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Red wine polyphenols increase calcium in bovine aortic endothelial cells: a basis to elucidate signalling pathways leading to nitric oxide production

[1]Sophie Martin, [1]Emile Andriambeloson, [1]Ken Takeda & *,[1]Ramaroson Andriantsitohaina

[1]Pharmacologie et Physico-Chimie des Interactions Cellulaires et Moléculaires, UMR CNRS 7034, Faculté de Pharmacie, Université Louis Pasteur, 67401 Illkirch, France

1 The present study investigates the mechanisms by which polyphenolic compounds from red wine elicit Ca^{2+} mobilization in bovine aortic endothelial cells (BAECs). Two polyphenol-containing red wine extracts, red wine polyphenolic compounds (RWPC) and Provinols™, and delphinidin, an anthocyanin were used.

2 RWPC stimulated a Ca^{2+}-dependent release of nitric oxide (NO) from BAECs accounting for the relaxation of endothelium-denuded rat aortic rings as shown by cascade bioassay.

3 RWPC, Provinols™ and delphinidin increased cytosolic free calcium ($[Ca^{2+}]_i$), by releasing Ca^{2+} from intracellular stores and by increasing Ca^{2+} entry.

4 The RWPC-induced increase in $[Ca^{2+}]_i$ was decreased by exposure to ryanodine (30 μM), whereas Provinols™ and delphinidin-induced increases in $[Ca^{2+}]_i$ were decreased by bradykinin (0.1 μM) and thapsigargin (1 μM) pre-treatment.

5 RWPC, Provinols™ and delphinidin-induced increases in $[Ca^{2+}]_i$ were sensitive to inhibitors of phospholipase C (neomycin, 3 mM; U73122, 3 μM) and tyrosine kinase (herbimycin A, 1 μM).

6 RWPC, Provinols™ and delphinidin induced herbimycin A (1 μM)-sensitive tyrosine phosphorylation of several intracellular proteins.

7 Provinols™ released Ca^{2+} *via* both a cholera (CTX) and pertussis toxins (PTX)-sensitive pathway, whereas delphinidin released Ca^{2+} only *via* a PTX-sensitive mechanism.

8 Our data contribute in defining the mechanisms of endothelial NO production caused by wine polyphenols including the increase in $[Ca^{2+}]_i$ and the activation of tyrosine kinases. Furthermore, RWPC, Provinols™ and delphinidin display differences in the process leading to $[Ca^{2+}]_i$ increases in endothelial cells illustrating multiple cellular targets of natural dietary polyphenolic compounds.
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Abbreviations: BAECs, bovine aortic endothelial cells; CTX, cholera toxin; NO, nitric oxide; PTX, pertussis toxin; RWPC, red wine polyphenolic compounds

Introduction

Natural dietary polyphenolic compounds that are present in a wide variety of plants are thought to protect against cardiovascular disease and cancer (Renaud & de Lorgeril, 1992; Hertog *et al*., 1993; Jang *et al*., 1997; Carbo *et al*., 1999). One of their therapeutically relevant effects on the cardiovascular system may be their ability to interact with the NO-generating pathway in vascular endothelium (Andriambeloson *et al*., 1997). This effect of polyphenols is of importance because NO has vasorelaxant and anti-aggregatory properties (Furchgott *et al*., 1984; Furchgott, 1984) and is able to limit the flux of atherogenic plasma proteins into the artery wall (Luscher, 1991). Previous studies (Andriambeloson *et al*., 1997) from our laboratory show that a polyphenol-containing extract from red wine (RWPC) induces an endothelium-dependent relaxation of rat thoracic aorta. This effect was mediated by an increase in aortic NO content due to enhanced NO synthesis rather than protection against its breakdown by oxygen radicals associated with the antioxidant properties of RWPC. Using EPR spectroscopy, NO formation was 1.17 ± 0.08 mmol g^{-1} wet tissue in the presence of RWPC but was undetectable in arteries without endothelium (Andriambeloson *et al*., 1997). We also reported that the endothelium-NO-dependent relaxation induced by polyphenolic compounds was produced through an extracellular Ca^{2+}-dependent mechanism (Andriambeloson *et al*., 1999). Amongst the different classes of polyphenolic compounds present in RWPC, anthocyanins and oligomeric condensed tannins had the same pharmacological profile as RWPC (Andriambeloson *et al*., 1998). Of different anthocyanins identified in wine, only delphinidin caused endothelium-dependent relaxation, although it was slightly less potent than RWPC (Andriambeloson *et al*., 1998).

We suggested that polyphenols affect intracellular calcium ($[Ca^{2+}]_i$) homeostasis and tyrosine kinase (TK) pathway in endothelial cells, thereby regulating biosynthesis of endothelial-derived vasoactive factors, especially NO (Luckhoff *et al*.,

*Author for correspondence at: Pharmacologie et Physico-Chimie, UMR CNRS 7034, Faculté de Pharmacie, 74 route du Rhin, BP 24, 67401 Illkirch, France; E-mail: nain@pharma.u-strasbg.fr

1988; Graier *et al*., 1990; Busse & Fleming, 1995). Increases in $[Ca^{2+}]_i$ are due to mobilization of Ca^{2+} from intracellular stores and Ca^{2+} entry *via* voltage-independent cationic channels (Takeda *et al*., 1987; Luckhoff *et al*., 1988). The action of Ca^{2+} mobilizing agonists is associated with the stimulation of phospholipase C (PLC) or phospholipase A_2 (PLA_2) pathways (Graier *et al*., 1994). Also, TK may play key roles in the biosynthesis of NO (Ayajiki *et al*., 1996). However, to date, no information is available concerning how polyphenolic compounds affect Ca^{2+} handling in endothelial cells, thereby leading to NO production.

The aim of this study was to elucidate the signalling mechanisms activated by polyphenolic compounds leading to increases in $[Ca^{2+}]_i$ and subsequent NO production in bovine aortic endothelial cells (BAECs). The sources of Ca^{2+}, the involvement of G-proteins, the role of PLC and the TK pathway were investigated using polyphenolic compounds contained in two extracts from red wine, RWPC and Provinols™, which contains similar types of polyphenols. Their effects were compared to those induced by delphinidin, an anthocyanin.

Methods

Materials

The RWPC dry powder extract from red wine (Cabernet-Sauvignon grapes) was provided by M. Moutonet (Institut National de la Recherche Agronomique, Montpellier, France) and Provinols™ by D. Ageron (Societé Française de Distilleries, Vallon Pont d'Arc, France). The method for production of RWPC dry powder has been previously reported (Andriambeloson *et al*., 1998). The composition of both polyphenol extracts has been previously identified by chromatography (Andriambeloson *et al*., 1998): RWPC contained 1.7% phenolic acids (including 19.5% caftaric acid), 18% oligomerized condensed tannins (including only 1% of each monomeric form of catechin and epicatechin), 1% flavonols, 16% anthocyanin-enriched fractions (including 36% malvinidin-3-glucoside) and 63.3% polymeric condensed tannins; polyphenols in Provinols™ were 1.8% hydroxycinnamic acids, 3.8% catechin, 1.4% flavonol, 8% anthocyanins (including 3.7% of delphinidin), 48% proanthocyanidols and 37% polymeric condensed tannins. Delphinidin chloride, an anthocyanin having the same pharmacological profile as RWPC, was purchased from Extrasynthèse (Genay, France). Delphinidin chloride dry powder was 99.93% pure, according to the manufacturer. All compounds were used at 10^{-2} g l^{-1}, the concentration eliciting maximal endothelium-dependent relaxation of pre-contracted rat aorta.

DMEM, Ham's F-12, FBS were purchased from BioWhittaker; fura 2-AM was from Sigma; the anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories; HRP-conjugated anti-mouse antibody was from Promega; herbimycin A and ryanodine were from Calbiochem. All other compounds were from Sigma.

Cell culture

BAECs were grown as described previously (Schini *et al*., 1988). Briefly, cells were cultured at 37°C in 5% CO_2 atmosphere in plastic flasks (Nunc) pre-coated with type I collagen (0.06 mg ml^{-1}) in a mixture of DMEM and Ham's F12 (1 : 1, v v^{-1}) with 10% FBS, 2 mM L-glutamine, 100 mg ml^{-1} heparin, antibiotics (100 u ml^{-1} penicillin and 100 u ml^{-1} streptomycin and 10 μM vitamin C. Cultures were used up to fifth passages during which no modifications of responses to agonists and polyphenolic compounds were observed. For bioassay experiments, BAECs were grown to confluence on microcarrier beads in stirrer bottles (2 min at 20 r.p.m., then 100 min rest). For Ca^{2+} experiments, cells were grown to confluence in 35 mm Petri dishes (Nunc) in which a 2 cm diameter hole had been cut in the base and replaced by a thin (0.07 mm) glass coverslip. For Western blots, cells were subcultured in 6-well plates (Nunc) until confluence.

Cascade bioassay

As previous data obtained in our laboratory showed that the polyphenol effects are endothelium-dependent and mediated by nitric oxide (Andriambeloson *et al*., 1997; Stoclet *et al*., 1999; Diebolt *et al*., 2001), bioassay experiments were performed. Microcarrier beads (Cytodex 3 from Sigma) with approximately 20×10^6 BAECs were packed into a 1 cm diameter column and perfused with physiological salt solution (PSS) (mM): NaCl 119, KCl 4.75, $CaCl_2$ 1.25, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25, glucose 11; 37°C; gassed with 95% O_2–5% CO_2) at 2 ml min^{-1} using a pump. The effluent from the column superfused a 2 mm length rat aortic ring (male Wistar rats, 12–14 weeks old) without endothelium that has been stretched under a passive wall tension of 2 g, used as a detector of NO production by BAECs. A second PSS perfusion line (2 ml min^{-1}) bypassed the BAECs column, allowing 0.2 μM noradrenaline to directly pre-contract the ring (60–70% maximum tone). Indomethacin (10 μM) was added to prevent prostacyclin formation. Polyphenolic compounds were given through the column; in separate experiments, 300 μM L-NAME was added to inhibit NO synthesis or $CaCl_2$ was removed from PSS. Relaxations were expressed as percentage decrease of the level of pre-contraction (2.08 ± 0.02 g; $n = 9$).

Measurement of $[Ca^{2+}]_i$

Cells were loaded with fura-2 AM (45 min at 37°C in culture medium), then washed and placed in PSS with 20 mM HEPES. For Ca^{2+}-free experiments, $CaCl_2$ was omitted from PSS and 0.5 mM EGTA was added 10 min before measurement. To assess Ca^{2+} release from stores, cells were pretreated with ryanodine (Ry, 30 μM) which acts on Ca^{2+} release-activated channels, and bradykinin (BK, 0.1 μM) or thapsigargin (TG, 1 μM) which affect SR Ca^{2+} stores *via* IP_3 generation and inhibition of Ca^{2+}-ATPase pumps, respectively. U73122 (3 μM) and neomycin (3 mM), which are inhibitors of PLC and phospholipid turnover (Yule & Williams, 1992; Lee & Wu, 1999) were pre-incubated for 1 h and 10 min respectively prior to stimulation with polyphenolic compounds. Herbimycin A (1 μM), an inhibitor of tyrosine kinase and protein tyrosine phosphorylation in response to various stimuli in endothelial cells (Kruse *et al*., 1994; Cohen *et al*., 1999) was pre-incubated for 1 h prior stimulation with polyphenolic compounds. G-protein involve-

ment was studied after 3 h incubation with *B. pertussis* toxin (PTX, 1 μg ml^{-1}) or *V. cholera* toxin (CTX, 1 μg ml^{-1}).

Cells were stimulated by injection of polyphenols in the bath using a standard pipette directly on the cells. Perfusion system was not used in order to avoid spontaneous Ca^{2+} oscillations linked to shear stress. This method is similar to that used in spectrofluorimetric experiments for non-adherent cell stimulation and ward off problems inherent to the use of a puffer pipette (i.e. continuous flow, pipette not blocked or run out of the compound ...). No modifications of $[Ca^{2+}]_i$ were observed when solvent alone was applied. Digital Ca^{2+} imaging was performed at 37°C on single cells viewed with a UV-fluor 20× objective (n.a. 0.75) on an inverted microscope (Nikon Diaphot, Tokyo, Japan). Fluorescence was measured at 510±20 nm using an amplified Darkstar-800 CCD camera (Photonics, Milham, U.K.). Ratiometric Ca^{2+} images were generated at 3 s intervals by using four averaged images at each wavelength (340±10 and 380±10 nm) and analysed with IMSTAR software. For each cell, $[Ca^{2+}]_i$ was averaged from pixels within manually outlined cell areas. We used the method of Grynkiewicz *et al*. (1985) to calibrate the $[Ca^{2+}]_i$ *in vitro*: $[Ca^{2+}]_i = (K_d)(\beta)[R - R_{min}]/[R_{max} - R]$. K_d represents the dissociation constant of Ca^{2+}-fura-2 complex (224 nM). R is the ratio of fluorescence due to excitation at 340 and 380 nm. R_{min} and R_{max} are the ratios measured as described elsewhere (Lynch *et al*., 1994), by addition of 10 μM of the Ca^{2+} ionophore, ionomycin, to Ca^{2+}-free (with 10 mM EGTA) solution or Ca^{2+} replete (10 mM $CaCl_2$) solution respectively. β is the ratio of the 380 nm signals in Ca^{2+}-free and Ca^{2+} replete solution. The values of R_{min}, R_{max} and β have been calculated as indicated above. Results were expressed in terms of absolute $[Ca^{2+}]_i$ in nM.

Western blot analysis

After incubation with pharmacological agents for different periods of time at 37°C, experiments were stopped by addition of 500 μl ml^{-1} of ice-cold buffer A (in mM): Tris 50, NaCl 250, $MgCl_2$ 8, 10 μg ml^{-1} of aprotinin, leupeptin and pepstatin (Roche), PMSF 1 (Roche), EDTA 5, EGTA 0.5, 2 Na orthovanadate. Cells were spun down and lysed for 1 h in 1 ml of ice-cold lysis buffer (buffer A plus 1% Triton X-100). After quantification by the Bradford method, 10 μg protein was resuspended in Laemmli's buffer, separated on 10% SDS–PAGE and Western blotted. The sample loading was verified by staining membranes with Ponceau red and amido black. Blots were probed with the anti-phosphotyrosine antibody, followed by the HRP-conjugated antibody. Blots were treated with enhanced chemiluminescence reagents for 10 min, exposed to CL-Xposure films (Kodak). Then, blots were scanned and densitometric analysis was performed on the scanning images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com). Results were expressed as the percentage of tyrosine phosphorylation increase in stimulated cells versus basal tyrosine phosphorylation in control cells and as the percentage of tyrosine phosphorylation inhibition in herbimycin A treated cells versus non treated cells.

Statistical analysis

Results are expressed as means±s.e.mean of n separate experiments. Analysis of variance (ANOVA) or unpaired Student's t-test were used for statistical analysis with $P<0.05$ being considered significant.

Results

RWPC-induced vasorelaxation via *endothelial NO production depends on extracellular Ca^{2+}*

Effluent from BAECs exposed to RWPC relaxed pre-contracted endothelium-denuded aortic rings by 25±2% (Figure 1a, $n=6$), while PSS perfusion alone was without effect. As a control, BAECs exposed to 10 μM ionomycin relaxed endothelium-denuded aortic rings by 36±5% ($n=6$). In other controls, no change in detector vessel tone was seen upon perfusion of PSS or RWPC through the column packed with endothelial cell-free beads (not shown). L-NAME almost completely inhibited the relaxation induced by RWPC, as was also the case for perfusion of the column with RWPC in Ca^{2+}-free PSS (Figure 1b, $n=5-9$). Together, these data indicate that RWPC induced a Ca^{2+}-dependent release of NO from BAECs, accounting for the relaxation of the endothelium-denuded aortic rings of the rat. These data also support the hypothesis that NO release either from rat (Andriambeloson *et al*., 1999) or bovine aortic endothelial cells involved an increase of intracellular Ca^{2+}.

Polyphenolic compounds increase $[Ca^{2+}]_i$ in BAECs

Typical traces showing increases in $[Ca^{2+}]_i$ induced by polyphenolic compounds in single BAECs are illustrated in Figure 2. After stabilization of $[Ca^{2+}]_i$ for few minutes, mean baseline values of 219±10 nM was measured in the presence of 1.25 mM of extracellular Ca^{2+} ($n=21-24$). Three profiles

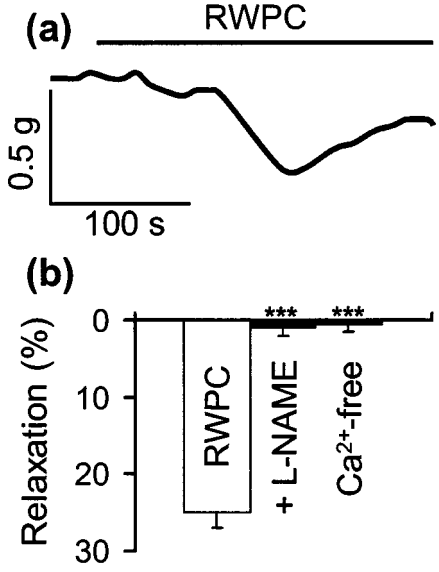

Figure 1 Bioassay of NO production by BAECs stimulated with RWPC. (a) Representative trace showing relaxation of an endothelium-denuded rat aortic ring upon application of RWPC (10^{-2} g l^{-1}) through the BAEC-containing column. (b) Relaxations to RWPC through the column are inhibited by L-NAME (300 μM) and in the absence of external Ca^{2+}. Values are the means±s.e.mean of 5–9 experiments. ***$P<0.001$.

of Ca^{2+} responses were observed: a single Ca^{2+} transient (left columns); Ca^{2+} oscillations (centre columns); a biphasic Ca^{2+} response consisting of an initial spike followed by a sustained plateau lasting ~400 s (right columns). Cells were classed into these three response profiles, which were equally frequent and similar for the three polyphenolic compounds tested (Figure 2). The resting $[Ca^{2+}]_i$ was not significantly different between each group or each compound. Increases in $[Ca^{2+}]_i$ (determined for all experiments at the peak of the first spike of each cell) reached a maximum at 300 ± 3, 313 ± 3 and 420 ± 6 nM for RWPC, Provinols™ and delphinidin respectively ($n=21-24$). At lower concentrations (10^{-4} and 10^{-5} g l^{-1}), delphinidin produced a smaller increase in $[Ca^{2+}]_i$ which peaks at respectively 247 ± 6 and 234 ± 4 nM ($n=3$).

Increases in $[Ca^{2+}]_i$ induced by polyphenolic compounds require extracellular Ca^{2+}

After stabilization of $[Ca^{2+}]_i$ for a few minutes, mean baseline values of 115 ± 10 nM was measured in the Ca^{2+}-free PSS ($n=15$). When polyphenolic compounds were applied in Ca^{2+}-free PSS, the mean peak increase in $[Ca^{2+}]_i$ was significantly reduced: for RWPC, Provinols™ and delphinidin, the decreases were $76\pm3\%$, $74\pm2\%$ and $70\pm2\%$ respectively, compared to those elicited in normal PSS (Figure 3). Increases in $[Ca^{2+}]_i$ reached a maximum at 134 ± 3 ($n=3$) and 140 ± 2 nM ($n=4$) and 160 ± 3 nM ($n=8$) for RWPC, Provinols™ and delphinidin respectively. BK (0.1 μM) was able to stimulate a mean peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i=35\pm8$ nM, $n=6$) when added after the polyphenolic compounds which is not significantly different from that observed in absence of pre-stimulation with polyphenols ($\Delta[Ca^{2+}]_i=39\pm10$ nM, $n=6$). Another difference was that the Ca^{2+} response profile for ~80% of cells consisted of a single Ca^{2+} transient (percentage are indicated under traces). These results suggest that Ca^{2+} influx plays a major role in the increase of $[Ca^{2+}]_i$ produced by the polyphenolic compounds.

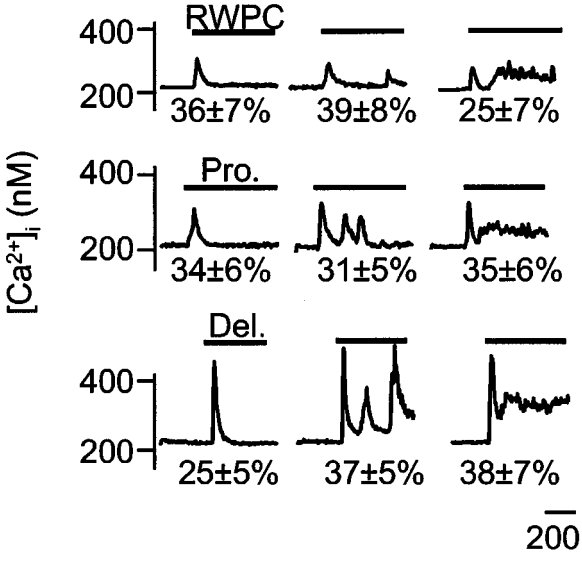

Figure 2 Polyphenolic compounds induce increases in Ca^{2+} in BAECs. Application of RWPC, Provinols™ and delphinidin (at 10^{-2} g l^{-1} in Ca^{2+}-containing PSS) produced increases in $[Ca^{2+}]_i$. Cells were classed into three groups according to their $[Ca^{2+}]_i$ response profile (left columns: single Ca^{2+} transient; centre columns: Ca^{2+} oscillations; right columns: biphasic Ca^{2+} response) and the percentage of cells displaying each type of response is given. No differences between the groups were found by ANOVA. Basal $[Ca^{2+}]_i$ was 219 ± 10 nM. On average, 75–97% of cells responded to stimulation with polyphenolic compounds. For each compound, 882–967 responsive cells from 21–24 separate experiments were analysed.

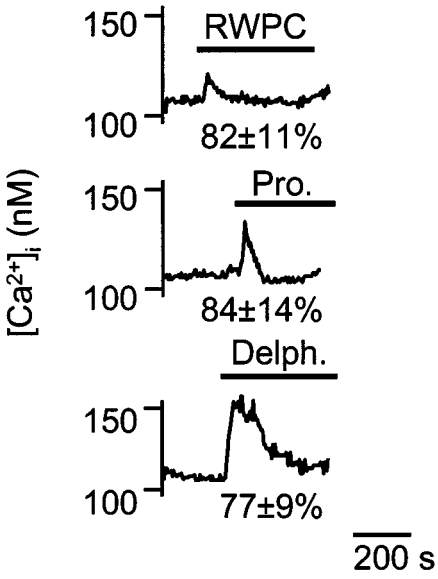

Figure 3 Ca^{2+} response induced by polyphenolic compounds is decreased in Ca^{2+}-free PSS. Representative traces showing changes in $[Ca^{2+}]_i$ induced by application of polyphenolic compounds (10^{-2} g l^{-1}) in Ca^{2+}-free PSS. Peak responses were smaller, and most cells responded with a single Ca^{2+} transient, as indicated. For each compound, 56–313 cells from 3–8 separate experiments were analysed.

Intracellular Ca^{2+} stores mobilized by polyphenolic compounds

The nature of intracellular Ca^{2+} stores involved in the response to polyphenolic compounds was examined by prior stimulation with different Ca^{2+}-mobilizing agents in normal PSS. Pre-treatment with Ry significantly attenuated the response to RWPC ($28\pm4\%$ inhibition) but did not affect the increase in $[Ca^{2+}]_i$ produced by Provinols™ or delphinidin (Figure 4a, $n=7$). In contrast, after BK or TG pre-treatment, responses to RWPC were significantly enhanced (by $19\pm6\%$ and $200\pm23\%$ respectively, $n=6-7$) whereas those elicited by Provinols™ and delphinidin were significantly reduced ($51\pm3\%$ and $26\pm6\%$ inhibition for Provinols™; $28\pm4\%$ and $28\pm7\%$ inhibition for delphinidin, $n=6-7$) (Figure 4b,c). It should be noted that TG but not Ry nor BK treatment significantly enhanced the basal Ca^{2+} values prior to stimulation to polyphenolic compounds (from 220 ± 1 to 318 ± 8, from 220 ± 1 to 305 ± 5 and from 220 ± 1 to 307 ± 5 for RWPC, Provinols™ and delphinidin respectively).

In addition, after pre-treatment of the cells with these Ca^{2+} mobilizing agents, we observed an increase in the percentage of cells having a single Ca^{2+} transient in response to polyphenolic compounds (51 ± 19, 34 ± 20, $29\pm10\%$ after Ry treatment, 81 ± 1, 61 ± 18, $38\pm16\%$ after BK treatment and 67 ± 29, 56 ± 28, $51\pm29\%$ after TG treatment for RWPC, Provinols™ and delphinidin respectively).

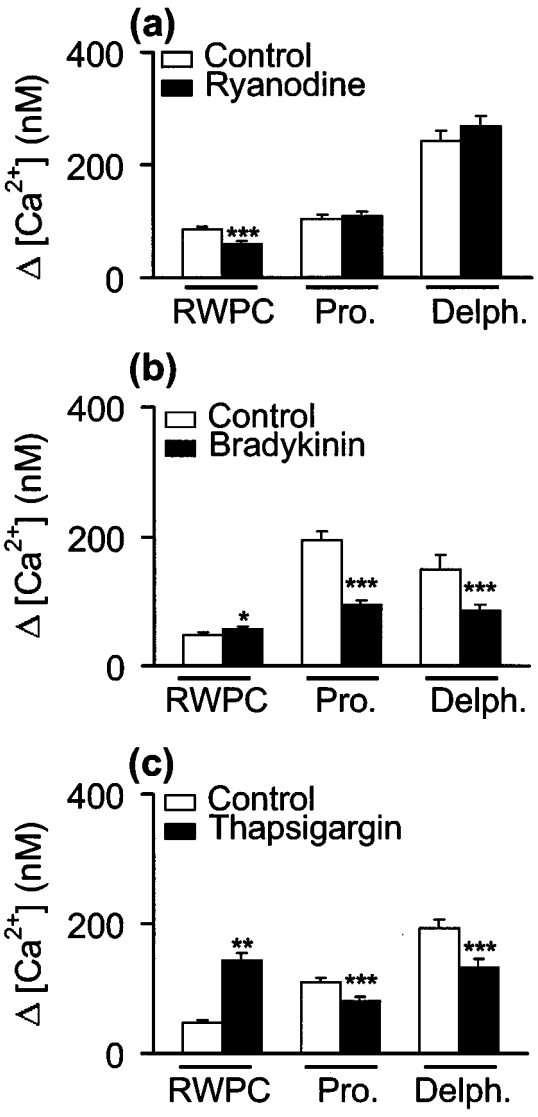

Figure 4 Intracellular Ca^{2+} stores are involved in Ca^{2+} responses to polyphenolic compounds. Histograms show the mean values of Δ increase in Ca^{2+} responses induced by polyphenolic compounds (10^{-2} g l^{-1}) alone (Control) or after prior stimulation (a) with 30 μM Ry (152–234 cells from seven experiments), (b) 0.1 μM BK (145–236 cells from seven experiments) or (c) 1 μM TG (125–177 cells from six experiments). Cells were stimulated with polyphenolic compounds only after the end of the responses to the Ca^{2+}-mobilizing agents. Note that RWPC responses were differently affected compared to those for Provinols™ (Pro.) and delphinidin (Delph.). $*P<0.05$, $**P<0.01$, $***P<0.001$.

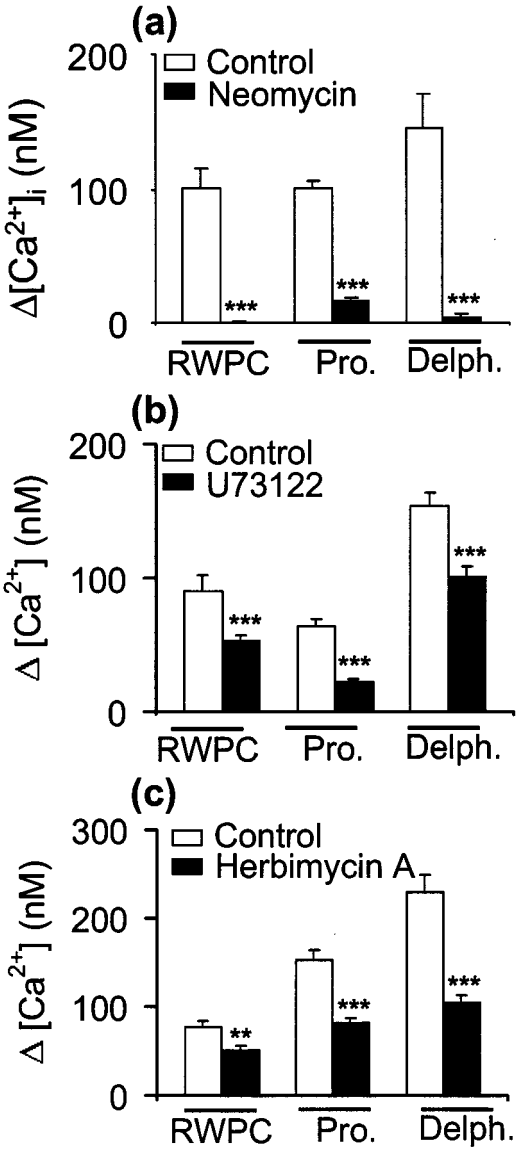

Figure 5 Involvement of PLC and TK in the Ca^{2+} response induced by polyphenolic compounds. Histograms show the mean values of Δ increase in Ca^{2+} responses induced by polyphenolic compounds (10^{-2} g l^{-1}) alone (Control) or after (a) 10 min prior treatment with 3 mM neomycin (20–40 cells from three experiments), (b) 1 h prior treatment with 3 μM U73122 (70–259 cells from six experiments), (c) 1 μM Herbimycin A (120–280 cells from five experiments). $**P<0.01$, $***P<0.001$.

Involvement of PLC and TK in Ca^{2+} responses produced by polyphenolic compounds

To investigate whether the polyphenolic compounds-induced $[Ca^{2+}]_i$ increase was mediated by PLC, endothelial cells were treated with the PLC inhibitors, neomycin (3 mM) and U73122 (3 μM), for 10 min and 1 h respectively prior to stimulation. As shown in Figure 5a, neomycin totally abolished the response to RWPC, Provinols™ and delphinidin ($n=3$), and U73122 significantly reduced Ca^{2+} responses produced by the three polyphenolic compounds (41 ± 3, 65 ± 3 and 37 ± 5% inhibition for RWPC, Provinols™ and delphinidin respectively, $n=6$; Figure 5b). As a control, the response to BK (0.1 μM), known to elevate $[Ca^{2+}]_i$ by the way of PLC activation and IP_3 production, was completely prevented by pretreatment of cells with both neomycin (3 mM) and U73122 (3 μM). The involvement of tyrosine kinases in the Ca^{2+} signalling activated by polyphenols was tested. We found that treatment of cells with 1 μM herbimycin A, a TK inhibitor, significantly inhibit Ca^{2+} responses produced by the three polyphenolic compounds (33 ± 6, 45 ± 3 and 54 ± 3% inhibition for RWPC, Provinols™ and delphinidin respectively, $n=5$; Figure 5c). Interestingly, the combination of both U73122 and herbimycin A did not produce further inhibition of Ca^{2+} responses to

RWPC and delphinidin, compared to the effect of either inhibitor alone (not shown). In contrast, the response to Provinols™ was not affected by the combination of both U73122 and herbimycin A (not shown). The later result are intriguing and further studies are needed to sort out the underlying mechanism. It should be noted that neither neomycin nor U73122, nor herbimycin A treatment enhanced the basal Ca^{2+} values prior stimulation to polyphenolic compounds (from 252 ± 2 to 252 ± 2, from 224 ± 5 to 217 ± 10 and from 268 ± 1 to 249 ± 10 for RWPC, Provinols™ and delphinidin respectively for neomycin, from 220 ± 1 to 221 ± 1, from 225 ± 5 to 217 ± 5 and from 217 ± 1 to 218 ± 5 for RWPC, Provinols™ and delphinidin respectively for U73122 and from 226 ± 3 to 221 ± 3, from 226 ± 1 to 227 ± 1 and from 224 ± 2 to 221 ± 5 for RWPC, Provinols™ and delphinidin respectively for herbimycin A). Finally, after treatment of the cells with U73122, herbimycin A or both, the percentage of cells responding with a single Ca^{2+} transient was increased.

Figure 6 illustrates the pattern of tyrosine phosphorylation induced by polyphenolic compounds in BAECs. A time-dependent increase in tyrosine phosphorylation of several proteins having molecular weights of ~30, 50, 60, 70, 90–110 kDa was observed. The identity of these proteins was not explored. Global increases in tyrosine phosphorylation were detectable at 30 s (82 ± 25, 171 ± 54, 78 ± 33% increase for RWPC, Provinols™ and delphinidin respectively, $n=8$) and reached a steady state at 3 min for RWPC, Provinols™ (89 ± 28, 154 ± 33%, $n=8$) but decreased for delphinidin (39 ± 23%, $n=8$). As herbimycin A was able to inhibit Ca^{2+} responses to RWPC, Provinols™ and delphinidin, we further tested its specific effect on tyrosine phosphorylation. As shown in Figure 7, tyrosine phosphorylation induced by the three polyphenolic compounds was inhibited by 1 μM herbimycin A (35 ± 6, 37 ± 8, 48 ± 6% inhibition for RWPC, Provinols™ and delphinidin respectively at 3 min, $n=5$).

G-protein involvement in Ca^{2+} responses to polyphenolic compounds

In normal PSS, the rise in $[Ca^{2+}]_i$ elicited by RWPC was not significantly affected by pre-treatment of BAECs with either PTX or CTX (Figure 8a,b, $n=4$). PTX treatment significantly reduced the responses to Provinols™ and delphinidin, with 40 ± 5 and 64 ± 3% inhibition, respectively (Figure 8a, $n=4$), while CTX treatment inhibited only the increase in $[Ca^{2+}]_i$ produced by Provinols™, by 58 ± 3% (Figure 8b, $n=4$). It should be noted that neither PTX nor CTX treatment enhanced the basal Ca^{2+} values prior stimulation to polyphenolic compounds (from 220 ± 1 to 220 ± 1, from 220 ± 1 to 220 ± 1 and from 218 ± 1 to 219 ± 1 for RWPC, Provinols™ and delphinidin respectively for PTX and from 236 ± 2 to 237 ± 1, from 226 ± 10 to 229 ± 2 and from 216 ± 1

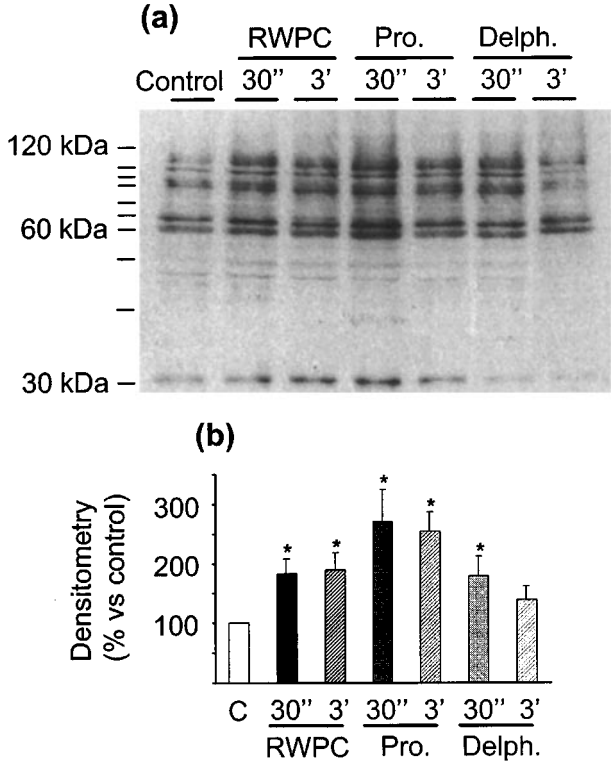

Figure 6 Polyphenolic compounds increase protein tyrosine phosphorylation in BAECs. (a) Representative gel showing the pattern of tyrosine phosphorylation of cells stimulated for 30 s and 3 min with RWPC, Provinols™ and delphinidin (10^{-2} g l^{-1}). Cell samples were processed for SDS–PAGE and immunoblotted (see Methods). (b) Histogram showing densitometric analysis from eight separate experiments.

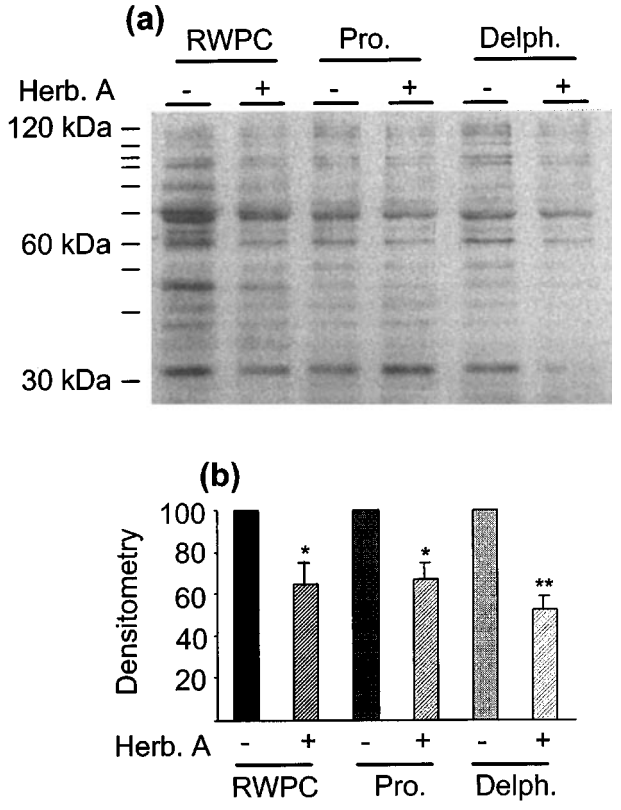

Figure 7 Tyrosine phosphorylation of polyphenolic compounds is inhibited by herbimycin A. (a) Representative gel showing the inhibition of tyrosine phosphorylation of cells stimulated for 3 min with RWPC, Provinols™ and delphinidin (10^{-2} g l^{-1}). Cell samples were processed for SDS–PAGE and immunoblotted (see Methods). (b) Histogram showing densitometric analysis from five separate experiments.

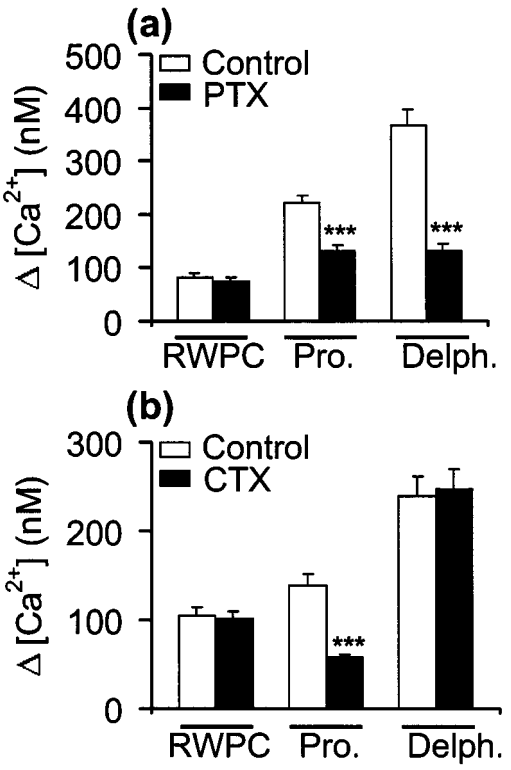

Figure 8 PTX- and CTX-sensitive G-proteins are involved in Ca^{2+} responses to polyphenolic compounds. Histograms show the mean values of Δ increase in Ca^{2+} responses induced by polyphenolic compounds (10^{-2} g l^{-1}) alone (Control) or after (a) a 3 h pre-treatment with 1 μg ml^{-1} PTX (76–95 cells from four experiments), or (b) 1 μg ml^{-1} CTX (99–167 cells from four experiments). ***$P<0.001$.

to 216±1 for RWPC, Provinols™ and delphinidin respectively for CTX). In Ca^{2+}-free PSS, the increases in $[Ca^{2+}]_i$ elicited by Provinols™ were also inhibited by PTX and CTX in a similar degree as in normal PSS (not shown). Taken together, these results suggest that the response to Provinols™ involves both PTX- and CTX- sensitive mechanisms whereas the rise in $[Ca^{2+}]_i$ produced by delphinidin is linked only to a PTX-sensitive pathway.

Discussion

The present study shows that RWPC, Provinols™ and delphinidin increase $[Ca^{2+}]_i$ in endothelial cells. This requires primarily the presence of extracellular Ca^{2+} although the three polyphenolic compounds are able to mobilize Ca^{2+} from intracellular stores and activate PLC and TK pathways. However, the mechanisms of Ca^{2+} handling are different for the three compounds with regards to the type of intracellular Ca^{2+} stores mobilized and the nature of G-proteins implicated. Most importantly is the finding that increase of $[Ca^{2+}]_i$ in endothelial cells was associated with the production of NO to promote vasorelaxation as shown by the bioassay experiments.

Polyphenols detected in human plasma are in the range of 2.5 μg ml^{-1} after 100 ml red wine intake (Duthie *et al*., 1998). These concentrations are comparable to the EC_{50} value for relaxation (Andriambeloson *et al*., 1998) of active polyphenolic compounds (0.5 μg ml^{-1} for RWPC and 10 μg ml^{-1} for delphinidin). Thus, the concentrations of these compounds used in this study (10 μg ml^{-1}) might be reached in plasma, suggesting that polyphenolic compounds from red wine may have physiological actions on the endothelium *in vivo*. This is also supported by our recent study showing that daily feeding of rats for one week with 20 mg kg^{-1} RWPC (a concentration 10 times greater than that producing maximal relaxation of rat aortic rings *ex vivo*) results in sufficient circulating concentrations of polyphenols to induce cardiovascular effects (Diebolt *et al*., 2001).

Cascade bioassay experiments showed that RWPC was able to stimulate the release of NO from BAECs to produce relaxation of endothelium denuded aortic rings of the rat. These results support the relevance of the findings on cultured endothelial cells effects on vascular tone in terms of NO release. Since, the release of NO from BAECs upon stimulation by RWPC primarily depends on the presence of Ca^{2+} in the medium (also shown by the cascade bioassay experiments), increase in intracellular Ca^{2+} plays a major role in this phenomenon. Therefore, the cellular mechanism involved in the release of NO from either rat (Andriambeloson *et al*., 1999) or bovine aortic endothelial cells involved an increase in intracellular Ca^{2+}.

Our data indicate that Ca^{2+} signalling in response to polyphenols is different compared to that induced by classical pharmacological agonists like histamine or by a physiological stimulus such as shear stress (Fleming *et al*., 1997; 1998). For the former, only the activation of the classical PLC pathway is involved whereas for the latter, a Ca^{2+}-independent process leading to tyrosine phosphorylation of proteins occurs. We found that the three polyphenolic compounds act *via* Ca^{2+}-dependent pathways involving the activation of both PLC and TK pathways. Neither the anti-oxidant nor the cyclic nucleotide phosphodiesterase inhibitory activities of polyphenols are likely to account for the effects on Ca^{2+} signalling observed here (Campos-Toimil *et al*., 2000).

The three polyphenolic compounds produce an asynchronous rise in $[Ca^{2+}]_i$ in BAECs due to both an influx of Ca^{2+} from extracellular medium (as reflected by Ca^{2+} oscillations and the biphasic response only observed when depleted intracellular Ca^{2+} stores were refilled) and to a release of Ca^{2+} from intracellular stores (as reflected by single Ca^{2+} spikes seen in Ca^{2+}-free medium and the inhibitory effect of agents interfering with intracellular Ca^{2+} stores). All three polyphenolic compounds mobilize Ca^{2+} from IP_3-sensitive stores as Ca^{2+} responses produced by the three compounds were decreased or abolished by PLC inhibitors, U73122 and neomycin. However, in addition to IP_3-sentitive stores, RWPC produces the release of Ca^{2+} from Ry-sensitive Ca^{2+} stores as shown by the inhibitory effect of Ry. The later may also contribute to the enhancement of the response to RWPC by BK and TG and suggests an interaction between Ry- and IP_3-sensitive Ca^{2+} stores, as observed in vascular smooth muscle cells (Tribe *et al*., 1994; Otun *et al*., 1996). Ca^{2+} released after depletion of IP_3-sensitive stores by either TG or BK would refill the Ry store, thus, more Ca^{2+} would be available for the response to RWPC. Further studies are needed to confirm the above hypothesis. In contrast, BK and TG but not Ry were able to inhibit the response to Provinols™ or delphinidin. These results suggest the absence of interaction between the Ry- and IP_3-sensitive stores in response to Provinols™ and

delphinidin. It is noteworthy that after depletion of intracellular Ca^{2+} by BK and TG or after removal of extracellular Ca^{2+}, the percentage of cells responding to stimulation by a single Ca^{2+} spike increased. As shown by cascade bioassay experiments, the residual Ca^{2+} increase, produced by polyphenolic compounds, is probably not sufficient to induce NO production and subsequent relaxation as it was abolished when Ca^{2+} was omitted from the extracellular medium. These results also indicate that the Ca^{2+} influx pathway plays a major role in the responses of BAECs to polyphenols for NO production in accordance with our previous studies (Andriambeloson *et al*., 1997; 1998). This hypothesis is supported by recent studies showing that Ca^{2+} influx, probably *via* the capacitative Ca^{2+} entry (CCE) pathway is necessary for sustained NO production (Lantoine *et al*., 1998; Taniguchi *et al*., 1999; Wang *et al*., 2000; Lin *et al*., 2000).

Ca^{2+} entry by the CCE pathway might occur subsequent to the release of intracellular Ca^{2+} by IP_3 after the activation of PLC, after stimulation of TK, or both. The use of specific inhibitors of each pathway showed that PLC and TK are both involved in the Ca^{2+} signalling elicited by RWPC, Provinols™ and delphinidin. The involvement of TK was reinforced by herbimycin A-sensitive tyrosine phosphorylation of several proteins produced by the three polyphenolic compounds. The latter results contrast with the commonly described property of polyphenols as tyrosine kinase inhibitors, as described for example for flavonoids (i.e. quercetin, luteolin, genistein) or resveratrol (Palmieri *et al*., 1999). Also, the results are in contrast with the activation of endothelial NO-synthase by shear stress (Fleming *et al*., 1997; Fleming & Busse, 1999) which is reported to involve the activation of a TK pathway but through a Ca^{2+}-independent process in endothelial cells. Taken together, these results highlight a mechanism for Ca^{2+} handling leading to endothelial NO production produced by polyphenols which involves the activation of both PLC and TK. Furthermore, tyrosine phosphorylation of endothelial NO-synthase has been reported to enhance its activity and thus NO production (Corson *et al*., 1996). In accordance with the later, we reported here that even though all three polyphenolic compounds tested were able to increase tyrosine phosphorylation, delphinidin is the less potent in maintaining this phosphorylation over a period of 3 min. Thus, these data might explain that delphinidin stimulates 2 fold greater increase of $[Ca^{2+}]_i$ than the RWPCs and cause 10 fold less potent endothelium-dependent, L-NAME-sensitive relaxation response (Andriambeloson *et al*., 1998).

The involvement of G-proteins in the Ca^{2+} signalling activated by polyphenolic compounds were investigated using PTX and CTX. G-proteins sensitive to these two toxins have been characterized in BAECs (Gil-Longo *et al*., 1993) and have been implicated in Ca^{2+} signalling and NO release (Ohno *et al*., 1993; Malek *et al*., 1999). The results suggest that neither PTX nor CTX affected the response produced by RWPC, suggesting that RWPC-induced Ca^{2+} increase is not mediated by PTX- and CTX-sensitive G-proteins in BAECs. These data are consistent with our previous study of RWPC-induced vasorelaxation due to endothelial NO production (Andriambeloson *et al*., 1997). The involvement of G-proteins insensitive to both PTX and CTX cannot be excluded. In contrast to RWPC, the Ca^{2+} response induced by Provinols™ was sensitive to both PTX and CTX, whereas that evoked by delphinidin was sensitive to PTX only. A similar pattern of inhibition of the Ca^{2+} response was obtained with the two toxins in Ca^{2+}-free medium for both Provinols™ and delphinidin. This suggests that G-proteins are also involved in the early signalling mechanism leading to intracellular Ca^{2+} store mobilization. It is noteworthy that RWPC, Provinols™ and delphindin which have different polyphenol content do not have the same G-protein coupling mechanism for Ca^{2+} signalling. Also, these results suggest that delphinidin is not the sole active polyphenolic compound that activates the rise in $[Ca^{2+}]_i$ in BAECs, inasmuch as it is present in small amounts in the wine extracts (~ 0.2 μg ml^{-1} delphinidin in Provinols™). The involvement of different G-proteins might help to resolve the identification of active polyphenols for endothelial NO production.

In conclusion, the present study demonstrates that natural dietary polyphenolic compounds contained in wine promote increase $[Ca^{2+}]_i$ in BAECs, and this may be an essential step for NO synthesis. The rise in $[Ca^{2+}]_i$ involves both Ca^{2+} release and Ca^{2+} entry. Finally, PLC and TK are both implicated in this Ca^{2+} signalling. However, differential mechanisms were observed for the three polyphenolic compounds with regards to the source of intracellular Ca^{2+} and the nature of G-proteins involved. One can advance the hypothesis that RWPC and Provinols™, when compared to delphinidin, might activate multiple targets in the process leading to Ca^{2+} signalling in BAECs. Elucidation of these mechanisms will provide important insights into the effects of red wine polyphenols on the synthesis and release of NO by vascular endothelium. This latter effect is important not only to maintain coronary artery patency but also to reinforce antithrombotic defenses that might contribute to the cardioprotective action of wine polyphenols.

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