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Ouabain-insensitive Na^+-ATPase activity is an effector protein for cAMP regulation in basolateral membranes of the proximal tubule

C. Caruso-Neves [a], L.B.A. Rangel [a], D. Vives [a], A. Vieyra [a,b], S. Coka-Guevara [b], A.G. Lopes [a,*]

[a] *Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, CCS, 21949-900 Rio de Janeiro, RJ, Brazil*
[b] *Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, CCS, 21949-900 Rio de Janeiro, RJ, Brazil*

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Abstract

This study describes the modulation of the ouabain-insensitive Na^+-ATPase activity from proximal tubule basolateral membranes by cAMP. An increase in dibutyryl-cAMP (d-cAMP) concentration from 10^{-8} to 5×10^{-5} M stimulates the ouabain-insensitive Na^+-ATPase activity. The ATPase activity increases from 6.0 ± 0.4 to 10.1 ± 0.7 nmol Pi mg^{-1} min^{-1}, in the absence and presence of 5×10^{-6} M d-cAMP, respectively. Similarly, the addition of cholera toxin (CTX), forskolin (FSK) or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) also increases the Na^+-ATPase activity in a dose-dependent manner, with maximal effect at 10^{-8} M, 10^{-6} M and 10^{-7} M, respectively. The effect of 10^{-8} M CTX is not additive to the effect of GTPγS, and is completely abolished by 200 μM guanosine 5′-*O*-(2-thiodiphosphate). The stimulatory effects of CTX and FSK on the Na^+-ATPase activity are accompanied by an increase in cAMP formation by the basolateral membranes of the proximal tubule cells. Furthermore, 10^{-8} M protein kinase A peptide inhibitor (PKAi) completely abolishes the stimulatory effect of 5×10^{-6} M d-cAMP or 10^{-4} M FSK on the Na^+-ATPase activity. Incubation of the basolateral membranes with [γ-^{32}P]ATP in the presence of d-cAMP or FSK increases the global hydroxylamine-resistant phosphorylation and especially promotes an increase in phosphorylation of protein bands of approximately 100 and 200 kDa. This stimulation is not seen when 10^{-8} M PKAi is added simultaneously. Taken together these data suggest that activation of a cAMP/PKA pathway modulates the Na^+-ATPase activity in isolated basolateral membranes of the proximal tubule.

Keywords: Na^+-ATPase; cAMP; Furosemide; Proximal tubule

1. Introduction

Sodium reabsorption in renal proximal tubules is one of the processes involved in the regulation of plasma Na^+ and volume of the extracellular compartment. The reabsorption of Na^+ in proximal tubules involves several transporters, including primary active pumping mechanisms resident in the basolateral membrane [1]. Two sodium pumps are expressed

Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, (*N*-[2-hydroxyethyl]-piperazine-*N′*-[2-ethanesulfonic acid]); Tris, [tris (hydroxymethyl)-aminomethane]; FSK, forskolin; CTX, cholera toxin; d-cAMP, dibutyryl-cAMP; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate)

* Corresponding author. Fax: +55-21-280-8193;
E-mail: agilopes@chagas.biof.ufrj.br

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in the basolateral membrane of the renal proximal tubule cells: the ouabain-sensitive (Na^++K^+)ATPase and the ouabain-insensitive, furosemide-sensitive Na^+-ATPase [2–5]. Although several aspects of the structural and kinetic properties of the ouabain-insensitive Na^+-ATPase remain to be elucidated, it is well known that it transports Na^+ against an electrochemical gradient and that it is not stimulated by K^+ [4,6]. Recently, it was shown that the Na^+-ATPase is a P-ATPase, able to form a phosphorylated intermediate during the catalytic cycle, with a molecular weight of about 100 000, and that phosphorylation is stimulated by furosemide and is insensitive to K^+ [7]. With respect to its physiological role it has been observed that the natriuretic compounds adenosine and bradykinin inhibit the Na^+-ATPase activity, while angiotensin II, an antinatriuretic compound, stimulates it in isolated basolateral membrane of the proximal tubule and cortex homogenate from pig [5,8,9]. In the same conditions, the (Na^++K^+)ATPase activity does not change. These observations suggest that the primary active transport target involved in the regulation of sodium reabsorption in the proximal tubule by natriuretic and antinatriuretic compounds is the ouabain-insensitive Na^+-ATPase.

Since cAMP has been described to mediate some of the actions of several natriuretic and antinatriuretic compounds [1,10–13], we investigated its effects on the Na^+-ATPase activity and on the hydroxylamine-resistant phosphorylation profile of basolateral membranes from proximal tubule cells. The data presented here indicate that activation of a cAMP pathway modulates the ouabain-insensitive Na^+-ATPase.

2. Materials and methods

2.1. Materials

ATP, ouabain, furosemide, azide, mannitol, dibutyryl-cAMP (d-cAMP), protein kinase A peptide inhibitor (PKAi) and cholera toxin (CTX) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Percoll was from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity available. ^{32}Pi was obtained from the Institute of Energetic and Nuclear Research (São Paulo, Brazil). Distilled water deionized with the Milli-Q system of resins (Millipore Corp., Marlborough, MA, USA) was used in the preparation of all solutions. CTX and PKAi were diluted in pure water and 10 mM HCl, respectively. [γ-^{32}P]ATP was prepared as described by Maia et al. [14].

2.2. Preparation of cortex homogenates and purified basolateral membranes

The basolateral membranes were prepared from adult pig kidney by the Percoll gradient method as described elsewhere [5,8,9,15]. Controls for enrichment and contamination with other membranes were carried out as previously described [16]. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 10 mg of protein ml^{-1} and stored at −4°C. The (Na^++K^+)ATPase activity, a marker for basolateral membranes, was 69.2 ± 7.2 nmol Pi mg^{-1} min^{-1}, 8.9 times higher than the activity found in cortex homogenate (7.8 ± 0.3 nmol Pi mg^{-1} min^{-1}). Protein concentration was determined by the Folin phenol method [17] using bovine serum albumin as a standard.

2.3. Measurement of Na^+-ATPase activity

Except where otherwise noted, the standard assay medium (0.2 ml) contained: 10 mM $MgCl_2$, 5 mM [γ-^{32}P]ATP (0.75 μCi μmol^{-1}), 20 mM HEPES–Tris (pH 7.0), 5 mM Na^+-azide, and 6 mM NaCl for the measurement of the Na^+-ATPase activity.

The ATPase activity was measured according to the method described by Grubmeyer and Penefsky [18]. The reaction was started by the addition of purified basolateral membranes to a final protein concentration of 0.3–0.5 mg ml^{-1}. The reaction was stopped after 30 min by the addition of HCl-activated charcoal (0.1 N). The ^{32}Pi released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for 10 min at 3000 rpm in a clinical centrifuge. Spontaneous hydrolysis of [γ-^{32}P]ATP was measured simultaneously in tubes where the membranes were added after the acid. The Na^+-ATPase activity was calculated by the difference between the ^{32}Pi released in the absence and in the presence of 2 mM furosemide, both in the presence of 1 mM ouabain [3,5,6,8,

9,19]. Each experiment was repeated at least six times using different preparations of basolateral membranes. The data were analyzed by two-way analysis of variance (ANOVA), considering the treatments as factors. The significance of the differences was verified by the Bonferroni *t*-test. Statistical analysis was made and the results are expressed using absolute values. When the data are expressed as a percentage of the control values, the statistical test was applied to the absolute values.

2.4. Measurement of hydroxylamine-resistant phosphorylation of basolateral membranes of renal proximal tubules

To measure the incorporation of ^{32}Pi from [γ-^{32}P]ATP into isolated basolateral membranes, we measured the radioactivity bound to an insoluble protein fraction. The reaction was initiated with the addition of the membrane preparation (final concentration of 1.5 mg ml^{-1}) to a reaction medium containing 1 mM [γ-^{32}P]ATP (disodium salt, 16 μCi μmol^{-1}), 10 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0), 6 mM NaCl, 1.1 M hydroxylamine (pH 5.5) and 1 mM EGTA. After 20 min, the reaction was stopped with 1.5 ml of an ice-cold solution (0.25 M perchloric acid, 1 mM ATP and 4 mM sodium phosphate). The mixture was centrifuged for 1 h at 47 000 rpm (rotor 65 Ti) and the pellet was resuspended in 100 μl of Laemmli buffer [20]. An aliquot was used to quantify the radioactivity by liquid scintillation counting and another aliquot was analyzed by SDS–polyacrylamide gel (10%) electrophoresis.

2.5. Electrophoresis analysis

SDS–polyacrylamide gel (10%) electrophoresis was performed as described by Laemmli [20], with the following modifications: (1) the proteins were stained with Coomassie brilliant blue R-250 for 1 h; (2) the gel was destained with methanol 50% (v/v) and acetic acid 10% (v/v). The intensities of the bands in the autoradiographies were quantified by densitometry, using the program Sigma-Gel.

2.6. cAMP determinations

The membranes were preincubated at 37°C for 60 min in the presence of sucrose and test substances, and then mixed with the reaction media containing 5 mM ATP as described above. After 30 min the samples were boiled for 10 min and then centrifuged at $14\,000 \times g$ for 5 min. A radioimmunoassay was used to measure cAMP formation in the supernatant [16,21].

3. Results

3.1. Determination of the PKA activity in the basolateral membrane preparation

PKA activity was assayed in two ways: (1) by measurement of the total hydroxylamine-resistant ^{32}Pi incorporation from [γ-^{32}P]ATP into membranes in the absence or presence of d-cAMP, forskolin (FSK) and PKAi; (2) SDS–PAGE and autoradiography of the phosphorylation profile of the membranes incubated with [γ-^{32}P]ATP in the presence of d-cAMP and PKAi. It was observed that total hydroxylamine-resistant phosphorylation of the membranes increased approximately 50% after addition of 5×10^{-6} M d-cAMP or 10^{-4} M FSK (Fig. 1). Furthermore, both effects were completely reversed by 10^{-8} M PKAi. Fig. 2 shows the phosphorylation

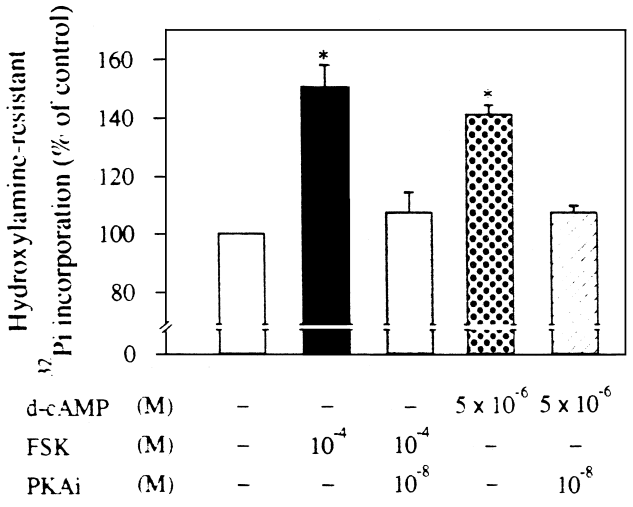

Fig. 1. Hydroxylamine-resistant protein phosphorylation in basolateral membranes of renal proximal tubules. Protein phosphorylation was assayed with or without FSK, d-cAMP and PKAi, in the combinations specified on the abscissa, as described in Section 2 (mean ± S.E.M.). *Statistically significant ($P < 0.05$) when compared to control ($n = 3$).

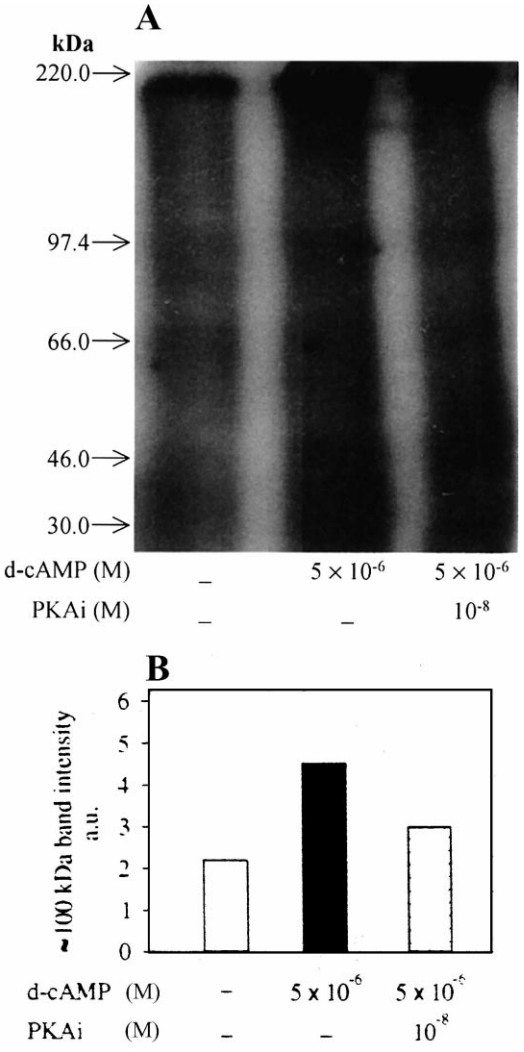

Fig. 2. (A) Analysis of protein phosphorylation profile by autoradiography after SDS–PAGE. Aliquots of phosphorylated proteins from basolateral membranes of renal proximal tubules (50 μg of protein samples incubated in the presence of the compounds shown on the abscissa) were analyzed by SDS–PAGE. The molecular weight markers (myosin, phosphorylase β, bovine serum albumin, ovalbumin and carbonic anhydrase) were electrophoresed in a neighboring lane of the same gel (not shown) and their corresponding positions are indicated on the autoradiogram. (B) Densitometry of the ~100 kDa band in the same conditions ($n=3$).

profile of basolateral membranes following incubation with [γ-^{32}P]ATP and 5×10^{-6} M d-cAMP. The addition of d-cAMP increased the phosphorylation of several proteins with different molecular weights, and this effect was also reversed by 10^{-8} M PKAi (Fig. 2A). Besides phosphorylation of low molecular weight proteins it can be seen that the addition of d-cAMP increases phosphorylation of bands at ~100 and ~200 kDa. Since the phosphorylation signals are not completely defined, the representative band intensity at ~100 kDa was quantified by densitometry (Fig. 2B). This revealed an increase of more than 100% in the phosphorylation of this region in the presence of d-cAMP. Taken together these data indicate that the basolateral membrane preparation contains an activatable PKA activity.

3.2. Increase in cAMP concentration modulates the Na^+-ATPase activity

To verify the effect of cAMP formation on the Na^+-ATPase activity, experiments were performed in the presence of d-cAMP. The results are shown in Fig. 3. As d-cAMP concentration varied from 10^{-10} to 5×10^{-5} M, the ATPase activity increased from 6.0 ± 0.4 to 10.1 ± 0.7 nmol Pi mg^{-1} min^{-1}, with maximal stimulation at 5×10^{-6} M. The half-maximal stimulation was obtained at about 10^{-8} M d-cAMP.

3.3. Activation of adenylyl cyclase modulates the Na^+-ATPase activity

In many systems, an increase in cAMP concentration involves the activation of adenylyl cyclase by stimulatory G protein (G_s). To determine if G_s modulates the Na^+-ATPase activity, experiments were

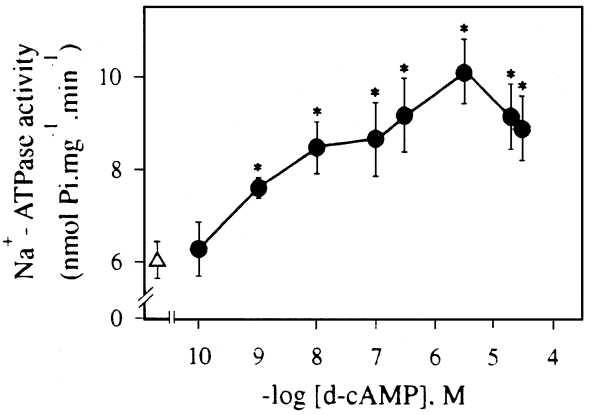

Fig. 3. Dependence of Na^+-ATPase activity on d-cAMP concentration. ATPase activity was measured as described in Section 2 (mean ± S.E.M.). Open symbol is the control value with no additions. *Statistically significant ($P<0.05$) when compared to control ($n=6$).

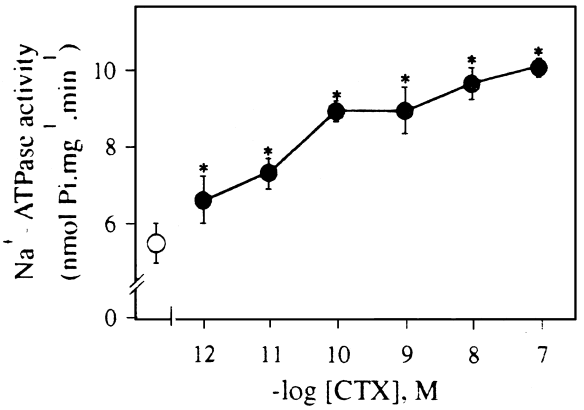

Fig. 4. Dependence of Na^+-ATPase activity on CTX concentration. ATPase activity was measured in the CTX concentrations shown on the abscissa. The data show the mean ± S.E.M. Open symbol is the control value with no toxin. *Statistically significant ($P < 0.05$) when compared to control ($n = 7$).

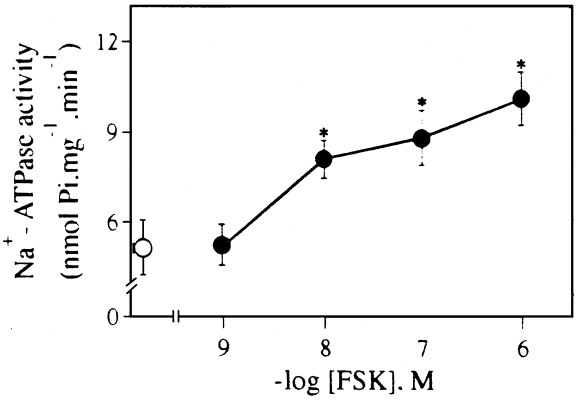

Fig. 6. Dependence of Na^+-ATPase activity on FSK concentration. ATPase activity was measured in the FSK concentrations shown on the abscissa. Open symbol is the control value. *Statistically significant ($P < 0.05$) when compared to control. The data show mean ± S.E.M. ($n = 6$).

performed in the presence of CTX, an activator of G_s. The results are summarized in Fig. 4. The rise in concentration of CTX from 10^{-12} to 10^{-7} M increased the Na^+-ATPase activity, with maximal effect at 10^{-8} M. The enzyme activity increased from 5.5 ± 0.1 in the absence to 9.6 ± 0.4 nmol Pi mg^{-1} min^{-1} in the presence of 10^{-8} M CTX. To confirm the involvement of G proteins, non-hydrolyzable guanine nucleotide analogues were tested: guanosine 5′-*O*-(3-thiotriphosphate (GTPγS), an activator of G protein, and guanosine 5′-*O*-(2-thiodiphosphate (GDPβS), an inhibitor (Fig. 5). The addition of 10^{-7} M GTPγS, a nucleotide that activates the Na^+-ATPase in a dose-dependent manner [9], increased the enzyme activity from 6.0 ± 1.2 to 11.7 ± 1.1 nmol Pi mg^{-1} min^{-1}, but he simultaneous addition of 10^{-8} M CTX had no additional effect. On the other hand, the addition of 2×10^{-4} M GDPβS reversed the activation of Na^+-ATPase activity promoted by 10^{-8} M CTX (see Fig. 4), but 2×10^{-4} M GDPβS alone did not alter the control levels of the enzyme activity.

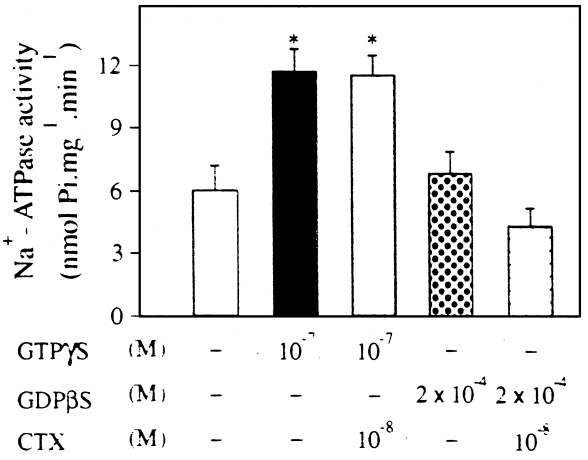

Fig. 5. Activation of the Na^+-ATPase activity via G protein ligands. GTPγS, GDPβS and CTX were added in the combinations shown. *Statistically significant ($P < 0.05$) when compared to control. The data show mean ± S.E.M. ($n = 6$).

Fig. 6 shows the effect of FSK, an activator of adenylyl cyclase, in Na^+-ATPase activity. An increase in FSK concentration from 10^{-9} to 10^{-6} M raised the ATPase activity from 5.2 ± 0.9 in the absence to 10.1 ± 0.9 nmol Pi mg^{-1} min^{-1} in the presence of 10^{-6} M.

3.4. Correlation between increase of endogenous cAMP and Na^+-ATPase activity

It is generally accepted that the CTX-sensitive G proteins are, in most cases, coupled to the adenylyl cyclase present in the cell membrane [22,23], and that the basolateral membranes of epithelial cells are rich in adenylyl cyclase [24]. The levels of endogenous cAMP in the presence of either 10^{-8} M CTX or 10^{-7} M FSK were correlated with Na^+-ATPase activity. Table 1 shows that both drugs stimulated cAMP formation, in parallel with an increase in

Table 1
Na^+-ATPase activity and cAMP formation

Addition	Na^+-ATPase activity[a] (nmol Pi mg^{-1} min^{-1})	cAMP formation[b] (pmol mg^{-1})
Control	5.42 ± 0.6	1167
10^{-8} M CTX	9.48 ± 0.4	3280
10^{-7} M FSK	9.91 ± 1.5	7360
10^{-8} M CTX+10^{-7} M FSK	8.91 ± 1.5	8355

Basolateral membranes were assayed for Na^+-ATPase activity and cAMP formation in the reaction media described in Section 2 with the additions specified in column 1.
[a]Mean ± S.E.M. of at least six determinations performed in duplicate, using different membrane preparations.
[b]Taken from Coka-Guevara et al. [16]. Mean of two determinations performed in duplicate, using different membrane preparations and cAMP assay kits. In every case the two measurements agreed within 10%.

Na^+-ATPase activity. The simultaneous addition of 10^{-8} M CTX and 10^{-7} M FSK did not have an additive effect on either the cAMP level or the Na^+-ATPase activity (Table 1).

3.5. *PKA is involved in the stimulation of Na^+-ATPase by d-cAMP*

In order to determine the involvement of PKA in the modulation of Na^+-ATPase activity by cAMP, experiments were performed in the presence of PKAi. In Fig. 7 it can be observed that the simultaneous addition of 10^{-8} M PKAi with 5×10^{-6} M d-cAMP or with 10^{-4} M FSK completely abolished the stimulation of Na^+-ATPase activity by d-cAMP or FSK, whereas the addition of PKAi alone did not change the enzyme activity.

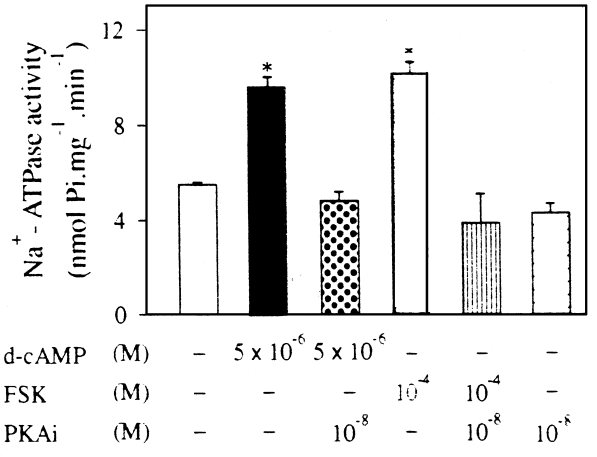

Fig. 7. Modulation of the Na^+-ATPase activity by PKA ligands. d-cAMP 5×10^{-6} M, PKAi 10^{-8} M and FSK 10^{-6} M were added in the combinations shown. The data show mean ± S.E.M. ($n=6$). *Statistically significant ($P<0.05$) when compared to control.

4. Discussion

In the present work, we studied the regulation of the Na^+-ATPase activity of the basolateral membranes from proximal tubules of pig kidney by cAMP. It was observed that activation of the cAMP/PKA pathway stimulates the Na^+-ATPase activity in the isolated basolateral membranes. The concentration of cAMP that modulates the enzyme activity is similar to that described for a number of known effects of cAMP [25].

Several transporters in proximal tubules have been described as being modulated by cAMP. However, the activities of both sodium pumps located in the basolateral membrane, (Na^++K^+)ATPase and Na^+-ATPase, are the limiting step for sodium reabsorption. The possibility that the effect of activating the cAMP/PKA pathway on ATP hydrolysis could be due to an effect on the (Na^++K^+)ATPase can be ruled out since the experiments were performed in the absence of K^+ and in the presence of 1 mM ouabain, a specific inhibitor of (Na^++K^+)ATPase [26].

In the present paper, it was observed that: (1) CTX stimulates the Na^+-ATPase activity in a dose-dependent manner, similarly to the effect obtained with d-cAMP; (2) the effects of d-cAMP are mimicked by GTPγS, an activator of G proteins, and reversed by GDPβS, an inhibitor of G proteins; (3) like d-cAMP itself, FSK, an activator of adenylyl cyclase, increases the Na^+-ATPase activity; (4)

FSK and CTX stimulate the Na^+-ATPase activity and also cause an increase in the endogenous level of cAMP; and (5) PKAi completely abolishes the effect of d-cAMP.

As shown in Table 1, 10^{-8} M CTX or 10^{-7} M FSK increased the Na^+-ATPase activity to the same magnitude, while the cAMP level was increased much more by FSK. However, in Fig. 1 it can be observed that the effect of d-cAMP on the Na^+-ATPase activity is maximal at 5×10^{-6} M, which rules out the possibility that the effects of CTX and FSK on the enzyme activity occur through different pathways. Taken together, these data suggest that the Na^+-ATPase of proximal tubules is modulated by the cAMP/PKA pathway and that G proteins participate in an early step of this signaling network.

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