Vanillin and Vanillin Analogs Relax Porcine Coronary and Basilar Arteries by Inhibiting L-Type Ca$^{2+}$ Channels

Gábor Raffai, Gilson Khang, and Paul M. Vanhoutte

Department of BIN Fusion Technology, Department of Polymer Nano Science & Technology, Chonbuk National University, Jeonju, South Korea (G.R., G.K., P.M.V.); and Key State Laboratory of Pharmaceutical Biotechnologies and Department of Pharmacology and Pharmacy, University of Hong Kong, Hong Kong SAR, People's Republic of China (P.M.V.)

ABSTRACT

Vanillin (VA) and vanillyl alcohol (VAA), components of natural vanilla, and ethyl vanillin (EtVA; synthetic analog) are used as flavoring agents and/or as additives by the food, cosmetic, or pharmaceutical industries. VA, VAA, and EtVA possess antioxidant and anti-inflammatory properties, but their vascular effects have not been determined. Therefore, we compared in isolated porcine coronary and basilar arteries the changes in isometric tension caused by VA, VAA, and EtVA. VA and its analogs caused concentration-dependent relaxations of both preparations during contractions from U46619 (9,11-dideoxy-11-$\alpha$-epoxymethanoprostaglandin F2$\alpha$, a thromboxane A$_2$ receptor agonist), and of coronary arteries contracted with KCl or endothelin-1. The order of potency was VAA < VA < EtVA. The relaxations were not inhibited by endothelium removal, by 4-hydroxy-TEMPO, tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1-4-nitro-L-arginine methyl ester hydrochloride; L-NAME, N-$\omega$-acetylcysteine, tiron). VA and its analogs inhibited contractions induced by Ca$^{2+}$ re-introduction in coronary arteries, and by an opener of L-type Ca$^{2+}$-channels (methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-1,4-dihydropridine-3-carboxylate [Bay K8644]) in coronary and basilar arteries. They inhibited contractions of coronary rings induced by the protein kinase C activator phorbol 12,13-dibutyrate to the same extent as the removal of extracellular Ca$^{2+}$ or incubation with nifedipine. Thus, in porcine arteries, relaxation from VA (and its analogs) is due to inhibition of L-type Ca$^{2+}$ channels. Hence, these compounds could be used to relieve coronary or cerebral vasospasms due to exaggerated Ca$^{2+}$ influx, but therapeutic efficacy would require exposures that far exceed the current levels obtained by the use of vanillin additives.

Introduction

Vanillin (VA; Fig. 1A) and vanillyl alcohol (VAA, Fig. 1B) are major components of natural vanilla (Vanilla planifolia) extracts (Shyamala et al., 2007), and ethyl vanillin (Fig. 1C) is a synthetic vanillin derivative (Gradoff and Murayama, 1982). They are commonly used as flavoring agents or as additives by the food, cosmetic, and pharmaceutical industries. VA and its analogs have antioxidant (Shyamala et al., 2007; Jung et al., 2010; Tai et al., 2011; Kwon et al., 2013; Lee et al., 2013), anti-inflammatory (Jung et al., 2010; Kwon et al., 2013; Lee et al., 2013), antiproliferative, and antiangiogenic (Fukuoka et al., 2004; Lirdprapamongkol et al., 2009; Jung et al., 2010) properties.

This work was supported by Brain Korea 21 PLUS Project, National Foundation of Korea and National Research Foundation of Korea [Grant 2012M3A9C6050204]. dx.doi.org/10.1124/jpet.114.217935.

ABBREVIATIONS: Bay K8644, methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-1,4-dihydropridine-3-carboxylate; DMSO, dimethylsulfoxide; EtVA, ethyl vanillin; HC-030031, theophylline-7-[(4-isopropylphenyl) acetamide; L-NAME, N-$\omega$-nitro-$\omega$-arginine methyl ester hydrochloride; NOS, nitric oxide synthase(s); ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one [ODQ]), K$_{Ca}$ (1-[2-chlorophenyl]diphenylmethyl)-1H-pyrazole [TRAM-34], 6,12,19,20,25,26-hexahydro-5,27:13,18,21,24-triethyleno-11,17-metheno-7H-dibenzo[b,n][1,5,12,16]tetraazacyclotricosine-5,13-diamid ditrifluoracetate hydrate; UCL 1684, 6,12,19,20,25,26-hexahydro-5,27:13,18,21,24-triethyleno-11,17-metheno-7H-dibenzo[b,n][1,5,12,16]tetraazacyclotricosine-5,13-diamid ditrifluoracetate hydrate; VA, vanillin; VAA, vanillyl alcohol.
Coronary and Basilar Arterial Relaxations to Vanillin

Materials and Methods

Tissue Preparation

Hearts and brains from 6-month-old pigs weighing approximately 110 kg were obtained from the local slaughterhouse (NH Livestock Cooperation Association, Nonsan City, Chungnam Province, South Korea) and were transported to the laboratory in ice-cold Krebs-Ringer bicarbonate buffer with the following composition (in mM): 123 NaCl, 4.7 KCl, 5.5 glucose, 1.2 MgSO_4, 1.6 CaCl_2, 1.2 KH_2PO_4, 21 NaHCO_3, and 0.03 Na_2EDTA (control solution). Circumflex coronary and basilar arteries were dissected free, cleaned of adherent fat and connective tissue, and cut into approximately 3-mm long rings and 4-mm long segments, respectively. In certain preparations, the endothelium was removed mechanically (Furchgott and Zawadzki, 1980). The mechanical removal of the endothelium was performed by inserting a cotton thread repeatedly to scrub the endothelial surface. This procedure allows effective removal of the endothelium (Raffai, et al., 2014). This tissue, and cut into approximately 3-mm long rings and 4-mm long segments, respectively. In certain preparations, the endothelium was removed mechanically (Furchgott and Zawadzki, 1980). The mechanical removal of the endothelium was performed by inserting a cotton thread into the lumen of the coronary artery rings that were moved along the thread repeatedly to scrub the endothelial surface. This procedure allows effective removal of the endothelium (Raffai, et al., 2014). This was tested by adding 10^{-7} M bradykinin during contractions to 2 \times 10^{-7} M U46619 (9,11-dideoxy-11,9α-epoxymethanoprostaglandin F_20). Under those conditions, coronary artery rings with endothelium relaxed by approximately 80%, but successful removal of the endothelium resulted in less than 10% relaxation to bradykinin.

Isometric Tension Recording

Coronary Arteries. Recording of isometric tension in coronary rings was performed in a multichannel organ bath system (Panlab S.L.U., Barcelona, Spain). The rings of coronary arteries were suspended in organ chambers filled with 10 ml of control solution bubbled with 5% CO_2 and 95% O_2 and maintained at 37°C between a stationary and an adjustable stainless steel hook. Changes in isometric force were measured by an isometric force transducer (Harvard Apparatus, Holliston, MA). The measured force was recorded and analyzed with an iWorx Acquisition system (model IX/408) with Labscribe2 software (iWorx Systems, Dover, NH). The initial tension was increased to 2.5 g gradually during a 1-hour incubation period. Reference contractions were obtained by exposing the coronary rings to 60 mM KCl buffer solution made by equimolar substitution of NaCl with KCl twice at the beginning of the actual experiment.

The rings were incubated with pharmacologic agents for 30 minutes, and concentration-dependent responses to VA, VAA, and EtVA were measured in quiescent preparation or in rings contracted with 2 \times 10^{-7} M stable thromboxane A_2 mimetic U46619, with 40 mM KCl buffer solution (equimolar substitution for NaCl), or with 2 \times 10^{-8} M endothelin-1. Agonist concentrations were selected to reach approximately 50% of the reference contraction obtained with 60 mM KCl.

In a subset of experiments, contractions to increasing concentrations of Ca^{2+} after incubation in Ca^{2+}-free 40 mM KCl Krebs-Ringer bicarbonate buffer to activate the L-type calcium channels or to increasing concentrations of the pharmacologic activator of L-type Ca^{2+} channels Bay K8644 [methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]-1,4-dihydropyridine-3-carboxylate] (Schramm et al., 1983) were obtained in the presence of different concentrations of VA, VAA, and EtVA.

In another series of experiments, the effects of VA, VAA, and EtVA were compared on the contractions to the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (Leach and Blumberg, 1985).

Basilar Arteries. Isometric tension recording of the basilar segments was performed in a multiwire myograph system (model 620M; Danish Myo Technology A/S, Aarhus, Denmark). The measured force was recorded and analyzed with a data acquisition system (model AIF-01) and with SPEL Advanced Kymograph software (Experimetric, Budapest, Hungary). The initial tension was set to 0.2 g at the beginning of a 1-hour incubation period. All experimental procedures were performed as described previously for coronary preparations except that 10^{-7} M U46619 was used to pre-contract the arteries and 10^{-4} M papaverine was applied to obtain complete relaxation at the end of the relaxation experiments.

Drugs. N-Acetyl-l-cysteine, apocynin, barium chloride, catalase, TRAM-34 (1-[(2-chlorophenyl)liphidinylmethyl]-1H-pyrazole), tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate), glibenclamide, UCL 1684 [6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7H-dibenzo[b,n]tetrazacyclotricosine-5,13-diium ditrifluoroacetate hydrate], Tempol (4-hydroxy-TEMPO), indomethacin, L-NAME ([N^\text{-}nitro-l-arginine methyl ester hydrochloride), and vanillin were purchased from Sigma-Aldrich (St. Louis, MO), Bay K8644, endothelin-1, phorbol 12,13-dibutyrate, and U46619 were purchased from Tocris Bioscience (Bristol, UK). ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one) was purchased from Cayman Chemical (Ann Arbor, MI). Ruthenium red was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Iberiotoxin was purchased from Bachem AG (Bubendorf, Switzerland). Nifedipine was purchased from Tocris Bioscience (Bristol, UK).

Fig. 1. Chemical structures of (A) vanillin (3-methoxy-4-hydroxybenzaldehyde), (B) vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), and (C) ethyl vanillin (3-ethoxy-4-hydroxybenzaldehyde).

Fig. 2. Original recording showing the concentration-dependent decreases in isometric tension caused by vanillin in a porcine coronary artery ring (with endothelium) contracted with 2 \times 10^{-7} M U46619.

Table 1. Materials and Methods for Coronary and Basilar Arteries.
was purchased from Chungwa Chemical Synthesis & Biotech (New Taipei City, Taiwan). Vanillyl alcohol was purchased from Alfa Aesar (Ward Hill, MA), and ethyl vanillin was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Indomethacin was dissolved in 0.2 M Na₂CO₃, UCL 1684, TRAM-34, glibenclamide, Bay K8644, ODQ, nifedipine, and phosphol 12,13-dibutrate were dissolved in dimethylsulfoxide (DMSO). When DMSO was used as a solvent, the same dilution of DMSO was applied to obtain appropriate (solvent) control(s). All other drugs were dissolved in distilled water or in Krebs-Ringer bicarbonate buffer. The concentrations of drugs in the bath solution are given in molar.

**Calculations and Statistical Analysis.** Coronary artery rings or basilar artery segments from the same arteries were used for all the experimental groups comparing responses to VA, VAA, and EtVA or the control solution in the presence or absence of pharmacologic agents. Contractions are expressed as a percentage of the reference (solvent) control(s). All other drugs were dissolved in distilled water or in Krebs-Ringer bicarbonate buffer. The concentrations of drugs in the bath solution are given in molar.

**Results**

**Quiescent Preparations.** In quiescent coronary artery rings, VA, VAA, or EtVA did not cause a statistically significant concentration-dependent change in tension (Fig. 3).

**Contracted Preparations.** VA, VAA, and EtVA caused complete relaxation of coronary artery rings contracted with U46619, 40 mM KCl, or endothelin-1 (Fig. 4, A–C). Similar observations were made in U46619-contracted basilar artery segments (Fig. 5A). During contractions to U46619 in either vessel type or to endothelin-1 in coronary arteries, the order of relaxing potencies of vanillin and its analogs was VAA < VA < EtVA, which is also reflected by the EC₅₀ and area under the curve values (Table 1). The order of relaxing potencies for VA, VAA, and EtVA against different contractile agonists was U46610 = 40 mM KCl < endothelin-1, U46619 < 40 mM KCl = endothelin-1, and U46619 > 40 mM KCl < endothelin-1, respectively (Table 1). The relaxations caused by VA and its analogs were reversible (data not shown).

**Absence of Effect of Endothelium Removal and Inhibitors of Nitric Oxide Synthases, Cyclooxygenases, and Soluble Guanylyl Cyclase.** Relaxations induced by VA, VAA, and EtVA in U46619 contracted coronary artery rings were comparable in the presence or absence of endothelium (Fig. 6A; Table 2) and were largely insensitive to L-NAME (100 μM), an inhibitor of nitric oxide synthases (NOS) (Fig. 6B; Table 2), and indomethacin (10 μM), an inhibitor of cyclooxygenases, given alone or in combination with L-NAME (Table 2). Likewise, the inhibitor of the soluble guanylyl cyclase (sGC) ODQ (10 μM) had no statistically significant inhibitory effect on the VA, VAA, or EtVA-induced coronary relaxations (Fig. 6C; Table 2). Relaxations induced by VA in U46619-contracted basilar artery segments were also not significantly affected by L-NAME (data not shown).

**Absence of Effect of Inhibitors of Potassium and Transient Receptor Potential Receptor Vanilloid 3 Channels.** Inhibitors of small and intermediate (10 μM UCL 1684 and 10 μM TRAM-34, respectively) as well as large (0.1 μMiberiotoxin) conductance calcium dependent potassium channels between more than two groups, and Dunnett’s post hoc test was used to identify statistically significant differences compared with control. P < 0.05 was considered statistically significant. Data are shown as mean ± S.E.M.

Fig. 3. Changes of isometric tension in quiescent porcine coronary artery rings in response to increasing concentration of vanillin (n = 6), vanillyl alcohol (n = 10), and ethyl vanillin (n = 6). The initial tension was gradually increased to 2.5 g during the 1-hour incubation period. Data are shown as mean ± S.E.M. No statistically significant changes were observed.

Fig. 4. Relaxation of porcine coronary arteries to increasing concentrations of vanillin, vanillyl alcohol, and ethyl vanillin during contractions to (A) U46619 (2 × 10⁻⁶ M, n = 8–10), (B) KCl (40 mM, n = 8–9), and (C) endothelin-1 (2 × 10⁻⁶ M, n = 10–11). Data are shown as mean ± S.E.M. Statistically significant difference (P < 0.05), vanillin compared with *vanillyl alcohol and Ethyl vanillin groups.
had no significant effect on VA, VAA, or EtVA induced relaxations (Table 3). Likewise, 1 μM glibenclamide and 1 μM BaCl_2 (inhibitors of ATP-dependent and inwardly rectifying potassium channels, respectively) were also without significant effect (Table 3). Ruthenium red (10 μM), a nonspecific inhibitor of transient receptor potential cation channel receptor vanilloid 3 (TRPV3), also did not significantly affect the relaxations elicited by VA, VAA, or EtVA (Table 3).

**Absence of Effect of Antioxidants.** Relaxations to VA, VAA, and EtVA were not inhibited by nonenzymatic (1 mM tempol, 1 mM N-acetyl-L-cysteine, 1 mM tiron, 100 μM apocynin) or enzymatic (1000 U/ml catalase) antioxidants (Table 4).

**Effect of VA, VAA, and EtVA on Contractions Induced by Increases in Calcium Influx.** VA, VAA, and EtVA caused concentration-dependent inhibition of the contractions induced by Ca^{2+} reintroduction after incubation in 40 mM KCl Ca^{2+}-free solution in coronary arteries (Fig. 7A) or by increasing concentrations of the L-type Ca^{2+} channel opener Bay K8644 in both coronary artery rings (Fig. 7B) and basilar artery segments (Fig. 8B). These contractions were enhanced by Bay K8644 (Fig. 8C).

**PKC Activation and Protein Phosphatase(s) Inhibition.** The PKC activator phorbol 12,13-dibutyrate induced concentration-dependent contractions that were significantly reduced by VA, VAA, and EtVA (Fig. 8A) to a similar extent as by nifedipine or incubation in Ca^{2+}-free medium (Fig. 8B). These contractions were enhanced by Bay K8644 (Fig. 8C).

**Discussion**

The relaxing properties of VA and its analogs were compared in ring preparations of isolated coronary arteries and basilar artery segments contracted isometrically in response to the thromboxane A2 mimetic U46619 (Coleman et al., 1981) or the vasoconstrictor peptide endothelin-1 (Yanagisawa et al., 1988). These vasoconstrictor agonists were selected because, among other factors (Ginsburg et al., 1982; Kaski et al., 1986; Shepherd and Vanhoutte, 1986), both thromboxane A2 and endothelin-1 can contribute to the development of coronary (Ginsburg et al., 1982; Shepherd and Vanhoutte, 1986; Toyo-Oka et al., 1991) and cerebral (Tani et al., 1984; Nishizawa et al., 2000) vasospasm.

To judge from findings in pulmonary arteries, contractions induced by U46619 are associated with cell membrane depolarization (Cogolludo et al., 2003), but those by endothelin-1 in porcine coronary arteries are not (Kasuya et al., 1989). In addition, to directly open the L-type Ca^{2+} channels before determining relaxations to VA and its analogs in coronary arteries, high KCl depolarizing solution is applied (Kasuya et al., 1989).

VA, VAA, and EtVA caused concentration-dependent and reversible (data not shown) relaxations during contractions of either blood vessel type in the three cases with an order of potency of VAA < VA < EtVA. These results indicate that these compounds inhibit a common step(s) contributing to contractions induced via U46619, endothelin-1, or high KCl.

The order of relaxing potencies of VAA and its analogs may be related to their varying hydrophobicity which, to judge from findings in pulmonary arteries, contractions induced by U46619 are associated with cell membrane depolarization (Cogolludo et al., 2003), but those by endothelin-1 in porcine coronary arteries are not (Kasuya et al., 1989). In addition, to directly open the L-type Ca^{2+} channels before determining relaxations to VA and its analogs in coronary arteries, high KCl depolarizing solution is applied (Kasuya et al., 1989).

VA, VAA, and EtVA caused concentration-dependent and reversible (data not shown) relaxations during contractions of either blood vessel type in the three cases with an order of potency of VAA < VA < EtVA. These results indicate that these compounds inhibit a common step(s) contributing to contractions induced via U46619, endothelin-1, or high KCl.

The order of relaxing potencies of VA and its analogs may be related to their varying hydrophobicity, which, to judge from the elution time during reversed-phase high-performance liquid chromatography separation (Shyamala et al., 2007; Tai et al., 2011), follows the same ranking as observed in the present experiments. Such a difference in hydrophobicity of VA, VAA, and EtVA may be responsible not only for their different antioxidant activities/capacities (Tai et al., 2011) but also for their varying potencies in inhibiting L-type Ca^{2+} channels (as discussed herein), which are involved in contractions to KCl, U46619, and endothelin-1 (Goto et al., 1989; Kasuya et al., 1989; Sato et al., 2000; Nobe and Paul, 2001).

**Table 1**

<table>
<thead>
<tr>
<th>Contraction</th>
<th>Vanillin</th>
<th>Vanillyl Alcohol</th>
<th>Ethyl Vanillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log(EC50)</td>
<td>AUC</td>
<td>log(EC50)</td>
</tr>
<tr>
<td>U46619 (n = 8–10) coronary artery</td>
<td>3.31 ± 0.06</td>
<td>154.9 ± 14.9</td>
<td>2.49 ± 0.10*a</td>
</tr>
<tr>
<td>U46619 (n = 8) basilar artery</td>
<td>2.77 ± 0.15</td>
<td>118.9 ± 7.9</td>
<td>2.22 ± 0.29*a</td>
</tr>
<tr>
<td>40 mM KCl (n = 8–9) coronary artery</td>
<td>3.26 ± 0.03</td>
<td>151.2 ± 5.2</td>
<td>3.20 ± 0.07</td>
</tr>
<tr>
<td>Endothelin-1 (n = 10–11) coronary artery</td>
<td>3.64 ± 0.09</td>
<td>206.5 ± 12.2</td>
<td>3.30 ± 0.10*a</td>
</tr>
</tbody>
</table>

*aStatistically significant difference (P < 0.05) from vanillin group.
However, the present experiments provide no explanation as to why the relative inhibitory potencies of the individual compounds vary differently in function of the contractile agonist used.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Vanillin</th>
<th>Vanillyl Alcohol</th>
<th>Ethyl Vanillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$-\log(\text{EC}_{50})$</td>
<td>AUC</td>
<td>$-\log(\text{EC}_{50})$</td>
</tr>
<tr>
<td>Control ($n=9-10$)</td>
<td>3.08 ± 0.05</td>
<td>132.4 ± 4.0</td>
<td>2.49 ± 0.10</td>
</tr>
<tr>
<td>− Endothelium</td>
<td>3.13 ± 0.06</td>
<td>145.6 ± 5.8</td>
<td>2.42 ± 0.10</td>
</tr>
<tr>
<td>Control ($n=8-11$)</td>
<td>3.03 ± 0.05</td>
<td>130.7 ± 4.8</td>
<td>2.64 ± 0.20</td>
</tr>
<tr>
<td>+100 µM L-NAME</td>
<td>2.87 ± 0.05</td>
<td>126.9 ± 5.6</td>
<td>2.48 ± 0.20</td>
</tr>
<tr>
<td>+10 µM indomethacin</td>
<td>2.98 ± 0.09</td>
<td>138.4 ± 11.7</td>
<td>3.15 ± 0.23</td>
</tr>
<tr>
<td>+100 µM L-NAME</td>
<td>2.78 ± 0.07*</td>
<td>126.5 ± 8.3</td>
<td>2.59 ± 0.16</td>
</tr>
<tr>
<td>Control ($n=8$)</td>
<td>2.97 ± 0.04</td>
<td>124.9 ± 2.9</td>
<td>2.74 ± 0.11</td>
</tr>
<tr>
<td>+10 µM ODQ</td>
<td>3.01 ± 0.03</td>
<td>142.1 ± 4.5*</td>
<td>2.34 ± 0.13*</td>
</tr>
</tbody>
</table>

*Statistically significant difference ($P < 0.05$) from control.

**U46619 Contractions.** The basic mechanism underlying the relaxation to VA, VAA, and EtVA first was investigated during U44619 contractions. These experiments showed that these relaxations are independent of the endothelium and do...
nonspecific TRP channel blocker ruthenium red but not by and relax rat thoracic aortae contracted with phenylephrine or 2009). Vascular effects of TRPV3 agonists other than VA and its activate TRPV3 channels (Xu et al., 2006; Vriens et al., 2008, eugenol, and thymol), natural or synthetic VA or EtVA can permeable transient receptor potential channels that are highly calcium-dependent as well as ATP-dependent and inwardly (Wulff et al., 2000), and large (Galvez et al., 1990) conductance responses. Mediation by hyperpolarization resulting from ac-

TABLE 3
EC50 and area under the curve (AUC) values for vanillin, vanillyl alcohol, and ethyl vanillin concentration response curves measured in porcine coronary artery rings contracted with U46619 (2 × 10−7 M) and preincubated with the inhibitors of small, intermediate, and large conductance calcium-dependent potassium channels (UCL 1684, TRAM-34, and iberiotoxin, respectively) as well as of inward rectifier (BaCl2) and ATP-dependent (glibenclamide) potassium channels and TRPV3 channels (ruthenium red)

<table>
<thead>
<tr>
<th></th>
<th>Vanillin</th>
<th>Vanillyl Alcohol</th>
<th>Ethyl Vanillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−log(EC50)</td>
<td>AUC</td>
<td>−log(EC50)</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>3.32 ± 0.10</td>
<td>168.8 ± 14.0</td>
<td>3.09 ± 0.31</td>
</tr>
<tr>
<td>+10 µM UCL 1684 + 10 µM TRAM-34</td>
<td>3.37 ± 0.15</td>
<td>183.0 ± 19.5</td>
<td>3.46 ± 0.27</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>3.14 ± 0.10</td>
<td>141.9 ± 9.9</td>
<td>2.79 ± 0.15</td>
</tr>
<tr>
<td>+0.1 µM iberiotoxin</td>
<td>3.16 ± 0.10</td>
<td>154.2 ± 10.2</td>
<td>2.85 ± 0.15</td>
</tr>
<tr>
<td>Control (n = 5–10)</td>
<td>3.21 ± 0.08</td>
<td>150.0 ± 8.6</td>
<td>2.75 ± 0.12</td>
</tr>
<tr>
<td>+1 µM BaCl2</td>
<td>3.20 ± 0.07</td>
<td>157.1 ± 6.3</td>
<td>2.75 ± 0.07</td>
</tr>
<tr>
<td>Control (n = 7–8)</td>
<td>3.07 ± 0.09</td>
<td>134.3 ± 9.3</td>
<td>2.89 ± 0.10</td>
</tr>
<tr>
<td>+1 µM glibenclamide</td>
<td>3.15 ± 0.10</td>
<td>145.2 ± 11.5</td>
<td>2.95 ± 0.08</td>
</tr>
<tr>
<td>Control (n = 5)</td>
<td>2.99 ± 0.07</td>
<td>139.3 ± 5.2</td>
<td>1.75 ± 0.16</td>
</tr>
<tr>
<td>+10 µM ruthenium red</td>
<td>2.92 ± 0.06</td>
<td>137.6 ± 6.0</td>
<td>1.75 ± 0.16</td>
</tr>
</tbody>
</table>

not involve activation/products of NOS, cyclooxygenases, and sGC, as endothelium removal (Furchgott and Zawadzki, 1980), L-NAME (Rees et al., 1995), indomethacin (Botting, 2006), and ODQ (Garthwaite et al., 1995), respectively, did not inhibit the responses. Mediation by hyperpolarization resulting from ac-

TABLE 4
EC50 and area under the curve (AUC) values for vanillin, vanillyl alcohol, and ethyl vanillin concentration response curves measured in porcine coronary artery rings contracted with U46619 (2 × 10−7 M) and preincubated with various antioxidants

<table>
<thead>
<tr>
<th></th>
<th>Vanillin</th>
<th>Vanillyl Alcohol</th>
<th>Ethyl Vanillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−log(EC50)</td>
<td>AUC</td>
<td>−log(EC50)</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>3.10 ± 0.06</td>
<td>142.4 ± 7.3</td>
<td>2.77 ± 0.12</td>
</tr>
<tr>
<td>+1 mM tempol</td>
<td>3.12 ± 0.08</td>
<td>144.4 ± 5.8</td>
<td>2.70 ± 0.12</td>
</tr>
<tr>
<td>+1 mM N-acetyl-l-cysteine</td>
<td>3.22 ± 0.09</td>
<td>151.5 ± 9.2</td>
<td>3.02 ± 0.19</td>
</tr>
<tr>
<td>+1 mM tiron</td>
<td>3.28 ± 0.12</td>
<td>176.7 ± 13.5</td>
<td>3.30 ± 0.27</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>3.19 ± 0.08</td>
<td>141.7 ± 9.7</td>
<td>2.69 ± 0.11</td>
</tr>
<tr>
<td>+100 µM apocynin</td>
<td>3.31 ± 0.06</td>
<td>165.6 ± 8.2</td>
<td>3.27 ± 0.26</td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>3.33 ± 0.14</td>
<td>164.8 ± 16.5</td>
<td>2.81 ± 0.10</td>
</tr>
<tr>
<td>+1000 U/ml catalase</td>
<td>3.18 ± 0.06</td>
<td>148.2 ± 8.4</td>
<td>2.75 ± 0.10</td>
</tr>
</tbody>
</table>

*Statistically significant difference (P < 0.05) from control.
compounds on contractions induced by activation of L-type Ca$^{2+}$ channels. These channels are also involved in the agonist-induced contractions (e.g., endothelin-1, see the previous discussion) (Goto et al., 1989; Kasuya et al., 1989). Our experiments have demonstrated that VA and its analogs inhibit the contractions induced not only by Ca$^{2+}$ reintroduction after incubation in Ca$^{2+}$-free 40 mM KCl solution in coronary arteries, but also those elicited by Bay K8644, an opener of the L-type Ca$^{2+}$ channels (Schramm et al., 1983) in both coronary and basilar arteries. Complete inhibition was achieved with the concentrations that caused complete relaxations of precontracted preparations in both cases, an effect similar to that obtained with nifedipine serving as the reference dihydropyridine blocking L-type Ca$^{2+}$ channels (Vater et al., 1972). These results strongly suggest that vanillin and its analogs inhibit the activity of the L-type calcium channels facilitating relaxation.

PKC Activation. In coronary arterial smooth muscle, the contractions evoked by thromboxane A$_2$ (TP) receptor activation and endothelin-1 eventually involve PKC activation and/or Rho kinase–dependent Ca$^{2+}$ sensitization (Sato et al., 2000; Nobe and Paul, 2001). To investigate the effect of VA and its analogs on contractions mediated by PKC, we used an activator of the enzyme phorbol 12,13-dibutyrate (Leach and Blumberg, 1985). Phorbol 12,13-dibutyrate causes contractions without substantial changes in myoplasmic Ca$^{2+}$ concentration in porcine coronary arteries by increasing the sensitivity of the contractile apparatus to Ca$^{2+}$ (Miller et al., 1986; Mori et al., 1990). Contractions due to phorbol ester-induced Ca$^{2+}$ sensitization have been attributed to increasing myosin light chain phosphorylation resulting from inhibition of myosin light chain phosphatase (Itoh et al., 1993; Masuo et al., 1994; Zhao et al., 2005) without the involvement of Rho kinase (Fu et al., 1998; Zhao et al., 2005). The resulting contractions depend on the

Fig. 7. Effect of vanillin (left), vanillyl alcohol (middle), and ethyl vanillin (right) on contractions induced by (A) Ca$^{2+}$ reintroduction in 40 mM KCl Ca$^{2+}$-free solution ($n = 8$) and (B) the L-type Ca$^{2+}$ channel opener Bay K8644 ($n = 8$). Data are expressed as mean ± S.E.M. *Statistically significant difference ($P < 0.05$) from control.

Fig. 8. Effect of (A) vanillin, vanillyl alcohol, and ethyl vanillin ($n = 6$), (B) Ca$^{2+}$-free medium with 1 mM EDTA and nifedipine ($n = 6$–8), and (C) Bay K8644 ($n = 9$) on contractions to phorbol 12,13-dibutyrate in porcine coronary arterial rings. Data are expressed as mean ± S.E.M. Statistically significant differences ($P < 0.05$) of the different treatment groups from control: *vanillin, Ca$^{2+}$-free, Bay K8644, †vanillyl alcohol, nifedipine, and #ethyl vanillin.
availability of extracellular calcium and the opening of L-type calcium channels (Mori et al., 1990). Our experiments have confirmed this dual dependency, as the contractions caused by phorbol 12,13-dibutyrate were inhibited by either nifedipine and incubation in Ca²⁺-free solution, but were enhanced by stimulating Ca²⁺ influx with Bay K8644. They further demonstrate that contractions induced by the PKC activator are inhibited by VA, VAA, and EtVA at a concentration(s) that abolished contractions induced by the PKC activator. VA, VAA, and EtVA at a concentration(s) that abolished contractions induced by the PKC activator are as additives. 

**Conclusions and Therapeutic Potential.** The endothelium- and NOS-independent relaxations via VA and its analogs can be attributed to inhibition of Ca²⁺-entry via L-type Ca²⁺ channels in both coronary and basal arteries. Based on these properties, VA, VAA, and EtVA could be used to prevent or relieve exaggerated Ca²⁺ influx resulting in PKC activation in coronary (Ito et al., 1994; Kadokami, et al., 1996) or cerebral (Laher and Zhang, 2001) vasospasm and other cardiovascular disorders (Khalil, 2013). However, the concentrations of VA and its analogs needed to act as antioxidants (Shyamala et al., 2007; Jung et al., 2010; Kwon et al., 2013; Lee et al., 2013) or as vasodilators (present study) are larger than the measured plasma level [0.0005 M] reached after oral administration of 30 mg/kg VA in male ICR (imprinting control region) mice (Tai et al., 2011). This comparison implies that there is a therapeutically favorable coronary and/or cerebral vasodilator, larger doses of VA and its analogs would be required than with their use as additives.

**Authorship Contributions**

- Participated in research design: Rafsfi, Khang, Vanhoutte.
- Conducted experiments: Rafsfi.
- Contributed new reagents or analytic tools: Khang.
- Performed data analysis: Rafsfi.
- Wrote or contributed to the writing of the manuscript: Rafsfi, Vanhoutte.

**References**


