Homer2 Protein Regulates Plasma Membrane Ca\(^{2+}\)-ATPase-mediated Ca\(^{2+}\) Signaling in Mouse Parotid Gland Acinar Cells*

From the 4Department of Oral Biology, BK21 PLUS Project, Yonsei University College of Dentistry, Seoul 120-752, Korea, the 3College of Life Sciences and Graduate School of Biotechnology, Kyunghee University, Global Campus, Gyeonggi 446-701, Korea, and the 5Epithelial Signaling and Transport Section, Molecular Physiology and Therapeutics Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892

Background: Homer proteins bind multiple Ca\(^{2+}\)-signaling proteins to shape the Ca\(^{2+}\) signal by poorly understood mechanisms.

Results: Homer2 regulates PMCA expression and activity in parotid acinar cells.

Conclusion: Homer2 acts as a regulator of PMCA-mediated Ca\(^{2+}\) clearance.

Significance: Inhibition of Ca\(^{2+}\) clearance by Homer2 further clarifies its role in Ca\(^{2+}\) signaling.

Homer proteins are scaffold molecules with a domain structure consisting of an N-terminal Ena/VASP homology 1 protein-binding domain and a C-terminal leucine zipper/coiled-coil domain. The Ena/VASP homology 1 domain recognizes proline-rich motifs and binds multiple Ca\(^{2+}\)-signaling proteins, including G protein-coupled receptors, inositol 1,4,5-trisphosphate receptors, ryanodine receptors, and transient receptor potential channels. However, their role in Ca\(^{2+}\) signaling in nonexcitable cells is not well understood. In this study, we investigated the role of Homer2 on Ca\(^{2+}\) signaling in parotid gland acinar cells using Homer2-deficient (Homer2\(^{-/-}\)) mice. Homer2 is localized at the apical pole in acinar cells. Deletion of Homer2 did not affect inositol 1,4,5-trisphosphate receptor localization or channel activity and did not affect the expression and activity of sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase pumps. In contrast, Homer2 deletion markedly increased expression of plasma membrane Ca\(^{2+}\)-ATPase (PMCA) pumps, in particular PMCA4, at the apical pole. Accordingly, Homer2 deficiency increased Ca\(^{2+}\) extrusion by acinar cells. These findings were supported by co-immunoprecipitation of Homer2 and PMCA in wild-type parotid cells and transfected human embryonic kidney 293 (HEK293) cells. We identified a Homer-binding PPX XF-like motif in the N terminus of PMCA that is required for interaction with Homer2. Mutation of the PPX XF-like motif did not affect the interaction of PMCA with Homer1 but inhibited its interaction with Homer2 and increased Ca\(^{2+}\) clearance by PMCA. These findings reveal an important regulation of PMCA by Homer2 that has a central role on PMCA-mediated Ca\(^{2+}\)signaling in parotid acinar cells.

Ca\(^{2+}\) is a common second messenger with roles in fertilization, muscle contraction, neurotransmitter release, exocytosis, learning, and memory. In addition, it regulates critical functions of polarized secretory gland cells (1, 2). Secretory cell Ca\(^{2+}\) signaling is evoked primarily by activation of G protein-coupled receptors (GPCRs), which are coupled to G\(_{q}\) or G\(_{i}\). These cells are an excellent model system to study Ca\(^{2+}\) signaling and the role of scaffolding proteins in the function of various components of the Ca\(^{2+}\) signal, including plasma membrane Ca\(^{2+}\)-ATPase and sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pumps, the inositol 1,4,5-trisphosphate (IP\(_{3}\)) receptors (IP\(_{3}\)Rs), Ca\(^{2+}\) release channels in the endoplasmic reticulum (ER), and Ca\(^{2+}\) influx channels in the plasma membrane (3).

Several GPCRs possess binding motifs that associate with members of the Homer family of scaffolding proteins. The Homer family, which includes Homer1, Homer2, and Homer3, as well as several splice variants, was discovered when homer1a was cloned as an immediate early gene product expressed in neurons upon prolonged stimulation (4–7). With the exception of Homer1a, all Homer proteins (i.e. Homer1b/c, 2, and 3; referred to as “long Homers”) are expressed constitutively throughout the central nervous system (CNS). These proteins are composed of an N-terminal Ena/VASP homology 1 protein-binding domain and a C terminus that folds into a coiled-coil multimerization domain and leucine zipper (7, 8). The Ena/VASP homology 1 domain is a protein-protein binding module that recognizes the proline-rich motifs PPXXF, PPXF, and LPSSP (9–12) and binds to several GPCRs, canonical transient receptor potential channels, IP\(_{3}\)Rs, ryanodine receptors, and the Shank family of scaffolding proteins (10, 12–16). Homer proteins play a central role in Ca\(^{2+}\) signaling via regulation of

---

*This work was authored, in whole or in part, by National Institutes of Health staff. This work was supported by the grant (R01-2012R1A1A1003487) from the Korea government (MSIP).

To whom correspondence should be addressed: Dept. of Oral Biology, Yonsei University College of Dentistry, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, Korea. Tel.: 82-2-2228-3051; Fax: 82-2-364-1085; E-mail: dmshin@yuhs.ac.
Regulation of PMCA-mediated Ca\(^{2+}\) Signaling by Homer2

neuronal transcription activity and thereby regulate dendritic spine morphogenesis, synapse remodeling, and synaptic clustering of CNS neurons (9, 17–19). Previous research found that Homer1 regulates Ca\(^{2+}\) influx by associating IP\(_3\)Rs with transient receptor potential channels (12). Homer2 tunes GPCR stimulus intensity by regulating the regulator of G protein signaling proteins and phospholipase C\(\beta\)-promoting guanosine triphosphatase by G\(\alpha\) in pancreatic acinar cells. Moreover, Homer2 and Homer3 bind nuclear factor of activated T cells by competing with calcineurin in T lymphocytes (14, 20). However, the role of Homer proteins in Ca\(^{2+}\) signaling in nonexcitable cells remains poorly characterized.

In this study, the role of Homer2 on Ca\(^{2+}\) signaling in parotid gland acinar cells was investigated using Homer2 knock-out (Homer2\(^{-/-}\)) mice. We report that Homer2 interacts with PMCA (particularly PMCA isoform 4) in model systems and findings suggest a mechanism by which Homer proteins can regulate PMCA expression and PMCA-mediated Ca\(^{2+}\) efflux in parotid acinar cells. We suggest that by inhibiting transient receptor potential channel-mediated Ca\(^{2+}\) influx (12) and by differentially modulating Ca\(^{2+}\) extrusion by PMCA (present data), the Homers serve to protect the cells from Ca\(^{2+}\) toxicity by facilitating cytosolic Ca\(^{2+}\) clearance to limit the Ca\(^{2+}\) signal duration.

EXPERIMENTAL PROCEDURES

Antibodies and DNA Constructs—Anti-Homer1a (M-13), anti-Homer1 (D-3), anti-Homer2, and anti-PMCA (5F10) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-IP\(_3\)R3 antibodies were purchased from Cell Line Bank, South Korea) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 100 units/ml penicillin and streptomycin in a 5% CO\(_2\) incubator. Approximately 1–5 \(\times\) 10\(^5\) cells were seeded onto coverslips in 60-mm dishes and then incubated in antibiotic-free medium. The next day, DNA was mixed with Lipofectamine reagent (Invitrogen) and OptimEM, incubated for 20 min at room temperature, and then added to the cell culture media. The transfected cells were assayed at 38–48 h after post-transfection.

Measurement of Intracellular Ca\(^{2+}\) Concentration ([Ca\(^{2+}\)]\(_i\))—Parotid acinar cells from WT and Homer2\(^{-/-}\) mice were loaded with 5 \(\mu\)M Fura-2/AM (Teflabs Inc., Austin, TX) and 0.05% pluronic F-127 for 60 min in physiologic salt solution. Fura-2 fluorescence was measured at the appropriate excitation wavelengths (340/380 nm) and emission at 510 nm wavelengths (ratio = \(F_{340}/F_{380}\)) using a Molecular Devices (Downingtown, PA) imaging system. The emitted fluorescence was monitored with a charge-coupled device camera (Photometrics, Tucson, AZ) attached to an inverted microscope. Fluorescence images were obtained at 2-s intervals. All data were analyzed using MetaFluor software (Molecular Devices).

Immunocytochemistry—The immunostaining procedure was described previously (14). In brief, parotid acinar cells from WT and Homer2\(^{-/-}\) mice were stained with antibodies against Homer2 (1:50); IP\(_3\)R1, -2, or -3 (1:100); SERCA2, PMCA, or PMCA4 (1:200). Staining was detected using goat anti-rabbit or anti-mouse IgG conjugated to fluorescein or rhodamine. Images were collected with confocal LSM 510 and LSM 710 laser scanning microscopes (Zeiss, Göttingen, Germany).

Western Blotting—Protein extracts were prepared from parotid, submandibular gland, and pancreatic acini from WT and Homer2\(^{-/-}\) mice. Acinar cells were lysed in a buffer containing 150 mM NaCl, 10 mM Tris (pH 7.8 with HCl), 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and protease inhibitors (i.e. 2 mM Na\(_2\)VO\(_4\), 10 mM NaF, 10 \(\mu\)g/ml aprotonin, 10 \(\mu\)g/ml leupeptin, and 10 \(\mu\)g/ml PMSF). The extracts were spun at 13,000 \(\times\) g for 15 min, and protein concentration was measured using a Bio-Rad protein assay. The samples (50 \(\mu\)g of protein/well) were separated to 6–8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, blocked with 5% skimmed milk, and incubated overnight with the specific primary antibody, an anti-SERCA2b (1:500), anti-PMCA4 (1:9, 1:500), and anti-PMCA (5F10, 1:500), at 4 °C. The blots were exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h and detected by chemiluminescence (Amer sham Biosciences).

Immunoprecipitation—The immunoprecipitation procedure was modified from Shin et al. (22) and Kim et al. (23). In brief, parotid and HEK293 microsomes were prepared by homogenizing a minced parotid and harvested HEK293 in a buffer containing 20 mM Mops (pH 6.7 with KOH), 250 mM sucrose, 1 mM EDTA, 1 mM MgCl\(_2\), 10 mM benzamidine, and 0.2 mM PMSF. The homogenized samples were centrifuged at 400 \(\times\) g for 10 min. The supernatants were collected and centrifuged at 900 \(\times\) g for 10 min at 4 °C. To avoid protein degradation by digestive enzymes, immunoprecipitation was initiated immediately after completion of microsomal preparation. Microsomes were lysed in a buffer containing 50 mM Tris (pH 6.8 with HCl), 150 mM NaCl, 3 mM EDTA, 2 mM EGTA, and 0.5% Triton X-100 supplemented with protease inhibitors. The lysates were cleared by centrifugation at 14,000 \(\times\) g for 15 min. About 150 \(\mu\)l of the extract (300 \(\mu\)g of protein/sample) was
Regulation of PMCA-mediated Ca\(^{2+}\) Signaling by Homer2

incubated with 10 µl of anti-PMCA (5F10) or 10 µl of anti-FLAG antibodies for 2 h by rocking at 4 °C. Protein A/G-agarose (Thermo Fisher Scientific Inc., Waltham, MA) was added to each mixture, and rocking was continued overnight at 4 °C. Protein A/G-agarose was pelleted at 1,000 × g for 10 s, and the beads were quickly washed with cold PBS. The immunoprecipitated proteins were separated by SDS-PAGE and probed with anti-FLAG (M2, 1:1,000) by overnight incubation at 4 °C. Protein A/G-agarose was pelleted at 1,000 × g for 10 s, and the beads were quickly washed with cold PBS. The immunoprecipitated proteins were separated by SDS-PAGE and probed with anti-FLAG (M2, 1:1,000) by overnight incubation at 4 °C.

Measurement of [Ca\(^{2+}\)] Eflux—To measure directly the rate of Ca\(^{2+}\) efflux by PMCA, we measured the appearance of Ca\(^{2+}\) in the external medium using the procedure published by Zhao et al. (24) with slight modifications. Intact parotid acini from WT and Homer2\(^{-/-}\) mice were permeabilized with SLO and allowed to reduce [Ca\(^{2+}\)] in the incubation media to approximately 75 nM. Next, Ca\(^{2+}\) release was measured by adding increasing concentrations of IP3 (solid arrows) and 1 mM carbachol (dotted arrows). C, quantification of the results shown in B. D, parotid acini were stained for SERCA2b, total PMCA, and PMCA isoform 4 (PMCA4). Expression of PMCA increased in the apical membrane of Homer2\(^{-/-}\) cells. Data are presented as the mean ± S.E.

RESULTS

Homer2 Deletion Does Not Affect the Polarized Expression of IP\(_3\)Rs, IP\(_3\)-mediated Ca\(^{2+}\) Release, or SERCA Activity—Comparison of the receptor-evoked Ca\(^{2+}\) signaling in wild-type (WT) and Homer2\(^{-/-}\) acini revealed the altered signaling in the Homer2\(^{-/-}\) acini. To further examine this phenomenon, we analyzed the expression and activity of key Ca\(^{2+}\) transporters. IP\(_3\)Rs are established binding partners of Homer proteins (10, 12). Therefore, we first examined the localization and expression of Homer2 and IP\(_3\)Rs in parotid acinar cells from WT and Homer2\(^{-/-}\) mice. In WT parotid acinar cells, Homer2, and IP\(_3\)Rs, positive staining was primarily observed in the apical pole. As expected, Homer2 staining was not detected in Homer2\(^{-/-}\) acini, and the expression and localization of all IP\(_3\)R isoforms remained unchanged in Homer2\(^{-/-}\) acini (Fig. 1A).

We next examined whether Homer2 deletion affects Ca\(^{2+}\) uptake into the ER, as well as the activity of IP\(_3\)Rs and their response to IP\(_3\). Parotid acinar cells from WT and Homer2\(^{-/-}\) mice were permeabilized with SLO within 10–15 s, and the [Ca\(^{2+}\)] in the incubation media was reduced to 50–80 nM within 2 min at 37 °C by SERCA-mediated Ca\(^{2+}\) uptake into the IP\(_3\)-mobilizable pool, similar to the method used with pancre-
Regulation of PMCA-mediated Ca^{2+} Signaling by Homer2

The rate and extent of Ca^{2+} uptake into the ER was similar in WT and Homer2^{-/-} cells, providing the first indication that, unlike in the pancreas (14), Homer2 deletion did not affect parotid acinar cell SERCA activity. The addition of increasing concentrations of IP3 and the muscarinic agonist carbachol resulted in a similar increase in [Ca^{2+}] due to

FIGURE 2. Expression of Ca^{2+} pumps in WT and Homer2^{-/-} cells. A, total PMCA and SERCA2b levels were analyzed by Western blot prepared from parotid, submandibular gland, and pancreatic acini from four WT and four Homer2^{-/-} mice. Band intensity was analyzed by densitometry. B, quantitation of Western blot data in A. C, PMCA4 expression increased in the parotid of Homer2^{-/-} mice. Quantitation is shown in the bar graph. Data were normalized to expression levels in WT cells and are presented as the mean ± S.E. *, p < 0.05; ***, p < 0.001 (compared with WT).

FIGURE 3. Characterization of Ca^{2+} signaling in WT and Homer2^{-/-} cells. A, parotid acini from WT and Homer2^{-/-} mice were stimulated with 1 mM carbachol while inhibiting SERCA with 100 μM CPA to elevate [Ca^{2+}], and prevent Ca^{2+} uptake by the ER. When [Ca^{2+}], stabilized at a steady state representing a balance of Ca^{2+} efflux and influx, Ca^{2+} clearance was initiated by removing external Ca^{2+} and adding of 10 μM atropine. B, average rate of Ca^{2+} clearance was determined from the slope of [Ca^{2+}] decline as a measure of PMCA activity. Results were normalized to the slope obtained from WT cells and are presented as the mean ± S.E. The rate of Ca^{2+} clearance increased significantly in Homer2^{-/-} cells. C, WT and Homer2^{-/-} parotid acinar cells in lightly Ca^{2+}-buffered media were exposed to 1 mM carbachol while measuring [Ca^{2+}]. Homer2^{-/-} cells exhibited an increased rate of [Ca^{2+}]_o, indicating a higher rate of PMCA in Homer2^{-/-} than cells from WT mice. D, quantitation of multiple experiments similar to those shown in C. The average rate of increased [Ca^{2+}])_o was significantly higher in Homer2^{-/-} cells. Data are depicted as the mean ± S.E. *, p < 0.05; **, p < 0.01 (compared with WT).
Ca\(^{2+}\) release from stores of SLO-permeabilized WT and Homer2\(^{-/-}\) cells (Fig. 1, B and C). Thus, no compensatory effects in expression, localization, and IP\(_3\)R activity were observed in Homer2\(^{-/-}\) mice.

**PMCA Expression Is Selectively Increased in Specific Tissues from Homer2\(^{-/-}\) Mice**—Next, we examined the expression of the SERCA and PMCA pumps. Because both PMCA1 and PMCA4 are expressed in salivary gland cells, we used a pan anti-PMCA antibody, 5F10, which detects both isoforms, to determine PMCA expression. To verify this PMCA expression, we used the anti-PMCA antibody JA9, which is specific for PMCA4 (14, 24). Notably, immunostaining experiments suggested increased PMCA expression in the apical region of Homer2\(^{-/-}\) parotid acinar cells, whereas the SERCA2 expression remained unaffected in these mice (Fig. 1D). To further analyze how Ca\(^{2+}\) pump expression was affected by Homer2 deletion, we examined protein expression using Western blotting analyses and parotid membranes prepared from WT and Homer2\(^{-/-}\) parotid acinar cells. As shown in Fig. 2, A and B, PMCA expression in parotid acinar cells from Homer2\(^{-/-}\) mice increased significantly to 2.5 \pm 0.1-fold greater than parotid acinar cells from WT mice (n = 4, p < 0.001). However, the expression of SERCA2b (1.2 \pm 0.2-fold of WT, n = 4) remained unchanged in the parotid membranes. Similar results were obtained with submandibular gland membranes. Interestingly, however, opposite results were observed in pancreas membranes, suggesting a tissue-specific adaptive response to Homer2 deletion. Furthermore, the protein levels of the PMCA4 isoform were higher in Homer2\(^{-/-}\) parotid membranes compared with WT (3.7 \pm 0.6-fold of WT, n = 4, p < 0.05, Fig. 2C).

**Rate of [Ca\(^{2+}\)]\(_{\text{efflux}}\) Is Increased in Homer2\(^{-/-}\) Parotid Cells**—The major routes for Ca\(^{2+}\) clearance in nonexcitable cells, such as parotid acinar cells, are Ca\(^{2+}\) uptake into the ER by the SERCA pumps and Ca\(^{2+}\) efflux across the plasma membrane by PMCA (25–27). The results shown in Figs. 1B and 2 suggest that Homer2 deletion does not affect SERCA expression and activity but increases the PMCA expression. To determine whether increased PMCA protein expression translates to increased PMCA activity in intact cells, we examined PMCA-mediated Ca\(^{2+}\) clearance in WT and Homer2\(^{-/-}\) parotid acini. In the first protocol, cells were stimulated with a high concentration of carbachol and treated with the SERCA inhibitor CPA to release ER Ca\(^{2+}\) and maximally activate Ca\(^{2+}\) influx to cause a large increase in cytoplasmic Ca\(^{2+}\). Ca\(^{2+}\) clearance was then initiated by terminating cell stimulation with atropine while simultaneously inhibiting SERCA activity. Fig. 3A shows that the addition of 10 \textmu M atropine in a Ca\(^{2+}\)-free solution resulted in an immediate clearance of Ca\(^{2+}\) primarily by PMCA (Fig. 3A). Comparing the slope of Ca\(^{2+}\) clearance revealed that Ca\(^{2+}\) clearance in Homer2\(^{-/-}\) cells is 1.5-fold faster compared with WT cells (1.5 \pm 0.3-fold, n = 4, p < 0.05, Fig. 3B). In a second protocol, we assayed PMCA activity by measuring the change in [Ca\(^{2+}\)]\(_i\) in cells incubated in media with low external Ca\(^{2+}\) concentration and stimulated with 1 mM carbachol. As shown in Fig. 3C, [Ca\(^{2+}\)]\(_i\) increased significantly in response to carbachol stimulation in both cell types. Importantly, the change in

![FIGURE 4. Interaction of Homer proteins with the PPXXF-like motif and PDZ-binding domain of PMCA]
Regulation of PMCA-mediated Ca\(^{2+}\) Signaling by Homer2
Regulation of PMCA-mediated Ca\textsuperscript{2+} Signaling by Homer2

A previous study suggests that exogenous expression of Homer2 affects PMCA activity. Overexpression of Homer2 in HEK293 cells increased native PMCA activity, whereas overexpression of Homer1 or Homer2, had no effect (Fig. 5A). These findings suggest that the level of endogenous long Homers is saturating with respect to PMCA in HEK293 cells, and thus further expression had no inhibitory effect. Accordingly, Homer1 likely relieved the tonic inhibition by the long Homers to activate the native PMCA activity.

To determine whether these mutants interact with other Homer proteins, we searched for a PPXXF-like motif in PMCA that may interact with the Ena/VASP homology 1 domain of Homer proteins (16). The only such potential motif is present in the N-terminal 91–98 residues of several PMCA subtypes; however, proline and phenylalanine are separated by more than two residues (Fig. 4A). To determine whether this region functions as a Homer-binding motif to mediate the PMCA–Homer2 interaction, we first examined whether Homer2 selectively binds PMCA in parotid acinar cells from WT mice using co-immunoprecipitation assays. As shown in Fig. 4B, PMCA co-immunoprecipitates with endogenous Homer2 suggesting that endogenous Homer2 associates with PMCA.

PMCA isoforms 2b and 4b have PDZ domain-binding ligands in their C terminus that interact with various scaffolding proteins such as membrane-associated guanylate kinase, NHERF2 (Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor-2), NOS-1 (nitric-oxide synthase 1), and Homer1 (Ania-3) (28–31), some of which interact with the Homers. Therefore, we examined whether mutation of the PDZ ligand of PMCA4 (marked Del_PMCA4) affected the interaction of Homers with PMCA.

Co-expression of Homers and Del_PMCA4 showed reduced interaction of Homer1a and Homer1c with Del_PMCA4 compared with their interaction with WT PMCA4. However, interaction of Homer2 was unaffected (Fig. 4C). These results indicate that the PDZ ligand of PMCA4b may function to mediate Homer1 variant interaction with PMCA.

A previous study suggests that exogenous expression of Homer1a, Homer1c, or Homer2a had no effect on endogenous PMCA function, whereas knockdown of Homer1 slowed PMCA-mediated Ca\textsuperscript{2+} clearance in neuronal cells (44). We re-examined these effects in nonexcitable cells by expressing the Homers in HEK293 cells and measured native PMCA activity. Transfection of Homer1a enhanced native PMCA activity, whereas transfection of the long Homers, Homer1c or Homer2, had no effect (Fig. 5A). These findings suggest that the level of endogenous long Homers is saturating with respect to PMCA in HEK293 cells, and thus further expression had no inhibitory effect. Accordingly, Homer1 likely relieved the tonic inhibition by the long Homers to activate the native PMCA activity.

Therefore, to examine the relationship between the Homer-binding motif and PMCA activity, we measured the effect of Homer proteins on Ca\textsuperscript{2+} extrusion by expressed WT and mutant PMCA pumps. Overexpression of WT PMCA pumps increased Ca\textsuperscript{2+} clearance, and expression of the PPXXF-like mutants additionally accelerated Ca\textsuperscript{2+} clearance (Fig. 5, B and E). Co-transfection of Homer2 and WT PMCA pumps significantly slowed Ca\textsuperscript{2+} clearance. This inhibitory effect was abolished when Homer2 was co-expressed with the PMCA mutants (Fig. 5, C and E). However, Homer1a did not increase PMCA activity when cells were co-transfected with WT and mutant PMCA pumps compared with the overexpression of Homer1a or WT and mutant PMCA pumps (Fig. 5, D and E). These results indicate that Homer2 interaction with the PPXXF-like motif on PMCA is essential to regulate Ca\textsuperscript{2+} clearance and Ca\textsuperscript{2+} signaling in nonexcitable cells.

DISCUSSION

In this study, we demonstrate a novel interaction between Homer2 and PMCA in native parotid acinar cells. Furthermore, we provide evidence in support of a critical role for Homer2 in modulating PMCA activity and thus Ca\textsuperscript{2+} signaling. Previous reports that focused on the molecular structure of Homer proteins in the CNS indicated that these proteins bind GPCRs, such as mGluR1/5, and IP\textsubscript{3}Rs, as well as act as scaffolding proteins for assembling Ca\textsuperscript{2+}-signaling complexes in cellular microdomains (8–10, 13, 32). The apical region of exocrine cells is equivalent to the CNS synaptic region, where signaling complexes are clustered to form a “trigger zone” from which all forms of Ca\textsuperscript{2+} signals, including Ca\textsuperscript{2+} waves, are initiated (33, 34). Consistently, immunocytochemical studies have demonstrated that all IP\textsubscript{3}R types are highly enriched in the apical region (14, 35, 36). Accordingly, we found that Homer2 and IP\textsubscript{3}Rs are expressed in the apical pole of parotid acinar cells. However, Homer2 deletion had no effect on the expression or function of any of the IP\textsubscript{3}R isoforms, suggesting that Homer2 may have another role on Ca\textsuperscript{2+} signaling in parotid acinar cells.

Further analyses revealed that Homer2 affected PMCA expression in the apical membranes of parotid acinar cells. Hence, the most interesting finding from our study is the adaptive increase in PMCA protein expression in Homer2-deficient parotid and submandibular gland acinar cells. Like SERCA, the PMCAcs have a crucial role in maintaining Ca\textsuperscript{2+} homeostasis.

![Figure 5. Effects of the Homers on Ca\textsuperscript{2+} clearance by PMCA in HEK293 cells.](http://example.com/figure5)
Regulation of PMCA-mediated \( \text{Ca}^{2+} \) Signaling by Homer2

(37). Previous studies suggested that PMCAs are expressed in both the lateral and apical regions of pancreatic, submandibular gland, and parotid cells, as well as in the brain (31, 38–41). In addition, PMCAs co-localize with mGluR1, IP\(_3\)R1, and Homer proteins, including Homer1a, Homer1b/c, and Homer3, in neurons (31, 40, 41). Here, we discovered that Homer2 interacts with PMCA and decreases the rate of \([\text{Ca}^{2+}]_i\) clearance in native parotid acinar cells, which is absent in Homer2-deficient mice. These results are similar to previous findings showing that native PMCA was increased by overexpression of short Homer1a, Homer1b/c, and Homer3, in neurons (31, 40, 41). However, unlike a previous report, the \( \text{Ca}^{2+} \) clearance rate by native PMCA was increased by overexpression of short Homer1a but not by overexpression of long Homers (Fig. 5A) (44). These experiments suggest that the level of long Homers in HEK293 cells (and perhaps other nonexcitable cells) is saturating with respect to PMCA, and thus additional expression of the long Homers caused no further inhibition. A prediction of this interpretation is the deletion of the long Homers, and their inhibition by the short Homer1a should accelerate PMCA activity and \( \text{Ca}^{2+} \) clearance. This was indeed the case, in which deletion of Homer2 in mice or expression of Homer1a in HEK293 cells increased the native PMCA activity.

In summary, the key findings of the our study are as follows: 1) Homer2 binding to the PPXXF-like motif of PMCAs inhibited PMCA activity, and 2) expression and binding of Homer1 to the PDZ domain of PMCAs increased their activity. Thus, PMCAs undergo dual regulation by Homer proteins, i.e., inhibition by the Homer2 and stimulation by Homer1. In this manner, Homer proteins can regulate the duration of the \( \text{Ca}^{2+} \) signal in parotid acinar cells to either extend or shorten the signal by inhibition or stimulation of PMCA activity, respectively.

Acknowledgments—We thank Dr. Paul F. Worley for sharing the Homer2 \(^{-/-} \) mice and Boryung Park for excellent technical support for the immunoprecipitation experiment.

REFERENCES

1. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. 1, 11–21
2. Petersen, O. H., and Tepikin, A. V. (2008) Polarized calcium signalling in exocrine gland cells. Annu. Rev. Physiol. 70, 273–299
3. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat. Rev. Mol. Cell Biol. 4, 517–529
4. Fagni, L., Chavis, P., Ango, F., and Bockaert, J. (2000) Complex interactions between mGluRs, intracellular \( \text{Ca}^{2+} \) stores and ion channels in neurons. Trends Neurosci. 23, 80–88
5. Szumlinski, K. K., Kalivas, P. W., and Worley, P. F. (2006) Homer proteins: implications for neuropsychiatric disorders. Curr. Opin. Neurobiol. 16, 251–257
6. Kiselkov, Y., Shin, D. M., and Mulleim, S. (2003) Signalling specificity in GPCR-dependent \( \text{Ca}^{2+} \) signalling. Cell. Signal. 15, 243–253
7. Worley, P. F., Zeng, W., Huang, G., Kim, J. Y., Shin, D. M., Kim, M. S., Yuan, J. P., Kiselkov, Y., and Mulleim, S. (2007) Homer proteins in \( \text{Ca}^{2+} \) signaling by excitable and non-excitible cells. Cell Calcium 42, 363–371
8. Fagni, L., Worley, P. F., and Ango, F. (2002) Homer as both a scaffold and transduction molecule. Sci. STKE 2002, re8
9. Kato, A., Ozawa, F., Saitoh, Y., Fukazawa, Y., Sugiyama, H., and Inokuchi, K. (1998) Novel members of the Vesl/Homer family of PDZ proteins that bind metabotrophic glutamate receptors. J. Biol. Chem. 273, 23969–23975
10. Tu, J. C., Xiao, B., Yuan, J. P., Lanahan, A. A., Leoffert, K., Li, M., Linden, D. J., and Worley, P. F. (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP\(_3\) receptors. Neuron 21, 717–726
11. Brakeman, P. R., Lanahan, A. A., O’Brien, R., Roche, K., Barnes, C. A., Huganir, R. L., and Worley, P. F. (1997) Homer: a protein that selectively binds metabotrophic glutamate receptors. Nature 386, 284–288
12. Yuan, J. P., Kiselkov, Y., Shin, D. M., Chen, J., Scheynikov, N., Kang, S. H., Dehoff, M. H., Schwarz, M. K., Seeburg, P. H., Mulleim, S., and Worley, P. F. (2003) Homer binds TRPC family channels and is required for gating of TRPC1 by IP\(_3\) receptors. Cell 114, 777–789
13. Xiao, B., Tu, J. C., Petralia, R. S., Yuan, J. P., Doan, A., Breder, C. D., Ruggiero, A., Lanahan, A. A., Wenthold, R. J., and Worley, P. F. (1998) Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins.
Regulation of PMCA-mediated Ca\(^{2+}\) Signaling by Homer2

Zhao, X. S., Shin, D. M., Luo, X., Kang, S. H., Tu, J., Nayak, S. K., Ross, E. M., Worley, P. F., and Muallem, S. (2003) Homer 2 tunes G protein-coupled receptors stimulus intensity by regulating RGS proteins and PLC\(\beta\) GAP activities. J. Cell Biol. 162, 293–303

Feng, W., Tu, J., Yang, T., Vernon, P. S., Allen, P. D., Worley, P. F., and Pessah, I. N. (2002) Homer regulates gain of ryanodine receptor type 1 channel complex. J. Biol. Chem. 277, 44722–44730

Tu, J. C., Xiao, B., Naisbitt, S., Yuan, J. P., Petralia, R. S., Brakenp, P., Doan, A., Aakalu, V. K., Lanahan, A. A., Sheng, M., and Worley, P. F. (1999) Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron 23, 583–592

Sala, C., Roussignol, G., Meldolesi, J., and Fagni, L. (2005) Key role of the postsynaptic density scaffold proteins Shank and Homer in the functional architecture of Ca\(^{2+}\) homeostasis at dendritic spines in hippocampal neurons. J. Neurosci. 25, 4587–4592

Gray, N. W., Fourgeaud, L., Huang, B., Chen, J., Cao, H., Oswald, B. J., Hémar, A., and McNiven, M. A. (2003) Dynamin 3 is a component of the postsynapse, where it interacts with mGluR5 and Homer. Curr. Biol. 13, 510–515

Bottai, D., Guzowski, J. F., Schwarz, M. K., Kang, S. H., Xiao, B., Lanahan, A., Worley, P. F., and Seeburg, P. H. (2002) Dynactin activity-induced conversion of intrinsic to exonic sequence in Homer 1 immediate early gene expression. J. Neurosci. 22, 167–175

Huang, G. N., Huso, D. L., Bouyain, S., Tu, J., McCorkell, K. A., May, M. J., Zhu, Y., Lutz, M., Collins, S., Dehoff, M., Kang, S., Whartenby, K., Powell, J., Leahy, D., and Worley, P. F. (2008) NFAT binding and regulation of T cell activation by the cytoplasmic scaffolding Homer proteins. Science 319, 476 –481

Zeng, W., Lee, M. G., Yan, M., Diaz, J., Benjamin, I., Marino, C., Kopito, R., Freedman, S., Cotton, C., Mullem, S., and Thomas, P. (1997) Immuno and functional characterization of CFTR in submandibular and pancreatic acinar and duct cells. Am. J. Physiol. 273, C442–C455

Shin, D. M., Zhao, X. S., Zeng, W., Mozhayeva, M., and Mullem, S. (2000) The mammalian Sec6/8 complex interacts with Ca\(^{2+}\) signaling complexes and regulates their activity. J. Cell Biol. 150, 1101–1112

Kim, J. Y., Zeng, W., Kiselyov, K., Yuan, J. P., Dehoff, M. H., Mikoshiba, K., Worley, P. F., and Muallem, S. (2006) Homer 1 mediates store- and inositol 1,4,5-trisphosphate receptor-dependent translocation and retrieval of TRPC3 to the plasma membrane. J. Biol. Chem. 281, 32540–32549

Zhao, X. S., Shin, D. M., Liu, L. H., Shull, G. E., and Mullem, S. (2001) Plasticity and adaptation of Ca\(^{2+}\) signaling and Ca\(^{2+}\)-dependent exocytosis in SERCA2\(^{–/–}\) mice. EMBO J. 20, 2680–2689

Bruce, J. I., Yule, D. I., and Shuttleworth, T. J. (2002) Ca\(^{2+}\)-dependent protein kinase–a modulation of the plasma membrane Ca\(^{2+}\)-ATPase in parotid acinar cells. J. Biol. Chem. 277, 48172–48181

Homann, V., Kinne-Saffran, E., Arnold, W. H., Gaengler, P., and Kinne, R. K. (2006) Calcium transport in human salivary glands: a proposed model of calcium secretion into saliva. Histochem. Cell Biol. 125, 583–591

Gorr, S. U., Venkatesh, S. G., and Darling, D. S. (2005) Parotid secretory granules: crossroads of secretory pathways and protein storage. J. Dent. Res. 84, 500–509

DeMarco, S. J., and Strehler, E. E. (2001) Plasma membrane Ca\(^{2+}\)-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

DeMarco, S. J., Chicka, M. C., and Strehler, E. E. (2002) Plasma membrane Ca\(^{2+}\)-ATPase isoform 2b interacts preferentially with Na\(^+\)/H\(^+\) exchanger regulatory factor 2 in apical plasma membranes. J. Biol. Chem. 277, 10506–10511

Schuh, K., Uldrijian, S., Telkamp, M., Rothlein, N., and Neyes, L. (2001) The plasma membrane calmodulin-dependent calcium pump: a major regulator of nitric oxide synthase I. J. Cell Biol. 155, 201–205

Spambato-Faure, V., Xiong, Y., Berke, J. D., Hyman, S. E., and Strehler, E. E. (2006) The Homer-1 protein Ania-3 interacts with the plasma membrane calcium pump. Biochem. Biophys. Res. Commun. 343, 630–637

Kammermeier, P. J., Xiao, B., Tu, J. C., Worley, P. F., and Ikeda, S. R. (2000) Homer proteins regulate coupling of group I metabotropic glutamate receptors to N-type calcium and M-type potassium channels. J. Neurosci. 20, 7238–7245

Lee, M. G., Xu, X., Zeng, W., Diaz, J., Wojcikiewicz, R. J., Kuo, T. H., Wuytack, F., Racymaekers, L., and Muallem, S. (1997) Polarized expression of Ca\(^{2+}\) channels in pancreatic and salivary gland cells. Correlation with initiation and propagation of [Ca\(^{2+}\)]\(^{\text{waves}}\). J. Biol. Chem. 272, 15765–15770

Tojo, Y., Tanimura, A., and Matsumoto, Y. (1997) Imaging of intracellular Ca\(^{2+}\) waves induced by muscarinic receptor stimulation in rat parotid acinar cells. Cell Calcium 22, 455–462

Zhang, X., Wen, J., Bidasee, K. R.,Besch, H. R., Jr., Wojcikiewicz, R. J., Lee, B., and Rubin, R. P. (1999) Ryandine and inositol trisphosphate receptors are differentially distributed and expressed in rat parotid gland. Biochem. J. 340, 519–527

Takemura, H., Yamashina, S., and Segawa, A. (1999) Millisecond analyses of Ca\(^{2+}\) site invocations evoked by muscarinic receptor stimulation in exocrine acinar cells. Biochem. Biophys. Res. Commun. 259, 656–660

Floyd, R., and Wray, S. (2007) Calcium transporters and signalling in smooth muscles. Cell Calcium 42, 467–476

Baggaley, E., McLaren, S., Demeter, I., Varga, G., and Bruce, J. I. (2007) Differential regulation of the apical plasma membrane Ca\(^{2+}\)-ATPase by protein kinase A in parotid acinar cells. J. Biol. Chem. 282, 37678–37693

Lee, M. G., Xu, X., Zeng, W., Diaz, J., Kuo, T. H., Wuytack, F., Racymaekers, L., and Muallem, S. (1997) Polarized expression of Ca\(^{2+}\) pumps in pancreatic and salivary gland cells. Role in initiation and propagation of [Ca\(^{2+}\)]\(^{\text{waves}}\). J. Biol. Chem. 272, 15771–15776

Kurnellas, M. P., Lee, A. K., Li, H., Deng, L., Ehrlich, D. J., and Elkahab, S. (2007) Molecular alterations in the cerebellum of the plasma membrane calcium ATPase 2 (PMCA2)-null mouse indicate abnormalities in Purkinje neurons. Mol. Cell. Neurosci. 34, 178–188

Sandonà, D., Scilarì, A., Mikoshiba, K., and Volpe, P. (2003) Subcellular distribution of Homer 1b/c in relation to endoplasmic reticulum and plasma membrane proteins in Purkinje neurons. Neurochem. Res. 28, 1151–1158

Genazzani, A. A., Carafoli, E., and Guerini, D. (1999) Calcineurin controls inositol 1,4,5-trisphosphate type 1 receptor expression in neurons. Proc. Natl. Acad. Sci. U.S.A. 96, 5797–5801

Guerini, D., García-Martin, E., Gerber, A., Volbracht, C., Leist, M., Merino, C. G., and Carafoli, E. (1999) The expression of plasma membrane Ca\(^{2+}\) pump isoforms in cerebellar granule neurons is modulated by Ca\(^{2+}\). J. Biol. Chem. 274, 1667–1676

Salm, E. J., and Thayer, S. A. (2012) Homer proteins accelerate Ca\(^{2+}\) clearance mediated by the plasma membrane Ca\(^{2+}\) pump in hippocampal neurons. Biochem. Biophys. Res. Commun. 424, 76–81