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Digitalis-induced cell signaling by the sodium pump: On the relation of Src to Na^+/K^+-ATPase

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ABSTRACT

In addition to performing its essential transport function, the sodium pump also activates multiple cell signaling pathways in response to digitalis drugs such as ouabain. Based mainly on cell-free studies with mixtures of purified Src kinase and Na^+/K^+-ATPase, a well-advocated hypothesis on how ouabain initiates the activation of signaling pathways is that there is a preexisting physiological complex of inactive Src bound to the α-subunit of Na^+/K^+-ATPase, and that ouabain binding to this subunit disrupts the bound Src and activates it. Because of the published disagreements of the results of such cell-free experiments of two other laboratories, our aim was to attempt the resolution of these discrepancies. We reexamined the effects of ouabain, vanadate, and oligomycin on mixtures of Src, Na^+/K^+-ATPase, Mg^{2+}, and ATP as specified in prior studies; and assayed for Src-418 autophosphorylation as the measure of Src activation. In contrast to the findings of the proponents of the above hypothesis, our results showed similar effects of the three inhibitors of Na^+/K^+-ATPase; indicating that Src activation in such experiments is primarily due to the ATP-sparing effect of the ATPase inhibitor on the mixture of two enzymes competing for ATP. We conclude that there is no solid evidence for direct molecular interaction of Src with Na^+/K^+-ATPase under physiological conditions.

1. Introduction

Na^+/K^+-ATPase is the energy transducing enzyme of the plasma membrane that pumps Na^+ and K^+ against their physiological gradients in higher eukaryotic cells [1,2]. Two subunits of the enzymes (α and β) are necessary for this function [2], but the ATP site, the ion binding sites and the transport routes are within the α-subunit [3]. A third subunit (FXYD protein) regulates the pumping function and the Na^+/K^+-ATPase activity of the enzyme [2].

In addition to its main transport function, Na^+/K^+-ATPase may also act as a signal transducer. When intact cells are exposed to digitalis drugs (e.g., ouabain and digoxin) that are specific inhibitors of this enzyme [1,2], various cell signaling pathways are activated leading to highly cell-specific down-stream consequences [4].

That the ubiquitous tyrosine kinase Src is activated in response of an intact cell's exposure to ouabain was first reported by Haas et al. [5] who showed that the addition of nontoxic concentrations of ouabain to cultured neonatal cardiac myocytes or A7r5 smooth muscle cells rapidly activated Src, and that an activated EGFR/Src–Ras–ERK½ pathway was involved in the previously noted gene regulatory and growth-related ouabain effects on cultured cells [4–6]. Although at the time the mechanism of the linkage of ouabain-inhibited Na^+/K^+-ATPase to EGFR/Src was left open [5], subsequent studies led to the hypothesis advanced by Tian et al. [7] that ouabain-induced cell signaling may be due to the existence of an inactive pool of cellular Src that is bound to intracellular domains of the α-subunit of Na^+/K^+-ATPase, and that the known ouabain binding to the extracellular domains of the α-subunit causes conformational changes leading to disinhibition of this Src; thus allowing the activation of the EGFR/Src–Ras–ERK½ pathway. This hypothesis has not been fully tested despite ample claims to the contrary [e.g., 8–10].

The critical part of the evidence in support of a molecular complex of Src and Na^+/K^+-ATPase as the receptor for ouabain-induced signaling came from two sets of cell-free experiments [7]. First, in which the addition of ouabain to a mixture of purified preparations of Na^+/K^+-ATPase, Src, and the appropriate substrates of the two enzymes led to significant activation of Src. Second, similar experiments in which the addition of vanadate (an inhibitor of Na^+/K^+-ATPase with a site of action different from that of ouabain) did not activate Src; therefore, indicating the specificity of ouabain as the activator of the receptor for ouabain-induced cell signaling

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[7]. More recently, Weigand et al. [11] have questioned the validities of findings and conclusions of the cell-free experiments of Tian et al. [7]. Noting that either digoxin or vanadate activates Src when added to a mixture of purified Src and Na^+/K^+-ATPase, Weigand et al. [11] suggested that any inhibitor of Na^+/K^+-ATPase is expected to do the same in such experiments due to the competition of the two enzymes for ATP. Considering that Tian et al. [7] indeed used vanadate to rule out this possibility, we deemed it necessary to reevaluate the effect of this Na^+/K^+-ATPase inhibitor in order to further our understanding of the mechanism of the signaling function of the sodium pump. Here we present our findings on the effects of ouabain, vanadate, and oligomycin (another reversible inhibitor of Na^+/K^+-ATPase) on cell-free mixtures of Src and Na^+/K^+-ATPase, the resulting Src activations, and the implications of our findings for the presumed direct molecular contact of Src with Na^+/K^+-ATPase.

2. Materials and methods

Membrane-bound Na^+/K^+-ATPase was purified from the pig kidney outer medulla by standard procedures as cited before [7]. The steady-state ouabain-sensitive activity was assayed in a solution containing 100 mM NaCl, 25 mM KCl, 2 mM ATP, 3 mM $MgCl_2$, 1 mM EGTA, and 20 mM Tris–HCl (pH 7.4) in the presence and absence of 1 mM ouabain [7]. Specific activities of the preparations used were in the range of 800–1200 μmol of Pi/mg/h. Purified recombinant full-length Src was obtained from Millipore (Bellerica, MA). Autophosphorylation of Src-418, assayed by antibodies to pY-418 Src (Invitrogen Corporation, Camarillo, CA, cat: 44660G) and Src (Santa Cruz Biotechnology, Inc, Dallas, Texas, cat: sc-8056), and expressed as the ratio of phosphorylated Src/total Src, was assumed to indicate Src activation [7]. For quantitations of the immunoblots, multiple exposures were used to ensure that comparisons were made within the linear range of the assay [5]. Ouabain, sodium orthovanadate, and oligomycin were purchased from Sigma–Aldrich Corporation (St. Louis, MO).

In experiments of Figs. 1 and 2, all reaction conditions and the order of additions of the reagents were strictly followed as specified by Tian et al. [7]. Unless otherwise stated, in experiments of Figs. 3 and 4a, reaction conditions and order of reagent additions were according to Ye et al. [12].

Data were expressed as mean ± SEM. All analyses including curve fitting were performed on GraphPad Prism 5.0 software (La Jolla, CA). Paired Student's *t*-test was used. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Comparison of the effects of ouabain and vanadate on autophosphorylation of Src-418 in the cell-free mixtures of Src and Na^+/K^+-ATPase

Attempting to resolve the above-mentioned discrepancies between the findings of Tian et al. [7] and Weigand et al. [11], we noted that somewhat different procedures for the purification of the pig kidney Na^+/K^+-ATPase and significantly different ratios of Na^+/K^+-ATPase/Src had been used in the two studies. Therefore, we prepared Na^+/K^+-ATPase according to Tian et al. [7], and faithfully conducted the experiments by their published procedures to compare the effects of ouabain and vanadate on Src activation in a mixture of Na^+/K^+-ATPase, Src, and ATP.

The results of the first set of experiments summarized in Fig. 1 showed that (a) in agreement with Tian et al. [7], Src-418 phosphorylation was reduced when both Na^+/K^+-ATPase and Src were present in the reaction mixture; and (b) addition of 10 μM ouabain partially antagonized the inhibitory effect of Na^+/K^+-ATPase on Src phosphorylation.

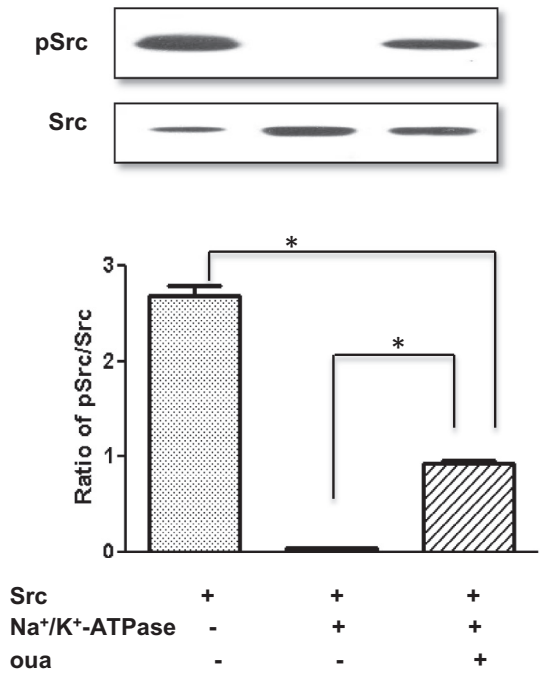

Fig. 1. Inhibitory effect of Na^+/K^+-ATPase on Src autophosphorylation, and antagonism of this inhibition by 10 μM ouabain. Cell-free experiments with mixtures of purified Na^+/K^+-ATPase and Src were performed, and Src phosphorylation was assayed as described in Section 2. $n = 3$ $^*P < 0.05$ for indicated comparison.

In stark contrast to the findings of Tian et al. [7], however, our results of Fig. 2 showed that (a) vanadate also increased Src phosphorylation when added to the reaction mixture; and (b) the highest vanadate concentration used was as effective as 10 μM ouabain in activating Src. Clearly, our findings of Figs. 1 and 2 support the

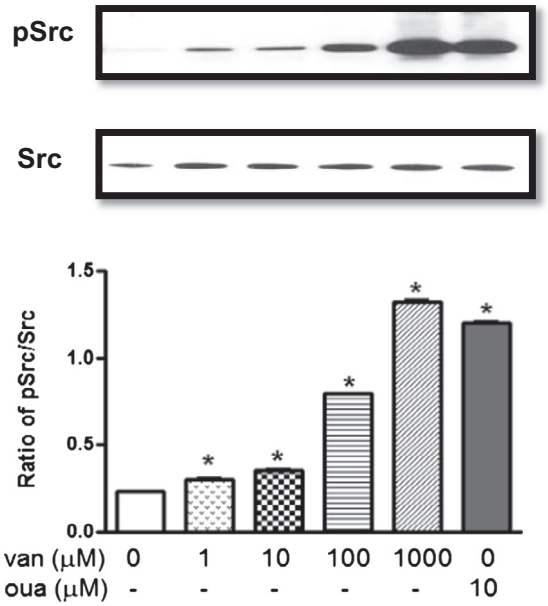

Fig. 2. Stimulation of Src autophosphorylation in cell-free mixtures of Src and Na^+/K^+-ATPase by varying concentrations of vanadate. Experiments and assays were done as specified in Section 2. $n = 3$, $^*P < 0.05$ compared to absence of vanadate and ouabain.

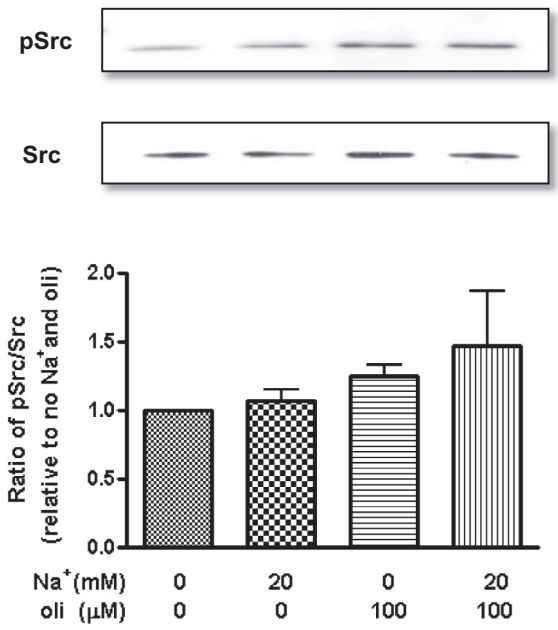

Fig. 3. Lack of effect of Na^+ and/or oligomycin in the absence of K^+ on Src autophosphorylation in the mixtures of Src and Na^+/K^+-ATPase. Experimental conditions and assays were as specified in Section 2. $n = 3$, all P values were greater than 0.05 compared to absence of Na^+ and oligomycin.

findings and the main conclusions of Weigand et al. [11], indicating that activations of Src by ouabain and vanadate in such cell-free experiments are due to the ATP-sparing effects of the ATPase inhibitors.

In spite of the above-indicated disagreements between our findings and those of Tian et al. [7], it is appropriate to ask if their original report contains findings other than those of the cell-free experiments to indicate the existence of direct molecular contact between Src and Na^+/K^+-ATPase. We think not. Their FRET and BRET analyses suggest proximities of Src and Na^+/K^+-ATPase in intact cells, but do not establish direct contact [13,14]. Additionally, their report also includes various immunoprecipitation and pull-down experiments involving detergent-solubilized Na^+/K^+-ATPase. However, the detergents used and the indicated solubilization conditions denature Na^+/K^+-ATPase. At best, therefore, these studies suggest interactions between Src and the unfolded Na^+/K^+-ATPase subunits.

3.2. Comparison of the effects of ouabain and oligomycin on autophosphorylation of Src-418 in the cell-free mixtures of Src and Na^+/K^+-ATPase

Assuming the validity of the proposal of Tian et al. [7] that there is direct molecular contact between Src and Na^+/K^+-ATPase, the same laboratory [12] has subsequently modified the proposed model to suggest that different conformations of Na^+/K^+-ATPase (E_1 and E_2) are bound to Src differently to regulate its kinase activity. E_1 and E_2 are the Na^+-bound and the K^+-bound forms of Na^+/K^+-ATPase that are phosphorylated and dephosphorylated at Asp-369 of the α-subunit during the well-known Albers-Post scheme of ATP hydrolysis [1,2]. In their recent studies, Ye et al. [12] pretreated purified Na^+/K^+-ATPase with inhibitors that were presumed to favor either E_1 or E_2 conformations, then mixed them with Src and ATP, and measured Src activation. Based on these results, they concluded that Src bound to E_1 is inactive, but Src bound to E_2 is active [12]. Following their experimental conditions, we repeated their cell-free experiments on oligomycin, which is a long-established

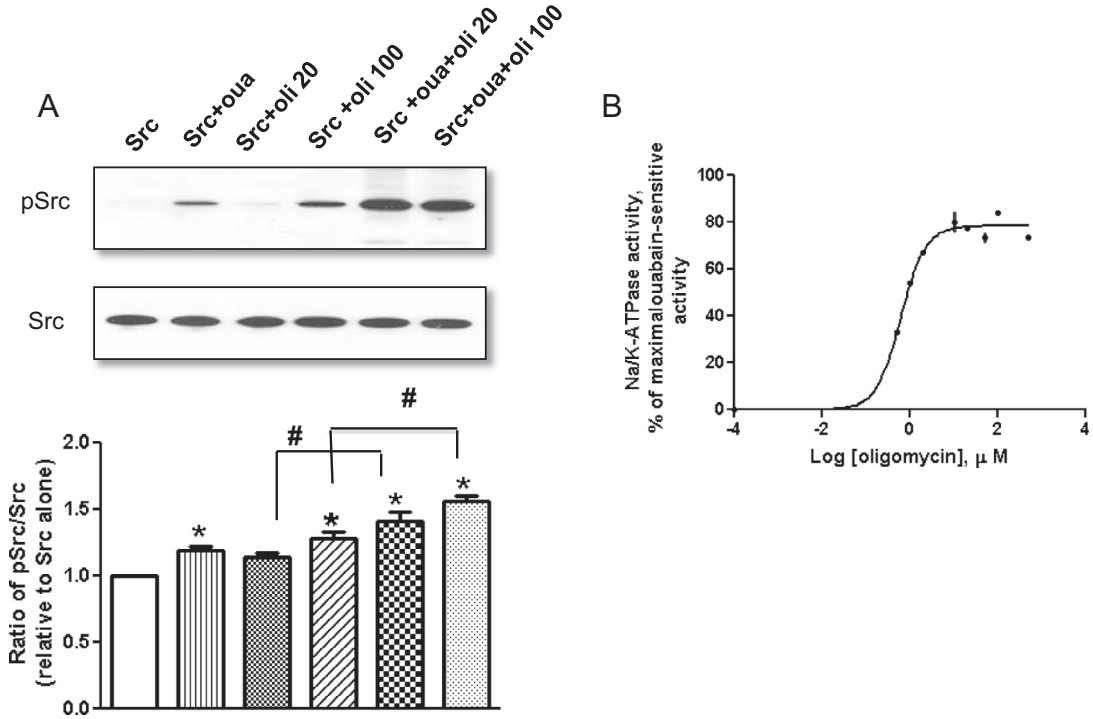

Fig. 4. Comparison of the effects of oligomycin and ouabain on Src autophosphorylation in mixtures of Src and Na^+/K^+-ATPase in the presence of Na^+ and K^+. (A) Experimental conditions were the same as in Fig. 3, except that 100 mM Na^+ and 20 mM K^+ were added to obtain near-maximal activity of Na^+/K^+-ATPase. Ouabain concentration was 10 μM, and oligomycin concentrations were 20 or 100 μM. $n = 3$ $^*P < 0.05$ compared to Src alone. $^\#P < 0.05$ for the indicated comparisons. (B) Effect of varying concentrations of oligomycin on Na^+/K^+-ATPase activity. Purified enzyme was pre-incubated with inhibitors for 30 min before the assay. Maximal steady-state Na^+/K^+-ATPase activity was assayed as described in Section 2. At each oligomycin concentration ($n = 2$–3), the activity was calculated relative to the maximal activity. The indicated line is the fit of the data to the 4-parameter logistic nonlinear regression model. R^2 (goodness of fit) is 0.97.

inhibitor of Na^+/K^+-ATPase with known mechanism and locus of binding to the E_1 conformation [15–20]. As in experiments of Fig. 2C of Ye et al. [12], we added oligomycin to Na^+/K^+-ATPase in a K^+-free medium containing Na^+, then mixed this with Src and ATP, and assayed for Src phosphorylation. Our results were in distinct contrast to those of Ye et al. [12]. Whereas they reported that either Na^+ alone or Na^+ plus oligomycin significantly reduced Src phosphorylation, our results indicated no effect of Na^+ or Na^+ plus oligomycin (Fig. 3); thus questioning the suggested regulation of Src binding to E_1 [12]. On the other hand, our data of Fig. 3 are consistent with the proposal [11] that in such cell-free mixtures of Src and Na^+/K^+-ATPase it is the ATP-sparing effect of the ATPase inhibitor that affects Src activity. While Na^+/K^+-ATPase does have a Na^+-ATPase activity in the absence of K^+, the maximal specific activity of Na^+-ATPase is about 2–5% of the maximal Na^+/K^+-dependent activity [21]. Hence, with the ATP concentrations used in our experiments of Fig. 3, and those of the Fig. 2C of Ye et al. [12], there is little or no significant change in ATP concentration due to Na^+-ATPase.

Effects of oligomycin on mixtures of Src and Na^+/K^+-ATPase in the presence of both Na^+ and K^+ were not reported by Ye et al. [12]. Therefore, we conducted experiments similar to those of Fig. 3, but in the presence of Na^+ and K^+. The results (Fig. 4a) showed that oligomycin, like ouabain, increased Src phosphorylation, clearly indicating that inhibitors of Na^+/K^+-ATPase have similar effects on Src in such cell-free studies regardless of inhibitors' selectivity for E_1 or E_2 conformation.

Experiments of Fig. 4b on the effects of varying concentrations of oligomycin on Na^+/K^+-ATPase activity of the preparations used here confirmed previous findings [15,18] that oligomycin, unlike ouabain, is a partial inhibitor of this ATPase and that it causes no more than about 80% of the activity. This is consistent with the binding site of oligomycin being within the lipid phase of the enzyme as previously suggested [17] and shown by the recent crystal structure analysis of the oligomycin-bound enzyme [20]. More pertinent to the present findings, the fact that oligomycin is a partial inhibitor of the enzyme also explains why ouabain and oligomycin exerted additive effects on Src activation (Fig. 4a).

3.3. Implications for the proposed direct contact between Src and the α-subunit of Na^+/K^+-ATPase under physiological conditions

We began this work to see if we could resolve the conflicting findings of two laboratories [7,11] relevant to the proposed binding of Src to Na^+/K^+-ATPase and the role of Src in the digitalis-induced cell signaling. Our findings on vanadate (Fig. 2) agreed with those of one laboratory [11], but not with those of the other [7]. We were also unable to reproduce the findings of the latter laboratory [12] on oligomycin effects in cell-free mixtures of Src and Na^+/K^+-ATPase (Fig. 3). Taken together, our present findings and those of Weigand et al. [11] seriously question the validity of the once attractive hypothesis that a molecular complex of Src and the sodium pump is the receptor for digitalis-induced cell signaling pathways. This is reinforced by our recent report indicating that activations of some digitalis-induced pathways and their growth consequences occur even in cells that are devoid of Src [22].

Acknowledgments

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References

[1] J.C. Skou, M. Esmann, The Na^+/K^+-ATPase, J. Bioenerg. Biomembr. 24 (1992) 249–262.
[2] J.H. Kaplan, Biochemistry of Na^+/K^+-ATPase, Annu. Rev. Biochem. 71 (2002) 511–535.
[3] M. Laursen, L. Yatime, P. Nissen, N.U. Fedosova, Crystal structure of the high-affinity Na^+/K^+-ATPase-ouabain complex with Mg^{2+} bound in the cation binding site, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 10958–10963.
[4] Z. Xie, A. Askari, Na^+/K^+-ATPase as a signal transducer, Eur. J. Biochem. 269 (2002) 2434–2439.
[5] M. Haas, A. Askari, Z. Xie, Involvement of Src and epidermal growth factor receptor in the signal-transducing function of Na^+/K^+-ATPase, J. Biol. Chem. 275 (2000) 27832–27837.
[6] M. Peng, L. Huang, Z. Xie, W.H. Huang, A. Askari, Partial inhibition of Na^+/K^+-ATPase by ouabain induces the Ca^{2+}-dependent expressions of early-response genes in cardiac myocytes, J. Biol. Chem. 271 (1996) 10372–10378.
[7] J. Tian, T. Cai, Z. Yuan, H. Wang, L. Liu, M. Haas, E. Maksimova, X.Y. Huang, Z.J. Xie, Binding of Src to Na^+/K^+-ATPase forms a functional signaling complex, Mol. Biol. Cell 17 (2005) 317–326.
[8] J. Tian, Z.J. Xie, The Na^+/K^+-ATPase and calcium-signaling microdomains, Physiology (Bethesda) 23 (2008) 205–211.
[9] Z. Li, Z. Xie, The Na^+/K^+-ATPase/Src complex and cardiotonic steroid-activated protein kinase cascades, Pflugers Arch. 457 (2009) 635–644.
[10] J. Liu, Z.J. Xie, The sodium pump and cardiotonic steroids-induced signal transduction protein kinases and calcium-signaling microdomain in regulation of transporter trafficking, Biochim. Biophys. Acta 2010 (1802) 1237–1245.
[11] K.M. Weigand, H.G. Swarts, N.U. Fedosova, F.G. Russel, J.B. Koenderink, Na, K-ATPase activity modulates Src activation: a role for ATP/ADP ratio, Biochim. Biophys. Acta 2012 (1818) 1269–1273.
[12] Q. Ye, Z. Li, J. Tian, J.X. Xie, Z. Xie, Identification of a potential receptor that couples ion transport to protein kinase activity, J. Biol. Chem. 286 (2011) 6225–6232.
[13] B.A. Pollok, R. Heim, Using GFP in FRET-based applications, Trends Cell Biol. 9 (1999) 57–60.
[14] B.A. Corgiat, J.C. Nordman, N. Kabbani, Chemical crosslinkers enhance detection of receptor interactomes, Front. Pharmacol. 7 (2014) 171.
[15] J.D. Robinson, L.J. Robinson, N.J. Martin, Effects of oligomycin and quercetin on the hydrolytic activities of the Na^+/K^+-dependent ATPase, Biochim. Biophys. Acta 772 (1984) 295–306.
[16] M. Esmann, J.C. Skou, Occlusion of Na^+ by the Na^+/K^+-ATPase in the presence of oligomycin, Biochem. Biophys. Res. Commun. 127 (1985) 857–863.
[17] M. Esmann, Oligomycin interaction with Na^+/K^+-ATPase: oligomycin binding and dissociation are slow processes, Biochim. Biophys. Acta 1064 (1991) 31–36.
[18] T. Arato-Oshima, H. Matsui, A. Wakizaka, H. Homareda, Mechanism responsible for oligomycin-induced occlusion of Na+ within Na^+/K^+-ATPase, J. Biol. Chem. 271 (1996) 25604–25610.
[19] M. Nyblom, H. Poulsen, P. Gourdon, L. Reinhard, M. Andersson, E. Lindahl, N. Fedosova, P. Nissen, Crystal structure of Na^+/K^+-ATPase in the Na^+-bound state, Science 342 (2013) 123–127.
[20] R. Kanai, H. Ogawa, B. Vilsen, F. Cornelius, C. Toyoshima, Crystal structure of a Na^+-bound Na^+/K^+-ATPase preceding the E1P state, Nature 502 (2013) 201–206.
[21] M. Esmann, ATPase and phosphatase activity of Na^+/K^+-ATPase: molar and specific activity, protein determination, Methods Enzymol. 156 (1988) 105–115.
[22] J. Wu, E.E. Akkuratov, Y. Bai, C.M. Gaskill, A. Askari, L. Liu, Cell signaling associated with Na^+/K^+-ATPase: activation of phosphatidylinositide 3-kinase IA/Akt by ouabain is independent of Src, Biochemistry 52 (2013) 9059–9067.