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The effect of lactate on myometrial contractility

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by

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List of abbreviations

Ab	Antibody
ANOVA	Analysis of Variance
ATP	Adenosine-5'-triphosphate
AUC	Area under the curve
BK_{Ca}	Large conductance calcium-activated potassium channel
but	Sodium butyrate
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
Carboxy-SNARF AM	Carboxy-SNARF acetoxymethyl ester
CICR	Calcium induced calcium release
Cl^-	Chloride ions
CS	Caesarean section
DMSO	Dimethyl sulphoxide
EC_{50}	Half maximal effective concentration
ECL	Enhanced chemiluminescence
HBSS	Hanks balanced salt solution
High K^+	40mM potassium chloride
ICC	Interstitial cells of Cajal
Indo-1 AM	Indo-1 acetoxymethyl ester
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
K^+	Potassium ions
K_{ATP}	ATP-sensitive potassium channel
KCl	Potassium solution

kDa	Kilo daltons
Kir	Inward-rectifier potassium channels
Km	Michaelis constant
Kv	Voltage-gated potassium channel
lac	Sodium lactate
M	Moles
MCT	Monocarboxylate transporter
mg	Milligrams
Mg^{2+}	Magnesium ions
$Mg_2SO_47H_20$	Magnesium sulphate
MgADP	Magnesium adenosine-5'-diphosphate
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRTB	Myometrial research tissue bank
mV	Millivolts
Na^+	Sodium ions
NCX	Sodium-calcium exchanger
NaCl	Sodium chloride
NHS	National Health Service
O_2	Oxygen
OT	Oxytocin
OTR	Oxytocin receptor
pH_i	Intracellular pH

pH_o	Extracellular pH
PIP_2	Phosphatidylinositol 2,5-biphosphate
pKa	Dissociation constant
PLC	Phospholipase C
PMCA	Plasma membrane Ca^{2+}-ATPase
PMT	Photomultiplier tube
prop	Sodium propionate
pryv	Sodium pyruvate
RCOG	Royal College of Obstetricians and Gynaecologists
RyR	Ryanodine receptors
SDS	Sodium doecyl sulphate
s.e.m.	Standard error of the mean
SERCA	Sarcoplasmic reticulum
SK_{Ca}	Small conductance calcium-activated potassium channels
SOCC	Store operated calcium channels
SOCE	Store operated calcium entry
SR	Sarcoplasmic reticulum
STOCs	Spontaneous transient outward currents
TBS	Tris buffered saline solution
TBS-T	Tris buffered saline solution with Tween-20
VOCC	Voltage operated calcium channel
WHO	World Health Organisation

The effect of lactate on myometrial contractility

Jacqui-Ann Hanley

Abstract

Strong coordinated uterine contractions are needed in labour and the mechanisms that govern this are well understood. However when these contractions are weak or uncoordinated, labour cannot progress normally. This is termed dysfunctional labour and it accounts for ~20% of all caesarean sections (CS) in the UK. Currently the only treatment available is oxytocin, however this does not reduce the incidence of CS. Lactate is significantly increased in myometrial capillary blood during dystocia suggesting it may be impairing force production, but there are no functional data on the effect of lactate on myometrial contractility.

The aims of this work were to a) investigate the effect of lactate and its mechanism of action on myometrial contractility using both human myometrium and an animal model. The effect on spontaneous and oxytocin-stimulated contractions was also examined and b) investigate the presence of lactate transporters MCT-1 and MCT-4 in rat myometrium throughout gestation and in human myometrium was looked at.

Lactate, dose-dependently and significantly, decreased spontaneous contractility in rat and human myometrium. Weak acids butyrate, propionate, and pyruvate also significantly reduced contractions in a dose-dependent manner. The effects of lactate were reduced in the presence of oxytocin but not abolished. The effect on pregnant myometrium was greater than in non-pregnant, and was shown to increase towards term in the animal model. In labouring myometrium, there was little effect of lactate on contractility. Using the animal model to investigate lactate's mechanism in the myometrium, tissue strips were loaded with either Indo-1 AM (a Ca^{2+} sensitive indicator) or Carboxy SNARF-AM (a pH sensitive indicator). Lactate inhibited Ca^{2+} transients and had little effect on force when a Ca^{2+} channel agonist was present. Lactate decreased pH_i in a dose dependent manner and this drop in pH_i was reduced when the tissue was stimulated by oxytocin or in labour. Both MCT-1 and MCT-4 were found to be present in rat and human myometrium, with MCT-1 at a higher amount in pregnant myometrium.

From this work I have shown that lactate in the physiological range potently decreases spontaneous contractility in both rat and human myometrium. The effects of lactate were reduced in the presence of oxytocin or labour but still produced significant decreases and this may be due the difference in alteration of pH_i. Other weak acids produce similar effects to lactate suggesting its mechanism of action is not via lactate's role in metabolism. Lactate inhibited Ca^{2+} transients, which could be due to a fall of pH_i, as it has previously been demonstrated that intracellular acidification decreases Ca^{2+} current through L-type Ca^{2+} channels. The difference in MCT-1 and MCT-4 expression between non-pregnant and pregnant tissue may indicate a role in labour. I suggest that differences in myometrial lactate in women can lead to accumulation of extracellular lactate, which as we have shown, will reduce myometrial contractions and could therefore contribute to dysfunctional labour.

Chapter 1

Introduction

Chapter 1

Introduction

1.1 Uterus

1.1.1 Anatomy

The human uterus is a pear-shaped muscular organ, which is hollow and thick-walled. In nulliparous women of reproductive age the uterine wall is 1.5cm thick. It is small, 7.5cm long with a maximum diameter of 5cm. The non-pregnant uterus weighs around 30-40g but in pregnancy, it can increase to 1kg at term (Gray 2000). In its normal position the uterus bends anteriorly near its base. The broad ligament and three pairs of suspensory ligaments stabilise it in position and limits its range of movement.

There are two regions of the uterus - the body forming the upper two-thirds of the organ and the cervix, which is narrower and more cylindrical in shape.

The uterine body or the 'corpus' is the largest portion of the uterus. The fundus is the rounded portion of body and the body ends at a constriction known as 'isthmus' (Figure 1.1). The cervix is the inferior portion that extends from the isthmus to the vagina.

The uterus receives blood from uterine arteries, which arise from branches of the internal iliac arteries, and from ovarian arteries which arise from the abdominal aorta inferior to the renal arteries. The uterus is extensively interconnected so as to have a reliable blood flow.

The uterus is a myogenic organ. The smooth muscle is able to contract without input hormonal or nervous input (Wray 1993). The basic processes of excitation and contraction

reside in the smooth muscle cells (Wray et al. 2001). Hormones can affect contraction, they can be powerful modulators and are used clinical to induce and augment labour.

The uterus has a thick outer fibromuscular layer called the myometrium. There is a thin inner glandular layer called the endometrium. The perimetrium is the “layer of peritoneum that covers the uterus except at the sides where it extends to form the broad ligaments” (Gray 2000).

1.1.2 Rat uterus

The uterus of the Wistar rat ‘Rattus norvegicus’ is a duplex uterus which enables multiple offspring. The ovaries are located at the distal ends of the horns and are connected to the uterus by oviducts. Both horns lead to a shared vagina (Figure 1.2). The anatomy at the myometrial level is similar to human tissue.

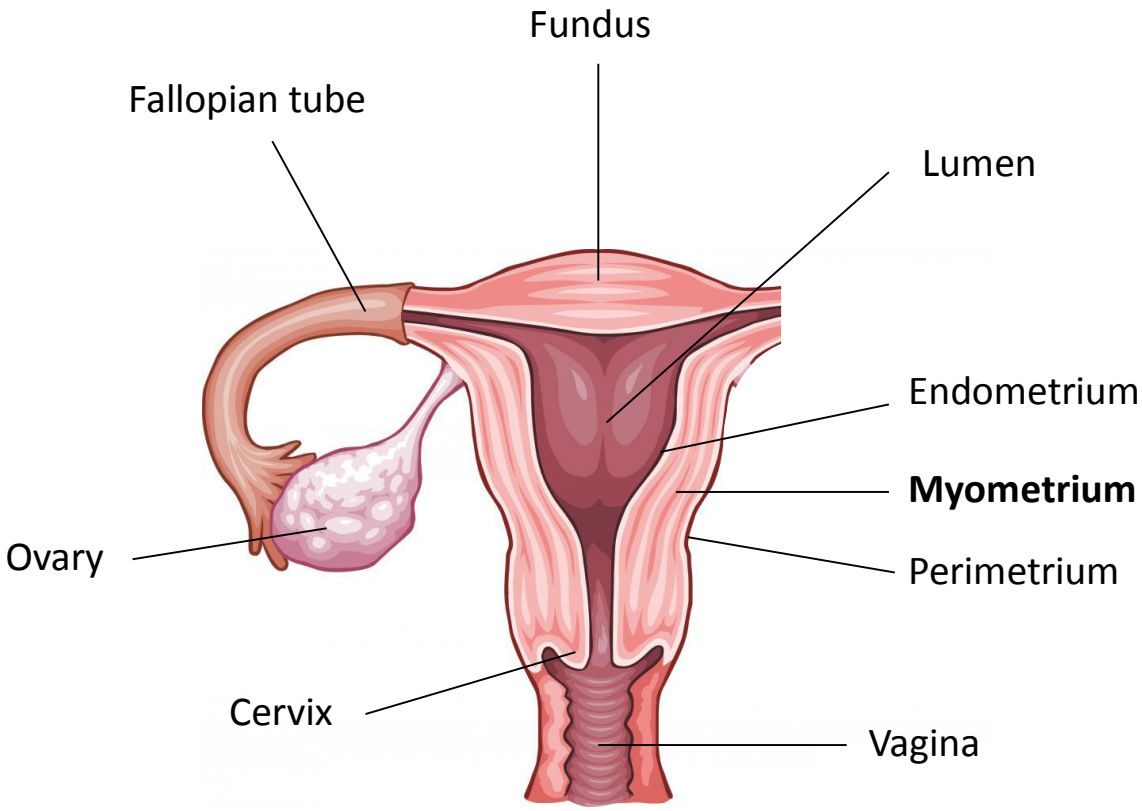


Figure 1.1 Anatomy of the human uterus

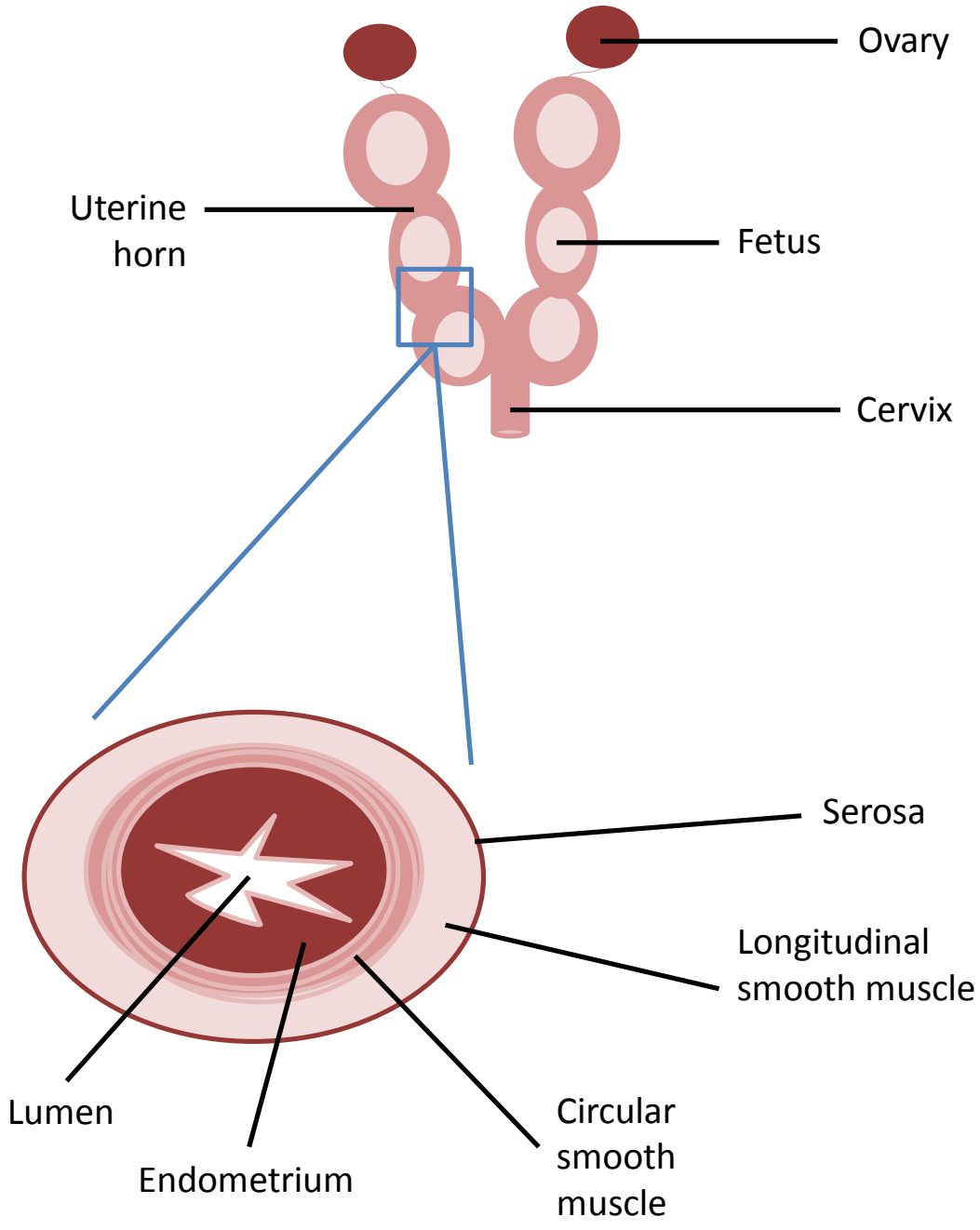


Figure 1.2 Anatomy of the rat uterus

1.2 Myometrium

1.2.1 Overview

The myometrium accounts for 90% of mass of uterus (the endometrium accounts for 10%). It is a smooth muscle with longitudinal, circular and oblique muscle layers in women, which provides force to expel the fetus out of uterus during labour.

The myometrium has 4 distinct layers:

1. Internal layer- ‘stratum submucosum’ composed mostly of longitudinal and some oblique smooth muscle. Oblique muscle runs ‘criss cross’ and constrict blood vessels when the uterus contracts.
2. External to that ‘stratum vasculare’ zone rich in blood vessels and longitudinal muscle.
3. ‘stratum supravasculare’ predominately circular muscle.
4. Thin longitudinal layer ‘stratum subserosum’ adjacent to perimetrium.

Contraction of the myometrium shortens the uterus and decreases the size of the lumen.

During pregnancy there is an increase in vascularity and tissue fluid of the myometrium, together with myometrial growth. Cell proliferation is the cause of the increased size from the nulliparous to the parous uterus. This increased growth is driven by a combination of mechanical stretching and oestrogen stimulation. The smooth muscle mass of myometrium increases throughout pregnancy by hypertrophy with some hyperplasia in early pregnancy.

1.2.2 Myometrial machinery

Myometrial cells are elongated and fusiform with tapering ends, which is typical of most visceral smooth muscle. Cells are varied in length and 5 to 10µm diameter. Up to 90% of their volume is filled with myofilaments, with the remaining space taken up by the nucleus, sarcoplasmic reticulum and mitochondria.

The myometrial force apparatus is made up of thick and thin filaments- called myosin and actin respectively (Gabella 1984).

Myosin filaments measure 15nm in diameter and are more heterogeneous in diameter and longer than that of myosin in striated muscle. Smooth muscle myosin is 470kDa in size, with two globular head groups joined by 150nm long tail. Associated with each head are 20 and 17kDa chains. The 20kDa chain is involved in calcium dependent regulation of contraction (see **Chapter 1.3.1**).

In the myometrium there are thin filaments, of which actin is a major component. Actin is circular and 7nm in diameter. It is organised into orderly packed bundles with a lattice-like appearance. Actin monomers organise as double-stranded helix to form filamentous actin.

Intermediate filaments are not directly involved in contractile processes. They form a cytoskeleton to support the myofilaments. These are composed mainly of desmin (Leoni, Carli & Halliday 1990).

The pattern of myofilaments in the myometrium does not constitute well-defined myofibrils and sarcomeres as found in striated muscle. Myosin and actin filaments do not run parallel to each other. There are also more thin than thick filaments found in smooth muscle compared to striated muscle (Cavaille 1985).

1.2.3 Resting membrane potential

Resting membrane potential in term pregnant human myometrium is around -40 to -70mV (Nakajima 1971) and this is similar in rat myometrium (Kuriyama & Suzuki 1976). The resting membrane potential becomes more negative towards mid-pregnancy (-60mV) and increases near term and parturition to -45 mV (Anderson, Kawarabayashi & Marshall 1981; Kuriyama & Suzuki 1976).

Depolarisation of the plasma membrane is caused by voltage and time dependant changes in membrane ion permeabilities. In smooth muscle there is an influx of Ca^{2+} which is caused by the activation of voltage-gated Ca^{2+} channels. This inward current occurs when the threshold of -40mV is reached and gives rise to the action potential. The time course for the action potential is highly variable and dependent on species, reproductive state and the region of measurement (Parkington & Coleman 1988). The peak of this upstroke is reached in 10ms and is followed by repolarisation. This is due to inactivation of voltage-, time- and calcium-activated channels and the activation of fast (voltage-gated) and slow (calcium-activated) K^+ channels, allowing the outward K^+ current (Figure 1.3).

Frequency, amplitude and duration of contractions are determined by the frequency of electrical bursts, number of cells active during bursts and the duration of bursts. Gap junctions provide the connection between cells to allow electrical coupling and increase conductance. They are composed of connexion proteins and grouping provides channels of low electrical resistance between cells. There channels are low in number throughout pregnancy, which decreases the coupling and conductance of cells. This favours quiescence.

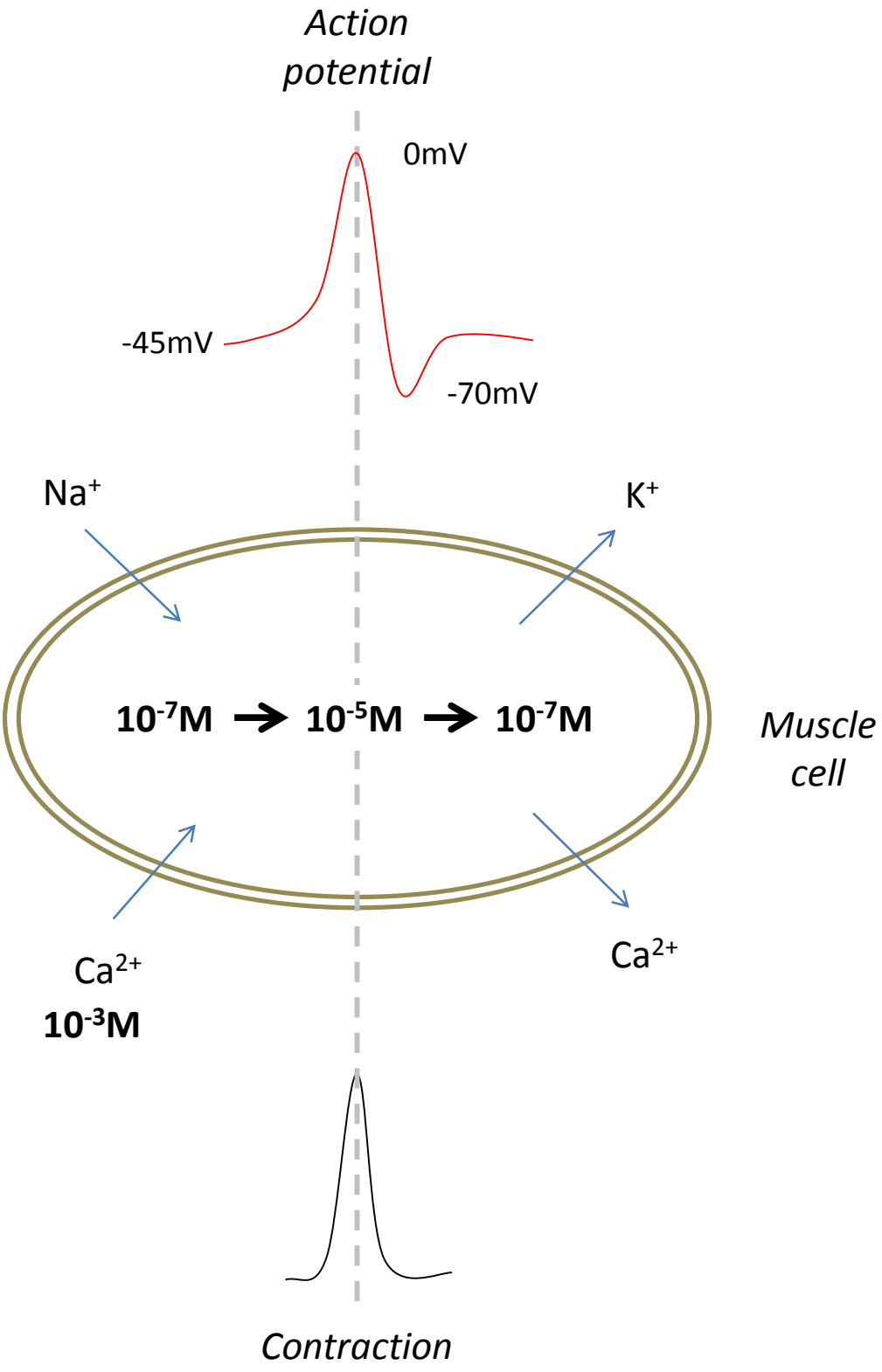


Figure 1.3 Membrane potential, Ca^{2+} flux and muscle contraction

However at term, junctions increase and allow the coordination of cells for contractions. Gap junctions are controlled by oestrogen and progesterone.

The myometrium is a spontaneously active and highly excitable muscle (*The Uterus* 1994). Pacemaker cells are thought to be responsible for the spontaneous initiation of activity. They are autonomously active. Action potentials arise in pacemaker cells and are transmitted over the whole organ. These cells are not localised to a specific region and it has been said that "all muscle cells are capable of becoming pacemakers" (*The Uterus* 1994). Although these cells have not been characterised in the myometrium, it is thought that they are similar to ICC cells as found in the gut (Duquette & Wray 2001). Stretching of the uterus has also been shown to induce activity (Lodge & Sproat 1981).

1.2.4 Ca^{2+} channels

The main function of Ca^{2+} channels in the myometrium is to provide a route of entry for Ca^{2+} down its concentration gradient into the myometrial cells for contraction. There are two types of calcium channel: Voltage-gated (VCCC) and ligand-gated channels. Voltage-gated channels divided into three main groups: Cav1 (L-type) Cav2 (P-N-and R-type) and Cav3 (T-type) (Ertel et al. 2000; Lipscombe 2002). L-type and T-type channels have been identified in the myometrium (Lee, Ahn & Lee 2009; Wray et al. 2003; Young, Smith & McLaren 1993). T-type currents have been recorded in human myometrium; they produce transient, low-voltage currents which are implicated in action potential initiation and modulation for spontaneous Ca^{2+} transient frequency (Lee, Ahn & Lee 2009).

-L-type Ca^{2+} channels

L-type Ca^{2+} channels are the main type of VCCC in the uterus. They are crucial for excitation-coupling (Lipscombe, Helton & Xu 2004). These channels produce high voltage, long lasting currents through Ca1.2 channels, which is the primary calcium entry route. L-type channels account for majority of calcium current measured in the myometrium (Shmygol et al. 2007) and main route for Ca^{2+} entry in the myometrium. Membrane depolarisation is key for activation of these channels. L-type Ca^{2+} channels are also subject to hormonal control. Auxiliary subunits allow modulation of these channels via protein-kinase A-,C- and G-mediated phosphorylation (Shmygol et al. 2007). The levels of these channels alter throughout gestation suggesting a role in parturition (Young, Smith & McLaren 1993).

1.2.5 K^+ channels

Potassium efflux from the myometrium results in membrane repolarisation and this is responsible for maintaining the resting membrane potential (Brainard, Korovkina & England 2007). They also contribute to uterine quiescence during pregnancy.

-Ca^{2+}-activated potassium channels

There are three subclasses of Ca^{2+}-activated potassium channels: large (BK) intermediate (IK) and small (SK) conductance channels (Noble et al. 2009). These channels are activated when $[Ca^{2+}]_i$ is elevated (BlatzMagleby1987). BKCa channels are the most extensively studied in the uterus due to their abundance and significant current (Brainard, Korovkina & England 2007). Their expression has been shown to decrease at the time of parturition indicating a role in the switch from quiescence to labour (Noble et al. 2009). However BKCa channels have little functional effect in rat uterus (Aaronson et al. 2006). Hormones can

regulate myometrial tone via these channels- sex hormones and hormones of the hypothalamus-pituitary-adrenal axis (Benkusky et al. 2002).

SK channels recently been shown to be expressed in myometrium (Noble et al. 2010). They have a similar structure to voltage gated potassium channels (Maylie et al. 2004) and contribute to outward current and regulate membrane potential. SK channels appear to contribute more to quiescence that BK channels.

-Voltage-gated K^+ channels

Voltage-gated K^+ (Kv) channels are widely expressed and also contribute to resting membrane potential in the myometrium. K^+ efflux occurs via these channels, in response to depolarisation which induces repolarisation of the membrane. Two types of Kv current have been identified in human myometrium: delayed rectifiers and rapidly inactivating current (Knock, Smirnov & Aaronson 1999; Wang et al. 1998).

The role of two pore domain K^+ channels is unresolved in the myometrium but they are thought to be involved in setting resting membrane potential similar to K_{ATP} and Kv channels. They are inward rectifying channels sensitive to pH, hypoxia and stretch (Brainard, Korovkina & England 2007). Isoforms of these channels have been identified in human pregnant myometrium (Bai et al. 2005) but not fully elucidated.

-K_{ATP} channels

ATP-sensitive inward rectifying K^+ channels (K_{ATP}) maintain basal membrane potential and contribute to uterine quiescence throughout pregnancy (Brainard, Korovkina & England 2007). They are expressed at high levels in non-pregnant myometrium and are down regulated in late pregnancy which facilitates myometrial activity necessary for labour

(Curley et al. 2002). K_{ATP} channels are inhibited by intracellular ATP and stimulated by MgADP, coupling cell excitability to metabolic state (Dunne & Petersen 1986a, 1986b).

Lactate will rise during metabolic stress and during hypoxic conditions and intracellular ATP will decrease. This will increase the opening of these channels and lead to reduced uterine activity (due to change in membrane potential).

1.3 Myometrial force production

1.3.1 Contraction via MLCK, calcium influx

To initiate a contraction, calcium needs to enter the cell. This is down its concentration gradient: from outside (10^{-3}M) to inside (10^{-7}M) the cell. $[Ca^{2+}]_i$ must reach 10^{-6}M to initiate a contraction. Calcium can also be released from the sarcoplasmic reticulum (SR) via reversal of SERCA pump or release via IP_3 receptors. Calcium can also enter the cell when a hormone e.g. oxytocin binds to its receptor **(Chapter 1.4.3)**.

Calcium inside the cell is bound by calmodulin (Figure 1.4). This complex activates myosin light chain kinase (MLCK) by binding to its binding site. This induces a conformational shift on inactive MLCK whereby the auto regulatory inhibitory sequence of MLCK moves away from the catalytic site activating its enzymatic activity (Olson et al. 1990). MLCK is a serine/threonine kinase that phosphorylates myosin regulatory light chains (MLC20) at serine-19 (and threonine-18 at high-agonist concentrations). This initiates actin-myosin ATPase on myosin heavy chains generating mechanical energy for contraction (Kamm et al. 1989).

1.3.2 Relaxation via MLCP, calcium efflux

Relaxation of the myometrium is achieved when $[Ca^{2+}]_i$ is reduced inside the cell. Removal occurs via the plasma membrane or sarcoplasmic reticulum. On the plasma membrane, there are two channels which operate to remove Ca^{2+}: the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX). The NCX extrudes one Ca^{2+} for three Na^+ ions. It uses the energy stored in the electrochemical gradient of Na^+: Na^+ flows down its concentration

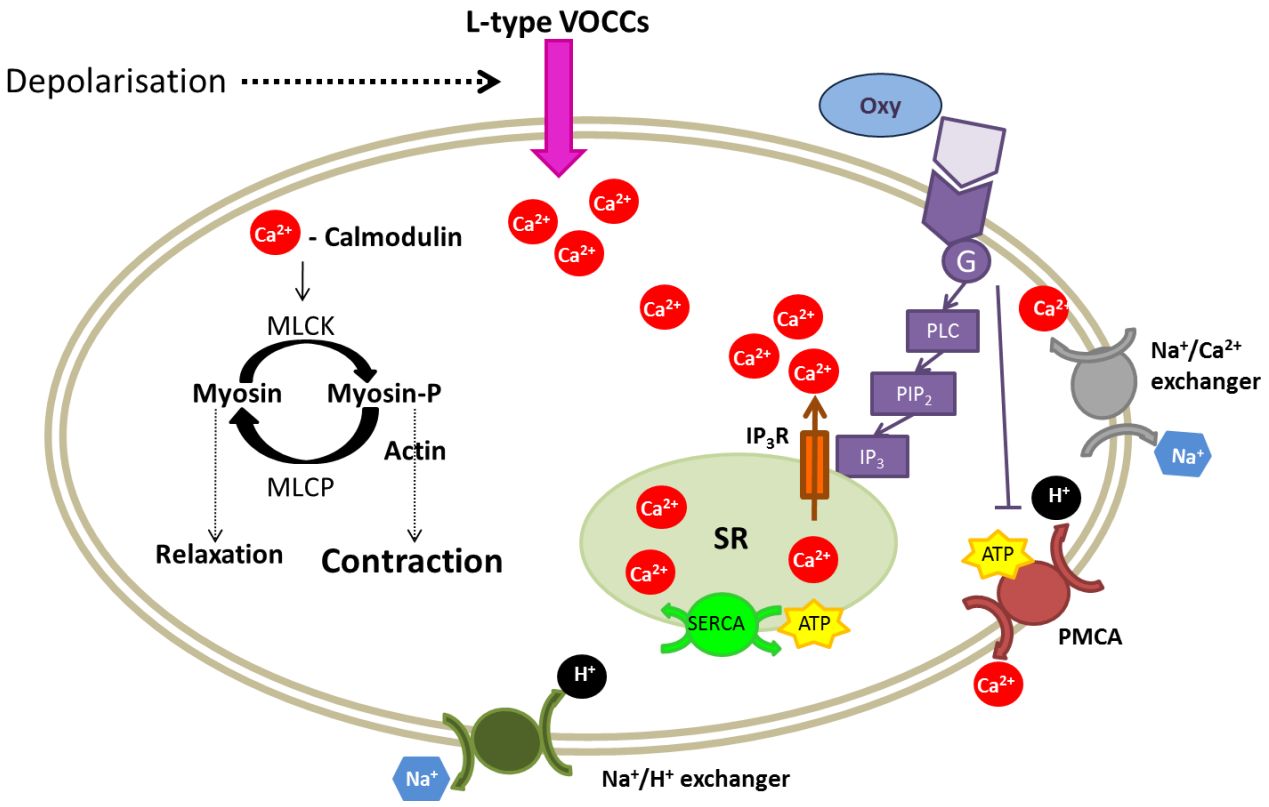


Figure 1.4 Excitation-contraction coupling in the myometrium

gradient and counter transports Ca^{2+}. The gradient is maintained by the Na^{+}/K^{+} ATPase. This can be reversed if the gradient is reversed i.e. move Ca^{2+} into the cell. PMCA is electron neutral as Ca^{2+} pumped out is exchanged for two protons (Sanders 2001). PMCAs are activated by calmodulin binding, which removes auto-inhibition and increases its affinity for Ca^{2+} (Marin et al. 1999). Both PMCA and NCX make a significant contribution to Ca^{2+} efflux in the myometrium- 70% and 30% respectively (Matthew, Shmygol & Wray 2004).

L-type Ca^{2+} channels close when the membrane repolarises. This is caused by K^{+} channels opening by the action potential. Activity of the Na^{+}/K^{+} ATPase produces a negative charge: 3 Na^{+} leave the cell and 2 K^{+} enter it and this also produces a direct effect on excitability.

Dephosphorylation of MLC20 occurs by myosin light chain phosphatase (MLCP). This phosphatase can also be activated by small G-protein coupled protein kinase cascade (Kimura et al. 1996).

1.3.3 Sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is the internal Ca^{2+} store in all smooth muscle cells. It has a Ca^{2+}-ATPase (SERCA) that takes up Ca^{2+} from the cytoplasm to the lumen of the SR against its concentration gradient (at the expense of ATP). Expression of the pump is increased in labouring myometrium versus non-labouring indicating increase Ca^{2+} accumulation takes place in labouring myometrium (Tribe, Moriarty & Poston 2000). Within the organelle, calsequestrin and calreticulin proteins bind the Ca^{2+}.

Calcium is released from the SR via Ca^{2+} channels in the membrane. Agonists, such as oxytocin, act to increase $[Ca^{2+}]_i$ by generating intracellular second messengers, such as IP_3 which activate these channels. Two types of release channels have so far been identified:

inositol 1,4,5-triphosphate (IP_3) receptors and ryanodine receptors (RyR). It has been demonstrated that RyR are non-functional in the myometrium (Noble et al. 2009).

Store-operated Ca^{2+} entry (SOCE) is defined as 'Ca^{2+} influx through specific store-operated Ca^{2+} channels as a result of SR Ca^{2+} depletion' (Noble et al. 2009). It has recently been established that STIM1, a Ca^{2+} sensor and Orai1, a pore-forming subunit of the SOC channel are involved. However store-operated Ca^{2+} currents have not been reported in the myometrium.

1.3.4 Modulation of force

- Hormonal

Oxytocin binds to its receptor (OTR) on the plasma membrane. OTR is a G-protein coupled receptor (GPCR) coupled to several G proteins including Gαq. This activates phospholipase C (PLC). This hydrolyses plasma membrane phosphoinositide biphosphate (PIP_2) to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to receptors on sarcoplasmic reticulum stimulating release of calcium and therefore increase intracellular calcium concentration. DAG activates PKC. Both OTR and PKC activate the MAPK cascade which leads to prostaglandin production and therefore enhanced contractile activity. Other hormones signal through receptor tyrosine kinases, activating PLCγ generating the same intermediates (Taggart & Morgan 2007).

- Calcium sensitization

Force can be promoted by Ca^{2+}-sensitising agents that act to increase myosin activity in constant $[Ca^{2+}]_i$. These agents act to modulate either MLCP or MLCK. They inhibit MLCP by

two mechanisms: RhoKinase phosphorylation of MLCP and inhibition by CPI-17 (Ito et al. 2004). Activation of Rho, a small G protein, activates Rho-kinase (ROCK) which activates myosin by direct phosphorylation or through inhibition of MLCP. It does this by phosphorylating the subunit on MLCP. Inhibition of this cascade has only a small effect on force (Kupittayanant, Burdyga & Wray 2001). Protein kinase C-potentiated inhibitor protein of 17 kDa (CPI-17) when phosphorylated by PKC, inhibits MLCP by binding to its catalytic subunit (Somlyo & Somlyo 2000).

1.3.5 Buffering capacity/pH in uterus

Buffering systems help to maintain pH within physiological range. It is important to maintain pH stability as fluctuations in $[H^+]$ are dangerous to cells. Rises in $[H^+]$ may disrupt cellular function and affect enzyme activity. The mean resting value of pH_i in pregnant rat myometrium is 7.18 (± 0.04). Human myometrial tissue has similar pH values; pH_i in non-pregnant myometrium is 7.06 which rises to 7.14 in pregnant myometrium (Parratt, Taggart & Wray 1995a, 1995b).

There are three important buffering systems in the uterus: bicarbonate, phosphate and protein. The function of these systems is to neutralise H^+ produced by metabolism to prevent alterations in function. Proteins can accept or donate protons due to their structure (*Acid-base balance* 1986). Inorganic phosphate is an effective buffer at physiological pH. Monohydrogen phosphate (HPO_4^{2-}) can bind H^+ to form dihydrogen phosphate ($H_2PO_4^-$).

$$H^+ + HPO_4^{2-} \rightleftarrows H_2PO_4^-$$

In the bicarbonate buffering system, carbon dioxide (CO_2) combines with water (H_20) to form carbonic acid (H_2CO_3). This dissociates to bicarbonate (HCO_3^-) and protons (H^+). When $[H^+]$ is increased this reaction is shifted; excess H^+ will bind to HCO_3^- to form H_2CO_3 then CO_2 and H_20. This acts to reduce acidity (*Acid-base balance* 1986).

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+$$

Small transient acidifications (0.04 pH unit) have been seen to follow spontaneous uterine contractions (Taggart & Wray 1993b). This has been attributed to a reduction in blood flow and therefore a build-up of H^+. Alkalinisation occurs within the uterus within last few weeks of pregnancy (Parratt, Taggart & Wray 1995a).

If an acid or base is added to contracting myometrium it results in a change in contractility (Parratt, Taggart & Wray 1995b; Pierce et al. 2003). These solutions initially cause a pH_o change which has been demonstrated to affect the myometrium via pH_i changes (Pierce et al. 2003). Acidification decreases whilst alkalinisation increases force and $[Ca^{2+}]_i$ both in human myometrium (Parratt, Taggart & Wray 1995b) and rat myometrium (Taggart & Wray 1993a). It has also been demonstrated that acidification acts at the level of cross bridge cycling; H^+ and Ca^{2+} compete for binding of the cross bridges (Pierce et al. 2003).

The myometrial capillary of women in labour has been shown to be high (7.49), consistent with alkalinisation of the uterus (Quenby et al. 2004). Women experiencing a dysfunctional labour had a decreased blood pH of 7.35. In the same study, it was demonstrated that, in vitro, reduce the extracellular pH to 7.3 reduced amplitude and frequency of contractions.

1.4 Lactate

1.4.1 Production

Lactic acid is a carboxylic acid with the formula $C_3H_60_3$. At physiological pH, it is dissociated into a lactate anion and hydrogen ion. Lactic acid is a weak monocarboxylic acid with a pKa 3.86. Lactic acid is a chiral molecule and has two optical isomers: L-(+)-lactic acid or D-(-)-lactic acid.

Aerobic respiration is the oxidation of glucose in sufficient oxygen supply (Figure 1.5). In the first stage- glycolysis- glucose is converted to two 3-carbon molecules of pyruvate. 2 molecules of ATP are consumed to initiate the process and 4 molecules of ATP and 2 molecules of NADH are produced. Acetyl coenzyme A is formed when each pyruvate molecule is oxidised to carbon dioxide and a 2-carbon acetyl group. This binds to coenzyme A and enters the mitochondria. Carbon dioxide is released as a waste product. In the citric acid cycle the 2-carbon acetyl groups gradually become oxidised. The protons are bound to NAD and FAD to form NADH and $FADH_2$ respectively. 2 molecules of ATP are synthesised for each glucose molecule (i.e. two pyruvate molecules) entering the mitochondria. In the electron transport chain (ETC) the electrons produced in the previous steps are pumped across the inner membrane of the mitochondria. They flow through ATP synthase enzyme molecules and release energy. 34 ATP molecules are formed from this step. In total 36 molecules of ATP are synthesised.

The first step in this respiration process in anaerobic. Oxygen is not used in any of the steps. However oxygen needs to be present for citric acid cycle in the mitochondria to continue. When there is little/no oxygen, pyruvate oxidation is impaired and is instead reduced by lactate dehydrogenase (LDH) to lactate (Figure 1.6). This step involves oxidation of NADH to

NAD^+. NADH generated in the initial steps of glycolysis which is re-oxidised into NAD^+. Anaerobic respiration only produces 2 ATP molecules compared to 36 ATP in aerobic respiration.

Glycolysis in the cytoplasm

Citric acid cycle in the mitochondria

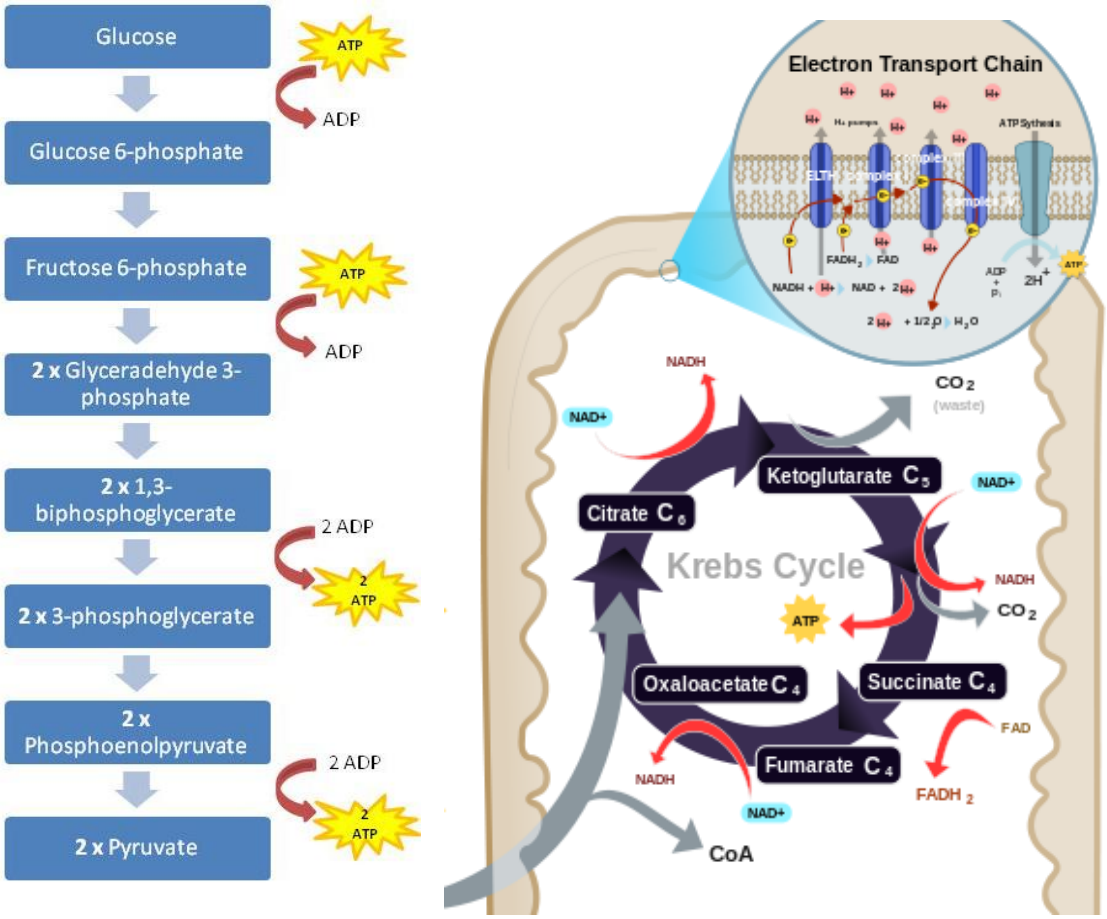


Figure 1.5 Aerobic respiration

Including all four stages of respiration : glycolysis, acetyl coenzyme A formation, citric acid cycle and the electron transport chain.

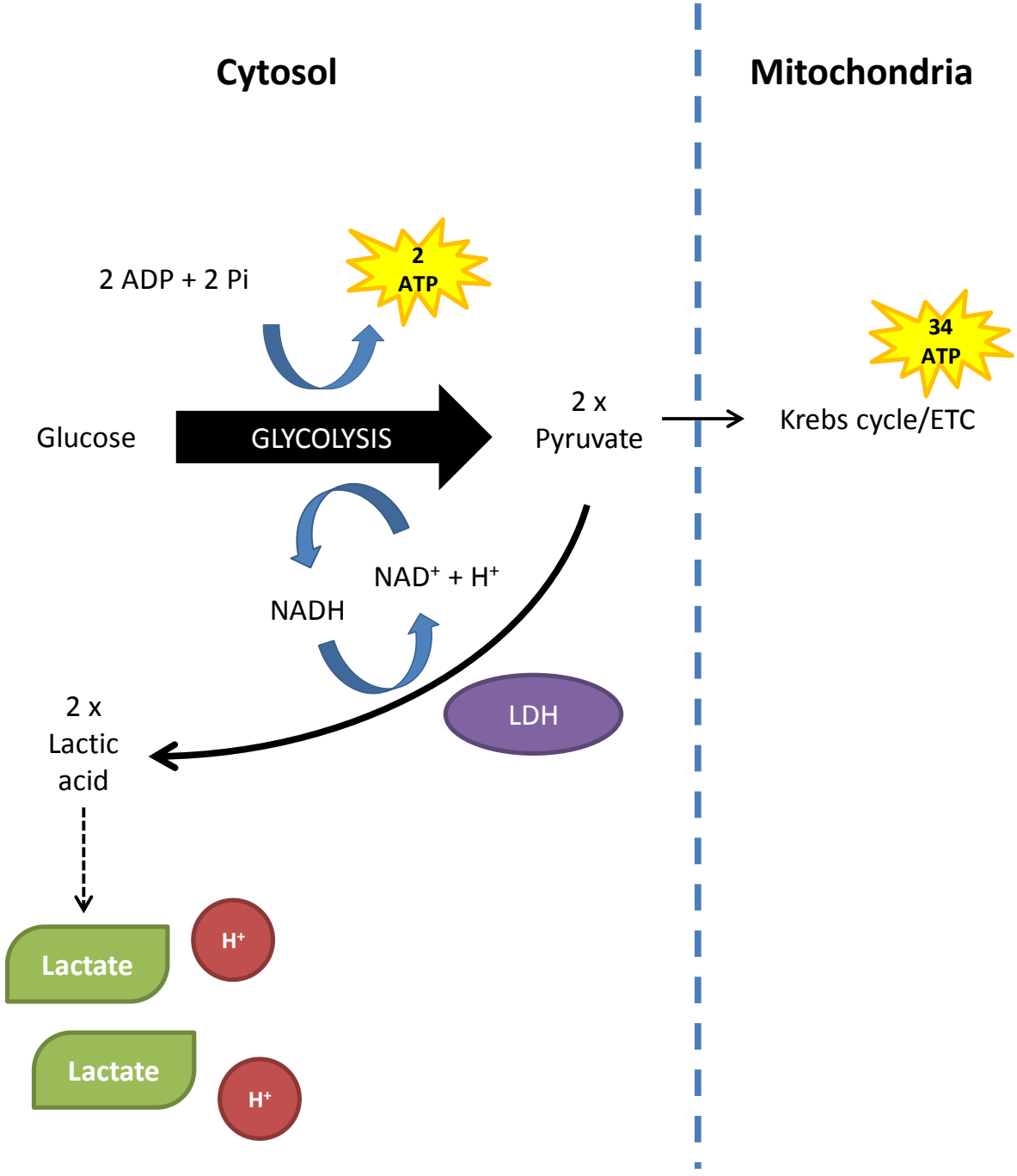


Figure 1.6 Lactate production in anaerobic respiration

1.4.2 Use of lactate and breakdown

Lactate is transported via the blood to the liver where it is involved in the Cori cycle. Lactate is oxidised by LDH to pyruvate and glucose is formed in gluconeogenesis. Glucose is released back into the blood where it can travel to muscle cells and be utilised in respiration.

Lactate was originally thought of as a waste product of anaerobic respiration due to its presence in hypoxic conditions and was thought to be the primary cause of fatigue. However in the last decade, Brooks has changed the role of lactate in metabolism. His group determined it is helpful during exercise when oxygen levels are low.

The idea of cell to cell lactate shuttle was introduced by Brooks in 1985. Skeletal muscle is thought to be a major component of this shuttle due to its size and capacity. It is able to produce lactate as well as uptake and utilise it. In normal conditions, lactate is released into the blood. During short-term high-intensity exercise, lactate is rapidly produced and its clearance from muscle is slow. During recovery or long periods of exercise, there is a net uptake of lactate from the blood by resting muscles. Muscles that originally released lactate may uptake it. Lactate from glycolytic fibres can diffuse to oxidative fibres and lactate can be oxidised for respiration. Cardiac muscle is highly oxidative and a high lactate consumer (Brooks et al. 2006). As oxygen volume increases lactate becomes preferred fuel for the heart. 60% of substrate is utilised (Stanley 1991). In smooth muscle, there is limited evidence for this. Smooth muscle is not highly oxidative therefore it is unlikely this mechanism is predominant (Steingrimsdottir et al. 1995).

The blood is the route for lactate to be transported other tissues. Lactate moves via monocarboxylate transporters (MCTs) although there is a small amount of diffusion of lactate not via transporters. Lactic acid can be up-taken by red blood cells in three ways

(Gladden 2004) including MCTs and this is the cells primary pathway. In plasma it is then taken up by RBC. Leaves plasma and enters interstitial fluid down concentration gradient.

1.4.3 Monocarboxylate Transporters

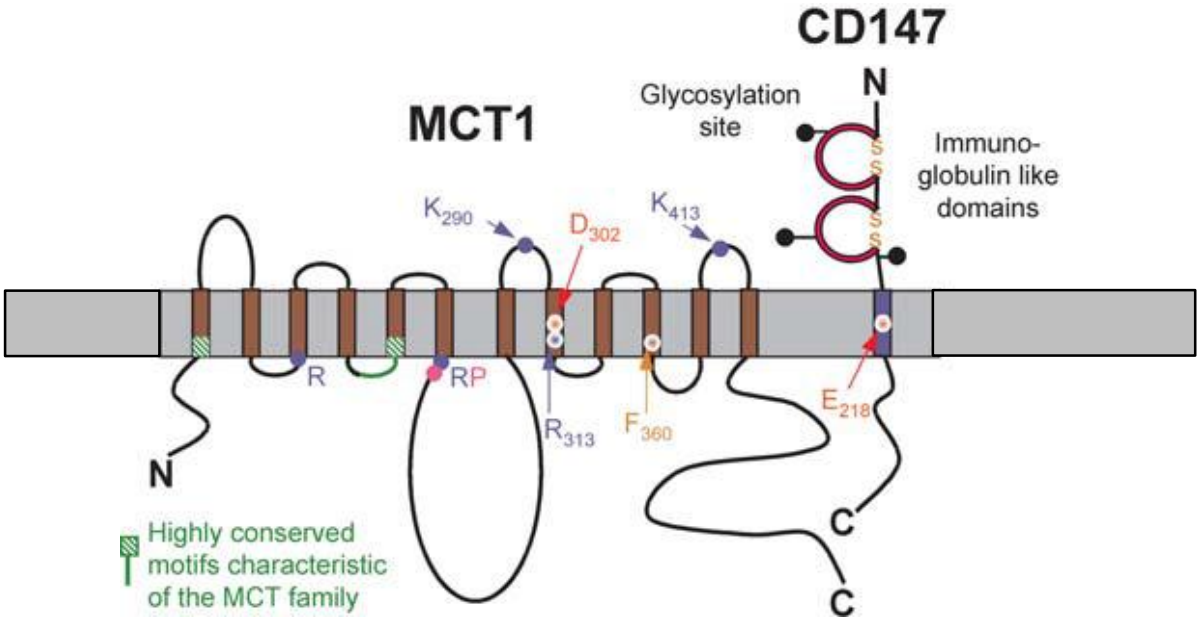
Monocarboxylate transporters (MCTs) are part of SLC 16 family (solute carrier) of proteins which has 14 members. Their structures are very similar and they share conserved sequence motifs. These transporters consist of 12 transmembrane (TM) helices with an intracellular N and C terminus (Figure 1.7). There is a large cytosolic loop between TM 6 and 7 (Halestrap & Meredith 2004).

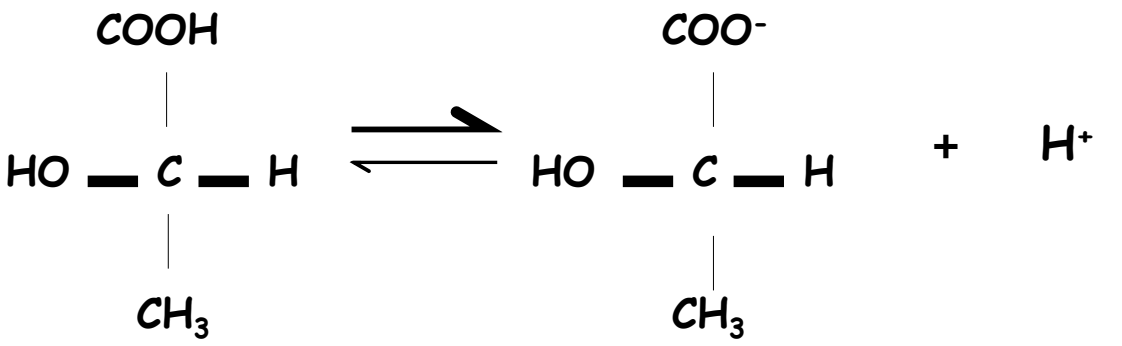
Only MCTs 1-4 have been shown to transport monocarboxylates, after which they were named; molecules such as L-lactate, pyruvate, ketone bodies (acetoacetate, beta hydroxybutrate). They are also confirmed to be proton-linked. Other members of the MCT family include MCT-8 (SLC16A2) a thyroid hormone transporter and MCT-10 (known as TAT-1 or SLC16A10) an aromatic amino acid transporter.

- **MCT-1**

This was the first MCT discovered and is found in majority of tissues. The transport of lactate and other monocarboxylates is UNIdirectional- it depends on the intracellular/extracellular concentration and pH gradient across the plasma membrane. MCT-1 can also exchange 1 monocarboxylate for another without net H+ transport (Deuticke 1982; Deuticke, Beyer & Forst 1982).

When transporting lactate across the plasma membrane, MCT-1 (and other MCTs) first binds H^+ (Km 0.2μM) followed by lactate anion (Km 3-5mM) on extracellular matrix side. There is a protein conformational change (to the closed formation) which translocates





L-(+)-lactic acid *L-(+)-lactate* *Proton*

Figure 1.7 Monocarboxylate transporter-1 and lactate

Adapted from Halestrap and Meredith 2004

the substrate across the membrane. This exposes lactate and H^+ to the opposite side of membrane. Lactate is released first then H^+. Finally returning to an open conformation without bound substance is a rate-limiting step. Transport via MCT can be stimulated by drop in pH in cytoplasm (which decreases Km for lactate, increasing affinity for MCT) or increased pH on extracellular side

It has been demonstrated that MCT-1 is up-regulated in response to intense exercise/training in skeletal muscle and in the heart in response to hypertrophy (Bickham et al. 2006; Bonen 2001).

- **MCT-4**

MCT-4 is widely expressed especially in glycolytic tissue such as white skeletal muscle fibres, white blood cells and astrocytes (Halestrap & Meredith 2004). It is therefore important in tissues that rely on glycolysis. For example, the adult heart has no MCT-4 present yet in the neonate heart, MCT-4 is predominant. This is due to the neonate heart being more glycolytic than the adult heart (Hatta et al. 2001). MCT-4 is strongly expressed in placenta where it exports lactic acid rapidly from fetal to maternal circulation. It has a lower affinity for substrate and inhibitors than MCT-1: the Km value for pyruvate = 150mM. This is to avoid pyruvate's loss from cell, which would prevent removal of NADH formed when pyruvate turned into lactate. If this were to occur, glycolysis would cease. The Km for lactate is 30mM.

MCT-4 is up-regulated in other cells during hypoxia (Perez de Heredia, Wood & Trayhurn 2010) and is adapted to efflux over influx of monocarboxylate/H^+.

- **Other MCTs**

There is lower expression of MCT-2 compared to MCT-1 however they are similar as they both have higher affinities for pyruvate over lactate. However the tissue distribution of these two transporters is different (Garcia et al. 1995; Jackson et al. 1997) and when they are expressed in the same tissue, their location within it is different. MCT-2 is strongly expressed in the liver (Halestrap & Wilson 2012). The capacity to transport lactate is lower in MCT-2 compared to MCT-1 (Broer et al. 1999).

MCT-3 is only found in retinal pigment epithelial cells (Halestrap 2013) and choroid plexus epithelia (Philp, Yoon & Lombardi 2001). It has a Km value for lactate of 6mM.

The concentration of lactate is ~3mMol-L in the myometrium. This would indicate that MCT-1 would be the most important lactate transporter in the uterus, if present.

- Ancillary proteins

Basigin (CD147) is a glycoprotein that acts as a chaperone for MCTs (Figure 1.7). It is widely expressed, unlike its closely related protein, Embigin, which is not found in many tissues. MCT-1 and MCT-4 co localise with Basigin as it is its preferred binding partner. However if there is no Basigin, MCT1 will bind to Embigin. In the same way, MCT-2 needs embigin but can also bind basigin. MCTs need these proteins to be expressed on the plasma membrane; otherwise they would accumulate in the Golgi apparatus. These glycoproteins have a single transmembrane domain with a short intracellular C terminus and large glycosylated extracellular domain.

1.4.4 Lactate in skeletal muscle and heart

Oxidative fibres use lactate as fuel and there is an increase in lactate during exercise. Lactate also transported into cells for gluconeogenesis (generation of glucose from non-carbohydrate molecules) and lipogenesis (glucose to lipids) – liver and kidney or oxidised in heart, skeletal muscle and brain.

In cardiac muscle, lactate increases cardiac cell excitability by increasing the maximal inward sodium current which shifts the voltage dependence of voltage- gated sodium channels (Na_v) towards hyperpolarised potentials (Guo, Wasserstrom & Rosenthal 1994; Tanaka et al. 1994). Lactate has also been shown to reduce chloride conductance, increasing excitability of rat skeletal muscle (de Paoli et al. 2010).

1.4.5 Lactate in the uterus and other smooth muscles

The myometrium produces lactate in both non-pregnant and pregnant states with myometrial contractions shown using ^{31}P NMR (Wray 1990). Lactate production was increased in the presence of cyanide, a metabolic inhibitor, which increased lactate efflux. Higher amounts of lactate produced in normoxic conditions when compared to other tissues due to a higher rate on anaerobic respiration (Shimizu et al. 2000).

In vascular smooth muscle, it has been shown that lactate can cause vasodilation and depressed responsiveness to agonists (Barron & Nair 2003) and it is suggested this is due to depressed Ca^{2+} permeability. Lactate has also been shown to induce arterial relaxation by the activation of Ca^{2+}-activated K^+ channels (Mori et al. 1998).

1.4.6 Lactate and pregnancy and parturition

Quenby *et al* recently examined lactate levels in myometrial capillary blood of women undergoing caesarean sections. Higher lactate was found in dysfunctionally labouring women: 2mmol/L in women contracting normally and this was raised to 3.5mmol/L in dysfunctionally labouring women (Quenby et al. 2004). This is also in conjunction with a lower blood pH.

Measurements of lactate in the amniotic fluid of women during parturition and is currently being used for dysfunctional labour predictor. Lactate levels in amniotic fluid are higher than myometrial capillary blood: 8.9mmol/L spontaneous vaginal delivery and 10.9mmol/L labour dystocia (Wiberg-Itzel et al. 2010).

1.5 Pregnancy and Labour

Normal pregnancy lasts around 39-41 weeks. Pre-term pregnancy is classed as delivery of the baby before 37 weeks and prolonged pregnancy is usually classed as beyond 41 weeks 3 days. Some define prolonged pregnancy as more than 42 weeks.

1.5.1 Onset of labour

The precise time and sequence of events preceding the onset of labour is unknown. The trigger for commencement and factors involved are still to be determined (Figure 1.8). The uterus is relaxed throughout pregnancy and becomes more contractile towards late pregnancy. A series of genes encoding contraction-associated proteins (CAPs) is suggested by Challis and Lye (Challis J 1994). This includes the gap junction protein connexion-43 and the oxytocin receptor. Oxytocin levels have been shown to remain the same (Fuchs et al. 1984). Progesterone is essential in promoting uterine quiescence throughout pregnancy. Functional progesterone withdrawal is thought to control onset of parturition; plasma levels of progesterone are maintained but the reduction of progesterone receptors lead to increased uterine activity (Shmygol et al. 2007).

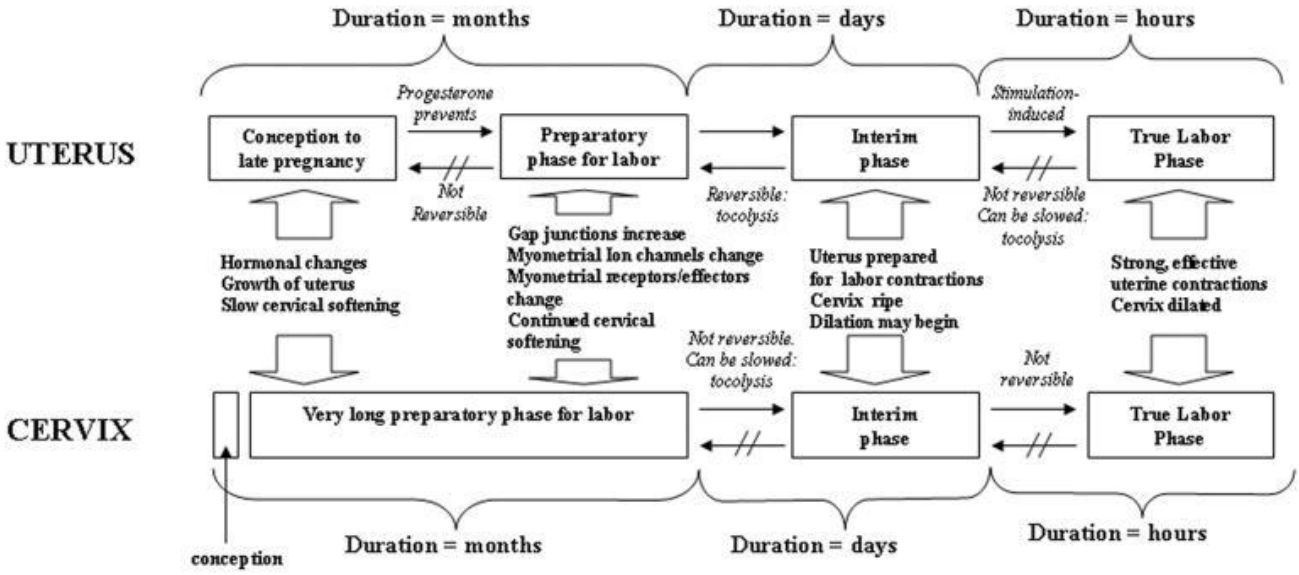


Figure 1.8 Adaptations during pregnancy

Adapted from Garfield and Maner 2007

1.5.2 Stages of labour

1. First stage- Onset of contractions and cervical dilation from 0 to 10 cm.
    i. Latent (up to 4cm)
    ii. Active (from 4-10cm)

    Cervix dilates rapidly, minimum 1cm per hour (Friedman 1955) normal rate of progress. Frequency and duration of contractions increase to become more powerful.
2. Second stage- expulsive contractions lead to the delivery of baby
    i. Passive

    Presenting part of the baby descends through pelvis till it reaches the muscle of the pelvic floor and there is a maternal urge to bear down.
    ii. Active

    Once the mother is pushing or 'bearing down', there is further expulsive forces leading to the complete delivery of infant.
3. Third stage- the time from the delivery of the baby to the delivery of placenta and membranes.

-Dysfunctional labour

- **Definition**. Dystocia defined as abnormal labour resulting from abnormal power, abnormal passage or abnormal passenger. Abnormal power from uterine contractions or maternal expulsive forces is the commonest cause of poor progress in labour. Dysfunctional labour is not diagnosed until the active phase of labour is achieved. Active labour is defined by a cervix opened to 3-4cm with an advanced thinning of the cervix (effacement).

The definition of dysfunctional labour is controversial. O'Driscoll in 1969 defined it as less than 1cm per hour. However recent NICE guidelines changed this to less than 0.5cm per hour ((NICE) 2008).

The following signs suggest that labour is not progressing as planned/expected: less than 3 contractions in 10 minutes lasting less than 40 seconds each, less than 0.5cm change in cervical dilation per hour for 2 hours in a row or no change in baby's descent into birth canal after 3 hours of pushing if the cervix is fully dilated (10 cm).

- Prolonged **latent** phase *NO LONGER THAN 8 HRS
    - Cervical dilation up to 4cm
    - Regular painful contractions
- Prolonged **active** phase *NO LONGER THAN 12 HRS
    - Cervical dilation more than 4cm
    - Regular painful contractions

- **Prevalence**

Up to 1/3 of women fail to achieve 'normal' contractions to deliver the fetus. Dysfunctional labour "is the second leading cause of Caesarean section rates, which in developed countries are around double the rate recommended by the World Health Organisation" (Pervolaraki 2013). It is associated with significant increased maternal mortality and morbidity. These labours account for 20% of all caesarean sections in England and Wales.

- **Treatment**

The administration of oxytocin is currently the only treatment for dysfunctional labour. However its use has been questioned as it has no significant effect on reducing caesarean

section rates (Bugg et al. 2006). The concentration and length of administration varies between hospitals, and high-dose regimes are associated with a reduction in labour duration, reduction in CS rates and increase in vaginal deliveries (Mori 2013). However high concentrations of oxytocin are not commonly used as it leads to sustained tonic contractions and fetal hypoxia (Bugg, Siddiqui & Thornton 2013). If oxytocin is unsuccessful in starting/maintain frequent contractions of sufficient power, a caesarean section is needed to deliver the baby safely. Additional risks are involved with surgery and with dysfunctional labour there is significant increase of morbidity and mortality for both mother and baby.

It has been suggested that increasing intravenous (IV) fluids during labour, to keep women adequately hydrated, may reduce the duration of labour as "adequate hydration and… supplemental glucose is required to maintain endurance and muscle efficiency during the process of labour and parturition" (Dawood 2013). It has also been postulated that administration of these fluids may reduce CS in women experiencing prolonged labours (Garite 2000). When compared to oral intake alone, the administration of IV fluids did decrease labour duration, however this did not significantly reduce the number of CS carried out (Dawood 2013).

Dysfunctional labour is an enormous health and economic issue that needs to be addressed. Scientific insight is needed into these labours to develop treatments which will eventually lead to a cure. Lactate appears to be increased in these labours, indicating it could be involved. However, there is no direct evidence of this as yet. My hypothesis is that more lactate is being produced in these labour compared to normal labours. This increase causes

a drop in pH_i which has been show to affect Ca^{2+} channels. Ca^{2+} transients are inhibited and therefore myometrial contractility is affected.

1.6 Aims

As there are no data that examines the functional effect of lactate on myometrial contractility or its mechanism of action, the focus of this work was to investigate the effect of lactate on myometrial contractility. The aims of this study were to:

i) To investigate the effect of lactate on spontaneous and oxytocin-driven contractile activity in rat and human myometrial contractility

ii) to compare the effects of other weak acids on spontaneous and oxytocin-driven contractions on rat myometrium

iii) to investigate the mechanistic action of lactate, looking at its effect on intracellular pH and calcium signalling and how the response to lactate is altered with gestational state.

iv) To investigate the presence of MCT-1 and 4 in the myometrium

Chapter 2

Methods

Chapter 2

General Materials and Methods

This chapter will give a general description of the materials and methods used throughout the thesis. More detailed explanations and relevant chemicals will be found in the relevant chapters.

2.1 Animal Tissue

Use of animal tissue

Animal tissue is more readily available than human myometrial tissue and smooth muscle from the rat is commonly used as a model for human tissue (Wray 1993, 2001) as it behaves in a similar manner to the uterus. Animal tissue also allows different time-points in pregnancy to be obtained; similar time-points are unavailable in human tissue.

Female virgin Wistar rats (Charles River, Kent UK) were time-mated with the male rats going in/taken out of the cage being classed as Day 0/1. When pregnant, rats at different gestational ages were killed and dissected accordingly (e.g. 11 day gestation at Day 11). Term tissue was taken from rats that were within 24 hours of labour (Day 22/23). Labouring tissue was taken on Day 23 when the rat had begun to litter and at least one pup had been delivered. Non-pregnant uterine tissue was taken from virgin Wistar rats.

Collection

Animals were culled with full compliance with the code of practice for the humane killing of animals under schedule 1 of the Animals (Scientific Procedures) Act 1986. Rats were placed in a culling chamber and subjected to a rising CO_2 concentration. Death was confirmed by cervical dislocation. Pups were delivered manually by caesarean section and culled via cervical dislocation. The uterus was then removed and placed straight into fresh physiological saline solution (PSS solution, composition given below). If tissue was being used for protein extraction and western blotting, tissue was collected on ice-cold PSS. The tissue was used for contractility experiments and/or frozen on day of collection.

Dissection

The whole rat uterus was placed on a dissection dish and covered with fresh PSS. A 1cm chunk of uterus was cut from the ovarian end of either uterine horn, with the rest of the uterus was placed back into PSS and stored at 4^{o}C. The 1cm piece was then cut longitudinally and opened out, with the inner endometrium side facing down. Serosal cells were carefully cleaned away using cotton buds soaked in PSS. Longitudinal myometrial strips of 1mm x 5mm in size were dissected from the uterine portion. If the tissue was to be used for protein extraction and western blotting, strips were weighed and flash frozen in liquid nitrogen. They were stored at -80 ^{o}C until enough samples were collected to commence extraction. If myometrial strips were to be used in contractility experiments, aluminium clips were attached at each end.

2.2 Human tissue

Although rat smooth muscle is commonly used as a human model, some experimental manoeuvres may not yield similar results in both animal and human tissue. For example in rodents, progesterone levels decrease to initiate parturition whereas in humans, this isn't the case (Mitchell & Taggart 2009). This is why human myometrial samples have been used in some experiments in this thesis.

Collection and storage

A full thickness myometrial biopsy measuring 1cm x 1cm was taken at the end of lower segment caesarean section, from the upper lip of the uterine incision. Tissue was removed after the baby had been delivered and before oxytocin injection, which is administered to prevent post-partum haemorrhage. Biopsies of non-pregnant myometrium were taken from women undergoing hysterectomies and were removed immediately after the uterus had been removed from the patient. Biopsies were taken from the lower half of the anterior uterine wall, in the same position as pregnant biopsies.

All biopsies were placed in Hank's balanced salt solution and transported to the Physiology department. Portions of tissue were taken and fixed in neutral buffered formalin (NBF) or flash frozen in liquid nitrogen and stored at -80^{o}C for future experiments. Tissue strips were dissected from biopsies and used on the day of collections or the next day after overnight storage at 4^{o}c. It has been shown that human myometrial tissue stored at room temperature for up to 18 hours did not behave differently to freshly collected tissue (Popat & Crankshaw 2001; Senchyna & Crankshaw 1999).

Types of biopsies

All studies relating to the use of human tissue had approval of the local ethics committee and written, informed consent from each patient taking part in the study. A further application was submitted and accepted for use of tissue in the Myometrial Research Tissue Bank (MRTB) at Liverpool Women's hospital. Patients were consented by research midwives at pre-operative clinic or by clinicians in the hospital's Delivery Suite.

All pregnant myometrial tissue was obtained from caesarean sections performed at term (37-40 weeks). In regards to non-pregnant tissue was obtained from women undergoing hysterectomies at Liverpool Women's hospital. Those that were on medication, those that had abnormal smears or were having hysterectomies for endometriosis were excluded from this project.

Dissection

Biopsies were pinned out on a dissection plate in PSS at room temperature. Samples were cleaned of blood and endometrium using PSS soaked cotton buds. Blunt dissection, by opening scissors in a gap between bundles, was done to expose myometrial bundles. This was done to reduce the trauma to tissue. As the majority of fibres run in the same direction in pregnant tissue, it was easier to dissect than non-pregnant tissue that has interwoven muscle fibres. Small myometrial strips 1 x 5mm in size were removed and treated in the same way as animal tissue.

2.3 Solutions and chemicals

Unless stated, all chemicals were obtained from Sigma (Poole, Dorset, UK).

a. Salts – all salt solutions were freshly prepared on a daily basis

i. Physiological saline solution (PSS)

Sodium chloride (NaCl)	154mM	9.00 g/L
Potassium chloride (KCl)	5.6mM	0.42 g/L
Magnesium sulphate heptahydrate ($MgSO_4$)	0.12mM	0.29 g/L
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES)	10.9mM	2.60 g/L
Glucose	8mM	2.10 g/L
Calcium chloride ($CaCl_2$)	2.0mM	2ml/L
Distilled H_20 (dH_20)		1000mL

The pH of the solution was adjusted to 7.40 using sodium hydroxide (NaOH). HEPES is an effective buffer at pH between 6.8 and 8.2. If solutions were needed below pH 6.8, 2-(N-morpholino)ethanesulfonic acid (MES) buffer was used. For solutions above 8.2, N-cyclohexyl-3-aminopropanesulfonic acid (CAPSO) buffer was used.

ii. High potassium PSS solution (known as High K^+) was made by isosmotic replacement of NaCl with KCl:

NaCl	114mM	6.99 g/L
KCl	40mM	2.98 g/L

All other ingredients were identical to normal PSS.

iii. Sodium Lactate (and other weak acids) was made by isosmotic replacement of NaCl with sodium lactate, or other weak acid:

NaCl	134mM	7.80 g/L
Sodium Lactate (Lac)	20mM	2.24 g/L

NaCl	134mM	7.80 g/L
Sodium Propionate (Prop)	20mM	1.92 g/L

NaCl	134mM	7.80 g/L
Sodium Butyrate (But)	20mM	2.20 g/L

NaCl	134mM	7.80 g/L
Sodium Pyruvate (Pyrv)	20mM	2.20 g/L

All other ingredients were identical to normal PSS.

Lower concentrations of weak acid solutions were made by diluting in PSS.

iv. High potassium lactate (KCl-Lac) solution:

NaCl	94mM	5.50 g/L
KCl	40mM	2.98 g/L
Lac	20mM	1.92 g/L

All other ingredients were identical to normal PSS.

v. Ammonium chloride (NH_4Cl) solution:

NaCl	149mM	8.71 g/L
NH_4Cl	5mM	0.27 g/L

All other ingredients were identical to normal PSS.

vi. Ammonium chloride lactate (NH_4Cl-Lac) solution:

NaCl	144mM	8.42 g/L
NH_4Cl	5mM	0.27 g/L
Lac	5mM	0.56 g/L

All other ingredients were identical to normal PSS.

b. Oxytocin

Oxytocin was dissolved in dH_20 at a concentration of 10mM. Desired concentrations were obtained by diluting the stock solution with PSS. For animal tissue, it was used at [0.1nM]. For human, [0.5nM] oxytocin was used.

2.4 Tension measurements

Calibration of force

Force was calibrated in Newtons (N) and done so by comparing force traces to those obtained from a known amount of force. This was achieved by suspending known weights from the force transducer and measuring the deflection. 0.1g weight is equal to 1mN (N is $kg.ms^{-2}$).

Control contractions

Tissue was attached to the fixed hook and force transducer using the aluminium clips in 1ml perfusion bath (Figure 2.1). The tissue strips were then stretched by 2mN. Strips were continually superfused with PSS solution. The force transducer was attached to a digidata acquisition package and Axoscope software. In animal tissue, if contractions appeared within 20 minutes, KCl was applied to gain the tissue's maximal activity and used in future analysis. A further 5 minutes of control contractions (of equal amplitude and duration) were needed before an experiment would begin. If no contractions appeared, KCl would be applied to test viability and check amount of stretch applied.

Human tissue was attached in a similar manner and stretched to 2mN. If contractions appeared within 3 hours, at least 20 minutes or 4 contractions of equal amplitude were needed before an experiment would start. If no contractions were seen, KCl was used to test viability. If a good KCl response, oxytocin was applied. If no/ little KCl, strip was discarded.

Tissues were able to produce stable, regular contractions for many hours (Figure 2.1).

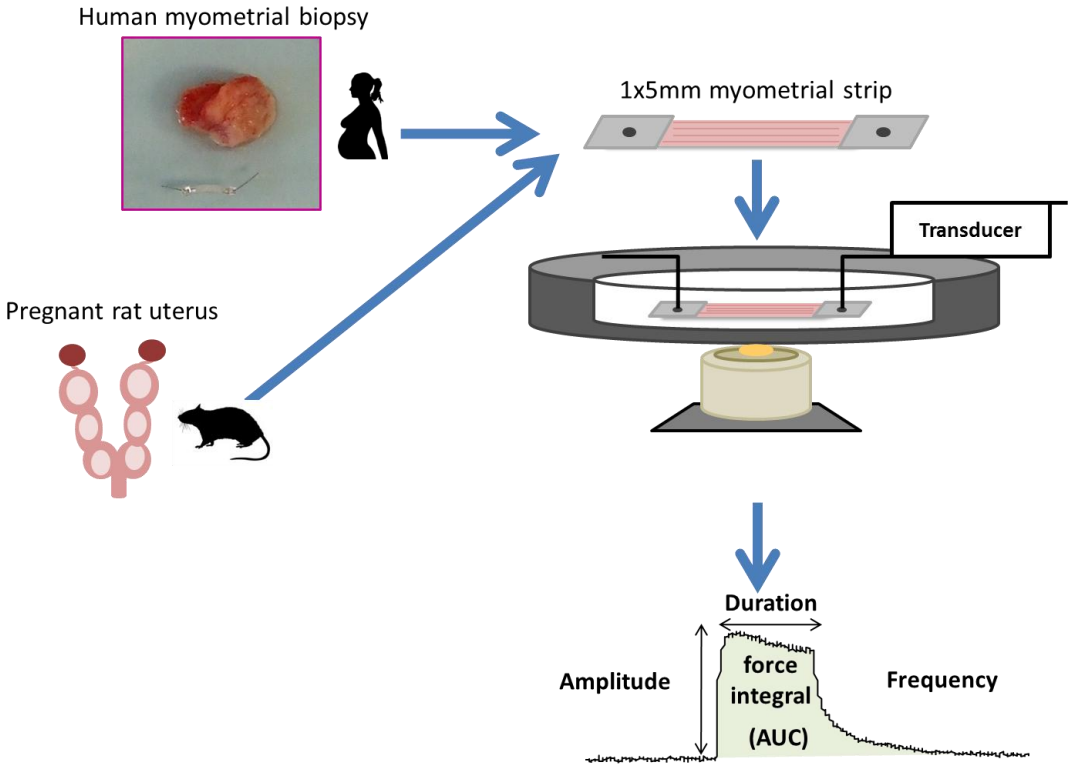


Figure 2.1 Measurement of contractile activity

Myometrial strips , measuring 1x5mm in size, are dissected from either rat uterus or human myometrial biopsies. Using aluminium clips, tissue was attached to a fixed hook and force transducer. This was superfused with physiological saline solution at 37^{o}C, pH 7.4. Contractility was then measured.

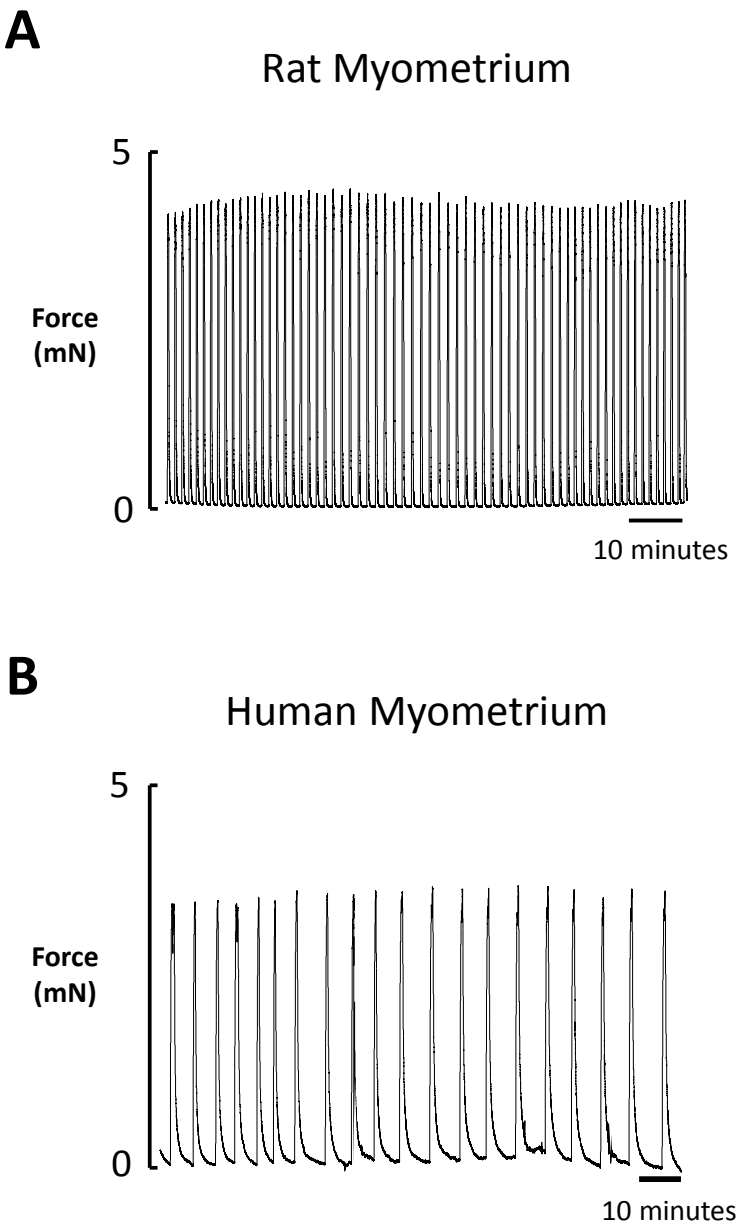


Figure 2.2 Control contractility traces

Two hours of contractions from small myometrial strips from:
A. Term pregnant rat
B. Term pregnant human showing no decline in force.

Analysis of contractility

Four main parameters that were analysed from all contractility experiments using Origin Pro 8.6 software (Figure 2.2).

Force amplitude

Force amplitude (mN) is calculated by subtracting the baseline from the peak of contraction. An average of 5 minutes of contractions (in rat) or 20 minutes or 4 contractions in (human tissue) were needed before drug/treatment could be added. This was termed 'control'. The control period was compared to an average of 5 minutes (rat) or 20 minutes or 4 contractions (human) in the presence of a drug or treatment. The difference in time measured between species is to account for slower rate of contractions seen in human myometrium. If there were no contractions, it was recorded as '0'.

Duration

Duration is the measured time (minutes) of contraction at half maximal amplitude (t50). The average time of all contractions in control or treatment period were used in statistical analysis. Where contractions ceased, they were removed from analysis.

Frequency

The interval between the start of one contraction and the start of the next was measured for all contractions in the control or treatment period. The time in the period being analysed (e.g. 5 minute control) was divided by the average time per contraction to give an accurate number of contractions in the set time.

Area under the curve (A.U.C.)

The AUC is also known as mean force integral. This gives a good indication of the overall effect of all the other parameters together. A period of 5 minutes (rat) or 20 minutes (human) was selected and area under the curve measured using Origin Pro 8.6 software. In some cases, the last 2 minutes (rat) or 5 minutes (human) on a given control or treatment period were analysed to give an accurate measure of the effect of the treatment. If there were no contractions, it was recorded as '0'.

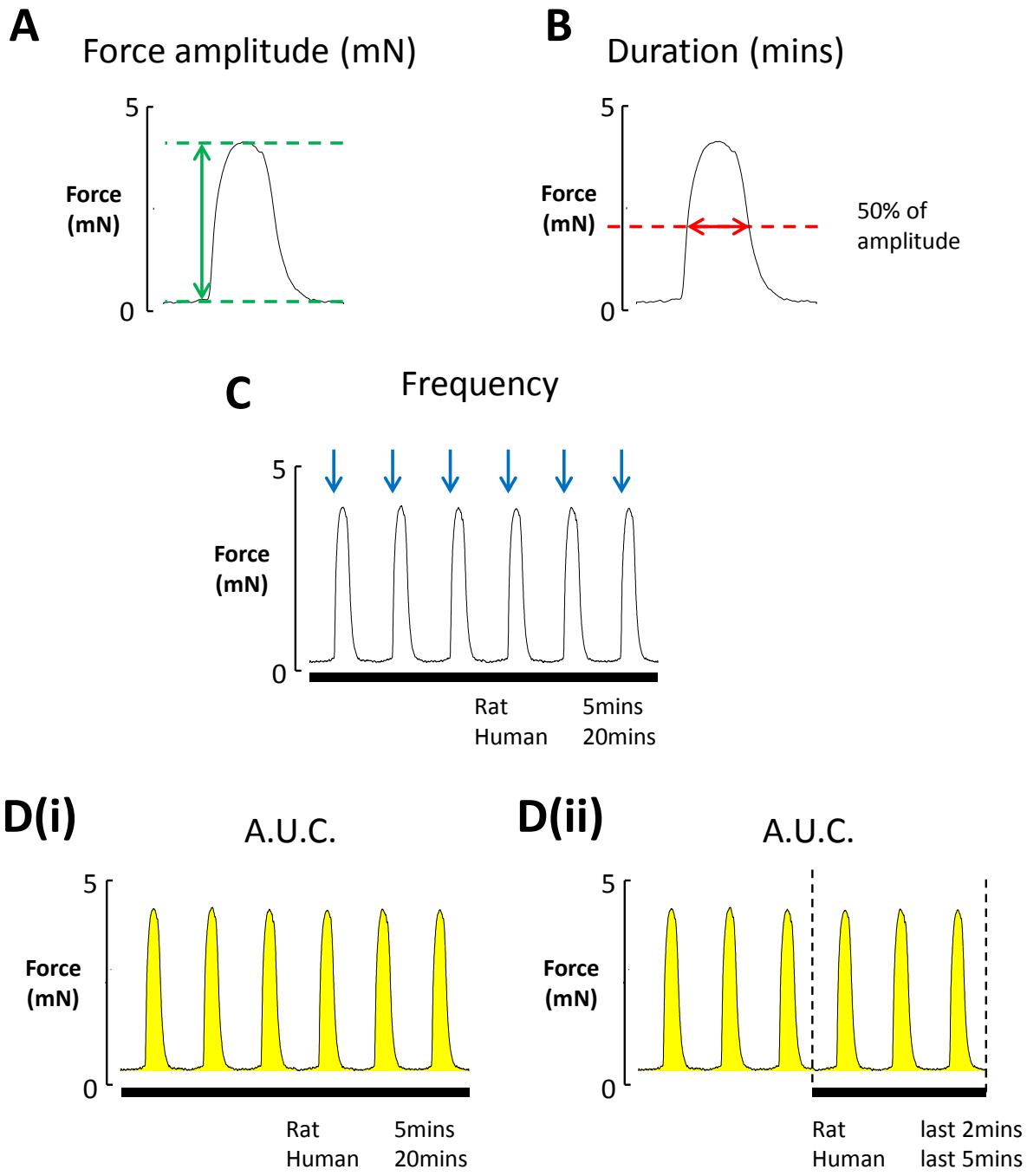


Figure 2.3 Measured parameters of contractility

A. Amplitude is measured from baseline to peak of contraction (mN).
B. Duration is measure at half maximal amplitude (minutes).C
C. Frequency is the number of contractions in a given time period. For rat data this is 5 minutes, for human, 20 minutes.
D. (i) Area under the curve (AUC) is measured using Origin Pro 8.6 software in a given time period. In some instances the last 2 minutes (rat) or last 5 minutes (human) were analysed (ii).

2.5 Fluorescent indicators

The concentration of intracellular ions is important in the regulation of cell function. Changes in the concentration of ions in the cytosol affects things like membrane potential, osmoregulation and can act as second messengers. Intracellular calcium is the main ion responsible for the contraction of smooth muscle. However, variations in pH_i are also responsible for enzymatic function, protein structure and ionic species. Fluorescent probes are useful tools for investigating intracellular ions. Reasons for this include their ability to be incorporated into tissue without damaging the plasma membrane, they can be used in single cells or multicellular preparations and they allow the free concentration of ions or their activity to be measured (Tsien 1989).

Fluorescence allows the detection of particular ions in tissue by the use of fluorescent probes. These are designed to respond to a stimulus. The fluorescence process occurs in three stages:

1. Excitation
2. Excited-state
3. Emission

In stage 1, a photon of energy is supplied by an external source (e.g. xenon lamp) and absorbed by the fluorophore. This creates an excited electronic singlet state. The excited state exists for a finite time (stage 2). The fluorophore undergoes conformational changes and its energy is partially dissipated. In stage 3, a photon of energy is emitted returning the fluorophore back to its ground state. Because of the energy dissipation during its excited state, the energy of the photon is lower (and therefore a longer wavelength). The difference in energy or wavelength is called the Stokes Shift (Invitrogen Mol Probes).

Ratiometric indicators are used within this thesis and are based on the use of a ratio between two fluorescent intensities. Fluorophores can be dual excitation, where excitation alternates between two different wavelengths. The fluorescence emission is captured at a single wavelength. Fura-2 is an example of a dual excitation fluorescent indicator. It is excited at 340nm and 380nm and emission is measured at ~510nm (ShmigolJPhysiol2001). Dual emission indicators are excited at one wavelength, which is true for Indo-1, which is excited at 350nm and emission fluorescence is measured at 400nm and 500nm simultaneously. The ratio of the emission fluorescence is obtained by dividing the 400 intensity by the 500 intensity. Ratiometric indicators allow for the correction of artefacts due to photobleaching, focus changes and variations in laser intensity and uneven dye loading or dye retention.

Measurement of intracellular calcium and pH

Indo-1 AM was developed by Roger Tsien and collaborators and allows the measurement of intracellular calcium (Grynkiewicz, Poenie & Tsien 1985). Its structure is based on 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), an EGTA homologue with a high selectivity for calcium and is more pH insensitive (Harrison & Bers 1987). This is due to the presence of carboxylic acid groups which are able to bind calcium ions (Figure 2.3). When excited at 350nm, the emission shifts from ~500nm without calcium to 400nm with calcium (Figure2.3).

1,5-(and 6)-carboxyseminaphtorhodafluor-1-acetoxymethy ester (Carboxy-SNARF) is a ratiometric pH-sensitive fluoroprobe (Figure 2.4).It is excited at 540nm and the emission collected at 590nm and 640nm wavelengths, where it shifts toward 590nm when acidic (Figure 2.5). The value is dependent on dye concentration, path length and excitation

intensity but this dual-emission dye is better than the dye previously used- BCECF, a dual excitation fluoroprobe, which is excited sequentially. The signal-to-noise ratio worse as one wavelength is weaker and more pH-insensitive than the other (Buckler & Vaughan-Jones 1990) Carboxy-SNARF is used as it pKa value is close to physiological pH of cell (Bond & Varley 2005).

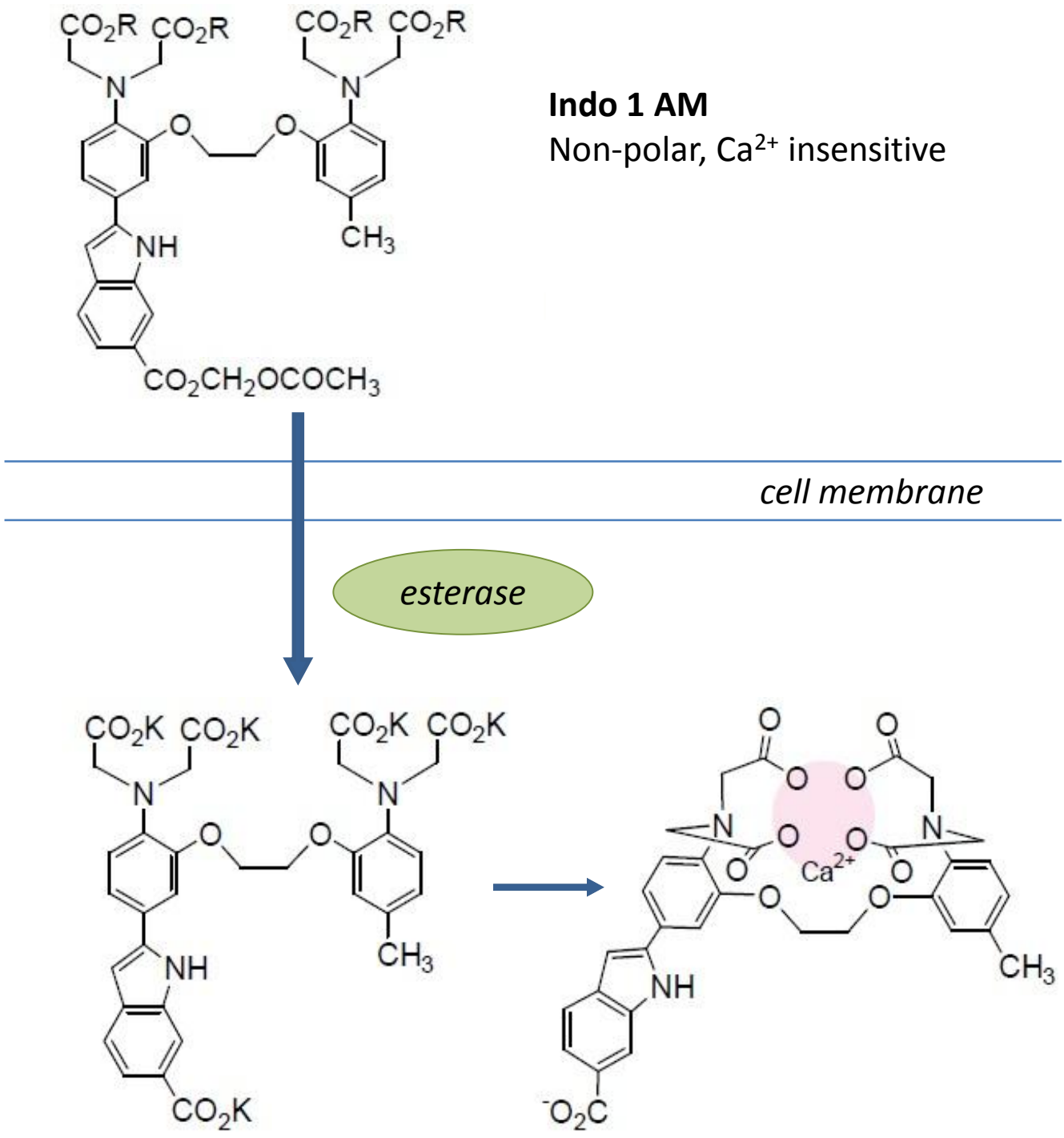
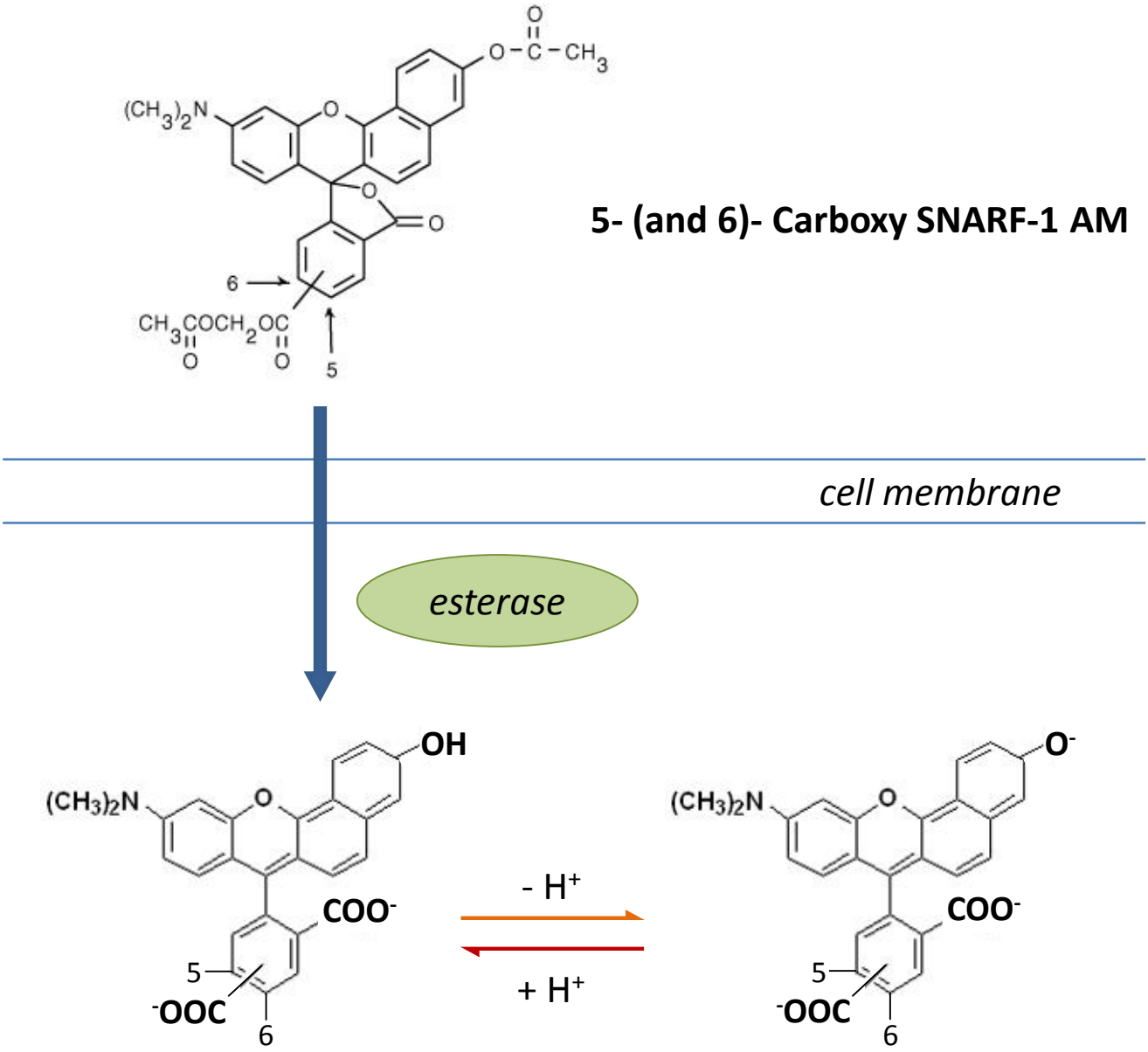


Figure 2.4 Indo-1 AM structure

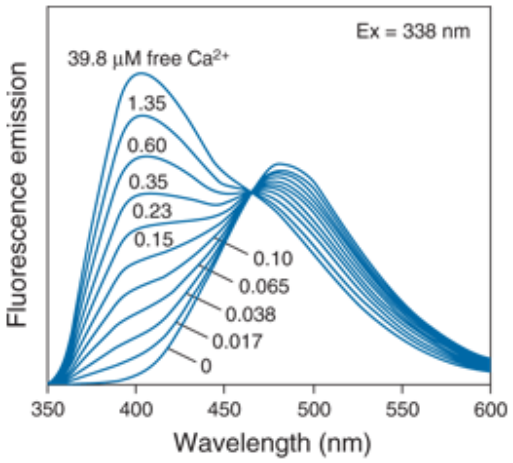
The ester form of Indo-1 is able to pass freely across the cell membrane. Once inside the cell, esterases cleave the ester group and it is unable to freely leave the cell. Here is it able to bind calcium.(Adapted from dojindo.com)



5- (and 6)- Carboxy SNARF-1

pH sensitive

Figure 2.5 Carboxy SNARF-1 AM structure



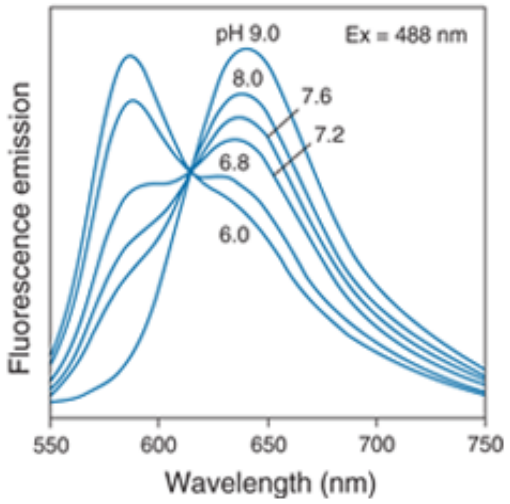


Figure 2.6 Fluorescence emission spectrum for Indo-1 AM and Carboxy SNARF-1 AM

The acetoxymethyl ester (AM) ester form of the dye is used to allow the dye to permeate the cell membrane. This is because this form of the indicator is uncharged and hydrophobic and can therefore cross the lipid membranes (Bootman et al. 2013). Once inside the cell, endogenous cytosolic esterases cleave ester groups, charging the molecule. The carboxyl groups in the indicator are also essential for the indicator to sense Ca^{2+} or H^{+} so the AM groups need to be removed. This form of the indicator leaks out of the cell more slowly (Invitrogen).

Loading of fluorescent indicators into tissue can be increased by using Pluronic F-127, a dispersing agent. This is a mild non-ionic detergent that facilitates cell loading of water-insoluble dyes (Bootman et al. 2013). The indicator is reconstituted in an anhydrous dimethylsulfoxide (DMSO) to form a DMSO-Pluronic mix. Gentle warming is needed to assist getting the detergent to dissolve into DMSO.

1. Calibration of Carboxy SNARF-1 for pH_i measurements

Nigericin

Nigericin is H^+ K^+ antiporter. It is used to 'clamp' pH_i at the external PH (pHo) by equilibrating the potassium concentration. When internal and external potassium concentrations are equal, protons can move freely across the plasma membrane so therefore pH_i should equal pH_o (Thomas et al. 1979).

Nigericin calibration solution (dissolved in EtOH):

KCl	140mM
$MgCl_2$	117mM
Nigericin	10µM

20mM buffer was added to the calibrations solution as follows and adjusted to desire pH at 37^oC with NaOH:

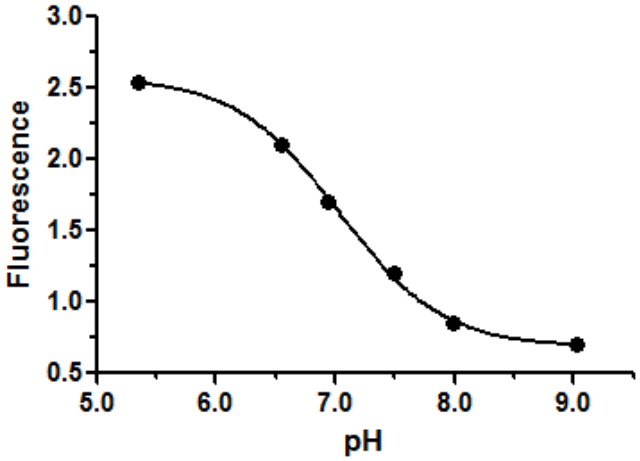
pH 5.5	MES
pH 6.5, 7.0, 7.5, 8.0	HEPES
pH 9.0	CAPSO

Mock intracellular solution

Using a mock intracellular solution:

NaCl	10mM
KCl	146mM
$MgSO_4$	4mM
ATP	4.5mM
HEPES	11mM

1µM free acid Carboxy SNARF-1was added and pH adjusted to known values (pH 5.0, 6.5, 7.0, 7.5, 8.0, 9.0). Fluorescence was measured using these solutions and a calibration graph using the ratio vs pH.



From this, the Rmin, Rmax and PkA can be calculated and used in the equation below to calculate pH.

$$pH = pKa + \log [R\text{-}Rmin/(Rmax\text{-}R)]$$

Nigericin has been shown to be unreliable in tissues/multicellular smooth muscle preparations as the ionophore is slow to diffuse (Taggart & Wray 1993a) and can persist in tubing and tissue baths (Buckler & Vaughan-Jones 1990). There is good agreement between the two calibration methods (Austin & Wray 1993).

Advantages and disadvantages of using fluorescent indicators

Photobleaching

Photobleaching is the destruction of the fluorophore under high-intensity illumination conditions. It can be prevented by only subjecting the loaded tissue to illumination for the shortest time possible. The use of neutral density filters placed in front of the excitation light is also encouraged to reduce the intensity of light exciting the tissue (Tsien 1989). Although the de-esterified indicator is essentially membrane impermeant, some anion transporters in the plasma membrane can cause clearance of fluorescent indicators. This loss is temperature sensitive (Kao 1994; Thomas et al. 2000). In the experiments conducted in this thesis, tissue loaded with fluorescent indicators were not in focus (i.e. away from direct light excitation) until an experiment is ready to begin.

Autofluorescence

Autofluorescence is the natural emission of light by structures that have absorbed light, without the presence of fluorphores. The most common autofluorescencing molecules include NADPH, riboflavin and FAD (Takahashi et al. 1999). The extracellular matrix can also contribute to this phenomenon. The contribution of autofluorescence to the overall fluorescence signal can be measured by quenching with manganese (Hesketh et al. 1983). The measured emission is subtracted from total signal.

Subcellular compartmentalisation of fluorophores

Fluorescent indicators can become compartmentalised by organelles within the cell such as the sarcoplasmic reticulum and mitochondria (Thomas et al. 2000). Indo-1 is

less prone to compartmentalisation than Fura-2, although it is also less resistant to photobleaching (Scheenen et al. 1996).

2.6 Force and calcium or pH measurements

Both fluorescent indicators used in these experiments were prepared using dissolved DMSO/Pluronic F-127 mix. There is no significant effect on contractility at the concentration used in these experiments (Taggart et al. 1997).

Loading Indo-1 AM

To monitor changes in intracellular calcium signalling, 50µg of Indo-1 AM was dissolved in 50µL of solution of 200µL DMSO and 0.05g Pluronic acid. This mix was added to 4mL PSS and vortexed. This creates a concentration of 12.5µM Indo-1 AM. The Indo-1 AM-PSS mix was split between two 5mL vials and dissected myometrial strips were added to each- no more than 6 strips per vial. Vials were placed on a rotator plate for 3 hours at room temperature. Loaded strips were washed with fresh PSS before use in experiments.

Loading Carboxy-SNARF

The ratiometric pH indicator Carboxy SNARF-1 AM was used to monitor changes in intracellular pH. 50µg of the indicator was dissolved in 50µL of solution of 200µL DMSO and 0.05g Pluronic acid. This was added to 4mL PSS and vortexed. This creates a concentration of 12.5µM. The Indo-1 AM-PSS mix was split between two 5mL vials and dissected myometrial strips were added to each- no more than 6 strips per vial. Vials were placed on a rotator plate for 2 hours at room temperature. Loaded strips were washed with fresh PSS before use in experiments.

Simultaneous measurement of force and calcium or pH

Tissue in bath attached to transducer resting tension 2mN. Once contracting, focused on tissue and excited by U.V. illumination is provided by a xenon lamp (Figure 2.6). A heat filter, a neutral density filter are placed in the light path to prevent excess heat and reduce light intensity reaching the tissue. The appropriate excitation wavelength is selected by way of interference filter. For calcium measurements using Indo-1 AM, the excitation wavelength is 345nm; for pH measurements using Carboxy-SNARF, the excitation wavelength is 450nm. An electromagnetic shutter blocks the path of light to reduce illumination time of the tissue and prevent photobleaching. This is used until a control period of contractions is established. The excitation light is reflected and directed upwards by a dichroic mirror (DM1) and focused onto the tissue by the microscope objective. The light emitted by the fluorescent indicator is passed back through the objective and transmitted by the dichroic mirror (DM1) reflected by the sliding mirror. The light hits a second dichroic mirror (DM2) at 610nm, mounted at a 45o which directs longer wavelength emitted light (>610nm) to form an image on the video camera which relays the image to the TV monitor. The shorter wavelength light (<610nm) is directed towards the photomultipliers (PMTs). The shorter wavelength light is split by another dichroic mirror (DM3) and passes to either PMTs. In front of each PMT is an appropriate emission filter to select the correct wavelength of light. For calcium measurements, this is 400nm and 500nm and for pH measurements this is 590nm and 640nm.

The signals detected by the PMTs and force transducer provide a recording on the Axoscope software (Figure 2.7). The rise and fall of intracellular calcium or pH are measured from the ratio of the two emission signals.

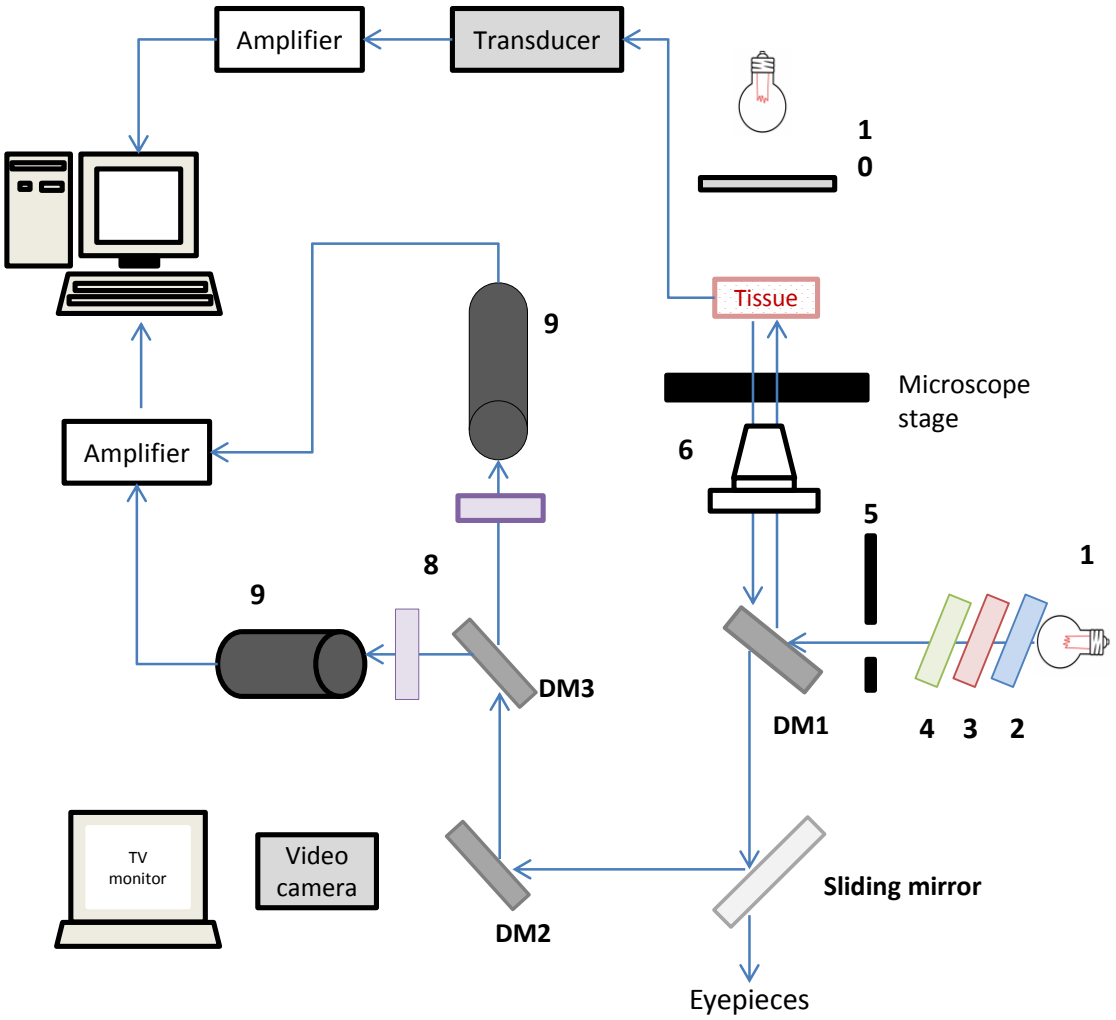


Figure 2.7 Schematic diagram of the inverted microscope used to make simultaneous force/calcium measurements.

U.V. illumination is provided by a xenon lamp (1). A heat filter (2) neutral density filter (3) are placed in the light path to prevent excess heat and reduce light intensity reaching the specimen. The appropriate excitation wavelength is selected by way of interference filter (4). An electromagnetic shutter (5) block the path of light to reduce illumination time of the tissue and prevent photobleaching. The excitation light is reflected and directed upwards by a dichroic mirror (DM1) and focused onto the tissue by the microscope objective (6). The light emitted by the fluorescent indicator is passed back through the objective and transmitted by the dichroic mirror (DM1) reflected by the sliding mirror. The light hits a second dichroic mirror (DM2) at 610nm, mounted at a 45o which directs longer wavelength emitted light (>610nm) to form an image on the video camera which relays the image to the TV monitor. The shorter wavelength light (<610nm) is directed towards the photomultipliers (PMTs, 9). The shorter wavelength light is split by another dichroic mirror (DM3) and passes to either PMTs. In front of each PMT is an appropriate emission filter to select the correct wavelength of light. Microscope lamp (10)

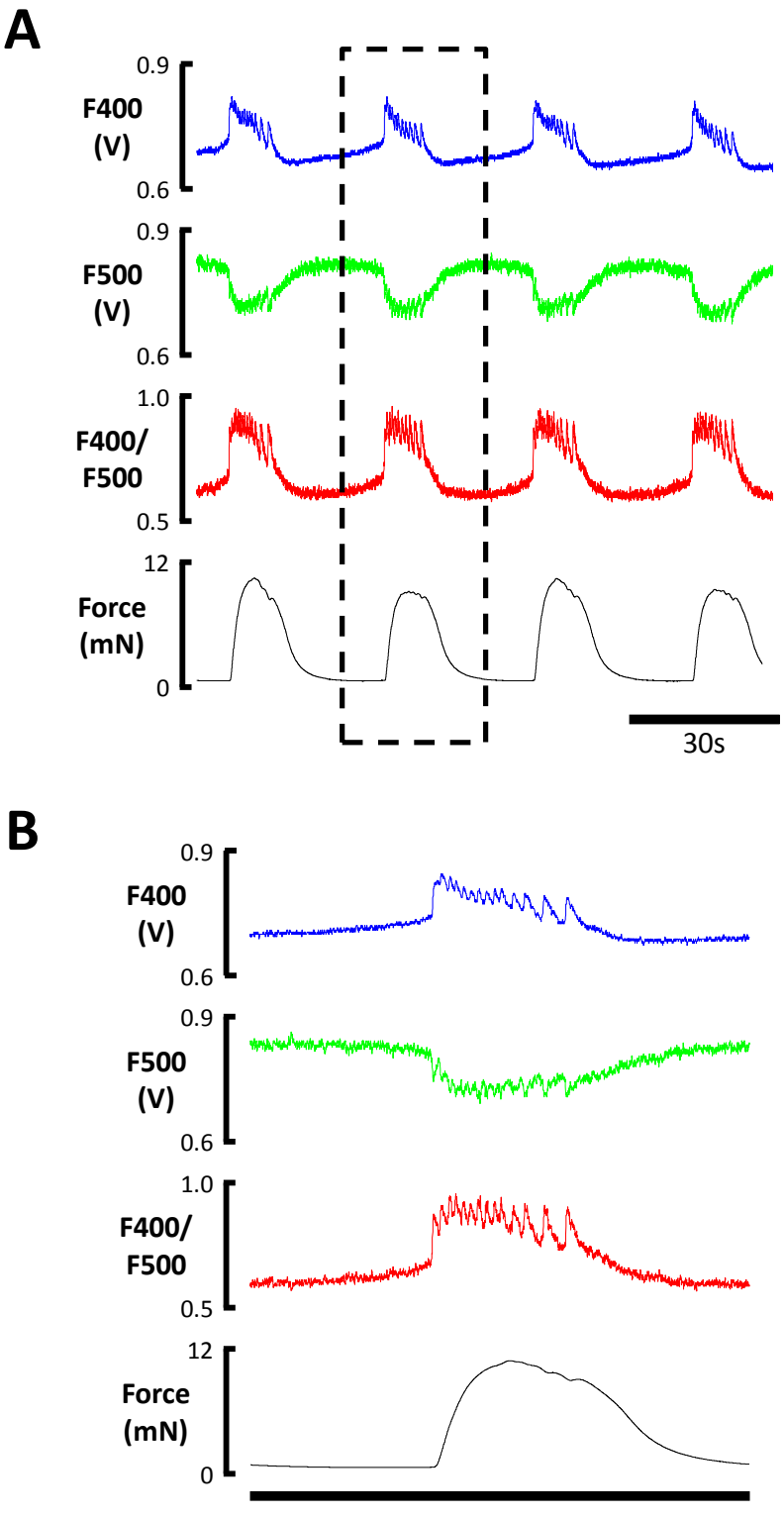


Figure 2.8 Simultaneous force and calcium trace

A. A typical trace showing separated F400 and F500 channel, ratio of the two and force.
B. One contraction showing rise in intracellular calcium before force begins to increase.

2.7 Western blotting

Protein extraction

Radio-Immunoprecipitation assay (RIPA) buffer was used to allow efficient cell lysis and protein solubilisation. It also avoids protein degradation as it contains phosphatase and protease inhibitors, and does not interfere with biological activity. 3mL RIPA buffer per 1g tissue was used.

PBS tablets	2 tablets
IGEPAL	1%
Sodium deoxycholate	0.5%
SDS	0.1%

Small chunks of tissue from time-mated animals or human myometrial biopsies, that had been previously snap frozen in liquid nitrogen and stored at -80^{o}C, were homogenised using a pestle and mortar and liquid nitrogen until it was a fine powder. RIPA buffer was added and the sample re-ground. Samples were centrifuged at 10000 RCF (g) at 4^{o}C for 10 minutes. The pellet was discarded and the supernatant frozen before quantification using in a protein assay.

Protein quantification

Bio-Rad detergent compatible (DC) protein assay was used to quantify the amount of protein present in the samples. This is because the RIPA buffer used to extract the proteins contains SDS, a detergent. The colourmetric assay is based on the Lowry method, which uses the reaction of cupric ions with peptide bonds under alkaline conditions, using a Folin-Ciocalteu reagent. This is a modified-Lowry based assay with the following improvements: 90% of the maximum reaction colour occurs within 15mins and colour changes no more

than 5% in 1hour. The same RIPA buffer that was used for the protein extractions was used in the assaying of the samples. 2mg/ml protein standard (0.01g bovine serum albumin (BS) in 5mL RIPA buffer) was made, from which a set of protein standards were also made (0, 0.1, 0.25, 0.3, 0.5 and 1mg/mL). 20µl reagent S was added to each mL of reagent A. This was added to the protein standards and the proteins of interest. All solutions were vortexed and left to develop for 15 minutes. The solutions were transferred to cuvettes and the absorbance of each sample recorded at 750nm. From this data a standard curve was plotted and the unknown protein quantifications could be determined.

Preparation of protein samples

25µg of protein was added to 3x Laemmli buffer (0.125M Tris-HCl pH6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue, 10% β-mercaptoethanol). These samples were boiled at 100^{o}C for 3 minutes, vortexed, spun in a mini centrifuge and placed on ice before loading.

SDS_PAGE and Immunoblotting

Spacer plates were cleaned with dH_20 and 70% EtOH and assembled into casting kit ready for gel loading.

As the proteins of interest in this project were between 17-65 kDA, a 12% resolving gel was used (30% acrylamide:bis-acrylamide, 1.5M Tris HCl, pH 8.8, 0.4% SDS, 10% APS, TEMED). Gel was poured and overlaid with propylalcohol. This was left for 30 minutes to polymerise. The alcohol was then washed off with dh_20. 4% stacking gel is added above the resolving gel (30% acrylamide:bis-acrylamide, 0.5M Tris HCL, pH 6.8, 0.4% SDS, 10% APS, TEMED). Combs were inserted into the stacking gel before polymerisation to create wells for protein loading.

20µl prepared protein samples were loaded alongside 5µL protein ladder (SeeBlue® Plus2 Pre-stained standard, Invitrogen) which is used during at a later stage to show efficient transfer and allow recognition of the protein of interest. Gels were run at 150V for 2 hours, surrounded by running buffer on ice. Gels were removed, moved to transfer cassettes and assembled with nitrocellulose membrane (Whatman Protran). Transfer buffer containing 10% methanol was poured in the tank covering the cassettes. Gels were transferred at 40v for 1 hour. Membranes were blocked for 1 hour at room temperature in 5% BSA (in TBS- 0.1% Tween). Membranes were then placed in primary antibodies (Table 2.1) overnight at 4^{o}c on a rocker. The next day, membranes were washed with TBS- 0.1% Tween for 40 minutes. A secondary antibody was added for 1hr at room temperature. A further 1 x 15min and 4 x 5min of TBS-0.1% Tween washes. Membranes were incubated in chemiluminescent ECL reagent for Horseradish peroxidase (HRP) enzyme for 5 minutes (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). Membranes were developed Kodak processing chemicals developer and fixer in a dark room onto film (CL-Xposure Film, Thermo Fisher Scientific).

PRIMARY

Antibody	Species	Mono/Poly?	Concentration	Diluent	Code
MCT1	Rabbit	Polyclonal	1:500	5% BSA	Ab85021
SLC16A3	Rabbit	Polyclonal	1:500	5% BSA	Ab74109
β-actin	Mouse	Monoclonal	1:4000	5% BSA	Ab8224

SECONDARY

Antibody	Species	Mono/Poly?	Concentration	Diluent	Code
Anti-rabbit	Donkey	Polyclonal	1:10,000	5% BSA	Ab16284
Anti-mouse	Goat	Polyclonal	1:80,000	5% BSA	

Table 2.1 Antibodies used in western blotting experiments

Analysis

Experiments were analysed using ImageJ. All blots were scanned at 200dpi and saved as in the TIFF format. Files were opened in ImageJ and the background subtracted. Multiple large rectangular boxes were drawn around the bands. These were long enough to incorporate free space above and below the bands to assess background. All boxes were the same size. Once all lanes were selected, signal intensities were plotted. These are visualised as peaks and troughs. Using the line drawing tool, the areas of each peak (which corresponds to each band) were enclosed. Each enclosed area was selected by the 'wand' tool to display the area under the curve (AUC) for each band. These were exported into Excel for further calculations.

2.8 Statistics

Graphs and tables were constructed using Origin Pro 8.6, Microsoft Excel 2010 and GraphPad Prism 5. Stastistical analysis of data was performed in SPSS Statistics 20.
If data was normally distributed, the standard deviation and standard error of the mean were calculated. Student's t-test and One-way ANOVA were used as detailed in the relevant chapters. If the data was not normally distributed, Wilcoxon Signed-Ranks test, Mann-Whitney U test and Kruskal-Wallis test were used in the relevant chapters.

For all tests significance was taken as a p value less than 0.05 ($p<0.05$*)

Chapter 3

The effect of lactate and other weak acids on rat myometrial contractility and their mechanism of action

Chapter 3

The effect of lactate and other weak acids on rat myometrial contractility and their mechanism of action

3.1 Abstract

Lactate is produced during anaerobic respiration in all cells during hypoxic conditions but has also been shown to be produced during normoxic conditions in the uterus. Lactate has been previously shown to increase in the myometrium during dysfunctional labour but currently no work has been done to investigate the effect of lactate on the myometrial contractility. Myometrial strips from time-mated Wistar rats at various gestational time points were dissected and contractility measured. 1mM -20mM sodium lactate (and other weak acids: butyrate, propionate and pyruvate) were added to spontaneously contracting and oxytocin-driven (0.1nM) tissue strips. In some cases, tissue was loaded with Indo-1 AM or Carboxy-SNARF AM to simultaneously measure force and intracellular calcium or pH_i respectively. 40mM KCl solution was used to look at the effect of lactate beyond membrane excitability. Lactate significantly decreased spontaneous contractility (*n*=7-8). A dose dependent decrease in all contractile parameters was seen, which was significant at concentrations of 5mM and above (relative to control 100%). Other weak acids also significantly reduced contractions in a dose dependent manner. There was no significant difference between weak acid responses. The effects of lactate were significantly reduced in the presence of oxytocin (*n*=3-6). Lactate decreased intracellular pH and reduced Ca^{2+} transients. The effect of

5mM lactate was more potent at late pregnant myometrium but had little effect on early pregnant and labouring tissue (*n*=4-8). Lactate in the physiological range potently decreases spontaneous contractility in rat myometrium. Lactate also decreases oxytocin-stimulated contractions but its sensitivity is decreased. Other weak acids produce similar effects to lactate suggesting its mechanism of action is not via metabolism. Lactate inhibited Ca^{2+} transients, which could be due to a fall of intracellular pH and I suggest this is the mechanism whereby lactate and similar weak acids reduce myometrial contractions.

3.2 Introduction

Lactic acid is produced by glycolysis in all cells and is dissociated into lactate and a hydrogen ion at physiological pH (Philp, Macdonald & Watt 2005). During glycolysis, glucose is split into two molecules of pyruvate, yielding two ATP molecules and two molecules of NADH. Pyruvic acid progresses through glycolysis and the citric acid cycle (Kreb's cycle) in the mitochondria, if there is ample oxygen. In hypoxic conditions, pyruvic acid is reduced by lactate dehydrogenase to lactic acid, which becomes dissociated to lactate and H^+. It has been shown that the uterus is highly glycolytic even under normoxic conditions (Steingrimsdottir et al. 1995; Wray 1990). Lactate is transported out of the cell and intracellular pH (pH_i) maintained within normal limits and this is mediated by a family of proton-linked monocarboxylate transporters (MCTs) (Bonen et al. 2000a; Bonen et al. 2000b). Lactate and H^+ are co-transported down their concentration gradients.

Our group have previously shown that there is increased lactate in myometrial capillary blood from women suffering dysfunctional labour and that this blood is of a reduced pH (Quenby et al. 2004). Recent work by others has also reported an increase in lactate in the amniotic fluid of women suffering dysfunctional labour (Akerud, Ronquist & Wiberg-Itzel 2009; Wiberg-Itzel et al. 2008). Thus increased lactate may be a significant cause of dysfunctional labour.

Intracellular and extracellular pH plays a vital role in all tissues and must be tightly regulated within a certain range. In the myometrium, both intracellular and extracellular pH changes occur during labour due to vascular occlusion at the peak of uterine contraction (Wray et al. 1992) and it has already been shown that intracellular acidification can affect contractility of

the uterus (Shmigol et al. 1995). Intracellular acidification has been demonstrated to inhibit L-type Ca^{2+} channels and reduce inward Ca^{2+}current (Taggart & Wray 1993b) and cause membrane hyperpolarisation thus causing a decrease in force of contraction.

There are no data that examines the functional effect of lactate on myometrial contractility or its mechanism of action. In vascular smooth muscle, it has been shown that lactate can cause vasodilation and depressed responsiveness to agonists (Barron & Nair 2003) and it is suggested this is due to depressed Ca^{2+} permeability. Lactate has also been shown to induce arterial relaxation by the activation of Ca^{2+}-activated K^{+} channels (Mori et al. 1998). However in cardiac muscle, Lactate increases cardiac cell excitability by increasing the maximal inward sodium current which shifts the voltage dependence of voltage- gated sodium channels (Na_v) towards hyperpolarised potentials (Guo, Wasserstrom & Rosenthal 1994; Tanaka et al. 1994). Lactate has also been shown to reduce chloride conductance, increasing excitability of rat skeletal muscle (de Paoli et al. 2010). Although in smooth muscles, it is unclear if the myometrium behaves in a similar way.

I hypothesise that an increased amount of lactate (and H^{+}) in the myometrium will alter contractility and may be a cause of dysfunctional labour. The aims of the study were:

v) To investigate the effect of lactate on spontaneous contractile activity in rat myometrial contractility

vi) to compare the effect of lactate under oxytocin-driven contractions

vii) to compare the effects of other weak acids on spontaneous and oxytocin-driven contractions

viii) to investigate the mechanistic action of lactate, on intracellular pH and calcium

signalling

ix) to investigate how the response to lactate is altered with gestational state.

3.3 Methods

3.3.1 Tissue

Pregnant Wistar rats at term (within 24hours of expected labour) were humanely killed using CO_2 anaesthesia and cervical dislocation. The uterus was removed, cleaned and longitudinal myometrial strips (5mm X 1mm) dissected. Individual strips were mounted between a fixed support and force transducer using aluminium clips in a 1ml bath and were continuously superfused with PSS (pH 7.40) at a rate of 2mL/min and maintained at 37°C.

For measurement of intracellular pH or calcium, myometrial strips were loaded with fluorescent indicator Carboxy-SNARF AM or Indo 1-AM respectively (12.5µM, 3 hours at room temperature.) After loading, the strips were washed for 30mins in physiological saline solution (PSS) and mounted.

3.3.2 Force and calcium/pH_i measurements

Carboxy-SNARF loaded strips were excited at 530nm using a Xenon lamp and emitted light was detected by photomultipliers at 590 and 650nm. For Indo-1 AM loaded tissue, the wavelengths were 350nm (excitation) 400 and 500nm (emission). Both were digitally recorded at a sampling rate of 100 Hz and the ratio of these two signals was used. The pH_i recordings were calibrated as described in **Chapter 2**. A 40mM high potassium (KCl) solution was used to test tissue viability and measure maximal force contraction. Contractile activity in rat myometrial strips was observed for ~30mins after the application of 40mM KCl for 1 minute until steady contractions appeared. Once stable contractions were established, contractility was manipulated with elevating sodium lactate solutions (1-20mM, pH 7.40) and other weak acids: sodium butyrate, sodium propionate and sodium pyruvate.

3.3.3 Solutions

Buffered physiological saline solution (PSS) composed of (mM): NaCl 154, KCL 5.6, $MgSO_4$ 1.2, $CaCl_2$ 2, Glucose 8 and HEPES 10.2. 40mM KCL was made by isoosmotic replacement of sodium chloride. Oxytocin was added to PSS at a final concentration of 0.1nM (rat) 0.5nM (human.) All chemicals were obtained from Sigma, Poole, Dorset, unless otherwise stated. Carboxy-SNARF and Indo 1-AM were obtained from Molecular probes.

3.3.4 Analysis and statistics

Data was analysed using Origin 8.6 (Microcal) and *n* is the number of myometrial tissue strips from different animals. Amplitude, duration and area under the curve (a.u.c.) of each contraction was measured and the average in the last two minutes of solution application was used. Frequency was measured as the number of contractions in the total lactate application. Data is presented as % of control period (taken as 100%). The data was found to be normally-distributed and statistical differences were tested using parametric statistical tests in SPSS Statistics 20. Significance was taken as $P<0.05$.

3.4 Results

3.4.1 Effect of lactate on spontaneous contractility

5mM lactate was applied for 10 minutes to spontaneously contracting rat myometrium (*n*=3) (Figure 3.1). As shown in Figure 3.1, the response to lactate plateaued after 5 minutes. Therefore, for future experiments, an application time of 5 minutes was chosen. Increasing concentrations of sodium lactate (1-20mM) were applied extracellularly to myometrial strips for 5 minutes and the effects on contractility determined (*n*=7-8) (Figure 3.2). Lactate caused a reduction and abolition of contractile activity and this response was dose dependent (Figure 3.2 Ai-v). Once lactate was removed and the myometrium returned to PSS, contractions resumed and returned to control values. There was a significant decrease in contractile parameters- amplitude, frequency and A.U.C. of contractions when compared to the preceding control period (Paired students t-test, $p<0.05$) (Table 3.1). The amplitude and frequency of contractions were significantly reduced at 5mM lactate and higher. A.U.C was significantly decreased at concentrations ≥ 3mM. There was a significant difference between lactate concentrations in these three parameters (One-way ANOVA with Bonferroni *Post-hoc* test) (Figure 3.3 Ai, ii and iv). It should be noted that 5mM lactate completely abolished contractions in 4 out of total 8 tissue strips tested (Figure 3.4 Ai).

A.U.C. values were used to plot a dose response curve as percentage reduction of a.u.c. compared to control period (Figure 3.3 Biv). From this dose-response curve the EC_{50} for lactate (the concentration at which contractile activity is reduced by 50%) was calculated. The EC_{50} on spontaneously contracting rat tissue was 3.9 ± 0.3 mM.

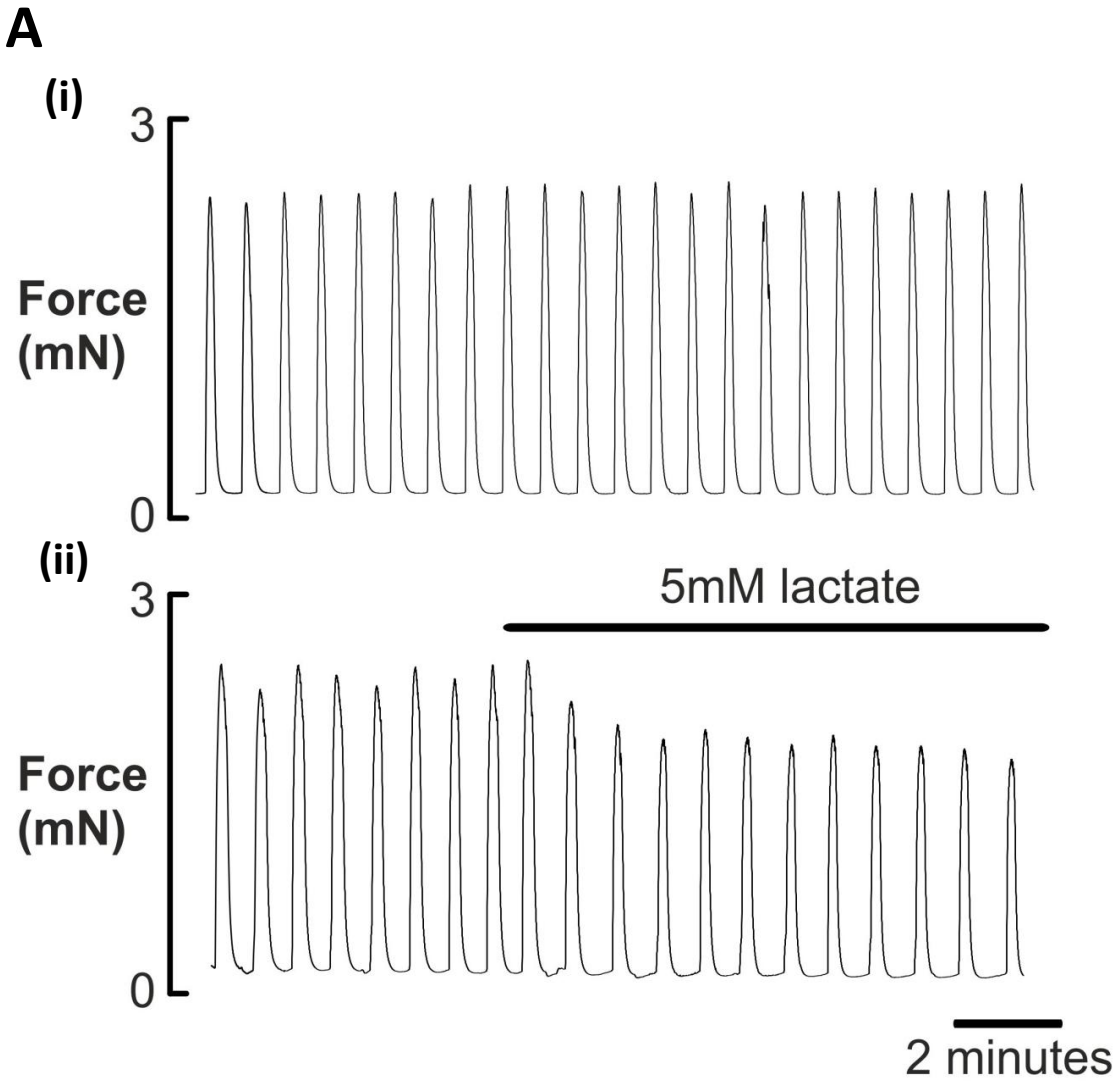


Figure 3.1 Effect of 10 minute application of 5mM lactate on rat myometrium

A: Isometric recording of the application of 5mM lactate to spontaneously contracting rat myometrium for 10 minutes (i) and a paired tissue strip exposed to PSS for the same length of time (ii) (*n*=3)

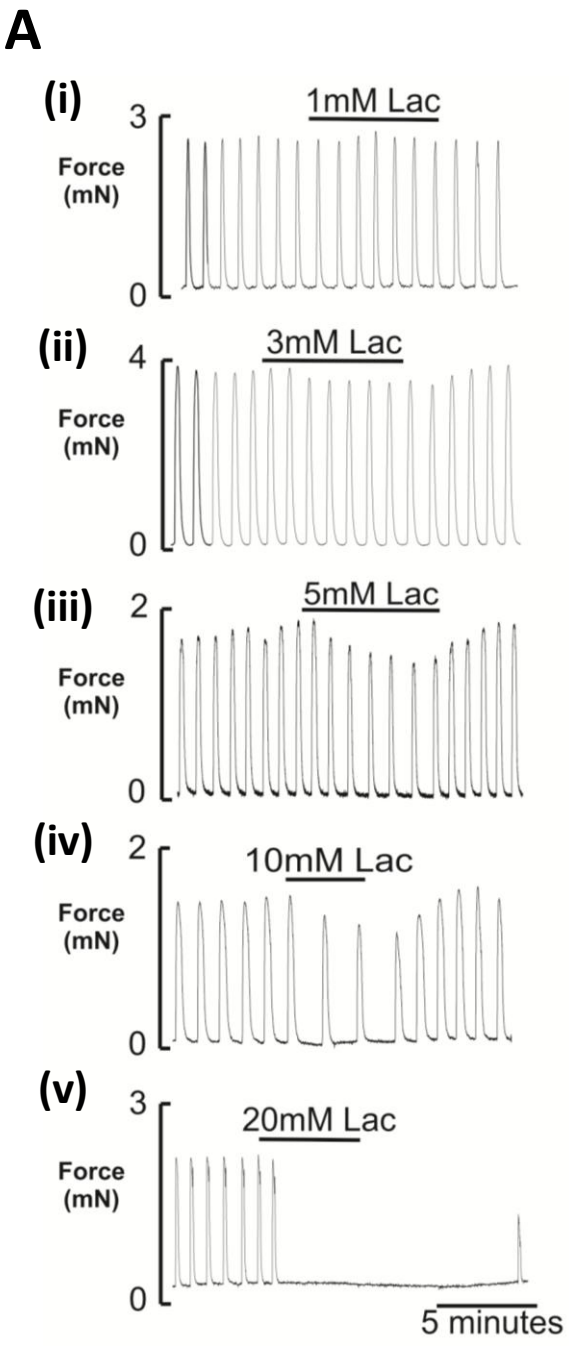


Figure 3.2 Dose dependency of sodium lactate in pregnant rat myometrium

A. Representative force recordings of spontaneously contracting myometrial strips during 5 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM and (v) 20mM lactate.

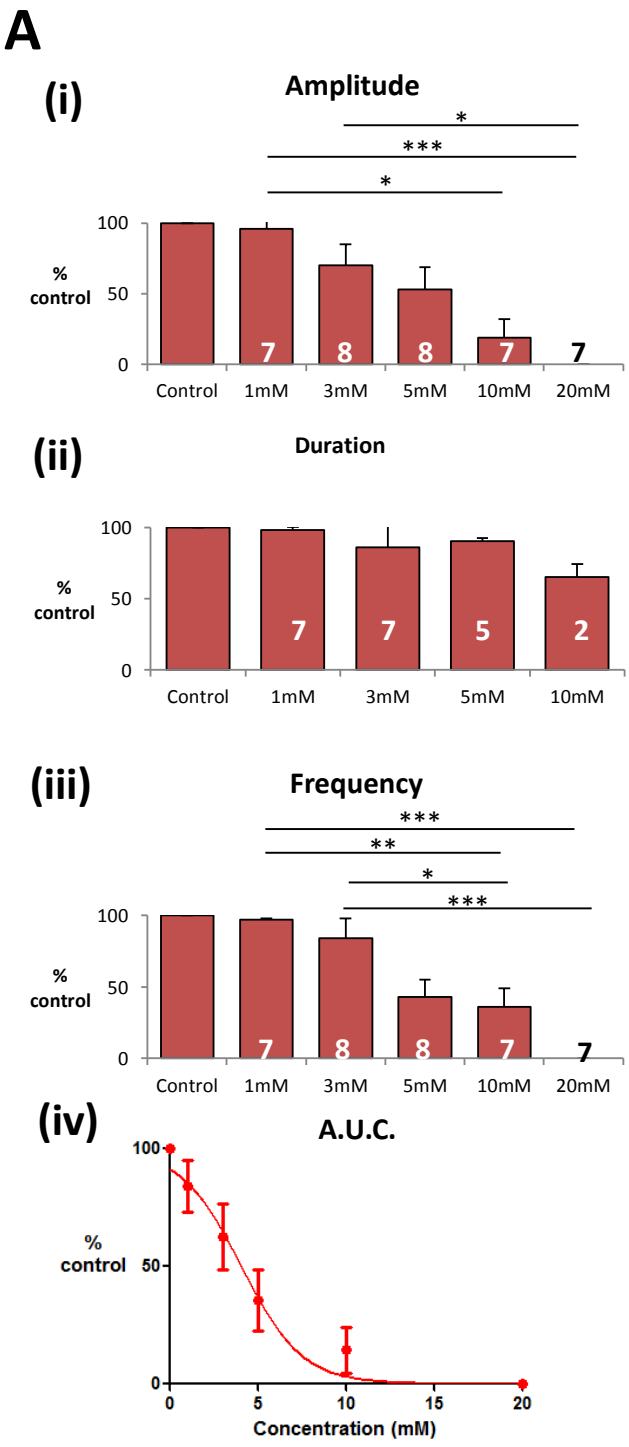


Figure 3.3 Dose dependency of sodium lactate in pregnant rat myometrium

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to lactate. Using the dose-response curve plotted using the AUC data, the IC50 value for lactate is 4.83mM. N numbers are denoted by values within bars. A significant difference in activity was found using ANOVA with Bonferroni * denotes $p<0.05$ **$p<0.01$ ***$p<0.001$ significance.

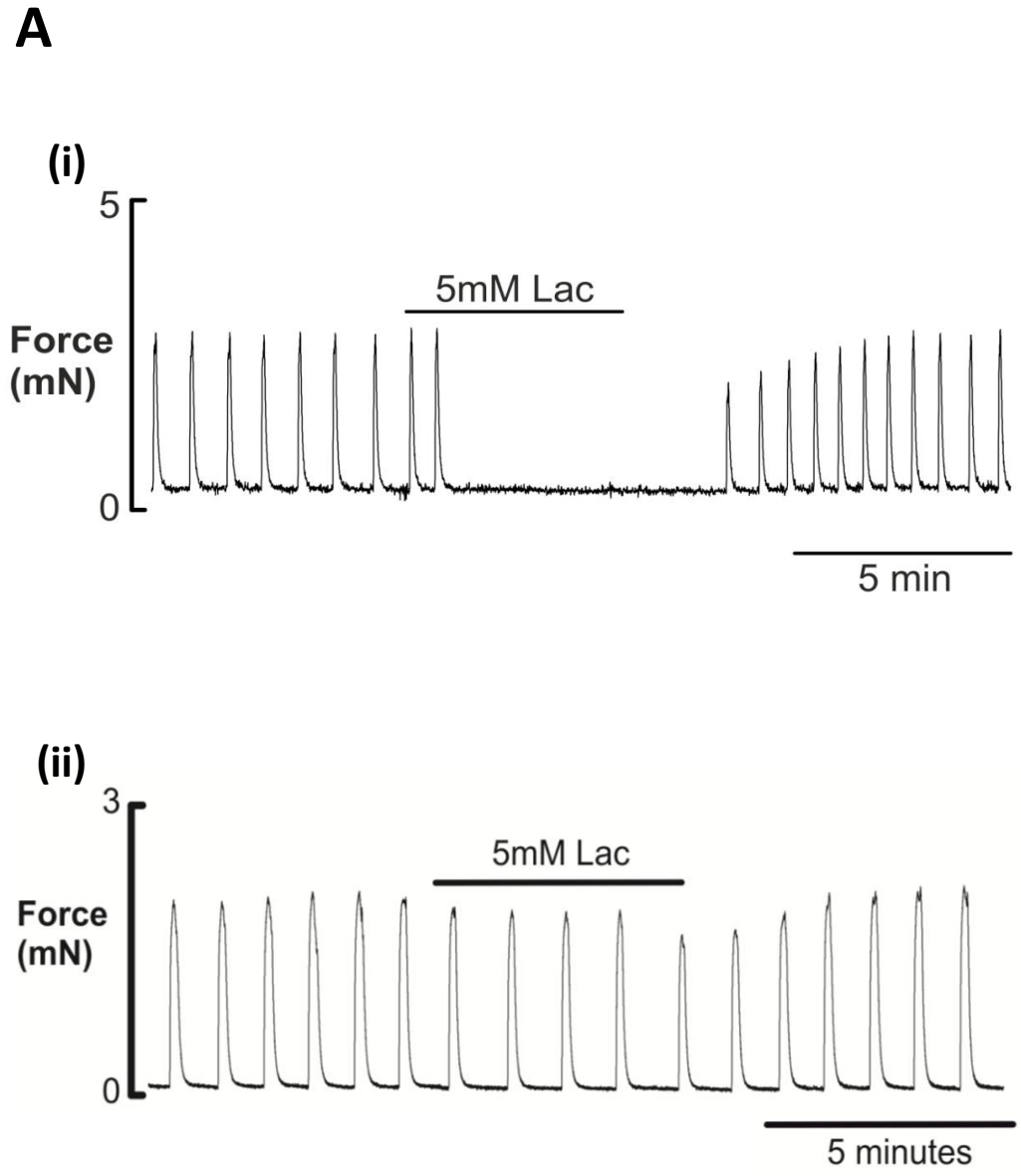


Figure 3.4 The effect of 5mM lactate on spontaneous rat myometrium

A. Representative paired isometric recordings of contracting myometrial strips in the presence of 5mM lactate. Out of 8 myometrial strips tested, 4 continued contracting under lactate (i) and in 4 strips contractions were abolished (ii).

Contractile parameters	1mM lac (% ± S.E.M.) *n*= 7	3mM lac (% ± S.E.M.) *n*= 8	5mM lac (% ± S.E.M.) *n*= 8	10mM lac (% ± S.E.M.) *n*= 7	20mM lac (% ± S.E.M.) *n*= 7
Amplitude	96% ± 10%	70% ± 15%	53% ± 16%*	19% ± 13%*	0% ± 0%*
Duration	98% ± 2%	86% ± 16%	90% ± 2%*	65% ± 9%	N/A
Frequency	97% ± 1%	84% ± 14%	43% ± 12%**	36% ± 13%*	0% ± 0%**
A.U.C.	84% ± 11%	62% ± 14%*	35% ± 13%*	14% ± 10%**	0% ± 0%**

Table 3.1 Changes in contractility in response to 1-20mM sodium lactate in term pregnant rat myometrium

Term (within 24 hours of labour) rat myometrial strips were used from 7-8 animals. Control baseline contractions were obtained 5minutes before lactate application. Tissue strips were superfused with the solutions indicated for 5 minutes. The parameters of contractions were re-measured and expressed as a percentage of control values (i.e. Paired) for the last two minutes of lactate application. Frequency is for the total 5 minute application. Values are means ± standard error of the mean (S.E.M.)
* represents significant differences in contractility compared to preceding control period $p<0.05$ **$p<0.01$ ***$p<0.001$

3.4.2 Oxytocin-induced contractions

To investigate if lactate can affect force under agonist stimulation, the effects of lactate on oxytocin-induced contractions were studied. 0.1nM oxytocin was added to PSS and applied to myometrial strips. This concentration was chosen as it caused an increase in force which could be sustained by the tissue and avoided tonic activity.

Increasing concentrations of lactate (1-40mM) were applied to contracting tissue. This produced a decrease in contractile activity as seen in spontaneous contractions (Figure 3.5, *n* = 3-6). However lactate in the presence of oxytocin and at concentrations previously used, did not abolish force (Figure 3.5 Ai-v). Only 40mM lactate abolished contractility (Figure 3.5 Aiv).

Amplitude, frequency and a.u.c. were significantly decreased at 20mM and 40mM, with duration and a.u.c. of 3mM lactate response also being significantly reduced (Paired students t-test, $p<0.05$). There were significant differences between concentrations and in all parameters (Figure 3.6 Ai-iv). The EC_{50} for lactate in the presence of oxytocin, taken from the a.u.c dose response curve, is 11.1 ± 1.7 mM (Figure 3.6 Aiv). Figure 3.7 compares the effect of 10mM lactate in both spontaneous and oxytocin-driven activity and the greater effect on spontaneous contractility is clearly seen.

Lactate affected contractility to a lesser extent than with spontaneously contracting tissue as shown by mean data (+s.e.m) in Figure 3.8. This was significant at 10mM lactate (Figure 3.7) for amplitude (Figure 3.8 Ai), frequency (Figure 3.8 Aiii) and a.u.c (Figure 3.8 Aiv). The dose response curve for lactate +oxytocin, when plotted against spontaneous activity, is shifted to the right, indicating a higher EC_{50} value.

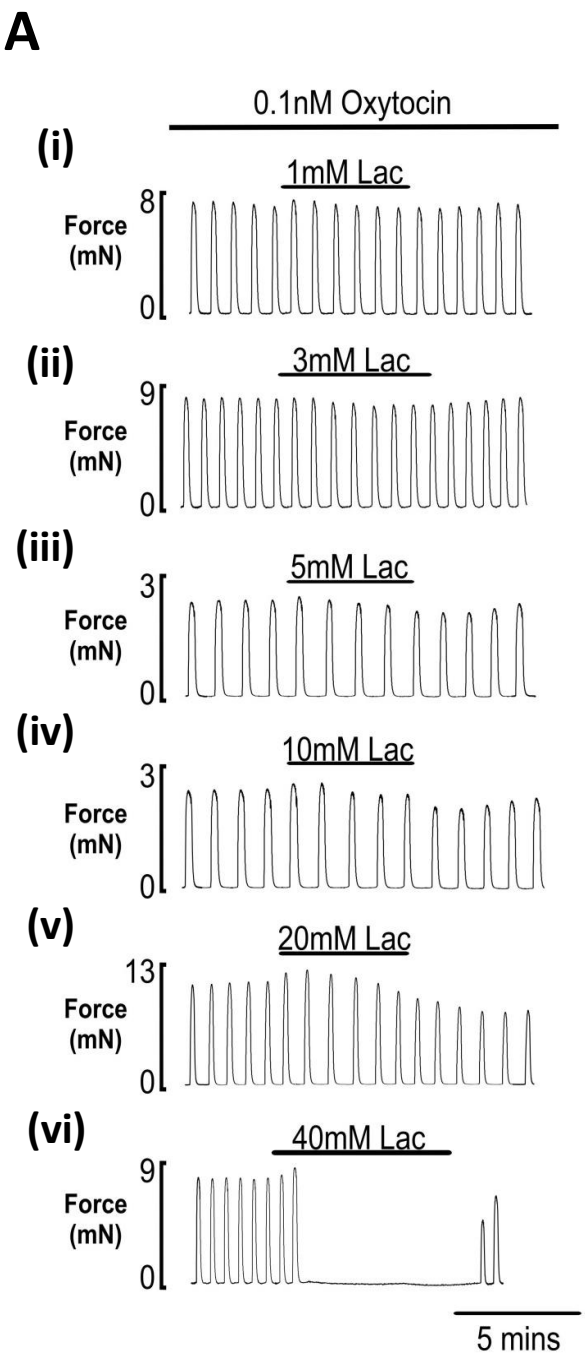


Figure 3.5 Dose dependency of sodium lactate in oxytocin-driven activity

A. Representative isometric recordings of contracting myometrial strips in the presence of oxytocin during 5 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM (v) 20mM (vi) 40mM lactate.

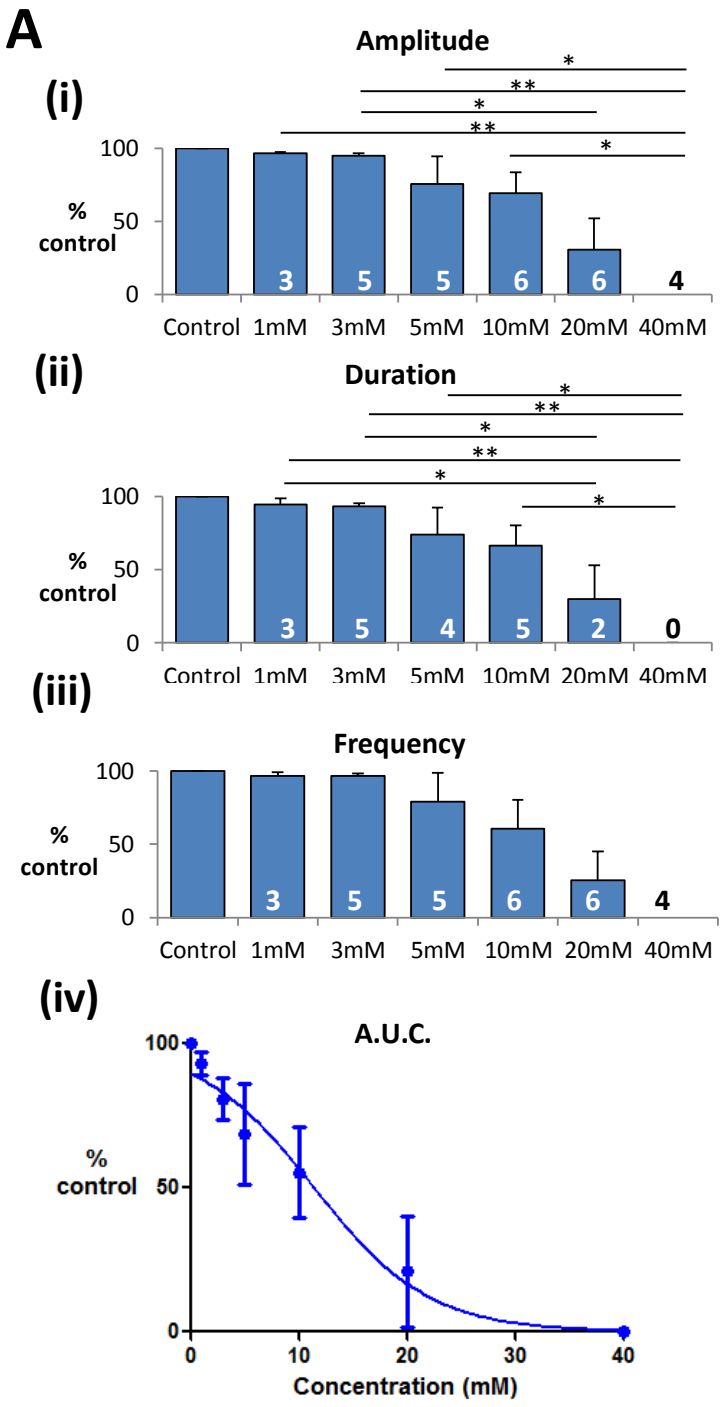


Figure 3.6 Dose dependency of sodium lactate in oxytocin-driven activity

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to lactate. N numbers are denoted by values within bars. A significant difference in activity was found using ANOVA with Bonferroni. * denotes a significance $p<0.05$ **$p<0.01$

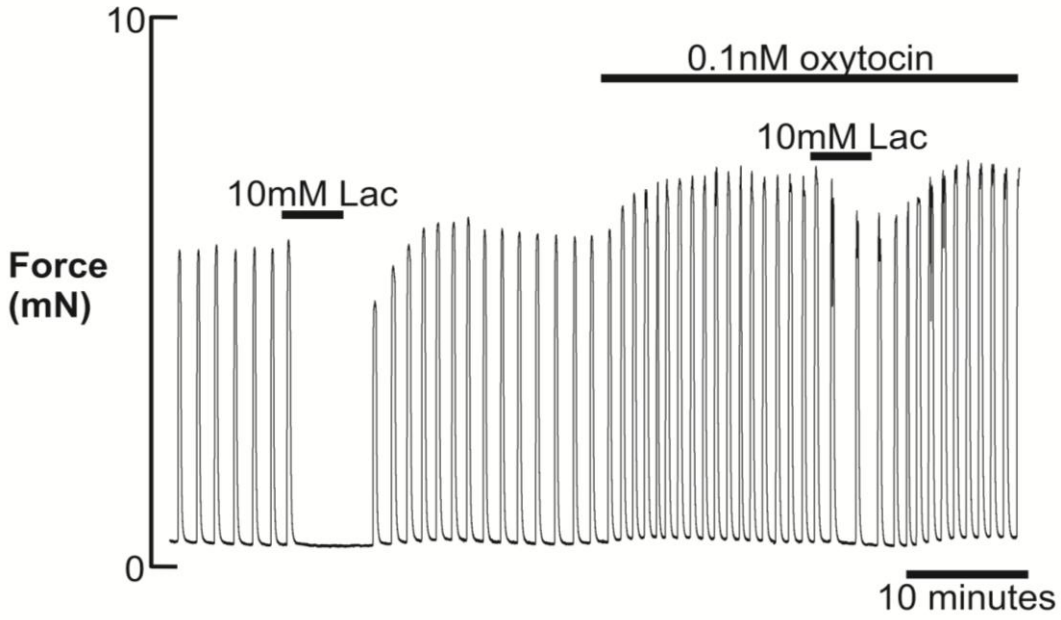


Figure 3.7 A comparison of response to lactate in spontaneous and oxytocin activity

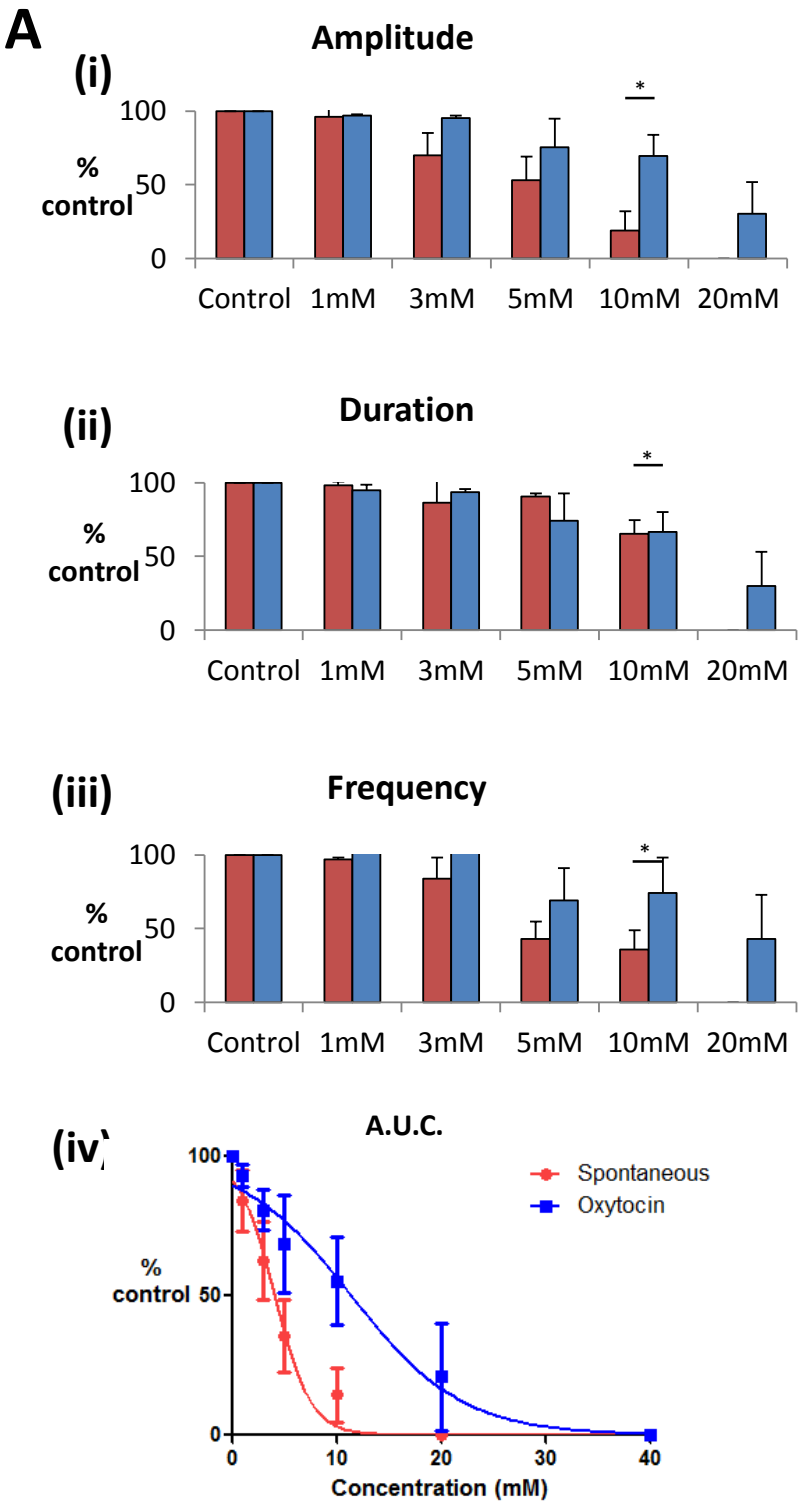


Figure 3.8 A comparison of lactate response in spontaneous and oxytocin activity

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to lactate, when applied to spontaneously contracting term pregnant rat myometrial strips (red bars) and in the presence of 0.1nM oxytocin (blue bars). A significant difference in activity was found using Paired Samples T-Test *denotes $p<0.05$

3.4.3 Comparative effects of other weak acids

To determine if myometrial response were specific to lactic acid, three other weak acids were tested; butyric acid, propionic acid and pyruvic acid at 5mM concentration. These weak acids have pKa values (log quantitative measure of strength of an acid in solution) of 4.87 (propionic acid) 4.82 (butyric acid) and 2.50 (pyruvic acid). These are similar to lactate which has a pKa of 3.86.

These weak bases were also applied to spontaneously contracting tissue for 5 minutes after a control period. The three weak acids were found to produce effects similar to that of lactate: a decrease in force (Figure 3.9). Contractions under sodium propionate (*n*=6) sodium butyrate and sodium pryuvate (*n*=3) decreased in amplitude (Figure 3.9 Ai), duration (Figure 3.9 Aii), frequency (Figure 3.9 Aiii) and A.U.C. (Figure 3.9 Aiv). There was no significant difference between these weak acid responses. Because of this, only one weak acid (sodium propionate) was used to repeat the full range of concentrations used in the previous lactate experiment in **Chapter 3.4.1**.

1-20mM propionate was applied to spontaneously contracting tissue and there was a dose-dependent decrease in force (*n*=6) (Figure 3.10 A). 10mM and 20mM propionate was significantly reduced when compared to control (100%) in all four parameters. Frequency under 3mM and 5mM propionate was significantly reduced (58.5% ± 16.3% p=0.03, 36.3% ± 18.6% p=0.01 respectively, students paired t-test). Force amplitude was also significantly reduced in 1mM propionate (92.5% ± 2.6% p=0.025) Mean data for all four parameters shows there were significant differences between concentrations (Figure 3.11 Ai-iv). The EC_{50} for propionate on spontaneous tissue, measured from the dose-response curve, is 3.2±0.9 mM (Figure 3.11 Aiv).

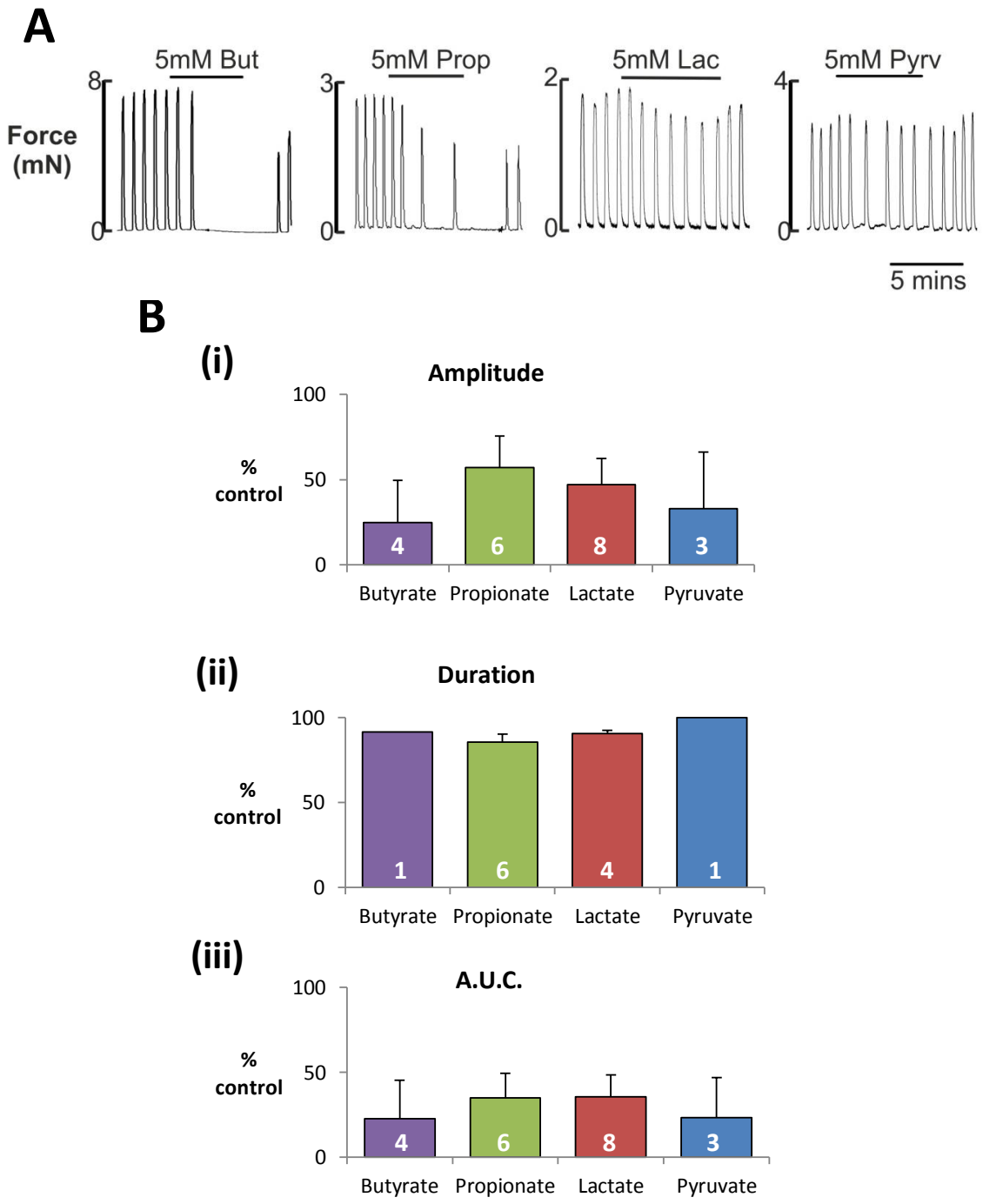


Figure 3.9 A comparison of weak acids

A. Representative traces for 5 minute application of four weak acids tested.
B. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to 5mM sodium butyrate (purple bars), sodium propionate (green), sodium lactate (red) or sodium pyruvate (blue), when applied to spontaneously-contracting term pregnant rat myometrial strips. N numbers are denoted by values within bars. There was no significant difference between the weak acid responses.

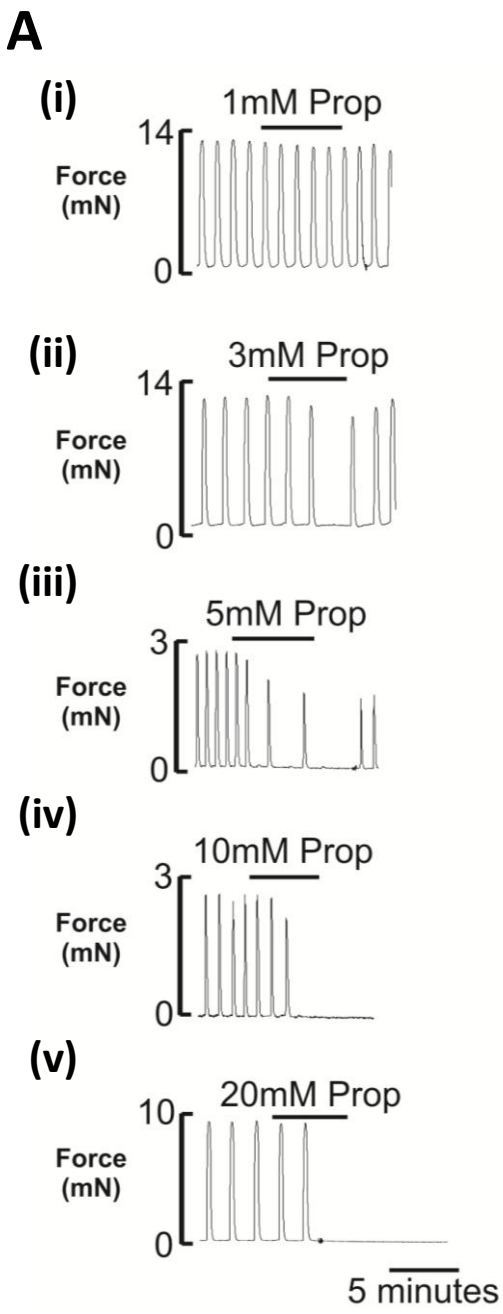


Figure 3.10 Dose dependency of sodium propionate in pregnant rat myometrium

A. Representative isometric recordings of spontaneously contracting myometrial strips during 5 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM and (v) 20mM propionate.

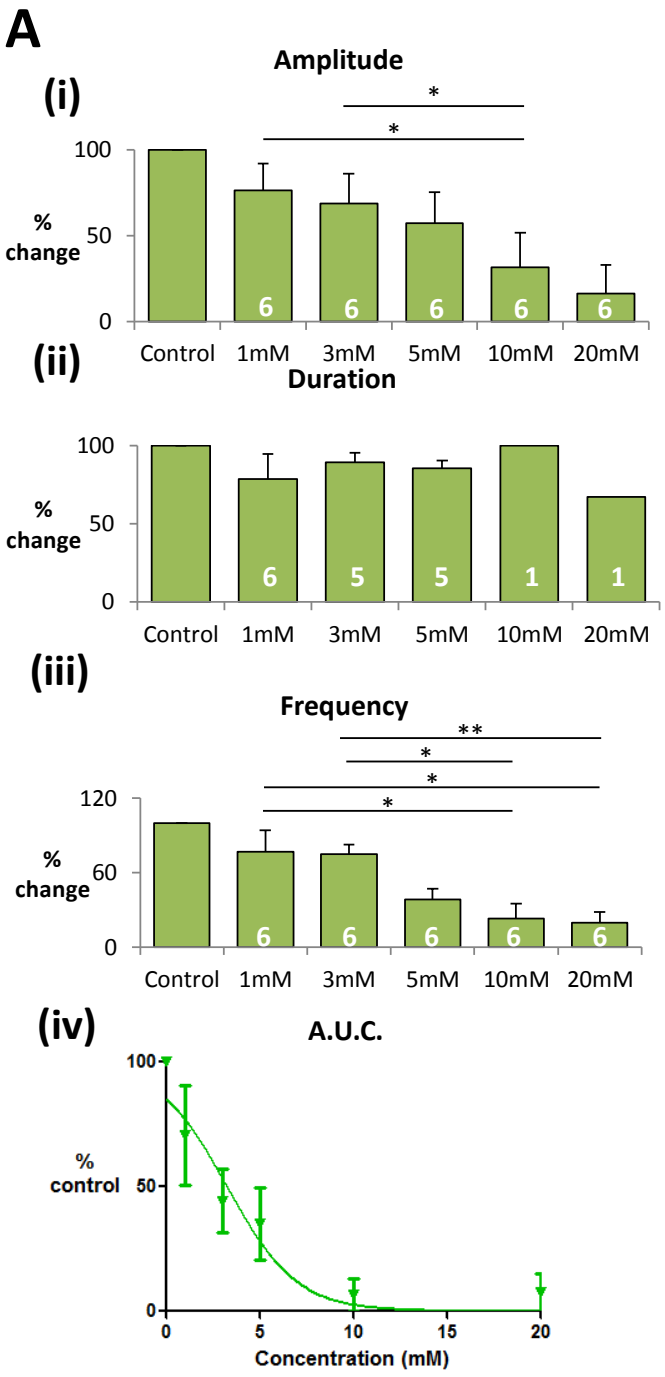


Figure 3.11 Dose dependency of sodium propionate in pregnant rat myometrium

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to propionate. N numbers are denoted by values within bars. A significant difference in activity was found using ANOVA with Bonferroni. * denotes a significance $p<0.05$ **$p<0.01$

Propionate was also tested on oxytocin-driven activity (Figure 3.12 and 3.13). This reduced contractions in all preparations (*n*=3-6) and this response was similar to lactate under oxytocin stimulation. Amplitude, duration, frequency and a.u.c. were significantly decreased at 20mM, with duration and a.u.c. of 10mM lactate and duration of contractions in 3mM lactate also being significantly reduced (Paired students t-test, $p<0.05$). The EC_{50} for propionate under oxytocin is 9.6±1.1 mM (Figure 3.13 Aiv).

Mean data for lactate and propionate was plotted together to compare the two weak acid responses. There is no significant difference between lactate and propionate on spontaneous activity, in amplitude (Table 3.2 Ai), duration (Table 3.2 Aii), frequency (Table 3.2 Aiii) or a.u.c. (Table 3.2 Aiv). There was also no significant difference in responses between propionate and lactate in the presence of oxytocin for all parameters (Table 3.3).

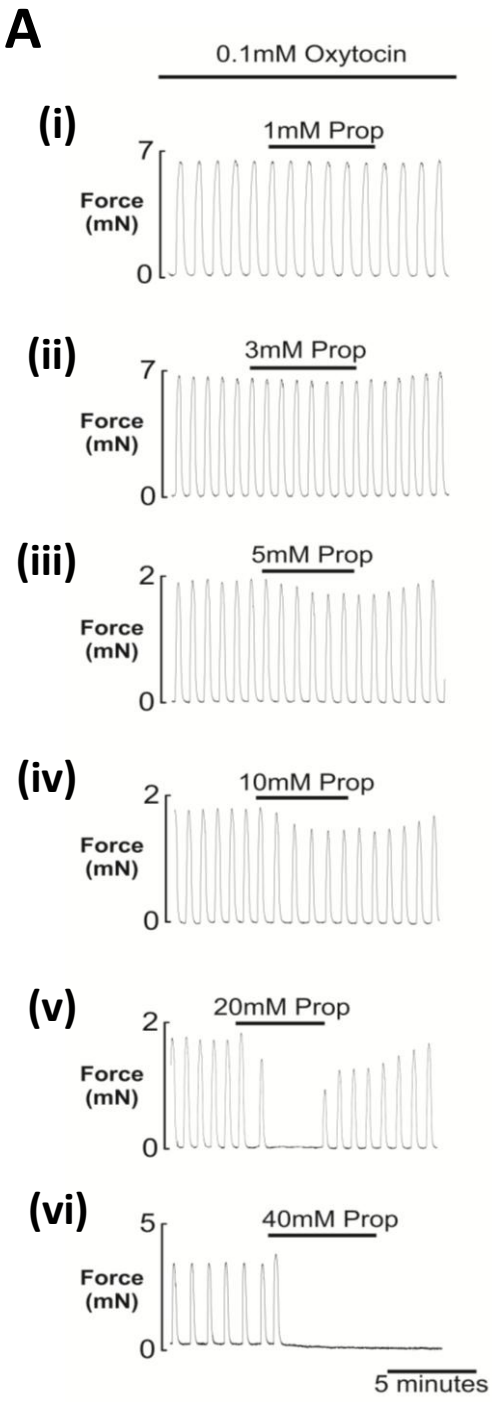


Figure 3.12 Dose dependency of sodium propionate in oxytocin-driven activity

A. Representative isometric recordings of contracting myometrial strips in the presence of oxytocin during 5 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM (v) 20mM (vi) 40mM propionate.

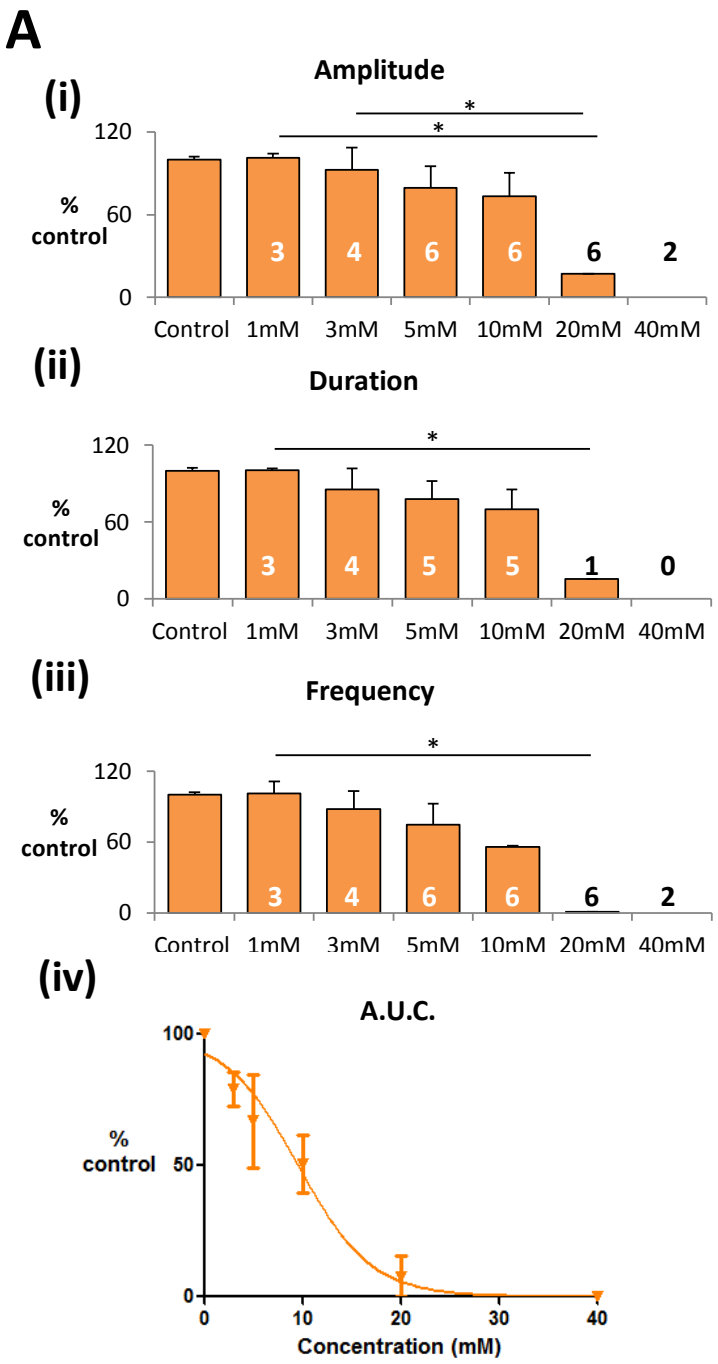


Figure 3.13 Dose dependency of sodium propionate in oxytocin-driven activity

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to propionate. N numbers are denoted by values within bars. A significant difference in activity was found.

A

(i) Amplitude

	Lactate	Propionate	P value
1mM	96.0±2.0%	76.5±15.5%	0.28
3mM	69.8±15.3%	68.9±17.3%	0.90
5mM	47.3±15.3%	57.3±18.3%	0.68
10mM	19.5±12.7%	31.6±20.3%	0.78
20mM	0	5.0±0%	-

(ii) Duration

	Lactate	Propionate	P value
1mM	98.3±2.2%	78.5±16.0%	0.36
3mM	86.2±15.9%	89.2±6.3%	0.88
5mM	90.5±2.2%	85.6±4.8%	0.07
10mM	65.3±9.1%	100. 0±0%	0.19
20mM	0	67.1±0%	-

(iii) Frequency

	Lactate	Propionate	P value
1mM	96±2%	76.7±17.4%	0.20
3mM	80±20%	75.0±7.4%	0.67
5mM	39±13%	38.4±8.5%	0.58
10mM	33±15%	22.8±12.1%	0.71
20mM	0	19.6±8.5%	-

(iv) A.U.C.

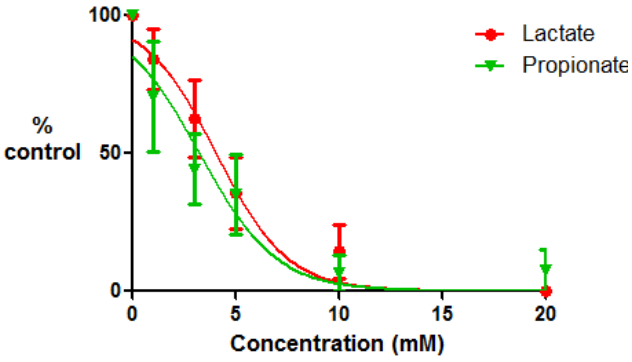


Table 3.2 A comparison of lactate and propionate response in spontaneous activity

A. Mean data ± s.e.m. showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency and (iv) AUC in response to lactate and propionate, when applied to spontaneously contracting term pregnant rat myometrial strips. There was no significant difference between the two weak acids.

A

(i) **Amplitude**

	Lactate	Propionate	P value
1mM	96.7±0.8%	101.3±2%	0.18
3mM	95.0±1.9%	92.7±3.2%	0.45
5mM	75.6±19.1%	79.7±16.2%	0.87
10mM	69.5±14.2%	73.3±15.3%	0.86
20mM	30.4±21.5%	17.2±17.2%	0.63

(ii) **Duration**

	Lactate	Propionate	P value
1mM	99..4±5.6%	100.2±2.2%	0.92
3mM	93.5±2.0%	82.8±2.8%	0.01*
5mM	92.5±1.2%	93.4±6.84%	0.88
10mM	79.9±3.8%	83.8±3.3%	0.87
20mM	89.6±12.1%	91.5±15.3%	0.56

(iii) **Frequency**

	Lactate	Propionate	P value
1mM	96.7±2.6%	101.0±2%	0.12
3mM	96.7±1.8%	87.9±10.2±	0.69
5mM	78.9±19.9%	74.5±15.5%	0.62
10mM	60.8±19.3%	56.0±17.8%	0.97
20mM	25.4±19.7%	1.0±0%	0.80

(iv) **A.U.C.**

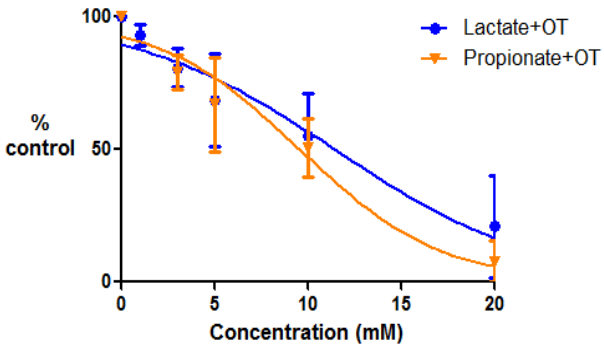


Table 3.3 **A comparison of lactate and propionate response in oxytocin-driven activity**

A. Mean data ± s.e.m. showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency and (iv) AUC in response to lactate and propionate, when applied to term pregnant rat myometrial strips in the presence of oxytocin. There was no significant difference between the two weak acids.

3.4.4 Mechanisms underlying the effect of lactate

3.4.4.1 Intracellular pH

Using the fluorescent indicator Carboxy-SNARF allows for the simultaneous measurement of intracellular pH (pH_i) and force. Loading myometrial tissue with this indicator has been shown not to alter contractile activity (Taggart & Wray 1993a). The application of 5mM lactate to loaded myometrial strips, either spontaneously active or quiescent, altered the pH_i (Figure 3.14). Lactate significantly decreased the internal pH by 0.13±0.01 pH units (*n*=10, p=0.002 Paired students t-test). Values obtained for those continuing to contract under 5mM lactate and those that stopped contracting were similar. When lactate was removed there was a rebound alkalinisation and pH_i returned to control values. Increasing concentrations of lactate caused a significant dose dependent decrease in pH_i (Figure 3.14): 10mM caused a 0.19± 0.02 pH unit drop (*n*=5, p=0.02) and 20mM lactate decreasing pH_i by 0.24±0.03 pH unit drop (*n*=5, p=0.001). There was a significant difference between 5mM and 20mM lactate (Figure 3.14).

5mM lactate was applied in the presence of 0.1nM oxytocin (Figure 3.15). There are small notches in the baseline, in both spontaneous and oxytocin-driven activity, which represents normal acidification associated with contractions and has been shown in previous work (Taggart & Wray 1993b). Contractions reappear when lactate is removed and pH_i increases. Lactate caused a pH drop of 0.09±0.01pH units (*n*=4, p=0.001) which was significantly different to the drop in spontaneous contractions (p=0.04, unpaired students t-test) (Figure 3.15).

5mM butyrate was used in some experiments as a reference marker, as this is a more widely

used and studied weak acid in pH experiments, and has been shown before to decrease pH_i in the myometrium (Wray). Butyrate caused a similar drop in pH_i to lactate (0.13±0.02 pH units, p=0.002) (Figure 3.16) which is in agreement with previously seen values (Taggart & Wray 1993b). This was not significantly different to the drop seen with 5mM lactate (p=0.98, students unpaired t-test) (Figure 3.16).

To reinforce the theory that drop in pH_i is causing the decrease in force; I used a weak base simultaneously with lactate to 'null' the pH change. Ammonium chloride is a commonly used weak base and has been shown previously to cause intracellular alkalinisation of the myometrium (Parratt, Taggart & Wray 1995b). 5mM NH_4Cl was simultaneously applied with 5mM lactate to spontaneously contracting pregnant rat myometrium for 5 minutes (*n*=2). As seen in Figure 3.17 A, contractions continued in the presence of the weak base and acid and pH_i fluctuated only slightly. This effect on contractility is also seen using 5mM butyrate (Figure 3.17 B)

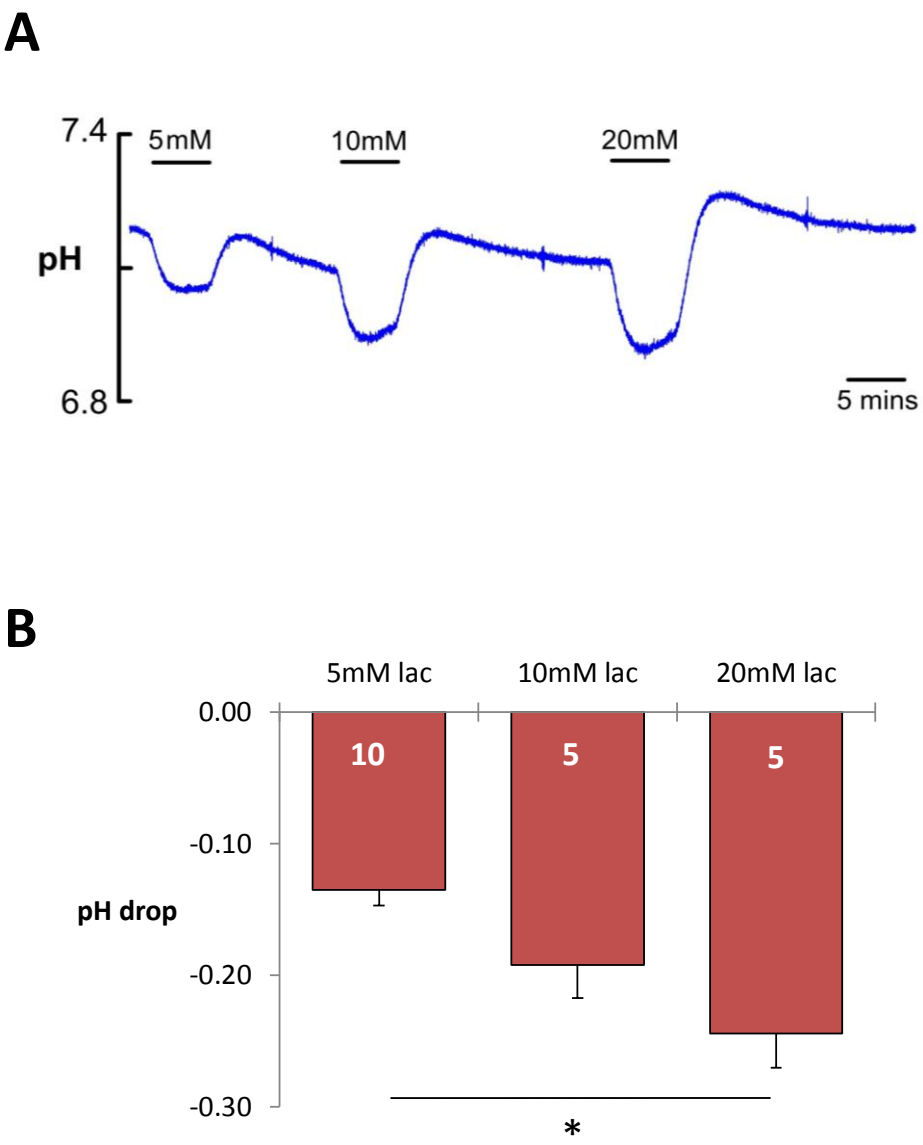


Figure 3.14 The effect of lactate on intracellular pH

All term pregnant myometrial strips were loaded with Carboxy-Snarf 1 AM, excited at 540nm and emitted fluorescence recorded at F590:F640.

A: Significant dose dependent decrease in pH_i in response to 5mM, 10mM and 20mM sodium lactate (5 minutes). Force not shown (*n*=5-8).
B: Mean (+ s.e.m.) data for pH drop of lactate (5mM-20mM, red bars). *N* numbers are shown in their respective bars. There was a significant difference between 5mM lactate and 20mM lactate (p=0.002, unpaired students t-test)

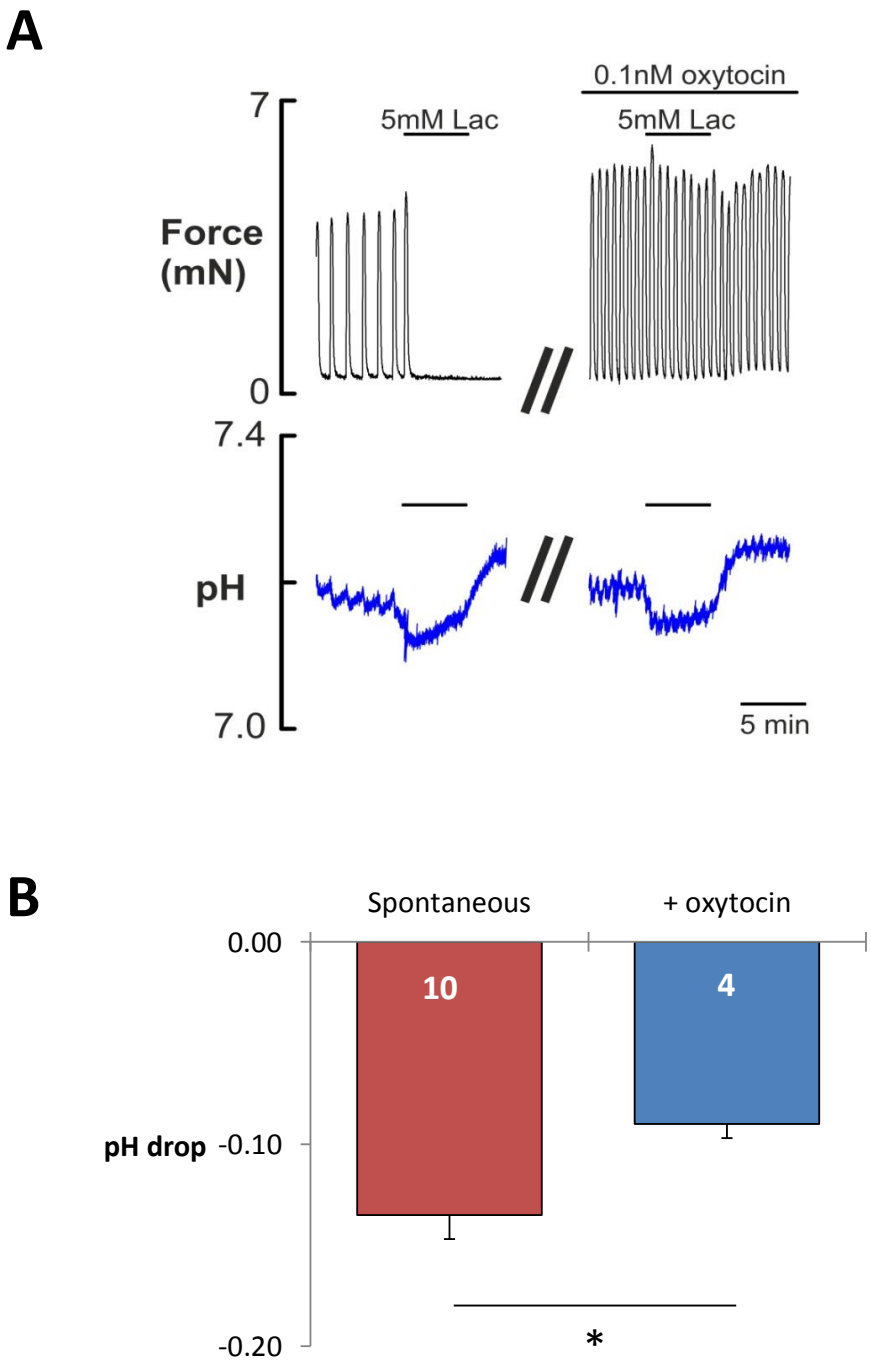


Figure 3.15 The effect of lactate on intracellular pH of oxytocin-driven contractions

A: Simultaneous recording of force and pH_i in the presence of 5mM lactate (5 minutes) on spontaneous and oxytocin-driven activity (*n*=4). Small notches in baseline are associated with normal contractions.
B: Mean (+ s.e.m.) data for pH drop of 5mM lactate on spontaneously active tissue (red bar) compared to the drop in the presence of 0.1nM oxytocin (blue bar). *N* numbers are denoted by values within bars. The pH drop in oxytocin was significantly different to spontaneous activity (p=0.042, unpaired student t-test)

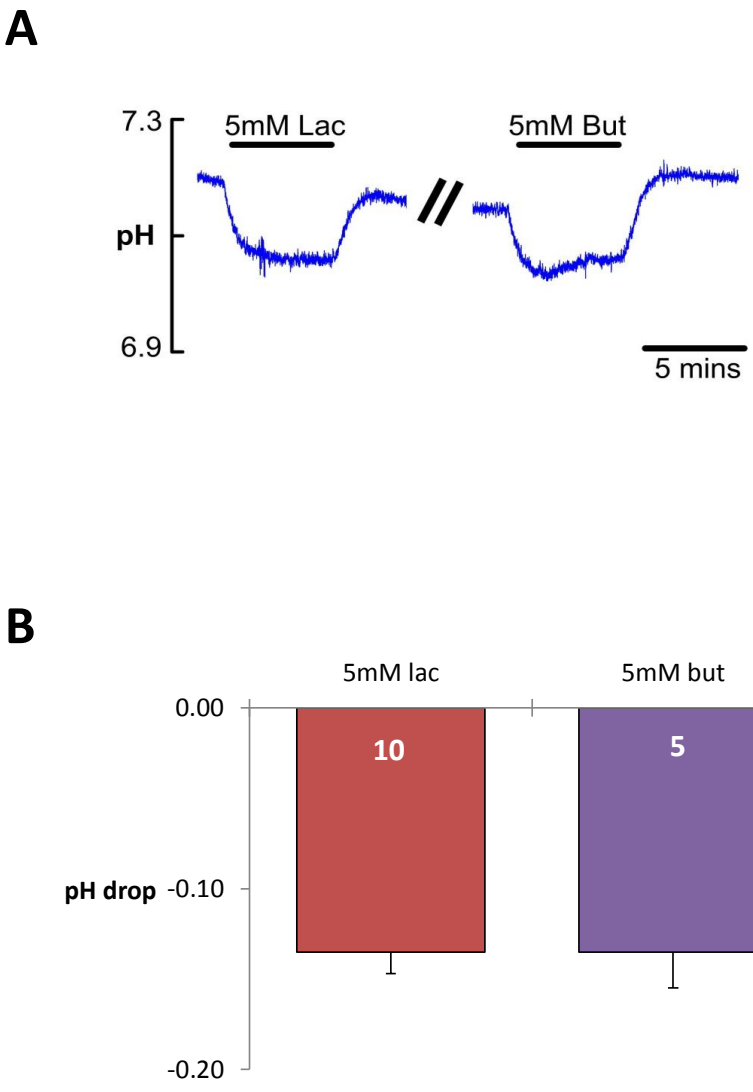


Figure 3.16 The effect of lactate and butyrate on intracellular pH

A: 5mM butyrate decreased pH_i to the similar extent as lactate. Force not shown. (*n*=5)
B: Mean (+ s.e.m.) data for pH drop of lactate (5mM, red bar) and 5mM butyrate (purple bar). *N* numbers are shown in their respective bars. There was no significant difference between lactate and butyrate.

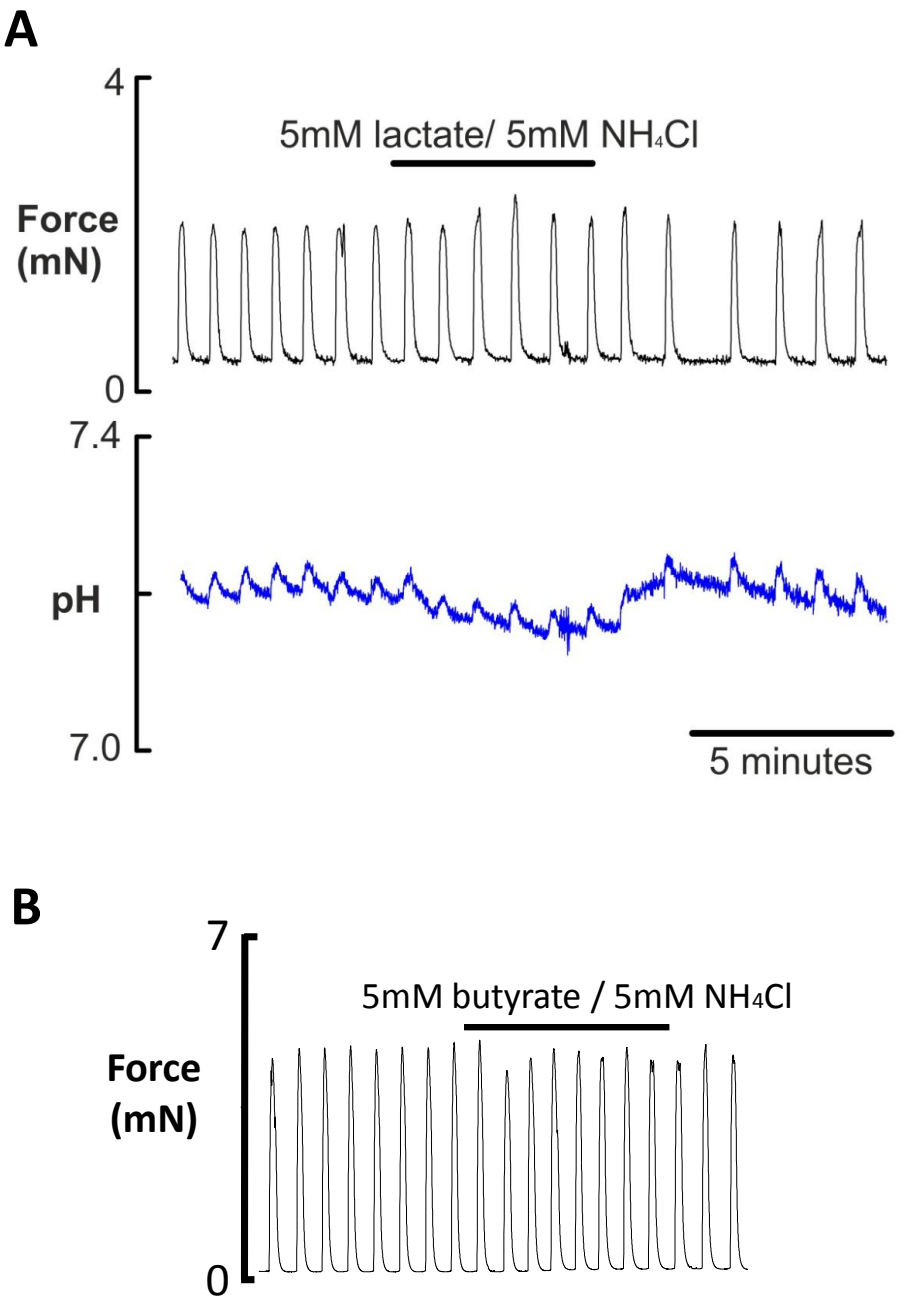


Figure 3.17 pH null experiment using lactate and ammonium chloride

A: Simultaneous force and pH_i measurements of spontaneously contracting term rat myometrium. 5mM lactate and 5mM ammonium chloride were applied simultaneously for 5 minutes (*n*=2).
B: Force only measurements using 5mM sodium butyrate and 5mM ammonium chloride (*n*=2)

3.4.4.2 Intracellular Ca^{2+}

The effect of lactate on intracellular calcium signalling was investigated by using Indo-1 AM. 7 strips were loaded and 5mM lactate applied for 5minutes. 3/7 of strips continued contracting in the presence of lactate and were used in statistical analysis. In the presence of lactate, calcium transients decreased in amplitude (88.8%± 2.6%) (Figure 3.18 A). This was mirrored in the force. Once abolished, transients (and therefore contractions) were inhibited throughout lactate application. Calcium transients reappeared when lactate was removed with increasing amplitude and this was reflected in the force measurements, which returned to previous control levels. Duration (in the last two minutes of lactate application) and frequency (in the total application) of calcium transients were significantly decreased when compared to control contractions (100%): Duration to 79.5 ±2.0% ($p=0.01$); frequency to 77.8±0.6% ($p=0.01$). Amplitude was not significantly reduced ($p=0.12$). Further analysis of the last contraction under lactate, when compared with control, shows the decrease in duration is due to the number of spikes present in the calcium transient (Figure 3.18 B). Calcium spikes are caused by calcium entry triggered by individual action potentials and triggers phasic contraction (Burdyga, Wray & Noble 2007). The mean number of spikes present in the transient significantly falls from 20±6 to 13±5 in the presence of lactate ($n=3$, $p>0.05$)

In order to determine if the effects of lactate were due to inhibition of L-type Ca^{2+} channels, Bay K-8644, a Ca^{2+} channel agonist was applied to spontaneously contracting tissue ($n=6$). In all preparations application of Bay K-8644 stimulated spontaneous contractions (Figure 3.19 A) consistent with its ability to increase the opening probability of L-type Ca^{2+} channels (Yoshino, Nishio & Yabu 1988). 5mM Lactate was applied in the presence of Bay K-8644 for

10minutes. This had a small but significant effect on contractility as shown in the mean data (Figure 3.19 B) when compared to preceding contractions in Bay K-8644 (control 100%). Note the x-axis on the graph starts at 85%. Duration of contractions significantly decreased to 92.5±1.9% (p=0.01 paired students t-test) and overall A.U.C. decreased to 93.9±2.3% (p=0.04). Amplitude and frequency of contractions were not significantly altered (99.7±0.7% p=0.73, 100.9±2.2% p=0.69 respectively)

When compared to the effect of lactate on spontaneous activity, amplitude frequency and a.u.c increased in the presence of lactate and Bay K8644 when the last 2 minutes of activity were compared. Amplitude was significantly increased by 110.7% (p=0.01, Student unpaired t-test), frequency increased by 139.4% (p=0.01) and a.u.c. by 150.6% (p=0.03). Duration of contractions did not differ (p=0.84).

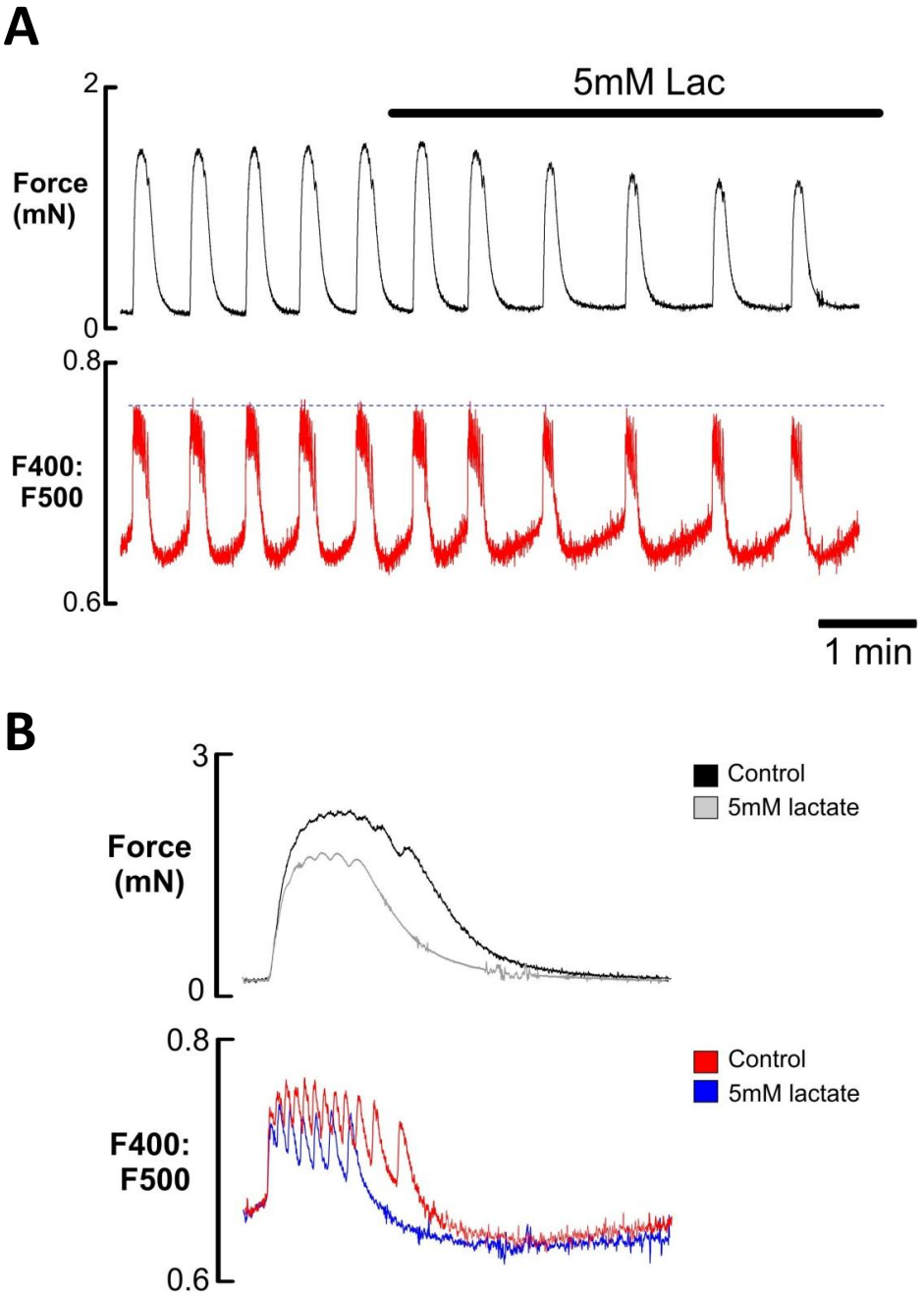


Figure 3.18 Effect of lactate on intracellular Ca^{2+} signalling in the myometrium

A. Simultaneous force and intracellular Ca^{2+} recording, using Indo-1 AM, in spontaneously contracting rat myometrium in the presence of 5mM lactate. Emitted fluorescence is shown as F400:F500 (*n*=3).
B. The last contraction in the control period (black line) overlaid with the last contraction in the presence of lactate (grey line) with the corresponding intracellular Ca^{2+} signal (red and blue respectively)

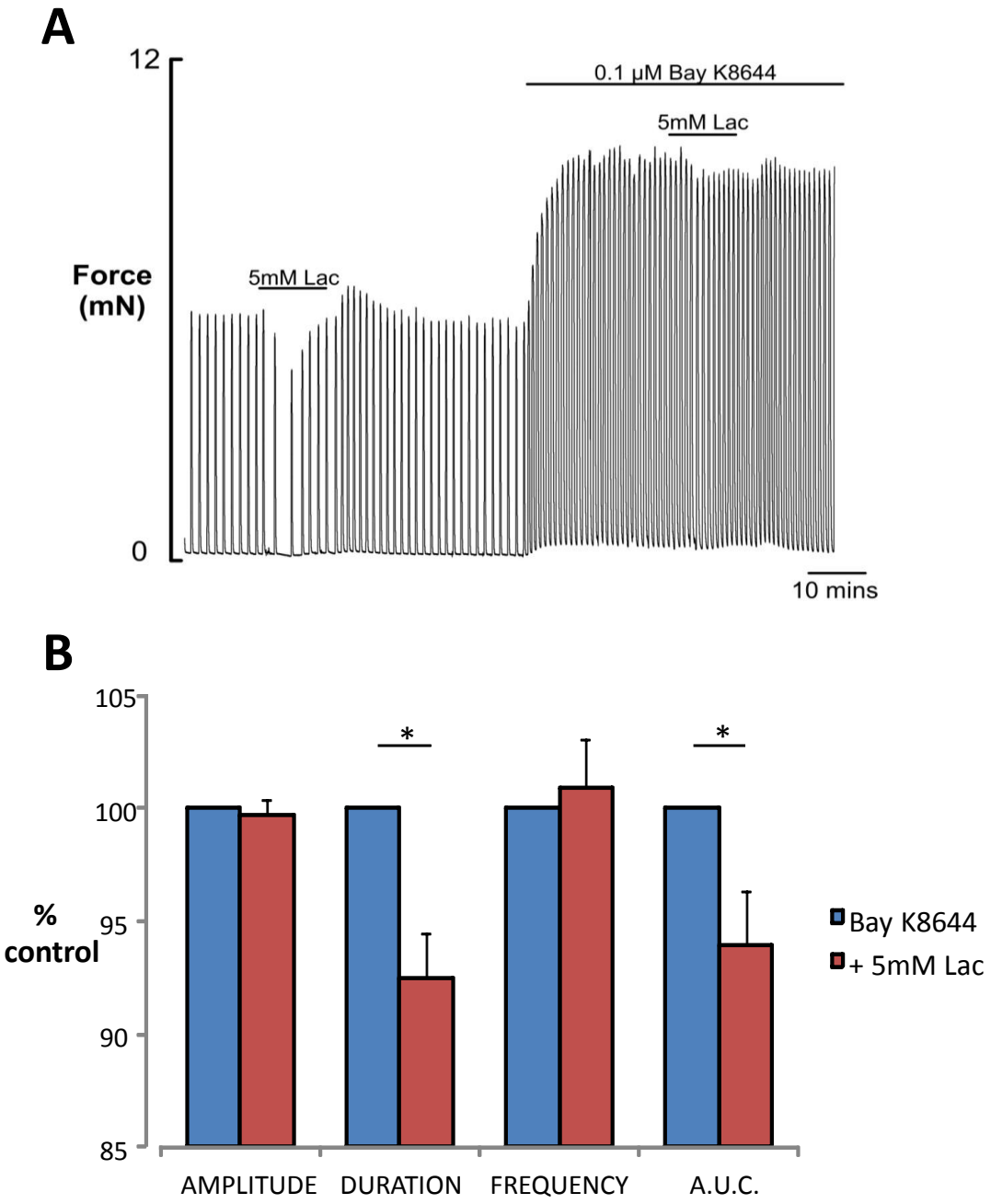


Figure 3.19 The effect of lactate in the presence of Bay K8644, a Ca^{2+} channel agonist

A. A representative force trace to show 5mM lactate (10 minutes) effect on spontaneously contracting tissue in comparison to its effect in the presence of 0.1 µM Bay K8644.
B. Mean data (± s.e.m.) for all four contractile parameters analysed before (blue bar) and during (red bar) lactate application. (*n*=6). There was a significant difference in duration and overall a.u.c. of contractions with lactate.

3.4.4.3 KCl

Lactate was applied to depolarised myometrium. The depolarization was produced by applying 40mM KCl solution. These experiments were performed to investigate if the effects of lactate are beyond surface membrane excitability i.e. can lactate still reduce force if the membrane is held depolarized and Ca^{2+} entry maintained?

Application of KCl solution produced a rapid increase in force (to the peak amplitude) which then declined to a level and plateau throughout the application of solution (Figure 3.20). This was a repeatable response that was seen in all preparations and there was no difference between peak amplitudes or a.u.c. of all KCl responses (*n*=8, p=0.7 unpaired t-test) (Figure 3.20 B). 10mM lactate, in the presence of KCl, was applied after 2.5mins of KCl application for 5 minutes (*n*=6) (Figure 3.20 A). Force in the presence of lactate was, surprisingly, significantly *increased* by 18.9±4.5 % when compared to force at 2.5minutes, directly before lactate was applied (p=0.044, unpaired t-test). Force decreased when lactate was removed and returned to basal levels when KCl was removed. However the a.u.c. did not reach significance with lactate (p=0.54) Simultaneous Ca^{2+} measurements show an increase in intracellular Ca^{2+} when lactate is applied.

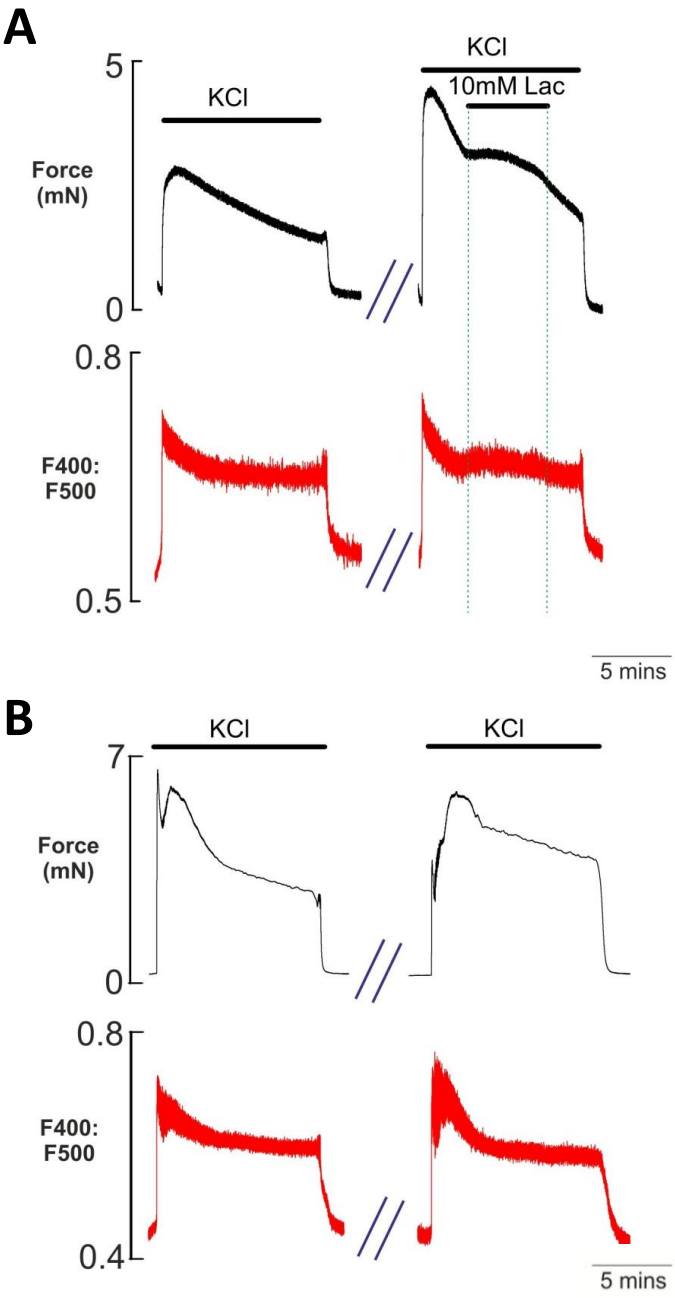


Figure 3.20 Effect of lactate on Ca^{2+} entry

Depolarisation of the membrane using 40mM KCl (10 minutes) in term pregnant myometrial strips loaded with Indo-1 AM.

A. Simultaneous force and intracellular Ca^{2+} recording of the effect of 10mM lactate in the presence of KCl after an initial 10minute application of KCl. (*n*=6)
B. KCl was applied for 10 minutes after the first KCl application as a control. (*n*=2)

3.4.5 Gestational dependent effects of lactate

The above data were all obtained on late-pregnant myometrium. To investigate if the lactate data were specific to the animal being close to labour, I investigated if the effect of lactate changed during pregnancy or with the onset of labour. Lactate was applied to spontaneously contracting myometrial strips from rat uterus at varied gestational states; non-pregnant (*n*=6), 11day pregnant (*n*=4), 18day pregnant (*n*=6), term pregnant (*n*=8) and labouring myometrium (*n*=5) (Figure 3.21).

After a control period of 10minutes spontaneous activity, 5mM lactate was applied extracellularly to tissue strips for 10minutes (Figure 3.21 A). This was in contrast to previous experiments as the frequency of contractions in non-pregnant and 18d tissue was low i.e. ≤2 contractions in 5 minutes. A longer application time was used to allow the full effect of lactate on frequency to be seen. In analysis of contractility, amplitude, duration and A.U.C. were measure from the last 5 minutes of application. This is so the data can be compared to term pregnant rat tissue that was exposed to 5 minutes of lactate in **Chapter 3.4.1**. The last 2 minutes of lactate in term tissue was used in analysis.

Lactate had little effect on non-pregnant and 11day myometrium (Figure 3.21 Ai, Aii respectively). As gestation progressed to term, lactate had increasing effects on contractility (Figure 3.21 Aiii-iv). At 18day gestation, 5mM lactate had a significant effect on force amplitude (34.8±21.4% p=0.05) and frequency (47.4±10.0%, p=0.04). After labour was initiated, surprisingly, lactate had little effect on contractility, similar to non-pregnant tissue (Figure 3.21 Av). Mean data shows amplitude, duration, frequency and a.u.c. reduced under lactate at gestations >11day until labour onset (Figure 3.21 B).

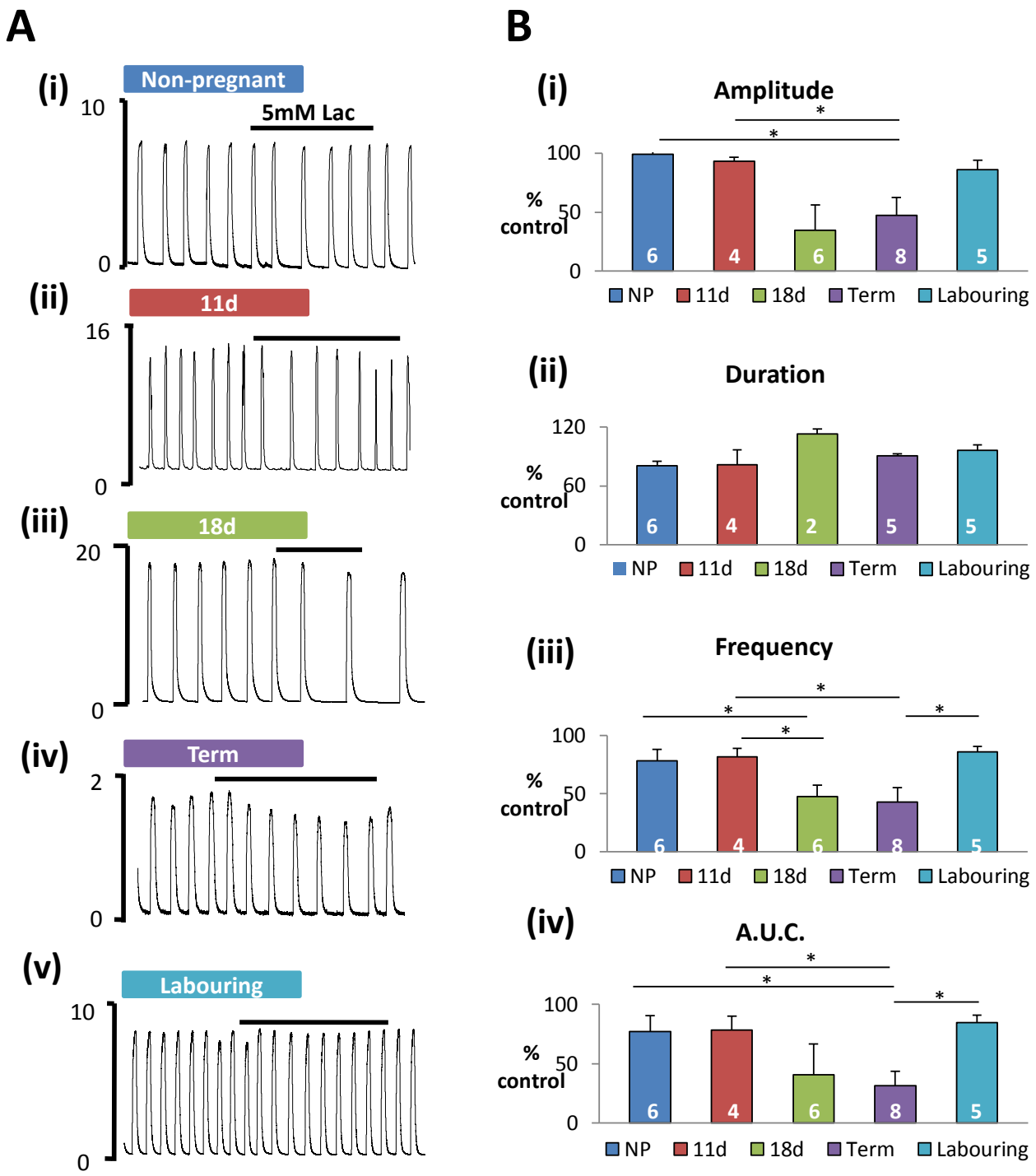


Figure 3.21 The effect of lactate on rat myometrium throughout gestation

A. Representative isometric recordings of spontaneously contracting myometrial strips from (i) non-pregnant (ii) 11d gestation (iii) 18d gestation (iv) term pregnant and (v) labouring uterus during 5 minutes application of lactate.
B. Mean data ± s.e.m. (denoted by error bars) for (i) amplitude (ii) duration (iii) Frequency and (iv) A.U.C. for all gestations. N numbers are denoted by values within bars. A significant difference in activity was found using Anova with Bonferroni. * denotes $p<0.05$ significance.

There was a significant difference in amplitude (Figure 3.21 Bi), frequency (Figure 3.21 Biii) and a.u.c (Figure 3.21 Biv) between gestations ($p>0.05$, Anova with Bonferroni *post-hoc* test).

When comparing 5mM lactate response between non-pregnant myometrium and term pregnant myometrium, there was a significant difference in amplitude ($p=0.02$, unpaired students t-test) and a.u.c. ($p=0.05$) (Figure 3.22 Ai and iii respectively). There was no significant difference in duration ($p=0.10$) or frequency ($p=0.08$).

There was a significant difference in response to 5mM lactate between term pregnant myometrium and labouring tissue. Contractions under lactate were significantly increased in labouring myometrium (99.7%±4.8% when compared to term-pregnant 100%) ($p=0.02$) and overall a.u.c. was increased to 167.5%±6.6% ($p=0.01$). Amplitude, although not significant, was increased in labouring tissue ($p=0.09$). There was little difference in duration between the two tissues (labouring tissue 6.5%±5.4% larger duration than term, $p=0.36$). I performed a dose-response experiment on labouring tissue and found higher concentrations were needed to abolish contractions ($n=2$) (Figure 3.22 A). To investigate why there was a dramatic difference between these two gestations, I loaded labouring myometrial strips with Carboxy-Snarf to investigate intracellular pH ($n=2$) (Figure 3.23 B). Figure 3.23 Bi shows how, in the presence of lactate, there is a drop in pH_i and contractions continue. When this drop is compared with that seen in term pregnant tissue, it appears the drop is less significant (0.09±0.02) (Figure 3.23 Bii). Repeat experiments are needed before statistical analysis can be performed.

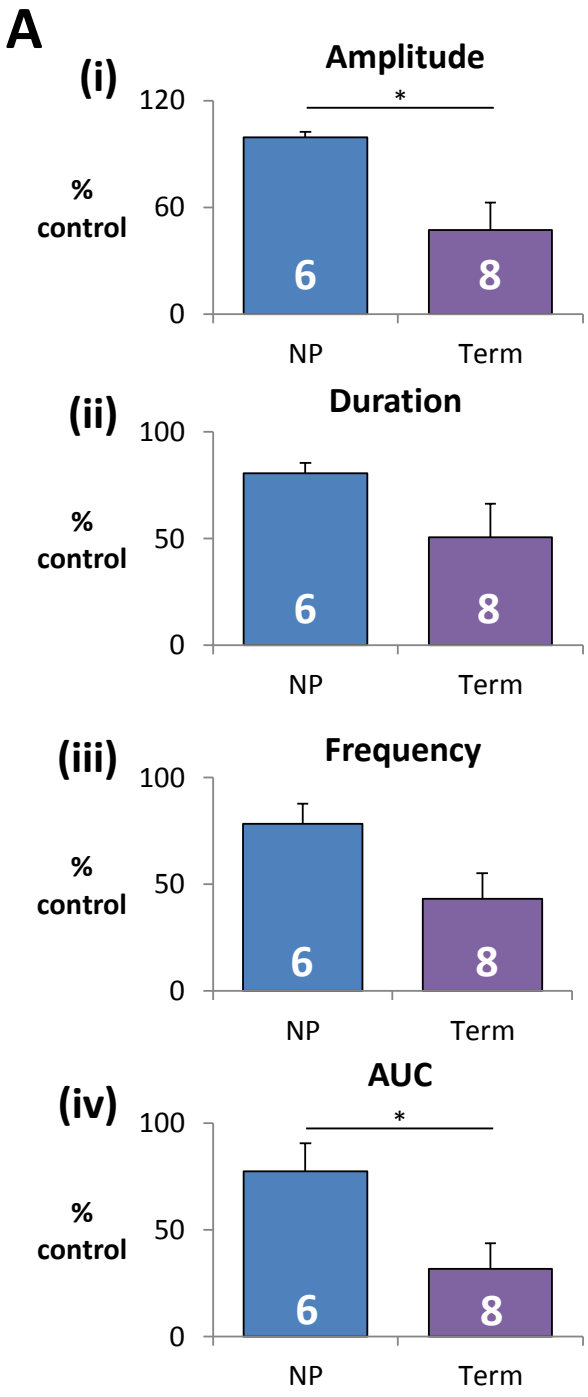


Figure 3.22 Comparison of response to 5mM lactate between non-pregnant and pregnant myometrium.

A. Mean data (± s.e.m.) for (i) amplitude (ii) duration (iii) frequency and (iv) A.U.C. for non-pregnant myometrium (blue bar) and term pregnant myometrium (purple bar). N numbers are denoted in the relevant bars. * denotes $p<0.05$ significance value found by unpaired students t-test.

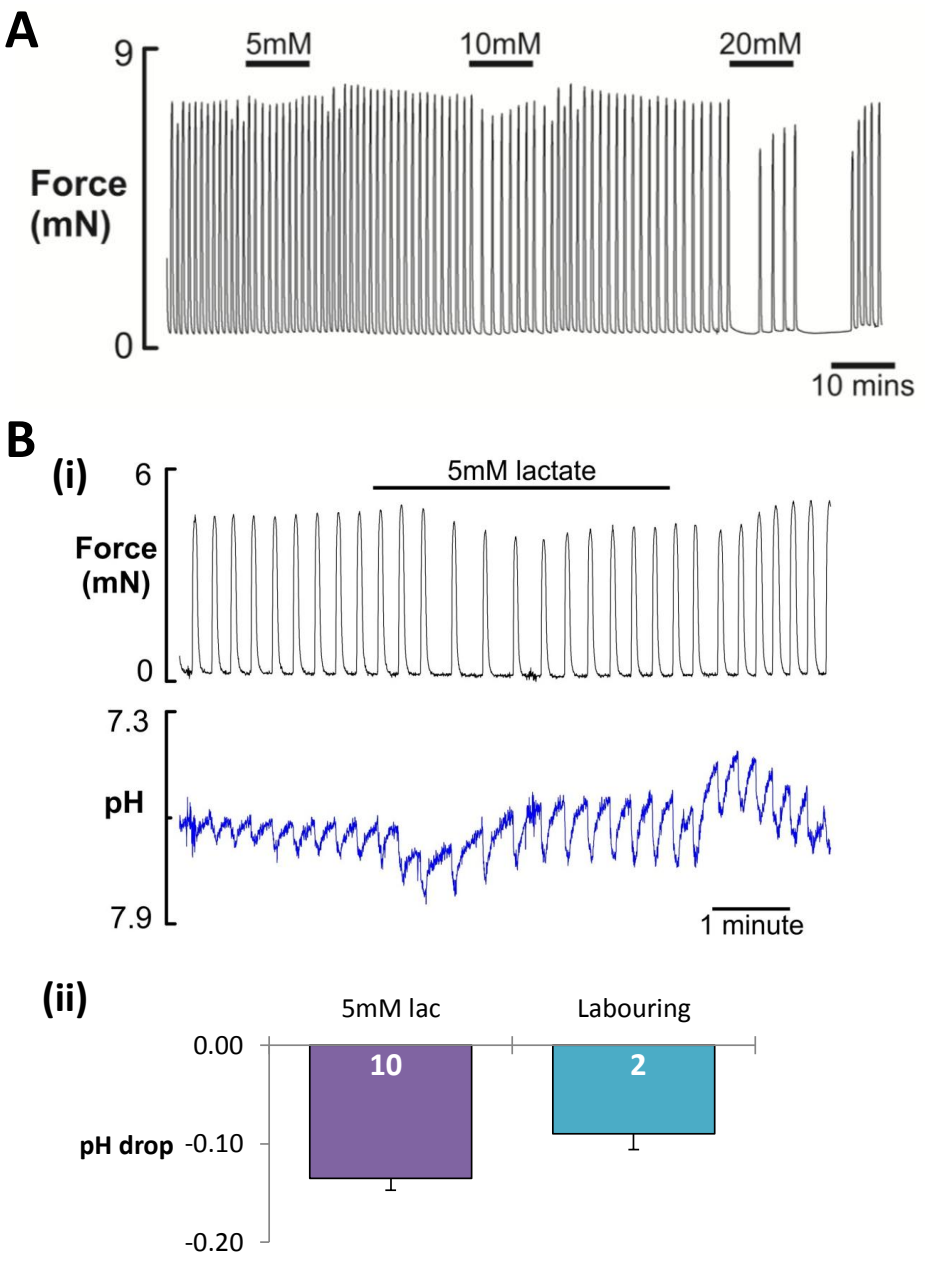


Figure 3.23 The effect of lactate on intracellular pH of labouring rat myometrium

A: Force recording of the application of lactate spontaneously contracting labouring myometrium for 10 minutes (5mM-20mM) (*n*=2).
B: (i) Simultaneous force and pH_i measurement of the effect of 5mM lactate (5 minutes application) on labouring rat myometrium.
(ii) Mean (+ s.e.m.) data for pH drop of 5mM lactate on spontaneously active tissue (purple bar) compared to the drop on labouring tissue (turquoise bar). *N* numbers are denoted by values within bars.

3.5 Discussion

Previous work has indicated lactate may play a role in dysfunctional labour; however no previous functional studies have investigated the effect of lactate on myometrial contractility. This study shows that the application of lactate and other weak acids to rat myometrium causes a decrease and, at high enough concentrations, a cessation of contractions. The response seen is not specific to lactate; other weak acids produce a similar decrease and cessation of contractility, on both spontaneous and oxytocin-driven activity. Therefore the effect seen with lactate is not due to its role in metabolism. The effect of lactate throughout gestation is not consistent; its effects are greater closer to term and lactate has little effect on labouring tissue. Loading with the fluorescent indicators Carboxy-SNARF and Indo-1 have confirmed that lactate causes the intracellular pH of the myometrium to fall and calcium transients are affected. Depolarisation of myometrial tissue with KCl solution indicates lactate may affect membrane potential.

Lactates ability to induce relaxation of vascular smooth has previously been documented. There is also agreement that lactate causes a significant decrease in pH_i. Although my data shows a similar concept, I found EC_{50} value of ~5mM, whereas other groups have seen used >10mM (Barron & Nair 2003), with one group finding an EC50 value of 26mM (McKinnon et al. 1996). However this could be due to tissue-specific differences.

The reason for lactates effect on term pregnant tissue could be an in-built mechanism to prevent excessively powerful tonic-like uterine contractions during labour, which could clamp down blood vessels and reduce blood flow (and therefore oxygen) to the fetus and cause fetal distress. Reduced blood flow also reduces the ability to clear lactate and H^+, thus causing a decrease in pH_i. As lactate (and H^+) levels increase in the myometrium, L-type Ca^{2+}

channels are inhibited (Figure 3.24). It is known that H^+ displacement affects L-type calcium channel (Klockner & Isenberg 1994). H^+ and Ca^{2+} are cations and therefore there may also be competition between the two ions at intracellular binding sites. H^+ could displace Ca^{2+} bound to myofilaments and therefore decrease tone. As Ca^{2+} levels in the myometrial cell are reduced or displaced from the myofilaments, contractility is abolished and restriction to blood flow is removed. Blood flow to the myometrium is restored and lactate, through its transporters, can leave the cell down its concentration gradient. Once lactate levels have been reduced, L-type Ca^{2+} channels are no longer inhibited by protons, and Ca^{2+} entry is restored and with it, contractions.

Non-pregnant and early pregnant myometrium appear to be resistant to the effects of 5mM lactate compared to late-pregnant tissue. This could be due to differences between the myometrium, suggested in Figure 3.25. The expression of transporters may be up regulated towards term, allowing for the eventual increase in both aerobic and anaerobic respiration in labour. The levels and activity of lactate dehydrogenase (the enzyme responsible for the production/oxidation of lactate) may also be altered. There was surprisingly little inhibitory effect of lactate on labouring myometrium, especially compared to the effects seen in late pregnant rats. Higher concentrations of lactate are needed to affect labouring tissue. Initial experiments loading labouring tissue with Carboxy-Snarf suggests 5mM lactate does not cause the same pH_i drop as seen in late pregnant tissue. This may be due to increased buffering power in labouring myometrium (Figure 3.25). However further experiments are needed to confirm this. The mechanism of how lactate is acting on the myometrium, suggested previously in this chapter, may also not be applicable in this animal model due to differences in the duration of labour. Experiments

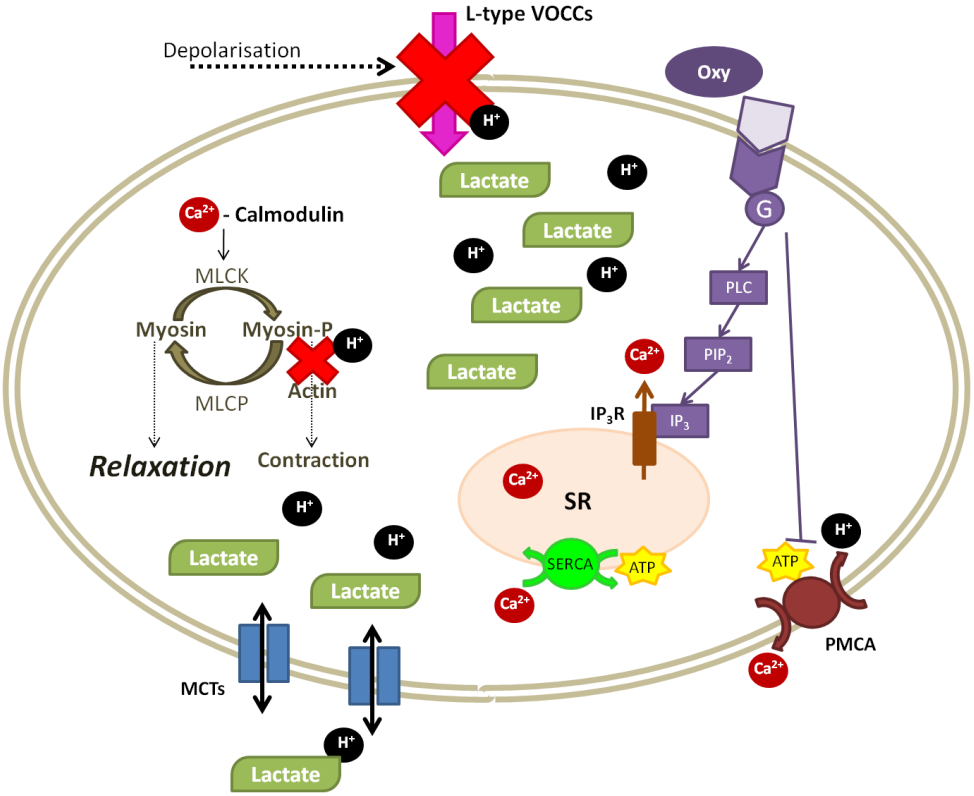


Figure 3.24 **Suggested mechanism of action for lactate in term pregnant tissue**

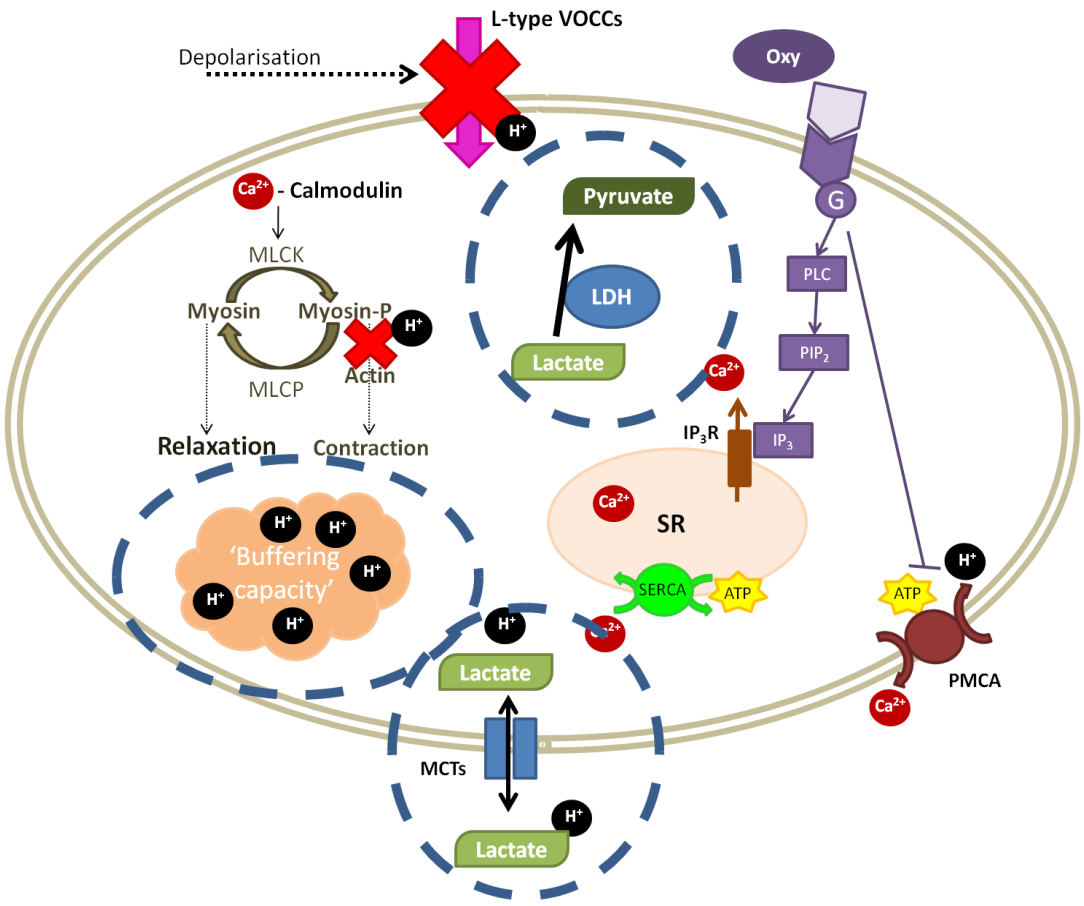


Figure 3.25 **Suggested differences between late-pregnant and non-pregnant/ labouring myometrium**

need to be carried out on human myometrium, in particular labouring myometrium, to see if this mechanism can be elucidated.

KCl is used to directly open L-type Ca^{2+} channels. Lactate did not significantly reduce force in the presence of KCl, indicating its effects are via these channels. The increase in force when lactate was applied indicates it is able to increase cytosolic free Ca^{2+}. This is possibly due to Ca^{2+} release from the mitochondria (Pierce 2003).

When lactate is removed, pH_i rises. There is a rebound alkalinisation due to mechanisms that restore resting pH and because of this the frequency and amplitude of contractions increase to above control levels. When the resting pH is reached, contractions return to control values. In rat myometrium, oxytocin has some rescue effect when a weak acid is applied. This is more noticeable at higher acid concentrations (10mM and 20mM). In 5mM lactate the drop in pH_i is significantly reduced. Oxytocin can increase intracellular [Ca^{2+}] by stimulating Ca^{2+} entry, releasing Ca^{2+} from the SR and decreasing Ca^{2+} efflux from the cell. (Soloff & Sweet 1982; Wray 2007). Oxytocin has also been shown to sensitise myofilaments to Ca^{2+} (Somlyo & Somlyo 1998). However it is not enough to overcome the fall in pH caused by lactate application and contractions are still affected. This is seen clinically where administration of oxytocin does stimulate a dysfunctional- contracting uterus in all cases.

The response of the myometrium to 5mM lactate needs to be investigated further. The reasons for some tissue strips continually contracting under lactate and others being abolished is unclear. This response could also be due to the natural variability of myometrial tissue-perhaps there are differences in LDH levels or MCT expression in these tissues therefore effecting lactate entry into the myometrial cell and its movement and breakdown. As every care was taken to dissect strips of similar size and weight, variation in dissection of

these tissue strips is unlikely to be a factor.

In the rat myometrium, an initial increase in force of contraction is produced when lactate is applied. More investigation is need but an initial theory may be that Ca^{2+} increase when lactate applied or the activation of ASIC channels by the increase in H^{+}. Further work includes the calibration of Carboxy-SNARF emission ratios to obtain actual pH values of the myometrium and to analyse how much the pH changes when lactate is added.

In summary, the data in this chapter shows the effect of lactate on rat myometrial contractions and how its effects are most potent at term, possibly as a natural failsafe mechanism if contractions become too strong or powerful. Future investigations into the transporters and enzymes involved in the lactate response will shed further insight.

3.5.1 Limitations of study

- There is no direct evidence of an effect on calcium channels. This would need to be addressed in future work.

3.6 Summary

Lactate in the physiological range potently decreased spontaneous contractility in rat myometrium. Lactate also decreases oxytocin-stimulated contractions but its sensitivity was decreased. Lactate caused a larger decrease in pH_i in spontaneously-contracting term pregnant myometrium than oxytocin-stimulated activity or labouring myometrium indicating the reason for the decrease in lactate sensitivity in these tissues. Lactate inhibited

Ca^{2+} transients, which could be due to a fall of intracellular pH as shown in previous studies. I suggest this is the mechanism whereby lactate and similar weak acids reduce myometrial contractions.

Chapter 4

The effect of lactate on human myometrial contractility

Chapter 4

The effect of lactate on human myometrial contractility

4.1 Abstract

Labour dystocia accounts for 20% of all caesarean sections (CS) in the UK. The only treatment available is application of oxytocin, which only works in around 50% of cases. Lactate has been shown to be significantly increased in myometrial capillary blood during labour dystocia suggesting it may be impairing force production, but there are no functional data on the effect of lactate on the myometrium. Therefore I have investigated its effect on contractility of human myometrium.

Myometrial strips were taken from biopsies obtained (following written informed consent) from women undergoing either a caesarean section (CS) at term (pregnant) or a hysterectomy (non-pregnant). The effects on spontaneous contractility in response to sodium lactate and sodium propionate (1-20mM) were recorded. Oxytocin-stimulated contractions (0.5nM) were also investigated to increase the physiological and clinical relevance of data obtained. Statistical differences were tested using appropriate tests and significance taken as $p<0.05$.

Lactate significantly decreased spontaneous contractility (*n*=5-6). A dose dependent decrease in all contractile parameters was seen, relative to the preceding control period. Propionate also significantly reduced contractions in a dose-dependent manner. There was no significant difference in the effects of these weak acids on human myometrium. The effects of lactate were significantly reduced in the presence of oxytocin (*n*=4-6).

When compared to spontaneous contractility, there was a significant difference at 10mM in amplitude ($p=0.01$) and frequency of contractions ($p=0.04$). Non-pregnant myometrium was resistant to lactate: 5mM lactate significantly decreased duration and overall a.u.c of contractions in spontaneously-contracting non-pregnant tissue. Amplitude and frequency of contractions were not significantly affected.

Lactate potently decreases human myometrial contractility. The effects of lactate were reduced in the presence of oxytocin. Another weak acid, propionate, produced similar effect to lactate. I suggest accumulation of extracellular lactate, which as I have shown, will reduce myometrial contractions, could therefore contribute to labour dystocia.

4.2 Introduction

For successful labour to occur the uterus needs to produce strong coordinated contractions. The processes and pathways that achieve this are well characterised, as detailed in **Chapter 1**. However when contractions are weak and uncoordinated, labour cannot progress successfully. Dysfunctional labours (or dystocia), in particular those due to inefficient uterine activity in the first stage, are common but there are limited therapeutic options available to these women. The administration of oxytocin only works in 50% of all cases (Wray 2007) and because of this, dysfunctional labour is a frequent cause of non-elective caesarean sections (Thomas J. 2001). Oxytocin has not reduced the number of sections in the UK (Bugg, Siddiqui & Thornton 2013). Caesarean sections are associated with increased morbidity for mother (Saunders, Paterson & Wadsworth 1992).

Our group have previously shown that there is increased lactate in myometrial capillary blood from women suffering dysfunctional labour and that this blood is of a reduced pH (Quenby et al. 2004). In the myometrium, both intracellular and extracellular pH changes occur during labour due to vascular occlusion at the peak of uterine contraction (Wray et al. 1992) and it has already been shown that intracellular acidification of human myometrial strips can affect contractility and it has been demonstrated to inhibit L-type Ca^{2+} channels (Pierce et al. 2003).

There are no data that examines the functional effect of lactate on human myometrial contractility. Lactate has been shown to be produced by cultured myometrial cells (Akerud, Ronquist & Wiberg-Itzel 2009) but its effect on contractility has not been investigated. The aims of the study were:

x) To investigate the effect of lactate on spontaneous contractile activity in human

myometrial contractility

xi) to compare the effect of lactate under oxytocin-driven contractions

xii) to compare the effects of another weak acid, propionate on spontaneous and oxytocin-driven contractions

xiii) to investigate the effect of lactate on non-pregnant human myometrium

4.3 Methods

4.3.1 Tissue

Human myometrium was obtained from women undergoing elective lower segment caesarean section at term (34-41 completed weeks) or hysterectomy at Liverpool Women's hospital with local ethical committee approval and written, informed consent. Non-pregnant biopsies were taken from the lower part of the anterior wall of the uterus, to correspond with the pregnant biopsy area.

A total of 42 samples were used in this study. The reasons for section included previous traumatic vaginal delivery (8) or previous CS (23), breech (8) and patient choice (3). Those who had failed IOL or who had commenced labour at the time of section were not included. 4 women were prescribed inhalers for asthma. The mean age of women undergoing caesarean delivery was 32 years old (range 22- 44 years), mean BMI of these women was 27.3(range 18.0- 50.7) and the mean gestation was 38 completed weeks. The mean parity was 1 (range 0- 4).

For non-pregnant tissue, those that were on medication, those that had abnormal smears or were having hysterectomies for endometriosis were excluded from this study. A total of 6 non-pregnant samples were used. Indications for surgery included menorrhagia (4) fibroids (1) and ovarian cancer prevention (1). The median age of women was 43 years old (range 34- 49 years), the median BMI was 28.0 (range 21.2- 30.8) and the median parity of these women was 2 (range 0- 4).

1mm x 5mm human myometrial strips were dissected from myometrial biopsies. Individual strips were mounted between a fixed support and force transducer using aluminium clips in

a 1ml bath and were continuously superfused with PSS at a rate of 2mL/min and maintained at 37^{o}C. Contractile activity was seen in all myometrial strips within 3 hours. Stable contractions were observed for 60 minutes before an experiment begun. Force was recorded as described in detail in **Chapter 2**.

4.3.2 Solutions

All solutions were created as described in **Chapter 2**. Physiological saline solution was composed of NaCl 154mM, KCl 5.6mM, $MgSO_4$ 10.9mM, Glucose 8mM, $CaCl_2$ 2mM at pH 7.4. 40mM high K^+ solution was used to test tissue viability and provide maximal activity. Oxytocin was used at a concentration of 0.5nM in PSS. 20mM Lac solution was made and diluted in PSS to provide 1mM-10mM solutions. Other weak acid solutions were made in the same way.

4.3.3 Statistics

Data was analysed using Origin 8.6 (Microcal) and *n* is the number of myometrial tissue strips from different animals. Amplitude and duration of each contraction was measured and the average in the 20 minutes of solution application was used. Frequency was measured as number of contractions within 20 minutes. If there were no contractions during lactate application, amplitude and frequency were recorded as '0' and excluded from duration analysis. Integral force (AUC) was measured during 20 minutes of solution application or control period. All data is presented as % of control period (100%). Normality tests were carried out on the data and the appropriate statistical tests used in SPSS Statistics 20. Significance was taken as $P<0.05$.

4.4 Results

4.4.1 Dose response of lactate on human myometrium

As an increased amount of lactate has been suggested as a cause of dysfunctional labour, the effect of lactate on human myometrial contractility was tested. Tissue strips were able to produce stable, regular contractions for many hours. A 20 minute application time was chosen as the average time for 4 contractions (Figure 4.1). 1-20mM sodium lactate was applied extracellularly for 20 minutes and the effects on contractility determined (n=4-6) (Figure 4.2). Lactate caused a reduction and abolition of contractile activity and this response was dose dependent (Figure 4.2 Ai-v). Contractions reappeared when lactate was removed and the myometrium returned to PSS. There was a significant decrease in contractile parameters- amplitude, frequency and A.U.C. of contractions in the presence of lactate when compared to the preceding control period (Paired students t-test, $p<0.05$). Amplitude of contractions were significantly reduced at 20mM ($p=0.04$). Frequency and a.u.c of contractions were significantly affected at 3mM lactate and higher. There was no significant difference between duration of contractions in the presence of lactate. When comparing mean data (Figure 4.3) there was a significant difference in amplitude (Figure 4.3 Ai) duration (Figure 4.3 Aii) and a.u.c (Figure 4.3 Aiii) between lactate concentrations (Anova with Bonferroni Post-hoc test, $p<0.05$). From the dose response curve plotted from a.u.c data, the EC_{50} of lactate on human spontaneous contractions is 3.8mM (± 1.4mM).

It can also be seen that there was a dose-dependent drop in baseline tension seen in human myometrium (Figure 4.4). This was measured as the difference between the lowest point during lactate was compared to the baseline during control contractions. This drop was significant for concentrations ≥ 3mM (Paired student t-test, $p<0.05$). Baseline and

spontaneous force returned to control levels upon removal of lactate.

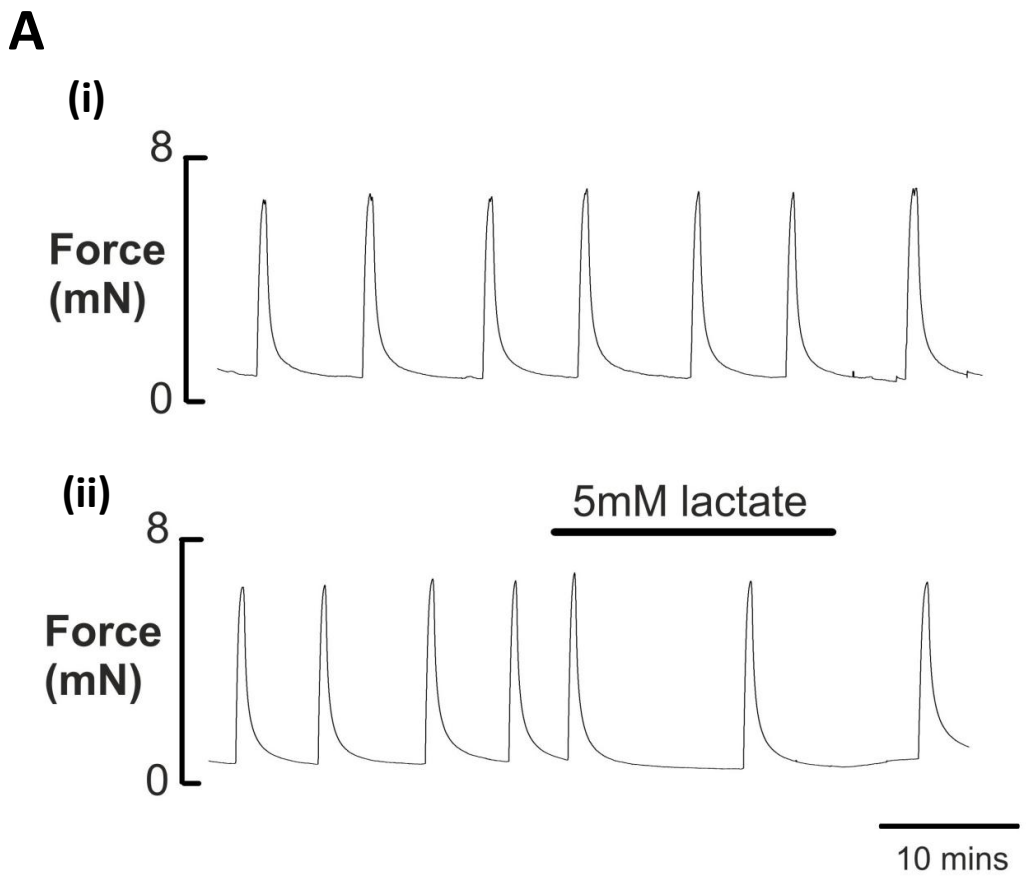


Figure 4.1 The effect of sodium lactate in pregnant human myometrium

A: Isometric recording of the application of 5mM lactate to spontaneously contracting human myometrium for 20 minutes (i) and a paired tissue strip exposed to PSS for the same length of time (ii) (*n*=7)

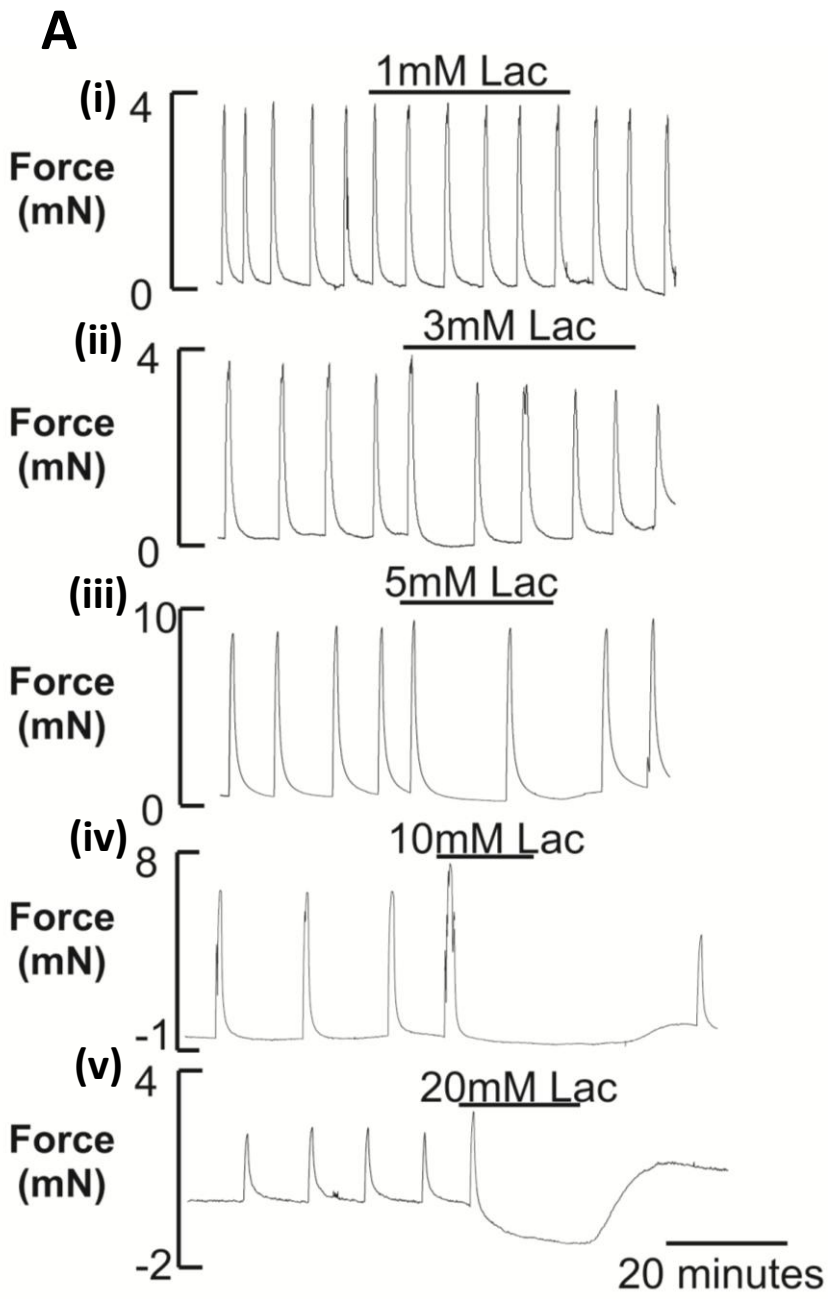


Figure 4.2 Dose dependency of sodium lactate in pregnant human myometrium

A. Representative force recordings of spontaneously contracting myometrial strips during 20 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM and (v) 20mM lactate.

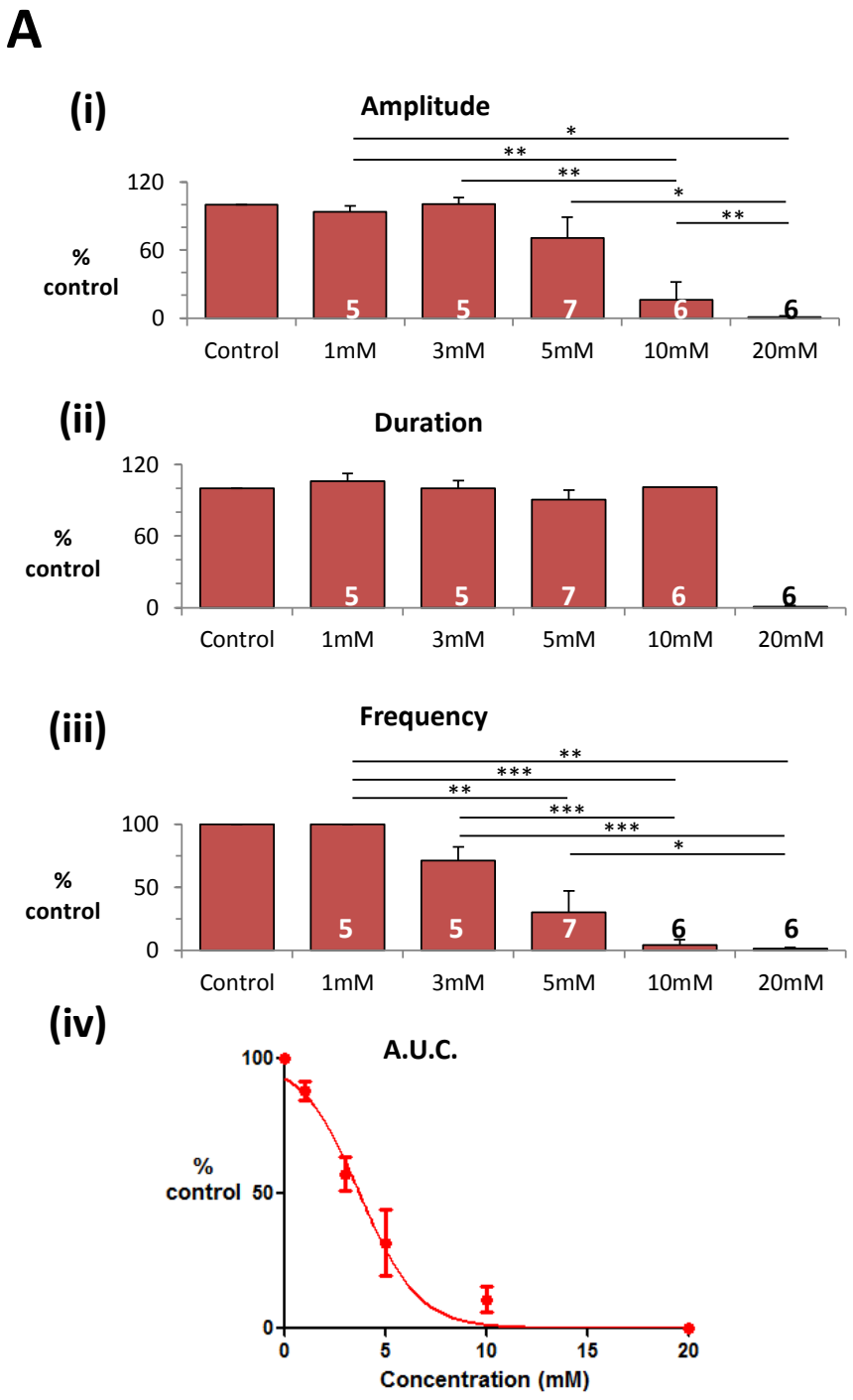


Figure 4.3 Dose dependency of sodium lactate in pregnant human myometrium

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) AUC in response to lactate. Using the dose-response curve plotted using the AUC data, the EC50 value for lactate is 4.83mM. N numbers are denoted by values within bars. A significant difference in activity was found using ANOVA with Bonferroni *post-hoc* test * denotes p<0.05 **p<0.01 ***p<0.001 significance.

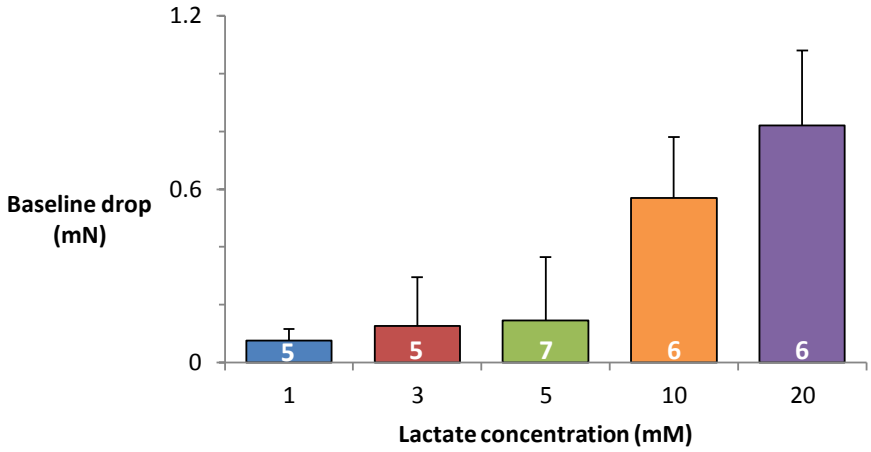


Figure 4.4 Drop of baseline tension in the presence of lactate

A dose dependent drop in baseline tension of spontaneously contracting human myometrium in response to 1mM (blue bar) 3mM (red) 5mM (green) 10mM (orange) and 20mM lactate (purple). *N* numbers are denoted by values within bars.

4.4.2 Oxytocin-induced contractions

The administration of oxytocin is used clinically to augment contractions in labour (Bernal 2001) and this is seen *in vitro*. To investigate if lactate can affect force under agonist stimulation, the effects of lactate on oxytocin-induced contractions were studied. 0.5nM oxytocin was added to PSS and applied to myometrial strips (Figure 4.5). This concentration was chosen as it caused an increase in force which could be sustained by the tissue and avoided tonic activity and has been used in previous experiments in our laboratory (Robinson & Wray 2012).

The response to lactate (1-20mM) when applied to contracting myometrium, in the presence of oxytocin, was a decrease in contractility (Figure 4.6 Ai-v). This is similar to what was seen during spontaneous activity in previously. When compared to the adjacent control period, frequency of contractions were significantly decreased at ≥5mM (Students paired t-test, $p<0.05$). The a.u.c. was significantly reduced in all concentrations used. There was no significant difference in amplitude or duration of contractions. There were significant differences between lactate concentrations and in amplitude, frequency and a.u.c (Figure 4.7 Ai, iii, iv). The EC_{50} for lactate in the presence of oxytocin, taken from the a.u.c dose response curve, is 5.4mM ± 1.2mM (Figure 4.7 Aiv).

The drop in baseline tension seen with lactate on spontaneous activity is still seen in the presence of oxytocin (Figure 4.8). This drop in tension was significant at lactate concentrations of ≥5mM (paired students t-test, $p<0.05$). There was no significant difference between the drop in tension in spontaneous activity or oxytocin-driven contractions.

Figure 4.9 compares the effect of lactate between spontaneous and oxytocin-driven activity. As shown by the mean data (+s.e.m) there is a significant difference at 10mM lactate in

amplitude (p=0.01) and frequency (p=0.04) of contractions (Figure 4.9 Ai and iii respectively). There is no difference between duration and a.u.c of contractions. The dose response curve for lactate +oxytocin, when plotted against spontaneous activity, is shifted to the right, indicating a higher EC_{50} value.

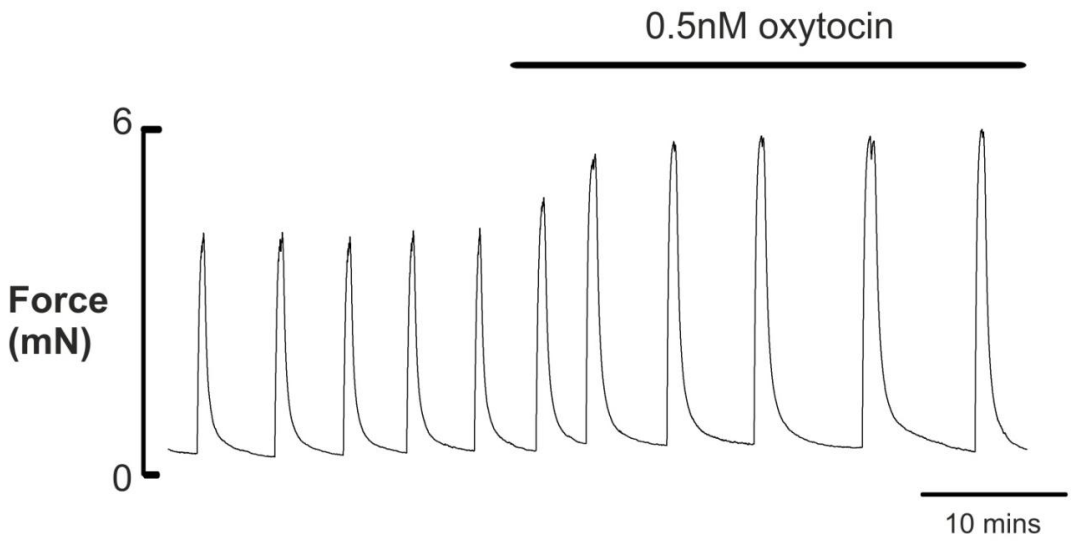


Figure 4.5 The effect of oxytocin on contracting human myometrium

Isometric recording of the application of 0.5nm oxytocin to spontaneously contracting human myometrium.

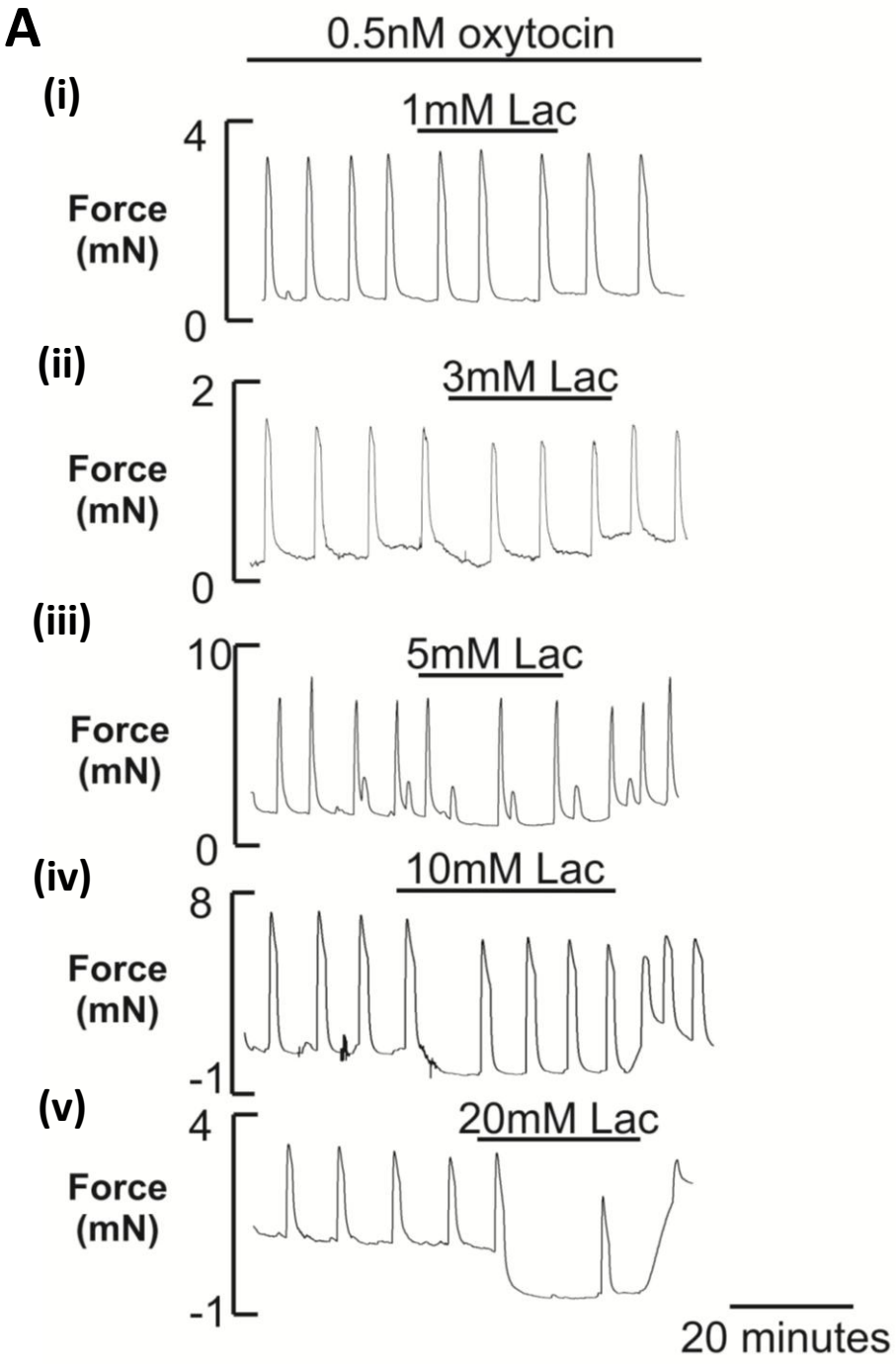


Figure 4.6 Dose dependency of sodium lactate in oxytocin-driven activity

A. Representative isometric recordings of contracting myometrial strips in the presence of 0.5nM oxytocin during 5 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM (v) 20mM lactate.

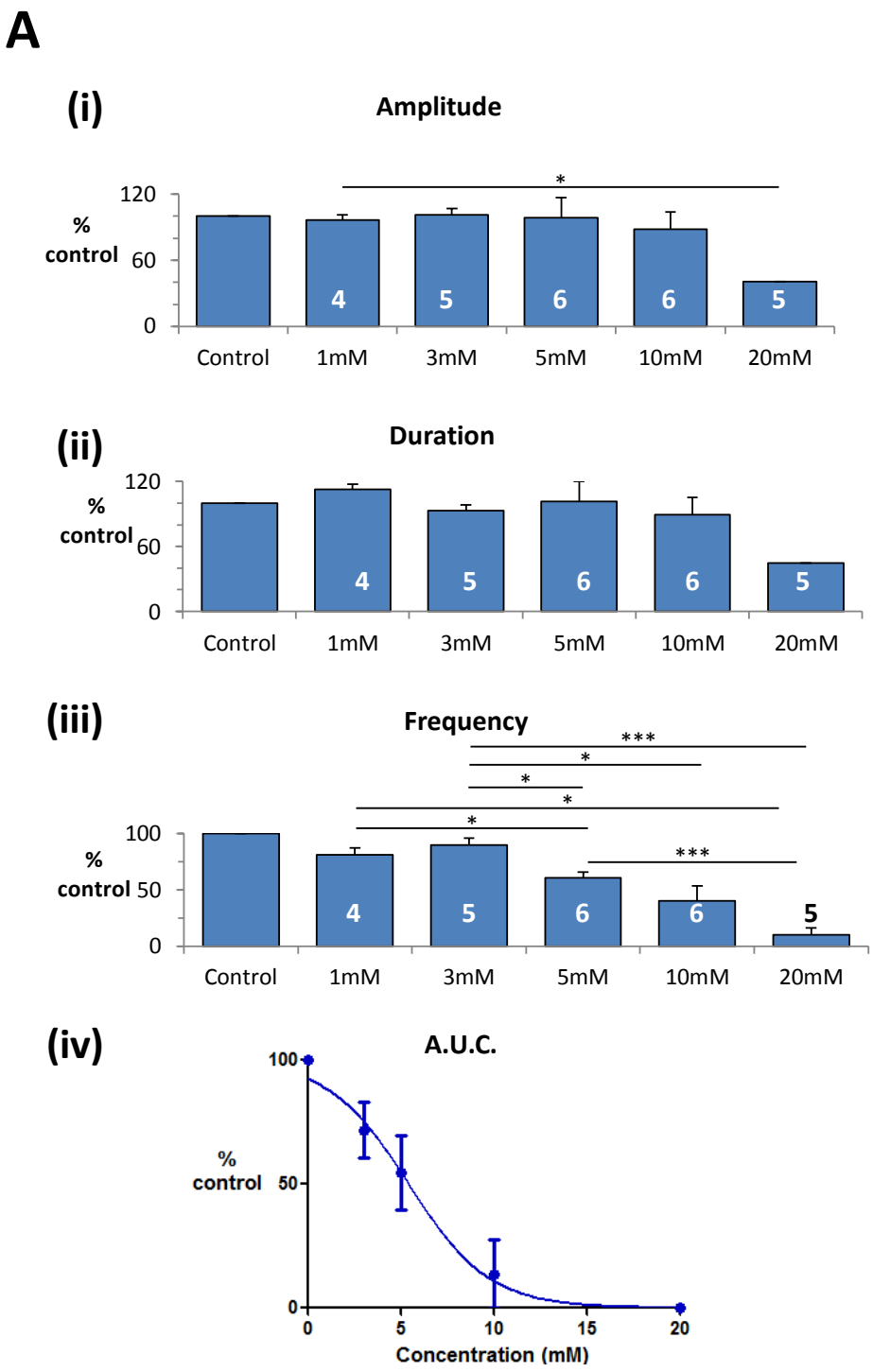


Figure 4.7 Dose dependency of sodium lactate in oxytocin-driven activity

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) AUC in response to lactate. N numbers are denoted by values within bars. A significant difference in activity was found using ANOVA with Bonferroni *post-hoc* test * denotes $p<0.05$ **$p<0.01$ ***$p<0.001$ significance.

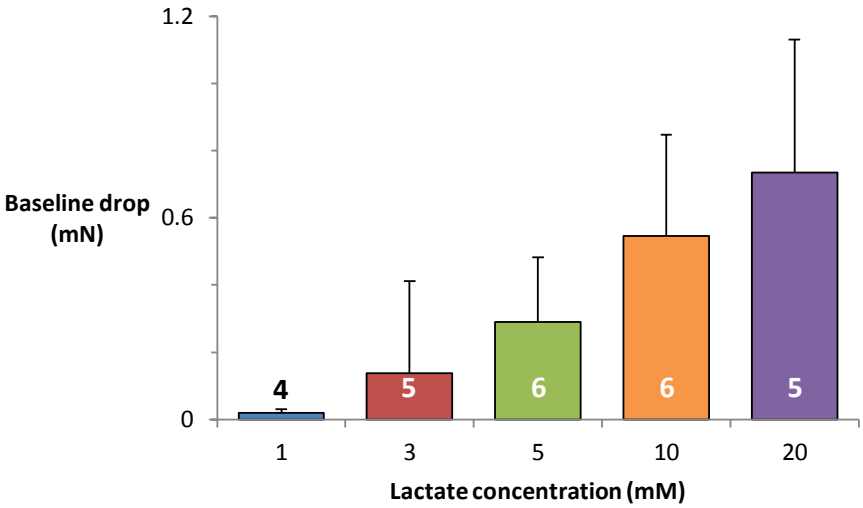


Figure 4.8 Drop of baseline tension in the presence of lactate and oxytocin

A dose dependent drop in baseline tension of oxytocin-driven (0.5nM) contracting human myometrium in response to 1mM (blue bar) 3mM (red) 5mM (green) 10mM (orange) and 20mM lactate (purple). *N* numbers are denoted by values within bars.

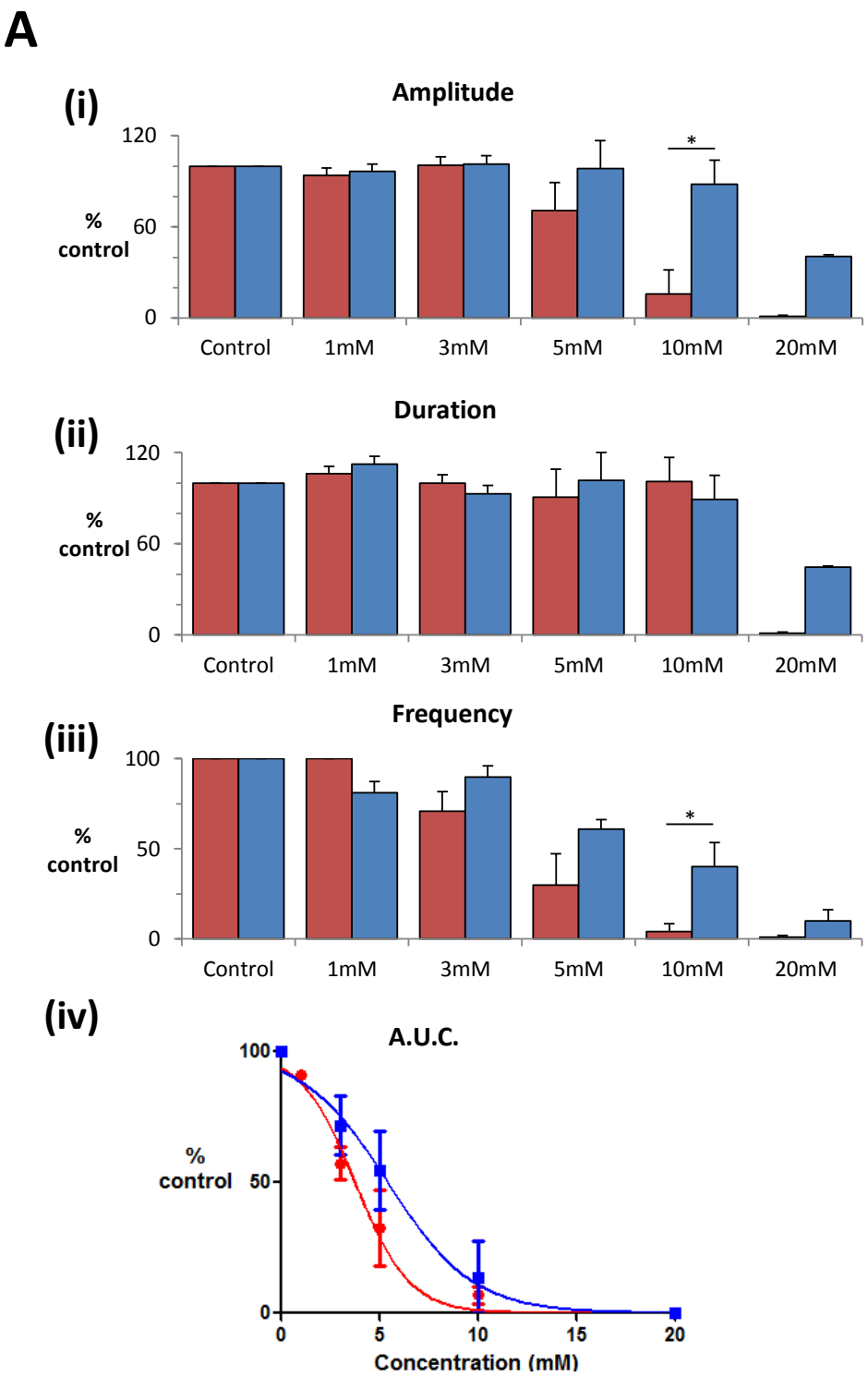


Figure 4.9 A comparison of lactate response in spontaneous and oxytocin activity

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) AUC in response to lactate, when applied to spontaneously contracting term pregnant non-labouring human myometrial strips (red bars) and in the presence of 0.5nM oxytocin (blue bars). A significant difference in activity was found using Paired Samples T-Test *denotes $p<0.05$

4.4.3 Comparative effects of sodium propionate

Sodium propionate was used to test the specificity of the lactate response in human tissue. 1-20mM propionate was applied to spontaneously contracting myometrial strips for 20 minutes and contractile parameters measured (*n*= 4-6) (Figure 4.10 Ai-v). Contractions were reduced in a dose-dependent manner, as seen with lactate, which returned to control values when in PSS. There was a significant difference in amplitude of contractions in 1mM lactate (Paired students t-test). Frequency and a.u.c. were significantly reduced at concentration ≥3mM. There was no difference in duration of contractions in the presence of propionate. As shown in Figure 4.11, there is a significant difference between propionate concentrations when observing amplitude (Figure 4.11 Ai) frequency (Figure 4.11 Aii) and a.u.c. (Figure 4.11 Av). The EC_{50} for propionate in spontaneously contracting human myometrium is 3.9mM ± 1.2mM.

There was also a dose-dependent drop in baseline tension, as seen with lactate (Figure 4.12). The drop was significantly increased for concentrations ≥ 5mM propionate. When compared to the drop seen with lactate, there was no significant difference between the two weak acids (paired student t-test, $p>0.05$)

The effect of propionate in the presence of oxytocin was also investigated. 1-20mM propionate was applied to tissue contracting under the stimulation of 0.5nm oxytocin (Figure 4.13 Ai-v). This response was the similar to lactate on oxytocin-driven activity. Frequency was significantly reduced at concentrations ≥10mM (paired students t-test, $p<0.05$), a.u.c. was significantly affected at 3mM, 10mM and 20mM propionate. Amplitude was only significantly affected in 3mM propionate. The EC_{50} value generated from a.u.c. data is 5.2mM ± 1.0mM (Figure 4.14).

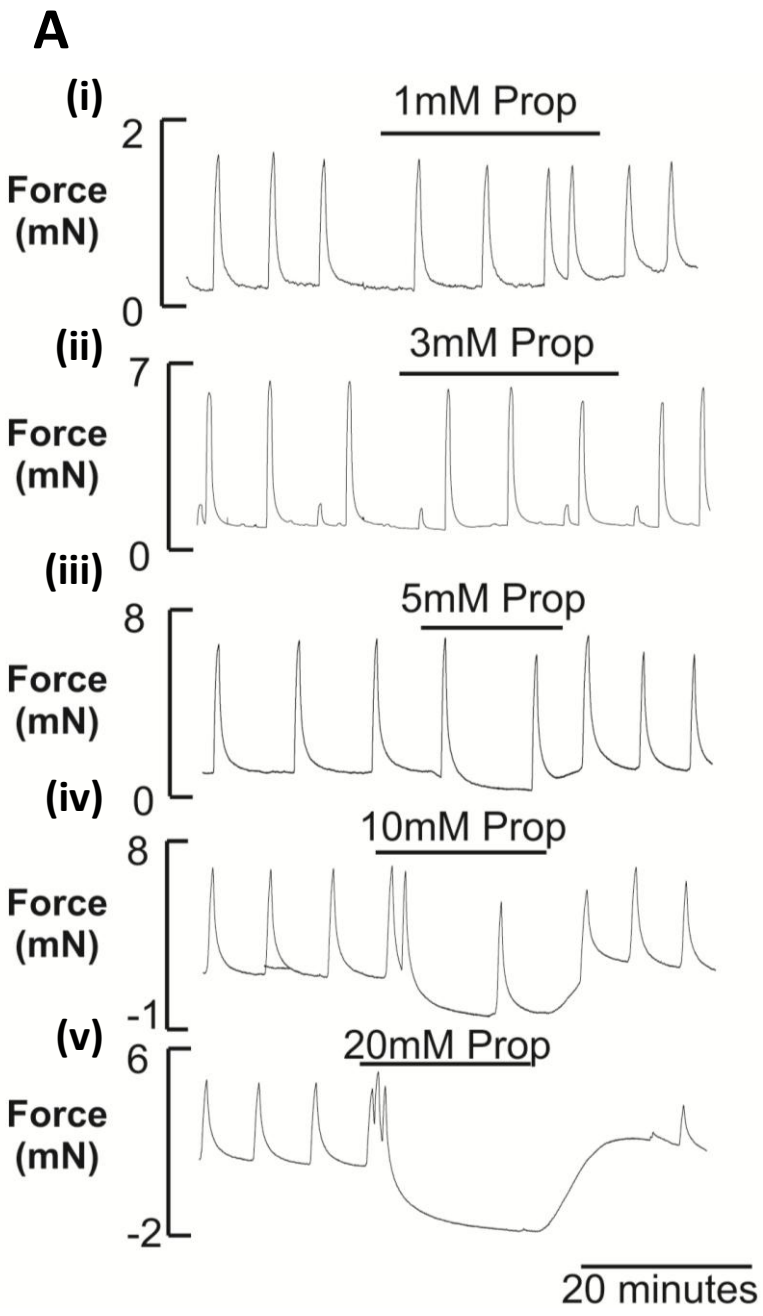


Figure 4.10 Dose dependency of sodium propionate in pregnant human myometrium

A. Representative isometric recordings of spontaneously contracting myometrial strips during 20 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM and (v) 20mM propionate.

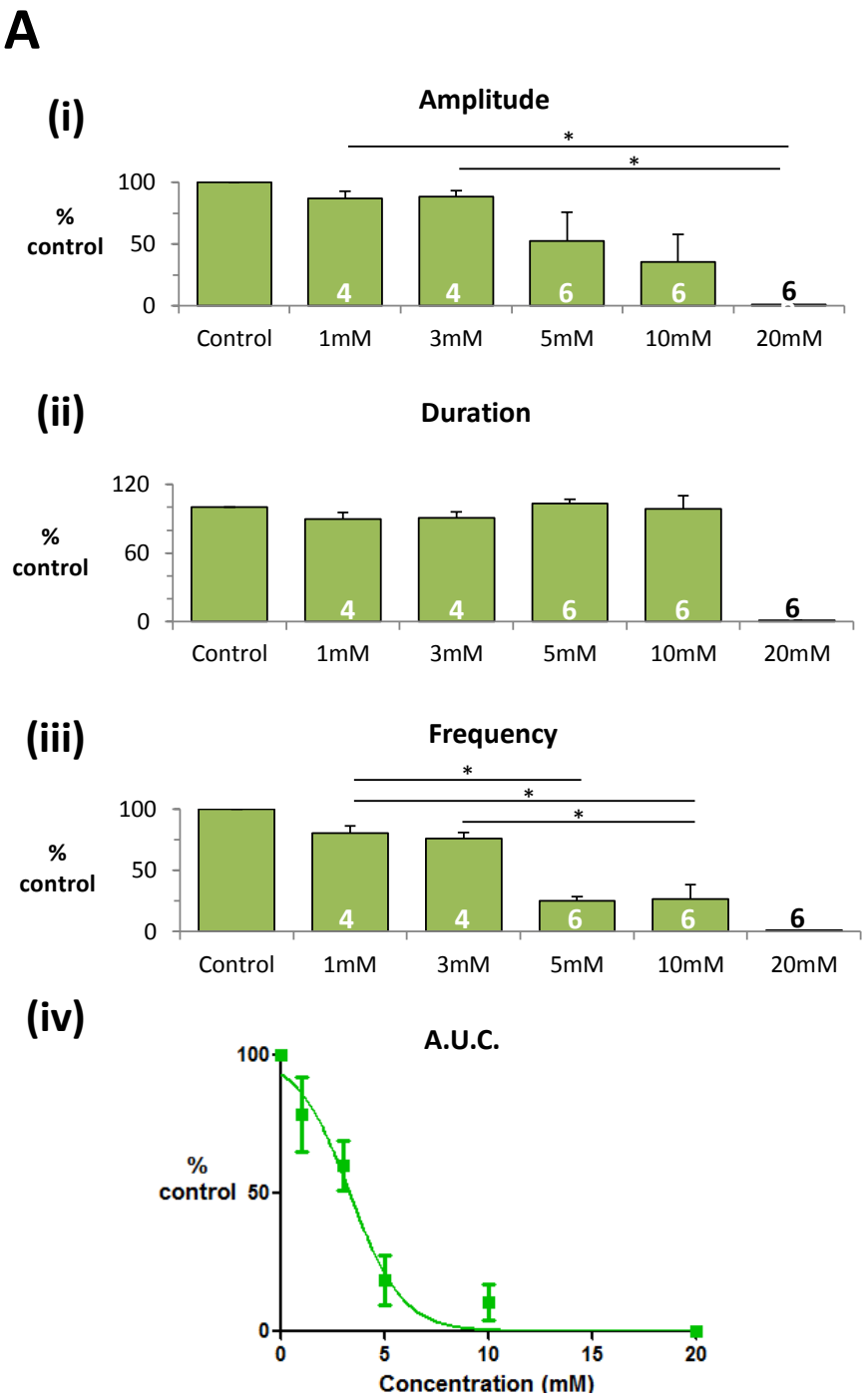


Figure 4.11 Dose dependency of sodium propionate in pregnant human myometrium

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to propionate. N numbers are denoted by values within bars. A significant difference in activity was found using ANOVA with Bonferroni *post-hoc* test * denotes $p<0.05$.

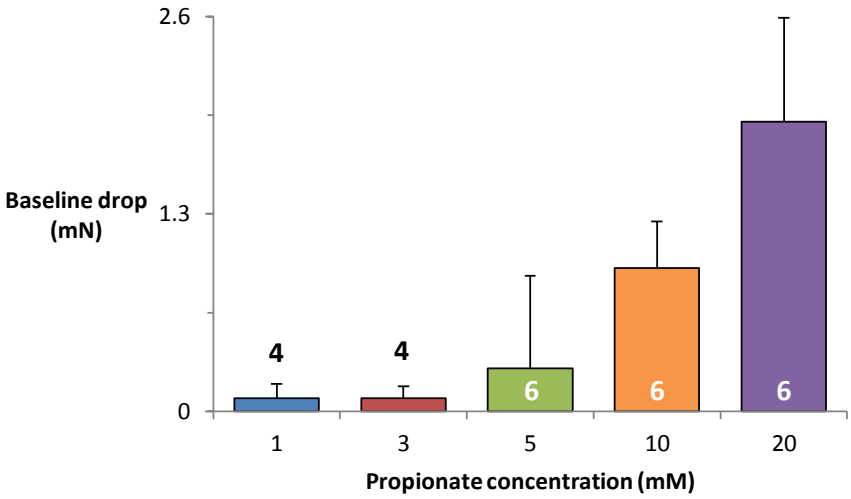


Figure 4.12 Drop of baseline tension in the presence of sodium propionate

A dose dependent drop in baseline tension of spontaneously contracting human myometrium in response to 1mM (blue bar) 3mM (red) 5mM (green) 10mM (orange) and 20mM propionate (purple). *N* numbers are denoted by values within bars.

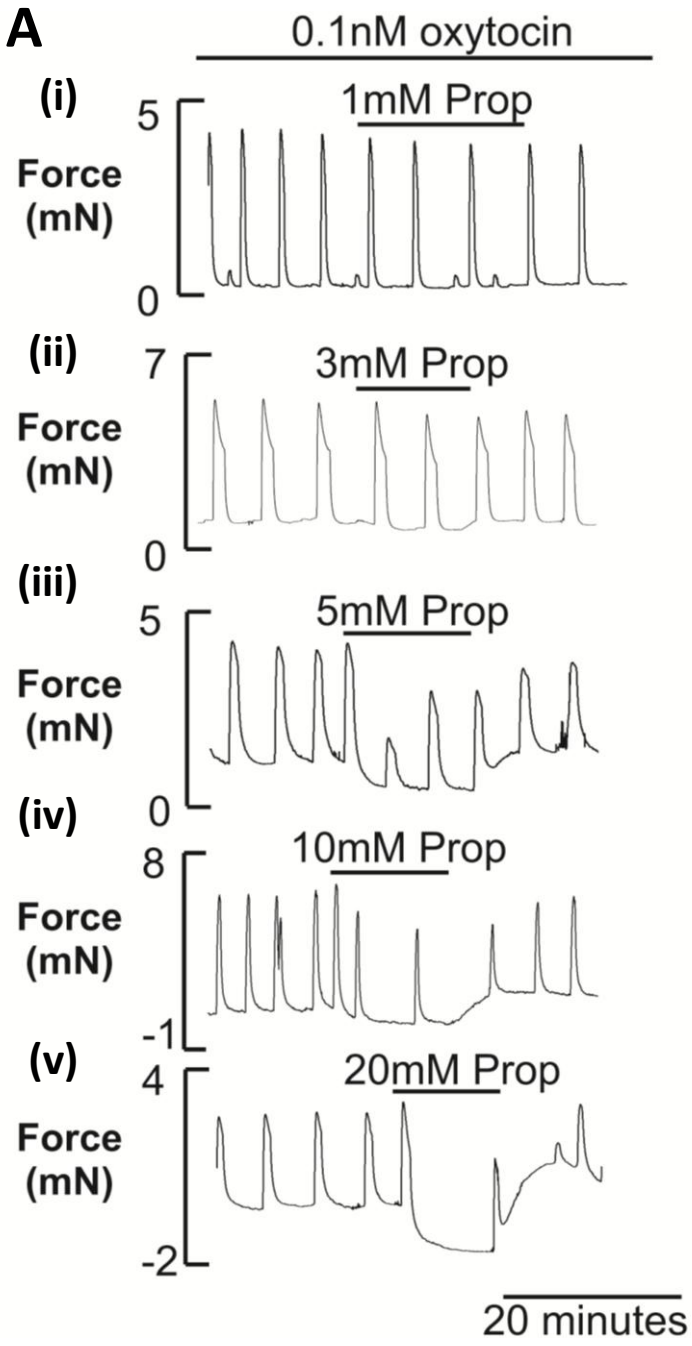


Figure 4.13 Dose dependency of sodium propionate in oxytocin-driven activity

A. Representative isometric recordings of contracting myometrial strips in the presence of oxytocin during 5 minutes application of (i) 1mM (ii) 3mM (iii) 5mM (iv) 10mM (v) 20mM propionate.

Propionate caused a significant baseline tension drop (Figure 4.15). This was significant in 3mM, 10mM and 20mM propionate, in the presence of oxytocin. When compared to the drop seen in lactate in the presence of oxytocin, this was not significantly different.

Mean data for lactate and propionate was plotted together to compare the two weak acid responses. There is no significant difference between lactate and propionate on spontaneous activity, in amplitude (Table 4.1 Ai), duration (Table 4.1 Aii), frequency (Table 4.1 Aiii) or a.u.c. (Table 4.1 Aiv).

There was also no significant difference in responses between propionate and lactate in the presence of oxytocin for all parameters (Table 4.2 Ai-iv).

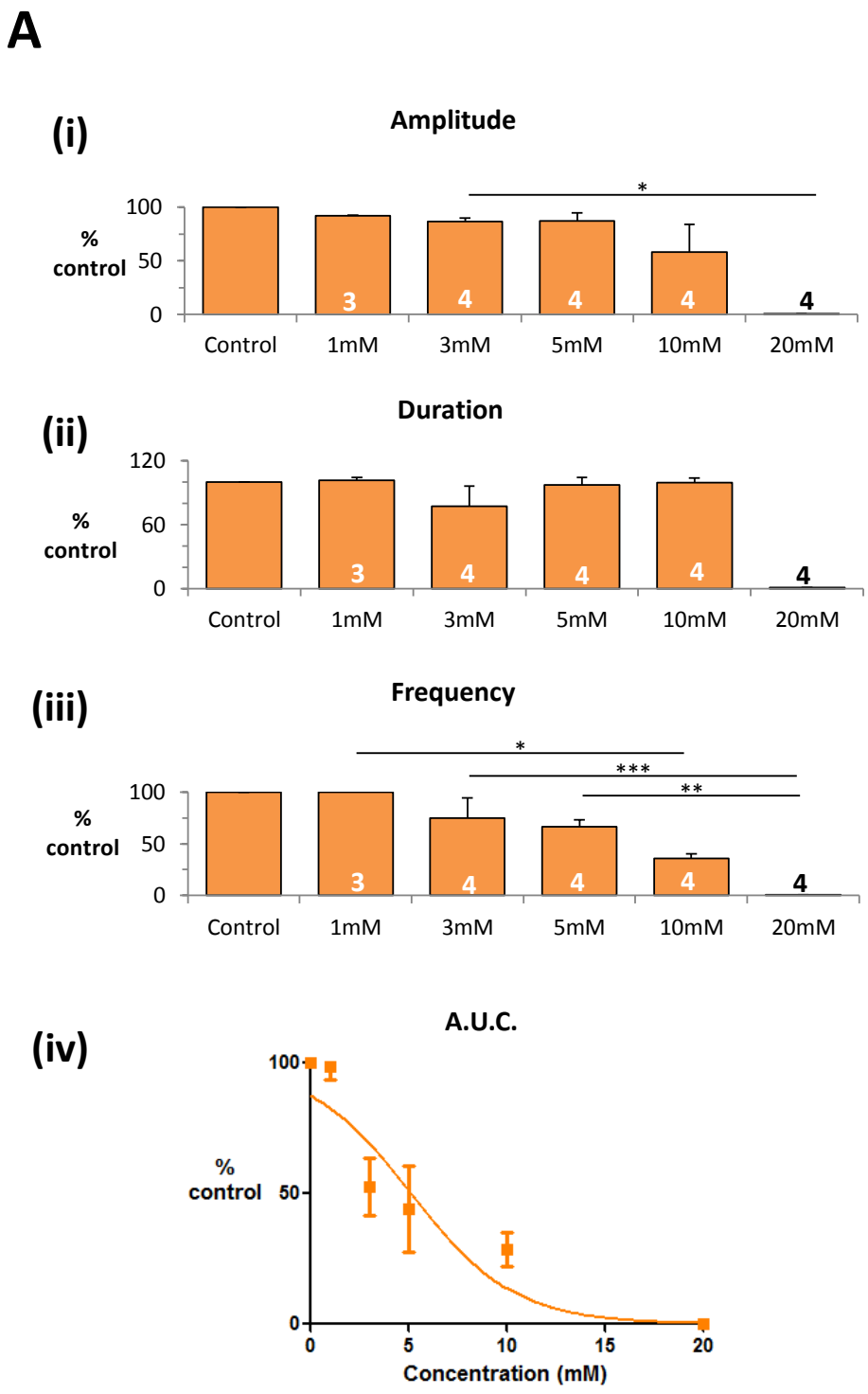


Figure 4.14 Dose dependency of sodium propionate in oxytocin-driven activity

A. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) AUC in response to propionate. N numbers are denoted by values within bars. A significant difference in activity was found using ANOVA with Bonferroni *post-hoc* test * denotes $p<0.05$ **$p<0.01$ ***$p<0.001$ significance.

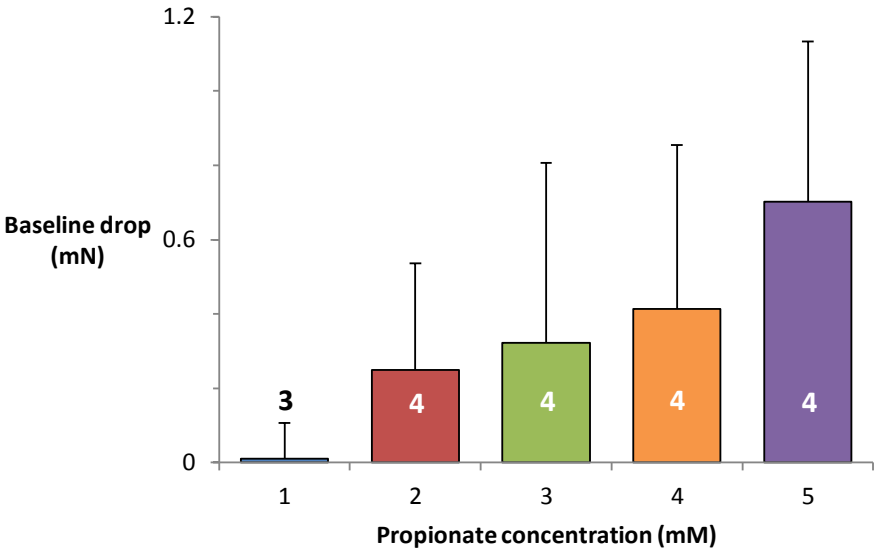


Figure 4.15 Drop of baseline tension in the presence of propionate and oxytocin

A dose dependent drop in baseline tension of oxytocin-driven contracting human myometrium in response to 1mM (blue bar) 3mM (red) 5mM (green) 10mM (orange) and 20mM propionate (purple). *N* numbers are denoted by values within bars.

A

(i) Amplitude

	Lactate	Propionate	P value
1mM	93.9±4.9%	86.7±5.9%	0.38
3mM	100.5±5.4%	88.4±4.7%	0.147
5mM	70.7±18.4%	52.4±23.5%	0.55
10mM	15.9±15.9%	35.3±22.3%	0.50

(ii) Duration

	Lactate	Propionate	P value
1mM	106.1±6.4%	90.0±5.7%	0.11
3mM	99.9±6.6%	90.9±5.3%	0.34
5mM	90. 8±7.6%	103.5±3.4%	0.41
10mM	100.9±0%	98.7±11.7%	0.96

(iii) Frequency

	Lactate	Propionate	P value
1mM	100±0%	80.2±10.7%	0.16
3mM	71.0±10.9%	75.7±10.2%	0.77
5mM	30.0±4.2%	25.0±14.7%	0.47
10mM	4.2±4.2%	26.4±12.1%	0.11

(iv) AUC

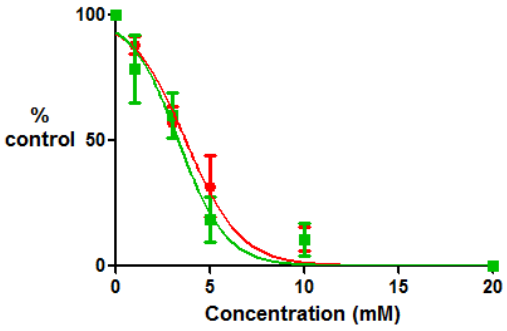


Table 4.1 A comparison of lactate and propionate response in spontaneous activity

A. Mean data ± s.e.m. showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency and (iV) AUC in response to lactate, and propionate when applied to spontaneously contracting term pregnant non-labouring human myometrial strips. 20mM response was excluded in the tables as both abolished contractility.
There was no significant difference between the two weak acids.

A

(i) Amplitude

	Lactate	Propionate	P value
1mM	96.5±4.6%	92.3±0.7%	0.57
3mM	101.4±9.0%	86.9±3.4%	0.21
5mM	98.5±1.9%	87.2±7.5%	0.25
10mM	88.1±20.5%	58.6±25.4%	0.37
20mM	40.7±24.9%	0	0.25

(ii) Duration

	Lactate	Propionate	P value
1mM	112.5±5.9%	101.5±2.6%	0.64
3mM	92.9±4.1%	77.0±18.9%	0.45
5mM	101.6±3.7%	97.4±6.6%	0.69
10mM	89.2±21.0%	99.2±4.4%	0.89
20mM	44.5±27.3%	0	0.90

(iii) Frequency

	Lactate	Propionate	P value
1mM	81.2±6.2%	100±0%	0.05
3mM	90.0±6.1%	75.0±10.2%	0.23
5mM	60.8±5.3%	66.7±11.8%	0.62
10mM	40.3±13.2%	35.8±16.3%	0.84
20mM	10.0±6.1%	0	0.19

(iv) AUC

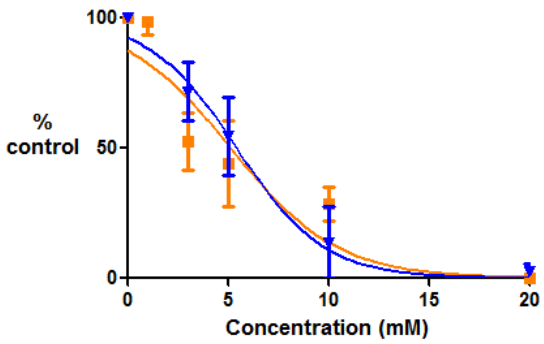


Table 4.2 A comparison of lactate and propionate response in oxytocin-driven activity

A. Mean data ± s.e.m. showing dose-dependent decrease in (i) amplitude (ii) duration (iii) AUC in response to lactate and propionate, when applied to term pregnant non-labouring human myometrial strips. There was no significant difference between the two weak acids.

4.4.4 Non-pregnant myometrium

The effect of 5mM sodium lactate on non-pregnant myometrium was determined. Lactate was applied for 20 minutes to spontaneously contracting myometrial strips (*n*=6) (Figure 4.16 A). When compared to the preceding control period, there was a decrease in contractile activity (Figure 4.16 B). Amplitude decreased to 80.4% ± 16.2% (paired student t-test, p=0.29) (Figure 4.16 Bi), duration significantly decreased to 83.1% ± 8.8% (p=0.03) (Figure 4.16 Bii), frequency to 55.1% ± 14.7% (p=0.08) (Figure 4.18 Biii) and a.u.c significantly decreased to 61.2% ± 14.4% (p=0.04) (Figure 4.16 Biv).

Mean data from lactate's effect on human term pregnant myometrium was compared to non-pregnant lactate response (Figure 4.17). There is little difference between amplitude (Figure 4.17 Ai) (students unpaired t-test, p=0.70) and duration (Figure 4.17 Aii) (p=0.53). There is a marked difference between frequency of contraction (Figure 4.17 Aiii) (p=0.48) and a.u.c (Figure 4.17 Aiv) (p=0.16). However these differences were not statistically significant.

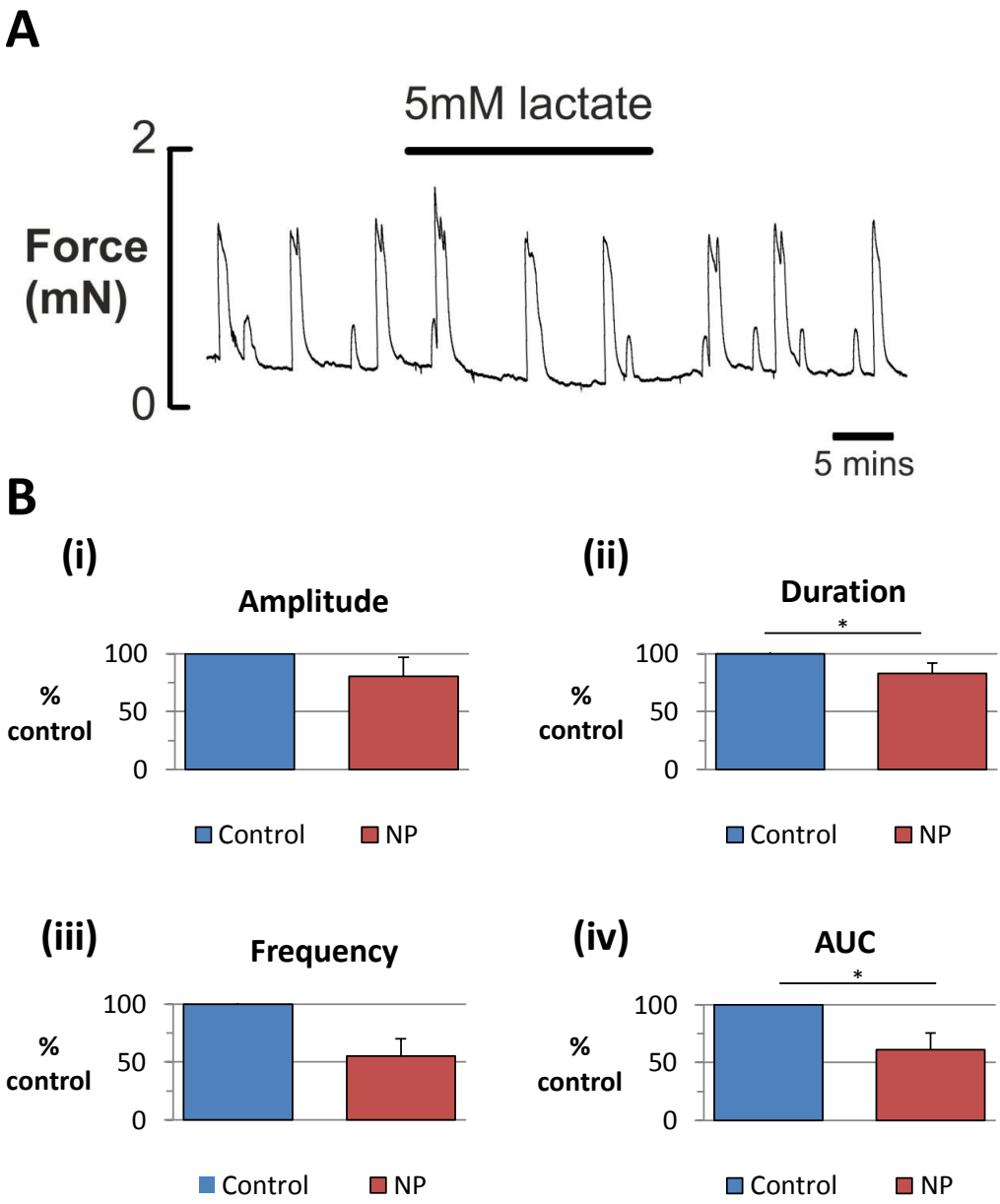


Figure 4.16 The effect of sodium lactate on non-pregnant human myometrium

A. Representative isometric recordings of spontaneously contracting myometrial strips during 20 minutes application of 5mM sodium lactate on spontaneously-contracting non-pregnant myometrium *(n*=6).
B. Mean data ± s.e.m. (denoted by error bars) showing dose-dependent decrease in (i) amplitude (ii) duration (iii) frequency (iv) AUC in response to 5mM lactate (red) when compared to the preceding control (blue). N numbers are denoted by values within bars. There was a significant difference in duration and a.u.c (students paired t-test, p= 0.03and p=0.047 respectively)

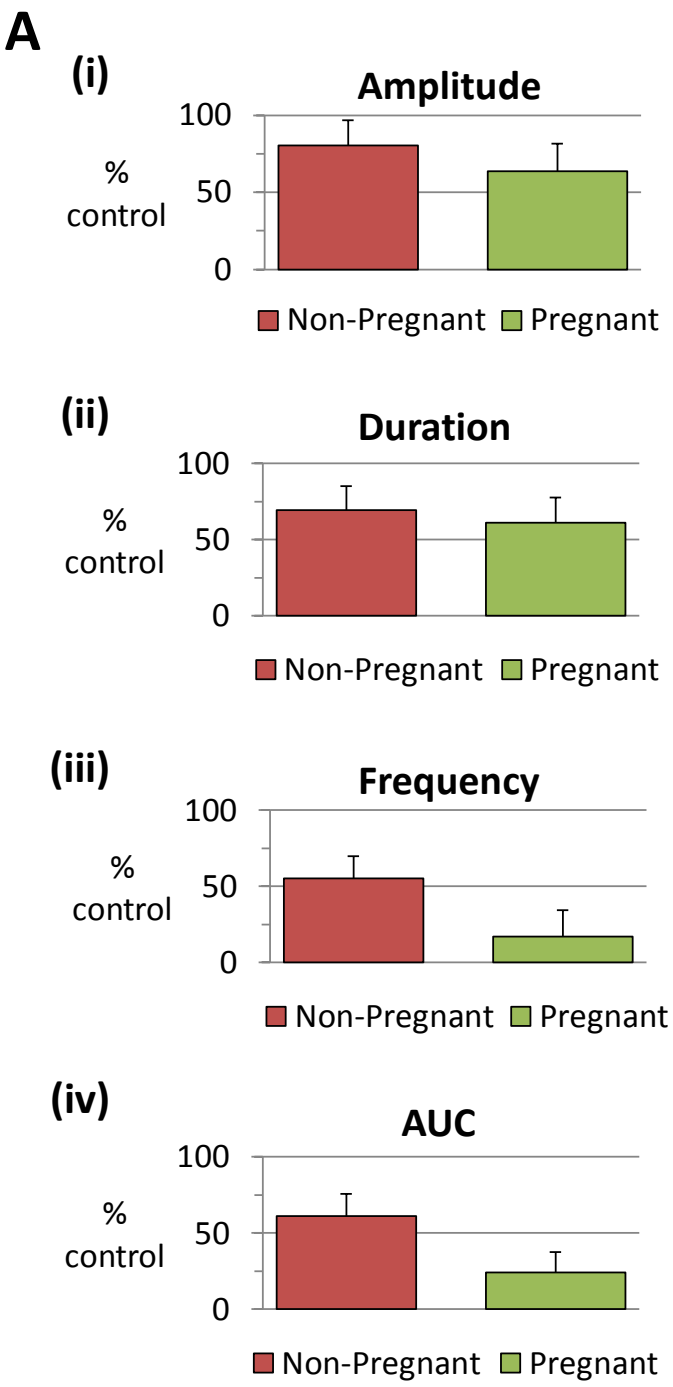


Figure 4.17 Comparison of lactate response between non-pregnant and pregnant human myometrium

A. Mean data (i) amplitude (ii) duration (iii) frequency and (iv) a.u.c. of the response of 5mM lactate in non-pregnant human myometrial tissue (red bar) and pregnant myometrium (green). There was no significant difference between the two gestational states.

4.4.5. Comparison of rat and human lactate data

As the same experimental procedures were used to test the effect of lactate on human and rat myometrium and both tissues were the same gestation (term non-labouring), I can directly compare the two tissues. Mean data for 1-20mM lactate on all four parameters of contractility for spontaneously contracting pregnant myometrium were plotted together (Figure 4.18) and statistical analysis between groups performed. The EC_{50} values for these two tissues were 3.9 ±0.3mM (rat) and 3.8 ±0.7mM (human). There was no significant difference between rat and human myometrium in amplitude (Figure 4.18 Ai) duration (Figure 4.18 Aii) frequency (Figure 4.18 Aiii) or a.u.c (Figure 4.18 Aiv) (Table 4.3) (Anova with Bonferroni post-hoc test, $p>0.05$).

Mean data for rat and human pregnant myometrium in the presence of oxytocin was also compared (Figure 4.19). The EC_{50} values for these two tissues were 11.1 ±1.7mM and 5.4 ±1.2mM respectively. There was no significant difference between rat and human myometrium in amplitude (Figure 4.19 Ai) duration (Figure 4.19 Aii) frequency (Figure 4.19 Aiii) or a.u.c (Figure 4.19 Aiv) (Table 4.4) (Anova with Bonferroni post-hoc test, $p>0.05$).

Non-pregnant tissue when challenged with 5mM lactate, from both rat and human myometrium, was also compared (Figure 4.20). There was no significant difference between these two tissue types (Students unpaired t-test, $p>0.05$).

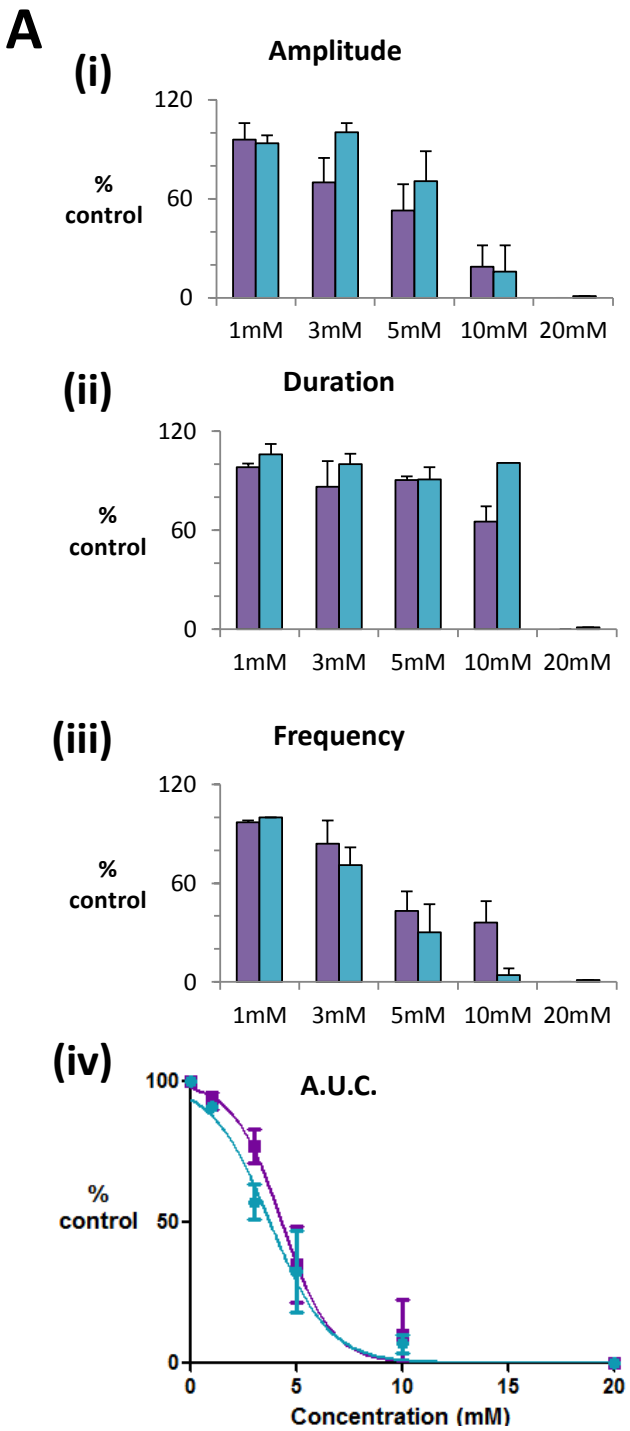


Figure 4.18 Comparison of lactate response between rat myometrium and human myometrium

A. Mean data for (i) amplitude (ii) duration (iii) frequency and (iv) a.u.c. comparing rat myometrium (purple) and human myometrium (turquoise) on spontaneously contracting tissue. There was no significant difference between the two tissues.

A

(i) Amplitude

	Rat	Human	P value
1mM	96±10%	93.9±4.9%	0.66
3mM	70±15%	100.5±5.4%	0.09
5mM	53±16%	70.6±18.4%	0.34
10mM	19±13%	15.9±15.9%	0.62

(ii) Duration

	Rat	Human	P value
1mM	98.3±2.2%	106.1±6.4%	0.30
3mM	86.2±15.9%	99.9±6.6%	0.31
5mM	90.5±2.2%	90.7±7.6%	0.04*
10mM	65.3±9.1%	100.9±0%	0.05

(iii) Frequency

	Rat	Human	P value
1mM	97±1%	100%	0.08
3mM	84±14%	71.1±10.9%	0.42
5mM	43±12%	30.1±17.2%	0.69
10mM	36±13%	4.2±4.2%	0.31

(iv) AUC

	Rat	Human	P value
1mM	84.1±11.2%	88.1±3.3%	0.74
3mM	62.6±14.1%	57.2±6.3%	0.78
5mM	35.5±12.9%	33.3±12.1%	0.92
10mM	14.5±9.7%	5.0±5.0%	0.43

Table 4.3 Comparison of lactate response between rat myometrium and human myometrium

A. Mean data for (i) amplitude (ii) duration (iii) frequency and (iv) a.u.c. comparing rat myometrium and human myometrium on spontaneously contracting tissue. There was no significant difference between the two tissues. 20mM response was excluded from the table as contractions were completely abolished.

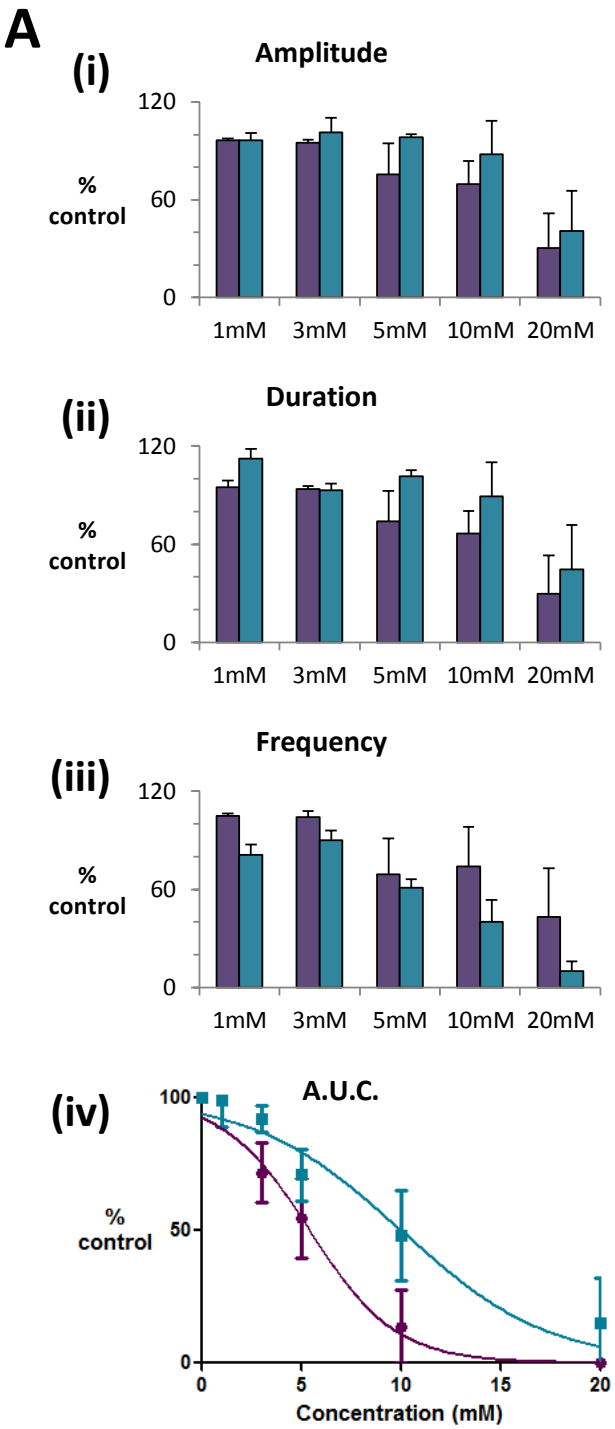


Figure 4.19 Comparison of lactate response between rat myometrium and human myometrium in the presence of oxytocin

A. Mean data for (i) amplitude (ii) duration (iii) frequency and (iv) a.u.c. comparing rat myometrium (purple) and human myometrium (turquoise) on oxytocin-stimulated tissue. There was no significant difference between the two tissues.

A

(i) Amplitude

	Rat	Human	P value
1mM	96.7±0.8%	96.5±4.6%	0.61
3mM	95.0±1.9%	101.4±9.0%	0.65
5mM	75.6±19.1%	98.5±1.9%	0.63
10mM	69.5±14.2%	88.1±20.5%	0.17
20mM	30.4±21.5%	40.7±24.9%	0.99

(ii) Duration

	Rat	Human	P value
1mM	94.7±4.1%	112.5±5.9%	0.31
3mM	93.5±2.0%	93.0±4.2%	0.40
5mM	74.0±18.5%	101.6±3.7%	0.17
10mM	66.6±13.7%	89.2±21.0%	0.69
20mM	29.9±23.3%	44.5±27.3%	0.20

(iii) Frequency

	Rat	Human	P value
1mM	105±1.5%	81.2±6.2%	0.23
3mM	104±4%	90.0±6.1%	0.85
5mM	69±22%	60.8±5.3%	0.09
10mM	74±24%	40.3±13.2%	0.23
20mM	43±30%	10.0±6.1%	0.25

(iv) AUC

	Rat	Human	P value
1mM	94.9±3.0%	80.8±1.3%	0.18
3mM	80.7±7.3%	71.7±11.4%	0.27
5mM	68.5±17.3%	55.2±12.9%	0.38
10mM	48.3±15.9%	13.8±10.4%	0.16
20mM	20.9±19.3%	2.8±2.4%	0.41

Table 4.4 Comparison of lactate response between rat myometrium and human myometrium in the presence of oxytocin

A. Mean data for (i) amplitude (ii) duration (iii) frequency and (iv) a.u.c. comparing rat myometrium and human myometrium on oxytocin-stimulated tissue. There was no significant difference between the two tissues.

A

(i) Amplitude

	Rat	Human	P value
1mM	96.7±0.8%	96.5±4.6%	0.61
3mM	95.0±1.9%	101.4±9.0%	0.65
5mM	75.6±19.1%	98.5±1.9%	0.63
10mM	69.5±14.2%	88.1±20.5%	0.17
20mM	30.4±21.5%	40.7±24.9%	0.99

(ii) Duration

	Rat	Human	P value
1mM	94.7±4.1%	112.5±5.9%	0.31
3mM	93.5±2.0%	93.0±4.2%	0.40
5mM	74.0±18.5%	101.6±3.7%	0.17
10mM	66.6±13.7%	89.2±21.0%	0.69
20mM	29.9±23.3%	44.5±27.3%	0.20

(iii) Frequency

	Rat	Human	P value
1mM	105±1.5%	81.2±6.2%	0.23
3mM	104±4%	90.0±6.1%	0.85
5mM	69±22%	60.8±5.3%	0.09
10mM	74±24%	40.3±13.2%	0.23
20mM	43±30%	10.0±6.1%	0.25

(iv) AUC

	Rat	Human	P value
1mM	94.9±3.0%	80.8±1.3%	0.18
3mM	80.7±7.3%	71.7±11.4%	0.27
5mM	68.5±17.3%	55.2±12.9%	0.38
10mM	48.3±15.9%	13.8±10.4%	0.16
20mM	20.9±19.3%	2.8±2.4%	0.41

Table 4.4 Comparison of lactate response between rat myometrium and human myometrium in the presence of oxytocin

A. Mean data for (i) amplitude (ii) duration (iii) frequency and (iv) a.u.c. comparing rat myometrium and human myometrium on oxytocin-stimulated tissue. There was no significant difference between the two tissues.

4.5 Discussion

No previous functional study has investigated the effect of lactate on myometrial contractility. Previous work has indicated lactate may play a role in dysfunctional labour. This study shows that the application of lactate and another weak acid, propionate, cause a decrease in human myometrial contractility in a dose-dependent manner. This occurs in both spontaneously contracting tissue and in the presence of oxytocin, although oxytocin provides a 'rescue effect' as seen in the dose-response curve (which is shifted to the right). 5mM lactate affects contractility in non-pregnant myometrium, but to a lesser extent than term pregnant tissue.

I propose that lactate is affecting human myometrium in the same way as rat myometrium as suggested in **Chapter 3**. Although intracellular pH was not investigated using human tissue, it is likely to be the cause of its ability to decrease contractions, via its action on L-type Ca^{2+} channels. The inhibition of these channels caused by intracellular acidification in human myometrium has already been shown (Pierce et al. 2003). Also women experiencing a dysfunctional labour have been shown to have a lower myometrial blood pH than women experiencing a normal labour (Quenby et al. 2004) indicating acidification.

There was a notable drop in baseline tension when either lactate or propionate was applied, regardless of the presence of oxytocin. This may be due to the fact H^+ and Ca^{2+} are cations and therefore there may also be competition between the two ions at intracellular binding sites. H^+ could displace Ca^{2+} bound to myofilaments and therefore decrease tone. This loss of tension was not seen in rat myometrial tissue in **Chapter 3**. However larger tissue strips from rat uterus that contain both circular myometrium and endothelium have been shown to lose tension when challenged with hypoxic conditions (Personal communication: Dr Sarah

Arrowsmith). Because of this the effect of weak acids on myometrial tissue may be underestimated in smaller strips as they have a smaller mass (and therefore the number of muscle bundles is significantly lower).

Non-pregnant myometrium appears to be more resistant to the effect of 5mM lactate when compared to term pregnant human myometrium. Differences between the two gestational states could be responsible for this. There may be a difference in lactate transporters between non-pregnant and pregnant myometrium i.e. lower in non-pregnant myometrium and therefore in these experiments, less lactate could enter the myometrial cells. Cells in non-pregnant myometrium have not undergone hypertrophy which occurs during pregnancy. This may be the case as the non-pregnant myometrial strips are harder to dissect from biopsies than pregnant biopsies as the tissue is firmer, more compact and harder to blunt dissect. When lactate's effect on human non-pregnant myometrium is compared with non-pregnant rat tissue, there is no significant difference between the two. This is good indication that the animal model is robust for future experiments.

The effect of lactate on rat and human contractility was compared and no significant differences were found between the two. This is another good indication that the animal model is ideal for future lactate experiments. Although lactate in both tissues produced a dose-dependent decrease in contractility, there were slight differences between the two. Frequency of normal control contractions was lower in human tissue, hence the longer lactate application time. Amplitude and duration of contractions in human tissue are higher. These three parameters may be linked. Every care was taken to dissect tissue strips of similar size to avoid differences in contractility cause by differences in size. The dose response curve for lactate in human tissue stimulated by oxytocin was shifted further to the

right than rat tissue as indicated by the EC50 values. This shows the 'rescue effect' of oxytocin and why it is used in clinical practice as a treatment of dysfunctional labour. The concentrations of oxytocin were chosen as they have been used previously in myometrial contractility experiments I could speculate that at higher concentrations of oxytocin, lactate would be ineffective at decreasing contractility. However in vivo this may produce a large tonic contraction upon application. Other in vivo differences e.g. acidic extracellular environment and hypoxia may occur that would decrease the effectiveness of oxytocin.

4.5.1 Limitations of study

- This study has low *n* numbers. Human tissue is, in its nature, more variable than an animal model and the tissue is harder to acquire. My conclusions on the effects of lactate however appear to be robust as the effects are large and reproducible. More experiments would increase confidence in the conclusion between non-pregnant and pregnant myometrium.
- No experiments were performed on labouring human myometrium therefore it is still unclear the effect of lactate on this tissue and how it relates to dysfunctional labour.
- No mechanistic work looking at intracellular pH or Ca^{2+} was included in this study. These experiments need to be done to confirm the mechanistic action of lactate in human myometrium, although these have been performed for rat myometrium and I anticipate that the conclusions would be similar concerning mechanisms.

4.6 Summary

Lactate has profound effects on the contractility of human myometrium. It caused a dose-dependent decrease in contractility that was not overcome by the application of oxytocin. If lactate is involved in dysfunctional labour, this would correlate with clinical observations. There was no difference between lactates response in human or rat myometrium, indicating the animal model is robust to use for future experiments concerning lactate and mechanistic data obtained in rat myometrium would be comparable to human myometrium.

Chapter 5

The expression of monocarboxylate transporters (MCTs) in rat and human myometrium

Chapter 5

The expression of monocarboxylate transporters (MCTs) in rat and human myometrium

5.1 Abstract

Monocarboxylate transporters (MCTs) are responsible for transporting lactate and other monocarboxylates, including pyruvate and ketone bodies, across the plasma membrane. Monocarboxylates, together with a proton, are transported down the concentration gradient via MCTs 1 to 4. They are widely expressed in many tissues, including skeletal muscle, where it has been shown that MCT-4 is up-regulated in hypoxic conditions. There is limited evidence for the expression of MCTs in the myometrium. The aim of this study was to investigate the expression of MCT-1 and MCT-4 in both rat and human myometrium. Proteins were extracted from myometrium of rats throughout gestation, including non-pregnant and labouring myometrium and human non-pregnant and pregnant myometrial tissue. Western blots were performed using anti-MCT-1 and anti-MCT-4 antibodies with anti-β-actin antibody used as an internal loading control. Experiments show that both transporters are present in rat and human myometrium. The levels of MCT-1 and MCT-4 change throughout gestation of rat myometrium with MCT-1 being at its highest in mid-gestation (*n*=2), and MCT-4 being at its lowest (*n*=4). Expressions of both transporters are higher in pregnant myometrium compared to non-pregnant tissue (*n*=4). A small 25kDa band was seen when probed with anti-MCT-4 antibody. Initial experiments agree with previous studies that MCT-1 and MCT-4 are present in the myometrium. It is the first study

to investigate their expression throughout gestation and compare with expression in non-pregnant myometrium. Future work will determine the localisation of these transporters in the myometrium.

5.2 Introduction

Monocarboxylates such as lactate, pyruvate and ketone bodies are involved in a number of metabolic pathways, including lipogensisis, gluconeogenesis, and anaerobic glycolysis. They are transported across the plasma membranes of cells by proton-linked monocarboxylate transporters (MCTs) (Halestrap 2012).

MCTs are part of the solute carrier (SLC) 16 family. Out of a total of 14 family members, only four have been shown to facilitate proton-linked monocarboxylate transport: MCTs 1-4.

The structure of MCTs is similar to other SLC16 family members and they share conserved sequence motifs (Halestrap & Meredith 2004) (Figure 5.1). The transporters consist of 12 transmembrane helices with an intracellular N and C terminus. They first bind H^+ followed by a lactate anion. There is a conformational change of the protein to a closed formation which exposes lactate and H^+ to the opposite side of the membrane and the two molecules are released. This transport can be stimulated by a drop in cytoplasmic pH or increased pH on extracellular side, which decreases Km (defined as substrate concentration at half the maximum velocity) for lactate.

Ancillary proteins are needed for the activity of MCTs on the cell surface. Embigin (GP70) and Basigin (CD147) both have a single transmembrane domain with a large glycosylated extracellular domain. These proteins need to be expressed for their respective MCTs to be expressed on the plasma membrane. If this does not happen, the transporters accumulate in the Golgi apparatus i.e. they act as a chaperone proteins (Halestrap & Wilson 2012). MCTs 1 and 4 co-localise with Basigin, although MCT-1 will bind Embigin if there is no Basigin. MCT-2 can bind Basigin if there is no Embigin.

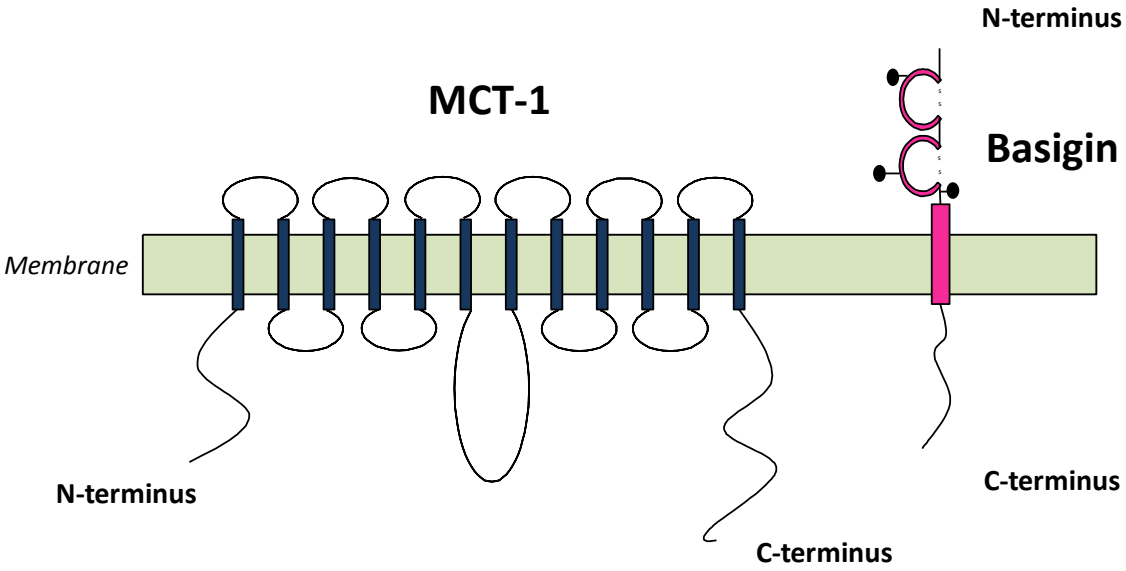


Figure 5.1 Monocarboxylate transporter-1 and its ancillary protein, Basigin

Monocarboxylate transporters (MCTs) consist of 12 transmembrane helices with an intracellular N and C terminus. Ancillary proteins (e.g. Basigin) are needed for the expression of these transporters on the plasma membrane.

MCTs bind H^+ followed by a lactate ion. There is a conformational change which exposes lactate and H^+ to the opposite side of the membrane.

MCT- 1 is found in the majority of tissues across a wide range of species. It has been shown to be up-regulated in response to exercise in skeletal muscle, i.e. conditions when lactate may be anticipated to rise. (Halestrap 2012)

MCT-4 is widely expressed in glycolytic tissue e.g. white skeletal muscle fibres and white blood cells. In the neonate heart, MCT-4 is dominant whereas in the adult heart there is no MCT-4. This is because it is less glycolytic than the neonate heart. MCT-4 is also strongly expressed in placenta to export lactate and H^+ rapidly from fetal circulation to maternal blood. It has a high Km value for pyruvate (therefore low affinity) to avoid its loss from the cell. This prevents removal of NADH which is formed when pyruvate is converted to lactate and allows the continuation of glycolysis.

MCT-2 is similar to MCT-1 as they both have higher affinities for pyruvate over lactate. However the tissue distribution of these two transporters is different (Garcia1995) and the capacity to transport these acids is lower in MCT-2 compared to MCT-1 (Broer1999). MCT-3 is only found in retinal pigment epithelial cells (Halestrap & Meredith 2004).

The only evidence for presence of MCTs in the myometrium is from Akerud *et al* 2009. This group used immunohistochemistry techniques on cultured myometrial cells from human pregnant myometrial biopsies. They found MCT-1 and MCT-4 to be present, at the cell membrane and in the stroma (MCT-4 only). MCT-4 was found at lower levels than MCT-1 (Akerud, Ronquist & Wiberg-Itzel 2009).

The aim of this study was to investigate the expression of MCT-1 and MCT-4 in rat and human non-pregnant and pregnant myometrium, and in rat myometrium throughout

gestation. These transporters were chosen due to their proven role in exercise and lactate transport when glycolysis is increased.

5.3 Methods

5.3.1 Tissue and protein extraction

Myometrium was dissected from non-pregnant virgin rats, 11 day gestation, 18 day, 22 day, 23 day and labouring rats. 23 day pregnant animals were due to deliver within 4 hours and were used to determine if expression of transporters change immediately prior to labour. Human myometrial biopsies, both non-pregnant and pregnant, were used. Non-pregnant samples were age (mean 44 years ±1year) and BMI-matched (28.7 ±0.5). Pregnant samples were closely matched by age (mean 32 years ±0years), BMI (22.4 ±1.4), gestation (39weeks+1day ±1day), parity (1 ±0) and reason for section (previous traumatic vaginal delivery). Four samples of non-pregnant tissue and pregnant tissue were selected, to indicate natural differences. Tissue strips were dissected and weighed and flash frozen in liquid nitrogen and stored at -80^{O}C until sufficient samples had been collected for all experiments. Tissue from rat skeletal muscle, liver and brain were dissected and frozen for use as positive controls. Proteins were extracted in RIPA buffer using liquid nitrogen. Protein samples were quantified using Bio-Rad DC protein assay. Due to the low amount of protein in 3/8 human samples (2 non-pregnant, 1 pregnant), these were not used in experiments. 25µg of protein was added to 3x Laemmli buffer (0.125M Tris-HCl pH6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue, 10% β-mercaptoethanol) and boiled for 3minutes.

5.3.2 SDS_PAGE and Immunoblotting

12% resolving gel and 4% stacking gel were cast using OMNI-Page kit. 20µl prepared protein was loaded. 5µL protein ladder (SeeBlue® Plus2 Pre-stained standard, Invitrogen) was also loaded to show efficient transfer and allow recognition of proteins. Gels were run at 120V for 2hours. Gels assembled into transfer cassettes with nitrocellulose paper and transferred

at 40V for 1 hour. Membranes were incubated in primary antibodies overnight at 4^{o}c. After washed in TBS-0.1% tween for 40minutes, membranes were incubated in secondary antibodies for 1 hour at room temperature. After a further 1x15minute and 4x5minute wash, Membranes were incubated in chemiluminescent ECL reagent for Horseradish peroxidase (HRP) enzyme for 5 minutes (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). Negative controls were incubated overnight or for 1 hour on Day 2 in 5% BSA. Blots were quantified using Image J and presented at % of β-actin internal control expression.

5.4 Results

5.4.1 Expression of MCT-1

5.4.1.1 Rat myometrium

To address the question of whether there are monocarboxylate transporters in the myometrium, western blotting using antibodies against MCT-1 was performed on rat myometrium to investigate its expression (Figure 5.2). Protein (25µg) from myometrium of rats at defined stages of gestation was used, along with skeletal muscle as a positive control. Due to the high amount of expression of MCTs in skeletal muscle, 12.5µg of this protein was loaded. This was taken into consideration during analysis. Figure 5.2 A shows a representative blot for MCT-1 (*n*=2). MCT-1 was detected in all of these tissues. There are two bands in skeletal muscle (Figure 5.2 A). Using the same experimental protocol and tissues, simultaneous blots were probed with anti β-actin antibody as a loading control. β-actin is a highly conserved protein and a major constituent of contractile apparatus. Because of this it is a commonly used control and has been used and validated previously in my group. There was no significant difference between β-actin expression from the rat myometrium tested, indicating even protein loading (Students unpaired T-test, $p>0.05$).

Densitometric quantification of MCT-1 expression was performed and displayed at percentage of β-actin internal control expression (Figure 5.2 B). Analysis of signal intensity shows there appears to be a small increase in expression towards mid-gestation which decreases in labouring tissue, to lower than non-pregnant levels. MCT-1 in skeletal muscle was 1572% times higher than in the myometrium. Due to the low *n* numbers for this experiment, statistical analysis was not performed on the signal intensity of MCT-1.

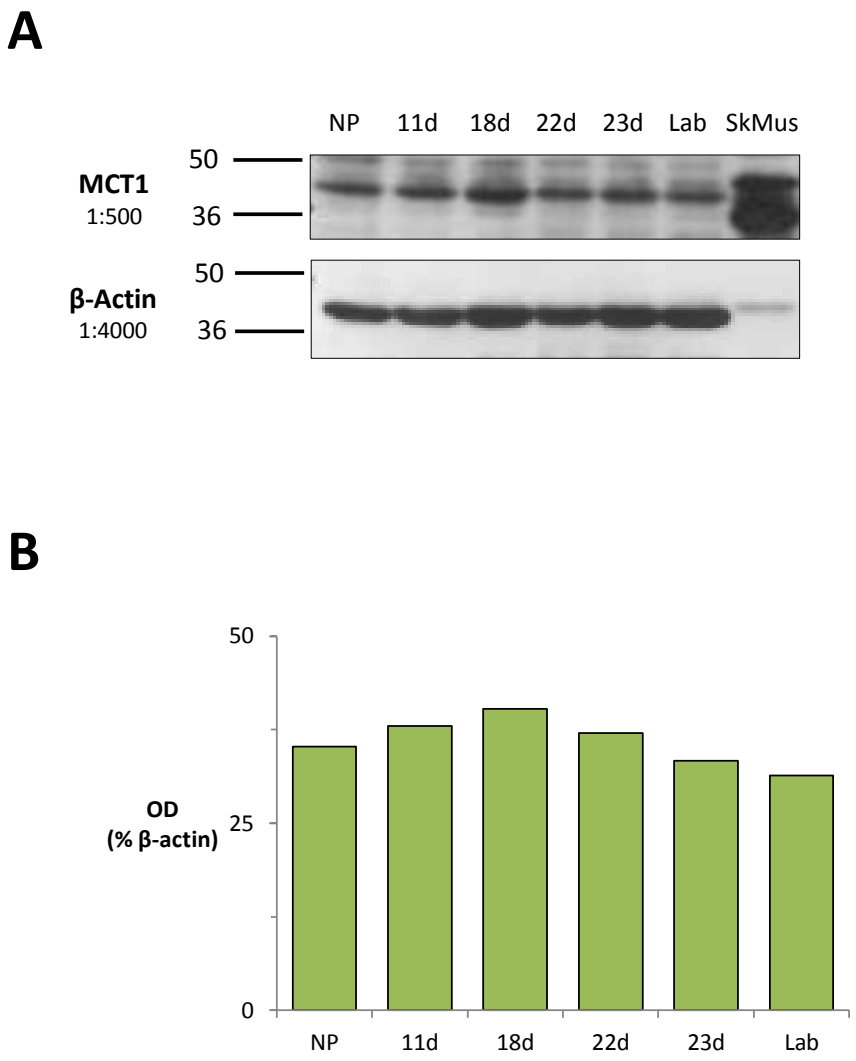


Figure 5.2 Presence of MCT1 in rat myometrium

A: Representative image of blots for MCT1 in rat myometrium throughout gestation (*n*=2). 25µg protein was loaded for all gestations and positive controls, except skeletal muscle where 12.5µg was used.
B: Quantification of MCT1 expression. Densitometric quantification of western blots for all gestations, displayed as % of matched β-actin internal control expression.

5.4.1.2 Human myometrium

As I had demonstrated that there was MCT-1 in rat myometrium, expression in human non-pregnant and pregnant myometrium was tested. An initial experiment with MCT-1 antibody provided a positive result (Figure 5.3 A). However when the same experiment was repeated omitting the primary anti- MCT-1 antibody, the same bands were seen (Figure 5.3 B). This indicated the secondary antibody (goat anti-rabbit) was binding non-specifically to proteins in the samples and potentially obscuring true MCT-1 expression. Another secondary antibody, donkey anti-rabbit, was used and this provided a cleaner blot with no bands seen (Figure 5.3 C)

Figure 5.4 A shows a representative blot for MCT-1 in human myometrium (*n*=4). MCT-1 was detected in both non-pregnant and pregnant tissues. Using the same experimental protocol and tissues, simultaneous blots were probed with anti β-actin antibody as a loading control. There was no significant difference between β-actin expression (Students unpaired T-test, $p>0.05$)

Quantification of MCT-1 expression in human myometrium was performed and displayed at percentage of β-actin internal control expression (Figure 5.4 B). There is an increase in signal intensity in pregnant tissue when compared to non-pregnant tissue, but there was no significant difference between the two tissue types ($p=0.35$).

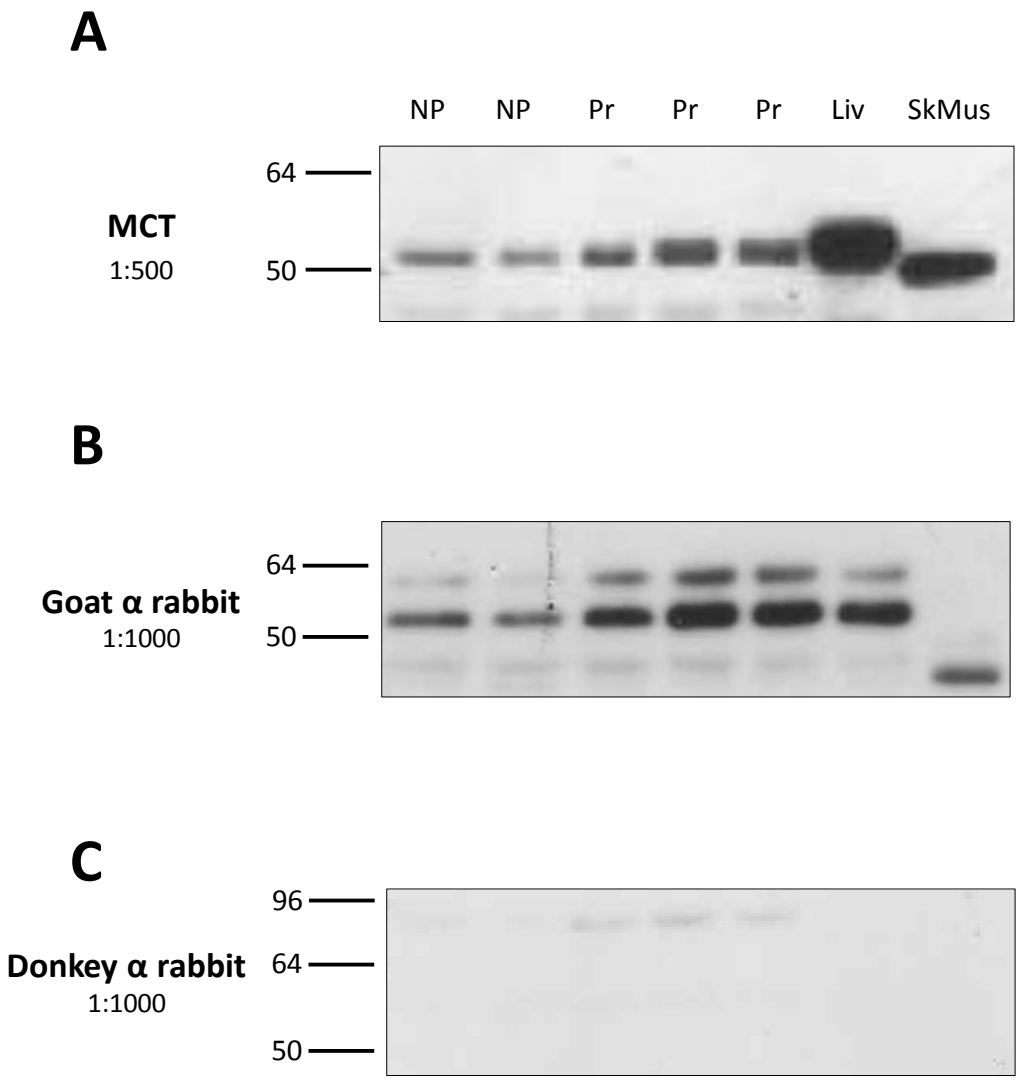


Figure 5.3 Non-specific binding of the secondary antibody in human myometrium

A: Representative western blot probed with anti- MCT-4 antibody. Bands are seen at expected kDa weight.
B: Control western blot probed with secondary goat anti-rabbit antibody. Bands are seen are expected kDa size of MCT proteins.
C: Control western blot probed with secondary donkey anti-rabbit antibody. No bands are seen at MCTs expected size.

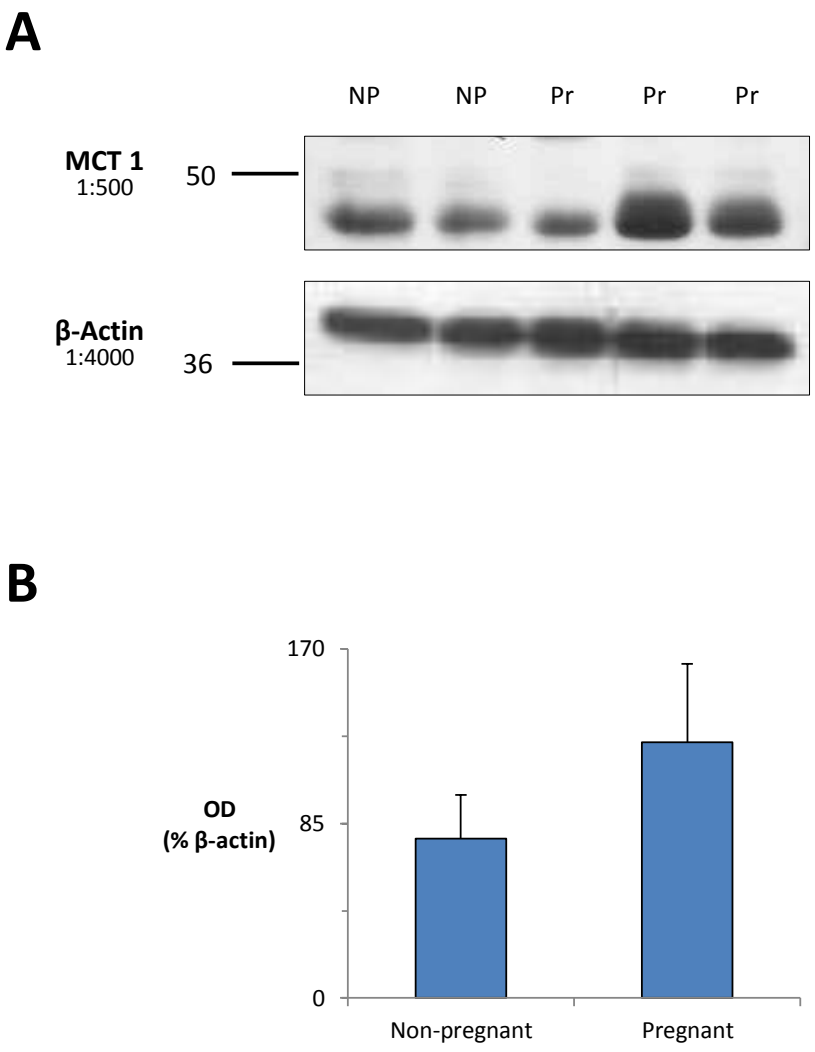


Figure 5.4 Presence of MCT1 in human myometrial tissue

A: Representative image of blots for MCT1 in human non-pregnant and pregnant myometrium (*n*=4). 25µg protein was loaded for all gestations and positive controls (brain and liver)
B: Optical density of MCT1, displayed as % of matched β-actin control.

5.4.2 Expression of MCT-4

5.4.2.1 Rat myometrium

MCT-4 expression in rat myometrium through gestation was investigated (Figure 5.5) Figure 5.5 A shows a representative blot for MCT-4 in rat myometrium (*n*=4). MCT-4 was detected in all tissues; non-pregnant, 11 day, 18 day, 22 day and 23 day gestation and labouring tissue. Rat skeletal muscle was used as a positive control for the antibody. There are two bands at the expected molecular weight of MCT-4 (45 kDa). Using the same experimental protocol and tissues, simultaneous blots were probed with anti β-actin antibody as a loading control. There was no significant difference between β-actin expression (Students unpaired T-test, $p>0.05$)

Quantification of MCT-4 expression was performed and displayed at percentage of β-actin internal control expression (Figure 5.5 B). Initially the intensity of both bands was combined to give one single reading for each gestation. There is a decrease in signal as the myometrium increases in gestation. There is a small increase in 23 day pregnant myometrium and a bigger increase when the tissue is labouring. This is larger than the signal for non-pregnant tissue. Despite the change, there was no significant difference between the different tissues ($p>0.05$).

As there were two distinct bands, separate analysis was performed to see if the bands changed throughout gestation. The intensity of the higher weight band (defined 'Top band') was compared against the intensity of the lower band ('Bottom band') as a percentage of total band intensity (Figure 5.6). The bottom band in non-pregnant and 11 day myometrium is higher than the bottom band and this pattern shifts in 18 day onwards- top band has a higher intensity than the bottom band.

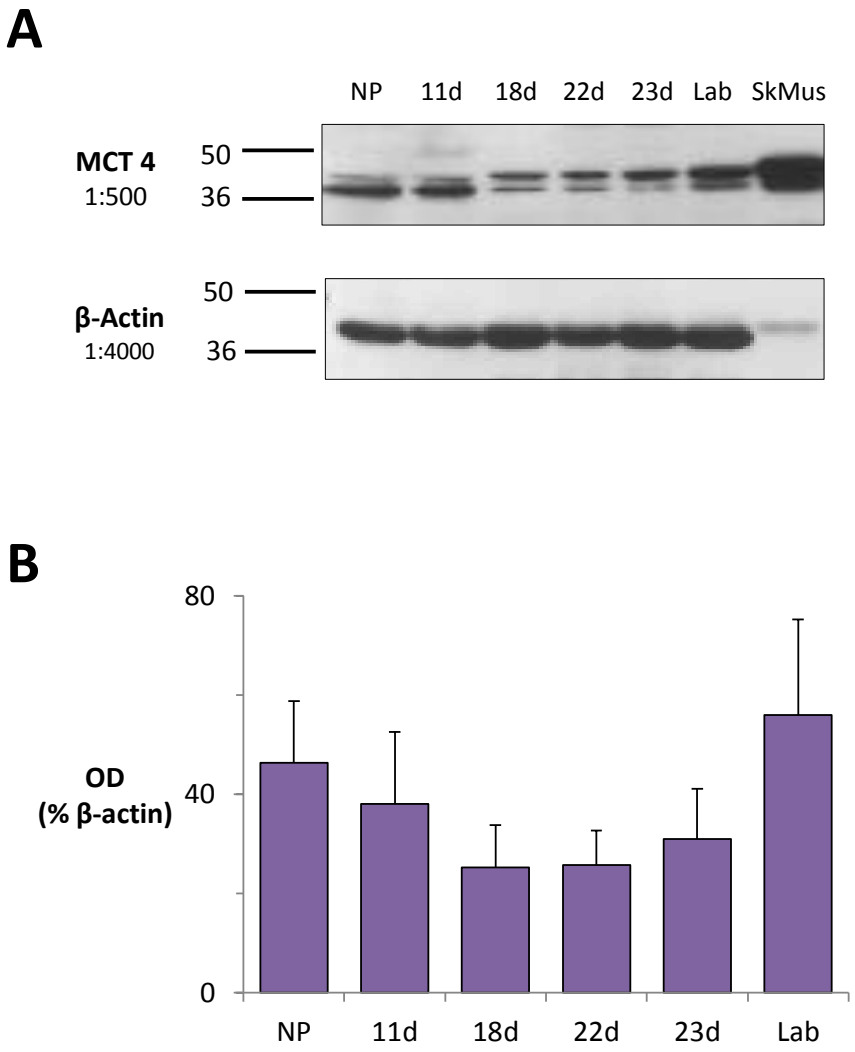


Figure 5.5 Presence of Monocarboxylate transporter 4 (MCT4) in rat myometrium

A: Representative image of blots for MCT4 in rat myometrium throughout gestation (*n*=4). 25µg protein was loaded for all gestations and positive controls, except skeletal muscle where 12.5µg was used.
B: Optical density of MCT4, displayed as % of matched β-actin control.

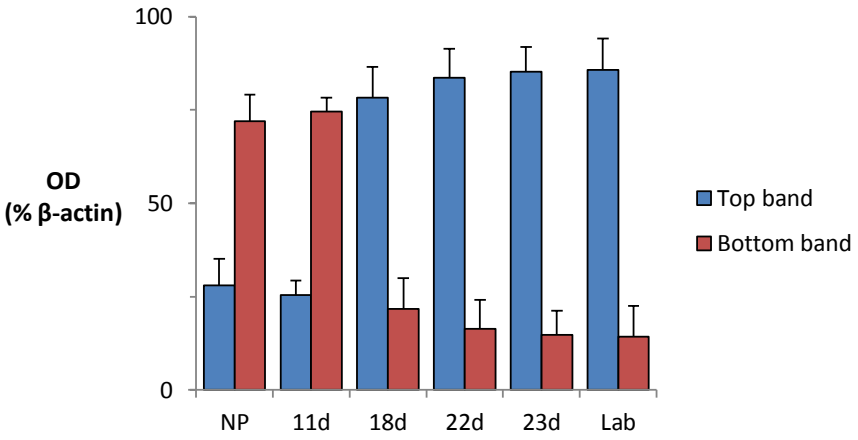


Figure 5.6 Quantification of multiple bands of MCT4 in rat myometrium

5.4.2.2 Human myometrium

The expression of MCT-4 was examined in human myometrium (*n*=4) (Figure 5.7). At the predicted molecular weight, there were no bands in either non-pregnant or pregnant samples. A band was seen in skeletal muscle, the positive control (Figure 5.6 A). Bands were seen at 25kDa in all three pregnant samples (Figure 5.7 A, red circle). Simultaneous blots were probed with anti β-actin antibody as a loading control and there was no significant difference between β-actin expression (Students unpaired T-test, $p>0.05$).

Quantification of MCT-4 expression in human myometrium was performed and displayed at percentage of β-actin internal control expression. The area of predicted molecular weight was analysed and there was no increase in signal intensity. When comparing that result for non-pregnant myometrium to the lower weight bands seen in pregnant myometrium (Figure 5.7 B) there is an increase in signal intensity in pregnant tissue. This difference in signal intensity between the two samples was significant ($p=0.006$).

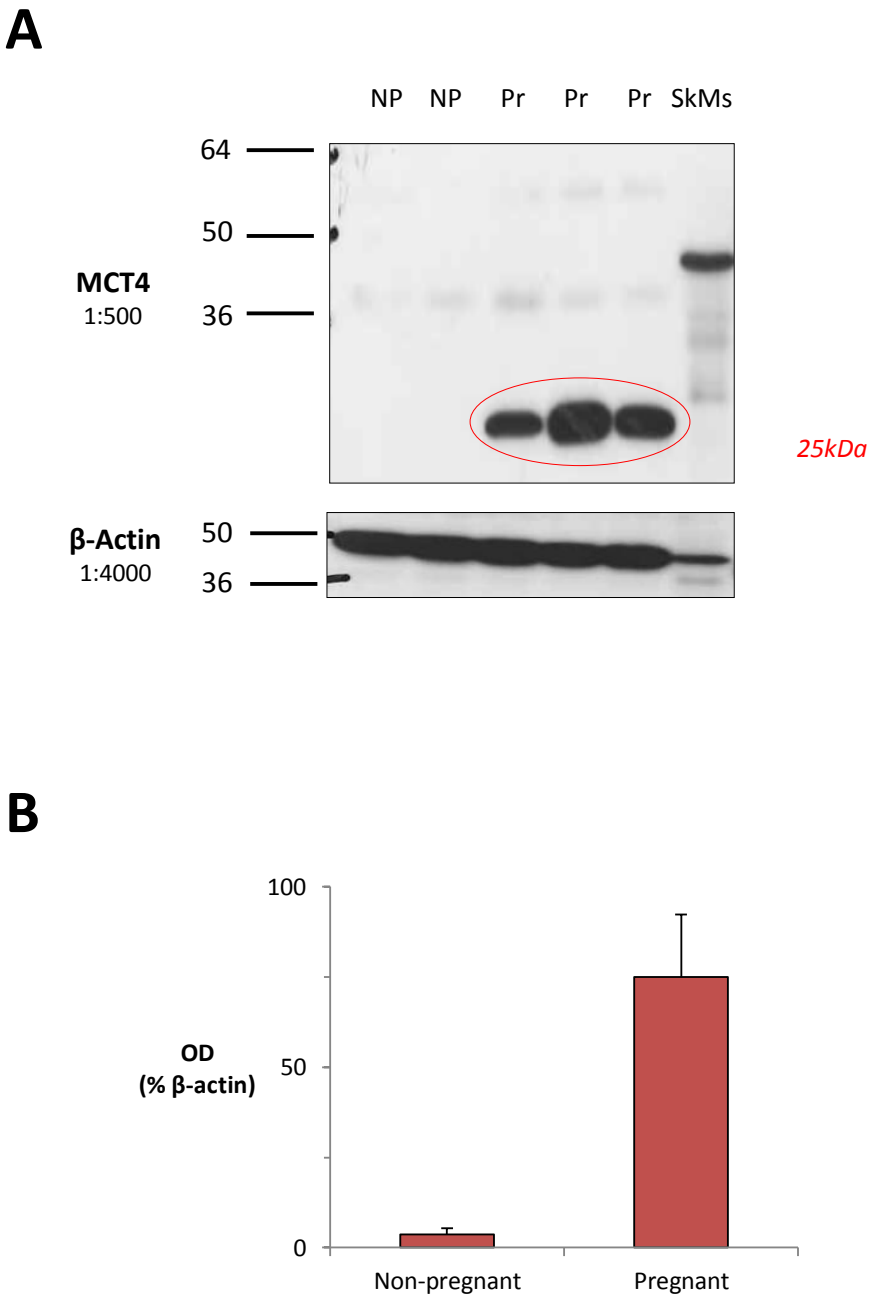


Figure 5.7 Presence of MCT4 in human myometrial tissue

A: Representative image of blots for MCT4 in human non-pregnant and pregnant myometrium (*n*=4). 25µg protein was loaded for all gestations and positive controls, except skeletal muscle where 12.5µg was used.
B: Optical density of MCT4, displayed as % of matched β-actin control.

5.5 Discussion

There is little previous evidence for the expression of monocarboxylate transporters in the myometrium, as only one previous study appears to have been published. One study has shown, by immunohistochemistry that MCTs 1 and 4 are present in cells for pregnant myometrial biopsies but their expression was not quantified or expression throughout pregnancy investigated. The aim of this study was to investigate MCT expression in both rat and human myometrium. Initial experiments have indicated that both transporters are present and are expressed at varying levels from non-pregnant myometrium throughout gestation and in term and labouring myometrium.

β-actin expression was used as an internal loading control and in the experiments performed in this study; the expression in the myometrium was not significantly different between samples. This indicates protein loading was even throughout experiments and that any differences seen in MCT signal intensity was not be due to protein loading. Levels of β-actin were higher in myometrium compared to skeletal muscle. This is because skeletal muscle expresses α-actin.

Bands corresponding to MCTs ran at a lower weight to their predicted molecular weight. This has been noted before in the literature (Hussien & Brooks 2011). Post-translational modifications can occur and if the protein has a higher molecular weight than predicted, this could be due to glycosylation, phosphorylation or multimer formation. Proteins may be a lower molecular weight due to cleavage or degradation.

When the anti-MCT-1 antibody used, multiple bands were detected at different molecular weights. This could be due to the fact “tissue extracts tend to contain more background bands and degradation products than cell line extracts due to connective tissue” (Cell

Signalling). The data sheet supplied with the antibody also detected other bands. Multiple bands may also indicate non-specific binding of either the primary or secondary antibodies. As in the case of human myometrium, the initial secondary antibody used (goat anti-rabbit) used showed bands at ~50kDa where MCTs 1 and 4 were expected to be. This non-specific binding has also been noted in other western blots in our laboratory using human protein samples and this secondary antibody (Personal communication: Dr. Sarah Arrowsmith). A different secondary antibody, donkey anti-rabbit, did not bind non-specifically. The reason for this result is unclear and requires further investigation. The antibody was not previously adsorbed with human proteins, which can cause non-specific binding in human protein samples.

Double bands were seen in rat myometrium when probed for MCT-4. The intensity of two bands changed throughout pregnancy. The reason for this is unknown but it could be due to phosphorylation of protein. This would cause the protein to run at a higher molecular weight that un-phosphorylated protein. Immunoprecipitation could be performed on these samples using the anti-MCT-4 antibody and then probed using phospho-ser- or thr- antibodies. This would determine if the protein is phosphorylated.

In pregnant human myometrial samples, a small 25kDa band was seen when blots probed for MCT-4. It is half the size of normal MCT-4 protein (~50kDa) and was an unexpected result. However this has been seen before in a breast cancer cell line when probed with the same antibody (Hussien & Brooks 2011). Unfortunately there has been no other information published. One reason for this smaller band could be alternative splicing of the MCT-4 gene which may influence the structure and function of the gene.

Akerud found MCT-4 in two locations in the myometrium (cell membrane and stroma). Although not using the same antibodies or same protocol, the two bands could correlate to the two separate locations. The difference in size could be due to a 'tag' that targets the transporter to a specific location.

The use of anti-MCT-1 antibody on rat myometrium proved tricky, hence the low *n* number for this experiment. This was due to antibody (and the secondary antibody) not being fully optimised in this tissue type, despite several months of work. Further repeat experiments are needed to optimise these antibodies.

Little difference in MCT expression was seen between 22 day and 23 day pregnant tissue. This demonstrates that changes in expression occur in mid-gestation, whether the protein is up regulated (MCT-4) or down regulated (MCT-1). Utilising this animal model, myometrium from every day of gestation could be tested for MCT expression which would give a clearer idea of when these changes occur and in what quantity.

5.5.1 Limitations of study

- The expression of transporters was not investigated for labouring or dysfunctionally human myometrium. It is interesting to speculate how their expression and localisation may be different compared with non-labouring myometrium.

- The anti- MCT-1 antibody not fully optimised as multiple bands were seen on the blots. Further repeat experiments are needed to optimise the antibody in both rat and human tissue.

5.6 Summary

This is the first body work to show the expression of MCT-1 and MCT-4 in rat myometrium at defined stages of gestation. My work suggests they are expressed and play an important role in the myometrium. This is in agreement with initial experiments using the inhibitor alpha-cyano-4- hydroxycinnamate. I believe they will be physiologically important when lactate rises within the myometrium and there may be decreased expression of these transporters within the myometrium of women labouring dysfunctionally. It would be useful to look at the expression of the accessory proteins that chaperone the transporters to analyse their involvement during pregnancy and in labour.

Chapter 6

Final discussion

Chapter 6

Final discussion

The aim of this work in this thesis was to investigate the effect of lactate on myometrial contractility and to explore the mechanism by which it occurs. The ultimate aim is to understand what occurs during dysfunctional labour: how it is different from normal labour and thus modulate the process to improve outcomes for both mother and baby. I believe the work described here goes some way to help us understand what may be affecting contractility in these labours.

6.1 Lactate and pH

This is the first functional study of the effect of lactate on myometrial contractility. In both human myometrial tissue and in an animal model, lactate reduced contractile activity in a dose-dependent manner. Comparison of the two tissue responses showed there was no significant difference between the two, indicating that the animal model is representative of human myometrium. To investigate its mechanism of action using the animal model, tissue strips were loaded with the pH-sensitive indicator Carboxy-Snarf AM. This allowed me to see lactate decreasing pH_i of the myometrium. There is conflicting data in the literature about

lactates ability to cause a pH decrease (Brooks 2010). In some studies it is reported to alkalinise pH of the cell by buffering protons and leaving the cell via its transporters (Brooks et al. 2006). The pH measurements and null experiments performed in this study confirm that lactate is decreasing pH in the myometrium, and by using a weak base to 'null' the pH change, the effect on contractility is overcome. The use of butyrate, another weak acid that is commonly used in pH studies, produced the same effect on pH_i and contractility as lactate. Labouring myometrium loaded with Carboxy-Snarf AM demonstrated that the drop in pH_i when lactate is applied is less compared to term pregnant myometrium. This suggests it is likely that the buffering capacity of human labouring tissue is increased. This is turn suggests an adaptation, probably to cope with increased metabolites and protons due to increased work and metabolism during labour. It is interesting to speculate this may be a difference that occurs in dysfunctional labour: i.e. the buffering capacity is reduced and therefore unable to resist the effect of increased lactate (and H^+).

My data showing acidification and its effect on contractility correlates with previous studies that have shown intracellular acidification of the myometrium decreases contractility (Wray et al. 1992). It should be noted however that the lactate molecule itself may have an effect on the myometrium and the effect of pH on contractility may be masking this effect. It has been seen that lactate *per se* can affect chloride channels in skeletal muscle (de Paoli et al. 2010).

6.2 Effects of H^+ on the myometrium

The effects of H^+ on the pregnant myometrium have been previously documented. The reason for lactates effect on term pregnant tissue could be an in-built mechanism to prevent powerful tonic-like uterine contractions during labour, which could clamp down

blood vessels and reduce blood flow (and therefore oxygen) to the fetus and cause fetal distress. My data indicates H^+ acts upon Ca^{2+} entry to the myometrial cell and may affect the contractile apparatus, as shown by the drop in baseline tension in human tissue samples.

Throughout gestation the effect of lactate (and therefore H^+) increased. It had little effect on non-pregnant myometrium. It is known Ca^{2+} channels increase towards parturition (Mershon, Mikala & Schwartz 1994) and as experiments using Ca^{2+} indicator Indo-1 AM has shown, H^+ can affect L-type Ca^{2+} channels (Pierce et al. 2003). Intracellular pH of human myometrium is known to increase towards late pregnancy; non-pregnant myometrium is more acidic pH_i than late-pregnant (Parratt, Taggart & Wray 1995a). Perhaps non-pregnant myometrial tissue is able to cope with acid load as already acidic? There is a change in intracellular conditions during pregnancy meaning it is less likely to cope with an acid load and that this is reversed in labour, where lactate also has little effect on contractility.

Oxytocin is used to augment uterine contractions and it works to increase $[Ca^{2+}]_i$ available for the excitation-coupling pathway. The administration of oxytocin does not overcome lactate's inhibitory effects on contractility. This indicates the increase in Ca^{2+} by oxytocin is not enough to continue contractions. This correlates to clinical observations where administration of oxytocin for dysfunctional labour does not increase the incidence of vaginal delivery (Bugg, Siddiqui & Thornton 2013).

6.3 Lactate and MCTs

This is the first known study investigating the expression of monocarboxylate transporters 1 and 4 throughout gestation and in non-pregnant tissue. The results from this study correlate with other published studies (Akerud, Ronquist & Wiberg-Itzel 2009; Fishbein,

Merezhinskaya & Foellmer 2002). How does it correlate lactates response? There is not enough evidence to suggest if a change in expression of transporters is affecting contractility. It is an interesting result in double bands of MCT-4 in rat tissue and small band in human pregnant tissue. In some studies, two bands for MCT-1 and MCT-2 are seen in cattle but not MCT-4 (Koho et al. 2008).

6.4 Dysfunctional labour

The direct link between lactate and dysfunctional labour still needs to be established even though higher lactate levels have been seen in dysfunctionally labouring women *in vivo* (Quenby *et al.* 2004).The lack of human labouring and dysfunctionally-labouring myometrial samples makes this difficult. Rat myometrium is more readily available and the experiments in this thesis show that it generally behaves in a similar way to human myometrium. Although it is reasonable to extrapolate the labouring data to human myometrium, there may be species differences that are not obvious when comparing in non-pregnant or pregnant tissue. There is also no animal model for dysfunctional labour, only 'normal' labouring. Analysis of dysfunctionally-labouring samples is imperative to confirm the link between lactate and problematic labours. Preliminary data with Dr Annabelle Kendrick suggests lactate does have an effect on labouring myometrium.

The clinical approach to treat dysfunctional labours is to augment uterine contractions using an agonist. If dysfunctional labour is caused by increased lactate in the myometrium, the agonist will need to overcome the effects of acidification that occur. In this study oxytocin was unable to overcome this effect indicating it is unsuitable for treatment of these labours. It has already been shown to be ineffective in clinical practice where it does not reduce the number of caesarean sections needed, although commonly recommended and

used. The pH null experiments in this thesis are promising as the addition of a weak base reduced the effect of lactate and normal contractions resumed. However the idea of using a weak base to 'null' the effect of the weak acid is long way from clinical practice as key considerations, such as how to get the base into the myometrium and how to avoid it having detrimental effects on the mother and baby, need to be considered and resolved.

6.5 Future work

6.5.1 Lactate generation

Exploring lactate generation in the myometrium is fundamental to understanding the mechanism behind its action. With the use of a colorimetric assay, lactate generation can be determined in contracting myometrial tissue strips. Differences in lactate generation between labouring and dysfunctionally labouring myometrium in response to oxytocin stimulation and hypoxia would shed light on these problematic labours and offer insight into new treatments. Initial experiments using the assay shows lactate is produced in increasing quantities when challenged with oxytocin, possibly as it is contracting more forcefully and there are therefore longer periods of hypoxia, reducing lactate clearance.

Preliminary results using this technique with Dr Annabelle Kendrick have shown higher levels of lactate are released from human *labouring* myometrium under oxytocin stimulation compared to spontaneous activity (Figure 6.1). However there was not enough time to investigate this fully.

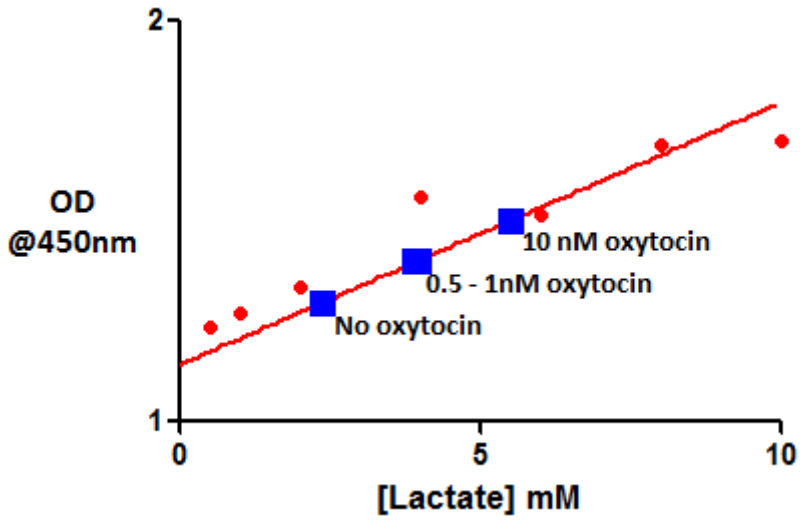


Figure 6.1: Lactate assay standard curve

Lactate assay standard curve (red line) plotted using [lactate] efflux from human *labouring* myometrium under different contractile conditions: no oxytocin, 0.5-1nM oxytocin and 10nM oxytocin.

6.5.2 Lactate and MCTs

Further investigation into MCTs is needed. qRT-PCR could be used to measure gene transcription and protein synthesis by mass spectrometry to establish if levels of the mRNA transcript for the transporters are altered or if only protein translation is changed in dysfunctionally labouring women. Determination of the MCTs location in myometrial cells is also vital: their location may change through gestation or when labour commences. Also it is vital to look at the chaperone proteins Embigin/Basigin to analyse their regulation during pregnancy and labour, and if they are altered in problematic labours.

LDH enzyme expression has never been quantified in labouring human myometrium. Variation in expression may lead to a difference in lactate production between myometrial samples and be accountable for higher lactate levels seen in women with dysfunctional labour. Analysis of expression would shed more light on the differences that predispose to slow, inefficient uterine contractions.

Gene expression profiling allows the measurement of expression levels of a large number of genes simultaneously. This technique could be employed to compare gene expression between 'normal' labouring and dysfunctionally labouring myometrium and could identify new genes that have yet to be investigated and may contribute to problematic labours.

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