Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2015-0577.R2
Manuscript Type:	Article
Date Submitted by the Author:	25-May-2016
Complete List of Authors:	Lopatina, Ekaterina; Federal Almazov North-West Medical Research Centre, Department of the Experimental Physiology and Pharmacology; Russian Academy of Sciences, Laboratory of the Excitable Membrane Pavlov Institute of Physiology Kipenko, Anna; Federal Almazov North-West Medical Research Centre, na V. Kipenko aDepartment of the Experimental Physiology and Pharmacology; Pavlov Institute of Physiology of Russian Academy of Sciences, Laboratory of the Excitable Membrane Pasatetskaya, Natalia; Federal Almazov North-West Medical Research Centre, Department of the Experimental Physiology and Pharmacology; Pavlov Institute of Physiology of Russian Academy of Sciences, Laboratory of the Excitable Membrane Penniyaynen, Valentina; Pavlov Institute of Physiology of Russian Academy of Sciences, Laboratory of the Excitable Membrane Krylov, Boris; Pavlov Institute of Physiology of Russian Academy of Sciences, Laboratory of the Excitable Membrane
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MODULATION OF THE TRANSDUCER FUNCTION OF Na^+, K^+ -ATPase: NEW MECHANISM OF HEART REMODELING

Ekaterina V. Lopatina^{a,b}, Anna V. Kipenko^{a,b}, Natalia A. Pasatetskaya^{a,b}, Valentina A. Penniyaynen^b, Boris V. Krylov^b

^aDepartment of the Experimental Physiology and Pharmacology Federal Almazov North-West Medical Research Centre, 2 Akkuratova St., 197341 St. Petersburg, Russia

^bLaboratory of the Excitable Membrane Pavlov Institute of Physiology of Russian Academy of Sciences, 6 Makarova Emb., 199034 St. Petersburg, Russia

Corresponding author: Ekaterina V. Lopatina (e-mail: evlopatina@yandex.ru).

ABSTRACT

Endogenous digitalis-like factors were found in the mammalian and human blood. It was the starting point for the elucidation of the new non-pumping function of the Na^+, K^+ -ATPase. It was previously well-known that Na^+, K^+ -ATPase is a pharmacological target receptor for cardiac glycosides (Skou 1957). We have investigated the trophotropic effects of such agents as ouabain, epinephrine, norepinephrine, atenolol and comenic acid using the organotypic tissue culture combined with the reconstruction of optical cross sections and confocal microscopy. It was shown that the growth zone of organotypic culture forms a multidimensional structure. Our results indicate that the cardiac glycoside ouabain applied in endogenous concentrations (10^{-8} , 10^{-10} M) can modulate transducer function of Na^+, K^+ -ATPase and control the cell growth and proliferation. It was also shown that Src-kinase is involved in “endogenous” ouabain activated intracellular pathways as a series unit. Epinephrine (10^{-9} – 10^{-14} M) and comenic acid (10^{-6} – 10^{-10} M) were demonstrated to modulate the growth of 10-12-day-old chicken embryo cardiac tissue explants in a dose-dependent manner. Epinephrine and comenic acid regulate growth and proliferation of the cardiac tissue via receptor-mediated modulation Na^+, K^+ -ATPase as a signal transducer. The trophotropic effects of the investigated agents specifically control the heart remodeling phenomenon.

Keywords: organotypic culture, cardiac tissue explants, epinephrine, comenic acid, Na^+, K^+ -ATPase.

Introduction

Digitalis has been used to treat chronic heart failure for a long time. It was not clear how phytochemicals regulate functions in the human body. Endogenous ouabain was discovered in the blood of warm-blooded animals and humans only in the 90s of the 20th century (Hamlyn et al. 1991). Na^+, K^+ -ATPase represents the only target for digitalis-like factors. After the discovery of endogenous digitalis-like agents it has become evident that Na^+, K^+ -ATPase has functions that are not directly related to ion transport (Aperia 2007).

Therefore, Na^+, K^+ -ATPase has been considered as a pharmacological receptor for endogenous digitalis-like factors and cardiac glycosides (Doris and Bagrov 1998; Hamlyn et al. 1991). Na^+, K^+ -ATPase is involved in the modulation of nociceptive signals (Lopatina et al. 2012). A new mechanism of membrane signaling was discovered in the membrane of sensory neurons dorsal root ganglia of mammals. It was proved that the Na^+, K^+ -ATPase functions as a signal transducer in the system: opioid-like receptor- Na^+, K^+ -ATPase- $\text{Na}_v1.8$ -channel (Krylov et al. 2000). Using the patch clamp method was found that comenic acid is able to selectively modulate the voltage sensitivity of $\text{Na}_v1.8$ channels. Comenic acid does not switch on the opioid-controlled signaling cascades. It activates a novel additional nociceptive opioid-like-receptor-coupled mechanism that does not involve G-proteins. Patch-clamp and quantum-chemical data indicate that comenic acid interact with the opioid-like receptor in a form of Ca^{2+} chelate complex, thus decreasing the voltage sensitivity of $\text{Na}_v1.8$ channels. A decrease in excitability of primary and secondary neuron membranes due to receptor-coupled action of comenic acid results in pain relief at both spinal and supraspinal levels, as shown with the hot-plate and formalin tests. A new analgesic anocetpin containing comenic acid as an active substance. (Plakhova et al. 2014).

It was supposed, that ouabain is a new stress hormone (Schoner and Schiener-Bobis 2005), since ouabain synthesis is increased in the hypothalamus, pituitary and adrenal cortex in response to stress (Bagrov and Shapiro 2008).

The term signal transducer describing the possible involvement of Na^+, K^+ -ATPase in the regulation of cell growth and expression of different genes was first used in 1999 (Xie 2001). The interaction ouabain with Na^+, K^+ -ATPase leads to activation of Src kinases, the formation of the receptor-tyrosinekinase complex and the launch of various signaling cascades, including that leading to increase of reactive oxygen species (Aperia 2007). Being a signal transducer, Na^+, K^+ -ATPase controls cell growth and proliferation (Kipenko et al. 2009; Lopatina et al. 2005, 2008b; Panniaänen et al. 2003; Schoner 2002a, 2002b; Xie 2001). In low concentrations ouabain increase the synthesis of proteins, cell size, stimulates the process of cell division. Effect of ouabain revealed similarities with other hypertrophy factors that regulate cardiac growth (Doris and Bagrov 1998; Ferrandi et al. 2004). Catecholamines and angiotensin II modulate the synthesis of endogenous ouabain in the adrenal cortex (Dmitrieva and Doris 2003). Specific modulatory effects of catecholamines on the cell growth and proliferation have never been thoroughly investigated.

The purpose of the research: To investigate the non-pumping function of Na^+, K^+ -ATPase in heart remodeling. To demonstrated elucidation of trophotropic effects of endogenous and exogenous substances on membrane receptor or transducer. To demonstrate elucidation of trophotropic effects of endogenous and exogenous substances on membrane receptor or transducer.

MATERIALS AND METHODS

Animal preparation

The 10-12-day old *White Leghorn* chicken embryos provided by Sinyavino Poultry (Leningrad Region, Russia) were used in the study. Cardiac tissue explants were obtained by extirpation from the ventricle of heart.

The study was approved by the local Ethics Committee (N 140113), in compliance with the Helsinki Convention for the protection of rights of animals.

Organotypic tissue culture

Studies were performed on 2200 explants (110 chicken embryos; 20 explants from every ventricle of heart). Cardiac tissue explants were cultured for three days on collagen supports in Petri dishes at 37 °C with 5% CO₂ in the standard CO₂-incubator (Sanyo, Japan). Each Petri dish contained approximately 20 explants.

The growth of explants in tissue culture was controlled on vital preparations using a confocal laser scanning microscope LSM-710 (Carl Zeiss, Germany). The preparations were also visualized by a television microscope attachment Axiostar Plus (Carl Zeiss, Germany).

In the course of vital microscopic examination two zones could be observed in the explants after three days of culturing: the central zone (more dense) and a less optically dense growth zone, which surrounds the central zone (Fig. 1). In both control (Fig. 2) and experimental explants the growth zone comprised mostly proliferating cardiomyocytes (65–70%), as well as fibroblasts (up to 35%). We have further investigated the influence of endogenous (epinephrine, norepinephrine, ouabain) and exogenous (comenic acid) signaling compounds on the growth of chicken embryos cardiac tissue explants.

The area index (AI) was used to estimate the explants growth. It was calculated in relative units as a ratio of total explant area to the central zone area (Fig. 1). The AI value was taken as 100% in the control experiments.

Culture medium

pH 7,4; 45% – Hank's solution; 40% – Eagle's medium; 15% – fetal cow serum; supplemented with glucose (0,6%), insulin (0,5 ED/ml), gentomycin (100 ED/ml), glutamine (0,35%) (Lopatina et al. 2005).

Drugs

Epinephrine, norepinephrine, ouabain, Src-kinase inhibitor PP2 (Sigma, USA), comenic acid (was kindly provided by Prof. Ivan Domnin, St.Petersburg, Russia) were added to the experimental Petri dishes in appropriate concentrations. Control explants were cultured in a standard culture medium free of drugs.

Differential interference contrast (DIC) microscopy

A PlasDIC-contrast was used for better visualization of organ cultures growth in plastic Petri dishes. The PlasDIC-contrast method, also known as DIC-microscopy, in experimental work with plastic dishes. For the best possible image contrast the correction for Petri dish bottom thickness was set at 1 mm (objective lens Carl Zeiss) was experimentally established. Standard protocol (for glass dishes) – correction was set at 0.17 mm. We used inverted optical microscope Axio Observer Z1 (Carl Zeiss, Germany).

The condenser of a microscope is equipped with a slit diaphragm with the size of numerical aperture of 0.55 and a working distance of 0.26 mm. The microscope is equipped with a set of lenses with achromatic objective (A-Plan) and semi-apochromatic (Plan-Neofluar) type of optical correction and long working distance: A-Plan 10x/0.25 (№ 441030-0000-000); LD Plan-Neofluar 20x/0.4 Korr (№ 421350-9970-000); LD Plan-Neofluar 40x/0.6 Korr (№ 421360-9970-000); LD Plan-Neofluar 63x/0.75 Korr (№ 421380-9970-000). The optical objectives with the magnification ratio equal to, or exceeding, 20x are also equipped with adjusting frame mechanism, allowing for image quality correction, facilitating switching between plastic dishes with different glass cover thicknesses, which might differ from the cover with standard thicknesses of 0.17 mm.

Confocal microscopy

A laser scanning confocal microscope (LSM-710, Carl Zeiss, Germany) was integrated with a Axio Observer Z1 inverted optical microscope (Carl Zeiss, Germany). For accumulation, storage and processing of experimental data software package for analysis and image visualization ZEN_2014 (Carl Zeiss, Germany) was used.

Intravital staining of cardiac tissue explants was carried out using phalloidin, conjugated with a fluorescent dye Texas Red (Texas Red®-X phalloidin, Life Technologies, USA) for 45 min at 37°C with 5 units of Texas Red®-X Phalloidin. Phalloidin specifically binds with F-actin and allows to evaluate actin cytoskeleton of cells in the growth zone. Texas Red was excited at $\lambda = 591$ nm, the dye emitted fluorescence in the red region of the spectrum ($\lambda = 608$ nm).

The nuclei of cells were counterstained with 0.1 ug/ml 4',6'-diamidino-2-phenylindole (DAPI; Sigma, USA) for 10 minutes. Fluorescence caused by embedding the dye in the minor groove of double stranded DNA molecule. The luminescence excited by a diode laser with a wavelength of 405 nm, fluorescence was detected at $\lambda = 450-490$ nm.

Method of the optical cross-section reconstruction

The use of confocal microscope allows not only for 3D reconstruction of the investigated tissue explants, but also registers the formation of cell layers at different levels, to change the thickness of the emerging growth area, and to compare the data obtained in pharmacological effects with the data of control experiments.

For data analysis we used a special criterion – the thickness of the growth zone, measured in microns and the method of reconstruction of optical cross-sections. Other approaches are not usable to assess synthetic processes in the developing growth zone of layer-by-layer growth. It should be noted that until recently it was believed that the cells of the growth zones represent a monolayer

(Lopatina et al. 2015). The thickness of the growth zone in control experiments was approximately 15 μm (Fig. 2).

Statistical analysis

Cardiac tissue explants growth was quantified using the ImageJ software.

The data were processed with STATISTICA 8.0 and Student's *t*-test at $p < 0.05$.

RESULTS

Epinephrine was tested in a wide range of concentrations from 10^{-9} M to 10^{-14} M. The effect of epinephrine was dose-dependent (Fig. 3). Maximum trophotropic effect of the epinephrine was manifested in concentration 10^{-12} M. AI exceeded the reference values by 68%. Epinephrine (10^{-13} M) stimulated the growth of the cardiac tissue on the 50% (Fig. 3). The thickness of the growth zone did not differ from control values (15 μm).

Our previous studies have shown that norepinephrine stimulates the growth of cardiac tissue explants in effective concentration 10^{-12} M (Lopatina et al. 2005; Tsyrlin et al. 2006). Growth zone thickness was 45 μm .

In the presence of β_1 -adrenoreceptors blocker atenolol (10^{-4} M) the stimulatory effect of catecholamines was absent (Fig. 4). We hypothesized that β_1 -adrenoreceptors were involved in mediation of adrenergic trophotropic effects observed in our experiments (Fig. 4).

The results of experiments with the culture medium containing epinephrine (10^{-12} M) or norepinephrine (10^{-12} M) together with ouabain (10^{-8} M) indicate that the trophotropic effect of epinephrine and norepinephrine is likely to result from modulation of Na^+, K^+ -ATPase signaling function via β_1 -adrenoreceptors (Fig. 5). Probably, catecholamines control the non-pumping function of Na^+, K^+ -ATPase in receptor-coupled manner via β_1 -adrenoreceptors (Fig. 6).

Our previous studies have shown that ouabain (10^{-8} to 10^{-13} M) dose-dependently regulates the growth of explants of cardiac tissue (Lopatina et al. 2005). Ouabain (10^{-8} M) totally inhibited the growth and proliferation of the cardiac tissue explants. However, in concentration 10^{-10} M ouabain stimulated the growth of explants by 33% (Lopatina et al. 2005).

Src inhibitor PP2 in wide range concentration (60×10^{-6} – 10^{-8} M) dose-dependently regulated the growth of explants of cardiac tissue (Fig. 7a). Src inhibitor PP2 (10^{-8} M) has no effect on the growth of the cardiac tissue explants. In the presence of Src inhibitor PP2 (10^{-8} M) stimulating effect of ouabain (10^{-10} M) was absent. Src-kinase is involved in ouabain-induced modulation of heart remodeling (Fig. 7a, b).

Effect of comenic acid on the growth of cardiac tissue explants was evaluated in a concentration range from 10^{-6} to 10^{-10} M (Fig. 8a). At a concentration of 10^{-6} M comenic acid increased the growth of explants by 40% (Fig. 8a). In the growth zone cardiac tissue explants of 10-12-day old chicken embryo cardiomyocytes and fibroblasts were observed as in control experiments (Fig. 9). Comenic acid (10^{-7} M) stimulated the growth of explants of the cardiac tissue by 17% in relation to control value. In concentration of 10^{-8} M and 10^{-10} M comenic acid on the growth of explants of the cardiac tissue is not affected. Therefore, the action of a substance was a dose-dependent.

In order to clarify the role of Na^+, K^+ -ATPase in the observed effects of the comenic acid a special series of experiments was carried out. Ouabain (10^{-8} M) was added to the culture medium together with comenic acid (10^{-6} M). While 10^{-8} M ouabain alone completely blocked the growth of explants, comenic acid eliminated this inhibitory effect of ouabain. Area index of the experimental explants did not differ from the control value (Fig. 8b). The latter indicates that the comenic acid modulates the non-pumping function of Na^+, K^+ -ATPase not directly, but indirectly through the receptor.

Conclusions

Endogenous ouabain in chelate complex with Ca^{2+} ion directly modulates the non-pumping function of Na^+, K^+ -ATPase (Lopatina et al. 2008; Rogachevskii 2008). The remainder rhamnose of ouabain plays a very important role in the realization of its physiological effects (Kipenko et al. 2009; Lopatina et al. 2008a; Rogachevskii et al. 2011). Src-kinase is involved in ouabain-induced modulation of the heart remodeling. It is shown by the method of optical cross sections reconstruction that the growth zone in organotypic culture forms a multidimensional structure. Trophotropic effects of catecholamines are mediated by modulating the non-pumping function of Na^+, K^+ -ATPase via β_1 -adrenoreceptors. Endogenous ouabain directly modulates the non-pumping function of Na^+, K^+ -ATPase, while comenic acid and catecholamines affect the ATPase indirectly. Probably, Na^+, K^+ -ATPase forms a complex with the receptor for comenic acid. The signal to initiate non-pumping function is binding of the ligand (comenic acid) with receptor of the membrane of excitable cells. Directional modulation of growth and proliferation of cardiomyocytes and fibroblasts in the embryonic period of ontogenesis may be associated with the signaling function of the Na^+, K^+ -ATPase. The trophotropic effects of the investigated agents specifically control the heart remodeling phenomenon.

Acknowledgement

The experiments were carried out using the equipment of shared-use Centre “Confocal microscopy” at Pavlov Institute of Physiology, Russian Academy of Sciences.

The authors are grateful to Professor Ivan Domnin.

This work was supported by the Russian Science Foundation (№14-15-00677 to Boris V. Krylov).

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Legend

Fig. 1. Microphotograph of 10-12-day old chicken embryo cardiac tissue explant. Control experiments (3rd day of culturing, lens 10, LSM-710, Carl Zeiss). Plas-DIC contrast.

a – Central zone (more dense)

b – The growth zone

Fig. 2. The fragment of the growth zone cardiac tissue explant of 10-12-day old chicken embryo. Microphotograph. Control (3rd day of cultivation, lens 40, LSM-710, Carl Zeiss). Red – F-actin (phalloidin, conjugated with Texas Red). Blue – nucleus (DAPI). 3D imaging, step size of optical cross-section 0.2 μm . The thickness of the growth zone 15 μm .

a – Cardiomyocytes

b – Fibroblasts

Fig. 3. Trophotropic effect of epinephrine.

The Area Index value was taken as 100% in the control experiments.

* – $p < 0.05$. Significant differences from control

Fig. 4. β_1 -adrenoreceptors are involved in trophotropic effects of epinephrine and norepinephrine.

Epinephrine (10^{-12} M), norepinephrine (10^{-12} M), atenolol (10^{-4} M).

The Area Index value was taken as 100% in the control experiments.

* – $p < 0.05$. Significant differences from control

Fig. 5. Epinephrine and norepinephrine receptor-mediated modulates the transducer function of Na^+ , K^+ -ATPase. Epinephrine (10^{-12} M), norepinephrine (10^{-12} M), ouabain (10^{-8} M).

The Area Index value was taken as 100% in the control experiments.

* – $p < 0.05$. Significant differences from control

Fig. 6. Catecholamines control the non-pumping function of Na^+ , K^+ -ATPase in receptor-coupled manner via β_1 -adrenoreceptors.

L – Catecholamines

Fig. 7. Src-kinase is involved in ouabain-induced modulation of heart remodeling.

a – Src inhibitor PP2 (60×10^{-6} – 10^{-8} M) dose-dependently regulated the growth of explants of cardiac tissue.

b – In the presence of Src inhibitor PP2 (10^{-8} M) stimulating effect of ouabain (10^{-10} M) was absent.

The Area Index value was taken as 100% in the control experiments.

* – $p < 0.05$. Significant differences from control

Fig. 8. Comenic acid modulates the non-pumping function of Na^+ , K^+ -ATPase indirectly, through the receptor.

a – Comenic acid (10^{-6} to 10^{-10} M) stimulated the growth of explants of the cardiac tissue dose-dependent.

b – Comenic acid (10^{-6} M) eliminated inhibitory effect of ouabain (10^{-8} M).

The Area Index value was taken as 100% in the control experiments.

* – $p < 0.05$. Significant differences from control

Fig. 9. Fragment of the growth zone cardiac tissue explant of 10-12-day old chicken embryo. Microphotograph. After comenic acid 10^{-6} M staining (3rd day of cultivation, lens 40, LSM-710, Carl Zeiss). Red – F-actin (phalloidin, conjugated with Texas Red). Blue – nucleus (DAPI). 3D imaging, step size of optical cross-section $0.2\ \mu\text{m}$. The thickness of the growth zone $20\ \mu\text{m}$.

a – Cardiomyocytes

b – Fibroblasts

Draft

Draft