Effect of mitogen lectin on lymphocyte or brain cortex cell activation

Hana Kovaru [1], Frantisek Kovaru [1], Marek Pav [1], Rudolf Dvorak [2], Miloslava Palikova [2], Jiri Raboch [1], Jarmila Zidková [3]

1 1st Faculty of Medicine, Charles University, Prague, Czech Republic
2 University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic
3 Institute of Chemical Technology in Prague, Czech Republic

Correspondence to: Prof. RNDr. Hana Kovářů, DSc.
1st Faculty of Medicine in Prague, Charles University,
Ke Karlovu 11, 120 00 Prague 2, Czech Republic.
e-mail: hkova@lf1.cuni.cz; hkovaru@cesnet.cz

Submitted: 2010-04-01 Accepted: 2010-05-05 Published online: 2010-06-30

Key words: **mitogen lectin; PHA; T lymphocytes; brain cortex cells; Na^+/K^+-ATPase activation; cell proliferation**

Neuroendocrinol Lett 2010; **31**(3):325–329 **PMID:** |20588247 NEL310310A11

Abstract

OBJECTIVES: We studied a) mitogen lectin (PHA) evoked changes of Na^+/K^+-ATPase activity in functionally different lymphocytes or brain cortex cells and b) quantitative relationship between PHA- evoked early enzyme activation and late lymphocyte proliferation were analyzed.
MATERIALS AND METHODS: We performed biochemical analyses of Pi released from ATP by Na^+/K^+-ATPase activity. Lymphocyte proliferation was assayed by ^3H-thymidine incorporation.
RESULTS: We demonstrated PHA stimulated Na^+/K^+-ATPase activity of mouse spleen lymphocytes or freshly isolated brain cortex cells. Besides this, we estimated high stimulation of Na^+/K^+-ATPase activity and subsequent late ^3H-thymidine incorporation into pig lymphocytes as both PHA dose and K^+ ion concentration dependent.
CONCLUSIONS: Thus, early PHA dose-dependent stimulation of Na^+/K^+-ATPase activity is a more general response in different animal species and functionally different cells. We measured both cell type- and PHA-dose dependent enzyme activity stimulation. We can suggest that intensity of early PHA induced Na^+/K^+-ATPase activation could be in relationship to subsequent elevated level of T lymphocyte proliferation. The Na^+/K^+-ATPase can be a part of mitogen lectin evoked signal transduction mechanisms.

ORIGINAL ARTICLE

INTRODUCTION

Phytohemagglutinin (PHA), lectin from Phaseolus vulgaris, has N-acetylglucosamine saccharide binding specifity. The PHA binding specifity was used in studies of β1,6-*N*-acetylglucosamine-bearing N-glycans in human gliomas and brain cells (Yamamoto *et al.* 2000, Kitamura *et al.* 2005). Furthermore, there are many in vitro reactions to study T lymphocytes, activated from quiescent state to proliferation, e.g. using PHA (Severini *et al.* 1987; Kozáková *et al.* 1997; Anner and Volet 1999; Gridley *et al.* 2009). Increase of Na^+/K^+-ATPase mRNA levels was detected in PHA activated human lymphocytes by real time-PCR method after 24-h. incubation, supporting data

about Na^+/K^+-ATPase role in T cell activation (Vereninov *et al.* 1993; Marakhova *et al.* 2005).

The Na^+/K^+-ATPase carries out the coupled extrusion and uptake of Na^+ and K^+ ions across the plasma mebrane in most higher eukaryotic cells (Kaplan 2002). Important physiological role of Na^+/K^+-ATPase is maintaining the balance of electrolytes and fluids at the whole organism level as reviewed by Tian and Xie (2008). The noncovalently linked α and β Na^+/K^+-ATPase subunit isoforms exist in different combinations among cell types, organ tissues and various developmental stages (Tiang and Xie 2008). Generally, α catalytic subunit is tightly cooperating with β subunit, participating in the plasma membrane targeting of α subunit and stability of Na^+/K^+-ATPase complex (Kaplan 2002; Vagin *et al.* 2006; El-Beialy *et al.* 2010). Regulatory β-1 subunit of Na^+/K^+-ATPase may be involved in mouse neural cell interactions (Kitamura *et al.* 2005; Vagin *et al.* 2006).

In the central nervous system, Na^+/K^+-ATPase can participate on neurite growth, as well as mutations of Na^+/K^+-ATPase cause familial hemiplegic migraine in humans and rapid-onset dystonia-Parkinsonism (Desfrere *et al.* 2009). In the immune system, the role of Na^+/K^+-ATPase in immunity is supported by findings of social stress effect on decreased cell immunity responses (Entringer *et al.* 2008; Gridley *et al.* 2009). Abnormal chemokine receptor modulation with impaired cationic ion transporter and Na^+/K^+-ATPase expression on polymorphonuclaer cells was described in active systemic lupus erythematosus, autoimmune disorder (Hsieh *et al.* 2008).

Thus widening knowledge is supporting neuroimmunomodulatory aspects of the central nervous system (CNS) and immune system, working and cooperating by both physiological mechanisms and regulations based on neurotransmitters, cytokines, hormones and their receptor expression on immunocytes or brain cells (Franco *et al.* 2007; Maes 2008; Kovářů *et al.* 2009).

The aim of this study was to estimate a quantitative relationship between PHA concentration induced Na^+/K^+-ATPase activation and degree of intensity in T pig lymphocyte stimulation at early stage (first 60 min) up to late interval of cell proliferation, which is also potentiated by K^+ ions. Another point of interest was comparison of early PHA effect on Na^+/K^+-ATPase activity for 15 min incubation in different animal species and functionally different cells, i.e. mouse spleen lymphocytes and brain cortex cells, freshly isolated and functionally active.

MATERIAL AND METHODS

Experimental animals

We used 30-day-old pigs (n=8) of Czech white improved breed from three litters. Piglets were reared under physiological conditions. For other details see Kovářů *et al.* (2002).

Two-month-old male mice of SPF inbred CBA/J strain were purchased from Anlab (Prague). The total amount was 30 animals. For other details see Kovářů (1980), Kovářů *et al.* (1997).

Experimental animal care and procedures were performed in accordance with the European convention for care and use of laboratory animals, and with the Czech law (246/1992 Coll. and later regulations).

Preparation of thymus or spleen lymphocytes

Thymus or spleen, small tissue fragments were dissociated in Potter-Elvehjem homogenizer (Corning) in MEM medium, 10 mM HEPES, pH 7.3, washed out repeatedly. In spleen cell suspension, erythrocytes were eliminated by hypotonic lysis and cells of reticula by clumping and sieving in Ca^{2+}/Mg^{2+} free isotonic buffer. Viability of lymphocytes was 94–96%, and erythrocyte contamination 0.6–0.8% (Kovářů *et al.* 1997; Kovářů *et al.* 2002).

Brain cortex cell suspension

Brain cortex tissue fragments were dissociated by mechanical sieving through nylon sieves with decreasing pore diameter in 7.5% (w/v) polyvinylpyrrolidone (Serva)-containing isolation medium, washed out repeatedly into appropriate isotonic incubation media and immediately used in experiment. Freshly prepared brain cortex cells fullfiled metabolical and functional criteria: high resynthesis of energy reserves – ATP and creatinphosphate up to first 10 min of incubation, high O_2 uptake as well as high K^+ ion evoked stimulation of O_2 uptake and Na^+/K^+-ATPase activity and decreased ATP (Kovářů 1980; Kovářů *et al.* 1997; Walz 2000).

Enzyme activity

Na^+/K^+-ATPase activity was estimated by spectrophotometric semimicromethod of released inorganic phosphate estimation. To reach optimal enzyme actiity, buffers were of various compositions in mM: Na^+/K^+-ATPase - 120 (100) NaCl, 20 (10) KCl, 4(3) $MgCl_2$ for brain cells (lymphocytes), respectively, with 30 mmol/L Tris-HCl, pH 7.3, 1 mM ATP (Serva, synthetic), +/– 0.3 mM ouabain (mouse cells) or +/– 0.1 mM ouabain (pig lymphocytes). Ouabain sensitive Na^+/K^+-ATPase activity was estimated as a difference between total ATPase and ouabain resistant Mg^{2+}-ATPase. Optimal ion concentration of enzyme buffer for both lymphocytes or brain cortex cells was used including species dependent ouabain cocentration for inhibition of enzyme activity. We used purified preparate of PHA (Serva). For other details see papers Kovářů (1980); Kovářů *et al.* (1997; 2002).We used purified preparate of PHA (Serva).

^{3}H-thymidine incorporation

Lymphocytes (5×10^5 per well) were cultured in 96 flat-bottom microwell plates in serum-free medium MEM supplemented with antibiotics, 2 mmol/L glutamine

(Serva), 5 mM pyruvate (Serva), 5 mM KCl (Aristar) and 10 mM HEPES, pH 7.3 (Serva) under standard conditions in 5% CO_2 humidified atmosphere at 37 °C. For last 16 h of total 48-h. cultivation ^{3}H-thymidine (Amersham) was added. Then, incorporated radioactivity of harvested cells was measured. For details see Kovářů and Pospíšil (1980) and Kozáková *et al.* (1997).

Protein determination

We used both modified rapid biuret method at UV region and Lowry method (Kovářů 1980; Kovářů *et al.* 1997).

Statistical analysis

The data are expressed as arithmetical mean values ± S.E.M. The differences between experimental samples were evaluated by the Student´s t-test for unpaired values.

RESULTS AND DISCUSSION

We focused our attention on cell biochemistry of mitogen lectin activated T lymphocytes or functionally different brain cortex cells.

Increased PHA concentration induced elevation of Na^+/K^+-ATPase activity (Table 1). It could be suggested that optimal PHA doses for mitogen stimulation of pig T lymphocytes were 6.25–12.5 μg/ml, indicated by higher enzyme activation for 15–60 min and subsequent cell proliferation. Besides this, we demonstrated quantitative relationship between early and late potentiation of both enzyme activity followed by ^{3}H-thymidine incorporation which was PHA dose and 10mM K^+ concentration dependent. From qualitative point of view, our data are in accordance to PHA activated enzyme of human and mouse T lymphocytes from resting state to subsequent DNA synthesis (Kozáková *et al.* 1997; Marakhova *et al.* 2005).

In our experiments, we presented PHA induced ouabain sensitive Na^+/K^+-ATPase activity stimulation only. Our results indicate that ouabain resistant Mg^{2+}–ATPase activity was not stimulated significantly with purified PHA, i.e. in both pig or mouse cells, enzyme activity was in range 89–112% of control values. We showed here that purified PHA-induced Na^+/K^+-ATPase activation only was accompanied by late DNA synthesis. However, partially purified PHA stimulated non-specifically other ATPases (Kovářů *et al.* 1997; Kovářů *et al.* 2002).

Main finding, summarized in Table 2, is PHA induced activation of mouse brain cortex cell as well as spleen lymphocyte Na^+/K^+-ATPase activity. It is evident that enzyme activity was 5× times higher on brain cortex cells in comparison with spleen lymphocytes. Intensity of PHA induced Na^+/K^+-ATPase activity stimulation was similar in pig and spleen lymphocytes at 12.5 μg/ml PHA concentration. Thus, PHA-evoked Na^+/K^+-ATPase activation is a more general cell reaction, as evident from pig and mouse T lymphocytes or brain cortex cells.

Some data as possible aspect of pleiotropic function of Na^+/K^+-ATPase will be mentioned below. Formerly, it was reported that Na^+/K^+-ATPase can participate in phylogenetically conserved self versus non-self recognition events of immunocytes (granulocytes) of Limulus polyphemus based on N-acetylglucosamine saccharide specificity of highly glycosylated β chain of enzyme molecule with regulatory properties linked to α catalytic subunit of the enzyme (Gupta *et al.* 1991).

It is known that mitogen lectin studies can be taken as model T lymphocyte stimulation from quiescent state more often then allogeneic cell reaction dependent on major histocompatibility complex (MHC) differences *in vitro*. We demonstrated mouse (H-2) allogeneic activation between mouse lymphocytes, alloreactive brain cortex enriched glial fractions of brain cortices (Kovářů

Tab 1. Early and late events in lymphocytes as function of PHA concentration.

PHA μg/ml	Na^+/K^+-ATPase activity (S.I.[1]) preincubation			^{3}H-thymidine incorporation (S.I.[1]) cultivation 48 h.	
	15 min	30 min	60 min		
	K^+ions			K^+ions	
	10 mM	10 mM	10 mM	10 mM	5.8 mM
2.50	n.d.	1.09±0.12	1.22±0.19	3.27±0.41	1.25±0.28
6.25	n.d.	1.45±0.14*	1.70±0.22*	5.80±0.49**	2.09±0.36
12.50	1.83±0.28*	1.91±0.16*	2.17±0.19*	13.78±0.98*	7.50±0.86*
25.00	2.44±0.17**	2.25±0.19*	1.78±0.17*	11.37±1.33*	12.94±1.23*

Basal values: Na^+/K^+-ATPase activity: 88±9 nmoles Pi/1 mg protein × h^{-1}.
^{3}H-thymidine incorporation: 1.332 cpm ± 189. S.I.[1] stimulation index (control value = 1).
Values are mean of 14-16 measurements for each interval ± S.E.M. in three experiments.
Statistical significance: *p<0.01, **p<0.05. n.d. means not determined.

Tab. 2. PHA stimulated Na^+/ K^+-ATPase activity of mouse spleen lymphocytes and brain cortex cells.

PHA µg/ml	Na^+/ K^+-ATPase activity (µmoles P_i/1 mg protein × h^{-1})			
	lymphocytes	%	brain cells	%
0	0.83±0.08	100	4.28±0.24	100
2.5	1.43±0.13*	172.86	5.21±0.49**	121.71
12.5	1.34±0.14*	162.44	6.07±0.31*	141.78

PHA incubation: 15 min. For other details see Table 1.

1980; Kovářů *et al.* 1997). Furthermore, we estimated inhibitory effect of MHC Class II antibody on Na^+/K^+-ATPase activity or its external part of ouabain-sensitive K^+-dependent p-nitrophenyl phospatase (p-NPPase) activities by K^+ ion- and antibody dose-dependent manner. Interestingly, it has been described in other paper, when anti-MHC Class II antibody was added to co-cultured T cells and astrocytes, primary astrocyte expression of ion transport proteins and lactate transporter was found (Korn *et al.* 2005).

We can not excluded that mentioned types of Na^+/ K^+-ATPase complex activation are a part of signal transduction mechanisms. Finally, multiple functions of Na^+/K^+-ATPase, ubiquitous enzyme, are suggested in relationship to signalling cooperation with number of kinases (tyrosin kinase Src**,** mitogen-activated protein kinase MAPK, phosphoinositide 3´kinase PI3K, etc) as well as G protein coupled receptors, G proteins, glutamate and lactate transporters and transcription factors (Tian and Xie 2008; Desfrere *et al.* 2009). Future efforts will provide insights to newly appreciated functions of Na^+/K^+-ATPase, not only ion-transporting but also cell signalling, and their roles in celular biology and animal physiology (Tian and Xie 2008, El-Beialy *et al.* 2010).

ACKNOWLEDGEMENTS

This study was supported by grant of Academy of Sciences of Czech Republic, No. IAA 601680801 and grants of Ministery of Education of Czech Republic, No.MSM 6215712403 and No.MSM 0021620849.

REFERENCES

1 Anner BM, Volet B (1999). Uptake of reconstituted Na,K-ATPase vesicles by isolated lymphocytes measured by FACS, confocal microscopy and spectrofluorimetry. Cell Biochem Biophys. **30:** 437–454.
2 Desfrere L, Karlsson M, Hiyoshi H, Malmersjö S, Nanou E, Estrada M, Miyakawa A, Lagercrantz H, El Manira A, Lal M, Uhlén P (2009). Na,K-ATPase signal transduction triggers CREB activation and dendritic growth. Proc.N.Y.Acad. Sci. **106:** 2212–2217.
3 El-Beialy W, Galal N, Deyama Y, Yoshimura Y., Suzuki K, Tei K, Zotsuka Y (2010). Regulation of human and pig renal ATPase by tyrosine phosphorylation of their subunits. J Membrane Biol. **233:**119–126.
4 Entringer S, Kumsta R, Nelson EL, Hellhammer DH, Wadhwa PD, Wüst S (2008). Influence of prenatal psychosocial stress on cytokine production in adult women. Dev Psychobiol. **50**: 579–587
5 Franco R, Pacheco R, Lluis C, Ahern GP,O´Connell PJ (2007).The emergence of neurotransmitters as immune modulators. Trends Immunol. **28:** 400–407
6 Gridley DS , Slater JM, Luo-Owen X, Rizvi A, Chapes SK, Stodieck LS, Ferguson VL, Pecaut MJ (2009). Spaceflight effects on T lymphocyte distribution, function and gene expression. J Appl Physiol. **106**: 194–202.
7 Gupta AP, Orenberg SD, Das YT, Chattopadhyay K (1991). Lectin-binding receptors, Na^+/ K^+-ATPase and acetylcholinesterase on immune plasma membrane of Limulus polyphemus. Exp Cell Res. **194:** 83–86.
8 Hsieh SC, Wu TH, Tsai CY, Li KJ, Lu MC, Wu CH, Yu CL (2008). Abnormal in vitro CXCR2 modulation and defective cationic ion transporter expression on polymorphonuclear neutrophils responsible for hyporesponsiveness to IL-8 stimulation in patients with active systemic lupus erythrematosus. *Rheumatology*.**47**: 150–157.
9 Kaplan JH (2002). Biochemistry of Na,K-ATPase. Annu Rev Biochem.**71**:511–535.
10 Kitamura N, Ikekita M, Sato T, Akimoto Y, Hatanaka Y, Kawakami H, Inomate M,Furukawa K (2005). Mouse Na^+/ K^+-ATPase beta1-subunit has a K^+-dependent cell adhesion for beta-GlcNAc-terminating glycans. Proc N Y Acad Sci. **102:** 2796–2801.
11 Korn T, Magnus T, Jung S (2005). Interaction with antigen-specific T cells regulates expression of the lactate transporter MCT1 in primary rat astrocytes: specific link between immunity and homeostasis. Glia. **49**: 73–83.
12 Kovářů H (1980). Effect of genetic differences on K^+-induced metabolic changes in allogeneic murine brain cortical cells incubated in vitro. Med Biol. **58**: 273–280.
13 Kovářů H, Pospíšil M (1980). Effect of low molecular factors released during allogeneic interactions of leucocytes or brain cells on PHA- and LPS-induced DNA synthesis in lymphocytes. Lymphology. **13:** 30–33.
14 Kovářů H, Kovářů F, Hašková V (1997). Cell surface enzymes of brain cortex cells or lymphocytes during early allogeneic reaction. Physiol Res. **46:** 137–144.
15 Kovářů H, Kovářů F, Fišerová A, Zelníčková P, Matalová E (2002). Biochemical markers of lymphocyte maturation. Acta Vet Brno. **71:** 503–508.
16 Kovářů H, Páv M, Kovářů F, Raboch J, Fišerová A(2009). Cell signalling in CNS and immune immune system in depression and during antidepressant treatment:focus on glial and natural killer cells. Neuroendocrinol Lett. **30:** 421–428.

17 Kozáková H. Kovářů H. Mareš V. Kovářů F. Šiman P. (1997). Peptide cytokines in CNS and the immune system. *Phys Res*. **46:** 145–153.
18 Maes M (2008). The cytokine hypothesis of depression: inflammation, oxidative& nitrosative stress(IO&NS) and leaky gut as new targets for adjunctive treatments in depression. Neuroendocrinol Lett. **29**: 287–291.
19 Marakhova I, Karitskaya I, Aksenov N, Zenin V, Vinogradova T (2005). Interleukin–2–dependent regulation of Na/K pump in human lymphocytes. FEBS Lett. **579:** 2773–2780
20 Severini A, Prasad KV, Almeida AF, Kaplan JG (1987): Regulation of K^+-pump after mitogenic activation of lymphocytes. Biochem Cell Biol. **65:** 95–104.
21 Tian J, Xie Z (2008). The Na,K-ATPase and calcium-signalling microdomains. Physiology.**23:** 205–211.
22 Vagin O, Tokhtaeva E, Sacha G (2006). The role of beta1 subunit of the Na,K-ATPase and its glycosylation in cell-cell adhesion., *J* Biol Chem. **281:** 39573–39578.
23 Vereninov AA, Marakhova II, Osipov VV, Toropova FV(1993). Expression of mRNA encoding the alpha1 and the beta1 subunits of Na^+/ K^+-ATPase in human lymphocytes activated with phytohaemagglutinine. FEBS Lett. **316:** 37–40.
24 Walz W (2000): Role of astrocytes in the clearance of excess extracellular potassium. Neurochem Inter. **36:** 291–300.
25 Yamamoto H, Swoger J, Greene S, Saito T, Hurh J, Sweeley C, Leestma J, Mkrdichian E, Cerullo L, Nishikawa A, Ihara Y, Taniguchi N, Moskal JR (2000). β1,6-N-acetylglucosamine-bearing N-glycans in human gliomas: implications for a role in regulating invasivity. Cancer Res. **60:** 134–142.