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SHORT COMMUNICATION

Resveratrol Interferes with Fura-2 Intracellular Calcium Measurements

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Abstract Resveratrol, a naturally occurring polyphenol found in some fruits and especially in grapes, has been reported to provide diverse health benefits. Resveratrol's mechanism of action is the subject of many investigations, and some studies using the ratiometric calcium indicator Fura-2 suggest that it modulates cellular calcium responses. In the current study, contradictory cellular calcium responses to resveratrol applied at concentrations exceeding 10 μM were observed during in vitro imaging studies depending on the calcium indicator used, with Fura-2 indicating an increase in intracellular calcium while Fluo-4 and the calcium biosensor YC3.60 indicated no response. When cells loaded with Fura-2 were treated with 100 μM resveratrol, excitation at 340 nm resulted in a large intensity increase at 510 nm, but the expected concurrent decline with 380 nm excitation was not observed. Pre-treatment of cells with the calcium chelator BAPTA-AM did not prevent a rise in the 340/380 ratio when resveratrol was present, but it did prevent an increase in 340/380 when ATP was applied, suggesting that the resveratrol response was an artifact. Cautious data interpretation is recommended from imaging experiments using Fura-2 concurrently with resveratrol in calcium imaging experiments.

Keywords Ca^{2+} signaling · Fura-2 · Fluo-4 · YC3.60 · BAPTA-AM · Resveratrol

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Introduction

Resveratrol (3,4′,5-trihydroxystilbene) is a naturally occurring polyphenol that is produced in grapes, peanuts, and other food crops, and has been proposed to protect against a variety of aging-related diseases including cardiovascular disease, cancer, neurogenerative disease, and type 2 diabetes [1, 2]. The mechanism of action of this compound has been the subject of intensive research. Resveratrol was reported to directly inhibit cAMP-specific phosphodiesterases [3], alter voltage-gated calcium current [4], and modulate store operated calcium entry [5, 6]. Uncertainty remains about whether resveratrol initially elicits calcium or cAMP signals, and if these secondary signal responses are similar in all cell types or are cell-type specific.

Calcium is an essential second-messenger signal in all cell types, influencing a wide variety of cellular processes. Calcium signaling is accomplished by the fine control of intracellular calcium concentration gradients [7]. These calcium gradients and their shifts can be visualized using indicator dyes or genetically coded biosensors that bind Ca^{2+} specifically [8–10]. Intracellular calcium concentration changes at the nanomolar scale can be detected in live cells with either dyes or biosensors.

Fura-2 is a fluorescent ratiometric dye that is widely considered the standard for quantitative intracellular calcium measurements [11]. The peak absorbance shifts from 340 nm when bound to Ca^{2+}, to 380 nm when it is not bound, and it fluoresces maximally at approximately 510 nm when excited at either wave length. The typical procedure for in vitro imaging experiments using Fura-2 involves incubating cells with a 1–5 μM solution of the acetoxy-methyl ester form of the dye, which is membrane-permeable, at 37 °C for 15–30 min. Dye enters the cells and the acetoxy-methyl ester side chain is then cleaved by the cellular non-specific esterase activity, trapping the dye in the cells [8].

Investigations into calcium signaling induced by resveratrol application using Fura-2 have suggested that resveratrol causes release of intracellular calcium stores [5, 12–15]. The current study was initiated to determine how resveratrol affects calcium signaling in human epithelial breast cancer cells (MDA-MB-231). Initial experiments with Fura-2 suggested that resveratrol caused a dose-dependent increase in intracellular calcium. However, unusual responses observed using Fura-2 caused concern about the reliability of the data. The overall hypothesis for the experiments reported here is that in vitro calcium measurements made with Fura-2 dye are biased by the presence of resveratrol at 10 μM or higher concentration.

Methods

Cell Culture

MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum and 100 units/ml penicillin G and streptomycin sulfate. Cultures were maintained at 37 °C and 5 % CO_2. Cells were plated on sterile glass 25 mm diameter cover slips in 6-well plates the day before imaging.

The majority of experiments reported here were completed with calcium indicator dyes, but some experiments used calcium biosensor YC3.60 [16] as an alternative. YC3.60 was introduced into MDA-MB-231 by adenovirus transduction. Cells were grown to approximately 80 % confluence on 25-mm glass cover slips as described above. Growth medium was removed and replaced with DMEM supplemented with 2 % (v/v) fetal bovine serum and Adenovirus YC3.60 using a multiplicity of infection of 500. Cells were incubated for approximately 4 h with the virus before it was removed and replaced with the 10 % FBS growth medium described above. Transduced cells were grown for approximately 48 h prior to use in imaging experiments.

Imaging

In experiments with Fura-2, cells were loaded with 1 μM Fura-2 acetoxymethyl ester (Molecular Probes Inc.) by incubating for 15 min at 37 °C and 5 % CO_2 in Krebs Ringers Bicarbonate HEPES buffer (pH 7.4) supplemented with 10 mM D-(+)-glucose (KRBH10) and 0.015 % Pluronic F127 (Molecular Probes Inc). Fluo-4 (Molecular Probes Inc.) was substituted for Fura-2 in tests with that indicator. In some experiments, cells were incubated with 10 μM BAPTA-AM (Molecular Probes Inc.) for 15 min in KRBH10 plus 0.015 % Pluronic F127 at 37 °C and 5 % CO_2, followed by removal of the BAPTA-AM loading solution, and cells were loaded with Fura-2 as above.

Cover slips were placed in a 37 °C microperifusion chamber mounted on the specimen stage of an inverted fluorescence microscope. Cells were continuously superfused with KRBH10 (4 ml/min) at 37 °C and treatments, including resveratrol (R5010, Sigma, St. Louis, MO), were introduced through the perfusion system. Experiments with Fura-2 were completed with an Olympus IX81 inverted microscope (Olympus America, Center Valley, PA, USA). Fura-2 dual excitation and emission was accomplished using 340- and 380-nm excitation filters and a 510 nm emission filter, and cells were visualized with an Olympus UPlan FLN 40X 1.3 NA oil immersion objective. Light was supplied by a Lambda XL (Sutter Instrument Company, Novato, CA, USA) using a variable aperture to achieve 65–75 % attenuation. Digital images (25–50 ms exposure) were recorded with a Hamamatsu EM charge-coupled device camera (Hamamatsu Photonics, Japan) at 10 s intervals.

Cells that were loaded with Fluo-4 or expressed YC3.60 were visualized on a Nikon TE-2000U inverted microscope. Fluo-4 excitation (485 nm) and emission (520 nm) was accomplished with a GFP cube (Chroma Technologies) and cells were visualized with a Nikon Plan Fluor 20X objective. Cells expressing FRET biosensor YC3.60 were visualized as described previously [17] with minor deviations. A Nikon Super Fluor 40X 1.30 NA oil immersion objective was used with 90 % light attenuation by neutral density filters. Excitation was at 440 nm and dual emission ratio imaging at 485 and 535 nm was accomplished with a high speed filter wheel (Sutter Instrument Co., Novato, CA). An Xcite 120 light source (EXFO Life Science Industries, Mississauga, ON, Canada) was used for Fluo-4 and YC3.60 experiments. Digital images (200–250 ms exposure) were captured with a 16-bit Cascade 650 digital camera (Roper Instruments) at 10-s intervals.

Imaging data acquisition and analyses on both microscope systems were accomplished using MetaFluor software (Universal Imaging Corp) and OriginPro 7E (OriginLab Corp.). Emission intensities were background subtracted. Fura-2 data are expressed as the ratio of 340/380 normalized to average resting values in the 60 s prior to the application of the first experimental treatment. Data from YC3.60 experiments are expressed as the fold-change in ratio of FRET acceptor and donor emission (535/485 nm ratio) normalized to resting values. Fluo-4 data are expressed as fold-change in emission intensity relative to resting values. All experiments were replicated at least twice, and means and standard errors were calculated using pooled data from relevant experiments.

Results

MDA-MB-231 cells loaded with Fura-2-AM showed an apparent increase in the 340/380 ratio when treated with 100 μM resveratrol, suggesting a rise in intracellular calcium concentration (Fig. 1a). However, review of the intensities of the

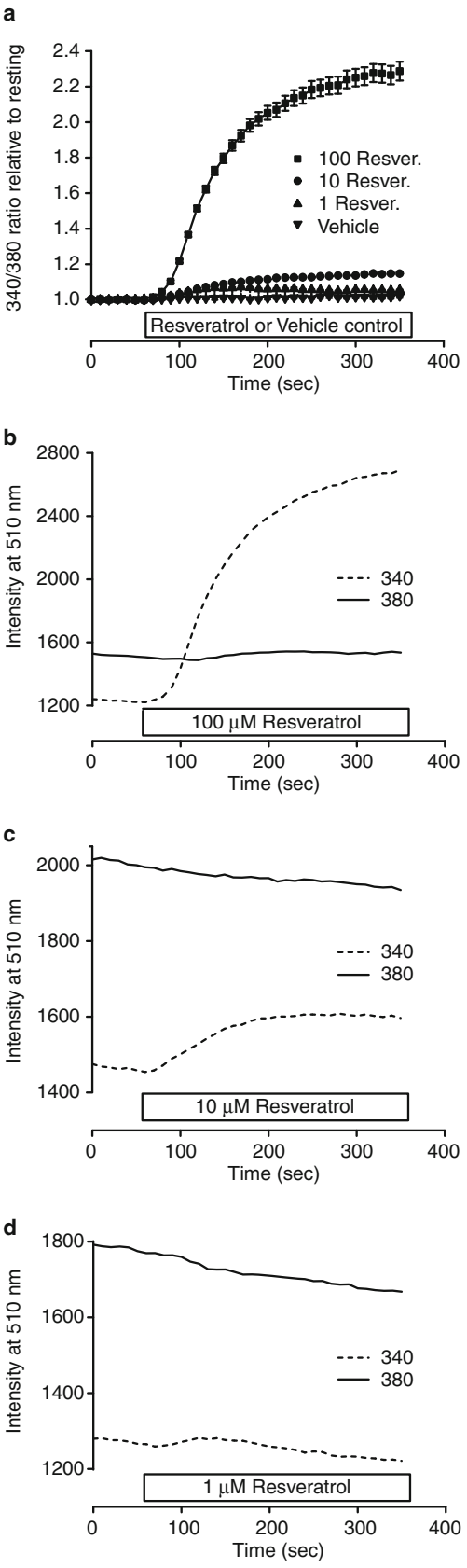

Fig. 1 Intracellular calcium response of MDA-MB-231 cells to resveratrol ▶ based on Fura-2 measurements. **a** Average and standard error of 340/380 ratio relative to resting values for resveratrol applications (5 min.) at 100, 10, and 1 μM concentrations, plus DMSO vehicle control. Measurements were from a minimum of 89 cells and three independent experiments. **b**, **c**, **d** Average 340 and 380 traces for cells treated with 100, 10, and 1 μM resveratrol, respectively. Note the increase in intensity upon resveratrol addition with excitation at 340 nm but no concurrent decline at 380 nm

individual 340 and 380 nm traces shows a rise with excitation at 340 nm upon resveratrol introduction but no corresponding decline with excitation at 380 nm (Fig. 1b). Similarly, resveratrol applied at 10 and 1 μM caused increases in the 340/380 ratios, but individual traces of 340 nm and 380 nm revealed intensity increases with 340 nm excitation without the expected concurrent declines at 380 nm (Fig. 1a, c–d).

Tests with agonists known to cause store-release and store operated calcium entry showed the expected Fura-2 response. Application of 100 μM ATP caused a large increase in the 340/380 ratio (Fig. 2a). The individual plots of 340 and 380 for the ATP response showed an increase at 340 and a concomitant decline in 380 (Fig. 2b). Similarly, application of 50 μM ionomycin, a calcium ionophore, resulted in a large increase in 340/380 ratio, and the individual 340 and 380 traces showed the expected large increase at 340 and concurrent decline at 380 (Fig. 2c and d). These data indicate a normal response by Fura-2 to increases in cytosolic Ca^{2+} elicited by ATP and ionomycin, but resveratrol caused a response that is not characteristic for calcium binding with Fura-2.

MDA-MB-231 cells loaded with Fluo-4 and then treated with 100 μM resveratrol followed by 100 μM ATP showed no response to resveratrol, but a large response to ATP (Fig. 3a). Similarly, MDA-MB-231 cells expressing the cytosolic calcium indicator YC 3.60 that were treated with 100 μM resveratrol followed by a subsequent application of 100 μM ATP showed no response to the resveratrol but a large increase in FRET ratio due to ATP application (Fig. 3b). Collectively, these data demonstrate that the Fluo-4 dye and YC 3.60 calcium biosensor in MDA-MB-231 cells were capable of responding to an agonist that is known to increase cytosolic calcium, but no response to 100 μM resveratrol application was detected by these calcium indicators.

When cells were incubated with BAPTA-AM and Fura-2-AM followed by treatment with 100 μM resveratrol, a large increase in 340/380 ratio was observed (Fig. 4a). Cells treated with only Fura-2 and 100 μM resveratrol showed an increase in 340/380 ratio that was slightly larger than the BAPTA-treated cells (Fig. 4a). Area under the curve calculations indicated that BAPTA treatment reduced the magnitude of the resveratrol response by approximately 29 % compared with cells incubated with only Fura-2-AM and 100 μM resveratrol (Fig. 4b). However, MDA-MB-231 cells incubated with BAPTA-AM and Fura-2-AM and then treated with 100 μM ATP exhibited a large reduction in 340/380 ratio

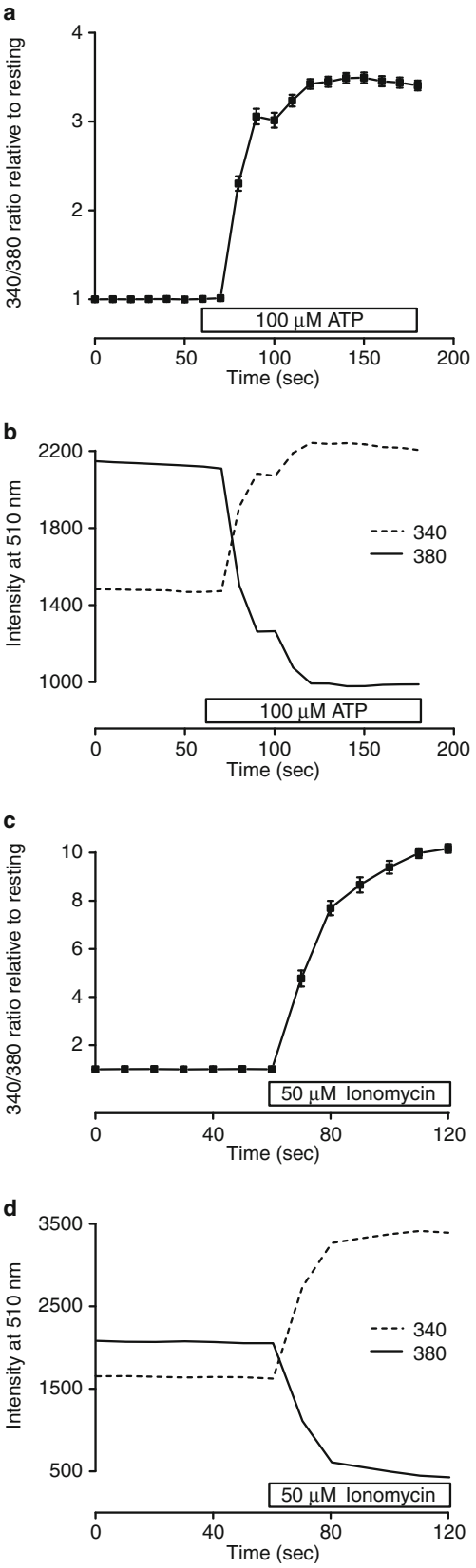

Fig. 2 Intracellular calcium response by MDA-MB-231 cells elicited by ▶ treatments known to induce store operated calcium entry based on Fura-2 measurements. **a** 2 min application of 100 μM ATP. Average and standard error of 340/380 ratio relative to resting were from 176 cells and seven independent experiments. **b** Average 340 and 380 traces for cells treated with 100 μM ATP. **c** 2 min application of 50 μM ionomycin. Measurements were from 77 cells and three independent experiments. **d** Average 340 and 380 traces for cells treated with ionomycin. Note the increase in 340 intensity and the concurrent decline in 380 intensity for both ATP and ionomycin treatments

compared with cells incubated with only Fura-2-AM (Fig. 4a). The reduction in area under the curve for the ATP response due to BAPTA-AM treatment was approximately 97 % (Fig. 4b).

Discussion

Our data provide strong evidence that resveratrol at concentrations exceeding 10 μM in an imaging buffer interferes with the function of Fura-2-AM dye used in in vitro calcium imaging experiments. Normally, an increase in cytoplasmic calcium in Fura-2-loaded cells causes an increase in the emission at 510 nm upon excitation at 340 nm and a concomitant decline in the 510 emission with 380 nm excitation [8]. The typical decline in the 510 emission upon excitation at 380 does not occur in the presence of high concentrations of resveratrol, and even 1 μM of resveratrol caused a small 340 response but no 380 response. The abnormal 380 excitation response was the initial evidence that caused our laboratory to question data being generated which indicated a strong, rapid, and easily reversible calcium response elicited by resveratrol.

Agreement of the Fluo-4 and YC3.60 calcium indicators in their lack of response to resveratrol application, combined with the abnormal 380 excitation response by Fura-2 in the presence of resveratrol, suggests that the apparent increase in cytosolic calcium elicited by resveratrol in Fura-2 experiments may in fact be experimental artifact. Fluo-4 is a calcium indicator with excitation and emission peaks at 488 and 520 nm, respectively [18]. YC3.60 is a FRET-based genetically encoded cameleon biosensor targeted to the cytosol, with excitation at 440 nm and emission peaks at 485 and 535 nm [16]. These indicators function in very different ways from Fura-2 and from each other and there is no evidence that they functioned abnormally in these experiments. The observation that cells loaded with Fluo-4 could respond to ATP after resveratrol treatment suggests that the resveratrol did not modify the function of Fluo-4. Similarly, the observation that cells expressing YC3.60 responded to ATP after exposure to resveratrol suggests that the biosensor function was not impaired by resveratrol.

BAPTA-AM incubation prior to Fura-2 loading prevented intracellular calcium release due to ATP addition as expected, but it did not prevent a rise in the 340/380 ratio when resveratrol was applied. The observation that cells treated with BAPTA-AM did not respond to ATP, a known agonist of store

depletion and store-operated calcium entry, suggests that BAPTA-AM was functioning as expected. Failure of BAPTA-AM to prevent an increase in the 340/380 ratio with resveratrol suggests the possibility of an interaction between resveratrol and Fura-2 that causes fluorescence upon 340 excitation, and this fluorescence is not associated with an increase in cytosolic calcium concentration.

The current study does not support the results of several reports in the literature involving Fura-2 calcium imaging measurements when resveratrol was present an imaging buffer. Several reports in the literature describe imaging experiments using Fura-2 with resveratrol at concentrations exceeding 10 mM [12, 14, 15, 19, 20]. The failure of BAPTA-AM treatment to prevent an increase in the 340/380 ratio upon resveratrol addition in the current study contradicts the report of Sareen et al. [14]. Explaining the discrepancies between the current study and several literature reports is difficult since independent 340 and 380 traces were not reported in other studies and there are many differences in experimental protocols among the studies. Altered performance of Fura-2 in the presence of resveratrol at concentrations greater than 10 μM was reported previously [5], suggesting that results of the current study are not unprecedented.

Our laboratory no longer uses Fura-2 in the presence of resveratrol at concentrations of 10 μM or higher, but it is uncertain if results reported here can be generalized. All of our experiments were completed with a single source of resveratrol and the same imaging buffer. We completed tests with Fura-2 and resveratrol on our Nikon instrument and achieved the same result as on our Olympus system (data not shown). We recommend that investigators use caution when

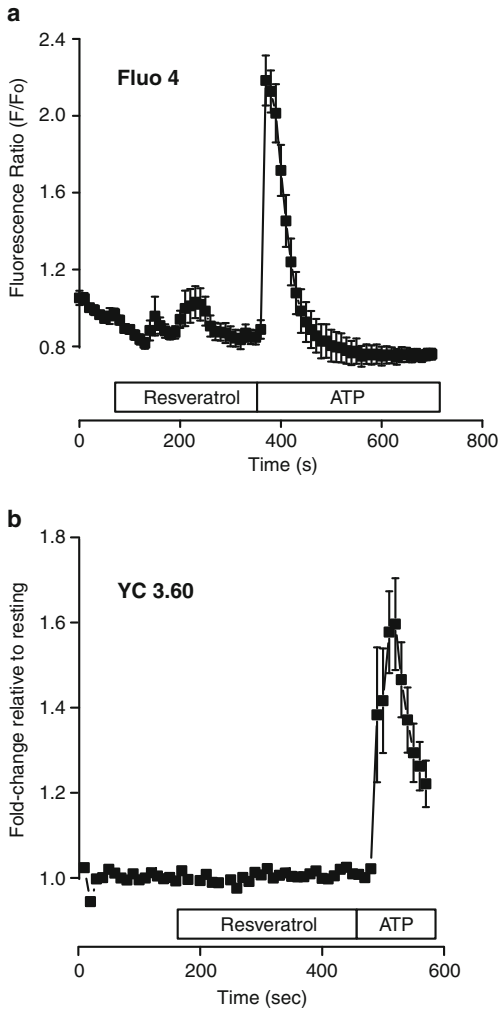

Fig. 3 Effect of 100 μM resveratrol application on intracellular calcium release in MDA-MB-231 cells based on measurements with Fluo-4 and YC3.60 calcium indicators. **a** and **b** Cells loaded with Fluo-4 (**a**) or expressing YC3.60 (**b**) treated with 100 μM resveratrol followed by 100 μM ATP. Fluo-4 and YC3.60 measurements were completed on 40 and 10 cells, respectively, both in two independent experiments

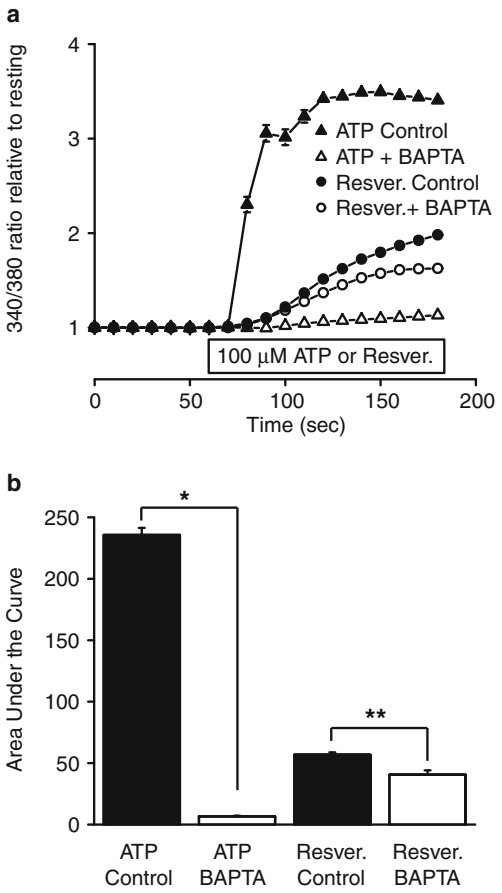

Fig. 4 Effect of 10 μM BAPTA pretreatment on intracellular calcium response in MDA-MB-231 cells loaded with Fura-2 and treated with 100 μM ATP or 100 μM resveratrol. **a** Pretreatment with 10 μM BAPTA prevented an increase in the 340/380 ratio relative to cells loaded only with Fura-2 upon application of 100 μM ATP. BAPTA reduced the increase in 340/380 ratio upon 100 μM resveratrol application, but the reduction was much less compared with the effect of BAPTA on the ATP response. **b** Area under the curve for the graphs shown in Fig. 4a. Statistically significant ($p<0.003$) differences were detected between BAPTA-treated and control cells for both ATP and resveratrol application. Measurements were completed on a minimum of 50 cells in two independent experiments. *, $p<0.001$; **, $p=0.003$

interpreting data from imaging studies using buffers containing both Fura-2 and resveratrol at concentrations exceeding 10 μM.

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