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EFFECTS OF METFORMIN ON CONTRACTILITY OF ISOLATED RAT THORACIC AORTA

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เมทฟอร์มินเป็นยาลดระดับน้ำตาลในเลือดที่มีฤทธิ์ลดความดันโลหิต จึงทำการศึกษาผลของเมทฟอร์มินต่อความสามารถในการทำงานของหลอดเลือดแดงใหญ่หนูขาวที่แยกจากกายในภาวะที่มีและไม่มี endothelium โดยวัดการหดตัวของกล้ามเนื้อหลอดเลือด พบว่าเมทฟอร์มินมีผลลดการหดตัวของหลอดเลือดที่กระตุ้นด้วย noradrenaline และ caffeine อย่างมีนัยสำคัญทางสถิติในสารละลายที่มีแคลเซียม รวมทั้งเมทฟอร์มินมีผลลดการหดตัวที่ถูกกระตุ้นด้วย noradrenaline ในสารละลายที่ปราศจากแคลเซียม นอกจากนี้เมทฟอร์มินที่ความเข้มข้นสูงทำให้กล้ามเนื้อหลอดเลือดคลายตัวทั้งในภาวะที่มีและไม่มี endothelium โดยพบว่าเมทฟอร์มินทำให้หลอดเลือดคลายตัวในภาวะที่มี endothelium ได้มากกว่าภาวะที่ไม่มี endothelium อย่างมีนัยสำคัญ (มีendothelium: 32.96 $\pm$ 2.84 %,n = 16; ไม่มี endothelium 14.93 $\pm$ 3.07 %, n = 7) ยิ่งไปกว่านั้น Methylene blue, atropine และ L-NAME สามารถยับยั้งฤทธิ์ของเมทฟอร์มินในการคลายหลอดเลือดอย่างมีนัยสำคัญ ในขณะที่ indomethacin, propranolol, TEA, 4-AP และ glibenclamide ไม่มีผลต่อฤทธิ์ในการคลายหลอดเลือดของเมทฟอร์มิน จึงสรุปได้ว่าเมทฟอร์มินมีผลต่อการเคลื่อนที่ของแคลเซียมจากแหล่งเก็บภายใน และทำให้หลอดเลือดคลายตัวได้ทั้งในภาวะที่มีหรือไม่มี endothelium และกลไกการออกฤทธิ์คลายหลอดเลือดของเมทฟอร์มินในภาวะที่มี endothelium อาจเกี่ยวข้องกับการกระตุ้น cholinergic receptor ใน NO-cGMP pathway

ภาควิชา	เภสัชวิทยา	ลายมือชื่อนิสิต ……..
สาขาวิชา	เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา……..
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SARAPEE THRORARITH: EFFECTS OF METFORMIN ON CONTRACTILITY OF ISOLATED RAT THORACIC AORTA. THESIS ADVISOR: ASST. PROF. SUREE JIANMONGKOL, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. PRASAN DHUMMA-UPAKORN, Ph.D., 71 pp. ISBN 974-17-5893-6.

Metformin is antihyperglycemic drug with antihypertensive property. In this study, the effects of metformin on the contractility of vascular smooth muscle of isolated rat aorta in the presence and absence of endothelium were investigated. The contactile response were measured isometrically.

These results showed that metformin significantly inhibited the contraction induced by noradrenaline and caffeine.

In addition, metformin directly caused significant vasodilation effect ($p< 0.05$). The percentage of maximal relaxation in endothelium-intact (32.96 $\pm$ 2.84 %, n = 16) was significantly higher than that of endothelium-denuded (14.93 $\pm$ 3.07 %, n = 7) ($p< 0.05$). In addition, methylene blue, atropine and L-NAME significantly inhibited the relaxant effect of metformin in endothelium-intact segment ($p< 0.05$). However indomethacin, propranolol, TEA, 4-AP and glibenclamide had no effect on metformin-mediated relaxation. In conclusion, metformin may affect vascular contractility by induced endothelium-dependent and endothelium-independent relaxation. Metformin-induced relaxation may be mediated direct activation of cholinergic receptor on the endothelium to initiate the NO-cGMP pathway.

Department	Pharmacology	Student's signature ……
Field of study	Pharmacology	Advisor's signature ……
Academic year	2004	Co-advisor's signature ……..

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LIST OF ABBREVIATIONS

Ca^{2+}	calcium ion
$[Ca^{2+}]_I$	intracellular calcium concentration
$[Ca^{2+}]_c$	cytosolic calcium concentration
AC	adenylate cyclase
GTP	guanosine 5′-triphosphate
PLC	phospholipase C
IP_3	inositol1,4,5-triphosphate
$Ins(1,4,5)P_3R$	inositol1,4,5-triphosphate receptor
RyR	ryanodine receptor
DAG	diacylglycerol
ATP	adenosine 5′-triphosphate
cAMP	cyclic adenosine 3′,5′-monophosphate
cGMP	cyclic guanosine 3′,5′-monophosphate
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
EDRF	endothelium-derived relaxing factor
EDCF	endothelium-derived constricting factor
NO	nitric oxide
SR	sarcoplasmic reticulum
MLC	myosin light chain
MLCK	myosin light chain kinase
ROC	receptor-operated Ca^{2+} channel
VOC	vlotage-operated Ca^{2+} channel
CAMKII	Ca^{2+} calmodulin-dependent protein kinase II
NA	noradrenaline

K^+	potassium ion
KCl	potassium chloride
TEA	tetraethylammonium
Ach	acetylcholine
L-NAME	N^G-nitro-L-arginine methyl ester
DMSO	dimethyl sulphoxide
M	molar
mM	millimolar
μM	micromolar

CHAPTER I

INTRODUCTION

Metformin is now approved to use as an alternative to sulfonylurea for long-term treatment of hyperglycemia in type 2 diabetic patients (Hermann, *et al*., 1994; Defronzo, *et al*., 1995; UKPDS, 1995). In addition, metfromin is effective on glycemic control in combination with insulin, sulfonylureas (Haupt, *et al*., 1991; Reaven, *et al*., 1992; Riddle, *et al*., 2000), and thiazolidinediones (Fonseca, *et al*., 2000). In conjunction with diet, metformin reduces fasting glucose concentration by 2.78 to 3.90 mmol/L (50 to 70 mg/dL), which corresponds to a 1.3% to 2.0% reduction in hemoglobin A_{1c} values (Defronzo, *et al*., 1995).

In diabetic patients, metformin appears to provide cardiovascular protection that cannot be attributed only to its antihyperglycemic effects (UKPDs, 1995). These additional cardioprotective effects in the patients may be related to its intrinsic antihypertensive property (Dmitri, *et al*., 2002).

Metformin has been shown to lower blood pressure in diabetic patients (Landin, *et al*., 1991; Giugliano, *et al*., 1993). Furthermore, an antihypertensive effect of metformin has been shown in spontaneously hypertensive and insulin resistance rats (Peterson, *et al*., 1996; Prasad, *et al*., 2000). In addition, metformin rapidly relaxed the contractions produced by phenylephrine in isolated rat tail arterial rings, suggesting that this drug directly cause vasodilation (Peuler, *et al*., 1997).

Moreover, the vasorelaxant effect of metformin has been linked to the generation of nitric oxide (Prasad, *et al*., 2000). In addition to the NO-pathway, the relaxation can be resulted from other pathways including activation of hyperpolarization, modulation of potassium channel and activation of β-adrenoceptors. This study aimed to investigate the effect of metformin on the contractility of vascular smooth muscle as well as its mechanism of action in mediating vasorelaxation, using the in *vitro* model of isolated rat thoracic aorta.

Hypothesis

Metformin has direct effect on the contractility of vascular smooth muscle by produced vasorelaxation through NO-dependent pathway of isolated rat thoracic aorta.

Objectives

1. To investigate the effect of metformin on the contractility of vascular smooth muscle.
2. To investigate the effect of metformin on the relaxation of smooth muscle in the presence or absence of endothelium.
3. To investigate the mechanism of metformin-induced vasorelaxation through NO-dependent pathway.

Significants

This study will provide new pharmacological information of the effects of metformin and the mechanism of action on the functionallity of vascular smooth muscle.

CHAPTER II

LITERATURE REVIEWS

Metformin is an insulin-sensitizing biguanide which has been approved for the treatment of type 2 diabetes. The formular of metformin is $C_4H_{11}N_5$ HCl (figure 1). The antihyperglycemic properties of metformin are mainly attributed to suppression of hepatic gluconeogenesis, and promotion of insulin sensitivity in peripheral tissue. In addition to antihyperglycemic effect, metformin has been shown its ability to reduce blood pressure in diabetic patients as well as in experimental animal models of hypertension (Verma, *et al*., 1994).

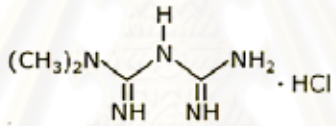


Figure 1 The structure of metformin

<u>Antihypertensive effects of metformin</u>

Metformin has been shown to reduce blood pressure in several diabetic patients. For example, metformin significantly increased the reduction in blood pressure after L-arginine infusion in newly diagnosed type 2 dibetic patients with mildly fasting hyperglycemia. The maximal decrease in systolic blood pressure was enhanced from 8 $\pm$ 2.5 mmHg to 12 $\pm$ 3.4 mmHg and diastolic blood pressure was enhanced from 4.5 $\pm$ 1.9 mmHg to 9.5 $\pm$ 2.4 mmHg (Marfella, *et al*., 1996). In addition, the studies on the effect of metformin in normotensive type 2 dibetic patients was associated with significant reduction in diastolic blood pressure. The reduction in diastolic blood pressure was enhanced from 4.4 % to 14.9 % (Juliana, *et al*., 1993). Another study

showed that systolic blood pressure decreased by 40 ± 19 mmHg (p<0.001) and diastolic blood pressure decreased by 24 ± 5 mmHg (p<0.001) after 6 weeks treatment with metformin in hypertensive patient (Landin, *et al*., 1991).

Furthermore, metformin was shown its ability to reduce blood pressure in spontaneously hypertensive rats. It was found that metformin treatment significantly attenuated the increase in systolic blood pressure from 196 ± 9.0 mmHg to 157 ± 6.0 mmHg in the spontaneously hypertensive rat, but it had no effect in normotensive rats (Verma, *et al*., 1994). Moreover, the long-term treatment of metformin prevented significanty enhancement of systolic blood pressure in the fructose-metformin group. The systolic blood pressure reduced from 157 ± 5 mmHg to 140 ± 3 mmHg (Verma, *et al*., 1996). Another study was done to investigate the antihypertensive effect of metformin in the insulin resistance rats. It was found that mean arterial pressure significantly decreased after 2 weeks of metformin treatment in insulin resistance rats compared with insulin resistance-placebo rats (Prasad, *et al.,* 2000). There were several studies to investigate the mechanism of metfromin-reduced blood pressure. These studies have reported that the antihypertensive effect of metformin involved the contractility of vascular smooth muscle.

Effects of metformin on vascular smooth muscle

It has been reported that metformin significantly reduced the maximal tension induced by NA in fructose metformin-treated superior mesenteric arterial rings from 0.82 ± 0.05 g/mm^3 to 0.70 ± 0.03 g/mm^3 of endothelium-intact aortic strips and from 0.99 ± 0.05 g/mm^3 to 0.83 ± 0.04 g/mm^3 of endothelium-denuded aortic strips (Verma, *et al.,* 1996). In addition, metformin significant reduced the maximal tension induced by NA in noral rat from 0.89 ± 0.05 g/mm^3 to 0.72 ± 0.03 g/mm^3 of endothelium-intact aortic strips and from 0.95 ± 0.03 g/mm^3 to 0.85 ± 0.04 g/mm^3 of endothelium-denuded aortic strips. Furthermore, the study of the effect of metformin on vascular smooth muscle revealed that acute effect of high concentrations of metformin (>10 mmol/L) relaxaed

intact deendothelialized rat tail artery (Chen, *et al*., 1997). The study of metformin in endothelium-intact of rat tail artery, at the range of 0.2-20 mmol/L of metformin rapidly relaxed half-maximal contractions induced by either phenylephrine or noradrenaline (JiHun, *et al.,* 1998). It also relaxed the contraction mediated by serotonin, although to a lesser extent. Although, the relaxant effect of metformin was independent of the contractile agonist in the contraction period, removal of adrenergic nerve endings significant enhanced relaxant effects of metformin. However, the acute relaxation of rat tail arterial smooth muscle by metformin did not mediated through K^+ channels (JiHun, *et al*., 1998). Furthermore, it had been found that methylene blue (an inhibitor of guanylate cyclase enzyme) did not inhibit metformin-induced relaxation of phenylephrine-induced contraction in rat tail artery

There was the *in vivo* study shown that the maximal relaxation to ACh was enhanced in insulin resistance-metformin (92 $\pm$ 2 %) compared with insulin resistance-placebo rats (44 $\pm$ 4 %) ($p < 0.05$) after a 2-week treatment with metformin (Prasad, *et al.,* 2000). In addition, the relaxation in the presence of *N*-nitro-L-arginine was greater in insulin resistance-metformin (33 $\pm$ 4 %) than in insulin resistance-placebo rats (12 $\pm$ 4 %) but remained depressed compared with control rats (68 $\pm$ 5 %). *In vitro*, metformin (10^{-4} M) pretreatment of insulin resistance mesenteric arteries enhanced ACh-induced relaxation compared with insulin resistance arteries without metformin. Furthermore, at the high concentration of metformin (>10^{-3} M) can induce direct vascular smooth muscle relaxation in mesenteric arteries of normal and insulin-resistance rats. These data suggest that metformin improved ACh-induced relaxation in insulin resistance rats through nitric oxide-dependent relaxation

Control of vascular smooth muscle tone

Contraction of vascular smooth muscle is regulated by intracellular calcium concentration and the sensitivity of the contractile elements to an increase of calcium (Karaki, *et al*., 1997). The mechanism by which an increase in intracellular calcium

stimulates vascular smooth muscle contraction is illustrated in the figure 2. The free calcium binds to a special calcium binding protein called calmodulin. The calcium-calmodulin complex then activates myosin light chain kinase (MLCK), an enzyme that is capable to phosphorylates myosin light chains (MLC) in the presence of ATP. Then phosphorylated myosin interacts with actin to induce contraction (Westfall, *et al*., 1998).

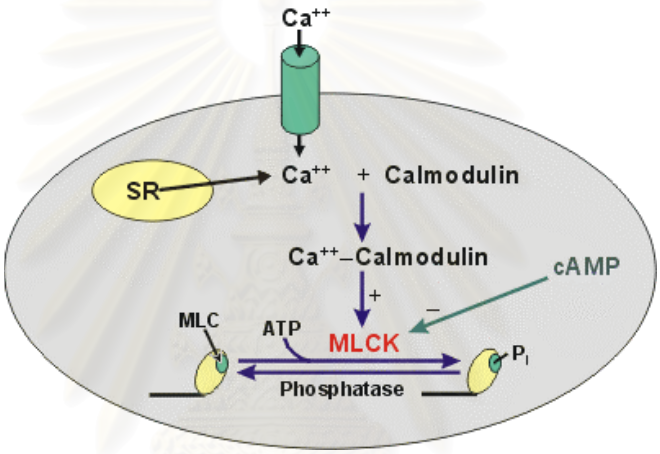


Figure 2 The mechanism by which an increase in intracellular calcium stimulates vascular smooth muscle contraction

The concentration of intracellular calcium depends upon the balance between the calcium that enters the cells, the calcium that is released by intracellular storage sites (e.g., sarcoplasmic reticulum), and removal of calcium either back into storage sites or out of the cell (Karaki, *et al*., 1997). Calcium enters the cell through voltage-operated Ca^{2+} channels, which open when the cell is depolarized, and partly through receptor-operated channels (Rang, *et al*., 1999). The voltage-operated Ca^{2+} channels open under influence of second messenger produced in response to receptor activation. Many vasoconstrictors (e.g. noradrenaline) cause both a depolariztion by increasing the membrane permeability to cations such as Na^{+} and Ca^{2+} and a further

increase in Ca^{2+} uptake through distinct receptor-operated channels (Rang, *et al*., 1999). Norepinephrine (NA) acting via $alpha_1$-adrenoceptors activate phospholipase C (PL-C) causing the formation of inositol triphosphate (IP_3) and diacylglycerol from phosphatidylinositol (PIP_2). The IP_3 then stimulates the sarcoplasmic reticulum (SR) to release calcium (Karaki, *et al*., 1997). The formation of diacylglycerol (DAG) activates protein kinase C (PK-C) which can also contribute to vascular smooth muscle contraction via protein phosphorylation (Silverthorn, 1998). Calcium is removed from the cytosol partially by a Ca^{2+} -Na^{+} antiport exchanger and partially by a Ca^{2+}-ATPase (Karaki, *et al*., 1997). The sodium–calcium exchanger uses the energy of the sodium gradient across the plasma membrane, whereas ATP-dependent calcium pumps utilize energy derived from the hydrolysis of ATP to move calcium out of the cell (Katz. 1997). Caffeine induces a transient contraction which is attributable to the release of calcium from internal stores (Karaki, *et al*., 1997).

Some vasoconstrictors induce contraction via other mechanisms such as membrane depolarization. High potassium depolarizes the membrane, initiating conformation changes that open voltage–operated calcium channels, which allow calcium to diffuse rapidly into the cell, resulting in an increase of intracellular calcium (Karaki, *et al*., 1997). Caffeine induces a transient contraction which is attributable to the release of calcium from internal stores (Karaki, *et al*., 1997).

Relaxation of smooth muscle is usually initiated by a fall in $[Ca^{2+}]_i$ which leads to dephosphorylation of myosin light chain via myosin phosphatase (Rang, *et al*., 1999) or

directly on the contractile machinery. Increase in the concentration of cAMP are also associated with smooth muscle relaxation. When the concentration of cAMP is elevated, cAMP-dependent protein kinase (PKA) is activated. The relaxation may result from decrease intracellular calcium secondary to reduce influx of Ca^{2+} uptake into the sarcoplasmic reticulum, or enhanced Ca^{2+} extrusion through the cell membrane. PKA may also phosphorylate and inhibit myosin light chain kinase, thus inhibiting contraction.

β-adrenergic receptor agonists, such as isoproterenol cause vasodilation via the information of cAMP (Rang, *et al*., 1999). Stimulation of β- receptors activates

adenylate cyclase, which catalyzes the generationof cAMP from ATP. Drugs that inhibit phosphodiesterases enzymes that metabolize cAMP and cGMP, promote smooth muscle relaxation by elevating concentrations of these second messengers. cAMP inhibits MLCK leading to decrease MLC phosphorylation, thereby decreasing the interaction between actin and myosin (Rang, *et al*., 1999).

Agents cause vasodilation by opening the potassium channels, causing hyperpolarization, and thus preventing voltage-operated Ca^{2+} channel from opening (Vanhoutte, *et al*., 1996).

Furthermore, it is realized that vascular endothelium acts as a source of potent chemical mediators which actively control the contraction of underlying smooth muscle (Rang, *et al*., 1999). ACh-induced vasorelaxation is mediated by activating of muscarinic receptor leading to release NO or increase sensitivity of vascular smooth muscle to NO (Furchott and Zawadzki 1980). In addition, endothelial cells release prostaglandin derived from cyclooxygenase pathway and leading to vasorelaxation (Furchott and Zawadzki 1980).

NO-cGMP system plays an importance role in regulating vascular smooth muscle tone. When NO is formed by an endothelial cell, for example, it readily diffuses out of the cell and into smooth muscle cells where is binds to a heme moiety on guanylyl cyclase and activates this enzyme. NO activate guanylyl cyclase causing increased formation of cGMP (Westfall, *et al*., 1998). cGMP can inhibit calcium entry into the vascular smooth muscle and produced vasorelaxation (figure 3). Increased cGMP activates a kinase that subsequently leads to the inhibition of calcium influx into the smooth muscle cell, and decreased calcium-calmodulin stimulation of myosin light chain kinase (MLCK). The overall control of vascular smooth muscle is shown in figure 4.

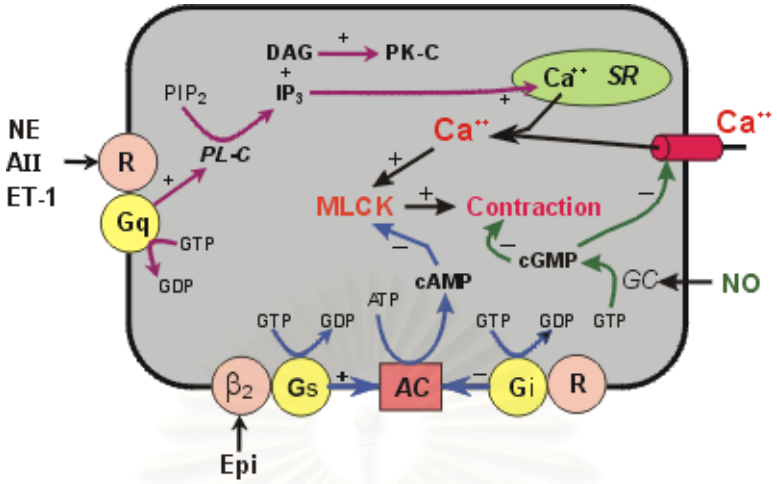


Figure 3 The signal transduction mechanisms that modulate intracellular calcium concentration

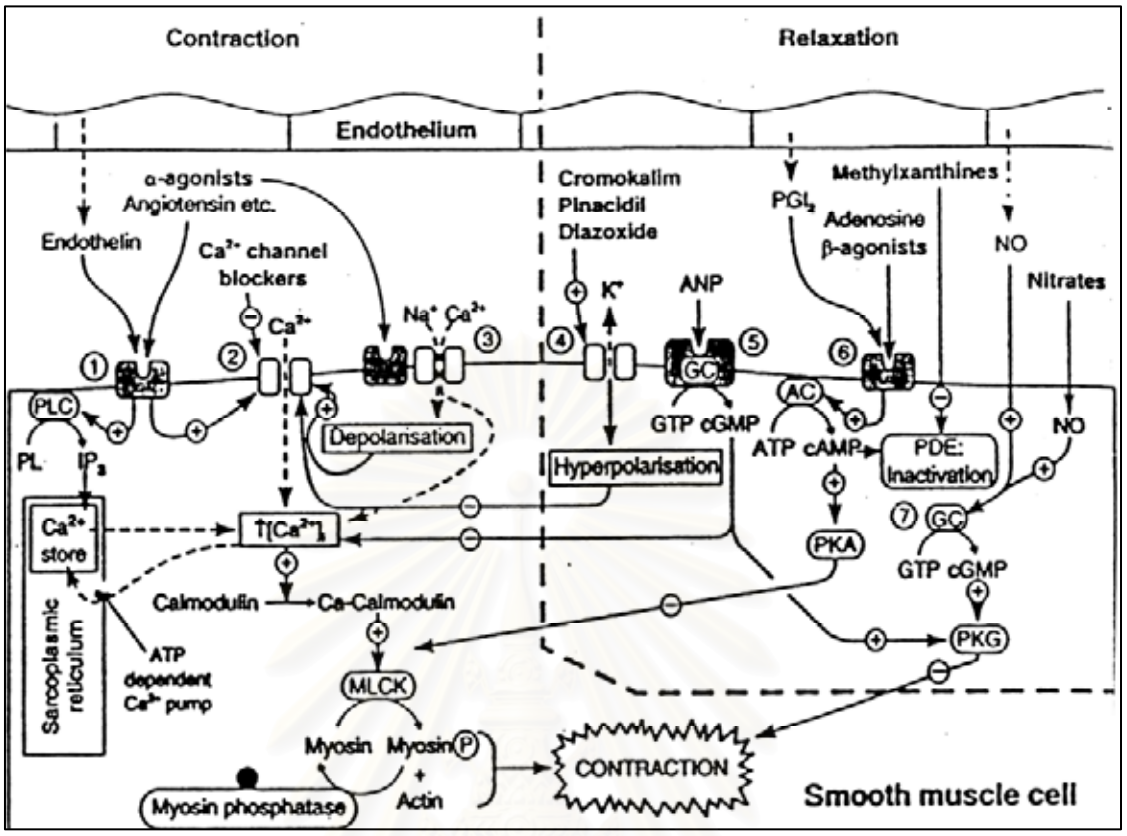


Figure 4 Control of vascular smooth muscle. (Rang, *et al.,*1999)

Agents elicit contraction by increasing $[Ca^{2+}]i$, or increasing the sensitivity of myofilament toCa^{2+}.

$[Ca^{2+}]i$ is increased by

- Receptor coupled to phospholipase C (PLC), which lead to IP_3, production and release of store Ca^{2+}.
- Voltage-gated Ca^{2+} channels, which open in response to depolarization.
- Receptor-operated channels, which allow Ca^{2+} entry and also cause depolarization. The other way to increase $[Ca^{2+}]i$ is decreasing in myosin phosphatase activity, then cause contraction via Ca^{2+} sensitization.

Agents that cause relaxation may effect by reducing $[Ca^{2+}]_i$, or directly on the contractile machinery.

- K^+ channel (sensitive to intracellular ATP) openers, cause hyperpolarisation, and thus prevent opening of voltage-operated Ca^{2+} channel.
- ANP occupied a receptor that is directly coupled to membrane-bound guanylyl cyclase.
- Receptors (e.g.for PGI_2, adenosine) coupled to adenylate cyclase, activation of which cause increased camp production. This acts via protein kinase A (PKA) and myosin light chain kinase (MLCK) to inhibit contraction. Inhibitors of phosphodiesterase (PDE) protect camp or cGMP from degradation.
- Stimulation of soluble guanylate cyclase by NO increase cGMP formation.

(Enzymes:AC=adenylate cyclase; GC=guanylate cyclase ; MLCK=myosin light chain kinase; PKA=cAMP-dependent protein kinase; PKG=cGMP-dependent protein kinase)

CHAPTER III

MATERIALS AND METHODS

Experimental animals

Adult male Wistar rats of body weight between 250-300 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The animals were housed in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University under the standard conditions and acclimatized for 1-2 weeks before the experimentation. This study was approved by The Ethical Committee of Faculty of Pharmaceutical Science, Chulalongkorn University.

Preparation of isolated rat aorta

1. Male wistar rats (250-300 g) were sacrificed by cervical dislocation and the thoracic aorta quickly removed. After cleaning of fat and connective tissue, the aorta was helically cut into a strip (figure 6).
2. In some preparation, endothelium layer was removed mechanically by gently rubbing the entire intimal surface. The absence of a functional endothelium was shown by the absence of relaxant response after addition of acetylcholine (10^{-6} M) to the strips pretreated with noradrenaline 10^{-6} M.
3. Each aorta strip was suspened in a 15 ml organ bath containing physiological solution, maintained at 37 °C and gassed with 95% O_2 and 5% CO_2
4. An initial load of 1 g was applied to each strip and maintained throughout the experiment.
5. The aoric strips were incubated in Ca^{2+}-containing solution for 60-90 minute until the tension was stable before starting the experiment.
6. Tension is recorded isometrically on a polygraph via force-displacement transducers.

7. At the end of the experiment, the contractant was added to induce contraction to test the viability of smooth muscle.

Figure 5 Thoracic aorta and preparing of isolated rat aorta

Chemicals

1. **Reference compounds**

Acetylcholine (Ach)	endothelium-dependent relaxant
Noradrenaline (NA)	α-adrenoceptor agonist
Sodium nitroprusside	endothelium-independent relaxant
Atropine	muscarinic antagonist
Isoproterenol	β-adrenoceptor agonist
Propranolol	β- adrenoceptor antagonist
Indomethacin	an inhibitor of cyclooxygenase
Methylene Blue	an inhibitor of soluble guanylyl cyclase
Tetraethylammonium	an inhibitor of calcium-sensitive potassium channels
Glibenclamide	an inhibitor of ATP-sensitive potassium channels
4-Aminopyridine	a delayed rectifier potassium channel blocker
Caffeine	Ca^{2+} -ATPase pump agonist

N^G-nitro-L-arginine methyl ester	an inhibitor of nitric oxide synthase
Dimethyl sulphoxide (DMSO)	solvent
Potassium chloride (KCl)	membrane depolarizer

All were purchased from Sigma, St. Louis, MO, U.S.A., except for KCl which was purchased from APS Chemicals, Australia.

2. Testing compounds

Metformin (Sun Pharmaceutical industries) was generously given by Utopian CO., Ltd. The impurity was 0.02 % W/W. Other chemicals include NaCl, KCl, $CaCl_2$, $MgCl_2$, KH_2PO_4, $NaHCO_3$, D-glucose and EDTA were purchased from APS Chemicals, Australia.

All drugs were dissolved in distilled water, with the exception of glibenclamide, which was dissolved in dimethyl sulphoxide. Metformin was freshly prepared on the day of the experiments.

Experimental instruments

1. Double–walled organ bath (figure 5). The organ bath was made of glass comprises an inner and outer chamber. An inner chamber with capacity of 25 ml is for suspending the isolated tissue in physiological solution. The reservoir should also be constantly aerated with 95% O_2 + 5% CO_2. An outer chamber is for temperature control of the inner chamber.
2. Water bath and thermoregulation water pump
3. Isometric transducer of Washington transducer (Harvard Apparatus Ltd., England)
4. Recorder Universal Oscillograph (Harvard Apparatus Ltd., England)
5. Recorder with electrical disperser Gilson N_2 (Harvard Apparatus Ltd., England)
6. Tank of Carbogen gas (95% O_2 + 5% CO_2) (T.I.G., Thailand)

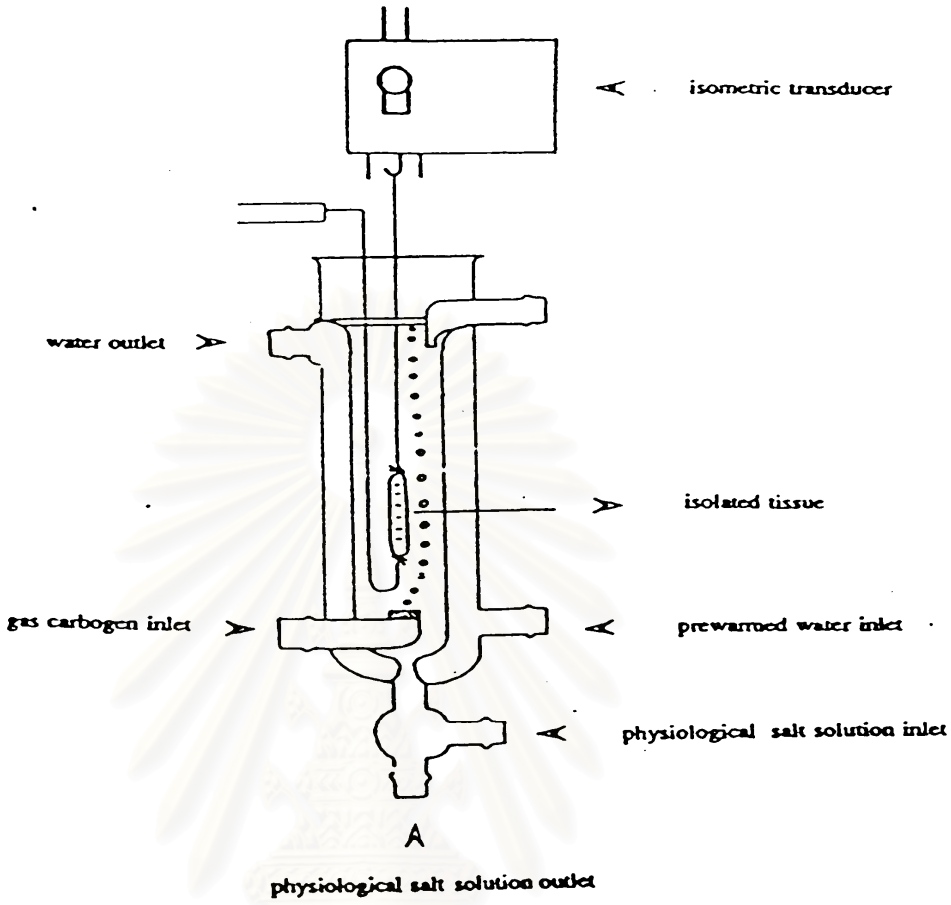


Figure 6 Illustration of instrument and organ bath for isolated rat aorta

Experimental procedures

1. Effects of metformin on aortic contraction of isolated rat aorta

1.1 Effect of metformin on aortic contraction in Ca^{2+}- containing solution

After the equilibration period, noradrenaline (NA) at the concentration of 10^{-9} M to 10^{-6} M was added cumulatively to induce contraction of endothelium-denuded aortic strip in Ca^{2+}-containing solution. The tension was recorded for 15 minutes prior to washing the tissue with Ca^{2+}-containing solution 3 times. The effect of metformin on NA-induced contraction in Ca^{2+}-containing solution was studied by incubating metformin at the concentration of 10 μM or 100 μM for 30 minutes prior to addition of NA. The contractions were expressed as tension (g) and calculated as a percentage of maximal contraction induced by NA in Ca^{2+}- containing solution. Another experiment with the same procedure was carried out in the absence of metformin and was used as a control group.

Furthermore, the effect of metformin on aortic contraction induced by NA $5x10^{-8}$ M, NA 10^{-6} M, KCl $4x10^{-2}$ M and caffeine 10^{-3} M were also performed. The experimental procedure was similar to those abovementioned. The effect of metformin on contraction in Ca^{2+}- containing solution was studied by incubating metformin at the concentration of 1 μM, 10 μM, 50 μM or or 100 μM for 30 minutes prior to addition of NA, KCl or caffeine.

In addition, the effect of endothelium on the contraction induced by NA and KCl was investigated. The similar experimental procedures as abovementioned were performed but, using endothelium-intact aortic strips instead of the endothelium-denuded aortic strips.

1.2 Effect of metformin on aortic contraction in Ca^{2+}-free medium

After the equilibration period, NA (10^{-6} M) was added to induce contraction of endothelium-denuded aortic strip in Ca^{2+}- containing solution. The tension was recorded 15 minutes prior to washing tissue. Then the tissue was treated with Ca^{2+}-free EDTA-

containing medium for 30 minutes, followed by addition of NA (10^{-6} M). The effect of metformin on NA-induced contraction in Ca^{2+}-free medium was studied by incubating metformin at the concentration of 10 μM for 30 minutes prior to addition of NA in Ca^{2+}-containing solution. After that, the tissue was treated with Ca^{2+}-free EDTA-containing medium for 30 minutes prior to incubating metformin for 30 minutes and then NA was applied. The contractions induced by NA in Ca^{2+}-free medium were expressed as a percentage of NA-induced contraction in Ca^{2+}-free medium. Another experiment with the same procedure was carried out in the absence of metformin and was used as a control group.

1.3 Effects of metformin on aortic contraction induced by addition of calcium to calcium-free depolarizing solution.

The method described by Hof and Vuorela (1993) was used to determine the effect of metformin on $CaCl_2$–induced contraction in high K^+-Ca^{2+}-free solution. After the equilibration period, the tissue was treated with Ca^{2+}-free depolarizing for 30 minutes. Then $CaCl_2$ at the concentration of $1x10^{-5}$ M to $1x10^{-2}$ M was added cumulatively to induce contraction of endothelium-denuded aortic strip in high K^+-Ca^{2+}-free solution. The tension was recorded for 15 minutes prior to washing the tissue with Ca^{2+}- containing solution 3 times. The effect of metformin on $CaCl_2$–induced contraction in Ca^{2+}-free depolarizing solution was studied by incubating metformin at the concentration of 10 μM or 100 μM for 30 minutes prior to addition of $CaCl_2$. The contractions by $CaCl_2$ were expressed as a percentage of the maximum contraction induced by maximum concentration of $CaCl_2$. Another experiment with the same procedure was carried out in the absence of metformin and was used as a control group.

2. Effects of metformin on relaxation of isolated rat aorta

2.1 Effects of metformin on endothelium-dependent vasorelaxation

The present study was carried out to investigate the effect of metformin on vasorelaxation induced by activation of β-adrenoceptor and muscarinic receptor. In

order to study the metformin effect on β-adrenoceptor activation mediated relaxation. The relaxation response to cumulative concentration of isoproterenol in endothelium-intact aortic strips preconstricted with a sub-maximal concentration of NA (EC_{80}) were obtained. At this concentration of NA was sufficient enough to sustain the contraction for further relaxation studies. After the equilibration period, NA at the EC_{80} of 10^{-7} M was added to induce contraction of endothelium-intact aortic strips in Ca^{2+}-containing solution. When the contraction reached plateau, isoproterenol at the concentration of 10^{-9} M – 10^{-4} M were added to produce relaxation. The tension was recorded for 10 minutes prior to washing the tissue with Ca^{2+}-containing solution 3 times. The effect of metformin on the β-adrenoceptor-mediated relaxation produced by isoproterenol in Ca^{2+} -containing solution was studied by incubating metformin at the concentration of 100 μM for 30 minutes prior to addition of NA and then isoproterenol was added. The relaxation was expressed as a percentage of the NA-induced contraction in Ca^{2+} -containing solution.

In order to study the effect of metformin on muscarinic receptor activation mediated relaxation on the endothelium-dependent relaxation produced by ACh in Ca^{2+} -containing solution was studied. The similar experimental procedures as abovementioned were performed using ACh 10^{-6} M to induce vasorelaxation. The procedure involved incubating metformin at the concentration of 1 μM, 10 μM, 50 μM or 100 μM for 30 minutes prior to addition of NA ($5x10^{-8}$ M), and then ACh was added. The relaxation responses were expressed as a percentage of the NA-induced contraction in Ca^{2+} -containing solution. The relaxation profiles of vascular smooth muscle before and after addition of metformin were compared.

Furthermore, the effect of metformin on endothelium-independent relaxation produced by sodium nitroprusside (SNP) of endothelium-denuded aortic strips in Ca^{2+}-containing solution was studied. The similar experimental procedures as abovementioned was performed, using SNP $6x10^{-8}$ M instead of ACh.

2.2 Effects of metformin as the relaxant to NA-induced contraction

The intrinsic property of metformin as vasorelaxant was also tested in this study. After the equilibration period, NA at the EC_{80} of 10^{-7} M was added to induce contraction in endothelium-denuded aortic strip. When the contraction reached plateau, metformin at the concentration ranging from 0.1 μM to 1500 μM was added cumulatively to produce relaxation. The tension was recorded for 10 minutes prior to washing the tissue with Ca^{2+}-containing solution 3 times. The relaxations were expressed as a percentage of the NA-induced contraction in Ca^{2+}-containing solution. Another experiment with the same procedure was carried out in the absence of metformin, and was used as a control group.

In addition, the role of endothelium on the relaxation induced by metformin was investigated. The similar experimental procedures as abovementioned were performed with the use of endothelium-intact aortic strips.

2.3 The endothelium-dependent relaxation mechanism of metformin

Other series of experiments were performed to elucidate the mechanism of metformin-induced relaxation. Several possible mechanisms, including its possible agonistic action on the muscarinic receptor or β-adrenoceptor were investigated. In addition, the role of cycloxygenase enzyme, guanylyl cyclase enzyme, nitric oxide synthase enzyme or potassium channel in endothelium-dependent relaxation to metformin was evaluated. After the equilibration period, various vasorelaxant inhibitors including methylene blue (10^{-5} M), atropine (10^{-4} M), L-NAME (10^{-4} M), propranolol (10^{-5} M), indomethacin (10^{-5} M), TEA (10^{-3} M), 4-AP (10^{-4} M) or glibenclamide (10^{-5} M) were applied 30 minutes prior to start metformin-induced vasorelaxation as abovementioned. The concentration of vasorelaxant inhibitor was selected base on the significant inhibition of ACh (10^{-5} M)-induced relaxation. The relaxations were expressed as a percentage of the NA-induced contraction in Ca^{2+}-containing solution. Another experiment with the same procedure was carried out in the absence of metformin, and was used as a control group.

Statistical analysis

Data are presents as means ± S.E.M. for *n* separated experiments. Statistical significances were tested either by Student's *t*-tests for unpaired data or by one-way ANOVA followed by a post-hoc Dunnett tests, where approprate. The *p* values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

1. Profiles of aortic contraction of isolated rat aorta

1.1 Aortic contraction in Ca^{2+}-containing solution.

As shown in figure 7, the profiles of contractile response of rat aortic strips evoked by NA consisted of two phases: an initial phasic and a continuing tonic phase. The maximum contractile response induced by NA 10^{-6} M was 0.75 $\pm$ 0.03 g (n=33) for endothelium-intact aortic strips, and 0.60 $\pm$ 0.02 g (n=40) for endothelium-denuded aortic strips. These responses decreased in the lesser concentration of NA (table 1). Similar contractile responses were obtained when $4x10^{-2}$ M KCl was used instead of NA (figure 7). However, the magnitude of contraction was less than that elicited by NA. The maximum contractile response induced by KCl $4x10^{-2}$ M was 0.51 $\pm$ 0.04 g (n=26) in endothelium-intact aortic strips and 0.45 $\pm$ 0.02 g (n = 24) in endothelium-denuded aortic strips. The contractions induced by NA 10^{-6} M and KCl $4x10^{-2}$ M in endothelium-intact were significantly higher than those in endothelium-denuded aortic strips. The contraction profiles induced by caffeine were markedly different from the others. The caffeine-induced contraction profile consisted of a rapid transient contraction with the magnitude of 0.20 $\pm$ 0.02 g (n = 16), followed by a decrease of muscle tension to a level below a resting tone (figure 8).

1.2 Aortic contraction in Ca^{2+}-free medium.

As seen in figure 9, addition of 10^{-6} M NA to endothelium-denuded aortic strips in Ca^{2+}-free medium also induced a biphasic contraction like those observed in Ca^{2+}-containing solution. However, the magnitude of contraction was only 27.94 $\pm$ 3.35 % (n = 18) of the maximum response observed in Ca^{2+}-containing solution. In Ca^{2+}-free medium, caffeine could not evoke an observable contraction.

2. Effects of metformin on the contraction of isolated rat aorta

2.1 Effects of metformin on contraction induced by contractants in Ca^{2+}-containing solution

The contractile profiles of the effect of metformin on cumulative addition of NA to induce contraction were shown in figure 10. Metformin at the concentration of 10 μM and 100 μM can suppress the contractile response curve of NA in both endothelium-intact and endothelium–denuded aortic strips (figure 11 and 12). Moreover, metformin at the concentration of 1 μM significantly inhibited contraction induced by NA $5x10^{-8}$ M from 108.20 ± 2.18 % to 85.93 ± 4.13 % (n=6) in endothelium-intact aortic strips and from 103.74 ± 1.79 % to 86.12 ± 2.17 % (n=8) in endothelium–denuded aortic strips. The inhibitory effects were also observed when increasing the contraction of metformin to 10^{-5} M. The contraction was significant suppressed from 108.20 ± 2.18 % to 86.14 ± 4.95 % (n=5) in endothelium-intact aortic strips and from 103.74 ± 1.79 % to 73.80 ± 2.08 % (n=7) in and endothelium–denuded aortic strips respectively (figure 13).

However, metformin at the higher concentration of 50 μM and 100 μM had no influence on NA ($5x10^{-8}$ M)-induced contraction. In addition, upon increasing the concentration of NA to 10^{-6} M, metformin at any given concentration in this experiment could not suppress the contraction (figure 14).

In addition, metformin had no effect on the contraction evoked by KCl $4x10^{-2}$ M(figure 15).

Furthermore, the contraction profiles of the effects of metformin at the concentration of 10 μM on caffeine-induced contraction are shown in figure 16. Metformin at the concentration of 10 μM significantly inhibited the contraction induced by caffeine from 99.31 ± 1.58 % (n=4) to 86.92 ± 2.93 % (n = 9) (figure 18).

2.2 Effects of metformin on contraction induced by NA in Ca^{2+}-free medium.

The contraction profiles of the effect of metformin on NA-induced contraction of endothelium-denuded aortic strips in Ca^{2+}-free medium were shown in figure 17.

Metformin at the concentration of 10 μM significantly inhibited the contraction induced by NA (10^{-6} M) from 83.80 ± 3.79 % (n=4) to 58.45 ± 5.38 % (n = 8) (figure 18).

2.3 Effects of metformin on contraction induced by adding calcium to a calcium-free depolarizing solution.

The contractile profiles of the effects of metformin on $CaCl_2$-induced contraction were shown in figure 19. Metformin 10 μM had no effect on $CaCl_2$-induced contraction. However, in the presence of meformin at the concentration of 100 μM, the contractile response was significantly higher than the control group at the concentration of $CaCl_2$ higher than $3x10^{-3}$ M (figure 20). The maximum contraction in the presence of 100 μM metformin was 147.09 ± 16.03 % (n = 8).

3. Effects of metformin on the relaxation of rat thoracic aorta

3.1 Effects of metformin on endothelium-dependent relaxation

The relaxation profiles of isoproterenol-induced relaxation was shown in figure 21.1. Metformin at the concentration of 100 μM significant increased the relaxation induced by isoproterenol to 127.80 ± 9.64 % (n=7) in endothelium-intact aortic strips (figure 22). When propranolol at the concentration of 10^{-5} M was applied in place of metformin, the relaxation significant decrease to 71.94 ± 6.10 % (n=8) in endothelium-intact aortic strips (figure 22).

Furthermore, the relaxation profiles of metformin on endothelium-dependent relaxation-induced by ACh were shown in figure 23.1. The percentage of relaxation induced by 10^{-6} M ACh in endothelium-intact aortic strip was 58.34 ± 5.83 % (n=12). The effect of metformin at the concentration of 1 μM, 10 μM, 50 μM or 100 μM on the percentage of relaxation were 92.32 ± 13.76 % (n=6), 96.11 ± 15.87 % (n=5), 108.49 ± 10.97 % (n=5) and 91.43 ± 13.94 % (n=5) in endothelium-intact aortic strips, respectively. These results showed that metformin had no effect on the relaxation produced by ACh (figure 24).

In addition, the relaxation profile of metformin on endothelium-independent relaxation was shown in figure 23.2. The percentage of relaxation induced by sodium nitroprusside ($6x10^{-8}$ M) in endothelium-denuded aortic strip was 83.22 ± 3.34 % (n=12). The effects of metformin at the concentration of 1 μM, 10 μM, 50 μM or 100 μM on the percentage of relaxation were 100.06 ± 9.63 % (n=8), 84.66 ± 6.80 % (n=6), 108.21 ± 6.8 % (n=6) and 107.03 ± 7.88 % (n=6) in endothelium-denuded aortic strips respectively. These results had been shown that metformin had no effect on the relaxation produced by sodium nitroprusside (figure 24).

3.2 The relaxant effects of metformin on isolated rat aorta

The relaxation profiles in the presence or absence of metformin were shown in figure 25. Metformin directly caused vasodilation in both endothelium-intact and endothelium–denuded aortic strips. However, metformin elicited more vasorelaxation effect in endothelium-intact aortic strip than in endothelium-denuded aortic strip. The percentage of maximal relaxation in endothelium-intact aortic strip was 32.96 ± 2.84 % (n = 16) and in endothelium-denuded aortic strip was 14.93 ± 3.07 % (n = 7) (figure 26). These results showed that the relaxant effects of metformin can be observed in the absence or presence of endothelium.

3.3 Effects of vasodilation inhibitor on metformin–induced relaxation

Various inhibitors of vasorelaxation were used to probe the mechanism of metfromin-induced relaxation. Methylene blue 10^{-5} M was used to determine whether metformin induced vasorelaxation involved the guanylyl cyclase enzyme. The relaxation profiles of metformin in the presence and absence of methylene blue were shown in figure 27. Methylene blue at the concentration of 10^{-5} M caused the significantly inhibition of ACh (10^{-5} M)-induced relaxation by 85.12 ± 7.35 % (n=4) in endothelium-intact aortic strips. Methylene blue at the concentration of 10^{-5} M significant reduced the metformin-induced relaxation by 73.96 ± 4.82 % (n=6). The maximal percentage

relaxation was significant decreased from 32.96 ± 2.84 % to 8.58 ± 1.47 % (n=6) (figure 28).

In addition, atropine 10^{-4} M was used to determine whether metformin-induced-vasorelaxation involve the cholinergic receptor. The relaxation profiles of metformin in the presence and absence of atropine were shown in figure 29. Atropine at the concentration of 10^{-4} M produced the significant inhibition of ACh (10^{-5} M)-induced relaxation by 93.74 ± 8.15 % (n=4) in endothelium-intact aortic strips. Atropine (10^{-4} M) significantly reduced the metformin-induced relaxation by 42.19 ± 8.03 % (n=6). The maximal percentage relaxation decreased significantly from 42.33 ± 5.03 % to 24.47 ± 4.04 % (n=6) (figure 30).

Furthermore, L-NAME (10^{-4} M) was used to determine whether metformin induced vasorelaxation involve the production of nitric oxide. The relaxation profiles of metformin in the presence or the absence of atropine were shown in figure 31. L-NAME at the concentration of 10^{-4} M produced the complete inhibition of ACh (10^{-5} M)-induced relaxation in endothelium-intact aortic strips. L-NAME at the concentration of 10^{-4} M significantly reduced the metformin-induced relaxation by 59.11 ± 8.14 % (n=6). The maximal percentage relaxation decreased significantly from 9.61 ± 0.97 % to 3.93 ± 0.79 % (n=6) (figure 32).

Other studies to determine whether metformin induced vasorelaxation via β-adrenoceptor, cyclooxygenase enzyme or potassium channel were also performed. The results showed that neither propranolol (10^{-5} M), indomethacin (10^{-5} M), TEA (10^{-3} M), 4-AP (10^{-4} M) or glibenclamide (10^{-5} M) could interfere metformin-induced vasorelaxation in endothelium-intact aortic strips (figure 33 and figure 34).

Table 1 Force of contraction (g) of aortic strips induced by various contractants in Ca^{2+}-containing solution.

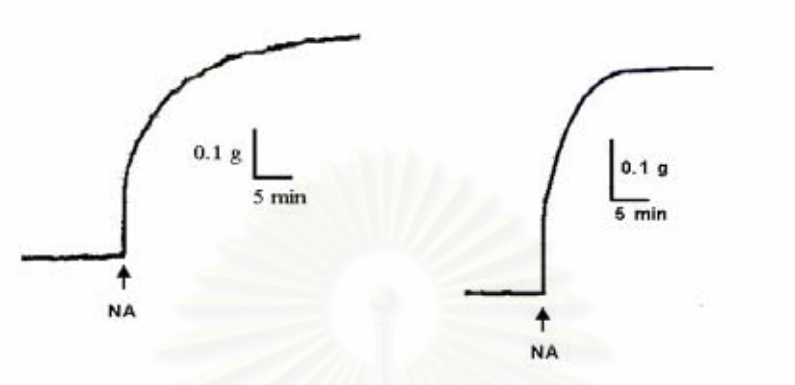
Contractants	Aortic strips			
	n	Endothelium-intact	n	Endothelium-denuded
Norepinephrine $5x10^{-8}$ M	25	0.61 ± 0.04	27	0.43 ± 0.01 *
Norepinephrine10^{-7} M	45	0.62 ± 0.03	17	0.59 ± 0.04
Norepinephrine10^{-6} M	33	0.75 ± 0.03	40	0.60 ± 0.02 *
KCl $4x10^{-2}$ M	26	0.51 ± 0.04	24	0.45 ± 0.02 *
Caffeine 10^{-3} M	-	-	16	0.20 ± 0.02

Data are presented as mean ± S.E.

*p< 0.05 shows significant difference between endothelium-intact and endothelium-denuded aortic strips

1a) Endothelium-intact

1b) Endothelium-denuded



2a) Endothelium-intact

2b) Endothelium-denuded

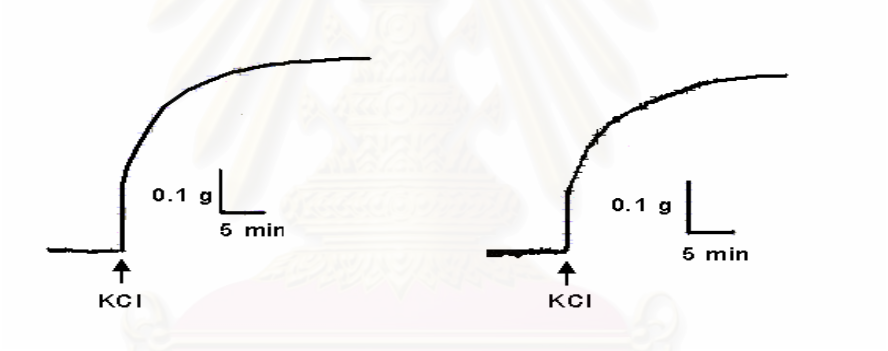


Figure 7 Representative tracing shows the aortic contraction in Ca^{2+}-containing solution induced by NA in endothelium-intact aortic strip (1a) and endothelium-denuded aortic strip (1b), KCl in endothelium-intact aortic strip (2a) and endothelium-denuded aortic strip (2b).

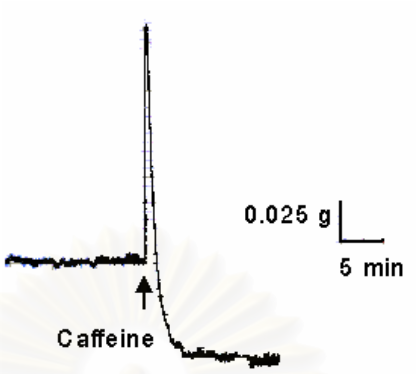


Figure 8 Representative tracing shows the aortic contraction of endothelium-denuded aortic strips induced by caffeine 10^{-3} M in Ca^{2+}-containing solution .

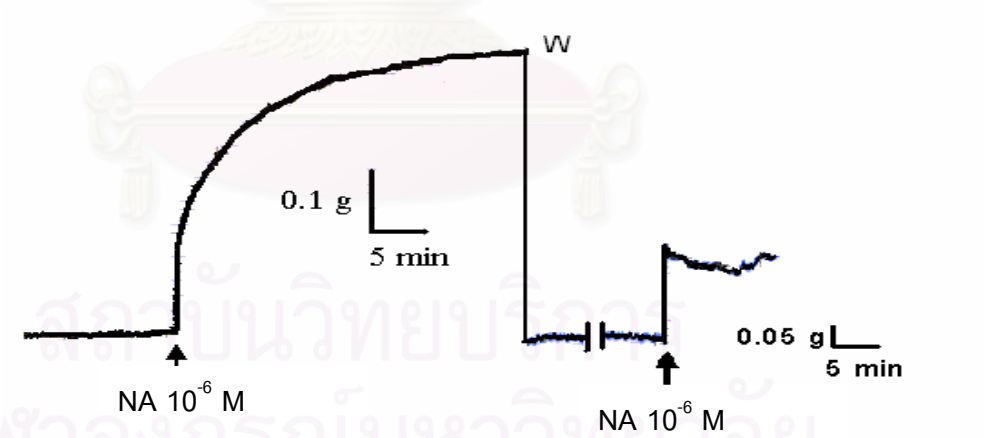
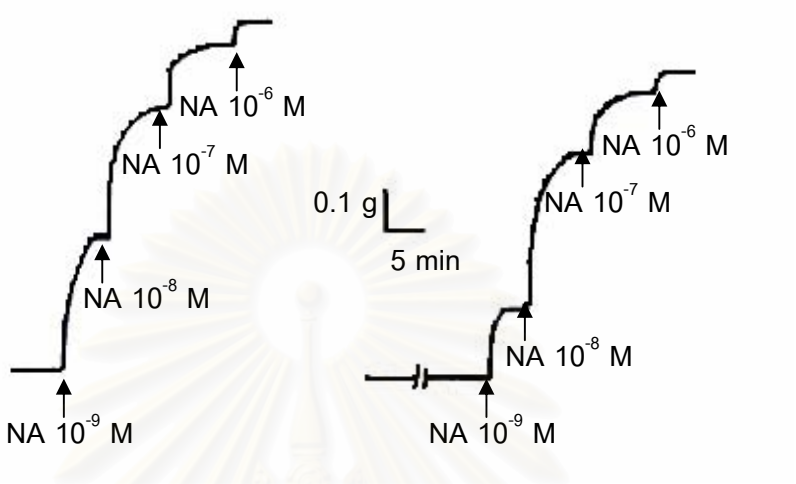


Figure 9 Representative tracing shows the aortic contraction of endothelium-denuded aortic strips induced by NA 10^{-6} M in Ca^{2+}-free medium.

1a) Control

1b) Metformin 10 μM



2a) Control

2b) Metformin 100 μM

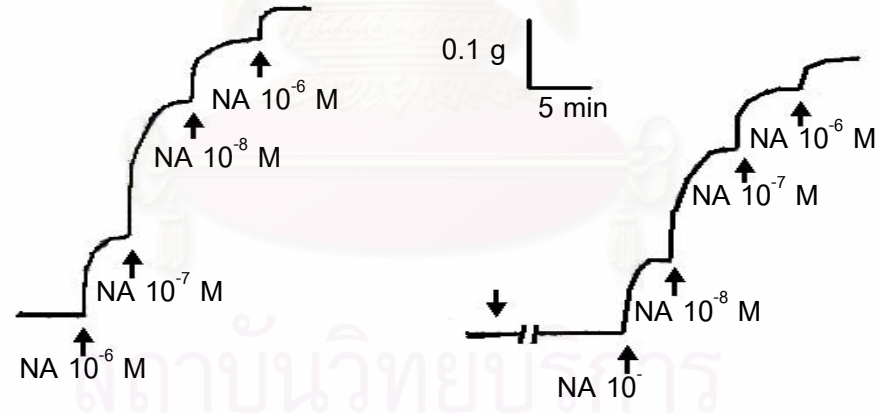


Figure 10 Representative tracing shows the effect of metformin on aortic contraction induced by cumulative addition of NA in Ca^{2+}-containing solution.

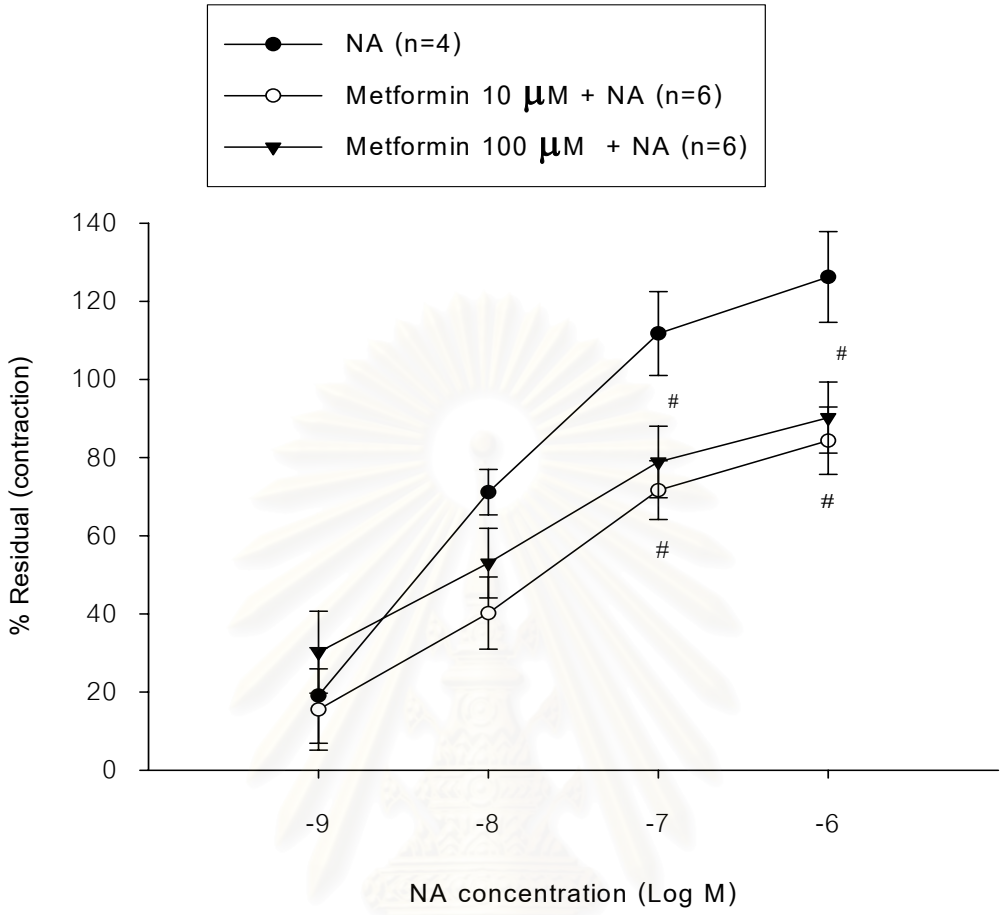


Figure 11 Effects of metformin on contraction in endothelium–intact aortic strips induced by cumulative addition of NA in Ca^{2+} -containing solution.
Data were presented as mean ± S.E.

# $p < 0.05$ shows significant difference of 10^{-4} M metformin-treated group from control.

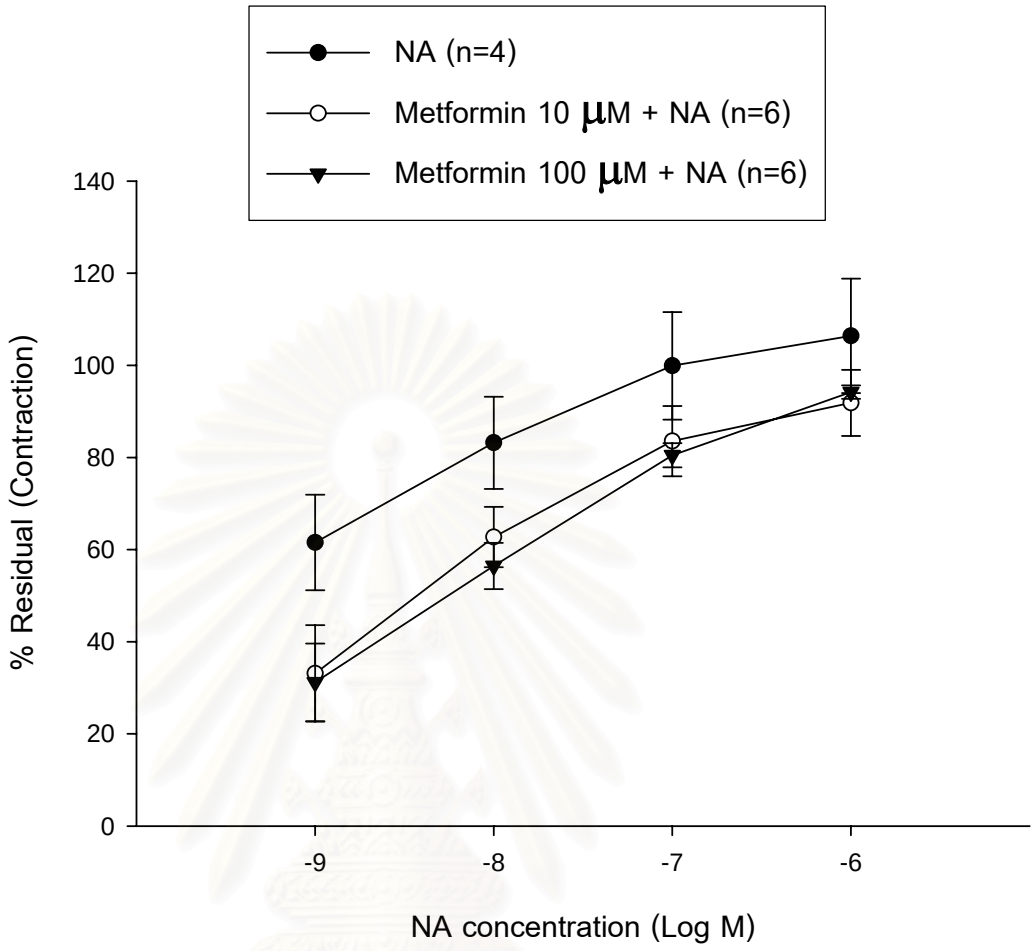


Figure 12 Effects of metformin on contraction of endothelium–denuded aortic strips induced by cumulative addition of NA in Ca^{2+}-containing solution.
Data were presented as mean ± S.E.

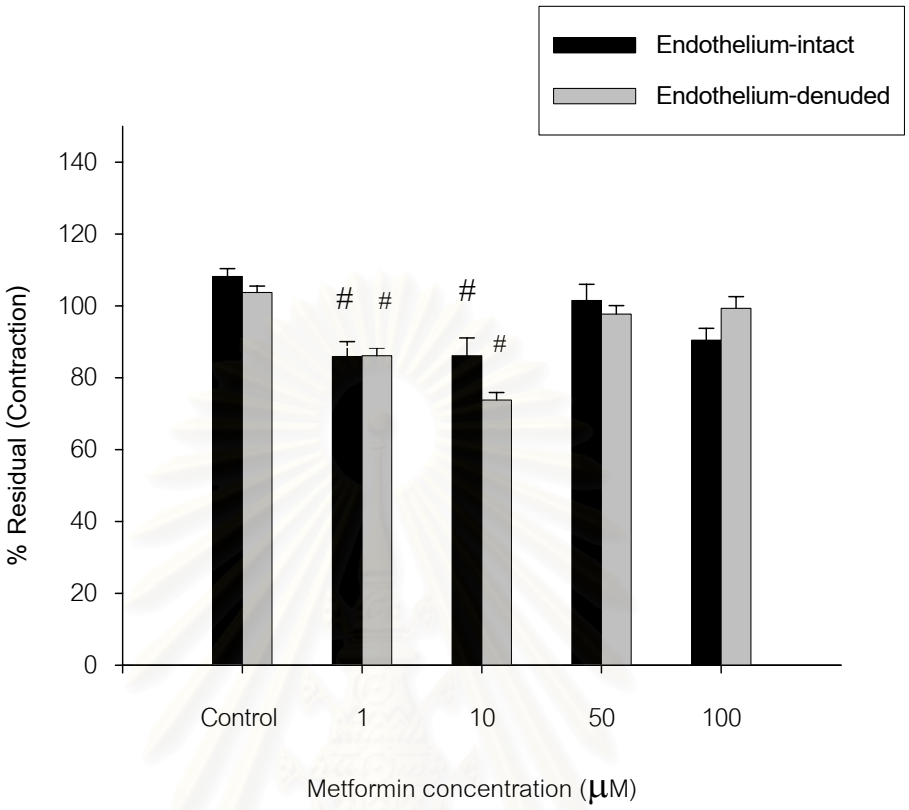


Figure 13 Effects of metformin on contraction of endothelium-intact and endothelium–denuded aortic strips induced by $5x10^{-8}$ M NA in Ca^{2+}-containing solution.

Metformin concentration = 1 μM, 10 μM, 50 μM or 100 μM

Data were presented as mean ± S.E., n = 4-8

# $p < 0.05$ shows significant difference of endothelium-denuded aortic strips from control.

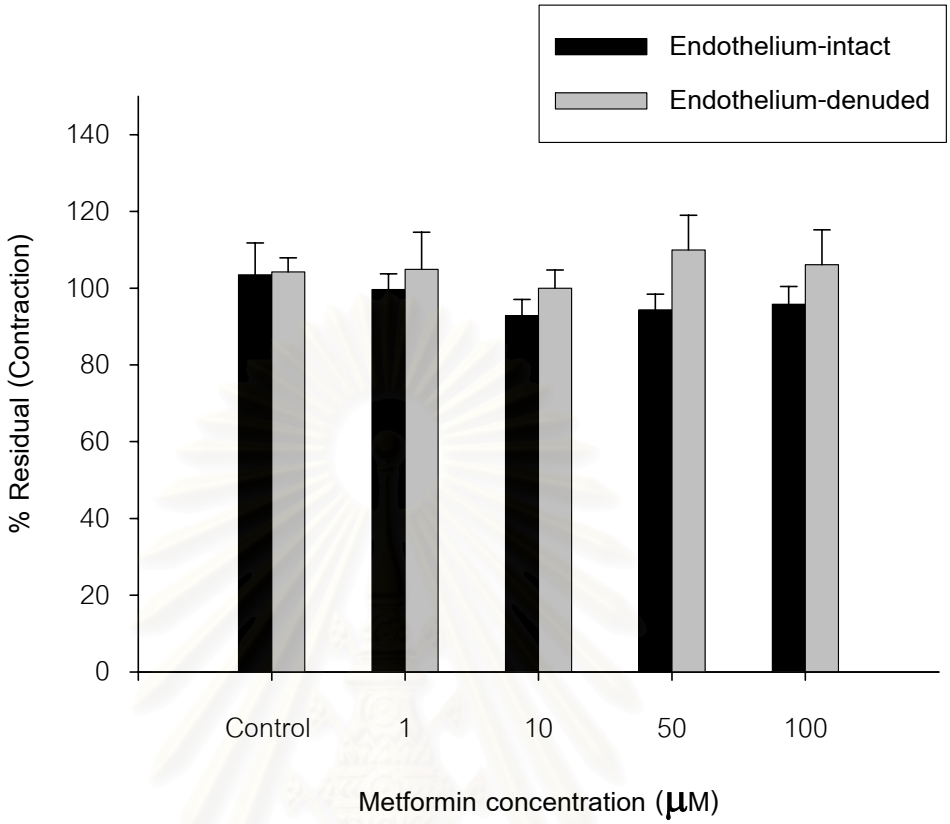


Figure 14 Effects of metformin on contraction of endothelium-intact and endothelium–denuded aortic strips induced by 10^{-6}M NA in Ca^{2+}-containing solution.

Metformin concentration = 1 μM, 10 μM, 50 μM or 100 μM

Data were presented as mean ± S.E., n = 4-8

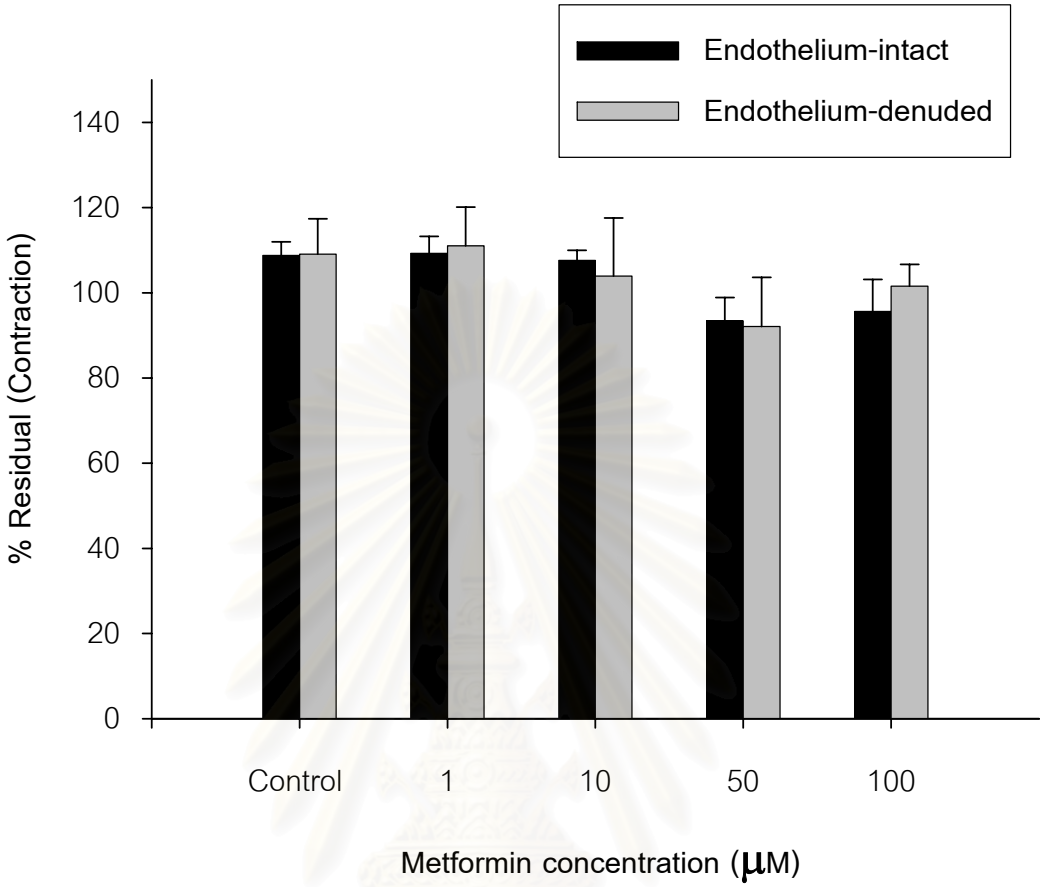


Figure 15 Effects of metformin on contraction of endothelium-intact and endothelium–denuded aortic strips induced by $4x10^{-2}$ M KCl in Ca^{2+}-containing solution.

Metformin concentration = 1 μM, 10 μM, 50 μM or 100 μM

Data were presented as mean ± S.E., n = 4-8

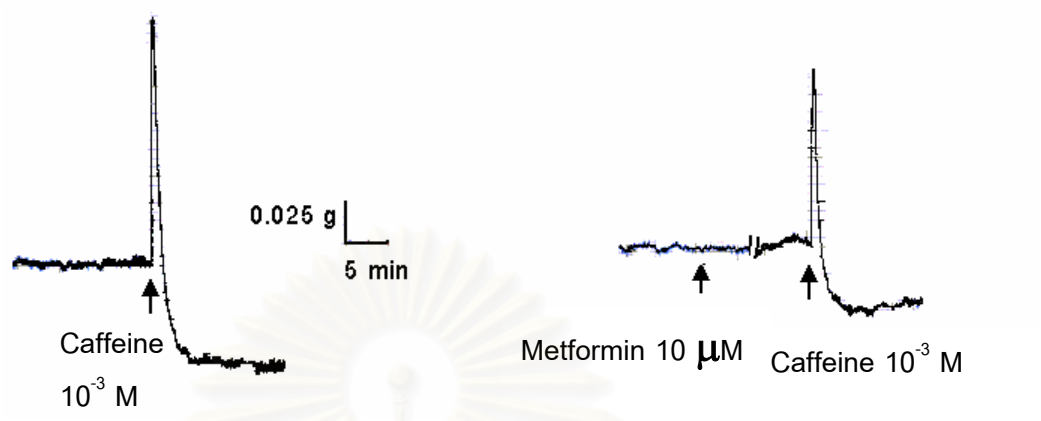
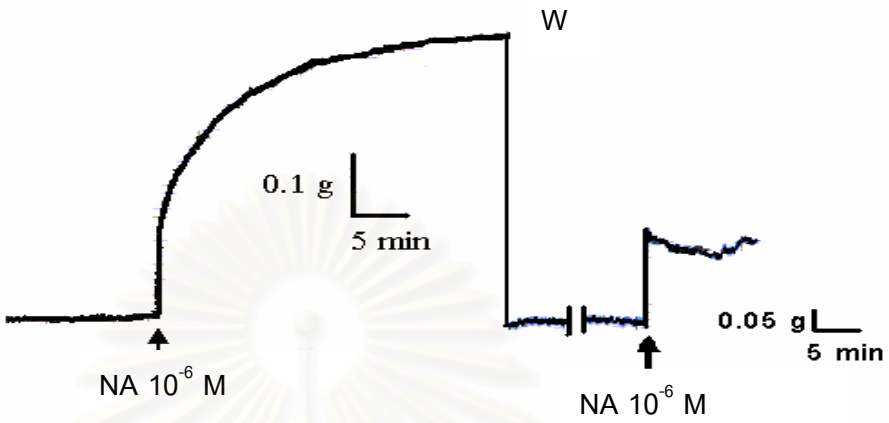


Figure 16 Representative tracing shows the effect of metformin on aortic contraction of endothelium-denuded aortic strips induced by 10^{-3} M caffeine in Ca^{2+}-containing solution.

1) Control



2) metformin 10 μM

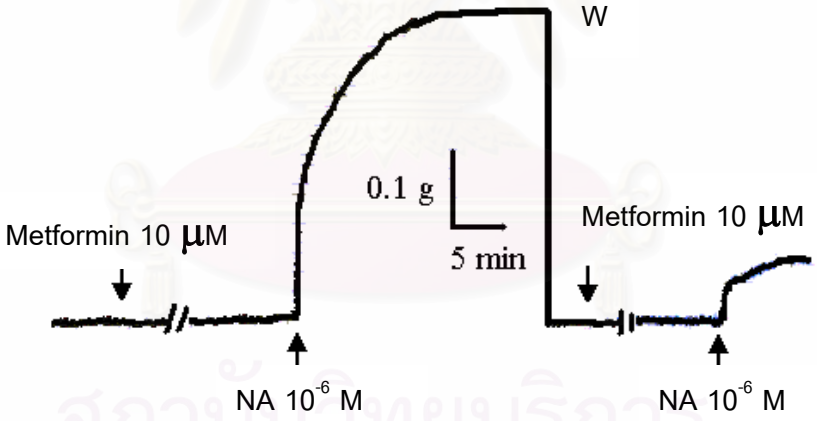


Figure 17 Representative tracing shows the effect of metformin on aortic contraction of endothelium-denuded aortic strips induced by NA 10^{-6} M in in Ca^{2+}-free medium.

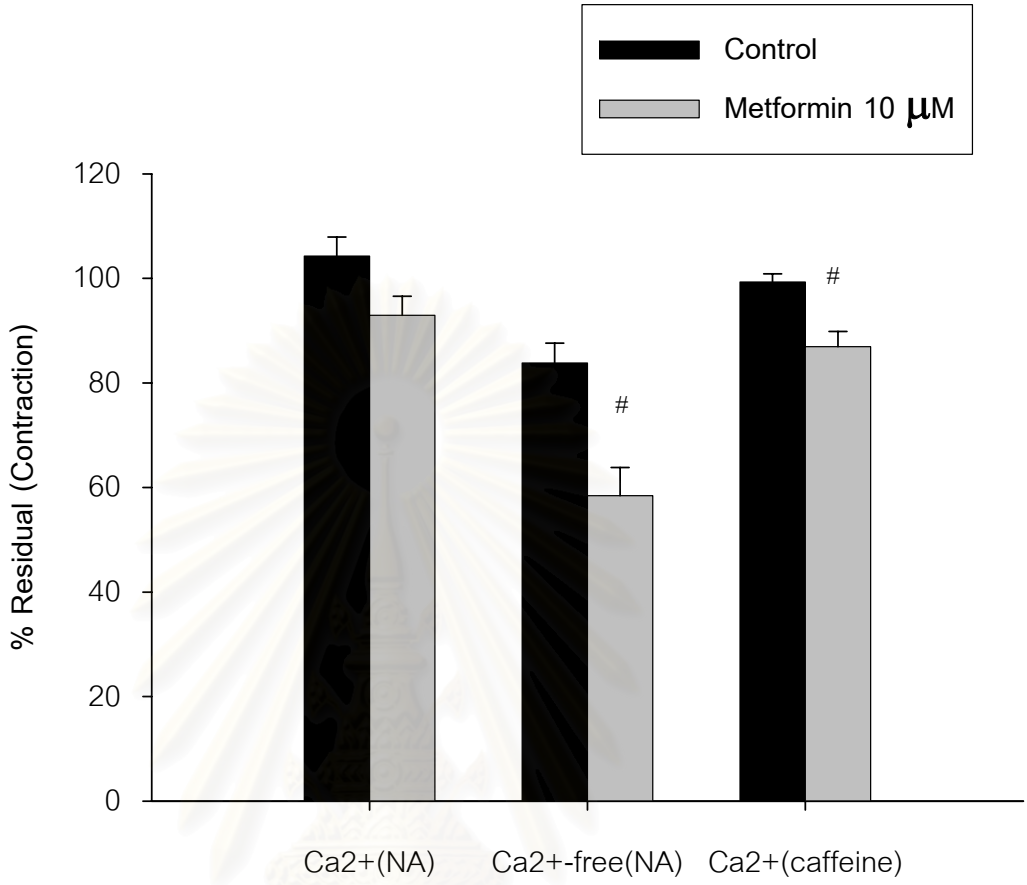
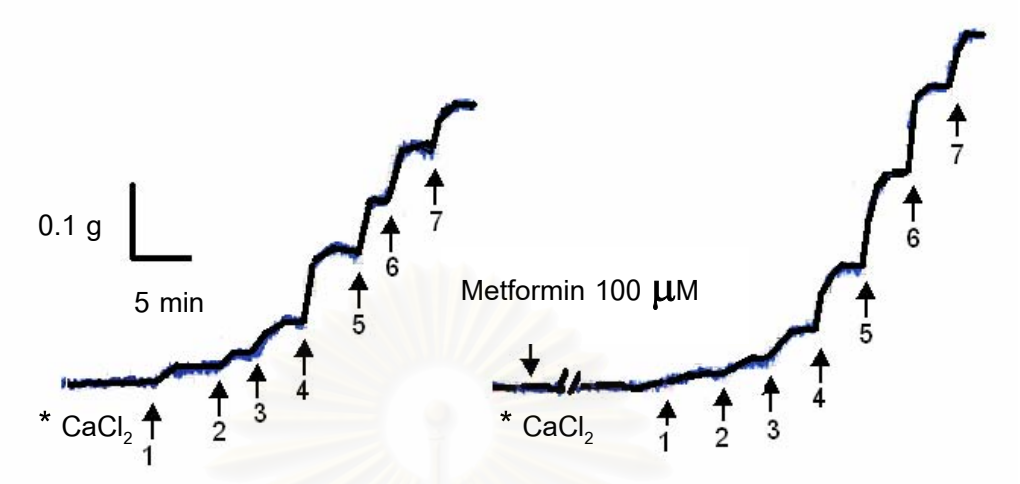


Figure 18 Effects of metformin on contraction of endothelium-denuded aortic strips induced by 10^{-3} M caffeine in Ca^{2+}-containing solution and 10^{-6} M NA-induced contraction in Ca^{2+}-free medium. Data were presented as mean $\pm$ S.E., n = 4-9

# $p < 0.05$ shows significant difference of metformin-treated group from control.



Control Metformin 100 μM+ $CaCl_2$

Figure 19 Representative tracing shows the effect of metformin on aortic contraction of endothelium-denuded aortic strips induced by cumulative addition $CaCl_2$ in Ca^{2+}-free depolarizing solution .

Concentration of $CaCl_2$ (M): 1= $1x10^{-5}$, 2= $3x10^{-5}$, 3= $1x10^{-4}$, 4= $3x10^{-4}$, 5=$1x10^{-3}$, 6= $3x10^{-3}$, 7= $1x10^{-2}$

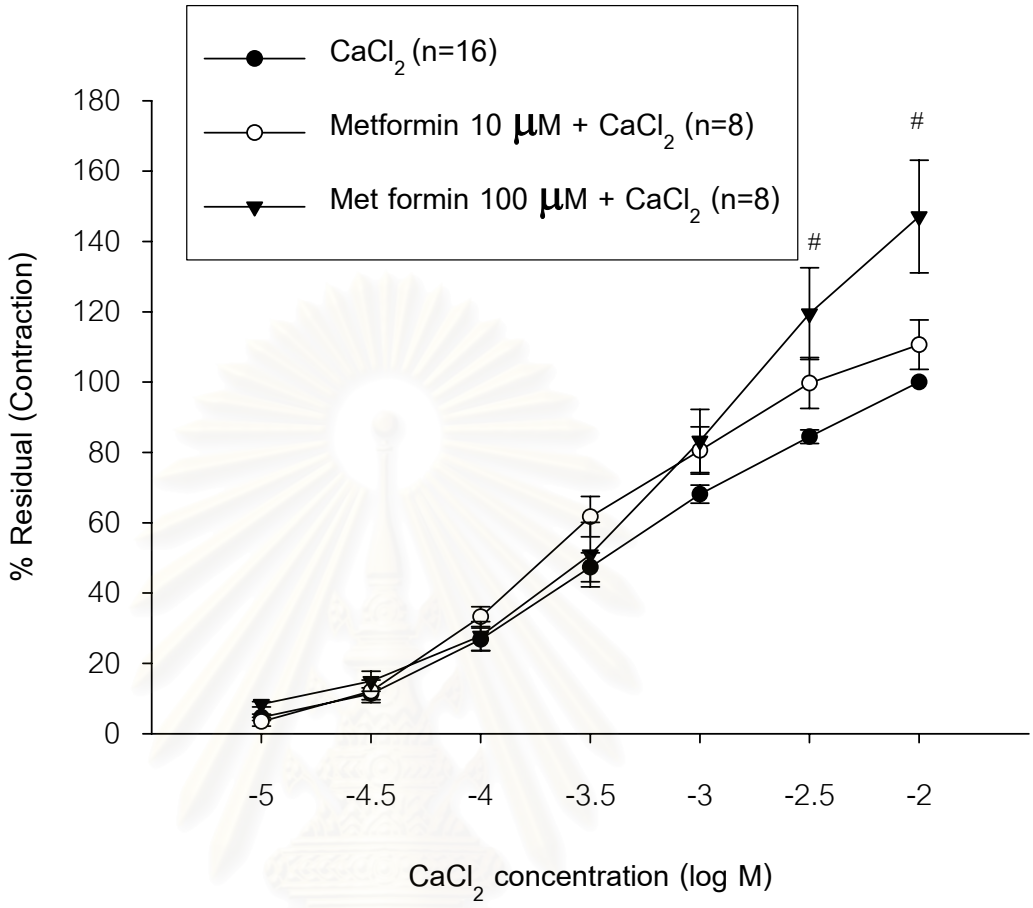
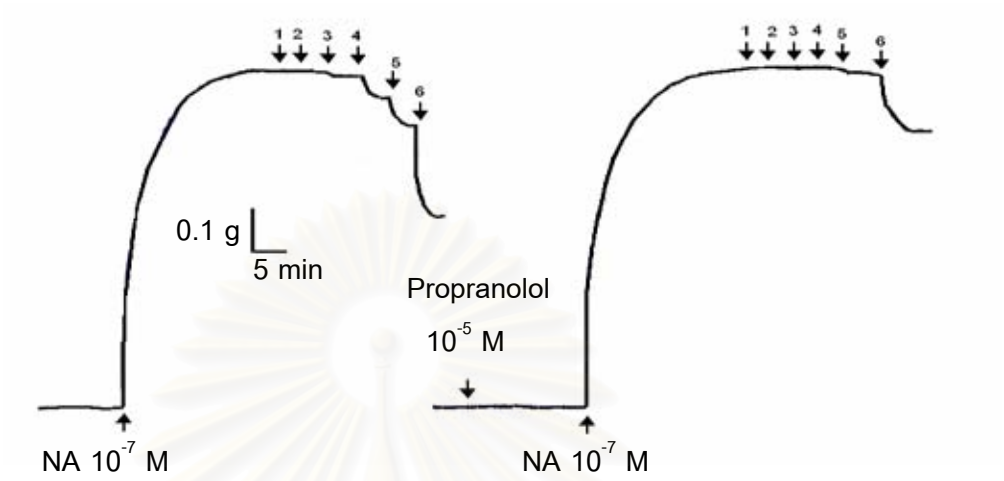


Figure 20 Effects of metformin on the contraction of endothelium-denuded aortic strips induced by cumulative addition of $CaCl_2$ in Ca^{2+}-free depolarizing solution.

Data were presented as mean ± S.E.

# $p<0.05$ show significant difference of 100 μM metformin-treated group from control group.

1) Control Propranolol 10^{-5} M + Isoproterenol



2) Control Metfromin 10 μM + Isoproterenol

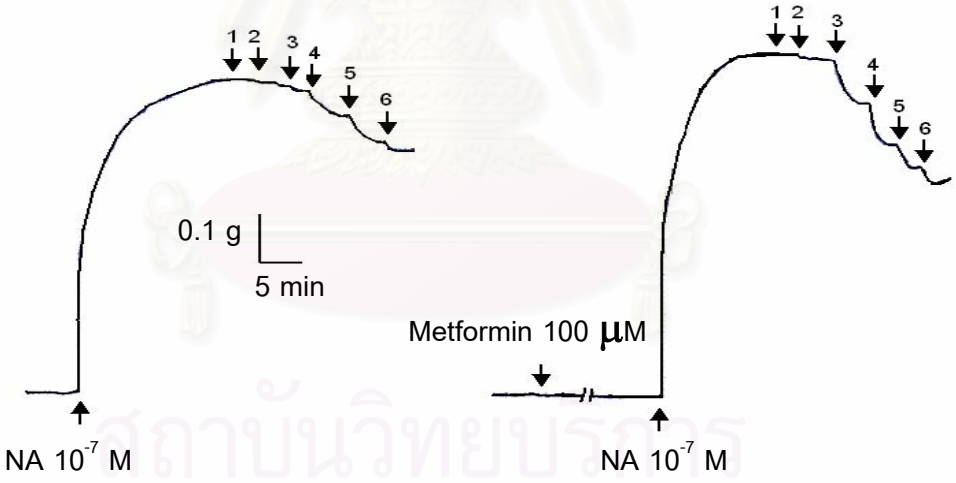


Figure 21 Representative tracing shows the aortic relaxation induced by cumulative addition of isoproterenol in endothelium-intact aortic strips in the presence of 10^{-5} M propranolol and 100 μM metformin. Isoproterenol concentration (M); 1= 10^{-9}, 2= 10^{-8}, 3= 10^{-7}, 4= 10^{-6}, 5= 10^{-5}, 6= 10^{-4}

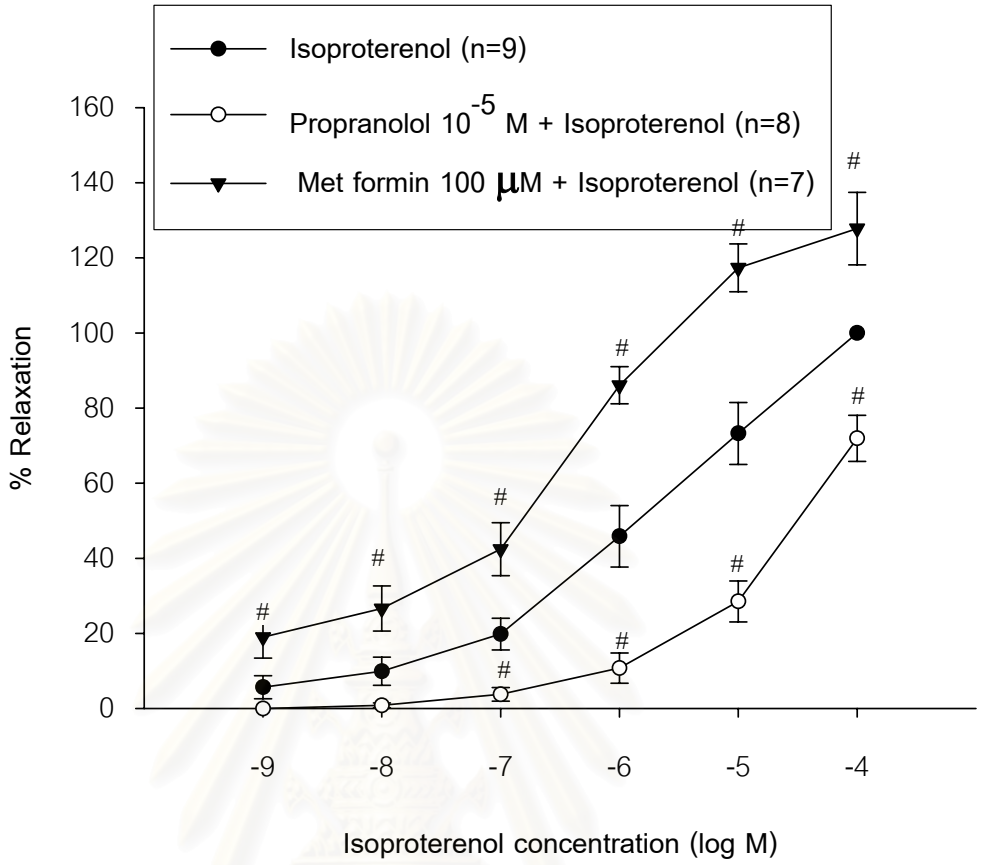
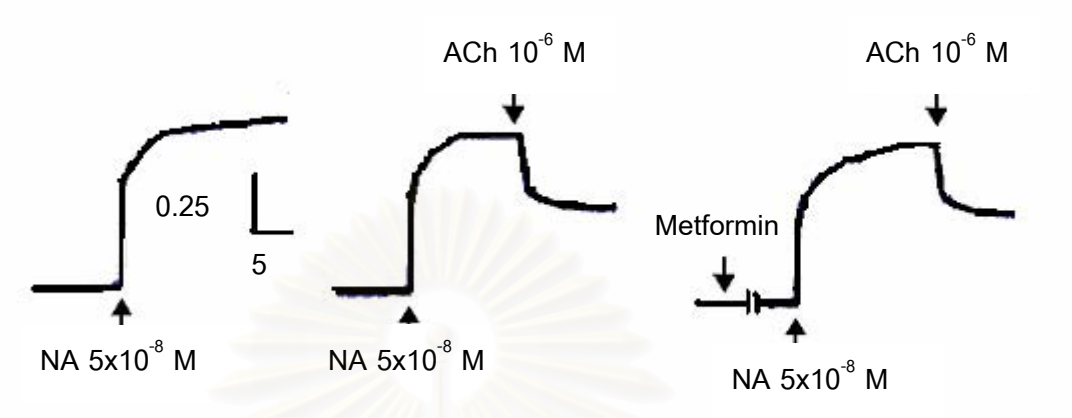


Figure 22 Effects of metformin (100 μM) and of propranolol 10^{-5} M on cumulative addition of isoproterenol to induce vasorelaxation of endothelium-intact aortic strip in Ca^{2+}-containing solution.
Data were presented as mean ± S.E.

# $p<0.05$ show significant difference from control group.

1) 10^{-6} M ACh-induced relaxation



2) $5x10^{-8}$ M Sodium nitroprusside

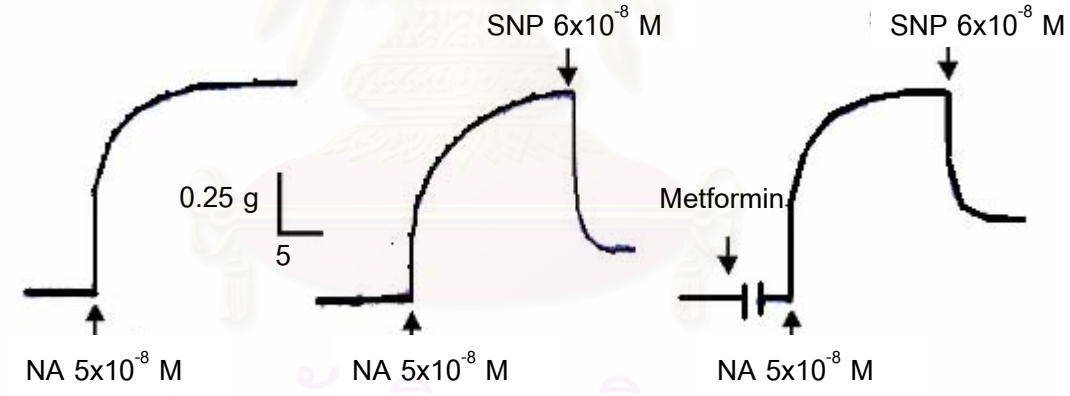


Figure 23 Representative tracing shows the aortic relaxation induced by ACh of endothelium-intact and sodium nitroprusside of endothelium-denuded aortic strips in Ca^{2+}-containing solution.

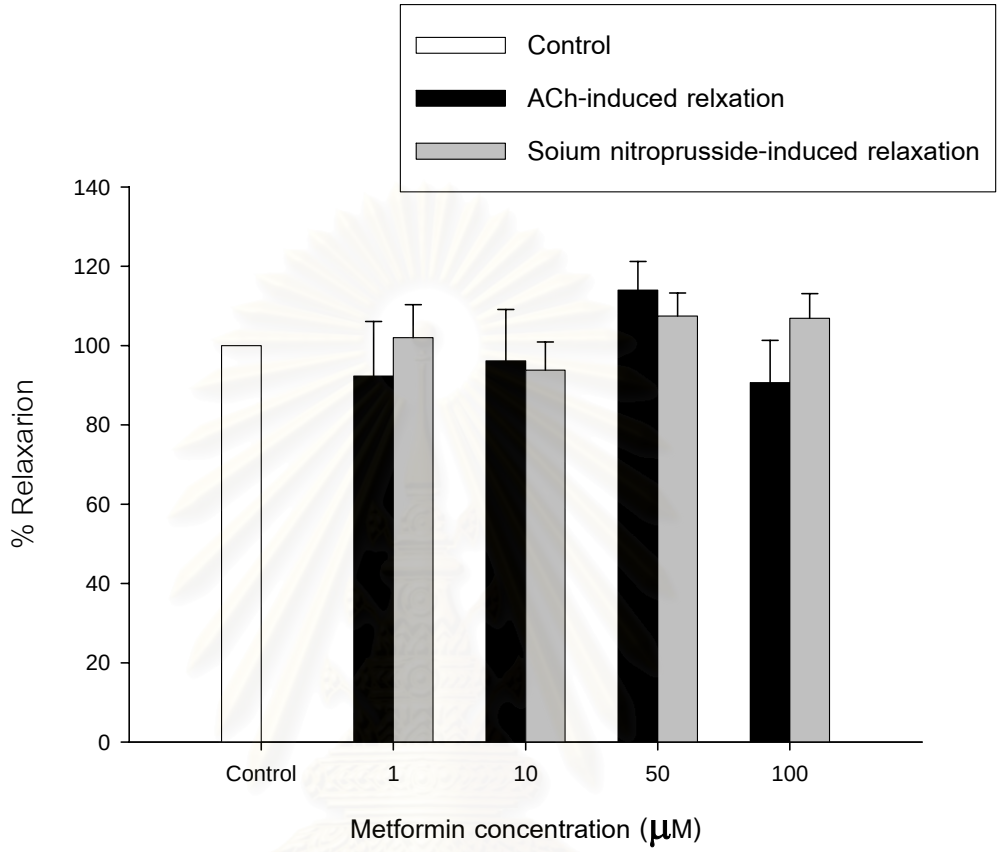
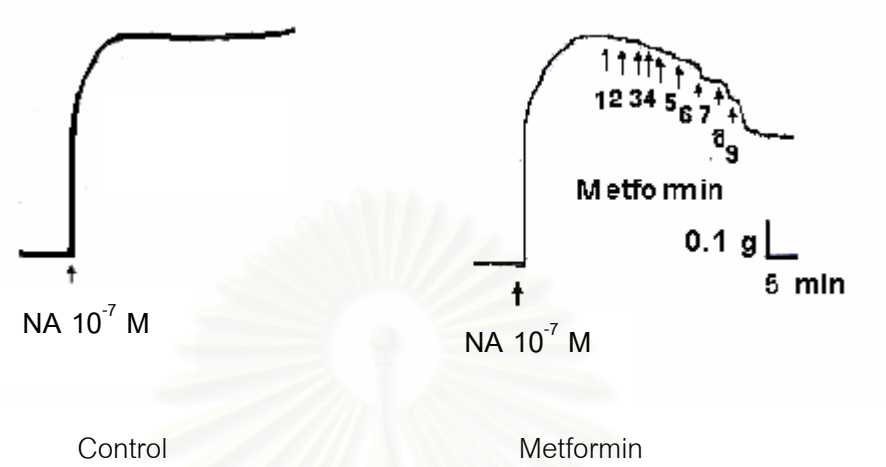


Figure 24 Effects of metformin on relaxation of endothelium-intact aortic strips induced by 10^{-6} M Ach and endothelium-denuded aortic strips induced by $6x10^{-8}$ M sodium nitroprusside in Ca^{2+}-containing solution.

Metfromin concentration = 1 μM, 10 μM, 50 μM or 100 μM

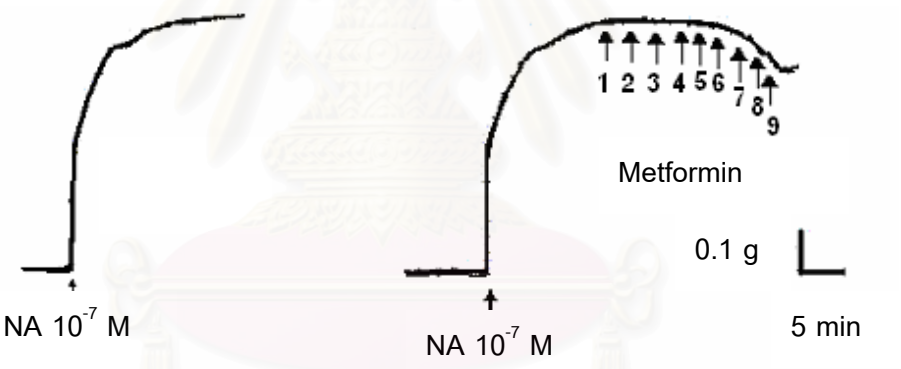
Data were presented as mean ± S.E., n = 4-8

1) Endothelium-intact



Control Metformin

2) Endothelium-denuded



Control Metformin

Figure 25 Representative tracing show the metformin-induced relaxation in endothelium-intact and endothelium-denuded aortic strips in Ca^{2+}-containing solution.

Metformin concentration (μM); 1= 0.1, 2= 1, 3= 10, 4= 50, 5= 100, 6= 200, 7=500, 8=1000, 9=1500

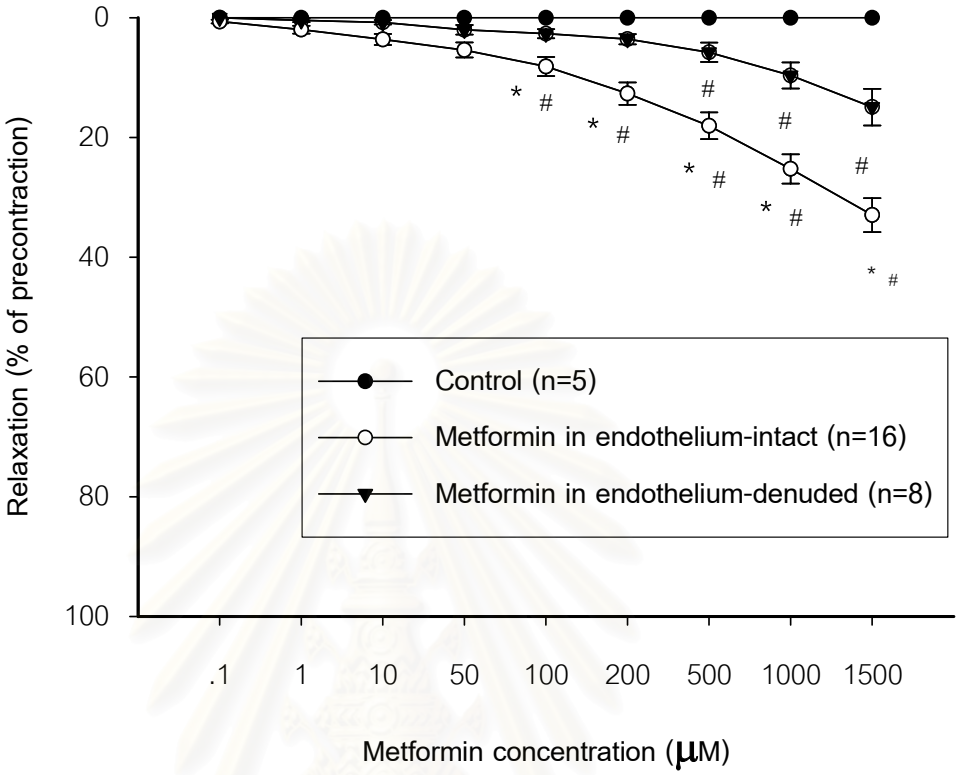


Figure 26 Relaxation-response curves for metformin of endothelium-intact aortic strips precontracted with NA (10^{-7} M) in the presence or absence of endothelium. Removal of endothelium did not affect the response to metformin.

Data were presented as mean ± S.E.

# $p<0.05$ show significant difference from control group.

*$p<0.05$ show significant difference between endothelium-intact and endothelium-denuded aortic strip

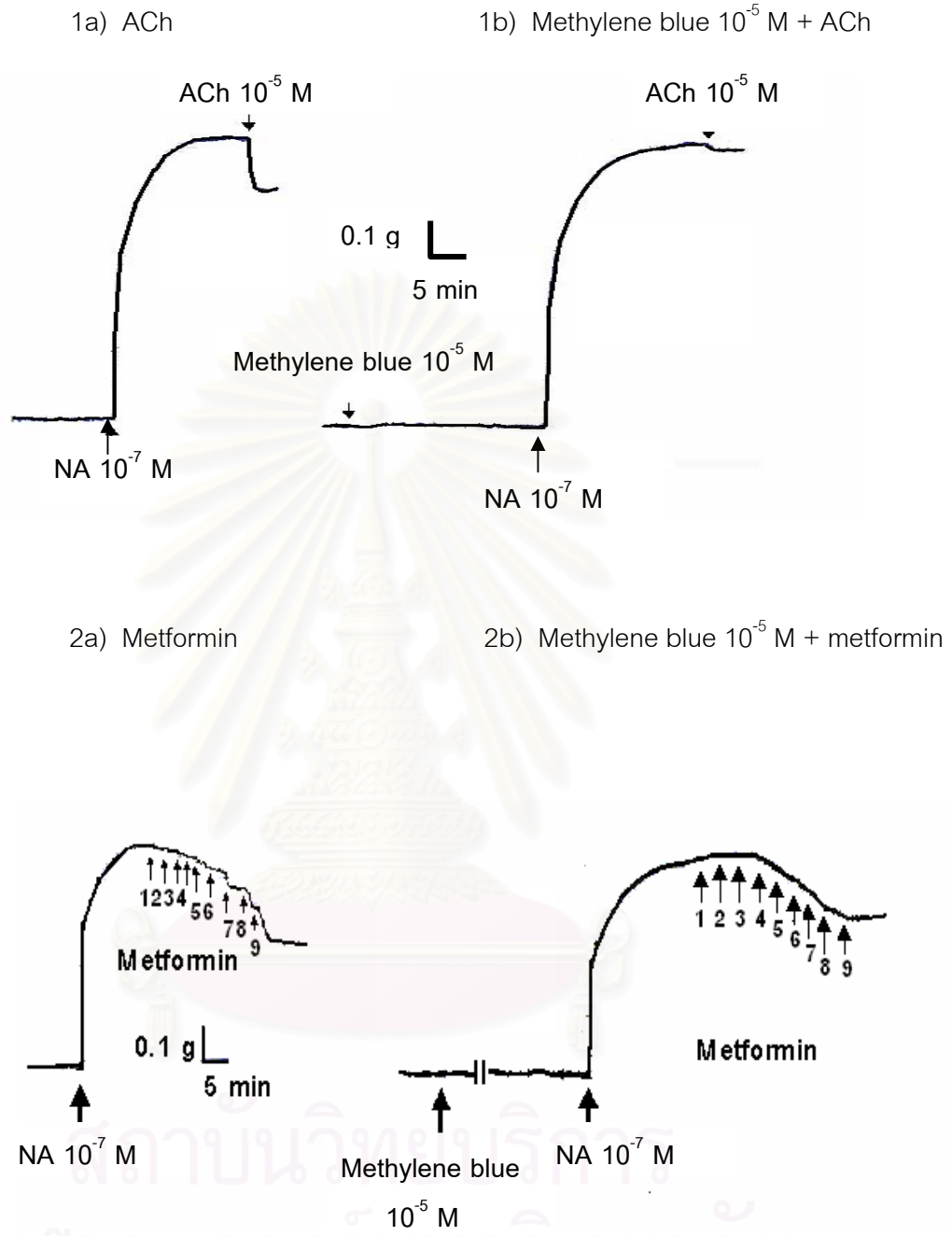


Figure 27 Representative tracing show the metformin-induced relaxation in the presence of methylene blue of endothelium-intact aortic strips in Ca^{2+}-containing solution.

Metformin concentration (μM); 1= 0.1, 2= 1, 3= 10, 4= 50, 5= 100, 6= 200, 7=500, 8=1000, 9=1500

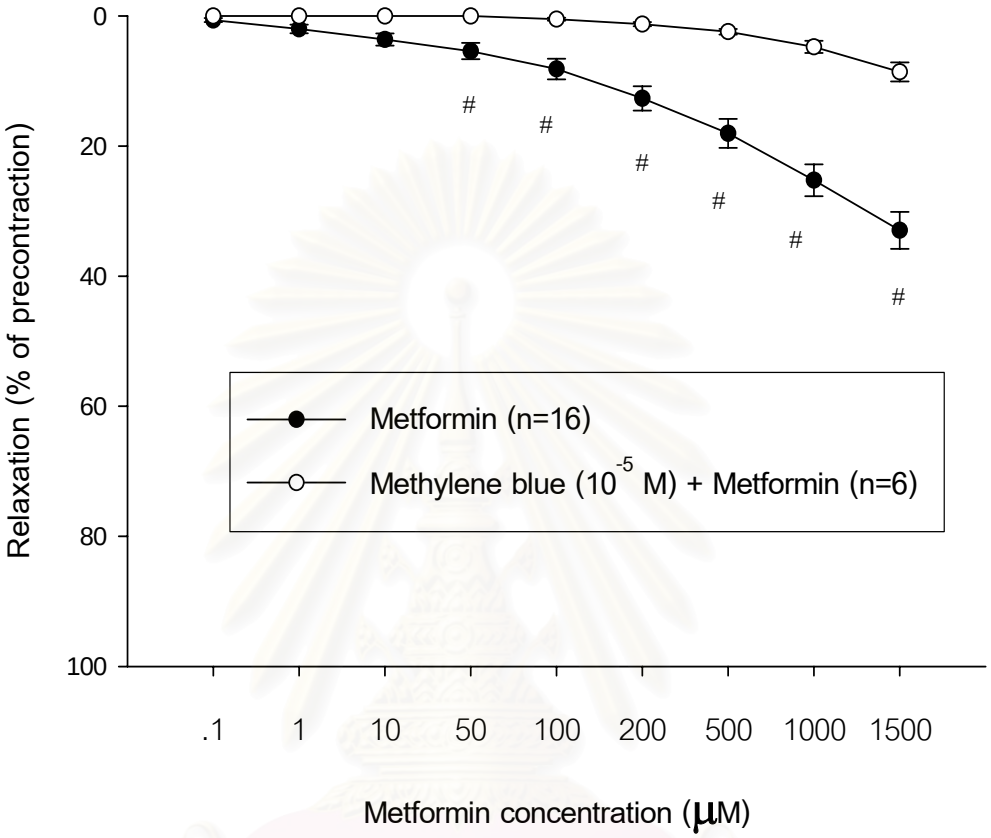
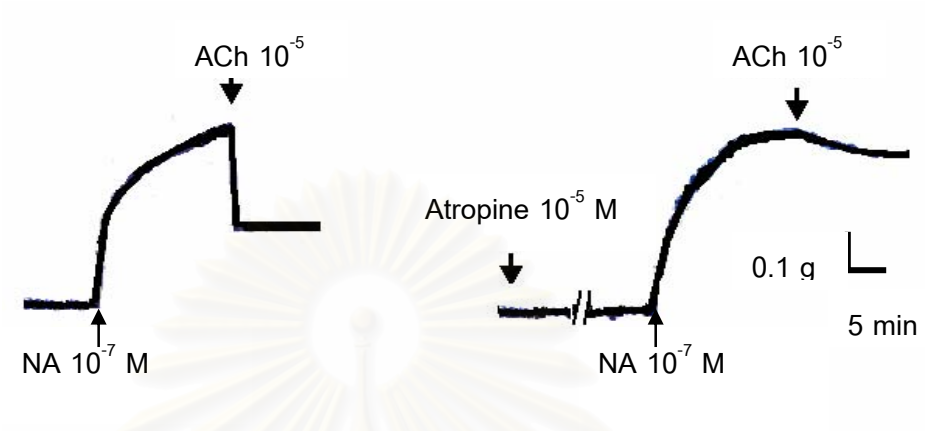


Figure 28 Relaxation-response curves for metformin of endothelium-intact aortic strips precontracted with NA in the presence or absence of 10^{-5} M methylene blue.
Data were presented as mean ± S.E.

# $p<0.05$ show significant difference from control group.

1a) NA+ACh

1b) Atropine+NA + ACh



2a) NA+Metformin

2b) Atropine+NA + metfromin

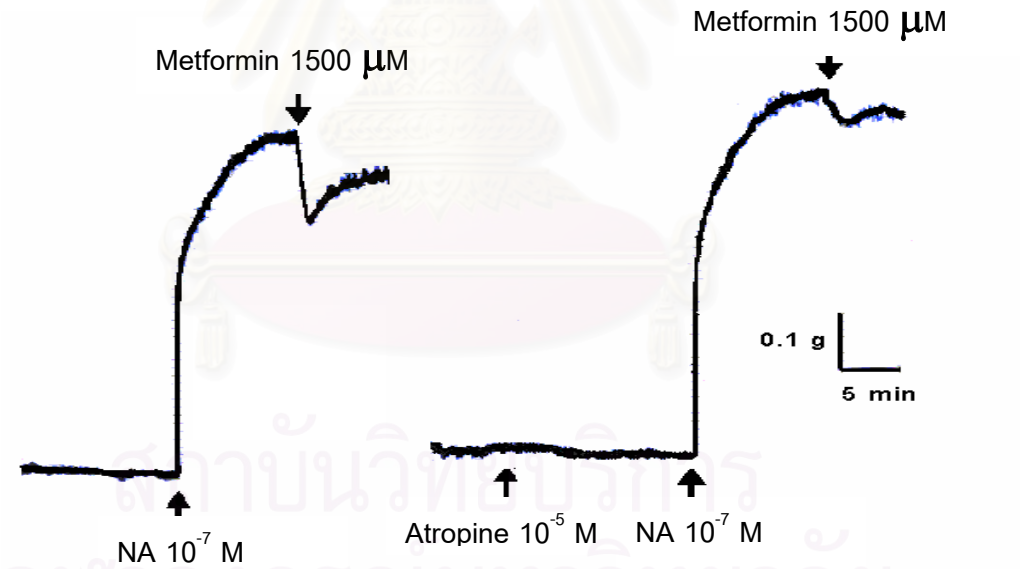


Figure 29 Representative tracing show the metformin-induced relaxation in the presence of 10^{-5} M atropine of endothelium-intact aortic strips in Ca^{2+}-containing solution.

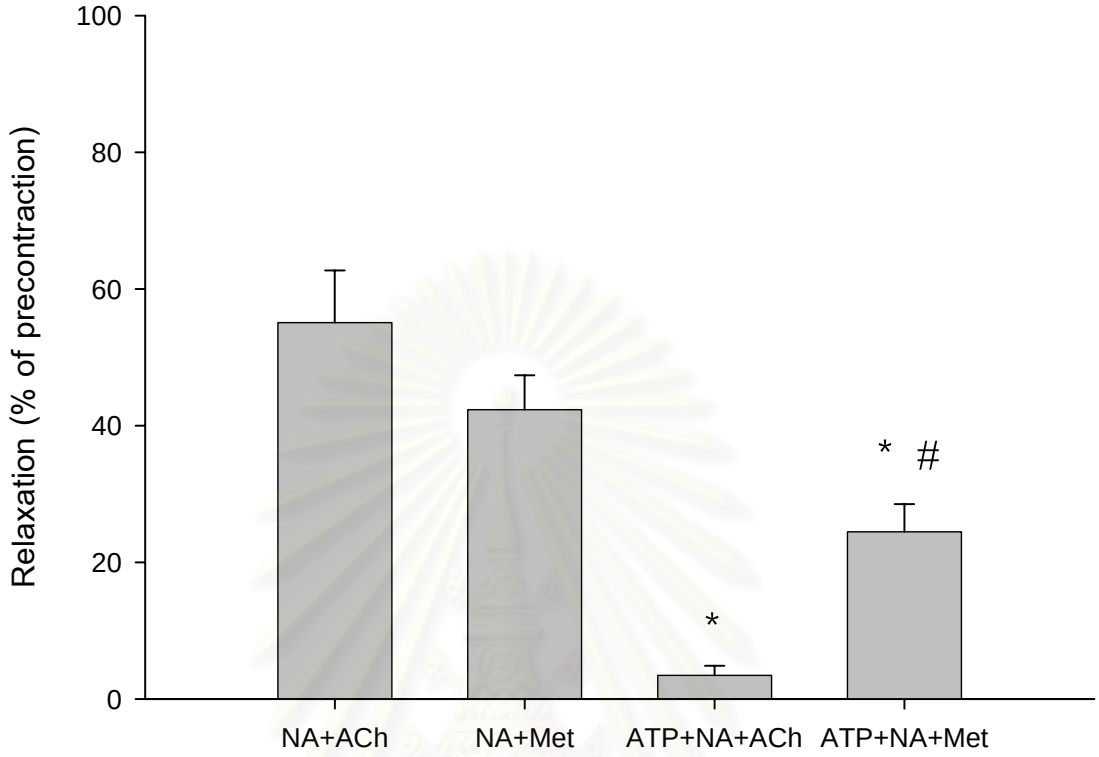


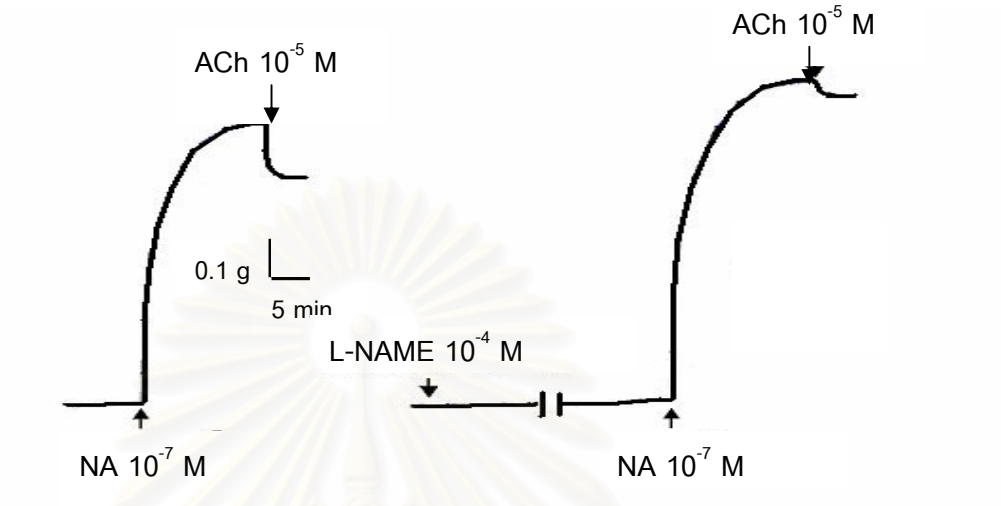
Figure 30 Effect of 10^{-5} M atropine on metformin-induced relaxation of endothelium-intact aortic strips in Ca^{2+}-containing solution . Data were presented as mean ± S.E., n = 4-8

*p<0.05 show significant difference from NA + ACh group.

# p<0.05 show significant difference from NA + metformin group

1a) ACh

1b) L-NAME + ACh



2a) Metformin

2b) L-NAME + Metformin

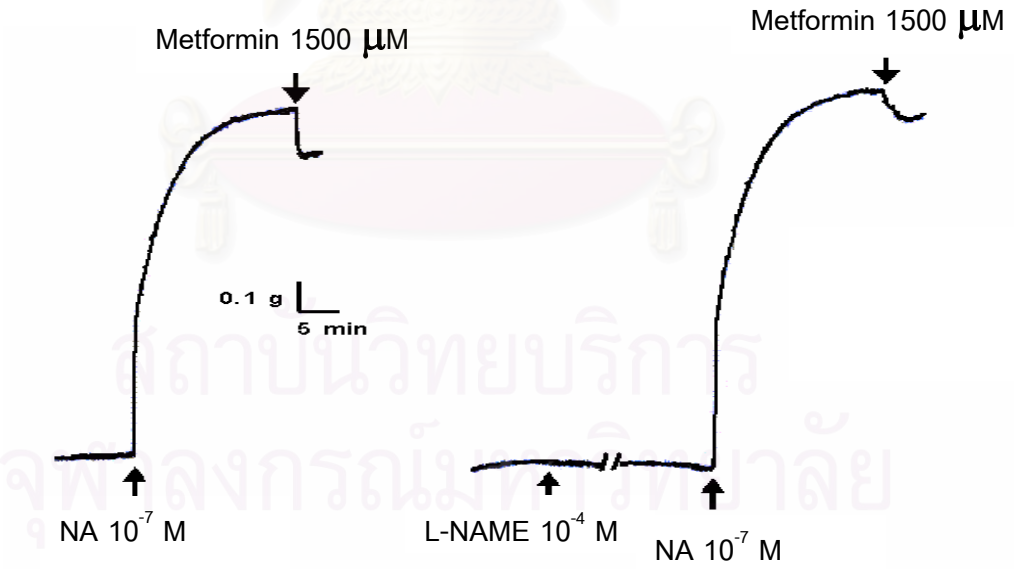


Figure 31 Representative tracing show the metformin-induced relaxation in the presence of 10^{-4} M L-NAME of endothelium-intact aortic strips in Ca^{2+}-containing solution.

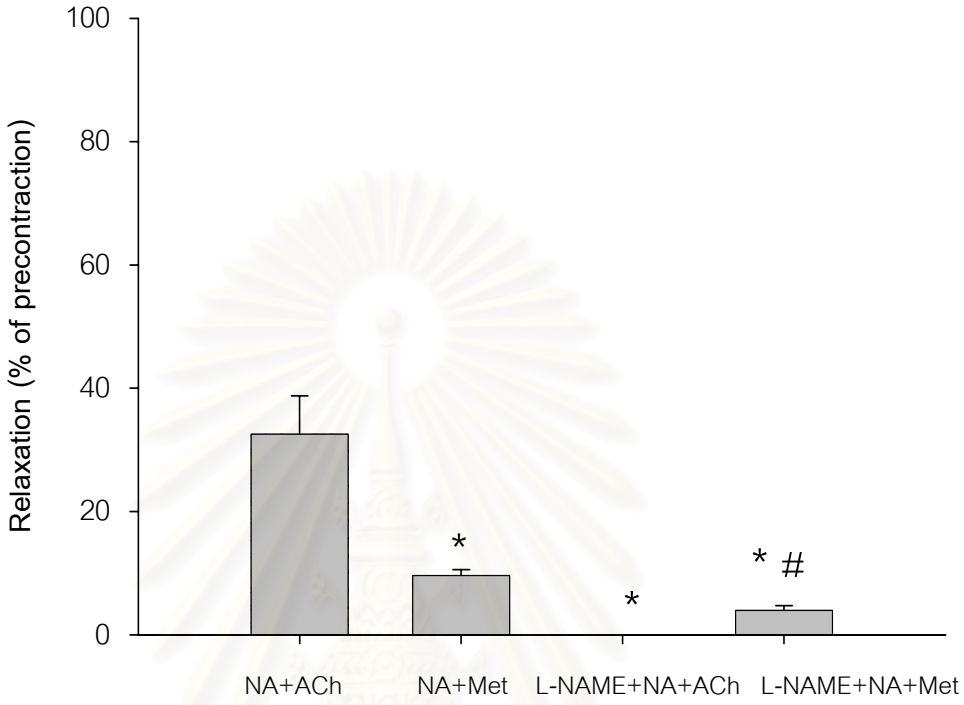


Figure 32 Effect of 10^{-4} M L-NAME on metformin-induced relaxation of endothelium intact aortic strips in Ca^{2+}-containing solution. Data were presented as mean ± S.E., n = 3-6

*p<0.05 show significant differenceof from NA + ACh group.

# p<0.05 show significant difference from NA + metformin group

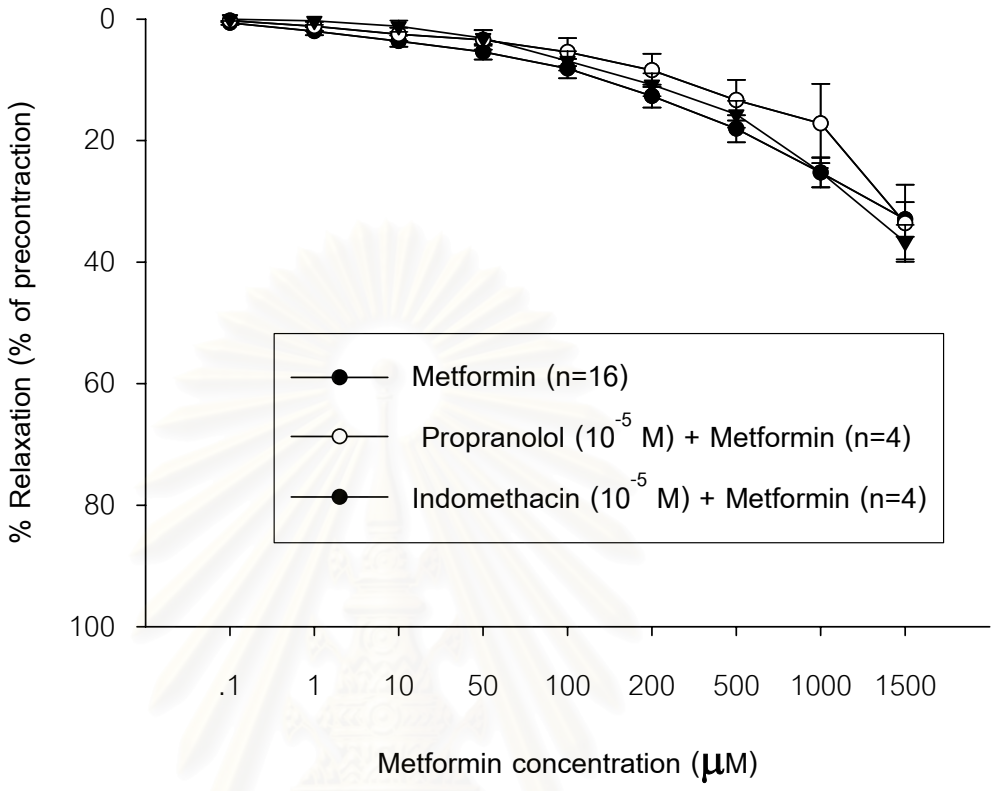


Figure 33 Relaxation-response curves for metformin of endothelium-intact aortic strips precontracted with NA in the presence or absence 10^{-5} M Indomethacin and 10^{-5} M propranolol.
Data were presented as mean ± S.E.

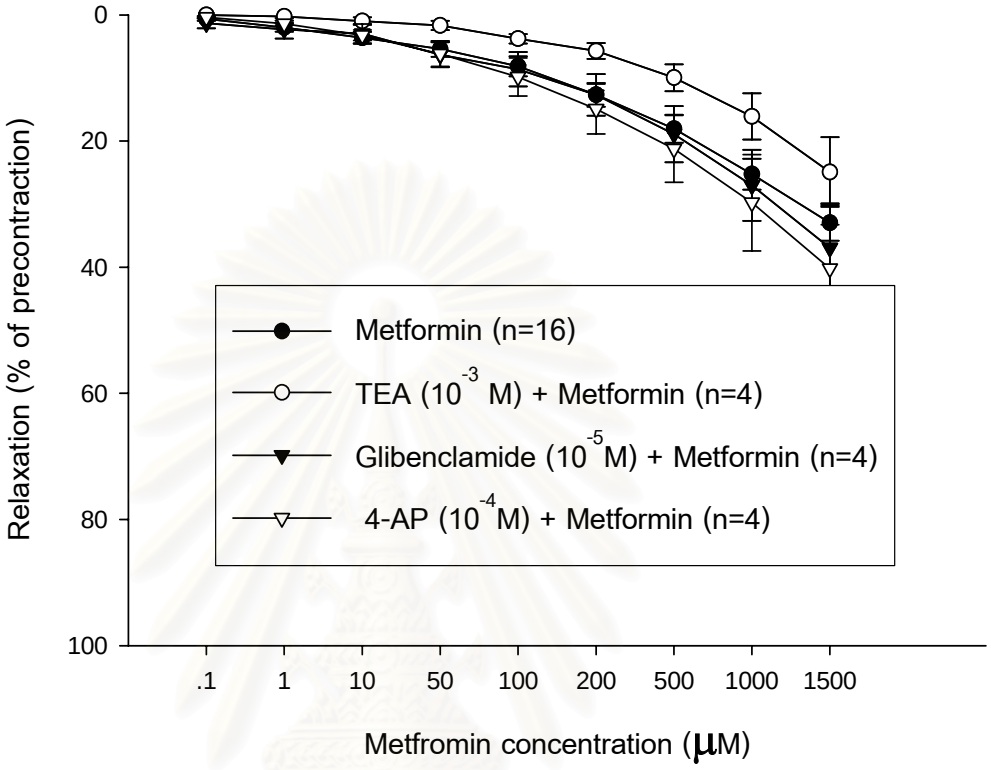


Figure 34 Relaxation-response curves for metformin of endothelium-intact aortic strips precontracted with NA in the presence or absence 10^{-3} M TEA, 10^{-3} M 4-AP and 10^{-5} M glibenclamde.
Data were presented as mean $\pm$ S.E.

CHAPTER V

DISCUSSION AND CONCLUSION

This study aimed to investigate the effects of metformin on the contractility of aortic smooth muscle and to characterize the mechanism of metformin-induced relaxation in endothelium-intact aortic strips. As known, intracellular calcium is very important in regulating smooth muscle contraction. NA triggers the contractile response by activation of the α-adrenoceptor leading to a release of Ca^{2+} from intracellular stores. In the continuing presence of NA, contraction is maintained and this phase is associated with an influx of Ca^{2+} into the cell (Noguera and D'Ocon, 1993).

In order to elucidate the action of metformin on vascular function, these studies applied various contractants to evoke contraction. Metformin significantly inhibited the aortic contraction induced by adding cumulatively concentration of NA in endothelium-intact aortic strips. These results suggested that metformin might reduce NA-induced contraction via blockage of α-adrenoceptor. However, this possibility was ruled out because the observed inhibitory effect of metformin on NA-induced contraction was dose-independent. The increase in concentration of metformin did not cause more inhibitory effect. On the contrary, the inhibitory effects vanished when the concentration of metformin increased. In addition, the inhibitory effects were obtained only when the low concentration of NA was applied in the study.

The mechanism of KCl-induced contraction involves membrane depolarization, leading to open L-type Ca^{2+} channels. Hence, treatment of KCl results in an increase of Ca^{2+} influx and leading to vascular smooth muscle contraction (Karaki, *et al*., 1997; Katz, *et al*., 1997). In this study, metformin did not alter the contraction induced by KCl in both endothelium-intact and endothelium-denuded aortic strips, suggesting that metformin action did not involve membrane depolarization. In addtion, in the present study, metformin at the concentration of 10^{-5} M did not inhibit calcium entering to cell through voltage-operated Ca^{2+} channels, thus, an inhibition of VOC was ruled out. However,

metformin at the higher concentration (10^{-4} M) caused increase the contraction induced by the concentration of $CaCl_2$ more than $3x10^{-3}$ M. This result may be described by the possibility that the contraction induced by NA was increased when the concentration of metformin was increased. The mechanisms other than VOC might be involved to the action of metformin. The mechanism of metformin on Ca^{2+} influx are remained unidentified and further research on this topic is needed.

Furthermore, metformin reduced the contraction induced by NA in Ca^{2+}-free medium and induced by caffeine in Ca^{2+}-containing solution. This finding suggested metformin weakly inhibited calcium release from intracellular stores.

Because metformin has no inhibitory action on the contraction of aortic strip, it is likeky that metformin may interfere the vasorelaxation. There are several mechanisms to be considered for the vasorelaxation. β-adrenoceptor - mediated relaxation is though to play an important role in the relaxation of vascular tone. β-adrenoceptor agonist relaxes vascular smooth muscle by stimulateβ-adrenoceptor (Rang, *et al*., 1999). In this study, metformin increased the relaxation induced by isoproterenol. Suggesting that metformin has the synergistic effect with β-adrenoceptor agonist.

Variuos mechanisms are attribute to the vasorelaxation. Vasodilator action of ACh is mediated by release of relaxant substance termed as EDRF such as NO from endothelial cell (Furchott and Zawadzki, 1980). To produce the vasodilatation effect, EDRF stimulate the activity guanylate cyclase and dilates vascular smooth muscle by increasing cAMP (Sato, *et al.,* 1988). However, relaxation response induced by ACh or NO donor such as sodium nitroprusside were not altered by metformin. These results suggested that metformin did not interfere neither endothelial release of NO by activation of muscarinic receptor or change the sensitivity of vascular smooth muscle to NO. These results supported the finding of an another studied which demonstrated that metformin had no effect on endothelium-dependent relaxation by pretreatment the aortic strips with metformin prior to addition ACh in normotentsive rats (Prasad, *et al*., 2000).

In this study, metformin has an intrinsic vasorelaxant effect on the isolated thoracic aorta. Metformin could relax the NA-precontracted muscle in both endothelium-

intact and endotheluim–denuded aortic strips. However, metformin-induced relaxation was reduced by endothelium removal. In addition, the previous experiments have demonstrated that high concentration of metformin (>10 mmol/L) induced vascular relaxation in rat tail artery (Chen, *et al*., 1997). Another studies using mesenteric arteries of normal and insulin-resistant rats showed that metformin at the concentration higher than 10^{-3} M induce vasorelaxation (Prasad, *et al*., 2000). In contrast, this study showed that 10^{-4} M also investigated much of vasorelaxant action of metformin. These results demonstrated that the isolated rat thoracic aorta is more sensitive to metformin than rat tail artery or mesenteric artery. Therefore, the thoracic aorta is suitable model for further characterized the mechanism of metformin-induced relaxation.

The present work demonstrated endothelium-dependent of metformin-induced vasorelaxation which may be mediated through NO pathway. To investigate the involvement of muscarinic receptor on metformin-induced relaxation, atropine was used. It had been found that atropine significantly reduced the metformin-induced relaxation, suggesting that metformin might be act mediated through muscarinic receptor to initiate and produce vasorelaxation because atropine is a selective antagonist to muscarinic receptor. However, this further prove by produce the concentration response curve of atropine.

In the ACh-NO pathway of vasorelaxation, NO is produced from L-arginine by NO synthase enzyme (Inarro, *et al.,* 1981), which is inhibited by specific analogues of L-arginine such as L-NAME. In these studies, a specific inhibitor of NO synthesis, L-NAME significantly inhibited the metformin-induced relaxation in the presence of endothelium. It is confirm to be related to the production of NO. In addition, metformin reduced the vasorelaxation in the presence of methylene blue, indicating the involvement of the enzyme guanylyl cyclase to the relaxant action of metformin in NO pathway (Gruetter, *et al.,* 1981).

It has been shown that endothelial cells release locally acting vasodilating prostaglandin. Indomethacin did not affect the relaxation to metformin, indicating that

the prostaglandins derived from COX pathway may not play a role in the relaxation to metformin in this study.

An other possible effects on vasorelaxation may involve K^+, the possibility that the repolarizing action of metformin is linked to an effect of metformin on membrane-bound potassium channel, resulting in K^+ efflux and reduction of smooth muscle tone (Karaki, *et al*., 1997). However, the results shown that three inhibitors of K^+ channels, TEA (non-selective K^+ channel blocker), 4-aminopyridine (slective voltage-operated K^+ channel blocker) or glibenclamide (ATP-activated K^+ channel blocker) did not antagonize the acute relaxant effect of metformin on NA-induced contraction. Therefore, mechanisms responsible for this acute relaxant effect were unrelated to potassium channel and their role in cell membrane repolarization. Furthermore, this study was consistented with that acute relaxation of rat tail arterial smooth muscle by metformin did not mediated through voltage- K channels (JiHun, *et al*., 1998)

In conclusion, metformin had a multieffect to a contractility of thoracic aorta. It could be suggested that metformin induced relaxation in both endothelium-intact and endothelium-denuded aortic strip. Metformin-induced relaxation in endothelium-intact aortic strip may be partially mediated through the cholinergic receptor on the endothelium to initiate the NO-cGMP pathway.

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APPENDICES

Table 2 The percentage of contraction induced by adding cumulatively NA in aortic strips.

2a. Endothelium-intact

Endothelium-intact	Noradrenaline concentration (M)			
	10^{-9}	10^{-8}	10^{-7}	10^{-6}
Control, n = 4	19.12 ± 12.23	71.15 ± 5.82	111.76 ± 10.73	126.23 ± 11.60
Metformin 10 μM, n = 6	15.55 ± 10.38	40.22 ± 9.22	71.67 ± 7.53	84.35 ± 8.61
Metformin 100 μM, n = 6	30.21 ± 10.47	53.01 ± 8.89	78.90 ± 9.16	90.24 ± 9.10

2b. Endothelium-denuded

Endothelium-denuded	Noradrenaline concentration (M)			
	10^{-9}	10^{-8}	10^{-7}	10^{-6}
Control, n = 3	61.56 ± 10.37	83.19 ± 10.02	99.89 ± 11.67	106.39 ± 12.39
Metformin 10 μM, n = 6	33.14 ± 10.46	62.72 ± 6.56	83.55 ± 7.64	91.85 ± 7.18
Metformin 100 μM, n = 6	31.21 ± 8.41	56.44 ± 5.01	80.49 ± 2.64	94.18 ± 1.45

Data are presented as mean ± S.E.

Table 3 The effect of metformin on the percentage of contraction induced by NA and KCl.

1) Endothelium-intact

Contractant	Metformin concentration (μM)				
	Control	1	10	50	100
NE $5x10^{-8}$ M	108.20 ± 2.18 (n=4)	85.93 ± 4.13 * (n=6)	86.14 ± 4.95 * (n=5)	101.5 ± 4.51 (n=4)	90.43 ± 3.31 (n=4)
NE 10^{-6} M	103.50 ± 4.15 (n=7)	99.64 ± 4.09 (n=6)	92.87 ± 4.18 (n=6)	94.35 ± 4.09 (n=7)	95.82 ± 4.63 (n=6)
KCl $4x10^{-2}$ M	108.78 ± 3.18 (n=5)	109.25 ± 3.97 (n=6)	107.59 ± 2.36 (n=8)	93.41 ± 5.46 (n=7)	95.60 ± 7.57 (n=8)

2) Endothelium-denuded

Contractant	Metformin concentration (μM)				
	Control	1	10	50	100
NE $5x10^{-8}$ M	103.74 ± 1.79 (n=4)	86.12 ± 2.17 * (n=8)	73.80 ± 2.08 * (n=7)	97.72 ± 2.39 (n=6)	99.35 ± 3.27 (n=6)
NE 10^{-6} M	107.46 ± 2.94 (n=7)	104.88 ± 3.70 (n=6)	99.96 ± 4.76 (n=11)	109.97 ± 9.03 (n=8)	106.12 ± 9.10 (n=7)
KCl $4x10^{-2}$ M	109.08 ± 8.31 (n=4)	111.04 ± 9.10 (n=6)	103.91 ± 13.65 (n=6)	92.11 ± 11.51 (n=6)	101.56 ± 5.10 (n=6)

Data are presented as mean ± S.E.

* $p<0.05$ show significant difference of metformin-treated group from control group.

Table 4 The percentage of contraction induced by adding cumulatively $CaCl_2$ in endothelium-denuded aortic strips.

$CaCl_2$ concentration (M)	Control (n = 16)	Metformin 10 μM (n = 6)	Metformin 100 μM (n = 6)
10^{-5}	4.65 ± 0.93	3.42 ± 1.24	8.38 ± 0.77
$3x10^{-5}$	11.34 ± 1.69	12.05 ± 3.22	14.91 ± 2.83
10^{-4}	26.79 ± 3.24	33.28 ± 2.84	27.77 ± 4.10
$3x10^{-4}$	47.32 ± 4.11	61.71 ± 5.72	50.90 ± 9.16
10^{-3}	68.12 ± 2.57	80.54 ± 6.71	83.24 ± 8.98
$3x10^{-3}$	84.46 ± 1.95	99.73 ± 7.24 *	119.44 ± 13.06 *
10^{-2}	100	110.65 ± 7.02 *	147.09 ± 16.03 *

Data are presented as mean ± S.E.

* $p<0.05$ show significant difference of metformin -treated group from control group.

Table 5 The percentage of relaxation induced by adding cumulatively isoproterenol in the presence of propranolol 10^{-5} M and metformin 100 μM in endothelium-intact aortic strips.

Isoproterenol concentration (M)	Isoproterenol (n = 16)	Propranolol 10^{-5} M (n = 6)	Metformin 100 μM (n = 6)
10^{-9}	5.65 ± 3.06	0	18.99 ± 5.56 *
10^{-8}	9.89 ± 3.74	0.84 ± 0.62	26.62 ± 5.99 *
10^{-7}	19.84 ± 4.22	3.76 ± 1.80 *	42.41 ± 7.04 *
10^{-6}	45.86 ± 8.16	10.78 ± 4.01 *	86.06 ± 4.95 *
10^{-5}	73.27 ± 8.24	28.53 ± 5.47 *	117.36 ± 6.36 *
10^{-4}	100	71.94 ± 6.10 *	127.80 ± 9.64 *

Data are presented as mean ± S.E.

* $p<0.05$ show significant difference from control group.

Table 6 The percentage of relaxation induced by metformin in endothelium-intact and endothelium–denuded aortic strips.

Mtformin concentration (μM)	Endothelium-intact (n=16)	Endothelium-denuded (n=8)
0.1	0.64 ± 0.32	0
1	2.00 ± 0.66	0.43 ± 0.28
10	3.62 ± 0.93	0.77 ± 0.38
50	6.39 ± 1.27	2.01 ± 0.81
100	8.16 ± 1.59 *	2.66 ± 0.76
200	12.67 ± 1.88 *	3.58 ± 0.87
500	18.05 ± 2.22 *	5.76 ± 1.61 *
1000	25.26 ± 2.45 *	9.63 ± 2.17 *
1500	32.96 ± 2.84 *	14.93 ± 3.07 *

Data are presented as mean ± S.E.

* $p<0.05$ show significant difference from control group.

Table 7 The effect of methylene blue on the percentage of relaxation induced by metformin in endothelium-intact aortic strips.

Metformin concentration (μM)	Control (n=16)	Methylene blue 10^{-5} M (n=6)	Indomethacin 10^{-5} M (n=4)	Propranolol 10^{-5} M (n=4)
0.1	0.64 ± 0.32	0	0	0
1	2.00 ± 0.66	0	0.29 ± 0.29	0.26 ± 0.26
10	3.62 ± 0.93	0	1.16 ± 1.16	1.18 ± 0.74
50	6.39 ± 1.27	0 *	3.12 ± 1.59	2.51 ± 1.10
100	8.16 ± 1.59	0.50 ± 0.22 *	6.89 ± 3.18	3.43 ± 1.61
200	12.67 ± 1.88	1.25 ± 0.31 *	10.80 ± 4.64	5.43 ± 2.32
500	18.05 ± 2.22	2.42 ± 0.44 *	15.67 ± 4.14	8.44 ± 2.73
1000	25.26 ± 2.45	4.75 ± 0.94 *	25.19 ± 6.66	13.35 ± 3.34
1500	32.96 ± 2.84	8.58 ± 1.47 *	36.68 ± 8.93	33.59 ± 6.33

Data are presented as mean ± S.E.

*$p<0.05$ show significant difference of metformin-induced relaxation in the presence of methylene blue in endothelium-intact from control group.

Table 8 The effects of TEA, 4-AP and glibenclamide on the percentage of relaxation induced by metformin in endothelium-intact aortic strips.

Metformin concentration (μM)	Control (n=16)	TEA 10^{-3} M (n=4)	4-AP 10^{-4} M (n=4)	Glibenclamide 10^{-5} M (n=4)
0.1	0.64 ± 0.32	0	0.37 ± 0.23	1.32 ± 0.81
1	2.00 ± 0.66	0.25 ± 0.25	1.39 ± 0.23	2.31 ± 1.43
10	3.62 ± 0.93	0.97 ± 0.59	3.23 ± 0.83	2.98 ± 1.51
50	6.39 ± 1.27	1.69 ± 0.73	6.21 ± 1.96	6.30 ± 1.98
100	8.16 ± 1.59	3.77 ± 0.74	9.84 ± 3.02	8.62 ± 2.72
200	12.67 ± 1.88	5.72 ± 1.26	14.92 ± 3.95	12.69 ± 3.31
500	18.05 ± 2.22	9.95 ± 2.14	21.24 ± 5.03	18.91 ± 4.45
1000	25.26 ± 2.45	16.10 ± 3.67	29.77 ± 7.63	27.01 ± 5.63
1500	32.96 ± 2.84	24.90 ± 5.52	40.19 ± 6.92	36.93 ± 7.05

Data are presented as mean ± S.E.

Table 9 Compound of Physiological solutions (mM/L)

Chemicals	Krebs-Henseleit Solution (KSH)	Ca^{2+}-free KHS	High K^{+}-depolarized
NaCl	119	119	27
KCl	4.7	4.7	100
$CaCl_2$	2.5	-	-
$MgSO_4$	1.0	1.0	-
KH_2PO_4	1.2	1.2	14.0
D-glucose	11.1	11.1	10
EDTA	-	0.1	-
$MgCl_2$	-	-	0.54
$NaHCO_3$	25	25	14

CURRICULUM VITAE

Miss Sarapee Throrarith was born in 1974 in Suratthani, Thailand. She graduated with Bachelor of Science in Pharmacy in 1997 from Faculty of pharmaceutical Sciences, Mahidol University, Thailand. After graduation, she worked as a pharmacist in Suratthani Hospital, Thailand, for 5 years.