The influence of Lyn kinase on Na,K-ATPase in porcine lens epithelium

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Bozulic, Larry D., William L. Dean, and Nicholas A. Delamere. The influence of Lyn kinase on Na,K-ATPase in porcine lens epithelium. Am J Physiol Cell Physiol 286: C90–C96, 2004.—Na,K-ATPase is essential for the regulation of cytoplasmic Na+ and K+ levels in lens cells. Studies on the intact lens suggest activation of tyrosine kinases may inhibit Na,K-ATPase function. Here, we tested the influence of Lyn kinase, a Src-family member, on tyrosine phosphorylation and Na,K-ATPase activity in membrane material isolated from porcine lens epithelium. Western blot studies indicated the expression of Lyn in lens cells. When membrane material was incubated in ATP-containing solution containing partially purified Lyn kinase, Na,K-ATPase activity was reduced by ~38%. Lyn caused tyrosine phosphorylation of multiple protein bands. Immunoprecipitation and Western blot analysis showed Lyn treatment causes an increase in density of a 100-kDa phosphotyrosine band immunopositive for Na,K-ATPase α1 polypeptide. Incubation with protein tyrosine phosphatase 1B (PTP-1B) reversed the Lyn-dependent tyrosine phosphorylation increase and the change of Na,K-ATPase activity. The results suggest that Lyn kinase treatment of a lens epithelium membrane preparation is able to bring about partial inhibition of Na,K-ATPase activity associated with tyrosine phosphorylation of multiple membrane proteins, including the Na,K-ATPase α1 catalytic subunit.

Na,K-ATPase is a ubiquitous active ion transport protein complex found in the plasma membrane of all animal cells. The functional Na,K-ATPase comprises a 112-kDa catalytic α-subunit together with a noncatalytic 35-kDa β-subunit and appears to exist as an α2β2 heterodimer (3). Na,K-ATPase couples the hydrolysis of one molecule of ATP to the outward translocation of three sodium ions and inward translocation of two potassium ions against their steep electrochemical gradients. Thus Na,K-ATPase maintains the normally high-potassium and low-sodium concentrations in the cytoplasm of animal cells (6). In the lens, regulation of cytoplasmic electrolyte concentration is vital for transparency. The majority of human cataractous lenses display an abnormally high sodium content, and the degree of lens opacification is more severe in lenses with higher sodium values (20). For normal lens function, it has been argued that Na,K-ATPase activity must be high in the monolayer of epithelium that covers the anterior lens surface (33). Fiber cells that make up the bulk of the lens do not lack Na,K-ATPase polypeptides but have a lower Na,K-ATPase activity than the epithelium (18). Na,K-ATPase-mediated ion transport by the epithelium appears to play a major role in regulating electrolyte composition for the entire lens. Regulation of Na,K-ATPase activity and function in lens epithelium has not been widely studied.

Regulation of Na,K-ATPase function can occur through several different protein kinase-mediated mechanisms. Studies on the effects of phosphorylation by PKA and PKC on Na,K-ATPase function reveal a wide range of response patterns in different tissue types, some stimulatory, some inhibitory (42). The activation of tyrosine kinases appears to stimulate Na,K-ATPase in the intact kidney proximal tubule, as judged by an increase of ouabain-sensitive 86Rb uptake (22, 36). The proximal tubule response may involve recruitment of tyrosine-phosphorylated Na,K-ATPase protein from the cytosol to the plasma membrane (36). In rat skeletal muscle, insulin causes tyrosine phosphorylation of Na,K-ATPase α1 and α2 protein (8) and stimulates Na,K-ATPase α1 and α2 translocation to the plasma membrane (2). In rat astrocytes, tyrosine kinase activation by insulin causes an increase of Na,K-ATPase activity associated with a selective increase in the synthesis of Na,K-ATPase α1 protein (34). In other tissues, however, tyrosine kinase activation appears to inhibit Na,K-ATPase function. In cultured rabbit nonpigmented ciliary epithelium, tyrosine kinase inhibitors were found to prevent the inhibitory action of dopamine and D-1 agonists on Na,K-ATPase-mediated ion transport (35). In the intact, organ-cultured lens, previous studies show genistein reduces the inhibitory effects of endothelin (ET-1) on ouabain-sensitive 86Rb uptake (38). Also in the lens, thrombin-induced inhibition of Na,K-ATPase-mediated active ion transport is suppressed by herbimycin A, an inhibitor of Src-family tyrosine kinases (37). Inhibition of Na,K-ATPase function occurs in parallel with increased tyrosine phosphorylation of multiple membrane proteins in the epithelium of thrombin-treated lenses.

Nonreceptor tyrosine kinases, including Src-family kinases, are activated in thrombin-treated tissues (23). In platelets, the Src-family kinase Lyn plays a key role in the response to thrombin (26). When platelets from Lyn-deficient mice are challenged with thrombin, normal platelet aggregation, thromboxane A2 production, and ADP secretion fail to occur (10). In the present study, experiments were conducted to test whether a change of intrinsic Na,K-ATPase activity is detectable after Lyn kinase-dependent tyrosine phosphorylation of isolated, partially purified lens epithelium membrane material. Lyn treatment caused a partial inhibition of Na,K-ATPase activity associated with tyrosine phosphorylation of multiple membrane proteins, including the Na,K-ATPase α1-catalytic subunit.

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**EXPERIMENTAL PROCEDURES**

*Tissues.* Porcine eyes and kidneys were obtained from Swift Meat Packing (Louisville, KY). The lens was isolated by dissecting open the posterior of the eye, cutting the suspensory ligaments, and transferring the lens to a petri dish. The lens capsule and attached monolayer of epithelial cells covering the anterior portion of the lens were removed and snap-frozen in liquid nitrogen. Material from 40–50 lenses was pooled. Kidneys were bisected and the dark red outer medulla was isolated and snap-frozen in liquid nitrogen (16).

**Membrane preparation.** Membrane preparations containing plasma membranes as well as intracellular membranes were obtained following methodology described by Okafor et al. (37). Previously frozen samples of lens capsule epithelium and kidney outer medulla were homogenized in ice-cold homogenization buffer A (150 mM sucrose, 4 mM EGTA, 5 mM HEPES, 800 μM dithiothreitol (DTT), pH 7.4) in the presence of protease inhibitors [100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 2 μg/ml aprotinin] using a glass homogenizer. The homogenate was centrifuged at 115,000 g for 60 min at 4°C. To remove extrinsic proteins, the membrane pellet was then resuspended in homogenization buffer A containing 600 mM KCl and subjected to centrifugation once again at 115,000 g for 60 min at 4°C (16). The membrane pellet was resuspended in homogenization buffer A and subjected to centrifugation a final time at 115,000 g for 60 min at 4°C. The final pellet containing epithelium or kidney membrane material was resuspended in buffer A and the protein content was measured using the BCA protein assay kit (Pierce, Rockford, IL).

**Lyn-dependent phosphorylation and Na,K-ATPase activity measurement.** Lens epithelium or kidney membrane material was incubated in kinase buffer containing 1 mM EGTA, 10 mM Tris, pH 7.2, 20 mM MgCl₂, 1 mM ATP, 0.2 mM sodium orthovanadate, 10 μg/ml pepstatin A, 10 μg/ml antipain, 10 μg/ml leupeptin, 1 mM PMSF, 5 mM DTT, and Lyn kinase (0.08 units/μg protein) (Upstate Biotechnology, Lake Placid, NY) for 20–30 min at 30°C. Treated material was then used for Western blot analysis, immunoprecipitation, or Na,K-ATPase activity measurements. Sodium orthovanadate, an inhibitor of Na,K-ATPase activity, was removed before Na,K-ATPase activity measurements. To remove sodium orthovanadate, the membrane material was centrifuged at 100,000 g for 3 min. The membrane pellet was resuspended twice in 100 μl of centrifugation buffer [10 mM Tris, pH 7.2, 5 mM DTT, 10% glycerol (wt/vol)] and centrifuged at 100,000 g for 3 min. The final pellet was resuspended in ~100 μl of Na,K-ATPase buffer and assayed immediately for Na,K-ATPase activity.

In some experiments, Lyn kinase–treated membrane material was subsequently incubated with protein tyrosine phosphatase 1B (PTP-1B) (0.5 U/μl) (Upstate Biotechnology, Lake Placid, NY) for 30 min at 37°C in PTP-1B assay buffer (25 mM HEPES, 50 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, pH 7.4). Ouabain, a specific inhibitor of Na,K-ATPase (46), was added to half the sample aliquots to a final concentration of 1 mM. Samples were preincubated for 15 min at 37°C with gentle agitation. ATP hydrolysis was initiated by the addition of ATP to a final concentration of 1 mM. The ATP hydrolysis reaction was carried out for 45 min at 37°C with gentle agitation. The reaction was stopped with the addition of 15% ice-cold trichloroacetic acid. ATP hydrolysis was quantified using a colorimetric method that measured the amount of inorganic phosphate released in each reaction sample (37). Less than 20% of the available substrate ATP was hydrolyzed. The difference in ATP hydrolysis in the presence and absence of ouabain was a measurement of Na,K-ATPase activity. The data were presented as nanomoles phosphate released per milligram protein per minute.

Because Na,K-ATPase activity was measured in samples of lens epithelium or membrane material that had been treated with buffer containing 0.2 mM sodium orthovanadate and then washed, separate studies were conducted to confirm that Na,K-ATPase activity was not inhibited by residual vanadate. Na,K-ATPase activity was 9.7 ± 0.4 nmoles Pi·mg protein¹min⁻¹ (mean ± SE; n = 5) in vanadate–treated samples, which was not significantly different from the activity of 10.2 ± 0.6 measured in control samples.

**86Rb uptake.** Ouabain-sensitive 86Rb uptake by the intact lens was used as an index of Na,K-ATPase-mediated active sodium-potassium transport. Intact lenses were bathed at 37°C in Krebs solution with the following composition (in mM): 119 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 1 MgCl₂, and 5.5 glucose at pH 7.4. It was assumed that the Na,K-ATPase mechanism transports 86Rb similarly to potassium. Lenses were preincubated for 10 min in Krebs solution containing test agents, and then 86Rb (100 μCi/ml) was added. Half of the lenses in each group also received 1 mM ouabain, added simultaneously with the 86Rb. The 86Rb uptake period was 30 min. Previous studies show that during this time, 86Rb uptake is linear. After the 30-min 86Rb uptake period, each lens was placed in a large volume of ice-cold nonradioactive Krebs solution for 2 min to wash 86Rb from extracellular space. After this, the lenses were weighed, lyophilized, and reweighed to determine water content. The dried tissue was digested in 30% nitric acid, and radioactivity in the acid solubilize was determined by 3-h counting in scintillation fluid. On the basis of the specific activity of 86Rb in the medium, the data were calculated as nanomoles potassium (86Rb) accumulated per gram of lens water per minute.

**Western blot analysis.** Membrane material was solubilized with Laemmli sample dilution buffer, and proteins were separated on a 7.5% gel by SDS-PAGE at 40 mA for 2 h using the Laemmli buffer system (31). Proteins were electrophoretically transferred to nitrocellulose sheets at 30 V for 16 h. The nitrocellulose membranes were blocked for 1 h with 5% dry milk in TTBS (30 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH 7.4). For immunodetection of Na,K-ATPase α₁, Lyn kinase, or tyrosine phosphoproteins, the nitrocellulose membranes were incubated at room temperature for 60 min with either a monoclonal antibody directed against Na,K-ATPase α₁ (Sigma, St. Louis, MO), Lyn kinase (Upstate Biotechnology), or anti-phosphotyrosine antibody PY20 (Transduction Lab, Lexington, KY) conjugated to horseradish peroxidase. Nitrocellulose membranes probed for Na,K-ATPase α₁ and Lyn kinase were washed with TTBS twice for 15 min and then three times for 5 min before being incubated for 60 min with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). Nitrocellulose membranes probed for PY20 were washed with TTBS twice for 15 min and then four times for 5 min at room temperature and then visualized with chemiluminescence substrate (Pierce, Rockford, IL). Nitrocellulose membranes were exposed to X-ray film.

**Immunoprecipitation.** Following a methodology modified from a technique described by Khundmiri and Lederer (29), lens epithelium (500 μg) and kidney membrane material (200 μg) were solubilized in sufficient immunoprecipitation buffer (10 μM deoxycholate, 100 mM Tris-mannitol, 5 mM Tris, pH 7.6, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, and 10 μg/ml pepstatin A) to bring the final protein concentration to 2 or 0.8 μg/ml, respectively. The membrane material was mixed on a rotating wheel at 4°C for 3 h. The insoluble material was then pelleted by centrifugation at 8,000 g for 15 min at 4°C. The supernatant (250 μl) was removed then preblocked with 20 μg of...
mouse IgG and 50 μl of immobilized protein G (ImmunoPure, Pierce, Rockford, IL) for 15 h on a rotating wheel at 4°C. The membrane material mixture was then centrifuged at 1,000 g for 3 min at 4°C. The supernatant was removed and precleared once again with rabbit IgG and 50 μl of immobilized protein A (ImmunoPure) for 3 h on a rotating wheel at 4°C. The supernatant was transferred to a fresh microcentrifuge tube, and 10 μg of polyclonal antibody directed against Na,K-ATPase α1 polypeptide (RD1, Flanders, NJ) was added. The membrane material mixture was incubated on a rotating wheel for 15 h at 4°C. After this, 50 μl of immobilized protein A (ImmunoPure) were added and mixed for an additional 3 h on a rotating wheel at 4°C. The mixture was then washed with 200 μl of PBS, pH 7.4, and then centrifuged at 1,000 g for 3 min at 4°C. The wash procedure was repeated two more times, and then immunoprecipitated Na,K-ATPase α1 polypeptide was dissociated from the protein A and antibody mixture by being incubated in 45 μl of Laemmli sample dilution buffer for 20 min at 65°C. The samples were centrifuged at 4,000 g for 5 min. The supernatant was then subjected to SDS-PAGE, followed by Western blot analysis. In some experiments, a different immunoprecipitating antibody was used. The immunoprecipitation was carried out using 2 μg of monoclonal antibody directed against tyrosine phosphoproteins (PY99) (Santa Cruz Biotechnology, Santa Cruz, CA).

In some experiments, Lyn kinase-treated membrane material was incubated with PTP-1B (100 μM/l) for 30 min at 37°C in PTP-1B assay buffer. The Lyn-PTP-1B-treated membrane material was subjected to immunoprecipitation, followed by SDS-PAGE. Resolved proteins were analyzed for tyrosine phosphoproteins by Western blot. Statistical analysis. Student’s t-test was used for statistical analysis.

RESULTS

Figure 1 illustrates tyrosine phosphorylation of membrane proteins in the intact lens. Membrane material isolated from lenses exposed to thrombin exhibited a marked increase in the density of several tyrosine phosphoprotein bands (Fig. 1). The phosphorylation increase was abolished by herbimycin A, a recognized inhibitor of Src-family tyrosine kinases. Among the many tyrosine phosphoprotein bands, a 100-kDa band appeared to comigrate with Na,K-ATPase α1 subunit. In some studies, the 100-kDa band was excised, subjected to in-gel tryptic digestion, and analyzed using mass-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Database search analysis resulted in the identification of peptides covering ~20% of the Na,K-ATPase α1 protein sequence corresponding to ~16 peptide matches for each analyzed sample (data not shown). Similar to findings reported earlier (37), exposure of the intact lens to thrombin was observed to cause a 35% decrease in the rate of ouabain-sensitive potassium (86Rb) uptake to 19.5 ± 1.8 nmoles/g lens water−1 min−1 from a control value of 30.1 ± 1.4 (mean ± SE: n = 6 lenses; significant P < 0.01), whereas in the presence of herbimycin + thrombin no reduction in the rate was apparent (35.3 ± 1.0).

Thrombin induces the activation of nonreceptor tyrosine kinases (11, 30, 32), including Lyn, a Src tyrosine kinase family member (10, 26). Lyn kinase is expressed in lens tissue. When lens epithelium membrane material was isolated and used for Western blot analysis, two immunopositive bands corresponding to the two known isoforms of Lyn kinase were detected (Fig. 2), although the results do not signify the degree to which Lyn is activated. To determine the effects of tyrosine kinase-mediated phosphorylation on lens epithelium membrane proteins in vitro, isolated lens epithelium membrane material was incubated with active, partially-purified Lyn kinase (0.08 units Lyn/μg membrane material) in ATP-containing kinase reaction buffer. After this, tyrosine phosphorylation and Na,K-ATPase activity were examined. Western blot analysis revealed a marked increase in several phosphotyrosine protein bands (Fig. 3). A smaller increase of phosphotyrosine band density observed in the presence of ATP but absence of added Lyn may signify the activity of endogenous Lyn and other tyrosine kinases. No increase was observed in the absence of both ATP and Lyn (data not shown). Na,K-ATPase activity was reduced significantly in lens epithelium material subjected to Lyn pretreatment (Table 1). To compare the effects of Lyn on a different tissue, Na,K-ATPase activity was also measured in membrane material isolated from kidney medulla. Lyn pretreatment was found to cause a ~20% reduction of Na,K-ATPase activity measured in kidney membrane material (Table 1).

Studies were conducted to determine whether tyrosine phosphorylation of the Na,K-ATPase α1 polypeptide occurs. Na,K-ATPase α1 protein was first isolated from lens epithelium membrane samples by immunoprecipitation using a polyclonal antibody directed against the Na,K-ATPase α1-subunit, and then the immunoprecipitated Na,K-ATPase α1 protein was subjected to treatment with Lyn kinase. The Lyn-treated immunoprecipitates were resolved by SDS-PAGE and subjected to Western blot analysis (Fig. 4). A dense, 100-kDa tyrosine phosphoprotein band was observed in Lyn-treated Na,K-ATPase α1 immunoprecipitates. The 100-kDa tyrosine phosphoprotein band was undetectable in Na,K-ATPase α1 immunoprecipitates that were not treated with Lyn. For technical

Fig. 2. Detection of Lyn kinase in lens epithelium membrane material. Lens epithelium membrane material (50 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against Lyn kinase. Lysate (20 μg) of nonstimulated A431 cells, a human epidermoid carcinoma line, was used as a positive control for Lyn kinase expression.

Fig. 1. The influence of thrombin on tyrosine phosphorylation of lens epithelium membrane proteins. Intact lenses were exposed to 2 units/ml thrombin (T) or vehicle (control; C) or exposed to thrombin in the presence of 7.5 μM herbimycin A (T + H) or herbimycin A alone (H) for 45 min and then used as a source of membrane material for Western blot to probe for phosphotyrosine residues. The phosphotyrosine blot (top) was stripped and reprobed for Na,K-ATPase α1 (bottom).
reasons, Na\textsubscript{+}-K-ATPase activity could not be determined reliably in the immunoprecipitates.

To examine the effects of Lyn on Na,K-ATPase in the nonsolubilized membrane, lens epithelium membrane material was treated first with Lyn before Na,K-ATPase \(\alpha_1\) protein was isolated by immunoprecipitation. Western blot analysis revealed a 100-kDa phosphotyrosine band in immunoprecipitates isolated from lens epithelium membrane material that had been subjected to Lyn treatment (Fig. 5A). A similar result was observed in membrane material isolated from porcine kidney medulla, a non-lens tissue in which the major Na,K-ATPase isofrom is \(\alpha_1\) (Fig. 5B). Phosphotyrosine bands were not detectable in immunoprecipitates isolated from either lens or kidney membrane material that was not treated with Lyn.

The results suggest Na,K-ATPase \(\alpha_1\) polypeptide is subject to tyrosine phosphorylation by Lyn. To confirm this idea, studies were conducted to test whether Na,K-ATPase \(\alpha_1\) protein could be immunoprecipitated from Lyn-treated lens membrane material using a monoclonal antibody directed against tyrosine phosphoproteins. A 100-kDa phosphotyrosine band immunopositive for Na,K-ATPase \(\alpha_1\) protein was observed in immunoprecipitates isolated from Lyn-treated lens membrane material (Fig. 6). Neither Na,K-ATPase \(\alpha_1\) nor phosphotyrosine bands were detected in immunoprecipitates isolated from non-Lyn treated lens epithelium membrane material or control samples in which the immunoprecipitating antibody was substituted for mouse IgG.

In some studies, lens epithelium membrane material was first treated with Lyn kinase and then subjected to tyrosine phosphatase treatment with PTP-1B. Treated membrane material was then immunoprecipitated with a polyclonal antibody directed against Na,K-ATPase \(\alpha_1\). A 100-kDa phosphotyrosine band was observed in the Na,K-ATPase \(\alpha_1\) immunoprecipitate obtained from Lyn kinase-treated lens epithelium membrane material but not from membrane material that had subsequently been treated with Lyn kinase and then subjected to tyrosine phosphatase treatment with PTP-1B.

Table 1. The influence of Lyn kinase on Na,K-ATPase activity

<table>
<thead>
<tr>
<th>Na,K-ATPase Activity After Lyn Kinase Treatment, % control</th>
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<tr>
<td>Lens epithelium (+ ATP − Lyn)</td>
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<tr>
<td>Lens epithelium (+ ATP + Lyn)</td>
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<td>Kidney medulla (+ ATP − Lyn)</td>
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Data are presented as means ± SE of results obtained from 25 measurements made using 6 different pools of Lyn-treated and non-Lyn-treated membrane material (\(n = 6\); lens epithelium) or from 33 measurements made using 8 different pools of Lyn-treated and non-Lyn-treated membrane material (\(n = 8\); kidney medulla). Control Na,K-ATPase specific activity was 9.1 ± 1.7 and 230.8 ± 13.8 nmoles Pi/mg protein/30 min, respectively, in lens epithelium and kidney medulla preparations. Data are shown as means ± SE. *Significant difference from control (\(P < 0.01\)).
been treated with PTP-1B (Fig. 7). Na,K-ATPase activity measured in Lyn-treated lens epithelium membrane material increased from 7.1 ± 1.7 nmoles P\text{\textsubscript{\text{-}}}mg protein\textsuperscript{-1}\text{\text{-}}min\textsuperscript{-1} to 12.3 ± 2.4 by subsequent PTP-1B treatment. This represents a 39.5 ± 14% increase in Na,K-ATPase activity (data as mean ± SE; n = 5; P < 0.05). In comparison, PTP-1B failed to change Na,K-ATPase activity in lens epithelium membrane material that was not pretreated with Lyn (data not shown).

DISCUSSION

The results suggest that tyrosine phosphorylation of isolated, partially purified lens epithelium membrane material can partially inhibit Na,K-ATPase activity. Lyn treatment caused Na,K-ATPase inhibition, and PTP-1B reversed the effect. The findings add to previous evidence for an association between tyrosine phosphorylation and inhibition of Na,K-ATPase activity in the intact lens (37, 38). The Na,K-ATPase inhibition response does not appear unique to lens cells because similar findings were observed in kidney medulla. Because multiple membrane proteins, including the Na,K-ATPase α\textsubscript{1} catalytic subunit polypeptide, were subject to tyrosine phosphorylation by Lyn, it is not possible to specify the extent to which the observed change of Na,K-ATPase activity depended on tyrosine phosphorylation of a specific protein.

Na,K-ATPase α\textsubscript{1} is the main isoform expressed in porcine lens cells, and neither Na,K-ATPase α\textsubscript{2} or α\textsubscript{3} isoforms are detectable by Western blot (19). Immunoprecipitation using antibodies directed against Na,K-ATPase α\textsubscript{1} and against phosphotyrosine residues confirmed tyrosine phosphorylation of Na,K-ATPase α\textsubscript{1} polypeptide in Lyn-treated membrane material. It was also demonstrated that Na,K-ATPase α\textsubscript{1} polypeptide could be isolated from lens epithelium first by immunoprecipitation and then subjected to Lyn treatment to elicit tyrosine phosphorylation detectable by Western blot. Further, recombinant protein tyrosine phosphatase PTP-1B was found to cause reversal of Lyn-induced tyrosine phosphorylation. Taken together, the results suggest Lyn causes tyrosine phosphorylation of Na,K-ATPase α\textsubscript{1} polypeptide.

Exogenous Lyn was used in this study as a means of causing tyrosine phosphorylation in isolated lens epithelium membrane material. Endogenous Lyn kinase was detected in lens epithelium, but the Western blot results do not provide information on its activation state. Lyn kinase, a member of the Src-family of tyrosine kinases, is a membrane-associated nonreceptor tyrosine kinase that is also expressed in myeloid and B lymphoid hematopoietic cells (17). Lyn has also been detected in human endometrium and brain endothelium, where it is thought to play an important role in human reproduction and blood-brain barrier development, respectively (1, 13). It is also expressed in intestinal crypt cells (39). Alternative splicing of the Lyn gene results in the expression of Lyn A (56 kDa) and Lyn B (53 kDa) forms of Lyn (40). Except for a 20-amino acid deletion, Lyn B is identical to Lyn A (47). Lyn kinase is thought to be involved in signal transduction mechanisms after activation of B cell antigen receptors, FcE high-affinity receptors (25), and interleukin-3 receptors (44). Lyn kinase phosphorylates many substrates, including phosphoinositol-3 kinase (PI3-K), ras GTPase-activating protein (GAP), and mitogen-activating protein kinase (MAPK) (12).

Several studies have shown that the Na,K-ATPase α\textsubscript{1}-subunit is phosphorylated by PKC and PKA on serine-threonine residues (5, 9). However, the existence of additional phosphorylation sites was suspected because neither the deletion of the known Na,K-ATPase α\textsubscript{1}-subunit serine-threonine sites nor treatment with PKC or PKC inhibitors was able to fully suppress residual levels of Na,K-ATPase α\textsubscript{1} phosphorylation (4). This fits with the notion that Na,K-ATPase α\textsubscript{1} can be subject to tyrosine phosphorylation. To examine a possible tyrosine phosphorylation site, Feraille et al. (22) analyzed Na,K-ATPase pump activity in opossum kidney (OK) cells transfected with mutant Na,K-ATPase α\textsubscript{1} in which tyrosine-10
was substituted either by alanine or glutamate. Insulin-induced stimulation of Na,K-ATPase function was suppressed in cells expressing the Tyr-10 substitutions. Consistent with the idea of phosphorylation of the Na,K-ATPase α1-subunit at Tyr-10 in the presence of a Src-family tyrosine kinase, this region of the Na,K-ATPase α1 protein exhibits a Src kinase consensus phosphorylation sequence composed of multiple acidic residues (50). Interestingly, the gastric H+<sup>-</sup>,K+<sup>-</sup>-ATPase proton pump is also subject to phosphorylation at Tyr-10 (43).

Lyn kinase-induced inhibition of Na,K-ATPase activity in isolated, partially purified lens epithelium and kidney medulla membrane material observed in the present study differs from the response to insulin and other agonists in intact astrocytes, proximal tubule, and skeletal muscle in which tyrosine phosphorylation is associated with stimulation of Na,K-ATPase function (8, 21, 22, 34, 36). This may reflect differences in the cascade of events triggered by insulin and partially purified Lyn, differences in the response of intact cells in which changes in Na,K-ATPase synthesis or recruitment to the plasma membrane may occur (2, 21, 34, 36), differences in cell-specific regulatory mechanisms, or differences in Na,K-ATPase α<sub>1</sub> isofrom characteristics. In the porcine lens, the Na,K-ATPase α<sub>1</sub> isofrom is predominant, although long-term changes in Na,K-ATPase activity might occur through the upregulation of the α<sub>2</sub>-subunit in response to alteration of cytoplasmic ion balance (19).

The lens epithelium is specialized for active sodium-potassium transport. Na,K-ATPase-mediated ion transport by the epithelial monolayer is essential for maintenance of electrolyte homeostasis in the mass fiber cells that constitute the bulk of the lens (33). The results of the present study suggest that changes in the activity of Lyn or other tyrosine kinases could lead to modulation of Na,K-ATPase function in lens epithelium. Modulation of Na,K-ATPase activity as the result of Lyn kinase activation has not previously been reported, and although there is strong evidence from several different cell types indicating the susceptibility of Na,K-ATPase to tyrosine phosphorylation, the identity of the tyrosine kinases that influence Na,K-ATPase in intact tissues is not known (21, 38, 48). In gastric mucosa, there is evidence suggesting that plasma membrane H<sup>+</sup>-,K<sup>-</sup>-ATPase is subject to tyrosine phosphorylation (27), and chromatographic separation of detergent-solubilized membrane material revealed an endogenous Src-family kinase at ~60 kDa (28). Sarc family kinases are known to influence other ion transporters. In platelets, for example, phosphorylation of plasma membranes by pp60<sup>Src</sup> kinase resulted in significant inhibition of calcium ATPase activity that correlated with the degree of PMCA tyrosine phosphorylation (15). In mouse erythrocytes, activation of Src family tyrosine kinases appears to modify K<sup>+</sup>-,Cl<sup>-</sup>-cotransporter function (14). In the lens, Lyn kinase is likely to be just one of several nonreceptor tyrosine kinases, and it is possible that other tyrosine kinases also influence Na,K-ATPase activity. The present experiments did not permit us to identify the tyrosine kinases activated by thrombin.

Several studies have identified Src family tyrosine kinases in the lens. It has been suggested that nonreceptor tyrosine kinases play an essential role in differentiation with inhibition of Src family tyrosine kinases acting as one of the events required for lens epithelial cells to withdraw from the cell cycle and commence differentiation toward the lens fiber cell phenotype (45). When the intact lens is maintained in organ culture, inhibition of Src family tyrosine kinases with PP1 appears to prevent opacification (49).

In summary, the results of the present study suggest that Na,K-ATPase activity in lens epithelium is susceptible to modulation by tyrosine phosphorylation. The significance of modulating Na,K-ATPase activity in lens epithelium remains to be determined. It has been proposed that spatial localization of high-Na,K-ATPase activity to specific regions of the lens surface is essential to support circulation of electrical currents that work via electroosmosis to speed solute movement through the tortuous extracellular space between the tightly packed lens cells (33). Although Na,K-ATPase protein is abundant in all lens cells (16, 18), Na,K-ATPase activity is higher at the epithelium than the fibers and highest in epithelium at the equator of the lens (7, 24, 41). To establish the circulating currents, there may be a need for mechanisms that modulate Na,K-ATPase activity to produce unequal activity in different parts of the lens.

**GRANTS**

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**C95LENS TYROSINE PHOSPHORYLATION**

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