Functional regulation of reconstituted Na,K-ATPase by protein kinase A phosphorylation

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Abstract Reconstituted Na^+,K^+-ATPase from either pig kidney or shark rectal glands was phosphorylated by cAMP dependent protein kinase, PKA. The stoichiometry was ~0.9 mole P_i/mole α-subunit in the pig kidney enzyme and ~0.2 mol P_i/mol α-subunit in the shark enzyme. In shark Na^+,K^+-ATPase PKA phosphorylation increased the maximum hydrolytic activity for cytoplasmic Na^+ activation and extracellular K^+ activation without affecting the apparent K_m values. In contrast, no significant functional effect after PKA phosphorylation was observed in pig kidney Na^+,K^+-ATPase.

Key words: Protein kinase A (PKA); Regulation; Na^+,K^+-ATPase; Reconstitution; Phosphorylation

1. Introduction

There is a consensus in the literature that the Na^+,K^+-ATPase under certain conditions can be phosphorylated in vitro both by the cAMP dependent protein kinase, PKA, and by protein kinase C, PKC [1–7] and it has been hyphotesized that this forms the molecular basis for its regulation, however, a direct link to the physiological regulation of Na^+,K^+-ATPase in vivo is still missing. Conflicting results of the effects of protein kinase phosphorylation on enzyme function have been reported (cf. [2,6]). For PKA phosphorylation this is especially true due to the requirement for detergents [1–3] which probably exposes the target site for PKA, but makes it inaccessible to functional studies due to inhibition of the Na^+,K^+-ATPase activity. This necessity for detergents also makes it difficult to assess the sidedness of PKA action.

The purpose of this paper is to demonstrate that after reconstitution of solubilized Na^+,K^+-ATPase it can be phosphorylated by PKA in the absence of detergents. With this preparation it is, therefore, for the first time possible to investigate both the functional effects and the sidedness of PKA phosphorylation in vitro.Here, phosphorylation by PKA of Na^+,K^+-ATPase from pig kidney and shark rectal glands reconstituted into liposomes is demonstrated. The stoichiometry of phosphorylation was significantly different in the two species: ~0.9 mol/mol α-subunit for pig kidney and ~0.2 mol/mol α-subunit for shark rectal gland, a difference also found in purified enzyme preparations prior to reconstitution. PKA phosphorylation of inside-out oriented Na^+,K^+-ATPase from shark (α_3 isoform from immunological measurements [4]) increases the maximum rate of hydrolysis (V_{max}), but had no effect on the pig kidney enzyme (mainly α_1 isoform, [8]). In both preparations phosphorylation by PKA left $K_{0.5}$ unchanged both for activation by cytoplasmic Na^+ and by extracellular K^+.

2. Materials and methods

Preparation of purified Na^+,K^+-ATPase from shark rectal glands and from pig kidney was as previously described [9,10]. The specific activities at 37°C were 1800–2000 μmol/mg·h (30–33 U/mg) for shark and about 1600 μmol/mg·h (27 U/mg) for pig kidney. The amount of phosphoenzyme formed from ATP-hydrolysis, determined as described in [11], was 2.72 ± 0.03 nmol/mg (mean ± S.E.M., $n = 11$) and 2.36 ± 0.03 nmol/mg (mean ± S.E.M., $n = 4$) in shark and pig, respectively. Solubilization of shark Na^+,K^+-ATPase using $C_{12}E_8$ was as previously described [12]. Pig kidney Na^+,K^+-ATPase was solubilized using CHAPS: to 2.4 mg/ml enzyme in 30 mM histidine and 0.5 mM EDTA, 45 mM CHAPS is added. After incubation at 0°C for 10 min the solution was centrifuged at 170.000×g for 60 min at 4°C and the supernatant recovered. The specific activity of shark Na^+,K^+-ATPase at 23°C after solubilization is 600–900 μmol/mg·h and of pig kidney Na^+,K^+-ATPase 300–400 μmol/mg·h.

Reconstitution of Na^+,K^+-ATPase into lipid vesicles containing either 130 mM Na^+ (Na^+ vesicles), or 260 mM sucrose (sucrose vesicles) was obtained by complete detergent elimination using Biobeads, either from a mixed suspension of protein/lipid/detergent (shark), or from a mixture of preformed liposomes destabilized by 4 mM CHAPS and solubilized enzyme (pig [13]). In both cases the final protein/lipid weight ratio was 1:20, and the proteoliposomes contained phosphatidylcholine/cholesterol in a molar ratio of 60:40. The protein orientation after reconstitution was determined in each preparation from functional tests [14]. For both shark and pig preparations an asymmetric orientation of reconstituted Na^+,K^+-ATPase was found with about 10% of the total protein oriented inside-out for shark and 16% for pig enzyme. ATPase activity was measured with [γ-^{32}P]ATP using the method of Lindberg and Ernster [15]. For solubilized shark enzyme $C_{12}E_{10}$ and $C_{12}E_8$ was included in the assay medium [16], whereas for pig kidney only $C_{12}E_{10}$ (0.375 mg/ml) was present. Measurement of hydrolytic activity of reconstituted enzyme oriented exclusively inside-out was assured by addition of ATP to the medium and preincubation with MgPi + ouabain. The former is inaccessible to the right side out oriented enzyme, the latter completely inhibits enzyme reconstituted with both sides exposed (non-oriented), as previously described [14]. Na^+ activation (cytoplasmic) of hydrolysis was achieved by varying the [Na^+] in the medium by isosmotic replacement with sucrose, whereas in the case of (extracellular) K^+ activation, nigericin (7 μM) was included to equilibrate the proteoliposomes with medium K^+ [11]. Protein was determined by a modified Lowry-method [17].

Reconstituted Na^+,K^+-ATPase was phosphorylated by PKA after incubation at 23°C of 50 μl proteoliposomes (≈0.2 mg protein/ml) in 450 μl reaction medium containing HEPES (50 mM, pH 7.5), $MgCl_2$ (10 mM), EGTA (1mM), and PKA (80 mU). The reaction was initiated by addition of 100 μM ATP, and after 30 min the samples were cooled on ice and EDTA added (2 mM). A control identical in all other respects except for PKA was processed in parallel. After incubation, the vesicles (PKA treated and controls) were passed

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through a Penefsky column [18] to remove the small amount of K^+ introduced by the PKA solution (≈2 mM). Purified membrane preparations of shark and pig kidney were phosphorylated by PKA as described for reconstituted preparations with the addition of 0.1% Triton X-100 (shark) or 18 mM CHAPS (pig). For measurements of the stoichiometry of PKA phosphorylation the proteoliposomes were incubated with PKA as described above in the presence of 100 μM [γ-^{32}P]ATP (~2000 cpm/pmol) and the reaction stopped by addition of SDS sample buffer [19]. The samples were analyzed by gel electrophoresis using 7.5% SDS-polyacrylamide gels. The gels were stained with Coomassie blue, destained and dried, followed by autoradiography analysis. The bands corresponding to the α-subunit were excised from the gels and the radioactivity measured. The catalytic subunit of PKA was purchased from Boehringer.

3. Results and discussion

Fig. 1 illustrates phosphorylation of reconstituted shark and pig kidney Na^+,K^+-ATPase α-subunit by PKA in the absence of detergents. This is in contrast to results using purified membrane preparations of the enzymes where Triton X-100 is necessary in order to obtain phosphorylation by PKA [1–3,6]. Probably, the dissociation from the native membranous constraints and the subsequent dilution into the liposomes makes the PKA target site accessible. For pig Na^+,K^+-ATPase the stoichiometry of PKA phosphorylation ranges from 0.40–1.15 mol P/mol α-subunit (0.86 ± 0.16, mean ± S.E.M., $n = 5$) which is within the same range as recently determined by Fechenko and Sweadner [6]. The stoichiometry is calculated on the basis of the measured maximum ATP phosphorylation number (≈6.1 nmol/mg, cf. [11]) and by correcting the total protein content for the measured inside-out fractions of the Na^+,K^+-ATPase after reconstitution, since right-side out oriented Na^+,K^+-ATPase is not exposed to PKA phosphorylation. Enzyme oriented with both sides exposed (n-o) was not phosphorylated by PKA since incubation with ouabain in the medium which inhibits PKA phosphorylation in shark membrane preparations (not shown, [6]) did not decrease the PKA phosphorylation in reconstituted preparations. Moreover, by mild treatment with Triton X-100 phosphorylation increased exactly in proportion equivalent to the fraction of n-o enzyme, indicating the demasking of this fraction. For shark Na^+,K^+-ATPase the stoichiometry was significantly lower, ranging from 0.08–0.30 mol P/mol α-subunit (0.19 ± 0.04, mean ± S.E.M., $n = 5$). In the presence of H-89, a potent inhibitor of PKA, phosphorylation was barely detectable (data not shown). The difference in phosphorylation stoichiometry between the two preparations was also present prior to reconstitution since purified membrane preparations from pig and shark (in the presence of detergents) gave 1.19 ± 0.05 ($n = 4$) and 0.40 ± 0.02 ($n = 8$) mol P_i/mol α-subunit, respectively. Since both enzyme preparations were purified to the same high specific activity and the phosphoenzyme contents were similar, the variation is not due to variable impurities but could be caused by a more favourable exposure of the PKA target site in pig kidney Na^+,K^+-ATPase. The difference is present even though the RRxS consensus sequence is conserved in all three isoforms including the Ser-938 which in rat α_1 is the site for PKA phosphorylation [4–6]. A less possible explanation is that the shark Na^+,K^+-ATPase could have been purified in a state already phosphorylated. The other possible cause, the presence of phosphatases is also considered unlikely since previous attempts to detect phosphatase activity in various Na^+,K^+-ATPase preparations have been unsuccesfull [6].

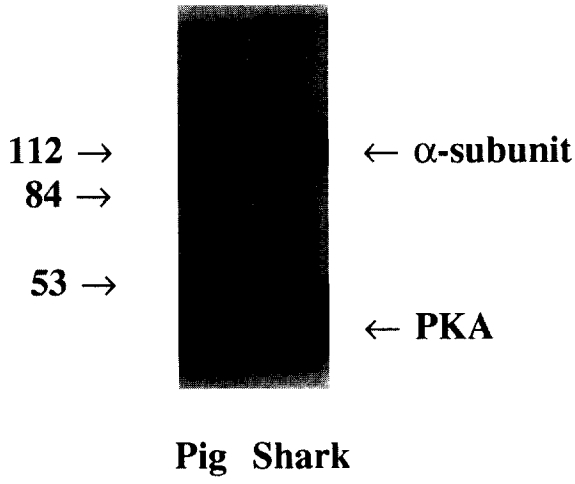

Fig. 1. Phosphorylation of shark rectal gland Na^+,K^+-ATPase and pig kidney Na^+,K^+-ATPase by cAMP dependent protein kinase, PKA. Autoradiograms of ^{32}P-labelled proteins after SDS-gel electrophoresis.

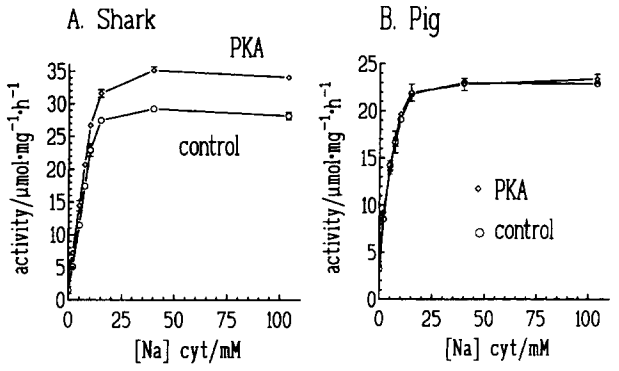

Fig. 2. Activation by cytoplasmic Na^+ of hydrolytic activity associated with $Na^+:Na^+$-exchange of inside-out reconstituted Na^+,K^+-ATPase from shark (A) and pig kidney (B). Proteoliposomes contained 130 mM Na^+ and no K^+. Panel A demonstrates the increased V_{max} by about 22% after PKA phosphorylation of shark Na^+,K^+-ATPase, whereas activation by PKA phosphorylation of pig kidney is absent (panel B). For shark fitting of a sigmoid curve to the data gave K_m values of 9.1 ± 0.3 mM and 8.4 ± 1.1 mM for controls and after PKA phosphorylation, respectively. Temperature was 23°C.

To assess possible functional consequences of PKA phosphorylation on inside-out reconstituted Na^+,K^+-ATPase we measured the cytoplasmic Na^+ activation of hydrolytic activity associated with (i) the physiological $Na^+:K^+$-exchange, (ii) the analogous $Na^+:Na^+$-exchange, and (iii) the uncoupled Na^+-efflux. Cytoplasmic Na^+ activation of $Na^+:K^+$-exchange was achieved by variation of the medium [Na^+] after exposing the extracellular sites (inside the liposome) to optimal [K^+] by permeabilizing the liposomes to K^+ with nigericin. Na^+ activation of $Na^+:Na^+$-exchange and uncoupled Na^+-efflux was accomplished using liposomes containing either 130 mM Na^+, or 260 mM sucrose, respectively. The activation by extracellular K^+ was measured by varying the concentrations in the medium in the presence of nigericin keeping the sum of [Na^+] plus [K^+] constant.

In Fig. 2 Na^+ (cytoplasmic) activation of inside-out oriented Na^+,K^+-ATPase from shark and pig reconstituted

into liposomes containing Na^+ after PKA phosphorylation is shown and compared with controls. In this Na^+:Na^+-exchange extracellular Na^+ acts as a K^+-congener resulting in a lower turnover (about 20% of Na^+:K^+-exchange) than in the physiological Na^+:K^+-exchange in an otherwise identical reaction [20].

As seen from Fig. 2A, PKA phosphorylation of shark enzyme increased V_{max} by up to 30% and decreased K_m slightly as calculated from a non-linear fitting of a sigmoid curve to the data. The calculated Hill coefficients are identical in the two cases, amounting to 2.2. In experiments where the hydrolysis is associated with Na^+:K^+-exchange or with uncoupled Na^+-efflux identical activations were obtained (not shown). In contrast, the cytoplasmic Na^+-activation of reconstituted pig kidney Na^+,K^+-ATPase engaged in either Na^+:K^+-exchange, Na^+:Na^+-exchange, or in uncoupled Na^+-efflux was unaffected by phosphorylation with PKA, as exemplified for Na^+:Na^+-exchange in Fig. 2B.

In Fig. 3A the effects of PKA phosphorylation on the K^+-activation of inside-out oriented shark enzyme reconstituted into sucrose-vesicles is shown as a Scatchard plot. V_{max} (Y-intercept) increases slightly by about 13% whereas K_m (-slope) is similar before and after PKA phosphorylation. Similar experiments using pig kidney enzyme showed no significant effects on either V_{max} or K_m (Fig. 3B). We are currently studying if functional changes other than hydrolytic activity and cation affinity could be associated with PKA phosphorylation of the pig kidney Na^+,K^+-ATPase, or if the functional effects of PKA phosphorylation may be isoform dependent. Fig. 4 summarizes results of cytoplasmic Na^+-activation and extracellular K^+-activation from 5 independent experiments using shark enzyme. In a ping-pong model generally assumed for the Na^+,K^+-ATPase reaction V_{max} and K_m are expected to vary in parallel [21]. In shark enzyme the ratio K_m/V_{max} decreases for extracellular K^+ and cytoplasmic Na^+ after PKA phosphorylation, this could indicate an increased cytoplasmic Na^+-affinity and extracellular K^+-affinity upon PKA phosphorylation. In previous experiments with membrane bound shark enzyme PKA phosphorylation in the presence of Triton X-100 has been reported to decrease the Na^+,K^+-ATPase hydrolytic activity under certain conditions [2]. Therefore, the effect of detergent and PKA on hydrolytic activity of membrane bound Na^+,K^+-ATPase was controlled under the present conditions. As seen from Fig. 5 the combined effect of Triton and PKA is an inhibition of hydrolytic activity in accord with Bertorello et al. [2]. However, we consider it unlikely that this can be ascribed to the PKA phosphorylation per se, since (i) in membrane preparations of both shark and pig kidney 0.1% Triton X-100 strongly inhibit the Na^+,K^+-ATPase activity by itself (Fig. 5A), and (ii) in shark enzyme PKA protected this detergent inhibition considerably stimulating the residual Na^+,K^+-ATPase activity. In contrast, in

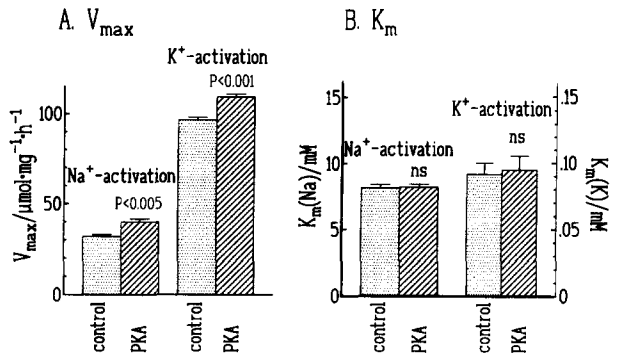

Fig. 4. Comparison of computed values for V_{max} (panel A) and K_m (panel B) in cytoplasmic Na^+ activation and extracellular K^+ activation of reconstituted shark Na^+,K^+-ATPase in controls (speckled) and after PKA phosphorylation (hatched). Mean values for 7 (Na^+-activation) or 5 (K^+-activation) independent experiments ±S.E.M. are shown. PKA phosphorylation significantly increased V_{max} for both cytoplasmic Na^+ activation ($P<0.005$) and extracellular K^+ activation ($P<0.001$), whereas the corresponding K_m values differ insignificantly (ns), as determined by Students t-test.

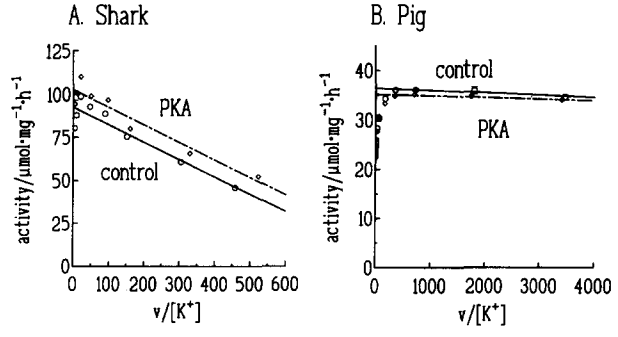

Fig. 3. Scatchard plot of hydrolytic activity of inside-out reconstituted Na^+,K^+-ATPase from shark (A) and pig kidney (B) as a function of increasing K^+ in the medium, the sum of Na^+ plus K^+ held constant. The proteoliposomes containing 260 mM sucrose and no alkali cations are permeabilized to K^+ by the addition of 0.5 μg/ml (~7 μM) nigericin therefore the initial activation represents the high-affinity activation of extracellular K^+. PKA phosphorylation increases V_{max} by about 13% for shark, but not for pig kidney Na^+,K^+-ATPase. The straight lines indicate linear regression analysis of the data for [K^+] which does not induce inhibition. Their slopes indicate identical K_m values for shark Na^+,K^+-ATPase of 0.10±0.01 mM in controls and after PKA phosphorylation. For pig Na^+,K^+-ATPase the slopes correspond to K_m values of 0.4±0.1 μM and 0.31±0.08 μM, in controls and after PKA phosphorylation, respectively. Temperature was 23°C.

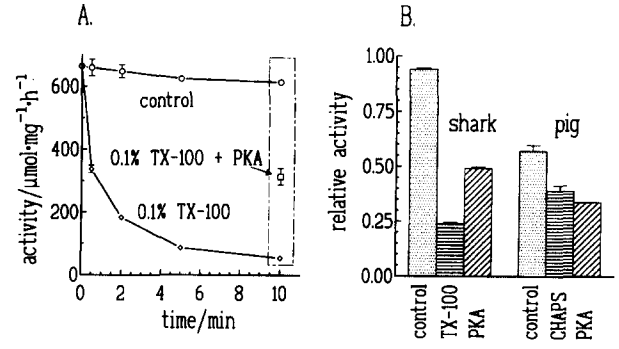

Fig. 5. Hydrolytic activity of purified Na^+,K^+-ATPase (membrane bound preparations) in controls compared to samples incubated either with addition of 0.1% Triton X-100 or with 0.1% Triton X-100 plus PKA. The maximum hydrolytic activity was measured with 130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+} in the presence of 3 mM ATP. Temperature was 23°C. In panel A a typical experiment with membrane preparations from shark shows an almost 90% inhibition after 10 min incubation with Triton X-100 (0.1%) which is partially released by PKA. The points are mean±S.D. ($n=3$). In panel B membrane preparations from shark and pig kidney are compared after 10 min incubations corresponding to the framed data in panel A. The hydrolytic activity from pig kidney rapidly deteriorates in controls and in 5 mM CHAPS, but no protection by PKA is observed in contrast to shark enzyme. Means of 3 experiments are shown.

pig kidney enzyme no protection by PKA was observed (Fig. 5B).

The finding that PKA phosphorylation activates the hydrolytic activity of shark Na^+,K^+-ATPase but does not affect the functional state of α_1 from pig, although the former is phosphorylated to a lower stoichiometry, is unexpected. It may indicate not only isoform dependent differences of PKA effects but also variations between the same isoforms from different species. This is in keeping with previous findings where stimulation of Na^+,K^+-ATPase activity was observed in isolated shark rectal gland cells by cAMP [22], whereas in rat kidney Na^+,K^+-ATPase (α_1) Feschenco and Sweadner were unable to demonstrate effects of PKA phosphorylation on hydrolytic activity using the mild detergent CHAPS [6]. Yet, in COS cells transfected with wild type α_1 stimulation of PKA caused a small (19%) inhibition of the Na^+,K^+-ATPase activity, whereas $K_{0.5}$ increased [4]. It is still, therefore, an open question if the effects seen by in vivo PKA stimulation are elicited exclusively by the Ser-938 phosphorylation, or if additional phosphorylations by other kinases at alternative sites are also taking place.

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