Induction of Tyrosine Phosphorylation and Na\(^+\)/H\(^+\) Exchanger Activation during Shrinkage of Human Neutrophils*

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The ubiquitous isoform of the Na\(^+\)/H\(^+\) exchanger (NHE1) is essential for the regulation of cellular volume. The underlying molecular mechanism, which is poorly understood, was studied in human polymorphonuclear leukocytes (PMN). Suspension of PMN in hypertonic medium induced rapid cellular shrinkage and activation of NHE1, which is measurable as a cytosolic alkalinization. Concomitantly, hypertonic stress also induced extensive tyrosine phosphorylation of several proteins. Pretreatment of PMN with genistein, a tyrosine kinase inhibitor, prevented not only the tyrosine phosphorylation in response to a hypertonic shock but also the activation of NHE1. The signal elicited by hyperosmolarity that induces activation of tyrosine kinases and NHE1 was investigated. Methods were devised to change medium osmolarity without altering cell volume and vice versa. Increasing medium and intracellular osmolarity in normovolemic cells failed to activate tyrosine kinases or NHE1. However, shrinkage of cells under iso-osmotic conditions stimulated both tyrosine phosphorylation and NHE1 activity. These findings imply that cells detect alterations in cell size but not changes in osmolarity or ionic strength. The identity of the proteins that were tyrosine-phosphorylated in response to cell shrinkage was also investigated. Unexpectedly, the mitogen-activated protein kinases SAPK, p38, erk1, and erk2 were not detectably phosphorylated or activated. In contrast, the tyrosine kinases p59\(^{flk}\) and p56\(^{lck}\) were phosphorylated and activated upon hypertonic challenge. We propose that cells respond to alterations in cell size, but not to changes in osmolarity, with increased tyrosine phosphorylation, which in turn leads to the activation of NHE1. The resulting changes in ion content and cytosolic pH contribute to the restoration of cell volume in shrunken cells.

The Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1) is a ubiquitously expressed cation antipporter that is involved in the regulation of cell volume and intracellular pH (pH\(_i\)). NHE1 is nearly quiescent in resting cells but becomes activated upon cytosolic acidification or by treatment of the cells with a variety of hormones and growth factors (see Ref. 1 for review). Phosphorylation of the exchanger was suggested to induce its activation, since treatment with growth promoters was found to increase the phosphoserine content of NHE1 (2, 3). Moreover, increased phosphorylation and functional activation were also induced by inhibitors of Ser/Thr phosphatases, such as okadaic acid (3).

NHE1 is also rapidlystimulated when cells are made to shrink in hypertonic solutions (4). It is unclear whether increased osmolarity or reduced cell volume are the signals that trigger activation of the exchanger. The osmotic stimulation of Na\(^+\)/H\(^+\) exchange requires intracellular ATP and is not additive with that induced by growth factors (5). These observations suggested that phosphorylation was also involved in the osmotic activation of NHE1. However, the phosphorylation state of the exchanger was found to be unaffected during osmotic challenge (4). Moreover, osmotic stimulation could still be observed following truncation of all the putative phosphorylation sites of NHE1 (6). Thus, the mechanism responsible for osmotically induced stimulation of the exchanger remains unclear. It is possible that phosphorylation of ancillary regulatory proteins is involved. In this context, calcineurin B homolog protein (CHP), a substrate of Ser/Thr kinases, was reported to bind to the cytosolic tail of the antipporter (7). Also, a polypeptide of ~24 kDa, the approximate size of CHP, is constitutively associated with NHE1 in several cell types (8).

Osmotic shrinkage of mammalian cells is a powerful stimulant of MAPK including the stress kinases p38 and SAPK (JNK) (9, 10) and in some instances Erk (11). MAPK have recently been invoked as possible regulators of the activity of NHE1 in platelets (12) and fibroblasts (13) treated with various agonists. The precise mechanism whereby shrinkage stimulates the kinases is unknown, as is their relationship to the osmotic stimulation of NHE1.

In this report, we investigated the relationship between the stimulation of protein kinases and the activation of NHE1, and we attempted to determine whether reduced cell volume or increased cytosolic osmolarity were the signals leading to the activation of these effectors. To this end we used human blood neutrophils, which express NHE1 (14) and are known to respond vigorously to changes in medium osmolarity (15).

**EXPERIMENTAL PROCEDURES**

Materials—Dextran T-500 and Ficoll-Paque were from Pharmacia Biotech Inc. Genistein and erbastin analog were from Calbiochem. BCECF was from Molecular Probes Inc. Nystatin was from Sigma and was freshly dissolved in dimethyl sulfoxide before each experiment. All other chemicals used were of the highest purity available. The enhanced chemiluminescence detection system and horseradish peroxidase-coupled anti-rabbit and anti-mouse antibodies were from Amersham Life Science. GST, glutathione S-transferase; PVDF, polyvinylidene difluoride; SAPK, stress-activated protein kinase.
sham Corp. Phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology Inc. Polyclonal anti-paxillin antibody was from Zymed Inc. and anti-c-cbl was from Transduction Laboratories Inc. Polyclonal antibody against p38 was the generous gift of Dr. Brent Zanke (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada). A GST-c-Zanke (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada). A GST-c-Jun construct was provided by Dr. Joseph B. Bolen (DNAX Research Institute, Palo Alto, CA).

A GST-c-Jun was provided by Dr. James Woodgett (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada). Phospho-specific Erk polyclonal antibody was from New England Biolab. lyn, fgr, and hck polyclonal antibodies were generously provided by Dr. Joseph B. Bolen (DNAX Research Institute, Palo Alto, CA).

Solutions—Bicarbonate-free RPMI 1640 was buffered to pH 7.4 with 10 mM Hepes. Isotonic NaCl buffer contained (in mM) 5 KCl, 10 glucose, 140 NaCl, 1 CaCl2, 1 MgCl2, 10 Hepes, pH 7.4. Isotonic KCl buffer contained 10 glucose, 145 KCl, 1 CaCl2, 1 MgCl2, and 10 Hepes, pH 7.4. Hypertonic NaCl buffer contained 5 KCl, 10 glucose, 240 NaCl, 1 CaCl2, 1 MgCl2, and 10 Hepes, pH 7.4. Hypertonic KCl buffer was similar to hypertonic NaCl buffer, except that NaCl was replaced with KCl. Hypertonic NaCl buffer contained 5 KCl, 10 glucose, 50 NaCl 1 CaCl2, 1 MgCl2, and 10 Hepes, pH 7.4. Iso-osmotic sucrose buffer contained 5 KCl, 10 glucose, 280 sucrose, 1 CaCl2, 1 MgCl2, and 10 Hepes, pH 7.4. Ca++ and Mg++ were omitted from all buffers that were used during permeabilization with nystatin. The iso-osmolar buffers were adjusted to 290 ± 5 mOsm with either water or the major salt. All buffers used for cell incubations were nominally HCO3–-free. Laemmli sample buffer (LSB) contained 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.025% bromophenol blue, 62.5 mM Tris, pH 6.8. Nonidet P-40 buffer contained 1% Nonidet P-40, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris, pH 8.0.

Cells—Human PMN were isolated from fresh blood drawn by venipuncture into heparinized tubes. Isolation of cells was performed using dextran sedimentation and centrifugation on Ficoll-Paque cushions as described previously (16). Cells were resuspended in Hepes-buffered RPMI 1640 and kept on a rotary shaker at room temperature until use. When immunoprecipitation was performed, PMN were pretreated with 1 mM diisopropylfluorophosphate for 30 min to minimize proteolysis. Cell volume and counts were assessed with a Coulter Counter (model ZM) equipped with a Channelizer.

Immunoprecipitation and Immunoblotting—Treatments were stopped by the addition of 2 volumes of ice-cold buffer of the corresponding osmolarity, and the PMN were rapidly sedimented in a microcentrifuge. For experiments where whole cell anti-phosphotyrosine blotting was performed, the cell pellet was resuspended in hot LSB and boiled for 10 min. For immunoprecipitation, the cell pellet was dissolved in ice-cold Nonidet P-40 buffer containing protease and phosphatase inhibitors (1 mg/ml phenylmethylsulfonfyl fluoride, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µM sodium vanadate, and 1 mM NaF) and kept on ice for at least 10 min. Immunoprecipitation and immunoblotting were performed as described previously (17). Samples were subjected to 10% SDS-PAGE, transferred to poly(vinylidene difluoride) membranes, and blotted with the appropriate antibody.

Kinase Assays—Tyrosine kinase activity was assayed in vitro using enolase as the substrate, as described previously (18). SAPK assays using GST-c-Jun as a substrate were performed essentially as described (19). Reaction products were separated by 10% SDS-PAGE, and incorporated 32P was quantified with a PhosphorImager equipped with ImageQuant software (Molecular Dynamics Inc.).

Intracellular pH Measurements—PMN (107/ml) were incubated with 2 µM of the acetoxymethyl form of BCECF for 15 min at 37 °C, sedimented, and resuspended (at 2 × 106/ml) in the appropriate buffer. Where indicated, the cells were pretreated with nystatin (50 µg/ml) to increase the permeability of the plasmalemma to small monovalent ions (see “Results”). An aliquot of the cell suspension (106 cells) was added to 1 ml of prewarmed (37 °C) buffer of the required osmolarity in the cuvette compartment of a spectrofluorimeter (Perkin-Elmer model 650-40). Measurements of BCECF emission and the calibration of fluorescence versus pH, were performed as described previously (20).

Statistical Analysis—All experiments were performed at least in triplicate. Data are presented as means ± S.E. or illustrated as representative traces or blots. Significance was assessed using Student’s paired t test. A score of p < 0.05 was considered significant.

RESULTS

Correlation between Tyrosine Phosphorylation and NHE1 Activation

We tested the effect of hypertonic solutions on PMN volume, measured electronically, and pHi, estimated from the fluorescence of BCECF. Increasing the osmolarity of the medium from 290 to 475 mOsm by addition of 100 mM NaCl caused a rapid reduction of median cell volume from 327 ± 3 fl to 273 ± 2 fl (means ± S.E., n = 5, p < 0.01).2 As shown in Fig. 1A, hypertonic stress also induced an alkalization of the cytosol ranging from 0.2 to 0.3 pH units, which was evident at 30 s and stabilized within 5 min. As in other cells, this alkalization was mediated by the NHE, since it was abolished by omission of external Na+ (not shown) or by addition of the specific inhibitor compound HOE694 (see below).

Phosphorylation of tyrosine residues is one of the earliest events in a variety of signaling cascades. We questioned whether tyrosine phosphorylation was also involved in signaling the osmotic activation of NHE1. To address this possibility, the content of tyrosine-phosphorylated proteins was assayed by immunoblotting with anti-phosphotyrosine antibody (4G10). Results in A and B are representative of three separate experiments.

2 Note that PMN shrink in response to osmolarity less than predicted for a perfect osmometer by the Van’t Hoff relationship. This is due to the presence of a sizable osmotically unresponsive volume within the cells, likely the secretory granules and/or the nucleus.
proteins, which was clearly apparent at 30 s, attained maximum levels by 2 min, and persisted for up to 30 min. Polypeptides of ~210, 125, 74, 60, 42, and 40 kDa were consistently tyrosine-phosphorylated in all of our experiments.

We next investigated whether tyrosine phosphorylation was a consequence or the cause of NHE1 activation. To determine if activation of Na\(^+/H^+\) exchange was required for induction of tyrosine phosphorylation, PMN were pretreated with 2 mM HOE694, a concentration predicted to produce almost complete inhibition of NHE1 (21) and subjected to a hypertonic shock. As shown in Fig. 2, while the inhibitor largely eliminated the cytosolic alkalinization, the accumulation of phosphotyrosine induced by hyperosmolarity was unaffected. A comparable degree of tyrosine phosphorylation was also obtained in cells suspended in a hypertonic KCl (Na\(^+\)-free) medium. The absence of Na\(^+\), the external substrate for NHE, precluded cytosolic alkalinization (results not shown). These experiments imply that stimulation of tyrosine phosphorylation by hyperosmolar solutions is not a consequence of activation of NHE1.

We therefore considered whether tyrosine phosphorylation was instead the cause of NHE1 activation. Cells were pretreated with 100 μM genistein, a potent tyrosine kinase inhibitor, and subjected to hypertonicity. Under these conditions, both the cytosolic alkalinization (Fig. 3A) and tyrosine phosphorylation were inhibited (Fig. 3B). Similar results were obtained by pretreating PMN with 10 μg/ml erbstatin analog, a structurally unrelated tyrosine kinase inhibitor (not shown). These findings suggest that phosphotyrosine accumulation is required for the hypertonic activation of NHE1.

Role of Osmolarity in the Induction of Tyrosine Phosphorylation

We next investigated the signal that triggers phosphotyrosine accumulation in cells exposed to hypertonic media. In principle, the response could be initiated by osmosensors that detect the change in medium or intracellular tonicity. Alternatively, the signal for phosphorylation could be the cellular shrinkage that results from the net loss of cytosolic water. The experiments described below were designed to discern between these alternative models.

Fig. 4A illustrates the protocol used to increase the intracellular osmolarity while keeping the cellular volume constant. PMN were suspended in isotonic KCl medium and treated with 50 μg/ml nystatin, a pore-forming molecule that allows the
passage of small monovalent ions across the plasma membrane (22). Sucrose (50 mM), which cannot permeate through nystatin, was added to the medium to prevent swelling due to the presence of impermeant osmolytes within the cells (20). After 9 min, the time required for adequate permeabilization, an additional 125 mM KCl was introduced to render both the extracellular and intracellular solutions hyperosmotic. Because both K⁺ and Cl⁻ permeate readily through nystatin, cell shrinkage is minimal (step III in Fig. 4A). Cells were then washed at 37 °C to remove extracellular as well as membrane-associated nystatin, resulting in rapid and effective resealing of the membrane, and the hypertonic KCl was replaced with hypertonic NaCl (step IV). Sizing with the Coulter-Channelizer confirmed that, following nystatin treatment in the hypertonic buffer, the volume of the cells was then measured after 5 min using the Coulter Counter. The roman numerals in B and C identify the conditions with the diagram in A. Data are means ± S.E. of three experiments, counting a minimum of 2 × 10⁴ cells per experiment. Data are normalized to the volume of untreated PMN in isotonic solution (290 mOsm), which averaged 327 ± 3 fl. C, cells were treated as in B except that aliquots of the suspension were boiled in LSB buffer and used for immunoblotting with anti-phosphotyrosine antibody. Results are representative of three separate experiments.

Fig. 4. Effect of increased osmolarity at constant cell volume on tyrosine phosphorylation. A, diagrammatic representation of the experimental protocols used to either shrink cells (II) or to increase intra- and extracellular osmolality while maintaining cell volume constant, using nystatin (III and IV). I, cells were initially in isotonic NaCl buffer; II, cells transferred to hypertonic KCl or NaCl buffer (475 mOsm) which causes shrinkage; III, cells resuspended an ice-cold isotonic KCl buffer (115 mM KCl + 50 mM sucrose) containing 50 μg/ml nystatin. After 9 min, an extra 125 mM KCl was added, making the medium hyperosmotic (475 mOsm); IV, the cells were then suspended in prewarmed (37 °C) hyperosmolar NaCl buffer (475 mOsm). B, PMN were treated under the conditions indicated at the foot of the figure, using the protocols detailed in A. The median volume of the cells was then measured after 5 min using the Coulter Counter. The roman numerals in B and C identify the conditions with the diagram in A. Data are means ± S.E. of three experiments, counting a minimum of 2 × 10⁴ cells per experiment. Data are normalized to the volume of untreated PMN in isotonic solution (290 mOsm), which averaged 327 ± 3 fl.

Role of Cell Shrinkage in the Induction of Tyrosine Phosphorylation

In the next series of experiments, we analyzed the contribution of cell volume changes to the induction of tyrosine phosphorylation. To this end, we attempted to induce cell shrinkage while maintaining iso-osmolar conditions. Fig. 6A illustrates
Fig. 5. Effect of hyperosmolar urea on tyrosine phosphorylation. A, diagrammatic representation of the experimental protocols used to either shrink cells (IV), to increase intra- and extracellular osmolarity while maintaining cell volume constant, or using urea (II) to shrink cells in the presence of urea (III). I, cells were initially in isotonic NaCl buffer (290 mOsm); II, 200 mM urea was added to increase osmolarity (475 mOsm). Urea rapidly equilibrated across the membrane, with no shrinkage in the steady state; III, 200 mM sucrose or 100 mM NaCl was then added to the urea-containing suspension, inducing sustained shrinkage; IV, cells transferred directly to medium made hypertonic (475 mOsm) with sucrose. B, PMN were treated under the conditions indicated at the foot of the figure, using the protocols detailed in A. The median volume of the cells was then measured using the Coulter Counter. The roman numerals in B and C identify the conditions with the diagram in A. Data are means ± S.E. of three experiments, normalized to the volume of untreated PMN in isotonic solution; C, tyrosine phosphorylation was assessed under the conditions described in A. Lanes I–IV correspond to conditions I–IV in A and B. Lanes 5 and 6 are identical to lanes 3 and 4, respectively, except that sucrose was replaced with 100 mM NaCl. Results are representative of three separate experiments.

A second method used to dissociate the effects of cell shrinkage and hypertonicity is illustrated in Fig. 7A. PMN were suspended in hypotonic NaCl buffer (~50% of the normal osmolarity), thereby causing the cells to swell (II in Fig. 7, A and B). This initial passive swelling was followed by a gradual loss of volume, reaching near normal size after approximately 30 min (III in Fig. 7, A and B). This secondary volume loss, known as regulatory volume decrease, is thought to be mediated by increased permeability to K+ and anions (23). Subsequent addition of 90 mM NaCl to the medium, which restored the osmolality to the initial (iso-osmotic, 290 mOsm) level, caused the cells to shrink (IV in Fig. 7). Such shrinkage under iso-osmotic conditions was accompanied by a marked phosphorysorine accumulation, usually exceeding that induced by comparable hypertonic shrinkage (Fig. 7C). The combined results of Figs. 6 and 7 demonstrate that tyrosine phosphorylation can be promoted in PMN by reducing the volume of the cells, regardless of the osmolality of the medium or cytosol.

Role of Cell Volume and Hypertonicity in the Activation of NHE1

The preceding data indicate that tyrosine phosphorylation was triggered by a reduction of the cell volume and not by hypertonicity per se. It was therefore of interest to define whether cell volume, as opposed to medium osmolarity, is responsible for activation of NHE1. Protocols like those employed above were used to differentially alter cell volume and osmolarity while measuring pH, to evaluate the state of activation of NHE1. Fig. 8 shows that a significant cytosolic alkalization, comparable to that observed during hypertonic stress, was caused by reducing cell volume isotonically using nystatin/sucrose, or by restoring iso-osmolarity after regulatory volume decrease. Conversely, increasing osmolality while keeping the volume constant, using either nystatin/KCl or urea, failed to activate the antiporter. This pattern correlates closely with that of tyrosine phosphorylation and is consistent with the notion that NHE1 activation lies downstream of phosphorysorine accumulation.

Identity of Tyrosine-phosphorylated Proteins in Shrunken PMN

MAPK—Because the activation of NHE1 appears to be dependent on phosphorysorine accumulation, we tried to identify
some of the proteins that become tyrosine-phosphorylated when PMN shrink. The stimulation of NHE1 by growth factors has recently been reported to be partially dependent on the erk1 and erk2 MAPK (p42/44MAPK) pathway (13). Moreover, it is well established that kinases of the MAPK family require phosphorylation on tyrosine residues to become active (24). Since hypertonic stress has been shown to induce the activation of erk1 and erk2 in other cell types (11, 13), we investigated whether these MAPK are the 40–42-kDa tyrosine-phosphorylated proteins observed in shrunken PMN. Cells were subjected to hypertonic stress for up to 30 min, and whole cell lysates were immunoblotted with an antibody that specifically recognizes the phosphorylated form of erk1 and erk2. Fig. 9A shows that neither erk1 nor erk2 were tyrosine-phosphorylated in PMN in response to hypertonic stress. The sensitivity of the phosho-specific antibody and the responsiveness of the cells were assessed by stimulation with 100 nM fMLP, a well documented activator of erk1 and erk2 in PMN (25, 26). As shown in Fig. 9A, comparable amounts of cell lysate revealed sizable amounts of phosphorylated erk1 and erk2 after treatment with the chemoattractant. We conclude that erk1 and erk2 are not phosphorylated during hypertonic challenge and are therefore unlikely to mediate the activation of NHE1.

Another member of the MAPK family, p38, has been shown to be activated by hypertonic stress in other cells (10) and was recently detected in fMLP-stimulated human PMN (17, 27). To test whether this kinase is phosphorylated and activated by shrinkage also in PMN we immunoprecipitated p38 and blotted the precipitates with anti-phosphotyrosine antibodies (Fig. 10A). Unlike other cells, PMN did not show evidence of p38 phosphorylation upon shrinking. As before, the effectiveness and sensitivity of the procedure were confirmed in parallel samples stimulated with fMLP (rightmost lane in Fig. 10A). That p38 was activated by chemoattractant but not by osmotic challenge was also confirmed in experiments where whole cell lysates were blotted with an anti-MAPKAPK-2 antibody (Fig. 10C). This kinase, a substrate of p38, undergoes an upward shift in electrophoretic mobility when phosphorylated (17). A distinct shift was noted for fMLP-stimulated samples but not in osmotically shrunken cells. We conclude that p38 is not phos-
FIG. 8. Dissociation of the effects of increased osmolarity and cell shrinkage on pH<sub>i</sub>. PMN were loaded with BCECF, and pH<sub>i</sub> was determined fluorimetrically as described under “Experimental Procedures.” The difference between the maximal pH<sub>i</sub> attained 5 min after application of the indicated treatment, and the basal pH<sub>i</sub>, is illustrated. First bar, cells suspended in iso-osmotic sucrose (10 min at 4°C), washed free of nystatin, and transferred to warm (37°C) isotonic NaCl buffer. Second bar, PMN were shrunken by permeabilization with nystatin in iso-osmotic sucrose (10 min at 4°C), washed free of nystatin, and transferred to warm (37°C) isotonic NaCl buffer. Third bar, PMN were suspended in hypertonic NaCl buffer and allowed to regulate their volume for 30 min. Next, they were induced to shrink by transfer to isotonic NaCl medium. Fourth bar, PMN were nystatin-permeabilized in isotonic KCl buffer at 4°C. Extra KCl (125 mM) was then added to render the medium hypertonic. Nystatin was then washed away, and finally, the cells were suspended in hypertonic NaCl medium. Fifth bar, PMN were suspended in a NaCl medium made hyperosmotic by addition of 200 mM urea. Values of ΔpH<sub>i</sub> are the means ± S.E. of the number of experiments indicated in parentheses.

phorylated or activated by hypertonic challenge in PMN.

Hypertonic stress activates SAPK in a number of cells (e.g. Ref. 9). To investigate if SAPK was similarly stimulated in PMN, this kinase was precipitated from cell lysates using GST-c-jun-coupled to Sepharose beads and its activity tested in vitro. SAPK failed to phosphorylate GST-c-jun following hypertonic stress in PMN (results not shown). It is unclear whether SAPK is not activated or not expressed by human PMN, since we were also unable to demonstrate activation upon treatment of these cells with anisomycin, a well known activator of SAPK.

Src Family Kinases—Kinases of the src family are themselves regulated by phosphorylation on tyrosine residues and may account for the phosphotyrosine accumulation in the 60-kDa range in shrunken PMN. We therefore investigated the ability of cell shrinkage to induce the phosphorylation and activation of three src family kinases that are comparatively abundant in PMN, namely fgr (59 kDa), hck (56/59 kDa), and lyn (59 kDa). PMN were osmotically stimulated for 1 min and lysed, and the three tyrosine kinases were individually immunoprecipitated. The immunoprecipitates were subsequently separated by SDS-PAGE and blotted with a phosphotyrosine-specific antibody. As shown in Fig. 11A, all three kinases were significantly phosphorylated in untreated cells, and cell shrinkage promoted increased tyrosine phosphorylation of fgr and hck, whereas a slight decrease was noted for lyn. The effect of volume changes on the activity of these kinase assays was also tested, performing in vitro assays with immunoprecipitates from control and shrunken cells. We assessed the ability of the kinases to autophosphorylate as well as to phosphorylate the exogenous substrate enolase. Consistent with the phosphotyrosine immunoblots of Fig. 11A, both auto-phosphorylation and enolase kinase activity increased for fgr and hck but decreased slightly for lyn (Fig. 11B).

The identity of other tyrosine-phosphorylated proteins was also probed using sequential immunoprecipitation and blotting as in Fig. 11A. We failed to detect tyrosine phosphorylation of paxillin (67 kDa) or c-ebl (120 kDa) in PMN stimulated hypertonically (results not shown).

DISCUSSION

PMN are exposed to a wide range of dynamic physical forces during their active life span, particularly during passage through narrow capillaries and across vascular walls and during chemotaxis. Such mechanical stress causes shape and volume alterations that need to be compensated in order for the cells to function optimally (28). Such regulation of shape and volume can occur in part via the movement of ions and osmotically obliged water across the cell membrane. The current study investigated the mechanism that regulates the activation of a major, volume-sensitive ion transporter in human PMN, namely NHE1. The salient observations were (i) that a moderate reduction of the cell volume (~16%) induced the tyrosine phosphorylation of several proteins and (ii) that such tyrosine phosphorylation is seemingly required for the activation of NHE1.

Several hypotheses exist regarding the mechanism(s) whereby cells detect osmotic stress (reviewed in Refs. 29–31). First, cells may sense the ionic strength or total osmolarity of the medium or of the intracellular milieu. This explanation cannot account for the observed phosphotyrosine accumulation in PMN for several reasons. Tyrosine phosphorylation could be induced by shrinkage at constant osmolarity and ionic strength (Figs. 6 and 7). Moreover, increasing the osmolarity and ionic strength at constant volume had minimal effect on phosphotyrosine formation (Figs. 4 and 5). It has also been suggested that changes in cytoskeletal architecture upon shrinking may mediate activation of the cells. While we cannot dismiss this
tyrosine kinases precedes and is necessary for activation of ion effects of genistein and erbstatin, suggests that stimulation of phosphorylation and of NHE1, together with the inhibitory clustering of Fc receptors, integrins, and/or other tyrosine kinases lead to the activation of the tyrosine kinases (33). In PMN, engagement and cross-linking of Fc receptors or receptor stimulation was found in the shrunken HeLa cells known to be crucial to their activation (34), and accordingly, despite the absence of their ligands. Clustering of receptors is be the aggregation of surface receptors recently reported by Rosette and Karin (33). These authors found that osmotic changes in cell volume can lead to large increases in the thermodynamic activity of macromolecules (32). One form of crowding, leading to such disproportionate increases in activity, may be the aggregation of surface receptors recently reported by Rosette and Karin (33). These authors found that osmotic shrinkage of HeLa cells induced clustering of interleukin-1, epidermal growth factor, and tumor necrosis factor receptors (35–38), which were also found to be stimulated osmotically in this study. It is tempting to speculate that shrinkage of PMN induces the activation of fgr and hck through clustering of Fc receptors, integrins, and/or other tyrosine kinase (associated) receptors.

The similarity in the pattern of osmotic activation of tyrosine phosphorylation and of NHE1, together with the inhibitory effects of genistein and erbstatin, suggests that stimulation of tyrosine kinases precedes and is necessary for activation of ion exchange. A causal relationship between these events has in fact been postulated for several cell types (e.g. Ref. 39) including PMN where phagocytic stimuli (14) and chemotactic peptides (40) regulate pH in a tyrosine kinase-dependent manner. In the context of macromolecular crowding, it is noteworthy that cross-linking of Fc receptors and integrins can in fact activate NHE1 in PMN and in other cells (14, 41, 42). It is, however, unlikely that NHE1 itself is the target of the tyrosine kinases for the following reasons. First, only serine residues have been found to be phosphorylated in this isoform (2, 3). Potential regulators of NHE1 include Ca2+/calmodulin, protein kinase C, phosphatidylinositol 3-kinase, and heterotrimeric G proteins (43–46). We found, however, that depletion of Ca2+ had no effect on either tyrosine phosphorylation or NHE1 activation in response to hypertonic stimulation. Moreover, pretreatment of PMN with bis-indolylmaleimide (a protein kinase C inhibitor), wortmannin (a phosphatidylinositol 3-kinase inhibitor), or pertussis toxin (a heterotrimeric G protein inhibitor) all failed to inhibit NHE1 or the tyrosine phosphorylation stimulated by hypertonic stress.3

Hooley et al. (44) demonstrated that RhoA was involved in the activation of NHE1 in fibroblasts. Interestingly, a connection between tyrosine kinases and RhoA had been previously established (47). It is therefore conceivable that the pathway

3 E. Krump, unpublished observations.
leading to osmotic activation of NHE1 involves stimulation of RhoA through src-related tyrosine kinases. The mechanism by which RhoA activates NHE1 is currently unknown, but some information can be gleaned from the recent identification of Rho-binding proteins. Of relevance, the phosphorylation of myosin light chain was found to be regulated by a RhoA-dependent kinase (48). This observation is important in that Shrode et al. (49) demonstrated that inhibitors of myosin light chain kinase (48). This observation is important in that Shrode and Lamara D. Shrode for helpful comments on the manuscript.

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