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The role of calcium in modulating the reactivity of the smooth muscle cells during ischemia/reperfusion. Part 1

Rola jonów wapnia w modulowaniu reaktywności mięśniówki gładkiej podczas niedokrwienia i reperfuzji. Część 1

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Summary

Background: Calcium ions regulate the function of cells in many ways, acting as first messengers of intercellular information and second messengers of intracellular information. Changes in cytoplasmic calcium levels depend on calcium influx from the extracellular space or calcium release from cellular stores. Increase in calcium ion concentration takes place in pathological situations, such as ischemia. In the present study the roles of calcium and G protein in contraction induced by angiotensin II (agonist of the metabotropic receptor AT1), phenylephrine (agonist of alpha1-adrenergic metabotropic receptor), and Bay K8644 (a calcium channel agonist) after ischemia/reperfusion were investigated.

Material/Methods: Experiments were performed on perfused male Wistar rats' tail arteries. Contraction induced by angiotensin II, phenylephrine, and Bay K8644 mediated by intracellular or extracellular calcium after ischemia/reperfusion and in the presence of the blocker of G protein Bordetella pertussis toxin (P 7208) was analyzed.

Results: Ischemia reduced while reperfusion augmented the response of vascular smooth muscle cells to angiotensin II and phenylephrine, but they did not change the effects of Bay K8644. P 7208 decreased the effects of phenylephrine mediated by intracellular and extracellular calcium and reduced the reactions of angiotensin II mediated only by intracellular calcium, but did not change the effects of Bay K8644.

Conclusions: Ischemia/reperfusion modulates vascular contraction induced by angiotensin II and phenylephrine. Both intracellular and extracellular calcium ions mediate the contraction induced by angiotensin II and phenylephrine. The results suggests that G protein modulates the effects of angiotensin II mediated by intracellular calcium ions while it plays a role in the reactions of phenylephrine mediated by calcium coming from both sources, intracellular and extracellular.

Key words: **angiotensin II • phenylephrine • ischemia • reperfusion • calcium • G protein**

Streszczenie

Wstęp: Jony wapnia regulują czynność komórki w wieloraki sposób, działając jako pierwszy przekaźnik informacji międzykomórkowej i drugi przekaźnik informacji wewnątrzkomórkowej. Zmiana stężenia jonów wapnia w cytoplazmie komórki może sie odbywać poprzez napływ z puli zewnętrznej lub uwolnienie z magazynów komórki. Wzrost stężenia jonów wapnia występuje również w sytuacjach patologicznych, na przykład w niedokrwieniu. W pracy oceniono udział jonów wapnia i znaczenie białka G w skurczu naczyń wyzwalanym przez angiotensynę II (agonistę receptora metabotropowego AT1), fenylefrynę (agonistę alfa1-adrenergicznego receptora metabotropowego) i Bay K8644 (agonistę dihydropirydynowych kanałów wapniowych), po niedokrwieniu i reperfuzji.

Materiał/Metody: Badania przeprowadzono na perfundowanych tętnicach ogonowych szczurów, samców szczepu Wistar. Badano skurcz wyzwalany przez angiotensynę II, fenylefrynę i Bay K8644, przy udziale wewnątrzkomórkowej i zewnątrzkomórkowej puli jonów wapnia po niedokrwieniu i reperfuzji oraz w obecności blokera białka G – toksyny krztuśca (P 7208).

Wyniki: Niedokrwienie osłabia, a reperfuzja wzmaga reakcję tętnic na angiotensynę II i fenylefrynę, ale nie zmienia działania Bay K8644. P 7208 obniża działanie fenylefryny wyzwalane przy udziale wewnątrz- i zewnątrzkomórkowej puli jonów wapnia, osłabia efekt angiotensyny II w obecności wapnia pochodzącego z magazynów komórkowych, a nie wpływa na działanie Bay K8644.

Wnioski: Niedokrwienie/reperfuzja modulują skurcz tętnic wyzwalany przez angiotensynę II i fenylefrynę. W działaniu angiotensyny II i fenylefryny pośredniczy wewnątrz- i zewnątrzkomórkowa pula jonów wapnia, ale białko G moduluje działanie angiotensyny II wyzwalane przy udziale wewnątrzkomórkowej puli jonów wapnia, podczas gdy odgrywa rolę w reakcjach skurczu wyzwalanego fenylefryną zależnych od obu źródeł jonów wapnia.

Słowa kluczowe: **angiotensyna II • fenylefryna • niedokrwienie • reperfuzja • jony wapnia • białko G**

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Introduction

Calcium ions regulate the function of the cells in many ways. They act as first messengers co-creating the signals at the cell membrane level. They are also second messengers of intracellular information through the activation of many enzymes involved in muscle contraction, blood clotting, and modulation of neuronal function [19]. As the level of free calcium ions is maintained within a very narrow range, its physiological function as a signaling system in the cell is provided. Alterations of Ca^{2+} ions in the cellular cytoplasm occurs through the influx of their extracellular pool or release from the cell's storage [11,18]. Alterations of Ca^{2+} ion concentrations affect such cell processes as the contraction and relaxation of smooth muscle and striated muscles, synthesis and release of hormones, neurotransmitters, cell cycle, gene expression, and apoptosis [20]. Increased calcium ion concentration also occurs in pathological situations, such as hypoxia or ischemia, whereby cells become energy deficient [21]. Calcium ions participate in cell death by apoptosis, ischemia/reperfusion (I/R), and excitotoxicity.

Excitotoxicity is the killing of nerve cells by an excessive influx of Ca^{2+} into their interior. Calcium ions are considered to be one of the main mediators in the process of cell death by excitotoxicity because of the augmentation of glutamate release and the activation of protease and lipase, leading to damage to the the cell membrane, the activation NOS, and the augmentation of arachidonic acid release, which leads to an increased production of free radicals and a reduction of the uptake glutamate [2,5,9]. Damage and CNS cell death after injury also appear to be the consequence of influx to the cell and increase in calcium ion concentrations in the cytoplasm, with the subsequent activation of enzymes that cause degenerative changes [27]. Calcium ions are also considered mediators of the effect called ischemic preconditioning (IPC), which found beneficial influence of short-term repeated episodes of ischemia/reperfusion of heart muscle on the consecutive tolerance to sustained ischemia [16,17].

Angiotensin II (ANG II) is a receptor agonist of angiotensin AT1 receptor and phenylephrine (PHE) stimulates alpha1-adrenergic receptor [4,6,12]. AT1 and alpha 1-adrenergic

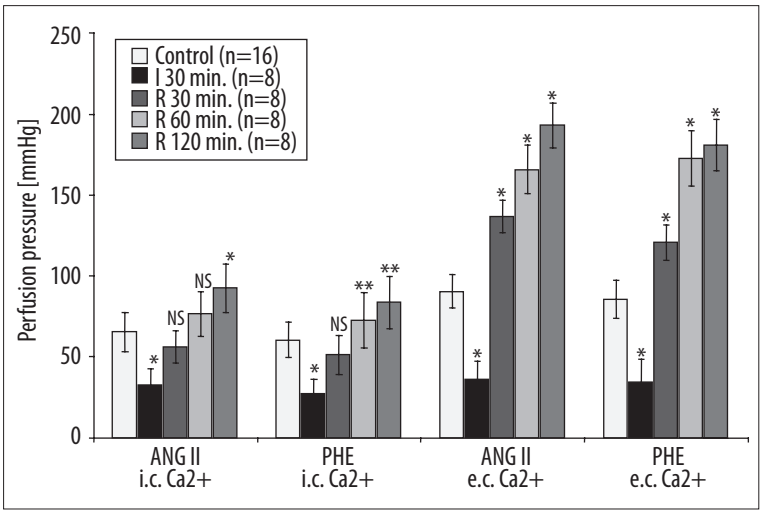

Fig. 1. Perfusion pressure induced by angiotensin II (ANG II) and phenylephrine (PHE) after ischemia (I)/reperfusion (R) mediated by intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) calcium ions (mean ±*SE*). * $p<0.0001$ vs. control, ** $0.05>p>0.0001$ *vs.* control, NS not statistically significant

receptors are metabotropic receptors functionally coupled with G proteins. The activation of phospholipase C and the synthesis of IP_3 and DAG are triggered by G-protein. These are the second messengers of ANG II and PHE action. IP_3 binds with IP_3R receptors on the membranes of the endoplasmic reticulum, causing the release of Ca^{2+} from intracellular pools and the contraction of vascular smooth muscle. Both factors, ANG II and PHE, trigger the contraction of vascular smooth muscle by activating the corresponding metabotropic receptors in the cell membrane and associated G protein [3]. The obtained increase in blood pressure depends on the increase in the concentration of free calcium ions in the cytoplasm due to their rapid release from intracellular stores and influx from the extracellular space through the calcium ion channels in the cell membrane (receptor-operated Ca^{2+} channels (ROC), activated by agonist – ANG II or PHE) [1,15,23]. Bay K8644 is a dihydropyridine calcium channel agonist which, by stimulating the influx of calcium into the cells, triggers the contraction of smooth muscle.

Cell damage caused by ischemia and the intensification of these changes after the restoration of flow is one of the major problems not only in transplantation, but is also observed in complexes of hypoxia (ischemic syndromes) in heart, liver, intestine, brain, and kidney [8,20,28]. The destructive effects of I/R start from the production of large amounts of reactive oxygen species (ROS) after reoxygenation, with direct tissue damage and the initiation of a cascade of harmful cellular responses, leading to inflammation, cell death, and organ failure [24].

The aim of this study was to determine the role of calcium ions (from intracellular stores and extracellular fluid) and G proteins in smooth muscle contraction reactions triggered by angiotensin II, phenylephrine, and Bay K8644 after ischemia and reperfusion. In Part 2, a study to assess the significance of the antioxidant system in arterial contraction triggered by ANG II after I/R with the participation of intracellular and extracellular calcium pools was carried out.

Material and Methods

The experiments were performed on isolated and perfused tail arteries of Wistar rats (250–350 g) which were euthanized by urethane injected intraperitoneally at a dose of 120 mg/kg. In order to examine the influence of I/R on the vascular smooth muscle reactivity of the selected agonists (ANG II 30 nM/l, PHE3 μM/l, Bay K8644 30 μM/l), a hemostat was put on the proximal part of the prepared artery for 30 or 60 minutes. Only after this time were the arteries cut off and a cannula was mounted and then connected with a perfusion system and a system which enabled measuring and registering the perfusion pressure.

After removing the surrounding tissue, the cannula was introduced in the proximal part of the severed artery of rat tail (length 2.5–3 cm) which was then assimilated with the perfusion system and with the measurement and registration system. After loading the distal end of the artery prepared with weights of 500 mg, the artery was placed in an upright position in the thermostated 20 ml container for the isolated organs filled with oxygenated normal saline at 37°C. Perfusion solution flow was gradually increased using peristaltic pump until 1 ml/min was reached.

To assess the contribution of the intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) pools of Ca^{2+} in the reactions triggered by the agonists under control conditions after ischemia and reperfusion and in the presence of the G protein blocker pertussis toxin (P 7208), the experiments were carried out using two types of Krebs fluid:

1. FPSS [free physiological salt solution]: Ca^{2+} free – EGTA – Krebs fluid. 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)
2. PSS [physiological salt solution] Ca^{2+} content – EGTA- Krebs fluid (standard). 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 the addition of EGTA (30 μM/l), after emptying the intracellular calcium pools.

Vessel constriction was induced by agonists and measured as increase in perfusion pressure. The results are expressed as average values and standard deviation. Statistical differences were assessed by Student's t test. Differences were regarded as statistically significantly with $p<0.05$. The

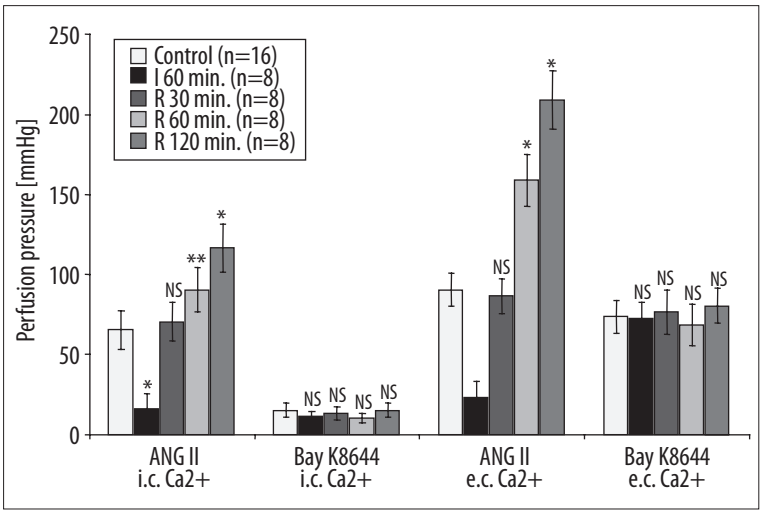

Fig. 2. Perfusion pressure induced by angiotensin II (ANG II) and Bay K8644 after ischemia (I)/reperfusion (R) mediated by intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) calcium ions (mean ±SE). * $p<0.0001$ vs. control, ** $0.05>p>0.0001$ *vs.* control, NS not statistically significant

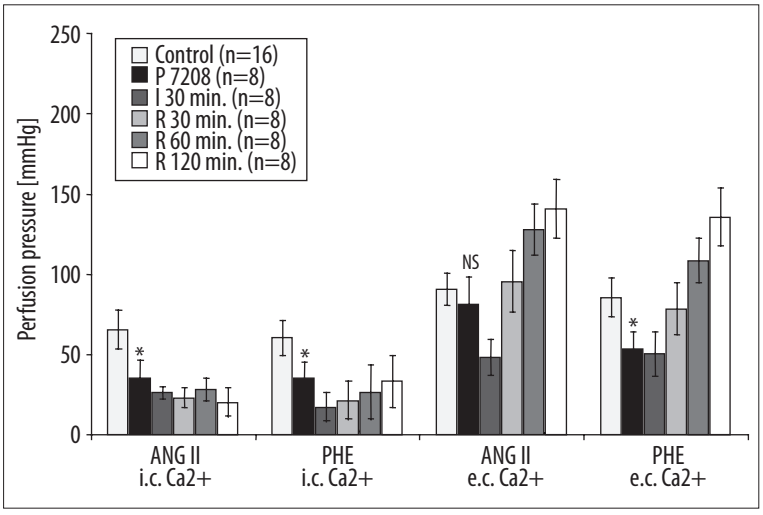

Fig. 3. Perfusion pressure induced by angiotensin II (ANG II) and phenylephrine (PHE) after ischemia (I)/reperfusion (R) mediated by intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) calcium ions (mean ±*SE*) in the presence of pertussis toxin (P 7208). * $p<0.0001$ *vs.* control, ** $0.05>p>0.0001$ *vs.* control, NS not statistically significant

calculations were carried out with the program Statistica 6.0PL.

Results

ANG II and PHE trigger increases in perfusion pressure in FPSS and PSS and the values were higher for both agonists in PSS (Fig. 1). After 30 min. of ischemia, decreases in the arteries' reactions to ANG and PHE in both types of experiment were observed. The maximal effects were statistically more significant in PSS after 30, 60, and 120 min. of reperfusion, whereas in FPSS only after 120 min. of reperfusion for ANG II and after 60 and 120 min. of reperfusion for PHE was there a significant increase in perfusion pressure. The obtained values of perfusion pressure triggered by ANG II and PHE after I/R in FPSS and PSS are shown the Figure 1.

Bay K8644 triggers contraction only in PSS, which, in contrast to the experiments with ANG II and PHE, is not modulated by I/R. Figure 2 presents the perfusion pressures obtained after stimulation by ANG II and Bay K8644 in FPSS and PSS after 60 min. of ischemia and 30, 60, and 120 min. of reperfusion.

Prolongation of ischemia time from 30 to 60 min. caused a statistically significant decrease of artery reactivity to ANG II. In the experiments carried out in FPSS after 30 min. of ischemia, perfusion pressure was 33±10 mmHg and after 60 min. was decreased to 17±9 mmHg (p=0.046). In the experiment carried out in PSS after 30 min. of ischemia, the perfusion pressure was 37±11 mmHg and after 60 min. it decreased to 24±10 mmHg (p=0.026). From the comparison of the perfusion pressure values obtained in FPSS after reperfusion it appears that prolongation of the ischemia time to 60 minutes heightens the amplifying influence of reperfusion on artery contraction triggered by ANG II. After 30 min. of reperfusion, the perfusion pressure was 57±10 mmHg after 30 min. of ischemia and 71±12 mmHg after 60 min. of ischemia (p=0.02). After 120 min. of reperfusion, the perfusion pressure was 93±15 mmHg after 30 min. of ischemia and 117±15 mmHg after 60 min. of ischemia (p=0.006). In the experiments carried out in PSS, one can note a tendency to a slower rise in contraction after reperfusion, which is particularly marked in the reaction after 30 min. of reperfusion because the perfusion pressure was 137±10 mmHg after 30 min. of ischemia compared with 87±11 mmHg after 60 min. of ischemia (p<0.0001).

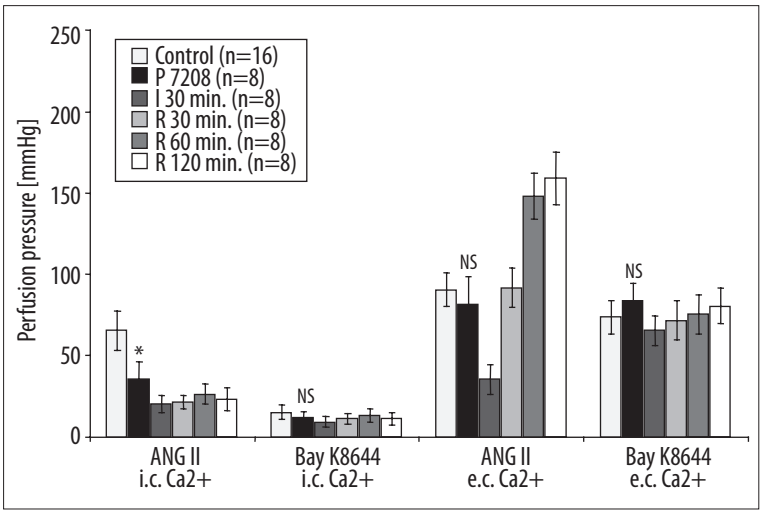

Fig. 4. Perfusion pressure induced by angiotensin II (ANG II) and Bay K8644 after ischemia (I)/reperfusion (R) mediated by intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) calcium ions (mean ±*SE*) in the presence of pertussis toxin (P 7208). * $p<0.0001$ *vs.* control, ** $0.05>p>0.0001$ *vs.* control, NS not statistically significant

In the presence of the G protein blocker pertussis toxin (P 7208), the reactions triggered by ANG II were reduced only in FPSS, while the contraction triggered by PHE was reduced in both types of experiments, i.e. FPSS and PSS (Fig. 3).

P7208 suppressed the modulating influence of I/R on artery contraction after ANG II and PHE application in FPSS. However, pertussis toxin limited the inhibitory effect of ischemia in PSS ($p<0.05$ vs. experiments without P 7208) and amplified the influence of reperfusion ($p<0.0001$ vs. experiments without P 7208) on the artery reaction triggered by ANG II and PHE. Figure 3 shows the perfusion pressure obtained after simulation by ANG II and PHE in FPSS and PSS after 30 min. of ischemia and 30, 60, and 120 min. of reperfusion in the presence of pertussis toxin.

P 7208 did not change the action of Bay K8644, also after I/R. Figure 4 shows a comparison of the perfusion pressures triggered by ANG II and Bay K8644 after 60 min. of ischemia and 30, 60, and 120 min. reperfusion in the presence of pertussis toxin.

Discussion

In the present study the influence of calcium ions and G protein on the contraction triggered by angiotensin II (receptor agonist angiotensin AT1), phenylephrine (receptor alpha1-adrenergic agonist), and Bay K8644 (a calcium channel agonist) was analyzed. Then the influence of ischemia and reperfusion on these reactions was studied. In order to determine the contribution of calcium ions (from intracellular stores and extracellular fluid), the experiments were carried out in a fluid without calcium (to evaluate the importance of intracellular pools) and in standard Krebs fluid after empting the storage of cellular calcium (extracellular pool of assessment participation).

The AT1 and alpha1-adrenergic receptors, according to experiments, are considered to be classic metabotropic receptors functionally coupled via G protein and IP3 synthesis. The analysis of smooth muscle reactions and Ca^{2+} ions alterations, according to the literature [10], is preceded by a biphasic increase in Ca^{2+} concentration. The first phase, according to the present studies with isolated smooth muscles cells, is IP_3 dependent and associated with the release of calcium from the endoplasmic reticulum, while the second phase, the slow accumulation of Ca^{2+} concentration, is determined by the influx of Ca^{2+} ions from the extracellular space.

In the present study, arteries were triggered by ANG II and PHE through both the intracellular and extracellular pools of calcium ions, while the reaction was higher in PSS.

In contrast to the alpha1-adrenergic receptor, the role of G protein in acting of ANG II via AT1 receptor was only partially confirmed by the experiments with pertussis toxin. The experiments indicate that only the perfusion pressure increase obtained with the participation of the intracellular IP_3-dependent calcium pools are closely associated with protein G. The results indicate indirectly (because the level of calcium was not determined and only contraction was measured) that perfusion pressure triggered by ANG II and mediated by calcium influx from the extracellular pool is partly independent of protein G. A similar observation appeared from studies on artery reactivity triggered by ANG II in which, after applying xestospongin C (IP_3 receptor antagonist), a reduction of perfusion pressure was achieved in the response to ANG II in FPSS which was not observed in experiments carried out in PSS after emptying the cellular calcium store [25].

In contrast to ANG II and PHE, Bay K8644 triggers perfusion pressure increases only in the standard Krebs fluid. This action of Bay K8644 is the result of direct activation of dihydropyridine calcium channels located in the cell membrane. Thus the perfusion pressure increase liberated by Bay K8644 uses only the extracellular pool of calcium ions and is independent of G proteins Confirmation of this view was also obtained in studies with pertussis toxin, which does not change the responses of arteries to Bay K8644. Similar results were noted in the studies of Liu et al. on a culture of cardiac muscle cells [13,14].

Seascholtz et al. [22] suggest the involvement of G proteins in disturbances caused by I/R in the reactions triggered by the alpha1-adrenergic receptors. The disorders be-

tween G-alpha$_q$ proteins and G-alpha$_i$ protein during I/R may be essential for the observed changes in the vascular reactivity of constrictory factors.

The present study demonstrated that under conditions of ischemia there is a vasoconstrictory response with ANG II and PHE, but after reperfusion, in a duration-dependent manner, a perfusion pressure increase is triggered by these agonists. The presented study indicates that the increase in artery responsiveness after reperfusion affects not only the activation of phospholipase C and the synthesis of IP_3 and Ca^{2+} release from intracellular pools of ions, as earlier studies suggested [7,10]. In the amplified action of reperfusion on arterial responses, both pools of Ca^{2+} ions are involved. A comparison of responses in standard and Ca^{2+}-free Krebs fluid shows that the dominant role in increasing perfusion pressure triggered by ANG II and PHE after reperfusion is played by the extracellular pool of calcium ions. Szadujkis-Szadurski et al. [26] showed that the inhibitory effect of ischemia on artery contraction is associated with the presence of endothelium, nitric oxide synthesis, and the activation of cGMP.

Pertussis toxin eliminates only the responses to ANG II in Ca^{2+}-free Krebs fluid, and only under these conditions removes the modulating effect of ischemia and reperfusion. This confirms the participation of G protein reactions triggered by ANG II. By contrast, responses to ANG II and the modulating effect of ischemia and reperfusion in the presence of pertussis toxin observed in the standard Krebs fluid, after having emptied the pool of intracellular calcium ions, are observed, but lower than before the application of pertussis toxin.

From the comparison of the values obtained by perfusion pressure triggered by ANG II, PHE, and Bay K8644, it appears that ischemia and reperfusion did not affect the responses triggered by the direct activation of dihydropyridine calcium channels by Bay K8644. No effect of ischemia and reperfusion on the reactions triggered by the ionotropic receptor agonist suggests that the modulating effect of ischemia and reperfusion involves the coupling or uncoupling of G protein receptor or effector. Similar results were obtained in studies with a depolarized concentration of KCl [29]. They stated that ischemia does not affect the responses of arteries to KCl. Thus both vasoconstriction triggered by Bay K8644 and as a result of depolarized KCl action is independent of G proteins.

The results showed that, despite the fact that both ANG II and PHE are metabotropic agonists, their intracellular signaling pathways are subject to different regulatory mechanisms. An explanation of the differences in the modulation of the blood vessels by G protein and phospholipase C in the artery reaction triggered by ANG II and PHE, with the participation of calcium from intracellular stores and calcium ions coming from the extracellular space, however, requires further study.

Conclusions

Two pools of calcium ions, intracellular and extracellular, mediate in the contraction triggered by ANG II and PHE. Ischemia reduces while reperfusion, in a duration- dependent manner, augments the response of arteries to ANG II and PHE, especially in the case of the participation of the extracellular calcium ion pool. The decrease in contraction by the use of pertussis toxin suggests that G protein modulates the action of ANG II triggered by the participation of the intracellular calcium ion pool and plays a role in the arterial responses to PHE, in which both calcium ions from the storage cell and those flowing from the extracellular space mediate.

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