Interferences of resveratrol with fura-2-derived fluorescence in intracellular free- Ca^{2+} concentration determinations

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Abstract Resveratrol (3,4',5-trihydroxy-trans-stilbene) is an antioxidant widely employed in cell physiology studies. It has been reported that it interferes with fura-2-derived fluorescence, making the employment of this dye nonviable. In this work, the interference of resveratrol with fura-2 determinations of intracellular free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) was examined. Solutions containing different concentrations of resveratrol, with or without fura-2, in the presence or in the absence of Ca^{2+} , were analyzed by spectrofluorimetry. AR42J tumor cells were employed to study the influence of resveratrol on fura-2 fluorescence in living cells, by single cell fluorimetry. Resveratrol impaired the detection of fura-2-fluorescence emission (510 nm) at the 340, 360 and 380 nm excitation wavelengths. Resveratrol emitted fluorescence at 510 nm when lighted at all three excitation wavelengths. In addition, resveratrol emitted fluorescence at 380 nm when excited at 340 nm. Our observations suggest that the employment of the ratiometric properties of fura-2 to follow changes in $[\text{Ca}^{2+}]_c$ in the presence of resveratrol is not viable. However, we think that the 380 nm excitation light could be employed. Results could be expressed as F_0/F_{380} , where F_0 is the resting fluorescence and F_{380} is

the value of fluoresce at a certain time point. We could follow changes in $[\text{Ca}^{2+}]_c$ evoked by CCK-8, and we also detected Ca^{2+} mobilization by 100 μM resveratrol in AR42J cells. This investigation presents evidence demonstrating that resveratrol interferes with fura-2 fluorescence spectra. Nevertheless, a chance still exists if the 380 nm excitation wavelength is employed in the middle or low micromolar concentrations of resveratrol.

Keywords Resveratrol · Fura-2 · Fluorescence · Calcium · Cholecystokinin · Thapsigargin

Abbreviations

CCK-8	Cholecystokinin octapeptide
$[\text{Ca}^{2+}]_c$	Intracellular free- Ca^{2+} concentration
EGTA	Ethylene glycol-bis(2-aminoethyl- ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
fura-2/AM	Fura-2 acetoxymethyl ester
resveratrol	3,4',5-trihydroxy- <i>trans</i> -stilbene
SERCA	Sarcoendoplasmic reticulum Ca^{2+} - ATPase
Tps	Thapsigargin

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Introduction

It is well known that calcium (Ca^{2+}) plays a pivotal role in the majority of cellular processes and ensures

the functionality of the organism. Regulated changes in its intracellular concentration ($[Ca^{2+}]_c$) represent signals ending in gene transcription, cell growth, differentiation, secretion, muscle contraction, cell survival and cell death (ample bibliography exists).

The effect of resveratrol on Ca^{2+} physiology and the consequences of Ca^{2+} mobilization by the antioxidant on cell fate have been studied along the preceding years. Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a phytoalexin naturally found in grapes and red wine, and is a redox-active compound (Holthoff et al. 2010). Evidences support that resveratrol plays a pivotal role in cell physiology protection. Namely, it activates cell defense pathways or it inhibits mechanisms underlying disease, leading then to protection and cell survival in different tissues (Carrasco et al. 2014; Faghihzadeh et al. 2014; Jha et al. 2012; Joe et al. 2015; Liu et al. 2014; Rouse et al. 2014; Sin et al. 2015; Wang et al. 2014). The antioxidant modulates $[Ca^{2+}]_c$ through a variety of mechanisms that control Ca^{2+} influx, store filling, release from intracellular stores, and downstream activation of Ca^{2+} -sensitive molecules (McCalley et al. 2014). Since most cellular activity is initiated by changes in $[Ca^{2+}]_c$, and other processes are downstream to Ca^{2+} mobilization, the analysis of resveratrol action on $[Ca^{2+}]_c$ is of particular interest.

Fluorescent Ca^{2+} indicators allow accurate measurement of $[Ca^{2+}]_c$. Fura-2 has been described as a dye with a high selectivity for Ca^{2+} binding, with respect to other intracellular ions. Ratiometric reading reduces the effects of photobleaching, leakage, unequal loading, and differences in cell thicknesses in populations, delivering sharper and reproducible results. Upon binding Ca^{2+} , fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at ~ 510 nm. In addition, it is practical to change excitation wavelengths (340 nm/380 nm). With these properties, fura-2 allows measurements at intracellular concentrations of dye unlikely to cause significant Ca^{2+} buffering. In addition, there is a value (at excitation with 360 nm wavelength), termed isosbestic point, at which the fura-2-emitted signal is independent of Ca^{2+} concentration. This fluorescence value can be employed as a control for ratiometric determinations or in fluorescence quenching studies. Due to its fluorescence properties fura-2 is particularly well-suited for ratio-imaging microscopy (Haugland 2002).

However, it has been shown that resveratrol interferes with fura-2 lines of fluorescence. In a previous work we first called the attention on this point (Garcia-Sanchez et al. 2012). Since then, new reports have provided evidences that agree with our observation (Kopp et al. 2014; Paudel et al. 2014).

The current study aimed to further analyze how resveratrol affects determinations of $[Ca^{2+}]_c$ employing fura-2. Physiological solutions containing Ca^{2+} , fura-2 and resveratrol were challenged at the excitation and emission wavelengths for fura-2-fluorescence detection. Pancreatic AR42J tumor cells were employed as a cellular model to monitor $[Ca^{2+}]_c$ in the presence or in the absence of resveratrol. Despite the antioxidant impaired ratiometric Ca^{2+} measurements with fura-2, we think that the dye still is suitable for Ca^{2+} determinations in the presence of resveratrol if the 380 nm excitation wavelength is employed.

Materials and methods

Cells and chemicals

AR42J cell line (ECACC No. 93100618) derived from exocrine pancreatic rat tumour was purchased from The European Collection of Cell Cultures (ECACC) (Porton Down, Salisbury, UK). Fetal bovine serum (FBS) was purchased from HyClone (Thermo Scientific, Erembodegem, Belgium). Glutamine, RPMI 1640 medium and penicillin/streptomycin were obtained from BioWhittaker (Lonza, Basel, Switzerland). Cholecystokinin fragment 26–33 amide (CCK-8), dexamethasone, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 3,4',5-trihydroxy-trans-stilbene (resveratrol) and thapsigargin were obtained from Sigma Chemicals Co. (Madrid, Spain). Fura-2 acetoxymethyl ester (fura-2/AM), fura-2 pentapotassium salt and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were obtained from Invitrogen (Barcelona, Spain). All other reagents were of analytical grade.

Preparation of AR42J cells cultures

Cultures were prepared following previously described methods (del Castillo-Vaquero et al. 2010). Briefly, AR42J cells (passages 8–15) were seeded onto glass cover slips ($\sim 10^5$ cells) placed in independent dishes

(35 mm diameter), and incubated in culture medium at 37 °C under a humidified condition of 95 % air and 5 % CO₂. Culture medium consisted of RPMI 1640 supplemented with 2 mM glutamine, 10 % FBS and antibiotics (0.1 mg/mL streptomycin, 100 IU penicillin). The experiments were carried out after 7–9 days after plating of the cells.

Study of resveratrol-derived fluorescence and interference with fura-2-determinations

Changes in resveratrol-derived fluorescence were monitored employing a fluorescence spectrofluorimeter (RF-5001-PC; Shimadzu, Kyoto, Japan). A Na-HEPES buffer (containing: 140 mM NaCl, 4.7 mM KCl, 2 mM MgCl₂, 10 mM Hepes, and 10 mM glucose; prepared in milli-Q water; pH was adjusted to 7.4) was employed.

For the study of resveratrol interference in fura-2-fluorescence the pentapotassium salt of the probe was used. Fura-2-derived fluorescence closely reports changes in Ca²⁺ concentration (Gryniewicz et al. 1985).

The potassium salt of fura-2 is a cell-impermeant probe that can be employed for calibration purposes of Ca²⁺ measurements (Haugland 2002). Fura-2 pentapotassium salt was added to the cuvette to yield a final concentration of 2.5 μM. Solutions containing different concentrations of resveratrol, alone or in combination with increasing concentrations of Ca²⁺ (0.001–1 μM), were placed in a cuvette in the spectrofluorimeter. Solutions were stirred continuously, and the experiments were performed at 37 °C. Samples were excited alternatively at 340 and 380 nm, and the fluorescence emission was measured at 510 nm. Resveratrol and CaCl₂ were dissolved in the Na-HEPES buffer, and were added directly to the cuvette to yield the final concentration required. Results show the fluorescence emission (510 nm) detected at the 340, 360 and 380 nm excitation lights. Data also show the ratio of the fluorescence emitted at 340 and 380 nm excitation wavelengths. Values for ratio fluorescence were normalized to the basal fluorescence (value measured prior to resveratrol addition).

Determination of intracellular free-Ca²⁺ concentration ([Ca²⁺]_c)

Monitoring of [Ca²⁺]_c was performed as previously described (Del Castillo-Vaquero et al. 2010). For

fura-2 loading of the cells the culture medium was replaced by a physiological solution containing: 130 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 1.2 mM KH₂PO₄, 10 mM glucose, 10 mM Hepes, 0.01 % trypsin inhibitor (soybean) and 0.2 % bovine serum albumin (pH = 7.4 adjusted with NaOH). Cultured cells were then loaded with the fluorescent Ca²⁺ indicator fura-2 by incubation with fura-2/AM (4 μM) at room temperature (23–25 °C) for 40 min. For monitoring of changes of fura-2-dependent fluorescence, the coverslip with cultured cells was mounted on an experimental perfusion chamber and placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY, USA). The cells were continuously superfused with a control Na-HEPES buffer containing (in mM): 140 NaCl, 4.7 KCl, 1 CaCl₂, 2 MgCl₂, 10 Hepes, 10 glucose (pH adjusted to 7.4). When Ca²⁺ free conditions were applied, the Na-HEPES buffer contained no added Ca²⁺ and was supplemented with 0.5 mM EGTA.

In order to determine fura-2-derived fluorescence, an image acquisition system was employed (Hamamatsu Photonics, Hamamatsu, Japan). Cells were excited alternatively at 340/380 nm, with light from a xenon arc lamp passed through a high-speed monochromator (Polychrome IV, Photonics, Hamamatsu, Japan). Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu, Japan) and recorded using dedicated software (Aquacosmos 2.5, Hamamatsu Photonics, Hamamatsu, Japan). Results show the fluorescence emission (510 nm) detected at the 340 and 380 nm excitation lights, and also show the ratio of the 510 nm fluorescence emitted at both excitation wavelengths (340/380). Data for ratio fluorescence were normalized to the basal fluorescence (value measured prior to resveratrol addition).

All stimuli were dissolved in the extracellular Na-HEPES buffer, with or without Ca²⁺, and were applied directly to the cells in the perfusion chamber. Experiments were performed at room temperature (23–25 °C), and different batches of cells were used for the studies. Cell viability was not significantly changed by the isolation procedure, as assayed by trypan blue exclusion test, and was >95 %. Under our experimental conditions, no apparent morphological changes of cells were observed that could introduce errors in Ca²⁺ measurements.

Results and discussion

Effect of resveratrol on the spectra of fura-2-lines of fluorescence

We filled in a cuvette with Na-Hepes solution and we added resveratrol (1–100 μM). Each sample was excited with light from 300 to 450 nm, and the fluorescence emission was measured at 510 nm. As shown in Fig. 1a, fluorescence emission was dependent on the concentration of resveratrol at all excitation wavelengths employed.

The maximal emission was detected at approximately 340 nm excitation light, a value that is employed for fura-2 imaging. Moreover, resveratrol emitted fluorescence at 510 nm when excited at 360 and 380 nm. The emission values were smaller compared with those obtained with excitation at 340 nm. Nearly negligible emission could be detected beyond 410 nm excitation wavelength. The excitation wavelength which gave maximal emission at 510 nm shifted, depending on the concentrations of resveratrol employed. This means that the higher the concentration of resveratrol it is used, the wider spectrum of wavelengths is found, in which resveratrol might be excited.

Having noticed that resveratrol emits fluorescence at 510 nm when it is excited at the three major excitation wavelengths employed for fura-2 determinations, the next steps were directed to find out the maximal emission wavelength of resveratrol. For this purpose, we performed a series of experiments in which resveratrol was excited at 340, 360 or 380 nm.

Resveratrol was excited at 340 nm and the emission (350–600 nm) was monitored (Fig. 1b). It called our attention that the emission detected was maximal around 380–390 nm, a wavelength commonly used to monitor fura-2 non-bound to Ca^{2+} . Fluorescence emission was also noted at 360 nm, a wavelength used to monitor the isosbestic point of fura-2. In addition, upon excitation with the 340 nm light, resveratrol further emitted fluorescence at 510 nm. The effects were dependent on the concentration of the polyphenol employed. These observations suggest that resveratrol emits fluorescence that will interfere with that of fura-2.

When the samples were excited at 360 nm and the resulting fluorescence emission was scanned (380–580 nm), we observed a maximal value around

460 nm (Fig. 1c). Emission of resveratrol at 510 nm was also noted, although it was lower compared with that detected at the excitation with 340 nm. The result was again dependent on the concentration of the polyphenol employed. These observations suggest that resveratrol emits fluorescence that could impair that of fura-2 at the isosbestic point. Moreover, fluorescence emission at 380 nm was observed.

When the samples were excited at 380 nm and the resulting fluorescence emission was scanned (400–600 nm), we detected a maximal value around 460 nm (Fig. 1d). Emission of resveratrol at 510 nm was also observed, although it was much lower compared with that detected at excitation with 340 nm or with 360 nm. As observed above, the result was dependent on the concentration of the polyphenol employed. These observations suggest that resveratrol emits fluorescence that will impair that emitted by fura-2 non-bound Ca^{2+} . As it occurred with the other excitation wavelengths, the result will be a resveratrol-dependent increase in the fluorescence detected at 510 nm that is added to that emitted by fura-2 when it is excited at 380 nm.

When the samples were excited from 300 to 370 nm an increase in the fluorescence emitted at 380 nm was observed, which was dependent on the concentration of resveratrol present in the cuvette (Fig. 1e). The maximal emission was observed around 330–335 nm excitation light. These results indicate that, at the excitation light of 340 nm, resveratrol emits fluorescence (at 380 nm) that could excite fura-2 non-bound to Ca^{2+} . In turn, the Ca^{2+} -free probe would emit fluorescence at 510 nm that could impair that emitted by fura-2 that is bound to Ca^{2+} , no matter whether Ca^{2+} is changing or not. With all up to now observations, upon addition of resveratrol to the medium an increase in the fluorescence emission at 510 nm should be observed when the excitation lights of 340 and 380 nm are employed (the excitation/emission wavelengths commonly employed in fura-2 determinations). The next set of experiments was directed to confirm this possibility.

The samples were excited alternatively at 340 and 380 nm, and the emission was measured at 510 nm. After a period (100 s) of “basal” determination, resveratrol (final concentration of 10 μM) was added to the cuvette. In the presence of the polyphenol an increase in the ratio of fluorescence was observed (Fig. 2a). After 100 s, an additional

Fig. 1 Effect of resveratrol on the spectra of fura-2-lines of fluorescence. A cuvette, placed in a fluorescence spectrofluorimeter, was filled in with 2 ml of a Na-HEPES buffer to which resveratrol was added. **a** Excitation scans were performed between 300 and 450 nm, and the emission was detected at 510 nm. The scans were repeated employing increasing concentrations of resveratrol (1–100 μM). **b–d** The samples were excited at 340, 360 and 380 nm, and the resulting

fluorescence emission was scanned between 350–600 nm, 380–580 nm and 400–600 nm respectively. The scans were repeated employing increasing concentrations of resveratrol (1–100 μM). **e** Analysis of the fluorescence emitted by resveratrol at 380 nm. The samples were excited from 300 to 370 nm. Excitation of resveratrol (1–100 μM) depicted a concentration-dependent increase in the fluorescence emitted ($n = 3$ independent experiments)

volume of a stock solution of resveratrol was added to the cuvette, to yield a final concentration of 50 μM . Following addition of resveratrol an additional increase in the ratio of fluorescence was noticed (Fig. 2a). The analysis of the fluorescence emission (510 nm) at each excitation wavelength revealed that addition of resveratrol resulted in an increase of the fluorescence (Fig. 2b). The increase was stronger at the 340 nm excitation wavelength, compared with that obtained at 380 nm. This is consistent with the observations described above,

and reveals that resveratrol emits fluorescence at both excitation wavelengths usually employed for fura-2-related Ca^{2+} determinations. The overall result is an interference with the fluorescence spectrum of the dye, being the 340 nm line the one that is affected to a major extent.

Effect of resveratrol on fura-2 fluorescence

We performed a series of experiments in which both resveratrol and fura-2 were present in the cuvette.

Fig. 2 Effect of resveratrol on the lines of fluorescence employed in fura-2-dependent Ca^{2+} determinations. Na-HEPES buffer (2 mL) was added to the cuvette placed in the spectrofluorimeter. The sample was excited alternatively at 340 and 380 nm, and the fluorescence emission was measured at 510 nm. Two concentrations of resveratrol were sequentially added to the cuvette (final concentration of 10 and 50 μM). **a** Upon addition of each concentration of resveratrol an increase in the ratio of fluorescence at 340/380 nm was observed. **b** The review of the fluorescence emission (510 nm) at each excitation wavelength revealed that addition of resveratrol resulted in an increase of fluorescence at both excitation wavelengths. The tests were performed in the absence of fura-2. An arrow indicates the time point at which the desired concentration of resveratrol was added to the cuvette ($n = 3$ independent experiments)

Additionally, the experiments were carried out in the presence of different concentrations of Ca^{2+} .

For this purpose, we loaded the cuvette with Na-HEPES buffer. Fura-2 pentapotassium salt (final concentration of 2.5 μM) was added. The tests were performed in the absence of Ca^{2+} (Na-HEPES with no added Ca^{2+} plus 10 mM EGTA) or in its presence (0.01 or 1 μM), and without resveratrol or in its presence (10 or 50 μM). The samples were then excited with light from 300 to 450 nm. The fluorescence emission at 510 nm was detected.

As shown in Fig. 3a, the fluorescence spectrum of fura-2 shifted towards the left in the presence of Ca^{2+} ,

compared with the fluorescence emitted in the absence of Ca^{2+} . This is the expected behavior of the probe-derived fluorescence when it is bound to the ion. The value of maximal excitation wavelength was detected at 340 nm, and the isosbestic point (i.p.) was found at 361 nm.

When resveratrol was present in the medium, the value for the maximal excitation wavelength was not affected at 10 μM resveratrol (Fig. 3b), but it shifted towards the right (348 nm) in the presence of 50 μM resveratrol (Fig. 3c), compared with the value obtained in the absence of the polyphenol. The isosbestic point (i.p.) shifted slightly towards the right in the presence of both concentrations of resveratrol (the value obtained was approximately 365 nm). In addition, the maximal value of fluorescence obtained increased in the presence of the polyphenol (horizontal dotted lines). Noteworthy, a slight increase in the fluorescence emission at the 380 nm excitation light could be noted in the presence of resveratrol, compared with the values observed in its absence. The effect was more noticeable in the presence of 50 μM resveratrol.

These results confirm the above observations, which show that resveratrol impairs the fluorescence detected at 510 nm with the 340, 360 and 380 nm excitation wavelengths. However, the effect observed at 380 nm is several orders of magnitude smaller compared with that noted at 340 nm excitation light. The effect is more noticeable at the high micromolar concentrations of resveratrol. As suggested above, the consequence is that the Ca^{2+} concentration will be overestimated at the 340 nm excitation wavelength, whereas it could be underestimated at the 380 nm excitation wavelength. Parallely, determinations of Ca^{2+} based on the isosbestic point of fura-2 could be impaired.

Analysis of intracellular free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) in cultured cells

AR42J cells contain functional Ca^{2+} stores from which the ion is released upon stimulation with Ca^{2+} -mobilizing agonists. Cells were loaded with fura-2 as described in the “Materials and methods” section, and the 340 nm/380 nm ratio of fluorescence (indicative of changes in $[\text{Ca}^{2+}]_c$; Grynkiewicz et al. 1985) was monitored. In a first stage we performed a series of experiments in which the cells were incubated in the

presence of 1 μM thapsigargin (Tps). Tps is a potent selective sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibitor, and is often used to estimate the

Fig. 3 Effect of resveratrol on fura-2 emitted fluorescence at 510 nm in the presence of several concentrations of Ca^{2+} . A cuvette was loaded with 2 mL of Na-HEPES buffer. Fura-2 pentapotassium salt (final concentration of 2.5 μM) was added. The samples were excited with light from 300 to 450 nm, and fluorescence emission was detected at 510 nm. The tests were performed in the absence of Ca^{2+} (Na-HEPES with no added Ca^{2+} plus 10 mM EGTA) or in its presence (0.01 or 1 μM). **a** Resveratrol was not present in the solution. **b** 10 μM resveratrol was added. **c** Experiments carried out in the presence of 50 μM resveratrol. Horizontal dotted lines indicate the maximal value of fluorescence emitted at 510 nm in the presence of a certain concentration of Ca^{2+} ($n = 3$ independent experiments; i.p., isosbestic point of fura-2)

Ca^{2+} pool content (Nielsen et al. 1995). Due to the inhibition of SERCA, Tps leads to Ca^{2+} release from endoplasmic reticulum (ER) (Dallwig and Deitmer 2002). To avoid the contribution of extracellular Ca^{2+} to Tps-induced Ca^{2+} responses, cells were stimulated in the absence of Ca^{2+} in the extracellular medium (medium containing 500 μM EGTA). Now Ca^{2+} responses will depend on the release of the ion from intracellular stores.

In the presence of Tps (1 μM) a transient increase in the fura-2 ratio of fluorescence was observed, which decreased towards the prestimulation level. This response reflects a release of Ca^{2+} from the ER. The additional stimulation of cells with resveratrol (100 μM) still was able to induce a further increase in the fura-2 ratio of fluorescence (Fig. 4a; $n = 3$ experiments). In a first instance, the change in the ratio of fluorescence evoked by resveratrol should be regarded to as an additional release of Ca^{2+} from the ER. Review of the intensities of the individual 340 and 380 nm traces showed a rise of the emitted light with excitation at 340 nm upon Tps addition (Fig. 4b), and a corresponding decline with excitation at 380 nm (Fig. 4c). However, upon introduction of resveratrol a rise in the emission with excitation at 340 nm (Fig. 4b) was noted, but not the corresponding decline with excitation at 380 nm (Fig. 4c). Conversely, a slight increase in fluorescence emitted was detected. This means that the increase in $[\text{Ca}^{2+}]_c$ evoked by resveratrol was an artifact. Therefore, the ratio of fluorescence usually employed in fura-2 determinations is not suitable to monitor of $[\text{Ca}^{2+}]_c$ properly when resveratrol is used.

It has been previously suggested that, when working with dual wavelength-excited fluorescent dyes, if one of the lines is affected by the experimental procedures, the wavelength not affected can be successfully employed for the determinations (Gonzalez et al. 2005). Following these directions, data of fluorescence at 380 nm excitation light were normalized to the basal (resting) fluorescence values. Results were expressed as F_0/F_{380} , where F_0 is the basal (resting) fluorescence and F_{380} is the value of fluorescence at a certain time point. With this transformation, increases in $[\text{Ca}^{2+}]_c$ are represented as an increase in F_0/F_{380} ratio. Employing this tool, the artifact induced by resveratrol on $[\text{Ca}^{2+}]_c$ was minimized (Fig. 4d).

We think that the approach that we have described here (i.e., the sole monitoring of the 510 nm emission

Fig. 4 Time course of changes in $[Ca^{2+}]_i$ evoked by thapsigargin (Tps; 1 μ M) and the subsequent addition of 100 μ M resveratrol. The graphics show the fura-2 ratio of fluorescence at 340/380 nm excitation lights (**a**), the intensities of the individual traces at 340 nm (**b**) and 380 nm (**c**) excitation wavelengths, and the F_0/F_{380} normalization of the fluorescence emitted at 380 nm

excitation light (**d**). Fluorescence emission was measured at 510 nm. All tests were performed in the absence of extracellular Ca^{2+} (medium containing 0.5 mM EGTA). Horizontal bars show the time during which the stimuli and Ca^{2+} free medium were applied to the cells. The traces are typical of 3 independent experiments

of fura-2 at the 380 nm excitation light) can be employed to monitor $[Ca^{2+}]_i$ in response to physiological agonists. As examples, AR42J cells were challenged with the pancreatic agonist CCK-8. Incubation of fura-2-loaded AR42J cells with 1 nM CCK-8 in the presence of extracellular Ca^{2+} led to the typical transient Ca^{2+} mobilization evoked by the agonist. Ca^{2+} mobilization consisted of an initial increase, followed by a decrease of $[Ca^{2+}]_i$ towards a value over the prestimulation level ($n = 3$ experiments). The behaviour of fura-2-derived fluorescence, the ratio (340/380 nm) of fluorescence and the F_0/F_{380} nm transformation are shown in figure Fig. 5. Moreover, detection of physiological oscillations in $[Ca^{2+}]_i$ in response to 20 pM CCK-8 could be resolved (Fig. 6). These patterns of changes in $[Ca^{2+}]_i$ have been previously observed in both tumor and non-tumor pancreatic cells (Del Castillo-Vaquero et al. 2010; Fernandez-Sanchez et al. 2009; Gonzalez et al. 1997).

We have previously shown that resveratrol releases Ca^{2+} from intracellular stores, probably involving the ER (Garcia-Sanchez et al. 2012). Due to the interference of the polyphenol with fura-2 fluorescence, it might be feasible to state that our observations were the result of an artifact, and that Ca^{2+} mobilization could not be studied employing resveratrol. Nevertheless, this objective might be achievable as long as the fluorescence characteristics of the compound, together with its influence in fura-2-derived fluorescence, were taken into account. The approach that we have described here could be employed. In agreement with this, when AR42J cells (loaded with fura-2) were incubated with 10 μ M resveratrol in the absence of extracellular Ca^{2+} (medium containing 500 μ M EGTA), we could not detect any change in $[Ca^{2+}]_i$. However, subsequent stimulation of cells with CCK-8 (1 nM) evoked a typical Ca^{2+} response (Fig. 7; $n = 4$ experiments).

Fig. 5 Time course of changes in $[Ca^{2+}]_c$ evoked by a supramaximal concentration of CCK-8 (1 nM). The *graphics* show the fura-2 ratio of fluorescence at 340/380 nm excitation lights (a), the intensities of the individual traces at 340 nm (b) and 380 nm (c) excitation wavelengths, and the F_0/F_{380} normalization of the fluorescence emitted at 380 nm excitation light (d). Fluorescence emission was measured at 510 nm. A horizontal bar shows the time during which CCK-8 was applied to the cells. The traces are typical of 3 independent experiments

Fig. 6 Time course of changes in $[Ca^{2+}]_c$ evoked by a physiological concentration of CCK-8 (20 pM). The *graphics* show the fura-2 ratio of fluorescence at 340/380 nm excitation lights (a), the intensities of the individual traces at 340 nm (b) and 380 nm (c) excitation wavelengths, and the F_0/F_{380} normalization of the fluorescence emitted at 380 nm excitation light (d). Fluorescence emission was measured at 510 nm. A horizontal bar shows the time during which CCK-8 was applied to the cells. The traces are typical of 3 independent experiments

In another set of experiments, cells were stimulated with 100 μ M resveratrol in the absence of Ca^{2+} in the extracellular solution (medium containing 500 μ M EGTA). Interestingly, the compound induced an increase in $[Ca^{2+}]_c$ ($n = 3$ experiments). Subsequent

stimulation of cells with CCK-8 (1 nM) failed to additionally induce Ca^{2+} mobilization. Review of the intensities of the individual 340 and 380 nm traces revealed a rise of the fluorescence emission with excitation at 340 nm upon resveratrol introduction,

Fig. 7 Effect of resveratrol on $[Ca^{2+}]_i$ and subsequent addition of a supramaximal concentration of CCK-8. Cells were perfused with 10 μ M resveratrol and later with 1 nM CCK-8 in a Ca^{2+} free medium (containing 0.5 mM EGTA). The *graphics* show the fura-2 ratio of fluorescence at 340/380 nm excitation lights (**a**), the intensities of the individual traces at 340 nm (**b**) and

380 nm (**c**) excitation wavelengths, and the F_0/F_{380} normalization of the fluorescence emitted at 380 nm excitation light (**d**). Fluorescence emission was measured at 510 nm. The *horizontal bars* indicate the time during which the stimuli and Ca^{2+} free medium were applied to the cells. The traces are typical of 4 independent experiments

and a decline of the emitted fluorescence with excitation at 380 nm (Fig. 8). Bearing in mind the effect of resveratrol on fura-2 excitation and emission lines, the effect of the polyphenol on $[Ca^{2+}]_i$ could be resolved by analyzing the 380 nm fluorescent line. Employing this approach, we could avoid the artifact that resveratrol induces in $[Ca^{2+}]_i$ determination when fura-2 340/380 nm ratio is used, simply discarding data provided by the 340 nm excitation line. Therefore, our observations suggest that resveratrol effectively induces Ca^{2+} mobilization, in agreement with our previous results (Garcia-Sanchez et al. 2012).

In summary, our investigations present further evidences demonstrating that resveratrol interferes with fura-2-fluorescence spectrum. This is in agreement with the existing observations. The effect of resveratrol is concentration-dependent. We suggest

that despite the usefulness of fura-2 for quantitative intracellular Ca^{2+} measurements, the dye can be rather employed for qualitative Ca^{2+} determinations in the presence of resveratrol. In this case, the emission at 380 nm excitation wavelength should be employed. Concentrations of resveratrol in the high micromolar range should be avoided, although not completely discarded. If a calibration should be needed, then an approximation could be performed using previously described methods for Ca^{2+} determinations with non-ratiometric dyes (Cheng et al. 1993; Kao et al. 1989; Minta et al. 1989; Plank and Sussman 2003). Unavoidably, it is important to bear in mind that $[Ca^{2+}]_i$ could be underestimated. Alternatively, other probes for Ca^{2+} determinations exist, whose fluorescence is not affected by resveratrol (Kopp et al. 2014; Paudel et al. 2014).

Fig. 8 A high micromolar concentration of resveratrol releases Ca^{2+} from agonist-sensitive Ca^{2+} stores. The *graphics* show the time course of changes in $[\text{Ca}^{2+}]_c$ evoked by resveratrol (100 μM) and the subsequent addition of 1 nM CCK-8 in a Ca^{2+} free medium (containing 0.5 mM EGTA). **a** Fura-2 ratio of fluorescence at 340/380 nm excitation lights. **b** and **c** Intensities of the individual traces at 340 and 380 nm excitation

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Conflict of interest The authors declare that there is no conflict of interest.

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wavelengths, respectively. **d** F_0/F_{am} normalization of the fluorescence emitted at 380 nm excitation light. Fluorescence emission was measured at 510 nm. The *horizontal bars* indicate the time during which the stimuli and Ca^{2+} free medium were applied to the cells. The traces are typical of 3 independent experiments

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