A Novel Function of Lactate Transporter MCT1 in Gastric Restitution

Lisa Marrone

under the direction of
Dr. Susan J. Hagen
Beth Israel Deaconess Medical Center

Research Science Institute
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Abstract

We investigated the function of the monocarboxylate transporter MCT1 in restitution of gastric surface mucous cells. Restitution is the repair mechanism by which cells migrate to cover an injured area. Cell migration after injury is dependent upon glycolysis, which under some conditions yields the waste product lactate. We used the bioflavenoid phloretin, a known inhibitor of MCT1, to block lactate transport in migrating cells and determine the overall effect of MCT1 on restitution. We found that inhibition of MCT1 with phloretin significantly and dose-dependently inhibited restitution. Furthermore, under HCO_3^--free conditions that simultaneously inhibit the bicarbonate transporter NBC, there was a complete inhibition of restitution after injury with no effect on viability. These results suggest that MCT1 functions along with NBC to regulate intracellular pH and provide a mechanism for lactate efflux during glycolysis. Thus, this newly discovered role of MCT1 in gastric restitution could lead to novel strategies for both facilitating restitution in gastrointestinal cells after injury and inhibiting metastasis of cancer and other cells that utilize glycolysis for migration.

1 Introduction

The layer of epithelial cells that lines the inner surface of the body's digestive system forms a barrier between digestive fluids and surrounding tissues and organs in the body cavity [1]. In the stomach, the integrity of this layer is necessary to prevent acidic gastric fluids from permeating into surrounding tissues, causing inflammation and widespread tissue damage. Because it is particularly prone to injury from food and/or ingested drugs [2], the body has evolved a repair mechanism called *restitution* that restores the structure and function of injured stomach lining [3].

Surface mucous cells comprise most of the epithelium that lines the stomach lumen. The mucus secreted by these cells protects the stomach from digested substances as well as from its own secreted hydrochloric acid (HCl). Other protective mechanisms exist, such as the secretion of bicarbonate, which neutralizes the acid, and the formation of tight junctions between cells, which form the basis of the epithelial barrier [2]. Surface cells are arranged at the top and upper sides of tubular gastric glands that extend downwards from the surface towards underlying muscle layers (Figure 1) [4]. Injury occurs in those cells that line the stomach lumen, where surface cells are most exposed [1]. Although all surface cells prior to injury are columnar in shape, in the first step of restitution, uninjured cells from the gastric pit transform into flat cells. These flattened cells are then able to migrate across the apical mucosal surface to cover the wounded area. The new cells at the surface repolarize to form a confluent layer by reestablishing the tight junctions that hold cells together in a tissue layer. Restitution is complete when the flattened cells have completely polarized into columnar epithelial cells. These newly populated cells are identical in structure and function to those that were lost from the apical surface upon injury [3]. Most importantly, this repair is rapid, taking place in two hours or less [2].

In the presence of oxygen, cells create energy in the form of ATP through a pathway

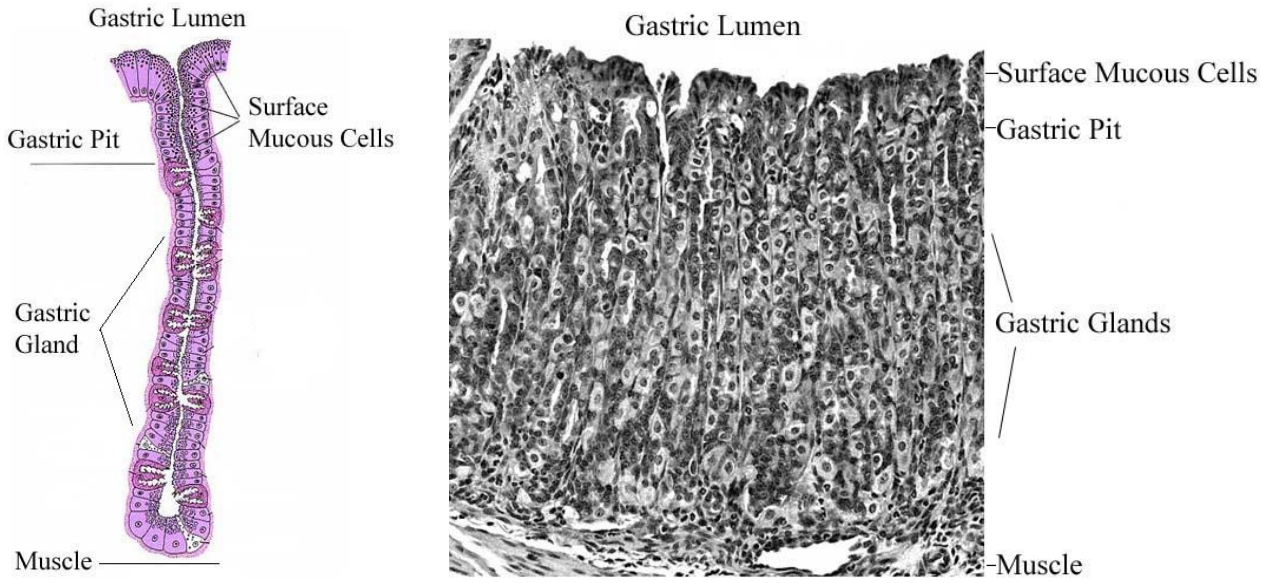

Figure 1: Left: Morphology of the gastric pit and gastric gland. After injury at the surface, cells at the bottom of the gastric pit migrate upwards to replenish denuded areas. Right: gastric pits open into the lumen of the stomach, while gastric glands extend downwards towards underlying smooth muscle.

beginning in the cytoplasm and ending in the mitochondrion. In the cytoplasm, glucose is broken down through a process called *glycolysis*, which yields two net ATP molecules. Upon completion of glycolysis, glucose has been converted into a molecule called pyruvate. Although glycolysis can occur under either aerobic or anaerobic conditions, cellular respiration in mitochondria will only take place if oxygen is present. Under low O_2 conditions, anaerobic metabolism of pyruvate takes place, where pyruvate is reduced to lactate. [5]

Although it was initially thought that restitution would be dependent on energy from mitochondrial respiration, recent studies showed that, in fact, glycolysis drives restitution. Cell migration after injury is dependent on glycolysis, while the reformation of tight junctions and cell polarization after migration is dependent on both glycolysis and respiration. Thus, restitution can occur even under anaerobic conditions. Interestingly, cell migration in the absence of oxygen occurs in many different cells, including tumor cells. [1]

Although glycolysis provides the energy needed for restitution, acidification due to the

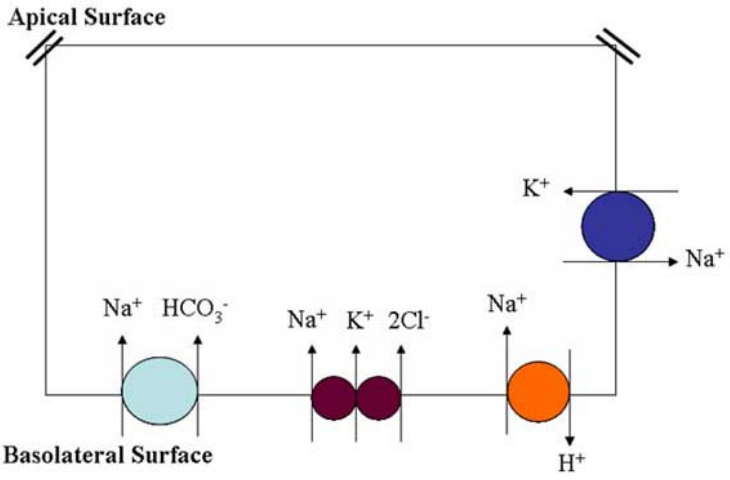

Figure 2: Gastric surface cells contain four known ion channels. Of these four, the Na^+/H^+ exchanger and the Na^+/HCO_3^- cotransporter are thought to play a role in pH regulation.

waste product lactate can poison migrating cells [3]. When intracellular pH becomes too low, glycolysis is inhibited, cells are unable to migrate, and they quickly undergo programmed cell death [6]. Thus, combined with an already high hydrogen ion (H^+) concentration in the stomach lumen, the presence of lactate inside the cell makes it imperative that ion transporters are present to regulate intracellular pH and to reduce intracellular lactate concentrations [7].

It is believed that surface cells have ion transporters to regulate intracellular pH by rapidly transporting H^+ out of the cell or by rapidly transporting bicarbonate (HCO_3^-) into the cell [3]. Currently, four distinct ion transporters have been identified within the gastric surface cell: Na^+/H^+ exchangers (NHE), Na^+/K^+ ATPases, Na^+/HCO_3^- cotransporters (NBC), and Na^+-K^+-Cl^- contransporters (Figure 2) [4]. Of these transport mechanisms, NHE and NBC especially are thought to play a role in maintaining intracellular pH [3]. However, recent studies demonstrated that other pH regulatory mechanisms must be activated during restitution, because blockade of existing transporters had little effect on restitution. A study by Hagen *et. al.* [3] showed that the compound DIDS, a potent inhibitor of NBC, completely inhibits restitution. Contradictory to this result, cells incubated in HCO_3^--free

conditions, which would also inhibit NBC, successfully undergo restitution, although slightly less effectively than under standard conditions. This suggests the presence of another ion transport mechanism that is inhibited by DIDS but not dependent upon HCO_3^-.

With high intracellular lactate concentrations predicted during restitution, we hypothesized that a lactate transporter must be required for lactate efflux during restitution. Lactate cannot freely cross a cell's plasma membrane, but instead must be transported through a special channel called a monocarboxylate transporter (MCT), which interestingly has H^+-coupled transport properties and thus also regulates intracellular pH [6]. The first MCT cloned, MCT1, was shown to be present in surface mucous cells at the basolateral membrane and is potently inhibited by bulky aromatic compounds such as the bioflavenoid phloretin [7]. To date, however, no function for this MCT has been identified in surface mucous cells.

In this study, we set out to discover the first known role of MCT1 in surface mucous cells. To do this, we inhibited MCT1 with phloretin, a known inhibitor of MCT1 [7], and showed a significant reduction in restitution under standard conditions with no reduction in cell viability. To test the hypothesis that DIDS blocks both NBC and MCT1 to inhibit restitution, we also used phloretin in HCO_3^--free conditions. This particular experiment showed complete inhibition of restitution demonstrating the dependence of restitution on both basolateral ion transport systems.

2 Materials and Methods

2.1 Preparation of RGM1 Cell Cultures

The cells used in this experiment were rat gastric mucosal (RGM1) cells, which are non-transformed and immortalized surface cells. RGM1 cells were established by Dr. H. Matsui of the Institute of Physical and Chemical Science (RIKEN) Cell Bank, Tsukuba, Japan [8]. Cells were grown in six-well plates and cultured in DMEM-F12 (1:1) supplemented with

10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 μg/ml amphotericin B [9]. All cells were starved in serum-free medium (DMEM-F12 containing 15 mM HEPES at pH 7.4) for 24 hours prior to experimentation. Cells were incubated at 37°C under 5% CO_2 in air. Experiments were performed in either standard (STD) buffer or bicarbonate-free (BF) buffer at pH 7.4.

2.2 Preparation of Buffer Solutions

The experimental medium was STD buffer [Appendix A]. To determine the role of HCO_3^- in the recovery of wounds, mannitol and HEPES were substituted for HCO_3^- to create a bicarbonate-free (BF) buffer [Appendix A]. STD buffer contained (in mM): 147 Na^+, 5 K^+, 131 Cl^-, 1.3 Mg^{2+}, 1.3 $SO_4{}^{2-}$, 2 Ca^{2+}, 15 HEPES, 20 D-Glucose, and 25 HCO_3^-. Total osmolarity of STD buffer was 347.6 mOsm. BF buffer contained (in mM): 147 Na^+, 5 K^+, 131 Cl^-, 1.3 Mg^{2+}, 1.3 $SO_4{}^{2-}$, 2 Ca^{2+}, 25 HEPES, 20 D-Glucose, and 15 Mannitol. Total osmolarity of BF buffer was 347.6 mOsm.

2.3 Preparation of Phloretin Solutions

In order to investigate the role of monocarboxylate transporters (MCT) in wound repair, wounded gastric cells were treated with phloretin, a known inhibitor of MCT1. Phloretin solutions were prepared in 100% DMSO and then diluted to 0 μM, 30 μM, and 60 μM in STD or BF buffer. The final concentration of DMSO was 0.1%.

2.4 Wounding

In order to mimic gastric injuries that heal through the process of restitution, small circular wounds were made in confluent monolayers of RGM1 cells. The wounds were made in each well of the cell culture plates using a pencil-type mixer with Teflon tip. After gentle

wounding, cells were washed twice with 1.5 mL STD or BF buffer. Buffer was then replaced with phloretin solutions. Two wells received 1.5 mL of 0 μM phloretin in STD of BF buffer, two wells received 1.5 mL of 30 μM phloretin in STD of BF buffer, and two wells received 1.5 mL of 60 μM phloretin in STD of BF buffer. Following wounding, cell plates were imaged and then returned to 37°C.

2.5 Microscopy

Wounds were imaged 0, 4, and 8 hours after the addition of phloretin. Cell plates were placed in the incubator chamber of a Nikon TE300 Inverted Microscope and photos were taken using a Hamamatsu Orcha digital camera. Wound area was measured using IPLab, purchased from Scanalytics, Inc.

2.6 Crystal Violet Viability Assay

Following the 8 hour experiment, a viability assay was conducted to ensure that the experimental conditions did not effect cell viability. Culture medium from each well was aspirated into a separate conical tube and reserved for pH measurements using a pH meter. All cells were washed twice with 1.5 mL PBS. Cells were fixed with 100% ice cold methanol and incubated for 15 min. at room temperature. Cells were stained with crystal violet (1% crystal violet, 9% methanol in PBS). The stain was incubated for 5 min. at room temperature before cells were washed 10 times with warm water. Cells were air dried overnight. Each well was solubilized with 1.5 mL 0.5% SDS for 30 min at room temperature using an environmental shaker. 50 μL of lysate from each well of the six-well plates was then transferred to a well of a 96-well plate. 200 μL of 0.5% SDS was added to each well of the 96-well plate. Absorbance was read at an excitation of 590 nm and emission of 640 nm. Data were calculated as percent viability when compared to cultures that received no treatment.

2.7 Statistical Analysis of Results

Statistical analysis was done with SigmaStat software (Jandel Scientific Software, San Rafael, CA). Data from wound area experiments (n=6/treatment) and viability experiments (n=6/ treatment) were averaged and reported as means ± standard error. A one-way ANOVA test was performed to compare controls to each level of drug concentration, with differences regarded as statistically significant at $P<0.05$. A t-test was performed to compare STD and BF conditions, with differences regarded as statistically significant at $P<0.05$. For all data presented, statistical differences were highly significant at $P<0.001$.

3 Results

3.1 Restitution in Control RGM1 Cells

Control cells were grown in STD buffer and thus contained functioning NBC and MCT transporters. After wounding and incubation for 4 hours in STD buffer, wound area was (85.1 ± .7)% of initial wound area. By 8 hours, control wound area was (73.2 ± .6)% of initial wound area (Figure 3A and C).

3.2 Restitution in HCO_3^--free Conditions

HCO_3^--free (BF) conditions were used to test the hypothesis that blockade of NBC inhibits restitution in RGM1 cells. After a 4 hour incubation, the wounds of cells grown in BF buffer in the absence of phloretin had restituted to (88.8 ± .5)% of initial wound area. By eight hours, BF wounds had restituted to (80 ± 1)% of initial wound area (Figure 3A and C). That cultures incubated in BF conditions restituted at a rate significantly slower than that of cultures in STD buffer demonstrates that the presence of a HCO_3^- transport mechanism, such as NBC, is required for restitution in RGM1 cells ($t=-6.031$, $P<0.001$).

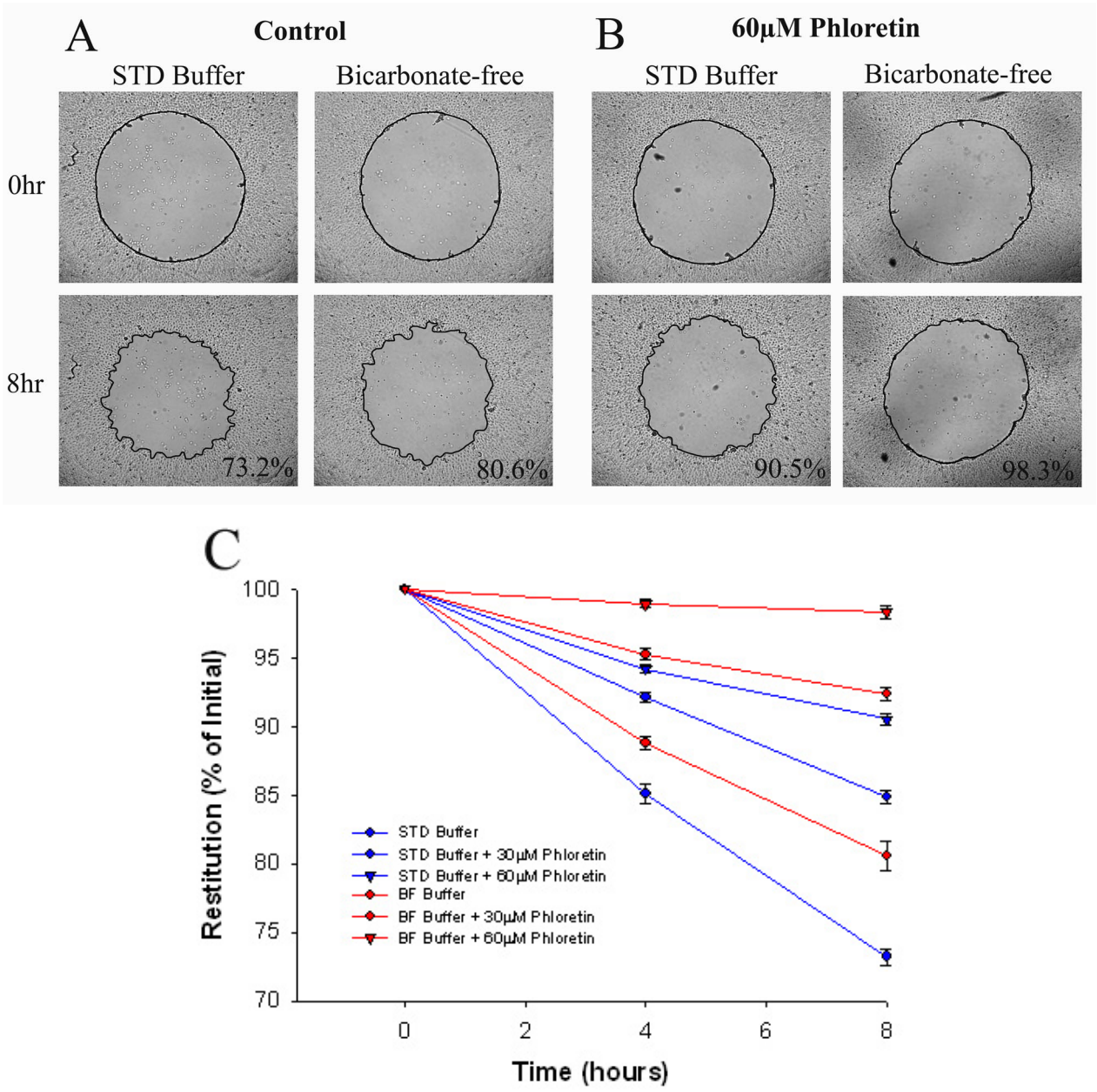

Figure 3: A: Images of wounded RGM-1 cells under both STD and BF conditions at time 0 hours and 8 hours. B: Images of wounded RGM-1 cells incubated with 60μM phloretin under both STD and BF conditions at time 0 hours and 8 hours. C: A graph of the effect of phloretin solutions on restitution in cultured cells (n=6/treatment, P<0.001).

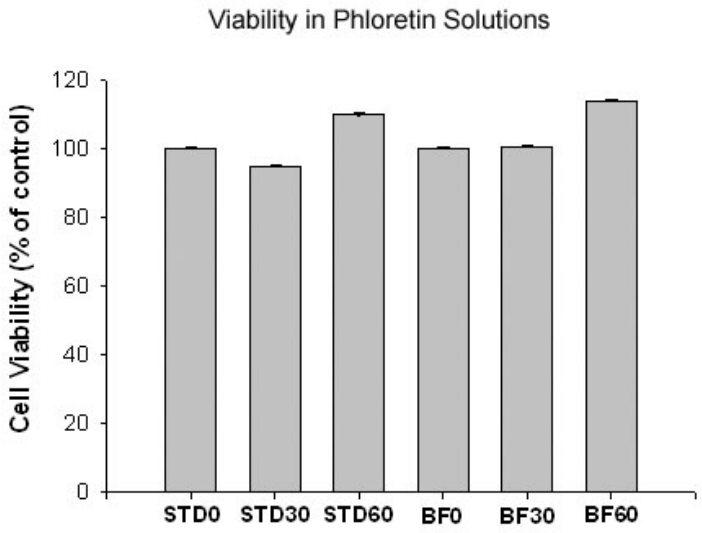

Figure 4: Cell viability data in STD and BF buffer with varying concentrations of phloretin. It was determined that neither phloretin nor HCO_3^--free conditions compromised cell viability (n=6/treatment, $p<0.05$).

However, considering the lack of the full inhibition of restitution that occurs when DIDS is added to inhibit NBC, other DIDS-inhibitible transporters, such as MCT1, must be required for restitution.

3.3 Restitution in Phloretin Solutions

Phloretin was used in varying concentrations to test the effect of MCT1 inhibition on restitution in cultured cells (Figure 3B). At a concentration of 30μM phloretin in STD buffer, restitution was (92.1 $\pm$ .3)% at 4 hours and (84.9 $\pm$ .5)% at 8 hours. At a concentration of 60μM phloretin, restitution was (94.2 $\pm$ .3)% at 4 hours and (90.5 $\pm$ .5)% at 8 hours (Figure 3B and C). At a concentrations of 30μM phloretin in BF buffer, restitution was (95.3 $\pm$ .4)% at 4 hours and was (92.3 $\pm$ .5)% at 8 hours (Figure 3C). 60μM phloretin in BF buffer completely inhibited restitution, with only (1.7 $\pm$ .4)% recovery by 8 hours (Figure 3B and C). Results at each phloretin concentration were statistically significant from control levels

under both STD and BF conditions ($P<0.001$). That inhibition of MCT1 with phloretin significantly and dose-dependently inhibits restitution shows that MCT1 in conjunction with NBC is critical for maximum wound repair in RGM1 cells.

3.4 Cell Viability

A crystal violet viability assay was conducted in order to ensure that the experimental conditions did not affect cell viability. It was found that cells were confluent and 100% viable after 8 hours of incubation with all experimental solutions (Figure 4).

4 Discussion

In this study, bicarbonate-free conditions were used in conjunction with phloretin to test the hypothesis that DIDS inhibits restitution by blocking both MCT1 and NBC, rather than NBC alone. Using bicarbonate-free buffer to inhibit NBC and phloretin to inhibit MCT1, it was found that although inhibition of NBC has an effect on restitution, the effect of MCT1 inhibition was three times greater. This result suggests that MCT1 activity is essential for attaining the maximum rate of restitution. These results are novel and have not been reported for other migration or wounding studies.

Although phloretin potently inhibits MCT1 at low concentrations [7], the drug is non-specific and blocks other ion transporters, channels, and intracellular signaling pathways. First among these are members of the protein kinase C (PKC) family [10], protein kinases that have been implicated in a variety of cellular functions including proliferation, apoptosis, differentiation, motility and inflammation [11]. PKC is found in a variety of cell types, including the gastric epithelia [11]. Recent experiments have demonstrated that inhibition of PKC inhibits restitution in guinea pig gastric mucosa [12], rabbit gastric epithelial cells [13], and colonic epithelial cells [14]. However, inhibition of PKC has been demonstrated to have

an inhibitory effect on restitution only at concentrations of 200-300μM. In fact, phloretin is most specific for inhibiting PKC at concentrations of 200-250μM [15], about 4-fold higher than those used in this experiment. Although it is unlikely that our concentrations of 30 and 60 μM phloretin were sufficient to inhibit restitution via PKC inhibition, positive control studies should be conducted using GF 109203X, another known PKC inhibitor [16].

Phloretin is also known to activate large conductance Ca^{2+}-activated-K^+ channels (BK(Ca)) [17]. However, although there is evidence that BK(Ca) channels are present in smooth muscle [18] [19] and neurons [20], there is no literature to support the presence of BK(Ca) in gastric epithelial cells. Positive control studies could be conducted with 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifluoromethyl-2H-benzimidazol-2-one, another known activator of BK(Ca) [21], to rule-out involvement of this channel in restitution.

Lastly, phloretin is a known inhibitor of many isoforms of glucose channels [22]. Although the specific glucose channels present in the gastric mucosa are not known, a study in frog gastric tissue conducted by Hagen *et al.* [1] showed that glucose-free conditions had no inhibitory effect on restitution. Preliminary data in RGM1 cultured cells for this study also indicate no inhibitory effects of glucose-free buffer on restitution.

Thus, given that the other known effects of phloretin are unlikely to account for our results, we conclude that the inhibition we see in this experiment is due to phloretin inhibition of MCT1.

The discovery that MCT1 is critically involved in restitution could support a novel model for both the regulation of energy and pH regulation in migrating gastric surface cells after injury. Although cell migration in restitution is driven by glycolysis, which under some conditions yields the waste-product lactate, accumulation of lactate within the cell can actually inhibit migration and lead to programmed cell death. As an H^+-coupled lactate transporter, MCT1 can transport lactate molecules through the otherwise impermeable cellular membrane and additionally prevent intracellular acidification by the efflux of H^+, allowing cell

migration to continue. We hypothesize that phloretin's potent inhibitory effects on restitution are demonstrative of its interference with MCT's pH regulation mechanism. However, further studies will be needed to fully elucidate the effects of MCT1 on restitution by specific blockade of MCT1 using siRNA technology.

Our results are immediately applicable to the study of maintenance of the gastric mucosa. Rapid restitution is required both in daily life and in disease to prevent acidic gastric juices from causing tissue damage and ulcers. We show that the rapid and efficient restitution that drives both daily maintenance and repair after disease is dependent upon MCT1 activity. In addition, the newly discovered role of MCT1 in migrating cells during gastric restitution can potentially extend to existing models of migration in other cell types, especially those that migrate under either aerobic or anaerobic conditions by utilizing glycolysis, such as the repair of skin epithelial cells and the migration of lymphocytes and cancer cells. In this way, our work in gastric restitution could lead to novel strategies for both facilitaing restitution in skin cells after burn injury and inhibiting metastasis of cancer.

5 Conclusion

We have found that inhibition of MCT1 with phloretin and concurrent inhibition of NBC with HCO_3^--free conditions results in complete inhibition of restitution after injury. In individual experiments, HCO_3^--free conditions had only a minimal effect on restitution, suggesting that bicarbonate transport by NBC is not particularly critical for restitution. However, the significant inhibition of restitution effected by phloretin in standard physiological conditions suggests that MCT1 is essential to attain maximum rates of restitution. Although phloretin is nonspecific, inhibiting other ion transporters, channels, and intracellular signaling pathways, we eliminated such nonspecific effects as explanations for our results through analysis of dosage thresholds and preliminary data on the inhibition of other ion

channels. Our results show that maintenance and repair after disease of the gastric mucosa is dependent upon MCT1 activity. In addition, this novel role of MCT1 can possibly extend models of migration in other cell types, especially those that require glycolysis to migrate under aerobic or anaerobic conditions.

6 Acknowledgments

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A Buffer Recipes

Compound	mM	FW	1L (g)	100mL (g)	500mL (g)
NaCl	122	58.44	7.13	0.713	3.57
KCl	5	74.56	0.373	0.0373	0.187
$MgSO_4$	1.3	246.5	0.320	0.032	0.16
$CaCl_2$	2	147.02	0.294	0.0294	0.147
HEPES	15	238.31	3.58	0.358	1.79
D-glucose	20	180.16	3.603	0.36	1.80
$NaHCO_3^-$	25	84.01	2.1	0.455	2.28

Table 1: Recipe for standard buffer

Compound	mM	FW	1L (g)	100mL (g)	500mL (g)
NaCl	122	58.44	7.13	0.713	3.57
KCl	5	74.56	0.373	0.0373	0.187
$MgSO_4$	1.3	246.5	0.320	0.032	0.16
$CaCl_2$	2	147.02	0.294	0.0294	0.147
Na^+-HEPES	25	260.3	6.51	0.651	3.255
D-glucose	20	180.16	3.603	0.36	1.80
Mannitol	15	182.2	2.73	0.273	1.37

Table 2: Recipe for bicarbonate-free buffer