Role of endothelium, acetylocholine and calcium ions in Bay K8644- and KCl-induced contraction

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Abstract. The aim of this study was to establish the involvement of acetylcholine (Ach) and calcium ions in modulating contractions induced by Bay K8644 (an agonist of calcium channels located in the cell membrane) and KCl (at depolarizing concentrations), and also to examine the importance of the vascular endothelium in the activity of Bay K8644. The study was performed on perfused Wistar rat tail arteries. Contraction induced by Bay K8644 with the participation of intracellular (in calcium-free physiological salt solution, FPSS) and extracellular (in physiological salt solution, PSS, following the emptying of the cellular Ca^{2+} stores) pools of Ca^{2+} and the addition of nitro-L-arginine (L-NNA; nitric oxide synthase inhibitor) or 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; an inhibitor of soluble guanylyl cyclase) was studied. In addition, the effect of Ach on the contraction response was analyzed and the results were compared with the depolarizing action of KCl. The effects of 8Br-cGMP on the artery contraction induced by Bay K8644 prior to and following removal of the endothelium were compared. Bay K8644 and KCl in PSS induced vascular contraction, which was reduced with the addition of Ach. The spasmolytic Ach action did not occur in the presence of L-NNA and ODQ. 8Br-cGMP reduced the contraction of arterial walls (with and without endothelium) induced by Bay K8644. The increase in vascular tone induced by Bay K8644 and KCl was independent of the intracellular calcium ion pool. The relaxant effect of Ach on the responses stimulated by Bay K8644 and KCl indicated the participation of nitric oxide in modulating the reactivity of the arteries to the factors examined, resulting in an influx of Ca^{2+} into the cell.

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Introduction

The structure of the arteries is important as serve as a target for a number of substances that regulate smooth muscle tension. The endothelium is a cell layer that lines the inside of blood vessels, and also produces and releases mediators that modulate the contraction of arteries (1,2).

Endothelial damage occurs in the course of various pathological processes, particularly atherosclerosis, and leads to vascular disorders with regard to diameter regulation, which potentially result in occlusion of the vessels. Although platelet aggregation is a natural recovery process following the injury of blood vessel walls, activation of the coagulation system following plaque rupture may result in a reduction or complete elimination of blood flow. Nitric oxide (NO) is a vasodilator agent that is produced by the endothelial cells by constitutive NO synthase (3). In smooth muscle cells, NO activates soluble guanylyl cyclase (GC) and the resulting increase in cyclic guanosine monophosphate (cGMP) levels leads to blood vessel dilatation (4,5). This effect may be as a result of the reduction in the concentration of Ca^{2+} in the cytoplasm (due to the influx of calcium into the cell or the inhibition of the release of calcium from intracellular stores), dephosphorylation of myosin light chains or interaction with the contractile system (6-10). The release of NO occurs under physiological conditions; however, it may be further stimulated by various factors, such as acetylcholine (Ach), bradykinin and histamine (11).

The aim of this study was to determine the significance of the signaling pathway associated with Ach in the contraction induced via the extracellular pool of Ca^{2+} by Bay K8644 (an agonist of calcium channels located in the cell membrane) and KCl (at depolarizing concentrations), and also to determine the importance of the vascular endothelium in the activity of Bay K8644.

Materials and methods

Reagents. The study was performed on perfused male Wistar rat tail arteries. Rats, weight 250-350 g, were narcotized by

Table I. EC_{50} values for Bay K8644 in the presence of 8Br-cGMP (experiments performed on arteries with/without endothelium).

Group	EC_{50} arteries with endothelium	EC_{50} arteries without endothelium
Bay K8644 (control)	$5.97(\pm0.21)x10^{-8}$	$1.37(\pm0.24)x10^{-8}$
8Br-cGMP (10 μM/l)	$4.07(\pm0.29)x10^{-7}$	$3.47(\pm0.21)x10^{-8}$
8Br-cGMP (30 μM/l)	$5.67(\pm0.26)x10^{-7}$	$2.17(\pm0.26)x10^{-7}$
8Br-cGMP (100 μM/l)	$7.12(\pm0.32)x10^{-7}$	$6.12(\pm0.22)x10^{-7}$

Values are presented as the mean ± SD. EC_{50}, half maximal effective concentration; cGMP, cyclic guanosine monophosphate.

intraperitoneal injection of 120 mg/kg of body mass. After being dissected and cleared from the surrounding tissue, 2.5-3-cm long segments of rat tail arteries were cannulated and connected to perfusion apparatus. Perfusion pressure was measured continuously. The distal part was weighted with a 500 mg weight and placed in a 20 ml container filled with oxygenated Krebs solution at 37˚C. The perfusion solution flow was gradually increased using a peristaltic pump until 1 ml/min was reached.

Two types of Krebs fluid were used in this study to determine the importance of the intracellular and extracellular pools of Ca^{2+} in the reactions induced by Bay K8644 (30 μM/l) and KCl (110 mM/l) under control conditions, following the addition of nitro-L-arginine (L-NNA; nitric oxide synthase, 10 μM/l) or 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; an inhibitor of soluble guanylyl cyclase; 10 μM/l), and in the presence of increasing Ach concentrations. The two Krebs solutions were as follows: i) fluid without Ca^{2+}-EGTA [Krebs (no calcium); calcium-free physiological salt solution, FPSS]; and ii) fluid with Ca^{2+}-EGTA [Krebs (normal); physiological salt solution, PSS]. The composition of the fluid without Ca^{2+}-EGTA was as follows: NaCl (71.8 mM/l), KCl (4.7 mM/l), $NaHCO_3$ (28.4 mM/l), $MgSO_4$ (2.4 mM/l), KH_2PO_4 (1.2 mM/l), glucose (11.1 mM/l) with the addition of EGTA (30 μM/l). The composition of the fluid with Ca^{2+}-EGTA was as follows: 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), glucose (11.1 mM/l) with addition of EGTA (30 μM/l), following the emptying of the intracellular pool of Ca^{2+}. All reagents were purchased from Sigma-Aldrich (Poznań, Poland).

Removal of the endothelium. In a number of cases, the endothelium of the arteries was removed using compressed air to determine the importance of the endothelium in the responses induced by Bay K8644 and following the addition of increasing concentrations of 8Br-cGMP (12).

Concentration-response curves (CRCs). CRCs were determined using the van Rossum method of increasing concentrations (12). Dose-dependency was determined for arteries with and without endothelium under control conditions and in the presence of 8Br-cGMP (10, 30 and 100 μM/l). The concentration of 8Br-cGMP that resulted in the half maximal effective concentration (EC_{50}) was determined using the method of linear regression for 20-80% maximal effect. Vessel contraction was measured as increase in perfusion pressure.

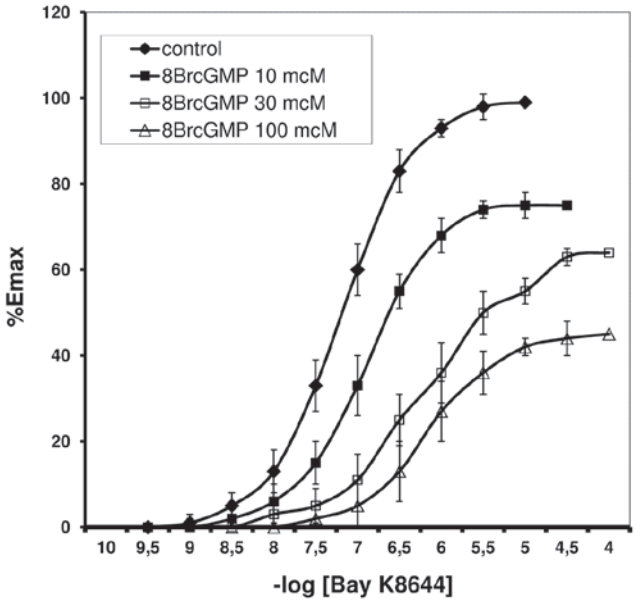

Figure 1. Concentration-response curves for Bay K8644 demonstrating the effect of 8Br-cyclic guanosine monophosphate (cGMP) on contraction of arteries with endothelium. Mean ± SD, n=8.

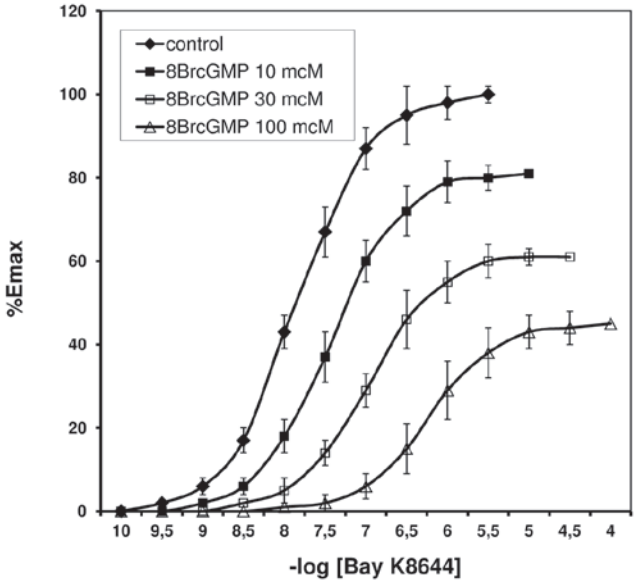

Figure 2. Concentration-response curves for Bay K8644 demonstrating the effect of 8Br-cyclic guanosine monophosphate (cGMP) on contraction of arteries without endothelium. Mean ± SD, n=8.

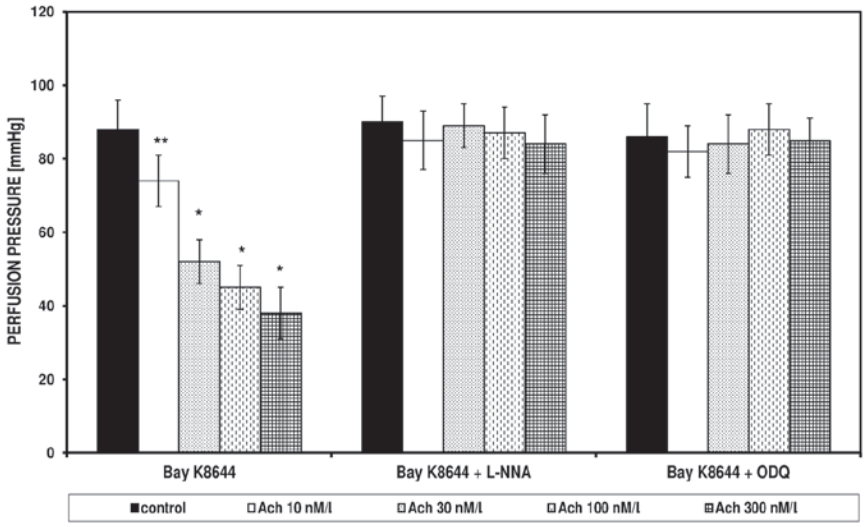

Figure 3. The influence of acetylocholine on the contraction induced by Bay K8644 (30 mM/l) in physiological salt solution (PSS), in the presence of nitro-L-arginine (L-NNA) (10 μM/l) and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) (10 μM/l); (mean ± SD, n=12); *P<0.0001 vs. control and **0.0001<P<0.05 vs. control.

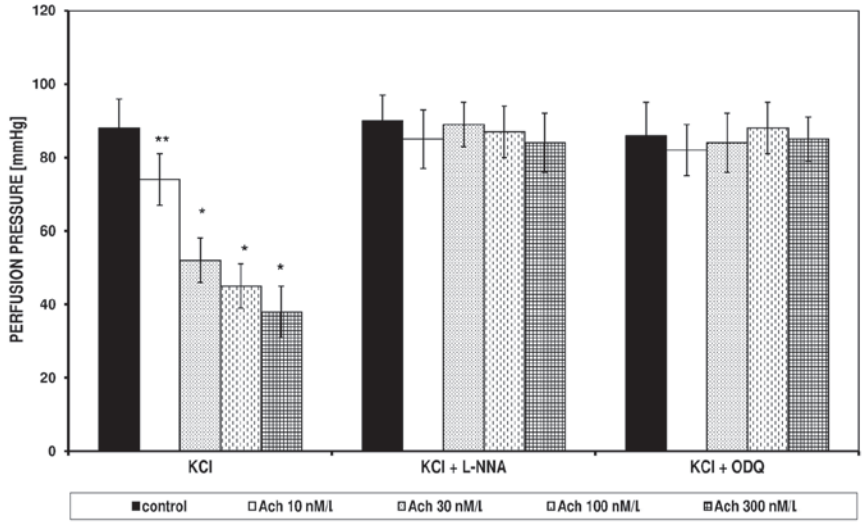

Figure 4. The effect of acetylocholine on the contraction induced by KCl (110mM/l) in physiological salt solution (PSS), in the presence of nitro-L-arginine (L-NNA) (10 μM/l) and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) (10 μM/l); (mean ± SD, n=12); *P<0.0001 vs. control and **0.0001<P<0.05 vs. control.

Ethical compliance. The Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences as well as specific national law were followed. The study was approved by the Ethics Committee for the Affairs of Experiments on Animals in Bydgoszcz (no. 1/2008-4).

Statistical analysis. Results are presented as the mean ± standard deviation. Statistical differences were analyzed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Bay K8644 induced contraction of the rat tail arteries with and without endothelium. CRCs for Bay K8644, in the presence of 8Br-cGMP are shown in Figs. 1 and 2. The resulting EC_{50} values are listed in Table I.

In the FPSS group, perfusion pressure under the influence of Bay K8644 and KCl did not change; the pressures were 14±6 and 15±5 mmHg, respectively (data not shown). Bay K8644 and KCl in the PSS induced an increase of perfusion pressure, which was significantly reduced in the presence of Ach (Figs. 3 and 4). The spasmolytic Ach action did not occur in the presence of L-NNA and ODQ. Figs. 3 and 4 present the effect of increasing concentrations of Ach on the perfusion pressure induced in the PSS by Bay K8644 and KCl, respectively, in the presence of L-NNA and ODQ.

Discussion

Arterial tension is dependent upon the structure and function of each layer of the arterial wall, the availability of Ca^{2+}

and the presence of certain vasodilator and vasoconstrictor factors. Endothelial NO is important in the induction of vasodilation (1), and acts through a signaling system involving cGMP (4,5). Bay K8644 and KCl are agents that stimulate blood vessel contraction by inducing an influx of Ca^{2+} into the cells through channels in the cell membrane. In the present study, the role of the endothelium in vascular contraction induced by Bay K8644 and the involvement of the Ach/NO/cGMP cascade in Bay K8644 and KCl activity were investigated.

Arterial spasm induced by Bay K8644 was demonstrated to be reduced by 8Br-cGMP, in a dose-dependent manner. The CRCs were shifted to the right with increasing 8Br-cGMP concentrations. Similar changes in the shape and position of the CRCs were demonstrated in the experiments performed in arteries without endothelium, thus the reaction was determined to be independent of this layer of the vessel wall. Similar findings were observed in previous studies, which utilized angiotensin II (ANG II) as a substance that stimulated vessel contraction (13,14). These studies also demonstrated that in arteries without endothelium, in contrast to vessels with an intact inner layer, no inhibition of ANG II-stimulated contraction following ischemia was observed. Numerous studies have confirmed that ischemia/reperfusion results in an impaired endothelial function, which reduces the synthesis of endothelial vasodilatators, such as prostaglandins and NO (15-18).

Under these conditions, vasodilatation stimulated by Ach, bradykinin, adenosine-5'-diphosphate (ADP), serotonin and thrombin, among others, is disturbed; however, the effect of NO donors remains to be observed (17,19). In the present study, experiments in FPSS and PSS demonstrated that Bay K8644 and KCl induce an increase in the perfusion pressure, due to an influx of Ca^{2+} from outside of the cell, as observed in previous studies (20-23). Other studies have indicated that KCl (at depolarizing concentrations) and Bay K8644 contract smooth muscle by opening the Ca^{2+} L-type channels located in the cell membrane, and by increasing concentrations of free Ca^{2+} in the cytoplasm. This effect may be eliminated in the presence of calcium channel Ca^{2+} antagonists, nifedipine and diltiazem (24-27). Subsequent experiments demonstrated that the contraction induced by Bay K8644 and KCl in the PSS was reduced at increasing Ach concentrations, and this effect was eliminated in the presence of L-NNA (nitric oxide synthase inhibitor) or ODQ (an inhibitor of soluble GC). In addition, similar observations have been demonstrated in studies of human mesenteric arteries (22). Ji *et al* demonstrated that Ach blocked phenylephrine-triggered contraction of endothelium-intact rat aorta, but did not affect the responses of arteries without endothelium (28).

The studies have also indicated that L-NNA and methylene blue (an inhibitor of soluble GC) may abolish the spasmolytic action of Ach. It was demonstrated that KCl stimulates the influx and increase of the calcium ion concentration in the cytoplasm, and also increases the myosin light chain kinase activity (29-31). However, the cellular mechanism of contraction induced by KCl remains to be elucidated.

This mechanism of action was the basis for the use of KCl in the present study investigating cell signaling as a factor in cell membrane depolarizing (electromechanical coupling), compared with agents that stimulate muscle contraction when combined with the corresponding receptors (pharmacomechanical coupling) (31,32). Other studies have demonstrated that KCl may lead to the release of Ca^{2+} from intracellular stores (33,34). It was also shown that membrane depolarization alone led to the sensitivity to Ca^{2+} (35). Studies have indicated that KCl also blocked the myosin light chain phosphatase through RhoA kinase activation, which led to the sensitivity of Ca^{2+} (36-38).

Signal pathways that control smooth muscle tension remain an important focus of research, as the mechanisms regulating vascular contraction may contribute to the understanding of physiological and pathological processes in the circulatory system, and may also be beneficial in determining novel treatment methods for cardiovascular diseases.

In conclusion, it was demonstrated that the increase in vascular tone induced by Bay K8644 and KCl was independent of the intracellular pool of Ca^{2+}. The relaxant effect of Ach on the responses stimulated by Bay K8644 and KCl indicated that NO was involved in modulating the reactivity of the arteries to the examined factors, which contributes to the influx of Ca^{2+} into the cell.

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