Biophysical Chemistry 254 (2019) 106246

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: www.elsevier.com/locate/biophyschem

A perspective on the modulation of plant and animal two pore channels (TPCs) by the flavonoid naringenin

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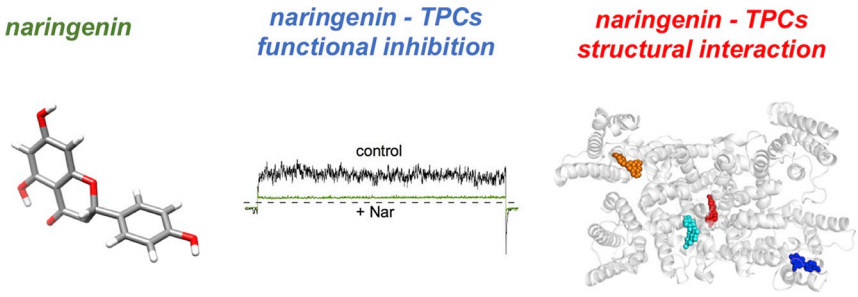
HIGHLIGHTS

- Naringenin inhibits the functional activity of plant and human TPC channels.
- Naringenin impairs VEGF-induced neoangiogenis.
- Molecular docking defines specific binding regions between naringenin and TPCs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
TPC channels
Plant vacuole
Animal lysosome
Patch-clamp
Fluorescence
Molecular docking

ABSTRACT

The inhibitory effect of the flavonoid naringenin on plant and human Two-Pore Channels (TPCs) was assessed by means of electrophysiological measurements. By acting on human TPC2, naringenin, was able to dampen intracellular calcium responses to VEGF in cultured human endothelial cells and to impair angiogenic activity in VEGF-containing matrigel plugs implanted in mice. Molecular docking predicts selective binding sites for naringenin in the TPC structure, thus suggesting a specific interaction between the flavonoid and the channel.

1. Introduction

The first functional recordings of TPC channels were performed by Rainer Hedrich, in the Erwin Neher Nobel laureate's laboratory, on sugar beet vacuoles and were published in 1987 [1].

Among the various differences between animal and plant cells, there is the presence in the latter of a large compartment, the vacuole, which can occupy 90% of the intracellular space. It is generally difficult to apply the patch-clamp technique to plant protoplasts, probably because, due to the removal of the cell wall, the plasma membrane is not

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https://doi.org/10.1016/j.bpc.2019.106246
Received 29 June 2019; Received in revised form 3 August 2019; Accepted 3 August 2019
Available online 09 August 2019
0301-4622/

entirely clean; to improve success rate, tricks, such as for instance the use of trivalent ions in the external solution, can be exploited [2–5]. Since lanthanum (La^{3+} at 1 mM concentration) and gadolinium (Gd^{3+} at 100–200 μM concentration) may act as blockers [6–8], their action on the channel under study must be experimentally verified. On the contrary, vacuole is easy to isolate and patch-clamp technique results to be very effective on this type of preparation [9]. As a consequence, similar to the oocyte system for plasma membrane channels (see as examples [10,11]), the vacuole was used as an expression system for plant vacuolar channels ([12,13] or animal lysosomal channels and transporters, see below) or as a convenient model to characterize the functional properties of channel-forming peptides [14–16]. The plant TPC currents have outward rectification, *i.e.* they are activated by positive (respect to the vacuolar lumen) cytosolic potentials and have cation selectivity [17,18]. Potassium and sodium have similar permeability [19]. Calcium ion permeability has been the subject of strong discussions but it is now consolidated that Ca^{2+} can permeate, together with potassium ions, the plant TPC channels [20,21]. When depolarizing potentials are applied, plants TPC currents are activated slowly, reaching the steady state in times of the order of seconds; for this reason, this type of channels has been given the name of Slow Vacuolar, or SV channel [1]. Edgar Peiter, in 2005, definitively discovered that SV currents are mediated by one protein, TPC1, which in Arabidopsis is encoded by a single gene [22]. TPC1 protein consists of two covalently linked subunits. Each subunit, similar to the shaker-type subunit, a fundamental unit of the voltage dependent channels, is formed by six transmembrane segments, with a so-called P-type loop (P stands for pore) between the S5 and S6 segments. Since four P-type segments form a permeation pore, the AtTPC1 channel has a dimeric form. It should be noted that it has only one permeation pore and therefore the name two pore channel is misleading; for TPCs one should actually talk about two pore loop forming domain channels. S4 and S10 segments give to the channel the voltage dependence due to the presence of multiple arginine residues. In the cytosolic portion, which binds the two shaker-like subunits, two EF-hands domains responsible for the dependence of the channel on cytosolic calcium can be observed [23]. In addition to cytosolic calcium, plant TPC channels have a variety of modulations among which we recall:, reducing and oxidising agents [17,24–26], magnesium [27], nickel [28–30], antibiotics [31], voltage protocol [32,33], polyunsaturated fatty acids [34]. Interestingly, the phosphoinositides $PI(3,5)P_2$, recently discovered as a modulator of the vacuolar anion/proton antiporter AtClCa [35], is a powerful activator of animal TPCs but has no effect on plant AtTPC1 [36].

It should be noted that inside the vacuole there is a high free calcium concentration larger than 50–100 μM; given the remarkable physiological effects of intracellular calcium, calcium permeation pathways on tonoplast are expected to be strongly controlled. Accordingly, it is found that TPC channels are normally closed and they open at calcium concentrations and tonoplast potentials far from the conditions that should be present *in vivo* [20]; therefore, an unknown modulation factor that would allow the channel to open at physiological conditions should exist. This is the reason which led us to investigate the effects of Naringenin (Nar), a hydrophobic molecule produced in the cytosol of plant cells, on TPC channels activity. However, the experimental results indicate that Nar is an inhibitor of the activity of plant and animal TPC channels.

2. Naringenin

Flavonoids have become an interesting object of research thanks to their biological properties supported by epidemiological studies that propose the existence of a correlation between the consumption of foods rich in flavonoids and low in fatty acids and a reduced risk of certain diseases in humans such as cardiovascular disease, diabetes and cancer. Because of their antioxidants properties, flavonoids seem to play a key role in countering the aging process, tissue damage and inflammatory processes [37]. Moreover, they exert a potential anti-invasive and/or anti-metastatic activity in a wide range of cancer types [38]. Nar (5,7-Dihydroxy-2-(4-hydroxyphenyl)chroman-4-one), whose structure is shown in Fig. 1, is a flavanone, a type of flavonoid, predominantly found in grapefruit but also in a variety of vegetables and fruits, whose consumption through the diet is associated to a reduced incidence of metabolic and chronic-degenerative diseases [39,40]. Nar has been pharmacologically evaluated as a potential anti-inflammatory, chemo-preventive, antioxidant and anti-degenerative agent [41]. The potential of Naringenin, and its glycosylated form Naringin, for the treatment of metabolic and cardiovascular disorders has been assessed by pre-clinical studies [40]. Moreover, Nar has been proved to have anticancer activity as it is able to block the progression and the formation of metastasis [42]. It is known that Nar affects the activity of several calcium-activated or calcium-permeable ion channels and hence to have healing effects by modulating their activity [43]. Despite the evidence of the pharmacological effectiveness of Nar, a complete view of its mechanism of action is still lacking.

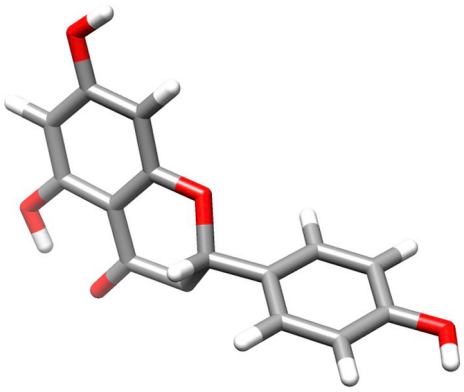

Fig. 1. Naringenin structure in sticks. Carbon atoms in grey, hydrogen atoms in white and oxygen atoms in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Inhibition of plant TPC by naringenin

We applied the patch-clamp technique to mechanically isolated carrot vacuoles and imposed defined ionic solutions both inside the pipette (corresponding to the vacuolar lumen) and in the extracellular bath solution (corresponding to the cytosolic side), so as to record the SV currents mediated by TPC channels. In Fig. 2a, it can be observed that the application of Nar 200 μM from the cytosolic side inhibited the current elicited by a potential of +80 mV by approximately 50%. Increasing Nar concentration to 1 mM led to a complete inhibition of the current, as shown in Fig. 2b. TPC cationic currents were composed by two components, due to the permeation of potassium and calcium. In order to quantify the contribution of both ions, Gradogna et al. (2009, [44]) had combined the patch-clamp with detection of Ca^{2+} variations by using the fura-2 fluorescent probe. This technique was called FLEP: FLuorescence combined with Excised Patch. The experiments were conducted in the cytosolic side-out excised patch configuration, focusing the photomultiplier on the tip of the recording pipette where the fluorescent dye was present. This excised configuration has two advantages with respect to the whole-vacuole mode: 1) a lack of delay in loading the fluorophore inside the vacuolar lumen; 2) the absence of photobleaching due to a quasi-infinite fluorophore reservoir inside the pipette. Moreover, the high density of SV channels allows recording macroscopic currents also in such a configuration. Using a symmetrical potassium concentration of 105 mM and a cytosolic calcium concentration of 2 mM, necessary to activate the channel, it was calculated that the fractional calcium current (I_{Ca} / (I_{Ca} + I_K)) (where I_{Ca} and I_K were the contribution to the current due to the calcium and potassium

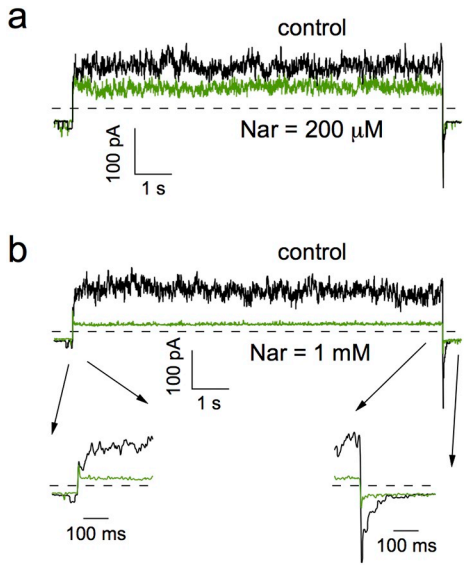

Fig. 2. Effects of naringenin on SV currents recorded on isolated carrot vacuoles.
(a) SV currents responses upon voltage stimulation to +80 mV from a holding potential of −80 mV in control and in the presence of Nar = 200 μM. (b) Effect of 1 mM naringenin on SV currents. The traces below, displayed on an expanded time scale, showed the typical activation, left, and deactivation, right, of the TPC mediated currents. Dotted lines in both panels indicated the zero current level. Pipette and bath solution as in [45].

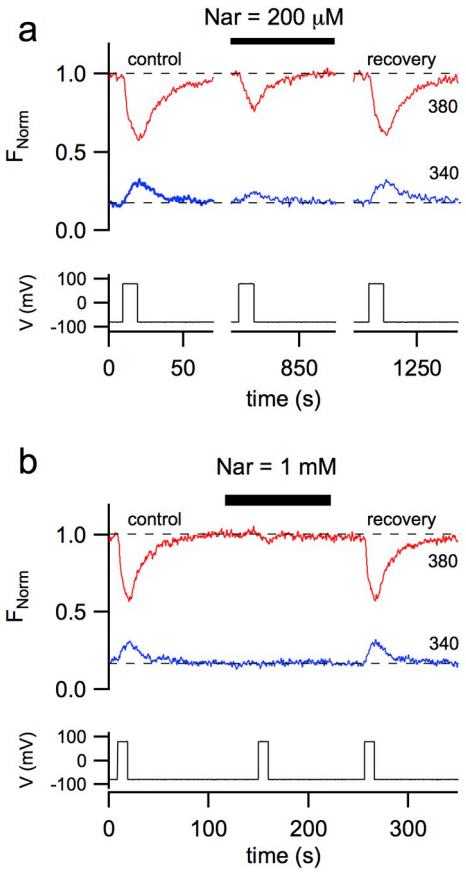

Fig. 3. Naringenin inhibited calcium permeation through carrot SV channels.
(a) Changes in fura-2 fluorescence in control conditions were elicited by a voltage step to +80 mV from a holding potential of −80 mV (see lower panel illustrating the applied voltage). The fluorescence signals at 380 and 340 respectively decreased and increased due to the increase of calcium concentration in the tip of the recording pipette caused by the permeation of Ca^{2+} through carrot SV channels. In the presence of 200 μM naringenin fluorescence signals were reduced of about 0.5 when compared to the control condition. The effect was fully reversible after washout of naringenin (recovery). (b) No fluorescence responses were present when Nar = 1 mM was applied.

ion respectively) was equal to 10% at high positive potentials. This ratio decreased to about 5% when cytosolic calcium was decreased to 0.5 mM [45]. We repeated this kind of measurements in order to quantify the effects of Nar on the passage of calcium through TPC channels. In Fig. 3a, it is possible to observe that Nar 200 μM halved the fluorescence signal due to the passage of Ca^{2+} through carrot TPC channels, while there was a complete inhibition of the fluorescence signals when Nar concentration was increased to 1 mM, Fig. 3b. Taken together, data in Figs. 2 and 3 indicated that Nar inhibited carrot TPC channels with an affinity of about 200 μM and its inhibition did not interfere with the ion permeation mechanism through the selective pore.

Since the *in vivo* concentration of flavonoids in the cytosol is considered to be in the micromolar range [46] therefore much lower than the affinity that Nar presents for TPC channels, it seems unlikely that the effects of Nar on TPC channels can occur under physiological conditions. Nevertheless, flavonoids are accumulated in the vacuole in glycosylated form at concentrations larger than 1 mM; under stress conditions endogenous β-glucosidases may produce high aglycone (deglycosilated form of the relative flavonoid) concentrations [47]. Therefore, Nar inhibition of TPC channels could play a role in the metabolic response of the plant to the stress.

4. Inhibition of human TPCs by naringenin

While plant TPC1 is a tonoplast protein, the human counterparts, namely TPC1 and TPC2, are *endo*-lysosomal channels [48]. Human TPC channels are involved in different pathologic conditions such as in neoangiogenesis processes linked to vascularization of solid tumors [49], in neurodegenerative Parkinson's disease [50], in Ebola virus infections [51] and in melanoma metastasis [52]. While hTPC2 expression is predominantly lysosomal, hTPC1 presents a wider distribution within the endolysosomal system, being localized in lysosomes, early and recycling endosomes.

The ability of Nar to modulate hTPCs channel activity, was tested in a recent study by Pafumi et al. (2017, [53]) by using a novel heterologous system [54,55], *i.e.* Arabidopsis vacuoles lacking endogenous TPCs. Electrophysiological measurements performed by Pafumi et al. [53] clearly demonstrated that the addition of 500 μM Nar to cytosolic solution led to a strong voltage-independent decrease in the activity of both hTPC1 and TPC2 currents. Interestingly, Nar inhibition was fully reversible. Moreover, Nar was able to dampen intracellular Ca^{2+} responses of human endothelial cells stimulated with VEGF, histamine or NAADP-AM, but was ineffective upon application with NAADP-independent stimuli, such as ATP and Angiopoietin-1. Since hTPC2 but not hTPC1 was known to be involved in this pathway, data from Pafumi et al. [53] strongly suggested that the reduced Ca^{2+} release was due to the inhibition of hTPC2 by Nar. In order to explore the anti-angiogenic effects of Nar, VEGF-dependent angiogenesis was evaluated in different experimental models [53]. HUVECs are able to form capillary-like tubes *in vitro*, characteristic usually considered as representative of later, differentiative steps of angiogenesis. This *in vitro* assay could be exploited to analyse the anti-angiogenic effects of Nar. Endothelial cells plated onto matrigel matrix adhered, migrated and, within a few hours, differentiated into capillary-like structures; in cells stimulated with VEGF in the presence of 500 μM Nar there was a significant reduction in the number of closed polygons compared with samples stimulated with VEGF alone. Again, Nar had no significant effect in limiting polygon formation when cells were challenged with Angiopoietin-1. These results by Pafumi et al. [53] highlighted the

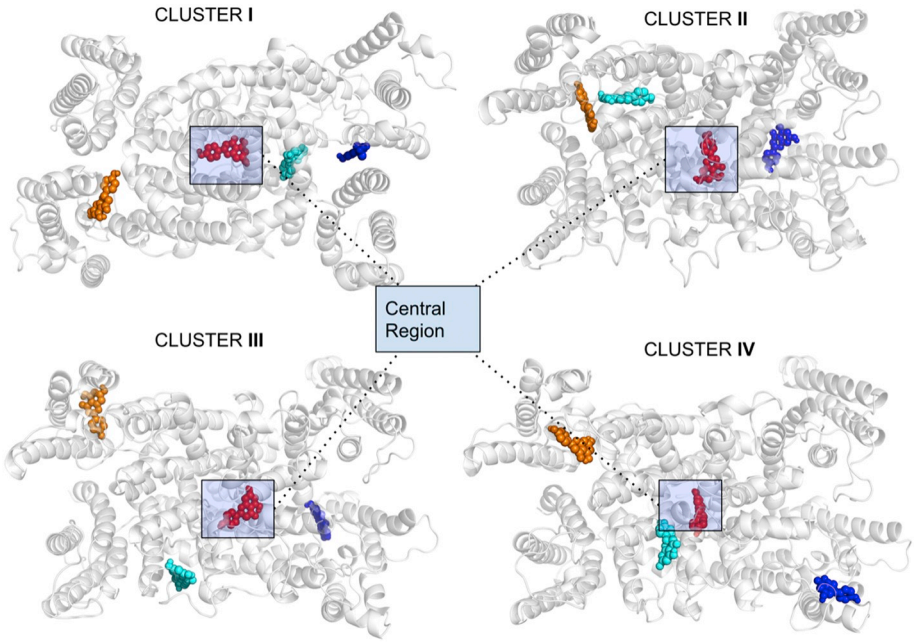

Fig. 4. Poses of naringenin from blind docking on the four main hTPC2 clusters sampled during a 400 ns all-atom Molecular Dynamics simulation. In all four clusters a pose occupy the central region near the ligand-gated hydrophobic region.

Table 1
Naringenin *vs* verapamil binding properties.

	Naringenin		Verapamil	
	Pore center	VSD regions	Pore center	VSD regions
Initial structure	−7.68	−7.46	−5.44	−5.93
Energy kcal/mol	−8.25	−8.23	−7.29	−7.89
Ki predicted (μM)	0.899 (experimental: 180 ± 20 μM)	0.932	4.56	1.64
Hbonds	ASN1406, SER1434	GLY766, GLU1168	ASN305, ASN1439	No hydrogen bond formed
Closed contacts with a distance cutoff of 3.2 Å	VAL651, THR1023, VAL1438, VAL1435, VAL652, TRP1430, TRP1407, ASN653, THR1022, GLN1408, VAL1404, ALA1024, SER1434, ASN1405 MET1056, ASN1406	PR0788, LEU1114, VAL1117, ALA792, LEU1118, GLU1168, GLY766, TYR1169, PR01167 VAL786 VAL786	VAL1435, THR271, MET304, ASN305, VAL1438, SER300, LEU1021, TRP1430, LEU1442, VAL1403, LEU1059, ASN1439, LEU301, SER1434, MET1059	LEU1302, PHE1290, PHE295, LEU229, PHE1256, ILE298, PHE291, ARG1306, PHE1305, SER1291, VAL1328, PHE230, LEU303, HIS226, VAL294, ILE 1303

ability of Nar to regulate VEGF-induced capillary-type formation *in vitro* specifically inhibiting the NAADP/TPC2-mediated Ca^{2+} signalling. In an *in vivo* murine model [49], it was demonstrated that VEGF-treated plugs implanted in the flank of wild type mice were not vascularized both if treated with Ned-19, a membrane-permeant non-competitive antagonist of NAADP, and also, more strikingly, in TPC2 knockout mice. Furthermore, vascularization was not inhibited in plugs from TPC1 knockout mice, thus supporting the specific role of TPC2. The inhibitory effect of Nar on neoangiogenis was verified in this model [53]. It came out that while VEGF alone induces significant vascularization, plugs treated with VEGF plus Nar remain virtually avascular. Interestingly, these *in vivo* results by Pafumi et al. [53] open a new outlook toward a potential therapeutic use of Nar although the possibility that targets other than TPC2 may contribute to this anti-angiogenic effect cannot be excluded.

5. Simulation of the binding of naringenin to TPC channels

In order to dissect at molecular level possible interactions of flavonoids and TPC channels one can employ molecular docking. Widely used, the molecular docking procedure is being able to provide a list of the possible binding sites with computational costs not too high, compatible with a virtual screening [56]. The list of binding sites can be either paired with the already existing experimental knowledge representing a valuable asset in understanding of rather complex ligand-protein interactions, or used as a predictive tool to guide and prepare *ad-hoc* experiments. The knowledge of the TPC literature can be used to guide docking in specific regions, such as selectivity filter & voltage sensitive domain (VSD) regions. Mutational studies have shown [48] that a perturbation of the pore selectivity filter can lead to slowing of the ion permeation or inactivation of the channel [57]. Multiple instances of inhibitors are known to bind in the selectivity filter or in

hydrophobic gate and effectively block the channel activity [58–60].

Although coming at the affordable computational cost, downside of the docking procedure lies in its initial conditions: too frequently the docking is performed on a single structure. This can lead to undesired artefacts and possible report of artificial binding sites or even more so, overestimation of particular amino-acid residues importance. The latter can specifically be the case when homology modeling is being used as a tool for grasping the target protein structure. Therefore, if one wants to elevate the precision of the docking, the target protein structure should be an ensemble that samples intrinsic and subtle changes of protein conformation. Unfortunately, this can be true only for the structures obtained *via* NMR. An X-ray structure represents an average, which can indeed provide misleading docking results. Our approach is therefore to combine docking to Molecular Dynamics (MD) simulations, which supply us with the necessary flexibility of proteins and statistics [61]. Together, homology modeling, MD simulations and molecular docking, represent a potent theoretical toolbox that can tackle ligand-protein interactions in general and provide solid and trustful results. We obtained the hTPC2 model coordinates with modeller [62] using as homology protein the X-ray structure of atTPC1 (pdbid: 5TUA, sequence identity 25%) [63]. A high sequence identity is observed in the pores filters that are highly conserved in TPC channels.

Thus we performed a 400 ns molecular dynamics simulations on the hTPC2 embedded in a phospholipid bilayer and solvated with water with the code ACEMD [64]. We classified the sampled conformations with the cluster analysis employing the Root Mean Square Distance as parameter. We performed blind docking of Nar on the four most populated clusters, covering 99% of all the conformations, with the code Autodock4.6 [65]. Fig. 4 gives an overview of the blind docking results obtained. The binding site in the central region is present in all clusters analyzed, and potentially can block the activity of the channel inhibiting the diffusion of ions. Nar is in contact with the hydrophobic residues that constitute the gate of the pore, creating an additional barrier to the unselective passage of ions, as demonstrated with electrophysiology. Further investigation will focus on the new ligand-activated X-ray structures available [66].

Another request for a good inhibitor is its selectivity. In order to investigate it, we performed docking with verapamil, belonging to Phenylalkylamines (PAAs) class of Ca^{2+} channels inhibitors. We observe that verapamil binds in the same regions as Nar, however a one-to-one comparison showed that the binding energy is always higher for Nar. The overview of the naringenin *vs* verapamil binding properties is given in Table 1. We observe that the nature of ligand-protein interactions is very similar with an exception in VSD regions where verapamil doesn't engage in H-bonding interaction with the TPC.

6. Conclusion

By means of electrophysiological and fluorescence measurements we were able to demonstrate the inhibition of plant TPC by naringenin. Nar was also effective to impair the activity of human TPCs. In particular, the inhibition of hTPC2 correlated with the ability of Nar to act as anti angiogenic factor in different experimental models. A computer-driven simulation approach determined selective binding sites for Nar in hTPC2 structural composition. Future studies, *i.e.* site-directed mutagenesis combined with electrophysiological functional characterisation, are needed to definitely confirm if the recorded effects are due to a specific interaction between naringenin and TPCs.

Acknowledgement

This work was supported by the Italian “Progetti di Ricerca di Interesse Nazionale” (2015795S5W).

References

[1] R. Hedrich, E. Neher, Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles, Nature 329 (1987) 833, https://doi.org/10.1038/329833a0.
[2] M. Bregante, A. Carpaneto, F. Pastorino, F. Gambale, Effects of mono- and multivalent cations on the inward-rectifying potassium channel in isolated protoplasts from maize roots, Eur. Biophys. J. 26 (1997) 381–391, https://doi.org/10.1007/s002490050092.
[3] A. Carpaneto, R. Magrassi, E. Zocchi, C. Cerrano, C. Usai, Patch-clamp recordings in isolated sponge cells (Axinella polypoides), J. Biochem. Biophys. Methods 55 (2003) 179–189.
[4] A. Costa, A. Carpaneto, S. Varotto, E. Formentin, O. Marin, E. Barizza, M. Terzi, F. Gambale, F. Lo Schiavo, Potassium and carrot embryogenesis: are K+ channels necessary for development? Plant Mol. Biol. 54 (2004) 837–852, https://doi.org/10.1007/s11103-004-0236-9.
[5] M. Bregante, Y. Yang, E. Formentin, A. Carpaneto, J.I. Schroeder, F. Gambale, F. Lo Schiavo, A. Costa, KDC1, a carrot shaker-like potassium channel, reveals its role as a silent regulatory subunit when expressed in plant cells, Plant Mol. Biol. 66 (2008) 61–72, https://doi.org/10.1007/s11103-007-9252-x.
[6] A. Carpaneto, A. Accardi, M. Pisciotta, F. Gambale, Chloride channels activated by hypotonicity in N2A neuroblastoma cell line, Exp. Brain Res. 124 (1999) 193–199.
[7] A. Carpaneto, A. Naso, A. Paganetto, L. Cornara, E.-R. Pesce, F. Gambale, Properties of ion channels in the protoplasts of the Mediterranean seagrass Posidonia oceanica, Plant Cell Environ. 27 (2004) 279–292, https://doi.org/10.1111/j.1365-3040.2003.01139.x.
[8] M. Bregante, A. Carpaneto, V. Piazza, F. Sbrana, M. Vassalli, M. Faimali, F. Gambale, Osmoregulated chloride currents in Hemocytes from Mytilus galloprovincialis, PLoS One 11 (2016) e0167972, , https://doi.org/10.1371/journal.pone.0167972.
[9] M. Festa, L. Lagostena, A. Carpaneto, Using the plant vacuole as a biological system to investigate the functional properties of exogenous channels and transporters, Biochim. Biophys. Acta 1858 (2016) 607–612, https://doi.org/10.1016/j.bbamem.2015.09.022.
[10] A. Carpaneto, H. Koepsell, E. Bamberg, R. Hedrich, D. Geiger, Sucrose-and H+-dependent charge movements associated with the gating of sucrose transporter ZmSUT1, PLoS One 5 (2010) e12605.
[11] G. Nicastro, G. Orsomando, E. Ferrari, L. Manconi, F. Desario, A. Amici, A. Naso, A. Carpaneto, T.A. Pertinhez, S. Ruggieri, Solution structure of the phytotoxic protein PcF: the first characterized member of the Phytophthora PcF toxin family, Protein Sci. 18 (2009) 1786–1791.
[12] A. Rocchetti, T. Sharma, C. Wulfetange, J. Scholz-Starke, A. Grippa, A. Carpaneto, I. Dreyer, A. Vitale, K. Czempinski, E. Pedrazzini, The putative K(+) channel subunit AtKCO3 forms stable dimers in Arabidopsis, Front. Plant Sci. 3 (2012) 251, https://doi.org/10.3389/fpls.2012.00251.
[13] J. Böhm, M. Messerer, H.M. Müller, J. Scholz-Starke, A. Gradogna, S. Scherzer, T. Maierhofer, N. Bazihizina, H. Zhang, C. Stigloher, P. Ache, K.A.S. Al-Rasheid, K.F.X. Mayer, S. Shabala, A. Carpaneto, G. Haberer, J.-K. Zhu, R. Hedrich, Understanding the molecular basis of salt sequestration in epidermal bladder cells of Chenopodium quinoa, Curr. Biol. CB. 28 (2018) 3075–3085.e7, https://doi.org/10.1016/j.cub.2018.08.004.
[14] A. Carpaneto, M. Dalla Serra, G. Menestrina, V. Fogliano, F. Gambale, The phytotoxic lipodepsipeptide syringopeptin 25A from Pseudomonas syringae pv syringae forms ion channels in sugar beet vacuoles, J. Membr. Biol. 188 (2002) 237–248.
[15] M. Dalla Serra, G. Menestrina, A. Carpaneto, F. Gambale, V. Fogliano, A. Ballio, Molecular mechanism of action of syringopeptins, antifungal peptides from Pseudomonas syringae pv. Syringae, pore-form, Pept. Proteins Toxins. (2003) 272–295.
[16] G. Menestrina, M. Coraiola, V. Fogliano, A. Fiore, I. Grgurina, A. Carpaneto, F. Gambale, M. Dalla Serra, Antimicrobial lipodepsipeptides from Pseudomonas spp: A comparison of their activity on model membranes, Pseudomonas Syr. Relat. Pathog, Springer, 2003, pp. 185–198.
[17] J. Scholz-Starke, A. Naso, A. Carpaneto, A perspective on the slow vacuolar channel in vacuoles from higher plant cells, J. Chem. Inf. Model. 45 (2005) 1502–1506, https://doi.org/10.1021/ci050218a.
[18] R. Hedrich, I. Marten, TPC1-SV channels gain shape, Mol. Plant 4 (2011) 428–441, https://doi.org/10.1093/mp/ssr017.
[19] A. Carpaneto, A.M. Cantu, H. Busch, F. Gambale, Ion channels in the vacuoles of the seagrass Posidonia oceanica, FEBS Lett. 412 (1997) 236–240.
[20] R. Hedrich, T.D. Mueller, D. Becker, I. Marten, Structure and function of TPC1 vacuole SV Channel gains shape, Mol. Plant 11 (2018) 764–775, https://doi.org/10.1016/j.molp.2018.03.017.
[21] V. Demidchik, S. Shabala, S. Isayenkov, T.A. Cuin, I. Pottosin, Calcium transport across plant membranes: mechanisms and functions, New Phytol. 220 (2018) 49–69, https://doi.org/10.1111/nph.15266.
[22] E. Peiter, F.J.M. Maathuis, L.N. Mills, H. Knight, J. Pelloux, A.M. Hetherington, D. Sanders, The vacuolar Ca2+ −activated channel TPC1 regulates germination and stomatal movement, Nature 434 (2005) 404–408, https://doi.org/10.1038/nature03381.
[23] C. Schulze, H. Sticht, P. Meyerhoff, P. Dietrich, Differential contribution of EF-hands to the Ca^{2+}-dependent activation in the plant two-pore channel TPC1, Plant J. Cell Mol. Biol. 68 (2011) 424–432, https://doi.org/10.1111/j.1365-313X.2011.04697.x.
[24] A. Carpaneto, A.M. Cantù, F. Gambale, Redox agents regulate ion channel activity in vacuoles from higher plant cells, FEBS Lett. 442 (1999) 129–132.
[25] J. Scholz-Starke, A. De Angeli, C. Ferraretto, S. Paluzzi, F. Gambale, A. Carpaneto,

Redox-dependent modulation of the carrot SV channel by cytosolic pH, FEBS Lett. 576 (2004) 449–454.
[26] J. Scholz-Starke, F. Gambale, A. Carpaneto, Modulation of plant ion channels by oxidizing and reducing agents, Arch. Biochem. Biophys. 434 (2005) 43–50.
[27] A. Carpaneto, A.M. Cantù, F. Gambale, Effects of cytoplasmic mg 2+ on slowly activating channels in isolated vacuoles of Beta vulgaris, Planta 213 (2001) 457–468.
[28] A. Paganetto, A. Carpaneto, F. Gambale, Ion transport and metal sensitivity of vacuolar channels from the roots of the aquatic plant Eichhornia crassipes, Plant Cell Environ. 24 (2001) 1329–1336.
[29] A. Carpaneto, Nickel inhibits the slowly activating channels of radish vacuoles, Eur. Biophys. J. EBJ. 32 (2003) 60–66, https://doi.org/10.1007/s00249-002-0268-5.
[30] S. Corem, A. Carpaneto, P. Soliani, L. Cornara, F. Gambale, J. Scholz-Starke, Response to cytosolic nickel of slow vacuolar channels in the hyperaccumulator plant Alyssum bertolonii, Eur. Biophys. J. EBJ. 38 (2009) 495–501, https://doi.org/10.1007/s00249-008-0400-2.
[31] J. Scholz-Starke, A. Carpaneto, F. Gambale, On the interaction of neomycin with the slow vacuolar channel of Arabidopsis thaliana, J. Gen. Physiol. 127 (2006) 329–340.
[32] F. Gambale, A.M. Cantu, A. Carpaneto, B.U. Keller, Fast and slow activation of voltage-dependent ion channels in radish vacuoles, Biophys. J. 65 (1993) 1837–1843, https://doi.org/10.1016/S0006-3495(93)81241-4.
[33] A. Carpaneto, A cyclic model for bimodal activation of calcium activated potassium channels in radish vacuoles, Cycl. Model Bimodal Act. Calcium Act. Potassium Channels Radish Vacuoles. (2001) 1000–1022, https://doi.org/10.1400/22851.
[34] P.V.K. Gutla, A. Boccaccio, A. De Angeli, F. Gambale, A. Carpaneto, Modulation of plant TPC channels by polyunsaturated fatty acids, J. Exp. Bot. 63 (2012) 6187–6197.
[35] A. Carpaneto, A. Boccaccio, L. Lagostena, E. Di Zanni, J. Scholz-Starke, The signaling lipid phosphatidylinositol-3,5-bisphosphate targets plant CLC-a anion/H+ exchange activity, EMBO Rep. 18 (2017) 1100–1107, https://doi.org/10.15252/embr.201643814.
[36] A. Boccaccio, J. Scholz-Starke, S. Hamamoto, N. Larisch, M. Festa, P.V.K. Gutla, A. Costa, P. Dietrich, N. Uozumi, A. Carpaneto, The phosphoinositide PI(3,5)P_2 mediates activation of mammalian but not plant TPC proteins: functional expression of endolysosomal channels in yeast and plant cells, Cell. Mol. Life Sci. CMLS. 71 (2014) 4275–4283, https://doi.org/10.1007/s00018-014-1623-2.
[37] D. Barreca, G. Gattuso, E. Bellocco, A. Calderaro, D. Trombetta, A. Smeriglio, G. Laganà, M. Daglia, S. Meneghini, S.M. Nabavi, Flavanones: citrus phytochemical with health-promoting properties, BioFactors Oxf. Engl. 43 (2017) 495–506, https://doi.org/10.1002/biof.1363.
[38] C.-J. Weng, G.-C. Yen, Flavonoids, a ubiquitous dietary phenolic subclass, exert extensive in vitro anti-invasive and in vivo anti-metastatic activities, Cancer Metastasis Rev. 31 (2012) 323–351, https://doi.org/10.1007/s10555-012-9347-y.
[39] E. Giovannucci, Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature, J. Natl. Cancer Inst. 91 (1999) 317–331, https://doi.org/10.1093/jnci/91.4.317.
[40] M.A. Alam, N. Subhan, M.M. Rahman, S.J. Uddin, H.M. Reza, S.D. Sarker, Effect of citrus flavonoids, naringin and naringenin, on metabolic syndrome and their mechanisms of action, Adv. Nutr. Bethesda Md. 5 (2014) 404–417, https://doi.org/10.3945/an.113.005603.
[41] I.A. Mir, A.B. Tiku, Chemopreventive and therapeutic potential of “naringenin,” a flavanone present in citrus fruits, Nutr. Cancer 67 (2015) 27–42, https://doi.org/10.1080/01635581.2015.976320.
[42] M.S.I. Abaza, K.Y. Orabi, E. Al-Quattan, R.J. Al-Attiyah, Growth inhibitory and chemo-sensitization effects of naringenin, a natural flavanone purified from Thymus vulgaris, on human breast and colorectal cancer, Cancer Cell Int. 15 (2015) 46, https://doi.org/10.1186/s12935-015-0194-0.
[43] S. Saponara, L. Testai, D. Iozzi, E. Martinotti, A. Martelli, S. Chericoni, G. Sgaragli, F. Fusi, V. Calderone, Naringenin as large conductance ca(2+)-activated K+ (BKCa) channel opener in vascular smooth muscle cells, Br. J. Pharmacol. 149 (2006) 1013–1021, https://doi.org/10.1038/sj.bjp.0706951.
[44] A. Gradogna, J. Scholz-Starke, P.V.K. Gutla, A. Carpaneto, Fluorescence combined with excised patch: measuring calcium currents in plant cation channels, Plant J. 58 (2009) 175–182.
[45] A. Carpaneto, A. Gradogna, Modulation of calcium and potassium permeation in plant TPC channels, Biophys. Chem. 236 (2018) 1–7, https://doi.org/10.1016/j.bpc.2018.02.006.
[46] G. Agati, E. Azzarello, S. Pollastri, M. Tattini, Flavonoids as antioxidants in plants: location and functional significance, Plant Sci. Int. J. Exp. Plant Biol. 196 (2012) 67–76, https://doi.org/10.1016/j.plantsci.2012.07.014.
[47] J. Le Roy, B. Huss, A. Creach, S. Hawkins, G. Neutelings, Glycosylation is a major regulator of Phenylpropanoid availability and biological activity in plants, Front. Plant Sci. 7 (2016) 735, https://doi.org/10.3389/fpls.2016.00735.
[48] A.F. Kintzer, R.M. Stroud, On the structure and mechanism of two-pore channels, FEBS J. 285 (2018) 233–243, https://doi.org/10.1111/febs.14154.
[49] A. Favia, M. Desideri, G. Gambara, A. D'Alessio, M. Ruas, B. Esposito, D. Del Bufalo, J. Parrington, E. Ziparo, F. Palombi, A. Galione, A. Filippini, VEGF-induced neoangiogenesis is mediated by NAADP and two-pore channel-2-dependent Ca2+ signaling, Proc. Natl. Acad. Sci. U. S. A. 111 (2014), https://doi.org/10.1073/pnas.1406029111 E4706-4715.
[50] L.N. Hockey, B.S. Kilpatrick, E.R. Eden, Y. Lin-Moshier, G.C. Brailoiu, E. Brailoiu, C.E. Futter, A.H. Schapira, J.S. Marchant, S. Patel, Dysregulation of lysosomal morphology by pathogenic LRRK2 is corrected by TPC2 inhibition, J. Cell Sci. 128 (2015) 232–238, https://doi.org/10.1242/jcs.164152.
[51] Y. Sakurai, A.A. Kolokoltsov, C.-C. Chen, M.W. Tidwell, W.E. Bauta, N. Klugbauer, C. Grimm, C. Wahl-Schott, M. Biel, R.A. Davey, Ebola virus. Two-pore channels control Ebola virus host cell entry and are drug targets for disease treatment, Science 347 (2015) 995–998, https://doi.org/10.1126/science.1258758.
[52] A. Favia, I. Pafumi, M. Desideri, F. Padula, C. Montesano, D. Passeri, C. Nicoletti, A. Orlandi, D. Del Bufalo, M. Sergi, E. Ziparo, F. Palombi, A. Filippini, NAADP-dependent ca(2+) signaling controls melanoma progression, metastatic dissemination and Neoangiogenesis, Sci. Rep. 6 (2016) 18925, , https://doi.org/10.1038/srep18925.
[53] I. Pafumi, M. Festa, F. Papacci, L. Lagostena, C. Giunta, V. Gutla, L. Cornara, A. Favia, F. Palombi, F. Gambale, A. Filippini, A. Carpaneto, Naringenin impairs two-Pore Channel 2 activity and inhibits VEGF-induced angiogenesis, Sci. Rep. 7 (2017) 5121, https://doi.org/10.1038/s41598-017-04974-1.
[54] A. Costa, P.V.K. Gutla, A. Boccaccio, J. Scholz-Starke, M. Festa, B. Basso, I. Zanardi, M. Pusch, F.L. Schiavo, F. Gambale, The Arabidopsis central vacuole as an expression system for intracellular transporters: functional characterization of the cl-/H+ exchanger CLC-7, J. Physiol. 590 (2012) 3421–3430.
[55] L. Lagostena, M. Festa, M. Pusch, A. Carpaneto, The human two-pore channel 1 is modulated by cytosolic and luminal calcium, Sci. Rep. 7 (2017) 43900, , https://doi.org/10.1038/srep43900.
[56] D.B. Kitchen, H. Decornez, J.R. Furr, J. Bajorath, Docking and scoring in virtual screening for drug discovery: methods and applications, Nat. Rev. Drug Discov. 3 (2004) 935–949, https://doi.org/10.1038/nrd1549.
[57] D.J. Posson, R. Rusinova, O.S. Andersen, C.M. Nimigean, Calcium ions open a selectivity filter gate during activation of the MthK potassium channel, Nat. Commun. 6 (2015) 8342, https://doi.org/10.1038/ncomms9342.
[58] M.D. Cahalan, W. Almers, Interactions between quaternary lidocaine, the sodium channel gates, and tetrodotoxin, Biophys. J. 27 (1979) 39–55, https://doi.org/10.1016/S0006-3495(79)85201-7.
[59] Z. Chen, B.H. Ong, N.G. Kambouris, E. Marbán, G.F. Tomaselli, J.R. Balser, Lidocaine induces a slow inactivated state in rat skeletal muscle sodium channels, J. Physiol. 524 (Pt 1) (2000) 37–49, https://doi.org/10.1111/j.1469-7793.2000.t01-1-00037.x.
[60] E. Kutluay, B. Roux, L. Heginbotham, Rapid intracellular TEA block of the KcsA potassium channel, Biophys. J. 88 (2005) 1018–1029, https://doi.org/10.1529/biophysj.104.052043.
[61] M.A. Scorciapino, G. Malloci, I. Serra, S. Milenkovic, L. Moynié, J.H. Naismith, E. Desarbre, M.G.P. Page, M. Ceccarelli, Complexes formed by the siderophore-based monosulfactam antibiotic BAL30072 and their interaction with the outer membrane receptor PiuA of P. aeruginosa, Biometals Int. J. Role Met. Ions Biol. Biochem. Med. 32 (2019) 155–170, https://doi.org/10.1007/s10534-018-00166-0.
[62] A. Fiser, A. Sali, Modeller: generation and refinement of homology-based protein structure models, Methods Enzymol. 374 (2003) 461–491, https://doi.org/10.1016/S0076-6879(03)74020-8.
[63] J. Guo, W. Zeng, Q. Chen, C. Lee, L. Chen, Y. Yang, C. Cang, D. Ren, Y. Jiang, Structure of the voltage-gated two-pore channel TPC1 from Arabidopsis thaliana, Nature 531 (2016) 196–201, https://doi.org/10.1038/nature16446.
[64] M.J. Harvey, G. Giupponi, G.D. Fabritiis, ACEMD: accelerating biomolecular dynamics in the microsecond time scale, J. Chem. Theory Comput. 5 (2009) 1632–1639, https://doi.org/10.1021/ct9000685.
[65] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, A.J. Olson, AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility, J. Comput. Chem. 30 (2009) 2785–2791, https://doi.org/10.1002/jcc.21256.
[66] J. She, J. Guo, Q. Chen, W. Zeng, Y. Jiang, X.-C. Bai, Structural insights into the voltage and phospholipid activation of the mammalian TPC1 channel, Nature 556 (2018) 130–134, https://doi.org/10.1038/nature26139.