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Inhibition of Na,K-ATPase activity by cGMP is isoform-specific in brain endothelial cells

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Abstract cGMP has been shown to either activate or inhibit Na,K-ATPase activity. Using mouse brain endothelial cells which express both ouabain-resistant α1 and ouabain-sensitive α2 and α3 isoforms, we show that cGMP reduces total Na,K-ATPase activity to about 58%. The inhibition is prevented by the protein kinase G (PKG)-specific inhibitor KT5823, indicating that cGMP-mediated activation of PKG leads to inhibition of the pump. A similar extent of inhibition is obtained with nitric oxide. cGMP-induced inhibition acts mainly on α1 isoforms but hardly affects α2/α3 isoforms. These data suggest that inhibition of Na,K-ATPase activity by cGMP occurs in an isoform-selective manner in brain endothelial cells.

Key words: Na,K-ATPase; Regulation; cGMP/protein kinase G; Endothelial cell

1. Introduction

The Na,K-ATPase is responsible for maintaining the electrochemical gradient of sodium and potassium ions and plays an important role in the regulation of the ionic homeostasis in tissues and cells [1]. The enzyme is composed of a catalytic α subunit and a β subunit glycoprotein which is essential for enzyme maturation [2]. For each subunit three isoforms, encoded by individual genes, have been discovered. This multiplicity allows the formation of, theoretically, nine different isoenzymes.

All α and β subunit isoforms exhibit a complex tissue-specific expression pattern [3]. The temporal and spatial expression in brain even demonstrates that multiple α and β isoforms are simultaneously expressed in the same cell type [4–6], suggesting the concurrent presence of several sodium pump isoenzymes. For example, brain microvessels and ependymal cells of the choroid plexus express (at least) five isoforms [7]. Endothelial cells of microvessels and ependymal cells form the barriers between the brain and the blood and the cerebrospinal fluid, respectively. These structures are implicated in the tight control of nutrient and ion fluxes from and into the brain parenchyma [8] and therefore depend on high Na,K-ATPase activity. Pump activity in microvessels was first localised at the abluminal [9] and then at both the luminal and abluminal side [10]. Interestingly, in this latter study low affinity ouabain binding sites, characteristic for the α1 isoform were assigned to the luminal membrane fraction and high affinity binding sites, defining α2 and α3 isoforms, to the abluminal one. This result provides evidence for an asymmetric distribution of Na,K-ATPase α subunit isoforms in blood vessels.

The regulation of Na,K-ATPase activity by exogenous factors has been widely studied. It is mediated e.g. by hormones, salts and neurotransmitters [11]. Intracellularly, many of these signals are likely to modulate phosphorylation of the α subunit by protein kinases A and C and protein phosphatases [12–14]. A direct linkage between neurotransmitter modulation and α subunit phosphorylation was demonstrated e.g. in the choroid plexus: serotonin reduced pump activity and increased the amount of the phosphorylated α subunit [15]. Studies on regulation of Na,K-ATPase activity by protein kinase G (PKG) provided evidence that cGMP inhibited the pump in kidney [16,17] and activated it in Purkinje neurons [18]. Since kidney predominantly expresses the α1 isoform gene, whereas in Purkinje neurons α3 is mainly found, a tissue- and/or isoform-specific response to cGMP/PKG activation might underlie these opposite responses. To address this subject, we have chosen mouse brain endothelial cells which simultaneously express α1, α2 and α3 isoforms and investigated whether modulation of Na,K-ATPase activity could occur in an isoform-specific manner in endothelial cells.

2. Materials and methods

2.1. RNA isolation and reverse transcription

For preparation of total RNA, bEnd4 cells were grown to confluence in a 75-cm^2 culture flask. RNA was isolated by the GTC method [19]. After dissolution in water, RNA was reextracted with phenol/chloroform and reprecipitated with sodium acetate and ethanol. The concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm and the quality was controlled by gel electrophoresis. Five μg total RNA were used for oligodT-primed single-stranded cDNA synthesis with the superscript preamplification system (Gibco-BRL). After RNase treatment, the cDNA was stored at −20°C in aliquots without further treatment.

2.2. PCR amplification of Na,K-ATPase subunit isoforms

PCR primers were deduced from mouse (β1 and β2) or rat (α1, α2, α3, β3) sequences. For each primer pair the optimal amplification conditions were determined by using the cloned subunit-specific cDNA as template. The optimisation of the β3 reaction was performed with single stranded cDNA derived from rat C6 glioma cells. The isoform-specific primers did not crosshybridise with the cDNA templates coding for the other isoforms and their amplification efficiencies were comparable. No signals were obtained when omitting the reverse transcription. The final PCR conditions were: 0.5 μl cDNA reaction mix, 30 s denaturation at 94°C, annealing for 1 min at the optimal temperature (between 57 and 60°C), extension at 72°C for 60–90 s. The cycles were repeated 40 times, which allowed to detect a weak signal for α3. For all reactions 1.5 mM $MgCl_2$ was the optimal concentration. The primer concentrations were 10 μM

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Abbreviations: 8-Br-cGMP, 8-bromo-cyclic GMP; DARPP-32, dopamine-cAMP-regulated phosphoprotein; HBS, HEPES-buffered saline; NO, nitric oxide; PKG, protein kinase G

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each. The reactions were initiated with a hot-start by addition of 0.2 μl Taq-Polymerase (Promega, 5 U/μl).

2.3. Determination of Na,K-ATPase activity

bEnd4 cells, an SV40-transformed mouse brain capillary endothelioma cell line [20], were grown in DMEM/10% FCS and split with 0.02% trypsin/1 mM EDTA twice a week. For determination of Na,K-ATPase activity, 0.3×10^5 cells were seeded per well of a 24-well plate and incubated for 16 h to reach about 60% confluence. Then the cells were washed twice with HEPES-buffered saline (HBS) (50 mM HEPES, pH 7.4, 127 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$) and equilibrated in 200 μl of HBS for 15 min at 37°C. Then the buffer was replaced with 200 μl of HBS containing the additives 4 mM 8-bromo-cyclic GMP (8-Br-cGMP), 3 mM nitroso-glutathione, or 2 μM KT5823, as indicated in the figure legends. Maximal effects of 8-Br-cGMP and nitroso-glutathione were obtained with these concentrations. Cells were incubated for 15 min at 37°C and then rapidly washed three times with buffer without additives. Next the cells were equilibrated for two min in buffer containing ouabain (15 mM or 10 μM, respectively, to distinguish between low and high affinity binding sites), followed by the addition of 1.5 μCi/ml [^{86}Rb]-chloride (Amersham, sp. act. 100 Ci/mmol) and incubation for 15 min. Uptake of Rb^+ was linear under these conditions for at least 40 min. The reaction was stopped by aspiration of the buffer and four wash steps with ice-cold 300 mM sucrose solution. The cells were lysed with 200 μl 10 mM NaOH, 0.1% SDS and transferred into an Eppendorf tube. Radioactivity was determined by Cerenkov counting and protein concentration determined according to Bradford.

3. Results

3.1. Expression of Na,K-ATPase isoforms in brain endothelial cells

Brain endothelial cells forming microcapillaries have both high and low affinity ouabain binding sites [10] and express at least five different isoforms of the sodium pump [7]. To investigate the expression pattern of Na,K-ATPase isoforms in bEnd4 cells, a mouse cell line derived form microcapillaries, we performed RT-PCR analysis, using primers specific for all known isoforms. Fig. 1 shows the results of the RT-PCR analysis in bEnd4 cells. We found highest expression for α1, β1 and β3, moderate expression of β2, somewhat lower expression of α2 and a hardly detectable signal for α3. Provided all proteins are translated from these transcripts, bEnd4 cells have the potential to generate at least six (eventually nine) different isoenzymes.

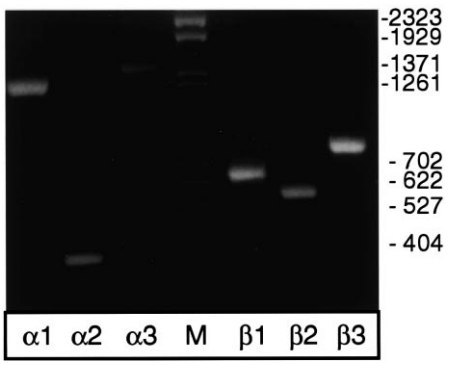

Fig. 1. Expression of Na,K-ATPase α and β subunit isoform genes in bEnd4 cells. The PCR products were resolved on a 1% agarose gel and visualised with ethidium bromide. The lengths of the products (in bp) are: α1, 1305; α2, 364; α3, 1441; β1, 619; β2, 569; β3, 792. Size markers (in bp) are indicated at the right margin. M = marker lane.

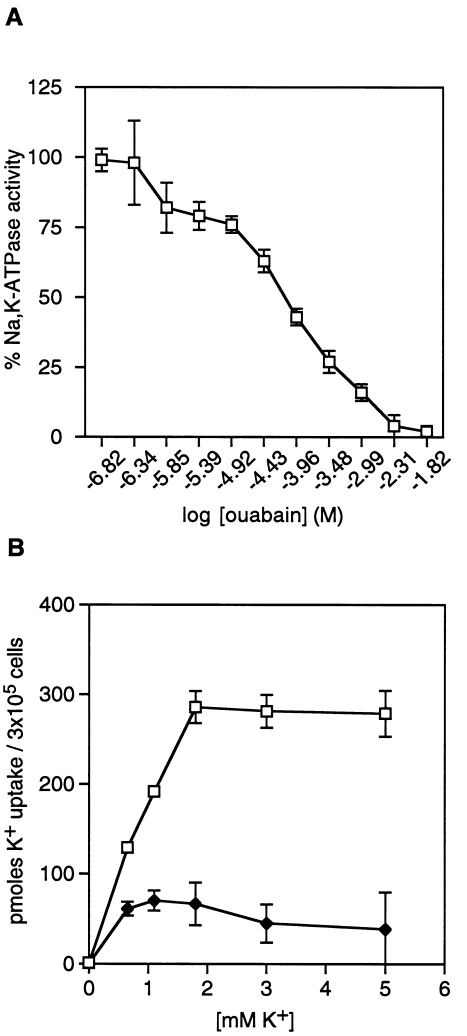

Fig. 2. Ouabain concentration-dependent Na,K-ATPase activity in bEnd4 cells. A: Ouabain titration experiment. bEnd4 cells in 24-well plates were preincubated with increasing concentrations of ouabain for 2 min. $^{86}Rb^+$ uptake was measured for 15 min in the presence of the inhibitor. Na,K-ATPase activity is expressed in % of the total ouabain-inhibitable ATPase activity. The results are the mean ± S.D. of a quadruplicate determination of one experiment. B: K^+ activation of ouabain-sensitive Na,K-ATPase activity. bEnd4 cells were washed with K^+-free HBS and then incubated in buffer containing increasing amounts of K^+. Na,K-ATPase activity was determined in the presence of either 15 mM to inhibit both low and high affinity α subunit isoforms, or 10 μM ouabain to inhibit the high affinity α2 and α3 isoforms. The fraction of the low affinity α1 isoform was calculated by subtracting the high affinity activity from total Na,K-ATPase activity. The activity is given in %. Data represent the mean ± S.D. of a quadruplicate determination of one experiment repeated three times. Open symbols: α1 activity; closed symbols: α2/3 activity.

3.2. Ouabain sensitivity of brain endothelial cells

The weak expression of the α2 and α3 isoforms made it necessary to determine whether these isoforms significantly contributed to the total Na,K-ATPase activity in bEnd4 cells. Since rodent α1 subunits have a several orders of magnitude lower ouabain affinity than α2 and α3 subunits, it is possible to distinguish between these two classes by selectively inhibiting high affinity pump activity only. We, therefore, performed an ouabain titration experiment as shown in Fig. 1A. The

biphasic shape of the curve indicates that two affinity populations are indeed present in bEnd4 cells. About 75–80% of the activity can be assigned to the low affinity α1 subunit-containing enzymes with a K_{i50} of 1.1×10^{-4} M. The high affinity fraction accounts for the remaining 20–25% of ouabain inhibitable Na,K-ATPase activity with an apparent K_{i50} of 3×10^{-7} M. Thus, at about 10 μM ouabain concentration α2/3-containing isoenzymes should be completely inactivated whereas isoenzymes with α1 still retain nearly full activity. The assumption was reinforced by similar experiments done with human and porcine endothelial cells which have only high affinity ouabain binding sites: 10 μM ouabain completely inhibited Na,K-ATPase activity (data not shown). To demonstrate the fractional proportion of isoform-specific pump activities under such conditions by another approach, we performed a K^+ activation experiment. bEnd4 cells were incubated either in the presence of increasing concentrations of extracellular K^+. High and low affinity ouabain-sensitive Na,K-ATPase activity was determined with 10 μM or 15 mM ouabain, respectively (Fig. 1B). In agreement with the ouabain titration, isoenzymes consisting of the α1 isoform account for about 80% of total pump activity.

3.3. Regulation of Na,K-ATPase activity by cGMP

To study the influence of cGMP on Na,K-ATPase activity in bEnd4 cells, the membrane-permeable cGMP analogue 8-Br-cGMP was used. Maximal inhibition of total ouabain-sensitive Na,K-ATPase activity to 58% of the control was obtained with 4 mM 8-Br-cGMP after 20 min of incubation at 37°C (Fig. 2). When we added the PKG-specific inhibitor KT5823 at 2 μM concentration to the cells prior to incubation with 8-Br-cGMP, the inhibitory effect of cGMP was antagonised. Incubation with KT5823 alone had no effect on pump activity. At the used concentration, this inhibitor has a more than 8-fold selectivity for inhibition of PKG compared with protein kinase A, indicating that the cGMP-induced inhibition is mediated through activation of PKG.

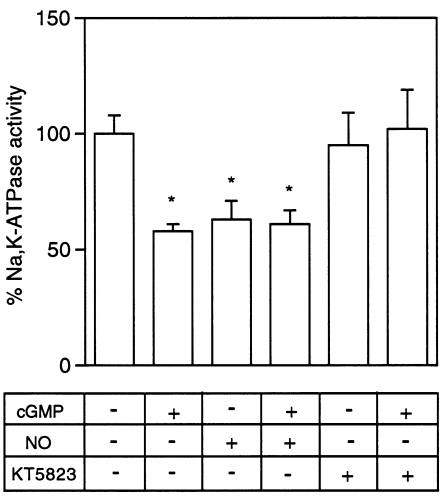

Fig. 3. Modulation of Na,K-ATPase activity in bEnd4 cells. bEnd4 cells were incubated for 15 min in HBS in the absence or presence of 4 mM 8-Br-cGMP, 1 mM nitrosoglutathione, or 2 μM KT5823, respectively, in the presence or absence of 15 mM ouabain. Ouabain-sensitive ATPase activity is expressed in % of the control activity measured with 15 mM ouabain without other additives. The results are the mean ± S.D. of a quadruplicate determination. The experiment was repeated two times. $^*P<0.05$ compared with the control using Student's *t*-test.

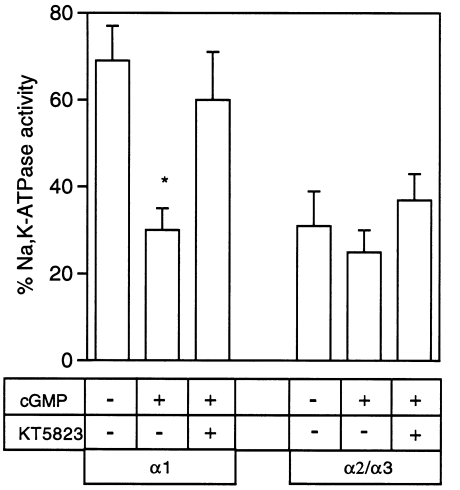

Fig. 4. Isoform-specific inhibition of Na,K-ATPase activity by cGMP. bEnd4 cells were incubated for 15 min in HBS in the absence or presence of 4 mM 8-Br-cGMP, with or without 2 μM KT5823, respectively, in the presence or absence of 15 mM or 10 μM ouabain. Ouabain-sensitive ATPase activity is expressed in % of the control activity measured with 15 mM ouabain without other additives. The fraction of the low affinity α1 isoform was calculated by subtracting the high affinity activity from total Na,K-ATPase activity. The results are the mean ± S.D. of a quadruplicate determination derived from two experiments. $^*P<0.05$ compared with the control using Student's *t*-test.

cGMP is mainly produced by the soluble guanylate kinase, which is the major receptor of nitric oxide (NO). We therefore used the NO donor nitroso-glutathione to study whether NO can also modulate Na,K-ATPase activity. Similarly to 8-Br-cGMP, NO reduces pump activity to 62% of the control level. Addition of both nitroso-glutathione and 8-Br-cGMP at maximally effective doses does not lead to a further inhibition of Na,K-ATPase activity, indicating that the effect of NO goes through activation of guanylate cyclase.

In the next series of experiments the question of an isoform-specific influence of 8-Br-cGMP on ouabain-sensitive Na,K-ATPase activity was addressed. bEnd4 cells were incubated with 4 mM 8-Br-cGMP alone or in the presence of 10 μM or 15 mM ouabain, respectively. To determine the α1 subunit-associated Na,K-ATPase activity, the Na,K-ATPase activity measured with 10 μM ouabain was subtracted from the activity obtained with 15 mM ouabain. α1-associated activity was reduced to 45% of the control and could be prevented by preincubation with 2 μM KT5823 (Fig. 3). We did not observe any significant influence of 8-Br-cGMP and KT5823 on the activity of the α2/3 fraction, indicating that the effect of cGMP/PKG is restricted to the α1 isoform in bEnd4 cells (Fig. 4).

4. Discussion

cGMP, activating PKG, reduces Na,K-ATPase activity in kidney, a tissue expressing predominantly the Na,K-ATPase α1 isoform, and increases pump activity in Purkinje neurons, which mainly express α3. We therefore investigated whether such opposite responses could be due to isoform-specific regulation of Na,K-ATPase activity. For these purposes we used

a brain endothelial cell line that simultaneously expresses different subunit isoforms.

The presence of three α and two β subunit isoforms in microvessels has been demonstrated on the protein level [7]. The lesser amount of β proteins than α subunit proteins led these authors to propose that β subunits could be rate limiting, allowing to regulate the number of Na,K-ATPase isoenzymes in microvessels by solely controlling β subunit gene expression. Our data suggest that β3, which was not yet known at that time, might compensate the apparently lower amount of β isoforms. Deduced from the expression level of β isoform genes in bEnd4 cells, one would even predict an excess of β over α proteins. However, our data on mRNA expression do not directly demonstrate the quantities of the respective protein. It is noteworthy that Zlokovic et al. [7] measured an excess of β over α protein when quantifying subunit isoforms in total brain extracts, suggesting that β subunits are not rate-limiting in these tissues.

Little is known about the subunit composition of Na,K-ATPase isoenzymes in cells expressing several isoforms simultaneously. Taking the relative strength of our PCR signals and the ouabain sensitivity as a measure, the predominant isoenzymes would be α1/β1 and α1/β3. In vitro reconstitution experiments demonstrated that any combination of an α with either β1 or β2 led to the generation of functionally active enzymes with only subtly different enzymatic activities [21,22], an indication that a cell's isoenzyme repertoire is a reflection of the isoform expression level. However, reconstitution experiments have provided biochemical evidence that the interaction of β1 is stronger with α1 than with α2 or α3 [22] and β2 preferentially assembles with α2 rather than with β1 [22,23]. Supporting these data is the observation that β2 is found predominantly in association with α2 when isolated from mouse brain [24]. Furthermore, in neurons, epithelial and endothelial cells a compartmentalised distribution of α subunit isoforms was observed [18,25,26]. In contrast, neuronal cells in culture develop polarisation, but apparently distribute α isoforms in a random manner [27]. Thus, a selective assembly and distribution of isoenzymes appears possible, but may also depend on extracellular cues that direct pump distribution. The large extracellular domain of β subunit isoforms might play a role for such a mechanism (see e.g. [24,28]).

The extent of inhibition of Na,K-ATPase activity by the cGMP analogue 8-Br-cGMP and the NO donor nitroso-glutathione is similar to the inhibitory action these compounds exert in kidney [16,29]. Since kidney expresses predominantly the α1 subunit isoform and the inhibition in bEnd4 cells is restricted to the same isoform, a specific inhibitory activity of cGMP/PKG on α1 appears plausible, whereas the activity of α2/α3 is not influenced. Owing to the very weak expression of α3 it is likely that most of the activity is generated through isoenzymes containing α2. The activating effect of cGMP seen in Purkinje neurons [18] supports a differential behaviour of the α2 and α3 isoforms too, since these cells predominantly have α3. Alternatively, the effects may include cell-specific pathways.

An important role in such pathways plays DARPP-32, a dopamine- and cAMP-regulated phosphoprotein that, when phosphorylated, inhibits protein phosphatase-1, ultimately leading to inhibited Na,K-ATPase [30]. DARPP-32 is abundantly present in renal tubule cells and involved in the dopamine-controlled regulation of Na^+ excretion. Phosphorylation of DARPP-32 by NO and cGMP was demonstrated in the brain [31] and by hormones and cGMP in the choroid plexus [32]. Whether it is expressed in bEnd4 cells is currently not known. Its presence has been demonstrated in ependymal cells of circumventricular organs [33], thus providing indirect evidence for the presence of DARPP-32 in tissues which control ion and fluid passage. We did not observe an effect of exogenously added cGMP and PKG on Na,K-ATPase activity when using a purified plasma membrane fraction, an indication that pump inhibition is unlikely to be the result of a direct action of PKG.

In conclusion, an isoform-specific regulation of Na,K-ATPase observed in endothelial cells, if existing in other cell types with a complex isoform expression pattern, e.g. neurons, may allow especially such cells accurate and perhaps locally restricted control of ion gradients that are required to regulate cell volume, fluid secretion and excitability.

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