Reviews and Commentaries

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A commentary on the inhibition of human TPC2 channel by the natural flavonoid naringenin: Methods, experiments, and ideas

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Abstract: Human endo-lysosomes possess a class of proteins called TPC channels on their membrane, which are essential for proper cell functioning. This protein family can be functionally studied by expressing them in plant vacuoles. Inhibition of hTPC activity by naringenin, one of the main flavonoids present in the human diet, has the potential to be beneficial in severe human diseases such as solid tumor development, melanoma, and viral infections. We attempted to identify the molecular basis of the interaction between hTPC2 and naringenin, using ensemble docking on molecular dynamics (MD) trajectories, but the specific binding site remains elusive, posing a challenge that could potentially be addressed in the future by increased computational power in MD and the combined use of microscopy techniques such as cryo-EM.

Keywords: TPC channels, naringenin, flavonoids, plant vacuole, patch-clamp

Abbreviations

Nar	naringenin
$PI(3,5)P_2$	phosphatidylinositol-(3,5)-bisphosphate
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
cryo-EM	cryogenic electron microscopy

Introduction

The TPC channel family

TPC family channels are intracellular channels found in animal [1] and plant cells [2]. In plant cells, the channels are localized on the vacuolar membrane and generally encoded by a single gene, which in the model plant *Arabidopsis thaliana* is called AtTPC1 [3]. In human cells there are two genes: hTPC1, which is localized on the membrane of endosomes, and hTPC2 located on the lysosomal membrane [1].

From a structural point of view, TPC channels are dimers [4]. Each dimer consists of two shaker-type subunits. The shaker-type subunit, the structure underlying all voltage-dependent channels, is composed of six transmembrane alpha-helices. The arginine-rich S4 segment, ARG is a positively charged amino acid, imparts voltage dependence to the channel. The terminal part of segments S5 and S6 is the channel "gate" since it is involved in the closing and opening mechanism of the channel. The segment between S5 and S6 is called the P-loop: the interaction of four P-loops generates the channel selectivity filter. Therefore, four shaker-type subunits are required to form a functional voltage-dependent channel. In potassium channels, the

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interaction of these four subunits is non-covalent. In TPC channels two subunits are covalently bound, in animal sodium and calcium channels all four subunits are covalently bound. In this context, it is useful to note that the name TPC is misleading, the channel in fact has only one permeation pore and should be called two pore loop forming domain channel.

A marked difference in the structure between plant and animal TPCs is the presence of two cytosolic EF-hands in the segment that covalently binds the two shaker subunits in the plant TPCs [5]. Consequently, plant TPC channels are activated by an increase in cytosolic calcium (see below).

Methods and results

Plant vacuole as a heterologous system suitable for studying the functional properties of endo-lysosomal channels and transporters

The initial recordings of TPC channels were performed using patch-clamp experiments applied to vacuoles of sugar beet plants [6]. The vacuole is an intracellular compartment in plant cells that can occupy up to 90% of the volume in a mature cell. In whole-vacuole configuration, an outward rectifying cation channel was recorded, which was active for cytosolic positive voltages [6]. The single-channel conductance was large, on the order of 100 pS, and exhibited typical characteristics of a single-file, multi-ion pore [7]. Due to its slow activation and deactivation times on the order of seconds, the channel was named Slow Vacuolar (SV) channel [6]. The channel is activated by an increase in cytosolic calcium, in line with its structural features. However, the voltage and calcium values that activate it are not compatible with physiological conditions [8]. Consequently, researchers are still trying to identify a "helper factor" that allows the channel to be open under physiological conditions. There are several factors that modulate the channel [9], such as reducing molecules, which act as activators [10–13]. In 2005, the TPC gene was associated with SV currents [3], and in 2016, the high-resolution structure of the channel was resolved [14,15].

Plasma membrane channels and transporters are usually expressed and characterized in Xenopus oocytes [16–20] or in plant/animal cell lines [21,22]; it is interesting to observe how the vacuole can be used as a heterologous system to study the functional properties of animal endo-lysosomal TPC channels [23–25], in addition to other techniques reviewed in the study by Festa et al. [26]. Endosomes and lysosomes are submicrometric compartments that are difficult to access for the application of electrophysiological techniques such as patch-clamp. Plant vacuoles are easy to isolate, the patch-clamp can be successfully applied in all possible configuration modes [27–30], background currents can be controlled and minimized [31–33], the lumen of the vacuole can be loaded with a specific fluorophore through the patch-pipette [34–39] and the cytosolic solution is the external solution of the vacuole [27], which can be easily changed to investigate cytosolic modulators [40,41]. When human TPCs are expressed in mesophyll vacuoles of Arabidopsis mutant plants lacking endogenous TPCs, both members, hTPC1 and 2, reach the membrane of the vacuole and are activated by the phosphoinositide $PI(3,5)P_2$, which, however, has no effect on the endogenous plant AtTPC1 channel [23]. The $PI(3,5)P_2$ binding site of the human TPC2 channel was validated by expression of mutated channels in vacuoles from Arabidopsis *tpc1-2* mutant (AtTPC1 null background [3]); site-specific mutations of TPC2 identified by a bioinformatics approach induced a highly significant change in channel affinity for $PI(3,5)P_2$ [42]. The binding site was subsequently confirmed by cryo-EM experiments [43].

Naringenin is a TPC2 inhibitor: searching for the binding site

The flavonoid naringenin, found in citruses and tomatoes, is able to inhibit hTPC2 activity, when the channel is expressed in vacuoles knockout for the endogenous channel [44]; the application of 200 μM naringenin in cytosolic solution, outside the vacuole, induces a reduction of the current recorded by patch-clamp of more than 50%; the effect is voltage-independent and reversible. Inhibition of TPC2 by naringenin has been linked to severe human diseases such as development of solid tumors through inhibition of the neoangiogenesis mechanism [44,45], melanoma [46], and Sars-CoV-2-mediated viral infection [47,48]. Therefore, it is important to characterize the molecular basis of the interaction between TPC2 and naringenin. In our previous work, to identify the amino acids that mediate the interaction between TPC2 and naringenin, we used a structure of TPC2 obtained by homology from the high-resolution structure of AtTPC1 [49]. In this study, we performed molecular dynamics (MD) simulations on three structural configurations obtained from cryo-EM experiments [43]: the structure lacking $PI(3,5)P_2$ in a closed non-conductive state (apo-closed), the structure with $PI(3,5)P_2$ bound also in a closed state (holo-closed), and the structure with $PI(3,5)P_2$ in an open conductive state (holo-open). Molecular simulations were successfully used to identify the mechanism of

conduction of sodium ions [50,51]. Starting from the three high-resolution structures, we embedded them in a POPC symmetric bilayer and solvated with water and NaCl 0.15 mM. The details of the preparation and equilibration are described in details in previous studies [50,51]. We carried out ten independent 300 ns MD simulations for each of the three structural configurations of the channel [51], we clusterized the structures of each simulation and then performed ensemble docking calculations with naringenin on each of the three representative structures from the MD simulation clusters. The details of docking procedures are described in ref. [52]. The results are presented in Figure 1, showing the four most stable binding sites of naringenin based on their binding energies. Naringenin, a hydrophobic small molecule, is capable of inserting itself into various different regions of the dimeric structure. Among the 12 identified binding sites (Table S1), only one, indicated by an arrow in Figure 1a and b, is common to the apo- and holo-closed structures. Each site is characterized by the interaction of at least nine amino acids, most of which are hydrophobic. This approach demonstrates how naringenin can interact with different structural parts of TPC2; however, no single preferred binding site emerges. We again performed docking calculations by mutating *in silico* three amino acids, F452A, T544G, and N548A, and found that naringenin does not bind anymore to that specific site.

We performed the aforementioned site-specific mutations (*in vitro*), in the common putative binding site of the apo and holo-closed structures (Figure 2a). The mutant channel, fused with an EGFP at its C-terminus, was expressed in vacuoles isolated from mesophyll leaves of Arabidopsis plants lacking the endogenous TPC1. The mutant channel exhibited functional activity, as evidenced by the increase in current induced by the application of 100 nM $PI(3,5)P_2$ in the cytosolic solution external to the vacuole (Figure 2b). However, the addition of 200 µM Nar to the cytosolic solution inhibited the current in a similar manner to what occurs in the wild-type channel (Figure 2b–d). Therefore, the selected molecular region on hTPC2 does not appear to be the binding site for Nar.

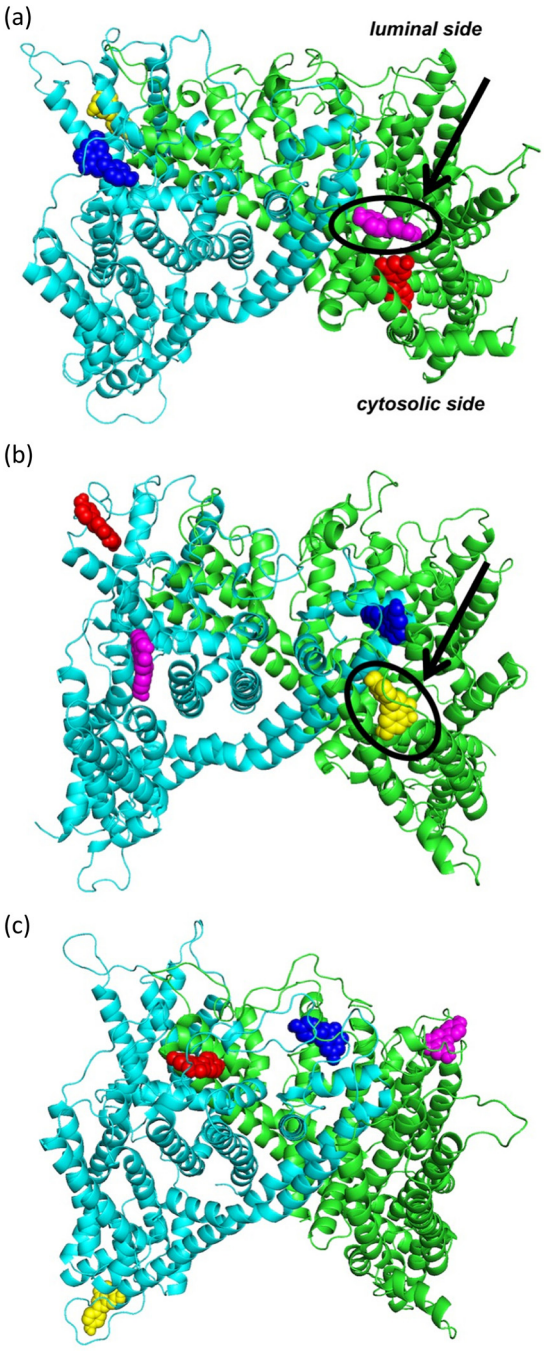

Figure 1: Docking poses. The most stable pose is in red, then blue, magenta, and yellow: (a) apo-closed, (b) holo-closed, and (c) holo-opened. The arrows indicate two identical poses found in apo-closed structure (panel a, naringenin in magenta) and holo-closed structure (panel b, naringenin in yellow).

Conclusion

Flavonoids are secondary metabolites in plants that have been extensively studied for their potential health effects. However, the molecular mechanisms through which flavonoids exert their effects are mostly unknown. Our simulations indicate that naringenin interacts with the hydrophobic region of the channel. Since the protein undergoes a conformational change in its opening mechanism, the insertion of naringenin could potentially block the channel in its closed conformation. However, our analysis suggests that finding the binding site is challenging. Naringenin interacts with multiple areas of the channel, involving hydrophobic amino acids, and multiple mutations may be necessary to significantly alter its

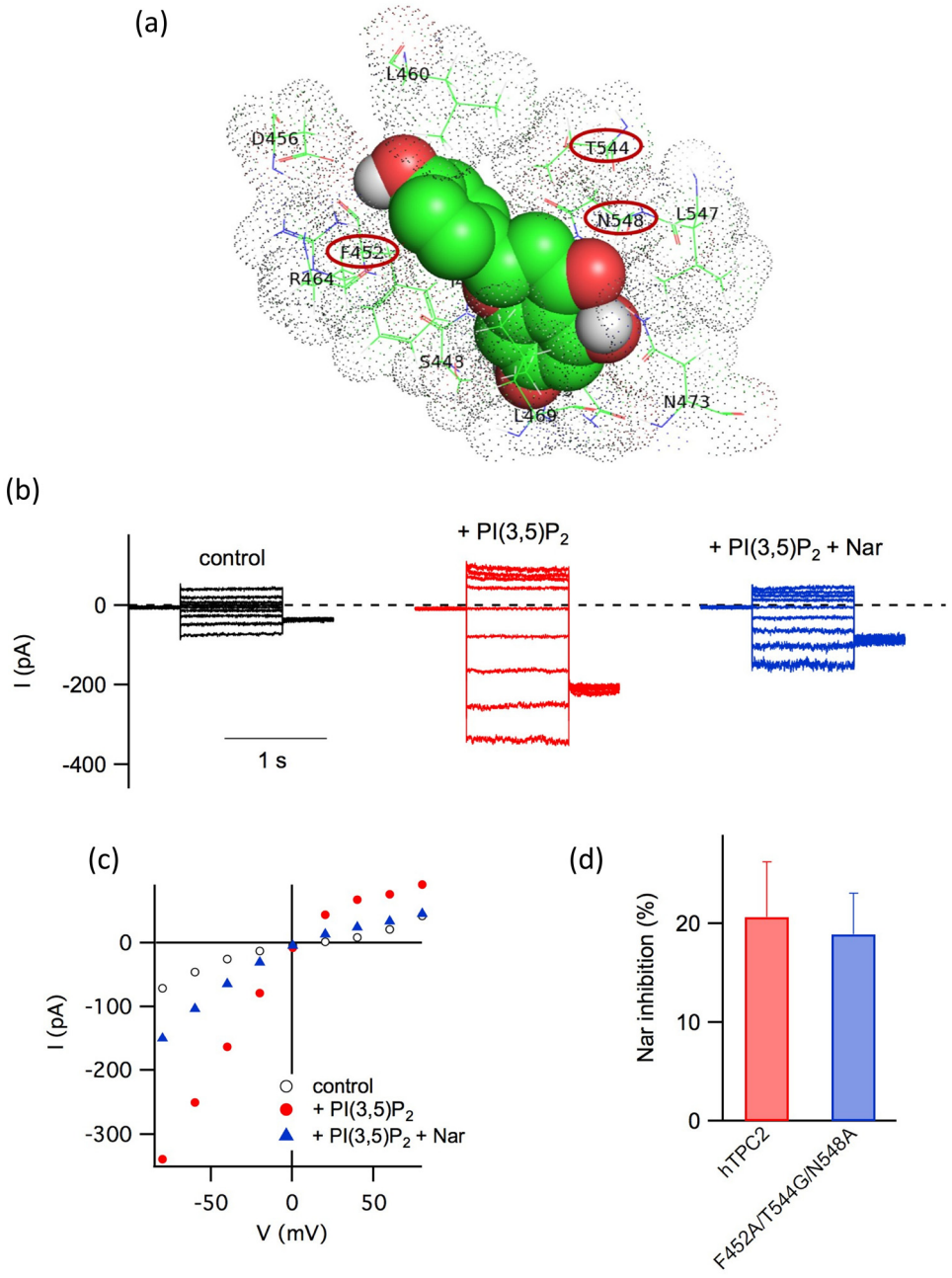

Figure 2: Site-directed mutagenesis and patch-clamp experiments. (a) Selected putative binding site for naringenin (indicated by the arrows in Figure 1). Naringenin is shown with van der Waals (vdW) spheres and the nearby amino acids with sticks and vdW dots: oxygen atoms in red, nitrogen atoms in blue, carbon atoms in green, and hydrogen atoms in white. Circled in red, the amino acids that were changed in hTPC2 by site-directed mutagenesis approach using Kit,QuikChange Site-Directed, Agilent Technologies. On the hTPC2 coding sequence (NM_139075.4) the TTC codon for F452 was changed with GCC coding for Alanine, the ACC codon for T544 was replaced with GCC coding for Glycine and the codon ACC for N548 was replaced with GCC coding for Alanine. (b) Currents mediated by the hTPC2 mutant channel expressed in Arabidopsis vacuoles lacking the endogenous TPC in control condition (left, control), induced by the application of 100 nM $PI(3,5)P_2$ in the cytosolic bath solution (middle, + $PI(3,5)P_2$) and inhibited by 200 µM cytosolic naringenin (left, + $PI(3,5)P_2$ + Nar). Holding voltage: 0 mV. Voltage pulses of 1 s from −80 to +80 mV in +20 mV steps. Tail voltage of 0.5 s at −50 mV. Cytosolic bath solution (in mM): 100 NaCl, 2 $MgCl_2$, 10 Hepes, 280 Sorbitol, pH 7.2 (NaOH). Luminal pipette solution (in mM): 100 NaCl, 2 $MgCl_2$, 10 MES, 260 Sorbitol, pH 5.6 (NaOH). $PI(3,5)P_2$ was purchased as dioctanyl ester (diC8) from AG Scientific or Echelon Biosciences Inc. (USA) and prepared as 1 mM stock solution, stored at −20°C. Naringenin, from Merck, Germany, was prepared fresh before use as 200 mM stock in DMSO. DMSO alone had no effect on channel activity [44]. (c) Current–voltage relationships of data displayed in (b). (d) Current inhibition induced by 200 µM Nar added in the cytosolic solution containing 100 nM $PI(3,5)P_2$, at −40 mV. Data, shown as mean ± s.e.m. (hTPC2, $n = 3$; mutant channel, F452A/T544G/N548A, $n = 3$), were not statistically significant.

affinity for Nar. The increase in computational power will allow for the simulation of the conformational change of the channel. This, combined with the application of other structural approaches such as cryo-EM, will ultimately enable the precise identification of the elusive molecular mechanism underlying the interaction between TPC channels and naringenin.

Conflict of interest: Authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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