European Journal of Pharmacology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

# L-type $Ca^{2+}$ channel blockers inhibit the window contraction of mouse aorta segments with high affinity

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ARTICLE INFO

*Article history:*
Received 4 December 2013
Received in revised form
20 May 2014
Accepted 21 May 2014

*Keywords:*
Vascular reactivity
L-type calcium channel
Calcium channel blocker
Depolarization
Contraction

*Chemical compounds studied in this article:*
Nifedipine (PubChem CID: 4485)
Amlodipine (PubChem CID: 2162)
Diltiazem HCl (PubChem CID: 62920)
Verapamil HCl (PubChem CID: 62969)
BAY K8644 (PubChem CID: 2303)

ABSTRACT

L-type calcium channel blockers (LCCBs) reduce blood pressure more effectively in hypertensive than in normotensive subjects and are more effective in vascular smooth muscle (VSM) than in cardiac muscle. This has been explained by the depolarized resting potential of VSM in comparison with heart muscle cells and by the hypertension because both favor the "high affinity" inactivated state of the L-type calcium channel (LCC). Depolarized resting potentials, however, also increase $Ca^{2+}$ influx via window, non-inactivating LCC. The present study investigated whether these channels can be effectively blocked by nifedipine, verapamil or diltiazem, as representatives of different LCCB classes. C57Bl6 mouse aortic segments were depolarized by 50 mM $K^+$ to attain similar degree of inactivation. The depolarization evoked biphasic contractions with the slow force component displaying higher sensitivity to LCCBs than the fast component. Removal of the fast force component increased, whereas stimulation of $Ca^{2+}$ influx with the dihydropyridine BAY K8644, a structural analog of nifedipine, decreased the efficacy of the LCCBs. Addition of LCCBs during the contraction caused concentration-dependent relaxation, which was independent of the presence of a fast force component, but still showed lower sensitivity in the presence of BAY K8644. Our data suggest that steady-state contractions by depolarization with 50 mM $K^+$ are completely due to window $Ca^{2+}$ influx, which is preferentially inhibited by LCCBs. Furthermore, results point to interactions between the LCCB receptors and $Ca^{2+}$ ions or BAY K8644. The high affinity for open, non-inactivating LCC may play a dominant role in the anti-hypertensive effects of LCCBs.





## 1. Introduction

$Ca^{2+}$ influx via L-type $Ca^{2+}$ channels (LCCs) plays a dominant role in blood pressure regulation and development of hypertension (Moosmang et al., 2003; Pesic et al., 2004; Rhee et al., 2009; Zhou et al., 2008). Inhibition of this $Ca^{2+}$ influx with L-type $Ca^{2+}$ channel blockers (LCCBs) causes vascular smooth muscle (VSM) cell relaxation and consequential vasodilatation. Three classes of LCCBs with different chemical structures are clinically used: phenylalkylamines (e.g. verapamil), benz(othi)azepines (e.g. diltiazem) and dihydropyridines (e.g. nifedipine). They exhibit unique features by reducing blood pressure more effectively in hypertensive than in normotensive subjects (Leonetti et al., 1982; Godfraind et al., 1984; Godfraind, 2005; Striessnig et al., 1998). LCCBs decrease the aortic pulse wave velocity, central aortic pressure and augmentation index, suggesting that in baseline conditions L-type $Ca^{2+}$ influx contributes to compliance of conduit arteries (Koumaras et al., 2012; Kum and Karalliedde, 2010; Mackenzie et al., 2009; Williams et al., 2006; Zulliger et al., 2004).

Several attempts have been made to explain the unique features of LCCBs in their action on blood pressure and arterial stiffness. The increased response to vasoconstrictors in hypertension may not only be due to an increased number of voltage-gated LCCs (Godfraind, 2005; Pesic et al., 2004; Pratt et al., 2002), but also due to depolarization of the resting potential of VSM cells (Morel and Godfraind, 1994; Pesic et al., 2004). Depolarization

*Abbreviations:* LCCBs, L-type calcium channel blockers; LCCs, L-type calcium channels; KR, Krebs Ringer; 0Ca, calcium-free solution; 0Ca/+Ca, re-addition of external $Ca^{2+}$ to calcium-free solution; L-NAME, $N^{\Omega}$-nitro-L-arginine methyl ester; L-NNA, $N^{\Omega}$-nitro-L-arginine; NO, nitric oxide; $IC_{50}$, drug concentration for 50% inhibition; VSM, vascular smooth muscle

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http://dx.doi.org/10.1016/j.ejphar.2014.05.036
0014-2999/

leads to an increased proportion of inactivated LCCs, which according to the "modulated receptor theory" may have higher affinity for LCCBs than channels in the resting state (Bean et al., 1986; Godfraind, 2005; Morel and Godfraind, 1987). Furthermore, depolarization of the VSM cell resting potential by hypertension not only favours the inactivated state of the channels, but is also expected to increase L-type $Ca^{2+}$ influx via the channel window (Fleischmann et al., 1994).

Recently, we presented a new view on how contraction of depolarized mouse aortic segments is caused by a time-independent, non-inactivating $Ca^{2+}$ influx. Whenever the resting potential of VSM cells resides in the voltage window of the LCC, there is a continuous $Ca^{2+}$ influx that leads to a sustained, tonic contraction (Fransen et al., 2012a). Hence, depolarization of the resting potential by hypertension not only favours the inactivated state of the channels, but is also expected to increase window LCC $Ca^{2+}$ influx (Fleischmann et al., 1994). Therefore, the present study investigated whether nifedipine/amlodipine, verapamil or diltiazem as representatives of the different LCCB classes also block the window contraction with high affinity in depolarized mouse aortic segments.

## 2. Materials and Methods

### 2.1. Aortic segments

The studies were approved by the Ethical Committee of the University of Antwerp, and conform with 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 (food and water ad libitum, 12/12 light–dark cycle) were used at the age of 4 to 7 months. After anesthesia (sodium pentobarbital, 75 mg $kg^{-1}$, i.p.) and sacrificing the animals by perforating the diaphragm, the thoracic aorta was carefully removed, stripped of adherent tissue and dissected systematically. Starting at the diaphragm, the descending thoracic aorta was cut in segments of 2 mm width (5–6 segments). Vessels were immersed in Krebs Ringer solution (KR solution, 37 °C, 95% $O_2$/5% $CO_2$, pH 7.4) with (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, CaEDTA 0.025, and glucose 11.1.

### 2.2. Isometric tension measurements

Aortic segments were mounted in 10 ml organ baths as previously described (Van Hove et al., 2009). Isometric force was reported in mN. In all experiments, endothelial cells were present but basal NO formation was inhibited with a combination of 300 μM $N^{\Omega}$-nitro-L-arginine methyl ester (L-NAME) and 300 μM $N^{\Omega}$-nitro-L-arginine (L-NNA) and to avoid any vasomotor interference due to prostanoids, indomethacin (10 μM) was present.

### 2.3. Experimental conditions

Before or after incubation with LCCB, window contractions were induced by depolarization of the aortic segments with 50 mM $K^+$ in three different experimental conditions (Fransen et al., 2012a) (protocol, Fig. 1). In control conditions, contractions were elicited by replacing normal $K^+$ with high $K^+$ solution. Here, a transient increase of intracellular $Ca^{2+}$ due to activation and inactivation of a fast population of LCC causes transient contraction and is followed by a tonic contraction due to $Ca^{2+}$ influx via non-inactivating LCC (window contraction). Secondly, to avoid the fast component of contraction, segments were first depolarized in the absence of extracellular $Ca^{2+}$ (0Ca) and then contracted by addition of 3.5 mM $Ca^{2+}$. In the third condition, the window contraction was modulated by addition of the LCC agonist, 30 nM BAY K8644. These experiments were performed with pre-incubation of the segments with LCCBs and repeated for addition of cumulative concentrations of LCCBs on top of the 50 mM $K^+$-induced contractions to induce relaxation (Fig. 1A–C versus D–F).

High $K^+$-solution was prepared as KR solution, in which NaCl was replaced by KCl in equimolar concentrations. $Ca^{2+}$-free solution (0Ca) was prepared by omitting $Ca^{2+}$ from the KR solution and adding 1 mM EGTA as chelator. To restore extracellular $Ca^{2+}$, 3.5 mM $Ca^{2+}$ was added to 0Ca (0Ca/+Ca condition). To obtain $Ca^{2+}$-free KR solution with different $K^+$ concentrations, NaCl in the 0Ca solution was replaced by equimolar amounts of $K^+$.

### 2.4. Data analysis

All results are expressed as mean ± S.E.M.; $n$ represents the number of mice. Concentration–response curves were fitted with sigmoidal concentration–response equations with variable slopes, which revealed maximal contraction or relaxation responses ($E_{max}$) and the negative logarithm of the concentration resulting in 50% of the maximal contraction or relaxation ($pEC_{50}$) for each vessel segment. Two-way ANOVA analysis with Bonferroni multiple comparison post-hoc test and paired or unpaired $t$-test (GraphPad Prism, version 5, GraphPad Software, San Diego, California, USA) were used to compare means of the different experimental groups. A 5% level of significance was selected.

### 2.5. Materials

Sodium pentobarbital (Nembutal®) was obtained from Sanofi (Brussels, Belgium), indomethacin from Federa (Belgium), L-NNA, L-NAME, and nifedipine from Sigma (Bornem, Belgium), verapamil hydrochloride, diltiazem hydrochloride, amlodipine, and (±) BAY K8644 from TOCRIS (Bristol, United Kingdom).

## 3. Results

### 3.1. Nifedipine, verapamil and diltiazem inhibit contractions elicited at depolarized membrane potentials

Aortic segments were depolarized with 50 mM external $K^+$ in control conditions, after stimulation of LCCs with 30 nM BAY K8644 or following re-addition of external $Ca^{2+}$ to segments depolarized with 50 mM $K^+$ in 0Ca. In the absence of LCCB, basal force increased significantly with the addition of 30 nM BAY K8644 (+2.14 ± 0.99 mN, $P < 0.001$, Fig. 1B), but decreased in 0Ca conditions (−0.37 ± 0.06, $P < 0.001$, results not shown). Near steady-state contractions induced by depolarization with 50 mM $K^+$ were 10.8 ± 0.2 mN in control, 10.9 ± 0.9 mN in BAY K8644 and 10.7 ± 0.7 mN in 0Ca/+Ca and were not significantly different ($n = 4$, $P > 0.05$, Fig. 2).

Pre-incubation of the aortic segments with $1 \times 10^{-8}$ M nifedipine, $3 \times 10^{-6}$ M verapamil or $1 \times 10^{-6}$ M diltiazem inhibited $K^+$-induced contractions, but the efficacy of the LCCBs was more dependent on the condition. In control conditions, LCCBs inhibited mainly the slow component of force development (Fig. 2A). The increase of basal force by 30 nM BAY K8644 was inhibited by pre-incubation of the segments with LCCB. After compensation for the BAY K8644-mediated increase in basal force (Fig. 2B, right axis), it was observed that none of the LCCBs significantly inhibited the subsequent, depolarization-induced contraction. Finally, when window contractions were elicited in the 0Ca/+Ca condition, inhibition of the depolarization-induced contraction was almost complete for the LCCBs (Fig. 2C). Hence, the inhibitory effects of



Please cite this article as: Michiels, C.F., et al., L-type $Ca^{2+}$ channel blockers inhibit the window contraction of mouse aorta segments with high affinity. Eur J Pharmacol (2014), http://dx.doi.org/10.1016/j.ejphar.2014.05.036