## Physiology in Press

# Characterization of the molecular and electrophysiological properties of the T-Type calcium channel in human myometrium

Andrew M Blanks, ZhengHang Zhao, Anatoly Shmygol, Gilles Bru-Mercier, Shirley Astle and Steve Thornton

*J. Physiol.* published online Apr 19, 2007;

DOI: 10.1113/jphysiol.2007.132126

Physiology in Press; published online on April 19, 2007 as 10.1113/jphysiol.2007.132126

# Characterization of the molecular and electrophysiological properties of the T-Type calcium channel in human myometrium.

Andrew M. Blanks[1,3*], Zheng-Hang Zhao[1,2*], Anatoly Shmygol[1], Gilles Bru-Mercier[1], Shirley Astle[1], & Steven Thornton[1]

1. Clinical Sciences Research Institute, Division of Clinical Sciences, Warwick Medical School, University of Warwick, Coventry CV4 7AL.

2. (Current address) Department of Pharmacology, Medical School, Xian Jiatong University, China.

3. Corresponding Author: Dr Andrew M. Blanks

Clinical Science Research Institute

Division of Clinical Sciences

Warwick Medical School

Coventry, United Kingdom.

CV4 7AL

Tel: +44(0)2476968703 Fax:+44(0)2476524311

Email:Andrew.Blanks@warwick.ac.uk

* A.M Blanks and ZH. Zhao should be considered joint first author.

Key Terms: Myometrium, Parturition, Calcium, Uterus, T-Type, Labour

Total word count: 4572

Funding: This work was supported by Wellbeing of Women grant (BB434/02) and BBSRC (BB/D016630/1) grants. AMB is supported by Research Councils United Kingdom by an RCUK fellowship in reproduction.

**Abstract:**

Rises in intracellular calcium are essential for contraction of human myometrial smooth muscle (HMSM) and hence parturition. The T-type calcium channel may play a role in this process. The aim was to investigate the role of the T-Type calcium channel in HMSM by characterizing mRNA expression, protein localization, electrophysiological properties and function of the channel subunits $Ca_v3.1$(α1G), $Ca_v3.2$(α1H), and $Ca_v3.3$(α1I). QRT-PCR, immuno-histochemistry, electrophysiology and *invitro* contractility were performed on human myometrial samples from term, preterm, labour and not in labour. QRT-PCR analysis of $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ demonstrated expression of $Ca_v3.1$ and $Ca_v3.2$ with no significant change ($P>0.05$) associated with gestation or labour status. Immuno-histochemistry localized $Ca_v3.1$ to myometrial and vascular smooth muscle cells whilst $Ca_v3.2$ localized to vascular endothelial cells and invading leukocytes. Voltage clamp studies demonstrated a T-type current in 55% of cells. Nickel block of T-type current was voltage sensitive ($IC_{50}$ of 118.57±68.9 μM at -30mV). Activation and inactivation curves of $I_{Ca}$ currents in cells expressing T-type channels overlapped demonstrating steady state window currents at the resting membrane potential of myometrium at term. Current clamp analysis demonstrated that hyperpolarizing pulses to a membrane potential greater than -80mV elicited rebound calcium spikes that were blocked reversibly by 100μM nickel. Contractility studies demonstrated a reversible decrease in contraction frequency during application of 100μM nickel ($P<0.05$). We conclude that the primary T-type subunit expressed in some MSMCs is $Ca_v3.1$. We found that application of 100 μM nickel to spontaneously contracting human myometrium reversibly slows contraction frequency.

## Introduction

The contractile activity of the uterus undergoes major modifications during the transition from the relative quiescence of the majority of gestation to the highly coordinated contractions of delivery. The transition of the myometrium into a contractile phenotype has been termed the "activation"(Challis *et al.*, 2000) or "preparatory phase"(Garfield *et al.*, 1998). This process, under the stimulation of oestrogen and a reduction in the influence of progesterone involves molecular changes that manifest in an increase in contraction frequency and a depolarized resting membrane potential in term myometrium(Parkington *et al.*, 1999a) when compared to mid-gestation.

Although much research has been dedicated to the mechanism by which the sex steroids mediate their influence on myometrium little is known about the physiological mechanism or the molecular identity of currents that manifest spontaneous contractions at term. Some investigations have explored the role of calcium activated $K^+$ channels (Anwer *et al.*, 1993; Khan *et al.*, 1993; Perez *et al.*, 1993; Khan *et al.*, 1997) and voltage activated $K^+$ channels (Knock *et al.*, 1999) in control of resting membrane potential and repolarisation. However the molecular identity or physiology of a pacemaker current remains elusive.

What is clear is that control of resting membrane potential and rises in intracellular calcium are essential for contraction in human myometrial smooth muscle (MSM)(Parkington *et al.*, 1999b). Calcium is not only an important second messenger for the generation of force via myosin light chain kinase, but also depolarizes the plasma membrane allowing for activation of other voltage-dependent ion channels. This latter

property is an important function for T-type or low voltage activated (LVA) calcium channels, which are responsible for generating low threshold spikes that in neurons lead to burst firing and oscillatory behavior(Kim *et al.*, 2001). The sub-family of T-type calcium channels currently comprises of 3 differing alpha subunits termed $Ca_v3.1$(α1G), $Ca_v3.2$(α1H), and $Ca_v3.3$(α1I)(Cribbs *et al.*, 1998; Perez-Reyes *et al.*, 1998; Lee *et al.*, 1999a; Monteil *et al.*, 2000) . $Ca_v3.1$ and $Ca_v3.2$ demonstrate similar activation and inactivation kinetics but can be differentiated by sensitivity to nickel and recovery from inactivation. $Ca_v3.3$ by contrast is easily distinguishable by its slower activation and inactivation kinetics.

In a recent molecular study in the rat it was demonstrated that both $Ca_v3.1$ and $Ca_v3.2$ were expressed in circular and longitudinal layers of myometrium and that the relative expression profile of these channels differed, dependent on gestational age, layer and subunit(Ohkubo *et al.*, 2005b).

It has previously been demonstrated in electrophysiological studies that a T-type like current is present in human myometrium (Young *et al.*, 1993) and that it is larger in magnitude than the more extensively investigated L-Type current. In a recent study it was demonstrated that administration of Mibefradil, a partially selective T-type inhibitor, reduced the force generated during a contraction whilst decreasing the magnitude of the initiation spike of tissue level electrical activity (Young & Zhang, 2005). This suggests that the T-type calcium channel may be involved in the initiation of action potentials in uterine smooth muscle. We sought to investigate the role of the T-Type calcium channel

1 in human myometrium by characterizing the expression, electrophysiological properties

2 and function of channel subunits $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$.

## Subjects and Methods

### *Subject Criteria and Selection*

All procedures were conducted within the guidelines of "The Declaration of Helsinki" and were subject to local ethical approval (REC-05/Q2802/107). Prior to surgery, informed written consent for sample collection was obtained. Subjects were recruited into two groups, spontaneous labour (LAB) and elective cesarean section not in labour (NIL) between 32-40wks gestation. Term was defined as >37 completed weeks gestation and pre-term labour defined as <37 completed weeks. The LAB group was undergoing cesarean section for reasons of presumed fetal distress. LAB was defined as regular contractions (<3mins apart), membrane rupture, and cervical dilatation (>2cm) with no augmentation.

### *Sample collection*

At cesarean section, samples were collected before Syntocin administration by knife biopsy from the lower uterine segment incision. Samples were washed briefly in saline and flash-frozen in liquid nitrogen for mRNA/immuno-histochemistry. Samples for cell isolation were placed in ice cold modified Krebs-Henseleit solution (see below) and utilized the same day.

### *Solutions*

Fresh samples were stored in ice cold modified Krebs-Henseleit (m-KHS) solution containing (mmol/L): NaCl, 133; KCl, 4.7; TES, 10; Glucose, 11.1; $MgSO_4$, 1.2;

$KH_2PO_4$, 1.2; $CaCl_2$, 2.5; adjusted to pH 7.4 at 25°C with 5M NaOH. Krebs-Henseleit (KHS) solution contained : NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; Glucose, 11.1 $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; $CaCl_2$, 2.5; pH 7.4 was maintained by constant aeriation with 5% $CO_2$/95% $O_2$ at 37°C. $Ca^{2+}$ -free Tyrode's solution contained : NaCl, 136; KCl, 5.4; $MgSO_4$, 1.0; $NaH_2PO_4$, 0.33; glucose, 5; HEPES, 10; adjusted to pH 7.4 at 25°C with 5M NaOH. Digestion solution (DS) contained: $Ca^{2+}$ -free Tyrode's; Sigma type IX collagenase, (1790IU/mg)1.25 mg $ml^{-1}$; Sigma type IA collagenase, (535 IU/mg) 1.25 mg $ml^{-1}$; Bovine Serum Albumin, 1 mg $ml^{-1}$. Kraftbrühe (KB) solution was as described in Klökner & Isenberg(Klockner & Isenberg, 1985). $Ca^{2+}$-free physiological salt solution (PSS) contained: NaCl, 130; KCl, 5; $MgCl_2$, 1.2; HEPES, 10; and glucose, 10; adjusted to pH 7.4 at 25°C with 5M NaOH. The electrode (interal) solution for voltage clamp contained: CsCl,135; $MgCl_2$, 2.5; MgATP, 5; HEPES, 10; and EGTA, 10; adjusted to pH 7.2 with CsOH. The bath solution was composed of : NaCl,120; CsCl, 1.0; tetraethylammonium chloride (TEA-Cl), 4.0; $MgCl_2$, 1.2; $CaCl_2$, 2.0; HEPES, 10.0; and glucose, 10.0; the pH was adjusted to pH 7.4 with 5M NaOH. For current clamp experiments bath solution was m-KHS. Electrode solution used in these experiments contained: NaCl, 12; KCl, 40; K-Glutamate, 90; TES, 10;Na-Pyruvate, 1; MgSO4, 1; EGTA, 0.2; CaCl2, 0.0803; pH 7.2. Nifedipine stock solution was 10 mmol/L in DMSO. Nickel stock solution was 100mmol/L in water. Final DMSO concentrations were less than 0.01%. All reagents were obtained from Sigma (Sigma-Aldrich Co., Dorset, UK) unless otherwise stated.

*Cell Isolation*

Strips of myometrium (2×2×4~5 mm) were incubated for 30 min in $Ca^{2+}$-free Tyrode's followed by 40~45 min at 37°C in DS. Digestion was terminated by dilution in pre-warmed $Ca^{2+}$-free Tyrode's followed by centrifugation for 10 mins at 250g. Cells were dispersed by slow tritutration through a wide bore fire polished glass pipette in KB solution. Single myometrial cells were filtered through a 200-μM gauze and stored in KB solution for at least 1 hour before experimental procedures. All experiments were performed within 6 hours after isolation.

*Electrophysiology*

Voltage Clamp

A drop of myometrial cell suspension was placed in a cell chamber (1.0ml) onto a glass coverslip mounted on the stage of an inverted microscope (IX51, Olympus). After settling (approx 20min) cells were superfused with bath solution for 10 min at a rate of 1~2 mL/min at room temperature. Transmembrane currents were recorded with an amplifier (Axopatch 200B; Axon Instruments). Patch pipettes were fabricated (MODEL P-87; Sutter Instruments, Novato, CA) from 1.5mm glass capillaries with a resistance of 2.0–4.0MΩ when filled with pipette solution. Liquid junction potential was zeroed prior to seal formation.

Following the formation of gigaseal, the membrane was ruptured by gentle suction obtaining the whole-cell voltage-clamp configuration. Membrane capacitance and series resistance were compensated after membrane rupture. Inward current was elicited by depolarising voltage steps at a frequency of 0.1Hz from a holding potential (HP) of

either-50mV or -80mV to +60mV in 10mV increments. Currents were filtered at 1 kHz and sampled at 2 kHz. Voltage protocols were delivered via a Digidata 1320 computer interface using pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA). Passive leakage currents were subtracted using a Positive/Negative (P/N) protocol. All experiments were carried out at 22–24 °C.

Current clamp

Cells were treated similarly to voltage clamp experiments. The resting potential and action potentials were recorded using 4-6 MΩ pipettes connected to a headstage of a discontinous voltage/current clamp amplifier (SEC-05, npi electronic GmbH, Tamm, Germany) operated in discontinous current clamp mode. The current and voltage outputs of the amplifier were recorded and the current stimuli delivered via an ITC-18 computer interface (InstruTECH, Port Washington, NY, USA) controlled by PatchMaster software (HEKA Electronik GmbH, Lambrecht, Germany). Liquid junction potential of 15 mV was subtracted from the obtained records. All experiments were carried out at 35 °C.

*RNA analysis*

RNA was isolated and reverse transcribed into cDNA for PCR analysis as described previously(Astle *et al.*, 2005). The expressions of r18s, $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ were verified by real-time RT-PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. TaqMan gene expression assay primer/probe identification numbers were $Ca_v3.1$ (Hs00367969_m1), $Ca_v3.2$ (Hs00184168_m1), and $Ca_v3.3$ (Hs00184168_m1) (Applied

Biosystems, Foster City, CA). Expression was normalized using the ΔΔcT method to r18s and non-pregnant myometrium. Amplification efficiency was determined over a linear cDNA titration for each probe set.

*Immuno-histochemistry*

Sample preparation

Frozen sections (8μm) of myometrium were briefly air dried and fixed for 30min in ice cold 4% paraformaldeyde in phosphate buffered saline. Isolated myocytes were plated onto cover slips in HBSS and placed overnight in a culture incubator. Plated cells were fixed for 5 min in ice cold 4% paraformaldeyde in phosphate buffered saline.

Antibody incubation

Detection of $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ was performed according to manufacturer's instruction using a 1:200 dilution (Alomone labs, Jerusalem, Israel).

Secondary antibody (anti-rabbit Alexa 635) (Molecular Probes, Invitrogen Ltd, Paisley, UK) was used according to manufacturer's instruction using a 1:200 dilution. Positive staining was compared with secondary antibody alone. F-actin staining was determined by addition of Phalloidin-488 (Molecular Probes, Invitrogen Ltd, Paisley, UK) according to manufactures instruction

Confocal Microscopy

After preparation, tissue sections or cells were examined using a Zeiss 510 META confocal microscope with X40 (NA 1.3, Oil DIC ) & X63 (NA 1.4, Oil DIC) objectives. Pinhole was set to 1 Airy unit and data was captured in 512x512 format.

*Myometrial Contractility*

Contractility experiments were performed as described previously (Woodcock *et al.*, 2006). Myometrial strips were allowed a 2 hour equilibration period to establish spontaneous contractions prior to addition of 100 μmol/L NiCl2.

*Data Analysis & Statistics*

All raw electrophysiological, expression and contractility data was imported directly into IGOR PRO v5 (Wavemetrics Inc, Portland, OR) for graphical and statistical analysis. Statistical significance was determined by one-way ANOVA (Newman Keuls test) for expression data and one-tailed unpaired student T-Test for contractility & voltage clamp.

**Results**

*Expression $Ca_v3$ alpha subunit mRNA*

Quantitative RT-PCR analysis of the expression of $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ in human myometrium demonstrated subunit-dependent differences. Expression of $Ca_v3.1$ and $Ca_v3.2$ were detected in myometrial cDNA from all gestational ages whilst $Ca_v3.3$ remained below the limit of detection in all samples tested. The level of expression of $Ca_v3.1$ (Fig. 1A) and $Ca_v3.2$ (Fig. 1B) demonstrated no significant change ($P>0.05$) between pre-term not in labour (n=9), pre term labour (n=5), term not in labour (n=12) and term labour (n=10).

*Localization of $Ca_v3.1$ and $Ca_v3.2$ protein*

After determining that mRNA encoding $Ca_v3.1$ and $Ca_v3.2$ was expressed throughout gestation in myometrial samples we sought to establish the cell types in which the proteins were expressed. Immuno-histochemistry utilizing an antibody raised to a twenty

two amino acid epitope of the rat $Ca_v3.1$ demonstrated specific binding in myometrial (Fig. 2A) and vascular smooth muscle cells (Fig. 2D). In contrast, antibody raised to a fifteen amino acid epitope of the rat $Ca_v3.2$ demonstrated specific binding in vascular endothelial cells (Fig. 2E) and invading leukocytes (Fig. 2H). The precise localization of $Ca_v3.1$ was further investigated by high magnification confocal microscopy in dissociated myometrial smooth muscle cells (MSMC). Detailed scans demonstrated localization of $Ca_v3.1$ protein in discreet punctate regions of the plasma membrane and in the peri-nuclear area of the sarcoplasmic reticulum (Figs. 2J, K, L).

*Characterization of the low voltage activated (LVA) $I_{Ca}$ current in myometrial smooth muscle*

To determine the LVA $I_{Ca}$ in freshly dissociated MSMCs we employed conventional patch clamp techniques combined with pharmacological maneuvers designed to separate the LVA and HVA $I_{Ca}$. Depolarizing pulses above -40mV elicited an inward current in all cells tested. In 54 of 98 cells tested, an inward current became apparent at voltages positive to -60mV indicating the presence of LVA $I_{Ca}$. In a number of control cells (n=12 for HVA expressing cells and n=12 for HVA/LVA expressing cells) we utilized two current-voltage (I-V) protocols from a holding potential of -50mV and -80mV respectively to distinguish the LVA and HVA components. From the resultant I-V plots we determined the approximate contribution of LVA and HVA channels to the overall inward current (Fig 3 A,B,C). This analysis yielded a population of cells with HVA current (Fig 3A) and a different population of cells demonstrating both LVA and HVA current (Fig 3B). After determination of the LVA $I_{Ca}$ by I-V plot at HP -50 and HP -80

we added 1μM nifedipine to remove the dihydropiridine sensitive component of the HVA current (Fig 3 D,E,F) (Fig 4A(a),(b)) and repeated the I-V protocol. This established that the peak LVA response in the presence of nifedipine could be estimated in a more rapid protocol by an assay for current density at -30mV from a holding potential of -80mV. Under these conditions LVA current was present in only 55% of cells tested (54/98 cells) which was in contrast to 100% (98/98 cells) for the HVA current. The mean cell capacitance of HVA expressing cells (140 ± 15 pF) was not significantly different to HVA/LVA expressing cells (142 ± 13 pF) ($P>0.05$).

The LVA $I_{Ca}$ was then further characterized using the divalent ion $Ni^{2+}$ which has previously been demonstrated to block $Ca_v3$ subunits. $Ca_v3.2$ can be discriminated from $Ca_v3.1$ and $Ca_v3.3$ by a 20-fold greater sensitivity to inhibition by $Ni^{2+}$(Lee *et al.*, 1999b). In agreement with the immuno-histochemistry data suggesting that $Ca_v3.1$ is the predominant LVA $I_{Ca}$ in MSMCs, the majority of cells with LVA $I_{Ca}$ (53/54) tested were relatively insensitive to 120μM nickel. Furthermore, a dose dependent response to nickel (Fig. 4A(c), B) demonstrated an $IC_{50}$ of 118.57±68.9 μM (n=3) at -30mV which is similar to that (167±15 μM) in Xenopus oocytes expressing $Ca_v3.1$(Lee *et al.*, 1999b). The inhibition of LVA current by nickel was voltage dependent with $IC_{50}$ decreasing to 73.741±17.5 at -40mV. In separate control experiments prior to nifedipine addition 100 μM nickel did not significantly inhibit HVA $I_{Ca}$ (Fig. 4A(b)) (mean inhibition versus control at -30mV from -80mV holding potential = 0.5% ± 0.01 (n=3), $P>0.05$).

After establishing that the LVA $I_{Ca}$ in human MSMCs was most likely to be $Ca_v3.1$ we attempted to determine the physiological function of the current. The precise function of a LVA $I_{Ca}$ in smooth muscle has remained the subject of much conjecture due to the

resting membrane potential lying near steady state inactivation of these channels. This property, could theoretically lead to the establishment of a "window" current at the resting membrane potential, which may either contribute to calcium dependent cellular processes or lead to a slow depolarisation. We therefore utilized a two-pulse protocol to measure the parameters of steady state activation and inactivation of both LVA and HVA $I_{Ca}$ in MSMCs to determine the "window" current. Cells were held at HP of -80mV followed by incremental pulses of 10mV for 500 msec (conditioning pulses) followed by re-polarization to -80mV and finally a 60 msec pulse to 0mV (test pulse). To evaluate the steady-state inactivation, peak currents recorded during the test pulse to 0mV after each conditioning pulse were normalized to a maximum and plotted as a function of the conditioning pulse amplitude. Steady-state activation was determined from the peak amplitude of inward current during conditioning pulses. The mean and standard error of 6 cells expressing both HVA and LVA currents are shown in Figure 4C. Boltzmann fit revealed haf-activation of inward current at -46±0.4 mV with a slope factor of approximately 5 mV. Half-inactivation of inward current was observed at -47±0.5 mV with 9mV slope factor .A pronounced overlap between steady-state activation and inactivation curves was evident, peaking at approximately -50 mV. At the reported resting membrane potential of approximately -55mV in human myometrium at term(Parkington *et al.*, 1999a) this overlap would lead to a persistent inward current. A well established role for $Ca_v3.1$ in the central nervous system is to participate in burst firing whereby a combination of $I_h$ and $Ca_v3.1$ elicit repetitive depolarization through low threshold calcium spikes(Kim *et al.*, 2001) upon which fast sodium spikes are superimposed. In our hands, under standard current clamp conditions, there was no

repetitive firing of action potentials in human isolated myocytes, which is in contrast with both rat and mouse myocytes. A typical current clamp recording of isolated human MSM in response to 15 second 10pA depolarizing pulse is depicted in Fig. 5A. A depolarizing current pulse of 15 second duration resulted in a single action potential with a threshold around -40mV. However a subsequent 5 second hyper-polarization to a potential greater than -80mV lead to a rebound (anode break) action potential at a threshold of approximately -65mV. This rebound LVA spike was completely inhibited by 100μM nickel and recovered after subsequent washout.

Finally, we sought to establish the role of the LVA $I_{Ca}$ current in contractions of freshly isolated MSM. A previous study in humans using the partly selective T-type inhibitor Mibefradil demonstrated an inhibition of contractile activity that was associated with a decrease in the magnitude of the initiation spike of the tissue level action potential (Young & Zhang, 2005). However, a number of studies have drawn into question the selectivity of Mibefradil (Viana *et al.*, 1997; Gomora *et al.*, 1999; Eller *et al.*, 2000) and subsequent conclusions about *in vivo* effects; most recently culminating in the demonstration of specific inhibition of HVA calcium channels in the cardiovascular system(Moosmang *et al.*, 2006). Given our electrophysiology data, and a reported $K_i$ value of 800μM (Zamponi *et al.*, 1996) for the HVA L-type subunit $\alpha_{1C}$ (with calcium as the permeant ion) we utilized 100μM nickel to inhibit the LVA $I_{Ca}$ in spontaneously contracting myometrial strips.

In these experiments addition of 100μM nickel increased mean contraction interval by 3.5-fold over a 30-50 minute period of application ($P<0.05$; $n=4$) (Fig. 6A,C) an effect that was reversible on washout (Fig. 6B). In addition to an effect on contractile

frequency, a small but significant ($P<0.001$) increase was observed in activity integral, but there was no significant effect on maximum contractile force (Fig. 6C).

**Discussion**

In this study we have investigated the expression of the three T-Type alpha sub-units, $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ in human myometrium. Initially, we established that the $Ca_v3.1$ and $Ca_v3.2$ isoforms are expressed in human myometrial samples. This was in agreement with a previous study utilizing RT-PCR that demonstrated both $Ca_v3.1$ and $Ca_v3.2$ are differentially expressed throughout gestation in the different layers of rat myometrium(Ohkubo *et al.*, 2005b). In our study we used a panel of cDNA derived from patients at different times of gestation and labour status to establish if there were gestation related changes in $Ca_v3$ isoforms. In contrast with the rat study we found no significant difference in expression in the $Ca_v3$ isoforms with either gestation or with labour. Following RT-PCR analysis we sought to establish whether there were different types of cells expressing $Ca_v3.1$ and $Ca_v3.2$. Utilizing immuno-histochemistry we found that $Ca_v3.1$ was expressed in some MSMCs and in vascular smooth muscle cells. In contrast, $Ca_v3.2$ demonstrated positive staining in vascular endothelial cells and leukocytes. This is the first description of the cellular distribution of $Ca_v3$ isoforms in the human uterus and demonstrates that the expression of $Ca_v3.2$ splice variants as described by Ohkubo et al(Ohkubo *et al.*, 2005a) is unlikely to be physiologically relevant to smooth muscle cells but is more likely to be relevant to invading leukocytes. Interestingly, block of the T-type current in leukocytes has been demonstrated to inhibit adhesion (and subsequent invasion) to vascular endothelial cells by inhibiting the calcium dependent expression of β2-integrins and L-selectin(Nebe *et al.*, 2002). Given the

importance of invading leukocytes in the process of parturition this may warrant further investigation.

We sought to characterize the function of the T-type current and establish the density of $Ca_v3.1$ current in myometrial cells by electrophysiology. Utilizing a combination of biophysical parameters , nifedipine and nickel, we were able to establish that there was LVA current in 55% of myometrial cells, which was in contrast to 100 % of cells demonstrating a HVA current. Our voltage-clamp data was in good agreement with that of Young et al (Young *et al*., 1993) and Knock & Aaronson (Knock & Aaronson, 1999) who demonstrated that both LVA and HVA $Ca^{2+}$ currents were present in freshly dissociated MSMCs.

The LVA current was blocked, in a voltage-dependent manner, by nickel with an $IC_{50}$ at -30mV that is consistent with recordings made from oocytes expressing $Ca_v3.1$ (Lee *et al*., 1999b). This further supported the immuno-histochemistry data suggesting that $Ca_v3.1$ is the predominant isoform in MSM.

Our data suggests that LVA $I_{Ca}$ is present in approximately 50% of cells which raises the question of whether there is a gestation dependent shift in the proportion of cells expressing LVA $I_{Ca}$? Unfortunately we were unable to address this question by assessing current density per patient because it is not technically possible to assay a sufficient population of cells from any given patient. However, if the overall ratio of cells expressing LVA $I_{Ca}$ was changing with gestation and assuming that mRNA reflects protein levels and that a single biopsy is a sample of millions of cells one would expect to see an overall change in expression of $Ca_v3.1$ mRNA. Since this was not the case in our

study, and in the absence of good population current density data, we assume there to be no overall change in ratio.

The precise physiological role of the T-type calcium channel in smooth muscle remains the subject of debate (Perez-Reyes, 2004; Fry *et al.*, 2006). This is largely because, unlike some neurons where resting membrane potentials of -70 to -80mV and/or contributions of hyperpolarizing currents lead to LVA spikes from the T-type, smooth muscle cells have resting membrane potentials within the range for steady-state inactivation of the T-type channel. However, due to the particular biophysical properties of the channel there remains a possibility that a small population of channels may contribute to a window current (Perez-Reyes, 2003) at the resting membrane potential of the smooth muscle cell and thereby contribute to either a slow wave depolarisation or other calcium-dependent intracellular processes. To elucidate the potential for both a contribution to a LVA spike or window current we undertook a combination of voltage clamp and current clamp experiments. Voltage-clamp experiments to determine the steady state activation and inactivation kinetics of the combined LVA and HVA inward currents demonstrated a window current between -60mV to 0mV. This is particularly interesting since the resting membrane potential of the myometrium becomes steadily depolarized throughout gestation from -80mV at mid-gestation to -55mV at labour and delivery (Parkington *et al.*, 1999a). At term therefore, due to the resting membrane potential being within the window current, a slow depolarisation or calcium "leak" may occur within smooth muscle cells expressing the T-type channel.

To explore the possibility of the T-type mediating a LVA spike in myometrial cells we tested cells under current clamp conditions by an initial depolarization to ensure the

initiation of an action potential followed by a hyper-polarization to negative potentials sufficient to de-inactivate the T-type. Consistent with the classical role of the T-type, hyper-polarization (to greater than -80mV) was followed by a rebound LVA spike that was abolished by nickel and recovered upon wash out. This suggests that $Ca_v3.1$ in MSMCs can elicit LVA spikes although it is unclear as to what the identity of the endogenous hyperpolarizing current might be that could drive de-inactivation. The physiological role of this process requires further investigation.

If there is a slow depolarisation via the T-type current in MSMCs, or that a steady calcium leak contributed to the activation of a calcium dependent inward conductance, one would hypothesize that an inhibition of this current would slow the frequency of contractions since any slow depolarisation/activation of an inward calcium conductance would increase the chances of a threshold event. Utilizing a concentration of nickel consistent with specific block of the T-type and not L-Type current in this study and well below the $K_i$ (800μM) for recombinant L-type subunit $\alpha_{1C}$(Zamponi *et al.*, 1996) we determined that the frequency of spontaneous contractions of myometrial strips was significantly reduced. Furthermore, the effect was reversible on wash out and caused no significant decrease in maximal contractile force. We observed a small but significant increase in activity integral that was due to an increase in duration of contraction. Whether this was a direct result of T-type blockade or an effect due to reduced contraction frequency remains to be determined. It should be noted that there are a number of plausible hypotheses other than slow depolarisation that may lead to the T-type channel affecting contraction frequency. For example, a steady calcium leak may contribute to a slow filling of the sarcoplasmic reticulum, the filling status of which, will

determine spontaneous release and subsequent activation of the calcium sensitive $I_{Cl-(Ca)}$current, previously demonstrated to affect contraction frequency (Jones *et al.*, 2004). The effect of nickel on other conductances that may contribute to contraction frequency cannot be ruled out. However, the concentration of nickel used in this study are well below the millimolar concentrations required for block of Na/Ca exchange or Na-K ATP-ase and addition of ouabain actually increases contraction frequency (Parkington *et al*., 1999b) suggesting a non-specific effect on a sodium pump is unlikely.

We conclude that the primary T-type subunit expressed in some MSMCs is $Ca_v3.1$. We show that the LVA $I_{Ca}$ conductance is heterogeneously expressed in human myometrial smooth muscle cells and that cells expressing both LVA $I_{Ca}$ and HVA $I_{Ca}$ demonstrate an extended window current. We found that 100 µM nickel reduces the contraction frequency of spontaneously contracting human myometrium.

**Acknowledgements**

We thank Prof DC Spanswick for advice, use of equipment and critical reading of the manuscript. We gratefully acknowledge the hard work and dedication of the UHCW labour ward staff for the collection of samples.

## Reference


Anwer K, Oberti C, Perez GJ, Perez-Reyes N, McDougall JK, Monga M, Sanborn BM, Stefani E & Toro L. (1993). Calcium-activated K+ channels as modulators of human myometrial contractile activity. *Am J Physiol* **265,** C976-985.

Astle S, Thornton S & Slater DM. (2005). Identification and localization of prostaglandin E2 receptors in upper and lower segment human myometrium during pregnancy. *Mol Hum Reprod* **11,** 279-287.

Challis JRG, Matthews SG, Gibb W & Lye SJ. (2000). Endocrine and paracrine regulation of birth at term and preterm. *Endocr Rev* **21,** 514-550.

Cribbs LL, Lee JH, Yang J, Satin J, Zhang Y, Daud A, Barclay J, Williamson MP, Fox M, Rees M & Perez-Reyes E. (1998). Cloning and characterization of alpha1H from human heart, a member of the T-type Ca2+ channel gene family. *Circ Res* **83,** 103-109.

Eller P, Berjukov S, Wanner S, Huber I, Hering S, Knaus HG, Toth G, Kimball SD & Striessnig J. (2000). High affinity interaction of mibefradil with voltage-gated calcium and sodium channels. *Br J Pharmacol* **130,** 669-677.

Fry CH, Sui G & Wu C. (2006). T-type Ca2+ channels in non-vascular smooth muscles. *Cell Calcium* **40,** 231-239.

Garfield RE, Saade G, Buhimschi C, Buhimschi I, Shi L, Shi SQ & Chwalisz K. (1998). Control and assessment of the uterus and cervix during pregnancy and labour. *Hum Reprod Update* **4,** 673-695.

Gomora JC, Enyeart JA & Enyeart JJ. (1999). Mibefradil potently blocks ATP-activated K(+) channels in adrenal cells. *Mol Pharmacol* **56,** 1192-1197.

Jones K, Shmygol A, Kupittayanant S & Wray S. (2004). Electrophysiological characterization and functional importance of calcium-activated chloride channel in rat uterine myocytes. *Pflugers Arch* **448,** 36-43.

Khan RN, Smith SK, Morrison JJ & Ashford ML. (1993). Properties of large-conductance K+ channels in human myometrium during pregnancy and labour. *Proc Biol Sci* **251,** 9-15.

Khan RN, Smith SK, Morrison JJ & Ashford ML. (1997). Ca2+ dependence and pharmacology of large-conductance K+ channels in nonlabour and labour human uterine myocytes. *Am J Physiol* **273,** C1721-1731.

Kim D, Song I, Keum S, Lee T, Jeong MJ, Kim SS, McEnery MW & Shin HS. (2001). Lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures in mice lacking alpha(1G) T-type Ca(2+) channels. *Neuron* **31,** 35-45.

Klockner U & Isenberg G. (1985). Action potentials and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig). *Pflugers Arch* **405,** 329-339.

Knock GA & Aaronson PI. (1999). Calcium antagonistic properties of the cyclooxygenase-2 inhibitor nimesulide in human myometrial myocytes. *Br J Pharmacol* **127,** 1470-1478.

Knock GA, Smirnov SV & Aaronson PI. (1999). Voltage-gated K+ currents in freshly isolated myocytes of the pregnant human myometrium. *J Physiol* **518,** 769-781.

Lee JH, Daud AN, Cribbs LL, Lacerda AE, Pereverzev A, Klockner U, Schneider T & Perez-Reyes E. (1999a). Cloning and expression of a novel member of the low voltage-activated T-type calcium channel family. *J Neurosci* **19,** 1912-1921.

Lee JH, Gomora JC, Cribbs LL & Perez-Reyes E. (1999b). Nickel block of three cloned T-type calcium channels: low concentrations selectively block alpha1H. *Biophys J* **77,** 3034-3042.

Monteil A, Chemin J, Leuranguer V, Altier C, Mennessier G, Bourinet E, Lory P & Nargeot J. (2000). Specific properties of T-type calcium channels generated by the human alpha 1I subunit. *J Biol Chem* **275,** 16530-16535.

Moosmang S, Haider N, Bruderl B, Welling A & Hofmann F. (2006). Antihypertensive effects of the putative T-type calcium channel antagonist mibefradil are mediated by the L-type calcium channel Cav1.2. *Circ Res* **98,** 105-110.

Nebe B, Holzhausen C, Rychly J & Urbaszek W. (2002). Impaired mechanisms of leukocyte adhesion in vitro by the calcium channel antagonist mibefradil. *Cardiovasc Drugs Ther* **16,** 183-193.

Ohkubo T, Inoue Y, Kawarabayashi T & Kitamura K. (2005a). Identification and electrophysiological characteristics of isoforms of T-type calcium channel Ca(v)3.2 expressed in pregnant human uterus. *Cell Physiol Biochem* **16,** 245-254.

Ohkubo T, Kawarabayashi T, Inoue Y & Kitamura K. (2005b). Differential expression of L- and T-type calcium channels between longitudinal and circular muscles of the rat myometrium during pregnancy. *Gynecol Obstet Invest* **59,** 80-85.

Parkington HC, Tonta MA, Brennecke SP & Coleman HA. (1999a). Contractile activity, membrane potential, and cytoplasmic calcium in human uterine smooth muscle in

the third trimester of pregnancy and during labour. *Am J Obstet Gynecol* **181,** 1445-1451.

Parkington HC, Tonta MA, Davies NK, Brennecke SP & Coleman HA. (1999b). Hyperpolarization and slowing of the rate of contraction in human uterus in pregnancy by prostaglandins E2 and f2alpha: involvement of the Na+ pump. *J Physiol* **514 ( Pt 1),** 229-243.

Perez-Reyes E. (2003). Molecular physiology of low-voltage-activated t-type calcium channels. *Physiol Rev* **83,** 117-161.

Perez-Reyes E. (2004). Paradoxical Role of T-type Calcium Channels in Coronary Smooth Muscle. *Mol Interv* **4,** 16-18.

Perez-Reyes E, Cribbs LL, Daud A, Lacerda AE, Barclay J, Williamson MP, Fox M, Rees M & Lee JH. (1998). Molecular characterization of a neuronal low-voltage-activated T-type calcium channel. *Nature* **391,** 896-900.

Perez GJ, Toro L, Erulkar SD & Stefani E. (1993). Characterization of large-conductance, calcium-activated potassium channels from human myometrium. *Am J Obstet Gynecol* **168,** 652-660.

Viana F, Van den Bosch L, Missiaen L, Vandenberghe W, Droogmans G, Nilius B & Robberecht W. (1997). Mibefradil (Ro 40-5967) blocks multiple types of voltage-gated calcium channels in cultured rat spinal motoneurones. *Cell Calcium* **22,** 299-311.

Woodcock NA, Taylor CW & Thornton S. (2006). Prostaglandin F(2alpha) increases the sensitivity of the contractile proteins to Ca(2+) in human myometrium. *Am J Obstet Gynecol.*

Young RC, Smith LH & McLaren MD. (1993). T-type and L-type calcium currents in freshly dispersed human uterine smooth muscle cells. *Am J Obstet Gynecol* **169,** 785-792.

Young RC & Zhang P. (2005). Inhibition of in vitro contractions of human myometrium by mibefradil, a T-type calcium channel blocker: support for a model using excitation-contraction coupling, and autocrine and paracrine signaling mechanisms. *Journal of the Society for Gynecologic Investigation,* **12,** e7-12.

Zamponi GW, Bourinet E & Snutch TP. (1996). Nickel block of a family of neuronal calcium channels: subtype- and subunit-dependent action at multiple sites. *J Membr Biol* **151,** 77-90.

**Figure Legends.**

**Fig 1.** mRNA expression of CAV3.1 (Panel A) and CAV3.2 (Panel B)in myometrial samples taken from patients Preterm not in labour (PTNIL; circle), Preterm in labour (PTL; square), Term not in labour(NIL, triangle), Term labour (TL, Inverted triangle). Each point represents individual patients normalized by $2^{-\Delta\Delta CT}$ method to r18S and non-pregnant myometrium. Patients group means were not significantly different P>0.05 One-way ANOVA (Neuman-Keuls) test.

**Fig. 2.** Immunohistochemistry for $Ca_v3.1$ & $Ca_v3.2$. Channels were visualized by confocal microscopy using secondary antibodies conjugated to Alexa 633 and are depicted in red. Nuclei were counterstained with DAPI and are depicted in blue. $Ca_v3.1$ (Alexa 633, Red; Nuclei DAPI, Blue) localised to myometrial smooth muscle cells (Panel A, bar 50µM) and vascular smooth muscle cells (Panel D, bar 50µM) but not leukocytes (Panel G, bar 50µM) in myometrium. $Ca_v3.2$ (Alexa 633, Red; DAPI, Blue) by contrast did not localize to myometrial smooth muscle cells (Panel B, bar 50µM) but to vascular endothelium (Panel E, bar 50µM) and leukocytes (Panel H, bar 50µM). Panel C, F, & I correspond to negative control (Secondary Ab only). In isolated myocytes detailed scans localized $Ca_v3.1$ to punctate regions of the plasma membrane and the sarcoplasmic reticular region (Panel J, Bar 20 µM). For contrast F-Actin cytoskeleton is highlighted by Phalloidin-488 (green) (Panel K) and Panel J & K are presented in overlay in Panel L.

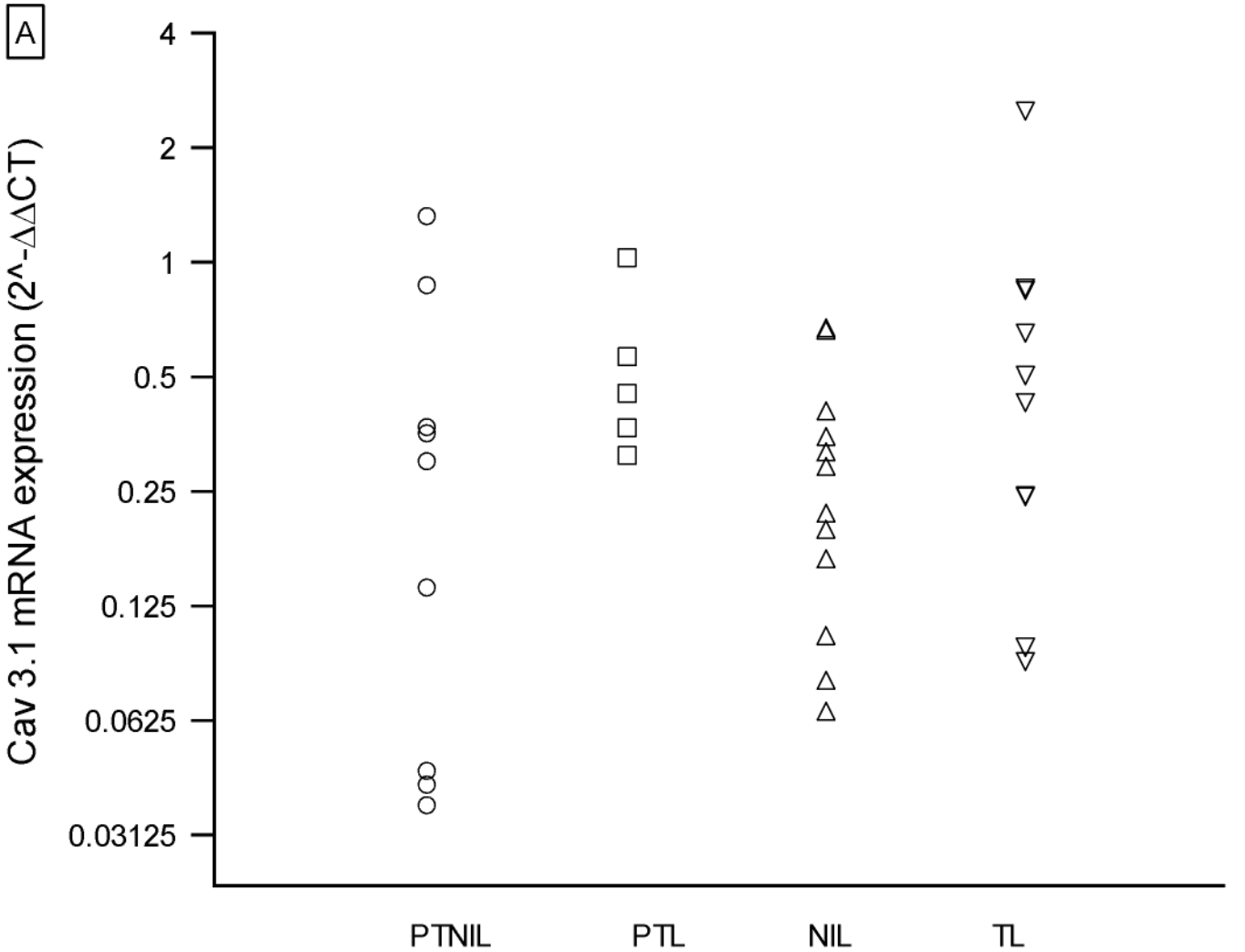
**Fig. 3.** Current-voltage relationship of $I_{Ca}$ in HVA and HVA/LVA expressing cells. Panel A. HVA expressing cells (mean± SE (n=12 per point)) current density *vs* step voltage from a holding potential of -50mV (closed circle) and -80mV (closed square). Panel B HVA/LVA expressing cells (mean± SE (n=12 per point)) current density *vs* step voltage from a holding potential of -50mV (closed circle) and -80mV (closed square). Panel C. Mean current density at holding potential -50mV subtracted from mean current density at holding potential -80mV *vs* step voltage. Subtracted current density of HVA expressing cells (closed circle) and HVA/LVA expressing cells (closed square) are significantly different at step potentials of -50, -40, -30 and -20mV only. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Panel D. HVA expressing cells (mean± SE (n=4 per point)) current density *vs* step voltage from a holding potential of -50mV in the absence (closed circle) and presence (closed square) of 1µM nifedipine. Panel E. HVA/LVA expressing cells (mean± SE (n=4 per point)) current density *vs* step voltage from a holding potential of -50mV in the absence (closed circle) and presence (closed square) of 1µM nifedipine. Panel F. Panel E. HVA/LVA expressing cells (mean± SE (n=4 per point)) current density *vs* step voltage from a holding potential of -80mV in the absence (closed circle) and presence (closed square) of 1µM nifedipine.

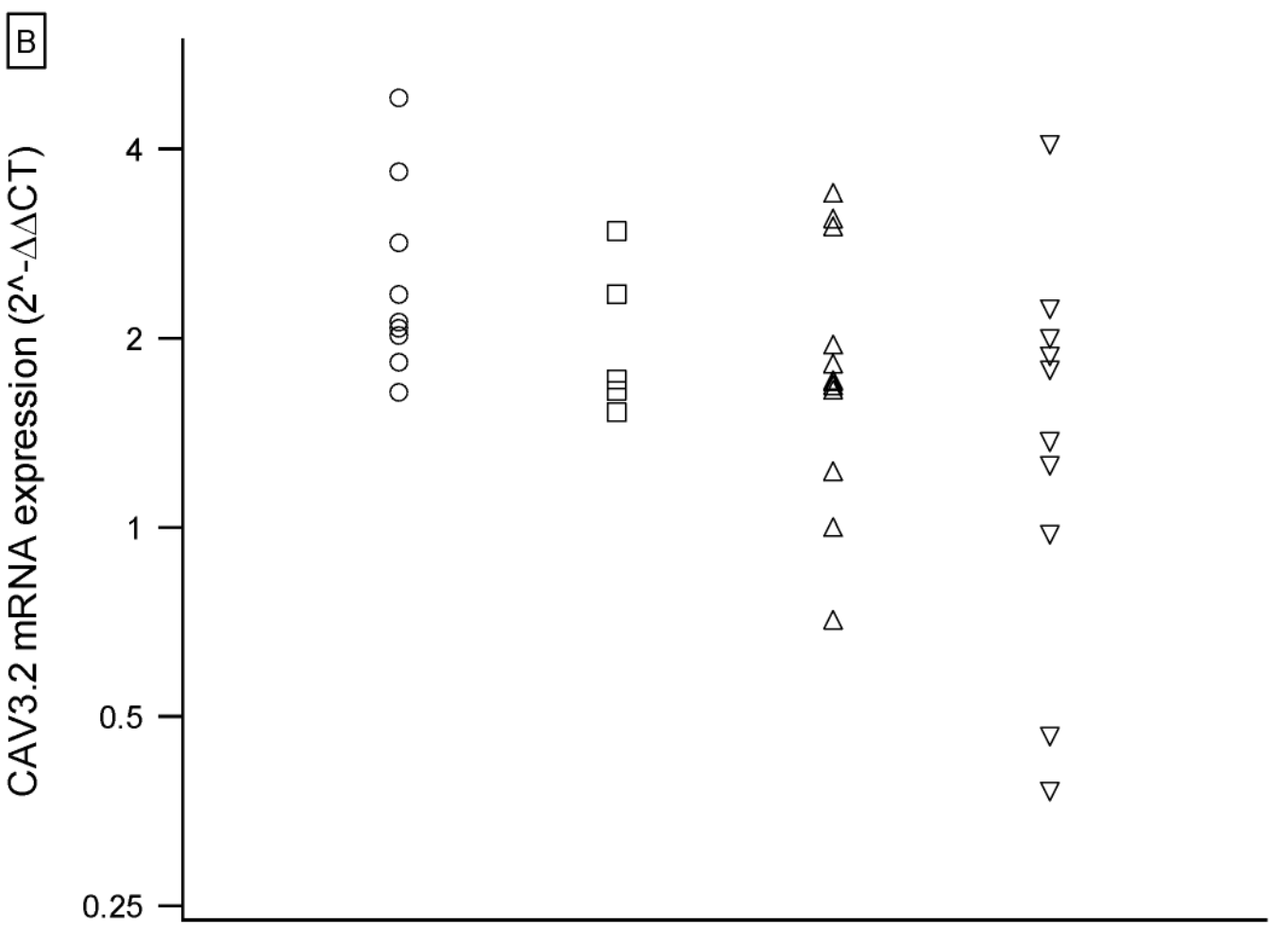
**Fig. 4.** Panel A. Effect of nifedipine and nickel on HVA and LVA $I_{ca}$ . A*a*. Effect of increasing concentrations of nifedipine on LVA $I_{Ca}$ elicited by voltage step to 0mV from -50mV holding potential in a HVA expressing cell. A*b*. Effect of

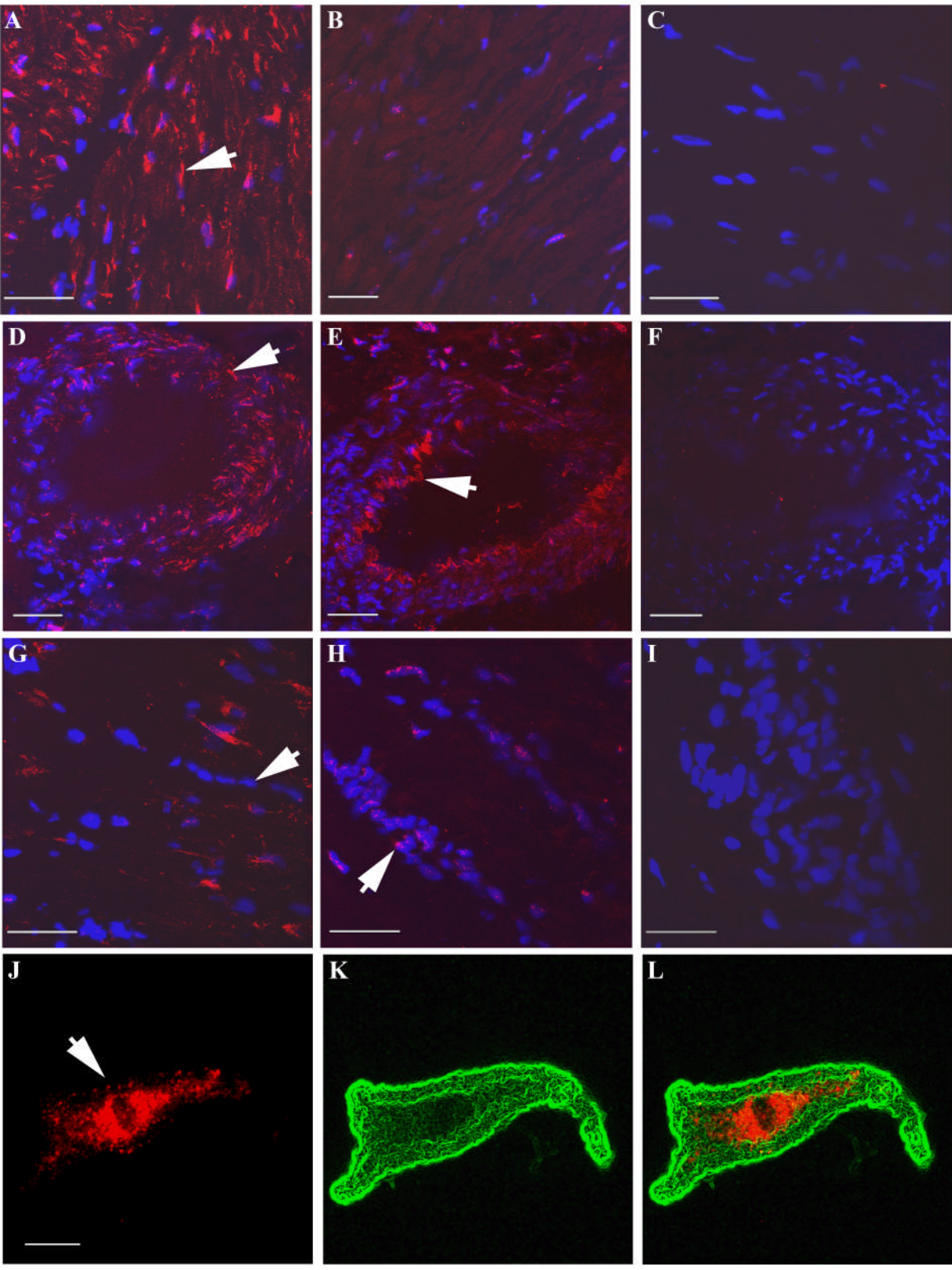
100µM nickel followed by 1µM nifedipine on HVA $I_{Ca}$ elicited by voltage step to 0mV from -50mV holding potential in a HVA/LVA expressing cell. A*c*. Effect of 1µM nifedipine followed by increasing concentrations of nickel on LVA $I_{ca}$ elicited by voltage step to -30mV from -80mV in a HVA/LVA expressing cell. Panel B. Dose dependent inhibition of LVA $I_{Ca}$ current by nickel chloride. Values represent mean±SE of percentage maximum current (n=3) Panel C. Steady state activation/inactivation curves determined from cells expressing both LVA and HVA current. Values represent mean±SE (n=6). Curves were fitted using the standard Boltzmann function.

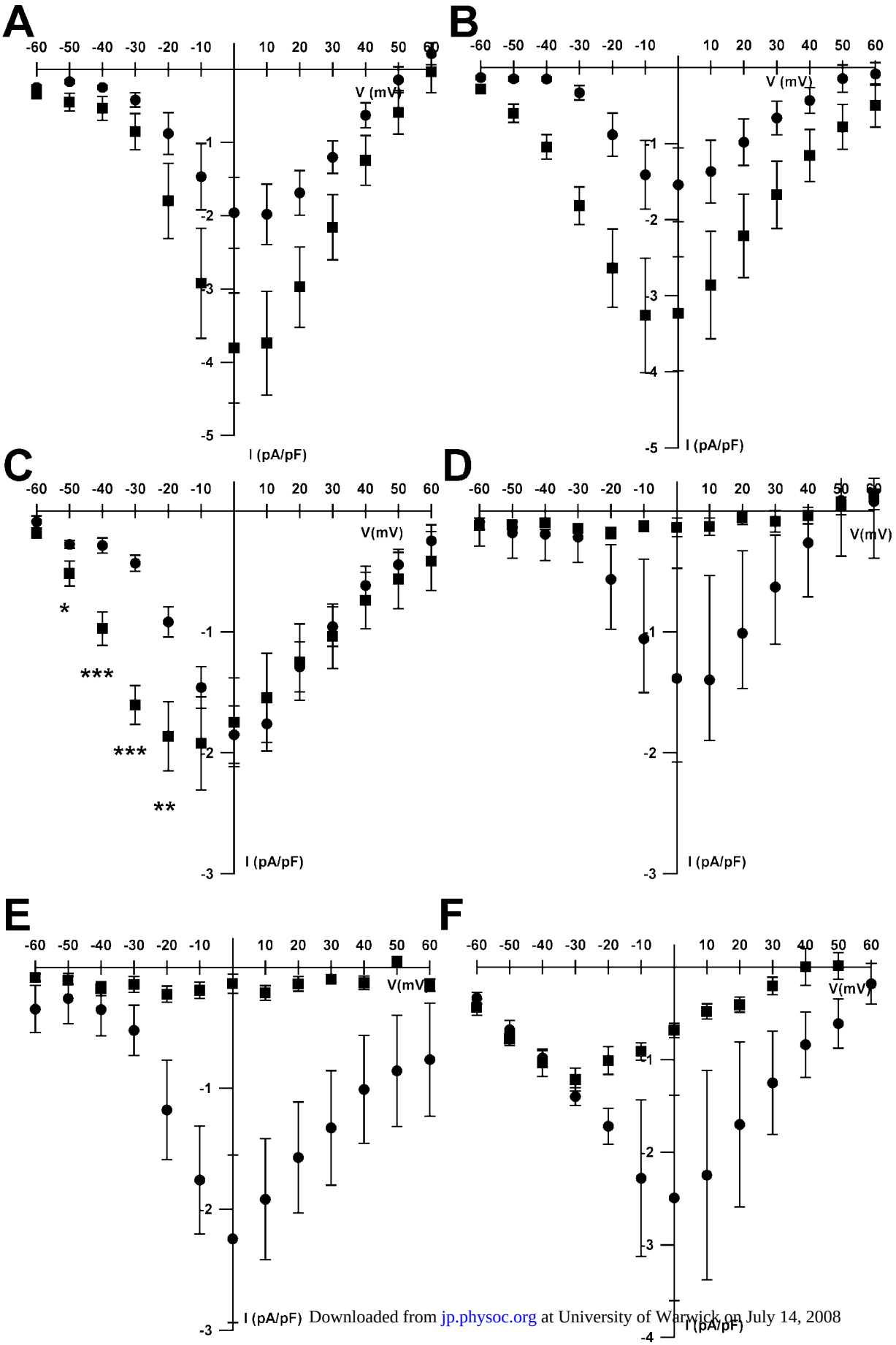
**Fig. 5.** Panel A. Current clamp trace depicting a single action potential response to a 15 second 10 pA depolarizing pulse followed by an LVA spike in response to a 5 second 10 pA hyperpolarizing pulse. Panel B. Time-scale expansion of the hyperpolarizing part of the protocol depicted in panel A. LVA spikes are elicited at approximately -65mV after a hyperpolarizing pulse and can be reversibly blocked by 100µM nickel chloride.

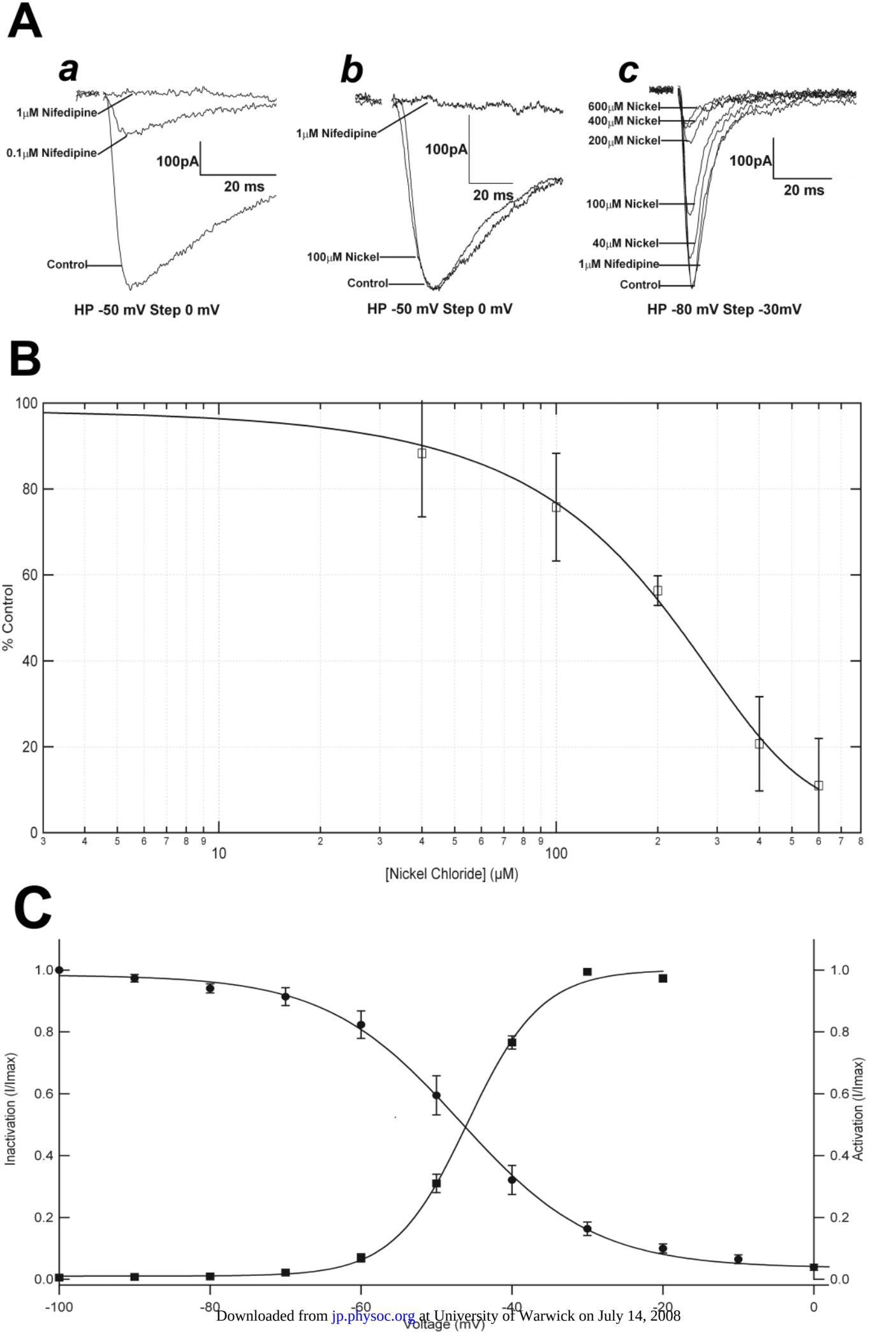
**Fig. 6.** Panel A. Spontaneous contractions of myometrial strips are slowed in frequency by application of 100 µM nickel chloride. Panel B. Effect on frequency is gradual in onset and reversible on washout. (100 µM nickel choride; circle) (Time matched vehicle control; square). Panel C. Effect of 100µM nickel chloride on maximal contractile force (M-M), activity integral (AI) and contraction interval (CI). Values represent mean±SE (n=4). $P<0.05$ = *; $P<0.001$=***

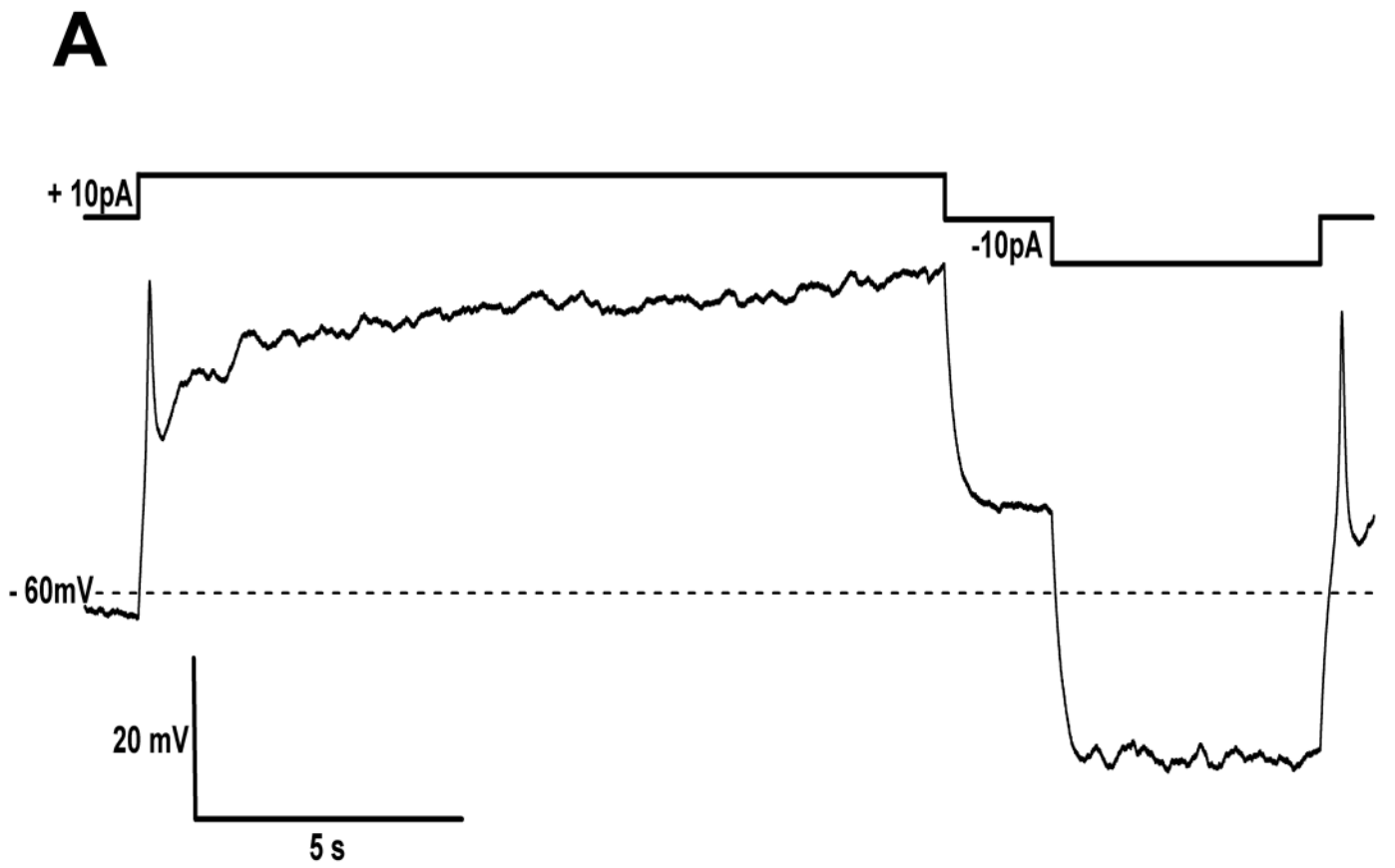


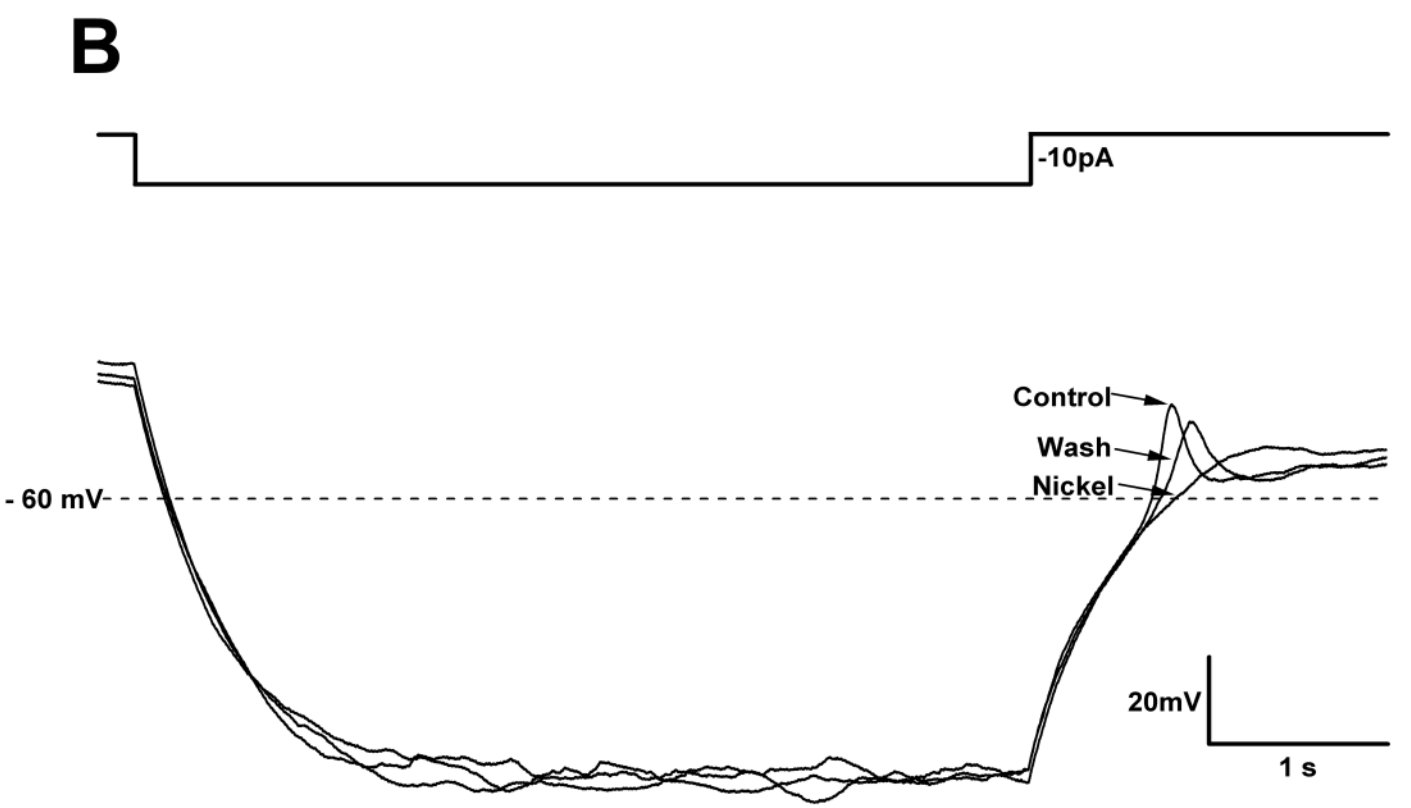



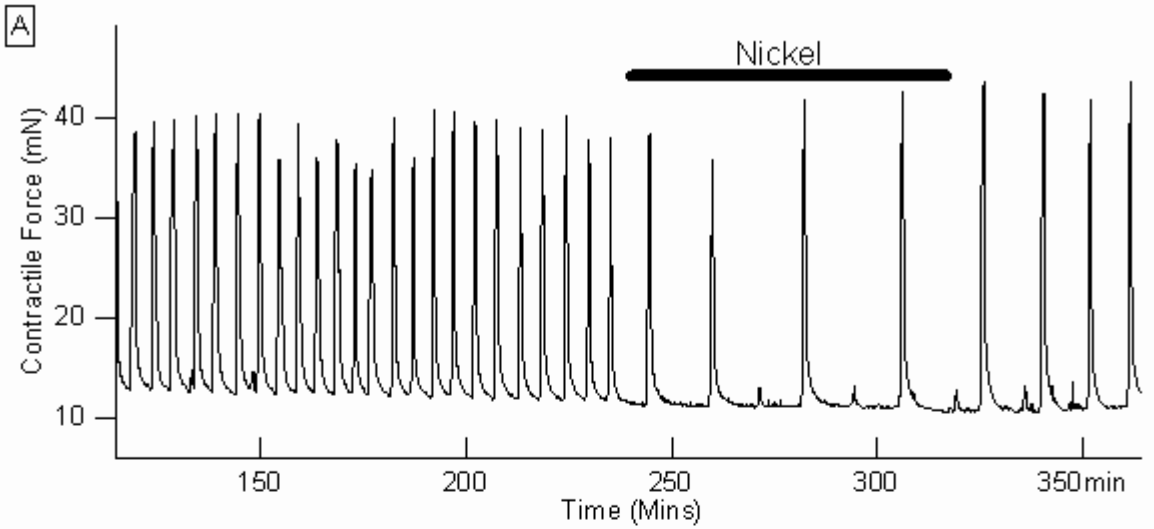


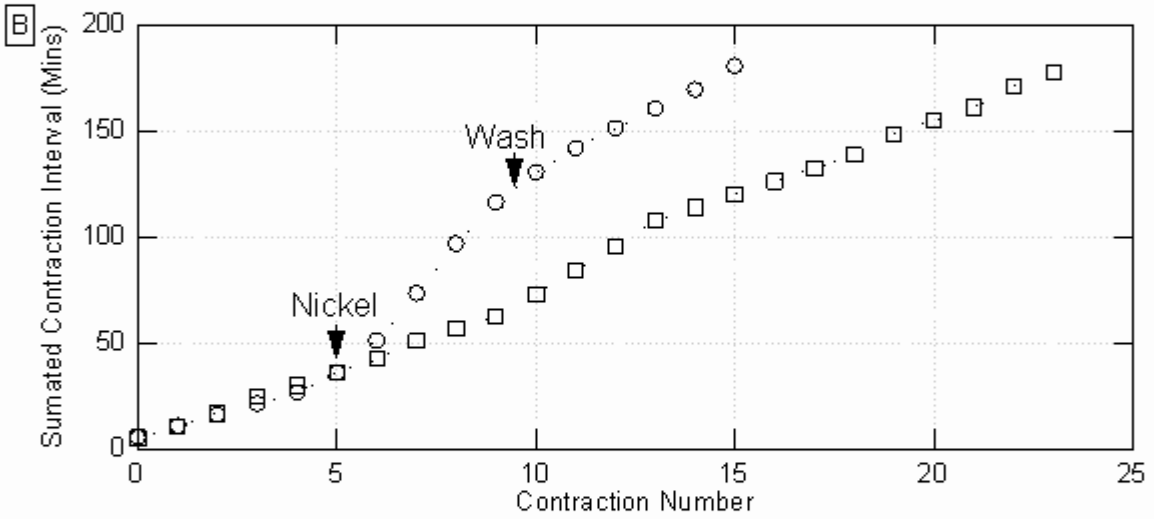


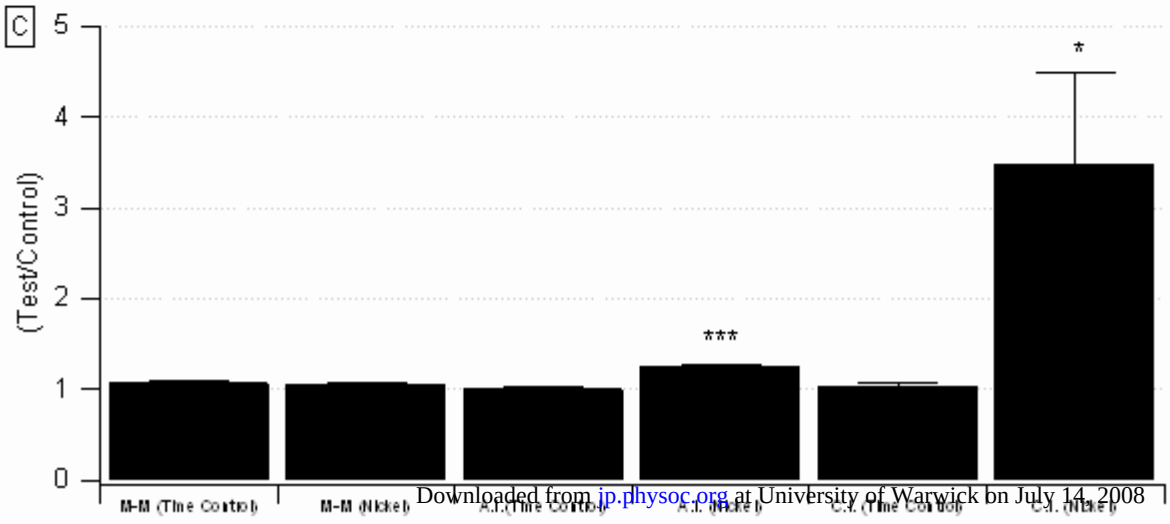






**Characterization of the molecular and electrophysiological properties of the T-Type calcium channel in human myometrium**

Andrew M Blanks, ZhengHang Zhao, Anatoly Shmygol, Gilles Bru-Mercier, Shirley Astle and Steve Thornton

*J. Physiol.* published online Apr 19, 2007;

DOI: 10.1113/jphysiol.2007.132126