Extracellular Zinc Triggers ERK-dependent Activation of Na\(^+\)/H\(^+\) Exchange in Colonocytes Mediated by the Zinc-sensing Receptor*

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Extracellular zinc promotes cell proliferation and its deficiency leads to impairment of this process, which is particularly important in epithelial cells. We have recently characterized a zinc-sensing receptor (ZnR) linking extracellular zinc to intracellular release of calcium. In the present study, we addressed the role of extracellular zinc, acting via the ZnR, in regulating the MAP kinase pathway and Na\(^+\)/H\(^+\) exchange in colonocytes. We demonstrate that Ca\(^{2+}\) release, mediated by the ZnR, induces phosphorylation of ERK1/2, which is highly metal-specific, mediated by physiological concentrations of extracellular Zn\(^{2+}\) but not by Cd\(^{2+}\), Fe\(^{2+}\), Ni\(^{2+}\), or Mn\(^{2+}\). Desensitization of the ZnR by Zn\(^{2+}\), is followed by ~90% inhibition of the Zn\(^{2+}\)-dependent ERK1/2 phosphorylation, indicating that the ZnR is a principal link between extracellular Zn\(^{2+}\) and ERK1/2 activation. Application of both the IP\(_3\) pathway and PI 3-kinase antagonists largely inhibited Zn\(^{2+}\)-dependent ERK1/2 phosphorylation. The physiological significance of the Zn\(^{2+}\)-dependent activation of ERK1/2 was addressed by monitoring Na\(^+\)/H\(^+\) exchanger activity in HT29 cells and in native colon epithelium. Preincubation of the cells with zinc was followed by robust activation of Na\(^+\)/H\(^+\) exchange, which was eliminated by cariporide (0.5 \(\mu M\)); indicating that zinc enhances the activity of NHE1. Activation of NHE1 by zinc was totally blocked by the ERK1/2 inhibitor, U0126. Prolonged acidification, in contrast, stimulates NHE1 by a distinct pathway that is not affected by extracellular Zn\(^{2+}\) or inhibitors of the MAP kinase pathway. Desensitization of ZnR activity eliminates the Zn\(^{2+}\)-dependent, but not the prolonged acidification-dependent activation of NHE1, indicating that Zn\(^{2+}\)-dependent activation of H\(^+\) extrusion is specifically mediated by the ZnR. Our results support a role for extracellular zinc, acting through the ZnR, in regulating multiple signaling pathways that affect pH homeostasis in colonocytes. Furthermore activation of both, ERK and NHE1, by extracellular zinc may provide the mechanism linking zinc to enhanced cell proliferation.

Arrested cell proliferation is a hallmark of zinc deficiency. This is particularly true in gastrointestinal cells (1–4), where insufficient dietary zinc attenuates the renewal of the epithelium leading to severe diarrhea (1). Extracellular zinc has been shown to regulate cell proliferation via the MAP1 kinase pathway in several cell types (5–7). Although the mitogenic and anti-apoptotic effects of zinc are well recognized (8, 9), and the treatment of severe diarrhea by addition of dietary zinc is common, the direct link between this ion and the cellular mechanisms regulating proliferation is not well understood. It has been suggested that a decrease in intracellular zinc may lead directly to a reduction in activity of various metalloenzymes involved in transcription and cell metabolism (1, 10).

Several studies, however, indicate that extracellular zinc acts as a signaling molecule. In tracheal cells, for example, extracellular zinc, through activation of Src, leads to transactivation of EGFR and subsequently to activation of ERK1/2 (11). In fibroblasts, extracellular zinc has been shown to trigger the activation of the PI3K pathway, subsequently leading to the activation of AKT and the S6 kinase (12). The signaling pathways linking extracellular zinc to these proteins and to subsequent regulation of cellular ion, pH or volume homeostasis, however, remain poorly understood.

We have recently identified and characterized an extracellular zinc-sensing receptor (ZnR) that triggers, upon exposure to extracellular zinc, the release of Ca\(^{2+}\) from intracellular stores by activation of the IP\(_3\) pathway (13). The pharmacological profile of the calcium response triggered by the ZnR, particularly its sensitivity to the PLC inhibitor, U73122, and the inhibitory effect of the IP\(_3\) receptor blocker, 2-APB, indicates that the ZnR is a G\(_{q}\)-coupled receptor (GPCR). Both the Go and the G\(_{q}\) dimer of various GPCRs have been linked to activation of the MAP kinase via multiple intracellular pathways (14–16).

In intestinal cells, furthermore, the muscarinic receptor has been shown to play a key role in promoting ion transport through activation of MAP and PI 3-kinase signal transduction (17, 18). A role for the ZnR in activation of the MAP kinase pathway is demonstrated in this work.

The ZnR was initially characterized in the colonocytic cell line, HT29, where a robust calcium signal was generated following activation of the receptor by changes in the concentration of extracellular Zn\(^{2+}\) (13). Importantly, the Ca\(^{2+}\) response induced by the ZnR in HT29 cells is triggered by ~80 \(\mu M\) zinc, i.e. within the physiological range of zinc concentrations found in the digestive tract (19, 20). Interestingly, activation of the ZnR also resulted in activation of Na\(^+\)/H\(^+\) exchange in colonocytes. Distinct NHE isoforms are expressed in the colonic mucosa, each playing a unique physiological role in colonic physiology (21, 22). It was not known, however, which of the NHE

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¶The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; ZnR, zinc-sensing receptor; PI, phosphatidylinositol; PLC, phospholipase C; PKC, protein kinase C; IP, inositol phosphate; CaM, calcium-calmodulin; PMA, phorbol 12-myristate 13-acetate.

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isoforms is regulated by the ZnR. The first NHE isoform, NHE1, is mostly expressed on the basolateral membrane of colonocytes and is involved in regulating the pH homeostasis (22–25). Such activity is of prime importance considering the major changes in osmolarity and the acid load imposed by the permeation of short chain fatty acids generated by bacterial fermentation (24, 26). Activation of NHE1, moreover, enhances cell survival by inhibiting caspase activity and by enhancing the PI3K pathway (27). The other NHE isoforms expressed in the colon are NHE3 which is involved in vectorial solute transport, but is not expressed in HT29 cells (25), and NHE2 which has been suggested to mediate butyrate-stimulated sodium absorption (22, 28). In the current study, we sought to identify the principal pathways activated by extracellular Zn²⁺ and to determine what role, if any, ERK1/2 plays in mediating the above described zinc-dependent regulation of the Na⁺/H⁺ exchanger.

Our results indicate that extracellular Zn²⁺, acting through the ZnR, triggers activation of ERK and PI 3-kinase pathways. Subsequently, the zinc-dependent activation of ERK1/2 leads to a robust and specific activation of NHE1 in HT29 colonocytes and similarly in murine native colon epithelium. Thus, our results provide a cellular mechanism linking the well known effects of zinc to cellular proliferation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HT29-Cl cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) as previously described (29, 30). Cells grown on 60-mm plates were serum-starved for 24 h, and then washed in Ringer’s solution for 30 min. The cells were subsequently stimulated in Ringer’s solution supplemented with ZnSO₄ (80 μM), for 10 min. The zinc chelator, Ca-EDTA (100 μM), was added to the Ringer’s solution to reduce residual zinc in control cultures. Agonists and inhibitors used for the analysis of the various signal transduction pathways were added prior to the addition of zinc, during the wash in Ringer’s solution, for the indicated times. Cells were then harvested into lysis buffer as previously described (31), in the presence of protease inhibitor mixture (Complete, Roche Applied Science) and centrifuged for 30 min (14,000 rpm). Supernatants (cytosolic fraction) were collected, SDS sample buffer was added, the samples were boiled for 5 min and then frozen at −80 °C until used.

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**Tissue Preparation**—Wistar rats (70–150 g, n = 4) were killed, and the colon removed and washed using Parsons solution as previously described (32). Briefly, a longitudinal incision was made along the colon wall and about 5 tissue samples were cut from the distal part of the colon. The tissue was spread, keeping the mucosal-luminal side upwards, on coverslips using cyanoacrylate glue (24). The tissue samples...
were kept at 37 °C in high K + solution for no longer than 3 h (32). In each experimental group, the results are the mean of at least 15 cells from three independent experiments from each animal.

**MAP Kinase Activation**—Cell samples containing the cytosolic fraction (20 µg) were separated on 7.5% SDS-PAGE followed by immunoblotting (29, 30). Antibodies against doubly phosphorylated ERK1/2 (33) and total ERK1/2 or phosphorylated AKT and total AKT (Sigma and Cell Signaling) were detected digitally using ChemiImager 5 (Alpha-Innotech, Labtrade), and the blots quantified using the Chemi-Imager software. Phospho-ERK1/2 or AKT levels were normalized against the total ERK1/2 or AKT protein, respectively. Phosphorylation of ERK1/2 is presented as a percentage of the effect triggered by application of 80 µM zinc. Each graph represents an average of at least three independent experiments. Statistical analysis was performed using unpaired Student’s t test assuming equal variance, comparing each treatment to zinc: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Fluorescent Imaging**—Fluorescent imaging measurements were acquired as previously described (13). Briefly, for [Ca 2+] i and [Zn 2+] i measurements, cells were incubated with 5 µM Fura-2 AM (TEF) for 30 min in 0.1% bovine serum albumin in Ringer’s solution. For pH measurements, cells were loaded with 1.25 µM BCECF-AM for 12 min.

Following dye loading, the coverslips were mounted in a flow chamber. For pH calibration, nigericin was added to KCI Ringer’s (120 mM KCl replacing NaCl) solution at pH 6.8, 7, and 7.2, the relative fluorescence was monitored, and a linear calibration curve produced (34, 35). The NH 4 Cl prepulse paradigm was applied to estimate Na + /H + exchanger activity. Briefly, cells were washed with Ringer’s containing NH 4 Cl (30 mM), and an intracellular equilibrium of NH 4 + and NH 3 was reached. Replacing the extracellular buffer with Na+-free Ringer’s (iso-osmotically replaced by NMG) caused rapid acidification of the cells. Na + /H + exchanger activity was estimated by calculating the rate of recovery (d pH/dt) following addition of Na + to the Ringer’s solution. The results shown are the means of 4–6 independent experiments, with averaged responses of 30 cells in each experiment. Statistical analysis was performed as described above, completing the activity of NHE in treated cells versus control cells.

For imaging experiments of the native colonic tissue, the coverslips were incubated with BCECF (5 µM) in the presence of 0.05% pluronic acid for 30 min and then washed with 0.1% bovine serum albumin in Ringer’s solution for 10 min before mounting on the stage of an Olympus BX-50 microscope. The pH measurements were performed only using crypts that the in epithelial cells clearly surrounded the lumen. The NH 4 Cl prepulse paradigm and analysis was performed as described above.

**RESULTS**

**Specificity and Temporal Dependence of ERK1/2 Phosphorylation by Zinc**—To determine if zinc, at physiological concentrations shown previously to activate the ZnR, will activate the MAP kinase pathway, analysis of time dependence was performed. Phosphorylation of ERK1/2 was monitored by Western blots using antibodies recognizing the doubly phosphorylated, active form of ERK1/2 in serum-starved HT29 cells. To determine the time course of ERK phosphorylation, zinc (80 µM) was applied for the indicated times and phosphorylation of ERK1/2 monitored (Fig. 1 A). Zinc-dependent phosphorylation of ERK1/2 was apparent already 2 min following application, peaking at 30 min. Phosphorylation was reduced after 60 min by about 30%, and approached resting levels after 2 h. Only very slight staining of the two bands was detected when extracellular residual zinc was chelated by Ca-EDTA. No significant change was detected in the total-ERK1/2 following addition of zinc. Application of the MEK1/2 inhibitors (U0126, PD98095) for 10 min in Ringer’s solution, inhibited Zn 2+-dependent ERK1/2 phosphorylation (Fig. 1B), indicating that the effect is mediated through activation of MEK1/2.

The MAP kinase pathway can be activated by a number of different heavy metals (6). We sought, therefore, to determine if ERK1/2 activation in colonocytes is metal-specific. Cells were exposed to the indicated heavy metals (100 µM, 10 min), and the resulting phosphorylation of ERK1/2 was compared with the effect triggered by zinc (Fig. 1 C). As shown, application of Mn 2+, Ni 2+, Cd 2+, or Fe 2+ failed to trigger activation of ERK1/2. In contrast, Zn 2+ was highly potent in triggering ERK phosphorylation, indicating that in colonocytes, zinc plays a prominent and highly specific role in the activation of this important intracellular signaling pathway.

**ERK1/2 Is Activated by Extracellular Zinc via the ZnR**—While the above results suggest that zinc specifically triggers ERK1/2 phosphorylation, they do not specify the role of extracellular zinc. To address this, we examined whether cellular Zn 2+ influx is apparent at concentrations and time intervals required for ERK1/2 phosphorylation in HT29 cells loaded with Fura-2 AM. This dye, known primarily as a Ca 2+-sensitive indicator, has a 100-fold higher affinity to Zn 2+ (K d [Zn 2+] 1/2 ~2 nm) (36), that is, at least 10-fold higher than the zinc probe previously used to monitor zinc in HT29 cells, Mag-fura (5, 37). Thus, Fura-2 AM is a highly sensitive dye for monitoring even minute changes in intracellular zinc concentrations. Potential

![Fig. 2. Extracellular zinc triggers ERK1/2 phosphorylation. A, zinc concentration was assessed using a single-cell imaging system. The cells were loaded with Fura-2 AM and then superfused with Ringer’s solution, at the time marked, 100 µM zinc was applied. Colonocytes were pretreated (not shown) with thapsigargin (200 nm) to deplete the intracellular calcium stores and thereby to inhibit the zinc-dependent calcium rise. Subsequently zinc was added to the perfusing solution (indicated). No change in fluorescence was observed in HT29 colonocytes. For comparison, Min-6 insulinoma cells loaded with Fura2 AM were treated with zinc, and a significant fluorescence rise is observed. B, dose dependence plot. HT29 cells were challenged with the indicated concentrations of zinc for 10 min. Our results show that a brief exposure to zinc, at physiological concentrations, triggers ERK1/2 phosphorylation.](http://www.jbc.org/content/383/29/51806)
interference by intracellular Ca\(^{2+}\) was eliminated prior to application of zinc by depleting the intracellular Ca\(^{2+}\) stores with thapsigargin (200 nM, not shown) and by the use of nominally Ca\(^{2+}\)-free solutions. As shown in Fig. 2A, there was no apparent rise in Fura-2 fluorescence when HT29 cells were perfused with zinc-containing Ringer’s solution for 10 min following Ca\(^{2+}\) store depletion. The sensitivity of this approach is demonstrated by monitoring Zn\(^{2+}\) influx, induced by opening of L-type calcium channels by depolarization, of Min6 insulinoma cells. Application of the intracellular zinc chelator, TPEN (50 \(\mu\)M), reduced the fluorescent signal to resting levels (not shown), indicating that it is indeed triggered by zinc. At time intervals sufficient to trigger a robust activation of ERK, therefore, no zinc influx was apparent in HT29 cells, supporting our hypothesis that ERK1/2 phosphorylation is mediated by extracellular Zn\(^{2+}\).

To study the physiological significance of ERK1/2 activation by zinc, a dose response analysis was performed. Previous studies have employed zinc in the presence of serum (5, 38). A major drawback of this approach is that the fraction of the zinc ions bound to serum components is difficult to estimate, the result being that the free zinc concentration under such conditions is unknown. We therefore used zinc in Ringer’s solution, such that the free zinc concentrations obtained (calculated using Geochem software, Ref. 13) are similar to those present in the digestive tract (19, 20). Phosphorylation of ERK1/2 was
monitored in cells exposed to the indicated concentrations of free zinc for 10 min (Fig. 2B) and was already apparent using 40 μM zinc. The effect of zinc on ERK phosphorylation was maximal at 80 μM and at 150 μM was still 10 ± 0.2-fold higher than controls. This was reduced by ~30% at higher zinc concentrations (200 μM). The results of this analysis show that zinc at concentrations found in the digestive tract (19, 20, 39), which induce Ca²⁺ release through the ZnR (13), also trigger phosphorylation of ERK1/2.

We next sought to determine the specific role played by the ZnR in mediating zinc-dependent ERK1/2 phosphorylation. To do this, we exploited the fact that the ZnR undergoes a profound functional desensitization that remains refractory for 3 h following exposure to 100 μM Zn²⁺ for 30 min. The ZnR-dependent Ca²⁺ release following reapplication of Zn²⁺ recovered to ~10 and 20% after 4 and 6 h, respectively (Fig. 3A). As shown in Fig. 3B, zinc desensitization of the ZnR was also followed by a similar pattern of inhibition of the zinc-dependent phosphorylation of ERK1/2. The inhibition of ERK1/2 phosphorylation, monitored 3 h after the desensitization, was almost complete (90 ± 5%) and was still apparent even after 6 h, yielding an ~50% inhibition. Phosphorylation of ERK1/2 induced by PMA...
(Fig. 3C) was used as a positive control to determine if the MAPK pathway, following zinc desensitization, remained responsive. No significant changes were detected in ERK1/2 phosphorylation induced by PMA in cells that were desensitized versus control cells, indicating that the ERK1/2 pathway was functional. The striking effect of ZnR desensitization, and the similarity between the patterns of the Ca$^{2+}$ response and the zinc-dependent ERK1/2 activation suggest that a functional ZnR is essential for mediating zinc-dependent activation of the MAP kinase pathway.

**ERK1/2 Activation by Extracellular Zn$^{2+}$ Is Mediated by Ca$^{2+}$-dependent and -independent Pathways—**Activation of ERK has previously been demonstrated to be mediated by intracellular Ca$^{2+}$ released via the IP$_3$ pathway, such as that
triggered by zinc via the ZnR in epithelial cells, ATP in astrocytes or by the cholinergic agonist, carbachol, in intestinal cells (18, 40–42). However, cell signaling upstream or distinct from the IP3 pathway may also play a role in Zn2+-mediated ERK activation. We have, therefore, addressed the role of the Ca2+ rise in ERK activation by chelating intracellular Ca2+ using BAPTA-AM (25 μM, applied for 15 min prior to addition of zinc). As shown in Fig. 4A, BAPTA partially reduced zinc-dependent ERK1/2 phosphorylation, resulting in 60 ± 3% inhibition of Zn2+-dependent activation. To further elucidate the role of upstream components of the IP3 pathway, cells were pretreated with the PLC inhibitor, U73122 (4 μM, applied for 15 min). Application of the PLC inhibitor resulted in 40 ± 4% inhibition of ERK phosphorylation (Fig. 4A), similar to the effect of Ca2+ chelation. These results indicate that a calcium rise, triggered by IP3 pathway activation, plays a role in mediating zinc-dependent ERK activation in HT29 cells. Calcium-calmodulin (CaM) kinase II is activated by intracellular calcium rise and has been suggested to trigger MAP kinase activation, possibly by inhibiting Ras-GTPase-activating protein (RAS-GAP), thereby leading to Ras activation (43–45). We studied the role of CaM kinase II in Zn2+-dependent phosphorylation of ERK1/2 using the CaM kinase II inhibitors, KN93 and KN62 (applied for 30 min in Ringer’s solution). As shown in Fig. 4A, the inhibitory effect of CaM kinase II inhibitors (40 ± 2%) was similar to that of the upstream inhibitors, U73122 and BAPTA, suggesting that calcium-dependent ERK1/2 phosphorylation by the ZnR is mediated by CaM kinase II.

A calcium rise like that mediated by the ZnR may trigger PKC activation. However, the PKC inhibitor bisindolylmaleimide I (BD) did not attenuate the zinc-dependent activation of ERK (Fig. 4B), suggesting that Zn2+-dependent activation of ERK1/2 is not mediated by PKC in colonocytes. This finding is consistent with previous studies demonstrating that PKC does not participate in ERK1/2 activation in colonocytes (46).

The residual phosphorylation of ERK1/2 following inhibition of the [Ca2+]i rise, suggests that other signaling pathways, distinct from the IP3 and CaM kinase II pathways, mediate zinc-dependent ERK1/2 phosphorylation in the colonocytes. We have focused on the PI 3-kinase pathway because of its key role in promoting colonocyte proliferation (47). As shown in Fig. 5A, application of zinc at concentrations that activate ZnR-mediated Ca2+ release and ERK1/2 phosphorylation, also resulted in phosphorylation of AKT. Application of wortmannin, an inhibitor of PI 3-kinase, resulted in complete elimination of AKT phosphorylation, indicating that the PI 3-kinase pathway is mediating zinc-dependent AKT activation. We next sought to determine the role of this pathway in linking zinc to the activation of ERK1/2. As shown in Fig. 5B, application of wortmannin (30 nM) inhibited the Zn2+-dependent ERK1/2 phosphorylation by ~40%.

To determine if the PI 3-kinase pathway and the calcium-dependent pathway act additively, cells were co-treated with wortmannin and KN93 (100 nM and 50 μM, respectively for 30 min in Ringer’s solution). Inhibition of these two pathways resulted in elimination of Zn2+-dependent ERK1/2 phosphorylation by ~75% (Fig. 5C), supporting our contention that both the PI 3-kinase and IP3 pathways play a major role in linking extracellular Zn2+ to phosphorylation of ERK1/2 in colonocytes.

The Role of Zinc-dependent ERK Activation in Regulating Na+/H+ Exchange—The Na+/H+ exchanger in the colon plays a key role in solute transport and pH homeostasis. The latter function is of particular importance considering the high con-
centration of short chain fatty acids generated by bacterial fermentation in the gut lumen (25). In a previous study, we demonstrated that the ZnR activates Na\(^+/H^+\) exchange (13). In the present work, we sought to determine: 1) whether zinc regulates Na\(^+/H^+\) exchange in native colon epithelium; 2) which NHE isoform is regulated by zinc; 3) through which signal transduction pathway ZnR activates the NHE.

Initially, HT29 cells were loaded with the pH-sensitive dye, BCECF, and imaged for activity of the Na\(^+/H^+\) exchanger using the NH\(_4\)Cl prepulse paradigm, following preexposure to zinc (2 min, 100 \(\mu\)M). As shown in Fig. 6, A and B, preincubation with zinc was followed by a robust stimulation (20-fold) of H\(^+\) efflux mediated by Na\(^+/H^+\) exchange in the HT29 colonocytes. The stimulatory effect of zinc, reported here is greater than that observed in our previous paper (13), presumably because the paradigm used for activation of the exchanger in the present study was consistent with the temporal pattern of ERK activation by zinc. We then used the same experimental paradigm to study the effect of Zn\(^{2+}\) using a rat colonic tissue preparation (see "Experimental Procedures"). As shown in Fig. 6, C and D, application of Zn\(^{2+}\) in this preparation resulted in a 7-fold activation of Na\(^+/H^+\) exchange.

To determine which NHE isoforms are regulated by zinc in HT29 cells and in the native rat colonic epithelium, the specific NHE1 inhibitor (cariporide, 0.5 \(\mu\)M) was applied (48). As shown in Fig. 7, in both the HT29 cells and in the native epithelium the application of cariporide totally blocked the activation of Na\(^+/H^+\) exchange triggered by zinc in HT29 cells and colon epithelium, indicating that zinc regulates the activity of NHE1. *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\) versus pH recovery in control cells.

FIG. 7. Zinc activates the NHE1 isoform. HT29 cells were treated with Zn\(^{2+}\) as described in Fig. 6, and the NH\(_4\)Cl prepulse paradigm was performed. The rate of the Na\(^+/\) dependent H\(^+\) efflux was determined in the presence of various concentrations of zinc or in the presence of the NHE1 inhibitor, cariporide (0.5 \(\mu\)M). An example for the effect of cariporide is shown in A and the averaged rates of pH recovery from five independent experiments in each group are shown in B. The same experimental paradigm was applied to colon epithelium in the presence of cariporide (0.5 \(\mu\)M) (C and D). Cariporide completely eliminated the activation of Na\(^+/H^+\) exchange triggered by zinc in HT29 cells and colon epithelium, indicating that zinc regulates the activity of NHE1. *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\) versus pH recovery in control cells.
We next sought to determine whether the zinc-dependent activation of NHE1 is mediated by ERK1/2 in colonocytes. To determine the role of the MAP kinase pathway in zinc-dependent exchange activity, we added the specific ERK1/2 inhibitor U0126 (1 μM) while applying Zn2+. As shown in Fig. 8, the inclusion of the inhibitor reduced the stimulatory effect of Zn2+ to rates similar to the non-stimulated HT29 cells. This indicates that the MAP kinase pathway is sufficient for mediating zinc-dependent activation of Na+/H+ exchange.

Recently, a mechanism describing the effect of chronic acidification on the stimulation of the Na+/H+ exchanger has been demonstrated to be mediated by ERK in cardiomyocytes (49). Because of the profound and prolonged acidification occurring in colonocytes under physiological conditions (25), it was important to determine whether the same mechanism applies to these cells, and whether it shares the same signaling pathway with zinc. We first compared the rate of Na+/H+ exchange following brief acidification (30 s) versus prolonged acidification (4 min). As shown in Fig. 9, prolonged acidification resulted in a 20-fold stimulation of exchanger activity, similar to the effect of zinc. Application of zinc followed by prolonged acidification did not further enhance the rate of Na+/H+ exchange. The application of the specific NHE1 inhibitor (cariporide 0.5 μM) completely eliminated the effect of the acidification following prolonged acidification (Fig. 9D), indicating that the acidification-enhanced Na+/H+ exchange is also mediated by NHE1.

We then assessed the role of ERK1/2 in mediating the stimulatory effect of prolonged acidification on the Na+/H+ exchange. To our surprise, application of U0126 (1 μM) followed by the prolonged acidification paradigm did not have any effect on the stimulation of the NHE1 (Fig. 9E). This indicates that in colonocytes, in contrast to cardiomyocytes, the effect of prolonged acidification on NHE is not mediated by the MAP kinase pathway (49). Our results further suggest that Zn2+ and prolonged acidification, in colonocytes, act through distinct signaling pathways both culminating in stimulation of the same Na+/H+ exchanger.

**Activation of NHE1 Is Mediated by the ZnR**—The results thus far indicate that zinc, through the regulation of ERK1/2, activates the Na+/H+ exchanger. To address the role of the ZnR in linking zinc to the activation of the exchanger we determined its effect on Na+/H+ exchange following zinc desensitization of the ZnR. Desensitization was performed using the same experimental paradigm described in Fig. 3. The rate of H+ efflux in the presence of Na+ was determined 2 h following desensitization, by briefly re-exposing the cells to 100 μM zinc (Fig. 8). While zinc activated NHE1 20-fold, the stimulatory effect of zinc on Na+/H+ exchange was eliminated following desensitization of the ZnR. The striking inhibition of Na+/H+ exchange following ZnR desensitization indicates that the latter is essential for mediating zinc-dependent activation of NHE1. We further demonstrated the specificity of the ZnR-dependent effect by monitoring the rate of Na+/H+ exchange following prolonged acidification of ZnR-desensitized cells. In contrast to the effect of desensitization on the zinc-dependent NHE1 stimulation, ZnR desensitization by zinc had no effect on the pH recovery following prolonged acid load.

Altogether, our results indicate that extracellular zinc, acting through the ZnR, activates the ERK1/2 pathway which is sufficient for stimulating Na+/H+ exchange mediated by the NHE1. A distinct, Zn2+- and ERK1/2-independent pathway leads to stimulation of the NHE1 following prolonged acidification.
A growing body of evidence suggests that in addition to its well known functions in protein structure and physiology, zinc acts as a signaling molecule (9, 50). A recent study has shown that in colonocytes, zinc is linked to regulation of cell proliferation via the MAPK pathway (5), though the mechanism by

**DISCUSSION**

A growing body of evidence suggests that in addition to its well known functions in protein structure and physiology, zinc acts as a signaling molecule (9, 50). A recent study has shown that in colonocytes, zinc is linked to regulation of cell proliferation via the MAPK pathway (5), though the mechanism by
which this occurs is unknown. The results presented here show that IP3 and PI 3-kinase pathways, both of which are important in regulating cell proliferation and ion transport, are activated by extracellular zinc. Using a highly sensitive fluorescent zinc probe (Fura-2 AM), we did not detect any change in intracellular zinc when cells were superfused with physiological concentrations of free-zinc for a time interval required for activation of these pathways. The functionally identified ZnR (13) is activated at these concentrations of extracellular zinc, suggesting that it may link extracellular zinc to the ERK1/2 pathway. Such a role for the ZnR is further supported by the specificity of the Zn2+/H+ -dependent activation of ERK. Thus, other heavy metals, which failed to activate ZnR-dependent Ca2+/H+ release, also failed to trigger the phosphorylation of ERK1/2 in colonocytes. A physiological consequence of zinc-dependent activation of ERK is a strong zinc-dependent enhancement of Na+/H+ exchange activity in colonocytes (both the cell line and native colon epithelium) mediated by the NHE1 isoform. Enhanced activation of NHE1 in colonocytes suggests an intriguing link between zinc, available in the colon either from bile secretion (51) or dietary sources (52), and a key membrane transporter involved not only in pH and cell volume homeostasis but also in the regulation of cell proliferation and apoptosis (53). Finally, desensitization of the ZnR largely prevented the zinc-dependent phosphorylation of ERK1/2, demonstrating that the ZnR constitutes a principal link between extracellular zinc and phosphorylation of ERK1/2. As expected from the role of the ZnR in mediating zinc signaling, desensitization of the ZnR abolished the zinc-dependent effect on NHE1. Hence, although cloning of the ZnR is still in progress, the above results strongly implicate this receptor in linking extracellular zinc to major cell signaling pathways (Fig. 10).

Intracellular calcium changes have a pleiotropic effect on numerous signal transduction pathways. The activation of the IP3 pathway, leading to intracellular calcium rise, is now accepted to be a key pathway linking intracellular Ca2+ rise to ERK1/2 phosphorylation (15). Subsequent activation of CaM kinase II has also been linked to ERK1/2 activation in keratinocytes (14, 54, 55). Similarly, in the brain, this kinase plays a fundamental role in neuronal development and function (56–59). The CaM kinase has been shown to regulate the activity of ion channels in colonocytes (60–63), though its role in inducing ERK activation has not been described. Extracellular zinc also specifically activates the PI 3-kinase in 3T3 fibroblasts, leading to activation of P70S6 kinase and subsequently, to progression of cells from G1 to S phase of the cell cycle (12). Many studies have shown functional interaction between the PI 3-kinase and MAP kinase pathways, involving Rac/CDC42 or direct interaction with Ras (64). This pathway not only plays a key role in normal cell growth, but also has an important pathophysiological role in colonocyte tumorigenesis (65, 66). We show that inhibition of the IP3 pathway (Fig. 4) or the application of the

![Fig. 10. Zinc, via the ZnR, activates the Na+/H+ exchanger.](http://www.jbc.org/)

The ZnR was desensitized by application of zinc (30 min, as shown in Fig. 3), and then the NH4Cl paradigm was applied and the rate of pH monitored. Desensitization that resulted in attenuation of ERK1/2 phosphorylation (A, Zn 100 μM and B, 100 μM Zn applied following desensitization) also resulted in inhibition of the zinc-dependent stimulation of the Na+-dependent H+ efflux. For the sake of clarity immunoblots are shown, and are the same as those shown in Fig. 3. The prolonged acidification paradigm was performed, following ZnR desensitization (C). Prolonged acidification of the cells, even following ZnR desensitization, induced stimulation of Na+/H+ exchange, indicating that it is mediated by a mechanism independent of the ZnR. Quantification of the rate of change in pH, following the above treatments (D). ***, p < 0.001 versus pH recovery in control or Zn-desensitized cells.

**ZnR Triggers ERK-dependent Activation of NHE**

![Graphs](http://www.jbc.org/)

**A.** pH with NH4Cl and Na+ addition.

**B.** pH with desensitized ZnR and Na+ addition.

**C.** pH with desensitized ZnR and prolonged acidification.

**D.** Quantification of pH change.

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**FIG. 10.**

Zinc, via the ZnR, activates the Na+/H+ exchanger. The ZnR was desensitized by application of zinc (30 min, as shown in Fig. 3), and then the NH4Cl paradigm was applied and the rate of pH monitored. Desensitization that resulted in attenuation of ERK1/2 phosphorylation (A, Zn 100 μM and B, 100 μM Zn applied following desensitization) also resulted in inhibition of the zinc-dependent stimulation of the Na+-dependent H+ efflux. For the sake of clarity immunoblots are shown, and are the same as those shown in Fig. 3. The prolonged acidification paradigm was performed, following ZnR desensitization (C). Prolonged acidification of the cells, even following ZnR desensitization, induced stimulation of Na+/H+ exchange, indicating that it is mediated by a mechanism independent of the ZnR. Quantification of the rate of change in pH, following the above treatments (D). ***, p < 0.001 versus pH recovery in control or Zn-desensitized cells.
CaM kinase II inhibitor have a similar inhibitory effect (~50%) on zinc-dependent ERK1/2 phosphorylation. The zinc-dependent effect on ERK phosphorylation is significantly lowered by inhibiting both the IP$_3$ and PI 3-kinase pathways. Activation of NHE1 was recently shown to enhance epithelial cell survival by interaction with ezrin/radixin/moesin (ERM), leading to activation of the anti-apoptotic kinase, AKT (27). Our results show that Zn$^{2+}$, acting via the ZnR, strongly activates NHE1 and also leads to activation of AKT. The regulatory effect of ZnR on cellular signaling promoting cell proliferation is consistent with the known role of zinc in promoting the growth of digestive tract cells rather than promoting vectorial solumate transport (9, 67, 68). We suggest that this mode of regulation may have therapeutic implications in the treatment of diarrhea resulting from the loss of colon epithelial cells. Indeed, the observation in the present study regarding the importance of zinc in the activation of pathways linked to anti-apoptotic processes is intriguing in light of the fact that erosion of the colon epithelium and acute diarrhea are particularly severe when even moderate zinc deficiencies are combined with bacterial infections or with other nutrient deficiencies (69). The non-toxic nature of orally administered zinc implies that it may be used as an efficient, available and inexpensive agent in the treatment of this very common syndrome.

Considering the profound effect of extracellular zinc on major signaling and transport pathways linked to normal as well as neoplastic growth, it is important to also consider the possible participation of zinc in tumorigenesis (1). The activation of NHE1 which has a well characterized mitogenic effect on multiple cell types (70, 71) may suggest another aspect of the effect mediated by the ZnR on cell proliferation. This could be mediated by dietary zinc or, alternatively by cellular release of zinc. Indeed, release of zinc from intracellular stores has been shown to be regulated by the nitric oxide pathway and may lead to dynamic changes in intracellular levels of this ion (72). It would be intriguing to determine if at least part of the therapeutic effect of metal chelators used as carriers of radioactive metals for diagnosis or radiotherapy (73, 74), can be attributed to chelation of zinc released during cell signaling.

Finally, colon cells are faced with an acid load that is arguably among the largest in epithelial cells, following the permeation of short chain fatty acids generated by bacterial fermentation in the colon. Na$^+$/H$^+$ exchange, particularly that mediated by NHE1, plays a key role in catalyzing the removal of protons in colon epithelium (75–77). In the present study, we describe two different regulatory mechanisms resulting in the stimulation of NHE1 activity in colonocytes. The first mechanism for Na$^+$/H$^+$ exchange regulation is mediated by extracellular zinc, which triggers the activation of Na$^+$/H$^+$ exchange via the activation of ERK1/2. A role for the ZnR is strongly supported by the demonstration that desensitization of the ZnR is followed by elimination of Na$^+$/H$^+$ exchange activation.

The second mechanism activating Na$^+$/H$^+$ exchange is prolonged (4 min) intracellular acidification that leads to activation of NHE1. A seemingly similar acidification-dependent regulation of Na$^+$/H$^+$ exchange has recently been described in cardiomyocytes (49). Although in heart muscle the effect is mediated by the ERK pathway, we do not find evidence linking activation ERK1/2 to the prolonged acidification-induced stimulation of Na$^+$/H$^+$ exchange activity in colonocytes. The physiological basis for this striking difference may rely on the fact that acidification is a normal process during the vectorial transport of short chain fatty acids in the colon. In cardiomyocytes, however, prolonged acidification and subsequent NHE activation are pathophysiological processes linked to ischemia (78). In contrast, in colonocytes, acidification requires fast activation of NHE1 but does not involve the activation of ERK1/2.

Nevertheless, the acid load generated by the transport of short chain free fatty acids in the colon is considered to be (76). Hence, a major physiological disadvantage of the prolonged acidification-induced mechanism in colonocytes is the relatively long interval during which the cells are exposed to the acid load. Dietary zinc acting via the ZnR-dependent mechanism may have a preemptive role in up-regulating NHE1 prior to permeation of short chain fatty acids. Subsequently, the absorption of free, short chain fatty acids will be met with a much more rapid response mediated by the exchanger. Since zinc is not hydrolyzed and only 10% of dietary zinc is absorbed (19), this ion is present at high concentrations along the entire digestive tract (19, 20, 39) and is thus ideally suited for a signaling role there.
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36. Attar, D., Backx, P. H., Appel, M. M., Gao, W. D., and Marban, E. (1995) J. Biol. Chem. 270, 2473–2477
37. Sensi, S. L., Canzoniero, L. M., Yu, S. P., Ying, H. S., Koh, J. Y., Kerechner, G. A., and Choi, D. W. (1997) J. Neurosci. 17, 9554–9564
38. Thamilselvan, V., Fomby, M., Walsh, M., and Basson, M. D. (2003) J. Surg. Res. 110, 255–265
39. Knudsen, E., Jensen, M., Solgaard, P., Sorensen, S. S., and Sandstrom, B. (1995) J. Nutr. 125, 1274–1282
40. Neary, J. T., Kang, Y., Willoughby, K. A., and Ellis, E. F. (2003) J. Biol. Chem. 278, 2348–2356
41. Cullen, P. J., and Lockyer, P. J. (2002) Nat. Rev. Mol. Cell. Biol. 3, 339–348
42. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
43. Egea, J., Epinet, C., Soler, R. M., Peiro, S., Rocamora, N., and Comella, J. X. (2000) Mol. Cell. Biol. 20, 1931–1946
44. Chen, H. J., Rojas-Soto, M., Oguni, A., and Kennedy, M. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 557–561
45. Ginnan, R., and Singer, H. A. (2002) Am. J. Physiol. Cell Physiol. 282, C754–C761
46. Keely, S. J., Uribe, J. M., and Barrett, K. E. (1998) J. Biol. Chem. 273, 27111–27117
47. Sheng, H., Shao, J., Townsend, C. M., Jr., and Evers, B. M. (2003) Gut 52, 1472–1478
48. Masereel, B., Pochet, L., and Laeckmann, D. (2003) Eur. J. Med. Chem. 38, 547–554
49. Haworth, R. S., McCann, C., Snaithies, A. K., Roberts, N. A., and Avkiran, M. (2003) J. Biol. Chem. 278, 31676–31684
50. Maret, W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13225–13237
51. Chen, S. M., Liu, J. F., Luo, C. D., and Ho, L. T. (2004) Nephron Physiol. 96, p113–p120
52. Vallee, B. L., and Falchuk, K. H. (1993) Physiol. Rev. 73, 79–118
53. Putney, L. K., Denker, S. P., and Barber, D. L. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 527–552
54. Prakova, M., Kalenderova, S., Miteva, L., Pousmay, Y., and Mitev, V. (2002) Exp. Dermatol. 11, 344–348
55. Prakova, M., Kalenderova, S., Miteva, L., Pousmay, Y., and Mitev, V. (2002) Arch. Dermatol. Res. 294, 198–202
56. Szaio, G., Vianna, M. R., McEachan, J. L., Medina, J. H., and Izuropo, I. (2003) Hippocampus 13, 53–58
57. Cammarota, M., Bevilacqua, L. R., Viola, H., Kerr, D. S., Reichmann, B., Teixeira, V., Bulla, M., Izuropo, I., and Medina, J. H. (2002) Cell Mol. Neurobiol. 22, 259–267
58. Elgersma, Y., Fedorov, N. B., Ikonen, S., Choi, E. S., Elgersma, M., Carvalho, O. M., Giese, K. P., and Silva, A. J. (2002) Neuron 36, 493–505
59. Miller, Y., Yaouatta, M., Coats, J. K., Jones, Y., Martone, M. E., and Mayford, M. (2002) Neuron 36, 507–519
60. Koh, S. D., Perrino, B. A., Hatton, W. J., Kenyon, J. L., Sanders, K. M., Xie, W., Solomons, K. R., Freeman, S., Kaelzel, M., A., Buzo, K. S., Nelson, D. J., Shears, S. B., Worell, R. T., and Frizzell, R. A. (1999) J. Physiol. 517, 75–84
61. Worrell, R. T., and Frizzell, R. A. (1991) Am. J. Physiol. 260, C877–C882
62. Robinson, N. C., Huang, P., Kaelzel, M., A., Buzo, K. S., Nelson, D. J., and Shears, S. B. (1998) J. Physiol. 510, 661–673
63. Xie, W., Solomons, K. R., Freeman, S., Kaelzel, M., A., Buzo, K. S., Nelson, D. J., and Shears, S. B. (1998) J. Physiol. 510, 661–673
64. Yart, A., Chap, H., and Raynal, P. (2002) Biochim. Biophys. Acta 1582, 267–111
65. Philips, W. A., St Clair, F., Uden, A. D., Thomas, R. J., and Mitchell, C. A. (1996) Cancer Res. 56, 41–47
66. Philip, A. J., Campbell, I. G., Leet, C., Vincan, E., Rockman, S. P., Whitehead, R. H., Thomas, R. J., and Phillips, W. A. (2001) Cancer Res. 61, 7426–7429
67. Lawson, M. J., Butler, R. N., Goland, G. J., Jarrett, I. G., Roberts-Thomson, I. C., Partick, E. J., and Dreosti, I. E. (1988) Biol. Trace Elem. Res. 15, 115–112
68. Chimenti, F., Aouffen, M., Favier, A., and Seve, M. (2003) Curr. Drug Targets 4, 323–338
69. Wapnir, R. A. (2000) J. Nutr. 130, 1388S–1392S
70. Winter, D. C., Taylor, C., O'Sullivan, G., and Harvey, B. J. (2000) Br. J. Surg. 87, 1684–1689
71. Takewaki, S., Kuro-o, M., Hiroi, Y., Yamazaki, T., Noguchi, T., Miyagishi, A., Nakahara, K., Aikawa, M., Manabe, I., and Yazaki, Y. (1995) J. Mol. Cell Cardiol. 27, 739–742
72. Bossy-Wetzel, E., Talantova, M. V., Lee, W. D., Scholzke, M. N., Harrop, A., Mathews, E., Got, T., Han, J., Ellisman, M. H., Perkins, G. A., and Lipton, S. A. (2004) Neuron 41, 351–365
73. Davitillard, C., Ponelle, T., Carvau, C., Piard, P., Romanet, P., and Chauffert, B. (2004) Anticancer Drugs 15, 295–299
74. McKearn, T. J. (1993) Cancer 71, 4302–4313
75. Busche, R., Bartels, J., Genta, K., and von Engelsdorf, H. W. (1997) Comp. Biochem. Physiol. A Physiol. 118, 395–398
76. Montrose, M. H., and Chu, S. (1997) Comp. Biochem. Physiol. A Physiol. 118, 389–393
77. Velazquez, O. C., Lederer, H. M., and Rombeau, J. L. (1997) Adv. Exp. Med. Biol. 427, 123–134
78. Wang, Y., Meyer, J. W., Ashraf, M., and Shull, G. E. (2003) Circ. Res. 93, 776–782
Extracellular Zinc Triggers ERK-dependent Activation of Na\(^+\)/H\(^+\) Exchange in Colonocytes Mediated by the Zinc-sensing Receptor
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