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EFFECT OF PHOSPHOLIPASE C INHIBITION ON VASCULAR SMOOTH MUSCLE CONTRACTION

WPŁYW INHIBITORA FOSFOLIPAZY C NA KURCZLIWOŚĆ MIĘŚNI GŁADKICH NACZYŃ

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Summary

Phospholipase C is the key enzyme responsible for the hydrolysis of membrane phospholipids phosphatidylinositol 4,5-bisphosphate (PIP_2) to two intracellular transmitters - diacylglycerol (DAG) and inositol triphosphate (IP_3). PLC activation of smooth muscle leads to an increase in the concentration of IP_3 and DAG which initiates the increase in the concentration of calcium ions into the cytoplasm as a result of the flow from the intracellular and then from extracellular pool.

Main aim of this study was to evaluate the effect of PLC inhibitor U-73122 on vascular smooth muscle contraction stimulated pharmacologically with phenylephrine (PHE, α_1-adrenoceptor agonist) and arg-vasopressin (AVP, vasopressin receptor agonist).

Material and methods. Experiments were performed on isolated and perfused tail artery of Wistar rats. Contraction force in our model was measured by increased level of perfusion pressure with a constant flow.

Results. Reactivity of vascular smooth muscle cells to PHE (10^{-9} - 10^{-3} M/L), and AVP (10^{-10} – 10^{-4} M/L), in the control group and in the presence of U-73122 – phospholipase C inhibitor at concentrations 1, 3 and 10 μM/l was analyzed. EC_{50} values calculated for PHE and AVP were 7.52 (±0.97) $x10^{-8}$ M/L and 1.84 (±0.6) $x10^{-8}$ M/L, respectively. The concentration-response curves obtained for PHE and AVP in the presence of U-73122 were shifted to the rightward with a decrease in maximal responses. In the presence of U-73122 the significant and dose dependent reduction in perfusion pressure was found for both agonists for calcium influx from intracellular calcium stores and from extracellular space.

Conclusion. Results of our experiments suggest that in the presence of phospholipase C inhibitors, contraction induced by G-protein coupled receptors activation may by effectively inhibited by reduction in calcium influx from intra and extracellular calcium stores.

Streszczenie

Fosfolipaza C jest kluczowym enzymem odpowiedzialnym za hydrolizę błonowego fosfolipidu fosfatydyloinozyto-lodwufosforanu (PIP_s) do dwóch wewnątrzkomórkowych wtórnych przekaźników – diacyloglicerolu (DAG) oraz trójfosforanu inozytolu (IP_3). Aktywacja PLC zlokalizowanej w mięśniach gładkich naczyń prowadzi do wzrostu stężenia IP_3 i DAG inicjując napływ jonów wapnia do cytoplazmy z puli początkowo wewnątrzkomórkowej, a następnie zewnątrzkomórkowej.

Celem niniejszej pracy była ocena wpływu inhibitora PLC (U-73122) na skurcz mięśni gładkich naczyń, stymulowanych farmakologicznie fenylefryną (PHE, agonistą α1-adrenergicznego) i arg-wazopresyną (AVP, agonistą receptora wazopresynowego).

Materiał i metody. Badania przeprowadzono na izolowanych i perfundowanych tętnicach szczurów szczepu Wistar.

Wykładnikiem siły skurczu mięśni gładkich tętnicy było ciśnienie płynu perfuzyjnego.

Wyniki. Oceniano reaktywność mięśni gładkich naczyń stymulowanych Phe (10^{-9} - 10^{-3} M/L) i AVP (10^{-10} - 10^{-4} M/L), w grupie kontrolnej oraz w obecności U-73122 -

inhibitora fosfolipazy C w stężeniach 1, 3 i 10 μM/L. Wartości EC_{50} obliczone dla Phe i AVP wynosiły odpowiednio 7,52 (±0,97) $x10^{-8}$ M/L i 1,84 (± 0,6) $x10^{-8}$ M/L. Krzywe zależności odpowiedzi od stężenia otrzymane dla Phe i AVP w obecności U-73122 były przesunięte w stronę prawą z jednoczesną redukcją odpowiedzi maksymalnych. W obecności U-73122 stwierdzono także istotne i zależne od stężenia obniżenie ciśnienia perfuzyjnego w przypadku zarówno napływu wapnia z magazynów wewnątrzkomórkowych jak i z przestrzeni zewnątrzkomórkowej.

W n i o s k i . Wyniki doświadczeń wskazują, że obecność fosfolipazy C, skutecznie zmniejsza reaktywność mięśni gładkich naczyń krwionośnych po stymulacji receptorów sprzężonych z białkiem G i prowadzi do ograniczenia napływu wapnia do cytoplazmy zarówno z wewnątrzkomórkowych, jak i zewnątrzkomórkowych magazynów.

Key words: phospholipase C, adrenoceptors, vasopressin receptor
Słowa kluczowe: fosfolipaza C, receptor adrenergiczny, receptor wazopresynowy

INTRODUCTION

Phospholipase C is the key enzyme responsible for the hydrolysis of membrane phospholipids phosphatidylinositol 4,5-bisphosphate (PIP_2) to two intracellular transmitters - diacylglycerol (DAG) and inositol triphosphate (IP_3). Transduction based on membrane phospholipids is responsible for providing information about the stimulation of cells by more than 100 of extracellular agonists. An important fact is that intracellular signal is not only the increase in concentration of IP_3 and DAG but also lowering the membrane concentration of PIP_2, which is an activator of phospholipase D and phospholipase A_2 and then determines the activity of membrane proteins as ion channels or transport proteins [1, 2, 3]. Currently, 11 subtypes of PIP_2 specific phospholipase C are classified into 4 basic groups: β (subtypes β1, β2, β3 and β4), γ (γ1 and γ2 subtypes), δ (subtypes δ1, δ2, δ3 and δ4), ε (one type - ε). PLC-β is activated by the α subunit of $G_{q/11}$ protein of adrenergic receptors α1-type, angiotensin II type 1, V1 type vasopressin, bombesin, bradykinin, histamine H1, muscarinic receptors (M_1, M_3, and M_5) and the protein βγ subunit of G-coupled receptors: muscarinic M_2-type and receptors for interleukin 8. Furthermore, the activation of α_1-adrenergic receptor or receptors for thromboxane or oxytocin activates PLC-δ. PLC-γ is activated by tyrosine kinases, cytokine receptors, or fibroblast growth factor and others. In the cell membrane of the smooth muscle cells of the rat tail artery the PLC-β is present. PLC-δ and PLC-γ subtypes were found only in the cytoplasm [4, 5].

PLC activation of smooth muscle leads to an increase in the concentration of IP_3 and DAG which initiates the increase in the concentration of calcium ions into the cytoplasm as a result of the flow from the intracellular, and then from extracellular pool [1, 6, 7]. Non-selective PLC inhibitors are edelfosine - ET-18-OCH3 [8] and U-73122 [9, 10], which reduce smooth muscle contraction efficiency by reducing the influx of calcium into the cytoplasm. In addition to receptor activation, it is possible to directly activate the phospholipase C and additionally increase calcium concentration in the cytoplasm using m-3F3FBS [11], but the selectivity of this action is still debated [6, 12, 13]. Moreover, beyond simply increasing influx of calcium in response to increased levels of IP_3 and DAG, the induction of apoptosis in monocytic leukemia cells lines may suggest the therapeutic effect of activators of phospholipase C [14].

Main aim of this study was to evaluate the effect of PLC inhibitor U-73122 on vascular smooth muscle contraction stimulated pharmacologically with phenylephrine (α_1-adrenoceptor agonist) and arg-vasopressin (vasopressin receptor agonist).

MATERIALS AND METHODS

Animals

The experiments were performed on isolated, perfused Wistar rat tail arteries. Animals were housed under a 12 h light/12 h dark cycle and had unlimited access to food and water. Rats weighing 250-350 g were anesthetized by intraperitoneal injection of 120 mg urethane per 1 kg of body mass, stunned and then sacrificed by cervical dislocation. The study protocol was approved by the Local Ethics Committee and all experiments were carried out in accordance with the United States NIH guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication No. (NIH) 85-23: Office of Science and Health Reports, DRR/NIH, Bethesda, MD, U.S.A.].

Drugs and solutions

Krebs solution contained NaCl (71.8 mM/l), KCl (4.7 mM/l), $CaCl_2$ (1.7 mM/l) $NaHCO_3$ (28.4 mM/l), $MgSO_4$ (2.4 mM/l), KH_2PO_4 (1.2 mM/l) and glucose (11.1 mM/l). All reagents were purchased from Sigma Aldrich Chemical Co.

Study design and conduction

After dissection from surrounding tissues, 2.5 to 3.0 cm long segments of rat tail arteries were cannulated and connected to a perfusion device. The distal part was weighted with a 500 mg weight and the arteries were placed in a 20-mL container filled with oxygenated Krebs solution at 37°C. The perfusion pressure was continuously measured. Perfusion fluid flow was increased gradually to 1 ml/min. The increase in pressure of the perfusate in the experimental system was an exponent of vessel spasm [15]. Investigations were performed on TSZ-04 system, Experimetria Ltd. Budapest. Perfusion pressure was measured on BPR-01 and BPR-02 transducers connected with digital recorder Graphtec midi Logger GL820. All transducers used in our experiments were made by Experimetria Ltd, Budapest. Peristaltic pump was made by ZALIMP.

Data analysis and statistical procedures

Concentration-response curves (CRCs) were calculated according to the van Rossum method. The maximal response of tissue (Emax) was calculated as a percent of the maximal response for PHE. Half maximal effective concentration (EC_{50}) was estimated using classical pharmacologic methods with pD_2 the negative logarithm of the EC_{50}. We used the number of the CRC and Emax in all calculations estimating the statistical significance.

Results were presented as means ± standard deviations. The Shapiro-Wilk test was used to determine normal distribution of the investigated variables. Statistical analysis was performed using the Newman-Keuls and ANOVA test for multiple comparisons of means. A two-sided difference was considered significant at $p<0.05$.

RESULTS

Effect of U-73122 on the contractility of vascular smooth muscle contraction

In this part of our experiments, the reactivity of VSMC to PHE (10^{-9} - 10^{-3} M/L), a preferential α_1-adrenoceptor agonist, and AVP (10^{-10} – 10^{-4} M/L), a non-selective vasopressin receptor agonist in the control group and in the presence of U-73122 – phospholipase C inhibitor at concentrations 1, 3 and 10 μM/l was analyzed. EC_{50} values calculated for PHE and AVP were 7.52 (±0.97) $x10^{-8}$ M/L and 1.84 (±0.6) $x10^{-8}$ M/L, respectively. The CRCs obtained for PHE and AVP in the presence of U-73122 were shifted to the rightward with a decrease in maximal responses (Figure 1 and 2). Under these conditions EC_{50} values for PHE were 1.74 (±0.69) $x10^{-7}$ M/l ($p<0.0001$), 2.45 (±0.82) $x10^{-7}$ M/l ($p<0.0001$) and 4.83 (±0.9) $x10^{-6}$ M/l ($p<0.0001$), and for AVP 7.63 (±0.64) $x10^{-8}$ M/l ($p<0.0001$), 1.88 (±0.77) $x10^{-7}$ M/l ($p<0.0001$) and 8.25 (±1.0) $x10^{-7}$ M/l ($p<0.0001$) respectively (Tab I and II, Fig. 3 and 4).

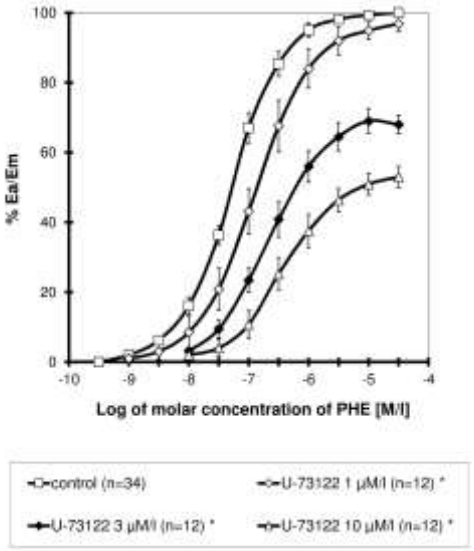


Fig. 1. *Concentration response curves for phenylephrine in the control and in the presence of U-73122, phospholipase C inhibitor. (* - p<0.05 vs. control for all points for effect range 20-80%)*

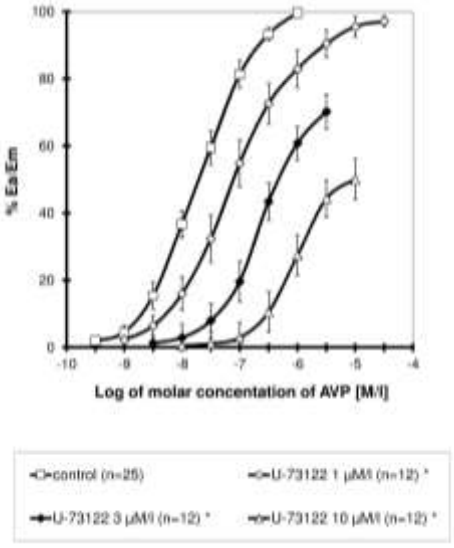


Fig. 2. *Concentration response curves for arg-vasopressin in the control and in the presence of U-73122, phospholipase C inhibitor. (* - p<0.05 vs. control for all points for effect range 20-80%)*

Table I. *EC_{50}, maximal response and relative potency for phenylephrine for controls and in the presence of U-73122, phospholipase C inhibitor*

	N[1]	%E_{max}[2]	EC_{50} [M/l]	pD_2	RP[3]	P[4]
controls	34	100	7.52 (±0.97) x10^{-8}	7.12±0.06	1.000	—
U-73122 1 μM/l	12	98±1	1.74 (±0.69) x10^{-7}	6.76±0.09	0.432	<0.0001
U-73122 3 μM/l	12	68±5	2.45 (±0.82) x10^{-7}	6.61±0.12	0.307	<0.0001
U-73122 10 μM/l	12	54±1	4.83 (±0.9) x10^{-7}	6.32±0.09	0.156	<0.0001

[1]- number of concentration-response curves used for calculations, [2] – Emax – calculated as a percent of maximal response for controls, [3] – RP - relative potency – calculated as EC_{50} for controls / EC_{50}, [4] - p-value calculated in comparison to control values

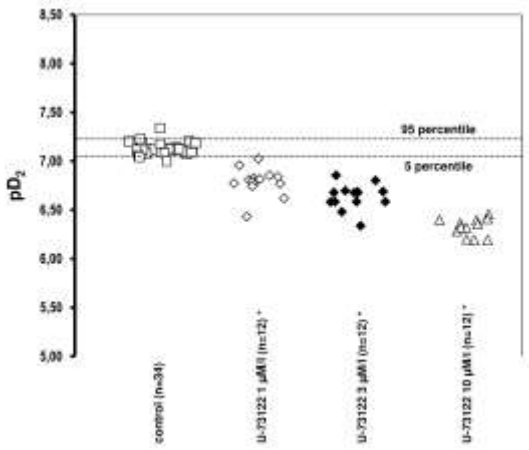


Fig. 3. *EC_{50} for phenylephrine induced in the control and in the presence of U-73122, phospholipase C inhibitor*

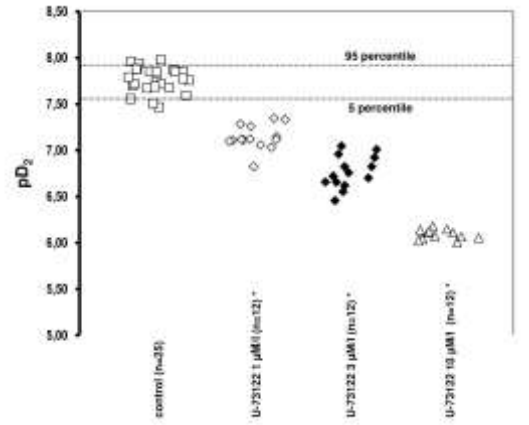


Fig. 4. *EC_{50} for arg-vasopressin induced in the control and in the presence of U-73122, phospholipase C inhibitor*

Table II. *EC_{50}, maximal response and relative potency for arg-vasopressin for controls and in the presence of U-73122, phospholipase C inhibitor*

	N[1]	%E_{max}[2]	EC_{50} [M/l]	pD_2	RP[3]	P[4]
controls	25	100	1.84 (±0.60) x10^{-8}	7.74±0.11	1.000	—
U-73122 1 μM/l	12	97±4	7.63 (±0.64) x10^{-8}	7.12±0.14	0.241	<0.0001
U-73122 3 μM/l	12	69±3	1.88 (±0.77) x10^{-7}	6.73±0.14	0.097	<0.0001
U-73122 10 μM/l	12	51±5	8.25 (±1.0) x10^{-7}	6.08±0.09	0.022	<0.0001

[1]- number of concentration-response curves used for calculations, [2] – Emax – calculated as a percent of maximal response for controls, [3] – RP - relative potency – calculated as EC_{50} for controls / EC_{50}, [4] - p-value calculated in comparison to control values

Influence of U-73122 on mobilization of Ca^{2+} ions from endoplasmic reticulum and extracellular space

In the performed experiment, contraction of arteries was triggered separately by PHE (10^{-5}M/l) and AVP (10^{-5} M/l). Values of pressure changes were presented as mean ± standard deviation. Investigation was made in a fluid without Ca^{2+}. The mean values of perfusion pressure, induced by PHE and AVP were 58.2 (±7.1) mmHg and 61.9 (±4.5) mmHg. In the presence of U-73122 the significant and dose dependent reduction in perfusion pressure was found for both agonists (Tab 3, 4). Second part of the experiment was made in Krebs solution containing Ca^{2+} to measure tissue answer on calcium influx from extracellular calcium stores. The mean value of perfusion pressure, induced by PHE and AVP was 94.1 (±7.6) mmHg and 103.4 (±6.1) mmHg, respectively. In the presence of U-73122 the significant and dose dependent reduction in perfusion pressure was found for both agonists (Tab 3, 4, Figure 5, 6).

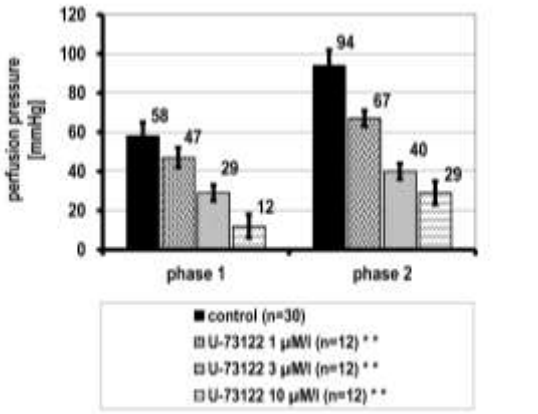
Table III. *Maximal perfusion pressure phenylephrine induced contraction activated by calcium influx from intracellular (phase 1) and extracellular calcium stores (phase 2), for controls and in the presence of U-73122, phospholipase C inhibitor*

	Intracellular calcium Phase 1		Extracellular calcium Phase 2	
	n	**perfusion pressure (±SE) [mmHg]**	**n**	**perfusion pressure (±SE) [mmHg]**
controls	**30**	**58.2 (±7.1)**	**30**	**94.1 (±7.6)**
U-73122 1 μM/l	**12**	**47.4 (±5.0)***	**12**	**67.2 (±4.4)***
U-73122 3 μM/l	**12**	**28.9 (±4.7)***	**12**	**39.6 (±4.2)***
U-73122 10 μM/l	**12**	**12.1 (±5.4)***	**12**	**28.8 (±5.5)***

* - p<0.0001 vs. control

Table IV. *Maximal perfusion pressure for arg-vasopressin induced contraction activated by calcium influx from intracellular (phase 1) and extracellular calcium stores (phase 2), for controls and in the presence of U-73122, phospholipase C inhibitor*

	Intracellular calcium Phase 1		Extracellular calcium Phase 2	
	n	perfusion pressure (±SE) [mmHg]	n	perfusion pressure (±SE) [mmHg]
controls	**32**	**61.9 (±4.5)**	**32**	**103.4 (±6.1)**
U-73122 1 μM/l	**12**	**39.1 (±4.6)***	**12**	**81.0 (±6.2)***
U-73122 3 μM/l	**12**	**28.1 (±4.3)***	**12**	**59.3 (±6.2)***
U-73122 10 μM/l	**12**	**13.2 (±4.4)***	**12**	**27.1 (±6.1)***



* - p<0.0001 vs. control

Fig. 5. *Impact U-73122 on perfusion pressure triggered with intra- and extracellular calcium pool in comparison to the controls for phenylephrine induced contraction*

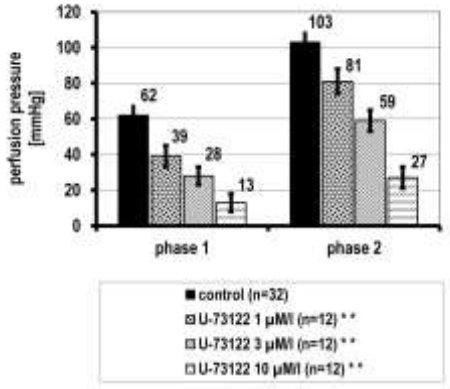


Fig. 6. *Impact U-73122 on perfusion pressure triggered with intra- and extracellular calcium pool in comparison to the controls for arg-vasopressin induced contraction*

DISCUSSION

Non-competitive inhibition is not related to blocking of the receptor function by competing with physiological receptor agonist binding site, but it is associated with a reduction in response triggered by an agonist receptor stimulation. For these reasons, non-competitive inhibition leads to a concentration-dependent reduction in response to receptor antagonist. The corner stone in the pathway of activation of phospholipase C via $G_{q/11}$ protein is a separation of pathway activation. The arm associated with the inositol triphosphate is responsible for the activation of calcium influx from the intracellular space, whereas the arm diacylglycerol-mediated activation is associated with the influx of calcium from the extracellular space. This last process is partly dependent on a prior increase in calcium concentration in the cytoplasm derived from the intracellular space, since protein kinase type C enzyme directly responsible for the activation of calcium channel is calcium ions dependent [7, 16, 17].

Our experiments using phospholipase C inhibitor U-73122 resulted in a non-competitive reduction of arterial smooth muscle reactivity to stimulation by either phenylephrine and arginine vasopressin. Analyzing a perfusate pressure in the artery treated with U-73122 we observed concentration-dependent inhibition of smooth muscle function with the proportional reduction of the perfusion pressure to both formed after activation of calcium influx and intracellular space extracellular space.

Phospholipase C also plays an important role in inducing apoptosis. In the vascular endothelial cells is observed conflicting reactions - inhibition of phosphatidylinositol-specific phospholipase C induces apoptosis while inhibiting of phospholipase C-specific to phosphatidylcholine inhibits apoptosis [18, 19]. Phospholipase C is also involved in the activation of potassium channels of the membrane of B cells [20], and also is a vital link leading to an increase in calcium ion concentration in the cytoplasm of vascular endothelial cells treated with bradykinin [21].

The inhibition of calcium influx from the endoplasmic reticulum leads to a marked limitation of perfusion pressure prevailing in the artery, in the first phase muscle contraction, whereas inhibition of calcium influx through the channels of the membrane leads to reduced perfusion pressure, resulting in the second contraction phase - phase slow influx of calcium into the cytoplasm from the extracellular space [7, 16, 17, 22]. Physiological effect similar to inhibiting the inflow of outside and intracellular space can be achieved by impairing activation of both or one of the processes. The phenomena occurring in the case

of competitive inhibition will always lead to a reduction of shrinkage of the two phases, so it will inhibit the influx from both intracellular and extracellular space. Additionally, inhibition of both phases will occur in a proportionate manner, as is inhibited formation of all secondary relay as a result of the activation mechanisms of contraction [23].

CONCLUSIONS

Results of our experiments suggest that in the presence of phospholipase C inhibitors, contraction induced by G-protein coupled receptors activation may by effectively inhibited by reduction in calcium influx from intra and extracellular calcium stores.

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