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Murrayafoline A modulation of rat vascular myocyte $Ca_V1.2$ channel: functional, electrophysiological and molecular docking analysis

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Running title: $Ca_V1.2$ channel modulation by murrayafoline A

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Authorship contribution statement

M Durante, P Mugnai, O Spiga and F Fusi performed the experimental work

F Fusi and NM Cuong designed the research study

M Durante, O Spiga and F Fusi analysed the data

F Fusi, S Saponara, O Spiga and G Sgaragli wrote the paper

Tran Thu Huong isolated murrayafoline A

Pham Ngoc Khanh purified murrayafoline A

Ninh The Son collected plant

Abstract

BACKGROUND AND PURPOSE

The carbazole alkaloid murrayafoline A (MuA) has been shown to enhance contractility and $Ca_v1.2$ Ca^{2+} currents [$I_{Ca(L)}$] of rat cardiomyocytes. As only few drugs are known to stimulate $I_{Ca(L)}$, this study was designed to analyse MuA effects on vascular $Ca_v1.2$ channels.

EXPERIMENTAL APPROACH

Vascular activity was assessed in rat aorta rings mounted in organ baths. $Ca_v1.2$ Ba^{2+} current [$I_{Ba(L)}$] was recorded in single rat aorta and tail artery myocytes by the patch-clamp technique. Docking into the rat α_{1c} central pore subunit was simulated *in silico*.

KEY RESULTS

MuA relaxed depolarised rat aorta rings: spasmolysis was more evident in 60 mM K^+- than in 30 mM K^+-stimulated rings, where, however, low drug concentrations caused an increase of K^+ induced tone. MuA, at concentrations ≤ 14.2 µM, shifted to the left the concentration-response curve to K^+ whereas, at concentrations > 14.2 µM, antagonized (S)-(-)-Bay K 8644-induced contraction. In single myocytes, MuA stimulated $I_{Ba(L)}$ in a concentration-dependent, bell-shaped manner, and inhibited it at high concentrations; stimulation was stable, incompletely reversible upon drug wash out and accompanied by a leftward shift of the voltage-dependent activation curve. MuA, nifedipine and Bay K 8644 docked to the α_{1C} subunit central pore with comparable free energy binding values and interacting with the same amino acid residues. Neither Bay K 8644-induced stimulation nor nifedipine-induced block of $I_{Ba(L)}$ were modified by MuA.

CONCLUSIONS AND IMPLICATIONS

MuA is a naturally-occurring vasoactive agent capable to modulate $Ca_v1.2$ channels and to dock at the α_{1C} subunit central pore.

Abbreviations

$I_{Ba(L)}$, $Ca_v1.2$ Ba^{2+} current; $I_{Ca(L)}$, $Ca_v1.2$ Ca^{2+} current; MuA, murrayafoline A; PSS, modified Krebs-Henseleit saline solution

Introduction

Many drugs are known to block $Ca_v1.2$ channels, whereas only a few predominantly stimulate them. Either types of compounds by probing specific pore regions, represent valuable pharmacological tools for the study of the channel structure-function relationship. While Ca^{2+} channel antagonists have achieved major prominence as therapeutic agents, Ca^{2+} channel agonists are still confined to the investigational field. Naturally occurring agents such as high molecular weight heparins (Knaus *et al.*, 1990) and certain animal toxins (e.g. atrotoxin and maitotoxin; Hamilton and Perez, 1987) stimulate $Ca_v1.2$ channels in various mammalian tissues. Among the synthetic Ca^{2+} channel ligands, some dihydropyridine derivatives such as Bay K 8644 (Hess *et al.*, 1984), CGP 28392 (Kokubun and Reuter, 1984), RS 30026 (Patmore *et al.*, 1990), (+) 1,4-dihydro-2,6-dimethyl-5-nitro-4-(benzofuran-5-yl)pyridine-3-carboxylate (Visentin *et al.*, 1999), and the benzoylpyrrole derivative FPL 64176 (Rampe and Lacerda, 1991) have been shown to possess agonist-like activity.

In this laboratory the flavonoids quercetin and myricetin have been shown to stimulate $Ca_v1.2$ Ca^{2+} current [$I_{Ca(L)}$] in rat tail artery myocytes (Saponara *et al.*, 2002; Fusi *et al.*, 2005) and the key structural requirements of 24 flavonoids for $I_{Ca(L)}$ modulatory activity have been analysed by the molecular modelling technique (Saponara *et al.*, 2011): OH groups in 5 and 7 position of the flavonoid scaffold proved to be important for either the agonistic or the antagonistic activity and an interaction with positively charged groups of the channel protein was hypothesized to facilitate their docking at the $Ca_v1.2$ channel.

A carbazol alkaloid, murrayafoline A (MuA; Figure 1B), extracted from the root of *Glycosmis stenocarpa* (Drake) Guilt. (Bhattacharyya and Chowdhury, 1985; Cuong *et al.*, 2004) has been shown recently to enhance contractility and increase Ca^{2+} influx in single rat ventricular myocytes (Son *et al.*, 2014), behaving like an $Ca_v1.2$ channel stimulator. Therefore, in view of its possible therapeutic use it would be interesting to know its effects on vascular function. To this end, an in

depth analysis of MuA effects on rat vascular $Ca_V1.2$ channel was performed *in vitro* both on intact vessels and single myocytes and *in silico* on α_{1c} subunit pore model of the channel. MuA was proven to dock at the α_{1C} subunit central pore, modulate $I_{Ca(L)}$ by sharing some features of dihydropyridines and exert a bimodal effect on aorta ring contractility.

Methods

Aorta ring preparation

All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and were approved by the Animal Care and Ethics Committee of the Università di Siena, Italy (08-02-2012). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 74 animals were used in the experiments described here. Aorta rings (2-mm wide), either endothelium-intact or -denuded, were prepared from male Wistar rats (300-400 g; Charles River Italia, Calco, Italy), anaesthetized (i.p.) with a mixture of Ketavet® (30 mg kg^{-1} ketamine; Intervet, Aprilia, Italy) and Xilor® (8 mg kg^{-1} xylazine; Bio 98, San Lazzaro, Italy), decapitated and exsanguinated, as described elsewhere (Cuong *et al.*, 2014). The endothelium was removed by gently rubbing the lumen of the ring with the curved tips of a forceps. Each arterial ring was mounted over two rigid parallel, L-shaped stainless steel bars, one fixed in place and the other attached to an isometric transducer (Fort 25, WPI, Berlin, Germany). Contractile tension was recorded with a digital PowerLab data acquisition system (PowerLab 8/30; ADInstruments, Castle Hill, Australia) and analysed by using LabChart 7.3.7 Pro (Power Lab; ADInstruments). The preparations were allowed to equilibrate for 60 min in order to overcome mechanical stress. During equilibration, the modified Krebs-Henseleit saline solution (PSS; see below for composition) was renewed every 15 min and tension, if diminished by more than 25% within the first 15 min, was restored to its initial value.

The presence of functional endothelium was assessed in all preparations by testing the ability of acetylcholine (10 μM) to reduce the 0.3 μM phenylephrine-induced contraction by a value ≥ 75%; on the contrary, a relaxation < 10% was considered representative of the lack of the endothelial layer. Control preparations were treated with the drug vehicle only.

Effect of murrayafoline A and Bay K 8644 on aorta rings depolarized with high K^+ concentrations

The effects of MuA and Bay K 8644 were studied on the contraction induced by high K^+ concentrations to determine the involvement of $Ca_v1.2$ channels in their vascular activity. Steady tension was evoked in endothelium-deprived rings by either 30 mM or 60 mM K^+; thereafter the drug under investigation was added cumulatively. At the end of each experiment, 10 μM nifedipine followed by 100 μM sodium nitroprusside were added to test muscle functional integrity. Muscle tension was evaluated as a percentage of the initial response to K^+, taken as 100%.

Effect of murrayafoline A on the concentration-response curve for K^+ of aorta rings

To study the sensitization to K^+ induced by MuA, a cumulative concentration-response curve to K^+ was constructed in rings preincubated for 15 min with vehicle or drug. Responses were evaluated as percentage of the contraction induced by 0.3 μM phenylephrine in PSS, taken as 100%.

Functional interaction between murrayafoline A and Bay K 8644

Any potential interaction between MuA and Bay K 8644 at the $Ca_v1.2$ channel was assessed in depolarised, endothelium-denuded rings. Rings were stimulated with 60 mM K^+ for 15 min and then washed for 90 min with a Ca^{2+}-free PSS containing 1 mM EGTA. The preparations were then challenged with 0.3 μM phenylephrine to empty the intracellular Ca^{2+} stores. The spasmogenic response to 3 mM Ca^{2+} was assessed on rings depolarized with Ca^{2+}-free 60 mM K^+ PSS and preincubated for 30 min with the drug or vehicle. At the plateau of the Ca^{2+}-induced contraction, 10 nM Bay K 8644 followed by 100 μM sodium nitroprusside were added to test $Ca_v1.2$ channels as

well as smooth muscle functional integrity. The response was evaluated as a percentage of the initial response to 60 mM K^+, taken as 100%.

Smooth muscle cell isolation procedure and whole-cell patch clamp recordings

Smooth muscle cells were freshly isolated from either the aorta, according to Zhao *et al.* (2001), or the main tail artery (Mugnai *et al.*, 2014). Briefly, a 3-mm long thoracic section of aorta was incubated at 37°C in 2 ml of Ca^{2+}-free external solution (see below) containing 20 mM taurine (prepared by replacing NaCl with equimolar taurine), 1 mg ml^{-1} bovine serum albumin, 0.75 mg ml^{-1} papain, and 1 mg ml^{-1} DL-dithiothreitol, and gently bubbled with a 95% O_2 - 5% CO_2 gas mixture, for 20-30 min. After removing the adventitia, the aorta was cut into small pieces and transferred into a Ca^{2+}-free external solution containing 20 mM taurine, 1 mg ml^{-1} collagenase (type XI), and 1 mg ml^{-1} hyaluronidase for 10 min at 37°C. Single cells were released by gentle trituration of minced, proteolysed tissue, through a Pasteur pipette, stored at 4°C in the Ca^{2+}-free external solution containing 20 mM taurine, and used on the same day of the preparation.

Smooth muscle cells were freshly isolated from a 5-mm long piece of main tail artery incubated at 37°C in 2 ml of 20 mM taurine and 0.1 mM Ca^{2+} external solution containing 1 mg/ml collagenase (type XI), 1 mg/ml soybean trypsin inhibitor, and 1 mg/ml BSA, gently bubbled with a 95% O_2 - 5% CO_2 gas mixture, as previously described (Fusi *et al.*, 2001). Cells, stored in 0.05 mM Ca^{2+} external solution containing 20 mM taurine and 0.5 mg/ml BSA at 4°C under normal atmosphere, were used for experiments within two days after isolation (Mugnai *et al.*, 2014).

Whole-cell patch-clamp recordings

Cells were continuously superfused with external solution containing 0.1 mM Ca^{2+} and 30 mM tetraethylammonium using a peristaltic pump (LKB 2132, Bromma, Sweden), at a flow rate of 400 µl/min. The conventional whole-cell patch-clamp method (Hamill *et al.*, 1981) was employed to voltage-clamp smooth muscle cells. Recording electrodes were pulled from borosilicate glass

capillaries (WPI, Berlin, Germany) and fire-polished to obtain a pipette resistance of 2-5 MΩ when filled with internal solution (see below). An Axopatch 200B patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. At the beginning of each experiment, the junction potential between the pipette and bath solution was electronically adjusted to zero. Current signals, after compensation for whole-cell capacitance and series resistance (between 70-75%), were low-pass filtered at 1 kHz and digitized at 3 kHz prior to being stored on the computer hard disk. Electrophysiological responses were assessed at room temperature (20-22°C).

$I_{Ba(L)}$ and $I_{Ca(L)}$ recordings

The nomenclature of the ion channel targeted by MuA conforms to the BJP's Concise Guide to Pharmacology (Alexander *et al.*, 2013). $I_{Ba(L)}$ or $I_{Ca(L)}$ was always recorded in external solution containing 30 mM tetraethylammonium and 5 mM Ca^{2+} or Ba^{2+} (tail artery) or 10 mM Ba^{2+} (aorta). Current was elicited with 250 ms clamp pulses (0.067 Hz) to 0 mV from a V_h of -50 mV (tail artery) or to 10 mV from a V_h of -80 mV (aorta). Data were collected once the current amplitude had been stabilized (usually 7-10 min after the whole-cell configuration had been obtained) by using pClamp 8.2.0.232 (Molecular Devices Corporation, Sunnyvale, CA, USA). At this point, the various protocols were performed as detailed below. $I_{Ba(L)}$ and $I_{Ca(L)}$ did not run down during the following 40 min under these conditions (Fusi *et al.*, 2012).

Steady-state inactivation curves were obtained using a double-pulse protocol. Once various levels of the conditioning potential had been applied for 5 s, followed by a short (5 ms) return to the V_h, a test pulse (250 ms) to 0 mV was delivered to evoke the current. The delay between the conditioning potential and the test pulse allowed full or near complete deactivation of the channels simultaneously avoiding partial recovery from inactivation.

Activation curves were derived from the current-voltage relationships (as shown in Figure 4B). Conductance (G) was calculated from the equation $G = I_{Ba(L)} / (E_m - E_{rev})$, where: $I_{Ba(L)}$ is the peak current elicited by depolarizing test pulses in the range -50 to 20 mV from V_h of -50 mV; E_m is the membrane potential; and E_{rev} is the reversal potential (181 mV, as estimated with the Nernst equation). G_{max} is the maximal Ba^{2+} conductance (calculated at potentials ≥5 mV). The ratio G/G_{max} was plotted against the membrane potential and fitted with the Boltzmann equation.

K^+ currents were blocked with 30 mM tetraethylammonium in the external solution and Cs^+ in the internal solution. Current values were corrected for leakage using 10 µM nifedipine, which completely blocked $I_{Ba(L)}$ and $I_{Ca(L)}$.

Solutions and chemicals

PSS contained (in mM): NaCl 118; KCl 4.75; KH_2PO_4 1.19; $MgSO_4 \cdot 7H_2O$ 1.19; $NaHCO_3$ 25; glucose 11.5; $CaCl_2 \cdot 2H_2O$ 2.5; gassed with a 95% O_2 - 5% CO_2 gas mixture to create a pH of 7.4. PSS containing KCl at a concentration greater than 4.75 mM was prepared by replacing NaCl with equimolar KCl.

External solution contained (in mM): 130 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 $MgCl_2 \cdot 6\,H_2O$, and 5 Na-pyruvate; pH 7.4. The internal solution contained (in mM): 100 CsCl, 10 HEPES, 11 EGTA, 1 $CaCl_2 \cdot 2H_2O$ (pCa 8.4), 2 $MgCl_2 \cdot 6\,H_2O$, 5 Na-pyruvate, 5 succinic acid, 5 oxalacetic acid, 3 Na_2-ATP, and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH.

The osmolarity of the tetraethylammonium- and Ca^{2+} or Ba^{2+}-containing external solution (320 mosmol) and that of the internal solution (290 mosmol; Stansfeld and Mathie, 1993) was measured with an osmometer (Osmostat OM 6020, Menarini Diagnostics, Florence, Italy).

Phenylephrine, acetylcholine, collagenase (type XI), trypsin inhibitor, bovine serum albumin, papain, DL-dithiothreitol, hyaluronidase, tetraethylammonium chloride, EGTA, HEPES, taurine, (S)-(-)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (Bay K 8644), and nifedipine were from Sigma Chimica (Milan, Italy); sodium nitroprusside from

Riedel-De Haën AG (Seelze-Hannover, Germany). MuA was isolated from the dried powdered roots of *Glycosmis stenocarpa* (Drake) Guilt. as previously described (Cuong *et al.*, 2004). MuA (473 mM stock solution), dissolved directly in DMSO, Bay K 8644 and nifedipine, dissolved in ethanol, were diluted at least 1000 times prior to use. All these solutions were stored at -20°C and protected from light by wrapping containers with aluminium foil. The resulting concentrations of DMSO and ethanol (below 0.1%, v v^{-1}) failed to alter the response of the preparations. Phenylephrine was dissolved in 0.1 M HCl. Sodium nitroprusside was dissolved in distilled water. All other substances used were of analytical grade and used without further purification.

Statistical analysis

Analysis of data was accomplished by using pClamp 9.2.1.8 software (Molecular Devices Corporation) and GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA). Data are reported as mean ± SEM; n is the number of cells or rings analysed (indicated in parentheses), isolated from at least three animals.

Statistical analyses and significance as measured by one-way or repeated measures ANOVA (followed by either Dunnett or Bonferroni post-test), one sample *t* test or Student's *t* test for paired or unpaired samples (two tailed) were obtained using GraphPad InStat version 3.06 (GraphPad Software, USA). Post-tests were performed only when ANOVA found a significant value of F and no variance in homogeneity. In all comparisons, $P < 0.05$ was considered significant. The pharmacological response to each substance was described in terms of either pEC_{50} or pIC_{50}.

Docking experiments

Construction of the model

The rat $Ca_V1.2$ channel α_{1C} subunit sequence (NP_036649.2) was retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/protein/). This has four repeats, each containing six transmembrane

helices (S1–S6), and a P-loop between S5 and S6 (Cheng *et al.*, 2010). The quality of a homology model is given by the accuracy of the sequence alignment and the resolution of the template structures used. A PSI-BLAST search (Altschul *et al.*, 1997) for rat α_{1C} subunit sequences was firstly performed in order to obtain the best template of the unit and the tetrameric portion of the model. Subsequently, the sequences were aligned as previously reported (Zhorov and Tikhonov, 2004; Cheng *et al.*, 2010): here the disposition of both P-loops and inner helices were derived from earlier structure templates. Therefore, complete, suitable templates turned out to be the K_vAP (1ORQ pdb) and the K_vAP (2R9R pdb) for the reconstruction of the unit and the tetramer, respectively.

When viewed from the extracellular side, the repeats I-IV turned out to be arranged clockwise around the central pore (Dudley *et al.*, 2000). This channel model was built using the SwissPdbViewer-DeepView v.4.1 (Guex and Peitsch, 1997), which enabled also to define the consistency of bond distances, bond angles, and torsion angles with the values of standard proteins. The structure of the channel model was energetically minimized using the Gromacs package (Berendsen *et al.*, 2012) equipped with the AMBER force field (Sorin and Pande, 2005) till a final convergence of 0.01 kcal/mol Å was achieved. The stereochemical quality of the final structure was assessed by means of PROCHECK program (Laskowski *et al.*, 1993). With this test no severely disallowed atomic contacts were detected, suggesting essentially good stereochemistry, with 86.1% and 11.0 % of the residues in the most favoured and additionally allowed regions of the Ramachandran plot.

Docking Simulations

Docking of ligands (nifedipine, Bay K 8644, and MuA) was simulated with AutoDock/Vina version 1.1 (Trott and Olson, 2010). This program used an Iterated Local Search Global Optimizer Algorithm based on a succession of steps which consisted of mutation and local optimization. Ligand structures were retrieved from the PubChem database

(http://www.ncbi.nlm.nih.gov/pcsubstance/) and pdbqt files were generated by using scripts included in the Molecular Graphics Laboratory (MGL) tools (Morris *et al.*, 2009). The generation and affinity grid maps, viewing of docking poses, and analysis of virtual screening results were done by using AutoDock plug-in of PyMOL. The dimensions of the box for docking calculation (60Å x 60Å x 60Å) were sufficiently great to include not only the active docking site, as previously suggested (Cosconati *et al.*, 2007), but also significant portions of the surrounding surface.

Results

Effect of murrayafoline A on aorta rings contracted by high K^+ concentrations

To determine the involvement of $Ca_V1.2$ channels in the vascular activity of MuA, its effect was evaluated on the contraction induced by both 30 mM and 60 mM K^+ in endothelium-denuded rings. As shown in Figure 1A, MuA caused a concentration-dependent relaxation of the preparations. Rings contracted by 60 mM K^+ relaxed fully in the presence of 473 µM MuA with a pIC_{50} value of 4.22 ± 0.13 (n = 6) and an AUC value of 109.6 ± 13.0. Furthermore, maximal relaxation was significantly greater than that recorded in endothelium-denuded preparations contracted by phenylephrine ($P < 0.05$, Student's t test for unpaired samples). When rings were depolarized with 30 mM K^+, the concentration-response curve was shifted upward (Figure 1A), showing an AUC value of 180.5 ± 25.3 (n = 6; $P < 0.05$ Student's t test for unpaired samples). MuA, at concentrations ≤ 47.3 µM, caused an increase in K^+-induced vascular tone while, at higher concentrations, partially reverted the contraction, showing a relative pIC_{50} value of 4.07 ± 0.10 µM (n = 6).

The contractile responses to K^+ were significantly potentiated by MuA (Figure 1B). In the presence of various concentrations of MuA, in fact, the K^+ concentration-response curve was shifted to the left with a change of the pEC_{50} value for K^+ from 1.61 ± 0.04 (2.1 mM DMSO; n = 12) to 1.67 ± 0.04 (1.4 µM MuA, n = 13; $P > 0.05$, Dunnett post-test), 1.78 ± 0.04 (4.7 µM MuA, n = 14; P <

0.05), and 1.80 ± 0.05 (14.2 μM MuA, n = 14; $P < 0.05$). Potentiation of responses to K^+ by 14.2 μM MuA was greater at15 mM K^+, being 409.6% of control, as compared to that observed at higher K^+ concentrations (157.0% and 124.3% at 30 mM and 60 mM K^+, respectively). The maximal response to K^+, however, was not modified by the drug.

Effect of Bay K 8644 on aorta rings contracted by high K^+ concentrations and its interaction with murrayafoline A

To interpret the effects of MuA on aorta rings, the same experiments described above were repeated with the $Ca_v1.2$ channel agonist Bay K 8644 as a substitute for MuA. When the effect of Bay K 8644 on the contraction induced by high K^+ concentrations was evaluated in endothelium-denuded rings, a concentration-dependent, marked increase of muscle tone in preparations contracted by 30 mM K^+ was observed whilst in rings contracted by 60 mM K^+ this was considerably smaller (Figure 2A). The AUC values were 214.3 ± 36.7 (n = 7) and 91.1 ± 20.6 (n = 6; $P < 0.05$ Student's t test for unpaired samples).

Any potential pharmacological interactions between MuA and Bay K 8644 were assessed in endothelium-denuded rings contracted by the addition of 3 mM Ca^{2+} to the Ca^{2+}-free, 60 mM K^+-containing PSS. In rings pre-treated with DMSO, 10 nM Bay K 8644 increased Ca^{2+}-induced contraction by about 40% (Figure 2B). MuA, at concentrations of 47.3 μM and 142 μM, significantly antagonized the Bay K 8644-induced increase.

Ca^{2+} influx stimulated by $Ca_v1.2$ channel agonists may be completely buffered by the superficial sarcoplasmic reticulum or even impeded if channels are not pre-activated with low K^+ concentrations. However, addition of MuA to endothelium-denuded rings either bathed in normal PSS or pre-treated with 1 μM thapsigargin or 15 mM K^+ failed to induce mechanical responses (data not shown). When this assay was performed with Bay K 8644, in normal PSS a concentration-dependent contraction was recorded in 7 out of 17 preparations (pEC_{50} value of 7.53 ± 0.24, n = 7). In rings pre-incubated with 15 mM K^+ or 1 μM thapsigargin, the concentration-response curves to

Bay K 8644 were shifted to the left (pEC_{50} value of 8.33 ± 0.19, n = 11, P > 0.05, and 9.13 ± 0.41, n = 6, P < 0.05, Dunnett post-test); in the former case (15 mM K^+), an increase in drug efficacy was also observed (data not shown).

Effect of murrayafoline A on $I_{Ba(L)}$ and $I_{Ca(L)}$

Contribution of $Ca_v1.2$ channel modulation to MuA effects on vascular rings was assessed on $I_{Ba(L)}$ recorded in isolated aorta myocytes. At V_h of -80 mV, MuA stimulated the current in a concentration-dependent manner with a pEC_{50} value of 5.33 ± 0.08 (n = 7) (Figure 3A). At 47.3 µM this stimulatory effect was less evident, whilst at 473.4 µM MuA clearly inhibited $I_{Ba(L)}$. Similar results (pEC_{50} value of 5.44 ± 0.03, n = 9; P>0.05) were obtained in tail artery myocytes. Therefore, an in depth analysis of MuA effects on $I_{Ba(L)}$ was performed on these cells, whose biophysical and pharmacological properties are well characterised (Mugnai *et al.*, 2014 and references therein). MuA modulation of the current through $Ca_v1.2$ channels did not depend on the charge carrier. In fact, when equimolar Ca^{2+} replaced Ba^{2+} in the external solution, the stimulatory potency (pEC_{50} 5.60 ± 0.12, n = 5; P>0.05) was not modified.

$I_{Ba(L)}$ evoked at 0 mV from a V_h of -50 mV activated and then declined with time courses that could be fitted by a mono-exponential function. MuA did not affect significantly both the τ for inactivation and that for activation at all concentration tested (data not shown).

Figure 3B shows the time course of the effects of MuA on $I_{Ba(L)}$ recorded from V_h of -50 mV to a test potential of 0 mV. After the current had reached steady values, the addition to bath solution of 14.2 µM MuA produced a gradual increase of the current that reached a plateau in about 4 min and was only partially reversible upon drug wash out. Furthermore, MuA-induced stimulation of $I_{Ba(L)}$ was stable for about 30 min.

The current-voltage relationships recorded at V_h of -50 mV (Figure 4A) show that 14.2 µM MuA significantly increased the peak $I_{Ba(L)}$ without altering both the maximum at 10 mV and the

threshold at approximately -30 mV. Also MuA-induced stimulation of the current-voltage relationship was incompletely reversed upon drug wash out.

Effects of murrayafoline A on steady-state inactivation and activation curves for $I_{Ba(L)}$

The voltage dependence of MuA inhibition was further investigated by analysing the steady-state inactivation and activation curves for $I_{Ba(L)}$. MuA (14.2 µM) failed to affect both the 50% inactivation potential (-17.88 ± 1.27 mV, control, and -19.96 ± 1.66 mV, MuA; $n = 6$) and the slope factor (-7.57 ± 0.16 mV and -7.62 ± 0.64 mV) of the steady-state inactivation curve (Figure 4B). The activation curves, calculated from the current-voltage relationships showed in Figure 4A, were fitted to the Boltzmann equation. MuA significantly decreased the 50% activation potential (-6.65 ± 1.24 mV, control, and -9.47 ± 1.18 mV, MuA, $n = 6$; $P < 0.05$, repeated measures ANOVA and Dunnett post-test) without changing the slope factor (6.65 ± 0.51 mV and 6.32 ± 0.32 mV; Figure 4B). This effect was completely reversed upon drug wash out (50% activation potential -7.07 ± 1.08 mV and slope factor 6.14 ± 0.28 mV; $P > 0.05$).

Modelling and docking

To determine in silico the interaction of MuA with the channel protein, the structure of the central pore of $Ca_v1.2$channel of the rat α_{1c} subunit has been reconstructed. The structural correctness of the pore model in the open-channel configuration resulted to be in accordance with other authors (Cheng *et al.*, 2010). Random docking simulation and the best pose of nifedipine, Bay K 8644 and MuA at the binding pocket are represented in Figure 5. The estimated interaction energy of the α_{1C} subunit central pore with the two dihydropyridines (Table 1) was comparable to that previously reported by Cosconati *et al.* (2007); noticeably, with MuA a similar value was found. Thirteen surrounding amino acid residues of the binding pocket turned out to be constantly involved in the binding of each out of the three ligands (Table 1).

Functional interaction between murrayafoline A and Bay K 8644 or nifedipine on $I_{Ba(L)}$

Since docking analysis suggested that MuA binds the $Ca_v1.2$ channel binding pocket at a site that can bind also nifedipine and Bay K 8644, the functional interaction between this alkaloid and the two dihydropyridines was investigated. Bay K 8644 (100 nM) stimulated $I_{Ba(L)}$ in the range -30 mV to 50 mV and shifted the maximum of the current-voltage relationships by 10 mV in the hyperpolarizing direction (Figure 6A). The activation curves, calculated from the current-voltage relationships showed in Figure 6A, were fitted to the Boltzmann equation. Bay K 8644 significantly decreased the 50% activation potential (-5.72 ± 0.72 mV, control, and -13.95 ± 0.82 mV, Bay K 8644, n = 6; $P < 0.05$, repeated measures ANOVA and Dunnett post-test) and changed the slope factor (from 6.22 ± 0.30 mV to 4.67 ± 0.26 mV; $P < 0.05$) (Figure 6A inset). The subsequent addition of 14.2 µM MuA did not modify Bay K 8644-induced effects on both $I_{Ba(L)}$ amplitude and activation curve (50% activation potential -13.71 ± 0.57 mV and slope factor 5.20 ± 0.20 mV; $P > 0.05$).

Under control conditions the current evoked at 0 mV from a V_h of -50 mV activated and then declined with time courses that could be fitted by mono-exponential functions (Figure 6B). Bay K 8644 (100 nM) significantly prolonged the τ for activation and reduced that for inactivation: the subsequent addition of MuA brought only the τ for activation almost to control values without affecting that for inactivation.

The antagonistic effect of nifedipine was determined under control conditions as well as in myocytes pre-treated with MuA or Bay K 8644. Nifedipine inhibited $I_{Ba(L)}$ in a concentration-dependent manner with an pIC_{50} value of 7.67±0.06 (n = 6; Figure 7A,D). Similar results were obtained in the presence of 14.2 µM MuA (pIC_{50} value of 7.60±0.10, n = 6; $P > 0.05$, one-way ANOVA and Dunnett post-test; Figure 7B,D). On the contrary, when $I_{Ba(L)}$ was stimulated with 100 nM Bay K 8644, the concentration-response curve to nifedipine was shifted to the right (pIC_{50} value of 6.42 ± 0.08, n = 7; $P < 0.05$, one-way ANOVA and Dunnett post-test; Figure 7C,D).

Discussion

MuA has been shown recently to enhance contractility and increase Ca^{2+} influx in single rat ventricular myocytes (Son *et al.*, 2014), behaving like a $Ca_v1.2$ channel stimulator. However, its effects on vascular function are unknown. The present investigation demonstrates that MuA is capable to either stimulate or inhibit contraction of vascular smooth muscle by activating or blocking $Ca_v1.2$ channels, respectively, depending on the concentration used. This conclusion is supported not only by indirect, functional observations but also by direct electrophysiological data and docking analysis.

The mechanical and electrophysiological effects of MuA were compared to those of the synthetic $Ca_v1.2$ channel activator Bay K 8644 (Hess *et al.*, 1984). Vascular smooth muscle active tone and $I_{Ba(L)}$ stimulation induced by MuA shared some basic features with those sustained by Bay K 8644. In fact, MuA, like Bay K 8644, stimulated the active tone of aorta rings depolarized with 30 mM K^+, this effect disappearing when K^+ concentration raised up to 60 mM (i.e. in fully activated preparations); furthermore, it shifted the K^+ concentration-response curve to the left without changing its maximum (for Bay K 8644 see Fusi *et al.*, 2003). Finally, at low concentrations both drugs stimulated $I_{Ba(L)}$ in a nifedipine-sensitive manner while exhibiting, at high concentrations, $Ca_v1.2$ channel block (see Su *et al.*, 1984). Collectively, these findings suggest that MuA, like Bay K 8644, affected the vascular $Ca_v1.2$ channel protein.

MuA, added either before or after K^+, enhanced tissue responses to low, but not to high depolarising stimuli. This "sensitization" to K^+ is generally observed with drugs, like Bay K 8644, that facilitate the voltage-dependent activation of $Ca_v1.2$ channels (this paper), thus shifting the curve relating tension development and depolarising stimulus (i.e. membrane potential) to lower K^+ concentrations (i.e. more negative values; see Fusi *et al.*, 2003). This hypothesis was confirmed by the Boltzmann analysis (activation curve) of the current-voltage relationship, showing that MuA, similarly to Bay K 8644, significantly decreased the 50% activation potential of $I_{Ba(L)}$.

Ca^{2+} channel activators, such as Bay K 8644, are capable to evoke full contractile, tonic responses in vascular smooth muscle preparations, mainly when they are depolarized with low K^+ concentrations (this paper; Usowicz *et al.*, 1995; Fusi *et al.*, 2003) or when the Ca^{2+} buffering activity of the superficial sarcoplasmic reticulum is impaired (this paper; Asano and Nomura, 1999). In fact, on one hand $Ca_V1.2$ channel activation is voltage-dependent and therefore channels have to be activated in order to respond to Ca^{2+}-agonist drugs. On the other hand, Ca^{2+} influx triggered by the Ca^{2+}-agonist drug can induce a maximum muscle contraction only in the absence of a functional sarcoplasmic reticulum. MuA, however, did not elicit significant mechanical responses in rat aorta rings either under control conditions or in the presence of thapsigargin or moderate concentrations of K^+, thus suggesting that its potency and efficacy were much lower than those of Bay K 8644.

When used at high concentrations, MuA acted mostly as a Ca^{2+} channel blocker, similarly to Bay K 8644 (Su *et al.*, 1984). Several pieces of evidence concur to this conclusion. First, MuA reverted the contraction induced by high K^+, in agreement with data already published by Wu *et al.* (1998), the relaxant effect becoming more pronounced at higher depolarization levels (i.e. depending on membrane potential; Bean, 1984; Kuriyama *et al.*, 1995). Vasorelaxation induced by Ca^{2+} channel blockers, in fact, is directly correlated to the extracellular concentration of K^+, as is the case with nifedipine, whose potency increases as the membrane voltage (i.e. the concentration of extracellular K^+) increases (McDonald *et al.*, 1984). Second, MuA-induced relaxation was lower when phenylephrine, instead of a high concentration of K^+, was employed to contract the vessel (data not shown), in line with the observation that $Ca_V1.2$ channels play only a secondary role in this type of contraction (see McFadzean and Gibson, 2002) and similarly to nifedipine (Gurney, 1994). Third, it inhibited $I_{Ba(L)}$ in single myocytes isolated from both aorta and tail artery. Since this biphasic effect was observed independently of the charge carrier used, a Ca^{2+}-dependent inactivation of the channel subsequent to current stimulation can be ruled out.

In the computational study, where a model of the α_{1C} subunit central pore region was reconstructed *in silico* to perform a molecular docking analysis, Bay K 8644, nifedipine, and MuA showed significant and comparable data of free-energy of binding to the α_{1C} subunit central pore; in particular, those related to dihydropyridines are in agreement with already published data (Cosconati *et al.*, 2007). On one hand, they confirmed the validity of the model constructed and, on the other, clearly indicate that MuA can be considered a novel ligand of $Ca_v1.2$ channels capable to bind as strongly as the test ligands to the pore-forming α_{1C} subunit. Noticeably, the docking analysis indicated 13 amino acid residues in the binding pocket region specifically involved in the binding interaction with the dihydropyridines as well as with MuA. This prompted us to study the functional interaction between these channel modulators. Findings indicate that MuA shared with the dihydropyridines a similar mechanism of action, although probably interacting at a distinct site within the same binding pocket. In fact, although assessed at concentrations two orders of magnitude higher, $I_{Ba(L)}$ stimulation by MuA did not add to that of Bay K 8644; moreover, when used alone, MuA displayed a much lower potency and efficacy. Nonetheless, MuA prevented Bay K 8644-induced facilitation of extracellular Ca^{2+} influx and partly reverted its effect on current kinetics, though failing to alter nifedipine blocking potency in contrast to Bay K 8644, which significantly and competitively antagonised it. Finally, Bay K 8644, at variance with MuA, affected $I_{Ba(L)}$ kinetics and stimulated $I_{Ba(L)}$ maximally at weak depolarization values, causing a leftward shift in the maximum of the current-voltage relationship (Wang *et al.*, 1989; Mc Donald *et al.*, 1994; Saponara *et al.*, 2008; this paper).

Interesting similarities emerged when the effects of MuA on vascular and cardiac (Son *et al.*, 2014) $Ca_v1.2$ channels were compared. In both tissues, in fact, MuA induced a concentration-dependent, nifedipine-sensitive stimulation of $I_{Ba(L)}$, without altering the current kinetics. Additionally, current stimulation was bell-shaped, though the highest concentration assessed in cardiomyocytes was only 200 µM, i.e. 2.5 fold lower than that tested in vascular myocytes. On the contrary, vascular preparations seemed more sensible to MuA as the pEC_{50} value was one order of magnitude lower

than that recorded in cardiomyocytes. Finally, in cardiomyocytes MuA stimulation of Ca^{2+} sparks and Ca^{2+} transients depends on PKC activation (Kim *et al.*, 2014), while its modulatory activity on rat tail artery $Ca_V1.2$ channel, where PKC plays a stimulatory role (Reference), was not significantly affected by the PKC inhibitors GF109203X and Gö6976 (Supplementary). This once more suggests that MuA exerted a direct activity on the vascular $Ca_V1.2$ channel.

In conclusion, the present findings show that the carbazole alkaloid MuA can be included among the molecules of natural origin capable of stimulating the voltage-dependent $Ca_V1.2$ channel, not only in cardiac (Son *et al.*, 2014) but also in vascular myocytes. Vietnamese medicinal plants represent a valuable source for the discovery of novel pharmacological agents that can be instrumental to probe the basic structure and function of $Ca_V1.2$ channel.

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Conflicts of interest

The authors declare no conflict of interest.

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Table 1

Murrayafoline A, nifedipine, and Bay K 8644 docking into the channel pore of rat $Ca_V1.2$ channel α_{1C} subunit

<table>
<tr><th>Molecule</th><th>ΔG_{bind}</th><th>Surrounding residues</th></tr>
<tr><td>Murrayafoline A ($C_{14}H_{13}NO$)</td><td>-7.4</td><td rowspan="2">MET392(IP), PHE424(IS6), ASN428(IS6), LEU431(IS6), GLY432(IS6), SER435(IS6), TYR772(II), ASN776(II), LEU779(IIP), ALA780(IIP), VAL783(IIP)</td></tr>
<tr><td>Nifedipine ($C_{17}H_{18}N_2O_6$)</td><td>-6.7</td></tr>
<tr><td>Bay K 8644 ($C_{16}H_{15}F_3N_2O_4$)</td><td>-6.7</td><td>THR1143(IIIP), PHE1144 (IIIP) , GLU 1145(IIIP), PHE1185(IIIS5),ASN1188(IIIS5), VAL1191(IIIS5), GLY1192(IIIS5), ILE1195(IIIS5), TRP 1381(IVS5), GLY1445(IVP), MET1491(IVS6), PHE1495(IVS6), ASN1499(IVS6)</td></tr>
</table>

ΔG_{bind} is the free-energy of binding estimated from the top of 20 cluster results and given in kcal/mol. Surrounding residues refer to aminoacid residues of the four repeats (in parentheses) in the binding pocket region interacting with and located within 10Å from any atom of the docked ligands. In bold are represented the residues of the segments typically engaged in the binding pocket (Cosconati *et al*. 2007) and specifically involved in the binding interaction according to the best poses of the different, calculated cluster solutions.

Legends for Figures

Figure 1 Effect of murrayafoline A on high K^+-induced contraction of rat aorta rings. (A) Effect of the drug on endothelium-denuded rings depolarized with either 30 mM or 60 mM K^+. In the ordinate scale, response is reported as a percentage of the initial tension induced by 30 mM or 60 mM K^+, taken as 100%. Data points are mean ± SEM (n = 6). * P < 0.05 maximum effect at 60 mM K^+ vs. that at 30 mM K^+, Student's t test for unpaired samples. Inset: trace (representative of 6 experiments) of responses to cumulative concentrations of MuA added on a ring precontracted with 30 mM K^+. The effect of 100 µM sodium nitroprusside (SNP) is also shown. (B) Concentration-response curves for K^+ in the absence (2.1 mM DMSO) or presence of various concentrations of MuA. Data points are mean ± SEM (n = 12-14) and represent the percentage of the response to 0.3 µM phenylephrine (phe), taken as 100%. The maximal responses to 80 mM K^+, recorded under the four experimental conditions, were not significantly different (NS), one-way ANOVA and Dunnett post-test. Inset: chemical structure of MuA.

Figure 2 Effect of Bay K 8644 on high K^+-induced contraction of rat aorta rings and its functional interaction with MuA. (A) Concentration-response curves of Bay K 8644 in endothelium-denuded rings depolarized with 30 mM or 60 mM K^+. In the ordinate scale, response is reported as percentage of the initial tension induced by 30 mM or 60 mM K^+, taken as 100%. Data points are mean ± SEM (n = 6-7). * P < 0.05 maximum effect at 30 mM K^+ vs. that at 60 mM K^+, Student's t test for unpaired samples. (B) Effect of 10 nM Bay K 8644 on Ca^{2+}-induced vascular tone of depolarized rings treated with either vehicle (DMSO) or MuA. Columns are mean ± SEM (n = 7-10) and represent the percentage of the response to 60 mM K^+, taken as 100%. * P < 0.05 vs. control, Student's t test for paired samples; # P < 0.05 vs. DMSO +Bay K 8644, one-way ANOVA and Dunnett post-test.

Figure 3 Murrayafoline A modulation of $I_{Ba(L)}$ of single rat aorta myocytes. (A) Concentration-dependent effect of MuA at the peak of $I_{Ba(L)}$ trace in aorta myocytes. The curve shows the best fit of the points. On the ordinate scale, response is reported as percentage of control. Data points are mean ± SEM (*n* = 7). * $P < 0.05$ vs. control (100%), one sample *t* test. Inset: average traces (recorded from 7 cells) of conventional whole-cell $I_{Ba(L)}$ elicited with 250-ms clamp pulses to 10 mV from a V_h of -80 mV (see schematic diagram), measured in the absence (control) or presence of various concentrations (µM) of MuA. (B) Time course of $I_{Ba(L)}$ stimulation induced by murrayafoline A and drug wash out. MuA (14.2 µM) was applied at the time indicated by the arrow and current was recorded during a typical depolarization from -50 mV to 0 mV, applied every 15 s (0.067 Hz), and subsequently normalized upon the current recorded just prior to MuA addition. Drug wash out allowed for partial recovery from stimulation. $I_{Ba(L)}$ suppression by 10 µM nifedipine is also shown. Data points are mean ± SEM (*n* = 7-9). Inset: average traces (recorded from 7 cells) of conventional whole-cell $I_{Ba(L)}$ elicited with 250-ms clamp pulses to 0 mV from a V_h of -50 mV, recorded in the absence (control) or presence of 14.2 µM MuA as well as after drug wash out.

Figure 4. Effect of murrayafoline A on $I_{Ba(L)}$-voltage relationship as well as on $I_{Ba(L)}$ activation and inactivation curves. (A) Effect of MuA on the current-voltage relationship. Current-voltage relationships, recorded from a V_h of -50 mV, were constructed prior to the addition of drug (control), in presence of 14.2 µM MuA as well as after drug wash out. Data points are mean ± SEM (*n* = 7). * $P < 0.05$ vs control, $^{\#}$ $P < 0.05$ vs MuA, repeated measures ANOVA and Bonferroni post-test. (B) Steady-state inactivation curves recorded from V_h of -50 mV, obtained in the absence (control) and presence of 14.2 µM MuA, were fitted to the Boltzmann equation. The current measured during the test pulse is plotted against membrane potential and expressed as relative amplitude. Activation curves were obtained from the current-voltage relationships of panel A and fitted to the Boltzmann equation (see Methods section). Data points are mean ± S.E.M. (*n* = 6-7).

Figure 5. Docking of murrayafoline A, nifedipine, and Bay K 8644 at the $Ca_v1.2$ channel α_{1C} subunit model. The pore channel is displayed as molecular surface, with repeats I, II, III, and IV colored cyan, orange, violet, and green, respectively. (A) Nifedipine, (B) Bay K 8644, and (C) MuA are displayed in bold sticks, and key binding site residues are shown as thin sticks.

Figure 6. Effects of Bay K 8644 and murrayafoline A on $I_{Ba(L)}$-voltage relationship and kinetics in rat tail artery myocytes. (A) Current-voltage relationships constructed prior to the addition of drugs (control), in the presence of 100 nM Bay K 8644 as well as in presence of Bay K 8644 plus 14.2 µM MuA. Data points are mean ± SEM (*n* = 6). * $P < 0.05$ vs. control, repeated measures ANOVA and Bonferroni post-test. Inset: steady-state activation curves obtained from the current-voltage relationships of panel A and fitted to the Boltzmann equation (see Methods section). (B) Average traces (recorded from 6 cells) of conventional whole-cell $I_{Ba(L)}$ elicited with 250-ms clamp pulses to 0 mV from a V_h of -50 mV, recorded in the absence (control) or presence of 100 nM Bay K 8644 (Bay) and Bay K 8644 plus 14.2 µM MuA (Bay+mur). Control and Bay K 8644 plus MuA traces are magnified so that the peak amplitude matched that of Bay K 8644. Inset: time constant for activation (τ_{act}) and for inactivation (τ_{inact}) measured in the absence (control) or presence of Bay K 8644 (Bay) and Bay K 8644 plus MuA. Columns represent mean ± SEM (*n* = 6). * $P < 0.05$ vs. control, repeated measures ANOVA and Bonferroni post-test.

Figure 7. Effects of nifedipine on Bay K 8644 or murrayafoline A-induced stimulation of $I_{Ba(L)}$ in rat tail artery myocytes. (a-c) Average traces (recorded from 6-7 cells) of conventional whole-cell $I_{Ba(L)}$ elicited with 250-ms clamp pulses to 0 mV from a V_h of -50 mV and recorded after the addition of cumulative concentrations of nifedipine (A) in the absence (control) or presence of (B) 14.2 µM MuA and (C) 100 nM Bay K 8644. (D) Amplitude of the current was normalized upon that recorded under control conditions and in the presence of either MuA or Bay K 8644, taken as 100%. The curves show the best fit of the points. Data points are mean ± SEM (*n* = 6-7).