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RESEARCH ARTICLE

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Dual effect of nifedipine on pregnant human myometrium contractility: Implication of TRPC1

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Abstract

Nifedipine, an L-type voltage-gated Ca^{2+} channel (L-VGCC) blocker, is one of the most used tocolytics to treat preterm labor. In clinical practice, nifedipine efficiently decreases uterine contractions, but its efficacy is limited over time, and repeated or maintained nifedipine-based tocolysis appears to be ineffective in preventing preterm birth. We aimed to understand why nifedipine has short-lasting efficiency for the inhibition of uterine contractions. We used ex vivo term pregnant human myometrial strips treated with cumulative doses of nifedipine. We observed that nifedipine inhibited spontaneous myometrial contractions in tissues with high and regular spontaneous contractions. By contrast, nifedipine appeared to increase contractions in tissues with low and/or irregular spontaneous contractions. To investigate the molecular mechanisms activated by nifedipine in myometrial cells, we used the pregnant human myometrial cell line PHM1-41 that does not express L-VGCC. The in vitro measurement of intracellular Ca^{2+} showed that high doses of nifedipine induced an important intracellular Ca^{2+} entry in myometrial cells. The inhibition or downregulation of the genes encoding for store-operated Ca^{2+} entry channels from the Orai and transient receptor potential-canonical (TRPC) families in PHM1-41 cells highlighted the implication of TRPC1 in nifedipine-induced Ca^{2+} entry. In addition, the use of 2-APB in combination with nifedipine on human myometrial strips tends to confirm that the pro-contractile effect induced by nifedipine on myometrial tissues may involve the activation of TRPC channels.

KEYWORDS

L-type calcium channel, myometrial contraction, preterm labor, store-operated calcium entry channels, tocolysis

The confirmation of TRPC1 implication in nifedipine-induced contractions in human myometrial tissue could lead to the optimization of current tocolytic therapies with the clinical use of TRPC1 antagonists.

Marie Cohen and Begoña Martinez de Tejada contributed equally to this work and are co-last authors.

1 | INTRODUCTION

Preterm birth, defined as delivery before 37 weeks' gestation, is one of the leading causes of neonatal morbidity and mortality worldwide, with a global incidence of approximately 15 million infants per year (Keelan & Newnham, 2017; Purisch & Gyamfi-Bannerman, 2017). Among these, approximately 1 million die, representing 75% of perinatal mortality (Goldenberg et al., 2008; Keelan & Newnham, 2017), and most are at risk for short- and long-term complications (Purisch & Gyamfi-Bannerman, 2017). Neonatal outcomes and the severity of preterm birth-associated complications are related to the gestational age at the time of delivery, with each additional week of gestation conferring a survival benefit. Spontaneous preterm deliveries are due to the onset of uterine contractions (preterm labor) and/or pre-labor rupture of the membranes (Keelan & Newnham, 2017; Purisch & Gyamfi-Bannerman, 2017). Tocolytic agents are drugs used to inhibit myometrial smooth muscle cell contractions by either targeting pathways involved in smooth muscle contraction or promoting smooth muscle relaxation. However, currently available tocolytic agents have short-term efficacy as they do not prevent delivery for more than 7 days or significantly improve perinatal outcomes (Gyetvai et al., 1999; Petraglia & Visser, 2009).

Nifedipine is one of the most frequent drugs used as a tocolytic agent in women with a threat of preterm delivery. The contraction of myometrial smooth muscle cells requires an increase in the intracellular Ca^{2+} level, which is triggered by both electrochemical and endocrine stimulations. Electrochemical-dependent Ca^{2+} entry involves the opening of voltage-gated Ca^{2+} channels (VGCC) due to plasma membrane depolarization (Alotaibi, 2014), while endocrine induction of myometrial contractions results from the activation of several converging pathways involving different factors, such as oxytocin or prostaglandins, and leading to an increased intracellular Ca^{2+} level (Hertelendy & Zakár, 2004). Nifedipine is a first-generation dihydropyridine (DHP), known to block the L-type VGCC (L-VGCC) and limit Ca^{2+} entry into smooth muscle cells in general and myometrial cells in particular, therefore inhibiting muscular contraction (Godfraind, 2017). Despite nifedipine efficacy as a tocolytic drug when given for 48 h, repeated or maintained nifedipine-based tocolysis appears to be ineffective in preventing preterm birth and thus reducing neonatal complications and hospital stay (Aggarwal et al., 2018).

Our study aimed to understand the effects and action mechanisms of nifedipine on uterine contractions to help improve indications for its use. To address this, we first evaluated its effect on ex vivo pregnant human myometrium contractility and then investigated in vitro the nifedipine-activated mechanisms leading to differential effects on the myometrium.

2 | MATERIALS AND METHODS

2.1 | Human myometrial tissue collection

Myometrial biopsies were collected from 23 term-pregnant non-laboring women undergoing an elective cesarean delivery at the Maternity Unit of Geneva University Hospitals (Switzerland). The study was approved by the local ethics committee (project number 2018-01018) and informed written consent was obtained from all participating women. Participants were uncomplicated singleton pregnant women delivering between 37 and 40 completed gestation weeks. Exclusion criteria were: women on labor or with multiple pregnancies; those having medication for preterm labor earlier during pregnancy; and women with human immunodeficiency virus, hepatitis B or hepatitis C. Tissues were collected immediately after delivery from the upper edge of the lower uterine segment at the site of the cesarean incision and immediately placed in cold Hank's Balanced Salt Solution (HBSS; Gibco) until processing. Fresh pieces of tissue were used for myometrial contractility experiments and purification of primary myometrial cells, while other pieces were frozen for gene expression analysis.

2.2 | Myometrial tissue contractility

Measurement of ex vivo human myometrial contractility was adapted from Arrowsmith et al. (2018). Following tissue collection and excision of decidua and adherent fetal membranes, myometrial biopsies were washed in fresh HBSS to remove blood. Myometrial strips (≈1 × 2 × 8 mm, from 1 to 4 strips/biopsy) were then dissected from myometrial muscle layers and placed in an organ bath (emkaBATH2; Emka Technologies) containing Krebs buffer, pH 7.4, heated at 37°C and bubbled with a gas mix (95% oxygen and 5% carbon dioxide). One extremity of each strip was attached to a fixed hook, while the other extremity was connected to a force transducer. Myometrial strips were stretched at 1 g under passive resting tension and then allowed to equilibrate for 1 h. Following equilibration, strips were challenged with a high-K^+ solution (40 mM KCl) and allowed to equilibrate until spontaneous contraction. For the experiments with cumulative doses of nifedipine (n = 26 strips from 13 patients), strips were then subjected to increasing concentrations of nifedipine (from 10^{-10} to 10^{-7} M). For the experiments with low doses of nifedipine (n = 14 strips from 6 patients), strips were subjected to successive treatments with nifedipine at 10^{-10} and 10^{-9} M. The 14 strips were then stored at −80°C until processing for messenger RNA (mRNA) extraction and RT-qPCR. For the 2-APB experiments (n = 16 strips from 4 patients), strips were subjected either to 75 µM 2-APB alone (n = 8), or successive treatments with 10^{-9} M nifedipine and 10^{-9} M nifedipine + 75 µM 2-APB (n = 8). Each treatment was applied for at least 15 min. Two strips were tested simultaneously. Data were recorded with iox.2 acquisition software (Emka Technologies).

2.3 | Purification of primary human myometrial cells

Primary myometrial cells (pPM) were used for the analysis of Ca^{2+} channel expression (RT-qPCR) and function (analysis of intracellular Ca^{2+} in response to the high-K^+ solution). Cells were purified from myometrial biopsies collected from three term-pregnant women

(mean gestational age, 38.9 ± 0.9 weeks), using a method adapted from Aguilar et al. (2010). Briefly, the tissues were cut into small pieces and digested in a digestion buffer (HBSS, 1% antimycotic/antibiotic [Gibco], collagenase A 2 mg/ml [Roche] and DNase I 1.25 mg/ml [Roche]) for 1 h at 37°C in a water bath. The supernatants were then discarded and the remaining tissues were then incubated overnight at 37°C in a water bath, with fresh digestion buffer. Tissue lysates were then filtered on 100 µm cell strainers washed with HBSS and centrifuged at 2000*g* for 5 min and washed three times with HBSS. The purified myometrial cells, named pPM1, pPM2, and pPM3, were cultured until passage 6 in Smooth Muscle Cell Growth Medium 2 (PromoCell GmbH), supplemented with 1% antimycotic/antibiotic at 37°C with 5% CO_2. Purified cells were characterized by analyzing the cell morphology and the mRNA expression of several markers by RT-PCR (OXTR, ESR1, ESR2, VIM, CNN1, ACTA2, and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) and immunofluorescence (α-smooth muscle actin, connexin 43, and tropomyosin) (see Supporting Informaion Materials and Methods and Figure S1).

2.4 | Myometrial cell line culture

The pregnant human myometrial 1-41 (PHM1-41) cell line was used to investigate the in vitro effect of nifedipine on myometrial cells (Ca^{2+} assay) and the implication of the store-operated Ca^{2+} entry (SOCE) channels using pharmacological inhibitors and small interfering RNA (siRNA). This cell line, derived from a single term-pregnant woman, has been previously described by Monga et al. (1996). PHM1-41 cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX™-I (Gibco) containing G-418 (0.1 mg/ml; Roche), 10% fetal calf serum (FCS) and gentamicin (50 µg/ml; Gibco).

2.5 | Calcium assay

Ca^{2+} assays were performed on PHM1-41 cells to measure the effect of nifedipine and pharmacological inhibitors of SOCE channels on the intracellular Ca^{2+} level. PHM1-41 cells were seeded in black wall 96-well plates with a clear bottom (Corning, Chemie Brunschwig AG) in a complete culture medium (1.2×10^4 cells/well) and allowed for attachment overnight. Cells were then starved by replacing the complete culture medium with G-418-free, FCS-free, phenol red-free DMEM for 24 h. The starvation medium was then discarded and cells were incubated with Fluo-8 No Wash dye (AAT Bioquest, LubioScience GmbH) according to the manufacturer's instructions for 30 min at 37°C with 5% carbon dioxide. The cells were transferred to a Functional Drug Screening System (FDSS µCell; Hamamatsu Photonics) to measure the fluorescence baseline for 1 min (1000 points/s, Ex480:Em540), followed by the addition of either 20 µl of 5× nifedipine (Fagron) solutions in 2.5% dimethyl sulfoxide (DMSO) (nifedipine final concentrations: from 10^{-10} to 10^{-4} M in 0.5% DMSO) or 20 µL of 5× SOCE channel inhibitors (final concentrations: GSK7975A 10 µM, BTP2 1 µM, and 2-APB 75 µM), followed by the addition of 20 µl of 6× nifedipine (final concentration: 10^{-4} M). The fluorescent signal was normalized to baseline max-min relative fluorescence units (RFU).

2.6 | Calcium imaging

Ca^{2+} imaging was performed on both PHM1-41 and pPM cells to study their response to a high-K^+ solution. Cells were loaded with 2 µM of Fura-2 AM and 0.1% pluronic acid (Pluronic F-127) in the dark at room temperature for 40 min in buffer containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05 mM EGTA, 10 mM HEPES, 10 mM glucose, with pH adjusted at 7.4 with 1 N NaOH. Cells were washed twice and allowed de-esterification of the dye for 20 min in the same buffer. High-K^+ solution (70 mM KCl) was then applied to the cells. Fluorescence was recorded using an inverted microscope (Axio Observer, Carl Zeiss) equipped with a Lambda DG4 illumination system (Sutter Instrument), which rapidly changed the excitation wavelengths between 340 nm (340AF15; Omega Optical) and 380 nm (380AF15; Omega Optical). Emission was collected through a 415 DRLP dichroic mirror and a 510WB40 filter (Omega Optical) by a cooled 12-bit CCD camera (CoolSnap HQ, Ropper Scientific). Image acquisition and analysis were performed with the Metafluor 6.3 software (Universal Imaging). The high-K^+ solution contained 70 mM KCl and 65 mM NaCl to maintain osmolarity.

2.7 | RT-qPCR

The expression of genes encoding for the α_1 subunit of the L-VGCC and the SOCE channels from the Orai and the transient receptor potential-canonical (TRPC) families was analyzed by RT-qPCR. Total RNA was extracted from the cells and tissues using the PureLinkRNA Mini Kit (AMBION, Life Technologies). Reverse transcription was performed with 0.5 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). Detection of the real-time qPCR product was performed using the KAPA SYBR FAST qPCR Kit Master Mix (Kapa Biosystems, Sigma-Aldrich) on an Eco Real-Time PCR System (Illumina). The relative expression of the different genes was normalized to the three housekeeping genes GAPDH, cyclophilin A, and HPRT1. Gene expression levels were calculated using the Livak method (Livak & Schmittgen, 2001). The oligonucleotide primers for qPCR are described in Table 1.

2.8 | siRNA experiments

The genes encoding for the SOCE channels from the Orai and TRPC families were downregulated in PHM1-41 cells using siRNA to investigate their implication in the nifedipine response. PHM1-41 cells were seeded in a complete culture medium in black wall 96-well

TABLE 1 qPCR primer sequences

Gene name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
Orai1	GCTCATGATCAGCACCTGCAC	GGGACTCCTTGACCGAGTTG
Orai2	CTGCATCCTGCCCAATGTG	GAGTTCAGGTTGTGGATGTTGCT
Orai3	AGCTGTGAGCAACATCCACAAC	CCACGTAGCGGTGCAGTCT
TRPC1	ACAGCAAAGCAATGATACCTTCC	AAAGCAAAGCAGGTGCCAAT
TRPC3	TGTTCAATGCCTCAGACAGG	AGTGTCACTTCACTGAGGTC
TRPC4	CAGTTCTCTGCGTCTGATCTCACT	GGTCCCAGGTGAGAATTTGC
TRPC5	CCTCTCATCAGAACCATGCCAA	GCGTTTGCTTGATGACTCAGC
TRPC6	CCTTGCTGTTGCCATTGGA	TCTTCCCCATCTTGCTGCAT
TRPC7	TGGGCATGCTGAATTCCAAA	GCTGTCAGATTTTCAGAATTCCTCA
$Ca_v1.2$	GCAGGAGTACAAGAACTGTGAGC	CGAAGTAGGTGGAGTTGACCAC
$Ca_v1.3$	CTTCGACAACGTCCTCTCTGCT	GCCGTAGTTCTCTCCATTCGAG
Cyclophilin A	TACGGGTCCTGGCATCTTGT	CCATTTGTGTTGGGTCCAGC
GAPDH	CGACCACTTTGTCAAGCTCA	CCCTGTTGCTGTAGCCAAAT
HPRT1	ATGACCAGTCAACAGGGGAC	TGCCTGACCAAGGAAAGCAA

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative polymerase chain reaction.

plates with a clear bottom (7.5×10^3 cells/well) and in 6-well plates (1.25×10^5 cells/well) for Ca^{2+} assay and RT-qPCR analysis, respectively. Twenty-four hours after seeding, transfection of 10 nM of either siOrai1 (Microsynth), siOrai2 (Microsynth), siOrai3 (Microsynth), siTRPC1 (Qiagen), siTRPC4 (Santa Cruz Biotechnologies), siTRPC6 (Santa Cruz Biotechnologies), or control siRNA (siCTL, Microsynth) was carried out using InterferIN transfection reagent (Polyplus transfection). Cells were incubated with the transfection mix for 48 h before processing.

2.9 | Statistical analysis

Data are represented as means ± *SEM* for at least three different replicates. Statistical differences between samples or tested conditions were assessed using either Student's *t*-test (RT-qPCR) or one-way analysis of variance (Ca^{2+} assays). A $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism® 8 software.

3 | RESULTS

3.1 | Effect of nifedipine on ex vivo term-pregnant human myometrium contractility

We first investigated the effect of nifedipine on ex vivo pregnant human myometrium contractility. Twenty-six strips were dissected from myometrial biopsies collected from 13 term-pregnant women. Two types of response to nifedipine were observed: (1) an efficient dose-dependent inhibition of tissue contractility in 16 strips (≈61.5%; Figure 1a), and (2) enhanced contractility at the lowest tested concentrations (10^{-10} and 10^{-9} M) in 10 strips (≈38.5%; Figure 1b), going up to tetany of the tissue in two cases. This effect was not patient-dependent as both responses were observed in different strips dissected from the same biopsy. The pro-contractile effect of nifedipine was essentially observed in tissues with low and/or irregular spontaneous contractions. By contrast, nifedipine inhibited tissue contractility in strips displaying high and regular spontaneous contractions, which demonstrates that it may have a differential effect on myometrial contractility, depending on the contractile capacity of the tissue.

3.2 | In vitro effect of nifedipine on intracellular Ca^{2+} in term-pregnant human myometrial cells

To decipher the mechanisms activated by nifedipine leading to differential effects on human myometrium, we studied the in vitro effect of nifedipine on intracellular Ca^{2+} level in the PHM1-41 cell line. We observed an important dose-dependent increase in intracellular Ca^{2+} level after nifedipine treatment at the highest tested concentrations (10^{-6} to 10^{-4} M) (Figure 2a and 2c). The Ca^{2+} elevation was strongly affected by applying nifedipine in the absence of external Ca^{2+} (Figure 2b,c), showing that nifedipine-induced Ca^{2+} increase was due to Ca^{2+} influx. A similar Ca^{2+} influx was obtained by treating primary myometrial cells purified from term-pregnant human

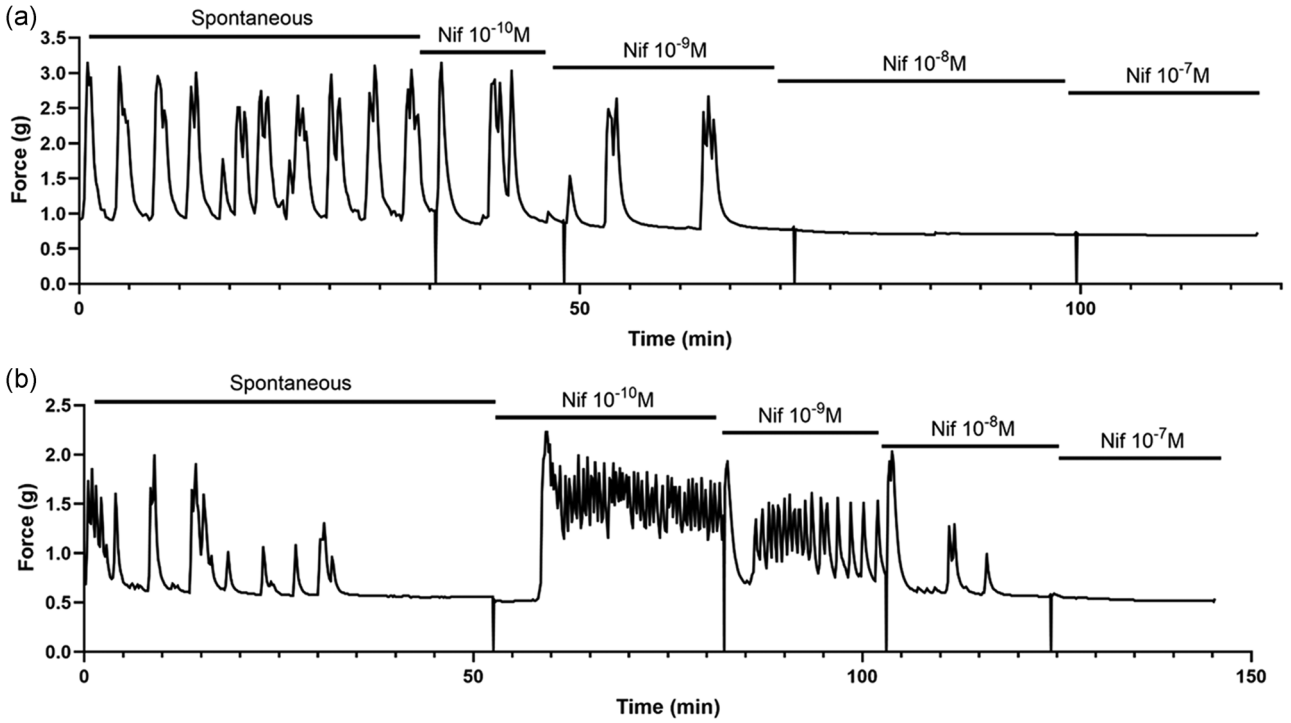

FIGURE 1 Effect of nifedipine on spontaneous myometrial contractions ex vivo. Spontaneously contracting myometrial strips were treated with increasing doses of nifedipine (Nif, from 10^{-10} to 10^{-7} M). Tissue contractions in response to nifedipine treatments differed, depending on the profile of spontaneous contractions. (a) Tissue displaying an inhibitory effect of nifedipine. (b) Tissue displaying a procontractile effect of nifedipine

myometrium with nifedipine (Figure S2) and PHM1-41 cells with nicardipine, another DHP (Figure S3).

Interestingly, the expression levels of $Ca_v1.2$ and $Ca_v1.3$, two genes encoding for the α_1 subunit that constitutes the ion-conducting pore of the L-VGCC, were dramatically decreased in both PHM1-41 and pPM cells, compared with tissue expression levels (Figure 3a). Western blot analysis performed on protein extracts from PHM1-41 cells, pPM cells, and myometrial tissues further confirmed the lowest $Ca_v1.3$ protein levels in both cell types (Figure S4). We thus hypothesized that L-VGCC are absent in these cells. This hypothesis was further reinforced by the absence of Ca^{2+} entry in response to membrane depolarization (70 mM KCl; Figure 3b,c), which would normally activate Ca^{2+} entry in cells expressing L-VGCC (Imanishi et al., 2006; Somlyo & Himpens, 1989).

3.3 | Role of store-operated Ca^{2+} channels in nifedipine-induced Ca^{2+} entry

Considering that the PHM1-41 cells do not express L-VGCC, the target of nifedipine, thus rendering them insensitive to membrane depolarization, we hypothesized that Ca^{2+} entry induced by nifedipine was due to the activation of voltage-independent channels and we investigated the role of SOCE channels. SOCE is an ubiquitous mechanism allowing Ca^{2+} entry following store depletion. The classical plasma membrane channels involved in SOCE belong to the Orai family of Ca^{2+} channels, but other nonselective cation channels were also shown to participate in SOCE (Prakriya & Lewis, 2015). Channels from the Orai and the TRPC families are known to play important roles in Ca^{2+} flux in the myometrium (Chung et al., 2010; Murtazina et al., 2011; Sanborn, 2000). In addition, a recent study identified that Orai channels could be activated by several types of VGCC blockers, including the DHP, and induced Ca^{2+} entry in vascular smooth muscle cells (Johnson et al., 2020). Here, we confirmed that both PHM1-41 and pPM cells express Orai1, 2, and 3 and, to a lesser extent, TRPC1, 4, and 6. The expression of TRPC3, 5, and 7 was very weak in these cells (Figure 4a). We thus investigated the implication of these SOCE channels by using different inhibitors: GSK7975A (Orai1 and 3), BTP2 (Orai channels), and 2-APB (Orai1 and 2, and TRPC family). 2-APB significantly reduced the amplitude of Ca^{2+} entry induced by nifedipine, while GSK7975A and BTP2 did not affect Ca^{2+} entry (Figure 4b,c), suggesting that nifedipine-induced Ca^{2+} entry in the PHM1-41 cells may be due to the activation of the TRPC channels, rather than the Orai channels. This was further confirmed by the knock-down of the genes encoding for Orai1, 2, and 3, and TRPC1, 4, and 6 using siRNA. The 57.8% downregulation of TRPC1 mRNA expression was sufficient to result in a significant reduction (36%) of Ca^{2+} entry in response to nifedipine. This response remained unaffected following the downregulation of Orai1, 2, and 3, and TRPC4 and 6 (Figure 5). The downregulation of TRPC1 expression in PHM1-41 cells also significantly reduced the response to

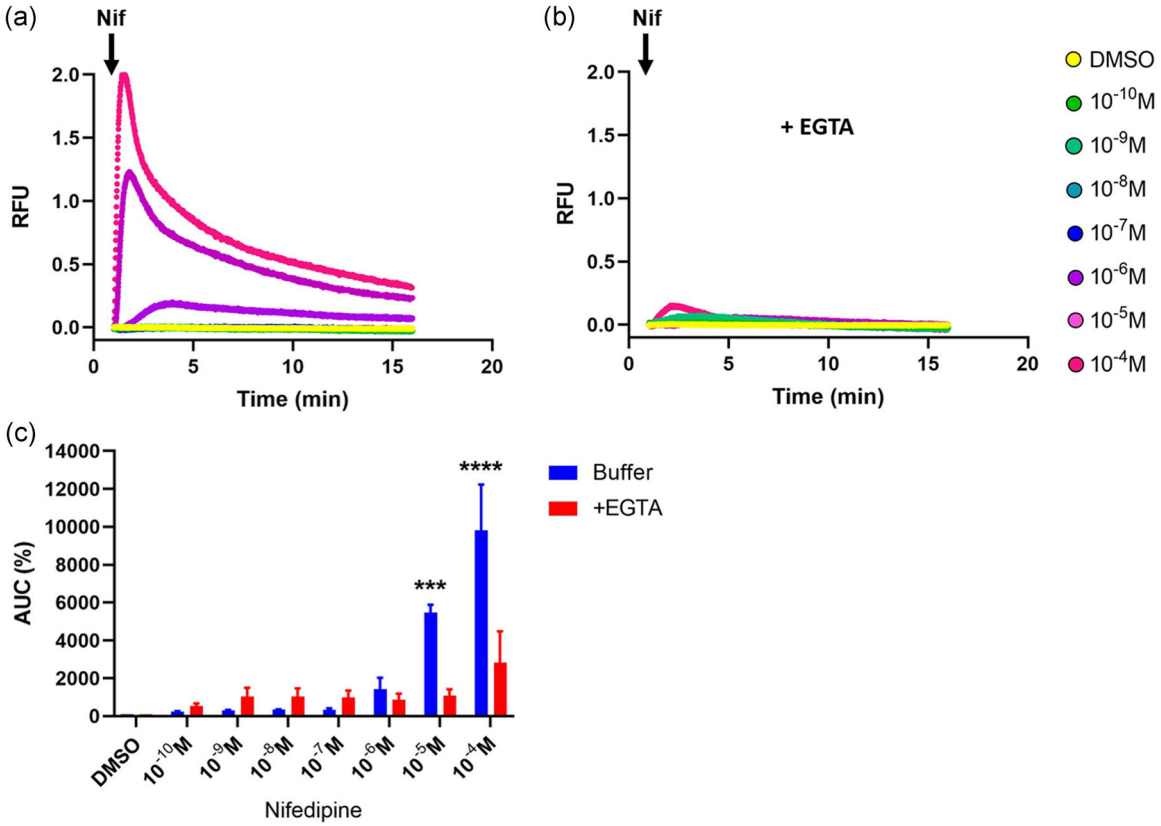

FIGURE 2 Evolution of intracellular Ca^{2+} in PHM1-41 cells in response to nifedipine. Cells were treated with different doses of nifedipine (10^{-10} to 10^{-4} M) or vehicle (DMSO) for 15 min. Nifedipine was added at 1 min (arrows). The intrinsic effect of nifedipine on Ca^{2+} entry was assessed by treating the cells with different doses of nifedipine alone (a) or combined with 10 mM EGTA (b). Data are expressed as relative fluorescent units (RFU). (c) Area under the curves (AUC) calculated from (a) and (b) between 1 and 16 min. Data are expressed as a percentage of the AUC calculated from DMSO-treated cells ±*SEM* ($n = 3$ independent experiments). ***$p < 0.005$, ****$p < 0.001$. DMSO, dimethyl sulfoxide

nicardipine (Figure S5), suggesting that the activation of TRPC1 could be due to a DHP property.

3.4 | Implication of L-VGCC and TRPC1 channel in ex vivo nifedipine-induced term pregnant human myometrium contractility

Given the results obtained in vitro, we then hypothesized that the differential effect of nifedipine on the ex vivo myometrial contractility could be due to differential expression levels for Ca^{2+} channels, and more specifically for L-VGCC and TRPC1. To test this hypothesis, 14 strips were incubated in the organ bath, treated with nifedipine at 10^{-10} and 10^{-9} M successively, and the tissue contractility in response to nifedipine was analyzed. Among the 14 strips, 8 of them had inhibited contractions, and the 6 other strips had enhanced contractions in response to nifedipine. The measurement of $Ca_v1.2$, $Ca_v1.3$, and TRPC1 genes expression levels by RT-qPCR showed a significantly lower $Ca_v1.2$ expression level in the strips displaying a pro-contractile effect of nifedipine (Figure 6a). However, the expression levels of $Ca_v1.3$ and TRPC1 were not statistically different between the two types of strips. This result tends to confirm that the effect of nifedipine on term pregnant human myometrium contractility is related to L-VGCC expression.

Then, to decipher if the pro-contractile effect induced ex vivo by nifedipine was due to the activation of TRPC1 observed in vitro, we tested the effect of the TRPC channel antagonist 2-APB on spontaneous contractions (Figure 6d–f) and on tissues pretreated with 10^{-9} M nifedipine (Figure 6g–i), independently of the profile of spontaneous contractions. 2-APB is known to be a multiple-target pharmacological agent, acting as a potential inhibitor of IP_3 receptor and Ca^{2+} entry (Bootman et al., 2002), with also opposite effects on SOCE, depending on its concentration (Zhang et al., 2020). In this study, we used this compound at 75 µM, based on our own in vitro (Figure 4b,c) data and data from the literature (DeHaven et al., 2008; Goto et al., 2010). Its ex vivo efficiency was further confirmed with a dose-response experiment performed on myometrial tissue (data not shown). The objective of the pretreatment with nifedipine was to observe the response pattern to nifedipine (inhibitory and procontractile effects) before adding the 2-APB. The group of nifedipine-treated strips was made of strips displaying both response patterns in equivalent proportions, as shown by the individual values in Figure 6g–i. In line with the inhibitory effect of 2-APB on spontaneous myometrial contractions independently on voltage-gated

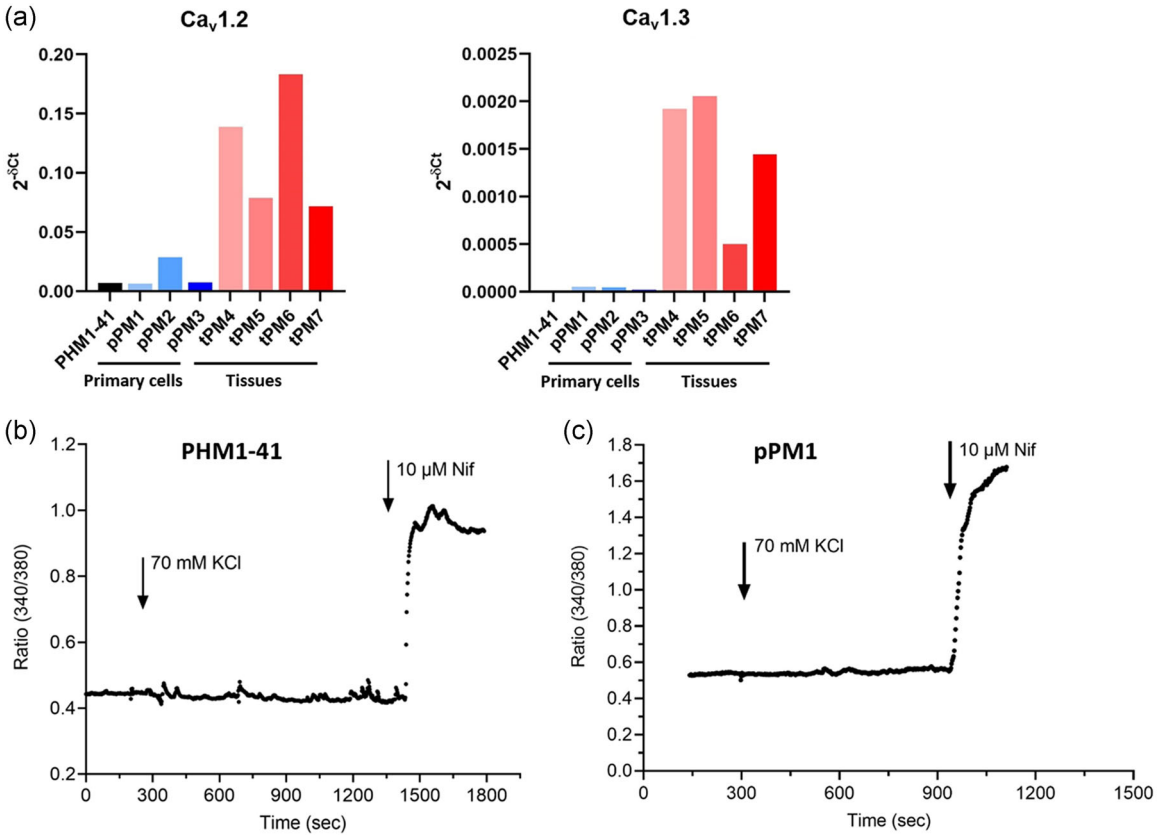

FIGURE 3 L-type voltage-gated Ca^{2+} channel activation and expression in human myometrial cells. (a) Quantification by RT-qPCR of the expression level of $Ca_v1.2$ and $Ca_v1.3$ in PHM1-41 cell lines, primary human myometrial cells pPM1, pPM2, and pPM3 at passage 4, and term-pregnant human myometrial tissues tPM4, tPM5, tPM6, and tPM7. (b, c) Cytosolic Ca^{2+} measurements on PHM1-41 cells (b) and primary myometrial cells pPM1 (c) treated with high K^+ solution (70 mM KCl) and nifedipine (10 µM Nif)

calcium channels (Ascher-Landsberg et al., 1999), treatment with 2-APB of spontaneously contracting strips showed an inhibitory effect on tissue contractility, even if not significant (Figure 6d–f). However, the addition of 2-APB on nifedipine-treated strips significantly reduced the mean force developed by the tissues (Figure 6g) and the frequency of contractions (Figure 6i) compared to the basal contractility and nifedipine alone, but did not affect the maximal amplitude of the contractions (Figure 6h). These results tend to confirm the implication of the TRPC channels in nifedipine-induced contractions in myometrial tissue. Furthermore, these results support that the combination of drugs targeting both L-VGCC and TRPC channels, overall improve the treatment efficacy for the inhibition of myometrial contractions, whatever to profile of spontaneous contractions.

4 | DISCUSSION

We report the capacity of nifedipine to induce ex vivo contractions in term-pregnant myometrial tissue. To our knowledge, this procontractile effect has not been described previously and is in clear contradiction with the use of nifedipine as a tocolytic for the prevention of uterine contractions in pregnant women with a threat of preterm delivery. Previous studies using this experimental model showed an inhibitory effect of nifedipine on myometrial contractions, either on oxytocin-induced contractions (Carvajal et al., 2017; Moynihan et al., 2008) or with high doses of nifedipine (10^{-6} to 10^{-5} M) (Noble et al., 2014). However, measurement of plasma nifedipine concentrations 4 h after starting a tocolytic treatment is approximately 10^{-7} M (Papatsonis et al., 2007), which very probably correlates with a nifedipine concentration below 10^{-7} M in myometrial tissue. Based on this observation, we showed that concentrations above 10^{-8} M efficiently inhibit myometrial contractility, but the lowest concentrations tested (10^{-10} and 10^{-9} M) had a differential effect on tissue contractility. Moreover, we demonstrated that in vitro nifedipine can activate the TRPC channels, especially TRPC1, leading to Ca^{2+} entry into the myometrial cells, even though this effect was observed only at high drug concentrations (>10^{-6} M). The in vitro activation of TRPC1 seems to be a common property of DHP as the same response was observed with nicardipine, another DHP.

The ex vivo procontractile effect of nifedipine was observed in tissues with low and/or irregular spontaneous contractions, suggesting that these tissues had low L-VGCC expression or activation.

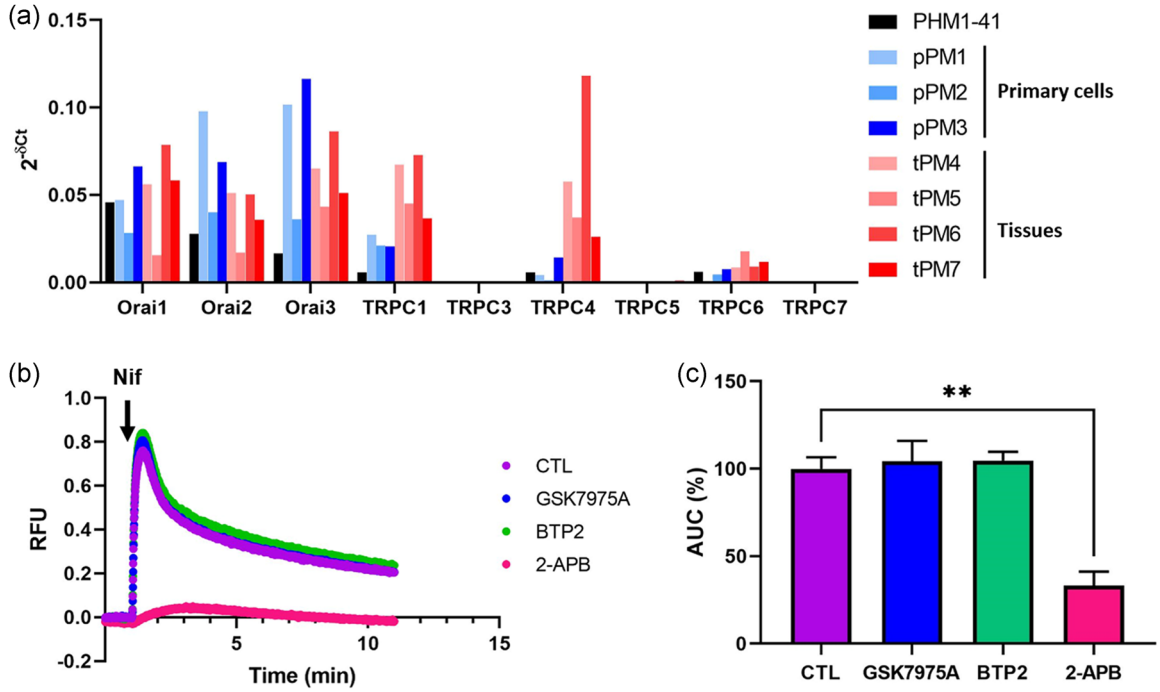

FIGURE 4 Involvement of channels from the Orai and TRPC families in the nifedipine-induced Ca^{2+} entry. (a) Quantification by RT-qPCR of the expression level of Orai1, 2, and 3, and TRPC1, 3, 4, 5, 6, and 7 in PHM1-41 cells, primary human myometrial cells pPM1, pPM2, and pPM3 at passage 4, and term-pregnant human myometrial tissues tPM4, tPM5, tPM6, and tPM7. (b) Evolution of intracellular Ca^{2+} in PHM1-41 cells in response to 10^{-4} M nifedipine (arrow). Cells were pretreated with the channel inhibitors GSK7975A, BTP2, and 2-APB, or untreated (CTL) for 15 min before the addition of nifedipine in all cells. Data are expressed as relative fluorescent units (RFU). (c) Area under the curve (AUC) calculated from (b). Data are expressed as a percentage of the AUC calculated from CTL cells ±*SEM* ($n = 3$ independent experiments). $^{**}p < 0.01$. AUC, area under the curve; TRPC, transient receptor potential-canonical

This hypothesis was supported by the decreased $Ca_v1.2$ mRNA level measured in tissues with low and/or irregular spontaneous contractions associated with a procontractile effect of nifedipine compared to tissues with regular spontaneous contractions. However, we failed to observe any significant difference in $Ca_v1.3$ and TRPC1 mRNA levels, and deeper investigations for the characterization of ion channels in both types of strips, with a larger strip number, would probably highlight differential expression for other ion channels. In vivo, myometrial L-VGCC expression is known to increase with pregnancy progression (Mershon et al., 1994; Ohkubo et al., 2005) and is associated with increased myometrial tissue contractility (Sanborn, 1995; Vrachnis et al., 2011). However, in our study, the differential response to nifedipine cannot be explained by gestational age-dependent contractility as all biopsies were collected from term-pregnant women and both relaxing and procontractile effects were observed in strips from the same biopsies. This tissue disparity in nifedipine sensitivity may probably come from a heterogeneous organization of the muscle fibers in not well-defined circular and longitudinal layers (Young, 2007). This hypothesis is supported by a differential expression of L-VGCC between longitudinal and circular muscle fibers described in pregnant rat myometrium (Ohkubo et al., 2005). Further molecular investigation of the different L-VGCC subunits, as well as a histological analysis of myometrial strips displaying the two response patterns to nifedipine would provide helpful information on tissue characteristics leading to either an inhibitory or a procontractile effect of nifedipine on myometrial contractility.

Interestingly, the in vitro effect of nifedipine that led to Ca^{2+} entry was observed in myometrial cells with no functional L-VGCC and thus insensitive to membrane depolarization. The two experimental models used in this study (PHM1-41 cell line and primary myometrial cells) were purified from term-pregnant myometrial tissues that may have the potential to express active L-VGCC. However, gene expression analysis and the measurement of intracellular Ca^{2+} entry in response to high-K^+ concentrations performed on both models demonstrate that these cells have lost the expression of L-VGCC, probably due to the transition from a contractile to a proliferative phenotype (Murtazina et al., 2011; Sanborn, 1995). Similar Ca^{2+} entries were reported in 2019 by Liu et al. (2019) in glioblastoma cells when treated with amlodipine, a third-generation DHP, and more recently by Johnson et al. (2020) in vascular smooth muscle cells in response to the three major classes of L-VGCC blockers used as anti-hypertensive drugs. Interestingly, in vascular smooth muscle cells, this effect seemed to be associated with a reduced expression of L-VGCC. These results suggest that nifedipine can

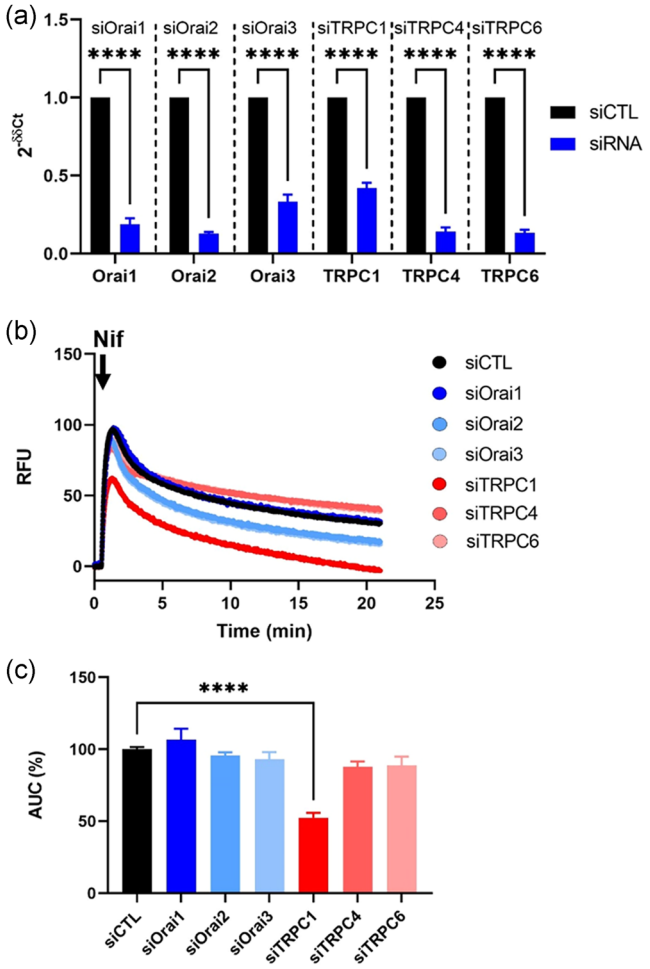

FIGURE 5 Role of SOCE channels in nifedipine-induced Ca^{2+} entry and myometrial contractility. (a–c) PHM1-41 cells were transfected with siRNA against Orai1, 2, and 3, TRPC1, TRPC4, and TRPC6 channels, or siCTL for 48 h. (a) The knockdown efficacies were verified by RT-qPCR. Gene expression levels are shown as relative expression to siCTL ± *SEM*. (b, c) Cells were treated with nifedipine 10^{-4} M 48 h after transfection and the intracellular Ca^{2+} level was monitored for 20 min. (b) Time-course of the evolution of intracellular Ca^{2+}. Nifedipine was added at 1 min (arrows). Data are expressed as relative fluorescent units (RFU). (c) Area under the curves (AUC) calculated from (b) between 1 and 21 min. Data are expressed as a percentage of the AUC calculated from siCTL cells ±*SEM* ($n = 3$ independent experiments). siRNA, small interfering RNA; SOCE, store-operated Ca^{2+} entry; TRPC, transient receptor potential-canonical

have a procontractile effect in myometrial tissue with low contractile capacity.

Using double and triple knock-out strategies for the SOCE channels Orai1, 2, and 3, and their partners STIM1 and STIM2 in the HEK293 cell line, Johnson et al. (2020) identified that this effect was mainly due to the indirect activation of Orai1 by STIM1. STIM2, as well as Orai2 and 3, seemed to be also involved, but to a lesser extent. We thus investigated the role of the Orai channels by first using pharmacological inhibitors. However, Orai channels are not the only Ca^{2+} entry channels expressed in human myometrial cells. RT-qPCR analysis conducted on our two models showed that several TRPC channels are also expressed (TRPC1, 4, and 6). Therefore, we also tested the effect of TRPC inhibitors. Of note, nifedipine-induced Ca^{2+} entry into the PHM1-41 cells was affected only by the pharmacological inhibition of the TRPC channels, using 2-APB. Nevertheless, this compound is known to target several channels and effectors of the Ca^{2+} signaling pathway with a differential effect, depending on the concentration (Bogeski et al., 2010). Indeed, we confirmed this result using siRNA against the three Orai channels and TRPC1, 4, and 6 channels, which are the most expressed TRPC channels in human myometrial cells. Despite an efficient downregulation of the genes encoding for all tested channels, the nifedipine-induced Ca^{2+} entry was significantly reduced only in cells with a downregulated expression of TRPC1. Thus, the rapid increase in intracellular Ca^{2+} observed in PHM1-41 cells in response to nifedipine primarily supports a direct action of the drug on TRPC1. However, TRPC1 overexpression in PHM1-41 cells did not significantly affect the nifedipine-induced Ca^{2+} entry (data not shown). Nicardipine seems to involve, at least partially, the same mechanism as TRPC1 downregulation also resulted in a significant reduction in nicardipine-induced Ca^{2+} entry, despite a slower kinetic of response than with nifedipine that might correspond to an indirect activation of the channels. Murtazina et al. (2011) also reported that TRPC1 plays an important role in the regulation of myometrial Ca^{2+} dynamics by allowing Ca^{2+} entry, following oxytocin-induced Ca^{2+} release from intracellular stores.

Finally, the treatment of myometrial strips with either 2-APB alone or in addition to nifedipine suggests that the procontractile effect induced by nifedipine ex vivo on myometrial tissues may involve the activation of TRPC channels. Indeed, the 2-APB inhibitory effect seems to be maintained even in tissues displaying reduced contractility in response to low concentrations of nifedipine. This suggests that the blockade of TRPC channels does not affect the inhibitory effect of nifedipine on L-VGCC, but efficiently reduces its activator effect on these channels. However, these findings have to be confirmed and specified by using more specific TRPC1 antagonists. If the causality between the ex vivo and in vitro nifedipine effects is confirmed, our results could explain the short-lasting efficacy of this drug when used as a tocolytic and its inefficacy when used as maintenance tocolysis.

In conclusion, we showed here that nifedipine, which normally inhibits myometrial contractions by blocking L-VGCC-mediated Ca^{2+} entry, can paradoxically enhance contractions. This procontractile effect seems to involve the activation of the nonselective cation channel TRPC1. If this mechanism is confirmed in human myometrial tissue, this finding could further lead to the optimization of nifedipine-based tocolytic therapies by combining nifedipine with a TRPC1 inhibitor.

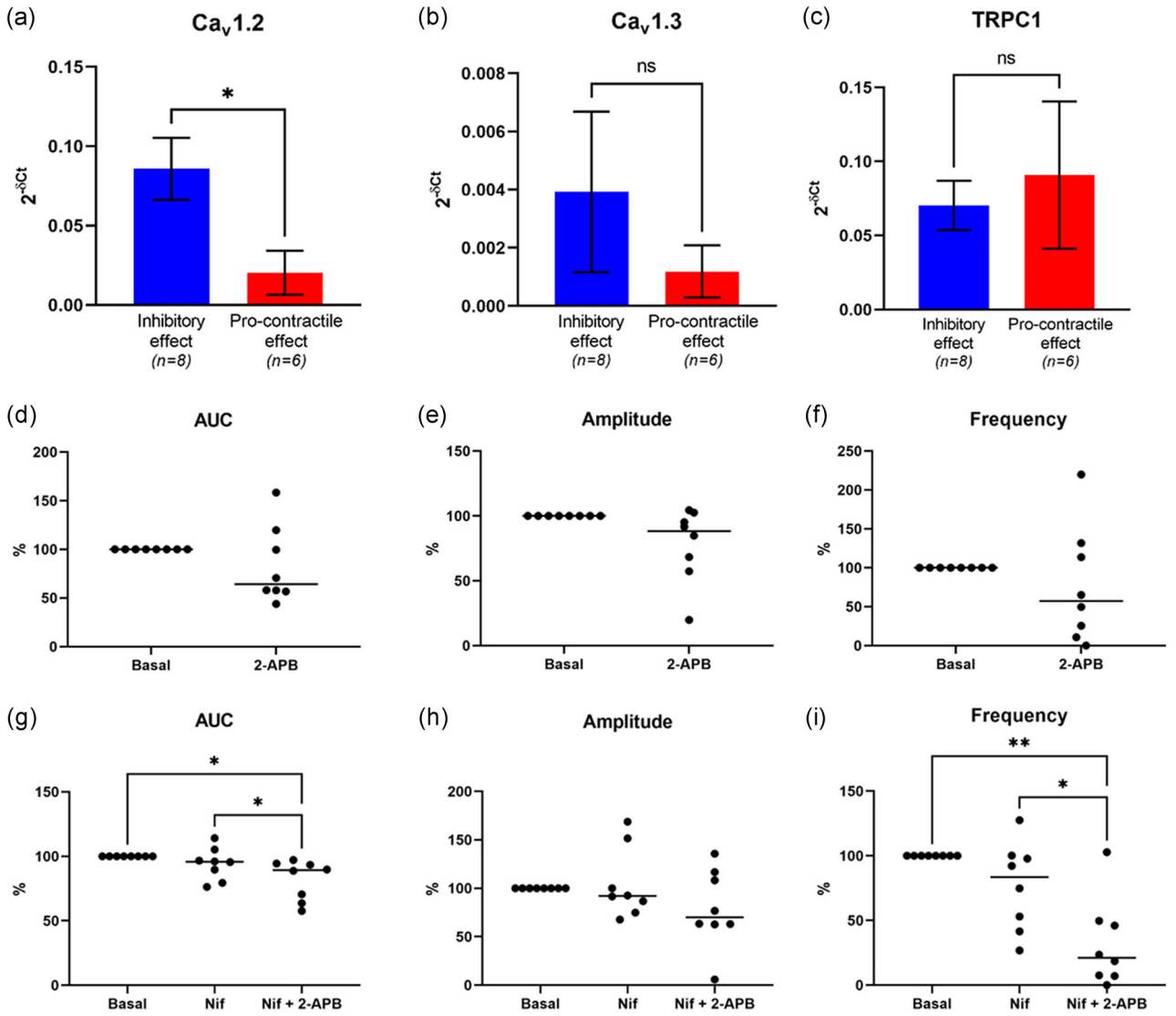

FIGURE 6 Implication of L-VGCC and TRPC1 in the nifedipine effect on ex vivo myometrial contractility. (a–c) Quantification of L-VGCC and TRPC1 genes expression in term pregnant human myometrial strips. $Ca_v1.2$ (a), $Ca_v1.3$ (b), and TRPC1 (c) gene expression levels were quantified by RT-qPCR in myometrial strips displaying either an inhibition (Inhibitory effect, $n = 8$ strips collected from 3 different patients) or an activation (procontractile effect, $n = 6$ strips collected from 3 different patients) of spontaneous contractions in response to nifedipine at 10^{-10} and 10^{-9} M, ex vivo. Data are expressed relative to the mean Ct of 3 reference genes ±*SEM*. (d–i) Spontaneously contracting myometrial strips were treated with either 75 µM 2-APB (d–f, $n = 8$ strips) or successive additional treatments of 10^{-9} M nifedipine (Nif), and 75 µM 2-APB (g–i, $n = 8$). The AUC, (d) and (g), the maximal amplitude of the contractions (e, h), and the frequency of contractions (f, i) were calculated for each treatment. Data are expressed as mean and individual percentages relative to the spontaneous contraction periods (Basal). $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.001$, ns: nonsignificant. AUC, area under the curve; L-VGCC, L-type voltage-gated Ca^{2+} channel; TRPC, transient receptor potential-canonical

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Lucile Yart, Marie Cohen, and Begoña Martinez de Tejada: conceived the study. **Lucile Yart, Marie Cohen, Maud Frieden, and Stéphane Konig:** designed the experiments. **Lucile Yart:** conducted all the experiments except Ca^{2+} imaging and analyzed the data. **Maud Frieden:** supervised Ca^{2+} imaging experiments. **Lucile Yart:** wrote the manuscript. All the authors gave input on writing the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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