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Angiotensin II Induced Tension Development in Rat-Tail Artery Involves PLD and PKC

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Abstract

Adrenergic and angiotensinergic receptors are coupled to biochemical pathways that cause a rapid and sustained vascular smooth muscle contraction, the interrelations of which are not completely understood. Our objective was to compare the contractile response of rat-tail artery to phenylephrine (PHE) and angiotensin II (Ang II). Isolated ring segments of tail arteries from male adult Sprague-Dawley rats were studied. Concentration-response relationships for PHE (EC_{50}=1 μM, E_{max}=10 μM) and Ang II (EC_{50}=10 μM, E_{max}=100 μM) revealed major differences in sensitivity to the agonists. PHE produced a greater, and faster contraction (2.6 g max tension, t_{50}=12 sec) than Ang II (0.5 g max tension, t_{50}=43 sec). Blockade of protein kinase C (PKC) with either calphostin C (IC_{50}=100nM), or chelerythrine chloride (IC_{50}=1μM), reduced the Ang II response by greater than 50%, but had no effect on

PHE induced tension. Thin-layer chromatography studies showed that Ang II in the presence of ethanol induced a greater than 3-fold increase in the production of Phosphatidylethanol (PEt), indicative of phospholipase D (PLD) activity. These data suggest that PKC plays a greater role in the contractile response mediated by angiotensin type I (AT_1) receptor, than α_1-adrenergic receptors (AR_1), and that α_1-AR_1 is the major receptor for mediating contraction in the rat-tail artery. The data further suggests that AT_1 receptor induced tension is linked to activation of PLD.

INTRODUCTION

Angiotensin II (Ang II) exerts a wide range of actions on the heart, blood vessels, adrenals, kidneys, and nervous system, and plays a major role in blood pressure maintenance and volume homeostasis [5]. Its effects are mediated mainly by plasma membrane receptors. At least two subtypes of Ang II receptors exist in smooth muscle cells, namely angiotensin type I (AT_1) and type 2 (AT_2) [11]. Pharmacologically, the antagonist Losartan binds with high affinity to AT_1 receptors while PD123177 and CGP 42112A [3] binds to AT_2. The AT_2 receptors are coupled to prostaglandin release; whereas, the AT_1 receptors are mainly responsible for the contractile response of rat tail artery to Ang II stimulation, and are coupled to metabolism of phosphoinositides and mobilization of intracellular calcium [12, 13]. Hydrolysis of phosphoinositides and mobilization of intracellular calcium are also linked to alpha$_1$-adrenergic (α_1-AR) receptors, which are also present in arterial smooth muscle [1, 4].

Vascular smooth muscle contraction involves multiple signal-transduction pathways, the interrelations of which are not completely understood. The classical pathway through which contractile stimuli induce development of tension in vascular smooth muscle is initiated by coupling of α_1-AR and AT_1 receptors to members of the G_q family of heterotrimeric G-proteins. This results in the activation of phospholipase Cβ (PLCβ), formation of inositol-1,4,5-trisphosphate (IP_3), diacylglycerol (DAG) and activation of protein kinase C (PKC) [4, 7]. In the last decade, several groups have demonstrated that AT_1 receptors mediate signal transduction via phospholipase D (PLD) activation by PKC to develop sustained smooth muscle contraction [22, 25]. There are however conflicting reports regarding the role of PKC in phospholipid metabolism and consequent contraction. Gu *et al*. [10] reported that norepinephrine stimulation of α_1-AR in rat tail artery resulted in activation of both PLC and PLD in a dose-dependent manner. They also showed that the ability of norepinephrine to stimulate phosphatidylcholine metabolism was independent of PKC, and was not affected by extracellular calcium depletion. In another study, Gu *et al.* [9] further showed that norepinephrine could stimulate the formation of inositol phosphates in tail artery within 3-5 s. However, LaBelle and

Murray [16] reported that norepinephrine also stimulated phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis in rat tail artery. One way by which PIP2 metabolism could affect hydrolysis of PC might involve PKC. The authors also showed that with respect to IP3 metabolism, tail artery was more active than aorta. In addition, rat-tail artery contains numerous types of receptors, and it is a good model to elucidate receptor mediated signal transduction response. In the present study we sought to compare the effects of α_1-adrenergic agonist phenylephrine (PHE) and Ang II (AT_1-agonist) on contraction in the rat-tail artery. Functionally, these agonists both cause contraction, but biochemically there are differences in how the result is achieved. In addition, we wanted to determine the role of protein kinase C (PKC) in (a) the contractile response of rat-tail artery to stimulation of the AT_1 receptor pathway and (b) activation of PLD.

METHODS

Tissue preparation

All protocols were approved by the Loma Linda University Animal Care and Use Committee.

Male adult Sprague-Dawley rats (200-300g) were decapitated. The ventral tail arteries were dissected out and cleaned of adipose and connective tissue. Arteries were cut into individual ring segments, 5 mm in length. To avoid the complications of endothelial-mediated effects, the endothelium was removed by carefully inserting a small wire 3 times into the lumen of the vessels. Two platinum wires were then inserted into the lumen of the segments, for mounting on a Grass PT-3 force transducer. The mounted vessels were immersed in 6.5 ml tissue bath containing Krebs physiological solution (KPS) at 37°C equilibrated with 95 %O_2-5 % CO_2. The composition of the KPS was (in mM): NaCl (118); KCl (5.4); $CaCl_2$ (2.5); $NaHCO_3$ (12.5); $MgSO_4$ (1.12); KH_2PO_4 (1.2); and glucose (10).

Vessel segments were equilibrated for 60 minutes. KPS was changed every 15 to 20 minutes. The preload that produced maximum response to contractile agonists was then determined [21]. The resulting electrical signals were digitized on a MacLab analog-digital converter, and were recorded by a Macintosh computer. After equilibration, the tissue reactivity was determined by the contractile response to 10 μM phenylephrine (PHE). The absence of a relaxation response to subsequent exposure to acetylcholine (10 μM) was taken as evidence that the vessel segments were functionally denuded of endothelium

Dose Response Relationships

After verifying that vessel segments were denuded of endothelium, artery segments were equilibrated for 1 hour, and then stretched slowly to the previously

determined preload of 1g, Dose-response curves of developed tension were then obtained using PHE (10^{-10} M to 10^{-3} M), an α_1-adrenergic agonist, and Ang II (10^{-10} M to 10^{-3} M), an AT_1- receptor agonist. Developed tension data were normalized relative to the maximum response to 106 mM potassium. The data were then fitted to a logistic equation (Sigma Plot software) to obtain pD_2 (-log EC_{50}) values, and the maximal contractile responses (E_{max}) for each agonist. The EC_{50} concentrations for the agonists were used in subsequent experiments unless otherwise stated.

Time-Tension Relationships

Time-tension relationship curves were constructed after treatment of vessel segments with E_{max} dose of PHE (10μM) and AngII (100μM) for 5 min. These doses were chosen as they gave the most consistent response.

Effect of PKC Inhibitors

In some experiments, vessels were incubated for 15 min with selective PKC inhibitors, calphostin-C (10 nM to 100 nM) or chelerythrine chloride (10 nM to 10μM) prior to addition of either PHE or Ang II.

Assay of PLD activity in rat-tail artery.

Preparation and treatment of arteries

Tail artery segments were treated by a modification of the method of Gu *et al* [10]. Briefly, artery segments were immersed in a 6 ml tissue bath containing HEPES (5 mM)-buffered Krebs physiological solution (HKPS) at 37° C, and equilibrated with 95% O_2 -5% CO_2 for 60 min. The segments were then transferred to tubes containing [^{3}H] myristic acid (50 μCi/0.5 ml) and1% serum albumin in HKPS, for an additional180 min. Segments were washed twice with 5 ml buffer, and then incubated three times in 1ml HKPS buffer for 30 min. Segments were incubated for 10 min with (1) buffer alone; (2) 2% ethanol alone; (3) Ang II (10 μM) alone; or (4) ethanol (2 %) + Ang II (10 μM). Treated segments were frozen for subsequent extraction and measurement of membrane lipids [9, 10].

Measurement of membrane lipids in rat-tail artery

Membrane lipid extraction was carried out according to the method by LaBelle and Murray [16]. Briefly, 0.4 ml of 2.4 M HCl was added to artery segments that had been frozen in a mortar that was immersed in liquid N_2. A chilled pestle was used to homogenize the frozen samples to powder. The powder was treated with 1.5 ml cold (0ºC) chloroform-methanol (1:2), and the extract transferred to a cold glass tube. The mortar was rinsed with 3 ml chloroform-methanol mixture, and the rinses were combined with the initial extracts. Combined extracts were treated with 2.3 ml HCl (2.4 M) and 1.5 ml chloroform. The resulting two phase mixture was clarified by centrifugation for 5 min at 1,000 g. The chloroform phases were transferred to clean, glass tubes, and the aqueous phases were washed with 2 ml chloroform. The combined chloroform phases were washed with 0.4 ml HCl and 3 ml methanol-water (1:1). The chloroform phases were dried under nitrogen, and the dried lipids were dissolved in 100 μl chloroform, and spotted on thin layer plates

(Linear K5, 150 Å; Whatman) along with phospholipid standards (phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid). Plates were developed with chloroform-methanol-4 M ammonium hydroxide (90-70-20:v-v-v). The developed thin layer plates were observed under UV light. Spots were marked and then scraped in to 5 ml scintillation vials. Scintillation fluid was added, and samples were counted in a liquid scintillation counter (Beckman) [10].

Drugs and Chemicals

Ang II, acetylcholine, PE, [^{3}H] myristate, and ingredients for Na and K Krebs and HEPES were obtained from Sigma (St. Louis, MO USA). Chelerythrine chloride and calphostin C were purchased from BIOMOL Research Labs, Inc (Plymouth Meeting, PA USA). All drugs and chemicals were dissolved in purified water.

Statistical analysis

All values were calculated as means ± SE. In all cases, N values refer to the number of rats used in each experiment. When appropriate, we used multiple range tests with the ANOVA to look at the differences between the means. P value < 0.05 was considered significant. GraphPad InStat and Prism 3.0 computer software were used for statistical analysis for all data values.

RESULTS

Both phenylephrine and AngII [Fig. 1], increased tension development in rat-tail artery, in a dose-dependent manner. Phenylephrine produced maximum responses at 10μM dose whereas AngII produced a maximum response at 100μM dose. The EC_{50} value for PHE (EC_{50} = 1 μM, E_{max}= 10 μM) was ten times less than that of AngII (EC_{50} = 10μM, E_{max}=100 μM). The linear portion of the PHE curve was much steeper than that of the Ang II curve, and differences in E_{max} values are clearly seen. These results are consistent with activity studies that identify adrenergic [1, 4] and angiotensinergic receptors in vascular tissue [3, 11]. The EC_{50} value for AngII was used in subsequent experiment as this value was consistent.

In comparing time-tension relationship for PHE and Ang II receptor activation in rat-tail artery segments (Fig. 2), PHE produced a greater and faster contraction (2.6 g maximum tension, t_{50}=12 sec) than Ang II (0.5 g maximum tension, t_{50}= 43 sec). T_{50} represents the time it takes for a ligand to develop 50% maximum tension.

After determining the dose response relationship between PHE and Ang II, and establishing EC_{50} and E_{max} doses, we wanted to investigate the influence of PKC on both receptor pathways. Calphostin C was determined to be a highly specific PKC inhibitor that interacts with the protein's regulatory domain, by competing at

the binding site for DAG and phorbol esters. Figure 3A shows that calphostin C (10 nM, 50 nM, and 100 nM) had no significant effect on tension development in tail artery caused by PHE. Figure 3B however showed that calphostin C caused a dose-dependent inhibition of tension development by Ang II, with an IC_{50} of 100 nM.

To further clarify the role of PKC in α_1-AR and AT_1 receptor pathways, we applied a second inhibitor of PKC, Chelerythrine Chloride, which is structurally unrelated to Calphostin C. Fig. 4A showed that PHE stimulation of arterial segments resulted in no significant dose-dependent inhibition in the presence of varying concentrations of Chelerythrine Chloride (10 nM, 100 nM, 1 μM, and 10 μM). On the other hand, Fig. 4B showed that Ang II stimulation of arterial segments resulted in a dose-dependent inhibition in the presence of Chelerythrine Chloride (100 nM, 1 μM, and 10 μM range) with an IC_{50} of 1 μM.

After determining PKC involvement in angiotensinergic-coupled contraction in rat-tail artery, biochemical studies were conducted to elucidate the role of phospholipase D (PLD). Fig. 5 showed that Ang II, in the presence of ethanol, induced a greater than 3-fold increase in the production of phosphatidylethanol (Pet), indicative of PLD activity.

DISCUSSION

The major focus of this study was to compare the contractile response of rat tail artery caused by activation of α_1-AR and AT_1 receptors, and to further determine the role of PKC in either pathway. The presence of both α_1-AR [1,4] and AT_1 [3,11] receptors in rat tail artery have been adequately described by others. Our data obtained in tail artery, treated with both Ang II and PHE, showed that both agonists produce sigmoidal concentration response curves, presumably through activation of AT_1 and α_1-AR receptors respectively (Figure 1). An examination of both curves reveals that maximum tension development caused by Ang II is less than that caused by PHE. This indicates that there are differences in the pathways leading to contraction that are activated by each agonist. This was further confirmed by examination of the time tension relationship for both Ang II and PHE which clearly showed (Figure 2) that PHE produced a greater and faster contraction compared to Ang II.

One previous study by Hong Gu *et al* [10] has shown that in rat tail artery, norepinephrine stimulates both PLC and PLD in a dose-dependent manner, and this effect is mediated through α_1-AR. They further showed that the ability of norepinephrine to stimulate phosphatidylcholine metabolism was independent of PKC, and was not affected by extracellular calcium depletion. However, LaBelle and Murray [16] reported that norepinephrine stimulated PIP2 hydrolysis in rat tail artery, and Gu *et al.* [10] reported that norepinephrine could stimulate the formation

of inositol phosphates in tail artery within 3-5 s. This suggests that NE may stimulate PC metabolism by PLD or PLC secondary to PIP2 metabolism. One way by which PIP2 metabolism could affect hydrolysis of PC might involve PKC. Two structurally unrelated PKC inhibitors were employed in our studies. Calphostin C is a highly specific PKC inhibitor that interacts with the protein's regulatory domain by competing at the binding site for DAG and phorbol esters. Chelerythrine Chloride acts on the catalytic domain of PKC. Our results showed that neither Calphostin C (Fig. 3A) nor Chelerythrine Chloride (Fig. 4A) had any significant effect on PHE induced tension development in tail arteries. Our results supported the conclusion of Gu e*t al*. [10] that PKC does not play a major role in α_1-AR mediated contraction of tail artery.

With regards to AT_1 receptors, several studies [14, 17, 20, 23] have shown that Ang II stimulation of AT_1 receptors, are coupled to PLC activation. Freeman *et al*. [6] have shown that in aortic vascular smooth muscle, Ang II activated PLD, and also stimulated tension development in a PKC dependent manner, subsequent to activation of PLC. We hypothesized that in rat tail artery, Ang II stimulation of tension development is coupled to PKC and PLD activation. We showed that, unlike PHE activated contraction, Ang II stimulated contraction was attenuated by both PKC inhibitors, Calphostin C (Fig. 3B) and Chelerythrine Chloride (Fig. 4B) in a dose-dependent manner. This suggests that Ang II contraction of rat tail artery is mediated in part by PKC. Since PKC is a known activator of PLD, we conducted biochemical studies to determine if PLD activation was coupled to AngII stimulation of rat tail artery. There is evidence that Ang II response is coupled to heterotrimeric G-protein $G_{\alpha 12\beta\gamma}$ activating PLD, and provides the cell a secondary source of DAG [15]. DAG is an important activator of PKC in vascular smooth muscle. Because there are multiple plasma membrane substrates (i.e., phosphatidylinositol and phosphatidylcholine) that are involved in the production of DAG, we wanted to use a technique that would isolate the specific activity of PLD.

Since transphosphatidylation is catalyzed by PLD, and not by PLC, this property provides the basis for a useful methodology to distinguish PLD from PLC. In several cells, PEt is formed when cells are exposed to specific agents in the presence of ethanol (0.1 to 2%), and when measured, PEt is formed with rates similar to those of choline and PA. Because of metabolic stability, PEt formation can be used to detect PLD activity in systems like the rat-tail artery [10] where PA does not accumulate due to rapid dephosphorylation. In Figure 5, we showed that in the presence of ethanol, Ang II induced a greater than 3-fold increase in the production of PEt, indicative of PLD activity. This suggests that Ang II induced tension is linked to the activation of PLD. Various authors have linked the biochemical activation of PLD in the plasma membrane via heterotrimeric G protein ($G_{\alpha 12\beta\gamma}$), as suggested by Ushino *et al* [24]. Also, Hong Gu *et al.* [10], showed similar PLD activity in the rat-tail artery by using norepinephrine, a non-selective α-adrenergic

agonist. With the use of angiotensin-(2-8)-peptide in the rat-aortic smooth muscle cells, Freeman and Tallant [6] here also showed PLD activation linked to AT_1 receptor activation. The fact that we conducted our experiments in intact rat-tail artery is significant. Most published studies dealing with PLD and PKC were conducted in HL-60 granulocytes [19], and lymphocytes [2], aortic cultured smooth muscle cells and other smooth muscle cell lines [4, 8, 18, 25].

CONCLUSIONS

The present study confirmed that rat-tail arterial smooth muscle contraction can be mediated by both α_1-AR and AT_1 receptors. Our results showed clear differences between the contractile response of tail artery to agonists Ang II and PHE, and that α_1-AR played a greater role than AT_1 receptors in mediating contraction in rat-tail artery. The data further showed that the role of PKC as a second messenger in rat tail artery contraction is more prominent for the AT_1 receptor pathway than the α_1-AR pathway. In addition, biochemical studies using thin layer chromatography, showed that Ang II, in the presence of ethanol, induced over three-fold increase in the production of PEt, indicative of PLD activity, which further suggest that AT_1 receptor induced tension is linked to activation of PLD.

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FIGURE LEGENDS

Figure 1: Comparison of concentration-response relationship for phenylephrine (PHE) and angiotensin II (Ang II)-induced tension development in rat-tail artery. Phenylephrine response is greater than the angiotensin II response, and increases with concentration up to the maximum, with both curves showing saturation. The EC_{50} value for PHE (EC_{50} = 1 μM, E_{max}= 10 μM) was ten times less than that of AngII (EC_{50} = 10μM, E_{max}=100 μM.Tissue was treated as described in Material and Methods. Data are the means ± SEM of six experiments (N=6).

Figure 2: Time-tension relationship for angiotensin II and phenylephrine-induced tension development in rat-tail artery. Phenylephrine produced a greater and faster contraction (2.6g max tension, t_{50}=12sec) than Ang II (0.5g max tension, t_{50}=43sec). Data are the means ± SEM of seven experiments (N=7).

Figure 3: Effect of addition of various concentrations of calphostin C on contractile response of tail artery to (A) Phenylephrine (10 μM) and (B) Angiotensin II (10 μM). Tissue was treated as described in Material and Methods. All contractile responses are expressed as % maximum response to 106 mM potassium. Calphostin C had no significant effect on phenylephrine-induced contraction of tail artery. Calphostin C caused a concentration-dependent(10 nM to 100 nM) inhibition of angiotensin II-induced contraction (IC_{50}=100 nM)..Data are the means ± SEM of five experiments (N=5 ; $P<0.05$)

Figure 4: Effect of addition of various concentrations of Chelerythrine Chloride on contractile response of tail artery to (A) Phenylephrine (10 μM) and (B) Angiotensin II (10 μM). Tissue was treated as described in Material and Methods. All contractile responses are expressed as % maximum response to 106 mM potassium. Chelerythrine Chloride had no significant effect on phenylephrine-induced contraction of tail artery. Chelerythrine Chloride caused a concentration-dependent

(100 nM to 10 :M) inhibition of angiotensin II-induced contraction (IC_{50}=1:m,. M). Data are the means ± SEM of five experiments (N=5 ; P<0.05)

Figure 5: Angiotensin II-induced activation of PLD in rat-tail artery. Segments of rat-tail artery were prelabelled with [^{3}H] myristic acid (50 μCi/0.5 ml), washed and then incubated for 10 min with either buffer alone; 2% ethanol alone; Ang II (10 μM) alone; or ethanol (2 %) + Ang II (10 μM). The formation of PEt was quantified as described in Materials and Methods. Ang II in the presence of ethanol induced over 3-fold increase in the production of PEt indicative of PLD activity. Data are the means ± SEM of three experiments (N=3).

Figure 6: Signal transduction mechanism of α_1-adrenergic and AT_1-angiotensinergic induced tension development in rat-tail artery. Protein kinase C involvement is greater via the AT_1 receptor than the α_1-adrenergic receptor pathway. PLC, phospholipase C; PIP, phosphoinositol phosphate; IP3, inositol trisphosphate; DAG, diacylglycerol; PA, phosphatidic acid; C, choline; PC, phosphatidylcholine; PLD, phospholipase D; PKC, protein kinase C; SR, sarcoplasmic reticulum, cmod, calmodulin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; CPI-17, myosin light chain phosphatase inhibitor; and Ca^{+2}, calcium. (I),inhibitory; (A),active.

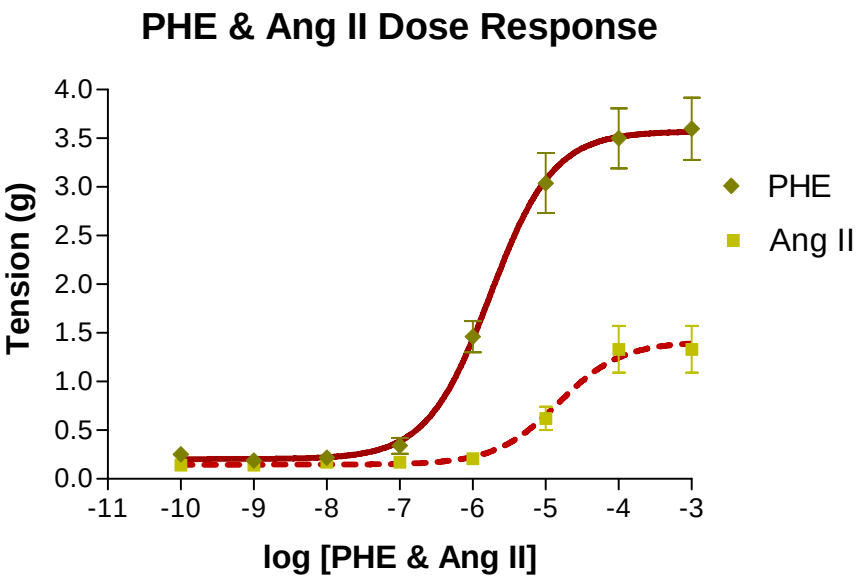

PHE & Ang II Dose Response
Tension (g)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-11
-10
-9
-8
-7
-6
-5
-4
-3
log [PHE & Ang II]
PHE
Ang II

Fig. 1

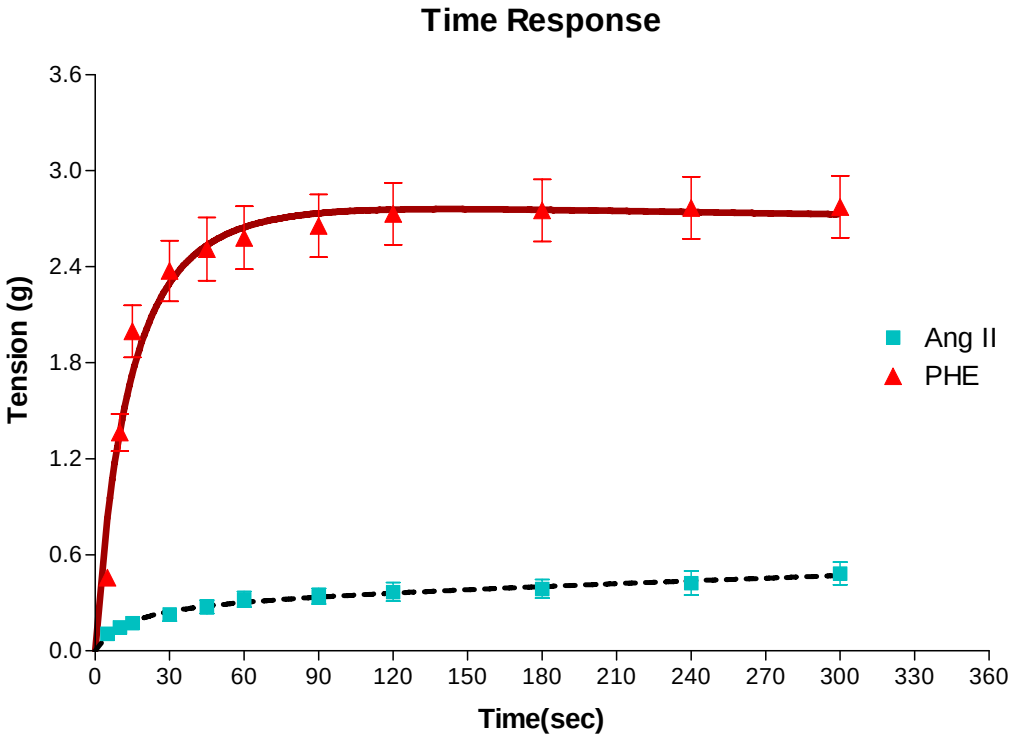
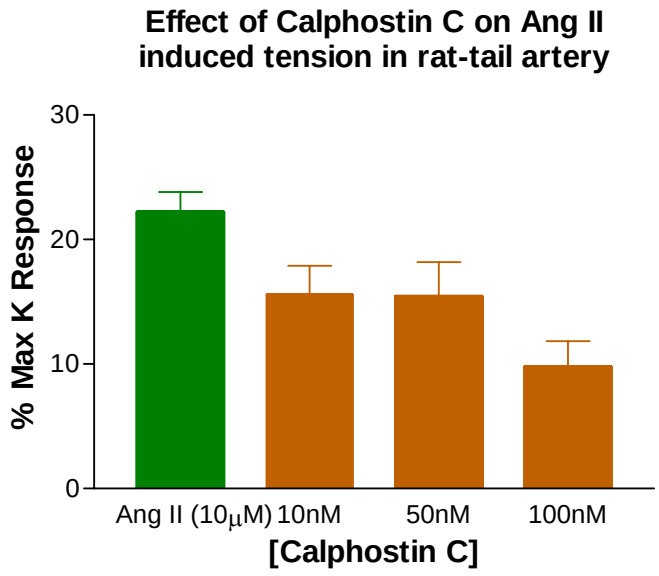
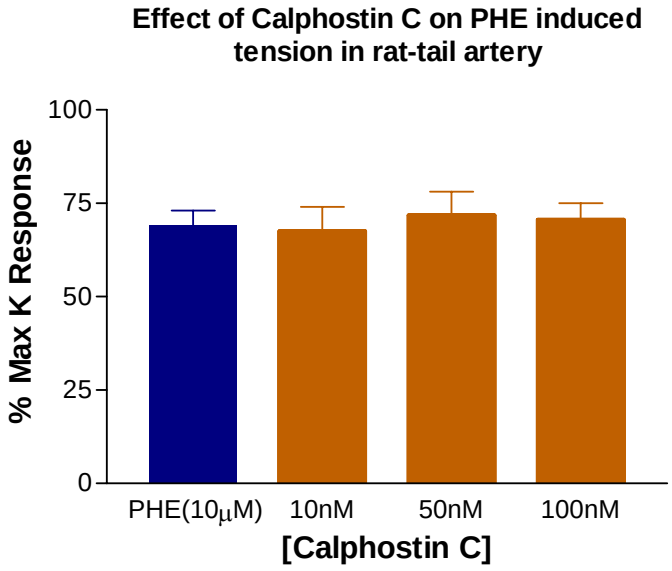

Time Response
Tension (g)
3.6
3.0
2.4
1.8
1.2
0.6
0.0
0
30
60
90
120
150
180
210
240
270
300
330
360
Time(sec)
Ang II
PHE

Fig. 2

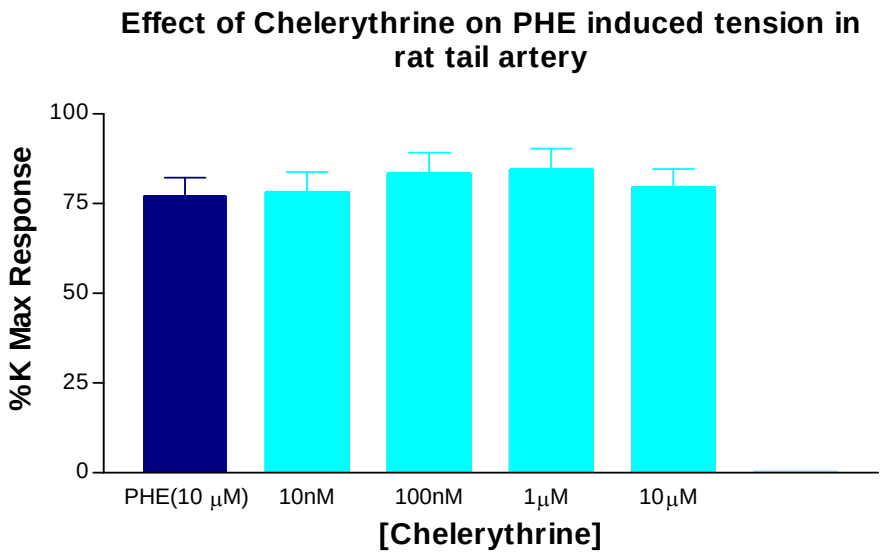
3A


Effect of Calphostin C on Ang II induced tension in rat-tail artery
% Max K Response
30
20
10
0
Ang II (10μM)
10nM
50nM
100nM
[Calphostin C]

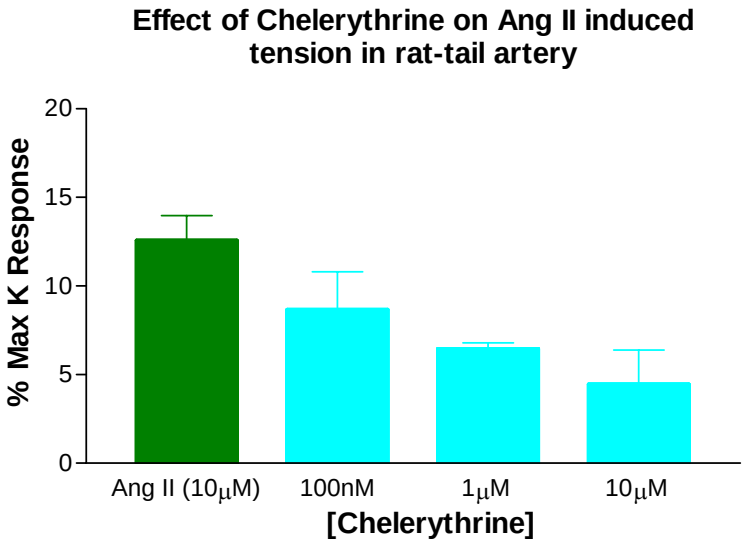
3B


Effect of Calphostin C on PHE induced tension in rat-tail artery
% Max K Response
100
75
50
25
0
PHE(10μM)
10nM
50nM
100nM
[Calphostin C]

4A


Effect of Chelerythrine on PHE induced tension in rat tail artery
%K Max Response
100
75
50
25
0
PHE(10 μM)
10nM
100nM
1μM
10μM
[Chelerythrine]

4B


Effect of Chelerythrine on Ang II induced tension in rat-tail artery
% Max K Response
20
15
10
5
0
Ang II (10μM)
100nM
1μM
10μM
[Chelerythrine]

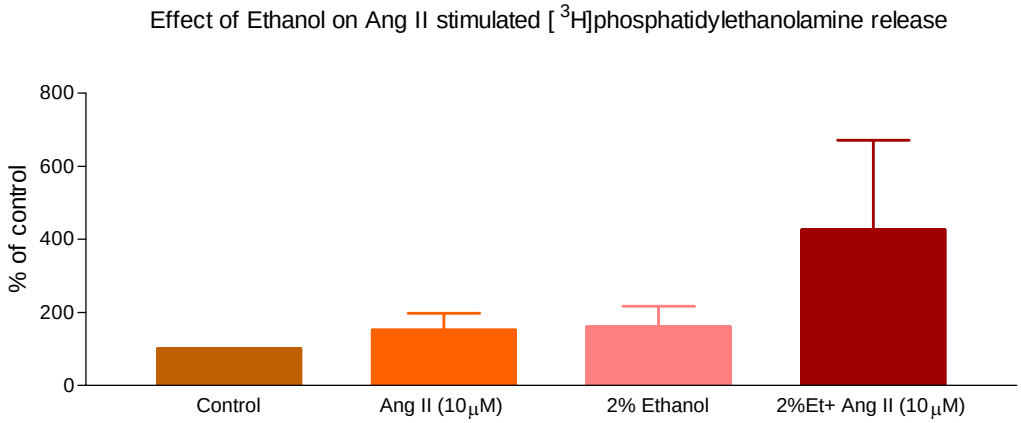

Effect of Ethanol on Ang II stimulated [3H]phosphatidylethanolamine release
% of control
800
600
400
200
0
Control
Ang II (10μM)
2% Ethanol
2%Et+ Ang II (10μM)

Fig 5

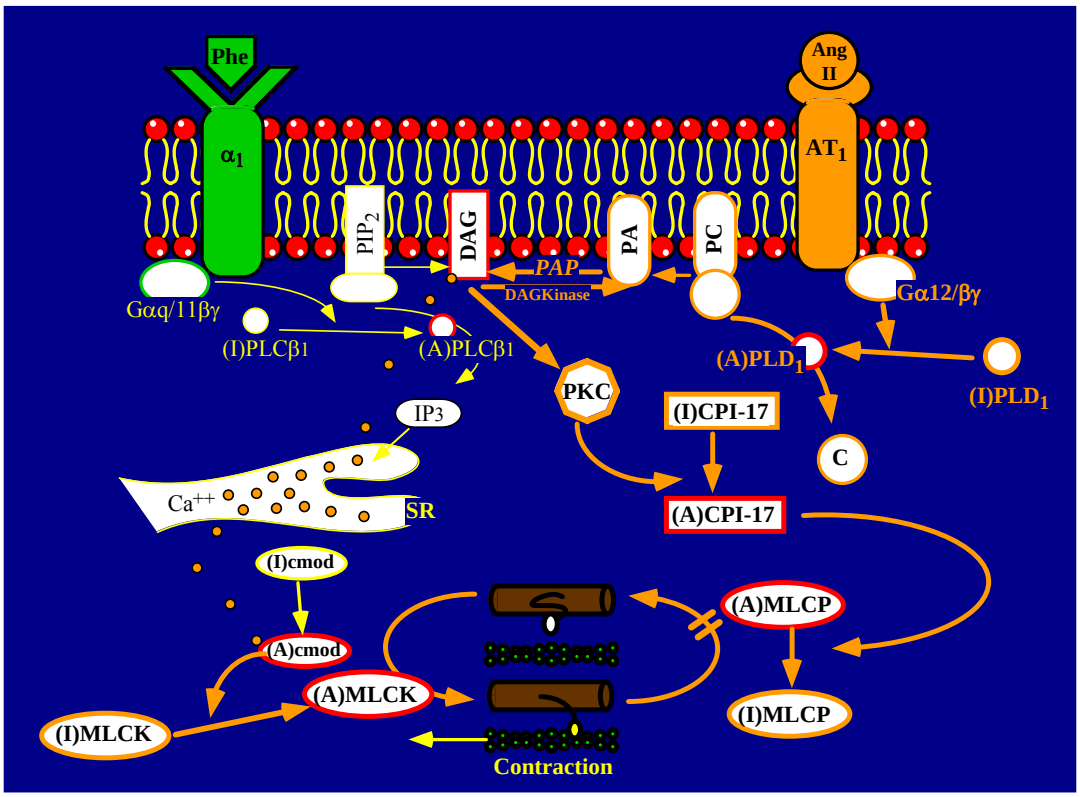


Fig.6

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