

RESEARCH ARTICLE

# Dissecting out the Complex $\text{Ca}^{2+}$ -Mediated Phenylephrine-Induced Contractions of Mouse Aortic Segments

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## Abstract

L-type  $\text{Ca}^{2+}$  channel (VGCC) mediated  $\text{Ca}^{2+}$  influx in vascular smooth muscle cells (VSMC) contributes to the functional properties of large arteries in arterial stiffening and central blood pressure regulation. How this influx relates to steady-state contractions elicited by  $\alpha$ 1-adrenoreceptor stimulation and how it is modulated by small variations in resting membrane potential ( $V_m$ ) of VSMC is not clear yet. Here, we show that  $\alpha$ 1-adrenoreceptor stimulation of aortic segments of C57Bl6 mice with phenylephrine (PE) causes phasic and tonic contractions. By studying the relationship between  $\text{Ca}^{2+}$  mobilisation and isometric tension, it was found that the phasic contraction was due to intracellular  $\text{Ca}^{2+}$  release and the tonic contraction determined by  $\text{Ca}^{2+}$  influx. The latter component involves both  $\text{Ca}^{2+}$  influx via VGCC and via non-selective cation channels (NSCC). Influx via VGCC occurs only within the window voltage range of the channel. Modulation of this window  $\text{Ca}^{2+}$  influx by small variations of the VSMC  $V_m$  causes substantial effects on the contractile performance of aortic segments. The relative contribution of VGCC and NSCC to the contraction by  $\alpha$ 1-adrenoreceptor stimulation could be manipulated by increasing intracellular  $\text{Ca}^{2+}$  release from non-contractile sarcoplasmic reticulum  $\text{Ca}^{2+}$  stores. Results of this study point to a complex inter-relationships between  $\alpha$ 1-adrenoreceptor-mediated VSMC contractile performance and  $\text{Ca}^{2+}$  release from contractile or non-contractile  $\text{Ca}^{2+}$  stores with concomitant  $\text{Ca}^{2+}$  influx. Given the importance of VGCC and their blockers in arterial stiffening and hypertension, they further point toward an additional role of NSCC (and NSCC blockers) herein.

## Introduction

Arterial stiffness and hypertension are common clinical conditions in the elderly that lead to increased cardiovascular risk. In the isolated aorta, in animal models of endothelial dysfunction and in humans, reduced nitric oxide (NO) bioavailability induces physiological alterations including arterial stiffness, vascular wall remodeling and hypertension [1,2]. Thereby, arterial smooth muscle cells in cross-talk with endothelial cells, determine arterial mechanics [3],

including the basal tonus of the blood vessel. Both L-type  $\text{Ca}^{2+}$  channels blockers and basal NO release from elastic arteries reduce arterial stiffness [4]. However, the specific role of L-type  $\text{Ca}^{2+}$  influx in arterial (de-)stiffening needs further investigation.

Isolated vascular smooth muscle cells (VSMC) display a window voltage range, in which a “time-independent” L-type  $\text{Ca}^{2+}$  influx or window  $\text{Ca}^{2+}$  current flows [5–8]. The physiological significance of this background window  $\text{Ca}^{2+}$  influx in multicellular preparations, and more specifically, in mouse aortic segments was evident in segments depolarised with elevated extracellular  $\text{K}^+$  [9–11]. How it contributes to contractions induced by  $\alpha_1$ -adrenoceptor stimulation is not clear. Elevated extracellular  $\text{K}^+$  and  $\alpha_1$ -adrenoceptor stimulation of VSMC causes contraction initially by intracellular  $\text{Ca}^{2+}$  release via  $\text{IP}_3$ -receptor-mediated  $\text{Ca}^{2+}$  release from sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  stores, followed by  $\text{Ca}^{2+}$  influx via  $\text{Ca}^{2+}$  permeable ion channels and concomitant  $\text{Ca}^{2+}$  sensitization [12–14]. Yet, even these contractions are associated with depolarisation [15–19]. Therefore, it is hypothesized that  $\alpha_1$ -adrenoceptor-stimulation with PE induces  $\text{IP}_3$ -mediated intracellular  $\text{Ca}^{2+}$  release, activates non-selective cation channels (NSCC), causes depolarization, opens L-type  $\text{Ca}^{2+}$  channels (VGCC) with concomitant background window  $\text{Ca}^{2+}$  influx and elicits contraction. The L-type  $\text{Ca}^{2+}$  influx is not only important for contraction of mouse aortic segments but also for their relaxation by endothelium-derived factors. Indeed, the relaxing efficacy of NO is dependent on the amount of  $\text{Ca}^{2+}$  influx via L-type  $\text{Ca}^{2+}$  channels [19].

In the present study, aortic segments of C57Bl6 mice were  $\alpha_1$ -adrenoceptor stimulated with phenylephrine (PE) to investigate the involvement of NSCC and VGCC  $\text{Ca}^{2+}$  influx in the isometric contraction, and more specifically, the potential role of the VGCC window  $\text{Ca}^{2+}$  influx herein. The relationship between  $\text{Ca}^{2+}$  mobilisation and isometric tension revealed that the contraction induced by intracellular  $\text{Ca}^{2+}$  release with PE could be isolated from the contraction evoked by  $\text{Ca}^{2+}$  influx. The latter, tonic contractile component involved both  $\text{Ca}^{2+}$  influx within the window voltage range of the VGCC and  $\text{Ca}^{2+}$  influx via NSCC. Modulation of the window  $\text{Ca}^{2+}$  influx by small variations of  $V_m$  of the VSMC caused significant effects on the contractile properties of aortic segments. Results of this study extend our knowledge of the important role of VGCC and NSCC (and their blockers) in arterial stiffening and hypertension, which is of major importance to develop therapeutical strategies for the treatment of arterial stiffness, hypertension and closely associated cardiovascular risk [4,20,21].

## Material and Methods

### Aortic segments

Ethics statement: The studies were approved by the Ethical Committee of the University of Antwerp, and the investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

C57Bl6 mice ( $n = 100$ , 80 males, 20 females, food and water ad libitum, 12/12 light-dark cycle) were used at the age of 4 to 7 months. Animals were euthanized by perforating the diaphragm under anaesthesia (sodium pentobarbital, 75 mg  $\text{kg}^{-1}$ , i.p.). The thoracic aorta was carefully removed, stripped of adherent tissue and dissected systematically. Starting at the diaphragm, the ascending thoracic aorta was cut in 5 to 6 segments of 2 mm width. Vessels were immersed in Krebs Ringer (KR) solution (37°C, 95%  $\text{O}_2$ /5%  $\text{CO}_2$ , pH 7.4) containing (in mM): NaCl 118, KCl 4.7,  $\text{CaCl}_2$  2.5,  $\text{KH}_2\text{PO}_4$  1.2,  $\text{MgSO}_4$  1.2,  $\text{NaHCO}_3$  25, CaEDTA 0.025 and glucose 11.1. High  $\text{K}^+$ -solution was prepared as KR solution, but NaCl was replaced with equimolar KCl. When  $\text{Ca}^{2+}$  was omitted from the KR solution, 1 mM EGTA was added (further named 0Ca) and, hence, to restore 2.5 mM free  $\text{Ca}^{2+}$  KR solution again, 3.5 mM  $\text{Ca}^{2+}$  was

added to the  $\text{Ca}^{2+}$ -free KR from a 1.75 M  $\text{CaCl}_2$  stock solution (further named +Ca). In the absence of external  $\text{Ca}^{2+}$ , the PE-induced contraction was transient and was called phasic contraction. The contraction upon re-admission of  $\text{Ca}^{2+}$  was permanent and was called tonic contraction. To avoid spontaneous emptying of the SR stores in 0Ca, PE was always added at 3 minutes after removal of external  $\text{Ca}^{2+}$ . To obtain 0Ca KR solution with different  $\text{K}^+$  concentrations, NaCl in the  $\text{Ca}^{2+}$ -free KR was replaced with equimolar amounts of  $\text{K}^+$ . Genders were randomly distributed over the experimental groups. In all experiments where both genders were used, there was no evidence for an influence of gender on the results.

Phenylephrine (PE) was added in concentrations of 1  $\mu\text{M}$ . Extracellular  $\text{K}^+$  was used to clamp the aortic segments at certain potentials [9].

### Isometric tension measurements

Aortic segments were mounted in 10 ml organ baths as described before [19]. Isometric force was acquired at 10 Hz and was reported in mN. To avoid any vasomotor interference due to prostanoids, 10  $\mu\text{M}$  indomethacin was present in all experiments. Endothelial cells were always present but the basal NO formation was inhibited by adding a combination of 300  $\mu\text{M}$   $\text{N}^{\text{G}}\text{-nitro-L-arginine methyl ester (L-NAME)}$  and 300  $\mu\text{M}$   $\text{N}^{\text{G}}\text{-nitro-L-arginine (L-NNA)}$ .

### Combined assay of isometric tension and VSMC $\text{Ca}^{2+}$

In myograph experiments, the endothelium was mechanically removed by rubbing the interior of the segment with a braided silk wax. Removal of the endothelium in these experiments was necessary to avoid interference of endothelial with VSMC  $\text{Ca}^{2+}$  signals [19]. The Fura-2 AM (10  $\mu\text{M}$ )-loaded segment was continuously perfused with KR (37°C) which was aerated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  (pH 7.4). The single emission (510 nm) ratio at dual excitation (340 and 380 nm) was used as a relative measure of free  $[\text{Ca}^{2+}]_i$  (relative units, RU) and was analysed with Felix software (PTI, USA). Tension was measured simultaneously, acquired at 1 Hz and reported in  $\text{mN mm}^{-1}$  [19].

### Data analysis

All results are expressed as mean  $\pm$  sem with n representing the number of mice. Time-force curves were fitted with a bi-exponential function revealing amplitudes and time constants of first (fast) and second (slow) components.  $Y = Y_0 + ((A_{\text{fast}} - Y_0) * (1 - \exp(-K_{\text{fast}} * (X - X_0))) + ((A_{\text{slow}} - Y_0) * (1 - \exp(-K_{\text{slow}} * (X - X_0))))$  with  $Y_0$ , the start amplitude, usually 0 mN;  $A_{\text{fast}}$ , the amplitude and  $K_{\text{fast}}$ , the rate constant of the fast component ( $K_{\text{fast}} = 1/\tau_{\text{fast}}$ );  $A_{\text{slow}}$ , the amplitude and  $K_{\text{slow}}$ , the rate constant of the slow component ( $K_{\text{slow}} = 1/\tau_{\text{slow}}$ );  $X_0$ , the time at which force increases.

Because tonic contractions by PE tended to increase for long (>15 minutes) periods of time, the contraction, measured at 650 s was considered as “steady-state” contraction. Concentration-response curves were fitted with sigmoidal concentration-response equations with variable slope, which revealed maximal responses ( $E_{\text{max}}$ ) and the logarithm of the concentration resulting in 50% of the maximal excitatory or inhibitory effect ( $\text{EC}_{50}$  or  $\text{IC}_{50}$ ) for each vessel segment. A two-way ANOVA with Bonferroni post test (concentration-response curves) and paired or unpaired  $t$ -test (GraphPad Prism) were used to compare means of the different experimental groups. A 5% level of significance was selected.

## Materials

Sodium pentobarbital was obtained from Sanofi (Brussels, Belgium), indomethacin from CERTA (Belgium),  $N^{\omega}$ -nitro-L-arginine methyl ester (L-NAME),  $N^{\omega}$ -nitro-L-arginine (L-NNA) and nifedipine from Sigma (Bornem, Belgium), Fura 2-AM from Molecular Probes (Invitrogen, Merelbeke, Belgium), 2-aminoethoxydiphenyl borate (2-APB), BAY K8644, lev-cromakalim, glibenclamide, cyclopiazonic acid (CPA), diltiazem, verapamil from TOCRIS (Bristol, United Kingdom).

## Results

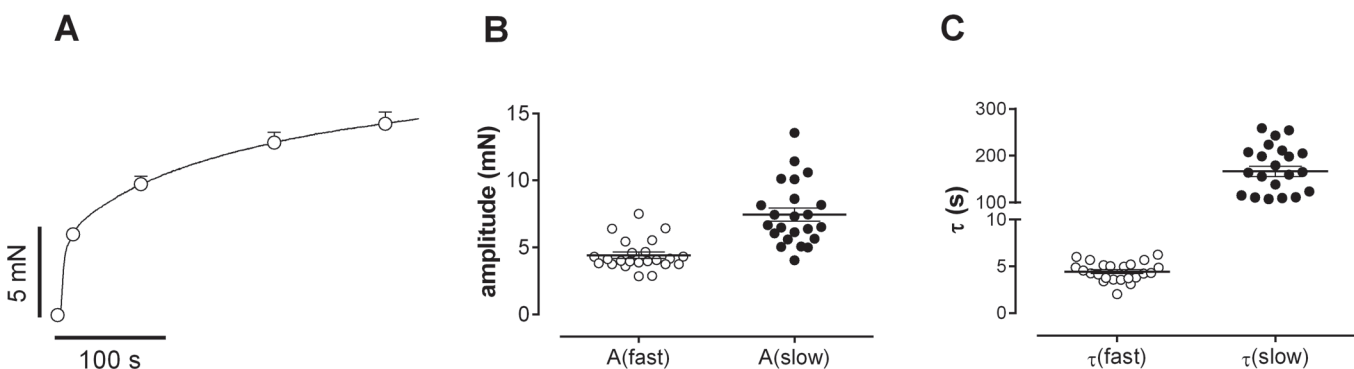
### PE-induced contractions

In the organ bath, 1  $\mu$ M PE caused isometric contractions, which followed a bi-exponential time course (Fig. 1, [22]). Besides a fast phase of contraction with an amplitude of  $4.43 \pm 0.42$  mN ( $37.7 \pm 0.6\%$  of total contraction) and a time constant of  $4.4 \pm 0.2$  s, there was a slow phase of contraction with an amplitude of  $7.47 \pm 0.49$  mN ( $62.3 \pm 0.6\%$  of total contraction) and a time constant of  $161 \pm 11$  s.

To correlate the fast and slow force components with intracellular  $Ca^{2+}$  signals in the VSMC,  $Ca^{2+}$  and isometric tension were measured simultaneously in endothelium-denuded aortic segments. The addition of 1  $\mu$ M PE increased both intracellular  $Ca^{2+}$  and tension, but the temporal relationship between both parameters was complex (Fig. 2A).  $Ca^{2+}$  showed an initial rapid spike followed by a slower increase and then a decrease to plateau values above baseline values. Tension increased to plateau values and then slightly decreased.

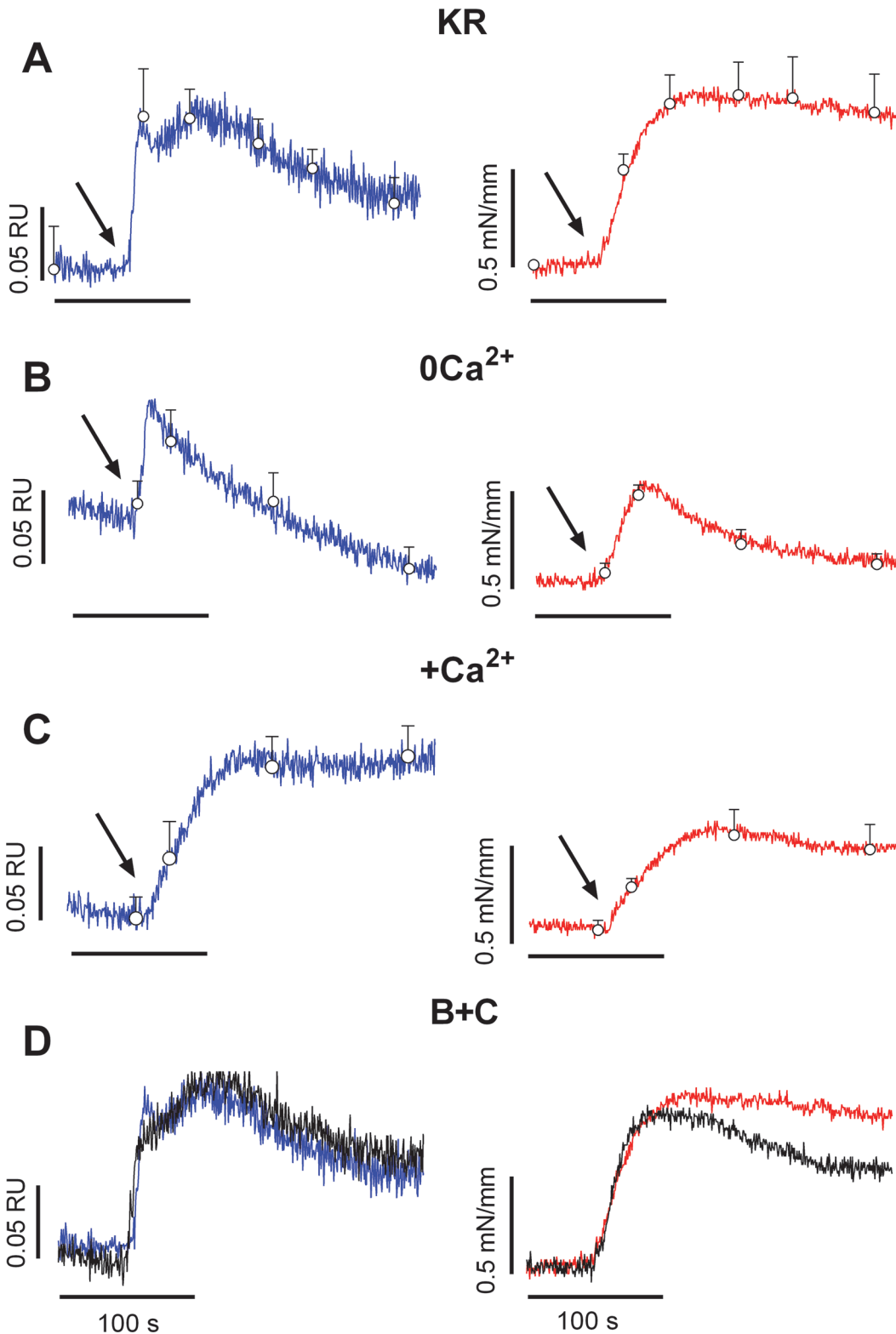
Removal of external  $Ca^{2+}$  (0Ca) caused a significant decrease of basal  $[Ca^{2+}]_i$  from  $0.91 \pm 0.03$  to  $0.81 \pm 0.02$  RU ( $p < 0.005$ ,  $n = 6$ ) and of basal tension from  $0.52 \pm 0.02$  to  $0.40 \pm 0.05$  mN/mm ( $p < 0.05$ ,  $n = 6$ ). Application of 1  $\mu$ M PE in 0Ca induced transient parallel  $[Ca^{2+}]_i$  and isometric tension increase (Fig. 2B). The peak  $[Ca^{2+}]_i$  was  $0.19 \pm 0.06$  RU and of similar magnitude as in the presence of external  $Ca^{2+}$  i.e.  $0.17 \pm 0.04$  RU ( $p > 0.05$ ). Peak PE-elicited tension of  $0.72 \pm 0.09$  mN/mm was significantly decreased compared with the peak tension of  $1.13 \pm 0.15$  mN/mm ( $p < 0.05$ ) in the presence of  $Ca^{2+}$ .

Re-admission of 3.5 mM  $Ca^{2+}$  to 0Ca containing PE (Fig. 2C) caused again a parallel time-dependent increase of  $[Ca^{2+}]_i$  and tension to values of  $0.18 \pm 0.02$  RU and  $0.78 \pm 0.11$  mN/mm. When the SR  $Ca^{2+}$  release and corresponding force development (Fig. 2B) as well as the  $Ca^{2+}$



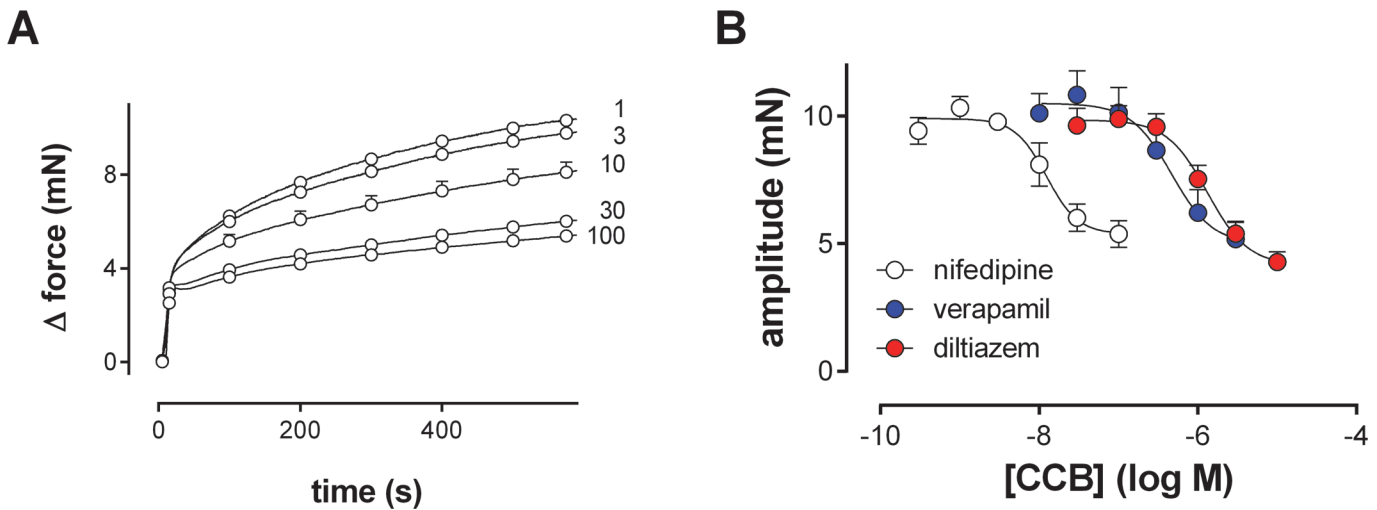
**Fig 1. Kinetic analysis of the isometric contractions of mouse aortic segments by 1  $\mu$ M PE.** Aortic segments mounted in organ baths produced isometric contractions as shown in (A). Bi-exponential fits of the contractions in A revealed amplitudes (B) and time constants (C) of fast and slow components. Data were acquired at 10 Hz, specific data points (open circles) are shown as mean  $\pm$  sem ( $n = 23$ ).

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**Fig 2. Temporal relationship between intracellular  $\text{Ca}^{2+}$  signal and isometric contraction by PE.**  $\text{Ca}^{2+}$  signal (left, blue) and corresponding isometric tension development (right, red) induced by  $1 \mu\text{M}$  PE (arrow indicates addition) in myograph-mounted endothelium-denuded mouse aortic segments in normal KR solution (A, KR), in  $\text{Ca}^{2+}$ -free KR (B,  $0\text{Ca}^{2+}$ ) and upon re-addition of  $3.5 \text{ mM}$   $\text{Ca}^{2+}$  to the  $\text{Ca}^{2+}$ -free KR solution containing  $1 \mu\text{M}$  PE (C,  $+\text{Ca}^{2+}$ ). (D) Sum (B+C) of PE-responses in the absence of  $\text{Ca}^{2+}$  and upon re-admission of  $3.5 \text{ mM}$   $\text{Ca}^{2+}$  (black) compared with the  $[\text{Ca}^{2+}]_i$  and tension signals by PE in control (blue and red). Data were acquired at  $1 \text{ Hz}$ , some data points (open circles) are shown as mean  $\pm$  sem ( $n = 6$ ).

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**Fig 3. Effects of VGCC blockers on isometric contractions by PE.** Nifedipine, verapamil and diltiazem partly inhibit isometric contractions induced by 1  $\mu$ M PE in organ bath-mounted aortic segments. A) Contractions induced by 1  $\mu$ M PE after incubating the segments with 1 to 100 nM nifedipine (n = 4); B)  $Ca^{2+}$  channel blocker (CCB) concentration-response curves for the inhibition of contractions induced by 1  $\mu$ M PE.

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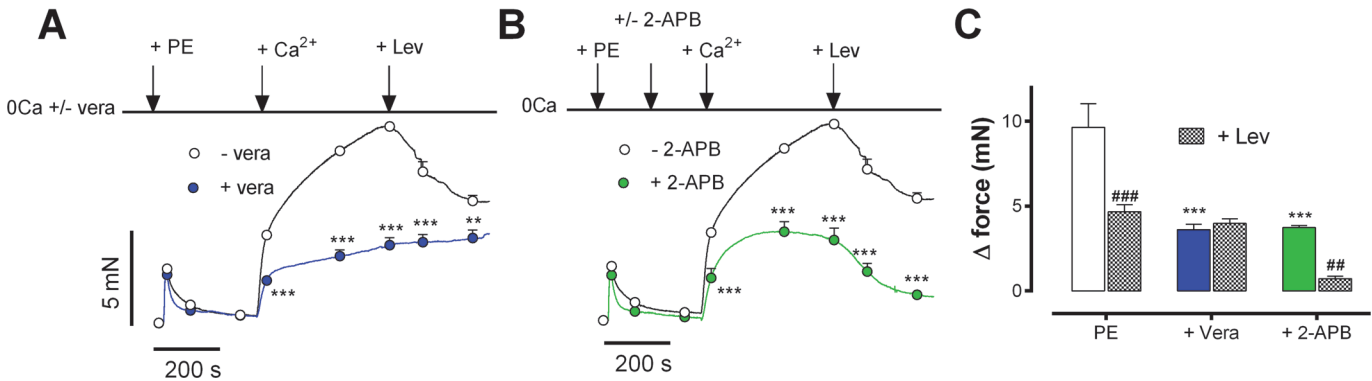
influx signal and corresponding contraction (Fig. 2C) were pair-wise summated,  $Ca^{2+}$  and tension signals as shown in Fig. 2D were very similar to the PE responses in control.

These results confirmed that the  $Ca^{2+}$  signals induced by 1  $\mu$ M PE in normal KR solution consisted of an initial release of  $Ca^{2+}$  from internal stores and a subsequent influx of extracellular  $Ca^{2+}$  and that both  $Ca^{2+}$  signals were paralleled by a temporally-related force signal [13].

### Analysis of the PE-elicited contraction

**L-type  $Ca^{2+}$  channels.** Nifedipine (1–100 nM), verapamil (30–3000 nM) or diltiazem (0.1–10  $\mu$ M), as representatives of the different classes of L-type  $Ca^{2+}$  channel blockers, did not reduce the fast phase of the PE (1  $\mu$ M)-induced contraction (Fig. 3A), but effectively reduced the slow phase of contraction. Nifedipine inhibited  $49 \pm 3\%$  of the steady-state contraction with an  $IC_{50}$  of  $-7.94 \pm 0.07$   $-\log M$ , diltiazem  $59 \pm 3\%$  with  $IC_{50}$  of  $-5.90 \pm 0.04$   $-\log M$  and verapamil  $57 \pm 5\%$  with an  $IC_{50}$  of  $-6.31 \pm 0.10$   $-\log M$ . These results again confirm that the fast phase of contraction is not due to extracellular  $Ca^{2+}$  influx via L-type  $Ca^{2+}$  channels, but most probably to intracellular  $Ca^{2+}$  release from the SR. The slow phase of the PE-contraction can be attributed for 50 to 60% to L-type  $Ca^{2+}$  influx of for 40 to 50% to  $Ca^{2+}$  influx via non-L-type  $Ca^{2+}$  channels.

**Non-selective cation channels (NSCC).** The contribution of  $Ca^{2+}$  influx via L-type and non-L-type  $Ca^{2+}$  channels to the contraction by PE was further investigated by incubation of the segments with the L-type  $Ca^{2+}$  channel blocker verapamil and/or the putative non-selective cation channel blocker 2-aminoethoxydiphenyl borate (2-APB) [23]. In  $0Ca^{2+}$ , 3  $\mu$ M verapamil did not affect the PE-induced phasic contraction, but significantly reduced the +Ca contraction (Fig. 4A). At 600 s, the contraction was inhibited by  $59 \pm 10\%$  (Fig. 4C). Because 2-APB inhibited the transient isometric contraction by PE in the absence of external  $Ca^{2+}$  in line with its capacity to block the SR  $IP_3$  receptor [23] (see also S1 Fig, S1 Text), it was applied after this transient contraction. The +Ca contraction in the presence of 100  $\mu$ M 2-APB typically increased to a maximum at 200 to 250 s and then decreased again (Fig. 4B). At 600 s, 100  $\mu$ M 2-APB suppressed the +Ca contraction by  $59 \pm 6\%$ . Finally, the combination of verapamil and 2-APB inhibited the +Ca contraction completely, indicating that the contraction remaining in

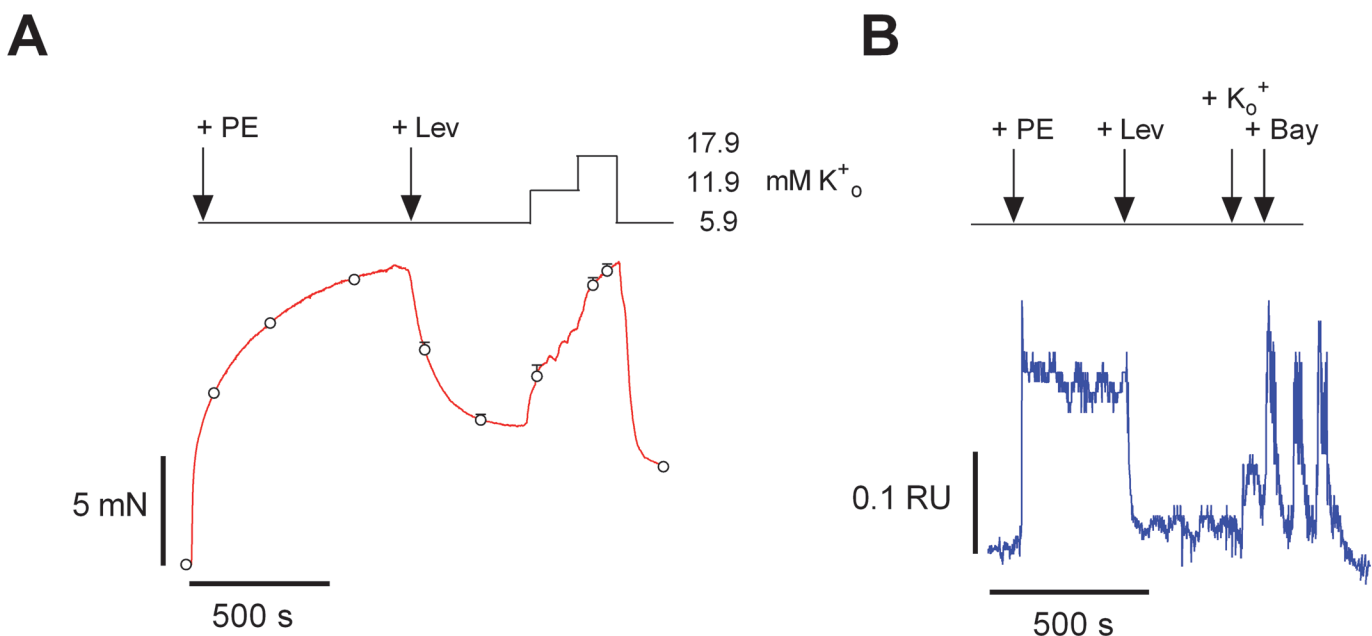


**Fig 4. VGCC and NSCC contribute to the Ca<sup>2+</sup> influx-mediated contraction elicited by PE.** The PE-induced contraction following addition of 3.5 mM Ca<sup>2+</sup> to 0Ca (n = 4) in the absence (black) and presence of 3 μM verapamil (Vera, blue) and B) in the presence of 100 μM 2-APB (green). Verapamil and 2-APB were added before and after the transient contraction by PE respectively. After 600 s, 100 nM levcromakalim (Lev) was added in each condition. C) Mean maximal contractions in control (PE), 3 μM verapamil (+Vera) and 100 μM 2-APB (+2-APB) in the absence (left bars) and presence (+ Lev, hatched bars) of 300 nM levcromakalim. \*\*\*: P<0.001, versus PE; ##, ###: P<0.01 and 0.001, + Lev versus control

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the presence of a maximally effective concentration of 2-APB was due to L-type Ca<sup>2+</sup> influx and the contraction remaining in the presence of a maximal dose of verapamil was caused by Ca<sup>2+</sup> influx via non-selective cation channels.

Levcromakalim, an ATP-dependent K<sup>+</sup> channel agonist, hyperpolarises the VSMC V<sub>m</sub> to the K<sup>+</sup>-equilibrium potential (E<sub>K</sub>: -80 to -85 mV) and decreases window Ca<sup>2+</sup> influx [9]. Applied on top of the PE contraction, levcromakalim relaxed PE-induced contractions (Figs. 4 and 5A) and decreased intracellular Ca<sup>2+</sup> (Fig. 5B). Remarkably, levcromakalim did not affect the contraction in the presence of 3 μM verapamil, but significantly reduced the contraction in



**Fig 5. Modulation of the PE-induced contraction by window VGCC Ca<sup>2+</sup> influx.** A) The relaxation of the isometric pre-contraction (1 μM PE, red, organ bath) with addition of 300 nM levcromakalim (+ Lev) is reversed by increasing extracellular K<sup>+</sup> (K<sub>o</sub><sup>+</sup>) to 11.9 and 17.9 mM (n = 4). B) Representative example of a myograph experiment, showing the increase of intracellular Ca<sup>2+</sup> (blue) with 1 μM PE (+ PE), decrease with 300 nM levcromakalim (+ Lev) and increase with supplementary addition of 10 mM external K<sup>+</sup> (+ K<sub>o</sub><sup>+</sup>) in combination with 30 nM BAY K8644 (+ Bay).

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the presence of 100  $\mu$ M 2-APB by  $81 \pm 4\%$  (Fig. 4B). The inhibition of  $\text{Ca}^{2+}$  influx and contraction could be completely reversed by adding 50  $\mu$ M glibenclamide (inhibitor of  $\text{K}^+$  (ATP) channels, data not shown), by increasing extracellular  $\text{K}^+$  from 5.9 to 12 and 18 mM (Fig. 5A) or, as shown for intracellular  $\text{Ca}^{2+}$ , by combining  $\text{K}^+$  increase with the L-type  $\text{Ca}^{2+}$  channel agonist BAY K8644 (Fig. 5B). These results suggest that the contraction in the presence of 2-APB is voltage-dependent and most probably due to L-type  $\text{Ca}^{2+}$  influx within the voltage range of its window.

**$\text{Ca}^{2+}$  influx-mediated contraction and resting membrane potential.** In basal, non-stimulated conditions, there is a time-independent, baseline  $\text{Ca}^{2+}$  influx via L-type  $\text{Ca}^{2+}$  channels, which can be inhibited by removal of extracellular  $\text{Ca}^{2+}$ , by hyperpolarization with levcromakalim or by L-type  $\text{Ca}^{2+}$  channel blockers [9,10]. Hence, if PE induces depolarisation and opening of L-type  $\text{Ca}^{2+}$  channels [15–19], the PE-elicited contraction of VSMC is expected to depend on shifts of  $V_m$  in the hyper- or depolarizing direction.

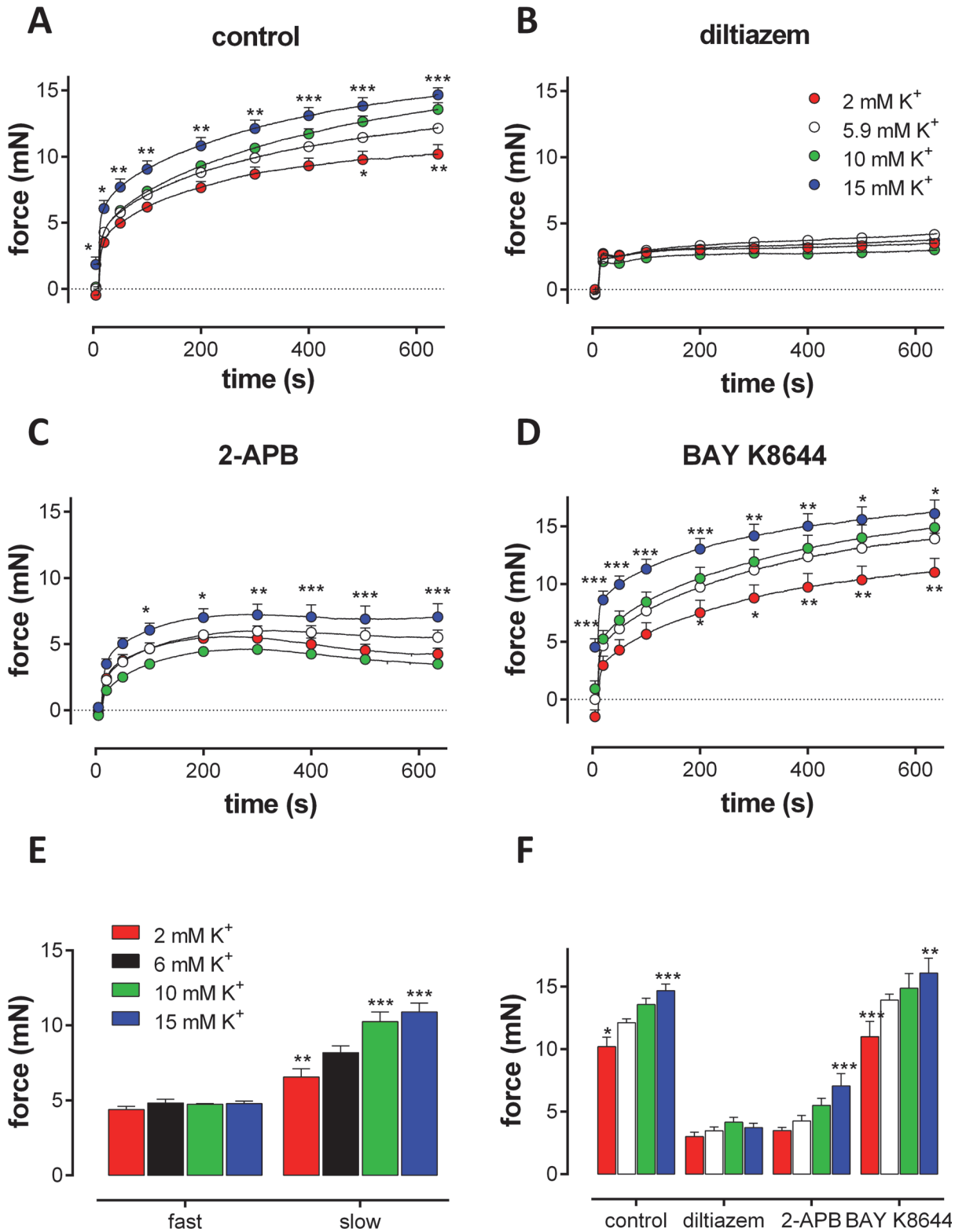
Hyper- or depolarization of  $V_m$  of VSMC, by changing the external  $\text{K}^+$  concentration from 2 to 15 mM, may promote or inhibit the window  $\text{Ca}^{2+}$  influx [9,24] and the PE-elicited contraction. It was observed that not only the PE-induced isometric force but also the basal force was  $\text{K}^+$ -dependent ( $-0.45 \pm 0.18$  mN at 2 mM  $\text{K}^+$ ;  $+0.14 \pm 0.20$  mN at 10 mM  $\text{K}^+$  and  $+1.85 \pm 0.58$  mN at 15 mM  $\text{K}^+$ ). PE-induced isometric contraction at the different  $\text{K}^+$  concentrations displayed a bi-exponential time course (Fig. 6A, see also Fig. 1). Whereas the fast contraction phase was  $\text{K}^+$ -independent, the slow phase changed with extracellular  $\text{K}^+$  and, hence, was voltage-dependent (Fig. 6E). The slow phase amplitude increased significantly from  $84 \pm 6\%$  at 2 mM  $\text{K}^+$  to  $112 \pm 5\%$  and  $121 \pm 6\%$  at 10 and 15 mM  $\text{K}^+$  with respect to 5.9 mM  $\text{K}^+$ .

As was shown in the levcromakalim experiments, the isometric contraction of PE following inhibition of the L-type  $\text{Ca}^{2+}$  influx with 30  $\mu$ M diltiazem was not dependent on the extracellular  $\text{K}^+$  concentration (Fig. 6B, F). However, in the presence of 100  $\mu$ M 2-APB, the tonic contraction increased with the external  $\text{K}^+$  concentration (Fig. 6C, F). Finally, when the L-type  $\text{Ca}^{2+}$  influx was stimulated with 30 nM BAY K8644, an agonist of L-type  $\text{Ca}^{2+}$  channels, the tonic PE contraction showed a similar  $\text{K}^+$ -dependency as in control or 2-APB conditions (Fig. 6D, F).

**Properties of non-selective cation channels (NSCC).**  $\text{Ca}^{2+}$  influx via non-selective cation channels could be increased by emptying CPA-sensitive  $\text{Ca}^{2+}$  stores before inducing PE-induced contractions. In aortic segments, 10  $\mu$ M CPA causes inhibition of the CPA-sensitive  $\text{Ca}^{2+}$  store  $\text{Ca}^{2+}$  pump [25] and leads to a large increase of intracellular  $\text{Ca}^{2+}$  in the VSMC (Fig. 7A). When VSMC intracellular  $\text{Ca}^{2+}$  and isometric contraction were measured simultaneously, it was observed that in the absence of extracellular  $\text{Ca}^{2+}$ , CPA induced a small and slow transient increase of intracellular  $\text{Ca}^{2+}$ , which was not accompanied by an increase of force (Fig. 7B). In contrast, 1  $\mu$ M PE caused a small and rapid transient increase of internal  $\text{Ca}^{2+}$ , which elicited a substantial phasic tension. Re-addition of  $\text{Ca}^{2+}$  (+Ca) caused a large influx of  $\text{Ca}^{2+}$  from the extracellular medium for CPA alone when compared with PE alone but this large  $\text{Ca}^{2+}$  influx was accompanied by a significantly smaller increase in force than by PE. Finally, even this large increase of intracellular  $\text{Ca}^{2+}$ , could in the presence of 1  $\mu$ M PE, not substantially increase the force induced by PE alone. As summarized in the calcium-force graph of Fig. 7C, CPA mainly causes  $\text{Ca}^{2+}$  release and influx without majorly affecting force, whereas  $\text{Ca}^{2+}$  release and influx by PE causes a significant temporally-related increase in force. When CPA and PE were combined, both a large  $\text{Ca}^{2+}$  influx and a large force increase were simultaneously present, but not significantly larger than these of CPA ( $\text{Ca}^{2+}$ ) or PE (tension) alone.

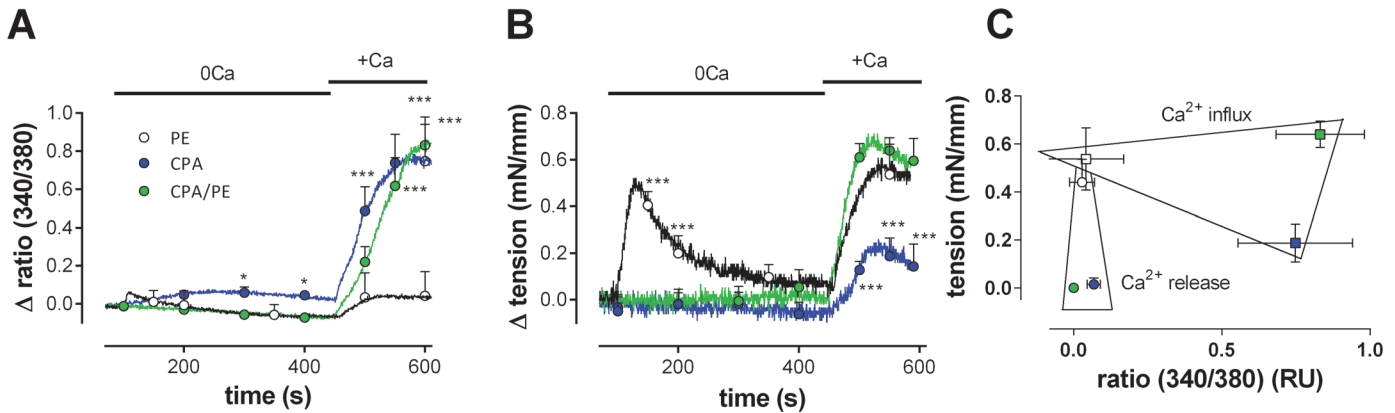
According to these data, CPA mobilizes  $\text{Ca}^{2+}$  from non-contractile  $\text{Ca}^{2+}$  stores and induces non-contractile  $\text{Ca}^{2+}$  influx. CPA did not significantly increase the tension induced by PE alone, but it empties  $\text{Ca}^{2+}$  stores, and thereby may affect the contraction induced by PE. Fig. 8A shows isometric contractions induced by 1  $\mu$ M PE following addition of extracellular





**Fig 6. External K<sup>+</sup> modulation of the PE-induced contraction.** Isometric contractions induced by 1  $\mu$ M PE at different extracellular K<sup>+</sup> concentrations in the organ bath. Contractions were measured at 2, 5.9, 10 and 15 mM extracellular K<sup>+</sup> in control (A) and in the presence of 35  $\mu$ M diltiazem (B), 100  $\mu$ M 2-APB (C) or 30 nM BAY K8644 (D). Bi-exponential analysis of the force development by PE revealed the amplitudes of fast and slow force components in control (E). The “steady-state” tonic contractions at 650 s in the absence (control) or presence of diltiazem, 2-APB or BAY K8644 are summarized in (F). \*, \*\*, \*\*\*:  $P < 0.05$ , 0.01, 0.001 versus 5.9 mM K<sup>+</sup> (n = 4–5).

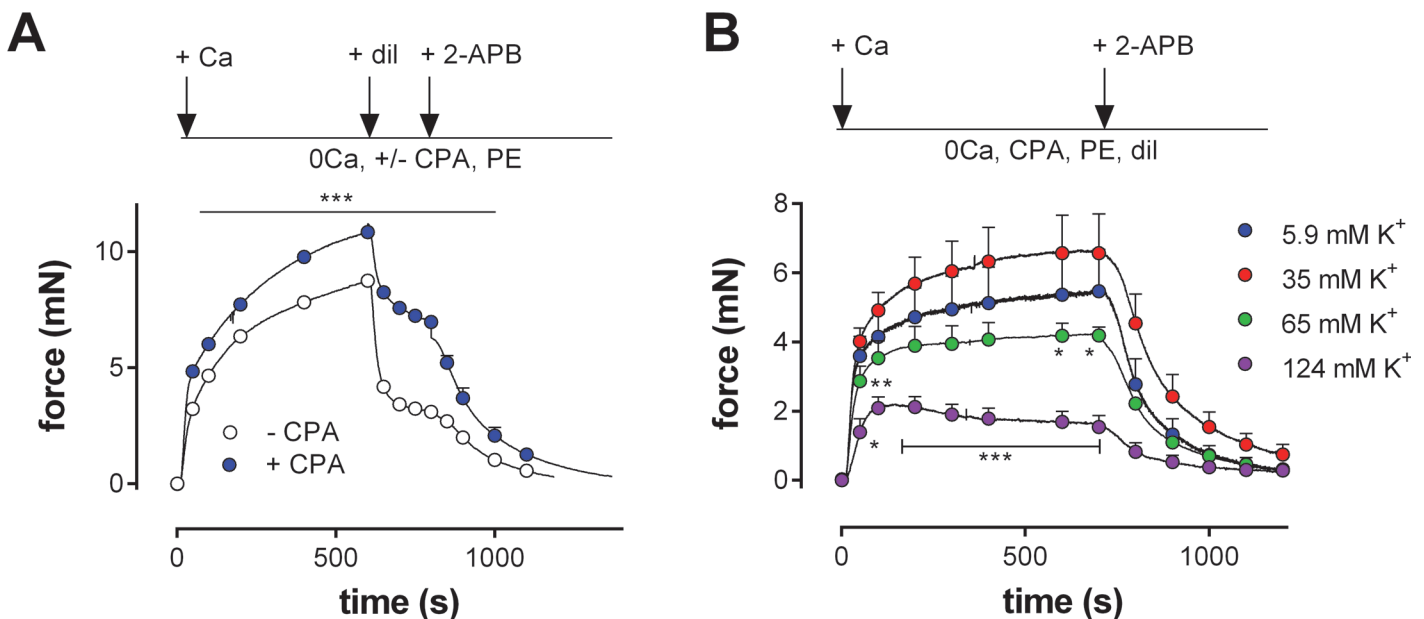
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**Fig 7. CPA and PE release intracellular  $\text{Ca}^{2+}$ , cause  $\text{Ca}^{2+}$  influx and produce force by different mechanisms.** Intracellular  $\text{Ca}^{2+}$  (A) and accompanying tension (B) were measured in aortic segments ( $n = 6$ ) in the absence (0Ca) and after re-addition of 3.5 mM  $\text{Ca}^{2+}$  (+Ca) in the presence of 1  $\mu\text{M}$  PE alone (black), in the presence of 10  $\mu\text{M}$  CPA alone (blue) or in the presence of the combination (green). C) Force-calcium graph for the data in A and B with the squares referring to the +Ca data ( $\text{Ca}^{2+}$  influx) and the circles to the 0Ca data ( $\text{Ca}^{2+}$  release). \*, \*\*\*,  $P < 0.05$ , 0.001 CPA or CPA/PE versus PE

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$\text{Ca}^{2+}$  (control) when segments were first incubated with 0Ca + 10  $\mu\text{M}$  CPA + 1  $\mu\text{M}$  PE to empty CPA-sensitive  $\text{Ca}^{2+}$  stores completely. In this condition, contraction increased to  $124 \pm 2\%$  ( $P < 0.001$ ,  $n = 4$ ) of the contraction in the presence of PE alone. Diltiazem (35  $\mu\text{M}$ ) inhibited this contraction by  $65 \pm 1\%$  in the absence and by  $36 \pm 2\%$  ( $P < 0.01$ ), in the presence of 10  $\mu\text{M}$  CPA. Although CPA caused the mean contraction by PE to increase with about 24%, the contraction changed from mainly VGCC-mediated to mainly NSCC-mediated. Combining diltiazem with 50  $\mu\text{M}$  2-APB inhibited the contraction completely, suggesting that in the presence of CPA the relative contribution of NSCC was significantly increased from 35 to 64%.



**Fig 8. Modulation of the PE-induced contraction by NSCC  $\text{Ca}^{2+}$  influx.** Isometric contractions induced by  $\text{Ca}^{2+}$  re-addition (+ Ca) to organ bath mounted aortic segments ( $n = 4$ ) incubated in 0Ca in the presence of 1  $\mu\text{M}$  PE A) with (blue) or without (white) 10  $\mu\text{M}$  CPA (blue) or B) with 10  $\mu\text{M}$  CPA and 35  $\mu\text{M}$  diltiazem (Dil) at 5.9 (blue), 35 (red), 65 (green) and 124 (purple) mM  $\text{K}^+$ . In A) 35  $\mu\text{M}$  diltiazem (+ dil) was added after 10 minutes to measure the relative amount of VGCC  $\text{Ca}^{2+}$  influx to the contractions. Finally, 50  $\mu\text{M}$  2-APB (+ 2-APB) was added to inhibit NSCC. \*\*\*,  $P < 0.001$  CPA versus control. In B) 50  $\mu\text{M}$  2-APB was added after 16 minutes to inhibit the contraction due to NSCC  $\text{Ca}^{2+}$  influx. \*, \*\*, \*\*\*,  $P < 0.05$ , 0.01, 0.001  $\text{K}^+$  versus control.

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The large contribution of NSCCs to the contraction by PE in the presence of CPA is further illustrated in [Fig. 8B](#), in which the voltage-dependent properties of the NSCC-mediated PE-induced contractions were investigated in more detail. Previous results suggested that the contraction by NSCC  $\text{Ca}^{2+}$  influx was nearly voltage-independent for  $\text{K}^+$  concentrations between 2 and 15 mM. Because NSCC have their reversal potential around 0 mV, contractions by 1  $\mu\text{M}$  PE were measured at more depolarized membrane potentials. These were calculated to be -66 mV at 5.9 mM  $\text{K}^+$ , -34 mV at 35 mM  $\text{K}^+$ , -19 mV at 65 mM  $\text{K}^+$  and -3 mV at 124 mM  $\text{K}^+$  [9]. Segments were incubated with 0Ca, 10  $\mu\text{M}$  CPA, 35  $\mu\text{M}$  diltiazem and 1  $\mu\text{M}$  PE at these extracellular  $\text{K}^+$  concentrations. Thereby, 35  $\mu\text{M}$  diltiazem prevented the activation of L-type  $\text{Ca}^{2+}$  influx upon re-addition of extracellular  $\text{Ca}^{2+}$  to the depolarized segments. Moreover, the relative contribution of NSCC was increased by emptying CPA-sensitive  $\text{Ca}^{2+}$  stores (see [Fig. 8A](#)). Hence, the isometric contractions shown in [Fig. 8B](#), are only due to  $\text{Ca}^{2+}$  influx via NSCC and, as such, could be completely inhibited by 50  $\mu\text{M}$  2-APB. The data show a voltage-dependent reduction in the isometric NSCC-mediated contraction at 65 mM  $\text{K}^+$  and 124 mM  $\text{K}^+$ , suggestive for NSCC activation in the presence of PE. That CPA directly stimulates NSCC is illustrated in [Figs. 9A and B](#). Isometric contractions were elicited by 1  $\mu\text{M}$  PE in the presence of 35  $\mu\text{M}$  diltiazem or 50  $\mu\text{M}$  2-APB, after which 10  $\mu\text{M}$  CPA was added. The contraction in the presence of diltiazem, which is attributed to NSCC  $\text{Ca}^{2+}$  influx, was increased by 10  $\mu\text{M}$  CPA. This contraction was completely inhibited by 50  $\mu\text{M}$  2-APB. On the other hand, the PE-induced contraction after incubation of the aortic segments with 2-APB, is mainly due to VGCC  $\text{Ca}^{2+}$  influx and is significantly decreased by 10  $\mu\text{M}$  CPA. This attenuated contraction was inhibited with 35  $\mu\text{M}$  diltiazem. When the segments were incubated with a combination of 50  $\mu\text{M}$  2-APB with 35  $\mu\text{M}$  diltiazem, 1  $\mu\text{M}$  PE caused a transient contraction, which was not increased upon subsequent addition of 10  $\mu\text{M}$  CPA ([Figs. 9C and D](#)).

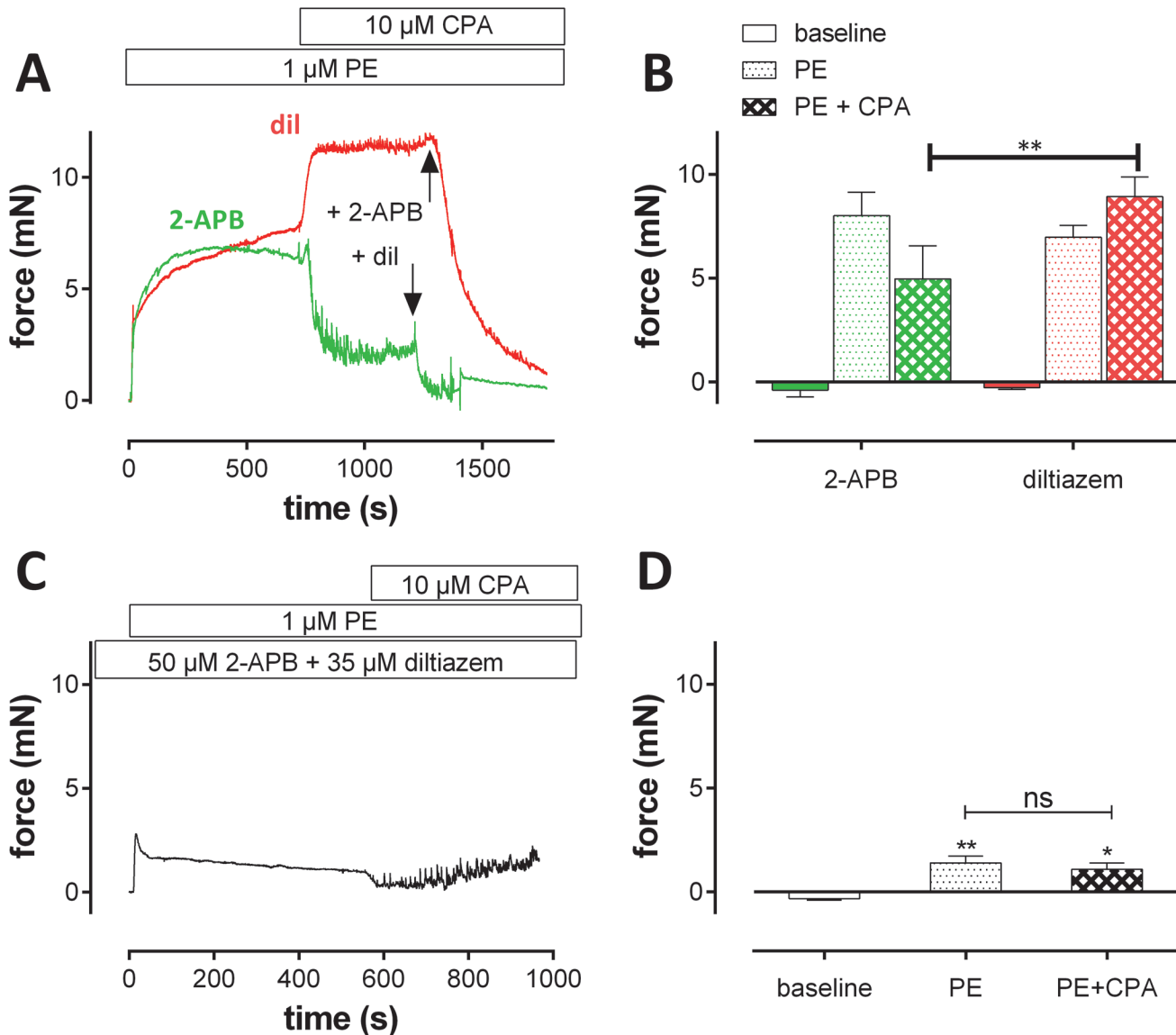
## Discussion

In this study, we show that isometric contractions of mouse aortic segments by  $\alpha_1$ -adrenoceptor stimulation with PE involve complex interactions between  $\text{Ca}^{2+}$  release from SR  $\text{Ca}^{2+}$  stores and concomitant activation of  $\text{Ca}^{2+}$  influx via VGCC and NSCC. The biphasic contractions display a fast and transient component, which corresponds to the intracellular  $\text{Ca}^{2+}$  release from the SR. The slow and sustained component is attributed to  $\text{Ca}^{2+}$  influx from the extracellular medium and determines the steady-state contraction.

Hyperpolarization, moderate depolarization or pharmacological VGCC stimulation affect the slow but not the fast force component of the PE-induced contraction, especially after inhibition of NSCC. This is in line with the properties of window L-type  $\text{Ca}^{2+}$  influx via VGCC. PE-induced contractions due to  $\text{Ca}^{2+}$  influx via NSCC are activated by causing  $\text{Ca}^{2+}$  release from non-contractile  $\text{Ca}^{2+}$  stores.

### Fast component of PE-elicited contraction

As demonstrated for depolarization with extracellular  $\text{K}^+$  elevation [9,10], PE-induced contractions displayed a bi-exponential time course with a fast and slow force component. Contrary to  $\text{K}^+$ -depolarized segments, where the fast, transient component corresponded to a population of L-type  $\text{Ca}^{2+}$  channels that quickly activated and completely inactivated [9], the fast and transient component of the PE-response was voltage-independent, independent of external  $\text{Ca}^{2+}$ , could not be inhibited with L-type  $\text{Ca}^{2+}$  channel blockers, but was sensitive to 2-APB. Experiments in the absence of external  $\text{Ca}^{2+}$  further indicated that the fast force component was temporally related to an increase of intracellular  $\text{Ca}^{2+}$ . This component probably corresponds to



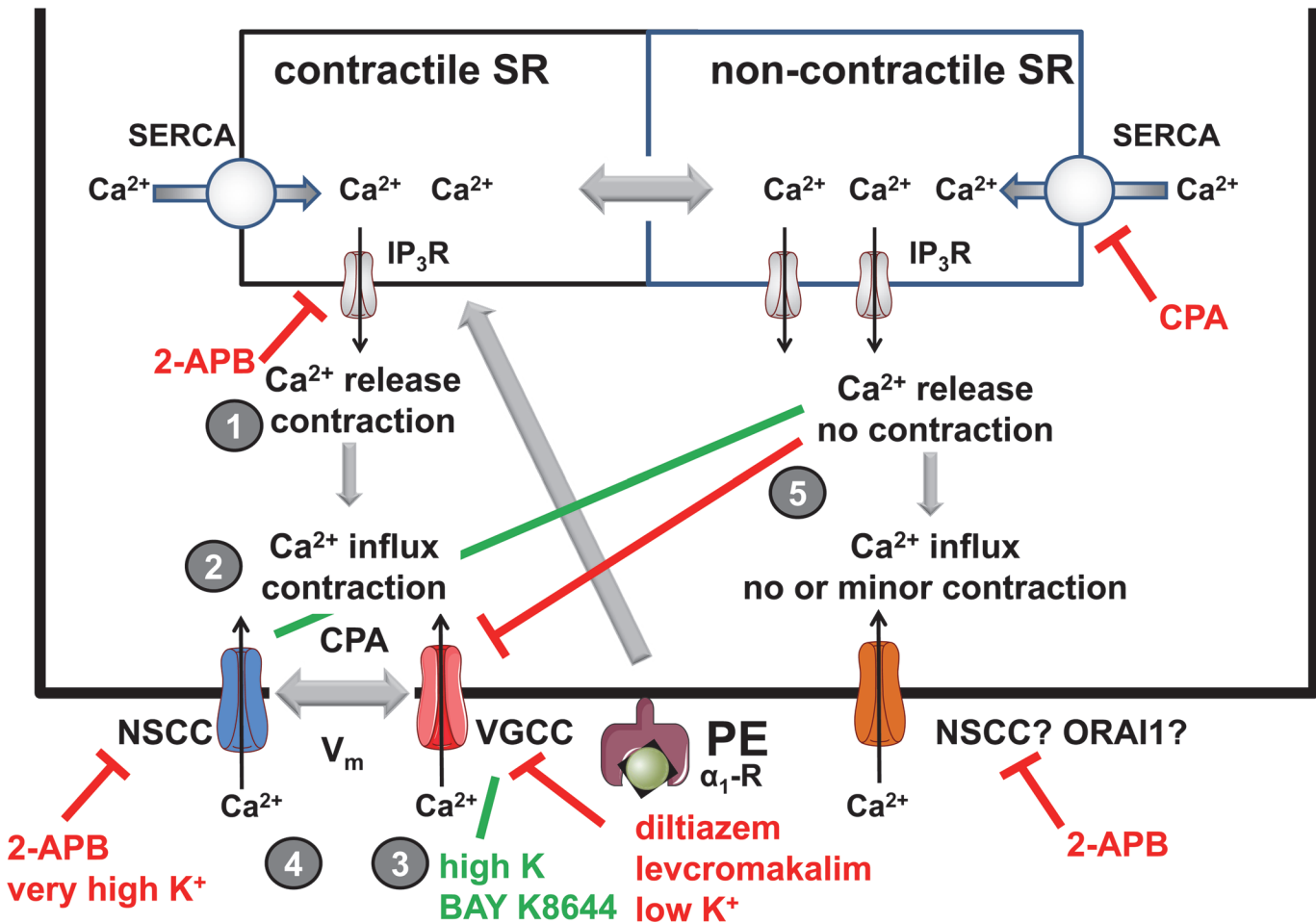
**Fig 9. Effects of intracellular  $Ca^{2+}$  increase by CPA on contractions by PE in the presence of diltiazem or 2-APB.** A) Representative example of PE-induced contractions after incubation of a segment with 35 μM diltiazem (red) or 50 μM 2-APB (green). 10 μM CPA was added in both conditions as indicated, after which 50 μM 2-APB (arrow, +2-APB) was added to the diltiazem condition and diltiazem (arrow, +dil) to the 2-APB condition. Figure B summarizes the results (n = 5). In C, the segments were incubated with 50 μM 2-APB and 35 μM diltiazem before the challenge with 1 μM PE. Subsequent addition of 10 μM CPA did not increase the PE-induced contraction. Part D summarizes the results with the change in isometric force by the diltiazem/2-APB in baseline conditions or following addition of 1 μM PE alone (PE) or in combination with 10 μM CPA (PE+CPA) (n = 4). \*, \*\*: p < 0.05, 0.01 2-APB versus diltiazem condition in B or PE/PE+CPA versus baseline in D.

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$Ca^{2+}$  release from contractile intracellular  $Ca^{2+}$  stores via  $IP_3$ -sensitive receptors (Fig 10, event 1) [15].

### Slow component of PE-elicited contraction

According to the kinetic and pharmacological analysis of PE-induced force development in mouse aorta, the slow component contributed for 60% to the total contraction. In rat aorta, this was about 25% [22], indicating that the store-dependent  $Ca^{2+}$  release and concomitant contraction is far more important in rat than in mouse aorta. From our experiments, however,



**Fig 10. Scheme showing the  $\text{Ca}^{2+}$  mediated processes involved in  $\alpha_1$ -adrenoreceptor stimulation of mouse aortic segments with PE.** PE causes phasic  $\text{Ca}^{2+}$  increase and concomitant contraction by releasing  $\text{Ca}^{2+}$  from the SR (event 1). This is accompanied by influx of  $\text{Ca}^{2+}$  via complex interactions between NSCC and VGCC and the steady-state contraction by PE is determined by the relative contribution of window  $\text{Ca}^{2+}$  influx via VGCC (very voltage-dependent) and  $\text{Ca}^{2+}$  influx via NSCC (less voltage-dependent) (event 2). Window VGCC  $\text{Ca}^{2+}$  influx and related contraction are inhibited by diltiazem, membrane potential repolarization with  $\text{K}^+$  or levcromakalim and  $\text{Ca}^{2+}$  release from non-contractile  $\text{Ca}^{2+}$  stores by CPA and stimulated by high  $\text{K}^+$  and BAY K844 (event 3). NSCC  $\text{Ca}^{2+}$  influx and related contraction are inhibited by 2-APB and very high  $\text{K}^+$  (strong depolarization to  $-20$  mV or less negative) and stimulated by  $\text{Ca}^{2+}$  release from non-contractile  $\text{Ca}^{2+}$  stores with CPA (event 4). CPA causes high  $\text{Ca}^{2+}$  release from a non-contractile compartment of the SR (event 5). Emptying of the non-contractile  $\text{Ca}^{2+}$  store with CPA causes large  $\text{Ca}^{2+}$  influx, which is accompanied with minor contraction in the absence of PE, but which turns the PE-induced contraction to one that is mainly mediated by NSCC. This points to a complex interaction between the non-contractile and contractile SR  $\text{Ca}^{2+}$  stores and their refilling via VGCC and/or different NSCC.

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it was clear that the slow force development, together with  $\text{Ca}^{2+}$  sensitizing mechanisms, determined the steady-state force. Hence, the relative contribution of fast and slow components to the total contraction provides information only on the amount of store-dependent  $\text{Ca}^{2+}$  release with respect to  $\text{Ca}^{2+}$  influx, but not on the steady-state contraction.

Force by high  $\text{K}^+$ -induced depolarization of mouse aortic segments [9] or by  $\alpha_1$ -adrenergic stimulation of rat renal artery [26] was completely inhibited by L-type  $\text{Ca}^{2+}$  channel blockers, suggesting that these contractions were completely due to VGCC  $\text{Ca}^{2+}$  influx. PE-induced contraction in mouse aortic segments was, however, only for 50% (nifedipine) to 60% (verapamil, diltiazem) inhibited by VGCC blockers, which indicates the occurrence of a non-L-type  $\text{Ca}^{2+}$  influx. The tonic component of PE-contracted segments was temporally related to a  $\text{Ca}^{2+}$  signal resulting from the influx of  $\text{Ca}^{2+}$  via different  $\text{Ca}^{2+}$  permeable ion channels (Fig. 10, event 2).

Similar results were obtained in penile small arteries of rat, where  $\alpha_1$ -adrenergic constriction with PE also involved  $\text{Ca}^{2+}$  entry via both L-type (50%) and 2-APB (50%)-sensitive receptor-operated channels, as well as  $\text{Ca}^{2+}$  sensitization mechanisms [27]. Of the four mechanisms at the basis of PE-induced contraction in rat tail artery, i.e. 1) depolarization and  $\text{Ca}^{2+}$  influx via VGCC; 2)  $V_m$ -independent  $\text{Ca}^{2+}$  influx probably via direct activation of VGCC; 3) increased  $\text{Ca}^{2+}$  sensitivity of the contractile elements and 4) release of  $\text{Ca}^{2+}$  from intracellular stores [28], the second mechanism was different in mouse aortic segments. The “voltage-independent” component occurred in the presence of VGCC blockers and could be inhibited with 2-APB, suggesting that it was mediated by NSCC permeable to  $\text{Ca}^{2+}$  [23]. One should, however, be cautious in interpreting the 2-APB data, because 2-APB has been described to block also VGCC (see S1 Fig, S1 Text) [29,30]. Nevertheless, the observations that 1) 2-APB completely inhibited the PE-induced contraction after eliminating the L-type  $\text{Ca}^{2+}$  influx with diltiazem and that 2) the contraction remaining in the presence of diltiazem was voltage-independent within a small voltage range or did not change with addition of levcromakalim, suggest that PE elicits  $\text{Ca}^{2+}$  influx via NSCC.

At physiological  $V_m$  or physiological  $\text{K}^+$  concentrations below 20 mM [31], the slow component of the contraction by PE increased gradually and significantly with small elevations of  $\text{K}^+$  or decreased with hyperpolarization induced by the ATP-dependent  $\text{K}^+$  channel opener levcromakalim (Fig. 10, event 3). Our results indicate that a small depolarization as with elevated  $\text{K}^+$  or a hyperpolarization as with lower  $\text{K}^+$  or with levcromakalim affects PE contraction because of an increase or decrease of the background window  $\text{Ca}^{2+}$  influx. This might be important in pathophysiological conditions such as hypertension or endothelial dysfunction where it has been described that VSMC are depolarised [24]. For example, mechanical stretch has been described to cause increase of intracellular  $\text{Ca}^{2+}$  via stretch-activated NSCC, depolarization and VGCC activation and these events may be amplified in hypertension [32–34].

CPA, an inhibitor of SERCA, causes intracellular  $\text{Ca}^{2+}$  increase without eliciting associated contractile responses. This may be due to the absence of a myofilament sensitizing effect of CPA, but can also be explained by assuming that the CPA-sensitive SERCA pump was predominantly expressed in the non-contractile SR  $\text{Ca}^{2+}$  store [25,35]. In mouse aortic segments, we confirmed that the intracellular VSMC  $\text{Ca}^{2+}$  elevation by SERCA inhibition with CPA, caused no or only small contractions (Fig. 10, event 4). Remarkably, although the contractile effect of CPA was negligible and the combination with PE did not or did only slightly increase the contractile effect of PE, it transformed the mainly VGCC-mediated PE-induced contraction to a mainly NSCC-mediated contraction. The PE-induced contraction mediated by VGCC  $\text{Ca}^{2+}$  influx, hence in the presence of 50  $\mu\text{M}$  2-APB, was not increased but inhibited by 10  $\mu\text{M}$  CPA. On the other hand, the PE-elicited contraction mediated by NSCC  $\text{Ca}^{2+}$  influx, hence in the presence of 35  $\mu\text{M}$  diltiazem, was stimulated by CPA. These data do not support the hypothesis that  $\text{Ca}^{2+}$  increase by CPA, similarly to PE, increases sensitivity of the myofilaments to  $\text{Ca}^{2+}$ . They suggest complex interactions between contractile and non-contractile SR  $\text{Ca}^{2+}$  stores and store-dependent influx of  $\text{Ca}^{2+}$  via ion channels (Fig. 10, event 3). Because these interactions might have important consequences for the re-filling of both  $\text{Ca}^{2+}$  stores via store-operated  $\text{Ca}^{2+}$  permeable ion channels and phenotypic switching of VSMC in pathological conditions [36,37], they need further investigation.

At 124 mM  $\text{K}^+$  the estimated  $V_m$  is close to 0 mV, which is the reversal potential for NSCC. Nevertheless, there was still a substantial contraction, suggesting that the reversal potential of the NSCC leading to the isometric contraction at 124 mM  $\text{K}^+$  displays preferential  $\text{Ca}^{2+}$  above  $\text{K}^+$  selectivity. The low voltage-dependency at moderate  $\text{K}^+$  concentrations is suggestive for outward rectification of the NSCC current involved in  $\alpha_1$ -adrenoceptor stimulation with PE. It should be mentioned, however, that changes of internal pH by exchange of  $\text{Na}^+$  for  $\text{K}^+$  ions in

the high  $K^+$  solution and partial inhibition of the  $Na^+$ -dependent acid extruders such as the  $Na^+/H^+$  exchanger or the  $Na^+/HCO_3^-$  transporter, may also affect isometric contractions in conditions of low external  $Na^+$ .

In conclusion,  $\alpha_1$ -adrenergic stimulation of mouse aortic segments causes transient contraction because of  $IP_3$ -mediated  $Ca^{2+}$  release from the SR and concomitant tonic contraction due to  $Ca^{2+}$  influx via VGCC and NSCC. At physiological  $V_m$  (physiological  $K^+$ ), the baseline and PE-induced force are voltage-dependent mainly because  $V_m$  of VSMC resides in the window voltage range of the VGCC. Hyperpolarization of  $V_m$  with levromakalim or low  $K^+$  avoids or diminishes activation of window  $Ca^{2+}$  influx and causes contraction by  $Ca^{2+}$  influx mainly via NSCC. By causing intracellular  $Ca^{2+}$  release from non-contractile  $Ca^{2+}$  stores with CPA, PE-induced contractions can be turned from mixed VGCC/NSCC-mediated to majorly NSCC-mediated. Results of this study emphasize the important role of VGCC and NSCC in mouse aortic performance and further indicate that although the contractile performance of aortic segments in different conditions may be the same, the relative contributions of VGCC or NSCC  $Ca^{2+}$  influx may significantly differ. The extreme voltage-dependent VGCC window contractions and the complex interactions with NSCC and non-contractile SR  $Ca^{2+}$  stores may have consequences for the development of arterial de-stiffening and antihypertensive therapies.

## Supporting Information

### S1 Text.

(DOCX)

### S1 Fig. Inhibition of PE(1 $\mu$ M)-mediated phasic (A) and tonic (B) contractions by 2-APB.

A. Phasic contractions by 1  $\mu$ M PE were measured 3 minutes after applying 0Ca. The concentration-response (area under the curve, AUC) curve in C revealed an  $IC_{50}$  of  $34 \pm 4$   $\mu$ M 2-APB. B. Tonic contractions by 1  $\mu$ M PE upon re-addition of 3.5  $\mu$ M  $Ca^{2+}$  to the 0Ca solution containing 1  $\mu$ M PE. The concentration-response (isometric force) curve in D revealed an  $IC_{50}$  of  $38 \pm 5$   $\mu$ M and was not significantly different from the  $IC_{50}$  for inhibition of the tonic contraction. (n = 5)

(TIF)

## Author Contributions

Conceived and designed the experiments: PF CVH AL. Performed the experiments: PF. Analyzed the data: PF. Contributed reagents/materials/analysis tools: PF CVH AL HB. Wrote the paper: PF AL WM GDM KL HB DS.

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