effect on PMCA activity would therefore definitely enhance nitric oxide accumulation and contribute to the control of vascular tone. This is just an example of the potential impact that the inhibition of Ca\textsuperscript{2+} efflux by GPER/GPR30 could have on Ca\textsuperscript{2+}-dependent activities.

Implications on GPER/GPR30 Studies—This study is the first to show a component of the Ca\textsuperscript{2+} signaling machinery can affect the function of GPER/GPR30. PMCA4b has been shown to affect vascular functions via PDZ domain-mediated interactions. Specifically, PMCA4b interacts with the PDZ domain of neuronal NOS, reducing Ca\textsuperscript{2+} available for the enzyme and leading to a decrease in NO production in the vascular smooth muscle cells (25). This study shows another instance in which PMCA4b potentially affects vascular function via its interaction with GPER/GPR30. The effect of PMCA4b to promote GPER/GPR30-mediated ERK1/2 phosphorylation likely plays a role in the initiation of more long term or genomic outcomes of GPER/GPR30 activation. In addition, GPER/GPR30 agonist G-1 has been shown to acutely trigger Ca\textsuperscript{2+} signals (50, 51). In many
Hetero-oligomeric Complex between GPER/GPR30 and PMCA4b

Sustained Ca\textsuperscript{2+} signals
\uparrow GPER/GPR30-mediated functions

FIGURE 11. Schematic of GPER/GPR30-PSD95-PMCA4b hetero-oligomeric complex and mutual functional impact. PDZ, PDZ domain; SH3, Src homology domain 3; GK, guanylate kinase domain. P, GPER/GPR30 activation-induced tyrosine phosphorylation of PMCA4b.

cases, these Ca\textsuperscript{2+} signals were used as a parameter of receptor activation (2, 3, 16). Nevertheless, the mechanisms of these signals remain unknown. Arguably, the inhibitory effect of GPER/GPR30 on Ca\textsuperscript{2+} efflux can contribute to the acute total Ca\textsuperscript{2+} signals and should be considered when using the total Ca\textsuperscript{2+} mobilization signal as a parameter for receptor activity. Likewise, experimental paradigms that involve a change in PMCA expression or signaling might interfere with GPER/GPR30 signaling. A schematic of our findings is provided in Fig. 11.

Acknowledgment—We thank Dr. Anthony Persechini (University of Missouri–Kansas City) for the gift of the DsRed\textsubscript{2} plasmid.

References
1. Filardo, E. J., Quinn, J. A., Bland, K. L., and Frackelton, A. R., Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol. Endocrinol. 14, 1649–1660
2. Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., and Thomas, P. (2007) Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology 148, 3236–3245
3. Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., and Prossnitz, E. R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307, 1625–1630
4. Prossnitz, E. R., and Barton, M. (2011) The G-protein-coupled estrogen receptor GPER in health and disease. Nutr. Rev. Endocrinol. 7, 715–726
5. Hammond, R., Nelson, D., and Gibb, R. B. (2011) GPR30 co-localizes with cholinergic neurons in the basal forebrain and enhances potassium-stimulated acetylcholine release in the hippocampus. Psychoneuroendocrinology 36, 182–192
6. Yates, M. A., Li, Y., Chlebeck, P. J., and Offner, H. (2010) GPR30, but not estrogen receptor-\(\alpha\), is crucial in the treatment of experimental autoinmune encephalomyelitis by oral ethinyl estradiol. BMC Immunol. 11, 20
7. Wang, C., Dehghani, B., Li, Y., Kaler, L. J., Proctor, T., Vandenbark, A. A., and Offner, H. (2009) Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. J. Immunol. 182, 3294–3303
8. Ullrich, N. D., Krust, A., Collins, P., and MacLeod, K. T. (2008) Genomic deletion of estrogen receptors ER\(\alpha\) and ER\(\beta\) does not alter estrogen-mediated inhibition of Ca\textsuperscript{2+} influx and contraction in murine cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol. 294, H2421–H2427
9. Delbeck, M., Golz, S., Vonk, R., Janssen, W., Hucho, T., Jensee, J., Schäfer, S., and Otto, C. (2011) Impaired left-ventricular cardiac function in male GPR30-deficient mice. Mol. Med. Rep. 4, 37–40
10. Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146, 624–632
11. Kanda, N., and Watanabe, S. (2003) 17\(\beta\)-estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting Bel-2 expression. J. Invest. Dermatol. 121, 1500–1509
12. Maggiolini, M., V gigantic, A., Fasanella, G., Recchia, A. G., Sisci, D., Pezzi, V., Montanaro, D., Musti, A. M., Picard, D., and Andò, S. (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17\(\beta\)-estradiol and phytoestrogens in breast cancer cells. J. Biol. Chem. 279, 27008–27016
13. Albanito, L., Madeo, A., Lappano, R., V gigantic, A., Rago, V., Carmino, A., Oprea, T. I., Prossnitz, E. R., Musti, A. M., Andò, S., and Maggiolini, M. (2007) G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17\(\beta\)-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. Cancer Res. 67, 1859–1866
14. V gigantic, A., Bonò, D., Albanito, L., Madeo, A., Rago, V., Carmino, A., Musti, A. M., Picard, D., Andò, S., and Maggiolini, M. (2006) 17\(\beta\)-Estradiol, genistin, and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the G protein-coupled receptor GPR30. Mol. Pharmacol. 70, 1414–1423
15. Brossel, S., Berg, K. A., Chavera, T. A., Kahn, R., Clarke, W. P., Olde, B., and Leeb-Lundberg, L. M. (2014) G protein-coupled receptor 30 (GPR30) forms a plasma membrane complex with membrane-associated guanylate kinases (MAGUKs) and AKAP5 that constitutively inhibits CAMP production. J. Biol. Chem. 289, 22117–22127
16. Bologna, C. G., Revankar, C. M., Young, S. M., Edwards, B. S., Arterburn, J. B., Kiselyov, A. S., Parker, M. A., Tkachenko, S. E., Savchuck, N. P., Sklar, L. A., Oprea, T. I., and Prossnitz, E. R. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. Nat. Chem. Biol. 2, 207–212
17. Rinaldi, G. J. (2005) Blood pressure fall and increased relaxation of aortic smooth muscle in diabetic rats. Diabetes Metab. 31, 487–495
18. Tran, Q. K., Black, D. J., and Persechini, A. (2003) Intracellular coupling via limiting calmodulin. J. Biol. Chem. 278, 24247–24250
19. Tran, Q. K., Black, D. J., and Persechini, A. (2005) Dominant affectors in the calmodulin network shape the time courses of target responses in the cell. Cell Calcium 37, 541–553
20. Wang, X., Reznick, S., Li, P., Liang, W., and van Breemen, C. (2002) Ca\textsuperscript{2+} removal mechanisms in freshly isolated rabbit aortic endothelial cells. Cell Calcium 31, 265–277
21. Streher, E. L., and Zacharias, D. A. (2001) Role of alternative splicing in the regulation of alternative splicing. J. Biol. Chem. 276, 23351–23357
22. Jarrett, H. W., and Penniston, J. T. (1977) Partial purification of the Ca\textsuperscript{2+} pump is slow and is changed by limiting calmodulin. J. Biol. Chem. 272, 1625–1630
23. Caride, A. J., Elwess, N. L., Verma, A. K., Filoteo, A. G., Enyedi, A., Bajzer, Z., and Penniston, J. T. (1999) The rate of activation by calmodulin of isoform 4 of the plasma membrane Ca\textsuperscript{2+} pump is slow and is changed by additional splicing. J. Biol. Chem. 274, 35227–35232
24. Schuh, K., Uldrijan, S., Telkamp, M., Rothlein, N., and Neyes, L. (2001) The plasma membrane calcium-dependent calcium pump: a major regulator of nitric oxide synthase I. J. Cell Biol. 155, 201–205
25. Schuh, K., Kauczking, T., Knauer, S., Hu, K., Kocak, S., Roethlein, N., and Neyes, L. (2003) Regulation of vascular tone in animals overexpressing the estrogen receptor coupled to a G protein in human breast cancer cells. J. Biol. Chem. 278, 41246–41252
26. Padányi, R., Pászty, K., Streher, E. E., and Enyedi, A. (2009) PSD-95 mediates membrane clustering of the human plasma membrane Ca\textsuperscript{2+} pump isoform 4b. Biochim. Biophys. Acta 1793, 1023–1032
27. Enyedi, A., and Streher, E. E. (2011) Regulation of apical membrane en...
richment and retention of plasma membrane Ca\textsuperscript{2+}-ATPase splice variants by the PDZ-domain protein NHERF2. *Commun. Integr. Biol.* 4, 340–343
28. DeMarco, S. J., and Strehler, E. E. (2001) Plasma membrane Ca\textsuperscript{2+}-ATPase isoforms 2b and 4b interact promiscuously and selectively with members of the membrane-associated guanylate kinase family of PDZ (PSD95/Dlg/ZO-1) domain-containing proteins. *J. Biol. Chem.* 276, 21594–21600
29. Watanebe, H., Takahashi, R., Tran, Q. K., Takeuchi, K., Kosuge, K., Satoh, H., Uehara, A., Terada, H., Hayashi, H., Ohno, R., and Ohashi, K. (1999) Increased cytosolic Ca\textsuperscript{2+} concentration in endothelial cells by calmodulin antagonists. *Biochem. Biophys. Res. Commun.* 265, 697–702
30. Tran, Q. K., and Vermeer, M. (2014) Biosensor-based approach identifies four distinct calmodulin-binding domains in the G protein-coupled estrogen receptor 1. *PLoS One* 9, e89669
31. Sattler, R., Xiong, Z., Lu, W. Y., Hafner, M., MacDonald, J. F., and Tymi-anski, M. (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* 284, 1845–1848
32. Brown, C. S., and Dean, W. L. (2007) Regulation of plasma membrane Ca\textsuperscript{2+}-ATPase in human platelets by calpain. *Platelets* 18, 207–211
33. Krapivinsky, G., Krapivinsky, L., Stotz, S. C., Manasian, Y., and Clapham, D. E. (2011) POST, partner of stromal interaction molecule 1 (STIM1), targets STIM1 to multiple transporters. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19234–19239
34. Dean, W. L., Chen, D., Brandt, P. C., and Vanaman, T. C. (1997) Regulation of platelet plasma membrane Ca\textsuperscript{2+}-ATPase by cAMP-dependent and tyrosine phosphorylation. *J. Biol. Chem.* 272, 15113–15119
35. Wan, T. C., Zabe, M., and Dean, W. L. (2003) Plasma membrane Ca\textsuperscript{2+}-ATPase isoform 4b is phosphorylated on tyrosine 1176 in activated human platelets. *Thromb. Haemost.* 89, 122–131
36. Strehler, E. E., Filoteo, A. G., Penniston, J. T., and Caride, A. J. (2007) Plasma-membrane Ca\textsuperscript{2+} pumps: structural diversity as the basis for functional versatility. *Biochem. Soc. Trans.* 35, 919–922
37. Otto, C., Rohde-Schulz, B., Schwarz, G., Fuchs, I., Klewer, M., Brittian, D., Langer, G., Bader, B., Prellé, K., Nubbemeyer, R., and Fritzemeier, K. H. (2008) G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. *Endocrinology* 149, 4846–4856
38. Pedram, A., Razandi, M., and Levin, E. R. (2006) Nature of functional estrogen receptors at the plasma membrane. *Mol. Endocrinol.* 20, 1996–2009
39. Peyton, C., and Thomas, P. (2011) Involvement of epidermal growth factor receptor signaling in estrogen inhibition of oocyte maturation mediated through the G protein-coupled estrogen receptor (Gper) in Zebrafish (*Danio rerio*). *Bioi Reprod.* 85, 42–50
40. Hart, D., Nilges, M., Pollard, K., Lynn, T., Patsos, O., Shiel, C., Clark, S. M., and Vasudevan, N. (2014) Activation of the G protein-coupled receptor 30 (GPR30) has different effects on anxiety in male and female mice. *Steroids* 81, 49–56
41. Haas, E., Meyer, M. R., Schurr, U., Bhattacharya, L., Minotti, R., Nguyen, H. H., Heigl, A., Lachat, M., Genoni, M., and Barton, M. (2007) Differential effects of 17β-estradiol on function and expression of estrogen receptor α, estrogen receptor β, and GPR30 in arteries and veins of patients with atherosclerosis. *Hypertension* 49, 1358–1363
42. Akama, K. T., Thompson, L. I., Milner, T. A., and McEwen, B. S. (2013) Post-synaptic density-95 (PSD-95) binding capacity of G-protein-coupled receptor 30 (GPR30), an estrogen receptor that can be identified in hippocampal dendritic spines. *J. Biol. Chem.* 288, 6438–6450
43. Ding, Q., Gros, R., Limbird, L. E., Chorazycewski, J., and Fieldman, R. D. (2009) Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30. *Am. J. Physiol. Cell Physiol.* 297, C1178–C1187
44. Dean, W. L. (2010) Role of platelet plasma membrane Ca-ATPase in health and disease. *World J. Biol. Chem.* 1, 265–270
45. Bozulic, L. D., Malik, M. T., and Dean, W. L. (2007) Effects of plasma membrane Ca\textsuperscript{2+}-ATPase tyrosine phosphorylation on human platelet function. *J. Thromb. Haemost.* 5, 1041–1046
46. Ferré, S., Baler, R., Bouvier, M., Caron, M. G., Devi, L. A., Durroux, T., Fuxe, K., George, S. R., Javitch, J. A., Lohse, M. J., Mackie, K., Milligan, G., Pfleger, K. D., Pin, J. P., Volkow, N. D., et al. (2009) Building a new conceptual framework for receptor heteromers. *Nat. Chem. Biol.* 5, 131–134
47. Lemhart, P. M., Broselid, S., Barrick, C. J., Leeb-Lundberg, L. M., and Caron, K. M. (2013) G-protein-coupled receptor 30 interacts with receptor activity-modifying protein 3 and confers sex-dependent cardioprotection. *J. Mol. Endocrinol.* 51, 191–202
48. Evans, N. J., and Walker, J. W. (2008) Sustained Ca\textsuperscript{2+} signaling and delayed internalization associated with endothelin receptor heterodimers linked through a PDZ finger. *Can. J. Physiol. Pharmacol.* 86, 526–535
49. Tran, Q. K., Leonard, J., Black, D. J., Nadeau, O. W., Boulatnikov, I. G., and Persechini, A. (2009) Effects of combined phosphorylation at Ser-617 and Ser-1179 in endothelial nitric-oxide synthase on EC50(Ca\textsuperscript{2+}) values for calmodulin binding and enzyme activation. *J. Biol. Chem.* 284, 11892–11899
50. Sheng, J. Z., Arshad, F., Braun, I. E., and Braun, A. P. (2008) Estrogen and the Ca\textsuperscript{2+}-mobilizing agonist ATP evoke acute NO synthesis via distinct pathways in an individual human vascular endothelium-derived cell. *Am. J. Physiol. Cell Physiol.* 294, C1531–C1541
51. Haas, E., Bhattacharya, L., Brailoiu, E., Damjanović, M., Brailoiu, G. C., Gao, X., Mueller-Guerre, L., Marjon, N. A., Gut, A., Minotti, R., Meyer, M. R., Amann, K., Ammann, E., Perez-Dominguez, A., Genoni, M., et al. (2009) Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circ. Res.* 104, 288–291