The Role of Calcium and Signalling Pathways in the Control and Modulation of Uterine Contraction; With Emphasis on Human Myometrium

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Sajeera Kupittayanant.

January 2003

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Abstract

Pre-term, dystocic and hyperactive labours remain common clinical problems, which often require surgical intervention and are significant causes of perinatal mortality. Whatever the causes of these clinical problems the final pathway is contraction of the myometrial cell. Thus an understanding of the physiological mechanisms that operate to control and modulate uterine smooth muscle contraction is important to ultimately improve reproductive health.

The aims of this work were to elucidate (**1**) how the sarcoplasmic reticulum (SR) contributes to uterine contraction, and (**2**) the importance of both the Ca-calmodulin-myosin light chain kinase (MLCK) pathway and non-Ca-calmodulin-MLCK pathways. The experiments were performed on intact strips of term pregnant non-labouring human myometrium. The strips were dissected from biopsies obtained, following informed consent, from women undergoing elective Caesarean section, and loaded with Indo-1 for intracellular Ca measurement. Intracellular calcium and force were simultaneously measured. In some experiments, phosphorylation of the 20-kDa regulatory myosin light chain (MLC_{20}) was also measured by urea gel electrophoresis and Western blot analyses.

In order to investigate the role of SR in the myometrium, the effects of exposing the strips to ryanodine (which abolishes calcium-induced calcium release (CICR), caffeine (which activates CICR) and cyclopiazonic acid (which depletes the SR Ca store) were examined and the relationship between force and intracellular Ca studied. The following results were found (**1**) Inhibiting the SR in human uterus does not decrease contraction. (**2**) There is no clear physiological role for CICR. (**3**) The SR acts normally to limit contraction. (**4**) Agonists can mobilise Ca from the SR via IP_3-induced Ca release, not Ca-induced Ca release.

In order to investigate the importance of both the Ca-calmodulin-MLCK pathway and non-Ca-calmodulin-MLCK pathways, the effects of exposing the strips to inhibitors of MLCK (wortmannin), Rho-associated kinases (Y-27632) and myosin light chain phosphatase (okadaic acid, cantharidin and calyculin A) were examined in spontaneous, high-K-depolarisation-induced and oxytocin-induced force. The relationship of force, intracellular Ca and MLC_{20} phosphorylation was studied. The following results were found (**1**) Little or no force is present in the uterus when MLCK is inhibited by MLCK inhibitor irrespective of how force was produced. (**2**) Y-27632 decreased force significantly under all three conditions, accompanied by decreases in MLC_{20} phosphorylation without changing intracellular Ca. However, the effects on force were only large when the uterus was producing force tonically rather than phasically. (**3**) There is a modulation of uterine contraction by myosin light chain phosphatase. However, there are important differences between the types of phosphatase in modulating the process of contractions. (**4**) When the contribution of the Ca-calmodulin-MLCK pathway and non-Ca-calmodulin-MLCK pathways in producing uterine force was simultaneously examined under physiological conditions, non-Ca-calmodulin-MLCK pathways are of secondary importance, compared with the Ca-calmodulin-MLCK pathway.

Acknowledgements

I am grateful to **The Royal Thai Government** for providing the financial support that enabled me to complete this study.

I would like to thank **Professor Susan Wray,** my supervisor, for her generous guidance and encouragement during my study. I also thank **Dr. Ted Burdyga** and **Dr. Tony Shmygol** for their advice and helping me learn many new techniques.

I would like to thank **Mr. David Gallacher** and **Dr. Gul Erdemli,** my advisory panels, for their discussion during the advisory panel interviews. I also thank **Dr. Rachel Tribe** and **Dr. Siobhan Quenby** for their discussion during the viva examination.

I would also like to thank **Dr. Duncan Smith, Dr. Jo Piece, Dr. Ebtsam Emonir, Zac Shabir, Karen Noble, Andy Matthew, Yathong Li, Dr. Karen Jones, Bill Franks, Collette Duvall, Dr. Eduard Babiychuk** and **Dr. Robert Duquette** for their help and friendship over the period of study.

I also thank all staffs on the labour ward of the Women's Liverpool Hospital for their help in obtaining the biopsies.

Thanks to **Dome,** my husband, who has helped and made my life easy and happy.

Finally, thanks to all my family and husband's family for their love and support.

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Abbreviations

ADP	Adenosine 5-diphosphate
ACh	Acetylchlorine
ATP	Adenosine 5-triphosphate
ATPase	Adenosine triphosphatase
AM	Acetoxymethyl ester
AUC	Area under the curve
Ba	Barium
^0C	Degree Celsius
Ca	Calcium
Ca-ATPase	Calcium adenosine triphosphatase
[Ca]	Calcium concentration
$[Ca]_i$	Cytosolic calcium concentration
$CaCl_2$	Calcium Chloride
CaM	Calmodulin
CaM kinase II	Calmodulin-dependent protein kinase II
cAMP	Adenosine 3':5'-cyclicmonophosphate
CCE	Capacitative calcium entry
cGMP	Guanine 3':5'-cyclicmonophosphate
CICR	Calcium-induced calcium release
Cl	Chloride
Cl_{Ca}	Ca-activated Cl channel
cm	centimetre, length
CO_2	Carbon dioxide
CPA	Cyclopiazonic acid
CRACs	Ca-release-activated Ca-channels
Cx	Connexin
DAG	Diacylglycerol
DDT	Dithiotheridol
DMSO	Dimethylsulphoside
ECL	Enhanced chemiluminecence

EGTA	Ethylene glycol bis (β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
GTP	Guanine triphosphate
h	Hour
HEPES	N-2-hydroxyethylpipeperazine-N'-2-ethanesulphonic acid
Hz	Hertz, frequency
ICC	Interstitial cell of Cajal
$I_{Cl(Ca)}$	Calcium-activated chloride currents
IICR	IP_3-induced Ca release
IK_{Ca}	Intermediate-conductance K_{Ca}
ILK	Integrin-linked kinase
IP_3	Inositol (1,4,5)-tris-phosphate
IP_3R	Inositol (1,4,5)-tris-phosphate receptor
K	Potassium
[K]	Potassium concentration
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	Calcium-activated potassium channel
kDa	KiloDalton
KCl	Potassium Chloride
K_{IR}	Inward rectifier potassium channel
K_V	Voltage-dependent potassium channel
l	litre, volume
L-Type	Calcium channel
M	Molar, concentration
MAP	Mitogen-activated protein
MBS	Myosin binding subunit
MEK	MAP kinase kinase
mg	milli-gram
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
min	minute, time

ml	millilitre, volume
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MLC_{20}	Regulatory 20-kDa of myosin light chain
mm	millimetre, length
mM	millimolar, concentration
mol	moles
mN	millinewton
$MnCl_2$	Manganese Chloride
mRNA	Messenger ribonucleic acid
ms	millisecond, time
mV	millivolt
n	Sample size
N	Newton
mA	milli Amp
Na	Sodium
Na/Ca exchange	Na/Ca pump
NaCl	Sodium Chloride
NaF	Sodium Fluoride
Na/K ATPase	Na/K adenosine triphosphatase
NaOH	Sodium Hydroxide
nM	nanomolar
NO	Nitric Oxide
P	Probability
pH	-log of hydrogen concentration
PG	Prostaglandin
pH_i	Intracellular pH
PIP_2	Phosphatidylinositol-4, 5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PMCA	Plasma membrane Ca-ATPase

pS	pico-Siemens
PP1	Type 1 myosin light chain phosphatase
PP2A	Type 2A myosin light chain phosphatase
RyR	Ryanodine receptor
RNA	Ribonucleic acid
ROCCs	Receptor-operated Ca channels
ROK	Rho-associated kinase
s.e.m	Standard error of the mean
Ser	Serine
SERCA	Sarcoplasmic reticulum Ca-ATPase
SK_{Ca}	Small-conductance K_{Ca}
SOCCs	Store-operated Ca channels
SR	Sarcoplasmic reticulum
STIC	Spontaneous transient inward current
STOC	Spontaneous transient outward current
TBS	Tris-base saline
TCA	Trichloroacetic acid
TEA	Tetraethylammonium
Thr	Threonine
TrpC	Transient receptor protein canoncial
T-type	Ca channel
µl	micro litre
µm	micro metre
µM	micro molar
VOCCs	Voltage-operated Ca channels
W	Watt
w/v	Weight per volume
Y-27632	ROK inhibitor

Chapter1

General Introduction

Chapter 1

General introduction

1.1The uterus

The activity of the smooth muscle of the pregnant uterus is controlled and modulated, so that it is relatively quiescent before term and then contracts powerfully at term. For a long time the mechanisms of the control and modulation of uterine contraction has been one of the classical objectives of physiological and clinical research, and this research area continues to fascinate scientists and clinicians. Though there is much interest in gaining a better understanding of how this control and modulation is achieved, much still remains unclear. The work described in this thesis was carried out to investigate some of the unanswered questions.

1.1.1 Anatomy of the uterus

The uterus is part of the female internal genitals. It is a hollow and muscular organ, normally situated in the lesser pelvis between the urinary bladder and rectum. Its upper part open into the uterine tubes, one on each side; whereas the lower part continues into the vagina. The uterus harbours the foetus through gestation and expels it during parturition.

There are morphological differences between the uteri of different species (For review see Broderick & Broderick, 1990). In the rat and other rodents a duplex uterus can be found, where two horns stem from the cervix. In human there is a single uterus that is much larger and described as simplex. In all mammalian species the uterine

structure is similar, and the uterus can be divided into three regions, the fundus, body and the cervix. Three layers constitute the uterine wall: the outer serosal layer, the inner endometrium and the myometrium in between.

The main arterial supply to the whole uterus is through the uterine artery, a branch of the internal iliac artery. Each uterine artery gives numerous branches, which enter the uterine wall. Once the uterine arteries have penetrated the myometrium they branch and form the arcuate vessels. The uterine arteries are greatly enlarged during pregnancy to support the foetus and placenta (Vorherr, 1972).

The uterus has a rich supply of afferent and efferent nerves (Glenister, 1977). The sympathetic and parasympathetic efferents are transmitted by way of the hypogastric and pelvic plexuses. In general, it is thought that activity of the sympathetic nerves produces uterine contraction, whereas that of the parasympathetic nerves produces uterine relaxation, but the effects of the innervation are complicated by hormonal influence (Glenister, 1977).

1.1.2 Structure of the myometrium

The myometrium is responsible for the contractile activity of the uterus. It is composed mainly of smooth muscle. In the duplex uteri, the myometrium consists of two distinct layers, an outer longitudinal layer where the bundles of smooth muscle are orientated in the long axis of the uterus, and an inner circular layer where the bundles are arranged concentrically around the longitudinal axis of the uterus. In the human simplex uteri, the myometrium is more complex. It is often described as having four more or less distinct layers, the most internal layer composed mainly of longitudinal fibres, the next layer containing vascular components and bundles of the muscle running in all directions, then a circular muscle layer and finally a thin longitudinal muscle

bundle layer (Schwalm & Dubrauszky, 1966; Bannister & Dyson, 1995). Recently, microanatomy of pregnant human myometrium has been Contraction of the myometrium results in shortening of the uterus and narrowing the size of the cavity (Broderick & Broderick, 1990).

1.1.3 Uterine myocyte ultrastructure

Uterine smooth muscle cells are typical of any smooth muscle cells. They are long, spindle shaped cells with tapering end (Broderick & Broderick, 1990). The cells are from 5 to 10 μm in diameter and from 300 to 600 μm in length (Broderick & Broderick, 1990). They are surrounded by connective tissues: collagen, elastin, glycoproteins and proteoglycans. These connective tissue elements are believed to contribute to the distribution of force development during contraction (Broderick & Broderick, 1990). To accommodate the growing of the foetus during pregnancy the myocytes become much enlarged, and the number of their gap junctions (see section 1.4.1.2) increasing greatly, indicating increased co-ordination of their contractility, and the amounts of connective tissue also increase. This is under the influence of hormonal factors (Broderick & Broderick, 1990).

Like other eukaryotic cells, the uterine myocytes plasma membrane shows the trilaminar appearance and measures approximately 8 nm in thickness. Several specialisation structures have also been found; i.e. the numerous vesicular inpocketings of the membrane known as caveolae, and the formation of cell-to-cell communications in the form of gap junction (Broderick & Broderick, 1990). Myometial cells contain a variety of other important intracellular organelles that contribute to contraction including the sarcoplasmic reticulum and mitochondria.

The myometrial sarcoplasmic reticulum (SR) is a well-developed organelle. It is responsible for the physiological regulation of cytoplasmic calcium in the cells by acting as a source and sink of calcium (Horowitz *et al.* 1996b). It occupies from 1.5 to 7.5 % of the smooth muscle cell volume (Broderick & Broderick, 1990). In the uterus, the SR can be found both close to the cell membrane (peripheral SR) and throughout the cytoplasm (central SR). It is reported to increase in pregnancy (Somlyo, 1985; Broderick & Broderick, 1990). Regions where the peripheral SR approaches the plasma membrane are called surface couplings. The gap between these two important structures is from 10 to 18 nm (Broderick & Broderick, 1990).

Mitochondria occupy 3 to 9% of the smooth muscle cell volume (Broderick & Broderick, 1990). In uterine cells, some mitochondria are clustered in the region near the nuclear pole whereas others are scattered through out the cytoplasm (Broderick & Broderick, 1990). It has been reported that they are closely associated with the sarcoplasmic reticulum and caveolae (Somlyo *et al.* 1974). Mitochondria are the site of oxidative metabolism in uterine smooth muscle but they may also have a role in the regulation of cytoplasmic calcium (Bond *et al.* 1985; Broderick & Somlyo, 1987).

1.2 Uterine plasmalemmal ion channels and pumps

1.2.1 Ion channels

Ion channels play an important role in the regulation of ion flow in and out of cells. In the myometrium, calcium (Ca), sodium (Na), potassium (K), and chloride (Cl) channels contribute to the regulation of the resting potential and the action potential. In addition, ion channel activity contributes both directly and indirectly to the regulation of intracellular free Ca ($[Ca]_i$), which affects the contractile apparatus and influence contraction and relaxation mechanisms. In this section I will describe the channel general properties and what is known in the uterus.

1.2.1.1 Ca channels

The classification of these channels is based on the way in which they are activated (McFadzean & Gibson, 2002). Voltage-operated Ca channels (VOCCs) are the channels that can be activated in response to a change in membrane potential. Also present in smooth muscles are two types of calcium-permeable channels that are non-voltage-operated, including the so-called receptor-operated Ca channels (ROCCs), activated by agonists acting on a range of G-protein-coupled receptors, and store-operated Ca channels (SOCCs), activated following depletion of the Ca stores within the sarcoplasmic reticulum. As for other smooth muscle cells, these channels have been reported in the myometrium (Sanborn, 2000; Tribe, 2001; Tribe *et al.* 2002).

Voltage-operated Ca channels

In neurons there are six subtypes of VOCCs: L-, N-, P-, Q-, R-, and T-type, based on the basic of their electrophysiological properties (Catterall, 1998). A number of VOCCs are expressed in the myometrium, including L-type ("L" for large and long

lasting") and T-type ("T for tiny and transient") Ca channels (Sanborn, 2000). L-types have a long lasting current when Ba is the current carrier, with a high voltage activation and a slow voltage dependent; inactivation T-type channels are so called because they carry a transient current, with a low voltage of activation and rapid inactivation (Young *et al.* 1993). The L-type Ca channel is considered to be the major Ca entry pathway in the uterus (see below) (Sanborn, 2000). The L-type channel is composed of a pore-forming subunit (α_1) with several auxiliary subunits (i.e., α_2, δ, β, γ (Klockner, 1996). The α_1 subunit also acts as the binding site for the channel blockers (Kass *et al.* 1991). During pregnancy, there is a progressive increase in channel subunit mRNA and/or channels density in rat myometrium (Tezuka *et al.* 1995).

The L-type Ca channel is activated by membrane depolarisation and inhibited by Ca channel blockers, with the dihydropyridines being the most effective and selective (Hollingsworth & Downing, 1988). All spontaneous activity in the myometrium is abolished if dihydropyridines such as nifedipine are applied to the uterus. Agonists initiate L-type calcium channel opening by inducing membrane depolarisation via activation of the non specific cation entry, inhibition of the K channel (Mironneau, 1994) and/or activation of the Cl channel (Arnaudeau *et al.* 1994). Furthermore, agonists may also open L-type Ca channels directly or indirectly through GTP-binding proteins in the absence of membrane depolarisation (Wray, 1993).

Receptor-operated Ca channels

These are located in the plasma membrane, and have binding sites for specific agonists, such as a neurotransmitter, a hormone or a paracrine substance. The channels open in response to the binding of an agonist to specific channel-associated receptors. There are two ROCCs activated solely by agonists. One is activated by ATP (Benham,

1989) and the other by acetylchlorine (ACh) (Inoue *et al.* 1987). They are both permeant to Na, K, and Ca and inhibited by the dihydropyridines. It has been noted that activation of ROCCs can change membrane potential and thus in turn activates or inhibits VOCCs (Wray, 1993).

Store-operated Ca channels

In many cell, the release of Ca from intracellular stores or the depletion of intracellular stores (see section 1.3) is coupled to activation of a Ca entry pathway (Putney & Ribeiro, 2000). This is known as store operated Ca entry or capacitative Ca entry (CCE). A recent report has proposed that members of the Trp family may involve the molecular entity responsible for CCE (Birnbaumer *et al.* 1996). Mammalian TrpC (Transient receptor protein canoncial) proteins have been cloned from a number of species; full or partial sequences are available for human TrpC 1, 3, 4, 5 and 6 (Birnbaumer *et al.* 1996; Zhu *et al.* 1996; Hofmann *et al.* 1999). Recently, the evidence of hTrpC1 mRNA, several splice variants of hTrpC1, and several other TrpC forms has been reported in a human myometrial cell line (Gupta *et al.* 1999; Dalrymple *et al.* 2002). Several findings also provide evidence for CCE operated either by releasing or depleting the stores in human myometrium (Monga *et al.* 1999, Tribe *et al.* 2002). These data are likely to be supported by the finding of TrpC protein expression in human myometrial cells (see above). However, it has been noted that the presence of TrpC proteins does not prove CCE exist, as individual TrpC proteins exhibit only some of the properties of CCE (Sanborn, 2000).

1.2.1.2 K channels

These channels contribute to repolarisation after an action potential and may play a role in setting the resting membrane potential. The electrochemical gradient for K ions is such that opening of K channels results in diffusion of this cation out of the cells and membrane hyperpolarisation. Closure of K channels has the opposite effect. Four major types of K channels have been recorded in smooth muscles (Nelson & Quayle, 1995), including voltage-dependent potassium (K_V) channels, calcium-activated potassium (K_{Ca}) channels, inward rectifier potassium (K_{IR}) channels, and ATP-sensitive potassium (K_{ATP}) channels. The channel subtypes have been characterised, based on their physiological and pharmacological properties. For example, three different subtypes of K_{Ca} have been described, large-conductance K_{Ca} (BK_{Ca}), intermediate-conductance K_{Ca} (IK_{Ca}), and small-conductance K_{Ca} (SK_{Ca}). A number of specific K channels and K currents have been described in myometrium, including BK_{Ca} and K_{ATP} channels (see below), a subtype of the K_V channels (Knock *et al.* 1999; Knock *et al.* 2001), a slowly activated delayed rectifier, the mRNA for which exhibits estrogen-dependent regulation, and the K_{IR} channels, the mRNA for which is highest in midpregnancy in rat uterus (Boyle *et al.* 1987; Folander *et al.* 1990; Pragnell *et al.* 1990; Lundgren *et al.* 1997).

BK_{Ca} Channels

The BK_{Ca} channel is one of the best-characterised and detected in myometrial cells from a number of species, including humans. Two other subtypes, IK_{Ca} and SK_{Ca} channels have not been described in any detail in the human myometrium. As with other smooth muscles, the myometrial BK_{Ca} channels comprise four α-subunits plus accessory β-subunit(s) (Khan *et al.* 2001). Both subunits are reported to be involved in

determining Ca sensitivity of the channels, and are present in both non-labour and labour (Khan *et al.* 2001).

The BK_{Ca} channel is the predominant K channel type encountered in non-pregnant (Tritthart *et al.* 1991; Erulkar *et al.* 1993; Perez *et al.* 1993) and pregnant human myometrium (Anwer *et al.* 1993; Khan *et al.* 1993, 1997). The channels share many pharmacological and physiological features with BK_{Ca} channels described in brain and other smooth muscle tissues (Khan *et al.* 2001). They display a large single-channel conductance of around 200 pS, and are Ca and voltage-dependent. The channels are blocked by tetraethylammonium (TEA) ions, 4-aminopyridine, barium (Ba), scorpion toxins and paxilline (Khan *et al.* 2001). In addition to Ca and voltage, protein kinases also modulate the activity of BK_{Ca} channels via phosphorylation of the channel protein (Schubert & Nelson, 2001). Protein kinase C (PKC) inhibits the BK_{Ca} channel in smooth muscle, whereas either cAMP-dependent protein kinase A (PKA) or cGMP-dependent protein kinase (PKG) activate the channel. However, this has not yet been observed in the myometrium. BK_{Ca} channels also open in response to an increase in $[Ca]_i$ close to the plasmamembrane as a result of a spontaneous transient release of calcium from SR store (Bolton *et al.* 1999). The resulting simultaneous opening of up to 100 BK_{Ca} channels is seen as a spontaneous transient outward current (STOC) in voltage-clamped visceral and vascular cells (Benham & Bolton, 1986). The opening of BK_{Ca} channel can play an important role in the responses to a number of inhibitory hormones and transmitters that cause their open probability to increase, producing hyperpolarisation (Bolton *et al.* 1999).

Inhibition of BK_{Ca} channels increases contractile activity in rat and human myometrium and depolarises human myometrial cells in association with an increase in $[Ca]_i$ as a result of VOCCs (Anwer *et al.*1993). The channels can influence both resting

membrane potential and the shape and duration of the action potential in myometrium (Anwer *et al.* 1993).

The BK_{Ca} channel activities are gestational stage dependent. It has been reported that BK_{Ca} channels in the pregnant human uterus appear to lose their Ca and voltage dependence with the onset of labour, which may permit an increase in $[Ca]_i$ without hyperpolarisation (Khan *et al.* 1993, 1997). In addition, in rat myometrium, a down regulation of both mRNA and protein for the α-subunits of the BK_{Ca} channels has been suggested as possible mechanism whereby uterine excitability is enhanced at term (Song *et al.* 1999).

K_{ATP} Channels

Although these channels have not been measured directly in myometrium, their action has been implicated in myometrial function. It has been shown that K_{ATP} channels activators can depress human uterine contractions (Morrison *et al.* 1993), and the effects of depression were more potent in nonpregnant than in pregnant (Cheuk *et al.* 1993; Downing & Hollingsworth, 1993). In rat myometrium, K_{ATP} channels activity can be increased following metabolic inhibition using cyanide and the effect can be partially inhibited by a K_{ATP} inhibitor (Heaton *et al.* 1993). Because dystocia is associated with metabolic inhibition, it has been suggested that activation of K_{ATP} channels may be associated with this condition (Heaton *et al.* 1993).

1.2.1.3 Cl channels

Chloride channels are important elements for a variety of cell functions relevant to the survival of human beings, including cell volume regulation and control of resting

membrane potential. Recent reports have provided evidence for the presence of chloride channels in a number of different smooth muscle cells, including myometrial smooth muscle cells (Pacaud *et al.* 1991; van Renterghem & Lazdunski, 1993; Large & Wang, 1996; Arnaudeau *et al.* 1994). More than two chloride channel types have been reported to be expressed in smooth muscle; a calcium-activated chloride (Cl_{Ca}) channel and a volume-regulated chloride (Cl_{VR}) channel (Nelson *et al.* 1997; Yamazaki *et al.* 1998). The mechanisms by which Cl channels are activated include changes in membrane potential; changes in intracellular calcium ions, cAMP (adenosine 3':5'-cyclicmonophosphate) concentrations, and pH; cell swelling; extracellular ligans; or a combination of those signals. Activation of chloride channels in smooth muscle cells leads to depolarisation, increasing entry of calcium through VOCCs, and contraction.

Like BK_{Ca} channels, Cl_{Ca} channels are activated by increases in $[Ca]_i$, and several studied have proposed that these channels participate in depolarisation (Large & Wang, 1996). Under physiological conditions, activation of Cl_{Ca} channels produces inward current and membrane depolarisation that can activate VOCCs, Ca influx, and contraction. Inactivation of Cl_{Ca} current could cause membrane repolarisation and inhibition of Ca influx, leading to relaxation. However, other investigators have argued that Cl_{Ca} channels will have little effect on membrane potential because of the high density of BK_{Ca} channels and their large conductance (Nelson, 1998). Interest has recently been generated in Cl_{VR} channels. Nelson *et al.* (1997) demonstrated that Cl_{VR} channel blockers dilated and hyperpolarised myogenically active cerebral arteries, which supports a role for Cl_{VR} channels in regulation of resting membrane potential and myogenic tone. The contribution of Cl_{Ca} in different tissues (i.e. myometrium) will depend on relative BK_{Ca} density, BK_{Ca} calcium sensitivity (i.e. related to gestation) and most importantly channel localisation. The evidence for Cl_{Ca} channels in the uterus is

limited but Arnaudeau *et al* (1994) showed that oxytocin in rat cells appeared able to stimulate these channels, and other investigators also find their activation via voltage-gated Ca entry (Jones *et al.* 2002).

Calcium-activated chloride currents ($I_{Cl(Ca)}$) have been recorded in many types of smooth muscle and it has been proposed that this conductance may be involved in agonist-induced contraction and some forms of spontaneous activity in smooth muscle (Large & Wang, 1996). Some insight into the gating of $I_{Cl(Ca)}$ has been obtained by studying macroscopic currents, and in particular spontaneous transient inward currents (STICs). These are randomly occuring calcium-activated chloride currents which are triggered by the sporadic release of Ca ion from the sarcoplasmic reticulum (Large *et al.* 1992).

1.2.1.4 Other ion channels

Na channels

Sodium channels have been detected in pregnant human and rat myometrium by some investigators (George *et al.* 1992; Young & Herndon-Smith, 1991; Sperelakis *et al.* 1992; Boyle & Heslip, 1994). They show a voltage-dependent inward current that is transient and sensitive to tetrodotoxin. The channels density is increased before parturition in the rat and may contribute to the rapid depolarisation of the membrane leading to Ca entry (Young & Herndon-Smith, 1991; Sperelakis *et al.* 1992).

Stretch-activated channels

Channels activated by stretch of the membrane have been reported in smooth muscles (Bolton *et al.* 1999). It is believed that the channels activation allow cations to pass, and their opening is facilitated by hyperpolarisation (Hisada *et al.* 1993).

However, some cation channels are inactivated by stretching (Wellner & Isenberge, 1993).

In the uterus stretch is not only an important stimulus for myometrium growth, but also a contractile stimulus (Wray, 1993). Loss of stretch after parturition appears to be the principal factor controlling initiation of uterine involution after parturition (Wray, 1982). The mechanism underlying stretch is not fully understood in the uterus, but it is believed that Ca or Na enter the cell through stretch-activated ion channels (Shmygol *et al*. 2002).

1.2.2 Plasma membrane pumps and exchanges

1.2.2.1 Na/K-ATPase

The Na/K-ATPase (Na/K adenosine triphosphatase) or sodium pump is present in the membrane of animal cells and represents a major pathway for Na and K transport across the plasma membrane. The inward movement of Na ions and the outward movement of K ions are passive and the reverse movements against the electrochemical gradients require the activity of the Na/K-ATPase (Rakowski *et al*. 1989). The sodium pump moves 3Na ions out of the cell at the same time as moving 2K ions into the cell. Thus, the pump is electrogenic; i.e., there is a net transfer of charge from one side of the membrane to the other (Rakowski *et al*. 1989).

The Na/K-ATPase is a member of the P-type ATPase pumps. That is, it transfers the energy of ATP to a high energy phosphorylated intermediate (aspartyl residue), the energy of which is then used in the ion transport step. The pump is composed of two subunits, α and β (Blanco & Mercer, 1998). The α subunit binds ATP and both Na and K ions, and contains the phosphorylation site. The smaller β subunit is necessary for activity of the complex. It appears to be critical in facilitating the plasma membrane

localisation and activation of the α subunit. Turi *et al.* (1992) examined the Na/K-ATPase activity and the expression of mRNAs encoding α and β subunits in the rat myometrium. The enzyme activity appeared to increase during pregnancy and reached the highest value at the 17th day. The expression of α1, α3 and β mRNAs was detectable, but not α2 mRNA. Like the enzyme activity, the expression of all three mRNAs increased with pregnancy. Furthermore, the expression of mRNA-β sharply decreased after the 17th day, while the level of α subunit mRNA barely changed. Unlike the rat myometrium, three α isoforms of the sodium pump have been found, and related to preeclampsia in human myometrium (Maxwell *et al.* 1998). There was a significant reduction of α2 mRNA level in myometrium of women with preeclampsia compared with normotensive pregnancies. This reduced sodium pump expression in preeclampsia may raise cell sodium, and thereby stimulate the Na/Ca exchange (see below), increase pressure sensitivity, or increase tone directly, which may contribute to hypertension in preeclampsia (Maxwell *et al.* 1998).

The sodium pump is regulated by several factors, including the intracellular Na concentration and a variety of hormones (Therien & Blostein, 2000). Most of the hormones that regulate the pump do so through signaling mechanisms that modulate the activities of a group of protein kinases, phospholipase, and phosphatases (Therien & Blostein, 2000). The activity of the pump can be either inhibited or activated by protein kinases depending on cell/tissue types. Phospholipase inhibits the activity of the sodium pump, whereas phosphatases activate its activity. Whether the hormones make a contribution to the sodium pump activity in the myometrium is still controversial. Indeed, it has been reported that the activity of the sodium pump is not dependent on the hormonal status of the rat myometrium (Missiaen *et al.* 1988). However, when the rat myometrial strips pretreated with estrogen and progesterone were incubated with

prolactin, there was a significant increase in the density of the sodium pump (Lipton *et al.* 1978). Because the sodium pump is the major determinant of cytoplasmic Na, it has an important role in regulating cytoplasmic Ca levels, through Na/Ca exchange (see below).

1.2.2.2 Na/Ca exchange

The Na/Ca exchange protein is a bidirectional, electrogenic ion antiporter that couples translocation of three Na ions against one Ca ion (Reuter, 1991; Philipson & Nicoll, 1994). This transport is driven by transmembrane electrochemical gradients, particularly the Na gradient (Blaustein & Lederer, 1999). In rat myometrium, Taggart & Wray (1997) reported that the exchange plays an important role in regulating $[Ca]_i$, by extruding Ca from the cell in the forward mode and by mediating Ca entry in its reverse mode. Na/Ca exchange proteins have various isoforms and are expressed in several cell types (Reithmeier, 1994), including the myometrium (Grover *et al.* 1981; Tribe *et al.* 2000).

1.2.2.3 Plasma membrane Ca-ATPase

Like the Na/K ATPase, the plasma membrane Ca-ATPase (PMCA) is a P-type ATPase. A structural model for the PMCA has been proposed, mostly based on the model for the Ca pump of the sarcoplasmic reticulum Ca-ATPase family (SERCA, see section 1.3.1.2). The pump contains a Ca binding region, an ATP binding region, and an aspartyl residue which is the phosphorylation site common to all P-type ATPases (Pederson & Carafoli, 1987). The pump uses energy from ATP to pump Ca up the steep electrochemical gradient from cytosol to extracellular space. This pump is thought to be electro neutral, because the Ca pumped to the extracellular space is exchanged for two

protons (Parkington & Coleman, 1990). Thus Ca extrusion results in uptake of a proton, and this has to be compensated for by transporters such as Na/H exchange.

The PMCA was first cloned by Shull & Greeb (1988), and there are four human isogenes (PMCA1-4), and also numerous spice variants (Carafoli, 1994). The PMAC isolated from the sarcolemal sheets of human myometrium appears very similar to the well-known PMCAs of erythrocyte membrane, heart sarcolemma or axolemma (Popescu & Ignant, 1983). Interestingly, PMCA protein was found to significantly increase in myometrium of women in labour compared with those not in labour (Tribe *et al*. 2000).

1.3 Intracellular Ca stores

1.3.1 The SR

Storage and release of Ca of cellular organelles is important for physiological regulation and signalling. Like other smooth muscles, the myometrial SR is capable of Ca uptake, storage, and specialised release. Calcium concentration in the SR is primarily determined by the balance between the release of calcium from the SR (either stimulated or spontaneous), via the SR release channels, and the reuptake, via the SR Ca-pump, of calcium back to the SR.

1.3.1.1 The SR Ca release channels

The SR has two different types of Ca release channels, i.e. ryanodine receptors (RyRs) and inositol 1, 4, 5-trisphosphate receptors (IP_3Rs). In general, both release channels mobilise Ca from the same internal store.

Ryanodine receptors

The RyR is so called due to the sensitivity of the receptor to ryanodine. Cytoplasmic Ca activates RyR channels, and thus they are referred to as Ca-induced Ca release (CICR) channels. At least three isoforms of RyRs have been cloned (RyR1-RyR3). RyR2 and RyR3 are the most widely expressed in smooth muscle but all three isoforms are found in some smooth muscles, including the uterus (Martin *et al.* 1999b). It has been reported in human myometrium that the mRNA RyR3 isoform was found in both non-pregnant and pregnant myometrial tissue samples whereas the mRNA RyR2 was found only in pregnant samples (Awad *et al.* 1997). The single channel properties of the three isoforms of RyR appear to be rather similar. They can be activated by sub-micromolar $[Ca]_i$, to form a large conductance channel, and can be inhibited by Mg and

millimolar $[Ca]_i$ and modulated by ryanodine. There are however reported differences between them, for example in their caffeine sensitivity. RyR1 and RyR2 are activated by caffeine (Meissner, 1994), whereas RyR3 is reported to be caffeine-insensitive (Giannini *et al.*1992; Murayama & Ogawa, 1996).

IP_3 receptors

Stimulation by a variety of agonists binding to G-protein-coupled receptors in smooth muscle results in activation of phospholipase C (PLC) and metabolism of phosphatidylinositol phosphate to IP_3. IP_3 activates Ca release via Ca release channels, known as IP_3 receptors. They are also referred to as IP_3-induced Ca release (IICR) channels.

Three IP_3R isoforms (IP_3R1-IP_3R3), which share up to 70% amino acid similarity, have been found. Each isoform is believed to assemble into tetrameric complexes of either the same or different subunits to form the channel (Patel *et al.* 1999). All IP_3Rs present three major functional domains; a large N-terminal domain, which lies freely in the cytoplasm with the IP_3 binding site located at its end, a short C-terminal hydrophobic domain, which anchors the protein in the membrane with four of the subunits combining to form the functional IP_3R channel, and an intervening regulatory domain, which includes phosphorylation sites and sites to which modulators such as ATP, Ca and calmodulin can bind (Patel *et al.* 1999). The receptor isoforms are differentially expressed in different cell types (Wojcikiewicz, 1995), within different subcellular locations (Nathanson *et al.*1994), and at specific developmental stages (Nakagawa *et al.* 1991). In human and rat myometrium, three IP_3R isoforms are present (Morgan *et al.* 1996; Crumb *et al.* 1997). In the rat myometrium, the levels of

expression increased, especially for the IP_3R3 isoforms, during the second half of gestation (Crumb *et al.* 1997).

In most instances, Ca release from IP_3R is triggered by IP_3 (see above). The opening of IP_3R is also modulated by Ca and in turn Ca release from IP_3R may stimulate RyR. Potentiation of openings of both IP_3R channels and RyR channels provides the possibility of interactions between Ca release mechanisms. If these channels are located close to each other in the SR membrane, then it is possible for release of Ca from one to stimulate release from the other. This interaction can be amplified by agonists that enhance IP_3 levels, and under some conditions can lead to regenerative Ca waves (Iino, 1999). However, the spatial distribution of IP_3R and RyR may account for differences in the responses of smooth muscle to stimulation (Young & Mathur, 1999).

Regulation of the IP_3R is complex and involves multiple regulatory proteins acting either directly or via intermediary proteins. The IP_3R has been shown to be regulated by several factors including PKA, PKG, PKC, Ca/calmodulin-dependent protein kinase II (CaM kinase II), and ATP (Patel *et al.* 1999). Changes in pH are important for IP_3 actions, increases in intracellular pH (pH_i) augment IP_3 ability to release Ca (Joseph *et al.* 1989).

1.3.1.2 SR Ca-pump

The SR membrane is not freely permeable to Ca. The SR Ca-ATPases, known as SERCA pumps, exist in the membrane and pump Ca into the SR. To translocate Ca from the cytoplasm to the lumen of the SR, SERCA pumps utilise the energy from ATP hydrolysis.

The pumps generate and maintain about a 10,000-fold Ca gradient between the SR lumen and the cytoplasm. After Ca is pumped into the SR, it is buffered by proteins, such as calreticulin and calsequestrin. These proteins can bind large amounts of Ca. As a result of high-affinity Ca uptake and intraluminal SR buffering, the actual Ca store is estimated to reach Ca concentrations of 10-15mM (van Breemen & Saida, 1989).

Three genes encode SERCA pumps; SERCA1, SERCA2, which gives rise to the SERCA2a and SERCA2b isoforms, and SERCA3 (Lytton *et al.* 1992). The SERCA2 and SERCA3 are inhibited by thapsigargin and cyclopiazonic acid (Darby *et al.* 1993). Most smooth muscles express SERCA2a (Lytton *et al.* 1989). However, in human myometrium, SERCA2a and SERCA2b are expressed and increased with pregnancy (Tribe *et al.* 2000).

The pump contains three major domains; a transmembrane domain containing the high affinity Ca binding site, a large cytosolic head which contains the ATP binding site, and the aspartyl residue which is the phosphorylation site common to all P-type ATPases, and a stalk region which links the membranous region to the cytosolic head (MacLennan *et al.* 1985; Brandl *et al.* 1986; Green & MacLennan, 1989). Unlike the PMCA pump, the SERCA does not have a calmodulin binding site but can be regulated by phospholamban, a small transmembrane protein found in the SR. Phospholamban control of the SR Ca-ATPases has been well studied in cardiac muscle, but not in smooth muscles (Inui *et al.* 1986). In the unphosphorylated form, it inhibits the SERCA. When phospholamban is phosphorylated by PKA, CaM kinase II, or PKC, the inhibition is relieved. The level of phospholamban in smooth muscles is much lower than in cardiac muscle; it is thought to correlate with the amount of the SERCA which is also much lower than in cardiac muscle (Raeymaekers & Jones, 1986).

1.3.2 Other sources

Mitochondria. Mitochondria can accumulate massive amounts of Ca, especially when there is sufficient inorganic phosphate (Carafoli, 1987). Isolated mitochondria can take up 100 nmol Ca /mg mitrocondrial protein (Carafoli, 1975). However, under *in vivo* conditions, mitochondria are likely to contain very much less (e.g.1 nmol/mg) (Carafoli, 1987). In the uterus, there is no evidence that the fluxes of Ca across the mitochondria make contributions to excitation-contraction coupling, and it has been proposed that mitochondria play only a minor role in Ca movements (Smith, 1996). However, recent studies in other smooth muscles have shown such a role for mitochondria in the regulation of $[Ca]_i$ (McCarron & Muir, 1999). Mitochondrial Ca transport may be important for increasing metabolism, to meet metabolic demands. In addition, in severe Ca overload, mitochondria may provide a temporary Ca store to protect the cytoplasm from very high Ca level (Kosterin *et al.* 1994).

Inner sarcolemmal surface. The surface of the sarcolemmal membrane facing the cytoplasm can also bind substantial Ca. In the myometrium, the Ca binding to these sites can be released upon acidification (Grover *et al.* 1983).

1.4 Excitation-contraction coupling

Smooth muscle contraction follows excitation at the plasma membrane. The link between these two events in excitation-contraction coupling is the rise in $[Ca]_i$. Excitation-contraction coupling in smooth muscle can be said to occur by two mechanisms; electromechanical and pharmacomechanical coupling (Somlyo & Somlyo, 1994). During the electromechanical coupling process, the primary drive for the rise in $[Ca]_i$ (and thus contraction of the muscle) is membrane depolarisation, with the consequential opening of VOCCs. Conversely, pharmacomechanical coupling does not depend on changes in membrane potential (although changes may occur). Rather, the rise in $[Ca]_i$ is brought about by a combination of calcium release from intracellular stores and calcium entry through VOCCs via activation of ROCCs.

1.4.1 Electromechanical coupling

An increase in $[Ca]_i$ is essential for the initiation of contraction in smooth muscles, including the myometrium. This calcium may be released from stores within the cell, but the most important source of calcium for contraction is from the extracellular space (see section 1.5.1). At the resting membrane potential, the influx of calcium is very small, but increases dramatically as the membrane becomes polarised (Parkington & Coleman, 1990). Thus, the membrane potential plays a crucial role in determining the level of contractility in uterine smooth muscle. In this section I will describe the electrical activity, underlying electromechanical coupling of the myometrium. Especially, I will focus on the spontaneous contractile activity, action potential, and coordination of contractile activity.

1.4.1.1 Electrical activity

Smooth muscles are often devided into the two categories; phasic and tonic smooth muscle (Horowizt *et al.* 1996a). Phasic muscles are referred to as those smooth muscles that maintain tone poorly, have relatively high shortening velocities, and are capable of displaying regenerative action potential. In contrast, tonic smooth muscles do not generally display action potentials or regenerative electrical activity under physiological conditions. They have slower shortening velocities but more effectively maintain tone.

The myometrium is a phasic smooth muscle. It is spontaneously active, and produces regular spontaneous contractions without the need for any nervous or hormonal stimulation. Changes in the myometrial membrane potential are fundamental to the control of uterine contractility. The spontaneous contractions are preceded by action potentials, and agonists can alter the contractions by affecting the frequency and duration of the action potentials (Wray, 1993). The basis of this myogenic mechanism is not known. Specialised pacemaker cells have been hypothesised as the initiators of activity (Poli *et al.* 1990), but have not been characterised (Wray, 1993). It has been reported that changes in the permeability of the membrane potential to a decrease in K and an increase in Na occur to give the slow depolarisation of the membrane potential preceding the action potential (Kuriyama & Suzuki, 1976; Parkington & Coleman, 1990). In gastrointestinal cells, many studies indicate that interstitial cells of Cajal (ICC) are the pacemaker cells that generate spontaneous electrical depolarisations, also known as slow waves (Langton *et al.* 1989; Sanders, 1996; Dickens *et al.* 1999). From ICCs, slow waves spread passively via gap junctions to neighbouring smooth muscle cells where depolarisation activates voltage dependent, L-type Ca channels (Horowitz *et al.* 1999).

The resting membrane potential of uterine smooth muscle is in the range -35 to –65 mV. In human, it has been shown to be in the rage -45 to –50 mV (Nakajima, 1971; Parkington & Coleman, 1990). The resting membrane potential in the rat becomes less negative with increasing gestation (Inoue *et al.* 1990), and falls to non-pregnant values after delivery (Casteels & Kuriyama, 1965). There is also a high degree of variable in the size and shape of the action potential, depending on the gestational state and species; it may be a simple spike or have a plateau. Generally, the upstroke of the action potential is due to an influx of Ca ions via VOCCs (Miyoshi *et al.* 1991), and repolarisation is due to inactivation of the Ca channels (Shmygol *et al.*1997) and K efflux (Miyoshi *et al.* 1991). Both Ca channels and K channels thus play an important role in myometrial force production (see section 1.2).

1.4.1.2 Gap junctions

For a multicellular muscle to contract as an organ, the contraction of individual cells must be coordinated by sequential depolarisation of cells. Recently, a model for human myometrial cell-to-cell communications has been proposed (For review see Young, 2000). Gap junctions are considered to be the low-resistance pathway between individual myometrial cells that allow this to happen (Cole *et al.* 1985). They are thought to function in the initiation of labor by allowing the propagation of electrical impulses throughout the myometrium, thus facilitating the synchronous muscle contractility of labor. During gestation, this coordination is far less effective than during labor, allowing gestation to be maintained.

Gap junctions are composed of a hexameric assembly of integral membrane proteins (connexins) arranged symmetrically around a central aqueous pore (Yeager & Nicholson, 1996). To date at least 18 connexin family proteins have been detected in

mammals, either on a protein or an mRNA level, including connexin-43 (Shibata *et al.* 2001). Connexin-43 (Cx-43), a 43 kDa protein, is the principal component of myometrial gap junctions (Lye *et al.* 1993). This protein is particularly abundant, but exhibits temporally distinct patterns of expression within the pregnant myometrium. The expression of Cx-43 is low throughout most of pregnancy, but increases dramatically immediately before the onset of labour, following by a rapid loss of the protein after delivery (Lye *et al.* 1993). Gap junctions appear to be under hormonal control (steroids and prostaglandins). The expression of Cx-43 is increased by estrogen and prostaglandins, but decreased by progesterone (Risek *et al.* 1990, Chow & Lye, 1994). Estrogen increases the expression more in circular than in longitudinal muscle. At parturition, however, Cx-43 expression is evenly distributed and present in both muscle layers of the rat uterus (Risek *et al.* 1990, Risek & Gilula, 1991), suggesting that widespread myometrial expression of gap junctions is important to parturition.

There is also evidence that stretch of the uterine wall also affects gap junction permeability, probably acting synergistically with estrogen (Wathes & Porter, 1982). Gap junctions can be regulated by phosphorylation processes via several protein kinases (Lampe & Lau, 2000). It has been reported that, for example, activation of PKA can enhance gap junction coupling in cardiomyocytes (Dhein, 1998). However, decreases in myometrial gap junction permeability were found in the presence of PKA or phosphodiesterase inhibitors (Cole & Garfield, 1986). The gap junction can also be regulated by small ions like protons, Ca and Na. These small ions have been reported to decrease cell-to-cell communication in cardiomyocytes (Dhein, 1998). The gap junction pores are closed by an increase in $[Ca]_i$ (mM) and $[Na]_i$, and by a decrease in pH_i. At the present, the regulation of these ions is still questionable in the myometrium.

1.4.2 Pharmacomechanical coupling

Pharmacomechanical coupling is the mechanism by which activation of agonists, such as neurotransmitters, hormones, and drugs can lead to contraction of the smooth muscles, through G-proteins and second messengers, without a change in membrane potential. The major mechanisms of pharmacomechanical coupling are Ca release by IP_3 generated by the phosphatidylinositol cascade and modulation of the sensitivity to Ca of the contractile proteins or their regulatory mechanisms.

The mechanism is started by the binding of agonist to its receptor at the plasma membrane. This activates a G-protein that stimulates PLC to cleave phosphatidylinositol-4, 5-bisphosphate (PIP_2) in membrane into IP_3 and diacylglycerol (DAG). IP_3 then causes Ca to be released from internal store, and thus $[Ca]_i$ is increased. DAG stimulates PKC and itself further hydrolysed to phosphatidic acid and arachidonic acid.

Binding of agonist to its receptor at the plasma membrane can also change membrane potential. Depolarisation can be induced by ROCCs (see section 1.2). This can activate VOCCs, and can have a strong impact on $[Ca]_i$.

The effects of agonists on contraction need not necessarily be due to changes in Ca concentration. Changes in the Ca sensitivity of the contractile proteins or their regulatory mechanisms (described in the subsequent section) can be brought about by agonists. In the uterus, however, agonist-induced contraction is usually characterised by an increase in the duration and/or frequency of spontaneous phasic activity and thus action potential alterations appear likely to have led to increased $[Ca]_i$ (Wray, 1993). In addition, the uterus belongs to that of phasic smooth muscles that may be less sensitive to cytosolic Ca than those of tonic smooth muscles (Somlyo & Somlyo, 1991).

1.5 Mechanisms of uterine contractions

The basis regulation of smooth muscle contraction

A rise in $[Ca]_i$ is the major trigger for the contraction of smooth muscle (Somlyo & Somlyo, 1994). This Ca is from the extracellular space via VOCCs or ROCCs, or from the SR via IP_3 or RyR receptor/Ca release channels. The elevation of $[Ca]_i$ leads to the binding of Ca to calmodulin (CaM). The Ca-CaM complex (four Ca ions per molecule of CaM) binds to myosin light chain kinase (MLCK) to form an active $(Ca)_4$-CaM-MLCK complex, and activates the enzyme. MLCK then phosphorylates the 20-kDa light chains of myosin (MLC_{20}) at Ser^{19} (Allen & Walsh, 1994). This phosphorylation leads to a conformational change in the myosin head that results in actin activation of myosin Mg-ATPase activity (Word, 1995). This phosphorylation reaction then triggers cycling of myosin cross-bridges along the actin filaments, which force development and shortening of the muscle (Walsh, 1991). Relaxation occurs following a return of $[Ca]_i$ to resting levels by extrusion of Ca from the cell by a sarcolemmal Ca pump or a Na/Ca exchange, or pumping of Ca into the SR by SERCA, resulting in dissociation of Ca from CaM, inactivation of MLCK and dephosphorylation of myosin catalysed by myosin light chain phosphatase (MLCP) (Hartshorne *et al.* 1998).

However, $[Ca]_i$ does not always parallel the extent of MLC_{20} phosphorylation and contraction. During tonic force maintenance, for example, the relationship between force and MLC_{20} phosphorylation levels can be modified or even dissociated (Somlyo & Somlyo, 1994). Thus, secondary regulatory pathways that can modify, independently of $[Ca]_i$, the activities of phosphorylating and dephosphorylating enzymes have been proposed to be functionally important in the control of smooth muscle contractility, and are referred to as Ca-sensitisation mechanisms (Somlyo & Somlyo, 1994). Several

mechanisms have been proposed to account for Ca-sensitisation including the regulation of PKC and the regulation of Rho-associated kinase (see section 1.5.4 and 1.5.7).

In addition to thick filament (myosin)-associated regulation of contraction, contractile regulation by thin filament-associated proteins (for example, caldesmon and calponin) has been proposed. It has been reported that they can alter myosin Mg-ATPase activity, possibly through their phosphorylation by mitogen-activated protein (MAP) kinase and/or other kinases (Somlyo & Somlyo, 1994).

The mechanisms of uterine muscle contraction are most likely the same as that of other smooth muscles (Barany & Barany, 1990). I will now describe the contractile process individually and schematically summarise the regulation of contraction in Fig.1.1-1.3.

1.5.1 Intracellular free calcium regulation

As mentioned in the above section, contraction of smooth muscle depends upon an increase in $[Ca]_i$. In the myometrium, there is clear temporal relationship between changes in $[Ca]_i$ and the development of force. It has been shown that $[Ca]_i$ rose and fell significantly before force during contraction and relaxation respectively (Szal *et al.* 1994; Taggart *et al.* 1996). The sources of calcium are 1) the plasmalemma, under the control of membrane depolarisation and agonists, and 2) the SR, controlled by second messengers. Generally, the concentration of calcium in the interstitial fluid is 10^{-3} M whereas inside the smooth muscle is 10^{-6} M in contraction and 10^{-7} M in relaxation. Thus, those sarcolemmal and SR membranes establish a calcium concentration gradient of about 10,000-fold. Most intracellular Ca is sequestered in organelles or bound to proteins and acidic phospholipids, only ~0.01% of total Ca is free (Smith, 1996). Studies using a fluorescent Ca indicator, fura-2, in human myometrial cells have shown

that $[Ca]_i$ in the resting smooth muscle to fall within the range of 80-120 nM and in the activated smooth muscle to be about 180-220 nM (Molnar & Hertelendy, 1990; Tasaka *et al.*1991). In the rat myometrial cells, the threshold for contraction was about 200 nM calcium with a peak contraction at 1,000-6,000 nM (Savineau *et al.* 1988).

1.5.1.1 Mechanisms for increasing in intracellular free Ca

In smooth muscle cells, four pathways exist at the level of plasma membrane for the entry of calcium in response to appropriate stimuli (Karaki *et al.* 1997). These include VOCCs, ROCCs, SOCCs, and the reverse mode of the Na/Ca exchange. As discussed already, in both human and rat myometrium calcium influx is predominately via VOCCs, L-type Ca channels, as dihydopyridine channel blockers such as nifedipine significantly attenuate or completely abolish spontaneous and agonist-induced contractions (Hollingsworth & Downing, 1988; Taggart *et al.* 1996; Parkington *et al.* 1999). At the level of the SR, activation of IP_3R and RyR mediate Ca release into the sarcoplasm. There is controversy, however, as to whether release of sarcoplasmic reticulum Ca plays a significant role in controlling uterine contractility. In the presence of pretreatment with a depolarising potassium solution, Luckas *et al.* (1999) have demonstrated that agonists, such as oxytocin, can elicit a single contraction in human term pregnant myometrium in the absence of extracellular calcium, suggesting that IP_3R plays a role in modulating agonist-induced contractions. In contrast, in the absence of a high potassium challenge, oxytocin does not stimulate a contraction in human term pregnant myometrium (Tribe, 2001). In the presence of extracellular Ca, a small contribution of SR-derived calcium to oxytocin-induced contractions is demonstrated as contraction amplitude is reduced by 14 % by depleting the SR Ca with an inhibitor of the SERCA (Tribe, 2001). However, it has been reported that the SR does not contribute

to spontaneous activity of the uterus as changes in the SR Ca cannot be detected during simultaneous measurements of changes in the SR and cytosolic [Ca] in rat uterine smooth muscle cells (Shmygol *et al.* 2001).

The release of Ca from the SR through RyR has been demonstrated in cultured pregnant and non-pregnant myometrial cells (Lynn *et al.* 1993; Morgan & Gillespie, 1995; Holda *et al.*1996). In non-pregnant myometrial cells oxcytocin-induced calcium transients are not inhibited by ryanodine (Holda *et al.* 1996), whereas Burghardt *et al.* (1999) show that RyR contribute to high frequency calcium oscillations induced by oxytocin in a pregnant human myometrial cell line. It has also been proposed that RyR may be important in the propagation of spontaneous and oxcytocin-induced calcium waves in cultured myometrial cells (Young & Zhang, 2001). In contracting myometrium, ryanodine enhanced contractions in 60 % of myometrium strips from term pregnant rats but not from non-pregnant rats (Taggart & Wray, 1998b). However, the effect of ryanodine on contracting pregnant human myometrium has not been elucidated.

1.5.1.2 Mechanisms for calcium removal

Contraction is terminated by a reduction in $[Ca]_i$. To maintain calcium homeostasis, calcium pumps move calcium against its concentration gradient across the plasma membrane and/or the membrane of the SR. The mechanisms responsible for this process are (see section 1.2.2 and 1.3.1.2); the PMCA which transports Ca out of the cell at the expense of ATP hydrolysis, the Na/Ca exchange which allows three Na ions to enter the cell in exchange for a Ca ion, and the SERCA which pumps Ca from the cytoplasm into the SR at the expense of ATP hydrolysis. The PMCA and Na/Ca exchange have been found in uterus of different species (Kosterin *et al.* 1994). It has

been suggested that both mechanisms made significant contribution to, and together were entirely responsible for, Ca extrusion from the uterine cell (Khan *et al.* 1997; Shmygol *et al.* 1998; Tribe *et al.* 2000). The mechanism responsible for removing calcium ions from the cytoplasm to the SR has recently been examined in pregnant rat myometrium (Shmygol *et al.* 1999). The above studies suggested that the SERCA contributes to the removal of intracellular Ca, but cannot function effectively in the absence of the PMCA and Na/Ca exchange.

An additional mechanism of Ca extrusion has been proposed (van Breemen *et al.* 1986). It was suggested that the SR might act as a 'superficial buffer barrier', by taking up a fraction of the Ca that enters the cell through the plasmalemma before it reaches the contractile machinery. This Ca is then released from the SR lumen into the narrow space between the SR and plasmalemma (referred to as 'vectorial Ca release'), from where the PMCA and Na/Ca exchange complete the extrusion process. Evidence supporting this hypothesis has been obtained in some smooth muscle cells (Chen & van Breemen, 1993; Petkov & Boev, 1996), including uterine smooth muscle cells (Shmygol *et al.* 1999).

Evidence from a variety of cell types suggests that mitochondria play an important role in calcium removal (Drummond & Fay, 1996; McGeown *et al.* 1996). Because mitochondria develop negative membrane potentials by extrusion of protons via the electron transport chain this can create a strong electrochemical gradient for Ca entry, and a Ca conductance in the inner membrane of mitochondria, the Ca uniporter, facilitates the uptake of Ca. It is, however, unlikely that mitochondria significantly contribute to the up take of Ca in uterine cells (Shmygol *et al.* 1999).

In addition to a reduction in $[Ca]_i$, another important mechanism for relaxation involves the activation of BK_{Ca} (Khan *et al.* 1997; Tribe *et al.* 2000; see also section

1.2.1.2), which results in membrane hyperpolarisation (Anwer *et al.* 1993; Khan *et al.* 1993). During labor, contractions cause uterine hypoxia, with a fall in pH_i and ATP concentration, and these metabolic changes prevent effective force production and lead to relaxation (Wray, 1993).

1.5.2 Proteins related to regulation of contraction

1.5.2.1 Contractile proteins

In uterine and all other smooth muscle cells, the two major contractile proteins have been described: thick and thin (Broderick & Broderick, 1990); myosin and actin respectively. Although the contractile proteins in the uterus and other smooth muscles do not have the orderly array seen in striated muscles, the same mechanism of contraction occurs due to the active sliding of thick and thin filaments relative to one another (Murphy, 1979).

Thick filaments

Myosin. Myosin is an ATPase enzyme with the unique property that the hydrolysis and release of inorganic phosphates and ADP is coupled to movement via a cyclic interaction between myosin and actin filaments (see below). Uterine myosin is approximately 15 nm in diameter and 160 nm in length. As in other smooth muscles it contains two heavy chains, each with a molecular weight of approximately 20 kDa. From the tail end, the two chains are wound around each other. The chains then separate to from the two heads. Each head contains a 20 kDa and a 17 kDa light chains. The head of myosin carries the ATPase and actin-binding sites. The 20 kDa is involved in the calcium dependent regulation of smooth muscle contraction (Kamm & Stull, 1985). It can be phosphorylated and dephosphorylated by specific enzymes (see section 1.5.2.1).

The role of the 17 kDa light chain in myosin is unknown (Bailin, 1986). Myosin has various forms that may reflect different biological activities. For example, it has been shown by two-dimensional gel electrophoresis that the 17 kDa myosin light chain of human and monkey uterus exist in two isoelectric forms, the more acidic one becoming progressively predominant at the end of pregnancy (Cavaille *et al.* 1986). Although change in myosin may accompany developmental and functional adaptation of muscle, it remains unclear whether changes in the 20 kDa light chain that also take place during pregnancy involved in the contractile activity of the muscle (Swynghedauw, 1986).

Thin filaments

Uterine thin filaments are about 7 nm in diameter and composed of filamentous actin (Barany & Barany, 1990). The length of the bundles has not been determined, but they are thought to extend for several micrometers along the cell length, branching and dividing along their length (Gabella, 1984).

Actin. A total of six actin isoforms have been identified, uterine smooth muscle contains α and γ forms (Barany & Barany, 1990). Mature uterus contains roughly equal quantities of α and γ isoforms; it has been suggested that the ratio of these two isoforms is related to whether the muscle is predominately phasic or tonic (Fatigati & Murphy, 1984). The isoform distribution changes during pregnancy, γ-actin increased early in pregnancy (Cavaille *et al.* 1986). In addition, it has been observed that α-actin mRNA is induced by estrogen (Hsu & Frankel, 1987).

Thin filament-associated proteins

Tropromyosin. Tropomyosin is a dimer made up with two peptides, each of which is virtually pure α helix, coiled around each other and is located in the grooves between

the two strands of the actin double helix. In smooth muscle, tropomyosin exists in two isoforms, α and β. The two isoforms are present in approximately equal quantities in uterus (Sanders & Millie, 1985). Uterine tropomyosin is not well identified but it is probably identical to other smooth muscle tropomyosins (Fatigati & Murphy, 1984).

Though the function of tropomyosin in smooth muscle is less clear, several lines of evidence suggest that tropomyosin functions to provide cooperativity in smooth muscle. Graceffa (1999) demonstrated that the binding of smooth muscle myosin heads to actin leads to the movement of smooth muscle tropomyosin in a highly cooperative fashion. Furthermore, it has been shown that the movement of smooth muscle tropomyosin by myosin binding is more easily facilitated by phosphorylated myosin than unphosphorylated myosin (Graceffa, 2000). Finally, tropomyosin is necessary for full inhibition of actomyosin ATPase activity by caldesmon (Chalovich *et al.* 1987; Smith *et al.* 1987).

Caldesmon. Smooth muscle caldesmon is a highly asymmetric monomer. It is the third most abundant protein component of smooth muscle thin filaments after actin and tropomyosin. Uterine caldesmon does not differ significantly from caldesmon in any other smooth muscle (Marston, 1989). Caldesmon is able to bind to actin, tropomyosin, and myosin. Besides binding these proteins, caldesmon also binds to calcium-binding proteins, calmodulin. Its in vitro properties have been recently reviewed (Horowitz *et al.* 1996b). Briefly, binding of the protein to actin can lead to an inhibition of myosin ATPase activity in vitro. This inhibitory action of caldesmon can be reversed by the binding of Ca/calmodulin and by phosphorylation. Interestingly, there is evidence suggesting that caldesmon plays an important role in the suppression of contractility during pregnancy and the initiation of labour at the end of pregnancy. Word *et al.*

(1993) have demonstrated that caldesmon levels are increased during pregnancy in human myometrium.

Calponin. Calponin is a smooth muscle specific protein, which is able to bind several proteins either in a Ca-dependent manner or a Ca-independent manner (Winder *et al.* 1998). Of these *in vitro* protein-protein interactions, only the calponin-actin interaction has been strongly implicated to have a physiological function (Winder *et al.* 1998). It has been reported that the binding of calponin to actin has an inhibitory effect on the actin-activated Mg-ATPase activity (Kake *et al.* 1995). This inhibitory effect is reduced through the phosphorylation of calponin by certain kinases such as PKC and CaM kinase II (Wider & Walsh, 1990). Recently, it has been reported that the cellular distribution of calponin changes during pregnancy (Cornwell *et al.* 2001).

1.5.2.2 Regulatory proteins

Calmodulin

Calmodulin is a low molecular weight, acidic, Ca binding protein which mediates many of the regulatory effects of Ca, including the contractile state of smooth muscle (Walsh, 1983). The principal function of CaM in smooth muscle is to activate crossbridge cycling and the development of force in response to a $[Ca]_i$ transient via the activation of MLCK and phosphorylation of myosin (see below (Walsh, 1983; Vogel, 1994). At low free Ca concentrations, such as exist in resting muscle sarcoplasm, CaM exists in the Ca-free form in which state it does not generally interact with a target protein. Following an appreciate stimulus, the free Ca concentration rises whereupon Ca binds to CaM which undergoes a conformational change enabling it to interact with a target protein(s). The overall result of this protein-protein interaction is a physiological

effect, e.g., Ca binding to CaM in smooth muscle allows it to interact with and activate MLCK which catalyses the phosphorylation of myosin. This reaction results in contraction of smooth muscle.

However, a new model for the activation of smooth muscle contraction by Ca and CaM has been proposed recently (Wilson *et al.* 2002). It was suggested that CaM at resting $[Ca]_i$ may contain two bound Ca ions and therefore interacts with, but does not activate, MLCK. Then it is the diffusion of Ca to the myofilaments, where it interacts with this permanently bound CaM, which is responsible for activating the kinase and triggering contraction.

The thin filament-associated proteins, caldesmon and calponin, which inhibit the actin-activated Mg-ATPase activity of smooth muscle myosin (the crossbridge cycling rate), appear to be regulated by CaM, either by direct binding of Ca/CaM or indirectly by phosphorylation catalysed by CaM kinase II (Walsh, 1994). Another level at which CaM can regulate smooth muscle contraction involves proteins which control the movement of Ca across the sarcolemmal and sarcoplasmic reticulum membranes and which are regulated by Ca/CaM, e.g. the sarcolemmal Ca-pump and the ryanodine receptor/Ca release channel, and others proteins which indirectly regulate $[Ca]_i$ via cyclic nucleotide synthesis and breakdown e.g. nitric oxide (NO) synthase and cyclic nucleotide phosphodiesterase (Wash, 1994).

Myosin light chain kinase

Myosin light chain kinase is a very specific regulatory protein for smooth muscle contraction, which acts by phosphorylating MLC_{20} to activate the myosin ATPase activity. The kinase specifically phosphorylates Ser^{19} of MLC_{20} (Horowizt *et al.* 1996). Phosphorylation of Thr^{18} also occurs in the presence of high MLCK

activations or extreme level of muscle activation (Ikebe *et al.* 1986), but dual phosphorylation is unlikely to have broad physiological significance in intact smooth muscle tissue (Ito *et al.* 1989).

Myosin light chain kinase contains the region homologous to the sequence surrounding Ser^{18} of MLC_{20} (Horowizt *et al.* 1996). This region acts to auto-regulate the kinase by acting as a pseudosubstrate prototype. Pearson *et al.* (1988) suggested that the kinase, in the inactive conformation, is folded such that the substrate binding domain is associated with the pseudosubstrate domain and thus does not have access to the true physiological substrate. They also suggested that the dissociation of the pseudosubstrate from the substrate binding site can be induced by MLCK upon biding to Ca/CaM.

MLCK can be phosphorylated in vitro by several kinases including PKA, PKC and CaM kinase II (Nishikawa *et al.* 1984; Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990; Stull *et al.* 1993). These kinases are able to phosphorylate MLCK at a specific serine residue located in the region of CaM-binding domain of MLCK reducing its affinity for Ca/CaM and consequently, its phosphorylating activity (Allen & Walsh, 1994).

Myometrial MLCK appears to be similar to that found in other smooth muscles (Higashi *et al.* 1983; Barany & Barany, 1990). Recently, three isoforms of MLCK of 218, 137, and 60 kDa have been detected in human myometrium (Moore & Bernal, 2001). Both the 218 and 137 kDa MLCK are equally expressed in human non-pregnant and term pregnant uterine smooth muscle whereas a MLCK at 60 kDa is only expressed in non pregnant myometrium, suggesting that its expression is inhibited during normal pregnancy in a hormonally dependent manner. The mechanisms by which expression of MLCK isoforms are altered during pregnancy and the functional consequences of these changes are unknown (Moore & Bernal, 2001).

Myosin light chain phosphatase

Myosin light chain phosphatase is physiologically responsible for the dephosphorylation of the MLC_{20}. The kinase is found to be bound tightly with myosin and is not dissociated from myosin under physiological ionic conditions, suggesting that under physiological condition the kinase is targeted for its substrate (Horowitz *et al.* 1996b).

Classification of MLCP is based on substrate specificity and sensitivity to a defined set of inhibitors and activators (Hartshorne *et al.* 1998; Herzig & Neumann, 2000). Several MLCP have been described and these were mostly type 1 (PP1) and type 2A (PP2A). There have been several reports on phosphatase preparations from different smooth muscles that were effective with phosphorylated light chains or phosphorylated myosin as substrates and these included PP1 and PP2A (Erdödi *et al.* 1996). However it is difficult to claim which phosphatase is effective physiologically.

Myosin light chain phosphatase comprises three subunits: a 37 kDa catalytic subunit, a 130 kDa myosin-binding subunit (MBS), also called myosin phosphatase target subunit (MYPT), and a 20 kDa subunit, whose function is not yet known (Hartshorne *et al.* 1998). Although all subunits are suggested to play a role in targeting the substrate, phosphorylated light chains or phosphorylated myosin, it is believed that the targeting domain of MLCP, MBS, binds to phosphorylated MLC_{20} or phosphorylated myosin and promotes catalytic activity of the 37 kDa subunit (Hartshorne *et al.* 1998). MBS was also shown to contain Ser and Thr residues, which, when phosphorylated, inhibit the ability of MBS to activate the catalytic subunit, thereby reducing activity of the MLCP holoenzyme (Hartshorne *et al.* 1998).

1.5.3 Thick filament regulation

As described above, the Ca-CaM-dependent phosphorylation of myosin is the key step in smooth muscle contraction. Binding of four Ca ions to one CaM molecule into a Ca/CaM complex results in the activation of MLCK, which, in turn, phosphorylates MLC_{20} on serine 19 site leading to an increase in actin-activated myosin Mg-ATPase activity and, crossbridge cycling (Somlyo & Somlyo, 1994). The relaxation of smooth muscle is brought about by resequestration of calcium and dephosphorylation of MLC_{20} by MLCP (Word *et al.* 1993). Little is known about the myometrial MLCP, whether type 1 or type 2A catalyses the dephosphorylation of MLC_{20} remains controversial. In permeabilised myometrium, the catalytic subunit of MLCP type 2A relaxed Ca-dependent contraction (Haeberle *et al.* 1985), whereas the catalytic subunit of MLCP type 1 was reported to be the dominant MLCP that catalyses the dephosphorylation of MLC_{20} in intact myometrium (Word *et al.* 1993).

1.5.3.1 Phosphorylation-dephosphorylation

The phosphorylation and dephosphorylation of myosin by the enzymes, MLCK and MLCP, regulates smooth muscle contraction. Unphosphorylated MLC_{20} in myosin is thought to inhibit the actin-activated myosin Mg-ATPase. Phosphorylation of MLC_{20} by MLCK induces a conformational change in the neck region of myosin; this results in activation of the myosin Mg-ATPase. Phosphorylation of MLC_{20} in smooth muscle generally serves two major functions (Horowitz *et al.* 1996b): 1) facilitates the ability of myosin monomers to assemble into filaments, and 2) it increases the ATPase activity of myosin. Dephosphorylation of MLC_{20} in the presence of physiological level of Mg-ATPase leads to disassembly of filaments.

A number of studies have demonstrated that development of uterine force is dependent on phosphorylation of MLC_{20}. Janis *et al.* (1980, 1981) and Word *et al.* (1994) demonstrated that the spontaneous activity of the uterus was accompanied by cyclic phosphorylation and dephosphorylation of MLC_{20}. Haeberle *et al.* (1985) and Mackenzie *et al.* (1990) also found that there was a linear relationship between the extent of uterine MLC_{20} phosphorylation and force, whether elicited by high K or uterotonic agents. However, dissociation between force and phosphorylation by which force may remain elevated while phosphorylation returns to baseline values was also shown (Csabina *et al.* 1987). The initial phase of contractile activity elicited by carbachol or oxytocin was related to MLC_{20} phosphorylation but not to the subsequent maintenance of force (Csabina *et al.* 1987).

Stretching of the uterus also elicits MLC_{20} phosphorylation (Csabina *et al.* 1986). This can occur in the presence or absence of a relaxing agent (EGTA). Without active tension, the phosphorylation induced by stretch is identical to the phosphorylation of uteri producing carbachol-elicited active force. These can lead to the conclusion that uterine light chain phosphorylation can occur without force development; however, force cannot develop without prior light chain phosphorylation (Barany & Barany, 1990).

1.5.3.2 Latch hypothesis

As discussed above, myosin phosphorylation correlates with the rise of tension following agonist stimulation, but subsequent sustained contractions are maintained while myosin phosphorylation level falls toward the relaxed level. In many tonic smooth muscles, this response is referred to as 'latch' (Dillon *et al.* 1981). It may be related to Ca sensitisation discussed above.

1.5.4 Thin filament regulation

In addition to thick filament regulation of contraction, the thin filament regulation of contraction has been proposed. As previously mentioned, smooth muscle thin filaments contain two specific proteins caldesmon and calponin (see section 1.5.2.2). Both proteins inhibit ATPase activity of acto-myosin and the effect is being reversed by phosphorylation of these proteins (Allen & Walsh, 1994).

Calponin and Caldesmon can be phosphorylated by PKCε, a Ca-independent isoform of PKC, directly or indirectly, respectively (Horowitz *et al.*1996). Such a phosphorylation alleviates the inhibitory action of these proteins on the actomyosin ATPase and thus increases the contraction at a fixed $[Ca]_i$. In addition, it has been shown in vascular smooth muscle that caldesmon is phosphorylated in intact canine aortic strips treated with phorbol ester, a PKC stimulator, at the same sites as those phosphorylated in vitro by MAP kinase (Adam & Hathaway, 1993). It is believed that activation of PKCε activates ras protein which converts raf protein from an inactive to an active state and induces sequential phosphorylation of MAP kinase leading to the phosphorylation of caldesmon (Adam & Hathaway, 1993).

1.5.5 PKC regulation

PKC is a protein serine/threonine kinase with wide substrate specificity. Its activation is shown in Fig.1.2. PKC is activated by diacylglycerol resulting from either hydrolysis of phosphatidylcholine by specific PLC, or the conversion of phosphatidic acid by phosphatidate phosphohydrolipase. Phosphatidic acid itself derives from hydrolysis of phosphatidylcholine by specific phospholipase D.

PKC exists in two main groups: a phorbol ester-sensitive PKC and a phorbol ester-insensitive PKC. The phorbol ester-sensitive PKC is divided into two isoforms, a

Ca-dependent isoform and a Ca-independent isoform. The phorbol ester-sensitive isoforms are activated by the physiological product of the phosphatidylinositol cascade, DAG. It has been shown that DAG can increase force at constant $[Ca]_i$ (Walsh *et al.* 1994). However, recent studies, as well as the known properties of DAG metabolism argue against a major, physiological role of the phorbol ester-sensitive PKC in Ca-sensitisation (Somlyo *et al.* 1999), although this remains controversial (Walsh *et al.* 1996). The phorbol ester-insensitive PKC has been proposed to implicate in Ca-sensitisation as it is activated by arachidonic acid that can also directly inhibit smooth muscle MLCP (Gong *et al.* 1992).

Unlike other smooth muscles, the regulation of PKC is less certain in the uterus because different mechanisms appear to contribute to its activation. The role of PKC in the uterus has been investigated with phorbol esters. They have been shown to exert stimulatory effects on contractile activity arising either spontaneously or by depolarisation (Fernandez *et al.* 1993; Kim *et al.* 1996). In the presence of agonists, the inhibitory effects were found (Fernandez *et al.* 1993; Kim *et al.* 1996). It is believed that phorbol esters potentiated spontaneous contraction by increasing the influx of Ca through L-type Ca channels because nifedipine, an inhibitor of L-type Ca channels, can inhibit the contraction (Fernandez *et al.* 1993). It is, however, unclear whether the effect on increasing of the influx of Ca through L-type Ca channels is direct or indirect. In vascular smooth muscle, it has been shown that closing K channels by phorbol esters causes membrane depolarisation, activation of Ca influx, and subsequent vascular smooth muscle contraction (Barman, 1999). To date, there is no evidence showing a relationship between PKC activation and K channel activity. The underlying mechanisms of the stimulatory effect during depolarisation have been reported to be a direct action on the contractile proteins (Fernandez *et al.* 1993; Kim *et al.* 1996). Two

mechanisms have been proposed to underlie the inhibitory effects during agonist-induced contraction. Firstly, PKC may play a negative feed back role by inhibiting the phosphatidylinositol cascade; thereby decreasing IP_3 mediated Ca release (Phillippe *et al.* 1994). Secondly, PKC may activate Na/K-ATPase activity because a Na/K-ATPase inhibitor can reverse the inhibitory effects of phorbol esters (Kim *et al.* 1996).

1.5.6 MAP kinase regulation

MAP kinase is another kinase that is activated upon stimulation with agonists or depolarisation with high K. The intracellular signalling pathways leading to MAP kinase activation are extremely complex (Horowitz *et al.* 1996b). It is thought that activation of MAP kinase is catalysed by MAP kinase kinase (MEK), which phosphorylates MAP kinase on threonine and tyrosine residues (Horowitz *et al.* 1996b). MEK is itself phosphorylated and activated by an upstream kinase that appear to require activation of the ras protooncogene, so called raf. Caldesmon is one of the substrates of MAP kinase (see section 1.5.2.2). It has been suggested that PKC may activate MAP kinase indirectly through ras (see also section 1.5.4). Activation of MAP kinase has been implicated in signaling pathways of smooth muscle. Nohara *et al.* (1995) have demonstrated that MAP kinase have some important roles in oxytocin-induced contraction of uterine smooth muscle.

1.5.7 Rho kinase regulation

It has become clear that Ca independent regulation occurs though the inhibition of MLCP and involves the monomeric GTP-protein Rho (Somlyo & Somlyo, 2000). Activation of Rho leads to the stimulation of Rho-associated kinase (Rho-kinase, identified as ROKα/ROCK2 and ROKβ/ROCK1) (Nobes & Hall, 1994). Rho-kinase, in

turn, phosphorylates the myosin binding-subunit of MLCP, which results in inhibition of the enzyme and subsequent enhancement of MLC_{20} phosphorylation (Somlyo & Somlyo, 2000).

At least ten members of the Rho family are present in mammals: Rho (isoforms A-E, and G), Rac (isoforms 1 and 2), Cdc42 and TC10. The functions of Rho have been most studied for RhoA (Nobes & Hall, 1994). The activation of RhoA-Rho-kinase pathway is summarised in Fig. 1.3.

Several studies have shown that Rho functions in response to various agonists whose receptors are coupled to some heterotrimeric G-proteins (Seasholtz *et al.* 1999). The α subunits from $G_{i\,(1\text{-}3)}$, $G_{q/11}$, and $G_{12/13}$, are postulated to activate Rho (Hart *et al.* 1998; Katoh *et al.* 1998; Kazasa *et al.* 1998; Seasholtz *et al.* 1999). For G_{13}, p^{115}-RhoGEF, a direct interaction with a specific Rho-guanine nucleotide exchange factor, is known to enhance activity and Rho-GTP binding (Hart *et al.* 1998; Kazasa *et al.* 1998).

In addition to GTP-binding proteins, arachidonic acid (Somlyo & Somlyo, 1998) and protein kinase C (Kitazawa *et al.* 2000; Koyama *et al.* 2000) might also be mediators of Ca sensitisation in smooth muscle contraction in vitro, although their physiological roles *in vivo* have yet to be determined. Recently, calponin was shown to be a putative substrate of Rho-kinase (Kaneko *et al.* 2000). These suggest that agonists use at least three pathways to activate Rho-kinase, and then induce Ca sensitisation. However, further analysis is necessary to elucidate which signalling pathway is physiologically significant in agonist-induced Ca sensitisation, particularly in the uterus.

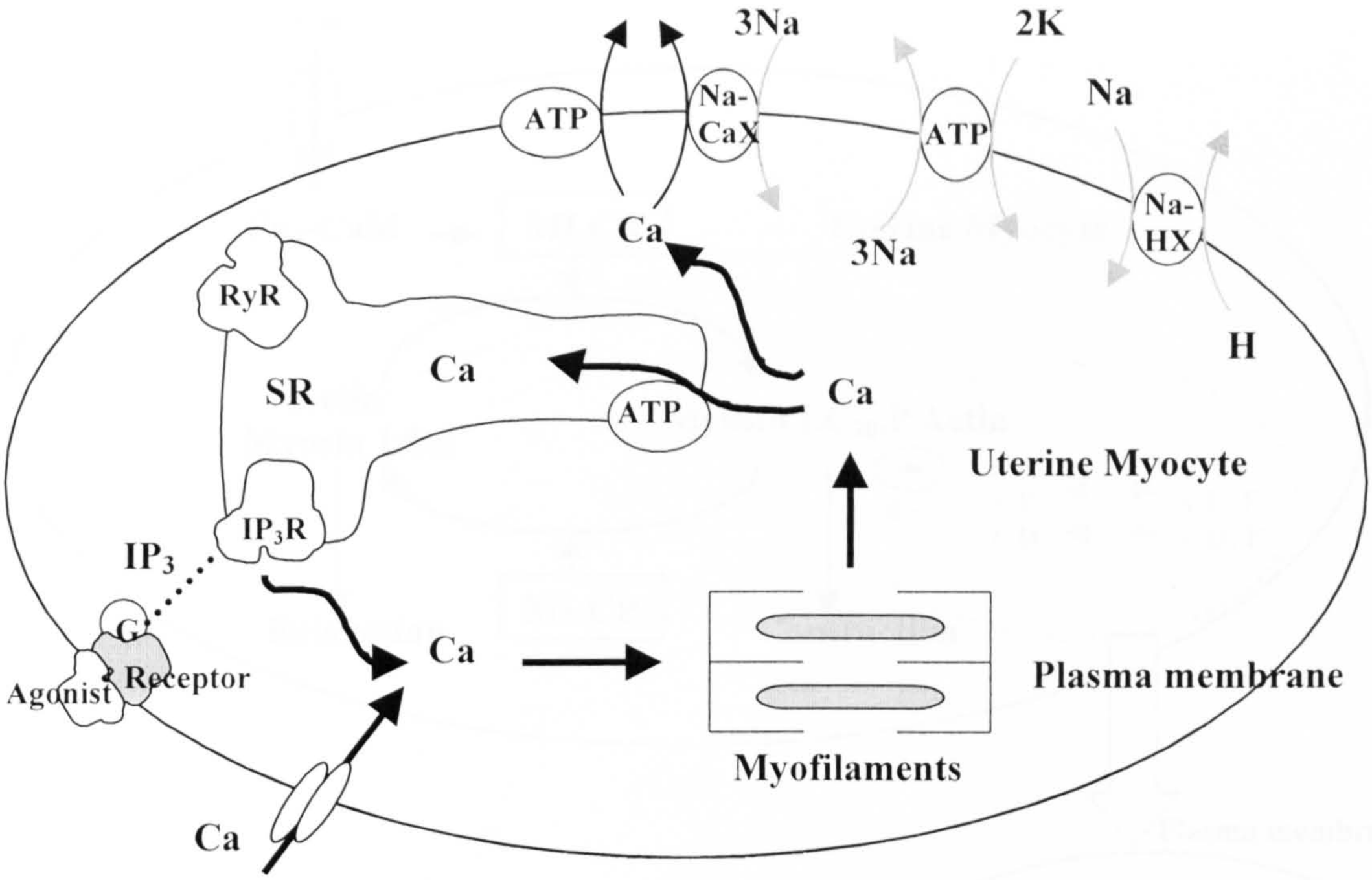


Figure 1.1 General scheme of Ca cycle in a uterine myocyte. Calcium can enter via L type Ca channels. An agonist can also increase $[Ca]_i$ by acting on-G-protein-coupled receptor to activate the phosphatidylinositol cascade and mediate the production of inositol (1,4,5)-triphosphate (IP_3) which induces Ca release from the sarcoplasmic reticulum (SR). Ca is removed from the myofilaments and cytosol by the SR Ca-ATPase pump, sarcolemmal Ca-ATPase pump, and Na/Ca exchange. Abbreviations: IP_3R, IP_3 receptor; RyR, ryanodine receptor; Na-CaX, Na/Ca exchange; Na-HX, Na/H exchange.

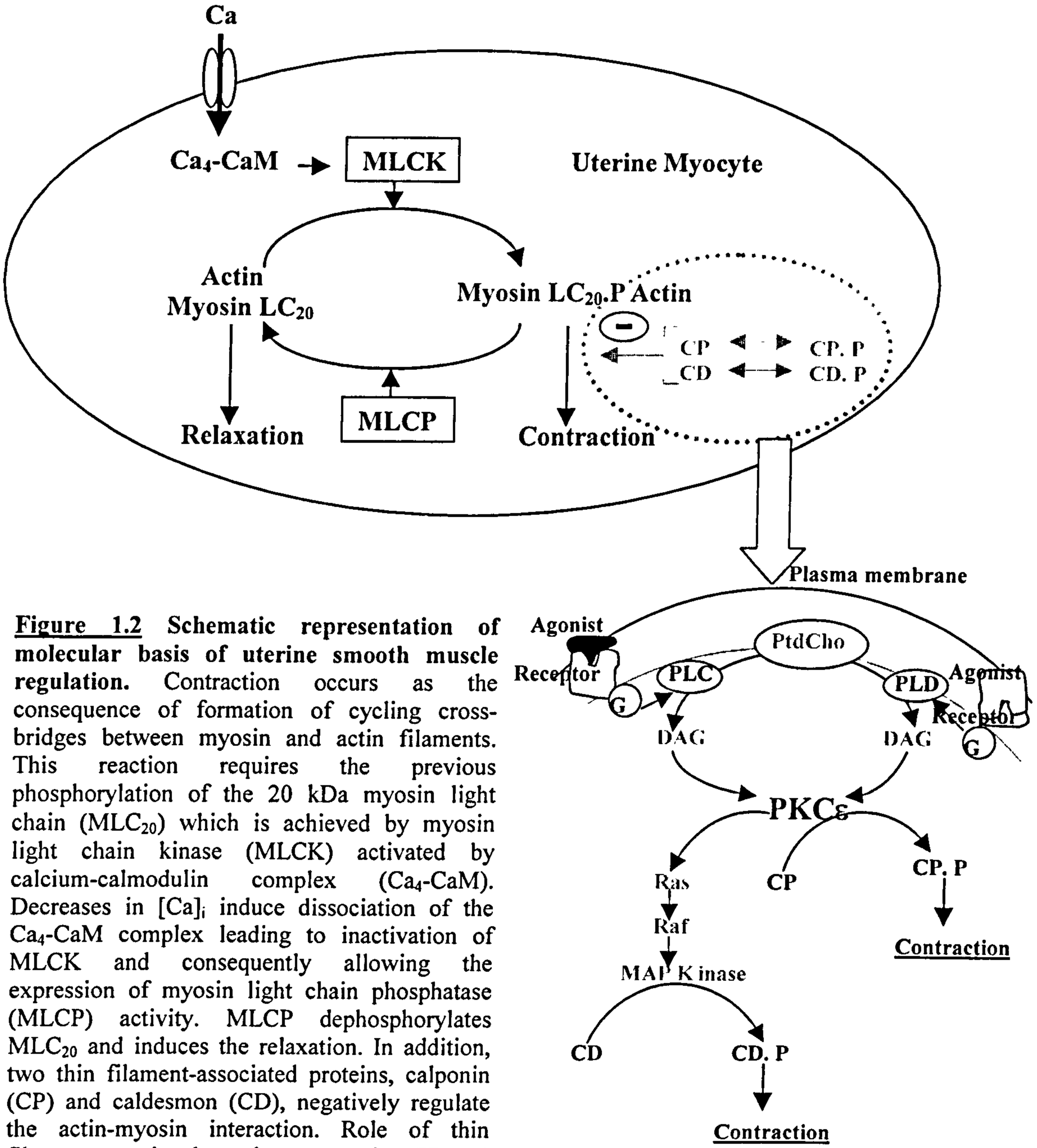


Figure 1.2 Schematic representation of molecular basis of uterine smooth muscle regulation. Contraction occurs as the consequence of formation of cycling cross-bridges between myosin and actin filaments. This reaction requires the previous phosphorylation of the 20 kDa myosin light chain (MLC_{20}) which is achieved by myosin light chain kinase (MLCK) activated by calcium-calmodulin complex (Ca_4-CaM). Decreases in $[Ca]_i$ induce dissociation of the Ca_4-CaM complex leading to inactivation of MLCK and consequently allowing the expression of myosin light chain phosphatase (MLCP) activity. MLCP dephosphorylates MLC_{20} and induces the relaxation. In addition, two thin filament-associated proteins, calponin (CP) and caldesmon (CD), negatively regulate the actin-myosin interaction. Role of thin filament-associated regulatory proteins CP and CD is also shown.

CP and CD can be phosphorylated by PKCε (a Ca-independent isoform of PKC) directly or indirectly, respectively. Direct phosphorylation of CP and indirect phosphorylation of CD via the mitogen-activated protein kinase (MAP kinase) alleviate the inhibitory action of these proteins on actomyosin-ATPase and thus increase the contraction at a fixed $[Ca]_i$. PKCε is activated by diacylglycerol (DAG) resulting from hydrolysis of phosphatidylcholine (PtdCho) by either specific phospholipase C (PLC) or D (PLD) upon agonist stimulation.

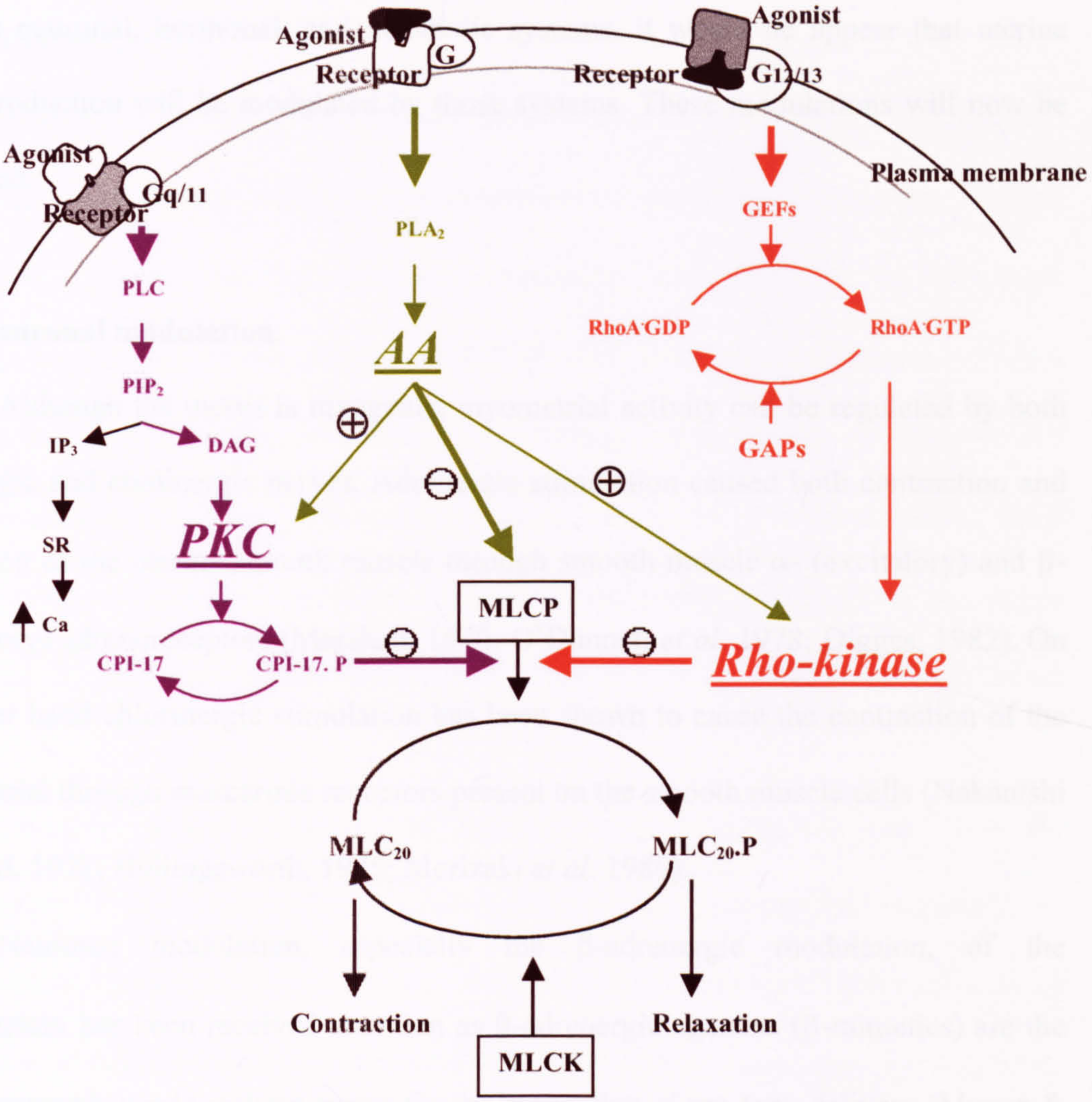


Figure 1.3 Ca-sensitisation in agonist-induced smooth muscle contraction. Each mechanism requires the presence of an active kinase that can phosphorylate Ser^{19} of the 20 kDa myosin light chain (MLC_{20}) and increase MLC_{20} activity by inhibiting myosin light chain phosphatase (MLCP) at constant $[Ca]_i$ (Ca-sensitisation). The first pathway (**Blue**) shows that enhanced MLC_{20} activity is through phosphorylation of CPI-17 by protein kinase C (PKC) leading to direct inhibition of the catalytic subunit of MLCP (PP-1C) by the phosphorylated CPI-17 (CPI-17.P). The mechanism operates in parallel with and independently of the activation of myosin light chain kinase (MLCK) by Ca released from the sarcoplasmic reticulum (see text). The second pathway (**Red**) shows that increases MLC_{20} activity is through activation of Rho-kinase by RhoA·GTP, resulting in inhibition of MLCP activity and increased MLC_{20} phosphorylation. The third pathway (**Green**) shows that increases in arachidonic acid (AA) can also activate Rho-kinase and also inhibit MLCP activity by dissociating the myosin-binding subunit from the PP-1C. AA can also act as a cofactor for PKC. Abbreviations: PLC, phospholipase C; PIP_2, phosphatidylinositol-bis-phosphate; IP_3, inositol (1,4,5) trisphosphate; PLA_2, phospholipase A_2; GEFs, guanine nucleotide exchange factors; GAPs, GTPase-activating proteins.

1.6 Modulation of Force

It is clear from the previous sections that force could be influenced at many loci. As uterine activity is regulated by complex interactions among physiological systems such as neuronal, hormonal, and metabolic systems, it would be appear that uterine force production will be modulated by those systems. These modulations will now be described.

1.6.1 Neuronal modulation

Although the uterus is myogenic, myometrial activity can be regulated by both adrenergic and cholinergic nerves. Adrenergic stimulation caused both contraction and relaxation of the uterine smooth muscle through smooth muscle α- (excitatory) and β- (inhibitory) adrenoreceptors (Marshall, 1970; O'Donnell *et al.* 1978; Digges, 1982). On the other hand chlorinergic stimulation has been shown to cause the contraction of the myometrial through muscarinic receptors present on the smooth muscle cells (Nakanishi & Wood, 1971; Hollingsworth, 1975; Morizaki *et al.* 1989).

Neuronal modulation, especially the β-adrenergic modulation, of the myometrium has been received attention as β-adrenergic agonists (β-mimetics) are the most commonly used tocolytic agents for the prevention of pre-term delivery (Monga & Creasy, 1995). The rationale for using these compounds is based on their ability to increase adenosine 3', 5'-cyclic monophosphate (cAMP) level in smooth muscle of the uterus through binding to specific receptors linked to the stimulatory guanine neucleotide-dependent regulatory protein ($G_{i(s)}$), which, in turn, leading to uterine relaxation (see below). Because pre-term birth remains the leading cause of perinatal mortality and morbidity among non anomalous infants (Monga & Creasy, 1995), an

understanding of the modulation may allow for a more efficient use of those drugs in the pharmacological management of pre-term labour.

1.6.1.1 Adrenergic

Adrenergic receptors have been classified into α and β receptors (Ahlquist, 1948). In general, stimulation of α-adrenoreceptors promotes contraction, whereas stimulation of β-adrenoreceptors causes relaxation. Four subtypes of adrenergic receptors are present on the myometrium (Bottari *et al.* 1985). These subtypes include α_1, α_2, β_1, and β_2. Concerning α-adrenoreceptor subtypes, it is only the α_1 receptors that cause contraction in at least some species (Hoffman *et al.* 1981). In most species, the β receptors responsible for uterine relaxation appear to be predominately of the β_2 subtype (Lands *et al.* 1967; O'Donnell *et al.* 1978; Johansson *et al.* 1980; Lefkowitz *et al.* 1983).

Changes in concentrations of estrogen and progesterone can alter the proportional of the α- and β-adrenoreceptors. In most species, the excitatory, α-adrenoreceptor-mediated responses to adrenergic agonists are enhanced under conditions of estrogen dominance, whereas the inhibitory, β-adrenoreceptor responses are more prominent under the influence of progesterone (Diamond, 1990). This is consistent with their role in increasing and decreasing uterine activity respectively.

In human and rat myometria, α_1 receptors are linked to PLC and IP_3 formation (Breuiller-Fouche *et al.* 1991; Limon-Boulez *et al.* 1997) via activation of $G\alpha_{q/11}$ protein (Limon-Boulez *et al.* 1997). It is of interest that α_1 receptors are more effective at increasing PLC activity at term than during pregnancy in the rat (Limon-Boulez *et al.* 1997), indicating an increased sensitivity of the myometrium to the stimulant action of adrenergic agonists near term.

Coupling of β-adenoreceptors to adenylate cyclase via $G_{i(s)}$ can lead to an elevation of cAMP. The rise in the cAMP level activates PKA, which, in turn phosphorylates a large range of substrate proteins (i.e., phospholamban, Ca-ATPase, IP_3 and MLCK) and causes relaxation through a decrease in $[Ca]_i$ and in the Ca sensitivity of contractile proteins (Ruth, 1999). Other mechanisms by which cAMP may promote relaxation of smooth muscle cells are 1) direct inhibition of VOCCs, 2) indirect inhibition of VOCCs following the activation of BK_{Ca} or the inhibition of Cl_{Ca}, 3) inhibition of receptor-mediated signal transduction pathway, and 4) enhancement of Ca uptake into intracellular Ca stores (Karakaki *et al.* 1997). The relation between cAMP, relaxation, β-stimulation, and hormonal state in the uterus is not fully understood. Although an activation of β receptors can increase the level of cAMP and can relax the uterus, reports of dissociation between β-agonists induced relaxation and changes in cAMP is also provided (Bartelstone *et al.* 1967; Do Khac *et al.* 1986). Furthermore, cAMP elevation by itself is not always sufficient to cause relaxation (Scheid *et al.* 1979). Thus, relaxation of the uterus by β-agonists is the result of both cAMP-independent and cAMP-dependent processes.

It has been suggested that another nucleotide, guanine 3', 5'-cyclic monophosphate (cGMP), also act as an intracellular mediator for relaxation in some types of smooth muscles. In contrast to the situation with adenylate cyclase, guanylate cyclase does not appear to be coupled to agonist receptors via G proteins. Cyclic GMP was initially believed to be a mediator of smooth muscle contraction but was later suggested to be involved in smooth muscle relaxation. Several types of agents (e.g. atrial natriuretic peptide, NO) have been suggested to exert their relaxant effects via elevation of cGMP (Waldman & Murad, 1987). These agents can act through a common intermediate or by releasing a smooth muscle relaxing factor that activates soluble

guanylate cyclase and increases tissue level of cGMP. In vascular smooth muscle, there is strong evidence suggesting that relaxation by cGMP-elevating agents is mediated via activation of PKG (Lincoln, 1989). The identity of the substrate proteins and the underlying mechanisms by which PKG leads to relaxation have not been elucidated, although several possibilities have been suggested (Ruth, 1999). Through phosphorylation of PKG, cGMP is thought to cause a decrease in cytosolic Ca and in the Ca sensitivity of contractile proteins. Another mechanism by which cGMP may promote relaxation of smooth muscle cells is membrane hyperpolarisation as a consequence of K channel activation (Zhou *et al.* 2000).

The role of cyclic nucleotides both cAMP and cGMP in uterine relaxation is supported by several lines of evidence (see below). In addition, it has been proposed that the maintenance of uterine quiescence during pregnancy is stimulated by cAMP and cGMP (Lopez Bernal *et al.* 1995; Telfer *et al.* 2001). In both animal and human myometrium, the adenylate cyclase/cAMP signalling pathway is gestationally regulated (Europe-Finner *et al.* 1994; Lopez Bernal *et al.* 1995; Lindeman *et al.* 2000). Evidence from rat and guinea pig studies suggests that myometrium is relatively insensitive to cGMP (Kuenzli *et al.* 1996; Hennan & Diamond, 1998; Bradley *et al.* 1998), particularly in comparison to vascular smooth muscle (Word & Cornwell, 1998). Other studies, in contrast, show that cGMP can modulate myometrial contractility (Diamond, 1990) and that atrial natriuretic peptide inhibits spontaneous contractions in myometrial strips from estrogen-treated (Potvin & Verma, 1990) and pregnant rats (Syal *et al.* 1998). In pregnant rat and human uteri at term, several reports also indicate that exogenous NO (via cGMP) can inhibit spontaneous contractions (e.g. Yallampalli *et al.* 1998), however, whether endogenous NO is an important modulator of myometrial tone remains controversial (Bartlett *et al.* 1999).

1.6.1.2 Cholinergic

In smooth muscle, five subtypes of muscarinic receptors have been identified (e.g., M_1, M_2, M_3, M_4, and M_5) (Caulfield & Birdsall, 1998). The expression of these receptors differs with species and tissue organs. In many smooth muscle tissues, including the uterus, a minor M_3 receptor expression mediates contraction, despite the presence of a larger M_2 receptor expression (Varol *et al.* 1989). It is well established that the 'odd-numbered' muscarinic receptors (M_1, M_3, and M_5) typically couple via the α subunit of $G_{q/11}$ family, whereas the 'even-numbered' members (M_2, M_4) couple via G_i and G_o α subunit (Caulfield & Birdsall, 1998).

Uterine contraction in response to cholinergic agonists was observed in several species, including rat (Parkington & Lipton, 1976; Varol *et al.* 1989), guinea pig (Marc *et al.* 1986; Matucci *et al.* 1996; Boxall *et al.* 1998), and human (Morizaki *et al.* 1989). This effect is mediated by the interaction of cholinergic agonists with muscarinic receptors present on uterine smooth muscle cells. Coupling of cholinergic agonists to M_3 receptors via $G_{q/11}$ then lead to phosphoinositide breakdown, IP_3 formation, and hence elevating $[Ca]_i$ (Varol *et al.* 1989). The receptor density and functional responsiveness appear to be influenced by hormonal state. The density was increased in the myometrial strips from estrogen-treated rats compared with progesterone-treated rats (Ruzycky & Crankshaw, 1988). An increase in PLC activity and IP_3 formation was also reported near term (Lajat *et al.* 1996). Furthermore, It was observed that cholinergic agonists decrease the accumulation of cAMP presumably through M_2 receptors coupled to G_i α subunit.

1.6.2 Hormonal modulation

It is clear from the previous section that many signals for contraction and relaxation are transduced by G proteins that interact with cell surface agonist receptors. The responsiveness of the myometrial cells to a hormone is also governed by the coupling between the hormone receptor and a G protein. It is widely believed that some hormones can act via a G protein-coupled receptor, which, in turn, activates PLC. The hydrolysis of PIP_2 by PLC then generates IP_3, which mobilises Ca from the sarcoplsmic reticulum.

Like other smooth muscle G proteins, the myometrial G proteins consist of three subunits (α, β, and γ), and are classified into subfamilies ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$) that couple to different agonists (Europe-Finner *et al.* 1996; Sanborn *et al.* 1998). The $G\alpha_s$ stimulates adenylyl cyclase (see section 1.6.1.1), whereas the $G\alpha_i$ and $G\alpha_q$ are postulated to activate the inositol phospholipid pathway. The subfamily members found in myometrium include $G\alpha_{s(1-4)}$, $G\alpha_{i(1-3)}$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$, and $G\alpha_{13}$ (Sanborn *et al.* 1998). The $G\alpha_q$ and $G\alpha_{11}$ belong to $G\alpha_q$ subfamily, whereas the $G\alpha_{12}$ and $G\alpha_{13}$ are the members of $G\alpha_{12}$ subfamily.

There are number of hormones that affect myometrial force production. These include oxytocin, prostaglandins, vasopressin, edothelin, and oestrogen, which stimulate uterine contractions, and progesterone and relaxin, which inhibit uterine contractility. I will consider two major and clinically relevant hormones, oxytocin and prostaglandins.

1.6.2.1 Oxytocin

Oxytocin is a potent and specific stimulant of uterine contraction. It can increase the force, frequency and duration of contractions (Wray, 1993). The receptor for oxytocin is a member of the G protein coupled receptor family. Soloff *et al.* (1979) first

demonstrated and characterised the receptor for oxytocin in rat myometrium, and the presence of the receptor was confirmed in human myometrium and decidua (Fuchs *et al.* 1982). Oxytocin receptor number in the uterus is upregulated by oestrogen during pregnancy, leading to an enhance sensitivity of the myometrium to oxytocin (Alexandrova & Soloff, 1980; Kimura *et al.* 1996; Akerlund *et al.* 1999). Oxytocin receptors are also upregulated in uterine decidual cells during pregnancy; these cells response to oxytocin by synthesising the prostaglandin $PGF_{2\alpha}$ (Hensby *et al.* 1986), which itself is a potent stimulator of uterine contraction (see section 1.6.2.2). Thus, the uterine response to oxytocin increases dramatically throughout pregnancy.

Oxytocin-induced myometrial contraction is associated with an increase in $[Ca]_i$. This has been directly demonstrated in both rat (Anwer & Sanborn, 1989) and human (Tasaka *et al.* 1991) myometrial cells. The rise in $[Ca]_i$ produced by oxytocin predominately depends on Ca influx from the extracellular space. The L-type Ca channels have been proposed to be a major way because Ca removal or application of Ca channel blockers such as nifedipine reduces the rise of $[Ca]_i$ to 16% compared with control (Tasaka *et al.* 1991). There is also evidence that oxytocin closes BK_{Ca} channels, thus enhancing depolarisation-induced Ca entry via the L-type Ca channels (Mironneau, 1994). Inoue *et al.* (1992) were, however, unable to detect a role of potassium channels in oxytocin-induced uterine contraction.

Binding of oxytocin to myometrial receptors also opens receptor-operated channels (Batra, 1986; Anwer *et al.* 1989), which can lead to an increase in membrane conductance (Suzuki & Kuriyama, 1975). Oxytocin has also been shown to accumulate $[Ca]_i$ either by inhibiting the SERCA pump (Magocsi & Penniston, 1991) or inhibiting the PMCA (Akerman & Wikstrom 1979; Magocsi & Penniston, 1991).

Another alternative mechanism for oxytocin-induced the elevation of $[Ca]_i$ is via Ca mobilisation from intracellular stores. It has been demonstrated that oxytocin activated a G-protein, leading to an increase in IP_3 production, as well as increasing contractile activity (Anwer & Sanborn, 1989). This agonist produced a rapid and transient rise in $[Ca]_i$ even in the absence of external Ca (Marc *et al.* 1986), indicating Ca mobilisation from the internal stores. Several line of evidence indicate that oxytocin stimulates myometrial PLC activity by coupling through its receptor with the $G\alpha_i$, $G\alpha_q$, and $G\alpha_{11}$ subfamily members (Phaneuf *et al.* 1996; Sanborn *et al.* 1998; Kimura & Ivell, 1999). In support of these studies, there is evidence that the level of $G\alpha_q$ and $G\alpha_{11}$ is low in early pregnancy but increase near term (Warsop *et al.* 1993), making the uterus more sensitive to hormones that stimulate the inositol phospholipid pathway.

In Ca-free containing solution, oxytocin has been shown to produce continued contraction in both rat (Sakai & Uchida, 1980; Uchida *et al.* 1989) and human (Szal *et al.* 1994) myometrium. The contraction amplitude of is only 5-15% of that obtained with Ca, but these contractions persist for over 10 hours (Sakai *et al.* 1985). These Ca-free contractions did not accompany any change in cytosolic Ca level as measured with fura-2 (Matsuo *et al.* 1989) and were not inhibited by Ca antagonists such as nicardipine (Matsuo *et al.* 1989; Uchida *et al.* 1989) and MLCK inhibitor ML-9 (Karibe *et al.* 1990), suggesting the possibility of Ca-independent oxytocin contraction. In human myometrium, PKC (Morrison *et al.* 1996) and MLCP (McKillen *et al.* 1999) have been shown to involve in oxytocin-induced contraction. There is also evidence that oxytocin may activate, in a calcium-independent fashion, signalling pathways involving MAP kinase in both rat (Nohara *et al.* 1996) and human (Ohmichi *et al.* 1995) myometrial cells.

1.6.2.2 Prostaglandins

Prostaglandins are naturally occurring fatty acids that are produced by many tissues in the body, including the uterus. They are synthesised from membrane-bound phospholipids that, under the influence of PLC enzyme, are converted to arachidonic acid. Cyclo-oxygenase or prostaglandin synthase then stimulates the conversion of arachidonic acid to a range of prostaglandins through unstable intermediaries. Change in the activity of this enzyme is responsible for the fluctuations in the amount of prostaglandins present in the uterus throughout the menstrual cycle and in pregnancy (Erkinheimo *et al.* 2000).

A number of uterotonic agents such as α-adrenergic agonists and oxytocin have also been demonstrated to stimulate the production of prostaglandins in pregnant myometrium (Quass & Zahradnik, 1985; Hensby *et al.* 1986). In the presence of these agonists, it is interesting that inositol phospholipid hydrolysis may also lead to an increase in prostaglandin precursor, arachidonic acid, after further breakdown of diacylglycerol product of PLC action.

Different prostaglandins have a wide range of physiological action, and the biological activity produced will depend on the interaction between the specific type of prostaglandin and its receptor. For example, prostaglandin E_2 can act on receptor subtypes EP_1-EP_4. EP_1 and EP_3 receptors are considered to cause contraction through increasing in phosphatidylinositol turnover and inhibiting of adenylyl cyclase respectively. EP_2 and EP_4 receptors, on the other hand, cause relaxation through stimulating of adenylyl cyclase. In the human uterus, it has been found that the levels of EP_1, EP_3 (Senior *et al.* 1991, 1993) and EP_4 (Erkinheimo *et al.* 2000) receptors are dependent on the physiological stage, i.e. non-pregnant or pregnant. Therefore, the

levels and the balance of these receptors are a possible trigger in the initiation of labour (Matsumoto *et al.* 1997; Dong & Yallampalli 2000, Slater *et al.* 2002).

Both prostaglandin E_2 and $PGF_{2\alpha}$ increase the contractions of the uterus, which are associated with a rise in $[Ca]_i$ as measured by fura-2 in both single cultured human myometrial cells (Mackenzie *et al.* 1990; Thornton *et al.* 1992) and human myometrial strips (Parkington *et al.* 1999). The rise in $[Ca]_i$ is due to Ca influx from extracellular space and release from internal stores, though the later is still controversial. Prostaglandin E_2 and $PGF_{2\alpha}$ have been shown to release Ca from stores by IP_3 production in cultured human myometrial myocytes (Asboth *et al.* 1996; Carrasco *et al.* 1996), in freshly isolated cells neither PGE_2 nor $PGF_{2\alpha}$ had any effect on IP_3 production (Molnar & Hertelendy, 1990). In strips of human myometrium, Luckas *et al.* (1999) have demonstrated that $PGF_{2\alpha}$ was able to produce a rise in $[Ca]_i$ when extracellular Ca was removed, whereas Schrey *et al.* (1988) showed no evidence of $PGF_{2\alpha}$-induced rise in $[Ca]_i$ in the absence of external Ca.

1.6.3 Metabolic Modulation

Hypoxia or pH_i alteration can affect uterine force production (Wray, 1993). The resulting effect on contraction may be harmful; e.g. the hypoxia-reoxygenation of the uterus accompanying the powerful uterine contractions during labour may lead to serious foetal handicap and death.

1.6.3.1 Hypoxia

Uterine hypoxia (induced by metabolic inhibition with cyanide or decreasing levels of oxygen) has been shown to inhibit force production either with or without altering the activity of $[Ca]_i$ (Taggart & Wray, 1998a). In phasically active uterine

smooth muscle, the decline in spontaneous force during hypoxic conditions is mostly due to a direct effect of hypoxia itself on the Ca channels, rather than via an increase in K conductance (Heaton *et al.* 1993). In tonically active smooth muscle, however, there is a dissociation between Ca and force (Taggart & Wray, 1998a). Because acidification and changes in cellular metabolites (increased inorganic phosphates, increased phosphocreatine, decreased ATP) are the responses seen during hypoxia, the contribution of these changes to the fall of force have also received attention (Wray, 1993). However, the decrease in tonic force appears not to be due to limitation of ATP (Harrison *et al.* 1994; Taggart & Wray 1997). A combination of those metabolic factors, including increased inorganic phosphates, acting directly on the sensitivity of myofilaments to Ca is the most likely cause (Crichton *et al.* 1993; Taggart & Wray 1997). Interestingly, the mechanism by which the contractile dysfunction is not entirely explained by changes in $[Ca]_i$ has also been shown in phasically active uterine smooth muscle (Monir-Bishty & Wray, 2002). Monir-Bishty & Wray (2002) showed that basal Ca was elevated during hypoxic conditions, although spontaneous contractions of the human myometrium, and their associated intracellular Ca transients were abolished. This also occurs in the presence of agonists such as oxytocin and carbachol. The cause of the persistent elevation of basal Ca is not clear, but could be due to impaired mitochondrial Ca handling or Ca efflux mechanisms (Monir-Bishty & Wray, 2002).

1.6.3.2 pH_i

Changes in pH_i have been shown to have profound effects on force production by the uterus (Wray, 1988). The effects of changes in pH_i are not only dependent on the particular tissues, but also the contraction conditions.

In both rat (Taggart & Wray, 1993) and human (Parratt *et al.* 1994) uterus, intracellular acidification decreases, and alkalinization increases, spontaneous force in intact preparations. It has been shown that the rise and fall of $[Ca]_i$ are likely to occur as a result of pH_i effects on Ca entry (Taggart *et al.* 1996; Shmygol *et al.* 1998). In depolarised myometrial preparations, the effects of intracellular acidification and alkalinisation became more complex. The effect appears to vary between species. In the rat uterus and in depolarised preparations, acidification increased tonic force and $[Ca]_i$ (Taggart *et al.* 1996). Because the increase in $[Ca]_i$ persisted in the absence of external Ca, an elevation of Ca from an internal store is thought to be involved (Taggart *et al.* 1996). However, in the human uterus, there is a dissociation between force and Ca under depolarisation conditions. The tonic force is decreased but $[Ca]_i$ increased with acidification, and force is increased while $[Ca]_i$ decreases with alkalinisation (Pierce *et al.* 2002). It has been suggested that maintained acidic pH_i desensitises the myofilaments sensitivity to Ca and the desensitisation or inhibition of the contractile machinery must overcome the elevated Ca which are occurring (Pierce *et al.* 2002).

1.7 Summary

In summary, the uterus is spontaneously active, without any neuronal or hormonal stimuli, a piece of isolated uterus will produce regular spontaneous contraction. The uterus is relatively quiescent during pregnancy but highly active at the end of gestation.

Uterine force can be affected at many loci and altered by physiological systems, i.e., neuronal, hormonal, metabolic, and mechanical. Our understanding of uterine contraction, and its control and modulation, is still incomplete. Many areas still require a better understanding so that inappropriate uterine contraction either pre-term or at term can be controlled.

1.8 Aims

The focus of my study was to examine some of the physiological mechanisms that operate to control and modulate uterine smooth muscle contraction.

The aims of this study were:

- To elucidate the contribution of the SR to uterine contraction.
- To determine the importance of both the Ca-CaM-MLCK and non-Ca-CaM-MLCK pathways.

Portions of this study have been published in some scientific papers. Copies of these publications are given in the **Appendix.**

Chapter 2

General Materials and Methods

Chapter 2

General Materials and Methods

This chapter will give a general description of the equipment, materials and methodology used in the work presented in this thesis. More details pertinent to each study are given in the individual chapter concerned.

All experiments were performed on myometrial multicellular strips. In chapters 5 and 6 experiments were performed either on human tissue or on animal tissue, to establish valid models for physiological mechanisms in the human.

2.1 Tissue preparation

2.1.1 Human tissue

Caesarean biopsies

For the work demonstrated in this thesis, human myometrial tissue was obtained from 127 women aged 18-40 years (mean 30.9 ± 0.6 years) undergoing elective caesarean section at term (37-41 completed weeks, mean 38.2 ± 0.1 weeks) before the onset of labour (defined as regular painful uterine contractions associated with progressive cervical change). One hundred and twenty-two of the 127 caesarean biopsies were performed under spinal analgesia and 5 while the women were under general anaesthesia. The indications for caesarean delivery were previous caesarean delivery (79 patients), foetal malpresentation (24 patients), patient choice (5 patients), multiple pregnancy (8 patients), previous traumatic delivery (6 patients), small pelvis (1 patient), fractured pelvis (1 patient), unstable lie (1 patient), symphysis separation (1 patient), and suspected foetal macrosomia (1 patient). Exclusion criteria were serious medical complications or use of medication likely to affect myometrial activity. Ethical

approval was granted by the Liverpool Research Ethics Committee and written informed consent was obtained from each patient (see the **Appendix**).

Collection and storage

Full thickness tissue biopsy specimens were obtained from the upper edge of the uterine incision at caesarean delivery. The tissue specimens were immediately immersed in buffered physiological (Krebs') solution (pH 7.40) containing (mM): 154 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 12 glucose; 2 $CaCl_2$; and 10 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] [HEPES]). The tissue was either used immediately or stored for a maximum of 12 hours at 4 °C; this did not alter the contractile ability of the myometrium (Luckas *et al.*1999). It has also been reported that strips of human myometrium that are prepared from tissue specimens that have been stored in physiological saline at room temperature for up to 18 hours do not appear to behave significantly differently to a number of agents that do freshly prepared strips (Hillock & Crankshaw, 1999; Senchyna & Crankshaw, 1999; Popat & Crankshaw, 2001).

Dissection

The biopsy was placed in a shallow dissecting dish containing Krebs' solution at room temperature under a microscope. Excess blood, peritoneum and any foetal membranes were carefully removed. Five or six strips of longitudinal myometrial fibres with dimension of approximately 1-3 mm x 1 mm x 5-10 mm (width x thickness x length) were dissected and placed in a clean pot containing Krebs' solution for loading (discussed later).

2.1.2 Animal tissue

Animal myometrial tissue was obtained from Wistar rats killed by cervical dislocation under CO_2 anaesthesia. Non-pregnant rats were chosen at 200 g and pregnant rats of approximately the same age were used between 18 and 21 days of gestation. The uterus was removed and the longitudinal layer was separated from the endometrium and circular layers. Five or six strips (1-2 mm x 0.5 mm x 10 mm) of longitudinal fibres were then dissected.

2.2 Chemicals

All chemicals used in the thesis were obtained from Sigma (Dorset, UK) unless stated otherwise. Details of stock solutions are given in the individual chapter concerned. Dissolved vehicles (e.g. DMSO, acetic acid) did not alter the myometrial contractile ability as judged by the peak tension, frequency of contractions, and contraction integral.

2.3 Measurements of intracellular Ca and tension

2.3.1 The fluorescent Ca indicator, Indo-1

Changes in intracellular calcium concentration $[Ca]_i$ was monitored with the florescent Ca indicator, Indo-1 (Molecular Probes, Oregon, USA). Indo-1 is a dual wavelength ratiometric dye; when excited by ultraviolet light at wavelength 340 nm, its emission of light at wavelength at 400 nm and 500 nm is $[Ca]_i$ sensitive. Increasing $[Ca]_i$ increases light emission at 400 nm and decreases emission at 500 nm. Thus, the ratio of these two wavelengths can be used as an indicator of changes in $[Ca]_i$. The use of this ratio method provides a measure depends on changes in $[Ca]_i$ and is therefore

independent of indicator concentration, the illumination intensity, or movement artefacts (Cobbold & Rink, 1987).

2.3.2 Tissue loading

Isolated myometrial strips from both women and rats were loaded with Indo-1 acetoxymethyl ester (Indo-1/AM, Molecular Probes, Oregon, USA) at 15 μM. The AM esters, once in the cytoplasm, are cleaved from the parent compound by intracellular non-specific esterases to release the free acid form of the dye which remains trapped within the cytosol (Tsien, 1981). A 1 mM stock solution of Indo-1/AM was dissolved in dimethyl sulphoxide (DMSO) containing 20% (w/v) of the non-ionic detergent pluronic acid (F-127 Molecular Probes, Oregon, USA) to aid dispersal of the AM esters in aqueous solution (Tsien, 1981). A 15 μM solution of Indo-1 was then prepared by adding the 30 μl stock solution to 2 ml of Krebs' solution. Dissected strips were placed in this solution and incubated at room temperature for 3-4 hours or overnight at 4 °C. Figure 2.1 shows an example of CCD image of myometrial strips loaded with 15 μM Indo-1 at room temperature for 3 hours. The use of Indo-1 to measure $[Ca]_i$ did not affect contractile activity of the uterus. There was no difference in contractile activity between tissue loaded with Indo-1 and tissue not loaded with Indo-1 and strips studied before or after storage overnight at 4 °C. The details of those observations are discussed more in chapter 3.

After loading, the strips were then placed in Krebs' solution and were allowed to de-esterify for at least 30 minutes prior to the onset of experimentation.

2.3.3 Simultaneous measurements of calcium and tension

A schematic diagram of the set up used for simultaneous measurements of calcium and tension, using Indo-1, is shown in Fig 2.2. All the experiments were performed inside a Faraday cage in a darkened room.

The strips loaded with Indo-1 were mounted in a small (200 µl) chamber on an inverted microscope (Nikon Diaphot) and viewed with a 10 x power fluor objective lens. The myometrial strips were attached at each end to metal hooks using silk knots or aluminium foil "T-clips". One hook was fixed to a tension transducer (Grass FT03, Massachusetts, USA). Using silk knots or aluminium foil clips as muscle attachments produced no difference in the temporal characteristics of the force development, but deliberate crushing of the ends of the preparation increased the compliance (Burdyga & Wray, 1997). At the beginning of the experiments the length at which steady passive force was maintained at 10-20% of the maximal isometric force was determined, and taken as a measure of L_0 (resting length). Normally at this length the preparations, showed maximal force response. The strips were then superfused with oxygenated Krebs' solution at rate of 8 ml/min and a temperature of 35 °C, unless stated otherwise.

The tissue was excited (100 W xenon lamp) with light of wavelength 340 nm. Light emitted at wavelengths 400 nm and 500 nm was measured with photomultipliers and digitally recorded. As intracellular calcium level increased, the 400 nm signal increased and the 500 nm signal decreased (see above). In all experiments analysed, changes in the Indo-1 ratio were accompanied by shifts in the opposite direction of the 400 nm and 500 nm emission signals. Sampling was performed at a rate of 10 Hz. Each experiment was ended by superfusing the strips with 5 mM $MnCl_2$ for 10-20 minutes (see below). Figure 2.3 illustrates an example of simultaneous measurement of force and Ca using Indo-1.

2.3.4 Conversion of the fluorescent signals into the Indo-1 ratios

The fluorescence signals were not calibrated to actual $[Ca]_i$ due to the well-recognised difficulties in obtaining minimum and maximum florescence ratios, particularly in intact strips, as ionomycin is not as effective as it is in single cells (Austin & Wray, 1995). The fluorescence signals were corrected for background fluorescence. A 5 mM of $MnCl_2$ was applied to the strip to quench fluorescence and measure the background fluorescence for both wavelengths at the end of each experiment (Luckas *et al.* 1999). This background fluorescence was then subtracted from the total fluorescence of both wavelengths and the ratio of emission signals at 400 and 500 nm obtained. The loaded myometrial strips gave fluorescence signals that were threefold to eightfold above background signals, which is similar to that reported by others (Himpens *et al.* 1990). It was previously shown that myometrial strips have autofluorescence (Lukcas, 2000) at 340 nm excitation which increases the 500 nm signal in response to an increase in $[Ca]_i$. This occurs naturally from cellular fluorophores (pyridine nucleotides and flavine nucleotides, Aubin, 1979), and it can be measured by recording the emission signal in an unloaded strip. However, the myometrial autofluorescence is small (< 5%, Luckas, 2000) and the signals changed in opposite directions, thus the ratios in this thesis were not corrected.

2.3.5 Calibrating force

The electrical signal from the transducer was amplified and converted to a digital signal and recorded on a computer using Chart software (Fig. 2.2). Force was calibrated by comparing force traces to the traces obtained from a known amount of force. Weights were suspended from the transducer and converted to force using the equation: $N = kg.ms^{-2}$ where 1 kg is 9.8 N.

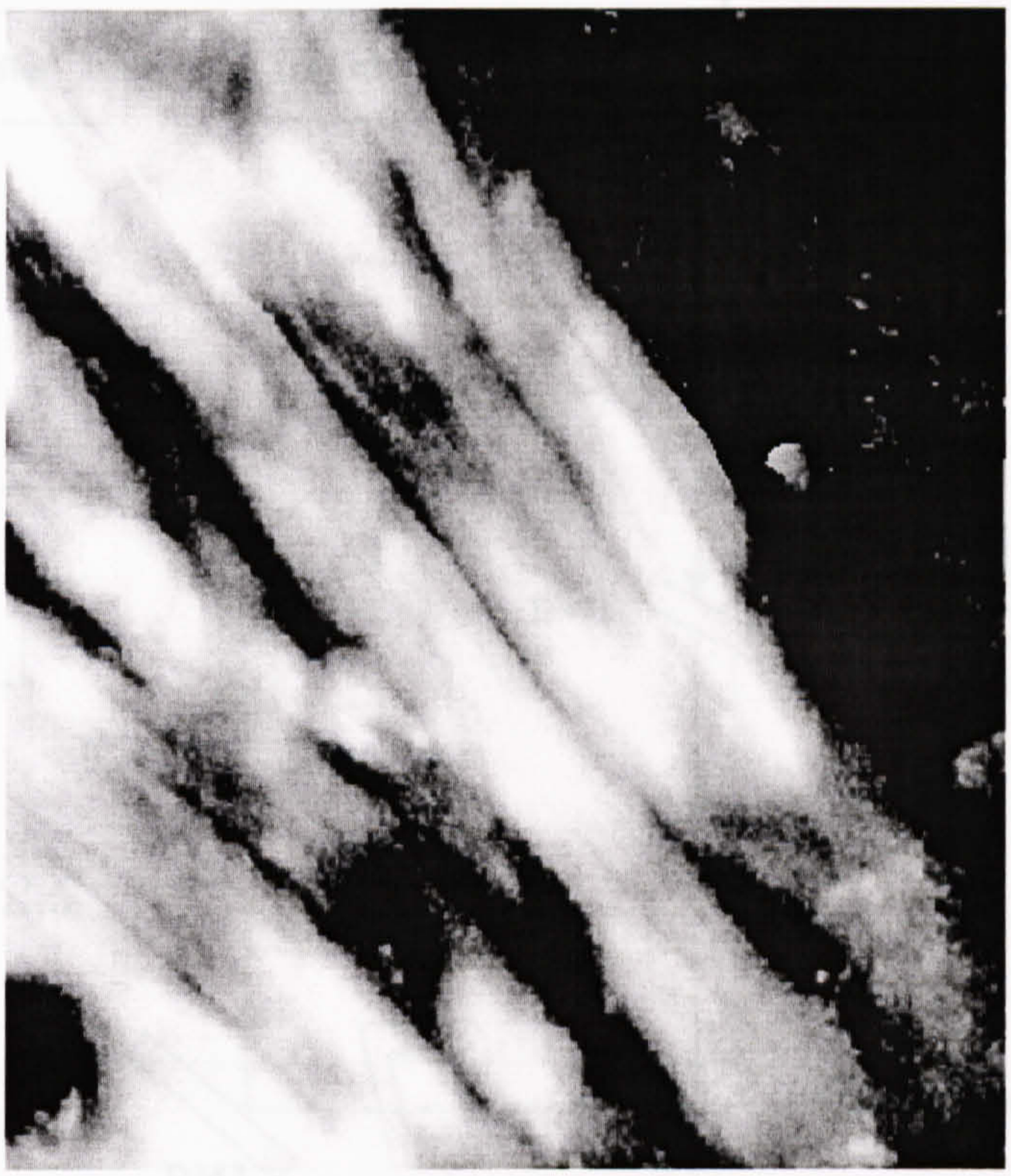


Figure 2.1 CCD image of smooth muscle cells taken from an intact strip isolated from human myometrium loaded with Indo-1. $\lambda_{ex} = 350$ nm, $\lambda_{em} = 500$ nm.

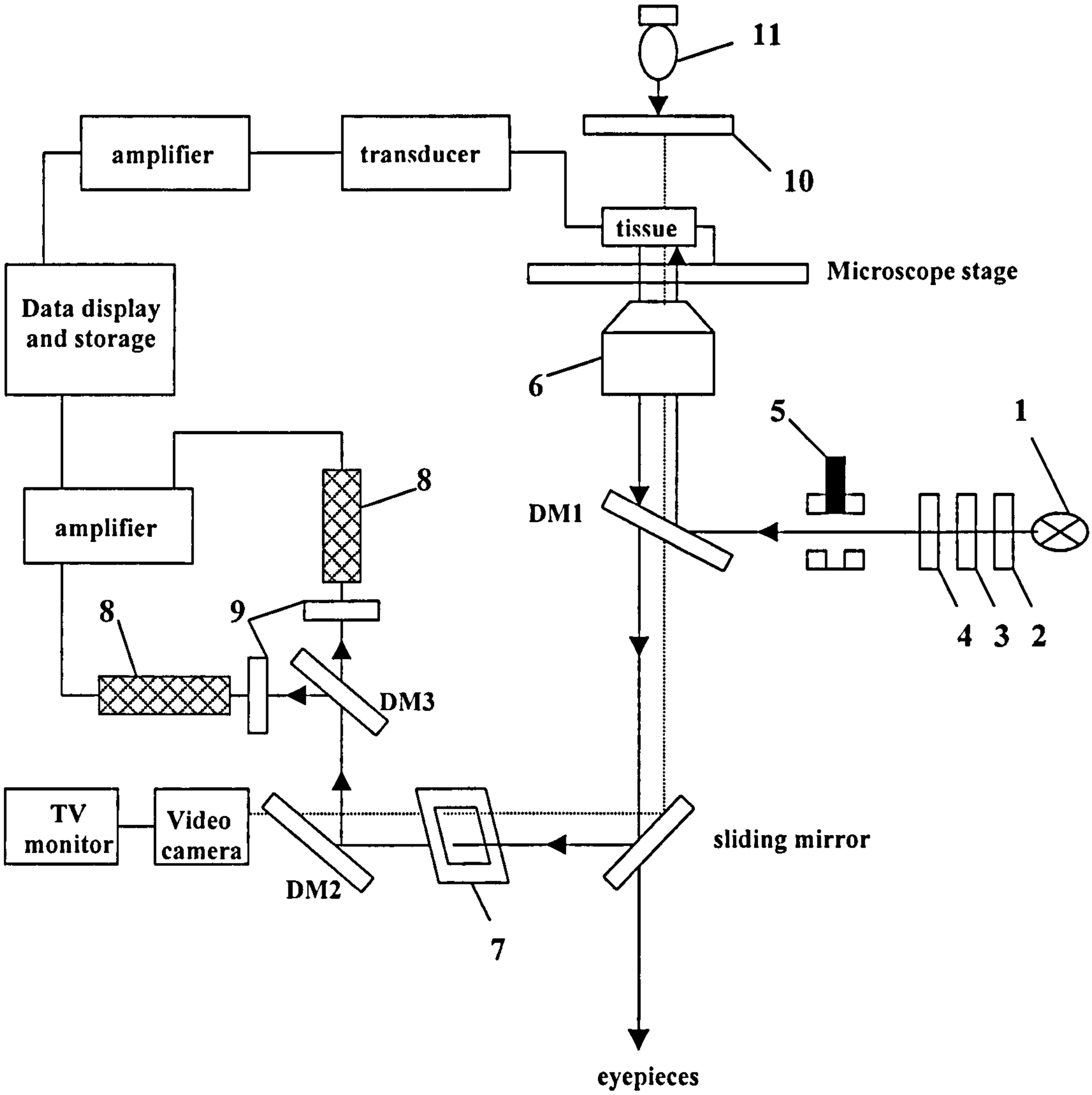


Figure 2.2 Schematic diagram of apparatus based around an inverted microscope. Ultraviolet illumination is provided by a xenon lamp (**1**). Heat filter (**2**) and natural density filter (**3**) are placed in the light path to prevent the excess of heat and to reduce the intensity of the excitation light respectively. The appropriate excitation wavelengths are selected by using an interference filter (**4**) centre at 340 nm. The illumination time of the tissue are kept to a minimum through the use of an electromechanical shutter (**5**) in the excitation path. The exciting light is then reflected and directed upward by a 400 dichroic mirror (DM1) and focused onto the preparation by a microscope objective (**6**). The light emitted by the fluorescent indicator passed back through the objective, is transmitted by the dichroic mirror (DM1), reflected by a sliding mirror and directed through an adjustable diaphragm (**7**). The light then hit a 610 nm dichroic mirror (DM2) mounted at 45 °, which directed the longer wavelength emitted light (greater than 610 nm) to form an image on a video camera which is relayed to a monochrome monitor, and shorter wavelength light to the photomultipiers (**8**, PMTs). The shorter wavelength light reflects and is split by a 450 nm dichroic mirror (DM3), passing to either the 400 nm or 500 nm PMTs. In front of each PMT there is an emission filter (**9**) centre at the appropriate wavelengths. To avoid interference from the microscope light with the fluorescence measurements a long pass filter (**10**) is placed in front of the microscope lamp (**11**).

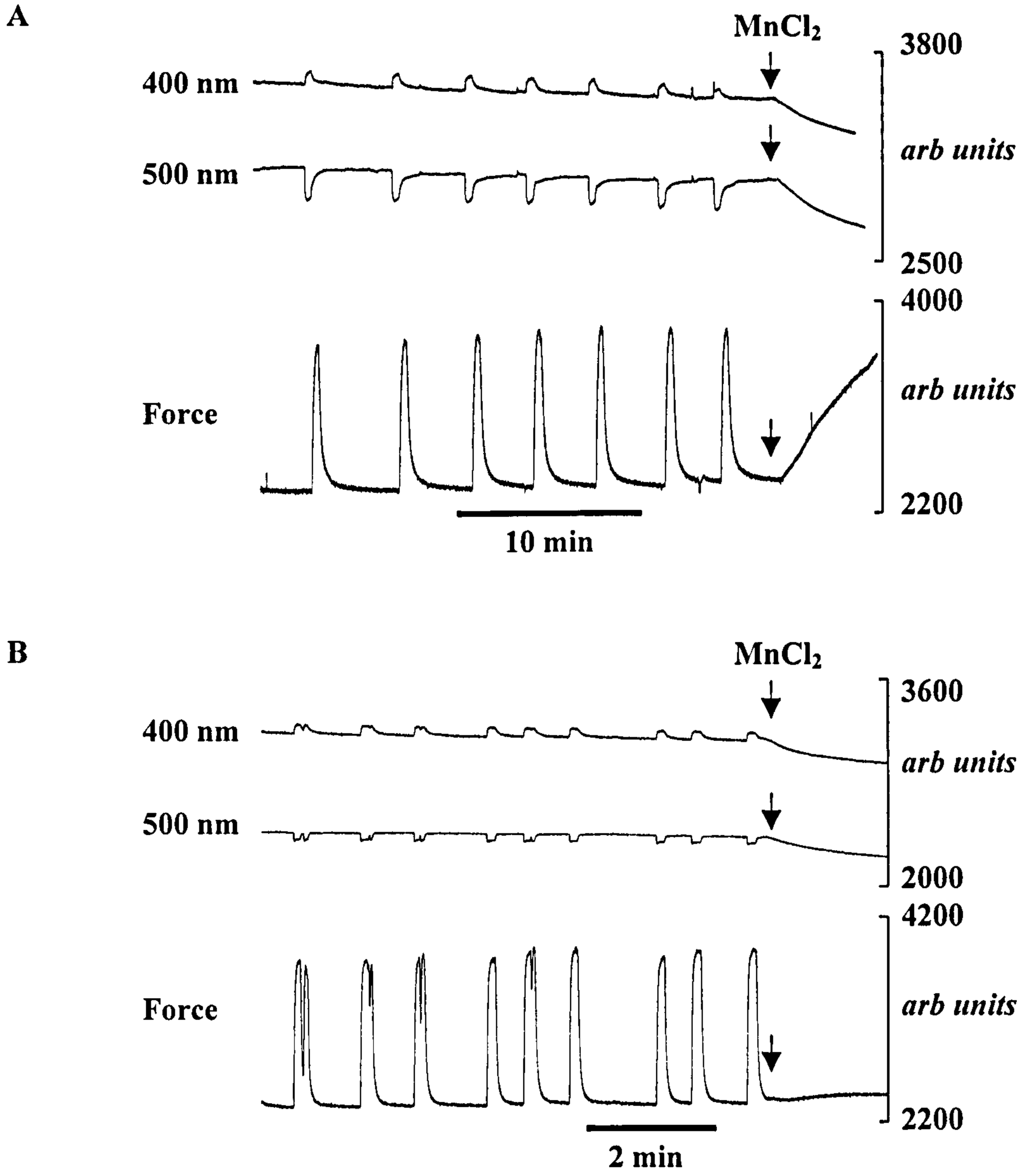


Figure 2.3 An original recording of simultaneous measurement of force and Ca using Indo-1. (A) Shows an example of the record taken from spontaneously active pregnant human (non labour) myometrium. At the end of the experiment 5 mM $MnCl_2$ was applied. The top trace is 400 and 500 nm emission fluorescence signal, showing opposite changes. The bottom trace is force. Both traces are represented with arbitrary units. (B) As in A, but taken from the rat.

2.4 Measurements of myosin light chain phosphorylation

The procedures have been described previously (Maass-Moreno *et al.* 2001; Mitchell *et al.* 2001). For freezing, muscle samples were mounted in a chamber in which they could be immersed in acetone at –80 °C. Measurements of regulatory 20-kDa myosin light chain (MLC_{20}) phosphorylation was quantified by an immunoblotting analysis after separation of the nonphosphorylated and phosphorylated forms of myosin light chain by urea/glycerol-polyacrylamide gel.

2.4.1 Principle of the method for freezing

Freezing apparatus

The apparatus for freezing thin strips of smooth muscle was designed by Dr. Ted Burdyga (University of Liverpool). A diagram of the apparatus is shown in Fig. 2.4A.

Muscle chamber

The strips were studied in the horizontal chambers ("a" and "b" in Fig. 2.4A) open at the top so that force could be recorded with a transducer (FT in Fig. 2.4A) above the chambers. These chambers were embedded in the chamber base ("c" and "d" in Fig. 2.4A). This chamber base was made of the top thick-glass slide ("c" in Fig. 2.4A) connected to the bottom glass slide ("d" in Fig. 2.4A) with four springs and so that the chamber base was sufficiently flexible and could be lowered and slided along by investigator hands. On the other side of the chamber base was the open round-shaped hanger ("e" in Fig. 2.4A) in which the acetone container ("f" in Fig. 2.4A) could be placed inside. To keep the chambers at 37 °C warm water was pumped by the water pump ("g" in Fig. 2.4A) and circulated underneath the bottom of the chamber by the tubes ("h" and "i" in Fig 2.4A) inside the top thick-glass slide.

Mounting and freezing muscle

The solution (200 µl) was placed inside the chambers. This solution was then warmed by the circulation of warm water underneath the chamber (see above). The muscle strips (see section 2.1) were immersed into the solution, and were held at both ends by aluminum foil "T-clip". Holes in the clips were passed over hooks to attach the strips to the apparatus. One hook was used to connect the muscle to a force transducer whereas the other was a piece of j-shaped metal wire firmly connected to the transducer ("j" in Fig. 2.4A). Thus by lowering and sliding along the chamber base (see above) the muscle attachments along with the strips were separated from one chamber and could be immersed into another chamber or the acetone container ("1", "2", or "3" in Fig 2.4A, respectively). The events were controlled by a computer ("k" in Fig. 2.4A) also used to record force, so that the time of freezing was precisely co-ordinated with the initiation of stimulation and force recording. Tests of this apparatus showed that ~ 100 ms elapsed between the time muscles were evacuated and the time the muscles were reached acetone, and phosphorylation reactions were arrested within this period (~ 200 ms, Maass-Moreno *et al.* 2001). Figure 2.4B shows a typical freezing procedure. The strip was immersed in Krebs' solution for 10 min. The chamber base was then lowered and slided to the left so that the muscle attachments along with the strip were separated from Krebs' solution ("1" in Fig. 2.4A and B). The chamber base was released so that the muscle attachments along with the strip were immersed in another chamber of Krebs containing Y-27632 (10 µM, "2" in Fig. 2.4A and B) and incubated for 10 minutes. Prior to freeze, the acetone container was in place and the strip was immersed and frozen in chilled acetone by the process described above ("3" in Fig. 2.4A and B). The acetone container was always kept in a foam box at the sublimation point of dry ice at – 80 °C.

2.4.2 Determination of myosin light chain phosphorylation

Protein extraction

After freezing, muscles were quickly transferred to similarly chilled acetone containing 5% TCA and 10 mM dithiotheridol (DDT), where they were stored at –80 °C until MLC_{20} extraction. Immediately before assay, tissues were allowed to thaw back slowly to room temperature, and then the acetone solution was siphoned off. TCA was removed from the samples by two washes at room temperature with 1.0 ml of acetone containing 10 mM DDT. With each wash, the tubes containing fixed muscle strips were mounted on a rotator and repeatedly inverted for 30 minutes. After the second wash, the tissues were cut from between the aluminum foil clips and placed in 9 M urea extraction buffer (200 µl/mg wet tissue wt) containing (mM): 10.0 DDT; 10.0 EGTA; 1.0 disodium EDTA; 5.0 NaF; 10.0 phenylmethylsulfonyl fluoride; 2.0 Tris base; 2.1 glycine and 0.04 % bromphenol blue. Samples were continually inverted on a rotator for 1.5 h at room temperature and then centrifuged at 15,000 g for 30 minutes.

Electrophoresis

Nonphosphorylated and phosphorylated MLC_{20} were separated by electrophoresis, using method modified from Hathaway and Haeberle (1985) and Persechini *et al.* (1986) for nondenaturing 1 mm minigels. A 30 µl of each sample was loaded on 3% acrylamide-urea stacking gels. Proteins were separated on 10% polycrylamide-glycerol gels until 45 min after the dye front exited (total time ~2.45 h). Electrophoresis was performed at 10 mA/gel through the stacking gel (~1.0 h) and at 16 mA/gel through the separating gel (~1.0 h). Running buffer (top and lower tanks) contained 100 mM glycerine and 50 mM Tris base, pH 8.7; 2-mercaptoethanol (2.0 µ l/ml buffer) was added to the top tank.

Protein transfer

Proteins were transferred to membranes using a semidry technique at 300 mA for 60 minutes. Transfer buffer contained 25 mM Tris.HCl, 192 mM glycine, and 20% methanol at pH 8.3. Membranes were blocked overnight at 4 °C in Tris-buffered saline (TBS), pH 7.6, containing 0.1% Tween 20 (TBS-T) and 3.0% nonfat dry milk.

Western blotting

On the next day, Western blotting was carried out using antibodies to MLC_{20}. Sheets were incubated at room temperature with 1:1,250 mouse monoclonal anti-MLC_{20} (Lot no. 128H4833, Sigma Chemical) for 60 min in TBS-T containing 1.0% nonfat dry milk and then washed three times (5, 10, and 15 min) with TBS-T-1.0% milk. Sheets were then incubated with a biotin-conjugated, F(ab)-specific anti-mouse secondary antibody (Sigma Chemical; 1:1,000) for 40 min at room temperature in TBS-T1.0% milk and washed three times (5, 10, and 15 min) with TBS-T. The blots were incubated for 40 min at room temperature with a streptavidin-horseradish peroxidase conjugate (Amersham; 1:5,000) and then rinsed twice (10 min each) in TBS-T and once (10 min) in TBS.

Chemilumigrams and data analysis

Excess TBS was removed from the blots, which were then developed with the Supersignal™ CL-HRP Substrate System (Pierce Chemical Co.) as described by the manufacturer. Typical lumigrams of measurement are shown in Fig. 2.5A. Two bands, representing nonphosphorylated (P_0-MLC_{20}) and monophosphorylated MLC_{20} (P_1-MLC_{20}), were seen in the same lane. On some occasions, third band, representing diphosphorylated MLC_{20} (P_2-MLC_{20}) was seen (shown later in the result chapters). The

band densities were measured using a Hewlett-Packard 4C scanner and ScanPlot software.

Percent phosphorylation was determined as 100 times the ratio of the density of the band containing monophosphorylated light chain to the sum of the density of the bands for phosphorylated and nonphosphorylated protein in the same lane, i.e. [P_1-MLC_{20}/(P_0-MLC_{20} + P_1-MLC_{20} + P_2-MLC_{20})] x 100%. Values are presented as mean ± s.e.m. Data were compared using appropriate *t* tests or ANOVA, as appropriate. A level of *P* values < 0.05 was considered to be statistically significant.

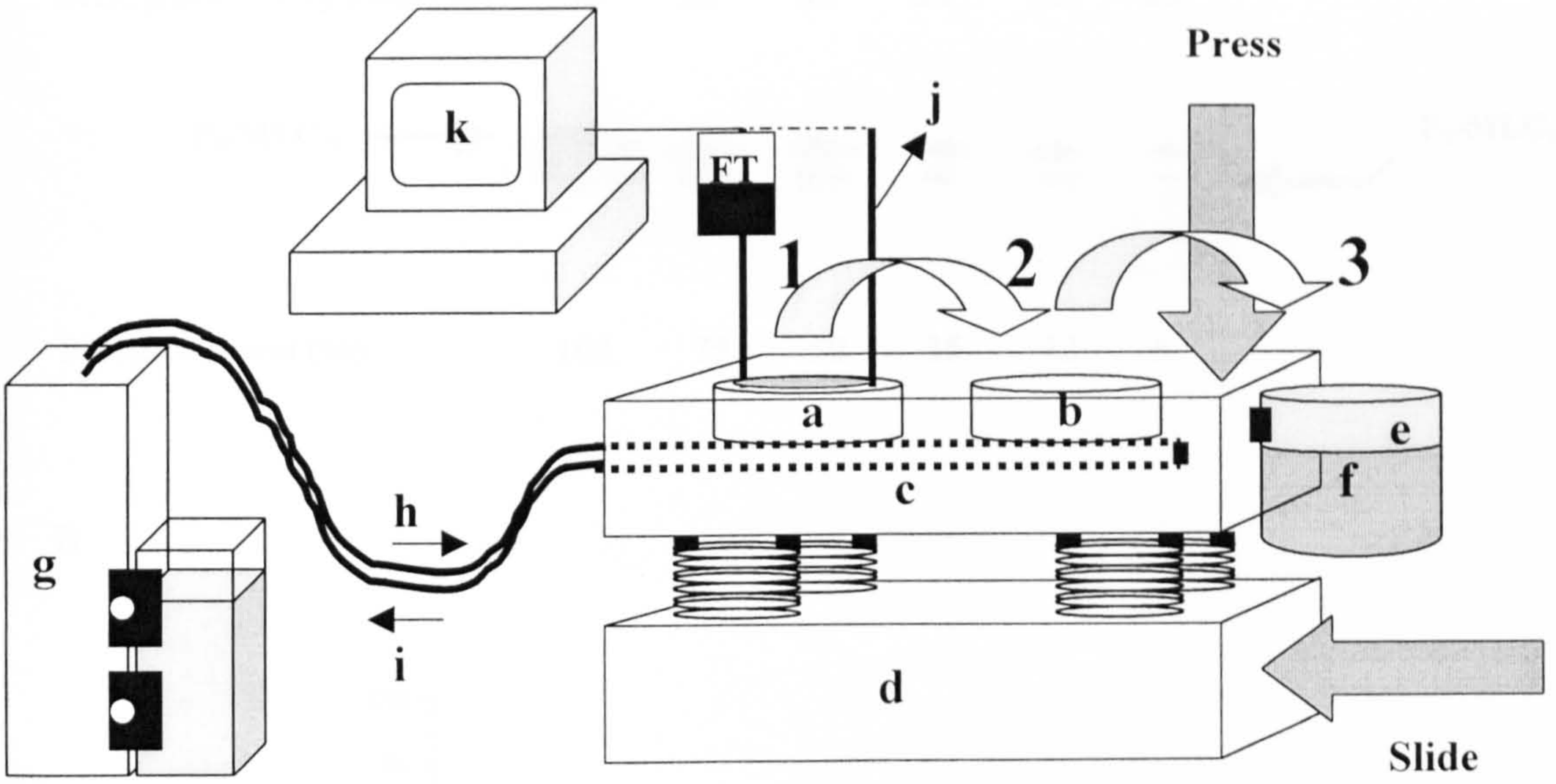
Validating methodology

A test for linearity of the assay was conducted separately in separate experiments. A serial dilution was made, and the percent phosphorylation determined for the more heavily loaded sample was compared with the less heavily loaded companion sample (Fig. 2.5A). Nonlinearity would produce a systematically greater level for one or the other loads. No systemic deviation was found because the assay was very nearly linear (Fig. 2.5B).

2.5 Analysis and statistics

For Ca and force measurements, the result data was analysed using Microcal Origin Software (Massachusetts, USA). The details of Ca and force analysis (e.g. analysed parameters) are discussed more in chapter 3. Data are given as mean ± s.e.m. and '*n*' represents the number of samples, each one from a different woman. Significance was tested using appropriate *t* tests or ANOVA and *P* values < 0.05 taken to be significant. Results are expressed as percentages of control contractions (i.e. the control is 100%) unless stated otherwise.

A



B

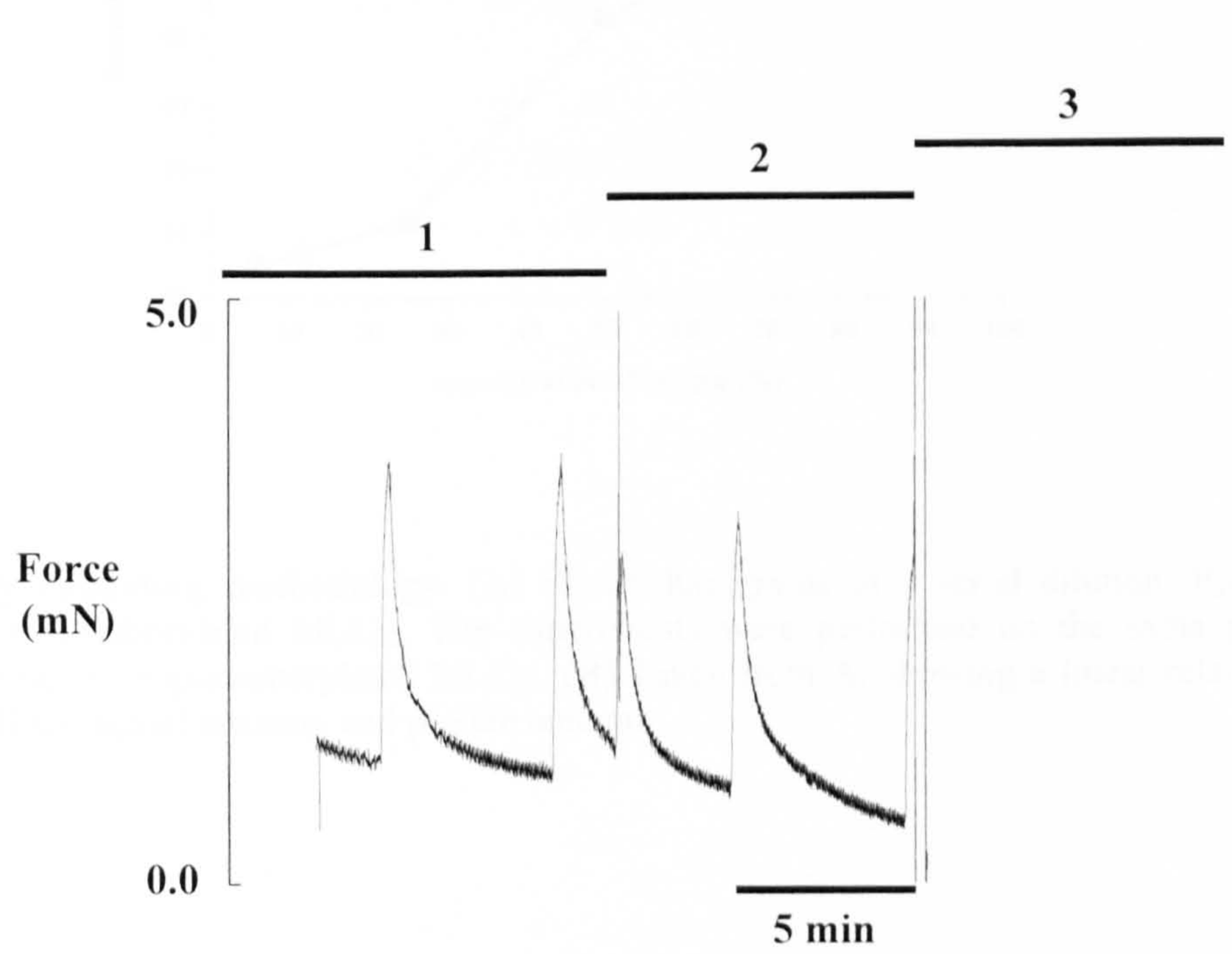
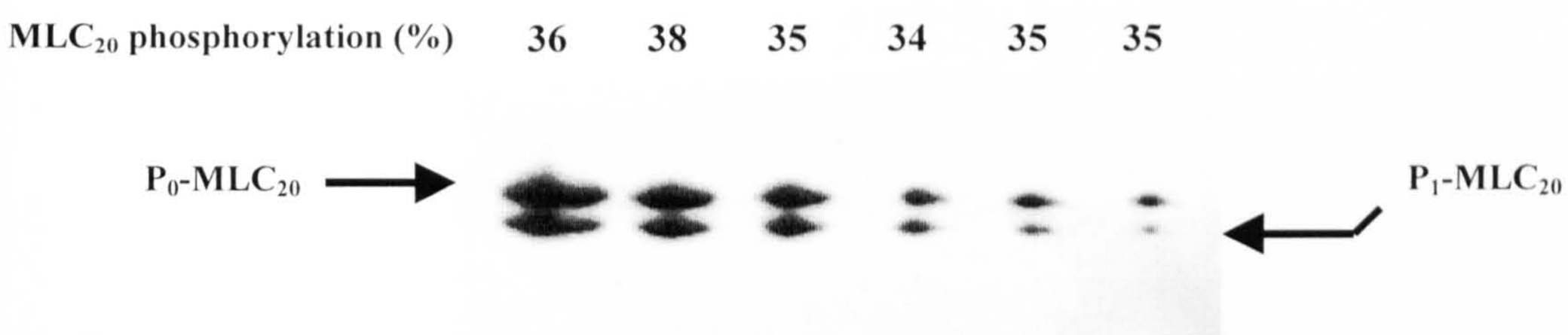


Figure 2.4 Principle of the method for freezing samples. (**A**) Chambers and their connections. (**B**) An original trace taken during freezing experiment.

A



B

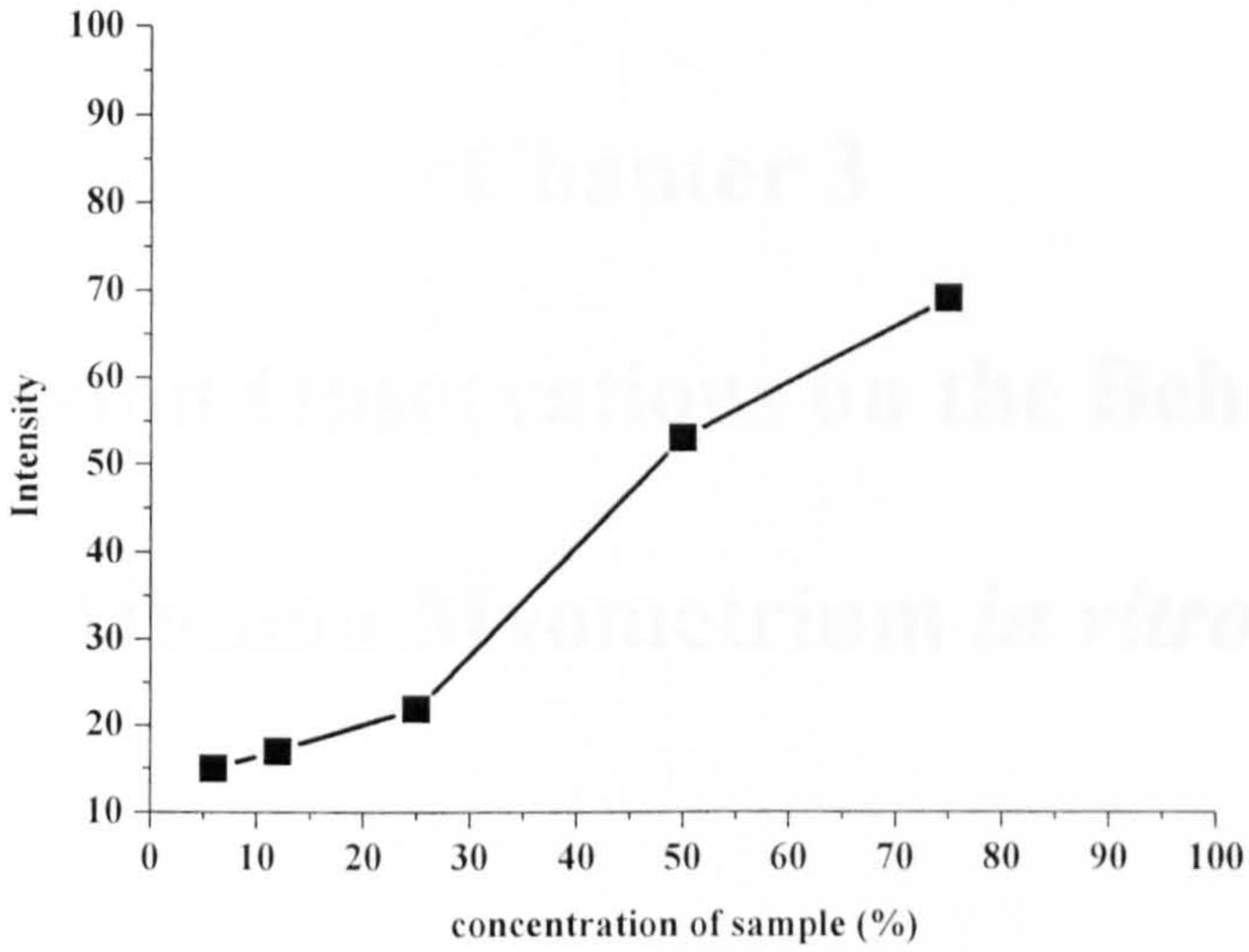


Figure 2.5 Validating methodology. (**A**) Shows lumigrams of a serial dilution. P_0-MLC_{20} denotes nonphosphorylated MLC_{20}. The experiments were performed on the same gel. P_1-MLC_{20} denotes monophosphorylated MLC_{20}. (**B**) Taken from A, showing a linear relationship between MLC_{20} signal intensity and protein amount.

Chapter 3

Background Observations on the Behaviour of Human Myometrium *in vitro*

Chapter 3

Background Observations on the Behaviour of Human Myometrium in vitro

3.1 Introduction

The aim of this chapter was to demonstrate general contractile activity of the human myometrium. Characteristics of spontaneous, high-K-depolarisation-induced and oxytocin-induced contractile activities are discussed. In particular, the experiments were designed to answer the questions: Does Indo-1 loading affect spontaneous activity? What is the relation between force and $[Ca]_i$ changes? What does elevated external K do to force and Ca? What is the effect of oxytocin-induced contraction?

The scope of this chapter did not cover the characteristics of contractile activity recorded in the rat myometrium, as this had well been established elsewhere (e.g. Taggart *et al.* 1996).

3.2 Materials and Methods

Simultaneous measurements of calcium and tension

Tissue preparation and simultaneous measurement of calcium and tension are essentially the same as those described in chapter 2.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK). Oxytocin was dissolved in 5% acetic acid at a concentration of 1 mM. Nifedipine was dissolved in DMSO at a concentration of 10 mM. These stock solutions were diluted to the desired concentrations with Krebs' solution. K was elevated and Na reduced to depolarise the membrane in some experiments. 0-Ca solutions were also used; Krebs' solution in which $CaCl_2$ had been omitted and 1 mM ethylene glycol bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA) added.

Analysis and statistics

Myometrial force and $[Ca]_i$ were recorded and analysed using Microcal Origin Software (Massachusetts, USA), as previously described in chapter 2. Parameters that were measured included maximum tension development of each contraction, the contraction integral (total tension developed in each contraction), contraction duration, and contraction frequency.

Data are given as mean and s.e.m. and '*n*' represents the number of samples, each one from a different woman. Significance was tested using appropriate *t* tests and P values < 0.05 taken to be significant. Results are expressed as percentages of control contractions (i.e. the control is 100%).

3.3 Results

Spontaneous contraction occurred in over 75% of samples taken from 127 women. There was no systematic difference due to age. The tissue samples were all taken from non-laboring women at term, 37 to 41 weeks, and thus represent a relatively homogenous group. The samples were taken from the lower uterine segment which has less smooth muscle and more connective tissue than upper segment. However Luckas & Wray (2000) have recently shown that there is no significant difference in contractile properties and response to agonists of these two regions. Thus the data obtained are representative of the response of the entire uterus.

3.3.1 Spontaneous contractions

Does Indo-1 loading affect spontaneous activity?

As mentioned in chapter 1, the uterus is myogenic organ, that is the smooth muscle contained within it is able to contract without nervous or hormonal input (Wray, 1993). Thus, a healthy piece of myometrium taken from the uterus will produce regular spontaneous contractions when it is placed in an appropriate condition, e.g. superfused with oxygenated Krebs' solution at rate of 8 ml/min and a temperature of 35 °C.

No significant difference for the mean time taken for spontaneous contractions to commence before or after loading with Indo-1 was found in paired strips (n = 15). The mean time to demonstrate spontaneous contraction (both loaded and unloaded) was 52.9 ± 3.6 minutes (n = 53). In paired comparisons of myometrial strips (n = 15), Indo-1 loading had no effect on spontaneous contractile activity (Fig. 3.1); the peak tension (110 ± 20% of control, $P > 0.05$), frequency of contractions (106 ± 15%, $P > 0.05$), and contraction integral (114 ± 12%, $P > 0.05$). In the majority (95%) of human myometrial strips (both loaded and unloaded), the amplitude and the frequency of spontaneous

contraction, particularly the first four contractions, gradually increased and then became more regularly by around 18.09 ± 1.0 minutes after the first response. The tissues then reached steady state and regularly contracted with the mean frequency rate of 0.33 ± 0.02 contractions per minute for several hours.

What is the relation between force and $[Ca]_i$ changes?

Rhythmical spontaneous contractions in the uterine strips occurred, which were accompanied by opposite changes in the 400-nm and 500-nm Indo-1 emission signals and an increase in the 400:500 nm ratio, indicative of an increase in $[Ca]_i$. A typical recording is shown in Fig. 3.2A. It can be seen that there is a close relationship between the rise and fall of the Indo-1 fluorescence ratio (Indo-1 ratio) and force production. The time peak was always faster for the Indo-1 ratio than for force. The $[Ca]_i$ transient can be seen to preceded the changes in force at all stages. This can be better seen in Fig. 3.2B where the $[Ca]_i$ transient and force were superimposed. For twenty preparations, the mean time to peak of the Indo ratio was found to be 11.25 ± 1.96 s, whereas peak force occurred around 3 s later (14.70 ± 1.84 s, significantly different). Removal of extracellular Ca or additional of nifedipine (L-type Ca channel blocker, 0.1-10 μM) abolished both spontaneous contractions and the changes in fluorescence ratio (Figure. 3.3).

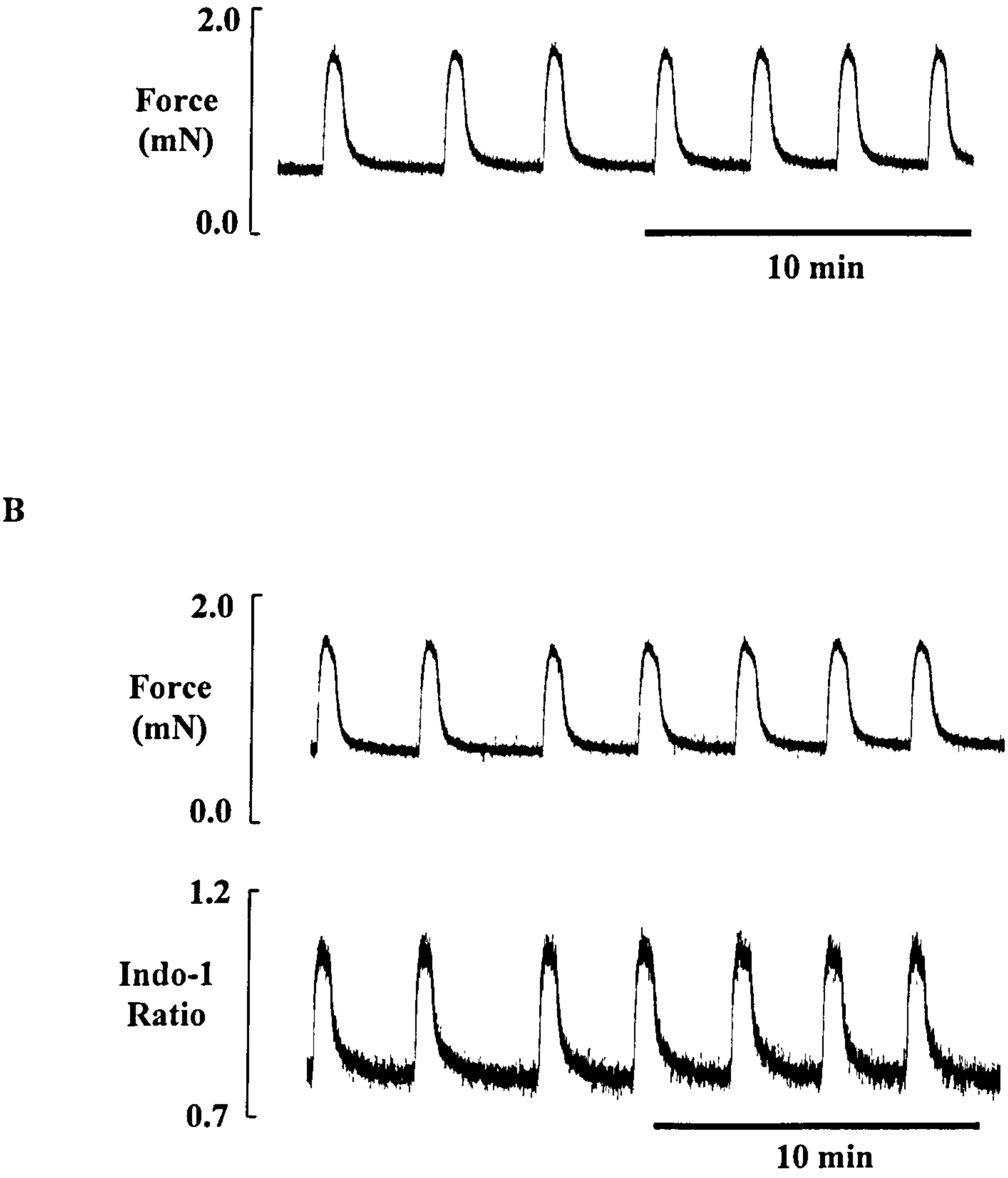
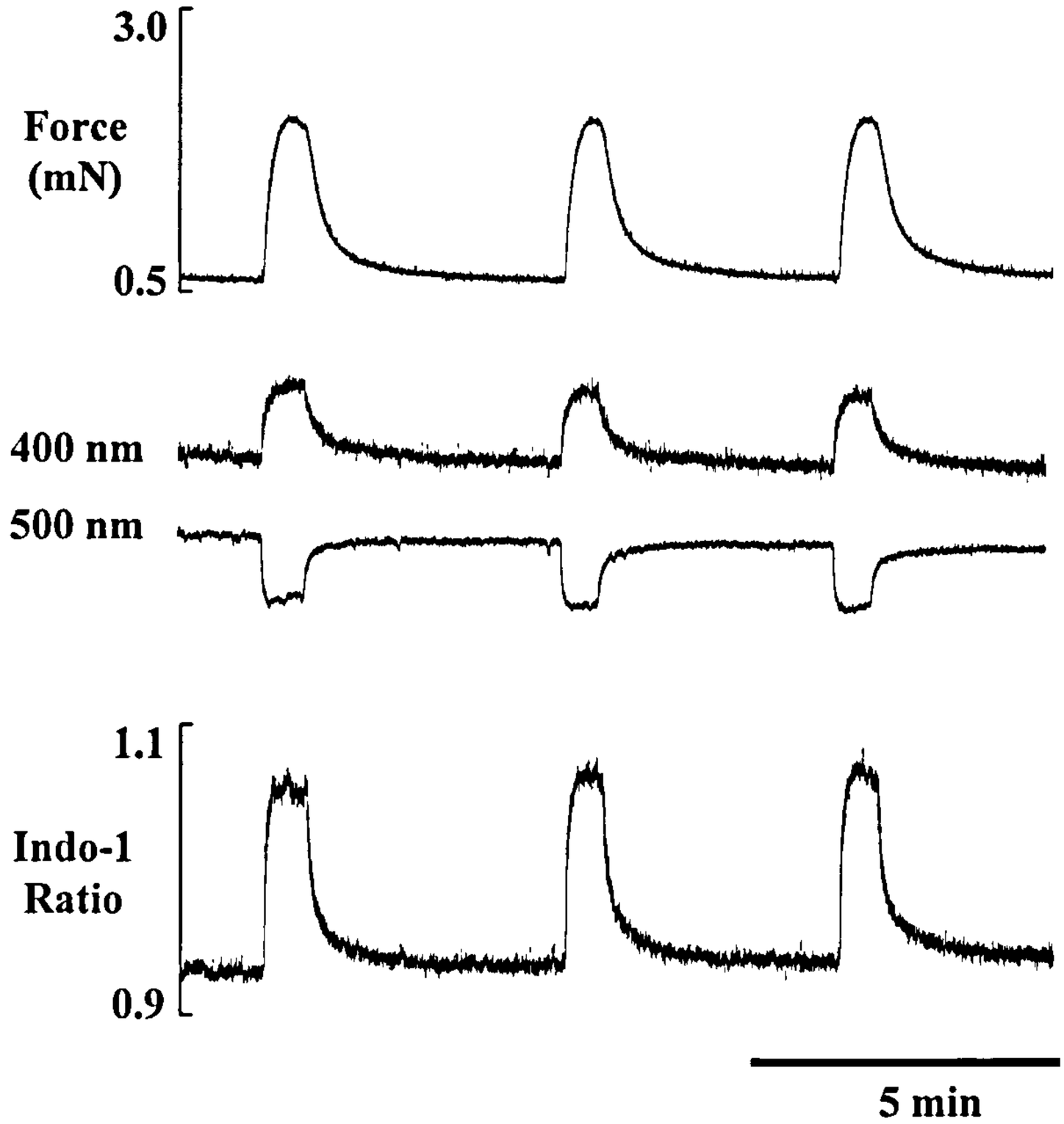


Figure 3.1 Spontaneous contractile activity of human myometrium. Shows the representative traces recorded before (**A**) and after (**B**) loading with 15 μM Indo-1 at room temperature for 3 hours.

A



B

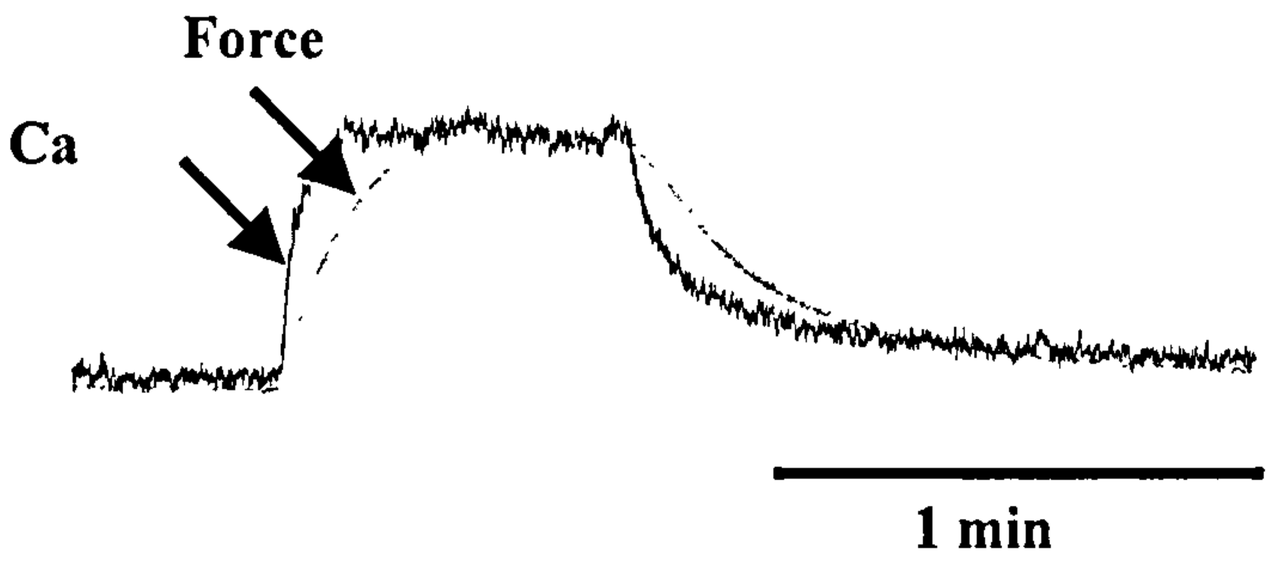


Figure 3.2 Spontaneous changes in human uterine force and Indo-1 signals. **(A)** The reciprocal changes in the 400-nm and 500-nm fluorescence signals can be seen and the bottom trace shows their ratio, which indicates the change in $[Ca]_i$. **(B)** Taken from A, superimposed traces of force (dotted line) and Ca ratio during contraction and relaxation. It can be seen that $[Ca]_i$ changes precede those of tension.

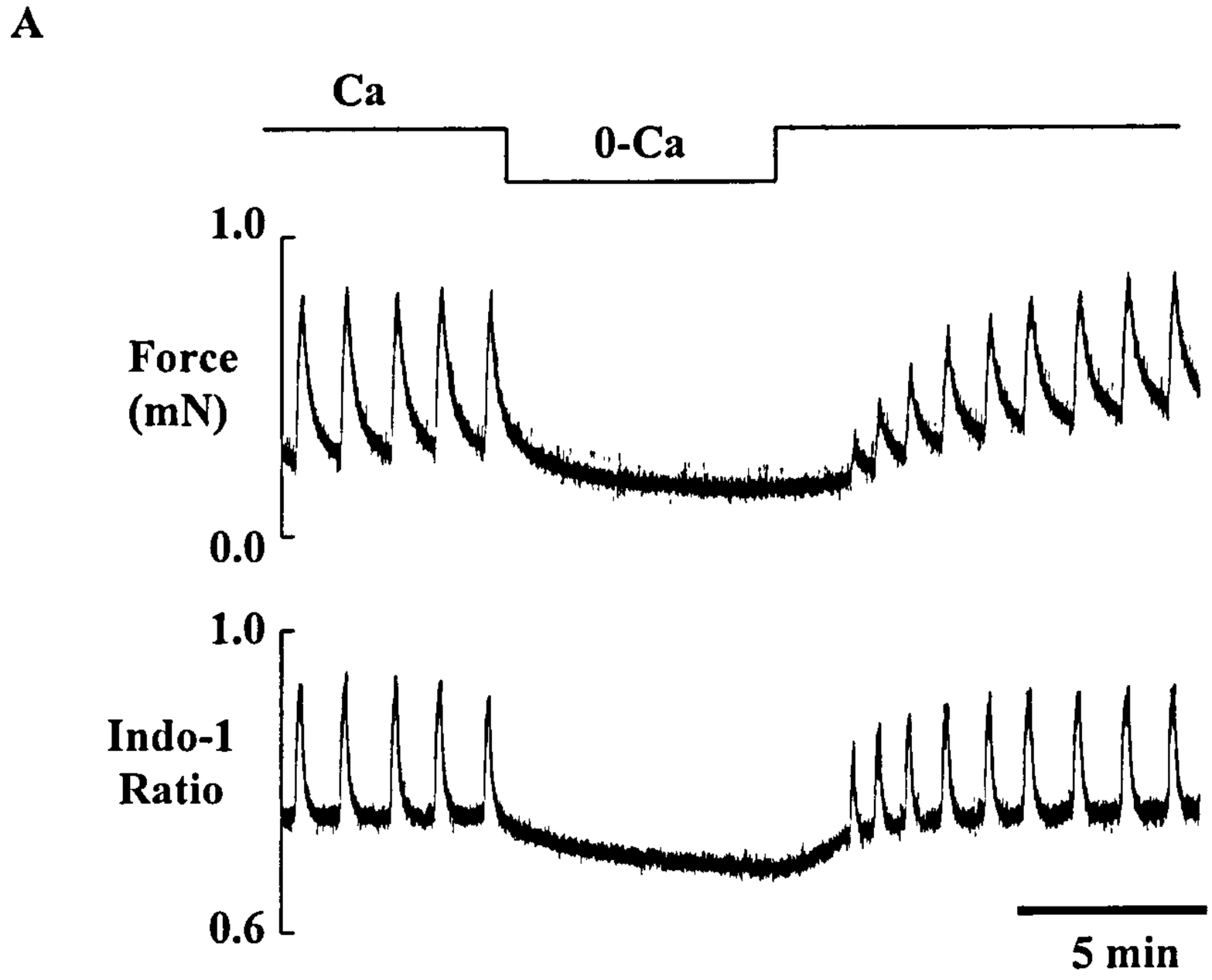


Figure 3.3 Spontaneous contractions were associated with changes in fluorescence ratio. **(A)** Spontaneous Ca transients and contractions in human myometrium recorded in the presence and absence of external Ca. **(B)** As in A but, recorded in the presence and absence of nifedipine (10 μM).

3.1.2 What does elevated external K do to force and Ca?

Depolarisation was produced by elevation of external [K] to 40 mM. This caused a rapid increase in force and Ca. Both force and Ca then declined and were then maintained at an elevated level (plateau) throughout the period of high K application. Relaxation occurred rapidly upon removal of high K. There were no significant differences ($P > 0.05$) between the first and second application of KCl in the human myometrium (e.g. the amplitude of peak force, the contraction integral). A typical (of 10 others) recording of the human myometrium in response to two applications is shown in Fig. 3.4A.

Depolarisation markedly altered the pattern of human uterine contractility compared to spontaneous contractions; as Fig. 3.4B shows, KCl produced maintained force in the uterus. The peak force and Indo ratio during KCl-induced contractions were always greater than for the preceding spontaneous contractions, the mean amplitude of contraction increased by 11 ± 2 % and the Indo ratio by 13 ± 2%.

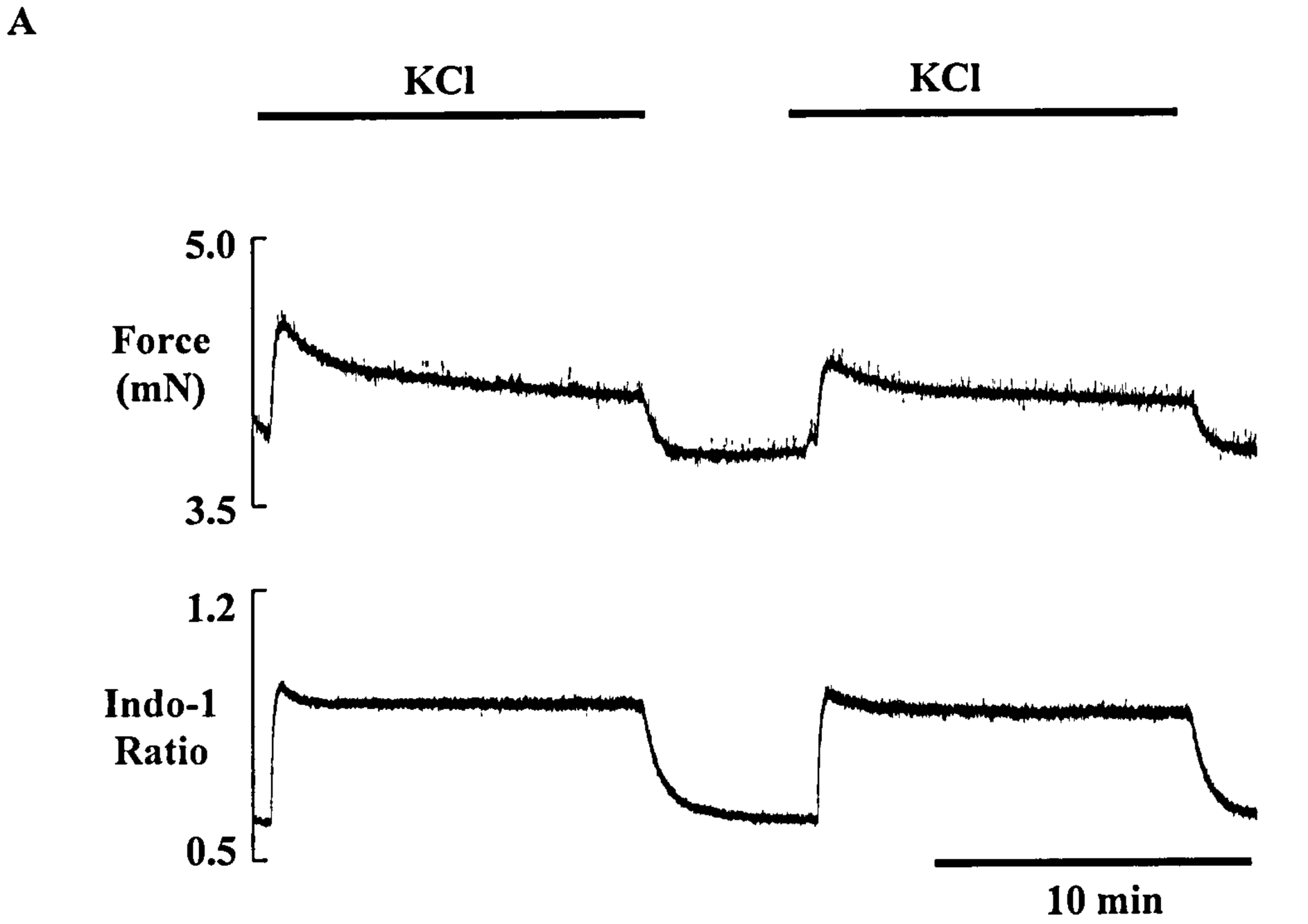


Figure 3.4 Force and intracellular calcium changes evoked by KCl (40 mM) in isolated term pregnant (non labour) human myometrial strips. (A) Sequential application of KCl. (B) An application of KCl to spontaneously active human myometrium.

3.1.3 What is the effect of oxytocin-induced contraction?

Oxytocin, as depolarisation with high K, can alter the influx of Ca across the sarcolemmal membrane, but in addition may also sensitise the contractile apparatus to Ca and/or release Ca from internal stores (Somlyo & Somlyo, 1994). Because the haft-maximal effect of oxytocin on myometrial contractility occurs when its concentration is ~10 nM (Szal *et al.* 1994; McKillen et *al.* 1999) this concentration was used in all subsequent experiments and chapters unless stated otherwise.

Figure 3.5 shows a record of force and the Indo ratio for oxytocin-stimulated myometrium. Application of oxytocin (10 nM) to spontaneously contracting myometrium elicits a significant increase in peak force and duration but no significant increase in the frequency of phasic contraction. The amplitude of both the force and Indo signal was increased. However, oxytocin more markedly increased tension than Indo-1 ratio. In the presence of oxytocin the mean values of contraction amplitude increased to 135 ± 6% ($P < 0.05$), and the Indo ratio to 105 ± 2% ($P > 0.05$), both higher compared to the preceding spontaneous contractions (100%, $n = 12$). Oxytocin had no significant effect on basal Ca or tension. Spontaneous phasic contractions rapidly reappeared upon removal of oxytocin. As can be seen in Fig. 3.5, there were no significant differences between the first and second application of oxytocin in the human myometrium (e.g. the amplitude of peak force and frequency).

An increase in frequency of the contraction was also found in response to the application of oxytocin (10 nM). An example of this effect is shown in Figure 3.6A. At a high dose (100 nM), oxytocin induced greater increased force amplitude, which was not associated with $[Ca]_i$ in the majority of the samples (Fig. 3.6B), and its action was particularly increased the frequency of the contractions. In the minority of the samples (5%), the effect was to produce tonic-like activity.

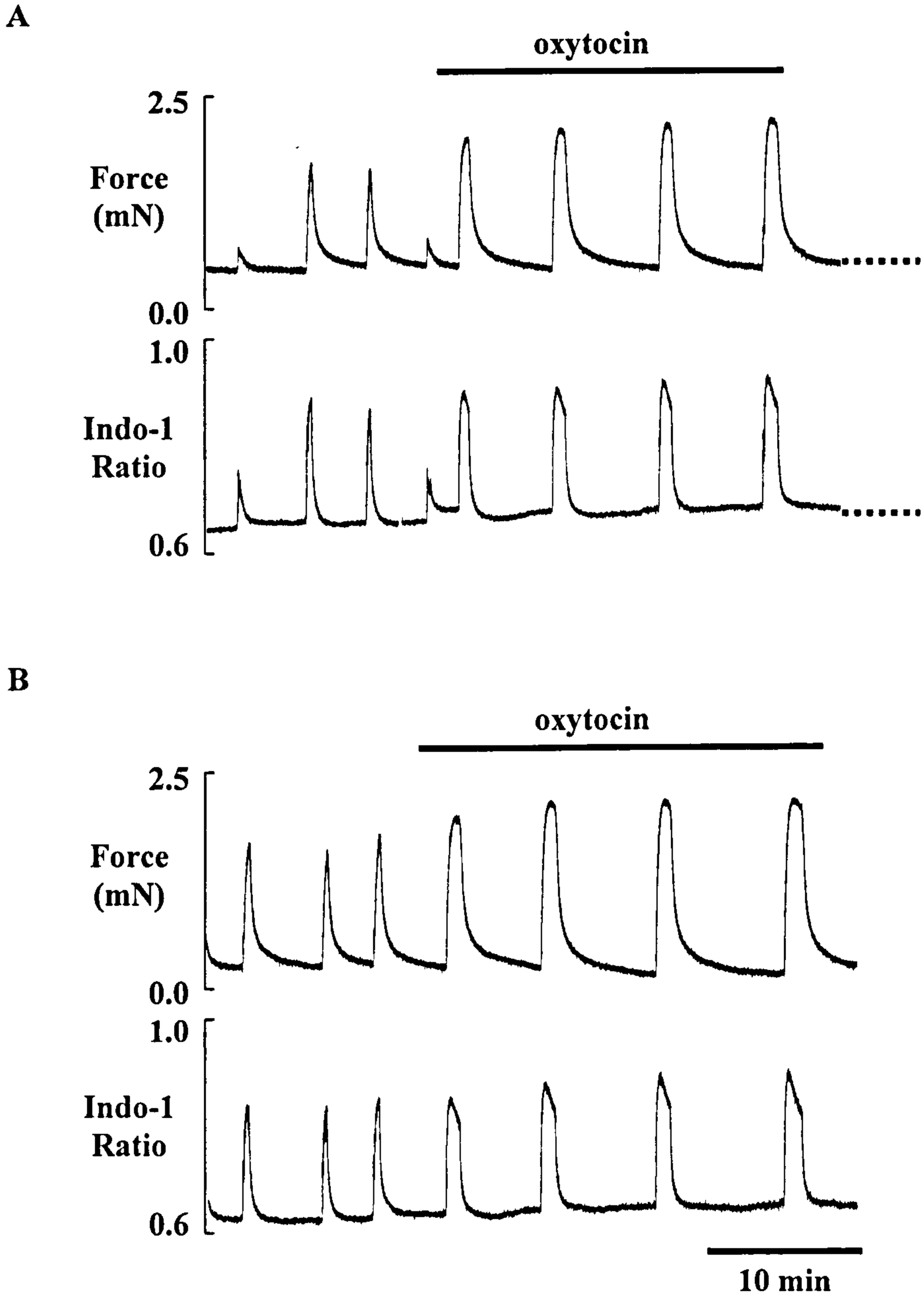
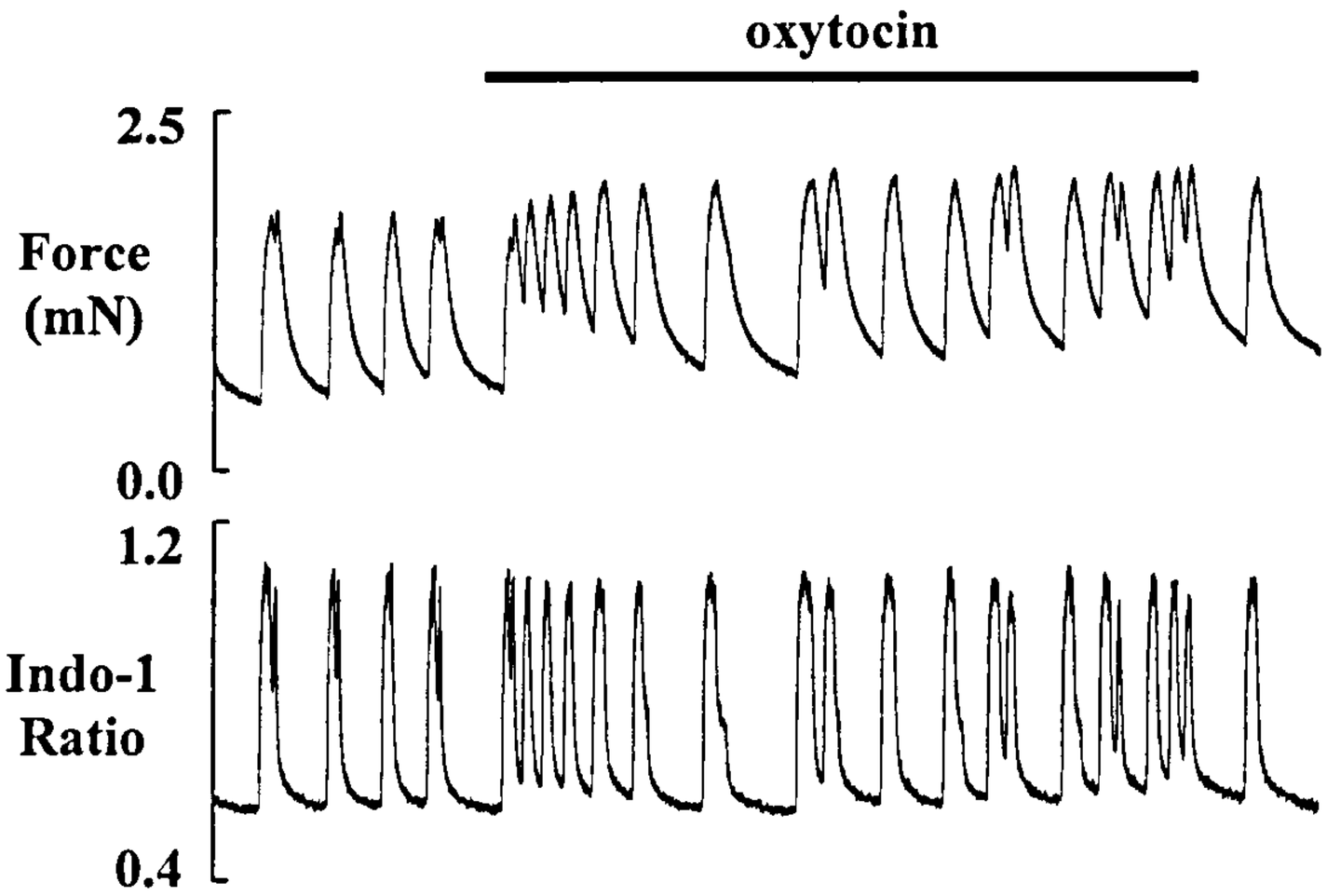


Figure 3.5 The response to oxytocin (10 nM) of the human myometrium. The first **(A)** and second application **(B)**. The experiments were performed on the same strip and recorded continually (dotted line).

A



B

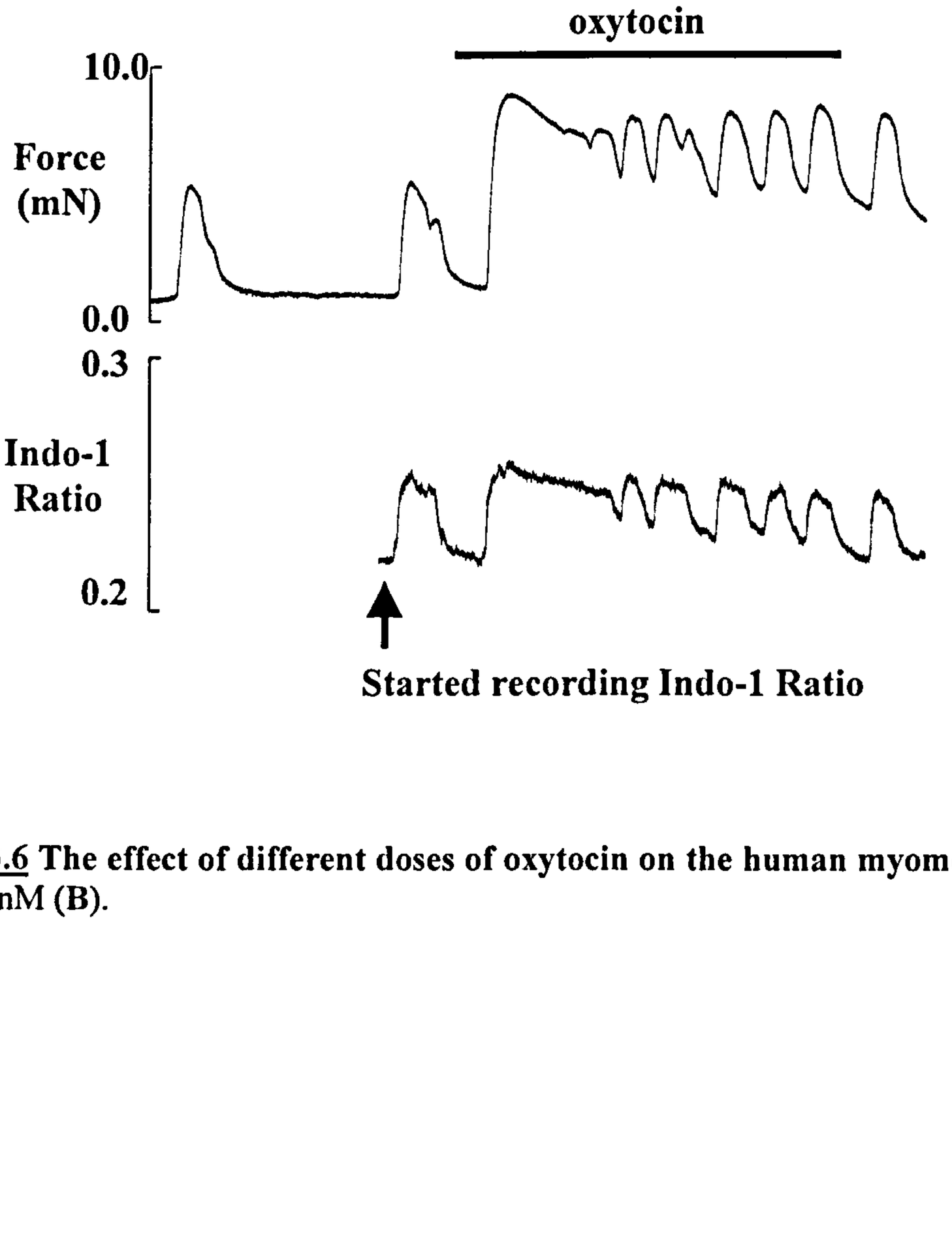


Figure 3.6 The effect of different doses of oxytocin on the human myometrium. 10 nM **(A)** and 100 nM **(B)**.

3.4 Discussion

In this chapter the Indo-1 fluorescence ratio was used to monitor changes in $[Ca]_i$ in the myometrium. The results indicate that Indo-1 can be used to quantify $[Ca]_i$ in spontaneously active human myometrium without interfering with its contractile behaviour (Fig. 3.1). There was a good correspondence between the rise in $[Ca]_i$ and force during the contractions of the uterus with the changes in $[Ca]_i$ preceding those contractions, consistent with the concept that the increase in $[Ca]_i$ causes the increase in force (Wray, 1993, Somlyo & Somlyo, 1994). The relationship between $[Ca]_i$ and force in response to high-K and oxytocin was in keeping with the results described by Szal *et al.* (1994) and McKillen *et al.* (1999), respectively. My data show that reproducible changes in tissue activity can be produced by successive applications of either agonist or high-K depolarisation. The effects observed were also similar to those recorded in rat myometrium, making it meaningful to compare the two tissues.

Having established the background observations on the behaviour of human myometrium *in vitro*, the next chapters present the results to the questions raised in my aims.

Chapter 4

The Effects of Inhibiting the Sarcoplasmic Reticulum on Spontaneous and Oxytocin-Induced Contractions of Human Myometrium

Chapter 4

The Effects of Inhibiting the Sarcoplasmic Reticulum on Spontaneous and Oxytocin-Induced Contractions of Human Myometrium

4.1 Abstract

The aims of this chapter were: 1) to assess the contribution of the sarcoplasmic reticulum (SR) calcium store in the generation of uterine smooth muscle contractions and 2) to evaluate the contribution of calcium induced calcium release (CICR) (ryanodine gated calcium channels), to myometrial force production. Myometrial strips were obtained from women undergoing elective prelabour caesarean section at term. The strips were loaded with the calcium sensitive indicator Indo-1, allowing simultaneous assessment of intracellular calcium concentration and force production. The effects of exposing the strips to ryanodine (which abolishes CICR), caffeine (which activates CICR) and cyclopiazonic acid (which depletes the SR Ca store) were examined. Exposure to ryanodine had no appreciable effect on either the amplitude or the duration of the myometrial calcium and force transients, but did increase the frequency of contractions (139 ± 5%). Caffeine did not potentiate force. Cyclopiazonic acid increased the frequency, duration and amplitude of both calcium and force transients. The ability of oxytocin to provoke calcium and force transients in the absence of extracellular calcium was abolished by cyclopiazonic acid but not by ryanodine. These results demonstrate that CICR does not play a significant role in intact human myometrium i.e. no functioning role for the ryanodine receptors in human myometrial tissue could be shown. These data suggest that the SR may act to limit contractions and act as a calcium sink, rather than to amplify contractions.

4.2 Introduction

As described in chapter 1, it is now well established that a rise in intracellular [Ca] $([Ca])_i$ is associated with contraction, via activation of myosin light chain kinase (MLCK) and phosphorylation of myosin. The rise of $[Ca]_i$ for myometrial contraction may come from two sources; these are: 1) extracellular Ca entry through voltage-gated Ca channels and 2) release of intracellular Ca held within the SR. The relative importance of these two sources remains unclear.

Calcium release from the SR store can occur through inositol trisphosphate (IP_3) gated channels or ryanodine (RyR) gated channels. IP_3 is generated when agonists such as oxytocin, bind to their receptors on the surface membrane. RyR channels are physiologically activated by Ca itself, giving rise to Ca-induced Ca release (CICR). Given the undoubted importance of the SR Ca to the rise of $[Ca]_i$ needed for contraction in striated muscles, it was more or less assumed that this would be its role in smooth muscle. However the contribution of the SR has been questioned following data obtained in non-pregnant rat myometrium. These data demonstrated no effect of SR inhibition on both spontaneous force and Ca transients (Taggart & Wray, 1998b). In addition, ryanodine, a plant alkaloid, can affect the SR by producing a long lasting sub-conductance state of the Ca sensitive Ca channels (low doses), or closing the channel at high doses, also had no effect. These data indicate a lack of functional CICR in rat myometrium (Taggart & Wray, 1998b). In pregnant rat myometrium a small effect was seen when CICR was inhibited with ryanodine, but thus was in the direction of potentiating, not reducing force and Ca (Taggart & Wray 1998). Subtle effects of ryanodine have however been noted, in single cells (Shmygol *et al.* 1998) including sensitivity to caffeine in about 30% of cells (Martin *et al.* 1999b).

In human myometrium the presence of both IP_3 and RyR receptors on the SR membrane, could be taken to indicate that both these Ca-release mechanisms would operate (Lynn *et al.* 1995; Martin *et al.* 1999a, 1999b). However the presence of channels cannot be taken as evidence of functional significance. Luckas & Wray (1999) have recently demonstrated agonist-induced release of Ca and contraction in the absence of external Ca, in human myometrium. These findings are consistent with a functional inositol trisphosphate-induced Ca release (IICR) (Luckas *et al.*1999), although 0-external Ca does not represent at a physiological condition for the tissue. In addition, the SR role seemed to be taking up Ca to aid relaxation, rather than providing Ca for contraction as has been reported in pregnant rat (Taggart & Wray, 1998b; Shmygol *et al.* 1999) and term pregnant human (Tribe *et al.* 2000) myometrium.

It is therefore important to assess the functional role of the SR store under physiological conditions, as factors which influence $[Ca]_i$ and contraction, are clearly important to the control of uterine contraction. The study was designed to evaluate in intact human myometrial strips: 1) the importance of CICR using ryanodine, and IICR using oxytocin, to produce production, 2) the importance of the SR to spontaneous activity, using cyclopiazonic acid (an inhibitor of sarcoplasmic reticulum Ca-ATPase and hence SR Ca filling) and 3) the contribution of the SR to oxytocin-stimulated contraction.

4.3 Materials and Methods

Tissue

For this chapter, human myometrial tissues were obtained from women undergoing elective lower-segment caesarean at term (37 to 39 completed weeks) before the onset of labor. Twenty-three of the 26 caesarean biopsies were performed under spinal analgesia and three while the women were under general anaesthesia. The indications for caesarean delivery were previous caesarean delivery (14 patients), foetal malpresentation (7 patients), patient choice (3 patients), small pelvis (1 patient), and suspected foetal macrosomia (1 patient). The mean age of women undergoing caesarean delivery was 29 years (range, 20-35 years).

Simultaneous measurements of calcium and tension

Tissue preparation and simultaneous measurement of calcium and tension are essentially the same as those described in chapter 2.

After regular spontaneous contractions had been established (0-60 minutes), modulators of the SR were applied; ryanodine, 10-50 μM (a dosage sufficient to inhibit CICR channels in uterine SR, Martin & Ashley, 1995); cyclopiazonic acid (CPA), 10 μM and 20 μM, and caffeine (10 mM). In some experiments 0-Ca solutions were used; Krebs' solution in which $CaCl_2$ had been omitted and 1 mM ethylene glycol bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA) added. In some experiments nifedipine (10 μM) a blocker of L-type Ca channels was used, and in others tetraethylammonium (TEA, 5 mM), a blocker of K channels, were used, as described in the text.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK), except for ryanodine which was from Calbiochem. Oxytocin was dissolved in 5% acetic acid at a concentration of 1 mM. CPA was dissolved in DMSO at a concentration of 20 mM. Nifedipine was dissolved in DMSO at a concentration of 10 mM. These stock solutions were diluted to the desired concentrations with Krebs' solution. Caffeine and TEA was added to Krebs' solution just before used.

Statistics

Data are given as mean and s.e.m. and '*n*' represents the number of samples, each one from a different woman. Significance was tested using appropriate *t* tests or ANOVA and P values < 0.05 taken to be significant. Results are expressed as percentages of control contractions (i.e. the control is 100%).

4.4 Results

4.4.1 The effects of ryanodine on Ca and force

Ryanodine was used to investigate the role of CICR in spontaneous force and Ca transients. The effects of 10 μM, 20 μM and 50 μM ryanodine applied for up to 40 minutes were examined. In all 14 preparations ryanodine had no inhibiting effect on either the Ca or force transient (Fig. 4.1). At each concentration ryanodine had no significant effect on the amplitude (103 ± 5%, $P > 0.05$) or duration (106 ± 2%, $P > 0.05$) of the contractions or accompanying Ca transients ($n = 14$), although in 5/14 there was a small (< 10%) increase in amplitude and in four a small prolongation of the contraction; these effects did not reach statistical significance. In four preparations ryanodine (10 μM) produced a small elevation of baseline Ca (103 ± 2%, $P > 0.05$, not shown). Ryanodine did, however, produce an increase in the frequency of the spontaneous contractions in 11/14 preparations (see Fig. 4.1), resulting in a significant increase in the mean frequency (139 ± 3% compared with control, $n = 14$).

In five preparations the effect of ryanodine on a high-K depolarization of the myometrium was examined. Ryanodine produced no significant effect on either force or Ca; a typical result is shown in Fig. 4.2.

Caffeine is an agonist for ryanodine Ca releasing channels. It has been previously reported to relax the myometrium (Savineau & Mironneau, 1990). For completeness in this study, 10 mM caffeine was applied to spontaneously contracting human myometrium. However the Indo-1 signal is quenched by caffeine (O'Neill *et al.* 1990), making it unreliable to use data on Ca transients in the presence of caffeine. In all cases ($n = 5$) the amplitude of the contractions was decreased and then abolished (Fig. 4.3).

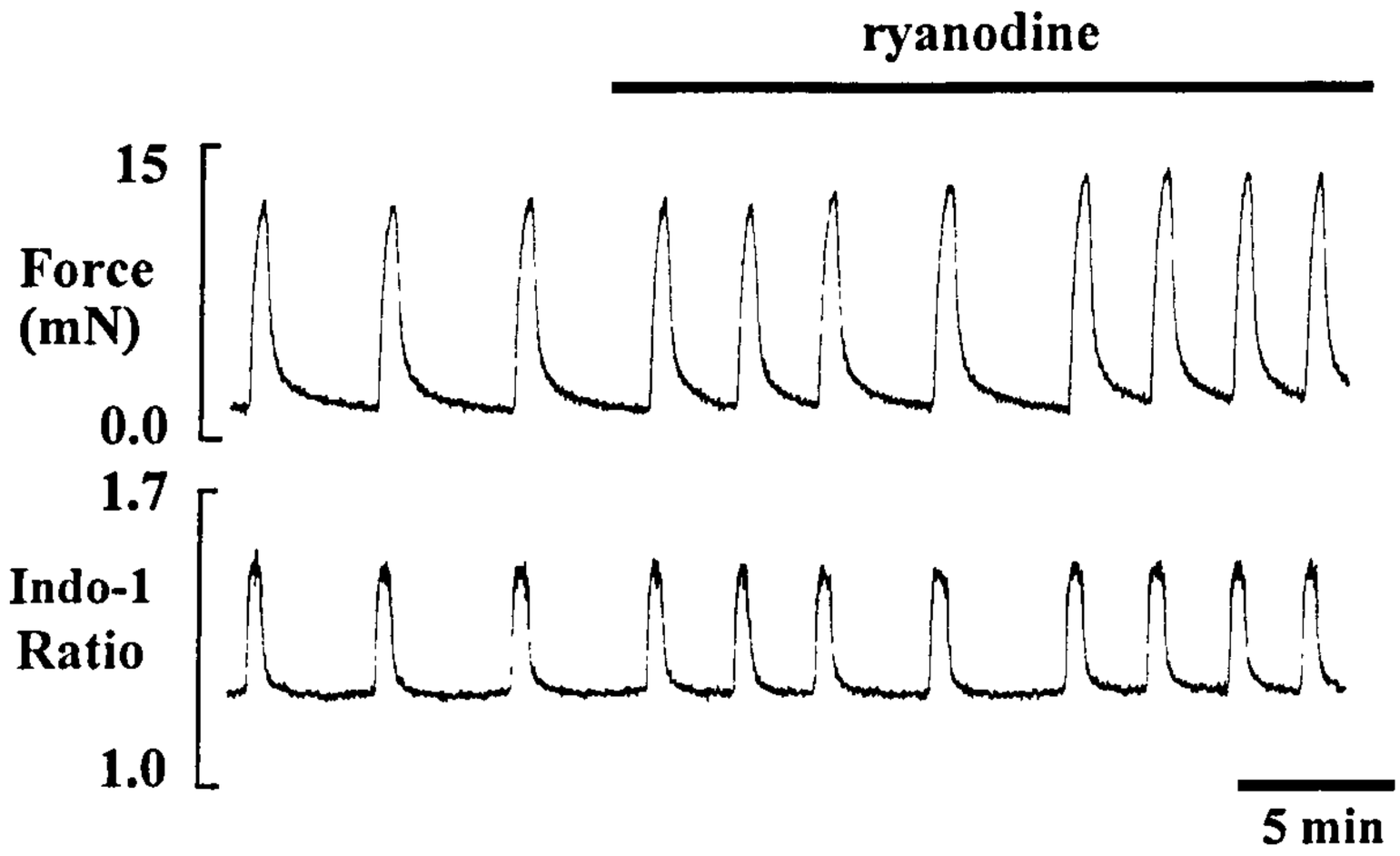


Figure 4.1 The effect of ryanodine on spontaneous contractions. Spontaneous Ca transients (Ratio) and contractions before and after 50 μM ryanodine application.

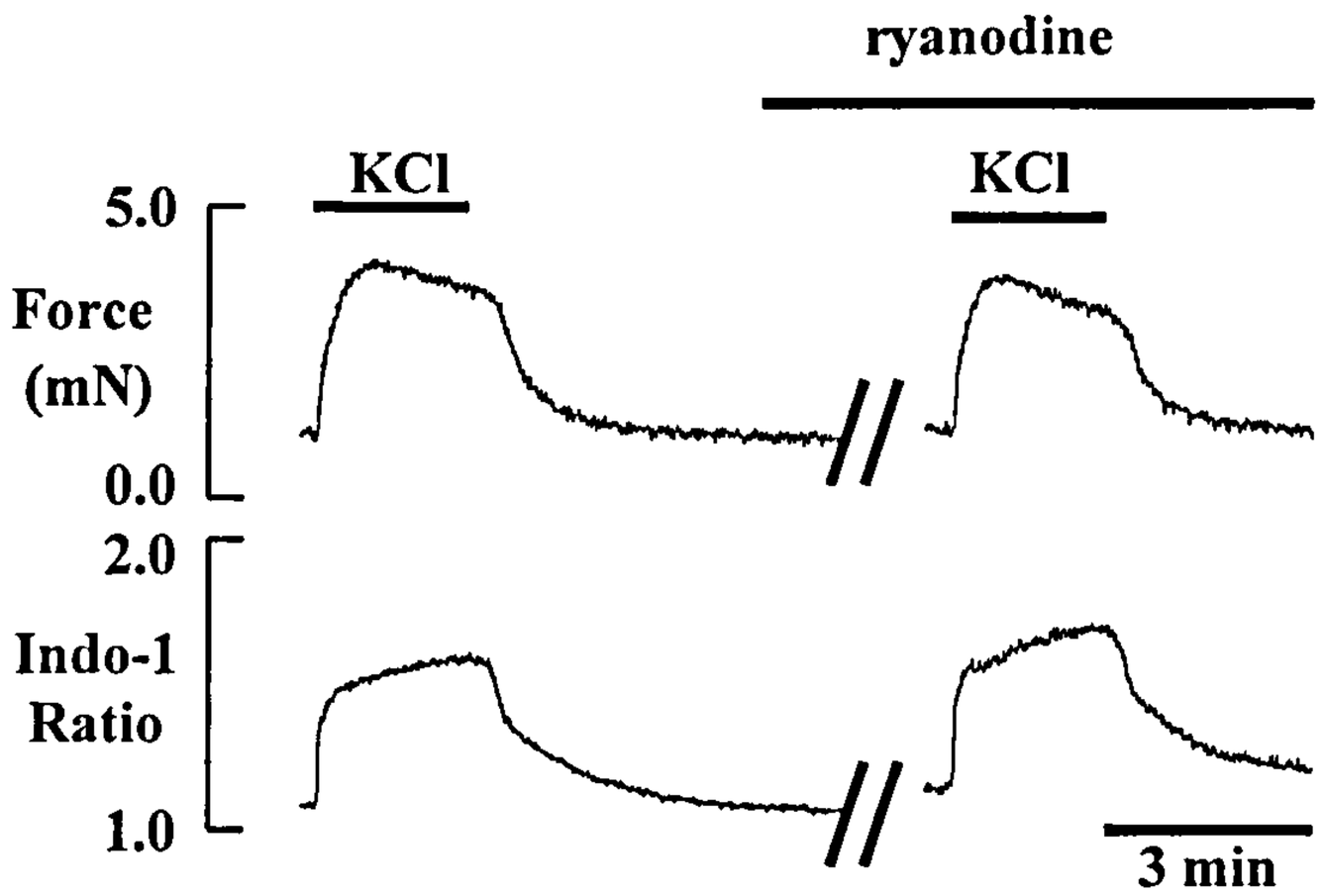


Figure 4.2 The effect of ryanodine on high-K depolarisation. Simultaneous recordings showing the response to high-K (40 mM) depolarisation in the absence and presence of ryanodine. After stable K-induced contraction was obtained, strip was relaxed and incubated for > 20 min treatment with 50 μM ryanodine on K-induced contraction of human myometrium. Breaks in the records represent 20 min periods during which excitation and data acquisition were turned off.

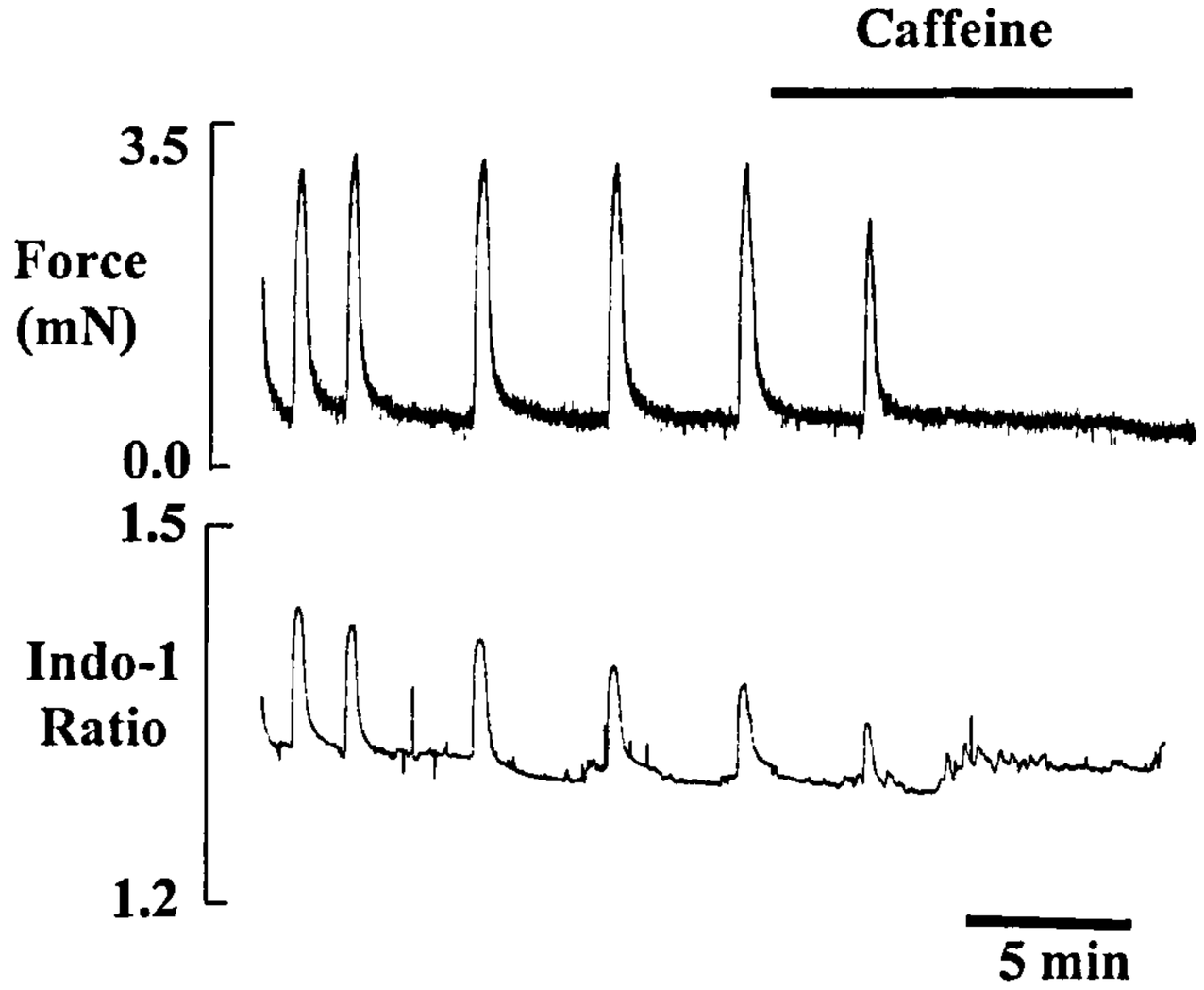


Figure 4.3 The effect of caffeine on uterine spontaneous contraction. Simultaneous measurements of spontaneous Ca transients (Ratio) and contractions before and after caffeine (10 mM) application are shown.

4.4.2 The effects of cyclopiazonic acid on Ca and force

Cyclopiazonic acid is a specific inhibitor of the myometrial SR Ca-ATPase (Kosterin *et al.* 1996), and thus its application disables the SR as it is no longer able to store Ca. Application of cyclopiazonic acid to the pregnant human myometrial preparations produced significant effects on spontaneous Ca and force-both were potentiated (n = 15). In the majority of these preparations, force amplitude (13/15) and duration (10/15) were significantly increased. The frequency of the contractions and Ca transients increased significantly to 131 ± 6%. The amplitude of force and Ca were also significantly increased; 121 ± 3% and 127 ± 4%, respectively, as was their duration; 133 ± 11% and 137 ± 4%, respectively, (all compared with control, 100%). A typical example is shown in Fig. 4.4A. It can be seen in Fig. 4.4B, where control and cyclopiazonic acid records have been expanded and overlapped, that there is a clear effect of cyclopiazonic acid to prolong the force and Ca transients; mostly due to an effect on the plateau phase, but also due to a significant slowing of the relaxation rate (117 ± 6%). In addition, cyclopiazonic acid consistently increased basal Ca (to 117 ± 3% but not force (see Fig. 4.4).

4.4.3 Are the effects of cyclopiazonic acid via K channels?

The increase of $[Ca]_i$ and force following inhibition of the SR by cyclopiazonic acid suggested an inhibitory role for the SR in the myometrium. As Ca release from the SR may activate Ca-activated K channels leading to hyperpolarisation of the membrane and decreased Ca entry and force, this could be the mechanism affected by cyclopiazonic acid (i.e. cyclopiazonic acid prevents this hyperpolarisation). The effects of cyclopiazonic acid on force also resembled those of K channel blockers, which prolong the action potential and thereby potentiate force (Burdyga & Wray, 1999). The

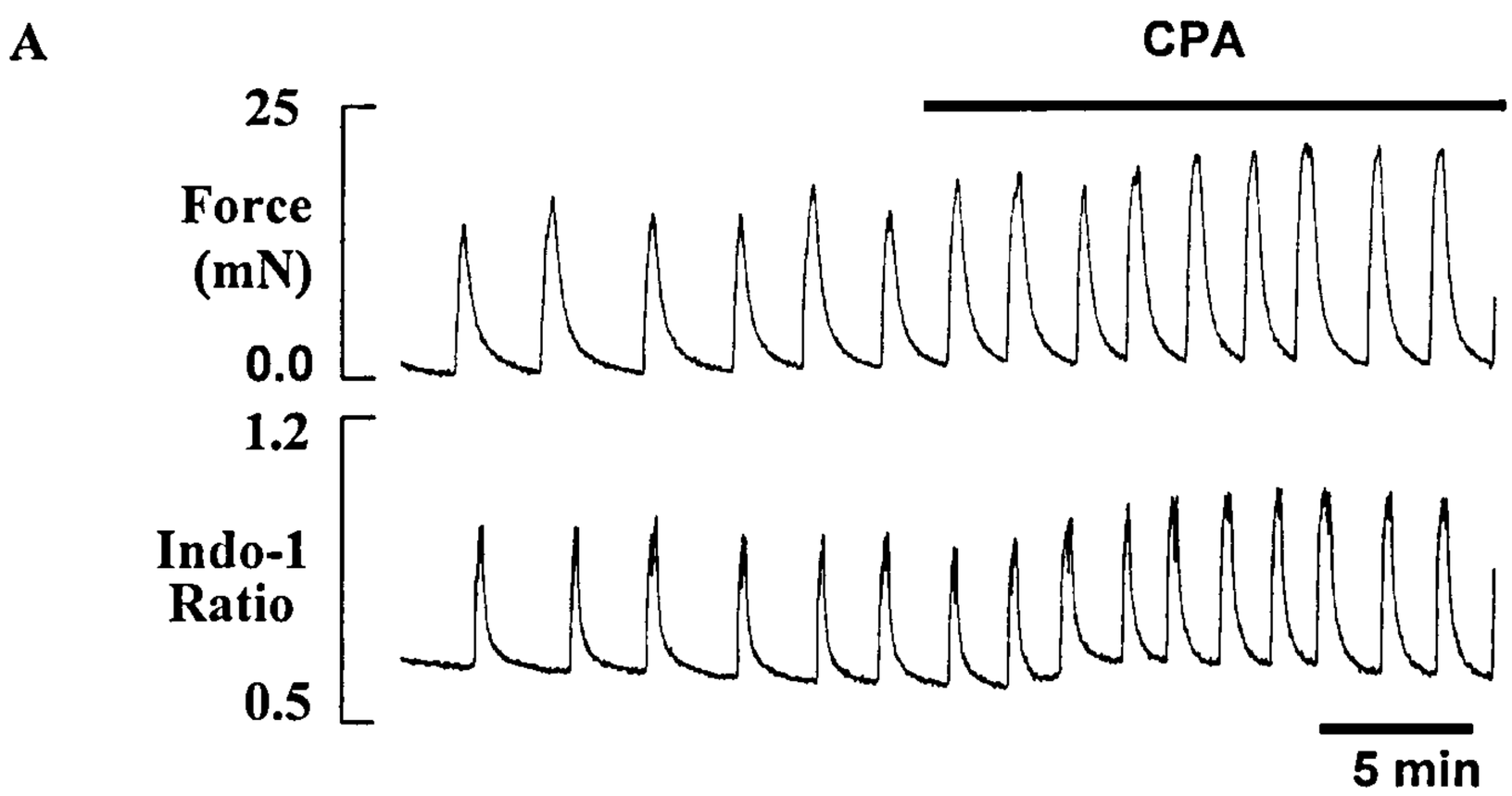


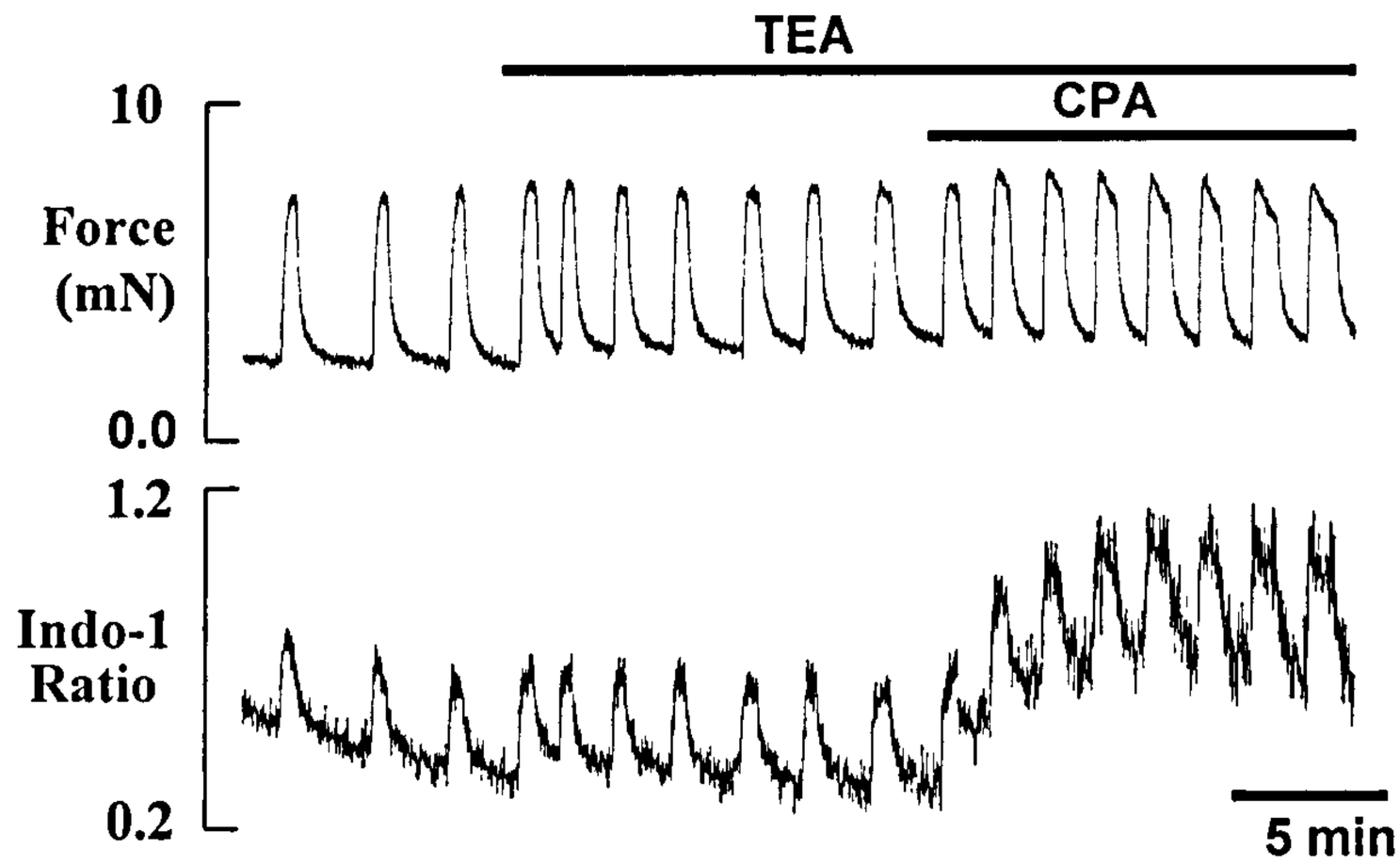
Figure 4.4 The effect of cyclopiazonic acid on contraction. (A) Spontaneous Ca transients and force before and during 20 μM cyclopiazonic acid (CPA) application; (B) superimposed force and Ca records taken from A, under control conditions and in the presence of cyclopiazonic acid (dotted trace).

next experiments were to investigate this possibility by blocking K channels, with TEA (5 mM) and studying the effects of cyclopiazonic acid application (n = 6). The potentiating effect of cyclopiazonic acid was not prevented by inhibition of K channels. The application of TEA increased force and Ca (151 ± 8% and 110 ± 7% compared with control integrated force), but a further increase occurred upon addition of cyclopiazonic acid in the continued presence of K channel inhibition (185 ± 9% and 246 ± 11%, Fig. 4.5A). Similarly if TEA was added after cyclopiazonic acid, it produced a further increase in Ca and force (Fig. 4.5B).

4.4.4 The effect of ryanodine on oxytocin-induced Ca release

The above results suggest that ryanodine has little effect on spontaneous Ca and force in the human uterus. To investigate whether ryanodine could influence agonist-induced Ca release, oxytocin was added in the absence of external Ca entry. This protocol was used to ensure that the only source of Ca was from the SR (Luckas *et al.* 1999). As can be seen in Fig. 4.6A, spontaneous contractions stopped and $[Ca]_i$ fell, upon changing to 0-Ca solution (n = 7). In the presence of the agonist oxytocin (10 nM), but in zero external Ca, some (< 10% of that found in the presence of Ca) force and Ca were produced (n = 7, Fig. 4.6B, in agreement with the previous data, Luckas *et al.* 1999). To investigate the contribution of CICR to the oxytocin-induced changes in force and Ca in zero external Ca, tissues (n = 7) were pre-incubated for up to 20 minutes with ryanodine (10 μM-50 μM) and then oxytocin added. It can be seen that $[Ca]_i$ and force were still produced by oxytocin despite the presence of ryanodine (Fig. 4.6C, n = 7).

A



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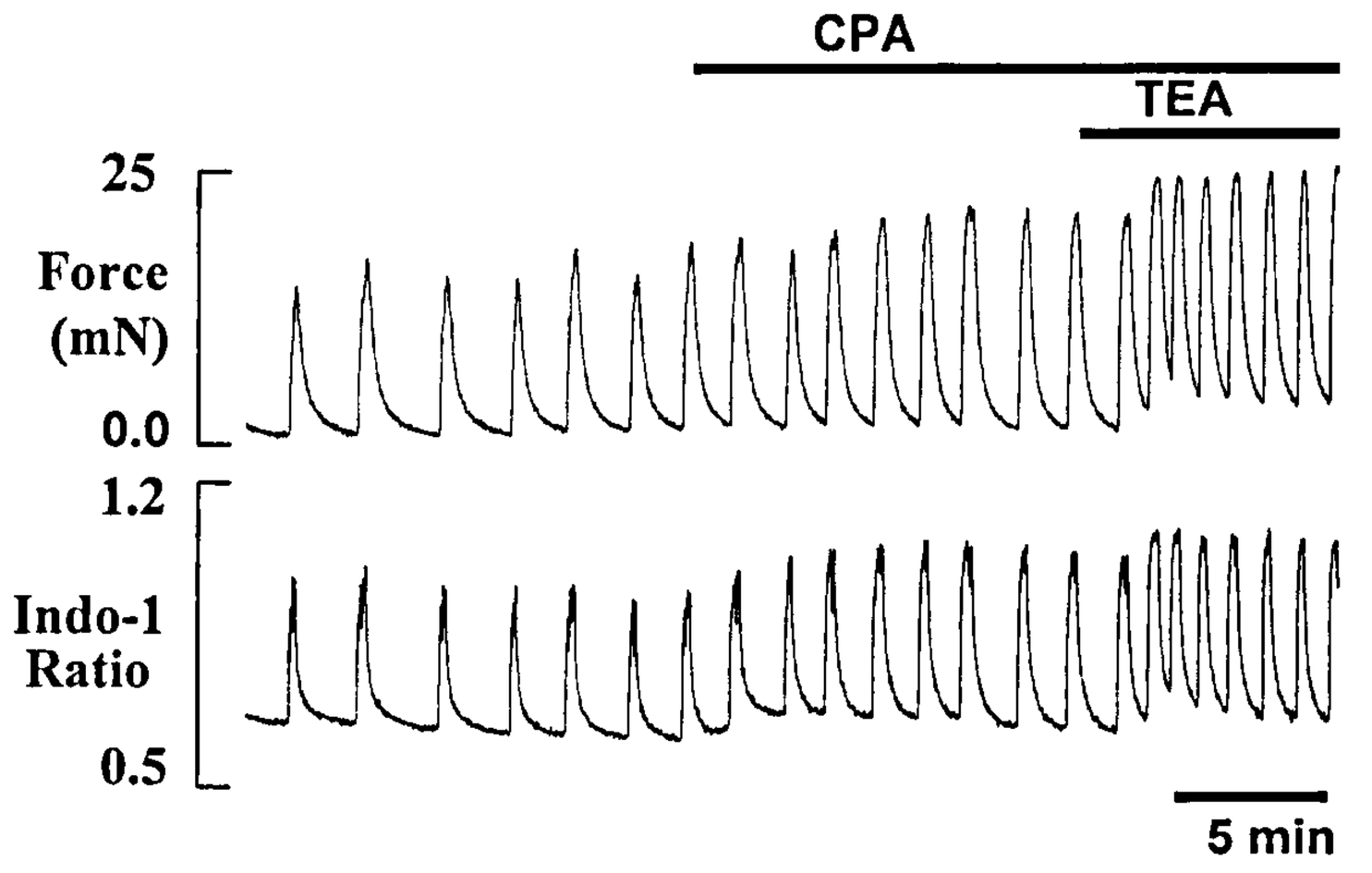


Figure 4.5 The effect of inhibiting the sarcoplasmic reticulum on contraction. Following control recordings the K channel blocker tetraethylamonium (TEA, 5 mM) was added before (**A**) or after (**B**) cyclopiazonic acid (CPA, 20 μM).

4.4.5 The effect of cyclopiazonic acid on oxytocin-induced Ca release

The same protocol as used above for ryanodine, was followed to investigate the effects of inhibiting the SR on oxytocin-induced Ca release. In contrast to ryanodine, cyclopiazonic acid was able to abolish the rise in Ca and force upon oxytocin application to the uterus in 0-Ca solution. An example of this is shown in Fig. 4.6D (typical of 6). Thus it is clear that in the absence of external Ca, force can only be produced by the application of agonist, to release Ca from the SR, and that this is likely to be via IICR and not CICR.

4.4.6 What is the contribution of the sarcoplasmic reticulum and voltage-gated entry in the oxytocin-induced force and Ca response?

From the above data it is clear that in the absence of external Ca, oxytocin can release Ca from the SR and produce force. It is also known that in the presence of external Ca, oxytocin produces Ca entry and potentiates contraction (Batra, 1986). In order to try and assess how much of this potentiation can be attributed to the SR, and how much to external Ca influx, the effect of oxytocin in the presence and absence of cyclopiazonic acid and nifedipine, a blocker of external Ca entry, were compared. Figure 4.7A shows a control response to oxytocin (i.e. in the presence of external Ca and without cyclopiazonic acid). After recovery from oxytocin, the effects of cyclopiazonic acid and then oxytocin were studied. As expected, following cyclopiazonic acid the response to oxytocin is different from control. Nevertheless there are clear and significant increases in force and Ca with oxytocin in the presence of cyclopiazonic acid (n = 4). When the blocker of L-type Ca channels, nifedipine (10 μ M), was added in the continued presence of oxytocin, the force and Ca transients were

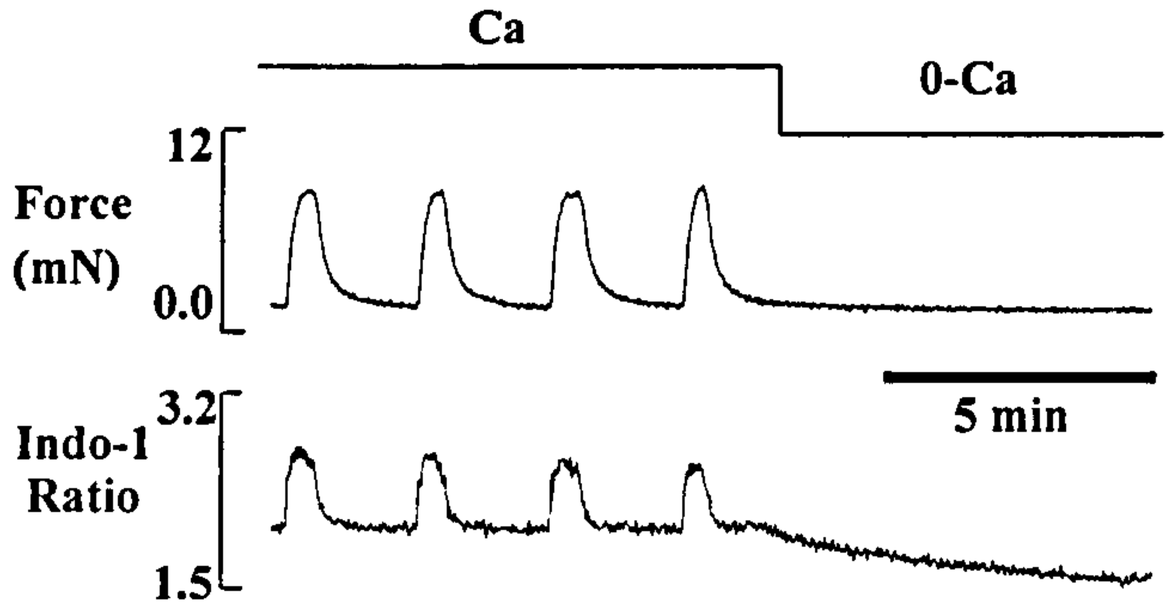
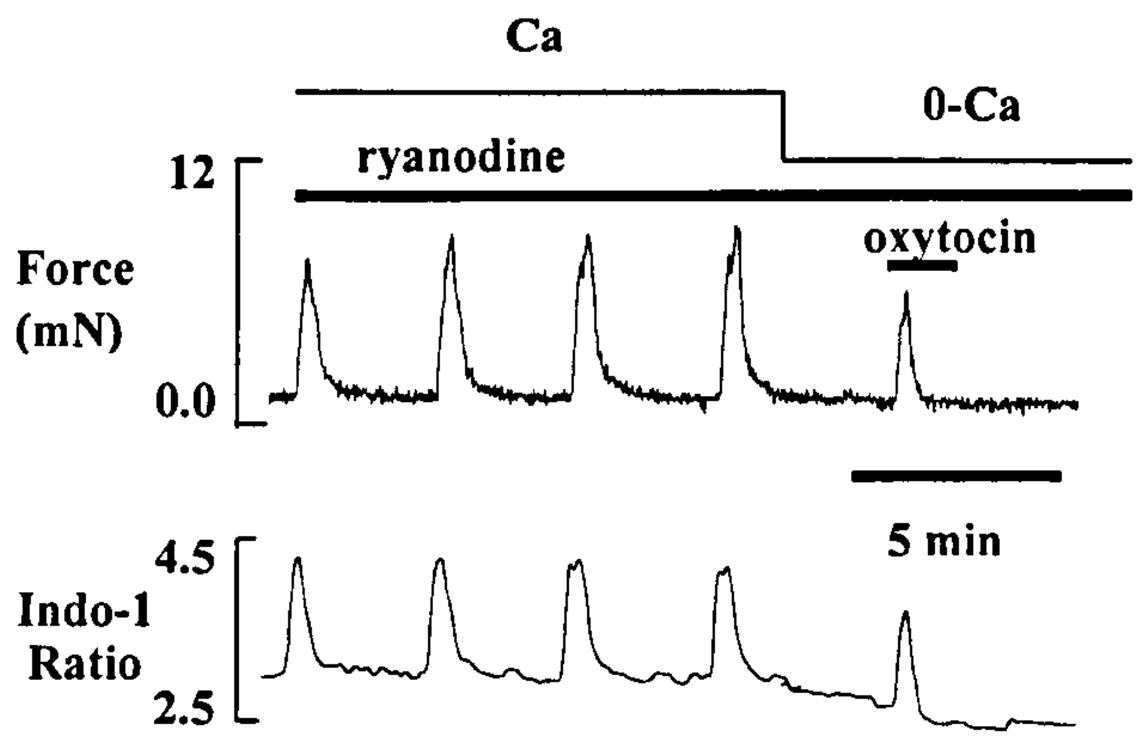


Figure 4.6 (A-B) The role of the sarcoplasmic reticulum in 0-Ca conditions. **(A)** The effects of removing external Ca on spontaneous Ca transients and contractions; **(B)** Oxytocin (10 nM) application in 0-Ca containing solution.

C



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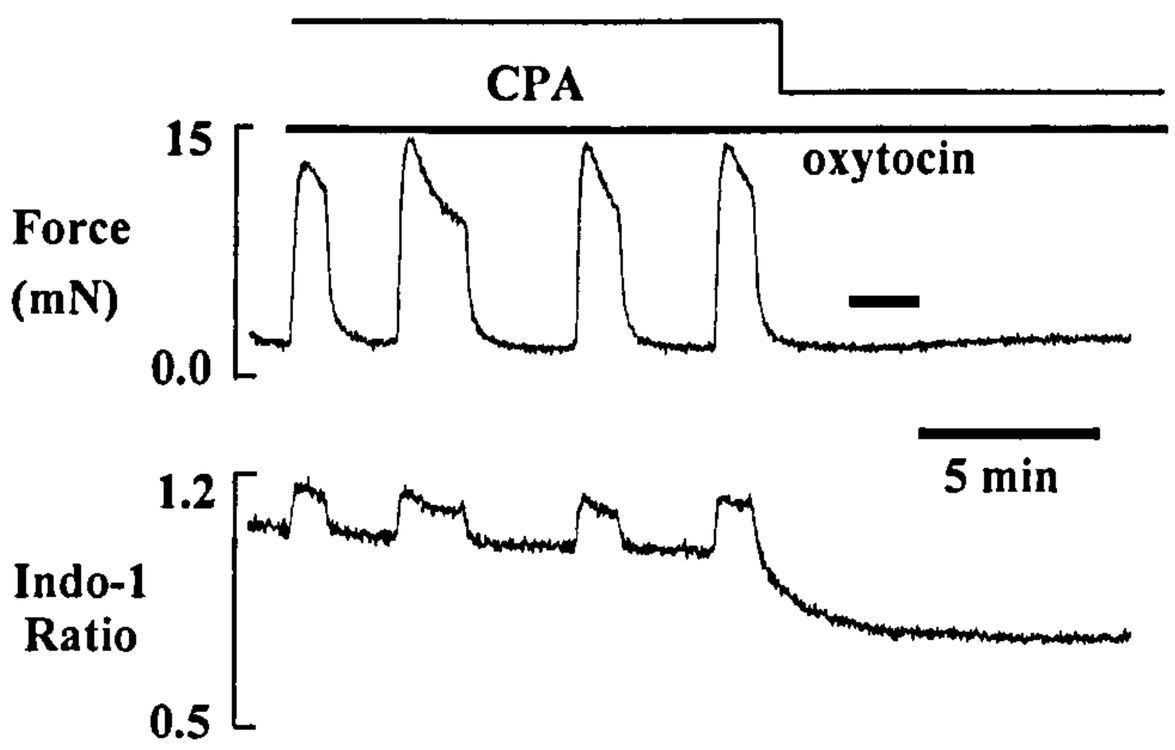


Figure 4.6 (C-D) The role of the sarcoplasmic reticulum in 0-Ca conditions. Application of oxytocin in 0-Ca containing solution after pre-treatment with **(C)** ryanodine or **(D)** cyclopiazonic acid (CPA).

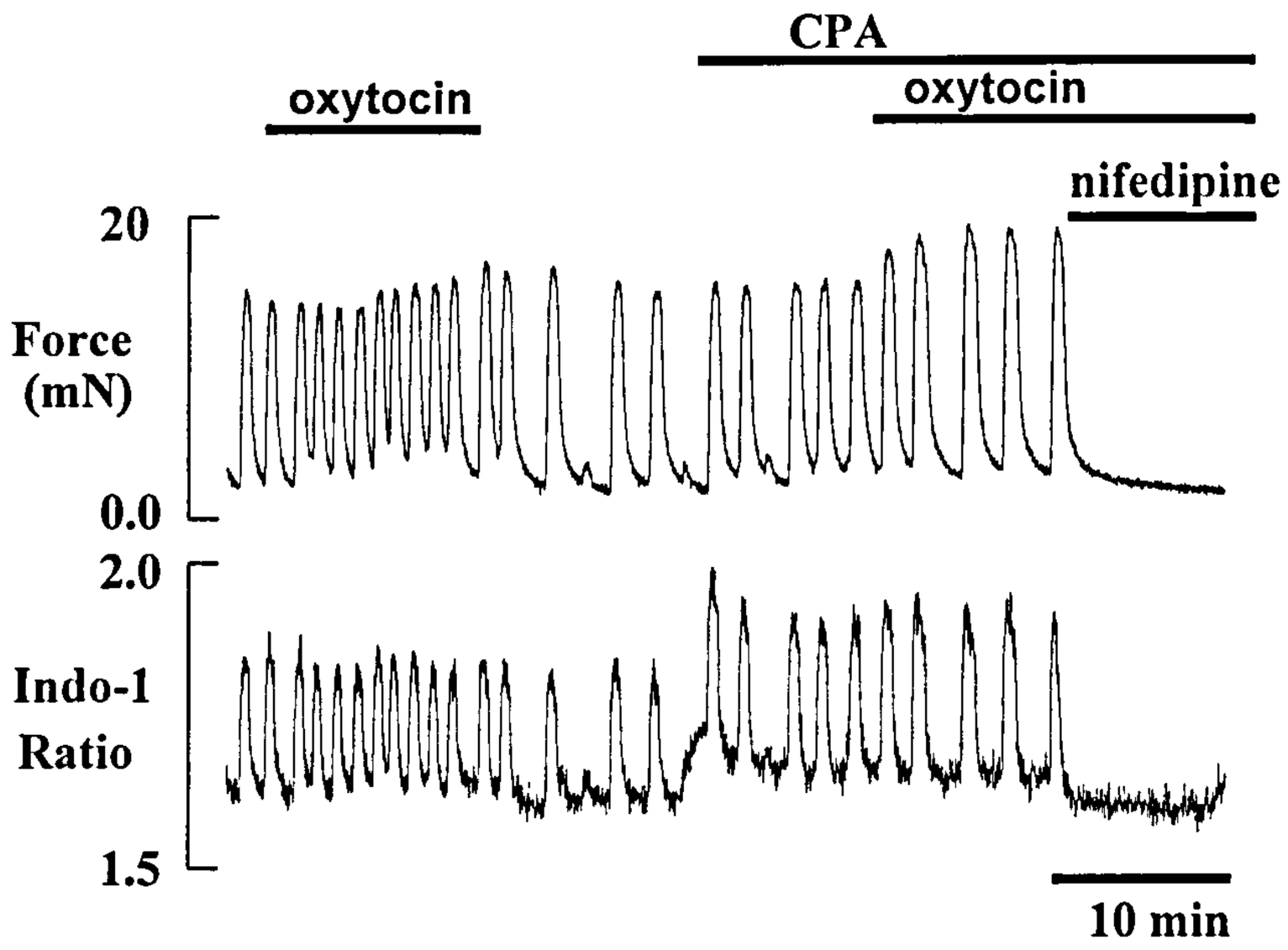


Figure 4.7 Contributions to the effects of oxytocin. Following a control application of oxytocin and recovery, oxytocin was added again following cyclopiazonic acid (CPA) pretreatment, and then nifedipine (10 μM) a blocker of L-type Ca channels.

rapidly abolished. When the protocol was changed so that the effects of nifedipine, without cyclopiazonic acid, on the oxytocin response, could be examined, it was found that very little if any force or Ca were produced (Fig. 4.8A, $n = 3$), despite the presence of the SR. This was the case irrespective of whether nifedipine was added to an oxytocin-induced contraction (Fig. 4.8A) or oxytocin was added after nifedipine (Fig. 4.8B).

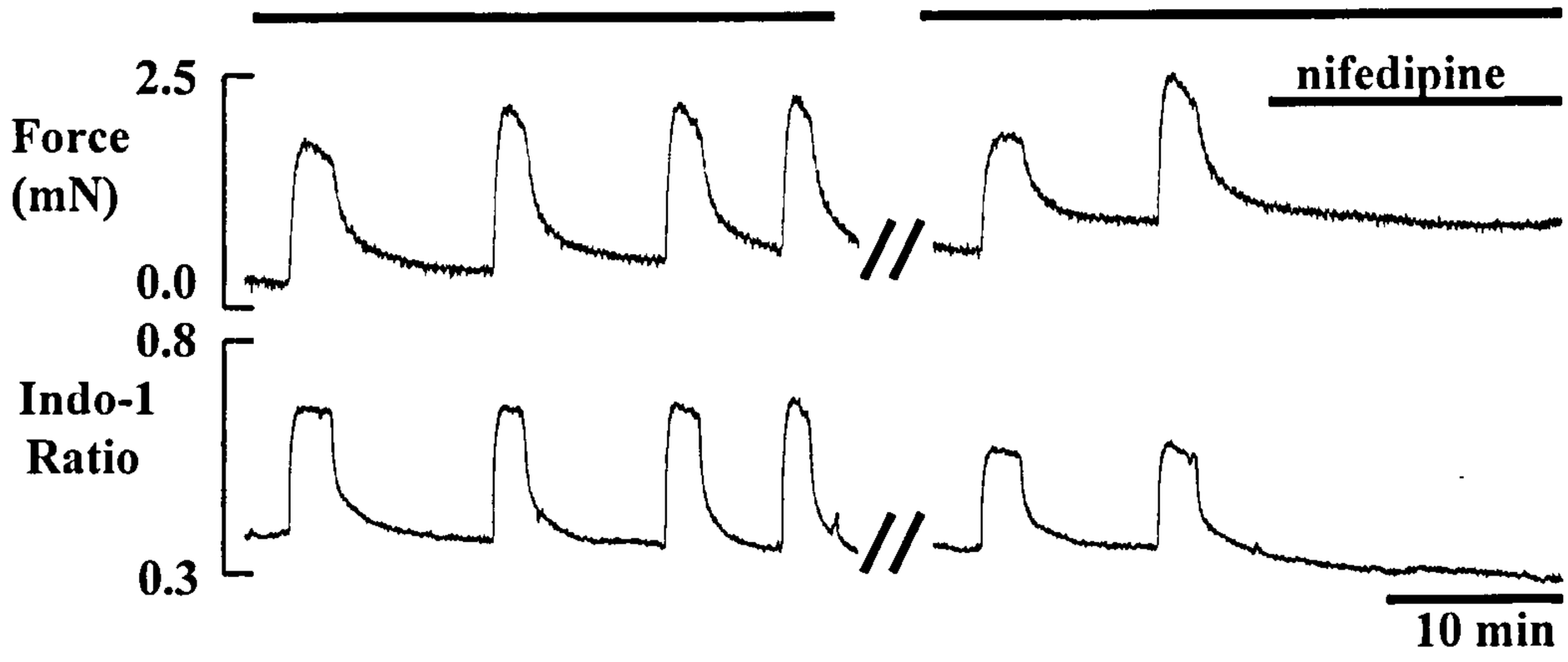


Figure 4.8 The effect of nifedipine on oxytocin-induced contraction. **(A)** Ca transients and contractions in response to oxytocin (10 nM) in the absence and presence of nifedipine (10 μM). **(B)** As in A, but, oxytocin was added following nifedipine pretreatment. Breaks in the records represent 10 min periods during which excitation and data acquisition were turned off.

4.5 Discussion

The aim of this chapter was to examine the role of SR in the human myometrium. The data show no role for CICR as determined by experiments using ryanodine and caffeine in both spontaneous and agonist induced activity in intact preparations. Confirming previous work (Luckas *et al.* 1999), an IP_3 mediated release of Ca and accompanying force can be demonstrated in the presence of agonist, but its contribution is small, compared with Ca influx. It was found that inhibition of the SR was associated with an increase in spontaneous force and Ca transients, suggesting that it is normally operating as a negative influence on contracting. This finding again confirms previous work shown by Tribe *et al.* (2000) in human myometrium. No force is produced in the absence of external Ca, indicating that this is the major source of Ca for spontaneous activity. In the presence of oxytocin a clear role for Ca entry in its mechanism of action was demonstrated.

Ryanodine

It is clear from the data that ryanodine (10-50 μM) had no inhibitory effect on the spontaneous activity or high-K depolarization-induced force, of the human myometrial samples, even after 40 minutes of application. A similar conclusion was reached in rat myometrium (Taggart & Wray, 1998b). In single cells from porcine myometrium, ryanodine also failed to release Ca or change $[Ca]_i$ (Zhu & Hsu, 1995). In addition, caffeine, an agonist of CICR was unable to increase force. These data are consistent with previous studies, which also showed an inhibitory effect of caffeine on myometrial preparations (Savineau & Mironneau, 1990). These inhibiting effects can be explained via a lack of response of the uterine SR to caffeine and an inhibition of phosphodiesterase by caffeine, leading to an elevation of cAMP and hence relaxation

(Chulia *et al.* 1995), and hence it is not straightforward to relate the effects of caffeine to CICR in smooth muscles.

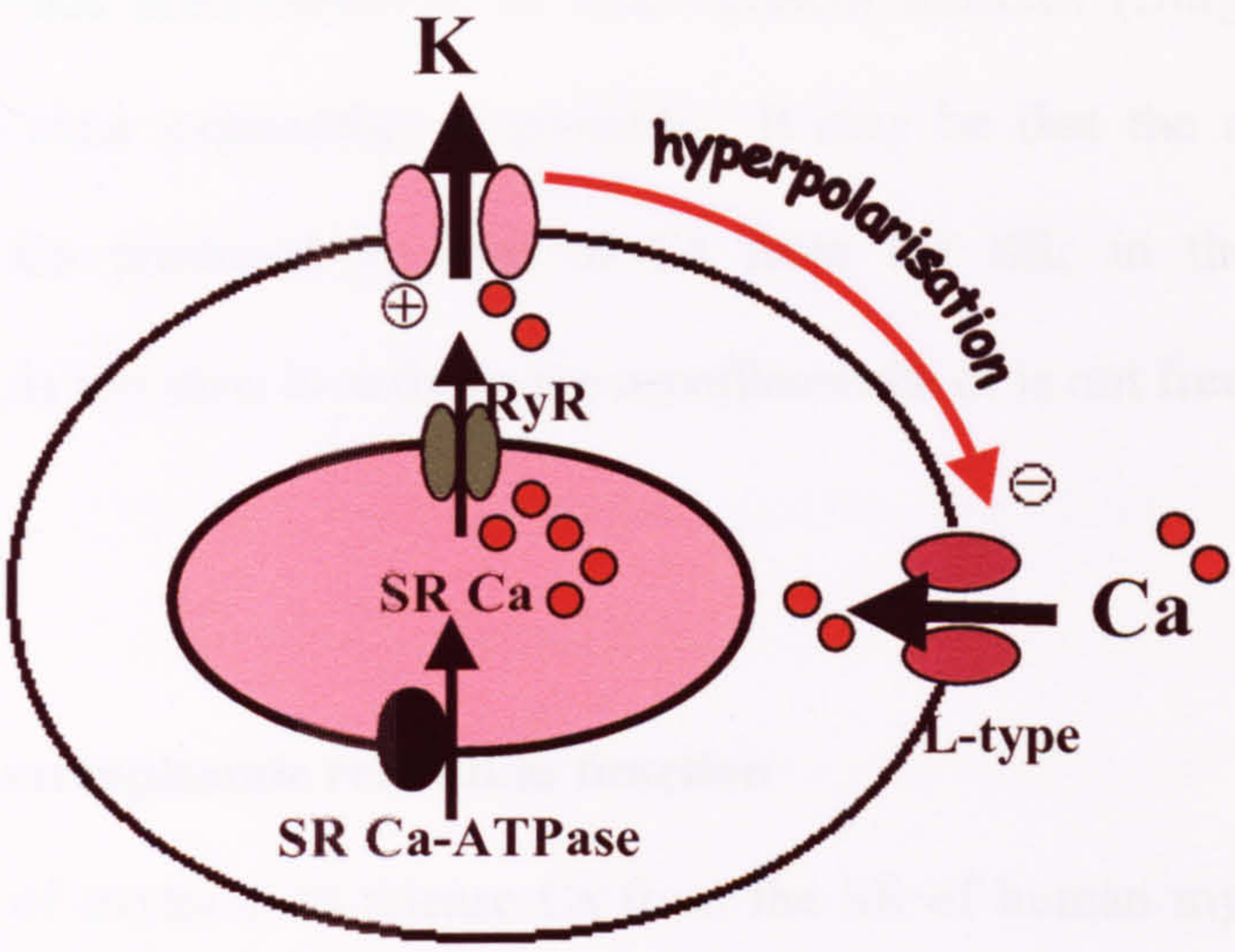
Thus in both human and rat myometrium, the data clearly show that inhibiting CICR does not reduce the rise in $[Ca]_i$, nor force. These data therefore suggest that there is no functional role or coupling of the ryanodine receptors, which have been identified on the SR membrane from myometrial preparations (Lynn *et al.* 1995; Martin *et al.* 1999b). It is not that the SR does not store Ca, because the data show Ca release following agonist stimulation (i.e. via IP_3 receptors), and there is an active Ca-ATPase (Kosterin *et al.* 1996; Shmygol *et al.* 1998). The data on intact preparations clearly represents the functional response from very many myocytes. Recent data on single uterine cells has shown that a response to ryanodine can be seen in around 30% of the cells (Martin *et al.* 1999b). Some evidence for CICR was also obtained electrophysiologically from single rat uterine myocytes (Shmygol *et al.* 1998). However, it is unclear at present what the physiological significance of the data on single cells is, as it does not appear to produce a functional effect in intact tissues. Variability in the expression of ryanodine receptors in late gestation may contribute to the differences reported above (Lynn *et al.* 1995; Martin *et al.* 1999a, 1999b). The potentiating effect of ryanodine on contraction frequency suggests some effect on electrical activity, which will be discussed alongside the data with cyclopiazonic acid below.

Cyclopiazonic acid

Cyclopiazonic acid has been directly demonstrated to inhibit the SR Ca-ATPase in myometrial preparations (Kosterin *et al.* 1996); thus it will functionally disable the SR. The data with cyclopiazonic acid are consistent with this; the Ca and force

transients took significantly longer to recover compared with controls. This suggests that the SR plays a role in lowering cytoplasmic [Ca] following stimulation, as shown in previous studies (Shmygol *et al.* 1999). Clearly the data also show that inhibition of the SR is not associated with a decrease in spontaneous uterine Ca or force transients; indeed they were both significantly augmented. Thus, as with the ryanodine data, there is no evidence in intact human myometrium that a functioning SR augments force production. In fact the question to be addressed is, how does inhibition of the SR increase Ca and force? The experiments shown in this chapter were designed to investigate whether Ca from the SR, released spontaneously, was activating K channels. Such stimulation of calcium activated-K channels (K_{Ca}) channels would prolong the plateau phase of the action potential and delay repolarization, and thereby increase Ca entry and force (Parkington & Coleman, 1990). Such a mechanism was shown to be functioning in ureteric and ileal myocytes, where similar effects of cyclopiazonic acid on force and Ca were found, to those reported here (Uyama *et al.* 1993; Burdyga & Wray, 1999). The data showed that additional effects of cyclopiazonic acid could be seen even after K channel inhibition with TEA, and TEA potentiated force following cyclopiazonic acid addition. Thus it is unlikely that all the effects of cyclopiazonic acid can be explained by such a mechanism. It remains to be established if emptying the SR can promote Ca influx via store-operated mechanisms in smooth muscle (Gibson *et al.* 1998), although it is possible that such a mechanism may be contributing (Tribe *et al.* 2000; Dalrymple *et al.* 2002). It may also be that the uptake of Ca into the SR plays a powerful role in curtailing the normal Ca transient, hence its prolongation in the absence of this uptake mechanism. Clearly direct measurements of SR Ca content, during normal activity and with cyclopiazonic acid would be useful (Shmygol *et al.* 2001). The proposed model for those mechanisms described above is given in Fig. 4.9.

A



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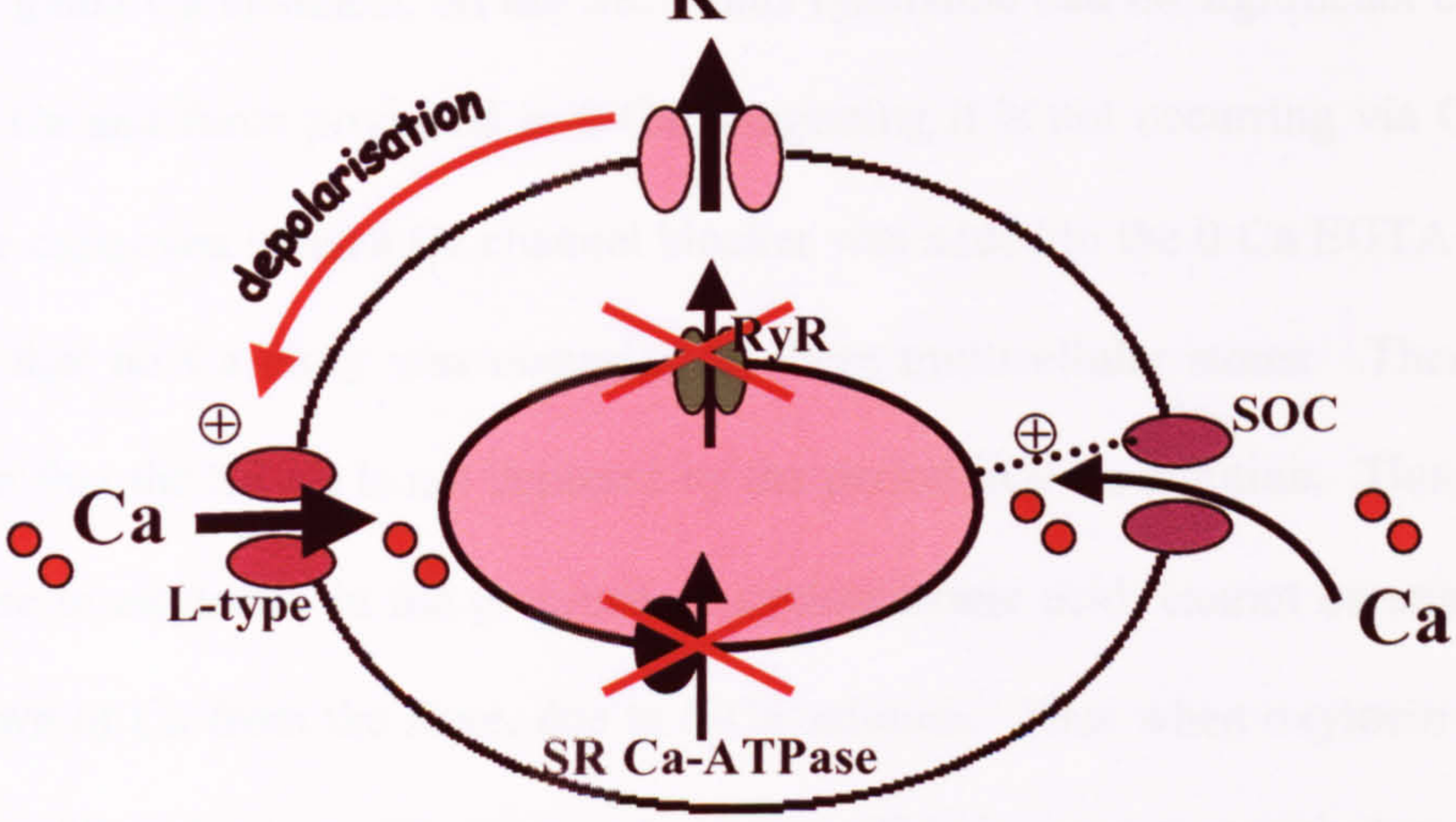

Figure 4.9 Proposed model for the contribution of the SR to uterine contraction. (**A**) With functioning SR. Ca release from SR ryanodine receptor (RyR) produces Ca sparks, which activate K channels. Activation of K channels leads to hyperpolarisation, decreasing entry of Ca through L-type Ca channels, and relaxation. (**B**) Without functioning SR. (e.g. in the presence of an inhibitor of the SR Ca-ATPase). Inactivation of K channels has the opposite effect. Also illustrated is a contribution of store-operated Ca (SOC) entry mechanism.

The increase in basal Ca with little or no change of basal tone, produced by cyclopiazonic acid has been observed in other smooth muscles (Burgyga & Wray, 1999). As yet no clear explanation is possible. It may be that the relatively slow increase in basal Ca produced by leak of Ca from the SR, in the presence of cyclopiazonic acid, is too slow to activate the myofilaments, or is not freely available to them.

Oxytocin and the sarcoplasmic reticulum function

The ability of oxytocin to release Ca from the SR of human myometrium and produce force, has been previously demonstrated (Kasai *et al.* 1994; Luckas *et al.* 1999; McKillen *et al.* 1999). The data obtained from this chapter show that the mechanism is via IP_3 gated Ca channels, on the SR. Thus ryanodine had no significant effect on the rise of Ca and force produced in 0-Ca, suggesting it is not occurring via CICR. This was the case even when a Ca channel blocker was added to the 0-Ca EGTA solution, to ensure that no Ca entry was occurring in these multicellular stores. These data also confirm that the SR Ca is not depleted by the period in 0-Ca solution. Thus the lack of response to oxytocin, in the presence of cyclopiazonic acid, cannot be explained by a run down of Ca from the store, due to 0-Ca solution. Thus when oxytocin releases Ca from the SR, it is via an IP_3 mediated process. This is consistent with data showing an increase in IP_3 when the myometrium is stimulated with oxytocin and that IP_3 can release Ca from myometrial SR preparations (Phaneuf *et al.* 1993; Izumi *et al.* 1995b; Holda *et al.* 1996).

Having demonstrated the ability of oxytocin to release Ca from the SR and produce uterine force, it is pertinent to ask what contribution this process makes to the augmentation of force seen with oxytocin, under physiological conditions (i.e. in the

presence of external Ca)? To address thee experiments with oxytocin and Ca in the presence and absence of cyclopiazonic acid were undertaken. Because cyclopiazonic acid itself alters force it is difficult to exactly compare the oxytocin responses before and after cyclopiazonic acid. However, the data have shown that the levels of force and Ca are approximately comparable. This data and those obtained with oxytocin in Ca-free solutions, showing very small effects on force and Ca, suggest that oxytocin effects on trans-sarcolemmal Ca movements contribute mostly to its stimulatory actions on the human uterus. There appear to be differences between labouring and non-labouring tissues (Riemer & Heymann, 1998; Tribe *et al*. 2000), but this could not be addressed in the current study.

Conclusion

Inhibition of SR function in intact myometrium from pregnant, but non-laboring women, does not decrease Ca or force. Indeed when the SR is emptied via cyclopiazonic acid, both are increased, indicating that under normal conditions the SR is functioning to limit the Ca and contraction. This may be partly due to the active uptake of Ca, partly due to stimulation of K_{Ca} channels and perhaps by some additional route, such as store-operated Ca uptake. Although the SR has RyR receptors, there is no functional role for them in this preparation. Finally oxytocin promotes force in the uterus by a variety of mechanisms which may include a contribution from Ca released by IP_3 from the SR, but which is predominantly on Ca entry.

Chapter 5

The Effects of Inhibiting Myosin Light Chain Kinase on Contraction and Calcium Signalling in Human and Rat Myometrium

Chapter 5

The Effects of Inhibiting Myosin Light Chain Kinase on Contraction and Calcium Signalling in Human and Rat Myometrium

5.1 Abstract

The effect of inhibiting myosin light chain kinase (MLCK) on contractions of human and rat myometrium has been investigated, to determine whether force can be produced independently of myosin phosphorylation. Wortmannin, a MLCK inhibitor was used, and its effect on spontaneous, high-K-depolarisation-induced and oxytocin-induced force studied. The inhibitor reduced and then abolished uterine force, irrespective of how it was produced; this was the case for both human and rat myometrium, and pregnant and non-pregnant tissue. The effect of wortmannin on intracellular [Ca] ($[Ca]_i$) was examined. The data showed that the reduction in force produced by wortmannin occurs without a reduction of $[Ca]_i$. It is concluded that, under normal physiological conditions, MLCK phosphorylation of myosin is essential for uterine force production and that there is little or no role for alternative force-producing pathways.

5.2 Introduction

Control of uterine smooth muscle is clearly important for preventing pre-term labour and ensuring strong and co-ordinated contractions during labour. As with other smooth muscles, changes in intracellular [Ca] ($[Ca]_i$) underlie uterine contractions but the relationship between $[Ca]_i$ and force may not be simple (Szal *et al.* 1994). Agonists in particular may act to alter the events subsequent to $[Ca]_i$ rising in the uterine cell. The main force-activating pathway is taken to be Ca-calmodulin (CaM) activation of myosin light chain kinase (MLCK) and phosphorylation of myosin light chains (Wray, 1993). An increase in phosphorylated myosin with contraction has been shown to occur in the human myometrium (Mackenzie *et al.* 1990). Recently, however, it has been become clear that agonists can modify this process (Izumi *et al.* 1995a). For example, MLCK and the phosphatase that dephosphorylates myosin can be phosphorylated and hence their activity decreased. In this way the relationship between force and Ca may be changed (Horowitz *et al.* 1996b). In addition alternative pathways have also been proposed (Somlyo & Somlyo, 1994) that depend upon kinases, such as RhoA, and initiate a cascade of events which may not require an elevation of $[Ca]_i$, culminating in force production. For example, mitogen-activated protein (MAP) kinase phosphorylation of thin-filament-associated proteins (calponin and caldesmon) leads to the removal of their inhibitory influence of actin-activated myosin ATPase activity (Nixon *et al.* 1995; Word, 1995; Horowitz *et al.* 1996b). Recently oestrogen and progesterone have been shown to modulate the levels of Rnd-1, a kinase that interacts with the Rho 1A pathway (Loirand *et al.* 1999). Thus it is tempting to speculate that such pathways may contribute to force production during labour.

Several authors have shown that oxytocin can elicit a contraction in 0-Ca solution (Matsuo *et al.* 1989; Oishi *et al.* 1991) and it has been suggested that this arises

from its stimulation of protein kinase C (PKC) (Karibe *et al.*1991; Oishi *et al.* 1991; Horowitz *et al.* 1996a). PKC in turn may activate MAP kinase (Horowitz *et al.* 1996b). It has been reported that this contraction occurs without myosin phosphorylation (Oishi *et al.* 1991).

Thus the question arises, "what is the relative importance in uterine contractions of the Ca-CaM-MLCK pathway compared to the alternative or modulatory pathway?" In order to investigate this, a selective inhibitor of MLCK, wortmannin (Nakanishi *et al.* 1992; Takayama *et al.* 1996) was used. The aims of this chapter were; 1) to determine the effects of inhibiting MLCK on uterine force produced spontaneously, by depolarisation with high-K solution and by the agonist oxytocin, in the presence and absence of Ca, 2) to investigate the effect of inhibiting MLCK on $[Ca]_i$, and 3) to compare the effects on uterine from women and rats, and pregnant and non-pregnant animals.

The results showing in this chapter were obtained in conjunction with E.R. Longbottom and M.J.M. Luckas.

5.3 Materials and Methods

Tissue

For this chapter, human myometrial tissues were obtained from women aged 17-38 years (mean 27 years) undergoing (elective) caesarean sections (37-41 weeks, mean 38.5 weeks) from the lower segment. Thirty-seven biopsies were performed under spinal analgesia. The indications for caesarean delivery were previous caesarean delivery (19 patients), foetal malpresentation (7 patients), patient choice (1 patient), multipregnancy (5 patients), and previous traumatic delivery (5 patients).

Animal tissues were obtained from non-pregnant rat at 200 g and pregnant rats of approximately the same age with 18 and 21 days of gestation.

Simultaneous measurements of calcium and tension

Tissue preparation and simultaneous measurements of calcium and tension are essentially the same as those described in chapter 2.

The tissue was continually perfused with Krebs or Krebs containing high-K, wortmannin (0.5-4 μM), or oxytocin (10 nM). K was elevated and Na reduced to depolarise the membrane in some experiments. In 0-Ca experiments $CaCl_2$ was omitted from the solution and 1 mM ethylene glycol bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA) added.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK). Oxytocin was dissolved in 5% acetic acid at a concentration of 1 mM. Wortmannin was dissolved in DMSO at a concentration of 5 mM. These stock solutions were diluted to the desired concentrations with Krebs' solution.

Statistics

The data were analysed and statistical significance was tested with the Student's *t*-test on paired samples, at 0.05 level. All values represent mean ± s.e.m, '*n*' is the number of samples.

5.4 Results

5.4.1 Effects of wortmannin on human myometrium

Spontaneous activity

Under control conditions spontaneous contractions of consistent amplitude and frequency could be recorded for several hours. Wortmannin (4 μM, for the concentration used see below) reduced force in all preparations studied (n = 15). Its effect was gradual, as can be seen in Fig. 5.1; a significant reduction occurred after 10 min (mean amplitude of contractions 37 ± 8%, compared to 100% control, $P < 0.05$) and by 20 min contractions were essentially abolished (2 ± 2%). After this exposure to wortmannin, neither high-K (40 mM) nor oxytocin (10 nM) was able to produce any force in the myometrium (n = 5, data not shown). Five preparations of human myometrium were left in control solution for 1 h following wortmannin application, to monitor resumption of contractile activity. Compared to control activity (100%), force amplitude recovered to 20% within this period.

Oxytocin-induced contractions

As expected, under control conditions, oxytocin (10 nM) increased contractile activity compared to spontaneous activity (Fig. 5.2, n = 4). As shown in Fig. 5.2, wortmannin abolished oxytocin-induced force.

High-K depolarisation

By elevating K and depolarising the surface membrane, the effects of wortmannin on force production, independent of pacemaker activity, could be examined. In addition the uniform response of tissues to high-K (40 mM) depolarisation

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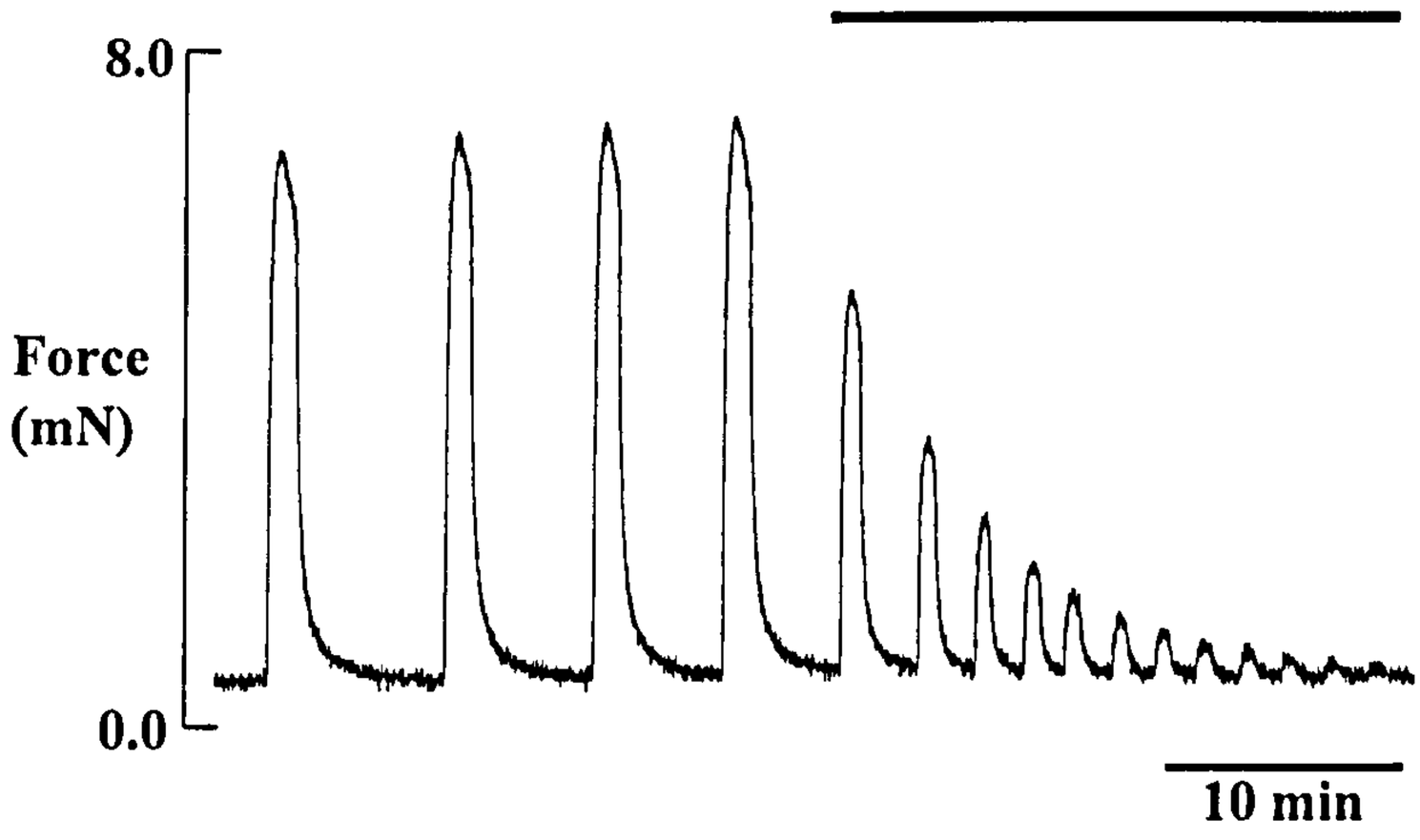

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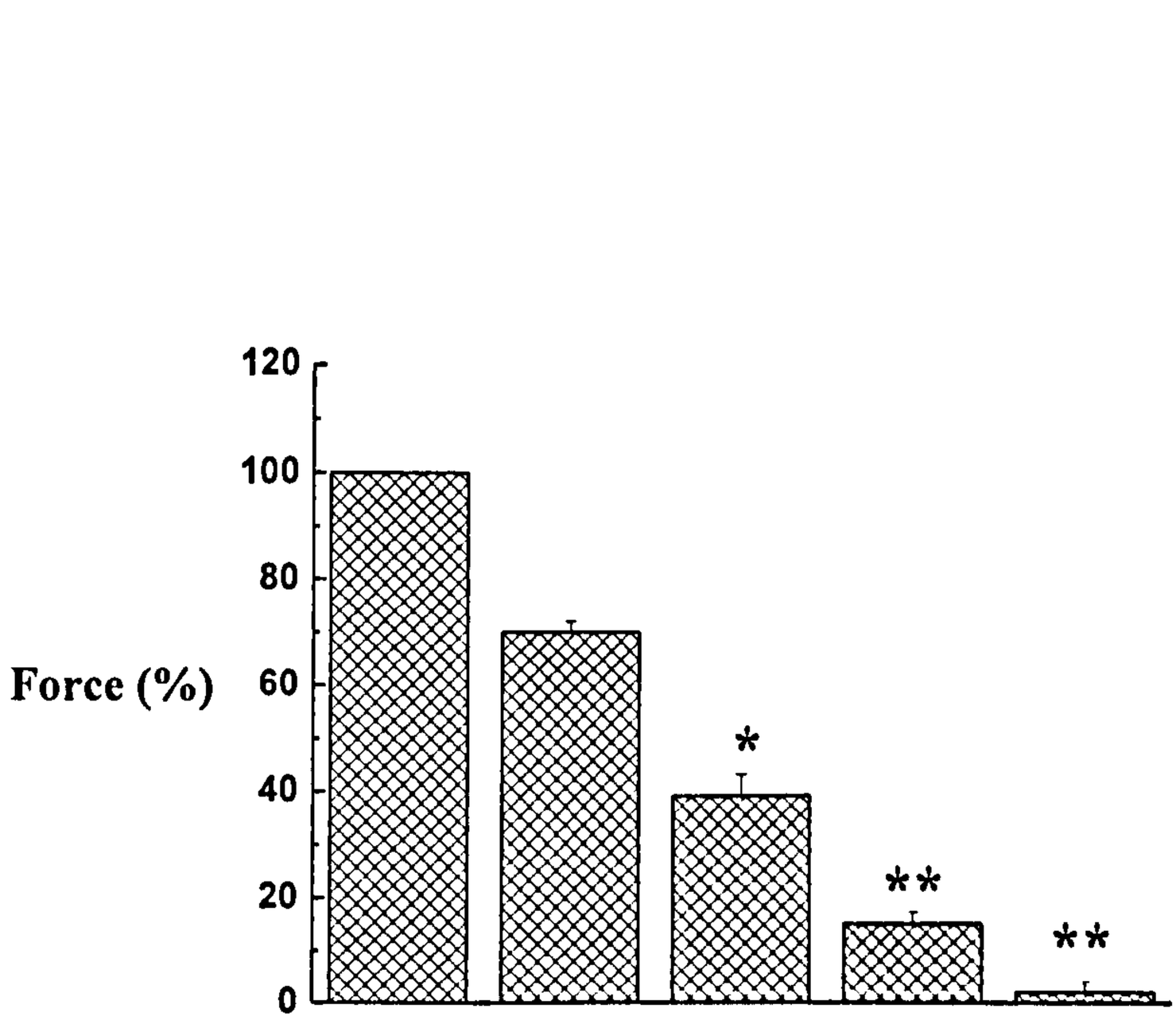

Figure 5.1 The effect of wortmannin on spontaneous contractions of human myometrium. (A) Spontaneous force transients in the absence (control conditions) and presence of wortmannin (4 μM). (B) Show the time courses of spontaneous contraction in the presence of wortmannin ($n = 4$). Force development is expressed as a percentage of the maximal force of the spontaneous phasic contraction without wortmannin (denoted as C). The force was measured 5, 10, 15, and 20 min (1, 2, 3, and 4 respectively) after wortmannin addition. * and **, significantly different from the force without wortmannin for $P < 0.05$ and $P < 0.01$, respectively.

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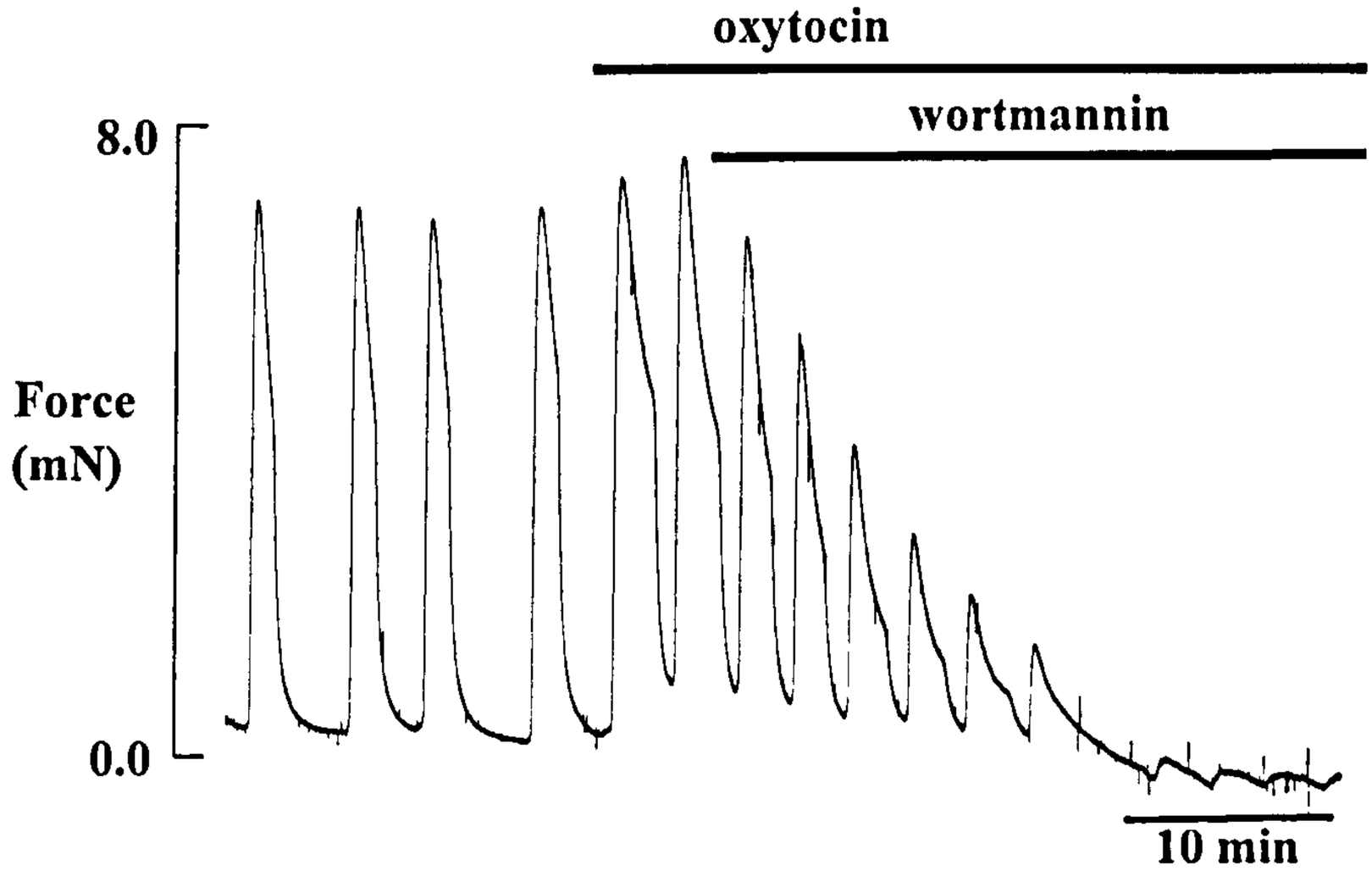

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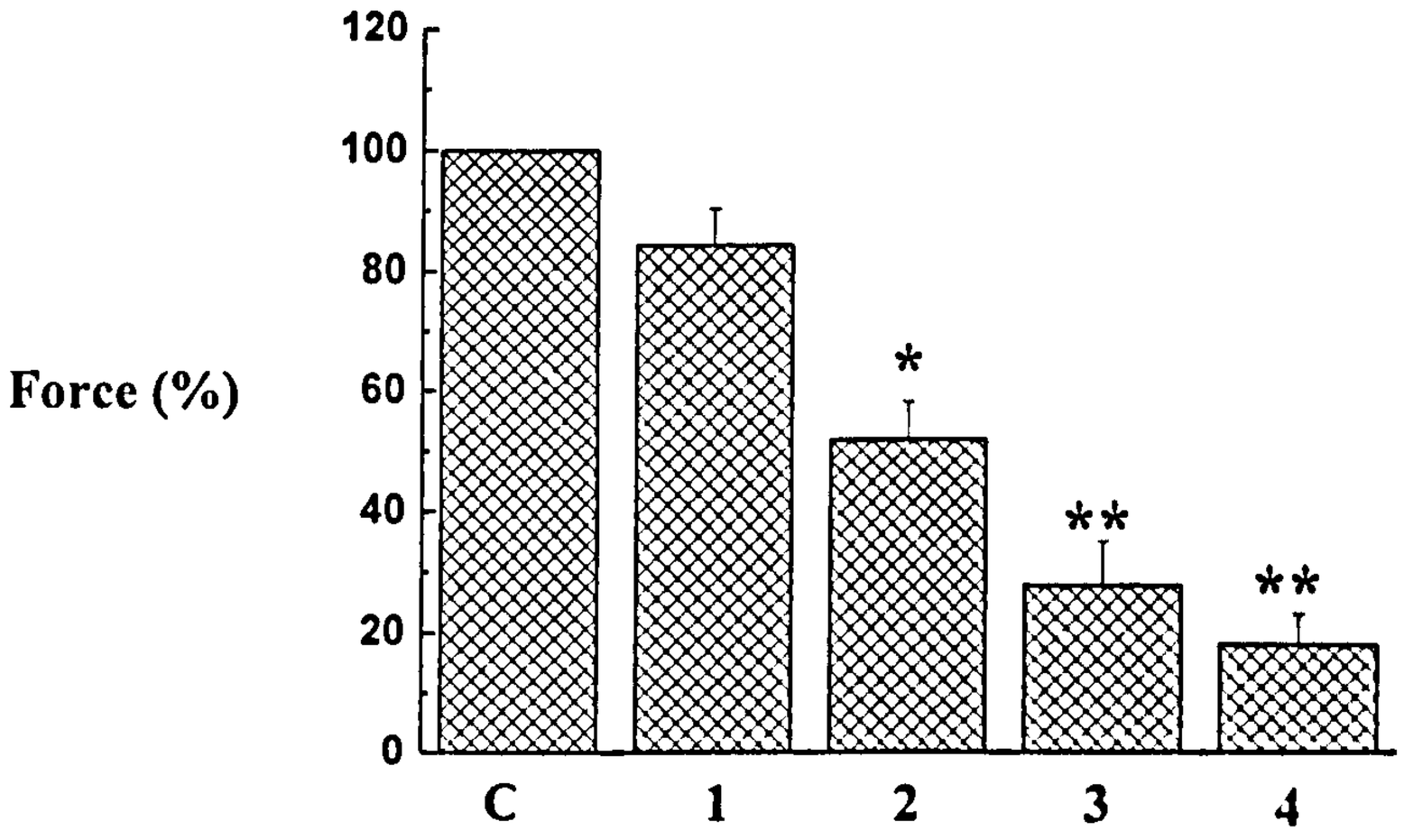

Figure 5.2 The effect of wortmannin on oxytocin-induced contraction of human myometrium. (A) Application of 10 nM oxytocin and then, in the continued presence of wortmannin (4 μM). (B) Show the time courses of oxytocin-induced contraction in the presence of wortmannin ($n = 4$). Force development is expressed as a percentage of the maximal force of the contraction developed in response to oxytocin without wortmannin (denoted as C). The force was measured 5, 10, 15, and 20 min (1, 2, 3, and 4 respectively) after wortmannin addition. * and **, significantly different from the force without wortmannin for $P < 0.05$ and $P < 0.01$, respectively.

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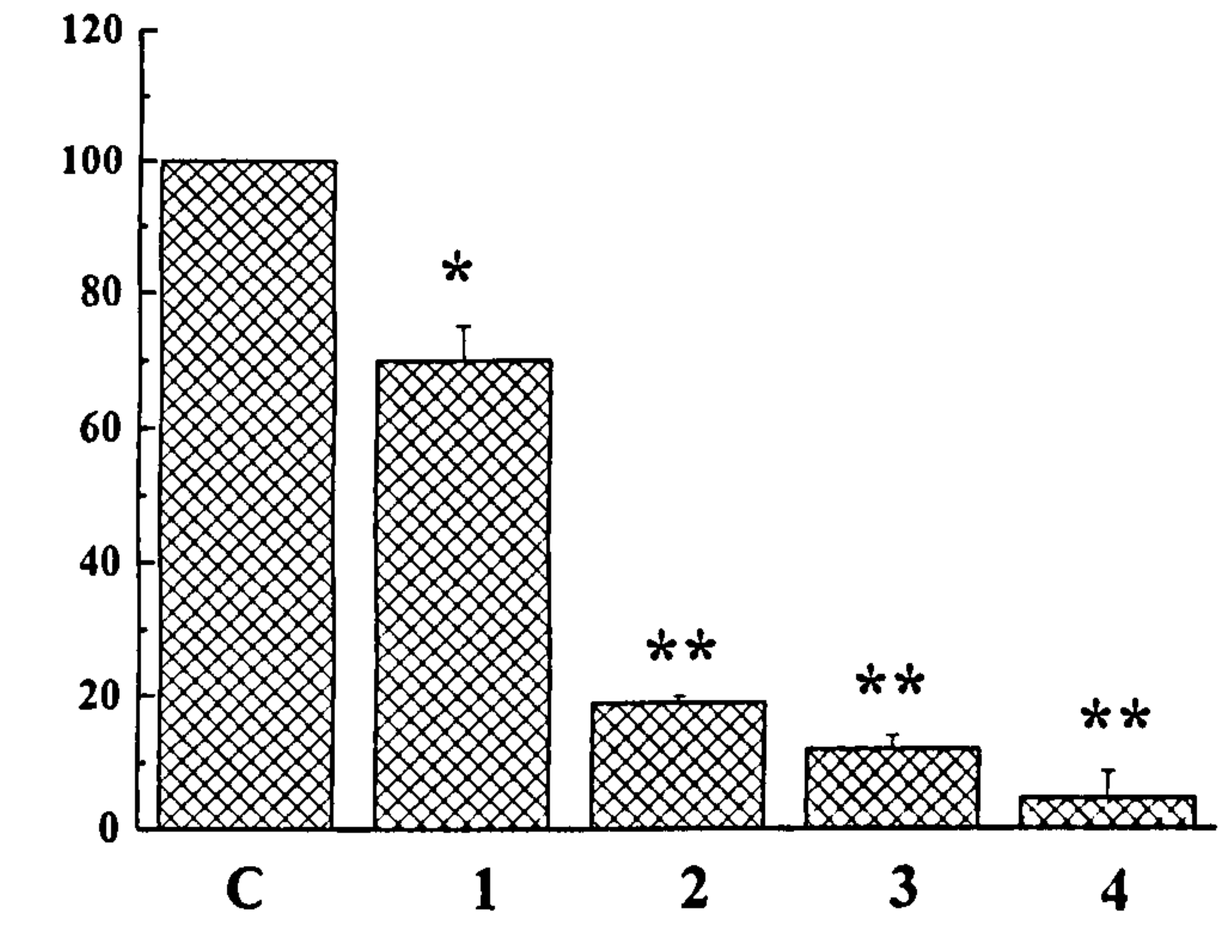

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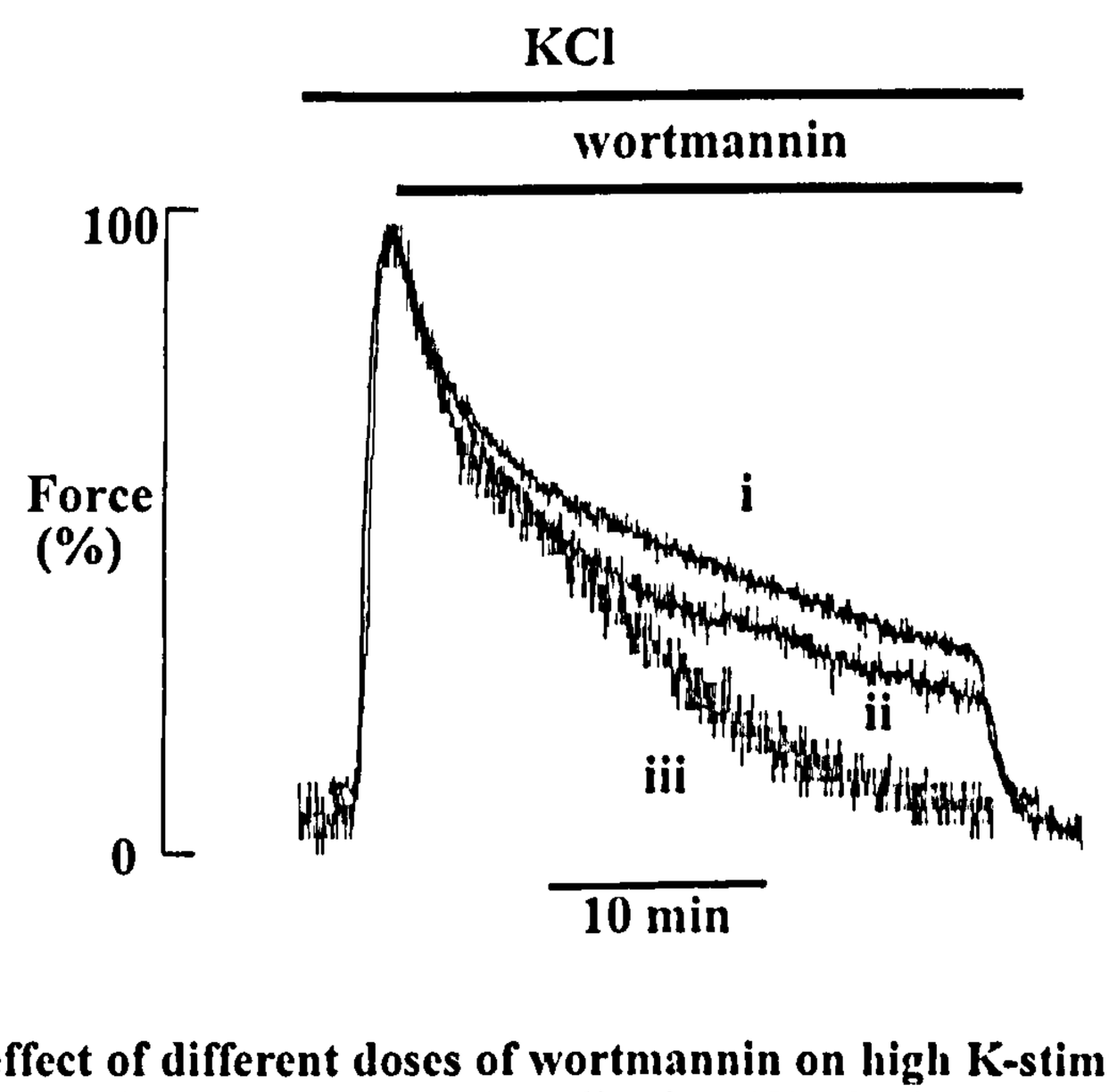


Figure 5.3 The effect of different doses of wortmannin on high K-stimulated contractions. (A) After a control period of 40 mM KCl application (C), the human myometrial strips were incubated with 0.5, 1, 2 or 4 μM wortmannin (1, 2, 3, and 4 respectively) for 10 min and then KCl added. Exposure to 0.5, 1, 2 or 4 μM wortmannin prior to application of high K significantly reduced force amplitude to 69.96 ± 8%, 18.88 ± 5%, 11.82 ± 7%, and 4.5 ± 2%, respectively (compared to 100% peak of control amplitude). (B) The effect of different doses of wortmannin after 15 min in the continued presence of high K; *i*, 0.5; *ii*, 1; *iii*, 4 μM. Exposure to 4 μM wortmannin resulted in a gradual reduction in force produced by high K to 3.98 ± 4% by 20 minutes (100% is amplitude of initial peak response to KCl). * and **, significantly different from corresponding response to KCl alone for $P < 0.05$ and $P < 0.05$, respectively.

allowed the effects of the different doses of wortmannin to be examined ($n = 4$). Fig. 5.3 shows the effect of four different doses of wortmannin, applied for 10 min to the high-K-induced contractions of human myometrium, and the effect of three doses of wortmannin in the continued presence of high-K, after a control period of 2 min. It can be seen that at each concentration (0.5-4 μM) wortmannin reduced force, and that the effect was dose dependent (Fig. 5.3).

The effect of wortmannin on intracellular Ca

As the above data clearly show that inhibiting MLCK can abolish uterine contractions in human myometrium, the next experiments were performed with wortmannin to determine its effects on Ca signalling.

Figure 5.4 shows a simultaneous recording of $[Ca]_i$ and spontaneous contraction in human myometrium (typical of four). It can be seen that under control conditions Ca transients underlie the phasic contractions. Wortmannin gradually inhibited these contractions but had little or no effect on the Ca transient (Fig. 5.4). Figure 5.5 shows a similar effect with oxytocin-induced force ($n = 6$), i.e. force falls but $[Ca]_i$ does not, in the presence of wortmannin. Similar data were also obtained with high-K-induced contraction (Fig. 5.6, $n = 4$).

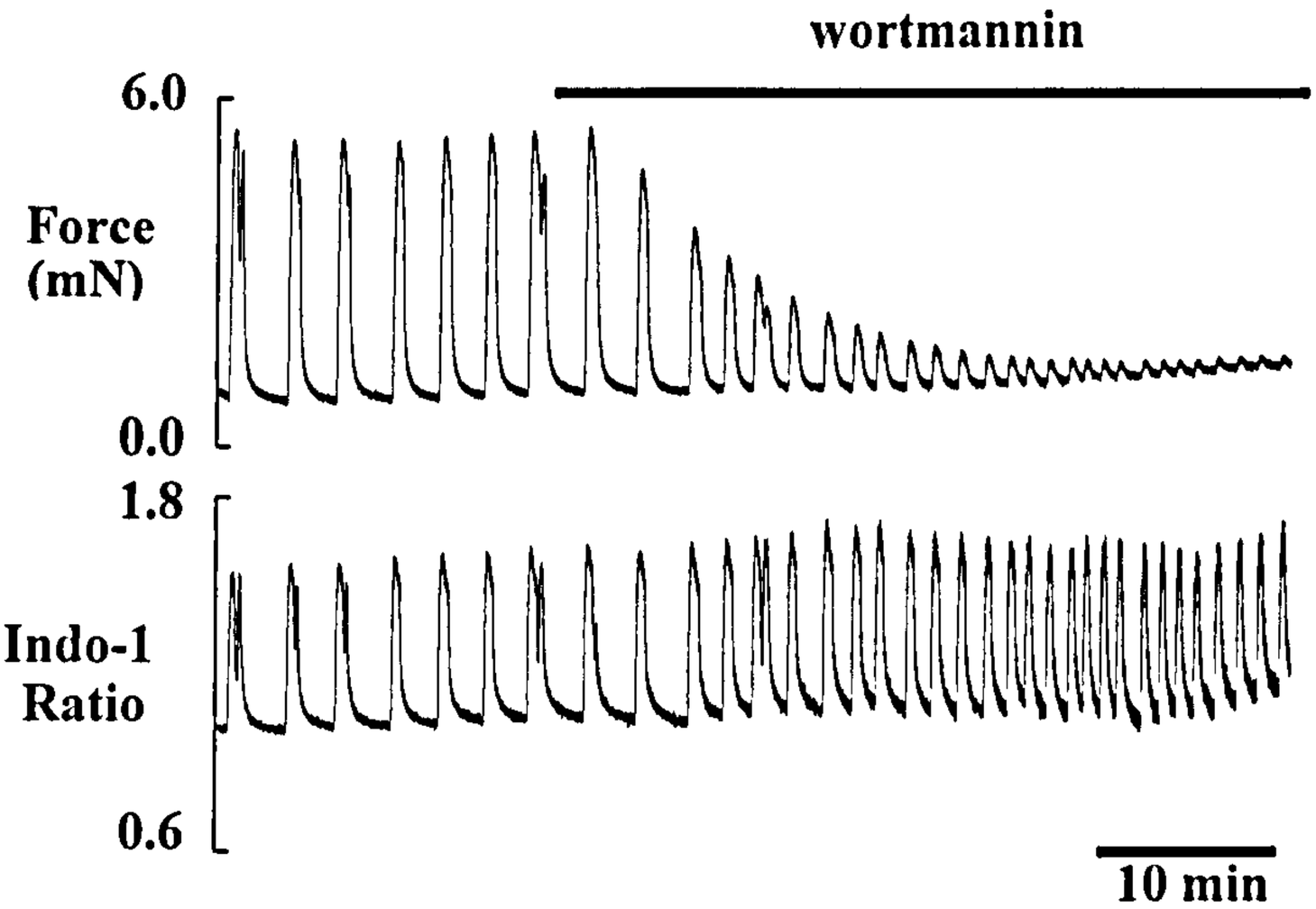


Figure 5.4 The effect of wortmannin on spontaneous force and $[Ca]_i$ transients of human myometrium. Simultaneous measurements of force (top) and intracellular Ca (bottom), showing the effect of wortmannin (4 μM).

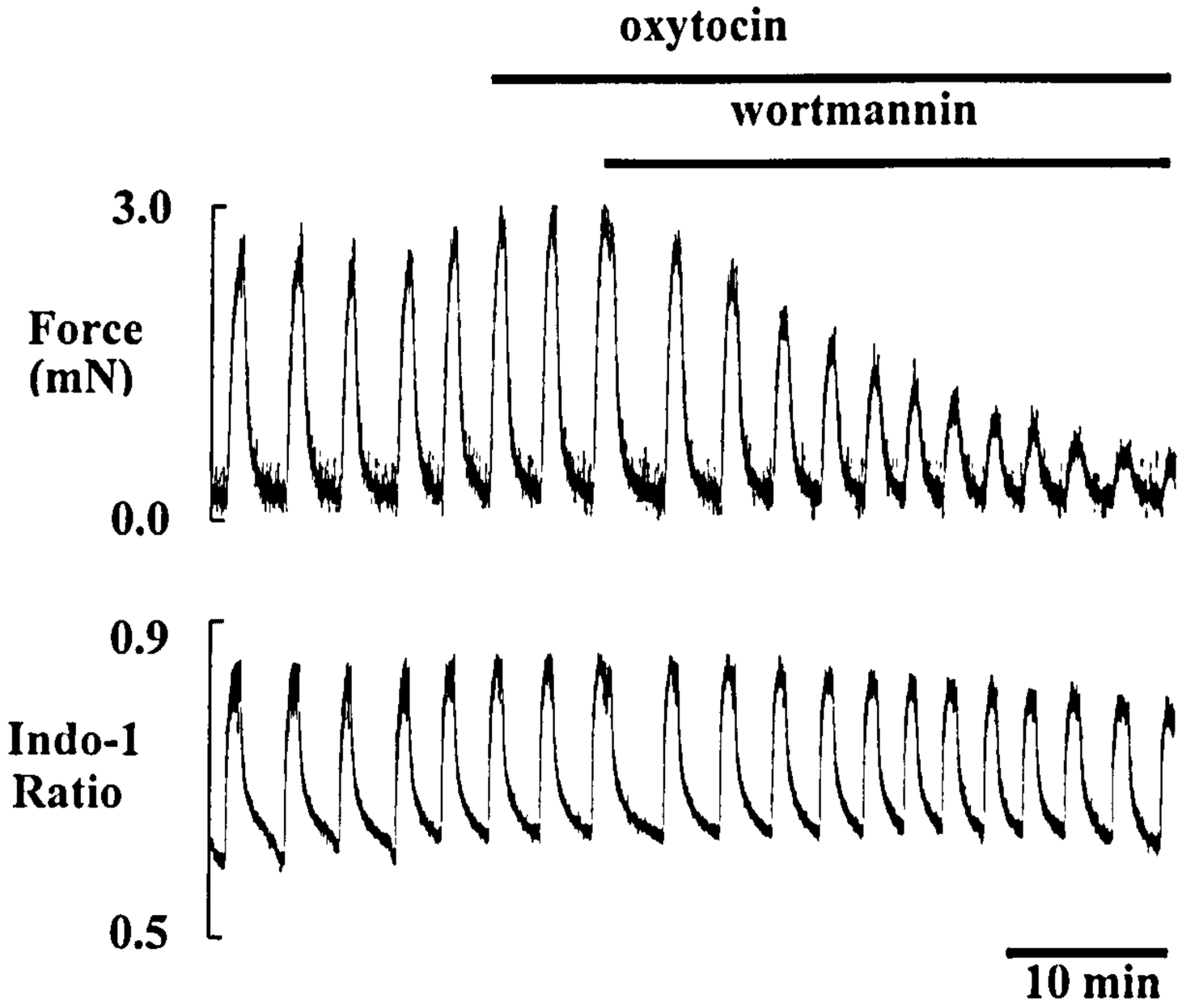
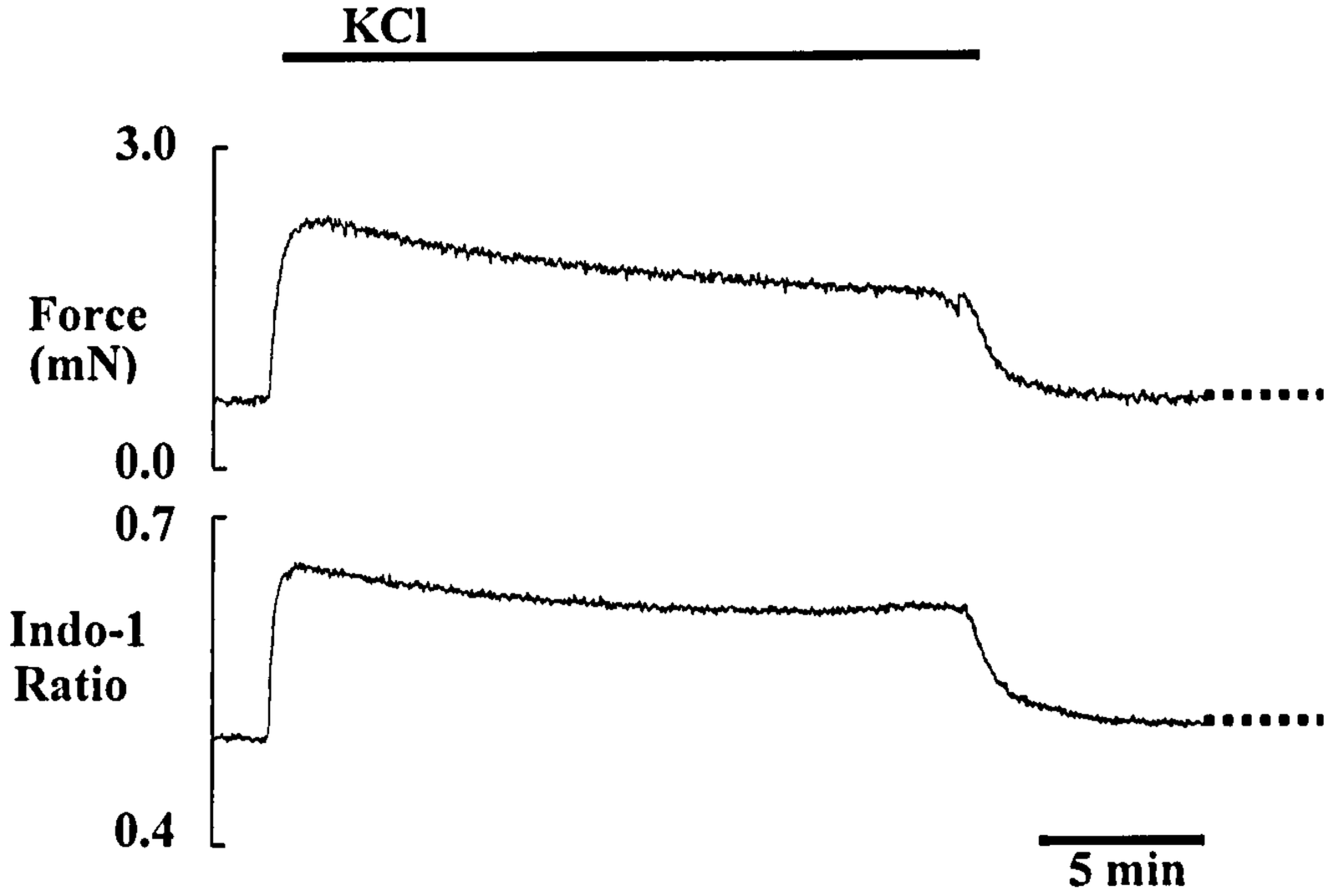


Figure 5.5 The effect of wortmannin on oxytocin-induced force and $[Ca]_i$ transients of human myometrium. After stable contractions were achieved, oxytocin (10 nM) was added and then wortmannin (4 μM).

A



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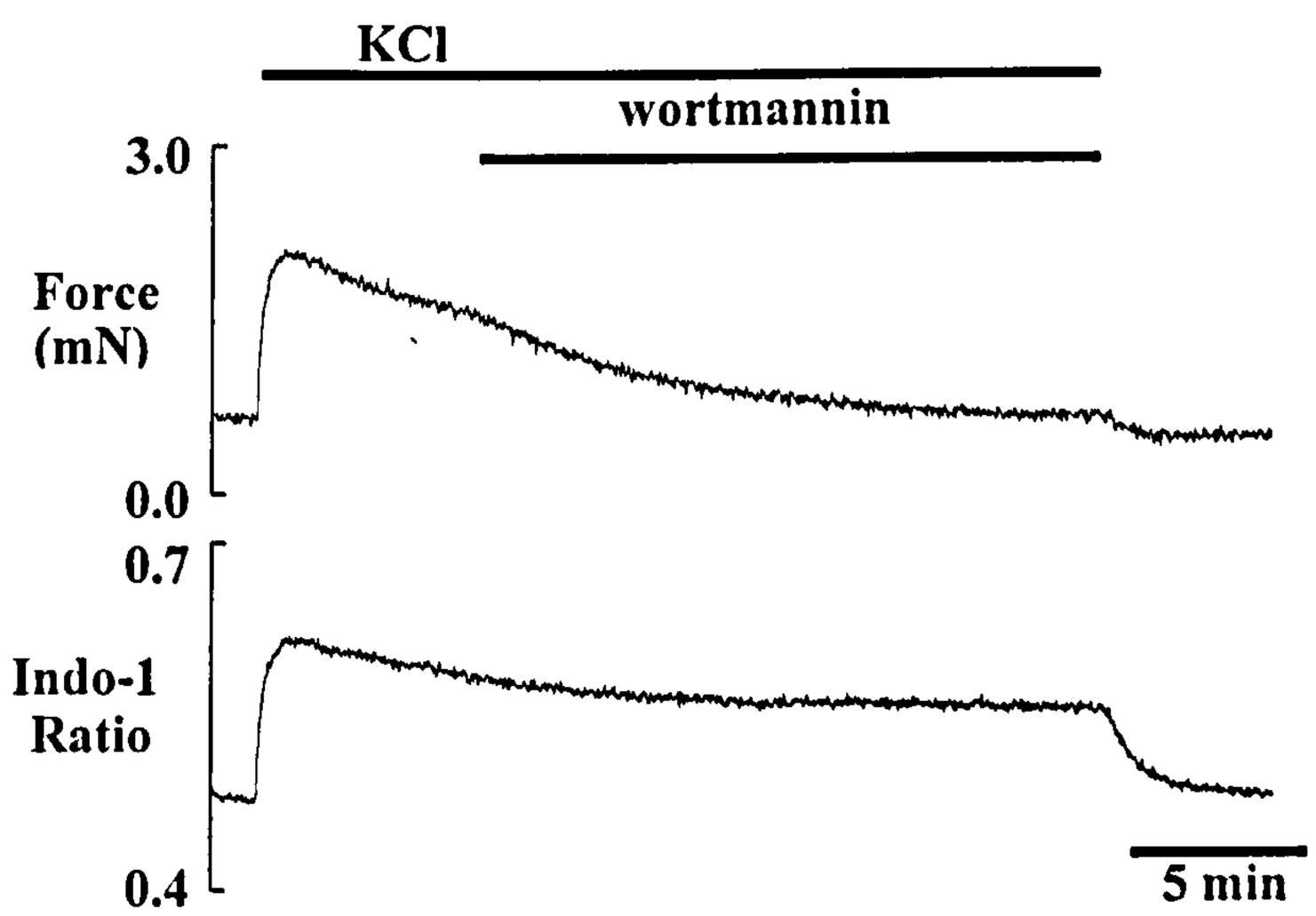


Figure 5.6 The effect of wortmannin on high-K-induced contractions of human myometrium. The control (**A**) and that with 4 μM wortmannin (**B**), applied after 5 min in high K, were obtained in the same strip and recorded continually (dotted line).

5.4.2 Effects of wortmannin on rat myometrium

In order to determine whether the above data are specific to human myometrium, wortmannin was also applied to pregnant and non-pregnant rat myometrium. No significant differences were found between data from pregnant and non-pregnant animals ($n = 6$) and so the data are combined in the following account.

As with human myometrium, spontaneous contractions could be recorded for many hours from the rat; their amplitude was comparable for strips of similar thickness (1-10 mN) but their frequency was higher (approximately one every 1-2 min compared to one every 3-5 min in human myometrium). Force per cross-sectional area was higher in human than rat myometrium (approximately 40 mN/mm^2, compared to 10 mN/mm^2). Wortmannin also abolished these spontaneous contractions (Fig. 5.7, $n = 6$). Its effect was significantly faster than in the human myometrium; thus force was reduced to 12 ± 1% after 10 min (compared to 37 ± 8% in human), which may be related to higher frequency of contractions (see Discussion). As with human myometrium, neither high-K or oxytocin was able to restore force once wortmannin had abolished it. Wortmannin (4 μM, 20 min) completely abolished high-K-elicited force in the rat myometrium ($n = 3$). Oxytocin produced similar effects on the rat as on human myometrium. Thus, as shown in Fig. 5.8A, the frequency and the amplitude of contractions were significantly increased compared to control. Two successive applications of oxytocin produced very similar effects on uterine force (Fig. 5.8A). In the presence of wortmannin (4 μM, Fig. 5.8B) the oxytocin-induced force was reduced and eventually abolished in all preparations ($n = 10$).

Measurement of $[Ca]_i$ in rat myometrium supported the data relating to human myometrium. Thus, wortmannin had little or no effect on the Ca transient

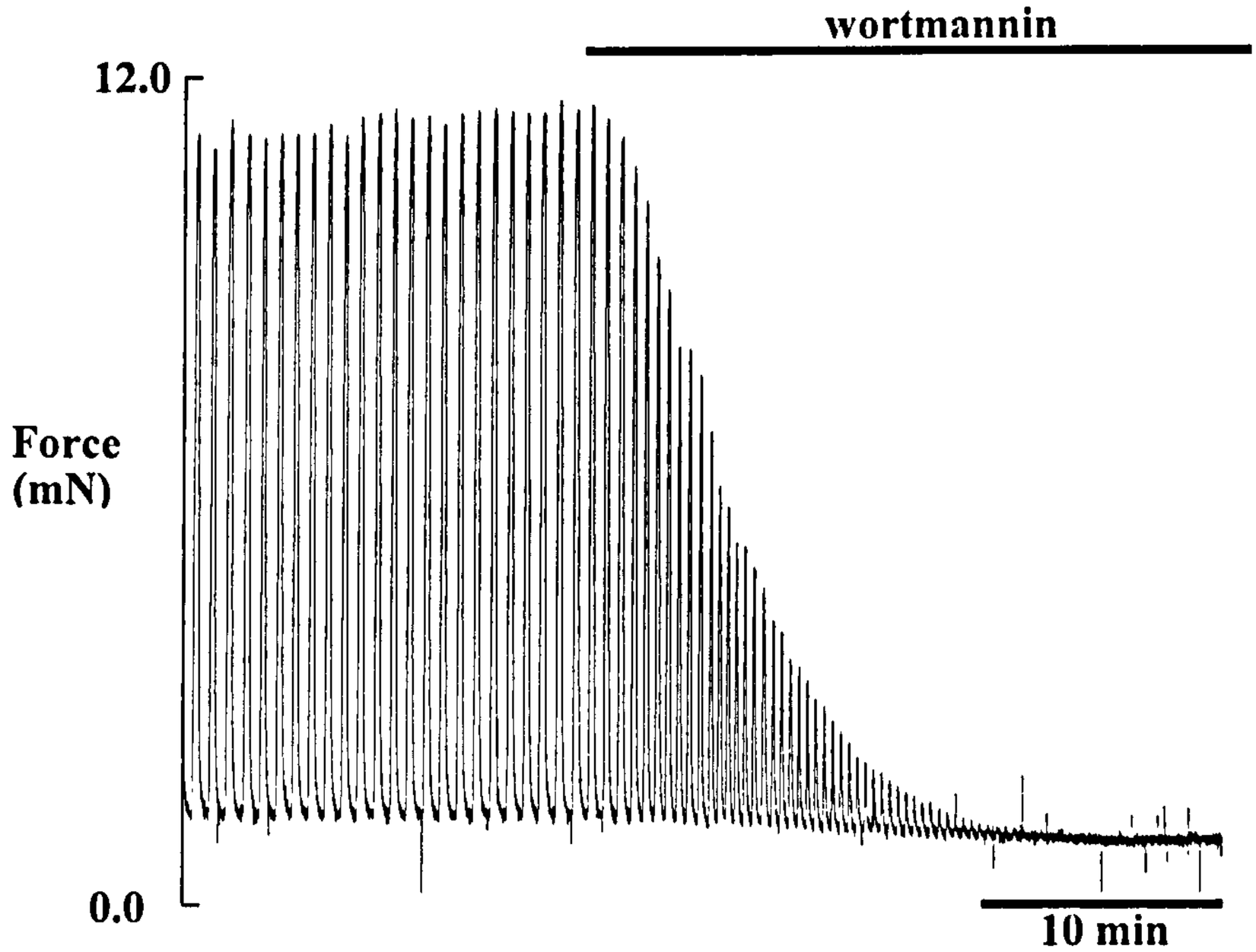
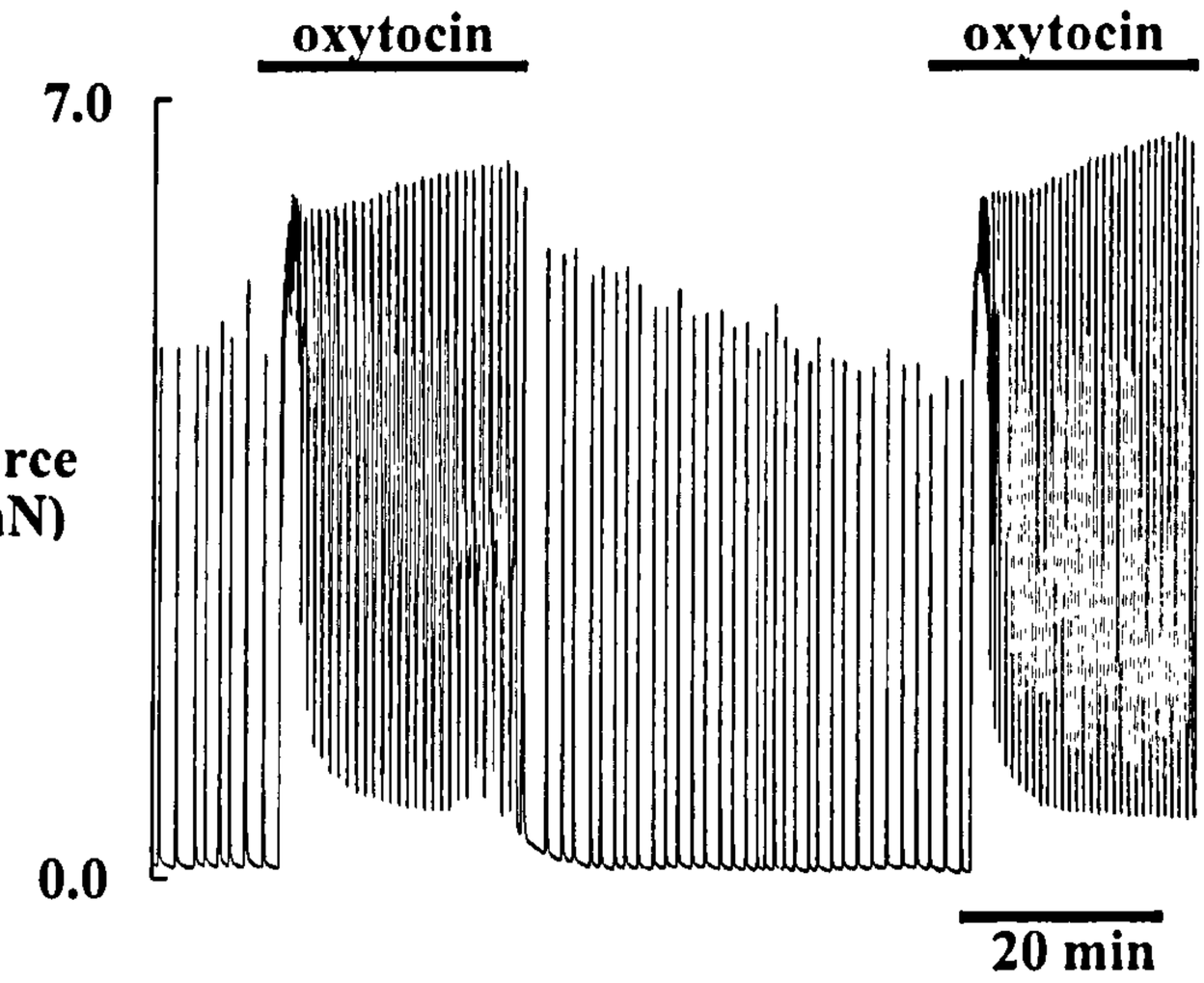


Figure 5.7 The effect of wortmannin on spontaneous contractions of non-pregnant rat myometrium. Show force in control conditions and in wortmannin (4 μM).

A



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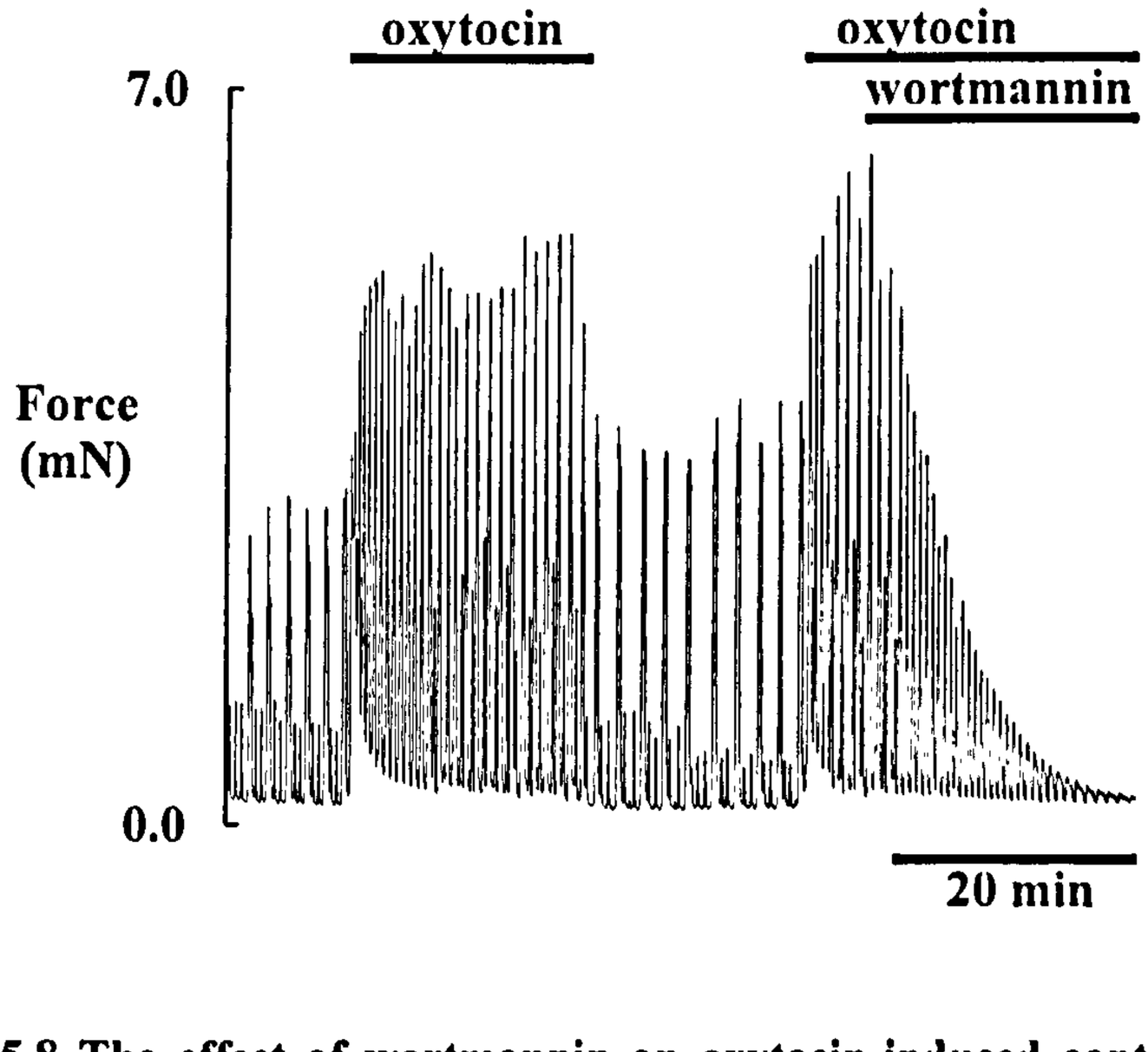


Figure 5.8 The effect of wortmannin on oxytocin-induced contractions of pregnant rat myometrium. (A) Control record showing two successive oxytocin (10 nM) applications. (B) Control oxytocin application followed by oxytocin and wortmannin (4 μM).

whether produced spontaneously ($n = 5$), by oxytocin ($n = 3$) or high-K ($n = 2$).

5.4.3 The effects of wortmannin on intracellular Ca and force in the absence of external Ca

In human ($n = 5$) or rat ($n = 5$) myometrium, oxytocin was applied in the absence of external Ca. A small (~5% of that found in the presence of Ca) tonic contraction gradually developed and was maintained as long as oxytocin was present (up to 30 min), as shown in Fig. 5.9A, which also shows a control oxytocin response. Application of wortmannin ($n = 4$) did not affect this contraction (Fig. 5.9B), irrespective of whether it was added before or during the oxytocin application. In three experiments $[Ca]_i$ was also measured (Fig. 5.10). Removing external Ca clearly reduced $[Ca]_i$, and addition of oxytocin produced a tonic contraction without any discernible rise in $[Ca]_i$ (Fig. 5.10A). Compared to control conditions (Fig. 5.10A), the contraction was unaffected by wortmannin (Fig.5.10B).

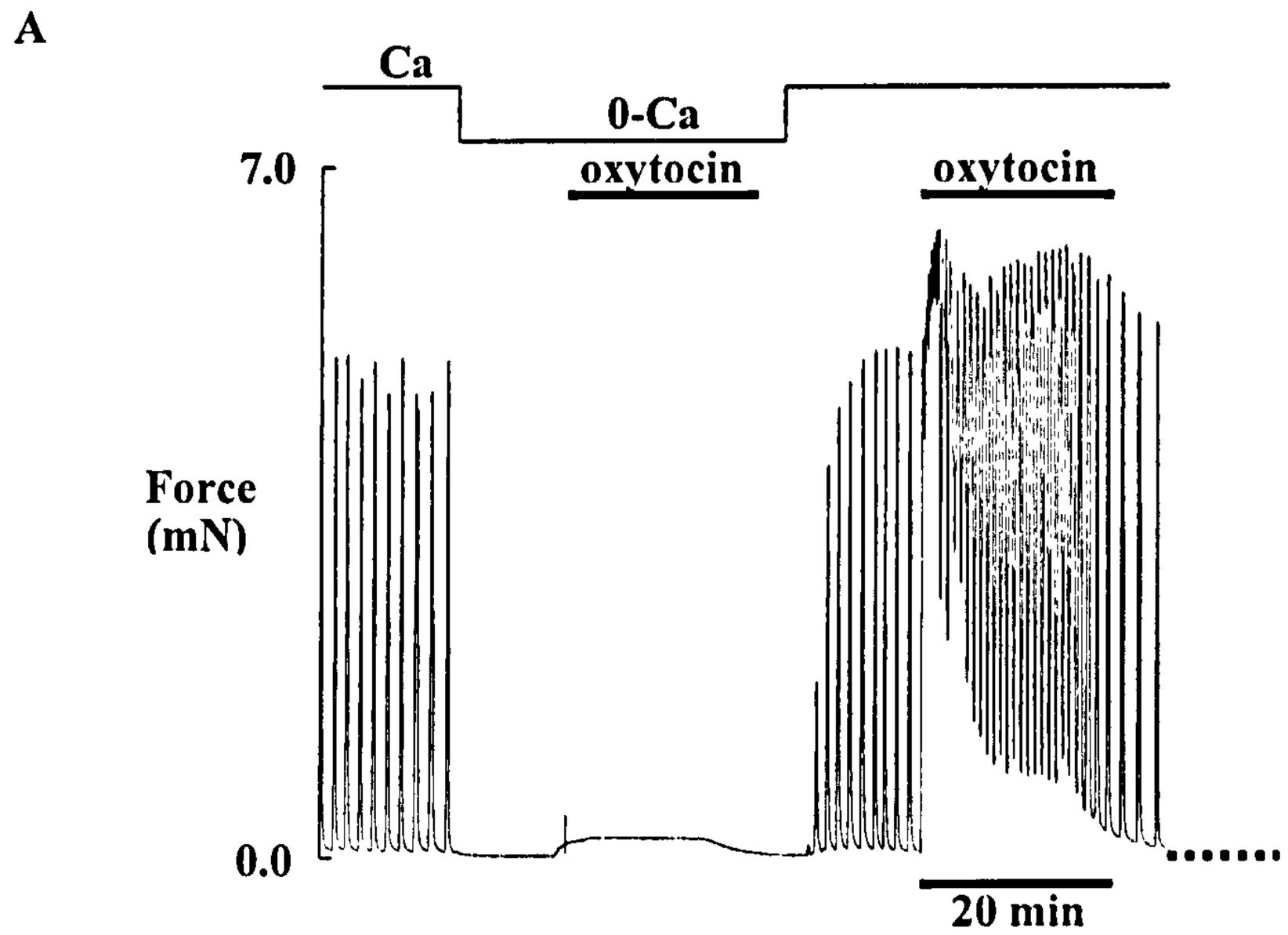
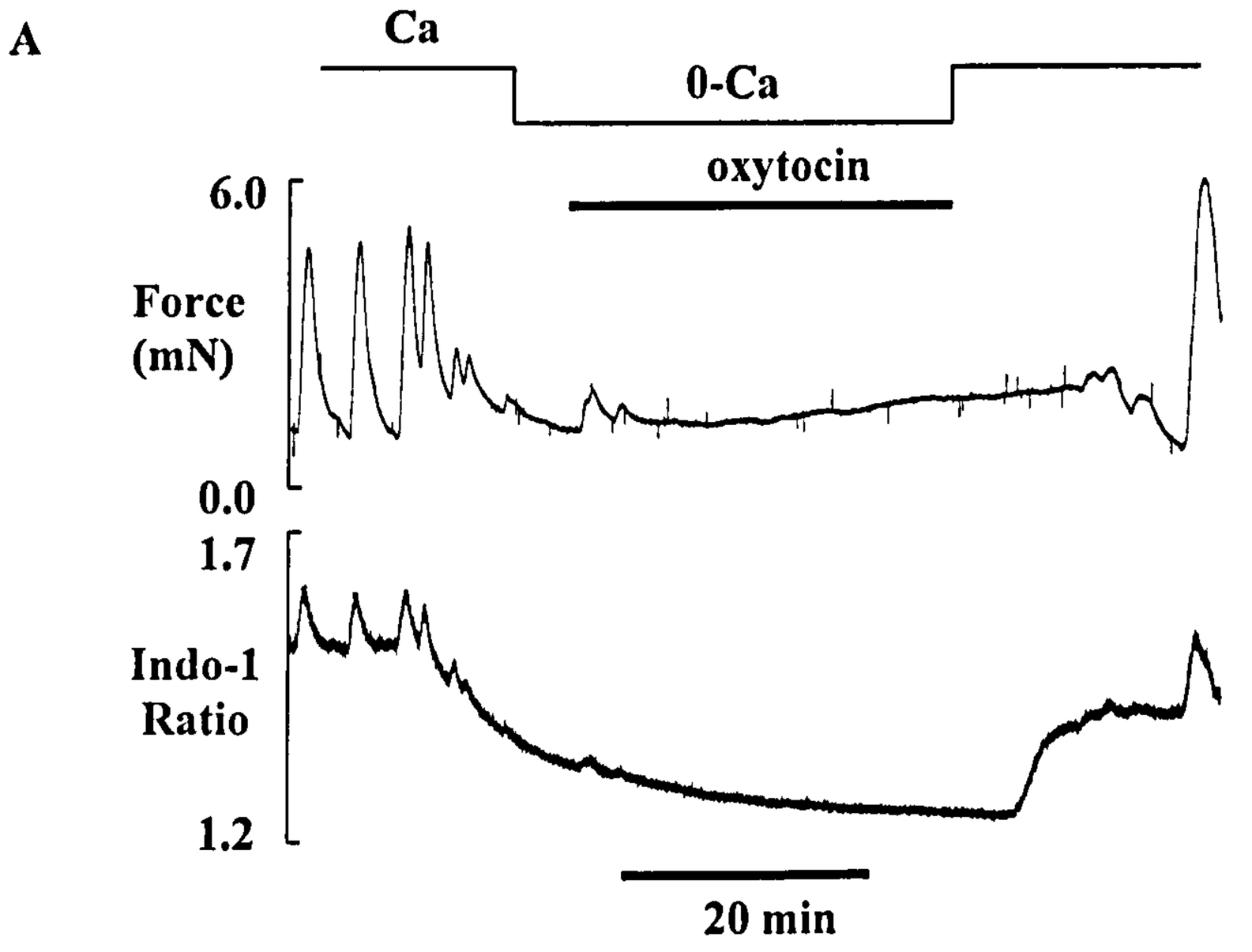


Figure 5.9 Ca-independent uterine contractions of the rat. (A) Following a control period of spontaneous activity in pregnant rat uterus, external Ca was removed. After 5 min oxytocin (10 nM) was added and then removed, and external Ca restored. Finally, the response of the tissue to oxytocin in the presence of Ca is shown. (B) The effects of wortmannin (4 μM) on the contraction evoked by oxytocin in the absence of external Ca are also shown. Oxytocin was added after a 5 min incubation with wortmannin. The experiments were performed on the same strip and recorded continually (dotted line).



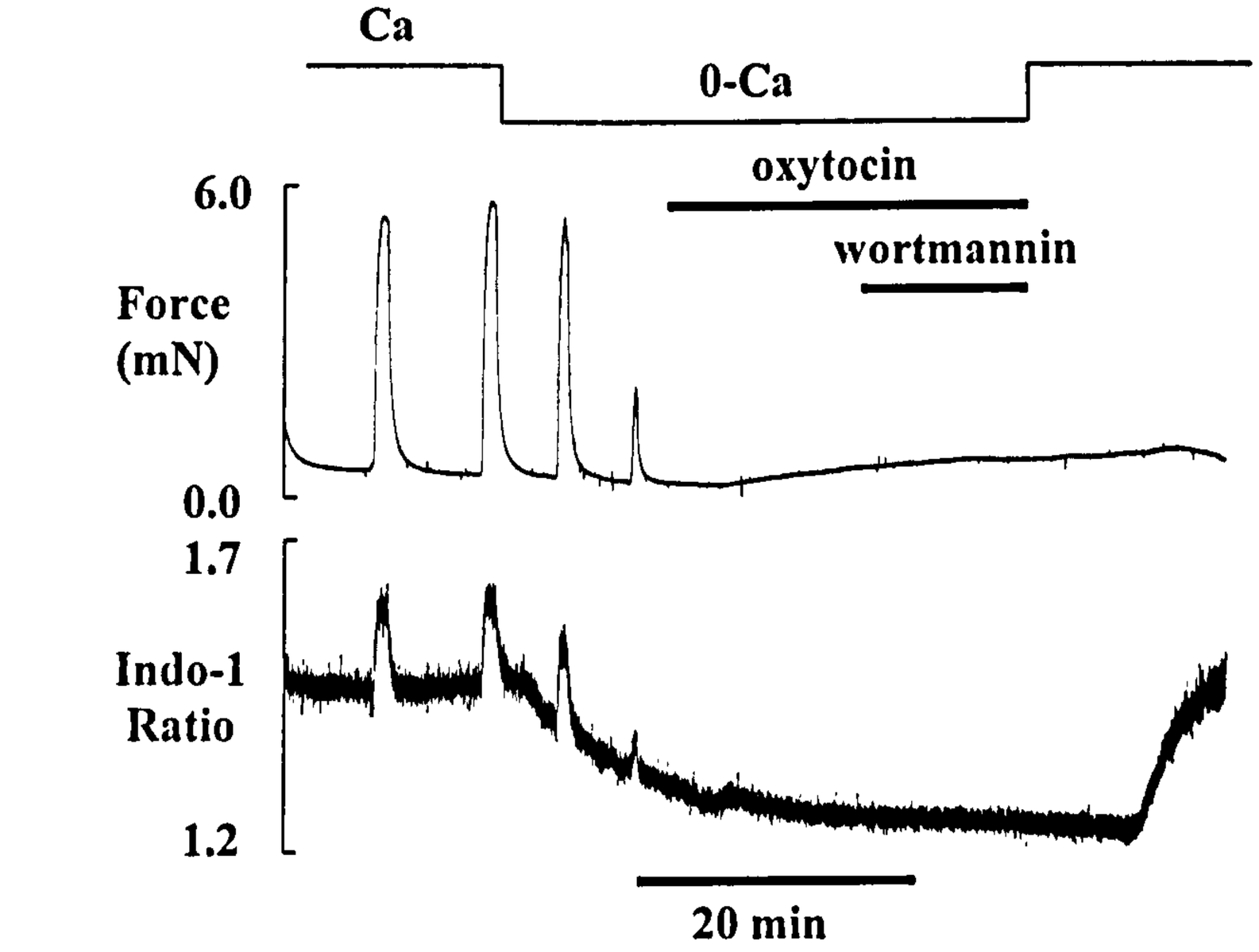


Figure 5.10 Ca-independent contractions of human myometrium. Simultaneous recordings of force (*top*) and Ca (ratio, *bottom*) in human uterus showing the response to 10 nM oxytocin in the absence of external Ca (**A**) and those with wortmannin (**B**).

5.5 Discussion

These data are the first to examine the effects of inhibiting MLCK on uterine contraction. They show that in the human and pregnant and non-pregnant rat uterus, inhibiting MLCK with wortmannin can completely inhibit force produced spontaneously, by a high-K-induced depolarisation or by oxytocin. This inhibition was accomplished with little or no effect on $[Ca]_i$. These data emphasise the crucial role of myosin light chain phosphorylation in uterine contraction and also question the importance of other force-producing pathways under physiological conditions.

Both human and rat myometrium were affected by inhibition of MLCK. There were no qualitative differences between the responses in the two tissues; in the presence of external Ca, force was reduced in both, irrespective of its mechanism of production and without effect on $[Ca]_i$. The only significant difference between the two was the speed of effect of wortmannin in reducing contraction. While some of this may be due to the slightly thicker human preparations, most of this relates to the significantly higher frequency of contractions in the rat. Thus in both tissues the effect is gradual and may reflect wortmannin's ability to access and inhibit the ATPase on MLCK only when the enzyme is activated (as $[Ca]_i$ rises). Thus if a tissue is contracting at a rapid rate, this access and inhibition will occur more rapidly than in a tissue contracting at a slow rate. The similarity in the effects of inhibiting MLCK in human and rat uterus are in agreement with previous data, suggesting that the basic mechanism of contraction is the same in both species (Wray, 1993). The fact that there were no significant differences between the pregnant and non-pregnant rat data also suggests that these basic mechanisms are not altered in pregnancy.

The potent inhibitory effect of wortmannin on uterine phasic force production agrees with earlier work on other phasic muscles (Meer & Eddinger, 1997; Burdyga &

Wray, 1998; Wingard & Murphy, 1999). Inhibition of tonic force in vascular smooth muscle has also been reported for wortmannin (Nakanishi *et al.* 1992; Takayama *et al.* 1996). Although not measured in the current study, as expected the inhibitor has been shown to reduce myosin phosphorylation in all tissues examined to date ([Saitoh *et al.* 1987; Nakanishi *et al.* 1992; Burke *et al.* 1996; Wingard & Murphy, 1999]; Burdyga & Mitchell, personal communication). Previous studies have recorded electrical activity in the presence of wortmannin and report no change in any of the parameters of the action potential (Burke *et al.* 1996; Burdyga & Wray, 1998). In the present study, force falls despite normal Ca transients; a result consistent with findings in studies of the ureter (Burdyga & Wray, 1998). Thus putting together the data from the present study and previous studies mentioned above, it is clear that wortmannin has no effect on the processes governing excitation and Ca rise in smooth muscle. This is consistent with its specific effect on MLCK, as a non-competitive inhibitor with respect to the ATPase site on the enzyme. Reports vary as to the reversibility of wortmannin (Burke *et al.* 1996). Some recovery can be seen in the uterus, in agreement with previous findings in a study of ureteric smooth muscle (Burdyga & Wray, 1998). Wortmannin is the most selective inhibitor of MLCK available. It has also been shown, in some tissues at least, to inhibit phosphatidylinositol 3-kinase (Pi3 kinase) (Arcaro & Wymann, 1993; Stephens *et al.* 1994). This effect however is at extremely low concentrations (1-10 nM) and is not compatible with the dose/response effects seen at wortmannin concentrations ranging from 0.5 to 4 μM. Furthermore, the involvement of this kinase in the absence of agonists is unlikely and thus again the data obtained relating to spontaneous and high-K contractions are not compatible with such an action of wortmannin. Burke *et al.* (1996) also showed Pi3 kinase to have little effect or no effect in gastric smooth muscle. Thus the major effect of wortmannin was to inhibit MLCK, and hence myosin

phosphorylation. Little or no force can be produced without phosphorylation of myosin by MLCK. The data also clearly show that wortmannin was effective in reducing force in the uterus, irrespective of the species or mechanism used to produce that force. Of particular note was the reduction of oxytocin-evoked contractions, as it is under these conditions that non-MLCK pathways would be expected to act (Ohmichi *et al.* 1995). The inability of a high-K contraction to overcome the inhibition of force, produced either spontaneously or by oxytocin, is consistent with the $[Ca]_i$ data. That is, the effect of wortmannin is not to cause a failure in the surface membrane mechanisms that lead to a rise in $[Ca]_i$, as the rise of $[Ca]_i$ were unaffected by wortmannin. These data are consistent with the failure of MLCK to phosphorylate myosin.

There is currently much interest in elucidating how agonists modulate force in smooth muscles. The data in this chapter suggest that any effects beyond myosin light chain phosphorylation are of limited importance in the human myometrium, at least under physiological conditions. The rat data also showed no differences in the effects of wortmannin on tissues from pregnant and non-pregnant animals, suggesting that the hormonal environment does not change this conclusion. Wingard and Murphy (1999) also concluded that there is no evidence for Ca- and MLCK-independent force production in arterial smooth muscle. It is so far unknown whether their contribution to contraction may be different under pathophysiological conditions, e.g. pre-term labour. In vascular smooth muscle, for example, it was found that inhibition of MAP kinase had no effect on the blood pressure of normotensive rats but reduced it in hypertensive rats (Uehata *et al.* 1997). In the absence of external Ca oxytocin can elicit a very small amount of force in the uterus (Matsuo *et al.* 1989; Oishi *et al.* 1991; Ishine *et al.* 1992; Ohmichi *et al.* 1995). The present study, as reported previously, shows that there is usually slow in developing and is tonic in nature. Wortmannin did not affect this

contraction, suggesting that it is not produced by myosin phosphorylated at serine 19 by MLCK. The lack of effect of wortmannin on this contraction also further supports the suggestion that wortmannin is acting to selectively inhibit MLCK. This is consistent with the report of Oishi *et al.* (1991) that such contractions occur without myosin phosphorylation. It has been suggested that this contraction arises via Ca-independent isoforms of PKC, possibly via thin filament disinhibition (Horowitz *et al.* 1996a). It is however unclear how relevant such mechanisms and such small levels of force are to the uterus *in vivo*, as external Ca will always be present, and, as shown in section 5.4.1, 5.4.2 under these circumstances little or no force is produced when MLCK is inhibited.

In summary the data show that wortmannin inhibits force production in human and rat myometrium irrespective of the mechanism used to elicit that force. This occurs despite unchanged $[Ca]_i$ and is consistent with MLCK phosphorylation of myosin being critical to force production in the uterus.

Chapter 6

The Effects of Inhibiting Rho-Associated Kinase with Y-27632 on Force, Intracellular Calcium, and Myosin Phosphorylation in Human Myometrium

Chapter 6

The Effects of Inhibiting Rho-Associated Kinase with Y-27632 on Force, Intracellular Calcium, and Myosin Phosphorylation in Human Myometrium

6.1 Abstract

Recent work has indicated that smooth muscle force production may be influenced by pathways not dependent upon the Ca-calmodulin phosphorylation of light chains. Few studies, however, have examined the importance of these pathways in intact muscles that contract phasically rather than tonically. Therefore, to determine whether the Ca-independent RhoA and associated kinase (ROK) pathway can affect contractions of the intact human myometrium, Y-27632 was used to inhibit ROK. Three types of contractile activity were examined: spontaneous and those elicited by oxytocin or depolarisation with high [K]. Y-27632 decreased force significantly under all three conditions, accompanied by decreases in myosin phosphorylation without changing intracellular [Ca]. However, the effects on force were only large when the uterus was producing force tonically rather than phasically. This suggests that the RhoA-ROK pathway may not be a potent modulator of force in the human myometrium under physiological conditions.

6.2 Introduction

The main force-producing pathway in the uterus is the Ca-calmodulin (CaM)-myosin light chain (MLC) kinase (MLCK) pathway (see chapter 5). However contraction can be regulated by the phosphorylation of the 20 kDa light chain of myosin (MLC_{20}), which depends on the balance between MLCK and myosin light chain phosphatase (MLCP) activities (Somlyo, 1999). Agonists, for example, activate the Ca-CaM-MLCK pathway but may also stimulate force production by inhibiting MLCP via ROK phosphorylation (Sward *et al.* 2000). It is unclear how important this pathway is for contractions of the intact myometrium, although RhoA has been reported in the myometrium and to translocate to the cell membrane from the peripheral upon muscarinic stimulation (Lee *et al.* 2001), and ROK expression increases with pregnancy (Niiro *et al.* 1997). Therefore, the aim of this chapter was to investigate the effect of inhibiting the RhoA pathway using Y-27632, a selective inhibitor of ROK (Kimura *et al.* 1996), on human myometrial contractions, arising either spontaneously or elicited by membrane depolarisation by high [K] or with oxytocin stimulation. In addition, intracellular [Ca] ($[Ca]_i$) was measured to determine if any functional effects of Y-27632 were occurring independently of changes in $[Ca]_i$. Some experiments were also performed on animal tissue, to establish valid models for physiological mechanisms in the human.

6.3 Materials and Methods

Tissue

For this chapter, human myometrial tissues were obtained from women undergoing elective caesarean section at term (37-41 weeks). Nineteen caesarean biopsies were performed under spinal analgesia. The indications for caesarean delivery were previous caesarean delivery (14 patients), foetal malpresentation (4 patients), and patient choice (1 patient). The mean age of women undergoing caesarean delivery was 31 years (range, 19-39 years).

Animal tissues were obtained from pregnant rats between 18 and 21 days of gestation.

Simultaneous measurements of calcium and tension

Tissue preparation and simultaneous measurement of calcium and tension are essentially the same as those described in chapter 2.

Measurements of myosin phosphorylation

Measurements of myosin phosphorylation was quantified by an immunoblotting analysis after separation of the nonphosphorylated and phosphorylated forms of myosin light chain (MLC_{20}) by urea/glycerol-polyacrylamide gel electrophoresis (as described in chapter 2). The experiments shown were performed on the same gel.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK) unless stated otherwise. Y-27632 was a kind gift from Yoshitomi Pharmaceutical Industries (Japan).

Y-27632 was dissolved in DMSO at a concentration of 10^{-2} M. Oxytocin was dissolved in 5% acetic acid at a concentration of 1 mM. Nifedipine was dissolved in DMSO at a concentration of 10 mM. These stock solutions were diluted to the desired concentrations with Krebs' solution. In some experiments K was elevated and Na reduced to depolarise the membrane in some experiments.

Statistics

Data were analysed with Student's (paired or unpaired) *t*-test; differences between means were assumed to be significant at $P < 0.05$. Data are given as mean ± s.e.m; *n* is the number of samples.

6.4 Results

6.4.1 Human myometrium

6.4.1.1 Effects of Y-27632 on spontaneous contractions and $[Ca]_i$

Adding Y-27632 (10 μM) to spontaneously active preparations of human myometrium for up to 20 min produced a small (16 ± 2%) but significant decrease in the amplitude of contractions compared with control (100%), as shown in Fig. 6.1A. There was however no significant change in the Ca transient amplitude (n = 4). The frequency of contractions also increased significantly to 162 ± 6%. The rate of relaxation of the contractions increased significantly to 123 ± 2% (n = 6), and thus the contractions were significantly shorter (Fig. 6.1B, $P < 0.05$). Y-27632 did not affect the haft-time for decay of $[Ca]_i$ (24.75 ± 0.9 s for Y-27632 compared with 24.25 ± 1.4 s for control, n = 4).

6.4.1.2 The effects of Y-27632 on contractions and $[Ca]_i$ induced by oxytocin

To investigate whether activation of MLCP could influence agonist-induced contraction, the effects of Y-27632 on the response to oxytocin (10 and 100 nM) were studied. As can be seen in Fig 6.2A, Y-27632 did not abolish the rise in $[Ca]_i$ or force produced by 10 nM oxytocin (n = 4); it only produced a small (12 ± 6%, $P < 0.05$) decrement of force. With 100 nM oxytocin, Y-27632 significantly reduced the tonic component of force (52 ± 2%, n = 3), without a decrease in $[Ca]_i$ (data not shown). Adding oxytocin (100 nM) to the uterus in the presence of KCl (Fig. 6.2B) potentiated force with a small transient rise of $[Ca]_i$, indicating release of Ca from internal stores. To establish whether this potentiation of force by oxytocin was due to the modulation of MLCP activity via inhibition of ROK, Y-27632 was applied. After 1-2 min, force began to fall markedly.

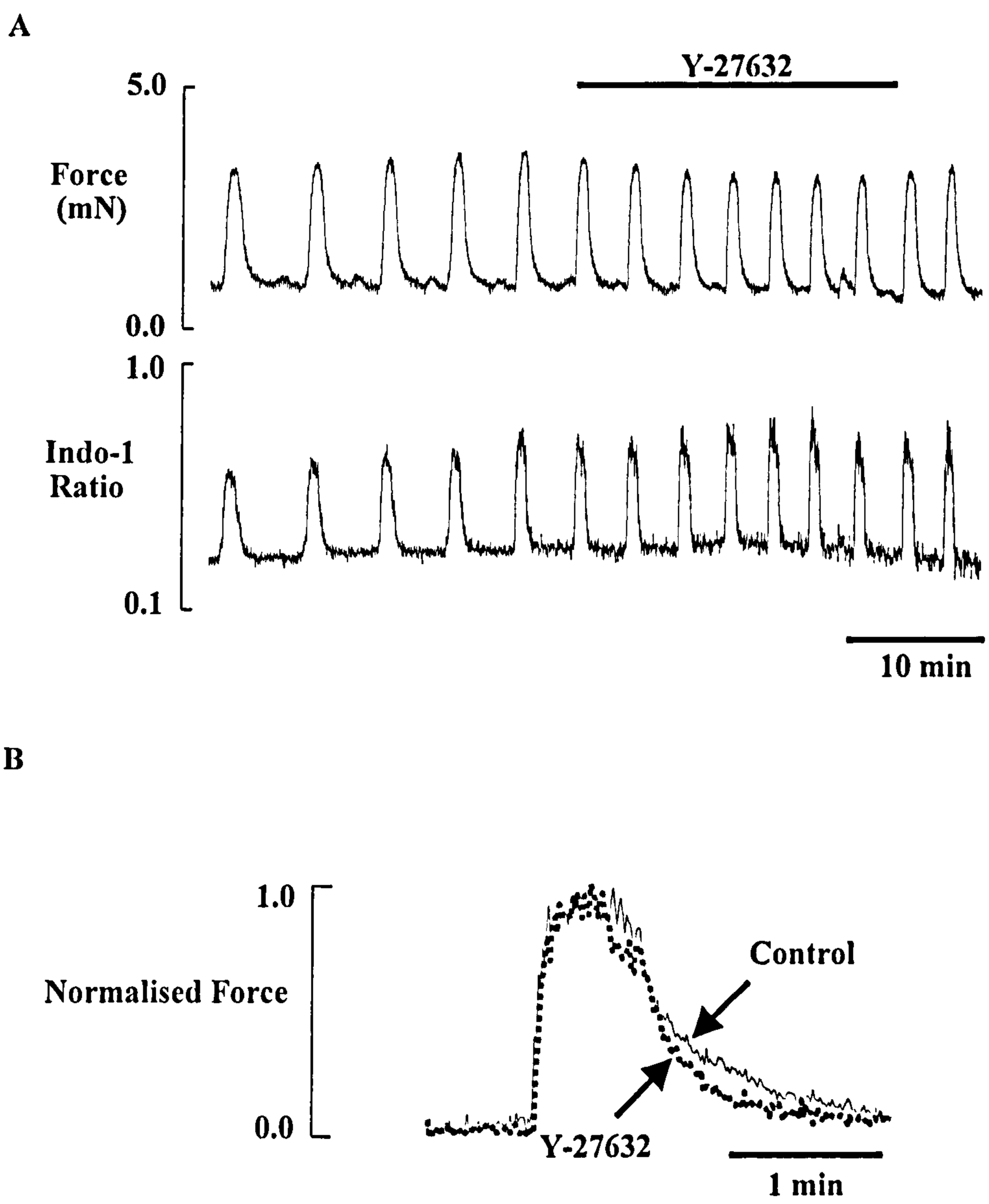


Figure 6.1 The effect of Y-27632 on spontaneous contraction. (A) Simultaneous measurements of spontaneous force (top trace) and Ca transients (bottom trace, measured with Indo-1), in pregnant human myometrium. (B) Normalised force taken from A in the absence and presence of Y-27632.

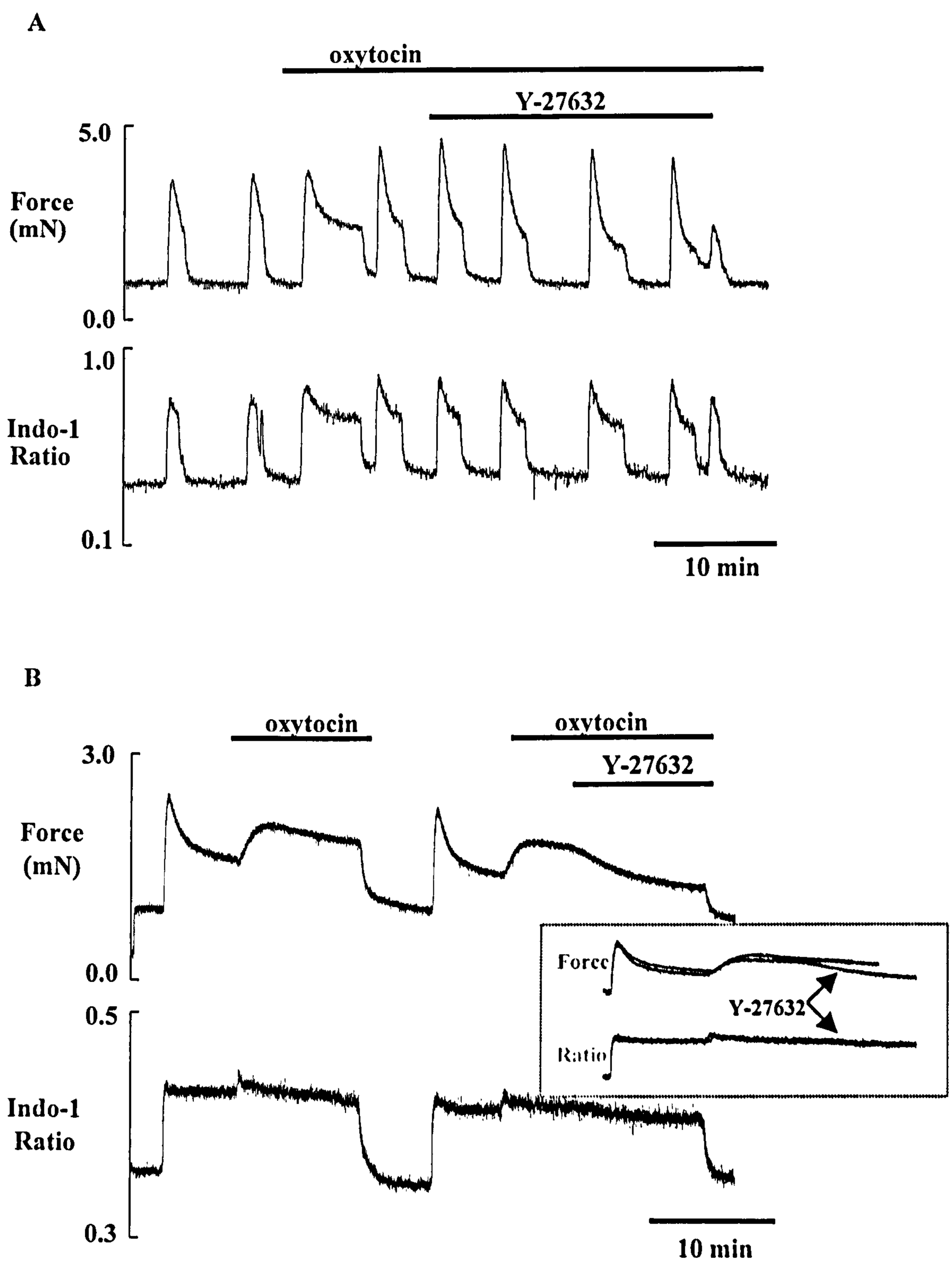


Figure 6.2 The effect of Y-27632 on oxytocin-induced contraction. (A) The effects of Y-27632 on force and Ca signal, in response to 10 nM oxytocin. (B) As in A, but with 100 nM oxytocin and the preparation depolarised by exposure to 40 mM KCl. The control and that with Y-27632, added after 5 min in oxytocin, were obtained in the same tissue and superimposed (the inset).

The superimposed traces show that Y-27632 produced a marked and decrease of force (85 ± 5%, $P < 0.05$, $n = 3$, compared with the peak amplitude in control traces). Again this occurred without a change in $[Ca]_i$.

6.4.1.3 The effects of Y-27632 on contractions and $[Ca]_i$ induced by KCl

The effect of Y-27632 on KCl-induced contractions was also examined. Figure 6.3A shows a control response to KCl (40 mM). Force and $[Ca]_i$ rose and were maintained until return to control solution. After recovery from KCl, the effects of Y-27632 were studied (Fig. 6.3B, superimposed trace). After 1-2 min exposure to Y-27632 (in the presence of KCl) there was a significant decrease in force without a change in $[Ca]_i$. The decrease in the mean maximum force was 52 ± 2% ($n = 8$, $P < 0.05$), compared with control.

6.4.1.4 The effects of Y-27632 on MLC_{20} phosphorylation

To investigate whether RhoA-ROK pathway is involved in the mechanism of spontaneous- and oxytocin induced-contraction via inhibition of MLC_{20} phosphorylation, the effects of Y-27632 on MLC_{20} phosphorylation were also studied. In the absence of external Ca, MLC_{20} phosphorylation level was undetectable (Fig. 6.4A, a). As shown in Fig. 6.4A, MLC_{20} phosphorylation level was increased during spontaneous contraction (b), and was reduced by Y-27632 (c). There is no significant difference between the level of MLC_{20} phosphorylation measured at the peak of spontaneous contraction and high-K-induced contraction (Fig. 6.4A (a) and Fig. 6.4B (b), respectively). However, oxytocin (100 nM) added under depolarisation significantly increased the level of MLC_{20} phosphorylation (Fig. 6.4B, c). Application of oxytocin in

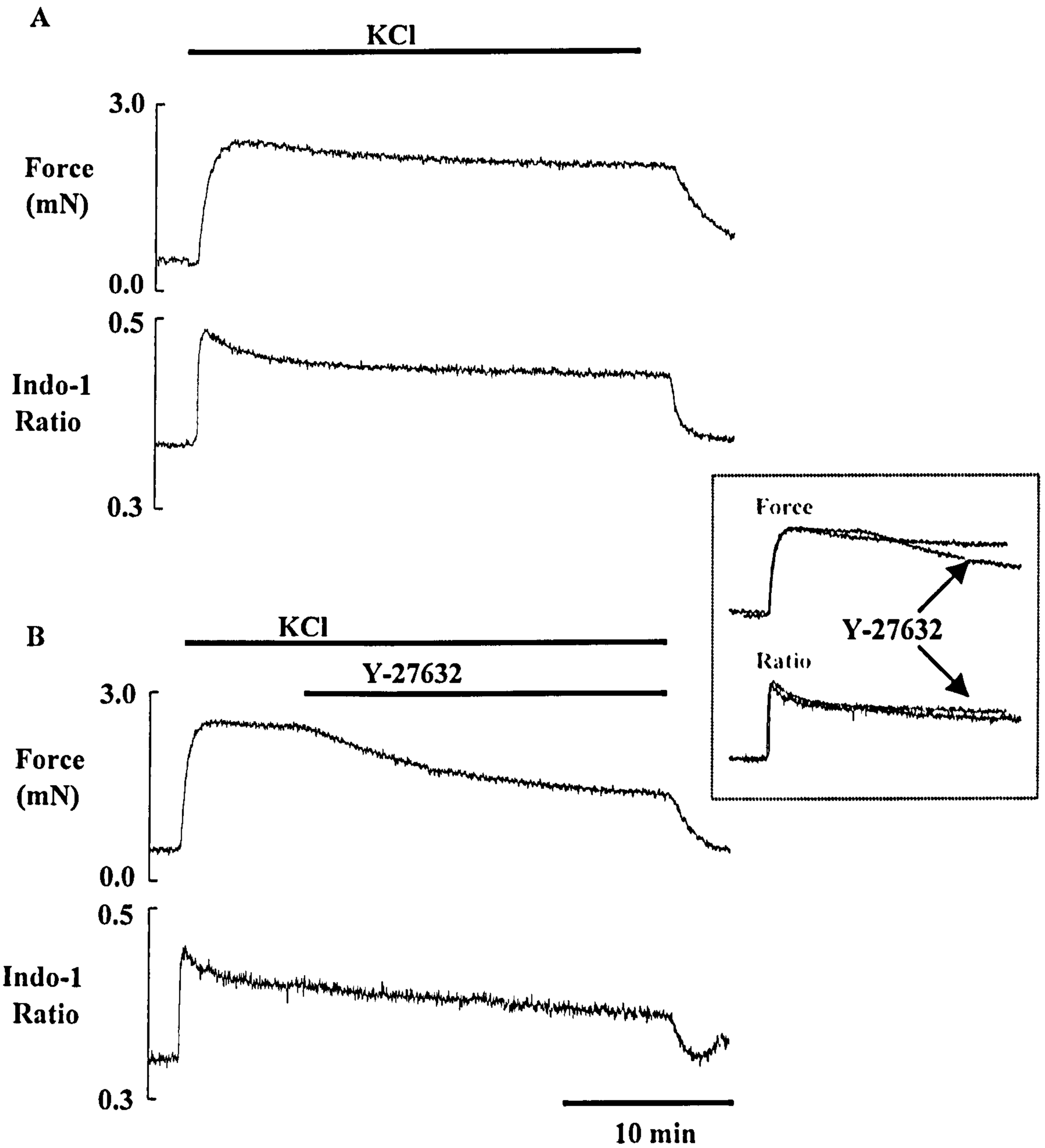
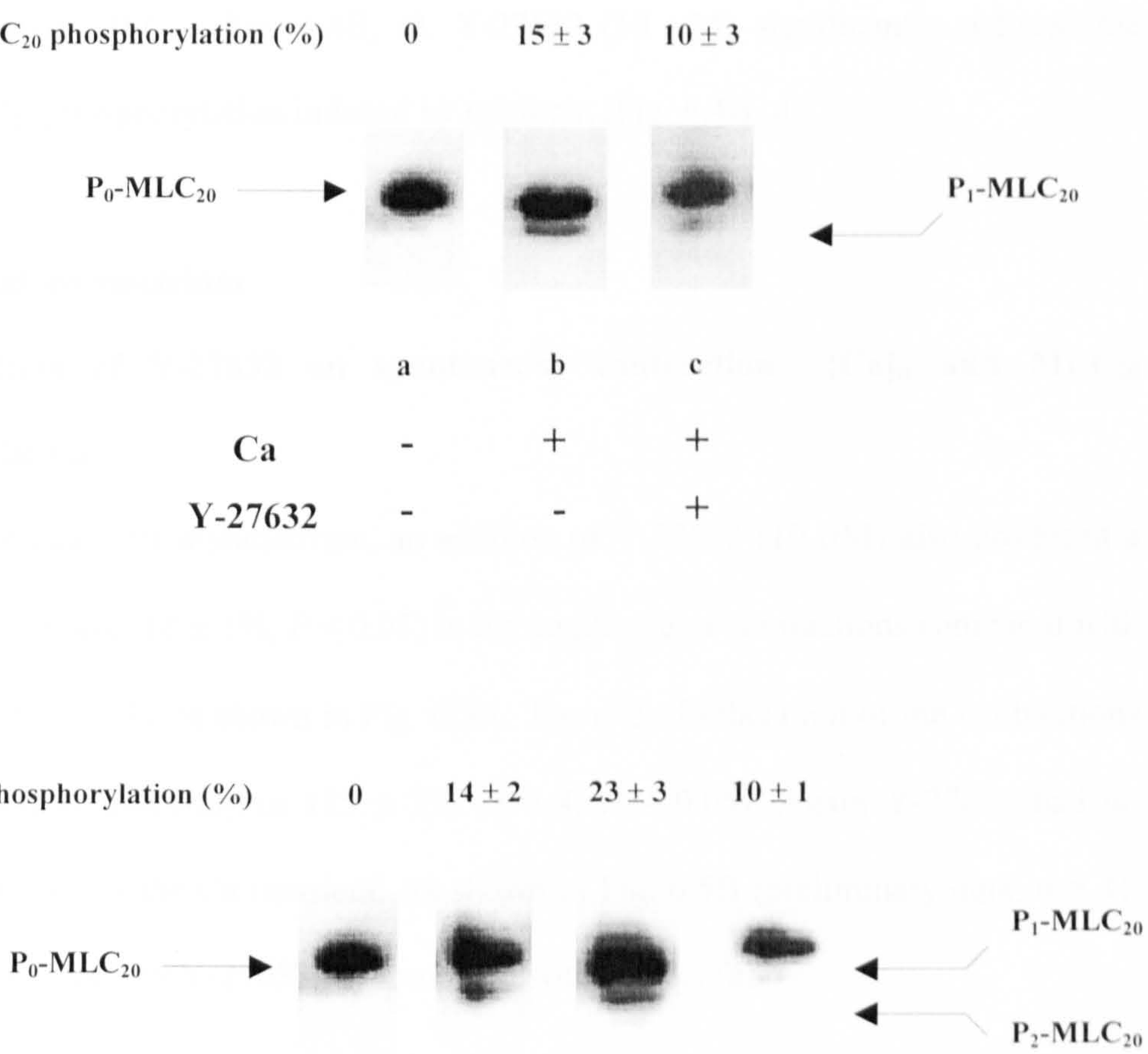


Figure 6.3 The effects of Y-27632 on the response of force and cytosolic Ca to depolarisation (40 mM KCl); control **(A)** and **Y-27632 (B)** response were obtained in the same tissue, and are superimposed (the inset).

A



B

Figure 6.4 The effects of Y-27632 on MLC_{20} phosphorylation. The level of MLC_{20} phosphorylation was measured in the absence and presence of Y-27632. Results shown are the mean ± s.e.m of the densitometric analysis (see the methods). (**A**) The level of MLC_{20} phosphorylation was measured in the absence of external Ca (a, n = 10), at the peak of spontaneous contraction in the absence (b, n = 4) and presence of Y-27632 (c, n = 3). (**B**) The level of MLC_{20} phosphorylation was measured in the absence of external Ca (a, n = 10), at the peak of KCl induced-contraction (b, n = 7), after a 5-7 min incubation of oxytocin added under depolarisation in the absence (c, n = 4) or presence of Y-27632 (d, n = 3). P_0-MLC_{20} denotes unphosphorylated MLC_{20}. P_1-MLC_{20} denotes monophosphorylated MLC_{20}. P_2-MLC_{20} denotes diphosphorylated MLC_{20}, n denotes the number of samples.

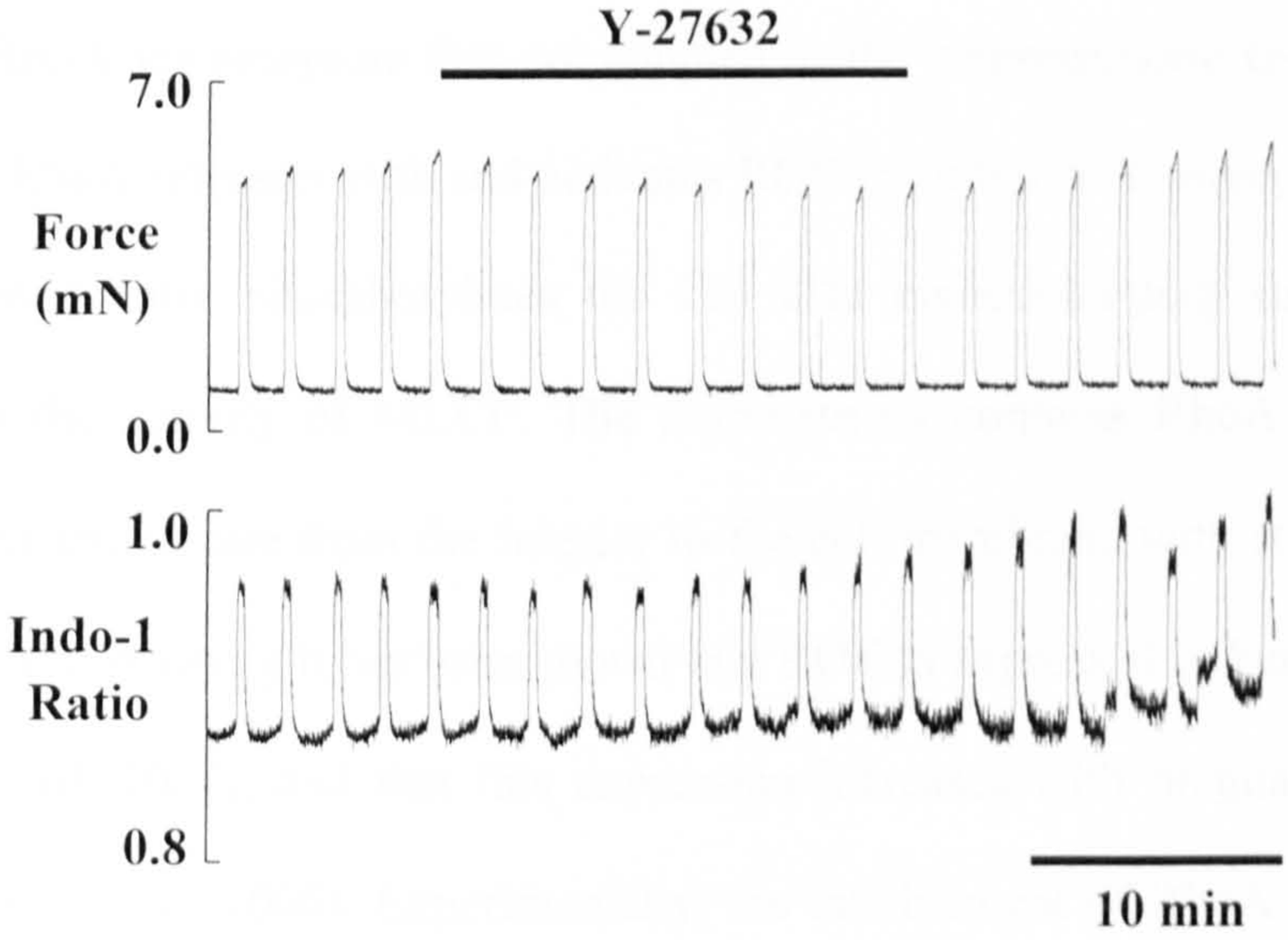
the continued presence of KCl not only produced monophoshorylated MLC_{20}, but also diphoshorylated MLC_{20} (Fig. 6.4B, c). Y-27632 (10 μM) significantly reduced the extent MLC_{20} phosphorylation induced by oxytocin (Fig. 6.4B, d).

6.4.2 Animal myometrium

6.4.2.1 Effects of Y-27632 on spontaneous contractions, $[Ca]_i$, and MLC_{20} phosphorylation

In pregnant rat myometrium, an addition of Y-27632 (10 μM) also produced a significant decrease (18 ± 3%, $P < 0.05$) in the amplitude of contractions compared with control (100%, $n = 4$), as shown in Fig. 6.5A. The rate of relaxation of the contractions also increased significantly to 125 ± 2% ($n = 4$, $P < 0.05$). Again Y-27632 had no significant effect on the Ca transient. As shown in Fig. 6.5B (preliminary data, $n = 1$), MLC_{20} was reduced by Y-27632 (b) compared with control (a).

A



B

MLC_{20} phosphorylation (%)	**24.00**	**16.00**

	a	**b**
Y-27632	**-**	**+**

Figure 6.5 Observations of the effects of Y-27632 on pregnant rat myometrium. (**A**) The effect on spontaneous force and Ca transients (n = 4). (**B**) The effect on myosin light chain (MLC_{20}) phosphorylation measured at spontaneous peak amplitude in the absence (a) and presence of Y-27632 (b) (n = 1). Results shown are the mean ± s.e.m of the densitometric analysis (see the methods). P_0-MLC_{20} denotes unphosphorylated MLC_{20}. P_1-MLC_{20} denotes monophosphorylated MLC_{20}. n denotes the number of samples.

6.5 Discussion

Ca-sensitisation of smooth muscle involves the small GTPase RhoA. Agonists activate RhoA via receptors that are coupled to the heterotrimeric G-proteins, and the activated RhoA interacts with and activates ROK (Somlyo & Somlyo, 2000). Activated ROK subsequently phosphorylates the 130 kDa myosin-binding subunit of MLCP, inhibiting the activity of MLCP. The myometrium contains RhoA and it has been reported to translocate from the interior to the cell membrane with stimulation (Lee *et al.* 2001). Furthermore it has been shown that ROK is expressed in human myometrium (Moran *et al.* 2002), and that this expression increases with pregnancy (Niiro *et al.* 1997; Moore *et al.* 2000). Experimentally, the involvement of RhoA-ROK-pathway in force modulation has been amenable to testing, due to an inhibitor of ROK; Y-27632 (Fu *et al.* 1998). At 10 μM, Y-27632 has been reported to inhibit agonist-induced contraction, without altering Ca, by inhibiting the kinase activity of Rho-kinase specifically in a manner competitive with ATP (Loirand *et al.* 1999; Ishizaki *et al.* 2000). The aim of the chapter was to investigate whether such pathway plays a potential role in human myometrial contraction using Y-27632 to inhibit ROK.

The data demonstrated that the amplitude and duration of the normal phasic contractions of pregnant non-labouring uterus decreased when ROK modulation of MLCP was inhibited by Y-27632. The degree of modulation was, however, only small, indicating that phasic activity is not greatly enhanced by this mechanism. This data are consistent with the previous findings that calcium entry and stimulation of MLCK is the predominant mechanism modulating spontaneous contractions (see chapter 5). The spontaneous contractions also shortened due to an increased rate of relaxation. This suggests that the MLCP was more active, consistent with ROK inhibition by Y-27632.

The augmentation of force and $[Ca]_i$ in response to physiological concentrations of oxytocin (10 nM) were little affected by Y-27632, consistent again with Ca entry playing a major role in this kind of contraction. However, when oxytocin was added under depolarised conditions or at supra-physiological concentrations (100 nM), and force production occurred with little rise of $[Ca]_i$, indicating that a change in the sensitivity to Ca is the major mechanism, then Y-27632 had profound effects. These conditions perhaps also occur in permeabilised preparations in which $[Ca]_i$ is maintained at elevated levels: under such circumstances Y-27632 also has large effects on force in rat myometrium (Lee *et al.* 2001). Interestingly, Y-27632 also decreased force in preparations depolarised by high K, indicating that MLCP modulation may play a role in this type of maintained contraction (Trujillo *et al.* 2000; Mita *et al.* 2002).

The above findings of human myometrium are accordance with observations on the pregnant rat myometrium (see Fig. 6.5). In animals, Y-27632 again produced a small reduction in force arising spontaneously (Fig 6.5A). The reduction of force was occurred along with decreases in the level of MLC_{20} without altering $[Ca]_i$ (Fig. 6.5B). The effect of Y-27632 on agonist-induced contraction of the pregnant rat myometrium was not be addressed in this chapter, however, Tahara *et al.* (2002) have gone on to show that Y-27632 decreased oxytocin-induced force with no changes in $[Ca]_i$ and that it reduced the phosphorylation of MLC_{20} produced by the agonist. Taken together, the similar effects of Y-27632 in human and rat myometrium, suggest that the mechanism of contraction elicited by the RhoA-ROK-pathway is the same in both species.

Smooth muscle contractile activity can be classified into two major types: phasic and tonic (Himpens *et al.* 1988). Phasic activity responds to depolarisation with a relatively rapid contraction that declines within minutes to an intermediate level, whereas, tonic activity, it is followed by sustained increases in force (Himpens *et al.* 1988). In this chapter, the data clearly show the different degree of Y-27632 inhibition between phasic and tonic activity (see below). The effects of Y-27632 were larger on the tonic activity produced by high K or oxytocin added under depolarisation (> 50% inhibition) than the phasic activity arising either spontaneously or with 10 nM oxytocin (< 20% inhibition). Similar results were obtained in other smooth muscles whereby the inhibitor had a relatively minor effect on the phasic response of agonist-induced contraction compared with the tonic response (Otto *et al.* 1996; Sward *et al.* 2000). The exact mechanism leading to the differences in responding to Y-27632 is unclear. However, it has been suggested that the Ca sensitivity might differ between phasic and tonic contraction (Kitazawa & Somlyo, 1990) and that MLCK and MLCP activities vary between those contractions (Mita *et al.* 2002). Mita *et al.* (2002) proposed that, during phasic contraction, $[Ca]_i$ is high enough to activate MLCK to a level that is much greater than that of MLCP. However, during the sustained contraction, maintained $[Ca]_i$ may not be high enough to activate MLCK to a level sufficient to overcome the phosphatase. In this case, inhibition of MLCP by ROK is required for the activity ratio of MLCK to MLCP to maintain MLC_{20} phosphorylation elevated.

It has become clear that agonists can increase the sensitivity for Ca in pregnant rat myometrium (Izumi *et al.* 1995a). It has also been shown that oxytocin can elicit uterine contraction in Ca-free solution in both rat (Matsuo *et al.* 1989; Oishi *et al.* 1991) and human myometrium (see chapter 5). The myometrium responds to oxytocin in a concentration-dependent manner. At a physiological concentration (10 nM), oxytocin

increased the phasic contractile activity (Fig. 6.2). Both force and Ca transients were potentiated by oxytocin, and were abolished by nifedipine (see chapter 4, Fig. 4.8) but not Y-27632 (Fig. 6.2A). At a high dose (100 nM), oxytocin can induce greater increased force, which is not associated with $[Ca]_i$ indicating increases in the sensitivity to Ca, then Y-27632 had profound effects. It is also clear that (1) oxytocin added under depolarisation was associated with enhanced MLC_{20} phosphorylation, which presumably resulted in the inhibition of MLCP and thereby induced contraction (2), RhoA mediated this MLC_{20} phosphorylation via activation of ROK and that the phosphorylation was inhibited by Y-27632 (Fig. 6.4B). Together, oxytocin exerts its effects via both Ca-CaM-MLCK and non-Ca-CaM-MLCK pathway, the results suggested that the later pathway is, at least in part, via RhoA-ROK-pathway and that the ability of oxytocin to increase the sensitivity to Ca of the uterus is concentration-dependent. The findings also fit with previous suggestions that the RhoA-ROK-pathway is one of the mechanisms whereby oxytocin produces its stimulation of the uterus (McKillen *et al.* 1999).

It is interesting to note, in the present study, that the diphosphorylation of MLC_{20} occurred upon oxytocin (100 nM) added under depolarisation and that it was more sensitive to Y-27632 than was the monophosphorylation. The diphosphorylation of MLC_{20} has been noted in intact smooth muscles such as cabachol-stimulated (Colburn *et al.* 1988) and neurally stimulated (Miller-Hance *et al.* 1988) bovine trachea and $PGF_{2\alpha}$-stimulated rabbit aorta (Seto *et al.* 1996) and in actively growing cultured SM-3 smooth muscle cells (Seto *et al.* 1990). Additionally, it has been shown that the diphosphorylation produced by $PGF_{2\alpha}$ was sensitive to other ROK inhibitors such as HA-1077 and HA-1100 (Sakurada *et al.* 1998). However, the function and generation

mechanism of diphosphorylation of MLC_{20} upon agonist stimulation is unknown (Sakurada *et al.* 1998).

As discussed in chapter 1, smooth muscle contraction is regulated by electromechanical, in addition to pharmacomechanical coupling mechanisms. Electromechanical coupling operates via changes in surface membrane potential, which affect $[Ca]_i$. Stimulation by K induces depolarisation of the cell membrane, which opens voltage-gated Ca channels causing Ca influx to increase $[Ca]_i$ (Bolton *et al.* 1999). Thus, high-K-induced contraction may depend entirely on the influx of extracellular Ca and is not mediated by receptors. Indeed, the contractile response to high-K-induced-contraction was found to be absolutely dependent on the influx of Ca through voltage-gated Ca channels, since it could be blocked by a Ca-channel blocker or by removal of extracellular Ca (Trujillo *et al.* 2000). As shown in chapter 5, treatment of myometrial strips with the MLCK inhibitor also inhibited the contraction induced by high K, without changes in $[Ca]_i$. In this chapter, Y-27632 inhibited the high-K-induced contractions of myometrial strips (Fig. 6.3). On the other hand, Y-27632 had no effect on high-K-induced changes in $[Ca]_i$ (see also Fig. 6.3). It therefore seems likely that maintained contraction in response to high K depends on both MLCK and ROK activities. The results shown here are similar to those obtained in guinea-pig intestine (Otto *et al.* 1996), human small arteries (Martinez *et al.* 2000), and rat tail arteries (Mita & Walsh, 1997; Uehata *et al.* 1999; Weber *et al.* 2000; Mita *et al.* 2002), though there are differences between the degree of inhibitory effects of Y-27632 on high K-induced contraction, probably due to differences in MLCP activities in different smooth muscles. Application of Y-27632 to those smooth muscle preparations before or after stimulation by high K partially (Otto *et al.* 1996; Uehata *et al.* 1999; Martinez *et al.* 2000) or completely (Mita & Walsh, 1997; Weber *et al.* 2000; Mita *et al.* 2002)

inhibited the high-K-induced contractions, but had no effect on the sustained increase in $[Ca]_i$ (Mita & Walsh, 1997; Uehata *et al.* 1999; Martinez *et al.* 2000; Weber *et al.* 2000; Mita *et al.* 2002).

In summary, the results indicate that inhibition of RhoA via Y-27632 significantly reduced the force produced by human either spontaneously, by oxytocin or high K depolarisation. However, the role of sensitisation during phasic activity is small compared to modulation of $[Ca]_i$. Thus the effect of MLCP modulation *in vivo* and its usefulness as a therapeutic tool will depend upon the relative importance of Ca-sensitising pathways, compared with Ca entry mechanisms.

Chapter 7

The Effects of Inhibiting Myosin Light Chain Phosphatase on Force, Intracellular Calcium, and Myosin Phosphorylation in Human Myometrium

Chapter 7

The Effects of Inhibiting Myosin Light Chain Phosphatase on Force, Intracellular Calcium, and Myosin Phosphorylation in Human Myometrium

7.1 Abstract

Smooth muscle force can be regulated by myosin light chain kinase and myosin light chain phosphatase (MLCP) activity. Modulation of MLCP activity has been shown to greatly influence force in permeabilised and tonic smooth muscles without changing calcium. To determine if such modulation influences contractions in an intact, phasic smooth muscle; the uterus, the effects of phosphatase inhibitors on force, calcium and myosin light chain (MLC_{20}) phosphorylation were studied. Application of okadaic acid and cantharidin, inhibitors with more specificity for the type 2A phosphatase than type 1 phosphatase, at 10^{-8} M produced no significant change in the amplitude of spontaneous force and calcium transients ($n > 5$ for each inhibitor). At 10^{-7} M, both okadaic acid ($n = 3$) and cantharidin ($n = 3$) decreased the amplitude of force and calcium transients, and at 10^{-6} M they abolished force and calcium ($n = 3$ for each). In contrast to the effects on spontaneous contraction, application of those inhibitors on high K-induced contraction resulting in an increase force accompanied by an increase in MLC_{20} phosphorylation without altering Ca. The most marked inhibition of MLCP on spontaneous contraction was seen on the application of calyculin A; an inhibitor with more specificity for the type 1 phosphatase than type 2A phosphatase. Calyculin A, at 10^{-8} - 10^{-7} M, markedly increased the baseline force, but not calcium ($n = 7$), and produced a significantly

slower relaxation (115 ± 6% slower compared to 100% control, $P < 0.05$, $n = 5$). The level of MLC_{20} phosphorylation was also increased with calyculin A. The results suggest that there is a modulation of pregnant, non-labouring uterus, by MLCP. However, there are important differences between the type 1 phosphatase and the type 2A phosphatase in modulating the process of contraction.

7.2 Introduction

As mentioned throughout this thesis the principal mechanism of regulation of smooth muscle contraction and relaxation involves myosin phosphorylation and dephosphorylation, respectively. Contractile stimuli often increase intracellular free calcium concentration ($[Ca]_i$) via Ca entry from the extracellular space through voltage-gated or receptor-operated Ca channels or Ca release from the sarcoplasmic reticulum through Ca release channels (inositol 1,4,5-trisphosphate receptors or ryanodine receptors). The increase in $[Ca]_i$ saturates the Ca-binding sites of calmodulin (CaM) leading to activation of myosin light chain kinase (MLCK). The active kinase (Ca_4-CaM-MLCK) then catalyses transfer of the terminal phosphoryl group of MgATP to serine 19 of the two 20 kDa light chain subunits of myosin (MLC_{20}). This simple phosphorylation reaction activates cross-bridge cycling, i.e. force development or shortening of the smooth muscle cell (Hoar *et al.* 1979; Walsh *et al.* 1982). Relaxation generally results following a return of $[Ca]_i$ to resting levels, due to extrusion of Ca from the cell by the sarcolemmal Ca ATPase and the Na/Ca exchanger or uptake by the sarcoplasmic reticulum membrane Ca-ATPase, resulting in dissociation of Ca from CaM, inactivation of MLCK and dephosphorylation of myosin catalysed by myosin light chain phosphatase (MLCP) (Hartshorne *et al.* 1998). Thus contraction and relaxation can be regulated by the phosphorylation state of MLC_{20}, which depends on the balance between MLCK and MLCP activities.

MLCK is a very specific protein kinase: its only known substrate *in vivo* is myosin (Gallagher *et al.* 1997). At high concentrations *in vitro*, MLCK can phosphorylate threonine 18 of MLC_{20} in addition to serine 19 (Ikebe *et al.* 1986), and diphosphorylation at serine 19 and threonine 18 has been described in stimulated intact smooth muscles (Colburn *et al.* 1988).

MLCP is a serine/threonine phosphatase composed of a 38 kDa catalytic subunit, a 130 kDa myosin-binding subunit that targets the phosphatase to the thick filaments and a 21 kDa subunit of unknown function (Hartshorne *et al.* 1998). Classification of MLCP is based on sensitivity to a defined set of inhibitors and activators (Herzig & Neumann, 2000). The main MLCP are type 1 (PP1) and type 2A (PP2A) (Hartshorne *et al.* 1998).

Although other mechanisms may exist (e.g. thin filament regulation, Morgan & Gangopadhyay, 2001), it is now clear that inhibition of MLCP is the main mechanism of Ca sensitisation (Somlyo & Somlyo, 1994). MLCP inhibition is responsible for an increase in the MLCK: MLCP activity ratio, MLC_{20} phosphorylation and contraction without a change in $[Ca]_i$. Three independent mechanisms have been proposed to account for MLCP inhibition. (1) Phosphorylation of the 130 kDa subunit of MLCP leads to inhibition of the phosphatase (Ichikawa *et al.* 1996). This may occur via activation of the small GTPase Rho-A, a member of the Rho subfamily of the Ras superfamily of monomeric GTPases, that activates Rho-associated kinase (ROK), which in turn phosphorylates the 130-kDa myosin-binding subunit of MLCP, inhibiting phosphatase activity, as discussed in chapter 6 (Kimura *et al.* 1996; Swärd *et al.* 2000). (2) Activation of protein kinase C (PKC) leads to inhibition of MLCP (Masuo *et al.* 1994). This may be mediated by a novel heat-stable protein, CPI-17, which becomes a potent inhibitor of MLCP upon phosphorylation by PKC (Li *et al.* 1998). (3) Arachidonic acid inhibits MLCP activity either directly (Gong *et al.* 1992), or via activation of an atypical PKC isoenzyme (Gailly *et al.* 1997) or ROK (Feng *et al.* 1999).

In the uterus, it is well documented that both MLCK and MLCP are modulated (see below). It has been shown in chapter 5 that MLCK is an important modulator in the pathway of force production. Inhibition of MLCP via ROK has been shown to greatly

influence force, without changing calcium in permeabilised and tonic smooth muscles (Somlyo & Somlyo, 2000), but less so in phasic smooth muscle (see chapter 6). However it is less clear how MLCP activity can be modulated via other pathways i.e. by its inhibitors. Therefore, the objective of this chapter was to determine if such modulation influences contractions in the uterus. The effect of MLCP inhibitors on force, calcium and phosphorylation of MLC_{20} were studied.

Phosphatase inhibitors such as okadaic acid, cantharidin and calyculin A have proved to be very useful tools in the study of the regulation of smooth muscle contraction due to their potency and specificity. In smooth muscle, the mechanism of action of those inhibitors is ascribed to their binding to the catalytic subunit of MLCP, leading to MLCP inhibition and then to an increase in the phosphorylation state of MLC_{20} (Herzig & Neumann, 2000). Several examples have been reported of MLCP inhibition of contractions of intact and permeabilised smooth muscles in response to okadaic acid (Shibata *et al.* 1982; Ozaki *et al.* 1987; Hartshorne *et al.* 1989; Hirano *et al.* 1989; Obara *et al.* 1989; Gong *et al.* 1992), cantharidin (Li & Casida, 1992; Erdodi *et al.* 1995; Knapp *et al.* 1998, Knapp *et al.* 2000), and calyculin A (Hartshorne *et al.* 1989; Ishihara *et al.* 1989; Suzuki & Itoh, 1993). Where investigated, these contractions correlate with an increase in MLC_{20} phosphorylation. In addition the relative contributions of PP1 and PP2A can be elucidated by tissue sensitivity to different MLCP inhibitors (Herzig & Neumann, 2000). PP2A is more sensitive to okadaic acid and cantharidin than PP1 (Knapp *et al.* 2000). Among MLCP inhibitors, calyculin A is also a potent inhibitor of PP1 and PP2A (Ishihara *et al.* 1989). It inhibits PP2A with similar potency to okadaic acid, but PP1 is inhibited with 10-fold to 100-fold greater potency.

Thus, to investigate the effect of MLCP inhibition in the uterus, those inhibitors were applied to contractions, arising either spontaneously or with agonist stimulation. The physiological role of PP2A and PP1 was particularly examined. To determine the relative importance of the MLCK and the MLCP in generating of uterine contractions, a MLCK inhibitor, wortmannin, was also used where necessary.

7.3 Material and methods

Tissue

For this chapter, human myometrial tissues were obtained from women undergoing elective caesarean at term (37-40 weeks). Forty-three of the 45 caesarean biopsies were performed under spinal analgesia and 2 while the women were under general anaesthesia. The indications for caesarean delivery were previous caesarean delivery (32 patients), foetal malpresentation (6 patients), multipregnancy (3 patient), unstable lie (1 patient), patient choice (1 patient), previous traumatic delivery (1 patient), and symphysis separation (1 patient). The mean age of women undergoing caesarean delivery was 31 years (range, 18-40 years).

Simultaneous measurements of calcium and tension

Tissue preparation and simultaneous measurements of calcium and tension are essentially the same as those described in chapter 2.

The tissue was continually perfused with Krebs or Krebs containing high-K, okadaic acid (0.01-0.1 μM), cantharidin (0.01-0.1 μM), calyculin A (0.01-0.1 μM), or oxytocin (10 nM). K was elevated and Na reduced to depolarise the membrane in some experiments.

Measurements of myosin phosphorylation

Measurements of myosin phosphorylation was quantified by an immunoblotting analysis after separation of the nonphosphorylated and phosphorylated forms of myosin light chain (MLC_{20}) by urea/glycerol-polyacrylamide gel electrophoresis (as described in chapter 2). The experiments shown were performed on the same gel.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK) unless stated otherwise. Okadaic acid, cantharidin, and calyculin A were purchased from Calbiochem. Oxytocin was dissolved in 5% acetic acid at a concentration of 1 mM. Okadaic acid, cantharidin, or calyculin A was dissolved in DMSO at a concentration of 100 μM. These stock solutions were diluted to the desired concentrations with Krebs' solution.

Statistics

All values are mean ± s.e.m; *n* is the number of samples, and significance was tested by Student's (paired and unpaired) *t*-test or ANOVA and significance taken at $P < 0.05$.

7.4 Results

7.4.1 The effects of PP2A inhibitors

7.4.1.1 Spontaneous contraction

Okadaic acid and cantharidin are MLCP inhibitors with specificity for PP2A (Hartshorne *et al.* 1998; Knapp *et al.* 1998; Herzig & Neumann, 2000; Knapp *et al.* 2000), and were therefore used to investigate the role of PP2A in spontaneous contraction.

Okadaic acid

In all preparations (n = 7), application of okadaic acid to spontaneous phasic activity of human myometrium caused a significant increase in the frequency of contractions to 161 ± 21% (compared to 100% control, $P < 0.05$). The increased frequency was mirrored in the calcium signal. In the majority of preparations (5/7), application of 10^{-8} M okadaic acid produced no significant change in the amplitude of spontaneous force and calcium transients (Fig. 7.1A). In 2/7 preparations there was a small (< 10%, $P > 0.05$) increase in the amplitude of the contractions but decrease in the amplitude of the Ca transients (Fig. 7.2). In all those preparations (n = 7), okadaic acid had no significant effect on the duration of contraction or accompanying Ca transients (For example, Fig. 7.1B). At 10^{-7} M, okadaic acid decreased the amplitude of force and calcium transients to 80 ± 2% and 83 ± 10%, respectively (n = 3, $P < 0.05$), and at 10^{-6} M it immediately abolished force and calcium (n = 3, Fig. 7.3).

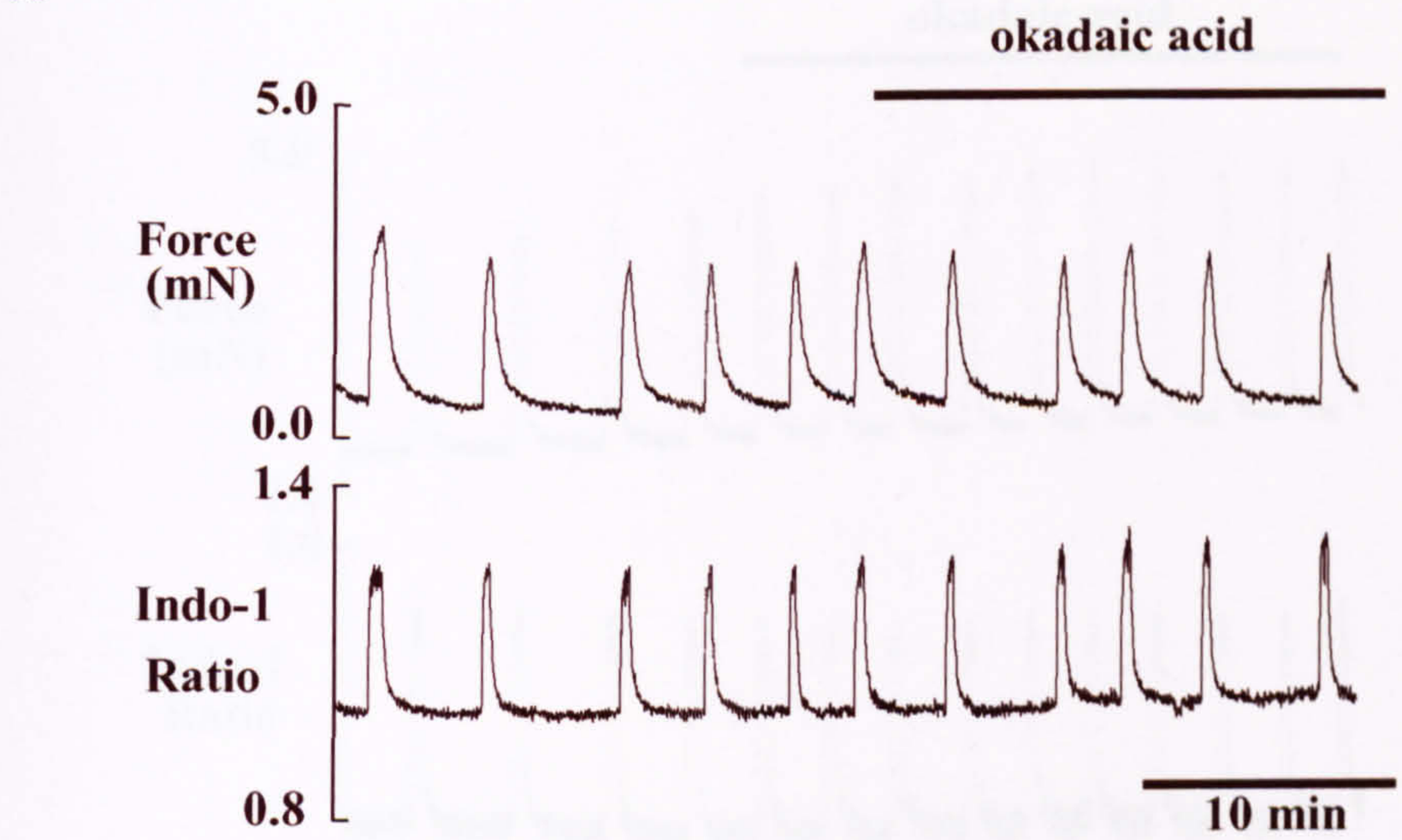


Figure 7.1 The effect of okadaic acid at a low dose on spontaneous force and calcium signal. (**A**) The effect of okadaic acid (10^{-8} M) on force (top panel) and Ca transients (bottom panel) obtained from pregnant human myometrium. (**B**) Taken from A, normalised force and Ca in the absence (black traces) and in the presence of okadaic acid (red traces).

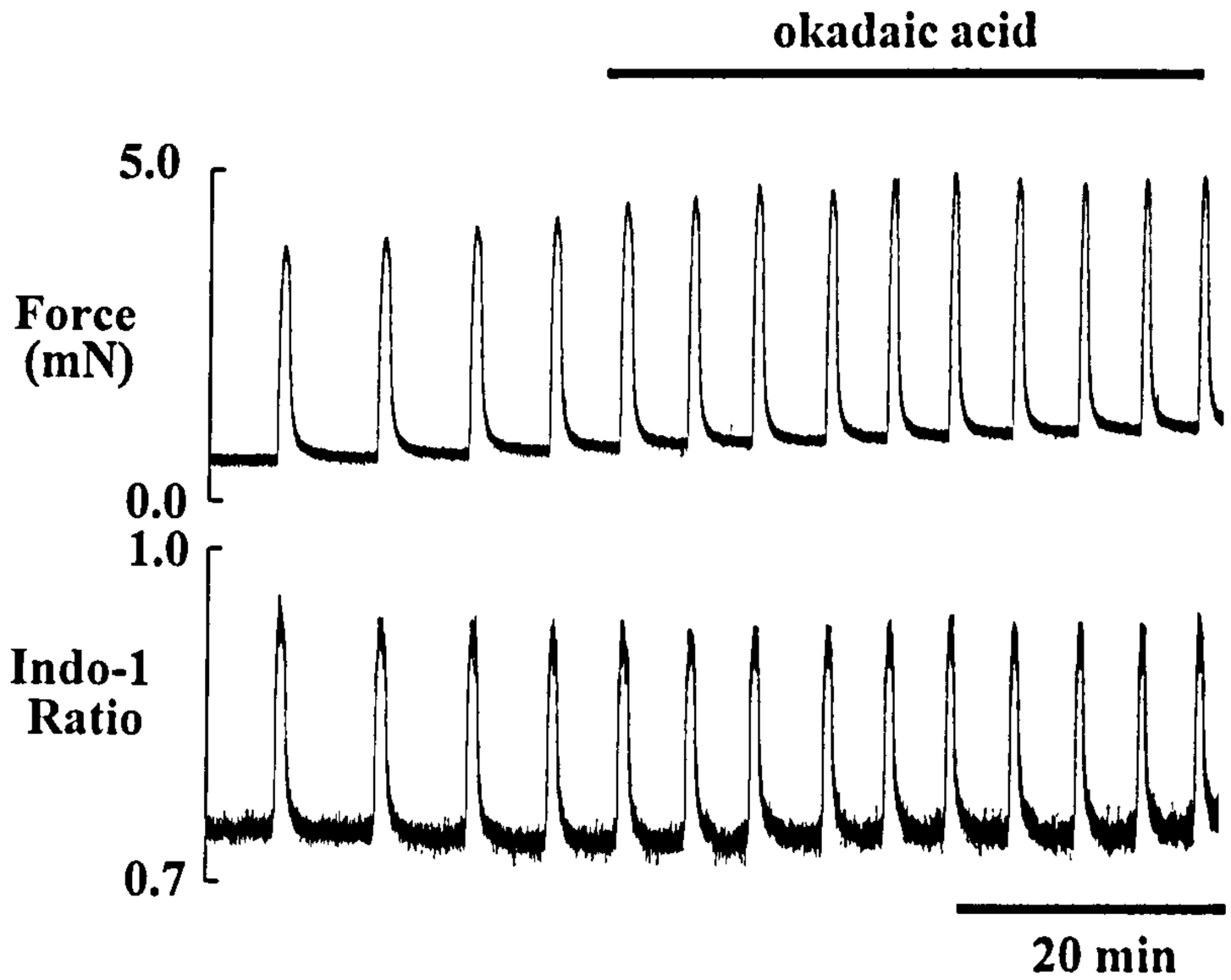


Figure 7.2 The effect of okadaic acid at a low dose on spontaneous force and calcium signal. Shows the recording of force (top panel) and Ca transients (bottom panel) in the absence and in the presence of okadaic acid (10^{-8} M). Okadaic acid increases tension, but decreases Ca.

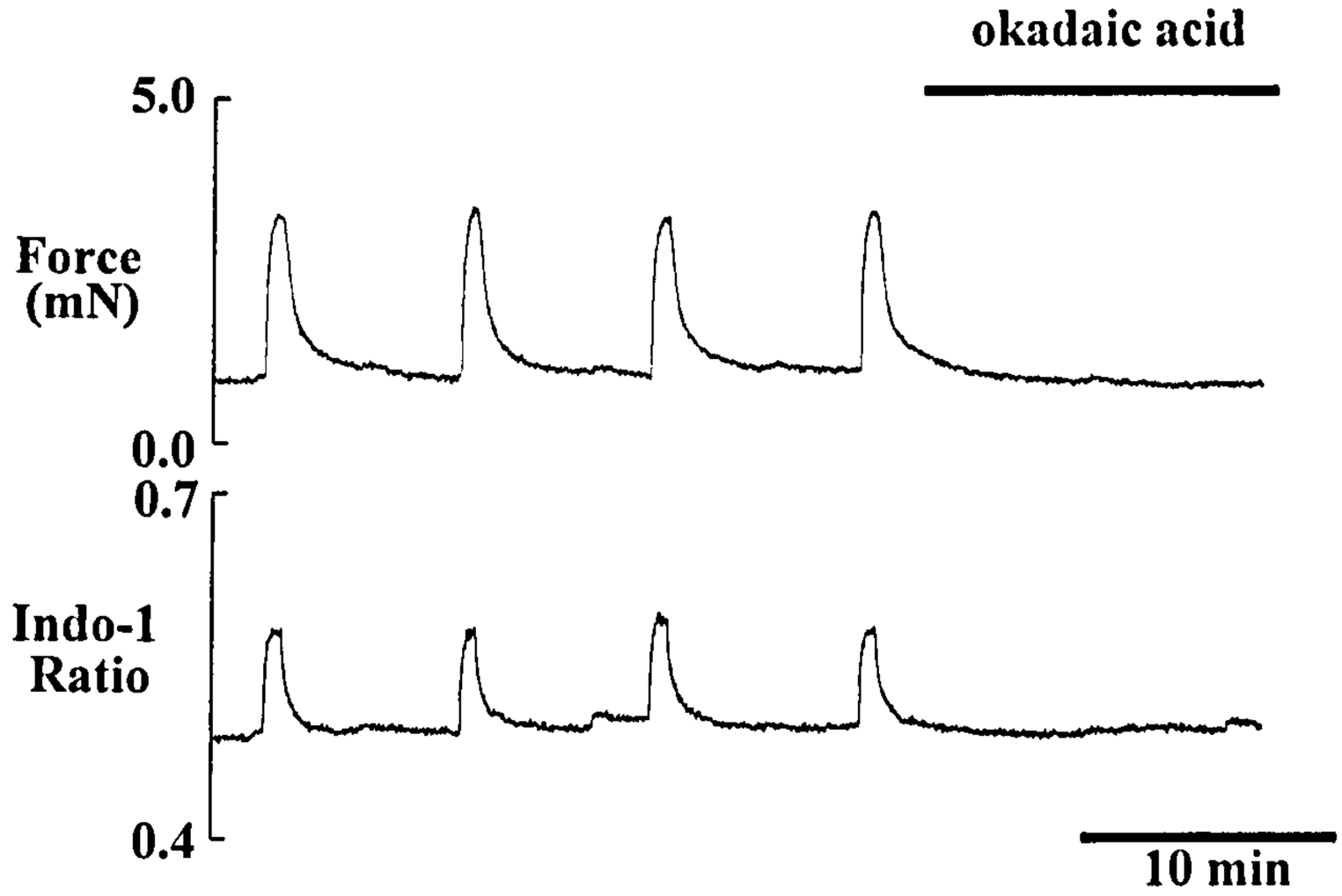


Figure 7.3 The effect of okadaic acid at a high dose on spontaneous force and calcium signal. Okadaic acid (10^{-6} M) was applied to spontaneously contracting pregnant human myometrium for up to 15 minutes.

Cantharidin

Similar effects to those of okadaic acid were seen with cantharidin, there was an increase in the frequency of contractions and Ca transients to 166 ± 15% ($P < 0.05$) upon the application of 10^{-8} M cantharidin (Fig. 7.4A, $n = 12$). At the same concentration, cantharidin produced no significant change in the amplitude of spontaneous force and calcium transients (Fig. 7.4A, $n = 12$). In 7/12 preparations, cantharidin had no significant effect on the duration (Fig. 7.4B), although in 5/12 preparations there was a prolongation of the contraction (123 ± 7%, $P < 0.05$). In 2/12 preparations the effects of cantharidin were to increase the amplitude of the phasic contractions but decrease the amplitude of Ca transients (Fig. 7.5). At 10^{-7} M, cantharidin decreased the amplitude of force and calcium transients to 77 ± 2% and 88 ± 6%, respectively ($n = 3$, $P < 0.05$), and both force and calcium were abolished at 10^{-6} M within 10 minutes ($n = 3$, Fig. 7.6).

In all experiments examined so far the effects of both inhibitors on spontaneous phasic activities were irreversible.

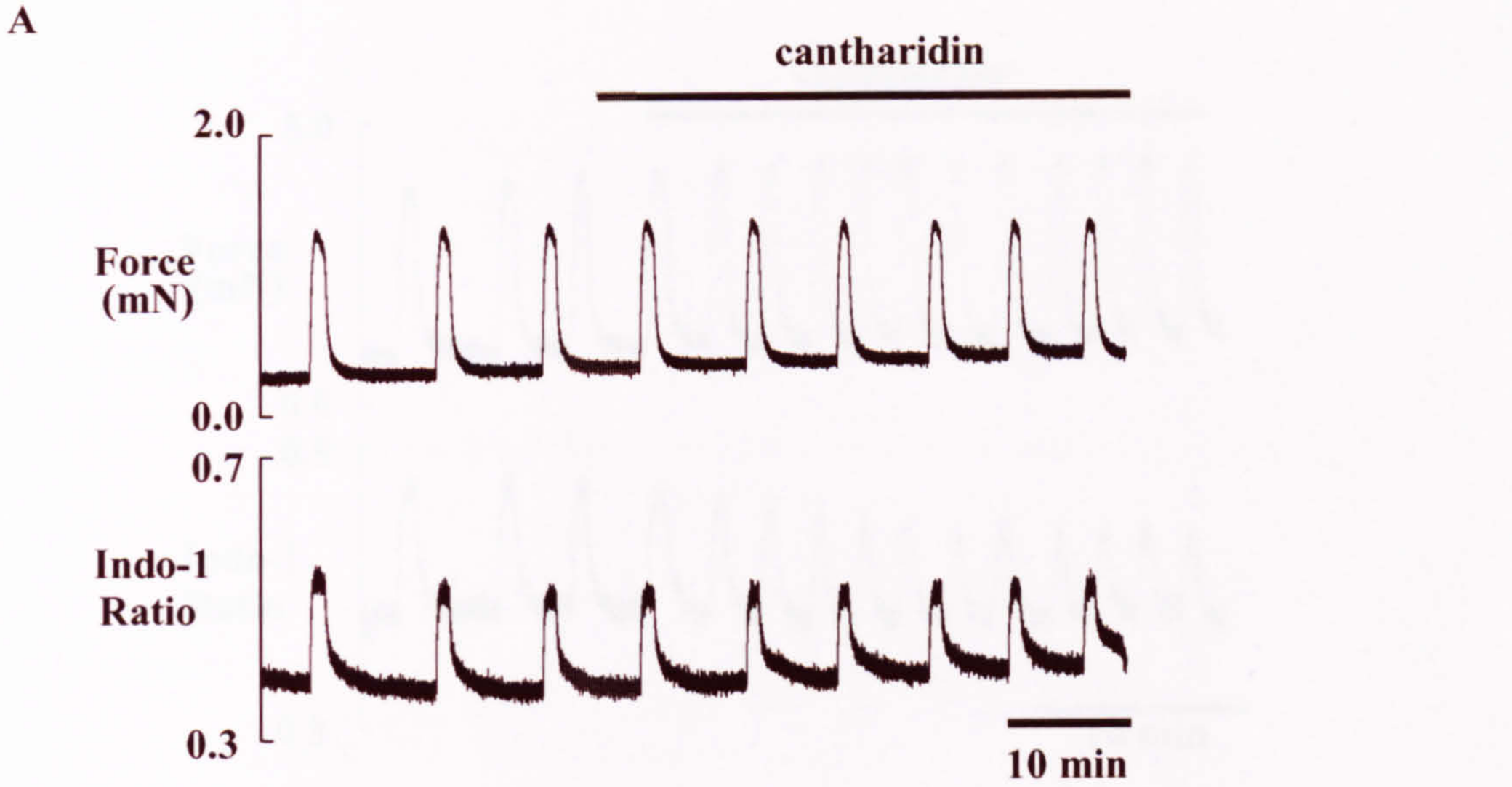


Figure 7.4 The effect of cantharidin at a low dose on spontaneous force and calcium signal. (**A**) The effect of cantharidin (10^{-8} M) on force (top panel) and Ca transients (bottom panel) obtained from pregnant human myometrium. (**B**) Taken from A, normalised force and Ca in the absence (black traces) and in the presence of cantharidin (red traces).

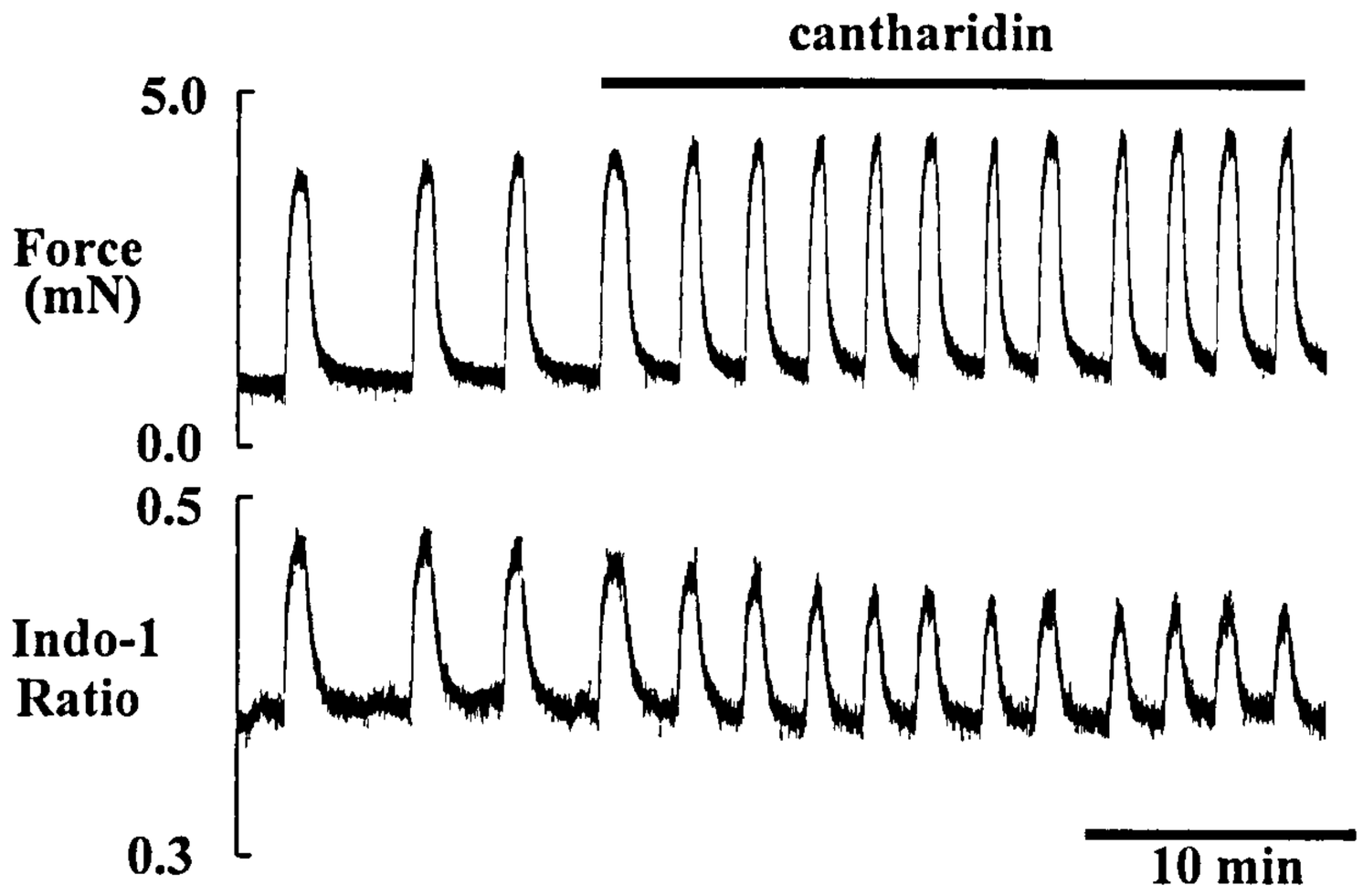


Figure 7.5 The effect of cantharidin at a low dose on spontaneous force and calcium signal. Shows the recording of force (top panel) and Ca transients (bottom panel) in the absence and presence of cantharidin (10^{-8} M). Cantharidin increases tension, but decreases Ca.

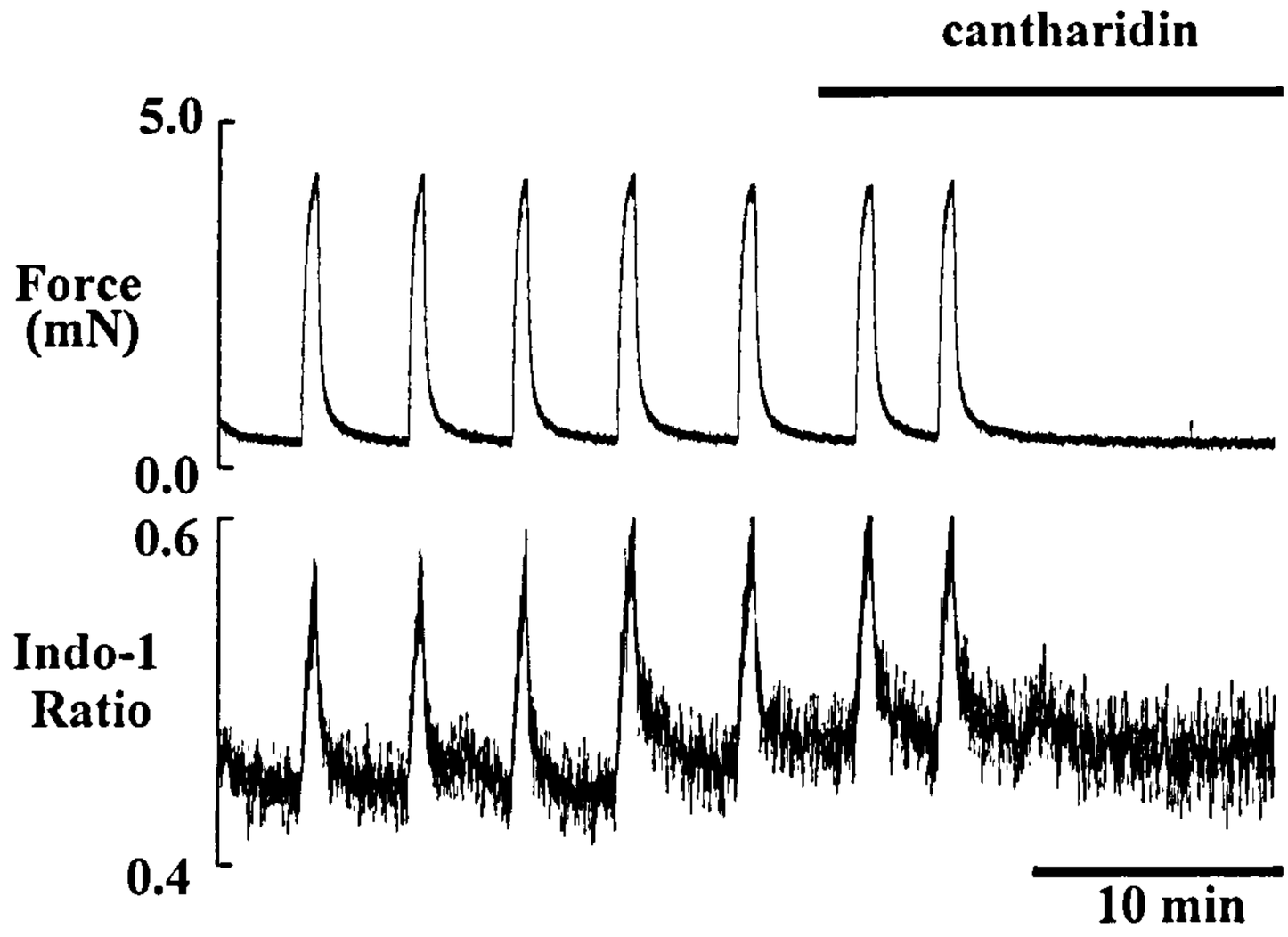


Figure 7.6 The effect of cantharidin at a high dose on spontaneous force and calcium signal. Cantharidin (10^{-6} M) was applied to the strip taken from human myometrium up to 15 minutes.

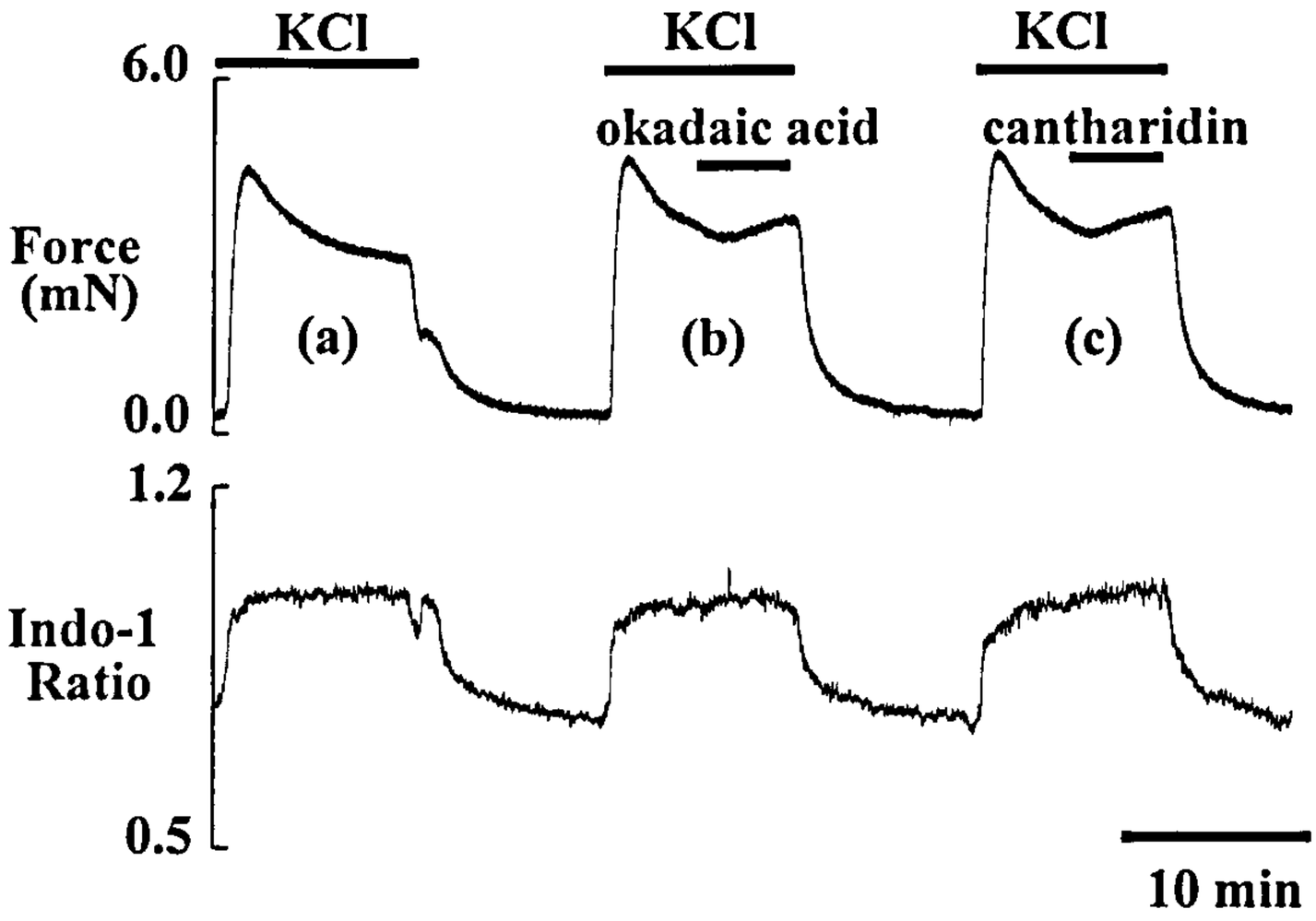
7.4.1.2 KCl-induced contraction

To determine whether membrane depolarisation-induced contraction of human myometrium could involve inhibition of PP2A, the effects of okadaic acid or cantharidin on a KCl-induced contraction were examined.

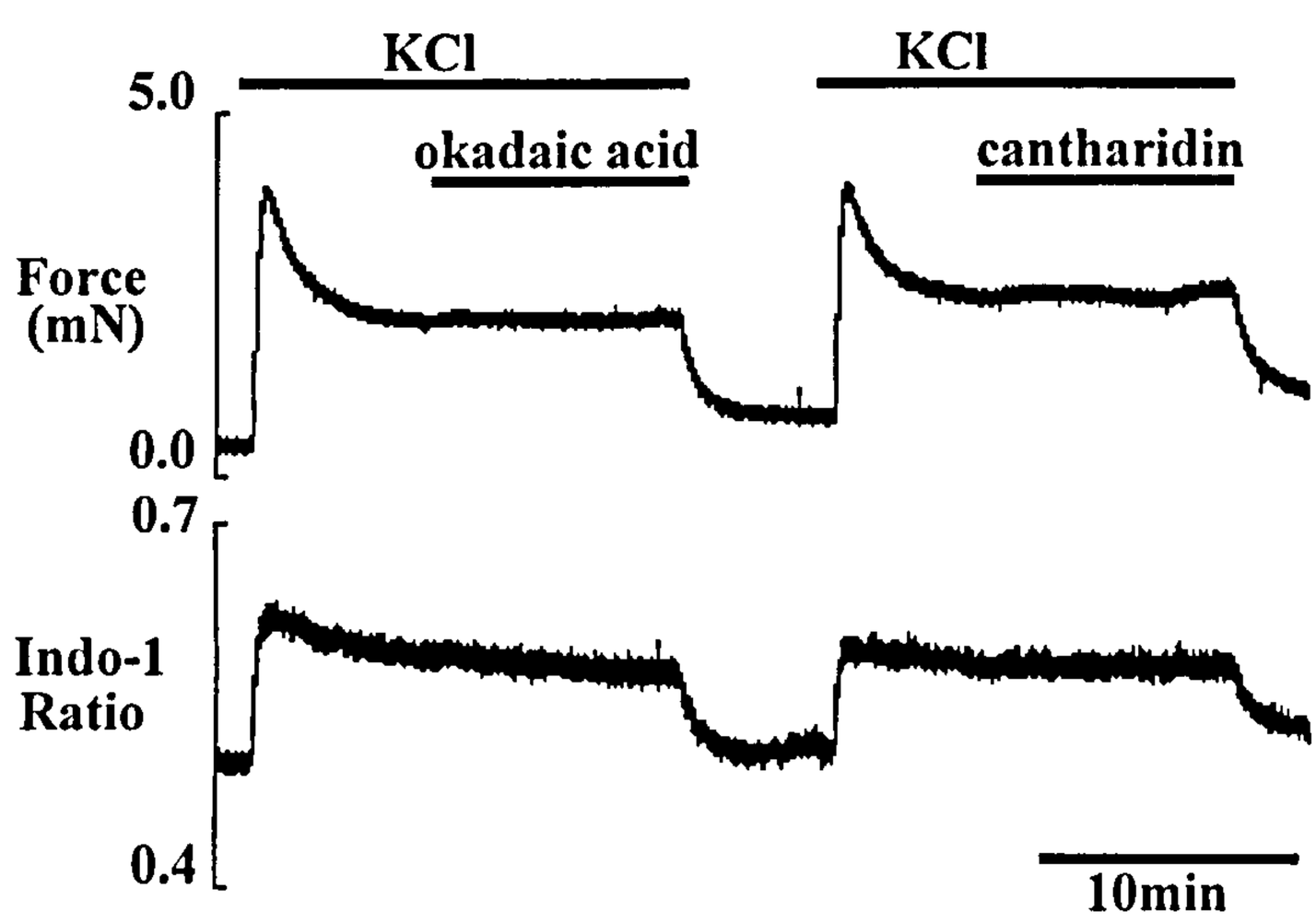
Figure 7.7A illustrates the effect of PP2A inhibitors on high-K-induced contractions. Application of KCl (40 mM) caused an increase in force and Ca (see "a" on Fig. 7.7A), which then rapidly declined and were then maintained at an elevated level (compared to resting levels) until return to normal solution. Addition of okadaic acid (at 10^{-7} M, $n = 5$) 5 minutes after the application of high K caused the tension to rise significantly to 110 ± 3% of control force development, 100% force is the level of maintained force produced during application of high K (see "b" on Fig.7.7A). Cantharidin (at 10^{-7} M, $n = 5$) added under depolarised contractions produced the same effect as okadaic acid; a significant increase in tension to 110 ± 3% of control values (see "c" on Fig. 7.7A). As can be seen there were no corresponding changes in the Ca signal throughout the entire period that both inhibitors were present. At 10^{-8} M, both okadaic acid and cantharidin also increased the level of maintained force produced by KCl ($n = 3$, $P > 0.05$), but the degree of increases in tension was lower, as can be seen in Fig. 7.7B, than that of at 10^{-7} M (Fig. 7.7A). Thus, the effect of PP2A inhibitors on high-K-induced contractions was concentration-dependent.

To investigate whether the potentiated force during the application of those inhibitors was due to phosphatase inhibition and to an increase in the phosphorylation state of MLC_{20}, the muscle was frozen during application of MLCP inhibitors in the continued presence of KCl and the measurement of MLC_{20} phosphorylation performed. As shown in Fig. 7.8 the level of MLC_{20} phosphorylation was significantly increased with those PP2A inhibitors at 10^{-7} M (Fig. 7.8).

A



B



<u>Figure 7.7</u> The effects of okadaic acid and cantharidin on KCl-induced contraction. (A) Application of KCl (40mM) in the absence (a), and the presence of okadaic acid (b) and cantharidin at 10^{-7} M (c). **(B)** The effects of okadaic acid and cantharidin (both at 10^{-8} M) on KCl-induced contraction.

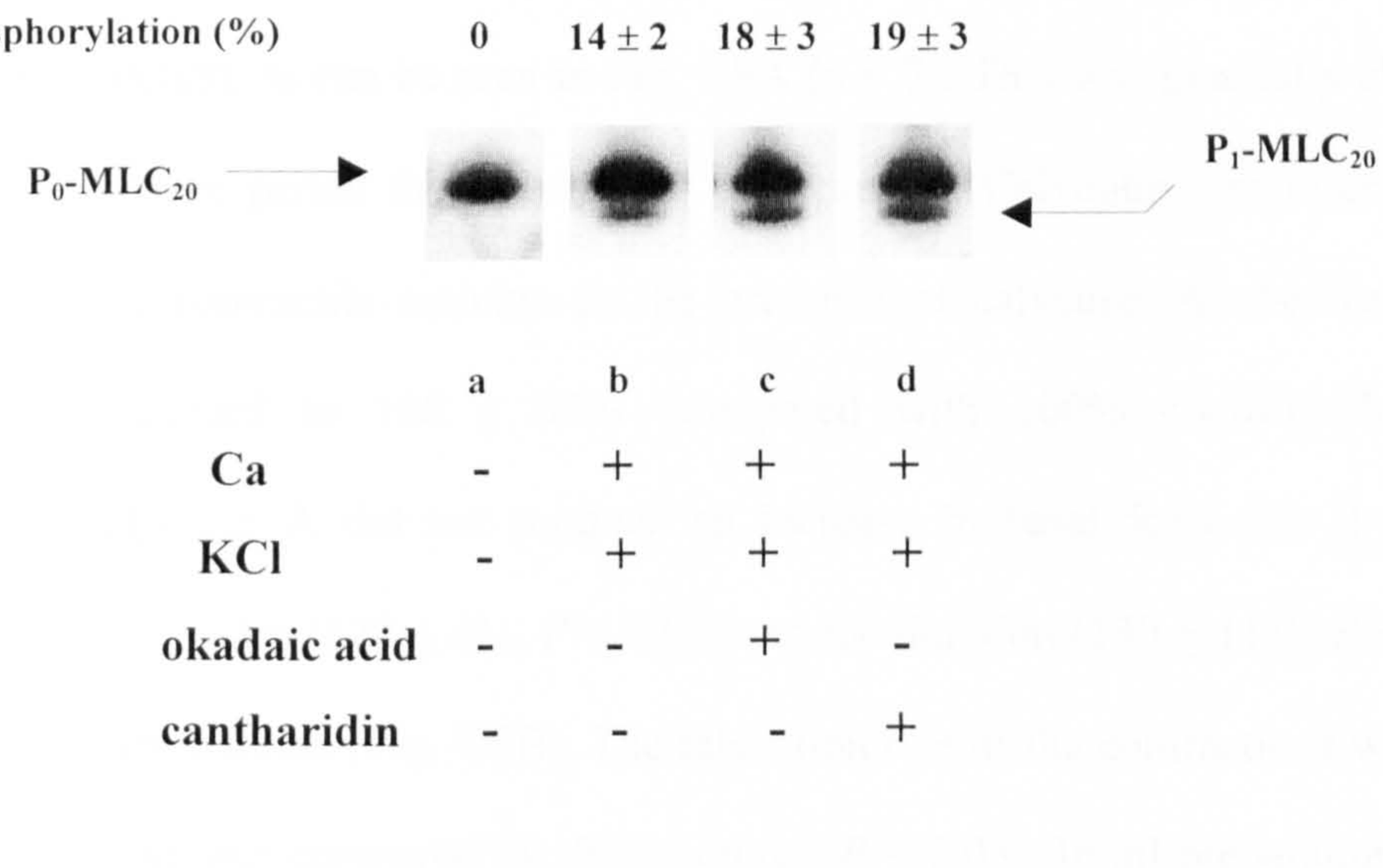


Figure 7.8 The effects of PP2A inhibitors (10^{-7} M) on MLC_{20} phosphorylation. Results of an immunoprecipitation experiment. Results shown are the mean ± s.e.m of the densitometric analysis (see the methods). The level of MLC_{20} phosphorylation was measured in the absence of external Ca (a, n = 10), after 10 min of KCl (40 mM, n = 7) application (b), and after 10 min of KCl with okadaic acid (c, n = 4) or cantharidin (d, n = 4). P_0-MLC_{20} denotes unphosphorylated MLC_{20}. P_1-MLC_{20} denotes monophosphorylated MLC_{20}. n denotes the number of samples

7.4.2 The effects of PP1 inhibitor

7.4.2.1 Spontaneous contraction

The most marked effect of inhibiting MLCP was seen on the application of calyculin A, an inhibitor of PP1 (Hartshorne *et al.* 1998; Herzig & Neumann, 2000), to spontaneously contracting uterus. Calyculin A, at 10^{-8} M, significantly increased basal force but not calcium, as can be seen in Fig. 7.9A ($n = 7$). This was gradually elevated throughout the entire period that calyculin A was present. Calyculin also affected the frequency of the contractile activity. In the presence of calyculin A, the frequency significantly increased to 166 $\pm$ 30% (compared with 100% control). In 5/12 experiments, calyculin A did not produce an increase in basal force but it clearly enhanced the amplitudes (120 $\pm$ 4%, $P < 0.05$) and the duration (130 $\pm$ 11%, $P < 0.05$) of spontaneous contraction (Fig. 7.9B). The relaxation rate of the contractions was also slow (115 $\pm$ 6% slower compared to 100% control, $P < 0.05$). In all preparations (n = 12), calyculin A produced a significant decrease (74 $\pm$ 3% compared to 100% peak of control Ca transients) in the amplitude of spontaneous Ca transients after 15 minutes of application (For example, Fig. 7.9A).

Application of calyculin A, 10^{-6} M, to spontaneous contracting strip resulted in a significant increase in the basal force without a change in basal Ca (Fig. 7.10). It gradually rose to an amplitude similar or exceeding that of the control phasic contractions. At this concentration, calyculin A caused a dramatic and significant fall in Ca amplitude. The amplitude was gradually reduced and finally abolished by around 20 minutes. When calyculin A (10^{-6} M) was applied to non-contracting uterus, only tonic contraction was observed ($n = 3$).

To investigate whether the actions of calyculin A were due to MLCP inhibition and to an increase in the phosphorylation state of MLC_{20}, the muscle was frozen during

application of calyculin A (10^{-8} M) and the measurement of MLC_{20} phosphorylation performed. As shown in Fig. 7.11, the level of myosin phosphorylation was significantly increased with calyculin A; however, it was apparent that there was a dual phosphorylation band.

As smooth muscle force can be regulated by the activity of both MLCP and MLCK, it is of interest to investigate the effect of MLCP inhibition in the presence of a MLCK inhibitor. Fig. 7.12 shows the effect of the MLCP inhibitor, calyculin A, in the presence of the MLCK inhibitor, wortmannin ($n = 5$). There is a clear effect of calyculin A to significantly increase basal force but not Ca transients (Fig. 7.12). Addition of wortmannin (4 μM), in the continued presence of calyculin A, did not prevent the rising of basal force caused by calyculin A. It significantly reduced the contractile amplitude to 40 ± 8% (compared with the highest peak amplitude in the presence of calyculin A alone), after 10 minutes exposure. By 20 minutes, the force fell to near to basal levels elevated by calyculin A. As shown in Fig. 7.12 (bottom trace), the amplitude of the Ca transients was also decreased to around 50% (compared to 100% amplitude of initial response without both inhibitors, $P > 0.05$) by 10 minutes. This decrease was presumably due to the effect of calyculin A as already shown in Fig. 7.9 (bottom trace). The MLCK inhibitor also significantly increased the frequency of contractions with a mean rate of 6.5 ± 2 per 10 minutes; this was significantly quicker than the frequency taken for spontaneous contraction with or without calyculin A.

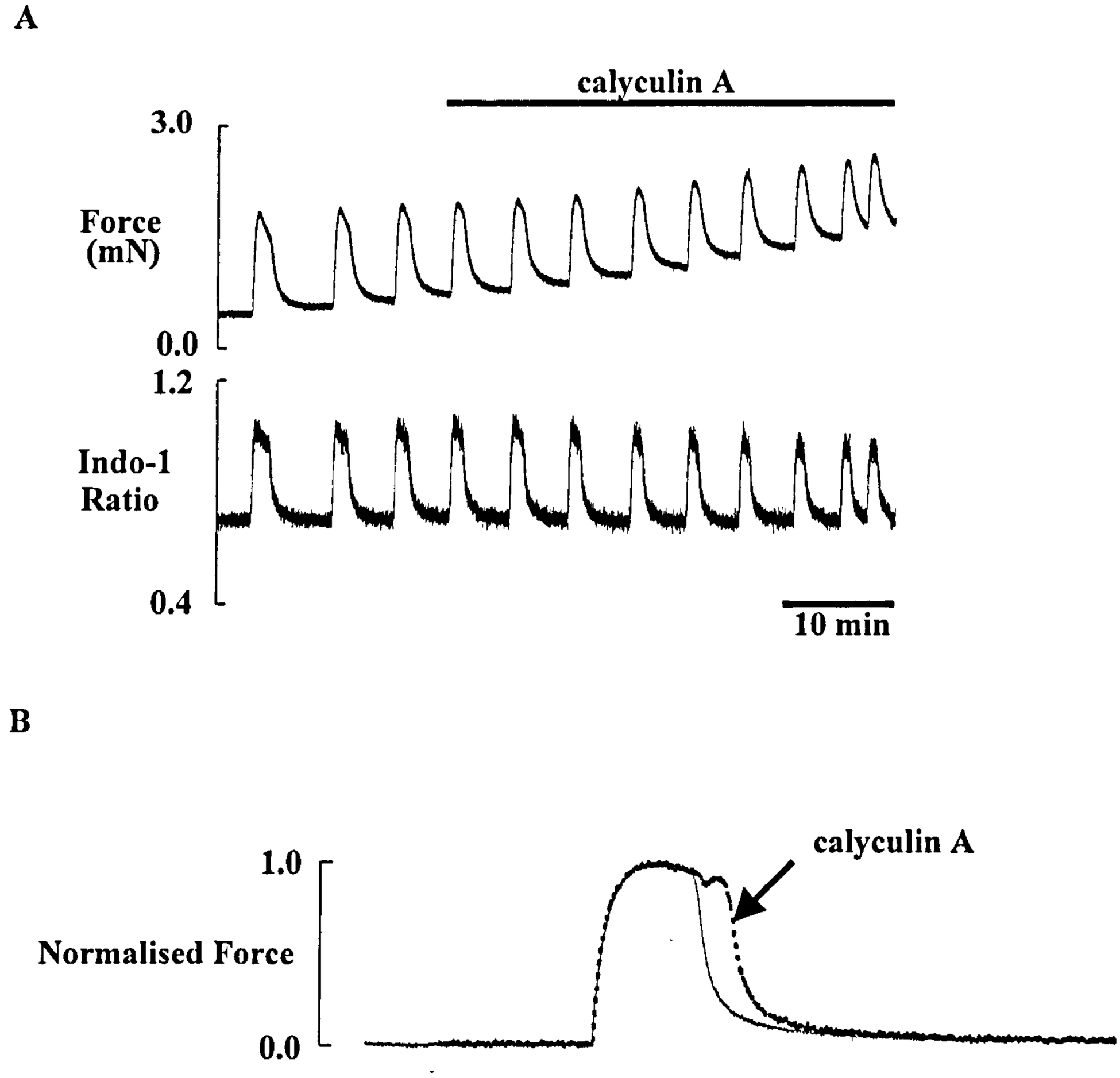


Figure 7.9 The effect of calyculin A on spontaneous force and calcium signal. **(A)** The effect of calyculin A (10^{-8} M) on force (top panel) and Ca transients (bottom panel) obtained from pregnant human myometrium. **(B)** Taken from another experiment, normalised force in the absence and presence of calyculin A.

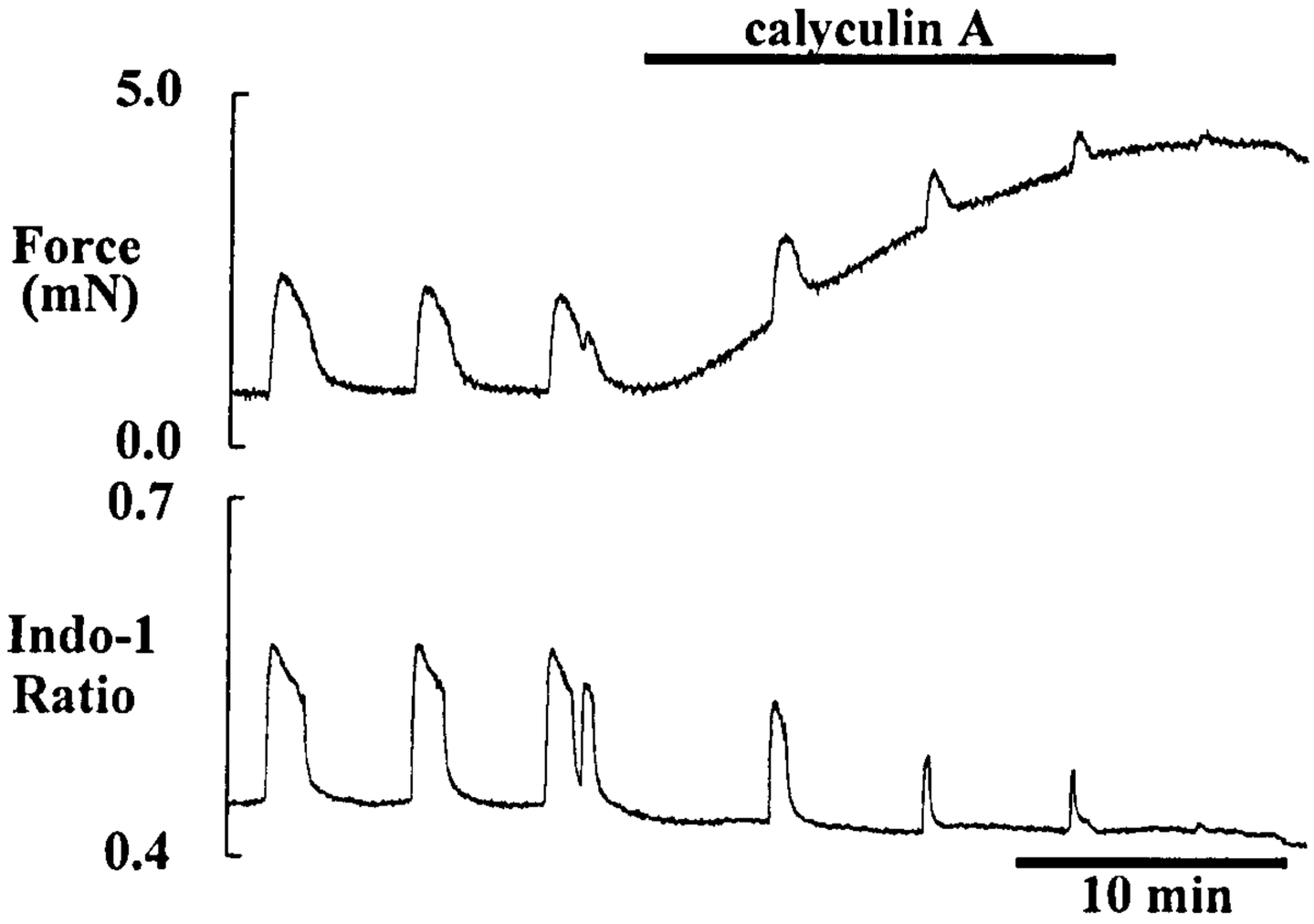


Figure 7.10 The effect of calyculin A at a high dose on spontaneous force and calcium signal. Simultaneous measurements recorded from pregnant human myometrium showing the effect on force (top panel) and Ca transients (bottom panel).

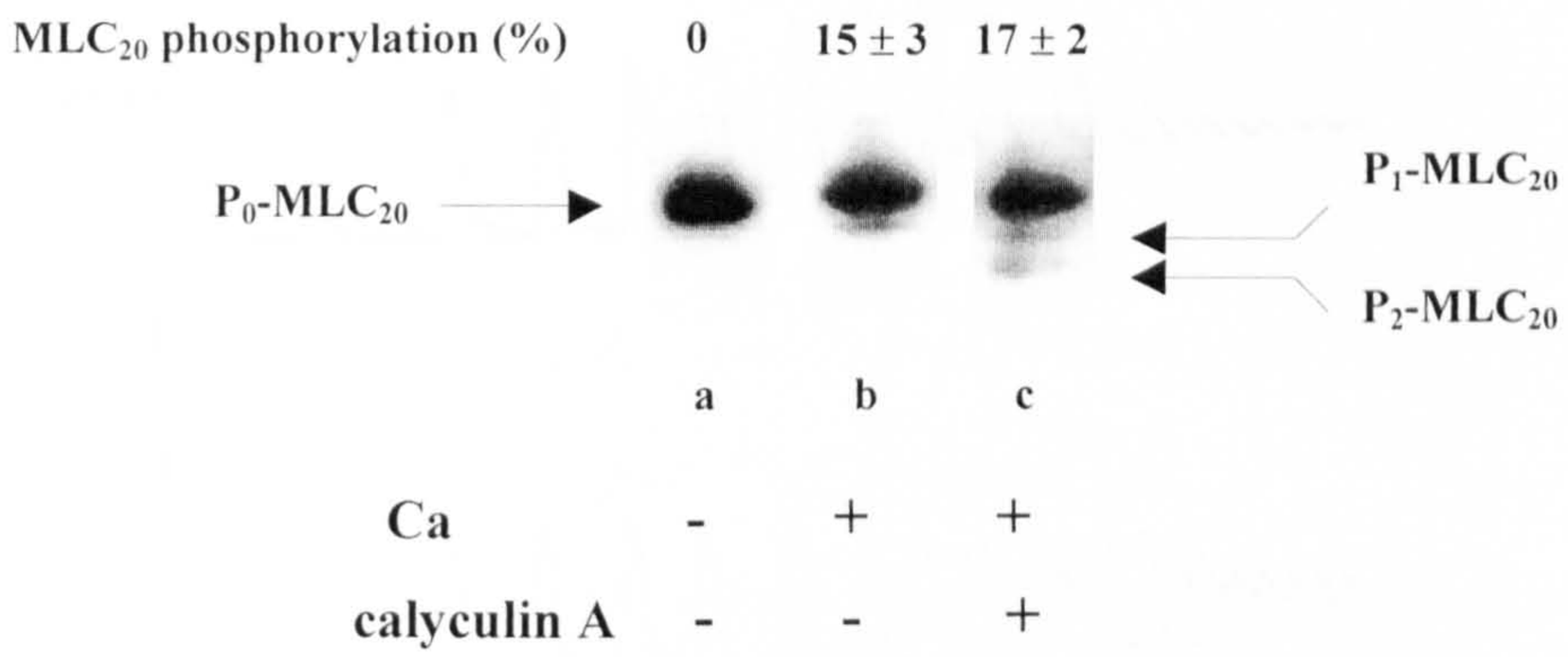


Figure 7.11 The effects of calyculin A on MLC_{20} phosphorylation in spontaneous contraction. Results shown are the mean ± s.e.m of the densitometric analysis (see the methods). The level of MLC_{20} phosphorylation was measured in the absence of external Ca (a, n = 10), at the peak of contraction in the absence (b, n = 4) and presence of calyculin A (c, n = 3). P_0-MLC_{20} denotes unphosphorylated MLC_{20}. P_1-MLC_{20} denotes monophosphorylated MLC_{20}. P_2-MLC_{20} denotes diphosphorylated MLC_{20}. n denotes the number of samples.

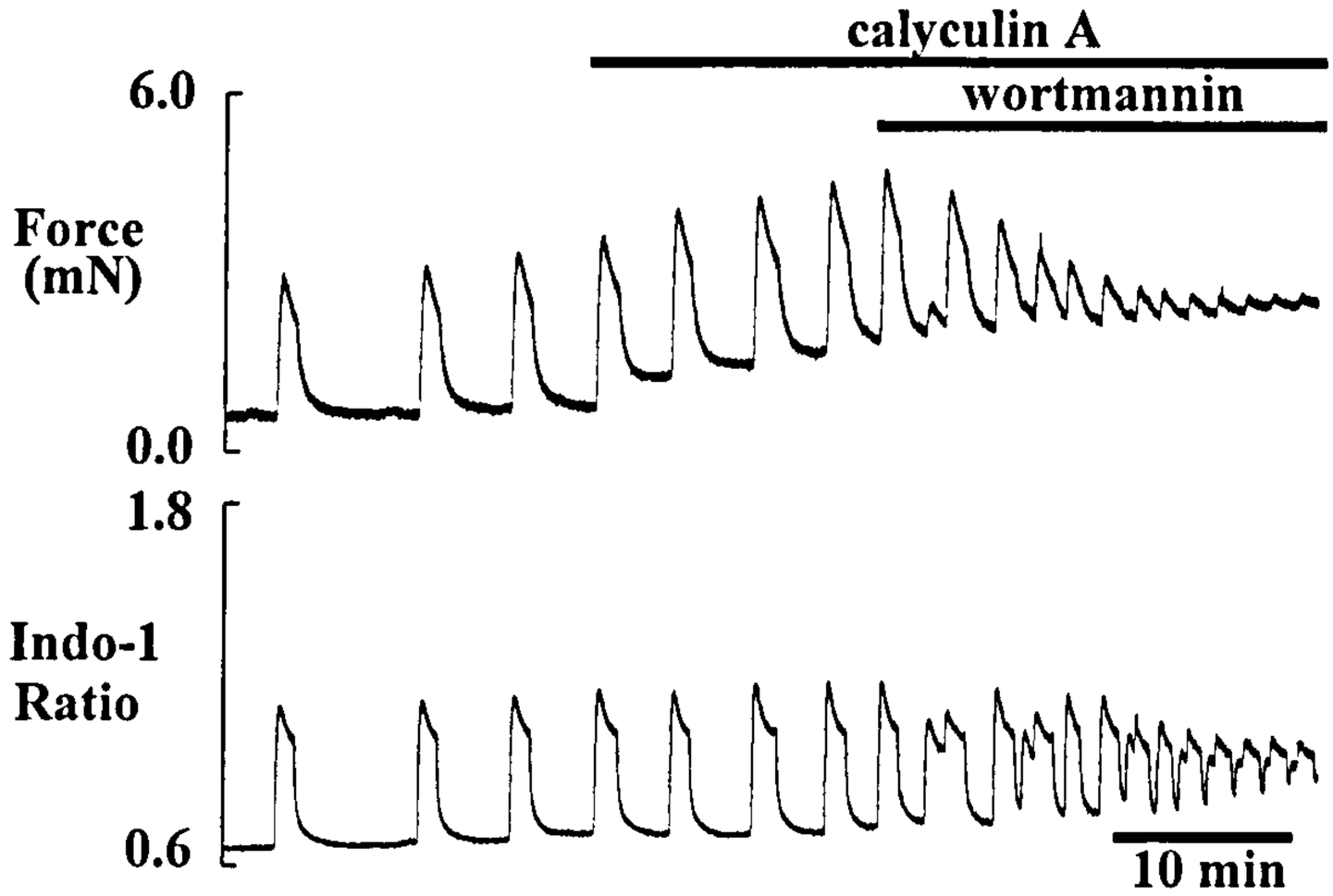


Figure 7.12 The effect of calyculin A in the absence and presence of wortmannin on spontaneous force (top panel) and calcium signal (bottom panel). After control recordings calyculin A (10^{-8} M) was added and then wortmannin (4 μM) added in the continued presence of calyculin A.

7.4.2.2 Oxytocin-induced contraction

The above data support a role for MLCP during spontaneous depolarisation, particularly PP1. To assess the influence of PP1 on agonist-induced force production, the effects of calyculin A on oxytocin-induced contraction were investigated. Application of oxytocin at 10^{-8} M produced an significant increase in force and Ca amplitude (n = 5). The frequency of contraction became more or less, but the duration of contraction and the basal force was significantly increased and maintained throughout the period of application (Fig. 7.13A, n = 5).

In 5 preparations, it can be seen that calyculin A (10^{-8} M) produced a further increase in the basal force but not Ca (For example, Fig. 7.13B, $P < 0.05$). In all preparation (Fig. 7.13B, n = 5) calyculin A added under oxytocin also had some significant inhibitory effects on Ca, the amplitude of the Ca transient was decreased to 80 ± 6% (100% is amplitude of initial peak response to oxytocin) by 15 minutes.

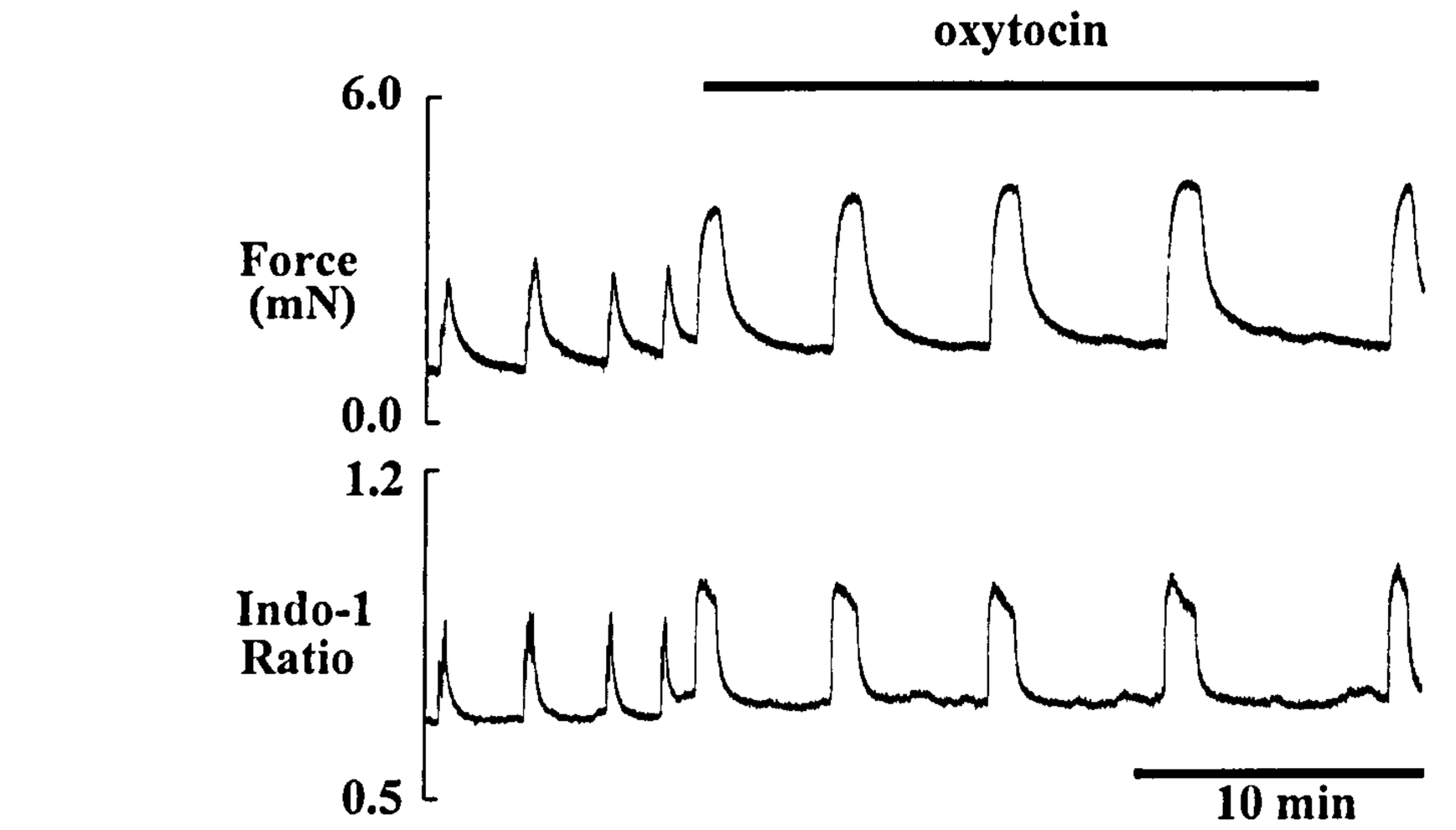


Figure 7.13 The effects of calyculin A on oxytocin-induced contraction. (A) Application of 10^{-8} M oxytocin on spontaneous force (top panel) and Ca transients (bottom panel) obtained from pregnant human myometrium. (B) Application of calyculin A (10^{-8} M) in the presence of oxytocin. The experiments were carried out in the same tissue.

7.4.2.3 KCl-induced contraction

As with okadaic acid and cantharidin, calyculin A (10^{-7} M) application slowly increased tension in all depolarised preparations to 115 ± 3% (n = 3, $P < 0.05$) of control values (Fig. 7.14). This was not accompanied by a rise in calcium ratio (Fig. 7.14). Removal of high-K and the inhibitor resulted in both force and Ca declining towards baseline levels. The effect of calyculin A (10^{-7} M) on depolarised preparations was also investigated, the muscle strips were frozen during KCl (40 mM) application with (n = 3) or without (n = 7) calyculin A (10^{-7} M). As can be seen in Fig. 7.15, calyculin significantly increased the level of MLC_{20} phosphorylation to 116 ± 3% (compared to 0% phosphorylation in the absence of Ca and 100% phosphorylation in the presence of KCl alone). As with spontaneous contraction, calyculin A added under depolarised preparations also produced diphosphorylation (Fig. 7.15, c).

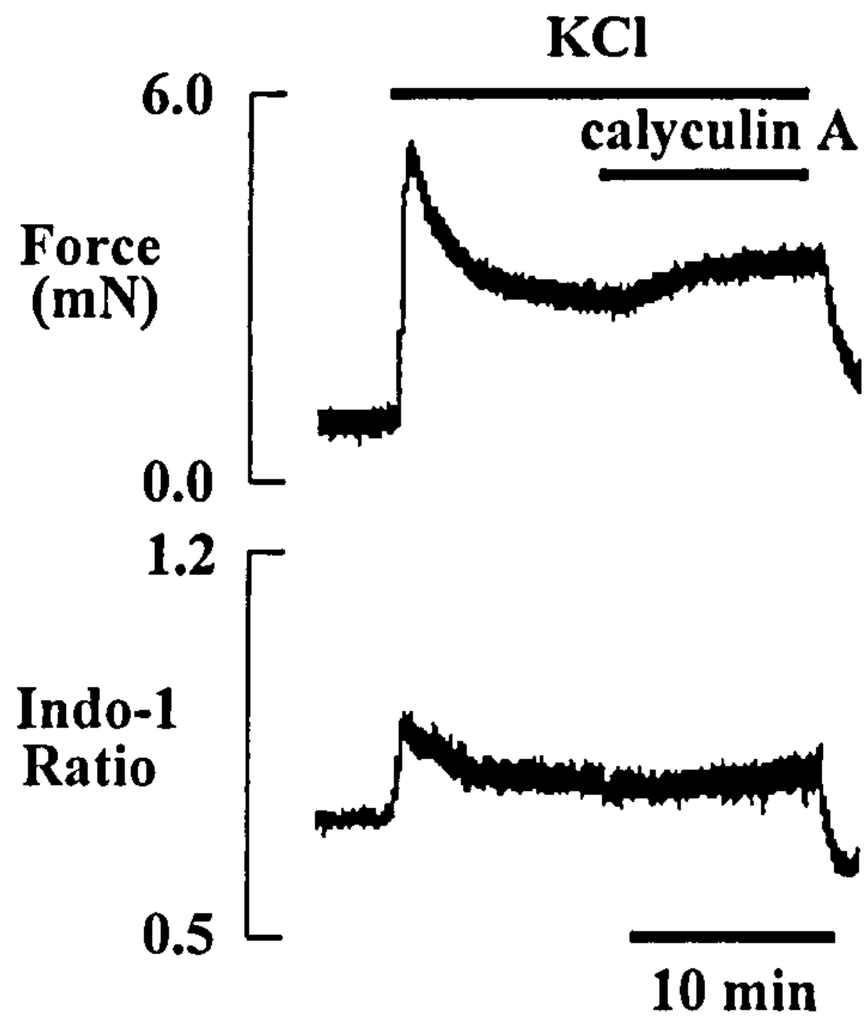


Figure 7.14 The effects of calyculin A on KCl-induced contraction. Application of KCl (40mM) in the absence, and the presence of calyculin A at 10^{-7} M.

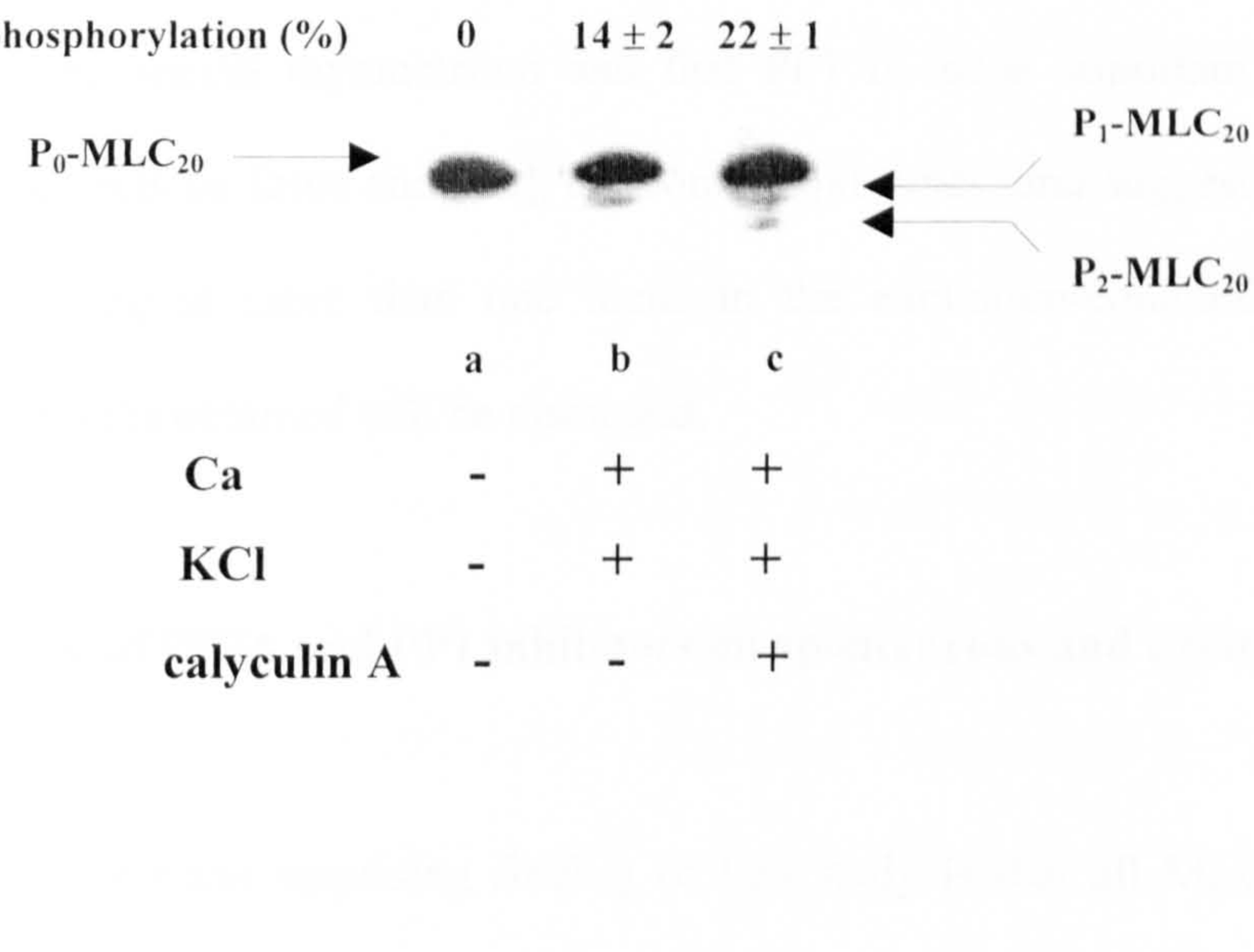


Figure 7.15 The effects of calyculin A on MLC_{20} phosphorylation in depolarisation preparations. Results shown are the mean ± s.e.m of the densitometric analysis (see the methods). The level of MLC_{20} phosphorylation was measured in the absence of external Ca (a, n = 10), after 10 min application of KCl (40 mM) without (b, n = 7) or with (c, n = 3) calyculin A. P_0-MLC_{20} denotes unphosphorylated MLC_{20}. P_1-MLC_{20} denotes monophosphorylated MLC_{20}. P_2-MLC_{20} denotes diphosphorylated MLC_{20}. n denotes the number of samples.

7.5 Discussion

The data presented in this chapter demonstrated the effects of inhibiting MLCP on contractility in the human myometrium, using inhibitors with specificity for PP2A or PP1. The effects on myosin phosphorylation clearly indicate that there is a modulation of MLCP in the human myometrium and that PP1 is more important than PP2A. However, the effects on force and $[Ca]_i$ are complex at times, and suggests that MLCP inhibition is acting at more than one locus in the excitation-contraction coupling pathway. The results obtained will be discussed.

7.5.1 The effects of PP2A and PP1 inhibitors on spontaneous and oxytocin-induced contraction

Perhaps the most surprising finding of this study is that all MLCP inhibitors affected Ca. Application of either okadaic acid, cantharidin or calyculin A to phasic activities arising either spontaneously or with oxytocin (calyculin A only) clearly have marked effects on calcium in human myometrium. The effect is unlikely to be due to some non-specific effects of the inhibitors, as this effect is not observed in tonic contraction i.e. KCl depolarisation. In some cases, falls in $[Ca]_i$ are accompanied by falls in tension (Fig. 7.3, 7.6). In others, however, there is a dissociation between Ca and force i.e. a fall in $[Ca]_i$, but an increase in tension (Fig. 7.2, 7.5, 7.9). To date, there appears to have little discussion about the inhibitory effect of those inhibitors on Ca, and the details of the mechanism are lacking, as their effects vary between species and tissues (Herzig & Neuman, 2000). By investigating force, calcium, and MLC_{20} phosphorylation it is, however, possible to draw conclusion about alterations of Ca during MLCP inhibition caused by those inhibitors.

A similar inhibitory effect of okadaic acid to that which I found, has been described in previous studies which did not measure $[Ca]_i$ in rat uterus (Candenas *et al.* 1992; Arteche *et al.* 1997a, 1997b) and in other smooth muscle tissues, i.e. bovine trachea (Tansey *et al.* 1990), and rabbit aorta (Karaki *et al.* 1989). This inhibitory effect of okadaic acid was observed on phasic activity arising either spontaneously or with agonists (Karaki *et al.* 1989; Tansey *et al.* 1990; Candenas *et al.* 1992; Arteche *et al.* 1997a, 1997b). The underlying mechanism is proposed to be attributed to an indirect activation of cyclic AMP- and/or cyclic GMP-dependent protein phosphorylation (Ashizawa *et al.* 1989; Karaki *et al.* 1989) and to a possible interaction with contractile elements (Ashizawa *et al.* 1989). Although the results shown in the present study are not sufficient to establish the mechanisms involved, simultaneous measurements of force and Ca along with calyculin A and cantharidin experiments revealed that the inhibitory effect of okadaic acid was due to inhibition of Ca influx. The proposed mechanisms will be discussed below.

As already discussed in chapter 3, membrane depolarisation and Ca entry are crucial in eliciting uterine phasic activities generated spontaneously or by agonists. These activities are abolished if external Ca is removed from the uterus. Similarly, although agonists can release Ca from the internal store, the majority of the Ca for contraction comes from the extracellular fluid. In turn voltage-gated, L-type, Ca channels require depolarisation to open and enable this Ca to enter. Thus it seems reasonable to suggest that the mechanisms governing depolarisation and L-type Ca channel activity are affected by MLCP inhibition. The same conclusion was reached in guinea-pig vas deference and human isolated bronchus where the $[Ca]_i$ measurements were performed and the inhibitory effects of okadaic acid observed (Shibata *et al.* 1991,

Naline *et al.* 1994). However, it is difficult to decide whether the effect on Ca influx is direct or indirect (see below).

As mentioned elsewhere, there are many different types of ion channels expressed on smooth muscle cells, and that alteration of these channels affects Ca entry. For example, stimulation of Ca-activated potassium (BK_{Ca}) channels leads to hyperpolarisation, decreasing entry of calcium through voltage-gated Ca channels, and hence relaxation. Inhibition of the channels has the opposite effect. In addition, it has been reported that inhibition of these channels can be modulated by dephosphorylation through PP2A but not PP1 in rat pulmonary artery (Archer *et al.* 1994) and neurohypophyseal (Sansom *et al.* 1997) cells. Thus, prevention of the phosphatase dephosphorylation, i.e. in the presence of MLCP inhibitors, would stimulate BK_{Ca} channel activity. Together, it remains to establish whether those mechanisms proposed (see above) are beyond the inhibition of MLCP in the human uterus.

So far the experiments show that in spontaneously contracting or oxytocin-induced pregnant human uterus, there is a modulation of MLCP. However, the effects of inhibiting MLCP are twofold: 1) to increase contraction due to inhibition of MLCP that dephosphorylates MLC_{20} and 2) to decrease $[Ca]_i$ due to inhibition of MLCP that dephosphorylate proteins (in addition to MLC_{20}) involved in Ca influx. As all MLCP inhibitors affected both PP2A and PP1 but in different degree of inhibition (Herzig & Neumann, 2000), it is difficult to claim which phosphatase (PP2A or PP1) it is that physiologically catalyses the dephosphorylation of MLC_{20} in the human myometrium or that dephosphorylate proteins involved in Ca influx. With okadaic acid and cantharidin experiments, all low concentrations used (0.01-1μM) are specific for PP2A inhibition. In addition, their effects on $[Ca]_i$ were large, and the effects on force were to decrease, not to increase. Thus, it seems reasonable to suggest that PP2A mostly act on

dephosphorylating proteins involved in Ca influx and that PP1 is the predominant MLCP that physiologically catalyses the dephosphorylation of MLC_{20} in the human myometrium. This is in agreement with previous suggestions that PP1 plays a major role in the human myometrium (Word *et al.* 1993) and that commonly catalyses the dephosphorylation of MLC_{20} in smooth muscles (Hartshorne *et al.* 1998).

7.5.2 The effects of PP2A and PP1 inhibitors on KCl-induced contraction

In contrast to the effect on spontaneous and oxytocin-induced contraction, both PP2A and PP1 inhibitors added under KCl depolarisation clearly increased force without interfering $[Ca]_i$. The increases were mirrored in the MLC_{20} phosphorylation, though a dual phosphorylation band was produced by calyculin A (discussed later). This indicates that depolarised contraction which $[Ca]_i$ is maintained inhibition of MCLP can be simultaneously activated. This again in agreement with the previous finding suggesting that MLCP modulates KCl-induced contraction (Trujillo *et al.* 2000; Mita *et al.* 2002; see also chapter 6). As the increased contraction was not only found in the presence of the PP1 inhibitor but also PP2A, suggesting that PP2A in maintained contraction is more sensitive to catalyse the dephosphorylation of MLC_{20} than that of in phasic contraction. Since okadaic acid, cantharidin and calyculin A are inhibitors of protein phosphatases (Herzig & Neumann, 2000), thus this increases in force were to inhibit MLCP and to an increase in the phosphorylation state of MLC_{20}.

The fact that MLCP inhibitor increased force without altering $[Ca]_i$ is also reported in vascular and intestinal smooth muscles (Shibata *et al.* 1982; Takai *et al.* 1987; Hirano *et al.* 1989). However, the results shown above contrast with those previously reported in some smooth muscles whereby okadaic acid inhibits contractions induced by KCl and other agonists such as noradrenaline (Ashzawa *et al.* 1989; Karaki

et al. 1989). Thus, taken together these data confirm that sensitivity to the inhibitors or perhaps MLCP modulation is related to the type of smooth muscle.

7.5.3 The effect of PP1 inhibitor on MLC_{20} phosphorylation

In spontaneously contracting uterus, it is clear that calyculin A increased basal force without altering basal Ca (Fig. 7.10). The increase was accompanied by an increased level of MLC_{20} (Fig. 7.11), suggesting that this was indeed due to PP1 inhibition. In some experiments, diphosphorylated MLC_{20} occurred. This could be due to MLC_{20} monophosphorylated at serine 19 being phosphorylated further by MLCK at threonine 18 to produce diphosphorylated MLC_{20}, as was demonstrated *in vitro* (Ikebe & Hartshorne, 1985; Ikebe *et al.* 1986), in intact (Colburn *et al.* 1988), and permeabilised smooth muscle (Haeberle *et al.* 1988). Diphosphorylated myosin exhibits a greater actin-activated MgATPase activity than myosin phosphorylated only at serine 19 (Ikebe & Hartshorne, 1985), but the velocity of movement of both forms of myosin in the *in vivo* motility assay is the same (Umemoto *et al.* 1989). It is interesting to note that, in the presence of agonists, diphosphorylated MLC_{20} can also be observed in intact arterial (Kitazawa *et al.* 2000) and human myometrial (see chapter 6) smooth muscle. In chicken gizzard smooth muscle, it has been shown that integrin-linked kinase (ILK) can also phosphorylate myosin light chain at serine 19 and threonine 18, raising the possibility that ILK may also play a role in regulation of Ca-independent contraction (Deng *et al.* 2001, 2002).

7.5.4 Modulation of MLCP and MLCK

Contraction and relaxation depend on the balance between MLCK and MLCP modulation (see 7.1). Of interest the present study was able to demonstrate the

modulation of both MLCK and MLCP at the same time. By doing so, it was possible to predict which enzyme is essential for normal uterine activity i.e. in the presence of extracellular Ca. The protocol was to apply calyculin A for ten minutes so that its effect could be clearly demonstrated and then, in its continued presence, to add wortmannin. Here wortmannin was used because it has been shown to be a highly specific inhibitor of the smooth muscle MLCK without altering Ca, by irreversibly binding at, or close to the ATP-binding site (Nakanishi *et al.* 1992; see also chapter 5). By blocking ATP binding, wortmannin prevents phosphorylation of MLC_{20}, binding of myosin to actin, and the subsequent regeneration of force. Interestingly, an inhibitory effect of wortmannin was found even when the human myometrium was under application of calyculin A (Fig. 7.12). The phasic contractions were abolished but not $[Ca]_i$, though a small decrement of the Ca transients was found presumably due the effect of calyculin A on PP2A (see section 7.6.1). However, wortmannin did not affect the basal force elevated by calyculin A. Thus, in investigating the important of those kinases to the uterine force production the data clearly show that modulation of MLCP is less importance compared with the modulation of MLCK. Rather the phosphatase acts to maintain the resting tone of spontaneous force under physiological condition i.e. in the presence of external Ca. This is in keeping in the previous results indicating that Ca-calmodulin (CaM)-MLCK pathway is essential for normal uterine activity, whereas non-Ca-CaM-MLCK pathway, possibly via MLCP inhibition, is secondary importance in comparison with the first pathway (see chapter 5, 6).

7.5.5 Conclusion

In conclusion, the data are the first to show inhibition of MLCP during Ca-dependent contraction in the human myometrium using PP2A and PP1 inhibitors. There

is a modulation of MLCP (both PP2A and PP1A) in generating force production irrespective of the mechanism used to generate that force. However PP1 is the most likely associate. Different mechanisms of contraction do not show the same sensitivity to MLCP inhibitors and may suggest the existence of differences between the mechanisms through which the inhibitors or protein phosphatases modulate the process of contraction and relaxation. The findings also indicate that MLC_{20} phosphorylation is not the only mechanism involved in the regulation of tension maintenance in the human myometrium.

Chapter 8

Final Discussion

Chapter 8

Final Discussion

The aim of the work described in this thesis was to gain a better understanding of how the control and modulation of uterine contraction is achieved. The role of calcium and signalling pathways was studied in various experimental conditions. The major findings can be summarised as follows:

8.1 The Contribution of the Sarcoplasmic Reticulum to Uterine Contraction

Ca-induced Ca release

Following excitation and the opening of voltage-gated Ca channels, $[Ca]_i$ rises. Thus this Ca could be the trigger for subsequent SR Ca release via Ca-induced Ca release (CICR), and stimulation contraction. By studying the role of CICR the conclusion is that CICR does not appear to play a role in intact human myometrium, despite the presence of ryanodine receptor. The experimental basis for this can be summarised as follows:

1. ryanodine application does not inhibit spontaneous contraction as was found in rat (Taggart & Wray, 1998) and mouse (Matthew & Wray, unpublished observations). There is no decrement in either force or Ca, nor was the time course of the force or Ca transients greatly affected. This was the case at all concentrations of ryanodine from 10-50 μM and even after 20 minutes of application.
2. caffeine (10 mM), an agonist for CICR, is unable to produce any rise of Ca or force.

IP_3-induced Ca release

A major component of agonist stimulation of smooth muscle involves their activation of phospholipase C (PLC) subsequent to them binding to their G-protein coupled receptors. PLC in turn leads to the production of IP_3 and diacylglycerol. Agonist stimulation can also lead to the opening of membrane ion channels and alteration of the cell membrane potential. In addition, agonists can initiate second messenger pathways, leading to alteration of the sensitivity of the force producing pathways in smooth muscle. However, it is their affects on the SR, which was the focus of my study. Unlike CICR, IP_3-induced Ca release (IICR) is clearly present in the uterus. If agonists are added in a solution containing zero external Ca, and thus preventing L-type Ca entry and any other Ca permeant pathway, a small release of $[Ca]_i$ and accompanying contraction can be seen. This released Ca is however only small compared to that occurring when Ca entry is taking place, which is the usual physiological condition.

The SR and uterine excitability

In the uterus, as in other smooth muscles, the SERCA pump can be inhibited by cyclopiazonic acid (CPA). This leads to a disruption of the SR Ca store and provides a mechanism for exploring its contribution to the contractile process. In the uterus when the SR is emptied by CPA, force and Ca transients are not inhibited. Furthermore it can be seen that both are slightly but significantly increased in amplitude. In addition the transients are prolonged. Thus in the uterus inhibition of the SR potentiates force and Ca increases, suggesting that in uterine smooth muscle the SR Ca store acts to feedback and limit contractions. Furthermore, I have found no evidence during spontaneous contractions for the SR contributing Ca to agonist contractions; as described above,

there is no inhibitory effect of ryanodine. This role of the SR to limit contraction rather than augment it, is similar in many ways to the feedback process occurring in vascular smooth muscle (Jaggar *et al.* 2000). Briefly Ca release from SR ryanodine receptor (RyR) produces Ca sparks, which activate K channels in the surface membrane, producing spontaneous transient outward currents (STOCs), and cell hyperpolarisation. Thus CICR and the SR act to maintain relaxation in vascular tissues, and inhibiting the SR leads to depolarisation and contraction, as I have shown in the uterus. The uterus possesses Ca-activated K (BK_{Ca}) channels, and their modulation and expression may alter with pregnancy and labour (Khan *et al.* 2001). Thus it is tempting to suggest a similar mechanism exists in the uterus and that it may play a role in governing contractility changes during labour. However, so far no reports of sparks in the myometrium have appeared. In addition CPA was still able to augment Ca and contraction when BK_{Ca} had been inhibited with tetraethylamonium (TEA, 5 mM). Thus SR activation of BK_{Ca} may be only a part of its inhibitory influence on uterine Ca and force. Additionally there may be a role for small conductance K_{Ca} channels or a role for capacitative Ca entry which remains to be established.

Another means whereby the SR may modulate excitability in the uterus could be via Ca-activated Cl (Cl_{Ca}) channels. Activation of Cl_{Ca} will produce spontaneous transient inward currents (STICs) and depolarisation. The evidence for Cl_{Ca} channels in the uterus is limited but Arnaudeau *et al* (1994) showed that oxytocin in rat cells appeared able to stimulate these channels. Recently, Jones *et al.* (2002) have also shown their activation via voltage-gated Ca entry. It remains a conundrum how the SR released Ca can be preferentially directed at K and Cl channels, given that to simultaneously activate both would produce opposing effects of STOCs and STICs.

In summary, The role of the SR in the uterus can be seen to incorporate excitability via Ca-activated channels, as well as releasing some Ca to support agonist-induced contraction.

8.2 The Importance of Both the Ca-Calmodulin-Myosin Light Chain Kinase Pathway and Non-Ca-Calmodulin-Myosin Light Chain Kinase Pathways

Pathways leading to contraction

Following excitation either arising from an action potential or due to agonist binding to a receptor, force rises within the myometrium. There is much debate about the Ca-dependent and -independent force producing pathways in smooth muscle. What is clear for the myometrium is that a rise of Ca and then stimulation of myosin light chain kinase (MLCK) via Ca-calmodulin (CaM) is essential for normal cyclic or phasic uterine activity. The evidence for this is that:

1. removal of external Ca prevents the rise of Ca needed for spontaneous contraction. Thus without a rise in $[Ca]_i$ no Ca-independent force producing pathway can elicit spontaneous contractions.
2. this pathway is also dependent upon MLCK as little or no force is present in the uterus when MLCK is inhibited by wortmannin or ML-9.

Thus under physiological conditions the Ca-CaM-MLCK pathway is predominant in producing uterine force.

Ca sensitisation

In other smooth muscles, particularly tonically active ones such as blood levels, Ca sensitisation has been described. This is when force can be produced without a

change in intracellular Ca. Many agonists are thought to be able to modify the reactions involved in the production of force. The two key targets of these mechanisms appear to be MLCK and MLCP. From the above it can be easily imagined that if the activity of MLCK is modified, then at a steady level of Ca, and CaM, force could be affected. The phosphorylation of MLCK by several kinases has been reported (Horowitz *et al.* 1996b). Such phosphorylation reduces its activity and thus desensitises the contractile machinery. It is now appreciated that the activity of MLCP can be modified (Somlyo & Somlyo, 1998) as this enzyme dephosphorylates myosin, it is usually associated with a decrease in force and relaxation. It can be phosphorylated, particularly by Rho-associated kinase (ROK), resulting in a decrease in its activity. This in turn would lead to an increase of force at constant $[Ca]_i$, i.e. an increase in Ca sensitivity. In considering the importance of such mechanisms to the uterus I have found that they are of secondary importance compared with the normal Ca-CaM-MLCK pathway under physiological conditions in intact preparations. Their importance may be increased during long lasting labour contractions, as was demonstrated under depolarisation or agonist-added under depolarisation. Such conclusion comes from the use of the inhibitor of both ROK and MLCP.

Further Work

So far I have elucidated some of the mechanisms underlying uterine contraction. However, my findings have questioned the role of other mechanisms, which requires definitive answers. To answer these questions, some possible future experiments are given below.

1. What is the contribution of capacitative Ca entry to uterine contraction under physiological condition?

Capacitative Ca entry (CCE) is a Ca entry pathway that is activated on depletion of intracellular Ca stores, providing an avenue for store refilling. The pathway is functionally related to the transient receptor potential (Trp) proteins that have been hypothesised to be structural components of Ca entry channels and to be activated by IP_3R in response to IP_3 or store depletion (Birnbaumer *et al* .1996). In term pregnant human myometrium, the presence of Trp in Ca entry channels has been proven (Dalrymple *et al.* 2002), however, the mechanism(s) by which the channels are activated has not yet been elucidated. Thus, it is tempting to investigate whether there is a physiological role for CCE in participating Ca for contraction. To do so, the SR will be depleted under physiological condition and the effects of a Ca entry channel inhibitor (e.g. SKF96365) on $[Ca]_i$ and contraction assessed. This will be done initially in intact preparations and then in single cells using patch-clamp techniques. In this way, with the membrane current information available, the question raised above can be addressed.

2. Is the role of the SR to help control excitability at the surface membrane?

I have shown that the SR Ca release, whether release spontaneously or following stimulation, may activate K and Cl channel. By hyperpolarising or depolarising the membrane (respectively) these channels will influence voltage-gated Ca entry, which is of major importance to the rise of $[Ca]_i$, and hence contraction. To investigate such a role the SR will be modified, and the ionic currents (both STOCs and STICs) will be identified and characterised in isolated smooth muscle cells of the human uterus. By obtaining data on the role of the SR in modulating excitability, along with the

information of release mechanisms and their contribution presented in this thesis a fuller picture of the function of the SR will be acquired.

3. Are calcium sparks present in human myometrium?

As described above, Ca sparks are considered to be due to spontaneous release of Ca from RyR. There have, however, been no reports or descriptions of sparks occurring in uterine cells. To investigate whether Ca sparks are present in human myometrium, the experiments will be studied using confocal microscopy and fluorescent Ca-binding indicators. This study, together with the studies outlined above will increase our understanding of how contractions are produced and can be controlled by the SR.

Using confocal microscopy and fluorescent Ca-binding indicators Burdyga & Wray (unpublished data) have recorded Ca sparks of the ureter *in situ*. I have recently started examining Ca sparks in the uterus using the same technique. Although a proper study has not been undertaken yet, my preliminary data showed that it is possible to record Ca sparks of the uterus (if present) *in situ*. As shown in Fig.8.1, some certain regions fluorescing more brightly; perhaps indicating Ca hot spots can be observed in an intact human myometrial strip loaded with Fluo-4 under confocal microscopy.

4. Relevance to human pregnancy and labour

The overall aim of my thesis was to increase our knowledge of the myometrium. How relevant however is the *in vitro* data to *in vivo* condition? To answer this will require knowing more about activity in labouring myometrium.

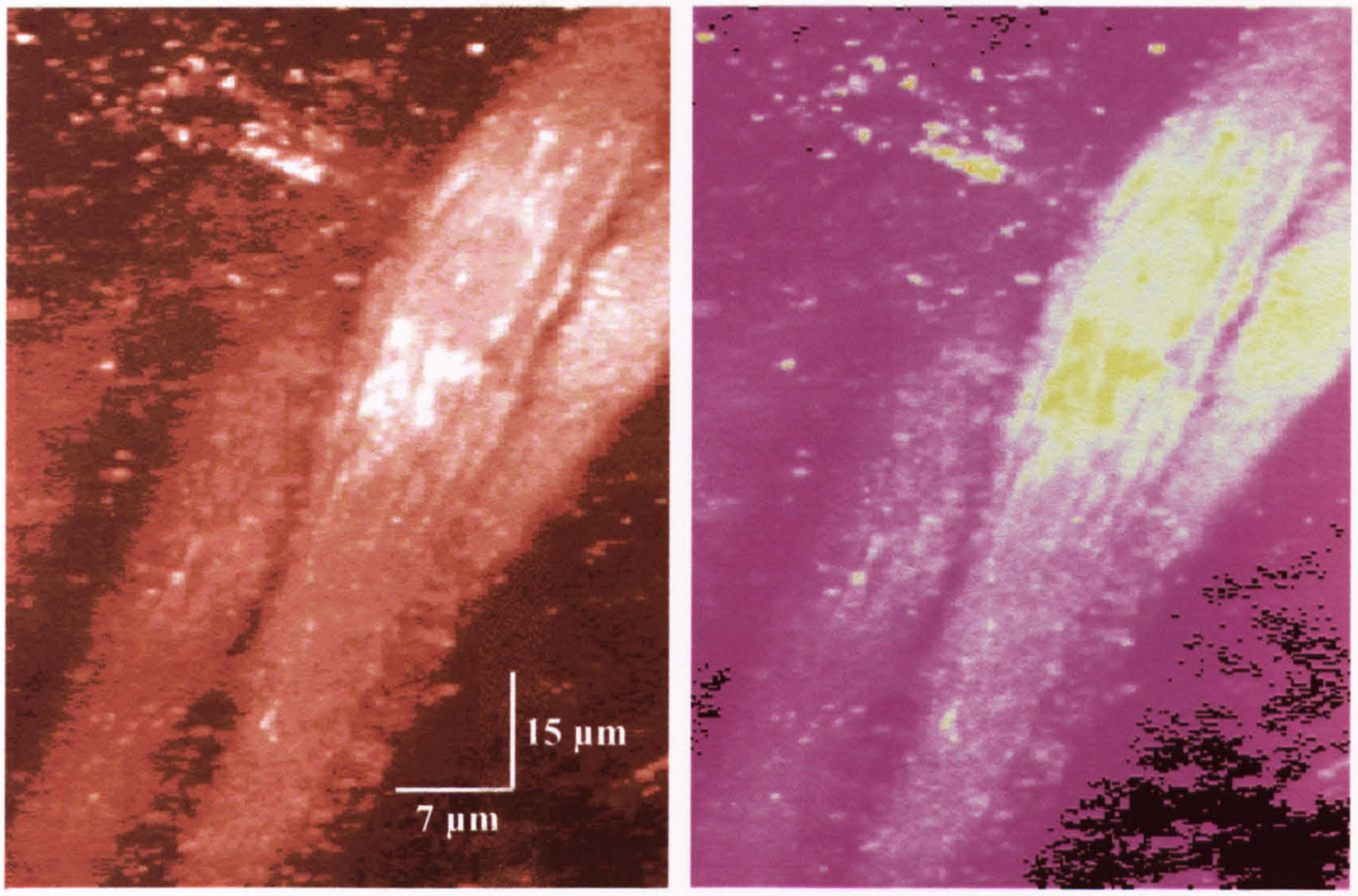


Figure 8.1 Confocal image of smooth muscle cells taken from an intact strip isolated from human myometrium loaded with Fluo-4. λ_{ex} = 488 nm, λ_{em} = 510 nm. (**A**) Grey scale. (**B**) Pseudo-colour.

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Appendix

Appendix

Lists of Publications

- LONGBOTTOM, E.R., LUKAS, M.J.M., **KUPITTAYANANT, S.**, BADRICK, E., SHMYGOL, A. & WRAY, S. (2000). The effect of wortmannin, an inhibitor of myosin light chain kinase (MLCK) on calcium and contraction in isolated human and rat myometrium. *European Journal of Physiology* **440**, 315-321.

- WRAY, S., **KUPITTAYANANT, S.**, SHMYGOL, A., SMITH, R.D. & BURDYGA, T. (2001). The physiological basis of uterine contractility: a short review. *Experimental Physiology* **86.2**, 239-246.

- **KUPITTAYANANT, S.**, BURDYGA, T. & WRAY, S. (2001). The effects of inhibiting Rho-associated kinase with Y-27632, on force and intracellular calcium in human myometrium. *European Journal of Physiology* **443**, 112-114.

- **KUPITTAYANANT, S.**, LUKAS, M.J.M. & WRAY, S. (2002). Effect of inhibiting the sarcoplasmic reticulum on spontaneous and oxytocin-induced contractions of human myometrium. *British Journal of Obstetrics and Gynaecology* **109**, 289-269.

Pflügers Arch – Eur J Physiol (2000) 440:315–321
Digital Object Identifier (DOI) 10.1007/s004240000305

ORIGINAL ARTICLE

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The effects of inhibiting myosin light chain kinase on contraction and calcium signalling in human and rat myometrium

Received: 26 October 1999 / Received after revision: 31 January 2000Accepted: 4 March 2000 / Published online: 14 April 2000

Abstract The effect of inhibiting myosin light chain kinase on contractions of human and rat myometrium has been investigated, to determine whether force can be produced independently of myosin phosphorylation. Two inhibitors were used, wortmannin and ML-9, and their effects on spontaneous, high-K-depolarization-induced and oxytocin-induced force studied. Both inhibitors reduced and then abolished uterine force, irrespective of how it was produced; this was the case for both human and rat myometrium, and pregnant and non-pregnant tissue. The effects of wortmannin on intracellular [Ca^{2+}] and inward Ca^{2+} current were examined. The data showed that the reduction in force produced by wortmannin occurs without a reduction of either the Ca^{2+} current or [Ca^{2+}]. It is concluded that, under normal physiological conditions, myosin light chain kinase phosphorylation of myosin is essential for uterine force production and that there is little or no role for alternative force-producing pathways.

Key words Ca^{2+}-calmodulin-MLCK pathway · ML-9 · Myosin light chain kinase · Uterine contraction · Wortmannin

Introduction

Control of uterine smooth muscle is clearly important for preventing pre-term labour and ensuring strong and coordinated contractions during labour. As with other smooth muscles, changes in intracellular [Ca^{2+}] ($[Ca^{2+}]_i$) underlie uterine contractions but the relationship between $[Ca^{2+}]_i$ and force may not be simple [23]. Agonists in particular may act to alter the events subsequent to $[Ca^{2+}]_i$ rising in the uterine cell. The main force-activating pathway is taken to be Ca^{2+}-calmodulin activation of myosin light chain kinase (MLCK) and phosphorylation of myosin light chains [28]. An increase in phosphorylated myosin with contraction has been shown to occur in the human myometrium [12]. Recently, however, it has been become clear that agonists can modify this process [8]. For example, MLCK and the phosphatase that dephosphorylates myosin can be phosphorylated and hence their activity decreased. In this way the relationship between force and Ca^{2+} may be changed [5]. In addition alternative pathways have also been proposed [21] that depend upon kinases, such as rho-A, and initiate a cascade of events which may not require an elevation of $[Ca^{2+}]_i$, culminating in force production. For example, MAP kinase phosphorylation of thin-filament-associated proteins (calponin and caldesmon) leads to the removal of their inhibitory influence of actin-activated myosin ATPase activity [5, 17, 27]. Recently oestrogen and progesterone have been shown to modulate the levels of Rnd-1, a kinase that interacts with the Rho 1A pathway [10]. Thus it is tempting to speculate that such pathways may contribute to force production during labour.

Several authors have shown that oxytocin can elicit a contraction in 0-Ca solution [13, 19] and it has been suggested that this arises from its stimulation of protein kinase C (PKC) [4, 9, 19]. PKC in turn may activate MAP kinase [5]. It has been reported that this contraction occurs without myosin phosphorylation [19].

Thus the question arises, "what is the relative importance in uterine contractions of the Ca^{2+}-calmodulin-MLCK pathway compared to the alternative or modulatory pathway?" In order to investigate this, we have used two chemically unrelated and selective inhibitors of MLCK, wortmannin [16, 24] and ML-9 [6, 15, 16, 24]. The aim of this work was threefold. First, we wanted to determine the effects of inhibiting MLCK on uterine force produced spontaneously, by depolarization with high-K^+ solution and by the agonist oxytocin, in the presence and absence of Ca^{2+}. Second, we wanted to investigate the effect of inhibiting MLCK on $[Ca^{2+}]_i$ and the Ca^{2+} current, and third to compare the effects on uteri

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from women and rats, and pregnant and non-pregnant animals.

A preliminary account of this work has been published [11].

Materials and methods

Tissue

Human myometrial tissue was obtained from women aged 17–38 years (mean 27 years) undergoing (elective) Caesarian sections (37–41 weeks, mean 38.5 weeks) from the lower segment. Ethics committee approval was given and written informed consent was obtained. Tissue was placed in Krebs solution and strips dissected along parallel muscle fibres, to give strips with dimensions of approximately 2–3 mm×1 mm×10 mm (width×thickness× length). Late-pregnant (19–21 days gestation, term day 22) or non-pregnant (200 g) rats were killed by cervical dislocation under CO_2 anaesthesia. The uterus was removed and small strips dissected, 1–2 mm×0.5 mm×10 mm. For both species, tension measurements were made from tissue with one end fixed and the other attached to an isometric force transducer (Grass FTO3) using silk threads. These measurements were made whilst the tissue was continually perfused with Krebs or "Krebs" containing high-K^+, wortmannin (0.5–4 μM), ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine 10–100 μM] or oxytocin (10 nM). Krebs solution contained (mM): NaCl, 154; KCl, 5.6; $MgSO_4$, 0.12; glucose, 11.7; HEPES, 10.9; $CaCl_2$, 2; pH 7.4 adjusted with NaOH at 37°C. K^+ was elevated and Na^+ reduced to depolarize the membrane in some experiments. In 0-Ca experiments CaCl was omitted from the solution and 1 mM EGTA added.

Simultaneous measurements of calcium and tension

Strips were loaded at room temperature for 3 h with the Ca^{2+}-sensitive fluorophore Indo-1 (7 μM Molecular Probes) containing 0.02% pluronic acid. Tissue was then placed on the stage of an inverted microscope and viewed with a 10× fluor objective. The tissue was excited at 340 nm (using a 75 W xenon arc lamp) and emitted light at 400 and 500 nm was detected (using photomultipliers Thorn EMI) and digitally recorded. After subtracting the background signal, obtained by adding Mn^{2+} at the end of experiment, the ratio of 400:500 nm emissions was used as an indicator of $[Ca^{2+}]_i$. The tissue was constantly perfused with oxygenated Krebs and the bath could be exchanged completely within 3 s.

Enzymatic isolation of human myometrial cells

Thin strips of tissue were dissected and then incubated in Hanks' solution (mM): NaCl, 137; KCl, 5; KH_2PO_4, 0.44; Na_2HPO_4, 0.26; glucose, 5.5; HEPES, 10 at pH 7.4 (NaOH) at 37°C for 30 min. This solution was then replaced with Hanks' containing 1 mg/ml collagenase 1A; 4 mg/ml BSA, fraction V; and 1 mg/ml trypsin inhibitor, type 1S and 50 μM $CaCl_2$ and incubated for ≅2 h at 37°C. The tissue was then triturated and the supernatant strained through a 80 nm nylon mesh and cells purified by centrifugation, and three washes in KB medium. KB medium composition was (mM): KCl, 40; K_2HPO_4, 10; taurine, 10; HEPES, 5; glucose, 11; pyruvate, 5; creatine, 5; succinate, 5; EGTA, 0.04; and K-glutamate, 100.

Patch-clamping studies

Patch-clamp studies were performed on human myometrial cells that were continuously perfused with Krebs solution at 37°C. The pipette solution contained (mM): CsCl, 130; NaCl, 5.0; $MgCl_2$, 2.0; NaH_2PO_4, 1.0; HEPES, 10.0; glucose, 5.0; oxalacetic acid, 2.5; pyruvic acid, 2.5; succinic acid, 2.5; Na_2ATP, 2.0; pH adjusted to 7.2. For whole-cell current experiments the membrane potential was stepped from –60 mV to +40 mV in 10-mV steps, from a holding potential of –80 mV. Wortmannin was applied by perfusion at a concentration of 4 μM in Krebs. The tip potential of the patch pipette was compensated before the gigaohm seal was obtained, and the pipette capacitance was compensated after a tight seal was established. pClamp software (Axon Instruments) was used for data acquisition and voltage-clamp protocol generation.

All chemicals were obtained from Sigma unless stated otherwise.

Statistics

Significant differences were tested using ANOVA and then Dunn's test.

Results

Human myometrium

Spontaneous activity

Under control conditions spontaneous contractions of consistent amplitude and frequency could be recorded for several hours. Wortmannin (4 μM) reduced force in all preparations studied. Its effect was gradual, as can be seen in Fig. 1A; a significant reduction occurred after 10 min (mean amplitude of contractions 37±8%, human compared to 100% control) and by 20 min contractions were essentially abolished (2±2%). After this exposure to wortmannin, neither high-K^+ nor oxytocin was able to produce any force in the myometrium (not shown). Five preparations of human myometrium were left in control solution for 1 h following wortmannin application, to

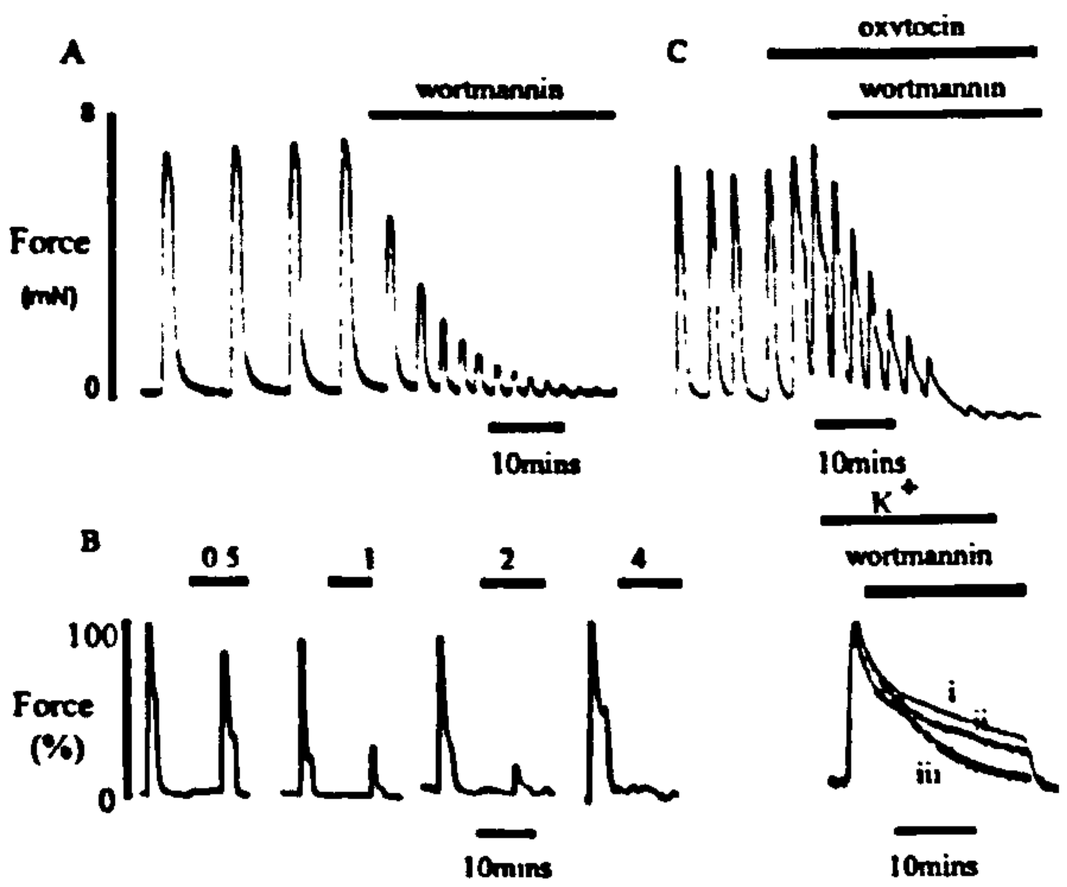

Fig. 1A–C The effect of wortmannin on human myometrial contractions. **A** The effect of 4 μM wortmannin on spontaneous contractions. **B** The effect of different doses of wortmannin on high-K^+-stimulated contractions, after a 10-min incubation with wortmannin (*left*) or after 15 min in the continued presence of high K^+ (*right*); *i*, 0.5; *ii*, 1; *iii*, 4 μM. **C** The effect on oxytocin-induced (10 nM) contractions. The *bars* in this and subsequent figures show the periods when drugs were applied

monitor resumption of contractile activity. Compared to control activity (100%), force amplitude recovered to 20% within this period.

High-K^+ depolarization

By elevating K^+ and depolarizing the surface membrane, the effects of wortmannin on force production, independent of pacemaker activity, could be examined. In addition the uniform response of tissues to high-K^+ depolarization allowed the effects of different doses of wortmannin to be examined. Figure 1B shows the effect of four different doses of wortmannin, applied for 10 min to the high-K^+-induced contractions of human myometrium, and the effect of three doses of wortmannin in the continued presence of high-K^+, after a control period of 2 min. It can be seen that at each concentration (0.5–4 μM) wortmannin reduced force, and that the effect was dose dependent.

Oxytocin-induced contractions

As expected, under control conditions, oxytocin increased contractile activity compared to spontaneous activity (Fig. 1C, n=4). As shown in Fig. 1C, wortmannin abolished oxytocin-induced force.

The effect of wortmannin on intracellular Ca^{2+} and Ca^{2+} current

As the above data clearly show that inhibiting MLCK can abolish uterine contractions in human myometrium, the next experiments were performed with wortmannin to determine its effect on Ca^{2+} signalling.

Figure 2A shows a simultaneous recording of $[Ca^{2+}]_i$ and spontaneous contraction in human myometrium (typical of four). It can be seen that under control conditions Ca^{2+} transients underlie the phasic contractions. Wortmannin gradually inhibited these contractions but had little or no effect on the Ca^{2+} transient (Fig. 2A). Figure 2B shows a similar effect with oxytocin-induced force (n=6), i.e. force falls but $[Ca^{2+}]_i$ does not, in the presence of wortmannin. Similar data were also obtained with high-K^+-induced contraction (n=4, not shown).

In single myometrial cells from human myometrium, inward Ca^{2+} current was recorded under voltage-clamp conditions and with Cs^+ replacing K^+ in the pipette solution (to remove outward currents). Upon stepping from a holding potential of –80 to –50 mV, inward current was activated (Fig. 2C). Maximal Ca current was observed at 0 mV. Removal of external Ca^{2+} resulted in the abolition of this inward current, and increasing Ca^{2+} potentiated the current (n=4, not shown). Having thus established that inward Ca^{2+} current was being recorded, the effect of wortmannin was then investigated. As can be seen in Fig. 2C wortmannin had no effect on the characteristics of the inward Ca^{2+} current in these cells.

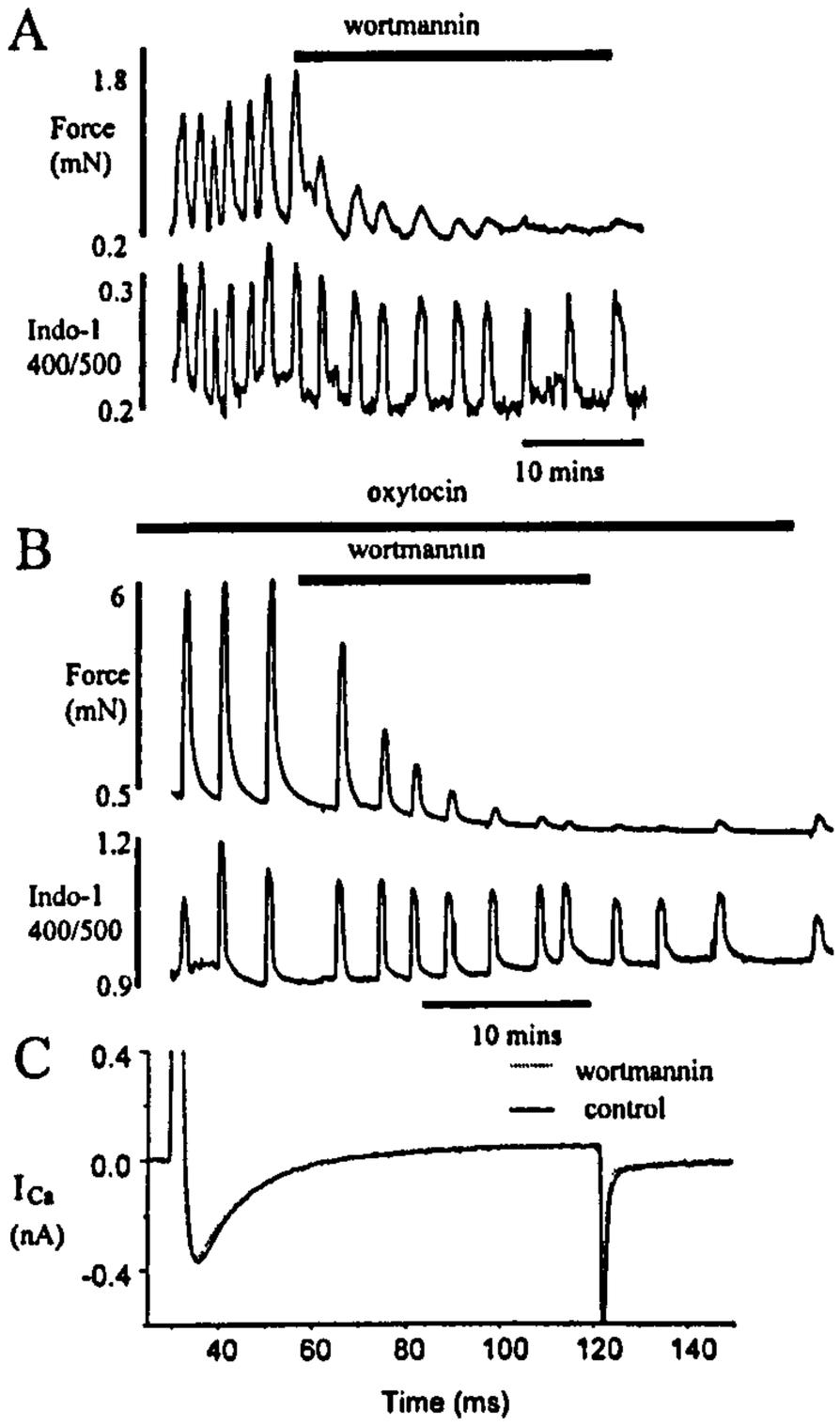

Fig. 2A–C The effects of wortmannin on $[Ca^{2+}]$. **A, B** Simultaneous recordings of force (*top*) and Ca^{2+} (Indo-1 ratio, *bottom*) in human myometrium contractility. Spontaneous contractions (**A**) and those in response to oxytocin application (**B**) are shown as well as the effect of wortmannin. **C** The Ca^{2+} current recorded upon stepping from –80 to 0 mV in voltage-clamped human uterine myocytes, under control conditions (*solid line*), and in the presence of 4 μM wortmannin (*dotted line*)

Effects of wortmannin on rat myometrium

In order to determine whether the above data are specific to human myometrium, wortmannin was also applied to pregnant and non-pregnant rat myometrium. No significant differences were found between data from pregnant and non-pregnant animals and so the data are combined in the following account.

As with human myometrium, spontaneous contractions could be recorded for many hours from the rat; their amplitude was comparable for strips of similar thickness (1–10 mN) but their frequency was higher (approximately one every 1–2 min compared to one every 3–5 min in human myometrium). Force per cross-sectional area was higher in human than rat myometrium (approximately 40 mN/mm^2, compared to 10 mN/mm^2). Wortmannin also abolished these spontaneous contractions (Fig. 3A, n=6). Its effect was significantly faster than in the human myometrium; thus force was reduced to 12±1% after 10 min (compared to 37±8% in human), which may be related to the higher frequency of contrac-

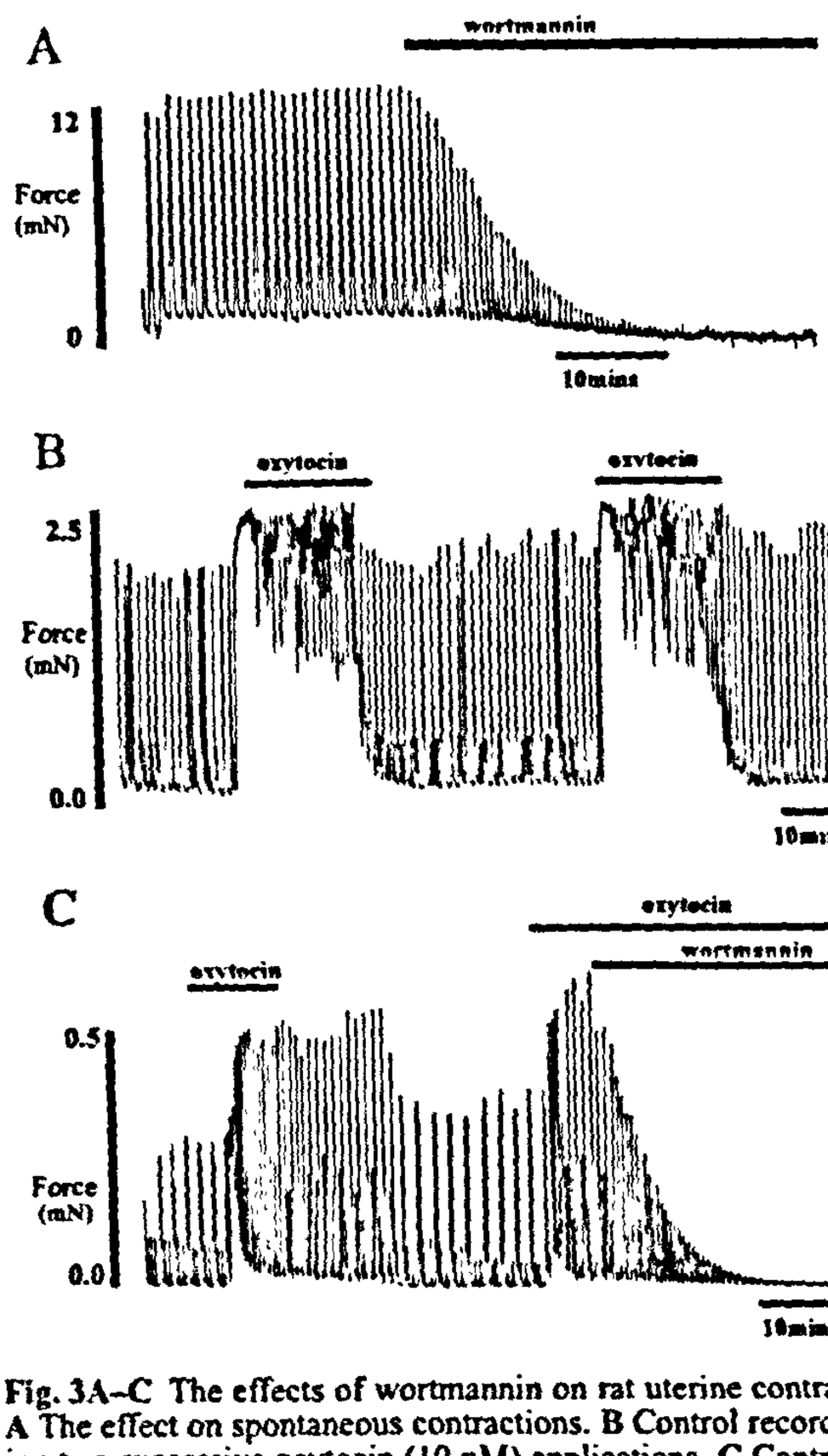

Fig. 3A–C The effects of wortmannin on rat uterine contractions. A The effect on spontaneous contractions. B Control record showing two successive ocytocin (10 nM) applications. C Control oxytocin application followed by oxytocin and wortmannin

tions (see Discussion). As with human myometrium, neither high-K^+ or oxytocin was able to restore force once wortmannin had abolished it (not shown). Wortmannin (4 μM 20 min) completely abolished high-K^+-elicited force in the rat myometrium (n=3, data not shown). Oxytocin produced similar effects on the rat as on human myometrium. Thus, as shown in Fig. 3B, the frequency and amplitude of contractions were significantly increased compared to control. Two successive applications of oxytocin produced very similar effects on uterine force (Fig. 3A). In the presence of wortmannin (4 μM, Fig. 3C) the oxytocin-induced force was reduced and eventually abolished in all preparations (n=10).

Measurement of $[Ca^{2+}]_i$ in rat myometrium supported the data relating to human myometrium. Thus, wortmannin had little or no effect on the Ca^{2+} transient whether produced spontaneously (n=5), by oxytocin (n=3) or high-K^+ (n=2), data not shown.

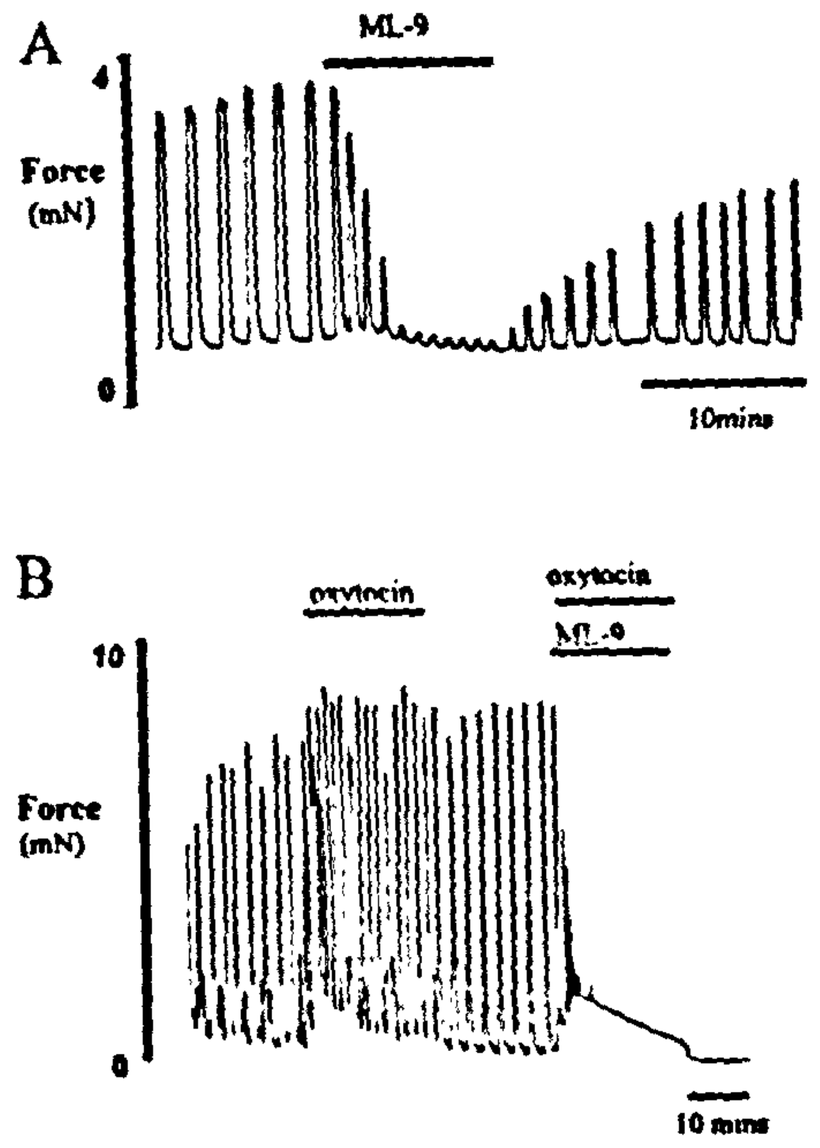

Fig. 4A, B The effect of ML-9 on rat myometrial contractions. A The effect on spontaneous contractions. B The effect on oxytocin-evoked contractions

The effects of ML-9

As there were no substantial differences between the effects of wortmannin on rat and human myometrium, the effects of ML-9, another inhibitor of MLCK, were investigated only in the rat.

ML-9 produced similar effects to wortmannin, i.e. spontaneous force was reduced in all preparations (n=4, Fig. 4A). The effects of ML-9 (50 μM) were significantly faster than those of wortmannin (force maximally reduced after 5 min), and full recovery occurred upon washout within 10 min. ML-9 also completely abolished high-K^+-induced force (n=3) and oxytocin-induced force (Fig. 4B).

The effect of wortmannin on intracellular Ca^{2+} and force in the absence of external Ca^{2+}

In rat (n=5) or human (n=5) myometrium, oxytocin was applied in the absence of external Ca^{2+}. A small (≅5% of that found in the presence of Ca^{2+}) tonic contraction gradually developed and was maintained as long as oxytocin was present (up to 30 min), as shown in Fig. 5A, which also shows a control oxytocin response. Application of wortmannin (n=4) or ML-9 (n=2, rat tissue only) did not affect this contraction (Fig. 5B), irrespective of whether it was added before or during the oxytocin application. In three experiments $[Ca^{2+}]_i$ was also measured. Removing external Ca^{2+} clearly reduced $[Ca^{2+}]_i$ (Fig. 5B). Compared to control conditions (i.e. external

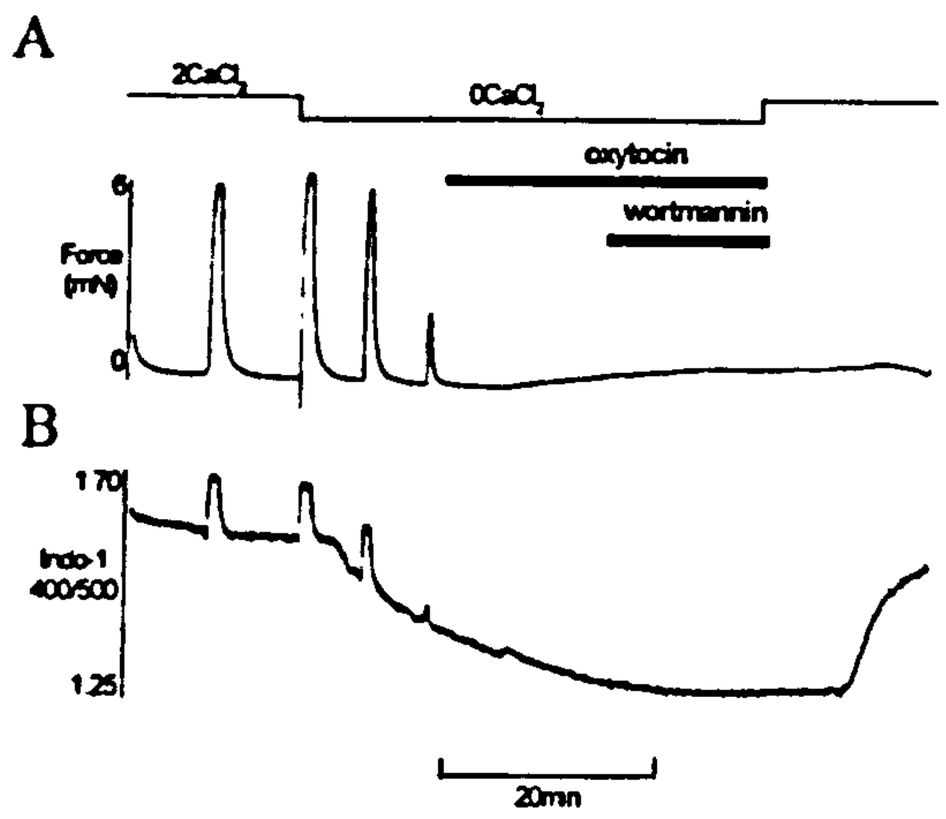

Fig. 5A, B Ca^{2+}-independent uterine contractions. **A** Following a control period of spontaneous activity in rat uterus, external Ca^{2+} was removed. After 5 min oxytocin was added and then removed, and external Ca^{2+} restored. Finally, the response of the tissue to oxytocin in the presence of Ca^{2+} is shown. **B** Simultaneous recording of force (*top*) and Ca^{2+} (Indo ratio, *bottom*) in human uterus showing the effects of wortmannin on the contraction evoked by oxytocin in the absence of external Ca2+

Ca^{2+} present) addition of oxytocin did not produce any discernible rise in $[Ca^{2+}]_i$, and wortmannin had no effect on $[Ca^{2+}]_i$.

Discussion

These data are the first to examine the effects of inhibiting MLCK on uterine contraction. They show that in the human and pregnant and non-pregnant rat uterus, inhibiting MLCK with two structurally unrelated compounds can completely inhibit force produced spontaneously, by a high-K^+-induced depolarization or by oxytocin. This inhibition was accomplished with little or no effect on either the inward Ca^{2+} current or $[Ca^{2+}]_i$. These data emphasize the crucial role of myosin light chain phosphorylation in uterine contraction and also question the importance of other force-producing pathways under physiological conditions.

Both rat and human myometrium were affected by inhibition of MLCK. There were no qualitative differences between the responses in the two tissues; in the presence of external Ca^{2+}, force was reduced in both, irrespective of its mechanism of production and without effect on $[Ca^{2+}]_i$. The only significant difference between the two was the speed of effect of wortmannin in reducing contraction. While some of this may be due to the slightly thicker human preparations, we suggest that most of this relates to the significantly higher frequency of contractions in the rat. Thus in both tissues the effect is gradual and may reflect wortmannin's ability to access and inhibit the ATPase on MLCK only when the enzyme is activated (as $[Ca^{2+}]_i$ rises). Thus if a tissue is contracting at a rapid rate, this access and inhibition will occur more rapidly than in a tissue contracting at a slow rate. The similarity in the effects of inhibiting MLCK in rat and human uterus are in agreement with previous data, suggesting that the basic mechanism of contraction is the same in both species [28]. The fact that there were no significant differences between the pregnant and non-pregnant rat data also suggests that these basic mechanisms are not altered in pregnancy.

The potent inhibitory effects of wortmannin and ML-9 on uterine phasic force production agrees with earlier work on other phasic muscles [2, 14, 26]. Inhibition of tonic force in vascular smooth muscle has also been reported for both wortmannin and ML-9 [16, 24]. Although not measured in the current study, as expected both inhibitors have been shown to reduce myosin phosphorylation in all tissues examined to date ([3, 16, 20, 26]; Burdyga and Mitchell, personal communication). Previous studies have recorded electrical activity in the presence of wortmannin and report no change in any of the parameters of the action potential [2, 3]. The present study is the only one to have shown directly that there is no effect of wortmannin on the inward Ca^{2+} current. In addition, we have shown that force falls despite normal Ca^{2+} transients; a result consistent with findings in studies of the ureter [2]. Thus putting together the data from our current and previous studies mentioned above, it is clear that wortmannin has no effect on the processes governing excitation and Ca^{2+} rise in smooth muscle. This is consistent with its specific effect on MLCK, as a non-competitive inhibitor with respect to the ATPase site on the enzyme. Reports vary as to the reversibility of wortmannin [3]. In the uterus we found some recovery, in agreement with previous findings in a study of ureteric smooth muscle [2]. Wortmannin is the most selective inhibitor of MLCK available. It has also been shown, in some tissues at least, to inhibit phosphatidylinositol 3-kinase (Pi3 kinase) [1, 22]. This effect however is at extremely low concentrations (1–10 nM) and is not compatible with the dose/response effects we saw at wortmannin concentrations ranging from 0.5 to 4 μM. Furthermore, the involvement of this kinase in the absence of agonists is unlikely and thus again the data we obtained relating to spontaneous and high-K^+ contractions are not compatible with such an action of wortmannin. Burke et al. [3] also showed Pi3 kinase to have little or no effect in gastric smooth muscle. Thus we conclude that in our study the major effect of wortmannin was to inhibit MLCK, and hence myosin phosphorylation. The data obtained using ML-9 were similar to those obtained using wortmannin. ML-9 is another potent and selective inhibitor of MLCK, and it competes with ATP for the active site on MLCK, where it has a direct effect [6, 20]. It also reduced and abolished uterine force in a dose-dependent manner; its effects were readily reversible. Wingard and Murphy [26] reported similar findings in arterial muscle with ML-9 and also reported attenuation of the aequorin light signal, indicating that it may also reduce Ca^{2+}. Thus, irrespective of the agent used to inhibit MLCK, the result is the

same; little or no force can be produced without phosphorylation of myosin by MLCK. The data also clearly show that wortmannin was effective in reducing force in the uterus, irrespective of the species or mechanism used to produce that force. Of particular note was the reduction of oxytocin-evoked contractions, as it is under these conditions that non-MLCK pathways would be expected to act [18]. The inability of a high-K^+ concentration to overcome the inhibition of force, produced either spontaneously or by oxytocin, is consistent with the $[Ca^{2+}]_i$ and Ca^{2+} current data. That is, the effect of wortmannin is not to cause a failure in the surface membrane mechanisms that lead to a rise in $[Ca^{2+}]$, as both the Ca^{2+} current and rise of $[Ca^{2+}]_i$ were unaffected by wortmannin. These data are consistent with the failure of MLCK to phosphorylate myosin.

There is currently much interest in elucidating how agonists modulate force in smooth muscle. The data in our paper suggest that any effects beyond MLC phosphorylation are of limited importance in the human myometrium, at least under physiological conditions. The rat data also showed no differences in the effects of wortmannin on tissues from pregnant and non-pregnant animals, suggesting that the hormonal environment does not change this conclusion. Wingard and Murphy [26] also concluded that there is no evidence for Ca^{2+}- and MLCK-independent force production in arterial smooth muscle. It is so far unknown whether their contribution to contraction may be different under pathophysiological conditions, e.g. pre-term labour. In vascular smooth muscle, for example, it was found that inhibition of MAP kinase had no effect on the blood pressure of normotensive rats but reduced it in hypertensive rats [25]. In the absence of external Ca^{2+} oxytocin can elicit a very small amount of force in the uterus [7, 13, 18, 19]. We found, as reported previously, that this is usually slow in developing and is tonic in nature. Wortmannin did not affect this contraction, suggesting that it is not produced by myosin phosphorylated at serine 19 by MLCK. The lack of effect of wortmannin on this contraction also further supports the suggestion that wortmannin is acting to selectively inhibit MLCK. This is consistent with the report of Oishi et al. [19] that such contractions occur without myosin phosphorylation. It has been suggested that this contraction arises via Ca^{2+}-independent isoforms of PKC, possibly via thin filament disinhibition [4]. It is however unclear how relevant such mechanisms and such small levels of force are to the uterus in vivo, as external Ca^{2+} will always be present, and, as shown in Figs. 1, 2, 3, under these circumstances little or no force is produced when MLCK is inhibited.

In summary our data show that wortmannin inhibits force production in human and rat myometrium irrespective of the mechanism used to elicit that force. This occurs despite unchanged Ca^{2+} currents and $[Ca^{2+}]_i$ and is consistent with MLCK phosphorylation of myosin being critical to force production in the uterus.

Acknowledgements We are grateful to the Wellcome Trust and Royal Thai Government for support.

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Experimental Physiology

Uterine Contractility Symposium

The physiological basis of uterine contractility: a short review

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In this review we discuss our current understanding of the cellular basis of uterine contractility, highlighting those areas requiring further study. It is clear that the basic processes of excitation–contraction coupling lie within the myometrial cell, and that these may be modified by agonists. Pacemaker acitivity, however, remains a mystery. The contribution of extracellular calcium entry to contraction is shown to be vital, whilst the role of the sarcoplasmic reticulum remains controversial. Much current experimental focus is on pathways controlling and regulating contraction, and we discuss sensitisation mechanisms and question their role in intact uterine preparations. *Experimental Physiology* (2001) **86.2**, 239–246.

Introduction

Much progress has been made in recent years in our understanding of the physiological basis of uterine contractility, at both the cellular and molecular level. The aim of this review is to outline where our understanding is, and to highlight those areas where progress is required. The ultimate aim is to be better able to control and manipulate uterine function, to prevent pre-term labours and hypo- or hypertonic term labours.

The uterus is a myogenic organ, that is the smooth muscle contained within it is able to contract without nervous or hormonal input (Wray, 1993). That is not to imply that agonists have no role in its contractility; indeed they are powerful modulators of contraction, but rather that the basic processes of excitation and contraction reside within the smooth muscle cells. Thus this is the area that we fill focus on in this article.

Excitation

It is still unclear which cells in the myometrium are pacemaker cells or indeed if all cells have pacemaker activity. The pacemaking cells, unlike for example those of the gut (Horowitz *et al.* 1999) are not anatomically distinct, nor fixed in location. It is interesting to note that specialised cells thought to be pacemakers have recently been found following enzymatic dissociation of the urethra (Sergeant *et al.* 2000). Perhaps similar cells remain to be discovered in the myometrium? It is also the case that the exact ionic nature of the pacemaker current remains to be established. Most of the work in this area has been undertaken using sharp microelectrodes (Parkington & Coleman, 1988, 1990). This group have reported electrophysiological differences between circular and longitudinal muscle bundles and their changes with gestational stage (Coleman *et al.* 1988). However, this information has not been developed into a more integrated understanding of the control of excitability in the whole tissue. It is clearly unsatisfactory that we do not have a proper characterisation of the channels and currents, which constitute pacemaker activity in the myometrium. Such knowledge may lead to the development of selective blockers or agonists needed to better control uterine contractility. The electrical activity of the uterus can be recorded via the electromyographic (EMG) signal from the abdominal surface (Devedeux *et al.* 1993). Such techniques provide for non-invasive detection of uterine activity, and may become useful in diagnosing term and pre-term labours (Garfield *et al.* 1998).

Investigations have been conducted to determine whether ion channels change in their type or expression with gestation. Little or no change in Ca^{2+} channel density or expression occurs in human myometrium throughout pregnancy (Thornton *et al.* 2000), although an increase was reported in rat myometrium (Tezuka *et al.* 1995). K^+ channels, however, have been reported to change. Khan *et al.* (1993) were the first to demonstrate changes in the expression of K^+ channel types in labouring compared with non-labouring human myometrium. Recently, Stefani's group showed a reduction in the number of maxi K^+ channels inserted in the membrane in late gestation (Song *et al.* 1999). However, Wang *et al.* (1998) reported a doubling of outward K^+ current density in

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rat myocytes at term. It may also be that there is a switch from Ca^{2+}-activated K^+ channels to voltage-activated K^+ channels at term (Coleman *et al.* 2000).

It is clear that depolarisation of the myometrial cell membrane leads to the opening of voltage-sensitive Ca^{2+} channels (Wray, 1993; Shmigol *et al.* 1998). This is illustrated in the left panel of Fig. 1, which shows the inward Ca^{2+} current and the increase in $[Ca^{2+}]_i$, which follows depolarisation in the voltage-clamped cell. Furthermore this Ca^{2+} entry, at least in rat uterus, occurs almost entirely via L-type Ca^{2+} channels. When nifedipine, a blocker of L-type Ca^{2+} channels, is present, no rise in intracellular Ca^{2+} or Ca^{2+} current occurs with depolarisation (Fig. 1, right panel). In human myometrium it has been suggested that there may be some T-type Ca^{2+} current activity present (Young *et al.* 1993) but the importance of this remains to be established. Clearly, however, if T-type Ca^{2+} channels were more represented in uterine myocytes than in other smooth muscle tissues, then block of them may form the basis of selective uterine relaxation.

Thus upon excitation and depolarisation of the myometrial cell membrane, Ca^{2+} influx occurs via voltage-gated Ca^{2+} channels. This in turn highlights the importance of external Ca^{2+}; without this Ca^{2+} there is no Ca^{2+} transient and no contraction, as illustrated in Fig. 2. Figure 2 shows records from an intact preparation of myometrium the contractions of which are occurring spontaneously, due to the pacemaker activity mentioned above. Clearly Ca^{2+} influx is vital to this form of uterine activity. It is also known that there is an internal Ca^{2+} store, the sarcoplasmic reticulum (SR), and its importance to uterine activity will be discussed next.

In summary, many fundamental questions concerning excitation in the uterus remain to be answered including the following. Which cells are pacemakers? What are the constituents of pacemaker current? How important is T-type Ca^{2+} current to human myometrium? It is clear, however, that external Ca^{2+} entry is vitally important to uterine contraction. The distribution of membrane channels expressed in the uterus is likely to alter at term and in labour, to allow better control of contractility. We do not yet have much detailed knowledge of the sequence either of these changes or of their quantitative importance to the contractile process (Coleman *et al.* 2000).

Role of the sarcoplasmic reticulum

The SR in the myometrium is found both close to the surface membrane (peripheral) and towards the centre of the cell (Broderick & Broderick, 1990). The peripheral SR may be in close apposition to special regions of the plasma membrane where caveolae (membrane invaginations) are found (Nixon *et al.* 1994). More detailed information on this will be found in Lee *et al.* (2001; this issue).

The SR takes up Ca^{2+} against a cytosolic Ca^{2+} gradient via an SR Ca^{2+}-ATPase, known as SERCA 2. Thus one way to investigate the role of the SR is to inhibit SERCA 2, with, for example, cyclopiazonic acid (CPA) or thapsigargin (Taggart & Wray, 1998; Shmigol *et al.* 1999*b*). Under these conditions the Ca^{2+} content of the SR will be discharged via leakage through the SR membrane. Ca^{2+} can also be released from the

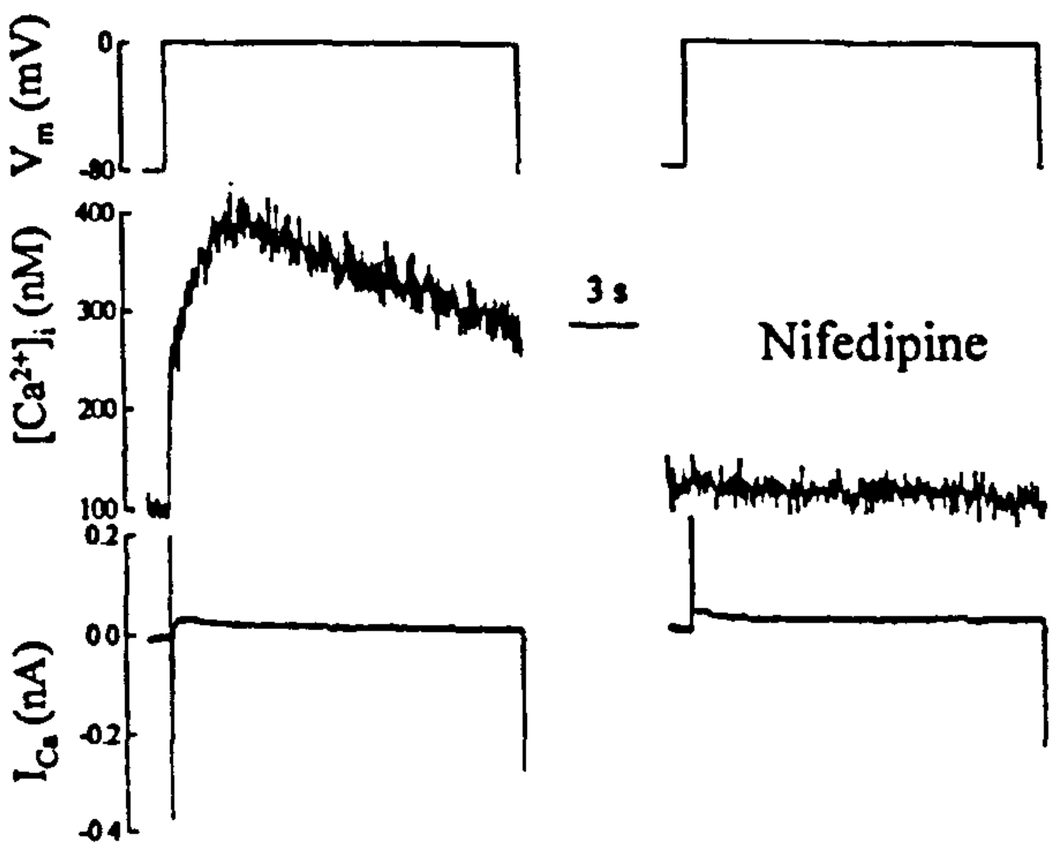

Figure 1

The effect of depolarisation on a voltage-clamped uterine myocyte from a pregnant rat, under control conditions (left panel) and in the presence of nifedipine (right panel). Upon depolarising the cell from −80 to 0 mV (top trace), an inward Ca^{2+} current (bottom trace) can be seen, which elicits a rise in intracellular Ca^{2+} concentration (middle trace). Nifedipine (10 μM), a blocker of L-type Ca^{2+} channels, prevents the inward current and there is no rise in $[Ca^{2+}]_i$.

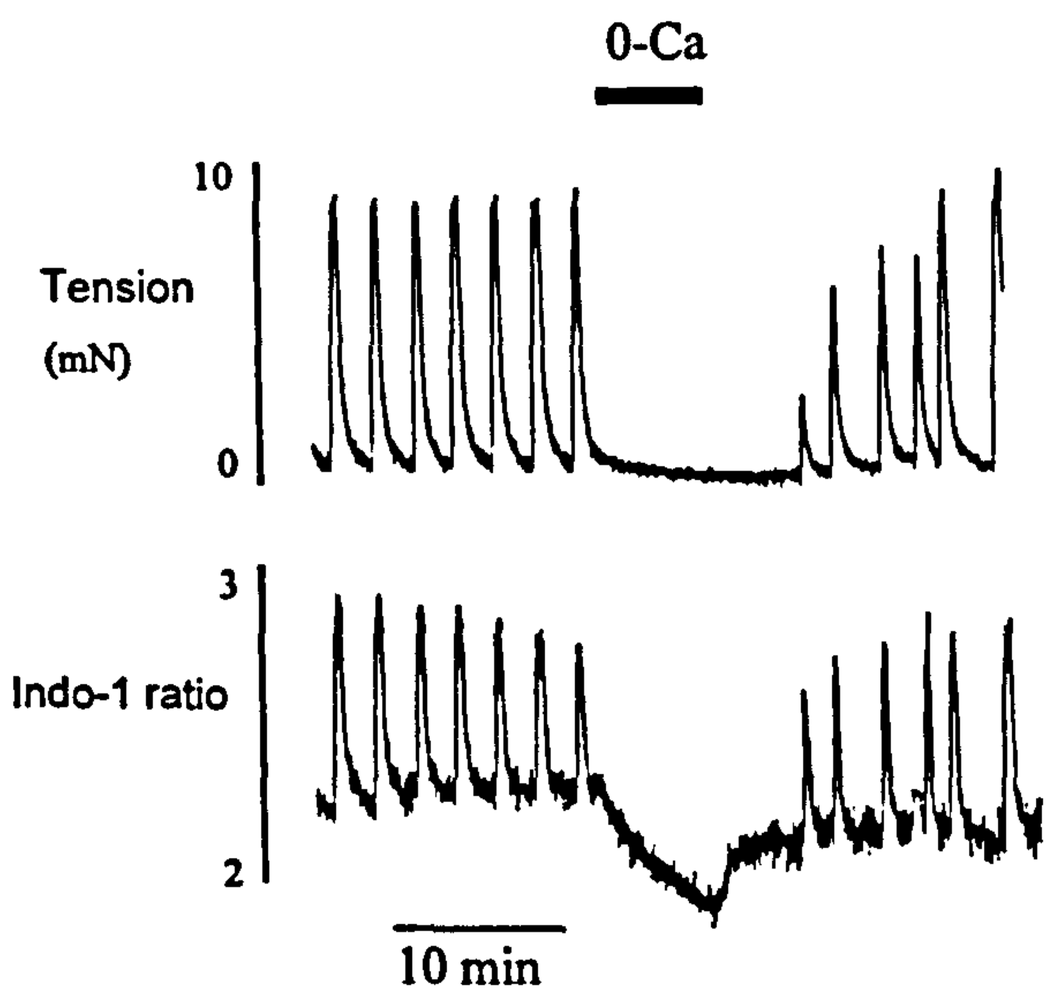

Figure 2

The effects of removing external Ca^{2+} on contraction. Spontaneous Ca^{2+} transients (measured using the fluorometric dye indo-1) and contractions in human myometrium recorded in the presence and then absence of external Ca^{2+}.

SR via two mechanisms. The first is via IP_3 binding to receptors on the SR membrane, known as agonist or IP_3-induced Ca^{2+} release (IICR). IP_3 is present in basal amounts in cells but its concentration rises rapidly when agonists bind to the cell membrane and cause the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2; Berridge, 1997). (Diacylglycerol is also formed.) Thus we can already see one mechanism whereby agonists can manipulate uterine force production, by raising [IP_3] and causing SR Ca^{2+} release and hence a rise in cytosolic Ca^{2+}. The second release mechanism is via Ca^{2+} itself. Thus there is a Ca^{2+} release channel on the SR gated by Ca^{2+}, leading to Ca^{2+}-induced Ca^{2+} release (CICR; Berridge, 1997). These Ca^{2+} release channels are known as ryanodine receptors, as ryanodine will bind to them, and change their gating. Caffeine, as well as Ca^{2+} acts as an agonist on these channels. Both IP_3 and ryanodine receptors have been reported on the myometrial SR membrane (Lynn *et al.* 1995; Morgan *et al.* 1996; Awad *et al.* 1997). Recent work has examined both isoform distribution (three isoforms are known for both receptor types) and whether changes with gestation arise. In rat and human myometrium all three ryanodine receptor (RyR) isoforms have been reported (Awad *et al.* 1997; Martin *et al.* 1999*a*,*b*). The predominant isoform is ryanodine 3 (RyR3), and recent work suggests no change with pregnancy in rats (Martin *et al.* 1999*b*). In human myometrium RyR3 was also the predominant form, but it was down-regulated at the end of pregnancy (Martin *et al.* 1999*a*). All three IP_3 receptors have been found in rat and human myometrium (Morgan *et al.* 1996), but their expression did not change in pregnancy. Before addressing the role of CICR and IICR in uterine contraction, the role of the SR in Ca^{2+} uptake and relaxation will be described.

So far we have discussed two mechanisms that can elevate [Ca^{2+}] within the myometrial cell; extracellular Ca^{2+} entry and SR Ca^{2+} release. Clearly for relaxation to occur the increased intracellular Ca^{2+} must be reduced to resting levels again. This occurs by transport out of the cell and uptake into the SR (Shmigol *et al.* 1998, 1999*b*). Both these mechanisms are active processes, as Ca^{2+} is being transported against its gradient. Ca^{2+} is extruded from the cell by both Na^+–Ca^{2+} exchange and a surface membrane Ca^{2+}-ATPase. These mechanisms are responsible for removing the bulk of Ca^{2+} from the cell. Indeed if they are both inhibited, then recovery of Ca^{2+} to resting levels is abolished (Shmigol *et al.* 1999*b*), indicating that the uptake of Ca^{2+} by the SR is not sufficient to significantly lower intracellular Ca^{2+}. However, if the SR is inhibited, then the recovery of Ca^{2+} is delayed. These data are consistent with the idea that Ca^{2+} is taken up into the SR and then released very close to the sarcolemmal Ca^{2+} extrusion sites, i.e. the SR works in series with the surface membrane extrusion mechanisms. This suggestion is consistent with the SR acting as a superficial barrier to Ca^{2+} (van Breemen & Saida, 1989) and also with there being specialised regions of the surface membrane where ion channels and pumps or caveolae are located and where the SR comes into close opposition.

Ca^{2+}-induced Ca^{2+} release in the myometrium

The data for and against CICR in rat or human myometrium are puzzling. As mentioned above, there is good evidence for RyRs on the SR. The question is, however, are these playing any functional role? On balance, in intact tissue the evidence suggests they are not. Thus no impairment of spontaneous contraction or Ca^{2+} release occurs when CICR is blocked by ryanodine (Taggart & Wray, 1998), as shown in Fig. 3, and no Ca^{2+} release or contraction is produced by caffeine application (Savineau & Mironneau, 1990; Holda *et al.* 1996). Different results may, however, occur at the single or sub-cellular level. Thus when myometrial RyRs are incorporated into lipid bilayers a cation-sensitive channel can be shown to be present (Martin & Ashley, 1995). We have also found evidence for a small amount of CICR in single cells from pregnant rat uterus (Shmigol *et al.* 1998). Recently Martin *et al.* (1999*b*), also in rat uterus, reported functional ryanodine and caffeine responses in 30 % of their cells. The physiological function, and importance of these data, on single cells, in relation to the activity of the intact organ, remains to be established.

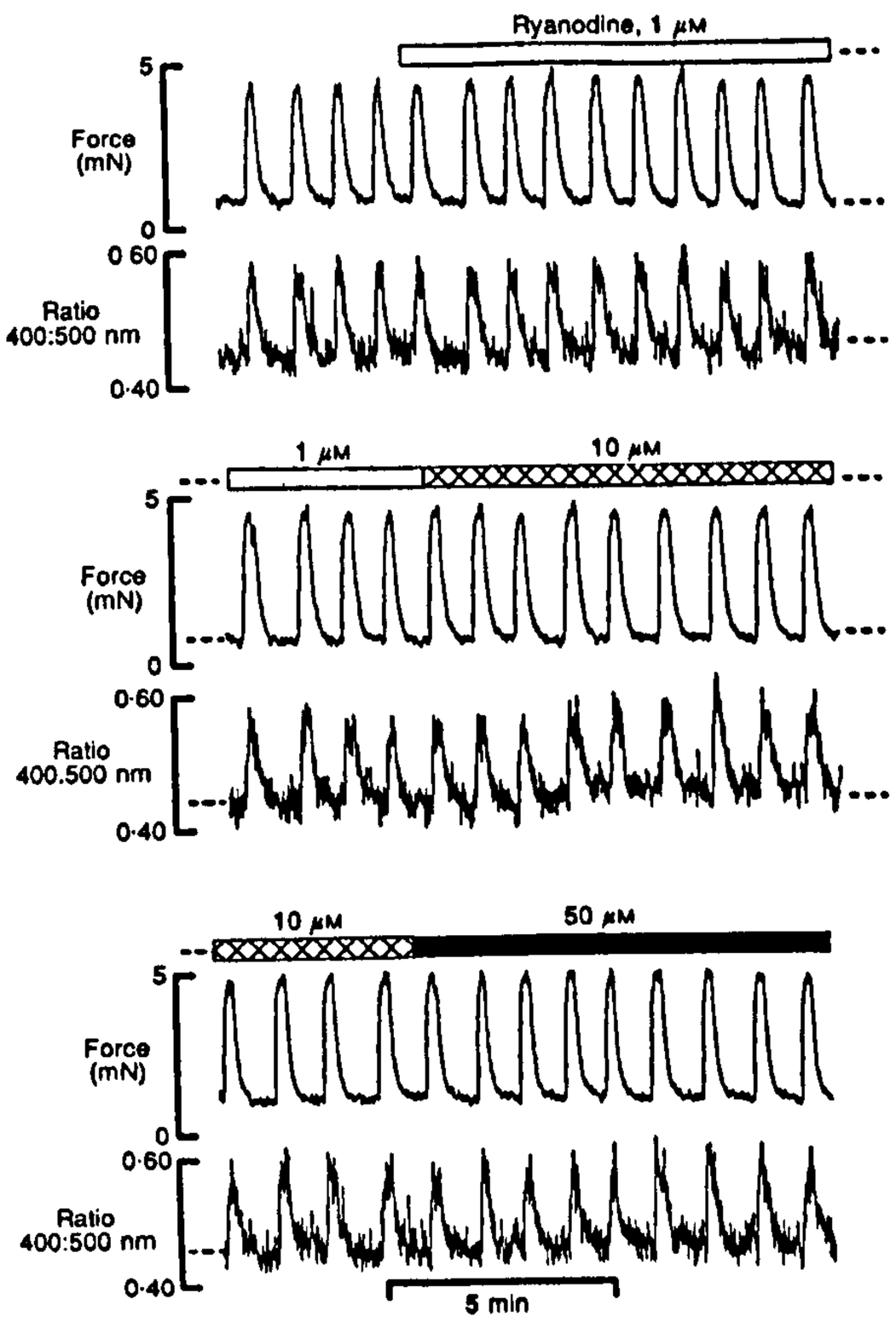

Figure 3

The effect of ryanodine on spontaneous force and Ca^{2+} transients recorded continuously in the presence of 1, 10 and 50 μM ryanodine in rat myometrium (taken from Taggart & Wray, 1998).

Role of IP_3-induced Ca^{2+} release in uterine contraction

It should of course be noted at the onset that probably all physiological agonists have more than one mechanism of action. Oxytocin, for example, will impair Ca^{2+} efflux from the cell, and alter Ca^{2+} entry, as well as releasing Ca^{2+} from the SR via IICR (Wray, 1993). Thus care should be exercised when attributing the effect of an agonist to one particular mechanism. As shown in Fig. 4*A* addition of oxytocin to intact human myometrium causes a significant rise in Ca^{2+} and force production. Addition of oxytocin in the absence of external Ca^{2+} (Fig. 4*B*) also elicits a small increase in Ca^{2+} and force. In these experiments the only source of Ca^{2+} is the SR. Other agonists, such as carbachol and prostaglandin $F_{2\alpha}$, induce similar Ca^{2+} rises and force production (Luckas *et al.* 1999). If the experiments are repeated in the presence of CPA to deplete the SR of Ca^{2+}, before addition of agonist, then no rise in Ca^{2+} occurs, again indicating that the SR is the source of Ca^{2+} (S. Kuppitayanant & S. Wray, unpublished observation). It is clear, however, that the rise in Ca^{2+} and contraction seen in Fig. 4*B* are small compared with the changes which occur in the presence of external Ca^{2+} (Fig. 4*A*). This indicates that *in vivo* oxytocin will stimulate both Ca^{2+} entry and SR Ca^{2+} release to promote force.

Figure 5 shows that the SR plays little role in spontaneous contractile activity in human myometrium. The spontaneous contractions and Ca^{2+} transients continue in the presence of CPA – indeed force and Ca^{2+} are slightly enhanced, and there is a rise in baseline $[Ca^{2+}]_i$ (Kupittayanant *et al.* 2000). One possible explanation for this is that the SR releases some Ca^{2+} spontaneously, which activates Ca^{2+}-activated K^+ channels (Bolton *et al.* 1999). This will increase the K^+ conductance and tend to hyperpolarise the cell. This in turn will make it less probable that Ca^{2+} channels will be opened and hence Ca^{2+} entry into the cell may decrease. Acting in this way the SR can be viewed as a negative regulator of contraction, rather than a stimulator. Thus if the SR is inactivated by CPA force will tend to increase. It is unclear why the rise in basal Ca^{2+} produced by CPA does not affect the contractile proteins. It is also possible that emptying of the SR stimulates Ca^{2+} entry via a capacitative or store-operated mechanism (Holda *et al.* 1998; Putney & McKay, 1999). Such mechanisms exist in non-excitable cells, which lack voltage-gated Ca^{2+} channels, to refill the SR. It is unclear, however, whether such mechanisms play an important role in excitable cells, such as uterine smooth muscle. It is also unclear whether spontaneous Ca^{2+} releases, so called Ca^{2+} sparks, occur in uterine myocytes. They have been demonstrated in several other smooth muscle tissues, particularly vascular, but so far not in the myometrium (Arnaudeau *et al.* 1996; Zhuge *et al.* 1998; Gordienko *et al.* 1998). Clearly their occurrence (or not) needs to be documented, along with the spontaneous transient outward currents (STOCs) associated with their activation of K^+ channels.

Recently we have made direct measurements of SR Ca^{2+} concentration by loading a low-affinity indicator, Mag-fluo 4, selectively into the SR (Shmigol *et al.* 1999*a*). This signal shows a large and rapid decline when agonists are added, and a rise in cytosolic Ca^{2+} concentration. Clearly by extending these studies to investigate SR Ca^{2+} content during spontaneous contractions and with SR function altered (e.g. high or low luminal Ca^{2+}), much useful, direct evidence of the SR's role in uterine contraction will be obtained (Golovina & Blaustein, 1997).

Another approach to obtaining information about the SR is to use immunohistological labelling of SR receptors and

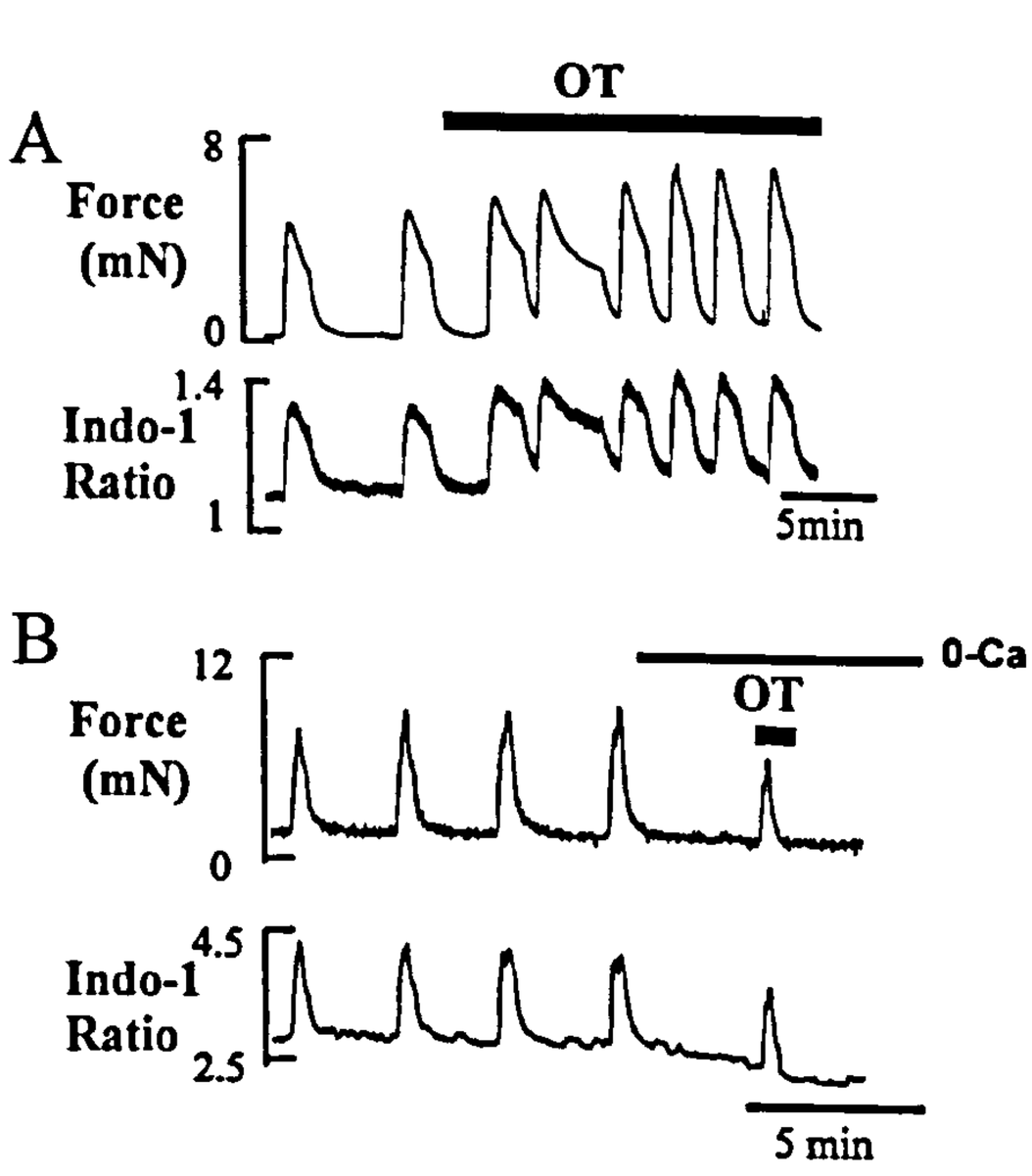

Figure 4

The stimulatory effect of oxytocin (OT, 10 nM) in the presence (*A*) and absence (*B*) of external Ca^{2+} on force (top traces) and intracellular Ca^{2+} (bottom traces, as assessed using indo-1) in human myometrium.

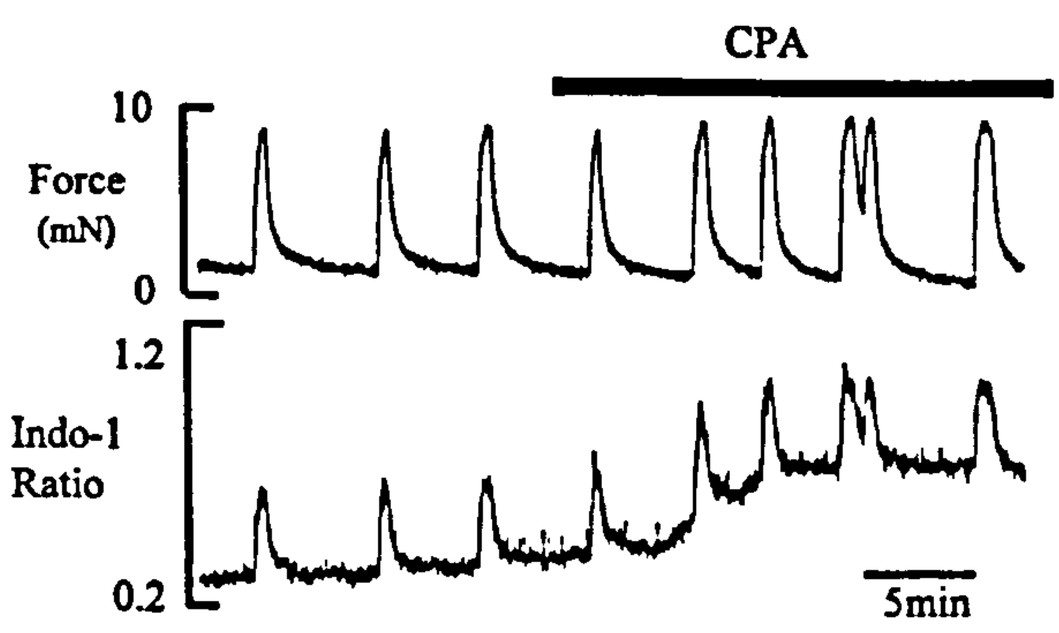

Figure 5

Simultaneous measurements of force (top) and intracellular Ca^{2+} (bottom), showing the effect of cyclopiazonic acid (CPA, 10 μM). Taken from Kupittayanant *et al.* (2000).

correlate their location with Ca^{2+} release events. The only studies to date on the myometrium appear to be those of Young *et al.* (1996) and Young & Mathur (1999). They have used confocal microscopy and found a lack of correlation between the Ca^{2+} release stores and the IP_3 and ryanodine receptors. Indeed they propose that both receptors are smoothly distributed throughout the SR and that SR Ca^{2+} release occurs via second messenger channels that are remote from the SR Ca^{2+} store (Young & Mathur, 1999). It would be worthwhile repeating this work in non-cultured cells, which will not have undergone any phenotypic changes, which make the uterine myocytes less contractile and more secretory in nature. Such techniques will in the near future allow determination of whether, for example, there are SR 'hot spots' for Ca^{2+} release (or indeed Ca^{2+} entry via the surface membrane) and communication between different compartments within the SR (van Breemen *et al.* 1995; Golovina & Blaustein, 1997; Gordienko *et al.* 1998).

Summary of SR in myometrium

From the above it is clear that there is much that distinguishes the myometrial SR from that of other smooth muscles. In particular its importance to the control of excitability via stimulation of Ca^{2+}-activated currents, its relation to other surface membrane microdomains, e.g. caveolae, and the relevance and importance of CICR to either spontaneous or agonist-induced force production.

Contraction

As with other smooth muscles, the rise of intracellular Ca^{2+} leads to cycling of actomyosin crossbridges, the hydrolysis of ATP and contraction. The content of actin and myosin rises in pregnancy and the uterus hypertrophies. Metabolic reserves to fuel contraction are also laid down during pregnancy; hence the concentration of phosphocreatine (PCr), the immediate backup for ATP, is increased, and glycogen and fatty acid deposits are increased (Dawson & Wray, 1985; Wray, 1990). The blood flow to the uterus during most of pregnancy rises in parallel with the growing products of conception, but then lags behind towards the end of pregnancy. Thus the uterus has been well developed throughout gestation to ensure that frequent and forceful contractions should occur during labour, to expel the fetus and placenta. Interestingly we and others have shown that during these contractions uterine blood flow is reduced, as the blood vessels within the myometrium are compressed (Larcombe-McDouall *et al.* 1999). This in turn, we have recently shown, leads to cyclic fluctuations in [ATP], [PCr] and pH, with each contraction (Larcombe-McDouall *et al.* 1999). We have found that the extent of these changes is proportional to the degree of vascular occlusion and that uterine force also falls directly with the reduction in blood flow (Larcombe-McDouall *et al.* 1998).

These changes may well feed back on the contraction to limit it, and thereby reduce uterine ischaemia and fetal hypoxia. In particular pH has been shown to be a potent modulator of uterine force, with intracellular acidification reducing or even abolishing contractions (Taggart & Wray, 1993; Parratt *et al.* 1994). We have previously found that when intracellular pH is lowered, Ca^{2+} transients are suppressed and intracellular Ca^{2} falls (Taggart *et al.* 1996). Measurement of membrane potential showed action potentials were also abolished by acidification (Taggart *et al.* 1996). Single cell studies revealed that the Ca^{2+} current but not the K^{+} current, was inhibited by acidification (Shmigol *et al.* 1995). Clearly this reduction in Ca^{2+} current will have profound effects on $[Ca^{2+}]$ and contraction in intact tissues. Thus if pH falls as a consequence of increased activity or hypoxia, it will act to limit contractions (Wray, 1988).

Pathways leading to contraction

Following excitation either arising from an action potential or due to agonist binding to a receptor, force rises within the myometrium. There is currently much debate about the Ca^{2+}-dependent and -independent force producing pathways in smooth muscle. What is clear for the myometrium is that a rise of Ca^{2+} and then stimulation of myosin light chain kinase (MLCK) via Ca^{2+}–calmodulin is essential for normal uterine activity. What do we mean by this and what is the evidence? By normal uterine activity we mean cyclic or phasic

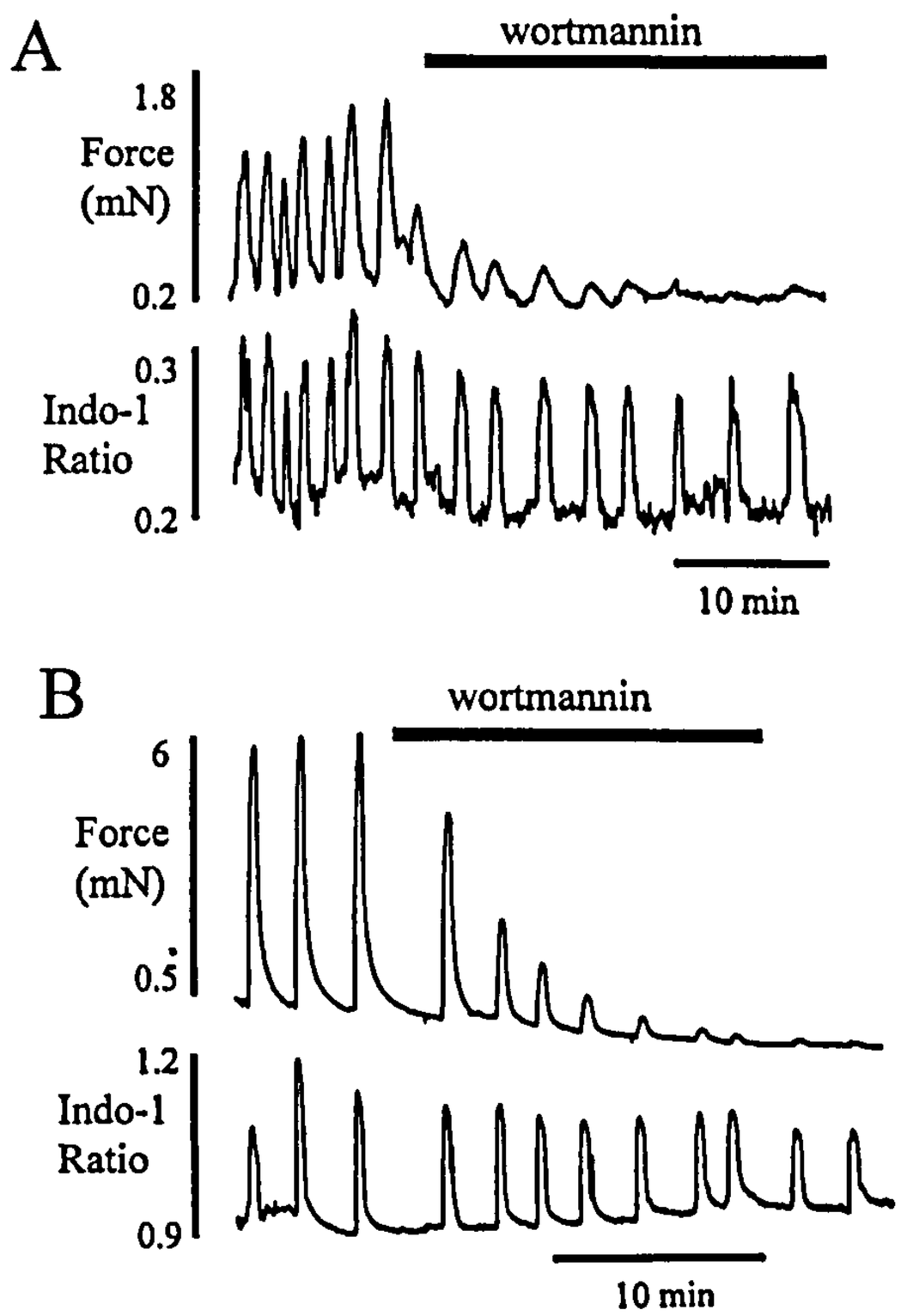

Figure 6

The effect of wortmannin (4 μM), an inhibitor of myosin light chain kinase, on force (top traces) and intracellular Ca^{2+} transients (bottom traces), arising either spontaneously (*A*) or stimulated by oxytocin (*B*, 10 nM). Taken from Longbottom *et al.* (2000).

contractions which last for around 1–3 min, not prolonged tonic activity for > 5 min. We have demonstrated already that removal of external Ca^{2+} prevents the rise of Ca^{2+} needed for spontaneous contraction (Fig. 2). Thus without a rise in [Ca^{2+}] no Ca^{2+}-independent force producing pathway can elicit spontaneous contractions. That this pathway is also dependent upon MLCK can be appreciated from Fig. 6. Here wortmannin was used to inhibit MLCK. It can be seen that the Ca^{2+} transients are unimpaired (as were the Ca^{2+} currents) but that contraction rapidly decreases (Longbottom *et al.* 2000). Thus in both rat and human myometrium we found no force when the Ca^{2+}–calmodulin–MLCK pathway was interrupted. In addition the experiments with wortmannin show that, if present, other force producing pathways dependent upon Ca^{2+}, could still work, as [Ca^{2+}] was maintained. Thus we conclude that under physiological conditions the Ca^{2+}–calmodulin–MLCK pathway is predominant in producing uterine force.

In other smooth muscles, particularly tonically active ones such as blood vessels, Ca^{2+} sensitisation has been described. This is when force can be produced without a change in intracellular [Ca^{2+}]. Many agonists are thought to be able to modify the reactions involved in the production of force. The two key targets of these mechanisms appear to be MLCK and MLC phosphatase (MLCP). From the above it can be easily imagined that if the activity of MLCK is modified, then at a steady level of Ca^{2+}, and calmodulin, force could be affected. the phosphorylation of MLCK by several kinases has been reported (Horowitz *et al.* 1996; Weber *et al.* 1999). such phosphorylation reduces its activity and thus desensitises the contractile machinery. It is now also appreciated that the activity of MLCP can be modified (Somlyo & Somlyo, 1998) as this enzyme dephosphorylates myosin, it is usually associated with a decrease in force and relaxation. It can be phosphorylated, particularly by rho-associated kinase, resulting in a decrease in its activity. This in turn would lead to an increase of force at constant [Ca^{2+}], i.e. an increase in Ca^{2+} sensitivity. In considering the importance of such mechanisms to the uterus we would suggest that they are of secondary importance compared with the normal Ca^{2+}–calmodulin–MLCK–MLCP pathway. Their importance may be increased during longer lasting labour contractions, but until their importance can be demonstrated under physiological conditions in intact preparations, their role remains unproven. Such data may come with the use of inhibitors of rho-A kinase *in vivo*, as was undertaken by Uehata *et al.* (1997) in rabbits and effects on blood pressure (tonic force) determined. An alternative approach could be in the use of antisense oligonucleotides (Suggs *et al.* 1999) to specifically disrupt putative modulators, or with knockout mouse models (Das *et al.* 1997).

In summary, although it is tempting to consider the modulation of uterine force by neurohormonal agonists to be due to mechanisms affecting sensitisation, a fuller understanding of how they modify excitability and Ca^{2+} homeostasis may be an equally profitable area for investigation.

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Acknowledgements

We are grateful to the MRC and Wellcome Trust for supporting this work. S.K. is supported by the Royal Thai government.

Pflügers Arch - Eur J Physiol (2001) 443:112–114
DOI 10.1007/s004240100668

ORIGINAL ARTICLE

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The effects of inhibiting Rho-associated kinase with Y-27632 on force and intracellular calcium in human myometrium

Received: 24 April 2001 / Revised: 9 June 2001 / Accepted: 11 June 2001 / Published online: 7 September 2001

Abstract Recent work has indicated that smooth muscle force production may be influenced by pathways not dependent upon the Ca^{2+}-calmodulin phosphorylation of light chains. Few studies, however, have examined the importance of these pathways in intact muscles that contract phasically rather than tonically. Therefore, to determine whether the Ca^{2+}-independent Rho-A and associated kinase (ROK) pathway can affect contractions of the intact human myometrium, we used Y-27632 to inhibit ROK. Three types of contractile activity were examined: spontaneous and those elicited by oxytocin and by depolarisation by high K^+. Y-27632 decreased force significantly under all three conditions, without changing intracellular [Ca^{2+}]. However, the effects on force were only large when the uterus was producing force tonically rather than phasically. This suggests that the Rho-A-ROK pathway may not be a potent modulator of force in the human myometrium under physiological conditions.

Keywords Uterus · Contraction · Signalling · Phosphatase

Introduction

The activity of the smooth muscle of the uterus is modulated so that it is relatively quiescent before term and then contracts powerfully at term. There is much interest in gaining a better understanding of how this modulation is achieved, so that appropriate activity, such as pre-term labour, can be controlled. The main force-producing pathway in the uterus is the Ca^{2+}-calmodulin-myosin light chain (MLC) kinase (MLCK) pathway [3]. However contraction can be regulated by the phosphorylation state of MLC_{20}, which depends on the balance between MLCK and MLC-phosphatase (MLCP) activation [6]. Agonists, for example, activate the Ca^{2+}-calmodulin-MLCK pathway but may also stimulate force production by inhibiting MLCP via Rho-associated kinase (ROK) phosphorylation [7]. It is unclear how important this pathway is for contractions of the intact myometrium, although RhoA has been reported in the myometrium and to translocate to the cell membrane from the periphery upon muscarinic stimulation [2], and ROK expression increases with pregnancy [5]. We therefore investigated the effect of inhibiting the Rho-A pathway using Y-27632, a selective inhibitor of ROK [1], on human myometrial contractions, arising either spontaneously or elicited by membrane depolarisation by high [K^+] or with oxytocin stimulation. In addition, we measured intracellular [Ca^{2+}] ($[Ca^{2+}]_i$) to determine if any functional effects of Y-27632 were occurring independently of changes in $[Ca^{2+}]_i$.

Materials and methods

Human, non-labouring, myometrial tissues were obtained from women undergoing elective Caesarean section at term (37–41 weeks). Exclusion criteria were serious medical complications or use of medication likely to affect myometrial activity. Written informed consent was obtained and Ethical approval granted. Tissue was placed in physiological saline (2 mM Ca^{2+} pH 7.4) and then strips (1×1×5 mm) of longitudinal myometrium dissected and incubated overnight in Krebs' solution containing 15 μM Indo-1 for Ca^{2+} measurements, as described in detail elsewhere [4]. The strips were then mounted in a small chamber on an inverted microscope, with one end attached to a Grass FT03 force transducer. Experiments were performed at 35 °C and tissues superfused with solution throughout. Paired data were analysed with Student's *t*-test; differences between means were assumed to be significant at $P<0.05$. Data are given as means±SEM for *n* samples. Y-27632 was a kind gift from Yoshitomi Pharmaceutical Industries.

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Results

Effects of Y-27632 on spontaneous contractions and $[Ca^{2+}]_i$

Adding Y-27632 (10 μM) to spontaneously active preparations of human myometrium for up to 20 min produced a small (16±2%) but significant decrease in the amplitude of contractions compared with control (100%), as shown in Fig. 1, but no significant change in the Ca^{2+} transient (n=4). The frequency of contractions also increased significantly to 162±6%. The rate of relaxation of the contractions increased significantly to 23±2% (n=6), and thus the contractions were significantly shorter.

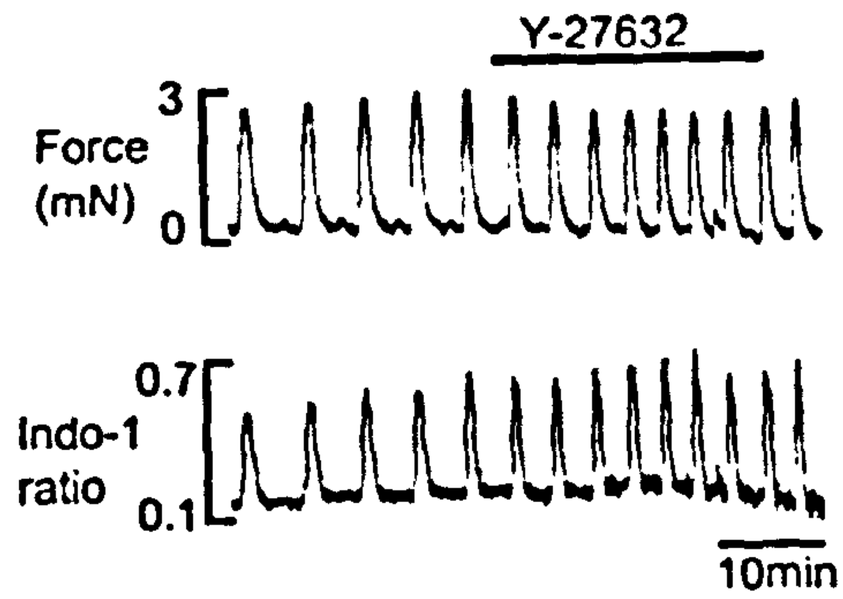

Fig. 1 The effect of Y-27632 on simultaneously measured spontaneous force (top trace) and Ca^{2+} transients (bottom trace, measured with Indo-1), in pregnant human myometrium

The effects of Y-27632 on contractions and $[Ca^{2+}]_i$ induced by oxytocin

To investigate whether activation of MLCP could influence agonist-induced contraction, the effects of Y-27632 on the response to oxytocin (10 and 100 nM) were studied. As can be seen in Fig. 2, Y-27632 did not abolish the rise in $[Ca^{2+}]_i$ or force produced by 10 nM oxytocin (n=4); it only produced a small (12±2%) decrement of force. With 100 nM oxytocin, Y-27632 significantly reduced the tonic component of force (54±2%, n=3), without a decrease in $[Ca^{2+}]_i$ (not shown). Adding oxytocin (100 nM) to the uterus in the presence of KCl (Fig. 2B) potentiated force with a small transient rise of $[Ca^{2+}]_i$, indicating release of Ca^{2+} from internal stores. To establish whether this potentiation of force by oxytocin was due to the modulation of MLCP activity via inhibition of ROK, we applied Y-27632. After 1–2 min, force began to fall markedly. The superimposed traces show that Y-27632 produced a marked decrease of force (85±5%, n=3, compared with the peak amplitude in control traces) to near basal levels without a change in $[Ca^{2+}]_i$.

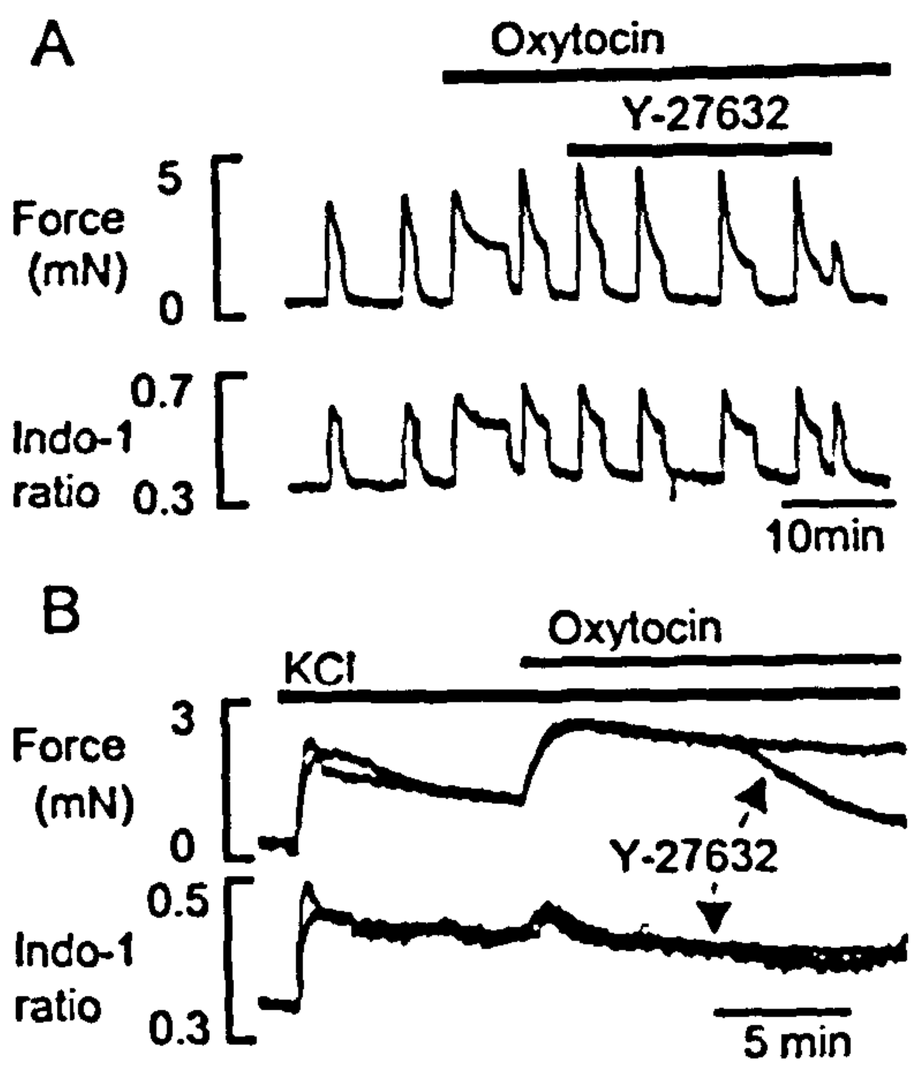

Fig. 2 A The effects of Y-27632 on force and Ca^{2+} signal, in response to 10 nM oxytocin. B As in A, but with 100 nM oxytocin and the preparation depolarised by exposure to 40 mM KCl. The control trace and that with Y-27632, added after 5 min in oxytocin, were obtained in the same tissue and are superimposed

The effect of Y-27632 on contractions and $[Ca^{2+}]_i$ induced by KCl

The effect of Y-27632 on KCl-induced contractions was also examined. Figure 3 shows a control response to KCl (40 mM). Force and $[Ca^{2+}]_i$ rose and were maintained until return to control solution. After recovery from KCl, the effects of Y-27632 were studied (Fig. 3, superimposed trace). After 1–2 min exposure to Y-27632 (in the presence of KCl) there was a significant decrease in force without a change in $[Ca^{2+}]_i$. The decrease in the mean maximum force was 53±2% (n=8), compared with control.

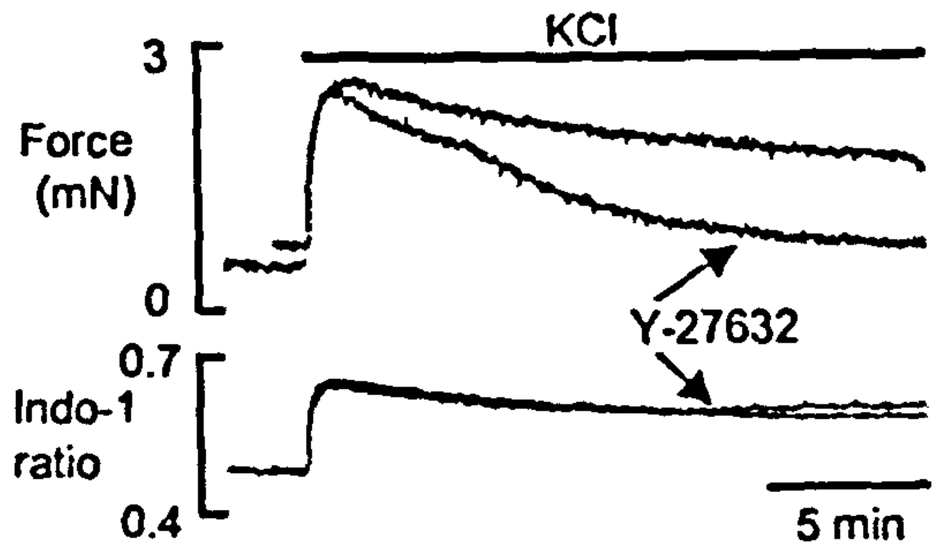

Fig. 3 The effects of Y-27632 on the response of force and cytosolic Ca^{2+} to depolarisation (40 mM KCl); control and Y-27632 responses were obtained in the same tissue and superimposed

Discussion

The amplitude and duration of the normal phasic contractions of pregnant non-labouring uterus decreased when ROK modulation of MLCP was inhibited by Y-27632. The degree of modulation was, however, only small, indicating that phasic activity is not greatly enhanced by this mechanism. These data are consistent with our previous findings that calcium entry and stimulation of MLCK is the predominant mechanism modulating spontaneous contractions [3]. The spontaneous contractions also shortened due to an increased rate of relaxation. This suggests that the MLCP was more active, consistent with ROK inhibition by Y-27632.

The augmentation of force and $[Ca^{2+}]_i$ in response to physiological concentrations of oxytocin (10 nM) were little affected by Y-27632, consistent again with Ca^{2+} entry playing a major role in this kind of contraction. However, when oxytocin was added under depolarised conditions or at supra-physiological concentrations (100 nM), and force production occurred with little rise of $[Ca^{2+}]_i$, indicating that a change in the sensitivity to Ca^{2+} is the major mechanism, then Y-27632 had profound effects. These conditions perhaps also occur in permeabilised preparations in which $[Ca^{2+}]$ is maintained at elevated levels: under such circumstances Y-27632 has large effects on force in rat myometrium [2]. Interestingly, Y-27632 also decreased force in preparations depolarised by high K^+, indicating that MLCP modulation may play a role in this type of maintained contraction [8]. Thus the effect of MLCP modulation in vivo and its usefulness as a therapeutic tool will depend upon the relative importance of Ca^{2+}-sensitising pathways, compared with Ca^{2+} entry mechanisms.

Acknowledgements We gratefully acknowledge the financial support of the MRC and the Royal Thai Government.

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BJOG: an International Journal of Obstetrics and Gynaecology
March 2002, Vol. 109, pp. 289–296

Effect of inhibiting the sarcoplasmic reticulum on spontaneous and oxytocin-induced contractions of human myometrium

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Objective 1. To assess the contribution of the sarcoplasmic reticulum calcium store in the generation of uterine smooth muscle contractions; 2. to evaluate the contribution of calcium induced calcium release or ryanodine gated calcium channels to myometrial force production.

Design Laboratory scientific study.

Methods Myometrial strips were obtained from women undergoing elective prelabour caesarean section at term. These were loaded with the calcium sensitive indicator Indo-1 allowing simultaneous assessment of intracellular calcium concentrations and force production. The effect of exposing the strips to ryanodine (which abolishes calcium induced calcium release), caffeine (which activates calcium induced calcium release) and cyclopiazonic acid (which abolishes the sarcoplasmic reticulum calcium store) was examined.

Results Exposure to ryanodine had no appreciable effect on either the amplitude or the duration of the myometrial calcium and force transients but did increase the frequency of contractions (139 ± 5%). Caffeine did not potentiate force. Cyclopiazonic acid increased frequency, duration and amplitude of both calcium and force transients. The ability of oxytocin to provoke calcium and force transients in the absence of extracellular calcium was abolished by cyclopiazonic acid but not by ryanodine.

Conclusions These results demonstrate that calcium induced calcium release does not play a significant role in human myometrium and that no functioning role for the ryanodine receptors in human myometrial tissue could be shown. These data suggest that the sarcoplasmic reticulum may act to limit contractions and act as a calcium sink, rather than to amplify contractions.

INTRODUCTION

Over recent years, great progress has been made in our understanding of the cellular and molecular mechanisms of uterine contraction. It is now well established that a rise in intracellular calcium ($[Ca^{2+}]_i$) is associated with contraction, via activation of myosin light chain kinase and phosphorylation of myosin. The rise of $[Ca^{2+}]_i$ for myometrial contraction may come from two sources, these are: 1. extracellular Ca^{2+} entry through voltage-gated Ca^{2+} channels and 2. release of intracellular Ca^{2+} held within the sarcoplasmic reticulum. The relative importance of these two sources remains unclear.

Calcium release from the sarcoplasmic reticulum store can occur through inositol trisphosphate gated channels or ryanodine gated channels. Inositol trisphosphate is generated when agonists such as oxytocin, bind to their receptors on the surface membrane. Ryanodine channels are physiologically activated by Ca^{2+} itself, giving rise to calcium induced calcium release. Given the undoubted importance of the sarcoplasmic reticulum Ca^{2+} to the rise of $[Ca^{2+}]_i$ needed for contraction in striated muscles, it was more or less assumed that this would be its role in smooth muscle. However the contribution of the sarcoplasmic reticulum has been questioned following data obtained in non-pregnant rat myometrium. These data demonstrated no effect of sarcoplasmic reticulum inhibition on both spontaneous force and Ca^{2+} transients[1]. In addition, ryanodine, a plant alkaloid that can disable the sarcoplasmic reticulum, by producing a long lasting sub-conductance state of the Ca^{2+} sensitive Ca^{2+} channels (low doses) or closing the channel at high doses, also had no effect. These data indicated a lack of functional calcium induced calcium release[1]. In pregnant rat myometrium a small effect was seen when calcium induced calcium release was inhibited with ryanodine, but this was in the direction of potentiating, not reducing force and Ca^{2+1}.

In human myometrium the presence of both inositol trisphosphate and ryanodine receptors on the sarcoplasmic reticulum membrane, could be taken to indicate that these Ca-release mechanism would operate[2–4]. However the presence of channels cannot be taken as evidence of functional significance. We have recently demonstrated agonist-induced release of Ca^{2+} and contraction in the absence of external Ca^{2+}, in human myometrium. These findings are consistent with a functional inositol trisphosphate-induced Ca-release (IICR)[5], although zero-external

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PII: S1470-0328(02)01110-2

Ca^{2+} does not represent a physiological condition for the tissue. A recent study reported an alteration in contractions of the human myometrium when the sarcoplasmic reticulum Ca-ATPase was inhibited[6]. As this ATPase transports Ca^{2+} into the sarcoplasmic reticulum, for subsequent release, and the same authors also found increased expression of this ATPase in labouring compared with non-labouring tissue samples[6], it suggests some functional role of the sarcoplasmic reticulum in calcium regulation in human myometrium. In rats, however, this role seemed to be taking up Ca^{2+} to aid relaxation, rather than providing Ca^{2+} for contraction[7].

We therefore considered it important to assess the functional role of the sarcoplasmic reticulum store under physiological conditions, as factors which influence $[Ca^{2+}]_i$ and contraction, are clearly important to our understanding of the control of uterine contraction. This study was designed to evaluate in intact human myometrial strips: 1. the importance of calcium induced calcium release using ryanodine, and IICR using oxytocin, to produce contraction, 2. the importance of the sarcoplasmic reticulum to spontaneous activity, using cyclopiazonic acid (an inhibitor of sarcoplasmic reticulum Ca-ATPase and hence sarcoplasmic reticulum Ca^{2+} filling) and 3. the contribution of the sarcoplasmic reticulum to oxytocin-stimulated contraction. Some of these data have been reported in abstract form[8].

METHODS

Human myometrial tissue was obtained from women undergoing elective lower-segment caesarean at term (37 to 39 completed weeks) before the onset of labour (defined as regular painful uterine contractions associated with progressive cervical change). Twenty-three of the 26 caesarean biopsies were performed under spinal analgesia and three while the women were under general anaesthesia. The indications for caesarean delivery were previous caesarean delivery (14 patients), fetal malpresentation ($n = 7$), patient choice ($n = 3$) small pelvis ($n = 1$) and, suspected fetal macrosomia ($n = 1$). The mean age of women undergoing caesarean delivery was 29 years (range 20–35 years). Exclusion criteria were serious medical complications or use of medication likely to affect myometrial activity. Written informed consent was obtained, and the local ethics committee granted ethical approval.

Full thickness tissue biopsy specimens were obtained from the upper lip of the uterine incision at caesarean delivery. The tissue specimens were immediately immersed in buffered physiological (Krebs') solution (pH 7.4) containing (mM): 154 NaCl; 5.4 KCl; 1.2 $MgSo_4$; 12 glucose; 2 $CaCl_2$; and 10 *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] [HEPES]). The tissue was either used immediately or stored for a maximum of 12 hours at 4°C; this did not alter the contractile ability of the myometrium.

Five or six strips of longitudinal myometrial fibers (1.5mm × 1mm × 5–8mm) were dissected and incubated for three to 15 hours (room temperature) in Krebs' solution containing 15μmol/L of the membrane-permeable form of the calcium-sensitive dye Indo-1 (Molecular Probes, Eugene, Ore). Previous work had shown no differences in the contractile properties of unloaded and Indo-1-loaded tissue, nor in force and Ca between tissues loaded for three or 15 hours.

The strips were mounted in a small (200μL) chamber on an inverted microscope (Nikon Diaphot) and viewed with a 10 power fluor objective lens. The myometrial strips were attached at each end to metal hooks and one hook was fixed to a tension transducer (Grass FT 03). The strips were superfused with oxygenated Krebs' solution at rate of 8mL/minute and a temperature of 35°C, which was used because it is close to physiological but allows better retention of the Indo-1 signal.

The tissue was excited (100W xenon lamp) with light of wavelength 340nm. Light emitted at wavelengths 400nm and 500nm was measured with photomultipliers and digitally recorded. As intracellular calcium level increased, the 400nm signal increased and the 500nm signal decreased[6]. In all experiments changes in Indo-1 ratio were accompanied by shifts in the opposite direction of 400-nm and 500-nm emission signals. Sampling was performed at a rate of 10Hz.

After regular spontaneous contractions had been established (0–60 minutes), modulators of sarcoplasmic reticulum were applied; ryanodine, 10–50μM (a dosage sufficient to inhibit calcium induced calcium release channels in uterine sarcoplasmic reticulum[9]); cyclopiazonic acid, 10μM and 20μM, and caffeine (10mM). In some experiments 0-Ca^{2+} solutions were used; Krebs solution in which $CaCl_2$ had been omitted and 1mM EGTA added. In some experiments nifedipine (10μM) a blocker of L-type Ca channels was used, and in others tetraethylammonium (5mM), a blocker of K channels, were used, as described in the text. All chemicals were purchased from Sigma, except for ryanodine which was from Calbiochem, and oxytocin, which was from Alliance pharmaceuticals (Chippenham).

Statistics

Data are given as mean and s.e.m. and '*n*' represents the number of samples, each one from a different woman. Significance was tested using appropriate *t* tests or ANOVA and *P* values >0.05 taken to be significant. Results are expressed as percentages of control contractions (i.e. the control is 100%).

RESULTS

Ryanodine was used to investigate the role of calcium induced calcium release in spontaneous force and Ca^{2+}

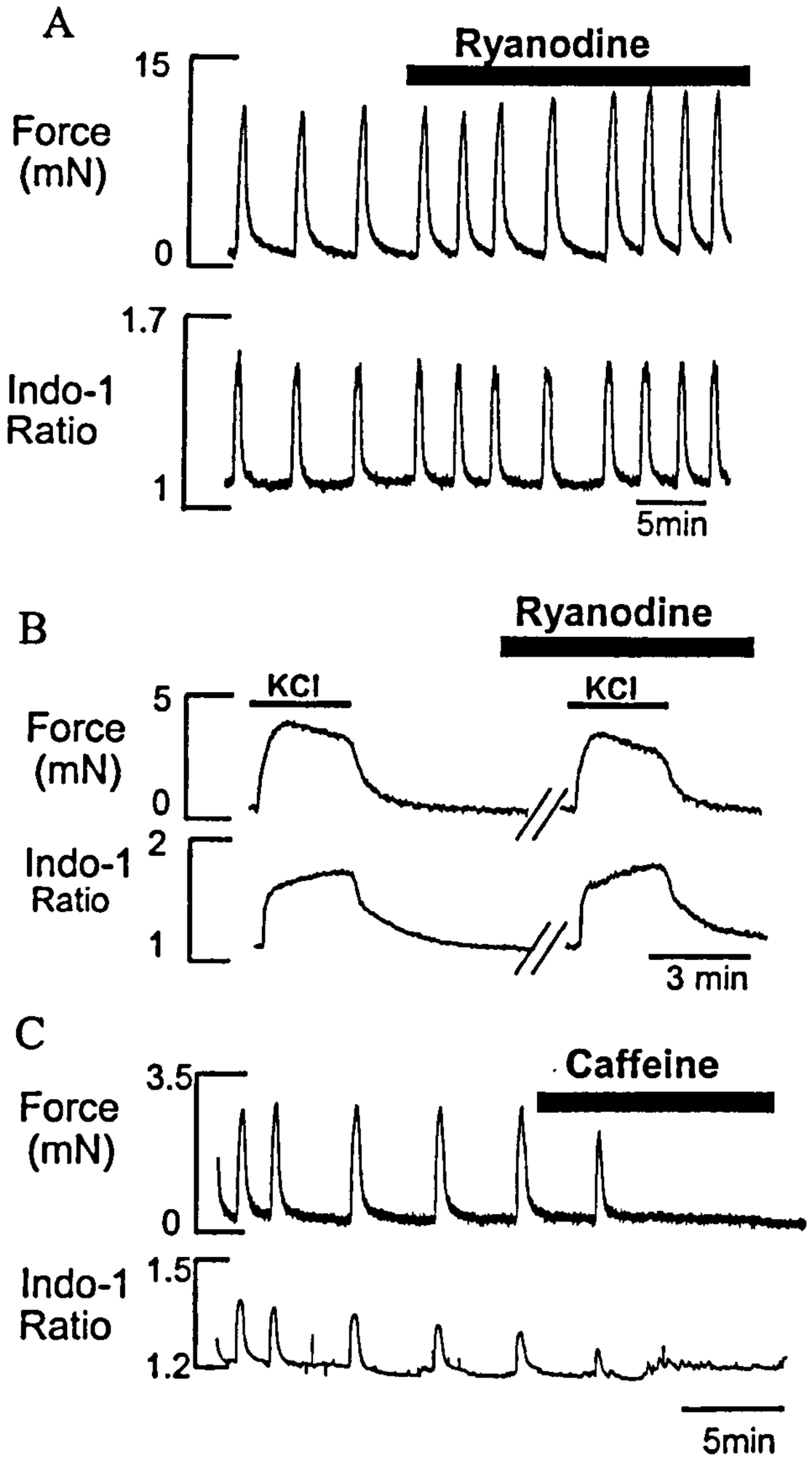

Fig. 1. The effect of modulating Ca^{2+}-induced Ca release on uterine contraction. (A) Spontaneous Ca^{2+} transients (Indo-1 ratio) and contractions before and after 50μM ryanodine application; (B) the response to high-K^+ (40mM) depolarisation in the absence and then presence of ryanodine; (C) the effect of caffeine (10mM) on spontaneous Ca^{2+} transients and force.

transients. The effects of 10μM, 20μM and 50μM ryanodine applied for up to 40 minutes was examined. In all 14 preparations ryanodine had no inhibiting effect on either the Ca^{2+} or force transient (Fig. 1a). At each concentration ryanodine had no significant effect on the amplitude or duration of the contractions or accompanying Ca^{2+} transients ($n = 14$), although in 5/14 there was a small (<10%) increase in amplitude and in four a small prolongation of the contraction; these effects did not reach statistical significance. In four preparations ryanodine produced a small elevation of baseline Ca^{2+} (not shown). Ryanodine did however produce an increase in the frequency of the spontaneous contractions in 11/14 preparations, resulting in a significant increase in the mean frequency (139 ± 3% compared with control, $n = 14$).

In five preparations the effect of ryanodine on a high-K^+ depolarization of the myometrium was examined. Ryanodine produced no significant effect on either force or Ca^{2+}; a typical result is shown in Fig. 1b.

Caffeine is an agonist for ryanodine Ca releasing channels. It has been previously reported to relax the myometrium[10]. For completeness in this study we applied 10mM caffeine to spontaneously contracting human myometrium. However the Indo-1 signal is quenched by caffeine[11], making it unreliable to use data on Ca transients in the presence of caffeine. In all cases ($n = 5$) the amplitude of the contractions was decreased and then abolished (Fig. 1c).

Cyclopiazonic acid is a specific inhibitor of the myometrial sarcoplasmic reticulum Ca^{2+}-ATPase[12], and thus its

application disables the sarcoplasmic reticulum as it is no longer able to store Ca^{2+}. Application of cyclopiazonic acid to the pregnant human myometrial preparations produced significant effects on spontaneous Ca^{2+} and force—both were potentiated (n = 15). In the majority of these preparations, force amplitude (13/15) and duration (10/15) were increased. The frequency of the contractions and Ca^{2+} transients increased significantly to 131 (6%). The amplitude of force and Ca^{2+} were also significantly increased; 121 (3%) and 127 (4%), respectively, as was their duration; 133 (11%) and 137 (4%), respectively, (all compared with control, 100%). A typical example is shown in Fig. 2a. It can be seen in Fig. 2b, where control and cyclopiazonic acid records have been expanded and overlapped, that there is a clear effect of cyclopiazonic acid to prolong the force and Ca^{2+} transients; mostly due to an effect on the plateau phases, but also due to a significant slowing of the relaxation rates. In addition cyclopiazonic acid consistently increased basal Ca^{2+} (to 117 (3%) but not force (see Fig. 2).

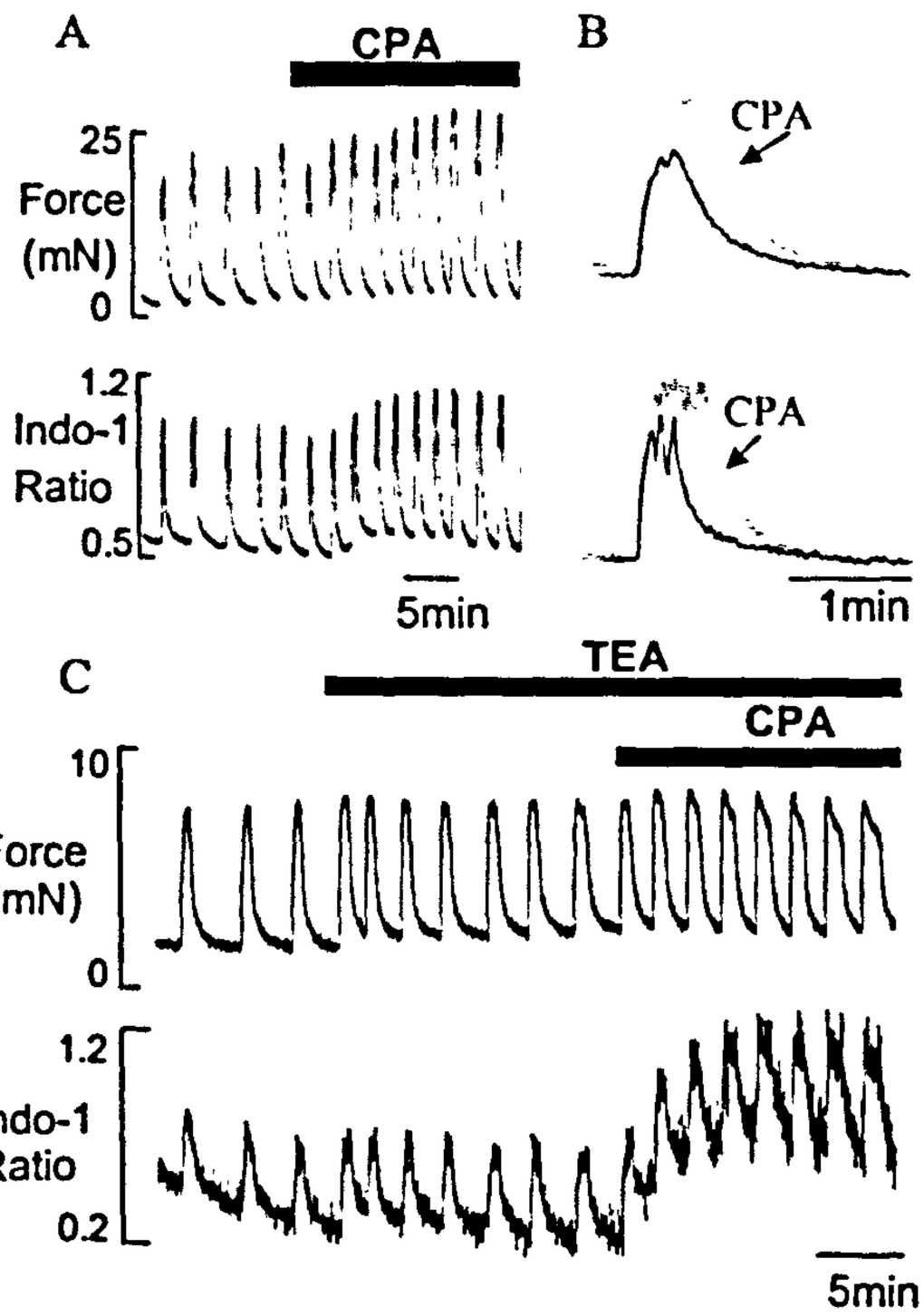

Fig. 2. The effect of inhibiting the sarcoplasmic reticulum on contraction. (A) Spontaneous Ca transients and force before and during 20μM cyclopiazonic acid (CPA) application; (B) superimposed force and Ca^{2+} records taken from A, under control conditions and in the presence of cyclopiazonic acid (dotted trace); (C) following control recordings the K^+ channel blocker tetraethylammonium (tetraethylammonium, 5mM) was added followed by cyclopiazonic acid.

The increase of $[Ca^{2+}]_i$ and force following inhibition of the sarcoplasmic reticulum by cyclopiazonic acid suggested an inhibitory role for the sarcoplasmic reticulum in the myometrium. As Ca^{2+} release from the sarcoplasmic reticulum may activate Ca-activated K^+ channels leading to hyperpolarisation of the membrane and decreased Ca^{2+} entry and force, this could be the mechanism affected by cyclopiazonic acid (i.e. cyclopiazonic acid prevents this hyperpolarisation). The effects of cyclopiazonic acid on force also resembled those of K^+ channel blockers, which prolong the action potential and thereby potentiate force[13]. We therefore next investigated this possibility by blocking K^+ channels, with tetraethylammonium (5mM) and studying the effects of cyclopiazonic acid application (n = 6). The potentiating effect of cyclopiazonic acid was not prevented by inhibition of K^+ channels. The application of tetraethylammonium increased force and Ca^{2+} (151 (8%) and 110 (7%) compared with control integrated force), but a further increase occurred upon addition of cyclopiazonic acid in the continued presence of K^+ channel inhibition (185 (9%) and 246 (11%), Fig. 2c). Similarly if tetraethylammonium was added after cyclopiazonic acid, it produced a further increase in Ca^{2+} and force (not shown).

The above results suggest that ryanodine has little effect on spontaneous Ca^{2+} and force in the human uterus. To investigate whether ryanodine could influence agonist-induced Ca^{2+} release, oxytocin was added in the absence of external Ca^{2+} entry. This protocol was used to ensure that the only source of Ca was from the sarcoplasmic reticulum[6]. As can be seen in Fig. 3a, spontaneous contractions stopped and $[Ca^{2+}]_i$ fell, upon changing to zero-Ca^{2+} solution. In the presence of the oxytocin (10nM), but in zero external Ca^{2+}, some force and Ca^{2+} were produced (Fig. 3b, in agreement with our previous data[6]). To investigate the contribution of calcium induced calcium release to the oxytocin-induced changes in force and Ca^{2+} in zero external Ca^{2+}, tissues were pre-incubated with ryanodine (10μM) and then oxytocin added. It can be seen that $[Ca^{2+}]_i$ and force were still produced by oxytocin, despite the presence of ryanodine (Fig. 3c, n = 7).

The same protocol as used above for ryanodine, was followed. In contrast to ryanodine however, cyclopiazonic acid was able to abolish the rise in Ca^{2+} and force upon oxytocin application to the uterus in 0-Ca solution. An example of this is shown in Fig. 3d (typical of 6). Thus it is clear that in the absence of external Ca, force can only be produced by the application of agonist, to release Ca from the sarcoplasmic reticulum, and that this is likely to be via IICR and not calcium induced calcium release.

From the above data it is clear that in the absence of external Ca^{2+}, oxytocin can release Ca^{2+} from the sarcoplasmic reticulum and produce force. It is also known that in the presence of external Ca^{2+}, oxytocin produces Ca^{2+} entry and potentiates contraction[14]. In order to try and assess how much of this potentiation can be attributed to the sarcoplasmic reticulum, and how much to external Ca^{2+}

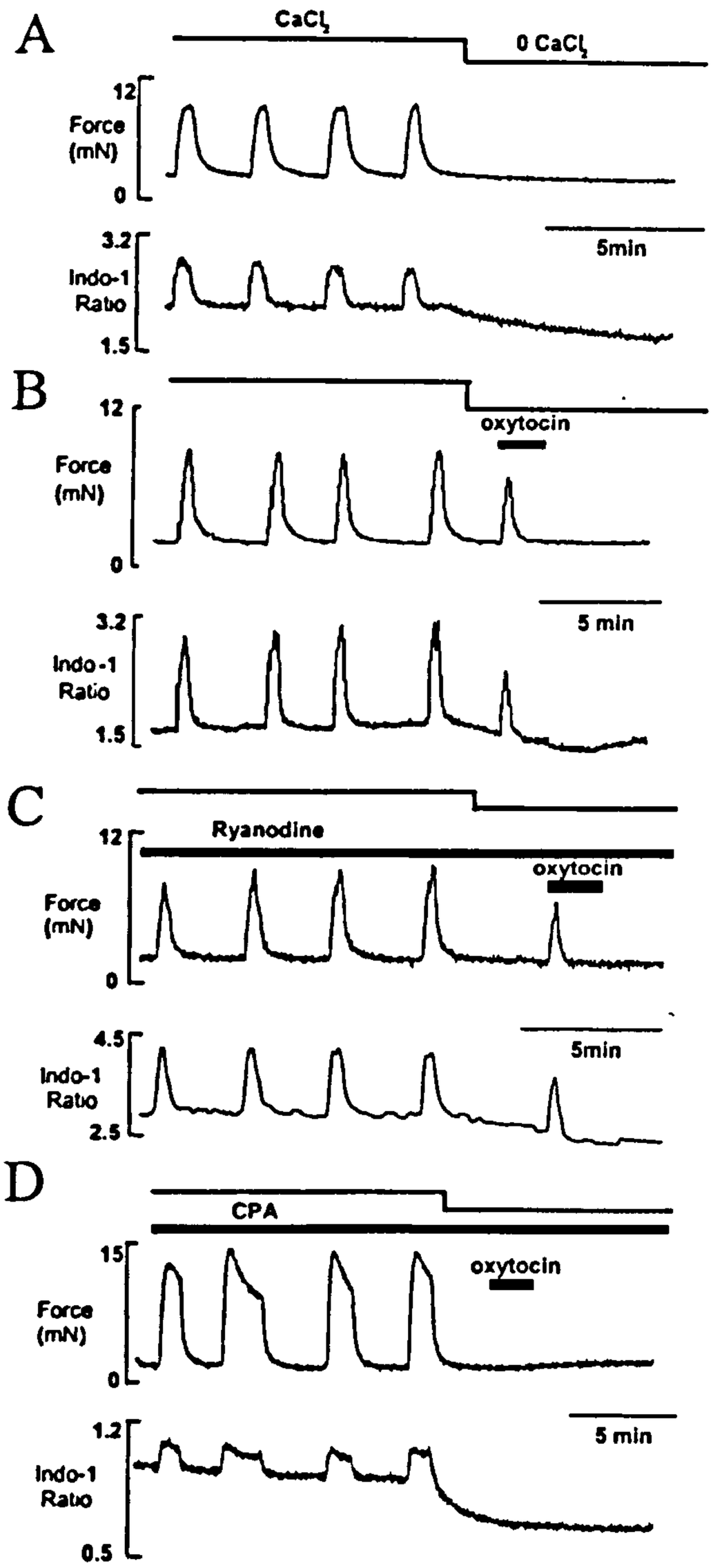

Fig. 3. The role of the sarcoplasmic reticulum in 0-Ca^{2+} conditions. **(A)** The effects of removing external Ca^{2+} on spontaneous Ca^{2+} transients and contractions; **(B)** oxytocin (10nM) application in zero-Ca containing solution. Application of oxytocin in zero-Ca containing solution after pre-treatment with **(C)** ryanodine or **(D)** cyclopiazonic acid (CPA).

influx, we compared the effect of oxytocin in the presence and absence of cyclopiazonic acid and nifedipine, a blocker of external Ca^{2+} entry. Fig. 4a shows a control response to oxytocin (i.e. in the presence of external Ca^{2+} and without cyclopiazonic acid). After recovery from oxytocin, the effects of cyclopiazonic acid and then oxytocin were studied. As expected, following cyclopiazonic acid the response to oxytocin is different from control. Nevertheless there are clear and significant increases in force and Ca^{2+} with oxytocin in the presence of cyclopiazonic acid ($n = 4$). When the blocker of L-type Ca channels, nifedipine (10μM), was added, it was clearly seen that force and Ca^{2+} transients were rapidly abolished. When the protocol was changed so that the effects of nifedipine, without cyclopiazonic acid, on the oxytocin response, could be examined, it was found that very little if any force or Ca^{2+} were produced (Fig 4b, $n = 3$), despite the presence of the sarcoplasmic reticulum. This was the case irrespective of whether nifedipine was added to an oxytocin induced contraction (Fig 4b) or oxytocin was added after nifedipine (not shown).

DISCUSSION

The aim of this work was to examine the role of sarcoplasmic reticulum in the human myometrium. The data show no role for calcium induced calcium release as determined by experiments using ryanodine in both spontaneous and agonist induced activity in intact preparations. Confirming previous work[5], an inositol trisphosphate 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. It was found that 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. No force is produced in the absence of external Ca^{2+}, indicating that this is the major source of Ca^{2+} for spontaneous activity. In the presence of oxytocin a clear role for Ca^{2+} entry in its mechanism of action was demonstrated.

The tissue samples were all taken from non-labouring women at term, 37 to 39 weeks, and thus represent a homogenous group. The samples were taken from the lower uterine segment which has less smooth muscle and more connective tissue than the upper segment. We have, however, recently shown that there are no significant differences in contractile properties and response to agonists of these two regions[15]. Thus we suggest that the data obtained are representative of the response of the entire uterus.

It is clear from our data that ryanodine (10–50μm) had no inhibitory effect on the spontaneous activity or high-K^+ depolarisation-induced force, of the human myometrial samples, even after 40 minutes of application. A similar conclusion was reached in rat myometrium[1]. In single cells from porcine myometrium, ryanodine also failed to release Ca^{2+} or change $[Ca^{2+}]$[16]. In addition, caffeine, an agonist of calcium induced calcium release was unable to increase force. These data are consistent with previous studies, which also showed an inhibitory effect of caffeine on myometrial preparations[10]. These inhibiting effects can be explained via lack of response of the uterine sarcoplasmic reticulum to caffeine and an inhibition of phosphodiester-

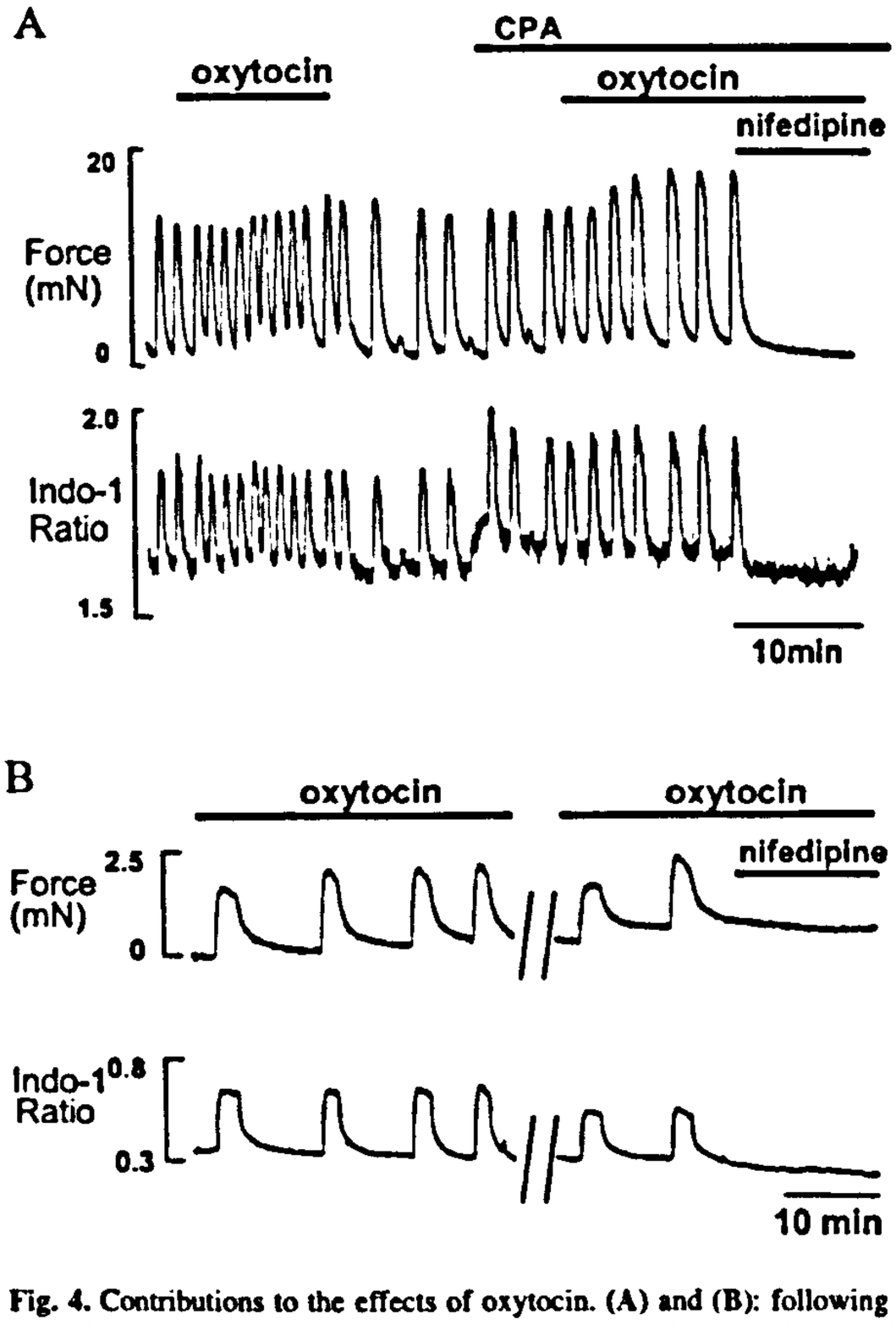

Fig. 4. Contributions to the effects of oxytocin. (A) and (B): following a control application of oxytocin and recovery, oxytocin was added again following cyclopiazonic acid pretreatment (CPA) (A), and then nifedipine (10μM) a blocker of L-type Ca^{2+} channels.

ase by caffeine, leading to an elevation of cAMP and hence relaxation[17], and hence it is not straightforward to relate the effects of caffeine to calcium induced calcium release in smooth muscles.

Thus in both human and rat myometrium we have data showing that inhibiting calcium induced calcium release neither reduces the rise in $[Ca^{2+}]_i$, nor force. These data therefore suggest that there is no functional role or coupling to the ryanodine receptors, which have been identified on the sarcoplasmic reticulum membrane from myometrial preparations[3,4]. It is not that the sarcoplasmic reticulum does not store Ca^{2+}, because our data show Ca^{2+} release following agonist stimulation (i.e. via inositol trisphosphate receptors), and there is an active Ca-ATPase[7,18]. The data on intact preparations clearly represents the functional response from very many myocytes. Recent data on single uterine cells has shown that a response to ryanodine can be seen in around 30% of the cells[4]. Some evidence for calcium induced calcium release was also obtained electrophysiologically from single rat uterine myocytes[19]. However, it is unclear at present what the physiological significance of the data on single cells is, as it does not appear to produce a functional effect in intact tissues. Variability in the expression of ryanodine receptors in late gestation may contribute to the differences reported above[2–4]. The potentiating effect of ryanodine on contraction frequency suggests some effect on electrical activity, which will be discussed alongside the data with cyclopiazonic acid below.

Cyclopiazonic acid has been directly demonstrated to inhibit the sarcoplasmic reticulum Ca-ATPase in myometrial preparations[18]; thus it will functionally disable the sarcoplasmic reticulum. Our data with cyclopiazonic acid are consistent with this; the Ca^{2+} and force transients took significantly longer to recover compared with controls. This suggests that the sarcoplasmic reticulum plays a role in lowering cytoplasmic $[Ca^{2+}]$ following stimulation, as shown in previous studies[7]. Clearly our data also show that inhibition of the sarcoplasmic reticulum is not associated with a decrease in spontaneous uterine Ca^{2+} or force transients; indeed they were both significantly augmented. This is also consistent with data obtained in the rat[1]. Thus, as with the ryanodine data, we can find no evidence in intact human myometrium that a functioning sarcoplasmic reticulum augments force production. In fact the question to be addressed is, how does inhibition of the sarcoplasmic reticulum increase Ca^{2+} and force? We investigated whether Ca^{2+} from the sarcoplasmic reticulum, released spontaneously, was activating K^+ channels. Such stimulation of K_{Ca} channels would prolong the plateau phase of the action potential and delay repolarisation, and thereby increase Ca^{2+} entry and force[20]. Such a mechanism was shown to be functioning in ureteric and ileal myocytes, where similar effects of cyclopiazonic acid on force and Ca^{2+} were found, to those reported here[13,21]. Our data showed that additional effects of cyclopiazonic acid could be seen even after K^+ channel inhibition with tetraethylammonium, and tetraethylammonium potentiated force following cyclopiazonic acid addition. Thus it is unlikely that all the effects of cyclopiazonic acid can be explained by such a mechanism. It remains to be established if emptying the sarcoplasmic reticulum can promote Ca^{2+} influx via store-operated mechanisms in smooth muscle[22], although it is possible that such a mechanism may be contributing[6]. It may also be that the uptake of Ca^{2+} into the sarcoplasmic reticulum plays a powerful role in curtailing the normal Ca^{2+} transient, hence its prolongation in the absence of this uptake mechanism. Clearly direct measurements of sarcoplasmic reticulum Ca^{2+} content, during normal activity and with cyclopiazonic acid, would be useful[23].

The increase in basal Ca^{2+} with little or no change of basal tone, produced by cyclopiazonic acid has been observed in other smooth muscles[13]. As yet no clear explanation is possible. It may be that the relatively slow increase in basal Ca^{2+} produced by leak of Ca^{2+} from the sarcoplasmic reticulum, in the presence of cyclopiazonic acid, is too slow to activate the myofilaments, or is not freely available to them.

The ability of oxytocin to release Ca^{2+} from the sarcoplasmic reticulum of human myometrium and produce force, has been previously demonstrated[5,24,25]. We show here that the mechanism is via inositol trisphosphate gated Ca^{2+} channels, rather than Ca^{2+} gated channels, on the sarcoplasmic reticulum. Thus ryanodine had no significant effect on the rise of Ca^{2+} and force produced in 0-Ca^{2+}, suggesting it is not occurring via calcium induced calcium release. This was the case even when a Ca^{2+} channel blocker was added to the zero-Ca^{2+} EGTA solutions, to ensure that no Ca^{2+} entry was occurring in these multicellular stores. These data also confirm that with our protocol, the sarcoplasmic reticulum Ca^{2+} is not depleted by the period in zero-Ca^{2+} solution. Thus the lack of response to oxytocin, in the presence of cyclopiazonic acid, cannot be explained by a run down of Ca^{2+} from the store, due to zero-Ca solution. When oxytocin releases Ca^{2+} from the sarcoplasmic reticulum we conclude that it is via an inositol trisphosphate mediated process. This is consistent with data showing an increase in inositol trisphosphate when the myometrium is stimulated with oxytocin and that inositol trisphosphate can release Ca^{2+} from myometrial sarcoplasmic reticulum preparations[26–28].

Having demonstrated the ability of oxytocin to release Ca^{2+} from the sarcoplasmic reticulum and produce uterine force, it is pertinent to ask what contribution this process makes to the augmentation of force seen with oxytocin, under physiological conditions (i.e. in the presence of external Ca^{2+})? To address this we undertook experiments with oxytocin and Ca^{2+} in the presence and absence of cyclopiazonic acid. Because cyclopiazonic acid itself alters force it is difficult to exactly compare the oxytocin responses before and after cyclopiazonic acid. However, our data have shown that the levels of force and Ca^{2+} are approximately comparable. These data and those obtained with oxytocin in Ca-free solutions, showing very small effects on force and Ca^{2+}, lead us to suggest that oxytocin effects on trans-sarcolemmal Ca^{2+} movements contribute mostly to its stimulatory actions on the human uterus. It may be that there would be differences between labouring and non-labouring tissues[6,29], but this could not be addressed in the current study.

CONCLUSION

Our data leads us to conclude that inhibition of sarcoplasmic reticulum function in intact myometrium from pregnant, but non-labouring women, does not decrease Ca^{2+} or force. Indeed when the sarcoplasmic reticulum is emptied via cyclopiazonic acid, both are increased, indicating that under normal conditions the sarcoplasmic reticulum is functioning to limit the Ca^{2+} and contraction. This may be partly due to the active uptake of Ca^{2+}, partly due to stimulation of K_{ca} channels and perhaps by some additional route, such as store-operated Ca^{2+} uptake. Although the sarcoplasmic reticulum has ryanodine receptors, we can find no functional role for them in this preparation. Finally oxytocin promotes force in the uterus by a variety of mechanisms which may include a small contribution from Ca^{2+} released by inositol trisphosphate from the sarcoplasmic reticulum, but which is predominantly on Ca^{2+} entry.

Acknowledgements

The authors would like to thank the MRC for supporting this work and the Royal Thai Government for support to SK.

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Accepted 20 July 2001

Copies of Ethics Letter and Consent Form

faxed 4/12/97

Your Ref:

Our Ref:

LIVERPOOL RESEARCH ETHICS COMMITTEE

4 December 1997

Our Ref: AAG/PM/97/190

Extension: 2097
Fax: 236 4493

Dr M Luckas
Liverpool Women's Hospital
Crown Street
Liverpool L8 7SS

Dear Dr Luckas

THE ROLE OF INTRACELLULAR IONS (ESPECIALLY CALCIUM AND MAGNESIUM) IN EXCITATION-CONTRACTION COUPLING IN HUMAN MYOMETRIUM

The above study was discussed at a meeting of the Liverpool Research Ethics Committee on 3 December 1997 and we are pleased to inform you that no ethical objection was found.

We remind you that the Trust or appropriate Health Service Authority must be asked for permission for the study to proceed. Please contact Mr D Richmond, Medical Director and Professor C Gosden, Chair -Research Sub-Committee, Liverpool Women's Hospital, Crown Street, Liverpool L8 7SS.

Any proposed amendments to this protocol must be notified to the Liverpool Research Ethics Committee for approval before implementation

Yours sincerely

P. Malone

Dr A A Gilbertson
Chairman
Liverpool Research Ethics Committee

Patient information sheet for myometrial biopsies.

It is becoming increasingly common to give birth by caesarean section. One of the most common reasons is because the womb does not contract properly during labour. Also one of the biggest threats to babies is being born prematurely as a result of the womb contracting too early.
Little is known about how the muscle of the womb contracts. A study is being done at Liverpool University by Dr Murray Luckas who is a Senior Registrar at the Liverpool Womens Hospital to try and understand better what produces these contractions. It is hoped that this will eventually lead to a better understanding of labour and to hopefully be able to help women in premature labour and those whose wombs do not contract properly.
We are asking for permission to take a small piece of the muscle from your womb during your caesarean section. This piece will be about 1cm x 0.5cm and is known as a biopsy. This will be done after the baby is born and will not make any difference to how the operation is done or how long it takes. These biopsies have been done previously on about 400 women with no complications.
The information obtained will not be used to change your care but hopefully will be able to improve things for women and their babies in the future.
If you have any problems do not hesitate to speak to Dr Luckas.

You are under no obligation to take part in this study and if you agree to do so, you can withdraw at any stage. Declining to take part or withdrawing will not affect your treatment in any way.

Dr Murray Luckas

Professor Susan Wray

Patient Consent form for myometrial biopsy

The patient should complete this sheet herself.

Have you read the patient information sheet? yes/no

Have you had an opportunity to ask questions & discuss the study? yes/no

Have you received satisfactory answers to all your answers? yes/no

Have you received enough information about this study? yes/no

Who have you spoken to?

Do you understand that you are free to withdraw from the study:

at any time
without having to give a reason for withdrawing
and without affecting your future medical care yes/no

I..... .....agree to take part in this study.

Signed Date

NAME IN BLOCK LETTERS

Your Ref:

LIVERPOOL RESEARCH ETHICS COMMITTEE

LIVERPOOL HEALTH AUTHORITY

Direct 12 June 2000

Our Ref: 97/190

Tel: 0151 285 2097
Fax: 0151 236 4493
E-mail: pauline.malone@gww.liverpool-ha.nwest.nhs.uk

Dr D Roberts
Department of Obstetrics & Gynaecology
First Floor
Liverpool Women's Hospital
Crown Street
Liverpool L8 7SS

Dear Dr Roberts

THE ROLE OF INTRACELLULAR IONS (ESPECIALLY CALCIUM AND MAGNESIUM) IN EXCITATION-CONTRACTION COUPLING IN HUMAN MYOMETRIUM

Thank you for your fax received on 9 June 2000 with which you enclosed a revised Patient Information Sheet and Consent Form for the above study. We understand that Dr Luckas is no longer an investigator.

I am pleased to inform you that I am able to grant approval by Chairman's Action. My decision will require ratification at our Committee Meeting on 5 July 2000. You will only receive further correspondence if ratification is not given.

Yours sincerely

P. Malone

Dr E J Tunn
Chairman
Liverpool Research Ethics Committee

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Patient information sheet for myometrial biopsies

It is becoming increasingly common to give birth by caesarean section. One of the most common reasons is because the womb does not contract properly during labour. Also one of the biggest threats to babies is being born prematurely as a result of the womb contracting too early.

Little is known about how the muscle of the womb contracts. A study is being conducted at Liverpool University to try and understand what produces these contractions. It is hoped that this will eventually lead to a better understanding of labour and help women in premature labour or with poorly contracting wombs.

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The information obtained will not be used to change your care but hopefully improve things for women and their babies in the future. Dr Devender Roberts is the doctor in charge of collecting the biopsies once they are performed and should you have any problems you are more than welcome to contact her on 708 9988

You are under no obligation to take part in the study and if you agree to do so, you can withdraw at any stage. Declining to take part or withdrawing will not affect your treatment in any way.

Dr Devender Roberts
Professor Susan Wray

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L8 7SS

Telephone: 0151 702 4100/4101
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Centre Number:

Study Number:

Pateinet Information Number fot this trial:

CONSENT FORM

Title of project: The role of intracellular ions (especially Calcium and Magnesium) in excitation-contraction coupling in the human myometrium

Name of researcher: Sajeera Kupit/Susan Wray

Patient addressograph

Please initial box

- **I confirm that I have read and understood the information sheet dated June 2000 (version 2) for the above study** ☐

- **I understand that my participation is entirely voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected** ☐

- **I understand that sections of any of my medical notes may be looked at by responsible individuals from University of Liverpool or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my rceords** ☐

- **I agree to take part in the above study** ☐

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

THE UNIVERSITY
of LIVERPOOL

J P Neilson, BSc, MD, FRCOG
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September 2001 (Version 3)

PATIENT INFORMATION SHEET FOR MYOMETRIAL BIOPSIES

It is becoming increasingly common to give birth by caesarean section. One of the most common reasons is because the womb does not contract properly during labour. Also, one of the biggest threats to babies is being born prematurely as a result of the womb contracting too early.

Little is known about how the muscle of the womb contracts. A study is being conducted at Liverpool University to try and understand what produces these contractions. It is hoped that this will eventually lead to a better understanding of labour and help women in premature labour or with poorly contracting wombs.

We are seeking for permission to take a small piece of muscle from your womb during your caesarean section. This piece is very small (about 1cm x 0.5cm) and is known as a biopsy. This will be done after the baby is born and will not make any difference to how the operation is done or how long it takes. These biopsies have previously been done on 400 women with no complications.

The information obtained will not be used to change your care but hopefully improve things for women and their babies in the future. Dr. Siobhan Quenby is the doctor in charge of collecting these biopsies once they are performed and should you have any problems you are more than welcome to contact her on 708 9988.

You are under no obligation to take part in the study and if you agree to do so, you can withdraw at any stage. Declining to take part or withdrawing will not affect your treatment in any way.

Dr. Siobhan Quenby
Professor Susan Wray

Cheshire and Merseyside NHS
Health Authority

LIVERPOOL RESEARCH ETHICS COMMITTEE
Chair: Dr T S Purewal, BSc., MD, FRCP
Vice Chair: Professor S Frostick, MA, DM, FRCS

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Laurie Lomax (Mr), Administrator, Liverpool LREC
Tel: 0151 285 2097, Fax: 0151 236 4493
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14 January 2003

Our Ref: 97/190

Dr S J Pierce
Physiological Laboratory
University of Liverpool
Crown Street
L69 3BX

Dear Dr Pierce

Re: THE ROLE OF INTRACELLULAR IONS (ESPECIALLY CALCIUM AND MAGNESIUM) IN EXCITATION-CONTRACTION COUPLING IN HUMAN MYOMETRIUM

Thank you for your letter dated 6 January 2003, requesting approval for amendments to the above study.

I confirm receipt of the following documents:

Letter informing change of lead clinician
Revised Patient Information Sheet version 4, dated January 2003

I am pleased to inform you that I find no ethical objection to the above document, as detailed in your letter. My decision, taken by Chairman's Action, will require ratification at our Committee Meeting on 5 February 2003. You will only receive further correspondence if ratification is not given.

Yours sincerely

Dr T S Purewal
Chairman
Liverpool Research Ethics Committee

THE UNIVERSITY
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Facsimile: 0151 702 4024

January 2003 (Version 4)

PATIENT INFORMATION SHEET FOR MYOMETRIAL BIOPSIES

It is becoming increasingly common to give birth by caesarean section. One of the most common reasons is because the womb does not contract properly during labour. Also, one of the biggest threats to babies is being born prematurely as a result of the womb contracting too early.

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The information obtained will not be used to change your care but hopefully improve things for women and their babies in the future. Dr. Siobhan Quenby is the doctor in charge of collecting these biopsies once they are performed and should you have any problems you are more than welcome to contact her on 708 9988.

You are under no obligation to take part in the study and if you agree to do so, you can withdraw at any stage. Declining to take part or withdrawing will not affect your treatment in any way.

Dr. Siobhan Quenby
Professor Susan Wray

THE UNIVERSITY
of LIVERPOOL

Place patient identification label here

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Facsimile: 0151 702 4024

CONSENT FORM

Project Title: The role of intracellular ions (especially Calcium and Magnesium) in excitation contraction coupling in the human myometrium.

Name of Researchers: _____

Please initial box

1. I confirm that I have read and under stand the information sheet dated ..... for the above study ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study ☐

Name of Patient	**Date**	**Signature**
Name of Person taking consent (if different from researcher)	**Date**	**Signature**
Researcher	**Date**	**Signature**

(top copy for patient, pink copy for research, blue copy to be kept with hospital notes)