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Importance of Ca^{2+} in Gastric Epithelial Restitution – new views revealed by real-time *in vivo* measurements

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Abstract

It has been a few decades since Ca^{2+} was identified as one of the important factors that can accelerate gastric wound repair as well as contribute to epithelial homeostasis and regulation of gastric secretions. The mechanistic basis has remained largely unexplored *in vivo* because it was not possible to track in real time either intracellular Ca^{2+} mobilization or wound repair in living tissues. Recent advances in technology, such as combining high resolution light microscopy and genetically encoded Ca^{2+} reporters in mice, now allow the monitoring of Ca^{2+} mobilization during gastric epithelial cell restitution. Ca^{2+} is a ubiquitous second messenger that influences numerous cellular processes, including gastric acid/bicarbonate secretion, mucus secretion, and cell migration. We have demonstrated that cytosolic Ca^{2+} mobilization within the restituting gastric epithelial cells is a central signal driving small wound repair. However, extracellular Ca^{2+} is also mobilized in the juxtamucosal luminal space above a wound, and evidence suggests extracellular Ca^{2+} is a third messenger that also promotes gastric epithelial restitution. Interplay between intracellular and extracellular Ca^{2+} is necessary for efficient gastric epithelial restitution.

Introduction

Two processes, proliferation and restitution, are responsible for maintaining epithelial continuity and consequently an epithelial cell barrier in the stomach. Proliferation requires mitosis and is therefore a fairly protracted process, while restitution is a much more rapid process dependent on cell migration. In response to acute disruption of the gastric epithelium, cell migration is always the first responder towards restoring epithelial continuity and barrier function [1].

Epithelial restitution is a highly regulated process that is energy-dependent and engages both intracellular and extracellular biomolecules to signal and drive repair (e.g. prostaglandins (PG), cytoskeletal rearrangements, ion transporters, and other cellular processes [2-5]). The Ca^{2+}-dependence of gastric epithelial restitution is not surprising since Ca^{2+} is a ubiquitous

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second messenger that influences numerous cellular processes [6,7]. In the field of gastric restitution, this broad set of roles has the potential to bring convergence to a disparate literature, as a common mediator for the wide range of factors that have been reported to stimulate restitution [8-11].

Despite this exciting potential, there are few reports measuring Ca^{2+} in the gastric epithelium. While the intracellular loading of conventional acetoxymethylester Ca^{2+} sensitive fluorescent probes (Fura2, Fluo4, etc) can be used for *in vitro* study, these fail to load effectively *in vivo* [12]. The reasons for this are unknown: the chemicals may be cleaved prematurely in the extracellular space, leak rapidly from gastric cells (like the SNARF probe [13]), or never get effectively cleaved to the active form in the intracellular space. Consequently, there was a 30 year interval with virtually no *in vivo* study exploring the role of intracellular Ca^{2+} in gastric epithelial cells. In 1997, genetically encoded yellow cameleon (YC) protein was developed in which cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are linked by calmodulin and a M13 calmodulin-binding domain [14]. Upon an increase in Ca^{2+} concentration, Ca^{2+} binds calmodulin and interacts with M13, which results in a conformational change of the protein that increases the efficiency of Förster resonance energy transfer (FRET) from CFP to YFP. A YC transgenic mouse has been created that allows direct observation of intracellular Ca^{2+} dynamics in real time *in vivo* [15]. There are even fewer reports evaluating the role of extracellular Ca^{2+}. The pioneering work of Curci's group using Ca^{2+} microelectrodes introduced the concept of Ca^{2+} being mobilized in the lumen of gastric glands as a signaling mechanism to regulate gastric secretion [16]. Our work and that of others has shown that there is also a pH microdomain adjacent to the surface epithelium, altered in the presence of epithelial damage [4,5,17,18]. Based on these advances, the conceptual and experimental foundation for evaluating luminal Ca^{2+} microdomains has solidified in recent years.

Restitution model using two photon confocal microscopy

The restitution process has been difficult to observe with high fidelity due to both the speed of the process and the inherent heterogeneity of damaged and healthy cells. Generally, *in vitro* cell culture studies have used a scratch-induced wound and start to measure the area that is covered by migrating cells at least 2 hr after wounding. This is a great tool to monitor cell migration, including cell shape changes [19-22]. Gastric restitution is classically measured morphologically in fixed tissue sections, or by transmucosal electrical resistance and potential difference between the luminal and serosal side using isolated gastric mucosa mounted in Ussing chambers. In such models, restitution required >4 hr to complete [2,22,23]. When gastric restitution was measured in *ex vivo* chambered stomach in anesthetized animals by detecting transmucosal potential difference, repair of damage was completed in 1-2 hr [24,25]. The different time courses may be related to different modes of inducing damage, or variable potency of tissue preparations to mount a robust damage response. Overall, these electrophysiological approaches are very reasonable models to monitor restitution since results monitor time-dependent closing of denuded/injured area of gastric epithelium. Since such outcomes also indicate that the restitution process starts within 10 min after induction of injury, the work also encourages exploration of approaches that allow higher resolution of early events in restitution.

Recent imaging advances led us to establish a new gastric restitution model that makes it possible to visualize both the repair progression and microenvironment changes using fluorescent probes. The microscopic precision of two-photon absorption allows the timed creation of microlesions (Photodamage) at any selected gastric surface epithelial region, followed by visualizing in real time of damage expansion, exfoliation of dead cells, and restitution (Figure 1A). Although the mechanism underlying two-photon-induced damage is still unknown, absorption of light energy by targeted fluorescent proteins or endogenous mitochondrial NAD(P)H results in photobleaching and potentially leads to induction of reactive oxygen species to cause cellular apoptosis or necrosis. Similar optical strategies are used in phototherapy to selectively kill tumor cells [26,27]. We have reported that two-photon absorption in response to high power laser scanning immediately bleaches NAD(P)H in a set of targeted gastric surface epithelial cells and damage expands to an area that is 5-10 times the original area of illumination [5]. Subsequently, exfoliation of dead cells and closing of the wound area were observed, and this entire process is completed within 15 min. Importantly, gastric restitution after this photodamage was inhibited in cyclooxygenase (COX) inhibitors or COX1 knockout mice [4,5,12], consistent with findings using different damage models [19,28].

The further advantage of this model is that while measuring restitution, additional parameters can be monitored using different fluorescent probes. For instance, several reports demonstrated that gastric luminal pH increased in response to damage [28,29], but these results obtained from measuring collected gastric fluid contents. In contrast, in the focal damage model after photodamage an increase of pH was observed only adjacent to the damage site [4,5]. Thus, the restitution model using two-photon microscope allows us to visualize the entire restitution process as well as microenvironmental changes in a real time setting.

Role of Intracellular Ca^{2+} in gastric epithelial restitution

Based on results from multiple tissue types, there is no doubt about the involvement of cytosolic Ca^{2+} mobilization in cell migration. In the stomach, one report using primary cultured rabbit gastric epithelial cells showed that intracellular Ca^{2+} was significantly higher in migrating cells at the edge of an imposed scratch wound at 2 hr after damage, and observed that verapamil, calmidazolium (Ca^{2+}/calmodulin complex inhibitor), and calphostin-C (protein kinase C : PKC inhibitor) significantly inhibited cell migration speed observed at 24 hr after monolayer wounding [21]. Until recently, there was no extension of this 2002 work to evaluate intracellular Ca^{2+} mobilization *in vivo*. This gap in knowledge was approached using the two-photon microscopy restitution model applied to transgenic mice with gastric expression of the YC Ca^{2+} sensor.

Use of high irradiance with 840 nm light caused two-photon light absorption by the CFP fluorophore, YC photobleaching, and induced cell death (Figure 1A). The intracellular Ca^{2+} selectively increased in the viable cells adjacent to fatally damaged cells (< 50 μm), in a verapamil or BAPTA/AM inhibitable manner, but not in the cells located far away from damage (> 100 μm) (Figure 1B) [12]. PLC inhibitor, IP3 receptor antagonist, or COX inhibitor slowed repair and also blocked the Ca^{2+} mobilization stimulated in restituting

gastric epithelial cells [12]. These drug effects are all likely to be mediated by inhibiting activation of the G_q protein. PLC is usually linked to G_q and its activation leads to IP3 turnover, by which Ca^{2+} is released from the endoplasmic reticulum (ER). Many receptors, such as the EP1 receptor subtype of the PGE receptor family, are coupled to G_q [30]. In the scheme of gastric mucosal protection, bicarbonate secretion is mediated by EP1 receptor in a verapamil inhibitable manner [31]. Even in the absence of imposed damage, we observed that PGE_2 stimulates intracellular Ca^{2+} in intact YC mouse gastric epithelial cells (Figure 2), suggesting this pathway is broadly available in an unperturbed epithelium but only selectively activated in some cells of the restituting epithelium. Furthermore, gastric damage induced by taurocholate is more severe in EP1 receptor knockout mice [28]. These data suggest that the increase of intracellular Ca^{2+} during restitution is likely mediated by PGE_2 /EP1 receptor acting downstream of PLC/IP3 activation. It should also be noted that *in vitro* studies of gastric epithelial cells reported that PGE_2 release is inhibited by a PLC inhibitor; suggesting that the increase of intracellular Ca^{2+} in response to damage will enhance PGE_2 production late in the repair cycle, predicted to stimulate repair while sustaining high Ca^{2+} levels [32,33]. However, because we observed that PLC inhibition blocked the intracellular Ca^{2+} increase in response to damage [12], another factor besides intracellular Ca^{2+} must be invoked as a mediator of any initial release of PGE_2 in response to damage.

The routes and mechanisms of stimulating Ca^{2+} influx from the extracellular compartment remain somewhat speculative in the gastric epithelium, with many potential candidates. Evidence (from use of inhibitors) suggests that some Ca^{2+} influx important to cell migration occurs through voltage-gated Ca^{2+} channels *in vivo* [12,21]. However, it has also been reported that *in vitro* the voltage-gated K^+ channel Kv1.1, is involved in rat gastric epithelial (RGM-1) cell migration [34] as well as intestinal epithelial cell migration [35], and the work showed that Kv1.1 causes Ca^{2+} influx independently of voltage-gated Ca^{2+} channels in these models [34,35]. It is possible that other Ca^{2+} channels such as transient receptor potential (TRP) channels regulate Ca^{2+} influx. TRPC seems to function as store-operated Ca^{2+} channel (SOC) in many cells, but TRP subtype expression profiles are still unknown in gastric surface epithelial cells [36,37]. Furthermore, the recently identified Ca^{2+} release-activated channel protein1 (Orai1), also activates in response to Ca^{2+} store depletion, which is mediated by translocation of a ER Ca^{2+} sensor, stromal interaction molecule 1 (STIM1) [38]. Recently, TRPC has been shown to associate with Orai1 and STIM1 in several models [39-41]. In the small intestine, it is reported that TRPC1 interacts with STIM1 in response to Ca^{2+} store depletion, resulting in stimulating Ca^{2+} entry and enhancing epithelial migration [42]. All these proteins remain viable candidates to mediate or modulate the Ca^{2+} influx that stimulates wound repair.

PKC inhibitors also slow gastric cell migration *in vitro* in response to wounding [21], and we further confirmed that blockade of PKC (by Chelerythrine 5 mg/kg, i.p.) inhibits photodamage-induced restitution *in vivo* (unpublished). Interestingly, PKC inhibitor treatment simultaneously inhibited mobilization of intracellular Ca^{2+}. Several studies showed that PKC regulates the action of SOCs including TRPC1 [43], suggesting that TRPCs/Orai1/STIM1 may be involved in gastric epithelial Ca^{2+} influx. However, lacking

studies in the gastric epithelial cells or most other regions of the gastrointestinal tract, the mechanisms of Ca^{2+} influx by gastric epithelial cells remains to be elucidated.

Extracellular Ca^{2+} in gastric epithelial restitution

A pivotal early observation was that extracellular Ca^{2+} chelation decreased gastric mucosal potential difference, and this drop was recovered by addition of excess extracellular Ca^{2+} [25], suggesting that gastric luminal Ca^{2+} is necessary to maintain integrity of gastric epithelial homeostasis. It has been reported more recently that Ca^{2+} release into the lumen of gastric glands can occur as part of the regulatory control of normal physiologic functions. Using Ca^{2+}- sensitive microelectrodes, it has been possible to monitor changes in local Ca^{2+} levels in the extracellular spaces surrounding amphibian gastric glands [44]. In our complementary observations in mice, the luminal environment adjacent to the surface epithelium also sustains a pH and Ca^{2+} microenvironment as a consequence of the surface unstirred layer [12,45] (Figure 3). It seems that an extracellular Ca^{2+} gradient exists in a variety of locations within the gastric luminal compartment, and this Ca^{2+} source has at least a physiologic role in promoting mucus and HCO_3^- secretion in intact tissue as one contribution to the first line of gastric defenses.

Extracellular Ca^{2+} also has a role in injured tissue. Critchlow et al. first demonstrated almost 30 years ago that an adequate extracellular Ca^{2+} level (changed conditions on the serosal side of isolated tissues in Ussing chamber) is required for complete restitution of isolated frog gastric mucosa after hyperosmolar injury, and that restitution occurs by migration of persisting viable gastric pit cells [2]. Similarly, in response to pervasive surface gastric damage caused by taurocholate, or 30% ethanol, the gastric luminal Ca^{2+} increased in the collected gastric effluent [24,46]. In the focal damage model after photodamage, we recently reported that luminal Ca^{2+} increased (Figure 1B) [12]. Importantly, the luminal Ca^{2+} increase was localized to areas adjacent to the focal damage. The increase of luminal Ca^{2+} during gastric restitution was inhibited by verapamil, COX inhibitor, or PLC inhibitor pretreatment which can also inhibit intracellular Ca^{2+} increase during restitution [12]. Furthermore, chelation of intracellular Ca^{2+} by BAPTA/AM inhibited the increase of luminal Ca^{2+} [12], suggesting that increase of luminal Ca^{2+} that benefits epithelial repair is dependent on the intracellular Ca^{2+} increase, likely the result of active Ca^{2+} efflux from the surviving epithelial cells that repair the epithelium.

In virtually all cells, there are only two classes of transporter that are candidates to extrude Ca^{2+} across the cell: plasma membrane Ca^{2+} ATPases (PMCA) and Na^+/Ca^{2+} exchangers (NCX). The latter class is not expressed in the gastric surface epithelium [12], focusing attention on the PMCAs. It has been reported that PMCA1 serves essential housekeeping functions for cellular Ca^{2+} homeostasis, whereas PMCA4 performs specialized physiological functions [47]. Caroppo *et al.* showed that Ca^{2+} extrusion into the gastric gland lumen is induced by carbachol and is mediated by PMCA [16]. Interestingly, (see Figure 3), PGE_2 failed to increase luminal Ca^{2+} release in PMCA1 (+/-) mouse stomach. We reported that PMCA1 plays an important role in gastric restitution and in regulation of extracellular Ca^{2+} following damage [12]. In contrast, full ablation of PMCA4 did not affect the speed of gastric restitution, although PMCA1 protein is upregulated, resulting in higher

basal luminal Ca^{2+} levels. These results suggest that although PMCA1 is responsible for Ca^{2+} efflux across the basolateral membrane of the gastric epithelium, either transmembrane or paracellular Ca^{2+} flux is allowing access of this pool of extracellular Ca^{2+} to the lumen even in a healthy epithelium. The tight junction protein claudin-16 has Ca^{2+} permeability as shown in the kidney and small intestine [48,49], but we could not detect claudin-16 mRNA in the stomach (unpublished). After imposing focal damage, PMCA1 (+/-) mice had a diminished increase in luminal Ca^{2+} compared to wild-type mice [12]. Since the lateral membranes of cells are exposed to the gastric lumen after photodamage and disruption of epithelial continuity, enhanced paracellular permeability is the simplest route predicted to cause the high Ca^{2+} concentrations we observe in the damage site microenvironment.

Cross-talk between intracellular and extracellular Ca^{2+}

One of the most striking findings from recent work is to reconcile long-standing scientific observations about the role for luminal Ca^{2+} as a necessary factor to promote gastric repair *versus* the lack of need to continually take Ca^{2+} supplements to promote health and sustain the gastric barrier. Observations in the focal damage model suggest that the resolution to this paradox is that (at least for modest injuries) the stomach is able to recruit endogenous Ca^{2+} to all the spaces (intracellular and extracellular) that are needed to promote efficient repair and epithelial barrier function. Exogenous luminal Ca^{2+} is helpful, but not necessary.

The distinct roles of extracellular versus intracellular Ca^{2+} on restitution remain uncertain. Lack of methods to create a pure separation of intracellular and extracellular Ca^{2+} mobilization requires cautious conclusions, but results do suggest that luminal Ca^{2+} plays a potentially independent role to stimulate the restitution. The two spaces display distinct time signatures of Ca^{2+} mobilization, in that extracellular Ca^{2+} has a slower time course (peak ~6 min) than intracellular Ca^{2+} levels (peak at ~1 min) after damage (Figure 1B) [12]. Additionally, luminal Ca^{2+} chelation by HEDTA partially blocked the increase of intracellular Ca^{2+} only at the late phase yet had a substantial effect to inhibit restitution [12]. Additionally, exogenous luminal $CaCl_2$ administration also promotes gastric repair of damage whether a pervasive damage model [25,46] or our focal damage model [12]. This supplemental luminal Ca^{2+} also rescued the inhibition of microscopic wound repair caused by verapamil. Importantly, Ca^{2+} supplement only partially restores intracellular Ca^{2+} responsiveness in the late phase; again suggesting separable roles of the Ca^{2+} acting from these two locations. Since a voltage-activated Ca^{2+} channel blocker also slows repair and blunts intracellular Ca^{2+} mobilization in the migrating cells, results suggest that luminal Ca^{2+} could have an important secondary role in later stimulation of Ca^{2+}-dependent signals in the restituting epithelial cells. We already know that PMCA1 efflux is a source of mobilized extracellular Ca^{2+}, but this would provide a second explanation of why it appears almost impossible to separate Ca^{2+} mobilization in the intracellular versus luminal compartments.

The route of extracellular Ca^{2+} action remains speculative, but there are some tantalizing possibilities. It has been reported that the Calcium sensing receptor (CaSR) and PMCA protein co-localize with H^+/K^+ ATPase in the oxynopeptic cells, and evidence has been provided for an autocrine feedback loop that involves luminal Ca^{2+} sensing to regulate alkali

and pepsinogen secretion, and drive water transport [16,44,50]. The mouse surface epithelium is likely to act differently. In Figure 4, CaSR clearly expresses in the apical membrane of gastric surface epithelial cells, distinct from PMCA1 localization. However it has been shown in other cell types that activation of the CaSR can lead to intracellular Ca^{2+} mobilization, providing yet another route for cross-talk between the two compartments. Extracellular Ca^{2+} may be physiologically important as it has been proposed that the protective effects of milk on the stomach are due to high Ca^{2+}, rather than buffering of pH [46]. We speculate that the gastric mucosal protective action of milk or Ca^{2+} administration on the stomach is mediated by CaSR. Some protein, such as casein also can stimulate CaSR leading to increase intracellular Ca^{2+} [51], while casein itself does not show gastric mucosal protection [46]. However, it is possible that casein potentiate Ca^{2+} effect on CaSR. Thus, a tempting speculation is that the CaSR may respond to the luminal Ca^{2+} signals, a topic to be pursued in further study.

Conclusion

In conventional signaling models, most physiological changes are triggered by intracellular second messengers, such as cAMP, cGMP and Ca^{2+}. All these signaling pathways are involved in the regulation of gastric acid secretion [52,53], pepsinogen secretion [54-56], and mucus secretion [57-59]. The gastric mucosal bicarbonate secretion is mediated by cGMP and Ca^{2+} [60,61]. it has been shown that cGMP-induced bicarbonate secretion is mediated by COX/PGE_2/EP1 receptor [62], so based on known signaling by this receptor, Ca^{2+} is likely to be the most downstream messenger to elicit receptor actions. In contrast, evidence from cultured cells suggests that neither cAMP nor cGMP plays any role in gastric epithelial cell migration [21,63]. Conversely, luminal Ca^{2+} likely stimulates cNOS-dependent nitric oxide/cGMP production resulting in facilitating gastric restitution due to inhibition of acid secretion or increase of bicarbonate secretion [24,64]. This set of results leaves space for Ca^{2+} and its signaling to take a central role in gastric epithelial cell restitution.

Overall, endogenous Ca^{2+} benefits gastric epithelial repair *in vivo*. Intact gastric epithelial cells respond to focal damage through the increase of intracellular Ca^{2+}, followed by the release of Ca^{2+} into the gastric lumen via PMCA1. Subsequently, luminal Ca^{2+} cooperates to complete restitution. Thus, we have expanded the concept that luminal Ca^{2+} acts as a 'Third messenger' [65] to provide an even more broad range of beneficial effects in both healthy and damaged stomach.

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Highlights

- Two-photon microscopy allows unique opportunities for monitoring of gastric events
- Two-photon damage model reveals functional microdomains of Ca^{2+} mobilization
- Intracellular Ca^{2+} increases selectively on restituting gastric epithelial cells
- Gastric luminal Ca^{2+} accelerates gastric epithelial cell restitution

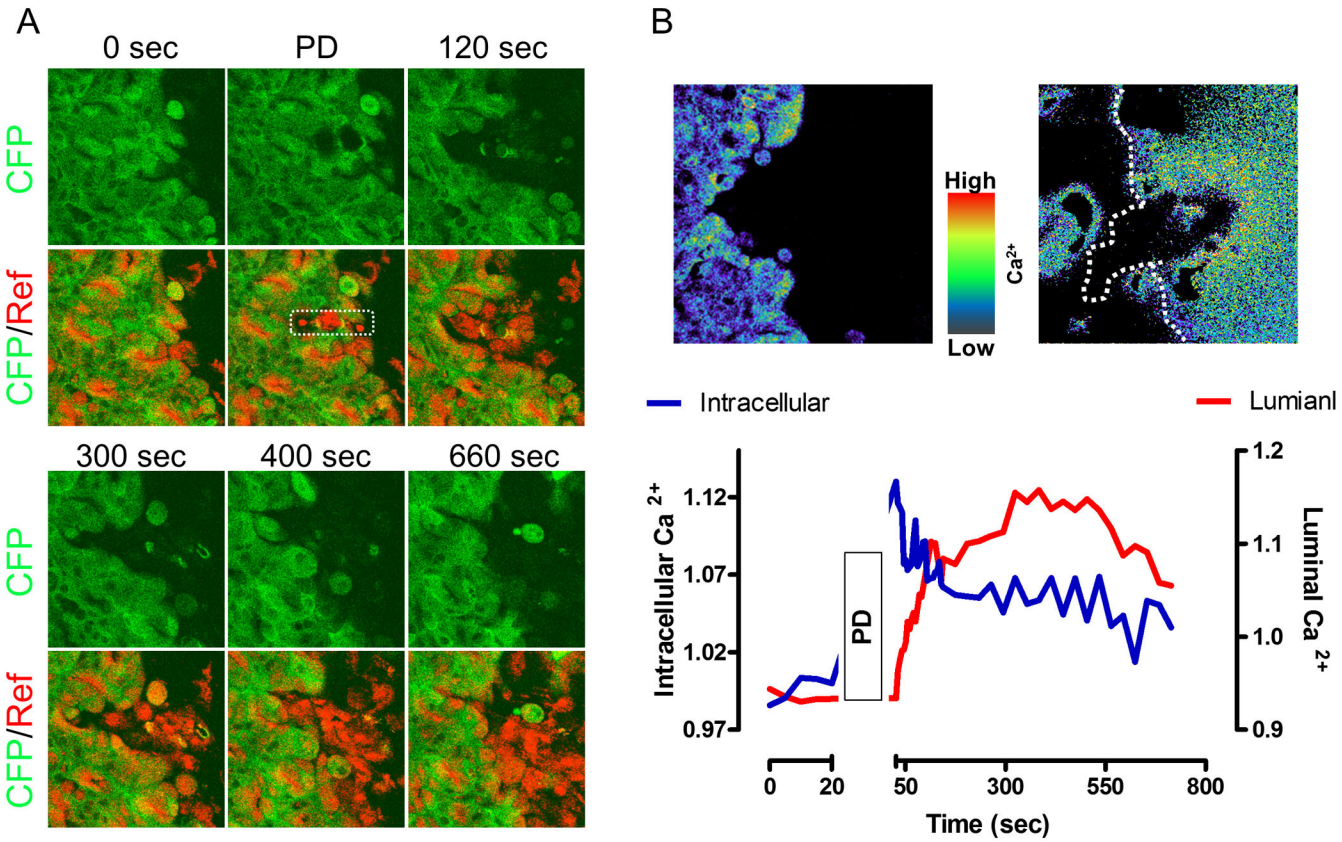

Figure 1. Change in intracellular and extracellular Ca^{2+} during gastric epithelial restitution induced by photodamage

Images were collected at the indicated times in anesthetized YC mouse stomach using dyes and imaging parameters as previously described [12]. **A**. CFP (Green) and reflectance (Red) images (Ex: two photon 840 nm) Gastric surface cells were photodamaged (PD) in the 258 μm^2 region marked by white rectangle. **B**. Pseudocolor FRET/CFP and Fura-Red F458/F488 images showed intracellular and luminal Ca^{2+} changes at 2 min after PD, respectively. In addition, the time course of intracellular (Blue line) and luminal (Red Line) Ca^{2+} changes was calculated from images. B: taken from [12].

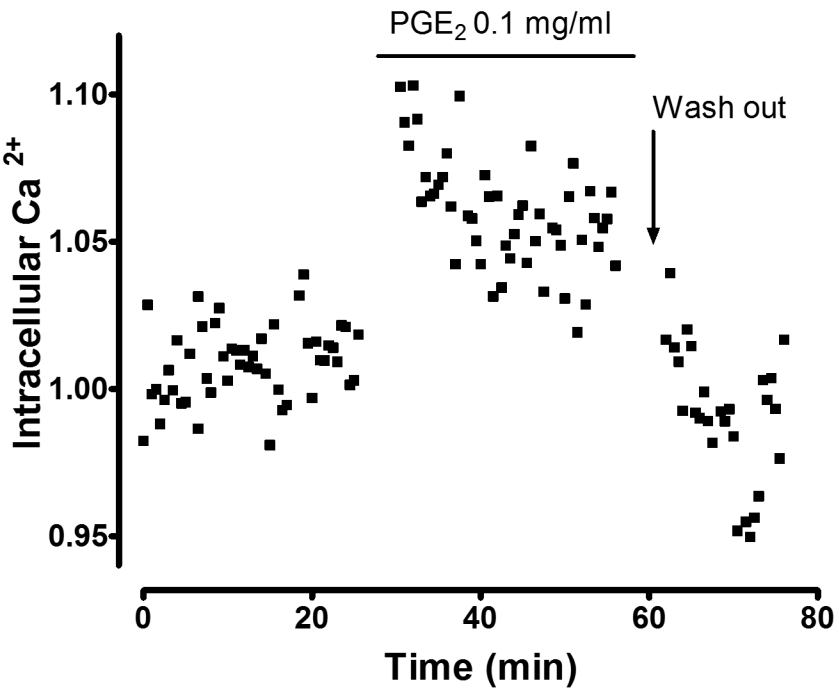

Figure 2. PGE_2stimulates intracellular Ca^{2+} mobilization in the intact stomach

Intracellular Ca^{2+} (Ratio FRET/CFP) was monitored in anesthetized YC mouse gastric surface epithelial cells as previously described [12]. PGE_2 (0.1 mg/ml) was topically applied to gastric tissue at the time indicated and then removed (washed out).

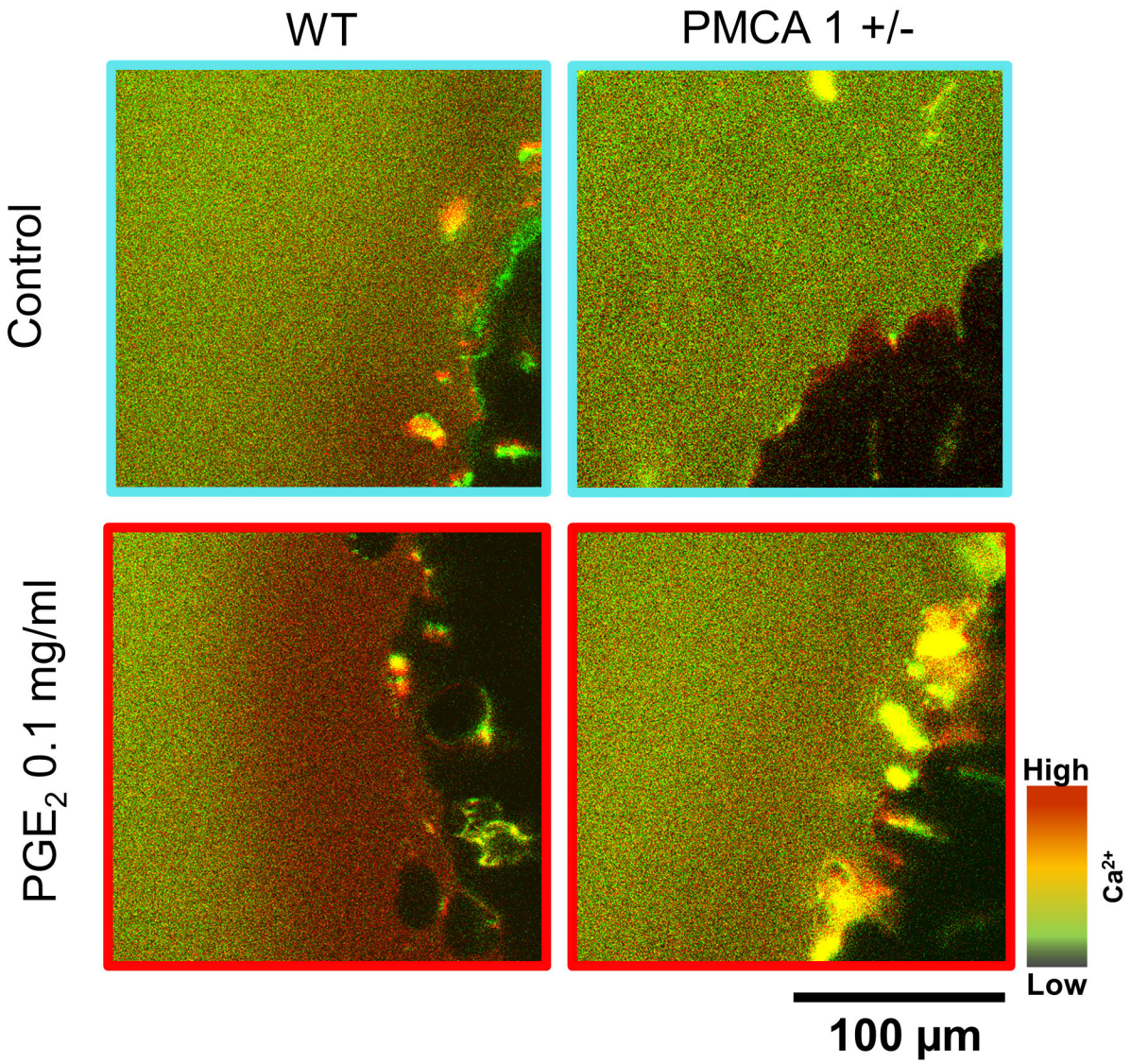

Figure 3. Luminal Ca^{2+} gradient in response to PGE_2

Luminal Ca^{2+} (Ratio F458/F488) was monitored by addition of 100 µM Fura-Red added to the gastric luminal perfusate. PGE_2 (0.1 mg/ml) was topically applied to gastric tissue.

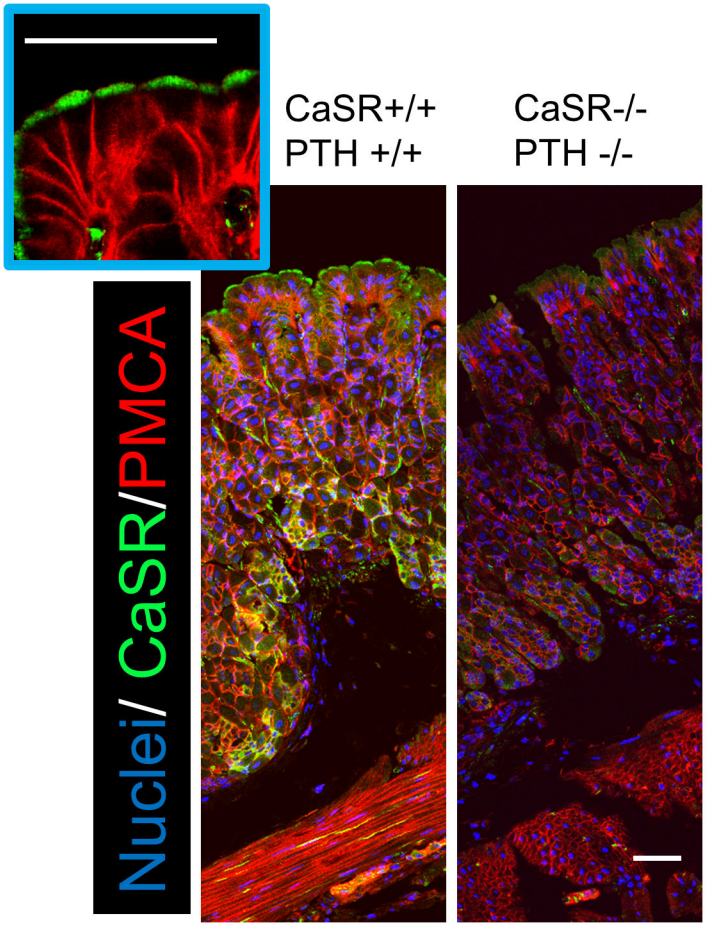

Figure 4. Immunofluoresence staining of Calcium sensing receptor in the stomach

Cryostat sections of stomachs from wild-type mouse are compared to sections from a dual knockout mouse lacking Calcium sensing receptor (CaSR -/-) and Parathyroid hormone (PTH -/-) [66]. were stained with primary antibodies that recognize all PMCA (5F10, Thermo Sci) and CaSR (Millipore). Both anti-CaSR or PMCA primary antibodies were used at 1:100 dilution and visualized with secondary antibodies at 1:100 dilution, Alexa Fluor 488-labelded goat anti-rabbit IgG or Alexa Fluor 555-labelded goat anti-mouse IgG2a, respectively. Nuclear staining was performed by incubation with Hoechst 33342 at 1 μg/ml. Scar bar = 50 μm.