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Na,K-ATPase activity modulates Src activation: A role for ATP/ADP ratio

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ABSTRACT

Digitalis-like compounds (DLCs), specific inhibitors of Na,K-ATPase, are implicated in cellular signaling. Exposure of cell cultures to ouabain, a well-known DLC, leads to up- or down regulation of various processes and involves activation of Src kinase. Since Na,K-ATPase is the only known target for DLC binding an *in vitro* experimental setup using highly purified Na,K-ATPase from pig kidney and commercially available recombinant Src was used to investigate the mechanism of coupling between the Na,K-ATPase and Src. Digoxin was used as a representative DLC for inhibition of Na,K-ATPase. The activation of Src kinase was measured as the degree of its autophosphorylation. It was observed that in addition to digoxin, Src activation was dependent on concentrations of other specific ligands of Na,K-ATPase: Na^+, K^+, vanadate, ATP and ADP. The magnitude of the steady-state ATPase activity therefore seemed to affect Src activation. Further experiments with an ATP regenerating system showed that the ATP/ADP ratio determined the extent of Src activation. Thus, our model system which represents the proposed very proximal part of the Na,K-ATPase-Src signaling cascade, shows that Src kinase activity is regulated by both ATP and ADP concentrations and provides no evidence for a direct interaction between Na,K-ATPase and Src.

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1. Introduction

More than 50 years ago, Skou suggested that an adenosine triphosphatase present in crab nerve is involved in the extrusion of sodium [1]. Following this hallmark publication, the enzyme involved in this process was shown to transport sodium and potassium ions across the cellular membrane, using ATP as a fuel source. Ouabain, a digitalis-like compound (DLC) [2], had already been reported to inhibit active ion transport in red blood cells [3]. By combining the findings mentioned above Post et al. [4] showed that in human red blood cells, Na^+ and K^+ activated transport is fuelled by ATP and inhibited specifically by ouabain. This Na,K-ATPase is now known to be one of the key players in cellular ion homeostasis, present in every human cell, and responsible for consumption of approximately 40% of the cellular ATP reserve [5].

It was suggested that in some cells, two pools of Na,K-ATPase are present on the cell surface: one dedicated to transport of Na^+ and K^+ over the cell membrane, whereas another pool resides in caveolae and is implicated in cellular signaling events [6]. Several signaling pathways have been linked to this pool; the Src – EGFR – RAS – p42/44 MAPK cascade [7], PLC-γ and IP3R microdomains involved in regulating Ca^{2+} homeostasis [8], and an AMPK-mediated response to cellular ATP reduction [9]. Recent experiments showed, however, that this strict division into ion-pumping and signaling pools of enzyme has to be abandoned. The detailed comparison of the properties of the caveolar and noncaveolar fractions of kidney Na,K-ATPase revealed no difference in either affinities to the specific ligands or molar activities of the enzymes [10].

In recent years the Src – EGFR – RAS – MAPK cascade has been studied extensively, resulting in a comprehensive knowledge of the consequences of activation of this cascade [7,11–13]. The first player, Src, is a well known non-receptor tyrosine kinase, which can be activated by (auto)phosphorylation of its kinase domain (SH1), more specifically tyrosine 418 [14]. Phosphorylation of another residue, tyrosine 529, results in a decrease in kinase activity. Src has been implicated in many different signaling pathways within the cell, and may be activated through different receptors including EGFR, PDGFR, HERBB2 and FGFR [14]. In addition, Src has been implicated in the Na,K-ATPase mediated cellular response to endogenous ouabain [7,13,15]. These studies suggest that Na,K-ATPase serves as an anchor for Src and restrains its kinase activity via two interactions. The first interaction is a constitutive binding (between the Na,K-ATPase CD2 and Src SH2-SH3 domains). The second, between the Na,K-ATPase CD3 and Src kinase/SH1 domain, is ouabain-dependent and exists only in the absence of ligand. Ouabain binding to Na,K-ATPase revokes the latter domain interaction, releases the kinase

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domain and thereby causes activation of Src with downstream regulation of proteins within the Src signal transduction cascade [13].

In this paper, an *in vitro* model resembling the proximal part of the Src-mediated signaling cascade is characterized in detail. Recombinant Src protein and purified Na,K-ATPase are used to form a complex. We observed that also in our hands Na,K-ATPase activity prevented Src activation. More importantly, any inhibition of Na,K-ATPase resulted in activation of Src: we found no difference between the inhibitors digoxin and vanadate. We found also that both the decrease in ATP level as well as the resulting increase in ADP level caused by Na,K-ATPase activity were unfavorable for Src activation. Since the concentrations of both ATP and ADP, and therefore also the ratio between these two, are heavily dependent on Na,K-ATPase activity, the activating effects of digoxin and vanadate on Src are of functional (inhibition of ATP hydrolysis) but not structural (induction of a certain conformation) origin. This provides an alternative hypothesis concerning the role of Src in DLC-induced signaling.

2. Methods

2.1. Materials

Rabbit polyclonal antibody for detection of activated Src protein (pY418) was purchased from Invitrogen (44660G). Protein was detected using a goat anti-rabbit antibody labeled with HRP obtained from Abcam (AB6721). Protein presence was detected using a Thermo Scientific Supersignal® West Pico Chemiluminescent kit (34080). Pig kidney Na,K-ATPase was purified according to Klodos et al. [16] to a specific activity of 1800 μmol $Pi \cdot mg^{-1} \cdot h^{-1}$. Recombinant full length Src protein obtained from Upstate (14–117) can be phosphorylated both at residues 418 or 529, rendering the active or inactive protein, respectively [14]. Creatine kinase and creatine phosphate were obtained from Roche, and ATP and ADP were purchased from Sigma-Aldrich.

2.2. In vitro Src phosphorylation

In a final volume of 40 μl, recombinant Src protein (0.5 μl = 1.5 units) was incubated for 30 min at 37 °C together with 610 ng of Na,K-ATPase in its buffer (250 mM sucrose, 20 mM histidine, 0.9 mM EDTA(II) pH 7.0), in 1 × PBS supplemented with 5.0 mM $MgCl_2$, or different when indicated. Phosphorylation was started by addition of 2.0 mM MgATP unless described otherwise. An ATP regenerating system, when added, consisted of 100 μg/ml creatine kinase and 10 mM creatine phosphate present with MgATP.

After 10 minutes of incubation at 37 °C, the phosphorylation reaction was stopped by incubation of samples with SDS sample buffer for 10 min at 65 °C, and subsequently the samples were loaded on a 10% SDS-PAGE gel. Proteins were blotted overnight onto a nitrocellulose membrane at 30 V. Then, the membranes were blocked for one hour at room temperature with TBS-T + 5% Elk. After washing with TBS-T, incubation of membranes with (1 : 1000) anti-pSrc-418 antibody was performed in TBS-T + 1% Elk + 1% BSA for two hours at room temperature. After three washes with TBS-T, incubation with (1 : 3000) goat-anti-rabbit HRP conjugate antibody was performed in TBS-T + 1% Elk + 1% BSA for one hour at room temperature. Afterwards, membranes were washed three times with TBS-T and subsequently three times with TBS before imaging was performed. Blots were imaged using a LAS-3000 scanner and images were subsequently analyzed using Image-J 1.42 software.

2.3. ATPase activity studies

In order to determine the extent of ATP hydrolysis under different experimental conditions, we tested the conversion of radiolabeled ATP, [γ-^{32}P]ATP (specific activity 100–500 $mCi \cdot mmol^{-1}$), which upon hydrolysis is converted into ADP and [^{32}P]Pi. The experimental procedure was identical to the *in vitro* Src phosphorylation experiments, except for the use of 2.0 mM radiolabeled ATP. After 10 min at 37 °C, the reaction was stopped by addition of 500 μl of 10% w/v charcoal in 6% trichloroacetic acid. After centrifugation of samples, 150 out of 540 μl final volume was subsequently analyzed for radioactivity using a liquid scintillation counter [17].

3. Results

3.1. Effect of Na,K-ATPase on Src activation

The effect of Na,K-ATPase on Src phosphorylation levels was studied by incubating a fixed amount of Src with increasing amounts of Na,K-ATPase. The level of Src activation was visualized on a Western blot (Fig. 1). In the absence of MgATP pSrc-418 was absent as well. In contrast, when Src was incubated with MgATP phosphorylation of Src was clearly visible. Addition of increasing amounts of Na,K-ATPase resulted in a gradual decrease of the pSrc-418 signal, indicating that Na,K-ATPase prevents Src activation. Presence of 610 ng Na,K-ATPase significantly reduced pSrc-418 levels, and therefore was used in further experiments, unless indicated otherwise.

To relate the extent of Src activation to the amount of ATP hydrolysis, we repeated the experiment using radiolabeled ATP. The level of ATP hydrolysis under the different experimental conditions is shown underneath the corresponding lanes of the Western blot image. As expected, the amount of ATP hydrolysed increased with the amount of Na,K-ATPase that was used to inhibit Src activation. In the presence of 610 ng of NKA, approximately 50% of the 2.0 mM ATP present was hydrolyzed during the time of experiment.

3.2. Digoxin induced Src activation

The effect of digoxin on Src phosphorylation was tested by incubation of Src with or without Na,K-ATPase in the absence or presence of digoxin. The results of seven independent experiments are shown in Fig. 2. The signal intensities of Src autophosphorylation are related to the control level reached in the presence of ATP alone (100%). Presence of Na,K-ATPase decreased the average signal intensity to approximately 20%, while simultaneous presence of Na,K-ATPase and digoxin resulted in a signal intensity indistinguishable from that in the control situation.

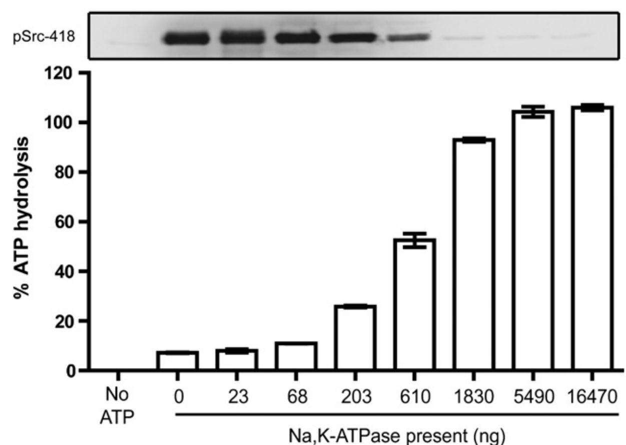

Fig. 1. Representative Western blot analysis of pSrc-418 formation after incubation of 1.5 units of Src with increasing amounts of Na,K-ATPase in PBS, supplemented with 5.0 mM $MgCl_2$. After 30 min, MgATP (final concentration 2.0 mM) or Milli-Q was added for 10 min to initiate Src phosphorylation. ATP hydrolysis for corresponding experimental conditions to Src activity levels visible on Western blot are shown in the graph below (mean ± SEM). These results were obtained from three experiments using identical methods as described above, except for the use of radiolabeled ATP.

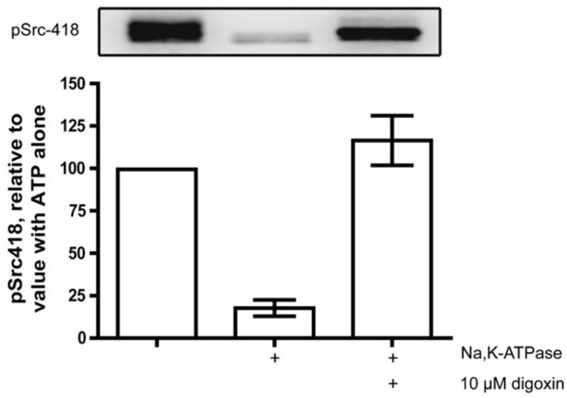

Fig. 2. Western blot showing the average signal intensities (± SEM) for seven independent experiments. 1.5 units of Src were incubated with or without the presence of (inactive) Na,K-ATPase in PBS for 30 min, subsequently MgATP (final concentration 2.0 mM) was added for 10 min and the amounts of pSrc-418 phosphorylation were quantified and related to pSrc-418 autophosphorylation signal intensities.

3.3. Role of sodium and potassium in Src inhibition by Na,K-ATPase

The effect of varying KCl or NaCl on Na,K-ATPase mediated inhibition of Src phosphorylation is depicted in Fig. 3. The left part of each panel represents the significant inhibition of Src autophosphorylation by 610 ng of Na,K-ATPase in PBS buffer essentially as described above. This inhibition was reversed by the addition of 10 μM digoxin. The right part illustrates how pSrc-418 levels increase as a result of both decreasing NaCl (150, 50, 15, 5.0 or 0 mM) (panel A) or KCl concentrations (4.5, 1.5, 0.50, 0.15 or 0 mM) (panel B). This modulation of Src activation by either Na^+ or K^+, the ligands of Na,K-ATPase, was unexpected.

The process of Src autophosphorylation itself caused only a limited decrease in the amount of ATP, whereas 55% of ATP was hydrolyzed when Na,K-ATPase was added. Digoxin prevented this drop in ATP concentration. When Src and Na,K-ATPase were incubated in a Tris buffer containing both NaCl and KCl, the ATP hydrolysis increased as expected (up to 75%), due to the absence of inhibitory inorganic phosphate. Lowering the NaCl or KCl concentrations, while maintaining ionic strength (using NMDG-Cl), decreased the level of ATPase consumption in a concentration dependent manner, in agreement with the finding described above showing that Na,K-ATPase activity is responsible for almost all ATP hydrolysis.

3.4. Role of ATP concentrations in Src inhibition

Since both Na^+ and K^+ are essential for Na,K-ATPase induced Src inhibition, we tested if the state (active vs. inactive) of the Na,K-ATPase was important for Src activation. For this purpose the classical P-type ATPase inhibitor vanadate was used. Fig. 4 shows that vanadate did not interfere with Src activity (lanes 1 and 2) in the absence of Na,K-ATPase, but did stimulate Src phosphorylation in the presence of Na,K-ATPase. Similar levels of Src activation were obtained by addition of saturating vanadate concentrations or digoxin, i.e. the presence of either inhibitor of Na,K-ATPase restored Src activity. The data presented in Fig. 4 reveal that the extent of ATP hydrolysis correlated inversely with the level of Src phosphorylation. This finding suggests that the two enzymes compete for the same substrate: ATP. Increasing amounts of vanadate clearly diminished ATP hydrolysis. Thus, Na,K-ATPase is responsible for most of the ATP conversion, whereas Src is responsible for <10% of ATP consumption in the absence of Na,K-ATPase (see also Fig. 3).

Fig. 5 confirms that the inactivation of Src in the presence of Na,K-ATPase under steady-state conditions of enzyme activity is due to ATP depletion. In the presence of a low amount (305 ng) of Na,K-ATPase a

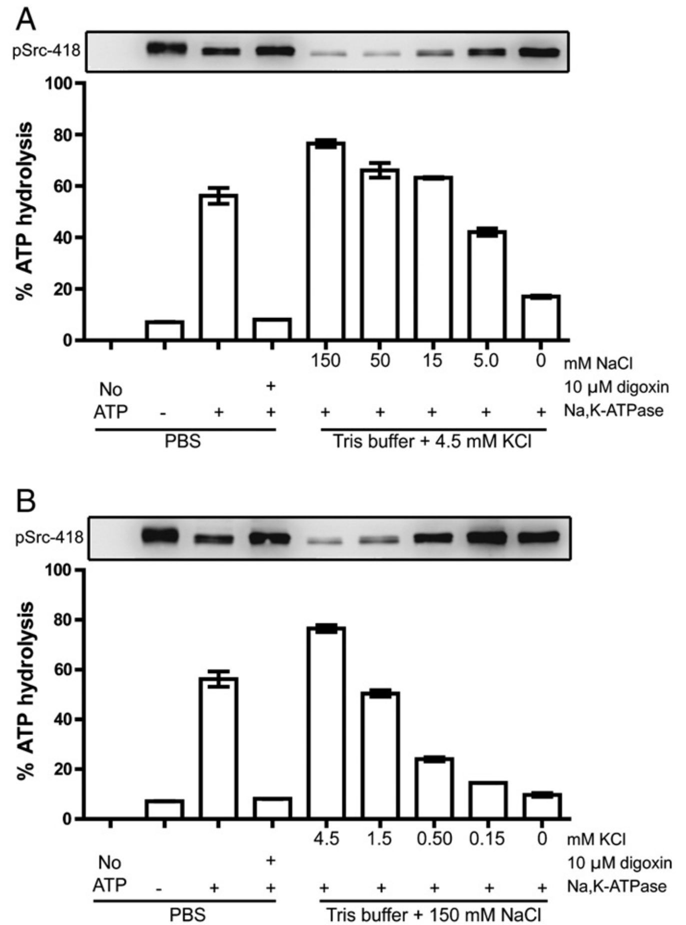

Fig. 3. Representative Western blot analysis of pSrc-418 formation under different buffer conditions. 1.5 units of Src was incubated with or without 610 ng of purified Na,K-ATPase in PBS or 50 mM Tris–HCl (pH 7.4) with or without NaCl or KCl. Panel A shows the effect of decreasing NaCl concentrations (150, 50, 15, 5 and 0 mM) while maintaining a constant KCl concentration, whereas panel B shows the result of decreasing KCl concentrations (4.5, 1.5, 0.50, 0.15 and 0 mM) under a constant NaCl concentration. After 30 min preincubation, the reaction was started by addition of 2.0 mM MgATP, and was allowed to take place for 10 min. For both experiments (panels A and B), the average amount of ATP hydrolysis (mean ± SEM) of three experiments is depicted below the corresponding lane of the Western blot image. Again, the methods were essentially the same as described above, except for the use of radiolabeled ATP to measure its hydrolysis.

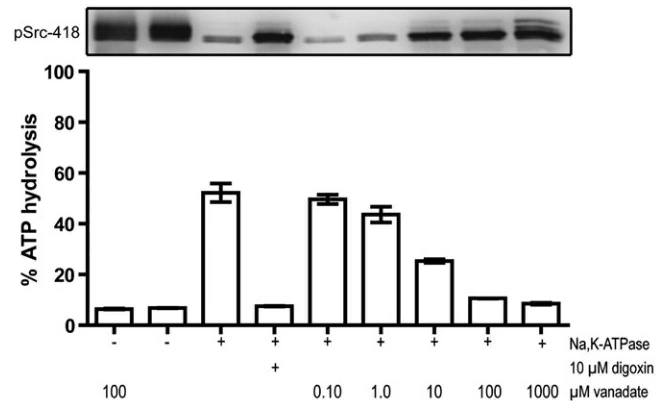

Fig. 4. Representative Western blot analysis of the effect of vanadate on Na,K-ATPase induced Src inactivation. 1.5 units of Src was incubated with or without 610 ng of purified Na,K-ATPase in combination with vanadate or digoxin, as indicated. After 30 min preincubation, 2.0 mM MgATP was added for 10 min to allow Src activation. The mean (± SEM) amount of ATP hydrolysis of three independent experiments is shown below the corresponding lane of the Western blot image. The amount of ATP hydrolysis was determined under similar experimental conditions as used for determining Src activity, except for the use of radiolabeled ATP to measure its hydrolysis.

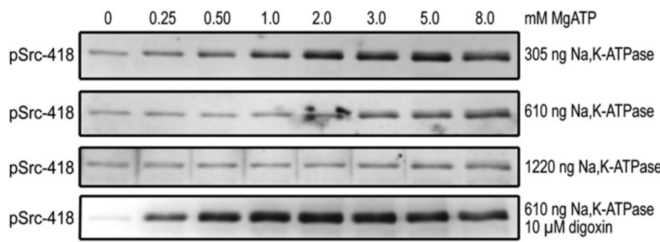

Fig. 5. Representative Western blot analysis of Src activation after stimulation of fixed amounts of Na,K-ATPase (with or without digoxin) with increasing concentrations of MgATP, as indicated. After 30 min of incubation of 1.5 units of Src with the indicated amounts of Na,K-ATPase in the presence or absence of digoxin, MgATP was added for 10 min at the concentration as indicated.

significant level of Src activation is observed already at low ATP concentrations (0.50–1.0 mM). Increasing the amount of Na,K-ATPase present (610 and 1220 ng) resulted in higher ATP demands in order to reach the same level of Src phosphorylation. In contrast, only 0.25 mM ATP was required for Src activation when Na,K-ATPase activity was inhibited by addition of 10 μM digoxin.

3.5. Effect of ADP in Src activation

ADP, the product of the Na,K-ATPase reaction cycle, is known to inhibit Src activity [18]. Therefore, the effect of increasing ADP concentrations on the level of Src phosphorylation was investigated (Fig. 6). Increasing ADP concentrations attenuated Src phosphorylation. The effect of an ATP regenerating system, which converts ADP and Pi into ATP, points in the same direction. The presence of Na,K-ATPase did not influence these results (Fig. 6, panel B), since the regenerating system maintained high ATP concentrations essentially in the absence of ADP.

4. Discussion

Na,K-ATPase, in parallel to its transport function, serves as a receptor in the ouabain signaling pathway [11,15,19,20]. It was shown that exposure of cell cultures to digitalis-like compounds leads to up- or down-regulation of various processes (e.g. gene activation, motility, cell–cell contact, cell proliferation, apoptosis) [21].

DLC binding to Na,K-ATPase always inhibits enzyme activity in *in vitro* experiments. On the cellular level, however, the interactions might result in diverse effects. Na,K-ATPase inhibition can cause changes in the ionic composition of the cytoplasm or energetic state of the cell. In addition, conformational changes of Na,K-ATPase, acting as a receptor and physically interacting with its downstream partners, might result in activation of signaling pathways. Several studies support the involvement of the non-receptor tyrosine kinase Src in DLC induced changes in cellular homeostasis [11–13,19]. The suggested mechanism implied a direct physical interaction between Src and Na,K-ATPase, where signal transduction is mediated via a specific conformational state of Na,K-ATPase. In short, Src is kept in an inactive state by a double intermolecular bond linking the enzyme to Na,K-ATPase [13]. Binding of ouabain induces the E_2P-ouabain bound conformation of Na,K-ATPase, relieving one of the two interactions (Src kinase domain–CD3 domain of Na,K-ATPase) followed by autophosphorylation of tyrosine 418. The phosphorylated Src then initiates diverse signaling pathways, eventually leading to changes in cellular homeostasis [13,22].

The attempts to interpret the stimulating effect of ouabain on Src activity as an indication of direct interactions between Na,K-ATPase and Src were earlier complicated by the discrepancies around the vanadate effect on the Na,K-ATPase – Src complex. Vanadate was first used to show that hydrolytic Na,K-ATPase activity does not interfere with Src activity [13], i.e. that vanadate, in contrast to ouabain, did not relieve the inhibitory effect of Na,K-ATPase on Src activity. In a recent publication [22], however, it was hypothesized that cellular signaling is activated via the E_2 conformations of Na,K-ATPase, conformations which are stabilized by ouabain binding. Vanadate binding shifts the E_1/E_2 equilibrium towards the E_2 conformation of Na,K-ATPase, and therefore should be expected to stimulate Src activity as well. As the experimental conditions for the *in vitro* experiments in both publications [13,22] are identical, as well as the enzymes used are of similar purity and specific activity, it is hard to find an explanation for the lacking vanadate effect observed by Tian et al. [13]. Moreover, the Src-stimulating effect of vanadate was also observed in a study involving intact cells and the viral homolog of Src [23]. Likewise, recent experiments showed endogenous Src activity in both caveolar and noncaveolar preparations of Na,K-ATPase and no additional Src activation by ouabain [10].

Our results confirm that active Na,K-ATPase causes inhibition of Src, while inhibited Na,K-ATPase was unable to inactivate Src kinase. This suggests another explanation for Src activation by binding of DLCs to Na,K-ATPase. Exposure of the presumed Na,K-ATPase-Src complex to different experimental conditions revealed that the magnitude of Na,K-ATPase activity affects the extent of Src phosphorylation. The first observation that evoked this association was the fact that Src phosphorylation was enhanced in response to diminished NaCl and KCl concentrations, both resulting in attenuated Na, K-ATPase activity, as was confirmed by ATPase studies (Fig. 3). Then, the Na,K-ATPase inhibitor vanadate stimulated Src activation in the same way as digoxin did (Fig. 4). Estimation of the amount of ATP present after termination of the reaction showed that 50–60% of ATP was hydrolyzed by Na,K-ATPase in the absence of vanadate, and that the vanadate induced decrease in the amount of ATP hydrolysis correlated with the degree of vanadate-dependent stimulation of Src phosphorylation (Fig. 4). Direct proof that ATP depletion is one of the reasons for Na,K-ATPase induced Src inhibition was obtained in experiments where this effect was counteracted by increasing ATP concentrations (Fig. 5). Finally, we observed that ADP has an inhibitory effect on Src phosphorylation both in the presence and absence of Na,K-ATPase. Src activity significantly decreased when ATP concentrations were below 2.0 mM or in the presence of more than 1.0 mM ADP (Fig. 6). In conclusion, the results of our *in vitro* experiments show that the hydrolytic activity of Na,K-ATPase determines ATP and ADP concentrations and thereby regulates Src activity.

In terms of whole cell physiology our results suggest that coupling between Src and Na,K-ATPase is based on the control of ATP and ADP concentrations, their common ligands. Inhibition of Na,K-ATPase activity by DLCs will increase the ATP/ADP ratio, activating Src

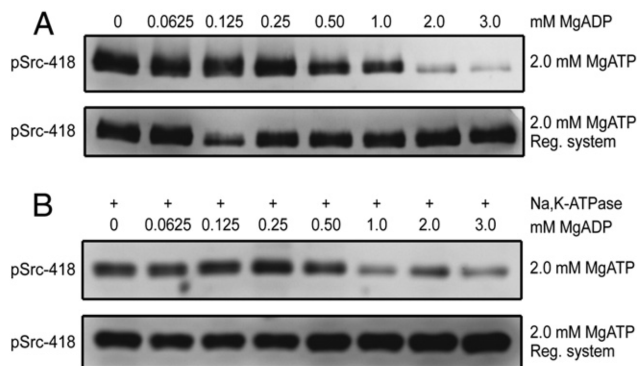

Fig. 6. Representative Western blot analysis of the effect of ADP together with ATP regeneration on Src activation. In short, 1.5 units of Src was incubated with (panel B) or without (panel A) 610 ng of purified Na,K-ATPase in the presence of concentrations of the MgADP concentrations as indicated, with or without the addition of 100 μg/ml creatine kinase in combination with 10 mM creatine phosphate, which together form ADP and Pi into ATP.

phosphorylation. The effect probably involves local changes in the concentrations of ligands, since DLC plasma concentrations are in the pico- and nanomolar range [24], too low to affect the housekeeping function of Na,K-ATPase and bulk concentrations of ATP and ADP. However, not much is known about DLC concentrations in caveolae, the cellular membrane invaginations playing an important role in cell signaling. It is possible that the increase in local DLC concentrations *in vivo* results in significant Na,K-ATPase inhibition.

The role of Na,K-ATPase as a regulator of Src kinase suggested above is in many aspects similar to its role in maintenance of cellular Ca^{2+} homeostasis. Inhibition of Na,K-ATPase activity affects the driving force for the Na/Ca-exchanger, resulting in gain of $[Ca^{2+}]_{CYT}$ and changes in contractility of heart muscle as well as in cellular signaling [25]. In this study, we report evidence that changes in the energetic status of the cell due to varying Na,K-ATPase activity may be involved in cellular signaling. This hypothesis is supported by the fact that in some tissues, Na,K-ATPase is responsible for almost 50% of ATP conversion. Thus, Soltoff and Hedden [9] showed that ouabain, via inhibition of Na,K-ATPase, increases the phosphorylation of AMPK, which subsequently results in phosphorylation of ERK1/2, converging on the same signaling pathway that has been reported for Src mediated signaling.

In conclusion, this study suggests another mode of DLC induced signaling than the proposed direct interaction between Na,K-ATPase and Src. We show that Na,K-ATPase activity regulates Src phosphorylation indirectly, via modulation of ATP and ADP concentrations. Src activation takes place when the ATP concentration is high and ADP concentration is low. The fact that vanadate, acting as an ATPase inhibitor, is also able to activate Src via this mechanism implies that other ligands are capable of stimulating Src activation by interfering with Na,K-ATPase activity.

Disclosure statement

The authors declare that there are no conflicts of interest.

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