K^+-p-Nitrophenylphosphatase Activity in Rat Brain and Liver

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Summary

K^+-p-nitrophenylphosphatase (K^+pNPPase) is the enzyme, which is considered to be involved in K^+-dependent hydrolysis of the phosphoenzyme in the reaction cycle of Na^+, K^+-ATPase. The aim of our present study was to characterize some features of K^+pNPPase in homogenates of the rat brain and liver. We determined p-nitrophenylphosphatase (pNPPase) activity in the presence of various ion combinations (Mg^{2+}+K^+, Mg^{2+}, K^+). We found a higher total pNPPase activity in the brain (0.8±0.079 nkat/mg protein) than in the liver (0.08±0.01 nkat/mg protein). Contrary to the liver, the main part of the total brain activity was K^+-dependent. The activity of K^+pNPPase was significantly higher in cerebral cortex homogenates (0.86±0.073 nkat/mg protein) in comparison to those of the whole brain (0.57±0.075 nkat/mg protein). The specific K^+pNPPase activity was 2-times higher in the isolated pellet fraction (0.911±0.07 nkat/mg protein), rich in synaptosomes, comparing to the whole brain homogenate (0.57±0.075 nkat/mg protein). Our results demonstrate the high activity of K^+pNPPase in the brain tissue and its distribution mainly into the pellet fraction, what might indicate a possible role of K^+pNPPase in specific structures of the brain, e.g. in synaptosomes.

Key words

Na^+, K^+-ATPase • K^+-p-nitrophenylphospatase • brain • liver

Introduction

Na^+, K^+-ATPase (E.C. 3.6.3.9), a sodium pump is the plasma membrane-bound enzyme responsible for maintaining electrochemical gradients of Na^+ and K^+ ions between cells and extracellular space, coupled with ATP hydrolysis. The ion pumping process is connected to a model of the enzyme reaction cycle with conformational changes of the catalytic α subunit. According to recent knowledge, Na^+, K^+-ATPase has two conformational states: E_1 and E_2. At the beginning of the Na^+, K^+-ATPase reaction cycle, Na^+ ions and ATP bind to E_1 conformational state. The formed NaE_1ATP

complex is phosphorylated to the high-energy phosphoenzyme (NaE_1P) and then transformed to the low-energy phosphoenzyme (E_2P), what is accompanied by Na^+ release. Afterwards, E_2P is K^+-dependently dephosphorylated to $E_2 + P_i$ (Homareda and Ushimaru 2005). The K^+- stimulated phosphatase activity of Na^+; K^+-ATPase is the most frequently determined by using p-nitrophenylphosphate (pNPP) as a substrate (Robinson *et al.* 1984). The enzyme is therefore also termed as K^+-p-nitrophenylphosphatase (K^+pNPPase).

The idea that K^+pNPPase activity represents a part of the Na^+, K^+-ATPase complex is supported by the following findings: i) both Na^+, K^+-ATPase and K^+pNPPase in the brain are inhibited by neurotensin (Lopez Ordieres and Rodriguez de Lores Arnaisz 2001); ii) both dephosphorylation of Na^+, K^+-ATPase and K^+pNPPase are dependent on the presence of K^+ in the medium. This idea is also consistent with the finding that K^+-binding sites, participating in activation of the dephosphorylation reaction of Na^+, K^+-ATPase as well as in activation of K^+pNPPase, exhibit similar properties of selectivity for K^+ ions (Homareda and Matsui 1982); iii) the isolated H_4-H_5 loop of Na^+, K^+-ATPase contains binding sites for ATP and pNPP (Krumscheid *et al.* 2004).

On the other hand, it was also reported that: **i)** pNPP is a structural analogue of a phosphoester but not of the compound with acylphosphate bond, which is produced during reaction of the P-type ATP-ases and undergoes K^+-dependent hydrolysis (Ziegelhöffer *et al.* 2000); **ii)** some inhibitors, such as oligomycine, 2,4,6-trinitrobenzenesulfonic acid and ions of bivalent metals, decrease the activities of Na^+, K^+-ATPase and K^+pNPPase to different degree (Vyskočil *et al.* 1981, Robinson *et al.* 1984, Fleary *et al.* 1985, Breier *et al.* 1987); **iii)** in contrast to vanadyl ions (IV) that are inhibitory to both Na^+,K^+-ATPase and K^+pNPPase, vanadate ions (V) exhibit no interference with Na^+,K^+-ATPase and inhibit only K^+pNPPase (Vyskočil *et al.* 1981). Although more K^+-dependent enzymes can participate in cleavage of pNPP, some of them need not be related to Na^+, K^+-ATPase activity.

It is well known, that Na^+, K^+-ATPase shows high activity in excitable tissues. However, it is not clear, if some other (? independent) form of K^+pNPPase might also exist in the tissue of central nervous system, except for the K^+pNPPase activity, that represents a part of the Na^+, K^+-ATPase reaction cycle. Our present study is designed to characterize some features of pNPPases present in homogenates of the excitable (the brain) and non-excitable (the liver) tissues and in the brain supernatant and pellet fraction, enriched in synaptosomes.

Methods

Experimental animals were adult male Wistar rats (300 – 400 g) from the Laboratory of Experimental Pharmacology of Slovak Academy of Science (Dobra Voda, Slovakia). The animals were fed on commercial rat diet and water *ad libitum*.

After decapitation, the brain and liver were removed and 1% homogenates (w/v) were prepared in Tris-HCl (30 mM, pH 7.5) at 0°C. Slices of cerebral cortex (400 μm thick) were prepared using a Stadie-Riggs tissue chopper. The slices were transferred to Tris-HCl (pH 7.5) at 0°C. Subcellular fractions were prepared from 10% brain homogenate (w/v) in 0.32 M sucrose by procedure of Gray and Whittaker (1962). After a centrifugation of the homogenate in K-23 centrifuge (4 500 x g,

10 min.), the sediment was resuspended twice in 1 ml of 0.32 M sucrose and centrifuged again under the same conditions. The supernatant fractions were mixed and centrifuged in Hettich-Universal 32R centrifuge (14 000 x g, 60 min., 4°C) for the separation of supernatant and pellet fraction containing mitochondria, synaptosomes and myelin.

P-nitrophenylphosphatase (pNPPase) activity was determined using the method of Stefanovic *et al.* (1974) in homogenates of the rat brain, cerebral cortex and liver, as well as in subcellular fractions of the brain (supernatant and pellet) by measuring of paranitrophenol (pNP) liberated from the hydrolysis of added p-nitrophenylphosphate (pNPP). The pNPPase activities were determined in the presence the following ions or their combinations: Mg^{2+}, Mg^{2+} + K^{+}, Mg^{2+} + EGTA (-Ca^{2+}), Mg^{2+} + K^{+} + EGTA (-Ca^{2+}). EGTA (0.1 mM) was used as a chelator of endogenous Ca^{2+} ions.

The reaction medium for assay of the basal enzyme activity contained: 3.4 mM pNPP and 30 mM Tris-HCl (pH 7.5). Individual enzyme forms of pNPPase activities ($Mg^{2+}$and $Mg^{2+}K^{+}$) were determined in the presence or combination of 3 mM $MgCl_2$ and 10 mM KCl. The K^{+}-dependent activity was calculated by subtracting of the Mg^{2+} activity from the total, i.e. ($Mg^{2+}K^{+}$) activity.

The enzyme reaction was started by the addition of a tissue homogenate to a reaction medium. The total volume of the reaction mixture was 0.5 ml. The reaction was stopped by the addition 0.1 M EDTA in 0.7 M NaOH, after 15 minutes of incubation at 37 °C. The released pNP was determined spectrophotometrically at 405 nm. Enzyme activities were expressed in nkat per mg of protein. Results were expressed as means of 6-14 experiments in triplicates ± S.E.M.

Protein concentration was determined according to Lowry *et al.* (1951), using bovine albumin as a standard. Results were statistically analyzed by Student's t-test. Differences were considered as significant when $p < 0.05$.

All chemicals were of the highest purity grades, available commercially.

Results

In brain homogenates, pNPPase activities were determined as well as effects of K^{+} and Ca^{2+} ions on them. K^{+}-dependent pNPPase represented 76% and Mg^{2+}pNPPase 24% of the total pNPPase activity. After chelation of Ca^{2+} ions, all pNPPase activities were increased in comparison with those in the presence of endogenous Ca^{2+} (Fig. 1).

In other experiments, the effect of 0.35 mM ATP on pNPPase activities was tested in rat brain homogenates (Fig. 1). ATP added to incubation medium significantly decreased the total (Fig. 1A) and K^{+}pNPPase (Fig. 1C) activities in the presence of endogenous Ca^{2+}, as well as after chelating of Ca^{2+} ions, in comparison with samples without added ATP. The Mg^{2+}pNPPase activity was not influenced by ATP, added to incubation medium (Fig. 1B).

Activities of individual forms of phosphatases were compared in rat brain and liver homogenates (Fig. 2). In the liver homogenates, all pNPPase activities were lower in comparison with those of brain homogenates. Contrary to the brain, the liver K^{+}pNPPase (Table 1) does not contain such a part of activity that was increased after chelating of Ca^{2+} ions in the brain (Table 2).

If we compared pNPPase activities in homogenates of the whole rat brain versus the brain cortex (Table 2), the activities were significantly higher in the brain cortex. The most significant difference was determined in the presence of K^+ ions (1.5-times higher activity in the brain cortex).

Moreover, activities of individual forms of pNPPases in brain tissue were determined in the separated pellet fraction containing mitochondria, synaptosomes and myelin, as well as in supernatant. The total Mg^{2+},K^+pNPPase activity was in the pellet fraction nine-times higher (1.157 nkat/mg protein) comparing to this activity in the supernatant (0.124 nkat/mg protein) (Fig. 3). The major part of the total pNPPase in the pellet was formed by K^+- activated pNPPase (0.911 nkat/mg protein), while this part of activity was very low in the supernatant (0.01 nkat/mg protein).

Discussion

In our study we confirmed that pNPPase activities were Mg^{2+}-dependent and regulated by K^+ and Ca^{2+} ions (Fig. 1). A similar inhibition of K^+pNPPase by high concentrations of Ca^{2+} ions (4 and 10 mM) was observed by Guerra Marichal *et al.* (1993) in synaptosomal membrane fraction from the rat brain cortex. The inhibitory effect of Ca^{2+} on K^+pNPPase, which represents the second part of the Na^+,K^+-ATPase complex, is explained either by competition between Ca^{2+} and Mg^{2+} or Ca^{2+} and K^+ for their binding sites at the enzyme (Guerra Marichal *et al.*, 1993). We found high content of K^+pNPPase from the total $Mg^{2+}K^+$pNPPase in the rat brain, (71% with endogenous Ca^{2+} and 64% without Ca^{2+}).

Moreover, we found a significant decrease of K^+pNPPase in rat brain homogenates after the addition of 0.35 mM ATP, which, however, had no effect on Mg^{2+}pNPPase activity. These results are in agreement with those demonstrating the specific inhibition of K^+pNPPase by 0.1-1.0 mM ATP and almost the total inhibition of this enzyme by ATP concentrations higher than 2 mM, which were reported by Guerra Marichal *et al.* (1993) in synaptosomal membranes of the brain cortex. ATP behaves as a competitive inhibitor of pNPPase activity in the absence of Na^+ and the presence of K^+ (Huang *et al.* 1985*)*. On the other hand, the combination of ATP and NaCl increased the affinity of pNPPase for K^+ and activated Na^+, K^+-ATPase activity (Homareda and Ushimaru 2005).

The important role of K^+pNPPase in the brain is supported by its high activity in this tissue in comparison to the liver (Fig. 2). This fact can be connected with different functions of these tissues. In excitable tissues, pNPPase might also play some another role, except for maintaining K^+ and Na^+ gradients, as a part of Na^+, K^+-ATPase. This role of K^+pNPPase in the brain (specifically in synaptosomal structures) may be supported by its higher activity in brain cortex versus whole brain (Table 2) and a different distribution this activity between the supernatant and pellet fraction, containing synaptosomes (Fig. 3). The specific K^+pNPPase activity was 2-times higher in the isolated pellet fraction comparing to the whole brain homogenate (Table 3).

We assume two different forms of the enzyme in brain tissue – Mg^{2+}pNPPase and K^+pNPPase. In the process of neurotransmission, the pNPPase activities might participate in restoring the resting state of Na^+, K^+ and Ca^{2+} distribution via dephosphorylation of Na^+, K^+-ATPase by K^+pNPPase (Beauge and Campos 1983, Homareda and Ushimaru 2005) as well as of Ca^{2+}-ATPase by Mg^{2+}pNPPase.

The other part of K^+pNPPase activity, modulated by Ca^{2+} ions, might play its specific functional role in synaptosomal structures. The activation of K^+pNPPase by Ca^{2+} chelating was detected also in our experiments in the brain, brain cortex and the pellet fraction of the brain, but not in the liver homogenate (K^+pNPPase activity was extremely low).

This presumption about synaptosomal localization of K^+pNPPase has to be supported by additional study using isolation of the enzyme in separated synaptosomes.

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Table 1. Individual enzyme forms of pNPPase in liver homogenate

	Activities in nkat/mg protein	
	With endogenous Ca^{2+}	After chelating of Ca^{2+}
Total ($Mg^{2+}K^{+}$pNPPase)	0.08±0.01	0.097±0.015
Mg^{2+}pNPPase	0.075±0.017	0.1±0.02
K^{+}pNPPase	0.002±0.008	0.0±0.01

Results are means ± S.E.M of 14 animals.

Table 2. Individual enzyme forms of pNPPase in whole brain and brain cortex homogenate

	Activities in nkat/mg protein			
	Brain homogenate		**Brain cortex homogenate**	
	with endogenous Ca^{2+}	after chelating of Ca^{2+}	with endogenous Ca^{2+}	after chelating of Ca^{2+}
Total ($Mg^{2+}K^{+}$pNPPase)	0.8±0.079	0.962±0.087	1.23±0.08 *	1.6±0.15 *
Mg^{2+}pNPPase	0.238±0.03	0.347±0.027	0.37±0.033	0.57±0.05
K^{+}pNPPase	0.57±0.075	0.616±0.064	0.86±0.073 *	0.99±0.12 *

Results are means ± S.E.M of 8 animals. * $p < 0.05$ vs. whole brain homogenate.

Table 3. Subcellular fractionation of K^{+}pNPPase from 10% rat brain homogenate

Subcellular fraction	**Volume (ml)**	**Content of proteins (mg)**	**Concentration of proteins (mg/ml)**	**Activity (pkat/ml)**	**Total activity (pkat)**	**Specific activity (pkat/mg protein)**	**Purification[a]**
homogenate	3	35.4	11.8	5740	17220	486	1
supernatant fraction	5	2.9	0.58	10	50	17.2	0.04
pellet fraction	1.5	0.9	0.62	652	978	1086.7	2.2

[a] specific activity of fraction / specific activity of homogenate

Fig. 1. Effect of ATP on individual enzyme forms of pNPPase in brain homogenates:

(A) $Mg^{2+}K^{+}$pNPPase

(B) Mg^{2+}pNPPase

(C) K^{+}pNPPase

Open columns: without ATP; full columns: with ATP.

**** significant difference (p<0.001) after addition of ATP versus without ATP*

Values are expressed as nkat/mg protein and represent the means ± S.E.M of 14 animals.

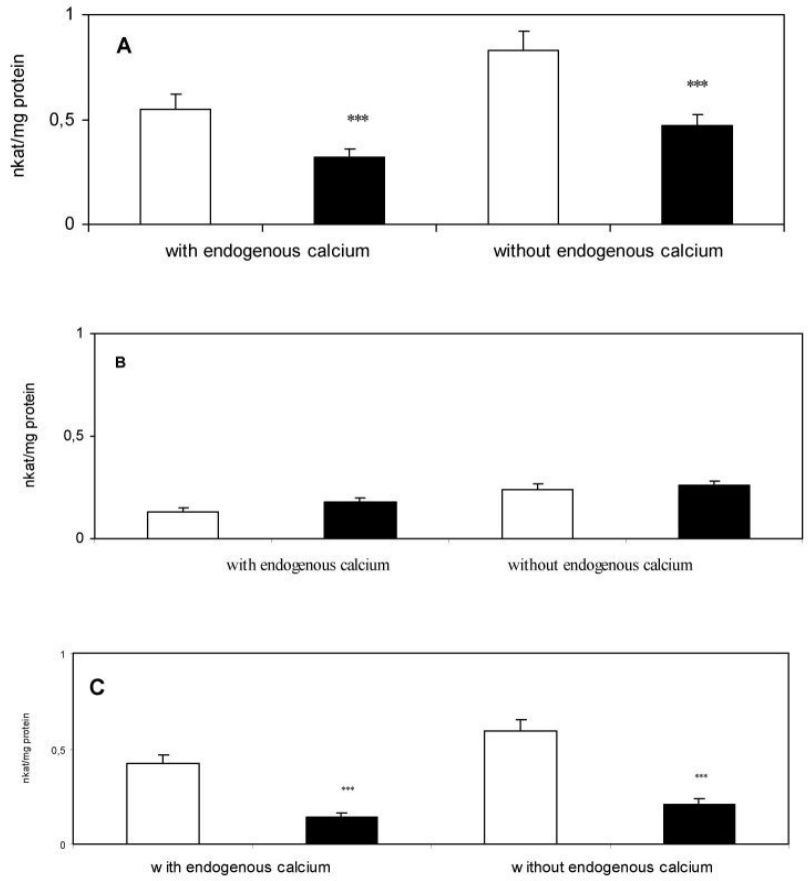

Fig. 2. Individual enzyme forms of pNPPase activities in whole rat brain and liver homogenates

**** significant difference (p<0.001) between liver and brain*

Values are expressed as nkat/mg protein and represent the means ± S.E.M of 14 animals.

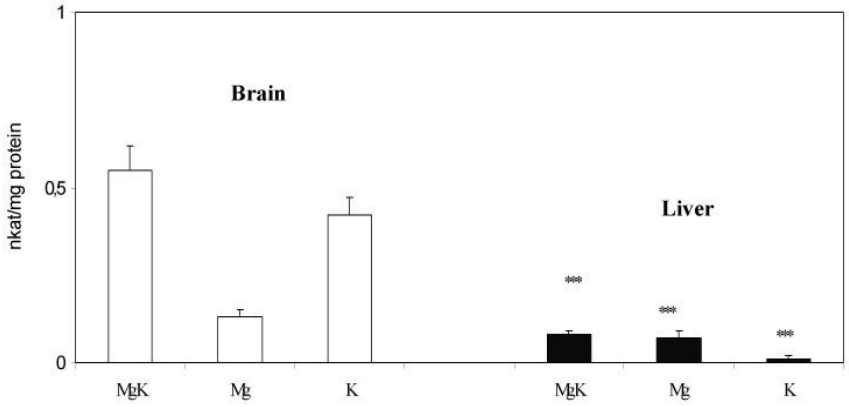

Fig. 3. Subcellular fractionation of rat brain pNPPases.

(A) supernatant (B) pellet

Open columns: with endogenous Ca^{2+}, hatching columns: after chelating of endogenous Ca^{2+} with EGTA.

Values are expressed as nkat/mg protein and represent the means ± S.E.M of 6 animals.