Effect of 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzene-sulfonamide on calcium influx in three contraction models

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Abstract. 2,4,6-Trimethyl-*N*-[3-(trifluoromethyl)phenyl] benzenesulfonamide (m-3M3FBS) activates phospholipase C and stimulates apoptosis; however, in smooth muscle cells it may increase the perfusion pressure. The main aim of the present study was to evaluate the physiological effect of direct stimulation of phospholipase C on vascular smooth muscle reactivity using three contraction models. Experiments were performed on the isolated and perfused tail artery of Wistar rats. The contraction force in the present model was measured by an increased level of perfusion pressure with a constant flow. Concentration-response curves (CRCs) obtained for phenylephrine, arg-vasopressin, mastoparan-7 and Bay K8644 presented a sigmoidal association. In comparison to the control curves, CRCs in the presence of m-3M3FBS were significantly shifted to the left except for Bay K8644. Analyses of calcium influx suggest that in the presence of m-3M3FBS the calcium influx from intra- and extracellular calcium stores was significantly higher. The results of the present experiments suggest that m-3M3FBS significantly increases the reactivity of vascular smooth muscle stimulated with metabotropic receptors or G-protein by an increase in calcium influx from intra- and extracellular calcium stores. The current knowledge regarding the apoptotic pathway shows the significance of calcium ions involved in this process, thus, m-3M3FBS may induce apoptosis by an increase of cytoplasmic calcium concentration; however, simultaneously, the use of this mechanism in therapy must be preceded by a molecular modification that eliminates a possible vasoconstriction effect.

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Introduction

Phospholipase C is the key enzyme responsible for the hydrolysis of membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two intracellular-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Transduction based on membrane phospholipids is responsible for providing information regarding the stimulation of cells by >100 of extracellular agonists. Notably, the intracellular signal is not only restricted to increasing the concentration of IP_3 and DAG, but also lowering the concentration in the membrane PIP_2, which is an activator of phospholipase D and phospholipase A_2, which subsequently determines the activity of a number of membrane proteins as ion channels or proteins conditioning the active transport (1-3). Currently, the key subtypes of phosphoinositide-specific phospholipase C were classified into 4 basic groups known as: β (subtypes β1, β2, β3 and β4), γ (γ1 and γ2 subtype), δ (subtypes δ1, δ2, δ3 and δ4) and ε (one type-ε). PLC-β is activated by the α subunit of the $G_{q/11}$ protein of the adrenergic receptors, α1-type receptors, angiotensin II type 1, type V1 vasopressin, bombesin, bradykinin, histamine H1, muscarinic receptors (M1, M2, and M3) and a subunit βγ of G-protein coupled with muscarinic M_2-type receptors and interleukin 8 receptors. In addition, the type of activation of the α_1 adrenergic receptor, the receptor for oxytocin or thromboxane, activates PLC-δ. PLC-γ can be activated by tyrosine kinases, cytokine receptors or fibroblast growth factor.

In the cell membrane of the smooth muscle cells of the rat tail artery, the PLC-β subtype was present, whereas PLC-δ and PLC-γ subtypes were identified only in the cytoplasm (4,5).

Activation of PLC smooth muscle leads to an increase in the concentration of IP_3 and DAG, which initiates the increase in the concentration of calcium ions in the cytoplasm as a result of the flow from the intracellular pool, and subsequently out of the extracellular pool (1,6). Non-selective inhibitors of PLC, such as edelfosine-ET-18-OCH3 (7) and U-73122 (8-10), reduce the efficiency of smooth muscle contraction by reducing the influx of calcium into the cytoplasm. In addition to receptor activation, it is possible to directly activate phospholipase C and this significantly increases the calcium ion concentration in the cytoplasm by 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)

phenyl]benzenesulfonamide (m-3M3FBS) (11,12); however, the selectivity of this action remains controversial (6,13,14). It was further found, beyond simply increasing the calcium influx by increased concentrations of inositol triphosphate and diacylglycerol, to be an effect of inducing apoptosis in monocytic leucaemia lines, which may suggest the possible therapeutic effect of activators phospholipase C (15). The study by Chen *et al* (16) observed the increased calcium influx and apoptosis in SCM1 human gastric cancer cells. Liu *et al* (17) observed a similar effect in HA59T human hepatoma cells. The modulatory effect of m-3M3FBS on smooth muscle reactivity in lipopolysaccharide-pretreated tissue was reported by Grześk (18). This protective effect of m-3M3FBS was confirmed by Kim *et al* (19).

To the best of our knowledge, there are no experiments directly analyzing the efficaciousness of m-3M3FBS stimulation in vascular smooth muscle cells, thus, the present study analyzed the real efficaciousness of m-3M3FBS in the modulation of vascular smooth muscle tone in an experimental model of small resistant artery.

Materials and methods

Animals. The study was performed on isolated, perfused arteries. Male Wistar rats 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 subsequently sacrificed by cervical dislocation. The study protocol was approved by the Local Ethics Committee of the University of Science and Technology (Kraków, Poland) and all the 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, USA].

Drugs and solutions. The experiments were performed to determine the role of intra- and extracellular calcium ions in contraction induced by phenylephrine and arg-vasopressin in the control conditions and in the pretreated arteries using two types of Krebs fluid: i) Ca^{2+}-free physiological salt solution (PSS) EGTA-Krebs with the following composition: NaCl (71.8 mM/l), KCl (4.7 mM/l), $MgSO_4$ (2.4 mM/l), $NaHCO_3$ (28.4 mM/l), KH_2PO_4 (1.2 mM/l) and glucose (11.1 mM/l) with the addition of EGTA (30 μM/l); and ii) PSS-fluid with Ca^{2+} EGTA-Krebs (normal) with the following composition: NaCl (71.8 mM/l), KCl (4.7 mM/l), $MgSO_4$ (2.4 mM/l), $NaHCO_3$ (28.4 mM/l), KH_2PO_4 (1.2 mM/l), $CaCl_2$ (1.7 mM/l) and glucose (11.1 mM/l) with the addition of EGTA (30 μM/l), subsequent to emptying the intracellular pool of calcium ions.

Study design and conduction. Segments (2.5 to 3.0 cm in length) of the rat tail arteries were gently dissected from surrounding tissues, and the proximal segment was cannulated and connected to the perfusion equipment. The arteries were placed in a 20-ml isolated organ bath filled with oxygenated Krebs solution at 37˚C. In the initial part of the experiment, perfusion fluid flow was increased gradually to 1 ml/min. The changes in continuously measured perfusate pressure in the experimental system were an exponent of arterial smooth muscle contractility. Investigations were performed using the isolated organ bath system (TSZ-04; Experimetria Ltd., Budapest, Hungary). Perfusion pressure was measured on BPR-01 and BPR-02 transducers (Experimetria Ltd.) connected with a Graphtec midi Logger GL820 digital recorder. The peristaltic pump was made by ZALIMP (Warsaw, Poland) (20,21).

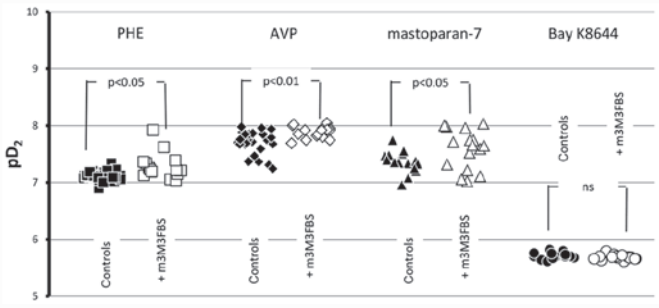

Figure 1. Half maximal effective dose values for phenylephrine, arg-vasopressin, mastoparan-7 and Bay K8644 in the control and in the presence of m-3M3FBS. n.s., not significant.

Data analysis and statistical procedures. The classical pharmacometric van Rossum method was used to calculate concentration-response curves (CRCs) (21,22). The maximal effect (E_{max}) of tissue stimulation was calculated as a percentage of the maximal response for the respective agonist. The half maximal effective dose (ED_{50}) was calculated using the classical pharmacological methods with pD_2, the negative logarithm of the ED_{50} (20-22). The number of the CRCs and E_{max} were used in all calculations estimating the statistical significance.

Data are presented as mean ± standard deviations. The Shapiro-Wilk test was used to determine the normal distribution of the investigated variables. Statistical analysis was performed using the Newman-Keuls and analysis of variance test for multiple comparisons of the means. A two-sided difference of $P<0.05$ was considered to indicate statistical significance.

Results

CRCs. The CRCs obtained for phenylephrine, arg-vasopressin, mastoparan-7 and Bay K8644 presented a sigmoidal association. The curve obtained for phenylephrine, arg-vasopressin and mastoparan-7 in the presence of m-3M3FBS were shifted to the left. For all the points for a relative effect of ≥20%, the differences were statistically significant. Calculated EC_{50} values were significantly lower (Fig. 1, Table I). The curves obtained for Bay K8644 in the presence of m-3M3FBS did not differ significantly compared to the control (Fig. 1). The calculated E_{max}, EC_{50} and pD_2 values are presented in Table I.

Presence of m-3M3FBS. Using the second experimental model, the maximal perfusion pressure following stimulation of the calcium influx from intracellular (phase 1) and extracellular (phase 2) calcium stores was measured for the control and in the presence of m-3M3FBS (10^{-5} M/l). In the presence of m-3M3FBS, a significant increase in calcium influx induced by phenyleprine, arg-vasopressin and mastoparan-7 from the intra- and extracellular space was observed. Artery

Table I. EC_{50}, maximal response and relative potency for phenylephrine, arg-vasopressin, mastoparan-7 and Bay K8644 for the controls and in the presence of phospholipase activator m-3M3FBS.

Treatment	n[a]	$\%E_{max}$[b]	EC_{50} [M/l]	pD_2	RP[c]	P-value[d]
PHE	30	100	$7.50\pm0.98x10^{-8}$	7.12±0.06	1.000	-
PHE+m-3M3FBS	16	111±11	$6.45\pm2.10x10^{-8}$	7.19±0.17	1.163	0.0182
AVP	25	100	$1.84\pm0.62x10^{-8}$	7.74±0.15	1.000	-
AVP+m-3M3FBS	16	117±11	$1.42\pm0.45x10^{-8}$	7.85±0.13	1.296	0.0071
Mastoparan-7	16	100	$4.48\pm2.36x10^{-8}$	7.34±0.21	1.000	-
Mastoparan-7+m-3M3FBS	16	101±12	$2.55\pm1.52x10^{-8}$	7.59±0.25	1.757	0.0112
Bay K8644	16	100	$1.96\pm0.26x10^{-6}$	5.71±0.08	1.000	-
Bay K8644+m-3M3FBS	16	99±9	$2.05\pm0.22x10^{-6}$	5.69±0.06	0.956	0.1824

[a]Number of concentration-response curves used for calculations; [b]$\%E_{max}$ calculated as a percent of maximal response for controls; [c]RP, relative potency calculated as EC_{50} for controls/EC_{50}; [d]P-value calculated in comparison to control values. EC_{50}, half maximal effect concentration; E_{max}, maximal tissue response; pD_2, negative logharitm of the EC_{50}.

Table II. Maximal perfusion pressure for phenylephrine, arg-vasopressin, mastoparan-7 and Bay K8644-induced contractions activated by calcium influx from intracellular (phase 1) and extracellular calcium stores (phase 2), for controls and in the presence of phospholipase activator m-3M3FBS.

	Intracellular calcium phase 1		Extracellular calcium phase 2	
Treatment	n	Perfusion pressure, mean mmHg ± SD	n	Perfusion pressure, mean mmHg ± SD
PHE	30	58.1±7.0	30	94.2±7.9
PHE+m-3M3FBS	16	67.3±6.2[a]	16	112.2±7.1[c]
AVP	32	62.5±6.5	32	103.4±5.9
AVP+m-3M3FBS	16	72.1±6.6[b]	16	118.2±7.5[c]
mastoparan-17	10	11.9±2.2[c]	10	11.2±2.6[c]
mastoparan-7	16	17.2±3.2	16	27.2±5.7
mastoparan-7+m-3M3FBS	16	21.2±3.2[a]	16	42.0±6.0[c]
Bay K8644	16	14.9±4.1	16	75.2±6.2
Bay K8644+m-3M3FBS	16	15.2±4.4	16	75.3±4.1

[a]P<0.05; [b]P<0.0002; [c]P<0.0001 vs. controls. n, number of concentration-response curves used for calculations; SD, standard deviation.

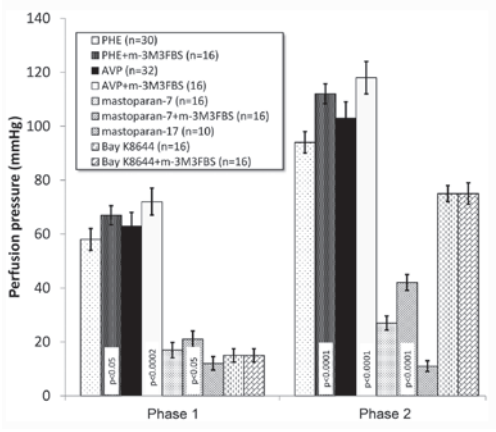

Figure 2. Impact of m-3M3FBS on perfusion pressure triggered with intra- and extracellular calcium pools in comparison to the controls.

contractility following stimulation with mastoparan-7 was significantly higher for both phases in comparison to the negative control, mastoparan-17 (Table II).

The presence of m-3M3FBS did not significantly change Bay K8644-induced contractions (Fig. 2, Table II).

Discussion

Activation of phospholipase C is a key link in numerous metabotropic receptors, including the receptors stimulated in the present study. Inhibition of the function of phospholipase C leads to a reduction in calcium ion concentration in the cytoplasm of cells activated by stimulation of α1-adrenergic receptors (23,24), endothelin type A (25) and angiotensin II receptor type-1 (26).

Previous studies have used biochemical methods to investigate the concentration of secondary messengers (DAG and IP_3) produced following phospholipase C activation and additionally the concentration of calcium ions in the cytoplasm (11,13). These early studies aided in the decision to use physiological and pharmacometric methods to evaluate the influence of these biochemical changes on vascular smooth muscle reactivity.

Certain results of biochemical experiments suggest the primary generation of free radicals by m-3M3FBS (11).

In response to m-3M3FBS, it is possibly the coexistence of several mechanisms, including primary free radical formation with secondary activation of ryanodinie receptors.

The results of the present study performed using the second experimental model indicate a balanced increase in calcium influx from an intracellular and extracellular calcium pool, suggesting a particular pathway activation at a stage no later than phospholipase C. Activation of the calcium influx following stimulation of the ryanodine receptor induces a calcium influx from the intracellular calcium stores only, and therefore the results suggest a direct activation of phospholipase C (27,28).

Contradictory results were reported in the study by Krjukova *et al* (13). In addition to the increase in cytoplasmic calcium concentration, the direct chemical markers of increased activity of phospholipase C were not observed. It was suggested that in the presence of m-3M3FBS the increase of membrane phospholipid metabolism is secondary to the production of oxygen free radicals.

Another experiment assessing m-3M3FBS was presented by Jansen *et al* (29). In studies of zinc metabolism in the cell, increased activity of phospholipase C manifested by an increase in cytoplasmic calcium concentration was observed in the presence of m-3M3FBS. A further study presented in 2005 confirmed these results (30).

The results confirming the study by Bae *et al* (11) were also reported in 2005 by Horowitz *et al* (6). All the exponents necessary to confirm the activation of phospholipase C were observed, and thus it was concluded that in the presence of $5x10^{-5}$ M/l m-3M3FBS, a significant activation of phospholipase C occurred. This may correspond to the reported increments in calcium concentration in the cytoplasm in previous studies (6,11,13,14). Furthermore, the increase of calcium concentration in the cytoplasm is important as it can lead to muscle contraction.

The increase in contractility of vascular smooth muscle cells in the presence of m-3M3FBS was reported in lipopolysaccharide-pretreated tissues (18). This protective effect of m-3M3FBS in sepsis was confirmed by Kim *et al* (19).

The results of the present experiments suggest that m-3M3FBS significantly increases reactivity of vascular smooth muscle stimulated with metabotropic receptors or G-protein by an increase in calcium influx from intra- and extracellular calcium stores. Current knowledge regarding the apoptotic pathway shows the significance of calcium ions involved in this process, thus, m-3M3FBS may induce apoptosis by an increase of cytoplasmic calcium concentration, but simultaneously, the use of this mechanism in therapy must be preceded by a molecular modification that eliminates a possible vasoconstriction effect.

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