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Specific Protein Phosphorylation Occurs in Molluscan Red Blood Cell Ghosts in Response to Hypoosmotic Stress

Alexander D. Politis* and Sidney K. Pierce
Department of Zoology, University of Maryland, College Park, Maryland 20742

Summary. The regulation of cellular volume upon exposure to hypoosmotic stress is accomplished by specific plasma membrane permeability changes that allow the efflux of certain intracellular solutes (osmolytes). The mechanism of this membrane permeability regulation is not understood; however, previous data implicate Ca^{2+} as an important component in the response. The regulation of protein phosphorylation is a pervasive aspect of cellular physiology that is often Ca^{2+} dependent. Therefore, we tested for osmotically induced protein phosphorylation as a possible mechanism by which Ca^{2+} may mediate osmotically dependent osmolyte efflux. We have found a rapid increase in $^{32}P_i$ incorporation into two proteins in clam blood cell ghosts after exposure of the intact cells to a hypoosmotic medium. The osmotic component of the stress, not the ionic dilution, was the stimulus for the phosphorylations. The osmotically induced phosphorylation of both proteins was significantly inhibited when Ca^{2+} was omitted from the medium, or by the calmodulin antagonist, chlorpromazine. These results correlate temporally with cell volume recovery and osmolyte (specifically free amino acid) efflux. The two proteins that become phosphorylated in response to hypoosmotic stress may be involved in the regulation of plasma membrane permeability to organic solutes, and thus, contribute to hypoosmotic cell volume regulation.

Key Words cell volume · protein phosphorylation · calcium · calmodulin · osmolyte efflux · invertebrate blood cells

Introduction

The importance of proper hydration to cellular function makes water balance a critical function in all cells. As a result, virtually all cells have some ability to regulate their volume. Cells that swell after exposure to hypoosmotic media respond by releasing intracellular osmotic solutes (osmolytes). These osmolytes can be organic (i.e., free amino acids or quaternary ammonium compounds) or inorganic (most commonly KCl), and in many cases both types are used. The efflux of osmolytes along with osmotically obligated water prevents lysis and results in at least a partial recovery from the swelling caused by hypoosmotic stress (Kregenow, 1971; Pierce, 1982; Chamberlin & Strange, 1989; Hoffmann & Simonsen, 1989).

The mechanisms of membrane permeability control that govern the osmolyte release are unknown; however, some common characteristics of the response have been established. For example, most osmolyte permeability changes are initiated by the imposition of an osmotic gradient across the plasma membrane and not the concomitant reduction in extracellular ion concentrations (Pierce & Greenberg, 1973; Hoffman & Hendil, 1976; Fugelli & Rohrs, 1980; Bui & Wiley, 1981; Grunewald & Kinne, 1989). In addition, external divalent cations, especially Ca^{2+}, play an important role in turning the osmolyte efflux on or off (Pierce & Politis, 1990). External concentrations of Ca^{2+} influence cell volume regulation and osmotically dependent free amino acid efflux in some systems, e.g., mussel (*Modiolus*) ventricle (Pierce & Greenberg, 1973); clam (*Noetia*) blood cells (Amende & Pierce, 1980; Smith & Pierce, 1987); and polychaete (*Glycera*) red coelomocytes (Costa & Pierce, 1983) and is required for organic osmolyte (sorbitol) efflux in rat kidney cells (Bevan, Theiss & Kinne, 1990). Hypoosmotically induced membrane permeability changes towards inorganic ion osmolytes also display a dependence upon Ca^{2+} in some cells: dog blood cells (Parker, 1983); human lymphocytes (Grinstein, Dupre & Rothstein, 1982); Ehrlich ascites cells (Hoffmann, Simonsen & Lambert, 1984); human red cells (Hoffman et al., 1980); *Amphiuma* red blood cells (Cala, 1983); and toad bladder (Wong & Chase, 1986). The Ca^{2+}-dependence of any organic osmolyte efflux in these systems has not been studied.

* *Present address:* Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814.

How and where Ca^{2+} exerts its effects on membrane permeability to osmolytes is unknown, but the possibility that the extracellular Ca^{2+} sensitivity of cell volume regulation results from changes in intracellular [Ca^{2+}] has been suggested by three types of experiments. First, divalent cation ionophores potentiate cell volume recovery in several cell types (*Amphiuma* red blood cells (Cala, 1983), thymocytes (Grinstein et al., 1983), Ehrlich Ascites cells (Hoffmann et al., 1984), *Glycera* blood cells (Pierce et al., 1988), most often attributed to an increase in inorganic ion permeability. Second, an influx of external Ca^{2+} occurs in response to hypoosmotic stress (toad bladder cells (Wong & Chase, 1986), *Amphiuma* red blood cells (Cala, Mandel & Murphy, 1986), choroid plexus epithelium (Christensen, 1987), *Glycera* and *Noetia* blood cells (Pierce et al., 1988). Third, pharmacological studies have implicated calmodulin in cell volume regulation (Grinstein et al., 1982; Cala, 1983; Hoffmann et al., 1984; Foskett & Spring, 1985; Cala et al., 1986; Wong & Chase, 1986; Pierce et al., 1989; Bevan et al., 1990).

One common mechanism by which Ca^{2+} influences cellular function is through the regulation of protein phosphorylation, which, in turn, regulates cellular metabolism and affects diverse physiological processes (Cohen, 1988). Thus, it is possible that Ca^{2+}, perhaps via calmodulin, affects volume-dependent membrane permeability changes by regulating the phosphorylation of cellular proteins. We have started to test this hypothesis in a marine invertebrate cell type, *Noetia ponderosa* red blood cells. These cells volume regulate under hypoosmotic stress in a Ca^{2+}- and calmodulin-dependent manner (Smith & Pierce, 1987; Pierce et al., 1988, 1989).

Our approach was to examine proteins from nucleated ghosts made from *Noetia* blood cells for changes in $^{32}P_i$ phosphorylation after the cells were exposed to a hypoosmotic stress. We have found at least two ghost protein phosphorylations that are stimulated by hypoosmotic stress. The osmotic challenge was important for the initiation of the phosphorylation of both proteins. Both phosphorylation events were dependent on extracellular Ca^{2+} and inhibited by the calmodulin antagonist, chlorpromazine.

Materials and Methods

Noetia Erythrocytes

Noetia ponderosa were obtained from Hog Island Bay on the east coast of the Delmarva Peninsula by watermen (Terry Bros., Willis Wharf, VA) and shipped to College Park by bus. The clams were maintained in aquaria containing artificial sea water (ASW, Instant Ocean; 935 mosM) at 10°C and acclimated for at least two weeks before use.

Red blood cells were collected from *N. ponderosa* by prying open the valves and slashing the mantle with a razor blade. The animal was placed over polyester fibers in a funnel, and blood was allowed to drain from the cut surfaces into a beaker. The blood was diluted with ASW (Costa & Pierce, 1983) (935 mosM) containing 5 mM MOPS (pH 7.4) and centrifuged at 160 × *g* for 10 min. The supernatant was discarded, the blood cells were resuspended in the buffered ASW and washed twice more as described above except that the last spin was done at 4000 × *g*. The supernatant and top layer of the pellet containing amoeboid cells and gametes were removed by careful aspiration. The cell pellet from the last wash was then resuspended in isosmotic ASW for use in the experiments.

Erythrocyte Ghost Production and Evaluation

Ghosts were produced by exposing *Noetia* erythrocytes to a lysing medium at 0°C (adapted from Nemhauser, Joseph-Silverstein & Cohen, 1983). The lysing medium contained (in mM): 50 Na_2PO_4 (pH 7.4), 2.5 EGTA, 10 EDTA, 50 NaF and 0.5 PMSF. The components of this medium stabilize phosphoproteins by inhibiting endogenous protein phosphatases (Lindemann et al., 1983; Adunyah & Dean, 1987) and proteases (Bond & Butler, 1987). The lysate was centrifuged at 500 × *g* for 10 min to pellet the ghosts, which were then washed twice in 50 volumes of lysing medium.

The ghost preparation was evaluated by transmission electron microscopy and by enzyme markers for plasma membrane and mitochondria. Na^+ + K^+-dependent ATPase was used as a plasma membrane marker and measured as ouabain-inhibited ATPase activity (Towle, Palmer & Harris, 1976). ATP hydrolysis by 20 to 100 μg of whole homogenate protein or ghost protein was measured in media with or without 1 mM ouabain. The medium without ouabain contained (in mM): 50 KCl, 250 NaCl, 5 $MgCl_2$, 5 ATP and 10 MOPS (pH 7.4). Inorganic phosphate was measured by adding molybdate and measuring the absorbance of phosphomolybdic acid which was reduced by 1-amino-2-naphthol-4-sulfonic acid (Peterson, 1978). The difference in the rate of inorganic phosphate liberation between the two media, representing ouabain-inhibited ATPase activity, was calculated. Protein was measured by a modification of the Lowry method (Peterson, 1977), and the Na^+ + K^+-dependent ATPase specific activity was expressed as μM P_i/hr/mg protein. Succinate cytochrome *c* reductase was used as a marker for mitochondria. The assay method spectrophotometrically monitored (600 nm) the reduction of the dye, DCIP to $DCIPH_2$ (King, 1967). From 20 to 100 μg of whole homogenate protein or ghost protein were added to a reaction buffer (25 mM Na_2PO_4 at pH 7.4, 50 μM DCIP, 25 mM Na-succinate and 2 mM NaCN), and DCIP reduction was monitored for 3 min at 22°C against a blank. CN^- was included in the reaction buffer to inhibit endogenous cytochrome oxidase activity, thereby preventing the reoxidation of $DCIPH_2$. The data are expressed as nmol DCIP reduced/min/mg protein.

Ghosts were prepared for transmission electron microscopy by fixing for 14 hr in glutaraldehyde (2.5%) in 5 mM MOPS (pH 7.4) and, after two buffer rinses, postfixed in 1% OsO_4. The ghosts were then dehydrated in ethanol, embedded in Epon 812, thin sectioned, and stained with 2% aqueous uranal acetate and lead citrate before being examined.

In Situ Ghost Protein Phosphorylation

Noetia erythrocytes (3×10^6 cells/ml) in ASW isosmotic to the salinity of acclimation (935 mosM) were incubated on a shaker table (22°C) with 0.4 mCi/ml $^{32}P_i$ (ICN, Irvine, CA) for 2 hr in order to label intracellular ATP pools. The cells were then washed twice in 30 volumes of unlabeled isosmotic ASW and exposed to isosmotic or hypoosmotic (560 mosM) ASW. At intervals after this exposure, aliquots of cells (about 2.4×10^7 cells) were removed and added to 60 volumes of lysing medium, producing ghosts as described above. In order to permanently protect the ghost phosphoproteins from endogenous protein phosphatases and proteases, as well as to prepare the samples for electrophoresis, the ghosts were immediately diluted with SDS-sample buffer (2.3% SDS, 63 mM Tris-HCl (pH 6.9), 12.5% glycerol) and boiled for 20 min. Detergent-insoluble material was removed from the samples by centrifugation (10,000 × *g* for 5 min). The supernatant containing SDS-dissolved ghost protein was analyzed for protein concentration (Bradford, 1976), and the quantity of ^{32}P incorporated into protein determined before electrophoresis. Incorporated ^{32}P was quantified by exposing aliquots of SDS-dissolved ghost protein to 6% TCA in the presence of 100 μg BSA carrier and pelleting precipitated protein by centrifugation (10,000 × *g* for 10 min). The pellet was dissolved in 0.1 N NaOH, and the radioactivity measured by liquid scintillation counting. Samples were diluted to obtain equal concentrations of TCA-precipitable counts, resulting in protein concentrations of 0.5–2.0 μg/μl. Up to 40 μl of these diluted samples were subjected to SDS polyacrylamide gel electrophoresis (SDS/PAGE; 0.8-mm thickness) containing a running gel of either 10% or a linear gradient of 7–10% acrylamide (Laemmli, 1970). Gels were dried over chromatography paper after staining in 0.1% Coomassie blue R-250 (Andrews, 1986) and exposed to X-ray film (Kodak X-Omat AR5) in the presence of an intensifying screen (Cronex Lightning Plus) at −70°C for 24–48 hr. Sample protein molecular weights were determined by comparing migration distances with molecular weight standards run in each gel (trypsinogen, 24 kDa; glyceraldehyde 3-phosphate dehydrogenase, 36 kDa; egg albumin, 45 kDa; bovine albumin, 66 kDa; and phosphorylase a, 97.4 kDa) (Sigma Chemical, St. Louis, MO). The intensities of the bands produced on the X-ray film were measured densitometrically (Gilford Response, Oberlin, OH), and the resulting peak areas digitized (Apple IIe and Bioquant, R&M Biometrics, Nashville, TN). Exposure was adjusted to provide band intensities within the linear range of the X-ray film. The band intensity or densitometric peak height at which film saturation begins was determined by subjecting different amounts of ^{32}P-labeled ghost proteins to SDS-PAGE and autoradiography. The digitized areas of the three proteins of interest were plotted as a function of the amount of ^{32}P-labeled protein loaded. Areas increased linearly with the amount of labeled protein loaded up to 700 mm^2, where it appeared to saturate. This corresponds to a peak height of 1.7 absorbance units.

Experimental Treatments

To distinguish whether the osmotic stress or the ionic dilution initiated the ghost protein phosphorylations, cells were preincubated in $^{32}P_i$ as described above and, in addition to isosmotic and hypoosmotic media, the cells were also exposed to a medium ionically equivalent to hypoosmotic ASW, but made isosmotic to the salinity of acclimation (935 mosM) by the addition of sucrose. After a 10-min exposure, the ^{32}P incorporation into ghost proteins was measured.

Two experiments were conducted to determine if the hypoosmotically induced protein phosphorylations were dependent upon $[Ca^{2+}]$. Both measured ghost protein phosphorylation 10 min after the cells were transferred to isosmotic (935 mosM) or hypoosmotic ASW (560 mosM) with or without the following treatments. First, the *Noetia* erythrocytes, after incubation with ^{32}P, were washed twice with Ca^{2+}-free, isosmotic ASW containing 1 mM EGTA. These cells were then transferred to isosmotic or hypoosmotic Ca^{2+}-free ASW, both containing 1 mM EGTA. In the second treatment, cells were exposed to the calmodulin antagonist, chlorpromazine (10 μM), for the last 20 min of $^{32}P_i$ incubation before transfer to isosmotic or hypoosmotic conditions containing the same concentration of the drug.

Cell Volume Measurements

Cell volume was measured using an electronic cell counter (Coulter Electronics, Hialeah, FL, model ZB) equipped with a cell sizer (Channelyzer, Coulter Electronics) which was calibrated against polystyrene beads. Channelyzer data were transferred to an Apple II+ computer for analysis. Red cells were collected and washed as described above and then diluted with isosmotic ASW to obtain a suspension of about 50,000 cells/ml. An aliquot of this suspension was used to determine initial cell volume, and additional aliquots were dispensed into the test solutions. The cells were then incubated at room temperature on a shaker table. At appropriate intervals, the volumes of about 25,000 cells were determined with the Coulter Counter.

Free Amino Acid (Taurine) Efflux Measurement

Noetia blood cells were prepared and dispensed into test solutions as described above. An aliquot of the cell suspension was taken for the determination of cell number using the Coulter Counter. The rest of the suspension was centrifuged (1000 × *g*). The supernatant was removed and mixed with an equal volume of 80% ethanol, brought to a boil and, after cooling, centrifuged to remove precipitated proteins (20,000 × *g*, 15 min). The supernatant from this last centrifugation was freeze-dried, the residue taken up in 0.2 N lithium citrate buffer (pH 2.2), and the amino acid content of that solution determined with an amino acid analyzer (Beckman, System Gold). Since it has been shown previously that taurine is the major amino acid in the bivalve cells, accounting for more than 60% of the total free amino acid pool, and is the major amino acid in the efflux (Amende & Pierce, 1980), taurine concentrations alone were measured. The results were expressed as taurine content, nmol/10^6 cells.

Statistics

The results were tested for significance using analysis of variance (ANOVA) with Student-Neuman-Keuls multiple range techniques (Steele & Torrie, 1960) and Student's *t* tests (Sokal & Rohlf, 1981). A probability level $P \leq 0.05$ was accepted as statistically significant.

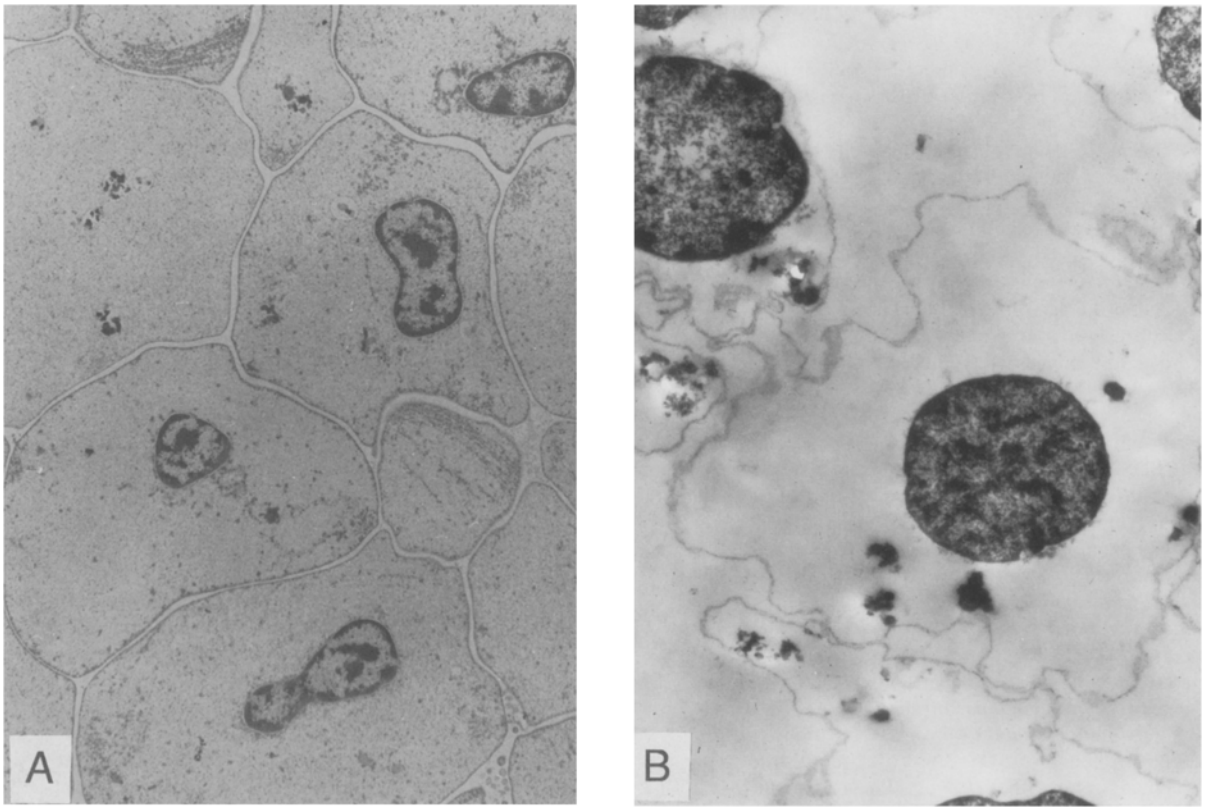

Fig. 1. Transmission electron micrographs of *Noetia* erythrocytes (*A*) (magnification ≈ 5700×) and nucleated ghosts (*B*) (magnification ≈ 11400×)

Results

Ghosts produced from *Noetia* erythrocytes are nucleated, 15–20 μm in diameter, devoid of hemoglobin and most other cytoplasmic components (Fig. 1), and reflect a substantial enrichment of plasma membrane. $Na^+ + K^+$-dependent ATPase in the ghosts is enriched 12-fold as compared with the whole-cell homogenate, while the activity of the mitochondrial marker, succinate cytochrome *c* reductase, is reduced by fourfold in the ghosts (Table).

Incubation of the blood cells with $^{32}P_i$ under isosmotic conditions results in the labeling of several ghost proteins. A consistently resolved, significantly labeled protein with a molecular weight of 58 kDa did not change in specific activity (film peak area/μg ghost protein) with hypoosmotic stress (Fig. 2). Thus, the ^{32}P incorporation into this protein was used as a constant to express the phosphorylation changes which occurred in other proteins. The data for each protein are expressed as its densitometric area divided by the area of the 58-kDa band in each sample.

Table Marker enzyme analysis of *Noetia* ghosts

	Enzyme-specific activity[a]	
	Succinate cytochrome *c* reductase[b]	$Na^+ + K^+$ ATPase[c]
Whole homogenate	23.0 (7.5)	0.06 (0.02)
Ghosts	5.3 (1.0)	0.74 (0.11)

[a] Average of 3–5 determinations (± SEM).
[b] nmol/min · mg^{-1} protein.
[c] μmol/hr · mg^{-1} protein.

At least two proteins consistently incorporate additional ^{32}P after cells are stressed hypoosmotically. The apparent molecular weights of these proteins are 63 and 34 kDa (Fig. 3). A major Coomassie blue stained band corresponds to the 34-kDa band, while the 63-kDa band is not visible with this staining method. Occasionally, additional proteins appeared to become phosphorylated in response to hypoosmotic stress; however, none were consistent among different experiments. Thus, although other phos-

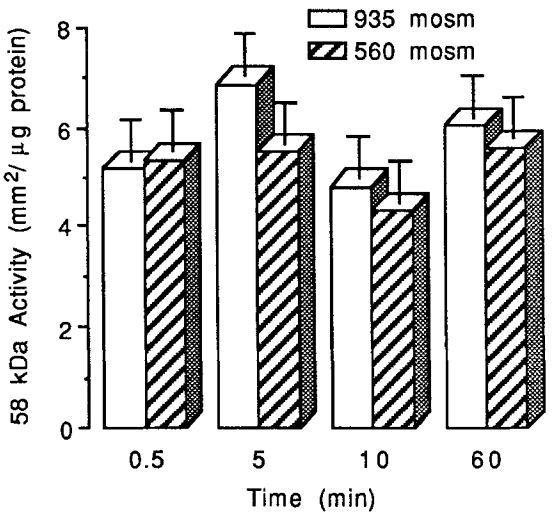

Fig. 2. The specific activity of ^{32}P-labeled 58-kDa ghost protein from cells exposed to isosmotic (935 mosM) or hypoosmotic (560 mosM) ASW. Specific activity is represented by densitometric area from the X-ray film (mm^2) per μg of protein loaded on SDS-PAGE. Histograms are means of five separate experiments. Error bars indicate SEM (ANOVA).

phoproteins may also respond to osmotic stimuli, only the 63- and the 34-kDa proteins were followed.

The changes in phosphorylation of the 34- and 63-kDa proteins are insignificant 30 sec after hypoosmotic stress. The ^{32}P content of both proteins then rises for at least 5–10 min, followed by a significant degree of dephosphorylation over the next 50 min (Fig. 4). The ^{32}P content of the 34-kDa band increases by 60% in the first 10 min and then partially returns to its prestress level of phosphorylation after an hour, ending 25% higher than isosmotic controls. The 63-kDa band rises by 120% in 10 min and decreases back to 20% above isosmotic levels after 1 hr.

Noetia red cells do not release taurine or change volume in response to a medium which has the ionic concentrations of hypoosmotic ASW, but is isosmotic to the salinity of acclimation (hypoionic-isosmotic ASW; 935 mosM) (Fig. 5). When cells are exposed to the ionic (but not the osmotic) stress in this way, the 34- and 63-kDa proteins incorporate significantly less ^{32}P than in hypoosmotic conditions (Fig. 6). The 34-kDa protein only becomes 15% more phosphorylated with the hypoionic-isosmotic treatment as compared to the 40% increase in phosphorylation with hypoosmotic exposure. The 63-kDa protein increases in phosphorylation by ~13% with hypoionic treatment instead of 125% with hypoosmotic exposure. The results suggest that, although hypoionic-isosmotic stress results in some phosphorylation, osmotic stress is an important component to the initiation of ^{32}P incorporation into both proteins.

The hypoosmotically induced phosphorylation of the 34- and 63-kDa proteins is Ca^{2+} dependent based on two experiments. The hypoosmotically induced phosphorylations of both proteins were significantly reduced by the Ca^{2+}-free treatment. The 34-kDa protein is phosphorylated at about 60% and the 63-kDa protein at about 35% of the control without extracellular Ca^{2+} (Fig. 6). Treatment of the erythrocytes with chlorpromazine also significantly inhibits the hypoosmotically induced phosphorylation of the 34- and 63-kDa bands (by 97 and 71%, respectively) (Fig. 6). Neither Ca^{2+}-free treatment nor exposure to chlorpromazine changed the ^{32}P content of the 34- and the 63-kDa ghost proteins when cells were maintained in isosmotic conditions.

Discussion

Two ghost proteins incorporate increased amounts of ^{32}P as a response to hypoosmotic exposure. This response is specific since at least one other protein (58 kDa) maintains a constant specific activity after the stress. The hypoosmotically induced increase in ^{32}P incorporation by the 34- and 63-kDa *Noetia* ghost proteins share several characteristics with the volume recovery response of the blood cells. First, the osmotic stress is important to the initiation of the phosphorylation, as it is to both the osmotically dependent taurine efflux reported here and the hypoosmotically induced Ca^{2+} influx we reported earlier (Pierce et al., 1988). Second, phosphorylation of both ghost proteins after hypoosmotic exposure is reduced by Ca^{2+}-free conditions. The same Ca^{2+}-free treatment also inhibits cell volume regulation in the *Noetia* blood cells by reducing taurine efflux, while leaving the KCl efflux unaffected (Smith & Pierce, 1987). Although these data indicate that extracellular Ca^{2+} is important for the osmotically induced phosphorylations and taurine efflux, results with the calmodulin antagonist provide evidence that the site of Ca^{2+} action is an intracellular one. The phosphorylations are inhibited by chlorpromazine at the same concentration that inhibits *Noetia* cell volume recovery and taurine efflux (Pierce et al., 1988). Chlorpromazine is a well-known calmodulin antagonist, but is not specific in its actions (Norman, Drummond & Moser, 1979; Kaczmarek, 1986; Greenberg, Carpenter & Messing, 1987). However, all of the other known targets for the drug (protein kinase C, voltage-dependent Ca^{2+} channels and nonspecific membrane effects due to anesthetic properties) have been ruled out of the cell volume recovery response in *Noetia* erythrocytes (Pierce et al., 1989).

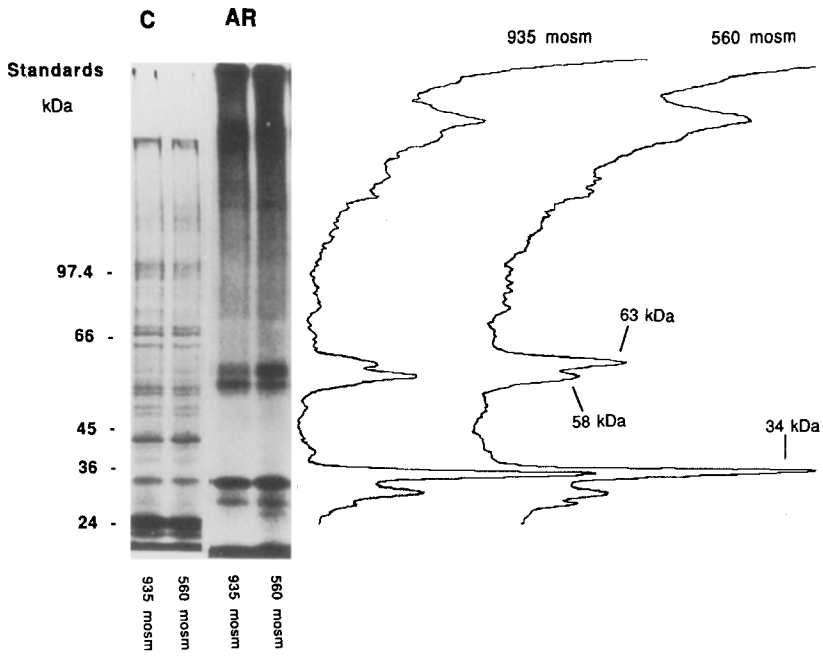

Fig. 3. The analysis of *in situ* protein phosphorylation. Erythrocytes were incubated with $^{32}P_i$, exposed for 10 min to isosmotic ASW (935 mosM) or hypoosmotic ASW (560 mosM) and lysed. The proteins from the resulting ghosts were separated by SDS-PAGE and stained with Coomassie blue R-250 (*C*). The numbers to the left of the gel represent the migration of molecular weight standards. The autoradiogram of the gel (*AR*) and the corresponding densitometric scan are also shown. The 63-, 58- and 34-kDa proteins are labeled on the 560-mosM scan.

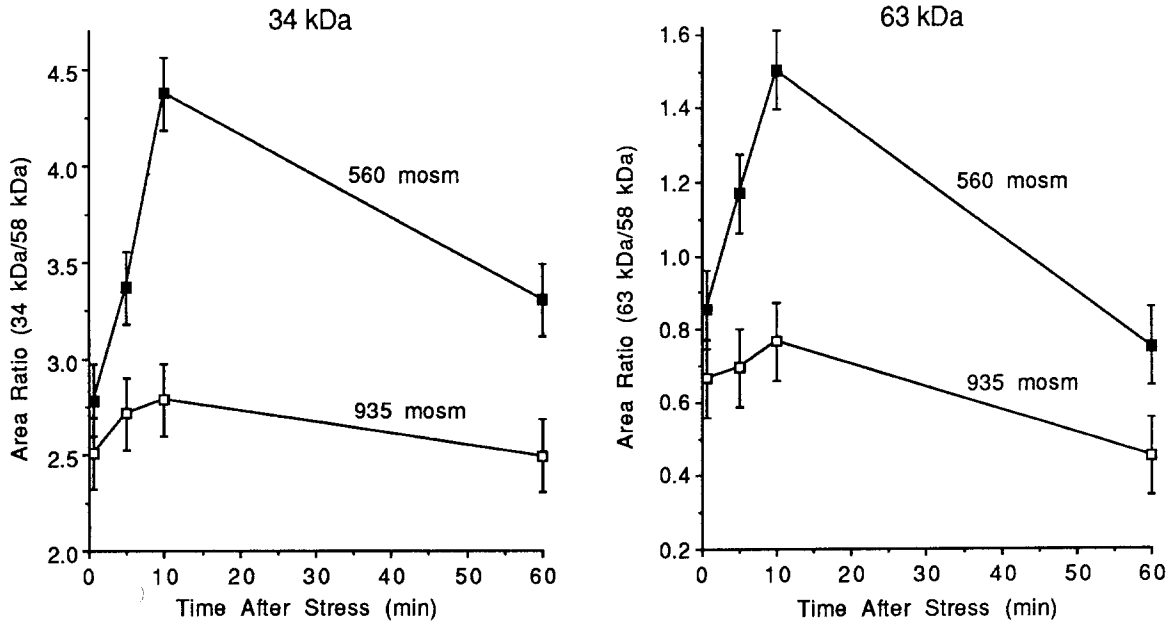

Fig. 4. Time courses of hypoosmotically induced phosphorylation of the 34-kDa (left) and the 63-kDa ghost protein (right) from *Noetia* red cells. ^{32}P-labeled cells were transferred to isosmotic (935 mosM) or hypoosmotic (560 mosM) ASW. The level of ^{32}P incorporation was measured over time and expressed as a ratio to the ^{32}P levels in the 58-kDa reference protein. Points represent the means of five separate experiments. Error bars indicate SEM (ANOVA).

The correlated effects of osmotic stimulation, extracellular Ca^{2+} dependence and the inhibition by chlorpromazine on both cell volume recovery and protein phosphorylation suggest that osmotically dependent taurine efflux may be initiated and/or regulated by these phosphoproteins.

A comparative time course of relevant events leading to the regulation of plasma membrane permeability to taurine and the contribution to cell volume recovery following hypoosmotic stress can now be suggested. First, within seconds of the hypoosmotic stress, a Ca^{2+} influx starts into the *Noetia*

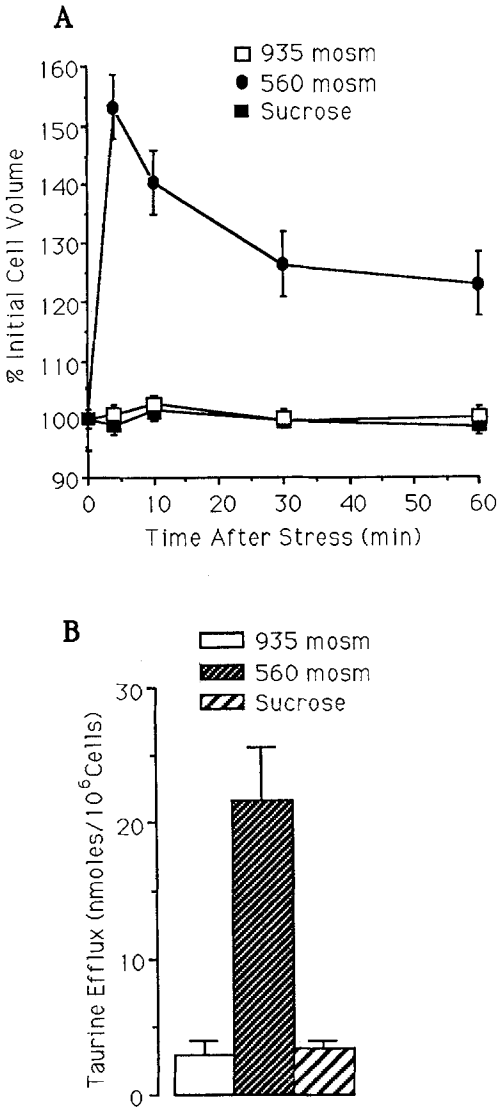

Fig. 5. Volume changes over time (*A*) and taurine efflux after one hr (*B*) of *Noetia* erythrocytes exposed to isosmotic ASW (935 mosM), hypoosmotic ASW (560 mosM) or ASW ionically equivalent to the hypoosmotic medium but maintained isosmotic with the addition of sucrose. Values represent means from three separate experiments. Error bars indicate SEM.

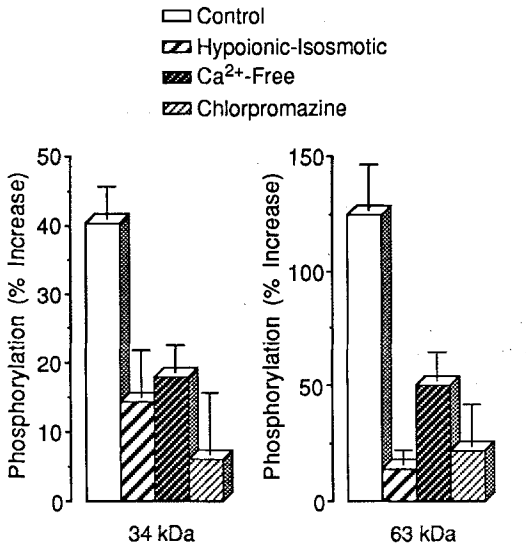

Fig. 6. The effects of three treatments on hypoosmotically induced phosphorylation of the 34-kDa (left) and the 63-kDa (right) ghost proteins from *Noetia* red cells. ^{32}P-labeled cells were incubated for 10 min in isosmotic or hypoosmotic ASW. "*Ca^{2+}-Free*" indicates medium without Ca^{2+} plus 1 mM EGTA. "*Chlorpromazine*" indicates treatment with 10 μM chlorpromazine. The data are expressed as % increase in ^{32}P incorporation by hypoosmotically stressed cells over cells exposed to the same treatment in isosmotic conditions. Cells were also exposed to "*Hypoionic-Isosmotic*" ASW, where phosphorylation was compared to cells exposed to isosmotic ASW without treatment. Histograms represent means of 4–8 separate experiments. Error bars indicate SEM.

blood cells, perhaps through a stretch-activated Ca^{2+} channel (Pierce et al., 1988). The rising intracellular [Ca^{2+}] activates calmodulin which, in turn, results in the activation of a kinase (Cohen, 1988), or the inactivation of a phosphatase (Cohen, 1989), causing the increased phosphorylation and activation of the specific proteins. The phosphorylation of the 34- and 63-kDa proteins occurs with a slightly later time scale relative to the stress as compared to the Ca^{2+} influx, requiring at least a few minutes to peak. Taurine efflux is evident by 10 min and is complete within 1 hr (Amende & Pierce, 1980; Smith & Pierce, 1987). This time course suggests that the activated proteins might positively influence taurine release. In any case, with time, the Ca^{2+} signal could be inactivated either by sequestration or Ca^{2+} extrusion, preventing additional phosphorylations and resulting in the dephosphorylation of the proteins, perhaps through the action of constitutive or reactivated phosphatases. Both proteins are then slowly dephosphorylated over the next 40–50 min. This reduces the taurine permeability and slows the efflux.

At present, our data do not prove that the proteins phosphorylated during osmotic stress are related to the efflux of osmolytes. However, the coincidence of phosphorylation changes after hypoosmotic stress, and the other characteristics of cell volume recovery suggest that these proteins may play a role in cell volume regulation. A role for protein phosphorylation in the response to hyperosmotic stress has been tested in at least two instances. Protein phosphorylation occurred in membranes from hyperosmotically stressed lymphocytes (Grinstein et al., 1986) where it was associated with Na^+/H^+ exchange. However, no protein phosphorylation was found in membranes from hyperosmotically stressed human and rat erythrocytes (Orlov et al., 1989).

The subcellular distribution of the 34- and 63-

kDa proteins is not yet clear, but they are present in nucleated ghosts, limiting their sites to either the plasma membrane or the nucleus. Phosphorylation of plasma membrane proteins is an obvious possibility for the mechanism that regulates osmolyte permeability. Although the matter has not been tested with regard to osmotic stress, membrane protein phosphorylation certainly modulates permeability under other conditions (James, Hiken & Lawrence, 1989; Weinman et al., 1989; McCann & Welsh, 1990; Toskulkao et al., 1990). Alternatively, protein phosphorylation within the nucleus might alter the expression of genes relevant to osmotic adaptation. Nuclear kinases are associated with the regulation of gene transcription (Nairn, Hemmings & Greengard, 1985), and the use of transcriptional regulation as a response to osmotic stress is well established in procaryotic organisms (Styrvold et al., 1986; Higgins et al., 1987; Eshoo, 1988). In any case, additional studies currently underway, including distribution, characterization and manipulation of the phosphoproteins, are required to define their role in osmolyte permeability.

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