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Effects of *trans*- and *cis*-resveratrol on Ca^{2+} handling in A7r5 vascular myocytes

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Abstract

Although the natural polyphenol resveratrol posses a direct vasorelaxant effect, its effects on cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular cells remain still unclear. Here, we have investigated the effects of the isomers *trans*- and *cis*-resveratrol on agonist- and high-K^+-induced $[Ca^{2+}]_i$ increases and on voltage-activated transmembrane Ca^{2+} fluxes using imaging and patch-clamp techniques in vascular A7r5 myocytes. Arginine vasopressin (AVP) or angiotensin II caused a biphasic increase in $[Ca^{2+}]_i$ that was reduced by preincubation with *trans*-resveratrol and *cis*-resveratrol. Both isomers also reduced the agonist-induced increase in $[Ca^{2+}]_i$ in absence of extracellular Ca^{2+}. In high-K^+ Ca^{2+}-free solution, reintroduction of Ca^{2+} caused a sustained rise in $[Ca^{2+}]_i$ that was reduced by preincubation with *trans*-resveratrol or *cis*-resveratrol. When the isomers were applied during the plateau phase of the agonist- or the high-K^+-induced response, a biphasic change in $[Ca^{2+}]_i$ was observed: a transient reduction of the plateau (<5 min) followed by an increase (>10 min). Finally, *trans*-resveratrol and *cis*-resveratrol inhibited voltage-dependent L-type Ca^{2+} currents ($I_{Ca(L)}$). In conclusion, resveratrol isomers exert a dual effect on $[Ca^{2+}]_i$ handling in A7r5 myocytes: 1) a blockade of $I_{Ca(L)}$ and 2) an increase in $[Ca^{2+}]_i$ by depletion of intracellular Ca^{2+} stores (which interferes with the agonist-induced release of intracellular Ca^{2+}) and influx of Ca^{2+}, mainly due to activation of capacitative Ca^{2+} entry, although other Ca^{2+}-permeable channels are also involved. Taken together, these effects may explain, in part, the endothelium-independent vasorelaxant effects of resveratrol in rat aorta.
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1. Introduction

Resveratrol (3,4′,5-trihydroxystilbene), is a polyphenolic component of a wide variety of plants which is present in grapes and wines (especially red wines) in significant amounts. Resveratrol has been reported to possess a wide range of biological activities, including anti-inflammatory, anticarcinogenic, antioxidant, estrogenic, platelet antiaggregatory, and antimicrobial properties (for review see, *e.g.*, Soleas et al., 1997; Aggarwal et al., 2004; Baur and Sinclair, 2006; Orallo, 2006b). Although resveratrol exists as *cis* and *trans* isomers, most studies to date on the biological effects of resveratrol have considered the *trans* isomer (Orallo, 2006a,b), possibly as a result of the fact that this isomer is the only one available commercially.

Many studies have also described properties of resveratrol that could be implicated in the prevention of cardiovascular diseases (for review see, *e.g.*, Burjonroppa and Fujise, 2006; Campos-Toimil et al., in press). In rats, *trans*-resveratrol has been shown to prevent increase in blood pressure in hypertensive rats (Mizutani et al., 2000). *In vivo* protection by *trans*-resveratrol against increase in systolic blood pressure, and subsequent cardiac hypertrophy, has also been reported by Liu et al. (2005a). Despite the above considerations, the *in vitro* vasorelaxant activity of resveratrol is presently somewhat controversial. For example, *trans*-resveratrol (at concentrations up to 100 μM), was unable to relax phenylephrine-precontracted smooth muscle of rat aortic rings (Fitzpatrick et al., 1993). On the other hand, Chen and Pace-Asciak (1996) reported that *trans*-resveratrol (>30 μM) concentration-dependently inhibited the contractile response to noradrenaline or phenylephrine in isolated endothelium-intact rat aorta. This inhibitory effect was blocked by pretreatment of the

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aortic rings with the nitric oxide synthase inhibitor *N*ω-nitro-L-arginine. These authors also shown that, at higher concentrations (>60 μM), resveratrol relaxed endothelium-denuded aortic rings, but this effect could not be reversed by *N*ω-nitro-L-arginine.

Similarly, we have reported that *trans*-resveratrol exhibits in rat aorta: a) a characteristic endothelium-dependent vasorelaxant effect (at concentrations <10 μM), probably mediated by an inhibition of vascular NADH/NADPH oxidase (Orallo et al., 2002) and b) an endothelium-independent vasodilator effect (at concentrations >50 μM), which appears to be partially mediated by an inhibition of protein kinase C (Orallo and Camiña, 1998).

Since vascular tone directly depends on $[Ca^{2+}]_i$ levels in smooth muscle cells, any action of resveratrol on calcium signalling in these cells may be related to the endothelium-independent vasorelaxant action of the drug. Surprisingly, in a previous study we have shown that *trans*-resveratrol and *cis*-resveratrol caused a sustained elevation in $[Ca^{2+}]_i$ in single A7r5 cells (a cell line derived from foetal rat aorta), although the resveratrol-induced increase in $[Ca^{2+}]_i$ seems to be unrelated to rat aorta contractility (Campos-Toimil et al., 2005). However, from our experiments we could not discard the possibility of resveratrol acting as an inhibitor of agonist-induced increases in $[Ca^{2+}]_i$.

The aim of the present study was to further elucidate the effects of resveratrol isomers on Ca^{2+} handling in vascular cells by evaluating, for the first time, their effects on the agonist- and the high-K^+-induced $[Ca^{2+}]_i$ changes using a fura-2 based Ca^{2+} imaging setup in A7r5 vascular myocytes. Additionally, using the whole-cell patch-clamp technique we have examined the role of resveratrol isomers in the activation of voltage-operated Ca^{2+} entry in these cells. In all the experiments we have compared *trans*-resveratrol and *cis*-resveratrol in order to investigate whether they have different actions or, on the contrary, the difference on their effects is merely quantitative.

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 (American Type Culture Collection; CRL1446; Rockville, MD, USA), were grown and kept in culture as described elsewhere (Campos-Toimil et al., 2005). The culture medium was Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1 v/v) supplemented with heat-inactivated foetal bovine serum (FBS, 10% v/v), L-glutamine (2 mM), penicillin G (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Cells kept in culture at 37 °C with 5% CO_2 in air. For imaging experiments, the cells were subcultured at low density (~1500 cells/cm^2) 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 allowed to grow for at least 24 h in culture medium and kept in culture for 2–5 days before the experiments.

For patch-clamp experiments, confluent cells were trypsinized and 50–100 μl of the cell suspension was transferred to a 15 ml plastic tube containing 3–4 ml of FBS-free culture medium that was kept at 4 °C for 2–5 h until the realization of the electrophysiological experiments. Then, 5–10 μl of the FBS-free cell suspension was added to a Petri dish, previously kept at 4 °C with bovine serum albumin (0.2% w/v), containing the patch-clamp extracellular solution (see below for composition).

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

$[Ca^{2+}]_i$ imaging experiments were carried out as previously described (Campos-Toimil et al., 2005). Briefly, A7r5 cells were incubated for 60 min at 37 °C in normal bathing solution (composition in mM: NaCl 140, KCl 5, $CaCl_2{\cdot}2H_2O$ 1.5, $MgCl_2$ 2, HEPES 10, glucose 11, pH 7.4) containing 2.5 μM fura-2 acetoxymethyl ester (fura-2 AM), then gently washed and allowed to rest for >15 min in the incubator. Cells were placed on an inverted microscope and continuously superfused at 0.5 ml/min 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. Pairs of fluorescence images at 340±10 nm and 380±10 nm were obtained every 2–20 s from isolated cells or small groups of dispersed cells (up to 6 cells). Images were digitally stored and analyzed using MetaFluor software (Universal Imaging Corporation, PA, USA).

Drugs (or vehicles, for the corresponding control experiments) were added in volumes of 10 to 50 μl to a final incubation volume of 2 ml of bathing solution. All procedures and experiments were performed at room temperature (~20 °C) to minimize compartmentalization and cell extrusion of the fluorescent dye.

2.3. Whole-cell patch-clamp recording

The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981; Verde et al., 1999) was used to record the voltage-dependent L-type Ca^{2+} current ($I_{Ca(L)}$) in A7r5 cells. The control extracellular solution contained (mM): NaCl 107.1, CsCl 40.0, $CaCl_2$ 1.8, $MgCl_2$ 1.8, Na–piruvate 5.0, $NaHCO_3$ 4.0, NaH_2PO_4 0.8, HEPES 10.0, glucose 5.0, pH 7.4 adjusted with NaOH. Patch pipettes (2–4 MΩ) were filled with intracellular solution (mM): CsCl 119.8, $CaCl_2$ 0.06, $MgCl_2$ 4.0, Na–ATP 3.1, Na–GTP 0.4, EGTA 5.0, HEPES 10.0, tetraethylammonium sodium salt 10.0, pH 7.4 adjusted with CsOH. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+. Solutions were filtered (0.22 μm) before use.

The cells were maintained at −80 mV holding potential (V_h) and routinely depolarised every 8s to 0 mV test potential during 500 ms. Currents were not compensated for capacitance and leak currents. All experiments were carried out at room temperature (21–25 °C) and the temperature did not vary by more than 1 °C in a given experiment. The cells were voltage clamped using an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc., Union City, CA, USA). Currents were sampled at a frequency of 10 kHz and filtered at 0.1 kHz using an analog-to-digital interface Digidata 1322A (Axon Instruments Inc.) connected to a

compatible computer with the Pclamp8 software (Axon Instruments Inc.). Control or drug-containing solutions were applied to the exterior by placing the cell at the opening of 250 μm inner diameter capillary flowing at a rate of 20 μl/min.

2.4. Drugs and chemicals

Angiotensin II, amphotericin B, arginine vasopressin (AVP), bovine serum albumin, dimethylsulfoxide (DMSO), *trans*-resveratrol, L-glutamine, ionomycin, nifedipine, penicillin G, tetraethylammonium sodium salt and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Thapsigargin was from RBI (Natick, MA, U.S.A.). Ryanodine was from ICN Biochemicals (Asse-Relegem, Belgium). Trypsin/EDTA was from Roche Diagnostics (Barcelona, Spain). Dulbecco's modified Eagle's medium/Ham's F-12 medium and FBS were from Gibco-Life Technologies (Grand Island, NY, USA). Fura-2 AM was from Molecular Probes (Eugene, OR, USA). *cis*-resveratrol was prepared at the *Departamento de Química Orgánica* (*Universidade de Santiago de Compostela*, Spain) following the method previously described (Leiro et al., 2004). All other reagents were of analytical grade.

Stock solutions of these compounds were prepared and stored at −20 °C as follows: angiotensin II (1 mM), AVP (1 mM), Ni^{2+} (10 mM) in deionized water; ionomycin (1 mM), nifedipine (10 mM), ryanodine (10 mM), thapsigargin (1 mM), *trans*-resveratrol (100 mM) and *cis*-resveratrol (100 mM) in DMSO. For patch-clamp experiments, stock solutions of nifedipine (10 mM), *trans*-resveratrol (100 mM) and *cis*-resveratrol (100 mM) were prepared in absolute ethanol. From these stock solutions, appropriate dilutions in distilled water or extracellular solution were freshly prepared every day as were needed for experiments. Final concentration of DMSO and ethanol never exceeded 0.01% and 0.1% (v/v), respectively. Fura-2 AM (5 μM) was prepared daily in physiological buffer containing DMSO (0.1% v/v).

Studies involving light-sensitive compounds (*trans*-resveratrol, *cis*-resveratrol and nifedipine) were carried out in the dark. For imaging experiments, appropriate precautionary measures were taken throughout the procedure to avoid degradation of light-sensitive compounds and extensive photobleaching due to the photosensitivity of the fura-2 molecule.

2.5. Data presentation and statistical analysis

Unless otherwise specified, results shown in the text and figures are expressed as mean±S.E.M. 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 or by one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test, where appropriate.

For each cell, $[Ca^{2+}]_i$ 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. The $[Ca^{2+}]_i$ was then calculated from the 340/380 nm fluorescence ratio as described by Grynkiewicz et al. (1985). For more details see Campos-Toimil et al. (2005). Basal $[Ca^{2+}]_i$ 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

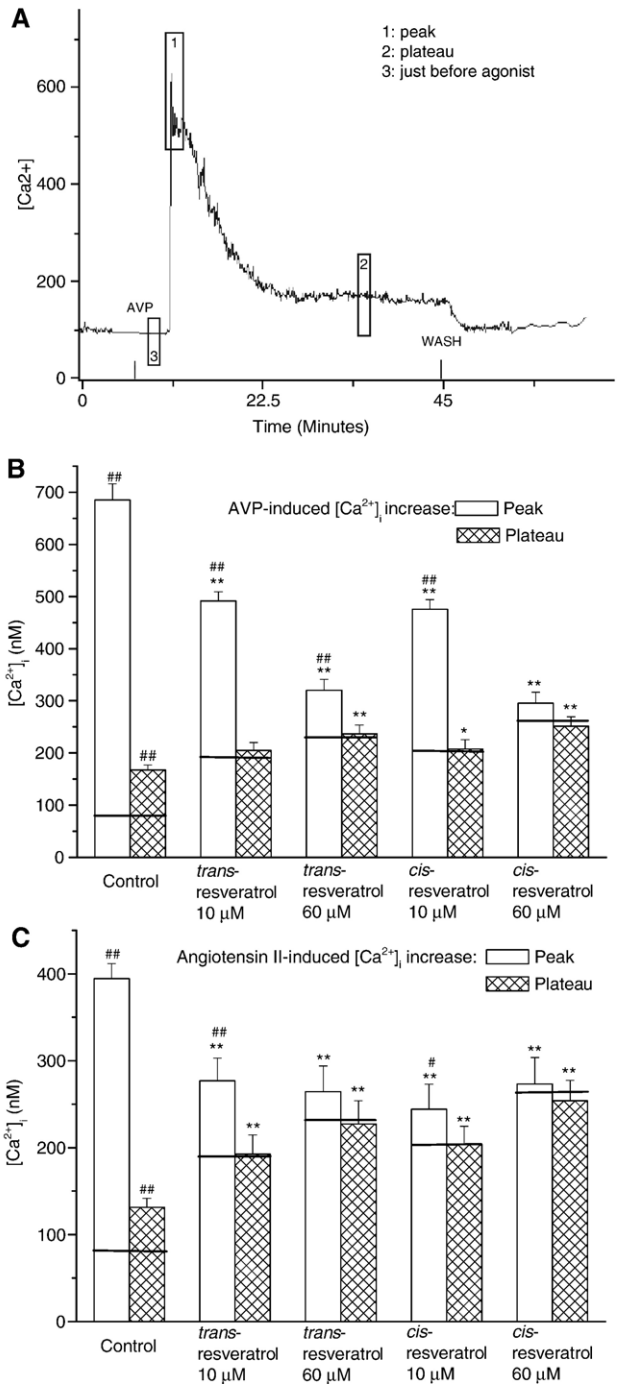

Fig. 1. (A) Original tracing illustrating the AVP-induced biphasic increase in $[Ca^{2+}]_i$ in a single A7r5 cell. Effects of the preincubation with *trans*-resveratrol or *cis*-resveratrol (20 min) on the increase in $[Ca^{2+}]_i$ induced by 0.5 μM AVP (B) or 0.1 μM angiotensin II (C) in A7r5 cells. Each bar represents the mean±S.E.M. (indicate by vertical lines) of, at least, 15 cells. $[Ca^{2+}]_i$ values just before the addition of AVP or angiotensin II in control and resveratrol-treated cells are indicated by horizontal lines (see also Table 1). Level of statistical significance: $^{*}P<0.05$ and $^{**}P<0.01$ with respect to the corresponding control value. $^{\#\#}P<0.01$ with respect to the corresponding $[Ca^{2+}]_i$ level before agonist application.

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.

$I_{Ca(L)}$ amplitudes were automatically calculated between the maximum current peak and the stable current plateau near the final of the pulse. $I_{Ca(L)}$ variations were studied in control extracellular solution containing or not drugs.

3. Results

3.1. AVP- or angiotensin II-induced increases in $[Ca^{2+}]_i$ in a 1.5 mM Ca^{2+}-containing medium

In a calcium containing solution, mean basal $[Ca^{2+}]_i$ was 80.8±4.4 nM (n=67), and, in absence of any treatment, it was unchanged throughout the experimental time course.

AVP (0.5 μM) caused a biphasic increase in $[Ca^{2+}]_i$ (Fig. 1A,B): first, a transient elevation (peak) in $[Ca^{2+}]_i$ within 1–2 min (maximal $[Ca^{2+}]_i$ reached: 685.2±30.9 nM, n=67, P<0.01 with respect to basal value) followed by a sustained plateau ($[Ca^{2+}]_i$: 167.7±9.5 nM, n=67, P<0.01 with respect to basal and peak values). This plateau was maintained for, at least, 45 min in the presence of the agonist and returned to basal value after wash. The plateau phase of the AVP-induced response was partially inhibited by 1 μM nifedipine ($[Ca^{2+}]_i$: 128.2±8.5 nM, n=18, P<0.05) and completely abolished by 300 μM Ni^{2+} ($[Ca^{2+}]_i$: 80.6±7.2 nM, n=7, P<0.01; P>0.05 with respect to basal value).

The application of angiotensin II (0.1 μM) induced a biphasic increase in $[Ca^{2+}]_i$ that was qualitatively similar to that induced by AVP (Fig. 1C), with a transient peak of 394.5±17.1 nM and a sustained plateau of 131.3±10.5 nM (n=31, P<0.01 with respect to basal value), which was stable for, at least, 45 min in the continuous presence of the agonist. The plateau phase of the angiotensin II-induced response was partially inhibited by 1 μM nifedipine ($[Ca^{2+}]_i$: 99.7±4.2 nM, n=18, P<0.05) and completely abolished by 300 μM Ni^{2+} ($[Ca^{2+}]_i$: 75.3±7.0 nM, n=10, P<0.01; P>0.05 with respect to basal value).

3.2. Effects of the preincubation with resveratrol isomers on the AVP- or angiotensin II-induced increase in $[Ca^{2+}]_i$ in a 1.5 mM Ca^{2+}-containing medium

In order to investigate the effects of *trans*- and *cis*-resveratrol (10, 60 μM) on the biphasic increase in $[Ca^{2+}]_i$ induced by AVP or angiotensin II, they were added to the bath 20 min before application of the agonists and caused a slow sustained elevation in $[Ca^{2+}]_i$ (Table 1). In these conditions, the peak phase of the increase in $[Ca^{2+}]_i$ elicited by the subsequent application of AVP (0.5 μM) was significantly reduced and the plateau phase was completely inhibited (Fig. 1B). Similar results were obtained using angiotensin II (0.1 μM) as agonist (Fig. 1C).

Table 1
Maximal $[Ca^{2+}]_i$ (nM) induced by *trans*-resveratrol and *cis*-resveratrol in 1.5 mM Ca^{2+}-containing solution (sustained increase) and in a high-K^+ (60 mM) Ca^{2+}-free solution (transient increase)

		1.5 mM Ca^{2+}	High-K^+/Ca^{2+}-free
trans-resveratrol	10 μM	190.4±9.9[b] (41)	101.6±13.1[a] (12)
	60 μM	231.5±9.8[b] (37)	145.4±13.2[b] (10)
cis-resveratrol	10 μM	203.1±10.3[b] (36)	116.9±9.9[b] (11)
	60 μM	263.8±10.5[b,c] (32)	152.2±15.8[b] (12)

Level of statistical significance: [a]P<0.05 or [b]P<0.01 with respect to basal values; [c]P<0.05 with respect to *trans*-resveratrol. The number of experiments (n) is given in brackets.

Table 2
Ratio peak/plateau for the absolute $[Ca^{2+}]_i$ levels induced by AVP (0.5 μM) and angiotensin II (0.1 μM) in the absence (control) and in the presence of resveratrol isomers

	Control	10 μM		60 μM	
		trans-resveratrol	*cis*-resveratrol	*trans*-resveratrol	*cis*-resveratrol
AVP	4.08	2.39	2.29	1.35	1.17
Angiotensin II	3.00	1.43	1.19	1.16	1.07

It is important to note here that, considering the absolute $[Ca^{2+}]_i$ values, the agonist-induced $[Ca^{2+}]_i$ peak is reduced by resveratrol isomers, while the plateau is increased, suggesting that the $[Ca^{2+}]_i$ rise induced by resveratrol preincubation is maintained after the application of AVP or angiotensin II (Fig. 1). To achieve a better understanding, we have also made a comparison between control and resveratrol-treated values using the ratio peak/plateau (considering the absolute $[Ca^{2+}]_i$ values), which was reduced in a concentration-dependent fashion by the isomers and it was lower for *cis*-resveratrol than for *trans*-resveratrol (Table 2).

3.3. Effects of the application of the resveratrol isomers on the plateau phase of the AVP- or angiotensin II-induced increase in $[Ca^{2+}]_i$ in a 1.5 mM Ca^{2+}-containing medium

The plateau phase of the AVP- and angiotensin II-induced rise in $[Ca^{2+}]_i$ in A7r5 cells is mainly due to influx of extracellular Ca^{2+} (see Discussion). To evaluate the effects of resveratrol isomers on Ca^{2+} influx during this phase, they were added to the bath once the $[Ca^{2+}]_i$ plateau was stabilized. When the isomers of resveratrol (60 μM) were applied during the plateau phase of the AVP- or angiotensin II-induced response, a biphasic change in $[Ca^{2+}]_i$ was observed: 1) an initial transient reduction of the agonist-evoked plateau that occurs during the first 5 min; 2) a sustained increase, starting ~10 min after the application of the isomers, that stabilizes ~15–20 min later in values similar or higher to those of the previous agonist-induced plateau (Fig. 2A–C).

Note that, at a concentration of 10 μM, only *cis*-resveratrol induced a significant increase of the AVP- or angiotensin II-induced $[Ca^{2+}]_i$ plateau after 20 min of incubation (Fig. 2B,C).

3.4. Effects of the resveratrol isomers on the AVP- or angiotensin II-induced increase in $[Ca^{2+}]_i$ in a Ca^{2+}-free medium

The agonist-induced $[Ca^{2+}]_i$ peak was significantly inhibited by resveratrol isomers (see above). To evaluate if they inhibit the release of Ca^{2+} from intracellular IP_3-sensitive Ca^{2+} stores, we have measured their effects on the AVP- and the angiotensin II-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution. In these

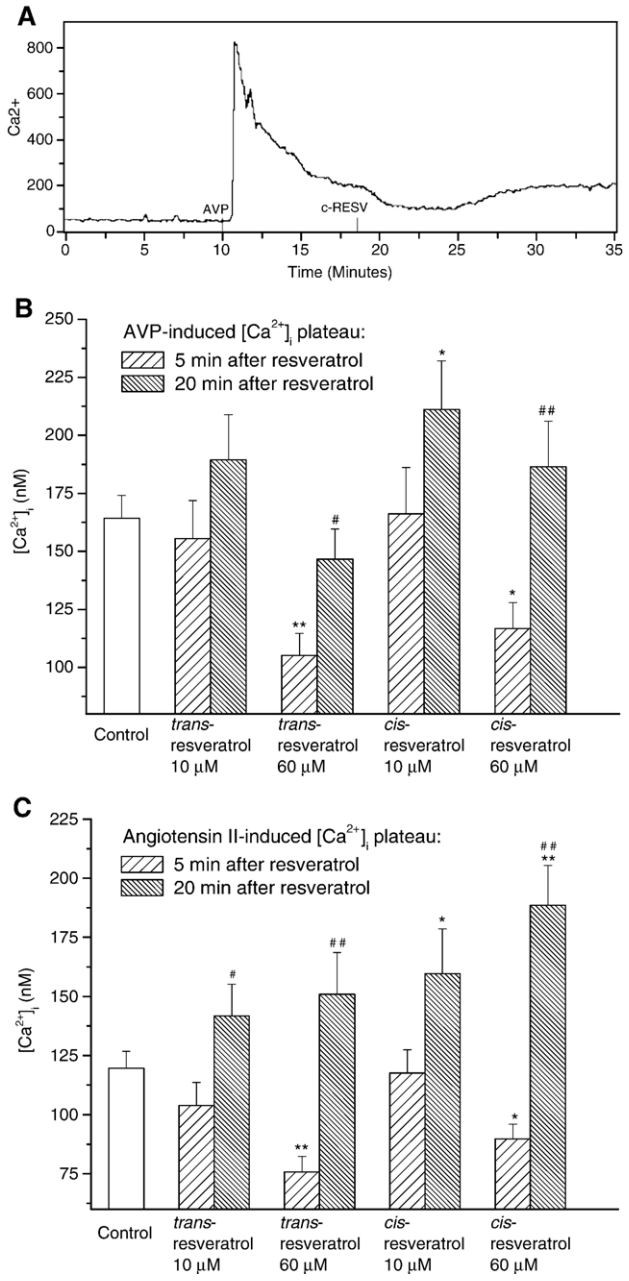

Fig. 2. (A) Representative recording illustrating the effects of *cis*-resveratrol (c-RESV, 60 μM) applied on the plateau phase of the 0.5 μM AVP-induced increase in $[Ca^{2+}]_i$. Changes on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by 0.5 μM AVP (B) or 0.1 μM angiotensin II (C) 5 or 20 min after the application of *trans*-resveratrol or *cis*-resveratrol. Each bar represents the mean±S.E.M. (indicate by vertical lines) of, at least, 14 cells. Level of statistical significance: $^{*}P<0.05$ and $^{**}P<0.01$ with respect to control values; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ with respect to the $[Ca^{2+}]_i$ measured 5 min after the application of the resveratrol isomers.

conditions, basal $[Ca^{2+}]_i$ was 67.3±3.0 nM ($n=121$; $P<0.05$ with respect to the basal value in extracellular 1.5 mM Ca^{2+}-containing solution). Treatment with AVP (0.5 μM) or angiotensin II (0.1 μM) induced an increase $[Ca^{2+}]_i$ consisting in a fast peak that returned to basal value in ~5 min (Fig. 3A,B). After that, substitution of the free-Ca^{2+} by a 1.5 mM Ca^{2+} external solution (normal bathing solution) induced an increase in $[Ca^{2+}]_i$ (Fig. 3A,C), probably due to Ca^{2+} entry through store-operated Ca^{2+} channels (Putney, 1990) although, according to previous data, nifedipine-sensitive Ca^{2+} channels may also be implicated (Campos-Toimil et al., 2005).

The addition of resveratrol isomers (10, 60 μM) at the beginning of the experiment induced a significant transient increase in $[Ca^{2+}]_i$ (Table 3) that returned to basal value after ~20–25 min. In these conditions, the increase in $[Ca^{2+}]_i$

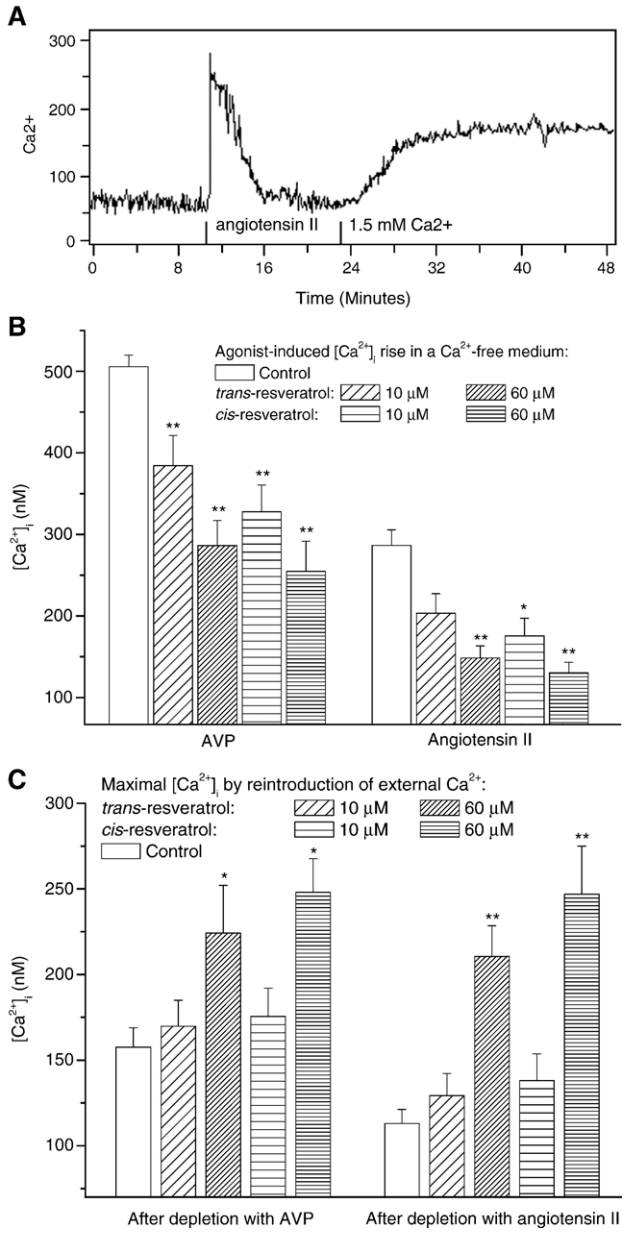

Fig. 3. (A) Representative recording illustrating the $[Ca^{2+}]_i$ rises induced by angiotensin II (0.1 μM) in Ca^{2+}-free solution and by the subsequent reintroduction of 1.5 mM Ca^{2+}-containing external solution. Effects of resveratrol isomers on the increases in $[Ca^{2+}]_i$ induced by AVP (0.5 μM) or angiotensin II (0.1 μM) in Ca^{2+}-free solution (B) and on the increase in $[Ca^{2+}]_i$ measured after reintroduction of Ca^{2+} (1.5 mM) in the extracellular solution (C). Each bar represents the mean±S.E.M. (indicate by vertical lines) of, at least, 10 cells. Level of statistical significance: $^{*}P<0.05$ and $^{**}P<0.01$ with respect to control values.

Table 3
Maximal $[Ca^{2+}]_i$ (nM) induced by *trans*-resveratrol and *cis*-resveratrol in Ca^{2+}-free medium before the application of any agonist, after the application of AVP (0.5 μM) or angiotensin II (0.1 μM) and after several consecutive applications of 0.5 μM AVP (after ≥3 AVP)

	trans-resveratrol		*cis*-resveratrol	
	10 μM	60 μM	10 μM	60 μM
Before agonist	109.7±11.8[b] (25)	156.6±11.1[b] (25)	118.8±10.4[b] (24)	167.5±15.8[b] (27)
After AVP	70.3±9.8 (12)	120.3±14.2[b] (12)	73.9±12.1 (12)	132.2±14.1[b] (14)
After ≥3 AVP	73.6±8.1 (10)	93.3±6.8[a] (12)	75.3±9.6 (9)	109.4±12.2[b] (9)
After angiotensin II	80.4±8.8 (10)	138.3±19.2[b] (12)	79.1±9.0 (11)	156.2±21.9[b] (10)

Level of statistical significance: [a]$P<0.05$ or [b]$P<0.01$ with respect to basal values. There were no significant differences between both isomers. The number of experiments (n) is given in brackets.

induced by the subsequent application of AVP (0.5 μM) or angiotensin II (0.1 μM) was significantly reduced (Fig. 3B).

After the agonist-induced $[Ca^{2+}]_i$ rise, *trans*-resveratrol or *cis*-resveratrol (60 μM, but not 10 μM) were still able to increase $[Ca^{2+}]_i$, which was maximal after 10 min (Table 3). The elevation in $[Ca^{2+}]_i$ measured after reintroduction of 1.5 mM Ca^{2+} was significantly potentiated in the presence of resveratrol isomers (60 μM, but not 10 μM) (Fig. 3C).

In order to achieve a complete depletion of AVP-sensitive Ca^{2+} intracellular stores, several administrations of this agonist (0.5 μM) were made in the absence of extracellular Ca^{2+}. Depending on the cell, the AVP-induced increase in $[Ca^{2+}]_i$ was completely absent after two or three consecutive applications. This lack of response to AVP was not due to receptor desensitization, since AVP was able to elicit an increase in $[Ca^{2+}]_i$ after reintroducing Ca^{2+} in the extracellular solution (data not shown). After depletion of AVP-sensitive Ca^{2+} stores, *trans*-resveratrol and *cis*-resveratrol (60 μM) or thapsigargin (0.5 μM) were still able to induce a significant increase in $[Ca^{2+}]_i$ (Table 3; maximal $[Ca^{2+}]_i$ reached with thapsigargin: 158.4±22.4 nM, $n=10$, $P<0.01$ with respect to basal value). On the other hand, *trans*-resveratrol and *cis*-resveratrol (10 μM) were without effect.

3.5. Effects of ryanodine on $[Ca^{2+}]_i$ in Ca^{2+}-free medium

The alkaloid ryanodine has been shown to activate specific receptors implicated in the mechanism of Ca^{2+} induced Ca^{2+} release. However, several authors have questioned the existence of such mechanism in A7r5 cells (see Discussion). To confirm or deny this lack of effect of ryanodine, we have evaluated its effects on $[Ca^{2+}]_i$ in Ca^{2+}-free medium. In these conditions, ryanodine (0.1–1 μM) did not induce significant changes in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ 3 min after application of 1 μM ryanodine: 68.9±8.8 nM, $n=18$, $P>0.05$ with respect to basal value). The subsequent application of *trans*-resveratrol or *cis*-resveratrol (60 μM) provoked an increase in $[Ca^{2+}]_i$ not significantly different from that induced in the absence of ryanodine (data not shown).

3.6. Effects of the resveratrol isomers on the high-K^+-induced increase in $[Ca^{2+}]_i$

The possibility of an action of resveratrol isomers on voltage-operated Ca^{2+} channels contributing significantly to their relaxant effects in vascular smooth muscle led us to carry out these experiments, in which cell membranes were depolarized by using a high-K^+ Ca^{2+}-free extracellular solution. The replacement of 1.5 mM Ca^{2+}-containing extracellular solution by a depolarizing high-K^+ (60 mM) Ca^{2+}-free solution induced a decrease in basal $[Ca^{2+}]_i$ levels (66.5±4.2 nM; $n=68$; $P<0.05$ with respect to the value in extracellular 1.5 mM Ca^{2+}-containing solution) which was maintained for, at least, 2 h. Reintroduction of Ca^{2+} (1.5 mM) in the extracellular medium provoked a sustained increase in $[Ca^{2+}]_i$ (maximal $[Ca^{2+}]_i$ reached: 227.9±22.1 nM; $n=16$; $P<0.01$ with respect to basal value) (Fig. 4A,B). This response was abolished in the continuous presence of nifedipine (1 μM) (maximal $[Ca^{2+}]_i$: 69.3±8.5 nM; $n=7$; $P>0.05$ with respect to basal value).

The incubation with *trans*-resveratrol or *cis*-resveratrol (10, 60 μM) provoked a significant transient increase in $[Ca^{2+}]_i$ that returned to basal value after ~20-25 min (Table 1). Then, reintroduction of Ca^{2+} (1.5 mM) in the extracellular solution, in the presence of both resveratrol isomers, induced an increase in $[Ca^{2+}]_i$ significantly lower than that provoked in the absence of the stilbenes (except for 10 μM *trans*-resveratrol; Fig. 4A,B).

The application of resveratrol isomers (10, 60 μM) during the $[Ca^{2+}]_i$ plateau induced by reintroduction of Ca^{2+} significantly reduced this plateau during the first 5 min. Then $[Ca^{2+}]_i$ starts to rise again until reaching values higher to those of the initial plateau (Fig. 4B,C). Note that the effects of 60 μM *cis*-resveratrol are significantly different from those obtained with the same concentration of *trans*-resveratrol.

3.7. Inhibition of $I_{Ca(L)}$ by the resveratrol isomers

This set of experiments was performed with the purpose of confirming the inhibition of voltage-operated Ca^{2+} channels by resveratrol isomers in A7r5 cells. In whole-cell clamped cells, *trans*-resveratrol (10–100 μM) and *cis*-resveratrol (30–100 μM) inhibited a voltage-dependent sustained $I_{Ca(L)}$ in a concentration-dependent way, *trans*-resveratrol being significantly more effective than the *cis* isomer. Lower concentrations of both isomers were without effect (Fig. 5).

4. Discussion

AVP or angiotensin II induced an increase in $[Ca^{2+}]_i$ in A7r5 cells that is mediated, as well as in other vascular myocytes, by activation of specific vasopressin V_1 and angiotensin AT_2 receptors, respectively (Thibonnier et al., 1991; Griendling

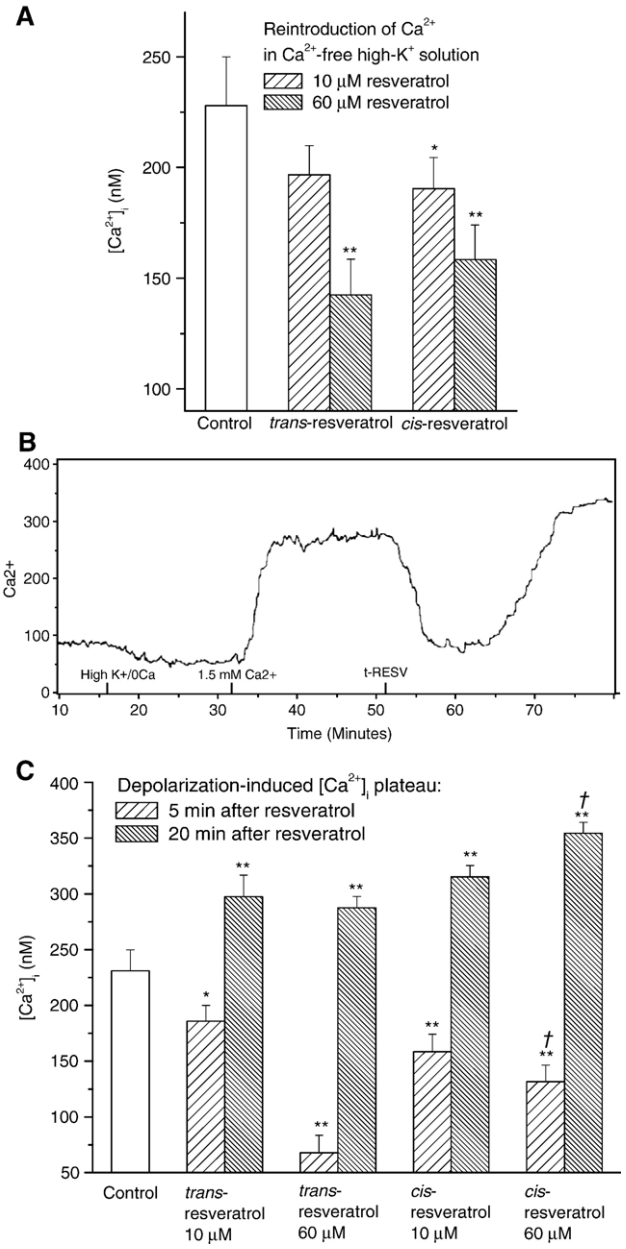

Fig. 4. In high-K^+ (60 mM) Ca^{2+}-free depolarizing medium, the reintroduction of $CaCl_2$ (1.5 mM) caused a sustained increase in $[Ca^{2+}]_i$ in A7r5 cells. (A) This increase in $[Ca^{2+}]_i$ was significantly inhibited by 20 min of preincubation with *trans*-resveratrol (60 μM) or *cis*-resveratrol (10, 60 μM). (B) Original tracing showing the effects of the application of *trans*-resveratrol (t-RESV, 60 μM) once the sustained $[Ca^{2+}]_i$ increase was stabilized. (C) $[Ca^{2+}]_i$ values measured 5 or 20 min after the application of *trans*-resveratrol or *cis*-resveratrol during the sustained plateau of this response (C). Each bar represents the mean±S.E.M. (indicated by vertical lines) of, at least, 11 cells; $^*P<0.05$ or $^{**}P<0.01$ with respect to control values; $^{\dagger}P<0.01$ with respect to *trans*-resveratrol.

et al., 1997). This response consists of a transient increase in $[Ca^{2+}]_i$, mainly due to the release of Ca^{2+} from intracellular stores, followed by a sustained plateau, mainly due to the influx of Ca^{2+} through several types of transmembrane Ca^{2+}-permeable channels (Thibonnier et al., 1991; Byron and Taylor, 1995; Jung et al., 2002). In vascular myocytes, Ca^{2+} is released from intracellular stores via IP_3-sensitive (IP_3 receptors) and ryanodine sensitive channels (ryanodine receptors). However, A7r5 cells used here do not have functional ryanodine receptors, since they did not respond to ryanodine, according to previous reports (Byron and Taylor, 1993; Missiaen et al., 1994) and contrary to the results of Berman et al. (1994).

Our results in absence of extracellular Ca^{2+} confirm that the peak of the agonist-induced response is mainly due to Ca^{2+} release from intracellular stores through IP_3 receptors, although this peak is significantly lower than the obtained in a Ca^{2+}-containing solution, agreeing well with the results reported by Hughes and Schachter (1994) using the same cell line. The previous depletion of intracellular stores by agonists significantly reduced the increase in $[Ca^{2+}]_i$ evoked by resveratrol isomers. Similarly, after a transitory increase in $[Ca^{2+}]_i$ induced by resveratrol isomers in absence of extracellular Ca^{2+}, the subsequent increase in $[Ca^{2+}]_i$ induced by application of AVP or angiotensin II was significantly reduced. However, after the complete depletion of IP_3-sensitive stores by consecutive applications of AVP, resveratrol isomers or thapsigargin were still able to increase $[Ca^{2+}]_i$.

From these results and taking in account our previous report (Campos-Toimil et al., 2005), the resveratrol-induced increase in $[Ca^{2+}]_i$ in absence of extracellular Ca^{2+} seems to be mediated by the depletion of Ca^{2+} from IP_3-sensitive intracellular stores. Additionally, resveratrol isomers may mobilize Ca^{2+} from a store that was not depleted by IP_3, which corresponds, in part, to the intracellular pool of Ca^{2+} depleted by thapsigargin. This fact supports the hypothesis of *trans*-resveratrol and *cis*-resveratrol interfering with sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase activity in a similar way that thapsigargin, although further experiments are required to verify this possibility. From our experiments we cannot discard the possibility of *trans*-resveratrol and *cis*-resveratrol activating a novel Ca^{2+}-induced Ca^{2+} release mechanism that is neither mediated by IP_3 receptors nor by ryanodine receptors (Kasri et al., 2003), which has been shown to be stimulated by disulfonated stilbene derivatives (Nadif et al., 2005).

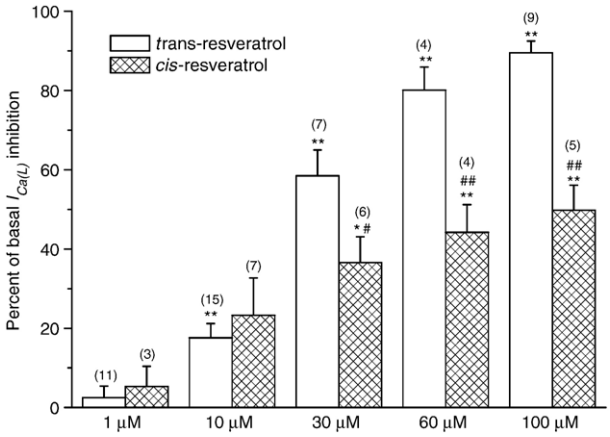

Fig. 5. Inhibition of the $I_{Ca(L)}$ by *trans*-resveratrol and *cis*-resveratrol measured in whole-cell patch-clamp experiments in A7r5 cells. Each bar represents the mean±S.E.M. (indicated by vertical lines) of the number of experiments indicated between brackets at the top of the bar; $^*P<0.05$ or $^{**}P<0.01$ with respect to control values with vehicle; $^{\#}P<0.05$ or $^{\#\#}P<0.01$ with respect to *trans*-resveratrol.

In a Ca^{2+}-containing solution, after the sustained increase in $[Ca^{2+}]_i$ evoked by *trans*-resveratrol or *cis*-resveratrol, both phases of the calcium response to AVP and angiotensin II were significantly modified, leading to a concentration-dependent reduction in the peak/plateau ratio calculated for the calcium response. A possible reason for the reduction of the peak phase is that the IP_3-sensitive stores were partially depleted by resveratrol isomers, as suggested by the experiments in Ca^{2+}-free solution (see above).

On the other hand, Ca^{2+} influx into A7r5 cells induced by agonists and resveratrol isomers may take place, in part, via similar mechanisms (an influx of extracellular Ca^{2+} activated by depletion of intracellular stores). However, the increase in $[Ca^{2+}]_i$ induced by resveratrol isomers is higher than the AVP- or angiotensin II-induced plateau in the controls, suggesting that not all the transmembrane Ca^{2+} channels activated by the agonist or the resveratrol isomers are precisely the same. In fact, the influx of Ca^{2+} induced by resveratrol isomers may be also mediated by Ca^{2+}-permeable channels that are not affected by Ni^{2+} (Campos-Toimil et al., 2005) while, in the present work, sustained agonist-induced Ca^{2+} influx was completely blocked by Ni^{2+}, in accordance with a previous report (Hughes and Schachter, 1994).

From our results in depolarizing high-K^+ Ca^{2+}-free solution, it seems that the increase in $[Ca^{2+}]_i$ provoked by the reintroduction of extracellular Ca^{2+} is mainly mediated through $I_{Ca(L)}$. Although it has been shown that Ca^{2+} influx through $I_{Ca(L)}$ may activate calcium-induced Ca^{2+} release in several preparations, including guinea pig aorta or rat portal vein (Ito et al., 1991; Ganitkevich and Isenberg, 1992), this is not the case for A7r5 cells in this study, since they lack of this mechanism, which is also activated by ryanodine (see above). In high-K^+ Ca^{2+}-free solution, incubation with *trans*-resveratrol or *cis*-resveratrol induced an increase in $[Ca^{2+}]_i$ which is not significantly different to that obtained in Ca^{2+}-free solution. Although it could be expected that resveratrol isomers will potentiate Ca^{2+} entry through $I_{Ca(L)}$ because of the additional capacitative Ca^{2+} influx after depletion of Ca^{2+} stores, the fact is that the increase in $[Ca^{2+}]_i$ after reintroduction of 1.5 mM Ca^{2+} was significantly reduced. This suggests that both stilbenes may act directly blocking Ca^{2+} entry through $I_{Ca(L)}$, a hypothesis also supported by our patch-clamp results. In good agreement, other authors have recently shown that *trans*-resveratrol may inhibit Ca^{2+} currents through $I_{Ca(L)}$ in rat ventricular myocytes (Liu et al., 2005b; Zhang et al., 2006; Chen et al., 2007). Furthermore diethylstilbestrol, a synthetic estrogen structurally related to resveratrol, have been shown to inhibit $I_{Ca(L)}$ in A7r5 cells (Nakajima et al., 1995).

Interestingly, resveratrol isomers displayed a biphasic effect when they were applied on a AVP-, angiotensin II- or high-K^+-induced stable $[Ca^{2+}]_i$ plateau. A possible explanation for the initial 5 min inhibitory effect is the above described inhibition of $I_{Ca(L)}$ since: i) nifedipine also partially inhibited the plateau phase of the calcium response induced by the agonists; ii) this inhibitory effect was stronger on the high-K^+-induced plateau compared to the agonist-induced plateau; and iii) the % of inhibition of $I_{Ca(L)}$ for each concentration of *trans*-resveratrol or *cis*-resveratrol correlates well with their ability to reduce the $[Ca^{2+}]_i$ plateau. On the other hand, at least three mechanisms could explain why $[Ca^{2+}]_i$ starts to rise again after >5 min: i) resveratrol isomers may exert a dual effect on some of the Ca^{2+} channels participating in the plateau phase, an inverse situation to that provoked by (−)-epigallocatechin-3-gallate on $I_{Ca(L)}$ (Campos-Toimil and Orallo, 2007); ii) an additional depletion of intracellular $[Ca^{2+}]_i$ followed by the subsequent potentiation of extracellular Ca^{2+} influx; and iii) the later activation of a type of channel that was not participating in the initial Ca^{2+} response: in this respect, it is interesting to note that the calcium response to resveratrol isomers occurs in two different phases: the first one (5–10 min) corresponding to the release of intracellular Ca^{2+} and store activated Ca^{2+} influx and the second one (15–20 min) takes place through a non-identified type of channels (Campos-Toimil et al., 2005; unpublished observations). A similar effect (simultaneous activation and blockade of different Ca^{2+} channels) has been previously described for other natural compounds, as the alkaloid rutaecarpine (Wang et al., 1999).

Throughout this study there were no qualitative differences between the effects of resveratrol isomers, in good agreement with most comparative studies on the biological effects of *cis*-resveratrol *vs.* *trans*-resveratrol (Campos-Toimil et al., 2005; Orallo, 2006a), suggesting that the different conformation does not modify markedly its interaction with the potential cellular targets involved in Ca^{2+} signalling in A7r5 cells.

To summarize, both resveratrol isomers exert two different actions on the Ca^{2+} signalling in A7r5 myocytes: i) a concentration-dependent inhibition of $I_{Ca(L)}$ and ii) a slow increase in $[Ca^{2+}]_i$ by depletion of IP_3-sensitive and IP_3-non-sensitive intracellular Ca^{2+} stores, that is potentiated by the activation of store-operated Ca^{2+} entry, although the later activation of another transmembrane Ca^{2+}-permeable channel may be also involved. As a result of these actions, resveratrol isomers reduce agonist- and high-K^+-induced increases in $[Ca^{2+}]_i$ and exert a biphasic effect when applied on the sustained plateau. The overall effects of resveratrol isomers described here may explain, at least in part, the endothelium-independent vasorelaxant effects exhibited by resveratrol isomers in isolated rat aortic rings (see Introduction), thus contributing to the cardioprotective effects induced by the long-term moderate wine consumption (Orallo et al., 2002). Bearing in mind these pharmacological properties and assuming that resveratrol isomers exhibit similar behaviour in humans *in vivo*, it can be concluded that they may have interesting potential as an original chemical model for the design and subsequent development of new drugs with cardioprotective properties.

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