The Influence of SRC-Family Tyrosine Kinases on Na,K-ATPase Activity in Lens Epithelium

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PURPOSE. Na,K-adenosine triphosphatase (ATPase) is essential for the regulation of cytoplasmic ion concentrations in lens cells. Earlier studies demonstrated that tyrosine phosphorylation by Lyn kinase, a Src-family member, inhibits Na,K-ATPase activity in porcine lens epithelium. In the present study, experiments were conducted to compare the ability of other Src-family kinases (Fyn, Src, and Lck) and Fes, a non-Src-family tyrosine kinase, to alter Na,K-ATPase activity.

METHODS. Membranes prepared from porcine lens epithelium were incubated with partially purified tyrosine kinases in buffer containing 1 mM adenosine triphosphate (ATP). ATP hydrolysis in the presence and absence of ouabain was used to measure Na,K-ATPase activity. Western blot analysis was used to examine phosphotyrosine-containing proteins and tyrosine kinase expression.

RESULTS. Fyn reduced Na,K-ATPase activity by ~30%. In contrast, Src caused a ~38% increase of Na,K-ATPase activity. Na,K-ATPase activity in membrane material treated with Lck or Fes was not significantly altered, even though Lck and Fes treatment induced robust tyrosine phosphorylation. Added exogenously, each tyrosine kinase induced a different pattern of membrane protein tyrosine phosphorylation. As judged by immunoprecipitation, Src, Fyn, Lyn, and Lck elicited tyrosine phosphorylation of the Na,K-ATPase α1 protein. Src, Fyn, Lyn, Lck, and Fes were each detectable in the epithelium by Western blot.

CONCLUSIONS. The results indicate considerable variation in the Na,K-ATPase activity response of lens epithelium to different tyrosine kinases. This could perhaps explain why inhibition of Na,K-ATPase activity is reported to be caused by tyrosine phosphorylation in some tissues, whereas stimulation of Na,K-ATPase activity is observed in other tissues. (Invest Ophthalmol Vis Sci. 2005;46:618 – 622) DOI:10.1167/iovs.04-0809

Na,K-adenosine triphosphatase (ATPase) is an active ion transport protein complex expressed in the plasma membrane of all animal cells. Acting as a pump, Na,K-ATPase couples the hydrolysis of ATP to the outward translocation of three sodium ions and the inward translocation of two potassium ions against steep electrochemical gradients. Regulation of cytoplasmic electrolyte concentration is essential for preserving the transparency of the lens. The monolayer of epithelium covering the anterior lens surface is required to maintain a high Na,K-ATPase activity.1–3 Regulation of Na,K-ATPase activity and function in the lens epithelium has not been widely studied.

Na,K-ATPase function can be regulated through several different protein-kinase-mediated mechanisms.4 In the lens, there is evidence that Na,K-ATPase activity may be regulated in part by tyrosine phosphorylation, since genistein, a tyrosine kinase inhibitor, decreases the inhibitory effects of endothelin-1 (ET-1) on ouabain-sensitive 86Rb uptake.5 Also in the lens, thrombin-induced inhibition of Na,K-ATPase–mediated active ion transport is suppressed by the Src-family tyrosine kinase inhibitor, herbimycin A.6 Inhibition of Na,K-ATPase function and increased tyrosine phosphorylation of multiple epithelium membrane proteins were found to occur at the same time in thrombin-treated lenses. In a recent study, the Src-family kinase, Lyn, was shown to induce tyrosine phosphorylation of lens epithelium membrane proteins, including the Na,K-ATPase α1 subunit.7 Lyn also was found to inhibit Na,K-ATPase activity in lens epithelium.

In some nonlens tissues, tyrosine kinase activation has been reported to stimulate Na,K-ATPase activity.8 86Rb uptake studies suggest this is the case in the intact kidney proximal tubule.9,10 In rat astrocytes, tyrosine kinase activation by insulin elicits an increase of Na,K-ATPase activity and an increase in the synthesis of Na,K-ATPase α1 protein.10 In other tissues, however, tyrosine kinase activation appears to inhibit Na,K-ATPase function.11,12 Genistein has been found to suppress the inhibitory action of dopamine and D-1 agonists on Na,K-ATPase–mediated ion transport in cultured rabbit nonpigmented ciliary epithelium.13 Under different experimental conditions or in different tissues, it appears that Na,K-ATPase activity is either stimulated or inhibited by tyrosine phosphorylation. In the present study, experiments were conducted to test whether different Src-family tyrosine kinases have different effects on Na,K-ATPase activity in lens epithelium.

MATERIALS AND METHODS

Tissues

Porcine eyes were kindly donated by the Swift Meat Packing Company (Louisville, KY). The posterior of the eye was dissected and the lens removed by cutting the suspensory ligaments. The lens was transferred to filter paper and the capsule-epithelium was removed and snap-frozen in liquid nitrogen. Material from 40 to 50 lenses was pooled.

Membrane Preparation

The membrane preparation was obtained according to the methodology described by Okafor et al.6 Frozen lens capsule-epiteliunm samples were homogenized in ice-cold homogenization buffer A (150 mM sucrose, 4 mM EGTA, 5 mM HEPES, 800 μM dithiothreitol [DTT], and 0.2 mM sodium orthovanadate [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, and 2 μg/ml aprotinin) with a glass homogenizer. The homogenate was then centrifuged at 115,000g for 60 minutes at 4°C. The membrane pellet was

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then resuspended in buffer A containing 600 mM KCl. The resuspended membrane pellet was subjected to centrifugation once again at 115,000g for 60 minutes at 4°C to remove extrinsic proteins. The membrane pellet was resuspended in buffer A one final time and subjected to centrifugation at 115,000g for 60 minutes at 4°C. The final pellet containing plasma membranes as well as intracellular membranes was resuspended in buffer A. The protein content was then measured using the BCA protein assay kit (Pierce, Rockford, IL).

**Tyrosine Kinase Treatment and Na,K-ATPase Activity Measurement**

Before measurement of Na,K-ATPase activity, lens epithelium membrane preparation was incubated in kinase reaction 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 Src, Lyn, Lck, or Fyn tyrosine kinases (0.04–0.12 U/g protein) or Fes tyrosine kinase (0.4 ng/µg protein) at 30°C. The tyrosine kinases were obtained from Upstate Biotechnology, Lake Placid, NY. Tyrosine kinase concentrations were selected to elicit a similar degree of tyrosine phosphorylation. Treated epithelium membrane preparation was then used either for Western blot analysis or Na,K-ATPase activity measurements. To prevent interference with the Na,K-ATPase activity assay, sodium orthovanadate and exogenous tyrosine kinases were removed by discarding the supernatant after subjecting the membrane preparation to centrifugation at 100,000g for 3 minutes. The membrane pellet was resuspended in centrifugation buffer (10 mM Tris, 5 mM DTT, and 10% [vol/vol] glycerol) and centrifuged at 100,000g for 5 minutes. This step was repeated, and the final pellet was resuspended in ~100 µL Na,K-ATPase buffer and assayed immediately for Na,K-ATPase activity.

Na,K-ATPase activity was determined according to a methodology described by Okafor et al. Aliquots of protein kinase-treated and untreated epithelium membrane preparation (~100 µg) were incubated in Na,K-ATPase buffer (100 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 1 mM EGTA, 40 mM histidine [pH 7.4]). Ouabain, a specific inhibitor of Na,K-ATPase, was added to half the samples at a final concentration of 1 mM. Samples were then preincubated with gentle agitation for 15 minutes at 37°C. ATP was added to a final concentration of 1 mM to initiate ATP hydrolysis. The ATP hydrolysis reaction was performed with gentle agitation for 45 minutes at 37°C. The reaction was then stopped by the addition of 15% ice-cold trichloroacetic acid. ATP hydrolysis was then quantified. The amount of inorganic phosphate released in each reaction sample was measured using a colorimetric method. Less than 20% of the available ATP was hydrolyzed. Na,K-ATPase activity was calculated as the difference between ATP hydrolysis in the presence and absence of ouabain.

As described previously, separate studies were conducted to confirm Na,K-ATPase activity was not inhibited by residual vanadate. Na,K-ATPase activity was 9.7 ± 0.4 nanomoles Pi/mg protein per minute (mean ± SE; n = 5) in samples that had been subjected to vanadate treatment and then washed. This was not significantly different from the activity of 10.2 ± 0.6 nanomoles Pi/mg protein per minute measured in control samples.

**Western Blot Analysis**

Membrane preparation samples were solubilized in Laemmli sample dilution buffer. Proteins were separated on a 7.5% gel by SDS-PAGE at 40 mA for 2 hours and then transferred to a nitrocellulose membrane by electrophoresis at 30 V for 16 hours. The nitrocellulose membranes were blocked for 1 hour with 5% dry milk in TTBS (30 mM Tris, 150 mM NaCl, and 0.5% Tween-20 [pH 7.4]). Tyrosine phosphoproteins or tyrosine kinases were detected by incubating the nitrocellulose membranes at room temperature for 60 minutes with a monoclonal antibody directed against Src, Fyn, Lyn, Lck, or Fes tyrosine kinase (Upstate Biotechnology) or the anti-phosphotyrosine antibody PY20 (Transduction Laboratory, Lexington, KY) conjugated to horseradish peroxidase.

Nitrocellulose membranes were probed for tyrosine kinases, then washed with TTBS two times for 15 minutes, and then three times for 5 minutes before being incubated for 60 minutes with a horseradish peroxidase–conjugated secondary antibody (Bio-Rad, Hercules, CA). The blots were visualized with chemiluminescence substrate (Pierce, Rockford, IL) and then exposed to x-ray film (Sigma-Aldrich, St. Louis, MO).

**Immunoprecipitation**

The methodology for immunoprecipitation of Lyn-kinase-treated membrane preparation has been described previously. In the current study, in a modification to improve efficiency, the immunoprecipitating antibody was first cross-linked to the immobilized protein A beads. The lens epithelium membrane preparation was treated with Src, Fyn, Lck, or Fes as described earlier to achieve an overall degree of tyrosine phosphorylation similar to that observed with Lyn. The kinase-treated membrane preparation (500 µg) was solubilized in immunoprecipitation buffer (30 mM Tris, 150 mM NaCl, 10 mM EGTA, 1.0% Triton X-100, 1 mM DTT, 1 mM PMSF, 0.2 mM leupeptin, 0.2 µg/mL pepstatin A, 10 µg/mL antipain, 10 µg/mL antipain, and pepstatin A [pH 7.4]) and brought up to a final protein concentration of 2 µg/µL. The membrane preparation was mixed for 1 hour on a rotating wheel at 4°C. The insoluble material was then pelleted by centrifugation at 10,000g for 15 minutes at 4°C. The supernatant (1 mL) was removed and preclarified with a 1:1 mixture of immobilized protein G (25 µL) and protein A beads (25 µL; Immuno-Pure; Pierce) for 2 hours on a rotating wheel at 4°C. The membrane mixture was then centrifuged at 1000g for 3 minutes at 4°C. The supernatant was removed and transferred to a microcentrifuge tube containing anti-Na,K-ATPase α1 polyclonal antibody (BDI, Flanders, NJ) cross-linked to immobilized protein A beads. The membrane and protein A-antibody mixture was then incubated on a rotating wheel for 15 hours at 4°C. The mixture was washed with 1 mL fresh phosphate-buffered saline (pH 7.4), and centrifuged at 1000g for 5 minutes at 4°C. The wash procedure was repeated three more times. Elution buffer (1 M glycine and 0.1% Triton X-100 [pH 2.6]) was added (45 µL), and immunoprecipitated Na,K-ATPase α1 polypeptide was dissociated from the protein A antibody mixture with agitation on a rotating wheel at 4°C for 30 minutes. The samples were then centrifuged at 4000g for 5 minutes. Finally, Laemmli sample dilution buffer was added to the supernatant, and the samples were subjected to SDS-PAGE followed by Western blot analysis.

**Statistical Analysis**

Student’s t-test was used for statistical analysis.

**Results**

To examine the effects of exogenous tyrosine kinases on Na,K-ATPase activity, membrane material isolated from porcine lens epithelium was incubated for 20 minutes with Src, Fyn, Lyn, Lck, or Fes in ATP-containing buffer. As described in the Methods section, the kinase-treated membrane preparation was subsequently washed to remove exogenous tyrosine kinases and buffer constituents. Na,K-ATPase activity was then measured in the treated samples. Na,K-ATPase activity was reduced by ~40% in lens epithelium preparations subjected to Lyn pretreatment (Fig. 1). Na,K-ATPase activity was also significantly reduced by Lyn pretreatment, but to a lesser degree. In contrast, Na,K-ATPase activity was increased by ~38% in membranes subjected to Src pretreatment. No significant change in Na,K-ATPase activity was observed in membranes subjected to Lck pretreatment or to pretreatment with Fes, a tyrosine kinase that is not a member of the Src family.

To examine tyrosine phosphorylation of membrane proteins, aliquots of a membrane preparation isolated from porcine lens epithelium were incubated in ATP-containing kinase reaction buffer with Src, Fyn, Lyn, Lck or Fes. The treated
samples were then subjected to Western blot analysis of tyrosine-phosphorylated proteins. After 20 minutes of tyrosine kinase treatment, phosphorylation was robust and individual phosphotyrosine bands were difficult to distinguish. Results of experiments with a shorter duration showed that each tyrosine kinase elicited a marked increase in the density of several phosphotyrosine protein bands, but the overall phosphotyrosine band pattern was different for each tyrosine kinase (Fig. 2). Tyrosine phosphorylation was not detected in membranes treated with kinase alone in the absence of ATP. ATP alone caused a much smaller increase of membrane protein phosphorylation (Fig. 2), suggesting that endogenous tyrosine kinase activity in the membrane preparation is low.

Because endogenous tyrosine kinase activity appeared low in the membrane preparation, Western blot analysis was used to determine whether the tyrosine kinases used in this study are present in the lens epithelium membrane preparation. A single 60-kDa band corresponding to the single known isoform of Src was detected (Fig. 3). Single Fyn-, Lck-, and Fes-immunoreactive bands were similarly detected. For Lyn, two immunopositive bands were observed, corresponding to the two known isoforms of Lyn kinase (Fig. 3). Similar Western blot results were obtained from lens epithelium lysates (data not shown). It should be noted that the detection of tyrosine kinase polypeptides does not signify the level of tyrosine kinase activity, since the antibodies used in this study do not discriminate between the active and inactive forms.

Previous studies have shown that Lyn elicits tyrosine phosphorylation of the Na,K-ATPase α1 subunit.7,17 Because different Src-family kinases have different effects on Na,K-ATPase activity (Fig. 1), studies were conducted to examine Na,K-ATPase α1 tyrosine phosphorylation by Src, Fyn, and Lck. Aliquots of lens epithelium membrane preparation were incubated for 20 minutes in ATP-containing kinase reaction buffer with Src, Fyn, Lyn, or Lck. Na,K-ATPase α1 protein was then isolated by immunoprecipitation and probed by Western blot analysis for tyrosine phosphoproteins. Detectable tyrosine phosphorylation of the α1 band was elicited by Src, Fyn, and Lck as well as Lyn (Fig. 4). Studies of Fes were inconclusive.
Src, Fyn, Lyn, and Lck tyrosine kinase–treated lens epithelium membrane preparation. Lens epithelium membrane preparation was incubated in the presence of Src, Fyn, Lyn, or Lck tyrosine kinases in ATP-containing buffer for 20 minutes. The treated membrane preparation was immunoprecipitated with a polyclonal antibody directed against Na,K-ATPase α1-subunit from Src, Fyn, Lyn, and Lck tyrosine kinase–treated lens epithelium membrane. In the control, the membrane preparation was not treated with exogenous tyrosine kinase or ATP. Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against tyrosine phosphoproteins (top). The phosphotyrosine blot was stripped and reprobed with a monoclonal antibody directed against Na,K-ATPase α1 (bottom).

**FIGURE 4.** Immunoprecipitation of Na,K-ATPase α1-subunit from Src, Fyn, Lyn, and Lck tyrosine kinase–treated lens epithelium membranes also was found to cause tyrosine phosphorylation of the lens epithelium Na,K-ATPase α1 protein. In the present study, Src, Fyn, Lyn, or Lck treatment of lens epithelium membranes also was found to cause tyrosine phosphorylation of multiple membrane proteins but only Fyn inhibited Na,K-ATPase activity in a manner similar to Lyn. Src caused the opposite response, stimulating Na,K-ATPase activity by ~40%. Even though Lck failed to alter Na,K-ATPase activity, it still caused detectable tyrosine phosphorylation of the Na,K-ATPase α1 subunit, as did Src, Fyn, and Lyn. The qualitative nature of the immunoprecipitation and Western blot methodology does not permit us to draw conclusions regarding similarity in the degree of α1 tyrosine phosphorylation caused by the different protein kinases. It remains to be tested whether Src, Fyn, Lyn, and Lck may phosphorylate the α1 protein at different sites. Because a number of membrane proteins are tyrosine phosphorylated, the extent to which changes of Na,K-ATPase activity depends on the phosphorylation of Na,K-ATPase α1 protein itself is not easily determined. It is noteworthy that no two kinases caused precisely the same overall pattern of membrane protein phosphorylation.

**DISCUSSION**

Different tyrosine kinases were found to have different effects on Na,K-ATPase activity. Stimulation or inhibition of Na,K-ATPase activity appears possible, depending on which tyrosine kinase was used to bring about tyrosine phosphorylation. In part, this could contribute to the variability of Na,K-ATPase responses to tyrosine phosphorylation elicited by different stimuli in different tissues. For example, Feraille et al. found that tyrosine phosphorylation in insulin-treated kidney cells suggests that endogenous tyrosine phosphorylation maintains fiber Na,K-ATPase in a partially inhibited state. Na,K-ATPase activity also may be changed subsequent to tyrosine phosphorylation that occurs after a stimulus such as G-protein–coupled receptor activation. Different stimuli may well activate different tyrosine kinases. In the present study, we showed that individual Src-family kinases cause unique patterns of membrane protein tyrosine phosphorylation. However, each of the four kinases tested elicited detectable phosphorylation of the Na,K-ATPase α1 subunit. For reasons yet to be explained, different kinases have different effects on Na,K-ATPase activity. The impact on net ion transport is likely to be complex, since other ion transport proteins could also be sensitive to Src kinase-mediated tyrosine phosphorylation. For example, activation of Src-family tyrosine kinases appears to modify K⁺/Cl⁻ cotransporter function and the TRPV4 calcium channel. Adding to the complexity of the response, the Src-family kinases are likely to be just a few of many nonreceptor tyrosine kinases in any given cell.

**References**


Lens epithelium was found to express Src, Fyn, Lyn, Lck, and Fes protein. Several of these nonreceptor tyrosine kinases have not been reported in the lens. Consistent with their involvement in a variety of cell functions, these tyrosine kinases are widely expressed and are known to be activated by a range of different stimuli. Under the conditions of our experiments, endogenous tyrosine kinase activity in lens epithelium was low, since incubation with ATP alone caused little detectable tyrosine phosphorylation. Lyn and Lck were first identified in myeloid B and lymphoid hematopoietic cells. Lyn has since been detected in the brain, in kidney distal convoluted tubule, and in kidney glomerular endothelial cells. Lck has been detected in distinct regions of the mouse brain and in mouse retinal neurons. Lyn and Src also are widely expressed. Lyn is expressed in T cells and brain, whereas Src is expressed in platelets and fibroblasts and in brain and mammary tissues. Fes, which is a member of a distinct subfamily of nonreceptor tyrosine kinases, also is distributed widely. Fes plays a role in immune responses and is involved in signaling cascades initiated by cell–cell and cell–matrix interactions.


