Influence of cyclic guanosine 3',5' monophosphate modulators on muscle contraction of rat aortas

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ABSTRACT

الأهداف: فحص تأثير البروبينيسيد، السيلدينافيل، واوكسيديازولوكينوكسالين على انقباض الشريان الأبهري للفئران المحفزة بمادة الفينيل افيرين.

الطريقة: أجريت هذه الدراسة في مركز بيتر هولتس لأبحاث علم الأدوية بجامعة جرايسفالد – ألمانيا، خلال الفترة مابين يوليو 2005م وحتى 30 سبتمبر 2005م. تم عزل 35 شريان أبهري من الفئران وقسمت كالتالي: مجموعة غير محفزة، مجموعة فينيل افيرين 10µM، مجموعة بروبينيسيد 10µM، مجموعة اوكسيديازولوكينوكسالين 10µM، مجموعة سيلدينافيل 50µM ، أُضيفت 10µM فينيل افيرين على المجموعات: الثالثة، الرابعة، والخامسة على التوالي. تم الكشف عن الفسفوريليتد MLC20 بواسطة مضاد سلسلة الفسفوميوسين الخفيفة2.

النَتائِج: جاءت نتائج 30 و 60 ثانية للمجموعة الأولى والثانية بنسبة 16.7:1 و 20.4:1 على التوالي، أما في الثانية الـ120 لوحظ النقصان في الفسفوريليشن بشكل ملحوظ، وكذلك 10 دقائق. بعد 30 ثانية من تحفيز المجموعتين الثالثة والخامسة بـ10µM من مادة فينيل افيرين تبين النقصان، بينما جاءت الزيادة في المجموعة الرابعة والمحفزة أيضاً بـ10µM من مادة فينيل افيرين لمدة 60 ثانية. وعند إزالة تأثير المواد على الشرايين المعزولة (washout–effect)، لم يتبين التأثير بشكل واضح.

خاتمة: أظهرت الدراسة علاقة بين (cGMP) ومغيراته، وانقباض عضلة الشريان الابهري للفئران، حيث قل الانقباض في حالة البروبينيسيد والسيلدينافيل بينما ازداد في حالة اوكسيديازولوكينوكسالين.

Objectives: The study examined the influence of probenecid (Pn), sildenafil (Sd) and oxidiazoloquinoxalin (ODQ) on contraction of phenylephrine (PhE) stimulated rat aortas.

Methods: The study was performed at Peter Holtz Research Center of Pharmacology and Experimental Therapeutics, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany, from 1st July to 30th September 2005. Thirty-five isolated rat aortas were stimulated with 10 µM PhE or preincubated for 30 minutes with 10 µM Pn, or 10 µM ODQ, or 50 µM Sd, and then incubated with 10 µM PhE in the presence or absence of the substances. The phosphorylated myosin light chain 20 was detected by using an antibody against phosphomyosin light chain 2.

Results: The ratio of PhE stimulated phosphorylation of aortas ($p<0.05$) to the untreated was 16.7:1 at 30 seconds and 20.4:1 at 60 seconds. The stimulation decreased significantly at 120 seconds then during the following 10 minutes. Pre-incubation with 50 µM Sd ($p>0.05$) or 10 µM Pn ($p<0.05$) reduced the phosphorylation induced by PhE that was added to each for 30 seconds. But pre-incubation with 10 µM ODQ increased the phosphorylation brought about by addition of PhE for 60 seconds, $p>0.05$. The washout-effect of these modulators was not significant after stimulation with PhE only.

Conclusion: The study demonstrates the involvement of cyclic guanosine 3',5' monophosphate and its modulators on muscle contraction of rat aortas. Sildenafil and Pn reduced while oxidiazoloquinoxalin increased the contraction of rat aortas.

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The contractile state of vascular smooth muscle controls the vessel lumen size. Impaired regulation of vascular tone plays an important role in the pathophysiology of cardiovascular diseases such as hypertension, vasospasm, and atherosclerosis.[1] Understanding the molecular mechanism regulating the vascular tone is thus, essential to establish regimens for the prevention and the treatment of vascular diseases. Smooth muscle contraction is activated primarily by an increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$). This is evoked by means of membrane depolarization, or vasoconstrictors such as alpha 1-adrenergic agonists, or stretch.[2] Calcium originates from extra cellular space and/or intracellular stores (especially the sarcoplasmatic reticulum [SR]). Then calcium binds to calmodulin,[3] which activates myosin light chain kinase (MLCK). It results in phosphorylation of the 20 kDa regulatory MLC[4] and triggers cross-bridge cycling[5] that ends with the development of force or contraction.[6,7] Phosphorylation can drop down while the steady state stress maintenance tone continues and even increases.[5] More than that during vascular smooth muscle relaxation, MLC phosphorylation decreases to resting state more rapidly than after stress.[8] Phosphorylation of MLC[9,10] is well recognized as the primary determinant of myosin ATPase activity and initiator of the contraction in arterial smooth muscle.[5,9,11] The regulation of the Ca^{2+} independent myosin light chain phosphatase (MLCP) activity is now considered to play a critical role in the regulating the myofilament Ca^{2+} sensitivity as well as maintenance of muscle tone.[12] Numerous protein kinases other than MLCK have been found to be involved in the regulation of MLC phosphorylation such as rho-kinase,[1,13] a protein kinase C potentiated inhibitor protein of 17 kDa (CP1-17),[14] integrin linked kinase[15,16] and protein kinase C.[2,17,18] All have the ability to phosphorylate MLC and activate the contraction in a Ca^{2+} independent manner. Additionally, they inhibit MLCP and subsequently further increase Ca^{2+} sensitivity.[16] Cyclic nucleotides (cyclic adenosine 3',5' monophosphate [cAMP] and cyclic guanosine 3',5' monophosphate [cGMP]) also contribute to cellular Ca^{2+} regulation.[19,20] Recently, cGMP has emerged as a principle focus in signal transduction. Physiological effects are caused by nitric oxide, all of the characterized effects of natriuretic peptides and guanylines are mediated by cGMP. The formation of cGMP through guanylyl cyclase (GC) leads to decreased contraction of smooth muscle.[21,22] Guanylyl cyclase appears to exist in both soluble and particulate fractions of most tissues.[23] There are several signal transduction mechanisms that modulate $[Ca^{2+}]_i$ and therefore the state of vascular tone. The most involved mechanisms are 1. Phosphatidylinositol pathway, 2. Gs protein coupled pathway, and 3. Nitric oxide cGMP pathway (**Figure 1**). The cellular level of cGMP can be regulated with phosphodiesterases, which degrade it[22,23] or transporters such as multi-drug resistance protein 5 (MRP5)[24] and MRP4,[25-28] as well as regulate its production.[19] Inhibition of such processes may lead to increment or decrement in the cellular cGMP content, which may display multiple actions and could affect the phosphorylation. This was included in the purpose of this study to find out indirectly through modulators its participation in myosin phosphorylation. Probenecid (Pn), an inhibitor of organic anion transports such as MRP4 and MRP5, has an effect on the cGMP level.[29] Sildenafil (Sd) potently inhibits a cGMP-binding cGMP-specific phosphodiesterase5[30] and increases its level, while oxidiazoloquinoxalin (ODQ) is the specific inhibitor of GC.[31] It blocks the production of cGMP resulting in reduction of the cellular level of this second messenger. The aim of this study was to demonstrate the phosphorylation time course in rat aorta after incubation with 10 µM phenylepherine (PhE). The effect of Pn, Sd, and ODQ on PhE induced myosin phosphorylation in rat aorta smooth muscles was also examined. The findings of this work might highlight the contribution of cGMP in myosin phosphorylation and muscle contraction.

Methods. Sildenafil was kindly provided by Dr. M. Baumhakel, Universitätskliniken des Saarlands, Homburg/Saar, Germany. Anti-MLCP 2 antibody was bought from Biolabs, Saar, Germany. Anti-glyceraldehyde 3-phosphate dehydrogenase (Anti-GAPDH) antibody was from Biodesign, Berlin, Germany. Oxidialzoloquinoxalin, PhE, and all substances were from Sigma, United States of America.

This study was performed at the Institute of Pharmacology Peter Holtz Research Center of Pharmacology and Experimental Therapeutics, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany from 1st July to 30th September 2005. Ethical approval was obtained from the Ethical Committee at Peter Holtz Research Center of Pharmacology and Experimental Therapeutics, Greifswald, Germany.

The rats (220-280 grams, 35 rats), obtained from Laboratories Science (Karlsburg) were anesthetized with 60 mg thiopental-sodium per kg then the aorta was rapidly excised and immersed in Rat Modified Krebs-Henseleit buffer (sodium chloride 113mM, potassium chloride 4.8mM, magnesium sulfate 1.3mM, potassium dihydrogen phosphate 1.2mM, sodium bicarbonate 25mM, calcium chloride 2.5mM, glucose 7mM). Loose

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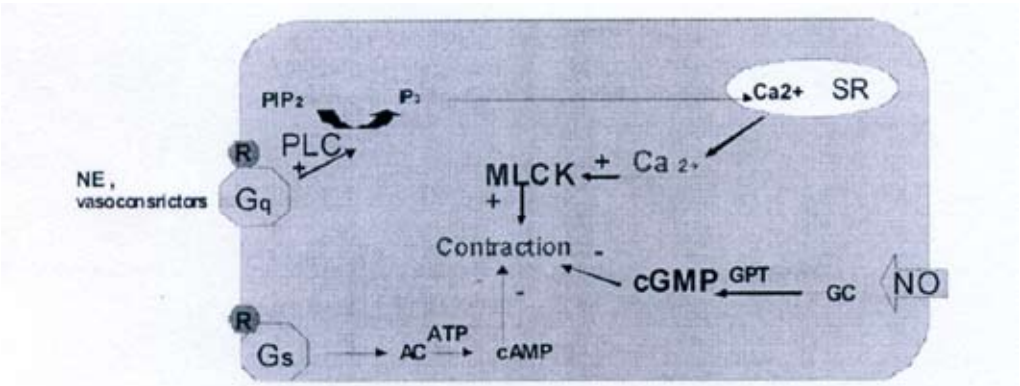

Figure 1 - Contraction and signal transduction system in smooth muscle including cyclic guanosine 3',5' monophosphate (cGMP). Vasoconstrictors and norepinephrine (NE) bind to receptors (G-protein coupled receptors) and activate phospholipase C (PLC) through protein Gq that releases inositol-3-phosphate (IP3). Inositol-3-phosphate releases calcium (Ca^{2+}) from sarcoplasmatic reticulum (SR). Calcium combines with calmodulin and activates myosin light chain kinase (MLCK) and induces contraction. Cyclic guanosine 3',5' monophosphate that is formed by nitric oxide (NO)-induced guanylyl cyclase (GC) as well as cyclic adenosine 3',5' monophosphate (cAMP) that is formed by Gs receptor activated adenylate cyclase (AC) inhibits the contraction. PIP_2 - phosphatidylinositol diphosphate, IP_3 - inositol-3-phosphate, GPT - guanosine 3',5'triphosphate, ATP - adenosine 3',5' triphosphate.

fat and connective tissues were carefully stripped. The aorta was stimulated with PhE 10-5 mole (M) for 30 or 60 minutes. Then it was preincubated with Pn 10-5 M, or ODQ) 10-5 M, or Sd 5.25 10-5 M for 30 minutes then PhE 10-5 M was added for 30 or 60 seconds. Unstimulated aorta was used as negative controls. The strips were rapidly removed from the medium, and frozen in liquid nitrogen and stored at -80°C. The aorta was homogenized in the presence of protease inhibitors (aprotinine, leupeptin, and phenylmethylsulfonyl fluoride). Then protein content was determined by using the bicinchoninic acid method with alkaline copper solution. By using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein 30 µg was separated on 15% polyacrylamide gale, and subsequently transferred to nitrocellulose membranes (0.2 µm; Schleicher and Schull, Dassel, Germany). Membranes were blocked with 10% Roti-block (Roth) in Tris Buffered saline with Tween (TBST) overnight. After washing, the membrane was incubated with anti-phosphomyosin at a dilution of 1:1000 overnight. Membranes were washed again with TBST buffer and blocked with 5% non-fat milk in TBST for 30 minutes. After washing, the blots were incubated with secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, at a dilution of 1:2000 for one hour at room temperature. Finally, the blot was developed with ECL-plus Western Blotting Detection System (Amersham). The phophorylation was measured by finding the ratio of the band intensity of the phosphorylation to its corresponding GADPH.

The data were analyzed using means and standard deviations as well as student's t-test. Statistical analyses were performed using the Statistical Package Social Science. The *p* value of =0.05 was taken as significant.

Results. To measure the phosphorylation of stimulated rat aortas, protein samples (30 µg) were loaded in each lane of SDS-PAGE. Equal loading was confirmed by Ponceau red staining as well as GAPDH immunoblotting. Measuring the ratio of the band intensity of the phosphomyosin to its corresponding GADPH showed signal differences between the control (unstimulated) and the PhE treated aortas for 30 and 60 seconds. With the use of 10 µM PhE, the stimulation of rat aorta tissue was significantly evident over 30 (p=0.05) and 60 (p=0.04) seconds through anti-phosphomyosin signal **Figure 2**. This stimulation decreased significantly (p=0.01) at 120 seconds. Then the decrease continued during the following 10 minutes. The ratio of stimulation with PhE (10 µM) to the untreated aortas was 16.7:1 at

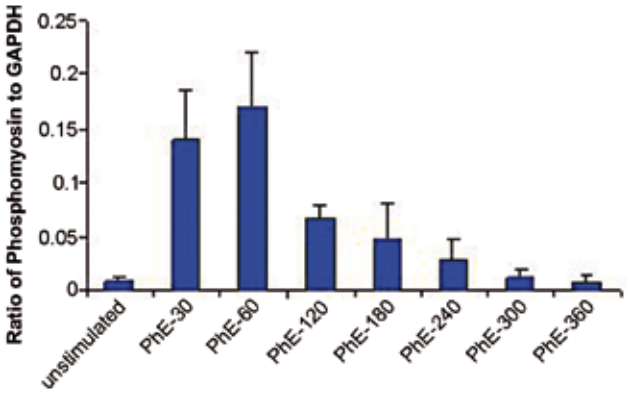

Figure 2 - Time course of myosin phosphorylation in rat aortas with phenylepherine (PhE) (10 µM). Rat aortas were incubated with PhE (10 µM) for 30, 60, 120, 180, 240, 300, and 360 seconds then frozen in liquid nitrogen -08°C. GAPDH - glyceraldehyde 3-phosphate dehydrogenase.

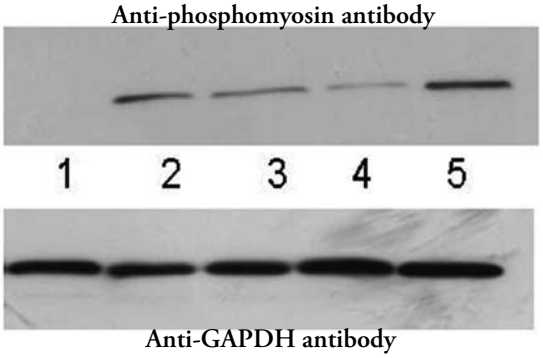

Figure 3 - Effect of cyclic guanosine 3',5' monophosphate modulators on myosin phosphorylation. Rat aorta tissues were pre-incubated for 30 minutes with 10 μM probenecid (3), 50 μM sildenafil (Sd) (4), or 10 μM oxidiazoloquinoxalin (ODQ) (5), and then 10 μM phenylepherine (PhE) was added for 30 seconds. Unstimulated aorta (1, negative control) and PhE (10 μM) stimulated tissue (2, positive control) are shown left. Phosphorylation of myosin light chain 20 was determined. Upper panel shows anti-phosphomyosin antibody and lower panel anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) antibody. 1=unstimulated rat aorta, 2=stimulated rat aorta with 10 μM PhE for 30 seconds.

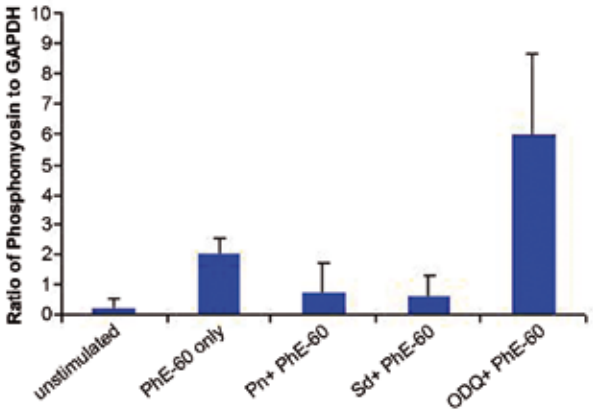

Figure 4 - Effects of cyclic guanosine 3',5' monophosphate modulators on phosphorylation of myosin light chain 20 in rat aortas stimulated with phenylepherine (PhE). Rat aorta tissues were pre-incubated for 30 minutes with 10 μM probenicid (Pn), 50 μM sildenafil (Sd), or 10 μM oxidiazoloquinoxalin (ODQ), and then 10 μM PhE was added (to the present modulator) for 60 seconds. Unstimulated aortas were showed left. Phosphorylation of MLC20 was determined. Ratio of phosphomyosin to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was calculated with standard error. Data were obtained from 5 experiments with different aortas.

30 seconds and 20.4:1 at 60 seconds.

To detect the effect of cGMP modulators on the phosphorylation state of the rat aorta, Pn (10 μM), Sd (50 μM) and ODQ (10 μM) were pre-incubated for 30 minutes with aortas, then for 30 or 60 seconds incubated with 10 μM PhE in their presence. **Figure 3** represents a western blot of cGMP modulators stimulated for 30 seconds with the presence of 10 μM PhE. Oxidiazoloquinoxalin showed a non-significant increment in the phosphorylation after adding 10 μM PhE for 30 (p=0.35) and 60 seconds (p=0.06), while Pn (p=0.18) and Sd (p=0.07) decreased it. Probenecid decreased it significantly at 30 second-stimulation p=0.03 (data not shown). **Figure 4** shows the non-significant effect of muscle treated with the modulators and then stimulated with 10 μM PhE for 60 seconds. The washout-effect of these modulators was not significant (p>0.05) after stimulation with PhE only, **Figure 5**.

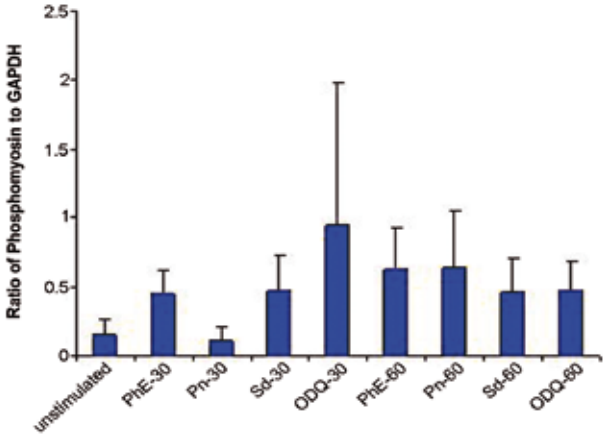

Figure 5 - Washout effect of cyclic guanosine 3',5' monophosphate (cGMP) modulators on myosin phosphorylation. Rat aorta tissues were pre-incubated for 30 minutes with 10 μM probenicid (Pn), 50 μM sildenafil (Sd), or 10 μM oxidiazoloquinoxalin (ODQ) and then stimulated with 10 μM phenylepherine (PhE) for 30 or 60 seconds in the absence of the modulators. Unstimulated aortas are shown left. Phosphorylation of myosin light chain 20 was determined. Ratio of phosphomyosin to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was calculated with standard error. Data were obtained from 3 experiments with different rat aortas.

Discussion. The results of this study showed that PhE (10 μM) stimulated rat aorta smooth muscle and caused myosin phosphorylation as it was detected by MLC 20 antibody. The phosphorylation course of aortas showed a significant increment in the first 60 seconds (p<0.05) and dropped down significantly at 120 seconds (p<0.05) then continued to decrease until 10 minutes. The ratio of myosin phosphorylation to untreated control was 16.7:1 at 30 seconds and 20.4:1 at 60 seconds stimulation.

Contraction in vascular smooth muscle (VSM) can be initiated by mechanical, electrical, and chemical stimuli. Passive stretching of VSM can cause myogenic contraction via unspecific phosphorylation and increment of Ca^{2+} sensitivity. The isolation technique can put the aorta under stress that may lead to initiation of

phosphorylation. However, this is one of the limitations of the study. In addition, different aortas exhibited various sensitivity. Therefore, incorporation of phosphomyosin anti-body in some un-stimulated samples could be caused by such unspecific phosphorylation. Such samples were excluded. Vasoconstrictors, such as sympathomemitic PhE that binds to G-protein-coupled receptors, produce contraction by increasing both the cytosolic Ca^{2+} concentration and the Ca^{2+} sensitivity of the contractile apparatus.[32] The increased sensitivity of VSM toward Ca^{2+} also results from inhibition of MLCP activity leading to increased MLC phosphorylation and tension at a constant Ca^{2+} concentration. The Ca^{2+} sensitizing effect of vasoconstrictors[16,32] is ascribed to the activation of the small 22-26-kDa GTPase RhoA that activates Rho kinase, which in turn, phosphorylates the regulatory subunit of MLCP and inhibits its activity.[1,33]

Thus, further insight into the pathogenesis of hypertension could be considered. Accumulated data suggested that various regulatory mechanisms are involved in initiation and maintenance of muscle contraction as well as phosphorylation.[34-36] Probenecid and Sd reduced myosin phosphorylation of aorta stimulated with PhE for 30 and 60 seconds. This finding was not significant, which may be associated with high biological variability of tissues from different rats (a limitation of the study). However, the reduction in myosin phosphorylation was evident. It may reflect down regulation of inositol 1, 4, 5-triphosphate receptors (IP3-r) due to their phosphorylation with dependent protein kinase (PKG), which is in turn activated by Pn and Sd induced accumulation of cGMP. Another remote explanation could include cAMP, which is cross activated by cGMP and it affects the phosphorylation negatively. There is a cross-activity between cAMP and cGMP, which leads to activation of PKG, which phosphorylates IP3-r.[37,38] The effect of cGMP and activation of PKG on the phosphorylation of IP3-r were examined in intact rat aorta.[37] Relaxation induced by PKG[34,39] can be attributed to reduction of free cytoplasmic Ca^{2+} concentration via inhibition of Ca^{2+} release by the SR, or inhibition of the IP3 formation by inhibition of phospholipase C (PLC) activation or inhibition of G protein coupling to PLC,[37] as well as Ca^{2+} desensitization. In spite of the presence of some contrary views, studies using confocal laser scanning microscopy to determine the cellular distribution of PKG suggest that PKG is found in SR where both the phospholamban (a protein that is phosphorylated in response to PKG activation and may contribute to the reduction in $[Ca^{2+}]_i$), and IP3 receptor are localized. Phosphorylation of these receptors by PKG plays a role in inhibition of agonist-evoked Ca^{2+} release from SR.[37] It was supposed that phophorylation may regulate Ca^{2+} gating or other regulating features of the protein.

This finding may be of benefit in the search for new antihypertensive agents acting on mechanisms different from the classical once based on unlimited pathogenesis of hypertension. Oxidiazoloquinoxalin blocks the activity of GC and therefore cGMP production.[40] Therefore, the level of this second messenger drops down. Pre-incubation of the aorta with this substance may reduce the availability of cellular cGMP, subsequently, cancel its negative influence on phosphorylation. Adding PhE (10 μM) to this solution increased the myosin phosphorylation, which was clear at 60 seconds after starting the stimulation. Depending on the state and level of phosphorylation, the effects of ODQ varied. After 30 seconds stimulation the activation of phosphorylation may not be so clear to produce a threshold concentration of cGMP allowing significant increment in myosin phosphorylation (data not shown). However, after 60 seconds stimulation, ODQ increased the phosphorylation in each experiment. This result agrees with Chinkers et al's[41] finding that atrial natriuretic peptide increases cGMP in a concentration- and time- dependent fashion.

The results show that PhE caused time dependent phosphorylation of rat aortas. Sildenafil and Pn reduced the phosphorylation and subsequently the contraction of rat aortas. Oxidiazoloquinoxalin potentiated the contraction. The effect of ODQ was depended on the level of myosin phosphorylation. Cyclic guanosine 3',5' monophosphate involved in smooth muscle contraction. The study demonstrates the importance of cGMP and its modulators in muscle contraction of rat aortas. Future studies should be directed to investigate the impact of cGMP and its modulators on other smooth muscle contraction such as bronchial smooth muscles.

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