Remifentanil induces L-type Ca^{2+} channel inhibition in human mesenteric arterial smooth muscle cells

[Le rémifentanil provoque l'inhibition des canaux calciques de type L dans les cellules musculaires lisses des artères mésentériques chez l'humain]

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Purpose: Remifentanil is known to cause vasodilation at standard anesthetic concentrations. The intracellular mechanisms underlying its vasodilator action may involve the activation of ion channels. The purpose of this study was to examine whether remifentanil inhibits L-type calcium channels ($Ca._L$) and provides dose-dependent effects on L-type calcium channel Ba^{2+} currents ($I_{Ba.L}$) in human mesenteric arterial smooth muscle cells.

Methods: Using the whole-cell patch-clamp method, an in-depth analysis of the mechanism of the $I_{Ba.L}$ induced by remifentanil was performed in cells which were enzymatically isolated from human mesenteric arterial smooth muscle. Ten millimolars Ba^{2+} was used to replace 1.5 mM Ca^{2+} to increase the amplitude of the inward current through Ca^{2+}channels. L-type calcium channel Ba^{2+} was elicited during 50 msec depolarizing test pulses (150 msec duration) to +80 mV (10 mV increments) from a holding potential of -60 mV. The effects of remifentanil on $Ca._L$ were observed at the following concentrations: 1.21, 4.84, and 19.4 nmol·L^{-1} and were compared with control.

Results: Remifentanil produced a concentration-dependent block of $I_{Ba,L}$ with IC_{50} values of $38.90 \pm 3.96 \times 10^{-3}$ μmol·L^{-1}. The L-type calcium channel blocker, nifedipine, antagonized these remifentanil-induced currents. Remifentanil, at all concentrations, shifted the maximum of the current-voltage relationship in the hyperpolarizing direction of $I_{Ba.L}$.

Conclusion: Remifentanil significantly inhibits $Ca._L$ channels in a concentration-dependent manner in human mesenteric arteriolar smooth muscle cells.

CAN J ANESTH 2008 / 55: 4 / pp 238–244

Objectif *: Il est connu que le rémifentanil provoque une vasodilatation à des concentrations anesthésiques standard. Les mécanismes intracellulaires sous-jacents à son action vasodilatatrice pourrait impliquer l'activation des canaux ioniques. L'objectif de cette étude était de déterminer si le rémifentanil inhibe les canaux calciques de type L ($Ca._L$) et procure des effets dose-dépendants sur les courants Ba^{2+} des canaux calciques de type L ($I_{Ba.L}$) dans les cellules musculaires lisses des artères mésentériques humaines.*

Méthode *: À l'aide de la méthode « patch clamp » en configuration de cellule entière, une analyse approfondie du mécanisme du $I_{Ba.L}$ provoqué par le rémifentanil a été réalisée sur des cellules isolées par action enzymatique du muscle lisse artériel mésentérique humain. Dix millimolaires Ba^{2+} ont été utilisés pour remplacer 1,5 mM Ca^{2+} afin d'augmenter l'amplitude du courant entrant à travers les canaux calciques. Le canal calcique de type L Ba^{2+} a été sollicité pendant des impulsions dépolarisantes de test de 50 msec (durée de 150 msec) jusqu'à + 80 mV (par paliers de 10 mV) à partir d'un potentiel de –60 mV. Les effets du rémifentanil sur le $Ca._L$ ont été observés aux concentrations suivantes : 1,21, 4,84, et 19,4 nmol·L^{-1} et ont été comparés aux données du groupe témoin.*

Résultats *: Le rémifentanil a produit un bloc de $I_{Ba,L}$ dépendant de la concentration avec des valeurs CI_{50} de $38,90 \pm 3,96 \times 10^{-3}$ µmol·L^{-1}. La nifédipine, un bloqueur des canaux calciques de type L, a opposé ces courants provoqués par le rémifentanil. Le rémifentanil, à toutes les concentrations évaluées, a déplacé le maximum de la relation courant-voltage dans la direction hyperpolarisante de $I_{Ba.L}$.*

Conclusion *: Le rémifentanil inhibe de manière significative les canaux calciques de type L ($Ca._L$) de façon dose-dépendante dans les cellules musculaires lisses de l'artère mésentérique humaine.*

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This study was supported by grant No. 2005CB522601 from the 973 Program, Beijing, China, and grant No. 30271259 from the National Research Foundation of Nature Sciences. Beijing, China.

Accepted for publication November 6, 2007.

Revision accepted January 9, 2008.

REMIFENTANIL, an ultra-short-acting μ-opioid receptor agonist, is unique from other opioids as a result of its esterase-based metabolism, minimal accumulation, and very rapid onset and offset of clinical action. Although widely used in a wide variety of balanced anesthetic techniques, results from experimental studies, in both animals and humans, reveal that remifentanil provokes a dose-dependent decrease in mean arterial pressure and systemic vascular resistance.[1–6] This cardiovascular response may result from inhibition of the central nervous system or stimulation of the centrally-mediated vagal tone,[7] as well as direct relaxation of blood vessels.[8–10] Although few studies have specifically examined the intrinsic mechanism of remifentanil-induced cardiovascular depression, sporadic laboratory investigations have demonstrated the involvement of ion channels. A study in rats by Unlugenc *et al.*[9] showed that remifentanil vasodilates smooth muscle via the suppression of voltage-sensitive Ca^{2+} channels. Thus, it is logical to hypothesize that, in human smooth muscle cells, remifentanil-induced vasodilatation is associated with alterations of Ca^{2+} channel currents. L-type Ca^{2+} channels ($Ca._L$), which are dominant ion conductive pathways in vascular muscle cells, have been shown to play a critical role in the generation and in the regulation of vascular tone.[10] In human mesenteric arteriolar smooth muscle cells (HMASMCs), the potential mechanisms underlying the vasodilatory effects of remifentanil and the contributions of $Ca._L$ channels have not been elucidated. Therefore, we undertook a study to further define the electrophysiological influence of remifentanil on $Ca._L$ channels using the conventional patch-clamp technique in freshly isolated HMASMCs. We specifically sought to investigate the direct effects and the concentration-response relations of remifentanil on the amplitude, current-voltage relationship curve of $Ca._L$ channels.

Methods

Arterial smooth muscle cell isolation

The West China Hospital of Sichuan University Medical Ethics Committee approved the use of discarded tissue segments, and patients provided consent for the use of their discarded tissues for research. Experiments were performed on single smooth muscle cells isolated from human mesenteric arteries. Arterial segments (outer diameter < 1.0 mm,1–3 cm in length) were immediately dissected from the fat and tissues adhering to mesentery during the course of abdominal operations (usually hemicolectomies or gastrectomies) on patients of both sexes, aged from 20–60 yr, who underwent elective surgery at West China Hospital, Sichuan University (Chengdu). The patients recruited in our study were all free of calcium-channel blocking drugs and had no hypertension, diabetes or other vascular diseases. The vessel segments were isolated and stored in physiological saline solution at 4°C until the experiment began.

The procedure of cell isolation is a modification of the technique previously described by Smirnov and Aaronson.[11] Briefly, dissected arteries were cut into small pieces (3 mm × 2 mm) and were placed into a calcium free, physiological salt solution (PSS) containing 0.13% papain, 0.17% bovine serum albumin (BSA), and 1 mM dithiothreitol. Incubated at 37° C for 12–15 min, with a gas mixture of $95\%O_2$–$5\%CO_2$, pieces of artery were then incubated in another calcium free PSS, containing 0.13% collagenase (type XI), 0.11% hyaluronidase (type I-S), and 0.17% BSA (all from Sigma Chemical Co, St. Louis, MO, USA) at 37°C for four to six minutes ($95\%O_2$–$5\%CO_2$). Arteries were then placed in non-enzyme, calcium-free PSS and were gently triturated to single smooth muscle cells by a Pasteur pipette. The cell suspension was stored in calcium-free PSS at 4°C for use within four hours.

Solutions and reagents

As suggested by Smirnov,[12] the replacement of 1.5 mM Ca^{2+} with 10 mM Ba^{2+} was used to augment the amplitude of the inward current through Ca^{2+} channels; the potential dependency of activation and inactivation were very similar to the results observed in 1.5 mM Ca^{2+}. To isolate the Ba^{2+} current (I_{Ba}), the following reagents were used for the external solution (mM): 130 NaCl, 1 cesium chloride (CsCl), 1.2 $MgCl_2$, 10 $BaCl_2$, 10 glucose, 4 tetraethylammonium(TEA) chloride, and 10 N-2-hydroxyethylpiperazine-N'- ethanesulphonic acid (HEPES). The pipette solution contained (mM): 135 CsCl, 2.5 $MgCl_2$, 2 Na_2ATP, 10 HEPES, and 10 ethyleneglycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), and the pH of the solution was adjusted to 7.2 with NaOH. CsCl and TEA were used to eliminate outward K^+ currents.[12]

Nifedipine (NF) was diluted in 100% dimethylsulfoxide (DMSO) for stock. The final DMSO concentration in the working solutions was 0.3% and was shown, through control experiments, to have no effect on membrane current.

Drugs

Basic chemicals were purchased from Shanghai Biochemistry Reagent Co. (China). HEPES, EGTA, CsCl, TEA, Na_2ATP, and nifedipine were obtained

from Sigma Chemical Co., St. Louis, MO, USA). Remifentanil (standard substance without glycin), kindly donated by YiChang Humanwell Pharmaceutical Co., Ltd., YiChang, Hubei, China, (Batch No: 040801), was dissolved in distilled water and then stored at -20°C until used. On the day of an experiment, an appropriate amount of this solution was thawed and diluted in the external solution to yield a final concentration.

Patch-clamp recording

The recording of whole cell $I_{Ba,L}$ was conducted at room temperature (20–24°C) using the patch-clamp technique. A drop of cell suspension was transferred into a Plexiglas chamber mounted on the stage of an inverted fluorescence microscope (Olympus IX 70, Tokyo, Japan). The suspension was then superfused with external solution. Cells were then allowed to settle to the bottom of the dishes. The selected cells were spindle-shaped and showed distinct borders and striations. Micropipettes, whose tip inside diameter were about 2 μm, were made using a micropipette puller (Flaming/brown P-97, Sutter instrument Co., Novato, CA, USA); and then the tip of the micropipette was heat-polished (micro forge MF-900, Marishige Co., Ltd, Tokyo, Japan). Electrode resistances ranged from 2–4 mΩ.

After the whole-cell configuration had been obtained, current signals were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Leak subtraction was performed using a P/6 protocol in pClamp, as described by Wilde *et al.*[13] Data acquisition and analysis were made by pClamp software package (version 8.1, Axon Instruments, Foster City, CA, USA). Additional analysis was conducted using ORIGIN version 5.0 software (Microcal Software Inc., Northampton, MA, USA).

Ba^{2+} currents through L-type Ca^{2+} channels ($I_{Ba.L}$) were recorded in 10 mM Ba^{2+}-containing external solution. $I_{Ba.L}$ was measured over a range of test potentials from -70 to +80 mV, during 150 msec from a holding potential of -60 mV. In our study, after a stable response had been attained, current antagonist was added to the solution to confirm the current attributes. Current–voltage (I/V) relationships were recorded before and after drug application.

Data analysis

Obtained whole cell configuration, $I_{Ba,L}$ were initially recorded for 15 min, to monitor time-dependent changes in peak current amplitude. Those cells which showed a significant change in current amplitude, because of "run down" phenomena (i.e., > 5%) during the stabilization period of 15–20 min, or which failed to show at least 80% recovery during washout, were not included in the data analysis.

Peak current amplitudes of $I_{Ba,L}$ were measured at each membrane potential and were converted to current density, which was calculated as the ratio of the current amplitude to the cell membrane capacitance, with the assumption of a specific membrane capacitance of 1 $\mu F/cm^2$. The cell membrane capacitance was determined from the area under the capacitive transients elicited by a 10-mV hyperpolarizing pulse of 5-msec duration.

Changes in current amplitude of peak $I_{Ba,L}$ were reported as percentage compared with control during depolarizing steps. Peak current amplitude and current-voltage (I-V) relationship were initially recorded in remifentanil free external solution (control) and then at the following concentrations of remifentanil; 1.21 $nmol \cdot L^{-1}$, 4.84 $nmol \cdot L^{-1}$, and 19.4 $nmol \cdot L^{-1}$. In order to demonstrate the extension of the concentration-response relations, we added extra two groups, including 77.4 $nmol \cdot L^{-1}$ and 310 $nmol \cdot L^{-1}$ according to geometric proportions.

The concentration-response curves, used to examine the relative sensitivity of $I_{Ba,L}$ during drug exposure, were generated by plotting the maximum reduction in peak current amplitude of $I_{Ba,L,}$ in the presence of five concentrations of remifentanil compared with control. Concentration-response curves were obtained by nonlinear regression to a sigmoidal equation:

$$B/M = 1/[1+(EC_{50}/[A])^{nH}] \qquad (1)$$

where B is a block by remifentanil, M is the maximum block by remifentanil, [A] is the concentration of remifentanil, EC_{50} is the concentration of half-maximal effect, and nH is the Hill coefficient. Steady-state activation for I_{Ba} was obtained by calculating conductance from:

$$g = I_{Ba,L}/(V\text{-}E_{rev}) \qquad (2)$$

where g is membrane conductance, I_{Ba} is the current amplitude, V is the test potential, and E_{rev} is the reversal potential for I_{Ba}, which was approximately 50-60mV during control conditions. The conductance was normalized to maximum conductance, g_{max}. Steady-state activation was fitted to a Boltzmann equation of the form:

$$g/g_{max} = 1/\{1+\exp[(V\text{-}V_{1/2})/k]\} \qquad (3)$$

where V is the membrane potential (for activation), $V_{1/2}$ is the voltage at which activation is half maximal, and k is the slope factor.

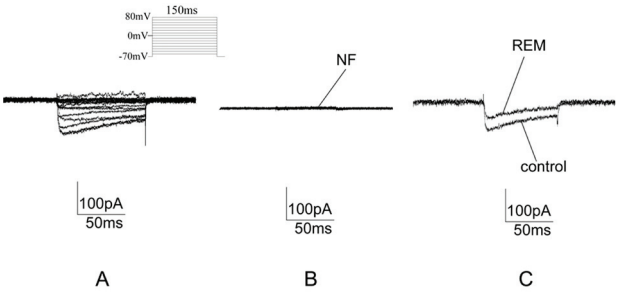

FIGURE 1 L-type Ca^{2+} channel Ba^{2+}currents ($I_{Ba,L}$) in isolated human mesenteric arterial smooth muscle cells. A) Original recordings of conventional whole-cell $I_{Ba,L}$ in human mesenteric artery vascular smooth muscle cells. Vertical bars were 100 pA, membrane potential was held at -60 mV, and the membrane potential was increased in 20 mV increments from a potential of -70 to +80 mV during 150 msec. B) Nifedipine (NF) inhibited $I_{Ba,L}$ in smooth muscle cell membranes at maximal activation membrane potentials. Superimposed current recorded before (control) and after the administration of 5 μmol·L^{-1} NF. C) The amplitude of the $I_{Ba,L,}$ recorded during a typical depolarization in the presence of 19.4 nmol·L^{-1} remifentanil (REM), compared with control at maximal activating potential (0–20 mV).

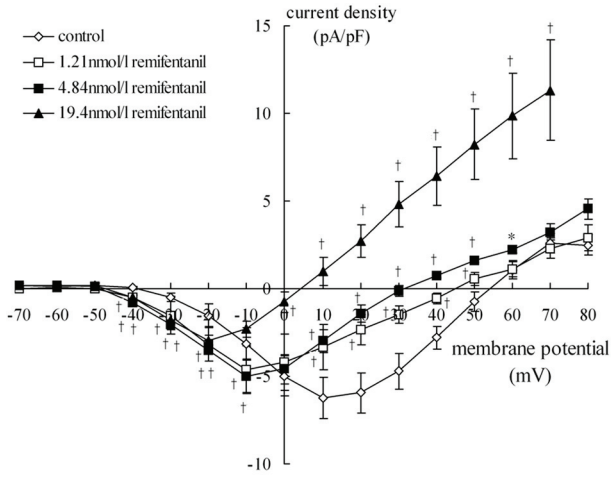

FIGURE 2 Current-voltage (I-V) relations for L-type Ca^{2+} channel Ba^{2+}currents in membrane patches obtained from smooth muscle cells of human mesenteric arteries. I-V curve is shown in the absence (control) and presence of three concentrations of remifentanil, during 150 msec depolarizations recorded from -70 to +80 mV with a holding potential -60 mV. Traces are superimposed. Data points are mean ± SEM. Compared with control, *$P < 0.05$ and †$P < 0.01$ were considered significant.

Statistical analysis

Acquisition and analysis of data were accomplished by using Clampex 8.0 and Clampfit 8.0 software (Axon Instruments, Foster City, CA, USA), and data were analyzed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA) software. Dependent samples were compared using a paired *t* test within groups and one-way analysis of variance between groups. If variances were equal, least-significant-difference *t* test was examined; otherwise, the Tamhane's T2 test was applied. Data are presented as mean ± SEM. Throughout the text and figures, number represents the number of cells tested. All statistical comparisons were performed at the 95% confidence level. A value of $P < 0.05$ was considered significant.

Results

Figure 1 summarizes the characteristics of $I_{Ba.L}$ in patches of HMASMCs membrane. Panel A shows a typical recording of $I_{Ba.L}$, which was a slowly decaying, inward current elicited beyond -40 mV, reaching a maximum at 10 mV (0–20 mV). The clamp pulse of 50 msec extended from -70 mV to +80 mV under control conditions in smooth muscle cells freshly isolated from human mesenteric arteriolar, at an external solution containing 10 mM Ba^{2+}. The response appeared to reverse between +40 mV and +50 mV, and was completely blocked by 5 μmol·L^{-1} L-type Ca^{2+} channel blocker nifedipine (Figure 1, Panel B). A representative whole cell $I_{Ba,L}$ trace is displayed in Figure 1, Panel C. The depression of $I_{Ba,L}$ by remifentanil was reversed after the washout of the anesthetic. ($P = 0.214$, $n = 5–6$).

Remifentanil attenuated $I_{Ba.L}$ in a concentration-dependent manner. At membrane potentials with maximal activation (0–20 mV), remifentanil at concentration of 1.21, 4.84, and 19.4 nmol·L^{-1} decreased the peak amplitude of the Ba^{2+} currents by 19.87 ± 4.20%, 30.81 ± 4.86%, and 47.37 ± 6.10% ($P = 0.04$, $P = 0.002$, or $P = 0.001$, $n = 5–6$), respectively. A higher concentration of remifentanil at 19.4 nmol·L^{-1} was associated with greater inhibition of peak currents compared with 1.21 nmol·L^{-1} remifentanil ($P = 0.035$, $n = 5–6$). The corresponding I-V relations, in the absence (control) and presence of remifentanil, were investigated in HMASMCs (Figure 2). Remifentanil shifted the I-V curve of the Ba^{2+} currents to the top left and induced a hyperpolarizing shift of $I_{Ba,L}$ toward negative membrane voltages under 0 mV, and, subsequently, attained a constant level at negative potentials when reaching maximal activation potential.

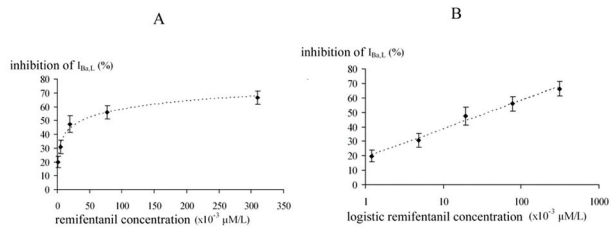

FIGURE 3 Concentration-response curves for L-type Ca^{2+} channel Ba^{2+}currents, in the presence of five concentrations of remifentanil in human mesenteric arteriolar smooth muscle cells at maximal activating potential (0–20 mV) during depolarization. The logistic concentration-response relationships and linear regression lines are displayed in A and B. Data points are mean ± SEM and were normalized to represent the percentage of the maximum reduction in peak current amplitude of $I_{Ba,L}$. Curves were fitted by nonlinear regression to a sigmoidal equation (1).

Remifentanil produced a concentration-dependent block of $I_{Ba,L;}$ and the logistic concentration of remifentanil was linear with its inhibition rate, as seen in Figure 3. The decrease in inward current was associated with increasing concentrations of remifentanil to a peak at 77.4 nmol·L^{-1}, at which point remifentanil inhibited channel currents in a steady state.

Discussion

This study provides convincing evidence for remifentanil-induced L-type Ca^{2+} channel inhibition in HMASMCs. Human mesenteric arterioles (diameter < 1 mm) are resistance vessels, whose smooth muscle tension is a critical determinant of vascular tone, correlating directly with systemic blood pressure. In our study, we isolated cells from human mesenteric arteriolar smooth muscle to obtain results which mimic the clinical setting. L-type calcium channels are particularly important in modulating vasomotion and myogenic reactivity in cell membrane, and, therefore, were examined in our study.

It has been demonstrated that the remifentanil plasma concentration, at which 50% of patients respond to skin incision (CP_{50}), is 4–6 ng·mL^{-1}.[14] Accordingly, in our study we tested escalating concentrations of remifentanil from 1.21 nmol·L^{-1} to 4.84 nmol·L^{-1} to 19.4 nmol·L^{-1}, equivalent to concentrations of 0.5–2.0–8.0 ng·mL^{-1} (0.1–0.4–1.6 CP_{50}) in whole blood. A 50% reduction of the minimal alveolar concentration of isoflurane is observed with 1.37 ng·mL^{-1} remifentanil, while concentrations exceeding 8 ng·mL^{-1} produce no further clinically relevant changes.[15] Recognizing that remifentanil is commonly combined with other volatile anesthetics to achieve the anesthetic state, the concentrations tested in this study were higher than the common dosing schemes adopted in general operations. The EC_{50} value for $I_{Ba,L}$ was 38.90 ± 3.96 × 10^{-3} μmol·L^{-1}, which would be equivalent to a concentration of 16.0 ng·mL^{-1} in whole blood.

A recent in vitro study of endothelium-intact and denuded rat thoracic aorta rings showed that remifentanil produces direct smooth muscle relaxation, either by an endothelium–dependent mechanism involving prostacyclin and nitric oxide release, or by an endothelium–independent mode, in which inhibition of voltage-sensitive Ca^{2+} channels was the major mechanism.[9] Consequently, we denuded the endothelium of HMASMCs to allow us to study the unique influence of the endothelium-independent vasodilation via Ca^{2+} channels. Our study shows that remifentanil significantly decreased $I_{Ba,L}$ in HMASMCs in a concentration-dependent manner, as observed in rat thoracic aorta rings.[9] It appears that remifentanil exerts a direct vasodilatory effect, in addition to alteration of sympathetic tone, to account for the summative effects on cardiovascular depression.

The signal pathways linking remifentanil to ion channel activity are not well characterized, but evidence from the present study confirms some important elements in this signalling cascade. We found that remifentanil, at concentrations of 1.21, 4.84, and 19.4 nmol·L^{-1}, resulted in a leftward shift of $I_{Ba.L}$ I-V curve in the hyperpolarizing direction, while decreasing the threshold. One possible mechanistic explanation is that remifentanil changes the $Ca._{L}$ channel characteristics by lowering activating voltage, while simultaneously inhibiting $Ca._{L}$ channel activity to decrease Ca^{2+} influx. Therefore, the blood vessel was ultimately dilated by inhibiting currents through calcium influx pathways to decrease systemic vascular resistance.

Our findings were consistent with recent studies on other μ-opioid agonists of [D-Ala 2, N-Me-Phe 4, Gly 5-ol] enkephalin, methadone, and morphine; they were all shown to inhibit I_{Ca} in AtT20 cells.[16] Similar effects were also found in neonatal rat dorsal root ganglion neurons,[17] and neuroblastoma glioma hybrid NG108-15 cells.[18] Meanwhile, a δ-opioid receptor agonist, leucine-enkephalin (0.01 μM) also reduced $I_{Ca.L}$ in rat ventricular myocytes.[19] In contrast, in cardiac myocytes from neonatal rats, morphine was shown to either induce transmembrane Ca^{2+} influx [20] or to increase $I_{Ca.L}$ in isolated rabbit ventricular myocytes.[21] Chronic exposure to sufentanil led to the activation of $I_{Ca.L}$ in guinea pig ileum.[22] The reasons

for these different effects of Ca^{2+} channels may be attributable to the different opioid receptor agonists and inter-species differences with various I_{Ca} expressions.

We acknowledge several limitations of this study. Firstly, the isolation procedure used in our study prevented determination of the effect of remifentanil on cardiac Ca^{2+} channel activity. As remifentanil and morphine were both shown to affect cardiac function via cardiac κ- and δ-opioid receptors in isolated rat hearts,[20,23] whether remifentanil affects Ca^{2+} channels in the cardiac system to induce hypotension should also be taken into consideration. Secondly, since the primary objective of this study was to assess whether remifentanil could affect Ca^{2+} channel current in HMASMCs, no functional studies were performed on its direct vasodilating effects. Nonetheless, this opioid has been shown to induce marked cardiovascular depression,[1–6] and our results further support these observations. Thirdly, it has been found that opioid receptors are involved in several signalling pathways, including G protein.[24] Further studies are warranted to more fully elucidate the precise mechanisms by which remifentanil influences vessel tone.

In summary, the present study provides electrophysiological evidence that remifentanil induces vasodilation through a direct effect of inhibiting L-type Ca^{2+} channels in a concentration-dependent manner. In addition, we have shown that these effects can be inhibited by the calcium channel blocker nifedipine. Although complete elucidation of the mechanism awaits further clarification, our findings provide a cellular mechanism that may help explain the molecular basis of how remifentanil induces endothelium-independent cardiovascular depression of human mesenteric arteries.

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