Role of acetylcholine and calcium ions in three vascular contraction models: Angiotensin II, phenylephrine and caffeine

KATARZYNA SZADUJKIS-SZADURSKA, GRZEGORZ GRZESK, LESZEK SZADUJKIS-SZADURSKI, MARTA GAJDUS and GRZEGORZ MATUSIAK

Department of Pharmacology and Therapeutics, Collegium Medicum Nicolaus Copernicus University, 85-094 Bydgoszcz, Poland

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Abstract. The aim of this study was to determine the role of acetylcholine and calcium ions in modulating the vascular contraction induced by angiotensin II (ANG II), phenylephrine (PHE) and caffeine. The study was performed on perfunded Wistar rat tail arteries. The contraction caused by ANG II, PHE and caffeine with the participation of intracellular [in free physiological salt solution (FPSS)] and extracellular [in physiological salt solution (PSS), after emptying the cellular stores] pools of calcium ions and the addition of L-NNA (NOSe inhibitor) or ODQ (GC inhibitor) was studied. Then the effect of acetylcholine on the contraction responses was analyzed. ANG II, PHE and caffeine induced an increase in perfusion pressure in PSS and FPSS. Acetylcholine reduced the contraction resulting from the presence of ANG II and PHE, but not caffeine. L-NNA and ODQ abolished the spasmolytic action of acetylcholine. Both pools of calcium ions mediated the action of ANG II and PHE, and caffeine induced the contraction with the participation of calcium released from intracellular stores. The spasmolytic effect of acetylcholine on responses stimulated by ANG II and PHE indicates the participation of nitric oxide in modulating the reactivity of the arteries on the studied agonists of the metabotropic receptors. No observed acetylcholine effect on caffeine suggests that the pathway associated with nitric oxide does not interfere with the contraction induced by the ryanodin receptor.

Introduction

The reactivity of blood vessels depends on their structure and the presence of calcium ions (1). It is modulated by numerous factors, which activate specific signaling pathways leading to contraction or relaxation of smooth muscle. The action of various vasodilatation substances may be altered under the influence of similar or quite different modulators. Vascular reactivity may vary with age, which probably is associated with endothelial dysfunction, leading to a reduction in nitric oxide synthesis (2-4). Angiotensin II (ANG II) triggers vasoconstriction via a metabotropic AT1 receptor (5). ANG II also regulates smooth muscle cell (SMC) growth, has an effect on apoptosis and migration and has proinflammatory action. In addition, it causes the production of other growth- and contraction-stimulating factors. It is, therefore, important both for maintaining the proper structure and function of blood vessels and may mediate pathophysiological processes leading to the development of cardiovascular diseases (6). Phenylephrine (PHE) is an agonist of the α1-adrenergic metabotropic receptor through which it induces vasoconstriction (7).

Both substances, ANG II and PHE, act through G proteins, which leads to stimulation of phospholipase C and the synthesis of secondary messengers: IP_3 and DAG (8-10). IP_3 binds to the endoplasmic reticulum membrane (ER) IP_3R receptors and causes the release of Ca^{2+} from intracellular pools. The ryanodin receptors (RyR), stimulated, among others, by caffeine (1,11,12) are an alternative way for the release of calcium from the ER. Contraction of vascular smooth muscle may also occur via calcium ions escaping from the extracellular space through channels in the cell membrane [receptor-operated Ca^{2+} channels (ROC)] activated by ligand ANG II or PHE (13).

Studies on vas deferens (human and rat) and rat tail artery have shown that receptor associated G-protein modulation may be influenced by sodium nitroprusside and 8Br-cGMP (14-16). Nitric oxide derived from endothelium is a major vasodilatation factor (17). Acetylcholine can stimulate the release of nitric oxide in a cGMP-mediated relaxing effect (18,19). However, studies using isolated human placental villous arteries found that NO donors and 8Br-cGMP did not cause relaxation of arteries contracted with caffeine. The mechanism of nitric oxide action on the cardiac calcium release channel (ryanodine receptor) (CRC) in canines was explored (20,21). Lim *et al* discussed various ways in which nitric oxide can modulate cardiac ryanodine receptor function and suggested the possibility of pharmacological strategies in heart failure, related to the considered mechanisms (22).

The aim of this study was to assess the role of acetylcholine and calcium ions in modulating the contraction induced by

Correspondence to: Dr Katarzyna Szadujkis-Szadurska, Department of Pharmacology and Therapeutics, Collegium Medicum Nicolaus Copernicus University, Skłodowskiej-Curie Street 9, 85-094 Bydgoszcz, Poland
E-mail: kataszsz@gmail.com

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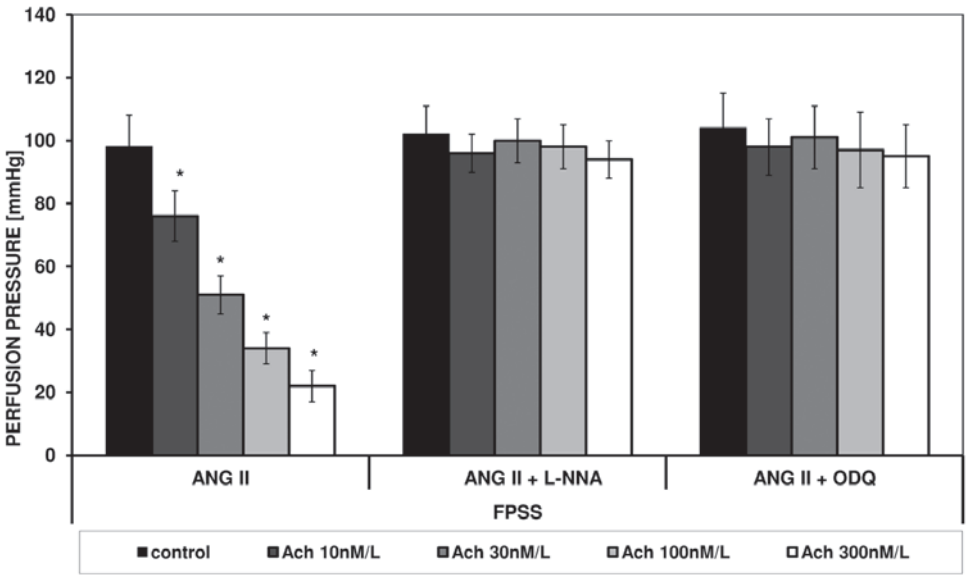

Figure 1. Influence of acetylcholine (Ach) on the vascular contraction induced by ANG II in FPSS, in the presence of L-NNA (10 μM/l) and ODQ (10 μM/l); (mean ± SE, n=12); *p<0.0001 vs. control. FPSS, free physiological salt solution.

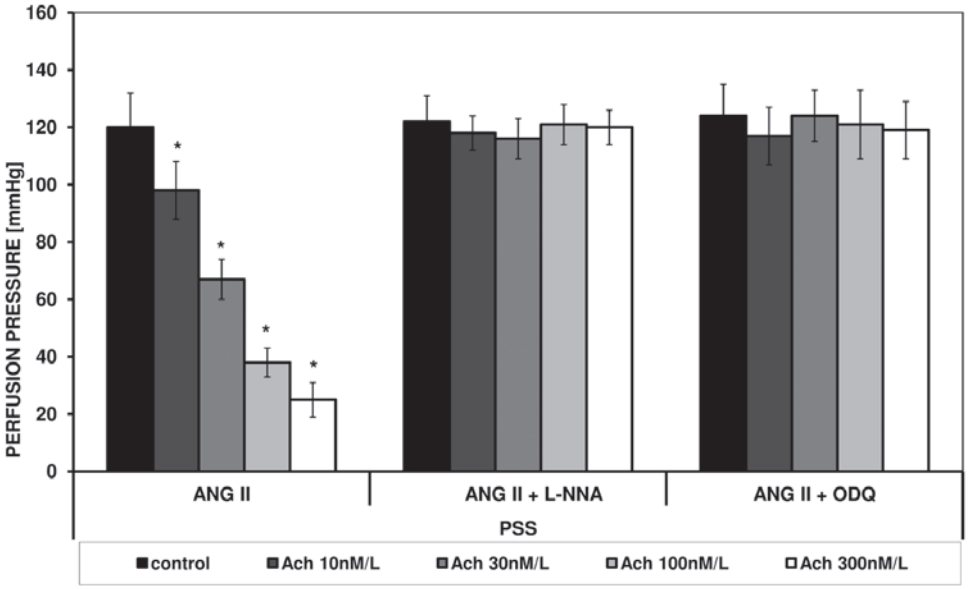

Figure 2. Influence of acetylcholine (Ach) on the vascular contraction induced by ANG II in PSS, in the presence of L-NNA (10 μM/l) and ODQ (10 μM/l); (mean ± SE, n=12); *p<0.0001 vs. control. PSS, physiological salt solution.

ANG II, PHE and caffeine. These substances acted together with the participation of calcium ions mobilized from intracellular stores, but also (as in the case of ANG II and PHE) using an extracellular pool of these ions.

Materials and methods

The study was performed on perfunded tail arteries of male Wistar weighing 250-350 g, euthanized with an intraperitoneally injection of urethane at the dose of 120 mg/kg. The cannula was introduced in the proximal section of rat tail artery (2.5-3 cm in length) and combined with a perfusion system and a set that allows constant measurement and recording of perfusion pressure. After loading the distal end of the isolated artery with a weight of 500 mg, the preparation was placed upright in a thermostated vessel for isolated organs 20 ml in volume and oxygenated with physiological fluid at a temperature of 37˚C. Perfusion fluid flow was increased gradually to 1 ml/min.

The experiments were carried out to determine the importance of intracellular and extracellular pools of Ca^{2+} in reactions induced by ANG II (30 nM/l), PHE (3 μM/l) and caffeine (100 μM/l) in control conditions and after addition of L-NNA (NOSe inhibitor) or ODQ (a soluble form of GC inhibitor) and in the presence of increasing concentrations of acetylcholine using two types of Krebs fluid: i) FPSS – Ca^{2+}-free EGTA-

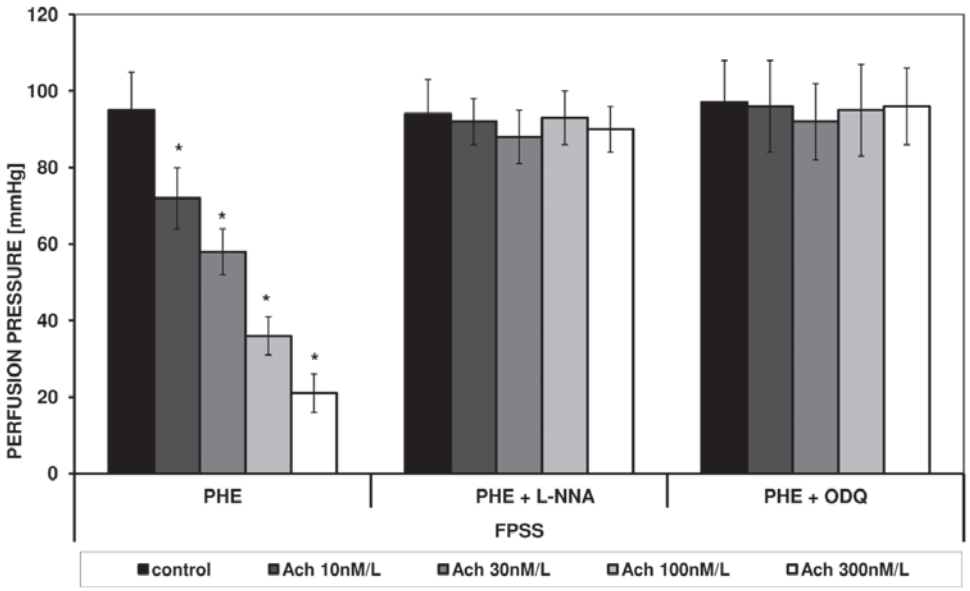

Figure 3. Influence of acetylcholine (Ach) on the vascular contraction induced by PHE in FPSS, in the presence of L-NNA (10 μM/l) and ODQ (10 μM/l); (mean ± SE, n=12); *p<0.0001 vs. control. PHE, phenylephrine; FPSS, free physiological salt solution.

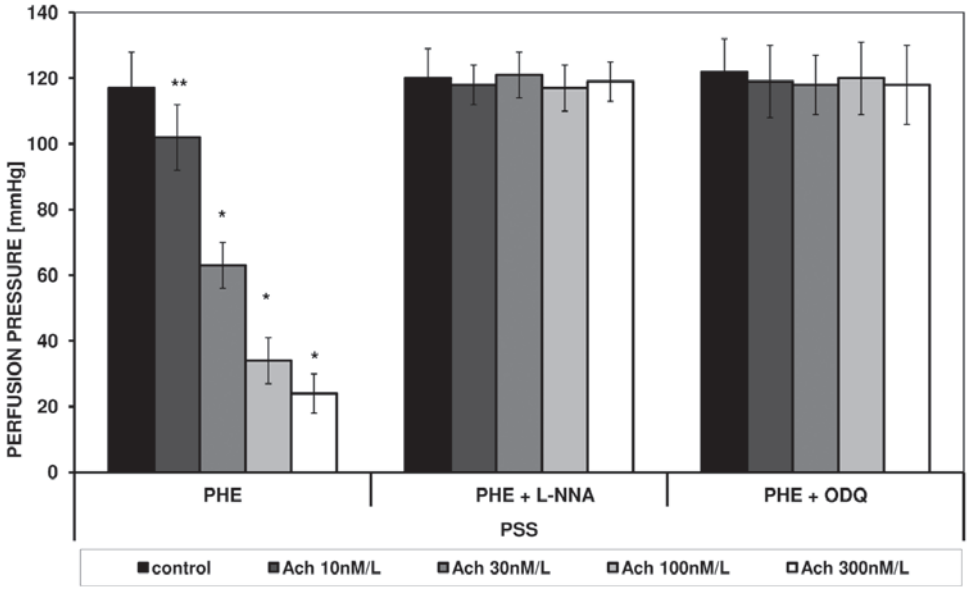

Figure 4. Influence of acetylcholine (Ach) on the vascular contraction induced by PHE in PSS, in the presence of L-NNA (10 μM/l) and ODQ (10 μM/l); (mean ± SE, n=12); *p<0.0001 vs. control; **0.05>p>0.0001 vs. control. PHE, phenylephrine; PSS, physiological salt solution.

Krebs with the following composition: 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); ii) PSS – fluid with Ca^{2+} EGTA-Krebs (normal) with the following composition: 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), after emptying the intracellular pool of calcium ions.

The increase in pressure of the perfusate in the experimental system was an exponent of vessel spasm. The Ethical Committee for the Affairs of Experiments on Animals in Bydgoszcz approved the protocol of the experiments undertaken (No. 1/2008-4).

Statistical analysis was performed by determining the mean and standard deviation. Statistical differences were evaluated by Student's t-test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

ANG II caused an increase in perfusion pressure in FPSS and PSS. Under the influence of increasing concentrations of acetylcholine a statistically significant reduction in perfusion pressure in both types of fluid was noted (Figs. 1 and 2).

In the presence of L-NNA and ODQ in both solutions no changes in contraction stimulated by ANG II or spasmolytic

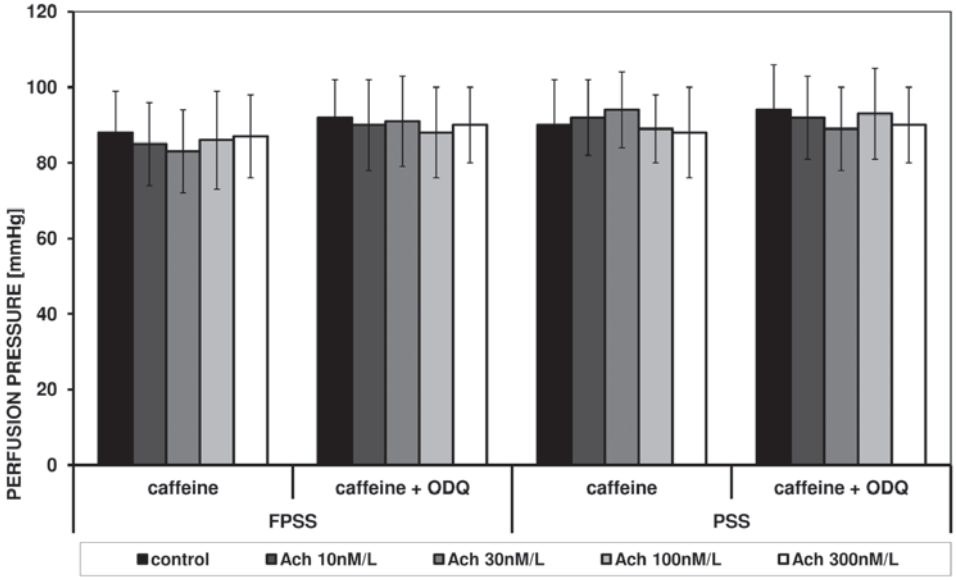

Figure 5. Influence of acetylcholine (Ach) on the vascular contraction induced by caffeine in PSS and FPSS, in the presence of ODQ (10 μM/l); (mean ± SE, n=12). PSS, physiological salt solution; FPSS, free physiological salt solution.

effect of acetylcholine were observed (Figs. 1 and 2). PHE, in a similar manner to ANG II caused contraction in FPSS and PSS, which was similarly modulated by acetylcholine. Figs. 3 and 4 present the effect of increasing concentrations of acetylcholine on the perfusion pressure induced by PHE in the presence of L-NNA and ODQ, respectively, in FPSS and PSS.

Caffeine induced an increase in perfusion pressure in FPSS and PSS, and these reactions were not altered under the influence of acetylcholine and ODQ. Fig. 5 shows the effect of increasing concentrations of acetylcholine on caffeine-triggered contraction in the presence of ODQ, FPSS and PSS.

Discussion

Calcium ions are an essential element in muscle contraction. Vascular tone can be adjusted by a variety of substances that stimulate the release of calcium from cellular stores, that cause the influx of calcium from the outside, and that stimulate sensitivity to calcium ions. A key role in regulating muscle function is maintaining the concentration of calcium ions within a very narrow range and regulating their ability for rapid increase (1). An increase in intracellular calcium levels precedes and induces the contraction of smooth muscle. Acetylocholine decreases arterial tension due to the release of NO from endothelial cells, thus it stimulates the NO/cGMP signaling pathway (23). Cyclic nucleotides, e.g., cAMP and cGMP, which regulate the function of ion channels and calcium levels in the cell through the appropriate protein kinases exhibit functional antagonism of calcium ions in smooth muscle (24,25). In experiments performed on mouse lung slices NO-induced relaxation was enhanced by selective inhibitors of cGMP-specific phosphodiesterase-5 (zaprinast or vardenafil), but was blocked by ODQ and by Rp-8-pCPT-cGMPS, an inhibitor of protein kinase G. NOC-5 (nitric oxide donor), cGMP analogues and selective PKG activators 8Br-cGMP and 8pCPT-cGMP were found to induce airway relaxation and decrease the frequency of Ca^{2+} oscillations (26). Studies using human mesenteric arteries showed that guanylate cyclase activators modulate vascular responses in conditions of ischemia/reperfusion (27). The aim of the present study was to determine the role of acetylcholine and calcium ions in modulating the vascular contraction induced by angiotensin II, phenylephrine and caffeine.

The experiments were carried out in PSS (after emptying the intracellular pool of calcium ions) and FPSS to determine the importance of the extracellular and the intracellular pools of calcium ions. Metabotropic receptor agonists, ANG II and PHE, led to an increase in perfusion pressure in both types of solutions; the reactions were stronger in PSS. Comparison of the results indicates that the vascular contraction induced by the studied substances was caused by the effect of calcium ions released from the endoplasmic reticulum (via the IP_3 receptor), entering the cell from the outside after opening the appropriate channels in the membrane. Similar observations were also derived from experiments on rat aorta and human mesenteric arteries (28,29).

The existence of a contraction mechanism independent of the intracellular calcium pool confirms previous experience using rat tail artery and human mesenteric arteries with xestospongin C, an IP_3 receptor antagonist (29,30). Acetylcholine reduced the vascular contraction stimulated by ANG II and PHE in a concentration-dependent manner. Additionally, in experiments using human mesenteric arteries such an effect of acetylcholine was observed (18). Ji *et al* in studies of rat aorta demonstrated that the inhibitory effect of acetylcholine was associated with the presence of endothelial cells and this effect was not present in experiments carried out in arteries denuded of endothelium (27). Another series of studies found that blocking NO synthase (after addition of L-NNA) and soluble guanyl cyclase (GC, after addition of ODQ) led to the elimination of the relaxing effects of acetylcholine. These results confirm the dependence of acetylcholine on NO synthesis and activation of GC.

A subsequent experiment was performed in FPSS and PSS using caffeine, agonist of ryanodin receptors in the endoplasmic reticulum, as a factor stimulating vascular contraction. Previous studies in human mesenteric arteries showed that

emptying the intracellular pool of Ca^{2+} and blockage of Ca^{2+}-ATPase (by specifying thapsigargin) caused the abolition of the response to caffeine (29). In contrast to the results of the metabotropic receptor agonists, acetylcholine did not inhibit caffeine-triggered contraction. This effect was consistent with previous reports (28,29). It was previously shown that the NPS, as a donor of NO, decreased rat artery contraction, induced by ANG II and PHE, but was not affected by caffeine (27). The effect of NO on Ca^{2+} sensitivity of airway SMCs was examined in mouse lung slices permeabilized to Ca^{2+} by treatment with caffeine and ryanodine. Neither NOC-5 nor 8pCPT-cGMP induced relaxation in agonist-contracted Ca^{2+}-permeabilized airways (26). Slupski *et al* showed that nitric oxide may reduce lung damage caused by increased vascular resistance and arterial pressure after ischemia/reperfusion (30).

In the present study the importance of calcium ions and acetylcholine, as an element in the Ach/NO/cGMP signaling pathway in the vascular contraction induced by ANG II and PHE through metabotropic receptors (AT1 and α1-adrenergic, respectively) was compared with the action of caffeine, a ryanodin receptor agonist, in ER. Ji *et al* explained that the lack of impact of acetylcholine and sodium nitroprusside on contraction caused by caffeine is likely due to the fact that NO can selectively block the release of calcium ions from the ER through an IP_3-dependent pathway (27). Perez-Zoghbi *et al* concluded that NO, acting via the cGMP-PKG pathway, induced airway SMC relaxation by predominately inhibiting the release of Ca^{2+} via the IP_3 receptor to decrease the frequency of agonist-induced Ca^{2+} oscillations (26).

The action of ANG II and PHE was mediated by two pools of calcium, and caffeine induced the contraction with the participation of calcium released from intracellular stores. The relaxing effect of acetylcholine on responses stimulated by ANG II and PHE indicates the participation of nitric oxide in modulating the reactivity of arteries to the studied metabotropic receptor agonists. No effect of acetylcholine on caffeine action suggests that the pathway associated with nitric oxide does not interfere with the vascular contraction induced by the ryanodin receptor.

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