Purinergic agonists stimulate lens Na-K-ATPase-mediated transport via a Src tyrosine kinase-dependent pathway

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Tamiya S, Okafor MC, Delamere NA. Purinergic agonists stimulate lens Na-K-ATPase-mediated transport via a Src tyrosine kinase-dependent pathway. Am J Physiol Cell Physiol 293: C790–C796, 2007. First published May 23, 2007; doi:10.1152/ajpcell.00579.2006.—The Na-K-ATPase is vital for maintenance of lens transparency. Past studies using intact lenses suggested the involvement of tyrosine kinases in short-term regulation of Na-K-ATPase. Furthermore, in vitro phosphorylation of a lens epithelial membrane preparation by Src family kinases (SFKs), a family of nonreceptor tyrosine kinases, resulted in modification of Na-K-ATPase activity. Here, the effect of purinergic agonists, ATP and UTP, on Na-K-ATPase function and SFK activation was examined in the rabbit lens. Na-K-ATPase function was examined using two different approaches, measurement of ouabain-sensitive potassium (86Rb) uptake by the intact lens, and Na-K-ATPase activity in lens epithelial homogenates. ATP and UTP caused a significant increase in ouabain-sensitive potassium (86Rb) uptake. Na-K-ATPase activity was increased in the epithelium of lenses pretreated with ATP. Lenses treated with ATP or UTP displayed activation of SFKs as evidenced by increased Western blot band density of active SFK (phosphorylated at the active loop Y416) and decreased band density of inactive SFKs (phosphorylated at the COOH terminal). A single PY416-Src immunoreactive band at ~60 kDa was observed, suggesting not all Src family members are activated. Immunoprecipitation studies showed that band density of active Src, and to a lesser extent active Fyn, was significantly increased, while active Yes did not change. Preincubation of the lenses with SFK inhibitor PP2 abolished the ATP-induced increase in ouabain-sensitive potassium (86Rb) uptake. The results suggest selective activation of Src and/or Fyn is part of a signaling mechanism initiated by purinergic agonists that increases Na-K-ATPase-mediated transport in the organ-cultured lens. Src kinase; receptors

Maintenance of ionic balance is important for physiological processes in tissues and cells. Disturbance of tissue ion levels often results in pathological conditions. This is the case in the ocular lens, in which proper ion balance is vital for maintaining transparency. Abnormally high sodium content has been reported for the majority of cataractous lenses from human patients (12). Na-K-ATPase plays a dominant role in the maintenance of ion balance by pumping out three sodium ions in exchange for two potassium ions, utilizing the energy from ATP hydrolysis. Na-K-ATPase-mediated transport also establishes the sodium gradient required for various co- and countertransport mechanisms such as sodium-calcium exchange, sodium-proton exchange, and sodium potassium chloride co-transport.

Various mechanisms are involved in the regulation of Na-K-ATPase function. While short-term regulation often involves changes in gene expression, previous studies have shown that short-term regulation can be achieved by phosphorylation events involving protein kinases (14, 42). The effects of serine/threonine kinases such as protein kinase C and protein kinase A have been studied extensively (3, 14, 42). In contrast, relatively little is known about the role of tyrosine kinases on Na-K-ATPase regulation, and research related to this topic has been emerging as an interesting area of study. Genistein, a general tyrosine kinase inhibitor, has been shown to abolish the change of Na-K-ATPase activity induced by various agonists in tissues, including kidney proximal tubule, lens, and ciliary epithelium (16, 32, 34, 35). Fairell and coworkers (15) have shown that the phosphorylation of Na-K-ATPase a1 catalytic subunit at Tyr10 is required for the insulin-induced stimulation of Na-K-ATPase activity in kidney proximal tubule cells.

Src family kinases (SFKs), a family of nonreceptor tyrosine kinases, have been shown to be involved in the regulation of Na-K-ATPase in the lens as well as several other cell types. Bozulic and colleagues (4) demonstrated that in vitro phosphorylation of a lens epithelial membrane and a kidney medulla membrane preparation by the SFK member Lyn results in decreased Na-K-ATPase activity. In alveolar epithelial cells, however, SFKs apparently have the opposite effect on Na-K-ATPase activity. Thyroid hormone increases alveolar cell Na-K-ATPase activity via SFK-dependent pathway (23). This fits with the finding that the direction of the change of Na-K-ATPase activity in a lens epithelium membrane preparation (decrease or increase) depended on the Src family kinase member used for the phosphorylation (5). This discovery points to a situation where activation of different SFKs could elicit different Na-K-ATPase responses. Here, we examined the Na-K-ATPase response of the lens to purinergic agonists.

Purinergic receptor signaling is involved in regulation of ion transport in various epithelial cell types (24). Furthermore, stimulation of P2Y receptors activates SFKs in various cell types (7, 19, 22, 26). Previous studies have shown that the lens expresses P2Y2 receptors (9–11) and that ATP is released under stress conditions from the lens as well as from ciliary epithelium, the tissue adjacent to the lens (13, 30). In the present study, effects of P2 receptor agonists on Na-K-ATPase activity and SFK activation in the organ-cultured rabbit lens

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were examined. Purinergic receptor agonists caused both an increase of the Na-K-ATPase activity and selective SFK activation. SFK activation was required for the change in Na-K-ATPase activity.

**MATERIALS AND METHODS**

**Rabbit lens organ culture.** Albino rabbit eyes, shipped overnight on ice, were purchased from Pelfreez Biologicals (Rogers, AR). Lenses were dissected from the posterior as previously described (40). Each lens was cultured in 20 ml of M199 (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and penicillin/streptomycin (100 U per ml/1 mg per ml). Damaged or detached lenses were omitted and only clear lenses were used for the study. To permit recovery from the dissection procedure, the lenses were cultured for 2 days. After 2 days, the lenses were transferred to 4 ml of Krebs solution composed of (in mM) 119 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 1 MgCl₂, and 5.5 glucose, pH 7.4, and preincubated for 1 h before each experiment. In some specified experiments, PP2 (Calbiochem, San Diego, CA), a selective inhibitor of Src family kinases, or PP3, an inactive analogue of PP2, was added (final concentration 10 μM) for 45 min during the 1-h preincubation period.

**Measurement of Rb uptake.** Ouabain-sensitive uptake of ⁸⁶Rb was used as an index of Na-K-ATPase-mediated potassium transport activity in organ-cultured lenses. One-half the lenses received ouabain 15 min before the addition of purinergic receptor agonists. Purinergic receptor agonists (final concentration 100 μM) were added to lenses in Krebs solution 5 min before ⁸⁶Rb. Each lens received ⁸⁶Rb (−0.1 μCi/ml) added to the Krebs solution for a 30-min uptake period. The lenses were then washed in ice-cold nonradioactive Krebs solution, blotted dry on a filter paper, weighed, and dried overnight in an oven. Dried lenses were reweighed and weight loss was assessed to calculate lens water content. Dried lenses were digested in 30% nitric acid for 4 h at 70°C. Radioactivity of acid digests was measured using a scintillation counter. An aliquot of the loading solution was counted. On the basis of the specific activity of the loading solution (cpm/moll potassium), Na-K-ATPase-mediated potassium transport was calculated as nanomoles potassium per gram lens water per 30 min. The ratio of ouabain-sensitive to ouabain-insensitive ⁸⁶Rb uptake was 75:25. In a time-course experiment, ouabain-sensitive ⁸⁶Rb uptake was linear up to 60 min with an r² value of 0.995.

**Measurement of Na-K-ATPase activity.** Ouabain-sensitive ATP hydrolysis was measured in homogenates of isolated lens epithelium. Lenses in Krebs solution were treated with or without 100 μM ATP for 5 min, followed by a 2-min wash in ice-cold Krebs solution. The lens capsule epithelium was then isolated from the fiber mass and homogenized in ice-cold ATPase assay solution composed of (in mM) 40 histidine, 100 NaCl, 10 KCl, 3 MgCl₂, and 1 EGTA (pH 7.4) with 10 μg/ml amalphacin in the presence of protease inhibitors (2 μM antipain, 2 μM leupeptin, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin). The protein concentration in the homogenate was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Ouabain-sensitive ATPase activity of the homogenates was measured as previously described (34). One-half of the samples received ouabain (final concentration 100 μM). Homogenate protein (100 μg) in ATPase assay solution was preincubated for 5 min at 37°C in the presence of amalphacin, an ionophore previously found to permeabilize cell membranes to ions and ATP (47). ATP (final concentration of 1 mM) was then added to initiate the assay. The reaction was stopped at 45 min by the addition of 15% ice-cold trichloroacetic acid. Colorimetric method was used to quantify the amount of inorganic phosphate released by the ATP hydrolysis reaction. Na-K-ATPase activity was defined as the ouabain-sensitive (the difference in the presence and absence of ouabain) ATP hydrolysis. The ratio of ouabain-sensitive to ouabain-insensitive ATP hydrolysis was 60:40. The data are presented as nmol phosphate release/100 μg protein/min.

**Immunoprecipitation and Western blot analysis.** Lenses were incubated in the presence or absence of purinergic receptor agonists (final concentration 100 μM) for 5 min. Then the capsule epithelium was isolated and placed in ice-cold lysis buffer composed of (in mM) 50 HEPES, pH 7.5, 150 NaCl, 1 EDTA, 10 sodium fluoride, 10 sodium pyrophosphate, 2 sodium orthovanadate, 1% Triton X-100, 1% sodium deoxycholate, and protease inhibitors (2 μM antipain, 1 μM leupeptin, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin) for Western blot analysis. For Western blot analysis, the lysates were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer (LICOR, Lincoln, NE) for 1 h at room temperature and incubated overnight at 4°C with the immunoprecipitation antibody: mouse monoclonal anti-Yes antibody (clone 1 from BD Bioscience, San Jose, CA) for Yes, mouse monoclonal anti-Src antibody (clone GD11) conjugated to agarose beads (Upstate Chemical, Temecula, CA) for Src, goat polyclonal anti-Fyn antibody (Fyn3 from Santa Cruz Biotechnology, Santa Cruz, CA) for Fyn and mouse monoclonal anti-Na-K-ATPase antibody (clone C464.6) conjugated to agarose beads (Santa Cruz Biotechnology). For unconjugated antibodies (Yes and Fyn), immobilized protein G beads (Pierce) were added and further incubated for 4 h at 4°C. Beads were collected by centrifugation and washed three times with PBS. SDS sample buffer was added to the beads and incubated at 60°C for 20 min. Beads were spun down and the supernatant was used for Western blot analysis.

For Western blot analysis, 50 μg protein was used per sample. Proteins were separated on a 7.5% gel by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer (LICOR, Lincoln, NE) for 1 h at room temperature and incubated overnight at 4°C with rabbit polyclonal anti-PY416 Src antibody or rabbit polyclonal anti-PYS27 Src (Cell Signaling Technology, Beverly, MA) or goat anti-Na-K-ATPase antibody (N-15 from Santa Cruz Biotechnology) for the Na-K-ATPase immunoprecipitations. The anti-PY416 Src antibody cross-reacts with SFK members phosphorylated at the active loop tyrosine (PY₆₋‐SFK) while the anti-PYS27 Src antibody cross-reacts with SFK members phosphorylated at the COOH-terminal inhibitory tyrosine (PY₇₋‐SFK). After three 5-min washes with Tris-buffered saline + Tween 20, the membrane was incubated for 1 h with anti-rabbit secondary antibody conjugated with IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA) or anti-goat secondary antibody conjugated with Alexa Fluor 680 dye (Invitrogen). The membrane was washed three times and the bands detected and quantified using an Odyssey infrared scanner (LICOR, Lincoln, NE). The same membrane was washed and incubated overnight at 4°C with mouse monoclonal anti-Src (clone GD11; Upstate, Charlottesville, VA), anti-Yes (clone 1 from BD Bioscience), anti-Fyn antibody [Fyn (15) from Santa Cruz Biotechnology], mouse anti-phosphotyrosine antibody (clone PY20 from BD Bioscience), or anti-β-actin antibody (clone AC-74; Sigma, St. Louis, MO). Three 5-min washes were followed by 1 h incubation with anti-mouse secondary antibody conjugated with Alexa Fluor 680 dye (Invitrogen) or anti-mouse secondary antibody conjugated with IRDye 800 (Rockland Immuno-
chemicals) for the Na-K-ATPase immunoprecipitates. After three washes, the membrane was scanned to detect and quantify the bands.

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

RESULTS

To investigate the effect of purinergic receptor stimulation on Na-K-ATPase activity, lenses were exposed to ATP (100 μM final concentration). ATP caused a significant increase in ouabain-sensitive potassium ($^{86}$Rb) uptake (Fig. 1A). UTP, a different purinergic receptor agonist also significantly increased ouabain-sensitive potassium ($^{86}$Rb) uptake (Fig. 1B). When lenses were exposed to suramin (100 μM) ATP failed to change the rate of ouabain-sensitive potassium ($^{86}$Rb) uptake: the rate was 1,663 ± 154 in the presence of suramin + 100 μM ATP and 1,767 ± 84 nmol potassium/g lens water per 30 min in the presence of suramin alone (means ± SE, $n = 11$).

Ouabain-sensitive potassium ($^{86}$Rb) uptake is a functional measure of Na-K-ATPase-mediated potassium transport. As such, it is dependent on the intracellular sodium concentration. To test whether the observed increase in ouabain-sensitive $^{86}$Rb uptake in ATP-treated lenses is associated with modification of intrinsic Na-K-ATPase activity, ouabain-sensitive ATP hydrolytic activity was measured under identical conditions in homogenized epithelium isolated from control lenses or lenses that had been exposed to 100 μM ATP for 5 min. As shown in Fig. 2, Na-K-ATPase activity was significantly increased in the epithelium of ATP-treated lenses. In contrast, Na-K-ATPase $\alpha_1$ protein isolated by immunoprecipitation from the epithelium of ATP-treated lenses showed no discernible difference in phosphotyrosine Western blot band density (Fig. 3).

Western blot analysis was performed to determine whether SFKs are activated in the organ-cultured lens following purinoceptor stimulation. Intact lenses were exposed for 5 min to ATP (100 μM) or UTP (100 μM) then the epithelium was isolated and subjected to Western blot analysis for phosphorylated SFKs. Both ATP and UTP activated SFKs, as evidenced by an increase in the band density of active Src family kinase (PY-A-SFK, phosphorylated at the active loop tyrosine) (Fig. 4). A decrease in band density of SFKs phosphorylated at the COOH-terminal inhibitory tyrosine (PY-T-SFK) was observed in the epithelium of lenses that had been exposed to both ATP

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**Fig. 1.** The effect of ATP (A) and UTP (B) on ouabain-sensitive potassium ($^{86}$Rb) uptake in organ-cultured rabbit lenses. Lenses were incubated for 5 min in the presence or absence (control) of ATP (100 μM). $^{86}$Rb was then added for a further 30 min. One-half of the lenses were also exposed to 100 μM ouabain 15 min before the addition of ATP or UTP. The data are the means ± SE (vertical bar) of results from 12 lenses for each condition. *$P < 0.05$, significant difference from control.

**Fig. 2.** The effect of ATP on Na-K-ATPase activity in lens epithelium. Rabbit lenses were incubated for 5 min in the presence or absence (control) of ATP (100 μM). The capsule-epithelium was then isolated from each lens and homogenized. Na-K-ATPase activity in control and ATP-treated lenses was measured under identical conditions. The data are the means ± SE (vertical bar) of 16 measurements made using 2 different pools of homogenate. *$P < 0.05$, significant difference from control.

**Fig. 3.** Tyrosine phosphorylation of Na-K-ATPase in lens epithelium. A: typical Western blot for phosphotyrosine (PY) and Na-K-ATPase $\alpha_1$ (NKA $\alpha_1$). B: scatter plot of PY vs. Na-K-ATPase $\alpha_1$ (NKA $\alpha_1$) Western blot band density in immunoprecipitates prepared from control (filled symbols) and ATP-treated lenses. Rabbit lenses were incubated for 5 min in the presence or absence (Ctrl) of ATP (100 μM). Epithelium was isolated from each lens and lysed. Na-K-ATPase catalytic subunit protein was immunoprecipitated from 500 μg of lysate protein, subjected to SDS-PAGE, transferred to nitrocellulose and probed with an antibody against phosphotyrosine (clone PY20). Total Na-K-ATPase protein served as loading control.
and UTP (Fig. 5). The activation of SFKs was transient as indicated by the increase in PYA-SFK band density, which displayed a peak at 5 min and returned to the basal level at 30 min (Fig. 6). Immunoprecipitation of SFK members Yes, Src, and Fyn was performed in an attempt to identify which family members are activated. Comparison of nonimmunoprecipitated samples with supernatants following immunoprecipitation revealed that immunoprecipitation efficiency was high for each SFK member with 80% of the target protein immunoprecipitated (data not shown). The band density of Yes protein phosphorylated at the active site was not significantly different in control and ATP-treated lenses, indicating that Yes is not activated by ATP treatment (Fig. 7A). In contrast, the abundance of active Src kinase was significantly increased (60%) in the ATP-treated lenses as evidenced by the increased band density of Src kinase phosphorylated at the active site (Fig. 7B). The abundance of Fyn kinase phosphorylated at the active site was increased, but to a lesser extent (25%) (Fig. 7C).

Previous studies from our laboratory (5) have shown that SFK members can modify Na-K-ATPase activity of isolated lens epithelial membrane material in vitro. PP2, a SFK inhibitor, inhibits SFK activity in lens epithelium homogenates (41). Here PP2 was utilized to determine whether the observed Src activation in the ATP-treated lenses was linked to the observed increase of ouabain-sensitive potassium (86Rb) uptake. ATP (100 μM) failed to increase ouabain-sensitive potassium (86Rb) uptake in lenses exposed to PP2 (Fig. 8). It is noteworthy that PP2 alone decreased ouabain-sensitive potassium (86Rb) uptake by ~20%. 86Rb uptake was unaffected by PP3, an inactive analogue of PP2 used as a control.

**DISCUSSION**

Both ATP and UTP significantly stimulated the Na-K-ATPase-mediated potassium (86Rb) uptake by the intact rabbit lens. In addition, both agonists significantly increased the abundance of active SFK phosphorylated at tyrosine residue 416 within the activation loop and diminished phosphorylation at the COOH-terminal inhibitory tyrosine residue. ATP and UTP had an approximately equipotent effect on the increase in Na-K-ATPase-mediated potassium uptake as well as the activation of SFK, suggesting the involvement of P2Y2 purinergic receptors in the Na-K-ATPase response. Consistent with this notion, suramin was found to abolish the stimulation of ouabain-sensitive potassium (86Rb) uptake by ATP. Studies to examine the effect of suramin on SFK activation by ATP were inconclusive because suramin itself was found to cause an increase in PYA-SFK band density (data not shown). A non-

**Fig. 4.** The effect of purinergic agonists on the abundance of active Src family kinases (PYA-SFK). Rabbit lenses were incubated for 5 min in the presence or absence (control) of ATP (A) and UTP (B; 100 μM). Epithelium was isolated from each lens and lysed. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed for active SFKs with an antibody against SFK phosphorylated at the active loop tyrosine (PY416-Src). β-Actin was used as a loading control. The data are means ± SE (vertical bar) of results from 7 pools of lysates for ATP and 4 pools of lysates for UTP. *P < 0.05, significant difference from control.

**Fig. 5.** The effect of purinergic agonists on the abundance of SFKs phosphorylated at the inhibitory COOH-terminal region tyrosine (PYT-SFK). Rabbit lenses were incubated for 5 min in the presence or absence (control) of ATP (A) and UTP (B; 100 μM). Epithelium was isolated from each lens and lysed. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed for inactive SFKs with an antibody against SFKs phosphorylated at the COOH-terminal region tyrosine (PY527-Src). β-Actin was used as a loading control. The data are means ± SE (vertical bar) of results from 7 pools of lysate for ATP and 4 pools of lysate for UTP. *P < 0.05, significant difference from control.

**Fig. 6.** Time course of ATP-induced change in the abundance of active SFKs. Rabbit lenses were incubated for specified time in the presence of ATP. Epithelium was isolated from each lens and lysed. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and probed for active SFKs with an antibody against SFKs phosphorylated at the active loop tyrosine (Fig. 4). β-Actin was used as a loading control.
specific effect of suramin on SFK activation has been reported earlier (33, 48).

Suramin abolished the potassium ($^{86}$Rb) uptake response to ATP but as a purinergic receptor antagonist suramin is nonselective, making it difficult to be certain what receptor subtype is involved. On the basis of what is known about purinergic receptor selectivity, however, the similar-sized responses to ATP and UTP fit the profile of P2Y2 and possibly P2Y4 purinergic receptor activation (38, 44). Rabbit lenses have been previously reported to express P2Y2 receptor mRNA (11). ATP induces a transient rise of cytoplasmic calcium in the epithelium of the intact rabbit lens (40) and pharmacological studies of calcium signaling suggested P2Y2 (previously known as P2U) is the functional lens receptor for ATP (9). The effect of ATP on Na-K-ATPase function was measured using two different approaches. Ouabain-sensitive $^{86}$Rb uptake and ATP hydrolysis both were increased. In the ATP hydrolysis assay, the ion concentration is defined and Na-K-ATPase activity is measured under identical $V_{\text{max}}$ conditions for both control epithelium and the epithelium taken from ATP-treated lenses. Therefore, the observed increase in Na-K-ATPase activity cannot be due to changes in sodium concentration. The increased Na-K-ATPase activity observed in the epithelium of ATP-treated lenses suggests that the activity of the Na-K-ATPase protein itself could have been modified following purinergic receptor stimulation in the intact lens. We cannot rule out, however, the possibility that the ATP-induced increase in Na-K-ATPase activity observed in this study is due in part to a shift in Na-K-ATPase protein from intracellular trafficking vesicles to the plasma membrane. Na-K-ATPase activity might go undetected in sealed membrane compartments and while alamethicin was added to the Na-K-ATPase assay solution to permeabilize membranes (47), the effectiveness of alamethicin in permeabilizing trafficking vesicles is currently unknown. In several cell types, the capacity for Na-K-ATPase-mediated ion transport is changed by translocation of Na-K-ATPase $\alpha$-subunit molecules into or out of the plasma membrane (14, 37). PKA and PKC have been shown to be involved in this process (21, 42). PKC is known to be activated following purinergic receptor stimulation (38). SFKs have been shown to be upstream of PKC in certain signaling pathways (39, 46). PKC delta has been reported to be phosphorylated and activated by SFKs (28). While SFKs are unlikely to directly phosphorylate other PKC isoforms, SFKs can indirectly activate PKCs. For example, conventional PKCs are activated following activation of phospholipase C (PLC) (28) and others have shown that SFKs can activate PLC-$\gamma$ (6, 29), resulting in indirect activation of conventional PKCs. SFKs have also been shown to be involved in the transactivation of several receptor tyrosine kinases such as the EGF-R, which in turn activates various signaling molecules, including the PKC (26, 46). Furthermore, Montiel et al. (31) proposed that SFK activation occurs upstream of PKC-$\zeta$ activation following P2Y receptor stimulation in the human umbilical vein endothelial cells. Further studies are required to determine whether PKC activation is involved in the chain of events between SFK activation and the regulation of Na-K-ATPase function in the ATP-treated intact lens.

Both ATP and UTP activated SFKs in lens epithelium as judged by an increase in the band density of PY416-Src immunoreactive proteins. The PY416-Src antibody does not discriminate between different Src family members but different Src family members have slightly different molecular weights (43). Despite this, a single PY416-Src immunoreactive band at $\sim$60 kDa was observed in the epithelium of ATP-treated lenses, suggesting that not all Src family members are activated. There was no evidence for activation of p53/Lyn or p56Lck because this would have given rise to PY416-Src bands that migrate to different positions. SFKs have been

**Fig. 8.** The effect of SFK inhibitor PP2 on ATP-induced stimulation of ouabain-sensitive potassium ($^{86}$Rb) uptake in rabbit lenses. Rabbit lenses in Krebs solution were preincubated for 45 min in PP3 (10 $\mu$M) (control) or PP2 (10 $\mu$M) before the addition of ATP (100 $\mu$M) for 5 min. Then $^{86}$Rb was added for a further 30 min. One-half of the lenses were also exposed to 100 $\mu$M ouabain 15 min before the addition of ATP. Data are means + SE (vertical bar) of results from 5 lenses for each treatment. *$P < 0.05$, significant difference from control.
shown to be activated following purinoceptor stimulation in various other cell types (7, 19, 22, 26). Members of the Src kinase family activated following purinoceptor stimulation appear to differ according to the tissue or cell type as well as the purinergic receptor. In neuroblastoma cells, stimulation of P2Y11 results in activation of Yes (22), while P2Y2 receptor stimulation results in the activation of Src in astrocytoma cells as well as hepatocytes (7, 26). In platelets, Src is activated by P2Y1 stimulation but not P2Y12 (19). Lens cells have been reported to express P2Y2 receptors (9–11). In the present study, Western blot analysis of immunoprecipitated SFK members revealed that ATP induces active site phosphorylation of Src, and to a lesser extent Fyn, in the epithelium of organ-cultured rabbit lenses. No significant change of Yes phosphorylation was detected. This suggests that in the lens epithelium ATP selectively activates Src and Fyn.

The ATP-induced increase in ouabain-sensitive potassium (86Rb) uptake was abolished by PP2, a SFK inhibitor previously shown to completely inhibit SFK activity of porcine lens homogenates in vitro (41). The ability of PP2 to suppress the 86Rb uptake response indicates that SFK activation is involved in the observed increase in 86Rb uptake. Results from this study point to the involvement of Src and/or Fyn. While Na-K-ATPase protein can be phosphorylated and the activity modified by SFK-induced phosphorylation in vitro (4, 5, 17), immunoprecipitation and Western blot analysis did not reveal a sustained increase in tyrosine phosphorylation of Na-K-ATPase α1 subunit in the epithelium of lenses that had been exposed to ATP. Thus, while the result with PP2 indicates that SFKs are involved in the signaling pathway leading to the increase in Na-K-ATPase function induced by ATP, it seems unlikely that the change of Na-K-ATPase activity is simply the result of SFK-induced tyrosine phosphorylation of Na-K-ATPase protein. Na-K-ATPase regulation is complex and involves the phosphorylation of the Tyr10 residue by thrombin or endothelin in the lens as well as by dopamine in the ciliary nonpigmented epithelial cells was relieved by the tyrosine kinase inhibitor genistein (32, 34, 35). SFK members have also been implicated in stimulation of Na-K-ATPase activity in alveolar epithelial cells and cortical neurons (23, 45). It is worth noting that different isoforms of the Na-K-ATPase α subunit may respond in different ways. Wang and Yu (45) have reported that Lyn causes an increase of Na-K-ATPase α1 isoform activity in rat cortical neurons while Bozulic et al. (4) observed inhibition of Na-K-ATPase α1 subunit activity in porcine lens. This adds further complication by demonstrating that not only can different SFK members affect a particular isoform of the α subunit differently (5) but also each isoform of the α subunit might react differently to a particular SFK member. In the rabbit lens used in the present study, α1 is the major isoform with low levels of α2 and α3 expression.

In summary, purinergic receptor stimulation results in increased activity of the Na-K-ATPase in the organ-cultured rabbit lens. The Src family of tyrosine kinases is involved in the chain of events between the purinergic receptor and the Na-K-ATPase. Results from the present study point to a role for Src and Fyn. Src has earlier been demonstrated to increase Na-K-ATPase activity in porcine lens membrane material (5). Previously, we have shown tyrosine kinase-dependent inhibition of Na-K-ATPase by thrombin and endothelin (34, 35). ATP and endothelin have been shown to be released by lens cells and therefore could be involved in the physiological regulation of the lens Na-K-ATPase in an autocrine fashion (13, 36). ATP is released under stress (13) and is likely to leak out of damaged or injured cells. The stimulation of Na-K-ATPase activity by ATP will reestablish the sodium gradient which is required for cotransport and countertransport of various molecules, such as osmoles, calcium, glucose, or amino acids, which are required for volume regulation following osmotic stress or for cell growth during wound healing after injury.

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