ผลของสารสังเคราะห์ CU 18-08, CU 18-10 และ CU 18-11 ต่อการเคลื่อนที่เข้าออกของแคลเซียมในกล้ามเนื้อเรียบของหลอดเลือดแดงใหญ่หนูขาวที่แยกจากกาย

นางสาว ปทุมมาศ พืชพรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECTS OF SYNTHETIC CU 18-08, CU 18-10 AND CU 18-11 COMPOUNDS ON Ca^{2+} MOBILIZATION IN SMOOTH MUSCLE OF ISOLATED RAT AORTA

Miss Patummas Puechprom

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy

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ปทุมมาศ พืชพรม: ผลของสารสังเคราะห์ CU 18-08, CU 18-10 และ CU 18-11 ต่อการเคลื่อนที่เข้าออกของแคลเซียมในกล้ามเนื้อเรียบของหลอดเลือดแดงใหญ่หนูขาวที่แยกจากกาย (EFFECTS OF SYNTHETIC CU 18-08, CU 18-10 AND CU 18-11 COMPOUNDS ON Ca^{2+} MOBILIZATION IN SMOOTH MUSCLE OF ISOLATED RAT AORTA) อ. ที่ปรึกษา: อ.ดร.สุรีย์ เจียรณ์มงคล, อ. ที่ปรึกษาร่วม: รศ.ดร.ประสาน ธรรมอุปกรณ์, 85 หน้า. ISBN 974-03-1172-5.

จากการศึกษาเบื้องต้นทางเภสัชวิทยาการออกฤทธิ์ของสารสังเคราะห์ CU 18-08, CU 18-10 ซึ่งเป็นอนุพันธ์ของ acyl aniline และ CU 18-11 ซึ่งเป็นอนุพันธ์ของ acyl aminopyridine ที่มีต่อการหดตัวของลำไส้เล็กส่วนduodenum ของหนูขาวพบว่าสารดังกล่าวยับยั้งการหดตัวที่เกิดขึ้นเองของลำไส้หนูขาวได้ ในการศึกษานี้ ศึกษาผลของสารสังเคราะห์ทั้ง 3 ตัวต่อแคลเซียมที่เข้าสู่เซลล์กล้ามเนื้อเรียบของหลอดเลือดแดงใหญ่ในหนูขาวหลังจากการถูกทำให้หมดไปโดยnoradrenaline นำหลอดเลือดแดงใหญ่ของหนูขาวพันธุ์ Wistar rats เพศผู้ น้ำหนักประมาณ 250-300 กรัม มาขูดเอาชั้นของ endothelium ออก และแขวนเนื้อเยื่อใน organ bath ที่บรรจุสารละลาย physiological buffer 15 มิลลิลิตร ไล่ที่แคลเซียมภายในเซลล์โดยใช้ noradrenaline 1 μM กระตุ้นการหดตัวของหลอดเลือดในสารละลายที่ปราศจากแคลเซียม หลังจากแคลเซียมภายในเซลล์ถูกไล่ที่ออกหมดแล้ว ล้างเนื้อเยื่อ 3 ครั้งด้วยสารละลายที่ปราศจากแคลเซียม หลังจากนั้นเมื่อเติมแคลเซียมเข้าไปจะพบว่าหลอดเลือดเกิดการหดตัวขึ้น (Resting tone, RT) ปรากฏการณ์นี้ถูกยับยั้งได้โดย prazosin (1 μM) ซึ่งเป็น α-adrenoceptor antagonist และ nifedipine (1 μM) ซึ่งเป็น Ca^{2+} entry blocker จากการศึกษาได้ผลว่า CU 18-08 (10 μM) and CU 18-10 (10 μM) ยับยั้งการหดตัวของหลอดเลือด อย่างมีนัยสำคัญทางสถิติ ในขณะที่ CU 18-11 ไม่มีผล แสดงว่า CU 18-08 และ CU 18-10 ออกฤทธิ์รบกวนกลไกการนำแคลเซียมเข้าสู่เซลล์ ทั้งนี้อาจเป็นไปได้ว่ากลไกการออกฤทธิ์ของสาร CU 18-08 และ CU 18-10 อาจเกี่ยวข้องกับ voltage-operated Ca^{2+}channels หรือ α-adrenoceptor ผลการศึกษาการหดตัวของหลอดเลือดโดยใช้ KCl 40 mM, TEA 1 mM และ แคลเซียมคลอไรด์แบบสะสมขนาดเป็นตัวกระตุ้น พบว่า CU 18-08 และ CU 18-10 ยับยั้งการหดตัวเมื่อกระตุ้นด้วย KCl 40 mM และ แคลเซียมคลอไรด์แบบสะสมขนาด ส่วน CU 18-10 ยับยั้งการหดตัวของหลอดเลือดเมื่อกระตุ้นด้วย TEA 1 mM ดังนั้น CU 18-08 และ CU 18-10 ออกฤทธิ์ผ่านการเคลื่อนที่ของแคลเซียมทาง voltage-operated Ca^{2+}channels นอกจากนี้ CU 18-11 มีฤทธิ์เพิ่มการหดตัวเมื่อกระตุ้นด้วย noradrenaline ซึ่ง CU 18-11 อาจมีฤทธิ์เพิ่มการเข้าเซลล์ของแคลเซียมผ่านทาง voltage-operated Ca^{2+}channels หรือ α-adrenoceptors

ภาควิชา	เภสัชวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2544	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4376588733 MAJOR: PHARMACOLOGY
KEY WORD: RAT AORTA/ INCREASE IN THE RESTING TONE/ CU 18-08/ CU 18-10/ CU 18-11

PATUMMAS PUECHPROM: EFFECTS OF SYNTHETIC CU 18-08, CU 18-10 AND CU 18-11 COMPOUNDS ON Ca^{2+} MOBILIZATION IN SMOOTH MUSCLE OF ISOLATED RAT AORTA. THESIS ADVISOR: SUREE JIANMONGKOL, THESIS COADVISOR: ASSOC. PROF. PRASAN DHUMMA-UPAKORN, 85 pp. ISBN 974-03-1172-5.

It has been shown that CU 18-08, CU 18-10, acyl aniline derivatives as well as CU 18-11, an acyl aminopyridine derivative suppressed the spontaneous contraction of rat duodenum. This study was to investigate the effect of these three synthetic compounds on the Ca^{2+} entry into aortic smooth muscle cells after noradrenaline-induced depletion of intracellular Ca^{2+}. Thoracic aortic strips were isolated from male Wistar rats (250-300g), denuded the endothelium layer, and suspended in a 15 ml-organ bath containing physiological solution. In order to deplete intracellular Ca^{2+}, noradrenaline (1 μM) was added to stimulate the aortic contraction in the Ca^{2+}-free medium. After the intracellular Ca^{2+} was completely depleted, the aortic strips were washed 3 times with Ca^{2+}-free medium. Upon addition of Ca^{2+}, the spontaneous contraction or resting tone (RT) of rat aortic strip was observed. This process was inhibited by prazosin (1 μM) and nifedipine (1 μM). Our results showed that CU 18-08 (10 μM) and CU 18-10 (10 μM) significantly inhibited the RT while CU 18-11 did not. These findings suggested that CU 18-08 and CU 18-10 interfered the mechanical Ca^{2+} refilling process into intracellular stores. It is possible that these two compounds may have pharmacological blocking effect on voltage-operated Ca^{2+}channels or Ca^{2+} entry due to α-adrenoceptor activation. CU 18-08 and CU 18-10 significantly inhibited contraction induced by KCl 40 mM and suppressed the cumulative dose response curve induced by $CaCl_2$ in Ca^{2+}-free depolarizing solution. CU 18-10 significantly inhibited contraction induced by TEA 1 mM. These findings suggest that CU 18-08 and CU 18-10 may interfere the entry of Ca^{2+} into intracellular space. In contrast, CU 18-11 potentiated the effect of noradrenaline in activation of contraction response. It is possible that CU 18-11 may increase Ca^{2+} influx through voltage-operated Ca^{2+} channels or α-adrenoceptors.

Department	Pharmacology	Student's signature.....
Field of study	Pharmacology	Advisor's signature.....
Academic year	2001	Co-advisor's signature.....

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LIST OF ABBREVIATIONS

Ca^{2+}	calcium ion
AC	adenylate cyclase
GTP	guanosine 5'-triphosphate
PLC	phospholipase C
IP_3	inositol 1,4,5-trisphosphate
DAG	diacylglycerol
ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
SR	sarcoplasmic reticulum
MLC	myosin light chain
MLCK	myosin light chain kinase
NA	noradrenaline
IRT	increase in the resting tone
CRAC	Ca^{2+} release-activated Ca^{2+} channel
CICR	Ca^{2+}-induced Ca^{2+} release
IICR	IP_3-induced Ca^{2+} release
CaMKII	Ca^{2+}-calmodulin-dependent protein kinase II
CaM	calmodulin
PPase	phosphatase
TEA	tetraethylammonium
K^+	potassium ion
KCl	potassium chloride
M	molar
mM	millimolar
ROC	receptor-operated Ca^{2+} channel
VOC	voltage-operated Ca^{2+} channel

CHAPTER I

BACKGROUND AND INTRODUCTION

Nowadays, cardiovascular disease has been recognized as one of the major causes of morbidity and mortality in many countries. Its prevalence is remarkably rising in the last decade (Koch-Weser and Vanhoutte, 1992). People in the countries of the South Asias, comprising more than one fifth of the global population, are highly susceptible to cardiovascular diseases (Whincup *et al*., 2002). Hence, in the foreseeable future, treatment of cardiovascular disease will be a major medical issue. Therapy for cardiovascular disease is aimed at relieving symptoms; reducing myocardial ischemia; improving left ventricular function; preventing progression of ventricular arrhythmia, acute myocardial necrosis, and coronary atherogenesis; and improving survival rates.

Other than the driving force of the heart, vascular smooth muscle plays an important role in regulating blood pressure. The changes in the contraction-relaxation cycle of smooth muscle tissues influence the myogenic resistance vascular smooth muscle tone (Kuriyama, Kitamura and Nabata, 1995).

CU 18-08, CU 18-10 and CU 18-11, which are acyl aniline derivatives and acyl aminopyridine derivatives (Thong and Boonwan, 2000) have been tested and found for its pharmacological actions. The preliminary data showed that these compounds could inhibit the contraction of smooth muscle of rabbit duodenum. It is possible that these three compounds may have pharmacological effects on vascular smooth muscle cells and alter the vascular tone, which may be useful for development of new cardiovascular agents. In addition, these compounds may be useful in cardiovascular research to study the regulation and function of vascular smooth muscle, which may be important in the pathophysiology of cardiovascular system.

Literature reviews

Control of vascular smooth muscle tone

Contraction of smooth muscle is regulated by intracellular Ca^{2+} concentration and the sensitivity of the contractile elements to an increase of Ca^{2+}. Like other muscle cells, vascular smooth muscle contracts when the intracellular Ca^{2+} rises. However, in vascular tissues the coupling between intracellular Ca^{2+} and contraction is less tight than the coupling in striated or cardiac muscles. Vasoconstrictors and vasodilators can affect the vascular tone by altering the level of intracellular Ca^{2+}, or the sensitivity of the contractile machinery to intracellular Ca^{2+} (Rang, Dale and Ritter, 1999).

Regulation of intracellular Ca^{2+}

The level of intracellular Ca^{2+} in vascular smooth muscle depends on the entry and removal of Ca^{2+} across the plasma membrane and on the sequestration of Ca^{2+} within the cell (Figure 1). Calcium enters the cell through voltage-operated Ca^{2+} channels and partly through receptor-operated channels.

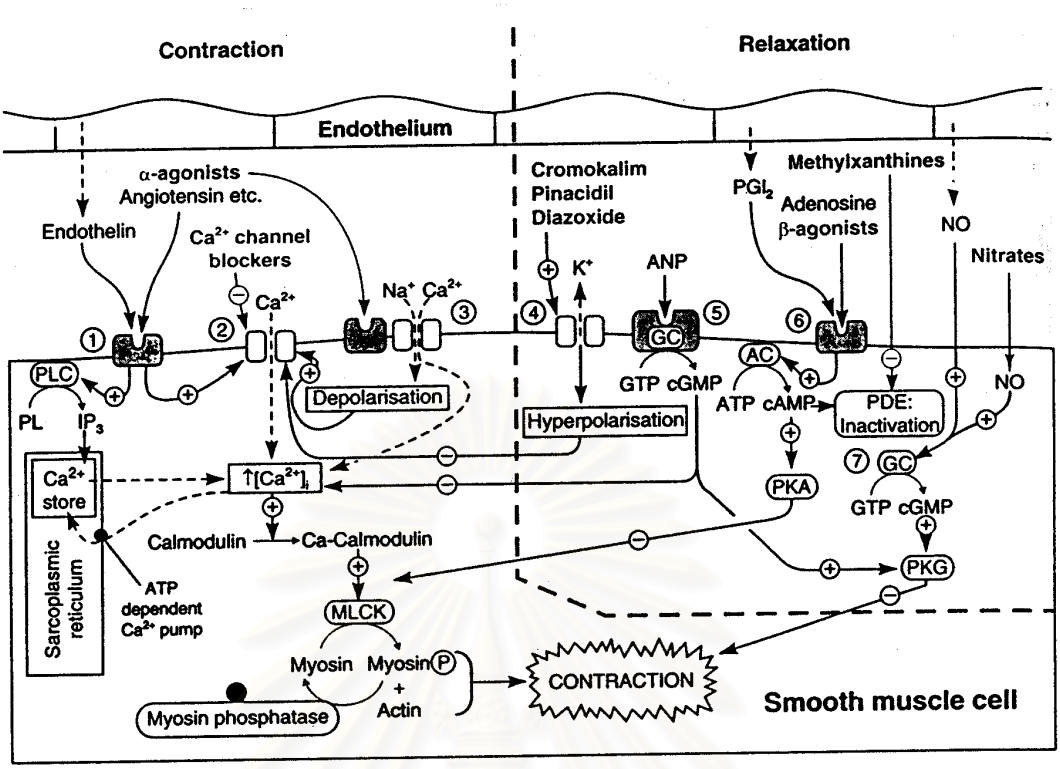


Figure 1 Contraction and relaxation of vascular smooth muscle (Rang *et al*, 1999). In contraction state, an increase of intracellular Ca^{2+} can be due to (1) Receptors coupled to phospholipase C (PLC) like, (2) Voltage-gated Ca^{2+} channels which open in response to depolarization. (3) Receptor-operated channels which allow Ca^{2+} entry and also cause depolarization. In relaxation state, a decrease of intracellular Ca^{2+} or a change in Ca^{2+} sensitization may also occur through contractile machinery. (4) K^{+} channels (sensitive to intracellular ATP) preventing voltage-gated Ca^{2+} channels from opening. (5) A receptor coupled to membrane-bound guanylate cyclase. (6) Receptors coupled to adenylate cyclase which links to PKA and MLCK. (7) Soluble guanylate cyclase stimulated by NO. (Enzymes: AC = adenylate cyclase; GC = guanylate cyclase; MLCK = myosin-light-chain kinase; PKA = cAMP-dependent protein kinase; PKG = cGMP-dependent protein kinase; + = stimulation; - = inhibition).

The voltage-operated Ca^{2+} channels have six subtypes: L-, N-, P-, Q-, R-, and T-type. In smooth muscle, only the L-type Ca^{2+} channel is considered to be a major Ca^{2+} influx pathway (Kuriyama *et al*., 1995). The voltage-operated Ca^{2+} channels open when the cell is depolarized. In addition, its opening can be under influence of second messengers produced in response to receptor activation. Several vasoconstrictors trigger the contraction via both membrane depolarization and receptor-operated channel pathways. For example, noradrenaline cause membrane depolarization by increasing the permeability to cations such as Na^{+} and Ca^{2+}, resulting in an opening of voltage-operated Ca^{2+} channels. Noradrenaline also binds to α_1-adrenoceptor activating the secondary messenger system linked to the Ca^{2+} channels, resulting in an opening of Ca^{2+} channels. Consequently, Ca^{2+} entry into the cells increase and the muscle contracts.

The receptor-operated channels function independently from the voltage-operated Ca^{2+} channels. The activation of the receptor-operated channels has been shown to occur in the fully-depolarization aortic strips suspended in isotonic potassium solution (Rang *et al*., 1999). Moreover, it is unaffected by dihydropyridine-type calcium antagonists which block voltage-operated Ca^{2+} channels (Rang *et al*., 1999). After the contraction, excess intracellular calcium can be removed from the cell via mechamism mediated by Na^{+}/ Ca^{2+} -ATPase and by Na^{+}/ Ca^{2+} exchanger.

The subtypes of α_1-adrenoceptors present in the rat thoracic aorta have been the subjects of extensive research (Hieble *et al*., 1995). It was demonstrated that in rat aorta, the functional activity of BMY 7378 which is a selective α_{1D}-antagonist correlates well with binding affinities for cloned α_{1D}-adrenoceptors (Hussain and Marshall, 1997).

Tetraethylammonium (TEA) was the first and is still the most frequently used K^{+}-channel blocker (Cook and Quast, 1990). TEA inactivates K^{+} channel nonselectivity, albeit with varying affinities, and cause membrane depolarized. This agent not only acts on the delayed rectifying K^{+} channel but also on the Ca^{2+}-sensitive and voltage-dependent K^{+} channel (Benham *et al*., 1985a, b). Therefore, this agent is often used

instead of K^+ in the patch-pipette to prevent K^+ permeability. In addition, TEA is a less permeable substrate because of its quaternary ammonium; however, it is a good tool when applied to intracellular media for the assessment of K^+ channel specificity.

Sequestration of intracellular Ca^{2+}

Intracellular Ca^{2+} in vascular smooth muscle is contained mainly in sarcoplasmic reticulum (Figure 1), which is the main storage site for releasable Ca^{2+}. Several vasoconstrictors activate a release of Ca^{2+} from sarcoplasmic reticulum via mechanism mediated through phospholipase C (PLC) and inositol 1,4,5-trisphosphate (IP_3) systems. IP_3 bind to receptors on the sarcoplasmic reticulum and activate a release of Ca^{2+} into the cytoplasm. Resequestration of the released Ca^{2+} is mediated by an ATP-driven active transport system, which is modulated by cAMP and cGMP.

The link between intracellular Ca^{2+} and contraction

Smooth muscle differs from striated and cardiac muscle in that it does not contain troponin. In smooth muscle, Ca^{2+}-calmodulin regulates myosin-light-chain kinase (MLCK), which phosphorylates myosin light chains; enabling myosin to interact with actin and thereby initiates contraction. Relaxation is usually initiated by a fall in intracellular Ca^{2+}, which leads to dephosphorylation of the myosin light chain via myosin phosphatase. The coupling between intracellular Ca^{2+} and contraction in smooth muscle allows for contraction to be regulated by mechanisms that increase or reduce the sensitivity of the contractile apparatus to intracellular Ca^{2+}. Decreased activity of myosin phosphatase or increased activity of myosin-light-chain kinase causes Ca^{2+} sensitization, while increased activity of myosin phosphatase or decreased activity of myosin-light-chain kinase causes Ca^{2+} desensitization.

Calcium movements in smooth muscle

Calcium movements in smooth muscle shown in Figure 2, the effects of high K^+ are to depolarize the membrane, resulting in an opening of the L-type Ca^{2+} channels, and an increase of intracellular Ca^{2+} (Karaki *et al*., 1997). Because the Ca^{2+} channel blockers inhibit the L-type Ca^{2+} channel and high K^+ does not increase the Ca^{2+} sensitivity of contractile elements, high K^+-induced contraction is inhibited by the Ca^{2+} channel blockers in corresponding to a decrease in intracellular Ca^{2+}.

In contrast, the effects of agonists such as noradrenaline are far more complicated than that was predicted from contraction data. Agonists activate five different mechanisms. The first mechanism is to release Ca^{2+} from the sarcoplasmic reticulum to induce initial transient contractions. The second mechanism is to open the L-type Ca^{2+} channel through the activation of GTP-binding protein, but not through membrane depolarization. The third mechanism is to open the nonselective cation channel. Since this channel is permeable not only to monovalent cations but also to Ca^{2+}, opening of this channel results in an increase in Ca^{2+} influx. This may be the mechanism of the previously suggested receptor-linked Ca^{2+} channel (Karaki *et al*., 1997). In addition, since opening of the nonselective cation channel depolarizes the membrane, the L-type Ca^{2+} channel is activated to further increase Ca^{2+} influx. The fourth mechanism is to activate of the non- L-type Ca^{2+} entry resulting from release of sarcoplasmic reticulum Ca^{2+} (CRAC). The last mechanism is to increase Ca^{2+} sensitivity of contractile elements which increases contractile force at a given intracellular Ca^{2+}.

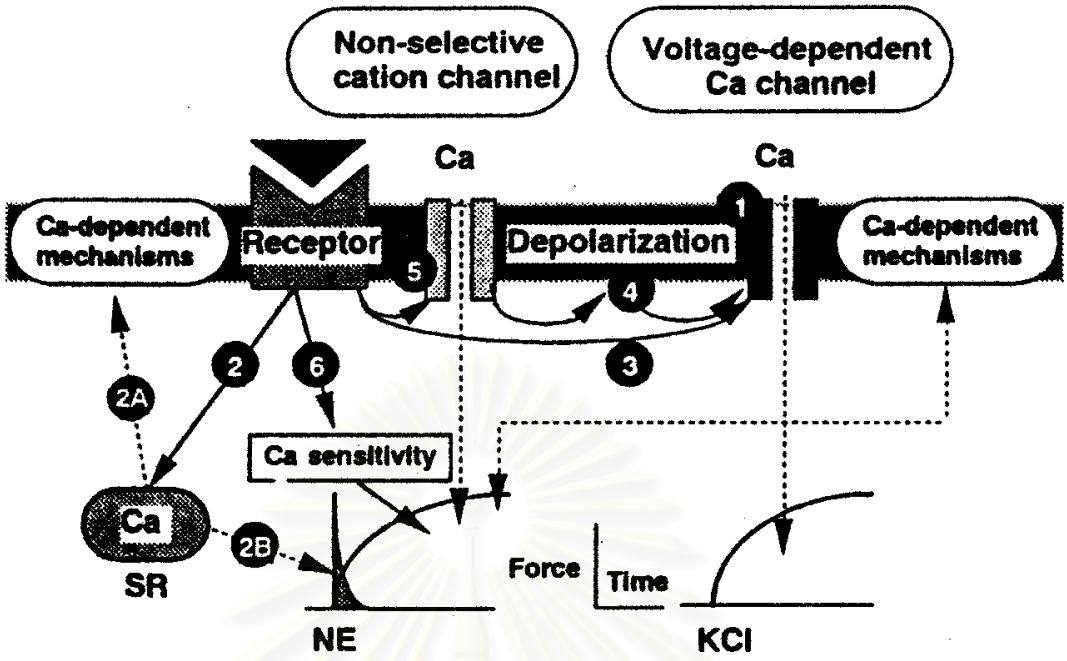


Figure 2 Calcium movements and muscle contraction (Karaki *et al.*, 1997). (1) The voltage-dependent Ca^{2+} channel increases Ca^{2+} influx, and elicits sustained contraction. (2A) Noradrenaline triggers Ca^{2+} release from the sarcoplamic reticulum (SR) toward the subplasmalemmal Ca^{2+} space (noncontractile comparment) to regulate membrane Ca^{2+}-dependent mechanisms and (2B) also toward the cytoplasm, where contractile proteins reside. (3) Noradrenaline also increases intracellular Ca^{2+} by opening the L-type Ca^{2+} channel directly or (4) indirectly through membrane depolarization induced by opening of nonselective cation channel, inhibition of K^{+} channel, or opening of Cl^{-} channels. (5) Nonselective cation channels are also permeable to Ca^{2+}. (6) In addition receptor activation may increase Ca^{2+} sensitivity of contractile elements.

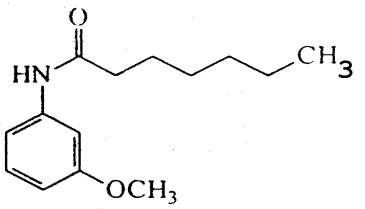
Vascular tone of the isolated rat aorta

In an experiment with isolated rat aorta, noradrenaline-induced contraction in smooth muscle has been characterized into two phases: the phasic and tonic phases (Figure 2). The initial phasic contraction is triggered by a rising of Ca^{2+} released from intracellular storage via phosphatidyl-inositol 1,4,5-trisphosphate (IP_3) pathway. The tonic contraction subsequently maintained by an influx of extracellular Ca^{2+} (Bolton, 1979; Bray *et al*., 1991). Noradrenaline through activation of α_1-adrenoceptors induces an IP accumulation, which releases Ca^{2+} from internal stores. The internal stores are further depleted by successive additions of noradrenaline in a Ca^{2+} -free medium (Graham *et al*., 1996). When emptied, the stores can be rapidly replenished by Ca^{2+} influx during the incubation in Ca^{2+} -containing solution in the absence of the agonist. This process manifests itself not only by the recovery of the response to noradrenaline in Ca^{2+}-free medium but also by the increase in the resting tone (Noguera *et al*., 1998).

Increase in the resting tone is closely related to α_1-adrenoceptors and not just to the emptying of intracellular Ca^{2+} pools is demonstrated by the fact that depletion of internal Ca^{2+} stores by methoxamine and phenylephrine also elicits an increase in the resting tone. Whereas clonidine, 5-hydroxytryptamine, caffeine, ryanodine, thapsigargine, and cyclopiazonic acid, which also depleted internal Ca^{2+} stores, did not elicit any increase in the resting tone (Noguera *et al*., 1998).

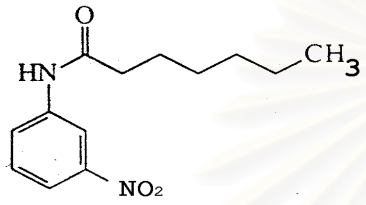
Treatment with α-adrenoceptor antagonist inhibited an increase in the resting tone. This indicates that α-adrenoceptor is involved in the increase in resting tone. The inhibition of this increase in tension by nifedipine seems to relate this mechanical response to Ca^{2+}-entry via voltage-operated Ca^{2+} channels. The great sensitivity of the increase in resting tone to nifedipine suggests that a change in membrane potential might be involved. The fact that the Ca^{2+} channel blocker, nifedipine also block the increase in the resting tone corroborates the involvement of a voltage-operated Ca^{2+} channel in this process (Noguera and D'Ocon, 1993).

The synthetic CU 18-08, CU 18-10 and CU 18-11 compounds



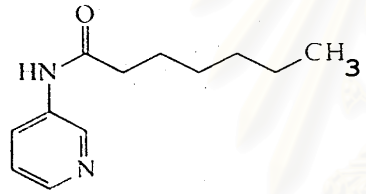
CU 18-08

N-heptanoyl-3-methoxyaniline, MW=235



CU 18-10

N-heptanoyl-3-nitroaniline, MW=250

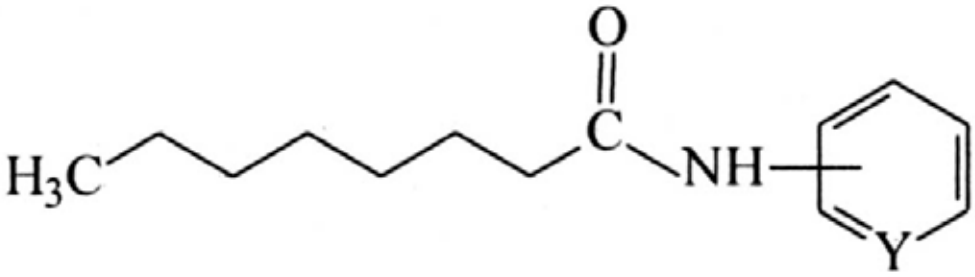


CU 18-11

N-heptanoyl-3-aminopyridine, MW=206

Figure 3 Structures of CU 18-08, CU 18-10 and CU 18-11 (Thong and Boonwan, 2000)

In 2000, Thong and Boonwan synthesized several acyl aminopyridine derivatives and acyl aniline derivatives including CU 18-08, CU 18-10 and CU 18-11. They found that these compounds inhibited the contraction of smooth muscle of rabbit duodenum. The compounds has been modified Alkyl (R), substitute group on benzene ring and type of ring (Y) the structure of 3,3-dimethylbutyryl aminopyridine derivatives to increase relaxation effects and to decrease mitochondria toxicity.



code	Y	Position of substitutions	Yeild (%)	Mp (^{o}C)
CU 18-08	C-OCH_3	Meta	98.62	-
CU 18-10	C-NO_2	Meta	100.0	-
CU 18-11	N	Meta	86.5	-

Because the contraction of vascular smooth muscle is dependent upon Ca^{2+} entry, this research is to investigate the pharmacological effect of CU 18-08, CU 18-10 and CU 18-11 on the Ca^{2+} entry into the vascular smooth muscle cell. This research emphasized on the mechanism of Ca^{2+} entry that is responsible for generating an increasing in the vascular resting tone during the refilling of Ca^{2+} pools. It is possible that the control of resting tone may be related to voltage-operated Ca^{2+}channels or Ca^{2+} entry due to α-adrenoceptor activation. These three compounds may affect Ca^{2+} movement into the vascular smooth muscle cells and alter the vascular tone.

Hypothesis

CU 18-08, CU 18-10 and CU 18-11 may alter the vascular smooth muscle resting tone by interfering the Ca^{2+} entry through the voltage-operated Ca^{2+}channels or on the Ca^{2+} entry due to α-adrenoceptor activation.

Specific aims

1. To investigate the mechanism by which these three synthetic compounds affect the Ca^{2+} influx on the resting tone of isolated aortic strip after noradrenaline-induced depletion of intracellular Ca^{2+}. It is possible that these three synthetic compounds may interfere Ca^{2+} entry at the voltage-operated Ca^{2+} channels or the Ca^{2+} entry mediated by α-adrenoceptor activation.

2. To investigate the effect of these three synthetic compounds on the Ca^{2+} entry mediated by α-adrenoceptor activation

3. To investigate the effect of these three synthetic compounds on depolarization of the cell membrane and on the delayed rectifying K^+ channel and on the Ca^{2+}-sensitive and voltage-operated K^+ channel

4. To investigate the effect of these three synthetic compounds on the entry of Ca^{2+} from the extracellular to intracellular pool upon depolarization stimulated by $CaCl_2$.

Experimental design

All of the experiments in this research project were done in *in vitro* isolated thoracic aorta, suspending in a 15 ml organ bath containing physiological solution. The contraction of vascular smooth muscle will be measured isometrically on a polygraph via force-displacement transducers. In order to determine the resting tone of isolated aorta, noradrenaline (1 μM) was added to stimulate the aortic contraction in the Ca^{2+}-free medium to deplete intracellular Ca^{2+}. Subsequently Ca^{2+} was added into the medium to generate the spontaneous contraction or resting tone of aortic strip.

Significance

1. This study will provide new pharmacological knowledge on the effects of CU 18-08, CU 18-10, and CU 18-11 and the mechanism of action on the contraction of vascular smooth muscle.

2. The information from this study will be useful for the new drug development especially for the cardiovascular agents.

CHAPTER II

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 and acclimatized for 1-2 week before the experimentation.

Chemicals

1. Reference compounds

Dimethyl sulfoxide (DMSO):	Solvent
Acetylcholine	
Noradrenaline:	α-adrenoceptor agonist
Nifedipine:	Ca^{2+} channel blocker
Prazosin:	α-adrenoceptor antagonist
Tetraethylammonium (TEA):	K^{+}-channel blocker
Potassium chloride (KCl):	membrane depolarization
Caffeine	

All were purchased from Sigma St. Louis MO, U.S.A.

2. Testing compounds

CU 18-08	MW = 235
CU 18-10	MW = 250
CU 18-11	MW = 206

All were synthesized by Assistant Professor Chamnan Patarapanich and colleagues at the department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

3. Other chemicals

NaCl, KCl, $CaCl_2$, $MgCl_2$, KH_2PO_4, $NaHCO_3$, glucose and EDTA.

All were purchased from APS Finechem, Australia.

Experimental instruments

1. Double-walled organ bath (Figure 4). The organ bath made of glass comprises an inner and outer chamber. An inner chamber with the 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-controlled water that is responsible for maintains the right temperature of the inner chamber.

2. Water bath and thermoregulation water pump

3. Isometric tranducer 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)

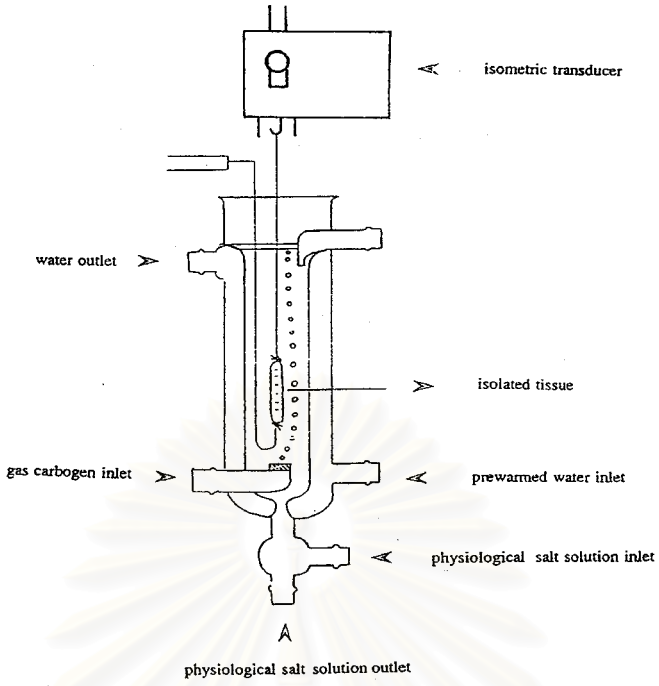


Figure 4 Illustration of instrument and organ bath for isolated rat aorta

Experimental methods

- **Preparation of isolated rat aorta**

1. Male Wistar rats (250-300 g) were sacrificed by sedating with ether and isolating the thoracic aorta out of the body. Then, the thoracic aorta were cleaned and helically cut into a strip (Figure 5).

2. The endothelium-denuded aortic strip was prepared for the experiment 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 (100 μM) to the strips pretreated with noradrenaline (1μM).

3. Each aortic strip was suspended 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 a 75-90 min equilibration period.

5. This tension was maintained constantly. However throughout the experiment a loss of tension (less than 10-15%) when the preparation was placed in Ca^{2+} -free medium.

6. Tension was recorded isometrically on a polygraph via force-displacement transducers. (Furchgott and Zawadzki, 1980)

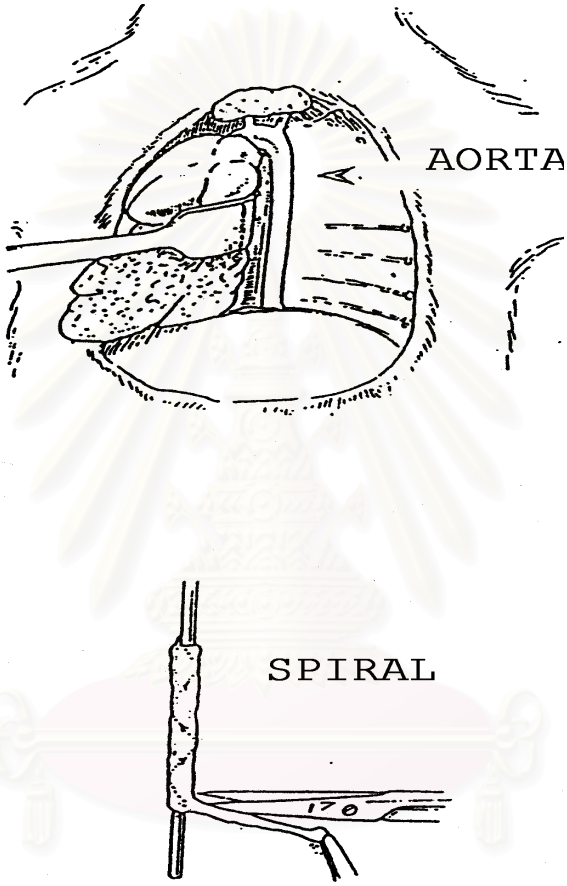


Figure 5 Thoracic aorta and preparing of isolated rat aorta

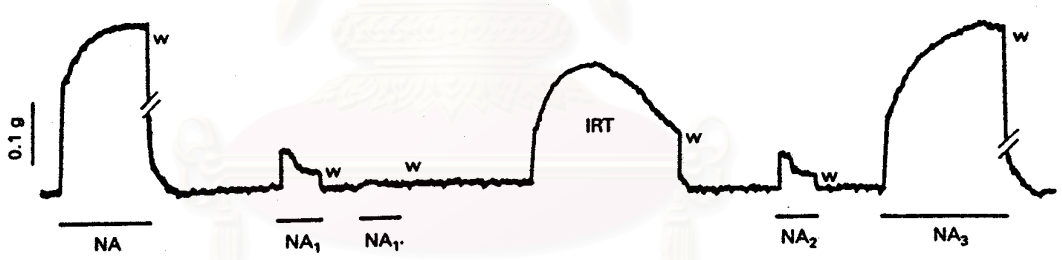
- **Experimental procedures**

1. **Determination of aortic contraction and increase in the resting tone of rat aorta (IRT)** (Noguera, Ivorra and D'Ocon, 1996)

The experimental procedure is designed to study the depletion of intracellular Ca^{2+} -stores sensitive to noradrenaline, caffeine or KCl in Ca^{2+} -free medium, and IRT

obtained by subsequent exposure to Ca^{2+} -containing solution during the refilling of these stores (Figure 6). Noradrenaline, caffeine or KCl was added in Ca^{2+} -containing solution at 37°C and then the tissue was treated with Ca^{2+} -free, EDTA- containing solution for 15 min. After this time, noradrenaline, caffeine or KCl was applied and washed until no contraction was induced, indicating complete depletion of internal Ca^{2+} -stores sensitive to the agonist. The tissue was incubated for 20 min in Ca^{2+} -containing solution to refill the intracellular Ca^{2+} -stores and a spontaneous increase in the resting tone of the aorta (IRT) would be observed. After washing and 15 min of loading in Ca^{2+} -free medium, a new addition of noradrenaline, caffeine or KCl would be made (NA_2).

At the end of the experiment, the aortic strips were tested its functionality by adding noradrenaline, caffeine or KCl to the tissue suspended in Ca^{2+}-containing solution. The contraction response in these conditions returned to the maximum level, which was comparable to those obtained at the beginning of experiment.



Ca (+)	Ca (-)	Ca (+)	Ca (-)	Ca (+)

Figure 6 The experimental procedure for IRT determination (Noguera *et al.*, 1996)

2. Effects of tested compounds (CU 18-08, CU 18-10 and CU 18-11) on aortic contraction and on the increase in the resting tone of rat aorta

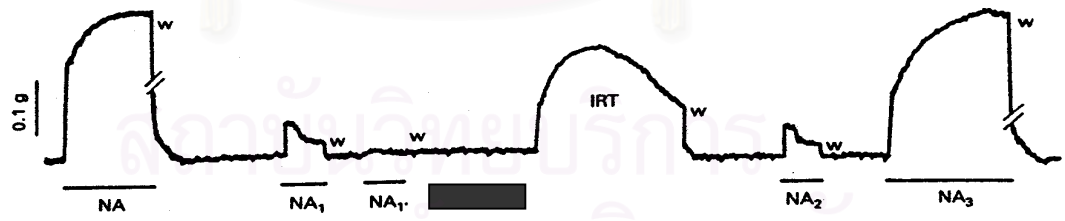
2.1 Effects on aortic contraction in Ca^{2+}-containing physiological solution. The aortic tissue is incubated in Ca^{2+}-containing solution until the tension is stable. Then noradrenaline is added to induce contraction. The tension is recorded for 20 minute prior to washing tissue with Ca^{2+}-containing solution 3 times. Incubated the tissue 60 minute until the tension is stabled similar to those observed before trial. The effects of DMSO, prazosin, nifedipine, CU 18-08, CU 18-10 and CU 18-11 on noradrenaline-induced contraction in Ca^{2+}-containing solution (NA) were studied by pretreated with DMSO 0.03% (v/v), prazosin $1x10^{-6}$ M, nifedipine $1x10^{-6}$ M, CU 18-08 $1x10^{-5}$ M, CU 18-10 $1x10^{-5}$ M and CU 18-11 $1x10^{-5}$ M 5 minute and then noradrenaline is added to induce contraction. Comparing the contraction of vascular smooth muscle between before and after adding testing agent in Ca^{2+}-containing solution (NA).

The aortic strip is prepared and incubated in Ca^{2+}-containing solution until the tension is stable. Then KCl or TEA is added to induce contraction. The tension is recorded for 20 minute prior to washing the tissue with Ca^{2+}-containing solution 3 times. The tissue is incubated 60 minutes with Ca^{2+}-containing solution until the tension is stabled similar to before trial. The effects of CU 18-08, CU 18-10 and CU 18-11 on KCl or TEA induced contraction in physiological solution were studied by pretreated with CU 18-08 $1x10^{-5}$ M, CU 18-10 $1x10^{-5}$ M and CU 18-11 $1x10^{-5}$ M 5 minute and then KCl or TEA is added to induce contraction. Comparing the contraction of vascular smooth muscle between before and after adding testing agent in Ca^{2+}-containing solution.

2.2 Effects on aortic contraction in Ca^{2+}-free medium. The aortic strip is prepared and incubated in Ca^{2+}-containing solution until the tension is stable. Then change the solution to Ca^{2+}-free medium and the tissue is incubated until the tension is stable and noradrenaline is added to induce contraction. The tension is recorded for 20 minute prior to washing tissue with Ca^{2+}- free medium 3 times. The tissue is incubated 60 minutes with Ca^{2+}-containing solution until the tension is stabled similar to those

observed before trial. Change solution to Ca^{2+}-free medium and the tissue is incubated until the tension is stable. The effects of DMSO, prazosin, nifedipine, CU 18-08, CU 18-10 and CU 18-11 on noradrenaline-induced contraction in Ca^{2+}-free medium (NA_1) were studied by pretreated with DMSO 0.03% (v/v), prazosin $1x10^{-6}$ M, nifedipine $1x10^{-6}$ M, CU 18-08 $1x10^{-5}$ M, CU 18-10 $1x10^{-5}$ M and CU 18-11 $1x10^{-5}$ M 5 minute and then noradrenaline is added to induce contraction. Comparing the contraction of vascular smooth muscle between before and after adding testing agent in Ca^{2+}-free medium (NA_1).

2.3 Effects on IRT. To test the effects of DMSO, prazosin, nifedipine, CU 18-08, CU 18-10 and CU 18-11 on an increase in the resting tone (IRT), the experimental procedure will be similar to that demonstrated in Figure 6, but the compound will be added into the Ca^{2+}-free medium 5 min (Figure 7). Then, the medium is changed to Ca^{2+}-containing solution and measures the IRT that permits the refilling of internal Ca^{2+} -stores previously depleted by noradrenaline. The magnitude of noradrenaline induces contraction in Ca^{2+}-containing solution and in Ca^{2+} -free medium in the presence of each compound were expressed as a percentage of the reference contraction. The magnitude of the IRT in the presence of each compound is expressed as a percentage of noradrenaline-induced contraction in Ca^{2+}-containing solution.



Ca (+)	Ca (-)	Ca (+)	Ca (-)	Ca (+)

Figure 7 The experimental procedure designed to assess the effect of agents on IRT.

3. Effects of tested compounds (CU 18-08, CU 18-10 and CU 18-11) on contractions induced by adding calcium to a calcium-free depolarizing solution.

The method described by Hof and Vuorela (1983) was used to determine the effects of CU 18-08, CU 18-10 and CU 18-11 on $CaCl_2$-induced contraction in high K^+-Ca^{2+} free solution.

The aortic strip is prepared and incubated in Ca^{2+}-containing solution until the tension is stable. Change the solution to Ca^{2+}-free depolarizing solution and incubates until the tension is stable. Then $CaCl_2$-cumulative dose ($1x10^{-5}$ M - $1x10^{-2}$ M) is added to induce contraction until the maximum contraction and then tissue is washed with Ca^{2+}-containing solution 3 times. The tissue is incubated 60 minutes with Ca^{2+}-containing solution until the tension is stabled similar to those observed before trial. Changed the solution to Ca^{2+}-free depolarizing and incubate the tissue until the tension is stable. The effect of nifedipine, CU 18-08, CU 18-10 and CU 18-11 on $CaCl_2$ induces contraction in Ca^{2+}-free depolarizing solution were studied by pretreated with nifedipine $1x10^{-6}$ M, CU 18-08 $1x10^{-5}$ M, CU 18-10 $1x10^{-5}$ M and CU 18-11 $1x10^{-5}$ M 5 minute and then $CaCl_2$ is added cumulatively to induce contraction. Comparing the contraction of vascular smooth muscle between before and after adding testing agent in Ca^{2+}-free depolarizing solution.

Statistical Analysis

Contractions in physiological solution were expressed in mg of developed tension and when elicited in Ca^{2+} -free medium, as a percentage of the noradrenaline-induced contraction obtained in normal physiological solution. Increases in the resting tone were also expressed as a percentage of the noradrenaline -induced contractions in normal physiological solution.

pA_2 value is calculated according to Van Rossum (1983).

Results are presented as the mean ± S.E. mean for N determinations obtained from different animals. (N = 6 separated experiments). Statistical significance was evaluated by Student's *t test* for paired or unpaired data. Differences were considered significant when *p* value is less than 0.05.

CHAPTER III

RESULTS

1. Profiles of aortic contraction and increase in the resting tone of rat aorta

1.1 Aortic contraction in Ca^{2+}-containing physiological solution. As shown in Table 1, the maximum contractile response of rat aortic tissues evoked by noradrenaline1 μM was 373.3 ± 51 mg (n =6). This response consisted of two phases, phasic and tonic (NA in Figure 8A). Similar results were obtained when 40 mM KCl was used instead of noradrenaline (Figure 8C). However, the magnitude of contraction was approximately 1.5 fold smaller than that elicited by noradrenaline. Caffeine 10 mM also generated aortic contraction but the patterns of contraction were markedly different from those stimulated by noradrenaline and KCl. The caffeine-induced contraction profile consisted of a rapid transient contraction (Caf) with the magnitude of 46.10 ± 6.10 mg, n = 6 (Table 1) and then muscle tension decreased to a level below a resting tone (Figure 8B).

1.2 Aortic contraction in Ca^{2+}-free medium. As shown in Figure 8A, addition of 1 μM noradrenaline to aortic strip suspended in Ca^{2+}-free medium also induced a biphasic contraction (NA_1) like that observed in experiment with Ca^{2+}-containing solution. However, the magnitude of contraction was only 37.88 ± 3.43 % of the maximum response (n =6). Further application of 1 μM noradrenaline could not evoke a contraction (NA_1') (Figure 8A). In contrast to the noradrenaline-experiment, 40 mM KCl produces a diminishing contraction in Ca^{2+}-free medium.

In Ca^{2+}-free medium caffeine could induce a transient contraction of aortic tissue (Caf_1) (Figure 8B). The amplitude of the contractions was 41.15 ± 1.83 % (n =6) of those observed in Ca^{2+}-containing physiological solution. Furthermore, a second treatment of caffeine in this Ca^{2+}-free medium could not evoke an observable contraction.

1.3 An increase in the resting tone (IRT). As shown in Figure 8A, upon changing the physiological solution from Ca^{2+}-free medium to a Ca^{2+}-containing solution, a spontaneous contraction was observed if that aortic strip was previously treated with noradrenaline. The amplitude of IRT was 55.92 $\pm$ 2.53 % (n = 6) relative to those of noradrenaline-induced contractions in physiological solution. But an IRT was not observed if the aortic strips were previously treated with caffeine or KCl. Subsequent treatment of noradrenaline in Ca^{2+}-free medium caused a contraction similar in size to that of the first contraction elicited in Ca^{2+}-free medium (NA_1). Although the IRT was not observed in caffeine experiment, treating the strip with caffeine in Ca^{2+}-free medium could produce a contraction with the magnitude similar to that of the first contraction elicited in Ca^{2+}-free medium (Caf_1).

At the end of the experiment, the aortic strips were tested for its functionality by adding noradrenaline, caffeine or KCl to the tissue suspended in Ca^{2+}-containing solution. The contraction response in these conditions returned to the maximum level, which was comparable to those obtained at the beginning of experiment.

2. Effects of tested compounds (CU 18-08, CU 18-10 and CU 18-11) on aortic contraction and on the increase in the resting tone of rat aorta.

2.1 Effects on aortic contraction in Ca^{2+}-containing physiological solution. Figure 9 shows the contraction profile of rat aortic strip in the presence of tested compounds in the Ca^{2+}-containing solution in comparison with that of the same strip before addition of those compounds. DMSO at the concentration of 0.03% (v/v), which was used as solvent throughout the studies, had no effect on noradrenaline-induced contraction in Ca^{2+}-containing solution (Figure 9A). Noradrenaline-induced contraction in Ca^{2+}-containing solution was completely inhibited by prazosin $1x10^{-6}$ M (Figure 9B). Under the same condition, nifedipine $1x10^{-6}$ M partially inhibits the contraction. The contraction in the presence of nifedipine was 87.61 $\pm$ 1.94% of the maximum response (Figure 9C).

CU 18-08 and CU 18-10 at the same concentration of $1x10^{-5}$ M partially inhibited noradrenaline-induced contraction (Figure 9D, 9E). In the presence of these two compounds, the maximum contraction was suppressed to 89.07 ± 1.35% by CU 18-08 and 86.29 ± 2.77% by CU 18-10 (Table 2). In contrast, CU 18-11 potentiated the effect of noradrenaline in activation of contraction response. As seen in Figure 9F, addition of CU 18-11 $1x10^{-5}$ M increases the percentage of noradrenaline-induced contraction to 150.0 ± 11.93 %.

As seen in Figure 10, KCl 40 mM evoked a contraction in Ca^{2+}-containing solution with the magnitude of 252.3 ± 25.25 mg (n = 6). These contractions could be significantly inhibited by CU 18-08 $1x10^{-5}$ M and CU 18-10 $1x10^{-5}$ M. The percentage of contraction was suppressed to 63.87 ± 4.45 % in the presence of CU 18-08 and 56.87 ± 2.65 % in the presence of CU 18-10 (Figure 10, 11). In contrast, CU 18-11 at the concentration of $1x10^{-5}$ M did not affect the KCl-induced contraction of rat aorta (Figure 12).

TEA $1x10^{-3}$ M exerted its stimulation effect on rat aortic contraction with the magnitude of 157 ± 42 mg (n = 6) (Figure 14). Only CU 18-10 but not CU 18-08 could exert its inhibitory effect on TEA-induced contraction. Unlike noradrenaline-induced contraction, CU 18-11 did not have any affect on TEA-induced contraction (Figure 14, 16, Table 3).

2.2 Effects on aortic contraction. As seen in Figure 18B, noradrenaline-induced contraction in Ca^{2+}-free medium was completely inhibited by prazosin $1x10^{-6}$ M. Nifedipine $1x10^{-6}$ M also significantly inhibited noradrenaline-induced contractions with the remaining percentage of contraction 28.25 ± 2.78 % (Figure 18C). None of the synthetic CU 18-08 $1x10^{-5}$ M, CU 18-10 $1x10^{-5}$ M, CU 18-11 $1x10^{-5}$ M compounds tested in these studies (Figure 18D-F, 18A) affect noradrenaline-induced contraction in Ca^{2+}-free medium.

2.3 Effects on IRT. Figure 8A shows the increase in the resting tone contraction profiles. As previously mentioned, the maximum IRT response was 55.92 ± 2.53 % relative to noradrenaline-induced contraction in Ca^{2+}-containing solution. However DMSO at the concentration of 0.03% (v/v), in contrast to the affect on aortic contraction in Ca^{2+}-containing solution, significantly inhibited the IRT. The percentage of IRT contraction was suppressed to 31.98 ± 4.81 %. Hence the contraction containing DMSO 0.03% (v/v) was used as the control group in these studied (Figure 19A).

Prazosin $1x10^{-6}$ M and nifedipine $1x10^{-6}$ M completely inhibited the IRT, which was similar to those reported in other studies (Noguera *et al*., 1996) (Figure 19B, 19C). CU 18-08 $1x10^{-5}$ M and CU 18-10 $1x10^{-5}$ M significantly inhibited contractions on IRT (Figure 19D-F), The IRT was suppressed to 9.26 ± 2.52 % in the presence of CU 18-08 and 7.79 ± 2.62 % in the presence of CU 18-10 (Table 2). CU 18-11 did not inhibit increase in the resting tone. CU 18-11 has an observable affected on IRT. The percentage of contraction was 36.30 ± 2.38 %, which was not statistically different from that of the DMSO control group.

3. Effects of tested compounds (CU 18-08, CU 18-10 and CU 18-11) on contractions induced by adding calcium to a calcium-free depolarizing solution.

As seen in Figure 21, nifedipine $1x10^{-6}$ M was able to completely suppress the contraction of aortic smooth muscle induced by cumulative addition of Ca^{2+} up to $3x10^{-3}$ M. The Schild plot of $CaCl_2$ versus percentage of contraction suggested noncompetitive effect of nifedipine. The apparent pD_2 value calculated according to Van Rossum (1963) was 6.29 ± 0.10. Among the three CU compounds, CU 18-08 and CU 18-10 were shown to inhibit the contraction of rat aortic strips in this condition. Unlike nifedipine, the Schild plot suggested competitive inhibiting effects of CU 18-08 and CU 18-10 on Ca^{2+} influx. The apparent pA_2 values of CU 18-08 and CU 18-10 at the concentration of $1x10^{-4}$ M were 4.61 ± 0.11 and 5.81 ± 0.28 respectively (Table 4). The apparent pA_2 values were slightly differing. However, data of pA_2 values suggested that CU 18-10 was more potent than CU 18-08 in the inhibition of Ca^{2+} entry. In contrast, CU 18-11 increased a

significantly contractions on $CaCl_2$ induced contraction of rat aorta in Ca^{2+}-free depolarizing solution (Figure 29, 30).

Table 1 Contractile responses to 1 μM noradrenaline (NA), 10 mM caffeine and 40 mM KCl in Ca^{2+}-containing, Ca^{2+}-free and increase in the resting tone (IRT) of rat aorta

Contractile response						
agonist	n	Ca^{2+}-containing agonist (mg)	Ca^{2+}-free $Agonist_1$ (%)	Ca^{2+}-free $Agonist_1'$ (%)	Ca^{2+}-containing (IRT) (%)	Ca^{2+}-free $Agonist_2$ (%)
noradrenaline	6	373.3 ± 51	37.88 ± 3.43	NR	55.92 ± 2.53	38.73 ± 4.6
Caffeine	6	46.10 ± 6.10	41.15 ± 1.83	NR	NR	40.65 ± 2.1
KCl	6	252.3 ± 25.25	NR	NR	R	NR

Increase in the resting tone and contraction in Ca^{2+}-free medium are expressed as a percentage of the noradrenaline-, caffeine-, or KCl-induced contractions in physiological solution respectively.

All values represent mean ± S.E. mean, n = number of experiments.

NR: no response.

Table 2 The percentage of agents tested on noradrenaline (NA)-induced contraction in Ca^{2+}-containing solution, noradrenaline (NA_1)-induced contraction in Ca^{2+}-free medium and the increase in the resting tone of rat aorta (IRT).

			NA (%) Ca^{2+}(+)		NA_1 (%) Ca^{2+} (-)		IRT (%) Ca^{2+} (+)	
Agent	μM	n	before	after	before	after	before	after
control	0.03%	6	100	105.28 ±8.9	36.28 ±3.42	34.48 ±2.72	60.25 ±3.53	31.98 ±4.81*
Prazosin	1	6	100	0*	37.12 ± 3.20	0*	59.20 ± 4.61	0*
Nifedipine	1	6	100	87.61 ±1.94*	41.50 ±2.56	28.25 ± 2.78*	58.86 ± 2.56	0*
CU 18-08	10	6	100	89.07 ±1.35*	36.64 ±2.81	28.62 ±2.76	59.20 ±4.61	9.26 ±2.52*
CU 18-10	10	6	100	86.29 ±2.77*	37.88 ±3.43	35.69 ±3.77	55.92 ±2.53	7.79 ±2.62*
CU 18-11	10	6	100	150.00 ±11.93*	36.96 ±1.06	40.84 ±3.54	57.13 ±2.31	36.30 ±2.38*

Increase in the resting tone (IRT) and contractions in Ca^{2+}-containing or Ca^{2+}-free medium are expressed as a percentage of the noradrenaline-induced contractions in physiological solution.

Before: before adding agent

After: after adding agent

Ca^{2+}(+) = Ca^{2+}-containing solution; Ca^{2+}(-) = Ca^{2+}-free medium. All values represent mean ± S.E. mean, n = number of experiments.

*$p<0.05$

Table 3 Effects of CU 18-08, CU 18-10 and CU 18-11 on KCl 40 mM and TEA $1x10^{-3}$ M induced contraction in Ca^{2+}-containing solution.

Agent	n	KCl 40 mM (%)	TEA $1x10^{-3}$ M (%)
control	6	100	100
CU 18-08	6	63.87 ± 4.45*	92.14 ± 3.47
CU 18-10	6	56.87 ± 2.65*	35.62 ± 5.8*
CU 18-11	6	97.4 ± 4.33	102.31 ± 1.48

All values represent mean ± S.E. mean, n = number of experiments.

*$p<0.05$

Table 4 Parameters of CU 18-08, CU 18-10 and nifedipine on $CaCl_2$ -induced contraction in potassium depolarizing solution.

Agent	Parameter
CU 18-08 $1x10^{-5}$ M	pA_2 = 5.18 ± 0.12
CU 18-08 $1x10^{-4}$ M	pA_2 = 4.61 ± 0.11
CU 18-10 $1x10^{-5}$ M	pA_2 = 5.69 ± 0.19
CU 18-10 $1x10^{-4}$ M	pA_2 = 5.81 ± 0.28
Nifedipine $1x10^{-6}$ M	pD_2' = 6.29 ± 0.10

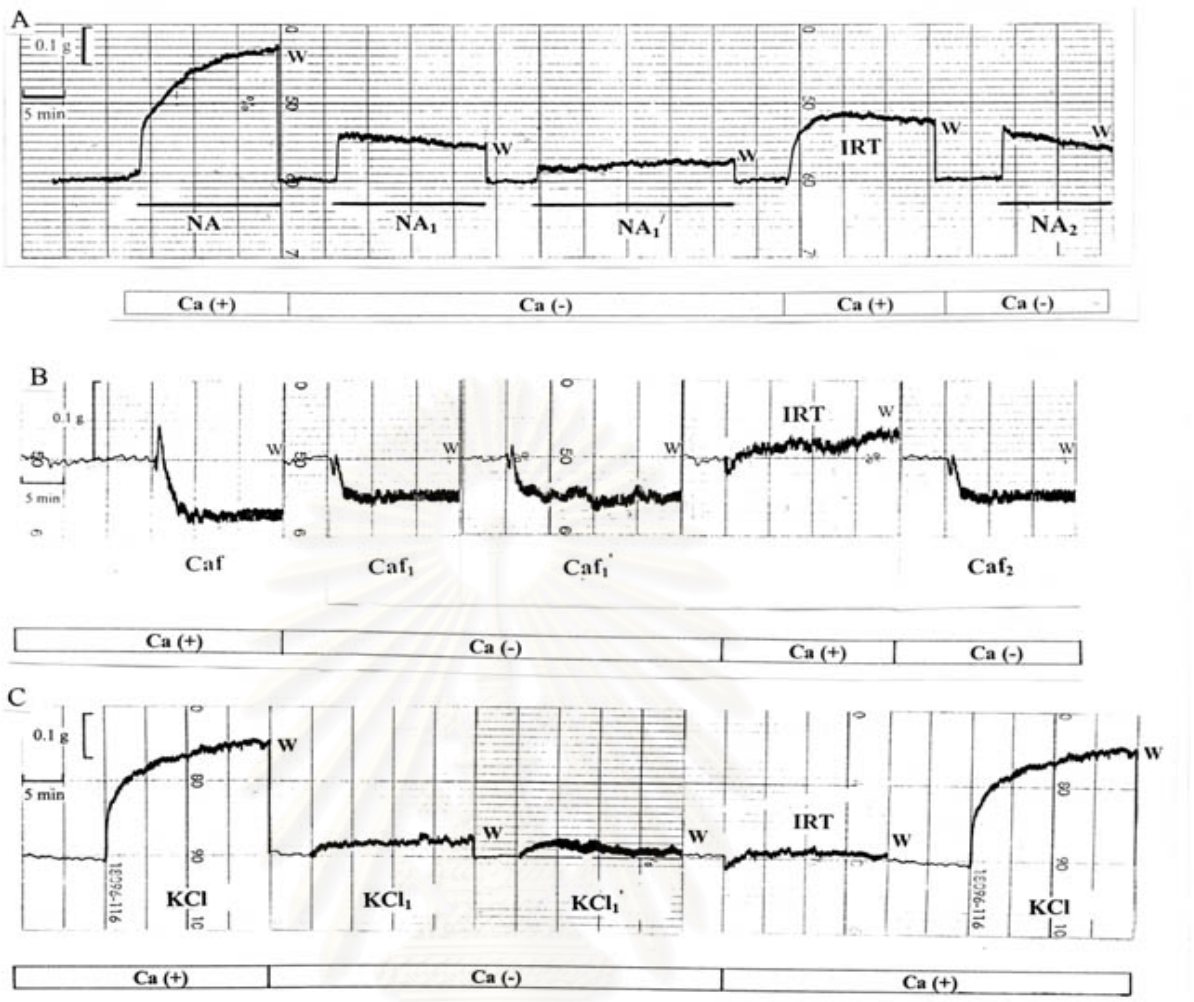


Figure 8 Contraction profiles of isolated rat aorta. The profiles also represent schematic of the experimental procedure designed to study the depletion of intracellular Ca^{2+}-stores sensitive to noradrenaline (NA), caffeine (Caf) or potassium chloride (KCl) in Ca^{2+}-free medium, and the increase in the resting tone obtained by subsequent exposure to Ca^{2+}-containing solution during the refilling of these stores. Agonist was added in Ca^{2+}-containing solution and then the tissue was treated with Ca^{2+}-free, EDTA-containing solution for 15 min. After this time the agonist was applied (NA_1, NA_1') and washed (W) until no contraction was induced, indicating complete depletion of internal Ca^{2+}-stores sensitive to the agonist. The tissue was incubated for 20 min in Krebs to refill the intracellular Ca^{2+}-stores and a spontaneous increase in the resting tone of aorta (IRT)

was observed. After washing and 15 min of loading in Ca^{2+}-free medium, a new addition of agonist (NA_2) was made.

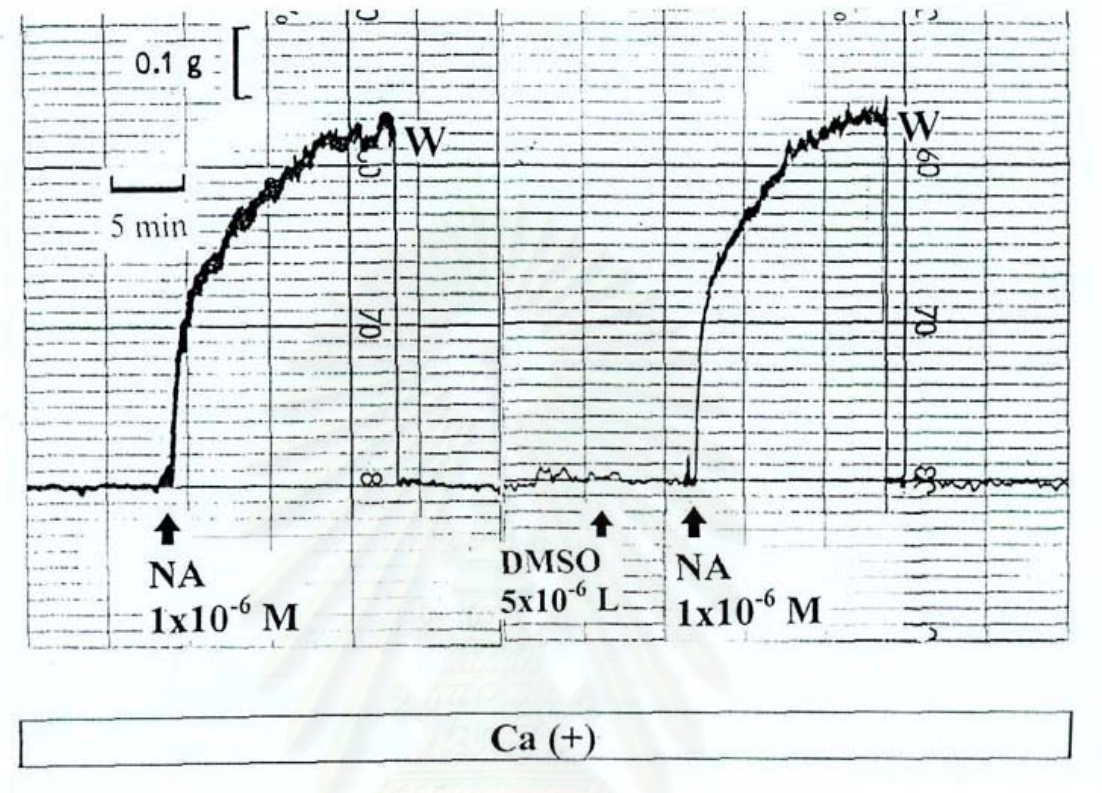


Figure 9A Contraction profiles showing the effect of DMSO 0.03% (v/v) on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}- containing solution. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. DMSO is added in Ca^{2+}-containing solution 5 min prior to induction of noradrenaline (as described in experimental procedure).

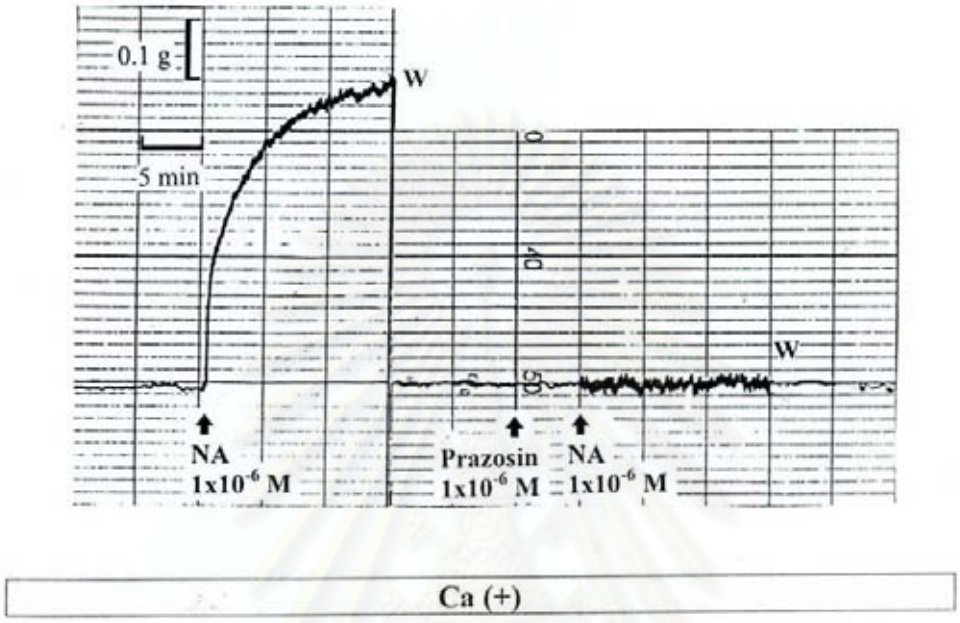


Figure 9B Contraction profiles showing the effect of prazosin $1x10^{-6}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}- containing solution. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. Prazosin is added in Ca^{2+}-containing solution 5 min prior to induction of noradrenaline (as described in experimental procedure).

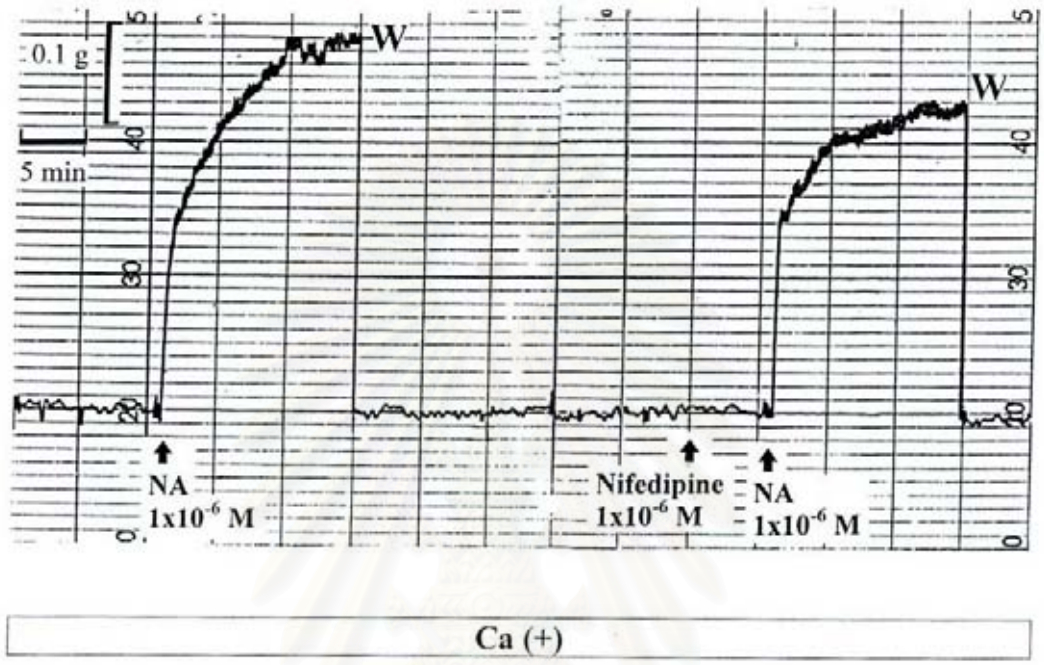


Figure 9C Contraction profiles showing the effect of nifedipine $1x10^{-6}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}- containing solution. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. Nifedipine is added in Ca^{2+}-containing solution 5 min prior to induction of noradrenaline (as described in experimental procedure).

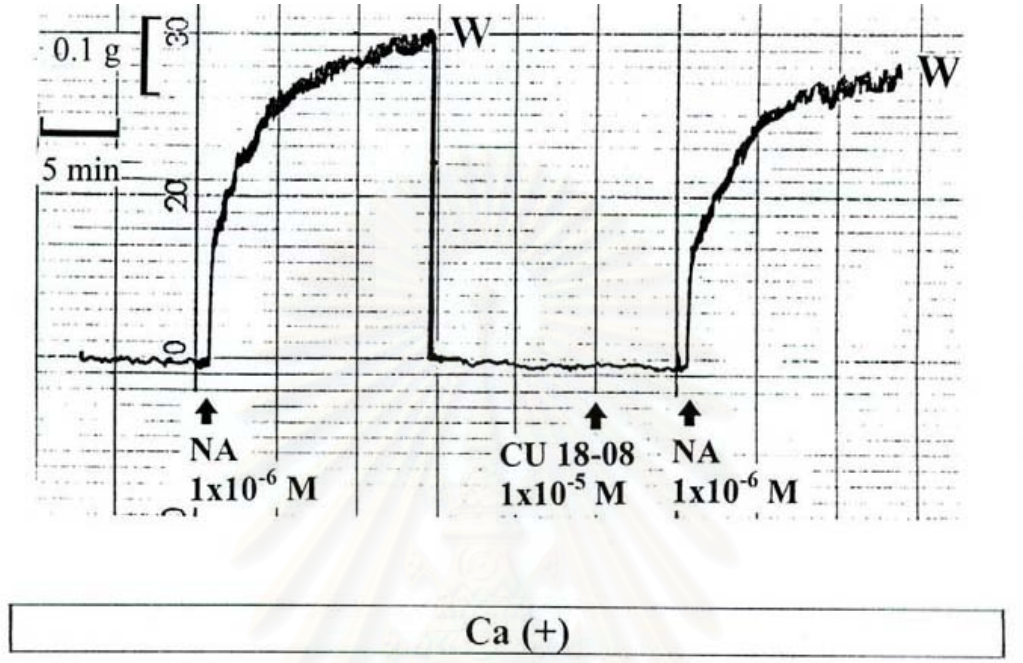


Figure 9D Contraction profiles showing the effect of CU 18-08 $1x10^{-5}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}- containing solution. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. CU 18-08 is added in Ca^{2+}-containing solution 5 min prior to induction of noradrenaline (as described in experimental procedure).

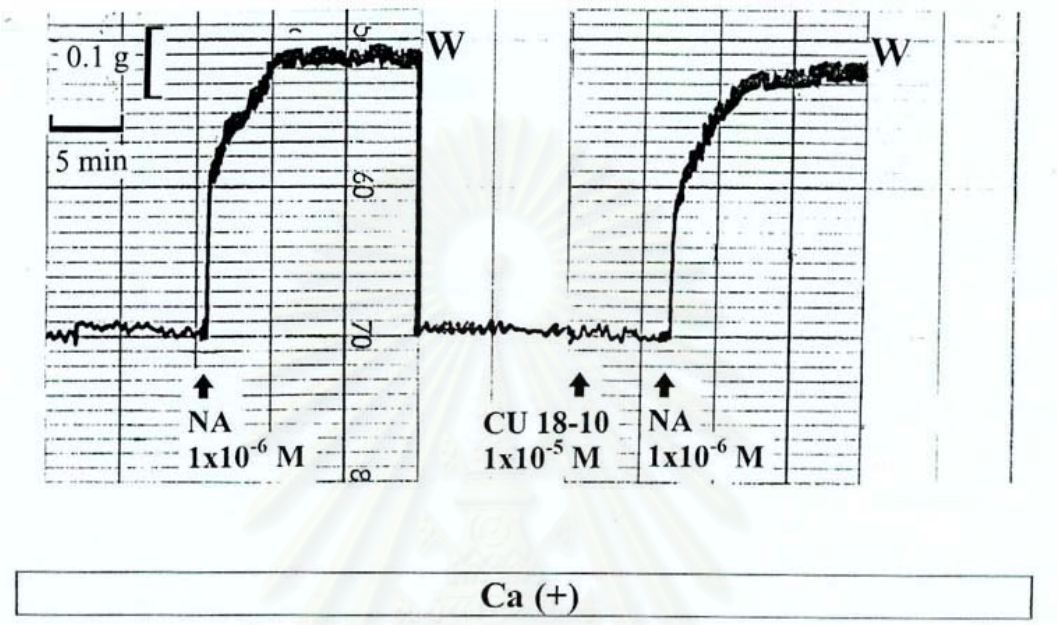


Figure 9E Contraction profiles showing the effect of CU 18-10 $1x10^{-5}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}- containing solution. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. CU 18-10 is added in Ca^{2+}-containing solution 5 min prior to induction of noradrenaline (as described in experimental procedure).

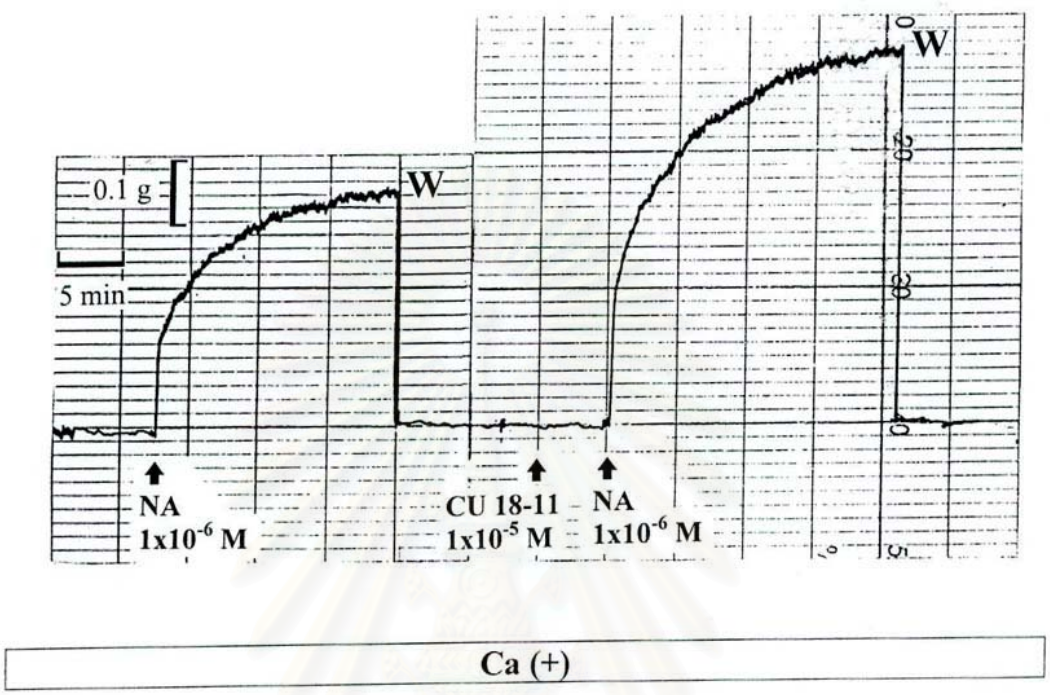


Figure 9F Contraction profiles showing the effect of CU 18-11 $1x10^{-5}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}- containing solution. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. CU 18-11 is added in Ca^{2+}-containing solution 5 min prior to induction of noradrenaline (as described in experimental procedure).

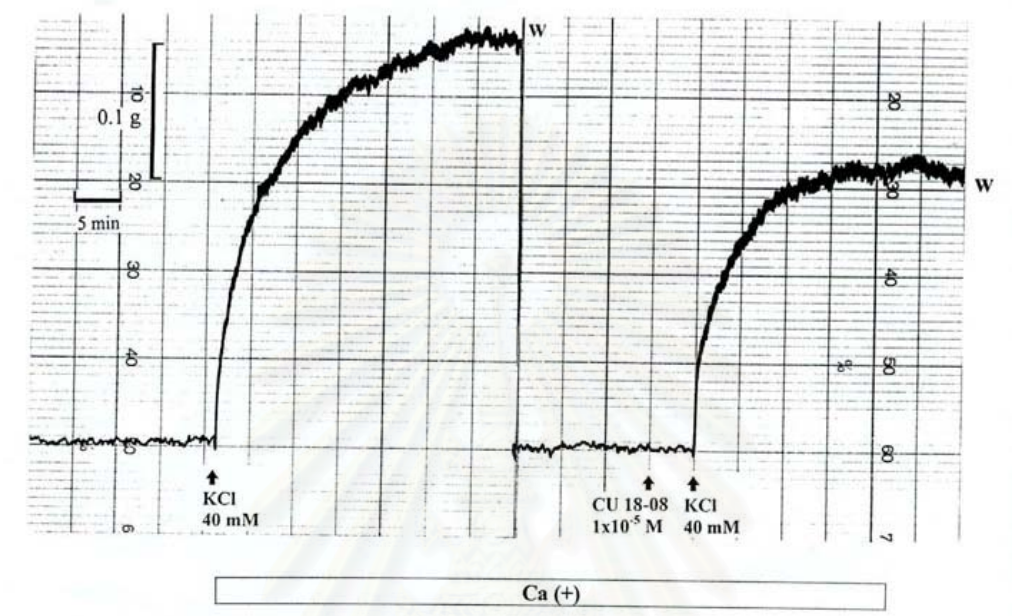


Figure 10 Contraction profiles showing the effect of CU 18-08 $1x10^{-5}$ M on the contraction of 40 mM KCl-induced contraction in Ca^{2+}-containing solution.

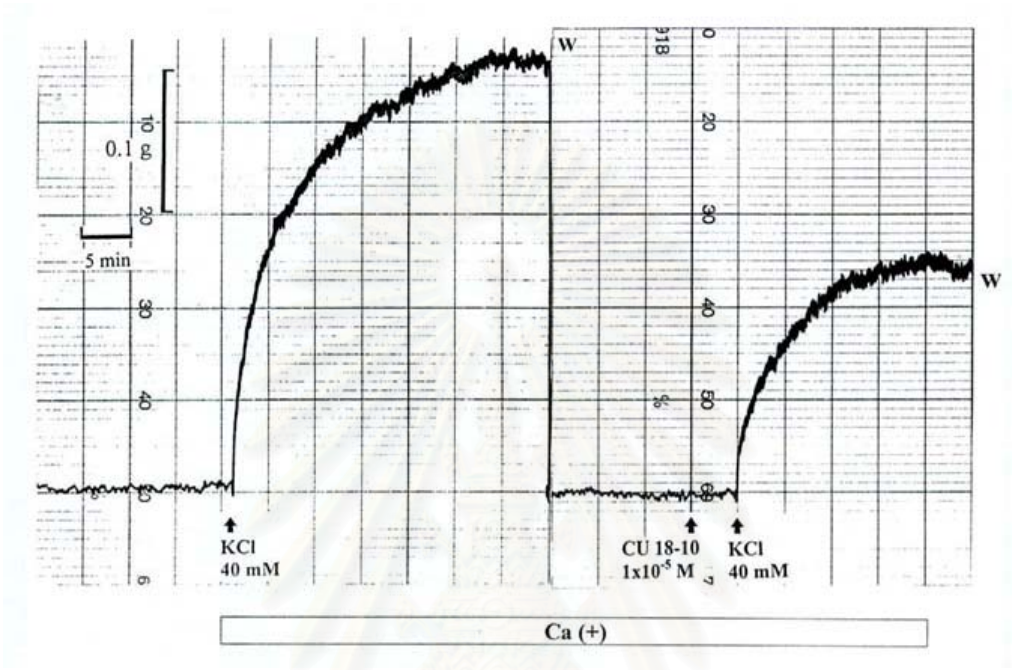


Figure 11 Contraction profiles showing the effect of CU 18-10 $1x10^{-5}$ M on the contraction of 40 mM KCl-induced contraction in Ca^{2+}-containing solution.

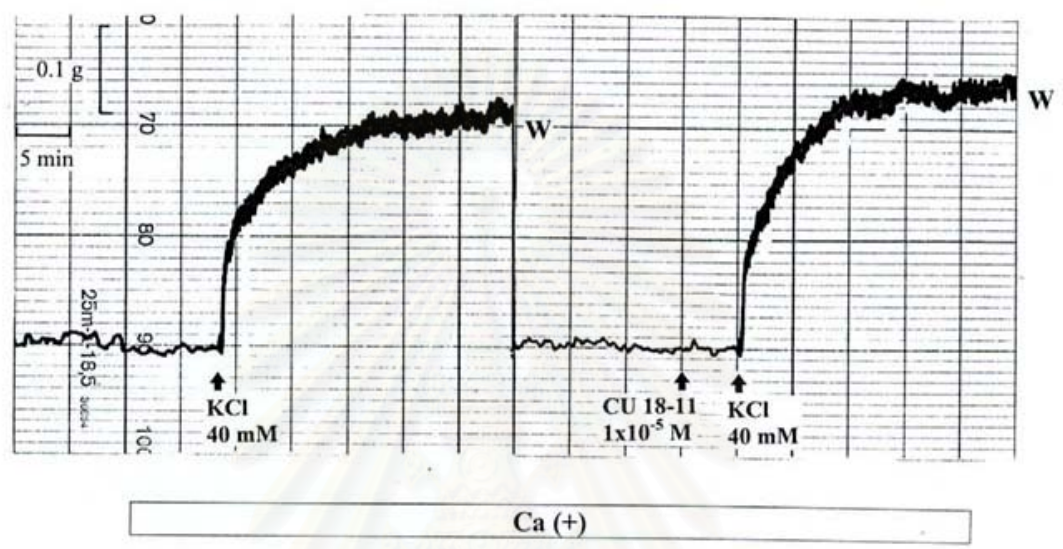


Figure 12 Contraction profiles showing the effect of CU 18-11 $1x10^{-5}$ M on the contraction of 40 mM KCl-induced contraction in Ca^{2+}-containing solution.

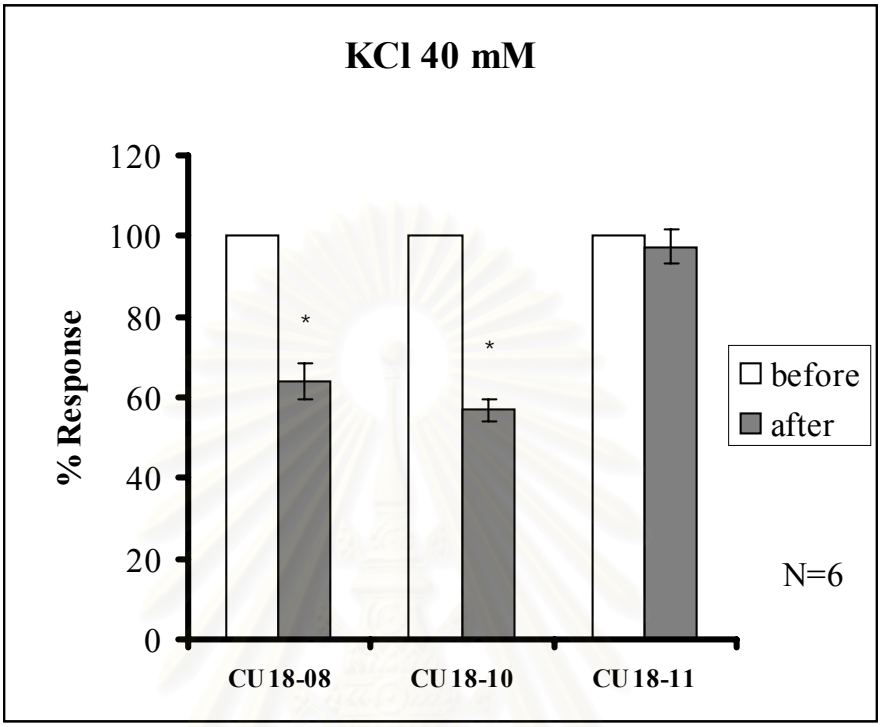


Figure 13 Effects of CU 18-08, CU 18-10 and CU 18-11 1x10^{-5} M on the contraction of 40 mM KCl-induced contraction in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*)

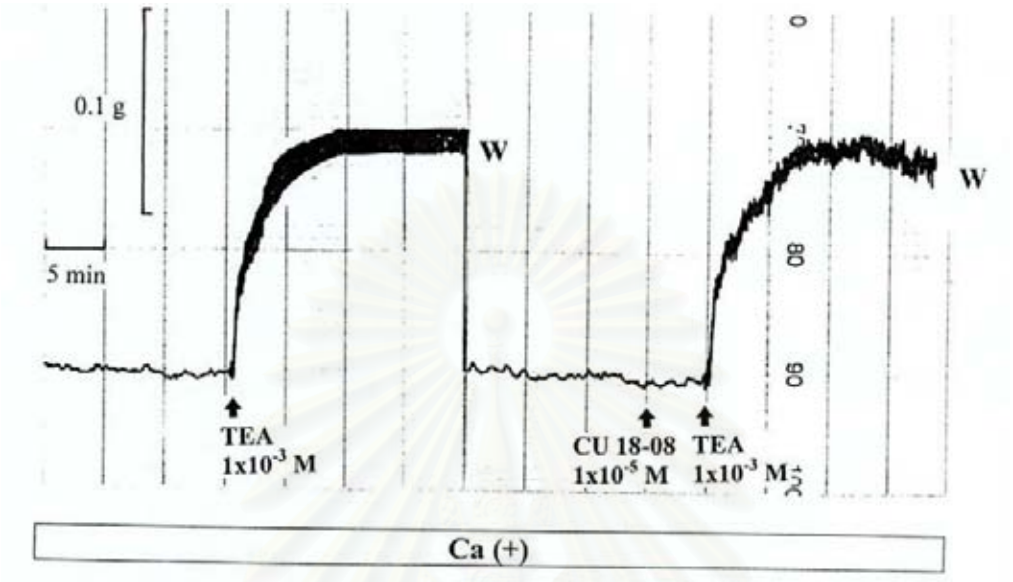


Figure 14 Contraction profiles showing the effect of CU 18-08 $1x10^{-5}$ M on the contraction of $1x10^{-3}$ M TEA-induced contraction in Ca^{2+}-containing solution.

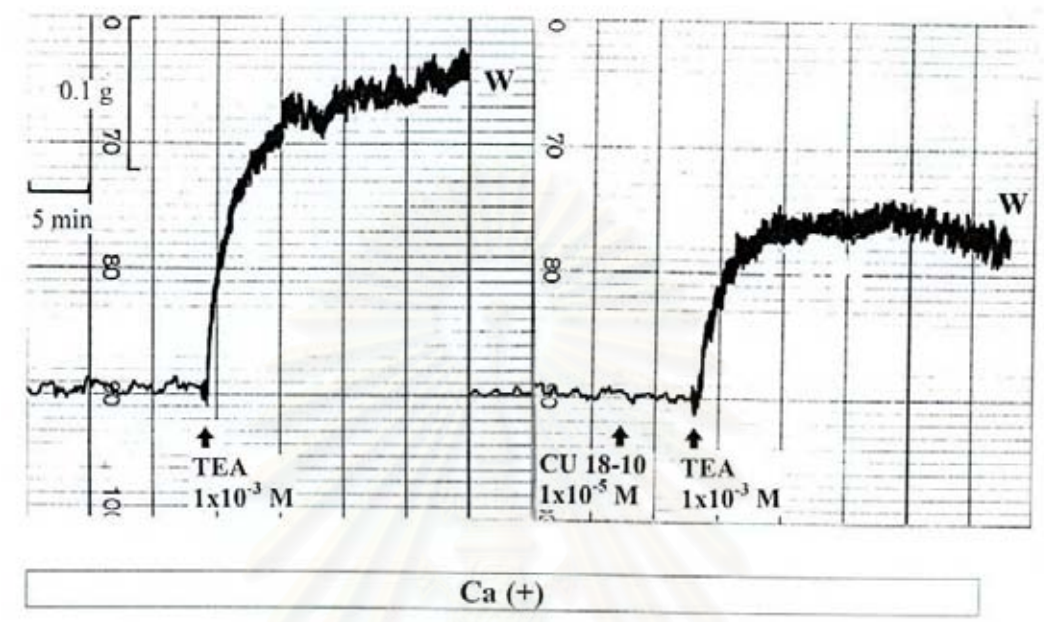


Figure 15 Contraction profiles showing the effect of CU 18-10 $1x10^{-5}$ M on the contraction of $1x10^{-3}$ M TEA-induced contraction in Ca^{2+}-containing solution.

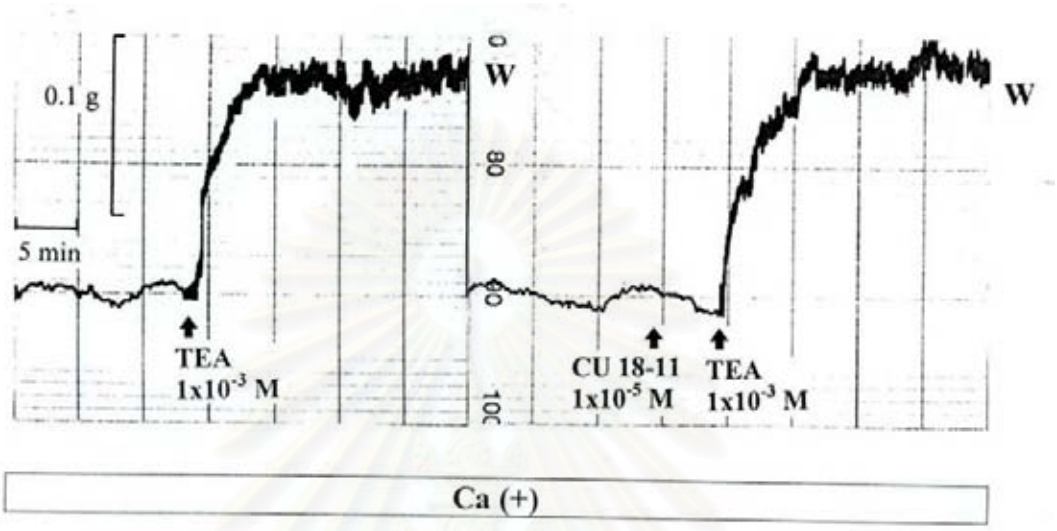


Figure 16 Contraction profiles showing the effect of CU 18-11 $1x10^{-5}$ M on the contraction of $1x10^{-3}$ M TEA-induced contraction in Ca^{2+}-containing solution.

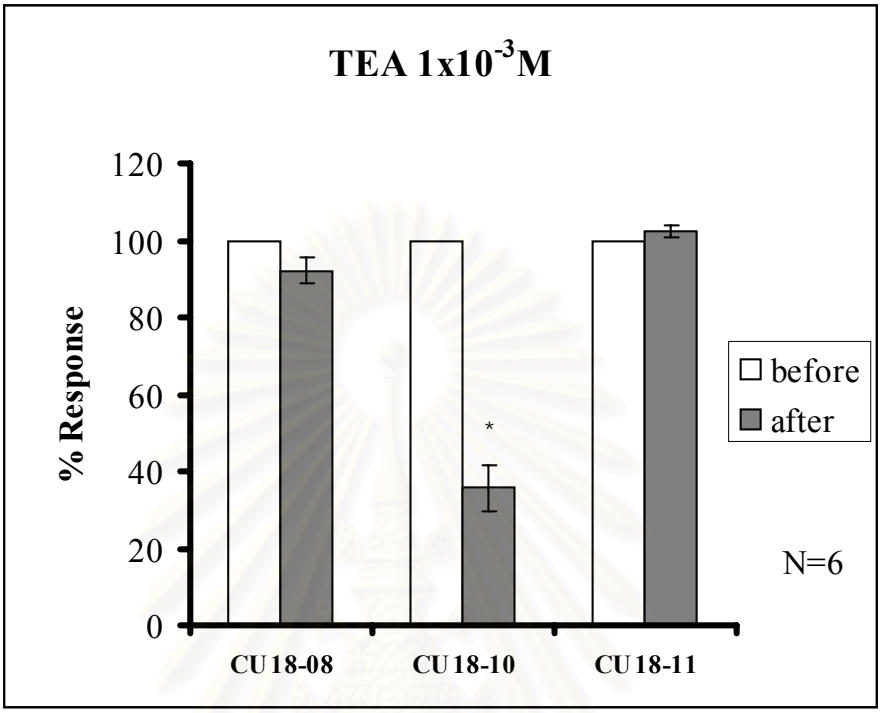


Figure 17 Effects of CU 18-08, CU 18-10 and CU 18-11 $1x10^{-5}$ M on the contraction of $1x10^{-3}$ M TEA-induced contraction in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*)

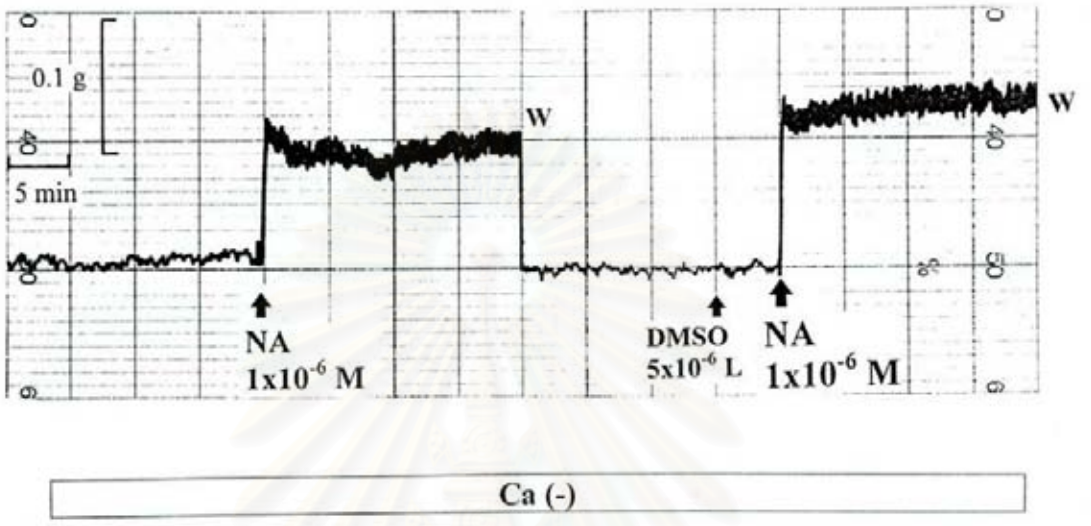


Figure 18A Contraction profiles showing the effect of DMSO 0.03% (v/v) on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}-free medium. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. DMSO is added in Ca^{2+}-free medium 5 min prior to induction of noradrenaline (as described in experimental procedure).

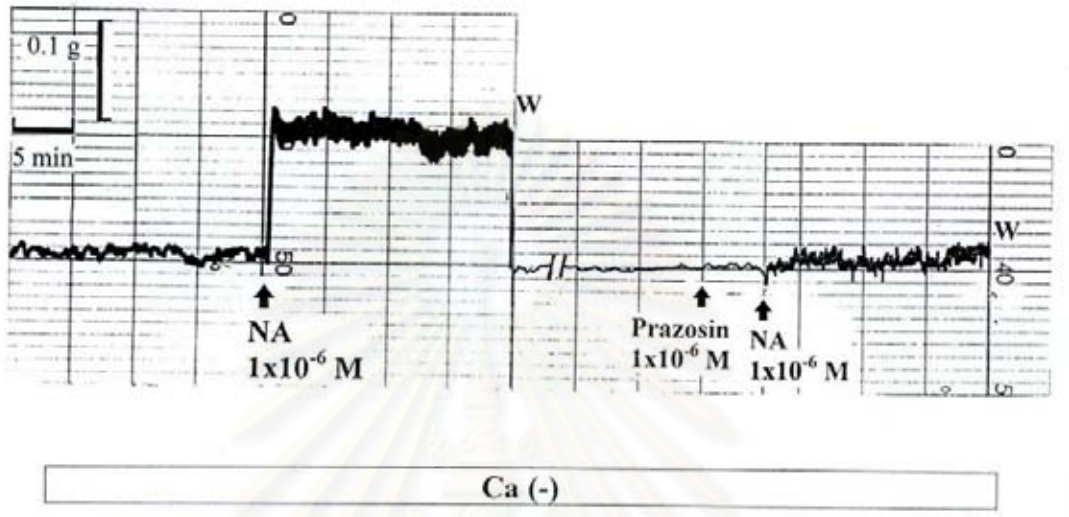


Figure 18B Contraction profiles showing the effect of prazosin $1x10^{-6}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}-free medium. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. Prazosin is added in Ca^{2+}-free medium 5 min prior to induction of noradrenaline (as described in experimental procedure).

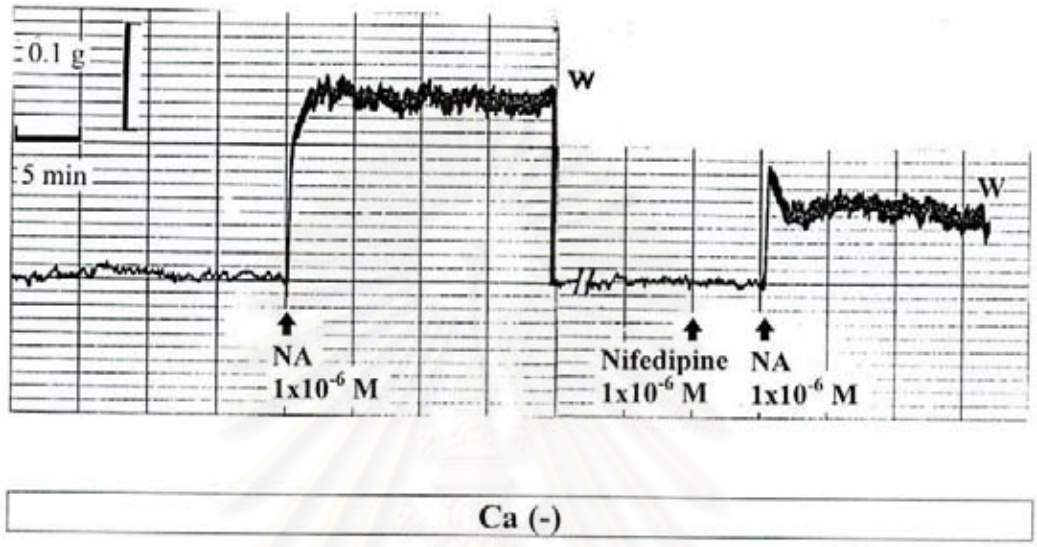


Figure 18C Contraction profiles showing the effect of nifedipine $1x10^{-6}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}-free medium. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. Nifedipine is added in Ca^{2+}-free medium 5 min prior to induction of noradrenaline (as described in experimental procedure).

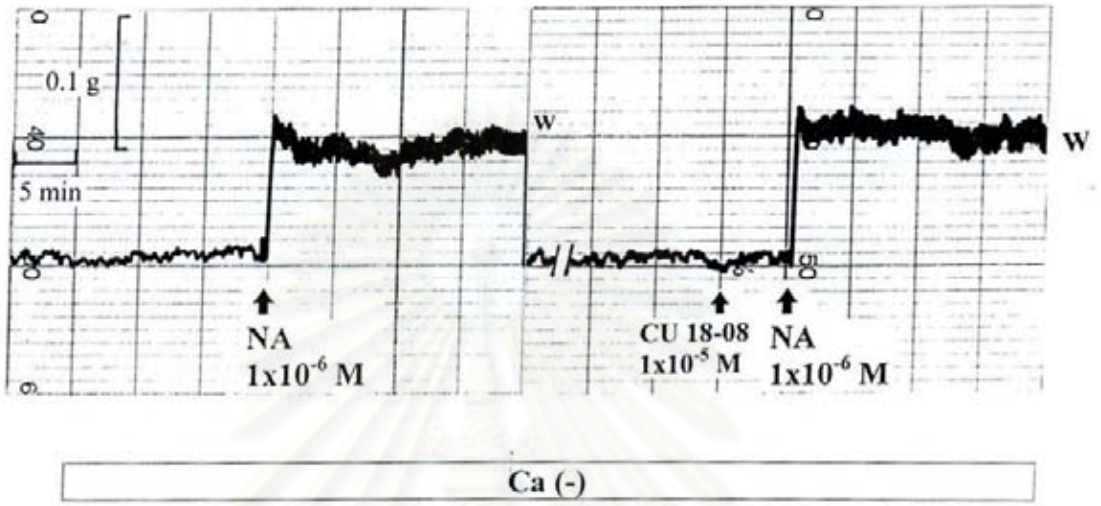


Figure 18D Contraction profiles showing the effect of CU 18-08 $1x10^{-5}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}-free medium. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. CU 18-08 is added in Ca^{2+}-free medium 5 min prior to induction of noradrenaline (as described in experimental procedure).

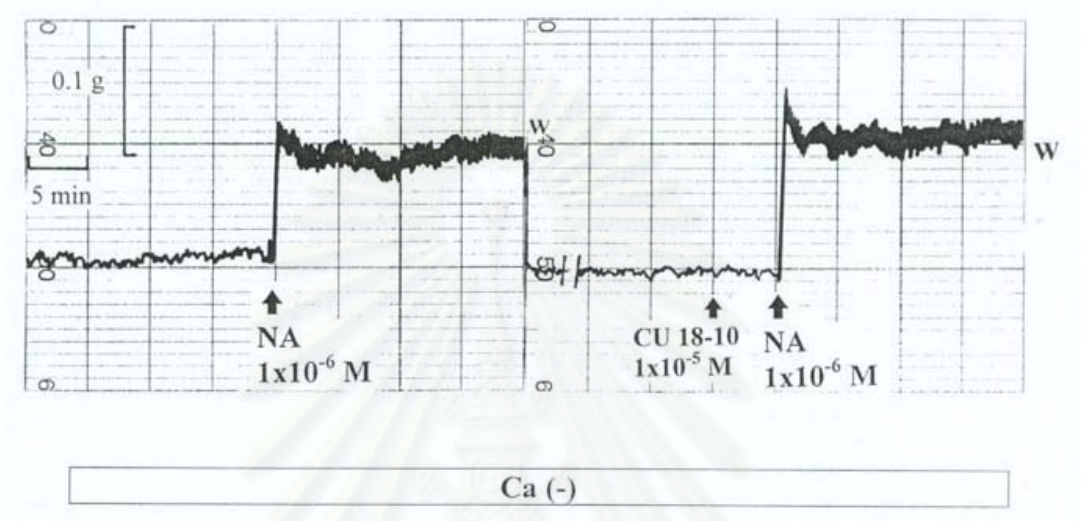


Figure 18E Contraction profiles showing the effect of CU 18-10 $1x10^{-5}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}-free medium. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. CU 18-10 is added in Ca^{2+}-free medium 5 min prior to induction of noradrenaline (as described in experimental procedure).

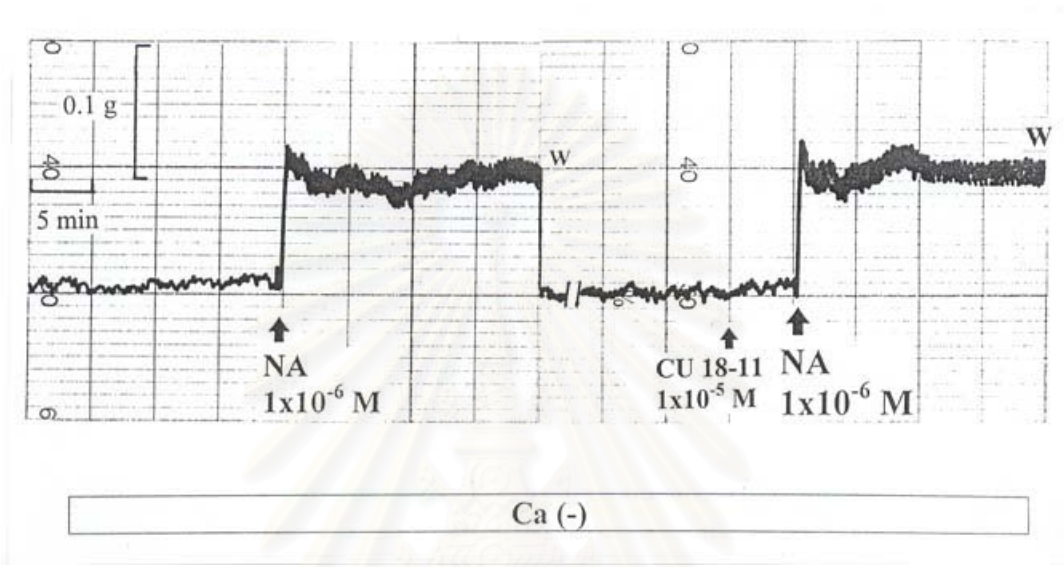


Figure 18F Contraction profiles showing the effect of CU 18-11 $1x10^{-5}$ M on the contraction of $1x10^{-6}$ M noradrenaline-induced contraction in Ca^{2+}-free medium. Contractions are expressed as a percentage of the noradrenaline-induced contraction in the Ca^{2+}-containing solution. CU 18-11 is added in Ca^{2+}-free medium 5 min prior to induction of noradrenaline (as described in experimental procedure).

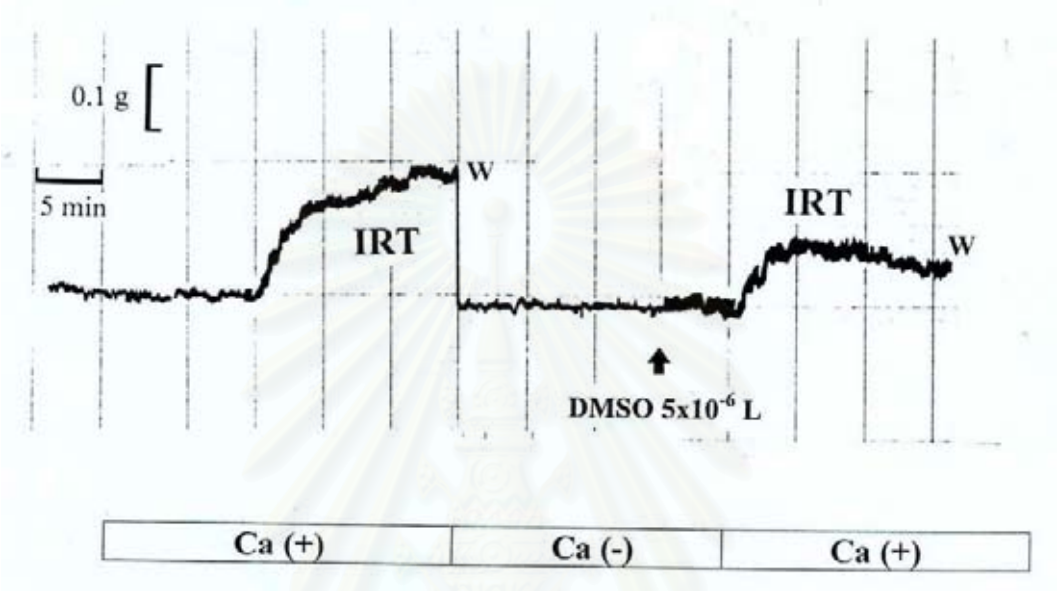


Figure 19A Contraction profiles showing the effect of DMSO 0.03% (v/v) on IRT. IRT is expressed as the spontaneous contraction in the Ca^{2+}-containing solution subsequent to depletion of intracellular Ca^{2+} sensitive to noradrenaline. DMSO is added in Ca^{2+}-free medium 5 min prior to induction of IRT (as described in experimental procedure).

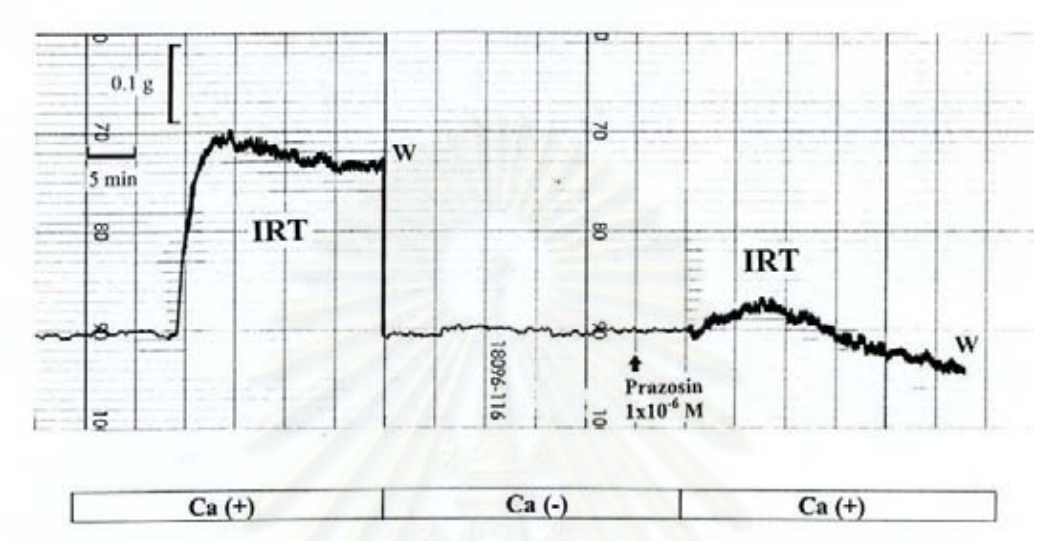


Figure 19B Contraction profiles showing the effect of prazosin $1x10^{-6}$ M on IRT. IRT is expressed as the spontaneous contraction in the Ca^{2+}-containing solution subsequent to depletion of intracellular Ca^{2+} sensitive to noradrenaline. Prazosin is added in Ca^{2+}-free medium 5 min prior to induction of IRT (as described in experimental procedure).

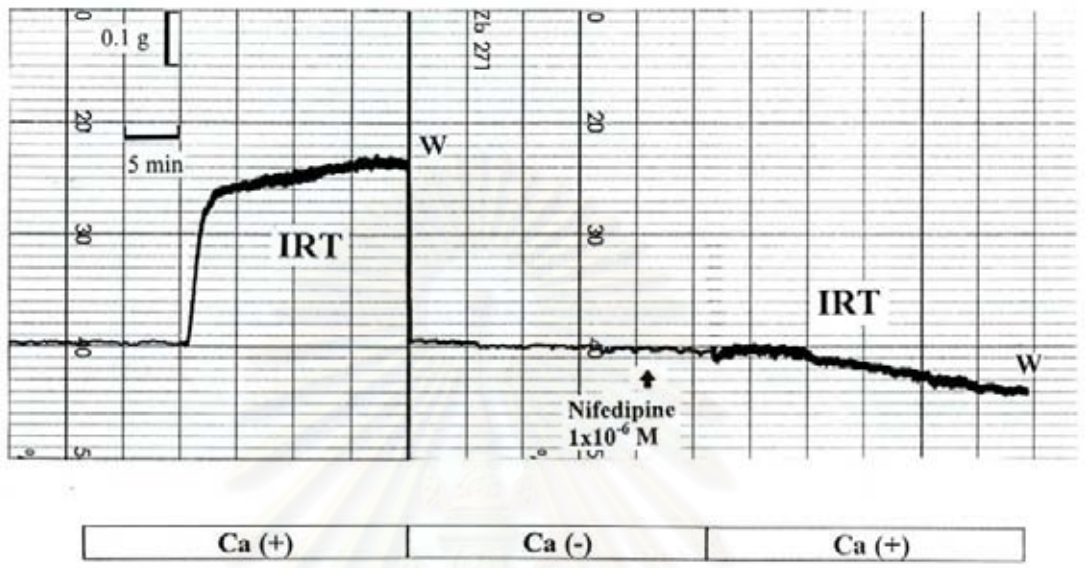


Figure 19C Contraction profiles showing the effect of nifedipine $1x10^{-6}$ M on IRT. IRT is expressed as the spontaneous contraction in the Ca^{2+}-containing solution subsequent to depletion of intracellular Ca^{2+} sensitive to noradrenaline. Nifedipine is added in Ca^{2+}-free medium 5 min prior to induction of IRT (as described in experimental procedure).

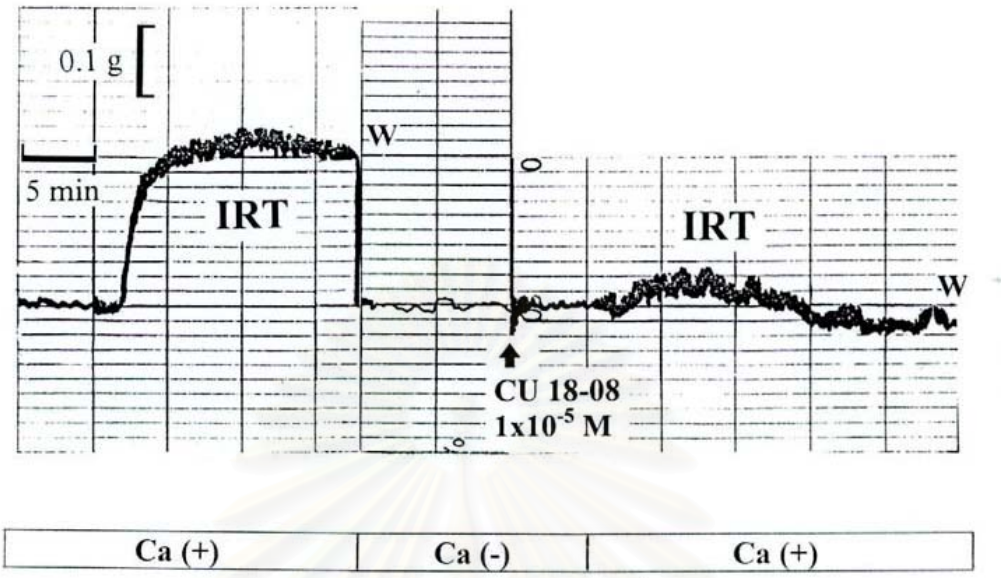


Figure 19D Contraction profiles showing the effect of CU 18-08 $1x10^{-5}$ M on IRT. IRT is expressed as the spontaneous contraction in the Ca^{2+}-containing solution subsequent to depletion of intracellular Ca^{2+} sensitive to noradrenaline. CU 18-08 is added in Ca^{2+}-free medium 5 min prior to induction of IRT (as described in experimental procedure).

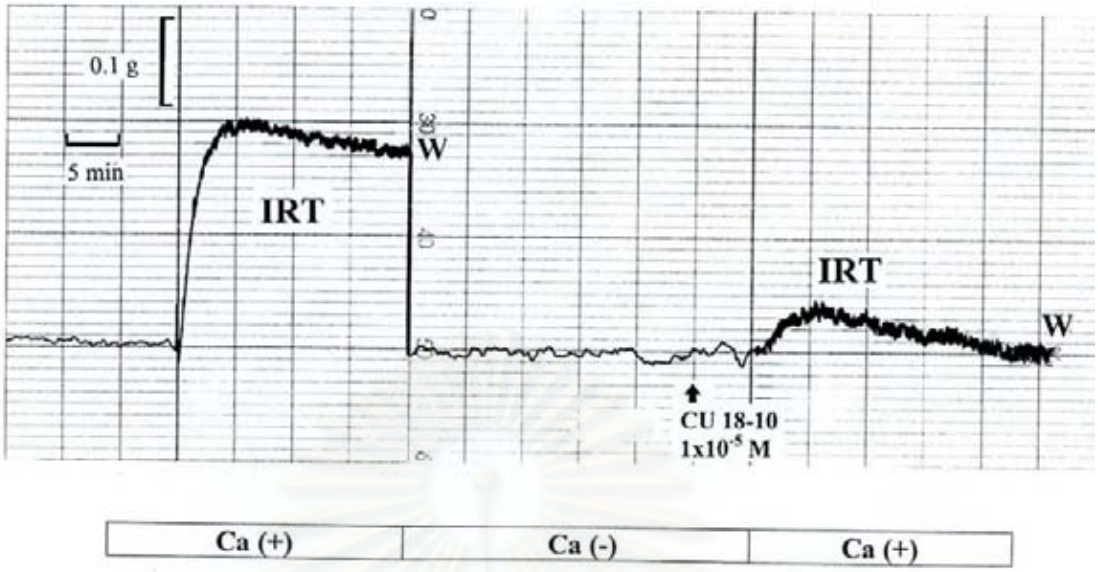


Figure 19E Contraction profiles showing the effect of CU 18-10 $1x10^{-5}$ M on IRT. IRT is expressed as the spontaneous contraction in the Ca^{2+}-containing solution subsequent to depletion of intracellular Ca^{2+} sensitive to noradrenaline. CU 18-10 is added in Ca^{2+}-free medium 5 min prior to induction of IRT (as described in experimental procedure).

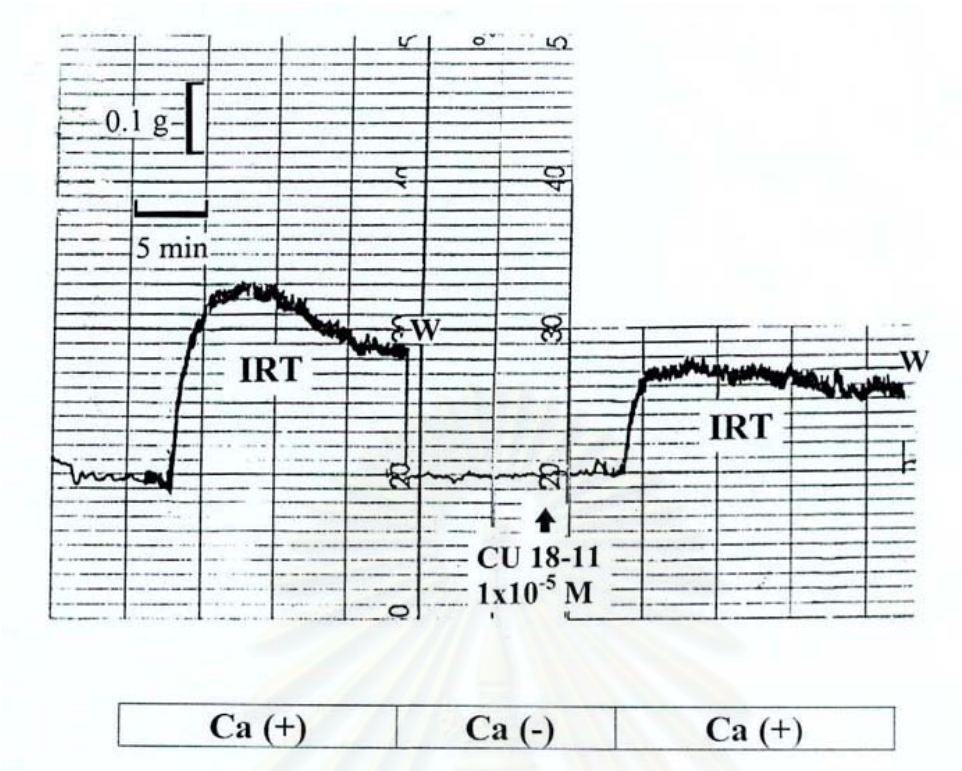


Figure 19F Contraction profiles showing the effect of CU 18-11 $1x10^{-5}$ M on IRT. IRT is expressed as the spontaneous contraction in the Ca^{2+}-containing solution subsequent to depletion of intracellular Ca^{2+} sensitive to noradrenaline. CU 18-11 is added in Ca^{2+}-free medium 5 min prior to induction of IRT (as described in experimental procedure).

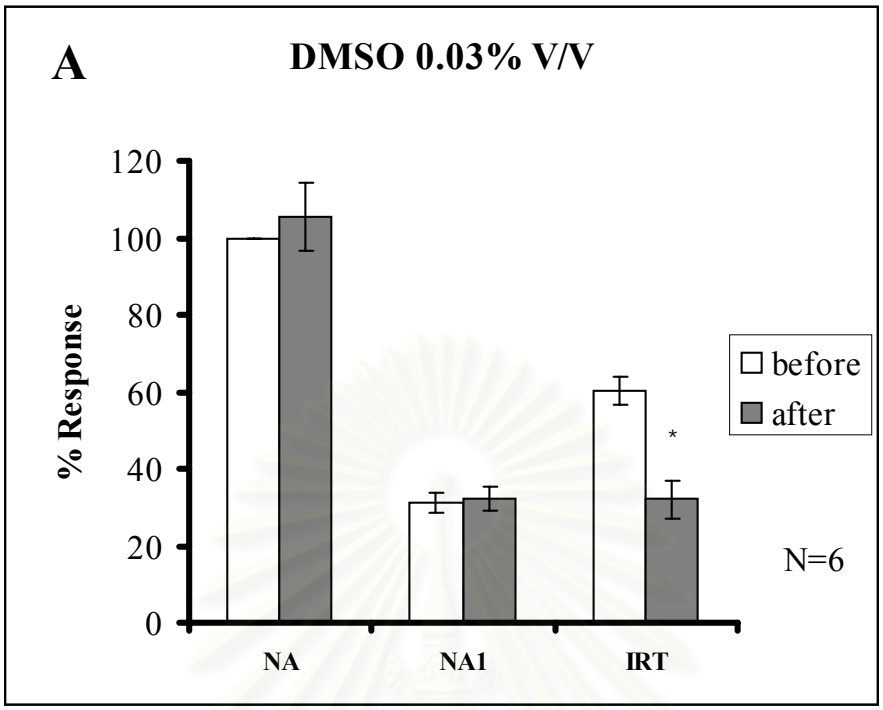


Figure 20A Effects of DMSO 0.03% (v/v) on noradrenaline-induce contraction in the Ca^{2+}-containing solution, in Ca^{2+}-free medium and on IRT. The IRT and contractions are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. NA: first addition of NA in Ca^{2+}-containing solution. NA_1: first addition of NA in Ca^{2+}-free medium. IRT: increase in the resting tone. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*).

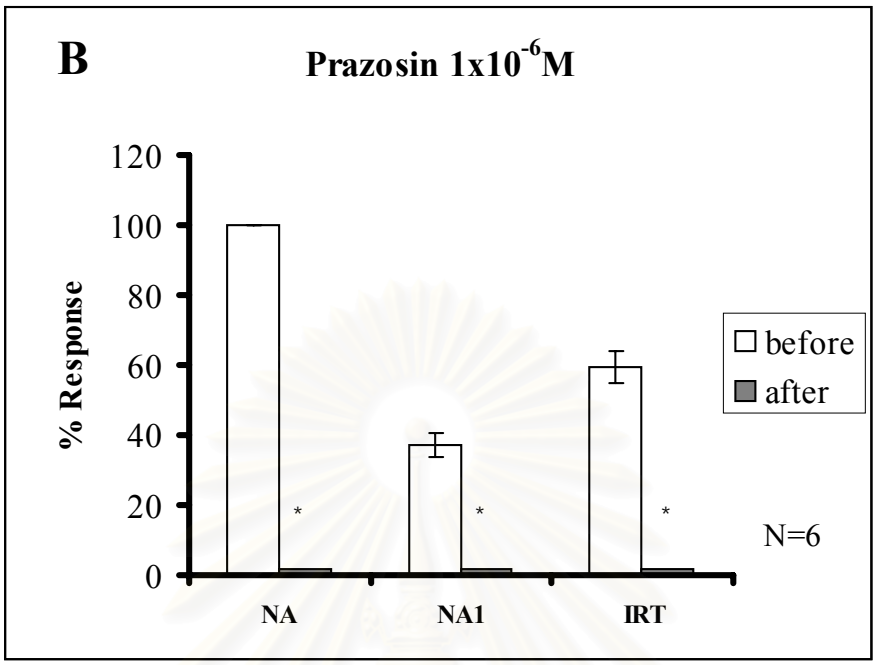


Figure 20B Effects of prazosin $1x10^{-6}$ M on noradrenaline-induced contraction in the Ca^{2+}-containing solution, in Ca^{2+}-free medium and on IRT. The IRT and contractions are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. NA: first addition of NA in Ca^{2+}-containing solution. NA_1: first addition of NA in Ca^{2+}-free medium. IRT: increase in the resting tone. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*).

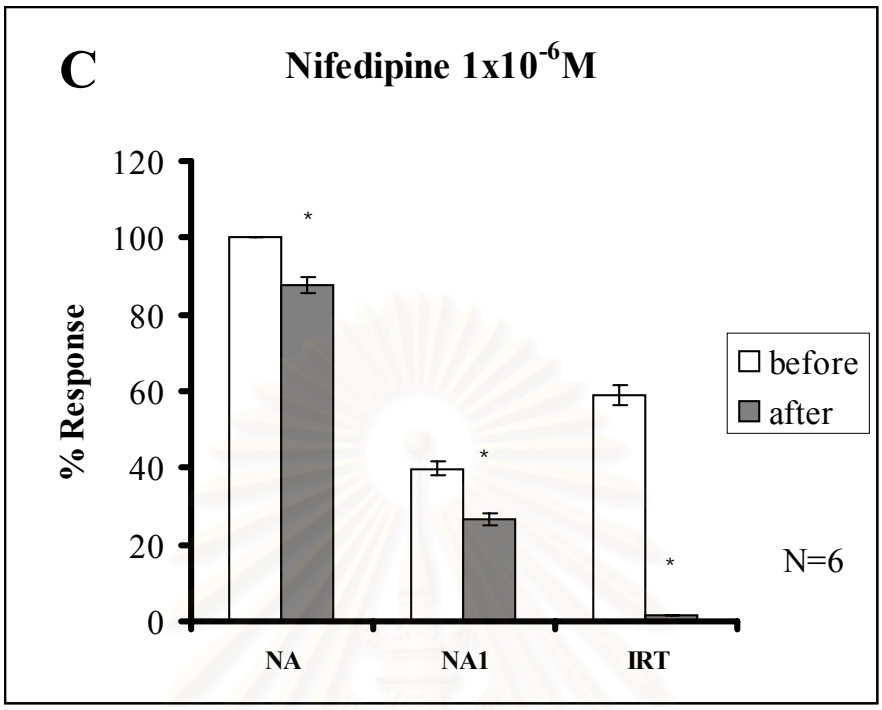


Figure 20C Effects of nifedipine $1x10^{-6}$ M on noradrenaline-induced contraction in the Ca^{2+}-containing solution, in Ca^{2+}-free medium and on IRT. The IRT and contractions are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. NA: first addition of NA in Ca^{2+}-containing solution. NA_1: first addition of NA in Ca^{2+}-free medium. IRT: increase in the resting tone. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*).

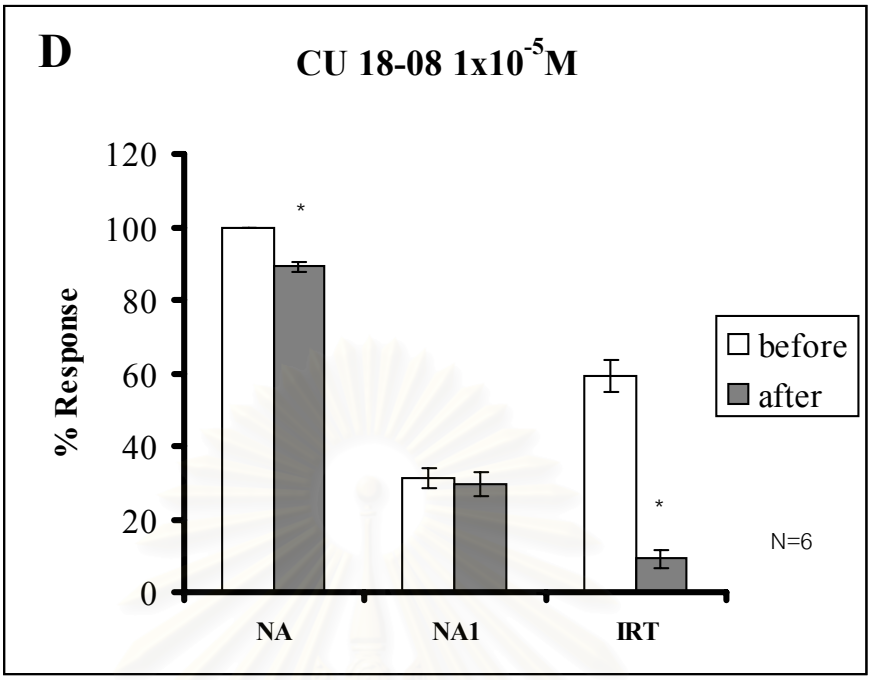


Figure 20D Effects of CU 18-08 $1x10^{-5}$ M on noradrenaline-induced contraction in the Ca^{2+}-containing solution, in Ca^{2+}-free medium and on IRT. The IRT and contractions are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. NA: first addition of NA in Ca^{2+}-containing solution. NA_1: first addition of NA in Ca^{2+}-free medium. IRT: increase in the resting tone. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*).

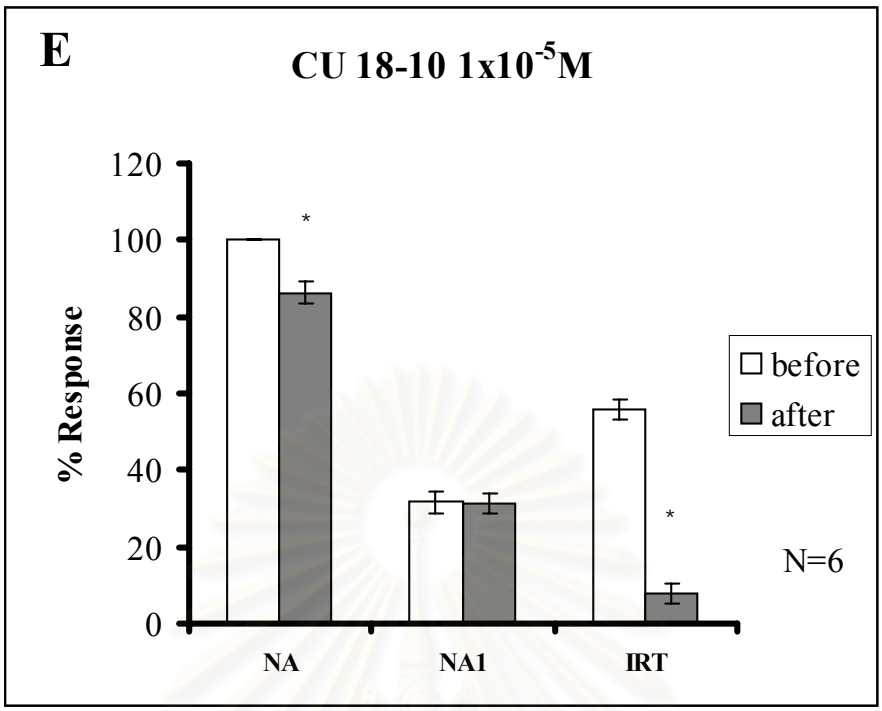


Figure 20E Effects of CU 18-10 $1x10^{-5}$ M on noradrenaline-induced contraction in the Ca^{2+}-containing solution, in Ca^{2+}-free medium and on IRT. The IRT and contractions are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. NA: first addition of NA in Ca^{2+}-containing solution. NA_1: first addition of NA in Ca^{2+}-free medium. IRT: increase in the resting tone. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*).

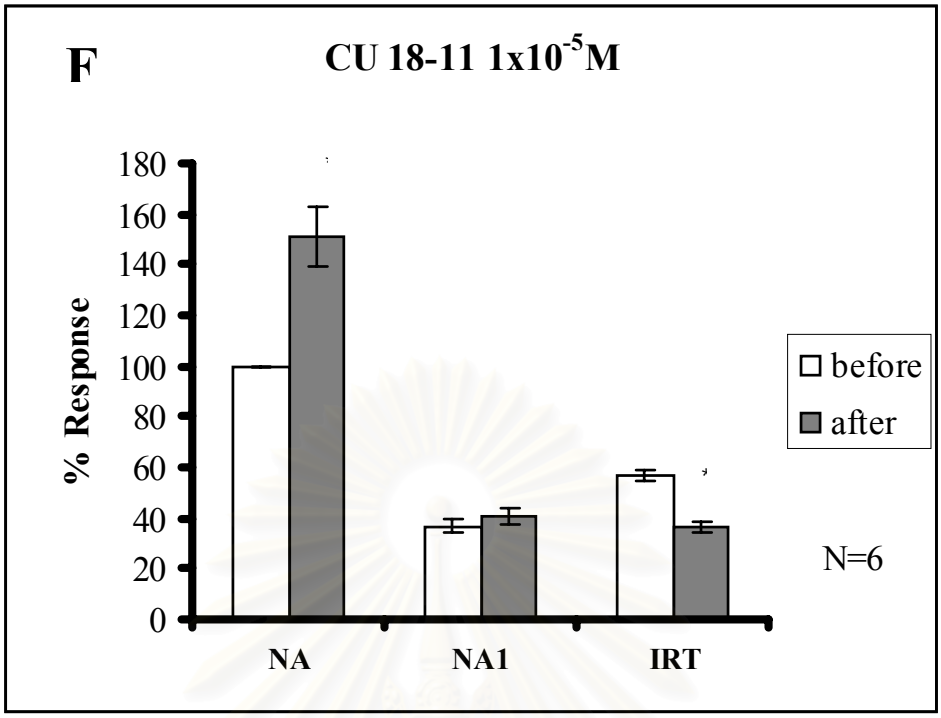


Figure 20F Effects of CU 18-11 $1x10^{-5}$ M on noradrenaline-induced contraction in the Ca^{2+}-containing solution, in Ca^{2+}-free medium and on IRT. The IRT and contractions are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. NA: first addition of NA in Ca^{2+}-containing solution. NA_1: first addition of NA in Ca^{2+}-free medium. IRT: increase in the resting tone. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*).

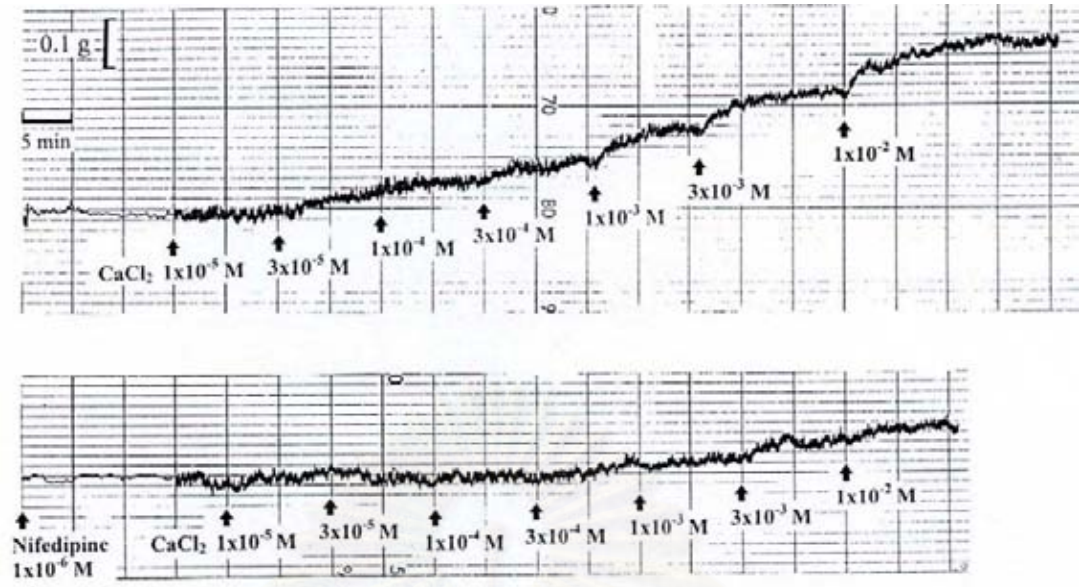


Figure 21 Contraction profiles showing the effect of nifedipine $1x10^{-6}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution.

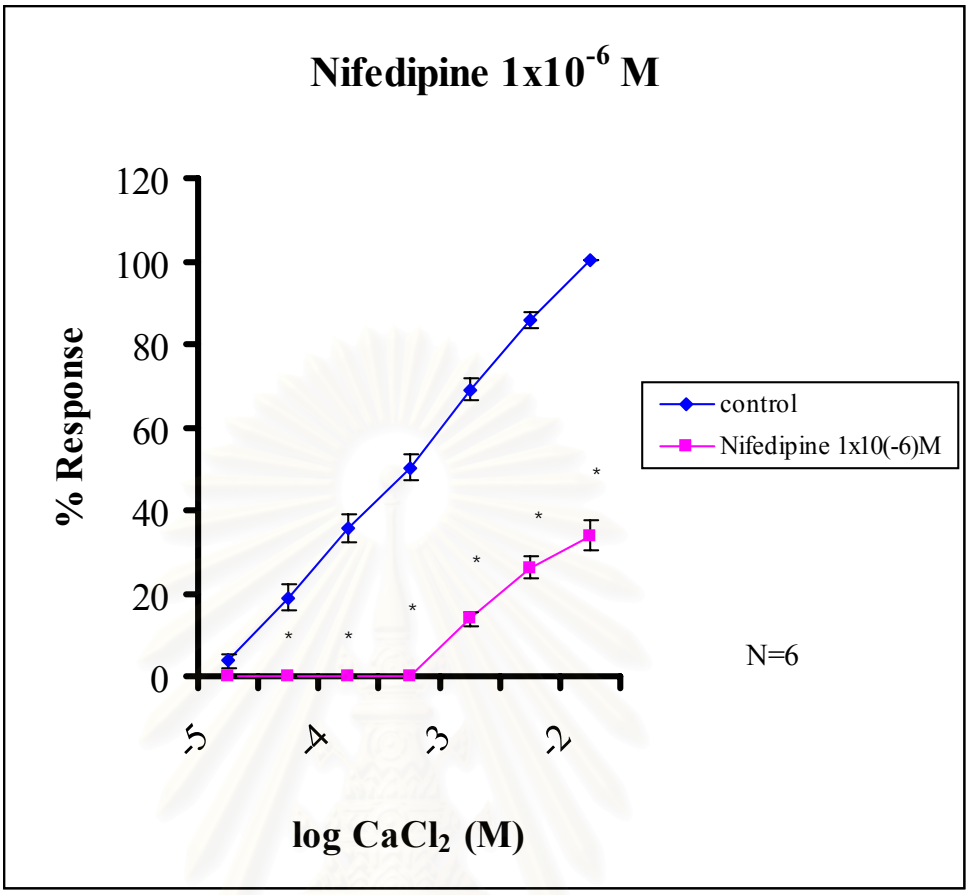


Figure 22 Effects of nifedipine $1x10^{-6}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution. All values represent mean ± S.E. mean, n=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*)

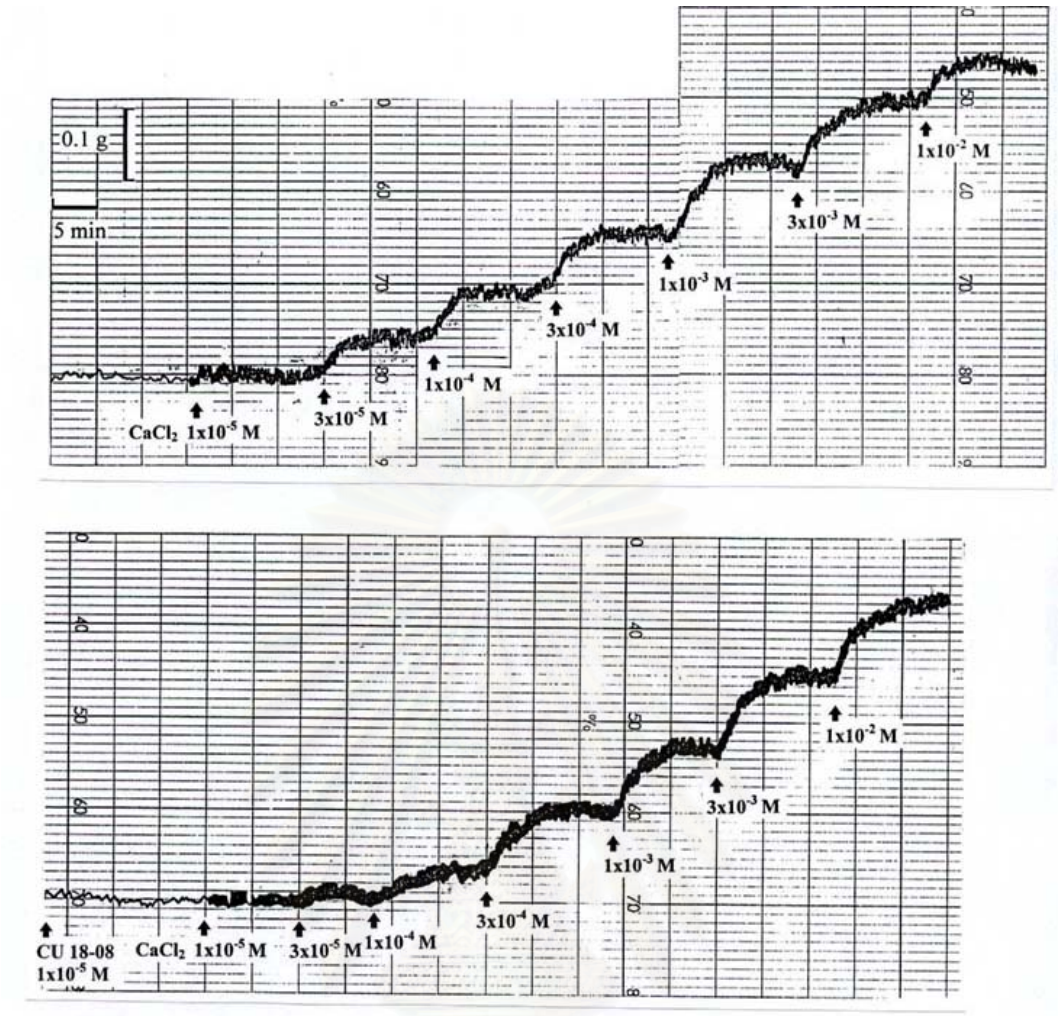


Figure 23 Contraction profiles showing the effect of CU 18-08 $1x10^{-5}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution.

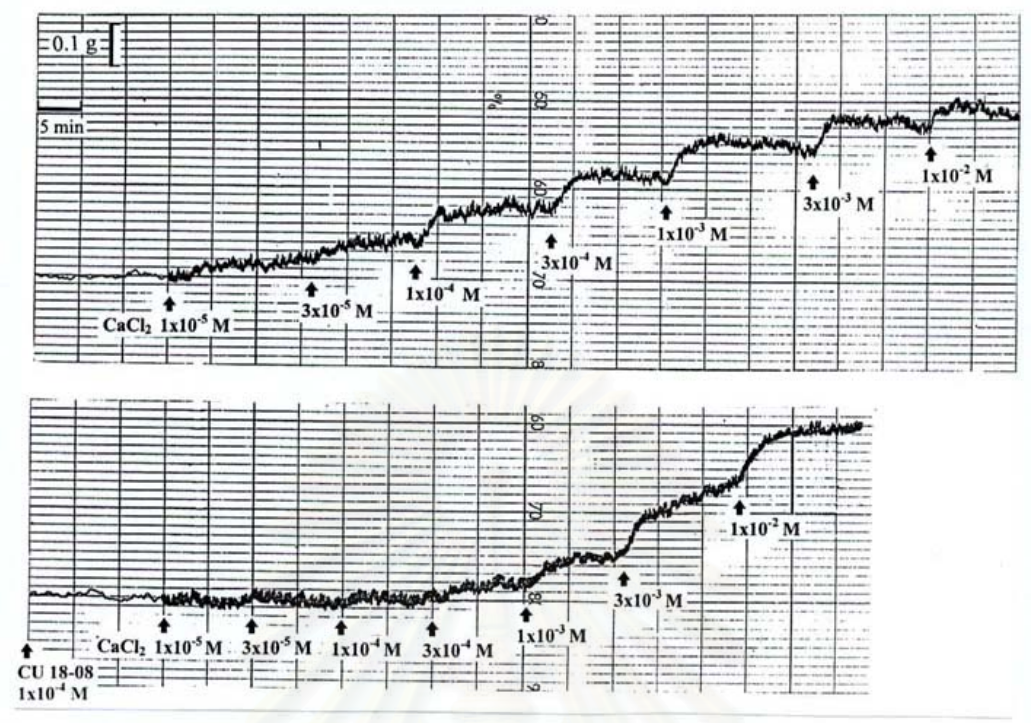


Figure 24 Contraction profiles showing the effect of CU 18-08 $1x10^{-4}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution.

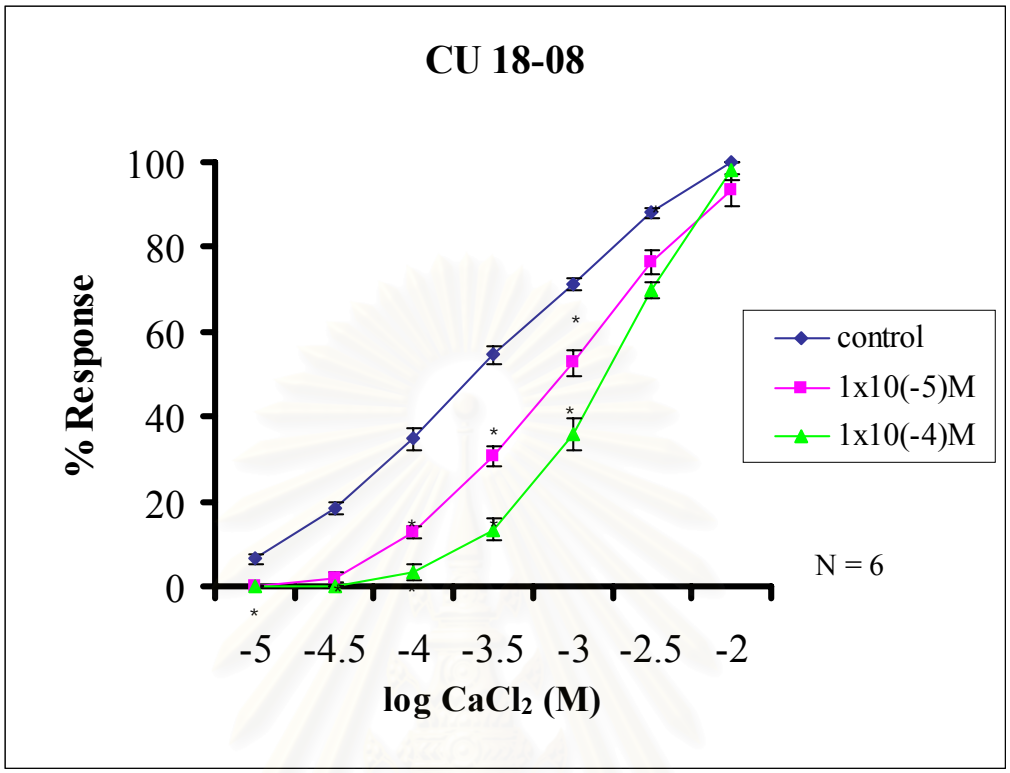


Figure 25 Effects of CU 18-08 $1x10^{-5}$ M and CU 18-08 $1x10^{-4}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*)

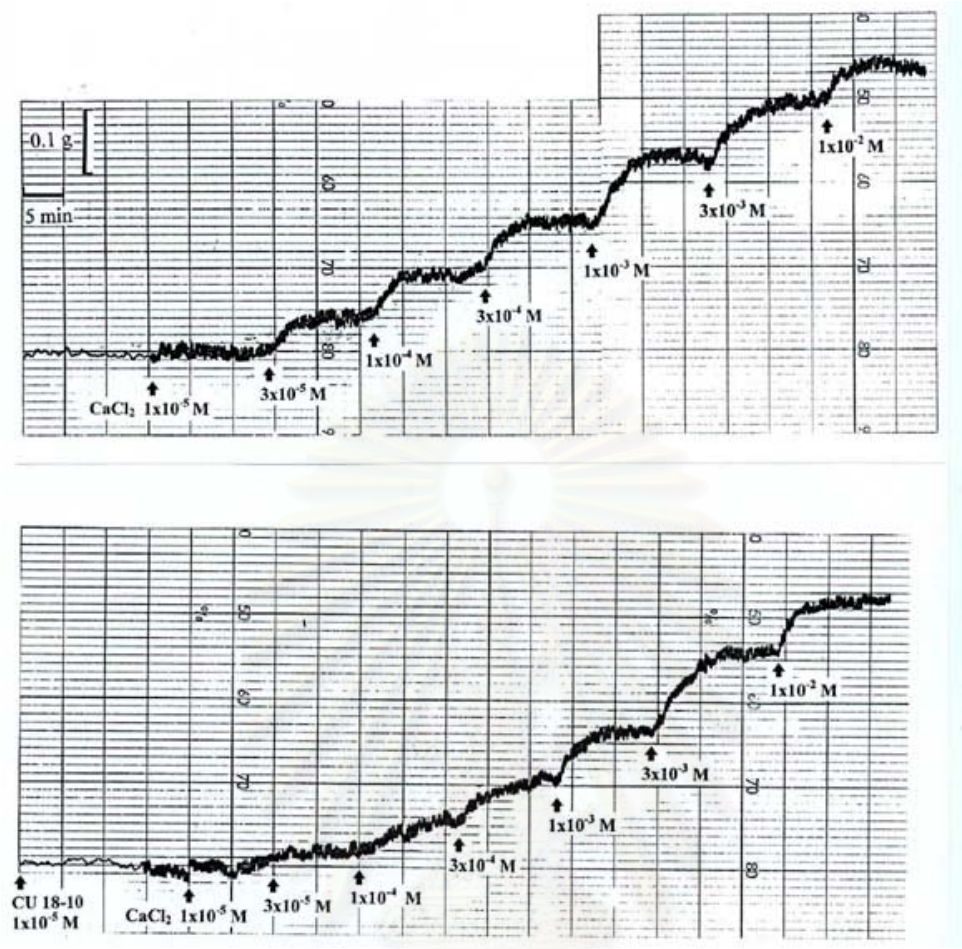


Figure 26 Contraction profiles showing the effect of CU 18-10 $1x10^{-5}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution.

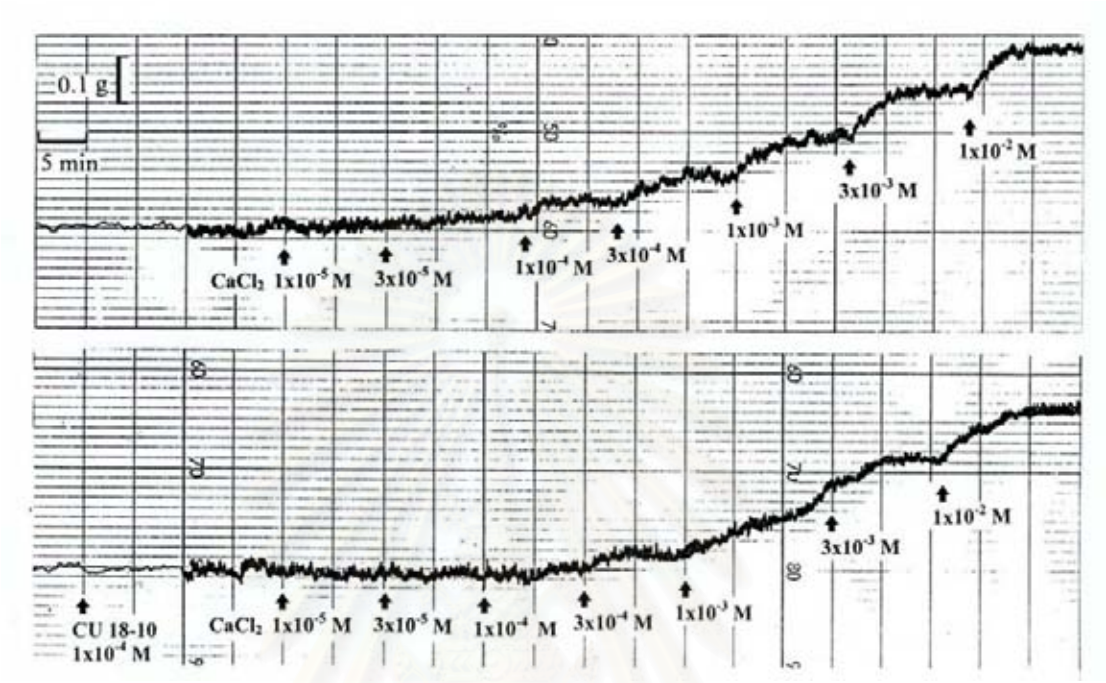


Figure 27 Contraction profiles showing the effect of CU 18-10 $1x10^{-4}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution.

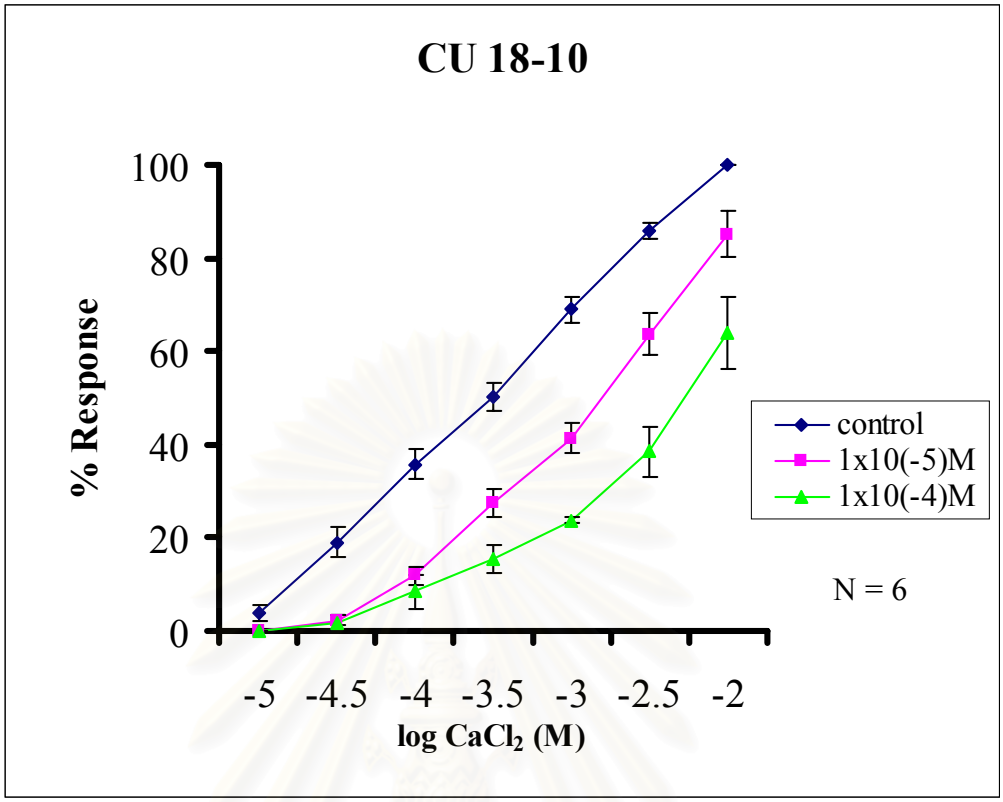


Figure 28 Effects of CU 18-10 $1x10^{-5}$ M and CU 18-10 $1x10^{-4}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*)

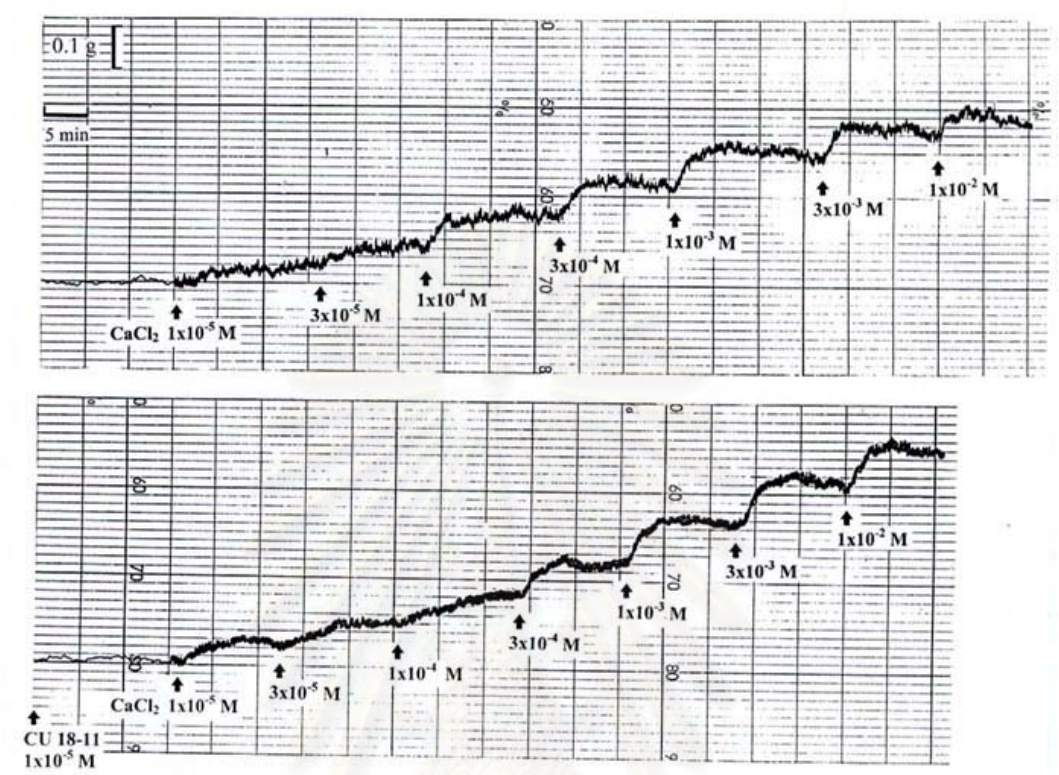


Figure 29 Contraction profiles showing the effect of CU 18-11 $1x10^{-5}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution.

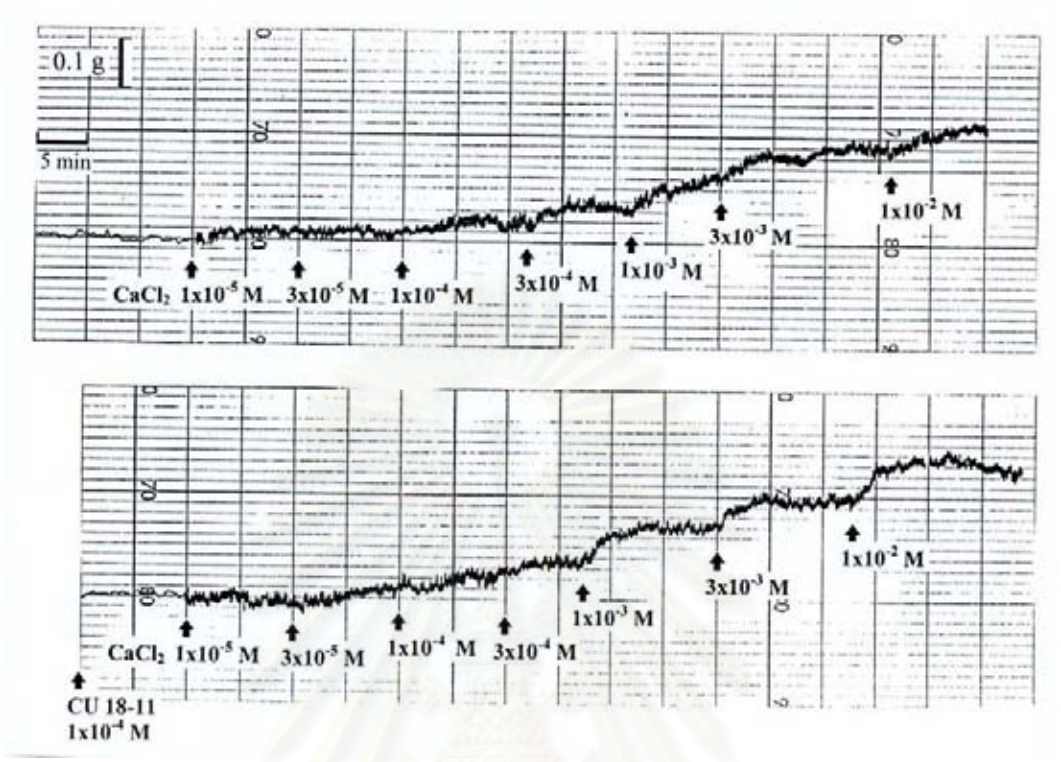


Figure 30 Contraction profiles showing the effect of CU 18-11 $1x10^{-4}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution.

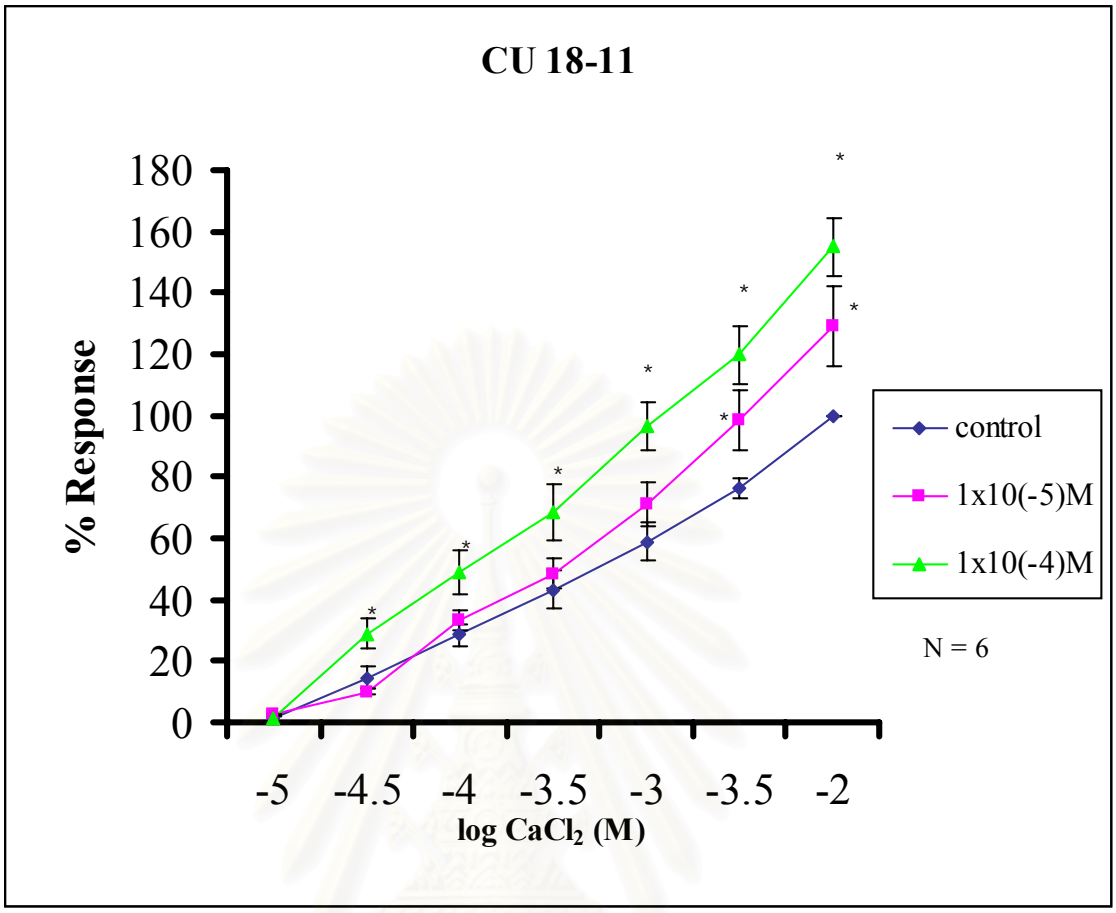


Figure 31 Effects of CU 18-11 $1x10^{-5}$ M and CU 18-11 $1x10^{-4}$ M on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution. All values represent mean ± S.E. mean, N=number of experiments, *$p<0.05$, significantly different from before addition of any tested compound (paired *t-test*)

CHAPTER IV

DISCUSSION AND CONCLUSION

The method to detect IRT that was used in this study has been proved to be useful in prediction of Ca^{2+} movement in vascular smooth muscle (Noguera and D'Ocon, 1992). In this study, the contraction induced by noradrenaline, caffeine and KCl as well as IRT response were performed to verify the research methodology. Our findings are in agreement with those previously reported by other studies (Noguera *et al*., 1996).

In Ca^{2+}-containing solution, noradrenaline generated a contractile response at the greater extent than KCl and caffeine. The difference in characteristic of the peak of contraction can be attributed to the mechanism by which each agonist triggers the contraction. Noradrenaline exerted its effects by releasing intracellular Ca^{2+} from the internal stores and by the Ca^{2+} influx through receptor coupled Ca^{2+} channel as well as voltage-operated Ca^{2+} channel (Rang *et al*., 1999). In case of K^{+}-induced contraction, the mechanism involves membrane depolarization, which cause an opening of the L-type Ca^{2+} channels, an increase of Ca^{2+} influx and intracellular Ca^{2+} (Karaki *et al*., 1997). KCl at the concentration of 40 mM cannot provoke a contraction in Ca^{2+}-free medium. It is possible that 40mM may be insufficient concentration to elevate intracellular Ca^{2+} in the absence of extracellular Ca^{2+}. In another study, the contraction of coronary arteries can be induced by 50 mM of KCl in Ca^{2+}-free medium was reported (Kalsner, 1997).

Contraction response of rat aortic strip to caffeine was markedly different from those induced by noradrenaline and KCl. Caffeine induced a transient smaller contraction. This is due to the effect of caffeine on release of Ca^{2+} from sarcoplasmic reticulum (Watanabe *et al*., 1992). In addition, the contraction profile also show that muscle tension in the presence of caffeine dropped to a level below a resting tone which may be due to an inhibitory effect of caffeine on cyclic AMP phosphodiesterase.

Consequently, caffeine leads to an increase of cyclic AMP in smooth muscle and causes muscle relaxation (Sato *et al*., 1988b, Ahn *et al*., 1988, Fredholm *et al*., 1979).

CU 18-10 inhibits the aortic contraction induced by noradrenaline, KCl and TEA in Ca^{2+}-containing solution. At the same concentration, CU 18-08 also inhibits aortic contraction induced by noradrenaline and KCl to the lesser extent, but not those induced by TEA. This finding suggests that CU 18-08 and CU 18-10 may interfere the entry of Ca^{2+} into intracellular space. In contrast to the inhibitory effect of CU 18-08 and CU 18-10, CU 18-11 potentiated the effect of noradrenaline in activating aortic contraction. However, the increase in contraction was not observed when KCl and TEA were used in place of noradrenaline. It is possible that CU 18-11 may increase Ca^{2+} influx or muscle sensitivity through noradrenaline mediated pathway either at α_1-adrenoceptors and voltage-operated Ca^{2+} channel. Moreover, it is possible that the concentration of CU 18-08 used in the study may not be sufficient to generate the observable effect on TEA-induced contraction. Further dose-response relation may be useful to investigate these effects.

None of the synthetic CU 18-08, CU 18-10, and CU 18-11 compounds at the contraction tested in these studies affected noradrenaline-induced contraction in Ca^{2+}-free medium. These findings suggested that the effects of these compounds were not link to the Ca^{2+} release from intracellular stores and α-adrenoceptor stimulation. In contrast, data suggested that CU 18-11 might potentiate Ca^{2+} influx from extracellular.

Activation of α_1-adrenoceptors promotes Ca^{2+} release from internal stores, which can cause the depletion of Ca^{2+}. These intracellular Ca^{2+} pools, when emptied, can be rapidly replenished, from the extracellular space, by incubation in Ca^{2+}-containing solution. In agreement with IRT hypothesis, this study show that only the aortic strips pretreated with noradrenaline but not KCl and caffeine resulted in subsequent IRT formation.

CU 18-08 and CU 18-10 inhibited the IRT but CU 18-11 did not. These findings suggested that CU 18-08 and CU 18-10 interfered the mechanical refilling process of Ca^{2+} into intracellular pools. It is possible that these two compounds may have pharmacological effect on membrane Ca^{2+}channels coupled to α-adrenoceptor. In addition, DMSO at the contraction of 0.03% (v/v), which has no observable effect on aortic contractions show a significantly inhibitory effect on IRT formation. The mechanistic effects of DMSO on aortic tissue are unclear and further research on this topic is needed.

To confirm the effects of the three compounds on Ca^{2+} influx, the method proposed by Hof and Vuorela (1983) was used. This method was to specifically induce Ca^{2+} entry from the extracellular to intracellular compartment by depolarizing membrane in the presence of $CaCl_2$. These experiments were able to show that CU 18-08 and CU 18-10 inhibited the rat aortic contraction by inhibiting Ca^{2+} entry to the cell. In contrast, CU 18-11 increased the rat aortic contraction, suggesting this compound increased Ca^{2+} entry to the cell.

In general, the plot of $CaCl_2$ and percentage of contraction (concentration-response curves) may suggest type of inhibition (Rang *et al*., 1999). In contrast to nifedipine, CU 18-08 and CU 18-10 show the characteristic of competitive inhibitor of Ca^{2+} influx.

In addition, the pA_2 values suggested that CU 18-10 was more potent than CU 18-08 in the inhibition of Ca^{2+} entry. In contrast, CU 18-11 significantly increased $CaCl_2$-induced contraction of rat aorta in Ca^{2+}-free depolarizing solution. Taken together, the results suggested that CU 18-08 and CU 18-10, but not CU 18-11, were Ca^{2+}-channel blocker. And the synergistic effect of CU 18-11 on noradrenaline-induced contraction, suggested that CU 18-11 was strongly related to an increase of Ca^{2+} entry to intracellular compartment.

These difference in functional group at meta-position and phenyl ring can contribute to the effect of these three synthetic compounds on aortic contraction. The change of functional group from -OCH_3 group to -NO_2 group on phenyl ring appears to increase the potency of the compound in inhibiting the Ca^{2+} influx. In addition, the pyridine ring instead of phenyl ring with functional group appears to increase Ca^{2+} influx.

Conclusions

CU 18-08 and CU 18-10 may suppress the vascular smooth muscle resting tone by interfering the Ca^{2+} entry through the voltage-operated Ca^{2+}channels. In contrast, CU 18-11 did not affect the vascular smooth muscle resting tone. CU 18-11 increased the aortic response to noradrenaline stimulation, which may be due to an increase in Ca^{2+} influx into the cell at voltage-operated Ca^{2+} channels.

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APPENDIX

Table 5 Compound of Physiological solutions (mM/L)

Chemical	Physiological solution		
	Kreb Henseleit	Ca^{2+}-free Krebs Henseleit	Potassium Depolarizing
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

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