In vivo epithelial wound repair requires mobilization of endogenous intracellular and extracellular calcium*

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Background: Calcium promotes gastric wound repair, but in vivo mechanisms are unknown.

Results: Signaling pathways sequentially mobilize intracellular and extracellular calcium as a requirement to repair gastric lesions. PMCA1 mediates extracellular calcium increases.

Conclusion: Endogenous calcium is an essential second and third messenger driving gastric repair.

Significance: Signaling and ion flux pathways are newly identified targets for enhancing gastric repair.

ABSTRACT

We report that a localized intracellular and extracellular Ca\(^{2+}\) mobilization occurs at the site of microscopic epithelial damage in vivo, and is required to mediate tissue repair. Intravital confocal/two-photon microscopy continuously imaged the surgically exposed stomach mucosa of anesthetized mice, while photodamage of gastric epithelial surface cells created a microscopic lesion that healed within 15 min. Transgenic mice with an intracellular Ca\(^{2+}\)-sensitive protein (yellow cameleon 3.0) report that intracellular Ca\(^{2+}\) selectively increases in restituting gastric epithelial cells adjacent to the damaged cells. Pretreatment with U-73122, indomethacin, 2-APB or verapamil inhibits repair of damage and also inhibits the intracellular Ca\(^{2+}\) increase. Confocal imaging of Fura-Red dye in luminal superfusate shows a localized extracellular Ca\(^{2+}\) increase at the gastric surface adjacent to damage, which temporally follows intracellular Ca\(^{2+}\) mobilization. Indomethacin and verapamil also inhibit the luminal Ca\(^{2+}\) increase. Intracellular Ca\(^{2+}\) chelation (BAPTA/AM) fully inhibits intracellular and luminal Ca\(^{2+}\) increases while luminal calcium chelation (HDETA) blocks increase of luminal Ca\(^{2+}\), and unevenly inhibits late phase intracellular Ca\(^{2+}\) mobilization. Both modes of Ca\(^{2+}\) chelation slow gastric repair. In plasma membrane Ca-ATPase (PMCA) 1 (+/−) mice, but not PMCA4 (−/−) mice, there is slowed epithelial repair and a diminished gastric surface Ca\(^{2+}\) increase. We conclude that endogenous Ca\(^{2+}\), mobilized by signaling pathways and transmembrane Ca\(^{2+}\) transport, causes increased Ca\(^{2+}\) levels at the epithelial damage site that are essential to gastric epithelial cell restitution in vivo.

Intracellular Ca\(^{2+}\) is a ubiquitous second messenger that influences numerous cellular processes, but recently extracellular Ca\(^{2+}\) has also been ascribed a role as a third messenger that can integrate and coordinate signals among diverse cells (1,2). Recognizing the possibility of more dispersed roles for Ca\(^{2+}\), in many biological systems there is now less certainty about the sites at which Ca\(^{2+}\) drives biological responses.

The greatest uncertainty is in the understanding of in vivo responses in mammals, because of the technical hurdles of measuring Ca\(^{2+}\) in such environments (3). There is long-standing evidence that Ca\(^{2+}\) plays an important role in regulating protective mechanisms of the gastric epithelium, including bicarbonate secretion and mucus secretion (4-6). However discrimination of Ca\(^{2+}\) roles is limited to amphibian and/or in vitro models. Despite this, some of the most elegant support for the third messenger hypothesis comes from study of amphibian gastric glands. In healthy tissue, carbachol increases both intracellular and luminal Ca\(^{2+}\) in amphibian gastric glands and this Ca\(^{2+}\) extrusion into the gastric gland lumen is mediated by the plasma membrane Ca\(^{2+}\)-ATPase (PMCA)(7). Furthermore, luminal Ca\(^{2+}\) is sufficient to elicit gastric secretions, independent of intracellular Ca\(^{2+}\) changes (7,8). Thus, luminal Ca\(^{2+}\) contributes to the regulatory control of normal physiologic functions in the amphibian stomach. In mammalian systems, reports have only defined the presence of agonist-stimulated intracellular Ca\(^{2+}\) mobilization in isolated gastric glands or isolated surface cells (7,9,10).

In contrast, even in the gastric system the roles of Ca\(^{2+}\) during the tissue response to injury are only roughly defined. Critchlow et al. (4) first demonstrated that an adequate extracellular Ca\(^{2+}\) level is required for restitution of isolated frog gastric mucosa after hyperosmotic injury, but the variable Ca\(^{2+}\) found in human diets raises questions about the physiologic significance of the finding. Conversely, in response to extensive gastric damage caused by application of taurocholate, 1 M NaCl, or 50% ethanol into rat stomach, investigators observe that gastric luminal Ca\(^{2+}\) increases in the collected gastric effluent (11-13). Since the luminal Ca\(^{2+}\) could be due to non-specific Ca\(^{2+}\) release from dying cells, it remains unclear if these early observations could be extrapolated to the more modest and punctuate
damage observed in response to physiologically relevant stressors (e.g., non-steroidal anti-inflammatory drugs, H. pylori) (14-16). For measurement of intracellular Ca\(^{2+}\) during gastric injury, only primary cultured rabbit gastric epithelial cells have been used to demonstrate intracellular Ca\(^{2+}\) mobilization during wound repair (17). Thus there is limited understanding of the roles of Ca\(^{2+}\) as either a second or third messenger during tissue injury of the stomach.

In the present study, intravital confocal and two-photon microscopy is used in vivo to measure intracellular Ca\(^{2+}\) in epithelial cells and extracellular Ca\(^{2+}\) in the space adjacent to the mouse gastric surface epithelium. At the site of microscopic lesions, we observe highly localized intracellular and extracellular Ca\(^{2+}\) mobilizations that are both the result of cellular signaling pathways and are required to promote repair of the epithelium.

**EXPERIMENTAL PROCEDURES**

**Animal husbandry and surgery** - Experiments used C57Bl/6 mice, YC3.0 transgenic mice on a 129J x C57Bl/6J background (18,19), PMCA1 (+/-) mice, PMCA4 (-/-) mice, and PMCA1(+/-)PMCA4(-/-) mice. For experiments examining knockout genotypes, wild-type controls were (+/+) genotypes from the PMCA1 or PMCA4 colony. All animals were used for experiments at 3-6 month of age, were fed a standard rodent chow diet, and had free access to water. All animal procedures were approved by the University of Cincinnati IACUC.

The surgical preparation of animals has been previously described (3,20,21). Briefly, mice were anesthetized with inactin (10mg/kg i.p., Sigma) and ketamine (50 mg/kg i.p.), then the exposed gastric mucosa protruded into a perfusion chamber on the stage of an inverted confocal/two-photon microscope (Zeiss LSM 510 NLO), with the microscope stage enclosed and heated to keep the animal’s body temperature at ~37°C. The mucosal surface was exposed to pH5 solution (150 mM NaCl, 4 mM homopipes; Research Organics). In some experiments, solutions also contained Fura-Red (100 μM, Invitrogen) and/or HEDTA (10 mM, Fluka), BAPTA/AM (250 μM, Calbiochem) or 2-APB (100 μM, Tocris Bioscience)(22). In some experiments, 10 mM NaCl was replaced with 10 mM CaCl\(_2\). U-73122 (30 mg/kg, i.p., Tocris Bioscience) (23-25), indomethacin (5 mg/kg, s.c., Sigma) (20) or verapamil (3 mg/kg, s.c., Sigma) (26), were administered 1 hr before damage induction.

**Live tissue quantitative imaging** - The surface epithelium of YC mouse gastric corpus was excited at 840 nm with a femtosecond-pulsed titanium sapphire laser for two-photon fluorescence excitation, using 90-100 mW light power into the scanhead. At each time point, images were collected simultaneously for directly excited CFP fluorescence (435-485 nm), FRET fluorescence from YFP (535-590 nm), and confocal reflectance (reflected 840 nm light to show cell/tissue structure). In experiments using tissue from other mouse strains, two-photon excitation at 730 nm was used to simultaneously monitor tissue autofluorescence (435-485 nm) and confocal reflectance. Fura-Red (Invitrogen) fluorescence was imaged (>560 nm) in response to alternating excitation at 458 nm or 488 nm. Imaging regions of the gastric surface were selected in which perfusion solutions could rapidly access the epithelial layer.

**Induction of laser-induced microlesion in superficial gastric epithelium** - The method for inducing photodamage (PD) in the gastric surface epithelium with a two-photon laser has been described previously (3,20,21). Briefly, after collecting a set of control images using minimal Ti-Sa (840 nm or 730 nm) laser power, a small rectangle region (~200 \(\mu\)m\(^2\)) of gastric mucosa was repetitively scanned at high laser power (550 mW or 350 mW average laser power, respectively), for 150 iterations (requiring 5-10 s). The damage-repair cycle was measured independently several times per animal in different locations of the corpus.

**Image analysis** - All image analysis was performed with Metamorph software (ver. 6.3, Molecular Devices). In the time course series of images collected from each experiment, we measured maximal damage area. As described previously, the changing size of the damaged area was used to estimate rates of epithelial restitution by fitting a single exponential decay curve to the 10 min of data subsequent to the time that maximal damage was observed. Results report the exponential rate constant, in units of sec\(^{-1}\) (20).

Intracellular free Ca\(^{2+}\) was measured semi-quantitatively using the YC mouse. FRET/CFP
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ratio was obtained by averaging 3-5 circular regions (diameter=16 µm) from the epithelial cells adjacent to the damaged area. The background-corrected FRET/CFP ratio image was calculated, and normalized to a value of 1 in the pre-damage baseline to compensate for daily instrument settings. To semi-quantitatively measure extracellular luminal Ca²⁺ values adjacent to the site of damage (<20 µm distance from the gastric surface), background-corrected 458/488 fluorescence ratio images were calculated and values were obtained by averaging 3-5 non-overlapping circular regions (diameter=16 µm) from every ratio image. Regions with dye binding to luminal mucus were excluded from analysis. Ratio values were normalized for daily instrument settings by comparison to Fura-Red dye in perfusate solution prior to tissue exposure.

Isolation of mRNA using Laser Capture Microdissection- Frozen sections (5 µm thick) were cut in a -20°C cryostat. Slides were dehydrated through an ethanol series then xylene and air dried. Using a laser capture microdissection microscope (Veritas model, Arcturus), 4 non-overlapping gastric cell populations were separately collected: surface epithelium (< 10 µm from gastric surface), mid-gland region, basal-gland region (final 50 µm at base of gastric glands), and muscle cells. Tissue regions were collected onto HS LCM Caps (Arcturus) and total RNA in the collected samples was extracted and isolated using the PicoPure RNA Isolation Kit (Arcturus).

Detection of mRNA using Reverse transcriptase PCR and Real Time RCR- Single-stranded cDNA was synthesized by iScript cDNA synthesis kit (Bio-Rad). Conventional reverse transcriptase polymerase chain reaction was performed using the Advantage 2 polymerase mixture (Clontech) on a thermal cycler (MJ Research). Real time PCR was used to compare the level of gene expression, by amplification with an ABI 7500 (Applied Biosystems) using SYBR green I Dye (Applied Biosystems) for the detection of PCR products. To analyze entire gastric mucosa separate from muscle layers, the PMCA knockout mouse corpus was blunt dissected under a stereo microscope, and RNA extracted by TRI reagent (Molecular Research Center). PMCA1 gene was amplified by QuantiTect Primer (Mm_Atp2b1_1_SG: Qiagen). Other PCR primers are described in Fig. S1.

Immunofluorescence- Cryosections (10 µm) were prepared. Antigen retrieval was performed for each antibody using antigen unmasking solution (Vector) for 10 min. To remove nonspecific binding, sections were first incubated with goat serum (20%) for 30 min, followed by Fab fragment goat anti-mouse IgG (0.13 mg/ml, Jackson ImmunoResearch) for 30 min. Sections were then incubated with the primary antibodies for 30 min in room temperature. The primary antibodies to PMCA (5F10: mouse monoclonal anti-mouse PMCA, Thermo Scientific) or PMCA4 (JA9: mouse monoclonal anti-mouse PMCA4) were used at 1:100 dilution. NHE1 antibody (S1A: polyclonal rabbit antisera) was co-incubated at 1:100 dilution (27). Secondary antiserum was used at 1:100 dilution (Alexa 555-labeled goat anti-mouse IgG2a, and Alexa 488-labeled goat anti-rabbit IgG, Invitrogen) and incubated for 30 min in room temperature. Nuclear DNA staining was performed by incubation with Hoechest 33342 (Invitrogen) at 1 µg/ml for 1 min.

Western blot- The mouse corpus was blunt dissected under a stereo microscope to separate gastric mucosa from muscle layer. Then 700 µl RIPA buffer (Sigma) with protease inhibitor (Roche) was added to isolated mucosa. Protein (30 µg) was separated on a 4-15 % gradient gel and transferred to a PVDF membrane (Millipore). Membranes were incubated 1 hr with Odyssey blocking buffer (Li-Cor Biosciences), incubated 1 hr at room temperature with 5F10 (1:1000 dilution), and then incubated 1 hr in goat anti-mouse secondary antibody (IRDye 800, 1:1000 dilution; Li-COR Biosciences). The antibodies were stripped (25 mM glycine-HCl pH 2 with 1 % SDS, 30 min), incubated 1 hr at room temperature with mouse monoclonal anti-mouse GAPDH (1:10,000 dilution; Sigma), and then incubated 1 hr in goat anti-mouse secondary antibody (IRDye 800, 1:10,000 dilution; Li-COR Biosciences). Blots were quantified using an Odyssey infrared imaging system (Li-Cor Biosciences).

Statistical analysis- All values are reported from representative experiments as the mean ± standard error of the mean (SEM) from multiple experiments. Statistical significance was
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determined using unpaired Student’s T-test, or one-way ANOVA with post-hoc Dunnett’s multiple comparison test. A p value of < 0.05 was considered significant.

RESULTS

In preliminary studies, conventional Ca\(^{2+}\) sensitive dyes (indo-1, fura-2 or fluo-4) could not be loaded into gastric tissue in vivo. Therefore, we measured free Ca\(^{2+}\) using yellow cameleon 3.0 (YC) transgenic mice. Two-photon excitation at 840 nm minimized tissue autofluorescence in wild-type C57BL/6 mouse stomach, efficiently excited CFP, and minimally excited YFP directly (data not shown). We used 840 nm excitation to estimate free Ca\(^{2+}\) from YC transgenic tissue, as the ratio of FRET-stimulated YFP fluorescence divided by directly stimulated CFP fluorescence.

The gastric surface epithelial cells of YC transgenic mice uniformly express YC fluorescence (data not shown). When a single gastric surface epithelial cell in the YC mouse was photodamaged in a diffraction-limited spot (< 0.5µm x 0.5µm in the plane of focus, marked by an ‘X’ in Fig 1), CFP fluorescence intensity dropped and YFP (FRET) intensity increased in the surrounding cytoplasm of the targeted cell, resulting in a rapid increase of FRET/CFP ratio (Fig. 1A). These dynamic fluorescence changes were blunted by intracellular calcium chelation with BAPTA/AM (Fig. 1B). Results show a measurable and local calcium mobilization occurs in response to damage. However, this highly focal damage did not result in cell exfoliation, so imposing damage on several cells was subsequently evaluated.

Mobilization of intracellular Ca\(^{2+}\) via signaling cascades during epithelial wounding-

Fig. 2A shows pseudocolored images of FRET/CFP ratio and qualitative effects of PD to 3-5 cells caused by (10 s) high intensity 840 nm excitation within the red rectangle region. We observe damage progression followed by restitution, qualitatively similar to previous findings using 700-730 nm PD (3,20,21). The size of damage was quantified by digital comparison of the area occupied by all cells (identified by reflectance) versus areas of dead/dying cells (no YC protein), and outcomes compiled in Fig. 2B. The damage area was maximal at 102 ± 15 sec after imposing damage (n=6 animals, 1908 ± 195 µm\(^2\) maximal damage area) and was almost fully repaired at ~1200 sec (70 ± 43 µm\(^2\)). The restitution rate (4.2 ± 0.5 10\(^{-3}\)sec\(^{-1}\)) was calculated as described in Methods.

FRET/CFP ratio (Ca\(_{\text{a}}\)) was measured in the restituting viable epithelial cells adjacent to the damaged area (Fig. 2A white circle), as well as cells >100 µm from the damaged area (Fig. 2A white rectangle). As shown qualitatively in Fig. 2A, and quantitatively in 2C, the FRET/CFP ratio selectively and rapidly increased in the cells surrounding the damaged region but was unchanged in cells far from damage. Statistical analyses of outcomes are based on the ratio change observed during the first 3 min as compiled in Fig. 2F. In subsequent analyses, multiple ratio time points are averaged to simplify graphical presentation and reduce experimental noise.

To ask if Ca\(^{2+}\) was released non-specifically by cellular damage or specifically by activation of signaling pathways, we investigated effects of the phospholipase C (PLC) inhibitor U-73122 or COX inhibitor indomethacin. As shown in Fig. 2D, the wound repair rates after administration of U-73122 or indomethacin (1.1 ± 0.2 10\(^{-3}\)sec\(^{-1}\)) were significantly slower (p<0.05) than control. Either U-73122 or indomethacin significantly blocked the intracellular Ca\(^{2+}\) increase during PD-induced wounding (Fig. 2E, F). We further tested the effect of 1,4,5-triphosphate, IP3 receptor antagonist, 2-APB on gastric repair (Fig. 3). 2-APB significantly slowed gastric repair (Fig. 3A, B, n=4) and the intracellular Ca\(^{2+}\) increase after PD-induced damage (Fig. 3C, D).

Extracellular Ca\(^{2+}\) mobilization in the gastric lumen during epithelial wounding-The ratio of Fura-Red in response to alternating 458nm and 488nm excitation (F458/F488) was dependent on Ca\(^{2+}\) concentration (28) and dye responsiveness was equivalent when pH was >pH 3.8 (Fig. S2A-C). Since we previously demonstrated that pH at the gastric surface was >pH 3.8 when the gastric lumen was perfused with pH 5 solutions (29,30), we used pH 5 superfusates in all subsequent experiments.

When the gastric surface of C57Bl/6 mice was exposed to a luminal pH 5 solution containing Fura-Red but no added Ca\(^{2+}\), PD caused a local and transient increase in F458/F488 ratio (Ca\(\text{L}_{\text{LU}}\)) adjacent to the site of damage, indicative of a local
increase in luminal Ca\textsuperscript{2+} fueled by endogenous Ca\textsuperscript{2+}. Fig. 4A shows a representative time course of F458/F488 ratio images. Simultaneously collected images of NAD(P)H autofluorescence confirm the site and extent of epithelial damage (indicated by arrow). The sharply bounded region of dye observed at the bottom of all ratio images in 4A is due to dye within a concave portion of the surface at the opening of a gastric pit. Compiled results from such experiments (Fig. 4B) document the localized extracellular Ca\textsuperscript{2+} increase as being larger in the luminal space near damage (< 50 µm, white circle in Fig. 4A) with the peak Ca\textsuperscript{2+} level occurring 5-6 min after imposing damage. Based on a Fura-Red calibration curve (Fig.S2A), calcium increased by 651 ± 173 nM at 5 min. U-73122 or indomethacin pretreatment inhibited the increase of luminal Ca\textsuperscript{2+} during gastric wounding (Fig. 4C and 4D), suggesting that non-specific cell destruction was not the source of mobilized extracellular Ca\textsuperscript{2+}.

Endogenous Ca\textsuperscript{2+} requirement for epithelial wound repair- Intracellular Ca\textsuperscript{2+} chelation by exposure to BAPTA/AM significantly inhibited both the repair of gastric damage (Fig. 5A, B) and the rise in intracellular free Ca\textsuperscript{2+} reported in YC transgenic mice after PD (Fig 5C, D). Extracellular Ca\textsuperscript{2+} chelation by luminal HEDTA significantly inhibited repair of gastric damage (Fig. 5A, B) and the luminal Ca\textsuperscript{2+} increase reported by Fura-Red after PD (Fig. 5E, F). In addition, the PD-induced intracellular Ca\textsuperscript{2+} increase was modestly blunted by HEDTA at later time points (Fig. 5C, D), but BAPTA/AM completely blocked the observed extracellular Ca\textsuperscript{2+} increase at all time points (Fig. 5E, F) Control in vitro experiments (Fig. S2D) confirmed that HEDTA did not reduce the free Ca\textsuperscript{2+} in fresh superfusate solution, suggesting the solution contained sub-micromolar [Ca\textsuperscript{2+}]. Results suggest that endogenous Ca\textsuperscript{2+} is essential to promote repair via interdependence between the mobilization of intracellular and extracellular Ca\textsuperscript{2+}.

Routing of the endogenous Ca\textsuperscript{2+} needed for restitution- We asked if cellular Ca\textsuperscript{2+} uptake was a source of mobilized Ca\textsuperscript{2+} in response to damage. Voltage-activated calcium channels are involved in Ca\textsuperscript{2+}-homeostasis of multiple gastric epithelial cells(26,31), including enterochromaffin-like cells(32), D cell(33), and G cell(10). A voltage-activated calcium channel blocker, verapamil, significantly inhibited the speed of gastric repair (Fig. 6A). As shown in Fig. 6B, and C, verapamil blocked the increase of intracellular and extracellular Ca\textsuperscript{2+} in response to PD. Given the confirmed absence of superfusate Ca\textsuperscript{2+} in this protocol, results suggest that basolateral Ca\textsuperscript{2+} uptake may be required for the Ca\textsuperscript{2+} mobilization that promotes repair of damage.

Raising luminal Ca\textsuperscript{2+} has been shown to enhance repair of extensive gastric damage, but the mechanism has not been examined (4). Addition of 10 mM Ca\textsuperscript{2+} into the luminal perfusate also significantly accelerated repair of microscopic gastric lesions (4.7 ± 0.2 10\textsuperscript{-3}sec\textsuperscript{-1}, n=6) in comparison to the absence of added luminal Ca\textsuperscript{2+} (3.6 ± 0.4 10\textsuperscript{-3}sec\textsuperscript{-1}, n=6) (Fig. 6A). Further, the inhibition of gastric restitution by verapamil (1.5 ± 0.2, n=8) was rescued in the presence of 10 mM luminal Ca\textsuperscript{2+} (4.0 ± 0.8 10\textsuperscript{-3} sec\textsuperscript{-1}, n=6) (Fig. 6A). In contrast, intracellular Ca\textsuperscript{2+} mobilization was not consistently increased by the extracellular Ca\textsuperscript{2+} supplement in either the absence or presence of verapamil (Fig 6B). Results suggest that luminal Ca\textsuperscript{2+} can stimulate repair by mechanisms that are downstream of intracellular Ca\textsuperscript{2+} mobilization.

We explored the mechanisms that could mediate the raise of extracellular Ca\textsuperscript{2+} after damage. Gene families of Na/Ca exchangers (NCX) and Ca-ATPases (PMCA) mediate Ca\textsuperscript{2+} extrusion from mammalian cells (34). In the stomach muscle layers, the expression and function of these transporters is well established, but there is no corresponding information about their distribution or roles in other regions of the gastric mucosa (35-37). To evaluate localized expression of mRNA for NCX and PMCA isoforms, four regions of the gastric tissue were separately collected using laser capture microdissection: surface epithelium (mucous cell region), mid-gland (parietal cell region), basal-gland (chief cell region), and muscle layer (Fig. 7A). Using reverse transcriptase PCR (Fig. 7B) or real-time PCR (Fig. 7C) applied to cDNA created from pooled mRNA from each tissue region, we confirmed RNA quality (ubiquitously expressed GAPDH and NHE1 mRNA) and cellular heterogeneity among microdissected regions (mucin MUC5ac mRNA was detected only in the surface epithelial layer, parietal cell specific H,K-ATPase α subunit was detected in the middle and basal-gland layers).
NCX1 mRNA was only observed in the basal-gland and muscle layers. Neither PMCA2, PMCA3, NCX2, nor NCX3 genes had measurable mRNA expression in the stomach (Fig. 7B). Because 2-APB inhibited gastric repair of damage (Fig. 3), we checked IP3 receptor subtype expression. We detected only IP3R type 3 expressed in the gastric surface epithelium (Fig. 7B). PMCA1 mRNA was found in all regions, while PMCA4 was expressed in muscle layer and less prominently in the basal-gland layer (Fig. 7B, C). Immunohistochemistry confirmed the site of PMCA expression in the gastric mucosa. Using an antibody that recognizes all PMCA isoforms (36,38), PMCA was observed in the wild-type (WT) gastric surface epithelium, lamina propria, and muscle layer (Fig. 8B, panels a, d, g, j). PMCA co-localized with a basolateral membrane protein (NHE1) in the gastric surface epithelium (Fig. 8A). Results suggest that PMCA1 is a candidate Ca\textsuperscript{2+} extrusion transporter in the gastric surface epithelium, although PMCA4 and NCX1 may play a role in other gastric epithelial cell types.

Since PMCA1(-/-) mice die before birth (37), we examined gastric PMCA protein expression in PMCA1(+/-) mice and PMCA4(-/-) mice. Under identical imaging conditions (and compared to WT stomach), PMCA staining of the gastric epithelium appeared diminished in PMCA1(+/-) mice (Fig. 8B, panel b) and elevated in PMCA4(-/-) mice (Fig. 8B, panel c). A PMCA4-specific antibody showed staining only in lamina propria and the muscle layer, and this signal was absent in PMCA4 (-/-) mouse stomach (Fig. 9), suggesting the pan-PMCA antibody was detecting another PMCA isoform elevated in the gastric epithelium of PMCA4 (-/-) mouse stomach.

Western blot analysis confirmed PMCA protein (~140 kDa as in (36,37)) was reduced in PMCA1 (+/-) gastric mucosal homogenates, while it was increased in corresponding PMCA4 (-/-) tissue. A representative blot is shown in Fig. 10A, and densitometric analysis from multiple experiments shown in Fig. 10B. Additionally, PMCA1 mRNA was increased in PMCA4 (-/-) gastric mucosa and this increase was diminished in PMCA1 (+/-)/PMCA4 (-/-) mouse stomach (Fig 10C). In PMCA1 (+/-) mice we observed reduction of PMCA protein but not PMCA1 mRNA, compared to WT. This is consistent with previous Northern blot findings in PMCA1 (+/-) mouse stomach (37). Results confirm that PMCA1 (+/-) mice have reduced amounts of the Ca\textsuperscript{2+} pump and suggest that PMCA4(-/-) mice have adaptive up-regulation of alternative PMCA isoforms (likely PMCA1) in the surface gastric epithelium.

Role of PMCA1 in epithelial restitution- The rate of gastric restitution was similar in PMCA4(-/-) and WT mice, but was significantly slowed in PMCA1(+/-) mice (Fig. 11A). In PMCA1(+/-) mice, the luminal Ca\textsuperscript{2+} level prior to damage was significantly lower compared with WT mice, and was not significantly elevated after damage (Fig. 11B). Results with PMCA1(+/-) mice suggest that diminishing the amount of this transporter in heterozygotes reduces Ca\textsuperscript{2+} flux into the gastric lumen. Conversely, in PMCA4(-/-) mice the resting gastric luminal Ca\textsuperscript{2+} level was elevated prior to damage compared to WT mice, and remained elevated after PD (Fig. 11B). These effects in PMCA4(-/-) mice were eliminated in PMCA1(+/-)/PMCA4(-/-) double knockout mice (Fig. 11C, D). Up-regulation of PMCA1 in surface cells of PMCA4(-/-) mouse stomach may explain the modified Ca\textsuperscript{2+} mobilization after damage in this tissue compared to WT. Results suggest that Ca\textsuperscript{2+} efflux via PMCA1 is required to generate the luminal Ca\textsuperscript{2+} increase that promotes wound healing.

DISCUSSION

Observations of the beneficial effects of Ca\textsuperscript{2+} on epithelial repair (4,11-13) have been limited because of the lack of mammalian models to examine Ca\textsuperscript{2+} dynamics in vivo. This paper establishes the conditions necessary for monitoring intracellular Ca\textsuperscript{2+} mobilization during gastric restitution in vivo in mice. Miyawaki et al. introduced YC proteins as genetically encoded calcium sensors in 1997 (18). There is only one prior report using isolated mouse cardiomyocytes that successfully used the YC3.0 transgene as a FRET-based calcium reporter in mouse tissue (39). Limitations of the YC3.0 sensor have been noted (low Ca\textsuperscript{2+} affinity, small FRET changes detected in wide field imaging), as have limitations of the YC transgenic mouse (mosaic expression of YC in some tissues) (19,40,41). While genetically encoded calcium sensors have been continuously improving, in vivo reports remain limited. The current work extends
outcomes to measure gastric intracellular Ca\(^{2+}\) dynamics \textit{in vivo}, and benefits from the relatively homogenous expression of the gastric YC protein and the increased FRET signal resolved by two-photon excitation. It was noted that higher magnification studies of immobilized tissue (Fig 1) resolve cell boundaries precisely, but do not allow for cell exfoliation due to limited free space above the epithelium and the required small size of damage. In contrast, when imaging a larger field of view with a clearly open perfusion space and unrestricted tissue (as in Fig 2 and all subsequent work), the imaging noise and even modest tissue motion dilutes resolution to analysis of the \textit{region directly adjacent to damage}. In this imaging mode, required to see exfoliation and restitution, it was not possible to study a single cell continuously and reliably. The Ca\(^{2+}\) mobilization we observe after microscopic damage is not derived from the non-specific release of Ca\(^{2+}\) from dead/dying cells. Either a phospholipase C inhibitor (U-73122), IP3 receptor antagonist (2-APB) or cyclooxygenase (COX) inhibitor (indomethacin) slowed gastric repair while simultaneously inhibiting the mobilization of intracellular Ca\(^{2+}\). It has been observed that prostaglandins produced from COX activity regulate gastric mucosal defenses via the EP1 receptor that utilizes a Ca\(^{2+}\)-dependent, verapamil-sensitive signal transduction pathway (26). All inhibitor effects are likely to be mediated by inhibiting activation of the G protein, G\(_q\), that regulates intracellular Ca\(^{2+}\) metabolism mediated by IP3 turnover (23,24). Further, we observed that intracellular Ca\(^{2+}\) selectively increased in the viable cells adjacent to fatly damaged cells, but not in the cells located far away from damage. This is consistent with work using cultured rabbit gastric epithelial cells, which showed elevated Ca\(^{2+}\) levels in migrating cells at the edge of an imposed wound (17). We previously reported that the repair of two photon-induced gastric damage was inhibited in COX-1 knockout or trefoil factor (TFF)2 knockout mice, and conversely was stimulated by exogenous application of prostaglandin or TFF (20,21). Although it is unclear how these factors increase in response to damage, evidence suggests that TFF2 and prostaglandins are candidate agonists to stimulate intracellular Ca\(^{2+}\) via the PLC/IP3 pathway. Results suggest that the signaling pathways activated by microscopic damage \textit{in vivo} converge on the Ca\(^{2+}\) mobilization pathway(s) as a common downstream target to promote tissue repair (Fig. 12). Evidence suggests that the intracellular Ca\(^{2+}\) mobilization occurring after damage is required to observe subsequent changes in extracellular Ca\(^{2+}\) in the juxtamucosal space of the lumen. Four compounds that are expected to have independent effects primarily on intracellular Ca\(^{2+}\) mobilization (the Ca\(^{2+}\) channel blocker verapamil, the COX inhibitor indomethacin, the PLC inhibitor U-73122, and the intracellular Ca\(^{2+}\) chelator BAPTA) all blunt intracellular Ca\(^{2+}\) mobilization as predicted, but also inhibit the subsequent luminal Ca\(^{2+}\) rise. After damage, the rapid increase in intracellular Ca\(^{2+}\) precedes the slower luminal Ca\(^{2+}\) change and mice with reduced PMCA1 have diminished luminal Ca\(^{2+}\) after damage. Combined, results suggest that the raised intracellular Ca\(^{2+}\) is a source and stimulus for PMCA1-mediated Ca\(^{2+}\) efflux, which could be the transport route by which endogenous Ca\(^{2+}\) reaches the lumen. Ca\(^{2+}\) efflux via basolateral PMCA1 in surface cells would need to access the lumen via the paracellular pathway (and likely the epithelial breach caused by damage). Alternatively, Ca\(^{2+}\) efflux via apical PMCA1 in gastric gland cells (7) could access to the lumen via transit up the gland lumen, but this alternative would also require a localized stimulation that created a luminal Ca\(^{2+}\) increase at the site of damage. Independent of mechanism, our results suggest that a linkage between intracellular and extracellular Ca\(^{2+}\) mobilization is important to promote efficient gastric repair.

Results suggest that the mobilization of endogenous Ca\(^{2+}\) is essential to observe efficient repair. In overview, Ca\(^{2+}\) chelation slows repair, and luminal Ca\(^{2+}\) supplementation accelerates repair. It is possible that some of the observed Ca\(^{2+}\) may actually promote damage expansion through toxic overloading of mitochondria and other intracellular stores. However, a variety of experimental conditions causing reduced or buffered calcium mobilization utilized in our study did not lead to reduction in maximum damage size (Fig S3). These results strongly suggest that the mobilized Ca\(^{2+}\) is not promoting the expansion of damage to adjacent cells. Since these conditions did delay the time to reach maximum damage size (Fig. S3), results suggest Ca\(^{2+}\) has a role in
accelerating the cell migration that leads to repair and potentially execution/expulsion of cells already fated to die. Our work extends the early findings of Critchlow et al. (4), Takeuchi et al. (11,12), and Koo et al. (13) who demonstrated that after a variety of insults causing pervasive surface damage, the luminal application of Ca\(^{2+}\) improved the gastric repair process while Ca\(^{2+}\) chelation delayed it. Our results extend these results to a focal damage model, and provide new information from directly measuring both intracellular and extracellular Ca\(^{2+}\) levels. These conditions did not selectively affect Ca\(^{2+}\) in only one of these spaces, so results support interactions between Ca\(^{2+}\) signals observed in the two environments. These results also make it difficult to separate the roles of Ca\(^{2+}\) in the intracellular vs. extracellular domain. However, HEDTA did not affect the early intracellular Ca\(^{2+}\) signal dynamics and so the observed repair rate in the presence of HEDTA (~40% of normal) may indicate the portion that is stimulated by this intracellular Ca\(^{2+}\) signal. Conversely, supplementing Ca\(^{2+}\) in the lumen accelerated repair in normal tissue ~30% despite only a small (one time point significant) increase in intracellular Ca\(^{2+}\) levels after damage. Finally, when intracellular Ca\(^{2+}\) mobilization is blocked by verapamil, supplemental luminal Ca\(^{2+}\) is able to fully rescue repair despite only partial restoration of intracellular Ca\(^{2+}\) levels towards normal values at later time points. Results support the speculation that there are separate beneficial effects of intracellular and extracellular Ca\(^{2+}\) to promote repair (Fig. 12).

There are only a few in vitro reports implicating the PMCA gene family of P-type ATPases in cell migration or wound healing. Dreal et al. reported that vanadate, a nonspecific ATPase inhibitor, impairs the migration of MDCK-F cells which express only PMCA1 (42), while Talarico used siRNA-knockdown to show the PMCA4 protein is necessary for rabbit corneal epithelial cell migration during wound healing (43). In the present study, results from PMCA1(+/−) mice suggest that PMCA1 plays an important role in gastric surface restitution and in regulation of extracellular Ca\(^{2+}\) following damage. We were surprised to observe such a pronounced phenotype in a PMCA1 heterozygote. In contrast, full ablation of PMCA4 did not affect the speed of gastric surface restitution, although this is the only other PMCA isoform detectable in any region of the gastric mucosa. We confirmed that PMCA protein was reduced in the gastric mucosa in PMCA1(+/-) and so results can be explained by the heterozygote cells dropping below the threshold of Ca\(^{2+}\) extrusion capacity that is required to fully activate migration of the restituting epithelial cells. Since the PMCA1(−/−) homozygote does not survive to birth, we could not evaluate the effect of full PMCA1 ablation.

Results suggest that PMCA contributes to luminal Ca\(^{2+}\) levels even in undamaged tissue. Prior to imposing damage, basal Ca\(^{2+}\) levels were higher in PMCA4 (-/-) mice that had a demonstrated increase in basolateral PMCA in the surface epithelium (most likely upregulated PMCA1). We confirmed in PMCA1(+/-)/PMCA4(-/-) mice that this high basal luminal Ca\(^{2+}\) level reversed, in parallel with changes in PMCA total protein and PMCA1 mRNA abundance. As stated earlier when talking about the response to damage, the basolateral Ca\(^{2+}\) exported from surface cells by PMCA1 would need paracellular transit to reach the lumen (Fig. 11). Elegant work from Hofer’s laboratory shows that PMCA (isoform unspecified) co-localizes with the H,K-ATPase found in the apical membrane of parietal cells, but also confirm our findings that PMCA is basolateral in surface epithelial cells and they observed that PMCA can affect Ca\(^{2+}\) levels in the gastric gland lumen of healthy tissue (7).

In conclusion, our results provide insight into the molecular and cellular mechanisms whereby endogenous Ca\(^{2+}\) benefits gastric epithelial repair in vivo. Results reveal unanticipated Ca\(^{2+}\) signals in the damage microenvironment that are required to repair epithelial function. Our results suggest that signal transduction pathway(s) activated in response to damage drive a PMCA-1 mediated interaction between intracellular and extracellular Ca\(^{2+}\) signals that is necessary for efficient repair of damage.
REFERENCES


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FOOTNOTES

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Abbreviations: plasma membrane Ca-ATPase (PMCA), N-(2-hydroxyethyl)-ethylenediamin-N,N,N′,N′-triacetic acid trisodium (HEDTA), 1,2-bis(o-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic Acid (BAPTA), Na/Ca exchanger (NCX), photodamage (PD), yellow cameleon 3.0 (YC), 2-APB (2-Aminoethoxydiphenylborane), trefoil factor (TFF)

FIGURE LEGENDS

FIGURE 1. Using YC3.0 transgenic mouse to detect calcium mobilization by two-photon FRET after damage to single gastric surface epithelial cell. Representative time course of images, and two-photon fluorescence data collected in control conditions (A: n=7) or after 250 µM BAPTA/AM incubation (B: n=9). Pseudocolor FRET/CFP ratio images were collected at times indicated, with damage imposed at time 5s. The first image in each series has overlay showing outlines of individual gastric surface epithelial cells. A single cell in the image was stimulated at 5 sec by high power two-photon light at a single pixel (X in image). Bar = 10 µm. Fluorescence intensity values (mean ± SEM) extracted from the damaged cell, normalized to values before imposing photodamage (at arrow) of CFP (O) and FRET fluorescence (□), as well as calculated FRET/CFP ratio values (●).

FIGURE 2. Gastric surface repair and intracellular calcium (Ca_{IN}) mobilization after photodamage. Experiments were performed using YC transgenic mice. U-73122 or indomethacin (Indo) was applied 1 hr before photodamage (PD). Averaged results are mean ± SEM, *, p<0.05 vs. control. (A) Pseudocolor FRET/CFP ratio images (2x2 median filtered) collected at the indicated times from a representative time course experiment. Gastric surface cells were photodamaged (red rectangle) at time zero. Bar = 50 µm. (B) The time course of damage size observed after inducing PD at time indicated by arrow. Inset shows expanded time scale. Values are mean ± SEM (n=6 mice). (C) FRET/CFP ratio was measured in viable cells adjacent to the damaged area (Near: white circles) or in viable cells > 100 µm from damage area (Far: white rectangles). Time of PD is indicated by arrow. Inset shows expanded time scale. n= 6 mice. (D) Exponential curve fits to results such as those in panel B were used to measure rates of repair, as described in Methods. Resulting rate constants are presented in the absence of drugs (control, n=6) or the presence of U-73122 (n=5) or indomethacin (Indo, n=5). (E) Using the animals evaluated in panel D, FRET/CFP ratio was calculated from cells adjacent to damage in the absence or presence of drugs as indicated. (F) Results show the calculated difference between FRET/CFP ratio at time zero and the indicated time point. Results are shown for the absence of drug (Near and Far cells as defined above, n=6), or Near cells in the presence of U-73122 (n=5) or indomethacin (n=5).

FIGURE 3. Effects of 2-APB on gastric surface repair. Compiled results (mean ± SEM) with YC transgenic mice (Control n=5, 2-APB n=4) showing (A) time course of damaged area, which was used to...
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derive (B) calculated repair rate. In the same conditions, (C) FRET/CFP ratio (CaIN) was calculated from cells adjacent to damage, and (D) the difference calculated between FRET/CFP ratio at time zero and the indicated time point. *, p<0.05 vs. control.

FIGURE 4. Extracellular Ca\textsuperscript{2+} (CaLU) mobilization adjacent to gastric surface damage. Experiments were performed using C57Bl/6 mice. (A) A representative experiment, with photodamage (PD) imposed directly after time zero (at site shown by arrow), and NAD(P)H autofluorescence and Fura-Red F458/F488 ratio images collected at the indicated times. Calcium levels in ratio images are pseudocolored per the color bar in the panel. Bar = 50 µm. (B) Time course of the measured damage area (left y-axis scale) and F458/F488 ratio (CaLU, right y-axis scale) measured in the luminal space directly adjacent (<50 µm) from the PD site (Near: white circles in A) or >100 µm from the damage site (Far: white rectangles in A). Values are mean ± SEM. n=6. Time course of F458/F488 ratio (CaLU) (C) and values directly prior to damage (Basal) and 5 min after damage (D) are shown in the absence of drug (n=6) or the presence of U-73122 (n=7) or indomethacin (n=4) as applied in Fig 2. *, p<0.05 vs. basal control. †, p<0.05 vs. control.

FIGURE 5. Effects of calcium chelation on gastric surface repair and calcium mobilization. Experiments tested effects of luminal HEDTA (\textordfervor or ) or BAPTA/AM (■ or ) on the response to photodamage (PD) compared with the absence of drug (control). Compiled results (mean ± SEM) with YC transgenic mice (Control n=4; HEDTA n=4; BAPTA/AM n=4) showing (A) time course of damaged area, which was used to derive (B) calculated repair rate. In the same conditions, (C) FRET/CFP ratio (CaIN) was calculated from cells adjacent to damage, and (D) the difference calculated between FRET/CFP ratio at time zero and the indicated time point. Using C57Bl/6 mice and Fura-Red in luminal perfusates (Control n=6, HEDTA n=4, BAPTA/AM n=5), the time course of F458/F488 ratio (CaLU) (E) and values directly prior to damage (Basal) and 5 min after damage (F) are shown. *, p<0.05 vs. control. †, p<0.05 vs. control at 5 min. Symbols in C and E are the same as A, while D and F are the same as B.

FIGURE 6. Effects of verapamil on gastric surface repair. Verapamil was given 1 hr before the photodamage (PD) applied at time zero. The gastric mucosa was superfused either with conventional perfusate solution with no added Ca\textsuperscript{2+} (Control: n=6, Verapamil, n=8) or with solution containing 10 mM Ca\textsuperscript{2+} (Control: n=4, Verapamil n=6). Compiled results from YC transgenic mice showing (A) calculated repair rate (*, p<0.05 vs. no Ca\textsuperscript{2+} control. †, p<0.05 vs. absence of verapamil), and (B) Change in FRET/CFP ratio (∆CaIN) calculated from cells adjacent to damage, where the basal ratio value before damage is subtracted from the ratio at the indicated time point (*, p<0.05 vs. time control. †, p<0.05 vs. absence of verapamil). (C) Using C57Bl/6 mice and Fura-Red in luminal perfusates (n=6), F458/F488 ratio values before (basal) and 5 min after PD (*, p<0.05 vs. basal control. †, p<0.05 vs. control at 5 min). Symbols in B and C are the same as A.

FIGURE 7. Site-specific expression of mRNA for PMCA, NCX and IP3R isoforms in stomach tissue. Laser capture microdissection collected four stomach regions: surface epithelium (S, <10 µm from lumen), middle (Mi, mid-way through glandular region), bottom (B, <50 µm from base of gastric glands) and muscle (Mu). (A) Image shows the 4 gastric regions collected: (B) Conventional PCR and agarose gel separation evaluated 14 genes as indicated. Brain section was positive control, and no cDNA (water) was negative control. (C) Real-time PCR was performed to compare expression levels among the 4 gastric regions. Data was normalized to GAPDH expression using ∆∆CT calculations. Mean ± SEM, n=3. ND = no detection.

FIGURE 8. PMCA localization in mice with selective PMCA isoform knockout. (A) C57Bl/6 mouse stomach stained with primary antibodies that recognize all PMCA isoforms (a), NHE1 (b), or merged image with nuclei stain (c). (B) Wild-type (WT), PMCA1(+/-) or PMCA4 (-/-) mouse stomach reacted with primary antibodies that recognize all PMCA isoforms (a-c) or NHE1 (d-f). These images were
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merged to compare labeling patterns (g-i), and compared to a lower magnification merged image that shows muscle layers (j-l). Scale bars = 50 µm.

FIGURE 9. PMCA4 localization in mouse stomach. The wild-type (WT) or PMCA4 (-/-) mouse stomach was reacted with primary antibodies for PMCA4 (a, e) or NHE1 (b, f). These images were merged to compare labeling patterns (c, g), and also compared to a lower magnification merged image that shows muscle layers (d, h). Scale bars = 50 µm.

FIGURE 10. PMCA protein and mRNA levels in mice with selective PMCA isoform knockout. (A) Representative western blot comparing extracts from gastric mucosal scrapings (30 µg). PMCA protein band was ~140 kDa, and GAPDH was ~37 kDa. (B) Results compile densitometry evaluation of PMCA band normalized to GAPDH. Bars = 50 µm. Mean ± SEM, n= 4 mice. *, p<0.05 vs. WT. (C) Real-time PCR was performed to compare PMCA1 mRNA expression levels in extracts from gastric mucosal scrapings. Data was normalized to wild type whole stomach (including muscle) PMCA1 expression using ∆∆CT calculations. Mean ± SEM, n=4-6. *, p<0.05 vs. WT. †, p<0.05 vs. PMCA1(+/-)/PMCA4(-/-).

FIGURE 11. Effect of PMCA gene deletion on gastric surface repair and luminal Ca\(^{2+}\) mobilization. Experiments with wild-type (WT, n=5), PMCA1 (+/-) (n=8), or PMCA4 (-/-) (n=6) mice, using luminal Fura-Red to report juxtamucosal Ca\(^{2+}\) levels. Compiled results showing (A) calculated repair rate, (B) Fura-Red F458/F488 ratio values before (basal) and 5 min after photodamage. Mean ± SEM. *, p<0.05 vs. WT Basal. †, p<0.05 vs. WT at 5 min. Experiments were performed using PMCA1 (+/-)/PMCA4 (-/-) (n=5), or PMCA1(+/-)/PMCA4 (-/-) (n=5) mice. Compiled results (mean ± SEM) are shown. (C) Calculated repair rate. *, p<0.05 vs. (+/-)/PMCA4 (-/-) basal. †, p<0.05 vs. PMCA1 (+/-)/PMCA4 (-/-) at 5 min.

FIGURE 12. Schematic illustration of calcium mobilization in response to two photon damage. Dotted lines indicate more speculative pathways. VOCC, voltage operated Ca\(^{2+}\) channel; TFF2, trefoil factor 2; PGE\(_2\), prostaglandin E\(_2\); PLC, phospholipase C; ER, endoplasmic reticulum; IP3R3, IP3 receptor isoform 3; PMCA1, plasma membrane Ca-ATPase isoform 1
Figure 1

**A** Control

![Control images](image1)

![Control graphs](image2)

**B** BAPTA/AM

![BAPTA/AM images](image3)

![BAPTA/AM graphs](image4)
Figure 2

A. Damage 1 min 2 min 4 min 6 min 10 min

B. Damage Area (μm²) vs. Time (s)

C. Ca IN (ratio) vs. Time (s)

D. Repair Rate (10⁻³ s⁻¹)

E. Time (s) vs. Ca IN (ratio)

F. Δ Ca IN (Δ ratio) for 1 min, 2 min, and 3 min
Figure 3

A

Damage Area (µm²)

0 20 50 100 150 200 250 300 350 400 450 500

Time (s)

Control
2-APB

B

Repair Rate (10⁻³ s⁻¹)

0 1 2 3 4

Control 2-APB

C

CaIN (ratio)

1.00 1.05 1.10

Time (s)

PD

D

Δ CaIN (Δ ratio)

0.02 0.04 0.06 0.08 0.10

1 min 2 min 3 min

Δ CaIN (Δ ratio)
Figure 4

A. NAD(P)H and Fura-Red images at different time points (sec).

B. Graph showing area damage over time (µm²).

C. Graph showing Ca²⁺ LU (ratio) over time.

D. Graph comparing control near and U-73122 vs. Indomethacin.

Legend:
- Near
- Far
- Control (Near)
- U-73122
- Indomethacin

* Basal 5 min
† †
Figure 5

**Damage**

**A**

- Graph showing Damage Area (µm²) over time (s) for Control, HEDTA, and BAPTA/AM.
- Damage Area increases over time and varies among the three groups.

**B**

- Bar graph showing Repair Rate (10⁻³ s⁻¹).
- Control group has a repair rate of 1, HEDTA shows a slightly increased rate, and BAPTA/AM has an even greater increase.

**Intracellular Calcium**

**C**

- Graph showing Intracellular Calcium (Caᵢⁿ (ratio)) over time (s) for Control, HEDTA, and BAPTA/AM.
- Caᵢⁿ (ratio) increases over time and varies among the three groups.

**D**

- Graph showing ΔCaᵢⁿ (Δ ratio) over time (s) for 1 min, 2 min, and 3 min.
- ΔCaᵢⁿ (Δ ratio) increases over time and varies among different time periods.

**Luminal Calcium**

**E**

- Graph showing Luminal Calcium (Ca₇ (Ratio)) over time (s) for Basal and 5 min.
- Ca₇ (Ratio) increases over time and varies among the two conditions.

**F**

- Graph showing Ca₇ (Ratio) over time (s) for Basal and 5 min.
- Ca₇ (Ratio) increases over time and varies among the two conditions.
Figure 6

A

Repair Rate (10^{-3} s^{-1})

10 mM Ca^{2+} - +

Control Verapamil

B

\(\triangle C_{a_{in}} (\triangle \text{ratio})\)

1 min 2 min 3 min

† *

C

\(C_{a_{lu}} (\text{ratio})\)

Basal 5 min

† *
Figure 9

PMCA4 Ab  NHE1 Ab  Merge  100x

PMCA4 (+/+)  

PMCA4 (-/-)
Figure 12

The diagram illustrates a cellular signaling pathway involving calcium concentration changes. The main reactions include:

1. **Gq** activates **IP3R3**, leading to increased calcium concentration ($\uparrow \text{Ca}^{2+}$).
2. **IP3R3** also activates **PLC**, which in turn activates **VOCC**.
3. **VOCC** opens voltage-dependent calcium channels, further increasing calcium concentration ($\uparrow \text{Ca}^{2+}$).
4. **PMCA1** pumps calcium out of the cell, maintaining the calcium concentration.

The elevated calcium concentration in the cell activates **TFF2** and **PGE_2**, which stimulate paracrine migration agonists leading to cell migration and restitution.

The damaged cell is depicted with a question mark, indicating an unknown mechanism or variable in the process.
In vivo epithelial wound repair requires mobilization of endogenous intracellular and extracellular calcium

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