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

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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\(^{\alpha}\)-hydroxy-TEMPO, a thromboxane A\(_2\) (TXA\(_2\)) 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 inhibitors of NO synthases (N\(^\alpha\) nitro-L-arginine methyl ester hydrochloride), cyclooxygenases (indomethacin), soluble guanylyl cyclase (1H-1,2,4-oxadiazolo[4,3-b]quinoxalin-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-trietheno-11,7-metheno-7H-dibenzo[b,n][1,5,12,16]tetraazacyclotrisocine-5,13-dium ditrifluoroacetate hydrate [UCL-1684]), by ibetaxel, by K\(_{ATP}\) (glibenclamide), by K\(_{Ca}\) (BaCl\(_2\)), by transient receptor potential receptor vanilloid 3 (TRPV3) channels (ruthenium red), or by antioxidants (catalase, apocynin, tempol, N-acetylcysteine, tiron). VA and its analogs inhibited contractions induced by Ca\(^{2+}\) rings 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-dihydropyridine-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 (Gradeff 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) and anti-inflammatory (Jung et al., 2010; Kwon et al., 2013) properties. They are commonly used as flavoring agents 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\(^{\alpha}\)-hydroxy-TEMPO, a thromboxane A\(_2\) (TXA\(_2\)) 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 inhibitors of NO synthases (N\(^\alpha\) nitro-L-arginine methyl ester hydrochloride), cyclooxygenases (indomethacin), soluble guanylyl cyclase (1H-1,2,4-oxadiazolo[4,3-b]quinoxalin-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-trietheno-11,7-metheno-7H-dibenzo[b,n][1,5,12,16]tetraazacyclotrisocine-5,13-dium ditrifluoroacetate hydrate [UCL-1684]), by ibetaxel, by K\(_{ATP}\) (glibenclamide), by K\(_{Ca}\) (BaCl\(_2\)), by transient receptor potential receptor vanilloid 3 (TRPV3) channels (ruthenium red), or by antioxidants (catalase, apocynin, tempol, N-acetylcysteine, tiron). VA and its analogs inhibited contractions induced by Ca\(^{2+}\) rings 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-dihydropyridine-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.

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ABBREVIATIONS: Bay K8644, methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-1,4-dihydropyridine-3-carboxylate; DMSO, dimethylsulfoxide; EtVA, ethyl vanillin; HC-030031, theophylline-7-(V-4-isopropylphenyl) acetamide; L-NNAME, N\(^\alpha\) nitro-L-arginine methyl ester hydrochloride; NOS, nitric oxide synthase(s); ODQ, 1H-1,2,4-oxadiazolo[4,3-b]quinoxalin-1-one; PKC, protein kinase C; sGC, soluble guanylyl cyclase(s); Tempol, 4-hydroxy-TEMPO; tiron, 4,5-dihydroxy-1,3-benzendisulfonic acid disodium salt monohydrate; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole; U46619, 9,11-dideoxy-11\(^{\alpha}\)-hydroxy-TEMPO, a thromboxane A\(_2\) receptor agonist; UCL 1684, 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7H-dibenzo[b,n][1,5,12,16]tetraazacyclotrisocine-5,13-dium ditrifluoroacetate hydrate; VA, vanillin; VAA, vanillyl alcohol; EtVA, ethyl 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 MgSO4, 1.6 CaCl2, 1.2 KH2PO4, 21 NaHCO3, and 0.03 Na2EDTA (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 was tested by adding 10^{-7} M bradykinin during contractions to 2 \times 10^{-7} M U46619 (9,11-dideoxy-11{\alpha}-epoxymethanoprostaglandin F_{2\alpha}). 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% CO2 and 95% O2 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 A2 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 (Experimeta, 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)diphenylmethyl]-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][1,5,12,16]tetraazacyclotrisocine,5,13-dilium ditrifluoroacetate hydrate], Tempol (4-hydroxy-TEMPO), indomethacin, L-NAME (N^\text{N}-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 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 phorbol 12,13-dibutyrate 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 response to 60 mM KCl (100%) obtained at the beginning of the experiment for coronary arteries or as a change in tension compared with the baseline for the basilar arteries. Relaxations are expressed as a percentage of the contractions to U46619, 40 mM KCl, or endothelin-1. To compare various treatments, area under the curve values (Table 1). The order of relaxing potencies for 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.

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).

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had no significant effect on VA, VAA, or EtVA induced relaxations (Table 3). Likewise, 1 μM glibenclamide and 1 μM BaCl₂ (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 Contraction Induced by Increases in Calcium Influx.** VA, VAA, and EtVA caused concentration-dependent inhibition of the contractions induced by Ca²⁺ re-introduction after incubation in 40 mM KCl Ca²⁺-free solution in coronary arteries (Fig. 7A) or by increasing concentrations of the L-type Ca²⁺ channel opener Bay K8644 in both coronary artery rings (Fig. 7B) and basilar artery segments (Fig. 5B).

**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 Ca²⁺-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 Ca²⁺ 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 Ca²⁺ 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**

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 x 10⁻⁷ M), 40 mM KCl, and endothelin-1 (2 x 10⁻⁸ M), and basilar artery segments contracted with U46619 (10⁻⁷ M).

<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*</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*</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*</td>
</tr>
</tbody>
</table>

*Statistically 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.

U46619 Contractions. The basic mechanism underlying the relaxation to VA, VAA, and EVa first was investigated during U46619 contractions. These experiments showed that these relaxations are independent of the endothelium and do

**TABLE 2**

EC\(_{50}\) and area under the curve (AUC) values for vanillin, vanillyl alcohol, and ethyl vanillin concentration response curves measured in porcine coronary artery rings with or without endothelium contracted with U46619 (2 × 10\(^{-7}\) M) and preincubated with the inhibitors of NOS (L-NAME), cyclooxygenases (indomethacin), and sGC (ODQ). Data are expressed as mean ± S.E.M. *Statistically significant difference (\(P < 0.05\) ) from control.

<table>
<thead>
<tr>
<th>Treatment</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 (\mu)M 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 (\mu)M indomethacin</td>
<td>2.98 ± 0.09</td>
<td>138.4 ± 11.7</td>
<td>3.15 ± 0.23</td>
</tr>
<tr>
<td>+10 (\mu)M indomethacin</td>
<td>2.78 ± 0.07*</td>
<td>126.5 ± 8.3</td>
<td>2.59 ± 0.16</td>
</tr>
<tr>
<td>+100 (\mu)M L-NAME</td>
<td>2.97 ± 0.04</td>
<td>124.9 ± 2.9</td>
<td>2.74 ± 0.11</td>
</tr>
<tr>
<td>Control ((n = 8))</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.
CARVACROL IS ENDOTHELIO-DEPENDENT AND INHIBITED BY THE TRP CHANNEL RECEPTOR ANKYRIN 1 (TRPA1) (EARLEY ET AL., 2010)

For example, both carvacrol and eugenol reduce the myogenic relaxation caused by vasopressin and its analogs (Nishijima et al., 1999; Damiani et al., 2009). Vascular effects of TRPV3 agonists other than VA and its analogs have been reported (Xu et al., 2006; Vriens et al., 2008, 2009). Like other natural compounds (e.g., carvacrol, eugenol, and thymol), synthetic VA or EtVA can activate TRPV3 channels (Xu et al., 2006; Vriens et al., 2008, 2009). Vascular effects of TRPV3 agonists other than VA and its analogs have been reported (Nishijima et al., 1999; Damiani et al., 2003; Earley et al., 2010; Peixoto-Neves et al., 2010, 2014). For example, both carvacrol and eugenol reduce the myogenic tone of cerebral arteries coexpressing endothelial TRPV3 and TRP channel receptor ankyrin 1 (TRPA1) (Earley et al., 2010) and relax rat thoracic aorta contractile with phenylephrine and KCl (Damiani et al., 2003), respectively. The relaxation to carvacrol is endothelium-dependent and inhibited by the nonspecific TRP channel blocker ruthenium red but not by the specific TRPA1 inhibitor HC-030031 [theophylline-7-(N-4-isopropylphenyl) acetamide]; it is accompanied by hyperpolarization of and reduction of the intracellular calcium concentration in the vascular smooth muscle cells (Earley et al., 2010).

Relaxations to eugenol are partially sensitive to endothelium removal, and inhibition of either NOS or sGC (Damiani et al., 2003). By contrast, in our study, relaxations to VA and its analogs in porcine coronary arteries were independent of the presence of the endothelium, resistant to inhibitors of NOS, sGC, or hyperpolarizing potassium channels (discussed earlier), and were not prevented by ruthenium red, which inhibits all TRPVs and other types of TRPs (Nilius et al., 2007; Vriens et al., 2009) including TRPV3 channels (Earley et al., 2010).

Hence, the mechanism underlying relaxations induced by VA, VAA, and EtVA in porcine coronary arteries is not likely to involve the opening of TRPV3 channels.

Treatment with antioxidants scavenging superoxide anions (Krishna et al., 1992; Heumuller et al., 2008; Luo et al., 2009) or catalase eliminating H2O2 (Gaetani et al., 1996) also did not alter the relaxations to VA and its analogs. Thus, the relaxations to VA and its analogs in porcine coronary arteries are not due to their antioxidant properties. Based on these observations, H2O2 scavenging experiments to test the effect of these antioxidants on the relaxations induced by VA, VAA, and EtVA were not done.
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 both 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 caused by either Ca²⁺ reintroduction or incubation in Ca²⁺-free solution, and thus can be attributed to the inhibitory effect of the compounds on the influx of extracellular calcium ion through L-type Ca²⁺-channels. However, this interpretation awaits confirmation with more direct electrophysiologic or molecular pharmacologic approaches.

**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 basilar 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 (Khālīl, 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 mM] reached after oral administration of 30 mg/kg VA in male ICR (imprinting control region) mice (Tai et al., 2011). This comparison implies that to obtain a therapeutically favorable coronary and/or cerebral vasodilator, larger doses of VA and its analogs would be required than with available with their use as additives.

**Authorship Contributions**

**Participated in research design:** Rafsai, Khang, Vanhouotte.

**Conducted experiments:** Rafsai.

**Contributed new reagents or analytic tools:** Khang.

**Performed data analysis:** Rafsai.

**Wrote or contributed to the writing of the manuscript:** Rafsai, Vanhouitte.

**References**


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