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Regulation of Endothelial Na^+-K^+-ATPase Activity by cAMP

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Using an *in vitro* cell system and Cs^+ NMR techniques we were able to show that porcine aortic endothelial cells (PAEC) reduce their Na^+-K^+-ATPase activity upon an increase in intracellular cAMP. Reduction in the pump rate was due to phosphorylation of the α-subunit of the ATPase as shown by immunoprecipitation. Apart from a pump inhibiton using 8-Br-cAMP and IBMX, we were also able to show that changes in the Na^+-K^+-ATPase activity could be mediated by the adenosine-A_2 and prostaglandin receptor agonists 5′-N-Ethylcarboxamidoadenosine and Iloprost, respectively. Parallel to a decrease in pump activity we also observed a decrease in intracellular Cs^+, indicating opening of K^+ channels. © 1998 Academic Press

The vascular endothelium plays a crucial role in the maintainance of blood pressure and flow. Recent evidence indicates that signals generated within the endothelium are important in the regulation of the endothelial barrier (1). Changes in the permeability of the barrier are correlated to changes in cell shape and/or volume (2). From studies on other cell types it is known that changes in the cellular cAMP concentration also results in a change of cell volume. Both cell swelling (3) and shrinking (4) have been observed due to an increase in cAMP.

An increase in intracellular cAMP concentration has, in different cell types, also been reported to result in an inhibition of Na^+-K^+-ATPase activity (5-8). Several studies on membrane preparates have shown that cAMP-dependent pump inhibition was a result of phosphorylation of the Na^+-K^+-ATPase α-subunit through protein kinase A (PKA) (7,9). Other reports in the literature indicate that the Na^+-K^+-pump is also involved, directly or indirectly, in vascular contractility and blood pressure control (10). We were interested if changes in intracellular cAMP in endothelial cells would also result in a change of Na^+-K^+-ATPase activity. For this reason we decided to study the effects of cAMP on the Na^+-K^+-pump in an *in vitro* system of PAEC grown on microcarrier beads using $^{133}Cs^+$ magnetic resonance.

Measuring Na^+-K^+-ATPase activity using $^{39}K^+$ fluxes in cellular systems is hampered by the very low sensitivity of this nucleus. Recently $^{133}Cs^+$ NMR has been used to study K^+ fluxes in biological tissue and shown to be 100% visible (11-13). Cs^+ NMR allows one to do multiple experiments, without the use of an exogenous shift reagent, on the same cell sample by simply washing-out the accumulated Cs^+ after determination of the rate of Cs^+ accumulation.

MATERIALS AND METHODS

Materials. 3-Isobutyl-1-methylxanthine (IBMX), 8-Br-cAMP, Protein A-sepharose and anti-mouse IgG antibody were obtained from Sigma, Deisenhofen, Germany. 5′-N-Ethyl-carboxamidoadenosine (NECA) was purchased from RBI, Natick MA, USA, and Immobilon-P from Millipore, Bedford, USA. Iloprost was a gift from Schering A. G., Berlin, Germany. Polyclonal rat Na^+-K^+-ATPase α-subunit, rabbit antibody was purchased from Paesel and Lorei, Hanau, Germany and monoclonal mouse antibody, specific for the α-subunit of the Na^+-K^+-ATPase, from Biomol, Hamburg, Germany. All other chemicals were obtained from Merck, Darmstadt, Germany, and were of analytical grade.

NMR experiments. PAEC were grown to confluency on microcarrier beads and perfused at 37 ± 0.3°C in a 10 mm NMR tube. Details of the cell culture have been described in a previous publication (14). All $^{133}Cs^+$ NMR experiments were performed at 52.48 MHz on a Bruker AMX 400 broadband variable temperature probe with 22.5 μs $\frac{\pi}{2}$ pulse lengths. For the $^{133}Cs^+$ NMR experiments KCl of the perfusion medium was replaced with CsCl. After switching to Cs^+ medium, 4k data points were accumulated every 10 min. in 64 scans. The Cs^+ accumulation was fitted to:

$$C_i(t) = C_i^{\infty} \cdot [1 - exp(-k \cdot t)], \quad (1)$$

in order to obtain the Na^+-K^+-ATPase activity. C_i represent the intracellular Cs^+ concentration and k the passive in- and efflux rate con-

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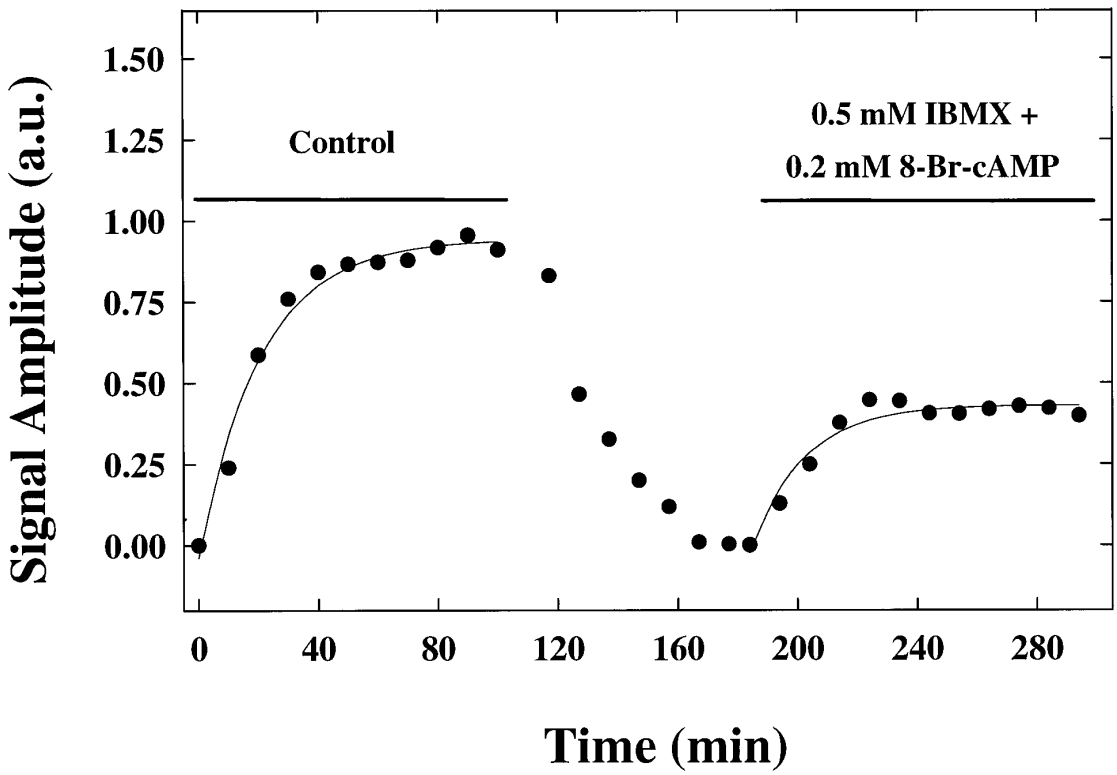

FIG. 1. Typical Cs^+ NMR data set for the determination of the Na^+-K^+-pump rate during control (first 100 min.) and pump inhibition using 0.5 mM IBMX + 0.2 mM 8-Br-cAMP (last 110 min). In the figure the intracellular Cs^+ signal amplitude is plotted versus time. These data were fitted to a three parameter fit to obtain the pump rate (see Methods).

stant which we assume to be equal. C_i^∞ represents the equilibrium Cs_i^+ concentration after Cs^+ loading. It should be noted that C_i^∞ is defined as (12,15),

$$C_i^\infty = \frac{V_{Cs} + k \cdot C_e}{k}, \qquad (2)$$

with C_e the time independent extracellular Cs^+ concentration and V_{Cs} the rate of active Cs^+ uptake. The Na^+-K^+-ATPase activity was defined as:

$$\frac{d}{dt} C_i(t)|_{t=0} = C_i^\infty \cdot k. \qquad (3)$$

Combining eqqns. 2 and 3 one obtains:

$$V_{Cs} = k \cdot C_i^\infty = \frac{d}{dt} C_i(t)|_{t=0}, \qquad (4)$$

when $C_i^\infty \gg [Cs^+]_e$. This is always the case under control conditions. The experimentally obtained Cs^+ accumulation curves were fitted to $C_i(t)$ + *constant*, with $C_i(t)$ defined as in eqn. 1. The constant was used to check for any baseline off-set. From the fit one obtains the parameters C_i^∞ and k, the product of these parameters determines the pump rate.

The experimental protocol was as follows. First the cells were perfused with Cs^+-medium to study Cs^+-uptake, followed by Cs^+-washout using a K^+ containing medium. After the washout the same cells were again perfused with Cs^+-medium, however, this time containing one of the substances supposed to increase intracellular cAMP. ^{23}Na NMR experiments were performed using $Dy(P_3O_{10})_2^{7-}$ as a shift reagent. Details have been described previously (14). Data points were collected every 10 min. as for Cs^+.

Immunoprecipitation and intact cell phosphorylation. Phosphorylation of the Na^+-K^+-ATPase α-subunit of PAEC was carried out as described (16). Composition of the phosphate-free buffer was (in mM); (140) NaCl, (3.8) KCl, (1.2) $MgSO_4$, (1) $CaCl_2$, (20) Hepes, pH 7.4 and 5% (v/v) Fetal Calf Serum (FCS), dialyzed for 2 days against phospate-free buffer without FCS (17). This medium was used to incubate each confluent cell monolayer grown in 35-mm dishes with $^{32}PO_4^{3-}$ (200 μCi/dish) for 2 hr. prior to treatment with IBMX, IBMX+8-Br-cAMP, NECA or Iloprost. The cell monolayer was washed three times with iced phosphate-free buffer. After adding 500 μL of precipitation buffer (in mM); (50) Tris-HCL, (5) EDTA, (150) NaCl, 1% (w/v) Nonidet P-40, pH 8.3, the cells were harvested and homogenized with 20 strokes in a glass-on-glass homogenizer. The suspension was incubated on ice for 20 min. and centrifuged 30 min. at 50.000 *g*. Amounts of supernatant protein were equalized based on the method of Lowry (18). Homogenates were cleared with 10 mg/sample Protein A-sepharose for 60 min. at 4°C. The cleared supernatant was incubated overnight with the polyclonal α-subunit of rat Na^+-K^+-ATPase rabbit antibody (1 μg/sample). Immune complexes were precipitated with 10 mg Protein A-sepharose. The pellet was resuspended, vortexed and washed four times with buffer (in mM); (50) Tris-HCl, (1) EDTA, (150) NaCl, 0.5% (w/v) Nonidet P-40, pH 8.3, at 4°C. After the final wash, the pellet was suspended in SDS sample buffer; 50 mM Tris/HCl, 10% (w/v) Glycerol, 2% (w/v) SDS, 10% (v/v) Mercaptoethanol, 0.01% (w/v) Bromophenolblue, pH 6.8, boiled for 5 min., and cleared by low speed centrifugation. The proteins were separated for 2 hr on 7.5% SDS-PAGE and tranferred

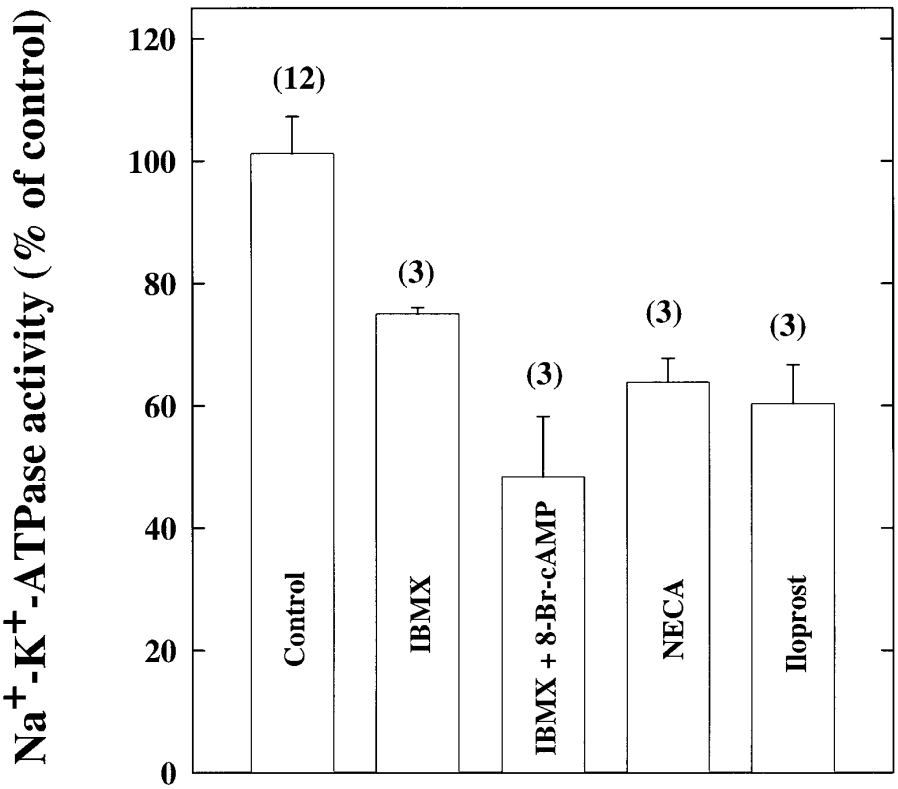

FIG. 2. The effect of 0.5 mM IBMX, 0.5 mM IBMX + 0.2 mM 8-Br-cAMP, 10 μM NECA and 10 μM Iloprost on the observed Na^+-K^+-pump rate. All substances caused a reduction in Na^+-K^+-ATPase activity. The numbers in brakets refer to the number of experiments on different cell samples.

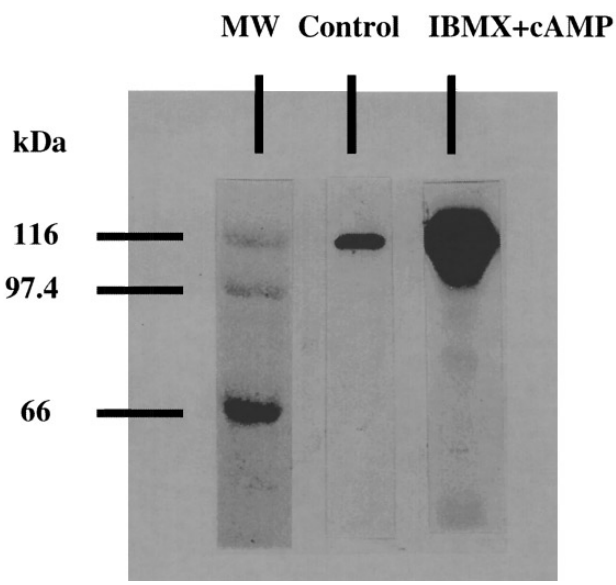

FIG. 3. Molecular Weight protein standard (MW) and auto-radiogramms (control and 0.5 mM IBMX+0.2 mM 8-Br-cAMP) from immunoprecipitation of the phosphorylated Na^+-K^+-ATPase α-subunit with ^{32}P in intact PAEC. Similar results were obtained in three separate experiments.

to an Immobilon-P membrane. with a semi-dry blotting system (2.5 mA/cm^2) (19). Blots were analyzed with autoradiography, washed with PBS containing 5% (w/v) low-fat milk for 45 min., PBS for 10 min., and incubated with the antibodies in PBS containing 1% low-fat milk for 2 hr. The used monoclonal antibody is specific for the Na^+-K^+-ATPase α-subunit and the blot was incubated for 1 hr, using a 1:3000 dilution. The primary antibody was washed for 45 min. with PBS/low-fat milk, and the secondary reaction was performed with alkaline phosphatase conjugated anti-mouse IgG antibody (dilution 1:30.000).

Statistics. Data in this study are presented as mean values ± standard deviation. The number data averaged is indicated by (*n*). To compare data obtained under different conditions a Student's *t*-test was used (SPSS for Windows, SPSS Inc. Illinois USA). $P<0.05$ was taken to indicate a significant difference.

RESULTS AND DISCUSSION

From a Cs^+ loading experiment the Na^+-K^+-ATPase activity could be determined under control conditions as shown in figure 1, representing a typical experiment. The pump activity obtained from the fit represents the slope of the Cs^+ accumulation curve at time zero. After Cs^+ loading, the cells were washed with a K^+ containing control medium. Following the washout, shown in figure 1, the cells were perfused with Cs^+-medium containing 0.5 mM IBMX + 0.2 mM 8-Br-cAMP. 8-Br-cAMP is a membrane permeable cAMP derivative. In

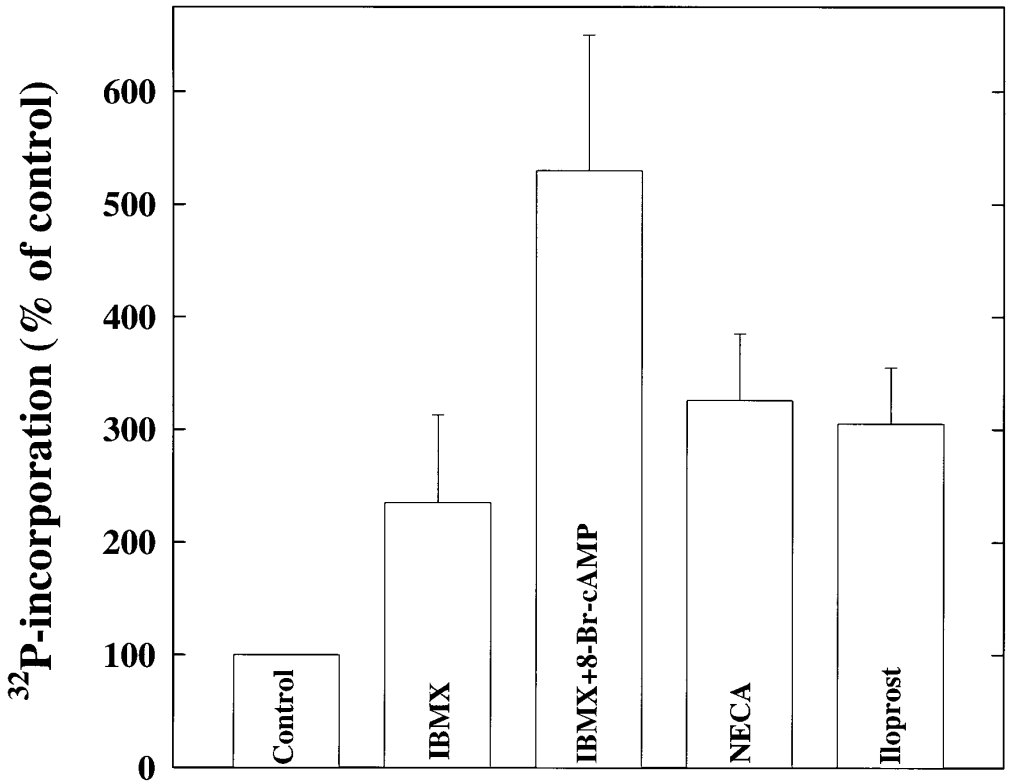

FIG. 4. The effect of 0.5 mM IBMX + 0.2 mM 8-Br-cAMP, 10 μM NECA, 10 μM Iloprost and 0.5 mM IBMX on the observed ^{32}P incorporation into the α-subunit of the pump (n=5).

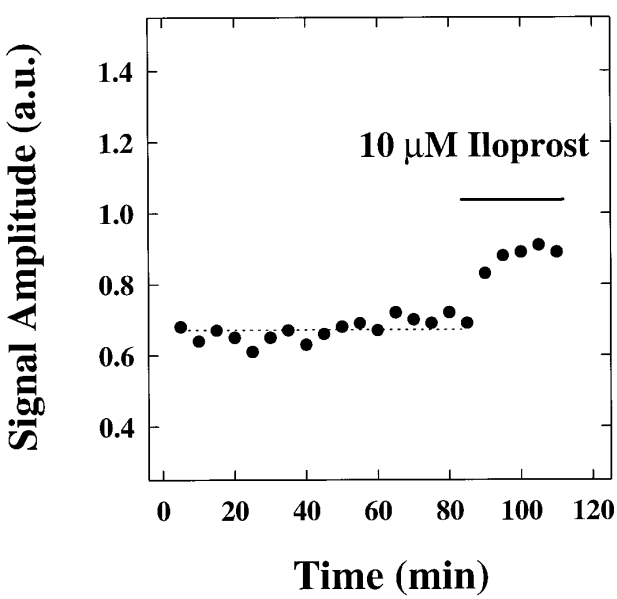

FIG. 5. Typical ^{23}Na NMR experiment with 10 μM Iloprost. During the 30 min. perfusion with Iloprost the amount of intracellular odium did show a small increase. The dashed line represents the average ^{23}Na level in PAEC during control.

this this figure one clearly observes a change in the Cs^+ accumulation rate. The fit to the data points showed a reduction of the pump activity to 48±10 % (n=3) (mean±S.D.) compared to control. Using 0.5 mM IBMX, an inhibitor of phosphodiesterase, we also observed an effect on the pump activity. The rate decreased compared to control to 75±1 % (n=3). This effect was significantly potentiated by 8-Br-cAMP (p=0.04), resulting in a pump inhibition of up to 50 %. Apparently an additional increase in the cellular cAMP concentration resulted in a larger inhibition of the Na^+-K^+-ATPase.

The intracellular cAMP concentration could also be increased through stimulation of adenylate cyclase (AC) activity. As AC stimulants we tested NECA, an adenosine-A_2 receptor agonist and Iloprost, a prostaglandin receptor agonist. Both NECA and Iloprost were applied at a concentration of 10 μM and resulted in a decreased pump activity of about 40 % as compared to control. For the NECA and Iloprost experiments we obtained a pump activity of 64±4 (n=3) % and 60±6 % (n=3), respectively. These data are summarized in figure 2. Our data indicates that short term regulation of endothelial Na^+-K^+-ATPase is coupled to an increase in intracellular cAMP. Such an increase in cAMP does not necessarily result in phosphorylation of the Na^+-K^+-ATPase α-subunit through PKA (20). Finally im-

muno blotting experiments were performed to determine whether a decreased pump activity was caused by a phosphorylation of the Na^+-K^+-pump.

In the presence of 8-Br-cAMP the α-subunit of the Na^+-K^+-ATPase was phosphorylated as shown by intact-cell phosphorylation experiments in figure 3. A significant increase in ^{32}P-incorporation (figure 4, n=5), indicating the involvement of AC in the regulation of Na^+-K^+-ATPase activity. Figure 4 shows the increase in ^{32}P-incorporation in the α-subunit in the presence of IBMX+8-Br-cAMP (530±120%), NECA (326±59%) and Iloprost (305±50%) as compared to control (100%).

An increase in cAMP also resulted in a decrease in the final amount of intracellular Cs^+ (see figure 1). This decrease indicates that the endothelial cells release some of their K^+/Cs^+ through K^+ channels, resulting in a hyperpolarization of the membrane. As such a release possibly could affect the cell's osmolarity, changes in volume and/or cell sodium content can be expected. ^{23}Na NMR experiments showed that during perfusion with Iloprost a small change in the total amount of endothelial ^{23}Na occured (figure 5). Similar results were obtained with 0.5 mM IBMX + 0.2 mM 8-Br-cAMP and NECA. Measurement of PAEC cytosolic volume did not show a significant decrease in volume. As a result the measured decrease in pump activity is due to pump phosphorylation and does not involve a major effect from a change in cell volume. Experimental evidence on other cell types suggests that protein phosphorylation/dephosphorylation by protein kinases is involved in volume regulation (21,22), however, PAEC do did not display this type of regulation in our experiments.

Recently Borin (5) also reported that cAMP evokes a rise in the sodium concentration of rat aortic smooth muscle cells parallel to a decrease in Na^+-K^+-pump activity. Applying Iloprost to dog artery smooth muscle cells, Siegel *et al.* (23) showed that this resulted in the opening of K^+ channels. The opening of K^+ channels resulted, in turn, in a hyperpolarization of the cell membrane and a rapid decrease in cellular K^+. These observations all fit into our explanation for the effect of cAMP on endothelial cells. Regarding the time scale of the observed processes it should be noted that for shark rectal gland the Na^+-K^+-ATPase activity dropped to about 70% of control within 5 minutes after the initial increase in cAMP (9).

In summary, our Cs^+ NMR and immunoprecipitation results indicate that PAEC undergo a rapid, short term decrease of their Na^+-K^+-ATPase activity upon an increase in intracellular cAMP. Parallel to this short term pump regulation endothelial cells were observed to expell some of their intracellular K^+.

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