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RESEARCH PAPER

F 15845, a new blocker of the persistent sodium current prevents consequences of hypoxia in rat femoral artery

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BACKGROUND AND PURPOSE

The persistent sodium current is involved in myocardial ischaemia and is selectively inhibited by the newly described 3-(*R*)-[3-(2-methoxyphenylthio-2-(*S*)-methylpropyl]amino-3,4-dihydro-2H-1,5-benzoxathiepine bromhydrate (F 15845). Here, we describe the pharmacological profile of F 15845 against the effects of hypoxia in femoral arteries *in vitro*.

EXPERIMENTAL APPROACH

Isometric tension measurement of rat isolated femoral arteries was used to characterize the protective effect of F 15845 against contraction of the vessels induced by veratrine (100 $\mu g \cdot mL^{-1}$) or hypoxia.

KEY RESULTS

Rat femoral artery expressed the $Na_v1.5$ channel isoform. When exposed to veratrine (100 $\mu g \cdot mL^{-1}$), vessels developed a rapid and strong contraction that was abolished by both absence of sodium and blockade of the Na^+/Ca^{++} exchanger by KB-R7943 (10 and 32 $\mu mol \cdot L^{-1}$) or treatment with F 15845. When used before veratrine exposure, the potency of F 15845 depended on the extracellular K^+ concentration (IC_{50} = 11 and 0.77 $\mu mol \cdot L^{-1}$ for 5 and 20 $mmol \cdot L^{-1}$ KCl, respectively), whereas its potency was unaffected by extracellular K^+ concentration when given after veratrine. F 15845 did not affect either KCl (80 $mmol \cdot L^{-1}$) or phenylephrine-induced femoral artery contraction. Moreover, endothelium disruption did not affect the protective effect of F 15845 against veratrine-induced femoral artery contraction, suggesting a mechanism of action dependent on smooth muscle cells. Finally, F 15845 prevented in a concentration-dependent manner rat femoral artery contraction induced by hypoxia.

CONCLUSION AND IMPLICATIONS

F 15845, a selective blocker of the persistent sodium current prevented vascular contraction induced by hypoxic conditions.

Abbreviations
F 15845, 3-(*R*)-[3-(2-methoxyphenylthio-2-(*S*)-methylpropyl]amino-3,4-dihydro-2H-1,5 benzoxathiepine bromhydrate; TTX, tetrodotoxin

Introduction

Although the role of voltage-gated sodium channels is well established in neurones, cardiomyocytes and skeletal muscle cells, they are generally not thought to play a role in the contractility of smooth muscles. Indeed, opening of voltage-dependent calcium channels mainly controls smooth muscle cell (SMC) membrane depolarization and contraction. So far, no convincing data contradict this calcium hypothesis even if numerous electrophysiological studies have demonstrated the presence of voltage-gated sodium currents in SMCs from portal vein (Mironneau *et al.*, 1990), pulmonary (Jo *et al.*, 2004;

Platoshyn *et al.*, 2005), coronary (Quignard *et al.*, 1997; Jo *et al.*, 2004) and mesenteric arteries (Berra-Romani *et al.*, 2005). The functional role of these sodium currents remains elusive. Moreover, the genes coding for these 'vascular' sodium channels have only been identified in cultured cells. Thus, Jo *et al.* (2004) failed to detect *SCN4A* and *SCN6A* genes in cultured myocytes from pulmonary and coronary arteries, but proposed *SCN9A* as the major gene product responsible for the tetrodotoxin (TTX)-sensitive current recorded in these cells. Deshpande *et al.* (2002)) provided evidence of $Na_v1.4$ channels in human oesophageal SMCs (channel nomenclature follows Alexander *et al.*, 2009). Cells maintained under culture conditions, however, differ from freshly dissociated myocytes. Despite evidence of the presence of the *SCN* gene in freshly dissociated myocytes from blood vessels (Shinjoh *et al.*, 1991; Holm *et al.*, 2002; Saleh *et al.*, 2005), there is still an urgent need to determine which voltage-gated sodium channels are expressed in freshly dissociated myocytes from normal, healthy arteries, and to characterize the physiological role of these channels. Conceivably, they contribute to the intracellular calcium pool by slowing or reversing the Na^+/Ca^{2+} exchanger, and so increase intracellular calcium. In support of this proposal, Arnon *et al.* (2000) reported that sodium influx via store-operated sodium channels reduced calcium extrusion in arterial myocytes. Recently, Saleh *et al.* (2005) have established for the first time the molecular identity of the voltage-gated Na^+ channels ($Na_v1.6$ and Na_v 1.7 in freshly dispersed SMCs), and have shown that these channels can modulate contractility possibly involving reverse mode Na^+–Ca^{2+} exchange. The objective of the present work was to investigate the functional presence of another Na channel subtype ($Na_v1.5$) and to evaluate whether it could similarly contribute to the contractility of the SMCs.

In non-smooth muscle systems such as cardiomyocytes, $Na_v1.5$ sodium channels working in persistent mode contribute to the sustained elevations in $[Ca^{2+}]_i$ through sodium-dependent calcium influx via the Na^+/Ca^{2+} exchanger operating in reverse mode (Hale *et al.*, 2008; Shryock and Belardinelli, 2008). In cardiomyocytes, it is well established that hypoxia constitutes a major trigger of the persistent mode of $Na_v1.5$ channels which in turn leads to Ca^{2+} overload (Saint *et al.*, 1992; Saint, 2008).

We recently described a new potent $Na_v1.5$ channel blocker, F 15845 (Le Grand *et al.*, 2008; Vacher *et al.*, 2009) that selectively interacts with the persistent sodium current in cardiomyocytes. It is unknown, however, whether blockade of the persistent sodium current mediated by $Na_v1.5$ channels in SMCs can influence the contractility of artery and prevent the development of hypoxia-induced contractures. To investigate these questions, we describe here the expression and activation of $Na_v1.5$ channel in rat femoral arteries, and the pharmacological profile of F 15845 on rat isolated femoral artery under normal and hypoxic conditions.

Methods

Animals

Animals were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care-accredited facility in strict compliance with all applicable regulations, and the protocol was carried out in compliance with French regulations and with local ethical committee guidelines for animal research.

Tissue preparation

Male OFA-SPF rats (Iffa-Credo, l'Arbresle, France) weighing 250–270 g were killed by injection of sodium pentobarbital (160 $mg \cdot kg^{-1}$, i.p.). The femoral arteries were quickly removed and placed in ice-cold Krebs solution (NaCl 118 $mmol \cdot L^{-1}$, KCl 4.7 $mmol \cdot L^{-1}$, $MgSO_4$ 1.2 $mmol \cdot L^{-1}$, $CaCl_2$ 2.5 $mmol \cdot L^{-1}$, KH_2PO_4 1.2 $mmol \cdot L^{-1}$, $NaHCO_3$, 25 $mmol \cdot L^{-1}$, glucose 10 $mmol \cdot L^{-1}$, pH 7.4). Arteries were cleaned of fat tissue and cut into 2 mm segments (rings).

The femoral artery rings were mounted in a 5 mL wire myograph for resistance arteries (DMT 610 M, Danish Myo Technology, Aarhus, Denmark) in a resting tension of 5 mN. This optimal resting tension was previously evaluated with successive challenges with KCl 80 $mmol \cdot L^{-1}$ for stepwise tensions, and a resting tension that gave the optimal contraction was chosen. The Krebs solution was maintained at 37°C and bubbled with 95% O_2/5% CO_2 to maintain the pH at 7.4 in each case. Vessels were then allowed to stabilize for half an hour. Artery viability was tested using a high K^+ solution (80 $mmol \cdot L^{-1}$), and reproducibility of the response was assessed with a second challenge of the vessels with high K^+ solution. For experiments with intact vessels, endothelial integrity was assessed by using 1 $\mu mol \cdot L^{-1}$ acetylcholine to induce vessel relaxation after a pre-constriction with 1 $\mu mol \cdot L^{-1}$ phenylephrine. For endothelium-free experiments, endothelium was destroyed by gently rubbing the inner surface of the vessel with a wire shaft. Under these conditions, no acetycholine (1 $\mu mol \cdot L^{-1}$)-induced relaxation was observed after phenylephrine (1 $\mu mol \cdot L^{-1}$) pre-constriction.

Isometric tension measurements

For the preventive treatments, the late sodium channel blocker F 15845, KB-R7943 or corresponding vehicle [dimethyl sulphoxide (DMSO) 0.1%] were pre-incubated with the rings 30 min before the veratrine-induced or hypoxia-induced femoral artery contraction. Hypoxia was induced by closing organ chambers with plexiglass covers and replacing 95% O_2/5% CO_2 bubbling by 95% N_2/5% CO_2. To determine whether F 15845 could relax tissue pre-contracted with veratrine, F 15845 or vehicle (DMSO 0.1%) was incubated after veratrine-induced contraction reached a plateau. The amplitude of the tension was measured irrespective of the time for each concentration. A single concentration of antagonist was tested per tissue. To modify the resting potential of the vessel, KCl (5, 7.5, 10 and 20 mmol·L^{-1}) concentration was modified 30 min before the veratrine treatment. For Na-free experiments, sodium was replaced by an equivalent concentration of *N*-methyl-D-glucamine to maintain the osmolarity of the Krebs solution.

Immunofluorescence analysis

Segments of femoral artery were mounted in embedding medium (Miles Laboratories Inc., Elkhart, IN, USA), frozen in isopentane pre-cooled in liquid nitrogen and stored at –80°C. Immunostaining of $Na_v1.5$ channel was performed on transverse cross sections (7 µm). Tissue sections were permeabilized with cold methanol for 10 min. After extensive washing, they were saturated with 10% BSA in phosphate-buffered saline (PBS) for 10 min and then incubated with $Na_v1.5$ antibody 1/100 (Alomone, Jerusalem, Israel) in PBS–BSA 1% for 1.5 h. After five additional washings, primary antibody was detected with goat anti-rabbit Alexa 488 antibody (Interchim, Montluçon, France; 1:200 in PBS–BSA 1%, for 1 h). Positive staining was visualized with a Nikon microscope and Lugia image software (Nikon France, Champigny-sur- Marne, France).

Quantitative RT-PCR

Total RNA was isolated from dissected rat heart left ventricle and rat femoral artery containing both endothelium and SMCs, frozen at –80°C in FastPrep tubes, by using QIAZOL and RNeasy Mini Kit according to the manufacturer's specification (MP Biomedicals, Cleveland, OH, USA and Qiagen, Valencia, CA, USA).

Contaminating DNA in RNA preparation was removed by DNAse I treatment on-column at room temperature for 15 min (according to the Qiagen protocol). The amount and quality of the extracted RNA were determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For reverse transcription, 500 ng of total RNA was used to generate cDNA by using Quantitec Reverse Transcription Kit (ref 205313) according to the manufacturer's specification (Qiagen) in total volume of 20 µL. This step contains a second DNAse treatment to ensure absence of genomic DNA contamination. Real-time PCR was carried out on the iCycler iQ Real Time PCR Detection System (Bio-Rad, San Diego, CA, USA) using gene-specific primers and iQ SYBR green Supermix (Bio-Rad). The sequences of the primers are as follows: rat $Na_v1.5$ (NM_013125) forward 5′- GCA GCT CTT CAT GGG CAA CCT -3′; rat $Na_v1.5$ (NM_013125) reverse 5′- CCG TCG GCC TCC ACG GA -3′; rat GAPDH (NM_017008) forward 5′- CGC CTG GTT ACC AGG GCT G-3′; rat GAPDH (NM_017008) reverse 5′- TGG AAC ATG TAG ACC ATG ATG TTG AGG TC-3′; rat Arbp (NM_022402) forward 5′- TGT TTC ATT GTG GGA GCA GAC A -3′; rat Arbp (NM_022402) reverse 5′- CCA TCA GCA CCA CAG CCT TC -3′; rat YWHAZ (NM_013011) forward 5′- CAA GCA TAC CAA GAA GCA TTT GA-3′; rat YWHAZ (NM_013011) reverse 5′- GGG CCA GAC CCA GTC TGA -3′. The PCR reactions were performed on duplicate on a final volume of 25 µL as follows: 5 min at 95°C to activate 'hot start' enzyme and 40 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by a fusion curve from 65 to 95°C (0.5°C every 10 s) to determine specific Tm of each amplicon. Rat GAPDH, Arbp and YWHAZ were used as internal reference. A geometric mean of *Ct* values of these three genes was used to normalize the $Na_v1.5$ Ct value for each structure (Vandesompele *et al.*, 2002).

Data analysis

Results are expressed as % mean (E_{max}) ± SEM. E_{max} was obtained for vehicle-treated groups. One-way ANOVA or one-way ANOVA on ranks (Kruskal–Wallis) followed by a Dunnett's or Dunn test, respectively, was performed to compare each group.

Materials

F 15845 was synthesized (Le Grand *et al.*, 2008) by the Division of Medicinal Chemistry I, Centre de Recherche Pierre Fabre. F 15845 was dissolved in DMSO with highest concentration of DMSO being 0.1%. Veratrine and *N*-methyl-D-glucamine were purchased from Sigma Chemicals (St Louis, MO, USA) and dissolved in distilled water. KB-R7943 was synthesized by the Resynthesis Department, Centre de Recherche Pierre Fabre.

Results

Characterization of $Na_v1.5$ channels in rat femoral artery

Quantitative RT-PCR allowed the detection of *SCN5A* mRNA in rat femoral artery. Left cardiac ventricle was chosen as positive control. Figure 1Aa details the relative expression in both tissue expressed in arbitrary units (see Methods). Similarly, Figure 1Ab shows that immuno-staining for $Na_v1.5$ protein was expressed in rat femoral artery. Immuno-labelling showed that $Na_v1.5$ was expressed both in endothelial cells and SMCs of the media.

Effects of veratrine on rat isolated femoral artery

Veratrine was used to activate the late sodium current through $Na_v1.5$ channels (Sunami *et al.*, 1993) in rat femoral artery. As shown in Figure 1B, veratrine (100 $\mu g \cdot mL^{-1}$) induced a contraction of the rat isolated femoral artery (Δ contraction 9.6 $\pm$ 0.9 mN, n = 19). However, in the presence of sodium-free medium, veratrine at the same concentration did not produce vessel contraction (Δ contraction of 0.1 $\pm$ 0.1 mN, n = 8). Thus, Figure 1C clearly demonstrates that veratrine induced a Na-dependent contraction of rat isolated artery. Veratrine-induced contraction of rat femoral artery was also abolished when artery was pre-incubated with a blocker of the reverse mode of Na^+/Ca^{++} exchanger, KB-R 7943, at 10 and 32 $\mu mol \cdot L^{-1}$ (Iwamoto *et al.*, 1996; Figure 1D), suggesting that the veratrine-induced contraction involved the Na^+/Ca^{++} exchanger. KB-R 7943 alone did not produce any effect on femoral artery tension when incubated over 30 min (6.0 $\pm$ 0.3 mN for baseline vs. 6.6 $\pm$ 0.3 mN, n = 8 after 30 min incubation with KB-R 7943 32 $\mu mol \cdot L^{-1}$).

Effects of F 15845 pretreatment on veratrine-induced contraction of rat femoral artery

Typical recordings showing the effects of F 15845 pretreatment on veratrine-induced contraction are shown in Figure 2A. Figure 2B summarizes the effects of F 15845 and TTX, sodium channel blockers, against veratrine-induced vasoconstriction of rat femoral artery. In normal Krebs solution (5 $mmol \cdot L^{-1}$ KCl), F 15845 caused a concentration-dependent reduction in the veratrine-induced contraction from 10 $\mu mol \cdot L^{-1}$ with maximal inhibition obtained at 32 $\mu mol \cdot L^{-1}$. F 15845 reduced the veratrine-induced femoral artery contraction by about 85% at 32 $\mu mol \cdot L^{-1}$ (Figure 2B,C). In these conditions (5 $mmol \cdot L^{-1}$ KCl), the IC_{50} value was 11 μM with 95% confidence limits of [3; 112]. Nevertheless, in the same conditions, F 15845 (up to 32 $\mu mol \cdot L^{-1}$) prevented neither high K^+ (80 $mmol \cdot L^{-1}$; n = 4–6, P = ns) nor phenylephrine- (1 $\mu mol \cdot L^{-1}$; n = 6, P = ns) induced femoral artery contractions (Figure 3A,B), confirming that F 15845 did not cause a generalized depression of smooth muscle contractility and acted principally in a Na_v-dependent mechanism. F 15845 was then evaluated in the presence of higher concentrations of extracellular KCl in order to test the effect on more depolarized cells. In the presence of 7.5, 10 and 20 $mmol \cdot L^{-1}$ KCl, F 15845 (10 $\mu mol \cdot L^{-1}$) completely prevented veratrine-induced femoral artery contraction (n = 7–8, $P < 0.001$) (Figure 2B,C). In the presence of 20 $mmol \cdot L^{-1}$ KCl, the IC_{50} value of F 15845 was 0.77 $\mu mol \cdot L^{-1}$ with 95% confidence limits of [0.32; 1.5], which is approximately 10 times lower than the IC_{50} value obtained in the presence of 5 $mmol \cdot L^{-1}$ KCl (see above). The non-selective Na^+ channel blocker TTX (0.1 $\mu mol \cdot L^{-1}$) completely prevented contractions for all KCl concentrations (n = 5–7, $P < 0.001$) (Figure 2B). These results demonstrate that the protective effect of F 15845 was more potent for high KCl concentrations (i.e. 7.5, 10 and 20 $mmol \cdot L^{-1}$) (Figure 2C). Thus, high extracellular KCl concentrations produced changes in resting membrane potential similar to those produced by hypoxia (Carmeliet, 1999) and rendered F 15845 more potent.

Effects of F 15845 pretreatment on veratrine-induced contraction of endothelium-free femoral artery

The effect of F 15845 pretreatment against veratrine was assessed on femoral artery in which endothelium was destroyed. Figure 3C shows a typical recording demonstrating the absence of responses to acetylcholine after removal of the endothelium. However, veratrine-induced contractions were similar between endothelium-free vessels [9.2 $\pm$ 1.7 mN (n = 6)] and intact vessels [9.6 $\pm$ 0.9 mN (n = 19), P = ns]. F 15845 markedly reduced the veratrine-induced femoral artery contraction in endothelium-free rings at 32 or 10 $\mu mol \cdot L^{-1}$ (Figure 3C). This set of experiments clearly showed that absence of the endothelium did not modify the effect of pretreatment with F 15845, strongly suggesting that F 15845 principally acted on vascular SMCs.

Relaxant effects of F 15845 on femoral artery pre-contracted with veratrine

Typical recordings showing the relaxing effect of F 15845 are presented in Figure 4A. As shown in Figure 4B, F 15845 reduced the veratrine-induced

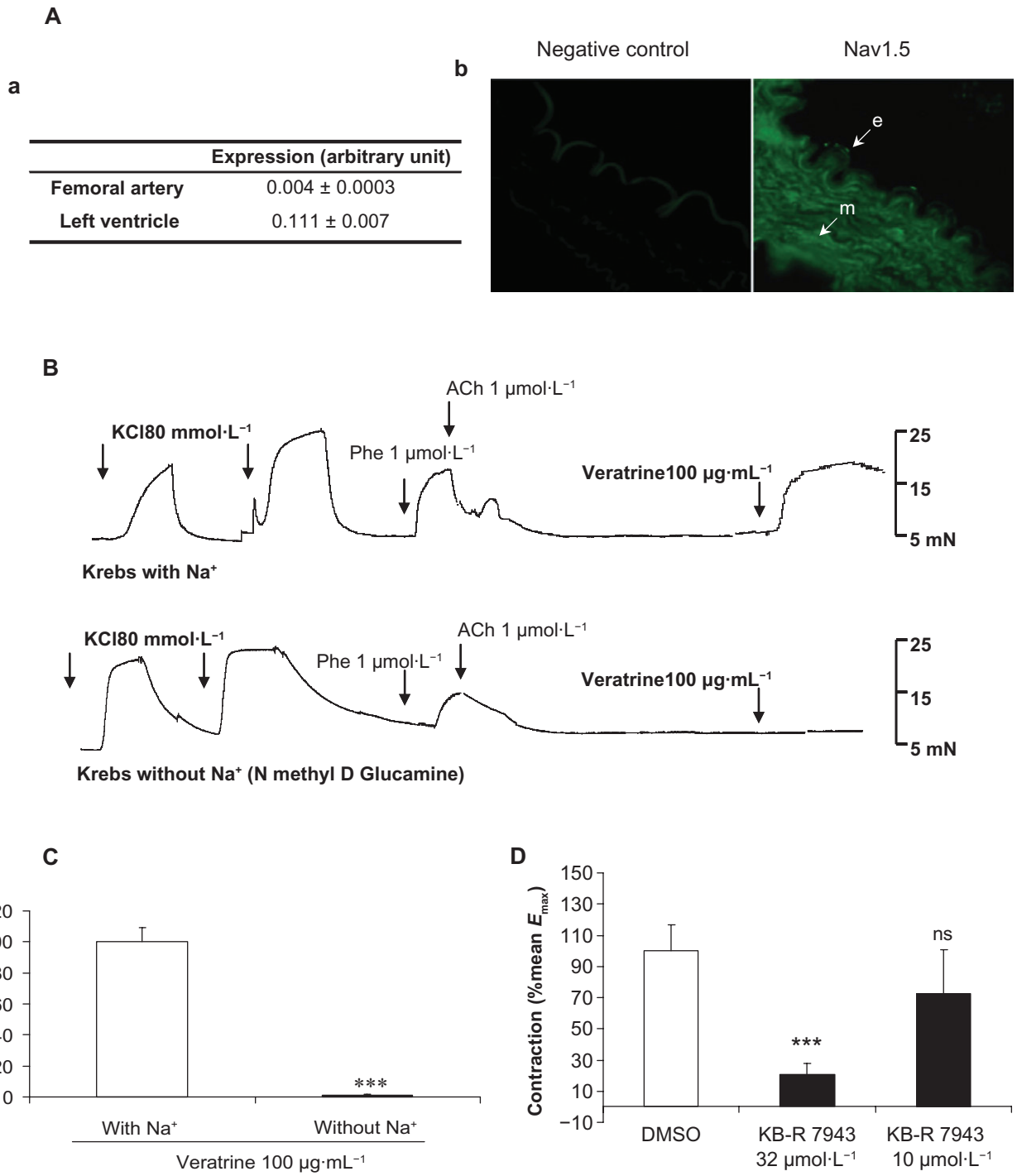

Figure 1

Expression and functional characterization of $Na_v1.5$ channel in rat femoral artery. (A) mRNA expression of $Na_v1.5$ in femoral artery and left ventricle (a) and typical photomicrograph of immunofluoresence labelling with an anti- $Na_v1.5$ antibody (b). Rat femoral artery expressed the $Na_v1.5$ channel in both endothelium (e) and media (m) of the vessel. (B,C) Typical recording and summary of isometric tension measurement experiments on rat femoral artery showing viability of vessels using KCl challenge, integrity of SMCs using contraction to phenylephrine (Phe) and integrity of endothelial cells using relaxation to ACh and finally the effects of veratrine: (C) veratrine (100 µg·mL^{-1}) induced a vessel contraction in the presence of Na^+ ($n = 19$). Absence of Na^+ abolished the veratrine-induced femoral artery contraction ($n = 9$) ***$P < 0.001$ versus medium containing Na^+. (D) Pretreatment of rat vessels with KB-R7943 reduced the veratrine-induced contraction ($n = 3$–4). Results are mean ± SEM, ***$P < 0.001$ versus vehicle.

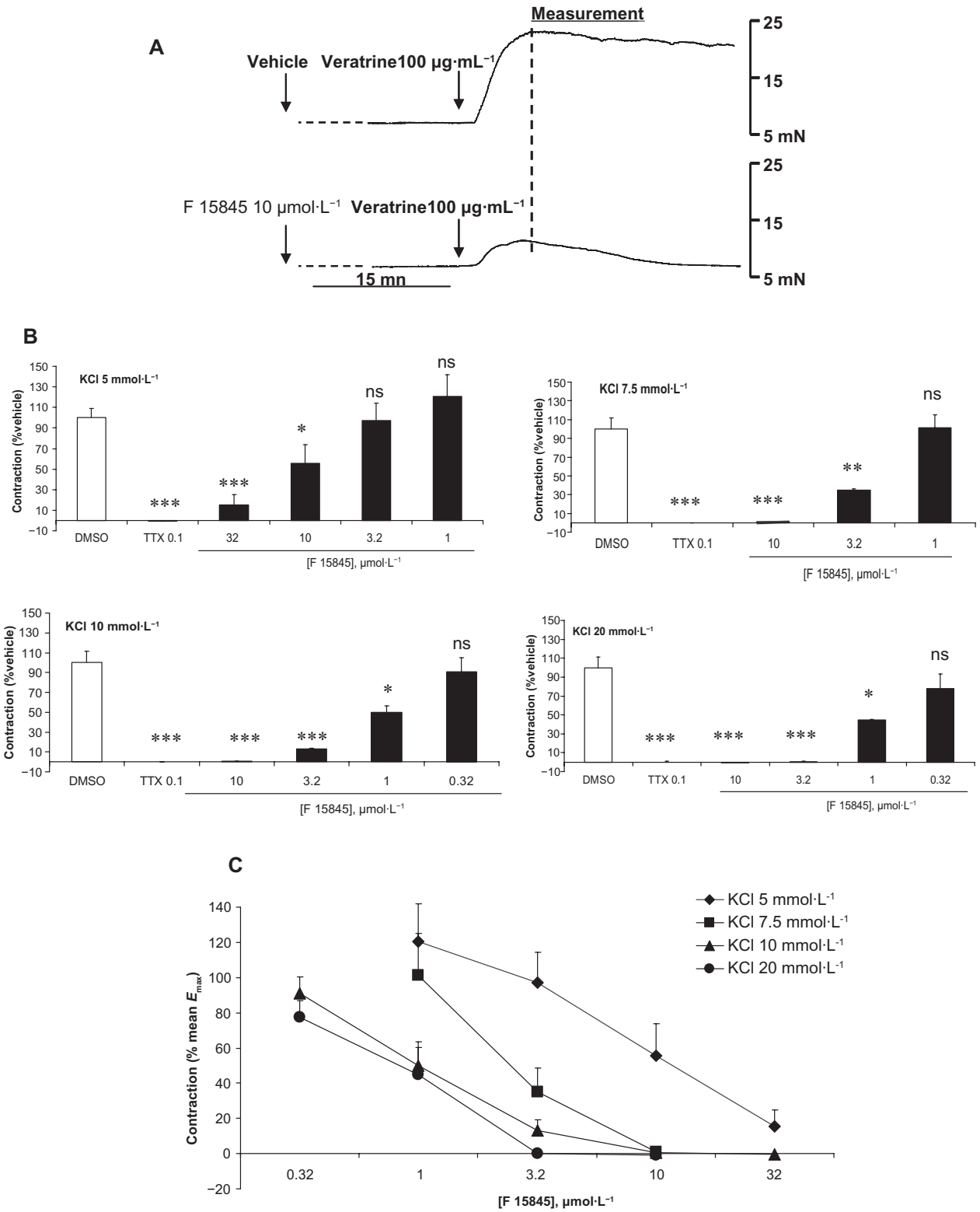

Figure 2

Effect of F 15845 pretreatment on veratrine-induced rat femoral artery contraction. (A) Typical recording of the effect of F 15845 (10 µmol·L^{-1}) on veratrine-induced femoral artery contraction. (B) Influence of KCl concentration on the effects of F 15845 against veratrine-induced femoral artery contraction. The effects of F 15845 increased with the extracellular KCl concentration, suggesting its action was dependent on resting potential. Results are mean ± SEM (B) *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle ($n = 5$–19). (C) Concentration–response curve of F 15845 in the presence of increasing extracellular KCl concentration.

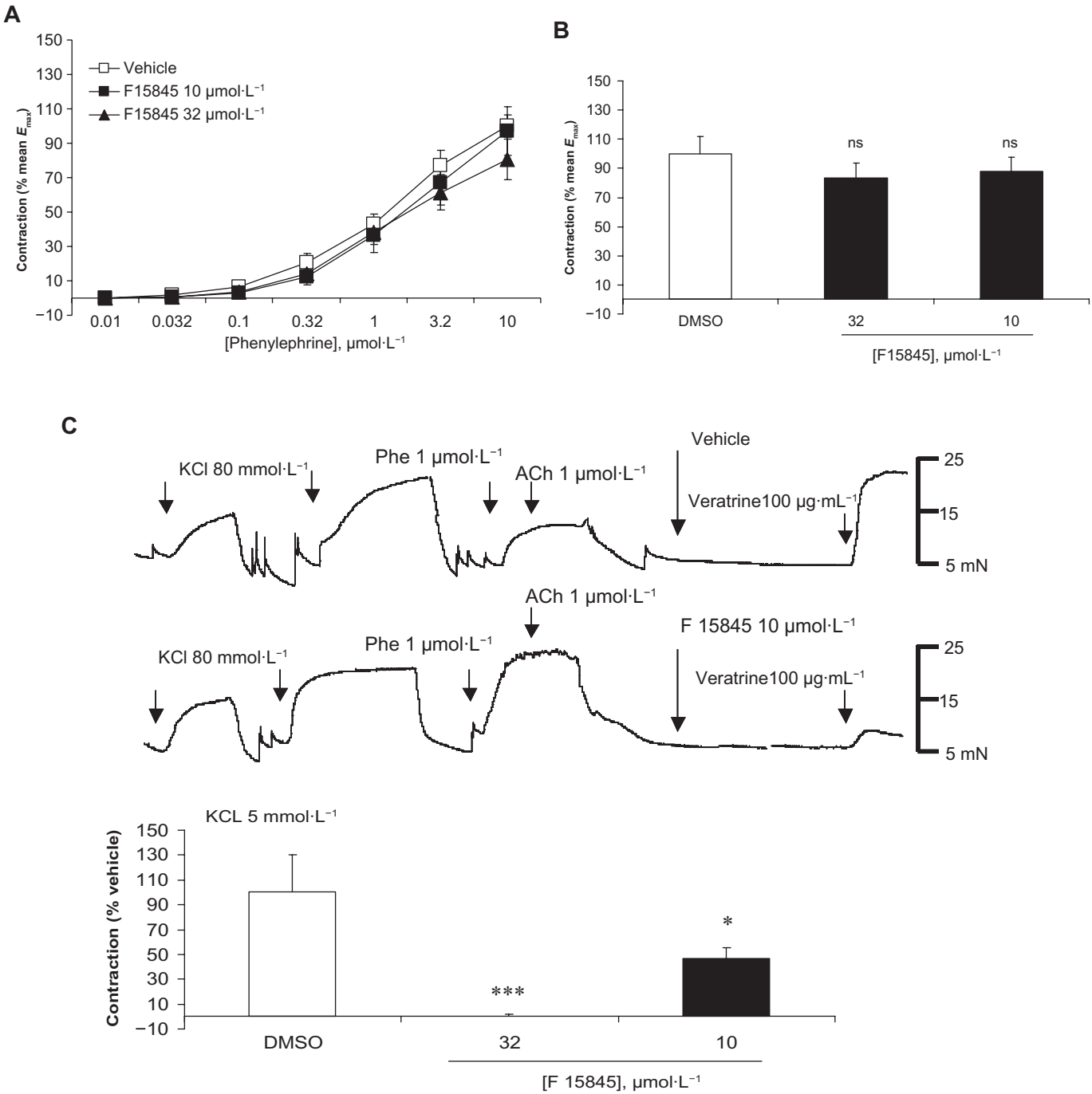

Figure 3

Effect of F 15845 pretreatment against contraction of intact femoral arteries induced by phenylephrine or KCl (80 mmol·L^{-1}) and on the veratrine-induced contraction in a femoral artery denuded of endothelium. (A,B) F 15845 (10 and 32 µmL·L^{-1}) did not antagonize either phenylephrine- (Phe; A) or KCl- (80 mmol·L^{-1}; B) induced femoral artery contraction. (C) Effect of F 15845 (10 and 32 µmol·L^{-1}) on veratrine-induced contraction in a femoral artery denuded of endothelium. Endothelium was destroyed by rubbing, and absence of response to ACh was taken as an index of endothelial loss, as shown on the typical recording. The inhibitory effect of F 15845 observed was similar to that in vessels with an intact endothelium, shown in Figure 2. Results are mean ± SEM. *P < 0.05, ***P < 0.001 versus vehicle, ns: non-significant.

contraction in a concentration-dependent manner when applied at the peak of the contraction of the vessel. In the presence of 5 mmol·L^{-1} KCl, a maximal relaxation of about 50% (Figure 4B, n = 4, P < 0.001) was obtained for F 15845 (10 µmol·L^{-1}). Elevation of extracellular KCl concentrations to 7.5 and 10 mmol·L^{-1} did not significantly increase the relaxation induced by F 15845 on contraction induced by

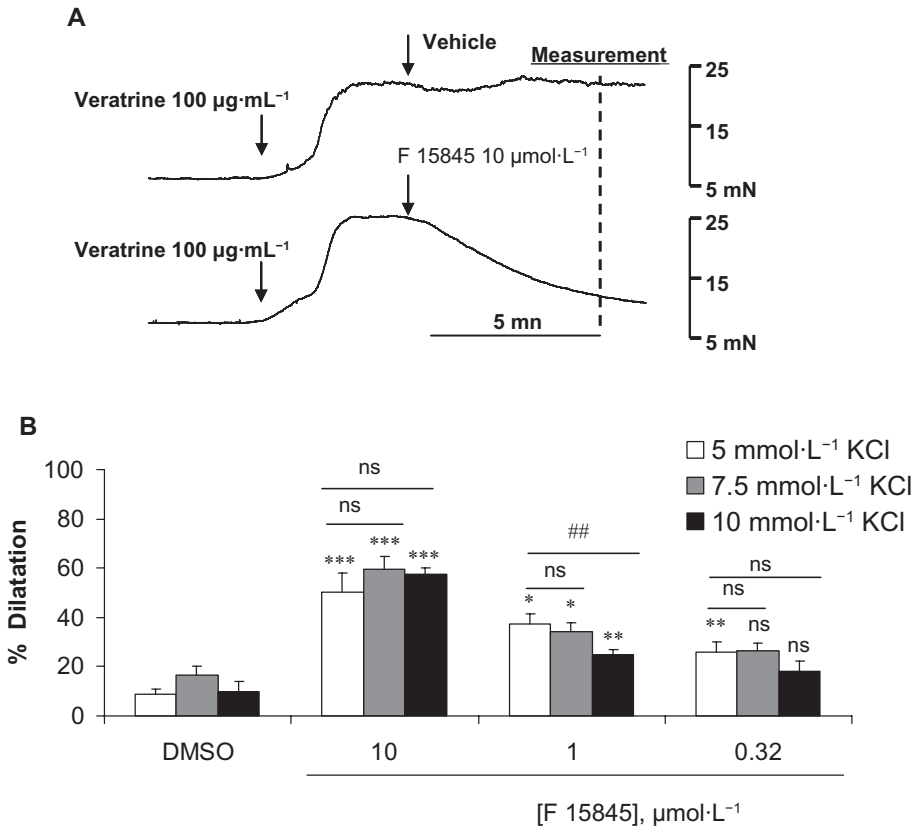

Figure 4

Vasorelaxant effect of F 15845 on rat femoral artery pre-contracted with veratrine. (A) Typical recording of relaxant effect of F 15845 10 µmol·L^{-1} on veratrine-induced femoral artery contraction. (B) F 15845 induced concentration-dependent relaxation of rat femoral artery contracted with veratrine. Compared to the protective effects, the relaxant effect of F 15845 was not dependent on the extracellular KCl concentration. Results are mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle, ##P < 0.01 versus 5 mmol·L^{-1} KCl (n = 4–12).

veratrine (P = ns) except for F 15845 (1 µmol·L^{-1}) in 10 mmol·L^{-1} KCl (P < 0.01).

Effect of F 15845 pretreatment on femoral artery contraction induced by hypoxia

The Krebs solution was bubbled with 95% N_2/5% CO_2 to induce hypoxia in the organ chamber without modifying the pH of the Krebs solution. Thus, 5 min after 95% N_2/5% CO_2 bubbling, a rapid contraction of rat femoral artery was observed (Figure 5A). Pretreatment with F 15845 (1, 10 and 32 µmol·L^{-1}) concentration-dependently decreased the hypoxia-induced femoral artery contraction with a maximal inhibition of about 85% at 32 µmol·L^{-1} (n = 9, P < 0.001; Figure 5B).

Discussion and conclusions

F 15845 is a selective, potent and voltage-dependent blocker of the persistent sodium current mediated by the $Na_v1.5$ channel isoform. The present study demonstrates that F 15845 can prevent veratrine- and hypoxia-induced contraction of femoral artery, most likely through blockade of the $Na_v1.5$ channel.

The functional significance of sodium currents generated in SMCs remains unknown, but may be explained by the following hypothesis. Influx of sodium through voltage-gated Na^+ channels may contribute to the regulation of the cytosolic calcium concentration $[Ca^{2+}]_{cyt}$. Thus, enhanced sodium current causes a transient increase in $[Na^+]_{cyt}$, which enhances $[Ca^{2+}]_{cyt}$ in the sub-sarcolemmal space via reverse-mode Na^+/Ca^{2+} exchange (Leblanc and Hume, 1990). This in turn triggers either further calcium release from the sarcoplasmic reticulum (SR) or replenishes SR calcium pools by SERCA-mediated calcium re-uptake (Santana *et al.*, 1998).

In cardiac cells, $Na_v1.5$ channels present an abnormal functioning mode, as they can open and close spontaneously. Reasons for this phenomenon are not completely understood. Late opening allows a persistent current of sodium ions to enter the cells throughout the systole (Saint, 2008). This current has been referred to as late, sustained or persistent to distinguish it from the peak, rapid or transient

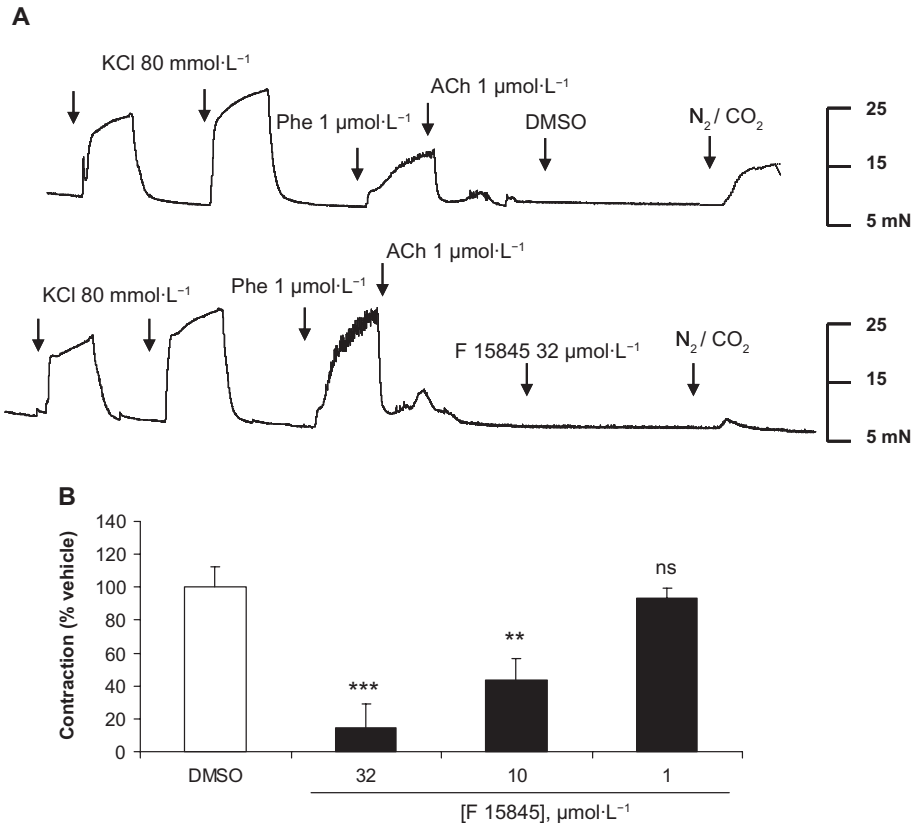

Figure 5

Effect of F 15845 pretreatment on hypoxia-induced contraction of rat femoral artery. (A) Typical recording of femoral artery contraction induced by hypoxia in the presence or absence of F 15845 (32 µmol·L^{-1}). (B) When femoral artery was pre-incubated with F 15845, hypoxia-induced contraction was not delayed (left panel), but was concentration-dependently decreased when measured at the contraction peak. Results are mean ± SEM, *P < 0.05, **P < 0.01 versus vehicle (n = 10–12), ns: non-significant.

sodium current. Although the amplitude of persistent sodium current is small, because it lasts for hundreds of milliseconds, the influx of sodium is substantial (Noble and Noble, 2006). Many voltage-gated Na^+ channel α and β subunits are expressed in SMCs (Saleh *et al.*, 2005). Therefore, the RT-PCR data are consistent with our immunochemistry and pharmacological experiments in which Na^+ channel activity was explored using veratrine in rat femoral artery. Although mRNA expression does not directly reflect expression of a functional channel, the results support the existence of functional voltage-gated $Na_v1.5$ channels in rat femoral artery. To date, however, there is no evidence that $Na_v1.5$ channels expressed in these SMCs can produce a persistent current similar to that observed in cardiac cells. The Na^+ channel blocker, F 15845, was used to assess the presence of such persistent sodium current (Le Grand *et al.*, 2008; Vacher *et al.*, 2009).

We have shown that, in cardiac cells, F 15845 selectively inhibits the persistent component of the Na^+ current and that its activity is enhanced under depolarizing conditions. During ischaemia, the persistent, inward sodium current (mediated by Na_v 1.5) increases, and this triggers a deleterious chain of events resulting in cell injury or death. By selectively blocking the persistent sodium current in depolarized ischaemic cardiac myocytes, F 15845 prevents or, at least, delays sodium overload and its consequences (Vacher *et al.*, 2009). Here, we show that F 15845 reduced veratrine-induced femoral artery contraction in a concentration-dependent manner. Veratrine intoxication of isolated vascular smooth muscle is a well-established model of modified Na^+ channel function-induced calcium loading and vascular contraction (Shinjoh *et al.*, 1991). Veratrine binds to the open state of the Na^+ channel and increases its probability of opening (Sunami *et al.*, 1993). The ensuing increase in sodium influx is accompanied by calcium loading and vascular contraction (Shinjoh *et al.*, 1991). The calcium loading is a consequence of the activation of the Na^+/Ca^{2+} exchanger in the reverse mode, and therefore it can be blocked by KB-R7943 (Saleh *et al.*, 2005; see also Figure 1D). Finally, F 15845 reduced contractions induced by veratrine, suggesting that F

15845 might act to reduce persistent sodium current in smooth muscle. However, this is only a possibility on the basis of our findings.

Another feature of F 15845 arises from the nature of its interaction with $Na_v1.5$ channels. The blockade of the persistent sodium current by F 15845 occurs in a defined, limited range of membrane potentials. Experimentally, we have demonstrated that inhibition of the persistent sodium current is most effective under depolarizing conditions (Vacher *et al.*, 2009). To test the effect of this voltage-dependent potency of F 15845 on femoral artery contraction, incremental concentrations of KCl were used to set varying levels of resting membrane potential in the SMCs. F 15845 was more effective against veratrine-induced contraction in the presence of higher concentrations of KCl.

Collectively, these data demonstrate, as previously observed in cardiac tissue (Vacher *et al.*, 2009), that the activity of F 15845 is optimal under depolarizing conditions which renders the compound 'ischaemia and/or hypoxia' selective. Nevertheless, this was not the case on veratrine pre-contracted vessels. We may hypothesize that increasing K^+ concentration in veratrine pre-contracted vessels depolarizes the resting membrane potential to a level at which some channels are in the inactivated state. In such a hypothesis, F 15845 would affect closed or activated channels only and would be devoid of any effect on inactivated channels (Vacher *et al.*, 2009)

Hypoxia is known to impair inactivation of Na^+ channels and to increase persistent sodium current (Saint, 2008). Tissue hypoxia and reperfusion generate metabolites (palmitoyl-L-carnitine and lysophosphatidylcholine, thrombin) and reactive oxygen/nitrogen species (hydrogen peroxide and nitric oxide) that increase persistent sodium current in ventricular myocyte (Tamareille *et al.*, 2002; Pinet *et al.*, 2008; Shryock and Belardinelli, 2008). The present results show that a pretreatment with F 15845 concentration-dependently decreased the hypoxia-induced femoral artery contraction, suggesting a direct blockade of the persistent sodium current in SMCs. In cardiomyocytes, this blockade of steady-state sodium influx is known to ensure fine regulation of the steady-state calcium influx. It is certainly an effective mechanism to regulate cell depolarization, to turn on the Na^+/Ca^{2+} exchanger activity and decrease sustained calcium influx and, in addition, to reduce elevated $[Na^+]_i$ (Saint, 2008). Thus, it could be easily hypothesized that such a fine regulation also existed in vascular SMCs.

The possibility that the persistent sodium current, through $Na_v1.5$ channels, is induced under certain pathophysiological conditions contributing to the basal arterial tonus, or other calcium- or sodium-dependent function(s), is worth considering and will be explored in the near future. However, the lack of information on the activities of the other sodium channel subtypes (i.e. $Na_v1.6$ and $Na_v1.7$) during vascular pathological situations probably constitutes a limitation of the present study. Finally, we believe that the voltage-dependent persistent sodium current blocker F 15845 may be very useful in the prevention of abnormal elevation of arterial tonus in pathological situations such as vessel hypoxia.

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Conflicts of interest

All the authors are employees of Institut de Recherche Pierre Fabre.

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