Effects of *Curcuma aeruginosa* Roxb. Extracts on Rat Uterine Contraction

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ชื่อวิทยานิพนธ์ ผลของสารสกัดว่านมหาเมฆต่อการบีบตัวของมดลูกในหนูแรท

ผู้เขียน นางสาวภัทรียา ตั้งเจริญ

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บทคัดย่อ

Curcuma aeruginosa Roxb. มีชื่อเรียกในภาษาไทยว่า ว่านมหาเมฆ หรือ กระเจียวแดง ในทางการแพทย์แผนไทยมีการนำเหง้าของพืชนี้มาใช้ในตำรับยาสตรีเพื่อทำให้มดลูกเข้าอู่ ลดอาการปวดประจำเดือน และอาการปวดมดลูกอื่นๆ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์และกลไกการออกฤทธิ์ของสารสกัดจากเหง้าของว่านมหาเมฆต่อการบีบตัวของมดลูกของหนูขาวที่แยกออกจากร่างกาย โดยทำการวัดและบันทึกการบีบตัวแบบ isometric ด้วย force transducer (รุ่น FT 03) ซึ่งต่อกับระบบคอมพิวเตอร์ MacLab เพื่อบันทึกและแสดงการบีบตัว ทำการสกัดสารด้วยคลอโรฟอร์ม เมทานอล และน้ำ โดยในการสกัดสาร จะทำการแบ่งผงพืชแห้งเป็นสองส่วน ส่วนแรกนำมาสกัดแบบลำดับขั้นด้วยคลอโรฟอร์ม และเมทานอล พืชอีกส่วนหนึ่งนำมาสกัดด้วยน้ำ จากนั้นนำสารสกัดมาทดสอบฤทธิ์ต่อการบีบตัวของมดลูกทั้งในขณะที่ไม่ถูกกระตุ้น และเมื่อถูกกระตุ้นให้บีบตัวด้วยสารกระตุ้นต่างๆ ผลการทดลองพบว่า ในขณะที่มดลูกไม่ถูกกระตุ้น สารสกัดน้ำ (10-1760 มคก./มล.) ทำให้มดลูกบีบตัวในลักษณะที่แปรผันตามขนาดของสารสกัด โดยทำให้มดลูกบีบตัวด้วยความแรงสูงสุด 2.93 กรัม และความถี่ 3 ครั้งใน 10 นาที แต่ความสามารถในการตอบสนองต่อสารสกัดของมดลูกจะลดลงอย่างรวดเร็ว ส่วนสารสกัดเมทานอล

และคลอโรฟอร์มไม่แสดงผลใดๆ ในทางตรงกันข้าม สารสกัดทั้งสามชนิดมีฤทธิ์ยับยั้งการบีบมดลูกที่เกิดจากการกระตุ้นด้วยสารต่างๆ สารสกัดน้ำมีความสามารถในการยับยั้งต่ำที่สุด โดยสามารถลดได้เฉพาะความถี่ในการบีบตัว (ประมาณ 25%) ที่เกิดจากการกระตุ้นด้วย oxytocin (0.1 มิลลิยูนิต/มล.) ส่วนสารสกัดคลอโรฟอร์มและเมทานอล (10-400 มคก./มล.) สามารถยับยั้งการบีบตัวของมดลูกที่เกิดจากการกระตุ้นด้วย oxytocin (1 มิลลิยูนิต/มล.), $PGF_{2\alpha}$ (0.5 มคก./มล.), ACh (3×10^{-6} โมลาร์) และ KCl (40 มิลลิโมลาร์) ได้อย่างสมบูรณ์ หรือยับยั้งได้บางส่วน โดยมีค่า IC_{50} (ในการลดความแรงในการบีบตัว) เท่ากับ 31.4, 58.59, 56.21, 29.28 มคก./มล. และ 57.79, 69.3, 223.8, 69.19 มคก./มล. ตามลำดับ ยามาตรฐาน verapamil ซึ่งมีฤทธิ์ปิดกั้นช่องทางแคลเซียมชนิดแอล สามารถยับยั้งการบีบตัวของมดลูกได้เช่นเดียวกับสารสกัด โดยมีค่า IC_{50} เท่ากับ 0.03, 0.25, 0.35, 0.04 มคก./มล. ตามลำดับ ส่วน IC_{50} ของ diclofenac ในการยับยั้งการบีบตัวที่เกิดจาก $PGF_{2\alpha}$ และ KCl มีค่าเท่ากับ 31.36 และ 28. 79 มคก./มล. ตามลำดับ เป็นที่ทราบกันดีว่าการบีบตัวที่เกิดจากตัวกระตุ้นต่างๆ ข้างต้น และ KCl เป็นผลมาจากการที่ตัวกระตุ้นเหล่านี้ทำให้มีการเปิดของช่องทางแคลเซียมชนิดแอล โดยทางตรงหรือโดยทางอ้อม นอกจากนี้สารสกัดคลอโรฟอร์ม สารสกัดเมทานอล และ verapamil ยับยั้งการบีบตัวของมดลูกที่เกิดจากการกระตุ้นด้วยแคลเซียมคลอไรด์ โดยทำให้เส้นกราฟในการตอบสนองต่อแคลเซียมคลอไรด์เคลื่อนไปทางขวาอย่างมีนัยสำคัญ ดังนั้นจึงอาจเป็นไปได้ว่าฤทธิ์ในการยับยั้งการบีบตัวดังกล่าวของสารสกัดทั้งสองชนิด เป็นผลมาจากการขัดขวางการนำแคลเซียมเข้าสู่เซลล์ผ่านทางช่องทางแคลเซียมชนิดแอลเช่นเดียวกับ verapamil การทดลองในครั้งนี้ยังแสดงให้เห็นว่าสารสกัดคลอโรฟอร์ม และสารสกัดเมทานอลยับยั้งการบีบมดลูกที่เกิดจากการกระตุ้นด้วย oxytocin ในสารละลายที่ปราศจากแคลเซียมและมี EDTA อยู่ด้วย และทั้งสารสกัดคลอโรฟอร์ม และเมทานอล

สามารถยับยั้งการหดตัวที่ถูกกระตุ้นด้วย vanadate ในสารละลายที่ปราศจากแคลเซียมและมี EDTA แสดงว่าส่วนหนึ่งในการออกฤทธิ์ของสารสกัดน่าจะเป็นกลไกภายในเซลล์ของกล้ามเนื้อมดลูก โดยอาจยับยั้งการหลั่งแคลเซียมอิออนจากแหล่งเก็บภายในเซลล์ ฤทธิ์ในการทำให้มดลูกคลายตัวของสารสกัดทั้งสองชนิดไม่ได้เกิดจากการกระตุ้นที่ β_2-adrenoceptors เนื่องจากเมื่อเปรียบเทียบกับยามาตรฐาน isoproterenol พบว่า propranolol ซึ่งเป็น non selective β-adrenoceptors antagonist ไม่สามารถยับยั้งฤทธิ์ของสารสกัดในการทำให้มดลูกคลายตัวได้ โดยลำดับความแรงในการออกฤทธิ์ยับยั้งการบีบตัวของมดลูกเมื่อเปรียบเทียบกับยามาตรฐานคือ verapamil > diclofenac > สารสกัดคลอโรฟอร์ม > สารสกัดเมทานอล >> สารสกัดน้ำ จากการที่สารสกัดทั้งสองชนิดสามารถยับยั้งการบีบมดลูกที่เกิดจาก oxytocin ได้ น่าจะมีประโยชน์ในการป้องกันการคลอดก่อนกำหนด การที่สารสกัดทั้งสองชนิดสามารถยับยั้งการบีบตัวที่เกิดจากการกระตุ้นด้วย $PGF_{2\alpha}$ แสดงว่าสารสกัดน่าจะมีประโยชน์ในการใช้รักษาอาการปวดประจำเดือน ส่วนฤทธิ์ในการกระตุ้นให้มดลูกบีบตัวของสารสกัดน้ำ น่าจะมีประโยชน์ในการป้องกันการตกเลือดหลังคลอดและช่วยเร่งให้มดลูกเข้าอู่ เมื่อเปรียบเทียบความแรงในการออกฤทธิ์ยับยั้งการบีบมดลูก พบว่า สารสกัดคลอโรฟอร์ม > สารสกัดเมทานอล >> สารสกัดน้ำ มีรายงานระบุว่าเหง้าของว่านมหาเมฆมีสารพวก monoterpene เช่น 1,8 cineol, α-pinene, β-pinene, และ sesquiterpene อีกหลายชนิด เช่น dehydrocurdione และมีผู้รายงานว่า α-pinene, β-pinene และ sesquiterpene มีฤทธิ์ในการทำให้ลำไส้เล็กที่ถูกกระตุ้นให้บีบตัวด้วย ACh และ KCl และมดลูกของหนูแรทที่ถูกกระตุ้นให้บีบตัวด้วย oxytocin และ KCl นั้นคลายตัว แสดงว่าฤทธิ์ในการคลายตัวของสารสกัดทั้งสามชนิดนี้อาจเกิดจากสาร monoterpene และ sesquiterpene เหล่านี้ แต่เนื่องจากสารเหล่านี้ละลายน้ำได้น้อย จึงอาจเป็นสาเหตุที่ทำให้สารสกัดน้ำมีความแรงในการออก

ฤทธิ์ต่ำที่สุด ส่วนฤทธิ์ในการที่สารสกัดน้ำทำให้มดลูกที่อยู่ในระยะพักบีบตัว อาจจะเกิดจาก 1,8 cineol, α-pinene, β-pinene ขนาดน้อยที่เป็นส่วนประกอบในสารสกัด เนื่องจากมีผู้รายงานไว้ว่าสารเหล่านี้มีฤทธิ์ในการทำให้ลำไส้เล็กของหนูขาวในระยะพักบีบตัวได้เช่นกัน

Thesis Title	Effects of *Curcuma aeruginosa* Roxb. Extracts on Rat Uterine Contraction
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ABSTRACT

Curcuma aeruginosa Roxb. (Zingiberaceae) is commonly known in Thai as Waan-Maa-Haa-Mek or Kajeawdang. Its rhizome has been used in Thai traditional medicine for postpartum uterine involution, treatment of dysmenorrhea and other uterine pains. The effects and plausible mechanism of action of the chloroform, methanol and water extracts from *C. aeruginosa* rhizome on the uterine contraction were investigated using isolated uterus strips from estrogen primed rats. Powder-dried rhizome was divided into two parts. One part was sequential extracted with chloroform and methanol. The other dried rhizome was extracted with water. The contractile responses were recorded isometrically with a Grass FT03 force transducer connected to a MacLab system. The experiments were carried out on both nonstimulated, agonist- and KCl-stimulated uteri. In the nonstimulated uterus, the water extract (10-1760 μg/ml) produced a concentration-dependent contraction of the uterus with the maximum increase in force and frequency = 2.93 g and 3 contractions/10 min, respectively. However, the effect was subjected to quick desensitization, whereas the chloroform and methanol extracts (10-400 μg/ml) had no significant stimulating effect. In contrast, in the agonist or KCl-stimulated uterus, the three extracts exhibited inhibitory effects. The water extract had a weak inhibitory effect and decreased the frequency (by about 25%) of the contraction-induced by 0.1 mU/ml oxytocin and showed no significant effect when the uterus was contracted by a higher concentration (1 mU/ml) of oxytocin. The chloroform and methanol extracts

exerted concentration-dependent and completely or partially inhibited the contraction induced by oxytocin (1 mU/ml), $PGF_{2\alpha}$ (0.5 μg/ml), ACh (3 μM) and KCl (40 mM) with the IC_{50} (in inhibition of force) of 31.40, 58.59, 56.21, 29.28 μg/ml and 57.79, 69.30, 223.80, 69.19 μg/ml, respectively. The reference L-type calcium channel blocker, verapamil exhibited similar pattern of inhibition with the IC_{50} of 0.03, 0.25, 0.35 and 0.04 μg/ml, respectively. IC_{50} of diclofenac against $PGF_{2\alpha}$ and KCl-induced contraction was 31.36 and 28.79 μg/ml, respectively. It is known that the contraction induced by agonists and KCl is mainly due to the calcium influx through the voltage-gated L-type calcium channels which opened indirectly or directly by agonist-receptor activation and KCl. Thus, it is speculated that the plant extracts might inhibit uterine contraction by interrupting the influx of Ca^{2+} probably through voltage-gated L-type calcium channels. This possibility was further substantiated by the ability of the extracts to shift the $CaCl_2$-contraction curves to the right. Our study also demonstrated that the methanol extract also reduced the contraction of oxytocin in Ca^{2+}-free EDTA solution. In addition, both chloroform and methanol extracts inhibited vanadate-induced contraction in Ca^{2+}-free EDTA solution. Thus, it is suggested that part of its action may involve an intracellular mechanism. The effect of the chloroform and methanol extracts did not involve the activation of β_2 –adrenoceptors since their effects were unaffected by propranolol. The order of potency of uterine relaxing action of both extracts compared with those of reference drug is verapamil > diclofenac > chloroform extract > methanol extract >> water extract. Based on the inhibitory effect of the chloroform and methanol extracts on the oxytocin-induced contraction, it is possible that the extracts might be useful as tocolytic agents for the prevention of preterm labour. Their effects on the inhibition of $PGF_{2\alpha}$-induced contractions also could be useful for the treatment of dysmenorrhea. In addition, the stimulating effect of water extract might be useful for the prevention of postpartum hemorrhage and accelerate uterine involution. There are reports by others that the plant rhizome contains 1,8 cineol, α-pinene, β-pinene, curzerenone and other sesquiterpenes. Furthermore, there is evidence that α-pinene, β-pinene and some sesquiterpenes possesses spasmolytic effects in the rat intestine and uterus, and that

may underlie the uterine relaxant effect in our study could be due to their actions. Because of the sequence of extraction, the methanol extract may contain smaller amount of terpene compounds. Thus, the methanol extract exhibited less relaxing potency. The weak inhibitory effect of water extract on the contraction-induced by lower concentration of oxytocin could also be due to lowest amount of monoterpene and sesquiterpenes. Nevertheless, The contractile effect of the water extract in nonstimulated uterus could be due to α-pinene, β-pinene, and 1,8 cineol which are monoterpene compounds previously reported to produce contraction in nonstimulated intestine and uterus.

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LIST OF ABBREVIATIONS AND SYMBOLS

5-HT	=	Serotonin
ACh	=	Acetylcholine perchlorate
AT	=	Atropine
ATPase	=	Adenosine triphosphatase
Ca^{2+}	=	Calcium ion
$[Ca^{2+}]_i$	=	Intracellular free calcium ion
Ca-CAM	=	Calcium-calmodulin complex
CAM	=	Calmodulin
CAMK II	=	Calmodulin kinase II
cAMP	=	Cyclic Adenosine monophosphate
cGMP	=	Cyclic guanosine monophosphate
CICR	=	Calcium induced calcium release
CIF	=	Calcium influx factor
CRAC	=	Calcium release activated calcium channels
C. aeruginosa	=	*Curcuma aeruginosa* Roxb.
DAG	=	Diacylglycerol
DMSO	=	Dimethylsulfoxide
E_{max}	=	Maximum effect
eNOS	=	Endothelial nitric oxide synthase
IC_{50}	=	50% Inhibitory concentration
DC	=	Diclofenac
InsP	=	Inositol phosphate
iNOS	=	Inducible nitric oxide synthase
IP_3	=	Inositol 1, 4, 5 triphosphate
$InsP_3Rs$ or IP_3Rs	=	Inositol triphosphate receptor
MLCK	=	Myosin light chain kinase
$[Na]_o$	=	Extracellular sodium ion

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

nNOS	=	Neural nitric oxide synthase
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NSAIDs	=	Non-steroidal anti-inflammatory drugs
OXY	=	Oxytocin
OXYR	=	Oxytocin receptor
PGE_2	=	Prostaglandin E_2
$PGF_{2\alpha}$	=	Prostaglandin $F_{2\alpha}$
PIP_2	=	Phosphatidyl inositol-4, 5-biphosphate
PLA_2	=	Phospholipase A_2
PLC	=	Phospholipase C
(PMCA)s	=	Plasmalemma Ca^{2+}-ATPases
ROCCs	=	Receptor-operated calcium channels
RyRs	=	Ryanodine receptors
SERCA	=	Sarcoplasmic reticulum Ca^{2+}-ATPases pump
SOCCs	=	Store-operated calcium channels
SR	=	Sarcoplasmic reticulum
VOCCs	=	Voltage-operated calcium channels

CHAPTER 1

INTRODUCTION

Nowadays, the most frequently used herbs derived from the Zingiberaceae family. In some countries they have been used either as spices in cooking or herbal drug in traditional medicines for centuries, especially in the Asian region. Medicinal plants in *Curcuma* genus of Zingiberaceae e.g. *Curcuma longa* Linn., *Curcuma domestica* Val., *Curcuma xanthorrhiza* Roxb. and *Curcuma zedoaria* Christm. Roscoe are gaining importance worldwide as a potential source of new drugs for combat a variety of ailments. The plants contain many chemical constituents credited with many pharmacological activities such as antioxidant, anti-inflammatory, antirheumatic, hypocholesterolemic, choleratic, antimicrobe, anti-diarrhea, antivirus, carminative, anti-inflatulent, antispasmodic, deliborative of digestive organs, hepatoprotective, anti-venom, antidiabetic, anticancer, cough relief as well as insect repellent. The turmeric oil of *Curcuma longa* is also used in aromatherapy and in the perfume industry. Furthermore some species have been used as common cosmetic, antieczema, antipruritic, poultice for acne, antidermatitis, wounds and ulcer scrubbing, and sprain relieving (Sirirugsa, 1992; Jantan *et al*., 2003; Asean Regional Centre for Biodiversity Conservation (ARCBC), 2004; Sasikumar, 2005)**.**

Curcuma aeruginosa (C. aeruginosa) **Roxb.** is one of the plants in Zingiberaceae family. The plant is native to Myanmar (Srivastava *et al*., 2006). It is distributed in Southeast Asia, including, Cambodia, Vietnam, Malaysia, Indonesia and Thailand (Sirirugsa, 1992; Newman *et al*., 2004). The plant is distinguished by red corolla lobes and ferruginous or greenish-blue rhizome, so it is named pink and blue ginger in English (Sirirugsa, 1992; Srivastava *et al*., 2006). In Thailand, it is commonly called Waan-Maa-Haa-Mek, Kajeawdang or Kha min dum.

C. aeruginosa has been used in traditional medicine in many countries. In 1966, Burkill reported in A Dictionary of the Economic Products of the Malay

Peninsular that it was used in the treatment of asthma, cough, dermatosis, insanity, parturition, purgative, scabies and scurf. It was also added into a beverage given to women in confinement to accelerate the lochia and decrease pain and inflammation of uterus (reviewed by Reanmongkol *et al*., 2006). It is present as an active ingredient in many Thai herbal preparations used as a tonic to alleviate female problems such as irregular, painful or excessive menstruation and for uterine pain or dysfunction (Wuthamawej, 1997; Asean Regional Centre for Biodiversity Conservation (ARCBC), 2004). In the study of medicinal plants in Khonsarn district, Chiyaphum province (II) carried out by interviewing the herbalists, it is reported that the rhizomes of *C. aeruginosa* have been applied locally as the remedy of snake and insect bites (Chuakul *et al*., 1995). The alcoholic macerate of dried *C. aeruginosa* rhizome has been used to hasten the uterine contraction in postpartum women. It is usually ingested once or twice or even thrice daily (reviewed by Matangkasombut *et al.*, 1983).

As the biological activity ascribed to *C. aeruginosa* is largely based on empirical data, more research is required to scientifically prove its action and efficacy. The present study aimed to investigate the effect and plausible mechanism of action of extracts from the plant on uterine contractions using isolated rat uteri. The experiments were carried out on both nonstimulated uterus tissues as well as uterine tissue contractions induced by some agonists (e.g. oxytocin, prostaglandin $F_{2\propto,}$ acetylcholine). The results will be useful as a scientific evidence to support its use in traditional medicine and for further *in vivo* study.

CHAPTER 2

LITERATURE REVIEWS

Curcuma aeruginosa Roxb.

C. aeruginosa is a perennial plant with roots or tubers oblong, leafy shoots are 45-60 cms high, a purplish patch along either side of the midrib on upper side. *C. aeruginosa* has the typical burgundy mid-stripe on the leaves as several other species, but the stripes do not fade with age as much as the others. This makes it an especially valuable foliage plant; because the tall arching leaves retain the color and shape of their midrib feather through the entire summer (Figure 2-1) (Skinner, 2005; Srivastava *et al*., 2006).

Figure 2-1 *C. aeruginosa* leaves with purple midrib; *a.* by Stamper, A. and *b.* by Skinner, D. Available at: www.gingersrus.com. [Accessed: Dec 25, 2006.]

Owing to the rhizomes which have pink tips and bluish-grey center (Figure 2-2), as distinct from the yellow or orange color of most species of the genus. It is named as pink and blue ginger (reviewed by Sirat *et al*., 1998; Skinner, 2005).

The plant is a spring bloomer, which occasionally blooms in summer and the pink flowers on a short inflorescence forth before the leaves emerge (Figure 2-3). Like other curcumas, it go into a natural dormancy in late fall; the leaves will yellow and die back. It is well growth in drained organic soil in light shade to part shade. Like the cultivated plant, it has enlarged rhizomes which are the source of spicy (Skinner, 2005). Fresh rhizomes emit the aroma ginger like and mildly aromatic. Transverse section of rhizome is circular in outline. Epidermal cells are rectangular in shape covers with thick cuticle; long unicellular trichomes present (Srivastava *et al*., 2006).

Figure 2-2 *C. aeruginosa* rhizome with pink tips and blue center. Available at: www.gingersrus.com [Accessed: Dec 25, 2006].

Figure 2-3 *C. aeruginosa* flower. Available at: www.thailandgeo-graphic.com [Accessed: Dec 25, 2006].

Pharmacological Studies

Postcoital contraceptive effect

The alcoholic macerate of *C. aeruginosa* has been reported to posses postcoital contraceptive effect in albino rats when orally administered from day-1 to day-5, and the efficacy increase with the increasing dose. But the water extract had no effect (Matangkasombut *et al.*, 1983).

Anti-HIV

Aqueous extract of *C. aeruginosa* rhizome from Indonesia was shown to be effective in inhibition of HIV protease and reverse transcriptase in HIV-1 induced cytopathogenicity of MT-4 cells (Otake *et al.,* 1995).

Hepatoprotective

The water extract of *C. aeruginosa* has significant protective effect on carbon tetrachloride induced liver injury in rat by decreasing the acute increase in serum enzyme levels. In the investigation of inducing immunological liver injury in mice, it effectively protected effect on liver cells (by measuring the level of GPT to indicate the extent of liver damage) and decrease mortality rate as compare to the control group (Hase *et al*., 1996).

Antimicrobial activity

Curdione, the constituent of petroleum ether extract from *C. aeruginosa* rhizome showed an inhibitory effect against *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Van *et al*., 1997).

Lethal effect

The methanolic extract of *C. aeruginosa* rhizome had strong lethal effects on *Callosobruchus chinensis* adults (Riyanto *et al.*, 1998).

Antioxidant activity

On β-carotene bleaching method and classification based on antioxidant content reported as butylated hydroxyanisole equivalent (BHA eq.), high antioxidative potency of *C. aeruginosa* flower methanolic extract (> 100 mg. BHA eq. in 100 g fresh vegetable) was analyzed (Trakoontivakorn and Saksitpitak, 2000).

Antiplatelet-activating factor

The methanolic extract of *C. aeruginosa* and other Malaysian medicinal plants were investigated for their inhibitory effects on platelet-activating

factor (PAF) binding to rabbit platelets. Some of them are potential sources of new PAF antagonists and the methanolic extract of *C. aeruginosa* showed significant inhibitory effects with IC_{50} values of 5.8 ± 3.1 μg/ml (Jantan *et al.*, 2005).

Antinociceptive activity

Reanmongkol *et al*., (2006) investigated the pharmacological activity of aqueous, chloroform and methanol extracts of *C. aeruginosa* rhizomes. They reported that oral administration of the chloroform and methanol extracts (100-400 mg/kg) of *C. aeruginosa* significantly decreased the number of writhing and stretching induced by acetic acid. Only the chloroform extract suppressed the licking activity of the late phase in the formalin test in mice. All extracts of *C. aeruginosa* rhizomes had no effects on heat-induced pain in mice, yeast-induced fever and carrageenin-induced edema in rats. The results suggest that the chloroform and methanol extract possess analgesic effects via a different mechanism from that of the aspirin. It is concluded that the extracts attenuated nociceptive responses to chemical stimuli through peripheral but not central mechanism(s) in the manner that of chloroform extract has higher potency than those of methanol extract.

Lethal Dose

The LD_{50} of the chloroform extract and methanol extract of *C. aeruginosa* in mice was 3.03 g/kg. No dead mouse was observed after oral administration of the aqueous extract at the dose of 10 g/kg (Reanmongkol *et al*., 2006).

Phytochemical data

Many countries in Southeast Asia cultivate and locally use *C. aeruginosa* in their traditional medicines. Its main chemical compositions are essential oils which have been isolated and reported (see Table 2-1).

Table 2-1 Essential oils of *C. aeruginosa*

	Country	Indonesia	China	Malaysia		Vietnam	Thailand		Vietnam	Malaysia	India		Vietnam
% of Essential oil		Rhizome										Leaves	
	β-pinene	6.5	-	-	-	-	7.7	-	-	-	-	-	-
	α-pinene	-	-	-	-	-	-	-	-	-	2.4	-	-
	dyhydrocurdione	9.4	-	-	-	-	-	-	-	R	-	-	-
	camphor	-	-	10.6	-	-	-	16.9	-	-	15.6	7.5	R
	1, 8-cineol	-	-	11.0	23.2	R	9.6	-	-	-	-	17.7	R
	curzerenone	-	H	24.6	28.4	R	41.6	16.8	R	-	-	10.5	R
	zedoarol	-	-	6.3	-	-	-	-	-	R	-	-	-
	curcumenol	9.9	-	5.6	-	-	-	-	R	-	-	4.3	R
	curcumenone	1.9	-	-	-	-	-	-	-	R	-	-	-
	epicurzerenone	-	-		-	-	-	3.5	-	-	-	-	R
	β-elemene	-	-	-	-	-	-	-	R	-	-	3.3	R
	furanogermenone	-	-	5.5	-	-	-	-	-	-	-	7.8	R
	furanodienone	-	-	-	-	-	-	-	R	R	-	5.1	R
	curdione	3.6	-	-	-	-	-	-	R	-	-	-	-
	humuladione	2.1	-	-	-	-	-	-	-	-	-	-	-
	β-eudesmol	6.5	-	-	-	-	-	-	-	-	-	-	-
	isocurcumenol	8.5	-	5.8	-	-	-	-	-	-	-	3.7	R
	Cur-cumanolides	11.4	-	-	-	-	-	-	-	-	-	-	-
	Other sesquiterpene	-	-	-	-	-	-		R	R	R	-	R
	Reference	Zwaving and Bos, 1990	Hongju *et al.*, 1982	Sirat *et al.*, 1998	Bin Jantan *et al.*,1999	Xuan Dung *et al.*, 1995	Jarikasem *et al.*, 2003	Aromdee *et al.*, 2007	Phan *et al.*, 1998	Sirat *et al.*, 1998	Srivastava *et al.*, 2006	Jirovezt *et al.*, 2000	Xuan Dung *et al.*, 1995

R= Reported, H = High

Uterine anatomy and physiology

Human uterus (Figure 2-4) lies deeply in the pelvic cavity between urinary bladder in front and the pelvic or sigmoid colon and rectum behind. It is flattened, thick-walled and is pyriform in shape, with the apex directed downward and backward. The uterus is about 7.5 cm. in length, 5 cm. in breadth, at its upper part, and nearly 2.5 cm. in thickness; it weighs from 30 to 40 gm (Soucasaux, 2001). It can be expanded about 3-6 times of normal size (Shier *et al*., 1999). The upper part of uterus is suspended by the broad and the round ligaments, while its lower part is imbedded in the fibrous tissue of the pelvis. The uterus is divisible into two portions; body and cervix, which the part of the body lies above a plane passing through the points of entrance of the fallopian tubes and is known as the fundus. The uterus is composed of three coats: an external or serous, a middle or muscular, and an internal or mucous (Soucasaux, 2001).

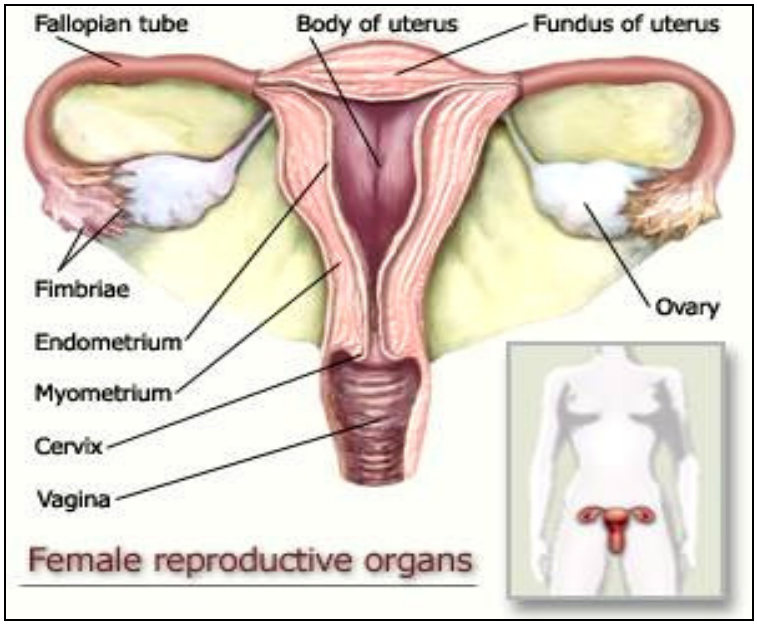

Figure 2-4 Human uteri. Available at: http://www.choicetolivewith.com/Fetaldevelopment/week1.html [Accessed: Sep 15, 2007]**.**

The perimetrium (mesometrium) is the outer serous coat that is supported by thin layer of connective tissue. The middle muscular coat, myometrium is thick in the middle of the body and fundus, and thin at the orifices of the uterine tubes. It consists of bundles of unstriped muscular fibers, disposed in layers, intermixed with areolar tissue, blood vessels, lymphatic vessels, and nerves. It is composed of three layers: external, middle, and internal. The external and middle layers constitute the muscular coat proper; while the inner layer is greatly hypertrophied muscularis mucosa. The external layer consists of fibers which pass transversely across the fundus, and, converging at each lateral angle of the uterus. The middle layer of fibers presents no regularity in its arrangement, being disposed longitudinally, obliquely, and transversely. It contains more blood vessels than either of the other two layers. The internal or deep layer consists of circular fibers arranged in the form of two hollow cones (Soucasaux, 2001). Endometrium is the inner mucous coat that firmly adheres to myometrium. It is changed following the menstruation cycles and regulated by the ovarian hormone (Figure 2-5) (Shier et al., 1999).

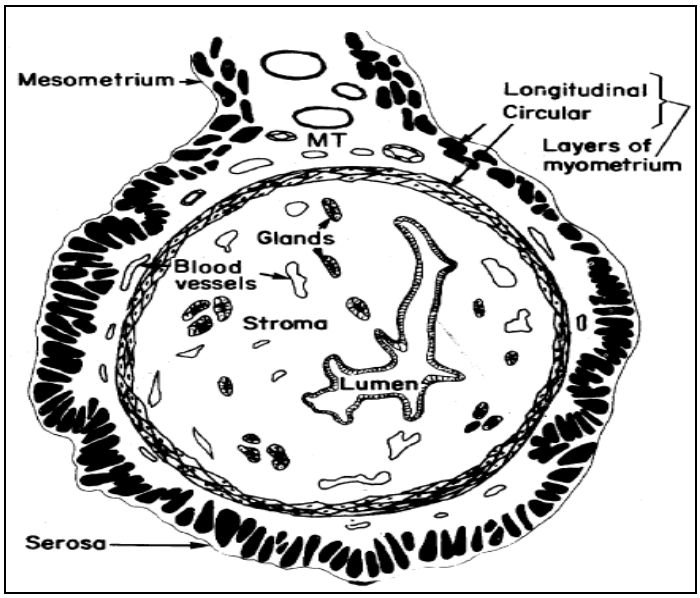

Figure 2-5 Uterine wall shows smooth muscle layers (Noble, 2006).

In rat, other rodents, and elephants, their uteri have different shapes from that of human. There are two separate uteri which known as a duplex uterus (Figure 2-6) (Wisenden, 2003). The rat uterus can be divided into 3 parts; right and left uterine horns and the body of the uterus (Figure 2-7) (Kittel, 2004). The uterine horns are lined with vascular and glandular mucosa and contain smooth muscle.

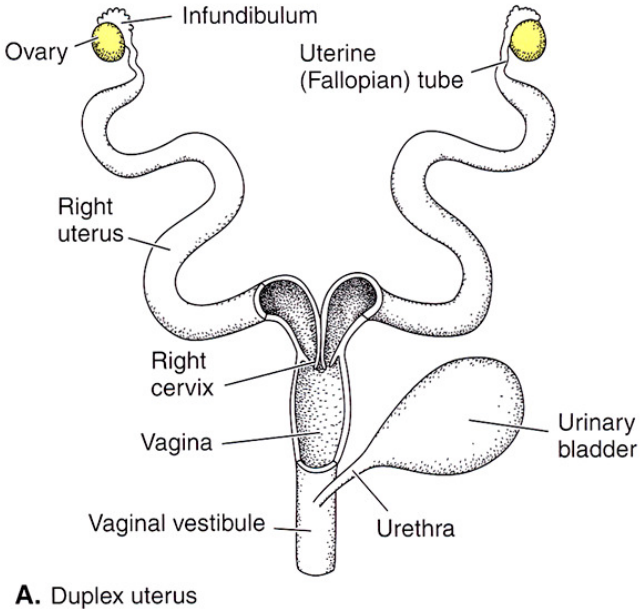

Figure 2-6 Duplex uteri (Wisenden, 2003).

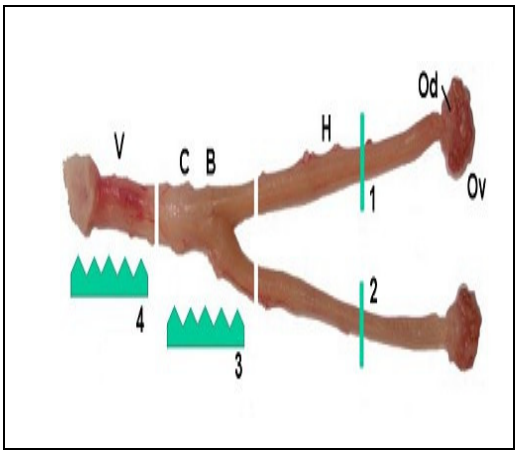

Figure 2-7 Rat uterus and vagina (V: vagina, C: cervix, B: body, H: uterine horn, Od: oviduct, Ov: ovary). Localization at 1 and 2 are the middle regions of rat uterine horns, 3 is uterine body and cervix and 4 is anterior portion of vagina (Kittel, 2004).

Circulation of the uterus

Blood supply of the uterus is from the abdominal aorta and hypogastric arteries. Blood from abdominal aorta is given off ovarian arteries, while the hypogastric artery branches into uterine and vaginal arteries. They are remarkable for their tortuous course in the substance of the organ, and for their frequent anastomoses. The termination of ovarian artery meets that of the uterine artery, and then forms an anastomotic trunk which branches are given off to supply the whole uterus (Figure 2-8). The veins are of large size, and correspond with the arteries. They end in the uterine plexuses.

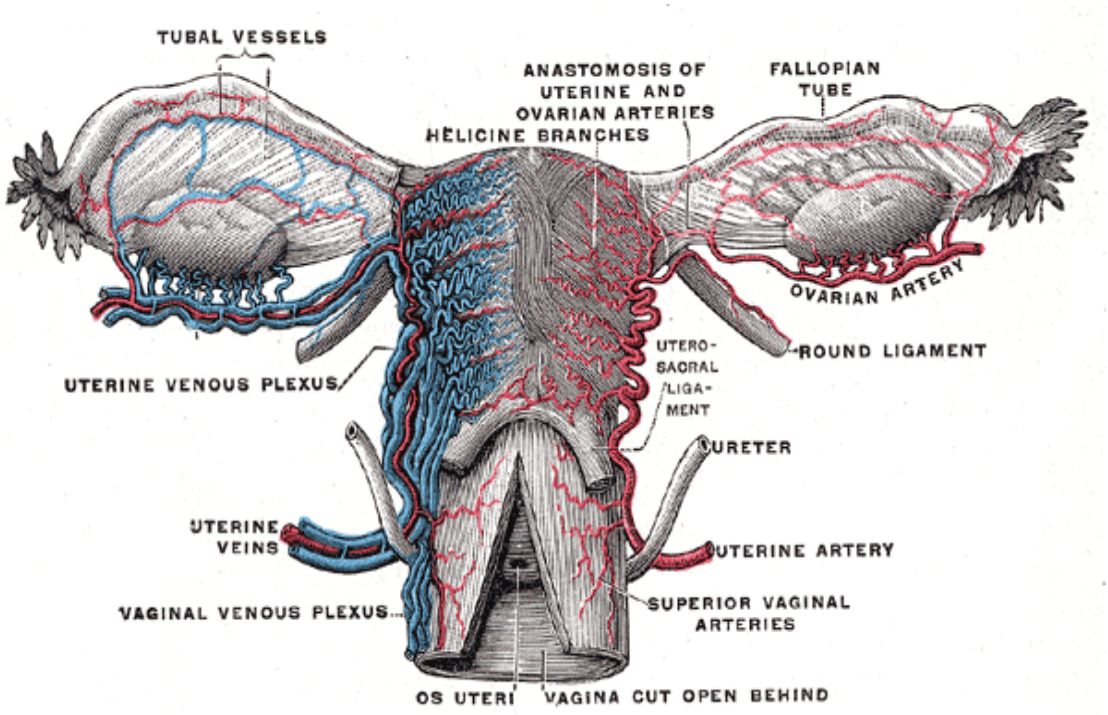

Figure 2-8 Vessels of the uterus and its appendages, rear view (Gray, 1918).

Innervations of the uterus

The contractile activity of smooth muscles is influenced by neurotransmitters released from autonomic nerve endings. Unlike skeletal muscles fibers, smooth muscle fibers do not have a specialized motor end-plate region. As the

axon of a postganglionic autonomic neuron enters the region of smooth muscle fibers, it divides into numerous branches, each branch containing a series of swollen regions known as varicosities. Each varicosity contains numerous vesicles filled with neurotransmitter (Guyton and Hall, 2006; Vander *et al*., 2001).

Uterine nerves are derived from the uterovaginal plexus; one of the pelvic plexuses that extends from inferior hypogastric plexus, which travels with artery at the junction of the base of peritoneal broad ligament and the superior part of the transverse cervical ligament. However, the sensations conveyed by the two nerves are different; hypogastric and pelvic nerves contain afferent and efferent fibers. The pelvic afferent fibers convey a wide range of information about innocuous and noxious vaginocervical stimulation, while hypogastric afferents convey uterocervical noxious information (Sato *et al*., 1996).

Sympathetic innervations originate from the lower thoracic spinal cord segments and pass through lumbar splanchnic nerves and the intermesenteric/hypogastric series of plexuses. The function of sympathetic nerves may involve vasodilatation and uterine relaxation.

Parasympathetic innervations originate from the S_2 through S_4 spinal segments and pass through the pelvic splanchnic nerves to the inferior hypogastric/ uterovaginal plexus, and are mainly project to the cervix and caudal regions of the uterine horns (Houdeau *et al*., 2003). The cholinergic nerve fibers are the dominant motor innervations of the uterus, because contractions evoked by transmural stimulation or in response to nerve stimulation (Sato *et al*., 1996) are abolished by atropine, a muscarinic antagonist (Houdeau *et al*., 2003). Layer-specific distributions of cholinergic nerve fibers are demonstrated, they are abundant in the circular muscle but scarce in the longitudinal muscle layer. In addition, marked decreases in the density of cholinergic fibers along the caudo-rostral axis of the organ also exist. Therefore, it might conclude that the cholinergic nerve control of uterine activity is layer specific and predominant in the caudal uterine horn and cervix (Houdeau *et al*., 2003).

In non-pregnant ovariectomized rats, electrical stimulation of a pelvic or hypogastric nerve evokes uterine contractions, which are partially reduced by the α-adrenergic blocker and totally blocked by hexamethonium (ganglionic blocker) and

atropine (postganglionic muscarinic blocker). The results suggest that efferent innervating the rat's uterus is predominantly post-ganglionic cholinergic, with some adrenergic components (Sato *et al*., 1996). Postganglionic fibers innervate both endometrium and myometrium layers of uterus, however, not appear to be close apposition of nerve ending at the myometrium cells. This is suggested that the neuronal effects which contribute to modulate the contractility of uterus, are less important than hormonal effect (Wray, 1993; Riemer and Heyman, 1998).

Menstrual cycle: Hormone and uterine contraction

Menstrual cycles are controlled by hormones. There is closely linked feedback system between many of the reproductive hormones which means that artificially altering in a single hormone will be likely to have an effect on one or more of the others.

Gonadotropin Releasing Hormone (GnRH): GnRH secreted by the hypothalamus, has a controlling influence over other reproductive hormones and is sensitive to negative feedback by steroid hormone, particularly estrogen and progesterone (Carr *et al*., 1998).

Follicle Stimulating Hormone (FSH): FSH is released from anterior pituitary gland by the stimulation of the pituitary gonadotropins releasing hormone. It stimulates the follicle in the follicular phase or proestrus in menstrual cycle. FSH acts mainly to regulate the final process by which androgens are aromatized to estrogen (Carr *et al*., 1998).

Luteinizing Hormone (LH): Increase in estrogen secretion exerts a positive feedback which leads to an exaggeration of the pulsatile release of LH from the anterior pituitary gland and eventually to menarche and ovulation. Some of the ovulation from the dominant follicle occur at 16 to 23 hour after the LH peak and some occur at 24 to 38 hour after the onset of the LH serge as the result of rupture of the follicular wall at the area of the stigma (Carr *et al*., 1998).

Estrogen: Naturally occurring estrogens are 18-carbon steroids characterized by an aromatic A ring, a phenolic hydroxyl group at C-3 and either a hydroxyl group (estradiol) or ketone (estrone) at C-17. Estrogen is synthesis from cholesterol and secreted by ovary. The most potent estrogen is estradiol which ovary is the main organ that produce it. In addition, estrone is also produced by the ovary, but it is mainly formed by extraglandular conversion of androstenedione in peripheral tissue. Estrogen promotes the development of the secondary sex characteristics in women and causes uterine growth, thickening of the vaginal mucosa, and thinning of the cervical mucus (Carr *et al*., 1998). Estradiol may increase uterine contractility by reducing the membrane potential of myometrial cells, increase gap junction formation. It may also stimulate prostaglandin production (Eagland and Cooper, 2001), increase myosin content, pacemaker activity, oxytocin and prostaglandin receptors. Estrogen increases myometrial sensitivity to acetylcholine by increase the amplitude of acetylcholine-induced contractions in response to nerve stimulation in ovariectomized rats (Sato *et al*., 1989), and chronic estrogen administration has been shown to increase the diameter and density of cholinergic nerve bundles in prepubertal animals (Richeri *et al*., 2002).

Progesterone: Progesterone, a 21-carbon steroid, is the principle hormone secreted by the corpus luteum and is responsible for progestational effects, i.e., induction of secretory activity in the endometrium, increase in the viscosity of cervical mucus, glandular development of the breast, and an increase in basal body temperature (thermogenic effect) (Carr *et al*., 1998). Progesterone has an inhibitory effect on cell communication by decreasing the formation of gap junctions; high circulating levels of progesterone in luteal phase may counteract estrogen-induced activation of cholinergic innervations.

Menstrual cycle is divided into 4 phases (Figure 2-9).

1. Proestrus or Follicular phase

In the first 14 days of menstrual cycle, high levels of FSH are released to stimulate the follicle and endometrium proliferation. There are increasing in

estrogen synthesis from ovary, increasing in size of gland cells (short and straight or slightly convoluted) and increasing in stromal edema and vascularity that will be conspicuous later.

2. Estrus or Ovulatory phase

In this phase, high estrogen level presents in the early, but after ovulation its level is decreased. During estrus, in vitro electrical field stimulation evoked contractions in the cervix and myometrium of the caudal horn, predominantly in circular muscle layer (Houdeau *et al*., 2003). But the muscarinic receptor-mediated contractions induced by carbachol occurred in all uterine regions and muscle layers.

3. Metestrus or Luteal phase

In about 90% of women, this phase last for 13-15 days. Corpus luteum proliferation and the production of sex hormone "progesterone" cause progressive proliferation of endometrium layer, increase in blood supply, the stromal edema, enlarge gland to cause cell hypertrophy, change to long and coiled or even saw-toothed and maybe with secretory product in lumen.

4. Diestrus or Menstrual phase

Physiological changes in this phase are the increasing in vascularity and cell edema. In case of non implantation, autolysis and hemorrhage of endometrium cell will present. Glands are disrupted and the stoma is filled with red blood cell. In diestrus rats, uterine contraction was not elicited in response to electrical field stimulation in the cervix and circular or longitudinal muscle from the caudal as well as rostral uterine horn. Addition of cumulative doses of carbachol failed to increase in a concentration-dependent manner of the frequency and amplitude of contraction in the cervix and myometrium layer from the caudal and rostral uterine horn. The distribution and density of cholinergic nerve fibers along the uterus and between the muscle layers did not differ from the oestrus stage; impaired of this nerve control from oestrus to diestrus stage occurs in a relation to a decrease in the myometrial sensitivity to muscarinic stimulation, not to a decrease in the density of cholinergic innervations (Houdeau *et al*., 2003).

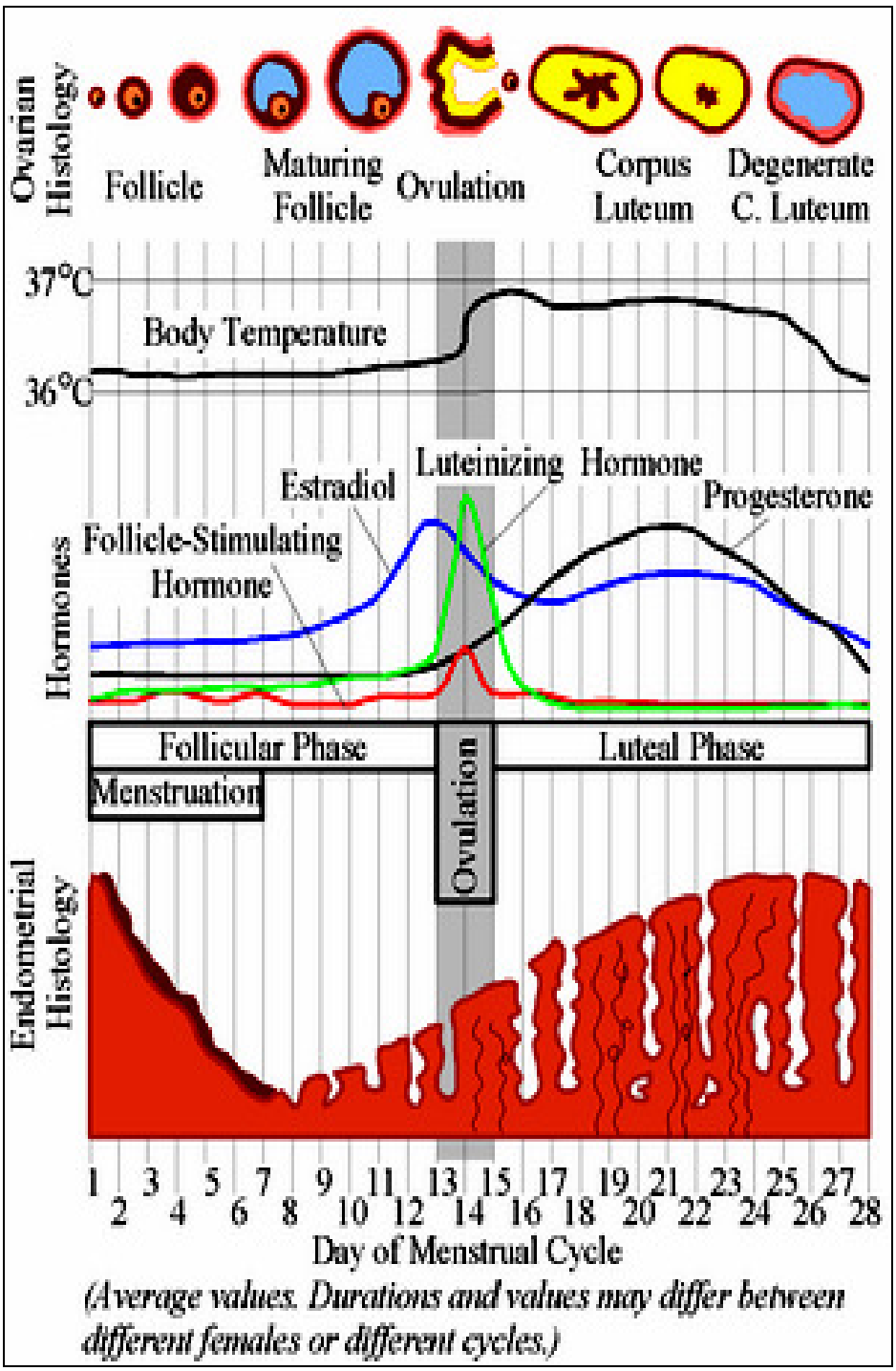

Figure 2-9 Menstrual cycle.

Available at: http://en.wikipedia.org/wiki/Image:MenstrualCycle2.png.

[Accessed: Oct 17, 2007].

Uterine contraction during menstrual cycle

Uterine contractions follow three characteristic patterns during the menstrual cycle. During the early follicular phase (menses), uterine contractions are vigorous, often perceived by women and sometimes painful. At this stage, contractions with antegrade (fundus-to-cervix) displacement uterine contractions predominate. This pattern is instrumental in the forward emptying of uterine content after sloughing of the endometrium occurs upon progesterone withdrawal. During the late follicular phase, uterine contractility increases under the influence of rising estrogen levels with progressive predominance of retrograde (cervix-to-fundus) displacement. Despite their relatively high frequency, uterine contractions at this stage of the cycle are typically painless. This pattern of uterine contractility (high frequency, predominantly retrograde) has been implicated in the rapid transport of sperm toward the distal end of the fallopian tubes that occurs after intercourse. During the luteal phase, uterine contractility characteristically decreases a phenomenon crucial for maximizing uterine receptivity to embryo implantation (reviewed by Ayoubi *et al*., 2001).

Uterine smooth muscle structure

The uterus consists of smooth muscle fiber. Based on the electrical characteristics of their plasma membrane, the uterus was classified as unitary or single-unit smooth muscles. This class undergoes synchronous activity; known as *syncytial* smooth muscle, both electrical and mechanical; that is, the whole muscle responds to stimulation as a single unit (Vander *et al*., 2001; Guyton and Hall, 2006).

The smooth muscle fibers which are composed of loosely organized spindle-shaped cell (Kuriyama *et al*., 1998; Lodish *et al*., 2000; Vander *et al*., 2001; Guyton and Hall, 2006) with small diameters (ranging from 2 to 10 μm) and from 200 to 600 μM in length (Kuriyama *et al*., 1998; Guyton and Hall, 2006). They usually are aggregated into sheets or bundles, and their cell membranes are adherent to one another at multiple points so that force generated in one muscle fiber can be transmitted to the next. In addition, this occurs because each muscle fiber is linked to adjacent fibers by gap junctions, through which action potential occurring in one cell is

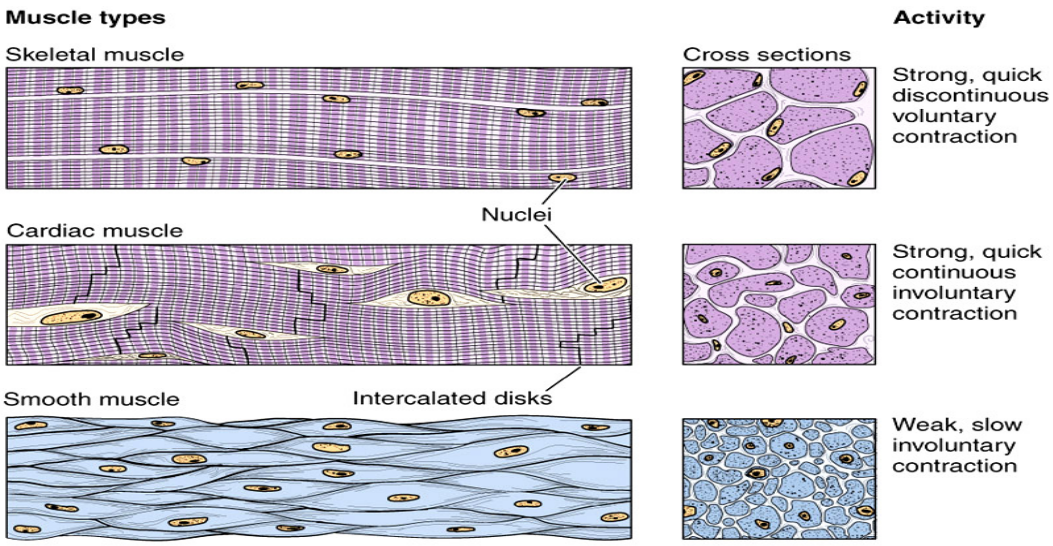

Figure 2-10 Schematic depiction of the three types of muscle found in the human body. Available at: http://www.mc.vanderbilt.edu/histology/labmanual2002/labsection1/Muscle03.htm. [Accessed: Oct 3, 2007].

propagated to other cells by local currents and ions flow freely from one cell to the next (Figure 2-10)(Vander *et al*., 2001; Guyton and Hall, 2006).

Gap junction

The uterine contraction happens when the action potential triggered in pacemaker regions spread throughout the uterus. Gap junction is a low resistance pathway which allows the spreading of action potential between individual myometrial cells to happen (Cole *et al*., 1985). Activity propagates from cell to cell through gap junctions forming an electrical syncytium (Figure 2-11) (Pauley, 2000). Gap junctions consist of pores which are formed from two hemichannel connexon, each composed of six connexin protein (Figure 2-12, 2-13 and 2-14) (Albert *et al*., 1994; Martin and Evan, 2004; Falk, 2000). The major functional constituent of myometrial gap junction is the 42-kDa protein, connexin 43 (Cx 43) (Miyoshi *et al*., 1998). Each gap junction may consist of a few to thousands of channels symmetrically aligned in an adjacent cell. These channels allow current and molecules up to 1000 Daltons to pass between cells (Monga and Sanborn, 1992; Evan and Martin, 2002).

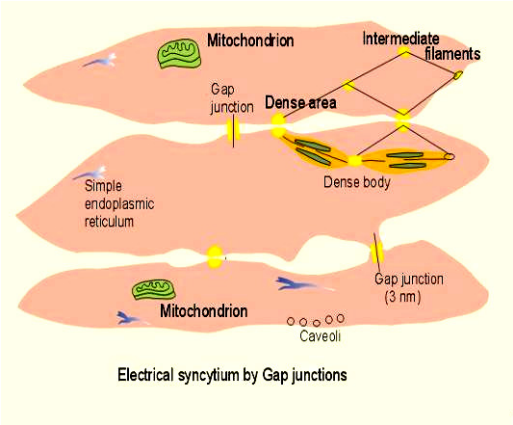

Figure 2-11 Single unit smooth muscle and gap junctions (Pauley, 2000).

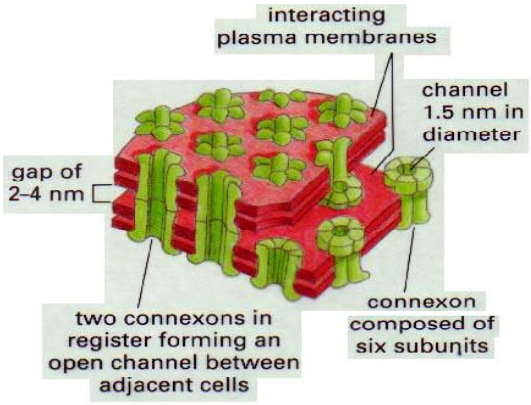

Figure 2-12 Models of gap junctions (Adapted from: Albert *et al*., 1994).

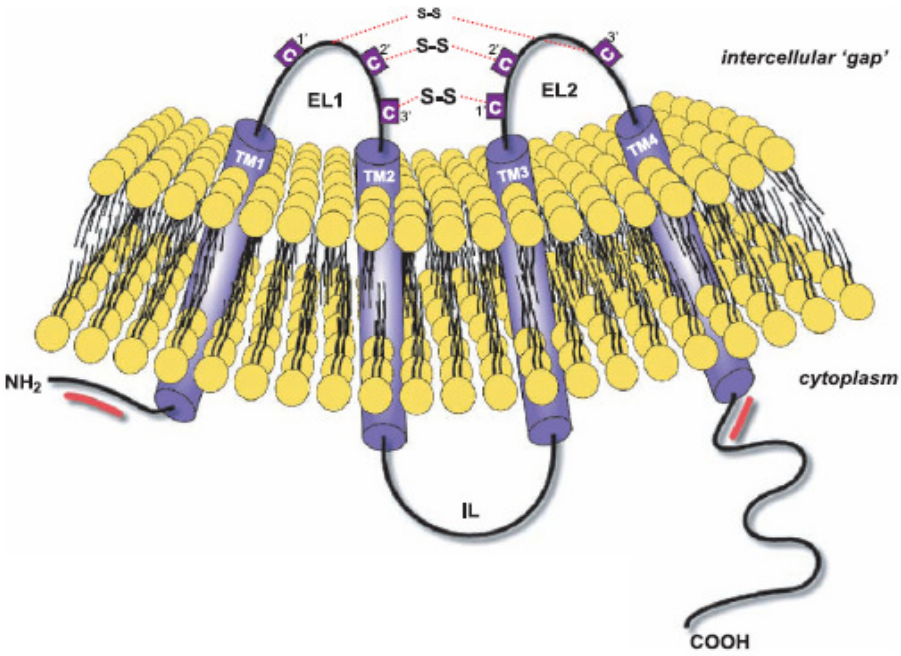

Figure 2-13 Functional maps of gap junction assembly determinants. Connexins span the membrane four times with two highly conserved extracellular loops (EL1 and EL2) facilitating docking and recognition of compatible connexins. The amino, intracellular loop (IL) and carboxyl termini (COOH) interface with the cytoplasm. The two calmodulin binding sites at the amino and carboxy termini and the six cysteine sites in the extracellular loops are indicated (Martin and Evan, 2004).

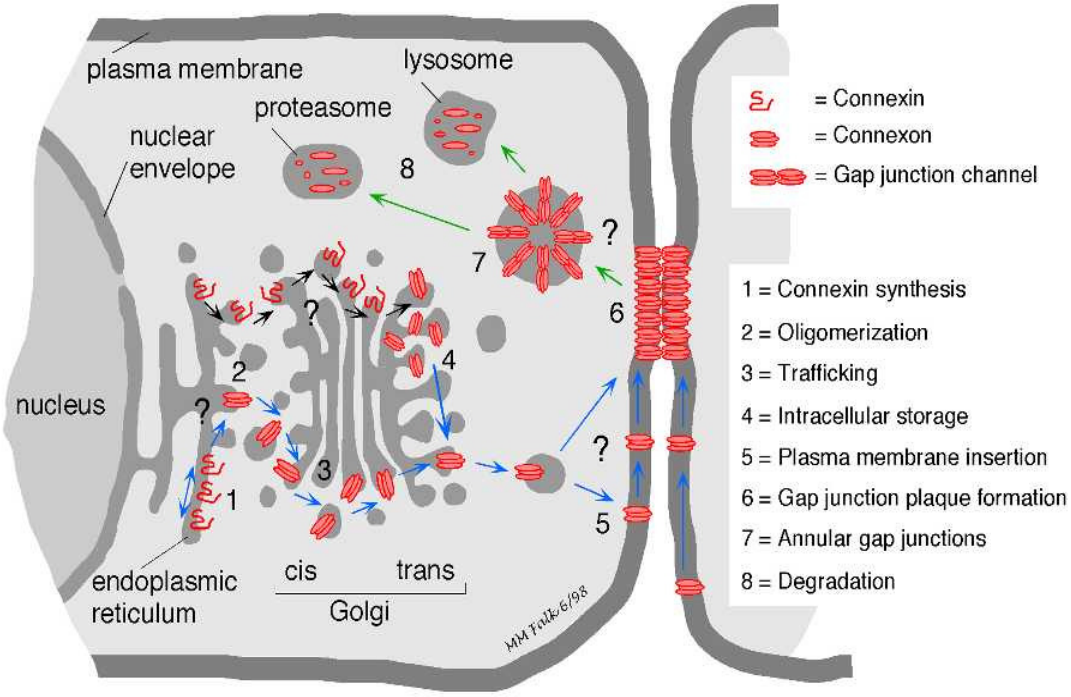

Figure 2-14 Schematic representation of gap junction formation 1) connexin synthesis and assembly in endoplasmic reticulum; 2) Oligomerization; 3) trafficking through Golgi (blue arrows); 4) intracellular storage; 5) plasma membrane insertion; 6) with gap junction plaque formation; 7) endocytosis, annular gap junction; 8) degradation in lysosomes and proteasomes (Folk, 2000).

The function of gap junction is regulated by the number of gap junction (structural coupling), their permeability (function coupling) and their degradation (Cole and Garfield, 1986). In human myometrium, the number of gap junction increases in woman in spontaneous labor when compare to the number in non pregnant or pregnant woman not in labor (Monga and Sanborn, 1992).

Both human and rat uterine myometrium contain connexin45 and connexin43. Co-expression of connexin45 with connexin43 may regulate gap junction coupling between these cells (Albrechta *et al*., 1996). In many species, progesterone appears to suppress the number and permeability of gap junction whereas estrogen acts to increase both of them (Cole and Garfield, 1986). The different temporal expression

patterns of Connexin43 messenger ribonucleic acid levels increased dramatically at term. In contrast, connexin45 messenger ribonucleic acid was present in nonpregnant myometrium, remained relatively constant early in gestation; fell just before term, and more than doubled post partum (Albrechta *et al.*, 1996). Gap junction rapidly disappears after delivery probably by internalization, endocytosis and digestion resulting in a decrease in excitability and contractile function of myometrium smooth muscle (Monga and Sanborn, 1992).

Uterine contraction and its control:

Calmodulin

Calmodulin (CAM) is the EF-hand protein (a helix-turn-helix structural domain), which is symmetrical dumbbell-shaped molecules containing two EF hand at each end, separated by an α-helical structure. As this protein binds to Ca^{2+}, they undergo the conformation changes responsible for activating their downstream targets. CAM is a multifunctional Ca^{2+} sensor responsible for activating a great variety of process (Figure 2-15).

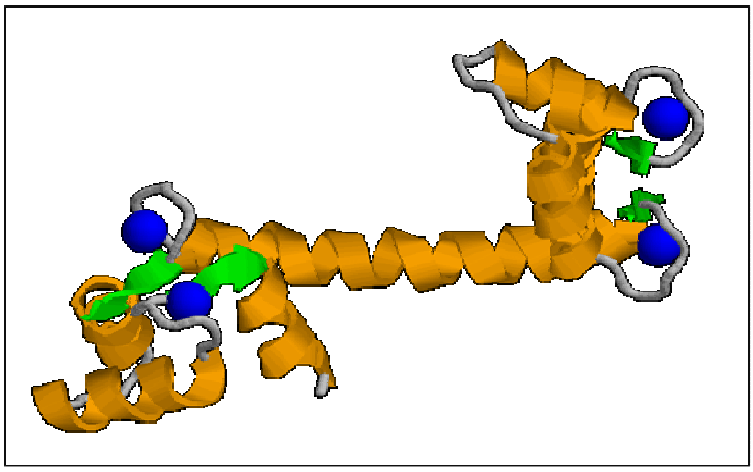

Figure 2-15 Structure of calmodulin, short loop region that usually binds Ca^{2+} (blue cycle). Available at: http://en.wikipedia.org/wiki/EF_hand [Accessed: Apr 7, 2008].

Calmodulin actions

1. Phosphorylation, one of the major actions of CAM is to stimulate a variety of protein kinases. Some of these have a broad substrate specificity (e.g. CAM kinase II), whereas other act on specific targets (e.g. phosphorylase kinase, myosin light chain kinase (MLCK) and elongation factor kinase).

2. Ca^{2+} transport, Ca^{2+} uses CAM to regulate its own extrusion, by activating plasma membrane Ca^{2+} ATPase pump (PMCAs).

3. Signaling cross-talk, a characteristic feature of signaling pathways that are frequently interact with each other at many different levels:

- Ca^{2+}-CAM can activate adenyl cyclase in the production of cyclic AMP.
- the intracellular inositol triphosphates ($InsP_3$) concentration is modulated by Ca^{2+}-CAM.
- nitric oxide (NO) synthesis is activated by Ca^{2+}-CAM (Berridge and Bootman, 1997).

In addition, Ca^{2+}-CAM also activates MLCK in the uterus. Studies in other smooth muscles have reported that recruitment of a slowly diffusible component of total cytosolic calmodulin takes around 200 milliseconds. Calmodulin can bind four Ca^{2+} ions but may have two already bound at the c-terminal binding sites under resting conditions (i.e., low $[Ca^{2+}]_i$). Indeed Wilson and colleagues (2002) recently suggested a novel scheme whereby a portion of calmodulin is tightly bound to the myofilaments, and the Ca^{2+} diffuses to this site. The Ca-calmodulin interaction introduces a significant delay between $[Ca^{2+}]_i$ increase and the ensuing increase of force. The activation of MLCK after Ca-calmodulin binding is also relatively slow and could be one of the rate-limiting steps in contraction along with recruitment and diffusion of calmodulin. There appear to be few studies using inhibitors of calmodulin in the uterus, but a reduction in force has been reported along with reduced gap junction communication and activity of Ca-activated K^+ channels (Wray *et al*., 2003).

Myosin Light Chain Kinase (MLCK)

With four Ca^{2+} bound the C-terminal of calmodulin will bind to MLCK at the N-terminal of MLCK's calmodulin binding domain and then causing

conformational changes which lead to the formation of activated MLCK. Tissues other than smooth muscle contain the smooth muscle MLCK gene, but it is found in the highest amounts in smooth muscle. The activity of MLCK is reported not to differ between pregnant and nonpregnant women. The regulatory light chains of myosin are the only target for MLCK; hence, it is extremely specific, unlike many kinases, and Ca-calmodulin binding is the only known physiologic activator. The phosphorylation of Ser-19 on myosin light chains is the end product of MLCK activity and leads to myosin ATPase activation by actin (Wray *et al*., 2003).

Physical basis for smooth muscle contraction

Two types of filaments are present in the cytoplasm of smooth muscle fibers: thick myosin-containing filaments and thin actin-containing filament. These filaments in smooth muscle are arranged in slightly diagonally to the long axis of the cell that is anchored either to the plasma membrane or to cytoplasmic structures known as dense bodies. Interspersed among the many actin filaments are a few myosin filaments. These filaments have diameter more than twice as great as that of the actin filaments (150 A˚ and 60 A˚ in diameters, respectively). Large numbers of actin filaments radiating from two dense bodies; these filaments overlap a single myosin filament located midway between the dense bodies (Figure 2-16). In fact, the dense bodies of smooth muscle serve the same role as the Z disc in skeletal muscle (Monga and Sanborn, 1992; Horowitz *et al*., 1996; Lodish *et al*., 2000; Vander *et al*., 2001; Guyton and Hall, 2006; Tapechum, 2007).

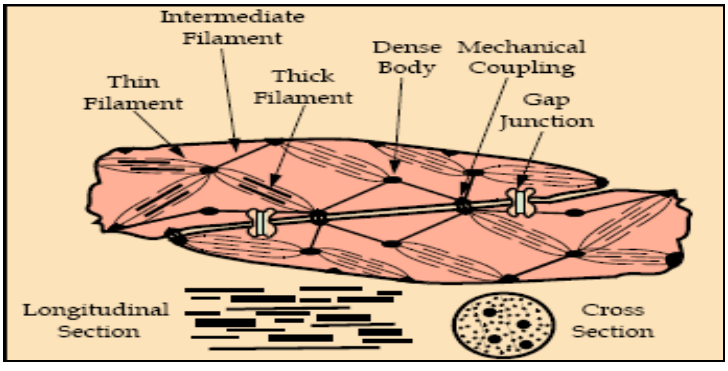

Figure 2-16 Physical basis for smooth muscle structure (Tapechum, 2007).

Myosin

The myosin is a diverse group of motor proteins which interact with microfilaments to produce force (Maciver, 2002). A myosin molecule is shaped a bit like a golf club, but with two heads. Many of these molecules stick together to form the thick filament, with the handles lying together to form the backbone and the heads sticking out in all directions to form the cross bridges (Figure 2-17) (Rothery, 2002).

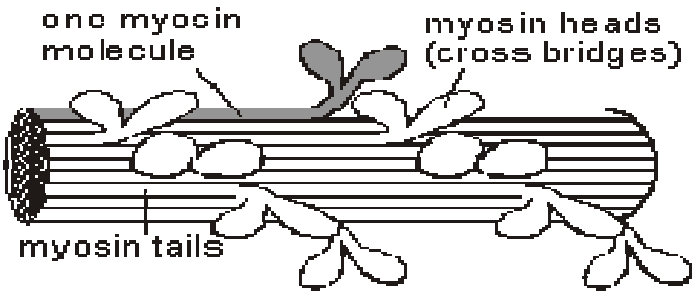

Figure 2-17 Myosin molecule is shaped a bit like a golf club (Rothery, 2002).

All types of myosin are composed of one or two heavy chains and several light chains. The globular head domain contains actin- and ATP-binding sites and is responsible for generating force; it is the most conserved region among the various myosins. Adjacent to the head domain lays the α-helical *neck region*, which is associated with the light chains. The latter regulate the activity of the head domain. Myosin II, the form that functions in muscle contraction, is composed of two heavy chains and two sets of light chains. The light chains are the essential light chains and regulatory light chains (Maciver, 2002). Myosin II is a dimer with a long rigid coiled-coil tail (Lodish *et al*., 2000), which polymerises with other molecules to give large bi-polar filaments in muscle and non-muscle cells. The assembly state is regulated by phosphorylation close to the tail end, whereas the contractile activity is controlled by phosphorylation of the regulatory light chain (Maciver, 2002). Smooth muscle myosin is a hexamer consisting of two heavy chain subunit (~200 kDa) and two pairs each of 20-kDa and 17-kDa light chains (Figure 2-18) (Lodish *et al*., 2000). The heavy chains from α-helices and two globular heads that contain actin-binding site and adenosine

triphosphate hydrolysis (ATPase) activity are present at the amino terminal end of the myosin molecule. Multiple myosin molecules interact via the α-helical tail in a coiled rod, forming the thick filament from which the globular head protrude (Monga and Sanborn, 1992; Horowitz *et al*., 1996). Myometrial actin molecules are globular (45-kDa) and polymerized into a double helical strand and associated proteins such as tropomyosin, caldesmon and calponin (Monga and Sanborn, 1992).

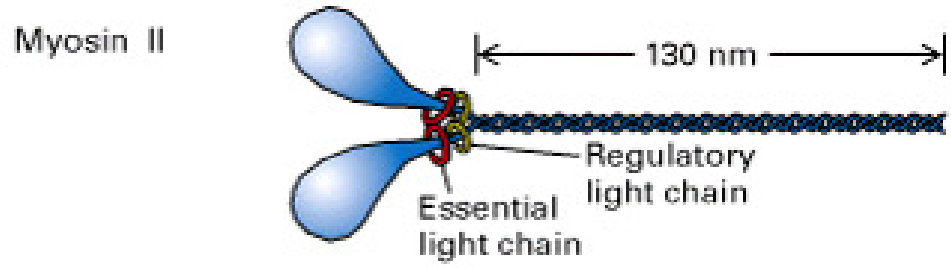

Figure 2-18 Myosin II (Adapted from: Lodish et al., 2000)

Tropomyosin

The regulatory proteins on the thin filament, tropomyosin is a long thin molecule and it can change its position on the thin filament. In a relaxed muscle it is on the outside of the filament, covering the actin molecules so that myosins cross bridges can't attach. This is why relaxed muscle is compliant: there are no connections between the thick and thin filaments. In a contracting muscle the tropomyosin has moved into the groove of the double helix, revealing the actin molecules and allowing the cross bridges to attach (Figure 2-19) (Rothery, 2002).

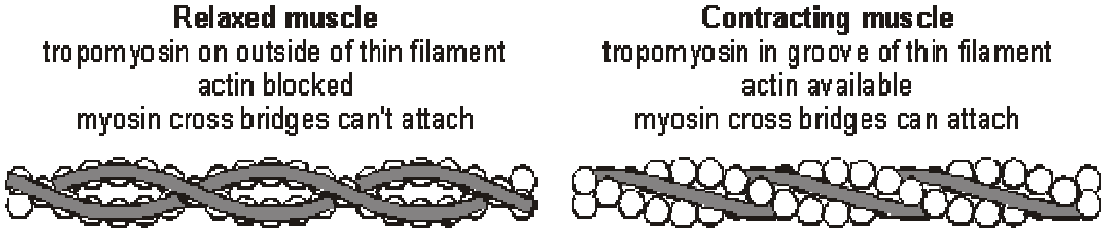

Figure 2-19 Tropomyosin; regularly protein on the thin filament (Rothery, 2002).

Chemical basis for smooth muscle contraction

The contractile process is activated by calcium ions, and adenosine triphosphate (ATP) which is degraded to adenosine diphosphate (ADP) to provide the energy for contraction (Guyton and Hall, 2006).

Cross-bridge activation and cycle

Change in cytosolic calcium concentration controls the contractile activity in smooth muscle fibers. Cross-bridge cycling in smooth muscle is controlled by a calcium-regulated enzyme that phosphorylates myosin. The following sequence of events occurs after a rise in cytosolic calcium (Figure 2-20) (Monga and Sanborn, 1992; Horowitz *et al*., 1996; Riemer and Heyman, 1998; Vander *et al*., 2001; Wray *et al*., 2003; Guyton and Hall, 2006):

1. Calcium ions bind to calmodulin.
2. The calcium-calmodulin complexes bind to a protein kinase, myosin light chain kinase, thereby activating the enzyme.
3. The active protein kinase then uses ATP to phosphorylate myosin light chains in the globular head of myosin. This phosphorylated correlates with increase in actomyosin ATPase activity, the enzyme that facilitates the actin-myosin interaction by increasing the flexibility of the head/neck junction.
4. The phosphorylated cross-bridge binds to actin.
5. Cross-bridge cycle produces tension and shortening

Processes of cross-bridge cycling

In the absence of bound nucleotide, a myosin head binds actin tightly in a “rigor” state. When ATP binds, it opens the cleft in the head, disrupting the actin-binding site. Weakening the interaction with actin and freed of actin. The myosin head then hydrolyzes ATP, causing a conformational change in the head that moves it to a new position, closer to the (+) end of the actin filament, where it rebinds to the filament. As phosphate (Pi) dissociates from the ATP-binding pocket, the myosin head undergoes a second conformational change the power stroke which restores myosin to its rigor conformation. Because myosin is bound to actin, this conformational change

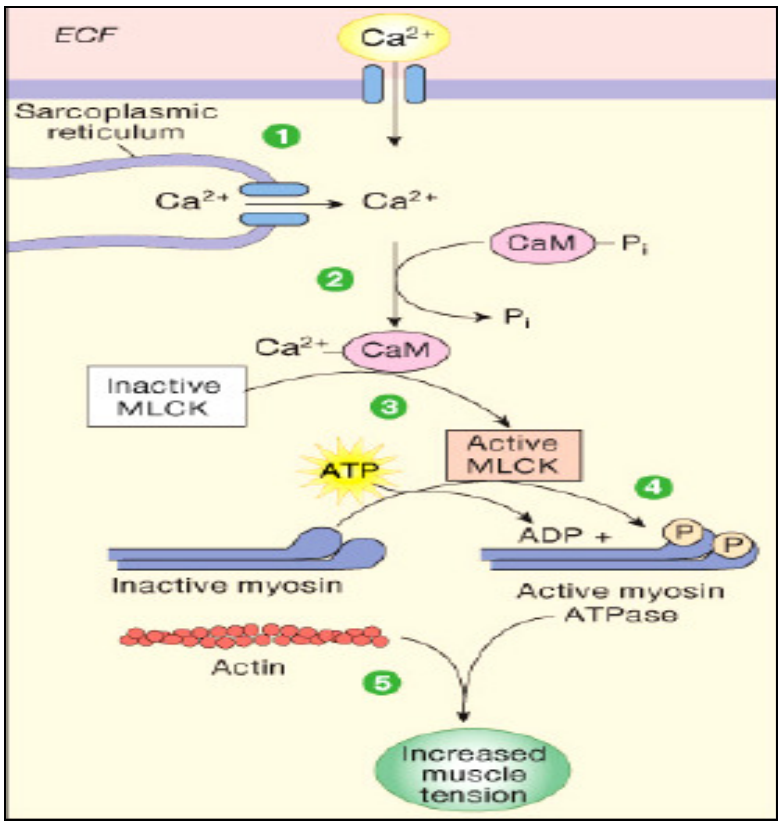

Figure 2-20 Cross-bridge activation; 1) Intracellular Ca^{2+} concentrations increase when Ca^{2+} enters cell and is released from SR, 2) Ca^{2+} binds to calmodulin (CaM), 3) Ca^{2+}-calmodulin activates MLCK, 4) MLCK phosphorylates light chains in myosin heads and increase myosin ATPase activity and 5) Active myosin cross-bridge slide along actin (Tapechum, 2007).

exerts a force that causes myosin to move the actin filament (Figure 2-21) (Monga and Sanborn, 1992; Lodish *et al.,* 2000; Maciver, 2002).

Slow cycling, energy requirement, slowness of onset and force of contraction

The rapidity of the cycling of the myosin cross-bridges in smooth muscle that is their attachment to actin and reattachment for the next cycle is much slower than in skeletal muscle. The frequency of cross-bridge cycling in smooth muscle compared to that of skeletal muscle is $^{1}/_{10}$ to $^{1}/_{300}$ (Vander, 2001; Guyton and Hall, 2006).

Energy required to sustain the same tension of smooth muscle contraction is much less than that required in skeletal muscle, because only one of molecule of ATP is required for each cycle, regardless of its duration (Guyton and Hall, 2006).

The slow onset of contraction in smooth muscle as well as the prolonged contraction is caused by the slowness of attachment and detachment of the cross-bridge with the actin filaments. Postulated from the prolonged period of attachment of the cross-bridges are resulting in the greater force of contraction of smooth muscle than that of skeletal muscle (Guyton and Hall, 2006).

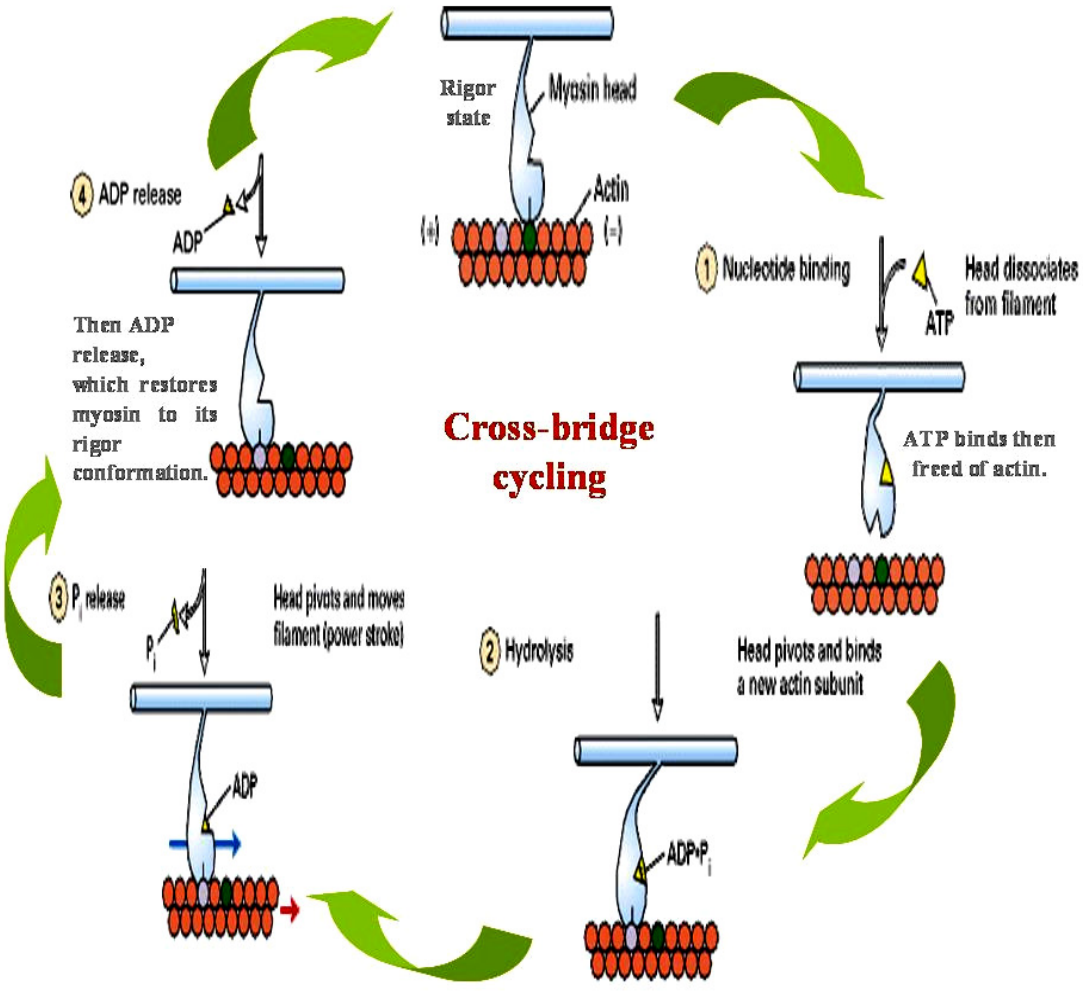

Figure 2-21 Cross-bridge cycling. (Adapteded from: Lodish *et al.*, 2000).

Latch mechanism for prolonged holding contractions of smooth muscle

The importance of latch mechanism is that it can maintain prolonged tonic contraction in smooth muscle for hour with little use of energy. Although, the degree of activation of the muscle usually can be reduced to far, less than the initial level (Guyton and Hall, 2006). At intermediate Ca^{2+} levels (Figure 2-22; orange portion of the cross-bridge cycle), dephosphorylation of some of the myosin heads while they are still attached to actin, may prolong attachment time, slow down cycling, and allow prolonged maintain of tone at a given length, without the use of large amounts of ATP; the latch mechanism (Figure 2-22) (Young, 2007).

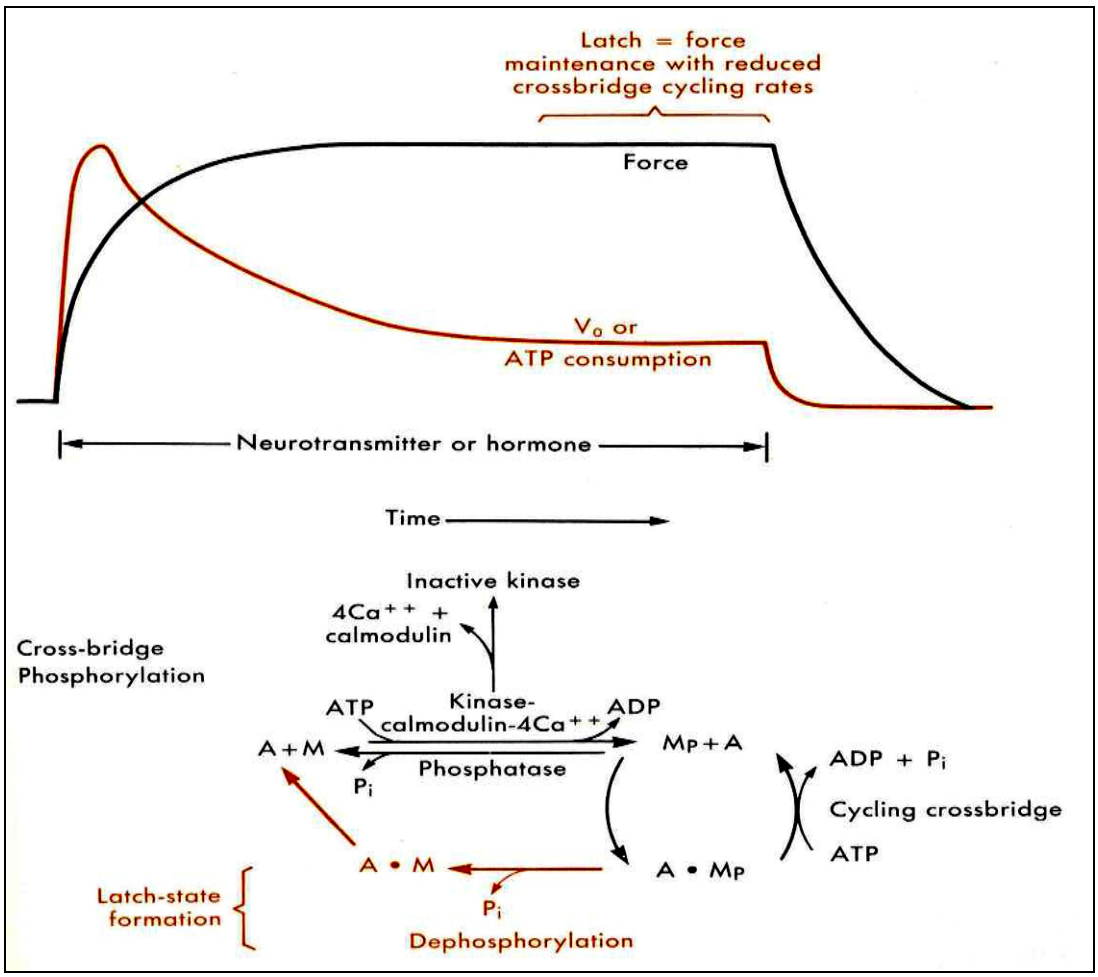

Figure 2-22 Latch mechanism (Young, 2007).

Cessation of contraction

When cytosolic calcium rises, the rate of myosin phosphorylation by the activated kinase exceeds the rate of dephosphorylation by the phosphatase, producing a rise in tension. When cytosolic calcium concentration decreases, the rate of dephosphorylation exceeds the rate of phosphorylation, and then relaxation occurs (Guyton and Hall, 2006). Warshaw *et al* (2007) demonstrated that if changes the state of myosin light chain phosphorylation acts only as a switch to turn rapid cross-bridge cycling on and off. The velocity of actin movement should be independent of the percentage of phosphorylated cross-bridges within the myosin filament. The observation that actin velocity depended on the ratio of phosphorylated to unphosphorylated myosin suggests that unphosphorylated myosin acts as a load to slow down the rate at which actin is moved by the faster cycling phosphorylated cross-bridges. Myosin that was chemically modified to generate a noncycling analogue of the "weakly" bound conformation was similarly able to slow down phosphorylated myosin.

Stress-relaxation of smooth muscle

Another important characteristic of smooth muscle is its ability to return nearly to its original force of contraction seconds or minutes after it has been elongated or shortened. This is known as plasticity and occurs because of a phenomenon called stress-relaxation and results from the loose arrangement of the actin and myosin filaments in smooth muscle. The filaments of a stretched muscle rearrange their bonds, causing sliding between the filaments. Within a few minutes, tension return to its previous level. The converse effect occurs when smooth muscle is shortened. All tension is lost when the muscle length is reduced, but tension gradually returns over a period of one minute or more (Guyton and Hall, 2006).

Effect of local tissue factors on smooth muscle contraction

The smooth muscle is highly contractile, responding rapidly to changes in local conditions in the surrounding interstitial fluid. Some of the specific control factors in the local tissue are causes of smooth muscle relaxation, including lack of oxygen, excess carbon dioxide, increased hydrogen ion concentration, acidity;

diminish Ca^{2+} concentration, decreased body temperature, paracrine agents. Nitric oxide (NO) is one of the most commonly encountered paracrine agents that produce smooth muscle relaxation. NO is released from some nerve terminals as well as a variety of epithelial cells. Because of the short life span of this reactive molecule, it acts as a paracrine agent, influencing only those cells that are very near its release site. Some smooth muscles respond by contracting when they are stretched (Vander *et al*., 2001).

Stretch-induced light chain phosphorylation

When visceral smooth muscle is stretched sufficiently, spontaneous action potentials are usually generated. According to Guyton and Hall (2006), it results from a combination of the normal slow wave potentials plus a decrease in the negative of the membrane potential caused by the stretch itself. Stretching opens mechanosensitive ion channels, leading to membrane depolarization (Vander *et al*., 2001), increases the numbers of gap junctions and connexins (Wray *et al*., 2003). Stretching of arterial or uterine smooth muscle induce light chain phosphorylation to the same extent as was observed in muscle contracted by K^+ or norepinephrine (Bárány, 2002). Muscle which were stretch 1.6 times their resting length did not develop tension, but contracted normally when the stretch was released and the muscles were allowed to return to their resting length. Importantly, this contraction was spontaneous, indicating that the stretch-induced activation carries all the information necessary for normal contraction. Mobilization of Ca^{2+} was necessary for the stretch-induced light chain phosphorylation and contraction to occur. When EGTA was added to the muscle bath, both the stretch-induced phosphorylation and the stretch-release-induced tension were inhibited; however, upon removal of EGTA by washings, both processes were fully restored. Treatment of the muscle with chlorpromazine (the calmodulin inhibitor) also abolished both the stretch-induced light chain phosphorylation and the stretch-release-induced tension development. These results suggest the presence of mechano-sensitive receptors in smooth muscle that are interacting with Ca^{2+} release channels in sarcoplasmic reticulum. Furthermore, 1.6 times stretched muscles, which are unable to contract (because there is no overlap between actin and myosin filaments), are able to phosphorylate their light chain.

Accordingly, smooth muscle contraction and light chain phosphorylation are not coupled. Time course experiment also demonstrated that light chain phosphorylation precedes tension development. Thus, light chain phosphorylation plays a role in the activation process but not in the contraction per se. K^+-contracted muscle maintains its tension for a prolonged time although its light chain becomes dephosphorylate (Bárány, 2002).

Membrane potentials

In the normal resting state of smooth muscle, the membrane potential is usually about -50 to -60 mV (Guyton and Hall, 2006). The resting membrane potential of human myometrium smooth muscle is approximately -40 to -50 mV (Parkington and Colemman, 1990), whereas in rat, is between -50 and -60 mV (reviewed by Okabe *et al*., 1999). The resting membrane potential of rat myometrium is larger in circular smooth muscle cells than in longitudinal muscle cells, both before and during the early stage of gestation. At midterm of gestation, the membrane potential was -60 mV for the inhibition of contractile response during pregnancy, whereas turn to approximately -45 mV at near term (Monga and Sanborn, 1992; Kuriyama *et al*., 1998).

Following terms are used to describe the direction of changes in the membrane potential relative to the resting potential (Vander *et al*, 2001):

Depolarize: membrane potential is less negative (closer to zero) than the resting level.

Overshoot: a reversal of the membrane potential polarity, that is, when the inside of a cell becomes positive relative to the outside.

Repolarize: a depolarized membrane returns toward the resting value.

Hyperpolarize: the membrane potential is more negative than the resting level.

Graded potentials

Graded potentials are changes in membrane potential that are signaling over short distances of muscle membranes, within 1-2 mm. of their set of origin. They are usually produced by some specific changes in the cell's environment acting on a

specialized region of the membrane. The potential change occurring during the spontaneous depolarization to threshold is known as a pacemaker potential (Vander *et al*., 2001), or slow wave potential (Guyton and Hall, 2006). The importance of this potential is that they can initiate action potentials (Guyton and Hall, 2006). Uterine contraction can occur spontaneously after action potentials which have been hypothesized that it is generated by the pacemaker cells (Lammers *et al*., 1994).

Pacemaker cell

Uterine smooth muscle can spontaneously generate action potentials in the absence of any neuronal or hormonal input. The single unit smooth muscle has pacemaker regions where contractions an exhibited spontaneously and rhythmically generated. These regions have a lower resting membrane potential known as pacemaker potentials (Eagland and Cooper, 2001; Vander *et al*., 2001). If a sequence of action potentials occurs; tonic state of contractile activity was produced (Vander *et al*., 2001). In uterus, the pacemaker cells are situated around the uterine insertion of the fallopian tube and have "Triple descending gradient" with the following characteristics: 1) The propagation of the contractile wave along the uterus has a descending direction. It happens because after starting in one of the "pacemakers", the contractile wave spreads throughout the uterine fundus and propagates downwards; 2) The systolic phase of the contraction lasts more at the uterine fundus and less at the inferior parts of the organ; 3) The contractions are stronger in the upper parts of the uterus than in the lower ones (Soucasaux, 2001).

Action potentials

Action potentials are rapid, large alterations in the membrane potential occurring with in one of two forms: spike potential or action potential with plateau.

1. Spike potential

Typical spike action potentials occur in most types of unitary smooth muscle. The duration of this type of action potential is 10 to 50 milliseconds. Spontaneous electrical activities in the muscle from the uterus are composed of intermittent bursts of spike action potentials. Such action potentials can be elicited in many ways, for example, by electrical stimulation, by the action of ovarian hormones

(principally estrogen), by the action of transmitter substances from nerve fibers, uterine volume (chronic stretch), or as a result of spontaneous generation in the muscle fiber itself (Guyton and Hall, 2006; Garfield and Maner, 2007) through their effect on resting membrane potentials. Single spikes can initiate contractions, but multiple, higher-frequency, coordinated spikes are needed for forceful and maintained contractions (Garfield and Maner, 2007).

2. Action potentials with plateaus

The onset of this action potential is similar to that of the typical spike potential. However, instead of rapid repolarization of the muscle fiber membrane, it is delayed for several hundred to as much as 1000 milliseconds. The importance of the plateau is that it can account for the prolonged periods of contraction that occur in uterus under some conditions (Guyton and Hall, 2006).

The action potentials in uterine smooth-muscle result from voltage- and time-dependent changes in membrane ionic permeability. In longitudinal and circular muscles of the uterus, the depolarizing phase of the spike is due to an inward current carried by Ca^{2+} and Na^{+}. In preterm uterine muscle, the well-known "plateau-type" action potential may be due to a combined effect of a sustained inward Ca^{2+} or Na^{+} current and a decrease in the voltage-sensitive outward current (Garfield et al., 2007).

Electrical activity: membrane potential and contraction

The uterus is very excitable and can generate spontaneous rhythmic contraction that varies in frequency and amplitude throughout the menstrual cycle (Lammers *et al*., 1994). Contractility of uterus is dependent on the resting membrane potential (Monga and Sanborn, 1992). The magnitude of the resting membrane potential is determined mainly by two factors:

1. Differences in specific ion concentrations in the intracellular and extracellular fluids.
2. Differences in membrane permeabilities to the different ions, which reflect the number of open channels for the different ions in the

plasma membrane (Vander *et al*., 2001).

Sodium, calcium and chloride ions are major ions in the extracellular fluid while K^+ is mainly in intracellular fluid (Monga and Sanborn, 1992; Sanborn, 2000). Na^+, K^+, Cl^- and Ca^{2+} are the most important ions, that can affect the membrane potential (Vander *et al*., 2001).

Rhythmic contraction of uterus involves the alteration of the membrane potential in the term of slow wave (Figure 2-23). Whenever the potential reaches threshold, a fast depolarization occurs to generate an action potential on the top of slow wave (Figure 2-24) (Parkington and Colemman, 1990; Tapechum, 2007). In smooth muscle in which action potentials occur, calcium ions, rather than sodium ions, carry positive charge into the cell during the rising phase of action potential that is, depolarization of the membrane opens voltage gated calcium channels, producing calcium mediated action potentials rather than sodium-mediated ones (Vander *et al*., 2001), and then lead to smooth muscle contraction (Figure 2-25) (Tapechum, 2007). The repolarization stage of the action potential is due to inactivation of calcium channel near the peak of the action potential, which causes them to close; and activation of potassium channel, lead to K^+ efflux and then the potential dropped to the resting stage (Riemer and Heyman, 1998; Vander *et al*., 2001).

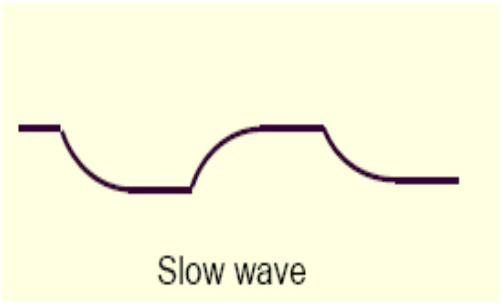

Figure 2-23 Slow wave
-Depolarization due to Ca^{2+} influx through VOCCs (L-type)
-Repolarization
- Delayed rectifier K^+ channel (K_{dl})
- Ca^{2+}-sensitive K^+ channel (K_{Ca})
(Tapechum, 2007).

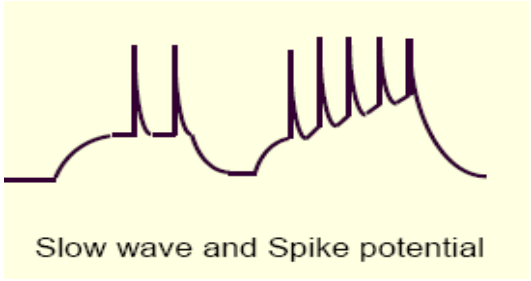

Figure 2-24 Spike potential
- Regenerative opening of VOCCs
(Tapechum, 2007).

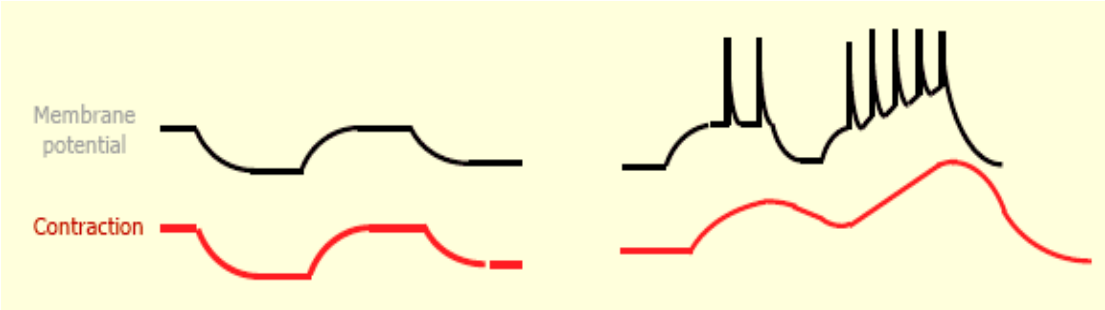

Figure 2-25 Calcium entry during slow wave and spike potential induced smooth muscle contraction (Tapechum, 2007).

Role of slow wave and spike potential on smooth muscle contraction

- The force of contraction depends on the amount of Ca^{2+} entry
- The frequency of slow wave determines the frequency of contraction
- Number of spikes indicates the amplitude of contraction (Tapechum, 2007).

Electromechanical and Pharmacomechanical coupling in smooth muscle

The excitation-contraction coupling in smooth muscle can be occurred by two overlapping mechanisms that are electromechanical and pharmacomechanical coupling. During electromechanical process, the primary drive for the rise in intracellular calcium (and thus contraction of the muscle) is membrane depolarization, with the consequential opening of voltage-operated calcium channels (VOCCs); neurotransmitters or hormones acting to depolarize the membrane will generally cause contraction whilst those producing membrane hyperpolarization will cause relaxation. Drugs which block calcium entry through VOCCs will inhibit electromechanical coupling (McFadzean and Gibson, 2002). Electromechanical coupling appears to play a predominant role in producing the initial peak or shortened peak of contraction in so-called phasic smooth muscle in which the membrane potential often displays marked oscillations upon which are superimposed calcium spikes (Bolton, 1979; Bárány, 1996; McFadzean and Gibson, 2002).

Pharmacomechanical coupling depends neither on change in membrane potential (although changes may occur) nor, consequently, on calcium entry via VOCCs. Rather the rise in intracellular calcium is brought about by a combination of calcium release from intracellular stores and calcium entry through non-voltage-operated channels, primary receptor-operated calcium channels (ROCCs) and store-operated calcium channels (SOCCs). The calcium signal produced following administration of an excitatory agonist to cells demonstrating pharmacomechanical coupling is often similar to that seen in many non-excitable cells, consisting of an initial rapid, but transient, rise in intracellular calcium followed by a smaller, but sustained, increase dependent upon calcium entry from the extracellular space. This latter influx, allied to the process of calcium sensitization whereby the contractile apparatus can be activated by near-resting levels of intracellular calcium, allows such muscles to maintain tone over prolonged periods in the presence of agonist. This process is energetically favorable and occurs when the initial peak reaches a maximum; it is slightly declined to a relative steady at low level. This steady stage is called tonic contraction (Bolton, 1979; McFadzean and Gibson, 2002).

Phasic and tonic smooth muscle contractions

There are two types of smooth muscle contractions; phasic and tonic. Phasic smooth muscle contractions, including those occurring in the myometrium, result from a transient rise in cytosolic-free Ca^{2+} concentration. During tonic smooth muscle contraction, the initial peak Ca^{2+} concentration does not return to baseline but reverts to a sustained lower level. This, in turn, results in a maintained contraction with considerably elevated tension (force), despite the relatively low calcium and phosphorylation (i.e., cross-bridge cycling) levels. This type of contraction is characteristic for a variety of vascular smooth muscles because of gradual depolarization (Bytautiene *et al.*, 2003).

Myometrium is a phasic smooth muscle, although tonic-like contractions may develop with high-frequency electrical stimulation during labor or after the use of some agonists such as oxytocin, prostaglandins, or endothelin (Bytautiene *et al.*, 2003).

Ion channels and contraction

In myometrium, Ca^{2+} entry into cells mediates myometrial membrane potential changes and serves as the internal signal for contraction. K^+ efflux is thought to promote repolarization after an action potential and to participate in setting the resting membrane potential. Ions cross the cell membrane through channels that have different regulated properties and selective. A number of myometrial Ca^{2+} channels have been described, including voltage-regulated L-type channels and Ca^{2+} entry in response to intracellular Ca^{2+} store depletion. Fast Na^+ channels may contribute to cation entry late in pregnancy. K^+ channels in myometrium include Ca^{2+}-activated channels, a delayed rectifier, and an inward rectifier. A Ca^{2+}-activated Cl^- channel is also present in myometrium. In addition to being regulated by Ca^{2+}, the activity of a number of these channels can be regulated by uterine contractants and relaxants. Regulation of ion channel activity can affect intracellular free Ca^{2+} concentrations in the myometrium. Therefore, control of ion channel activity represents one of several approaches for controlling myometrial contractile activity (Sanborn, 2000).

Voltage-gated sodium channel

Normally Na^+ participates little in the generation of the action potential in most smooth muscle (Guyton and Hall, 2006). Inoue and Sperelakis (1991) reported that the expression of these channels was found to progressively increase from midgestation until term. It has been found that the voltage-gated Na^+ channels mRNAs are express in pregnant human, rat tissues and cells (Sanborn, 2000). Inoue and Sperelakis (1991) also showed that the average current density of fast Na^+ channels increased markedly in the myometrium during gestation. The role of fast Na^+ channels in myometrial activity becomes more and more important as term approaches. They suggested that the fast Na^+ current may be involved in spread of excitation.

Calcium channels

The Ca^{2+} channel has a pore-forming α_1-subunit and several auxiliary subunit, including the β-subunit on the cytoplasmic side and an α_2/δ subunit located on the extracellular (Figure 2-26) (Wray *et al*., 2003; Sigma-Aldrich, 2003). Two

subtypes of Ca^{2+} channels are expressed in the myometrium including L-type and T-type Ca^{2+} channels (Riemer et al., 2000; Sanborn, 2000; Wray *et al*., 2003). However, only the L-type Ca^{2+} channel is considered to be a major Ca^{2+} influx pathway (Karaki *et al*., 1997). The role of Ca^{2+} channels in the myometrium will be described later.

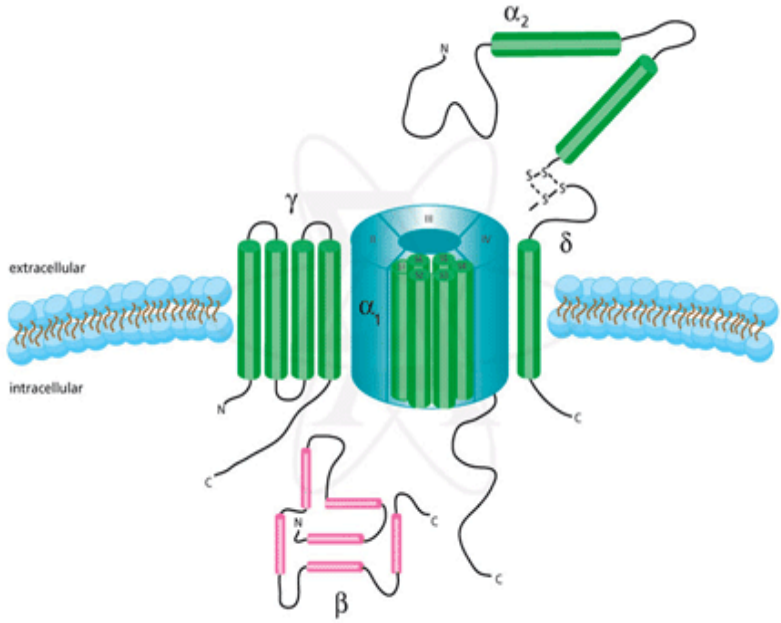

Figure 2-26 L-type Ca^{+} channel (Sigma-Aldrich, 2003).

Potassium channel

The repolarization stage of the action potential is due to inactivation of Ca^{2+} channel and activation of K^{+} channel, lead to K^{+} efflux and then the potential drops to the resting potential (reviewed by Riemer and Heyman, 1998). The efflux of K^{+} contributes to recovering stage (repolarization), maintaining, and/or enhancing (hyperpolarization) which results in a reduction of potential to resting membrane potential of the cells (Kuriyama *et al*., 1998).

Voltage-gated potassium channels (K_v channels), originally postulated by Hodgkin and Huxley (Varshney and Mathew, 2003). These have been shown to be tetrameric assemblies, each subunit consisting of six transmembrane segments and contributing a re-entrant Pore-lining loop (P-loop) to the ion conduction pathway. The fourth transmembrane segment (S4) is positively charged with arginine or lysine at every third position; and has been shown to move in response to changes in

transmembrane potential. The movements of this 'sensor' are transduced by an effector mechanism culminating in the rotation of S5 and S6 helices which opens the channel pore. The P-loop lines the aqueous pore and carries the elements of ionic selectivity. The N-terminus contains the T1 domain that tetramerizes in solution and is responsible for subfamily specificity of channel assembly (Figure 2-27) (reviewed by Varshney and Mathew, 2003).

The contribution of different types of K^+ channels to the regulation of myometrial contractility is incompletely understood. Electrophysiological studies at the whole-cell or single channel level have identified at least four types of K^+ currents or channels in human myometrium (Aaronson *et al*., 2006). There include the large conductance Ca^{2+}-activated K^+ channels (K_{Ca}) and three types of voltage-gated K^+ (K_V) currents including delayed rectifier K^+-channel (K_V), inward rectifier K^+-channel (K_{ir}) and 4-aminopyridine (4-AP)-sensitive, rapidly inactivating A-type current. In addition, pharmacological and biochemical evidences for myometrial ATP-activated K^+ (K_{ATP}) channels has also been presented (reviewed by Aaronson *et al*., 2006).

Wang *et al*., (1998) demonstrated that in rat myometrium smooth muscle cells, the K_{Ca} current coexists with at least three types of voltage-gated K^+ current. These delayed rectifier currents were present in myometrial smooth muscle cells from both nonpregnant and late pregnant animals, and were suppressed by both TEA ($\geq$ 0.5mM) and 4-AP ($\geq$ 0.4mM).

Ca^{2+}-activated K^+ channels (K_{Ca})

Depending upon the channel location and function, Ca^{2+}-activated K^+ channels (K_{Ca}) may be gated by depolarization and increase in intracellular Ca^{2+} (Figure 2-28) (Varshney and Mathew, 2003). Three subtypes of K_{Ca} channels have been described on the basis of their single-channel conductance and sensitivity to specific pharmacological blocker (Kuriyama *et al*., 1998). Maxi-K^+ (BK_{Ca}; big, high) is sensitive to charybdotoxin and iberiotoxin, intermediate conductance (IK_{Ca}) is blocked by high concentration of charybdotoxin and, the last, small conductance (SK_{Ca}) that are potently blocked by apamin. However, all the subtypes of Ca^{2+}-activated K^+ channels are also voltage-dependent K^+ channels (Kuriyama *et al*., 1998).

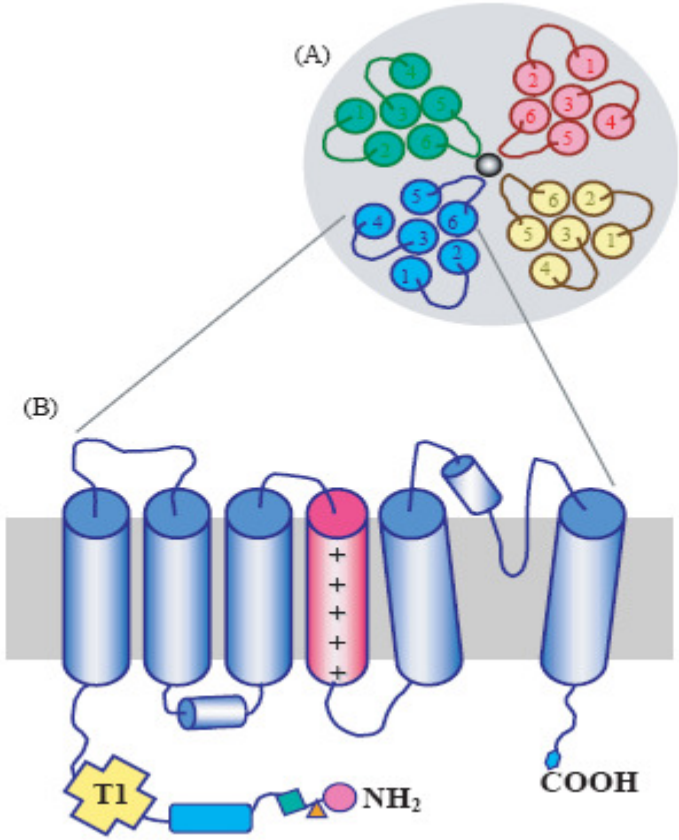

Figure 2-27 Schematic representation of K^+ channel. (A) The putative transmembrane tetrameric assembly of Kv channels, showing a K^+ ion at the central symmetrical axis. One of monomers has been shown in (B), containing six transmembrane segments and one re-entrant loop. The fourth segment is positively charged and functions as voltage sensor. On the cytoplasmic side, the known structural and functional domains have shown. The extreme N-terminus has a ball-like structure (pink circle), responsible for rapid inactivation. A secondary inactivation domain has also been reported for Kv1.4 channels (green diamond). The tetramerization domain, responsible for subunit–subunit interactions, has been depicted as yellow cross. In cardiac potassium channels (HERG), a PAS; gene products, domain (blue rectangle) is located in the N-terminus and shown to be important for channel deactivation. An N-type inactivation prevention (NIP) domain (orange triangle) has been shown to be necessary for protection against rapid inactivation in Kv1.6 channels. While the extreme C-terminus has a binding motif for PSD-95 class of proteins (cyan hexagon) and has been co-crystallized with them (Varshney and Mathew, 2003).

Anwer *et al*. (1993) demonstrated that the BK_{Ca} channel inhibitors iberiotoxin and tetraethylammonium increased the frequency of spontaneous myometrial contractions in myometrial strips from estrogen-primed rats, and also initiated spontaneous contractions in quiescent myometrial strips from term-pregnant women. Iberiotoxin increased contractile activity in rat, and human myometrium, and depolarize human myometrial cells in association with an increase in $[Ca^{2+}]_i$ as a result of activation of voltage-sensitive L-type Ca^{2+} channel (Anwer et al., 1993). Tetraethylammonium (TEA) a nonselective K^+ blocker also stimulates the mechanical activity of rat myometrial, most likely because of its ability to block K_{Ca} channel. Although during labor, the Maxi-K^+ is insensitive to voltage or Ca^{2+}, and many permit an increase $[Ca^{2+}]_i$ without hyperpolarization, has been describe in human myometrium (Sanborn, 2000).

Adenosine triphosphate-sensitive K^+ channels (K_{ATP})

K_{ATP} has been implicated in myometrial function, and measured directly (Sanborn, 2000). The primary structure of typical K_{ATP} channels contains two transmembrane domains with intracellular ATP binding site (Varshney and Mathew, 2003). Activation of K_{ATP} channel increases K^+ efflux, resulting in hyperpolarization and decreasing the likelihood of action potential generation in the cell (Monga and Sanborn, 1992). These channel inhibited by steady state-ATP concentration, and their activity is also modulated by nucleotide diphosphates and by phosphorylation (Sanborn, 2000). K_{ATP} channels openers, cromakalim and levcromakalim depressed the contractant-stimulated uterine contraction and were selective antagonized by glibenclamide. They were more potent in nonpregnant than pregnant rat and human myometrium (Sanborn, 2000).

Delayed rectifier K^+-channels (K_{dr} or $K_{V1.5}$)

The predominant K_V current in myometrium is a voltage-dependent, slowly activated delayed rectifier ($K_{V1.5}$) (Figure 2-28). This K^+-channel gene was exhibited estrogen-dependent regulation (Sanborn, 2000). In addition, The K_V current was maintained as the driving force for Ca^{2+} entry during activation of the resting cells. These delayed rectifier currents were present in myometrial smooth muscle cells from

both nonpregnant and late pregnant animals, and were suppressed by both TEA (≥ 0.5mM) and 4-aminopyridine (4-AP; ≥ 0.4mM, that exhibited slow recovery from inactivation) (Okabe *et al*., 1999; reviewed by Aaronson *et al*., 2006).

Inward rectifier K^+ channels (K_{ir})

The two transmembranes, inwardly rectifying K^+ channels (K_{ir}) have a different gating mechanism that involves the voltage-dependent plugging of the channel pore by intracellular Mg^{2+} and polyamines. The C-terminus of this class of channel has been shown to influence the gating and pore conductance. A negatively charged amino acid at the C-terminus of this channel is found to be critical for both inward rectification and ion permeation (Varshney and Mathew, 2003). The expression of the mRNA for (K_{ir}) is highest in midpregnancy (Sanborn, 2000).

Almost nothing is known regarding the contribution of the K_V and K_{ATP} channels in regulating myometrial contractility, although Hamada *et al*. (1994) suggested that the β_2-adrenoceptor agonist ritodrine activates the latter type of channel in human myometrium. There are, however, substantial evidences that BK_{Ca} channels are important in regulating myometrial function. For example, these channels have been shown to be activated by a variety of agents, which inhibit myometrial contractility; these include relaxin, human chorionic gonadotrophin, cAMP-generating agents (e.g. β-adrenergic agonist), cGMP-generating agents (e.g. NO) (reviewed by Aaronson *et al*., 2006). The mechanism which regulate the K^+ channel involve receptor-channel coupling, channel phosphorylation or modification of critical sufhydryl groups by oxygen radicals such as nitric oxide (NO) (Wray, 1993; Riemer and Heyman, 1998). Moreover, Khan and coworkers (1993) have presented evidence that BK_{Ca} channels in human myometrium from women in active labor lose their Ca^{2+} sensitivity, and have argued that this transition enhances myometrial contractility by preventing these channels from suppressing membrane excitability as $[Ca^{2+}]_i$ rises (reviewed by Aaronson *et al*., 2006). Aaronson *et al*. (2006) assessed the effects of various inhibitors of BK_{Ca} and K_V channels on spontaneous contractions in myometrial strips taken from nonpregnant rats, and also those in early, mid- and late pregnancy. They suggested that BK_{Ca} channels play little role in regulating these contractions at

any time, but inhibition of K_V channels causes marked effects on spontaneous activity. This implies that K_V channels, as in other types of rhythmically active smooth muscles, are important to the control of frequency and amplitude of spontaneous depolarization in rat myometrial tissue.

Chloride channel

Chloride channels are membrane proteins that mediate passive transport of chloride ions across the lipid bilayer by forming an aqueous diffusion pore (Pusch and Jentsch, 1994). The chloride equilibrium potential in many smooth muscles is usually more positive than the resting membrane potential. Thus, activation of Cl^- channels lead to depolarization and enhanced excitability and contraction (Carl *et al*., 1996). Another important class of Cl^- channels is activated by elevated intracellular calcium, calcium-activated chloride (Cl_{Ca}) channel (Figure 2-28). The Cl_{Ca} channels have been described in rat myometrium stimulated with oxytocin. These channels therefore may be activated by Ca^{2+} release from the sarcoplasmic reticulum and also activated by extracellular Ca^{2+} entry. Increasing the intracellular Ca^{2+} concentration in cells held under voltage clamp conditions gives rise to a chloride current proportional to the Ca^{2+} current. Activation of these channels will lead to depolarize the muscle and transiently open VOCCs, as Cl^- leaves the cell. Hence these channels may also play an important role in governing excitability of the myometrium (McFadzean and Gibson, 2002; Wray *et al*., 2003).

Cytosolic calcium and uterine contraction

Smooth muscle contraction is regulated by cytosolic Ca^{2+} level, and sensitivity to Ca^{2+} of the contractile elements in response to changes in the environment surround the cell (Karaki et al., 1997). An increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$ is an initial step for the cells to contract (Horowitz *et al*., 1996; Sanborn, 2000). For the uterine contractility, Ca^{2+} is considered to be the major charge carrier, except at the end of pregnancy which Na^+ may also play a role (Parkington and Colemman, 1990; Sanborn, 2000). The binding of an agonist (e.g. norepinephrine or oxytocin) to the surface receptor or smooth muscle induces a signal that spreads from

the outside to the inside of the plasma membrane and activates several effectors that ultimately initiate contraction.

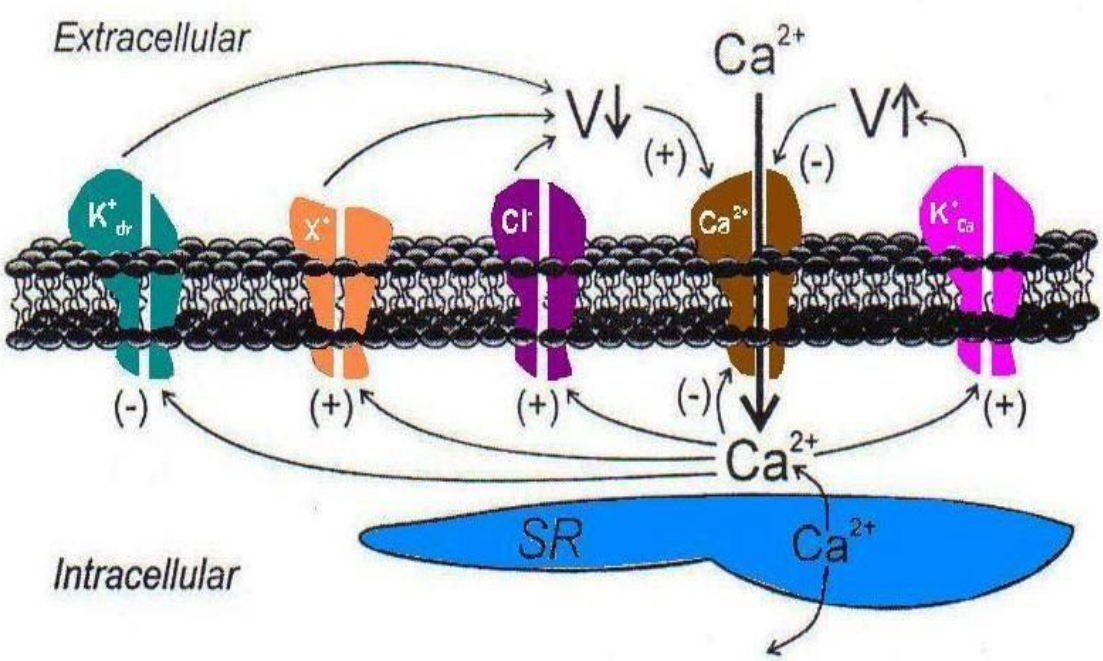

Figure 2-28 Positive and negative-feedback loops regulating Ca^{2+} entry. K^+_{dr}, Delayed rectifier K^+ channels; X^+, Ca^{2+}-facilitated nonselective cation channels; Cl^-, Ca^{2+}-activated Cl^- channels; Ca^{2+}, voltage-dependent Ca^{2+} channel; K^+_{Ca}, Ca^{2+}-activated K^+ channels. Effect of Ca^{2+} on K^+_{dr} , X^+, and Cl^- tend to decrease membrane potential (depolarize) and increase excitability (V↓). This enhances Ca^{2+} entry via voltage-dependent Ca^{2+} channels and generates positive feedback. Activation of K^+_{Ca} and inactivation of Ca^{2+} channels tend to increase membrane potential (hyperpolarization) and/or decrease excitability (V↑). These pathways constitute negative feedback. Major Ca^{2+} sources include entry through Ca^{2+} channels and release of Ca^{2+} from sarcoplasmic reticulum and either source can contribute to regulation of ion channels in the plasma membrane (Carl *et al*., 1995).

Two sources of calcium contribute to the rise in cytosolic calcium (Vander *et al*., 2001; Wray *et al.*, 2003; Guyton and Hall, 2006; Sanborn, 2007):

1. Extracellular calcium which enters the cell through plasmalemma calcium channels as follows:

a. Voltage-gated calcium channel (voltage-operated calcium channels; VOCCs); these channels are regulated by membrane depolarization.

b. Ligand-gated calcium channel which regulated by chemical substance e.g. neurotransmitters, hormone (Receptor-operated calcium channels; ROCCs) or by activation of chemical to the calcium channels.

c. Transient receptor potential channels (TRPC)/ nonselective cation channels. They are non voltage-gated calcium channel, including ROCCs and store operated calcium channels (SOCCs) (McFadzean and Gibson, 2002).

2. Releasing from the rapidly exchanging intracellular calcium stores (sarcoplasmic reticulum). There are two families of intracellular channels responsible for releasing Ca^{2+} from sarcoplasmic reticulum (Berridge and Bootman, 1997; McFadzean and Gibson, 2002):

a. Inositol 1, 4, 5-triphosphate ($InsP_3Rs$ or IP_3Rs or IP_3 receptor/ calcium release channels.

b. Ryanodine receptor/ calcium release channels

c. Passive leak from sarcoplasmic reticulum (SR)

In addition, in human myometrial cell, an IP_3 mediated release of Ca^{2+} and accompanying force can be demonstrated in the presence of agonist, but its contribution is small, compared with Ca^{2+} influx (Kupittayanunt *et al*., 2002). Therefore, the force of contraction of smooth muscle is highly dependent on the extracellular fluid calcium ion concentration. However, the more extensive the sarcoplasmic reticulum in the smooth muscle fiber, the more rapidly it contracts, presumably because calcium entry through the cell membrane is much slower than internal release of calcium ions from the sarcoplasmic reticulum (Guyton and Hall, 2006).

1. Extracellular calcium which enters the cell through plasmalemma calcium channels

1a. Voltage-operated calcium channels; VOCCs (Figure 2-29)

During the electromechanical coupling process, the primary drive for the rise in intracellular calcium (and thus contraction of the muscle) is membrane depolarization, with the consequential opening of VOCCs. Neurotransmitters or hormones acting to depolarize the membrane will generally cause contraction whilst that producing membrane hyperpolarization will cause relaxation (McFadzean and Gibson, 2002).

The smooth muscle cell membrane has far more voltage-gated calcium channels than does of skeletal muscle but few voltage-gated sodium channels. Therefore, sodium participates little in the generation of the action potential in smooth muscle. Instead, the influx of calcium ions through voltage-gated calcium channels is mainly responsible for the action potential. However, calcium channels open many times more slowly than do sodium channels, but they also remain open much longer (Guyton and Hall, 2006).

The characteristics of the Ca^{2+} conductance, especially the voltage and Ca^{2+} dependence of its inactivation parameters, have been determined in intact myometrial strips. It has been proposed that Ca^{2+} channel current inactivates by a mechanism that is both membrane potential and intracellular Ca^{2+} concentration (Amédée *et al*., 1987). Voltage-dependent calcium channels are a family of channels that open in response to membrane depolarization to mediate the selective entry of Ca^{2+}. The multiple types (L, T, N, P/Q and R types) are classified on the basis of their kinetics and pharmacological properties (Berridge, 2003). Tsien and colleagues (1985) extended the classification of dorsal root ganglion channels and proposed that these channels be called T type for transient, L type for long lasting, and N type for neither T nor L type (Perez-Reyes, 2002):

- L-type channels are activated by high voltage. They are found extensively in both myocardial and vascular tissues, as well as in the smooth muscle.
- T-type channels are activated by low voltage. They are found in smooth muscle, skeletal muscle and cardiac myocytic membranes.

- N-type channels are found only in neurons and are thought to play a direct role in release of neurotransmitter quanta packages into the synapse and are insensitive to calcium antagonists.
- P-type channels are found only in cerebellar purkinje neurons in the brain and, as such, are not discussed here.
- R-type channels are activated by high voltage (Berridge, 2003).

In human myometrium, two subtypes, L- and T-type calcium channels have been identified (Riemer et al., 2000; Sanborn, 2000; Miyoshi, 2004). A T-type Ca^{2+} channel has not yet been identified in rat myometrium (reviewed by Okabe *et al*., 1999; Wray *et al*., 2003). T-type Ca^{2+} channels were originally called low-voltage-activated (LVA) channels because they can be activated by small depolarization of the plasma membrane (Perez-Reyes, 2003). T-type Ca^{2+} channels are also voltage sensitive but open at more negative potential (-60 mV compared with -40 mV) and have a smaller conductance than L-type Ca^{2+} channels (only allow small amounts of ion passage; Perez-Reyes, 2002) and a short opening time (fast channels).

The presence of T-type Ca^{2+} channels is potentially very interesting as they have been associated with action potential transmission and pacemaker activity (Wray *et al*., 2003). In thalamic neuron, the depolarization of the plasma membrane by hyperpolarization-activated current (I_h) leads to activation of T-type currents (I_T), and a second phase of depolarization called the low-threshold Ca^{2+} spike. Riding on top of the low-threshold Ca^{2+} spike are a burst of Na^+ spikes mediated by fast voltage-gated Na^+ channels. High-threshold Ca^{2+} and K^+ currents can also be activated by the low-threshold calcium spikes. Ca^{2+} entry during the burst leads to activation of Ca^{2+}-activated K^+ currents, which in combination with voltage-gated K^+ channels are repolarize the membrane. T-type channels display a window current, i.e., there is a small range of voltages where they can open, but do not inactivate completely. This property may be particularly important in controlling intracellular Ca^{2+} levels (Jones, 1998; Perez-Reyes, 2002). T-type channels are thought to have a role in initiation of action potentials, but none in contractility (Breese, 1997).

Similar to other smooth muscles, L-type Ca^{2+} channels (now also referred to as Ca_v 1.2 channels) are considered to be a major Ca^{2+} influx pathway in

myometrium (Karaki *et al*., 1997; McFadzean and Gibson, 2002; Wray *et al*., 2003). They are opened by depolarization or during action potential or slow wave discharge (Bolton *et al*., 1999). Under resting condition (-40 to -50 mV), they are closed (Parkington and Colemman, 1990). Upon excitation, a rapid depolarization of cell membrane occurs; voltage-dependent Ca^{2+} channel is then opened. The opening leads to flood of Ca^{2+} into the cell and, ultimately, contraction. Thus, the major source of Ca^{2+} for contraction is the extracellular fluid. Entry of Ca^{2+} down its electrochemical gradient occurs when the membrane depolarizes to around -40 mV and L-type channels open. Inactivation of these channels (often by Ca^{2+} itself) and repolarization of the membrane by outward current due to K^+ channels opening terminates the Ca^{2+} entry, and relaxation will occur (Wray *et al*., 2003). It has long been known that high potassium solution can depolarized plasma membrane and opened calcium channel (Bolton, 1979). In smooth muscle, exposures to potassium-rich solution elicit contractions that are dependent on extracellular calcium (Godfraind *et al*., 1986). Agonists can open the L-type calcium channels by depolarizing the cell membrane through activation of the nonselective cation channels, inhibition of the K^+ channel and/or activation of the Cl^- channel (van Breemen and Saida, 1989; Carl *et al*., 1995; Karaki *et al*., 1997; Wray *et al*., 2003). Some agonists (such as Bay K8644, endogenous Ca^{2+}) have direct activation to L-type Ca^{2+} channels to activated mode, while Ca^{2+} channel blockers (nifedipine or verapamil) promotes in the inactivated (close) mode of Ca^{2+} channel (Vaghy, 1998; Wray *et al*., 2003).

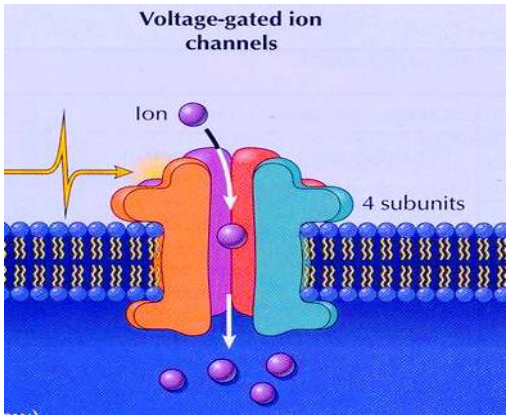

Figure 2-29 Voltage- operated Ca^{2+} channel (Adapted from Raffa *et al*., 2005)

1b. Receptor-operated calcium channels; ROCCs

Receptor activation could lead to calcium entry into smooth muscle cells by mechanisms independent of membrane depolarization (Bolton, 1979; van Breemen and Saida, 1989; Somlyo & Somlyo, 1994). ROCCs are coupled to excitatory receptors either directly (Figure 2-30) or via G proteins and open in response to receptor ligands (Cambell et al., 1999; Yousef *et al*., 2005). There are evidences that members of the subgroup of transient receptor potential (TRPC) channels (described below) constitute ROCCs and SOCCs in smooth muscle. ROCCs are activated in response to cell surface receptor (usually GPCR) stimulation which is independent of depletion of internal Ca^{2+} stores (Albert *et al*., 2007).

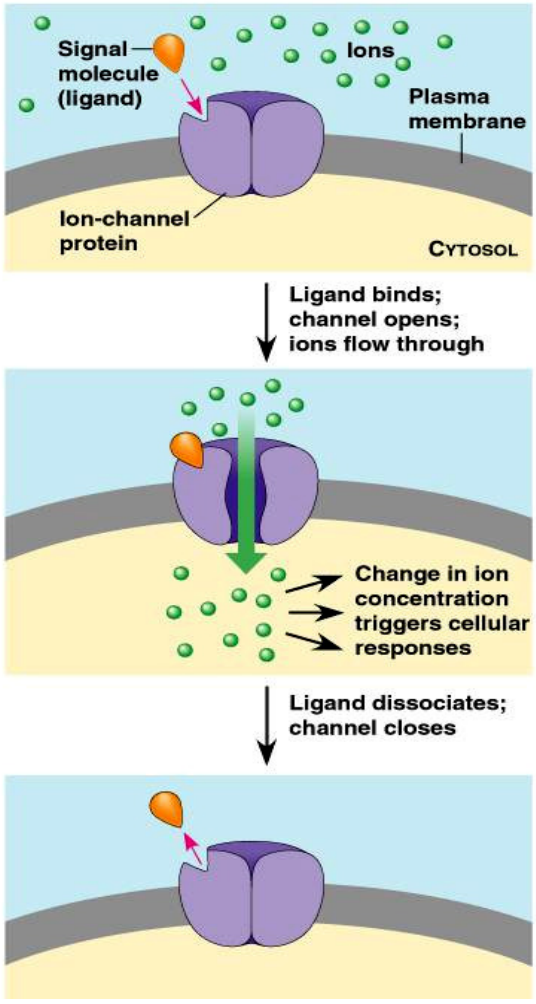

Figure 2-30 Ligand directly coupled to receptor (Cambell *et al*., 1999).

G-proteins

The guanine nucleotide binding proteins (G-proteins) are heterotrimers consisting of α-, β- and γ-subunits: a large α-subunit of 39-46 kDa, a β-subunit of 37 kDa and a γ-subunit of 8 kDa. The α-subunit has a binding site for GTP and GDP and an intrinsic GTPase activity. The β- and γ- subunits exist as a covalently bound complex and are only active in this form (Figure 2-31). The α-subunits appear to be most diverse and are believed to be responsible for the specificity of the interaction of different G-proteins with their effectors. In the basal state, the α-subunit contains bound GDP and association of α- and βγ-subunits is highly favored, keeping the G-protein in the inactive form. Stimulation of the G-protein results when it binds GTP rather than GDP. Receptors interact most efficiently with the heterotrimeric form of the G-protein and accelerate activation by increasing the rate of dissociation of GDP and enhancing the association of GTP. Activation of G-protein coupled receptor results in the dissociation of heterotrimeric G-proteins into α-subunits and βγ-dimers. Finally, the G-protein α-subunit has an intrinsic hydrolytic activity that slowly converts GTP to GDP and returns the G-protein to its inactive form (Bárány, 1996; Vander *et al*., 2001; Orun, 2006).

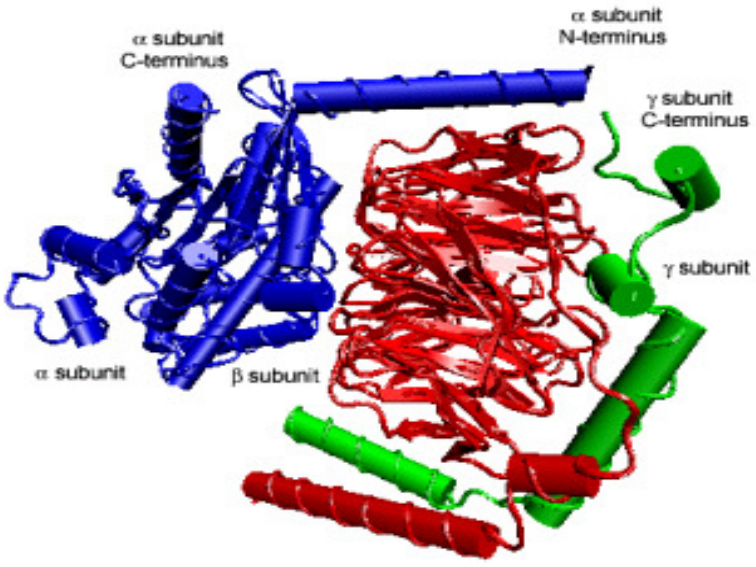

Figure 2-31 Overall structure of heterotrimeric G-proteins (Orun, 2006).

A G protein serves as a switch to “couple” a receptor to an ion channel or an enzyme in the plasma membrane. The G protein may cause the ion channel to open, with resulting generation of electric signals or, in the case of calcium channels, change in cytosolic calcium concentration. Alternatively, the G protein may activate or inhibit the membrane enzyme with which it interacts; these are enzymes that, when activated, cause the generation, inside the cell, of second messengers.

To illustrate some of the major points concerning G proteins, plasma-membrane effector proteins, second messengers and protein kinases. Following will describe one of the most important effector protein enzyme; phospholipase C-regulated by G proteins and the subsequent portions of the signal transduction pathways in which it participates (Figure 2-32 and 2-33) (Vander *et al*., 2001; Raffa *et al*., 2005).

Phospholipase C, Diacyglycerol, and Inositol Triphosphate

In this system, the relevant G protein (termed G_q), activates a plasma-membrane effector enzyme called phospholipase C. This enzyme catalyzes the breakdown of phospholipid known as phosphatidylinositol bisphosphate (PIP_2), to diacylglycerol (DAG) and inositol triphosphate (IP_3). Both DAG and IP_3 then function as second messengers but in very different ways (Figure 2-32) (Vander *et al*., 2001; Faber, 2003; Sanborn, 2007).

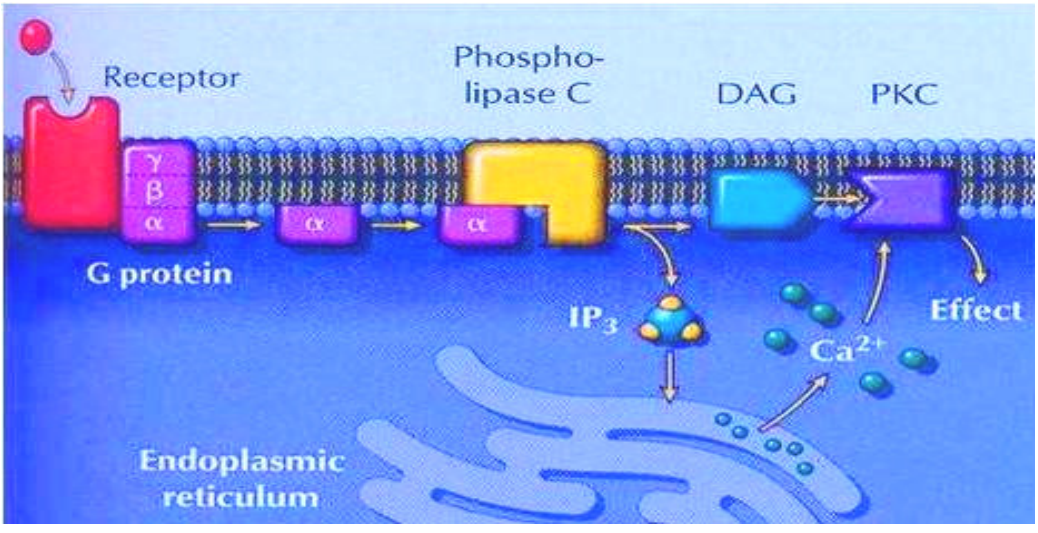

Figure 2-32 G-protein couple receptor and effector protein; phospholipase C (Adapted from: Raffa *et al*., 2005, p. 15).

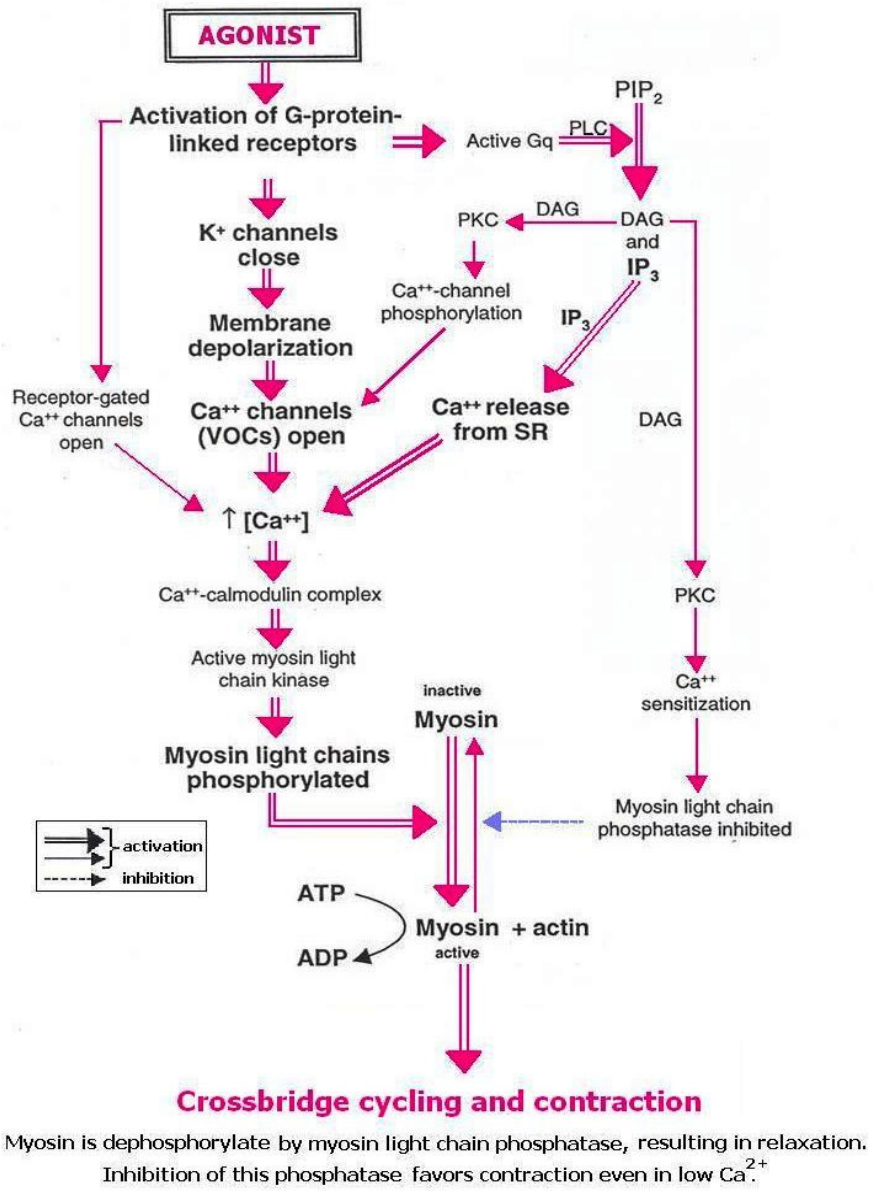

Figure 2-33 Mechanism of agonists-induced contraction via G-protein linked receptor (Adapted from: Faber, 2003).

Diacylglycerol

DAG activates a particular protein kinase known as protein kinase C (PKC), which then phosphorylates a large number of other proteins, leading to the cell's response (Figure 2-32) (Vander *et al.*, 2001). DAG together with Ca^{2+} activates PKC promote contraction by phosphorylation of L-type Ca^{2+} channels and subsequent augmentation of VOCCs conductance. In addition, PKC also phosphorylates other

proteins which further phosphorylate and decrease myosin light chain phosphatase activity (Figure 2-33) (Faber, 2003; Jin and Burnett, 2006).

Inositol 1, 4, 5-triphosphate

The inositol ring contains six hydroxyl residues; most of them can be phosphorylated by specific kinases. Inositol 1-monophosphate is the constituent of phosphatidylinositol (PI) one of the phospholipids in animal cell membranes and one of it hydrolytic product is inositol 1, 4, 5-triphosphate (IP_3). IP_3, in contrast to DAG, dose not exert its second messenger role by directly activating a protein kinase. Rather, IP_3, after entering the cytosol, binds to calcium channels on the outer membranes of the endoplasmic (sarcoplasmic) reticulum and opens them. Calcium then diffuses out of this organelle into the cytosol, significantly increasing cytosolic calcium concentration (Figure 2-32 and 2-33) (Vander *et al*., 2001). IP_3 is convert inside the cell to the (1,3,4,5) tetraphosphate, IP_4, by a specific kinase. The exact role of IP_4 remains unclear, but there is evidence that it too is involved in Ca^{2+} signaling. One possibility is that it facilitates Ca^{2+} entry through the plasma membrane, thus avoiding depletion of the intracellular stores as a result of the action of IP_3 (Rang *et al*., 2007).

1c. Transient receptor potential (TRP) channels/ nonselective cation channels

Although the best characterized calcium entry pathway utilizes voltage-operated calcium channels (VOCCs), however, there are several types of calcium-permeable channels which are non-voltage-gated, including the ROCCs and store-operated calcium channels (SOCCs), activated by depletion of the calcium stores within the sarcoplasmic reticulum (McFadzean and Gibson, 2002). According to those reviewed by Sanborn (2007), cells have additional mechanisms to allow Ca^{2+} entry. While, in the past, the focus was on the properties of the currents or stimuli resulting in changes in $[Ca^{2+}]_i$ {i.e., capacitative, receptor or store-operated, calcium-release activated calcium current (ICRAC), or non-selective cation currents}, as more of the proteins have been cloned, the focus has shifted to the properties of particular protein channel complexes; transient receptor potential (TRP) channel superfamily. The TRP channel superfamily is comprise of the TRPM, TRPC, TRPV, TRPA, TRPP, TRPML,

and TRPN subfamilies. A number of the proteins in this family have been shown, in many different cell types, to contribute to nonselective cation and Ca^{2+} entry in response to one or more signals. The TRPC responds to a variety of stimuli, including GPCR activation, IP_3 receptor activation, DAG and intracellular Ca^{2+} store depletion (Figure 2-34, 2-35) (Venkatachalam, 2002; Ramsey *et al*., 2006). Contraction evoked by agonists acting on, for example, α_1-adrenoceptors expressed in smooth muscle cell of aorta or acetylcholine receptors in the plasma membrane of airway smooth muscle cell initiate contraction by activating a calcium-dependent pathway. PLC_β activated by $G_{q/11}$ proteins cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and IP_3. While IP_3 acts on IP_3 receptors and releases calcium from internal stores (e.g. the ER), DAG activates ROC-like TRPC3/6/7 (Figure 2-34, 2-35). Recently, a carefully analysis of TRPC6 function supports a TRPC6 permeation model in which Ca^{2+} contributes only a small percentage (~4%) to whole-cell currents in the presence of extracellular Na^+. Regardless of their potential contribution of Ca^{2+} for contraction (stippled lines), TRPC3/6/7 channels do mediate all depolarization with ensuing activation of voltage-gated calcium channels ($Ca_V1.2$), entry of extracellular Ca^{2+} and contraction (Figure 2-34)(reviewed by Dietrich *et al*., 2006).

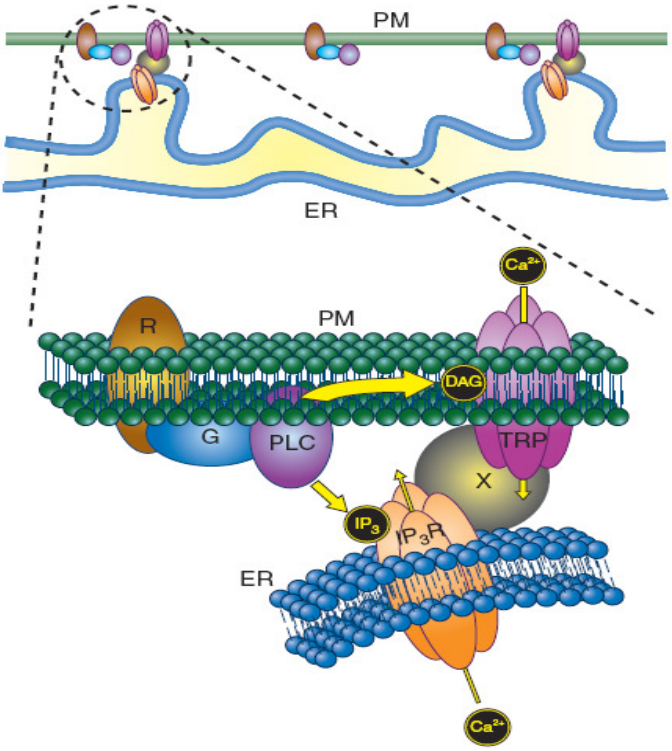

Figure 2-34 DAG and IP_3, the products of $PLC_\beta/G_{q/11}$ pathway, activates ROC-like TRPC3/6/7 (Adapted from: Venkatachalam, 2002).

Store-operated calcium channels (SOCCs)

The stimulation of G protein coupled receptors leads to a continued depletion of internal Ca^{2+} stores by the formation of IP_3 and results in activation of SOCCs which provides a constant influx of Ca^{2+} into the cytosol which may be capable of inducing a sustained contraction. The sustained contraction may be produced directly by the influx of Ca^{2+} through SOCCs and/or indirectly through VOCCs which may be opened as a result of depolarization produced by SOCCs activity. In addition, the SOCCs-evoked influx of Ca^{2+} may have an important role in refilling internal Ca^{2+} stores (Figure 2-34) (Albert and Large, 2003).

There are at least two, and probably more, distinct SOCCs in smooth muscle which have markedly different permeabilities to Ca^{2+}. One subtype of SOCCs, exhibits a low Ca^{2+}-selectivity, which in contrast to the high Ca^{2+}-selectivity exhibited by the CRAC subtype of SOCCs (Villereal, 2006; Albert and Large, 2007). Albert and Large, (2003) demonstrated that the calcium and sodium permeability ratio ($P_{Ca}{}^{2+}/P_{Na}{}^{+}$) of SOCCs in rat portal vein is about 50 whereas in aorta the estimated $P_{Ca}{}^{2+}/P_{Na}{}^{+}$ is 1. It is possible that high Ca^{2+} permeable SOCCs, ICRAC subtype, are not link to contractile response, but is well suited for refilling intracellular Ca^{2+} stores whereas low Ca^{2+} permeable SOCCs are important in mechanical effects. SOCCs may also represent a depolarizing mechanism as well as a Ca^{2+} influx pathway. These differences in Ca^{2+} permeability between SOCCs in smooth muscle and ICRAC in non-excitable cells provide further evidence that these channels are likely to possess different molecular structures.

To date, understanding of the functional role of TRPs in Ca^{2+} homeostasis and contraction has come largely from other smooth muscle systems. In aortic myocytes it has been proposed that depletion of [Ca^{2+}] SR releases a calcium influx factor (CIF) from the stores which displaces calmodulin (CaM) bound to membrane-delimited Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) which produces lysophospholipids to open SOCCs. This represents a classical store-dependent activation mechanism. In contrast, in rabbit portal vein and mesenteric artery myocytes, SOCCs evoked by both of SR Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA) and calcium chelator BAPTA-AM are almost completely blocked by inhibitors

of protein kinase C (PKC) indicating a pivotal role for this kinase in SOCCs activation (Albert *et al*., 2007).

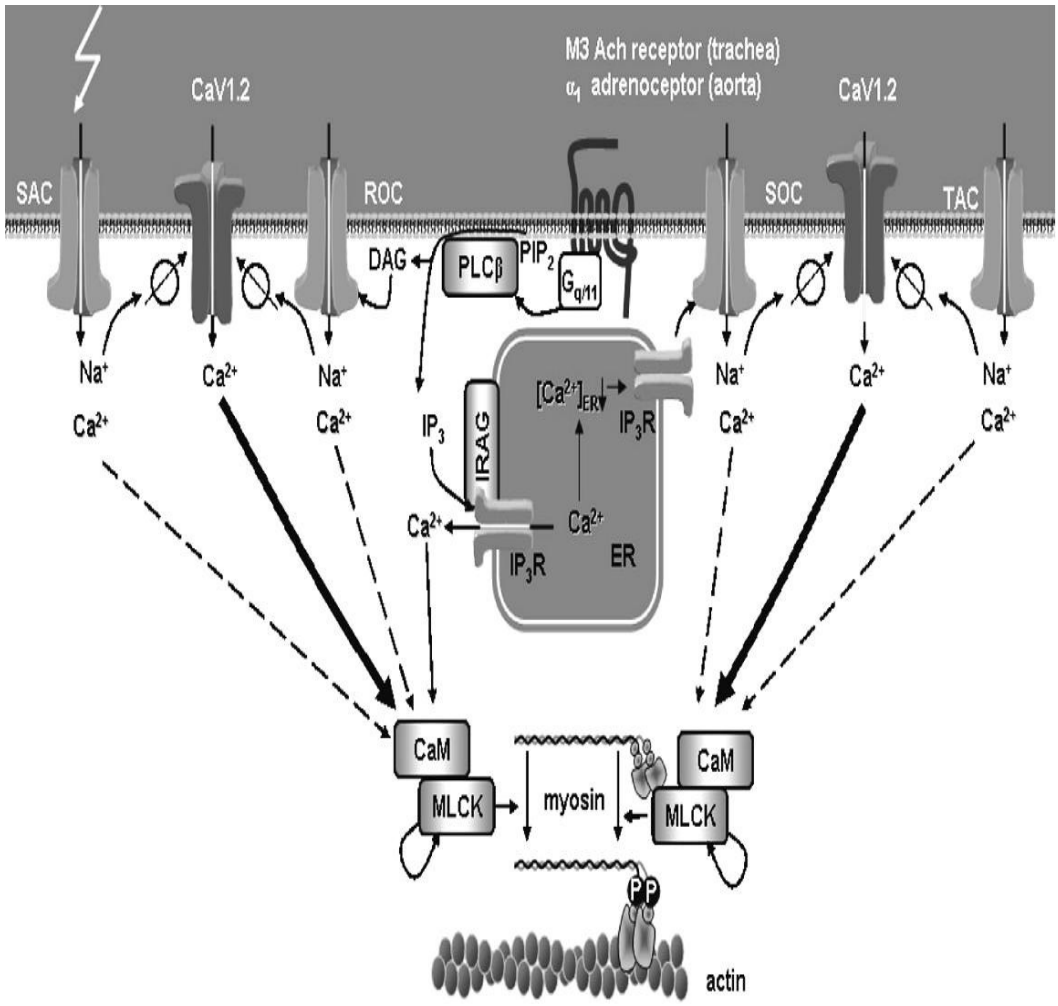

Figure 2-35 Ion channels in smooth muscle cells formed by members of the TRP superfamily. Light grey areas indicate high Ca^{2+} concentration (1–2 mM), white areas low Ca^{2+} concentrations (50–200 nM), M_3 ACh R=muscarinic acetylcholine receptor type 3, CaM=calmodulin, MLCK=myosin-light chain kinase, ER=endoplasmic reticulum, IP_3-R=IP_3 receptor, CaV1.2=voltage-gated calcium channel, SOC=store-operated channel, ROC=receptor-operated channel, SAC=stretch-activated channels, TAC=tonically active channels.(Dietrich *et al*., 2006).

A recent advance in the understanding of the potential molecular composition of SOCs has been the discovery of two families of transmembrane proteins, STIM and Orai, which have been proposed to mediate ICRAC in non-excitable cells with the Stim1 protein acting as an ER Ca^{2+} sensor/activator of Orai1 and Orai1 constituting the ICRAC channel/ion transport mechanism. As there are vast differences between the biophysical properties of ICRAC and many SOCCs, it seems unlikely that Orai proteins alone mediate the pore-forming subunits of all SOCs. However, recent studies have suggested that STIM1 and Orai1 may interact with TRPC proteins to modify their function. Huang *et al*. (2006) showed that over-expression of the cytosolic terminus of STIM1 increased TRPC1 activity and also demonstrated that STIM1 and TRPC1 proteins can associate with one another. In addition over-expression of Orai proteins was shown to enable thapsigargin to activate TRPC3 and TRPC6 activity through a STIM1-mediated mechanism which was not present in the absence of Orai proteins. These results indicate that STIM proteins may act as store-operated regulators of SOCs and also that Orai proteins may combine with TRPCs to produce functional store-operated channels either through acting as a pore-forming subunit or as a regulatory β-subunit (Albert *et al*., 2007).

Stim1 and Orai1 are expressed in human myometrium but their function there remains to be elucidated. An alternative store-operated mechanism involves production of a Ca^{2+} influx factor, Ca^{2+}-dependent phospholipase A_2 activation, and generation of lysophospholipids; this has not been studied in myometrium (Sanborn, 2007).

2. Releasing from the rapidly exchanging intracellular stores; sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is the physiological intracellular source and sink of intracellular Ca^{2+} in smooth muscle (Somlyo and Somlyo, 1994). The second messengers released from the plasma membrane or generated in the cytosol in response to the binding of extracellular chemical messengers to plasma-membrane receptors, can trigger the release of calcium from the more centrally located SR (Vander *et al*., 2001). The observation of spontaneous sporadic releases of packets of stored calcium made 20 years ago has opened up a number of new concepts in

smooth muscle physiology: (1) the calcium release sites are ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptor channels which contribute to cell-wide increases in $[Ca^{2+}]_i$ in response to cell depolarization, activation of IP_3-generating receptors, or other stimuli; (2) changes in $[Ca^{2+}]_i$ act back on the cell membrane to activate or modulate K^+, Cl^- and cation channel activity so affecting contraction, in arterial smooth muscle for example affecting blood pressure; (3) IP_3 production is voltage dependent and is believed to contribute to pacemaker potentials and to refractory periods which control the rhythmical motility of many hollow organs (Bolton, 2006). Similarly Wray and Schmygol (2007) also reported another role of the SR which has been; stimulating plasma ion channels and thereby altering membrane excitability.

Although the RyRs shares feature with the IP_3Rs, such as a homotetrameric structure, it is not activated by IP_3 (Horowitz et al., 1996; Fill and Copello, 2002). There is finding support that the ryanodine and IP_3-sensitive stores constitute physically distinct pools of readily releasable calcium and these two distinct intracellular calcium pools coexist in the smooth muscle from the longitudinal and circular layers (Oh et al., 1997).

2a. Inositol 1, 4, 5-triphosphate ($InsP_3$Rs or IP_3 receptor/ calcium release channels

Inositol 1, 4, 5-triphosphate ($InsP_3$Rs or IP_3 receptors) are Ca^{2+} channels assembled from four large subunits, each of ~2,700 residues and each with a single IP_3-binding site. They consist of three domains: an IP_3-binding amino-terminal domain, a regulatory domain containing ATP-binding and phosphorylation sites and a carboxy terminal domain containing six transmembrane regions. The binding of IP_3 to the amino-terminal domain induces a conformation change in the protein, leading to Ca^{2+} channel opening. The transmembrane regions are responsible for the aggregation of four subunits into the functional tetrameric receptor protein, and also serve to form the Ca^{2+} channel (four identical subunits contain an IP_3-binding site in the large N-terminal cytosolic domain. Binding of IP_3 to the amino-terminal induces a conformation change in the protein) (Berridge and Bootman, 1997; Taylor et al., 2004). Binding of IP_3 draws the two domains of the binding cleft together. Partial closure of the clam-like cleft brings together conserved acidic residues to form a Ca^{2+}-

binding site; the Ca-II patch. Binding of Ca^{2+} to this Ca-II site, which lies within a conserved surface and is suggested to mediate protein–protein interactions (P-II), disrupts an interaction between P-II and the suppressor region (Figure 2-36). An interaction between the suppressor region of one subunit and the C-terminal 'gatekeeper' region of another is proposed to keep the pore closed. Displacing the suppressor region by freeing it from its interactions with the IP_3-binding core would therefore allow the pore to open. Finally, the scheme suggests how Ca^{2+} inhibition of IP_3R, most probably mediated by calmodulin, is alleviated by IP_3 binding. It is suggested that Ca^{2+}-calmodulin locks the IP_3R in a closed state by bridging sites within the IP_3R. Irrespective of whether it has Ca^{2+} bound, calmodulin is tethered by one of its two lobes to residues within the N-terminal suppressor region. Binding of Ca^{2+} to the second lobe then allows calmodulin to crosslink to a site elsewhere in the IP_3R, effectively tying the N-terminal to it and reinforcing inhibition of the pore. By displacing the suppressor region, IP_3 moves the tethered calmodulin so that its second lobe can no longer reach to the Ca^{2+}–calmodulin-binding site to cause inhibition (Figure 2-37) (Taylor *et al*., 2004).

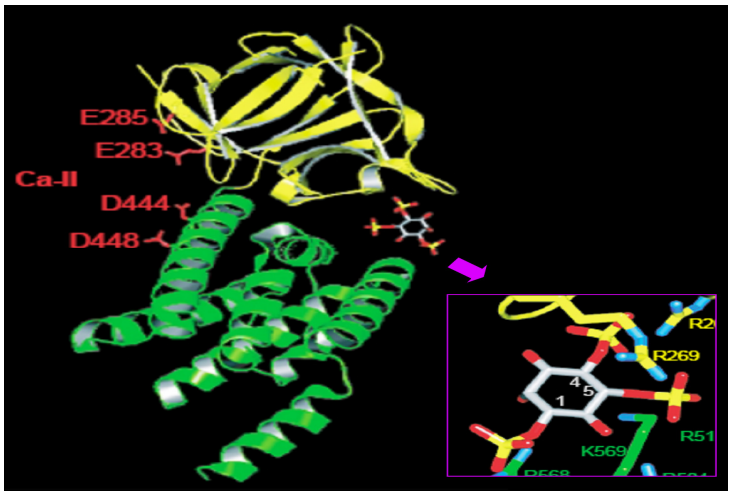

Figure 2-36 The IP_3-binding core shown to highlight four exposed acidic residues (Ca-II, red) that might come together to form a Ca^{2+}-binding site after IP_3 closes the 'clam' and stereoscopic view of contacts between IP_3 (phosphate groups numbered) and residues within the IP_3-binding core (Adapted from: Taylor *et al*., 2004).

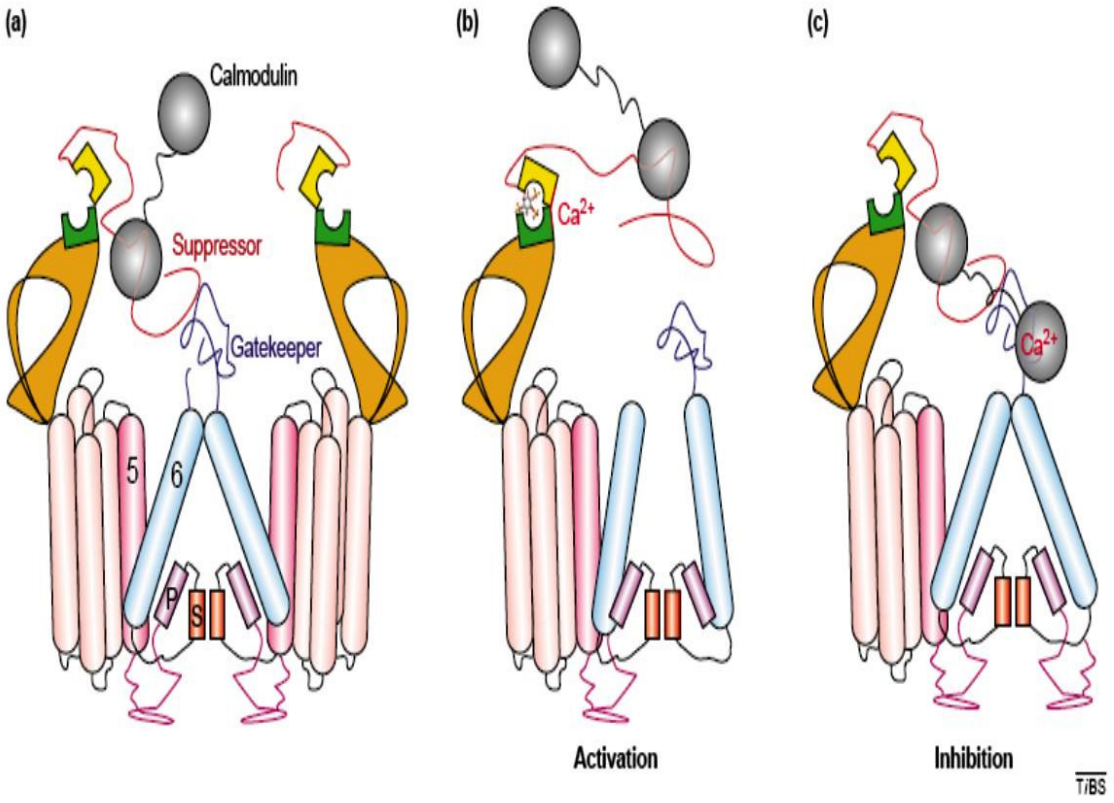

Figure 2-37 A model for activation of inositol (1,4,5)-trisphosphate receptor (IP_3R). This speculative scheme shows only two subunits of the IP_3R in its inactive state (a) with the N-terminal suppressor region holding the pore closed through its association with the C-terminal 'gatekeeper' region of a neighbouring subunit. As IP_3 binds (b), it pulls the 'clam'-like binding site closed and thereby brings together acidic residues near the hinge (red) to form a Ca^{2+}-binding site. Irrespective of the Ca^{2+} concentration, calmodulin is tethered by one of its lobes to the suppressor region (a). When Ca^{2+} binds to the second lobe, therefore, in the absence of IP_3, Ca^{2+} inhibits channel opening (c) (Taylor *et al*., 2004).

IP_3 and cytoplasmic Ca^{2+} synergize in opening the IP_3-receptor channel. The opening of calcium channel in response to IP_3 is potentiated by low (below 300 nM) and inhibited by high concentration (above 300 nM) of Ca^{2+} (Keraki et al., 1997; Bolton et al., 1999). The action of IP_3 on its receptor is blocked by heparin, which does not block and may potentiate the opening of RyRs (Bolton et al., 1999). IP_3Rs are more

physiologically important for its function. This sequestering and release of Ca^{2+} by the SR underlies its crucial role in (i) contributing to the maintenance of low resting $[Ca^{2+}]_i$, (ii) increasing $[Ca^{2+}]_i$ for cellular signaling mechanisms and (iii) promoting the decline of Ca^{2+} transient and relaxation (Wray and Schmygol, 2007).

In the myometrium, the IP_3 receptor mediated Ca^{2+} release is involved in oxytocin-, prostaglandin-, α-adrenergic and M-cholinergic-induced calcium signaling. Binding of agonists to their receptors on the surface membrane activates phospholypase C via GTP-binding proteins, which leads to the production of IP_3 and subsequent release of calcium from the SR (Wray and Shmygol, 2007). There are three isoforms of IP_3 receptors, namely type 1, type 2, and type 3. All three types share about 60–80% similarity in their amino acid sequences and are subject to elaborate regulation on cytoplasmic and luminal side of the SR membrane. Messenger RNA encoding for all three isoforms have been described in human and rodent myometrium with the type 1 being the predominant isoform (Wray and shmygol, 2007).

2b. Ryanodine receptor/ calcium release channels

Ryanodine receptors (RyRs) are homotetramers constructed from a subunit of molecular mass 565 kDa, making these receptors the largest known ion channels (Samsó *et al*., 1999; Fill and Copello, 2002). They represent another Ca^{2+} release mechanism in the SR of many cell types. The RyRs family comprises three major subtypes referred to as RyR1, RyR2, and RyR3. All three subtypes can be activated by Ca^{2+} itself, a process referred to as Ca^{2+}-induced Ca^{2+} release (CICR).

The role of RyR in uterine Ca^{2+} and contraction has not been completely defined; however, it has been proposed that RyR may play a role in oxytocin-induced Ca^{2+} oscillations (reviewed by Chini et al., 2002). Kupittayanunt et al. (2002) demonstrated that Ca^{2+} induced Ca^{2+} release does not play a significant role and that no functioning role for the RYRs in human myometrial tissue could be shown. They also suggested from their data that the sarcoplasmic reticulum may act to limit contractions and act as a calcium sink, rather than to amplify contractions Inhibition of the sarcoplasmic reticulum was associated with an increase in spontaneous force and Ca^{2+} transients, suggesting that it is normally operating as a negative influence on contraction.

Physiological modulation of ryanodine receptor

1. Ca^{2+}

The cytosolic Ca^{2+} has a major importance in the regulation of RyR activity and is considered to be its principal physiological activator. Because the other ligands are unable to activate the channel in the absence of Ca^{2+} or else they require it to exert a maximal effect. The three known isoforms have different sensitivity to Ca^{2+}. Whereas the activation of RyR1 by Ca^{2+} is bell-shaped, that of RyR2 and RyR3 is not. The minimal activation of RyR1 is obtained with ~5 μM, and decrease at $[Ca^{2+}]_i$ levels is mM range. This biphasic effect of Ca^{2+} suggests the presence of two different binding sites for Ca^{2+}: one with high affinity (Km > 1μM) which stimulates the opening of the channel, and another with low affinity (Km < 1mM) which inhibits the channel (Arredouani, 2004).

2. Cyclic adenosine 5'-diphosphoribose (Cyclic ADPribose; cADPr)

The synthesis of cADPr from nicotinamide-adenine dinucleotide (NAD^+) is catalysed by ADPr cyclase, which also catalyses its hydrolysis to ADPr (Arredouani, 2004). cADPr is a naturally occurring nucleotide implicated in the regulation of the gating properties of the ryanodine channel; in fact cADPr may be a second messenger that activates the ryanodine receptor. It potently activates Ca^{2+} release from ryanodine receptor. Chini et al. (2002) demonstrated that human myometrium contains all the components of the cADPr pathway including (1) cADP-activated microsomal Ca^{2+}release and (2) enzymes responsible for synthesis and degradation of cADPr and, furthermore, that intracellular levels of cADPr were detected in human myometrial tissue. They also concluded that the cADPr system is present and operational in human myometrial tissue. Furthermore expression of the ADP–ribosyl cyclase in both human and rat uterus is controlled by 17b-estradiol in the increasing manner (Chini *et al*., 2002). RYR-induced Ca^{2+} release is activated by caffeine and 4-chloro-*m*-cresol but is inhibited by ryanodine alkaloid; dantrolene (Berridge and Bootman, 1997), ruthenium red and the specific cADPr inhibitor 8-Br-cADPr, inhibited the cADPr-induced Ca^{2+} release. However, Ca^{2+} release induced by cADPr was not inhibited by 1 mg/ml heparin, a specific antagonist of the IP_3 channel (reviewed by Chini et al., 2002). It has been suggested that $[Ca^{2+}]_i$ must exceed 1μM

for CICR to occur (Berridge and Bootman, 1997; reviewed by Bolton et al., 1999).

3. Phosphorylation

The RyR is the substrate of many kinases (PKA, PKC, PKG and CaMKII) and potential phosphorylation sites by different kinases have been identified by analyzing the primary RyR structure. The effect of phosphorylation is isoform-dependent. The phosphorylation of RyR2 by PKA for instance increases its Ca^{2+} sensitivity. The CaMKII effects are variable, it activates or inhibits the RyR2. The phosphorylation of RyR1 is controversial. A serine at 2843 position has been reported to be phosphorylated by PKA, PKG and CaMKII. Other investigators have however observed only a very weak RyR1 phosphorylation by PKA or CaMKII (Arredouani, 2004). While RyR2 is phosphorylated by PKA, there is little information about phosphorylation of RyR3, which predominates in myometrium (Sanborn, 2007).

4. ATP

During excitation-contraction coupling, ATP augments the release of Ca^{2+} from the SR by increasing the Ca^{2+} sensitivity of the RyR (Laver et al., 2001). The presence of at least 2 ATP binding sites on RyR1 and 2-4 sites on RyR2 and RyR3 is suggested by the identification of consensus sequences in the primary structure of these proteins (Zucchi & Ronca-Testoni, 1997). The mechanism underlying the potentiation of CICR by ATP remains unclear. One possibility would be a diminution of the free Mg^{2+} activity (inhibitory effect, see below) caused by the increase in [ATP]. Other nucleotides such as ADP, AMP, cAMP, adenine and adenosine are also known to potentiate CICR (Arredouani, 2004).

5. Mg^{2+}

Experiments using $^{45}Ca^{2+}$ efflux from SR and measurements of lipid bilayers-incorporated RyR activity have showed an inhibitory effect of Mg^{2+} on CICR. The precise mechanisms of the Mg^{2+}-RyR interaction remain to be determined. The inhibitory effect of Mg^{2+} would result from a competitive displacement of Ca^{2+} from its high affinity stimulating site or from a binding to the low affinity inhibitory site. The mechanism underlying the inhibitory effect of Mg^{2+} on RyR in vivo is however is

unclear, as the physiological $[Mg^{2+}]_i$ is higher than 1mM. It would be related to the phosphorylation state of the RyR. The Mg^{2+} would inhibit only the non-phosphorylated form of RyR, and this inhibition would be removed by a CaM-dependent mechanism (Arredouani, 2004).

6. Protein-protein interaction

Calmodulin (CaM)

Many CaM binding sites (Figure 2-36) have been identified on RyR. It has been proposed that at rest up to 16 CaM molecules may be bound to RyR. During EC coupling, when $[Ca^{2+}]$i increases following the activation of RyR, the Ca^{2+} binds the CaM and it's the Ca^{2+}-CaM complex that maintains the channel in its inactivated form until most of the free Ca^{2+} is pumped back into the SR (Arredouani, 2004).

The FK506 binding protein (FKBP)

The FKBP is the cytosolic receptor of the immunosuppressor FK506. The dissociation of the FKBP/RyR1 complex induces an increase in the Ca^{2+} and the appearance of many subconductant states. It has also been suggested that the FKBP12 (Figure 2-36) would act a physical link between the VOCCs transverse tubules and RyR1during the EC coupling (Arredouani, 2004).

Calsequestrin (CSQ)

CSQ is a protein present in the lumen of the SR which plays an important role in the storage of Ca^{2+} because of its low affinity for the ion and its high binding capacity (Figure 2-38). Changes in $[Ca^{2+}]$SR would induce conformational changes in CSQ, which in turn would modulate RyR activity (Arredouani, 2004).

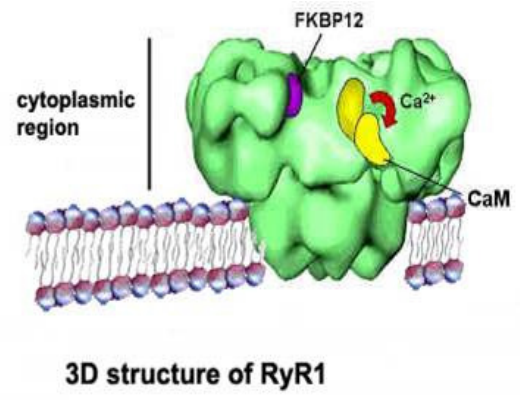

Figure 2-38 Three dimensional structure of the RyR1 receptor showing topology and calmodulin (CaM) and FKBP12 binding sites (Arredouani, 2004).

There are evidences suggest that the smooth muscle RyRs resembles that in cardiac muscle, there is no direct coupling as seen in skeletal muscle (reviewed by Bolton *et al*., 1999). Although RyR1, predominant in skeletal muscle (Figure 2-39; above), are normally activated by transmembrane voltage change (Δ*V*M) via a direct link between the dihydropyridine (DHPR) and RyR1 molecule (DHPR-RyR1 linkage in the cytosolic loop) sensitive Ca^{2+} channel. The membranes of the T-tubule and SR are juxtaposed and separated by a small 10-nm gap. The cytosolic domain of the RyR1 channel spans this narrow gap. Electron microscopy (EM) studies show that the skeletal DHPR in the T-tubules are arranged in clusters of four (tetrads). These tetrads are organized into distinct arrays. The RyR1 channels in the SR membrane are arranged in a corresponding fashion (Figure 2-40). The arrays of DHPR and RyR align in certain fast-twitch skeletal muscle such that every other RyR1 channel is associated with a DHPR tetrad. In cardiac muscle (Fig. 2-39; below), there is about 1 DHPR for every 5–10 RyR2 channels, and the DHPR and RyR2 channels are not aligned in such a highly ordered fashion (Figure 2-40). When a T-tubule Δ*V*M activates the DHPR and the resulting calcium entry triggers underlying RyR2 channels to open. During the long cardiac action potential (~100 ms), the DHPR Ca^{2+} channel has ample time to open and mediate a substantial Ca^{2+} influx. This Ca^{2+} influx is ultimate signal that activates the underlying RyR2 channels via the CICR process (Jones, 1998; reviewed by Bolton *et al*., 1999; Fill and Copello, 2002; Wray and Schmygol, 2007). Single RyR1 clearly turn off as the cytosolic free Ca^{2+}concentration is elevated toward the 1 mM mark. This low-affinity Ca^{2+} inhibition could come into play if local Ca^{2+} levels in the cell reach the 0.6–1 mM range. For the RyR2 channel, the situation is different. Single RyR2 channels turn off only after free Ca^{2+} concentrations reach much higher levels (5–10 mM). The RyR3 protein is also found in mammalian striated muscles, but at relatively low levels (Fill and Copello, 2002).

The uterine myocytes express all three types of RyR although RyR3 seems to be predominant, at least in the mouse myometrium. It appears that the expression of the RyR subtypes in the myometrium changes during different stages of pregnancy (reviewed by Chini *et al*., 2002). Interestingly, the very low level of expressions of RyR2 is up-regulated in pregnant uterus suggesting possible involvement of CICR in the regulation of uterine contractility in labour. There are

however numerous anomalies concerning the behavior of RyR in the uterus, which has led to the suggestion that they may be nonfunctional except perhaps at the end of pregnancy (Wray and Schmygol, 2007). Finally Ca sparks, small transient local releases of Ca^{2+} occurring through RyR have not been found in the uterus. Thus RyR3 may be expressed but not form functional release channels in the myometrium. Finally studies in RyR3 knock out mice suggested that they were acting to inhibit Ca^{2+} signals in the vascular myocytes. Clearly further work is required to determine the specific roles and expression of RyRs in uterine smooth muscle and to investigate their splice variants (Wray and Schmygol, 2007).

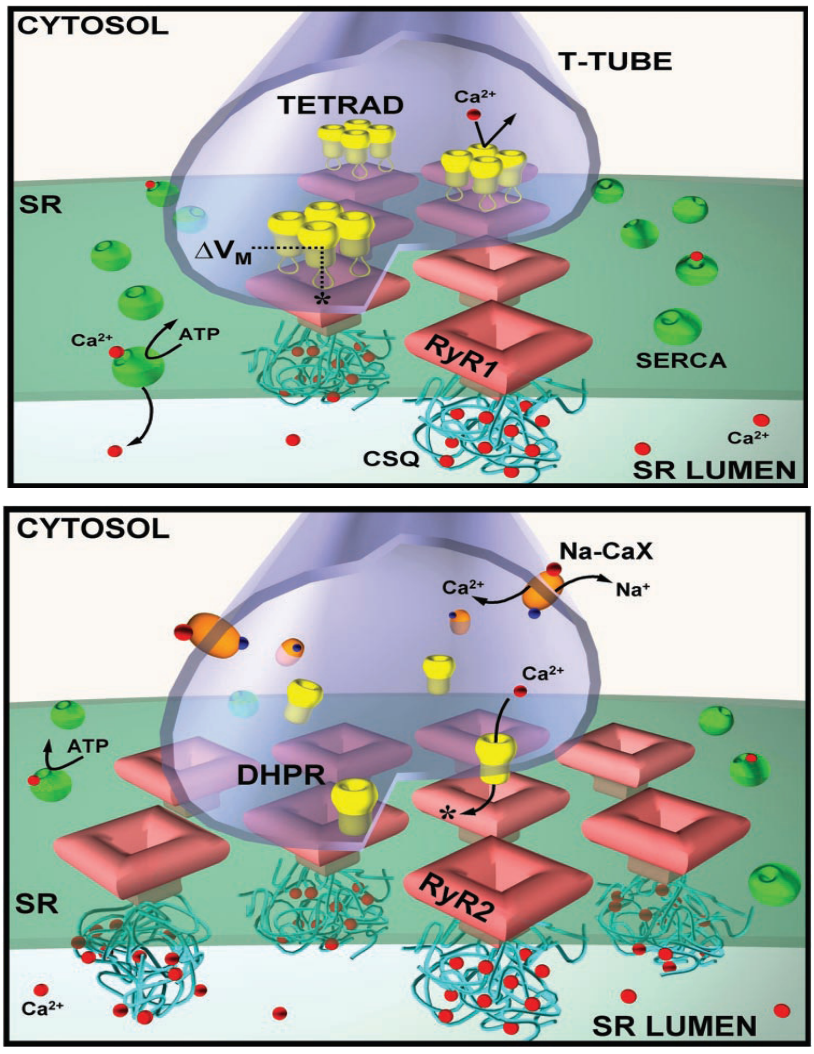

Figure 2-39 DHPR-RyRs interaction; RyR1 (above) are presented in skeletal muscle and RyR2 (below) in cardiac muscle. (Fill and Copello, 2002).

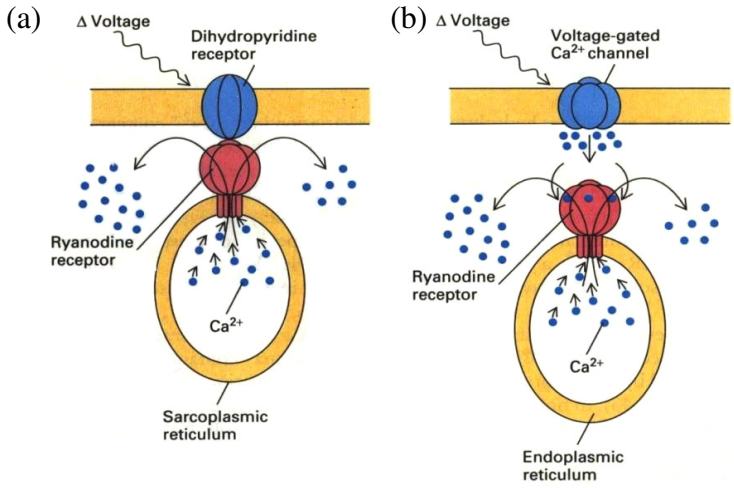

Figure 2-40 Models of ryanodine receptors (a) Voltage-sensitive RyRs which present in skeletal muscle, (b) Ca^{2+}-sensitive RyRs which present in cardiac muscle, neuronal and probably behave similarly to those of smooth muscle. (Lodish *et al*., 2000).

2C. Passive leak from sarcoplasmic reticulum (SR)

In parallel to the major Ca^{2+} release pathways (IP_3 receptors and RyRs), Ca^{2+} can also leave the SR lumen via passive leak (Shmigol *et al*., 1998; Wray and shmygol, 2007; Sanborn, 2007). The molecular identity of the leak channels is unknown and neither RyRs nor IP_3 receptors are involved. Passive Ca^{2+} leak was found to be stimulated by ATP at 0.375 – 4 mM concentration (Wray and shmygol, 2007). This partial release of the store stimulated an inward current, the activation of which was initially very slow (Kupittayanunt et al., 2002), but accelerated with time (Hofer *et al*., 1998); it may substantially deplete the SR store within a few minutes. This has to be taken into account when comparing the amplitudes of agonist-induced Ca^{2+} transients in the presence and in the absence of extracellular Ca^{2+}. Under normal conditions, the leak is counterbalanced by Ca^{2+} re-uptake from cytosol. The balance of these two Ca^{2+} fluxes is such that intraluminal Ca^{2+} concentration is approximately three orders of magnitude higher than cytosolic. The maintenance of relatively high concentration of intraluminal Ca^{2+} ($[Ca]_L$) is important for the "Ca^{2+} source" function of the SR (Wray and Shmygol, 2007). The leakage of calcium from the SR into a diffusionally restricted space could maintain SOCs in their closed state; after store-

emptying and concomitant decreased leak into the space, channel inhibition would be alleviated to allow calcium entry (Venkatachalam, 2006).

Thapsigargin is a potent and irreversible inhibitor of the ATP-dependent Ca^{2+} pump. It inhibited the oxytocin-induced $[Ca^{2+}]_i$ transient by inhibiting the SR Ca^{2+}-ATPase (Holda *et al*., 1996). Thapsigargin application results in the elevation of $[Ca^{2+}]_i$. This rise is not a direct action of Ca^{2+} pump inhibition but an indirect consequence of a basal leak of Ca^{2+} from the internal stores (Morgan *et al.*, 1996; Karaki *et al*., 1997)

Pathways of uterine relaxation

For relaxation to occur, the signal to Ca^{2+} entry should be terminated, and myosin light chain should become dephosphorylated. As previous mention, changes in membrane potential lead to the opening of L-type Ca^{2+} channels and Ca^{2+} entry. This entry is curtailed by at least two mechanisms, inactivation of the Ca^{2+} channels and repolarization of the membrane potential. These processes are interlinked; i.e., repolarization of the membrane will reduce Ca^{2+} entry because of deactivation of the channels even if no inactivation had occurred. Inactivation will reduce depolarization of the membrane because of a reduction in inward (depolarizing) current. There are two chief mechanisms of Ca^{2+} channel inactivation, Ca^{2+} dependent and voltage/time dependent. Both mechanisms are present in uterine cells. Repolarization will occur as Ca^{2+} entry decreases and K^+ channel activity is stimulated, as discussed earlier. Activation of Ca^{2+}-dependent K^+ channels by propagating Ca^{2+} waves has been recorded in human myometrial cells by using a combination of patch clamping and digital imaging. There was a time delay between the peak of the Ca^{2+} wave and the actual increase in the K^+ channel activity (Wray *et al*., 2003).

The increase in cytosolic Ca^{2+} is usually transient; once the "On mechanism" has generated a Ca^{2+} signal by introducing Ca^{2+} into the cytoplasm, the "Off mechanism" begins the process of recovery (so-called homeostatic mechanism; reviewed by Riemer and Heyman, 1998) by returning Ca^{2+} either to the stores or back to the external medium. These recovery pathways have to be extremely active because they need to remove not only the free cytosolic Ca^{2+}, but also the 100-fold large

amount that is bound to Ca^{2+} buffer or calcium binding protein such as parvalbumin (Berridge and Bootman, 1997). The cytosolic Ca^{2+} can be reduced, causing muscle relaxation, though several mechanism carried out by pump and exchangers. There are mainly three mechanisms for removal of Ca^{2+} from the cytosol; plasmalemma Ca^{2+}-ATPases, the Na^{+}/Ca^{2+} exchanger and the sarcoplasmic reticulum Ca^{2+}-ATPases (Matthew *et al*., 2004), furthermore, the leakage are also exhibited to extrude Ca^{2+} from cytosol (Anderson, 2006).

When $[Ca^{2+}]_i$ starts to decrease the Ca-calmodulin activation of MLCK is stopped as Ca^{2+} dissociates from calmodulin. The phosphorylated light chains are dephosphorylated by myosin light chain phosphatase (MLCP). Haeberle and colleagues (1985) were the first to demonstrate in skinned uterus that dephosphorylation of myosin light chains by MLCP produces relaxation. MLCP does not depend on Ca^{2+} for its activation, but its activity is regulated by phosphorylation of one of its three subunits, the 110–130-kDa myosin phosphatase targeting subunit (MYPT). The other two subunits are the 37-kDa catalytic subunit and a 20-kDa subunit of unknown function. Dephosphorylation of Ser-19 results in a much reduced (100 times) myosin ATPase rate, and hence cross-bridge cycling returns to basal levels. There is significant interest in the regulation of MLCP via phosphorylation of MYPT, as this is now considered a physiologically important route for calcium sensitization. MYPT has two isoforms that are developmentally regulated and tissue specific, but the importance of this for control of uterine activity is yet to be determined (Wray *et al*., 2003).

The mechanism of cytosolic Ca^{2+} removal

1. Plasmalemmal removal systems
    a. An ATP-dependent Ca^{2+}pump or plasmalemma Ca^{2+}-ATPases (PMCA)
    b. The sodium-calcium (Na^{+}/Ca^{2+}) exchanger
2. Sarcoplasmic reticulum removal systems: sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA)
3. The other; Leakage (Kuriyama, 1998; Berridge, 2003; Laporte *et al.*, 2004)

1. Plasmalemmal removal systems

The myometrial plasma membrane processes two distinct mechanisms for Ca^{2+} extrusion from the cytosol (Enyedi *et al*., 1988). That is the Ca^{2+} removal operated by two proteins that span the plasmalemma: the Ca^{2+}-ATPases (PMCAs) and the Na^{+}/Ca^{2+} exchanger (Matthew *et al*., 2004; Wray and Shmygol, 2007). It is the activity of these proteins that is responsible for the maintenance of the 10,000-fold concentration gradient across the plasmalemma. Differences in the properties of these proteins reveal the characteristics that define them, and are fundamental to their operation. The Na^{+}/Ca^{2+} exchanger has a lower affinity for Ca^{2+}, but is a higher capacity system, whereas the PMCAs extrudes Ca^{2+} at a lower $[Ca^{2+}]_i$. The PMCAs may be viewed as providing a "fine tuning" of resting $[Ca^{2+}]_i$, and the Na^{+}/Ca^{2+} exchanger having a role in the regulation of higher, stimulatory $[Ca^{2+}]_i$. Both efflux pathways were found to make a significant contribution to Ca^{2+} extrusion and myometrium relaxation, with the Na^{+}/Ca^{2+} exchanger accounts for approximately 15% of relaxation and 30% of Ca^{2+} removal, and the PMCAs is responsible for the remaining 85%, and 70%, respectively (Matthew *et al*., 2004).

1a. ATP-dependent Ca^{2+}pump or plasmalemma Ca^{2+}-ATPases (PMCAs)

As Ca^{2+}-ATPases are able to extrude large amount of Ca^{2+}, thus they may play the most important role of removing Ca^{2+} from smooth muscle cell (Wray and Shmygol, 2007). PMCAs utilize the energy of ATP to transport Ca^{2+} against the enormous electrochemical gradient that exists across the plasma membrane (Berridge and Bootman, 1997). Several PMCA isoforms have been described. There are four separate gene encoding PMCAs; PMCA1, PMCA2, PMCA3 and PMCA4 (Berridge and Bootman, 1997). PMCA1b, 4a and 4b mRNA/or protein are detectable in the rat uterus, and there are significant expression of PMCA1 and PMCA4b but not PMCA4a mRNA in human myometrial cells (Sanborn, 2007).

A characteristic of PMCA is its regulation by a variety of factors including calmodulin (CAM), acidic phospholipids and protein kinases such as cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC). Since CAM is a Ca^{2+} binding protein and increases pump activity, this molecule conveys positive feedback

on PMCAs, i.e. Ca^{2+}-stimulated Ca^{2+} extrusion (Berridge and Bootman, 1997).

1b. The sodium-calcium (Na^+/Ca^{2+}) exchanger

Na^+/Ca^{2+} exchange may be important for Ca^{2+} extrusion because, in the membrane fraction of bovine aortic smooth muscle, the Na^+/ Ca^{2+} exchanger has 3–6-fold transporting capacity than that of sarcolemmal Ca^{2+}-ATPase. However, Morel and Godfraind (1984) showed that Na^+/Ca^{2+} exchange had a lower capacity, a lower affinity, and a slower rate than the ATP-dependent Ca^{2+} pump in plasmalemmal vesicles isolated from guinea pig ileum and aorta. Furthermore, co-localization of the Na^+/ Ca^{2+} exchanger, Na^+-K^+ pump, and a marker of the SR, calsequestrin, has been suggested a linkage between Na^+/ Ca^{2+} exchange and Ca^{2+} release from the SR (Karaki *et al*., 1997).

The contribution of both of the Na^+/Ca^{2+} exchanger and the SR to Ca^{2+} homeostasis in smooth muscle has hitherto been unclear (Bradley *et al*., 2002). It is known that Ca^{2+} and Na^+ can move in the opposite direction across the plasma membrane. The direction of movement of these ions (either inward/outward) will depend upon the membrane potential and chemical gradient for the ions (Karaki *et al*., 1997). The transplasmalemmal Na^+ gradient, which is maintained (ultimately be limited) by the Na^+/K^+ ATPase (Matthew *et al*., 2004). The Na^+/Ca^{2+} exchanger relies upon a higher extracellular Na^+ $[Na^+]_o$ than intracellular Na^+ $[Na^+]_i$. When the uterus is in 0 Na^+ solutions, Na^+/Ca^{2+} exchanger will be inhibited. Furthermore, three Na^+ ions are exchanged for each Ca^{2+} (van Breeman *et al*., 1979).

Bradley et al. (2002) demonstrated that in guinea pig colonic smooth muscle cell, the increased SR Ca^{2+} content following depolarization returned to control values in approximately 12 min via Na^+/Ca^{2+} exchanger activity. In addition, inhibition of the Na^+/Ca^{2+} exchanger by removal of external Na^+ (by either lithium or choline substitution) prevented the increased SR Ca^{2+} content from returning to control levels. On the other hand, the Na^+/Ca^{2+} exchanger is not activated by a transient increase in bulk average cytosolic calcium ($[Ca^{2+}]_c$) directly since the rates of decline in $[Ca^{2+}]_c$, following either depolarization or the release of Ca^{2+} from the SR, were neither voltage nor Na^+ dependent. There is no evidence for short term (seconds) control of $[Ca^{2+}]_c$ by the Na^+/Ca^{2+} exchanger was found. The authors also suggested that the SR removes

Ca^{2+} from the cytosol after its elevation by depolarization. After overloading of the SR by depolarization, the Ca^{2+} released into the restricted space between the SR and the plasmalemma, and then they are removed from the cell by Na^{+}/Ca^{2+} exchanger. The Ca^{2+} extrusion by the exchanger requires the close proximity of the SR to activate its low Ca^{2+} affinity mechanism.

2. Sarcoplasmic reticulum removal system; **Sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA)**

Myometrial cells have an extensive sarcoplasmic reticulum (SR) network, which approaches very close to the plasma membrane, and is also abundant near the nuclear envelope. Uterine smooth muscle showed that both spontaneous force production and Ca^{2+} transients were increased when the SR was inhibited. This has led to the suggestion that the SR has a role in limiting contraction (reviewed by Noble and Wray, 2002). The sarcoplasmic reticulum Ca^{2+}-ATPases pump is located on the SR membrane (Berridge and Bootman, 1997). This pump is a protein, which has molecular weight ~100,000 with a hydrophobic region. SERCA is integrated into the lipidic bilayer of the SR and a hydrophilic region which protrudes into the cytosol. The hydrophobic region is comprised of 10 transmembrane segments (M1-M10) organized into a helices. The two regions are linked by a stalk which is made of 5 segments (S1-S5) linked to 5 transmembrane ones. The C and N termini are both cytosolic Oxygen-rich residues localized in M4, M5, M6 and M8 segments form two high sites with a high affinity for Ca^{2+}, forming a cavity responsible for the translocation of the calcium ion. The long loop between S4 and S5 of the stalk contains residues which are involved in the hydrolysis of ATP, and aspartate 351 (Asp 351) to which the hydrolysed ATP phosphate is transferred (Figure 2-41) (Arredouani, 2004). SERCAs play an important role for translocates Ca^{2+} in cytosol into SR lumen and preventing Ca^{2+} overload (Monga and Sanborn, 1992; Wray, 1993). This enzyme transports Ca^{2+} and using ATP as it energy source similar to PMCAs. The rate and extent of Ca^{2+} pumping into SR is sufficient to cause relaxation, the Ca^{2+} level in sarcoplasm must be returned near to the resting value (Somlyo and Somlyo, 1994).

Calmodulin kinase (CaMK) is the physiological modulator of SERCA isoform 2 (SERCA2). SERCA2 activity can be modulated by direct phosphorylation

by a CaMK associated with the SR by phosphorylating a Serine at position 38. This phosphorylation leads to a 50 to 70% increase of the maximum volume of Ca^{2+} reuptake. Furthermore, the best known inhibitor of the SERCA Ca^{2+} pump is thapsigargin (van Breeman, 2000; Arredouani, 2004), a potent and specific inhibitor of SERCA. Thapsigargin is extracted from the roots of *Thapsia garganica*, a plant of the umbelliferae family, and is widely used to deplete intracellular Ca^{2+} stores. There are however other known inhibitors of the SERCA family such as cyclopiazonic acid (Sprelakis, 1995, Matthew *et al*., 2004; Arredouani, 2004), 2,5-di(t-butyl) hydroquinone (DBHQ), vanadate (Nechay, 1984) and the thapsigargicin, but these are less potent than thapsigargin (Arredouani, 2004). Thapsigargin have been shown to stimulate calcium influx, and consequently raise $[Ca^{2+}]_i$., in a range of smooth muscles. In addition, these drugs have also been reported to produce contractions in a number of smooth muscles, suggesting that store-operated calcium entry may be important in regulating the contractile state of the tissues. An alternative interpretation is that the SERCA pump inhibitors are not directly initiating calcium entry, but rather, by disrupting the superficial buffer barrier function of the sarcoplasmic reticulum are allowing calcium entering by other routes, including poorly defined `leak' pathways, to gain access to the cell interior and the contractile proteins (McFadzean and Gibson, 2002) and consequence of a basal leak of Ca^{2+} from the internal stores (Morgan *et al.*, 1996; Karaki *et al*., 1997)

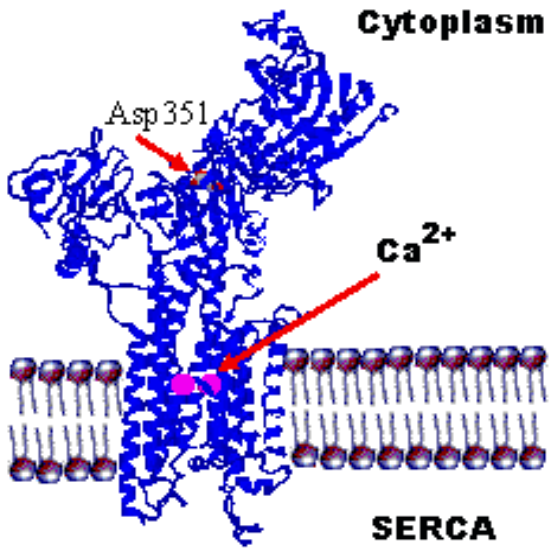

Figure 2-41 Structure and predicted topology of the SERCA showing Ca^{2+} and aspartate 351 (Asp 351) binding sites (Arredouani, 2004).

Matthew *et al*. (2004) have shown that if Na^+/Ca^{2+} exchanger and PMCAs are blocked, SERCA alone was not able to extrude Ca^{2+}. This leads to conclude that the SR is operating in series with the plasmalemmal Ca^{2+} extrusion mechanisms. The hypothesis was that the SERCA was bringing cytosolic Ca^{2+} into the SR lumen, and the SR then released its Ca^{2+} at regions of the SR closely opposed to the plasma membrane rich in Na^+/Ca^{2+} exchanger and PMCA, enhancing their ability to pump Ca^{2+} out of the cell.

3. The other; Leakage

Leakage current has been shown to be a linear function of voltage in the hyperpolarizing direction and for small depolarizing steps. On this basis it was assumed that leakage current was linear for all values of membrane potential. It is clear that under these conditions, an increase in membrane leakage current will decrease net inward current. It has been previously suggested that leakage current may limit the ability of the active transient conductance to depolarize the membrane. Kao et al. (1970) have also suggested that calcium may influence excitability in uterine smooth muscle by controlling membrane leakage permeability (Anderson et al., 2006).

Changes in calcium sensitivity

There are evidences showing that use of Ca^{2+} indicators to reveal the force/Ca^{2+} ratio is variable, and generally higher when activated by agonist than by a depolarizing-induced increase in $[Ca^{2+}]_i$, indicating that Ca^{2+}- sensitizing effect of agonists. ACh, oxytocin and the other agonists can increase force of smooth muscle in which $[Ca^{2+}]_i$ is clamped with chelators and intracellular Ca^{2+} compartmentalization and cytoplasmic Ca^{2+} gradients. Conversely, force can decline while cytoplasmic Ca^{2+} is maintained, indicating a decrease in Ca^{2+}-sensitivity. Such increase and decrease in force at constant Ca^{2+} are known to result from parallel changes in the activity of phosphorylating and dephosphorylating enzymes and, consequently, in 20kDa myosin light chain (LC_{20}) phosphorylation (Somlyo and Somlyo, 1994).

As above-mentioned, in contractile smooth muscle, Ca^{2+}-CAM activates MLCK, which phosphorylates myosin light chains. In relaxation,

dephosphorylation of myosin light chain via myosin phosphatase is usually initiated by a fall in $[Ca^{2+}]_i$. Indirect coupling between $[Ca^{2+}]_i$ and smooth muscle contraction regulated by mechanism involved that increase or decrease sensitivity of contractile apparatus to $[Ca^{2+}]_i$. The phenomenon of increased force at a low intracellular Ca^{2+} level is termed Ca^{2+} sensitization (Somlyo and Somlyo, 1994). Ca^{2+} sensitization is caused by a decrease in activity of myosin phosphatase or an increase in activity of MLCK, thus, Ca^{2+} desensitization, is produced in the contrast manner (Rang *et al*., 1999). However, the variation in the relation between $[Ca^{2+}]_i$ and concentration has explained the contraction in term of "Ca^{2+} sensitivity of phosphorylation" (Wray, 1993; Karaki *et al*., 1997; Riemer and Heyman, 1998). There are four proposed mechanisms for changes in the Ca^{2+} sensitivity of phosphorylation (Figure 2-41).

1. Activation of Ca^{2+}-calmodulin dependent protein kinase II; CAMKII

Phosphorylation of specific sites on MLCK affects its activity. In particular, phosphorylation of a serine residue by CAMKII, PKA, or PKC greatly reduces MLCK activity by increasing the amount of Ca-calmodulin required to activate it 10-fold. In this way the phosphorylation of myosin light chains will also be affected and, consequently, contraction (Wray *et al*., 2003). This phosphorylation occur in vivo, and is physiological mechanism of desensitization, although it requires a higher cytoplasmic $[Ca^{2+}]_i$ than those activation of MLCK, owing to the lower affinity of CAMKII compared with that of MLCK for Ca^{2+}-CAM (Somlyo and Somlyo, 1994). This phenomenon may be part of temporal feedback mechanism limiting contraction (negative feedback), as Ca^{2+} first activates MLCK via CAM and then inactivate it via CAMKII (Figure 2-42) (Monga and Sanborn, 1992; Wray *et al*., 2003).

2. Inhibition of myosin light chain phosphatase; MLCP

The state of myosin light chain phosphorylation by MLCK is further regulated by MLCP, which removes the high energy from the light chain of myosin to promote smooth muscle relaxation. Thus it plays an importance role in determining the sensitivity of the contractile apparatus and changing in $[Ca^{2+}]_i$. Inhibition of protein phosphatase is the main and perhaps only mechanism of G-protein couple Ca^{2+}-

sensitization. Inhibition of phosphatase is increase LC_{20} phosphorylation and hence the level of force at a given $[Ca^{2+}]_i$. It is proposed that agonists increase the Ca^{2+}-sensitivity of contractile elements by activating a GTP-binding protein since the sensitizing effect can be inhibited by GDP-βS, a competitive inhibitor of GTP, and mimicked by GTP-γS, a GTP analogue resistant to hydrolysis (Somlyo and Somlyo *et al*., 1994; Karaki *et al*., 1997).

Stimulation of G protein couple receptors (GPCR) leads to the activation both Ca^{2+} sensitization and Ca^{2+}-dependent pathways (Jin and Bernett, 2006). Protein kinase C (PKC) and arachinodic acid, the products from activation of GPCR to phospholipase C (PLC) and phospholipase A_2 (PLA_2), respectively, increase Ca^{2+}-sensitivity through the inhibition of dephosphorylation of myosin light chain (Itoh *et al*., 1993; Masuo *et al*., 1994; Gong *et al*., 1995; Horowitz *et al*., 1996; Karaki *et al*., 1997). After activation by DAG, PKC phosphorylates a smooth-muscle specific inhibitor called CPI-17 (Figure 2-42) (Jin and Burnett, 2006; Shmygol *et al*., 2006).

Phosphorylated CPI-17 has a high affinity for the catalytic subunit of MLCP and decreases MLCP activity through phosphorylation. Activation of GPCRs also stimulates RhoGEF activity, which facilitates the exchange of GTP for GDP on RhoA and dissociates RhoA from RhoGDI. The active RhoA-GTP translocates from the cytosol to the plasma membrane and activates Rho-kinase. Subsequently, Rho-kinase phosphorylates the targeting subunit of MLCP, leading to increased MLC phosphorylation. In addition, Rho-kinase has been shown to phosphorylate CPI-17 (Figure 2-43). Meanwhile, RhoGAP accelerates the intrinsic GTPase activity of RhoA and promotes hydrolysis of GTP; thus, inactive RhoA-GDP re-associates with RhoGDI and relocates to the cytosol (Jin and Burnett, 2006).

3. Increase free calmodulin concentration

It is well known that concentration of the Ca^{2+}-calmodulin complex can regulate the MLCK activity, and that Ca^{2+} concentration is regulated. However, it was postulated that the large intracellular pool of calmodulin is freely diffusible and saturating for kinase activity in living cells. Thus, the changes in calmodulin concentration determine the Ca^{2+} sensitivity of myosin light chain phosphorylation (Karaki *et al*., 1997).

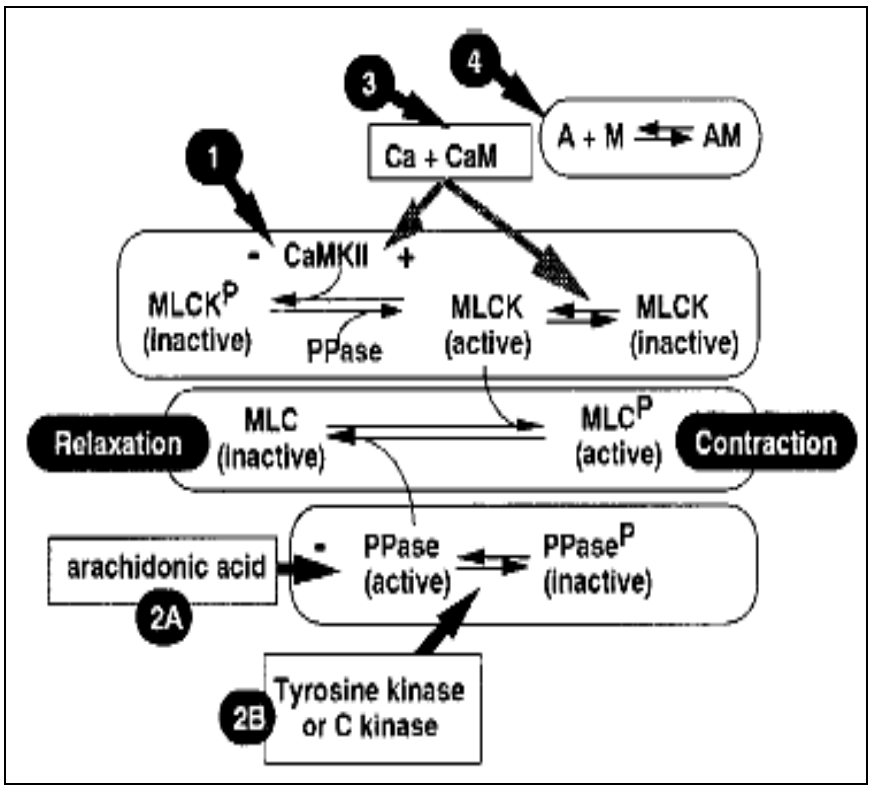

Figure 2-42 Mechanisms of agonist-induced Ca^{2+} sensitization in smooth muscle. Stimulation of a receptor increases $[Ca^{2+}]_i$, activates MLCK, phosphorylates myosin light chain, and induces contraction. This process is modulated by four different mechanisms. (1) The inhibition of Ca^{2+}-calmodulin dependent protein kinase II (CaMKII), which phosphorylates MLCK and inhibits its activity (2) Inhibition of MLCP (PPase); (2A) arachidonic acid produced by receptor-mediated activation of phospholipase A_2, may directly inhibit phosphatase, (2B) C kinase and tyrosine kinase may also inhibit phosphatase by inhibiting the endogenous inhibitor of phosphatase. (3) Increase free calmodulin concentration, (4) Activate actin independently of MLC phosphorylation (Karaki *et al*., 1997).

4. Activate actin independently of myosin light chain phosphorylation

Smooth muscle contraction is regulated not only by MLC phosphorylation but also by a phosphorylation-independent mechanism, possibly a mechanism linked to actin (Karaki *et al*., 1997). Actin-binding proteins, such as

caldesmon and calponin, also appear able to regulate contraction. Both proteins have an inhibitory effect on the myosin ATPase. The inhibitory actions of caldesmon and calponin can be reversed by phosphorylation. Caldesmon can be phosphorylated by PKC, p21-activated kinase (PAK), and extracellular-regulated kinase-1 (ERK1). In some cases this is associated with an increase in force. Calponin has been shown to associate with ERK and PKC during agonist stimulation. Thus, it is proposed that a part of the mechanism whereby agonists may modulate smooth muscle force is by reducing the inhibitory action of thin filament–associated proteins, and that this can occur by their phosphorylation and subcellular redistribution (Wray *et al*., 2003).

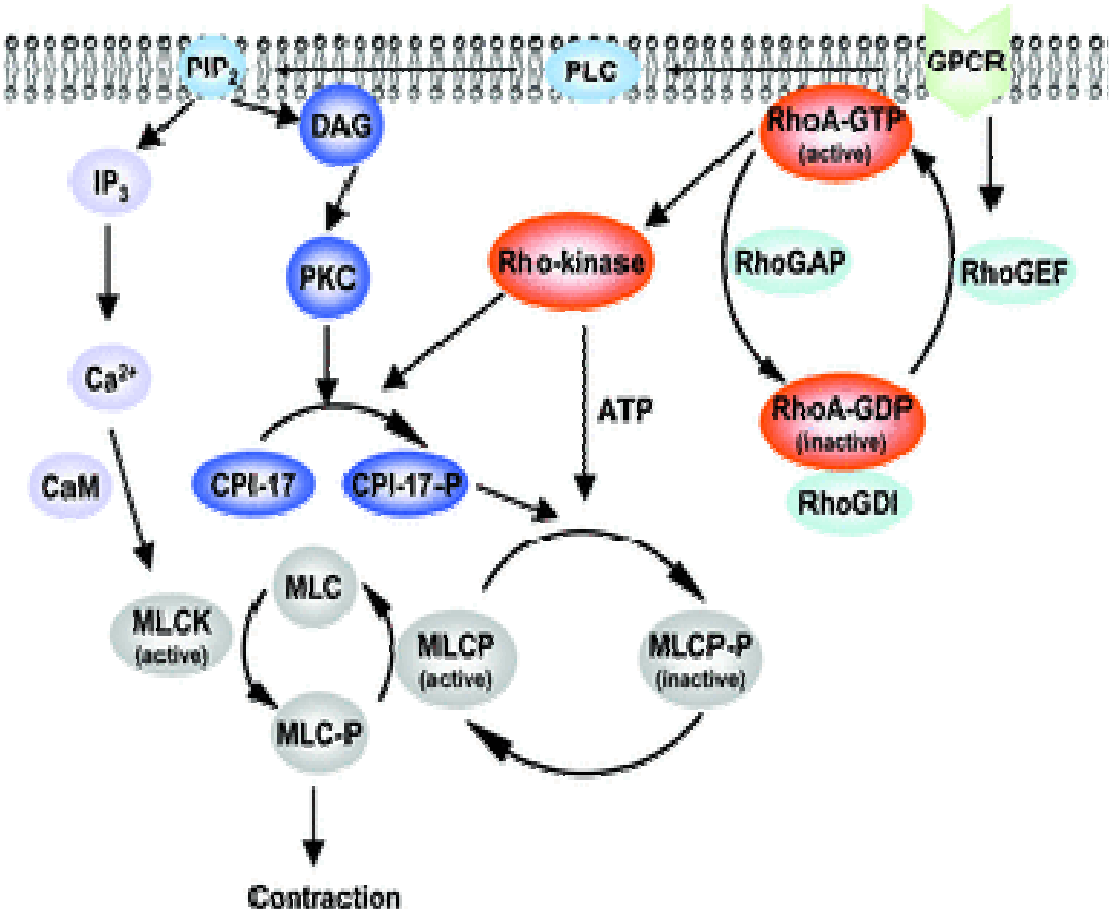

Figure 2-43 Ca^{2+} sensitization and Ca^{2+}-dependent pathways (Jin and Burnett, 2006).

The previous, many research focused on the role of MLCK in smooth muscle contractility, but recently the interest shifted to MLCP. It turned out that MLCP is composed of three subunits: a catalytic subunit of 37-38-kDa of the type 1 phosphatase, a subunit of about 20-kDa whose function is not known, and a larger 110-130-kDa subunit that targets MLCP to myosin. The phosphatase activity of the catalytic subunit is low and it is enhanced significantly by addition of the target subunit, its activating effect on the catalytic subunit is lost, and thereby the MLCP holoenzyme is inhibited.

Recent reports (Feng *et al*., 1999; Kaibuchi *et al*., 1999; Nagumo *et al*., 2000; Somlyo and Somlyo, 2000) indicate that in smooth muscle a Rho-regulated system of MLCP exists. Rho-kinase is the major player in this system; the enzyme phosphorylates the 130-kDa myosin binding subunit of MLCP and thereby inhibits MLCP activity. Due to the antagonism between MLCK and MLCP, inhibition of MLCP results in an increase in the phosphoryl content of light chain with concomitant increase in muscle force. Under these conditions, submaximal Ca^{2+}-levels are sufficient for maximal force, a phenomenon called increased Ca^{2+}-sensitivity (Somlyo and Somlyo, 1994). Specific inhibitors for Rho-kinase Y-27632 (Feng *et al*., 1999; Kaibuchi *et al*., 1999) and HA-1077 (Nagumo *et al*., 2000) are available. A rich array of secondary messengers regulate MLCP activity under physiological and pathological conditions through phosphorylation of either the targeting subunit of MLCP or CPI-17; 17-kDa myosin phosphatase inhibitor protein, which inhibits the catalytic subunit of MLCP and the holoenzyme MLCP (Kitazawa *et al*., 2000).

Summary of smooth muscle contraction

The consequences of increasing of $[Ca^{2+}]_i$ levels in smooth muscle cell are the binding of Ca^{2+} to calmodulin (CAM) to produce Ca^{2+}-CAM complex which then activates myosin light chain kinase (MLCK). The active MLCK then catalyzes the phosphorylation of serine at position 19 (Ser 19) of the regulatory light chain (20 kDa myosin light chain, MLC_{20}). Phosphorylation of Ser 19 of MLC_{20} allows the myosin ATPase to be activated by actin and the muscle to contract. A fall in cytosolic Ca^{2+} concentration by mechanisms mentioned above inactivates MLCK and permit dephosphorylation of LC_{20} by myosin phosphatase, thus deactivating the myosin ATPase and causing to myosin relaxation (Figure 2-44) (Somlyo and Somlyo, 1994; Karaki *et al*., 1997; Lodish *et al*., 1999; Wray *et al*., 2003).

Young (2007) has been posited the facts that are so obvious and as clearly accepted as facts that they probably are not even facts at all that are:

1. The human uterus is composed of longitudinal and circular layers of smooth muscle.
2. The functional cells of the uterus are the myocytes, which are a homogeneous cell type responsible for the generation of contraction forces, passage of action potentials, and control of contractility.
3. The phasic contractions of the uterus are typical for visceral smooth muscle.
4. The primary, and perhaps only, role of gap junctions is to allow passage of action potentials through the tissue.
5. Action potential propagation as the mechanism for global communication (over many centimeters throughout the uterus) is sufficient to recruit all regions and all myocytes of the uterus.
6. Slow waves pace the contractions of human myometrium.
7. Calcium-activated potassium channels are responsible for repolarization of the membrane potential that terminates each contraction.
8. Chloride channels are not important in uterine electrophysiology.

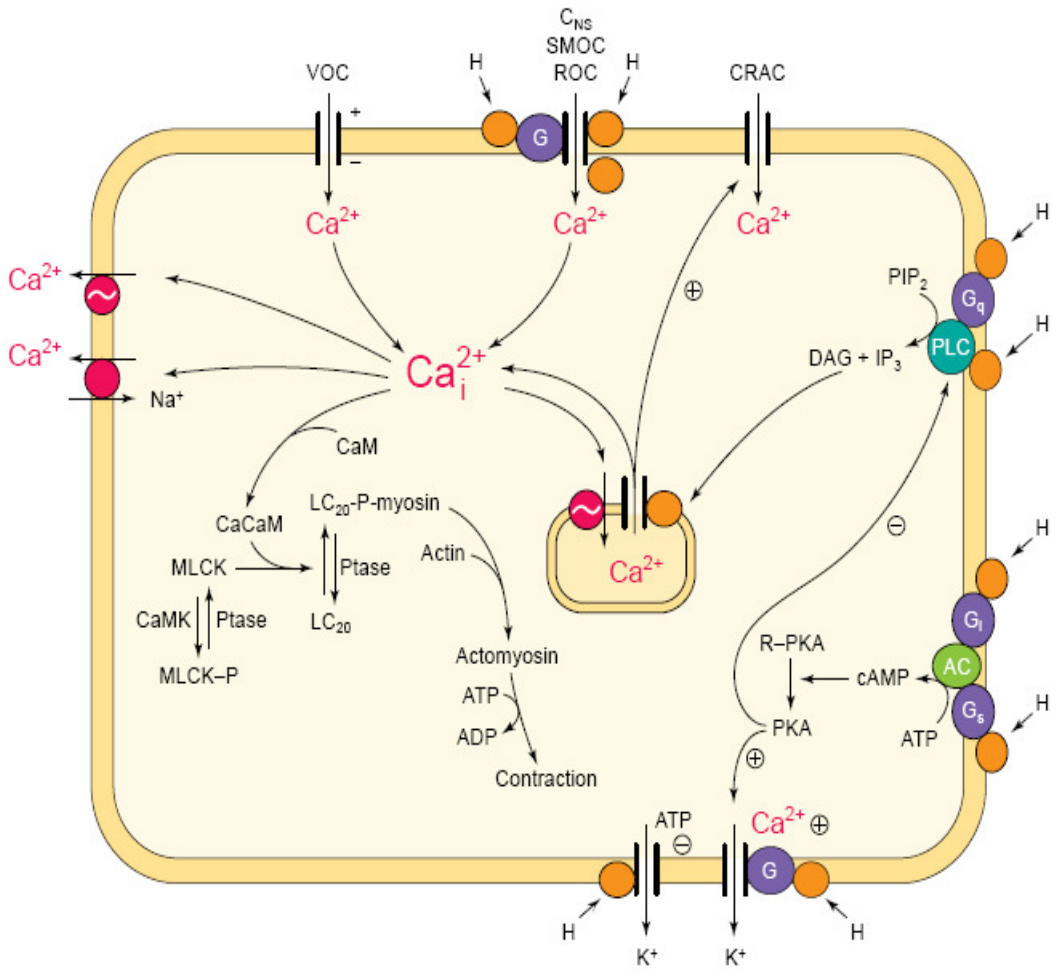

Figure 2-44 Pathways leading to control of $[Ca^{2+}]_i$ in smooth muscle. Components include hormones or other ligands (H), calmodulin (CaM), myosin light chain kinase (MLCK), myosin light chain (LC20), protein phosphatase (Ptase), a voltage-operated Ca channel (VOC), receptoroperated (ROC), nonselective (CNS) and second messenger-operated (SMOC) cation channels, a calcium release-activated calcium channel (CRAC), heterotrimeric G proteins (G), phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP3), diacylglycerol (DAG), adenylyl cylase (AC), protein kinase A (PKA), calcium calmodulin-dependent protein kinase (CaMK), phosphatidylinositol bis-phosphate (PIP_2), Ca transport ATPases, Na^+/Ca^{2+} exchanger, a calcium-activated potassium channel and ATP-sensitive potassium channel. Tyrosine kinase receptors stimulate PLCs directly, but not those of the PLCb class (Sanborn *et al*., 1996.)

Modulator of uterine contraction

Myometrium is a phasic smooth muscle, although tonic-like contractions may develop with high-frequency electrical stimulation during labor or after the use of some agonists such as oxytocin, prostaglandins, or endothelin (Bytautiene *et al*., 2003). Uterine contraction is modulated by several hormones, neurotransmitter, electrolyte, pharmacochemical. They affect the frequency, duration, and amplitude of contractions. The mechanisms of action of different modulators may be different. This section will review briefly some of hormones, autacoids and neurotransmitter in order to understand the way in which drugs act on the uterus.

Hormone

1. Estrogen and Progesterone

As describe earlier, estrogen plays a significant role in increasing the sensitivity of the contractile response. In general, during late pregnancy, estrogen promote uterine contractility via the effect of contractile protein, gap junction formation, and increase responsiveness of uterus to agonist such as oxytocin and prostaglandin $F_{2\alpha}$ by increase the number of receptor, increase receptor sensitivity, and gap junction expression in myometrium. Progesterone plays a role for maintaining the uterus in a quiescent stage which is thought to be mediated through suppression of the spontaneous generation and propagation of action potentials. Its effects may involve inhibition of gap junction formation and downregulation of oxytocin receptor (Cole and Garfield, 1986; Sato *et al*., 1989; Eagland and Cooper, 2001).

2. Oxytocin

Oxytocin is synthesized in the paraventricular and supraoptic nuclei as a large precursor molecule before being broken down to the active hypothalamic neuropeptide hormone. It has a biological half-life of only three to ten minutes and is broken down in the liver by specific peptidases and excretes by kidney. In pregnancy, it is degraded by placental oxytocinase (Sultatos, 1997; Eagland and Cooper, 2001). Oxytocin regulates a large number of reproduction-related processes in all species.

Particular importance is its ability to stimulate uterine contractility. This is achieved by multiple mechanisms involving sarcoplasmic reticulum Ca^{2+} release and sensitization of the contractile apparatus to Ca^{2+} (Shmygol *et al*., 2006).

Oxytocin can also induce uterine contraction in terms of Ca^{2+} independent-induced contraction (Wray, 1993). Oxytocin is generally assumed that it produces effect by (1) opening Ca^{2+} channels and/or (2) by liberating a signal molecule, which then releases Ca^{2+} from internal stores; and (3) by inhibiting of mechanisms responsible for Ca^{2+} extrusion from the cell (Enyedi *et al*., 1989). At least three distinct components can be discerned in the effect of oxytocin on human uterine smooth muscle: (1) increase in frequency of contractions; (2) initial transient increase in the basal tone (incomplete relaxation); and (3) long-lasting increase in the amplitude and duration of phasic contractions (Eagland and Cooper, 2001; Shmygol *et al*., 2006). It has been known for several years that oxytocin and some agonists, such as ACh, can cause continuous contraction of uterus in Ca^{2+} free, ethylene glycol-bis (β-aminoethyl-ester)-N,N,N',N'-tetra acetic acid (EDTA)-containing solution. The amplitude of contraction is only 5-15% of that obtained with Ca^{2+}, but nevertheless, this contraction is persistent for many hours. The contractions occur without measurable increase in $[Ca^{2+}]_i$ or myosin light chain phosphorylation. It is suggested that the contraction is probably due to a phosphorylation either a contractile or cytosol protein (Wray, 1993).

Oxytocin receptors are G-protein coupled receptor that binds with oxytocin on the myometrial cell membrane to facilitate in interaction with GTP-binding protein (G-$\alpha_{q/11}$ subfamilies) which stimulates phospholipase-Cβ. This leads to the generation of the second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Both second messengers are believed to be involved in the generation of the physiological response to oxytocin, although their relative contributions to the overall effect are unknown (Shmygol *et al*., 2006). In human myometrium, this receptor couples to Gq/11 also activate voltage-sensitive Ca^{2+} channels. Oxytocin also increases local prostaglandin production, via a MAPK-mediated increase in cyclooxygenase-2 production, which further stimulates uterine contractions (Zingg and Laporte, 2003; Park and Schimmer, 2006). The production of prostaglandin is caused by oxytocin. Oxytocin receptor concentration in myometrial

cells is increased by an elevation of estrogen and suppressed by progesterone (Monga and Sandborn, 1992). Uterine sensitivity to oxytocin is dependent on the receptor density, which increases during pregnancy. Although maternal serum oxytocin levels are not increased with the onset of labour, fetal, decidual and local uterine oxytocin may act on myometrial oxytocin receptors to initiate and maintain uterine contractions (Eagland and Cooper, 2001). In term, estrogen increases uterine sensitivity to oxytocin, but in post partum, oxytocin receptor densities rapidly decrease. The rat oxytocin receptor contains potential estrogen responsive elements in its promoter regions; however, the relative weak estrogen response of these promoters in transfection experiments cannot account for the strong estrogen-induced oxytocin receptor upregulation seen *in vivo*. The estrogen response probably involves additional factors and might be mediated indirectly or via other functional promoter elements (Zingg and Laporte, 2003).

Autacoids

1. Prostaglandins (PGs)

Prostaglandins play an important role in reproduction in mammals. Activation of prostaglandin receptors either increase or decrease smooth muscle contractile activity (Bolton, 1979; reviewed by Vedernikov *et al*., 2003). The actions of the PGs are mediated by specific receptors on the cell surface, which are classified into five types and four subtypes of receptors; DP, FP, IP, TP, and EP (subtypes: EP_1, EP_2, EP_3, and EP_4) receptors for PGD_2, $PGF_{2\alpha}$, PGI_2, TXA_2, and PGE_2, respectively (reviewed by Tsuboi *et al*., 2002). Generally, activation of DP and IP receptors increases the level of cyclic adenosine monophosphate (cAMP), which is associated with contractile inhibition, while activation of FP and TP receptors is associated with IP_3- mediated mobilization of intracellular calcium and increase in contractility. The most heterogeneous are EP receptors, activation of EP_1 generally leads to increase in intracellular calcium, while activation of inhibitory prostanoid receptors EP_2 or EP_4 leads to increase in cAMP level, and activation of EP_3 may either decrease cAMP or increase IP_3 turnover.

Both PGE_2 and $PGF_{2\alpha}$ have been considered to be important mediators and/or modulators of several processes in female reproduction. $PGF_{2\alpha}$ plays a crucial

role in the induction of parturition during late pregnancy and that PGE_2 plays an important role in ovulation and fertilization via the EP_2 receptor during early pregnancy (Tsuboi *et al*., 2002). There are increase numbers of myometrial receptors for PG at term. Binding of oxytocin to its receptor produce DAG to stimulate arachinodic acid release and, hence, PG synthesis (Wray, 1993). PGE_2 and $PGF_{2\alpha}$ stimulate uterine contractility and sensitize the uterus to oxytocin and may also induce gap junction formation. $PGF_{2\alpha}$ induces uterine contraction by generating phosphatidylinositol-signal pathway which results in an increase of Ca^{2+} release from IP_3 sensitive site. Furthermore, it may also open nonselective cation channel and then sodium influx into the cell which could cause depolarization of plasma membrane leading to Ca^{2+} influx (Bolton, 1979).

Pathophysiology of Primary Dysmenorrhea

Prostaglandins cause uterine contractions, which result in the expulsion of sloughed endometrial lining. It has been found that women with dysmenorrhea have twice higher prostaglandin activity in their menstrual fluid than women who do not experience menstrual pain (Durain, 2004; reviewed by Harel, 2006). The majority of dysmenorrhea in adolescents and young adults is primary (or functional), is associated with a normal ovulatory cycle and with no pelvic pathology, and has a clear physiologic etiology. After ovulation, there is a buildup of fatty acids in the phospholipids of the cell membranes. After the onset of progesterone withdrawal before menstruation, these omega-6 fatty acids, particularly arachidonic acids, are released, and a cascade of prostaglandins (PG) and leukotrienes (LT) is initiated in the uterus. The inflammatory response, which is mediated by these PG and LT, produces both cramps and systemic symptoms such as nausea, vomiting, bloating, and headaches. In particular, the prostaglandin $F_{2\alpha}$, cyclooxygenase (COX) metabolite of arachidonic acid, causes potent vasoconstriction and myometrial contractions, leading to ischemia and pain (Harel, 2006).

2. Histamine

Histamine increased spontaneous phasic myometrial contractions in a concentration-dependent manner, but significantly reduced tonic contractions of

uterine strips induced by indolactam V, a protein kinase C activator. The H_1-, but not H_2 receptor antagonist, inhibited the increase in uterine contractile activity in response to histamine. H_1 is a Ca^{2+}-mobilizing receptor, and its stimulation leads to activation of phospholipase C and formation of InsP and DAG. Histamine activates spontaneous phasic contraction, may increase uterine activity on the release of histamine from endogenous cellular source, such as mast cell and basophiles, during type I hypersensitivity reactions. When tonic contraction pattern develops (i.e., basal tension increase), histamine may partly counteract these change by relaxing the myometrium, this relaxation mechanism needs further investigation (Bytautiene *et al*., 2003).

Neurotransmitters and their receptors

1. Adrenergic receptor

Adrenergic signaling pathways are under the control of progesterone and estradiol, which regulate the expression of receptors (β-adrenergic receptor), heterotrimeric G proteins ($G_{s\alpha}$, $G_{i\alpha}$, and $G_{q\alpha}$), and PLC$_\beta$ enzymes. Hence, during pregnancy under progesterone dominance, norepinephrine stimulates β-adrenergic receptor and α_2-adrenergic receptor, which both activate adenylyl cyclase and increase intracellular concentrations of cAMP. The latter second messenger induces myometrial relaxation by inhibiting the pathways leading to Ca^{2+} increase. At term, when concentrations of estradiol rise, the β-adrenergic pathway becomes desensitized, and norepinephrine acts on α_2- adrenergic receptor and α_1- adrenergic receptor. At this time, α_2- adrenergic receptor shift to inhibit of adenylyl cyclase, whereas α_1-adrenergic receptor activates the G_q/PLC system and participates in the Ca^{2+} increase and uterine contraction. An intriguing observation is that α_1- adrenergic receptor is expressed throughout pregnancy, although they are not efficiently coupled to phosphoinositide hydrolysis due to down-regulation of $G_{q\alpha}$ and PLC$_\beta$ isoforms at this period (reviewed by Dupuis *et al*., 2004).

Although β-adrenoceptors have many subtypes, but Pennefather and Molenaar (1986) first identified the myometrial β-adrenoceptor as β_2-adrenoreceptor and demonstrated that this receptor was distributed homogeneously between the circular and longitudinal muscles in myometrium layer of uterus (reviewed by

Kitazawa *et al*., 2001). β-adrenoceptors are present mainly in the longitudinal muscle of rostral uterine horn (Houdeau *et al*., 1998). The proportion of these receptors will differ with hormonal state, species and muscle layer. Generally, high progesterone levels will increase in number and also produce the formation of high-affinity β_2-receptor. However, at term, there is rapid desensitization leading to a loss of myometrium response to its agonist. Intracellular cAMP increasing activates cAMP dependent kinase that phosphorylates protein and cause relaxation by decreasing Ca^{2+} sensitivity of contractile element. In such a case, the phosphorylated protein by cAMP dependent kinase may be MLCK. After that, it markedly decreases in its affinity to Ca-CAM leading to a reduction of phosphorylation of myosin light chain. In longitudinal smooth muscle from guinea pig ileum, isoproterenol suppressed the spontaneous increase in $[Ca^{2+}]_i$ measured with fura-2, and reduced the resting $[Ca^{2+}]_i$. These results suggest that cyclic AMP induced relaxation is caused by the cyclic AMP-mediated decrease in $[Ca^{2+}]_i$ due to indirect inhibition of the L-type Ca^{2+} channel, possibly mediated by activation of K^+ channels and resulting membrane hyperpolarization, and also inhibition of the receptor-coupled signal transduction (Karaki *et al*, 1997).

More recent study showed that both β_2- and β_3 –adrenoceptors mediated adrenergic stimulation-induced relaxation (Tomiyama *et al*., 2003). The activation of β-adrenoceptors cause inhibition of uterine contraction (Kitazawa *et al*., 2001) via regulation of the phospholipase C (PLC) system. It has been reported that pretreatment of myometrial strip, taken from midpregnant rat, with isoproterenol significantly reduced basal and agonist-stimulated inositol phosphate (InsP) production (Mhaouty-kodja *et al*., 2004).

The G_s proteins were activated when β-agonist receptors are stimulated, as this activates $G_{\alpha s}$ protein, and their $G_{\alpha s}$ subunit stimulates adenyl (A) cyclase. The activated adenylyl cyclase then catalyzes the conversion of some cytosolic ATP molecules to cyclic AMP (cAMP). Cyclic AMP acts as a second messenger; it binds to and activates an enzyme known as cAMP-dependent protein kinase A (PKA) (Figure 2-45). This enzyme can participate in reactions leading to such indirect gating by phosphorylate a plasma membrane ion channel including K^+

channel, thereby causing it to open and outward current (Vander *et al*., 2001; Faber, 2003; Wray *et al*., 2003). Furthermore, in smooth muscle that kinase phosphorylates Ca^{2+} channel subunits and activates the channel; this stimulation of the Ca^{2+} channel appears paradoxical. The small Ca^{2+} increase might activate K^{+} channels, as Ca^{2+} sparks from the sarcoplasmic reticulum do, and thereby promote relaxation rather than contraction (Figure 2-46)(Wray *et al*., 2003).

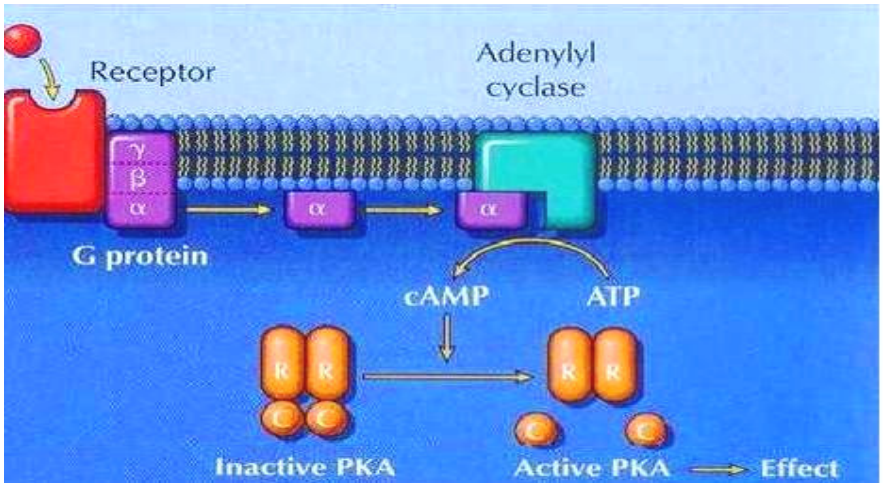

Figure 2-45 G protein couple receptor and adynylyl cyclase (Adapted from: Raffa *et al*., 2005, pp. 15).

Mechanisms of relaxant effects mediated by cAMP may be summarized as follows (Karaki *et al*., 1997):

1) inhibition of the receptor-mediated signal transduction resulting in the inhibition of all the effects of agonists including Ca^{2+} release, Ca^{2+} influx and Ca^{2+} sensitization
2) dissociation of contraction from MLC phosphorylation
3) increase in SR Ca^{2+} uptake
4) decrease in the Ca^{2+} sensitivity of MLC phosphorylation possibly by activating MLC phosphatase
5) increase in noncontractile $[Ca^{2+}]_i$ which may result in activation of K^{+} channels and membrane hyperpolarization. A part of these effects may be mediated by G kinase but not by A kinase.

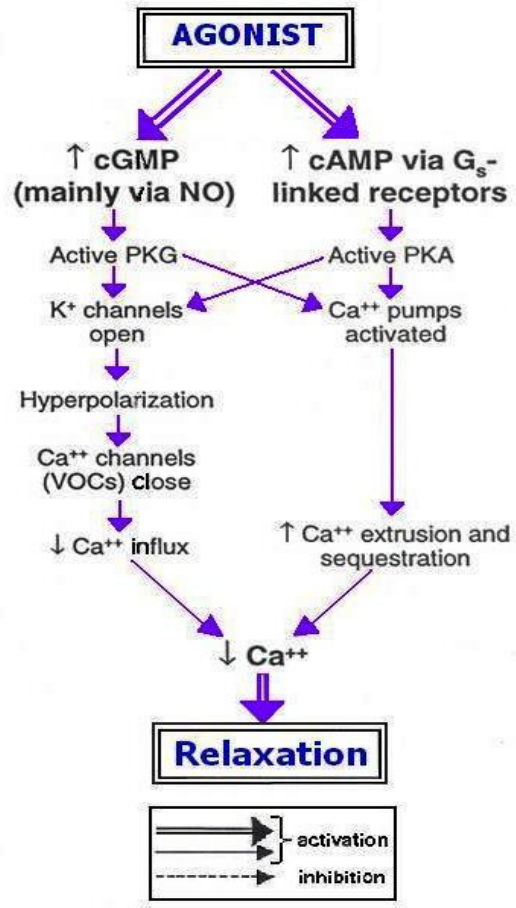

Figure 2-46 cAMP and cGMP activation (Adapted from: Faber, 2003).

2. Cholinergic receptor

Uterine contraction is stimulated by ACh, but it is well established that contraction and expulsion the fetus can be occurred in the absence of nerve activation. Thus the role of nerve in contractility of the uterus may involve only a small function such as a coordination activity (Wray, 1993).

Acetylcholine (ACh) is the principal excitatory neurotransmitter of the parasympathetic nervous system that produces smooth muscle contraction via activation of muscarinic receptors, those are known in many subtypes; M_1-M_5, which in uterine smooth muscle predominantly expressed of M_2, M_3 subtype (Bolton, 1979; Kitazawa *et al*., 1999). These receptors are G-protein coupled receptors, the stimulation of M_3 receptor is linked to activation of membrane bound enzyme PLC activity and accelerates the rate of (PIP_2) linked to phosphoinositide breakdown,

increase IP_3 and hence elevating $[Ca^{2+}]_i$ with produces contraction (Bolton, 1979). However extracellular Ca^{2+} is also required for the contraction and many mechanisms for elevating are $[Ca^{2+}]_i$ considerable similar to the action of oxytocin (Wray, 1993).

Vasoactive peptide: endothelin

Endothelin receptor has been present in amniotic, chorion, endometrium and myometrium (Monga and Sanborn, 1992). Three endothelin receptors (ET-1, ET-2 and ET-3) have been shown to cause uterine contraction both pregnant and non pregnant uterus (Bolton, 1979). These receptors are increase during delivery and may contribute to an enhancement of the contractile response to endothelin at term (Monga and Sanborn, 1992). Endothelin increases $[Ca^{2+}]_i$ by activating receptor-PLC coupling and also by increase Ca^{2+} influx via nonselective cation channels lead to activate voltage-dependent L-type calcium channels as result in depolarization (Sakata and Karaki, 1992), and then myosin phosphorylation was produced.

The nitric oxide-cyclic nucleotide system in myometrium

Nitric oxide is derived from L-arginine by the action of NO synthase (NOS), an enzyme existing in three isoforms. One of constitutive (cNOS) type [brain NOS (bNOS) or neuronal NOS (nNOS)] and one of endothelial NOS (eNOS) are together responsible for the continuous basal release of NO requiring Ca^{2+}-calmodulin. A third isoform is an inducible form (iNOS) which is expressed in response to cytokines and lipopolysaccharides but which is Ca^{2+} independent. NO was first recognized in the reproductive system by Ignarro et al. (1990) The presence of NOS (e.g. in human glandular epithelium, endometrial stroma and myometrial blood vessels) implies that NO plays a local role in the control of uterine function. It is possible that NO augments the vasodilatory effects of the uterine vascular bed in paracrine fashion and acts as an alternative inhibitor of endometrial platelet aggregation. Moreover, local synthesis of NO within the uterus may be important for regulating myometrial activity, i.e. spontaneous contraction and relaxation of the uterus. Buhimschi *et al*. (1996) demonstrated that the rat cervix expressed all three isoforms of NOS whereas the uterus expressed only iNOS and eNOS, but not nNOS

(reviewed by Rosselli, 1997). There is now considerable evidence albeit not conclusive suggesting that NO-cGMP system plays an important role in uterine and fetoplacental blood flow, quiescence of myometrium and ripening of cervix (Batra *et al*., 2003). NO exerts its smooth muscle relaxant effect by stimulating soluble guanylyl cyclase, therby increasing cGMP (Rosselli, 1997; Batra *et al*., 2003).

The cGMP content of myometrial strips was reported to be significantly higher in tissue from pregnant compared to non-pregnant women and the levels decreased in spontaneous labour (Longa *et al*., 2003; Bernal, 2007). The ubiquitous nitric oxide (NO), in addition to its numerous other activities, is also involved in maintaining normal uterine tone during gestation (Eagland and Cooper, 2001). Longa *et al*. (2003) demonstrated that the sensitivity of human myometrium to NO does not change in relation to gestational age or with the onset of preterm or term labour. These findings are consistent with the clinical effectiveness of these agents for management of preterm labour as well as for obtaining uterine relaxation in various obstetric emergencies such as retained placenta, difficult fetal extraction during caesarean delivery and uterine inversion.

Protein kinase G (PKG) is formed when guanyl cyclase (G-cyclase) is activated, e.g., by nitric oxide, and in turn produces PKG (Wray *et al*., 2003). Similar to A kinase, effects of G kinase on Ca^{2+} movements are diverse. There are some reports indicating that the G kinase mediated relaxation is due to a decrease in $[Ca^{2+}]_i$ (Karaki *et al*., 1997). In vascular smooth muscle, this is a well-documented route for relaxation, as PKG inhibits the Ca^{2+} channel by phosphorylation of the α_1-subunit (Wray *et al*., 2003). The relaxing effect of NO in myometrium may not be mediated via activation of guanylyl cyclase, thus, some results do not support a role for cGMP, but rather suggest an effect of NO on potassium channels (Bernal, 2007).

Mechanisms of relaxant effects mediated by cyclic GMP are similar to those of cyclic AMP (Karaki *et al*., 1997). Both cAMP and cGMP (Figure 2-46) increase the Ca^{2+} requirement for MLC phosphorylation (Ca^{2+} desensitization of MLC phosphorylation) either by inhibiting MLCK or activating MLCP. Phosphorylation of MLCK induced by cAMP-dependent protein kinase would decrease the affinity of MLCK for Ca^{2+}, resulting in a decrease of MLCK activity at a given Ca^{2+} in vitro. Recent work, however, demonstrated that the cAMP-induced phosphorylation of

MLCK is not the physiological mechanism for cAMP-induced smooth muscle relaxation. Recently, it has also been reported that cGMP inhibited Ca^{2+}-induced contraction accompanied by a decrease in MLC phosphorylation. Furthermore, these cyclic nucleotides also inhibited the contractions that are dependent neither on Ca^{2+} nor on MLC phosphorylation elicited by receptor agonists and phorbol esters in the absence of external Ca^{2+}. These results suggest that cyclic nucleotides inhibit not only MLC phosphorylation-dependent pathway but also -independent pathway regulating contractile elements, although the details of the inhibitory mechanisms are not yet understood (Karaki *et al*., 1997).

Difference between the effects of cyclic AMP and cyclic GMP are that;

1. Cyclic GMP augments Ca^{2+} extrusion by activating membrane Ca^{2+} pump.
2. Cyclic GMP does not increase but decreases the noncontractile Ca^{2+}.

Electrolyte

1. Potassium

An increasing in K^+ over the threshold level which is approximately 40 mM concentration leading to change membrane potential. Producing of cell membrane depolarization will increase probability of other important channels such as the Na^+, Cl^- and Ca^{2+} channels to open (reviewed by Riemer and Heyman, 1998).

High-K^+ solution induced uterine contraction with biphasic contractions. The initial peak of contraction is called phasic contraction. When the initial peak reaches a maximum, it is slightly declined to a relative steady at low level. This steady state is called tonic contraction (Bolton, 1979). The relative sizes of the phasic and tonic component vary, depending among other factors on the concentration of potassium applied. For example in taenia, low concentration of potassium (20-40 mM) caused a rise in tension that was maintained. Higher concentration produced contractions that were no greater but generally declined to about two third of the initial peak of tension, especially if isometric recording was used. Similar results were obtained in uterus (Bolton, 1979). It is plausible, although it has not been shown in detail, that the tension produced by high-K^+ declines in uterus after initial peak,

because depolarization is sufficient to abolish action potential discharge and maintained depolarization is less able to generate tension. Whereas, with low-K^+ concentration, action potential discharge is continuous and tension is better maintained (Bolton, 1979). Nevertheless, this contraction was totally abolished by removing external Ca^{2+} and also by agents that block Ca^{2+} channel.

Although high-K^+ mainly increases transmembrane Ca^{2+} influx via VOCCs, then increase $[Ca^{2+}]_i$ and results in contraction, some reports suggested an effect of KCl on the release of Ca^{2+} from intracellular storage and an increase in IP_3. It may be involved to a variety of enzymes such as PKC or PLA_2 (Somlyo and Somlyo, 1994). This activation can prolong effect on tonic contraction because the reduction of potassium gradient across the membrane (Bolton, 1979).

2. Calcium

As mentioned above, It has long been recognized that a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) is fundamental to smooth muscle contraction. The major route to contraction is a rise in intracellular $[Ca^{2+}]$, determined by the entry and efflux of Ca^{2+} and release and re-uptake into the sarcoplasmic reticulum (SR) (Matthew *et al*., 2004).

Vanadates

Protein tyrosine kinases (PTKs) play a critical role in regulating various cellular processes, including phosphoinositide diphosphate (PIP_2) hydrolysis, through tyrosine phosphorylation and the activation of phospholipase C gamma (PLC-γ) in a number of cell systems and tissues. Sodium orthovanadate, a protein tyrosine phosphatase (PTP) inhibitor increases tyrosine phosphorylation in smooth muscle by inhibiting PTPs, which dephosphorylate tyrosine (Palmier et al., 1999; Masui and Wakabayashi; 2001; Albert et al., 2001), elicits uterine contraction via protein tyrosine kinases (PTK)-dependent process associated with the generation of $InsP_3$, a major determinant of myometrial contractility. The increased degradation of PIP_2 was demonstrated to be due to the pervanadate-mediated PLC-γ1 phosphorylation on tyrosine residues (Palmier et al., 1999). In guinea-pig ileum, it has been proposed that

tyrosine phosphorylation may be involved in the stimulation of the muscarinic receptor operated non-selective cation channel (Albert et al., 2001).

Sodium vanadate induces contractions of vascular smooth muscles by increasing the Ca^{2+} permeability of the cell membrane and/or inhibiting the elimination of intracellular Ca^{2+}. In addition, vanadate also causes the release of Ca^{2+} from intracellular storage sites. Sodium vanadate at concentrations higher than 10^{-5} M induced elevations of basal tension and repetitive twitch-like contractions. The drug depolarized membrane only slightly but induced repetitive action potentials. Sodium vanadate potentiated phasic and tonic contractions of K^+-contracture. It also increased tension development of high amplitude in K^+-depolarized preparations. Sodium vanadate-induced twitch-like contractions were associated by repetitive action potentials and were blocked by Ca^{2+} removal or by verapamil, but elevated basal tension was depressed only slightly under these treatments. Tension development was induced in the absence of Ca^{2+} both in depolarized and K^+-depolarized preparations (Sunano et al., 1988).

Ca^{2+}-ATPase can be inhibited by vanadate (Ca^{2+}-ATPase inhibitor), in contrast with sarcoplasmic reticulum Ca^{2+}-ATPase, plasma membrane pump is generally very sensitive to inhibition by less than 10 μM vanadate, with half-inhibition occurring at about 3-5 μM vanadate. These results suggest that sodium vanadate increased mechanical activities by enhancing Ca^{2+} influx as well as releasing intracellular bound Ca^{2+} and/or inhibiting Ca^{2+} extrusion through cell membrane (Enyedi et al., 1988).

Drug acting on uterus

Oxytocic drug

1. Oxytocin

Oxytocin, marketed as Syntocinon®, is a synthetic nonapeptide used for induction or acceleration of labour. It is also used to facilitate contraction of the uterus after delivery for the prevention and treatment of postpartum hemorrhage (Sultatos, 1997; Eagland and Cooper, 2001).

2. Ergometrine

Ergometrine is used to treat postpartum uterine atony and bleeding, and was first used as a uterine-stimulating agent 400 years ago. Ergot is the product of a fungus that grows on rye and other grains. Its alkaloidal derivative, ergometrine, is now produced synthetically from the tetracyclic compound 6-methylergoline. Ergometrine is the most potent of the ergot alkaloids and increase contractile frequency and tone of uterine musculature. It has a direct action on uterine smooth muscle via α-adrenergic receptors and may also act as a partial agonist involving 5-HT receptors. Ergometrine is rapidly absorbed after oral or intramuscular administration with bioavailability of more than 50%. Its duration of action is up to one hour. It undergoes hepatic metabolism and is excreted in the feces (Eagland and Cooper, 2001).

Ergometrine is marketed as ergometrine maleate, and also as Syntometrine® in combination with Syntocinon® 5 units. The recommended dose of ergometrine for postpartum atony or haemorrhage is 500 μg intramuscularly. Intravenous administration is no longer recommended because of the intense cardiovascular effect (Eagland and Cooper, 2001).

3. Prostaglandins

Prostaglandin E_2 (Dinoprostone®) is used to induce abortion; evacuate the uterus after missed abortion, and to treat benign hydratiform mole. Administration of prostaglandin E_2 causes uterine contractions with an increased sensitivity with advancing gestational age. PGE_2 is metabolized in the lungs, liver and kidneys. It may be administered by intravenous, vaginal or extra-amniotic routes (Eagland and Cooper, 2001).

Carboprost (Hemabate®) is a synthetic 15-methyl analogue of prostaglandin $F_{2\alpha}$. It has a prolong action due to the presence of a methyl group, which delays inactivation by dehydrogenation. It has increased specificity for uterine smooth muscle and therefore less action on gastrointestinal muscle. It is used to treat postpartum haemorrhage after failure of conventional treatment but should be caution in patients with asthma, hypertension, renal and hepatic diseases (Eagland and Cooper, 2001).

Dinoprost tromethamine (Lutalyse®) is a synthetic $PGF_{2\alpha}$ tromethamine salt. Veterinary product for intramuscular use in estrus synchronization, treatment of unobserved (silent) estrus and pyometra (chronic endometritis) in cattle; for abortion of feedlot and other non-lactating cattle and for parturition induction in swine but not for human use (Pharmacia and Upjohn, 1997).

Tocolytic drug

1. β_2 Adrenergic receptor

Ritodrine is selective β_2- adrenergic agonist which used to inhibit, or prevent preterm labour, but chronotropic effects and peripheral vasodilation may be seen with therapeutic doses. It is contraindicated to heart disease, furthermore, their metabolic effect include a rise in blood glucose, free fatty acids and decrease in serum potassium. Ritodrine crosses the placenta and may cause neonatal hypoglycemia (Sultatos, 1997; Eagland and Cooper, 2001).

Salbutamol from a metered dose inhaler is also used to promote uterine relaxation in wakeful patients, as it may be required before delivery in caesarean section when there is a transverse lie. However, the technique of administration varies widely and the dose of drug reaching the systemic circulation is unpredictable (Eagland and Cooper, 2001).

Isoproterenol induced uterine relaxation via the same mechanism as described above, but not a selective drug to use because of the adverse effect to heart (Eagland and Cooper, 2001).

2. $MgSO_4$

Studies dating back to 1959 have demonstrated the ability of magnesium ions to inhibit myometrial contractions in a dose-related fashion (Phillippe, 1998). Bolton (1979) demonstrated that high extracellular Mg^{2+} concentration (10 nM) gradually inhibit Ca^{2+} influx into myometrial cells via L-type and T-type voltage operated calcium channel. They suggested that magnesium can rapidly produce repolarization or hyperpolarization resulting in uterine relaxation.

Magnesium ions significantly inhibit phasic myometrial contractions generated in response to several uterotonic agonists, all of which have been

previously reported to activate the phosphatidylinositol signaling pathway. These myometrial relaxants or tocolytic effects of magnesium were dose-related, rapid in onset, reversible with washout, and could be reversed with BAY K 8644, a calcium channel agonist. The inhibitory effects of magnesium appear to be produced by blockade of the membrane dihydropyridine-sensitive voltage activated calcium channels, along with inhibition of agonist-stimulated intracellular calcium release through the IP_3-receptor (Phillippe, 1998). In summary, magnesium inhibited extracellular calcium entry (Phillippe, 1998; Longa *et al*., 2003), intracellular calcium release, cytosolic calcium oscillations, and phasic contractions of myometrial smooth muscle (Phillippe, 1998).

Magnesium sulphate became widely used as a tocolytic agent in North America probably because obstetricians were familiar with it for the treatment of pre-eclampsia (Bernal, 2007). However, the evidence shows that magnesium sulphate is ineffective in delaying or preventing preterm birth and it increases the risk of infant mortality. Its use as a tocolytic agent should be discontinued (Bernal, 2007).

3. Oxytocin antagonist

Oxytocin antagonists (Atosiban®); Atosiban may act by competition with oxytocin at its receptor at the myometrial plasma membrane and inhibiting the second messenger system thereby preventing a rise in intracellular calcium. A second action may involve oxytocin receptors on the decidual and fetal membranes by preventing oxytocin mediated release of prostaglandins (Eagland and Cooper, 2001).

4. Calcium channel blocker

The Ca^{2+} channel blockers are selective inhibitors of the L-type Ca^{2+} channel (Godfraind *et al*., 1986). In various types of smooth muscle, Ca^{2+} channel blockers strongly inhibit the high K^+-induced increase in $[Ca^{2+}]_i$. In isolated smooth muscle tissues, Ca^{2+} channel blockers inhibited the increase in $[Ca^{2+}]_i$ induced by norepinephrine, α_2-adrenergic agonists, prostaglandin $F_{2\alpha}$. However, Ca^{2+} channel blockers did not inhibit the increase in $[Ca^{2+}]_i$ in smooth muscle tissues elicited by ATP. These results indicate that the increase in $[Ca^{2+}]_i$ is due to not only the L-type

Ca^{2+} channel, which is sensitive to Ca^{2+} channel blockers, but also to Ca^{2+} release and Ca^{2+} influx through non-L-type Ca^{2+}entry pathways in smooth muscle cells (Karaki *et al*., 1997).

Calcium channel blockers are known to relax the myometrium in vitro and being investigated for the use as tocolytic. It can inhibit Ca^{2+} influx via VOCCs leading to uterine relaxation. Nifedipine has few effects on cardiac conduction but has more specific effects on myometrial contractility than some other calcium channel blockers and hence has received most evaluation. However, this group of drug may reduce placental perfusion to the extent that fetal hypoxia and acidosis may be produced. Adverse effects of calcium channel blockers include dizziness, flushing, headache and peripheral edema. A starting dose of 20 mg nifedipine orally is common, but these do not appear to be a consensus on dosage or frequency of administration (Eagland and Cooper, 2001).

5. Prostaglandins (PG) synthetase inhibitor

Diclofenac and some non-steroid anti-inflammatory drugs (NSAIDs) cause a concentration-dependent inhibition of methacholine and prostaglandin $F_{2\alpha}$-induced contractions. They are relaxed in a concentration-dependent manner in tonic contractions induced by KCl. The smooth muscle relaxation by mechanisms independent of prostaglandin synthesis inhibition, but related to the inhibition of extracellular calcium influx through mechanisms related or unrelated to pertussis toxin sensible G proteins (Pérez Vallina *et al*., 1995). However, NSAIDs are not routinely used in gestation because of fears of premature ductus arteriosus closure and pulmonary hypertension in the fetus (Eagland and Cooper, 2001).

This group of drugs also used to treatment of dysmenorrhea. Dysmenorrhea is the most common gynecologic complaint. Non steroidal anti-inflamatory drugs (NSAIDS) are the most common pharmacologic treatment for dysmenorrhea (Harel, 2006). NSAIDS have also been found to reduce the amount of the menstrual flow, which may contribute to the level of dysmenorrhea (Durain, 2004). NSAIDs inhibit cyclooxygenase, leading to a reduction in prostaglandin production. The resulting lower levels of prostaglandin lead to less vigorous contractions of the uterus, and, therefore, to less discomfort. Not all adolescents and

young adults with dysmenorrhea respond to NSAIDs, and some of those who respond, report only partial relief. One possible explanation is that most NSAIDs inhibit only cyclooxygenase and do not affect the production of other inflammatory mediators such as leukotrienes. However, in a recent study, treatment with the leukotriene receptor antagonist montelukast (Singulair®), the FDA approved dose (for asthma) and commencing immediately before the menstrual period, failed to improve dysmenorrhea symptoms in adolescents (Harel, 2006).

6. Nitric oxide (NO) donor

Glyceryl trinitrate (GTN) has been used as a NO donor compound to facilitate rapid uterine relaxation (Eagland and Cooper, 2001). Interestingly NO donors inhibit the conversion of prostaglandin (PG) E_2 to $PGF_{2\alpha}$ in human fetal membranes, thus modulating the production of PGs associated with labour. Intravenous nitroglycerin has been used to relax the uterus in management of preterm labour (Longa *et al*., 2003) or during postpartum emergencies but the use of NO donors for routine tocolysis is not recommended (Bernal, 2007).

CHAPTER 3

MATERIALS AND METHODS

Plant material

Fresh rhizomes of *Curcuma aeruginosa* Roxb. (*C. aeruginosa*) were collected in February, 2003 from Songkhla Province, Thailand. The voucher specimen (number: SN 4601010) was identified by Associate Professor Dr. Sanan Subhadhirasakul, and was kept at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Plant extraction

Fresh rhizomes of *C. aeruginosa* were cleaned with distilled water. They were sliced and dried at 55˚C in hot air oven. Then they were pulverized, and extracted as follow. Powdered-dried rhizomes of *C. aeruginosa* (1.9 kg) were extracted with chloroform (10 L) for 7 days at room temperature and filtered; the maceration procedure was repeated 4 times. The solvent was evaporated under reduced pressure to produce the chloroform extract (81.5 g; yield 4.28%). The marc was dried at room temperature overnight and subsequently extracted with methanol by the same procedure as mentioned above to produce a methanol extract (122.1 g; yield 6.43%). Other Powdered-dried rhizomes of *C. aeruginosa* (3.5 kg) were extracted with boiling distilled water (15 L) for 1 hour. After filtration, the marc was repeatedly extracted twice by the same procedure. The solvent was evaporated under reduced pressure to produce the water extract (247.4 g; yield 7.07%). All extracts were stored in air tight bottle under 4°C until used.

Animal

Virgin female Wistar rats (200-300 g) were supplied by the Southern Laboratory

Animal Facility, Prince of Songkla University, Hat Yai campus, Songkhla, Thailand. The animals were maintained under a controlled environment (temperature = 25 ± 1 ^{o}C, light/ dark cycle was 12/12 h). Food and tap water were given *ad libitum*. All animal experiments were approved by the Institutional Committee for Ethical Use of Animals, Prince of Songkla University, Thailand (see Appendix I).

Isolated uterine preparations

The animals were pretreated with diethylstilbestrol (0.1 mg/kg, i.p.) 24 h prior to the experiment. They were then sacrificed by cervical dislocation and the uterine horns were isolated. Uterine strips (15 mm long) were dissected from both ovarian and cervical segments of each uterine horn. The strips were mounted under resting 1 g tension in Locke-Ringer filled organ bath aerated with carbogen (5 % CO_2 and 95 % O_2) and maintained at 37^{o} C. The contractile responses were recorded isometrically with a Grass FT03 force transducer connected to a MacLab system running chart v3.5- (ADI Instruments Pty Ltd., Castle Hill, NSW, Australia) and allowed to equilibrate for 60 min before starting the experiment, during which time the bathing solution was exchanged with fresh Locke-Ringer solution every 10 min.

Experimental Procedures

1. Effects of *C. aeruginosa* extracts on nonstimulated uterine contractions

After the equilibration period, the plant extract (chloroform and methanol extracts, 10-400 μg/ml or water extract, 10-1,760 μg/ml) was added into the bathing solution in a cumulative increase in concentration manner. In parallel control experiment, the effects of vehicle, DMSO were also performed on the contraction of uterus.

2. Effects on stimulant-induced uterine contractions

2.1 Determination of submaximal concentrations of oxytocin, acetylcholine, prostaglandin $F_{2\alpha}$ and KCl

After the equilibration period, uterine strips were induced to contract by oxytocin (0.01 - 10.0 mU/ml), acetylcholine (10^{-8}-3 x 10^{-4} M), prostaglandin $F_{2\alpha}$ (0.005 – μg 50 μg/ml) and KCl (10 - 60 mM) in a cumulative increase in concentration manner. Contractions induced by each drug were recorded. The concentration-response curve was then constructed. Approximately, 70 to 80% of maximum concentration was determined and will be used to induce uterine contraction in subsequent experiment.

2.2 Comparison of the effects of C. aeruginosa (chloroform, methanol and water) extractes with verapamil on oxytocin-induced uterine contraction

After the equilibration period, uterine strips were precontracted with oxytocin (0.1 or 1 mU/ml), after the stable responses to the oxytocin were achieved, the extracts (chloroform, methanol extracts of *C. aeruginosa,* 10-400 μg/ml or water extract, 10-1740 μg/ml) or verapamil (10^{-9}-3x10^{-5} M or 0.491 ng/ml to 0.491 μg/ml;) was added to the bathing solution in cumulative increasing concentration. The interval between successive additions was adjusted to allow the effect of each concentration on the agonist-induced contraction to develop fully. The control uterine strips were treated with equivalent volume of vehicle used for the preparation of the extracts. Force and frequency of uterine contractions were recorded and the concentration-response curves were prepared. Their IC_{50} values were then determined.

2.3 Comparison of the effects of C. aeruginosa (chloroform and methanol) extracts with verapamil and diclofenac on prostaglandin $F_{2\alpha}$ - and KCl- induced uterine contraction

In the other sets of experiments, uterine strips were precontracted with submaximal concentration of $PGF_{2\infty}$ (0.5 μg/ml) or KCl (40 mM). After the stable responses to the agonist or KCl were achieved, the extracts (chloroform and methanol extracts 10-400 μg/ml) or verapamil (10^{-9} -3x10^{-5} M) was added to the bathing solution in cumulative increasing concentration. The interval between successive additions was adjusted to allow the effect of each concentration of agonist-induced contraction to develop fully. The control uterine strips were treated with equivalent volume of vehicle used for the preparation of the extracts. Force and frequency of uterine contractions were

also recorded and the concentration-response curves were prepared. Their IC_{50} values were determined.

2.4 Comparison of the effects of C. aeruginosa (chloroform and methanol) extracts with verapamil and atropine on acetylcholine-induced uterine contraction

Time control experiment showed that the cumulative contraction produced by ACh was not sustained long enough to complete the study of the effects of a range of concentrations of the plant extracts. Therefore, the test agent had to be given to the tissue in a single concentration manner.

After the maximum contractile response to a single concentration of ACh ($3x10^{-6}$ M) was obtained, the uterine strip was then washed with fresh Locke-Ringer solution every 10 min until it returned to the resting level. The process was repeated two or three times until the stable response to ACh was obtained. Then the effects of the plant extracts (10-400 μg/ml), atropine (0.1 ng/ml-1 μg/ml) or verapamil (10^{-9}-$3x10^{-5}$ M) were determined in a single concentration manner by preincubating the uterine strip with single concentration of the extracts or drugs 15 min before the addition of ACh ($3x10^{-6}$ M). In control experiment, the uterine strips were treated with an equivalent volume of DMSO used to solubilize the extracts. Changes in uterine contraction were recorded and concentration-response curves were drawn. The IC_{50} values of the test agents were determined.

2.5 Comparison of the antagonistic effect of propranolol on the relaxation produced by isoproterenol (ISO) and C. aeruginosa (chloroform and methanol) extracts

The effects of isoproterenol (10^{-10}-10^{-6} M) or both plant extracts (10-400 μg/ml) were obtained on oxytocin (1mU/ml) precontracted uterine strips using the cumulative increase in concentration pattern. After completion of the cumulative concentration-response curve, the uterine strips were washed with fresh Locke-Ringer solution every 10 min until they returned to the resting level. Propranolol (10^{-8} M) was then added into the bathing solution, and 15 min later the effect of isoproterenol or the plant extracts on the oxytocin precontracted uterine strip were determined again. The

procedure was repeated with two higher concentrations of propranolol (10^{-7} and 10^{-6} M). In parallel control experiment, the effects of vehicle, DMSO were also tested. Changes in uterine contraction were recorded and concentration-response curves were prepared. The responses of the uterine strips to isoproerenol or plant extracts in the absence and presence of propranolol were then compared.

2.6 Comparison of the effect of C. aeruginosa extracts (chloroform and methanol extract) with verapamil on calcium chloride ($CaCl_2$) induced uterine contraction

The effects of the plant extracts (chloroform extract, 16-80 μg/ml and methanol extract, 80-400 μg/ml) and verapamil (10^{-8}-$3x10^{-5}$ M) on $CaCl_2$-induced uterine contractions were evaluated as follows. After 30 min equilibration in normal Locke-Ringer solution, the uterine strip was then exposed to high-K^+ calcium-free depolarizing solution for 60 min during which time it was washed every 10 min with the fresh depolarizing solution. Then the cumulative concentration-response relationship of $CaCl_2$ (0.01-50 mM) was obtained by addition to the bathing solution at 1 minute interval. The first concentration of the plant extracts or verapamil was added to the bath immediately after washing the tissue and 15 min later, a cumulative response curve of $CaCl_2$ was obtained again. The effects of subsequent concentration of the test agents were determined using the same procedure. In control experiment, pairs of concentration-response curves of $CaCl_2$ were performed with the addition of vehicle. Two concentrations of the extract or verapamil were used per uterine strip.

2.7 Effect of C. aeruginosa methanol extract on oxytocin-induced uterine contraction in Ca^{2+}- free solution

Using modified method of Perez-Guerrero *et al*. (1996), uterine strip was equilibrated in normal Locke-Ringer solution for 20 minutes and then the solution in the bath was replaced by Ca^{2+}-free solution containing 0.01 mM EDTA (see appendix II) and was equilibrated for 60 minutes. Subsequently the uterine strip was incubated with 10 μg/ml of the methanol extract, 15 min later, 10 mU/ml oxytocin was added to the organ bath in order to determine the effect of the methanol extract on the oxytocin-induced

contraction. The process was repeated four more times with higher concentrations (80-240 μg/ml) of the extract. Two concentrations of *C. aeruginosa* extracts were used per uterine strip. In parallel control experiment, the effects of the vehicle were performed on the oxytocin-induced contractions.

2.8 Effect of C. aeruginosa (chloroform and methanol) extracts on vanadate-induced uterine contraction in Ca^{2+}- free solution

Using modified method of Perez-Guerrero *et al*. (1996), uterine strip was immersed in Locke-Ringer solution and equilibrated for 20 min. Subsequently, the solution was replaced by Ca^{2+} free solution with 0.01 mM EDTA to chelate any contaminating extracellular Ca^{2+} (see appendix I), and the srip was incubated in this solution for 60 minutes. Then the uterine strip was induced to contract by vanadate (0.1-3 mM) in a cumulative increase in concentration schedule. The concentration-response curve of vanadate was constructed. The submaximal concentration (70-80 % maximum concentration) of vanadate was used in this experiment in order to determine the possible action of the plant extract on intracellular Ca^{2+}.

After equilibration period in Ca^{2+} free solution, a sustained contractile response to vanadate (0.3 mM) was obtained. Once the contraction was stable, plant extracts (chloroform or methanol extracts; 10-400 μg/ml) were added to the bath in a cumulative increase in concentration manner. The interval between successive addition was adjusted to allow the effect of each concentration of agonist-induced the plant extract to develop fully. In parallel control experiment, the effects of the vehicle were performed on the vanadate-induced contractions. Changes in uterine contraction were recorded and concentration-response curves were constructed. The IC_{50} values of the test agents were determined.

Drugs and Chemicals.

The drugs used were acetylcholine perchlorate, atropine sulfate, isoproterenol hydrochloride, diethylstilbestrol, DL-propranolol hydrochloride and verapamil hydrochloride (Sigma, St. Louis, USA); oxytocin (Gedeon Richer, Budapest,

Hungary); prostaglandin $F_{2\alpha}$ (Lutalyse®, Pharmacia, Rydalmere, Australia) and diclofenac sodium (Utopian, Samuthprakarn, Thailand). Most drugs were dissolved in distilled water, except diethylstilbestrol which was dissolved in olive oil.

Water extract of *C. aeruginosa* was dissolved in distilled water, while the methanol and chloroform extracts were dissolved in dimethylsulfoxide (DMSO; from Merck, Germany) to give a final concentration of 200 mg/ml and kept as stock solution at 4^{o} C until used. On each day of experiment, working solution was freshly diluted to appropriate concentration. The final concentration of DMSO in the organ bath was less than 0.5% which did not affect uterine contraction or relaxation.

The Locke-Ringer solution contained (mM): NaCl, 154; KCl, 5.63; $CaCl_2$, 2.16; $NaHCO_3$, 5.95; $MgCl_2$, 2.10 and D-glucose, 5.55. The high-K^+ calcium-free depolarizing solution contained (mM): NaCl, 99.63; KCl, 60; $NaHCO_3$, 5.95 and D-glucose, 2.77. The Ca^{2+}-free solution contained (mM)**:** NaCl, 154; KCl, 5.63; $NaHCO_3$, 5.95; D-glucose**,** 25.55 and EDTA 0.01 (see appendix II)**.** All chemicals were analytical grades.

Data Analysis

The results were expressed as mean ± standard error of mean (SEM). For each group, the log concentration-response curves were plotted. Regression lines were fitted to the linear portion of the log concentration-response curve by method of least squares. IC_{50} values (concentration required to produce 50% of the maximum effect) were determined using PHARM/PCS computer program version 4.0. Regression lines were tested for deviation from parallelism and relative potencies were determined.

Differences between means were analyzed using unpaired Student't-test or analysis of variance (ANOVA), when necessary. This was followed by Duncan multiple range test to determine individual differences. In some cases, the analysis was performed on the individual IC_{50} values obtained from each concentration-response curve for the test agent. A probability of < 0.05 was taken to indicate statistical significance.

CHAPTER 4

RESULTS

1. Effect of the plant extracts on nonstimulated uterine contraction

After 60 min of equilibration period, the spontaneous contraction of the uterus did not appear. Water extract of *C. aeruginosa* (10-1,760 μg/ml) produced significant uterine contraction in a concentration-dependent manner, with the maximum amplitude of contraction = 2.93 ± 0.35 g, and maximum frequency is 2.9 ± 0.5 contractions/10 minutes. The log-concentration response relationship is shown in Figure 4-1. In contrast, the chloroform and methanol extracts had no significant effect as compare to time control and solvent vehicle, DMSO (data not shown). However, the contractile activity of the water extract was susceptible to quick desensitization and the effect could not be reproduced when the extract was added again after washing.

2. Effect of the plant extracts and verapamil on oxytocin-induced uterine contraction

Oxytocin (0.1 and 1 mU/ml) caused rhythmic contractions of isolated rat uterus. Water extract in the same concentration range (10-1,760 μg/ml) which produced contraction of nonstimulated uterus, did not potentiate the contraction stimulated by either 0.1 or 1 mU/ml oxytocin. In contrast, it significantly decreased the frequency of contraction induced by low dose oxytocin) in a concentration dependent manner (decreased by 42.03 ± 5.77% at the highest concentration, 1,760 μg/ml) and had no effect on the force of contraction, while in the corresponding time control experiment (oxytocin alone) the frequency of contraction also decreased by 16.9 ± 2.86%. For the contraction induced by the higher dose of oxytocin (1 mU/ml), the water extract produced no significant effect on either force or frequency of contraction compared to time control (Figure 4-2).

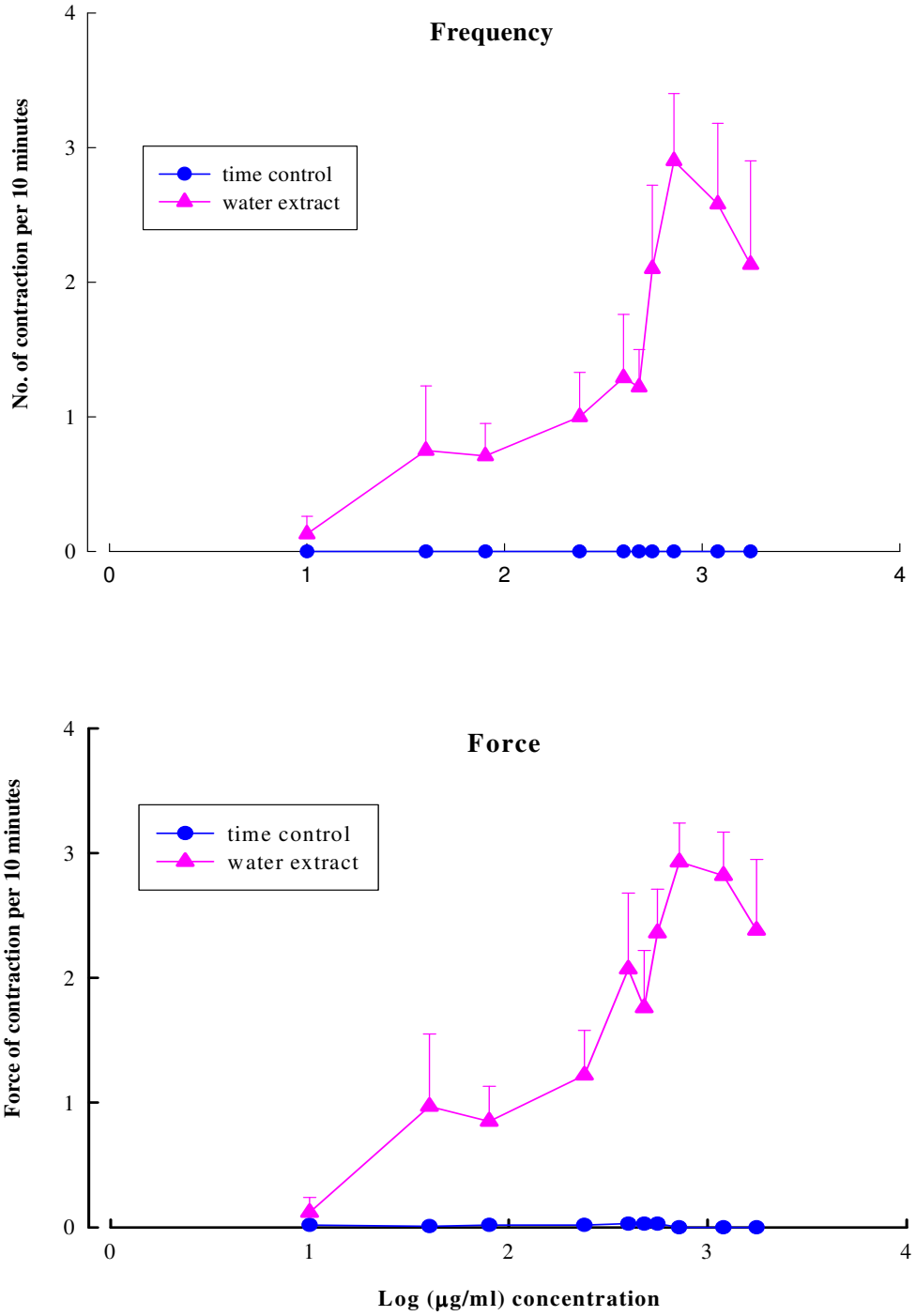

Figure 4-1 Frequency (above) and force (below) of contractions produced by *C. aeruginosa* water extract on nonstimulated isolated rat uterus in comparison to time control. Symbols represent means and the vertical lines show standard errors of means (n = 10-14).

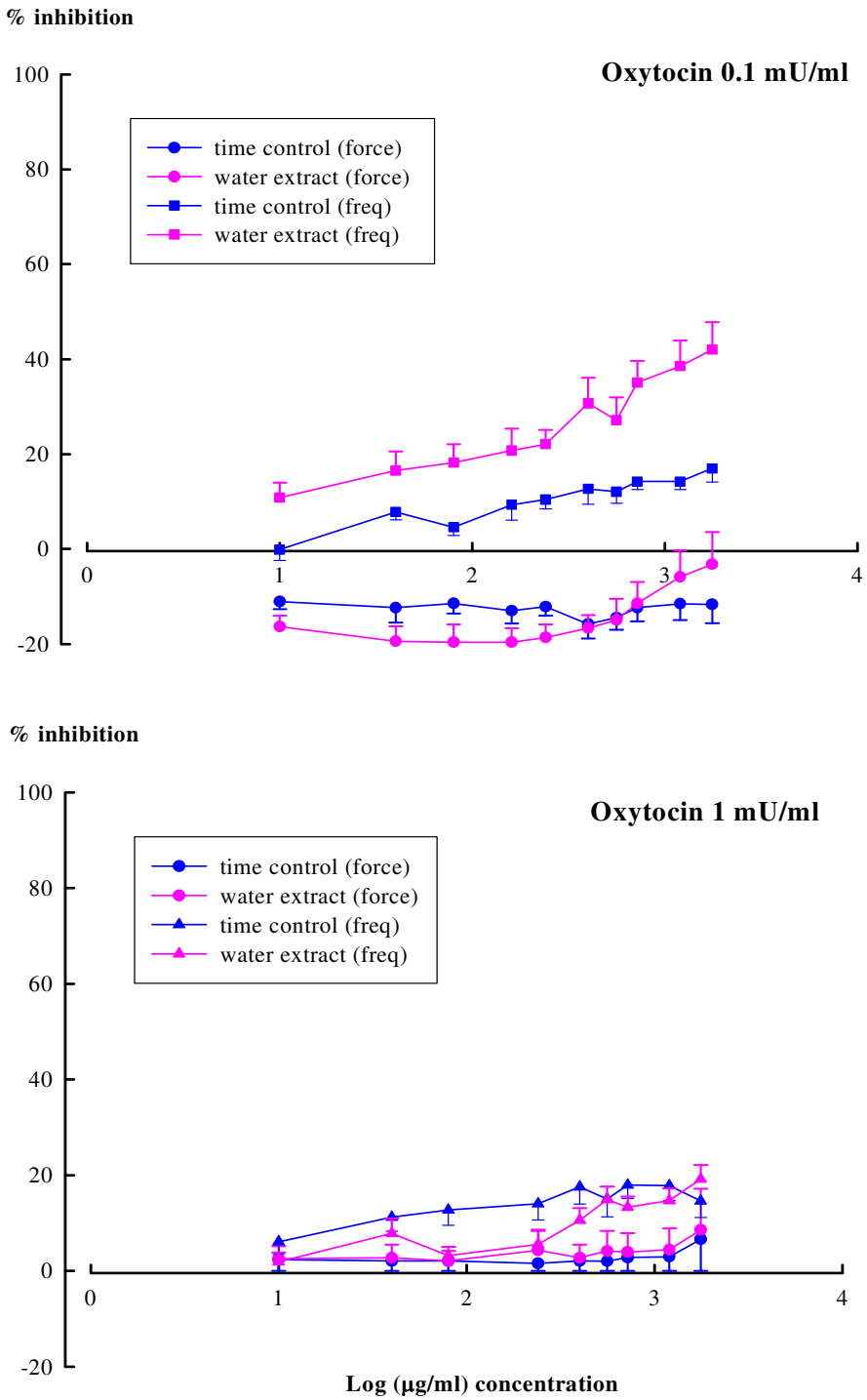

Figure 4-2 Inhibition of contraction induced by 0.1 mU/ml (above) and 1 mU/ml oxytocin (below) of *C. aeruginosa* water extract (10-1760 µg/ml). Symbols represent means and vertical lines show standard errors of means (n = 10-11).

However, the other two plant extracts exhibited the inhibitory effect. The chloroform and methanol extracts (10-400 μg/ml), and verapamil (0.49 ng/ml-0.49 μg/ml; 10^{-9}-10^{-6} M) caused significant inhibition on both amplitude and frequency of 1 mU/ml oxytocin-induced uterine contraction in the concentration dependent manner. All contractions were completely abolished by the highest concentration of the extracts or verapamil used. Typical tracing is shown in Figure 4-3 and data were summarized in Figure 4-4. Table 4-1 shows the IC_{50} of the plant extracts and verapamil.

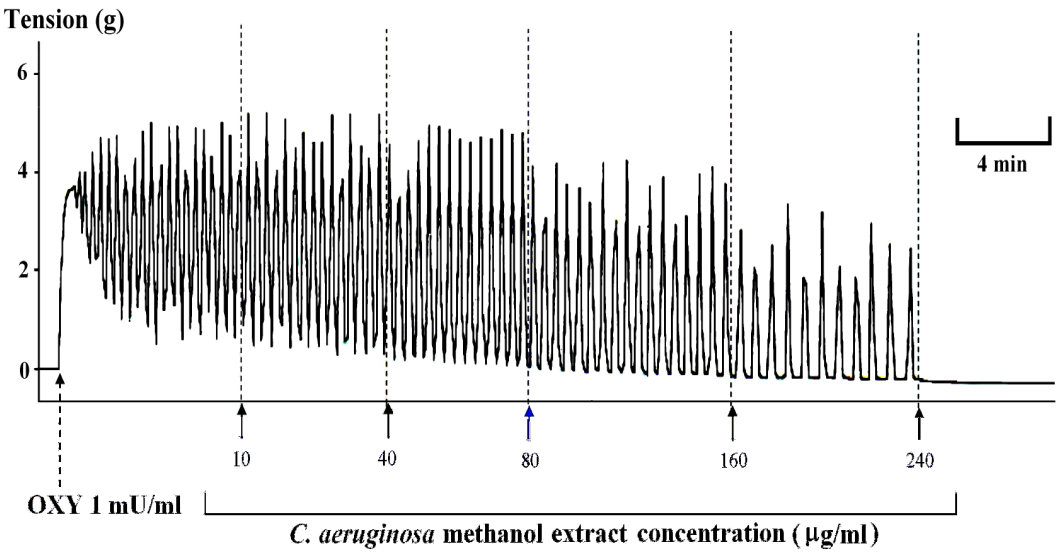

Figure 4-3 Effect of *C. aeruginosa* methanol extract given cumulatively on the oxytocin-induced contraction of isolated rat uterus.

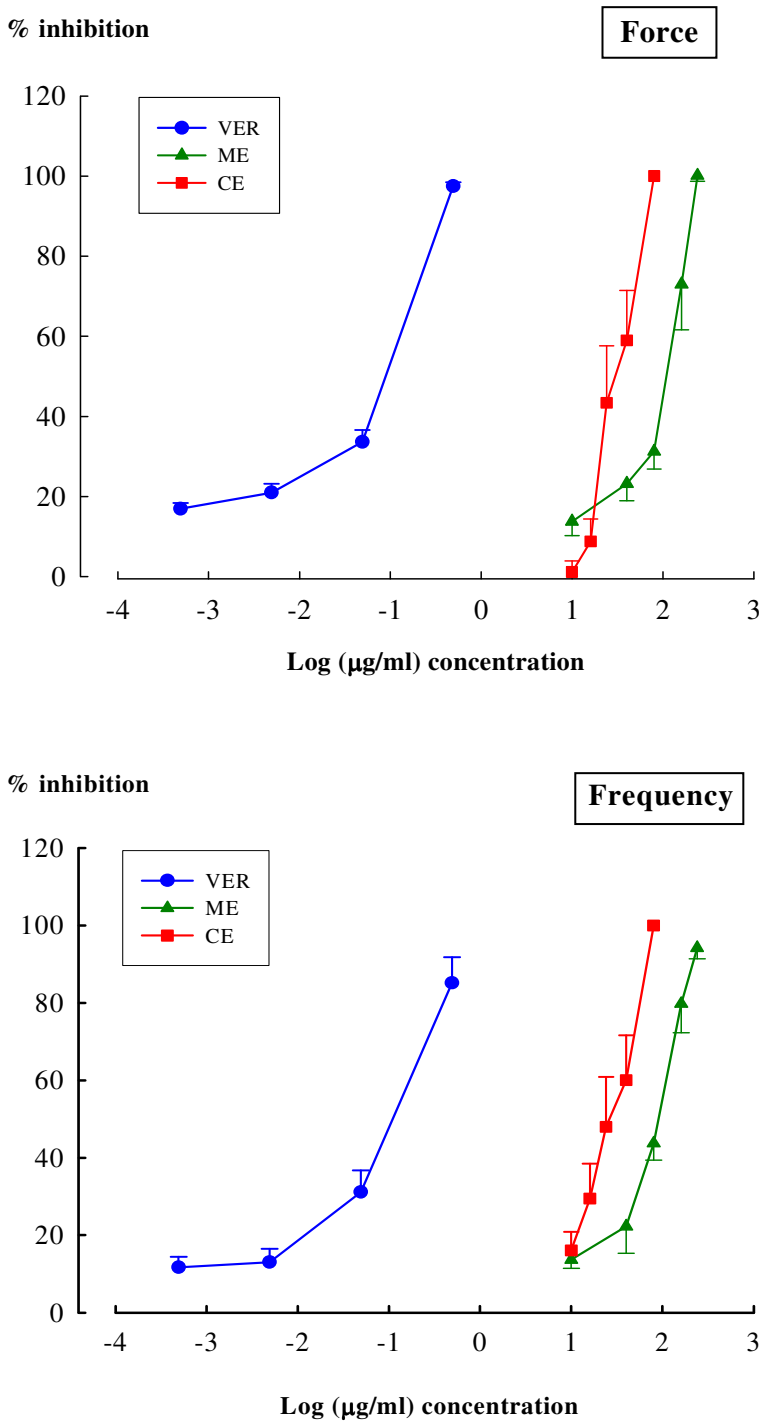

Figure 4-4 Inhibition of force (above) and frequency (below) of oxytocin-induced contractions of isolated rat uterus by verapamil (VER, 0.49 ng/ml-0.49 µg/ml), chloroform extract (CE, 10-400 µg/ml) and methanol extract (ME, 10-400 µg/ml). Symbols represent means and vertical lines show standard errors of means (n =10-11).

3. Effect of the plant extracts, verapamil and diclofenac on $PGF_{2\alpha}$-induced uterine contraction

The plant methanol and chloroform extracts (10-400 μg/ml), verapamil (0.49 ng/ml-4.91 μg/ml; 10^{-9}- 10^{-5} M) and diclofenac (0.25-250 μg/ml) exerted significant inhibitory effects on both amplitude and frequency of the contraction induced by 0.5 μg/ml. $PGF_{2\alpha}$ in a concentration dependent fashion which is completely abolished by the highest concentration of the test agents. Typical tracing is shown in Figure 4-5 and data were summarized in Figure 4-6. Table 4-1 shows the IC_{50} of the plant extracts, verapamil and diclofenac.

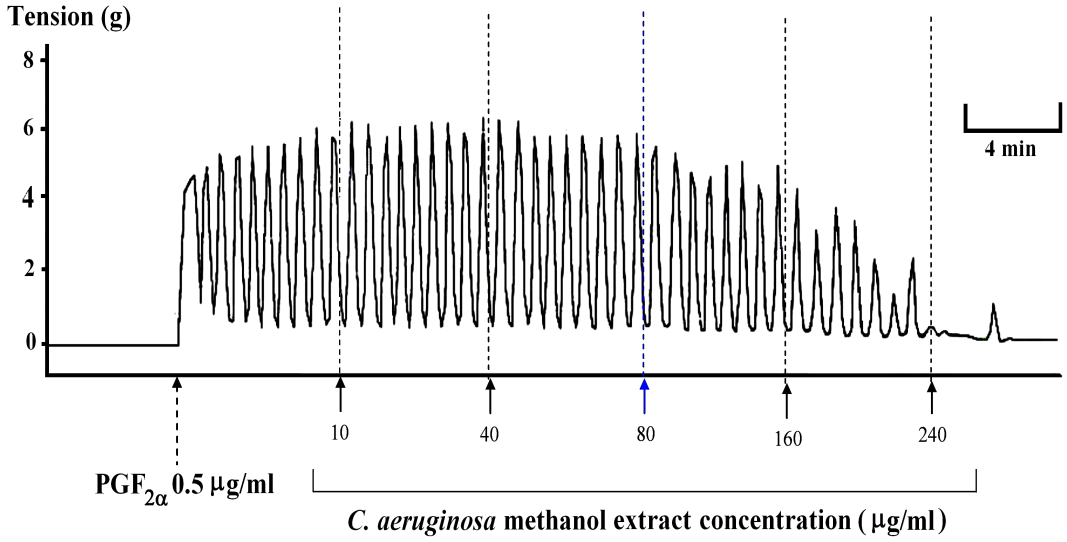

Figure 4-5 Effect of methanol extract of *C. aeruginosa* given cumulatively, on the prostaglandin $F_{2\alpha}$ -induced contraction of isolated rat uterus.

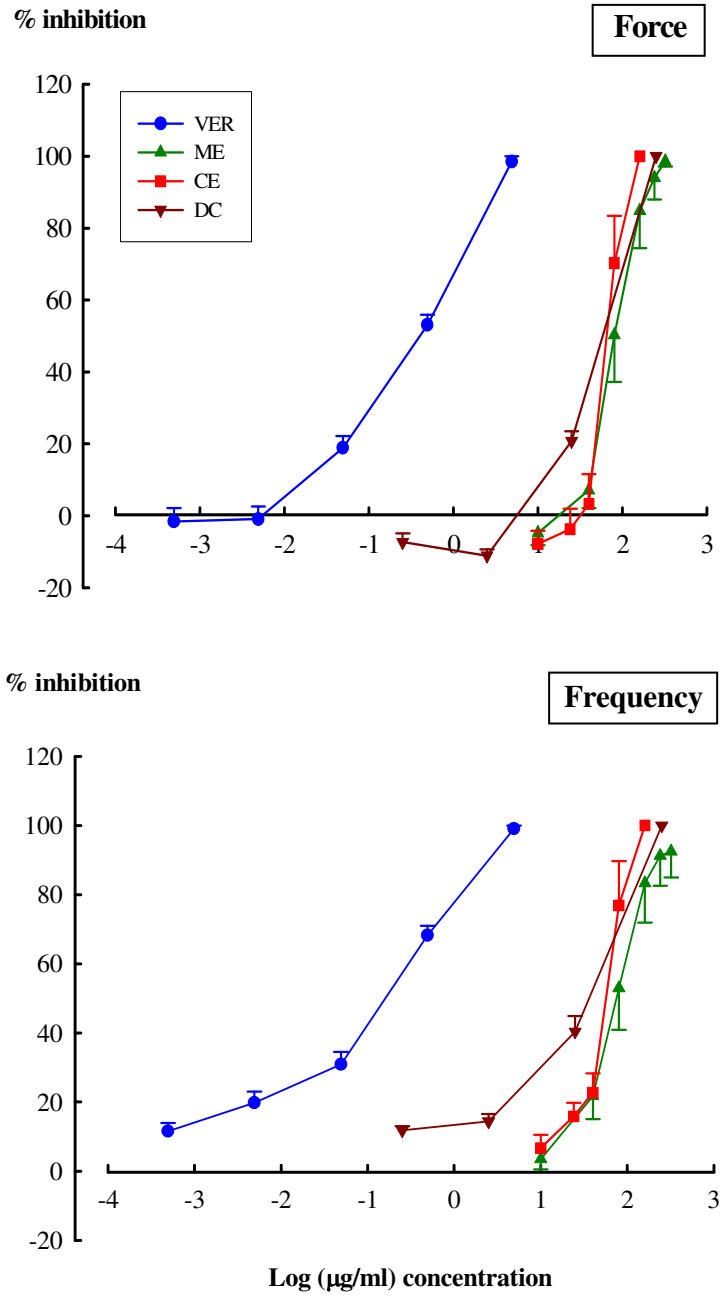

Figure 4-6 Inhibition of force (above) and frequency (below) of $PGF_{2\alpha}$-induced contractions of isolated rat uterus by verapamil (VER, 0.49 ng/ml-0.49 µg/ml), chloroform extract (CE, 10-400 µg/ml), methanol extract (ME, 10-400 µg/ml) and diclofenac (DC, 0.25-250 µg/ml). Symbols represent means and vertical lines show standard errors of means (n = 10) which are smaller than the size of symbols in some cases.

4. Effect of the plant extracts, verapamil and atropine on acetylcholine-induced uterine contraction

As shown in Figure 4-7, acetylcholine (3 μM) caused rhythmic contractions of isolated rat uterus similar to oxytocin (1 mU/ml) and $PGF_{2\alpha}$ (0.5 μg/ml). However, time control experiment showed that the contraction produced by ACh was not sustainable to complete the study on the cumulative effects of the plant extracts. Therefore, the test agent had to be given to the tissue in a single concentration manner. Both chloroform and methanol extracts (10-400 μg/ml), verapamil (4.91 ng/ml-14.73 mg/ml; 10^{-8}-3 x10^{-5} M) and atropine (0.1 ng/ml-1 μg/ml) caused concentration-dependent inhibition of force and frequency of the ACh-induced contraction. Data are summarized in Figure 4-8, verapamil and atropine completely abolished the contraction, whereas, the maximum inhibition produced by chloroform extract (240 μg/ml) were 79.94 ± 11.12% (for force) and 80.21 ± 12.37% (for frequency) and those produced by methanol extract (400 μg/ml) were 79.89 ± 3.30% (for force) and 46.70 ± 17.10% (for frequency). IC_{50} of the test agents were shown in Table 4-1.

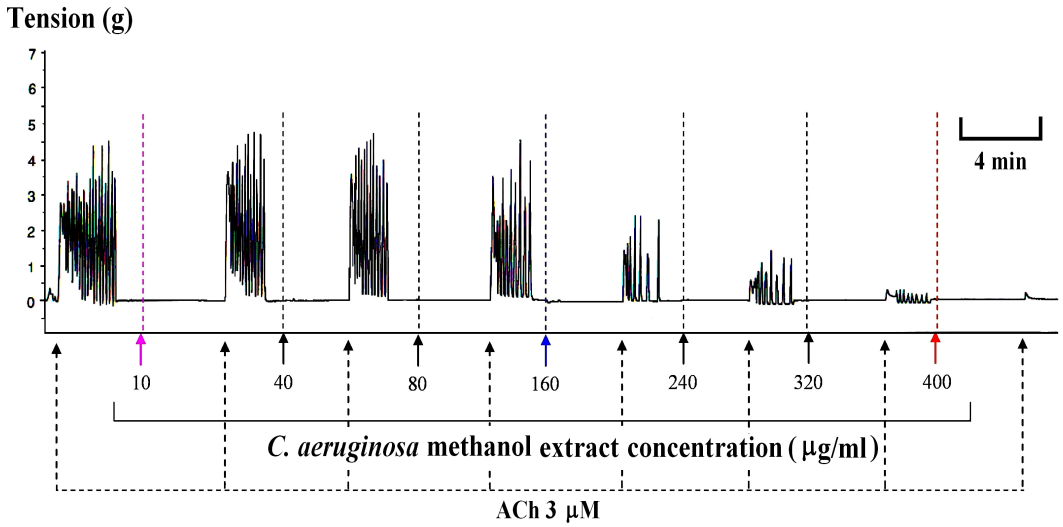

Figure 4-7 Effect of *C. aeruginosa* methanol extract on acetylcholine (ACh)-induced contraction of isolated rat uterus.

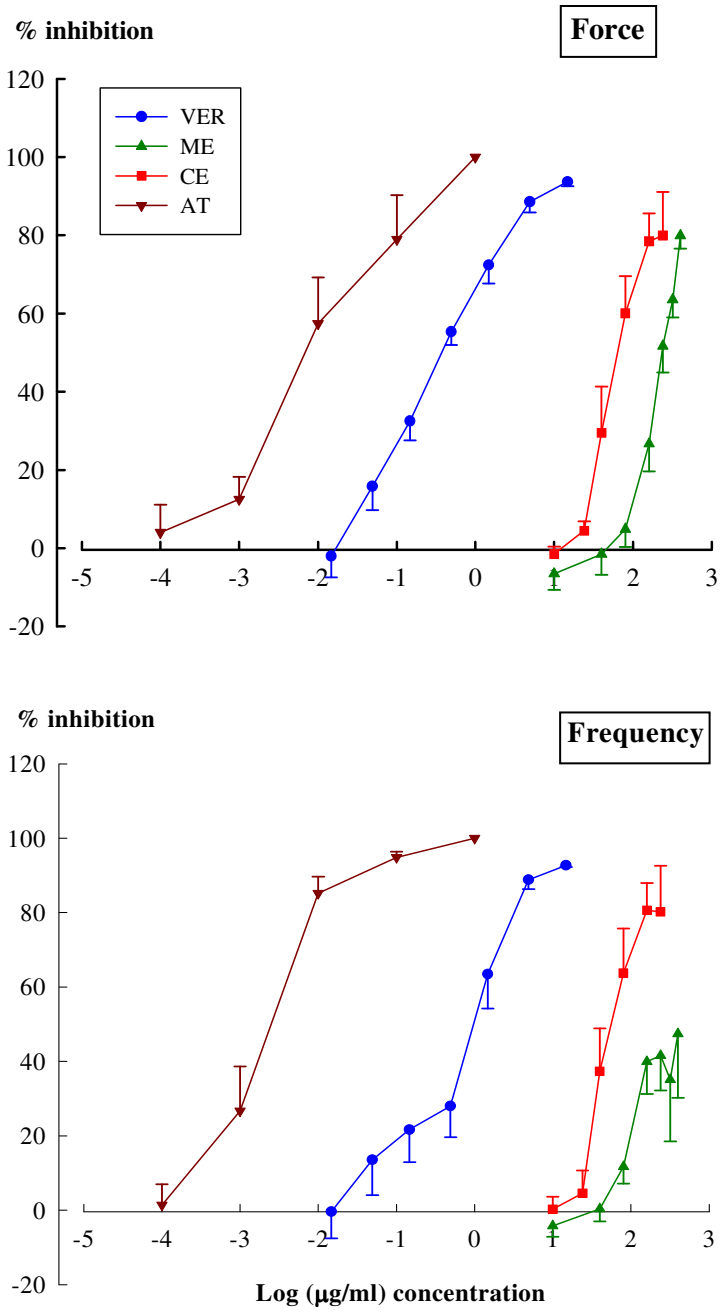

Figure 4-8 Inhibition of force (above) and frequency (below) of acetylcholine-induced contractions of isolated rat uterus by verapamil (VER, 4.91 ng/ml-14.73 µg/ml), chloroform extract (CE, 10-400 µg/ml), methanol extract (ME, 10-400 µg/ml) and atropine (AT, 0.1 ng/ml - 1 µg/ml). Symbols represent means and vertical lines show standard errors of means (n = 8-10) which are smaller than the size of symbols in some cases.

5. Effect of the plant extracts, verapamil and diclofenac on KCl-induced uterine contraction

The representative tracing of KCl (40 mM)-induced contraction of the isolated rat uterus (Figure 4-9) shows an initial rapid, phasic contraction followed by a sustained tonic contraction. Verapamil (VER, 0.49 ng/ml-4.91 µg/ml), the chloroform and methanol extracts (10-400 µg/ml) or diclofenac (0.25-500 µg/ml) added cumulatively during the sustained tonic contractions, exerted a dose dependent inhibition of the contractions which were completely blocked by the highest concentration of the test agent (Figure 4-10). Their IC_{50} were shown in Table 4-1.

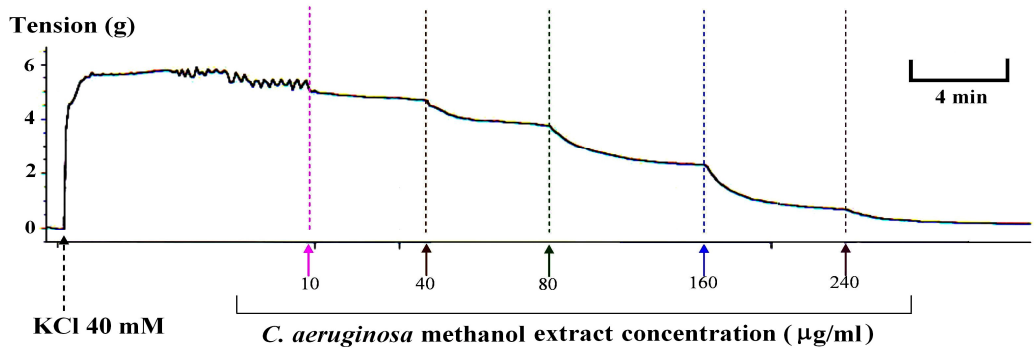

Figure 4-9 Effects of *C. aeruginosa* methanol extract in cumulatively increasing manner on KCl-induced contractions of isolated rat uterus.

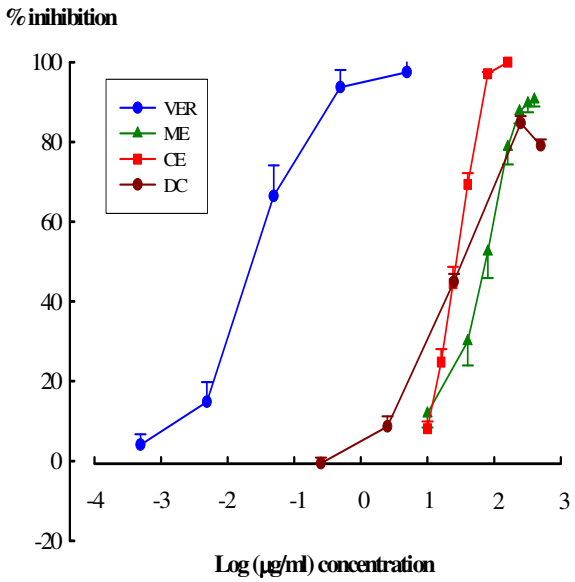

Figure 4-10 Inhibition of KCl-induced force of contractions of isolated rat uterus by verapamil (VER, 0.49 ng/ml-4.91 µg/ml), diclofenac (DC, 0.25-500 µg/ml), chloroform extract (CE, 10-400 µg/ml) and methanol extract (ME, 10-400 µg/ml). Symbols represent means and vertical lines show standard errors of means (n = 10-12).

6. Effect of propranolol on the relaxation produced by isoproterenol or the plant extracts

In isolated rat uterus precontracted by 1 mU/ml oxytocin, isoproterenol (0.0361 ng/ml-3.61 μg/ml; 10^{-10}- 10^{-6} M) added cumulatively into the bathing solution caused a concentration dependent inhibition of both force and frequency of the contraction. The inhibition by isoproterenol was blocked by preincubation the uterine strip with propranolol (10^{-8}-10^{-6} M) in a dose dependent manner (Figure 4-11). In contrast, neither the effect of plant methanol nor chloroform extracts was altered in the presence of propranolol (Figure 4-12).

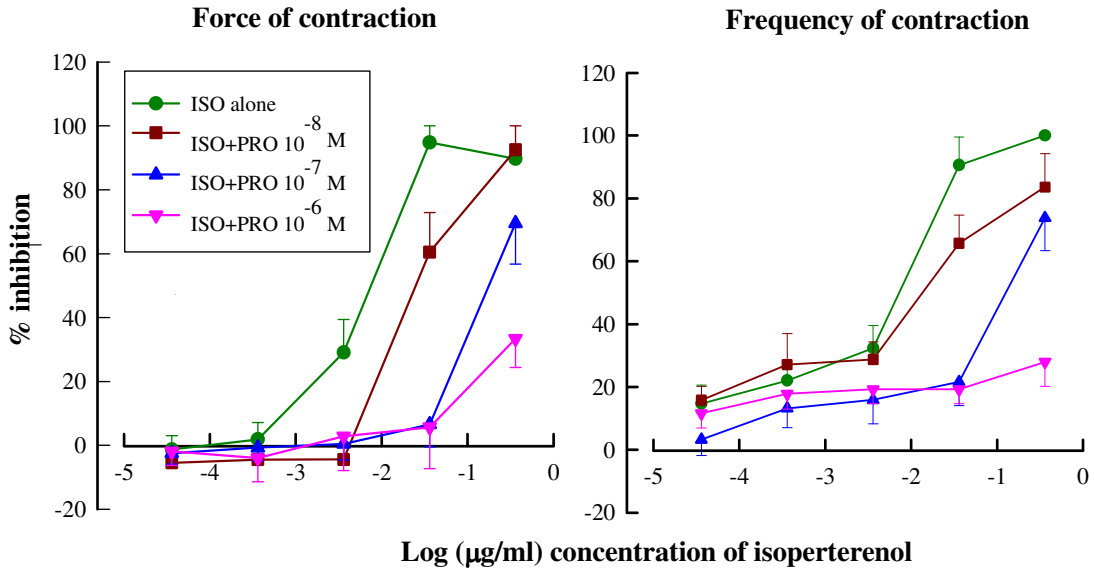

Figure 4-11 Cumulative concentration-inhibition curves of isoproterenol (0.0361 ng/ml-3.61 μg/ml) on the force (left) and frequency (right) of oxytocin-induced contractions of isolated rat uterus in the absence and presence of propranolol (10^{-8}-10^{-6} M). Symbols represent means. Vertical lines show standard errors of means (n = 6-11).

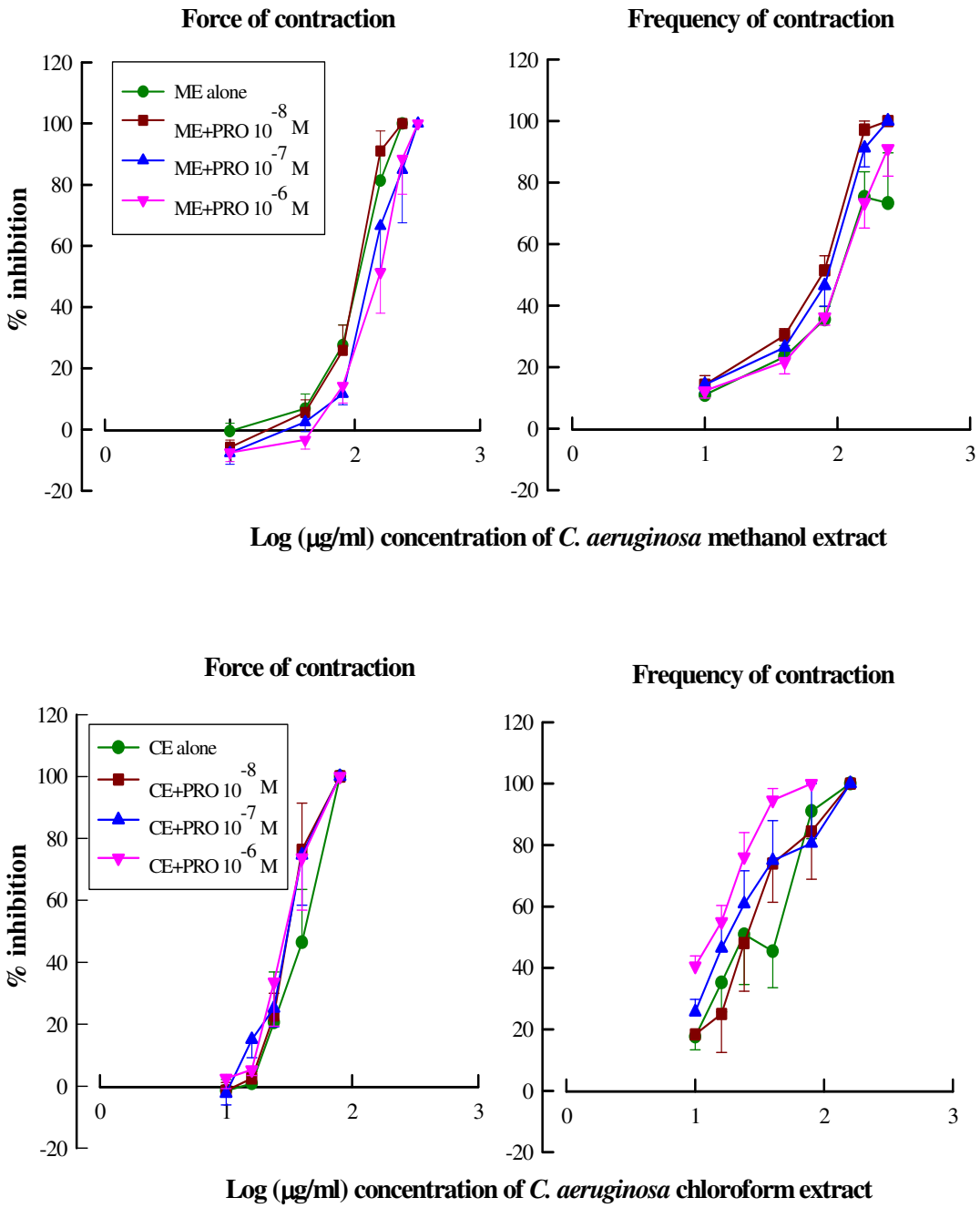

Figure 4-12 Cumulative concentration-inhibition curves of *C. aeruginosa* methanol extract (10-400 µg/ml, above) and chloroform extract (10-160 µg/ml, below) on the force (left) and frequency (right) of oxytocin-induced contractions of isolated rat uterus in the absence and presence of propranolol (10^{-8}-10^{-6} M). Symbols represent means. Vertical lines show standard errors of means (n = 6-12) which are smaller than the size of symbols in some cases.

7. Effect of the plant extracts and verapamil on $CaCl_2$-induced uterine contraction

$CaCl_2$ (0.1-50 mM) added cumulatively into the bathing solution produced a concentration dependent increase in contraction of the isolated rat uterus. The plant methanol extract (80-320 µg/ml), the chloroform extract (16-80 µg/ml) as well as verapamil (10^{-8}-$3x10^{-5}$ M) inhibited the contractions as demonstrated by the concentration dependent rightward shift of response curves to $CaCl_2$ (Figure 4-13 and 4-14).

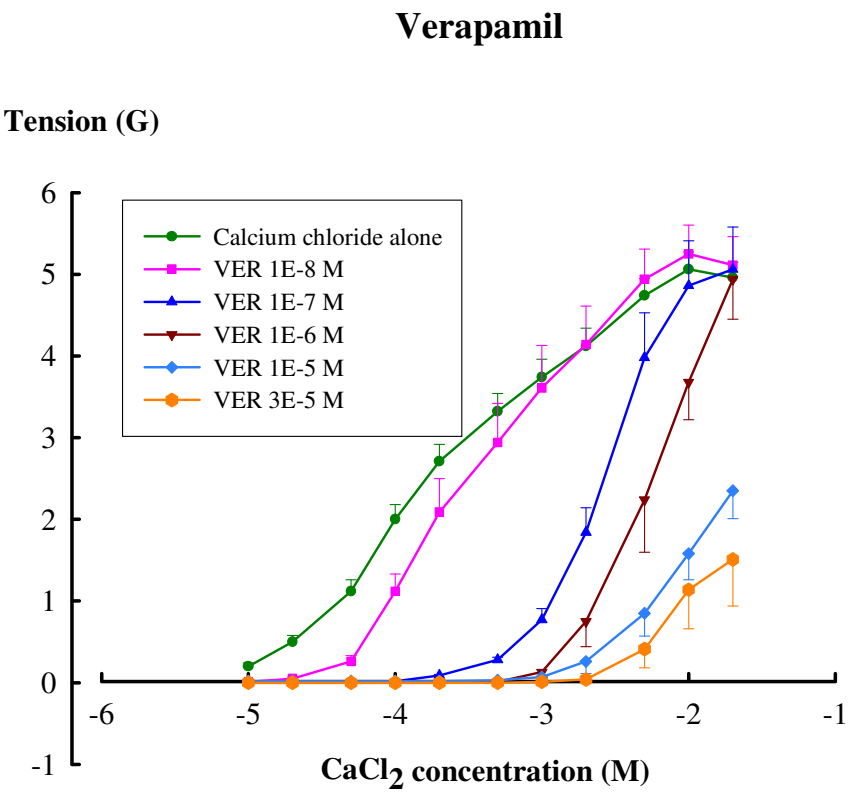

Figure 4-13 Cumulative concentration-effects of calcium chloride-induced contraction of isolated rat uterus and the inhibition by verapamil (VER, 10^{-8}-$3x10^{-5}$ M), The responses were compared to those of calcium chloride alone (time control). Vertical lines represent standard errors of means (n = 9) which are smaller than the size of symbols in some cases.

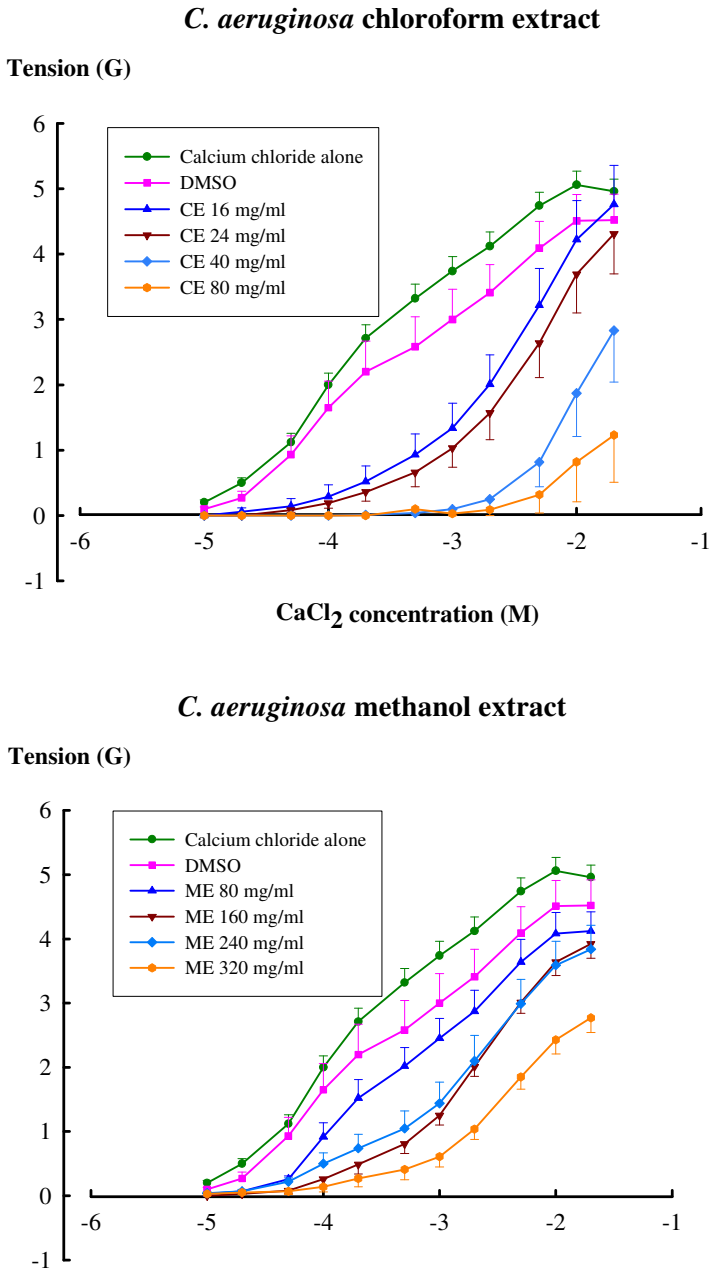

Figure 4-14 Cumulative concentration-effects of calcium chloride-induced contraction of isolated rat uterus and the inhibition by *C. aeruginosa* chloroform extract (CE, 16-80 µg/ml, above) and *C. aeruginosa* methanol extract (ME, 80-320 µg/ml, below). The responses were compared to those of calcium chloride alone (time control) and the equivalent amount of DMSO (vehicle control for the plant extracts). Vertical lines represent standard errors of means (n = 5-9) which are smaller than the size of symbols in some cases.

8. Effect of the methanol extract on oxytocin-induced uterine contraction in calcium free solution

In calcium free solution containing EDTA, the amplitude of contraction-induced by oxytocin 10 mU/ml is 8.64 ± 3.92% of those produced by 1 mU/ml of oxytocin in normal Locke-Ringer solution (data not shown). The plant methanol extract caused a significant concentration dependent inhibition of the contraction compared to time control and the vehicle control DMSO (Figure 4-15).

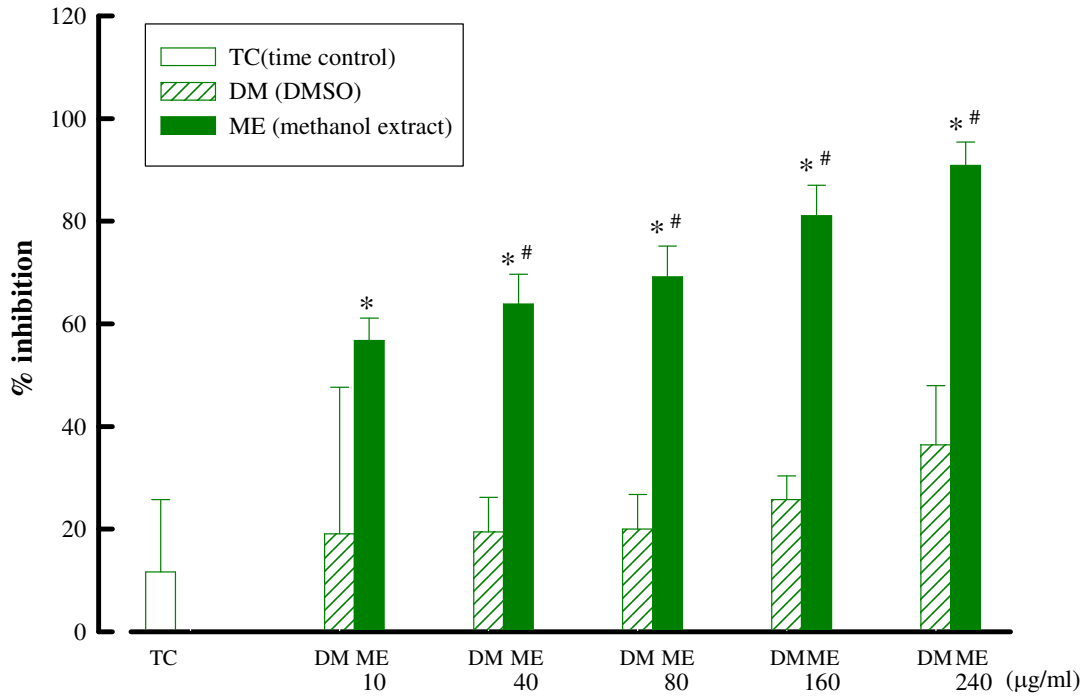

Figure 4-15 Inhibition of *C. aeruginosa* methanol extract on oxytocin-induced contractions of isolated rat uterus in Ca^{2+}-free EDTA containing solution. The responses were compared to those of time control (TC, oxytocin alone) and the vehicle control, DMSO. Bars represent mean responses (mean ± SEM) of 4-10 experiments. *indicates significantly greater than time control. #indicates significantly greater than vehicle control.

9. Effect of the plant extracts on vanadate-induced uterine contractions in calcium free solution

Vanadate (0.3 mM) induced a sustained uterine contraction. The plant chloroform (10-160 µg/ml) and methanol extracts (10-240 µg/ml) significantly inhibited the sustained contraction in a concentration dependent fashion as compare to time control and solvent vehicle DMSO (Figure 4-16).

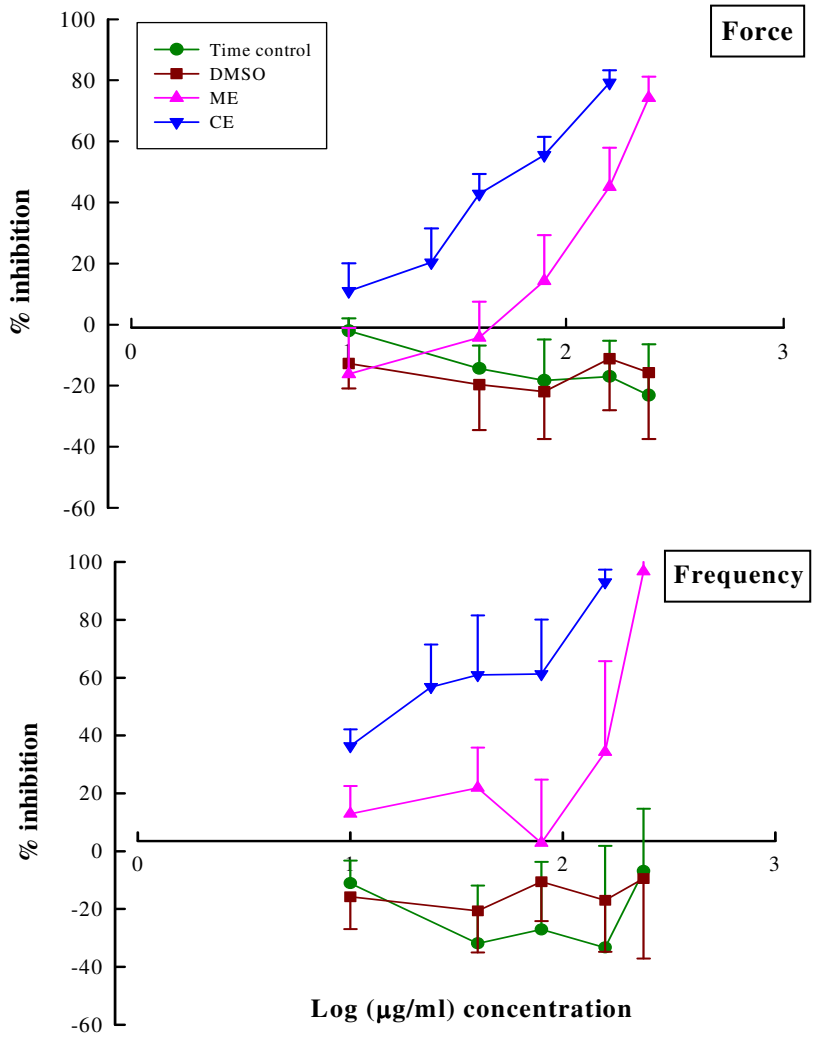

Figure 4-16 Inhibition of force (above) and frequency (below) of vanadate-induced contractions of isolated rat uterus in Ca^{2+}-free EDTA containing solution by chloroform extract (CE, 10-160 µg/ml), methanol extract (ME, 10-240 µg/ml) in comparison to time control and DMSO, solvent vehicle. Symbols represent means and vertical lines show standard errors of means (n = 4-5).

Table 4-1 Comparison of the IC_{50} values (concentration producing 50% of maximum inhibition of contractions induced by various spamogens on isolated rat uterus, n = 8-12) of verapamil, *C. aeruginosa* chloroform and methanol extracts, atropine and diclofenac.

Stimulant	IC_{50} (confidence limits)(μg/ml)				
	Force of contraction				
	Verapamil	**Chloroform extract**	**Methanol extract**	**Atropine**	**Diclofenac**
Oxytocin	0.03 (0.01-0.05) [a,d,e]	31.40 (24.05-40.99) [a,b,c,e]	57.79 (42.71-78.19) [a,b,c,d]	-	-
ACh	0.35 (0.24-0.49) [a,d,e]	56.21 (44.68-70.71) [a,b,c,e,f]	223.80 (191.75-261.21) [a,b,c,d,f]	0.01 (0.003-0.015) [a,c,d,e]	-
$PGF_{2\alpha}$	0.25 (0.17-0.37) [a,d,e,f]	58.59 (49.09-69.93) [a,b,c,f]	69.30 (49.32-97.38) [a,b,c,f]	-	31.36 (16.59-59.29) [a,c,d,e]
KCl	0.04 (0.02-0.07) [a,d,e]	29.28 (20.31-42.21) [a,b,c,e]	69.19 (57.26-83.60) [a,b,c,d],f	-	28.79 (21.35-38.81)[a,c,e]
Vanadate	-	53.07 (39.54-71.22) [a,b]	77.82 (12.16-497.89) [a,b]	-	-
	Frequency of contraction				
	Verapamil	**Chloroform extract**	**Methanol extract**	**Atropine**	**Diclofenac**
Oxytocin	0.04 (0.02-0.07) [a,d,e]	26.98 (18.88-38.55) [a,b,c,e]	56.28 (43.26-73.22) [a,b,c,d]	-	-
ACh	0.57 (0.23-1.44)[a,d,e,f]	67.91 (45.71-100.89) [a,b,c,e,f]	234.07 (147.94-370.36) [a,b,c,d,f]	0.002 (0.001-0.003) [a,c,d,e]	-
$PGF_{2\alpha}$	0.12 (0.08-0.17) [a,d,e,f]	47.07 (37.39-59.27) [a,b,c,f]	55.810 (40.58-76.75) [a,b,c,f]	-	15.70 (10.59-23.27) [a,c,d,e]
Vanadate	-	18.14 (7.87-41.80)[a,b]	65.24 (16.10-264.30)[a,b]	-	-

[a]: significantly different from time control ($p < 0.05$); [b]: significantly different from DMSO ($p < 0.05$)

[c]: significantly different from verapamil ($p < 0.05$); [d]: significantly different from *C.aeruginosa* chloroform extract ($p < 0.05$)

[e]: significantly different from *C.aeruginosa* methanol extract ($p < 0.05$); [f]: significantly different from atropine or diclofenac ($p < 0.05$)

CHAPTER 5

DISCUSSION AND CONCLUSION

The present study demonstrates the effects of *Curcuma aeruginosa* Roxb. (*C. aeruginosa*) rhizome extracts on rat uterine contraction. The uteri isolated from estrogen primed rats were used in this experiment because of the well established action of estrogen, which modulate uterine contractility. Estrogen may increase the uterine contractility by reducing the membrane potential of myometrial cells and increasing gap junction formation (Eagland and Cooper, 2001). An increase in gap junctions formation provides partial cytoplasmic continuity between coupled cells, and thus serve as an ideal anatomical substrate for coordinating tissue responses (Ramondt *et al*., 1994; Christ and Brink, 2000; Fanchin *et al*., 2001). Estrogen treatment of rats resulted in a down regulation of uterine cytosolic NOS activity (Batra *et al*., 2003). In addition, uterine responsiveness to oxytocin is highly dependent on estrogen, which increases the expression of the oxytocin receptor (Parker and Schimmer, 2006).

Both lower and upper segments of uterine horn were used in this study, because there were no significant differences between the contractile responses to either agonists, KCl or the plant extracts as previously observed in our preliminary study (data not shown). Thus, the data obtained are the representative of entire uterus responses.

This study reveals that only the water but not chloroform on methanol extracts of *C.aeruginosa* caused contraction of nonstimulated uterus. The water extract showed ~25% inhibitory effect on the frequency but had neither significant effect on the force of contraction, nor on the contraction induced by a higher concentration of oxytocin (1 mU/ml). In contrast, the chloroform and methanol extracts caused complete or partial inhibition of both the frequency and the force of contraction induced by various spasmogens in a concentration-dependent manner.

From the analysis of electrolytes in water extract (see appendix IV), it was found that the content of Ca^{2+}, K^+ and Na^+ were 138.5, 10,835, 1,256 mg/L, respectively. The final concentration of those electrolytes in bathing solution ($4x10^{-4}$, $3.1x10^{-2}$, $2.7x10^{-3}$ mM, respectively) was much less than those responsible for uterine contraction. Thus, the contraction did not caused by the electrolyte contained in water extract.

Jarikasem *et al*. (2005) reported that the essential oils extracted from *C. aeruginosa* rhizome by hydrodistillation were β-pinene (7.71%), 1, 8 cineol (9.4 %) and cuzerenone (41.63 %). Srivastava *et al*. (2006) reported that α-pinene (2.4%), camphor (15.6%) and other sesquiterpenes were found in *C. aeruginosa* rhizome. Sadraei *et al*. (2001) reported that neither α- nor β-pinene but the mixture of α- and β-pinene caused contraction of the ileum. The contractile activity of the mixture was susceptible to quick desensitization. Our study with the rat uterus demonstrated that *C. aeruginosa* water extract caused contraction of the nonstimulated uterus which was also susceptible to quick desensitization similar to the result which Sadaraei, *et al*. (2001) observed in the rat ileum. Mazzanti and Salvatore (1998) reported that 1, 8 cineol had no effect on the acetylcholine and $BaCl_2$-induced contraction of isolated guinea-pig ileum; in contrast, it showed a weak spasmogenic action. Thus, it is possible that α-pinene, β-pinene and 1,8 cineol which might be present in *C. aeruginosa* water extract contribute to this effect. However, there was no evidence to explain the effect of cuzerenone on smooth muscle contraction.

Sadaraei, et al. (2001) also demonstrated that α-pinene and β-pinene inhibited acetylcholine- and KCl-induced contractions of isolated rat ileum. They suggested that the plant extract containing α- and β-pinene (monoterpene compounds) exhibited spasmolytic activity when the rat ileum was stimulated to contract by ACh or KCl. Thus it is possible that the uterine relaxant effect obtained in our study could be due to these compounds. However, other components were also reported to present in the *C. aeruginosa* rhizome. Takano *et al*. (1995) isolated two guaiane sesquiterpene lactones, zedoalactone A and zedoalactone B from an n-butanol extract. Sirat *et al*. (1998) isolated six sequiterpenes: isofuradiene, furanodienone, dehydrocurdione, curcumenone, 13-hydroxygermacrone and zedoarol from a chloroform extract. Irie *et*

al. (2000) demonstrated that dehydrocurdione, a sesquiterpene isolated from *Curcuma zedoria* inhibited the contractile responses of guinea-pig ileum to ACh (0.01-10 μM) or histamine (0.03-1 μM). The dehydrocurdione significantly reduced the high K^+-stimulated increase in cytosolic Ca^{2+} level of Fura-2-loaded rat mesentery artery. The authors suggested that the inhibitory effects of dehydrocurdione on intestinal and vascular smooth muscle were mediated by blockade of Ca^{2+} entry from the extracellular space. As previously mentioned, it is reported that this dehydrocurdione was also found in the *C. aeruginosa* rhizome. In addition, it has been reported that two sesquiterpine lactones, glucolides D and E isolated from *Vernonia liatroids* relaxed both oxytocin- and KCl-induced contractions of rat uterus (Campos *et al*., 2003). Prakash *et al*. (2006) also demonstrated that mono- and sesquiterpene, the essential oils extracted from a plant (e.g. β-pinene, α-pinene and caryophyllene) relaxed both carbachol- and KCl-induced contractions of isolated rat duodenum. The sesquiterpene lactones found in the *C. aeruginosa* rhizome may participated in the effect similar to a sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, the sesquiterpene lactone extracted from a plant, *Thapsia garganica*. It has been reported that thapsigargin inhibited the frequency of contraction-induced by oxytocin, but did not abolish the oxytocin-induced increase in the amplitude of myometrial contractions (reviewed by Shmygol *et al*., 2006). However, both the frequency and amplitude of uterine contraction induced by oxytocin were inhibited by our methanol and chloroform extracts. As described earlier, not only α-pinene or β-pinene that have spasmolytic effect, the dehydrocurdione or other terpenes may potentiated this effect. Thus, we speculated that α-pinene, β-pinene and other terpenes contribute to the effect of the *C. aeruginosa* extracts on the isolated rat uterus in the present study.

These terpene compounds have very low solubility in water but they are soluble in alcohol, ether, benzene and chloroform. According to the sequence of extraction in the present study, the plant rhizome was extracted by chloroform prior to methanol; therefore, fewer amounts of terpenes were contained in the methanol than the chloroform extract. This could be the reason why the spasmolytic potency of the methanol extract in our study was lower than that produced by the chloroform extract. If the methanol extract contained very small amount of the terpenes, other compounds

different from those contained in chloroform extract might contribute to the actions of the methanol extract.

Different spasmogens were used to induce contraction in order to elucidate the effects and the plausible mechanisms of action of *C. aeruginasa* extracts. It is now well established that the mechanism of myometrium contraction is similar to other smooth muscles which are caused by a rise in intracellular Ca^{2+} ($[Ca^{2+}]_{i,}$ leading to the binding of Ca^{2+} to calmodulin. The Ca^{2+}-calmodulin complex then binds to the myosin light chain kinase, activating the enzyme that uses ATP to phosphorylate smooth muscle myosin cross bridges that binds to actin and causes muscle contraction (Vander *et al*., 2001). The rise of $[Ca^{2+}]_i$ for the myometrium contraction comes from two sources: the extracellular space and an intracellular store, the sarcoplasmic reticulum (SR). Extracellular Ca^{2+} enters the cell through: 1) voltage-operated calcium channels (VOCCs), which is regulated by membrane depolarization and 2) the other two channels which are non-voltage-gated: 2.1) receptor-operated calcium channels (ROCCs) which couple to excitatory receptor directly or indirect via G-protein and are activated by various agonists 2.2) store-operated calcium channels (SOCCs), which may be activated by calcium influx factor or by Orai1, the transmembrane signaling protein, that was released from the depleted intracellular Ca^{2+} store. At present, there are evidences suggest that ROCCs and SOCCs may be closely related, being formed from protein of the transient receptor potential channels (TRPC). Most TRPC are nonselective cation channels that are permeable to Na^+ and Ca^{2+}. Another source of Ca^{2+} is released from the SR by 1) the activation of inositol 1,4,5 triphosphate (IP_3) on IP_3 receptor 2) the activation of Ca^{2+} on ryanodine receptor and 3) passive leak from SR (Vander *et al*., 2001; McFadzean and Gibson, 2002; reviewed by Kupittayanant *et al*., 2002; Albert and Large, 2003; Faber, 2003; Dietrich *et al*., 2006; Jin and Burnett, 2006; Villereal, 2006; Sanborn, 2007; Albert *et al*., 2007).

The spasmogens in this experiment such as oxytocin and $PGF_{2\alpha}$ cause myometrium contractions by acting on oxytocin and prostaglandin FP receptors, respectively (Zingg and Laporte, 2003; Myatt and Lye, 2004; Shmygol *et al*., 2006). Activation of both receptors stimulate the G-protein, resulting in stimulation of phospholipase C (PLC) activity, increase in phosphoinositol turnover and the release

of Ca^{2+} from an intracellular store (Phillippe *et al*., 1997; reviewed by Asokan *et al*., 2002; Zingg and Laporte, 2003; Wray *et al*., 2003; Myatt and Lye, 2004; Shmygol *et al*., 2006). Besides the intracellular source, there is evidence showing that the influx of extracellular Ca^{2+} provides the major increase in intracellular Ca^{2+} since calcium channel blockers abolished the contractions of both agonists (Perusquia and Kubli-Gafias, 1992; Kawarabayashi *et al.*, 1997; Phillippe *et al*., 1977; Sultatos, 1997; Ruttner *et al*., 2002; Kupittayanant *et al*., 2002). There are evidences demonstrated how these agonists promote influx of Ca^{2+} through VOCCs. Shimamura *et al*. (1994) reported that in the experiment using a whole cell clamp technique, oxytocin induced an inward current through activation of nonselective cation channels. According to Carl *et al*. (1996), inward currents carried through nonselective cation channel could depolarize the plasma membrane and enhance excitability. The depolarization enhanced the probability of the voltage-dependent Ca^{2+} channels opening and increased the influx of Ca^{2+}. In addition, calcium-activated chloride (Cl_{Ca}) channels have also been described in oxytocin-stimulated rat myometrium. These channels therefore may be activated by Ca^{2+} released from the sarcoplasmic reticulum and extracellular Ca^{2+} entry. Activation of Cl_{Ca} channels leads to depolarization, as Cl^- leaves the cell. Thus, the channels may also play an important role in causing excitability of the myometruim and enhance Ca^{2+} entry through voltage-gated Ca^{2+}channels (Carl *et al*., 1996; Karaki *et al*., 1997; Wray *et al*., 2003). Similarly membrane depolarization causing Ca^{2+} entry may be caused by blocking of the delayed rectified potassium channels due to the increase in $[Ca^{2+}]_i$ by its release from the internal store and its entry from the extracellular site (Carl *et al*., 1996; Karaki *et al*., 1997).

Similar to the actions of oxytocin and $PGF_{2\alpha}$, acetylcholine also produces uterine contraction by activation of the muscarinic (M_3)-G protein coupled receptor on the myometrium, causing the release of intracellular Ca^{2+} through the PLC/IP_3 system (Pappano, 2007). However, the major sources of the increase in intracellular Ca^{2+} are also by influx from an extracellular source via voltage gated calcium channels because the calcium channel blocker reduces acetylcholine contraction by about 80 % (reviewed by Godfraind *et al*., 1986).

It has been known that a solution high in K^+ elicits membrane depolarization and thus opens the voltage-dependent L-type calcium channels to cause an influx of calcium ion and finally induce muscle contraction (Godfraind *et al*., 1986; Horowitz, *et al*., 1996). The K^+-induced contractions of rat uterus were abolished by a calcium entry blocker (Godfraind *et al*., 1986). The relative importance of electromechanical or pharmacomechanical coupling for any given smooth muscle preparation can be estimated simply by determining the effects of inhibitors of VOCCs on the contraction to agonists. In some tissues, for example the guinea-pig ileum, dihydropyridines such as nifedipine virtually abolish all contractions suggesting that electromechanical coupling predominates (McFadzean and Gibson, 2002). Thus, in this experiment, verapamil; one of the L-type calcium channel blocker was used as the standard drug to evaluate and compare the plausible mechanism of the plant extracts.

The methanol and chloroform but not water extract, as well as verapamil caused complete inhibition of the contraction-induced by oxytocin (1 mU/ml). The order of potency of the inhibitors were verapamil > chloroform extract > methanol extract. The two extracts also exerted inhibitory effects on the contractions produced by $PGF_{2\alpha}$ similar to those of verapamil and the nonsteroidal anti-inflammatory drug, diclofenac with the order of potency as follows: verapamil > diclofenac > chloroform extract = methanol extract. In a similar way atropine, the antimuscarinic drug, verapamil, and the two plant extracts inhibited the contraction-induced by acetylcholine with the following order of potency, atropine> verapamil > chloroform extract > methanol extract. Verapamil and atropine completely inhibited the ACh-induced contraction, while the two plant extracts produced about an 80% inhibition of the force of contraction. The chloroform and methanol extracts caused about 80% and 47 % inhibition, respectively, of the frequency of contractions. In addition to the inhibitory effects on the contraction induced by various agonists, they also inhibited the contraction produced by KCl and the order of potency was verapamil > diclofenac = chloroform extract > methanol extract.

The plant chloroform and methanol extracts both exhibited the inhibitory effects on various agonists (oxytocin, $PGF_{2\alpha}$ and ACh)-induced as well as the KCl-induced contractions which were similar to the effect of reference L-type

calcium channel blocker, verapamil. As previously mentioned, the contractile effects of all agonists and KCl are mainly due to the influx of calcium ions from the extracellular space. There are reports demonstrating that the nonsteroidal anti-inflammatory drugs; NSAIDs could produce their spasmolytic effect by inhibiting the influx of calcium (Vallina *et al*., 1995; Cantabrana *et al*., 1995). Our finding demonstrated that the relaxant effect of diclofenac (NSAIDs) on $PGF_{2\alpha}$-induced contraction related to the inhibition of extracellular calcium influx. This result is supported by the inhibitory effect of diclofenac on the isolated rat uterine contraction-induced by KCl. Cantabrana *et al*., (1995) has suggested that diclofinac might increase the level of cAMP in the myometrium. The increase in cAMP, would have a similar effect as a β-adrenoceptor agonist, resulting in a decrease in intracellular calcium by stimulation of its efflux from the cell as well as uptake by the sarcoplasmic reticulum (Sultatos, 1997). Thus, it is speculated that the two extracts might cause the relaxation of the isolated rat uterus through the inhibition of extracellular Ca^{2+} influx. This possibility was further substantiated by the inhibition and shifting of the $CaCl_2$-induced contractions to the right by the extracts and verapamil. Furthermore, it is unlikely that the extracts act through the activation of β_2-adrenoceptors as the inhibitory effect of both extracts could not be antagonized by the β-adrenoceptor antagonist, propranolol, while the relaxation of the isolated rat uterus by the β-adrenoceptor agonist, isoproterenol was abolished.

Many agonists induce sensitization of contractile apparatus to Ca^{2+} by inhibiting myosin phosphatase and decreasing the rate of myosin regulatory chain dephosphorylation. This prolongs myosin light chain phosphorylation, leading to enhanced tension without influencing $[Ca^{2+}]_i$ (Wray, 1993; Shmygol *et al*., 2006). It is suggested that the contraction is probably due to a phosphorylation of either a contractile or cytosol protein (Wray, 1993).

An inhibitor of protein tyrosine phosphatase, vanadate also induced contraction and increase in myosin light chain phosphorylation without increasing $[Ca^{2+}]_i$ in rat uterus. In rat uterus, however, contractions elicited by orthovanadate were not inhibited by genistein; the tyrosine kinase inhibitor (Karaki et al., 1997). Vanadate has been known to inhibit Ca^{2+}-ATPase or Ca^{2+} uptake of the plasma

membrane and also cause the release of Ca^{2+} from sarcoplasmic reticulum of vascular smooth muscle. This could be the cause of contraction by elevating the intracellular free Ca^{2+} concentration (Sunano *et al*., 1988). The Ca^{2+} pump of the plasmalemmal and sarcoplasmic reticulum vesicles could be differentiated by their different sensitivities to calmodulin. However, the two Ca^{2+}-transport ATPases did not differ by their sensitivity to vanadate nor by the energization of the Ca^{2+} transport by different nucleoside triphosphates (Raeymaekers et al., 1983). In the rat uterus, vanadate produced sustained contraction which could be initiated repeatedly after exposure to Ca^{2+} free solution for more than 1 hour, without a significant decrease (Mironneau *et al*., 1984). Similar contractions in the absence of Ca^{2+} have also been reported by Sunano et al. (1987). They have demonstrated that vanadate induced tension development in the absence of Ca^{2+} both in polarized and K^{+}-depolarized preparations, although the magnitude was small. They suggested that sodium vanadate increases mechanical activities by enhancing Ca^{2+} influx as well as releasing intracellular bound Ca^{2+} and/or inhibiting Ca^{2+} extrusion through cell membrane. In the present experiment, vanadate also produced sustained contraction in Ca^{2+}-free, EDTA solution. Furthermore, we also demonstrated that the plant methanol extract decreased the contraction–induced by oxytocin in a Ca^{2+}-free EDTA solution in rat uterus. Both chloroform and methanol extracts suppressed the vanadate-induced contraction, thus, it is suggested that parts of their effects were probably intracellulary mediated through the inhibition of the release of Ca^{2+} from the SR and/or modulation of contractile apparatus such as myosin light chain, calmodin and/or nucleoside triphosphates. The results are consistent with our finding that the methanol extract also reduced the contraction induced by oxytocin in Ca^{2+} free-EDTA solution.

Premature activation of the oxytocin system might be a leading cause of preterm labor; therefore the antagonists of oxytocin receptors have been extensively tested to evaluate their potential to inhibit premature uterine contraction (Shmygol *et al*., 2006). The inhibitory effect of the chloroform and methanol extracts on oxytocin-induced contraction observed in the present study, suggested that they have tocolytic effect on rat uterus which should be useful for the prevention of preterm labour. In addition, their inhibitory effects on $PGF_{2\alpha}$-induced contraction

suggested that they are useful for treatment of primary dysmenorrhea. These data support it's used in Thai folk medicine.

In summary, the present findings provide information of the extracts of *C. aeruginosa* rhizomes on the contraction of uterus. The water extract exhibited stimulating activity in nonstimulated (or basal tone of) isolated rat uterus. This effect was subjected to quick desensitization. It produced a weak inhibitory effect on the oxytocin-contracted uterus. These effects were similar to α-pinene, β-pinene and 1,8 cineol reported by other studies in the rat or guinea-pig ileum. In contrast, the other two extracts, chloroform and methanol extracts showed no significant effect on nonstimulated uterus. However, in the stimulated uterus, the two extracts produced concentration-dependent inhibition of the contractions profiles similar to those of reference drugs e.g. L-type calcium channel blocker, verapamil, the nonsteroidal anti-inflammatory drug, diclofenac and the antimuscarinic drug, atropine. It could be speculated that the plant extracts inhibit the contraction by interrupting the influx of Ca^{2+}. The inhibitory effect on stimulated uterus could be due to α-pinene and β-pinene which might be present in the extracts, however, other substances in the extract may also contribute to this effect or otherwise counteract the inhibition. Therefore, further study to separate and identify the constituents of the extracts as well as their actions should be performed in order to understand more about their mechanisms of action. However, based on the concentration of the extracts in the organ bath, we can compare the relative potency of the relaxing effect of the extract and other reference compounds as follows: chloroform extract > methanol extract >> water extract.

Furthermore, based on the inhibitory effect of the extracts on oxytocin-induced contraction observed in the present study, it is suggested that the extract of *C. aeruginosa* have a tocolytic effect on the rat uterus. Providing that the active constituents could be absorbed, the chloroform and methanol extracts should be useful for the prevention of preterm labour. In addition, their inhibitory effects on $PGF_{2\alpha}$-induced contraction suggested that they are useful for treatment of primary dysmenorrhea. The contractile activity of the water extract on nonstimulated uterus might be valuable for prevention of postpartum hemorrhage and accelerate uterine involution. However, in *vivo* studies are required to determine the therapeutic

efficacy. Further study is also needed to examine their effects on other smooth muscles and any undesirable effect on other organs. Nevertheless, the results of our study support the traditional use of the plant rhizome for the prevention of dysmenorrhea and use for uterine involution.

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APPENDIX I

วท-จส/46/19-4

ที่ ทม 1210/ ๗๙

คณะวิทยาศาสตร์
มหาวิทยาลัยสงขลานครินทร์
ตู้ ปณ 3 คอหงส์ 90112

หนังสือรับรองโครงการวิจัย

การศึกษาวิจัยที่ทำการทดลองในสัตว์ทดลองเรื่อง : " ผลของว่านมหาเมฆต่อฤทธิ์แก้ปวด แก้อักเสบ และปีบมดลูกในสัตว์ทดลอง "

หัวหน้าโครงการวิจัย : รศ.ดร.วันทนา เหรียญมงคล
ภาควิชา เภสัชกรรมคลินิก คณะเภสัชศาสตร์

ได้ผ่านการพิจารณาและเห็นชอบจาก : คณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง
มหาวิทยาลัยสงขลานครินทร์

ให้ไว้ ณ วันที่ 17 กรกฎาคม พ.ศ. 2546

(รองศาสตราจารย์นงพร โตวัฒนะ)
ผู้ช่วยคณบดีฝ่ายบัณฑิตศึกษา วิจัยและวิเทศสัมพันธ์ คณะวิทยาศาสตร์
ประธานคณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง
มหาวิทยาลัยสงขลานครินทร์

APPENDIX II

Chemical compositions of physiological salt solutions as follows:

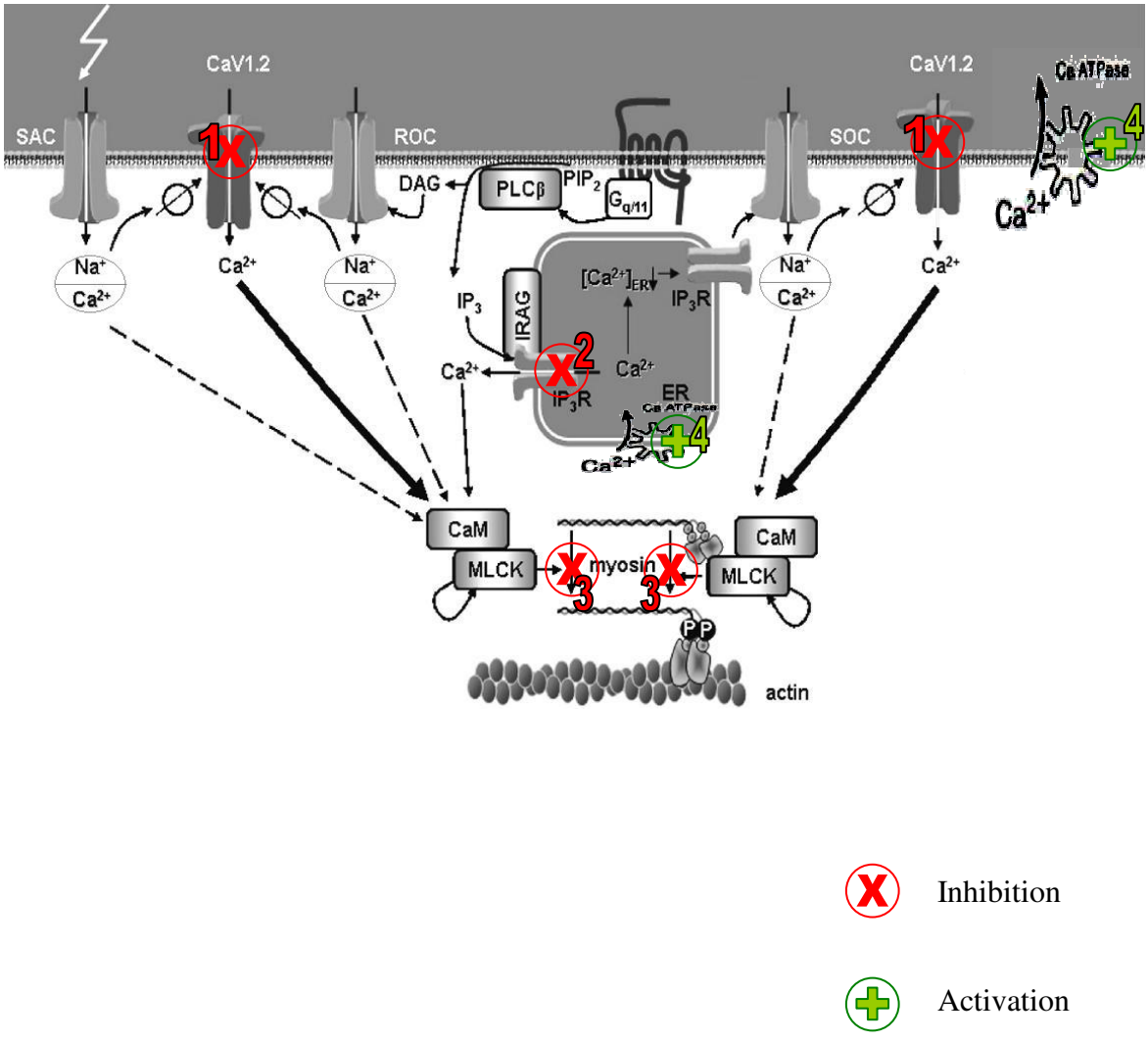
Chemical composition	MW	Physiological salt solutions					
		Locke-Ringer		High K^+ (56.3 mM) Ca^{2+} free (HKFCa)		Ca^{2+}-Mg^{2+} free EDTA (FCa)	
		mM	1x (g)	mM	1x (g)	mM	1x (g)
NaCl	58.44	154.00	9.00	99.60	5.82	154.00	9.00
KCl	74.55	5.63	0.42	56.30	4.47	5.63	0.42
$CaCl_2$	110.99	2.16	0.24	-	-	-	-
$NaHCO_3$	84.01	5.95	0.50	5.95	0.50	5.90	0.50
D(+) GLUCOSE	198.17	5.55	1.10	2.77	0.55	5.50	1.10
$MgCl_2$	95.30	2.10	0.20	-	-	-	-
EDTA	292.24	-	-	-	-	0.01	0.0029

(Source: Perez-Guerrers *et al*., 1996).

APPENDIX III

Plausible inhibitory mechanisms of *C. aeruginosa* extracts on rat uterine contraction

1. Interrupt calcium influx via voltage-gated channels
2. Inhibit release of Ca^{2+} from SR
3. Inhibit myosin light chain phosphorylation
4. Increase Ca^{2+}-ATPase activity



APPENDIX IV

An analysis of *C. aeruginosa* water extract by using ICP-OES*

Electrolyte	**Average concentration (mg/L)**
Calcium	138.5 (145, 132)
Potassium	10,835 (9,430, 12,240)
Sodium	1,256 (1,097, 1,415)

*Analyzed by Central Equipment Unit, Faculty of Science, Prince of Songkla University, Hat Yai Campus, Songkla, Thailand

Comparisons of electrolytes concentration between physiological salt solutions (PSS) without and with C. aeruginosa water extract (720 μg/ml) which produced maximal effects on rat uterine contraction

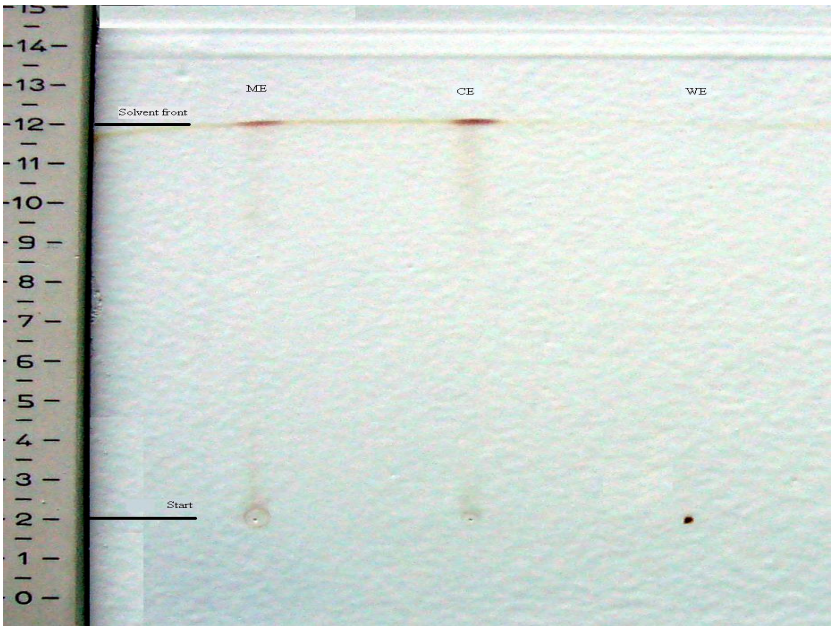
Electrolyte	**MW**	**Amount of electrolyte in water extract 720 μg/ml* (mg)**	**Final concentration in 20 ml organ bath (mM)**
Calcium	40.08	.00997	.0004
Potassium	39.098	0.78	.0305
Sodium	22.989	0.09	.0027

*volume .072 ml (from 200 mg/ml as stock solution)

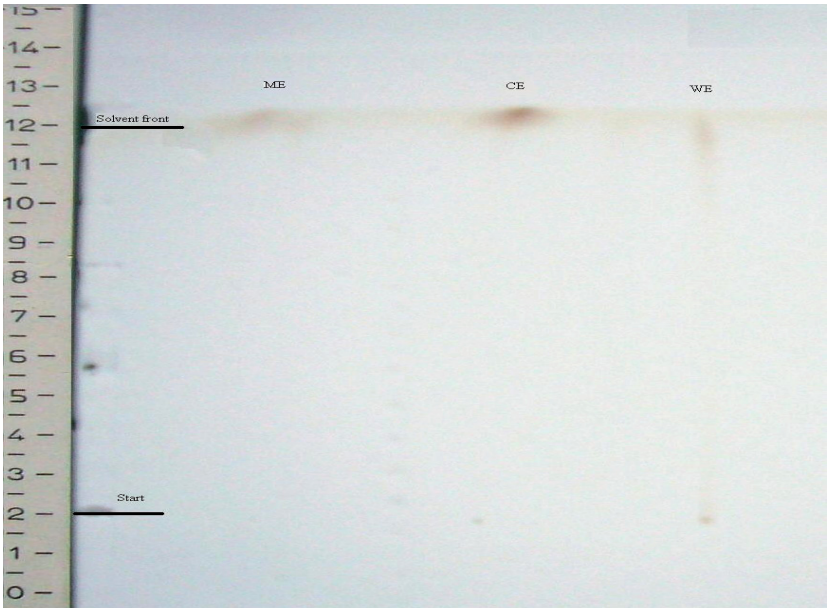
APPENDIX V

Thin layers chromatogram of *C. aeruginosa* extracts by using several developing system and spray with 20% H_2SO_4 in 95% ethanol

a) Solvent system: methanol 10: chloroform 90

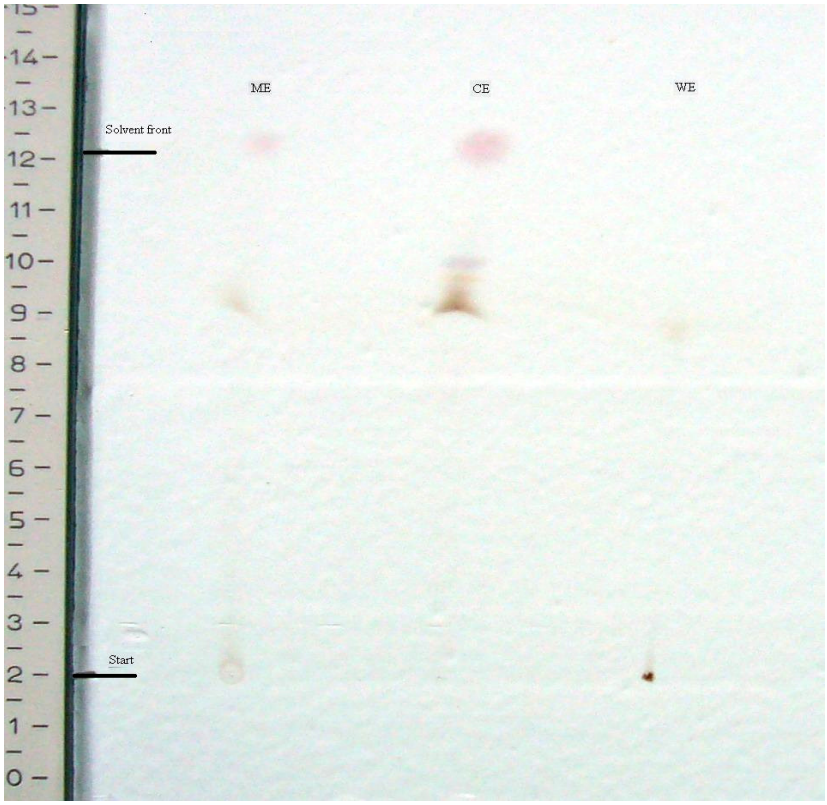


b) Solvent system: acetic acid 50: ethanol 30: water 20

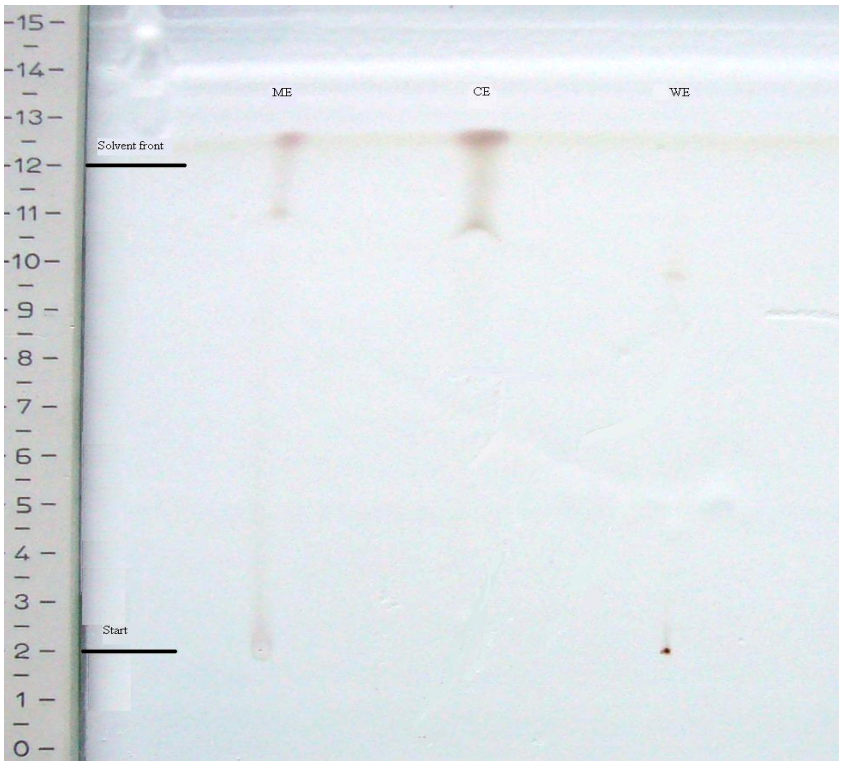


c) Solvent system: methanol 5: chloroform 95

(1)

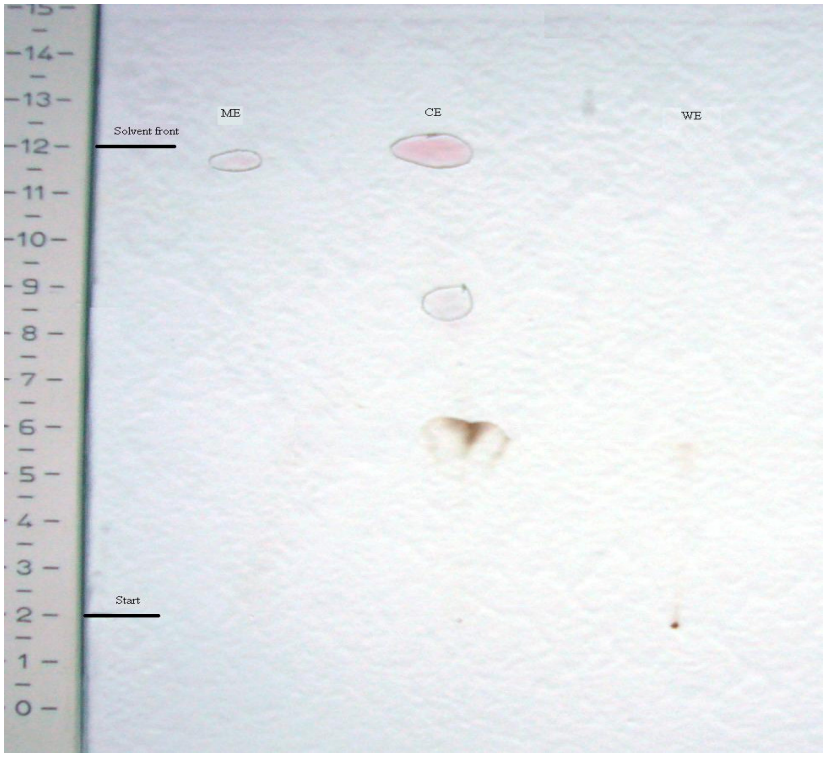


(2)



d) Solvent system: ethanol 30: hexane 70

(1)



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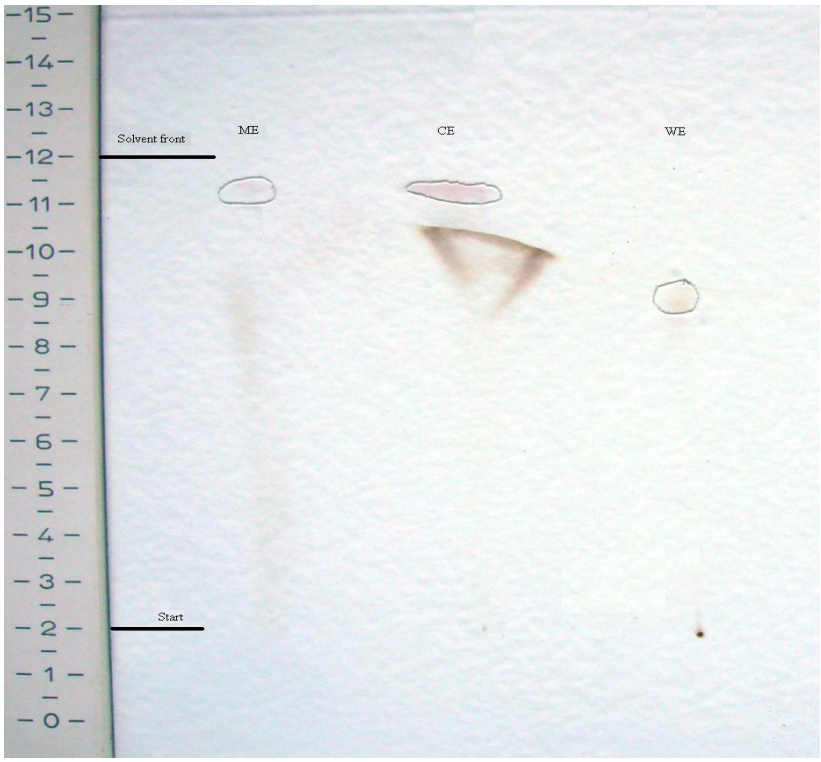

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List of Publication and Proceeding

1 Tungcharoen, P., Thaina, P., Wongnawa, M., Reanmongkol, W. and Subhadhirasakul, S. (2006). Responses of isolated rat uterus to methanolic extract of *Curcuma aeruginosa* Roxb. rhizome. "Proceeding of 28th Pharmacological and Therapeutic Society of Thailand Meeting". Thai Journal of Pharmacology 28(1), 73.

2 Thaina, P., Tungcharoen, P., Wongnawa, M., Reanmongkol, W. and Subhadhirasakul, S. (2007). Uterine relaxant effect of *Curcuma aeruginosa* Roxb. rhizome extracts. Proceedings 41st Annual Scientific Meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologist (ASCEPT), 10th Southeast Asian Western Pacific Regional Meeting of Pharmacologists (SEAWP-RMP) 12, poster 2-59.