

Trans- and cis-resveratrol increase cytoplasmic calcium levels in A7r5 vascular smooth muscle cells

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The effects of *trans*- and *cis*-resveratrol on cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were studied using fura-2 in vascular smooth muscle cells (A7r5). Both isomers of resveratrol caused a sustained elevation in $[\text{Ca}^{2+}]_i$, *cis*-resveratrol being significantly more effective than the *trans*-isomer. The resveratrol-induced increase in $[\text{Ca}^{2+}]_i$ was significantly potentiated by the previous application of low concentrations of thapsigargin, partially inhibited by nifedipine or Ni^{2+} , and not affected by SKF 96365. In the absence of extracellular Ca^{2+} , both isomers of resveratrol induced a transient, slow increase in $[\text{Ca}^{2+}]_i$, which was inhibited by the previous depletion of intracellular stores with thapsigargin and completely blocked by preincubation with TMB-8, an inhibitor of intracellular calcium release. Reintroduction of Ca^{2+} in the external solution after the resveratrol-induced release of Ca^{2+} activated the Ca^{2+} influx through store-operated calcium channels. The resveratrol-induced increase in $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} partially reduced the increase in $[\text{Ca}^{2+}]_i$ evoked by the subsequent application of thapsigargin. Our results suggest that *trans*- and *cis*-resveratrol induce a depletion of Ca^{2+} from the same intracellular stores released by thapsigargin and subsequent capacitative influx of Ca^{2+} . Additionally, a direct activation of transmembrane Ca^{2+} influx through another type of channel may be also implicated.

Keywords: A7r5 / Calcium / Fura-2 / Resveratrol / Thapsigargin

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1 Introduction

Resveratrol (3,4',5-trihydroxystilbene, RESV) is a natural phenolic component of *Vitis vinifera* L. (Vitaceae), abundant in the skin of grapes and present in wines, especially red wines. RESV is not unique to *Vitis* but is also present in at least 72 other plant species (distributed in 12 families and 31 genera; e.g., *Veratrum*, *Arachis*, *Morus*, and *Trifolium*), some of which are components of the human diet, such as mulberries and peanuts. In wines, RESV exists as *cis*- and *trans*-isomers [1, 2] (Fig. 1). To date most of the studies on the biological effects of RESV have considered the *trans*-isomer (*t*-RESV), which has shown a wide range of pharmacological actions including anti-inflammatory, anticarcino-

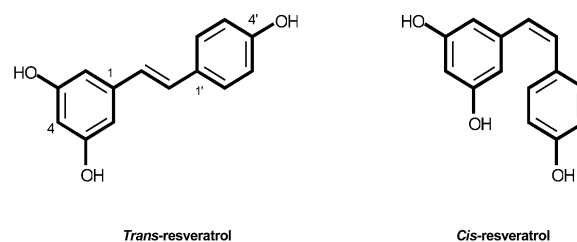


Figure 1. Chemical structures of *t*-RESV (3,4',5-trihydroxy-*trans*-stilbene) and *c*-RESV (3,4',5-trihydroxy-*cis*-stilbene).

genic, antioxidant, estrogenic, platelet antiaggregatory, and antimicrobial properties (for reviews, see e.g. [2–4]). Some of these activities have been implicated in the cardiovascular protective effects attributed to *t*-RESV and red wine [5, 6].

Despite the above described considerations, the *in vitro* vasodilator activity of *t*-RESV in rat aorta has surprisingly received little research attention. Thus, Fitzpatrick *et al.* [7] have reported that *t*-RESV (at concentrations up to 0.1 mM) is unable to relax rat aortic rings, whereas Chen and Pace-Asciak [8] have reported that *t*-RESV (>30 μM) induces relaxation of phenylephrine-precontracted endothelium-intact rat aorta, and at higher concentrations (> 60 μM) also

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Abbreviations: *c*-RESV, *cis*-resveratrol; fura-2 AM, fura-2 acetoxymethyl ester; SKF 96365, 1-(β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride; SOCC, store-operated calcium channel; *t*-RESV, *trans*-resveratrol; THAPS, thapsigargin; TMB-8, 3,4,5-trimethoxybenzoic acid 8-diethylamino-octyl ester 8

induces the relaxation of endothelium-denuded rat aorta. Similarly, we have reported that *t*-RESV exhibits in rat aorta: (i) a characteristic endothelium-dependent vasorelaxant effect which seems to be due to the inhibition of vascular NADH/NADPH oxidase and the subsequent decrease of basal cellular superoxide radicals ($\text{O}_2^{\cdot -}$) generation and, therefore, of nitric oxide (NO) biotransformation [5] and (ii) an endothelium-independent vasodilator effect, which appears to be mediated by an inhibition of protein kinase C (PKC) [9]. The endothelium-dependent component is probably the most relevant, since it is observed at lower drug concentrations ($< 10 \mu\text{M}$) than the endothelium-independent effect ($> 50 \mu\text{M}$).

Much less is known about the pharmacological activity of the *cis*-isomer (*c*-RESV) possibly as a result of the fact that this isomer (unlike *t*-RESV) is not commercially available. *c*-RESV significantly attenuates several components of the macrophage response to proinflammatory stimuli [10], and a few studies have demonstrated only quantitative differences in the activity of the two isomers; for example, in their ability to decrease the collagen-induced platelet aggregation [11], in their estrogenic/antiestrogenic and scavenging properties [12], or in the cyclooxygenase-1 assay [13].

Vascular tone directly depends on cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) levels in smooth muscle cells. Thus, any action of RESV on calcium signaling in these cells may be related to its endothelium-independent vascular actions. Again, there is little information on this subject. Orallo and Camiña [9], using $^{45}\text{Ca}^{2+}$ in rat aortic rings, have suggested that the vasorelaxant effect of *t*-RESV is not mediated by blockage of Ca^{2+} influx through transmembrane receptor-operated calcium channels. Rotondo *et al.* [14] reported a *t*-RESV-associated inhibition of agonist-induced $[\text{Ca}^{2+}]_i$ increases in polymorphonuclear leukocytes, and Dobrydenova *et al.* [15, 16] described that *t*-RESV inhibits Ca^{2+} influx in thrombin-stimulated human platelets.

Bearing in mind the above considerations and with the aim of providing new data on the effects of RESV on vascular smooth muscle cells, we here report the first detailed comparative study of the effects of both *t*-RESV and *c*-RESV on calcium signaling in single A7r5 cells (a cell line derived from fetal rat aorta), hoping to clarify how RESV affects smooth muscle cell $[\text{Ca}^{2+}]_i$ levels.

2 Materials and methods

2.1 Cell culture

A7r5 smooth muscle cells, a well-established vascular smooth muscle cell line obtained from embryonic rat aorta,

were obtained from the American Type Culture Collection (CRL1446; Rockville, MD, USA). The cells were grown in DMEM/F-12 (1:1) supplemented with heat-inactivated fetal bovine serum (10%), L-glutamine (2 mM), penicillin G ($100 \text{ U} \cdot \text{mL}^{-1}$), streptomycin ($100 \mu\text{g mL}^{-1}$), and amphotericin B ($0.25 \mu\text{g} \cdot \text{mL}^{-1}$). Cells were kept in culture at 37°C with 5% CO_2 in air. The cells were subcultured in 75 cm^2 tissue culture flasks and, for experiments, in 35 mm Petri dishes in which a 20 mm diameter hole had been cut in the base and replaced by a thin (0.1 mm) glass coverslip. Cells were seeded at low density ($\sim 1500 \text{ cells cm}^{-2}$) and allowed to grow for at least 24 h in culture medium (see above). Cells were kept in culture for 2–5 days before the experiments.

2.2 Measurement of $[\text{Ca}^{2+}]_i$ in isolated vascular smooth muscle cells

A7r5 cells (plated on Petri dishes as described above) were incubated for 60 min at 37°C in normal bathing solution (composition in mM: NaCl 140, KCl 5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.5, MgCl_2 2, HEPES 10, glucose 11, pH 7.4) containing $2.5 \mu\text{M}$ fura-2 acetoxymethyl ester (fura-2 AM), then gently washed twice with normal bathing solution and allowed to rest for > 15 min in the incubator, to allow the hydrolysis of fura-2/AM into Ca^{2+} -sensitive free acid form by cell esterases. Cells were placed on an inverted microscope (Zeiss Axiovert 135) and continuously superfused at $0.5 \text{ mL} \cdot \text{min}^{-1}$ with normal bathing solution except during the application of drugs. For experiments in extracellular Ca^{2+} -free medium, CaCl_2 was replaced by 10 mM EGTA. Measurements were made from isolated cells or small groups of dispersed cells (up to 8 cells) viewed with a $63 \times$ oil immersion objective (Zeiss Plan-Neofluar, $63 \times / 1.25$ oil) using a digital imaging system. Fura-2 was excited alternately at 340 ± 10 and 380 ± 10 nm (100 ms exposure time for both wavelengths) using a 75 W Xenon lamp (XBO 75W/2). Excitation light from the monochromator (P130/MLE/400 Optoscan; Cairn Research, UK) was reflected off a 400 nm dichroic mirror. Emitted fluorescence was collected through a 510 ± 20 nm emission filter and measured with an intensified CCD camera (C651-ICCD; Cairn Research). Ratiometric Ca^{2+} images were generated at 2–20 s intervals, using 4 averaged images at each wavelength. Images were digitally stored and analyzed using MetaFluor software (Universal Imaging Corporation, USA). The short exposition to the 340/380 nm never induced a partial isomerization of *t*-RESV to *c*-RESV. Drugs (or vehicles, for the corresponding control experiments) were added in volumes of 10–50 μL to a final incubation volume of 2 mL of bathing solution. All procedures and experiments were performed at room temperature ($\sim 20^\circ\text{C}$) to minimize compartmentalization and cell extrusion of the fluorescent dye.

2.3 Drugs and chemicals

Amphotericin B, DMSO, *t*-RESV, ionomycin, nifedipine, penicillin G, 3,4,5-trimethoxybenzoic acid 8-diethylamino-octyl ester 8 (TMB-8), 1-(β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride (SKF 96365), and streptomycin were purchased from Sigma (Alcobendas, Spain). Thapsigargin (THAPS) was from RBI (Natick, MA, USA). DMEM/F-12 and fetal bovine serum were from Gibco (Barcelona, Spain). Fura-2 AM was from Molecular Probes (Madrid, Spain). All other reagents were of analytical grade. *c*-RESV was prepared at the Departamento de Química Orgánica (Universidad de Santiago de Compostela) following the method previously described [10]. Stock solutions of these compounds were prepared and stored at -20°C as follows: SKF 96365 (10 mM) and TMB-8 (10 mM) in distilled water; ionomycin (1 mM), nifedipine (10 mM), *t*-RESV (100 mM), *c*-RESV (100 mM), and THAPS (1 mM) in DMSO. From these stock solutions, dilutions in physiological buffer were freshly prepared every day as were needed for experiments. The final concentration of DMSO never exceeded 0.01%. Fura-2-AM (2.5 μM) was prepared daily in physiological buffer containing 0.1% DMSO. Appropriate precautionary measures were taken throughout the procedure to avoid degradation of light-sensitive compounds (*t*-RESV, *c*-RESV, and nifedipine) and extensive photobleaching due to the photosensitivity of the fura-2 molecule.

2.4 Data presentation and statistical analysis

Unless otherwise specified, results shown in the text and figures are expressed as means \pm SEM. Significant differences between two means ($p < 0.05$ or $p < 0.01$) were determined by Student's two-tailed *t*-test for paired or unpaired data, where appropriate.

For each cell, $[\text{Ca}^{2+}]_i$ was averaged from pixels within manually outlined cell areas. Background compensation was performed by subtracting the illumination from an area of the image which contained no cells. $[\text{Ca}^{2+}]_i$ was then calculated from the 340/380 nm fluorescence ratio as described by Grynkiewicz *et al.* [17]. Ratios were converted to free Ca^{2+} by the equation: $[\text{Ca}^{2+}]_i = K_d (R - R_{\min}/R_{\max} - R)$ ($F_{\min 380}/F_{\max 380}$), where K_d is the affinity constant of fura-2 for Ca^{2+} , R is the 340/380 nm fluorescence ratio, R_{\min} and R_{\max} are the limiting ratios for minimal (0 mM Ca^{2+} and 10 mM EGTA in the bath) and saturating $[\text{Ca}^{2+}]_i$ (10 mM Ca^{2+} in the bath) both in the presence of 10 μM ionomycin. $F_{\min 380}/F_{\max 380}$ is the ratio of fluorescence of fura-2 at 380 nm in the presence of minimal to that of saturating $[\text{Ca}^{2+}]_i$. Basal $[\text{Ca}^{2+}]_i$ was determined by averaging resting Ca^{2+} values measured for 10 s on cells from different preparations. Only data obtained from cells that responded to

the Ca^{2+} ionophore ionomycin (0.5 μM), in the presence of 1.5 mM of external CaCl_2 , at the end of the experiments were used. From the cumulative-response curves, the 50% efficacy concentration (EC_{50}) for the effect of *t*-RESV or *c*-RESV was calculated using a sigmoidal curve-fitting analysis program (Origin 6.0).

3 Results

3.1 Effects of *t*-RESV and *c*-RESV on basal $[\text{Ca}^{2+}]_i$

In a calcium-containing external solution, the mean basal $[\text{Ca}^{2+}]_i$ level in A7r5 was 82.7 ± 4.3 nM ($n = 66$), and was unchanged throughout the experimental time course. Cumulative addition of *c*-RESV or *t*-RESV (0.1–100 μM) caused a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ (Fig. 2), which did not return to the resting level within 1 h, even after washing out the drugs. Maximal $[\text{Ca}^{2+}]_i$ levels reached were 246.7 ± 35.6 nM, $n = 14$ and 270.0 ± 27.9 nM, $n = 17$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them. EC_{50} : 16.5 ± 4.4 μM and 6.8 ± 1.3 μM , for *t*-RESV and *c*-RESV, respectively; $p < 0.05$. The

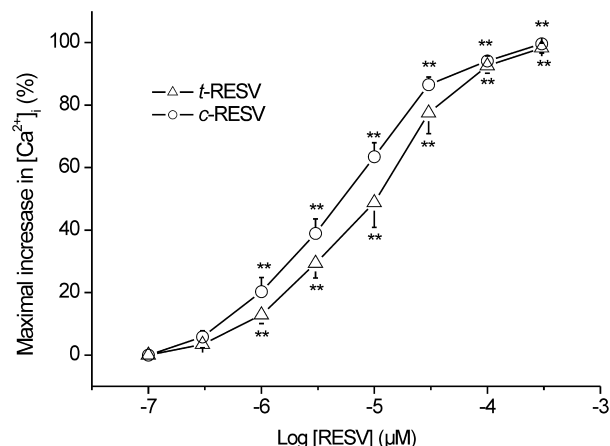


Figure 2. Cumulative concentration-response curves for *t*-RESV- or *c*-RESV-induced increases in $[\text{Ca}^{2+}]_i$ in a 1.5 mM Ca^{2+} -containing external solution in A7r5 cells. Responses are expressed as % of the maximal response obtained for each compound, considering the basal values as 0%. Each point represents the mean of 14 or 17 experiments for *t*-RESV and *c*-RESV, respectively, with SEM indicated by vertical bars. Statistical significance with respect to basal values: ** $p < 0.01$.

percentage of cells responding to *c*-RESV and *t*-RESV in a given culture did not vary greatly, and was on average ~90% of the cells that responded to 0.5 μM ionomycin at the end of the experiments.

3.2 Effects of Ni^{2+} , nifedipine, and SKF 96365 on RESV-induced rises in $[\text{Ca}^{2+}]_i$

A single application of *t*-RESV or *c*-RESV (10 μM) caused a sustained increase in $[\text{Ca}^{2+}]_i$ (maximal $[\text{Ca}^{2+}]_i$ levels: 201.9 ± 41.3 nM, $n = 19$ and 212.6 ± 39.8 nM, $n = 16$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them). Application of Ni^{2+} (300 μM) significantly inhibited the $[\text{Ca}^{2+}]_i$ -sustained plateau when it was administered 20 min after the application of *t*-RESV or *c*-RESV (reduction of the maximal response to both isomers of RESV: $\sim 50\%$; Fig. 3a). Similarly, application of nifedipine (1 μM) partially reduced the *t*-RESV- or *c*-RESV-induced increase in $[\text{Ca}^{2+}]_i$ (reduction of the maximal response to both isomers of RESV: $\sim 20\%$; Fig. 3a). However, SKF 96365 (30 μM) did not significantly modify the *t*-RESV- or *c*-RESV-induced $[\text{Ca}^{2+}]_i$ -sustained plateau (Fig. 3a).

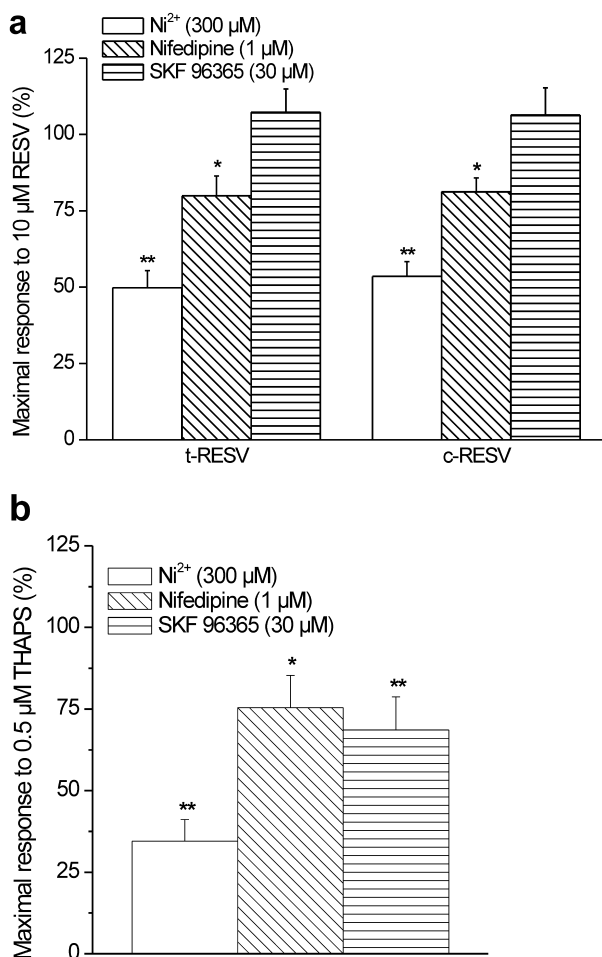


Figure 3. Effects of Ni^{2+} , nifedipine or SKF 96365 on the maximal increase in $[\text{Ca}^{2+}]_i$ (considered as the 100%) induced by (a) RESV or (b) THAPS in a 1.5 mM Ca^{2+} -containing external solution. Each bar represents the mean \pm SEM (indicated by vertical lines) of at least 12 cells. * $p < 0.05$ or ** $p < 0.01$ versus control values.

3.3 Interferences between THAPS- and RESV-induced increases in $[\text{Ca}^{2+}]_i$

3.3.1 Effects of previous administration of THAPS on RESV-induced increases in $[\text{Ca}^{2+}]_i$

In a 1.5 mM CaCl_2 -containing bathing solution, previous incubation with THAPS affects the *t*-RESV- and *c*-RESV-induced increase in $[\text{Ca}^{2+}]_i$, depending on the concentration used (Table 1). THAPS (10 nM) did not significantly modify basal $[\text{Ca}^{2+}]_i$ levels ($[\text{Ca}^{2+}]_i$ ~ 5 min after the application of THAPS: 82.0 ± 8.1 nM, $n = 10$; $p > 0.05$) and THAPS (100 nM) induced a slow transient increase of $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$ 5 min after the application of THAPS: 159.0 ± 15.4 nM, $n = 14$; $p < 0.01$). 10 min after the application of THAPS (10, 100 nM), *t*-RESV or *c*-RESV (10 μM) induced an increase in $[\text{Ca}^{2+}]_i$ that was significantly higher than in the absence of THAPS (Table 1). The subsequent application of a higher concentration of both isomers of RESV (30 μM) induced an additional increase in $[\text{Ca}^{2+}]_i$ in the presence of 10 nM THAPS but not in the presence of 100 nM THAPS (Table 1).

Higher concentrations of THAPS induced an increase in $[\text{Ca}^{2+}]_i$, consisting of an initial peak after 3–5 min ($[\text{Ca}^{2+}]_i$ ~ 5 min after the application of 0.5 or 1 μM THAPS: 332.6 ± 34.3 nM, $n = 16$ and 361.3 ± 36.9 nM, $n = 14$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them) that decreased to a sustained plateau ($[\text{Ca}^{2+}]_i$ ~ 15 min after the application of 0.5 or 1 μM THAPS: 143.1 ± 13.3 nM, $n = 16$ and 168.2 ± 16.8 nM, $n = 14$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them). The THAPS-induced plateau was significantly inhibited by Ni^{2+} (300 μM), nifedipine (1 μM) or SKF96365 (30 μM) (reduction of the maximal response to 1 μM THAPS: $\sim 65\%$; $\sim 25\%$ and $\sim 30\%$, respectively; Fig. 3b). The application of *t*-RESV or *c*-RESV (10, 30 μM) on the sustained plateau induced by THAPS (0.5, 1 μM) induced an additional increase in $[\text{Ca}^{2+}]_i$ which was not significantly higher, or even significantly reduced, compared to the values obtained in absence of THAPS (Table 1).

The additive increase in $[\text{Ca}^{2+}]_i$ induced by the application of THAPS and *t*-RESV or *c*-RESV was inhibited in part by Ni^{2+} (300 μM ; $\sim 60\%$ reduction) and nifedipine (1 μM , $\sim 20\%$ reduction), but not by SKF 96365 (30 μM) (data not shown).

3.3.2 Effects of previous administration of RESV on THAPS-induced increases in $[\text{Ca}^{2+}]_i$

In a 1.5 mM CaCl_2 -containing bathing solution, THAPS-induced rises in $[\text{Ca}^{2+}]_i$ were significantly affected by a previous administration of RESV. Lower concentrations of *t*-RESV or *c*-RESV (100 nM) did not induce by themselves significant increases in $[\text{Ca}^{2+}]_i$. Under these conditions, the increase in $[\text{Ca}^{2+}]_i$ induced by the subsequent administration

Table 1. Effects of the previous administration of THAPS on the *t*-RESV- and *c*-RESV-induced increases in $[Ca^{2+}]_i$ (nM) in a 1.5 mM Ca^{2+} -containing external solution in A7r5 cells

| | | <i>t</i> -RESV | | <i>c</i> -RESV | |
|----------------|-----------|--------------------|--------------------|--------------------|--------------------|
| | | 10 μ M | 30 μ M | 10 μ M | 30 μ M |
| Control | | 201.9 \pm 41.3 | 258.9 \pm 33.1 | 212.6 \pm 39.8 | 245.0 \pm 26.6 |
| Previous THAPS | 10 nM | 299.2 \pm 22.2** | 336.7 \pm 32.0** | 278.3 \pm 37.7* | 327.4 \pm 22.3** |
| | 100 nM | 343.4 \pm 31.1** | 345.2 \pm 44.9** | 326.8 \pm 26.4** | 319.1 \pm 25.8** |
| | 500 nM | 220.3 \pm 30.1 | 208.2 \pm 19.0* | 229.3 \pm 19.1 | 216.32 \pm 21.52 |
| | 1 μ M | 189.2 \pm 16.2 | 178.2 \pm 15.6** | 201.5 \pm 24.8 | 177.2 \pm 21.7** |

Each value is mean \pm SEM of at least 12 cells. Level of statistical significance: * $p < 0.05$ or ** $p < 0.01$ with respect to control values.

Table 2. Effects of the previous administration of *t*-RESV- and *c*-RESV on the THAPS-induced increases in $[Ca^{2+}]_i$ (nM) in a 1.5 mM Ca^{2+} -containing external solution in A7r5 cells

| | | THAPS | | | | | | | |
|-------------------------|------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| | | 10 nM | | 100 nM | | 500 nM | | 1 μ M | |
| | | Peak | Plateau | Peak | Plateau | Peak | Plateau | Peak | Plateau |
| Control | | 82.0 \pm 8.1 | 78.3 \pm 5.5 | 159.0 \pm 15.4 | 90.6 \pm 7.4 | 332.6 \pm 34.3 | 143.1 \pm 13.3 | 361.3 \pm 36.9 | 168.2 \pm 16.7 |
| Previous <i>t</i> -RESV | 100 nM | 129.8 \pm 21.2** | 85.9 \pm 7.1 | 208.1 \pm 31.1** | 116.4 \pm 15.0* | 395.0 \pm 24.4* | 177.3 \pm 19.0** | 377.9 \pm 28.9 | 184.3 \pm 23.4 |
| | 10 μ M | 198.4 \pm 30.0** | 135.3 \pm 16.9** | 215.2 \pm 28.9** | 142.2 \pm 28.1** | 256.3 \pm 22.0** | 122.1 \pm 19.5 | 235.4 \pm 33.7** | 119.8 \pm 15.1** |
| Previous <i>c</i> -RESV | 100 nM | 135.2 \pm 17.2** | 90.6 \pm 10.0 | 210.8 \pm 19.1** | 122.4 \pm 27.0 | 401.9 \pm 29.3** | 190.2 \pm 22.6** | 398.1 \pm 30.0 | 192.3 \pm 24.5 |
| | 10 μ M | 205.4 \pm 21.6** | 140.0 \pm 17.7** | 233.8 \pm 31.5** | 139.8 \pm 18.4** | 277.2 \pm 27.8** | 130.1 \pm 17.5 | 244.4 \pm 31.1** | 122.3 \pm 23.3** |

Each value is mean \pm SEM of at least 10 cells. Level of statistical significance: * $p < 0.05$ or ** $p < 0.01$ with respect to control values.

of THAPS (10, 100, 500 nM) was significantly potentiated (Table 2). However, the response to the higher concentration of THAPS (1 μ M) was not significantly modified (Table 2). A higher concentration of *t*-RESV or *c*-RESV (10 μ M), which induced a marked increase in $[Ca^{2+}]_i$ (see above), did significantly potentiate the increase in $[Ca^{2+}]_i$ induced by THAPS (10, 100 nM), but did reduce the increase in $[Ca^{2+}]_i$ induced by THAPS (0.5, 1 μ M) (Table 2).

3.4 Effects of *t*-RESV and *c*-RESV on basal $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

In a free-calcium external solution, the average basal $[Ca^{2+}]_i$ was 65.7 ± 5.7 nM ($n = 33$; $p < 0.05$ with respect to that obtained in 1.5 mM Ca^{2+} external solution), and was unchanged throughout the experimental time course. Addition of *c*-RESV or *t*-RESV (10 μ M) induced a transient increase in $[Ca^{2+}]_i$ that reaches its maximum after 8–10 min and returns to its basal levels after ~25 min. The maximal $[Ca^{2+}]_i$ level reached were 115.6 ± 12.5 nM, $n = 18$ and 111.2 ± 13.0 nM, $n = 15$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them. Repetitive and cumulative addition of both RESV isomers was able to induce new increases in $[Ca^{2+}]_i$ although the maximal level continued to be reduced until the complete absence of response after 3–4 applications.

After a single application of *t*-RESV or *c*-RESV (10 μ M), the replacement of the free- Ca^{2+} solution by a calcium-containing one (1.5 mM $CaCl_2$), induced an increase in $[Ca^{2+}]_i$ following the model of the classic capacitative entry pathway [18]. The maximal $[Ca^{2+}]_i$ levels reached were 163.1 ± 18.3 nM, $n = 9$ and 158.4 ± 26.1 nM, $n = 7$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them). *t*-RESV or *c*-RESV (100 μ M) induced an increase in $[Ca^{2+}]_i$ that was significantly higher than that induced by lower concentrations (10 μ M) of these agents. Under these conditions cells did not respond to a second application of the drugs (data not shown).

3.5 Interferences between THAPS- and RESV-induced rises in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

In a free-calcium external solution, the addition of THAPS (0.5 μ M) induced a transient increase in $[Ca^{2+}]_i$ that reached its maximum after 3–5 min (maximal $[Ca^{2+}]_i$ level: 184.3 ± 20.9 nM, $n = 12$; $p < 0.01$ with respect to basal values) and returned to its basal levels after ~10 min. After the application of THAPS, the replacement of the free- Ca^{2+} solution by a calcium-containing one (1.5 mM $CaCl_2$), induced a capacitative Ca^{2+} entry (maximal $[Ca^{2+}]_i$: 163.1 ± 25.1 nM, $n = 10$; $p < 0.01$ with respect to basal values).

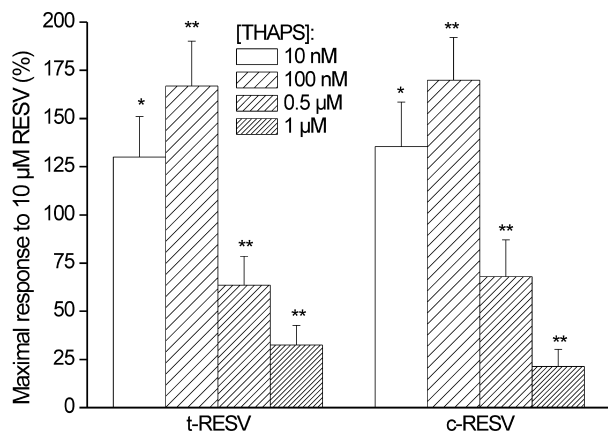


Figure 4. Effects of previous application of THAPS on the maximal increase in $[\text{Ca}^{2+}]_i$ (considered as the 100%) induced by *t*-RESV or *c*-RESV in the absence of extracellular Ca^{2+} . Each bar represents the mean \pm SEM (indicated by vertical lines) of at least 10 cells. * $p < 0.05$ or ** $p < 0.01$ versus control values.

The interactions between THAPS and both isomers of RESV correlates well with the results obtained in a 1.5 mM CaCl_2 -containing bath solution. Thus, the RESV-induced increases in $[\text{Ca}^{2+}]_i$ were potentiated by a previous application of low concentrations of THAPS (10, 100 nM). However, higher concentrations of THAPS (0.5, 1 μM) did significantly inhibit the *t*-RESV- or *c*-RESV-induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 4).

Furthermore, after partial depletion of intracellular Ca^{2+} stores with *t*-RESV or *c*-RESV (10 μM), THAPS-induced (0.5 μM) increases in $[\text{Ca}^{2+}]_i$ were significantly reduced (maximal $[\text{Ca}^{2+}]_i$: 111.9 ± 12.5 nM, $n = 11$ and 98.0 ± 14.2 nM, $n = 13$ after *t*-RESV and *c*-RESV, respectively; $p < 0.01$ with respect to values in the absence of RESV). Under the conditions when *t*-RESV or *c*-RESV were not able to release more intracellular Ca^{2+} (*i.e.*, after application of 100 μM *t*-RESV or *c*-RESV of after cumulative applications of 10 μM *t*-RESV or *c*-RESV), subsequent application of THAPS (0.5 μM) did not increase basal $[\text{Ca}^{2+}]_i$ levels.

3.6 Effects of TMB-8 on *t*-RESV-, *c*-RESV-, or THAPS-induced rises in $[\text{Ca}^{2+}]_i$

In the absence of extracellular Ca^{2+} , 5 min of preincubation with the intracellular Ca^{2+} antagonist TMB-8 (100 μM) did completely inhibit the $[\text{Ca}^{2+}]_i$ increases induced by THAPS (0.5 μM) or by both isomers of RESV (10 μM). However, after 5 min of preincubation with TMB-8 (100 μM) in the presence of 1.5 mM external Ca^{2+} , THAPS (0.5 μM), *t*-RESV (10 μM), or *c*-RESV (10 μM) were still able to induce a slow, sustained increase in $[\text{Ca}^{2+}]_i$ (maximal $[\text{Ca}^{2+}]_i$

levels: 142.5 ± 16.0 nM, $n = 9$, 129.1 ± 15.1 nM, $n = 12$ and 119.7 ± 18.3 nM, $n = 8$, respectively; $p < 0.01$ with respect to basal values), suggesting that these agents can induce a transmembrane Ca^{2+} influx by other mechanism than the activation of store-operated Ca^{2+} channels (SOCCs). These increases in $[\text{Ca}^{2+}]_i$ were not significantly inhibited by the application of Ni^{2+} (300 μM), nifedipine (1 μM), or SKF 96365 (30 μM).

4 Discussion

This is the first comparative study of the potential effects of both isomers of RESV on $[\text{Ca}^{2+}]_i$ in A7r5 cells. In our experiments, the pharmacological effects caused by *t*-RESV and *c*-RESV were qualitatively similar, *i.e.*, they directly increased $[\text{Ca}^{2+}]_i$, which suggests that the different spatial conformation of *c*-RESV (*versus* that of the *trans*-isomer) does not seem to modify markedly its interaction with the potential cellular targets in A7r5 cells. In this connection, it is interesting to note that, according to their EC_{50} values, *c*-RESV was slightly, but significantly more effective than *t*-RESV.

Since $[\text{Ca}^{2+}]_i$ variations directly regulate vascular contraction, it would be reasonable to speculate that the RESV-induced increase of $[\text{Ca}^{2+}]_i$ levels in A7r5 is correlated to an augmentation of the resting tone of isolated rat aorta. However, previous reports have shown that *t*-RESV directly relaxes rat aortic smooth muscle [8, 9] (see Section 1). These discrepancies could be due to the following reasons:

- A different sensitivity to RESV of the A7r5 cloned cell line compared to the rat aortic smooth muscle cells (RSMCs) isolated from the medial layer of rat aorta (for detailed method of preparation, see [19]). In order to exclude or confirm this possibility, further investigations are currently being conducted in our laboratory to establish whether RESV also induces increases in $[\text{Ca}^{2+}]_i$ in RSMCs.
- The increase in $[\text{Ca}^{2+}]_i$ measured by fura-2 imaging techniques in isolated RSMCs is not always accompanied by an augmentation of the basal tone in rat aortic rings. In fact, THAPS-induced transient rise in $[\text{Ca}^{2+}]_i$ in RSMC, in the absence of extracellular Ca^{2+} , is not accompanied by a contraction in rat aorta rings [20], suggesting that intracellular Ca^{2+} release by this drug in RSMCs is not coupled to muscular contraction. However, controversial results have been reported on the relationship between $[\text{Ca}^{2+}]_i$ elevation after inhibition of the sarcoplasmic reticulum Ca^{2+} -ATPase and contraction in vascular smooth muscle [20–22].
- The RESV-induced increase in $[\text{Ca}^{2+}]_i$ in A7r5 cells may be counteracted in the myocytes of isolated rat aortic preparations by other mechanisms contributing to the vasorelaxant effects of RESV, *i.e.*, the inhibitory effects of RESV

on NADH/NADPH oxidase enzymatic activity [5] or the capacity to inhibit some of the steps of the contractile effect induced by PKC [9] (see also Section 1). In this regard, the possibility of RESV acting as an inhibitor of agonist-induced increases in $[Ca^{2+}]_i$ by another mechanism (*i. e.*, a blockade of nonselective cation channels) can not be discarded from our experiments. In fact, in the present work, both *t*-RESV and *c*-RESV did significantly reduce the increases in $[Ca^{2+}]_i$ induced by the higher concentrations of THAPS (0.5–1 μ M). Furthermore, in another set of experiments performed in our laboratory, *t*-RESV did significantly decrease the plateau of the response to vasopressin, before inducing a slow increase in $[Ca^{2+}]_i$ in A7r5 cells (unpublished observations). Finally, after partial depletion of intracellular Ca^{2+} stores by RESV, the response to a subsequent application of an agonist acting through a phospholipase C and inositol 1,4,5-triphosphate-mediated mechanism, could be significantly reduced. Supporting this hypothesis, pre-incubation with low concentrations of *t*-RESV significantly reduces the vasopressin-induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} in A7r5 cells (unpublished observations). Thus, it seems that the effects of RESV isomers on $[Ca^{2+}]_i$ in A7r5 cells depend on the state of refilling of the intracellular stores and, under some conditions, an inhibitory effect may be more important than the $[Ca^{2+}]_i$ -increasing effect described here. Further studies are needed to confirm this possibility.

In our experiments in free- Ca^{2+} external solution, both *t*-RESV and *c*-RESV induced an increase in $[Ca^{2+}]_i$ as a consequence of the depletion of intracellular stores. TMB-8, an inhibitor of intracellular Ca^{2+} release that reduces Ca^{2+} availability in smooth and skeletal muscles by stabilizing Ca^{2+} binding to cellular Ca^{2+} stores [23], has been shown to transiently block THAPS-induced rise in $[Ca^{2+}]_i$ for about 20 min [24] and to abolish the initial peak of the contractile response induced by THAPS in rat aortic strips [25]. In the same work, Tepel *et al.* [25] have also shown that the second phase of the response to THAPS was not inhibited by TMB-8, but it was completely abolished in the absence of extracellular Ca^{2+} . According to this, in our experiments in the absence of extracellular Ca^{2+} , TMB-8 completely abolished the increase in $[Ca^{2+}]_i$ induced by THAPS. A similar effect of TMB-8 was observed on *t*-RESV- or *c*-RESV-induced increases in $[Ca^{2+}]_i$, suggesting that both stilbenes, as well as THAPS, release Ca^{2+} from intracellular stores which are sensitive to the action of TMB-8.

The interferences between THAPS- and RESV-induced responses in the absence of extracellular Ca^{2+} indicate that these drugs are probably acting on the same intracellular stores. Thus, after partial depletion of THAPS-sensitive intracellular stores, the *t*-RESV- and *c*-RESV-induced increases in $[Ca^{2+}]_i$ were significantly reduced. However, after applications of lower concentrations of THAPS

(which did not induce an increase of $[Ca^{2+}]_i$ by themselves), the response to both isomers of RESV was significantly potentiated. A similar situation was produced when the RESV isomers were administered before THAPS. The possibility of RESV inhibiting sarcoplasmic reticulum Ca^{2+} -ATPase in a similar way that THAPS can not be ruled out, since an inhibition of ATPase activity has already been described for *t*-RESV [26, 27].

It has been described that depletion of intracellular Ca^{2+} pools, either by membrane agonists or by intracellular agents, increases membrane permeability to Ca^{2+} in non-excitable cells, which, in turn, permits the refilling of depleted stores through SOCCs, in accordance with the so-called “capacitative model” [18, 28]. This Ca^{2+} influx can be inhibited with SKF 96365 [29]. However, the involvement of other channels, such as dihydropyridine-sensitive Ca^{2+} channels, is not clear, and contradictory results have been reported [30, 31].

Here, the release of Ca^{2+} from intracellular stores induced by THAPS, *t*-RESV, or *c*-RESV was followed by a transmembrane capacitative Ca^{2+} influx, since reintroduction of external Ca^{2+} in THAPS-, *t*-RESV-, or *c*-RESV-depleted cells elicited a sustained increase in $[Ca^{2+}]_i$. The RESV- and the THAPS-induced influx of Ca^{2+} through SOCCs was partially inhibited by Ni^{2+} , an inhibitor of capacitative Ca^{2+} entry or nifedipine, a voltage-dependent L-type Ca^{2+} channel blocker. However, SKF 96365, a nonselective Ca^{2+} channel inhibitor that also reduces Ca^{2+} influx through SOCC (see above), only inhibited the increase in $[Ca^{2+}]_i$ induced by THAPS, but not by *t*-RESV or *c*-RESV. This lack of effects of SKF 96365 could be explained on the basis that, under some conditions, this imidazole has been reported to increase intracellular $[Ca^{2+}]_i$ by releasing Ca^{2+} from intracellular stores in A7r5 cells [32]. Then, the inhibitory effect of SKF 96365 on SOCCs could be more evident on THAPS-induced capacitative Ca^{2+} influx, which was significantly higher than that induced by the RESV isomers.

Mechanisms other than the activation of SOCCs could also be implicated in the increase in $[Ca^{2+}]_i$ induced by both isomers of RESV, since in the presence of 1.5 mM extracellular Ca^{2+} TMB-8 was not able to completely inhibit the rise in $[Ca^{2+}]_i$, suggesting that, when the release of Ca^{2+} from intracellular stores was blocked, *t*-RESV or *c*-RESV were still able to induce a transmembrane Ca^{2+} influx, which was not significantly affected by Ni^{2+} , nifedipine or SKF 96365. A similar result was obtained for THAPS, in good agreement with the results of Tepel *et al.* [25] (see above).

In conclusion, we have described for the first time that *t*-RESV and *c*-RESV, either acting at the plasma membrane and/or by direct diffusion or active transport into the cytoplasm, induce an unexpected increase in $[Ca^{2+}]_i$; the *cis*-isomer being slightly but significantly more effective than the

trans-isomer. This increase in $[\text{Ca}^{2+}]_i$ is mediated by depletion of Ca^{2+} from the same intracellular stores released by thapsigargin, and subsequent influx of Ca^{2+} through Ni^{2+} - and nifedipine-sensitive SOCCs, although a direct activation of transmembrane Ca^{2+} influx through another type of channel that were not affected by Ni^{2+} , nifedipine, or by SKF 96365 may be also implicated.

Taking into account the previous reports on the effects of *t*-RESV in rat aortic preparations (see Section 1), the RESV-induced increase in Ca^{2+} seems to be not relevant for inducing contraction of rat aortic smooth muscle. However, a similar increase in $[\text{Ca}^{2+}]_i$ in endothelial cells could induce an increase of the NO release, and thus contribute to the endothelium-dependent vasorelaxant effect reported for *t*-RESV in rat aorta [5, 8]. In fact, substances that increase endothelial cell $[\text{Ca}^{2+}]_i$ potentially influence the production of endothelial factors (notably NO, because of the Ca^{2+} sensitivity of constitutive NO synthase in the endothelium) [33] and underlying vascular tone, as reported for the ionophore A23187 and for THAPS, which induce endothelium-dependent relaxation of Guinea-pig rat aorta [34, 35].

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5 References

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