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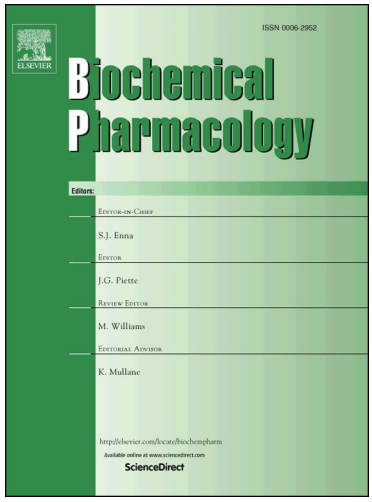

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PII: S0006-2952(19)30168-6
DOI: https://doi.org/10.1016/j.bcp.2019.05.004
Reference: BCP 13516

To appear in: *Biochemical Pharmacology*

Received Date: 4 January 2019
Accepted Date: 3 May 2019

Please cite this article as: M. Ontiveros, D. Rinaldi, M. Marder, M.V. Espelt, I. Mangialavori, M. Vigil, J.P. Rossi, M. Ferreira-Gomes, Natural flavonoids inhibit the plasma membrane Ca^{2+}-ATPase, *Biochemical Pharmacology* (2019), doi: https://doi.org/10.1016/j.bcp.2019.05.004

Natural flavonoids inhibit the plasma membrane Ca^{2+}-ATPase

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Abstract

Research on flavonoids from plant sources has recently sparked increasing interest because of their beneficial health properties. Different studies have shown that flavonoids change the intracellular Ca^{2+} homeostasis linked to alterations in the function of mitochondria, Ca^{2+} channels and Ca^{2+} pumps. These findings hint at plasma membrane Ca^{2+}-ATPase (PMCA) involvement, as it transports Ca^{2+} actively to the extracellular medium coupled to ATP hydrolysis, thus maintaining ion cellular homeostasis. The present study aims to investigate the effect of several natural flavonoids on PMCA both in isolated protein systems and in living cells, and to establish the relationship between flavonoid structure and inhibitory activity on PMCA.

Our results show that natural flavonoids inhibited purified and membranous PMCA with different effectiveness: quercetin and gossypin were the most potent and their inhibition mechanisms seem to be different, as quercetin does not prevent ATP binding whereas gossypin does. Moreover, PMCA activity was inhibited in human embryonic kidney cells which transiently overexpress PMCA, suggesting that the effects observed on isolated systems could occur in a complex structure like a living cell. In conclusion, this work reveals a novel molecular mechanism through which flavonoids inhibit PMCA, which leads to Ca^{2+} homeostasis and signaling alterations in the cell.

Keywords

Plasma membrane calcium pump, natural flavonoids, quercetin, gossypin, inhibition mechanism

1. Introduction

Flavonoids are natural compounds synthesized in plants by the phenylpropanoid pathway [1]. Due to their abundance in plants and vegetables, some flavonoids reach micromolar concentrations in human blood plasma [2]. Several studies have reported flavonoids anti-oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties, coupled with their capacity to modulate key cellular enzyme functions [3–8]. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization. The biochemical activity of flavonoids depends on their chemical structure. In particular, quercetin ((2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one), which is widely distributed in several vegetables and fruits (particularly onions, peppers, cranberries, blueberries, apples, cherries and grapes [9]), has extensive biological

activity related with the prevention of cancer, diabetes, asthma, hypertension and cardiovascular diseases [9–13]. Other flavone derivative such as gossypin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-8-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6 (hydroxymethyl)oxan-2-yl]oxychromen-4-one), which naturally occurs in various plants belonging to the family of *Malvaceae* [14], is reported to have antioxidant, anti-inflammatory, anticonvulsant and anti-cancer activity [15–18].

Intracellular calcium (Ca^{2+}) is tightly regulated. Cells display different mechanisms to maintain the low free Ca^{2+} concentrations (100-200 nM) needed to regulate its targets with optimal effectiveness. In non-excitable cells and under certain stimuli, Ca^{2+} release is induced from the endoplasmic reticulum (ER) Ca^{2+} stores, leading to a rise in intracellular Ca^{2+} which depletes the ER and induces the activation of store-operated channels (SOCs) [19]. This ion excess is removed from the cytoplasm through different mechanisms that include transport systems at the plasma membrane level (e.g. Ca^{2+}-ATPases, Na^{+}/Ca^{+} exchanger) and at certain intracellular organelle membranes (e.g. Sarco/endoplasmic reticulum Ca^{2+}-ATPase, SERCA). Evidence has shown flavonoids involvement in changes in intracellular Ca^{2+} concentration [20,21], which could be associated to their effect on Ca^{2+} transport systems [22,23].

Several studies suggest that certain flavonoids can inhibit a variety of ATPases, such as the oligomycin-sensitive mitochondrial F0/F1-ATPase, the Na^{+}/K^{+}-ATPase, the H^{+}/K^{+}-ATPase, and the Ca^{2+}-ATPases [22–27]. This inhibition may result from the interaction between flavonoids and the ATP binding sites on the protein, similarly to that observed in their interaction with kinases [28,29]. Some flavonoids have also been shown to inhibit SERCA, which could be related to the induction of apoptosis in cancer cells. Ogunbayo et al. [22] have suggested that apoptosis starts via the Ca^{2+}-dependent mitochondrial pathway, which can be activated through a sharp increase in cytosolic Ca^{2+}. This hypothesis is in agreement with the fact that certain flavonoids can induce

Ca^{2+} release from sarcoplasmic reticulum intracellular stores [30]. On the other hand, studies in HepG2 hepatoma cells show that quercetin induces a rise in intracellular Ca^{2+} which leads to autophagy [21]. In human breast cancer cells MDA-MB-231, quercetin also induces apoptosis by ER stress increasing cytosolic Ca^{2+} [20]. This evidence supports the idea that some flavonoids are related to changes in intracellular Ca^{2+} concentration and suggests that Ca^{2+} transport could be affected. However, the molecular mechanisms underlying these effects are still unclear.

The plasma membrane Ca^{2+} pump (PMCA) and SERCA are essential in the maintenance of low intracellular Ca^{2+} concentrations in most eukaryotic cells, as they use the energy obtained from ATP hydrolysis to transport Ca^{2+} from the cytoplasm to the extracellular medium or the reticulum lumen, respectively. As described above, SERCA can be inhibited by some natural flavonoids; however, the effect of these compounds on PMCA has not been established yet.

PMCA belongs to the P-type ATPase family, as it presents an acid-stable phosphorylated intermediate form during its reaction cycle. In humans, PMCAs are coded by four genes (PMCA 1–4), and additional isoforms are generated via alternative RNA splicing, which adds more isoform diversity [31]. The current kinetic model proposes that the enzyme exists in two main conformations, *E*1 and *E*2 (**Figure 1**). After the binding of intracellular Ca^{2+} to high-affinity sites, *E*1 can be phosphorylated by ATP with formation of the intermediate *E*1P, which results in occlusion of bound Ca^{2+}. After a conformational transition to *E*2P, Ca^{2+} is released to the extracellular medium from low-affinity sites, followed by the hydrolysis of the phosphoenzyme to *E*2 and a new conformational transition to *E*1 [32,33].

In this context, the present study aims to investigate the effect of different natural flavonoids on human PMCA isoform 4 (hPMCA4) activity. hPMCA4 is the most abundant in red blood cells and the most tightly regulated isoform, as well as being

ubiquitously distributed across tissues [31,34]. Thus, Ca^{2+}-ATPase activity was measured in the presence of several flavonoids selected on the basis of their correspondence to different basic structure types with varied substitutions (flavanone, flavone, flavonol, chalcone, aglycones and/or glycosides, **Table 1**), using purified hPMCA4 and sealed inside-out vesicle (IOV) systems obtained from human erythrocytes. Results show that flavonols quercetin and gossypin were the most potent inhibitors from this series of flavonoids. Moreover, our studies demonstrate that these compounds showed different mechanisms of inhibition. Additionally, the study of flavonoids effects on the activity of transiently overexpressed hPMCA4 in human embryonic kidney cells HEK293T also shows inhibition by both quercetin and gossypin.

2. Materials and Methods

2.1. Chemicals

HEK293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Lipofectamine LTX Reagent with PLUS Reagent and cell culture supplements were purchased from Invitrogen-Thermo Fisher Scientific (Carlsbad, CA, USA). Antibiotic/antimycotic solution was obtained from Life Technologies Inc. (Carlsbad, CA, USA). The fluorescent probe Fluo-4-AM was obtained from Molecular Probes (Eugene, OR, USA). PVDF blot membrane was obtained from BioRad Laboratories (Reinach, Switzerland). Anti-PMCA primary mouse monoclonal antibody 5F10 was from Thermo Fisher Scientific. Dimyristoyl phosphatidylcholine (DMPC) was from Avanti Lipids (Alabaster, AL, USA). Gossypin, flavanone and chalcone were obtained from Extrasynthese (Genay, France). Thapsigargin (TG), hesperidin, naringin, rutin, hesperetin, flavone, diosmetin, quercetin, apigenin, chrysin, 6-methylflavone, hesperidin methyl chalcone, kaempferol, galangin and hyperoside, polyoxyethylene (10) lauryl ether ($C_{12}E_{10}$) and all the other chemicals used in this work were of analytical grade and

were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human blood recently drawn at Fundación Fundosol (Buenos Aires, Argentina) was used for the isolation of PMCA. Donors provided informed consent for the donation of blood and for its subsequent legitimate use by the transfusion service.

2.2. Preparation of erythrocyte inside-out vesicles (IOVs)

IOVs were prepared from human erythrocyte plasma membrane as described previously [35]. IOVs are inverted vesicles obtained from membranes of human red blood cells. In this system, PMCA has the catalytic domains oriented toward the outside of the vesicle and maintains the native lipid environment. Isoforms present in human erythrocytes are PMCA1 and PMCA4, although hPMCA4 is the most widely expressed (80%) [34].

2.3. Purification of functional PMCA from human erythrocytes

PMCA was isolated from calmodulin CaM-depleted human erythrocyte membranes using a CaM-affinity chromatography column [36]. Briefly, membrane proteins were solubilized in a 0.5% $C_{12}E_{10}$-containing buffer. After centrifugation, the supernatant was loaded into a CaM-Sepharose column in the presence of 1 mM Ca^{2+}. The column was thoroughly washed with 0.05% $C_{12}E_{10}$-containing buffer. PMCA was eluted in 20% (w/v) glycerol, 0.005% $C_{12}E_{10}$, 120 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS-K (pH 7.4 at 4°C), 2 mM EGTA, 2 mM dithiothreitol. No phospholipids were added at any step along the purification procedure. Purified PMCA was assayed for protein concentration and homogeneity by SDS-PAGE (about 10 µg/ml, single band at Mr 134,000) and stored in liquid nitrogen until used. The purification procedure described preserves transport activity and the kinetic properties and regulatory characteristics of the enzyme in its native milieu [37].

2.4. Measurement of ATPase activity

PMCA ATPase activity in the samples was measured at 37 ºC by following the release of inorganic phosphate from ATP as previously described [38]. Briefly, hPMCA4 was resuspended in micelles containing 80 μM $C_{12}E_{10}$ and 38 μM DMPC. The determination of PMCA activity was performed at 37ºC in media containing 30 mM MOPS (pH 7.4), 120 mM KCl, 3.75 mM $MgCl_2$, 2 mM ATP, and the amount of $CaCl_2$ to give the desired final free Ca^{2+} concentration. The indicated flavonoid was added to the reaction medium at the beginning of the experimental at different concentrations (**Table1, Figure 2, Figure 3**) and the pump activity was measured. For PMCA ATPase activity measured with IOVs samples, blanks were included in an assay in the same medium without Ca2+ in the presence of 1 mM EGTA (without Ca^{2+}).

Free Ca^{2+} concentration in the incubation media was estimated using MaxChelator [39], a software for determining the free cation concentration in the presence of chelators or total metal given a desired free concentration (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/). The PMCA activity obtained at 80 μM free Ca^{2+} concentration was used as 100%.

2.5. Determination of phosphorylated intermediates

Phosphorylated intermediates (EP) were measured as the amount of acid-stable ^{32}P incorporated in the enzyme from [^{32}P] ATP after stopping the reaction with an ice-cold solution containing 10% trichloroacetic acid. The determination of EP was performed at 25ºC in media 30 mM MOPS (pH 7.4) buffer containing 120 mM KCl, 3.75 mM $MgCl_2$, 80 μM $C_{12}E_{10}$, 38 μM PC, 30 μM ATP, and the amount of $CaCl_2$ required to give the final free Ca^{2+} concentration of 80 μM. The flavonoid indicated was added to the reaction medium at the beginning of the experiment (100 μM gossypin, 25 μM quercetin; **Figure 4**). The isolation and quantification of the intermediate was performed according to the method described by Echarte et al. [40].

Flavonoids concentrations were selected to be more than 20 times the *Ki* for the PMCA (**Table 2**).

2.6. Cell culture and overexpression of hPMCA4

HEK293T cells were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution at 37°C in a 5% CO_2 atmosphere. Cells were transfected with EGFP-hPMCA4b [41] using Lipofectamine LTX with Plus Reagent as described in the manufacturer's protocol (Thermo-Fisher Scientific). HEK293T-mock cells (cells transfected with the empty vector) were cultured in parallel as control of expression and activity. hPMCA4 overexpression was verified by Western blot.

2.7. Kinetics of Ca^{2+} transport in HEK293T cells

HEK293T cells were seeded on sterile 96-well black-walled, clear-bottom plates (Corning Inc., Corning, NY, USA) at a density of 1.5 x 10^4 cells/well. After 18 h, cells were transfected with mock or EGFP-hPMCA4b plasmid, and Ca^{2+} transport was assayed 18 h after transfection using Fluo4-AM. Cells were loaded for 1 h at 37°C with 5 μM of the fluorophore in reaction buffer (RB) composed of 10 mM Hepes (pH 7.4), 120 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$ and 5 mM D-glucose. This buffer contained choline chloride instead of NaCl to minimize the effect of the Ca^{2+}/Na^+ exchanger. After dye loading, the plates were washed twice and 100 μl of RB was added to the samples. Cells were then incubated with 25 μM quercetin, 50 μM gossypin, DMSO (vehicle) or RB during 30 min. The flavonoids concentrations used were the ones at which cells maintained their adherence and shape. Fluorescence was measured at λ excitation 485±20 nm (λ emission: 528±20 nm) in a fluorometric plate reader (Synergy HT. Biotek, BioTek Instruments Inc., Winooski, VT, USA). Fluorescence signals were analyzed using software Gen 5.2.01. Stable baseline values were established for at least 3 min, and then 15 μl of RB with 1 μM TG was added.

Therefore, the fluorescence was recorded for at least 4 min, and then 15 µl of RB with 1 mM $CaCl_2$ was added. TG specifically inhibits the SERCA, which induces depletion of ER Ca^{2+} stores and the activation of SOCs [42]. Traces showing the time course of fluorescence intensity were normalized to the baseline and expressed as relative cytosolic Ca^{2+} ($[Ca^{2+}]_{CYT}$). The area under curve (AUC) is the integral of the rise in $[Ca^{2+}]_{CYT}$ from the moment of compound addition (TG or Ca^{2+}) until basal levels are reached (TG) or until the end of Ca^{2+} measurements. Activity of hPMCA4 was calculated using the AUCs and equation 1, as described by Dalghi et al. [43].

$$PMCA4\ activity = \frac{(AUC_{mock,treatment} - AUC_{PMCA4,treatment})}{(AUC_{mock,veh} - AUC_{PMCA4,veh})} \qquad \text{Eq. 1}$$

Flavonoids fluorescence did not interfere with FLUO4 fluorescence (data not shown).

2.8. Detection of PMCA expression in HEK293T cells

Cells were lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors (10 µg/ml aprotinin and 4 µg/ml leupeptin). Cells were then centrifuged at 5000 g during 15 min and the supernatant was collected. Protein content in the supernatant was determined using the Bradford protein assay [44]. Samples were separated using a 10% SDS gel (50 µg protein per lane in sample buffer containing 150 mM Tris-HCl (pH 6.5 at 14°C), 5% SDS, 5% DTT, 10% glycerol, and bromophenol blue), and proteins were transferred to a PVDF blot membrane (BioRad). After blocking, the blot was incubated with the anti-PMCA primary mouse monoclonal antibody 5F10 [45] (1:2000 dilution; Pierce) overnight at 4°C. Anti-mouse IgG, horseradish peroxidase-linked whole antibody (1:20000; Amersham, Buckinghamshire, UK) was used as the secondary antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence method (Kalium Technologies, Buenos Aires, Argentina). Digital images were obtained and quantified using the

ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life Sciences, Marlborough, MA, USA).

2.9. Detection of flavonoids in HEK293T cells

Cells cultured on coverslips were incubated with DMSO, 25 μM quercetin or 50 μM gossypin for 30 min in RB at 37°C. Cells were then fixed with 2% w/v formaldehyde in PBS for 10 min and nuclei were stained with 1 μM Hoechst. Coverslips were mounted with fluorescence mounting medium (Dako, Santa Clara, CA, USA) on glass slides and observed on a FluoView 1000 confocal microscope (Olympus, Tokyo, Japan). Flavonoid fluorescence was observed with excitation at 490 nm and emission at 510 nm.

2.10. Data analysis

Theoretical equations were fitted to experimental data by nonlinear regression based on the Gauss-Newton algorithm and using the commercial software Excel and Sigma-Plot 12.0 for Windows.

Equation 2 was used when the experimental data from Ca^{2+}-ATPase activity vs $[Ca^{2+}]$ (section 3.2) were described by the rectangular hyperbola

$$v = \frac{Vmax\,[Ca^{2+}]}{K_{0.5} + [Ca^{2+}]} \qquad \text{Eq. 2}$$

where *Vmax* is the Ca^{2+}-ATPase activity when the Ca^{2+} concentration ($[Ca^{2+}]$) tends to infinity, and $K_{0.5}$ represents the $[Ca^{2+}]$ at which the half-maximum effect is achieved.

One-way analysis of variance (ANOVA) followed by Bonferroni post-test was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software (San Diego, CA, USA). A probability (P) value < 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of PMCA Ca^{2+}-ATPase activity by natural flavonoids

The effects of different natural flavonoids on Ca^{2+}-ATPase activity were evaluated using two *in vitro* models: IOVs and purified hPMCA4.

Table 1 summarizes the Ca^{2+}-ATPase activity obtained in the presence of each flavonoid at 100 μM for purified hPMCA4 and IOV systems. The activity is expressed as the percentage of the value measured in the absence of flavonoids (presence of DMSO). Flavonoids showing less than 25% inhibition of activity were considered inactive. Results show that base structures (chalcone, flavone and flavanone) and all flavanones failed to inhibit PMCA activity. For flavones, only apigenin and diosmetin showed slight inhibition. In contrast, all three flavonol aglycones tested were able to inhibit PMCA, quercetin being the most potent, and gossypin being the most active flavonol glycoside. In addition, some flavonoids inhibitory effects were found to be higher on purified hPMCA4 than on IOVs, as can be seen for apigenin, diosmetin, quercetin, kaempferol and gossypin. Hyperoside, however, was only effective on purified hPMCA4.

Figure 2 shows the purified PMCA activity measured at different concentrations of the active flavonoids. The best fitting values of the apparent constant of inhibition (K_i) are shown in **Table 2**.

Results indicate that, in general, an increased number of -OH substitutions in the flavone nucleus led to an increase in the inhibitory effect. Consequently, flavonols resulted more potent than flavones. Thus, apigenin and diosmetin inhibited hPMCA4 with very low affinity, which could be related to the absence of the -OH in R3 position. Furthermore, gossypin and hyperoside have -OH in position R3' and R4', like quercetin, but their affinities were lower, which could be caused by the glycosides present in their structure. Moreover, the position of the glycoside could generate differences in the inhibition related to steric impediments at the interaction site.

As quercetin and gossypin proved to be the most active PMCA inhibitors, the subsequent studies were focused on these two flavonols.

3.2. Inhibition of PMCA by quercetin and gossypin regarding Ca^{2+}concentrations

To evaluate the type of inhibition exerted by these flavonoids on hPMCA4 regarding Ca^{2+}, the purified PMCA activity was measured as a function of Ca^{2+} concentration in the presence of different concentrations of quercetin or gossypin (**Figure 3A and D**, respectively). In both cases, experimental data were described by a rectangular hyperbola (Eq. 2). The best fitting values of $K_{0.5}$ for Ca^{2+} and *Vmax* were plotted as a function of quercetin (**Figure 3B and C**, respectively) or gossypin (**Figure 3E and F**, respectively).

For quercetin, results show that $K_{0.5}$ for Ca^{2+} was not significantly modified (**Figure 3B**) and *Vmax* decreased (**Figure 3C**), indicating that quercetin is a non-competitive inhibitor with respect to Ca^{2+}. For gossypin, both $K_{0.5}$ for Ca^{2+} and *Vmax* decreased as a function of gossypin (**Figure 3E** and **F,** respectively), while their relationship did not change with flavonoid concentration (***inset*** **Figure 3F**). These results suggest that gossypin is an uncompetitive inhibitor type with respect to Ca^{2+}.

3.3. Effect of quercetin and gossypin on steady state levels of phosphorylated intermediates

In order to obtain valuable information of ATP interaction with PMCA, we evaluated the effect of quercetin and gossypin on *EP*. The steady state levels of EP were measured during 30 s at 25°C in the absence (control) or in the presence of 25 μM quercetin, 100 μM gossypin or 500 μM $LaCl_3$. EP level obtained for control was established as 100%. La^{III} inhibits PMCA activity producing accumulation of *E*1P [46,47]. Current results show that, in presence of quercetin, hPMCA4 was phosphorylated at the same level as in the presence of La^{III} in conditions where the inhibition of PMCA activity was maximum. In contrast, gossypin caused a decrease in PMCA phosphorylation (**Figure 4**).

3.4. Effect of quercetin and gossypin on Ca^{2+} efflux mediated by hPMCA4 in HeK293T cells

To assess whether the effects of quercetin or gossypin observed on the purified hPMCA4 could occur in a living cell, the dynamics of cytoplasmic Ca^{2+} ($[Ca^{2+}]_{CYT}$) were studied in HEK293T cells overexpressing hPMCA4 according to the method previously described [43,48]. The dynamics of $[Ca^{2+}]_{CYT}$ were examined by studying the alterations in $[Ca^{2+}]_{CYT}$ generated by Ca^{2+} release from the ER, and by extracellular Ca^{2+} entry through SOCs. A typical time course experiment was conducted which comprised two phases: (i) a first phase involving the transient elevation in $[Ca^{2+}]_{CYT}$ generated by the addition of 1 μM TG, a SERCA inhibitor that causes Ca^{2+} release from the ER, and (ii) a second phase subsequent to the addition of 1 mM Ca^{2+} to the external medium, which induces an increase in $[Ca^{2+}]_{CYT}$ indicative of SOC activity (**Figure 5A**).

For each phase, the effect on the dynamics of Ca^{2+} was quantified as the AUC representing the overall $[Ca^{2+}]_{CYT}$ of each phase, so that a more effective mechanism of Ca^{2+} extrusion will be reflected in an overall AUC decrease. The AUC value obtained for HEK293T-mock cells was established as 100%. By performing ANOVA analysis on these data, we evaluated whether hPMCA4 overexpression affected the AUC compared to HEK293T-mock cells. Results show that AUC values for both phases were lower when cells overexpressed hPMCA4 (**Figure 5B and C**), suggesting that the Ca^{2+} extrusion mechanism was more effective in HEK293T-hPMCA4 cells. hPMCA4 overexpression was corroborated by western blot (**Figure 5D**).

To evaluate the effect of quercetin and gossypin on the dynamics of $[Ca^{2+}]_{CYT}$, HEK293T-mock and HEK293T-hPMCA4 cells were treated with 25 μM quercetin, 50 μM gossypin or vehicle for 30 minutes. The AUC values obtained for both phases were evaluated for HEK293T-mock and HEK293T-hPMCA4 cells in the presence of the different treatments. The AUC value obtained for HEK293T-mock cells treated with vehicle was established as 100%. By performing ANOVA on these data, we evaluated whether treatments affected the behavior of HEK293T-mock and HEK293T-hPMCA4

cells. Treatments revealed no significant effect on HEK293T-mock cells, which indicates that, in these conditions, Ca^{2+} release from the ER, Ca^{2+} entry through SOCs and Ca^{2+} removal from the cytosol remained unaltered. However, results obtained with HEK293T-hPMCA4 cells show that quercetin and gossypin treatments induced an increase in AUC values vs. vehicle treatment, suggesting that Ca^{2+} release from the ER or the cytosol was less effective in flavonoid-treated HEK293T-hPMCA4 cells (**Figure 6**). In order to evaluate to what extent this effect was due to hPMCA4, PMCA activity was obtained using equation 1 for both phases (**Table 3**). For the first phase, the control value for hPMCA4 activity was 100 ± 10%, whereas the values obtained for the treatment with quercetin and gossypin were 34 ± 14% and 10 ± 7%, respectively; however, Ca^{2+} extrusion mediated by hPMCA4 decreased ~66% and ~90%.

For the second phase, the control value for hPMCA4 activity was 100 ± 2%, whereas the values obtained for the treatment with quercetin and gossypin were 63 ± 2% and 34 ± 3%, respectively; however, Ca^{2+}extrusion mediated by hPMCA4 decreased ~37% and ~66%.

Finally, flavonoid fluorescence was assessed to determine the incorporation of these flavonoids in HEK293T cells. When cells were treated with 25 μM quercetin or 50 μM gossypin for 30 minutes, green fluorescence was observed inside the cells under the confocal scanning microscope, indicating that the two flavonoids were incorporated into HEK293T cells. In contrast, cells incubated with DMSO (vehicle control) showed no intracellular fluorescence (**Figure 7**). Images show that quercetin was widely distributed in the cytoplasm and nucleus, whereas gossypin was accumulated mainly around the nucleus.

4. Discussion

The present study reveals an inhibitory effect of natural flavonoids on the human isoform PMCA4 in different system levels: vesicles, isolated protein and living cells. Our results indicate that hPMCA4 activity is inhibited by some natural flavonoids both in IOVs and isolated protein. Apigenin, diosmetin, quercetin, kaempferol and gossypin showed an inhibitory effect on purified hPMCA4 which was higher than on IOVs, while hyperoside was only effective on purified hPMCA4 (**Table 1**). The current findings also show that a double bond between C2-C3 in the structure is key to flavonoid activity. Thus, chalcones and flavanones were not active, as they lack the double bond. Type, number and position of the substituents in the flavone nucleus are also determinants of activity. Consequently, hydroxylation in positions R5 and R7 of the A ring induced low inhibition (apigenin and diosmetin). Also, hydroxylation in R3 of the C ring had a moderate effect on activity (galangin vs chrysin, apigenin vs chrysin), while hydroxylation in positions R3' and R4' of the B ring significantly increased inhibition (quercetin and gossypin). On the other hand, glycosylation in position R7 (rutin and hyperoside) prevented inhibition in IOVs. These findings are in agreement with previous studies related to the effects of flavonoids on H^+,K^+-ATPase and Na^+,K^+-ATPase [11]. Conversely, studies on SERCA have revealed that the inhibitory activity of a flavonoid is not affected by hydroxylation in the B ring but does depend on hydroxylation in the A and C rings [9].

Quercetin and gossypin were the most potent inhibitors, as they inhibited the purified hPMCA4 with high apparent affinity (*K*i of 0.34 ± 0.01 and 4.8 ± 0.2 μM, respectively; **Table 2**). Both flavonoids have hydroxylation in R3' and R4' positions of the B ring; however, gossypin has a sugar in position 8' of the ring A, which could generate differences in inhibition related to steric impediments at the interaction site, causing a higher *K*i.

When Ca^{2+}-ATPase activity was measured as a function of quercetin (**Figure 2A and B**) or gossypin (**Figure 2C**) concentration, gossypin showed a hyperbolic inhibition curve, whereas quercetin exhibited a sigmoidal-shaped inhibition curve with Hill coefficient>1. This result indicates that quercetin inhibition was cooperative and suggests that hPMCA4 could have more than one site for quercetin binding. This behavior has been reported previously for other P-ATPases [23].

In turn, studies on flavonoids inhibitory effects regarding Ca^{2+} concentrations revealed that quercetin behaves as a non-competitive inhibitor for Ca^{2+} –that is, $K_{0.5}$ for Ca^{2+} unaltered and V_{max} reduced –, which suggests that quercetin binds to PMCA somewhere other than the Ca^{2+} site (**Figure 3A, B and C**). Also, this flavonoid induced an increase in phosphorylated intermediates, indicating that quercetin may interact with phosphorylated conformations, stabilize them, and prevent hPMCA4 dephosphorylation (**Figure 4**). Quercetin could interact with hPMCA4 through the nucleotide binding site after PMCA is phosphorylated by ATP. This assumption is in line with the fact that the phosphorylated intermediates –*E*1P and *E*2P– have nucleotide binding sites for ADP and ATP, respectively [49]. A further possibility is that the interaction occurs through a different site.

On the other hand, our results demonstrate that gossypin behaves as a non-competitive inhibitor for Ca^{2+} (**Figure 3D, E and F;** $K_{0.5}$ for Ca^{2+} and *Vmax* reduced and their relationship unaltered by flavonoid concentration), which suggests that gossypin may bind to the *E*1Ca conformation. This result is consistent with the fact that gossypin reduced the amount of phosphorylated intermediates by interfering with the binding of ATP (**Figure 4**). Therefore, we propose that gossypin binds to the *E*1Ca conformation through an interaction with the ATP binding site. Taken together, these results suggest that quercetin and gossypin inhibit hPMCA4 through different mechanisms, probably due to their structural differences. These findings are consistent with previous reports

showing flavonoids to interact with ATP binding sites [23,50]. However, the possibility that quercetin might interact with another site of the pump cannot be ruled out. Furthermore, identifying the generic modes of action with other target enzymes may help understand the molecular mechanism by which flavonoids inhibit the PMCA. Therefore, future studies should aim to elucidate the kinetic/structural mechanism by which flavonoids inhibit the PMCA.

In order to evaluate whether the flavonoid effects observed in isolated systems could occur in living cells, we studied the PMCA activity by measuring the dynamics of $[Ca^{2+}]_{CYT}$ in HEK293T cells. When Ca^{2+} penetrates the cytoplasm, either from intracellular stores or from the extracellular medium, there is an increase of cytoplasmic Ca^{2+} which is removed by the Na^+/Ca^{2+} exchanger and the PMCA, exported into the ER through the SERCA and into the mitochondria, and chelated by cytosolic proteins [51]. All these events allow to reestablish stationary $[Ca^{2+}]_{CYT}$ levels after a stimulus. In our model, we needed to enhance the role of PMCA in Ca^{2+} extrusion, thus SERCA activity was inhibited using TG, and the Ca^{2+}/Na^+ exchanger activity was minimized avoiding the presence of Na^+ in the extracellular media. However, under these experimental conditions, other mechanisms can produce Ca^{2+} extrusion besides PMCA. Since there are no specific inhibitors for the pump, we decided to overexpress hPMCA4 in the HEK293T cell line (HEK293T-hPMCA4 cells). In this case, results show that Ca^{2+} extrusion was more effective in HEK293T-hPMCA4 cells (**Figure 5**), which agrees with the presence of more enzyme units in the cells. These findings were previously described for the PMCA2 isoform [43].

Using this system to evaluate flavonoid effects on hPMCA4, we observed that quercetin and gossypin treatments showed no effect on the dynamics of $[Ca^{2+}]_{CYT}$ in HEK293T-mock cells, which indicates that the processes of Ca^{2+} release from the ER promoted by TG, Ca^{2+} entry through SOCs and Ca^{2+} removal from the cytosol remain unaffected in

these conditions (**Figure 6**). These findings in turn suggest that the endogenous transport systems involved in these processes were not affected. In HEK293T cells, endogenous PMCA is mainly PMCA1 isoform [52], which may mean that flavonoids do not inhibit this particular isoform. On the other hand, we observed that quercetin and gossypin treatments showed a significant inhibitory effect on the dynamics of $[Ca^{2+}]_{CYT}$ in HEK293T-hPMCA4 cells, indicating that Ca^{2+} removal from the cytosol was inhibited when hPMCA4 was overexpressed (>AUC values, **Figure 6**). In addition, studies on the extent to which these effects were due to hPMCA4 suggest that quercetin and gossypin treatments induced the inhibition of hPMCA4 activity in these experimental conditions (**Table 3**).

According to the results obtained with purified hPMCA4, the interaction of quercetin and gossypin with PMCA could occur in cytoplasmic domains (e.g. ATP binding site), which hints at flavonoids ability to cross the plasma membrane. Confocal microscopy assays performed on gossypin and quercetin capability to enter the cell revealed that quercetin was widely distributed in the cytoplasm and nucleus, whereas gossypin was accumulated mainly in the cytoplasmic-perinuclear region of the cell (**Figure 7**). Consequently, these flavonoids may be thought to interact with cytoplasmic domains of PMCA in the cells. In brief, the inhibitory effect of quercetin and gossypin observed with purified hPMCA4 could be comparable to that occurring in living cells. However, and considering the complexity of cellular systems, other underlying mechanisms cannot be ruled out.

Some reports suggest that quercetin inhibits cell migration [53,54], which is an essential cellular process involved in a variety of biological events such as embryonic development, angiogenesis, inflammation and in pathological conditions such as cancer metastasis [55]. This effect could occur at least in part by perturbing microtubule and

actin polymerization [54–56]. These cytoskeleton alterations could be affected by PMCA activity.

Our group previously reported a regulatory mechanism of PMCA activity by the actin cytoskeleton, showing that actin differentially regulates PMCA activity depending on its polymerization state: short actin oligomers increase PMCA activity while F-actin inhibits it [43,57,58]. Therefore, quercetin interacts with actin and induces depolymerization, which could be activated by PMCA although the inhibitory effect observed in the current study is more important. On the other hand, it has been shown that acetylated tubulin can bind and inhibit PMCA activity [59]; however, the effect of changes in microtubule dynamics on PMCA have not been addressed yet. Since quercetin inhibits microtubule polymerization, the tubulin pool may increase in the cell so that tubulin forms a complex with the pump and inhibits it. Our results in cells show that quercetin inhibited PMCA activity, an effect which might involve alterations in microtubule functionality.

In conclusion, this work reveals a novel inhibition mechanism through which flavonoids affect PMCA activity, which may occur in living cell systems. Our results indicate that some natural flavonoids inhibit purified hPMCA4 with different potency, which could be mainly due to the presence of -OH groups in R3' and R4' position of the B ring. Quercetin and gossypin were the most potent inhibitors, although their inhibition mechanisms seemed to be different; in the concentration range studied, quercetin did not prevent ATP binding whereas gossypin did. However, PMCA inhibition by quercetin may be independent of the ATP binding site. Moreover, both flavonoids were incorporated into the cells and PMCA activity was inhibited, suggesting that the effects observed on isolated systems may occur in a more complex structure like a whole cell.

Further studies are required to understand Ca^{2+} homeostasis alterations involving natural flavonoids in malignant cells and their relationship with PMCA. The

characterization of these mechanisms will allow us to develop new drugs for the prevention and/or treatment of diseases like cancer.

Acknowledgements

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica PICT 2015 #0067 and PICT 2014 #2014, Consejo Nacional de Investigaciones Científicas y Técnicas PIP 11220150100250CO and Universidad de Buenos Aires Ciencia y Técnica Grant 2014-2017: 20020130100254B and 2016-2018: 20020150100012BA (Argentina). Authors are grateful to Dr Osvaldo Rey for the generous gift of HEK293T cells and Qori Ontiveros for his kind help with the drawing of flavonoid structures (Table 1) and graphical abstract.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

$C_{12}E_{10}$: polyoxyethylene 10 lauryl ether; CaM: calmodulin; DMPC: dimyristoyl phosphatidylcholine; DMSO: dimethylsulfoxide; DTT: dithiothreitol; EGTA: ethylene glycol tetraacetic acid; IOVs: erythrocyte inside-out vesicles; ER: endoplasmic reticulum; MOPS: 3-(N-morpholino)propanesulfonic acid; DMPC: dimyristoyl phosphatidylcholine; PMCA: plasma membrane Ca^{2+} pump; hPMCA4: human plasma membrane Ca^{2+} pump 4 isoform; RB: reaction buffer; SERCA: sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SOCs: store-operated Ca^{2+} channels; TG: thapsigargin; AUC: area under the curve.

5. References

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Table 1. Effect of flavonoids on Ca^{2+}-ATPase activity in IOVs and purified PMCA. The table describes the name, classification and structure of flavonoids evaluated. Also, it shows the values of Ca^{2+}-ATPase activity obtained in the presence of 100 μM of each flavonoid for IOVs and PMCA purified. Activity measurements were carried out at 37°C in a reaction medium containing 50 μg/ml vesicles (IOVs) or 0.8 μg/ml purified PMCA and 100 μM free Ca^{2+} concentration. PMCA activity was related to the activity in the presence of DMSO (100%). Results are expressed as the mean ± S.E. (n≥3).

Table 2. *K*i values of the most active flavonoids.

Table 3. Effect of quercetin and gossypin on hPMCA4 activity in HEK293T cells. Activity values were obtained from data from Figure 6 as described in Materials and Methods.

FIGURE LEGENDS

Figure 1. Simplified kinetic model of the PMCA.

Figure 2. Effect of flavonoid concentration on PMCA Ca^{2+}-ATPase activity. Ca^{2+}-ATPase activity was measured using purified PMCA in the presence of increasing amounts of flavonols (A and B), flavonol glucosydes (C) and flavones (D). Measurements were carried out at 37°C in a reaction medium containing 0.8 µg/ml PMCA reconstituted in DMPC/$C_{12}E_{10}$ mixed-micelles (10 µg/ml and 70 µg/ml, respectively), 3.75 mM $MgCl_2$, 2 mM ATP and the $CaCl_2$ required to give concentrations of 100 µM $[Ca^{2+}]_{free}$. Experimental data were related to PMCA activity measured in the presence of DMSO. Data are expressed as the mean ± S.E. of 3 independent replicates in each experiment. Continuous lines in panels represent a hyperbolic decreasing function and discontinuous lines represent a Hill function.

Figure 3. Effect of Ca^{2+} on flavonoid inhibition of PMCA Ca^{2+}-ATPase activity. Ca^{2+}-ATPase activity was measured at different $[Ca^{2+}]$ in the presence of different concentrations of quercetin (A) and gossypin (D), phosphatidylcholine, and the amount of $CaCl_2$ required to give the desired final $[Ca^{2+}]_{free}$. In panel A, quercetin concentrations were: 0 (●); 0.17 (○); 0.25 (◆); 0.31 (△); 0.38 (■) and 0.42 (▽) µM. In panel D, gossypin concentrations were: 0 (●), 1 (○), 3 (◆), 5 (△), and 10 (■) µM. Measurements were carried out in a reaction medium containing 0.8 µg/ml PMCA, 120 mM KCl, 30 mM MOPS-K (pH 7.4 at 37 ºC), 3.75 mM $MgCl_2$, 70 µg/ml $C_{12}E_{10}$, 10 µg/ml. Data are expressed as the mean ± S.E. of 3 independent replicates in each experiment. Continuous lines represent rectangular hyperbolas. Best fitting values of $K_{0.5}$ and *Vmax* were obtained from A and D. $K_{0.5}$ values were plotted as a function of quercetin (B) and

gossypin (E). *Vmax* values were plotted as a function of quercetin (C) and gossypin (F). Inset in F: $K_{0.5}/Vmax$ ratio as a function of gossypin concentration.

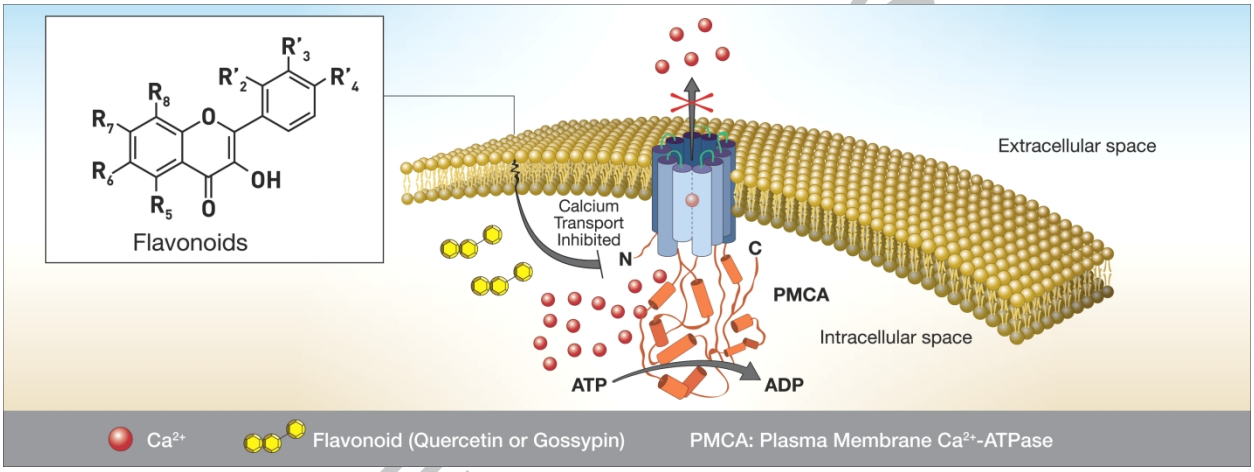
Figure 4. Quantification of phosphoenzyme in the presence of quercetin and gossypin. PMCA phosphorylation measurements were carried out in steady state at 25°C in a reaction medium containing 1µg/ml PMCA reconstituted in DMPC/$C_{12}E_{10}$ mixed-micelles (10 µg/ml and 70 µg/ml, respectively), 3,75 mM $MgCl_2$, 30 µM ATP, the $CaCl_2$ required to give 100 µM of free Ca^{2+} in the absence (control) or in the presence of 500 µM La^{III}, 25 µM quercetin (QT) or 100 µM gossypin (Gos) during 30 seconds. The EP level obtained for control was established as 100%. EP levels significantly different from controls (** p> 0.01; *** p> 0.001, one-way ANOVA with Bonferroni post-tests).

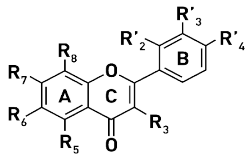
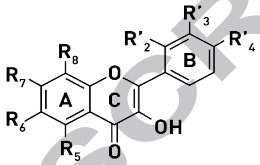
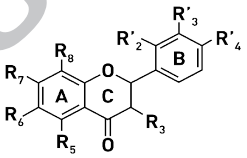
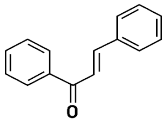
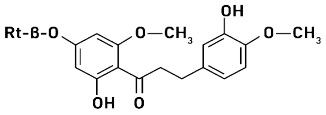
Figure 5. Dynamics of cytosolic Ca^{2+} in HEK293T cells. Pattern of Ca^{2+} transients in HEK293T cells induced by TG and SOCs. (A) Representative traces of the time course of $[Ca^{2+}]_{CYT}$ in HEK293T cells transiently overexpressing hPMCA4 or transfected with empty vector (control) (18 h post-transfection) upon addition of 1 µM TG in nominal free Ca^{2+} medium and after adding Ca^{2+} (1 mM) to the extracellular solution. The curves are representative of three independent experiments. B. Area under the curve of the $[Ca^{2+}]_{CYT}$ signal after TG (1 µM) addition (first phase). C) Area under the curve of the $[Ca^{2+}]_{CYT}$ signal after addition of Ca^{2+} (1 mM) to the extracellular solution (second phase). Significant differences from controls (*p<0.05 and ****p<0.0001, one-way ANOVA). AUC were calculated as indicated in Materials and Methods. (D) Western blot analysis was performed with whole-cell lysates using the 5F10 antibody.

Figure 6. Effect of quercetin and gossypin on hPMCA4. Areas under the curve of the $[Ca^{2+}]_{CYT}$ signal were obtained from similar experiments to **Figure 5** where cells were incubated at 37°C in the presence of DMSO (vehicle, Veh), (A and B) 25 µM quercetin (QT) or (C and D) 50 µM gossypin (Gos) during 30 minutes. AUC of the

$[Ca^{2+}]_{CYT}$ after addition of (A and C) TG (first phase), and (B and D) Ca^{2+} (second phase). AUC were calculated as indicated in Materials and Methods. Significant differences from controls (*p<0.05, **p<0.01, ***p<0.001, one-way ANOVA). Data were obtained from three independent experiments performed in n=6.

Figure 7. Intrinsic fluorescence of quercetin or gossypin. HEK293T cells were treated for 30 min with 25 μM quercetin, 50 μM gossypin or DMSO (control) according to Materials and Methods. Fluorescence was observed in a confocal microscope.



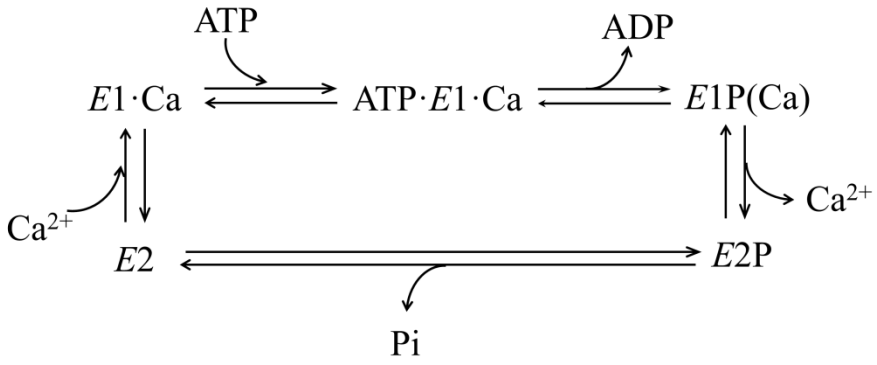
Flavonoid	Compound	Structure	R3	R5	R6	R7
Flavones	Flavone		H	H	H	H
	6-methylflavone		H	H	CH_3	H
	Chrysin (5,7-Dihydroxyflavone)		H	OH	H	OH
	Apigenin (4′,5,7-Trihydroxyflavone)		H	OH	H	OH
	Diosmetin (3',5,7-Trihydroxy-4′-methoxyflavone)		H	OH	H	OH
Flavonols	Galangin (3,5,7-Trihydroxyflavone)		OH	OH	H	OH
	Kaempherol (3,4′,5,7-Tetrahydroxyflavone)		OH	OH	H	OH
	Quercetin (3,3′,4′,5,7-Pentahydroxyflavone)		OH	OH	H	OH
	Rutin (3,3′,4′,5,7-Pentahydroxyflavone 3-rutinoside)		O-β-Rt	OH	H	OH
	Hyperoside (3,3′,4′,5,7-Pentahydroxyflavone 3-D-galactoside)		O-β-Gal	OH	H	OH
	Gossypin (3,3′,4′,5,7,8-Hexahydroxyflavone 8-glucoside)		OH	OH	H	OH
Flavanones	Flavanone		H	H	H	H
	Hesperetin (3′,5,7-Trihydroxy-4′-methoxyflavanone)		H	OH	H	OH
	Hesperidin (3′,5,7-Trihydroxy-4′-methoxyflavanone 7-rutinoside)		H	OH	H	O-β-R
	Naringin (4′,5,7-Trihydroxyflavanone 7-rhamnoglucoside)		H	OH	H	O-β-N
Chalcone	Chalcone					
	Hesperidin methyl chalcone					

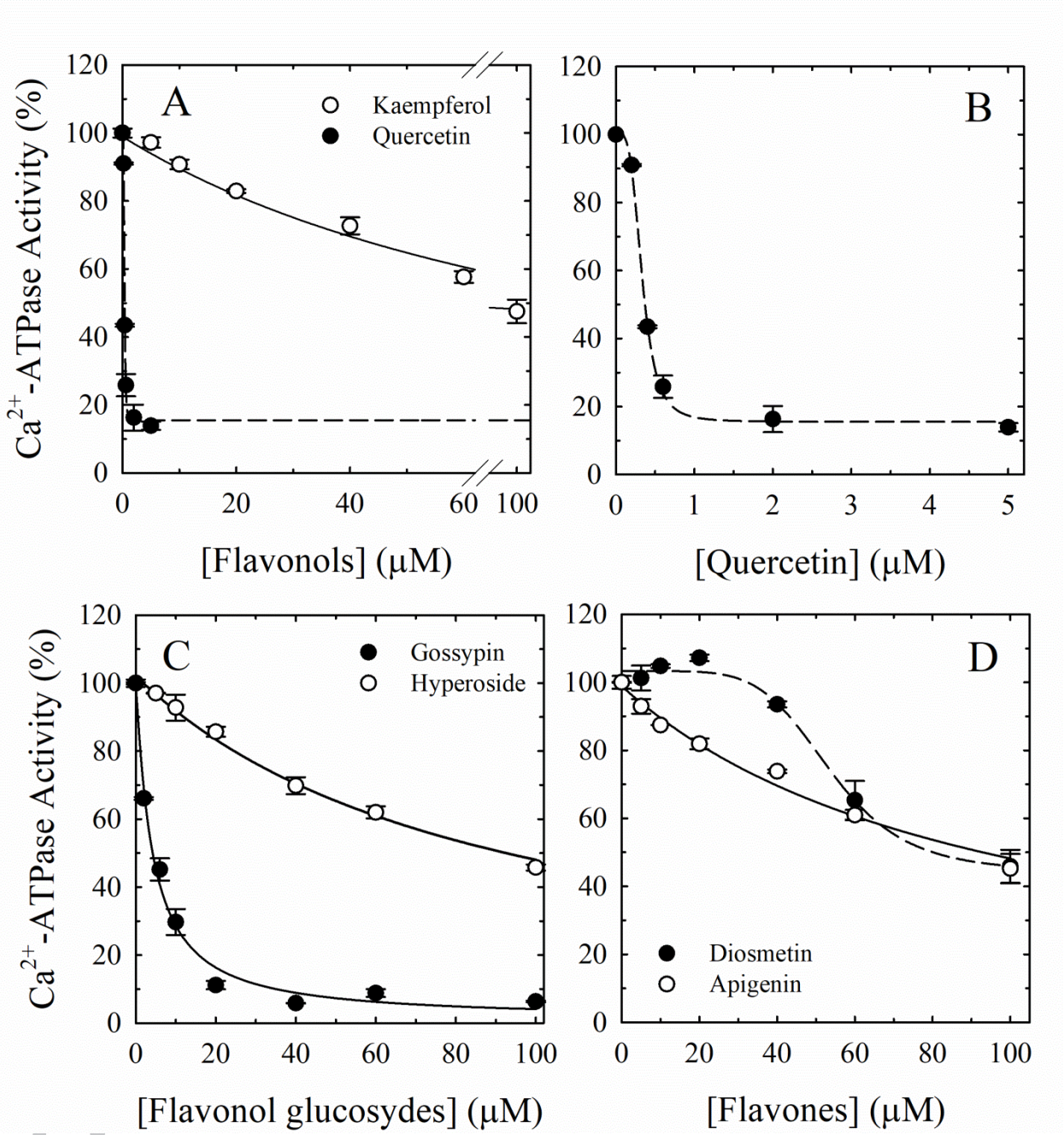
* Ca^{2+}-ATPase activity at 100 μM of flavonoid

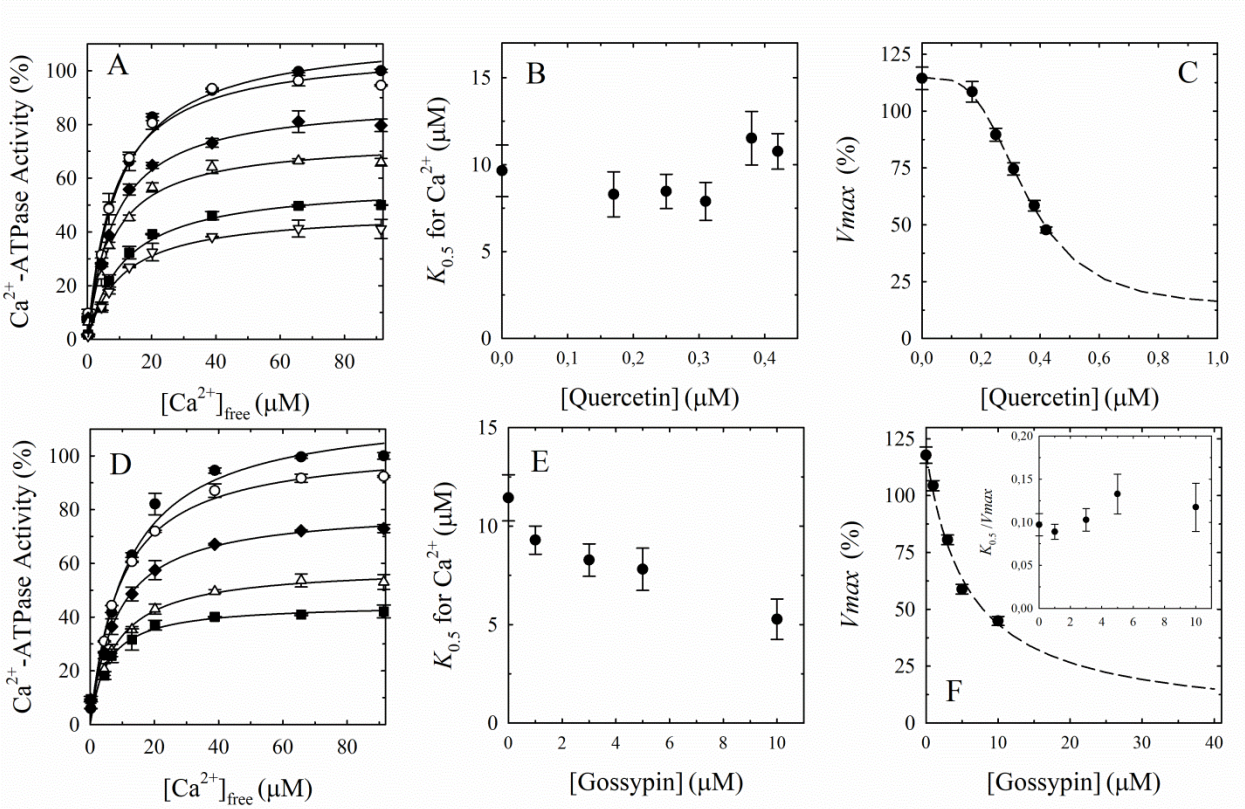
Sugar moieties: glucose (Glc), neohesperidose (O-L Rhamnosyl-(1/2)-glucose, Nh), rutinose (OL Rhamnosyl-(1/6)-glucose, Rt), galactose (Gal)

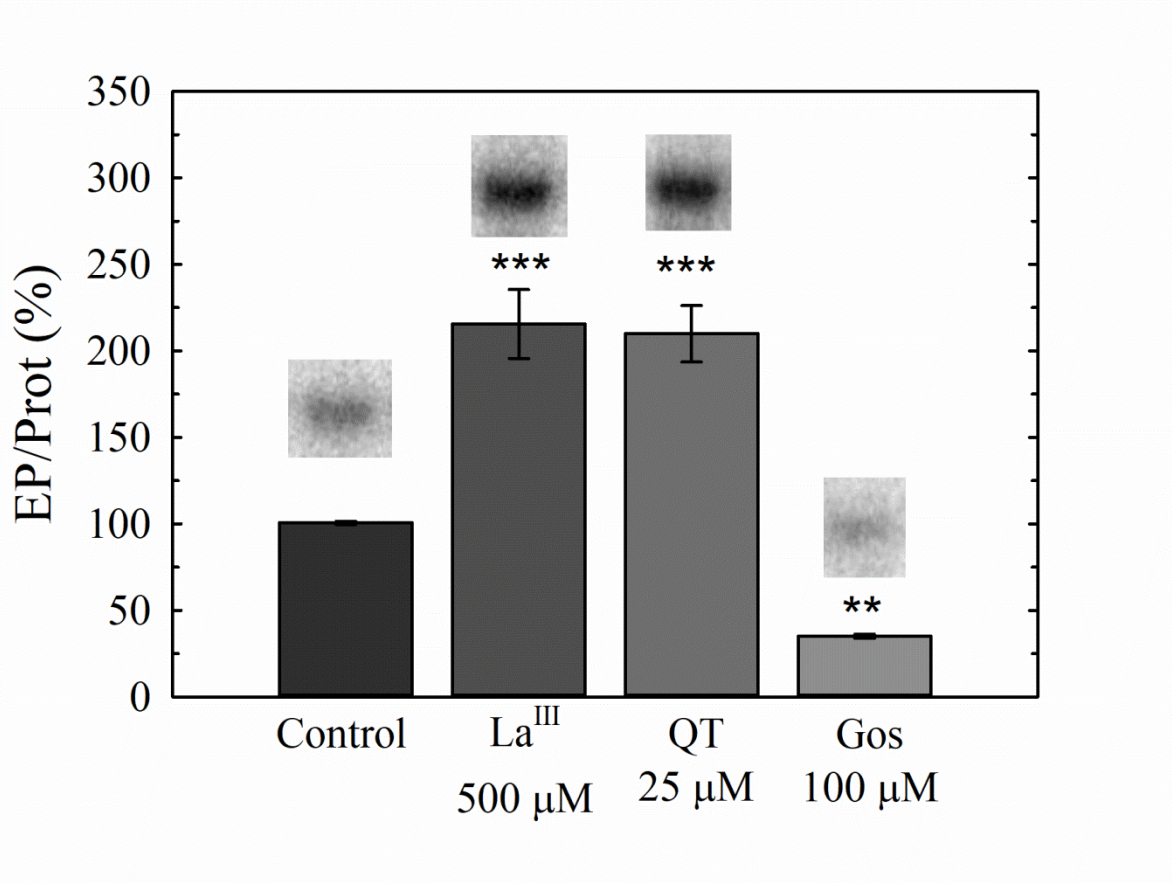
Treatment	hPMCA4 activity (mean ± S.E.M. in % of control)	
	First phase	Second phase
Vehicle (0.1% DMSO)	100	100
Quercetin 25 μM, 30 min	34 ± 14	63 ± 2
Gossypin 50 μM, 30 min	10 ± 7	34 ± 3

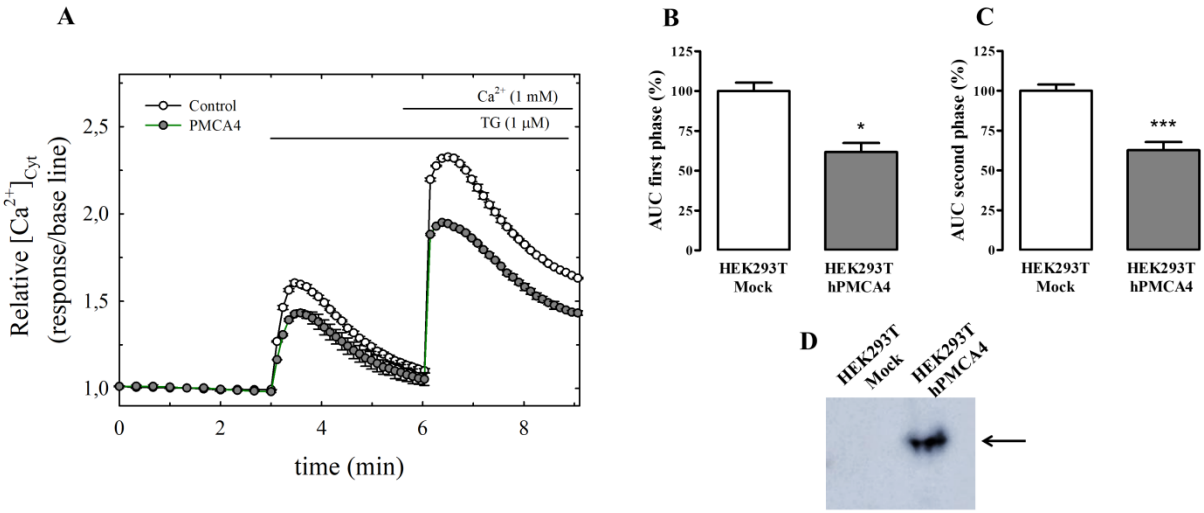
Flavonoid	K_i (μM)
Apigenin (4′,5,7-Trihydroxyflavone)	94.8 ± 7.9
Hyperoside (3,3′,4′,5,7-Pentahydroxyflavone 3-D-galactoside)	88.7 ± 5.1
Kaempferol (3,4′,5,7-Tetrahydroxyflavone)	88.5 ± 5.4
Diosmetin (3’,5,7-Trihydroxy-4′-methoxyflavone)	54.0 ± 2.9
Gossypin (3,3′,4′,5,7,8-Hexahydroxyflavone 8-glucoside)	4.8 ± 0.2
Quercetin (3,3′,4′,5,7-Pentahydroxyflavone)	0.34 ± 0.01

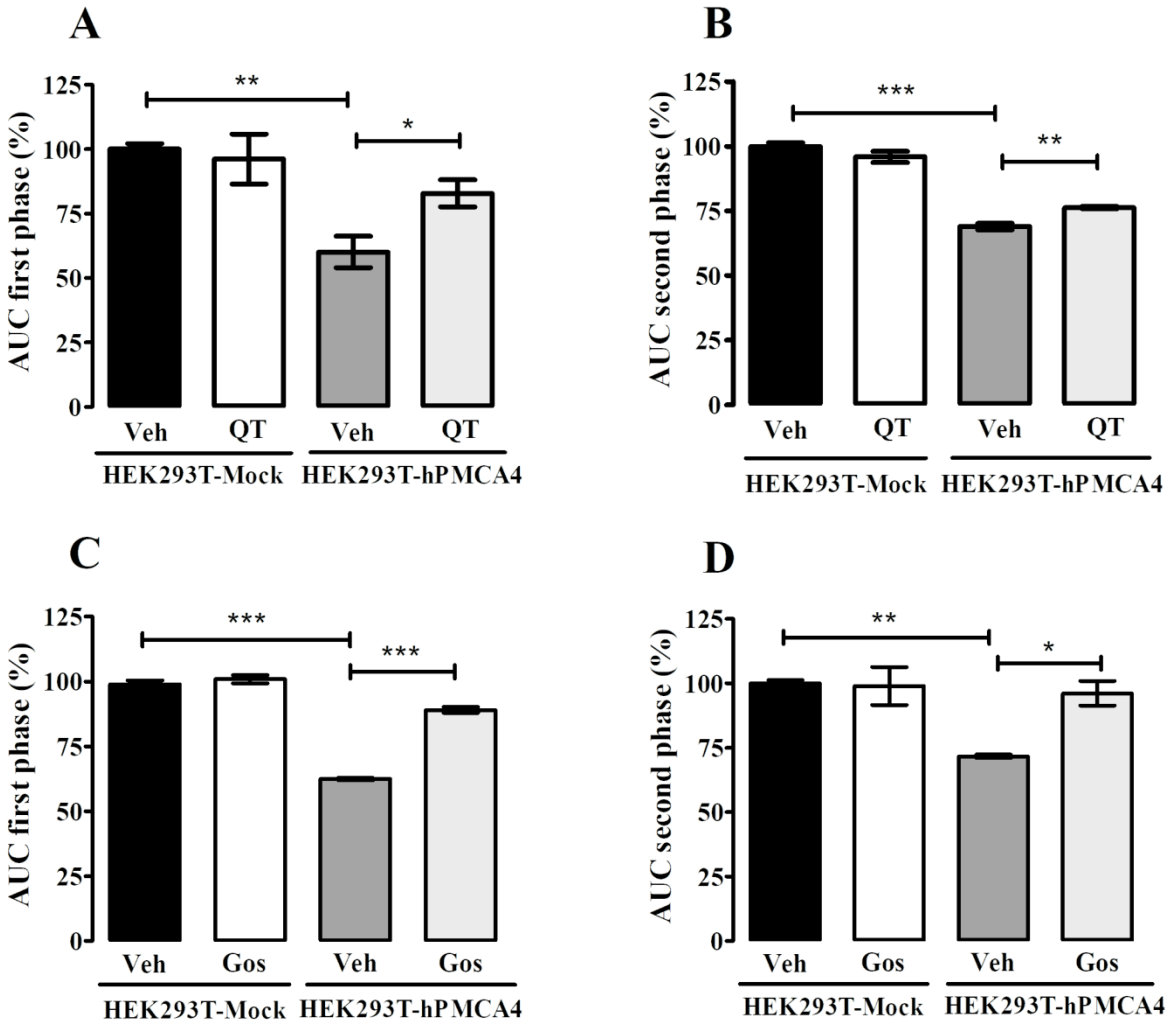



A
Kaempferol
Quercetin
Ca2+-ATPase Activity (%)
[Flavonols] (µM)
B
[Quercetin] (µM)
C
Gossypin
Hyperoside
[Flavonol glucosydes] (µM)
D
Diosmetin
Apigenin
[Flavones] (µM)


A
B
C
D
E
F
Ca2+-ATPase Activity (%)
[Ca2+]free (μM)
K0.5 for Ca2+ (μM)
[Quercetin] (μM)
Vmax (%)
[Gossypin] (μM)
K0.5 /Vmax


EP/Prot (%)
***
***
**
Control
La3+
500 μM
QT
25 μM
Gos
100 μM


A
Control
PMCA4
Ca2+ (1 mM)
TG (1 µM)
Relative [Ca2+]Cyt
(response/base line)
time (min)
B
AUC first phase (%)
HEK293T Mock
HEK293T hPMCA4
C
AUC second phase (%)
HEK293T Mock
HEK293T hPMCA4
D
HEK293T Mock
HEK293T hPMCA4


A
AUC first phase (%)
Veh
QT
Veh
QT
HEK293T-Mock
HEK293T-hPMCA4
B
AUC second phase (%)
Veh
QT
Veh
QT
HEK293T-Mock
HEK293T-hPMCA4
C
AUC first phase (%)
Veh
Gos
Veh
Gos
HEK293T-Mock
HEK293T-hPMCA4
D
AUC second phase (%)
Veh
Gos
Veh
Gos
HEK293T-Mock
HEK293T-hPMCA4