CELL AND MOLECULAR PHYSIOLOGY

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Electrophysiological characterization and functional importance of calcium-activated chloride channel in rat uterine myocytes

Abstract In order to better understand the mechanisms underlying excitation of the uterus, we have elucidated the characteristics and functional importance of Ca^{2+}-activated Cl^- currents (I_{Cl-Ca}) in pregnant rat myometrium. In 101/320 freshly isolated myocytes, there was a slowly inactivating tail current (162±48 pA) upon repolarization following depolarising steps. This current has a reversal potential close to that for chloride, and was shifted when $[Cl^-]$ was altered. It was activated by Ca^{2+} (but not Ba^{2+}) entry through L-type Ca^{2+} channels, enhanced by the Ca^{2+} channel agonist Bay K8644 (2 μM), and inhibited by the Cl^- channel blockers, niflumic acid (10 μM) and anthracene-9-carboxylic acid (9-AC, 100 μM). We therefore conclude that the pregnant rat myometrium contains Ca^{2+}-activated Cl^- channels producing inward current in ~30% of its cells. When these channels were inhibited by niflumic acid or 9-AC in intact tissues, the frequency of spontaneous contractions, was significantly reduced. Niflumic acid was also shown to inhibit oxytocin-induced contractions and Ca^{2+} transients. Neither 9-AC nor niflumic acid had any effect on high-K-invoked contractions. Taken together these data suggest that Ca^{2+}-activated Cl^- channels are activated by Ca^{2+} entry and play a functionally important role in myometrium, probably by contributing to membrane potential and firing frequency (pacemakers) in these cells.

Keywords Chloride channels · Contraction · Niflumic acid · Pacemaker · Smooth muscle

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Introduction

The uterus is a spontaneously active tissue, whose contractions have to be controlled and regulated for successful pregnancy and parturition. It is known that changes in myometrial membrane potential are essential for this uterine activity and this in turn is a function of ion movement across the membrane governed by ion channel activity [31, 35, 42]. The importance of electrical excitability and its essential role in the progression of pregnancy, and the onset of labour, is highlighted by the changes in K^+, Ca^{2+}, Na^+ channel density, that occur (see [16, 19, 31, 34, 38, 39]). However, there is much that is still unknown about what channel activity contributes to both the action potential and underlying pacemaker activity.

Ca^{2+}-activated chloride channels are a family of proteins of which four human forms have so far been cloned [5, 9, 32] and $CLCA_4$ may be the smooth muscle form. They are important for control of excitability as discussed here, and salt and water balance in the human body [33]. Ca^{2+}-activated Cl^- currents (I_{Cl-Ca}) have been reported in several smooth muscle cells [8, 29] and possible pacemaker cells [36], and have been shown to play an important role in the generation of the electrical activity underlying spontaneous contractile activity [13]. It has thus been suggested that such channels play a role in a pacemaker capacity. Very little, however, is known about Cl_{Ca} in the myometrium. The channels have been reported [7] and suggested to be activated following oxytocin stimulation of the uterus [3, 4]. It is not known, however, if Ca^{2+} entry via L-type Ca^{2+} channels can activate these channels, although there are reports that this occurs in other tissues [1, 11, 23]

There has been no study of the role of Ca^{2+}-activated Cl^- channels in contraction of the myometrium, apart from that of [43]. In this study, contraction, but not intracellular Ca^{2+}, was measured when two inhibitors of I_{Cl-Ca} were applied. They reported a significant decrease in force. However, as the inhibitors used may also inhibit Ca^{2+}

entry, which in turn modulates I_{Cl-Ca} as well as directly affecting force, this observation requires confirmation.

The aims of the present study were therefore (1) to identify and characterize I_{Cl-Ca} current, (2) to determine if L-type Ca entry can activate it, and (3) to elucidate its role in contraction of the myometrium. Freshly isolated myocytes from late-pregnant rat uterus and whole cell patch clamp techniques were used to characterize I_{Cl-Ca}. Simultaneous force and Ca^{2+} measurements were made to elucidate the functional effects of I_{Cl-Ca} inhibition. A preliminary account of this work has been presented [18].

Materials and methods

Pregnant female Wistar rats (18–21 days gestation) were killed humanely by cervical dislocation under CO_2 anaesthesia. The intrauterine contents were removed and the uterine horns placed in Krebs solution (140 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4 using NaOH). The tissue was dissected to obtain 12–16 strips of longitudinal muscle approximately 20 mm in length, which were incubated in 5 ml of low-Ca^{2+} (50 μM) Hanks balanced salt solution [HBSS, (35°C, 1 h)]. The strips were chopped into small pieces and incubated in enzyme containing solution (35°C, 45mins) comprised of HBSS (containing 50 μM Ca^{2+}) supplemented with 1 mg/ml collagenase (type 1A), 30 U/ml elastase (porcine pancreas, type 1, Worthington), 1 mg/ml trypsin inhibitor (soybean, type II-S) and 4 mg/ml bovine serum albumin (fraction V, essentially fatty acid free). At the end of the incubation the tissue was removed from the enzyme solution and triturated in HBSS (containing 50 μM Ca^{2+} and 1 mg/ml BSA) using a fire-polished glass Pasteur pipette of approximately 3 mm orifice. Remaining tissue was allowed to settle and the suspension obtained was filtered using an 80-nm nylon mesh. HBSS was added to the remaining tissue which was triturated for a final time, again the suspension was filtered through an 80 nm nylon mesh and the two aliquots were pooled. The cell suspension was centrifuged (2 min, 1,000 rpm), the supernatant removed and the cell pellet resuspended in KB media (40 mM KCl, 10 mM K_2HPO_4, 10 mM taurine, 10 mM TES, 11 mM glucose, 5 mM pyruvate, 5 mM creatine, 0.04 mM EGTA and 100 mM K-glutamate) supplemented with 1 mg/ml BSA and stored at 4°C until use.

Cells were used for approximately 8 h after dissociation. An aliquot of the cell suspension was placed in a perfusion chamber mounted on an inverted microscope (Nikon Diaphot 200). Cells were allowed to settle before continuous perfusion of the chamber with pre-warmed Krebs solution (35°C, 5 ml min^{-1}), of the following composition: NaCl, 130 mM; KCl, 5.8 mM; $CaCl_2$, 2.5 mM; MgCl2, 1.2 mM; HEPES, 10 mM; glucose, 11 mM.

The cell isolation procedure yielded a large quantity of cells, which appeared healthy and relaxed. The average cell capacitance was 105±3 pF (n=148, 39 separate experiments). Whole-cell voltage clamp was achieved using a PC-505B patch-clamp amplifier (Warner Instruments). Membrane currents were recorded and voltage-clamp protocols generated using an IBM-compatible computer through an ADC board (Digidata 1320A, Axon Instruments, Union City, USA). PClamp 8.0 software suite was used for data acquisition and analysis. Patch pipettes of 3–4 MΩ resistance containing 140 mM CsCl; 8 mM NaCl, 4 mM MgATP, 10 mM HEPES and 0.005 mM EGTA were used.

A tight seal was formed between the pipette and the cell membrane (>1 GΩ) before rupture of the membrane using negative pressure. Series resistance and the capacitive surge were usually uncompensated. All current records were filtered at 1 kHz and digitally corrected for passive leakage. Cells were maintained at a holding potential of −60 mV before application of depolarising voltage pulses. Ba^{2+}, Bay K8644 (2 μM), niflumic acid (10 μM in cells and 20 μM in tissue), 9-AC (100 μM) and low chloride

solutions were applied using a blunt pipette positioned close to the cell. In some experiments the external (Krebs) solution had [Cl$^-$] reduced by either 25% or 50% of normal by reducing NaCl to either 97 or 65 mM and replacing with Na-glutamate 33 or 65 mM, respectively; all other components of Krebs solution were unaltered, and the pipette solution remained unchanged.

Simultaneous force and $[Ca^{2+}]_i$ were made off tissue strips which had been loaded with 7 μM Indo-1 AM in the presence of 0.02% pluronic acid (Molecular Probes) for 3 h at room temperature. After rinsing in Krebs solution the tissue was transferred to the stage of an inverted microscope and viewed with a 10× fluor-objective. The tissue was excited at 350 nm, using a 75-W xenon lamp, and emitted light was detected at 400 and 500 nm, using a pair of photomultipliers (Thorn, EMI). Background signals were obtained by adding Mn^{2+} at the end of the experiment. After subtracting background signals, the ratio of 400:500 nm emissions was used as an indicator of $[Ca^{2+}]_i$. The tissues were superfused throughout at 5 ml min^{-1} and experiments performed at 35°C. In some experiments the strips were depolarised by increasing KCl to 60 mM by inorganic substitution of NaCl. All reagents were obtained from Sigma (Sigma-Aldrich, Dorset, UK) unless otherwise stated.

All results are expressed as the mean±SEM where appropriate. Statistical analysis was performed using the paired Student's t-test or ANOVA with $P<0.05$ being significant. The n values shown represent the number of cells, or animals, unless stated otherwise.

Results

Currents observed in freshly isolated uterine myocytes

Depolarising voltage steps in 10-mV increments from –50 mV to +60 mV from a holding potential of −60 mV were applied to freshly isolated cells (Fig. 1A). The application of these depolarising steps with CsCl pipette solution to inhibit outward current, gave rise to (1) an initial fast current attributed to Ca^{2+} entry through L-type Ca^{2+}-channels (Fig. 1A, filled squares); (2) with prolonged application (100 ms) of the depolarising steps, an outward current was seen (Fig. 1A, filled circles); and (3) upon repolarisation to a holding potential of –60 mV a slowly inactivating tail current was observed (Fig. 1A, open circles) in approximately one-third of the cells (n=101/320, 62 animals) The mean (n=30) I-V relationships for these currents are plotted in Fig. 1B.

The initial fast inward current had a peak amplitude of 695±58 pA (n=30). It was attributed to L-type calcium current (I_{Ca}), previously observed in uterine myocytes [37], as it activated within 1.9±0.2 ms (n=16) at approximately –40 mV, and peaked at 0 mV (Fig. 1B, I_{peak}, filled squares) and was blocked by nifedipine. A tail current (Fig. 1B, I_{tail}, open circles) upon repolarization was observed after voltage prepulses from −40 to −60 mV, with a mean peak amplitude of 162±48 pA, measured at −60 mV. The amplitude of the tail current correlated with that of the L-type Ca current, consistent with it being calcium activated. At the late stage of depolarisation (Fig. 1B, I_{late}, filled circles) the L-type Ca current will be inactivated (due to rising $[Ca^{2+}]$ and prolonged voltage application), and therefore can contribute little to the outward current observed at this time. The I-V relationship for this current is therefore attributed to the current
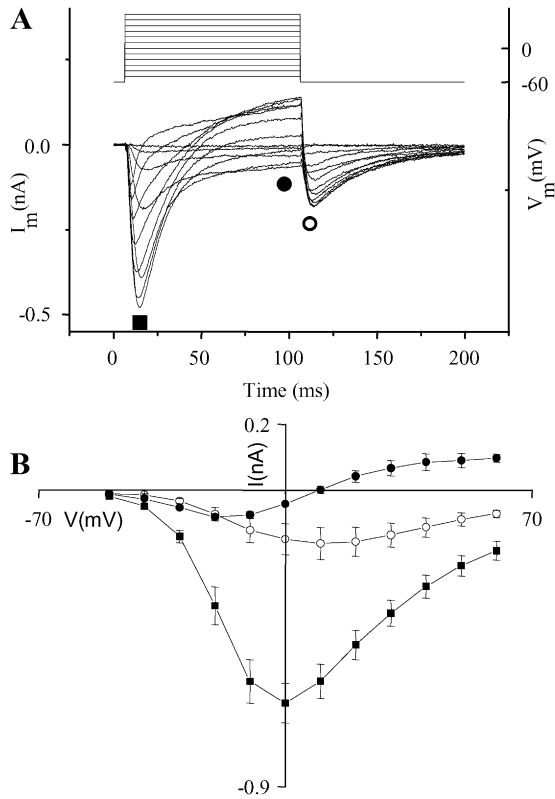
A

B

Fig. 1. **A** Currents observed in rat uterine myocytes using CsCl pipette solution with the application of depolarising steps in 10-mV increments from –50 mV to +60 mV. **B** Current-voltage relationships of peak calcium current (I_{Ca}; *filled squares*), peak tail current (I_{tail}; *open circles*) and late outward current (*filled circles*). Results represent the mean±SEM, $n=30$

responsible for the tail current. The s-shaped I-V curve for this late outward current, shows a change in direction of current, close to the reversal potential for chloride, under our ionic conditions, this suggests chloride channels are present and could be responsible for the tail current observed.

Reversal potential of the tail current

The reversal potential of the inward tail current was determined using voltage steps in 20-mV increments after an initial depolarising pulse to 0 mV from a holding potential of –60 mV, $n=18$, 5 animals (Fig. 2A). The tail current (I_{tail}) measured 10 ms after stepping the membrane potential from 0 mV to a value between −100 and 80 mV, had a reversal potential of –2.5±1.9 mV. This is within the expected range for Cl^- reversal potential under our conditions (Fig. 2B), which was calculated as –1.2 mV. When the $[Cl^-]$ of the extracellular solution was reduced by 25% or 50% by substitution with glutamate (Fig. 2B and C, respectively) the reversal potential shifted to a more positive value (9.5±2.1 mV, $n=4$ and 16.3±3.4 mV, $n=4$); again, this is within the range of the calculated values of 8.3 mV and 15.3 mV for our conditions.
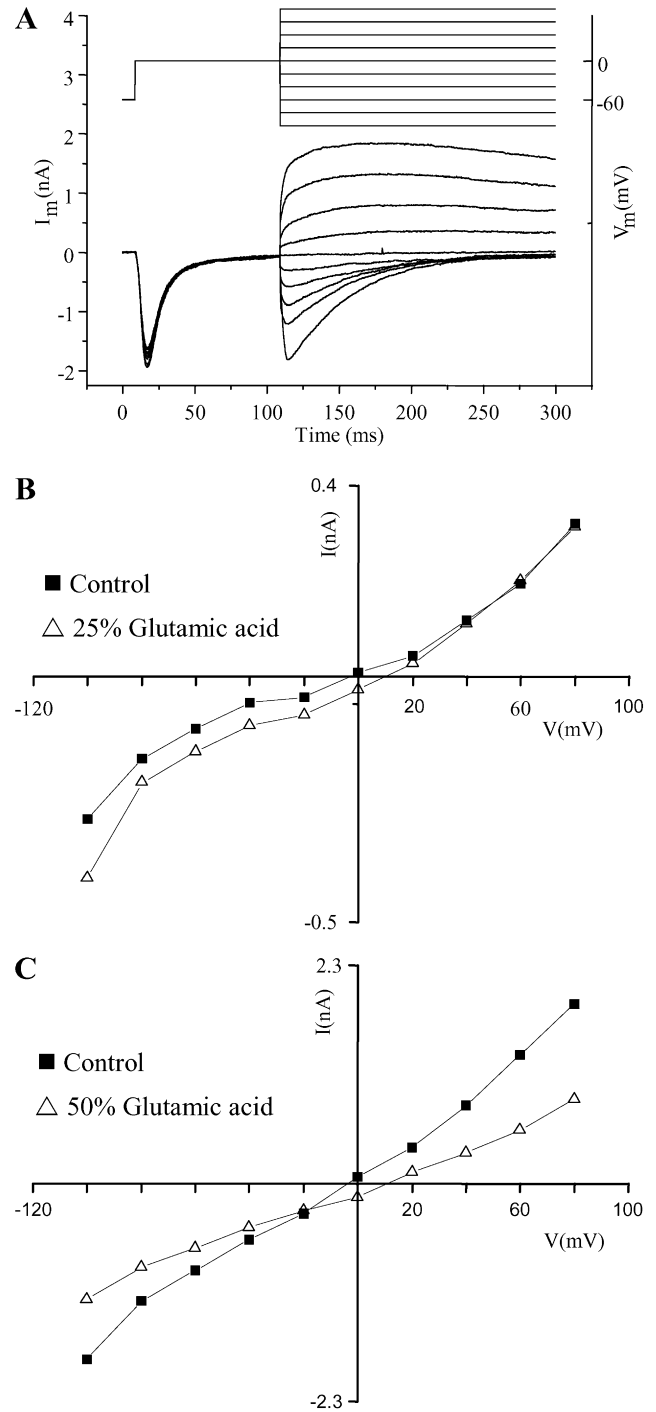

A

B

C

Fig. 2. **A** Currents observed in rat uterine myocytes using CsCl pipette solution with the application of depolarising steps in 20-mV increments from –100 mV to +20 mV. The reversal potential of Cl^- current in normal Krebs and with a 25% (**B**) and 50% (**C**) reduction in extracellular $[Cl^-]$

The time-course of the tail current was voltage dependent as evident from Fig. 2A. At negative values of V_m, the tail current decayed monoexponentially. At positive potentials its time course was more complex, comprising an initial slow rise above the value measured at 10th millisecond after the voltage jump, and a subsequent slow

decay. The time-constant of the decay at negative potentials was voltage dependent. Its value going from −100, -80, -60 and −40 mV increased, as follows, 34±2, 35±2, 42±2 and 66±7 ms, respectively.

The effect of replacing extracellular Ca^{2+} with Ba^{2+}

The I-V curves previously shown suggests the tail current observed is calcium activated. To confirm this, extracellular Ca^{2+} was replaced with Ba^{2+}, which can enter via L-type Ca^{2+} channels, but cannot activate Cl_{Ca} channels [30]. The effect of Ba^{2+} was determined by applying a depolarising step to 0 mV from a holding potential of –60 mV. This protocol was applied every 5 s to enable a comparison of control conditions and those with Ba^{2+}.

The application of a depolarising pulse in Ba^{2+} containing solution evoked an initial inward current as Ba^{2+} acted as the charge carrier. Its magnitude was 879 ±32 pA, (n=4, Fig. 3A). The rate of decay of this current was decreased compared to that elicited by Ca^{2+}, indicating the removal of the Ca-dependent components of inactivation. As can be clearly seen, with Ba^{2+} there was a very marked, significant decrease in inward tail current from 132±30 pA in Ca^{2+} and 26±1 pA in Ba^{2+} (n=4).

Relation between I_{Ca} and I_{Cl-Ca}

The I_{Cl-Ca} observed above in uterine myocytes is Ca^{2+} activated. Experiments were performed to determine the rate of rundown of both I_{Ca} and I_{Cl-Ca} and the correlation between these two currents. The current rundown was determined by applying a brief depolarising pulse to 0 mV every 5 s for approximately 8 min. The rate of run-down of peak I_{Cl-Ca} was faster than that of I_{Ca} (t=3.3±0.5mins versus 5.4±0.5 min, respectively, n=12, 9 animals; Fig. 3B). There was a strong correlation between the two (r=0.98±0.005, n=12,) suggesting chloride channels are tightly regulated by calcium.

The effects of Bay K8644

The observed tail currents were relatively small compared to L-type inward currents, and not observed in all cells. It was therefore possible that more cells were producing I_{Cl-Ca}, but that it was not being detected. As these currents were Ca^{2+}-activated, Bay K8644, a Ca^{2+} channel agonist was used to increase $[Ca^{2+}]_i$ and determine if this increased peak I_{Cl-Ca} and also to test if more cells exhibited I_{Cl-Ca}. Bay K8644 significantly increased peak I_{Ca}(n=37, 15 animals from 664±67 to 1116±86 pA) and peak tail current (from 84±13 to 144±20 pA; Fig. 4A, n=13, 11 animals). These changes were proportional (2.1 ±0.2-fold and 1.8±0.1-fold, respectively). However, the use of Bay K8644 did not reveal any tail current in cells initially observed without one (Fig. 4B), and the fraction
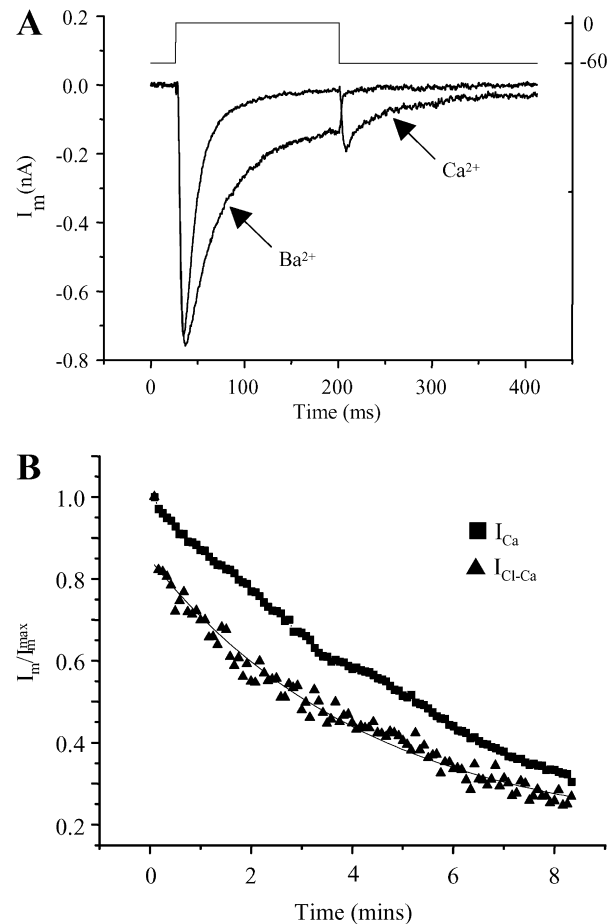

Fig. 3. A The effect of replacing extracellular Ca^{2+} with Ba^{2+} on inward currents activated on depolarisation and repolarization. **B** The decay of $I_{Ca}$and Ca^{2+}-activated Cl^- current (I_{Cl-Ca}) upon application of a depolarising pulse to 0 mV, which was repeated every 5 s. Results shown are the mean of 12 separate experiments

of cells possessing I_{Cl-Ca} remained around 30%, (13/37 cells).

The effects of chloride channel inhibitors

Niflumic acid and 9-AC, were used to inhibit chloride channels [28], to confirm the tail current observed was indeed a chloride current. Niflumic acid resulted in the loss of inward tail current (Fig. 4C). Although not significant, we did note a reduction in I_{Ca} in some preparations (n=3, 2 animals). When Bay K8644 was used to enhance I_{Ca}, in the presence of niflumic acid (n=4), the tail current was still absent (not shown). The application of 9-AC also resulted in a loss of inward tail current with no reduction in I_{Ca}, as shown in Fig. 4D (n=8, 4 animals).

Effects of blocking I_{Cl-Ca} on intracellular $[Ca^{2+}]$ and contractions

In order to determine if I_{Cl-Ca} could affect global $[Ca^{2+}]_i$ and uterine contractions, $[Ca^{2+}]_i$ and force were measured
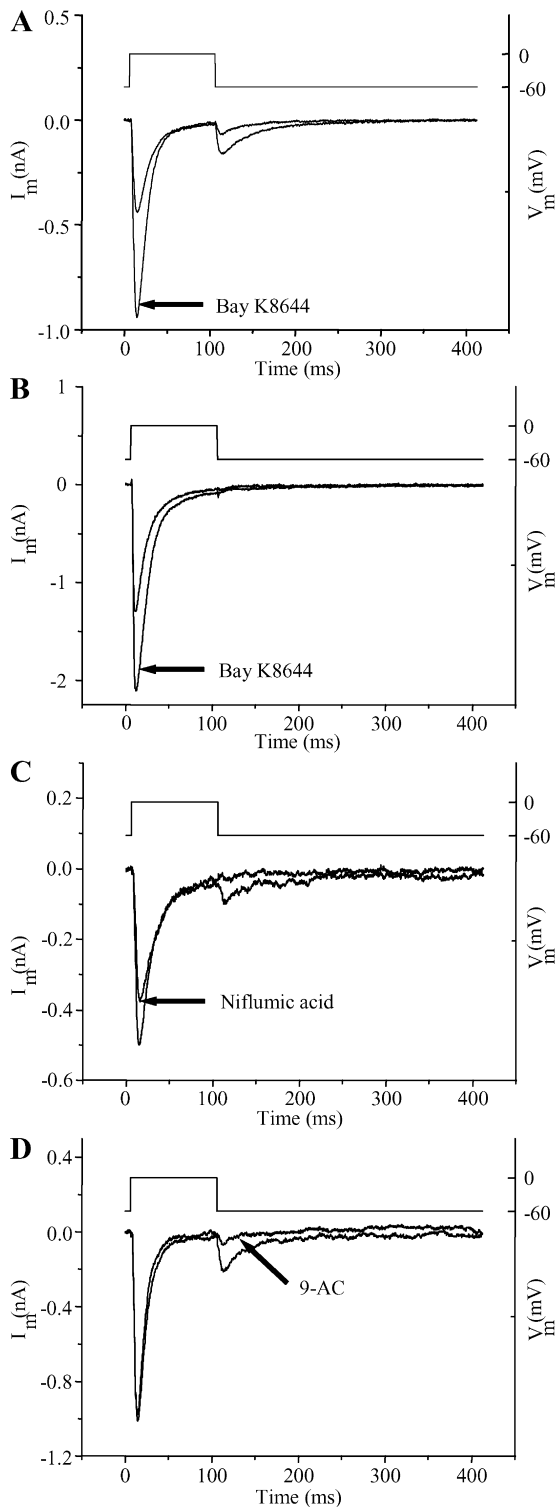
Fig. 4 . **A, B** The effects of Bay K (2 µM) in cells with and without expression of the tail current. **C** The effect of niflumic acid (10 µM) and **D** 9-AC (100 µM) upon tail current in uterine myocytes

simultaneously. Niflumic acid significantly decreased the frequency of Ca^{2+} transients and contractions in all preparations examined whether arising spontaneously (n=12, 5 animals, 58±5%) or with oxytocin (10 nM; n=6, 5 animals, 54±11%) as shown in Fig. 5A. Although

in some preparations there was a small change in the amplitude of either the Ca^{2+} or force transients, these did not reach significance. 9-AC was also shown to significantly decrease the frequency of spontaneous contractions (n=6, 3 animals, 53±3%; Fig. 5B). Because of its own fluorescence we were unable to measure calcium in the presence of 9-AC.

The effects of both inhibitors of high-K^+ depolarized preparations was also examined. High-K^+ (60 mM) produced a tonic rise in Ca^{2+} which gradually declined (Fig. 5C and D). Neither niflumic acid (n=6) nor 9-AC (n=6) had any significant effect on force under these conditions, as shown in Fig. 5C. The $[Ca^{2+}]$ was also measured in the experiments with niflumic acid, and it was also unchanged from controls in the same tissue.

Discussion

The main findings of this study are (1) that Ca-activated Cl channels are present in around 30% of freshly isolated pregnant rat myometrial cells, (2) the channels can be activated by Ca^{2+} entry through L-type Ca^{2+} channels and (3) that Cl_{Ca} channels contribute to both spontaneous and oxytocin-stimulated contractions in the myometrium, presumably by maintaining or initiating membrane depolarization.

Cellular heterogeneity

It is of interest that only around one-third of the freshly isolated cells produced inward tail currents. Due to the large number of cells studied (over 300) from a large number of animals (62) we are sure that this is not due to any variation in quality or number of cells produced by the isolation procedure, or time after isolation. Nor did increasing the size of the I_{Ca} have any effect on the number of cells exhibiting the tail current. In the study by Arnaudeau et al. [4], 80% of their cells, which were also from rats at the same stage of gestation, produced $I_{Cl\text{-}Ca}$ in response to oxytocin stimulation. Whether this represents a difference between activating the current via Ca^{2+} entry versus Ca^{2+} release, or is due to the period of culturing (10–36 h) used by Arnaudeau et al. remains to be determined. Differences between uterine myocytes, obtained from the same cell isolation, have also been reported by Martin et al. [26], who found about 30% of cells were sensitive to caffeine. In murine portal vein about two-thirds of the cells had Cl_{Ca} [5], but only a third of rabbit portal vein [12]. Heterogeneity has also been described in other smooth muscle cells [40].

Ca^{2+} entry stimulates Cl_{Ca}

The Ca^{2+} for activation of Ca^{2+}-activated Cl^- channels can come from either Ca^{2+} entry and/or Ca release from the SR. Local Ca^{2+} sparks due to spontaneous opening of

Fig. 5. A Simultaneous recording of force and intracellular Ca (indo-1 ratio) from pregnant rat myometrium, stimulated with oxytocin (10 nM). Niflumic acid (20 μM) was added as shown by the *bar*. **B** The effect of 9-AC (100 μM) on spontaneous uterine contractions. **C, D** The effects of niflumic acid (**C**) and 9-AC (**D**) on high-K (60 mM) depolarised tissue. Control and treated preparations are from the same tissue and superimposed
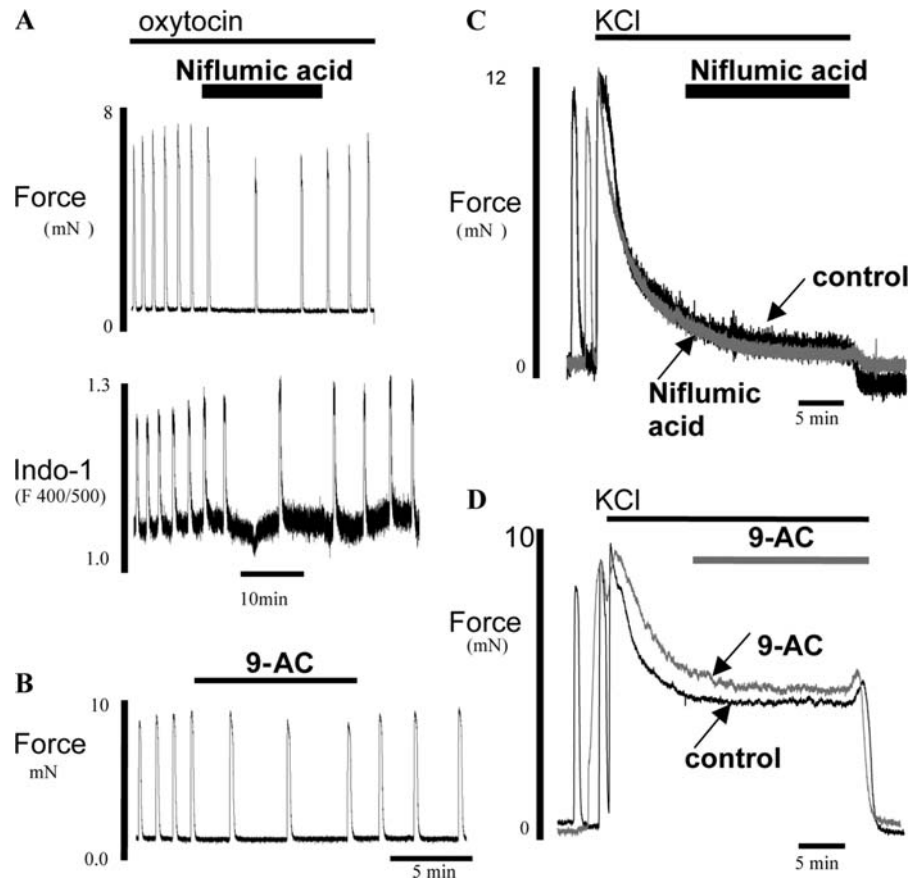
ryanodine receptors on the SR have been reported to activate Cl_{Ca} in tracheal and vascular myocytes [10, 45]. However, to date there are no reports of Ca^{2+} sparks or spontaneous transient inward currents in the myometrium, so it is not clear whether this mechanism is present. Agonists can trigger global rises of Ca^{2+} by releasing it from the SR via IP_3 receptors, and this has been associated with activation of Cl_{Ca} in the uterus [4], and other smooth muscles [6, 25] and been associated with rhythmicity [17] and oscillations [41]. The activation in turn will produce membrane depolarization and activation of L-type Ca^{2+} channels and Ca^{2+} influx [2, 4, 21, 28]. In this study we have determined that Ca^{2+} entry, in the absence of agonist, can also activate the channels, in myometrium, in agreement with reports on some other smooth muscles [1, 11, 23]. The evidence for this in the uterus is based on (1) close correlation between I_{Ca} and I_{Cl-Ca}, (2) the disappearance of the tail current in Ba^{2+}-containing solutions, and (3) Bay K8644 potentiation. The close correlation between I_{Ca} and I_{Cl-Ca} is in keeping with previous observations of a strict dependence on $[Ca^{2+}]_i$ for activation of I_{Cl-Ca} [6, 22, 25]. Inactivation may also correlate with $[Ca^{2+}]_i$ [14] as well as phosphorylation via CaM kinase II [12, 14, 22]. Ca^{2+}-activated Cl^- currents are voltage sensitive, activating slowly on depolarization and deactivating on hyperpolarization [25]. When Ba^{2+} is substituted for Ca^{2+}, the current is not seen despite large inward current carried by Ba^{2+} upon depolarization, as previously reported by [4]. Bay K8644 potentiated both

Ca^{2+} current and I_{Cl-Ca} but did not increase the fraction of cells showing a tail current.

Niflumic acid inhibits I_{Cl-Ca} by open channel blockade [8, 15, 20, 24, 28] and is the most potent blocker of I_{Cl-Ca} in smooth muscle. In rat portal vein it has been reported that niflumic acid is more potent in inhibition of I_{Cl-Ca} induced by Ca^{2+} influx, compared to that induced by agonist-stimulated SR Ca^{2+} release [28]. Niflumic acid was a potent blocker of myometrial I_{Cl-Ca}, without significantly affecting the magnitude or kinetics of I_{Ca}, in keeping with previous reports on its action [15, 28]. It slowed the time-course of I_{Cl-Ca} decay in agreement with previous reports [11, 23]. 9-AC has previously been reported to relax the rat uterus, but the mechanism was not investigated. The observed large reduction in tail current with 9-AC, without an effect on initial inward current, would therefore suggest that the tail current is a chloride current and is contributing to uterine activity. Although not significant we did note a reduction in the I_{Ca} with niflumic acid but not 9-AC, suggesting that at least in uterine smooth muscle 9-AC may be the better blocker to use.

Chloride current

Our data are consistent with the tail current being a Cl^- current. The current was slow to decay, taking into account the experimental temperature was 35°C, and the reversal potential for the tail current was close to 0 mV, the

equilibrium potential for Cl^- in our control solution. This value changed in a predictable manner when $[Cl^-]$ was altered outside the cell. In addition, the current was inhibited by niflumic acid and 9-AC known blockers of these channels. Taken together, these data, along with the I-V curves obtained led us to conclude that the inward tail current, and the late outward current, are due to I_{Cl-Ca}.

Functional effects

Despite being found in only one-third of uterine myocytes, inhibition of I_{Cl-Ca} produced a significant effect on the frequency of contractions in all preparations tested. Yarar et al. [43], using two other blockers of I_{Cl-Ca}, also reported an inhibition of frequency in myometrium but a decrease in contraction amplitude, although $[Ca^{2+}]_i$ was not measured. Similar findings have been reported for some vascular smooth muscles [20, 44]. When the uterus was depolarised with high-K^+ solution, neither inhibitor was effective. This suggests that the blocking of the Cl_{Ca} current is significant when the tissue is deolarized and that Cl_{Ca} would be more important at influencing excitability around normal resting potential values. These data also show that the inhibitors have little effect on Ca^{2+} entry, consistent with our electrophysiological data.

In summary, these data reveal a clear importance of Cl_{Ca} for uterine activity and suggest a role for them in contributing to pacemaker activity, as is known to be the case for spontaneously active smooth muscles (urethra [8]; portal vein [20]). Cl^--deficient solution removed the after depolarization that had previously followed the spike discharge in pregnant rat myometrium [27], suggesting a significant participation of Cl^- conductance to the membrane depolarization. These channels could therefore be useful targets for bringing about uterine relaxation for threatened pre-term labours. It remains however, to be established what their role is in human myometrium.

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